(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
27 September 2018 (27.09.2018)

WIPO | PCT

(10) International Publication Number
WO 2018/175315 A1

(51) International Patent Classification:
A61K 47/60 (2017.01) *A61P 17/00* (2006.01)
A61P 11/00 (2006.01) *A61P 27/02* (2006.01)
A61P 1/00 (2006.01)

(21) International Application Number:
PCT/US2018/023133

(22) International Filing Date:
19 March 2018 (19.03.2018)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

62/473,975	20 March 2017 (20.03.2017)	US
62/501,651	04 May 2017 (04.05.2017)	US
62/590,148	22 November 2017 (22.11.2017)	US
62/634,691	23 February 2018 (23.02.2018)	US

(71) Applicant: **SIENNA BIOPHARMACEUTICALS, INC.** [US/US]; 30699 Russell Ranch Road, Suite 140, Westlake Village, California 91362 (US).

(72) Inventors: **TRAVERSA, Silvio**; 30699 Russell Ranch Road, Suite 140, Westlake Village, California 91362 (US). **HARRIS, Todd James**; 30699 Russell Ranch Road, Suite 140, Westlake Village, California 91362 (US). **MAINERO, Valentina**; 30699 Russell Ranch Road, Suite 140, Westlake Village, California 91362 (US). **RAVA ROSSA, Luisa, Bertarione**; 30699 Russell Ranch Road, Suite 140, Westlake Village, California 91362 (US). **BAGNOD, Raffaella**; 30699 Russell Ranch Road, Suite 140, Westlake Village, California 91362 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ,

(54) Title: POLYMER CONJUGATES OF STAUROSPORINE DERIVATIVES HAVING REDUCED EXPOSURE

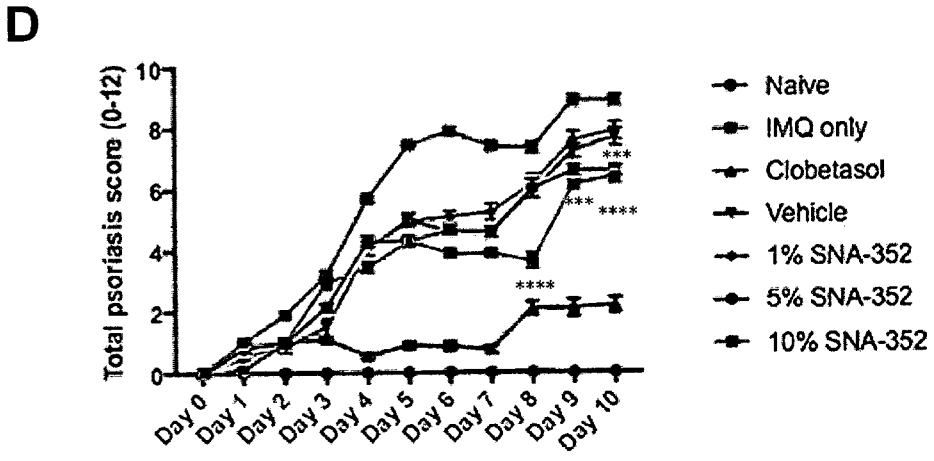


FIGURE 68

(57) Abstract: Disclosed herein are novel polymer conjugates, such as SNA-352, which comprise a Staurosporine derivative linked to a polymer. The disclosed polymer conjugates reduce exposure of the Staurosporine derivative to non-target sites. The conjugate inhibits kinase mediators of various inflammatory and other conditions.

WO 2018/175315 A1

[Continued on next page]

TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

POLYMER CONJUGATES OF STAUROSPORINE DERIVATIVES HAVING REDUCED EXPOSURE

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to US provisional patent application Serial No. 62/473,975 filed March 20, 2017, and claims priority to US provisional patent application Serial No. 62/501,651 filed May 4, 2017, and claims priority to US provisional patent application Serial No. 62/590,148 filed November 22, 2017, and claims priority to US provisional patent application Serial No. 62/634,691 filed February 23, 2018. Each of these applications is incorporated by reference in their entirety herein.

FIELD

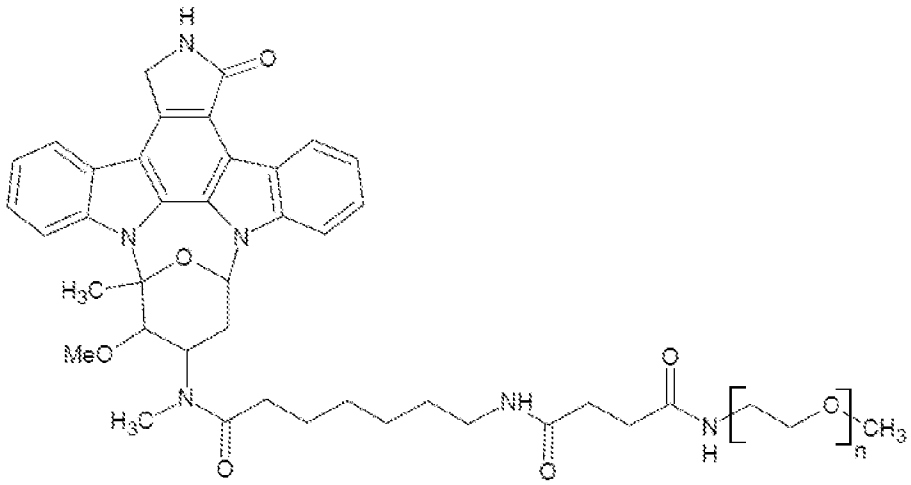
[0002] Disclosed herein are polymer conjugates, comprising active agents linked to polymers, and therapeutic uses thereof. More particularly, a polymer conjugate which exhibits reduced exposure to non-target sites and inhibits kinase mediators of various pathological conditions is described.

BACKGROUND

[0003] Staurosporine and derivatives thereof have been described for possible therapeutic use in the prevention, alleviation and treatment of kinase-associated pathologies. However, such compounds are associated with broad kinase specificity, as well as undesirable and toxic effects. Accordingly, strategies to render these active kinase inhibitors more specific and less toxic are needed.

SUMMARY OF EMBODIMENTS

[0004] In several embodiments, a polymer conjugate (such as SNA-352) is provided having the following structure:



[0005] Effective delivery of pharmacologically active agents may be hindered by unwanted exposure of those agents to non-desired locations (such as the systemic circulation and/or lymphatic system). For example, topical agents useful in treating various skin disorders may result in toxic side effects because of systemic exposure. One issue with delivering compositions comprising one or more active agents topically (or non-topically) is the concern that such agents may need to be delivered in an amount and at a location sufficient to have a therapeutic effect. At the same time however, exposure (e.g., absorption or longevity of the composition in the systemic circulation, lymphatic system, or other non-targeted sites) may not be desirable for multiple reasons, including, but not limited to, safety reasons. There remains an unmet need for compounds with reduced exposure at non-target sites that result in a clinically therapeutic effect.

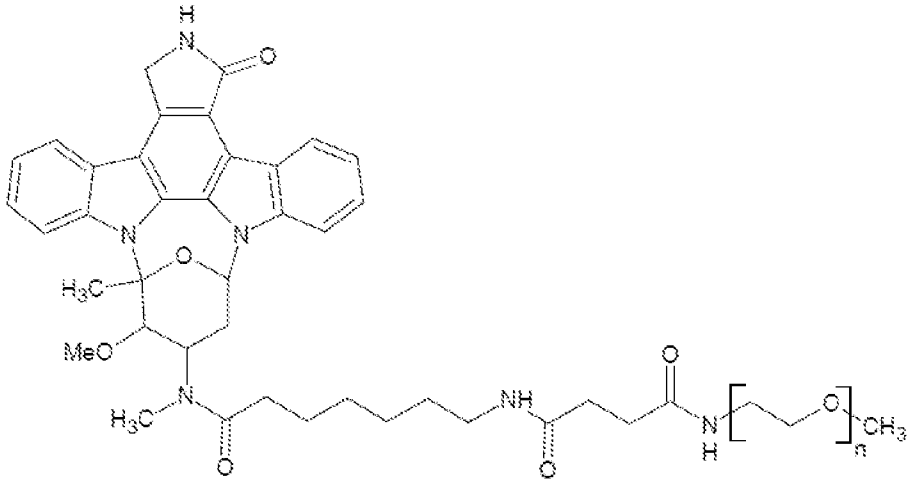
[0006] In several embodiments of the invention, the compositions described herein are both therapeutically efficacious and minimize non-target (e.g., systemic or bloodstream) exposure In some embodiments, the active agents are PEGylated or otherwise coupled to large molecules, and surprisingly, are effective in crossing biological membranes such that the active agents are effectively delivered to the target location. Although inflammatory bowel diseases are disclosed in several embodiments, other embodiments are used to treat dermal inflammation, as well as other several conditions (e.g., those conditions that would benefit from treatment with reduced exposure at non-target sites). For example, in some embodiments, the compositions and technology described herein are used in the gastrointestinal and pulmonary systems. Ophthalmic treatments are provided in some embodiments. In yet other embodiments, compositions for treating joints are provided. Treatment of the nose and

ear are provided in other embodiments. Inflammatory and non-inflammatory conditions are contemplated herein.

[0007] Reduced exposure compounds and compositions are provided in several embodiments. "Reduced exposure" compounds are those compounds that, when delivered to a target location, are formulated to act at the target location with reduced exposure (e.g., entry and/or longevity) in non-target sites. Exposure is reduced as compared to active agents not formulated according to the embodiments described herein. As a non-limiting example, a PEGylated topical dermal active agent has reduced exposure to the bloodstream as compared to the active agent alone. Reduced exposure compounds include topical compounds that can be delivered to body surfaces and cavities such as the skin, eyes, ears, nose, mouth, vagina, rectum, etc., as well as oral (e.g., enteric coated) compounds for oral delivery that treat the gastrointestinal system (e.g., the GI lining), inhalants that treat the lungs, injections for joints, and other modes of delivery that target one location with the goal of reducing exposure to a non-desired site. Non-desired target sites include, for example, the systemic system, the lymphatic system, non-target tissue, etc. "Reduced exposure compositions" comprise or consist essentially of one or more "reduced exposure compounds."

[0008] Reduced exposure topical compositions are provided in many embodiments. In some embodiments, a reduced exposure composition is delivered orally, e.g., for treatment of the gastrointestinal system. The active agent remains in the lining of the gastrointestinal tract and is able to achieve pharmacological specificity. Because the active agent is conjugated with PEG or another molecule as described herein, the active agent is absorbed more slowly into the non-target site (e.g., the systemic circulation and/or lymphatic system). In some cases, less or none of the active agent is absorbed into the non-target site (e.g., systemic circulation and/or lymphatic system). Further, once the composition enters the systemic circulation and/or lymphatic system, clearance (e.g., by the kidney) occurs at a much faster rate. One or more of the advantages of (i) *reduced* absorption into the non-target site (e.g., systemic circulation and/or lymphatic system), (ii) *slower* absorption into the non-target site (e.g., systemic circulation and/or lymphatic system), and (iii) *faster* clearance rates from the non-target site (e.g., systemic circulation and/or lymphatic system) are also achieved when using the compositions (formulated according to the methods described herein) for treating the eye (e.g., via eye drops), the lungs (e.g., via inhalants), the skin (e.g., via dermal topicals), joints (e.g., via injectables), nasal passageways, and the ear (such as the ear canal and other structures). Vaginal and rectal tissues are treated in some embodiments via, for example suppositories.

[0009] In several embodiments, there is provided in a reduced exposure composition, a polymer conjugate comprising a warhead (e.g., at least one active agent) linked to a polymer, wherein the warhead comprises an inhibitor, antagonist, or inverse agonist of, for example, a mediator of an inflammatory bowel disease. In some embodiments, at least one inhibitor, antagonist, or inverse agonist of a mediator of an inflammatory bowel disease comprises or consists of a composition that includes any one of compounds 1-40 (and derivatives thereof) disclosed herein in Table 1 coupled to a polymer. In some embodiments, the warhead of the polymer conjugate is compound 1. In some embodiments, the LSE polymer conjugate has the following formula below:



[0010] Dermal inflammation or other conditions may also be treated in some embodiments with compositions comprising these compounds. Non-inflammatory conditions may also be treated with some embodiments.

[0011] As described above, several embodiments disclosed herein provide reduced or minimized exposure (e.g., entry into and/or longevity in a non-target site such as the systemic circulation and/or lymphatic system). In some embodiments, exposure at a non-target site is less than 90%, 75%, 50%, 25%, 15%, 10%, 5% or 2% (or less) of the polymer conjugate as compared to a similar active entity that has not been produced according to the embodiments described herein. In some embodiments, desirable rate of clearance from the non-target site (e.g., systemic circulation and/or lymphatic system) for the compositions described herein is increased by at least 10%, 25%, 50%, or 75% or more as compared to non-conjugated controls. As an example, a PEGylated active agent described herein not only penetrates the desired membranes to reach a desired target, but has reduced non-target exposure by at least 20-80% or more as compared to

the non-PEGylated active agent. In some embodiments, blood concentrations measured post administration of the compositions described herein are less than about 0.1 ng/ml, less than 1 ng/ml, or less than 10 ng/ml after, e.g., 15 minutes, 30 minutes, 1 hour, 6 hours or 12 hours.

[0012] In some embodiments, reduced exposure at non-target sites contributes to enhanced efficacy. Efficacy may be enhanced because lower concentrations/amounts/dosing schedules are required to achieve the same or similar therapeutic efficacy at the target site (because, for example, the active ingredient stays at the desired target site for a longer time). In one embodiment, concentrations/amounts/dosing schedules are reduced by 25%-75% or more.

[0013] More rapid clearance rates of the active agent once in the non-target site(s) (such as systemic circulation and/or lymphatic system) are also beneficial because this may allow for a *higher* concentration or *more* doses to be delivered. This is especially beneficial for active agents in which a subject would benefit from a higher dose but cannot tolerate the higher dose due to toxicity at the non-target site (e.g., systemic toxicity). Faster clearance rates would permit the desired higher dose to be delivered according to the desired schedule. For example, a subject may be able to tolerate daily doses rather than weekly doses because of the reduced exposure.

[0014] In some embodiments, the active agents of the compositions described herein (e.g., the compounds in Table 1 conjugated e.g., with PEG or other polymers) are measured in non-target sites (e.g., the systemic circulation and/or lymphatic system) at less than amounts found when the active agent is delivered without conjugation (e.g., less than 0.5%, 1% or 2% after 6 or 12 hours, as compared with 3-15% (e.g., 3-6%) when the active agent is delivered without conjugation). In some embodiments, the active agents of the compositions described herein (e.g., the compounds in Table 1 conjugated e.g., with PEG or other polymers) are measured in non-target sites (e.g., the systemic circulation and/or lymphatic system) at less than 0.5%, 1% or 2% after 3-24 hours, as compared to an amount 2-20 times greater when the active agent is delivered without conjugation.

[0015] In some embodiments, clearance of the compositions (e.g., the conjugated polymer compounds) occurs within minutes of exposure to the non-target site (e.g., systemic circulation and/or lymphatic system), as opposed to hours. In other embodiments, 50% clearance of the conjugated polymer compounds occurs in less than 5 minutes, 15 minutes, 30 minutes, 1 hour, 6 hours, and 12 hours of exposure to the systemic circulation and/or lymphatic system. Clearance times of the conjugated polymer compounds are reduced by more than 25%, 50%, 75% and 90%, as compared

to the non-conjugated active agents or other formulations. These reduced clearance times are beneficial to reduce toxicity and undesired side effects.

[0016] In some embodiments, an active agent may be increasingly toxic as it is metabolized in the non-target site (e.g., systemic circulation and/or lymphatic system) because the metabolites exhibit more toxicity than the original agent. Thus, faster clearance rates, in some cases even before the toxic metabolites are created, are especially beneficial.

[0017] The term "active entity" as used herein should not be understood as limiting the participation of the polymer itself and/or the chemical linking moiety between the polymer and the warhead in defining the pharmacology of the polymer conjugate. In some embodiments, the polymer influences the selectivity and/or inhibitory activity of the polymer conjugate. In some embodiments, the chemical linking moiety between the polymer and warhead influences the selectivity and/or inhibitory activity of the polymer conjugate. In some embodiments, the polymer conjugates exhibit no change in selectivity or inhibitory activity against the therapeutic target in comparison with the unconjugated active agent. In some embodiments, the polymer conjugates exhibit a significant increase in selectivity against the therapeutic target in comparison with the unconjugated active agent. In some embodiments, the polymer conjugates exhibit a significant increase in inhibitory activity against the therapeutic target in comparison with the unconjugated active agent. In some embodiments, the polymer conjugates exhibit a significant increase in selectivity and inhibitory activity against the therapeutic target in comparison with the unconjugated active agent. In some embodiments, the increased selectivity and/or inhibitory activity of the polymer conjugate against the therapeutic target in comparison with the unconjugated active agent causes decrease in undesired biological effects. In some embodiments, the increased selectivity of the polymer conjugate is caused by an increase of the hydrodynamic volume resulting from the conjugated polymer chain. In some embodiments, the polymer chain creates a higher steric hindrance which allows discrimination among the diverse shapes and sizes of the binding sites of different proteins, thus improving selectivity with respect to the active agent alone.

[0018] In several embodiments, various inflammatory bowel diseases are treated. The inflammatory bowel disease comprises, in some embodiments, Crohn's disease, ulcerative colitis, collagenous colitis, lymphocytic colitis, ischemic colitis, diversion colitis, Behçet's syndrome, and indeterminate colitis.

[0019] Also provided herein, in several embodiments, are polymer conjugates wherein the polymer is polyethylene glycol (PEG) or methoxy-polyethylene glycol (m-PEG). In several embodiments, there is provided a pharmaceutical composition

comprising or consisting essentially of a polymer conjugate disclosed herein that is formulated for topical and non-topical administration. In several embodiments, methods of making and using the compositions described herein are provided.

[0020] In several embodiments, the invention comprises a reduced exposure composition comprising at least one active entity linked to at least one polymer, wherein the composition has reduced exposure at a non-target site as compared to the active entity delivered without the polymer. The non-target site comprises the systemic system, the lymphatic system and/or another non-target tissue site in some embodiments.

[0021] In some embodiments, the active entity comprises an inhibitor, an antagonist, or an inverse agonist. For example, the active entity may be an inhibitor, antagonist, or inverse agonist of a mediator of inflammation. In some embodiments, the active entity may be an inhibitor, antagonist, or inverse agonist of a mediator of an inflammatory bowel disease. In some embodiments, the active entity may be an inhibitor, antagonist, or inverse agonist of JAK and/or STAT family proteins. The active entity comprises or consists essentially of any one or more of compounds 1-40 in some embodiments. The active entity comprises compound 1 in some embodiments. The reduced exposure composition comprises CT352 in some embodiments.

[0022] The active entity binds to a JAK and/or STAT family protein in some embodiments. The binding may be partially or fully inhibitory or not.

[0023] In some embodiments, the polymer used in the reduced exposure compounds comprises polyethylene glycol (PEG) and/or methoxy-polyethylene glycol (m-PEG). In embodiments where the active entity has one or more carboxyl, hydroxyl, amino and/or sulfhydryl groups, the active entity is PEGylated (or conjugated/coupled to another polymer) at one or more of said carboxyl, hydroxyl, amino and/or sulfhydryl groups.

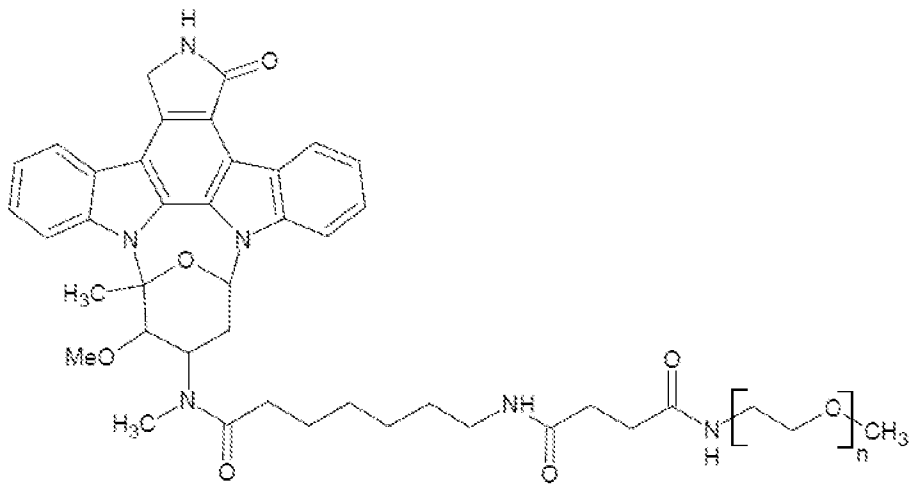
[0024] The reduced exposure compositions described herein are formulated for oral administration in several embodiments. Inhalants, injectables, eye drops, nasal sprays, topical administration etc. are provided in some embodiments. In several embodiments, methods of treating one or more of the following are provided: non-dermal inflammation, inflammatory bowel disease, inflammatory skin disease, wounds, scars, autoimmune disorders, and cancerous or pre-cancerous lesions. Kits comprising one or more compounds and devices for administration (syringes, containers, inhalers, etc.) as well as instructions for use, are provided in certain embodiments.

[0025] Compositions may be administered via at least two routes of administration, either simultaneously or sequentially according to some embodiments. In one embodiment, the composition is administered via a first (e.g. topical dermal) route to

a subject, wherein the subject further receives an additional agent via a second (e.g., non-dermal) route to achieve synergetic effects.

[0026] In several embodiments, the inventions comprises methods for reducing exposure of a composition at least one non-target site, wherein the method comprises applying a composition comprising at least one active entity linked to at least one polymer, wherein the combination of the active entity and polymer reduces exposure at the non-target site by more than 50% as compared to the active entity without the polymer. The composition may be applied topically, injected, inhaled, or administered orally. The non-target site includes non-target tissue at which pharmacological activity is not desired and/or not achieved. Non-target sites can include the bloodstream or systemic system. Non-target sites can also include the lymphatic system.

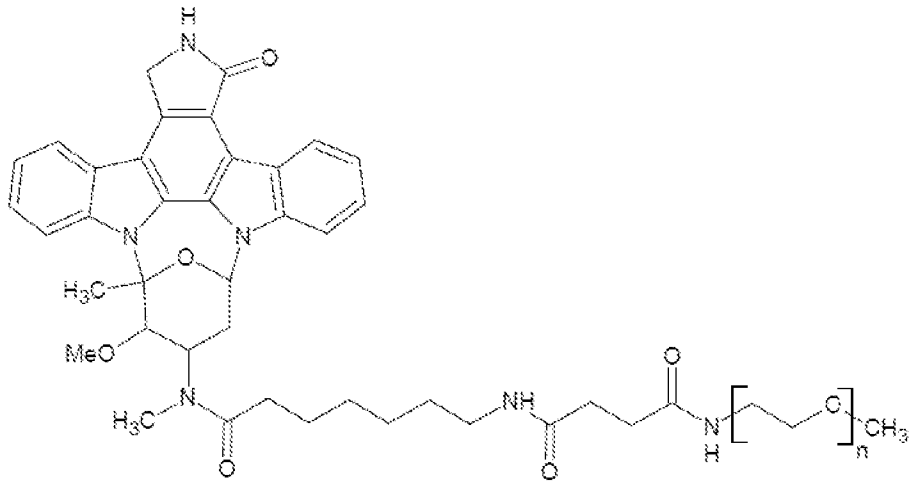
[0027] In several embodiments, a compound having the formula:



[0028] is provided. In some embodiments, n ranges from about 2 to about 2270 (e.g., 4-10, 10-20, 20-40, 40-60, 60-80, 80-100, 125-150-150-175, 175-200, 200-300, 300-400, 400-500, 500-600, 600-700, 700-800, 800-900, 900-1000, 1000-1100, 1100-1200, 1200-1300, 1300-1400, 1400-1500, 1500-1600, 1600-1700, 1700-1800, 1800-1900, 1900-2000, 2100-2200, 2200-2300, and overlapping ranges therein). There is provided, in some embodiments, stereoisomers, enantiomers, and/or pharmaceutically acceptable salts of the compound.

[0029] In several embodiments, a reduced exposure composition for treating a target site is provided. In one embodiment, the composition comprises or consists essentially of a conjugate comprising or consists essentially of an active entity coupled (e.g., linked) to at least one polymer. Two, three or more active entities or two, three or

more polymers may be used. In one embodiment, a pharmaceutically acceptable carrier formulated for delivering the conjugate to the target site is also provided. In several embodiments, the conjugate has reduced exposure at a non-target site as compared to the active entity delivered without the polymer. The non-target site includes for example the systemic system, the lymphatic system and/or other non-target tissue sites. In several embodiments, the non-target site comprises any site at which pharmacological activity is not desired and/or not achieved. In several embodiments, the conjugate has the formula:



[0030] In some embodiments, n ranges from about 2 to about 2270 (e.g., 4-10, 10-20, 20-40, 40-60, 60-80, 80-100, 125-150-150-175, 175-200, 200-300, 300-400, 400-500, 500-600, 600-700, 700-800, 800-900, 900-1000, 1000-1100, 1100-1200, 1200-1300, 1300-1400, 1400-1500, 1500-1600, 1600-1700, 1700-1800, 1800-1900, 1900-2000, 2100-2200, 2200-2300, and overlapping ranges therein). There is provided, in some embodiments, stereoisomers, enantiomers, and/or pharmaceutically acceptable salts of the conjugate.

[0031] In several embodiments, a reduced exposure composition for treating a cell within a target site is provided. Methods for treating diseases, conditions, and disorders are also provided. In one embodiment, the composition comprises or consists essentially of a conjugate comprising or consists essentially of an active entity coupled (e.g., linked) to at least one polymer. Two, three or more active entities or two, three or more polymers may be used. The active entity may be for example, an inhibitor, antagonist, or inverse agonist of a cellular kinase. In several embodiments, the active entity is one or more of compounds 1-40. In one embodiment, the composition comprises compound 1. In one embodiment, the composition comprises SNA-352. The polymer can include, for example, polyethylene glycol (PEG) and/or methoxy-

polyethylene glycol (m-PEG). In one embodiment, a pharmaceutically acceptable carrier formulated for delivering the conjugate to the target site is also provided. In several embodiments, the conjugate has reduced exposure at a non-target site as compared to the active entity delivered without the polymer. The non-target site includes for example the systemic system, the lymphatic system and/or other non-target tissue sites. In several embodiments, the non-target site comprises any site at which pharmacological activity is not desired and/or not achieved. In one embodiment, the conjugate can advantageously traverse plasma membranes of cells at the target site, thereby promoting interactions between the active entity and the cellular kinase This traversal may include the crossing of cellular lipid bilayers to, e.g., distribute the active entity among both lipophilic and hydrophilic cellular compartments. Membranes include the lipid bilayer, plasma membrane and the nuclear membrane as examples. In several embodiments, the conjugate interacts with a kinase associated with the plasma membrane, cytoplasm and/or nucleus. The conjugate may exhibit a depot effect across cellular compartments, thereby reducing the dose of the active entity required to inhibit the cellular kinase compared to the active entity without conjugation to the polymer.

[0032] In several embodiments, the cellular kinase may be a JAK family protein. In some embodiments, the JAK family includes one or more of JAK1, JAK2, JAK3, and Tyrosine kinase 2 (TYK2). In several embodiments, the cellular kinase may be a STAT family protein. In some embodiments, the JAK protein and/or STAT protein is bound and/or inhibited by the active entity. In some embodiments, the active entity has one or more carboxyl, hydroxyl, amino and/or sulfhydryl groups. In one embodiment, at least one polymer is conjugated to the active entity at the one or more carboxyl, hydroxyl, amino and/or sulfhydryl groups.

[0033] In some embodiments, the reduced exposure composition may be formulated for topical, oral, local ocular (e.g., eye drop), inhalation, injection or suppository delivery. Topical, oral, injection, inhalation, local ocular, and suppository administration is provided in several embodiments. In several embodiments, the administration is daily. In the methods of treatment, effective amounts of the active entity are delivered to a subject (e.g., human or veterinary). In several embodiments, the composition may be administered via at least two routes of administration, either simultaneously or sequentially. In some embodiments, the composition is administered via a topical route to a subject, and the subject further receives an additional agent via a non-topical route. In some such embodiments, this co-administration achieves synergetic effects. The composition may further comprise one or more additional ingredients, such as, for example, a protective agent, an emollient, an astringent, a humectant, a sun screening agent, a sun tanning agent, a UV absorbing agent, an antibiotic agent, an anti-

angiogenesis agent, a preventive or therapeutic agent for inflammatory bowel disease, a physiological cooling agent, an antifungal agent, an antiviral agent, an antiprotozoal agent, an anti-acne agent, an anesthetic agent, a steroidal anti-inflammatory agent, a non-steroidal anti-inflammatory agent, an antipruritic agent, an additional antioxidant agent, a chemotherapeutic agent, an anti-histamine agent, a vitamin or vitamin complex, a hormone, an anti-dandruff agent, an anti-wrinkle agent, an anti-skin atrophy agent, a skin whitening agent, and/or a cleansing agent.

[0034] In several embodiments, the active entity and/or conjugate may have a longer residence time within a cell or other tissue at the target site compared to the active entity without conjugation to the polymer. For example, the residence time of the active entity and/or conjugate within a cell or other tissue at the target site is, as compared to the active entity without conjugation to the polymer, (i) at least 25% (e.g., 25-50%, 50-75%, 75-100%, 100-150%, or higher and overlapping ranges therein) longer and/or (ii) at least 2-20 fold (e.g., 2-10 fold, 2-4 fold, 4-6 fold, 6-8 fold, 8-10 fold, 10-12 fold, 12-14 fold, 14-16 fold, 16-18 fold, 18-20 fold, 20-30 fold, 40-50 fold, 10-50 fold, 50-100 fold, and overlapping ranges therein) longer. In one embodiment, the residence time is over 100 fold longer.

[0035] In some embodiments, a smaller dose of the conjugate may be needed to achieve a therapeutic effect comparable to the active entity without conjugation to the polymer. For example, in several embodiments, the dose of the conjugate needed to achieve a therapeutic effect comparable to the active entity without conjugation to the polymer is at least 10% (e.g., 10-15%, 15-20%, 20-25%, 25-30%, 30-40%, 40-50%, 50-60%, 60-70%, 70-80%, 80-90%, 90-100%, 100%-125%, 125-150%, or higher and overlapping ranges therein) lower. In one embodiment, the dose is over 200% lower. In some embodiments, fewer doses and/or smaller doses of the conjugate are required as compared to the active entity delivered without the polymer.

[0036] In several embodiments, the active entity and/or conjugate may have an increased concentration, activity and/or bioavailability within a cell or tissue at the target site compared to the active entity without conjugation to the polymer. In some such embodiments, the therapeutically effective amount of the active entity is at the target site. For example, the concentration, activity and/or bioavailability within a cell or other tissue at the target site is, as compared to the active entity without conjugation to the polymer, at least 2-20 fold (e.g., 2-4 fold, 4-6 fold, 6-8 fold, 8-10 fold, 10-12 fold, 14-16 fold, 18-20 fold, 20-30 fold, 30-40 fold, 40-50 fold, 50-100 fold, and overlapping ranges therein) greater than within a cell or tissue at a non-target site (e.g., the systemic system, the lymphatic system, the circulatory system, bone marrow). In one

embodiment, the concentration, activity and/or bioavailability within a cell or tissue at the target site is over 100 fold greater.

[0037] In several embodiments, the active entity and/or conjugate may have reduced concentration, activity and/or bioavailability within a cell or tissue at a non-target site compared to the active entity without conjugation to the polymer. In several embodiments, the active entity and/or conjugate is present at a biologically inactive concentration within a cell or tissue at a non-target site. In several embodiments, reduced concentration, activity and/or bioavailability within a cell or tissue at a non-target site (e.g., the systemic system, the lymphatic system, bone marrow, the circulatory system) advantageously reduces toxicity and/or other side effects, such as, for example, immunosuppression. For example, in some embodiments, the active entity and/or conjugate has reduced systemic absorption and/or little or no systemic toxicity when the composition is formulated for oral delivery and is administered orally (e.g., a single administration, administration on a daily basis).

[0038] In several embodiments, the conjugate is amphiphilic and/or amphipathic. In some embodiments, the conjugate is more amphiphilic and/or amphipathic than the active entity without conjugation to the polymer. For example, in several embodiments, the conjugate, as compared to the active entity without conjugation to the polymer, is at least 25% (e.g., 20-25%, 25-30%, 30-40%, 40-50%, 50-60%, 60-70%, 70-80%, 80-90%, 90-100%, 100%-125%, 125-150%, or higher and overlapping ranges therein) more amphiphilic. In one embodiment, the amphiphilicity is over 200% greater. Additionally, in some embodiments, the conjugate is more hydrophilic than the active entity without conjugation to the polymer. For example, in several embodiments, the conjugate, as compared to the active entity without conjugation to the polymer, is at least 25% (e.g., 20-25%, 25-30%, 30-40%, 40-50%, 50-60%, 60-70%, 70-80%, 80-90%, 90-100%, 100%-125%, 125-150%, or higher and overlapping ranges therein) more hydrophilic. In one embodiment, the hydrophilicity is over 200% greater. In some embodiments, the greater hydrophilicity of the conjugate advantageously facilitates one or more of: non-compartmentalization within a cell or tissue at the target site; access to and activity in both the lipid bilayer and the cytosol of the cell; access to and/or activity in both the lipid bilayer and the cytoplasm of the cell; and/or access to and/or activity across the lipid bilayer. In some embodiments, the conjugate exhibits greater access to the kinase compared to the active entity without conjugation to the polymer.

[0039] In several embodiments, the method of treatment and/or use of the compositions described herein are provided for the prophylaxis or treatment of one or more of the following in a subject in need thereof: a joint, an eye, an autoimmune

disorder, the gastrointestinal system, a lung, a cancerous or pre-cancerous lesion, a scar, a wound, non-dermal inflammation, an inflammatory condition, an inflammatory skin condition, and/or an inflammatory skin disease.

[0040] In several embodiments, the method of treatment and/or use of the compositions described herein are provided for the prophylaxis or treatment of one or more of the following conditions: ulcerative colitis, Crohn's disease, inflammatory bowel disease, irritable bowel syndrome, small intestinal bacterial overgrowth Crohn's disease, ulcerative colitis, collagenous colitis, lymphocytic colitis, ischemic colitis, diversion colitis, Behçet's syndrome, and indeterminate colitis, alopecia, alopecia areata, androgenic alopecia, and/or dry eye.

BRIEF DESCRIPTION OF THE FIGURES

[0041] The Figures below are illustrative for some embodiments and should not be construed as overly limiting.

[0042] Figure 1 depicts the chemical structure of staurosporine.

[0043] Figure 2 depicts the synthesis scheme of CT352.

[0044] Figure 3 depicts the preparative HPLC chromatogram of CT352.

[0045] Figure 4 depicts the analytical HPLC chromatogram of purified CT352 at 292nm (Purity: 98.44%).

[0046] Figure 5 depicts the analytical HPLC chromatogram of purified CT352 (LES Detector).

[0047] Figure 6 depicts the MALDI-TOF spectrum of CT352.

[0048] Figure 7 depicts the comparison between the isotopic pattern of the peak at 2491 m/z and the theoretical one calculated for a CT352 molecule having 39 oxyethylenic units.

[0049] Figure 8 depicts the CT352 synthesis.

[0050] Figure 9 depicts the HPLC analysis of Staurosporine IRIS lot 02/12.

[0051] Figure 10 depicts the HPLC analysis of crude Intermediate 1.

[0052] Figure 11 depicts the UV profile (@292 and 254 nm) of Intermediate 1 purification by normal phase flash chromatography.

[0053] Figure 12 depicts the HPLC analysis of purified Intermediate 1.

[0054] Figure 13 depicts the HPLC analysis of reaction mixture/1 h aging.

[0055] Figure 14 depicts the HPLC analysis of crude Intermediate 2/1◦ solid.

[0056] Figure 15 depicts the HPLC analysis of crude Intermediate 2/2◦ solid.

[0057] Figure 16 depicts the HPLC analysis of crude CT352 from Intermediate 2/1◦ solid.

[0058] Figure 17 depicts the HPLC analysis of crude CT352 from Intermediate 2/2◦ solid.

[0059] Figure 18 depicts the UV profile (@292 and 210 nm) of CT352 purification by normal phse flash chromatography.

[0060] Figure 19 depicts the HPLC analysis of CT352 purified by by normal-phase flash chromatography.

[0061] Figure 20 depicts the UV profile (@292 and 210 nm) of CT352 purification by reversed phase flash chromatography.

[0062] Figure 21 depicts the HPLC analysis of final CT352 lot n◦ 2010CG02/S9.

[0063] Figure 22 depicts the NMR analysis of final CT352/ lot n◦ 2010CG02/S9.

[0064] Figure 23 depicts the Certificate of analysis of final CT352/ lot n◦ 2010CG02/S9.

[0065] Figure 24 depicts the CT352 synthesis.

[0066] Figure 25 depicts the HPLC analysis of reaction mixture/step 1/27h aging time.

[0067] Figure 26 depicts the HPLC analysis of crude Intermediate 1 in organic phase.

[0068] Figure 27 depicts the HPLC analysis of isolated Intermediate 1 dissolved in HCOOH/H2O/CAN 1:1:1.

[0069] Figure 28 depicts the HPLC analysis of reaction mixture/step 2/5h aging time.

[0070] Figure 29 depicts the HPLC analysis of crude Intermediate 2.

[0071] Figure 30 depicts the HPLC analysis of reaction mixture/step 3/27h aging time.

[0072] Figure 31 depicts the HPLC analysis of crude CT352.

[0073] Figure 32 depicts the HPLC analysis of CT352/P1 purified by normal-phase flash chromatography.

[0074] Figure 33 depicts the HPLC analysis of CT352/P2 purified by normal-phase flash chromatography.

[0075] Figure 34 depicts the HPLC analysis of final CT352 lot n◦ 2010RB15/S5.

[0076] Figure 35 depicts the NMR analysis of final CT352/lot n◦ 2010RB15/S5.

[0077] Figure 36 depicts the Certificate of analysis of final CT352/ lot n◦ 2010RB15/S5.

[0078] Figure 37 depicts (a) colitis development was evaluated monitoring colon shortening at mice sacrifice, (b) weight loss during the experiment, and (c) colitis clinical score at sacrifice. CT100 and CT300 data points refer to 100 and 300 mg/kg CT352 in vehicle. CsA data points refer to 25 mg/kg cyclsporin A (positive control).

[0079] Figure 38 depicts qRTPCR analysis of inflammatory cytokines and chemokines expression. RNA from whole proximal and distal colon was analysed and an increased expression of cytokines (IL6, IL17) and chemokines (MIP1a and MIP2) related to inflammation was detected in DSS treated mice, while this response was counteracted by CsA (positive control) and CT352 administration at the same time.

[0080] Figure 39 depicts histological analysis of colon samples. The extension and the degree of the colitis were determined in blind and the score assigned to each samples reported in the colitis clinical score graph (a). A representative sample image from each group was reported (4X magnification) (b).

[0081] Figure 40 depicts the BioMAP profile of SNA-352 in the Diversity PLUS Panel. The X-axis lists the quantitative protein-based biomarker readouts measured in each system. The Y-axis represents a log-transformed ratio of the biomarker readouts for the drug-treated sample (n = 1) over vehicle controls (n ≥ 6). The grey region around the Y-axis represents the 95% significance envelope generated from historical vehicle controls. Biomarker activities are annotated when 2 or more consecutive concentrations change in the same direction relative to vehicle controls, are outside of the significance envelope, and have at least one concentration with an effect size > 20% (|log10 ratio| > 0.1). Biomarker key activities are described as modulated if these activities increase in some systems, but decrease in others. Cytotoxicity is indicated on the profile plot by a thin black arrow above the X-axis, and antiproliferative effects are indicated by a thick grey arrow. Cytotoxicity and antiproliferative arrows only require one concentration to meet the indicated threshold for profile annotation. Other BioMAP profiles disclosed herein are also depicted in a similar manner.

[0082] Figure 41 depicts a Reference Benchmark Overlay of SNA-352 and Benchmark Cyclosporin A. Common biomarker readouts are annotated when the readout for both profiles is outside of the significance envelope with an effect size > 20% (|log10 ratio| > 0.1) in the same direction.

[0083] Figure 42 depicts the changes in secretion of (a) IL-17F (b) IgG, (c) IL-17A, and (d) TNFα in the BioMAP BT system mediated by SNA-352 (3.9 μM), Tofacitinib (3.3 μM), Apremilast (3.3 μM), SR2211 (3.3 μM), and Cyclosporin A (3.3 μM).

[0084] Figure 43 depicts an overlay of SNA-352 (3.9 μM) and Deferoxamine Mesylate (4.4 μM), which was the top similarity match from a search of the BioMAP Reference Database of > 4,000 agents. Common biomarker readouts are annotated

when the readout for both profiles is outside of the significance envelope with an effect size > 20% (|log10 ratio| > 0.1) in the same direction. Similarity search results are filtered and ranked. Profiles are identified as having mechanistically relevant similarity if the Pearson's correlation coefficient is ≥ 0.7.

[0085] Figure 44 depicts Mechanism HeatMAP Analysis for SNA-352. HeatMAP analysis of the 148 biomarker readouts (rows) within the Diversity PLUS panel by SNA-352 in comparison to 19 consensus mechanism class profiles (columns). Horizontal grey lines separate the 12 Diversity PLUS systems, while the vertical grey line separates SNA-352 from the 19 consensus mechanism profiles. Biomarker activities outside of the significance envelope are red if protein levels are increased, blue if protein levels are decreased and white if levels are within the envelope or unchanged. Darker shades of color represent greater change in biomarker activity relative to vehicle control.

[0086] Figure 45 depicts clustering of test agent profiles following pairwise correlation analysis and clustering of the most similar profiles. Each colored circle represents the BioMAP profile of a compound at a specific concentration, with larger circles representing higher concentrations.

[0087] Figure 46 depicts the (A) colon dissection diagram and (B) fields and scoring order employed in the oxazolone-induced colitis mouse study.

[0088] Figure 47 depicts the effect of SNA-125, SNA-352, tofacitinib, and prednisolone on the body weight of animals challenged with oxazolone. Compounds or vehicle controls were dosed BID as indicated intracecally (IC) or orally (PO). Figure 47A depicts percent body weight change from Day -1 to Day 4 of the study. Figure 47B depicts the area under the curve (AUC) of the percent weight change depicted in Figure 47A.

[0089] Figure 48 depicts the effect of SNA-125, SNA-352, tofacitinib, and prednisolone on the body weight of animals challenged with oxazolone according to last observation carried forward analysis. Compounds or vehicle controls were dosed BID as indicated intracecally (IC) or orally (PO). Figure 48A depicts percent body weight change from Day -1 to Day 4 of the study. Figure 48B depicts the area under the curve (AUC) of the percent weight change depicted in Figure 48A.

[0090] Figure 49 depicts the effect of SNA-125, SNA-352, tofacitinib, and prednisolone on the Day 2 endoscopy score of animals challenged with oxazolone by (A) bar chart and (B) dot plot. Compounds or vehicle controls were dosed BID as indicated intracecally (IC) or orally (PO).

[0091] Figure 50 depicts the effect of SNA-125, SNA-352, tofacitinib, and prednisolone on the Day 2 stool consistency score of animals challenged with oxazolone

by (A) bar chart and (B) dot plot. Compounds or vehicle controls were dosed BID as indicated intracecally (IC) or orally (PO).

[0092] Figure 51 depicts the effect of SNA-125, SNA-352, tofacitinib, and prednisolone on the Day 4 endoscopy score of animals challenged with oxazolone by (A) bar chart and (B) dot plot. Compounds or vehicle controls were dosed BID as indicated intracecally (IC) or orally (PO).

[0093] Figure 52 depicts the effect of SNA-125, SNA-352, tofacitinib, and prednisolone on the Day 4 stool consistency score of animals challenged with oxazolone by (A) bar chart and (B) dot plot. Compounds or vehicle controls were dosed BID as indicated intracecally (IC) or orally (PO).

[0094] Figure 53 depicts the effect of SNA-125, SNA-352, tofacitinib, and prednisolone on the disease activity index (DAI) score of animals at (A) Day 2 and (B) Day 4 following challenge with oxazolone. Compounds or vehicle controls were dosed BID as indicated intracecally (IC) or orally (PO).

[0095] Figure 54 depicts the effect of SNA-125, SNA-352, tofacitinib, and prednisolone on the colon weight/length ratio of animals challenged with oxazolone. Compounds or vehicle controls were dosed BID as indicated intracecally (IC) or orally (PO).

[0096] Figure 55 depicts the effect of SNA-125, SNA-352, tofacitinib, and prednisolone on the colon inflammation histopathology scores of animals challenged with oxazolone. Compounds or vehicle controls were dosed BID as indicated intracecally (IC) or orally (PO). Group means with standard error of the mean (SEM) bars are depicted.

[0097] Figure 56 depicts the effect of SNA-125, SNA-352, tofacitinib, and prednisolone on the colon edema histopathology scores of animals challenged with oxazolone. Compounds or vehicle controls were dosed BID as indicated intracecally (IC) or orally (PO). Group means with standard error of the mean (SEM) bars are depicted.

[0098] Figure 57 depicts the effect of SNA-125, SNA-352, tofacitinib, and prednisolone on the colon mucosal necrosis/loss histopathology scores of animals challenged with oxazolone. Compounds or vehicle controls were dosed BID as indicated intracecally (IC) or orally (PO). Group means with standard error of the mean (SEM) bars are depicted.

[0099] Figure 58 depicts the effect of SNA-125, SNA-352, tofacitinib, and prednisolone on the summed colon histopathology scores of animals challenged with oxazolone. Compounds or vehicle controls were dosed BID as indicated intracecally (IC) or orally (PO). Group means with standard error of the mean (SEM) bars are depicted.

[0100] Figure 59 depicts representative control animal H&E-stained colon histopathology micrographs at 40x and 100x magnifications.

[0101] Figure 60 depicts representative H&E-stained colon histopathology micrographs at 40x and 100x magnifications for animals administered BID (A) Vehicle PO, (B) 15 mg/kg Tofacitinib PO, (C) 1 mg/kg Prednisolone PO, (D) 400 mg/kg SNA-125 PO, and (E) 400 mg/kg SNA-352 PO. Moderate inflammation (unfilled black arrows), edema (filled red arrows) and multifocal ulceration (brackets) are indicated.

[0102] Figure 61 depicts representative H&E-stained colon histopathology micrographs at 40x and 100x magnifications for animals administered BID (A) Vehicle IC, (B) 1 mg/kg Tofacitinib IC, (C) 400 mg/kg SNA-125 IC, and (D) 400 mg/kg SNA-352 IC.

[0103] Figure 62 depicts the effect of SNA-125, SNA-352, tofacitinib, and prednisolone on the protein levels of IFNγ in colon tissue homogenate supernatants of animals following challenge with oxazolone. Compounds or vehicle controls were dosed BID as indicated intracecally (IC) or orally (PO). Group means with standard error of the mean (SEM) bars are depicted.

[0104] Figure 63 depicts the effect of SNA-125, SNA-352, tofacitinib, and prednisolone on the protein levels of TNFα in colon tissue homogenate supernatants of animals following challenge with oxazolone. Compounds or vehicle controls were dosed BID as indicated intracecally (IC) or orally (PO). Group means with standard error of the mean (SEM) bars are depicted, with outliers removed (A), or present (B).

[0105] Figure 64 depicts the effect of SNA-125, SNA-352, tofacitinib, and prednisolone on the protein levels of IL-6 in colon tissue homogenate supernatants of animals following challenge with oxazolone. Compounds or vehicle controls were dosed BID as indicated intracecally (IC) or orally (PO). Group means with standard error of the mean (SEM) bars are depicted, with outliers removed (A), or present (B).

[0106] Figure 65 depicts the effect of SNA-125, SNA-352, tofacitinib, and prednisolone on the protein levels of IL-10 in colon tissue homogenate supernatants of animals following challenge with oxazolone. Compounds or vehicle controls were dosed BID as indicated intracecally (IC) or orally (PO). Group means with standard error of the mean (SEM) bars are depicted, with outliers removed (A), or present (B).

[0107] Figure 66 depicts the SNA-352 kinase inhibition profile at test concentrations of 100nM and 200nM for the top inhibited kinases as well as those kinases in the middle in the inhibition spectrum.

[0108] Figure 67 depicts a schematic showing how the IMQ-induced psoriasis study was performed.

[0109] Figure 68 depicts the total psoriasis clinical scores over time for all groups (A), the SNA-101 group (B), the SNA-125 group (C), and the SNA-352 group (D). The mean score for each group is displayed for each day +/- SEM.

[0110] Figure 69 depicts the erythema scores over time for all groups (A), the SNA-101 group (B), the SNA-125 group (C), and the SNA-352 group (D). The mean score for each group is displayed for each day +/- SEM.

[0111] Figure 70 depicts the plaque scores over time for all groups (A), the SNA-101 group (B), the SNA-125 group (C), and the SNA-352 group (D). The mean score for each group is displayed for each day +/- SEM.

[0112] Figure 71 depicts the punctate redness/scabbing scores over time for all groups (A), the SNA-101 group (B), the SNA-125 group (C), and the SNA-352 group (D). The mean score for each group is displayed for each day +/- SEM.

[0113] Figure 72A depicts the weight of spleens upon experimental termination on day 10. Mean spleen weight for each group is displayed +/- SEM. Figure 72B depicts left ear thickness as measured with a caliper on days 0, 4, 6, 8, and 10. Mean thickness for each group is displayed for each day +/- SEM. Figure 72C depicts the daily weight of mice. Body weight changes are displayed for each day as a percent of their weight measured on day 0. Mean values for each group are displayed +/- SEM.

[0114] Figure 73 depicts the levels of IL-17F (A), TNF-α (B), IL-22 (C), and IL-17A (D) as measured in left ears biopunched on day 4. After tissue homogenization, the cytokine levels in tissue lysates were measured via multiplex and then normalized with total protein amounts. Mean values for each group are displayed +/- SEM.

[0115] Figure 74 depicts a schematic of the IL-23-induced psoriasis mouse model study.

[0116] Figure 75 depicts the effect SNA-120 and SNA-325 in an IL-23-induced psoriasis mouse model. Figure 75A depicts the total psoriasis clinical scores for each group over time. The mean score for each group is displayed for each day +/- SEM. Figure 75B depicts the right ear thickness of each group at the indicated time points. Mean thickness for each group is displayed for each day +/- SEM. Figure 75C the depicts body weight of each group over the course of the study. Body weight changes are displayed for each day as a percent of their weight measured on day 0. Mean values for each group are displayed +/- SEM.

[0117] Figure 76 depicts the inhibition of VEGF-induced proliferation following treatment with SNA-125 (A), SNA-352 (B), SNA-103 (C), and motesanib diphosphate (D). Data are presented as mean corrected counts per minute (CCPM) ± SEM, with n=6 for (A)-(C) and with n=4 for (D).

[0118] Figure 77 depicts the concentration vs. %Inhibition curves of staurosporine (A) and SNA-352 (B) against LIMK1. The calculated slope and IC50(M) are also depicted).

[0119] Figure 78 depicts the concentration vs. %Inhibition curves of staurosporine (A) and SNA-352 (B) against MAP2K6. The calculated slope and IC50(M) are also depicted).

[0120] Figure 79 depicts the concentration vs. %Inhibition curves of staurosporine (A) and SNA-352 (B) against MLK1. The calculated slope and IC50(M) are also depicted).

[0121] Figure 80 depicts the concentration vs. %Inhibition curves of staurosporine (A) and SNA-352 (B) against MLK3. The calculated slope and IC50(M) are also depicted).

[0122] Figure 81 depicts representative Day 2 endoscopy images of naïve control, vehicle control (PO), vehicle control (IC), and tofacitinib (15 mg/kg PO) animals. Animals underwent video endoscopy on Day 2 and colitis severity was scored on a scale of 0-4. Images were captured from each animal during the procedure and representative images from each treatment group are presented.

[0123] Figure 82 depicts representative Day 2 endoscopy images of tofacitinib (15 mg/kg IC), prednisolone (1mg/kg PO), SNA-125 (400 mg/kg PO), and SNA-352 (400mg/kg PO) animals. Animals underwent video endoscopy on Day 2 and colitis severity was scored on a scale of 0-4. Images were captured from each animal during the procedure and representative images from each treatment group are presented.

[0124] Figure 83 depicts representative Day 2 endoscopy images of SNA-125 (400 mg/kg IC) and SNA352 (400 mg/kg IC) animals. Animals underwent video endoscopy on Day 2 and colitis severity was scored on a scale of 0-4. n=8-15 per group. Images were captured from each animal during the procedure and representative images from each treatment group are presented.

[0125] Figure 84 depicts representative Day 4 endoscopy images of naïve control, vehicle control (PO), vehicle control (IC), and tofacitinib (15 mg/kg PO) animals. Animals underwent video endoscopy on Day 4 and colitis severity was scored on a scale of 0-4. n=8-15 per group . Images were captured from each animal during the procedure and representative images from each treatment group are presented.

[0126] Figure 85 depicts representative Day 4 endoscopy images of tofacitinib (15 mg/kg IC), prednisolone (1mg/kg PO), SNA-125 (400 mg/kg PO), and SNA-352 (400mg/kg PO) animals. Animals underwent video endoscopy on Day 4 and colitis severity was scored on a scale of 0-4. n=8-15 per group. Images were captured from each animal during the procedure and representative images from each treatment group are presented.

[0127] Figure 86 depicts representative Day 4 endoscopy images of SNA-125 (400 mg/kg IC) and SNA352 (400 mg/kg IC) animals. Animals underwent video endoscopy on Day 4 and colitis severity was scored on a scale of 0-4. n=8-15 per group. Images were captured from each animal during the procedure and representative images from each treatment group are presented.

[0128] Figure 87 depicts the SNA-352 synthesis scheme.

[0129] Figure 88 depicts the synthesis of Intermediate 2 trifluoroacetate.

[0130] Figure 89 depicts the plausible structure of 311 Da Impurity.

[0131] Figure 90 depicts the formation of guanidine side-product

[0132] Figure 91 depicts HPLC analysis of crude SNA-352 lot S12.

[0133] Figure 92 depicts HPLC analysis of Staurosporine.

[0134] Figure 93 depicts HPLC analysis of reaction mixture/step 1/24h aging time.

[0135] Figure 94 depicts HPLC analysis of isolated Intermediate 1.

[0136] Figure 95 depicts HPLC analysis of reaction mixture/step 2/3h aging time.

[0137] Figure 96 depicts HPLC analysis of reaction mixture/step 2/24h aging time.

[0138] Figure 97 depicts HPLC analysis of isolated Intermediate 2 salt.

[0139] Figure 98 depicts HPLC analysis of reaction mixture/step 3/24h aging time.

[0140] Figure 99 depicts HPLC analysis of crude CT352.

[0141] Figure 100 depicts the UV profile (@292 and 210 nm) of SNA-352 purification by normal-phase flash chromatography.

[0142] Figure 101 depicts HPLC analysis of SNA-352/Lot A purified by normal-phase flash chromatography.

[0143] Figure 102 depicts HPLC analysis of SNA-352/Lot B purified by normal-phase flash chromatography.

[0144] Figure 103 depicts the UV profile (@292 and 210 nm) of SNA-352 purification by reverse-phase flash chromatography.

[0145] Figure 104 depicts the UV profile (@292 and 210 nm) of SNA-352 purification by reverse-phase flash chromatography.

[0146] Figure 105 depicts HPLC analysis of final SNA-352/ lot n° 2017CG14/S19.

[0147] Figure 106 depicts ^{1}H-NMR analysis of final SNA-352/ lot n° 2017CG14/S19.

[0148] Figure 107 depicts the certificate of analysis of final SNA-352 lot n° 2017CG14/S19.

[0149] Figure 108 depicts HPLC analysis of final SNA-352/ lot n° 2017GC14/S7.

[0150] Figure 109 depicts ^{1}H-NMR analysis of final SNA-352/ lot n° 2017GC14/S7.

[0151] Figure 110 depicts the certificate of analysis of final SNA-352 lot n° 2017CG14/S7.

[0152] Figure 111 depicts HPLC analysis of final SNA-352/ lot n° 2017CG14/S14.

[0153] Figure 112 depicts ^{1}H-NMR analysis of final SNA-352/ lot n° 2017CG14/S14.

[0154] Figure 113 depicts the certificate of analysis of final SNA-352 lot n° 2017CG14/S14.

[0155] Figure 114 depicts HPLC analysis of final SNA-352/ lot n° 2017CG14/S18.

[0156] Figure 115 depicts ^{1}H-NMR analysis of final SNA-352/ lot n° 2017CG14/S18.

[0157] Figure 116 depicts the certificate of analysis of final SNA-352 lot n° 2017CG14/S18.

DETAILED DESCRIPTION

Platform Technology

[0158] Several embodiments relate to the use of agents that were developed using Applicant's proprietary Low Systemic Exposure™ ("LSE™") platform technology to generate LSE molecules (also generally referred to herein as polymer conjugates or compositions). In several embodiments, the LSE platform creates polymer conjugates optimized for topical applications. In several embodiments, the polymer conjugates developed by LSE or more generally the reduced exposure technology exhibit enhanced penetration. In still further embodiments, the enhanced penetration leads to delivery of a high local concentration of the drug. In further embodiments, the polymer conjugates show a limited non-target absorption upon topical administration due to their increased molecular size and amphiphilicity and/or amphipathicity. In still further embodiments, side-effects are minimized by limiting or eliminating non-target (e.g., systemic) absorption.

[0159] In several embodiments of the reduced exposure compositions/compounds, the polymer conjugate comprises a "warhead" linked to a polymer. In some embodiments, the warhead is a pharmacologically active entity selected according to the particular target or pathway of interest. As discussed herein, there are also provided, in several embodiments, polymer conjugates for use in the treatment of conditions (including but not limited to inflammatory bowel diseases). In several embodiments, the polymer is directly coupled to the warhead without a separate chemical linking moiety between the polymer and the warhead; such direct coupling may involve without limitation ester, ether, acetal, ketal, vinyl ether, carbamate, urea, amine, amide, enamine, imine, oxime, amidine, iminoester, carbonate, orthoester, phosphonate, phosphinate, sulfonate, sulfinate, sulfide, sulfate, disulfide, sulfinamide, sulfonamide, thioester, aryl, silane, siloxane, heterocycles, thiocarbonate, thiocarbamate, and phosphonamide bonds. In several embodiments, the linker is a separate chemical linking moiety between the polymer and the warhead. In several embodiments, the polymer is polyethylene glycol (PEG), wherein the terminal OH group can optionally be modified e.g. with C1-C5 alkyl or C1-C5 acyl groups, e.g., with C1-, C2- or C3-alkyl groups or C1-, C2- or C3 groups. In several embodiments, the modified PEG is a terminally alkoxy-substituted PEG. In several embodiments, the modified PEG is a methoxy-PEG (mPEG). In some embodiments, the polymer has a molecular weight ranging from about 100 to about 100,000 Da. In some embodiments, the polymer is polydisperse with respect to molecular weight (e.g., has a distribution of molecular weights) and the indicated molecular weight of the polymer represents an average molecular weight. In other embodiments, the polymer has a molecular weight ranging from about 200 to about 50,000 Da. In several embodiments, the polymer has a molecular weight ranging from about 500 to about 10,000 Da (e.g., 500-1000, 1000-2000, 2000-3000, 3000-5000,5000-7000, 7000-10,000 Da, and overlapping ranges therein).

[0160] In several embodiments, the polymer is a short-chain PEG, and in some embodiments a terminally alkoxy-substituted PEG, such as a mPEG with a molecular weight ranging from about 200 to about 4,000 Da, from about 400 to about 3,000 Da, from about 500 to about 2,000 Da, from about 700 to about 3,000 Da, from about 900 to about 4,000 Da, or from about 1,000 to about 5,000 Da. In several embodiments, the short-chain PEG or mPEG has an average molecular weight of about 1,000-3,000 Da. (e.g., 2,000 Da).

[0161] In some embodiments, the polymer is a long-chain PEG. The long-chain PEG may be a terminally alkoxy-substituted PEG, such as methoxy-substituted PEG, with a molecular weight ranging greater than about 4,000 Da. In several

embodiments, the molecular weight ranges from about 4,500-10,000Da (e.g., 4,500 to about 5,500 Da). In several embodiments, the long-chain PEG or mPEG has an average molecular weight of about 2,000 Da or of about 5,000 Da. In several embodiments, the polymer is of natural or semi-synthetic or synthetic origin. In several embodiments, the polymer has a linear or branched structure. In several embodiments, the polymer is selected from poly(alkylene oxides) or from (polyethylene) oxides. In several embodiments, the polymer selected may include, without limitation, one or more of the following: polyacrylic acid, polyacrylates, polyacrylamide or N-alkyl derivatives thereof, polymethacrylic acid, polymethacrylates, polyethylacrylic acid, polyethylacrylates, polyvinylpyrrolidone, poly(vinylalcohol), polyglycolic acid, polylactic acid, poly(lactic-co-glycolic) acid, dextran, chitosan, and hydroxyethyl starch.

[0162] In an embodiment, the polymer conjugates provided herein are administered to the skin by topical application.

[0163] In one embodiment, active agents useful for stimulating hair follicles (for hair growth) are provided as oral applications or topical applications for the scalp. Hair removal agents and ant-acne agents are provided in other embodiments. Hair growth, hair removal and anti-acne therapies can all involve active agents that, if exposed to the non-target site (e.g., systemic circulation and/or lymphatic system) for long periods, result in toxicity or undesired side effects. Thus, the reduced exposure compositions described herein provides benefits for these applications as well.

[0164] In alternative embodiments, the polymer conjugates configured for reduced exposure are administered to other areas of the body besides the skin. For example, in one embodiment, administration comprises treatment of the lung and respiratory conditions via inhalation of the polymer conjugates. Eye drops are provided in some embodiments to treat eye inflammation or ophthalmic disorders and diseases. Treatment to the joints to treat inflammation or other joint conditions is also provided. In yet another embodiment, administration comprises treatment of the gastro-intestinal tract via, for example, an enteric coated capsule comprising the polymer conjugates taken orally. Reduced exposure provides benefits in these applications. Applications for the nose and ear, such as inhalants, ointments and drops are provided in several embodiments. Treatment to the nasal passage to treat allergies or allergic rhinitis is also provided. Vaginal and rectal compounds are provided in some embodiments, including as suppositories, creams, ointments, etc. In one embodiment, the polymer conjugates provided herein treat inflammatory bowel diseases.

[0165] In some embodiments, conjugating the warhead to a polymer (e.g., PEG) in the disclosed molecular weight ranges may slow diffusion of the molecule in the tissue, thereby potentially increasing residence time of the molecule in the target tissue,

e.g. epidermis and dermis for skin, associated epithelial and sub-epithelial layers in other topical surfaces like gut, eye, mucosa, lungs etc. This "depot" effect may also lead to lower concentrations needing to be applied or for products to be applied with lower frequency, or both.

[0166] In other embodiments, conjugating the warhead to a polymer (e.g., PEG) in the disclosed molecular weight ranges may be useful in reducing the diffusion or extravasation of the molecule out of the circulatory system after it enters it via injection and or diffusion from the target tissue. Indeed, changes in the tissue distribution of polymer conjugates compared to unconjugated drug have been observed in IV injection studies. In general, the unconjugated drug tends to have a long half-life and a volume of distribution within tissues AND blood, suggesting that the unconjugated drug extravasates out of the blood vessels into the tissue prior to being cleared. Whereas, in some instances, the PEGylated drug has a volume of distribution that is largely restricted to the blood, indicating that very little extravasation occurs with the polymer conjugates prior to being renally cleared. This reduced extravasation may explain at least in part the observed shorter half-life for the polymer conjugates.

[0167] The compositions described herein may be combined with other modalities to achieve synergic effects. These other modalities include, but are not limited to, energy delivery (such as laser, radiofrequency, ultrasound, microwave, etc.), thermal therapy, light therapy, radiation, intravenous chemotherapy, and others. In some embodiments, the compositions are applied with pressure, heat, massage etc. to facilitate localization to the desired target site. In some embodiments, the compositions are administered in combination with one or more additional therapeutics that may not be reduced exposure compounds.

[0168] Santi et al. state that "permanent PEGylation is generally not applicable to small-molecule drugs because the bulky carrier usually prevents their binding to targets and cell penetration." (Proceedings of the National Academy of Sciences 109.16 (2012): 6211-6216). Further, Nakagami et al. state that "hydrophilic polymers on the surface of particles…prevents the close interactions between particles and target cell membranes, inhibiting the cellular uptake and, subsequently, preventing endosomal escape. All of these factors combine to decrease the biological efficacy of PEGylated particles" (Gene therapy (2013): 2-4). In several embodiments, the polymer conjugate exhibits unexpected permeability across the plasma membrane. In several embodiments, the polymer conjugate exhibits unexpected permeability across the nuclear membrane. In several embodiments, the polymer conjugate exhibits unexpected permeability across both the nuclear and plasma membranes.

[0169] The reduced exposure compounds, comprising a hydrophobic drug conjugated to a short chain PEG, exhibit surprising accessibility across cellular compartments, compared to the unconjugated drug. This accessibility is thought to result for the amphipathic nature of the conjugate, allowing it to traverse and distribute evenly among both lipophilic and hydrophilic cellular compartments. Accordingly, the conjugate can cross and reside within the lipid bilayer of the cell membrane, accumulate within the cytosol, and even traverse the nuclear envelope – thereby providing access both membrane, cytosolic and nuclear molecular targets. This property of the reduced exposure compounds result in excellent depo'ing, longer residence times within target cells, and relative non-compartmentalization. Consequently, these compounds are biologically active at lower concentrations and require less frequent dosing – thereby reducing potential drug toxicity.

Polymer Conjugates Targeting Mediators of Inflammatory Bowel Diseases

[0170] In several embodiments, the warhead employed in the LSE polymer conjugate is a small molecule targeting mediator(s) of inflammatory bowel diseases. There is also provided, in several embodiments, methods of treating an inflammatory bowel disease in a subject, the method comprising administering to the subject an effective amount of a polymer conjugate, wherein the warhead is a small molecule targeting mediator(s) of inflammatory bowel diseases. Non-limiting examples of inflammatory bowel diseases include Crohn's disease, ulcerative colitis, collagenous colitis, lymphocytic colitis, ischemic colitis, diversion colitis, Behçet's syndrome, and indeterminate colitis. In several embodiments, JAK and/or STAT family proteins are mediator(s) of inflammatory bowel diseases. In several embodiments, the warhead employed in the LSE polymer conjugate is a small molecule a targeting a JAK and/or STAT family protein.

[0171] The JAK kinase family is a cytoplasmic protein kinase family comprising the members JAK1, JAK2, JAK3 and TYK2. Various studies suggest that ligand binding to a receptor leads to receptor dimerization or oligomerization, which leads to JAK recruitment and activation either through autophosphorylation or phosphorylation by other JAK kinases or by other tyrosine kinases, which in turn leads to tyrosine phosphorylation of the receptors as well as downstream substrates of JAK. Growth factor or cytokine receptors that recruit JAK kinases include the interferon receptors, interleukin receptors (receptors for the cytokines IL-2 to IL-7, IL-9 to IL-13, IL-15, IL-23), various hormone receptors (erythropoietin (Epo) receptor, the thrombopoietin (Tpo) receptor, the leptin receptor, the insulin receptor, the prolactin (PRL) receptor, the

Granulocyte Colony-Stimulating Factor (G-CSF) receptor and the growth hormone receptor), receptor protein tyrosine kinases (such as EGFR and PDGFR), and receptors for other growth factors such as leukemia inhibitory factor (LIF), Oncastatin M (OSM), IFNα/β/γ, Granulocyte-macrophage colony-stimulating factor (GM-CSF), Ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-I) (See, Rane, S.G. and Reddy E.P., Oncogene 2000 19, 5662- 5679).

[0172] Many autoimmune diseases and disease associated with chronic inflammation, as well as acute responses, have been linked to excessive or unregulated production or activity of one or more cytokines, the signaling of which depend on JAK kinases. Such diseases include rheumatoid arthritis (RA) such as moderate to severe RA, systemic lupus erythematosus (SLE), multiple sclerosis (MS), Crohn's disease such as moderate to severe Crohn's disease, psoriasis such as moderate to severe chronic plaque psoriasis, ulcerative colitis such as moderate to severe ulcerative colitis, ankylosing spondilytis (AS), psoriatic arthritis, Juvenile Idiopathic Arthritis (JIA) such as moderate to severe polyarticular JIA, systemic lupus erythematosus (SLE), diabetic nephropathy, dry eye syndrome, Sjogren's Syndrome, alopecia areata, vitiligo, or atopic dermatitis.

[0173] Phosphorylated receptors serve as docking sites for other SH-2 domain containing signaling molecules that interact with JAKs such as the STAT family of transcription factors, Src family of kinases, MAP kinases PB kinase and protein tyrosine phosphatases (Rane S.G. and Reddy E.P., Oncogene 2000 19, 5662-5679). The family of latent cytoplasmic transcription factors, STATS, are the most well characterized downstream substrates for JAKs. The STAT proteins bind to phosphorylated cytokine receptors through their SH2 domains to become phosphorylated by JAKs, which event leads to their dimerization and release and eventual translocation to the nucleus where they activate gene transcription. The various members of STAT which have been identified thus far, are STAT1, STAT2, STAT3, STAT4, STAT5 (including STAT5a and STAT5b) and STAT6.

[0174] Signal transducer and activator of transcription 3 (STAT3), a member of the STAT protein family, is a transcription factor that regulates the expression of a variety of genes involved in many cellular processes such as cell growth, apoptosis, cell motility, and cytokine production. In response to cytokines and growth factors, STAT3 is activated by JAK kinases and translocates to the nucleus to act as a transcriptional activator. Studies have demonstrated that STAT3 plays a role in various immune disorders including the pathogenesis of inflammatory bowel disease (see, e.g., Sugimoto, World J. Gastroenterol., 14:5110-5114, (2008)).

[0175] Inflammatory bowel diseases (IBD) are diseases characterized by inflammation in the small intestine and colon. IBD is known to include two common autoimmune diseases in humans—Crohn's disease and ulcerative colitis—which share many of the same physiological, mechanistic, immune, inflammatory and genetic features, as well as common treatment strategies (such as TNF withdrawal therapy). Histopathologically and anatomically, these two conditions are distinct, with Crohn's disease characterized by transmural inflammation that can occur throughout the GI tract, and ulcerative colitis characterized by more superficial inflammation confined to the colon and rectum.

[0176] There is provided, in several embodiments, methods of treating an inflammatory bowel disease in a subject, the method comprising administering to the subject an effective amount of a polymer conjugate, wherein the warhead is a small molecule targeting mediator(s) of inflammatory bowel diseases. In several embodiments, the warhead employed in the LSE polymer conjugate is a small molecule targeting a JAK and/or STAT family protein. Non-limiting examples of inflammatory bowel diseases include Crohn's disease, ulcerative colitis, collagenous colitis, lymphocytic colitis, ischemic colitis, diversion colitis, Behçet's syndrome, and indeterminate colitis.

[0177] Compositions comprising compounds Nos 1-40 shown in Table 1 are used, in several embodiments, as inhibitors, antagonists, and inverse agonists of the mediator(s) of inflammatory bowel diseases disclosed herein. In several embodiments, compositions comprising compounds Nos 1-40 shown in Table 1 are used as inhibitors, antagonists, and inverse agonists of JAK and/or STAT family proteins. In some embodiments, the warhead of the polymer conjugate is compound 1. In some embodiments, the LSE polymer conjugate is CT352.

[0178] A growing body of research suggests that dry eye is the result of an underlying cytokine and receptor-mediated inflammatory process. There is provided, in several embodiments, methods of treating dry eye in a subject, the method comprising administering to the subject an effective amount of a polymer conjugate, wherein the warhead is a small molecule targeting a JAK and/or STAT family protein. In some embodiments the composition is formulated as an eye drop. In some embodiments, one or two drops of the composition are used per application. In other embodiments, three or four drops of the composition are used per application. In additional embodiments, six drops of the composition are used per application. In some embodiments, the composition is applied for a period of 60 seconds before flushing. In other embodiments, the composition is applied for a period of 120 seconds before flushing. In additional embodiments, the composition is applied for a period of 360 seconds before flushing. In

some embodiments, the composition may be administered one or more times a day. In some embodiments, the composition is administered daily. In some embodiments, the composition may be administered once a week.

[0179] In some embodiments, alopecia is treated. Non-limiting examples include androgenic alopecia and alopecia areata. Androgenic alopecia (also known as hereditary baldness, male pattern baldness, and seborrheic alopecia) is a non-scarring hair loss of telogen hairs caused by an excessive androgen effect in genetically susceptible men and women. Alopecia areata is known to be associated with autoimmune activities; hence, topically administered immunomodulatory compounds demonstrate efficacy for treating that type of hair loss.

[0180] There is provided, in several embodiments, methods of treating an alopecia in a subject, the method comprising administering to the subject an effective amount of a polymer conjugate, wherein the warhead is a small molecule targeting a JAK and/or STAT family protein. In some embodiments, hair regeneration compositions are in the form of a liquid. In other embodiments, hair regeneration compositions are in the form of a lotion. In additional embodiments, hair regeneration compositions are in the form of a cream. In some embodiments, hair regeneration compositions are in the form of a gel. In other embodiments, the hair regeneration composition is administered twice daily. In other embodiments, the hair regeneration composition is administered one daily. In additional embodiments, the hair regeneration composition is administered once weekly. In some embodiments, the hair regeneration composition is administered directly to the scalp. In some embodiments, the hair regeneration composition is administered directly non-scalp areas.

[0181] Allergic inflammatory diseases are characterized by an immune response against a sensitizing agent, such as an allergen, resulting in the release of inflammatory mediators that recruit cells involved in inflammation in a subject, potentially leading to tissue damage and sometimes death. Allergic inflammatory diseases of the eye, skin, upper and lower airways, and gastrointestinal tract, lung, including, but not limited to, atopic dermatitis, atopic keratoconjunctivitis, allergic conjunctivitis, asthma, and allergic rhinitis. There is provided, in several embodiments, methods of treating an allergic inflammatory disease in a subject, the method comprising administering to the subject an effective amount of a polymer conjugate, wherein the warhead is a small molecule targeting a JAK and/or STAT family protein.

[0182] There is also provided, in several embodiments, methods of treating the following conditions in a subject, the method comprising administering to the subject an effective amount of a polymer conjugate, wherein the warhead is a small molecule targeting a JAK and/or STAT family protein: nail dystrophy; seborrheic keratosis;

androgenic alopecia; contact dermatitis; actinic keratosis; acne; asthma; eczema (atopic derm); onychomycosis; sinusitis; allergic rhinitis; rosacea; COPD; pruritus; early AMD; urticaria; diabetic retinopathy; psoriasis; alopecia areata; dry eye; vitiligo; glaucoma; late AMD; ulcerative colitis; Crohn's disease; ocular rosacea; hair growth and cycling; skin neoplasias; squamous cell carcinoma; basal cell carcinoma; malignant melanoma; malignant cutaneous lymphomas; vascular tumors; angiosarcoma; kaposi's sarcoma; infantile hemangiomas; hemangioendothelioma; inflammatory dermatoses; dermatitis (atopic, contact); psoriasis; keloids; rosacea; bullous diseases; bullous pemphigoid; erythema multiforme; UV irradiation therapy; age-related macular degeneration; diabetic retinopathy; macular and corneal edema.

[0183] There is also provided, in several embodiments, methods of treating a respiratory disease in a subject via delivery of the polymer conjugates (wherein the warhead is a small molecule targeting a JAK and/or STAT family protein) to the lungs and/or airways. Delivery routes may include for example intratracheal instillation or inhalation. The formulation may include liquids, nebulized or aerosolized liquids or suspensions, dry powder, nanocomposites, nanoparticles or microparticles, etc. Respiratory disorders, include treatable obstructive, restrictive or inflammatory airways diseases of whatever type, etiology, or pathogenesis. Non-limiting examples of respiratory conditions include: acute bronchitis; acute laryngotracheal bronchitis; arachidic bronchitis; catarrhal bronchitis; croupus bronchitis; dry bronchitis; infectious asthmatic bronchitis; productive bronchitis; staphylococcus or streptococcal bronchitis; vesicular bronchitis; cylindric bronchiectasis; sacculated bronchiectasis; fusiform bronchiectasis; capillary bronchiectasis; cystic bronchiectasis; dry bronchiectasis; follicular bronchiectasis; chronic obstructive pulmonary disease (COPD), chronic obstructive lung disease (COLD), chronic obstructive airways disease (COAD) or small airways obstruction of whatever type, etiology, or pathogenesis, in particular chronic bronchitis, pulmonary emphysema, bronchiectasis, cystic fibrosis, bronchiolitis obliterans, organizing pneumonia (BOOP), chronic organizing pneumonia (COP), bronchiolitis fibrosa obliterans, follicular bronchiolitis or dyspnea associated therewith; cough of whatever type, etiology, or pathogenesis in particular idiopathic cough or cough associated with gastro-esophageal reflux disease (GERD), drugs, bronchial hyper-responsivity, asthma, COPD, COLD, COAD, bronchitis, bronchiectasis, pulmonary eosinophilic syndromes, pneumoconiosis, interstitial lung disease, pulmonary fibrosis, aspiration disorders, rhinitis, laryngitis or pharyngitis; pulmonary eosinophilic syndromes of whatever type, etiology, or pathogenesis, in particular acute eosinophilic pneumonia (idiopathic or due to drugs or parasites), simple pulmonary eosinophilia, Loeffler's syndrome, tropical pulmonary eosinophilia, chronic eosinophilic pneumonia, allergic

bronchopulmonary mycosis, allergic bronchopulmonary aspergillosis (ABPA), Churg-Strauss syndrome or idiopathic hypereosinophilic syndrome; asthma of whatever type, etiology, or pathogenesis, in particular asthma that is a member selected from the group consisting of atopic asthma, non-atopic asthma, allergic asthma, atopic bronchial IgE-mediated asthma, bronchial asthma, essential asthma, true asthma, intrinsic asthma caused by pathophysiologic disturbances, extrinsic asthma caused by environmental factors, essential asthma of unknown or inapparent cause, non-atopic asthma, bronchitic asthma, emphysematous asthma, exercise-induced asthma, allergen induced asthma, cold air induced asthma, occupational asthma, infective asthma caused by bacterial, fungal, protozoal, or viral infection, non-allergic asthma, incipient asthma and wheezy infant syndrome; alveolar hemorrhage of whatever type, etiology, or pathogenesis, in particular a member of the group consisting of idiopathic pulmonary hemosiderosis, alveolar hemorrhage due to drugs or other exogenous agents, alveolar hemorrhage associated with HIV or bone marrow transplant or autoimmune alveolar hemorrhage (e.g. associated with systemic lupus erythematosis, Goodpasture's syndrome, Wegener's granulomatosis, microscopic polyangiitis, Churg-Strauss syndrome, pauci-immune glomerulonephritis); pneumoconiosis of whatever type, etiology, or pathogenesis, in particular pneumoconiosis that is a member selected from the group consisting of aluminosis or bauxite workers' disease, anthracosis or miners' asthma, asbestosis or steam-fitters' asthma, chalicosis or flint disease, ptilosis caused by inhaling the dust from ostrich feathers, siderosis caused by the inhalation of iron particles, silicosis or grinders' disease, byssinosis or cotton-dust asthma and talc pneumoconiosis; interstitial lung diseases (ILD) or pulmonary fibrosis of whatever type, etiology, or pathogenesis, in particular idiopathic pulmonary fibrosis, crytogenic fibrosing alveolitis, fibrosing alveolitis, ILD or pulmonary fibrosis associated with connective tissue disease (systemic lupus erythematosis, mixed connective tissue disease, polymyositis, dermatomyositis, Sjórgen's syndrome, systemic sclerosis, scleroderma, rheumatoid arthritis), usual interstitial pneumonia (UIP), desquamative interstitial pneumonia (DIP), granulomatous lung disease, sarcoidosis, Wegener's granulomatosis, histiocytosis X, Langerhan's cell granulomatosis, hypersensitivity pneumonitis, extrinsic allergic alveolitis, silicosis, chronic eosinophilic pneumonia, lymphangiolyomatosis, drug-induced ILD or pulmonary fibrosis, radiation-induced ILD or pulmonary fibrosis, alveolar proteinosis, graft-versus-host-disease (GVHD), lung transplant rejection, ILD or pulmonary fibrosis due to environmental/occupational exposure, BOOP, COP, bronchiolitis fibrosa obliterans, follicular bronchiolitis, idiopathic acute interstitial pneumonitis (Hamman Rich syndrome) or alveolar hemorrhage syndromes; seasonal allergic rhinitis or perennial allergic rhinitis or sinusitis of whatever type, etiology, or

pathogenesis, in particular sinusitis that is a member selected from the group consisting of purulent or nonpurulent sinusitis, acute or chronic sinusitis and ethmoid, frontal, maxillary, or sphenoid sinusitis; Acute Respiratory Distress Syndrome (ARDS), adult respiratory distress syndrome or acute lung injury of whatever type, etiology, or pathogenesis; progressive massive fibrosis (PMF); pulmonary hypertension of whatever type, etiology or pathogenesis including primary pulmonary hypertension, essential hypertension, pulmonary hypertension secondary to congestive heart failure, pulmonary hypertension secondary to COPD, pulmonary venous hypertension, pulmonary arterial hypertension and hypoxia-induced pulmonary hypertension. Respiratory disorders also include, in some embodiments, malignancies and tumors of the respiratory system, non-limiting examples of which include lung adenocarcinoma, squamous cell carcinoma, large cell carcinoma, bronchioloalveolar carcinoma (BAC), pulmonary adenocarcinoma (AIS), non-small-cell carcinoma, small cell carcinoma, and mesothelioma.

[0184] Several embodiments relate to polymer conjugates of compounds 1-40, optimized for oral delivery to treat the gastrointestinal system while also minimizing side-effects caused by exposure at non-target sites (e.g., systemic absorption). Topical applications are provided in other embodiments.

[0185] In several embodiments, the warhead of the polymer conjugate is a small molecule disclosed in Table 1 targeting mediator(s) of inflammatory bowel diseases. There is also provided, in several embodiments, methods of treating an inflammatory bowel disease in a subject, the method comprising administering to the subject an effective amount of an LSE polymer conjugate wherein the warhead is a small molecule disclosed in Table 1 targeting mediator(s) of inflammatory bowel diseases. In several embodiments, JAK and/or STAT family proteins are mediator(s) of inflammatory bowel diseases. In several embodiments, the warhead employed in the LSE polymer conjugate is a small molecule targeting a JAK and/or STAT family protein. In some embodiments, the warhead of the polymer conjugate is compound 1. In some embodiments, the LSE polymer conjugate is CT352.

[0186] In some embodiments, for those compounds Nos 1-40 having one amino group, the compound is modified (e.g., PEGylated) at that location (e.g., a PEG or modified PEG is linked to the compound by reaction with the amino group). If two or more amino groups are present, either location is PEGylated in some embodiments. In other embodiments, the amino group located the furthest away from the moieties interacting with the target is used. In some embodiments, the amino group that causes the least hindrance on activity is used (whether or not it is located the furthest way from the moieties interacting with the target). The effect of conjugation on the activity of the compound can be determined based on various methods, such as bioassays, mass

spectroscopy, surface plasmon resonance, in vivo assays, clinical assays, and predictive in silico modeling programs.

[0187] In some embodiments, for those compounds Nos 1-40 having one sulfhydryl group, the compound is modified (e.g., PEGylated) at that location. If two or more sulfhydryl groups are present, either location is PEGylated in some embodiments. In other embodiments, the sulfhydryl group located the furthest away from the moieties interacting with the target is used. In some embodiments, the sulfhydryl group that causes the least hindrance on activity is used (whether or not it is located the furthest way from the moieties interacting with the target).

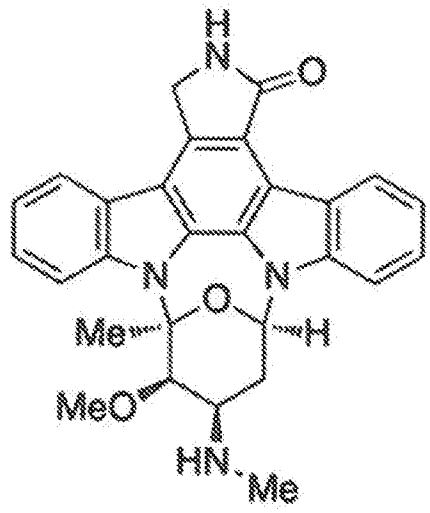
[0188] In some embodiments, for those compounds Nos 1-40 having one hydroxyl group, the compound is modified (e.g., PEGylated) at that location. If two or more hydroxyl groups are present, either location is PEGylated in some embodiments. In other embodiments, the hydroxyl group located the furthest away from the moieties interacting with the target is used. In some embodiments, the hydroxyl group that causes the least hindrance on activity is used (whether or not it is located the furthest way from the moieties interacting with the target).

[0189] In some embodiments, for those compounds Nos 1-40 having one carboxyl group, the compound is modified (e.g., PEGylated) at that location. If two or more carboxyl groups are present, either location is PEGylated in some embodiments. In other embodiments, the carboxyl group located the furthest away from the moieties interacting with the target is used. In some embodiments, the carboxyl group that causes the least hindrance on activity is used (whether or not it is located the furthest way from the moieties interacting with the target).

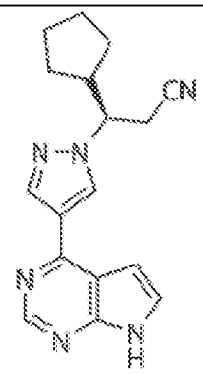
[0190] In some embodiments, for those compounds Nos 1-40 having two or more carboxyl, hydroxyl, amino and/or sulfhydryl groups, the compound is modified (e.g., PEGylated) at the site furthest away from the active site. In some embodiments, the site that causes the least hindrance on activity is used (whether or not it is located the furthest way from the moieties interacting with the target).

[0191] Methods for conjugating the PEG or modified PEG to the small molecule warheads in Table 1, through reaction between functional groups (or functionalized groups), including reaction with the above-mentioned functional groups (amino, sulfhydryl, hydroxyl, carboxyl) are used in several embodiments. Methods of conjugation can be found for example in "Bioconjugate Techniques" (3rd Edition) 2013 by Greg T. Hermanson (http://www.sciencedirect.com/science/book/9780123822390); incorporated herein in its entirety by reference. Although PEGylation is used as an example, other polymers are used in some embodiments.

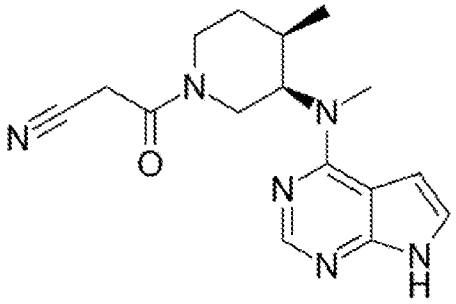
TABLE 1



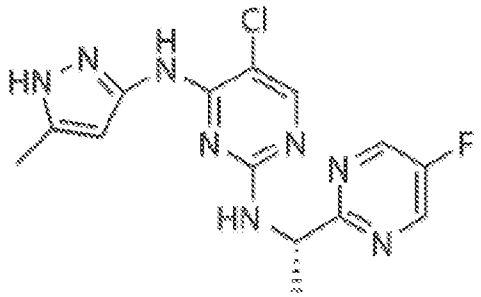
(1)



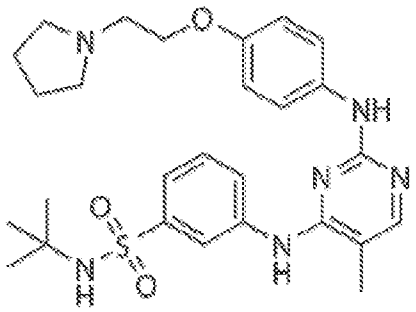
(2)



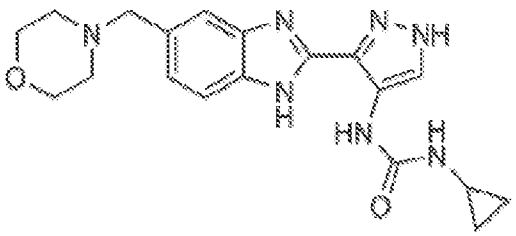
(3)



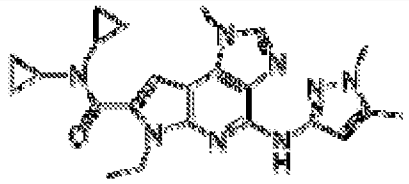
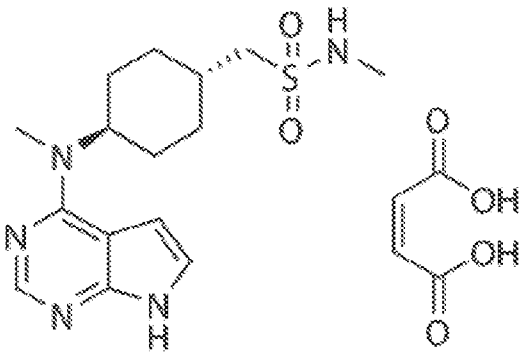
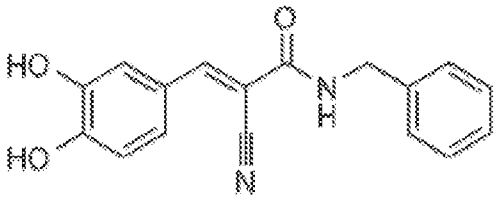
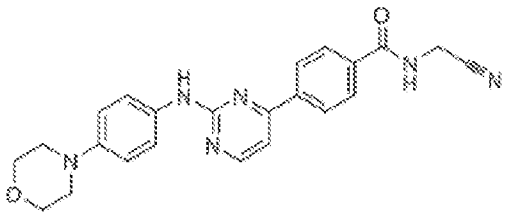
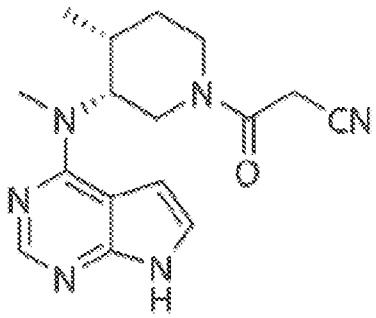
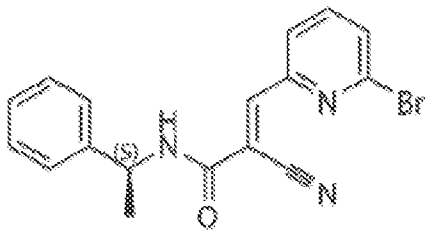
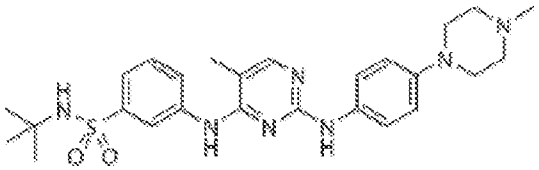
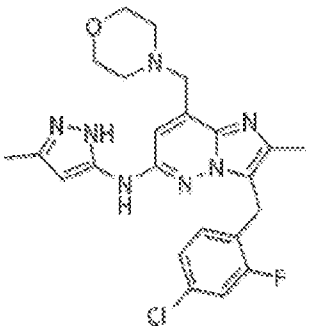
(4)

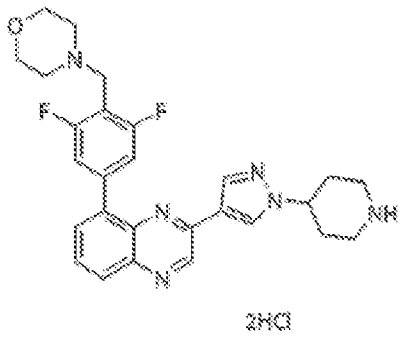


(5)

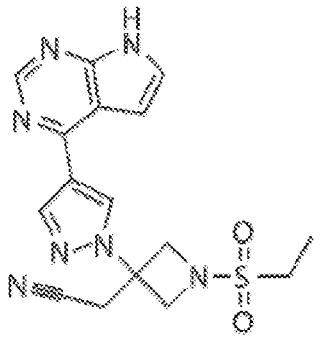


(6)

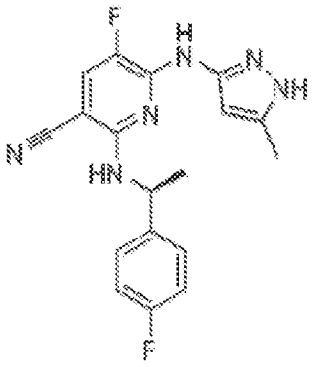
 (7)	 (8)
 (9)	 (10)
 (11)	 (12)
 (13)	 (14)



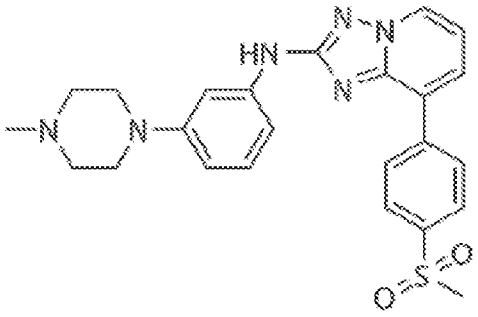
(15)



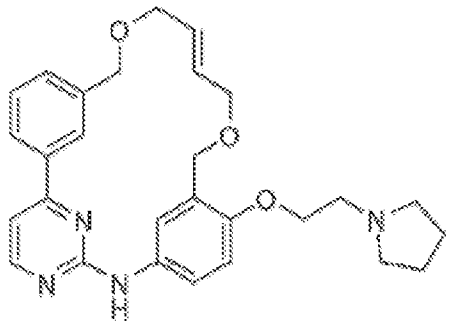
(16)



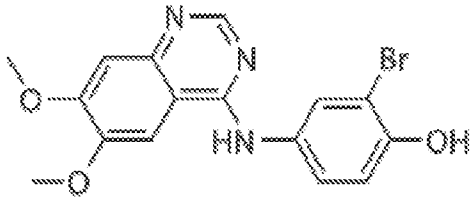
(17)



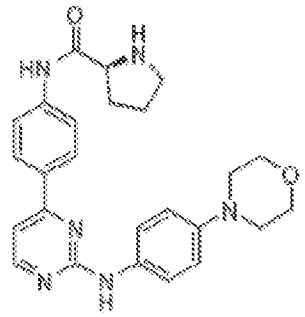
(18)



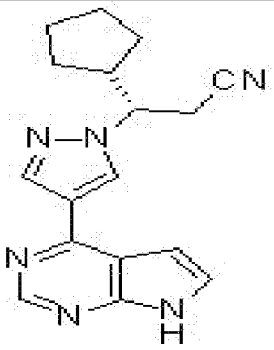
(19)



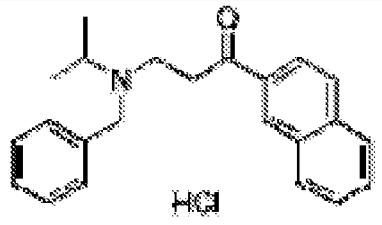
(20)



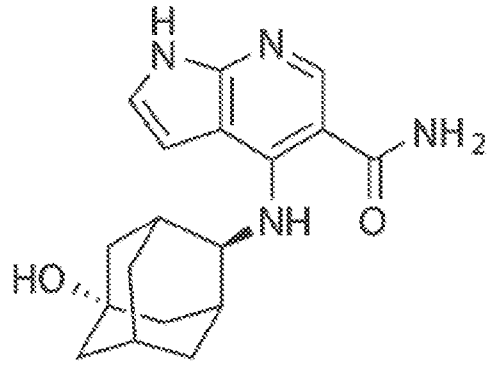
(21)



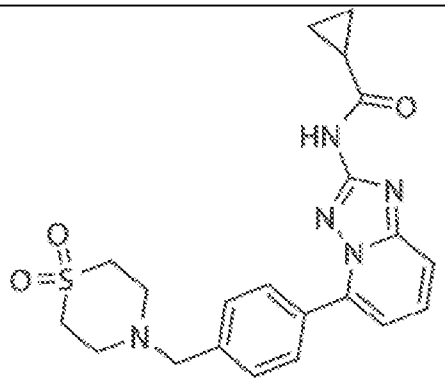
(22)



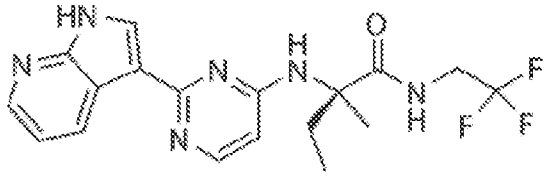
(23)



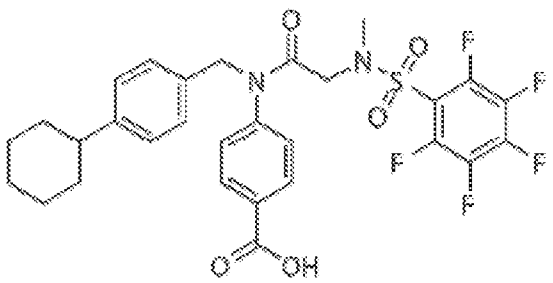
(24)



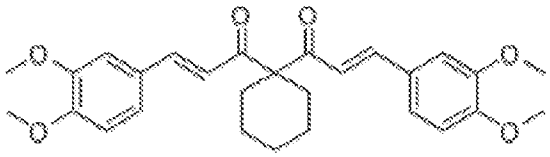
(25)



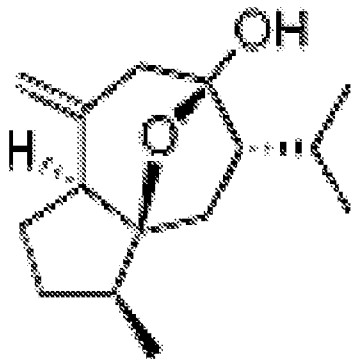
(26)



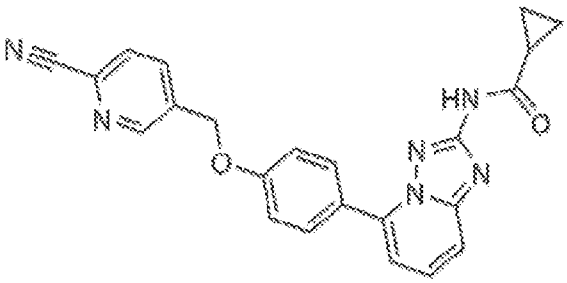
(27)



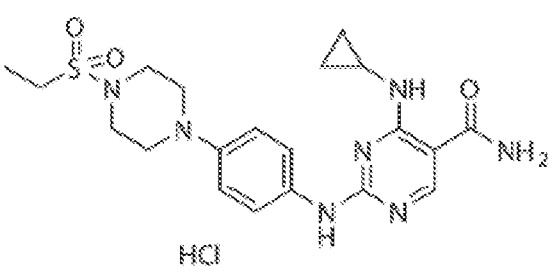
(28)



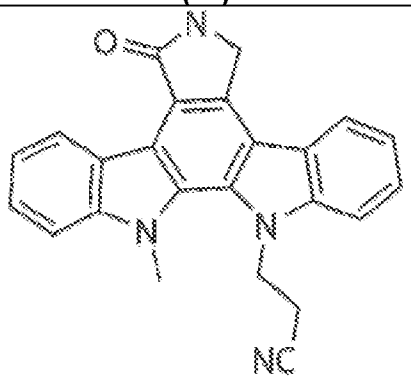
(29)



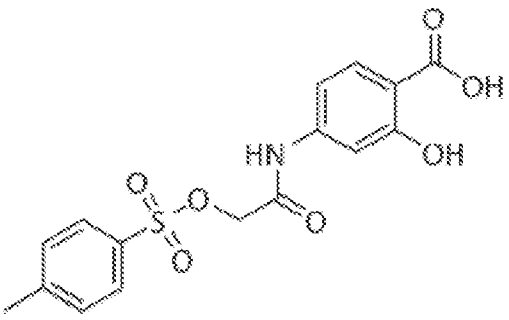
(30)



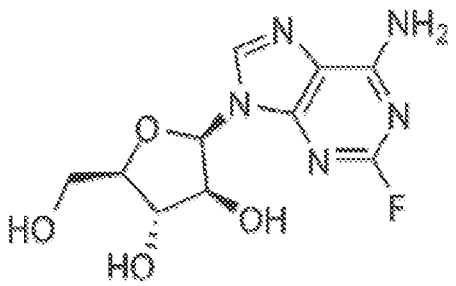
(31)



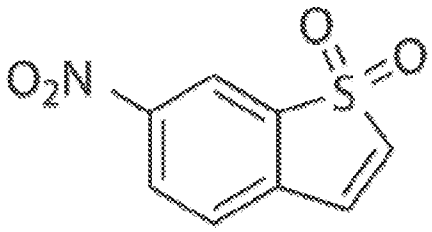
(32)



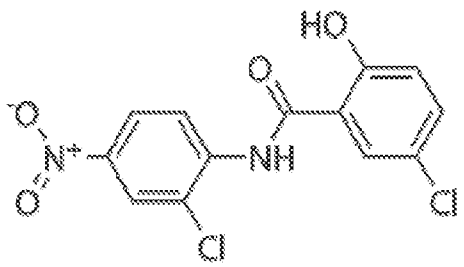
(33)



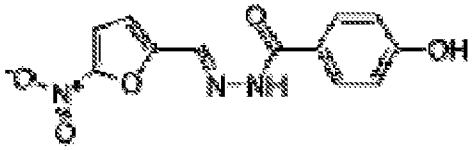
(34)



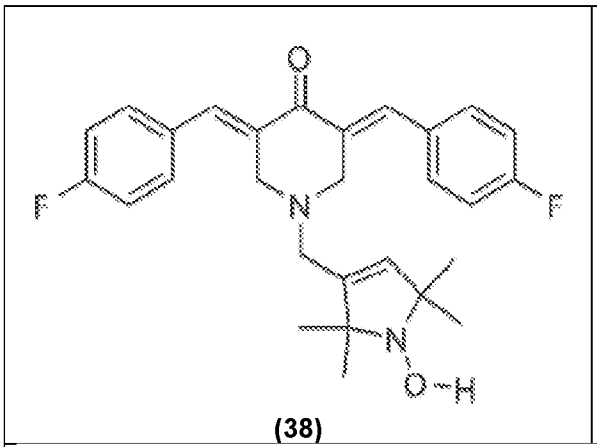
(35)



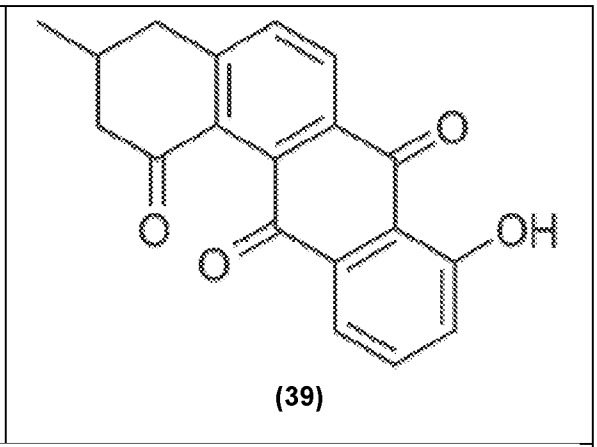
(36)



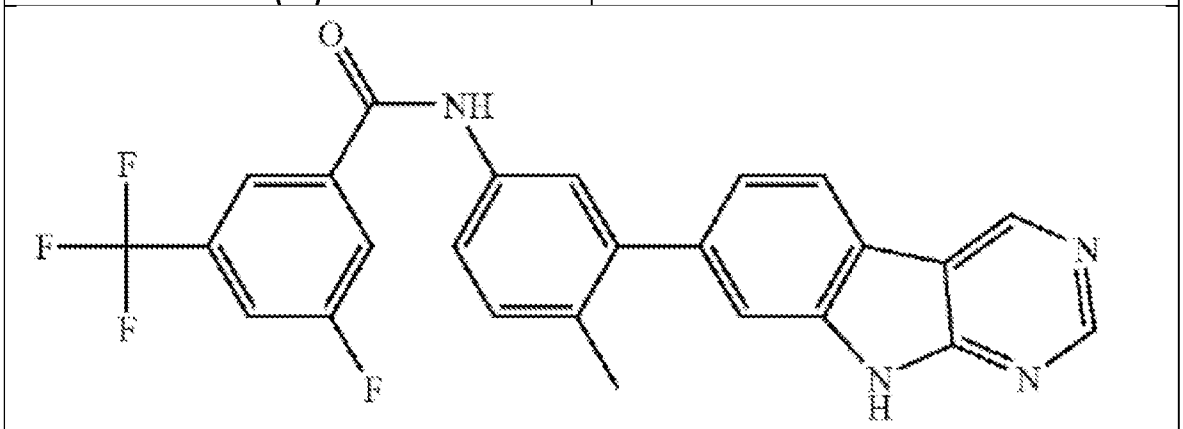
(37)



(38)



(39)



(40)

[0192] Non-limiting examples of conjugation sites according to some embodiments and chemistries for compounds in Table 1 are disclosed. For example, for structures 27 and 33 of Table 1, the existing carboxylic moiety (-COOH) could be conjugated to PEG-amine through formation of an amide bond using any one of several possible coupling agents (including, e.g., TBTU, HBTU, HOBt, DCC, and N-hydroxysuccinimide). Further, for example, for structures 1, 15, 21, and 34 of Table 1, the existing amino group (-NH2) could be conjugated to PEG-COOH through formation of an amide bond using any one of several possible coupling agents (including, e.g., TBTU, HBTU, HOBt, DCC, and N-hydroxysuccinimide). Further, for example, for structures 9, 20, 24, 29, 33, 34, 36, 37, 38, and 39 of Table 1, the existing hydroxyl moiety (-OH) could be conjugated to PEG-halide through formation of an ether bond in presence of a strong base (including, e.g. NaH, KH, and n-BuLi). These conjugation sites and chemistries are neither exhaustive nor limiting, and are included herein as examples only, and not intended to limit the scope of the embodiments described herein.

[0193] Identifying a conjugation site and developing a conjugation strategy and/or chemistry does not require that all the atoms and the structures of the starting compound are maintained. Once the active part of the compound has been identified or

hypothesized, some atoms, groups and structures of the compound can be removed or modified while maintaining sufficient or similar target site binding and activity in several embodiments.

[0194] In alternative embodiments, the warhead employed in the LSE polymer conjugate is a small molecule targeting a JAK and/or STAT family protein selected from one or more of the following: ruxolitinib; fedratinib; tofacitinib; baricitinib; pacritinib; decernotinib; xl019; azd1480; incb039110; ly2784544; bms911543; ns018; glpg0634; glpg0788; n-(cyanomethyl)-4-2-(4-morpholinoanilino)pyrimidin-4-yl)benzamide; cucurbitacin i, niclosamide, cryptotanshinone, sd 1008, stat3 inhibitor iii, wp1066, nifuroxazide, stat3 inhibitor, stattic, stat3 inhibitor, s3i-201; stat3 inhibitor viii, 5,15-dpp, 2-hydroxy-4-(((4-methylphenyl)sulfonyloxy)acetyl)amino)-benzoic acid (nsc74859); kahweol; pyrrolo[2,3-d]pyrimidine compounds; pyrrolopyridines; 2, 6-diamino-pyrimidin- 5-yl-carboxamides; deazapurines; quinazoline derivatives; benzisoxazole derivatives; 4-aryl-2-amino-pyrimidines or 4-aryl-2-aminoalkyl-pyrimidines; deazapurines; azaindoles; thiopyrimidine-based compounds; pyrrolo[2,3-d]pyrimidine compounds; 5-(2-aminopyrimidin-4-yl) benzisoxazoles; benzimidazo[4,5-f]isoquinolinone derivatives; phenyl amino pyrimidine compounds; macrocyclic compounds; 5, 7-substituted-imidazo [1, 2-c] pyrimidines; heterocyclyl pyrazolopyrimidine analogues; triazolopyridine jak inhibitor compounds; pyrazolopyrimidine jak inhibitor compounds; 2,4-pyrimidinediamine derivatives; n-(hetero)aryl-pyrrolidine derivatives of pyrazol-4-yl-pyrrolo[2,3-d]pyrimidines; pyrrol-3-yl-pyrrolo[2,3-d]pyrimidines; heterocyclic compounds; triazolopyridine jak inhibitor compounds; thiadiazoles; oxadiazoles; 3-[4-(7h-pyrrolo[2,3-d]pyrimidin-4-yl)-1h-pyrazol-1-yl]octane- or heptane-nitrile; retrometabolic compounds; pyrrolo[2,3-d] pyrimidine compounds; pyrrolo[2,3-d]pyrimidine compounds; tricyclic heteroaryl compounds; n-containing heteroaryl derivatives; pyrazole derivatives; pyridine derivatives; isoquinoline derivatives; azaindoles; piperidine inhibitors; imidazo [1,2-b] pyridazine and imidazo [4,5-b] pyridine derivatives; pyrrole six-membered heteroaryl ring derivative; pias molecules; imidazole-4, 5-dicarboxamide derivatives; heteroaryl imidazolone derivatives; macrocyclic compounds; peptide inhibitors; bicyclic diamines; tricyclic carbamate; heterocyclyl pyrazolopyrimidine analogues; ortho substituted pyrimidine compounds; pyrrolo [2, 3 - d] pyrimidine urea compounds; tricyclic lactones; heterocyclyl pyrimidine analogues; substituted 2-hydroxy-4-(2-(phenylsulfonamido)acetamido)benzoic acid analogs; phenyl amino pyrimidine bicyclic compounds; pyrimidine-2,4-diamine derivatives; geminally substituted cyanoethylpyrazolo pyridones; pyrrolo [2, 3 -d]pyrimidine derivatives; pyrazolo[4,3-c]pyridine derivatives; pyridin-2(1h)-one derivatives; substituted 2-(9h-purin-9-yl) acetic acid analogues; cycloalkyl nitrile pyrazolo pyridones; 5,7-substituted-imidazo[1,2-

c]pyrimidines; substituted pyrimidinyl kinase inhibitors; bicyclic oxa-lactam kinase inhibitors; n-(2-cyano heterocyclyl)pyrazolo pyridones; pyrazolo[4,3-c]pyridine derivatives (7h-pyrrolo[2,3-d]pyrimidin-2-yl)amine compounds; acyclic cyanoethylpyrazolo pyridones; acyclic cyanoethylpyrazoles; dihydropyrrolonaphtyridinone compounds; triazolopyridine compounds; azaindoles; pyrazolopyrimidin-2-yl derivatives; indazole derivatives; 4,6-substituted-pyrazolo[1,5-a]pyrazines; n-cyanomethylamides; 5-chloro-2-difluoromethoxyphenyl pyrazolopyrimidine compounds; naphthyridine compounds; sulfamide piperazine derivatives; sh2 stat3/stat1 peptidomimetices; 2-(pyrazolopyridin-3-yl)pyrimidine derivatives; 7h-pyrrolo[2, 3-d]pyrimidine derivatives; substituted n-(pyrrolidine-3-yl)-7h-pyrrolo[2,3-d]pyrimidine-4-amine compounds; nicotinamides; triazolopyridine compounds; aminopyrimidinyl compounds; ethyl n-boc piperidinyl pyrazolo pyridones; pyrrolo[2,3-d]pyrimidine derivatives; imidazolopyrimidin-2-yl derivatives; homopiperazine derivatives; 4-substituted pyrrolo[2,3-d]pyrimidine compounds; five-and-six-membered heterocyclic compounds; pyrrolo[2,3-d]pyrimidinyl, pyrrolo[2,3-b]pyrazinyl and pyrrolo[2,3-d]pyridinyl acrylamides and analogues; monoacetyldiglyceride compounds; and 2-methoxy-4-(3-(4-methoxyphenyl)prop-1-en-1-yl)phenol and derivatives.

[0195] Suitable protecting groups, in some embodiments, are for protecting functional groups during the conjugation of warhead and polymer. Various protecting groups as well as suitable means and conditions for protecting and deprotecting the substituents are used in several embodiments. The means and conditions of protecting and deprotecting employed depend on the nature of the involved functional groups. Protecting groups for hydroxy-, amino-, and/or carboxy residues are selected in several embodiments from acetonide, ethylidene methoxymethyl, 2-methoxyethoxymethyl, benzyloxymethyl, tetrahydropyranyl, methyl, ethyl, isopropyl, t-butyl, benzyl, triphenylmethyl, t-butyldimethylsilyl, triphenylsilyl, methoxycarbonyl, t-butyloxycarbonyl, benzyloxycarbonyl, fluorenylmethoxycarbonyl, acetyl, benzoyl, toluenesulfonyl, dimethoxybenzyl, nitrophenyloxycarbonyl, nitrobenzyloxycarbonyl, allyl, fluorenylmethyl, tetrahydrofuranyl, phenacyl, acetol, phenyl, trimethylsilyl, pyrrolidyl, indolyl, hydrazino and other protecting groups such as those that can be found in Greene T. W., et al., Protective Groups in Organic Synthesis, 4th ed., John Wiley and Son, New York, N.Y. (2007); incorporated herein in its entirety be reference. The reagents and conditions of protecting and deprotecting reactions are in particular selected for their suitability at selectively attaching and removing the protecting group without adversely affecting the rest of the compound.

[0196] The polymer conjugates disclosed herein may also be prepared as pharmaceutically acceptable salts including salts of inorganic acids such as hydrochloric,

hydroiodic, hydrobromic, phosphoric, metaphosphoric, nitric acid and sulfuric acids as well as salts of organic acids, such as tartaric, acetic, citric, malic, benzoic, glycolic, gluconic, succinic, aryl sulfonic, (e.g., p-toluene sulfonic acids, benzenesulfonic), phosphoric, malonic, and the like. Suitable acids for formation of pharmaceutically acceptable salts are used in some embodiments. Further, pharmaceutically acceptable salts of compounds may be formed with a pharmaceutically acceptable cation. Pharmaceutically acceptable cations include, but are not limited to, alkali cations (Li+, Na+, K+), earth alkali cations (Mg2+, Ca2+, Ba2+), ammonium and organic cations, such as quaternary ammonium cations.

Synthesis of Polymer Conjugates

[0197] The description above is not intended to be limiting and should be viewed as an example to guide the manufacture of the other compounds identified herein. The polymer conjugates may also be made as described in US Patent Nos. 8,673,347 and 8,926,955, both herein incorporated by reference. Several embodiments provide a method for the production of polymer conjugates of the active agents that result in a highly pure reaction product, obtained in high and consistent yields.

[0198] In one embodiment, the conjugation reaction of the process to synthesize a conjugate polymer compound is catalysed by a base in an organic solvent. The base may be a strong base. In one embodiment, the base is selected from the group of alkali metal hydrides, tertiary amines and/or alkoxide. In another embodiment, the base catalysing the polymer conjugation reaction is sodium hydride. Other bases, such as sodium methoxide, or triethylamine can also be used. In several embodiments, the molar ratio of the base catalyst to the compound is between about 1:1 and about 4:1, about 1:1 to about 1.5:1 and about 1:1. The reaction may be carried out in an organic solvent, such as in anhydrous conditions (e.g., in a dry organic solvent). The water content in the solution mixture of the conjugation process may be equal or less than 200 ppm. The organic solvent may be selected from the group of dichloromethane, chloroform, N,N-dimethylformamide. In certain embodiments, the organic solvent is dichloromethane or anhydrous dichloromethane.

[0199] The conjugation reaction may be carried out under inert gas atmosphere, such as nitrogen or argon atmosphere. The reaction of the process may be carried out at a temperature of about −10° to about 60° C, about 0° to about 25° C or at room temperature after an initial step at 0° C.

[0200] Following the production of the target compound, the polymer conjugate may then be separated and purified from the reaction mixture. In one embodiment, the compound is obtained by purification of the crude mixture by flash

chromatography. An automated gradient flash purification system may be used and may be equipped with a suitable column and solvent. The purification method may be selected from reverse phase and direct phase columns and the conditioning/elution solvent may be selected from dichloromethane, water, methanol, acetonitrile, ammonium formate buffer solution at different mixture ratios. In one embodiment, the compound is purified by a reverse phase flash chromatography equipped with a C18 cartridge and the purification is carried out by isocratic elution with acetonitrile/5 mM ammonium formate buffer (pH 3.5) 40:60. In one embodiment, the compound is purified by a normal phase flash chromatography.

[0201] The product may then be dried e.g. over sodium sulphate and filtered off and the solvent is removed by evaporation under reduced pressure at 25° C. Purification of the target product is carried out in several embodiments. After the purification step the resultant polymer compound has a purity of at least about 95%, about 96%, about 97%, about 98%, about 98.5%, about 99% or about 99.5%. The disclosed process results in an overall mass yield of the compound from about 40% to about 98% by weight, or from about 50% to about 95% by weight based on the weight of a reactant compound.

[0202] In several embodiments, the polymer moiety which is covalently attached to the active entity is biocompatible, can be of natural or semi-synthetic or synthetic origin and can have a linear or branched structure. The polymer may be selected from poly(alkylene oxides), or from (polyethylene) oxides. However, other polymers include without limitation polyacrylic acid, polyacrylates, polyacrylamide or N-alkyl derivatives thereof, polymethacrylic acid, polymethacrylates, polyethylacrylic acid, polyethylacrylates, polyvinylpyrrolidone, poly(vinylalcohol), polyglycolic acid, polylactic acid, poly(lactic-co-glycolic) acid, dextran, chitosan, hydroxyethyl starch.

[0203] In some embodiments, the above-mentioned polymer moiety can carry an amino functional end-group or can be functionalized to carry an amino functional end-group. Hence, the polymer moiety can be an amino-activated polymer of general formula X—NH2.

[0204] The reaction of formation of the compositions identified herein may be carried out at a temperature of about 10° to about 60° C., about 15° to about 25° C. or at room temperature. The polymer moiety X may be a polyethylene glycol (PEG) moiety, wherein the terminal OH group can optionally be modified e.g. with C1-C5 alkyl or C1-C5 acyl groups, such as with C1-, C2- or C3-alkyl groups or C1-, C2- or C3 groups. The modified polyethylene glycol may be a terminally alkoxy-substituted polyethylene glycol, including a methoxy-polyethylene-glycol (mPEG).

[0205] In other aspects, the conjugated polymer compounds may be used as active agents in a topical medicament useful for the prevention, alleviation and/or treatment of dermal pathologies. It has been shown that the conjugated polymer compounds described herein are very advantageously used as topical medicament since they do not show adverse or toxic effects (e.g. irritation) when dermally administered or any phototoxic effect (e.g. photomutagenicity, phototoxicity or photosensitisation) (as shown in the studies described in the following examples).

[0206] The dermal pathologies for such treatment may be pathologies characterized by hyperproliferation of the keratinocytes, such as psoriasis, atopic dermatitis, chronic eczema, acne, pitiriasis rubra pilaris, keloids, hypertrophic scars and skin tumors, such as keratoacanthoma, squamous cell carcinoma, basal cell carcinoma.

[0207] The compounds disclosed herein or pharmaceutically acceptable salts thereof can be administered as they are, or in the form of various pharmaceutical compositions according to the pharmacological activity and the purpose of administration. Yet another aspect is a pharmaceutical composition comprising an effective amount of at least one compound in Table 1 optionally together with pharmaceutically acceptable carriers, adjuvants, diluents or/and additives. Pharmaceutical carriers, adjuvants, diluents or/and additives are applied in the formulation of the pharmaceutical composition comprising a compound of embodiments identified herein.

[0208] The disclosed compounds can be employed as the sole active agent in a pharmaceutical composition. Alternatively, the compounds of Table 1 may be used in combination with one or several further active agents, e.g. other active pharmaceutical agents in the treatment of the conditions described herein.

[0209] In particular, the polymer conjugate compounds may be used in combination with at least one additional anti-IBD therapeutic agent, for example and not restricted to azathioprine, 6-mercaptopurine (6-MP), aminosalicylate, sulfasalazine, mesalamine, corticosteroid, prednisone, prednisone equivalent, budesonide, probiotic, methotrexate, cyclosporine, tacrolimus, metronidazole, ciprofloxacin, leflunomide, chloroquine, hydroxychloroquine, penicillamine, tocilzumab, anakinra, abatacept, rituximab, efalizumab, belimumab, tofacitinib, baricitinib, golimumab, vedolizumab, natalizumab, ustekinumab, etanercept, infliximab, adalimumab, certolizumab pegol

[0210] In particular, the polymer conjugate compounds may be used in combination with at least one steroidal anti-inflammatory drug and/or one further agent capable of inhibiting an early mediator of the inflammatory cytokine cascade, e.g. an antagonist or inhibitor of a cytokine selected from the group consisting of TNF, IL-1α, IL-1β, IL-Ra, IL-8, MIP-1α, MIF-1β, MIP-2, MIF and IL-6. Particularly useful anti-

inflammatory drugs are selected from alclometasone dipropionate, amcinonide, beclomethasone dipropionate, betamethasone, betamethasone benzoate, betamethasone dipropionate, betamethasone sodium phosphate, betamethasone sodium phosphate and acetate, betamethasone valerate, clobetasol butyrate, clobetasol propinate, clocortolone pivalate, cortisol (hydrocortisone), cortisol (hydrocortisone) acetate, cortisol (hydrocortisone) butyrate, cortisol (hydrocortisone) cypionate, cortisol (hydrocortisone) sodium phosphate, cortisol (hydrocortisone) sodium succinate, cortisol (hydrocortisone) valerate, cortisone acetate, desonide, desoximetasone, dexamethasone, dexamethasone acetate, dexamethasone sodium phosphate, diflorasone diacetate, diflucortolone valerate, fludrocortisone acetate, fludroxycortide, flumetasone pivalate, flunisolide, fluocinolone acetonide, fluocinonide, fluocortolone, fluorometholone, flurandrenolide, fluticasone propionate, halcinonide, halobetasol propionate, medrysone, methylprednisolone, methylprednisolone acetate, methylprednisolone sodium succinate, mometasone furoate, paramethasone acetate, prednisolone, prednisolone acetate, prednisolone sodium phosphate, prednisolone tebutate, prednisone, triamcinolone, triamcinolone acetate, triamcinolone acetonide, triamcinolone diacetate, triamcinolone hexacetonide. Useful antagonists or inhibitors of a cytokine are selected from infliximab, etanercept or adalimumab.

[0211] Further agents which can be used in combination with the polymer compounds are e.g. antagonists and/or inhibitors of RAGE, antagonists and/or inhibitors of HMGB1, antagonists and/or inhibitors of the interaction of a Toll-like receptor (TCR) with HMGB1, the functional N-terminal lectin-like domain (D1) of thrombomodulin and/or a synthetic double-stranded nucleic acid or nucleic acid analogue molecule with a bent shape structure as described in the international patent application WO 2006/002971 which is herein incorporated by reference.

[0212] The compositions described herein may be administered by a physician or other professional. Patients may also be able to self-administer. In several embodiments, administration of the composition may be performed dermally, via, for example, ointments, creams, oils, liposomes or trans-dermal patches, or wherein the polymer conjugates are incorporated into liposomes.

[0213] In some embodiments, at least one excipient is provided. Excipients can include a nonaqueous or aqueous carrier, and one or more agents selected from moisturizing agents, pH adjusting agents, strontium ions (Sr2+), deodorants, fragrances, chelating agents, preservatives, emulsifiers, thickeners, solubilizing agents, penetration enhancers, anti-irritants, colorants, surfactants, beneficial agents, pharmaceutical agents, and other components for use in connection with the compositions described herein (such as topical compositions for treatment of the skin). In several embodiments,

the composition is an anhydrous formulation to prevent skin irritation such as water-based irritant contact dermatitis or stinging sensation upon application to damaged skin. In another embodiment, the composition is formulated such that preservatives need not be employed (e.g., a preservative-free formulation) so as to avoid skin irritation associated with certain preservatives.

[0214] To facilitate application, the composition may be provided as an ointment, an oil, a lotion, a paste, a powder, a gel, or a cream. The composition may also include additional ingredients such as a protective agent, an emollient, an astringent, a humectant, a sun screening agent, a sun tanning agent, a UV absorbing agent, an antibiotic agent, an antifungal agent, an antiviral agent, an antiprotozoal agent, an anti-acne agent, an anesthetic agent, a steroidal anti-inflammatory agent, a non-steroidal anti-inflammatory agent, an antipruritic agent, an additional antioxidant agent, a chemotherapeutic agent, an anti-histamine agent, a vitamin or vitamin complex, a hormone, an anti-dandruff agent, an anti-wrinkle agent, an anti-skin atrophy agent, a skin whitening agent, a cleansing agent, additional peptides, additional modified peptides, and combinations thereof. In a further embodiment, the composition may avoid irritants (such as animal or cellular-based materials) to avoid skin irritation.

[0215] In some embodiments, the compositions may be administered by injection or infusion, in particular by intravenous, intramuscular, transmucosal, subcutaneous or intraperitoneal injection or infusion and/or by oral, topical, dermal, nasal, inhalation, aerosol and/or rectal application, etc.

[0216] In a further embodiment, the compositions are administered reversibly immobilized on the surface of a medical device, in particular by binding, coating and/or embedding the compositions on a medical device, such as but not limited to, stents, catheters, surgical instruments, cannulae, cardiac valves, or vascular prostheses. After contacting the medical device with body fluid or body tissue, the reversibly immobilized compounds are liberated. Consequently, the coated medical devices act as drug delivery devices eluting the medicament, whereby the drug delivery kinetics can be controlled, providing an immediate release or a controlled, delayed or sustained drug delivery, for example.

[0217] In some embodiments, the composition further comprises an enteric coating that resists degradation under the prevailing pH of the stomach and permits delivery to specific regions of the gastrointestinal tract.

[0218] The pharmaceutical compositions may also be used for diagnostic or for therapeutic applications. For diagnostic applications, the compound may be present in a labelled form, e.g. in a form containing an isotope, e.g. a radioactive isotope or an isotope which may be detected by nuclear magnetic resonance. In some embodiments,

a therapeutic application is, in the case of a topical application, the prevention, alleviation and treatment of psoriasis and dermatitis.

[0219] The concentrations of the compounds in the pharmaceutical composition can vary. The concentration will depend upon factors such as the total dosage of the drug to be administered, the chemical characteristics (e.g., hydrophobicity) of the compounds employed, the route of administration, the age, body weight and symptoms of a patient. The compounds typically are provided in an aqueous physiological buffer solution containing about 0.1 to 10% w/v compound for topical administration. Typical dose ranges are from about 1 µg to about 1 g/kg of body weight per day; a dose range may be from about 0.01 mg/kg to 100 mg/kg of body weight per day, or about 0.1 to 20 mg/kg once to four times per day. In some embodiments, the dosage of the drug to be administered is likely to depend on variables such as the type and extent of the progression of the disease or disorder, the overall health status of the particular patient, the relative biological efficacy of the selected compound and the formulation of the compound excipient, and its route of administration.

[0220] Although the foregoing has been described in some detail by way of illustrations and examples for purposes of clarity and understanding, it will be understood by those of skill in the art that modifications can be made without departing from the spirit of the present disclosure. Therefore, it should be clearly understood that the forms disclosed herein are illustrative only and are not intended to limit the scope of the present disclosure, but rather to also cover all modification and alternatives coming with the true scope and spirit of the embodiments of the invention(s).

[0221] Terms and phrases used in this application, and variations thereof, especially in the appended claims, unless otherwise expressly stated, should be construed as open ended as opposed to limiting. As examples of the foregoing, the term 'including' should be read to mean 'including, without limitation,' 'including but not limited to,' or the like.

[0222] The indefinite article "a" or "an" does not exclude a plurality. The term "about" as used herein to, for example, define the values and ranges of molecular weights means that the indicated values and/or range limits can vary within ±20%, e.g., within ±10%. The use of "about" before a number includes the number itself. For example, "about 5" provides express support for "5".

[0223] The phrases "active agent" and "active entity" are synonyms and can be used interchangeably.

[0224] The terms "CT352" and "SNA-352" are synonyms and can be used interchangeably.

EXAMPLES

[0225] Non-limiting examples are provided below.

Example 1: A first synthesis of CT352, a PEGylated variant of the kinase inhibitor, Staurosporine, by LSE Technology

[0226] Staurosporine (Figure 1), an indolo[2,3-a]carbazole alkaloid appears to possess inhibitory activity against fungi and yeasts, strong antihypertensive activity and pronounced *in vitro* activity against a number of experimental tumors as well as a cytotoxic effect on cancer cells.

[0227] The PEGylated variant, CT352, of Staurosporine was produced through LSE Technology. Staurosporine is conjugated to a linear mPEG-carboxylic acid chain (average MW 2 kDa) by means of an amide bond through a 6-(bis amido) succinyl hexylcarbonyl linker on the methyl amino group of the tetrahydropyran ring moiety.

[0228] The following materials were used:

- Acetonitrile for HPLC (Merck/VWR, Cat No 1.00030.2500)
- 2-(1H-Benzotriazole-1-yl)-1,1 ,3,3-tetramethyluronium tetrafluoroborate (TBTU) (Sigma Aldrich, Cat No 12806)
- 7-(Boc-amino)-heptanoic acid (Sigma Aldrich, Cat No 15295)
- 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) (Sigma Aldrich, Cat No 139009)
- Dichloromethane (VWR, Cat No. 23354.326)
- Dichloromethane, anhydrous (Sigma Aldrich, Cat No 270997)
- 2,5-dihydroxybenzoic acid (DHB) (Sigma-Aldrich, Cat No 1361740)
- Dimethylformamide, anhydrous (Sigma Aldrich, Cat No 227056)
- Iodine (Sigma-Aldrich, Cat No 03551-100G)
- Methanol (VWR, Cat No 20864320)
- mPEG-COOH 2015 Da (Iris Biotech GmbH, Cat No PEG1158, Lot No 128958)
- mPEG-COOH 840 Da (Iris Biotech GmbH, Cat No PEG1161, Lot No 1210326)
- $Na2SO_4$ anhydrous (VWR, Cat No 28114.296)
- $NaHCO_3$ (VWR, Cat No. 27780.291)
- Silica for chromatography (Merck/VWR, Cat No 1.09385.2500)
- Sodium Trifluoroacetate (NaTFA) (Fluka, Product No 17840-10G)
- Staurosporine (Iris Biotech GmbH, Cat No LS-106, Lot No 090801)

- THF, anhydrous (Sigma Aldrich, Cat No 401757)
- TLC (Fluka, Cat No 99577-1 EA)
- Trifluoroacetic acid (TFA) (Merck, Cat No. 8.08260.0025)

[0229] The following methods were used:

A) Analytical HPLC with UV detection at 292 nm and ELS detectors (Waters 2695, 2487, 2424) (Reference Method: 10 to 100 2%min ELSD 292)
HPLC analytical C18 column (eg. Phenomenex Jupiter C18 300Å, 5µm, 4.6×250mm, Cat No 00G-4053-EO)
Acetonitrile for HPLC
H_2O for HPLC

Time (min)	Flow (ml/min)	H_2O,0.1%TFA (%)	ACN,0.1%TFA (%)	Gradient
0	1	90	10	
5	1	90	10	
50	1	0	100	Linear gradient
55	1	0	100	
56	1	90	10	Linear gradient
70	1	90	10	

B) Analytical HPLC with UV detection at 292 nm and ELS detectors (Waters 2695, 2487, 2424) (Reference Method: 10 to 100 1%min ELSD)
HPLC analytical C18 column (eg. Phenomenex Jupiter C18 300A, 5µm, 4.6×250mm, Cat No 00G-4053-EO)
Acetonitrile for HPLC
H_2O for HPLC

Time (min)	Flow (ml/min)	H_2O,0.1TFA (%)	ACN,0.1%TFA (%)	Gradient
0	1	90	10	
5	1	90	10	
95	1	0	100	Linear gradient
100	1	0	100	
102	1	90	10	Linear gradient
115	1	90	10	

C) Preparative HPLC with UV detection at 292nm (e.g. AKTA Purifier 10)
HPLC semipreparative C18 column (e.g. Phenomenex Jupiter C18 300A, 5µm,

15×250mm, Cat No OOG-4053-AK)

Acetonitrile for HPLC

H_2O for HPLC

CV (endpoint)	H_2O (%)	ACN (%)	gradient
0	90	10	
1.2	90	10	
12	0	100	Linear gradient
15	0	100	
15.1	90	10	Linear gradient
18	90	10	

D) MALDI-TOF analysis using a Bruker Daltonics Ultraflex TOF/TOF spectrophotometer, in reflectron(+) mode (Reference Method: CREABILIS_REF)

Matrix: 2,5-dihydroxybenzoic acid (DHB)

Cationizing agent: Sodium Trifluoroacetate (NaTFA)

[0230] Figure 2 depicts a first synthesis scheme for CT352.

[0231] Step 1 – In a three neck round bottom flask dried under nitrogen, 55.12 mg of 7-(Boc-amino)heptanoic acid (MW: 245.32 g/mol; 1.05 eq, 0.2247 mmol) were dissolved in 15 ml of anhydrous dimethylformamide; then 72.15 mg of TBTU (MW: 321 .08 g/mol; 1.05 eq, 0.2247 mmol) and 35.8 mg of DBU (MW: 152.24 g/mol, d: 1.018 g/ml; 1.1 eq, 0.2354 mmol) were subsequently added under magnetic stirring at room temperature. After 1 hour, 100 mg of staurosporine (MW: 466.5 g/mol; 0.214 mmol) were added and the reaction vessel protected from direct daylight. The reaction mixture was then stirred under nitrogen atmosphere until complete conversion of staurosporine had occurred (silica gel TLC, 96/4 dichloromethane/methanol mixture as eluant, UV 254nm and I_2 vapors detection).

[0232] After 22 hours the reaction was quenched with 10 ml of water and extracted with dichloromethane. Organic layers were washed with water and dried over anhydrous sodium sulfate. Evaporation under vacuum yielded to 316.2mg of yellow residue.

[0233] This product was dissolved in 60 ml of anhydrous dichloromethane under nitrogen atmosphere and then cooled in ice bath. Then, 10 ml of trifluoroacetic acid were slowly added under stirring. After 3 hours the reaction mixture is quenched with 50ml of saturated solution of $NaHCO_3$ and extracted with dichloromethane. Organic layers were collected and dried over anhydrous sodium sulfate and evaporated, yielding

70.8 mg of yellow solid. The residue was then purified over 60 g of silica gel, eluted with a gradient of dichloromethane/methanol mixture from 96/4 to 60/40 (vol/vol), yielding 27.7 mg of solid intermediate (MW 593 g/mol; yield 22%).

[0234] Step 2 – In a three neck round bottom flask dried under nitrogen, 196.7 mg of mPEG-carboxylic acid (MW: 2015 Da; 1.5 eq, 0.0976 mmol) were dissolved in 20 ml of anhydrous dimethylformamide; then 31.34 mg of TBTU (MW: 321.08 g/mol; 1.5 eq, 0.0976 mmol) and 15.57 µl of DBU (MW: 152.24 g/mol, d: 1.018 g/ml; 1.6 eq, 0.1041 mmol) were subsequently added under magnetic stirring at room temperature. After 1 hour, 38 mg of the previously obtained intermediate (MW: 593 Da, 0.0651 mmol) were added and the reaction vessel protected from direct daylight. The reaction mixture was then stirred under nitrogen atmosphere until complete conversion of the intermediate had occurred (silica gel TLC, 96/4 dichloromethane/methanol mixture as eluent, UV 254nm and 12 vapors detection).

[0235] After 22 hours the reaction was quenched with 10 ml of water and extracted with dichloromethane. Organic layers were washed with water and dried over anhydrous sodium sulfate. Evaporation under vacuum yielded 260.4 mg of crude of reaction that were purified over 24 g of silica gel eluted with a gradient of dichloromethane/methanol mixture from 96/4 to *80120* (vol/vol). The resulting 153.3 mg of solid were dissolved in 2.4 ml of a 50/50 (vol/vol) water/tetrahydrofuran mixture and then purified by a series of injections of 0.8 ml on a reverse phase HPLC column (Phenomenex Jupiter C18, 300A, 15×250mm, Cat No. OOG-4053-AK) eluted with Method C. The main peaks registered at 292 nm were fractionated as showed in the sample chromatogram reported in Figure 3. Fraction collected were analyzed by HPLC (Phenomenex Jupiter C18 300A 4.6×250mm, Cat No. OOG-4053-EO) using Method A and those of interest evaporated of the acetonitrile and lyophilized, yielding 48.8 mg of white solid (final yield: 7.4%).

[0236] The product was characterized by analytical RP-HPLC, using Method A. In Figure 4 and 5 the chromatographic profiles at 292 nm and ELS are reported. Purity detected at 292 nm is 98.44% (RT 26.79 min).

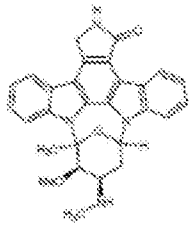
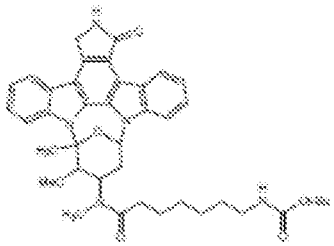
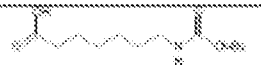
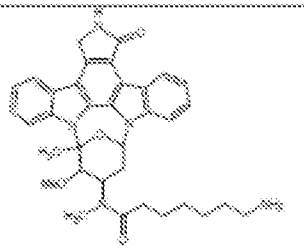
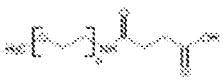
[0237] The exact mass of CT352 are estimated by MALDI MS spectroscopy, using Method D. The sample gave a strong positive ion MALDI-TOF mass spectrum (Figure 6) with a major series of singly-charged pseudomolecular ion cluster $[M+Na]^+$ observed between m/z 2006 and 3063, centered at approximately m/z 2490 and showing 44 Da differences. The evaluation of the isotopic pattern of each peak perfectly fits the theoretical abundance of a molecule having a structure analogue to that expected for a CT352 molecule with 39 oxyethylenic units (Figure 7). The final product was assigned

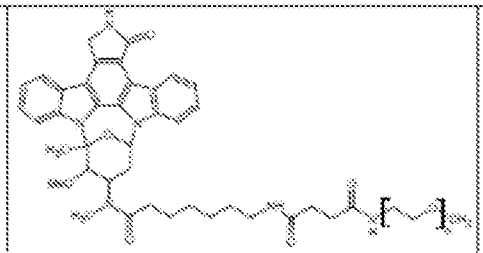
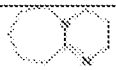
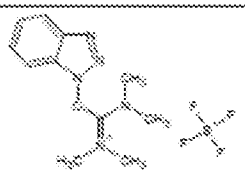
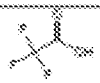
the mass of 2467 Da. The chemical structure of CT352 was confirmed by NMR analyses.

[0238] The above described synthesis scheme for the preparation of the staurosporine conjugate, CT352, resulted in acceptable yields and purities for the desired activities.

Example 2: A second synthesis of CT352, a PEGylated variant of the kinase inhibitor, Staurosporine, by LSE Technology

Abbreviations

IUPAC name (nickname)	Formula	MW
(9S,10R,11R,13R)-2,3,10,11,12,13-Hexahydro-10-methoxy-9-methyl-11-(methylamino)-9,13-epoxy-1H,9H-diindolo[1,2,3-gh:3',2',1'-lm]pyrrolo[3,4-j][1,7]benzodiazonin-1-one (Staurosporine)		466.53
(9S,10R,11R,13R)-2,3,10,11,12,13-Hexahydro-10-methoxy-9-methyl-11-[(7-[(tert-butoxycarbonyl)amino]heptanoyl)(methyl)amino]-9,13-epoxy-1H,9H-diindolo[1,2,3-gh:3',2',1'-lm]pyrrolo[3,4-j][1,7]benzodiazonin-1-one (Intermediate 1)		693.83
7-[(tert-butoxycarbonyl)amino]heptanoic acid		245.32
(9S,10R,11R,13R)-2,3,10,11,12,13-Hexahydro-10-methoxy-9-methyl-11-[(7-aminoheptanoyl)(methyl)amino]-9,13-epoxy-1H,9H-diindolo[1,2,3-gh:3',2',1'-lm]pyrrolo[3,4-j][1,7]benzodiazonin-1-one (Intermediate 2)		593.72
ω-Methoxy-ω-carboxylic acid poly(ethylene glycol) ($MeO\text{-}PEG_{n\text{-}COOH}$)		(for n=43) 2025.39 (Average MW) 2015

CT352		(for n=43) 2501.09 (Average MW) 2350
1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU)		152.24
O-(1H-benzotriazole-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate (TBTU)		321.09
N,N-dimethylformamide (DMF)		73.09
Dichloromethane (DCM)	CH_2Cl_2	84.93
Methanol (MeOH)	$H_3C—OH$	32.04
Trifluoroacetic acid (TFA)		114.02

[0239] A second synthesis scheme for CT352 is shown in Figure 8, and includes:

[0240] Step 1: Staurosporine amidation with 7-[(tert-butoxycarbonyl)amino]heptanoic acid, using TBTU as the coupling agent;

[0241] Step 2: removal of the Boc protective group of Intermediate 1 with trifluoroacetic acid;

[0242] Step 3: amidation of Intermediate 2 with MeO-PEG2000-COOH, using TBTU as the coupling agent.

[0243] Two preparations of CT352 were performed using respectively 50 mg and 500 mg of Staurosporine. The first synthesis at low scale was used to highlight any difficulties in the preparation process, while the higher scale preparation has been carried out to verify yield and quality of CT352 obtained according to the present process.

Step 1: Staurosporine amidation

[0244] The amidation reaction of Staurosporine with 7-[(tert-butoxycarbonyl)amino]heptanoic acid was carried out with TBTU as coupling agent and DBU as base, in DMF at 25°C. Reaction conditions are summed up in Table 2.

Table 2: Step 1 Reaction Conditions

Solvent	DMF
Coupling agent	TBTU
Base	DBU
Molar ratio Base / Staurosporine	1.7
Molar ratio TBTU/ Staurosporine	1.7
Molar ratio 7-[(tert-butoxycarbonyl)amino]heptanoic acid / Staurosporine	1.25

[0245] Reactions were monitored by withdrawing samples of reaction mixture at appropriate time intervals, which were then analyzed by HPLC. In nearly 20-24h reaction time, Staurosporine conversion was 98-99% and selectivity to Intermediate 1 was 93-95%, according to HPLC analysis (HPLC method MI CT352 001/section 8.1/page 38).

[0246] Only a partial conversion of Staurosporine was observed in a preliminary trial using DCM as solvent. The Intermediate 1 isolated after work up was used as such in the next step in the case of the test at reduced scale. Having been recognized impurities in the next step 2, attributable to the presence of the amino acid derivative, which is not UV visible, when the larger scale test was performed, the Intermediate 1 was previously purified by flash chromatography on silica gel, before using it in the next step 2.

[0247] Intermediate 1 with 95% HPLC purity was quantitatively isolated after purification by flash chromatography on silica gel, using mixtures of n-hexane and ethyl acetate as eluent phases.

Step 2: Boc cleavage of Intermediate 1

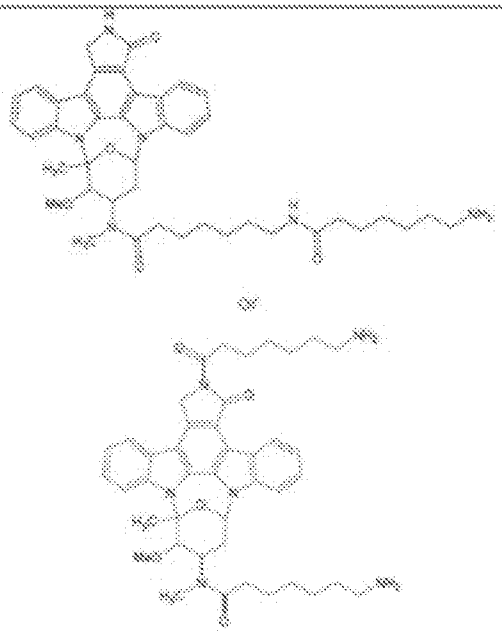
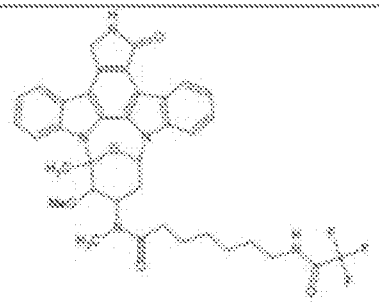
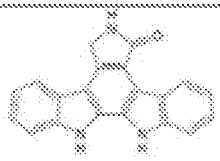
[0248] Boc cleavage of Intermediate 1 was achieved using trifluoroacetic acid in DCM as solvent. Complete Intermediate 1 conversion was observed in nearly 1.5 h reaction time, as well as high selectivity to Intermediate 2, according to HPLC analysis (HPLC method MI CT352 001/section 8.1/page 38)

[0249] Crude Intermediate 2 was isolated by neutralization with sodium hydrogen carbonate aqueous solution and extraction with DCM. This operation appeared

very ticklish, as several impurities were formed so that the crude Intermediate 2 from the larger scale test was isolated with reduced HPLC purity, 81%, as well as with low yield 40%.

[0250] The hypothesized structures for the main impurities, according to HPLC-MS, are listed in Table 3.

Table 3: Hypothesized Impurities

Nominal mass, Da	Structure
720	
699	
311	

[0251] The impurity with 720 Da molecular weight was not detected when the Intermediate 1 purified by flash chromatography was used as starting material.

[0252] The low yield was ascribed to the poor solubility of the intermediate 2, in the form of free base or, most likely, trifluoroacetate salt, as during the work up of the reaction an undissolved residue remained in the flask, which, analyzed by HPLC (by dissolving in acidic water/acetonitrile), turned out to be Intermediate 2, with 91% HPLC purity. This solid was collected and used in the next step.

Step 3: Intermediate 2 amidation

[0253] The amidation reaction of Intermediate 2 with MeO-PEG2000-COOH has been carried out with TBTU as coupling agent and DBU as base, in DMF at 25°C. TBTU was added as the last reagent. Reaction conditions are summed up in Table 4.

Table 4: Step 3 Reaction Conditions

Solvent	DMF
Coupling agent	TBTU
Base	DBU
Molar ratio Base / Intermediate 2	1.3
Molar ratio TBTU/ Intermediate 2	1.3
Molar ratio MeO-PEG_{2000}-COOH / Intermediate 2	1.3

[0254] Reactions were monitored by withdrawing samples of reaction mixture at time intervals, which were then analyzed by HPLC. In nearly 4h reaction time, Intermediate 2 conversion was 98- 99% and selectivity to CT352 was 84-86%, according to HPLC analysis (HPLC method MI CT352 001/section 8.1/page 25). When Intermediate 2 derived from the undissolved residue of the previous step was used, an excess of DBU was requires to get to almost complete conversion, thus supporting the hypothesis that the solid was in the form of trifluoroacetate salt.

[0255] CT352 isolated after work up was purified by flash chromatography on silica gel, using mixtures of dichloromethane and methanol as eluent phases. The purified CT352 sample from the larger scale test, according to NMR analysis, contained excess of MeO-PEG derivative (0.17 moles per CT352 mole), so it was further purified by reversed phase flash chromatography using water and acetonitrile as eluent phases.

[0256] The CT352 sample from the higher scale preparation was produced with 97.6% HPLC purity and 33% overall yield from Staurosporine. In this report, CT352 yield has been calculated assigning to CT352 *2590 Da* as the average molecular weight,

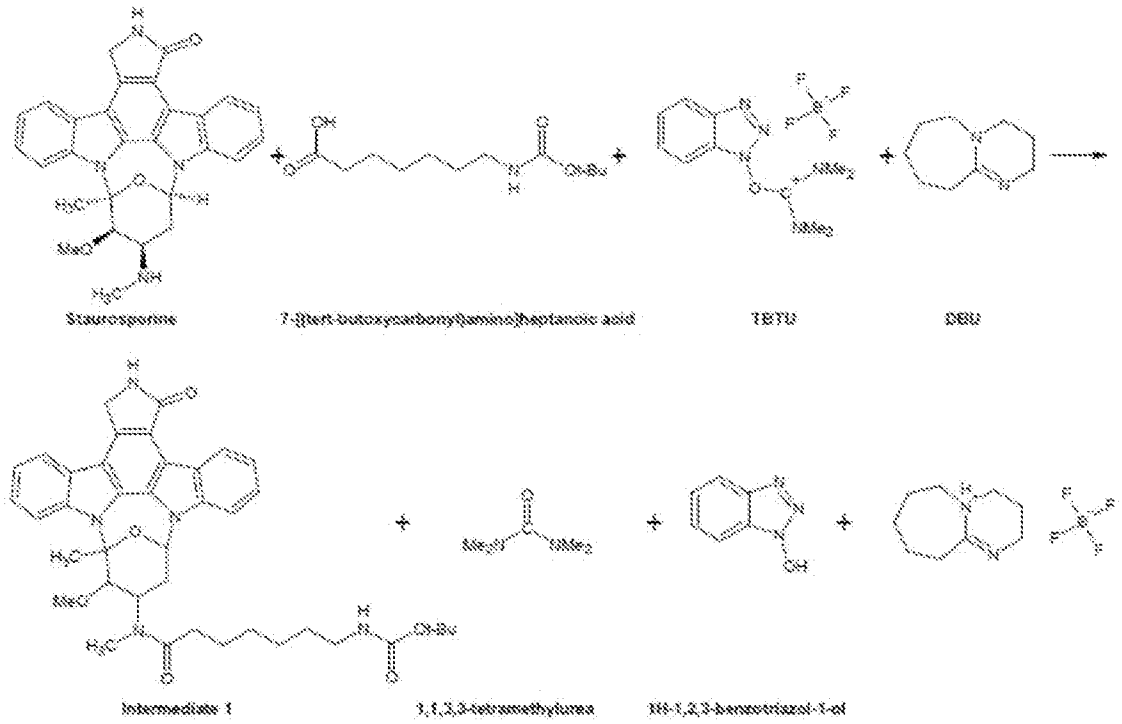
as the average molecular weight of MeO-PEG2000-COOH used for its preparation was *2015 Da*.

[0257] In alternative embodiments, step 2 of the process may be modified in order to facilitate the isolation of the Intermediate 2, either as the free base or as salt.

[0258] With respect to the amidation steps, 1 and 3, the reduction of the excess of 7-[(tertbutoxycarbonyl) amino]heptanoic acid in step 1 and MeO-PEG2000-COOH in step 3 simplifies the process and increases the yield, possibly avoiding the purification steps in both steps 1 and 3.

Experimental procedure for CT352 synthesis

Step 1: Staurosporine amidation



Material List

Reagents	PM	mmol	Assay, %	mg	d, g/ml	ml	ratio	
STAUROSPORINA	466,53	0,932	87	500,0			1	molar
7-[(TERT-BUTOXYCARBONYL)AMINO] HEPTANOIC ACID	245,32	1,166	98	291,8			1,25	molar
TBTU	321,08	1,585		508,9			1,7	molar
DBU	152,24	1,585	99	243,8	1,018	0,24	1,7	molar
DMF	73,09					20	0,04	ml/mg Staurosporina
PRODUCT								
INTERMEDIATE 1	693,83	0,932		646,9			1	molar

[0259] In a 100 ml three necked round bottom flask dried under nitrogen, equipped with magnetic stirrer bar, thermometer and condenser, 5 ml of DMF, 292 mg of 7-[(tertbutoxycarbonyl) amino], 240 ml of DBU and 509 mg of TBTU were placed at 25°C. The solution was aged under stirring at 25°C for 30 min., then 500 mg of Staurosporine dissolved in 15 ml of DMF were added. Figure 9 HPLC analysis of Staurosporine IRIS lot 02/12. The reaction mixture was aged at 25°C for 24 h under nitrogen atmosphere. The reaction progress was monitored by HPLC analyses taking samples during aging time. The final reaction mixture was transferred to a 250 ml separating funnel, diluted with 50 ml of DCM and washed with NaHCO3 aqueous saturated solution (1 x 50 g). The aqueous phase was extracted with 50 ml of DCM. The collected organic phases were washed with NaCl aqueous saturated solution (1 x 50 g), evaporated under reduced pressure at 40°C, affording 1,3 g of crude Intermediate 1 as a yellow liquid with 94.1% HPLC purity (Figure 10).

[0260] The crude Intermediate 1 was purified by normal-phase flash chromatography using a Biotage Isolera LS System equipped with a Biotage *SNAP-HP cartridge* packed with 50 g of HP-SIL (Silica). The cartridge was equilibrated at 50 ml/min. with 198 ml of n-hexane/ethyl acetate 50:50 v/v. Sample loading was performed using pre-packed *SNAP samplet cartridge* (Silica, 10 g). The *SNAP cartridge* was eluted with:

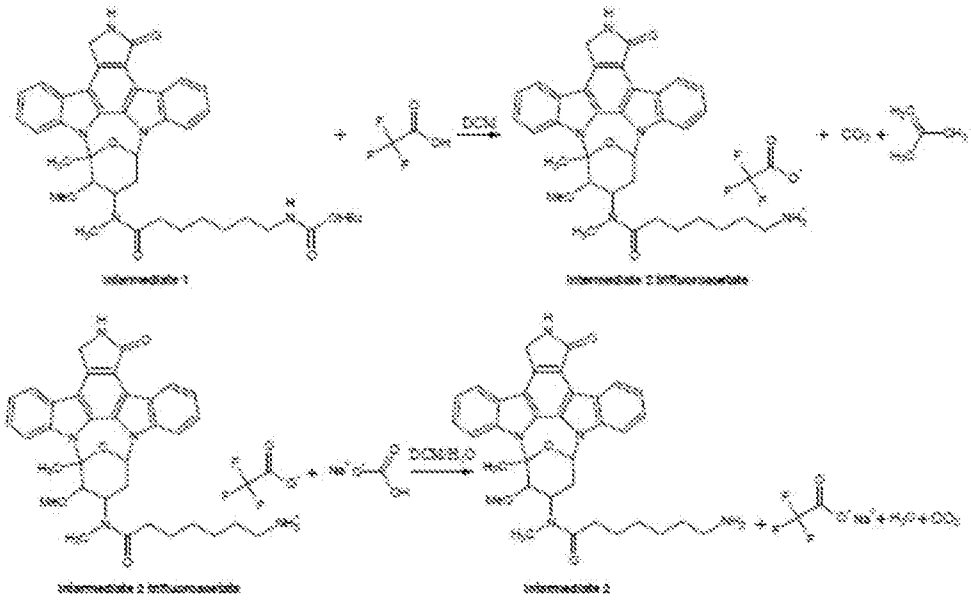
1. 132 ml n-hexane/ethyl acetate 50:50 v/v;
2. 66 ml from n-hexane/ethyl acetate 50:50 v/v to n-hexane/ethyl acetate 20:80 v/v;
3. 269 ml n-hexane/ethyl acetate 20:80 v/v;
4. 32 ml from n-hexane/ethyl acetate 20:80 v/v to n-hexane/ethyl acetate 10:90 v/v;

5. 160 ml n-hexane/ethyl acetate 10:90 v/v;
6. 15 ml from n-hexane/ethyl acetate 10:90 v/v to n-hexane/ethyl acetate 5:95 v/v;
7. 71 ml n-hexane/ethyl acetate 5:95 v/v;
8. 17 ml from n-hexane/ethyl acetate 5:95 v/v to n-hexane/ethyl acetate 0:100 v/v;
9. 480 ml n-hexane/ethyl acetate 0:100 v/v;

[0261] The first portion of eluate (132 ml) was sent to the waste, then the eluted solvent was collected in fractions of 50 ml each.

[0262] UV profile (@ 292 and 254 nm) of the purification is depicted in Figure 11. Collected individual fractions were analyzed by HPLC. Fractions from n° 10 to n° 18 were combined and evaporated under reduced pressure at 40°C to dryness, affording 660 mg of Intermediate 1 as a white solid, with 94.6% HPLC purity (Figure 12).

Step 2: Removal of the Boc protective group of Intermediate 1



Material List

REAGENTS	PM	mg	mmol	d, g/ml	ml	Ratio	
INTERMEDIATE 1	693,83	646,0	0,931				
DCM	84,93	120,3		1,33	150	0,23	ml/mg Intermediate 1
TFA	114,02	22187,4	194,6	1,48	15	209	molar
PRODUCT							
INTERMEDIATE 2	593,71	552,8	0,931			1,00	molar

[0263] In a 250 ml round bottom flask, equipped with magnetic stirrer bar, condenser, thermometer, 150 ml of DCM, and 660 mg of Intermediate 2 from the previous step were placed at 25°C. The mixture was stirred under nitrogen atmosphere for 15 min. obtaining a cloudy solution, which was cooled to 5°C. 15 ml of TFA were added in 30 minutes. The reaction mixture became a clear solution. The reaction mixture was aged for 1h at 25°C, monitoring the reaction progress by HPLC analyses (Figure 13).

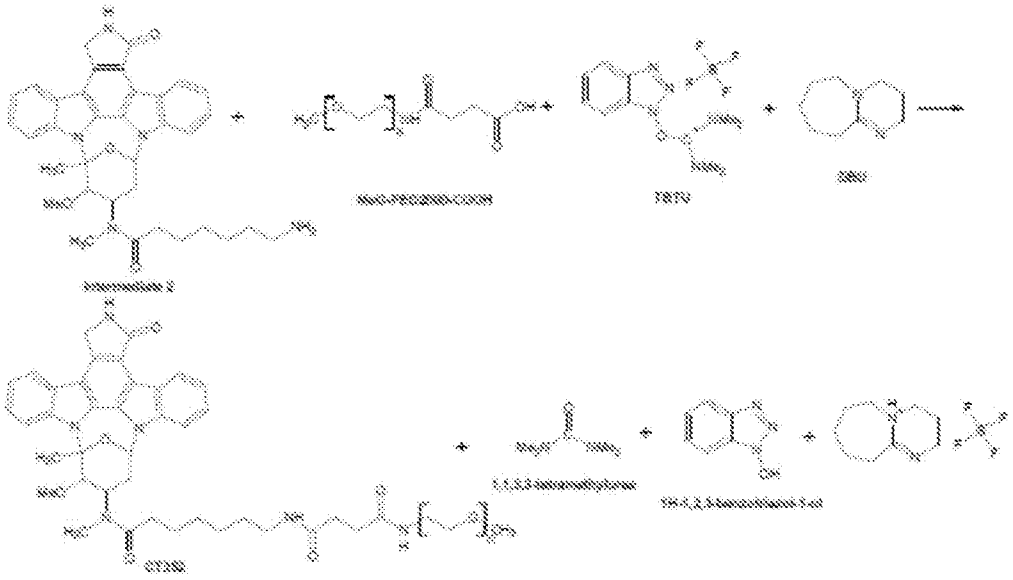
[0264] Excess of TFA was removed by evaporation under reduced pressure at 35°C. The green residual liquid in the flask was diluted with 50 ml of DCM and 40 ml of H2O, and cooled to 5 °C with an ice bath. A NaHCO3 aqueous saturated solution was added dropwise to pH ca. 8 (14.5 g), maintaining the temperature at 5 °C. The biphasic mixture was transferred to a 250 ml separating funnel, but a green solid remained on the bottom of the flask, which didn't dissolve in either DCM or in H_2O.

[0265] The organic phase in the separating funnel was separated. The aqueous phase was extracted with 25 ml of DCM. pH of the residual aqueous phase was 8. The combined organic phases were washed with NaCl aqueous saturated solution (48g). The organic phase was evaporated under reduced pressure at 40°C to dryness, affording 220 mg of Intermediate 2 as a white solid (1° solid), with 80.9% HPLC purity (Figure 14).

[0266] The undissolved solid in the flask (approximately 650 mg) according to HPLC analysis, was Intermediate 2 with 90.5% HPLC purity (2° solid) (Figure 15).

[0267] Both solids were separately used in the subsequent amidation reaction with MeO-PEG_{2000}-COOH.

Step 3: Intermediate 2 amidation



Material List

REAGENTS	PM	mg	Assay, %	mmol	d, g/ml	ml	Ratio	
INTERMEDIATE 2/1* SOLID	583,71	220	81*	0,30			1,00	
MeO-PEG$_{2000}$-COOH	2015	828	95	0,39			1,3	molar
TBTU	321,08	125		0,39			1,3	molar
DBU	152,24	60	99	0,39	1,02	0,06	1,3	molar
DMF	73,09				0,948	14	0,064	ml/mg intermediate 2
PRODUCT								
CT352	2590	777		0,30				molar

* HPLC purity.

REAGENTS	PM	mg	Assay, %	mmol	d, g/ml	ml	Ratio	
INTERMEDIATE 2/2* SOLID	583,71	333	90*	0,51			1,00	
MeO-PEG$_{2000}$-COOH	2015	1407	95	0,66			1,3	molar
TBTU	321,08	213		0,66			1,3	molar
DBU	152,24	102	99	0,66	1,02	0,100	1,3	molar
DBU	152,24	116	99	0,71	1,02	0,108	1,4	
DMF	73,09				0,948		0,04	ml/mg intermediate 2
PRODUCT								
CT352	2590	1322		0,51				molar

* HPLC purity.

[0268] In a 100 ml three necked round bottom flask, equipped with magnetic stirrer, condenser, thermometer, 828 mg of MeO-PEG-COOH, 3 ml of DMF, 220 mg of Intermediate 2/1° solid dissolved in 8 ml of DMF and 60 ml of DBU were placed at 25°C. To the slightly cloudy solution 125 mg of TBTU and 3 ml of DMF were added. The reaction mixture was aged at 25°C under nitrogen atmosphere for 4 h monitoring the reaction progress by HPLC analyses.

[0269] 64 g of water were added and the mixture, transferred to a 250 ml separating funnel, was extracted with DCM (2×25 ml). The combined organic phases were concentrated under reduced pressure at 40°C, affording 1,7 g of crude CT352 as a yellow-orange liquid with 70.1% HPLC purity (Figure 16).

[0270] The same procedure was repeated starting from Intermediate 2/2° solid. Being the theoretical quantity of Intermediate 2 from the previous step 553 mg and having isolated 220 mg as Intermediate 2/1° solid, it was assumed that the content of Intermediate 2 in solid 2 was approximately 333 mg.

[0271] The only change compared to the previous preparation was that a further addition of base in the course of the reaction was required to achieve complete conversion of Intermediate 2. 3,8 g of crude CT352 as a yellow-orange liquid with 81,3% HPLC purity (Figure 17) were obtained and combined with the crude CT352 previously obtained.

CT352 purification

A. Purification by normal-phase flash chromatography

[0272] Crude CT352 from the previous step was purified by normal-phase flash chromatography using a Biotage Isolera LS System equipped with a Biotage *SNAP-KP cartridge* packed with 100 g of KP-SIL (Silica). The cartridge was equilibrated at 50 ml/min. with 264 ml of dichloromethane. Sample loading was performed using pre-packed *SNAP samplet cartridge* (Silica, 10 g). The *SNAP cartridge* was eluted with:

1) 132 ml dichloromethane/methanol 95:5 v/v;
2) 396 ml from dichloromethane/methanol 95:5 v/v to dichloromethane/methanol 90:10 v/v;
3) 132 ml dichloromethane/methanol 90:10 v/v;
4) 26 ml from dichloromethane/methanol 90:10 v/v to dichloromethane/methanol 89:11 v/v;
5) 106 ml dichloromethane/methanol 89:11 v/v;
6) 224 ml from dichloromethane/methanol 89:11 v/v to dichloromethane/methanol 85:15 v/v;

7) 132 ml dichloromethane/methanol 85:15 v/v;

8) 264 ml from dichloromethane/methanol 85:15 v/v to dichloromethane/methanol 80:20 v/v;

9) 528 ml dichloromethane/methanol 80:20 v/v;

[0273] The first portion of eluate (264 ml) was sent to the waste, then the eluted solvent was collected in fractions of 60 ml each.

[0274] UV profile (@ 292 and 210 nm) of the purification is depicted in Figure 18. Collected individual fractions were analyzed by HPLC. Fractions from n° 7 and n° 8 were combined and evaporated under reduced pressure at 40°C to dryness, affording 1,2 g of CT352 as a white solid, with 97.8% HPLC purity.

[0275] According to NMR analysis of this sample, the ratio between the terminal methoxy group of the PEG unit and one of the aromatic protons of the molecule was 3.5:1 instead of 3:1, so a further purification by reversed phase flash chromatography was required.

[0276] Fractions n° 6, 9 and 10 were combined and evaporated under reduced pressure at 40°C to dryness, affording 0,9 g of CT352 with 81.3% HPLC purity, which were purified again as before affording 300 mg of CT352 with 97.3% HPLC purity and 250 mg of CT352 with 92.9% HPLC purity. Figure 19 shows HPLC analysis of CT352 purified by normal-phase flash chromatography.

B. Purification by reversed-phase flash chromatography

[0277] CT352 from the previous normal phase purification, divided in aliquots, was purified by reversed-phase flash chromatography using a Biotage Isolera LS System equipped with a Biotage *SNAP KP-C18-HS cartridge* packed with 120 g of KP-C18-HS Silica. The cartridge was equilibrated at 50 ml/min. with 396 ml of acetonitrile/water 80:20 v/v.

[0278] Sample loading was performed by dissolving the sample in H2O/ACN 2.5:1 and injecting it onto the cartridge through a syringe.

[0279] The *SNAP cartridge* was eluted with:

1. 792 ml from acetonitrile/water 80:20 v/v to acetonitrile/water 40:60 v/v;

[0280] The first portion of eluate (350 ml) was sent to the waste, then the eluted solvent was collected in fractions of 70 ml each. The UV profile (@ 292 and 210 nm) of the purification is depicted in Figure 20.

[0281] Collected individual fractions were analyzed by HPLC. Fractions with HPLC purity ≥ 97% were combined and concentrated under reduced pressure at 40°C to remove acetonitrile. The residual aqueous phase was extracted with DCM (5×100 ml). The combined organic phases were washed with 150 ml of NaCl aqueous saturated

solution and evaporated under reduced pressure at 40°C to dryness, affording 1 g of CT352 as a light yellow solid, which was added with 35 ml of cold diethyl ether. The mixture was cooled to 5°C and aged under stirring for 1.5 h. The precipitated solid was filtered over sintered glass filter (G3), washed with 10 ml of cold diethyl ether and dried under vacuum at 38°C for 2 h to afford 810 mg of CT352 as a light yellow solid (lot n° 2010CG02/S9).

[0282] Purity of CT352, determined by HPLC analysis (method MI CT352 001), was 97.6% (Figure 21). The product was characterized by 1H-NMR (Figure 22). Certificate of analysis of the CT352 sample is shown in Figure 23. The final CT352 was stored at –22°C.

Analytical methods

HPLC method MI CT352 001

[0283] The following method has been used both for reaction monitoring and for assessing chemical purity of isolated products.

Sample preparation	Reaction monitoring 50-150 µl of reaction mixture were diluted with 1 ml of .1% $HCOOH/H_2O$ v/v /acetonitrile 1:1 Isolated products 0,5-1,0 mg/ml solutions in a mixture of 0.1% $HCOOH/H_2O$ v/v /acetonitrile 1:1 were prepared. Fractions from normal phase flash chromatography 100-200 µl were evaporated under nitrogen flow and diluted with 2 ml of 0.1% $HCOOH/H_2O$ v/v /acetonitrile 1:1. Fractions from normal phase flash chromatography 100-200 µl were diluted with 1 ml of 0.1% $HCOOH/H_2O$ v/v/ acetonitrile 1:1.
Injected volume	5 µl
HPLC	Agilent 1100
Detector	UV 292 nm
Column	Zorbax Eclipse XDB-C18 Solvent Saver - 3 x 150 mm - 5 µm
Temperature	25°C
Flow	0.5 ml/min
Mobile phases	A: 0.1% $HCOOH/H_2O$ v/v B: 0.1% HCOOH/acetonitrile v/v

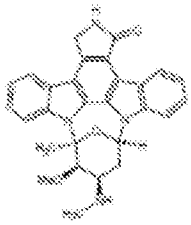
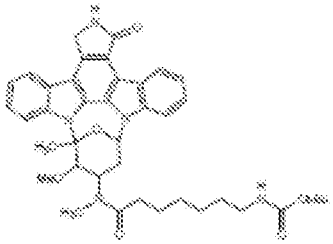
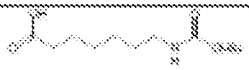
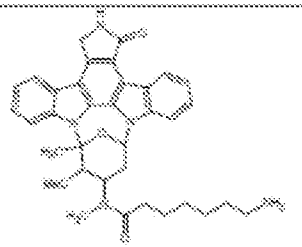
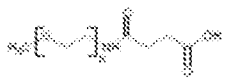
Gradient

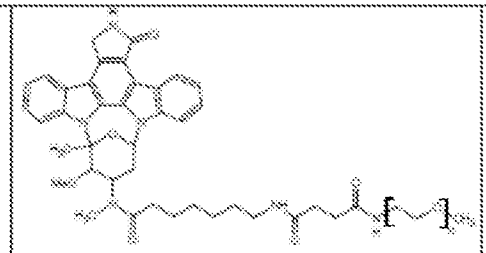
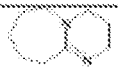
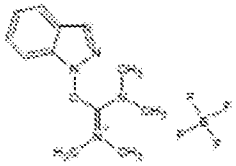
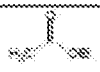
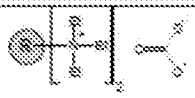
Time, min.	%A	B %
0	75	25
2	75	25
30	10	90
33	10	90
35	75	25

Staurosporine	Retention time	7.5 min.	RRT	0.53
Intermediate 1	Retention time	22.9 min.	RRT	1.62
Intermediate 2	Retention time	9.6 min.	RRT	0.68
CT352	Retention time	14.1 min.	RRT	1

Example 3: A third synthesis of CT352, a PEGylated variant of the kinase inhibitor, Staurosporine, by LSE Technology

Abbreviations

IUPAC name (nickname)	Formula	MW
(9S,10R,11R,13R)-2,3,10,11,12,13-Hexahydro-10-methoxy-9-methyl-11-(methylamino)-9,13-epoxy-1H,9H-diindolo[1,2,3-gh:3',2',1'-lm]pyrrolo[3,4-j][1,7]benzodiazonin-1-one (Staurosporine)		466.53
(9S,10R,11R,13R)-2,3,10,11,12,13-Hexahydro-10-methoxy-9-methyl-11-{[7-[(tert-butoxycarbonyl)amino]heptanoyl](methyl)amino}-9,13-epoxy-1H,9H-diindolo[1,2,3-gh:3',2',1'-lm]pyrrolo[3,4-j][1,7]benzodiazonin-1-one (Intermediate 1)		693.83
7-[(tert-butoxycarbonyl)amino]heptanoic acid		245.32
(9S,10R,11R,13R)-2,3,10,11,12,13-Hexahydro-10-methoxy-9-methyl-11-{[7-aminoheptanoyl](methyl)amino}-9,13-epoxy-1H,9H-diindolo[1,2,3-gh:3',2',1'-lm]pyrrolo[3,4-j][1,7]benzodiazonin-1-one (Intermediate 2)		593.72
α-Methoxy-ω-carboxylic acid poly(ethylene glycol) ($MeO\text{-}PEG_{\sim45}\text{-}COOH$)		(for n=43) 2028.39 (Average MW) 2015

CT352		(for n=43) 2601.09 (Average MW) 2590
1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU)		152.24
O-(1H-benzotriazole-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate (TBTU)		321.08
N,N-dimethylformamide (DMF)		73.09
Dichloromethane (DCM)	CH_2Cl_2	84.93
Methanol (MeOH)	$H_3C—OH$	32.04
Trifluoroacetic acid (TFA)		114.02
Ethyl acetate (AcOEt)		88.11
Acetonitrile (ACN)	$H_3C—C\equiv N$	41.05
Tetraalkylammonium carbonate, polymer-bound (MP-carbonate resin)		

[0284] A third synthesis scheme for producing CT352 is shown in Figure 24.

[0285] This synthetic procedure includes:

[0286] Step 1: Staurosporine acylation by 7-[(tert-butoxycarbonyl)amino]heptanoic acid, using TBTU as the coupling agent;

[0287] Step 2: removal of the Boc protective group of Intermediate 1 with trifluoroacetic acid;

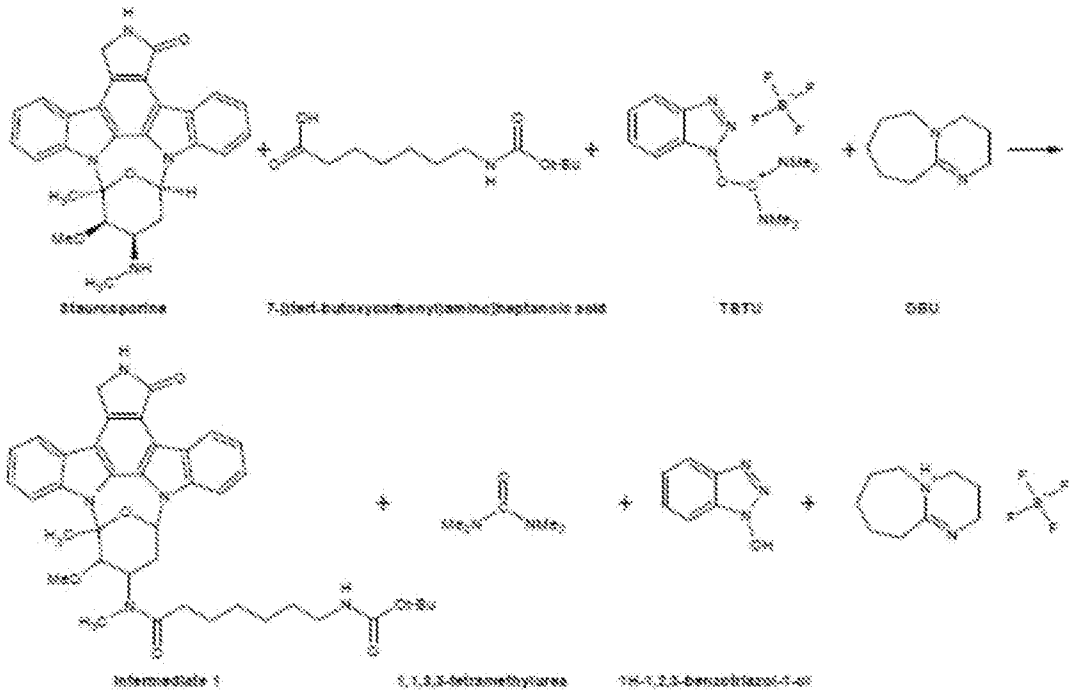
[0288] Step 3: acylation of Intermediate 2 by MeO-PEG_{2000}-COOH, using TBTU as the coupling agent.

[0289] The activity described in this report resulted in the preparation of 855 mg of CT352 with 97.2% HPLC purity and 25% overall yield from Staurosporine. CT352 yield has been calculated assigning to CT352 *2590 Da* as the average molecular weight, as the average molecular weight of MeO-PEG2000-COOH used for its preparation was *2015 Da*.

[0290] This preparation highlighted some optimization work, involving mainly the step 2 of the process, as already underlined in the previous Example.

Experimental procedure for CT352 synthesis

Step 1: Staurosporine acylation



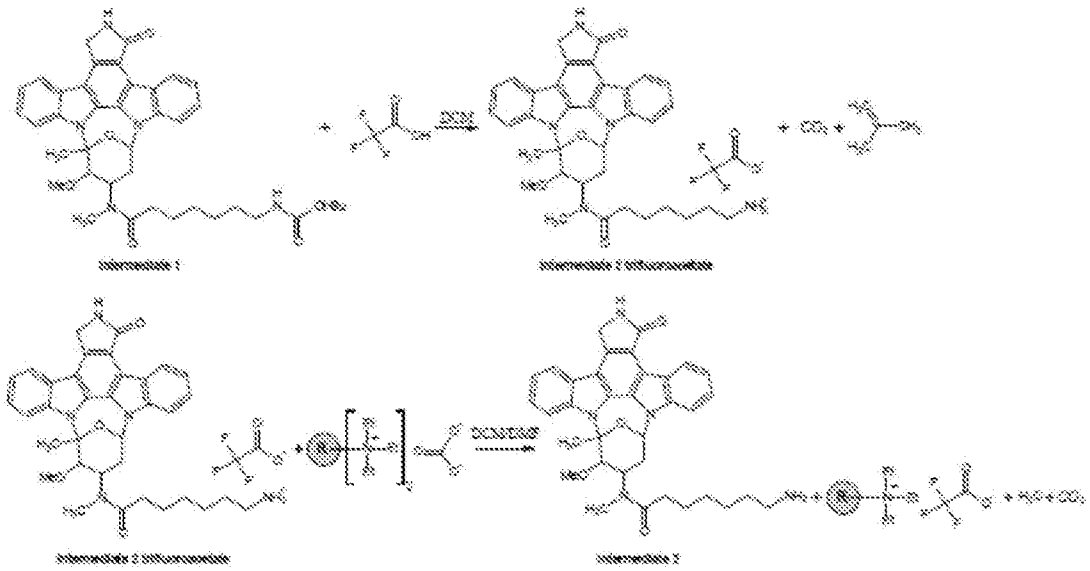
Material List

Reagents	Supplier Lot	PM	mmol	Assay %	mg	d g/ml	ml	ratio	
Staurosporine	INIS 02/12	466,53	1,305	87	700			1	molar
7-[(TERT-BUTOXYCARBONYL)AMINO] HEPTANOIC ACID	ABCR AB165739	245,32	1,632	98	408			1,25	molar
TBTU	Carbosynth FT070961101	321,08	2,219		712			1,7	molar
DBU	Alfa Aesar 61080085	152,24	2,219	99	341	1,018	0,335	1,7	molar
DMF	Sigma Aldrich STBC4508V	73,09					22	0,031	ml/mg Staurosporine
Product									
Intermediate 1		693,83	1,305		906			1	molar

[0291] In a 50 ml three necked round bottom flask wrapped in aluminum foil, dried under nitrogen, equipped with magnetic stirrer bar, thermometer and condenser, 5 ml of DMF, 408 mg of 7- [(tert-butoxycarbonyl)amino]heptanoic acid, 712 mg of TBTU and 341 mg of DBU were placed at 25°C. The solution was aged under stirring at 25°C for 25 min., then 700 mg of Staurosporine dissolved in 17 ml of DMF were added. The reaction mixture was aged at 25°C for 27 h under nitrogen atmosphere. The reaction progress was monitored by HPLC analyses taking samples during aging time (Figure 25). The final reaction mixture was transferred to a 250 ml separating funnel, diluted with 50 ml of DCM and washed with $NaHCO_3$ aqueous saturated solution (1 × 50 ml). The aqueous phase was extracted with 50 ml of DCM. The collected organic phases were washed with NaCl aqueous saturated solution (1 × 50 ml) (Figure 26), evaporated under reduced pressure at 40°C, affording 1,2 g of crude Intermediate 1 as a yellow solid.

[0292] Purification of crude Intermediate 1 by normal-phase flash chromatography was not feasible as the product didn't dissolve in suitable amounts of DCM, or mixtures of DMF/DCM. The solvents were removed again by evaporation under reduced pressure at 40°C. The residue was added with 7 ml of AcOEt and 8 ml of Et_2O, the solid was filtered over sintered glass filter (G4), and dried under vacuum at 40°C to afford 862 mg of crude Intermediate 1 as a light yellow solid. It was analyzed by HPLC (Figure 27) dissolving the solid in HCOOH/H_2O/ACN 1:1:1 v/v/v: due to the high content of HCOOH, used to obtain a homogeneous sample, some deprotection of Boc group occurred, so a mixture of Intermediate 1 and Intermediate 2 was revealed. HPLC purity (as sum of Intermediate 1 and Intermediate 2) was 97.2%. The yield (corrected for HPLC purity) from Staurosporine was 92.5%.

Step 2: removal of the Boc protective group of Intermediate 1



Material List

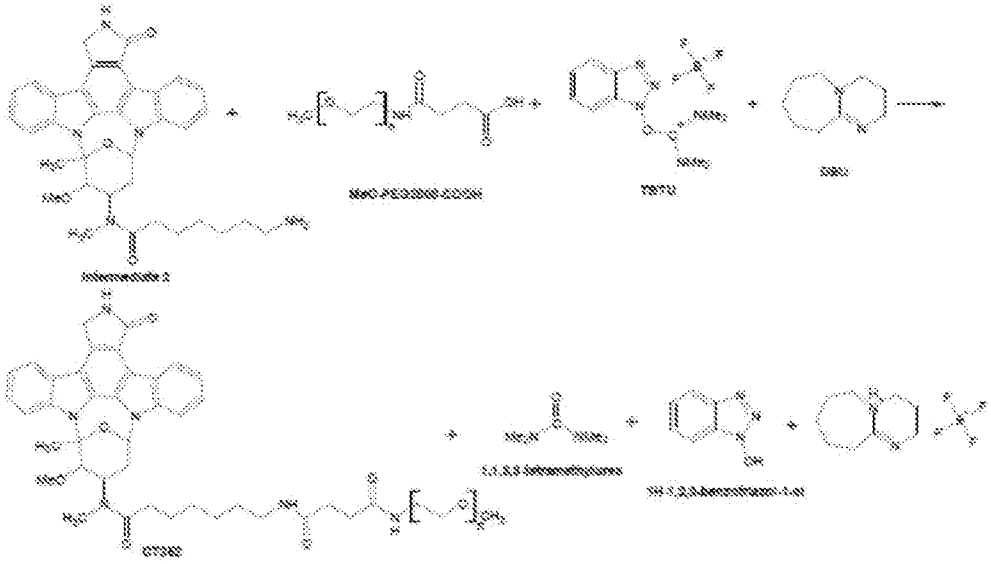
Reagents	Supplier Lot	PM	mg	Assay %	mmol	d, g/ml	ml	Ratio	
INTERMEDIATE 1		693,83	862	97,2*	1,308				
DCM	Panreac 614443	84,93				1,33	150	0,174	ml/mg Intermediate 1
TFA	Alfa Aesar 10161547	114,02	7435		65,210	1,48	5	56	molar
PRODUCT									
INTERMEDIATE 2		593,71	717		1,308			1,00	molar

* HPLC purity.

[0293] In a 250 ml round bottom flask, equipped with magnetic stirrer bar, condenser, thermometer, 150 ml of DCM, and 862 mg of Intermediate 1 from the previous step were placed at 25°C. The mixture was stirred under nitrogen atmosphere for 15 min. obtaining a cloudy solution, which was cooled to 5°C. 5 ml of TFA were added in 60 minutes. The reaction mixture became a clear solution once a few drops of TFA were added. The reaction mixture was aged for 2h at 5°C and for 3h at 25°C, monitoring the reaction progress by HPLC analyses (Figure 28). Page 14 of 28 Excess of TFA was removed by evaporation under reduced pressure at 35°C obtaining 1.5 g of residual liquid. Neutralization of the Intermediate 2 trifluoroacetate was carried out by dissolving the residue in 30 ml of DCM and adding 2.05 g of tetraalkylammonium

carbonate polymer supported (macroporous, 40-90 mesh, 2.5-3.5 mmol/g loading). The mixture was stirred by rotation through rotavapor. As some precipitation was observed, 2.5 ml of DMF were added and the liquid phase turned clear. After 2 h the resin was removed by filtration and washed with 4 ml of DMF. The liquid phase was evaporated under reduced pressure at 40°C to dryness, affording 1.5 g of Intermediate 2 as an orange liquid with 93.7% HPLC purity (Figure 29).

Step 3: Intermediate 2 acylation



Material List

Reagents	Supplier Lot	PM	mg	Assay, %	mmol	d, g/ml	ml	Ratio	
Intermediate 2		593,71	717	93,7*	1,132			1,00	
MeO-PEG$_{2000}$-COOH	IRIS 1211574 +1210298	2015	2761	95	1,301			1,151	molar
TBTU	Carbosynth FT070961101	321,08	442		1,377			1,217	molar
TBTU (further additions)	Carbosynth FT070961101	321,08	879		2,738			2,420	molar
DBU	Alfa Aesar 61080065	152,24	230		1,55	1,02	0,23	1,370	molar
DBU (further additions)	Alfa Aesar 61080065	152,24	445		2,925	1,02		2,585	
DMF	Sigma Aldrich STBC4903V	73,09				0,948	19,4	0,027	ml/mg Intermediate 2
Product									
CT352		2590	2931		1,132			1,00	molar

* HPLC purity.

[0294] In a 100 ml three necked round bottom flask, equipped with magnetic stirrer, condenser, thermometer, 1.5 g of Intermediate 2 from previous step (Maximum Intermediate 2 content is 717 mg. Accordingly the molar ratios were calculated.), 19 ml of DMF, 2,761 g of MeO-PEG-COOH, and 230 ml of DBU were placed at 25°C. To the slightly cloudy solution 442 mg of TBTU were added. The reaction mixture was aged at 25°C under nitrogen atmosphere. Reaction progress was monitored by HPLC analyses at time intervals. Due to the slow conversion of Intermediate 2, more TBTU and DBU during aging time were added. Altogether 879 mg of TBTU and 445 mg of DBU were further added. After 27 h aging time (Figure 30), the reaction mixture was diluted with 52 g of water, transferred to a 250 ml separating funnel, and extracted with DCM (2 × 35 ml). The combined organic phases were washed with NaCl aqueous saturated solution (A difficult to break down emulsion was formed) (1 × 50 ml), and concentrated under reduced pressure at 40°C, affording 4,7 g of crude CT352 as a yellow solid, with 79.0% HPLC purity (Figure 31).

CT352 purification

A. Purification by normal-phase flash chromatography

[0295] Crude CT352 from the previous step was purified by normal-phase flash chromatography using a Biotage Isolera LS System equipped with a Biotage *SNAP*

cartridge packed with 340 g of KP-SIL (Silica). The cartridge was equilibrated at 100 ml/min. with 705 ml of dichloromethane/methanol 95:5 v/v.

[0296] Sample loading was performed by dissolving crude CT352 in 10 ml of dichloromethane and applying to the pre-packed *SNAP sample cartridge* (Silica, 34 g), which was inserted into the *SNAP cartridge*.

[0297] The *SNAP cartridge* was eluted with a mixture of dichloromethane/methanol from 95:5 v/v to 80:20 v/v.

[0298] The eluted solvent was collected in fractions, which were analyzed by HPLC. Fractions with HPLC purity > 97.0 % were combined and evaporated under reduced pressure at 40°C to dryness, affording 1,51 g of CT352 as a white solid (CT352/P1) with 97.3% HPLC purity (Figure 32). According to NMR analysis of this sample, the ratio between the terminal methoxy group of the PEG unit and one of the aromatic protons of the molecule was 3.55:1 instead of 3.0:1, so a further purification by reversed phase flash chromatography was required.

[0299] The remaining fractions with HPLC purity > 90.0% were combined and evaporated under reduced pressure at 40°C to dryness, affording 1,24 g of CT352 as a white solid, with 91.0% HPLC purity, which were purified again by normal-phase flash chromatography as before affording 0,6 g of CT352 (CT352/P2) with 97.0% HPLC purity (Figure 33).

B. Purification by reversed-phase flash chromatography

[0300] CT352/P1 and CT352/P2 samples from the previous normal phase purification, divided in 4 aliquots, were purified by reversed-phase flash chromatography using a Biotage Isolera LS System equipped with a Biotage *SNAP KP-C18-HS cartridge* packed with 120 g of KP-C18-HS Silica. The cartridge was equilibrated at 50 ml/min. with 264 ml of water/acetonitrile 64:36 v/v. Sample loading was performed by dissolving the sample in H_2O/ACN 5:1 and injecting it onto the cartridge through a syringe. The *SNAP cartridge* was eluted with a mixture of water/acetonitrile from 64:36 to 46/54 v/v. The eluted solvent was collected in fractions, which were analyzed by HPLC. Fractions with HPLC purity ≥ 97% were combined and concentrated under reduced pressure at 40°C to remove acetonitrile. The residual aqueous phase was extracted with DCM (7 × 100 ml). The combined organic phases were washed with 150 ml of NaCl aqueous saturated solution and evaporated under reduced pressure at 40°C to dryness, affording 0.92 g of CT352 as a light yellow solid, which was added with 35 ml of cold diethyl ether. The mixture was cooled to 5°C and aged under stirring for 1.5 h. The precipitated solid was filtered over sintered glass filter (G3), washed with 10 ml of cold diethyl ether and dried

under vacuum at 38°C for 24 h to afford 855 mg of CT352 as a light yellow solid (lot n° 2010RB15/S5).

[0301] Purity of CT352, determined by HPLC analysis (method MI CT352 001), was 97.2% (Figure 34). The product was characterized by ^{1}H-NMR (Figure 35) Certificate of analysis of the CT352 sample is shown in Figure 36. Final CT352 was stored at –22°C.

Analytical Methods

[0302] The following method has been used both for reaction monitoring and for assessing chemical purity of isolated products.

Sample preparation	Reaction monitoring 50-150 µl of reaction mixture were diluted with 1 ml of Eluent A/acetonitrile 1:1 v/v. Isolated products 0,5-1,0 mg/ml solutions in 0.1% Eluent A/acetonitrile 1:1 v/v were prepared. Fractions from normal phase flash chromatography 100-200 µl were evaporated under nitrogen flow and diluted with 2 ml of Eluent A /acetonitrile 1:1 v/v. Fractions from normal phase flash chromatography 100-200 µl were diluted with 1 ml of Eluent A / acetonitrile 1:1 v/v.			
Injected volume	5 µl			
HPLC	Agilent 1100			
Detector	UV 292 nm			
Column	Zorbax Eclipse XDB-C18 Solvent Saver - 3 x 150 mm - 5 µm			
Temperature	25°C			
Flow	0.5 ml/min			
Mobile phases	A: 0.1% HCOOH/H_2O v/v B: 0.1% HCOOH/acetonitrile v/v			
Gradient	Time, min.	%A	B %	
	0	75	25	
	2	75	25	
	30	10	90	
	33	10	90	
	35	75	25	
Staurosporine	Retention time	7.3 min.	RRT	0.55
Intermediate 1	Retention time	22.4 min.	RRT	1.70
Intermediate 2	Retention time	9.2 min.	RRT	0.70
CT352	Retention time	13.2 min.	RRT	1

Example 4: Profiling study of CT352 against 311 kinases

[0303] CT352 and staurosporine (compound 1) were tested (at test concentrations of 0.2 µM) against a variety of target kinases.

Materials and Methods

Preparation of test compound solution

[0304] The test compounds were dissolved in and diluted with dimethylsulfoxide (DMSO) to achieve 100-fold higher concentration. Then the solutions were further 25-fold diluted with assay buffer to make the final test compound solutions. Reference compounds for assay control were prepared similarly.

Assay reagents and procedures

TK-ELISA

[0305] 1) The 10 μL of x4 compound solution, 10 μL of x4 Substrate/ATP/Metal solution, and 20 μL of x2 kinase solution were prepared with assay buffer (15 mM Tris-HCl, 0.01% Tween-20, 2 mM DTT, pH7.5) and mixed in a well of streptavidine-coated 96 well microplate (Perkin Elmer).

[0306] 2) The well was incubated for I hour at room temperature and then washed 4 times to stop the reaction.

[0307] 3) The well was blocked with blocking buffer containing 0.1% BSA and then 100 μL of the detection antibody (HRP conjugated PY20; Santa Cruz Biotechnology) solution was added and incubated for 30 minutes.

[0308] 4) After washing the well, 100 μL of TMB solution (MOSS Inc.) was added and incubated for 5 minutes. To stop the HRP reaction, 100 μL of 0.1 M sulfuric acid was added.

[0309] 5) The kinase reaction was evaluated by the absorbance at 450 nm of the well.

STK-ELISA

[0310] 1) The 10 μL of x4 compound solution, 10 μL of x4 Substrate/ATP/Metal solution, and 20 μL of x2 kinase solution were prepared with assay buffer (15 mM Tris-HCl, 0.01% Tween-20, 2 mM DTT, pH7.5) and mixed and incubated in a well of polypropylene 96 well microplate for 0.5 or 1 hour* at room temperature.(*; depend on kinase)

[0311] 2) 120 μL of 40 mM EDTA solution (pH 7.5) was added to the well, and then 120 μL of the mixture was transferred to the well of ELISA plate (see below table).

[0312] 3) After 30 minutes incubation, the well was washed 4 times, and blocked with blocking buffer containing O. I% BSA.

[0313] 4) 100 μL of the first antibody (see below table) solution was added to the well and incubated for 30 minutes.

[0314] 5) After 4 times washing of the well, 100 μL of the second antibody (sell below table) solution was added to the well, and incubated for 30 minutes.

[0315] 6) After washing the well, 100 μL of TMB solution (MOSS Inc.) was added and incubated for 5 minutes. To stop the HRP reaction, 100 μL of 0.l M sulfuric acid was added.

[0316] 7) The kinase reaction was evaluated by the absorbance at 450 nm of the well.

Kinase	ELISA plate and Antibody
LKB1	ELISA plate: Streptavidin-coated plate (PerkinElmer) 1st Ab: Rabbit phospho-threonine antibody (P-Thr-Polyclonal) (Cell Signaling Technology) 2nd Ab: HRP labeled goat anti-rabbit IgG Ab (Zymed)
JNK1	ELISA plate: Streptavidin-coated plate (PerkinElmer) 1st Ab: Monoclonal anti-phospho-ATF2 (Phosphothreonine,69,71) Clone ATF-22P (Sigma) 2nd Ab: HRP labeled goat anti- mouse IgG Ab (Zymed)
JNK2	ELISA plate: Streptavidin-coated plate (PerkinElmer) 1st Ab: Monoclonal anti-phospho-ATF2 (Phosphothreonine,69,71) Clone ATF-22P (Sigma) 2nd Ab: HRP labeled goat anti- mouse IgG Ab (Zymed)
JNK3	ELISA plate: Streptavidin-coated plate (PerkinElmer) 1st Ab: Monoclonal anti-phospho-ATF2 (Phosphothreonine,69,71) Clone ATF-22P (Sigma) 2nd Ab: HRP labeled goat anti- mouse IgG Ab (Zymed)
Erk5	ELISA plate: Streptavidin-coated plate (PerkinElmer) 1st Ab: Anti-phospho MBP(Thr98) antibody (Millipore) 2nd Ab: HRP labeled goat anti-mouse IgG Ab (Zymed)

MOS	ELISA plate: Glutathione coated 96 well plate (NUNC) 1st Ab: Anti-phospho MEK1/2(Ser217/221) antibody (Cell Signaling Technology) 2nd Ab: HRP labeled goat anti-rabbit IgG Ab (Zymed)
PLK4	ELISA plate: Streptavidin-coated plate (PerkinElmer) 1st Ab: Rabbit phospho-threonine antibody (P-Thr-Polyclonal) (Cell Signaling Technology) 2nd Ab: HRP labeled goat anti-rabbit IgG Ab (Zymed)
MAP3K1	ELISA plate: Glutathione coated 96 well plate (NUNC) 1st Ab: Anti-phospho MEK1/2(Ser217/221) antibody (Cell Signaling Technology) 2nd Ab: HRP labeled goat anti-rabbit IgG Ab (Zymed)
MAP3K2	ELISA plate: Glutathione coated 96 well plate (NUNC) 1st Ab: Anti-phospho MKK7(Ser271/Thr275) antibody (Cell Signaling Technology) 2nd Ab: HRP labeled goat anti-rabbit IgG Ab (Zymed)
MAP3K3	ELISA plate: Glutathione coated 96 well plate (NUNC) 1st Ab: Anti-phospho MKK7(Ser271/Thr275) antibody (Cell Signaling Technology) 2nd Ab: HRP labeled goat anti-rabbit IgG Ab (Zymed)
MAP3K4	ELISA plate: Glutathione coated 96 well plate (NUNC) 1st Ab: Anti-phospho-MEK3[pSer189/pThr193]/MEK6[pSer207/pThr211] (Sigma) 2nd Ab: HRP labeled goat anti-rabbit IgG Ab (Zymed)
MAP3K5	ELISA plate: Glutathione coated 96 well plate (NUNC) 1st Ab: Anti-phospho MKK7(Ser271/Thr275) antibody (Cell Signaling Technology) 2nd Ab: HRP labeled goat anti-rabbit IgG Ab (Zymed)
MAP2K1	ELISA plate: Glutathione coated 96 well plate (NUNC) 1st Ab: Anti-phospho p44/42 MAPK (Thr202/Tyr204) (E10) monoclonal antibody (Cell Signaling Technology) 2nd Ab: HRP labeled goat anti-mouse IgG Ab (Zymed)
MAP2K2	ELISA plate: Glutathione coated 96 well plate (NUNC) 1st Ab: Anti-phospho p44/42 MAPK (Thr202/Tyr204) (E10) monoclonal antibody (Cell Signaling Technology) 2nd Ab: HRP labeled goat anti-mouse IgG Ab (Zymed)
MAP2K3	ELISA plate: Glutathione coated 96 well plate (NUNC) 1st Ab: Anti-phospho p38 MAPK (Thr180/Tyr182) (28B10) monoclonal antibody (Cell Signaling Technology) 2nd Ab: HRP labeled goat anti-mouse IgG Ab (Zymed)
MAP2K4	ELISA plate: Glutathione coated 96 well plate (NUNC) 1st Ab: Anti-phospho JNK(Thr183/Tyr185, Thr221/Tyr223) rabbit Antibody (Millipore) 2nd Ab: HRP labeled goat anti-rabbit IgG Ab (Zymed)
MAP2K5	ELISA plate: Glutathione coated 96 well plate (NUNC) 1st Ab: Anti-phospho-ERK5(Thr218/Tyr220) antibody (Santa Cruz Biotechnology) 2nd Ab: HRP labeled goat anti-rabbit IgG Ab (Zymed)
MAP2K6	ELISA plate: Glutathione coated 96 well plate (NUNC) 1st Ab: Anti-phospho p38 MAPK (Thr180/Tyr182) (28B10) monoclonal antibody (Cell Signaling Technology) 2nd Ab: HRP labeled goat anti-mouse IgG Ab (Zymed)
MAP2K7	ELISA plate: Glutathione coated 96 well plate (NUNC) 1st Ab: Anti-phospho JNK(Thr183/Tyr185, Thr221/Tyr223) rabbit Antibody (Millipore) 2nd Ab: HRP labeled goat anti-rabbit IgG Ab (Zymed)
COT	ELISA plate: Streptavidin-coated plate (PerkinElmer) 1st Ab: Anti-phospho MEK1/2(Ser217/221) antibody (Cell Signaling Technology) 2nd Ab: HRP labeled goat anti-rabbit IgG Ab (Zymed)
LIMK1	ELISA plate: Glutathione coated 96 well plate (NUNC) 1st Ab: Anti-phospho-Cofilin 2 (Ser3) (Millipore) 2nd Ab: HRP labeled goat anti-rabbit IgG Ab (Zymed)
DLK	ELISA plate: Glutathione coated 96 well plate (NUNC) 1st Ab: Anti-phospho MKK7(Ser271/Thr275) antibody (Cell Signaling Technology) 2nd Ab: HRP labeled goat anti-rabbit IgG Ab (Zymed)
MLK1	ELISA plate: Glutathione coated 96 well plate (NUNC) 1st Ab: Anti-phospho MKK7(Ser271/Thr275) antibody (Cell Signaling Technology) 2nd Ab: HRP labeled goat anti-rabbit IgG Ab (Zymed)
MLK2	ELISA plate: Glutathione coated 96 well plate (NUNC) 1st Ab: Anti-phospho MKK7(Ser271/Thr275) antibody (Cell Signaling Technology) 2nd Ab: HRP labeled goat anti-rabbit IgG Ab (Zymed)

MLK3	ELISA plate: Glutathione coated 96 well plate (NUNC) 1st Ab: Anti-phospho MKK7(Ser271/Thr275) antibody (Cell Signaling Technology) 2nd Ab: HRP labeled goat anti-rabbit IgG Ab (Zymed)
TAK1-TAB1	ELISA plate: Glutathione coated 96 well plate (NUNC) 1st Ab: Anti-phospho MKK7(Ser271/Thr275) antibody (Cell Signaling Technology) 2nd Ab: HRP labeled goat anti-rabbit IgG Ab (Zymed)
BRAF	ELISA plate: Glutathione coated 96 well plate (NUNC) 1st Ab: Anti-phospho MEK1/2(Ser217/221) antibody (Cell Signaling Technology) 2nd Ab: HRP labeled goat anti-rabbit IgG Ab (Zymed)
BRAF [V600E]	ELISA plate: Glutathione coated 96 well plate (NUNC) 1st Ab: Anti-phospho MEK1/2(Ser217/221) antibody (Cell Signaling Technology) 2nd Ab: HRP labeled goat anti-rabbit IgG Ab (Zymed)
RAF1	ELISA plate: Glutathione coated 96 well plate (NUNC) 1st Ab: Anti-phospho MEK1/2(Ser217/221) antibody (Cell Signaling Technology) 2nd Ab: HRP labeled goat anti-rabbit IgG Ab (Zymed)
BMPR1A	ELISA plate: Glutathione coated 96 well plate (NUNC) 1st Ab: Anti-phospho Smad1(Ser463/Ser465) antibody (Santa Cruz Biotechnology) 2nd Ab: HRP labeled goat anti-rabbit IgG Ab (Zymed)

IMAP assay

[0317] 1) The *5* µL of x4 compound solution, *5* µL of x4 Substrate/ATP/Metal solution, and 10 µL of x2 kinase solution were prepared with assay buffer (20 mM HEPES, 0.01% Tween-20, 2 mM DTT, pH7.4) and mixed and incubated in a well of polystyrene 384 well black microplate for 1 hour at room temperature.

[0318] 2) 60 µL of IMAP binding reagent (IMAP Screening Express kit; Molecular Devices) was added to the well, and incubated for 30 minutes.

[0319] 3) The kinase reaction was evaluated by the fluorescence polarization at 485 nm for excitation and 530 nm for emission of the well.

[0320] Off-chip Mobility Shift Assay (MSA)

[0321] 1) The *5* µL of x4 compound solution, 5 µL of x4 Substrate/ATP/Metal solution, and 10 µL of x2 kinase solution were prepared with assay buffer (20 mM HEPES, 0.01% Triton X- 100, 2 mM DTT, pH7.5) and mixed and incubated in a well of polypropylene 384 well microplate for 1 or 5 hour(s)* at room temperature. (*; depend on kinase)

[0322] 2) 60 µL of Termination Buffer (QuickScout Screening Assist MSA; Carna Biosciences) was added to the well.

[0323] 3) The reaction mixture was applied to LabChip3000 system (Caliper Life Science), and the product and substrate peptide peaks were separated and quantitated.

[0324] 4) The kinase reaction was evaluated by the product ratio calculated from peak heights of product(P) and substrate(S) peptides (P/(P+S)).

[0325] Reaction Conditions -- The reaction conditions are depicted below:

Kinase	Platform	Substrate		ATP (uM)		Metal		Positive control
		Name	(uM)	Km	Assay	Name	(mM)	
ABL	MSA	ABLtide	1000	16	25	Mg	5	Staurosporine
ABL[E255K]	MSA	ABLtide	1000	17	25	Mg	5	Staurosporine
ABL[T315I]	MSA	ABLtide	1000	4	5	Mg	5	Staurosporine
ACK[1]	MSA	WASP peptide	1000	97	100	Mg	5	Staurosporine
ALK	MSA	Srctide	1000	57	50	Mg	5	Staurosporine
ALK[F1174L]	MSA	Srctide	1000	49	50	Mg	5	Staurosporine
ALK[R1275Q]	MSA	Srctide	1000	84	100	Mg	5	Staurosporine
ARG	MSA	ABLtide	1000	24	25	Mg	5	Staurosporine
AXL	MSA	CSKtide	1000	32	50	Mg	5	Staurosporine
BLK	MSA	Srctide	1000	62	75	Mg	5	Staurosporine
BMX	MSA	Srctide	1000	75	75	Mg	5	Staurosporine
BRK[1]	MSA	Blk/Lyntide	1000	250	250	Mg	5	Staurosporine
BTK	MSA	Srctide	1000	72	75	Mg	5	Staurosporine
CSK[1]	MSA	Srctide	1000	4.8	5	Mg+Mn	5+1	Staurosporine
CTK	TK-ELISA	Lyn substrate peptide	250	5.1	5	Mn	5	Staurosporine
DDR1 [1]	MSA	IRS1	1000	94	100	Mg	5	Staurosporine
DDR2[1]	MSA	IRS1	1000	38	50	Mg	5	Staurosporine
EGFR	MSA	Srctide	1000	2.7	5	Mg+Mn	5+1	Staurosporine
EGFR[d746-750]	MSA	Srctide	1000	19	25	Mg+Mn	5+1	Staurosporine
EGFR[L858R]	MSA	Srctide	1000	9.8	10	Mg+Mn	5+1	Staurosporine
EGFR[L861Q]	MSA	Srctide	1000	7.5	10	Mg+Mn	5+1	Staurosporine
EGFR[T790M]	MSA	Srctide	1000	0.9	1	Mg+Mn	5+1	Staurosporine
EGFR[d746-750/ T790M]	MSA	Srctide	1000	5	5	Mg+Mn	5+1	Staurosporine
EGFR[T790M/L858R]	MSA	Srctide	1000	1.9	2	Mg+Mn	5+1	Staurosporine
EML4-ALK[1]	MSA	Srctide	1000	43	50	Mg	5	Staurosporine
EPHA1	MSA	Blk/Lyntide	1000	22	25	Mg	5	Staurosporine
EPHA2	MSA	Blk/Lyntide	1000	67	75	Mg	5	Staurosporine
EPHA3	MSA	Blk/Lyntide	1000	170	150	Mg	5	Staurosporine
EPHA4	MSA	Blk/Lyntide	1000	52	50	Mg	5	Staurosporine
EPHA5	MSA	Blk/Lyntide	1000	56	50	Mg	5	Staurosporine
EPHA6	MSA	Blk/Lyntide	1000	27	25	Mg	5	Staurosporine
EPHA7	MSA	Blk/Lyntide	1000	58	50	Mg	5	Staurosporine
EPHA8	MSA	Blk/Lyntide	1000	69	75	Mg	5	Staurosporine
EPHB1	MSA	Blk/Lyntide	1000	29	25	Mg	5	Staurosporine
EPHB2	MSA	Blk/Lyntide	1000	86	100	Mg	5	Staurosporine
EPHB3	MSA	Blk/Lyntide	1000	49	50	Mg	5	Staurosporine
EPHB4	MSA	Blk/Lyntide	1000	56	50	Mg	5	Staurosporine
FAK[1]	MSA	Blk/Lyntide	1000	25	25	Mg	5	Staurosporine
FER	MSA	Srctide	1000	26	25	Mg	5	Staurosporine
FES	MSA	Srctide	1000	43	50	Mg	5	Staurosporine
FGFR1	MSA	CSKtide	1000	89	100	Mg	5	Staurosporine
FGFR2	MSA	CSKtide	1000	66	75	Mg	5	Staurosporine
FGFR3	MSA	CSKtide	1000	43	50	Mg	5	Staurosporine
FGFR3[K650E]	MSA	CSKtide	1000	41	50	Mg	5	Staurosporine
FGFR3[K650M]	MSA	CSKtide	1000	17	25	Mg	5	Staurosporine
FGFR4	MSA	CSKtide	1000	220	250	Mg	5	Staurosporine

FGFR4[N535K]	MSA	CSKtide	1000	30	25	Mg	5	Staurosporine
FGFR4[V550E]	MSA	CSKtide	1000	210	200	Mg	5	Staurosporine
FGFR4[V550L]	MSA	CSKtide	1000	160	150	Mg	5	Staurosporine
FGR	MSA	Srctide	1000	34	50	Mg	5	Staurosporine
FLT1	MSA	CSKtide	1000	140	150	Mg	5	Staurosporine
FLT3	MSA	Srctide	1000	94	100	Mg	5	Staurosporine
FLT4	MSA	CSKtide	1000	72	75	Mg	5	Staurosporine
FMS	MSA	Srctide	1000	26	25	Mg	5	Staurosporine
FRK	MSA	Srctide	1000	62	75	Mg	5	Staurosporine
FYN	MSA	Srctide	1000	50	50	Mg	5	Staurosporine
HCK	MSA	Srctide	1000	11	10	Mg	5	Staurosporine
HER2	MSA	Srctide	1000	9.4	10	Mn	5	Staurosporine
HER4	MSA	Srctide	1000	27	25	Mg	5	Staurosporine
IGF1R	MSA	IRS1	1000	63	75	Mg	5	Staurosporine
INSR	MSA	IRS1	1000	58	50	Mg	5	Staurosporine
IRR	MSA	IRS1	1000	64	75	Mg	5	Staurosporine
ITK	MSA	Srctide	1000	6.1	10	Mg	5	Staurosporine
JAK1 [300]	MSA	JAK1 substrate peptide	1000	68	75	Mg	5	Staurosporine
JAK2	MSA	Srctide	1000	13	10	Mg	5	Staurosporine
JAK3	MSA	Srctide	1000	3.5	5	Mg	5	Staurosporine
KDR	MSA	CSKtide	1000	24	75	Mg	5	Staurosporine
KIT[9]	MSA	Srctide	1000	370	400	Mg	5	Staurosporine
KIT[D816V][9]	MSA	Srctide	1000	14	10	Mg	5	Staurosporine
KIT[T670I][9]	MSA	Srctide	1000	100	100	Mg	5	Staurosporine
KIT[V560G][300]	MSA	Srctide	1000	460	450	Mg	5	Staurosporine
KIT[V654A][9]	MSA	Srctide	1000	220	250	Mg	5	Staurosporine
LCK	MSA	Srctide	1000	14	10	Mg	5	Staurosporine
LTK	MSA	Srctide	1000	49	50	Mg	5	Staurosporine
LYNa	MSA	Srctide	1000	14	10	Mg	5	Staurosporine
LYNb	MSA	Srctide	1000	18	25	Mg	5	Staurosporine
MER	MSA	CSKtide	1000	36	50	Mg	5	Staurosporine
MET	MSA	Srctide	1000	27	25	Mg	5	Staurosporine
MET[Y1235D]	MSA	Srctide	1000	71	75	Mg	5	Staurosporine
MUSK[9]	MSA	CSKtide	1000	14	10	Mg+Mn	3+1	Staurosporine
NPM1-ALK	MSA	Srctide	1000	57	50	Mg	5	Staurosporine
PDGFRa	MSA	CSKtide	1000	28	25	Mg	5	Staurosporine
PDGFRa[T674I][9]	MSA	CSKtide	1000	11	10	Mg	5	Staurosporine
PDGFRa[V561D]	MSA	CSKtide	1000	35	50	Mg	5	Staurosporine
PDGFRβ	MSA	CSKtide	1000	23	25	Mg	5	Staurosporine
PYK2	MSA	Blk/Lyntide	1000	56	50	Mg	5	Staurosporine
RET	MSA	CSKtide	1000	7.9	10	Mg	5	Staurosporine
RET[G691S]	MSA	CSKtide	1000	13	10	Mg	5	Staurosporine
RET[M918T]	MSA	CSKtide	1000	4.3	5	Mg	5	Staurosporine
RET[S891A]	MSA	CSKtide	1000	11	10	Mg	5	Staurosporine
RET[Y791F]	MSA	CSKtide	1000	29	25	Mg	5	Staurosporine
RON	MSA	Srctide	1000	27	25	Mg	5	Staurosporine
ROS	MSA	IRS1	1000	37	50	Mg	5	Staurosporine
SRC	MSA	Srctide	1000	31	50	Mg	5	Staurosporine
SRM	MSA	Blk/Lyntide	1000	38	50	Mg	5	Staurosporine
SYK	MSA	Blk/Lyntide	1000	26	25	Mg	5	Staurosporine
TEC	MSA	Srctide	1000	35	50	Mg	5	Staurosporine

TIE2	MSA	Blk/Lyntide	1000	94	100	Mg	5	Staurosporine
TNK1 [2]	MSA	CSKtide	1000	71	75	Mg	5	Staurosporine
TRKA	MSA	CSKtide	1000	65	75	Mg	5	Staurosporine
TRKB	MSA	Srctide	1000	80	75	Mg	5	Staurosporine
TRKC	MSA	Srctide	1000	47	50	Mg	5	Staurosporine
TXK [2]	MSA	Srctide	1000	110	100	Mg	5	Staurosporine
TYK2 [2]	MSA	Srctide	1000	18	25	Mg	5	Staurosporine
TYRO3	MSA	CSKtide	1000	80	75	Mg	5	Staurosporine
YES	MSA	Srctide	1000	13	10	Mg	5	Staurosporine
ZAP70 [2]	MSA	Blk/Lyntide	1000	1.7	1	Mg+Mn	5+1	Staurosporine
AKT1	MSA	Crosstide	1000	31	30	Mg	5	Staurosporine
AKT2	MSA	Crosstide	1000	110	100	Mg	5	Staurosporine
AKT3	MSA	Crosstide	1000	54	50	Mg	5	Staurosporine
AMPKa1/b1/g1	MSA	SAMS peptide	1000	130	150	Mg	5	Staurosporine
AMPKa2/b1/g1	MSA	SAMS peptide	1000	100	100	Mg	5	Staurosporine
AurA	MSA	Kemptide	1000	27	25	Mg	5	Staurosporine
AurA/TPX2 [20]	MSA	Kemptide	1000	1.7	2	Mg	5	Staurosporine
AurB/INCENP	MSA	Kemptide	1000	16	25	Mg	5	Staurosporine
AurC	MSA	Kemptide	1000	24	25	Mg	5	Staurosporine
BMPR1A	STK-ELISA	Smad1	125	19	30	Mg	5	Staurosporine
BRAF	STK-ELISA	MAP2K1	85	0.061	0.1	Mg	40	ZM336372
BRAF[V600E]	STK-ELISA	MAP2K1	85	3.2	5	Mg	40	ZM336372
BRSK1	MSA	CHKtide	1000	30	25	Mg	5	Staurosporine
BRSK2	MSA	CHKtide	1000	31	50	Mg	5	Staurosporine
CaMK1a [16]	MSA	G8 peptide	1000	750	1000	Mg	5	Staurosporine
CaMK1d [19]	MSA	Synapsin peptide	1000	11	10	Mg	5	Staurosporine
CaMK2a [8]	MSA	G8 peptide	1000	33	50	Mg	5	Staurosporine
CaMK2b [8]	MSA	G8 peptide	1000	19	25	Mg	5	Staurosporine
CaMK2g [8]	MSA	G8 peptide	1000	23	25	Mg	5	Staurosporine
CaMK2d [8]	MSA	G8 peptide	1000	6.3	5	Mg	5	Staurosporine
CaMK4 [8]	MSA	G8 peptide	1000	20	25	Mg	5	Staurosporine
CDC2/CycB1	MSA	Modified Histone H1	1000	34	50	Mg	5	Staurosporine
CDC7/ASK [3]	MSA	MCM2 peptide	1000	2.8	5	Mg	10	Staurosporine
CDK2/CycA2	MSA	Modified Histone H1	1000	27	25	Mg	5	Staurosporine
CDK2/CycE1	MSA	Modified Histone H1	1000	132	150	Mg	5	Staurosporine
CDK3/CycE1	MSA	Modified Histone H1	1000	1000	1000	Mg	5	Staurosporine
CDK4/CycD3 [3]	MSA	DYRKtide-F	1000	200	200	Mg	5	Staurosporine
CDK5/p25	MSA	Modified Histone H1	1000	10	10	Mg	5	Staurosporine
CDK6/CycD3 [3]	MSA	DYRKtide-F	1000	330	300	Mg	5	Staurosporine
CDK7/CycH/MAT1 [3]	MSA	CTD3 peptide	1000	32	50	Mg	5	Staurosporine
CDK9/CycT1 [3]	MSA	CDK9 substrate	1000	9.4	10	Mg	5	Staurosporine
CGR2 [4]	MSA	Kemptide	1000	34	25	Mg	5	Staurosporine
CHK1	MSA	CHKtide	1000	50	50	Mg	5	Staurosporine
CHK2	MSA	CHKtide	1000	51	50	Mg	5	Staurosporine
CK1a [3]	MSA	CKtide	1000	4.1	5	Mg	5	5-Iodotubercidin
CK1g1	MSA	CKtide	1000	6.3	5	Mg	5	5-Iodotubercidin
CK1g2	MSA	CKtide	1000	10	10	Mg	5	5-Iodotubercidin
CK1g3	MSA	CKtide	1000	3.2	5	Mg	5	5-Iodotubercidin

CK1δ	MSA	CKtide	1000	7.7	10	Mg	5	5-Iodotubercidin
CK1ε[4]	MSA	CKtide	1000	16	25	Mg	5	5-Iodotubercidin
CK2α1/β	MSA	CK2tide	1000	2.9	5	Mg	5	TBB
CK2α2/β	MSA	CK2tide	1000	2.1	5	Mg	5	TBB
CLK1	MSA	DYRKtide-F	1000	11	10	Mg	5	Staurosporine
CLK2	MSA	DYRKtide-F	1000	140	150	Mg	5	Staurosporine
CLK3	MSA	DYRKtide-F	1000	75	75	Mg	5	Staurosporine
COT	STK-ELISA	MAP2K1 peptide	250	7.3	10	Mn	10	K252b
CRIK[1]	MSA	Histone H3 peptide	1000	7.8	10	Mg	5	Staurosporine
DAPK1	MSA	DAPK1tide	1000	1.1	1	Mg	5	Staurosporine
DCAMKL2[1]	MSA	GS peptide	1000	120	150	Mg	5	Staurosporine
DLK	STK-ELISA	MAP2K7	72	38	20	Mg	0.5	Staurosporine
DYRK1A	MSA	DYRKtide-F	1000	16	25	Mg	5	Staurosporine
DYRK1B	MSA	DYRKtide-F	1000	59	50	Mg	5	Staurosporine
DYRK2	MSA	DYRKtide-F	1000	7.7	10	Mg	5	Staurosporine
DYRK3	MSA	DYRKtide-F	1000	6.8	5	Mg	5	Staurosporine
EEF2K[1][20]	MSA	EEF2Ktide	1000	12	10	Mg	5	NH125
Erk1	MSA	Modified Erktide	1000	34	50	Mg	5	5-Iodotubercidin
Erk2	MSA	Modified Erktide	1000	33	50	Mg	5	5-Iodotubercidin
Erk5	STK-ELISA	MBP	200	410	400	Mg	2.5	Staurosporine
GSK3α	MSA	CREBtide-p	1000	12	10	Mg	5	Staurosporine
GSK3β	MSA	CREBtide-p	1000	9.1	10	Mg	5	Staurosporine
Haspin	MSA	Histone H3 peptide	1000	140	150	Mg	5	Staurosporine
HGK	MSA	Moesin-derived peptide	1000	9.4	10	Mg	5	Staurosporine
HIPK1	MSA	DYRKtide-F	1000	4.4	5	Mg	5	Staurosporine
HIPK2	MSA	DYRKtide-F	1000	5.9	5	Mg	5	Staurosporine
HIPK3	MSA	DYRKtide-F	1000	7.3	5	Mg	5	Staurosporine
HIPK4	MSA	DYRKtide-F	1000	7	5	Mg	5	Staurosporine
IKKα	IMAP	IκBα peptide	100	41	40	Mg	10	Staurosporine
IKKβ	MSA	Modified IκBα-derived peptide	1000	16	25	Mg	5	Staurosporine
IKKε[1]	MSA	IκBα peptide	1000	9.5	10	Mg	5	Staurosporine
IRAK1	IMAP	SRPKtide	100	27	25	Mg	2.5	Staurosporine
IRAK4[16]	MSA	IRAK1 peptide	1000	917	1000	Mg	5	Staurosporine
JNK1	STK-ELISA	ATF2	250	0.66	1	Mg	5	JNK Inhibitor II
JNK2	STK-ELISA	ATF2	250	0.33	1	Mg	5	JNK Inhibitor II
JNK3	STK-ELISA	ATF2	250	0.50	1	Mg	5	JNK Inhibitor II
LATS2[17]	MSA	SGKtide	1000	381.00	400	Mg	5	Staurosporine
LIMK1	STK-ELISA	Cofilin2	250	22	25	Mg	5	Staurosporine
LKB1/MO25α/STRADα	STK-ELISA	LKBtide	250	120	150	Mg	5	Staurosporine
LOK[21]	MSA	Moesin-derived peptide	1000	100	100	Mg	5	Staurosporine
MAP2K1	STK-ELISA	Erk2	100	11	10	Mg	5	Staurosporine
MAP2K2	STK-ELISA	Erk2	100	13	15	Mg	5	Staurosporine
MAP2K3	STK-ELISA	p38α(9-352)	100	0.36	0.5	Mg	10	Staurosporine
MAP2K4[22]	STK-ELISA	JNK1	250	1.6	2	Mg	10	Staurosporine
MAP2K5[23]	STK-ELISA	Erk5	25	1.2	1	Mg	5	Staurosporine
MAP2K6	STK-ELISA	p38α(9-352)	100	0.56	0.5	Mg	10	Staurosporine
MAP2K7[24]	STK-ELISA	JNK1	250	2.7	3	Mg	10	Staurosporine
MAP3K1	STK-ELISA	MAP2K1	85	1.1	1	Mg	40	K252b
MAP3K2	STK-ELISA	MAP2K7	180	0.83	1	Mg	10	Staurosporine

MAP3K3	STK-ELISA	MAP2K7	180	1.6	2	Mg	10	Staurosporine
MAP3K4	STK-ELISA	MAP2K6	200	31	30	Mg	2.5	Staurosporine
MAP3K5	STK-ELISA	MAP2K7	180	2	2	Mg	5	Staurosporine
MAP4K2	MSA	S6k2 peptide	1000	93	100	Mg	5	Staurosporine
MAPKAPK2	MSA	GS peptide	1000	3.6	5	Mg	5	Staurosporine
MAPKAPK3	MSA	GS peptide	1000	13	10	Mg	5	K252b
MAPKAPK5	MSA	GS peptide	1000	12	10	Mg	5	Staurosporine
MARK1	MSA	CHKtide	1000	8	10	Mg	5	Staurosporine
MARK2	MSA	CHKtide	1000	8.8	10	Mg	5	Staurosporine
MARK3	MSA	CHKtide	1000	5	5	Mg	5	Staurosporine
MARK4	MSA	CHKtide	1000	12	10	Mg	5	Staurosporine
MELK[1]	MSA	GS peptide	1000	38	50	Mg	5	Staurosporine
MGC42105	MSA	CHKtide	1000	21	25	Mg	5	Staurosporine
MINK[1]	MSA	Modified Erktide	1000	36	50	Mg	5	K252b
MLK1	STK-ELISA	MAP2K7	180	1.7	2	Mg	5	Staurosporine
MLK2	STK-ELISA	MAP2K7	180	2.8	3	Mg	5	Staurosporine
MLK3	STK-ELISA	MAP2K7	180	5	5	Mg	10	Staurosporine
MNK1	MSA	RS peptide	1000	460	450	Mg	5	Staurosporine
MNK2	MSA	RS peptide	1000	110	100	Mg	5	Staurosporine
MOS	STK-ELISA	MAP2K1 (inactive mutant)	250	10	10	Mg	5	Staurosporine
MRCKα[1]	MSA	DAPKtide	1000	0.45	1	Mg	5	Staurosporine
MRCKβ	MSA	DAPKtide	1000	0.67	1	Mg	5	Staurosporine
MSK1	MSA	Crosstide	1000	13	10	Mg	5	Staurosporine
MSK2[1]	MSA	Crosstide	1000	40	50	Mg	5	Staurosporine
MSSK1[1]	MSA	DYRKtide-P	1000	56	50	Mg	5	Staurosporine
MST1[1][2]	MSA	IRS1	1000	50	50	Mg	5	K252b
MST2[1][2]	MSA	IRS1	1000	69	75	Mg	5	Staurosporine
MST3[1]	MSA	Moesin-derived peptide	1000	66	75	Mg	5	Staurosporine
MST4[1]	MSA	Moesin-derived peptide	1000	76	75	Mg	5	Staurosporine
MYO3A[1]	MSA	MGKtide	1000	12	10	Mg	5	Staurosporine
MYO3B[1]	MSA	MGKtide	1000	7.6	10	Mg	5	Staurosporine
NEK1[1]	MSA	CDK7 peptide	1000	64	75	Mg	5	Staurosporine
NEK2	MSA	CDK7 peptide	1000	65	75	Mg	5	Staurosporine
NEK4	MSA	GS peptide	1000	51	50	Mg	5	Staurosporine
NEK6[1]	MSA	CDK7 peptide	1000	69	75	Mg	5	PKR Inhibitor
NEK7[1]	MSA	CDK7 peptide	1000	40	50	Mg	5	PKR Inhibitor
NEK9[1]	MSA	CDK7 peptide	1000	190	200	Mg	5	Staurosporine
NuaK1	MSA	CHKtide	1000	39	50	Mg	5	Staurosporine
NuaK2	MSA	CHKtide	1000	26	25	Mg	5	Staurosporine
p38α	MSA	Modified Erktide	1000	150	150	Mg	5	SB202190
p38β	MSA	Modified Erktide	1000	63	75	Mg	5	SB202190
p38γ	MSA	Modified Erktide	1000	13	10	Mg	5	Staurosporine
p38δ	MSA	Modified Erktide	1000	5.8	5	Mg	5	Staurosporine
p70S6K	MSA	S6k2 peptide	1000	14	10	Mg	5	Staurosporine
p70S6Kβ	MSA	S6k2 peptide	1000	3.3	5	Mg	5	Staurosporine
PAK1	MSA	LIMKtide	1000	300	300	Mg	5	Staurosporine
PAK2	MSA	DAPKtide	1000	81	100	Mg	5	Staurosporine
PAK3	MSA	DAPKtide	1000	44	50	Mg	5	Staurosporine
PAK4[1]	MSA	SGKtide	1000	2.5	5	Mg	5	Staurosporine

PAK5	MSA	DAPK1tide	1000	1.9	1	Mg	5	Staurosporine
PAK6[3]	MSA	SGKtide	1000	3.7	5	Mg	5	Staurosporine
PASK[3]	MSA	GS peptide	1000	9.7	30	Mg	5	Staurosporine
PBK[3]	MSA	Histone H3 peptide	1000	35	50	Mg	5	Staurosporine
PDHK2[3]	MSA	PDHKtide	1000	28	25	Mg+K	5+3	DCA
PDHK4[3]	MSA	PDHKtide	1000	19	25	Mg+K	5+25	DCA
PDK1[100]	MSA	T308tide	1000	9.6	30	Mg	5	Staurosporine
PEK	IMAP	SRPKtide	100	13	30	Mg	5	Staurosporine
PGK[100]	MSA	Kemptide	1000	8.2	30	Mg	5	Staurosporine
PHKG1[3]	MSA	GS peptide	1000	71	75	Mg	5	Staurosporine
PHKG2	MSA	GS peptide	1000	8.1	30	Mg	5	Staurosporine
PIM1	MSA	86k2 peptide	1000	640	500	Mg	5	Staurosporine
PIM2[3]	MSA	86k2 peptide	1000	4	5	Mg	5	Staurosporine
PIM3	MSA	86k2 peptide	1000	150	150	Mg	5	Staurosporine
PKACa	MSA	Kemptide	1000	2.6	5	Mg	5	Staurosporine
PKACb	MSA	Kemptide	1000	4.7	5	Mg	5	Staurosporine
PKACg[3]	MSA	Kemptide	1000	4.5	5	Mg	5	Staurosporine
PKCa[3]	MSA	PKC q peptide (N-FL)	1000	19	25	Mg+Ca	5+0.05	Staurosporine
PKCb1[3]	MSA	PKC q peptide (N-FL)	1000	16	25	Mg+Ca	5+0.05	Staurosporine
PKCb2[3]	MSA	PKC q peptide (N-FL)	1000	15	30	Mg+Ca	5+0.05	Staurosporine
PKCg[3]	MSA	PKC q peptide (N-FL)	1000	23	25	Mg+Ca	5+0.05	Staurosporine
PKCd[3]	MSA	PKC q peptide (N-FL)	1000	11	30	Mg	5	Staurosporine
PKCe[3]	MSA	PKC q peptide (N-FL)	1000	14	30	Mg	5	Staurosporine
PKCz	MSA	PKC q peptide (N-FL)	1000	4.1	5	Mg	5	Staurosporine
PKCq[3]	MSA	PKC q peptide (N-FL)	1000	27	25	Mg	5	Staurosporine
PKCt[3]	MSA	PKC q peptide (N-FL)	1000	25	25	Mg	5	Staurosporine
PKCi	MSA	PKC q peptide (N-FL)	1000	24	25	Mg	5	Staurosporine
PKD1	MSA	GS peptide	1000	25	25	Mg	5	Staurosporine
PKD2	MSA	GS peptide	1000	26	25	Mg	5	Staurosporine
PKD3	MSA	GS peptide	1000	34	50	Mg	5	Staurosporine
PKN1	IMAP	S6K peptide	100	19	25	Mg	1	Staurosporine
PKR	IMAP	SRPKtide	100	13	30	Mg	5	Staurosporine
PLK1[3]	MSA	CDC25tide	1000	5.6	5	Mg	5	Staurosporine
PLK2	IMAP	CHK2 peptide	50	30	30	Mg	10	K252b
PLK3	MSA	CDC25tide	1000	6.8	5	Mg	5	K252b
PLK4	STK-ELISA	MBP	200	3.3	5	Mg	5	Staurosporine
PRKX[3]	MSA	Kemptide	1000	20	25	Mg	5	Staurosporine
QIK	MSA	AMARA peptide	1000	42	50	Mg	5	Staurosporine
RAF1	STK-ELISA	MAP2K1	85	0.39	0.5	Mg	40	ZM336372
ROCK1	MSA	LIMKtide	1000	3.1	5	Mg	5	Staurosporine
ROCK2	MSA	LIMKtide	1000	7.4	5	Mg	5	Staurosporine
RSK1	MSA	S6K peptide (N-FL)	1000	21	25	Mg	5	Staurosporine
RSK2	MSA	S6K peptide (N-FL)	1000	14	30	Mg	5	Staurosporine

RSK3	MSA	S6K peptide (N-FL)	1000	9.9	10	Mg	5	Staurosporine
RSK4	MSA	S6K peptide (N-FL)	1000	20	25	Mg	5	Staurosporine
SGK	MSA	SGKtide	1000	52	50	Mg	5	Staurosporine
SGK2	MSA	SGKtide	1000	58	50	Mg	5	Staurosporine
SGK3	MSA	SGKtide	1000	17	25	Mg	5	Staurosporine
SIK(SNPHLK)[2]	MSA	AMARA peptide	1000	47	50	Mg	5	Staurosporine
skMLCK[3]	MSA	MLCtide	1000	820	1000	Mg	5	Staurosporine
SLK[2]	MSA	Moesin-derived peptide	1000	36	50	Mg	5	Staurosporine
SRPK1	IMAP	SRPKtide	100	200	100	Mg	10	Staurosporine
SRPK2[2]	MSA	DYRKtide-F	1000	14	10	Mg	5	Staurosporine
TAK1-TAB1	STK-ELISA	MAP2K7	180	10	10	Mg	10	Staurosporine
TAOK2[10]	MSA	TAOKtide	1000	39	50	Mg	5	Staurosporine
TBK1	MSA	CEtide	1000	21	25	Mg	5	Staurosporine
TNIK	MSA	Moesin-derived peptide	1000	16	20	Mg	5	Staurosporine
TSSK1	MSA	GS peptide	1000	11	10	Mg	5	Staurosporine
TSSK2[2]	MSA	GS peptide	1000	8.8	10	Mg	5	Staurosporine
TSSK3[2]	MSA	GS peptide	1000	45	50	Mg	5	Staurosporine
TTK	TK-ELISA	Lyn substrate peptide	250	0.16	0.2	Mn	10	Staurosporine
WEE1	TK-ELISA	CDC2 peptide	250	7.7	10	Mg	5	Staurosporine
WNK1[2]	MSA	SPAKtide	1000	140	150	Mg+Mn	5+3	Staurosporine
WNK2[2]	MSA	SPAKtide	1000	48	50	Mg+Mn	5+3	Staurosporine
WNK3[2]	MSA	SPAKtide	1000	48	50	Mg+Mn	5+3	Staurosporine
PIK3CA/PIK3R1 [7][9]	MSA	Phosphatidyl-inositol	1000	58	50	Mg	5	PI-103
SPHK1	MSA	Sphingosine	1000	20	25	Mg	5	-
SPHK2	MSA	Sphingosine	1000	620	600	Mg	5	-

1) Reaction time is 5 hours.

2) Reaction time is 30 minutes.

3) $CaCl_2$, Calmodulin are added at the final concentration of 1 mM and 10 μg/ml, respectively.

4) cGMP is added at the final concentration of 5 μM.

5) Phosphatidylserine and Diacyl Glycerol are added at the final concentration of 50 μg/mL and 5 μg/mL, respectively.

6) Sodium orthovanadate is added at the final concentration of 25 μM.

7) Cantharidin is added at the final concentration of 10 μM.

8) PIPtide and Cantharidin are added at the final concentration of 2 μM and 20 μM, respectively.

9) Assay buffer is 20 mM HEPES(pH 7.5), 2mM DTT.
Sodium cholate, NaCl and cantharidine are added at the final concentration of 25 μM, 75 mM and 20 μM, respectively.

10) TPX2 peptide is added at the final concentration of 200 nM.

11) Cantharidin is added at the final concentration of 20 μM.

Data Analysis

[0326] The readout value of reaction control (complete reaction mixture) was set as a 0% inhibition, and the readout value of background (Enzyme(-)) was set as a 100% inhibition, then the percent inhibition of each test solution was calculated.

Results

[0327] The results are shown in Table 5 below.

Table 5: Target kinase inhibition by CT352 and staurosporine.

Kinase	% Inhibition CT352 (0.2μM)	% Inhibition Staurosporine (0.2μM)
ABL	-3. 6	68. 8
ABL(E255K)	-3. 7	50. 7
ABL(T315I)	0. 2	93. 8
ACK	88. 5	101. 8
ALK	0. 6	100. 5
ALK(F1174L)	-3. 1	97. 8
ALK(R1275Q)	-0. 6	100. 3
ARG	-3. 3	84. 2
AXL	19. 0	100. 1
BLK	6. 1	102. 2
BMX	5. 5	97. 4
BRK	-5. 4	35. 4
BTK	-2. 0	87. 8
CSK	-2. 6	77. 8
CTK	3. 6	27. 7
DDR1	91. 5	101. 6
DDR2	62. 4	100. 8
EGFR	-4. 2	75. 3
EGFR(d746-750)	-0. 1	96. 0
EGFR(L858R)	3. 6	93. 0
EGFR(L861Q)	-3. 3	73. 3
EGFR(T790M)	49. 8	103. 0
EGFR(d746-750/T790M)	76. 1	102. 3
EGFR(T790M/L858R)	59. 6	103. 1
EML4-ALK	-6. 3	98. 8
EPHA1	-1. 6	93. 5
EPHA2	-5. 0	57. 2
EPHA3	-7. 1	86. 6
EPHA4	-5. 0	77. 3
EPHA5	-6. 2	81. 6
EPHA6	3. 4	97. 7
EPHA7	-6. 2	74. 8
EPHA8	-12. 2	71. 3
EPHB1	-2. 0	77. 0
EPHB2	-5. 4	62. 0
EPHB3	-4. 3	8. 7
EPHB4	-7. 8	44. 5
FAK	-3. 0	89. 9
FER	66. 9	102. 0
FES	21. 0	103. 4
FGFR1	29. 2	99. 0
FGFR2	41. 7	99. 8
FGFR3	15. 8	99. 0
FGFR3(K650E)	25. 9	100. 5
FGFR3(K650M)	32. 8	100. 9
FGFR4	-1. 5	73. 4
FGFR4(N535K)	-2. 3	67. 8
FGFR4(V550E)	-0. 1	43. 4
FGFR4(V550L)	5. 8	95. 0
FGR	17. 0	103. 6

Kinase	% Inhibition CT352 (0.2μM)	% Inhibition Staurosporine (0.2μM)
FLT1	15. 7	99. 6
FLT3	99. 7	105. 4
FLT4	70. 8	101. 7
FMS	23. 9	101. 4
FRK	6. 9	101. 4
FYN	8. 9	98. 9
HCK	25. 9	99. 5
HER2	-2. 7	57. 4
HER4	-12. 5	86. 1
IGF1R	-2. 7	85. 9
INSR	0. 3	95. 6
IRR	-4. 6	96. 7
ITK	6. 5	104. 3
JAK1	10. 3	99. 9
JAK2	83. 8	104. 0
JAK3	101. 9	103. 8
KDR	29. 0	99. 3
KIT	2. 8	103. 0
KIT(D816V)	91. 6	103. 6
KIT(T670I)	29. 5	100. 4
KIT(V560G)	22. 1	98. 7
KIT(V654A)	-6. 9	99. 4
LCK	14. 2	102. 5
LTK	25. 3	101. 4
LYNa	21. 9	99. 9
LYNb	21. 3	100. 5
MER	25. 9	102. 0
MET	-7. 6	73. 6
MET(Y1235D)	-1. 5	69. 0
MUSK	36. 4	101. 6
NPM1-ALK	-1. 8	99. 1
PDGFRa	92. 5	101. 0
PDGFRa(T674I)	96. 8	100. 6
PDGFRa(V561D)	86. 2	101. 3
PDGFRb	94. 9	102. 7
PYK2	22. 7	101. 8
RET	14. 0	101. 0
RET(G691S)	13. 5	100. 1
RET(M918T)	12. 8	100. 2
RET(S891A)	43. 4	101. 7
RET(Y791F)	12. 1	100. 2
RON	-4. 7	84. 8
ROS	76. 1	102. 4
SRC	12. 9	99. 4
SRM	-3. 6	48. 6
SYK	53. 4	103. 6
TEC	-1. 6	88. 9
TIE2	-2. 4	71. 4
TNK1	61. 1	101. 2
TRKA	97. 6	102. 4
TRKB	82. 9	105. 8

Kinase	% Inhibition CT352 (0.2μM)	% Inhibition Staurosporine (0.2μM)
TRKC	90. 8	105. 4
TXK	-8. 6	69. 1
TYK2	14. 7	101. 1
TYRO3	-4. 7	100. 0
YES	24. 2	105. 3
ZAP70	25. 2	101. 1
AKT1	2. 3	98. 1
AKT2	1. 4	96. 7
AKT3	8. 6	96. 9
AMPKa1/b1/g1	16. 2	99. 6
AMPKa2/b1/g1	2. 1	98. 3
AurA	91. 5	101. 6
AurA/TPX2	57. 8	100. 5
AurB	19. 6	99. 4
AurC	62. 4	101. 3
BMPR1A	-2. 1	57. 4
BRAF	8. 6	-64. 3
BRAF(V600E)	-3. 2	-81. 0
BRSK1	35. 7	99. 5
BRSK2	24. 0	102. 7
CaMK1a	-1. 2	89. 8
CaMK1d	1. 7	102. 5
CaMK2a	5. 2	100. 3
CaMK2b	0. 9	100. 4
CaMK2g	11. 0	99. 5
CaMK2d	33. 1	101. 1
CaMK4	-5. 0	41. 9
CDC2	-1. 2	99. 1
CDC7	-7. 1	90. 1
CDK2/CycA2	-3. 9	100. 9
CDK2/CycE1	3. 5	100. 3
CDK3	0. 2	98. 4
CDK4	2. 0	91. 8
CDK5	-2. 9	99. 1
CDK6	-3. 4	75. 0
CDK7	-5. 5	92. 5
CDK9	-4. 1	97. 6
CGK2	95. 4	102. 5
CHK1	-1. 9	99. 9
CHK2	-5. 2	95. 1
CK1a	-2. 3	3. 9
CK1g1	-3. 5	5. 0
CK1g2	-4. 4	4. 6
CK1g3	-4. 4	5. 7
CK1d	-3. 6	3. 6
CK1e	3. 9	0. 8
CK2a1/b	-2. 8	0. 8
CK2a2/b	-4. 8	13. 9
CLK1	11. 1	100. 1
CLK2	1. 0	98. 8
CLK3	-2. 7	15. 6

Kinase	% Inhibition CT352 (0.2μM)	% Inhibition Staurosporine (0.2μM)
COT	2. 9	2. 8
CRIK	-3. 4	76. 1
DAPK1	-1. 7	94. 9
DCAMKL2	-6. 9	83. 8
DLK	-2. 9	22. 5
DYRK1A	11. 7	98. 4
DYRK1B	13. 2	99. 7
DYRK2	0. 2	35. 0
DYRK3	-0. 9	89. 2
EEF2K	0. 9	-0. 3
Erk1	-4. 1	8. 2
Erk2	2. 9	15. 9
Erk5	44. 9	77. 8
GSK3a	9. 1	93. 6
GSK3b	7. 6	94. 9
Haspin	0. 3	97. 2
HGK	48. 6	101. 1
HIPK1	1. 2	25. 0
HIPK2	3. 4	50. 6
HIPK3	2. 3	44. 6
HIPK4	5. 2	66. 5
IKKa	4. 9	41. 3
IKKb	-1. 8	38. 1
IKKe	14. 9	100. 0
IRAK1	9. 4	89. 3
IRAK4	-3. 8	96. 1
JNK1	0. 7	46. 5
JNK2	-1. 2	17. 9
JNK3	-3. 4	38. 9
LATS2	31. 4	100. 9
LIMK1	58. 1	99. 8
LKB1	1. 2	50. 1
LOK	22. 6	101. 4
MAP2K1	12. 8	99. 8
MAP2K2	17. 4	99. 7
MAP2K3	28. 4	99. 9
MAP2K4	1. 2	86. 9
MAP2K5	-25. 2	93. 4
MAP2K6	61. 8	100. 0
MAP2K7	-1. 0	88. 3
MAP3K1	12. 6	-12. 6
MAP3K2	22. 6	98. 4
MAP3K3	-7. 5	92. 1
MAP3K4	-3. 6	65. 4
MAP3K5	-0. 9	94. 4
MAP4K2	17. 1	97. 9
MAPKAPK2	7. 1	60. 0
MAPKAPK3	-3. 8	4. 3
MAPKAPK5	-0. 6	43. 3
MARK1	54. 7	100. 6
MARK2	64. 1	101. 2

Kinase	% Inhibition CT352 (0.2μM)	% Inhibition Staurosporine (0.2μM)
MARK3	63. 9	101. 5
MARK4	63. 1	102. 6
MELK	36. 9	99. 3
MGC42105	-3. 8	45. 2
MINK	14. 6	101. 6
MLK1	79. 6	98. 6
MLK2	13. 0	94. 5
MLK3	91. 9	99. 3
MNK1	-3. 2	86. 3
MNK2	1. 4	95. 8
MOS	3. 0	46. 2
MRCKa	-1. 8	100. 0
MRCKb	-3. 2	99. 1
MSK1	57. 0	100. 8
MSK2	10. 7	97. 3
MSSK1	-4. 0	3. 2
MST1	72. 3	100. 7
MST2	33. 6	101. 0
MST3	-6. 1	97. 7
MST4	-5. 5	96. 6
NDR1	-1. 5	100. 0
NDR2	-2. 7	99. 9
NEK1	-5. 1	65. 3
NEK2	-0. 1	6. 2
NEK4	-4. 0	41. 9
NEK6	-3. 3	1. 1
NEK7	-1. 0	0. 3
NEK9	4. 9	46. 4
NuaK1	73. 2	101. 4
NuaK2	62. 5	101. 0
p38a	-3. 4	-3. 1
p38b	-8. 2	-4. 2
p38g	-6. 5	58. 4
p38d	-5. 0	48. 5
p70S6K	19. 4	99. 9
p70S6Kb	7. 3	99. 7
PAK1	-0. 6	96. 9
PAK2	1. 8	95. 0
PAK3	27. 1	99. 4
PAK4	-10. 1	100. 8
PAK5	5. 7	98. 4
PAK6	4. 1	101. 1
PASK	-2. 5	90. 4
PBK	-3. 7	66. 6
PDHK2	-4. 7	3. 9
PDHK4	-1. 2	-0. 2
PDK1	25. 2	100. 1
PEK	9. 0	19. 3
PGK	74. 4	101. 5
PHKG1	72. 8	99. 4
PHKG2	0. 6	100. 1

Kinase	% Inhibition CT352 (0.2μM)	% Inhibition Staurosporine (0.2μM)
PIM1	7. 8	93. 5
PIM2	6. 1	98. 7
PIM3	6. 9	100. 5
PKACa	27. 9	101. 2
PKACb	30. 0	102. 3
PKACg	1. 0	99. 9
PKCa	59. 1	101. 3
PKCb1	38. 2	102. 6
PKCb2	67. 6	100. 8
PKCg	43. 9	100. 4
PKCd	35. 0	100. 8
PKCe	37. 4	102. 4
PKCz	-3. 3	69. 0
PKCh	13. 0	100. 3
PKCq	17. 6	101. 1
PKCi	11. 6	90. 0
PKD1	1. 6	98. 9
PKD2	2. 7	100. 1
PKD3	3. 1	98. 5
PKN1	83. 3	100. 8
PKR	11. 2	67. 7
PLK1	-6. 2	36. 5
PLK2	-4. 0	27. 0
PLK3	-10. 5	-3. 9
PLK4	42. 4	95. 0
PRKX	0. 0	100. 1
QIK	71. 7	102. 7
RAF1	-5. 3	-120. 7
ROCK1	-3. 5	101. 0
ROCK2	-3. 3	99. 1
RSK1	66. 2	101. 4
RSK2	83. 4	103. 6
RSK3	82. 3	102. 8
RSK4	87. 5	102. 8
SGK	-1. 8	94. 9
SGK2	2. 3	79. 6
SGK3	-1. 2	86. 4
SIK	71. 7	100. 3
skMLCK	2. 1	76. 2
SLK	9. 6	102. 2
SRPK1	2. 9	65. 8
SRPK2	-6. 6	22. 3
TAK1-TAB1	14. 6	90. 6
TAOK2	-6. 7	90. 7
TBK1	47. 2	101. 7
TNIK	36. 8	101. 7
TSSK1	43. 7	99. 1
TSSK2	2. 6	97. 0
TSSK3	-13. 1	79. 4
TTK	-8. 8	30. 8
WEE1	-2. 9	23. 7

Kinase	% Inhibition CT352 (0.2μM)	% Inhibition Staurosporine (0.2μM)
WNK1	-5. 7	-4. 1
WNK2	-6. 1	-2. 0
WNK3	-5. 9	-4. 4
PIK3CA	13. 9	38. 3
SPHK1	15. 4	6. 1
SPHK2	7. 4	2. 0

Example 5: CT352 bioavailability study in mice by intravenous and oral routes

Summary

Study Design

[0328] The purpose of this preliminary study was to investigate the bioavailability of CT352 in mice after a single oral administration, when compared to the intravenous route.

[0329] Two treatment groups, each composed of 27 males, were dosed by the oral or intravenous route with different concentrations of test item.

[0330] Clinical signs and body weight were the investigations performed during the in life phase of the study.

[0331] The pharmacokinetic profile was investigated in 3 animals/time point. Blood samples were collected at 9 time points after treatment as follows:

[0332] *Group 1*: Pre-dose, 10, 20 and 30 minutes, 1, 2, 4, 8 and 24 hours after dosing.

[0333] *Group 2*: Pre-dose, 15, 30 minutes, 1, 2, 4, 6, 8 and 24 hours after dosing.

[0334] The treatment schedule and sample collection are summarized below:

Group Number	Route of administration	Treatment (mg/kg)+	Mouse Numbers M (even)	Bleeding Time min/h post-dose	Mouse Numbers M (even)	Bleeding Time min/h post-dose	Mouse Numbers M (even)	Bleeding Time Hours post-dose
1	Intravenous	10	2, 4, 6 8, 10, 12 14, 16, 18	Pre-dose 10 min 20 min	20, 22, 24 26, 28, 30 32, 34, 36	30 min 1 h 2 h	38, 40, 42 44, 46, 48 50, 52, 54	4 h 8 h 24 h
2	Oral	100	56, 58, 60 62, 64, 66 68, 70, 72	Pre-dose 15 min 30 min	74, 76, 78 80, 82, 84 86, 88, 90	1 h 2 h 4 h	92, 94, 96 98, 100, 102 104, 106, 108	6 h 8 h 24 h
+: in terms of test item as supplied corrected for purity (97.6%)								

Mortality and clinical signs

[0335] No mortality occurred during the study and no signs of reaction to treatment were recorded.

Body weight

[0336] Body weights were within the expected range for this strain and age of animals.

Pharmacokinetic profile

[0337] The pharmacokinetic parameter values calculated from the mean plasma concentrations obtained after a single intravenous and oral administration of CT352 to male CD1 mice are shown in the Table 6 below.

Table 6

Group	Route of administration	Treatment (mg/kg)	C_{max} (ng/mL)	T_{max} (h)	$AUC_{(0-last)}$ (ng/mL*h)	AUC (ng/mL*h)	$t_{1/2\alpha}$ (h)	$t_{1/2\beta}$ (h)
1	Intravenous	10	-	-	24103.8	24178.9	0.6*	10.6*
2	Oral	100	63.26	2	311.81	398.884	3.3	3.3
*: Value obtained by compartmental analysis.								

[0338] Animals receiving a single oral administration of the test item showed C_{max} and AUC values lower than those detected after single intravenous administration.

[0339] On the basis of these results, it can be concluded that CT352, administered to mice after a single oral treatment, has a low bioavailability when compared to the intravenous route, with a relative value of 0.16%.

Introduction

[0340] The purpose of this preliminary study was to investigate the bioavailability of CT352 in mice after a single oral administration, when compared to the intravenous route.

Experimental Procedures

Choice of the species

[0341] The Hsd:ICR (CD-1) mouse was the species and strain of choice because it is accepted by many regulatory authorities and there is ample experience and background data on this species and strain

Choice of the route

[0342] The oral route was selected as it is one of the intended routes of administration of the test item in man. The intravenous route was selected as it presents the least barrier to absorption.

Test item

Identity

[0343] The test item (CT352) had a purity of 97.6% (area/area) and the storage conditions were 18°C, protected from light.

- Formulation procedure
- On the day of dosing, the test item was dissolved as follows:
- Vehicle: Water for injection
- Concentrations: 2 and 10 mg/mL
- Concentrations were calculated and expressed in terms of test item corrected for purity.

Methods

Animal management

Animal supply and acclimatization

Species and strain	:	Mice, Hsd: ICR(CD-1)
Sex	:	Males

Age and weight range (at order)	:	Approximately 4 weeks old, 20 to 22 grams
Supplier	:	Charles River Laboratories S.r.l., Calco (Lecco),
Breeder	:	Charles River Laboratories S.r.l., Calco (Lecco),
Date of arrival	:	07 March 2013
Weight range at arrival	:	20.9 to 22.4 grams
Acclimatisation period	:	6 days
Veterinary health check	:	After arrival

Caging

[0344] *No. of animals/cage*: up to 5, during acclimatisation, up to 3, during the study.

[0345] *Housing*: Polysulphone solid bottomed cages measuring 35.5x23.5x19 cm, with nesting material.

[0346] *Cage tray control*: Daily inspected and changed as necessary (at least 2 times/week).

Water and diet

[0347] *Water*: drinking water supplied to each cage via a water bottle.

[0348] Water supply: ad libitum

[0349] *Diet*: 4 RF21 (Mucedola S.r.l., Via G. Galilei, 4, 20019, Settimo Milanese (MI) Italy)

[0350] *Diet supply*: ad libitum throughout the study except for oral dosing procedure indicated in the Dosing section below.

[0351] Components present in the drinking water or diet are not at a level likely to interfere with the purpose or conduct of the study.

Housing conditions (parameter set)

Room lighting:	:	Artificial (fluorescent tubes), daily light/dark cycle of 12/12 hours
Air changes:	:	Approximately 15 to 20 air changes per hour
Temperature range:	:	22°C ± 2°C
Relative humidity range:	:	55% ± 15%

[0352] Actual conditions were monitored and recorded. No relevant deviations occurred.

Experimental Design

Allocation to treatment groups

Group composition

[0353] The required number of animals (27 males/group) was allocated to the study as follows:

Group Number	Route of administration	Treatment (mg/kg)+	Mice Numbers M (even)
1	Intravenous	10	2-54
2	Oral	100	56-108
+: in terms of test item as supplied corrected for purity (97.6%)			

Selection/Allocation

[0354] Selection/allocation was performed by computerised stratified randomisation to give approximately equal initial group mean body weights. The mouse numbers listed above formed the last digits of a computer generated 8 figure animal number (the remaining digits of the animal number were different for each concurrent study and served to ensure unique animal numbering for any study employing computerised data collection). The computerised system used in this study was the Xybion Path/Tox System, Version 4.2.2.

Animal Identification

[0355] Animal identification was carried out by a coloured mark on the tail, after arrival, and by ear notch and tattoo on the hind feet.

Dosing

[0356] Dosing was performed as follows:

Frequency of treatment	:	Once only, on the day of dosing (Day 1).
Dose calculation	:	Group 1: dose volume of 5 mL/kg of body weight for each animal. Group 2: dose volume 10 mL/kg of body weight for each animal.

Dosing methods	:	Group 1: by intravenous injection into the tail vein, at an approximate rate of 3 mL/minute, using a hypodermic needle attached to a syringe of suitable capacity. Group 2: by gavage, using a feeding plastic tube attached to a graded syringe.

In Life Observations

[0357] In life observations were performed as follows:

Mortality and morbidity	:	Twice daily.
Clinical signs	:	Day of dosing (prior to dosing and immediately after dosing).
Body weight	:	Allocation (Day –1) and on the day of dosing (Day 1).

Pharmacokinetic profile

Sample collection and analysis

- Blood samples were collected at 9 time points, as specified below.
- Group 1
- Pre-dose, 10, 20 and 30 minutes, 1, 2, 4, 8 and 24 hours after dosing.
- Group 2
- Pre-dose, 15, 30 minutes, 1, 2, 4, 6, 8 and 24 hours after dosing.

[0358] At each sampling time, approximately 0.5 mL blood samples were withdrawn under isofluorane anaesthesia from the abdominal vena cava of 3 animals of each group, each animal being sampled at one time point.

[0359] Samples were transferred into tubes containing K3EDTA anticoagulant, centrifuged at room temperature and the plasma frozen at –20°C pending analysis.

[0360] The plasma concentrations of CT352 were analysed based on a LC-Fluo method.

Assessment of pharmacokinetic data

[0361] The pharmacokinetic analysis was conducted according to a standard non-compartmental and compartmental analysis. The following pharmacokinetic parameters were obtained or calculated from the mean plasma values:

C_{max}	:	maximum (peak) observed concentration;	[ng/mL]
t_{max}	:	time to reach peak or maximum concentration;	[h]
$t_{1/2\alpha}$	:	distribution half-life;	
$t_{1/2\beta}$	:	elimination half-life associated with terminal slope (λ_z) of a semilogarithmic concentration-time curve;	[h]
$AUC_{(0\text{-}last)}$	:	area under the concentration-time curve calculated by the linear trapezoidal rule, from t=0 (pre-dose) to t_{last} (last quantifiable concentration);	[ng/mL•h]
AUC	:	area under the plasma concentration-time curve from time zero to infinity.	[ng/mL•h]

[0362] All the pharmacokinetic parameters were estimated or calculated by the Kinetica™, version 4.4.1, PK/PD Analysis (Thermo Electron Corporation Informatics, Philadelphia - USA) software.

[0363] Means and/or medians, standard deviations and coefficient of variations were obtained using a Microsoft Excel worksheet.

[0364] The results of the pharmacokinetic evaluation are presented in the Pharmacokinetic profile section below.

Terminal Studies

Termination: Day 2.

[0365] *Euthanasia method*: By exsanguination under isofluorane anaesthesia.

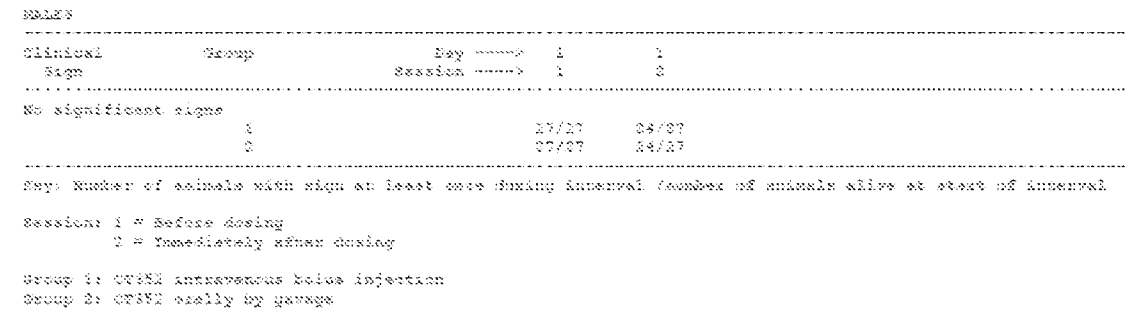
[0366] *Necropsy procedure*: No necropsy was performed.

Results

Mortality and clinical signs

[0367] No death occurred and no reaction to treatment was observed in the animals at the observations performed on the day of dosing (Table 7).

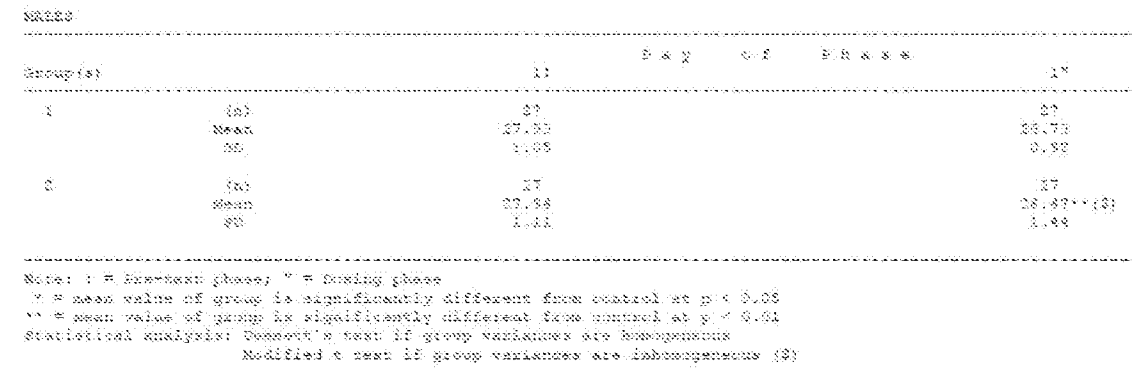
Table 7: Clincal signs – Group incidence



Body weight

[0368] Body weights were within the expected range for this strain and age of animals (Table 8).

Table 8: Body weight (g) – Group mean data



Pharmacokinetic profile

[0369] In Table 6 above, the kinetic parameter values for CT352, obtained after single intravenous administration at dose level of 10 mg/kg (Group 1) and after a single oral administration at 100 mg/kg (Group 2) are shown.

[0370] Comparing the two groups treated with the test item, the maximum plasma concentration (C_{max}) of CT352 was achieved at 2 hours after dosing in the animals of Group 2, receiving a single oral administration of CT352 at dose level of 100 mg/kg.

[0371] The analysis revealed low values of C_{max} and AUC following oral administration of CT352, when compared to the intravenous route. In fact, the relative bioavailability [$(AUC_{po}/Dose_{po})/(AUC_{IV}/Dose_{IV}).100\%$] for the oral route, calculated using

the intravenous route as reference, was 0.16%. Therefore, it can be concluded that CT352 administered to mice after a single oral treatment showed a low bioavailability.

Conclusion

[0372] The bioavailability of CT352 in mice after a single oral administration, when compared to the intravenous route, was investigated in this study.

[0373] Two treatment groups, each composed of 27 males, were included. The first group received a single intravenous administration of CT352 at a dose level of 10 mg/kg, while the second group was treated with a single oral administration of the test item at 100 mg/kg.

[0374] No mortality occurred and no signs of reaction to treatment were observed on the day of dosing. Body weights were within the expected range for this strain and age of animals.

[0375] The kinetic evaluation showed that CT352, administered to mice after a single oral treatment, has a low bioavailability when compared to the intravenous route, with a relative value of 0.16%.

Example 6: CT352 efficacy study in a DSS-induced murine acute colitis model

Aim of Study

[0376] The purpose of the present study was to evaluate the efficacy of CT352 to counteract acute colitis by repeated oral administration for seven consecutive days overlapping with the five days-DSS treatment in mice. Control groups were untreated, treated with test article vehicle (water) or with cyclosporine A (CsA, positive control).

[0377] This report shows the results of the in vivo experiments and the data obtained from ex vivo quantitative real time (qRT)-PCR and histology analysis performed on colon samples collected from all the groups.

Materials and Methods

Experimental Design

[0378] The experimental protocol was modified from Eckmann et al, PNAS 2008. Male Balb/c mice, 7-9 weeks old, with a mean 25 gr weight, were used for this experiment. Mice were kept in the animal house facility at the Molecular Biotechnology Center under standard conditions of temperature and light, and were fed with standard

laboratory chow and water ad libitum. Procedures were conducted in conformity with national and international laws and policies as approved by the Faculty Ethical Committee.

[0379] Mice were divided into the following experimental groups:

Group	Treatment	Animal ID
1 - Untreated	none	43-32-36
2 - Vehicle	3% DSS in drinking water + water by gavage	23-33-41-46-48-49-52
3 – CT100	3% DSS in drinking water + 100 mg/kg CT352 by gavage	21-35-37-39-31-47-51
4 – CT300	3% DSS in drinking water + 300 mg/kg CT352 by gavage	19-20-22-24-28-29-38
5 – Cyclosporine A	3% DSS in drinking water + 25 mg/kg CsA by gavage	26-34-40-42-45-50-53

[0380] 3% Dextrane Sodium Sulfate (DSS) (MP Biomedicals, m.w 35,000-50,000) in drinking water was administered for 5 days to all groups except group one, followed by regular water for further two days and sacrifice. Mice were daily monitored for colitis features (weight, bleeding and stool consistency). Mice colons were excised at sacrifice from the ileo-cecal valve to the rectum, rinsed with PBS, their length was measured as an indirect marker of inflammation and 0.5 cm sections from both the distal and the proximal colon ends were cut and snap frozen for the subsequent RNA analysis. The remaining colon was prepared as “Swiss Roll” for histological analysis (Moolenbeek and Ruitenberg Lab. Anim. 1981).

Colitis monitoring

[0381] The clinical course of the disease was followed daily and represented by colitis clinical score, that account for body weight, stool consistency and rectal bleeding. The colitis clinical score ranged from 0 to 12 and was the sum of scores given for body weight loss (scored as: 0, none; 1, 1–5%; 2, 5–10%; 3, 10–20%; 4, over 20%), stool consistency (scored as: 0, well formed pellets; 2, pasty and semiformed stools; 4, liquid stools) and presence or absence of fecal blood (scored as: 0, negative; 2, light bleeding; 4, gross bleeding). Accordingly, the clinical score ranged from 0 (healthy) to 12 (maximal activity of colitis).

Quantitative Real-time PCR

[0382] Total RNA was extracted from 0.5 cm proximal colon and 0.5 cm distal colon taken from sacrificed mice with the PureLink Micro-to-Midi total RNA Purification System (Invitrogen, Carlsbad, CA, USA) and its concentration was determined by

NanoDrop™ 1100 (NanoDrop Technologies, Wilmington, DE, USA). Samples were treated with DNase IAmp Grade (Invitrogen) before retrotranscription to eliminate genomic DNA contamination. To produce template cDNA, 125 ng of total RNA was reverse-transcribed using the RT High Capacity kit (Applied Biosystems) according to the manufacturer's protocol. qRT-PCR reactions were performed with the ABI Prism 7300 real-time PCR System (Applied Biosystems) using Platinum Quantitative PCR SuperMix-UDG with ROX (Invitrogen). The reactions were carried out in a total volume of 20 µl. The cytokines and chemokines were detected by using the Universal Probe Library system (Roche Italia, Monza, Italy) with the following primers: IL6, 5'-gctaccaaactggatataatcagga-3'; 5'-ccaggtagctatggtactccagaa-3'; CCL2, 5'-catccacgtgttggctca-3'; 5'-gatcatcttgctggtgaatgagt-3'; MIP2, 5'-aaaatcatccaaaagatactgaacaa-3'; 5'-ctttggttcttccgttgagg-3'; IFNg, 5'-atctggaggaactggcaaaa-3'; 5'-ttcaagacttcaaagagtctgaggta-3'; COX2, 5'-gatgctcttccgagctgtg-3'; 5'-ggattggaacagcaaggattt-3'; IL10, 5'-cagagccacatgctcctaga-3'; 5'-tgtccagctggtcctttgtt-3'; TNFa, 5'-tcttctcattcctgcttgtgg-3'; 5'-ggtctgggccatagaactga-3'; TGFb, 5'-tggagcaacatgtggaactc-3'; 5'-cagcagccggttaccaag-3'; IL1b, 5'-agttgacggaccccaaaag-3'; 5'-agctggatgctctcatcagg-3'; IL17, 5'-tgtgaaggtcaacctcaaagtc-3'; 5'-agggatatctatcagggtcttcatt-3'; MIP1a, 5'-agattccacgccaattcatc-3'; 5'-gccggtttctcttagtcagga-3'. Results were analysed with the $2^{-\Delta\Delta Ct}$ method using the 18S rRNA pre-developed TaqMan assay (Applied Biosystem) as an internal control. Expression of the target genes was calibrated against control non-colitic animals (group one, untreated mice).

Histological analysis

[0383] For histological analysis of the colon, samples were rinsed with PBS, prepared as "Swiss Roll", fixed overnight in formalin and then embedded in paraffin. Sections 5 µm in thickness were stained with haematoxylin/eosin and blinded analyzed by a pathologist who assessed the severity of colitis, expressed as a histology score that accounts for infiltration of inflammatory cells and epithelial structure, in a scale of 0 to 6: 0 = no damage; 1 = few inflammatory cells, no signs of epithelial degeneration; 2 = mild inflammation, few signs of epithelial degeneration; 3 = moderate inflammation, few epithelial ulcerations; 4 = moderate to severe inflammation, ulcerations in more than 25% of the tissue section; 5 = moderate to severe inflammation, large ulcerations of more than 50% of the tissue section; 6 = severe inflammation and ulcerations of more than 75% of the tissue section.

Statistical analysis

[0384] Data are expressed as mean ± S.D. An unpaired *t* test was used to calculate a *P* value for two groups. A *P* value of < 0.05 was considered statistically significant (GraphPad Software Inc., San Diego, CA, USA).

Results and Discussion

DSS-induced colitis evaluation

[0385] After five days-treatment with 3% DSS, mice were left two more days drinking water to recover before sacrifice. At sacrifice, colons were excised from the ileo-cecal valve to the rectum, abundantly rinsed with PBS and their lengths were measured, as an indirect marker of inflammation. As shown in Fig. 37a, the vehicle group displayed a reduction of colon length compared to the untreated group, due to a more prominent inflammatory response. Colon length increased in mice treated with CT352, both doses, and in mice treated with CsA, indicating that inflammation was reduced in these 3 groups in comparison with the vehicle group. None of these differences were statistically significant. During the experiment, mice were daily weighed and the weight loss of each day was compared to the initial weight of each mouse, to get the weight percentage reduction/increase. Considering the overall trend of each experimental group, the untreated mice increased their weight during the experiments, while the vehicle group animals lost weight during DSS treatment and only few animals started to recover weight right after the stimulus removal, while the majority of mice continued to lose weight or stayed stable. On the contrary, all DSS-treated mice lost weight during colitis induction, but while after DSS removal the vehicle group did not recover completely the weight, mice belonging to CT100, CT300 or CsA groups started to recover weight right after it (Fig. 37b). These data indicate that CT352 or CsA treatment lead to a quicker weight improvement than only vehicle administration. A more general and complete evaluation of the colitis response was given by the colitis clinical score that takes account not only of weight loss but also of bleeding and stool consistency. Untreated mice obviously displayed a minimal colitis clinical score, while vehicle group, although quite disomogeneous, showed a high score. Interestingly, CT352 clearly reduced the clinical colitis score in a dose-response manner, and its effect was more prominent than CsA's one (Fig. 37c).

[0386] These data indicate that vehicle group developed colitis compared to untreated group, and that the water gavage had no effect on colitis. On the contrary mice receiving CsA at the same time, developed a milder colitis (increased colon length, weight recovery after stimulus removal and low mean clinical colitis score), as shown by

previous papers. CT352 administration ameliorated colitis at both doses and with a dose-response when considering the colitis clinical score.

qRTPCR analysis of inflammatory cytokines and chemokines expression

[0387] The colonic expression of many cytokines and chemokines involved in the inflammatory process was analyzed by qRTPCR. We observed an increased expression of IL6, MIP1a and MIP2 in the vehicle group compared to the untreated group while CT352 treatment decreased their expression in a dose-dependent manner (Fig.38). Also IL17 expression was increased in the vehicle group and CT352 had no effect on reducing its expression. On the other hand the colonic levels of other inflammation-related mRNAs was affected neither by DSS alone nor by CT352 (IL1b, TNFa, CCL2, COX2, TGFb). We also analyzed IFNg expression that was expected to increase after DSS treatment, but instead, it was significantly decreased in vehicle group. Finally, IL10 expression was similar between untreated and vehicle group while it decreased in CT352 treated mice, maybe because a diminished inflammation could mediate a diminished expression of IL10. In many cases the high values obtained in the CsA group are due to one/two outlier mice that displayed an exacerbated response to the DSS treatment. These results suggest that while DSS treatment alone resulted in an inflammatory response to the colon, this inflammatory response was counteracted by the administration of CT352 or CsA at the same time.

Histological analysis of colon samples

[0388] At sacrifice, colons were excised and prepared for subsequent histological analysis. A blinded pathologist evaluated the degree of inflammatory infiltrate, epithelial damage and damage extension and attributed to each sample a colitis clinical score, reported on Fig. 39a. Control mouse colon sections showed intact epithelium, well defined crypts, no infiltrate and no ulcers or erosions and the mean score was 0 (Fig. 39a and 39b). DSS-treated mice showed severe inflammation throughout the mucosa, wide ulcers, shortening or complete loss of crypts, and an overall damage involving more than 75% of the total colon, being the distal colon the more involved part (Fig. 39b). This lead to a higher score in the majority of mice with the only exception of two mice showing an almost intact and not infiltrated mucosa (Fig. 39a). In CT352-treated mice, both infiltrate and epithelial alterations (ulcers, crypts loss, ...) were reduced, there was not a single damaged area involving the whole distal colon but from one to three limited damaged areas were detectable in the distal colon, all with the same damage degree. CT300 group displayed a milder histological colitis than the CT100 group (Fig. 39a and 39b). Five out of seven CsA-treated mice showed no evident

colitis features in their colon, only two mice (the same where cytokines and chemokines expression were increased) showed wide epithelial damage and massive inflammatory cells infiltration in the distal part of their colons (Fig. 39a and 39b).

Conclusions

[0389] These data demonstrate that CT352 administered at the same time with DSS reduced the colitis degree in this acute colitis model, with results similar and sometimes better that CsA, a treatment already known to be efficacious against DSS-induced colitis.The results obtained so far indicate that CT352 treatment could efficaciously counteract the DSS-induced colitis, leading to a reduced mucosa damage and to an almost completely absent manifestation of clinical symptoms such as weight loss, bleeding, and diarrhea.

Example 7: BioMAP Platform Analysis of SNA-352

Aim of Study

[0390] The goal of this study was to characterize SNA-352 in the BioMAP Diversity PLUS panel of 12 human primary cell-based systems. These systems are designed to model complex human tissue and disease biology of the vasculature, skin, lung and inflammatory tissues. Quantitative measurements of biomarker activities across this broad panel, along with comparative analysis of the biological activities of known bioactive agents in the BioMAP reference database are used to predict the safety, efficacy and function of these test agents.

Overview of BioMAP Technology Platform

[0391] BioMAP panels consist of human primary cell-based systems designed to model different aspects of the human body in an in vitro format. The 12 systems in the Diversity PLUS panel allow test agent characterization in an unbiased way across a broad set of systems modeling various human disease states. BioMAP systems are constructed with one or more primary cell types from healthy human donors, with stimuli (such as cytokines or growth factors) added to capture relevant signaling networks that naturally occur in human tissue or pathological conditions. Vascular biology is modeled in both a Th1 (3C system) and a Th2 (4H system) inflammatory environment, as well as in a Th1 inflammatory state specific to arterial smooth muscle

cells (CASM3C system). Additional systems recapitulate aspects of the systemic immune response including monocyte-driven Th1 inflammation (LPS system) or T cell stimulation (SAg system), chronic Th1 inflammation driven by macrophage activation (/Mphg system) and the T cell-dependent activation of B cells that occurs in germinal centers (BT system). The BE3C system (Th1) and the BF4T system (Th2) represent airway inflammation of the lung, while the MyoF system models myofibroblast-lung tissue remodeling. Lastly, skin biology is addressed in the KF3CT system modeling Th1 cutaneous inflammation and the HDF3CGF system modeling wound healing.

[0392] Each test agent generates a signature BioMAP profile that is created from the changes in protein biomarker readouts within individual system environments. Biomarker readouts (7 - 17 per system) are selected for therapeutic and biological relevance, are predictive for disease outcomes or specific drug effects and are validated using agents with known mechanism of action (MoA). Each readout is measured quantitatively by immune-based methods that detect protein (e.g., ELISA) or functional assays that measure proliferation and viability. BioMAP readouts are diverse and include cell surface receptors, cytokines, chemokines, matrix molecules and enzymes. In total, the Diversity PLUS panel contains 148 biomarker readouts that capture biological changes that occur within the physiological context of the particular BioMAP system.

[0393] Using custom-designed software containing data mining tools, a BioMAP profile can be compared against a proprietary reference database of > 4,000 BioMAP profiles of bioactive agents (biologics, approved drugs, chemicals and experimental agents) to classify and identify the most similar profiles. This robust data platform allows rapid evaluation and interpretation of BioMAP profiles by performing the unbiased mathematical identification of similar activities. Specific BioMAP activities have been correlated to in vivo biology, and multiparameter BioMAP profiles have been used to distinguish compounds based on MoA and target selectivity and can provide a predictive signature for in vivo toxicological outcomes (e.g., vascular toxicity, developmental toxicity, etc.) across diverse physiological systems.

Materials and Methods

Test Agent

[0394] SNA-352 was profiled in the BioMAP Diversity PLUS panel at concentrations of 3900 nM, 1300 nM, 430 nM, and 140 nM. Cyclosporin A was employed as the benchmark compound.

Methods for Diversity PLUS

[0395] Human primary cells in BioMAP systems are used at early passage (passage 4 or earlier) to minimize adaptation to cell culture conditions and preserve physiological signaling responses. All cells are from a pool of multiple donors (n = 2 to 6), commercially purchased and handled according to the recommendations of the manufacturers. Human blood derived CD14+ monocytes are differentiated into macrophages in vitro before being added to the /Mphg system. Abbreviations are used as follows: Human umbilical vein endothelial cells (HUVEC), Peripheral blood mononuclear cells (PBMC), Human neonatal dermal fibroblasts (HDFn), B cell receptor (BCR), T cell receptor (TCR) and Toll-like receptor (TLR).

[0396] Cell types and stimuli used in each system are as follows: 3C system [HUVEC + (IL-1β, TNFα and IFNγ)], 4H system [HUVEC + (IL-4 and histamine)], LPS system [PBMC and HUVEC + LPS (TLR4 ligand)], SAg system [PBMC and HUVEC + TCR ligands], BT system [CD19+ B cells and PBMC + (α-IgM and TCR ligands)], BF4T system [bronchial epithelial cells and HDFn + (TNFα and IL-4)], BE3C system [bronchial epithelial cells + (IL-1β, TNFα and IFNγ)], CASM3C system [coronary artery smooth muscle cells + (IL-1β, TNFα and IFNγ)], HDF3CGF system [HDFn + (IL-1β, TNFα, IFNγ, EGF, bFGF and PDGF-BB)], KF3CT system [keratinocytes and HDFn + (IL-1β, TNFα and IFNγ)], MyoF system [differentiated lung myofibroblasts + (TNFα and TGFβ)] and /Mphg system [HUVEC and M1 macrophages + Zymosan (TLR2 ligand)].

[0397] Systems are derived from either single cell types or co-culture systems. Adherent cell types are cultured in 96 or 384-well plates until confluence, followed by the addition of PBMC (SAg and LPS systems). The BT system consists of CD19+ B cells co-cultured with PBMC and stimulated with a BCR activator and low levels of TCR stimulation. Test agents prepared in either DMSO (small molecules; final concentration ≤ 0.1%) or PBS (biologics) are added at the indicated concentrations 1-hr before stimulation, and remain in culture for 24-hrs or as otherwise indicated (48-hrs, MyoF system; 72-hrs, BT system (soluble readouts); 168-hrs, BT system (secreted IgG)). Each plate contains drug controls (e.g., legacy control test agent colchicine at 1.1 μM), negative controls (e.g., non-stimulated conditions) and vehicle controls (e.g., 0.1% DMSO) appropriate for each system. Direct ELISA is used to measure biomarker levels of cell-associated and cell membrane targets. Soluble factors from supernatants are quantified using either HTRF® detection, bead-based multiplex immunoassay or capture ELISA. Overt adverse effects of test agents on cell proliferation and viability (cytotoxicity) are detected by sulforhodamine B (SRB) staining, for adherent cells, and alamarBlue® reduction for cells in suspension. For proliferation assays, individual cell types are cultured at subconfluence and measured at time points optimized for each system (48-

hrs: 3C and CASM3C systems; 72-hrs: BT and HDF3CGF systems; 96-hrs: SAg system). Cytotoxicity for adherent cells is measured by SRB (24-hrs: 3C, 4H, LPS, SAg, BF4T, BE3C, CASM3C, HDF3CGF, KF3CT, and lMphg systems; 48-hrs: MyoF system), and by alamarBlue staining for cells in suspension (24-hrs: SAg system; 42-hrs: BT system) at the time points indicated. Additional information can be found in previous descriptions.

Data Analysis

[0398] Biomarker measurements in a test agent-treated sample are divided by the average of control samples (at least 6 vehicle controls from the same plate) to generate a ratio that is then log10 transformed. Significance prediction envelopes are calculated using historical vehicle control data at a 95% confidence interval.

Profile Analysis

[0399] Biomarker activities are annotated when 2 or more consecutive concentrations change in the same direction relative to vehicle controls, are outside of the significance envelope and have at least one concentration with an effect size > 20% (|log10 ratio| > 0.1). Biomarker key activities are described as modulated if these activities increase in some systems, but decrease in others. Cytotoxic conditions are noted when total protein levels decrease by more than 50% (log10 ratio of SRB or alamarBlue levels < -0.3) and are indicated by a thin black arrow above the X-axis. A compound is considered to have broad cytotoxicity when cytotoxicity is detected in 3 or more systems. Concentrations of test agents with detectable broad cytotoxicity are excluded from biomarker activity annotation and downstream benchmarking, similarity search and cluster analysis. Antiproliferative effects are defined by an SRB or alamarBlue log10 ratio value < -0.1 from cells plated at a lower density and are indicated by grey arrows above the X-axis. Cytotoxicity and antiproliferative arrows only require one concentration to meet the indicated threshold for profile annotation.

Benchmark Analysis

[0400] Common biomarker readouts are annotated when the readout for both profiles is outside of the significance envelope with an effect size > 20% in the same direction. Differentiating biomarkers are annotated when one profile has a readout outside of the significance envelope with an effect size > 20%, and the readout for the other profile is either inside the envelope or in the opposite direction. Unless specified, the top non-cytotoxic concentration of both the test agent and benchmark agent are included in the benchmark overlay analysis.

Similarity Analysis

[0401] Common biomarker readouts are annotated when the readout for both profiles is outside of the significance envelope with an effect size > 20% in the same direction. Concentrations of test agents that have 3 or more detectable systems with cytotoxicity are excluded from similarity analysis. Concentrations of test agents that have 1 – 2 systems with detectable cytotoxicity will be included in the similarity search analysis, along with an overlay of the database match with the top concentration of the test agent. This will be followed by an additional overlay of the next highest concentration of the test agent containing no systems with detectable cytotoxicity and the respective database match. To determine the extent of similarity between BioMAP profiles of compounds run in the Diversity PLUS panel, we have developed a custom similarity metric (BioMAP Z-Standard) that is a combinatorial approach that has improved performance in mechanism classification of reference agents compared to other measures tested (including Pearson's and Spearman's correlation coefficients). This approach more effectively accounts for variations in the number of data points, systems, active biomarker readouts and the amplitude of biomarker readout changes that are characteristic features of BioMAP profiles. A Pearson's correlation coefficient (r) is first generated to measure the linear association between two profiles that is based on the similarity in the direction and magnitude of the relationship. Since the Pearson's correlation can be influenced by the magnitude of any biomarker activity, a per-system weighted average Tanimoto metric is used as a filter to account for underrepresentation of less robust systems. The Tanimoto metric does not consider the amplitude of biomarker activity, but addresses whether the identity and number of readouts are in common on a weighted, per system basis. A real-value Tanimoto metric is calculated first by normalizing each profile to the unit vector (e.g., $A = \frac{A}{\|A\|}$) and then applying the following formula: $\frac{A \cdot B}{\|A\| + \|B\| - A \cdot B}$, where A and B are the 2 profile vectors. Then, it is incorporated into a system weighted-averaged real-value Tanimoto metric in this calculation: $\frac{\sum W_i \cdot T_i}{\sum W_i}$. The calculation uses the real-value Tanimoto score for each *i*th system (T_i) and the weight of each *i*th system (W_i). W_i is calculated for each system in the following formula: $\frac{1}{1 + e^{-100 * (lr - 0.09)}}$, where *lr* is the largest absolute value of the ratios from the 2 profiles being compared. Based on the optimal performance of

reference compounds, profiles are identified as having mechanistically relevant similarity if the Pearson's correlation coefficient (*r*) ≥ 0.7. Finally, a Fisher r-to-z-transformation is used to calculate a z-score to convert a short tail distribution into a normal distribution as follows: $z = 0.5 \log_{10} \frac{1+r}{1-r}$. Then the BioMAP Z-Standard, which adjusts for the number of common readouts (CR), is generated according to the following formula: Z-Standard = $z \sqrt{CR-3}$. A larger BioMAP Z-Standard value corresponds to a higher confidence level, and this is the metric used to rank similarity results.

Cluster Analysis

[0402] Cluster analysis (function similarity map) uses the results of pairwise correlation analysis to project the "proximity" of agent profiles from multi-dimensional space into two dimensions. Functional clustering of the agent profiles generated during this analysis uses Pearson correlation values for pairwise comparisons of the profiles for each agent at each concentration, and then subjects the pairwise correlation data to multidimensional scaling. Profiles that are similar with a Pearson's correlation coefficient (r) ≥ 0.7 are connected by lines. Agents that do not cluster with one another are interpreted as mechanistically distinct. This analysis is performed for projects with 3 or more agents tested. Cytotoxic concentrations are excluded from cluster analysis.

Mechanism HeatMAP Analysis

[0403] Mechanism HeatMAP analysis provides a visualization of the test compound and 19 consensus mechanisms allowing comparison of biomarker activities across all compound concentrations and consensus mechanisms. The synthetic consensus profiles used in the Mechanism HeatMAP analysis are representative BioMAP profiles of the average of multiple compounds from structurally distinct chemical classes. Profiles were calculated by averaging the values for each biomarker endpoint for all profiles selected (multiple agents at different concentrations) to build the consensus mechanism profile.[8] Biomarker activities are colored in the heatmap for consensus mechanisms and compounds when they have expression relative to vehicle controls outside of the significance envelope. Red represents increased protein expression, blue represents decreased expression and white indicates levels that were unchanged or within filtering conditions. Darker shades of color represent greater change in biomarker activity relative to vehicle control. The Mechanism HeatMAP was prepared using R and the gplots package for R.

Assay Acceptance Criteria

[0404] A BioMAP assay includes the multi-parameter data sets generated by the BioMAP platform for agents tested in the systems that make up the Diversity PLUS panel. Assays contain drug controls (e.g., legacy control test agent colchicine), negative controls (e.g., non-stimulated conditions), and vehicle controls (e.g., DMSO) appropriate for each system. BioMAP assays are plate-based, and data acceptance criteria depend on both plate performance (% CV of vehicle control wells) and system performance across historical controls for that system. The QA/QC Pearson Test is performed by first establishing the 1% false negative Pearson cutoff from the reference dataset of historical positive controls. The process iterates through every profile of system biomarker readouts in the positive control reference dataset, calculating Pearson values between each profile and the mean of the remaining profiles in the dataset. The overall number of Pearson values used to determine the 1% false negative cutoff is the total number of profiles present in the reference dataset. The Pearson value at the one percentile of all values calculated is the 1% false negative Pearson cutoff. A system will pass if the Pearson value between the experimental plate's negative control or drug control profile and the mean of the historical control profiles in the reference dataset exceeds this 1% false negative Pearson cutoff. Overall assays are accepted when each individual system passes the Pearson test and 95% of all project plates have % CV <20%.

Results

BioMAP Profile

[0405] Figure 40 depicts the BioMAP profile of SNA-352 in the Diversity PLUS Panel. SNA-352 was found to be active with 8 annotated readouts, mediating changes in key biomarker activities listed by biological and disease classifications in Table 9 below. SNA-352 impacted inflammation-related activities (decreased SAA, sTNFα), immunomodulatory activities (decreased sIgG, sIL-10; increased CD69), and tissue remodeling activities (decreased TIMP-2, Collagen IV; increased uPAR).

Table 9: Key Biomarker Activities Impacted by SNA-352

Readouts in the Diversity Reference Category	Decreased activity	Increased activity
Inflammation-related activities	SAA, sTNFα	
Immunomodulatory activities	sIgG, sIL-10	CD69
Tissue remodeling activities	Coll-IV, TIMP2	uPAR

[0406] SNA-352 is antiproliferative to B cells, coronary artery smooth muscle cells, fibroblasts, and T cells (indicated by grey arrows in Figure 40).

Reference Benchmark Overlay

[0407] Figure 41 depicts an overlay of SNA-352 at 3.9 μM and the selected reference benchmark Cyclosporin A at 3.3 μM. Cyclosporin A is a calcineurin inhibitor widely used in organ transplantation to prevent rejection. There are 6 common activities that are annotated within the following systems: SAg (Prolif), BT (Prolif, sIL-17A, sIgG, sTNFα), and HDF3CGF (Prolif 72).

[0408] Differentiating biomarkers (not shown) are defined when one profile has a readout outside of the significance envelope with an effect size > 20% (|log10 ratio| > 0.1), and the readout for the other profile is either inside the envelope or in the opposite direction. There are 21 differentiating activities between the two compounds: 3C (uPAR), 4H (MCP-1, VCAM-1), LPS (sPGE2), SAg (CD38, CD40, CD69, E-selectin, IL-8, MCP-1, MIG), BT (sIL-17F, sIL-2, sIL-6), BE3C (MMP-9), CASM3C (SAA), MyoF (Collagen IV), and IMphg (CD69, E-selectin, IL-8, sIL-10).

BT System Secretion Profiles

[0409] Figure 42 depicts changes in the secretion of IL-17F, IgG, IL-17A, and TNFα in the BioMAP BT system mediated by SNA-352 (3.9 μM), Tofacitinib (3.3 μM), Apremilast (3.3 μM), SR2211 (3.3 μM), and Cyclosporin A (3.3 μM). Tofacitinib was found to be more active than SNA-352 in decreasing IL-17F secretion, displaying an activity similar to SR2211 (Figure 42A). SNA-352 and Tofacitinib were both very active in decreasing secreted IgG, with SNA-352 as active as Cyclosporin A (Figure 42B). Surprisingly, SNA-352 was found to be as active as tofacitinib in decreasing IL-17A secretion (Figure 42C). SR2211 decreased IL-17A secretion as expected, while Apremilast increased IL-17A secretion. Notably, SNA-352 was found to have remarkable activity with regards to reducing TNFα secretion (Figure 42D).

Top Database Search Result for SNA-352

[0410] In a search for mathematically similar compound profiles from the BioMAP reference database, SNA-352 (3.9 μM) was most similar to deferoxamine mesylate (4.4 μM) (Pearson's correlation, r = 0.879). The Pearson's correlation coefficient between these two profiles is above our determined threshold ($r \geq 0.7$) indicating these compounds share mechanistically relevant similarity. Deferoxamine mesylate is an iron chelator used to treat iron toxicity, and has been investigated as a

potential treatment for spinal cord injury. Figure 42 depicts an overlay of SNA-352 (3.9 μM) and Deferoxamine Mesylate (4.4 μM).

[0411] There are 8 common activities that are annotated within the following systems: SAg (Prolif), BT (Prolif, sIL-17A, sIgG, sTNFα), CASM3C (Prolif), HDF3CGF (Prolif 72), and MyoF (Collagen IV).

Top BioSeek Reference Database Matches for SNA-352

[0412] Table 10 depicts the top 3 similarity matches from a search of the BioMAP Reference Database of > 4,000 agents for each concentration of SNA-352. The similarity between agents is determined using a combinatorial approach that accounts for the characteristics of BioMAP profiles by filtering (Tanimoto metric) and ranking (BioMAP Z-Standard) the Pearson's correlation coefficient between two profiles. Profiles are identified as having mechanistically relevant similarity if the Pearson's correlation coefficient is ≥ 0.7.

Table 10: Top BioMAP Reference Database Matches for SNA-352

[illegible] SNA-352	Database Match	BioMAP Z-Standard	Pearson's Score	# of Common Readouts	Database Match (Mechanism Class)
	Deferoxamine Mesylate, 4.4 μM	18.582	0.879	148	Iron-chelating Agent
3.9 μM	YM155, 4.8 nM	19.348	0.878	148	Survivin Inhibitor
	Actinomycin D, 510 pM	18.344	0.876	148	RNA Polymerase Inhibitor
	Leflunomide, 11 μM	14.425	0.813	148	DHODH Inhibitor
1.3 μM	Zidovudine, 19 μM	14.257	0.823	148	RT Inhibitor
	Methotrexate, 3.7 μM	[illegible]	[illegible]	148	Dihydrofolate Reductase Inhibitor
	CGS 21680, 110 nM	5.432	[illegible]	160	A2A Agonist
430 nM	Aminoglutethimide, 10 μM	5.045	0.396	148	Antisteroidal Agent
	[illegible], 30 μM	4.892	0.385	148	Antiplatelet Agent
	Aminocaproic Acid, 3.3 μM	[illegible]	[illegible]	148	Plasminogen Inhibitor
140 nM	Pyridostigmine Bromide, 10 μM	[illegible]	0.341	148	AChE Inhibitor
	[illegible], 3.3 μM	[illegible]	[illegible]	[illegible]	PDE IV Inhibitor

[0413] The Pearson's correlation coefficient between profiles that is above our determined threshold (r ≥ 0.7) indicates these compounds share mechanistically relevant similarity. For profiles with a Pearson's correlation coefficient below our determined threshold (r < 0.7), the relevance of the similarity is unknown.

Mechanism HeatMAP Analysis of SNA-352

[0414] Figure 44 depicts Mechanism HeatMAP Analysis of SNA-352, with the 148 biomarker readouts within the Diversity PLUS panel compared to 19 consensus

mechanism class profiles. This analysis informs on the regulatory mechanisms controlling increases or decreases in each of the biomarker readouts.

Clustering of Project Profiles

[0415] Figure 45 depicts a clustering of tested agent profiles based on pairwise correlation analysis and clustering of most similar profiles. Profiles that are similar with a Pearson's correlation coefficient (r) ≥ 0.7 are connected by lines. Agents that do not cluster with one another are interpreted as mechanistically distinct. Cytotoxic concentrations are excluded from cluster analysis. Functional clustering of the agent profiles generated during this analysis uses Pearson's correlation values for pairwise comparisons of the profiles for each agent at each concentration, and then subjects the pairwise correlation data to multidimensional scaling. SNA-352 clusters internally at two concentrations. Internal clustering suggests the phenotypic signature of this compound is maintained across a range of concentrations, a characteristic commonly observed in marketed drugs.

Conclusions

[0416] In this study SNA-352 was characterized by profiling in the BioMAP Diversity PLUS panel of human primary cell based assays modeling complex tissue and disease biology of organs (vasculature, immune system, skin, lung) and general tissue biology. The Diversity PLUS panel evaluates the biological impact of test agents in conditions that preserve the complex crosstalk and feedback mechanisms that are relevant to in vivo outcomes. SNA-352 was active and non-cytotoxic in the Diversity PLUS panel, with broad antiproliferative effects observed for the top tested concentration of 3.9 μM. A robust decrease in sIgG and sTNFα was observed for the top two concentrations of SNA-352 in the BT system, consistent with inhibition of B cell function and systemic lupus erythematosus (SLE), chronic lymphocytic leukemia (CLL), or B-cell non-Hodgkin's lymphoma (B-NHL) as potential indications. At its top tested concentration, SNA-352 shared 6 common activities with the benchmark cyclosporin A, but overall, cyclosporin A is more active than SNA-352 in the SAg system, which is a model of T cell activation. The top database match for SNA-352 was deferoxamine mesylate.

Example 8: Efficacy Study of SNA-125 and SNA-352 in the Treatment of Oxazolone-Induced Colitis

Aim of Study

[0417] The goal of this study was to characterize the efficacy of SNA-125 and SNA-352 delivered by oral and intracecal routes for the treatment of colitis with the use of an oxazolone-challenged mouse model of colitis. For comparison, oxazolone-challenged mice were also treated with Tofacitinib and Prednisolone by oral and intracecal routes.

Study Design

[0418] Two hours following the Day 0 AM Treatment dose, colitis was induced in 114 male BALB/C mice by intrarectal administration of 100 µL of 2% Oxazolone (OXZ) under isoflurane anesthesia on day 0. One additional group of eight animals served as no-disease controls (Group 1). Animals were dosed with test article twice daily (BID) via oral gavage (PO) or intracecal (IC) as indicated in Table 11. All animals were weighed daily and assessed visually for the presence of diarrhea and/or bloody stool at the time of dosing. Mice had video endoscopy on Days 2 & 4 to assess colitis severity. Additionally, stool consistency was scored during endoscopy. Following endoscopy on day 4, all animals from each treatment group were sacrificed and blood collected. Following euthanasia, the colon was excised, rinsed, measured, weighed, and then trimmed to 6.0 cm in length and divided into 2 pieces as outlined in Figure 46A; the most distal 5.0 cm section was swiss rolled and placed in formalin for subsequent histological evaluation. The details of the study design are shown in Table 11.

TABLE 11 – Study Design

Group	Number of Animals	OXA Day 0	Treatment	Dose	Route	Dose Schedule (12 Hours Apart)
1	8	---	---	---	---	---
2	15	2%	Vehicle	---	PO	BID Days -1 to 4
3	15		Vehicle	---	IC	BID Days -1 to 4
4	12		Tofacitinib	15mg/kg	PO	BID Days -1 to 4
5	12		Tofacitinib	1mg/kg	IC	BID Days -1 to 4
6	12		Prednisolone	1mg/kg	PO	BID Days -1 to 4
7	12		Treatment A (SNA-125)	400mg/kg	PO	BID Days -1 to 4

8	12		Treatment B (SNA-352)	400mg/kg	PO	BID Days -1 to 4
9	12		Treatment A (SNA-125)	400mg/kg	**IC**	BID Days -1 to 4
10	12		Treatment B (SNA-352)	400mg/kg	**IC**	BID Days -1 to 4

Experimental Procedures

Disease Induction

[0419] Two hours following the Day 0 AM treatments, colitis was induced in 114 male BALB/C mice by intrarectal administration of 100 µL of 2% OXZ under isoflurane anesthesia on day 0. Mice were maintained in vertical position for 1 minute after intra-rectal administration to ensure the complete distribution of OXZ/vehicle throughout the colon.

Cecal/Colon Cannulation

[0420] All animals were allowed a minimum of 7 days to recover from surgery. Animals were placed under isoflurane anesthesia, and the cecum was exposed via a mid-line incision in the abdomen. A small point incision was made in the distal cecum through which 1-2 cm of the cannula (Norfolk Medical MMP-3S mouseport with a 3 french silicone catheter and 2 moveable beads for securing suture) was inserted and directed into the proximal colon.

Dosing

[0421] Animals were dosed with test article twice daily (BID) via oral gavage (PO) or intracecal (IC) as indicated in Table 11.

Endoscopy

[0422] Each mouse underwent video endoscopy on Days 2 & 4 using a small animal endoscope (Karl Storz Endoskope, Germany), under isoflurane anesthesia. During each endoscopic procedure still images as well as video were recorded to evaluate the extent of colitis and the response to treatment. Additionally, an image was captured from each animal at the most severe region of disease identified during endoscopy. Colitis severity was scored using a 0-4 scale as defined in Table 12. Additionally, stool consistency was scored during endoscopy using the parameters defined in Table 13.

TABLE 12 – Endoscopy Colitis Severity Scoring Criteria

Score	Description
0	Normal
1	Loss of vascularity
2	Loss of vascularity and friability
3	Friability and erosions;
4	Ulcerations and bleeding

TABLE 13 – Stool Consistency Scoring Criteria

Score	Description
0	Normal, well-formed pellet
1	Loose stool, soft, staying in shape
2	Loose stool, abnormal form with excess moisture
3	Watery or diarrhea
4	Bloody diarrhea

Sample Collection

[0423] Peripheral blood and colon tissue were collected at sacrifice on day 4 as follows. Blood was collected via cardiac puncture into KiEDTA-coated tubes and centrifuged at 4000x g for 10 minutes. Plasma was collected, flash frozen, and stored at -80°C. The colon was excised, rinsed, measured, weighed, and then trimmed to 6.0 cm in length and divided into 2 pieces; the most distal 5.0 cm section was swiss rolled and placed in formalin for subsequent histological evaluation (see Figure 46A). The proximal 1.0 cm portion was weighed, snap frozen, and stored at -80°C.

Histopathology

[0424] Each colon sample was rolled into a swiss roll. Tissues were embedded in paraffin and sectioned at approximately 5 microns. One slide for each colon was stained with hematoxylin and eosin and examined by a board-certified veterinary pathologist. The pathologist was blinded to the treatment that each group received at the time of assessment. Each slide containing one rolled colon was split into four approximately equal quarters. Each quarter was evaluated and scored for inflammation, edema, and mucosal necrosis, according to the scoring criteria listed

below in Tables 14 to 16. As depicted in Figure 46B, with the slide label to the left, quarters were evaluated starting at the top left and moving clockwise.

TABLE 14 –Inflammation Histopathologic Scoring Criteria

Score	Description
0	None present
1	Minimal change: Focal aggregate of a few cells of minimal diffuse inflammation
2	Mild change: Larger focal aggregates, multifocal small aggregates, or diffuse mild inflammation
3	Moderate change: Multifocal aggregates sometimes coalescing with one another or moderate diffuse change
4	Severe change: Marked diffuse inflammation

TABLE 15 – Edema Histopathologic Scoring Criteria

Score	Description
0	None present
1	Minimal change: Focal edema or minimal diffuse edema
2	Mild change: Larger focal areas of edema or diffuse mild edema
3	Moderate change: Multifocal areas of edema coalescing with one another or moderate diffuse edema
4	Severe change: Marked diffuse edema

TABLE 16 – Mucosal Necrosis/Loss Histopathologic Scoring Criteria

Score	Description
0	None present
1	Minimal change: Focal epithelial necrosis
2	Mild change: Larger focal areas of necrosis or multifocal areas of necrosis
3	Moderate change: Multifocal necrosis coalescing into larger areas
4	Severe change: Areas of erosion through the mucosa to the submucosa

Multiplex Analysis of Colon Tissue Homogenate Samples

[0425] Colon tissue homogenate supernatants were analyzed for protein levels of a panel of mouse inflammatory mediators: IFN-γ, IL-10, IL-6, & TNF-α using a multiplex system (MAGPIX, EMD Millipore).

Results

In-life Observations

[0426] Figure 47 depicts the effect of oral and intracecal administration of SNA-125, SNA-352, tofacitinib, and prednisolone on the body weight of animals challenged with oxazolone. Figure 48 depicts this data according to last observation carried forward analysis. A reduction of the body weight of animals treated with oxazolone was observed. A trend towards a decrease of the body weight loss was observed in the oral SNA-125 administration group.

Endoscopy Results

[0427] Figures 49 and 51 depict the effect of oral and intracecal administration of SNA-125, SNA-352, tofacitinib, and prednisolone on the Day 2 and 4 endoscopy scores, respectively, of animals challenged with oxazolone. At Day 2, oral and intracecal Tofacitinib administration yielded a 10-15% improvement in endoscopy scores. Surprisingly, orally administered SNA-125 demonstrated a 22% reduction of the Day 2 endoscopy score. At Day 4, oral and intracecal Tofacitinib adminstration yielded a 10-15% improvement in endoscopy scores. Surprisingly, orally administered SNA-125 yielded a 15% reduction of the endoscopy score. Figures 81 to 86 depict representative Day 2 and Day 4 endoscopy images.

[0428] Figures 50 and 52 depict the effect of oral and intracecal administration of SNA-125, SNA-352, tofacitinib, and prednisolone on the Day 2 and 4 stool consistency scores, respectively, of animals challenged with oxazolone. Oral SNA-125 showed a significant reduction (65%) in the stool consistency score on Day 2.

Disease Activity Index

[0429] Figure 53 depicts the effect of oral and intracecal administration of SNA-125, SNA-352, tofacitinib, and prednisolone on the disease activity index (DAI) score of animals at Days 2 and 4 following challenge with oxazolone.

Colon Weight/Length Ratio

[0430] Figure 54 depicts the effect of oral and intracecal administration of SNA-125, SNA-352, tofacitinib, and prednisolone on the colon weight/length ratio of animals challenged with oxazolone.

Histopathology Scoring Results

[0431] Oxazolone produced mild to moderate colitis characterized by multifocal inflammation, edema, and necrosis.

[0432] Figures 55-57 depict the histopathology scoring results for inflammation, edema, and mucosal necrosis/loss, respectively, while Figure 58 depicts the summation of these scores. Note that control animals not given oxazolone were essentially normal and were not included in the statistical analysis.

Inflammation Scoring

[0433] As shown in Figure 55, untreated control animals that did not receive oxazolone had minimal scattered background inflammation, while all other animals were given oxazolone and had varying degrees of inflammation. Inflammation tended to be mild to moderate with some regions more severely affected than others. When including all groups in the analysis, the treatment effect approached significance (one-way ANOVA, $p = 0.0656$). When oral and intracecal groups were analyzed independently treatment did significantly influence inflammation for oral groups (one-way ANOVA, $p = 0.0199$) but not for intracecal groups (one-way ANOVA, $p = 0.5970$).

[0434] In the oral groups, both SNA-125 and SNA-352 tended to reduce inflammation compared to vehicle, and this improvement was more noticeable than for either prednisolone or tofacitinib.

Edema Scoring

[0435] As shown in Figure 56, untreated control animals that did not receive oxazolone had minimal, random edema, while all other animals were given oxazolone and had varying degrees of edema that was generally associated with inflammation. Edema tended to be mild to moderate with some regions more severely affected than others. When including all groups in the analysis, treatment did not have a significant effect on edema (one-way ANOVA, $p = 0.2845$). When oral and intracecal groups were analyzed independently, the oral groups approached significance (one-way ANOVA, $p = 0.0855$) but not the intracecal groups (one-way ANOVA, $p = 0.4033$). In the oral groups, both SNA-125 and SNA-352 tended to reduce edema compared to vehicle and this improvement was more noticeable than for either prednisolone or tofacitinib.

Mucosal Necrosis Results

[0436] As depicted in Figure 57, untreated control animals that did not receive oxazolone did not have any mucosal necrosis, while all other animals were given oxazolone and had varying degrees of multifocal mucosal erosion and necrosis.

Necrosis tended to be regional with some areas more severely affected than others and some animals more severely affected than others.

[0437] In the oral groups, both SNA-125 and SNA-352 tended to reduce mucosal necrosis compared to vehicle and this improvement was more noticeable than for either prednisolone or tofacitinib.

Sum Score Results

[0438] As shown in Figure 58, summed histopathology scores (Inflammation + Edema + Mucosal Necrosis) were moderate in all animals administered oxazolone.

[0439] In the orally treated groups, both SNA-125 and SNA-352 tended to reduce the sum score compared to vehicle, and this improvement was more noticeable than for either prednisolone or tofacitinib. For inflammation, this treatment effect was statistically significant while for edema and the sum score, this effect approached significance

[0440] Overall, intracecal treatment was found to be less effective than an oral route of administration.

Analysis of Colon Histopathology Micrographs

[0441] Sections of colon were often thickened by inflammation and edema that variably extended into the lamina propria, submucosa and muscular wall. The inflammation was pyogranulomatous - composed of a mixture of neutrophils, macrophages, lymphocytes, and plasma cells. Mucosal necrosis was also variably present and characterized by partial or complete loss of the surface epithelium with erosion to underlying lamina propria or submucosa. There was multifocal peritonitis suggesting that there was multifocal full thickness erosion. Due to the multifocal distribution of these changes, the inflammation, edema, and mucosal necrosis were variable along the swiss rolled section. Representative photomicrographs are shown in Figures 59-61.

Control Animals

[0442] As seen in Figure 59, control animals in this study had basically normal colons without significant inflammation, mucosal necrosis, or edema.

Orally-treated Animals

[0443] Administration of oxazolone was associated with the development of multifocal mucosal ulceration, inflammation, and edema which was seen in all groups to

varying extent. Vehicle-treated animals had moderate inflammation (unfilled arrows) with edema (filled arrows) and multifocal ulceration (bracket).

[0444] Treatment with prednisolone or tofacitinib did not demonstrably reduce colitis. Animals still had a diffuse increase in background inflammation with multifocal pockets of more severe inflammation and multifocal mucosal ulceration (Figure 60).

[0445] Treatment with both SNA-125 and SNA-352 orally tended to reduce the severity of colitis. These animals tended to have a more mild increase in background inflammation without significant ulceration.

Intracecal-treated Animals

[0446] The intracecal vehicle group had relatively mild colitis compared to other groups. While there were some areas in some animals with inflammation, edema, and mild necrosis (inset), most of the colons had mild inflammation with mild edema and minimal to no necrosis (Figure 61). Animals treated with intracecal tofacitinib had diffuse inflammation with pockets of more severe inflammation and mucosal necrosis. Animals treated intracecal with SNA-125 were divided between mild colitis and severe colitis. Animals treated intracecally with SNA-352 had reduced colitis compared to tofacitinib treated animals, but since the vehicle group had such mild colitis there was no noticeable change compared to vehicle.

Multiplex Analysis of Colon Homogenates

[0447] Figures 62-65 depicts the effect of oral and intracecal administration of SNA-125, SNA-352, tofacitinib, and prednisolone on the levels of IFNγ, IL-10, IL-6 and TNFα in colon homogenates. Oral and intracecal SNA-352 administration decreased IFNγ. Intracecal SNA-125 significantly increased TNFα as compared to the intracecal vehicle control (with the intracecal control outlier removed). Oral tofacitinib significantly increased IL-10 levels, and a strong trend was also observed for the intracecal Tofacitinib group.

Conclusions

[0448] Oxazolone produced mild to moderate colitis characterized by multifocal inflammation, edema, and necrosis. Changes tended to be multifocal in nature with some areas affected more severely than others. It appeared that the distal colon was more severely affected than the proximal colon. Clear signs of efficacy for both SNA-125 and SNA-352 were observed. Overall, there was a clear trend for both SNA-125 and SNA-352 to improve colitis in this model. Both compounds showed comparable or even better results than tofacitinib and prednisolone. SNA-125 seems to work

differently if administered orally or intracecally (intracecal group data affected by animal loss).

[0449] Oral treatment with both SNA-352 and SNA-125 tended to reduce inflammation, edema, and necrosis compared to oral treatment with vehicle, tofacitinib, or prednisolone. For inflammation, this treatment effect was statistically significant while for edema and the sum score, this effect approached significance. In contrast, treatment with tofacitinib and prednisolone orally were ineffective at treating oxazolone-induced colitis in this study.

[0450] Intracecal treatment was less effective. Treatment with intracecal SNA-352 may have mildly reduced colitis compared to tofacitinib treatment and SNA-125; however any change was mild and not statistically significant.

Example 9: Profiling Study of SNA-352 against 274 Kinases

Aim of Study

[0451] The goal of this study was to measure the inhibitory activity of SNA-352 against 274 kinases.

Materials and Methods

Test Agent

[0452] SNA-352 (Lot # 2017GC14/S7) was used at a test concentration of 0.1 μM.

Target Kinases

[0453] ABL, ACK, ALK, ARG, AXL, BLK, BMX, BRK, BTK, CSK, DDR1, DDR2, EGFR, EPHA1, EPHA2, EPHA3, EPHA4, EPHA5, EPHA6, EPHA7, EPHA8, EPHB1, EPHB2, EPHB3, EPHB4, FAK, FER, FES, FGFR1, FGFR2, FGFR3, FGFR4, FGR, FLT1, FLT3, FLT4, FMS, FRK, HCK, HER2, HER4, IGF1R, INSR, IRR, ITK, JAK1, JAK2, JAK3, KDR, KIT, LCK, LTK, LYNa, LYNb, MER, MET, MUSK, PDGFRα, PDGFRβ, PYK2, RET, RON, ROS, SRC, SRM, SYK, TEC, TIE2, TNK1, TRKA, TRKB, TRKC, TXK, TYK2, TYRO3, YES, ZAP70, AKT1, AKT2, AKT3, AMPKα1/β1/γ1, AMPKα2/β1/γ1, AurA, AurA/TPX2, AurB, AurC, BRAF_Cascade, BRAF [V600E]_Cascade, BRSK1, BRSK2, BUB1/BUB3, CaMK1α, CaMK1δ, CaMK2α, CaMK2β, CaMK2γ, CaMK2δ, CaMK4, CDC2/CycB1, CDC7/ASK, CDK2/CycA2, CDK2/CycE1, CDK3/CycE1, CDK4/CycD3, CDK5/p25, CDK6/CycD3, CDK7/CycH/MAT1, CDK9/CycT1, CGK2, CHK1, CHK2, CK1α, CK1γ1, CK1γ2, CK1γ3,

CK1δ, CK1ε, CK2α1/β, CK2α2/β, CLK1, CLK2, CLK3, COT_Cascade, CRIK, DAPK1, DCAMKL2, DLK_Cascade, DYRK1A, DYRK1B, DYRK2, DYRK3, EEF2K, Erk1, Erk2, Erk5, GSK3α, GSK3β, Haspin, HGK, HIPK1, HIPK2, HIPK3, HIPK4, IKKα, IKKβ, IKKε, IRAK1, IRAK4, JNK1, JNK2, JNK3, LATS2, LOK, MAP2K1_Cascade, MAP2K2_Cascade, MAP2K3_Cascade, MAP2K4_Cascade, MAP2K5_Cascade, MAP2K6_Cascade, MAP2K7_Cascade, MAP3K1_Cascade, MAP3K2_Cascade, MAP3K3_Cascade, MAP3K4_Cascade, MAP3K5_Cascade, MAP4K2, MAPKAPK2, MAPKAPK3, MAPKAPK5, MARK1, MARK2, MARK3, MARK4, MELK, MINK, MLK1_Cascade, MLK2_Cascade, MLK3_Cascade, MNK1, MNK2, MOS_Cascade, MRCKα, MRCKβ, MSK1, MSK2, MSSK1, MST1, MST2, MST3, MST4, NDR1, NDR2, NEK1, NEK2, NEK4, NEK6, NEK7, NEK9, NIM1K, NuaK1, NuaK2, p38α, p38β, p38γ, p38δ, p70S6K, p70S6Kβ, PAK1, PAK2, PAK4, PAK5, PAK6, PASK, PBK, PDHK2, PDHK4, PDK1, PEK, PGK, PHKG1, PHKG2, PIM1, PIM2, PIM3, PKACα, PKACβ, PKACγ, PKCα, PKCβ1, PKCβ2, PKCγ, PKCδ, PKCε, PKCζ, PKCη, PKCθ, PKCι, PKD1, PKD2, PKD3, PKN1, PKR, PLK1, PLK2, PLK3, PRKX, QIK, RAF1_Cascade, ROCK1, ROCK2, RSK1, RSK2, RSK3, RSK4, SGK, SGK2, SGK3, SIK, skMLCK, SLK, SRPK1, SRPK2, TAK1-TAB1_Cascade, TAOK2, TBK1, TNIK, TSSK1, TSSK2, TSSK3, WNK1, WNK2, WNK3, SPHK1, SPHK2.

Results

[0454] Table 17 depicts the % Inhibition of the tested kinases at a SNA-352 test concentration of 0.1μM.

TABLE 17 –Percent Kinase Inhibition by SNA-352

ABL	2.0
ACK	86.5
ALK	2.2
ARG	2.2
AXL	12.4
BLK	3.3
BMX	8.4
BRK	0.0
BTK	3.5
CSK	-7.2
DDR1	76.6
DDR2	33.5
EGFR	-1.7
EPHA1	0.4
EPHA2	3.8
EPHA3	1.8
EPHA4	-3.1
EPHA5	2.2
EPHA6	2.4
EPHA7	4.4
EPHA8	-0.7
EPHB1	-1.1
EPHB2	2.6
EPHB3	2.2
EPHB4	-5.4
FAK	5.4
FER	53.0
FES	12.9
FGFR1	18.3
FGFR2	22.6
FGFR3	9.4
FGFR4	4.8
FGR	10.5
FLT1	5.9
FLT3	85.0
FLT4	50.8
FMS	12.8
FRK	6.1
HCK	12.1
HER2	-5.4
HER4	7.6
IGF1R	3.0
INSR	6.2
IRR	0.3
ITK	4.8
JAK1	9.6

TABLE 17 (continued) – Percent Kinase Inhibition by SNA-352

JAK2	71.3
JAK3	95.0
KDR	20.6
KIT	-3.3
LCK	32.1
LTK	16.2
LYNa	17.1
LYNb	14.1
MER	15.7
MET	10.8
MUSK	28.4
PDGFRα	81.1
PDGFRβ	87.8
PYK2	10.2
RET	12.8
RON	-2.3
ROS	63.3
SRC	6.4
SRM	4.1
SYK	39.0
TEC	1.2
TIE2	1.8
TNK1	54.2
TRKA	94.8
TRKB	63.4
TRKC	78.8
TXK	-3.6
TYK2	7.3
TYRO3	6.9
YES	11.4
ZAP70	3.4
AKT1	4.3
AKT2	0.9
AKT3	2.1
AMPKα1/β1/γ1	2.9
AMPKα2/β1/γ1	0.1
AurA	73.3
AurA/TPX2	35.0
AurB	14.2
AurC	55.5
BRAF_Cascade	-0.8
BRAF(V600E)_Cascade	4.4
BRSK1	24.2
BRSK2	15.0
BUB1/BUB3	-3.9
CaMK1α	-0.6
CaMK1δ	-1.2
CaMK2α	2.7
CaMK2β	1.5

TABLE 17 (continued) – Percent Kinase Inhibition by SNA-352

CaMK2γ	4.6
CaMK2δ	26.7
CaMK4	-6.9
CDC2/CycB1	4.5
CDC7/ASK	-0.3
CDK2/CycA2	-3.6
CDK2/CycE1	0.7
CDK3/CycE1	-3.7
CDK4/CycD3	2.5
CDK5/p25	-9.8
CDK6/CycD3	1.2
CDK7/CycH/MAT1	3.7
CDK9/CycT1	-2.7
CGK2	79.7
CHK1	8.8
CHK2	3.9
CK1α	-1.0
CK1γ1	-4.2
CK1γ2	-1.0
CK1γ3	-0.7
CK1δ	1.3
CK1ε	2.7
CK2α1/β	-1.6
CK2α2/β	-2.0
CLK1	6.5
CLK2	3.8
CLK3	0.7
COT_Cascade	36.1
CRIK	0.0
DAPK1	6.6
DCAMKL2	8.1
DLK_Cascade	5.4
DYRK1A	3.0
DYRK1B	-3.0
DYRK2	5.8
DYRK3	-3.7
EEF2K	4.2
Erk1	0.8
Erk2	-2.2
Erk5	-0.3
GSK3α	5.4
GSK3β	2.8
Haspin	0.5
HGK	33.4
HIPK1	-0.1
HIPK2	7.1
HIPK3	1.2
HIPK4	-0.7
IKKα	-3.0

TABLE 17 (continued) – Percent Kinase Inhibition by SNA-352

IKKβ	0.1
IKKε	19.9
IRAK1	2.5
IRAK4	-1.8
JNK1	1.2
JNK2	-0.6
JNK3	-0.9
LATS2	26.0
LOK	18.6
MAP2K1_Cascade	8.0
MAP2K2_Cascade	-0.8
MAP2K3_Cascade	-1.0
MAP2K4_Cascade	-1.9
MAP2K5_Cascade	-1.3
MAP2K6_Cascade	-2.1
MAP2K7_Cascade	-2.9
MAP3K1_Cascade	6.9
MAP3K2_Cascade	-0.3
MAP3K3_Cascade	17.8
MAP3K4_Cascade	2.6
MAP3K5_Cascade	15.6
MAP4K2	12.4
MAPKAPK2	-5.8
MAPKAPK3	-0.7
MAPKAPK5	-0.7
MARK1	38.8
MARK2	45.8
MARK3	44.1
MARK4	31.1
MELK	22.8
MINK	9.1
MLK1_Cascade	34.8
MLK2_Cascade	7.8
MLK3_Cascade	33.4
MNK1	2.1
MNK2	1.1
MOS_Cascade	0.3
MRCKα	-4.2
MRCKβ	-0.5
MSK1	40.1
MSK2	0.6
MSSK1	3.1
MST1	55.8
MST2	25.3
MST3	3.3
MST4	-2.4
NDR1	2.0
NDR2	5.3
NEK1	-8.2

TABLE 17 (continued) – Percent Kinase Inhibition by SNA-352

NEK2	10.4
NEK4	0.9
NEK6	0.4
NEK7	0.1
NEK9	0.6
NIM1K	-2.3
NuaK1	50.7
NuaK2	47.9
p38α	0.7
p38β	0.8
p38γ	3.4
p38δ	1.7
p70S6K	20.1
p70S6Kβ	3.0
PAK1	1.3
PAK2	7.9
PAK4	16.2
PAK5	17.3
PAK6	8.9
PASK	7.0
PBK	5.5
PDHK2	0.1
PDHK4	12.8
PDK1	24.6
PEK	-7.5
PGK	50.6
PHKG1	53.1
PHKG2	-1.5
PIM1	12.4
PIM2	-3.0
PIM3	5.6
PKACα	8.7
PKACβ	6.6
PKACγ	2.4
PKCα	25.1
PKCβI	14.1
PKCβ2	20.4
PKCγ	4.6
PKCδ	14.9
PKCε	29.3
PKCζ	22.3
PKCη	4.9
PKCθ	-3.1
PKCι	21.9
PKD1	3.6
PKD2	2.3
PKD3	-1.5
PKN1	60.0
PKR	-9.9

TABLE 17 (continued) – Percent Kinase Inhibition by SNA-352

PLK1	-0.5
PLK2	-0.5
PLK3	-9.5
PRKX	2.2
QIK	55.6
RAF1_Cascade	2.1
ROCK1	-11.7
ROCK2	-0.7
RSK1	48.0
RSK2	64.7
RSK3	59.6
RSK4	71.3
SGK	3.7
SGK2	8.8
SGK3	4.7
SIK	60.2
skMLCK	1.8
SLK	9.2
SRPK1	-2.2
SRPK2	0.1
TAK1-TAB1_Cascade	-1.8
TAOK2	-0.6
TBK1	27.3
TNIK	21.0
TSSK1	30.8
TSSK2	-2.3
TSSK3	-3.1
WNK1	0.6
WNK2	-3.9
WNK3	-1.3
SPHK1	-2.4
SPHK2	5.8

Example 10: Profiling Study of SNA-352 against 25 Kinases

Aim of Study

[0455] The goal of this study was to measure the inhibitory activity of SNA-352 against 25 kinases.

Materials and Methods

Test Agent

[0456] SNA-352 (Lot # 2017GC14/S7) was used at a test concentration of 0.1 μM.

Target Kinases

[0457] BMPR1A, BRAF, COT, DLK, LIMK1, LKB1, MAP2K1, MAP2K2, MAP2K3, MAP2K4, MAP2K5, MAP2K6, MAP2K7, MAP3K1, MAP3K2, MAP3K3, MAP3K4, MAP3K5, MLK1, MLK2, MLK3, MOS, RAF1, TTK, WEE1.

Results

[0458] Table 18 depicts the % Inhibition of the tested kinases by SNA-352 at a test concentration of 0.1μM.

TABLE 18 –Percent Kinase Inhibition by 0.1μM SNA-352

Kinase	% Inhibition by SNA-352 at 0.1μM
BMPR1A	-3.1
BRAF	0.9
COT	-1.9
DLK	-2.3
LIMK1	29.9
LKB1	0.9
MAP2K1	13.6
MAP2K2	6.1
MAP2K3	20.5
MAP2K4	2.5
MAP2K5	3.2
MAP2K6	15.3
MAP2K7	1.4
MAP3K1	0.8
MAP3K2	13.2
MAP3K3	4.3
MAP3K4	-4.6
MAP3K5	-6.3
MLK1	68.7
MLK2	13.6
MLK3	83.3
MOS	4.2
RAF1	0.3
TTK	4.8
WEE1	3.2

Conclusions

[0459] In Examples 9 and 10 the inhibition profile of SNA-352 at 100nM was profiled against 299 kinases, including tyrosine kinases (TK), serine/threonine kinases (STK) and mitogen-activated protein kinase (MAPK). It was found that 27 kinases of the 299 kinases were inhibited more than 50% by SNA-352. SNA-352 inhibited 7 kinases

(JAK3, TrkA, PDGFRβ, FLT3, MLK3, PDGFRα, and ACK) by more than 80%. Further, SNA-352 inhibited 6 kinases (CGK2, TrkC, DDR1, AurA, RSK4, JAK2) between 70 and 80%. Within the JAK family, 100nM SNA-352 inhibited JAK3, JAK2, JAK1 and TYK2 by 95.0%, 71.1%, 9.6%, and 7.3%, respectively. Within the Trk family, 100nM SNA-352 inhibited TrkA, TrkC, and TrkB by 94.8%, 78.8%, and 63.4%, respectively. Figure 66 depicts the SNA352 kinase inhibition profile at test concentrations of 100nM and 200nM for the top inhibited kinases as well as those kinases in the middle in the inhibition spectrum.

Example 11: Determination of IC_{50} of SNA-352 against 15 Kinases

Aim of Study

[0460] A literature search was performed to define the kinases linked to IBD and ophthalmologic diseases among the most inhibited by SNA-352: JAK3, TrkA, PDGFRβ, FLT3, MLK3, PDGFRα, ACK, AurA, JAK2, TrkB, and SIK were identified for IBD; PDGFRα, PDGFRβ, MLK3, DDR1, MLK1, FLT4, and LIMK1 were identified for ophthalmologic diseases. All kinases correlated to cancers (e.g. AurA) were excluded. All the members of the key families of kinases (JAK and Trk family) were included. The goal of this study was to assess the potency (IC_{50}) of SNA-352 against the 15 kinases identified by this analysis: JAK1, JAK2, JAK3, TYK2, TrkA, TrkB, TrkC, PDGFRβ, PDGFRα, FLT3, FLT4, MLK1, MLK3, ACK, SIK, MAP2K6, LIMK1, DDR1 and DDR2.

Materials and Methods

Test Agent

[0461] SNA-352 (Lot # 2017GC14/S7) was used at test concentrations of 10, 3, 1, 0.3, 0.1, 0.03, 0.01, 0.003, 0.001, 0.0003, 0.0001 μM.

Target Kinases

[0462] SNA-352 was used at test concentrations of 10, 3, 1, 0.3, 0.1, 0.03, 0.01, 0.003, 0.001, 0.0003 μM for ACK, DDR1, DDR2, FLT3, FLT4, JAK1, JAK2, PDGFRα, PDGFRβ, TRKB, TRKC, TYK2, and SIK.

[0463] SNA-352 was used at test concentrations of 3, 1, 0.3, 0.1, 0.03, 0.01, 0.003, 0.001, 0.0003, and 0.0001 μM for JAK3 and TRKA.

Results

[0464] Table 19 depicts the IC_{50} of SNA-352 and staurosporine for 15 kinases.

TABLE 19 – IC_{50} of SNA-352 and Staurosporine for 15 Kinases

Kinase	IC50 (M)	
	SNA-352	Staurosporine
ACK	4.09E-08	2.49E-09
DDR1	3.65E-08	3.09E-09
DDR2	1.79E-07	2.02E-09
FLT3	1.33E-08	1.21E-10
FLT4	1.09E-07	5.94E-10
JAK1	9.46E-07	5.68E-10
JAK2	5.36E-08	2.40E-10
JAK3	6.27E-09	2.70E-10
TYK2	1.58E-06	8.26E-10
PDGFRα	2.42E-08	2.02E-10
PDGFRβ	2.06E-08	1.49E-10
TRKA	8.89E-09	3.85E-10
TRKB	5.77E-08	3.02E-10
TRKC	2.88E-08	3.83E-10
SIK	6.68E-08	1.62E-09

Conclusions

[0465] SNA-352 is a potent inhibitor of kinases linked to IBD and ophthalmologic diseases.

Example 12: Mouse Model of IMQ-induced Psoriasis

Aim of Study

[0466] The objective of this study was to determine the efficacy of SNA-101, SNA-103, and SNA-352 as a therapeutic in the mouse model of IMQ-induced psoriasis.

Methodology

Test groups and experimental timing

[0467] Table 20 depicts the test groups and Figure 67 depicts the timing of the experiments performed in this study.

Table 20: Test Groups

Group Number	Group Size	Group Description	Disease Induction	TI/vehicle Route	TI/vehicle Dose Level	Dosing Route/ Regimen
1	*N=5*	Naïve	NA	NA	NA	NA
2	*N=10*	IMQ Only	5% IMQ on back and on both ears daily	NA	NA	NA
3	*N=10*	Clobetasol		Topical	50mg applied to backs, 5 mg applied to each ear	1×daily, 2 hrs post IMQ application
4	*N=10*	Vehicle		Topical	100 μl applied to back, 10 μl applied to each ear	2×daily, 2 and 8 hrs post IMQ application
5	*N=10*	1.0% SNA-352				
6	*N=10*	5.0% SNA-352				
7	*N=10*	10.0% SNA-352				
8	*N=10*	5.0% SNA-125				
9	*N=10*	10.0% SNA-125				
10	*N=10*	20.0% SNA-101				

Psoriasis Clinical Scoring

[0468] The animals were examined for signs of psoriasis on study day 0. These scores served as a baseline for the psoriasis clinical score parameter. Starting from IMQ cream application on day 0, psoriasis responses were examined daily until termination of the study.

[0469] Psoriasis reactions (erythema and plaques) were scored based on the parameters shown in Table 21 and recorded according to a 0-12 scale. The clinical score is determined by summing the score of each section.

Table 21: Psoriasis Clinical Scoring Parameters

Psoriasis Score					
Plaques	**Grade**	**Erythema**	**Grade**	**Punctate redness/scabbing**	**Grade**
Normal	**0**	Normal	**0**	No red dots	**0**
Very few plaques (cover about 5-10% of the back area).	**1**	Slight	**1**	Very few (1-10) red dots present on the entire back	**1**
Several plaques (cover about 20%-30% of the back area).	**2**	Moderate	**2**	Red dots are diffuse and cover ~50% of the back (10-25 red dots)	**2**
Moderate plaques (cover about 50% of the back area).	**3**	Marked	**3**	Red dots cover the entire back and are more concentrated (>25 red dots)	**3**
Spread plaques (cover over 90% of the back area).	**4**	Extreme	**4**		
Plaques are no longer detected, however the skin is irritated and no fur is growing on the skin	**5**				

Results

Psoriasis clinical score

[0470] The total psoriasis score was determined by summing the plaque score, the erythema score and the punctate redness/scabbing score. As seen in Figure 68, the difference between SNA-125 at 5% and the vehicle is statistically significant on

day 8 and 10, while for SNA-125 at 10% is significant from day 8 to day 10.Further, a statistically significant difference between SNA-352 at 5% and the vehicle on day 10 was found, as well as for SNA-125 at 10% from day 8 to day 10. Further, the difference between SNA-101 at 20% and the vehicle is statistically significant from day 8 to day 10.

Erythema scores

[0471] As shown in Figure 69, the differences between SNA-125 at 5% and 10% and the vehicle are statistically significant from day 8 to day 10. Further, statistically significant differences were found between SNA-352 at 5% and 10% and the vehicle from day 8 to day 10. Additionally, the difference between SNA-101 at 20% and the vehicle is statistically significant from day 8 to day 10

Plaque scores

[0472] As shown in Figure 70, the difference between SNA-125 at 5% and the vehicle is statistically significant from day 5 to day 8, while for SNA-125 at 10% it is significant at day 8. Additionally, a statistically significant difference between SNA-352 at 10% and the vehicle was observed from day 5 to day 8. Additionally, the difference between SNA-101 at 20% and the vehicle is statistically significant at day 8.

Punctate redness/scabbing scores

[0473] As shown in Figure 71, the difference between SNA-352 at 10% and the vehicle is statistically significant on day 9. Additionally, a statistically significant difference between SNA-101 at 20% and the vehicle was observed on day 9 and day 10.

Spleen weight and ear thickness

[0474] Topical application of the IMQ cream causes the enlargement of spleen and lymph nodes, and increased ear thickness. The commonly used antipsoriatic agent clobetasol almost completely attenuated these IMQ-induced changes. Neither treatments with vehicle or SNA-125, SNA-352, SNA-101 significantly modulated spleen weight (Figure 72A). There was no difference on ear thickness found between the vehicle and the different doses of SNA-125, SNA-352 and SNA-101 (Figure 72B). Figure 72C depicts the daily weight of mice throughout the study.

Cytokine analysis

[0475] Left ears were biopunched on day 4 and after tissue homogenization, the levels of cytokines IL-17F, TNF-α, IL-22, and IL-17A in the tissue lysates were

measured via multiplex and then normalized with total protein amounts. Mean values for each group are displayed in Figure 73.

Example 13: IL-23-induced Psoriasis Mouse Model

Aim of Study

[0476] The objective of this study was to determine the efficacy of SNA-120 and SNA-325 as a therapeutic in the mouse model of IL-23-induced psoriasis. The IL-23/Th17 pathway has been shown to play a major role in psoriasis, and injection of IL-23 into mice produces clinical features associated with psoriasis such as hyperproliferation of keratinocytes and thickened epidermis with infiltration of mononuclear cells. It has been found that an IL-23 mouse model simulates human AD (i.e. 37% homology with human AD transcriptome). In this model, tofacitinib administration has found to reduce ear swelling and inflammatory infiltrates in mouse skin in a dose-dependent manner.

Methodology

Test groups

[0477] Table 22 depicts the test groups and Figure 74 depicts a schematic of the timing of the experiments performed.

Table 22: Test Groups

Group Number	Group Size	Description	Disease Induction	Route	Dose Level (mg/kg)	Volume Dosage	Dosing Regime
1	*n=5*	Sham	10 µl of PBS on right ear 3×weekly	NA	NA	NA	NA
2	*n=5*	IL-23 Only	500 ng of IL-23 on right ear 3×weekly	NA	NA	NA	NA
3	*n=10*	Clobetasol		Topical	5 mg on both ears	NA	1×daily

4	*n=10*	Vehicle		Topical	NA	20 μl to right ear	BID
5	*n=10*	1.0% SNA-352		Topical	NA	20 μl to right ear	BID
6	*n=10*	5.0% SNA-352		Topical	NA	20 μl to right ear	BID
7	*n=10*	10.0% SNA-352		Topical	NA	20 μl to right ear	BID
8	*n=10*	10.0% SNA-120		Topical	NA	20 μl to right ear	BID

Psoriasis clinical scoring

[0478] The animals were examined for signs of psoriasis on study day 0. These scores served as a baseline for the psoriasis clinical score parameter. Starting from IL-23 injection on day 0, psoriasis responses were examined 3 times weekly until termination of the study. Psoriasis reactions (erythema and plaques) were scored using the parameters depicted in table 23 and recorded according to a 0-6 scale. The clinical score is determined by summing the score of each section.

Table 23: Psoriasis clinical scoring parameters

Psoriasis Score			
Erythema	**Grade**	**Plaques**	**Grade**
Normal	**0**	Normal	**0**
Slight	**1**	Very few plaques (cover about 5-10% of the ear area).	**1**
Moderate	**2**	Moderate plaques (cover about 50% of the ear area).	**2**
Severe	**3**	Spread plaques (cover about	**3**

		90% of the ear area).	

Results

[0479] Figure 75A depicts the total psoriasis clinical scores for each group over time. Figure 75B depicts right ear thickness for each group (measured with a caliper 3 times weekly) while Figure 75C depicts changes in body weight throughout the study.

Example 14: Prophetic Study – Acetone-diethyl-Ether-Water Model of Dry Skin Pruritus

[0480] Dry skin pruritus is common in the elderly, and the Acetone-diethyl-Ether-Water (AEW) model has become a recognized animal model of chronic itch. Studies have shown that AEW treatment causes dryness (measured as increased transepidermal water loss and decreased stratum corneum hydration). Further, studies indicate that increased secretion of NGF may induce dry skin itch. Old mice scratch more than young mice and AEW treatment induces more scratching compared to saline treatment both in young and old mice. It has further been reported that AEW treatment increases NGF levels in skin biopsies, with old mice having higher levels of NGF than young mice.

[0481] The efficacy of SNA-120, SNA-125, SNA-352 will be tested in the AEW model. AEW or 0.9% saline was administered topically BID. Additionally, SNA-120, SNA-125, SNA-352, or vehicle will be administered topically BID. Scratching measurements will be performed 14 hour after the last AEW treatment each day for one hour. Non-specific clinical signs and treatment site assessments will be performed from Day 1 to Day 6. All animals will be weighted on Day 1 and Day 6. On day 6 animals will be terminated and skin biopsies will be performed. Additionally, histology (H&E) and skin biopsies for NGF analysis were performed at study termination.

[0482] It is predicted that NGF and TrkA levels will be increased in old mice after AEW treatment, and that SNA-120, SNA-125, and SNA-352 administration will reverse this trend in a dose-dependent manner. It is predicted that SNA-120, SNA-125, and SNA-352 administration will reverse the AEW-induced skin thickening and skin dryness of young and old mice in a dose-dependent manner.

[0483] Parakeratosis is a mode of keratinization characterized by the retention of nuclei in the stratum corneum. In the skin, this process leads to the abnormal

replacement of annular squames with nucleated cells. Parakeratosis is associated with the thinning or loss of the granular layer and is usually seen in diseases of increased cell turnover, whether inflammatory or neoplastic. Parakeratosis also is seen in the plaques of psoriasis and in dandruff. It is predicted that in young mice treated with saline, a normal epidermis with keratinocytes organized as a single line will be observed. It is predicted that in old mice treated with saline, normal epidermis with keratinocytes frequently organized as a single line and a few signs of parakeratosis will be observed. In young mice treated with AEW, it is predicted that histology assessments will show normal epidermis with some increase in stratification. In old mice treated with AEW, it is predicted that histology assessments will demonstrate normal epidermis with increase in stratification and signs of parakeratosis. It is predicted that that SNA-120, SNA-125, and SNA-352 administration will reverse AEW-induced stratification and parakeratosis in a dose-dependent manner.

Example 15: Efficacy Analysis of SNA-125, SNA-352 and SNA-103 in a VEGF-induced Proliferation Assay Using HRMVEC Cells

Aim of Study

[0484] The aim of this study was to compare the efficacy of SNA-125, SNA-352 and SNA-103 in a VEGF-induced proliferation assay using Human Retinal Microvascular Endothelial (HRMVEC) cells. SNA-125 and SNA-352 have been observed to inhibit kinases in VEGF signaling pathway: ERK and RAF for SNA-125, and PCKa, PCKb2 and PKg for SNA-352. It is contemplated that these compounds could have an anti-angiogenic effect in addition to their anti-inflammatory effect.

Methodology

Experimental outline

[0485] Primary HRMVEC cells were seeded onto collagen-coated 96 well plates at a concentration of 2 or 5 x 10^3 and treated with VEGF at 10 or 50 ng/ml. As a control, cells without VEGF were included to give background levels of non-VEGF driven proliferation. Cells were incubated for 72 hours at 37°C with 5% CO2. Proliferation was measured by pulsing cells for the last 24 hours with tritiated thymidine. Plates were then harvested and assayed for tritiated thymidine incorporation. The ability of the lead compounds to inhibit proliferation was assessed by pre-incubation of cells with the lead compounds for 18 hours prior to stimulation with VEGF. Each lead compound was tested

at eight concentrations. Motesanib Diphosphate (AMG-706) was included as a positive control. Four concentrations were tested. Each condition was tested in sextuplicate. To readout cell proliferation, cells were pulsed with ^{3}H-Thymidine and harvested 24 hours later. Radiation was then quantified. IC_{50} values were calculated (where possible) for each compound.

Treatment Groups and Dosages

[0486] The doses of the test SNA compounds used were 300, 100, 33.3, 11.1, 3.7, 1.2, 0.41, and 0.14 μM. The doses of motesanib diphosphate used were 30, 10, 3.33, and 1.11 nM. The pre-incubation time was 18 hours. Table 24 depicts the treatment groups and dosages employed in this study.

Table 24: Treatment Groups and Dosages

Condition	Dose	Cell density	VEGF	Readout
Unstimulated	n/a	2×10^3	n/a	Cell proliferation ^{3}H-Thymidine incorporation
SNA-125	Doses 1-8		10 ng/mL 72 hours	
SNA-352	Doses 1-8			
SNA-103	Doses 1-8			
Motesanib Diphosphate	Doses 1-4			
SNA-125	Doses 1-8		50 ng/mL 72 hours	
SNA-352	Doses 1-8			
SNA-103	Doses 1-8			
Motesanib Diphosphate	Doses 1-4			
Unstimulated	n/a	5×10^3	n/a	
SNA-125	Doses 1-8		10 ng/mL 72 hours	
SNA-352	Doses 1-8			
SNA-103	Doses 1-8			
Motesanib Diphosphate	Doses 1-4			
SNA-125	Doses 1-8		50 ng/mL 72 hours	
SNA-352	Doses 1-8			
SNA-103	Doses 1-8			
Motesanib Diphosphate	Doses 1-4			

Results

[0487] Figure 76 depicts the inhibition of VEGF-induced proliferation following treatment with SNA-125, SNA-352, SNA-103, and motesanib diphosphate. The IC_{50} values calculated based on this analysis are shown in Table 25.

Table 25: IC50 Values for SNA-103, SNA-125, SNA-352

Cell density (cells/well)	VEGF concentration (ng/ml)	SNA-125 IC_{50} (μM)	SNA-352 IC_{50} (μM)	SNA-103 IC_{50} (μM)
2×10^3	10	7.638	4.405	122.2
	50	9.838	4.184	213.3
5×10^3	10	34.34	4.208	~103
	50	26.59	5.031	111.5

Conclusions

[0488] SNA-125 showed inhibition of VEGF-induced proliferation. Lower IC_{50} values were seen when treating the lower cell density (values between 7 and 9 μM). SNA-352 also showed consistent inhibition of VEGF-induced proliferation at both concentrations and cell densities tested, with IC_{50} values between 4 and 5 μM. SNA-103 appeared to have an effect on proliferation at the top concentration, with IC_{50} values between 100 and 200 μM. Motesanib diphosphate did not appear to inhibit proliferation with this cell line.

Example 16: Prophetic Study - Rabbit Model of Dry Eye

Study 1: Evaluate the Tolerability and Ocular Distribution of SNA-125 and SNA-352 after Topical Application in New Zealand White Rabbits

[0489] Rabbits will receive BID topical dose in both eyes in accordance with the treatment assignment and study schedule depicted in table 26.

Table 26: Study Schedule

Group	n	Treatment	Dose Route (OU)	Exams	Sample collection at termination
1	2	SNA-125	Topical: BID	Days 1-3: once per day	Tears and conjunctiva
2	2	SNA-352	Topical: BID	Days 1-3: once per day	Tears and conjunctiva

Study 1: Evaluate the Efficacy of SNA-125 and SNA-352 for Treatment of keratoconjunctivitis sicca (dry eye) in New Zealand White rabbits

[0490] Rabbits will be housed at ~ 20% humidity with increased airflow and administered daily with 50 ml of 1% atropine topically into both eyes until study conclusion. After disease induction and baseline exam, topical dose of test articles will be administered in both eyes for 21 days in accordance with the study schedule depicted in Table 27. In-life assessments to be performed are depicted in Table 28.

Table 27: Study Schedule

Group	n	Treatment	Dose Route (OU)	Euthanasia	Matrices collected
1	3	Vehicle (BSS)	Topical: BID	Day 21	AH, iris/ciliary body, cornea, conjunctiva, VH and the main lacrimal gland
2	3	Xdrop (Refresh ophthalmic solution)	Topical: BID	Day 21	
3	3	SNA-125	Topical: BID	Day 21	
4	3	SNA-352	Topical: BID	Day 21	

Table 28: In-life Assessments

Assessments	Frequency
Body weight	Baseline (prior to dosing) Treatment period: days 0 and 21 (all ± 2)
Tear collection	Treatment period: days 14 and 21 (all ± 2)
Blood collection	First day of dosing: pre-dose, 2, 4, 8, 24, 48 and 72 hours after treatment
Clinical ophthalmic exams (slit lamp only) with slit lamp photographs	
Schirmer tear test and fluorescein tear breakup tests (TBUs)	Baseline (prior to dosing) Induction period: days 3, 5, 14 (all ± 1) Treatment period: days 0, 7, 14 and 21 (all ± 2)
Fluorescein staining	
Lissamine green staining	

Example 17: Prophetic Study – DSS-induced colitis chronic model

[0491] Colitis will be induced in C57Bl/6 mice by exposure to 2% DSS in drinking water (3 cycles). The planned treatment groups and study schedule are depicted in Table 29. In-life observations (body weight, morbidity, presence of diarrhea and/or bloody stool) will be conducted at the indicated times. The disease activity index DAI (weigh loss, diarrhea and blood stool) will be determined for all study groups. Endoscopy will be performed on days 10, 21 & 34 and colitis severity will be scored using a 0-4 scale. In addition, colon histopathology (inflammation, edema & mucosal necrosis scoring) will be undertaken. Finally, multiplex analysis on colon homogenates will be performed for the following cytokines: IFN-γ, IL-10, IL-6 and TNFα.

[0492] Colitis will be induced by exposure of mice to 2% DSS in drinking water following a five days on, seven days off cycle for a period of 3 cycles (DSS will be administered on Days 0-4, 12-16, and 24-28). One additional group of eight animals will serve as no-disease controls (Group 1). Animals in Groups 2-4 & 6-11 will be dosed with vehicle or test article once daily (QD) via oral gavage (PO) as indicated in Table 29. Animals in Group 5 will be dosed by intraperitoneal injection (IP) every third day (Q3D) Days 0-30. All animals will be weighed daily and assessed visually for the presence of diarrhea and/or bloody stool at the time of dosing. The disease activity index will be scored daily, in addition to endoscopy DAI. Mice will undergo video endoscopy on Days 10, 21, & 34 to assess colitis severity. Images will be captured from each animal at the most severe region of disease identified during endoscopy. Additionally, stool consistency will be scored during endoscopy. Following endoscopy on day 34, all animals from each treatment group will be sacrificed and blood collected.

[0493] Following euthanasia, the colon will be excised, rinsed, measured, weighed, and then trimmed to 6.0 cm in length and divided into 2 pieces. The colon will be excised, rinsed, measured, weighed, and then trimmed to 6.0 cm in length and divided into 2 pieces; the most distal 5.0 cm section will be swiss rolled and placed in formalin for subsequent histological evaluation. The proximal 1.0 cm portion will be weighed and snap frozen in liquid nitrogen. Additionally, blood will be collected and prepared for plasma using K_2EDTA as the anti-coagulant. The details of the study design are shown in Table 29.

[0494] Colitis will be induced by exposure to 2% DSS in drinking water following a five days on, seven days off cycle for a period of 3 cycles (DSS will be administered on days 0-4, 12-16, and 24-28). For each five day dosing period, a fresh DSS/water solution will be prepared and used for the first three days. A fresh DSS/water solution will be prepared and used for the final two days of the five day dosing period. The DSS/water solution may be made more often if necessary. Animals in Groups 2-4 & 6-11 will be dosed with vehicle or test article once daily (QD) via oral gavage (PO) as indicated in Table 29. Animals in Group 5 will be dosed by intraperitoneal injection (IP) every third day (Q3D) Days 0-30.

[0495] Animals will be observed daily (weight, morbidity, survival, presence of diarrhea and/or bloody stool) in order to assess possible differences among treatment groups and/or possible toxicity resulting from the treatments. Animals will be monitored on a daily basis and those exhibiting weight loss greater than 30% will be euthanized, and will not have samples collected.

[0496] Each mouse will undergo video endoscopy on Days 10, 21, & 34 using a small animal endoscope under isoflurane anesthesia. During each endoscopic procedure still images as well as video will be recorded to evaluate the extent of colitis and the response to treatment. Additionally, we will attempt to capture an image from each animal at the most severe region of disease identified during endoscopy. Colitis severity will be scored using a 0-4 scale (0=normal; 1= loss of vascularity; 2= loss of vascularity and friability; 3= friability and erosions; 4=ulcerations and bleeding). Additionally, stool consistency will be scored during endoscopy.

[0497] The Disease Activity Index (DAI) of each mouse will be scored daily. These measurements will be combined to generate a daily DAI score. Additionally, DAI will also be calculated using the weight loss criteria, endoscopy colitis score, and endoscopy stool consistency score. All animals will be euthanized after endoscopy on day 34 and peripheral blood and colon tissue will be collected.

[0498] The proximal and distal colon samples will be trimmed into 6-8 equally spaced transverse sections. Tissues will be embedded in paraffin and sectioned at

approximately 5 microns. One slide for each animal, containing the distal and proximal colon samples (with all transverse sections per slide), will be stained with hematoxylin and eosin. Sections of colon will be scored for inflammation, edema and mucosal necrosis. Each of the transverse sections is scored for these parameters and the mean is reported for each animal for each parameter. Additionally, the mean sum score is calculated as the sum of inflammation, edema, and mucosal necrosis.

[0499] Colon tissue homogenate supernatants will be analyzed for protein levels of a panel of mouse inflammatory mediators: IFN-γ, IL-10, IL-6, & TNF-α using a multiplex system.

Table 29: Study

Group	Number of Animals	DSS Day 0-4, 12-16, & 24-28	Treatment	Dose	Route	Dose Schedule	Disease Activity Index	Endoscopy Schedule	Terminal Collection (Day 34)
1	8	---	---	---	---	---	Days 0-34	Days 10, 21, & 34	Blood: Plasma Colon: -Snap Frozen -Formalin
2	15	2%	Vehicle	---	PO	QD Days 0-34			
3	15		Vehicle	---	PO	QD Days 10-34			
4	12		Tofacitinib	30 mg/kg	PO	QD Days 10-34			
5	12		Anti-p40	10 mg/kg	IP	Q3D Days 0-30			
6	12		SNA-352	600 mg/kg	PO	QD Days 0-34			
7	12		SNA-352	400 mg/kg	PO	QD Days 10-34			
8	12		SNA-352	800 mg/kg	PO	QD Days 10-34			
9	12		SNA-125	800 mg/kg	PO	QD Days 0-34			
10	12		SNA-125	400 mg/kg	PO	QD Days 10-34			
11	12		SNA-125	800 mg/kg	PO	QD Days 10-34			

Example 18: TNBS-induced Colitis Mouse Model

[0500] Intra-rectal administration of 4 mg of TNBS in C57Bl/6 mice will provide a model of colitis in mice. Both oral (PO) and intracecal (IC) administration of the vehicle and test agents are planned. Contemplated treatment groups are SNA-125

(400mg/kg), SNA-352 (400 mg/kg), vehicle (water), prednisolone (2 mg/kg PO; positive control), and tofacitinib (15 mg/kg PO and 1 mg/kg IC). The vehicle, SNA-125, SNA-352 and tofacitinib will be administered BID Days 0-4 while prednisolone will be administered QD Days 0-4. In-life observations (body weight, morbidity, presence of diarrhea and/or bloody stool) will be conducted. The disease activity index DAI (weight loss, diarrhea and blood stool) will be determined for all study groups. Endoscopy will be performed on days 2 and 4, and colitis severity will be scored using a 0-4 scale. In addition, colon histopathology (inflammation, edema & mucosal necrosis scoring) will be undertaken. Finally, multiplex analysis on colon homogenates will be performed for the following cytokines: IFN-g, IL-10, IL-12(p40), IL12(p70), IL-13, IL-1b, IL-2, IL-6 and TNFα.

Example 19: Prophetic Study – DSS-induced Colitis Acute Model

[0501] Colitis will be induced by administration of 3% DSS on Days 0-5 in C57Bl/6 male mice. Both oral (PO) and intracecal (IC) administration of the vehicle and test agents are planned. Contemplated treatment groups are SNA-125 (400 mg/kg), SNA-352 (400 mg/kg), vehicle (water), Anti-p40 (10 mg/kg IP; positive control), and tofacitinib (15mg/kg PO and 1 mg/kg IC). The vehicle, SNA-125, SNA-352 and tofacitinib will be administered BID Days 0-19 while Anti-p40 will be administered Q3D Days 0-18.

[0502] In-life observations (body weight, morbidity, presence of diarrhea and/or bloody stool) will be conducted. The disease activity index DAI (weight loss, diarrhea and blood stool) will be determined for all study groups. Endoscopy will be performed on days 10, 14 and 19, and colitis severity will be scored using a 0-4 scale. In addition, colon histopathology (inflammation, edema & mucosal necrosis scoring) will be undertaken. Finally, multiplex analysis on colon homogenates will be performed for the following cytokines: IFN-g, IL-10, IL-12(p40), IL12(p70), IL-13, IL-1b, IL-2, IL-6 and TNFα

Example 20: Determination of IC_{50} of SNA-352 against 4 Kinases

Aim of Study

[0503] The goal of this study was to assess the potency (IC_{50}) of SNA-352 against the following kinases: LIMK1, MAP2K6, MLK1, and MLK3.

Materials and Methods

Test Agent

[0504] SNA-352 (Lot # 2017GC14/S7) was used at test concentrations of 10, 3, 1, 0.3, 0.1, 0.03, 0.01, 0.003, 0.001, 0.0003, μM.

Results

[0505] The IC50 value was calculated from concentration vs. %Inhibition curves by fitting to a four parameter logistic curve (Figures 77-80). Table 30 depicts the IC_{50} of SNA-352 and staurosporine for 4 kinases.

TABLE 30 – IC_{50} of SNA-352 and Staurosporine for 4 Kinases

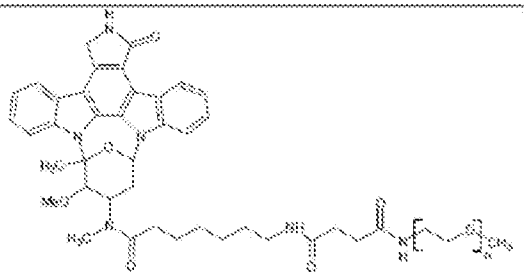
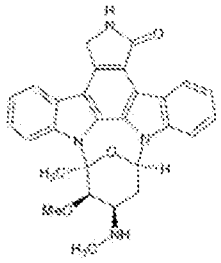
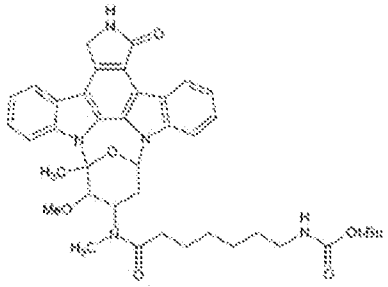
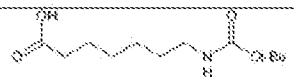
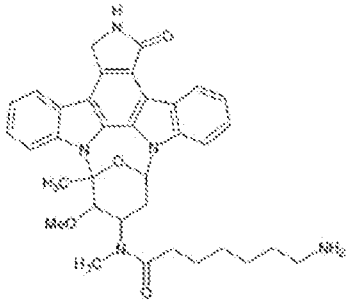
Kinase	IC50(M)	
	SNA-352	Staurosporine
LIMK1	1.49E-07	7.68E-10
MAP2K6	2.61E-07	5.20E-10
MLK1	3.52E-08	3.22E-09
MLK3	2.04E-08	1.68E-09

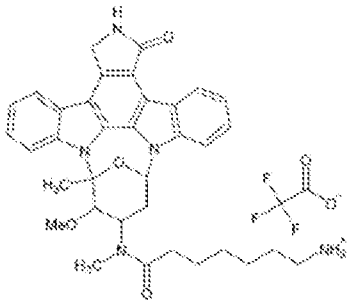
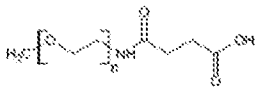
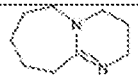
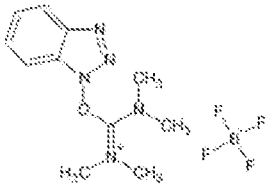
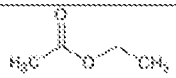
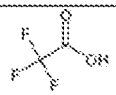
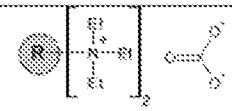
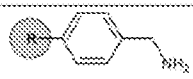
Conclusions

[0506] SNA-352 is a potent inhibitor of LIMK1, MAP2K6, MLK1, and MLK3.

Example 21: SNA-352 SYNTHETIC PROCESS OPTIMIZATION AND PRODUCTION

1. Abbreviations

IUPAC name (nickname)	Formula	MW
N-(7-{ *N*-[(9*S*,10*R*,11*R*,13*R*)-2,3,10,11,12,13-hexahydro-10-methoxy-9-methyl-1-oxo-9,13-epoxy-1*H*,9*H*-diindolo[1,2,3-*gh*:3',2',1'-*lm*]pyrrolo[3,4-*j*][1,7]benzodiazonin-11-yl](methyl)amino}-7-oxoheptyl)-*N*'-mPEG$_{2000}$butanediamide (SNA-352)		(for n=42) 2557.04 (MW 2556.7)
(9*S*,10*R*,11*R*,13*R*)-2,3,10,11,12,13-Hexahydro-10-methoxy-9-methyl-11-(methylamino)-9,13-epoxy-1*H*,9*H*-diindolo[1,2,3-gh:3',2',1'-lm]pyrrolo[3,4-j][1,7]benzodiazonin-1-one (Staurosporine)		466.53
(9*S*,10*R*,11*R*,13*R*)-2,3,10,11,12,13-Hexahydro-10-methoxy-9-methyl-11-[{7-[(tert-butoxycarbonyl)amino]heptanoyl}(methyl)amino]-9,13-epoxy-1*H*,9*H*-diindolo[1,2,3-gh:3',2',1'-lm]pyrrolo[3,4-j][1,7]benzodiazonin-1-one (Intermediate 1)		693.83
7-[(tert-butoxycarbonyl)amino]heptanoic acid		245.32
(9*S*,10*R*,11*R*,13*R*)-2,3,10,11,12,13-Hexahydro-10-methoxy-9-methyl-11-[(7-aminoheptanoyl)(methyl)amino]-9,13-epoxy-1*H*,9*H*-diindolo[1,2,3-gh:3',2',1'-lm]pyrrolo[3,4-j][1,7]benzodiazonin-1-one (Intermediate 2)		593.72

(9S,10R,11R,13R)-2,3,10,11,12,13-Hexahydro-10-methoxy-9-methyl-11-{(7-aminoheptanoyl)(methyl)amino}-9,13-epoxy-1H,9H-diindolo[1,2,3-gh:3',2',1'-lm]pyrrolo[3,4-j][1,7]benzodiazonin-1-one trifluoroacetate (Intermediate 2 trifluoroacetate)		707.73
α-Methoxy-ω-carboxylic acid poly(ethylene glycol) ($MeO\text{-}PEG_{5000}\text{-}COOH$)		(for n=42) 1981.34 (Mp) 1981
1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU)		152.24
O-(1H-benzotriazole-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate (TBTU)		321.08
N,N-dimethylformamide (DMF)		73.09
Ethyl acetate (AcOEt)		88.11
Dichloromethane (DCM)	CH_2Cl_2	84.93
Methanol (MeOH)	$H_3C—OH$	32.04
Trifluoroacetic acid (TFA)		114.02
Acetonitrile (ACN)	$H_3C—C\equiv N$	41.05
Tetraalkylammonium carbonate, polymer-bound (MP-carbonate resin)		
Benzylamine resin		

2. Introduction

[0507] This Example is related to the optimization of the CT352 synthetic procedure and to the production of a 20 g sample of SNA-352. The reference synthetic process is described in Figure 87.

[0508] The synthetic procedure consists of:

[0509] Step 1: Staurosporine acylation by 7-[(tert-butoxycarbonyl)amino]heptanoic acid, using TBTU as the coupling agent;

[0510] Step 2: removal of the Boc protective group of Intermediate 1 with trifluoroacetic acid; and

[0511] Step 3: acylation of Intermediate 2 by MeO-PEG_{2000}-COOH, using TBTU as the coupling agent.

[0512] The points of the process to be revised over the previous procedure were:

[0513] Step 1: isolation and purification of Intermediate 1, avoiding flash chromatography;

[0514] Step 2: isolation of Intermediate 2 (as free base or as trifluoracetate salt); and

[0515] Step 3: purification of final SNA-352 (normal and reverse phase chromatographic purifications required).

[0516] Four batches of SNA-352 were produced. The results are summed up in Table 31. In this report, SNA- 352 yield has been calculated by assigning it 2556.7 Da as molecular weight (SNA-352 molecular weight has been calculated according to the following formula: *SNA-352 molecular weight = Intermediate 2 molecular weight + MeO-PEG_{2000}-COOH molecular weight - 18*), as the molecular weight of MeO-PEG2000-COOH used for its preparation was 1981 Da (MeO-PEG_{2000}-COOH molecular weight was obtained from the certificate of analysis provided by the supplier).

[0517] The overall produced SNA-352 amounted to 25.9 g.

TABLE 31 – SNA-352 produced samples

SNA-352 lot n°	SNA-352 produced amount, g	SNA-352 yield from Staurosporine, %	SNA-352 HPLC purity @292 nm, %
2017CG14/S7	1.1	39	97.7
2017CG14/S14	2.7	56	98.2
2017CG14/S18	9.6	43	98.4
2017CG14/S19	12.5	40	97.5

3. Optimization studies

3.1 Step 1: Staurosporine acylation

[0518] The synthesis of Intermediate 1 was performed by Staurosporine acylation with 7-[(tert-butoxycarbonyl)amino]heptanoic acid, using TBTU as the coupling agent and DBU as base, in DMF as solvent.

[0519] Two experiments were performed starting from 1 g and 12 g of Staurosporine, obtaining Intermediate 1 with 83% and 81% yield, respectively.

[0520] The isolation procedure was slightly modified. The solid Intermediate 1 was precipitated directly by Et_2O addition to the crude product isolated after the work-up, which still contains DMF used in the reaction, so avoiding the use of AcOEt. The experiment on the larger scale (S10) is detailed below in section 4.1 of this example.

[0521] The step 1 outcome appears adequate, some improvement of the yield could be achieved by lowering the DMF content in the crude Intermediate 1 in order to reduce losses of product in mother liquor.

3.2 Step 2: Boc deprotection of Intermediate 1

[0522] Intermediate 2 was obtained by deprotection of Intermediate 1 using TFA in DCM as solvent (Figure 88).

[0523] In the reference procedure, the Intermediate 1 was reacted with excess of TFA (54 equivalents) in DCM, and then neutralized by treatment with a basic resin (tetraalkylammonium carbonate polymer supported) to obtain the free amine.

[0524] The reduction of the excess of TFA was tested and, working with 20 or 15 equivalents of TFA complete conversion was observed in 2-3 h.

[0525] Further reduction of TFA excess to 10 or 12 equivalents resulted in slower reaction rates (80- 90% Intermediate 1 conversion in 3-4 h), without improving reaction selectivity, so more TFA was usually added in order to complete Intermediate 1 conversion. It resulted that the kinetics of the reaction is related to TFA concentration in the reaction mixture.

[0526] In order to avoid the neutralization step, Intermediate 2 was isolated as trifluoroacetate salt by distillation of DCM and excess of TFA, and subsequent precipitation by Et2O addition. The use of Intermediate 2 directly as trifluoroacetate salt in the next step was successfully tested.

[0527] HPLC purity @ 292 nm of isolated Intermediate 2 was 86-90%. Main impurity, according to HPLC-MS, has 311 Da as MW. A plausible structure is depicted in Figure 89.

[0528] This impurity seems to increase during the concentration of the reaction mixture from about 2% in the reaction mixture to 4-6% in the isolated product. Results are shown in Table 32.

[0529] The experiment on the large scale (S15) is detailed in section 4.2 of this example below.

TABLE 32 – Step 2, Boc deprotection of Intermediate

Exp.	Starting Intermediate 1, g	Molar ratio Intermediate 1: TFA	Isolated crude Intermediate 2 HPLC Purity @ 292 nm		
			Intermediate 2	311 Da impurity	Others
S2	0.7	1:20	90.1%	4.6%	5.3%
S6	0.6	1:15	89.0%	5.5%	5.5%
S11	7.0	1: (10 + 2)	90.1%	4.3%	5.6%
S15	7.2	1: (12 + 3)	86.4%	5.8%	7.8%

3.3 Step 3: Intermediate 2 acylation

[0530] The synthesis of SNA-352, by intermediate 2 acylation with MeO-PEG_{2000}-CO_2H, was performed using TBTU as coupling agent and DBU as base, in DMF.

[0531] It was first verified the possibility of using directly Intermediate 2 as trifluoroacetate salt, in the presence of an excess of base. The first trials (Table 33, Exp. S3 and S4) were carried out, at 200-300 mg scale, to define the needed excess of the base and the addition sequence of reagents too, testing the addition of the base (Table 33, Exp. S3) or Intermediate 2 (Table 33, Exp. S4) as the last reagent.

TABLE 33 – Step 3, Intermediate 2 acylation

Exp.	Molar ratio Intermediate 2 salt : $mPEG_{2000}$-CO_2H : TBTU	Molar ratio Intermediate 2 salt : base	SNA-352 Yield[2] %	Normal-phase chromatography	SNA-352 HPLC purity[3] @ 292 nm, %	MeO-PEG derivate mol/SNA-352 mol[4]
S3	1:1.15:1.2	1: (2.4+4.8)	68	SNAP cartridge (50 μm irregular silica)	97.9	0.29
S4[1]	1:(1.15+0.6):(1.2+0.9)	1:(4+3)	65	SNAP cartridge (50 μm irregular silica)	97.3	0.27
S5	1:1.15:1.2	1:4	76	SNAP cartridge (50 μm irregular silica)	98.0	0.16
S8	1:0.95:1.1	1:4	48 + 23	SNAP ULTRA cartridge (25 μm spherical silica)	97.1 97.1	0.16 (S8A) 0.05 (S8B)
S9	1:0.95:1.1	1:4 (DIEA)	-	-		
S12	1:0.8:0.93	1:4	58	SNAP ULTRA cartridge (25 μm spherical silica)	96.2	0.06
S16	1:0.95:1.0	1:4	62	SNAP ULTRA cartridge (25 μm spherical silica)	96.5	0.07

[1] Intermediate 2 trifluoroacetate added as last reagent.

[2] Yield calculated from Intermediate 2, after purification by normal-phase chromatography.

[3] HPLC purity is referred to the SNA-352 samples purified by normal-phase chromatography.

[4] According to ^{1}H-NMR analysis.

[0532] In experiment S3 a further addition of the base was required to reach almost complete starting material conversion, while in experiment S4 further additions of the base or MeO-PEG_{2000}-CO_2H had no effect. Intermediate 2 conversion was achieved when more TBTU was added.

[0533] Following trials were performed adding the base as the last reagent, and, starting from the experiment S5, the base excess was set at 4 equivalents.

[0534] Crude SNA-352 samples, isolated after reaction work-up, were purified by normal-phase flash chromatography, using DCM/MeOH as eluent phases, but, according to ^{1}H-NMR analysis, the molar ratio between the terminal methoxy group of the PEG chain and one of the aromatic protons of SNA-352 in the purified samples was higher than expected.

[0535] In order to avoid a further purification by reverse-phase flash chromatography to reduce the content of MeO-PEG derivative in SNA-352 samples, the following tests (S8-S12-S16, Table 33) were carried out lowering the excess of MeO-PEG_{2000}-CO_2H. Simultaneously, in these experiments the purifications by normal-phase were performed using a new type of cartridge, Snap Ultra HP-Sphere 25 μm spherical silica, in order to improve the separation between SNA- 352 and the residual amounts of MeO-PEG derivate.

[0536] In the experiment S8 two separate lots were obtained and only a small amount of SNA-352 (lot S8B, 23% yield) was isolated with a low content of MeO-PEG derivate.

[0537] The use of a basic resin (tetraalkylammonium carbonate polymer-bound) to remove MeO-PEG derivate residues was tested, by treating a SNA-352 sample (S8A) in DCM as solvent, but it proved ineffective, so did the liquid-liquid extraction of a SNA-352 sample in DCM with a basic solution (5% NaOH).

[0538] In the experiment S9, DIEA was used instead of DBU, with no significant improvement in the reaction selectivity.

[0539] With a further reduction of MeO-PEG_{2000}-CO_2H equivalents to 0.8 (experiment S12), the reaction was not complete (about 3% of unreacted Intermediate 2) and the formation of a guanidine side-product (according to HPLC-MS), was observed (Figures 90 and 91). After purification, SNA- 352 was isolated with a poorer yield (58%), and a low content of MeO-PEG derivate.

[0540] In the experiment S16, to the mixture obtained at the end of the coupling reaction, once reached complete conversion of Intermediate 2, benzylamine resin and TBTU were added, in order to scavenge possible MeO-PEG_{2000}-COOH residues through a covalent bond to the resin. After purification, the content of MeO-PEG derivate was quite low, but not enough to avoid a reverse- phase purification.

[0541] In the end, conditions to limit the content of MeO-PEG derivate in the purified samples of SNA-352 were found, but, if the MeO-PEG compounds have to be almost completely removed, a reversed-phase purification may be used.

[0542] Combining SNA-352 samples (lots S3, S4, S5), a final sample was isolated after reversed-phase flash chromatography (lot 2017GC14/S7, Table 31) with the correct ratio, according to 1H-NMR analysis, between the terminal methoxy group of the PEG chain and one of the aromatic protons of SNA-352.

[0543] Two higher scale reactions, starting from 7.0 g (S13) and 7.2 g (S19) of Intermediate 2 respectively, were carried out.

[0544] An aliquot of crude SNA-352 from experiment S13 was directly purified by reverse-phase chromatography. Two trials were carried out (S17Pur1 and S17Pur2), using two different gradients and H_2O/acetonitrile as eluent phases. The results are depicted in Table 34.

TABLE 34 – Purification of crude SNA-352 by reverse-phase chromatography

Exp.	SNA-352 HPLC purity @ 292 nm, %	Yield* %	MeO-PEG derivate mol/SNA-352 mol
S17Pur1	95.2	75	0.11
S17Pur2	97.7	55	0.04

*Yield calculated from Intermediate 2.

[0545] While the first trial (S17Pur1) gave unsatisfactory results, in the second one, both HPLC purity and content of MeO-PEG derivate were at an acceptable level.

[0546] As no main advantage derives from this approach, the remaining crude samples of SNA-352 from both the preparations (S13 and S17) were first purified by normal-phase chromatography, then by reverse-phase chromatography using H_2O/acetonitrile as eluent phases. The results are depicted in Table 35.

TABLE 35 – purified SNA-352 samples

Exp.	SNA-352 HPLC purity @ 292 nm, %	Yield* %	MeO-PEG derivate mol/SNA-352 mol
S14	98.2	70	0.02
S18	98.4	54	0.02
S19	97.5	49	0.04

*Yield calculated from Intermediate 3.

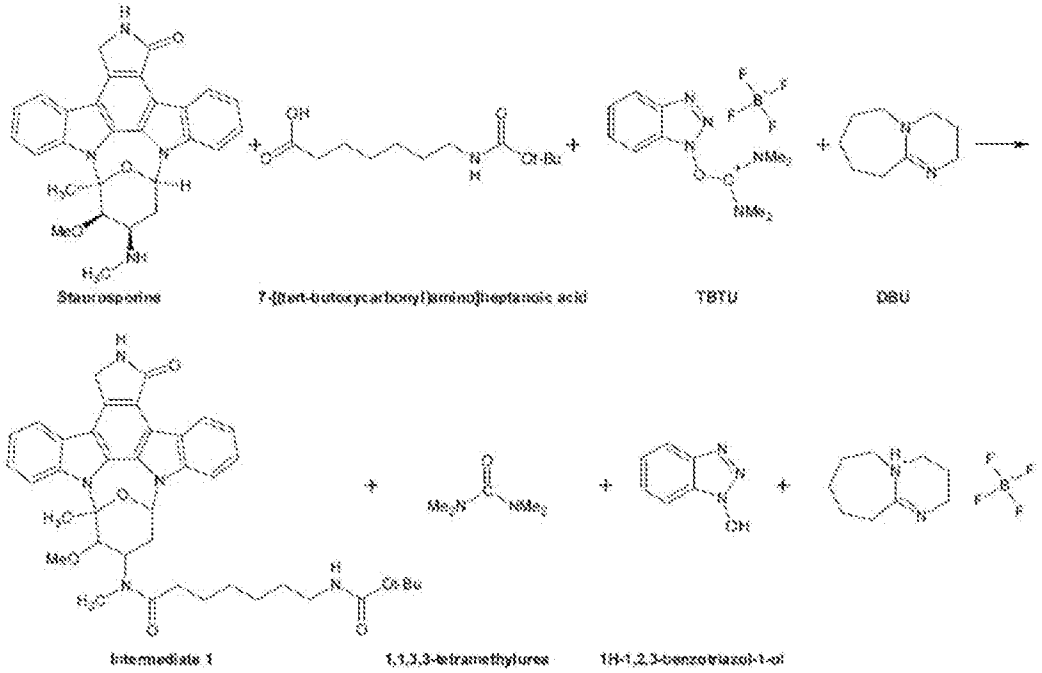
[0547] During the purification by reverse-phase (S14) some fractions attributed to MeO-PEG derivate (weak absorption only at 210 nm) were collected, isolated, and analyzed. According to 1H-NMR, no signals of MeO-PEG-COOH or MeO-PEG-NH_2 were detected, but it was not possible to identify the structure of the contaminant pegylated compound.

[0548] The reaction on the larger scale (S19) is detailed in sections 4.3 and 4.4 of this Example.

4. Experimental procedure for SNA-352 production

[0549] The experimental procedure used for the production of the SNA-352 sample, lot n° 2017CG14/S19 is hereinafter described.

4.1 Step 1: Staurosporine acylation



4.1.1 Material List

Reagents	Supplier, Lot	MW	mol	assay, %	g	density, g/mL	mL	ratio	
Staurosporine	Iris, 370188	466.53	0.0257	100	**12.0**			1	molar
Boc-7-aminoheptanoic acid	TCI, 2522O-TS	245.32	0.0322	98	8.0			1.25	molar
TBTU	Carbosynth, FT070961501	321.08	0.0437	98	14.0			1.7	molar
DBU	Aldrich, BCBQ4609V	152.24	0.0437	100	6.7	1.018	6.55	1.7	molar
DMF	Aldrich, SZBE0690V				227.8	0.949	240	20	mL/g Staurosporine
Product									
Intermediate 1		693.83	0.0257		17.85			1	molar

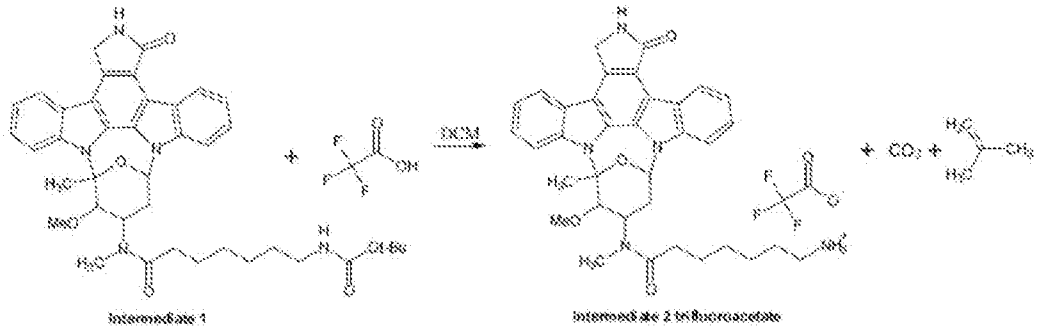
4.1.2 Procedure

[0550] In a 500 mL three necked round bottom flask wrapped in aluminium foil, dried under nitrogen, equipped with magnetic stirrer bar, thermometer and condenser, 130 mL of DMF, 8.0 g of 7-[(tert-butoxycarbonyl)amino]heptanoic acid, 14.0 g of TBTU and 6.55 mL of DBU were placed at 21°C. The yellow solution was aged under stirring at 25°C for 30 min., then 12 g of Staurosporine and 110 mL of DMF were added. The reaction mixture was aged at 21°C for 24 h under nitrogen atmosphere. The reaction progress was monitored by HPLC analyses taking samples during aging time

(Figures 92-93). The final heterogeneous reaction mixture was transferred to a 2 L separating funnel, diluted with 500 mL of DCM and washed with NaHCO3 aqueous saturated solution (1 x 500 mL). The aqueous phase was extracted with 100 mL of DCM. The combined organic phases were evaporated under reduced pressure at 40°C, affording 141 g of a beige suspension, which was treated with 200 mL of Et_2O and stirred for 30 min at 21°C. The beige solid was filtered over sintered glass filter (G3), washed with 2 x 25 mL of Et2O, and dried in an oven under vacuum at 30°C overnight.

[0551] 14.5 g of Intermediate 1 as a light white solid was isolated with HPLC purity @292 nm of 99.5% (Lot 2017CG14/S10) (Figure 94). The sample for HPLC analysis, due to its poor solubility, was prepared in a mixture of DMF/0.1% HCOOH in H_2O/ACN 0.5/0.75/0.75 v/v/v). The yield from Staurosporine was 81%.

4.2 Step 2: removal of the Boc protective group of Intermediate 1



4.2.1 Material List

Reagents	Supplier, Lot	MW	mol	assay, %	mg	density, g/mL	mL	ratio	
Intermediate 1	Serichim, 2017CG14/S10	693.83	0.010	100	7.22			1	molar
DCM	C. Erba, V7G9030378			98			144.4	20	mL/g Intermediate 1
TFA	C. Erba, Q7A113277C	114.02	0.125	98	14.24	1.48	9.6	12.0	molar
TFA	C. Erba, Q7A113277C	114.02	0.031	98	3.56	1.48	2.4	3.0	molar
Product									
Intermediate 2		593.71	0.010		6.18			1	molar
Intermediate 2 as trifluoroacetate salt		707.73	0.010		7.36			1	molar

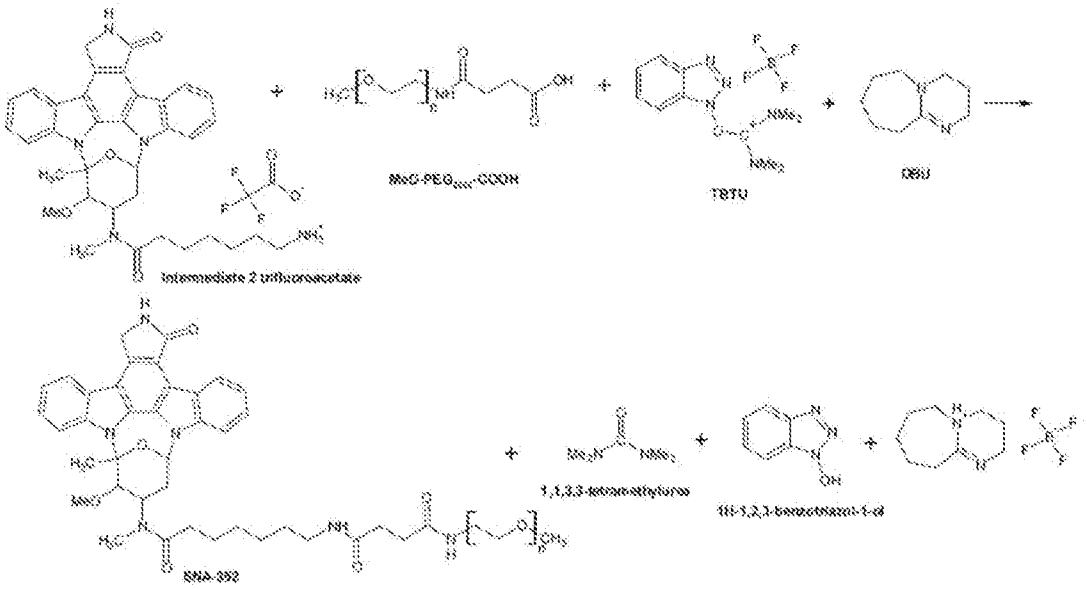
4.2.2 Procedure

[0552] In a 250 mL round bottom flask, equipped with magnetic stirrer bar, condenser, thermometer, 144 mL of DCM, and 7.22 g of Intermediate 1 were placed at 23°C. The mixture was stirred under nitrogen atmosphere for 15 min. obtaining a cloudy

solution, which was cooled to 2°C and 14.2 g of TFA were added in 10 min. The reaction mixture became a clear solution after the addition of about 4 g of TFA. The reaction mixture was aged for 15 min at 2-5°C, then heated to 23°C in about 2h. The reaction progress was monitored by HPLC analyses (Figure 95). After 3h, to complete the conversion of Intermediate 1, further 3.6 g of TFA were added, and the yellow-green mixture was stored in a refrigerator overnight (Figure 96). Excess of TFA was removed by evaporation under reduced pressure at 32-34°C in about 30 min. obtaining 15 g of residual yellow-green oil. The crude residue was treated with 100 mL of Et2O and stirred for 3h at 23°C. The yellow solid was filtered over sintered glass filter (G3), washed with 2 x 25 mL of Et2O, and dried in an oven under vacuum at 35-40°C for 2h.

[0553] 7.6 g of Intermediate 2 trifluoroacetate as a yellow solid were isolated with HPLC purity @ 292 nm of 86% (Lot 2017CG14/S15) (Figure 97). The maximum content of Intermediate 2 trifluoroacetate in the isolated solid was 7.3 g (assuming a quantitative yield), so the assay of Intermediate 2 trifluoroacetate in the isolated crude solid was estimated to be about 97%.

4.3 Step 3: Intermediate 2 acylation



4.3.1 Material List

Reagents	Supplier, Lot	MW	mol	assay, %	g	density, g/mL	mL	ratio	
Intermediate 2 trifluoroacetate	Serichim, 2017CG14/315	707.73	0.010	97	7.23			1	molar
MeO-PEG$_{2000}$-COOH	Iris, 1217393	1981	0.010	95	20.65			1.0	molar
TBTU	Carbosynth, FT070961501	321.08	0.010		3.38			1.0	molar
DBU	Aldrich, BCBQ4609V	152.24	0.040		6.03	1.02	5.9	4.0	molar
DMF	Aldrich, SZBE0690V				123.42	0.949	130.1	18	mL/g intermediate 2 trifluoroacetate
Product									
SNA-352		2556.71	0.010		25.32			1	molar

4.3.2 Procedure

[0554] In a 250 mL three necked round bottom flask, wrapped in aluminium foil, dried under nitrogen, equipped with magnetic stirrer, condenser, thermometer, 7.2 g of Intermediate 2 trifluoroacetate (molar ratios were calculated taking into account only the assay (97%) and not the HPLC purity (86%)), 130 mL of DMF, 20.6 g of MeO-PEG$_{2000}$-COOH, and 3.2 g of TBTU were placed at 22°C. To the clear yellow solution 5.9 mL of DBU were added and the reaction mixture was aged at 22°C under nitrogen atmosphere. The reaction progress was monitored by HPLC analyses (Figure 98). After 3.5 h, the conversion was almost complete. The reaction mixture was diluted with 1| of water, transferred to a 2 l separating funnel, and extracted with DCM (3 x 400 mL). It was noted that organic layers were clear, water phase was cloudy with a little solid at the interphase. The combined organic phases were concentrated under reduced pressure at 40°C in 1.5 h, affording 91.5 g of crude SNA-352 as a yellow liquid with 88% HPLC purity (Figure 99). Assuming a quantitative yield, the assay of SNA-352 in the isolated crude product was estimated to be about 28%.

4.4 SNA-352 Purification

4.4.1 Purification by normal-phase flash chromatography

[0555] Crude SNA-352 from the previous step was purified by normal-phase flash chromatography using a Biotage Isolera LS System equipped with a Biotage SNAP cartridge packed with 750 g of KP-SIL (50 μm Silica, 82x291 mm, column volume 990 mL). The cartridge was equilibrated at 200 mL/min. with 1485 mL of dichloromethane.

[0556] Sample loading was performed by pumping crude SNA-352 (91.5 g) directly onto the cartridge through a peristaltic pump.

[0557] The SNAP cartridge was eluted at 200 mL/min with:

[0558] 1. 990 mL of DCM;

[0559] 2. 2970 mL of DCM/MeOH 97:3 v/v;

[0560] 3. 4950 mL of DCM/MeOH 95:5 v/v;

[0561] 4. 990 mL from DCM/MeOH 95:5 v/v to DCM/MeOH 90:10 v/v;

[0562] 5. 2970 mL of DCM/MeOH 90:10 v/v;

[0563] 6. 1980 mL from DCM/MeOH 90:10 v/v to DCM/MeOH 85:15 v/v;

[0564] 7. 1881 mL of DCM/MeOH 85:15 v/v.

[0565] The first portion of eluate (6168 mL) was sent to the waste, then the eluted solvent was collected in fractions of 250 mL each. UV profile (@ 292 nm and 210 nm) of the purification is depicted in Figure 100. Collected individual fractions were analysed by HPLC. Fractions with HPLC purity ≥ 96% were combined and concentrated under reduced pressure at 40°C to dryness, affording 17.9 g of SNA-352 as a yellow oil (SNA-352/Lot A) with 95.8% HPLC purity (Figure 101). The remaining fractions with HPLC purity ≥ 93% were combined and evaporated under reduced pressure at 40°C to dryness, affording 6.0 g of SNA-352 as a yellow oil (SNA-352/Lot B), with 93.0% HPLC purity (Figure 102).

4.4.2 Purification by reversed-phase flash chromatography

[0566] SNA-352/Lot A sample (17.9 g) from the previous purification was dissolved in 40 mL of Milli-Q H_2O, and, divided into five portions, was purified by reversed-phase flash chromatography using a Biotage Isolera LS System equipped with a Biotage *SNAP KP-C18-HS cartridge* packed with 400 g of KP-C18-HS Silica (column volume 510 mL). The cartridge was equilibrated at 100 mL/min. with 1020 mL of acetonitrile/water 36:64 v/v.

[0567] Sample loading was performed by injecting the SNA-352 solution (about 11-12 g per purification) onto the cartridge through a syringe.

[0568] The SNAP cartridge was eluted at 100 mL/min with:

[0569] 1. 1020 mL of acetonitrile/water 36:64 v/v;

[0570] 2. 357 mL from acetonitrile/water 36:64 v/v to acetonitrile/water 43:57 v/v;

[0571] 3. 561 mL of acetonitrile/water 43:57 v/v;

[0572] 4. 255 mL from acetonitrile/water 43:57 v/v to acetonitrile/water 48:52 v/v.

[0573] The UV profile (@ 292 nm and 210 nm) of one of the purifications is depicted in Figure 103. The first portion of eluate (1020 mL) was sent to the waste, then the eluted solvent was collected in fractions of 100-200 mL each, which were analysed

by HPLC. Fractions with HPLC purity ≥ 97% were combined and concentrated under reduced pressure at 40°C to remove acetonitrile. The residual aqueous phase (about 1000 g) was extracted with DCM (3 x 400 mL). Brine (150 mL) was added during the first extraction to help phase separation. The combined organic phases were evaporated under reduced pressure at 35°C to dryness, affording 8.3 g of SNA-352 as a light yellow oil (HPLC purity @ 292 nm 97.7%).

[0574] Similarly, the remaining fractions with HPLC purity ≥ 95% were combined, concentrated and extracted with DCM affording after evaporation to 4.6 g of SNA-352/Rec 1 as a light yellow oil (HPLC purity @ 292 nm 96.4%).

[0575] The SNA-352/Lot B sample from the previous normal-phase chromatography (6.0 g, 93.0% HPLC purity) and SNA-352/Rec1 from the reverse phase chromatography (4.6 g, 96.4% HPLC purity) were further purified by reversed-phase flash chromatography. Four purifications were performed. The above procedure was applied with a slight modification of elution gradient:

[0576] 1. 1020 mL of acetonitrile/water 36:64 v/v;

[0577] 2. 1275 mL of acetonitrile/water 40:60 v/v;

[0578] 3. 357 mL from acetonitrile/water 40:60 v/v to acetonitrile/water 44:56 v/v.

[0579] UV profile (@ 292 nm and 210 nm) of one of the purifications is depicted in Figure 104.

[0580] The first portion of eluate (1020 mL) was sent to the waste, then the eluted solvent was collected in fractions of 100-200 mL each, which were analyzed by HPLC. Fractions with HPLC purity ≥ 97% were combined and concentrated under reduced pressure at 40°C to remove acetonitrile.

[0581] The residual aqueous phase (about 1200 g) was extracted with DCM (3 x 300 mL). Brine (150 mL) was added during the first extraction to help phase separation. The combined organic phases were evaporated under reduced pressure at 35°C to dryness, affording 7 g of SNA-352 as a light yellow oil (HPLC purity @ 292 nm 97.4%).

[0582] The two purified SNA-352 samples obtained from the reversed-phase chromatography were dissolved in 10 mL of DCM, combined and concentrated under reduced pressure at 35°C to dryness, affording 15 g of SNA-352 as a light yellow oil. The residue was treated with 280 mL of diethyl ether at 22°C, cooled to 5°C, and aged under stirring for 2h at 5°C. The precipitated solid was filtered over sintered glass filter (G3), washed with 2 x 55 mL of diethyl ether and dried under vacuum at 40°C for 21 h. 12.5 g of SNA-352 as a white-off solid were obtained (lot n°2017CG14/S19).

[0583] Final SNA-352 samples were stored at –22°C.

4.5 SNA-352 Characterization

4.5.1 SNA-352 lot n° 2017CG14/S19

[0584] The purity of SNA-352 sample, determined by HPLC analysis @ 292 nm (method MI CT352 001), was 97.5% (figure 105). The product was characterized by 1H-NMR analysis (figure 106). Certificate of analysis of the SNA-352 sample is shown in figure 107.

4.5.2 SNA-352 lot n° 2017GC14/S7

[0585] The purity of SNA-352 sample, determined by HPLC analysis @ 292 nm (method MI CT352 001), was 97.7% (figure 108). The product was characterized by ^{1}H-NMR analysis (figure 109). Certificate of analysis of the SNA-352 sample is shown in figure 110.

4.5.3 SNA-352 lot n° 2017CG14/S14

[0586] The purity of SNA-352 sample, determined by HPLC analysis @ 292 nm (method MI CT352 001), was 98.2% (figure 111). The product was characterized by ^{1}H-NMR analysis (figure 112). Certificate of analysis of the SNA-352 sample is shown in figure 113.

4.5.4 SNA-352 lot n° 2017CG14/S18

[0587] The purity of SNA-352 sample, determined by HPLC analysis @ 292 nm (method MI CT352 001), was 98.4% (figure 114). The product was characterized by ^{1}H-NMR analysis (figure 115). Certificate of analysis of the SNA-352 sample is shown in figure 116.

5.0 Analytical Methods

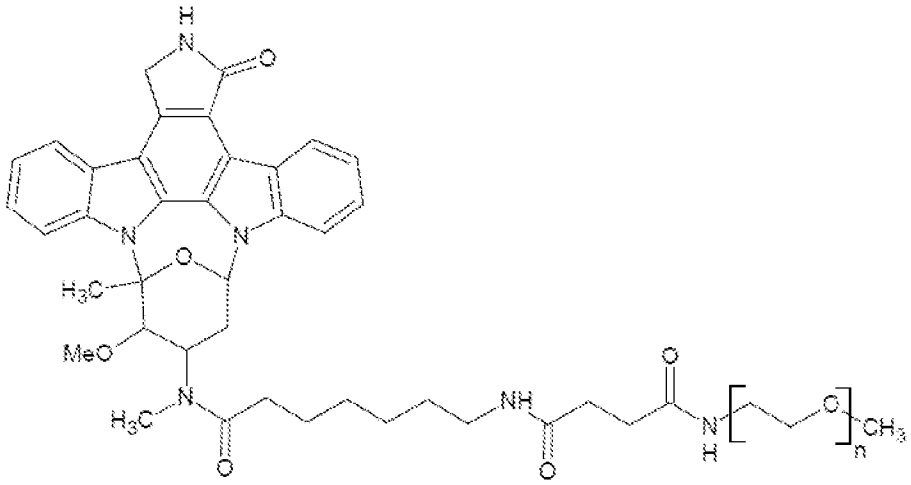
5.1 HPLC method MI CT352 001

[0588] The following method has been used both for reaction monitoring and for assessing chemical purity of final SNA-352 samples.

Sample preparation	Reaction monitoring About 2 drops of reaction mixture were evaporated under nitrogen flow and diluted with 4-6 mL of Eluent A/acetonitrile 1:1 v/v. Isolated products 0.5-1.0 mg/mL solutions in a mixture of H_2O/acetonitrile 1:1 v/v were prepared. Fractions from normal-phase flash chromatography 100-200 µl were evaporated under nitrogen flow and diluted with 2 mL of Eluent A /acetonitrile 1:1 v/v Fractions from reversed-phase flash chromatography 200 µl were diluted with 0.5-1.0 Eluent A/acetonitrile 1:1 v/v.		
Injected volume	5 µl		
HPLC	Agilent 1100		
Detector	UV 292 nm		
Column	Zorbax Eclipse XDB-C18 Solvent Saver - 3 x 150 mm - 5 µm		
Temperature	25°C		
Flow	0.5 mL/min		
Mobile phases	A: 0.1% v/v TFA in H_2O B: 0.1% v/v TFA in acetonitrile		
Gradient	Time, min.	%A	B %
	0	75	25
	2	75	25
	30	10	90
	33	10	90
	35	75	25
Staurosporine	Retention time	7.7 min.	
Intermediate 1	Retention time	22.8 min.	
Intermediate 2	Retention time	9.5 min.	
SNA-352	Retention time	13.6 min.	

WHAT IS CLAIMED IS:

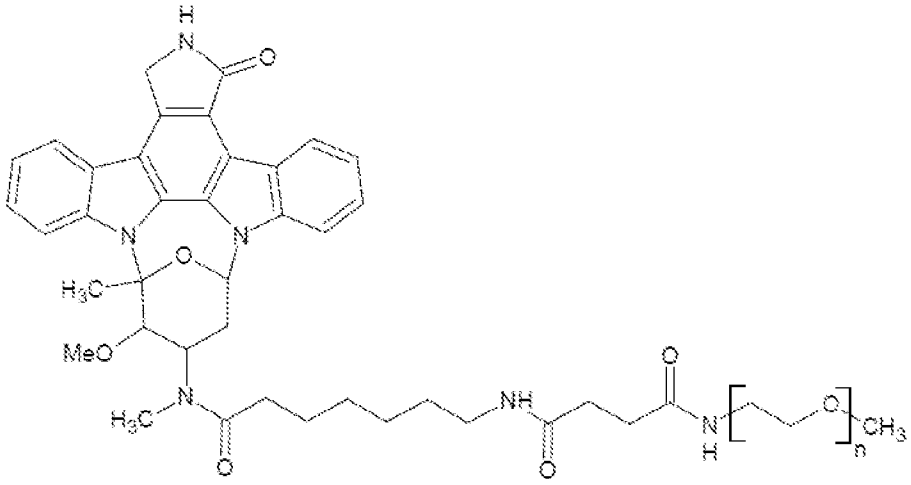
1. A compound having the formula:



wherein n is 2-2270;

and any pharmaceutically acceptable salt thereof.

2. A reduced exposure composition for treating a target site, comprising a conjugate comprising at least one active entity linked to at least one polymer, wherein the conjugate has reduced exposure at a non-target site as compared to the active entity delivered without the polymer, wherein the non-target site comprises the systemic system, the lymphatic system and/or another non-target tissue site, wherein the conjugate has the formula:



wherein n is 2-2270;

and any pharmaceutically acceptable salt thereof; and

a pharmaceutically acceptable carrier formulated for delivery of the conjugate to

the target site.

3. A reduced exposure composition for treating a cell within a target site, comprising a conjugate, the conjugate comprising an active entity linked to at least one polymer, and a pharmaceutically acceptable carrier formulated for delivery of the conjugate to the target site;

wherein the composition has reduced exposure at a non-target site as compared to the active entity delivered without the polymer;

wherein the active entity is an inhibitor, antagonist, or inverse agonist of a cellular kinase;

wherein the active entity comprises or consists essentially of any one or more of compounds 1-40;

wherein the at least one polymer is polyethylene glycol (PEG) or methoxy-polyethylene glycol (m-PEG); and

wherein the conjugate can traverse the cell membrane and distribute among both lipophilic and hydrophilic cellular compartments within the cell, thereby promoting interactions between the active entity and the cellular kinase.

4. The composition of claim 3, wherein the non-target site includes non-target tissue at which pharmacological activity is not desired and/or not achieved.

5. The composition according to any one of claims 3-4, wherein the active entity comprises compound 1.

6. The composition according to any one of claims 3-5, wherein the composition comprises SNA-352.

7. The composition according to any one of claims 3-6, wherein the cellular kinase is a JAK family protein or a STAT family protein.

8. The composition according to any one of claims 3-7, wherein the active entity binds to a JAK family protein.

9. The composition according to any one of claims 3-8, wherein the active entity inhibits a JAK family protein.

10. The composition according to any one of claims 3-9, wherein the JAK protein comprises one or more of JAK1, JAK2, JAK3, and Tyrosine kinase 2 (TYK2).

11. The composition according to any one of claims 3-10, wherein the active entity binds to a STAT family protein.

12. The composition according to any one of claims 3-11, wherein the active entity inhibits a STAT family protein.

13. The composition according to any one of claims 3-12, wherein the active entity has one or more carboxyl, hydroxyl, amino and/or sulfhydryl groups.

14. The composition of claim 13, wherein the at least one polymer is conjugated to the active entity at the one or more carboxyl, hydroxyl, amino and/or sulfhydryl groups.

15. The composition of any one of claims 3-14, wherein the conjugate has a longer residence time within the cell compared to the active entity without conjugation to the polymer.

16. The composition of claim 15, wherein the residence time of the conjugate is at least 25% longer as compared to the active entity without conjugation to the polymer.

17. The composition of claim 15, wherein the residence time of the conjugate is at least 2-20 fold longer as compared to the active entity without conjugation to the polymer.

18. The composition of any one of claims 3-17, wherein the conjugate exhibits greater access to the kinase compared to the active entity without conjugation to the polymer.

19. The composition of any one of claims 3-18, wherein the conjugate exhibits a depo effect across cellular compartments, thereby reducing the dose of the active entity required to inhibit kinase activity compared to the active entity without conjugation to the polymer.

20. The composition of claim 19, wherein the dose of the conjugate needed to achieve a comparable therapeutic effect is 10-90% lower as compared to the active entity without conjugation to the polymer.

21. The composition of any one of claims 3-20, wherein the activity entity has a concentration, activity and/or bioavailability at the target site that is at least 2-20 fold greater than at a non-target site, wherein the non-target site comprises the circulatory system.

22. The composition of any one of claims 3-21, wherein the activity entity has a concentration, activity and/or bioavailability at the target site that is at least 2-20 fold greater than at a non-target site, wherein the non-target site comprises the lymphatic system.

23. The composition of Claim 21 or 22, wherein the reduced concentration, activity and/or bioavailability reduces toxicity.

24. The composition of any one of claims 3-20, wherein the activity entity has a concentration, activity and/or bioavailability at the target site that is at least 2-20 fold greater than at a non-target site, wherein the non-target site comprises bone marrow.

25. The composition of Claim 24, wherein the reduced concentration, activity and/or bioavailability in the bone marrow reduces immunosuppression.

26. The composition of any one of claims 3-25, wherein the conjugate is present at a biologically inactive concentration at a non-target site.

27. The composition of any one of claims 3-26, wherein the conjugate is amphiphilic

28. The composition of any one of claims 3-27, wherein the conjugate is at least 25% more amphiphilic than the active entity without conjugation to the polymer.

29. The composition of any one of claims 3-28, wherein the conjugate is at least 25% more hydrophilic than the active entity without conjugation to the polymer, thus facilitating non-compartmentalization within the cell.

30. The composition of any one of claims 3-29, wherein the conjugate is at least 25% more hydrophilic than the active entity without conjugation to the polymer, thus facilitating access to and activity in both the lipid bilayer and the cytosol of the cell.

31. The composition of any one of claims 3-30, wherein the conjugate is at least 25% more hydrophilic than the active entity without conjugation to the polymer, thus facilitating access to and/or activity in both the lipid bilayer and the cytoplasm of the cell.

32. The composition of any one of claims 3-31, wherein the conjugate is at least 25% more hydrophilic than the active entity without conjugation to the polymer, thus facilitating access to and/or activity across the lipid bilayer.

33. The composition according to any one of claims 2-32, wherein the composition is formulated for topical administration.

34. The composition according to any one of claims 2-32, wherein the composition is formulated as an inhalant.

35. The composition according to any one of claims 2-32, wherein the composition is formulated as an injectable.

36. The composition according to any one of claims 2-32, wherein the composition is formulated as an eye drop.

37. The composition according to any one of claims 2-32, wherein the composition is formulated for oral administration.

38. The composition according to any one of claims 2-32, wherein said composition is administered via at least two routes of administration, either simultaneously or sequentially.

39. The composition according to any one of claims 2-32, wherein said composition is administered via a topical route to a subject, and wherein the subject further receives an additional agent via a non-topical route to achieve synergetic effects.

40. The composition according to any one of claims 2-39, the composition further comprising one or more additional ingredients from the group consisting of a protective agent, an emollient, an astringent, a humectant, a sun screening agent, a sun

tanning agent, a UV absorbing agent, an antibiotic agent, an anti-angiogenesis agent, a preventive or therapeutic agent for inflammatory bowel disease, a physiological cooling agent, an antifungal agent, an antiviral agent, an antiprotozoal agent, an anti-acne agent, an anesthetic agent, a steroidal anti-inflammatory agent, a non-steroidal anti-inflammatory agent, an antipruritic agent, an additional antioxidant agent, a chemotherapeutic agent, an anti-histamine agent, a vitamin or vitamin complex, a hormone, an anti-dandruff agent, an anti-wrinkle agent, an anti-skin atrophy agent, a skin whitening agent, and a cleansing agent.

41. A method for treating an inflammatory condition in a subject in need thereof, the method comprising administering to the subject an effective amount of a composition according to any one of claims 2-40.

42. A method for treating any one or more of inflammatory bowel disease, irritable bowel syndrome or small intestinal bacteria overgrowth in a subject in need thereof, the method comprising administering to the subject an effective amount of a composition according to any one of claims 2-40.

43. A method for treating ulcerative colitis in a subject in need thereof, the method comprising administering to the subject an effective amount of a composition according to any one of claims 2-40.

44. A method for treating Crohn's disease in a subject in need thereof, the method comprising administering to the subject an effective amount of a composition according to any one of claims 2-40.

45. A method for treating an inflammatory skin disease in a subject in need thereof, the method comprising administering to the subject an effective amount of a composition according to any one of claims 2-40.

46. A method for treating a wound in a subject in need thereof, the method comprising administering to the subject an effective amount of a composition according to any one of claims 2-40.

47. A method for treating a scar in a subject in need thereof, the method comprising administering to the subject an effective amount of a composition according to any one of claims 2-40.

48. A method for treating a cancerous or pre-cancerous lesion in a subject in need thereof, the method comprising administering to the subject an effective amount of a composition according to any one of claims 2-40.

49. A method for treating a lung in a subject in need thereof, the method comprising administering to the subject an effective amount of a composition according to any one of claims 2-40.

50. A method for treating the gastrointestinal system in a subject in need

thereof, the method comprising administering to the subject an effective amount of a composition according to any one of claims 2-40.

51. A method for treating an autoimmune disorder in a subject in need thereof, the method comprising administering to the subject an effective amount of a composition according to any one of claims 2-40.

52. A method for treating an eye in a subject in need thereof, the method comprising administering to the subject an effective amount of a composition according to any one of claims 2-40.

53. A method for treating a joint in a subject in need thereof, the method comprising administering to the subject an effective amount of a composition according to any one of claims 2-40.

54. Use of the composition of any one of claims 2-40 for treating non-dermal inflammation in a subject in need thereof.

55. Use of the composition of any one of claims 2-40 for treating any one or more of inflammatory bowel disease, irritable bowel syndrome or small intestinal bacterial overgrowth in a subject in need thereof.

56. Use of the composition of any one of claims 2-40 for treating ulcerative colitis in a subject in need thereof.

57. Use of the composition of any one of claims 2-40 for treating Crohn's disease in a subject in need thereof.

58. Use of the composition of any one of claims 2-40 for treating an inflammatory skin disease in a subject in need thereof.

59. Use of the composition of any one of claims 2-40 for treating a wound in a subject in need thereof.

60. Use of the composition of any one of claims 2-40 for treating a scar in a subject in need thereof.

61. Use of the composition of any one of claims 2-40 for treating a cancerous or pre-cancerous lesion in a subject in need thereof.

62. Use of the composition of any one of claims 2-40 for treating a lung in a subject in need thereof.

63. Use of the composition of any one of claims 2-40 for treating the gastrointestinal system in a subject in need thereof.

64. Use of the composition of any one of claims 2-40 for treating an autoimmune disorder in a subject in need thereof.

65. Use of the composition of any one of claims 2-40 for treating an eye in a subject in need thereof.

66. Use of the composition of any one of claims 2-40 for treating a joint in a

subject in need thereof.

67. Use of the composition of any one of claims 2-40 for treating or preventing one or more of the following conditions: inflammatory bowel disease, irritable bowel syndrome, small intestinal bacterial overgrowth Crohn's disease, ulcerative colitis, collagenous colitis, lymphocytic colitis, ischemic colitis, diversion colitis, Behçet's syndrome, and indeterminate colitis, alopecia, alopecia areata, androgenic alopecia, and dry eye.

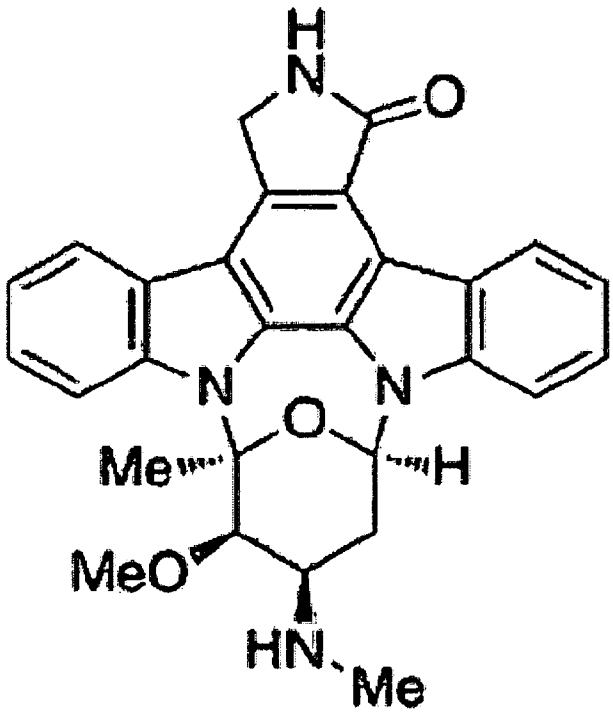


Figure 1

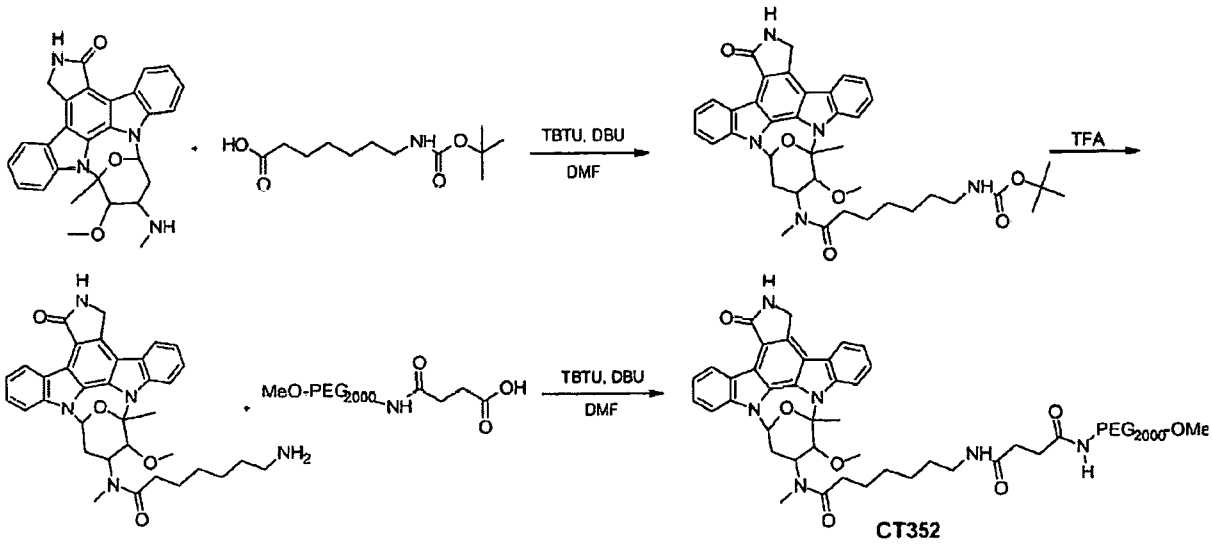


Figure 2

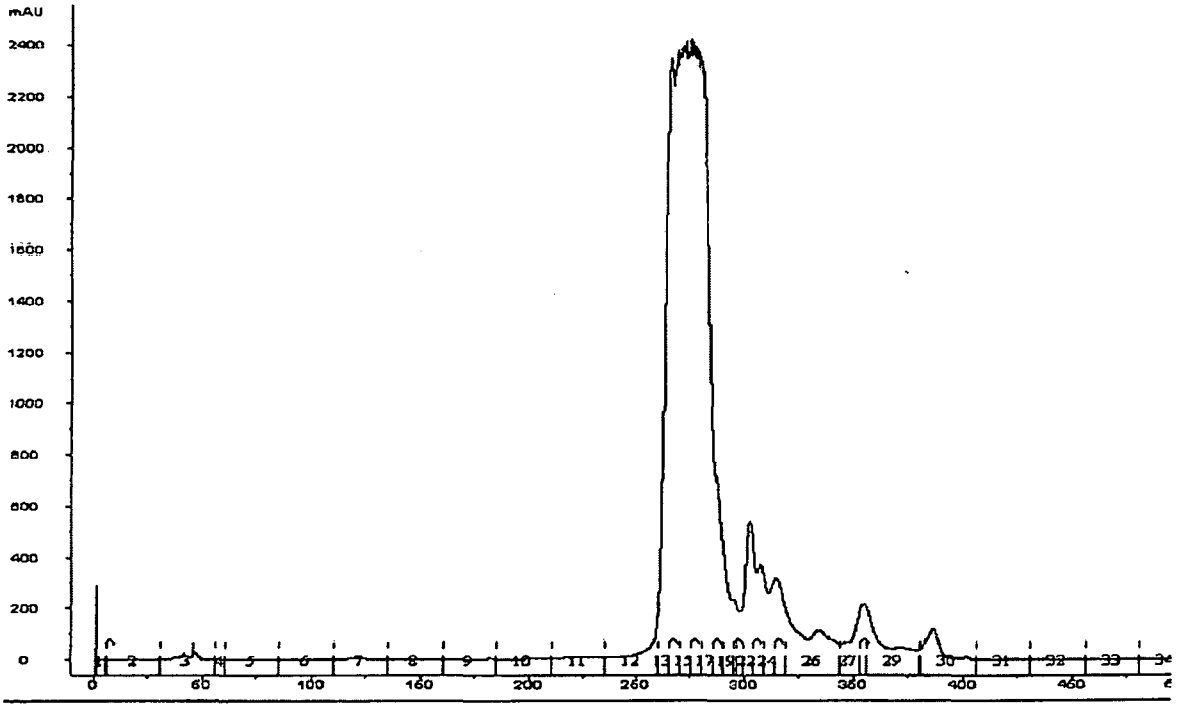

mAU
2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
0
60
100
160
200
260
300
360
400
460

Figure 3

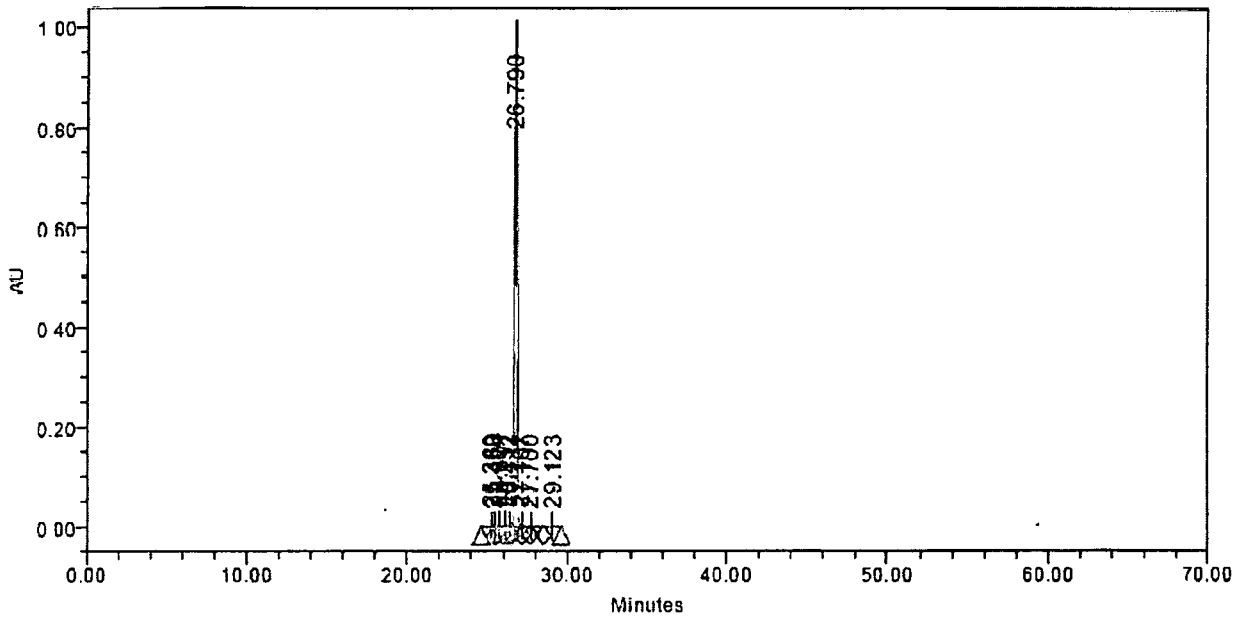

1.00
0.80
0.60
0.40
0.20
0.00
AU
26.790
29.123
0.00
10.00
20.00
30.00
40.00
50.00
60.00
70.00
Minutes

Figure 4

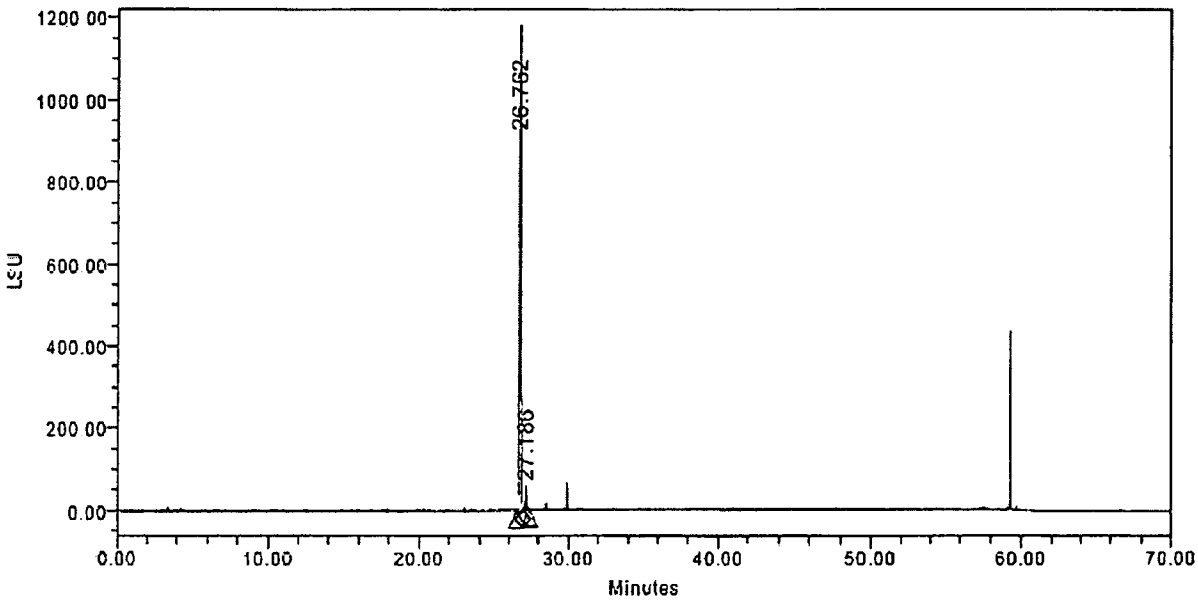

1200.00
1000.00
800.00
600.00
400.00
200.00
0.00
LSU
26.762
27.180
0.00
10.00
20.00
30.00
40.00
50.00
60.00
70.00
Minutes

Figure 5

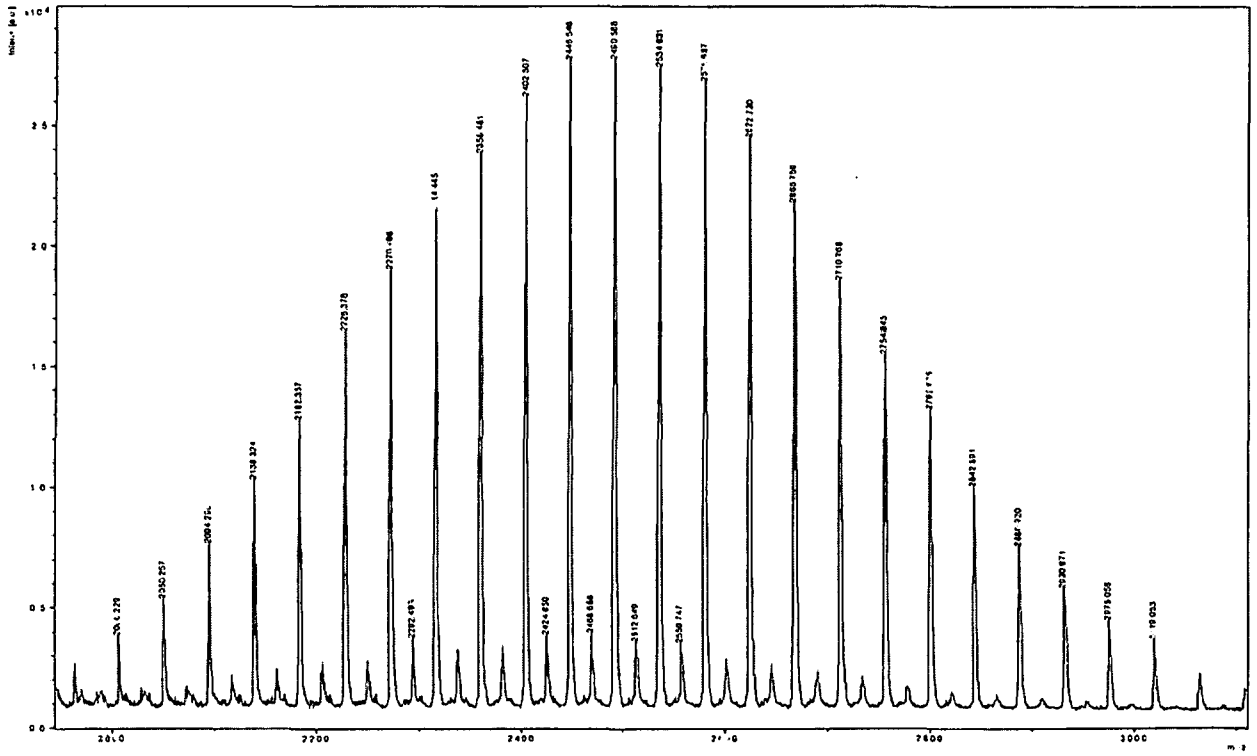


Figure 6

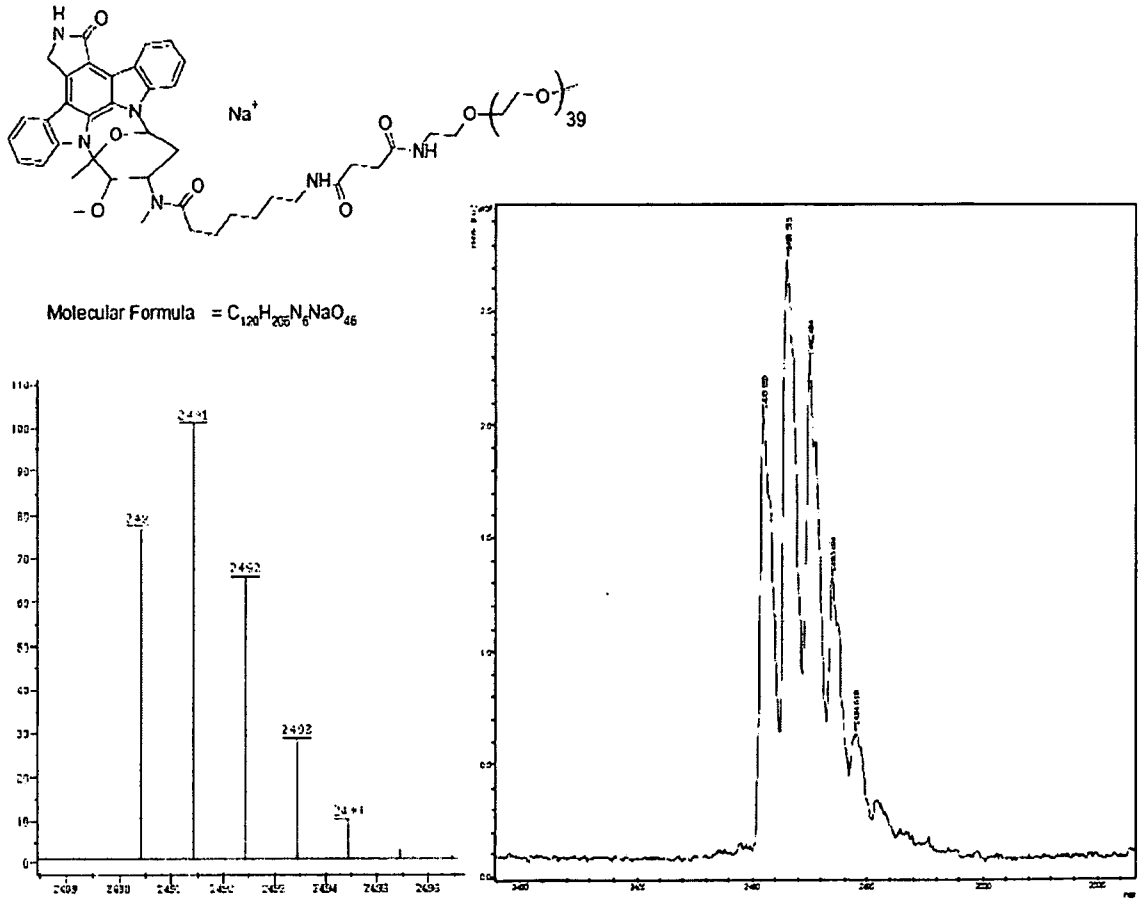


Figure 7

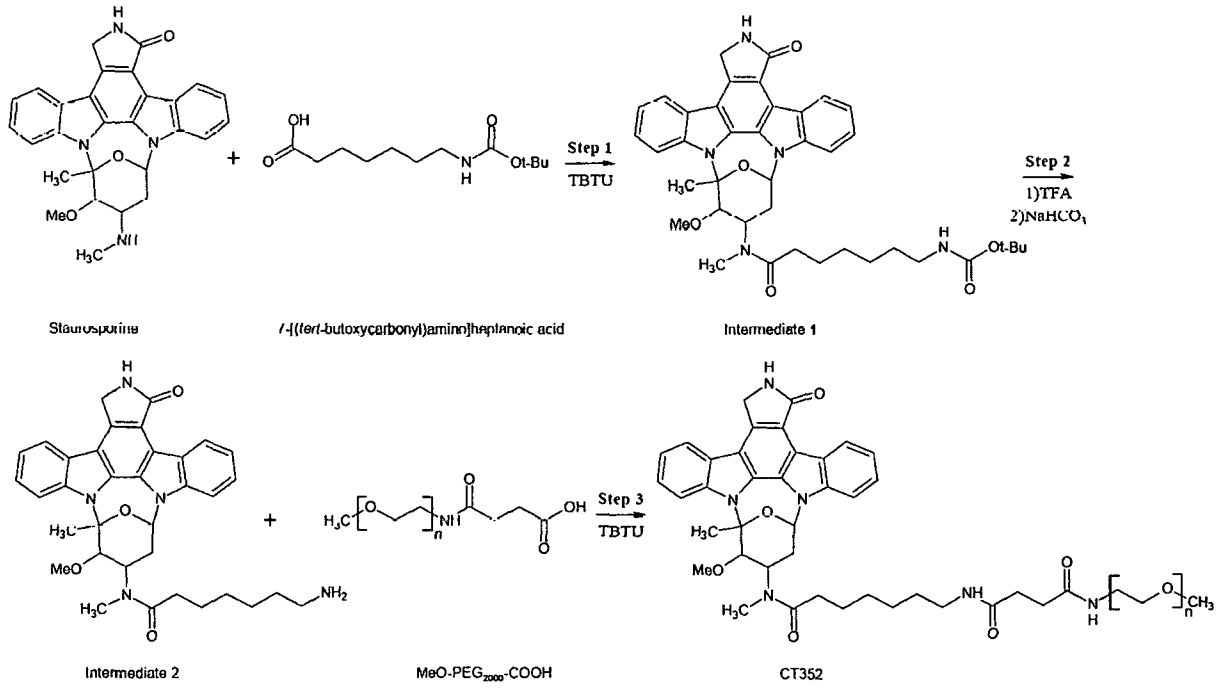
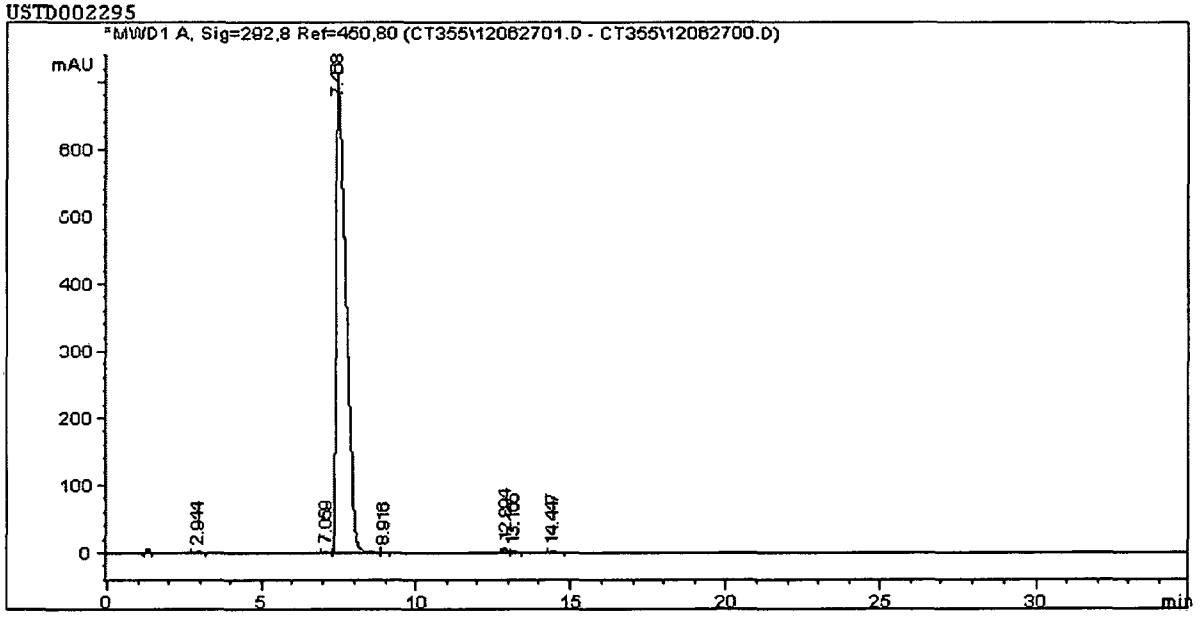

Step 1
TBTU
Step 2
1)TFA
2)NaHCO3
Step 3
TBTU
Staurosporine
7-[(tert-butoxycarbonyl)amino]heptanoic acid
Intermediate 1
Intermediate 2
MeO-PEG2000-COOH
CT352

Figure 8

S1 Staurosporina lot 02/12 in MeOH +0 .1% HCOOH in H2O/
ACN

```
==========================================================================
                                                    Seq. Line :   2
Sample Name      : S1                               Location : Vial 2
                                                        Inj :   1
                                                  Inj Volume : 5 µl
Acq. Method      : C:\HPCHEM\1\METHODS\CT355.M

Analysis Method : C:\HPCHEM\1\METHODS\CT355.M
```

CT355 Colonna Eclipse XDB-C18 Solvent Saver 3.0 x150 mm 5 micron P.N.993967-302; S.N. USTD002295




```
==========================================================================
                          Area Percent Report
==========================================================================

Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000

Signal 1: MWD1 A, Sig=292,8 Ref=450,80
 Signal has been modified after loading from rawdata file!
```

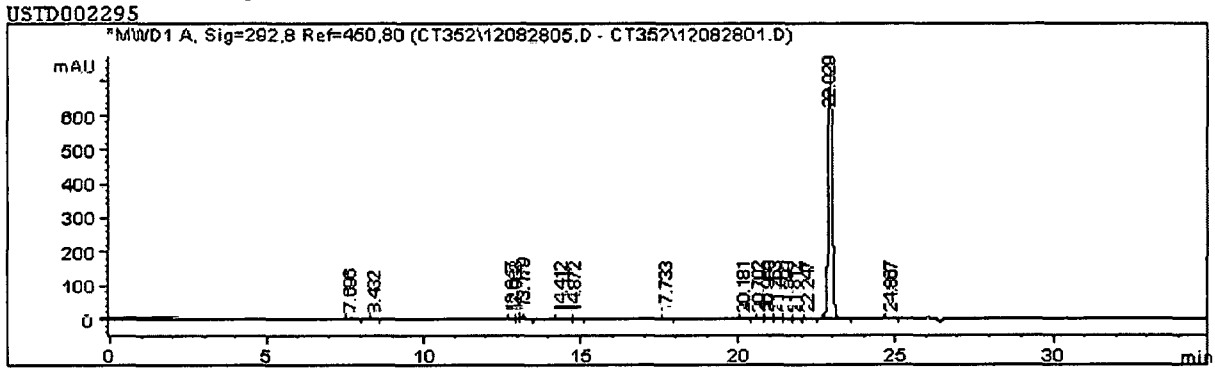
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	2.944	BB	0.1539	19.39532	1.91973	0.1248
2	7.059	BB	0.0936	21.65826	3.52674	0.1393
3	7.468	BB	0.3542	1.54086e4	707.11615	99.1087
4	8.916	BB	0.1717	12.28347	9.97600e-1	0.0790
5	12.894	PV	0.1005	44.49410	6.78552	0.2862
6	13.165	VB	0.1175	23.28148	2.96875	0.1497
7	14.447	BB	0.1688	17.46013	1.44626	0.1123
Totals :				1.55472e4	724.76075	

Figure 9

S7/C sintesi CT352 Step 1, Dopo work-up ed evaporazione
campione in 0.1% HCOOH in H2O/ACN 1/1

```
==========================================================================
                                                  Seq. Line :   3
Sample Name     : S7/C                             Location : Vial 26
                                                       Inj :   1
                                                 Inj Volume : 5 µl
Acq. Method     : C:\HPCHEM\1\METHODS\CT355.M

Analysis Method : C:\HPCHEM\1\METHODS\CT355.M
```

CT355 Colonna Eclipse XDB-C18 Solvent Saver 3.0 x150 mm 5 micron P.N.993967-302; S.N. USTD002295




```
==========================================================================
                          Area Percent Report
==========================================================================

Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000

Signal 1: MWD1 A, Sig=292,8 Ref=450,80
 Signal has been modified after loading from rawdata file!
```

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	7.696	BB	0.1193	49.89889	6.37614	0.7701
2	8.432	DB	0.0957	7.79769	1.23427	0.1203
3	12.847	PV	0.0973	18.23234	2.90086	0.2814
4	13.008	VV	0.0932	16.41774	2.61646	0.2534
5	13.179	VB	0.1118	90.06594	11.96988	1.3900
6	14.412	VB	0.1704	20.15851	1.69913	0.3111
7	14.872	BB	0.1049	10.82809	1.56087	0.1671
8	17.733	BP	0.1117	11.77978	1.64193	0.1818
9	20.181	BB	0.1225	19.63993	2.47606	0.3031
10	20.702	BV	0.1168	28.93373	3.88698	0.4465
11	20.960	VV	0.1378	18.19413	2.00689	0.2808
12	21.263	VV	0.1249	23.93188	2.94129	0.3693
13	21.590	VV	0.1344	16.40950	1.79990	0.2532
14	21.812	VB	0.1292	5.51248	6.35783e-1	0.0851
15	22.247	PP	0.1378	15.02051	1.68813	0.2318
16	22.929	BB	0.1279	6096.19922	741.33539	94.0827
17	24.867	BP	0.1289	30.60047	3.68398	0.4723
Totals :				6479.62084	790.45394	

Figure 10

Sample Name	S7		
Method	CT352 Step1		
Project			
Comment			
Cartridge	SNAP HP 50g	Detection Mode	UV1
Flowrate	50 ml/min	UV1 (Collection)	292 nm
Solvent A	n-Hexane	UV2 (Monitor)	254 nm
Solvent B	Ethyl acetate	Collect All	On
Rack Type	120 ml		
Max Fraction Volume	50 ml		
Dispense Order	S		
Initial Waste	2 CV		

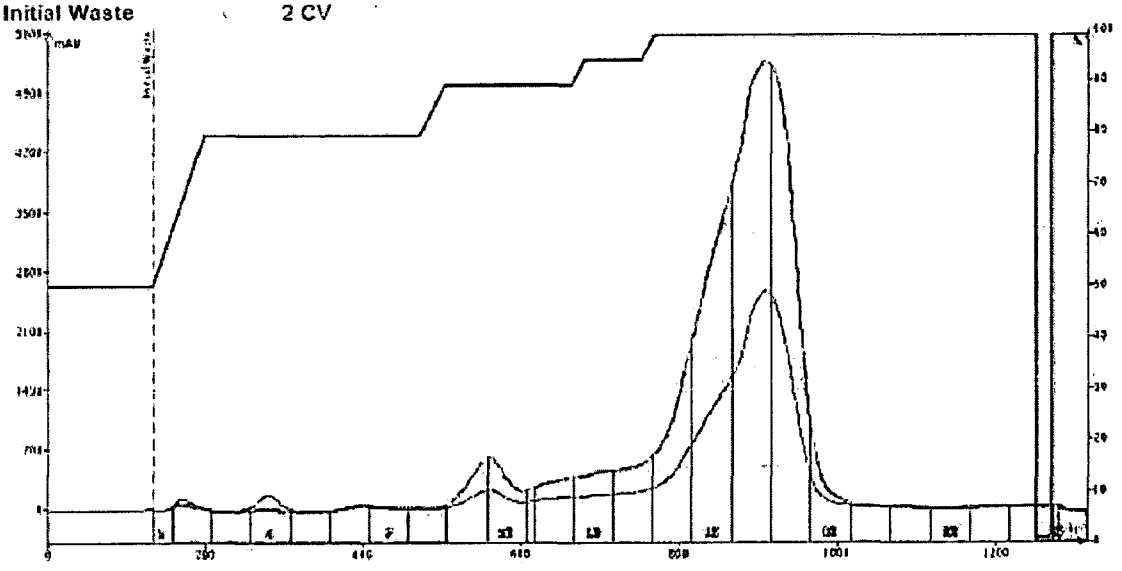
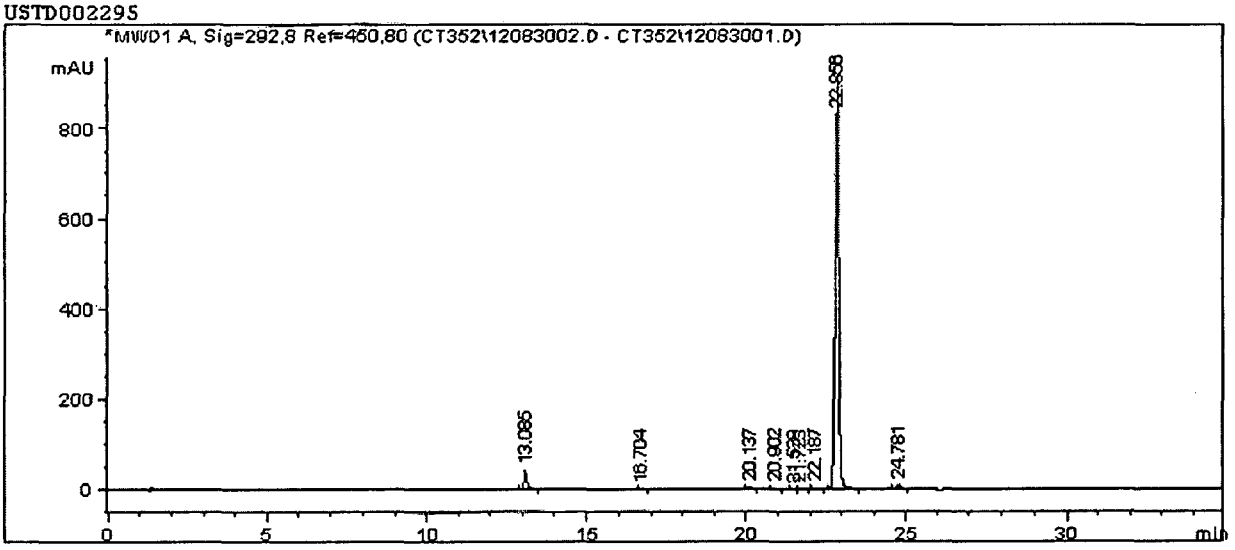


Figure 11

S7 Fr.10-18 dopo evaporazione e dissoluzione in DCM
in 0.1% HCOOH in H2O/ACN

```
==================================================================
                                                Seq. Line :   2
Sample Name     : S7 Fr.10-18                    Location : Vial 41
----- -------   . -----                            Inj :   1
                                                Inj Volume : 5 µl
Acq. Method     : C:\HPCHEM\1\METHODS\CT355.M

Analysis Method : C:\HPCHEM\1\METHODS\CT355.M

CT355 Colonna Eclipse XDB-C18 Solvent Saver 3.0 x150 mm 5 micron P.N.993967-302; S.N.
USTD002295
```




```
==================================================================
                        Area Percent Report
==================================================================

Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000

Signal 1: MWD1 A, Sig=292,8 Ref=450,80
 Signal has been modified after loading from rawdata file!
```

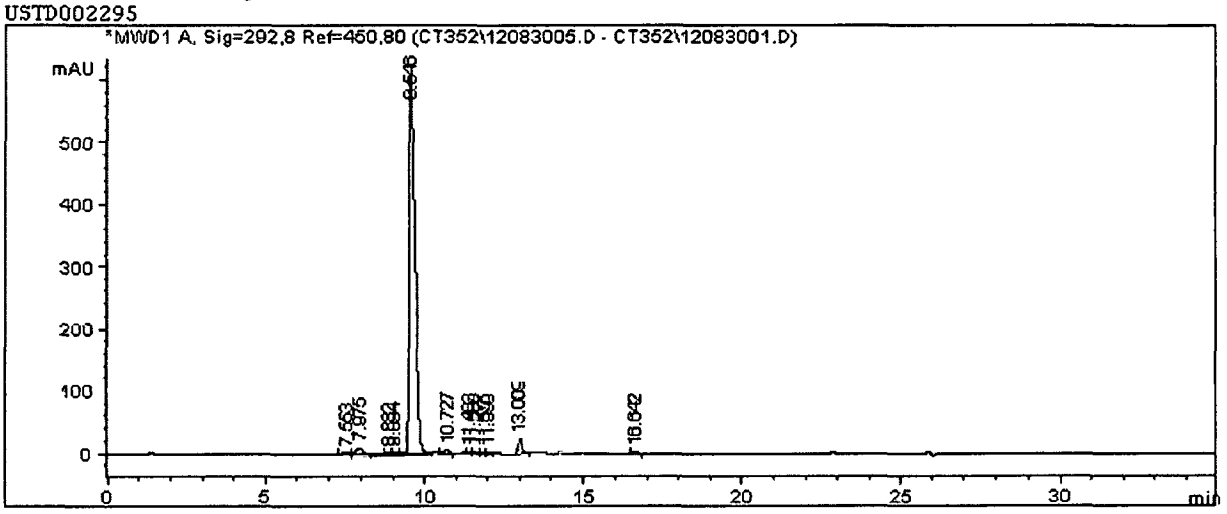
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	13.085	VB	0.1137	295.94769	39.35570	3.7324
2	16.704	VB	0.1289	6.05074	6.73259e-1	0.0763
3	20.137	BB	0.1177	21.11941	2.81025	0.2664
4	20.902	BP	0.1175	16.41666	2.14029	0.2070
5	21.539	BV	0.1172	9.04093	1.18282	0.1140
6	21.723	VP	0.1340	4.68822	5.15793e-1	0.0591
7	22.187	PP	0.1360	16.02187	1.85838	0.2021
8	22.856	BB	0.1276	7502.79834	915.71570	94.6231
9	24.781	BP	0.1345	57.05475	6.49561	0.7196
Totals :				7929.13862	970.74780	

Figure 12

0.1% HCOOH in H2O/ACN

```
=====================================================================
                                                  Seq. Line :   5
Sample Name      : S8/C                           Location : Vial 44
                                                      Inj :   1
                                                  Inj Volume : 5 µl
Acq. Method      : C:\HPCHEM\1\METHODS\CT355.M

Analysis Method : C:\HPCHEM\1\METHODS\CT355.M
```

CT355 Colonna Eclipse XDB-C18 Solvent Saver 3.0 x150 mm 5 micron P.N.993967-302; S.N. USTD002295




```
=====================================================================
                        Area Percent Report
=====================================================================

Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000

Signal 1: MWD1 A, Sig=292,8 Ref=450,80
 Signal has been modified after loading from rawdata file!
```

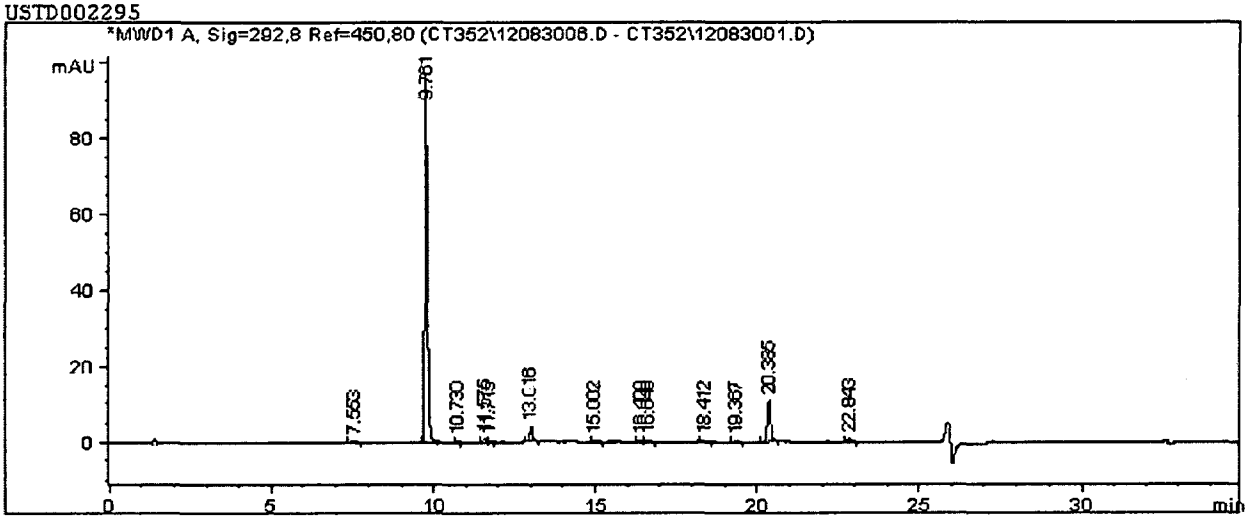
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	7.553	BV	0.1529	8.99737	8.27311e-1	0.1094
2	7.975	VB	0.1753	100.76027	9.09058	1.2251
3	8.882	BV	0.1265	19.27966	2.23835	0.2344
4	9.094	VV	0.1577	23.10063	2.07778	0.2809
5	9.546	VB	0.1919	7810.54541	607.97626	94.9679
6	10.727	VB	0.1126	38.26448	5.04020	0.4653
7	11.402	BV	0.1006	15.56134	2.36870	0.1892
8	11.568	VB	0.0998	8.64735	1.29748	0.1051
9	11.875	BV	0.0905	10.34258	1.76270	0.1258
10	11.990	VB	0.0902	8.73610	1.41216	0.1062
11	13.009	PB	0.1173	172.14793	22.50208	2.0931
12	16.642	BB	0.1125	8.02259	1.10719	0.0975
Totals :				8224.40571	657.70078	

Figure 13

Sintesi di INT2, dopo work up
0.1% HCOOH in H2O/ACN

```
==========================================================================
                                                    Seq. Line :   6
Sample Name      : S8/D                              Location : Vial 45
                                                         Inj :   1
                                                  Inj Volume : 5 µl
Acq. Method      : C:\HPCHEM\1\METHODS\CT355.M

Analysis Method : C:\HPCHEM\1\METHODS\CT355.M
```

CT355 Colonna Eclipse XDB-C18 Solvent Saver 3.0 x150 mm 5 micron P.N.993967-302; S.N. USTD002295




```
==========================================================================
                          Area Percent Report
==========================================================================

Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000

Signal 1: MWD1 A, Sig=292,8 Ref=450,80
 Signal has been modified after loading from rawdata file!
```

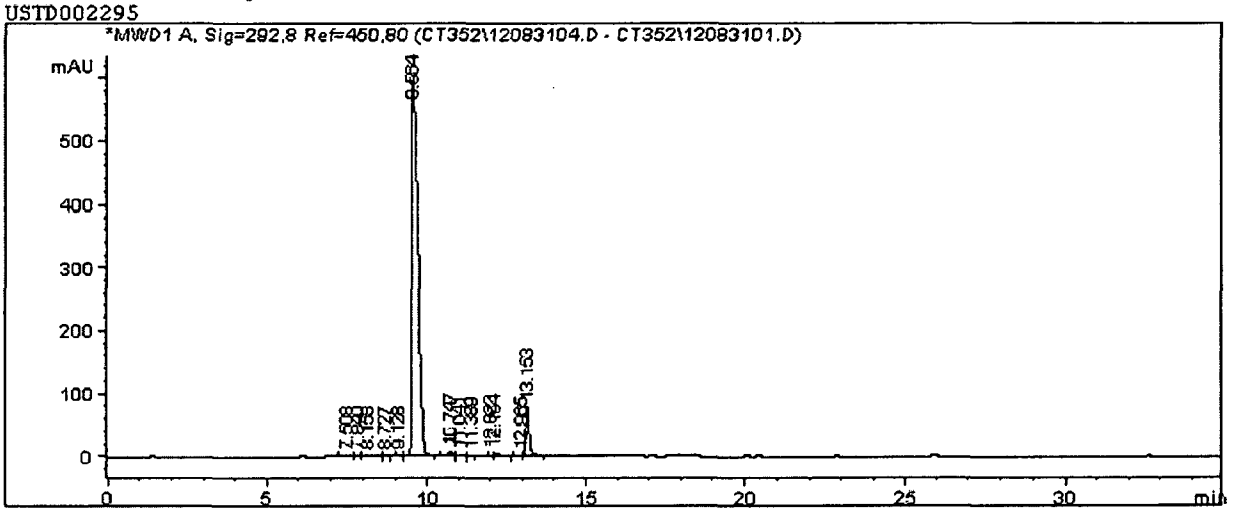
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	7.553	BP	0.1226	3.35987	3.89801e-1	0.4518
2	9.761	BB	0.0965	602.08746	96.84968	80.9537
3	10.730	BP	0.0864	2.51363	4.41339e-1	0.3380
4	11.575	BV	0.0920	5.49918	8.90750e-1	0.7394
5	11.715	VB	0.0894	1.47339	2.54986e-1	0.1981
6	13.016	BB	0.1147	27.82050	3.65890	3.7406
7	15.002	BB	0.1209	2.42088	2.91365e-1	0.3255
8	16.400	BV	0.1297	3.57477	3.87350e-1	0.4806
9	16.649	VB	0.1289	3.61409	4.17830e-1	0.4859
10	18.412	BP	0.1138	2.82269	3.83716e-1	0.3795
11	19.367	BB	0.1163	1.77186	2.02018e-1	0.2382
12	20.385	BB	0.1149	81.77750	10.98378	10.9954
13	22.843	BB	0.1287	5.00732	5.91889e-1	0.6733
Totals :				743.74314	115.74339	

Figure 14

0.1% HCOOH in H2O/ACN

```
==========================================================================
                                                    Seq. Line :   3
Sample Name      : S8/E                              Location : Vial 73
                                                         Inj :   1
                                                  Inj Volume : 5 µl
Acq. Method      : C:\HPCHEM\1\METHODS\CT355.M

Analysis Method : C:\HPCHEM\1\METHODS\CT355.M
```

CT355 Colonna Eclipse XDB-C18 Solvent Saver 3.0 x150 mm 5 micron P.N.993967-302; S.N. USTD002295

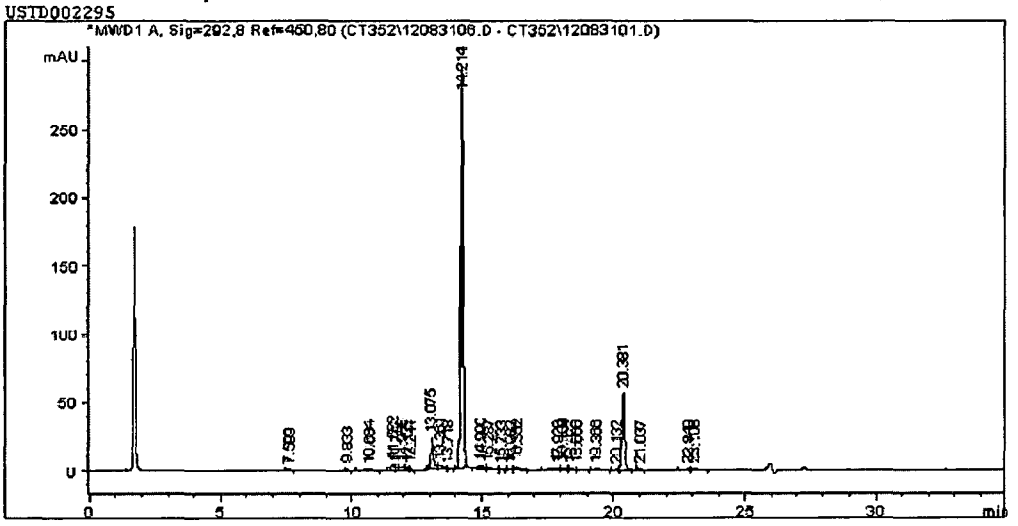



```
==========================================================================
                          Area Percent Report
==========================================================================

Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000


Signal 1: MWD1 A, Sig=292,8 Ref=450,80
 Signal has been modified after loading from rawdata file!

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]    [mAU*s]    [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1   7.508 BV    0.2311   20.12896    1.11233   0.2316
   2   7.840 VV    0.1547    8.50280 7.95758e-1   0.0979
   3   8.158 VP    0.1488   10.93581    1.02226   0.1259
   4   8.727 BV    0.0935    0.62557    1.44886   0.0993
   5   9.128 VP    0.0923    7.78606    1.29180   0.0896
   6   9.564 VB    0.1937 7868.28027  604.94421  90.5504
   7  10.747 BB    0.1168   64.18755    7.90134   0.7387
   8  11.041 BV    0.1000   13.11344    1.96220   0.1509
   9  11.389 VV    0.1186   14.70292    1.77922   0.1696
  10  12.023 VV    0.0857   13.27366    2.35695   0.1528
  11  12.164 VB    0.1342   48.85661    4.99278   0.5623
  12  12.965 PV    0.0921    9.27603    1.50152   0.1068
  13  13.153 VB    0.1128  601.69818   79.06605   6.9245

Totals :                   8689.39786  710.17529
```

Figure 15

Sample Name : S9/C
Seq. Line : 5
Location : Vial 75
Ini : 1
Inj Volume : 5 µl
Acq. Method : C:\HPCHEM\1\METHODS\CT355.M
Analysis Method : C:\HPCHEM\1\METHODS\CT355.M

CT355 Colonna Eclipse XDB-C18 Solvent Saver 3.0 x150 mm 5 micron P.N.993967-302; S.N. USTD002295



Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: MWD1 A, Sig=292,8 Ref=450,80
Signal has been modified after loading from rawdata file!

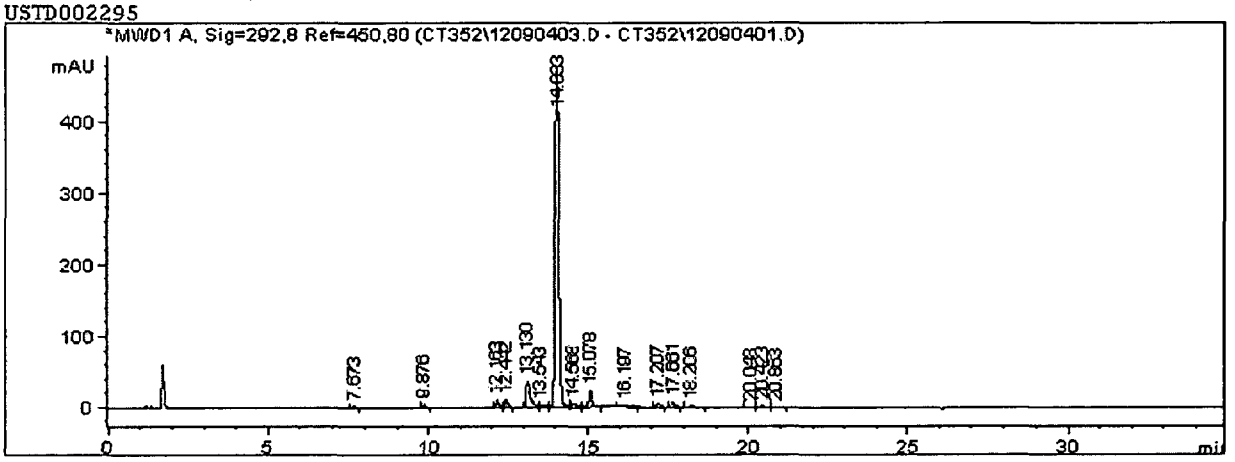
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	7.599	BB	0.1038	7.59977	1.13958	0.2498
2	9.833	BB	0.1008	10.02718	1.48509	0.3296
3	10.684	BV	0.4445	22.85130	6.16377e-1	0.7511
4	11.582	BV	0.0988	28.97993	4.39980	0.9526
5	11.743	VV	0.0936	11.43522	1.71997	0.3759
6	11.955	VV	0.2094	27.73810	1.93343	0.9117
7	12.125	VV	0.1050	15.40240	2.11368	0.5063
8	12.241	VB	0.0791	4.99569	9.53860e-1	0.1642
9	13.075	VV	0.1155	172.50424	22.48874	5.6701
10	13.361	VV	0.1961	32.35221	2.11091	1.0634
11	13.718	VB	0.1333	3.17372	3.05175e-1	0.1043
12	14.214	PB	0.1127	2132.54077	293.78217	70.0952
13	14.990	PV	0.1288	22.50480	2.50613	0.7397
14	15.287	VV	0.2286	12.24472	7.19161e-1	0.4025
15	15.733	VP	0.0982	3.87518	6.09331e-1	0.1274
16	16.082	BV	0.1154	2.99189	3.90332e-1	0.0983
17	16.352	VV	0.1997	18.23691	1.19746	0.5994
18	17.920	VV	0.3118	20.01883	7.94908e-1	0.6580
19	18.109	VV	0.1556	14.11302	1.31106	0.4639
20	18.426	VV	0.1296	8.46712	9.72420e-1	0.2783
21	18.660	VB	0.1618	3.03725	2.77291e-1	0.0998
22	19.366	PB	0.1452	9.96445	9.91620e-1	0.3275
23	20.132	PV	0.1086	4.19171	5.78313e-1	0.1378
24	20.381	VB	0.1186	427.95868	56.32845	14.0667
25	21.037	BV	0.1645	6.40902	5.47671e-1	0.2107
26	22.840	VV	0.1869	7.29907	5.56867e-1	0.2399
27	23.108	VP	0.1588	11.43440	1.08624	0.3758
Totals :				3042.34758	401.92402	

Figure 16

Sintesi di CT352, dopo work-up
0.1% HCOOH in H2O/ACN

```
==========================================================================
                                                  Seq. Line :   3
Sample Name      : S9bis/G                         Location : Vial 8
                                                       Ini :   1
                                                  Inj Volume : 5 µl
Acq. Method      : C:\HPCHEM\1\METHODS\CT355.M

Analysis Method : C:\HPCHEM\1\METHODS\CT355.M
```

CT355 Colonna Eclipse XDB-C18 Solvent Saver 3.0 x150 mm 5 micron P.N.993967-302; S.N. USTD002295




```
==========================================================================
                          Area Percent Report
==========================================================================

Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000

Signal 1: MWD1 A, Sig=292,8 Ref=450,80
 Signal has been modified after loading from rawdata file!
```

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	7.673	BP	0.0943	5.54913	8.95087e-1	0.1233
2	9.876	BB	0.0865	27.84885	5.03472	0.6190
3	12.163	PV	0.0886	56.69953	9.93552	1.2603
4	12.442	VP	0.0886	54.08538	9.47238	1.2022
5	13.130	BV	0.1262	315.11337	36.69934	7.0045
6	13.543	VV	0.1890	24.11346	1.72618	0.5360
7	14.033	VV	0.1207	3655.91016	470.28796	81.2655
8	14.568	VV	0.1724	51.25464	4.19991	1.1393
9	15.078	VB	0.1179	185.26355	23.02881	4.1181
10	16.197	BB	0.2846	9.44443	4.10166e-1	0.2099
11	17.207	BB	0.1134	32.40017	4.42693	0.7202
12	17.661	BB	0.1197	48.73643	6.20361	1.0833
13	18.206	BP	0.1983	10.77742	7.47291e-1	0.2396
14	20.048	PV	0.1473	5.25884	5.41731e-1	0.1169
15	20.423	VV	0.1895	10.02657	7.52425e-1	0.2229
16	20.863	VB	0.1634	6.24321	5.30180e-1	0.1388
Totals :				4498.72513	574.89224	

Figure 17

Sample Name	S9 Pur.1 diretta		
Method	CT355		
Project			
Comment			
Cartridge	SNAP 100g	Detection Mode	UV1
Flowrate	50 ml/min	UV1 (Collection)	292 nm
Solvent A	Dichloromethane	UV2 (Monitor)	210 nm
Solvent B	Methanol	Collect All	On
Rack Type	120 ml		
Max Fraction Volume	60 ml		
Dispense Order	S		
Initial Waste	2 CV		

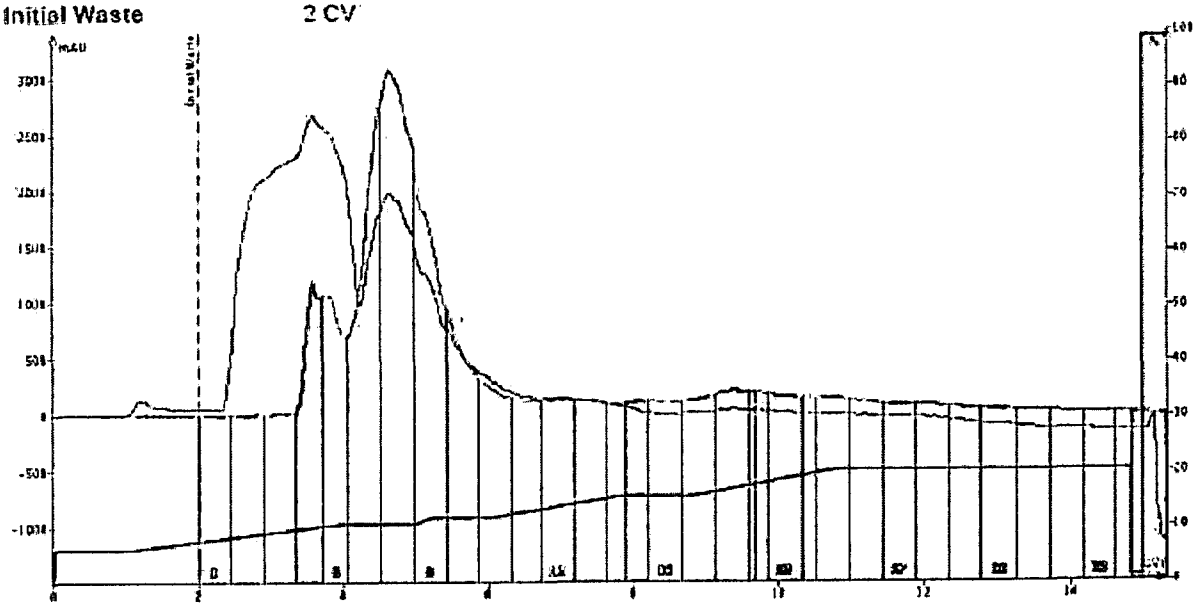
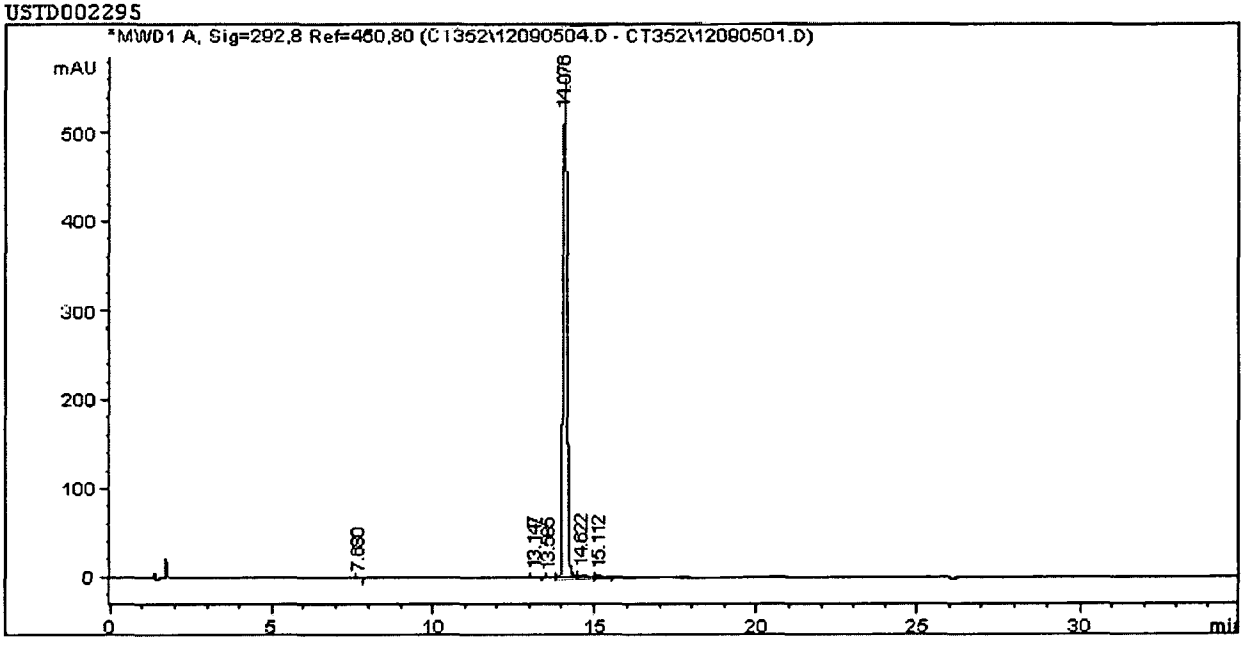


Figure 18

CT352 S9 Purl Fr riunite 7-8
0.1% HCOOH in H20/ACN

```
Seq. Line :   4
Sample Name      : S9 PurlFr7-8                    Location : Vial 60
                                                       Inj :   1
                                                Inj Volume : 5 µl
Acq. Method      : C:\HPCHEM\1\METHODS\CT355.M

Analysis Method : C:\HPCHEM\1\METHODS\CT355.M
```

CT355 Colonna Eclipse XDB-C18 Solvent Saver 3.0 x150 mm 5 micron P.N.993967-302; S.N. USTD002295



Area Percent Report

```
Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000

Signal 1: MWD1 A, Sig=292,8 Ref=450,80
 Signal has been modified after loading from rawdata file!
```

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	7.680	BP	0.0937	2.88061	4.96542e-1	0.0652
2	13.147	PB	0.1286	12.26159	1.39459	0.2774
3	13.585	BV	0.1427	5.82045	5.81739e-1	0.1317
4	14.076	VV	0.1201	4322.85449	559.63513	97.8113
5	14.622	VV	0.2454	53.50365	3.03963	1.2106
6	15.112	VB	0.1740	22.26524	1.77928	0.5038
Totals :				4419.50602	566.92692	

Figure 19

Sample Name	S9Pur6 Fr7-8-2a		
Method	SNAP C18 120g		
Project			
Comment			
Cartridge	SNAP C18 120g	**Detection Mode**	UV1
Flowrate	50 ml/min	**UV1 (Collection)**	292 nm
Solvent A	Water	**UV2 (Monitor)**	210 nm
Solvent B	Acetonitrile	**Collect All**	On
Rack Type	120 ml		
Max Fraction Volume	70 ml		
Dispense Order	S		
Initial Waste	2.65 CV		

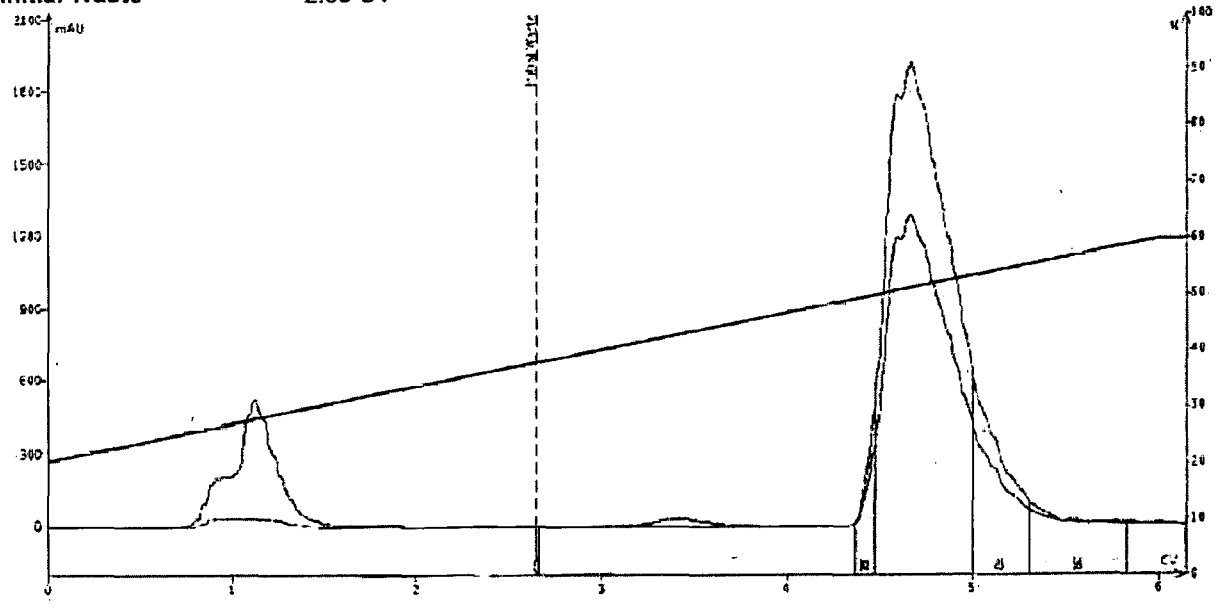
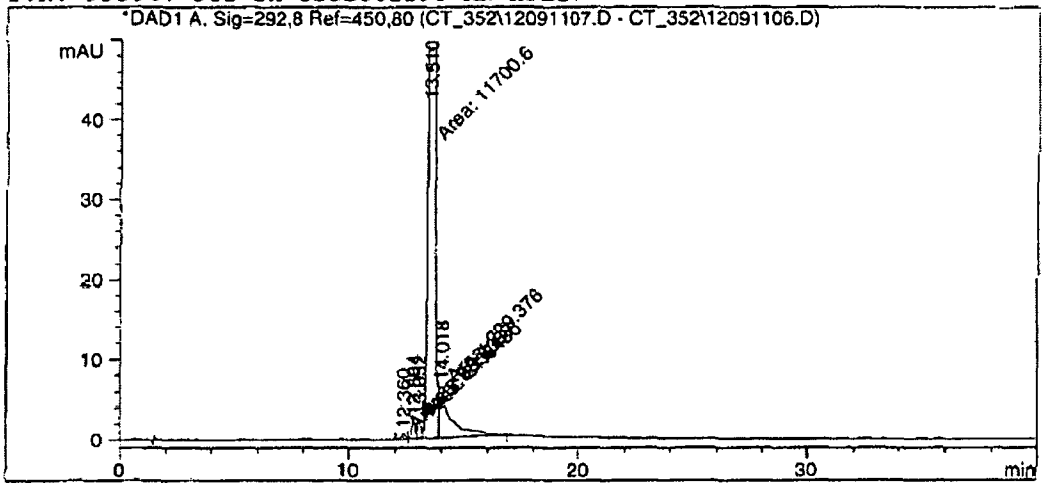


Figure 20

CT 352 Lot. 2012CG02/S9 sz. 0,9mg/mL
Gradiente 25/75 - 10/90 H2O+0,1% HCOOH/ACN+0,1% HCOOH

Sample Name	: CT 352	Seq. Line	: 5
		Location	: Vial 22
		Inj	: 1
Acq. Instrument	: HPLC 1	Inj Volume	: 5 µl
Acq. Method	: C:\HPCHEM\1\METHODS\CT352.M		
Analysis Method	: C:\HPCHEM\1\METHODS\CT352.M		

Metodo CT355
Colonna Zorbax Eclipse XDB-C18 Solvent Saver 3x150mmx5µm
P.N. 993967-302 sn USTD002295 AN-HPLC77



Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: DAD1 A, Sig=292,8 Ref=450,80
Signal has been modified after loading from rawdata file!

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	12.360	MF	0.1961	7.98574	6.78646e-1	0.0666
2	12.804	FM	0.1783	23.83873	2.22778	0.1989
3	13.052	FM	0.1525	21.04856	2.29998	0.1757
4	13.510	FM	0.1527	1.17006e4	1277.36951	97.6446
5	14.018	FM	0.5927	229.37633	6.44966	1.9142
Totals :				1.19828e4	1289.02557	

Results obtained with enhanced integrator!

Figure 21

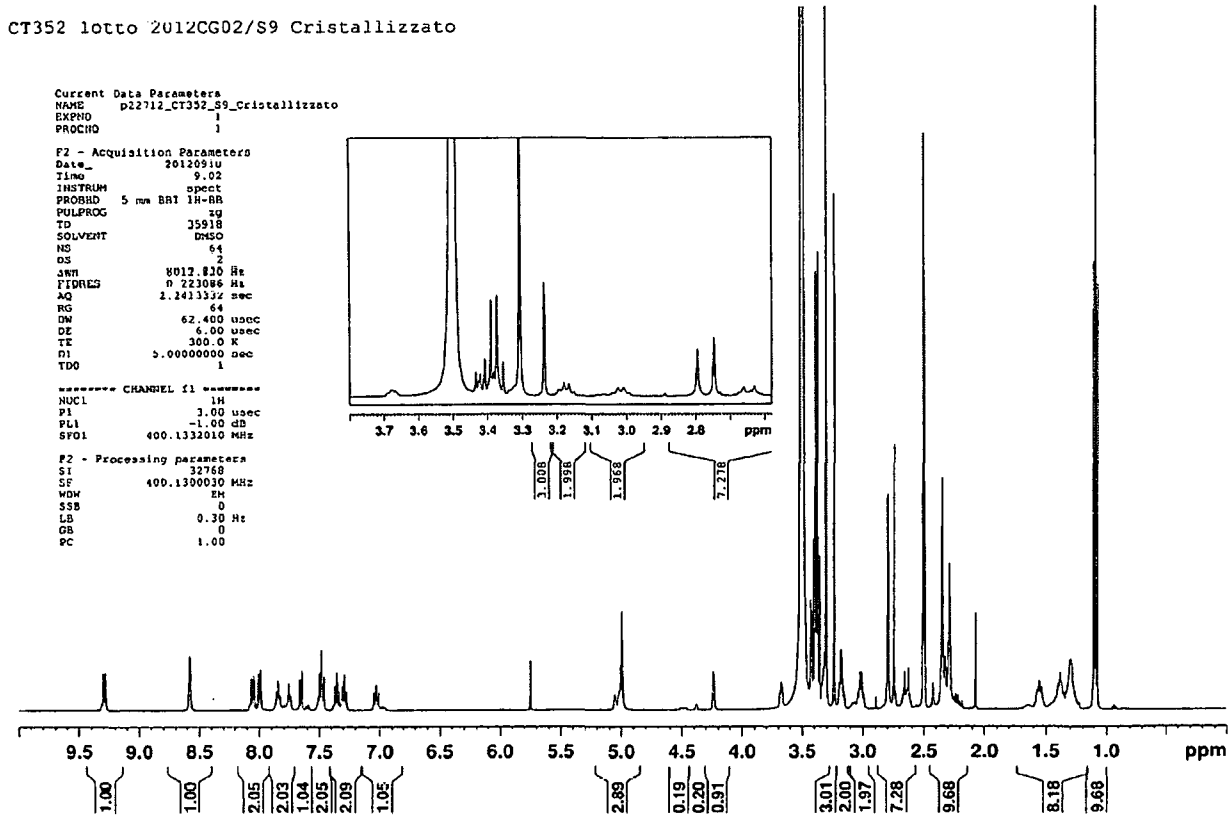

CT352 lotto 2012CG02/S9 Cristallizzato
Current Data Parameters
NAME p22712_CT352_S9_Cristallizzato
EXPNO 1
PROCNO 1
F2 - Acquisition Parameters
Date_ 20120911
Time 9.02
INSTRUM spect
PROBHD 5 mm BBI 1H-BB
PULPROG zg
TD 35918
SOLVENT DMSO
NS 64
DS 2
SWH 8012.820 Hz
FIDRES 0.223086 Hz
AQ 2.2413332 sec
RG 64
DW 62.400 usec
DE 6.00 usec
TE 300.0 K
D1 5.00000000 sec
TD0 1
======== CHANNEL f1 ========
NUC1 1H
P1 3.00 usec
PL1 -1.00 dB
SFO1 400.1332010 MHz
F2 - Processing parameters
SI 32768
SF 400.1300030 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00
3.7 3.6 3.5 3.4 3.3 3.2 3.1 3.0 2.9 2.8 ppm
1.006
1.996
1.968
7.278
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 ppm
1.00
1.00
2.05
2.03
1.04
2.05
2.09
1.05
2.89
0.19
0.20
0.91
3.01
2.00
1.97
7.28
9.68
8.18
9.68

Figure 22

TEST REPORT n. 2012/039

Product : **CT352**

Formula : **mPEG-$C_{39}H_{43}N_6O_6$** Mol. Weight: **2590 (*)**

CAS n°: ***** Quantity: **0,750 g**

Lot number : **2012CG02/S9**

Parameter	Unit	Results	Anal. Meth.
Aspect		Slightly yellow crystalline powder	visual
NMR Spectrum		conform to structure	
Purity *(HPLC, 292 nm)*	% area	≥ 97,6	MI CT352 001
Impurities *(HPLC, 292 nm)*			MI CT352 001
Unknown Rrt 0,91	% area	0,07	
Unknown Rrt 0,95	% area	0,20	
Unknown Rrt 0,97	% area	0,18	
Unknown Rrt 1,04	% area	1,91	

(*) Based on M_w = 2015 Da of MeO-PEG-COOH

Figure 23

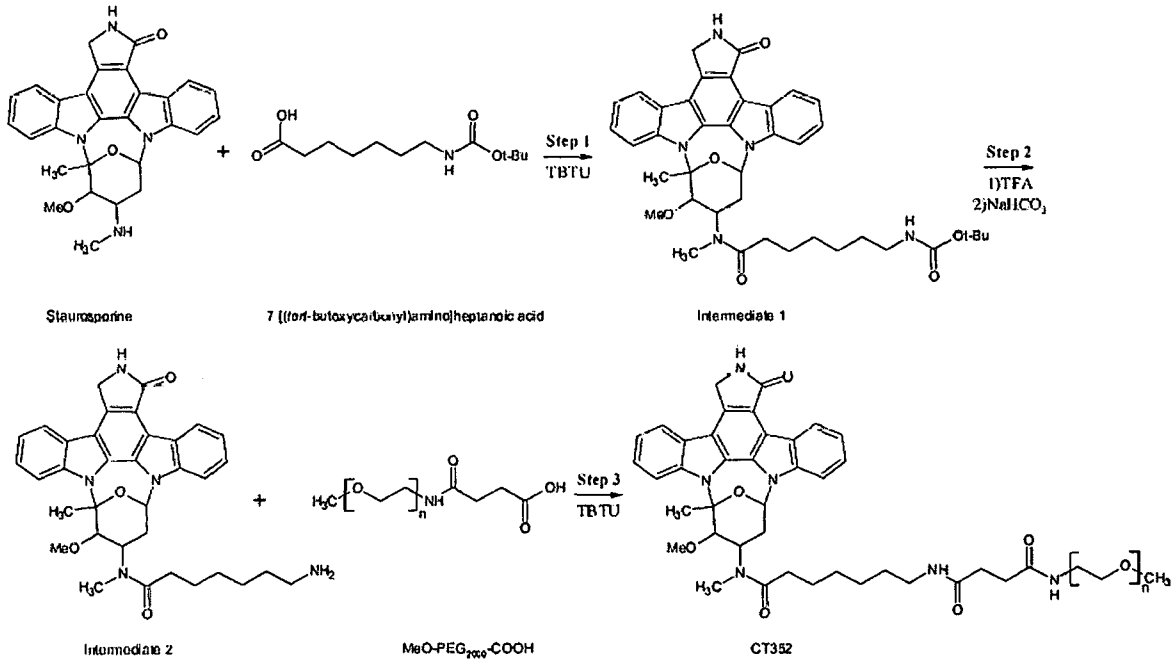
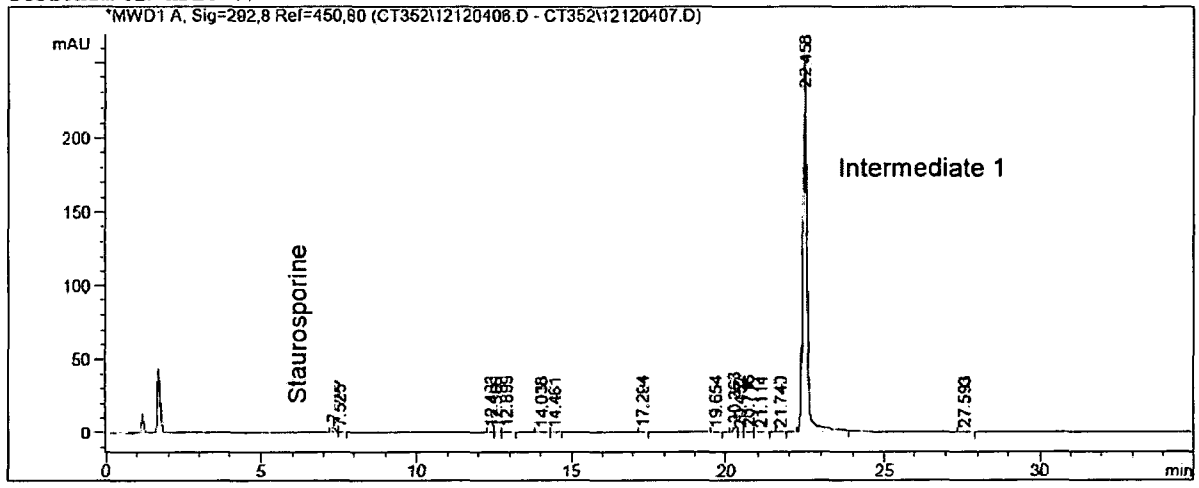

Staurosporine
7 [(tert-butoxycarbonyl)amino]heptanoic acid
Step 1
TBTU
Intermediate 1
Step 2
1)TFA
2)NaHCO3
Intermediate 2
MeO-PEG2000-COOH
Step 3
TBTU
CT352

Figure 24

Sample Name : S1/D 1step 27h
Acq. Method : C:\HPCHEM\2\METHODS\CT352.M
Analysis Method : C:\HPCHEM\2\METHODS\CT352.M

Seq. Line : 7
Location : Vial 14
Inj : 1
Inj Volume : 5 µl

CT355 Colonna Eclipse XDB-C18 Solvent Saver 3.0 x150 mm 5 micron P.N.993967-302; S.N.USTD002295
Serichim AN-HPLC-77



Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: MWD1 A, Sig=292,8 Ref=450,80
Signal has been modified after loading from rawdata file!

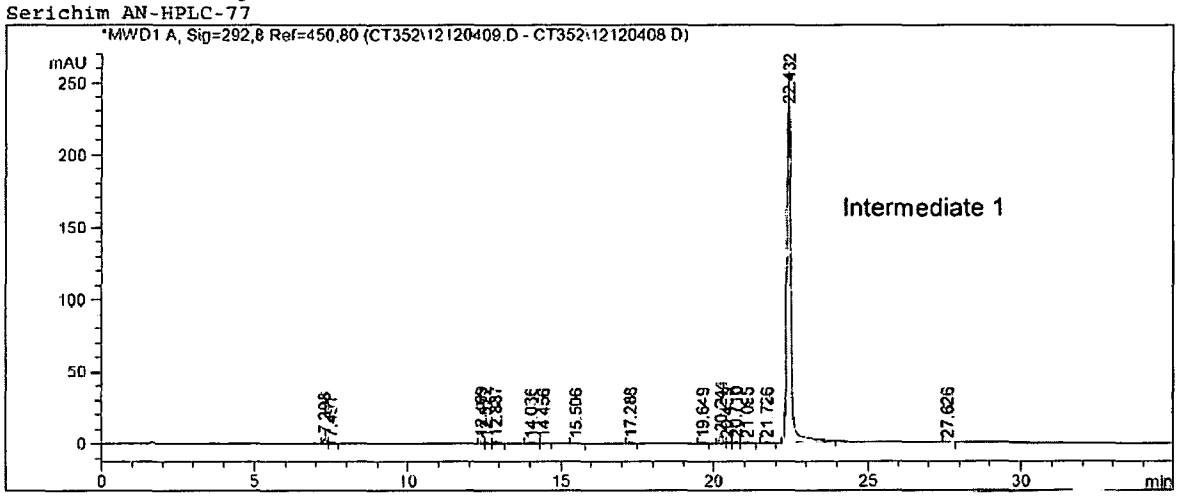
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	7.330	PV	0.1108	27.85367	3.83029	1.0767
2	7.525	VB	0.1191	8.27052	1.01511	0.3197
3	12.403	BV	0.1065	8.35359	1.18100	0.3229
4	12.566	VV	0.1082	2.69518	3.48677e-1	0.1042
5	12.899	VB	0.1412	10.01600	1.07039	0.3872
6	14.038	PV	0.1911	5.44156	3.79974e-1	0.2103
7	14.461	VB	0.1360	2.68724	2.95633e-1	0.1039
8	17.294	BB	0.1173	6.71978	9.19053e-1	0.2598
9	19.654	BB	0.1258	6.23270	7.28743e-1	0.2409
10	20.263	BV	0.1167	26.47814	3.48099	1.0235
11	20.452	VV	0.1081	3.43373	4.76207e-1	0.1327
12	20.716	VV	0.1343	9.01879	1.00913	0.3486
13	21.114	VB	0.1495	6.40493	6.36036e-1	0.2476
14	21.740	BV	0.1286	4.32426	4.91500e-1	0.1672
15	22.458	PB	0.1458	2454.67554	256.14789	94.8878
16	27.593	PB	0.1758	4.31834	3.15305e-1	0.1669
Totals :				2586.92396	272.32594	

Figure 25

Sample Name : S1 /E dopo lavag
Seq. Line : 10
Location : Vial 21
Inj : 1
Inj Volume : 5 µl
Acq. Method : C:\HPCHEM\2\METHODS\CT352.M

Analysis Method : C:\HPCHEM\2\METHODS\CT352.M

CT355 Colonna Eclipse XDB-C18 Solvent Saver 3.0 x150 mm 5 micron P.N.993967-302; S.N.USTD00229
Serichim AN-HPLC-77



==========

Area Percent Report

==========

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: MWD1 A, Sig=292,8 Ref=450,80
Signal has been modified after loading from rawdata file!

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	7.298	BV	0.1142	26.91773	3.64467	1.0293
2	7.491	VB	0.1218	9.83167	1.19603	0.3759
3	12.409	PV	0.1049	11.70797	1.68920	0.4477
4	12.577	VV	0.1132	3.86918	4.95180e-1	0.1479
5	12.887	VB	0.1322	15.01944	1.64954	0.5743
6	14.036	PV	0.2265	7.03549	5.05014e-1	0.2690
7	14.456	VP	0.1171	2.81455	3.52703e-1	0.1076
8	15.506	BB	0.1379	1.39148	1.33172e-1	0.0532
9	17.288	BP	0.1125	8.18635	1.10447	0.3130
10	19.649	BB	0.1291	7.58285	9.10889e-1	0.2899
11	20.244	BV	0.1156	36.61703	4.87664	1.4001
12	20.439	VV	0.1172	4.26815	5.58345e-1	0.1632
13	20.710	VV	0.1303	9.85625	1.14688	0.3769
14	21.095	VB	0.1409	7.19713	7.31604e-1	0.2752
15	21.726	PP	0.1370	5.91849	6.57641e-1	0.2263
16	22.432	PB	0.1433	2453.28052	257.25803	93.8062
17	27.626	BB	0.1882	3.77080	2.85346e-1	0.1442
Totals :				2615.26506	277.19625	

Figure 26

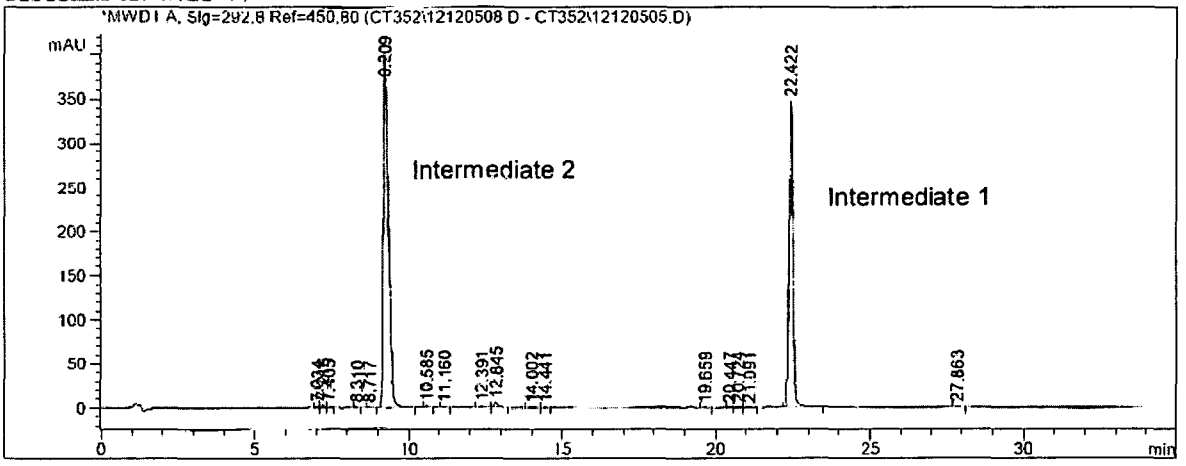
Sample Name : Sisolido crist.

Seq. Line : 1
Location : Vial 28
Inj : 1
Inj Volume : 5 µl

Acq. Method : C:\HPCHEM\2\METHODS\CT352.M

Analysis Method : C:\HPCHEM\2\METHODS\CT352.M

CT355 Colonna Eclipse XDB-C18 Solvent Saver 3.0 x150 mm 5 micron P.N.993967-302; S.N.USTD002295
Serichim AN-HPLC-77



==========
Area Percent Report
==========

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: MWD1 A, Sig=292,8 Ref=450,80
Signal has been modified after loading from rawdata file!

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	7.034	VV	0.0950	8.47431	1.35463	0.1055
2	7.216	VV	0.0982	25.02508	3.82944	0.3115
3	7.405	VV	0.1057	15.50346	2.16209	0.1930
4	8.310	BV	0.0948	7.66415	1.22794	0.0954
5	8.717	VB	0.0966	9.75884	1.52603	0.1215
6	9.209	VB	0.1823	4771.74951	402.41528	59.4050
7	10.585	PB	0.0964	14.79587	2.38445	0.1842
8	11.160	VP	0.0967	9.64854	1.50720	0.1201
9	12.391	PV	0.1201	16.70889	2.07120	0.2080
10	12.845	VB	0.1287	51.08748	5.92067	0.6360
11	14.002	VV	0.1946	11.96616	9.02755e-1	0.1490
12	14.441	VV	0.1221	6.75356	8.37097e-1	0.0841
13	19.659	BB	0.1245	6.36571	7.86011e-1	0.0792
14	20.447	VV	0.1264	5.17894	6.14211e-1	0.0645
15	20.724	VV	0.1338	12.03499	1.35234	0.1498
16	21.091	VB	0.1414	7.78126	8.14782e-1	0.0969
17	22.422	VB	0.1340	3037.73389	347.49631	37.8177
18	27.863	VB	0.1488	14.33490	1.50938	0.1785
Totals :				8032.56555	778.71182	

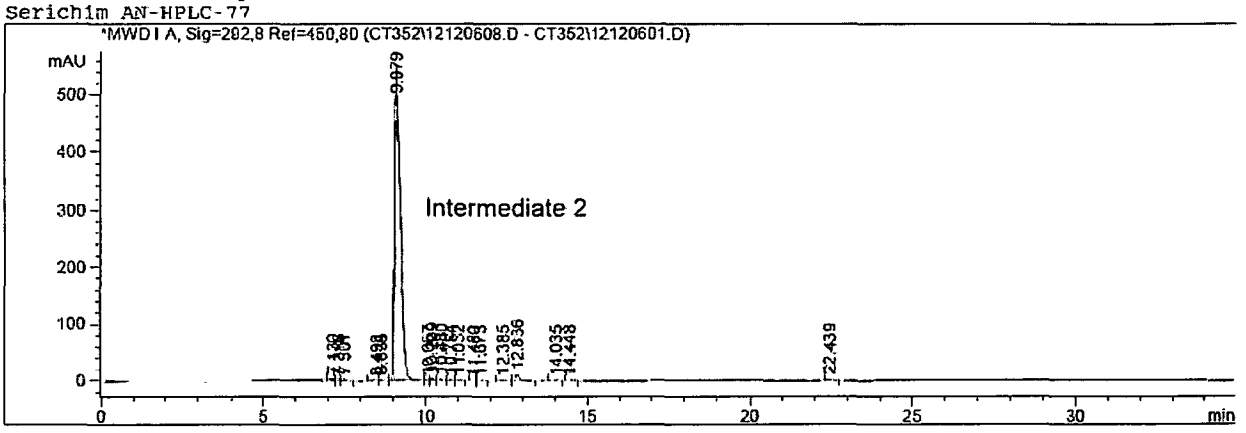
Figure 27

Sample Name : S2/E
Seq. Line : 9
Location : Vial 11
Inj : 1
Inj Volume : 5 µl

Acq. Method : C:\HPCHEM\2\METHODS\CT352.M

Analysis Method : C:\HPCHEM\2\METHODS\CT352.M

CT355 Colonna Eclipse XDB-C18 Solvent Saver 3.0 x150 mm 5 micron P.N.993967-302; S.N.USTD002295
Serichim AN-HPLC-77



Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: MWD1 A, Sig=292,8 Ref=450,80
Signal has been modified after loading from rawdata file!

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	7.130	BV	0.1103	24.51302	3.39150	0.3308
2	7.316	VV	0.1044	13.15192	1.86101	0.1775
3	7.501	VV	0.1393	31.73763	3.38764	0.4283
4	8.498	BV	0.1438	10.39282	9.79616e-1	0.1403
5	8.635	VV	0.1192	22.29611	2.67693	0.3009
6	9.079	VV	0.1959	7033.91650	547.51868	94.9273
7	10.067	VV	0.1094	10.30918	1.37539	0.1391
8	10.209	VV	0.1067	32.78475	4.51460	0.4425
9	10.480	VV	0.1059	29.26983	4.07165	0.3950
10	10.704	VV	0.1173	16.06295	2.05177	0.2168
11	11.032	VB	0.1185	12.25510	1.51222	0.1654
12	11.480	BV	0.1086	3.76307	5.19237e-1	0.0508
13	11.673	VB	0.1400	7.65049	7.69363e-1	0.1032
14	12.385	PP	0.1518	18.40398	1.92110	0.2484
15	12.836	VB	0.1267	100.26482	11.61131	1.3531
16	14.035	PV	0.1768	10.86477	8.29335e-1	0.1466
17	14.448	VP	0.1365	8.20661	8.98859e-1	0.1108
18	22.439	PB	0.1435	23.94774	2.59879	0.3232
Totals :				7409.79129	592.48899	

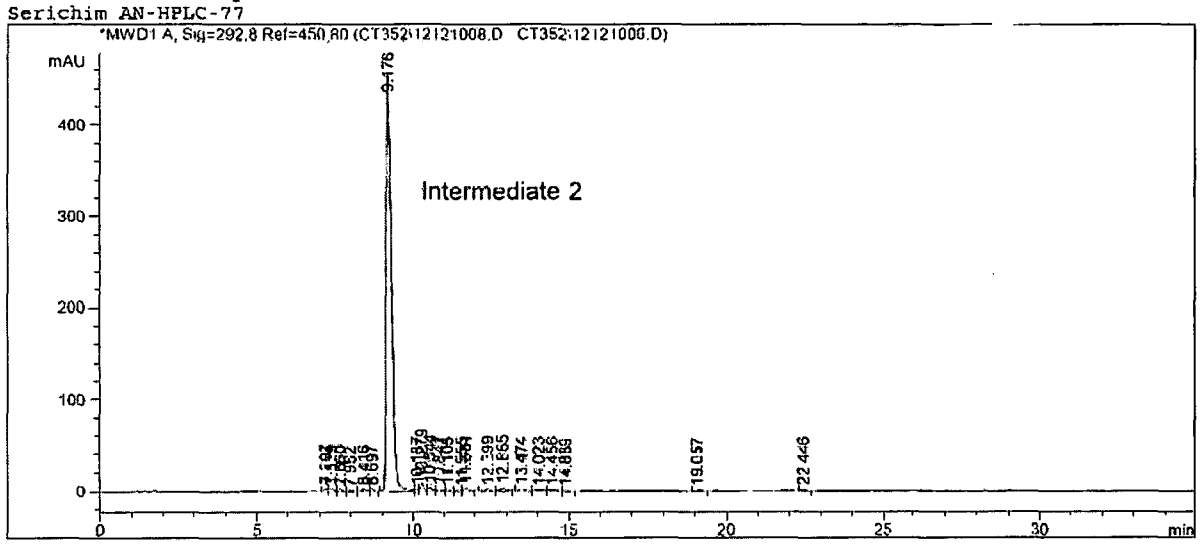
Figure 28

Sample Name : S2/G
Seq. Line : 9
Location : Vial 34
Inj : 1
Inj Volume : 5 µl

Acq. Method : C:\HPCHEM\2\METHODS\CT352.M

Analysis Method : C:\HPCHEM\2\METHODS\CT352.M

CT355 Colonna Eclipse XDB-C18 Solvent Saver 3.0 x150 mm 5 micron P.N.993967-302; S.N.USTD002295
Serichim AN-HPLC-77



Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: MWD1 A, Sig=292,8 Ref=450,80
Signal has been modified after loading from rawdata file!

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	7.207	BV	0.1238	25.88684	3.08838	0.4339
2	7.379	VV	0.1336	21.04380	2.28108	0.3527
3	7.660	VB	0.1393	16.70098	1.75109	0.2800
4	7.982	BV	0.1129	3.17185	4.06982e-1	0.0532
5	8.416	VV	0.1251	17.13240	1.93915	0.2872
6	8.697	VB	0.1064	14.34199	1.98301	0.2404
7	9.176	BB	0.1886	5588.30664	457.10202	93.6748
8	10.137	BV	0.0927	6.60384	1.05970	0.1107
9	10.279	VV	0.1002	62.35482	9.30595	1.0452
10	10.544	VV	0.1120	26.79437	3.55060	0.4491
11	10.841	VV	0.1538	18.03687	1.62107	0.3023
12	11.105	VV	0.1360	14.10326	1.44299	0.2364
13	11.555	VV	0.1346	5.98566	5.79270e-1	0.1003
14	11.681	VB	0.1216	30.77270	3.60513	0.5158
15	12.399	BV	0.1458	19.27883	1.84619	0.3232
16	12.865	VB	0.1480	32.54234	3.11017	0.5455
17	13.474	BV	0.1893	18.97106	1.42515	0.3180
18	14.023	VV	0.2140	13.46005	8.44293e-1	0.2256
19	14.456	VV	0.1936	11.95153	8.62631e-1	0.2003
20	14.889	VB	0.1595	3.00381	2.58709e-1	0.0504
21	19.057	BP	0.2005	3.83165	2.53342e-1	0.0642
22	22.446	BB	0.1352	11.36945	1.28506	0.1906
Totals :				5965.64474	499.60397	

Figure 29

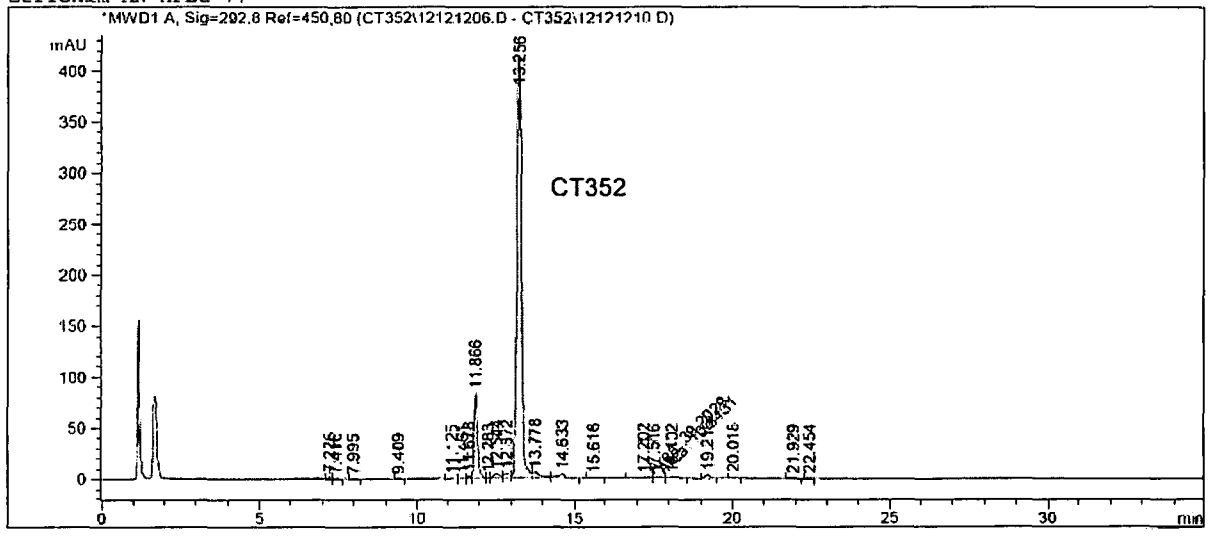
Sample Name : S5/G

Seq. Line : 7
Location : Vial 9
Inj : 1
Inj Volume : 5 µl

Acq. Method : C:\HPCHEM\2\METHODS\CT352.M

Analysis Method : C:\HPCHEM\2\METHODS\CT352.M

CT355 Colonna Eclipse XDB-C18 Solvent Saver 3.0 x150 mm 5 micron P.N.993967-302; S.N.USTD002295
Serichim AN-HPLC-77



Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: MWD1 A, Sig=292,8 Ref=450,80
Signal has been modified after loading from rawdata file!

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	7.226	BV	0.1174	17.50683	2.28414	0.3411
2	7.410	VB	0.1185	8.70533	1.09790	0.1696
3	7.995	PB	0.1113	2.33515	3.19386e-1	0.0455
4	9.409	BB	0.1058	5.45198	7.59231e-1	0.1062
5	11.125	VP	0.1170	6.08522	7.80106e-1	0.1186
6	11.461	VV	0.0902	4.52360	7.51430e-1	0.0881
7	11.678	VV	0.0924	17.30743	2.95275	0.3373
8	11.866	VV	0.1176	642.67224	83.71685	12.5234
9	12.283	VV	0.0958	8.68040	1.30237	0.1692
10	12.544	VV	0.1703	60.72813	4.77828	1.1834
11	12.872	VV	0.1593	58.02372	5.00449	1.1307
12	13.256	VV	0.1508	3997.44360	413.63025	77.8961
13	13.778	VV	0.2618	104.61684	5.55227	2.0386
14	14.633	VB	0.2050	66.72346	4.34970	1.3002
15	15.616	BP	0.1951	9.28546	6.40707e-1	0.1809
16	17.202	PM	0.5511	39.20291	1.18570	0.7639
17	17.516	PM	0.3126	10.84309	5.78027e-1	0.2113
18	18.102	VB	0.1958	8.60130	6.05231e-1	0.1676
19	19.216	PB	0.1312	36.47169	4.20523	0.7107
20	20.018	PB	0.1326	13.51087	1.56643	0.2633
21	21.929	PB	0.1690	2.75304	2.07237e-1	0.0536
22	22.454	BV	0.1325	10.29283	1.17187	0.2006

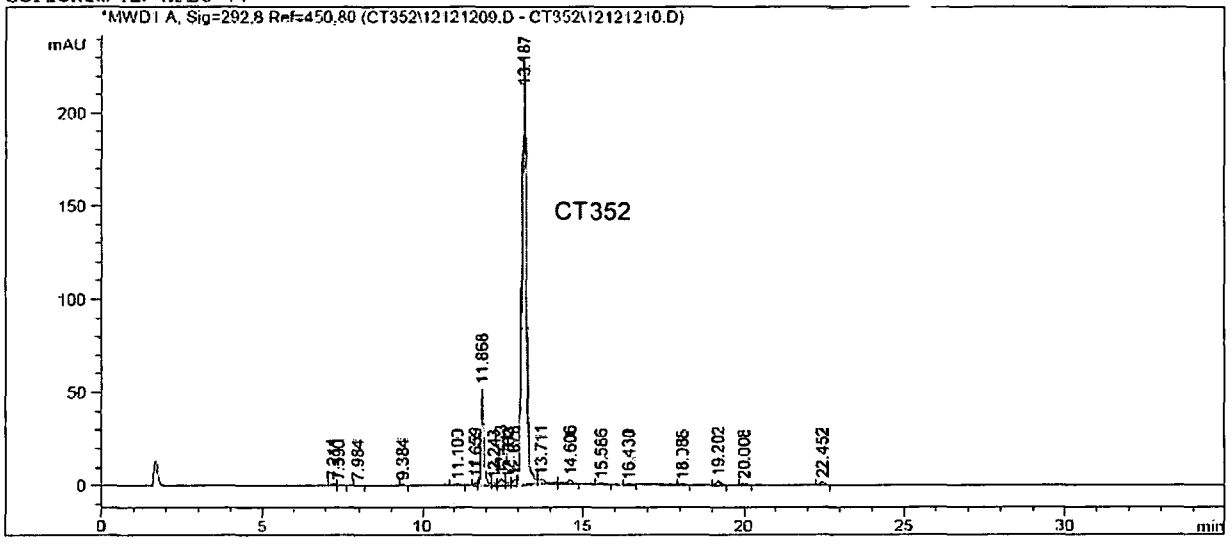
Totals : 5131.76514 537.43959

Figure 30

Seq. Line : 10
Sample Name : S5/H Location : Vial 10
Inj : 1
Inj Volume : 5 µl
Acq. Method : C:\HPCHEM\2\METHODS\CT352.M

Analysis Method : C:\HPCHEM\2\METHODS\CT352.M

CT355 Colonna Eclipse XDB-C18 Solvent Saver 3.0 x150 mm 5 micron P.N.993967-302; S.N.USTD002295
Serichim AN-HPLC-77



Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

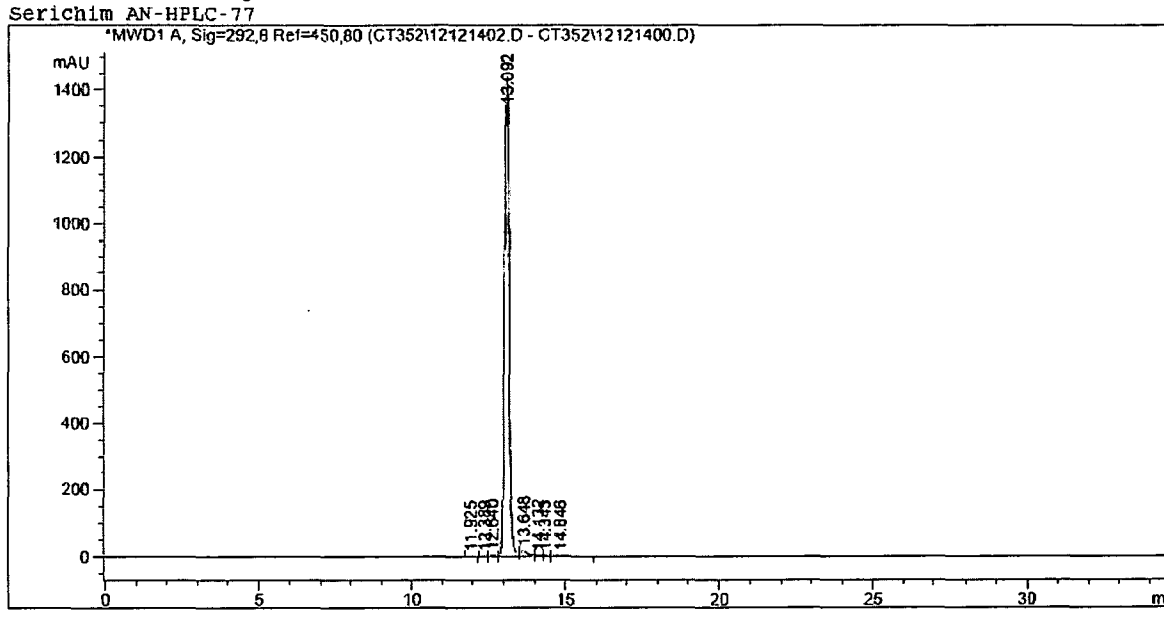
Signal 1: MWD1 A, Sig=292,8 Ref=450,80
Signal has been modified after loading from rawdata file!

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	7.211	BV	0.1293	7.85812	9.42602e-1	0.2732
2	7.390	VP	0.1195	2.23453	2.73085e-1	0.0777
3	7.984	PB	0.1063	1.32519	1.87836e-1	0.0461
4	9.384	PB	0.0957	3.97550	6.29085e-1	0.1382
5	11.100	VP	0.1126	3.53148	4.54966e-1	0.1228
6	11.659	BV	0.0948	9.61099	1.58483	0.3341
7	11.868	VV	0.1071	362.93497	52.22826	12.6165
8	12.243	VV	0.0994	5.25690	7.72108e-1	0.1827
9	12.473	VV	0.1563	34.54859	3.29597	1.2010
10	12.700	VV	0.1375	12.46425	1.37815	0.4333
11	12.878	VV	0.1289	22.39709	2.53844	0.7786
12	13.187	VV	0.1531	2273.71484	230.37323	79.0401
13	13.711	VV	0.2615	55.00721	2.84583	1.9122
14	14.606	VB	0.1872	31.78041	2.30074	1.1048
15	15.566	BP	0.1570	7.17835	6.59492e-1	0.2495
16	16.430	PB	0.1192	1.91140	2.55752e-1	0.0664
17	18.080	PB	0.1289	2.63525	3.10998e-1	0.0916
18	19.202	BB	0.1300	19.38854	2.26234	0.6740
19	20.008	PB	0.1295	7.24096	8.49322e-1	0.2517
20	22.452	PB	0.1394	11.66535	1.31578	0.4055

Totals : 3876.65994 305.45882

Figure 31

```
                                                    Seq. Line :   3
Sample Name      : S5/P1/Fr6-12                      Location : Vial 31
                                                         Inj :   1
                                                   Inj Volume : 5 µl
Different Inj Volume from Sequence !     Actual Inj Volume : 20 µl
Acq. Method      : C:\HPCHEM\2\METHODS\CT352.M

Analysis Method : C:\HPCHEM\2\METHODS\CT352RID.M

CT352 Colonna Eclipse XDB-C18 Solvent Saver 3.0 X150 mm 5 micron P.N.993967-302; S.N.USTD002295
Serichim AN-HPLC-77
```




```
================================================================
                      Area Percent Report
================================================================

Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: MWD1 A, Sig=292,8 Ref=450,80
 Signal has been modified after loading from rawdata file!
```

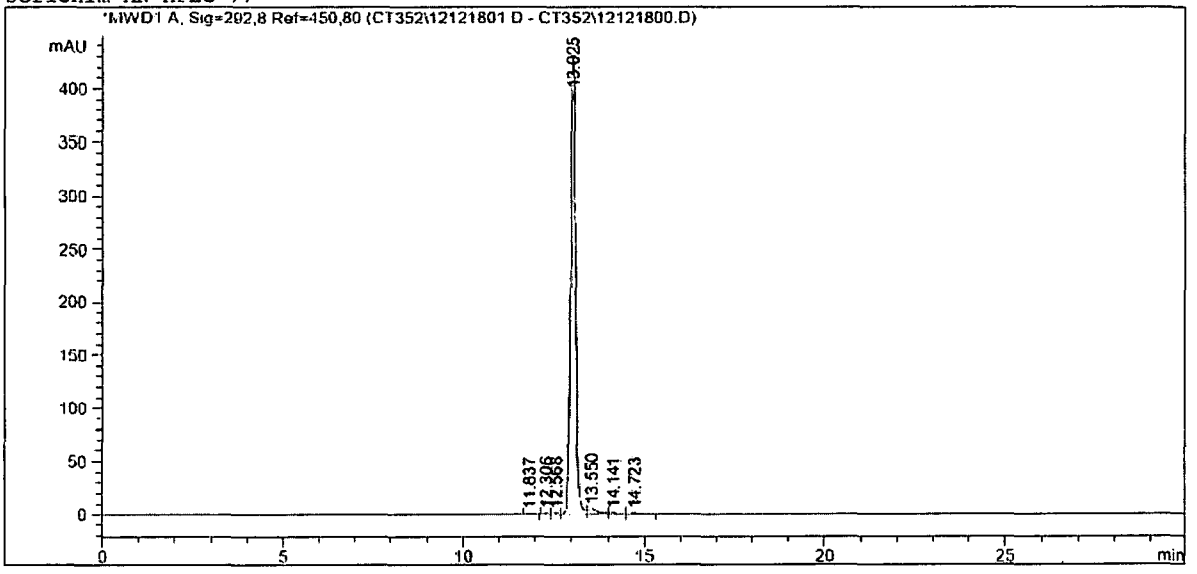
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	11.925	PP	0.1322	7.45134	0.67175e-1	0.0495
2	12.389	BV	0.1256	12.40352	1.51394	0.0823
3	12.640	VV	0.1322	32.23323	3.67962	0.2140
4	13.092	VV	0.1588	1.46554e4	1439.64856	97.2911
5	13.648	VV	0.2512	256.47552	14.87224	1.7026
6	14.132	VV	0.1759	30.20806	2.41612	0.2005
7	14.343	VV	0.1554	32.80045	2.03296	0.1519
8	14.846	VB	0.5229	46.39705	1.05544	0.3080
Totals :				1.50635e4	1466.08605	

Figure 32

Sample Name : S5P2 fraz buone
Seq. Line : 2
Location : Vial 51
Inj : 1
Inj Volume : 5 µl
Acq. Method : C:\HPCHEM\2\METHODS\CT352RID.M

Analysis Method : C:\HPCHEM\2\METHODS\CT352RID.M

CT352 Colonna Eclipse XDB-C18 Solvent Saver 3.0 x150 mm 5 micron P.N.993967-302; S.N.USTD002295
Serichim AN-HPLC-77



Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: MWD1 A, Sig=292,8 Ref=450,80
Signal has been modified after loading from rawdata file!

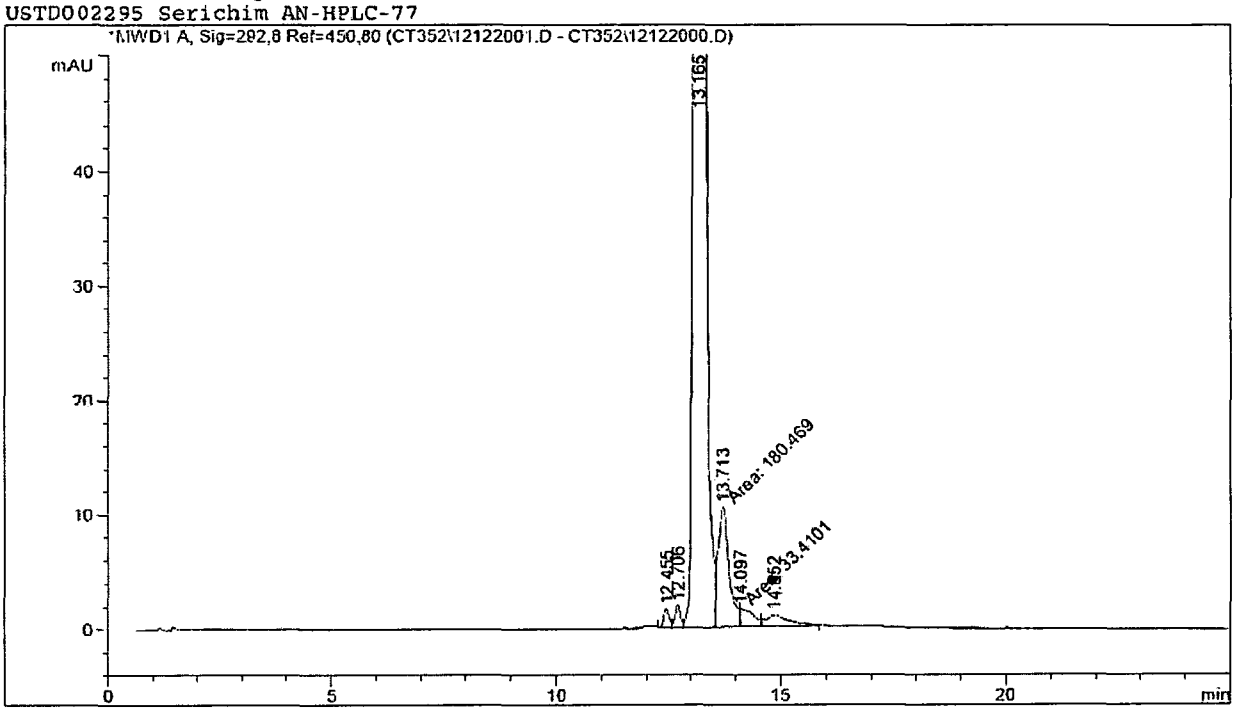
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	11.837	PB	0.1331	2.27188	2.22628e-1	0.0530
2	12.306	BV	0.1236	8.72041	1.08722	0.2033
3	12.568	VV	0.1318	9.70489	1.13397	0.2262
4	13.025	VV	0.1516	4163.22119	427.62067	97.0515
5	13.550	VB	0.2628	83.75877	4.50664	1.9526
6	14.141	BV	0.2133	11.80413	7.43228e-1	0.2752
7	14.723	VB	0.3346	10.22191	3.69662e-1	0.2383

Totals : 4289.70318 435.68402

Figure 33

Sample Name : CT352/S5/Finale
Seq. Line : 2
Location : Vial 21
Inj : 1
Inj Volume : 5 µl
Acq. Method : C:\HPCHEM\2\METHODS\CT352.M
Analysis Method : C:\HPCHEM\2\METHODS\CT352.M

CT355 Colonna Eclipse XDB-C18 Solvent Saver 3.0 x150 mm 5 micron P.N.993967-302; S.N. USTD002295 Serichim AN-HPLC-77



Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: MWD1 A, Sig=292,8 Ref=450,80
Signal has been modified after loading from rawdata file!

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	12.455	VV	0.1353	14.96157	1.69038	0.1465
2	12.706	VV	0.1255	16.62331	1.98812	0.1628
3	13.165	VV	0.1509	9930.74219	1025.90723	97.2458
4	13.713	MF	0.2854	180.46938	10.54016	1.7672
5	14.097	FM	0.3462	33.41013	1.60826	0.3272
6	14.852	BB	0.4313	35.79302	9.91097e-1	0.3505
Totals :				1.02120e4	1042.72524	

Figure 34

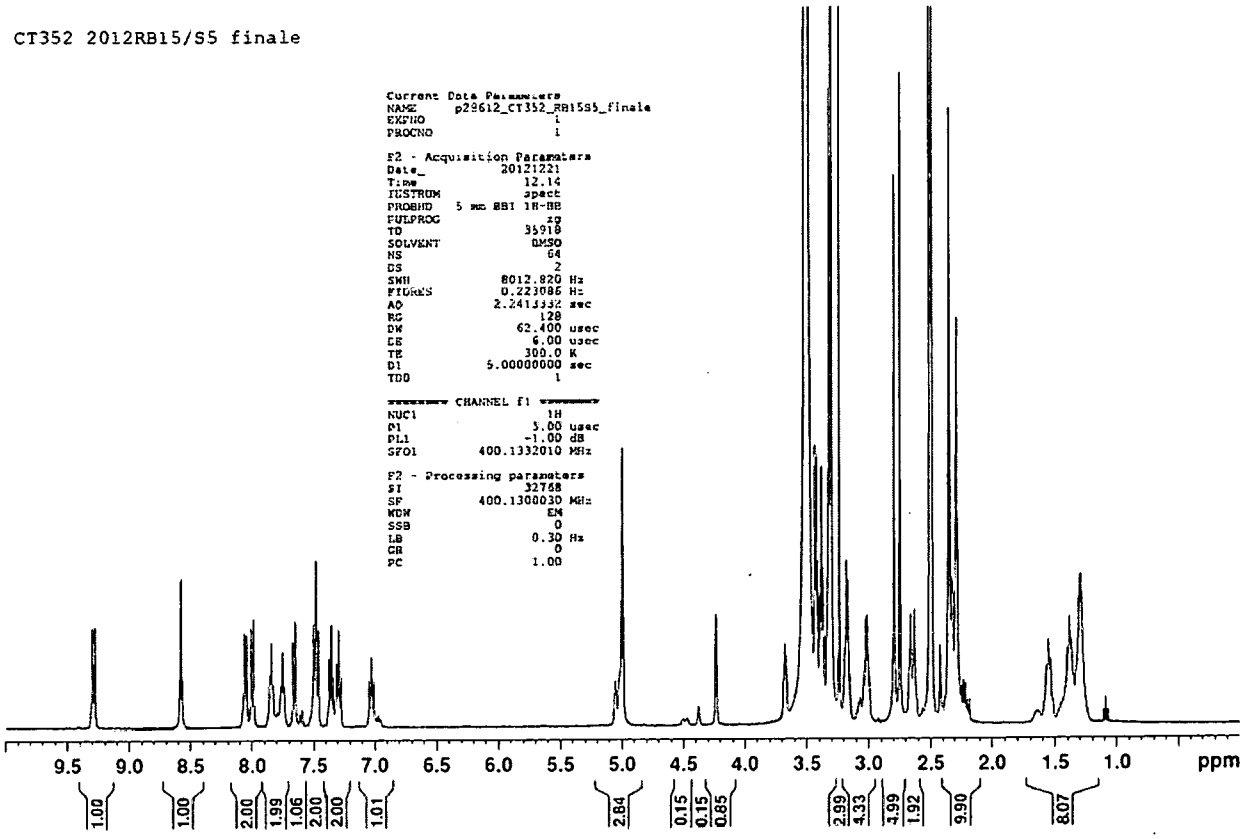

CT352 2012RB15/S5 finale
Current Data Parameters
NAME p29612_CT352_RB15S5_finale
EXPNO 1
PROCNO 1
F2 - Acquisition Parameters
Date_ 20121221
Time 12.14
INSTRUM spect
PROBHD 5 mm BBI 1H-BB
PULPROG zg
TD 35918
SOLVENT DMSO
NS 64
DS 2
SWH 8012.820 Hz
FIDRES 0.223086 Hz
AQ 2.2413332 sec
RG 128
DW 62.400 usec
DE 6.00 usec
TE 300.0 K
D1 5.00000000 sec
TD0 1
CHANNEL f1
NUC1 1H
P1 5.00 usec
PL1 -1.00 dB
SFO1 400.1332010 MHz
F2 - Processing parameters
SI 32768
SF 400.1300030 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 ppm
1.00 1.00 2.00 1.99 1.06 2.00 2.00 1.01 2.84 0.15 0.15 0.85 2.99 4.33 4.99 1.92 9.90 8.07

Figure 35

TEST REPORT n. 2013/001

Product : **CT352**

Formula : **mPEG-$C_{39}H_{43}N_6O_6$** Mol. Weight: **2590 (*)**

CAS n°: ***** Quantity: **0,800 g**

Lot number : **2012RB15/S5**

Parameter	Unit	Results	Anal. Meth.
Aspect		Slightly yellow crystalline powder	visual
NMR Spectrum		conform to structure	
Purity *(HPLC, 292 nm)*	*% area*	97,2	MI CT352 001
Impurities *(HPLC, 292 nm)*			MI CT352 001
Unknown Rrt 0,95	*% area*	0,15	
Unknown Rrt 0,97	*% area*	0,16	
Unknown Rrt 1,04	*% area*	1,77	
Unknown Rrt 1,07	*% area*	0,32	
Unknown Rrt 1,13	*% area*	0,35	

(*) Based on M_w = 2015 Da of MeO-PEG-COOH

Figure 36

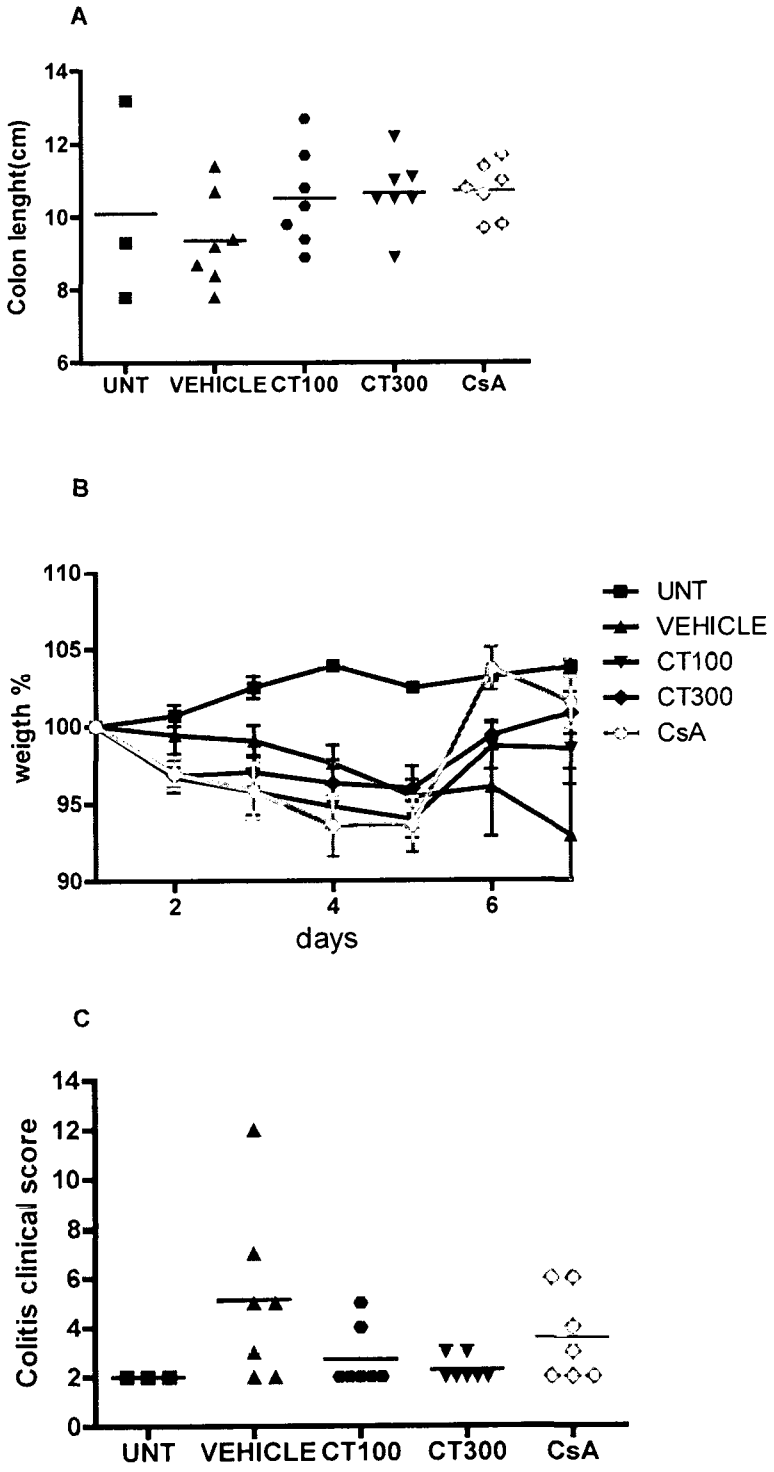

A
Colon lenght(cm)
14
12
10
8
6
UNT
VEHICLE
CT100
CT300
CsA
B
weigth %
110
105
100
95
90
2
4
6
days
UNT
VEHICLE
CT100
CT300
CsA
C
Colitis clinical score
14
12
10
8
6
4
2
0
UNT
VEHICLE
CT100
CT300
CsA

FIGURE 37

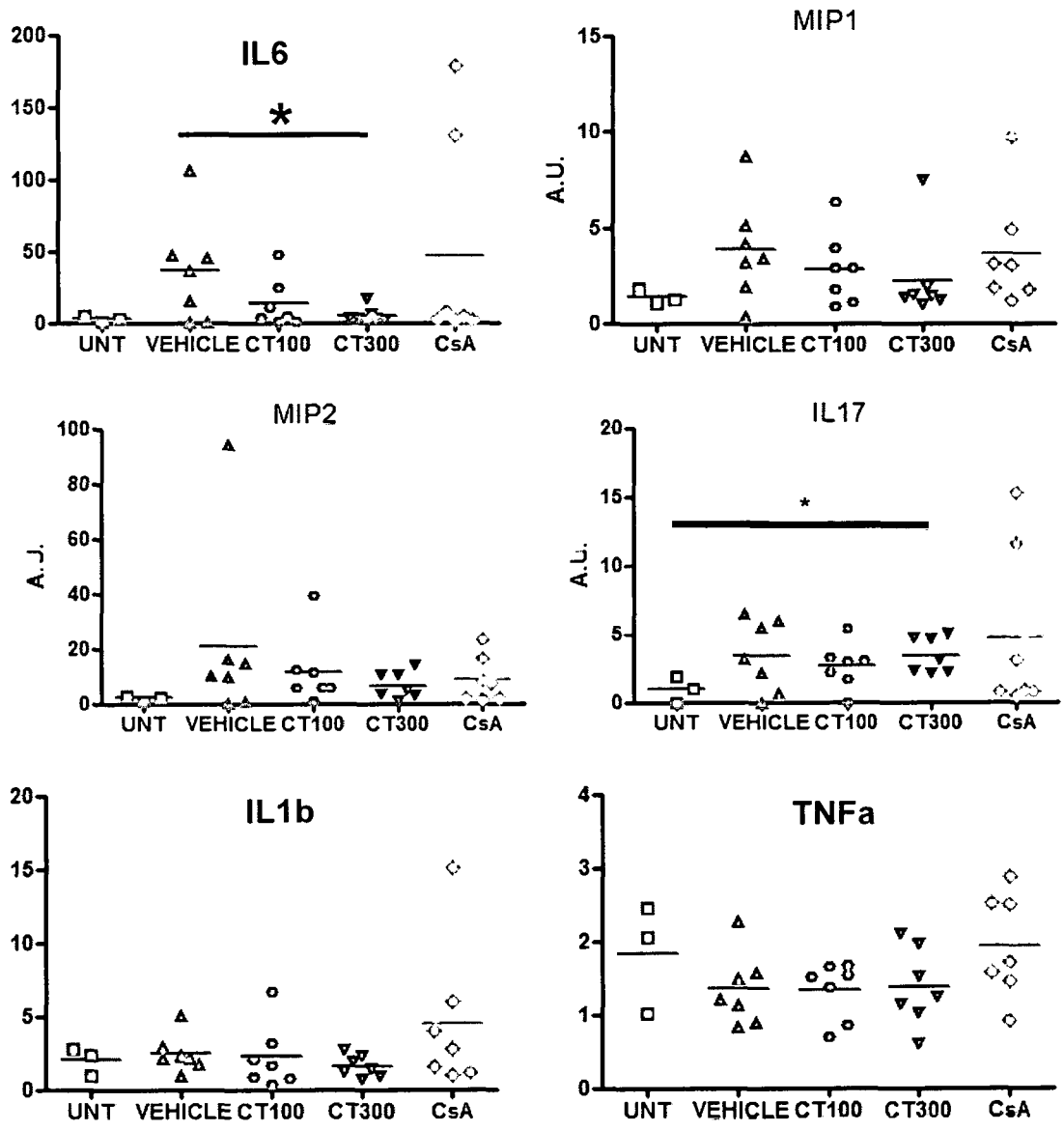

IL6
*
200
150
100
50
0
UNT VEHICLE CT100 CT300 CsA
MIP1
A.U.
15
10
5
0
UNT VEHICLE CT100 CT300 CsA
MIP2
A.U.
100
80
60
40
20
0
UNT VEHICLE CT100 CT300 CsA
IL17
*
A.U.
20
15
10
5
0
UNT VEHICLE CT100 CT300 CsA
IL1b
20
15
10
5
0
UNT VEHICLE CT100 CT300 CsA
TNFa
4
3
2
1
0
UNT VEHICLE CT100 CT300 CsA

FIGURE 38

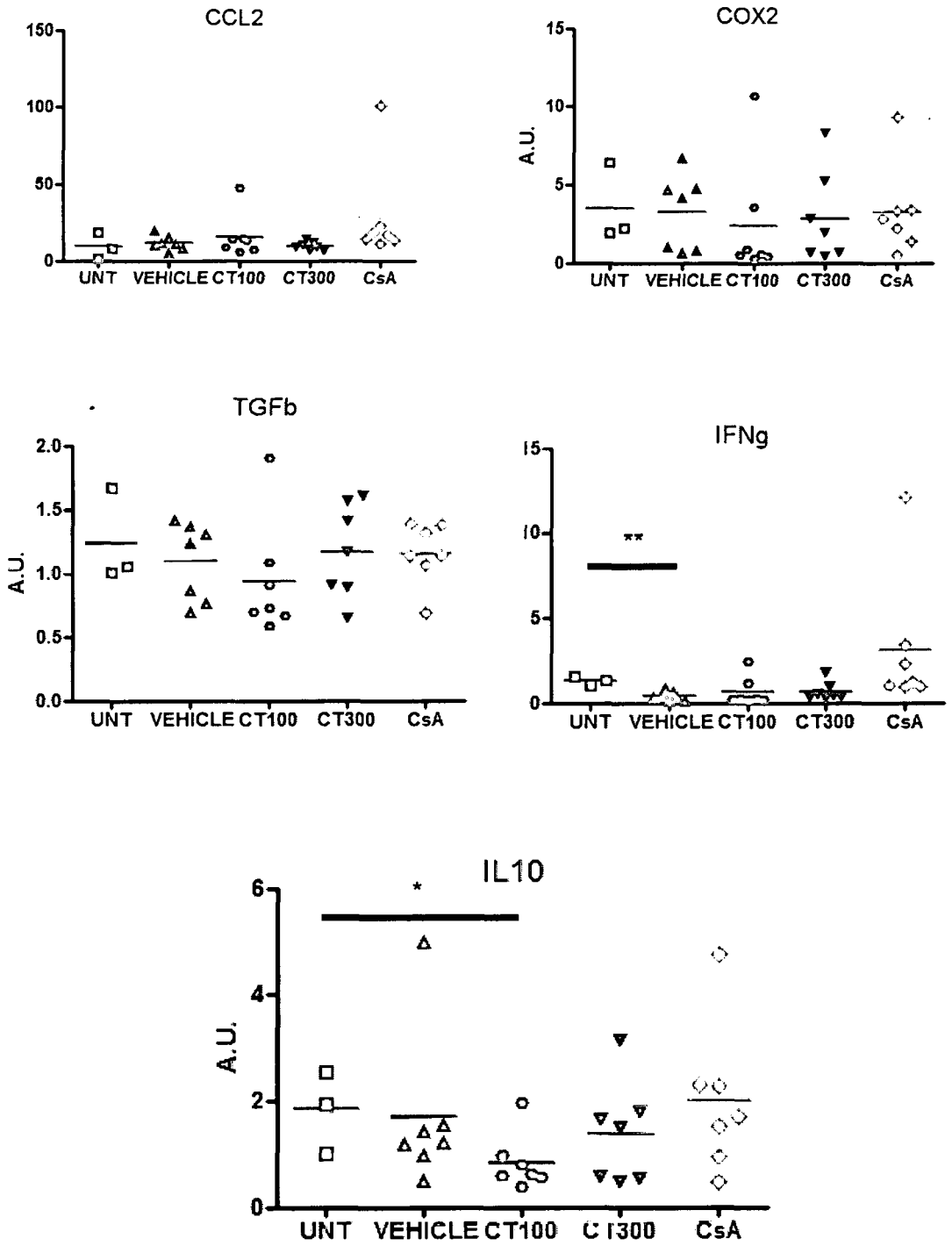


FIGURE 38 (continued)

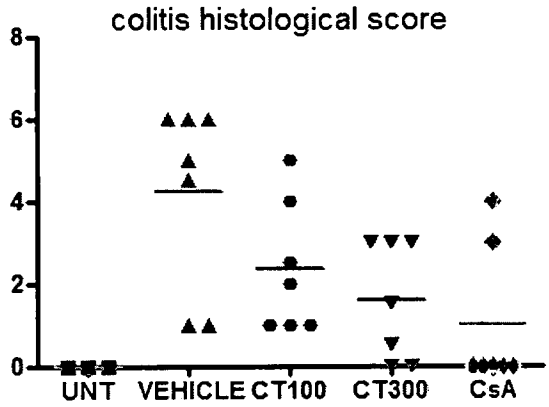

colitis histological score
8
6
4
2
0
UNT
VEHICLE
CT100
CT300
CsA

FIGURE 39A

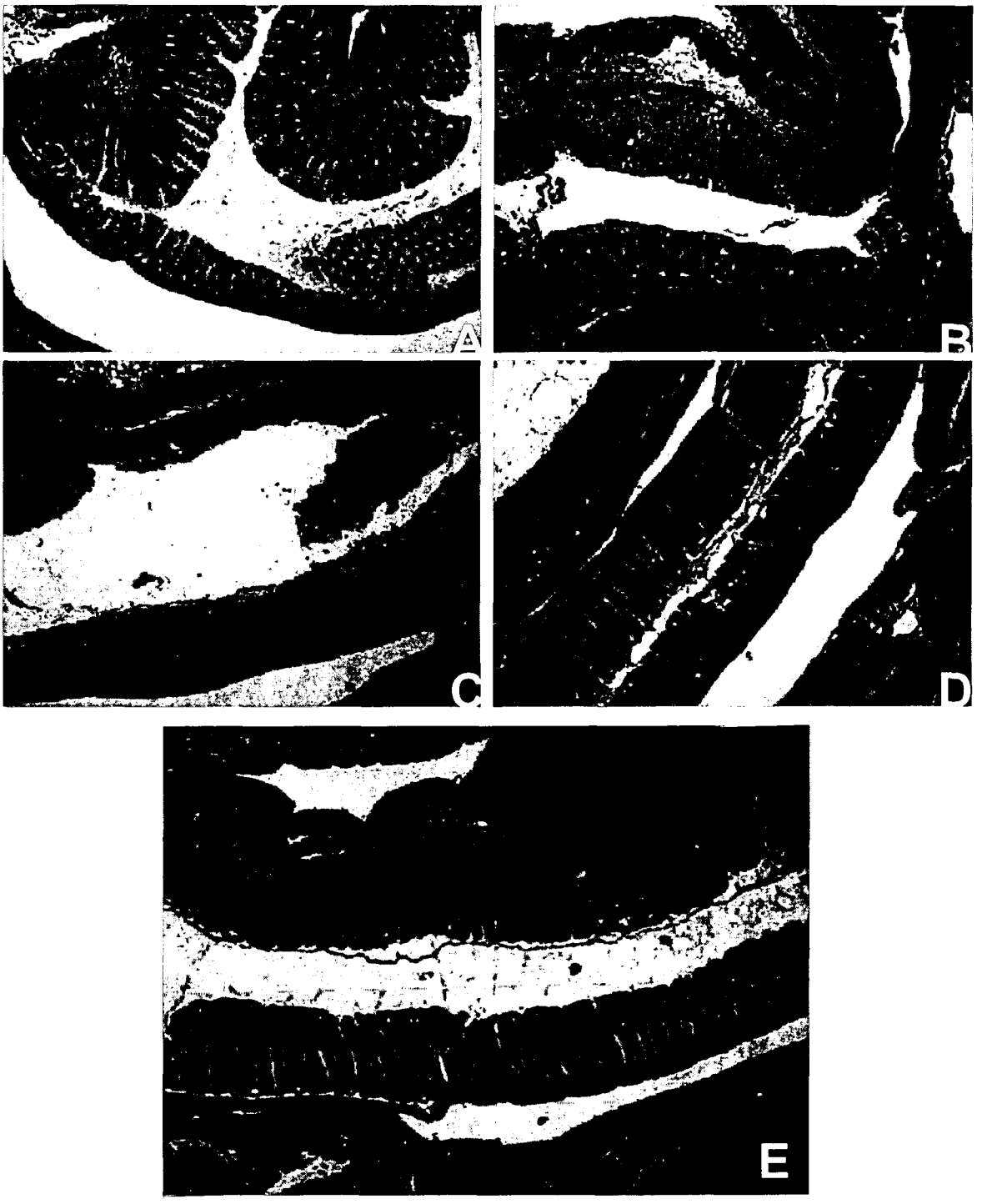


FIGURE 39B

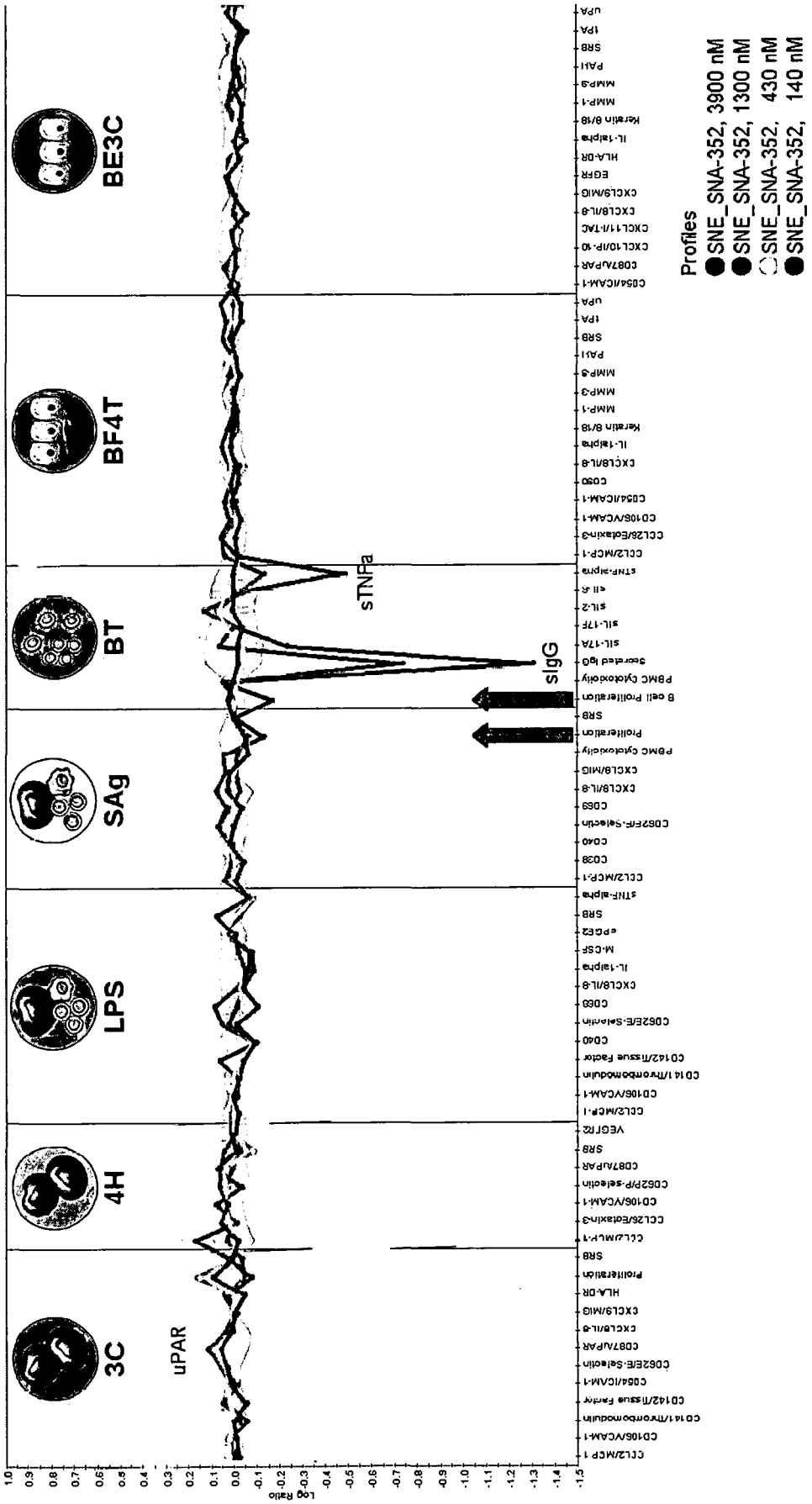

3C
4H
LPS
SAg
BT
BF4T
BE3C
uPAR
sTNFa
sIgG
Profiles
SNE_SNA-352, 3900 nM
SNE_SNA-352, 1300 nM
SNE_SNA-352, 430 nM
SNE_SNA-352, 140 nM
Log Ratio

FIGURE 40

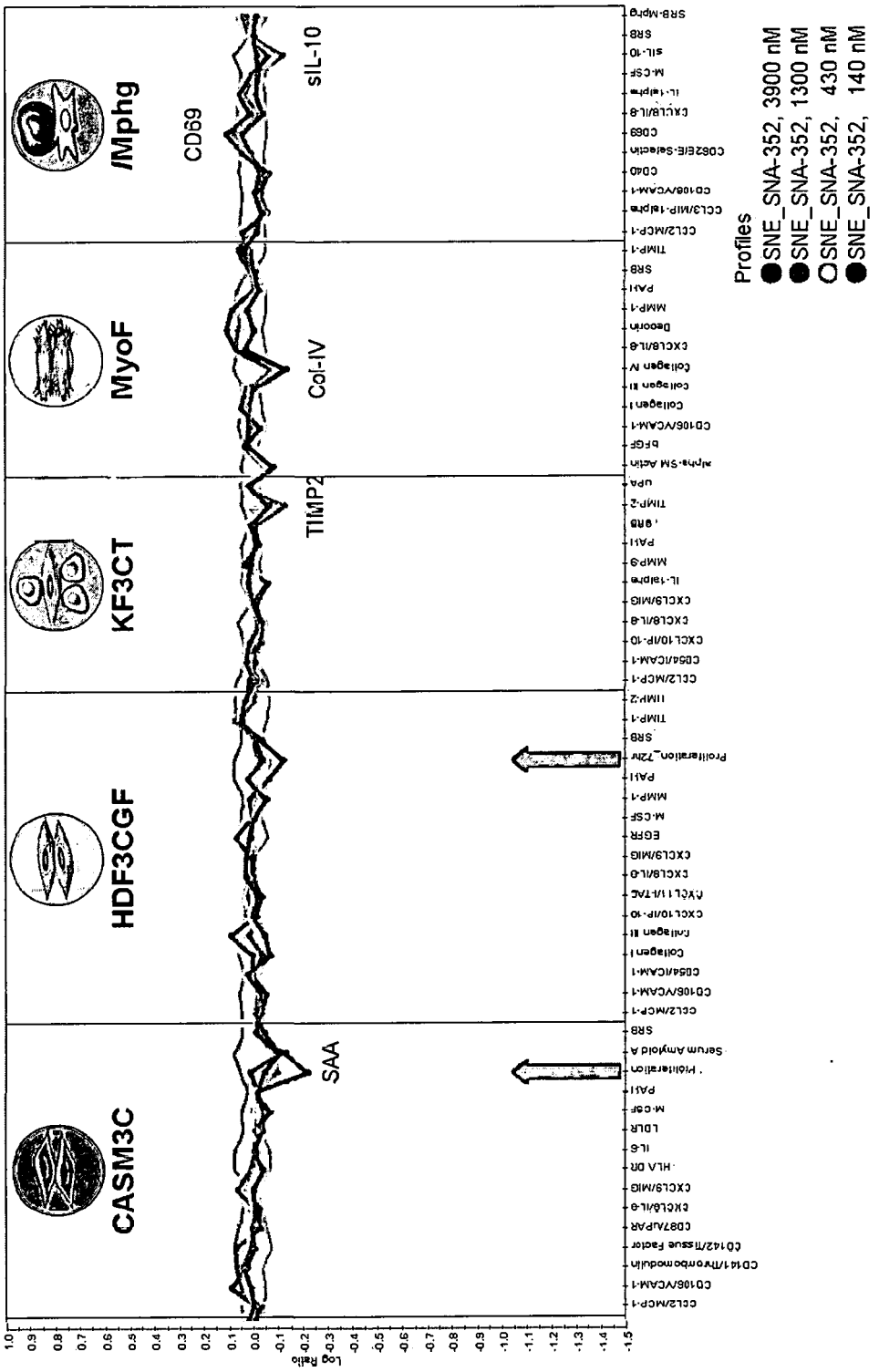


FIGURE 40
(CONTINUED)

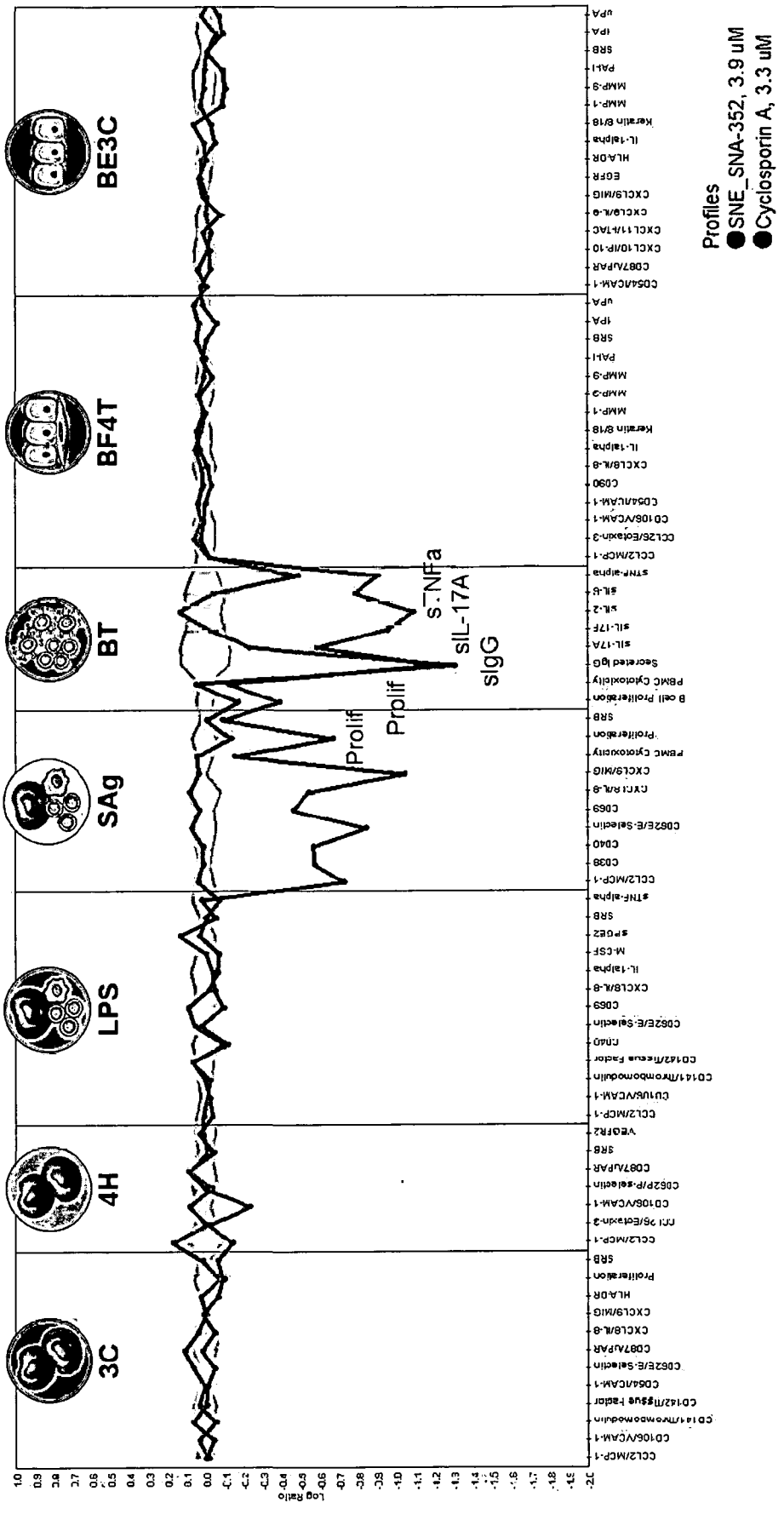

Profiles
SNE_SNA-352, 3.9 uM
Cyclosporin A, 3.3 uM
3C
4H
LPS
SAg
BT
BF4T
BE3C
Prolif
Prolif
s.NFa
sIL-17A
sIgG
Log Ratio

FIGURE 41

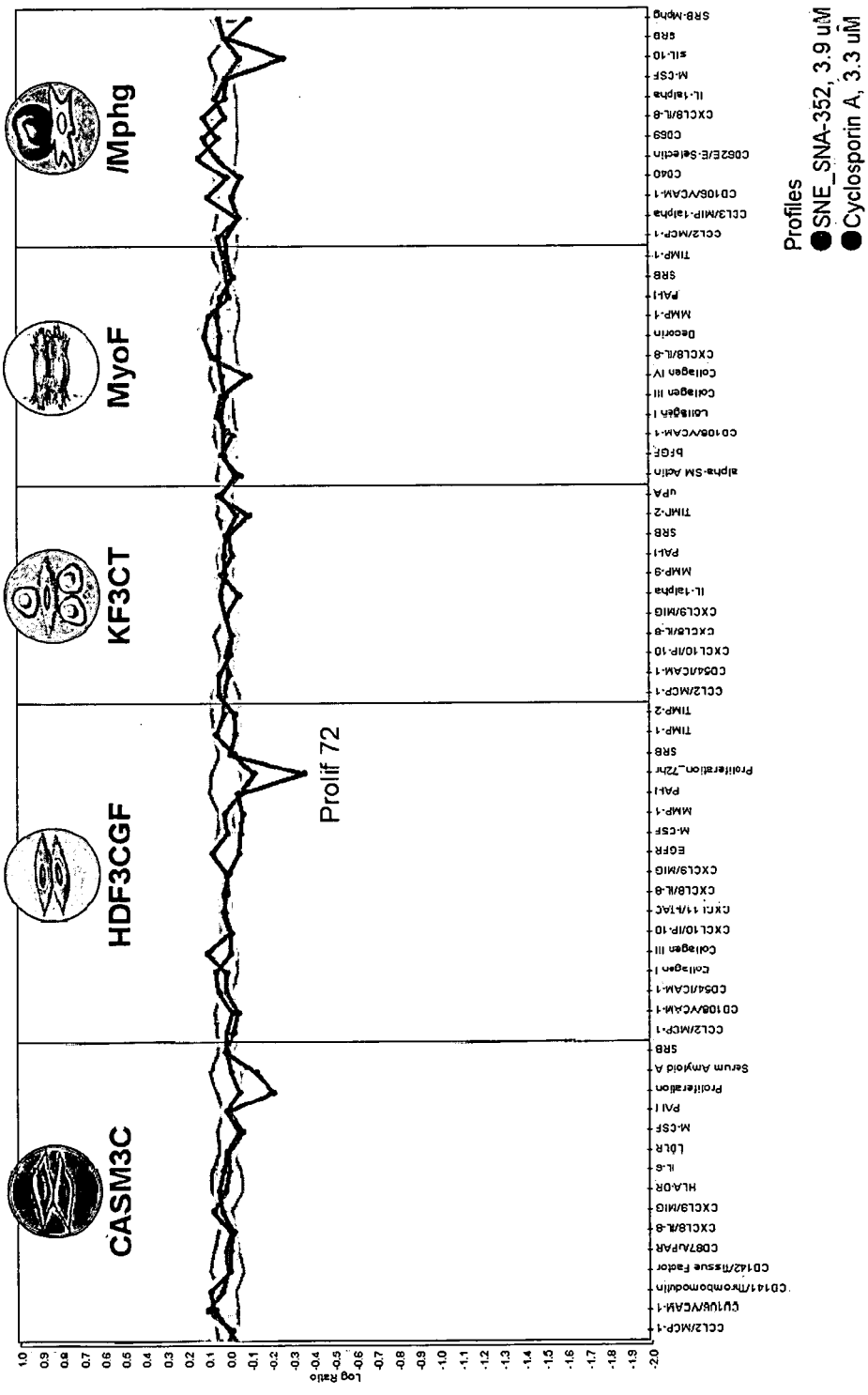
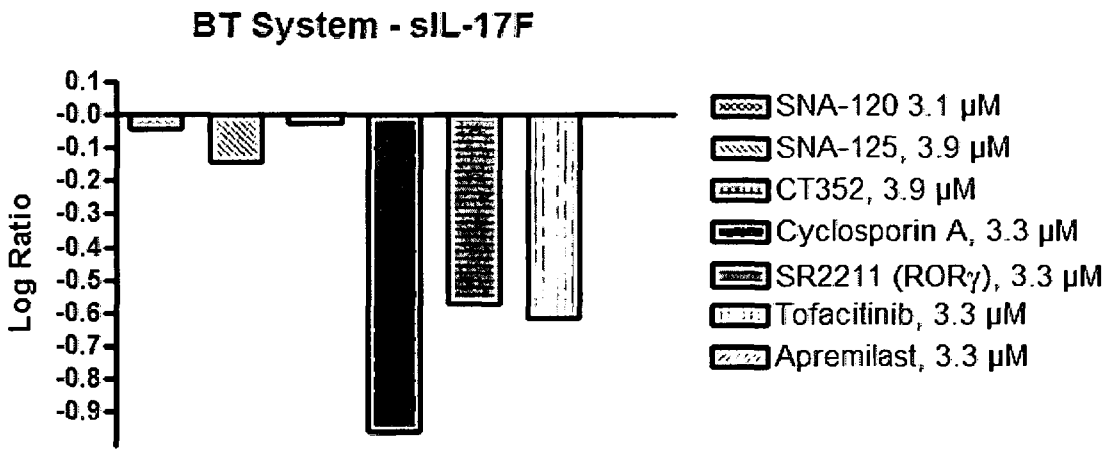


FIGURE 41
(CONTINUED)

A


BT System - sIL-17F
Log Ratio
0.1
-0.0
-0.1
-0.2
-0.3
-0.4
-0.5
-0.6
-0.7
-0.8
-0.9
SNA-120 3.1 µM
SNA-125, 3.9 µM
CT352, 3.9 µM
Cyclosporin A, 3.3 µM
SR2211 (RORγ), 3.3 µM
Tofacitinib, 3.3 µM
Apremilast, 3.3 µM

B

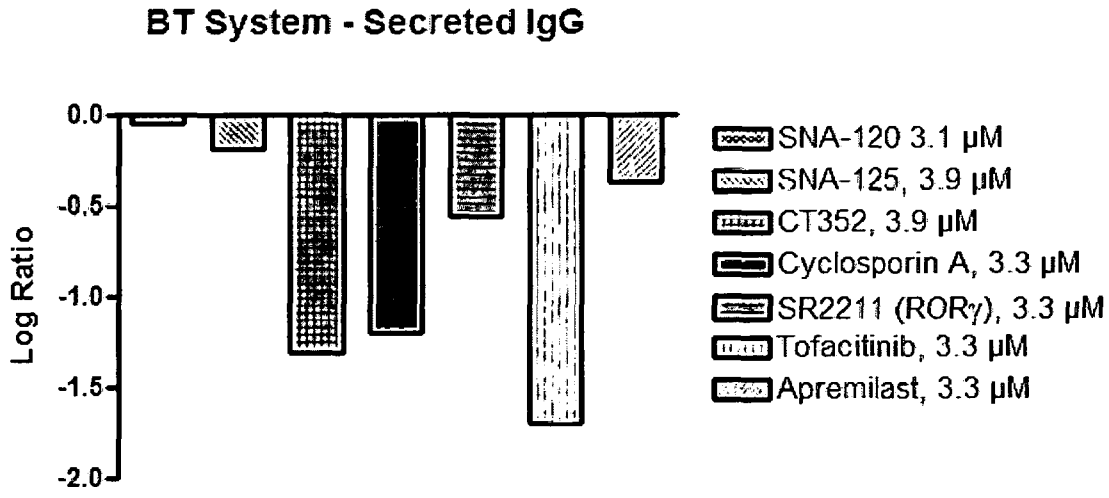
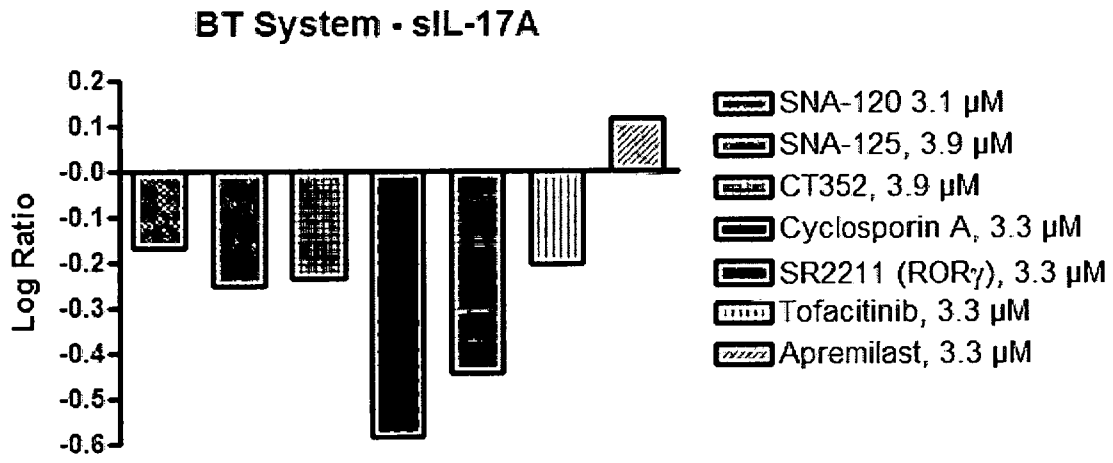

BT System - Secreted IgG
Log Ratio
0.0
-0.5
-1.0
-1.5
-2.0
SNA-120 3.1 µM
SNA-125, 3.9 µM
CT352, 3.9 µM
Cyclosporin A, 3.3 µM
SR2211 (RORγ), 3.3 µM
Tofacitinib, 3.3 µM
Apremilast, 3.3 µM

FIGURE 42

C



D

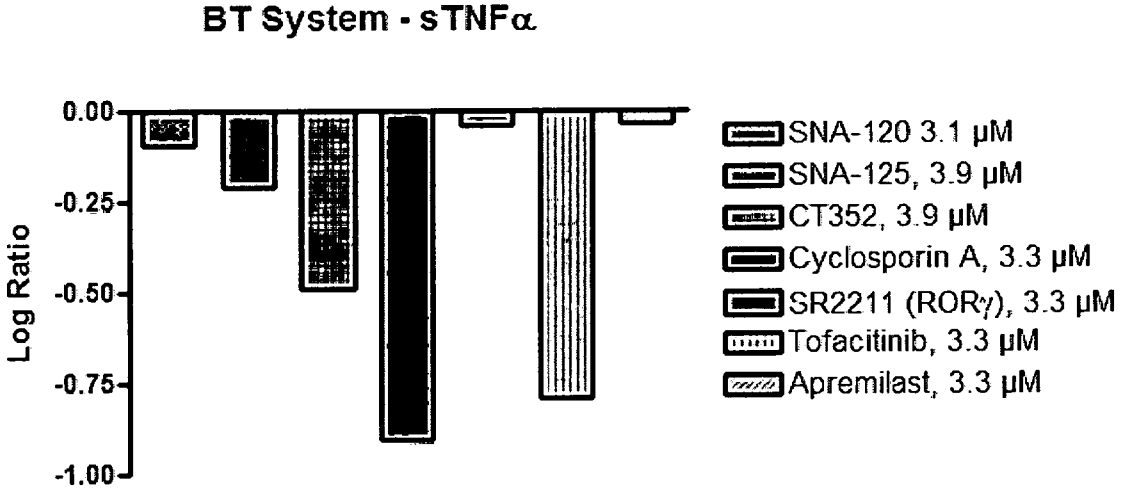


FIGURE 42 (CONTINUED)

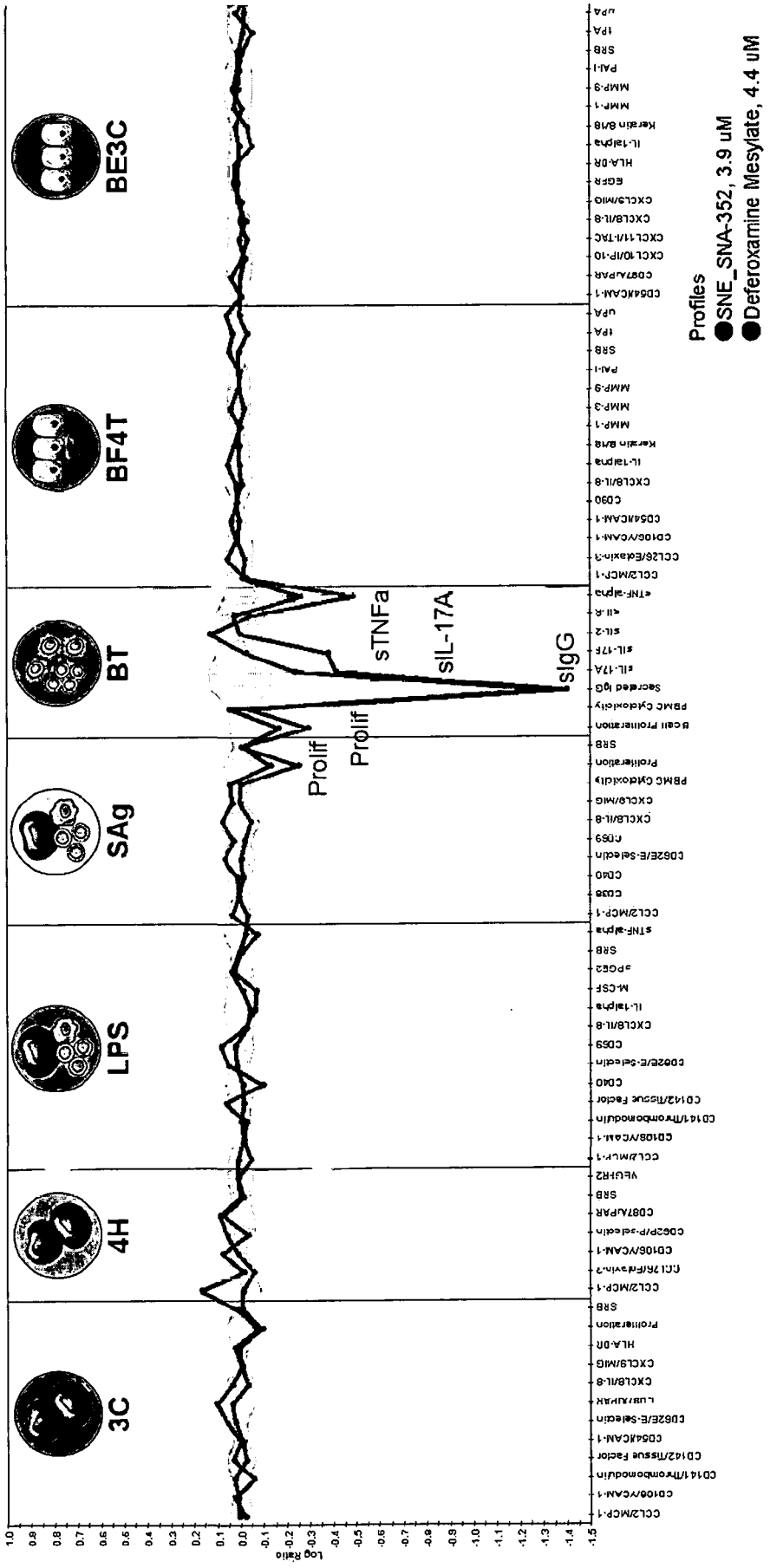

3C
4H
LPS
SAg
BT
BF4T
BE3C
Prolif
Prolif
sTNFa
sIL-17A
sIgG
Profiles
SNE_SNA-352, 3.9 uM
Deferoxamine Mesylate, 4.4 uM
Log Ratio

FIGURE 43

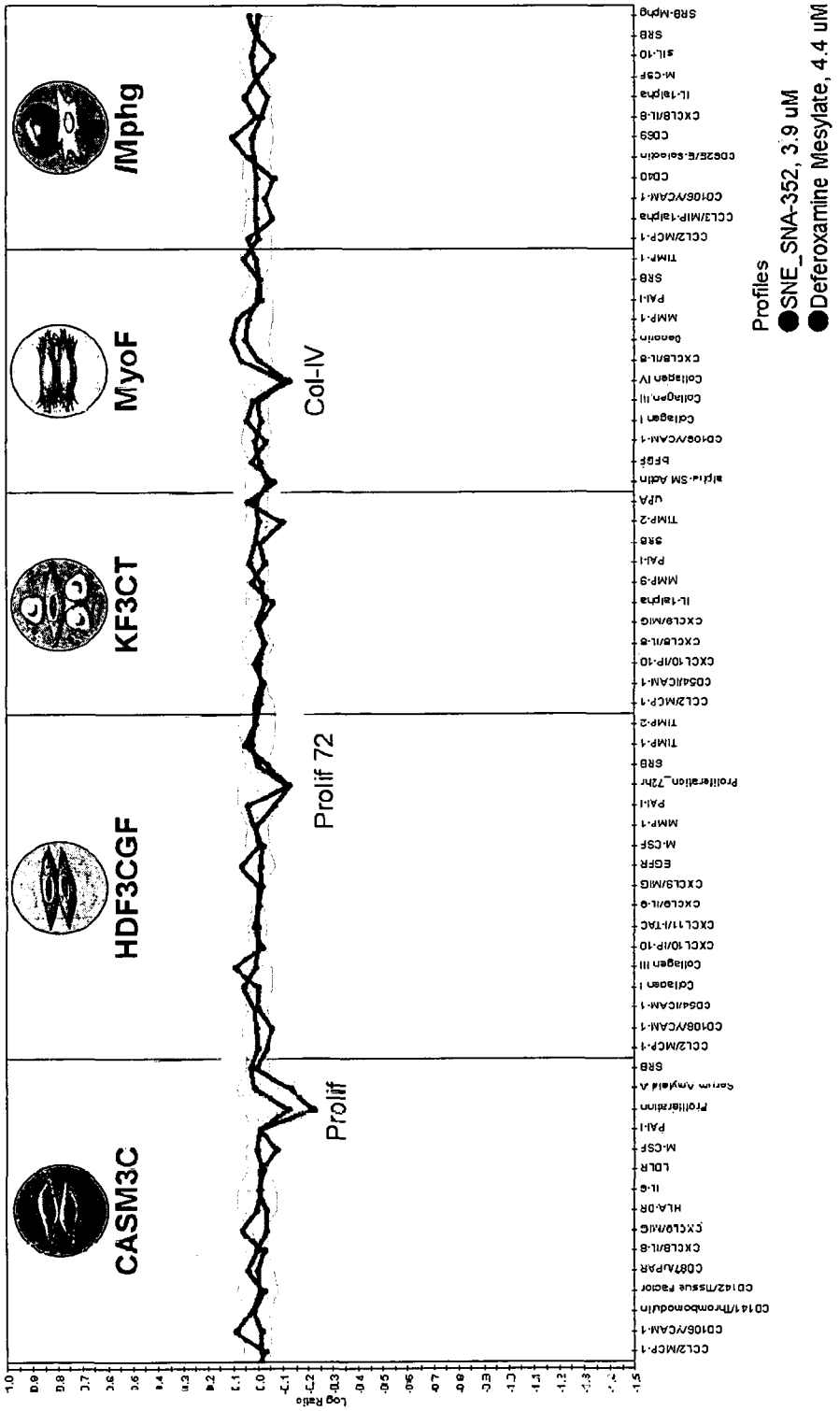


FIGURE 43
(CONTINUED)

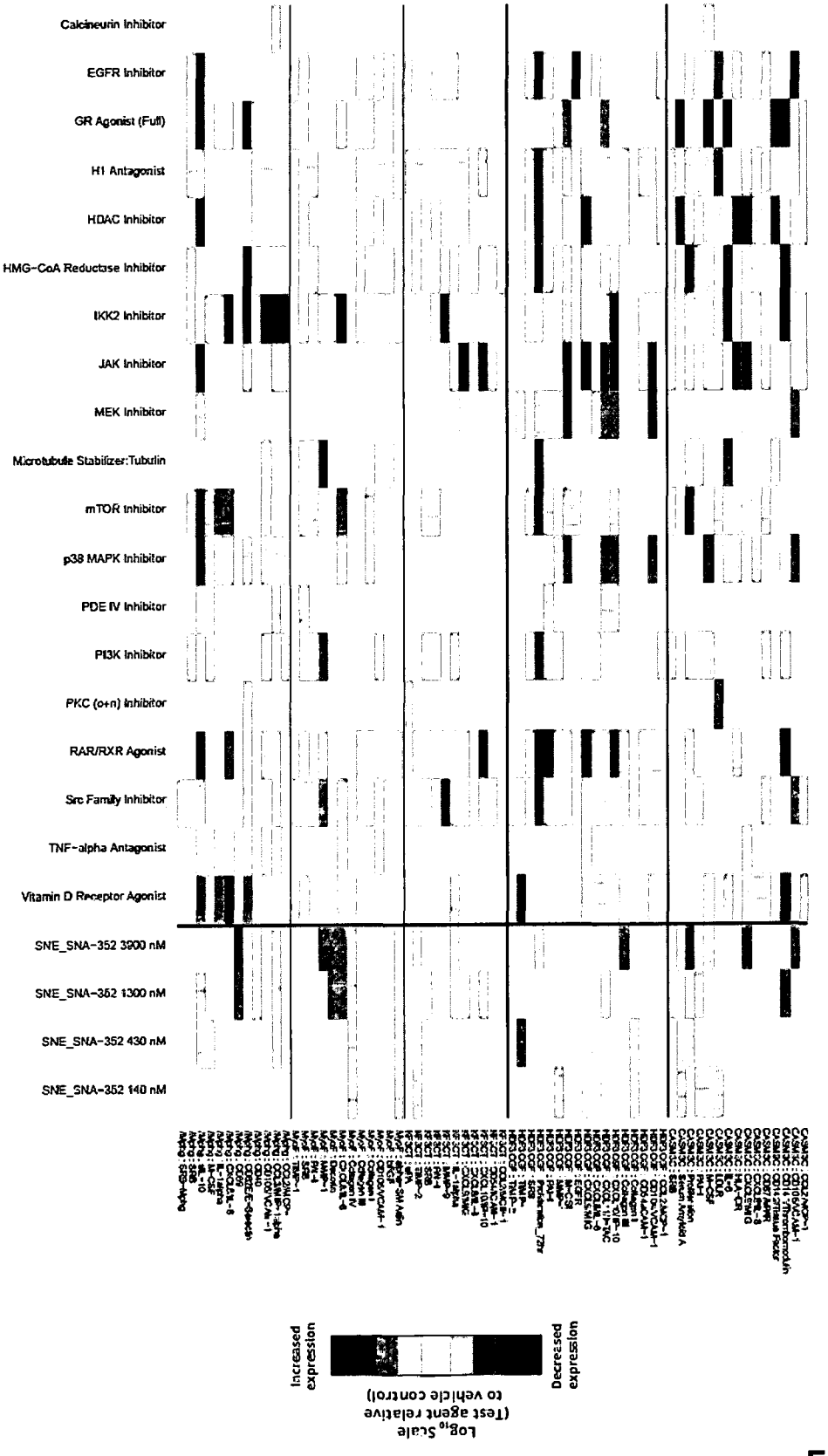

Calcineurin Inhibitor
EGFR Inhibitor
GR Agonist (Full)
H1 Antagonist
HDAC Inhibitor
HMG-CoA Reductase Inhibitor
IKK2 Inhibitor
JAK Inhibitor
MEK Inhibitor
Microtubule Stabilizer:Tubulin
mTOR Inhibitor
p38 MAPK Inhibitor
PDE IV Inhibitor
PI3K Inhibitor
PKC (α+η) Inhibitor
RAR/RXR Agonist
Src Family Inhibitor
TNF-alpha Antagonist
Vitamin D Receptor Agonist
SNE_SNA-352 3900 nM
SNE_SNA-352 1300 nM
SNE_SNA-352 430 nM
SNE_SNA-352 140 nM
Increased expression
Decreased expression
Log10 Scale (Test agent relative to vehicle control)

FIGURE 44

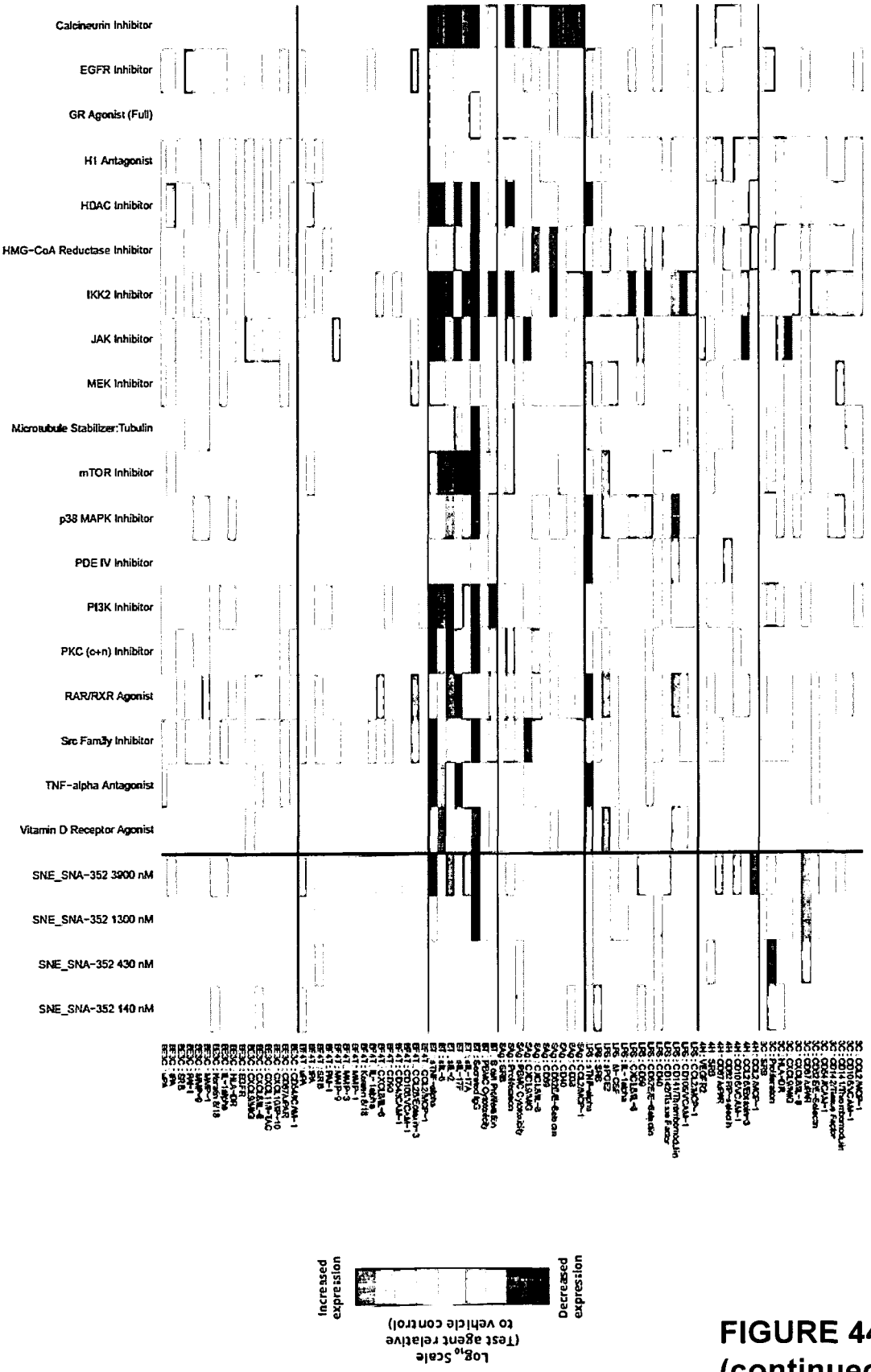


FIGURE 44 (continued)

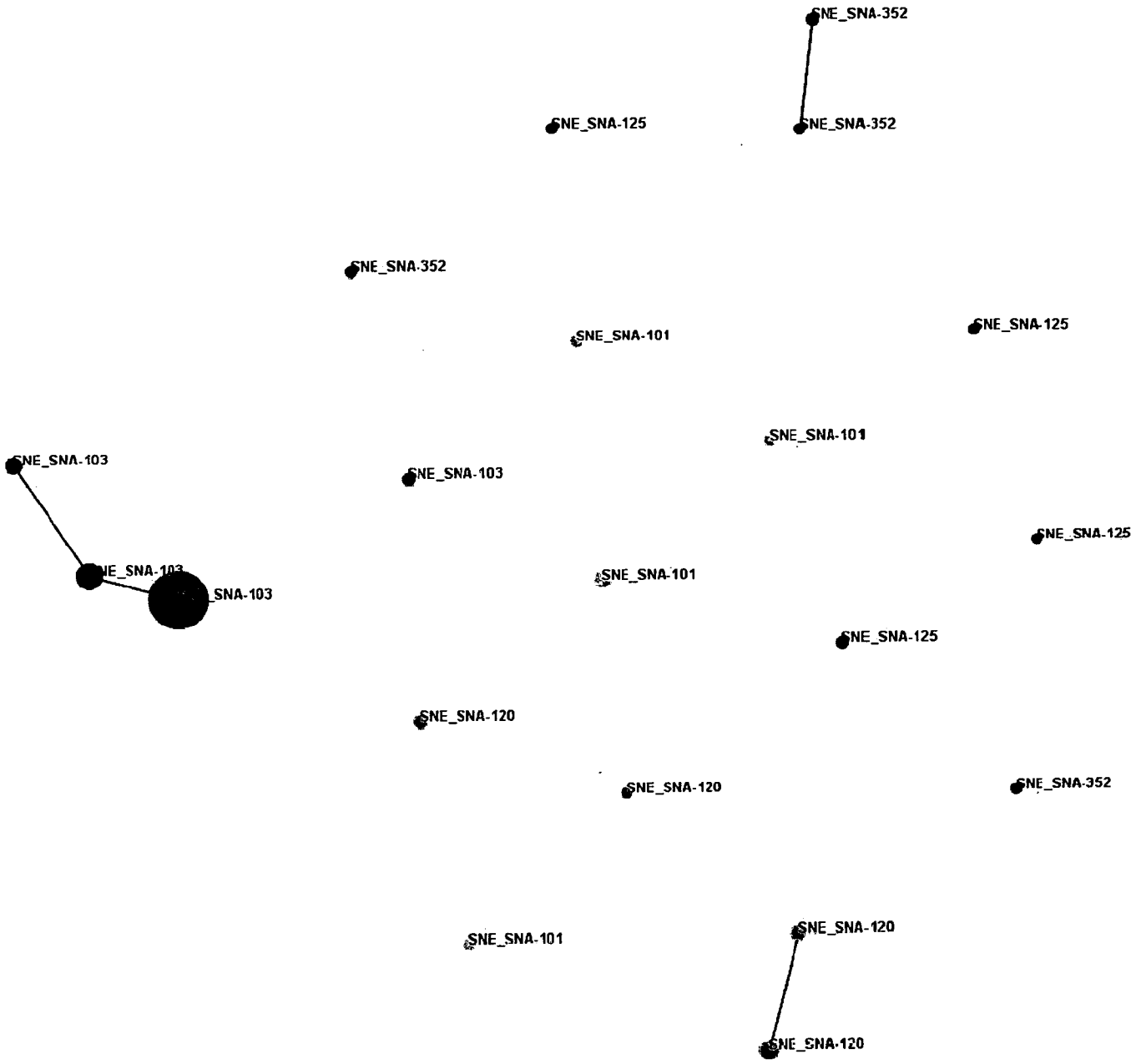
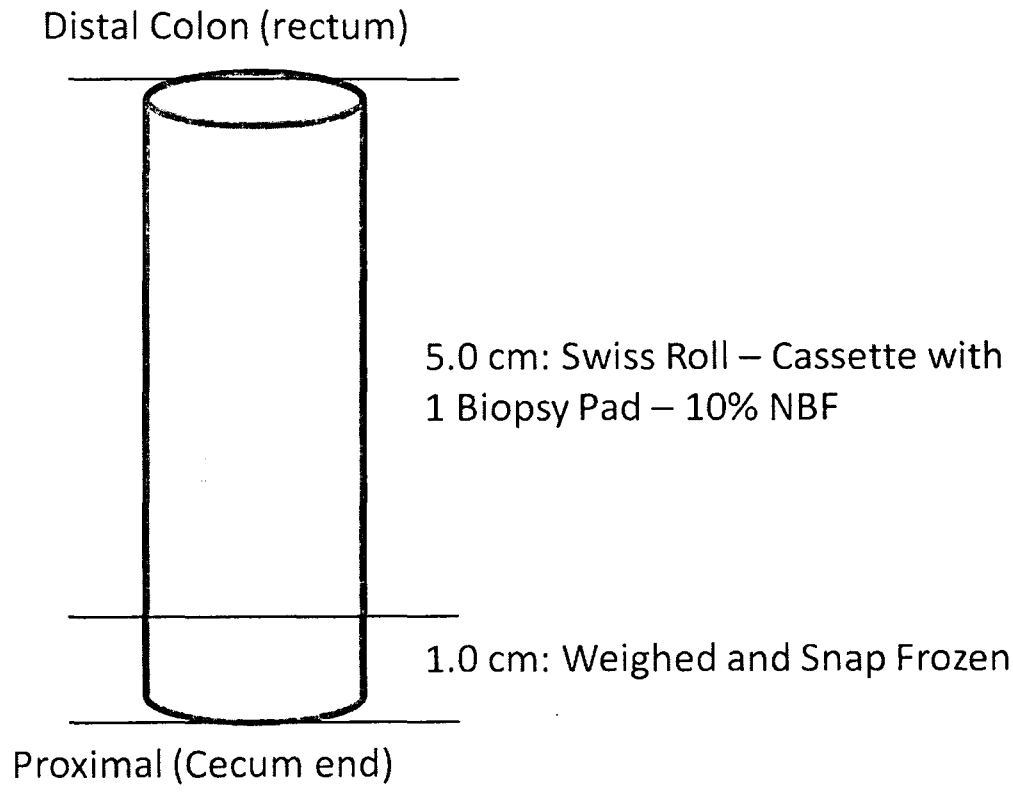

SNE_SNA-352
SNE_SNA-125
SNE_SNA-352
SNE_SNA-352
SNE_SNA-101
SNE_SNA-125
SNE_SNA-101
SNE_SNA-103
SNE_SNA-103
SNE_SNA-125
SNE_SNA-101
SNE_SNA-125
SNE_SNA-120
SNE_SNA-120
SNE_SNA-352
SNE_SNA-101
SNE_SNA-120
SNE_SNA-120

FIGURE 45

A


Distal Colon (rectum)
5.0 cm: Swiss Roll – Cassette with
1 Biopsy Pad – 10% NBF
1.0 cm: Weighed and Snap Frozen
Proximal (Cecum end)

B

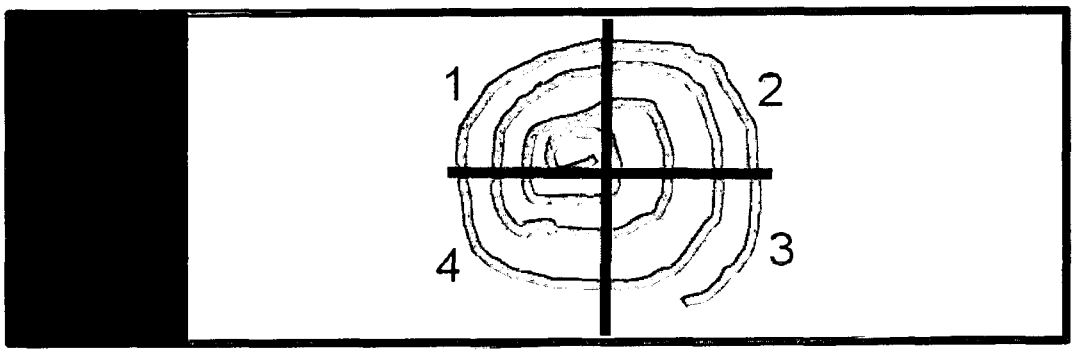

1
2
3
4

FIGURE 46

A

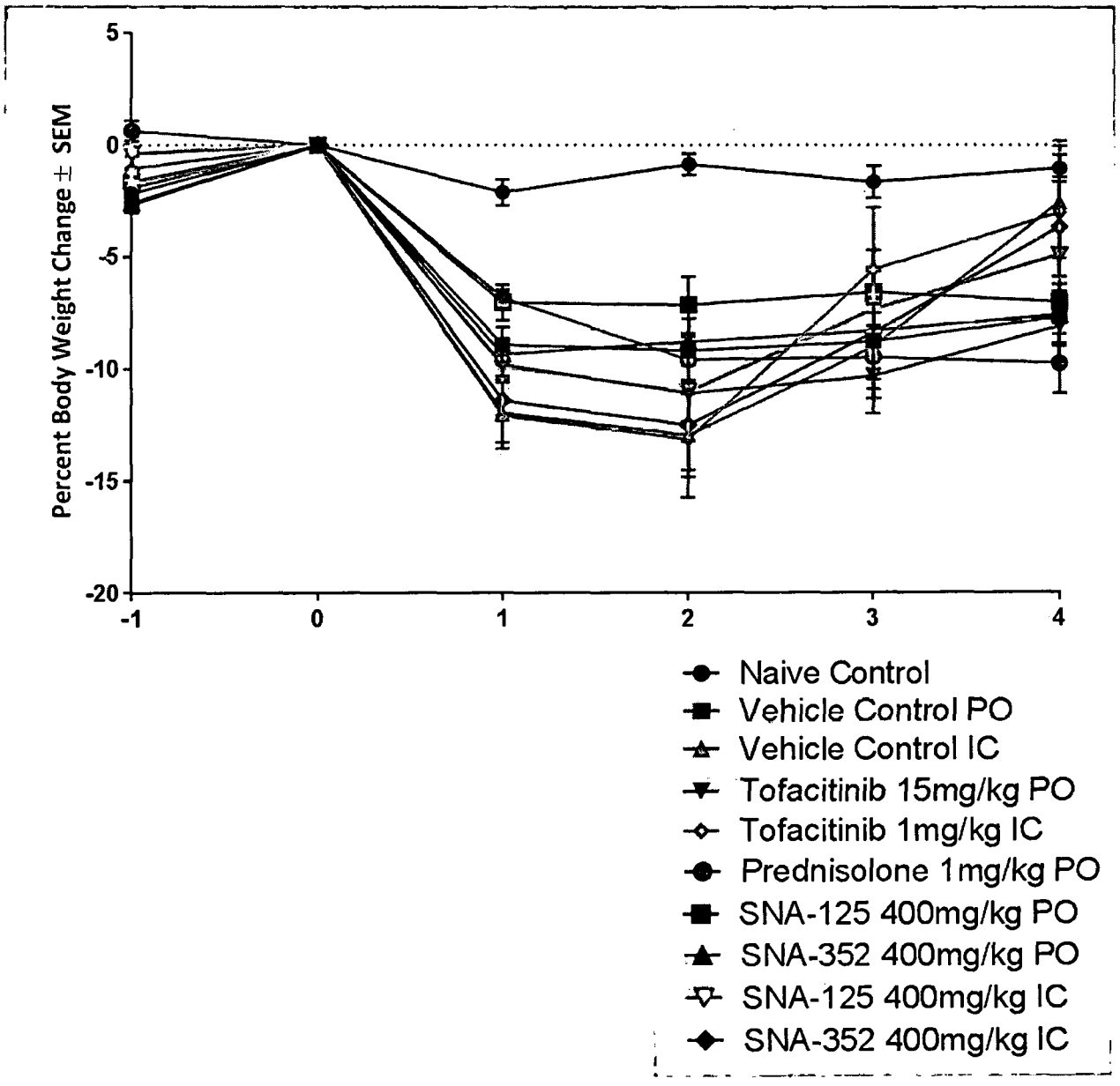

Percent Body Weight Change ± SEM
5
0
-5
-10
-15
-20
-1
0
1
2
3
4
Naive Control
Vehicle Control PO
Vehicle Control IC
Tofacitinib 15mg/kg PO
Tofacitinib 1mg/kg IC
Prednisolone 1mg/kg PO
SNA-125 400mg/kg PO
SNA-352 400mg/kg PO
SNA-125 400mg/kg IC
SNA-352 400mg/kg IC

FIGURE 47

B

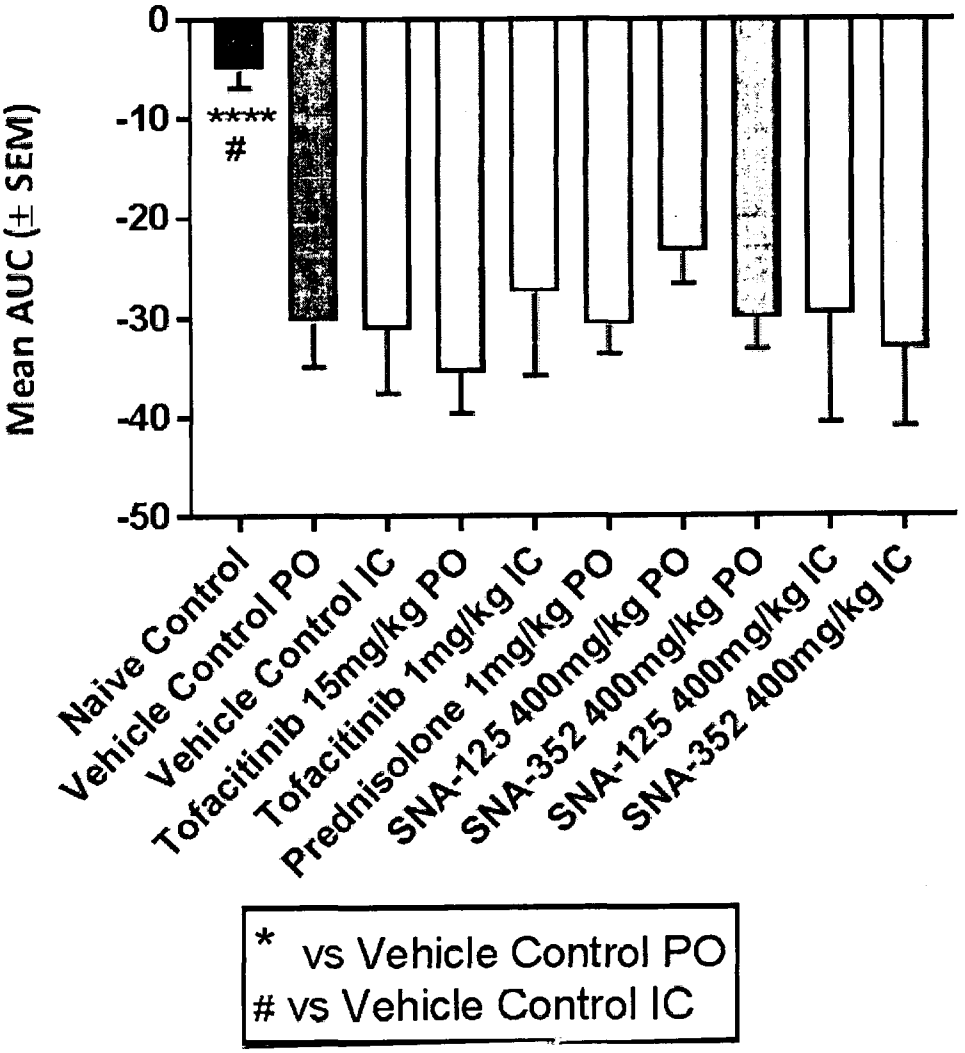

Mean AUC (± SEM)
0
-10
-20
-30
-40
-50
****
#
Naive Control
Vehicle Control PO
Vehicle Control IC
Tofacitinib 15mg/kg PO
Tofacitinib 1mg/kg IC
Prednisolone 1mg/kg PO
SNA-125 400mg/kg PO
SNA-352 400mg/kg PO
SNA-125 400mg/kg IC
SNA-352 400mg/kg IC
* vs Vehicle Control PO
# vs Vehicle Control IC

FIGURE 47

A

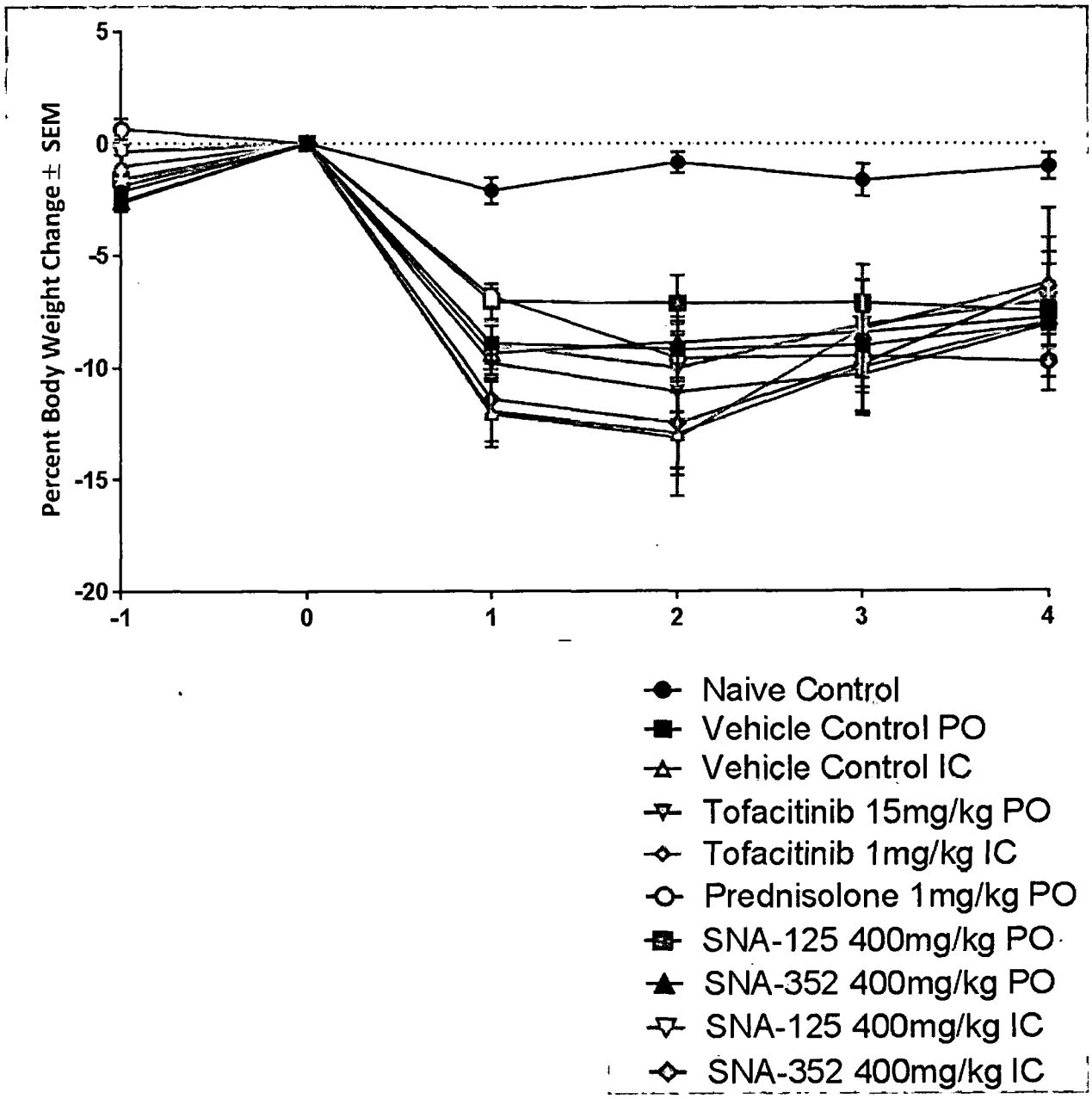

Percent Body Weight Change ± SEM
5
0
-5
-10
-15
-20
-1
0
1
2
3
4
Naive Control
Vehicle Control PO
Vehicle Control IC
Tofacitinib 15mg/kg PO
Tofacitinib 1mg/kg IC
Prednisolone 1mg/kg PO
SNA-125 400mg/kg PO
SNA-352 400mg/kg PO
SNA-125 400mg/kg IC
SNA-352 400mg/kg IC

FIGURE 48

B

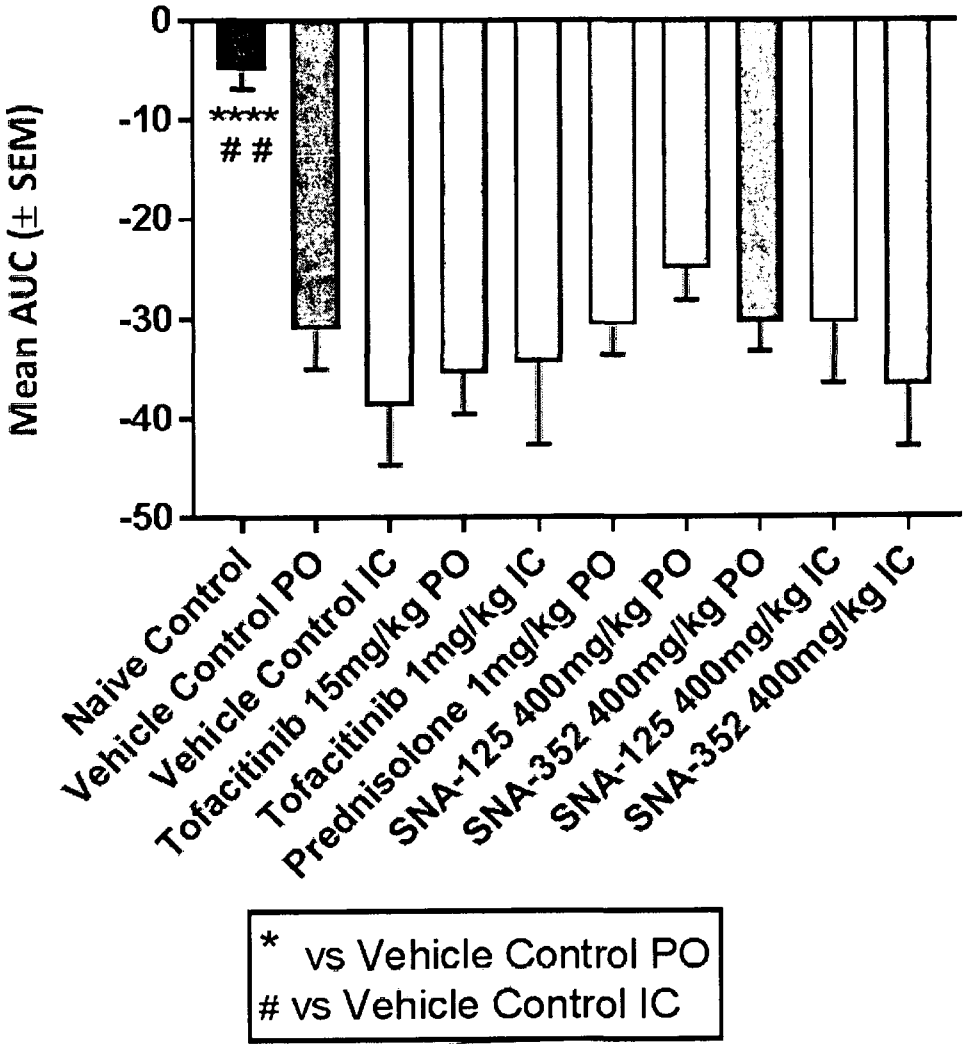

Mean AUC (± SEM)
0
-10
-20
-30
-40
-50
****
# #
Naive Control
Vehicle Control PO
Vehicle Control IC
Tofacitinib 15mg/kg PO
Tofacitinib 1mg/kg IC
Prednisolone 1mg/kg PO
SNA-125 400mg/kg PO
SNA-352 400mg/kg PO
SNA-125 400mg/kg IC
SNA-352 400mg/kg IC
* vs Vehicle Control PO
# vs Vehicle Control IC

FIGURE 48

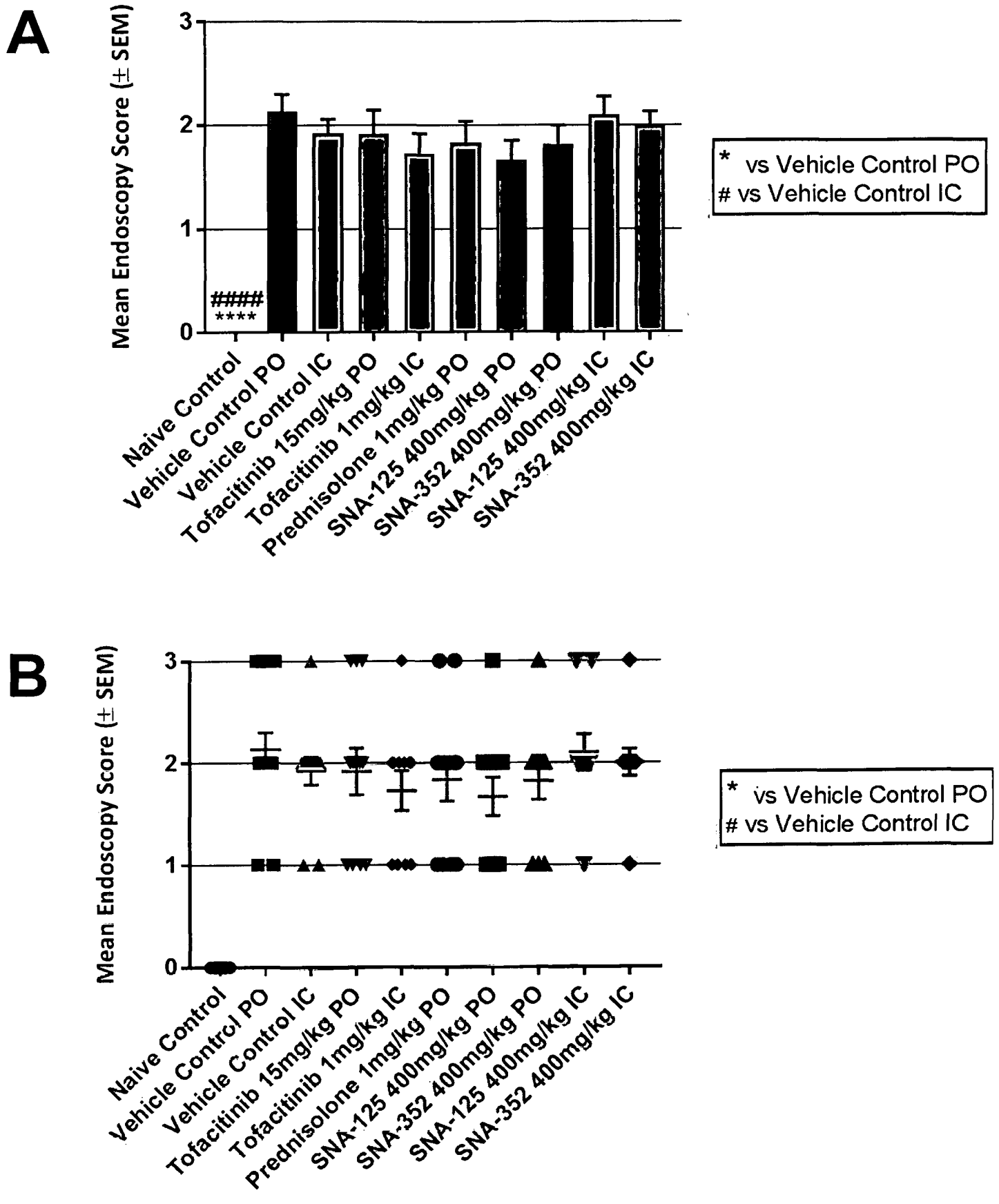
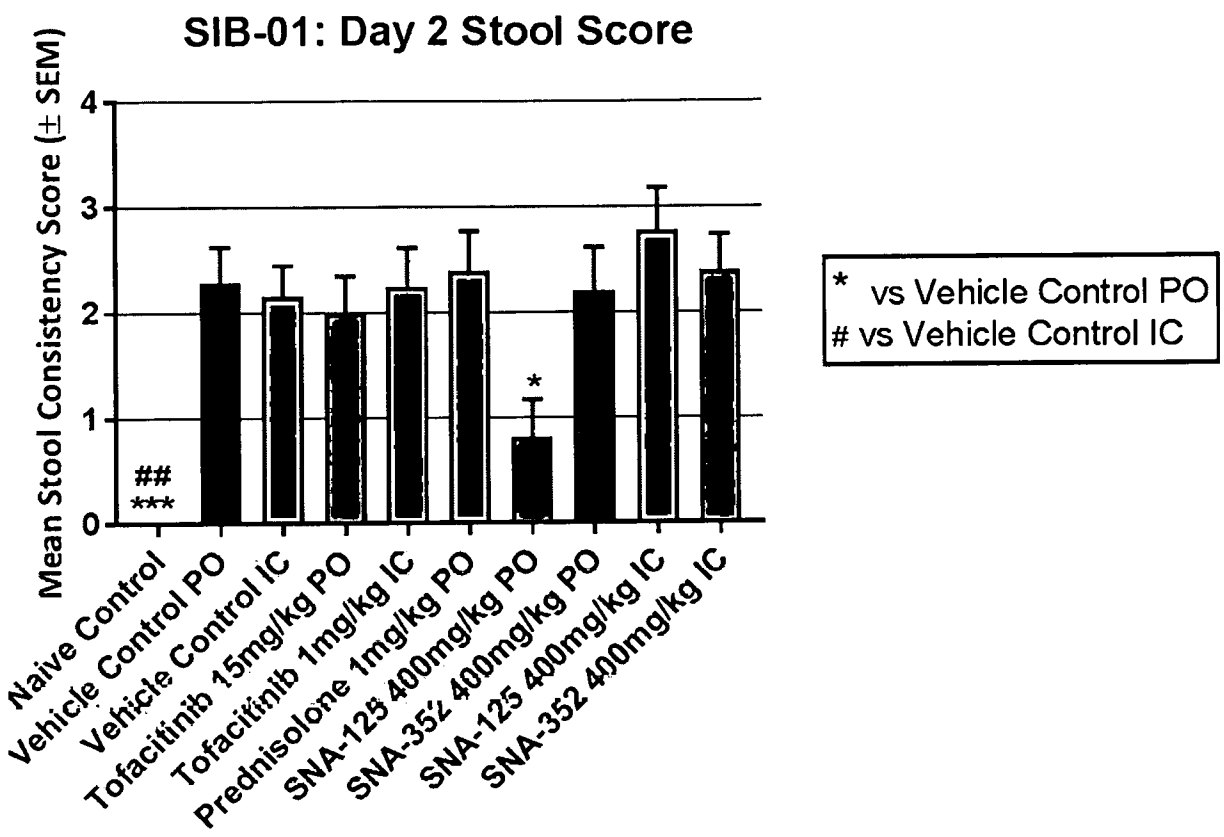

A
Mean Endoscopy Score (± SEM)
3
2
1
0
####
****
Naive Control
Vehicle Control PO
Vehicle Control IC
Tofacitinib 15mg/kg PO
Tofacitinib 1mg/kg IC
Prednisolone 1mg/kg PO
SNA-125 400mg/kg PO
SNA-352 400mg/kg PO
SNA-125 400mg/kg IC
SNA-352 400mg/kg IC
* vs Vehicle Control PO
# vs Vehicle Control IC
B
Mean Endoscopy Score (± SEM)
3
2
1
0
Naive Control
Vehicle Control PO
Vehicle Control IC
Tofacitinib 15mg/kg PO
Tofacitinib 1mg/kg IC
Prednisolone 1mg/kg PO
SNA-125 400mg/kg PO
SNA-352 400mg/kg PO
SNA-125 400mg/kg IC
SNA-352 400mg/kg IC
* vs Vehicle Control PO
# vs Vehicle Control IC

FIGURE 49

A


SIB-01: Day 2 Stool Score
Mean Stool Consistency Score (± SEM)
0
1
2
3
4
##
***
*
Naive Control
Vehicle Control PO
Vehicle Control IC
Tofacitinib 15mg/kg PO
Tofacitinib 1mg/kg IC
Prednisolone 1mg/kg PO
SNA-125 400mg/kg PO
SNA-352 400mg/kg PO
SNA-125 400mg/kg IC
SNA-352 400mg/kg IC
* vs Vehicle Control PO
# vs Vehicle Control IC

B

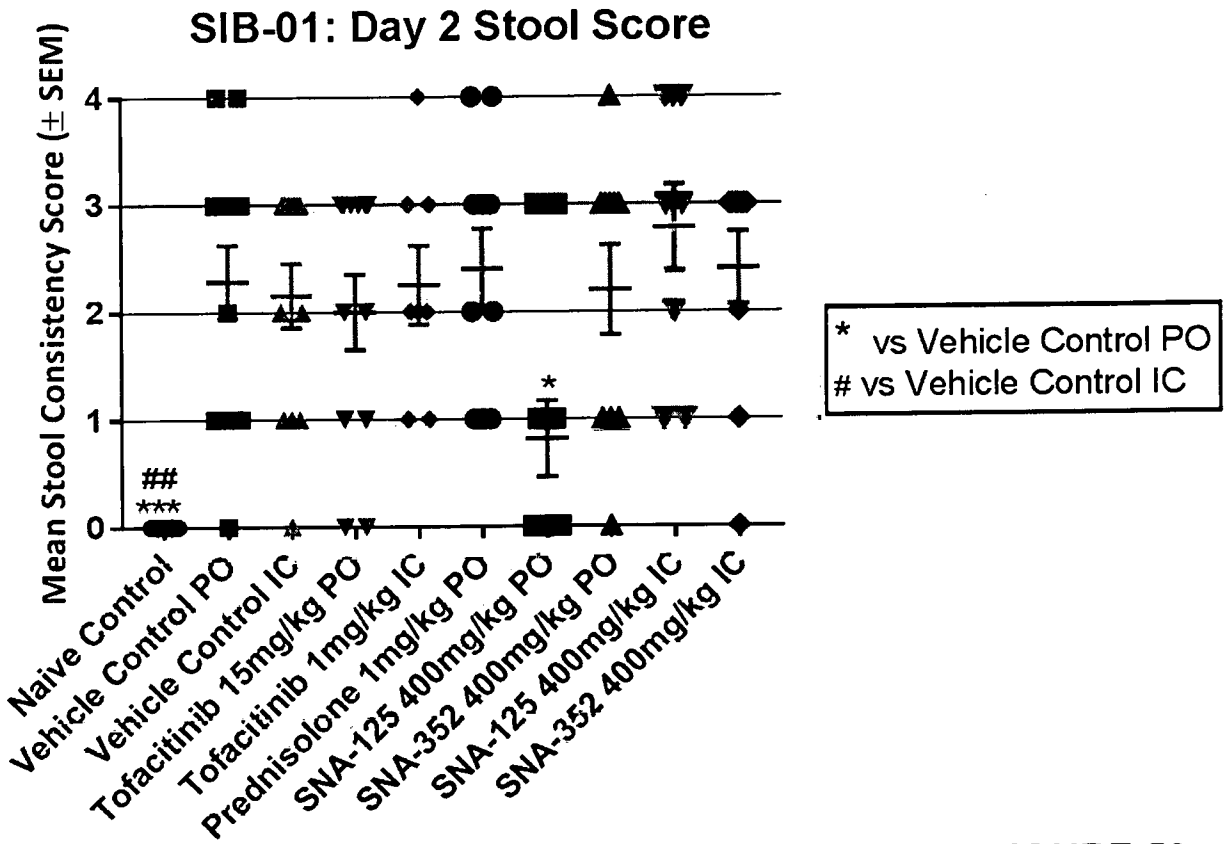

SIB-01: Day 2 Stool Score
Mean Stool Consistency Score (± SEM)
0
1
2
3
4
##
***
*
Naive Control
Vehicle Control PO
Vehicle Control IC
Tofacitinib 15mg/kg PO
Tofacitinib 1mg/kg IC
Prednisolone 1mg/kg PO
SNA-125 400mg/kg PO
SNA-352 400mg/kg PO
SNA-125 400mg/kg IC
SNA-352 400mg/kg IC
* vs Vehicle Control PO
# vs Vehicle Control IC

FIGURE 50

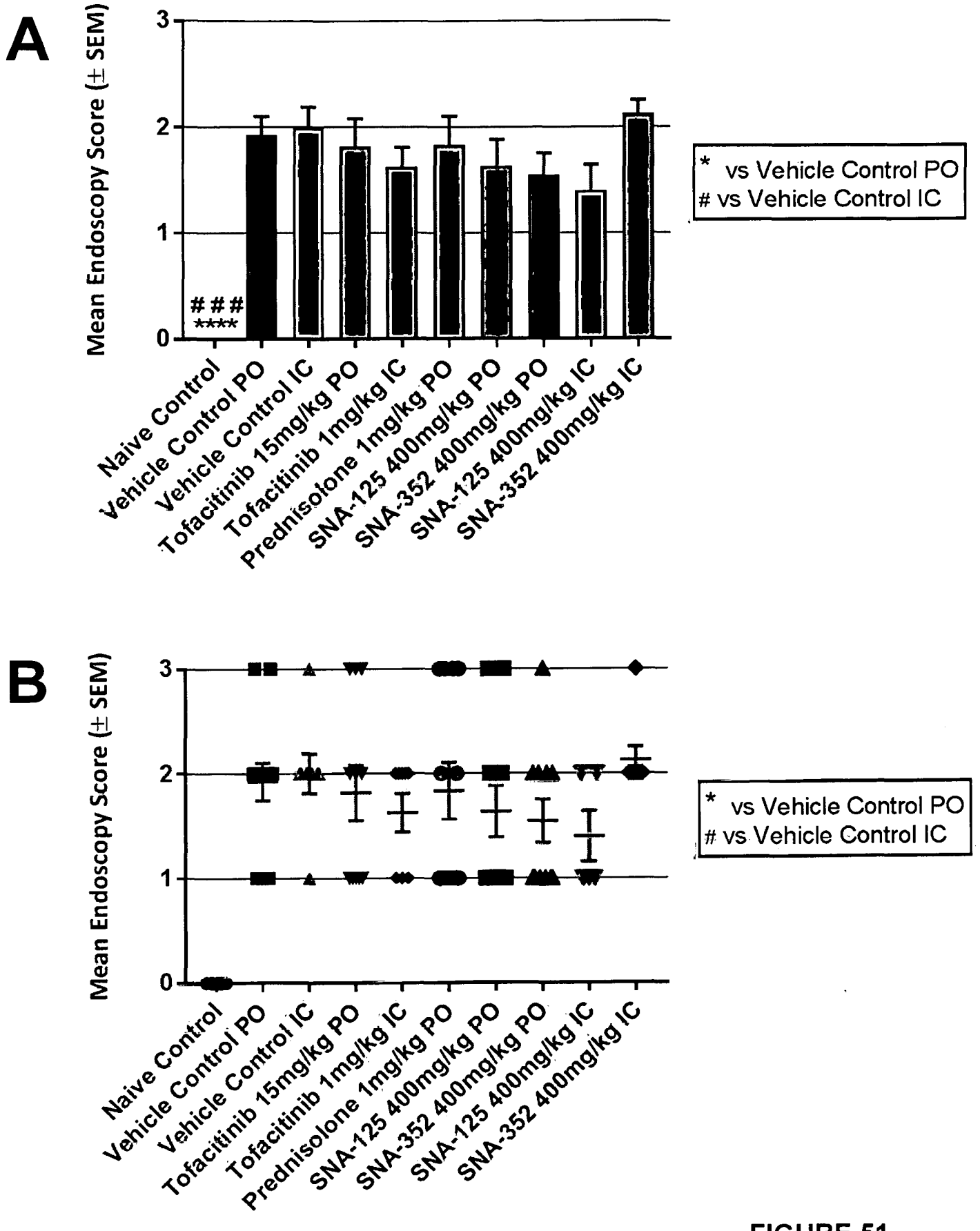
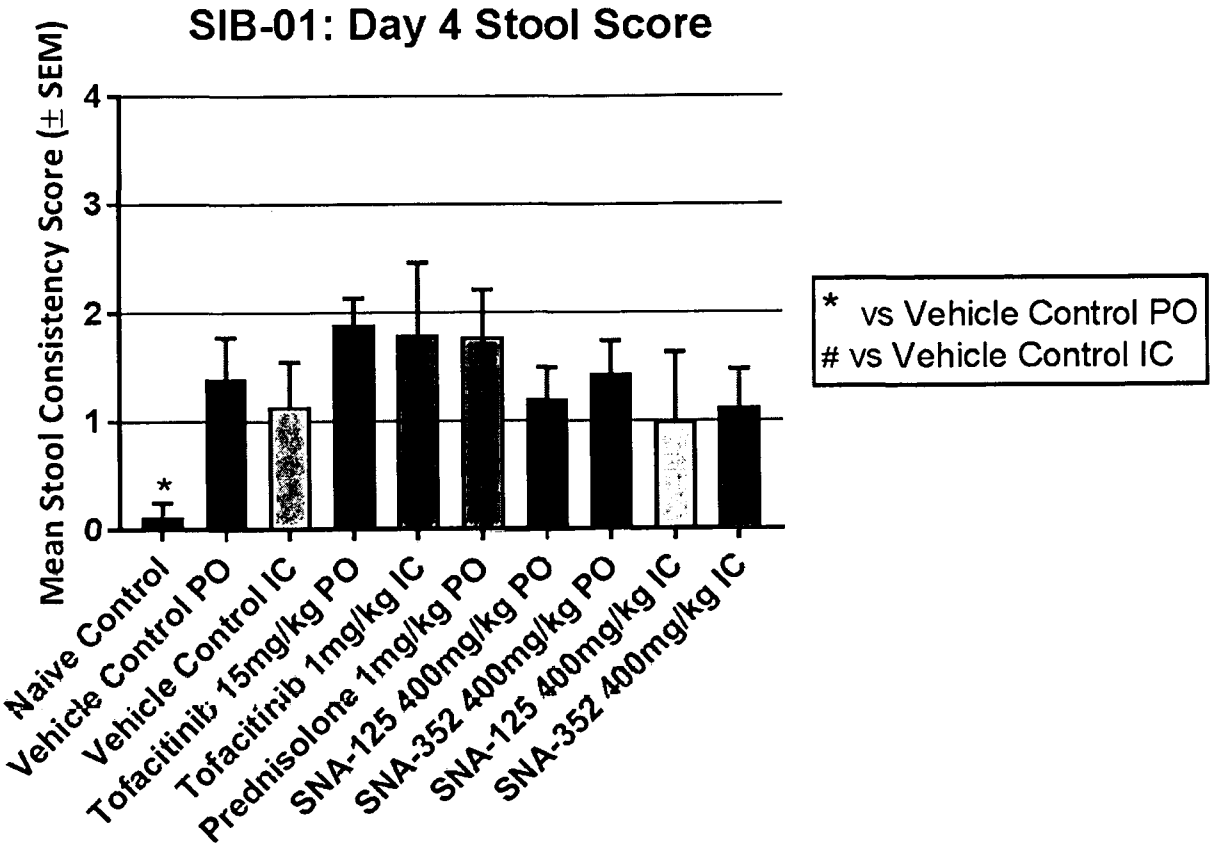

A
Mean Endoscopy Score (± SEM)
3
2
1
0
# # #
****
Naive Control
Vehicle Control PO
Vehicle Control IC
Tofacitinib 15mg/kg PO
Tofacitinib 1mg/kg IC
Prednisolone 1mg/kg PO
SNA-125 400mg/kg PO
SNA-352 400mg/kg PO
SNA-125 400mg/kg IC
SNA-352 400mg/kg IC
* vs Vehicle Control PO
# vs Vehicle Control IC
B
Mean Endoscopy Score (± SEM)
3
2
1
0
Naive Control
Vehicle Control PO
Vehicle Control IC
Tofacitinib 15mg/kg PO
Tofacitinib 1mg/kg IC
Prednisolone 1mg/kg PO
SNA-125 400mg/kg PO
SNA-352 400mg/kg PO
SNA-125 400mg/kg IC
SNA-352 400mg/kg IC
* vs Vehicle Control PO
# vs Vehicle Control IC

FIGURE 51

A


SIB-01: Day 4 Stool Score
Mean Stool Consistency Score (± SEM)
0
1
2
3
4
*
Naive Control
Vehicle Control PO
Vehicle Control IC
Tofacitinib 15mg/kg PO
Tofacitinib 1mg/kg IC
Prednisolone 1mg/kg PO
SNA-125 400mg/kg PO
SNA-352 400mg/kg PO
SNA-125 400mg/kg IC
SNA-352 400mg/kg IC
* vs Vehicle Control PO
# vs Vehicle Control IC

B

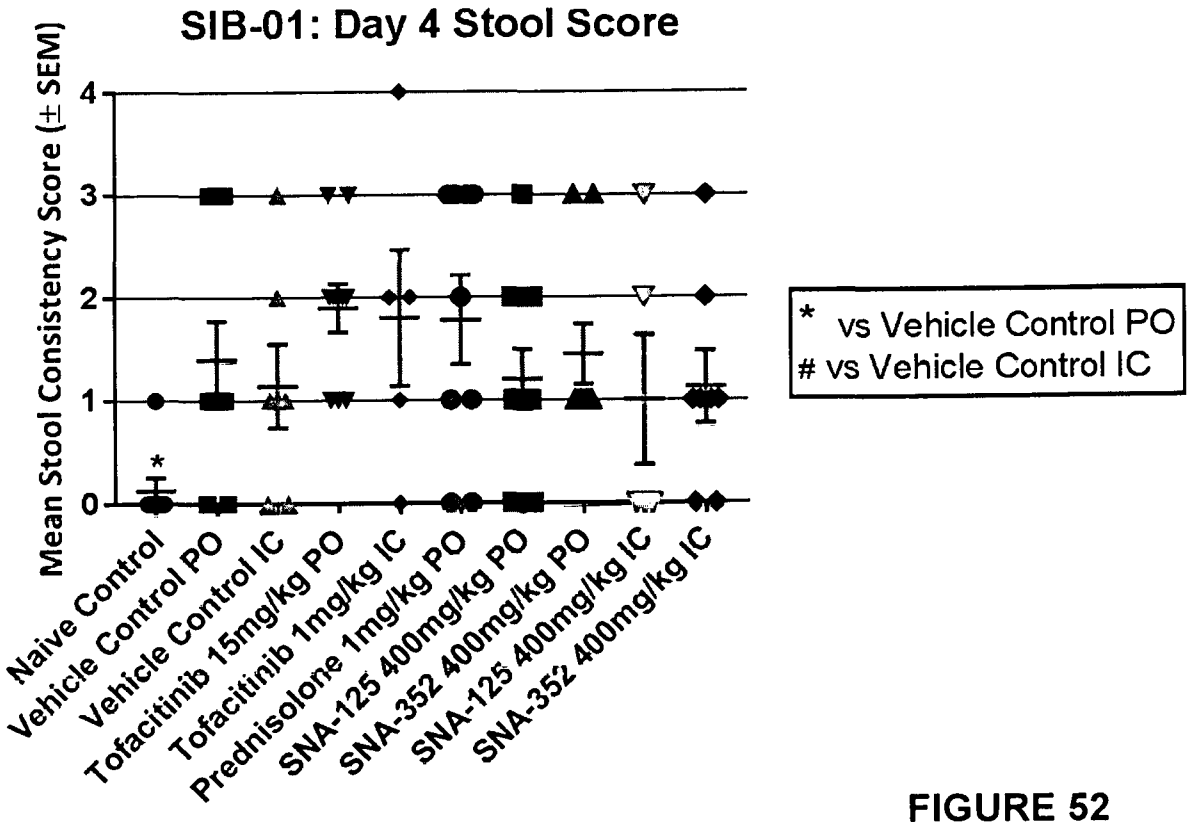
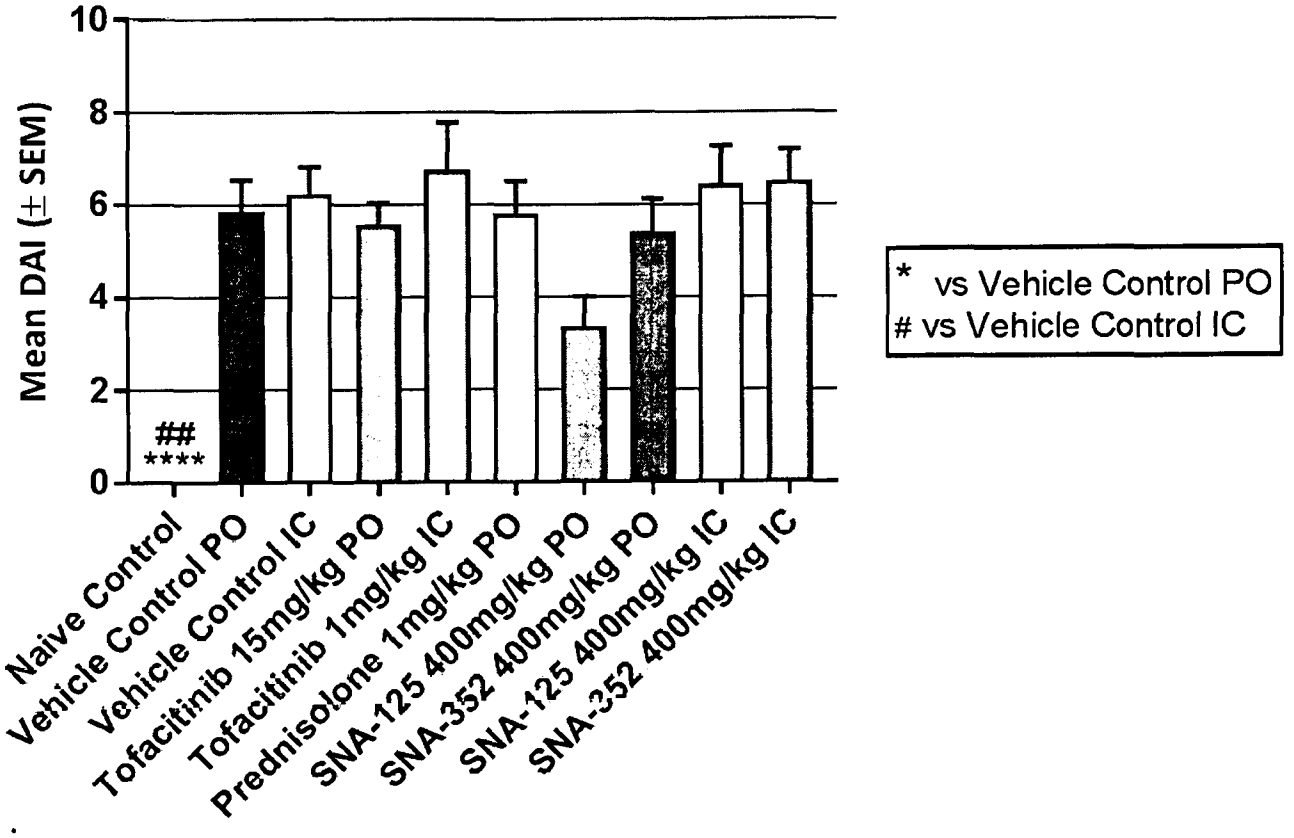

SIB-01: Day 4 Stool Score
Mean Stool Consistency Score (± SEM)
0
1
2
3
4
*
Naive Control
Vehicle Control PO
Vehicle Control IC
Tofacitinib 15mg/kg PO
Tofacitinib 1mg/kg IC
Prednisolone 1mg/kg PO
SNA-125 400mg/kg PO
SNA-352 400mg/kg PO
SNA-125 400mg/kg IC
SNA-352 400mg/kg IC
* vs Vehicle Control PO
# vs Vehicle Control IC

FIGURE 52

A


Mean DAI (± SEM)
10
8
6
4
2
0
##
****
Naive Control
Vehicle Control PO
Vehicle Control IC
Tofacitinib 15mg/kg PO
Tofacitinib 1mg/kg IC
Prednisolone 1mg/kg PO
SNA-125 400mg/kg PO
SNA-352 400mg/kg PO
SNA-125 400mg/kg IC
SNA-352 400mg/kg IC
* vs Vehicle Control PO
# vs Vehicle Control IC

B

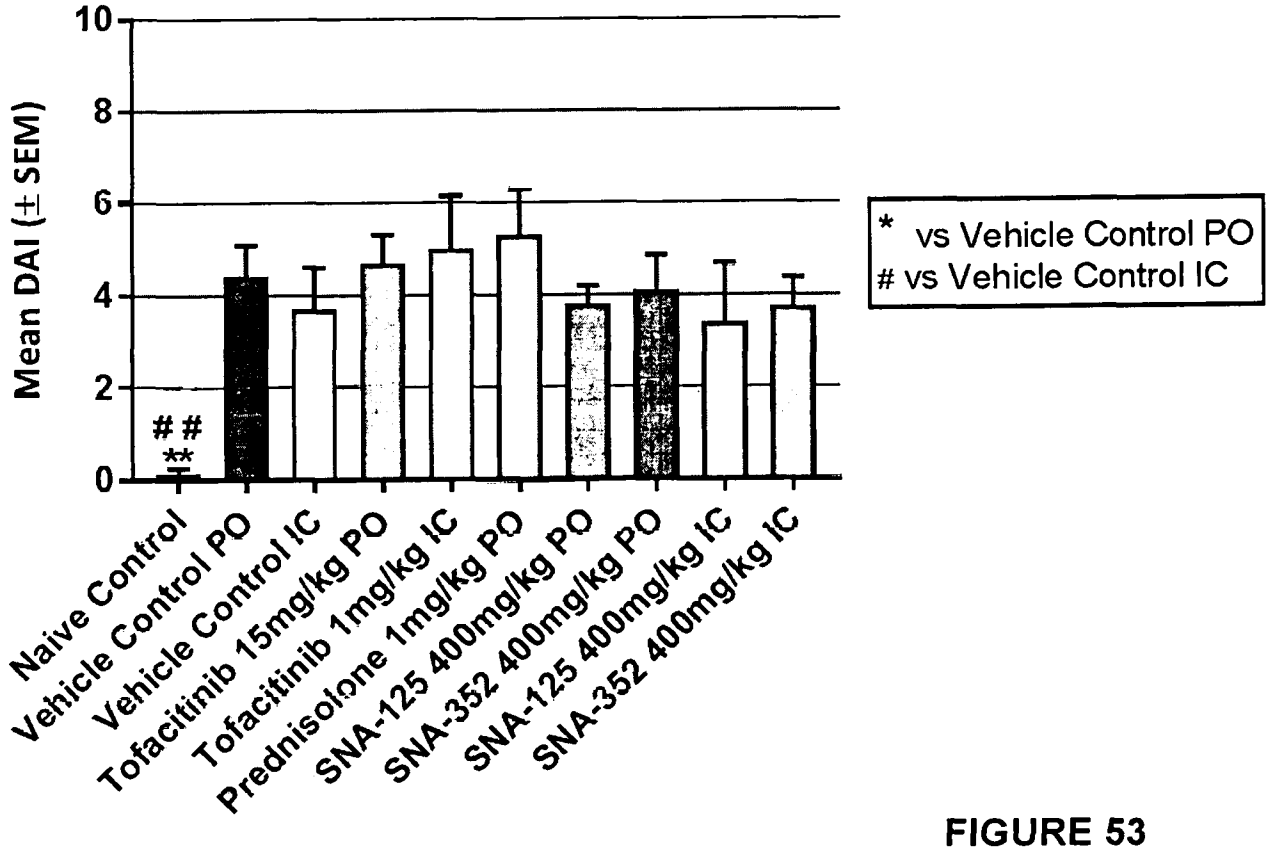

Mean DAI (± SEM)
10
8
6
4
2
0
##
**
Naive Control
Vehicle Control PO
Vehicle Control IC
Tofacitinib 15mg/kg PO
Tofacitinib 1mg/kg IC
Prednisolone 1mg/kg PO
SNA-125 400mg/kg PO
SNA-352 400mg/kg PO
SNA-125 400mg/kg IC
SNA-352 400mg/kg IC
* vs Vehicle Control PO
# vs Vehicle Control IC

FIGURE 53

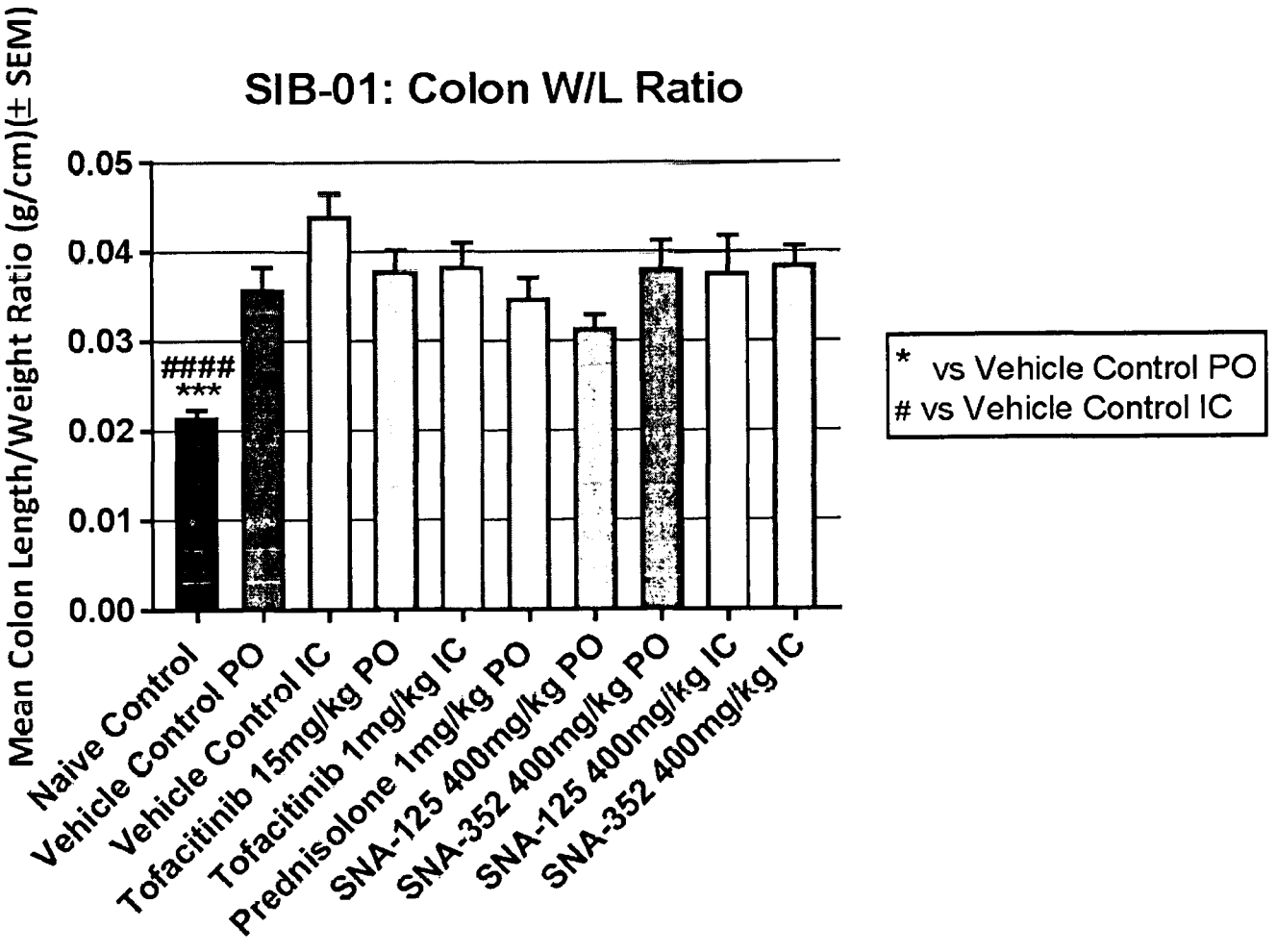

SIB-01: Colon W/L Ratio
Mean Colon Length/Weight Ratio (g/cm)(± SEM)
0.05
0.04
0.03
0.02
0.01
0.00
####
***
Naive Control
Vehicle Control PO
Vehicle Control IC
Tofacitinib 15mg/kg PO
Tofacitinib 1mg/kg IC
Prednisolone 1mg/kg PO
SNA-125 400mg/kg PO
SNA-352 400mg/kg PO
SNA-125 400mg/kg IC
SNA-352 400mg/kg IC
* vs Vehicle Control PO
# vs Vehicle Control IC

FIGURE 54

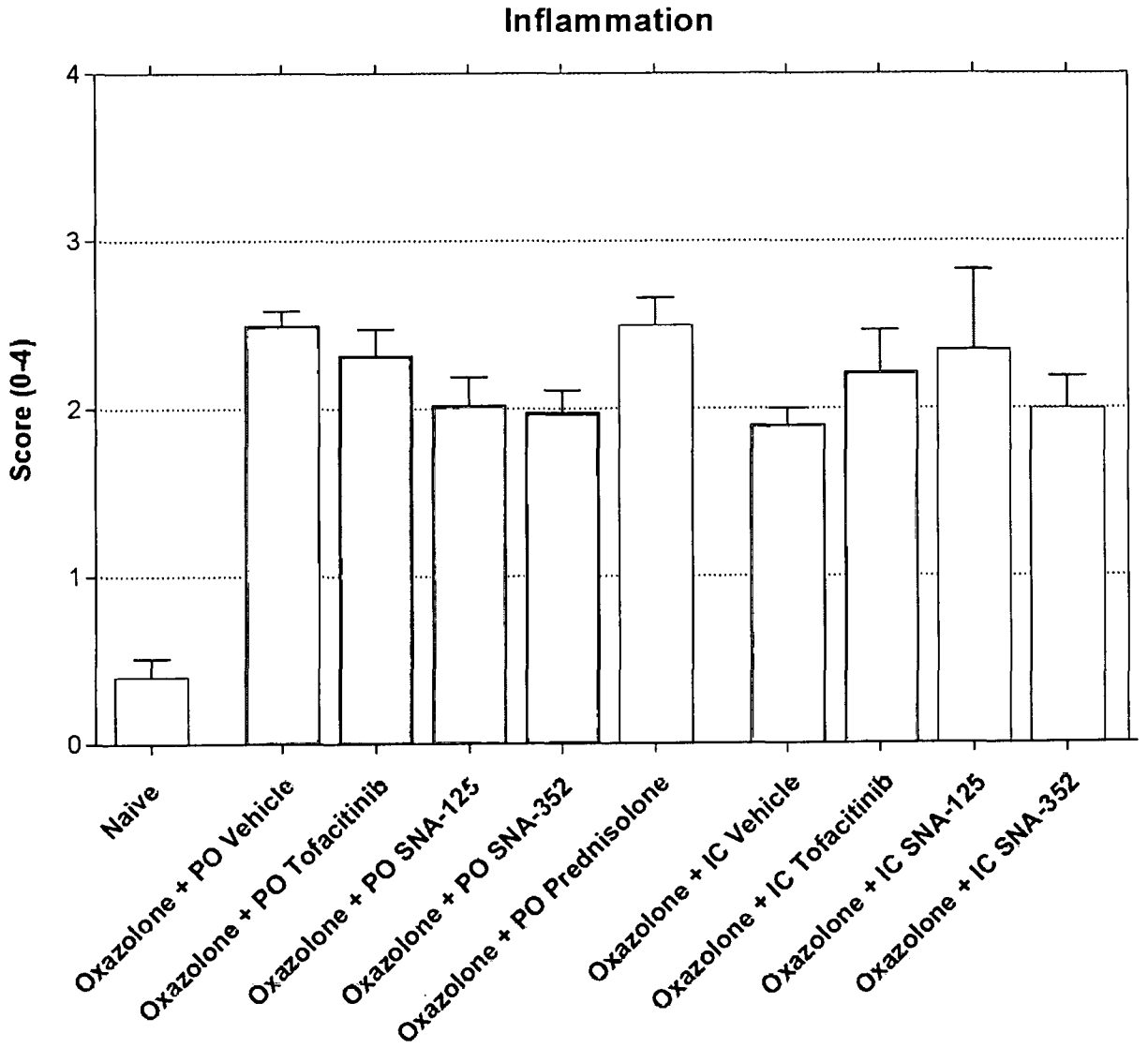

Inflammation
Score (0-4)
4
3
2
1
0
Naive
Oxazolone + PO Vehicle
Oxazolone + PO Tofacitinib
Oxazolone + PO SNA-125
Oxazolone + PO SNA-352
Oxazolone + PO Prednisolone
Oxazolone + IC Vehicle
Oxazolone + IC Tofacitinib
Oxazolone + IC SNA-125
Oxazolone + IC SNA-352

FIGURE 55

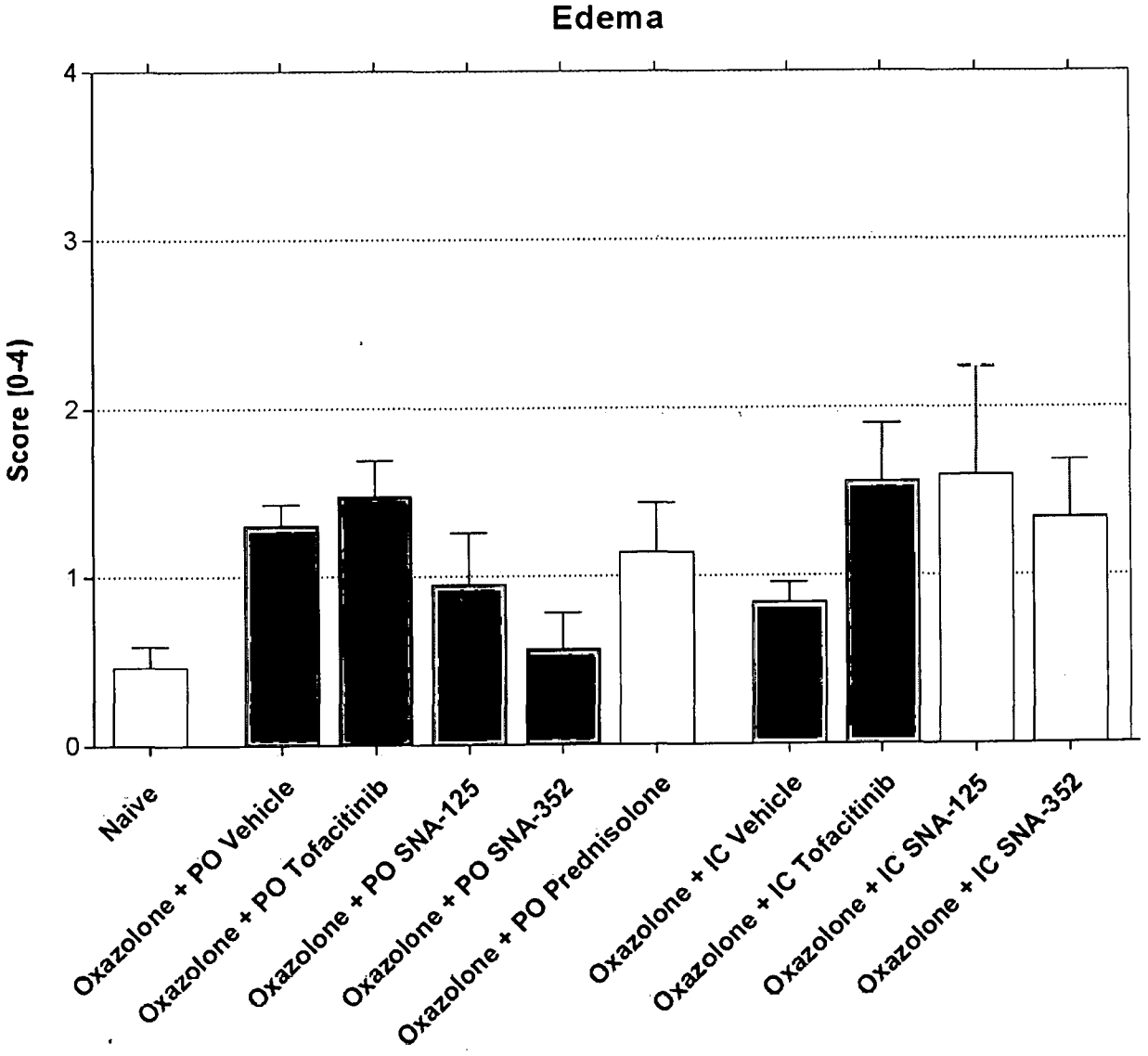

Edema
Score [0-4]
4
3
2
1
0
Naive
Oxazolone + PO Vehicle
Oxazolone + PO Tofacitinib
Oxazolone + PO SNA-125
Oxazolone + PO SNA-352
Oxazolone + PO Prednisolone
Oxazolone + IC Vehicle
Oxazolone + IC Tofacitinib
Oxazolone + IC SNA-125
Oxazolone + IC SNA-352

FIGURE 56

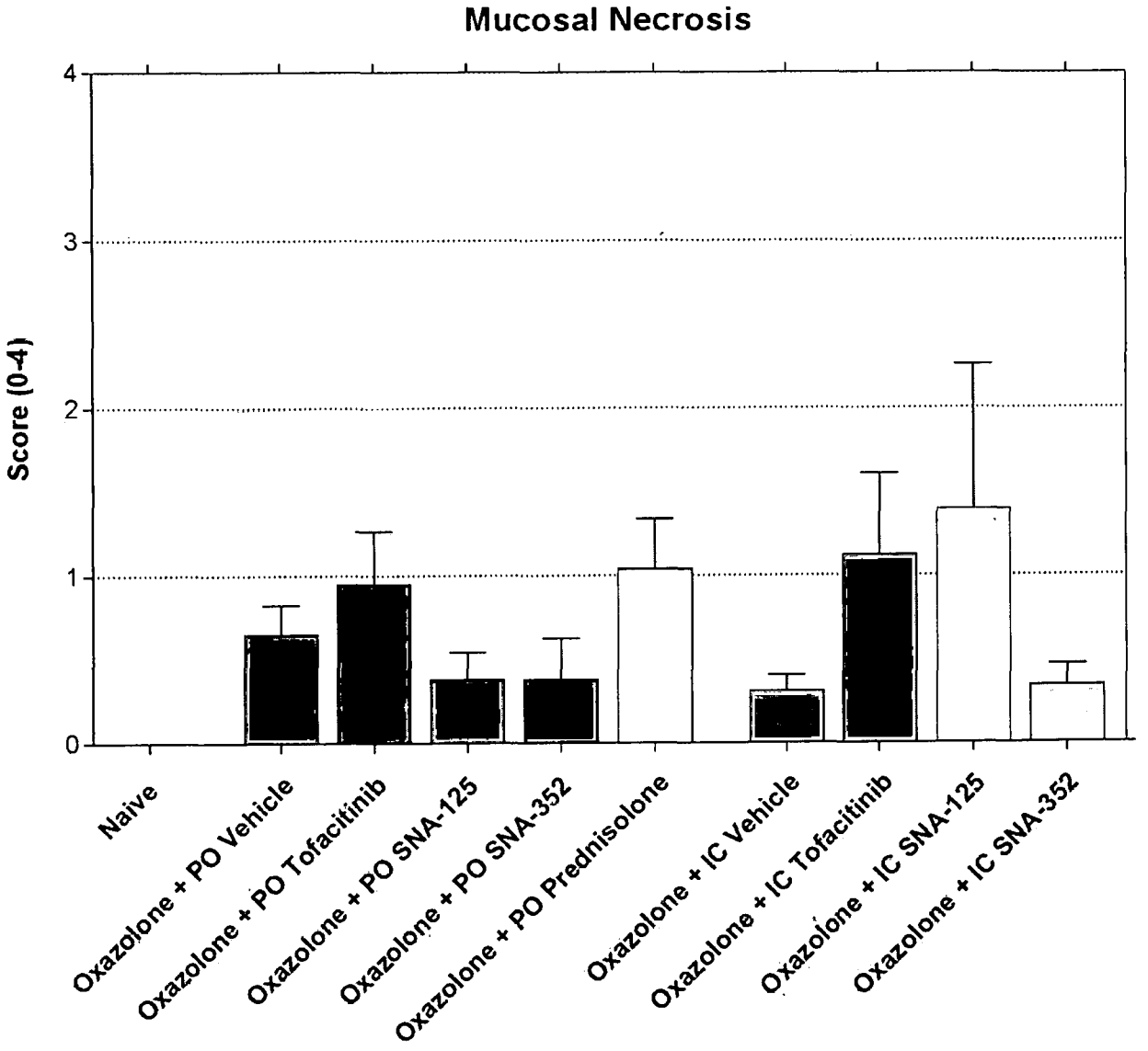

Mucosal Necrosis
Score (0-4)
0
1
2
3
4
Naive
Oxazolone + PO Vehicle
Oxazolone + PO Tofacitinib
Oxazolone + PO SNA-125
Oxazolone + PO SNA-352
Oxazolone + PO Prednisolone
Oxazolone + IC Vehicle
Oxazolone + IC Tofacitinib
Oxazolone + IC SNA-125
Oxazolone + IC SNA-352

FIGURE 57

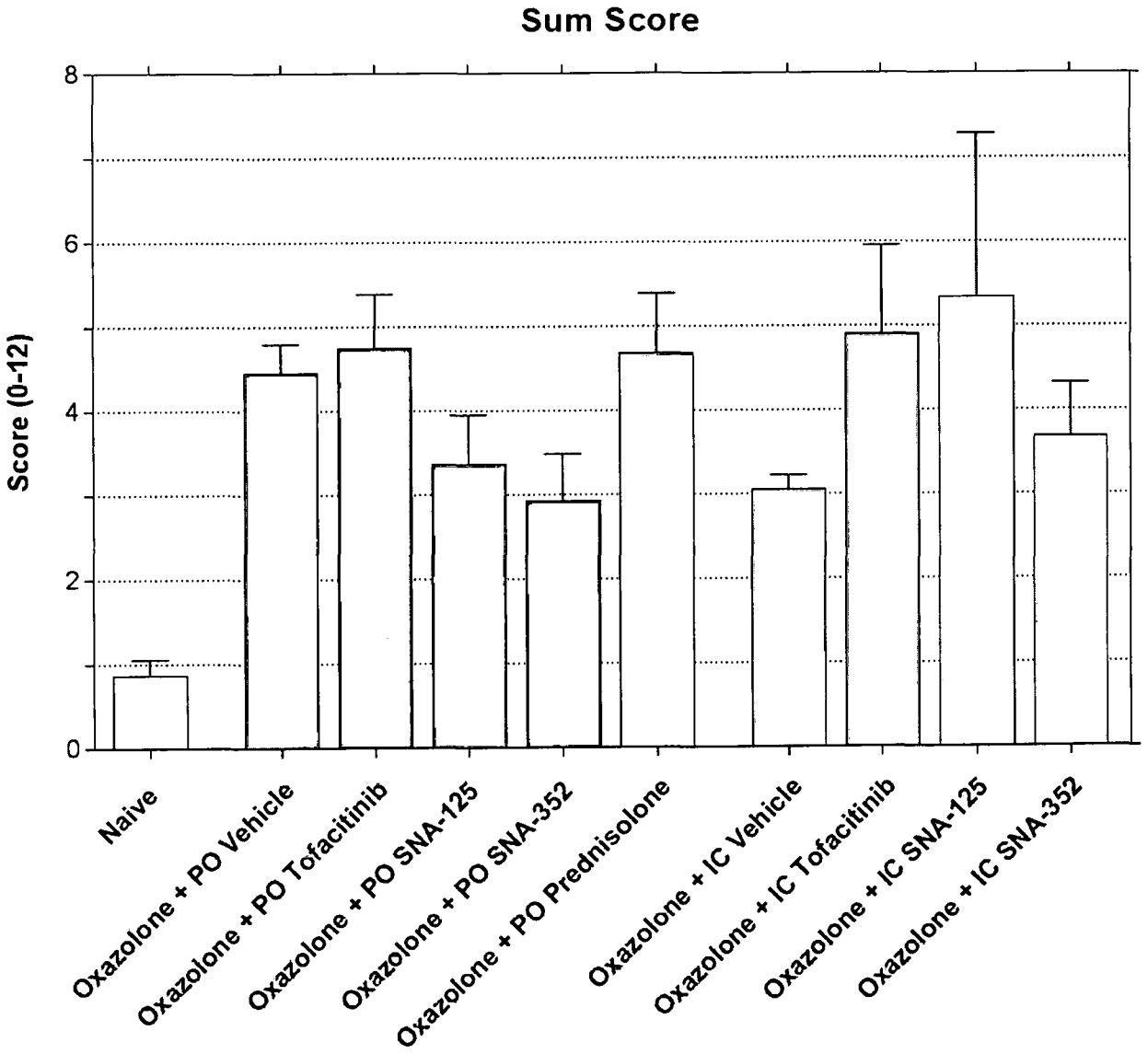

Sum Score
Score (0-12)
0
2
4
6
8
Naive
Oxazolone + PO Vehicle
Oxazolone + PO Tofacitinib
Oxazolone + PO SNA-125
Oxazolone + PO SNA-352
Oxazolone + PO Prednisolone
Oxazolone + IC Vehicle
Oxazolone + IC Tofacitinib
Oxazolone + IC SNA-125
Oxazolone + IC SNA-352

FIGURE 58

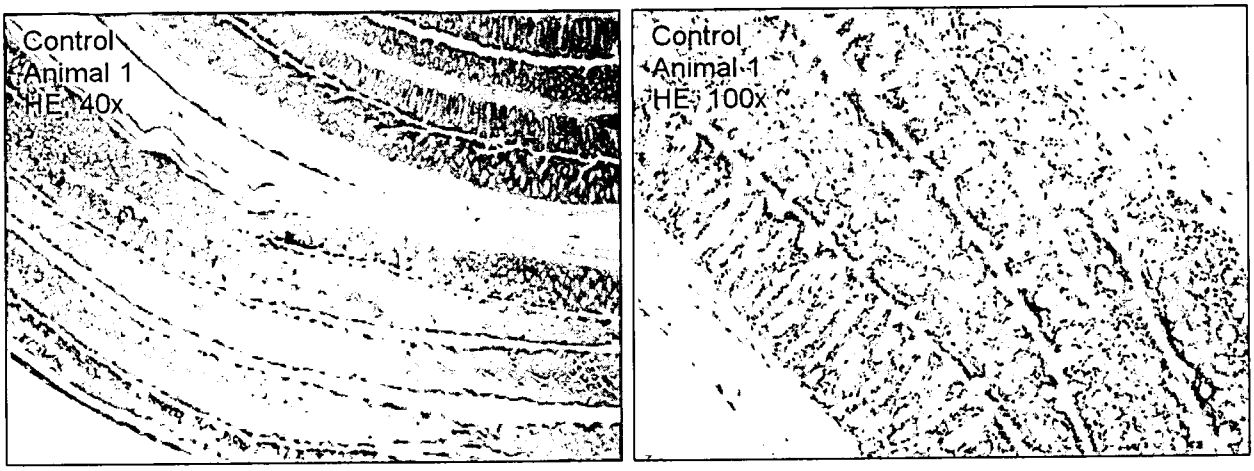
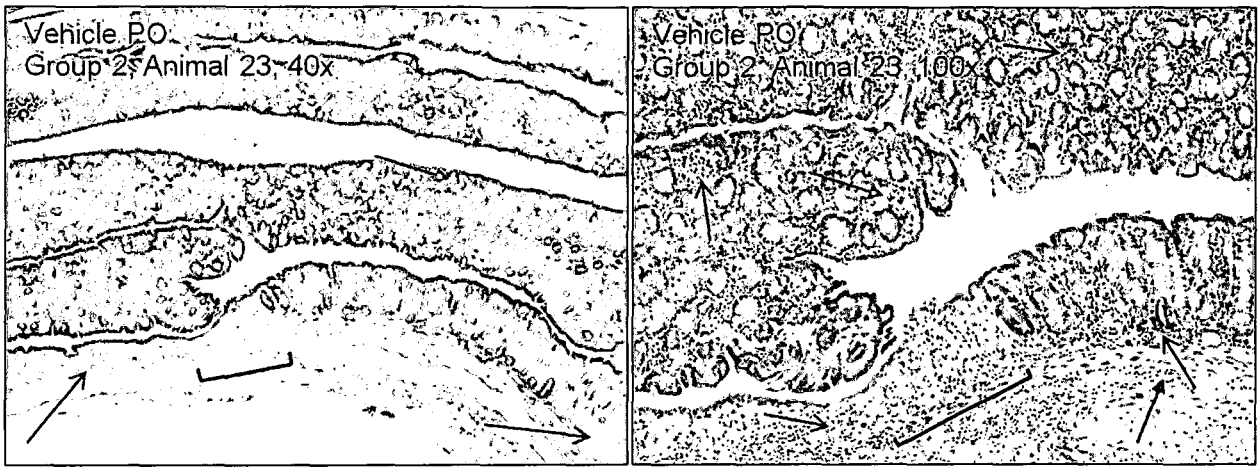


FIGURE 59

A



B

C

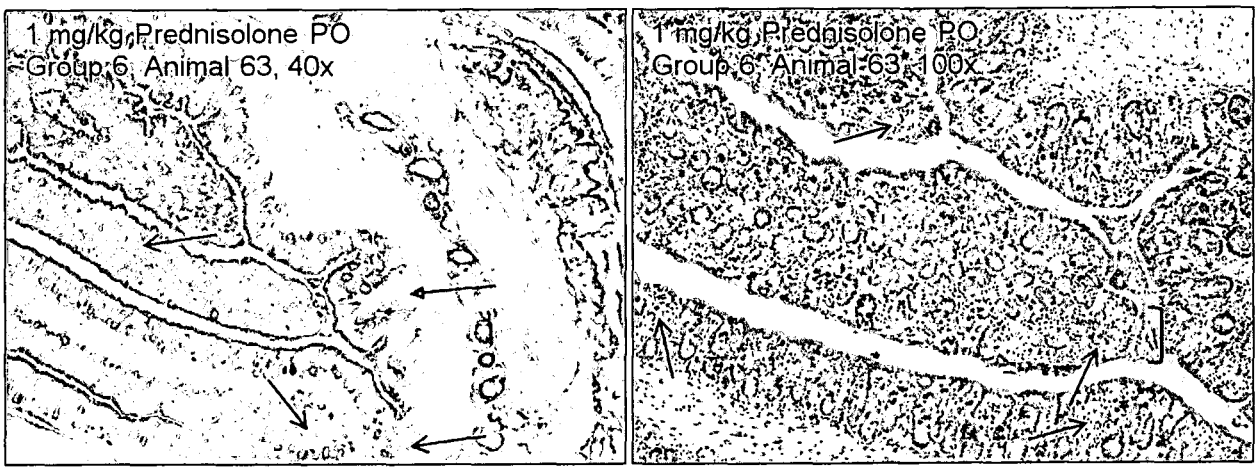
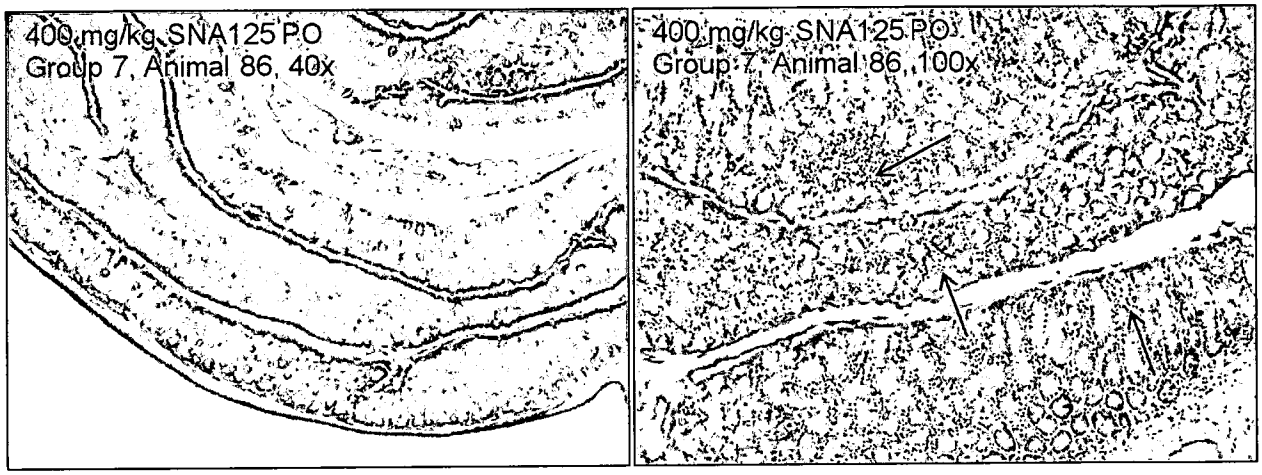


FIGURE 60

D


400 mg/kg SNA125 PO
Group 7, Animal 86, 40x
400 mg/kg SNA125 PO
Group 7, Animal 86, 100x

E

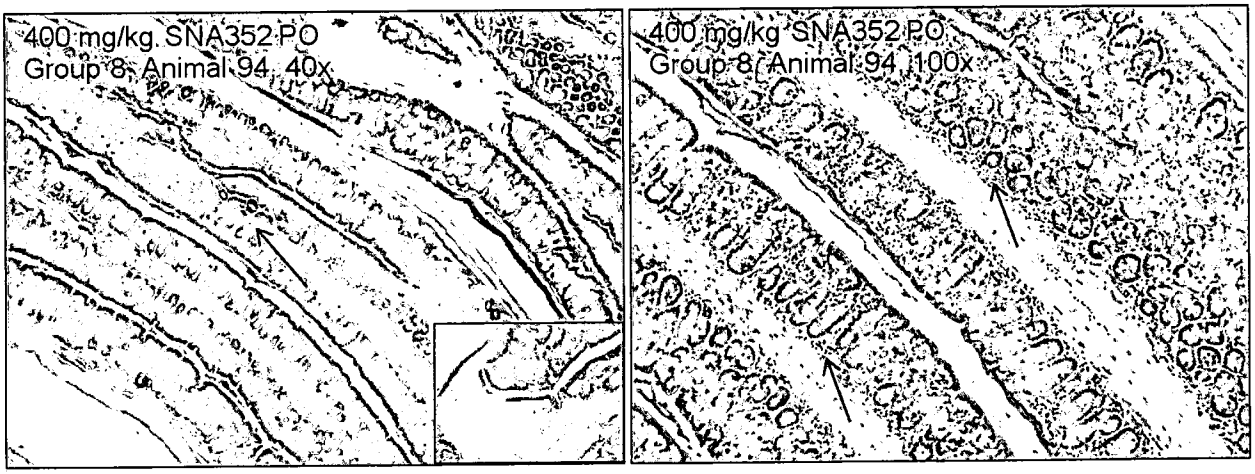
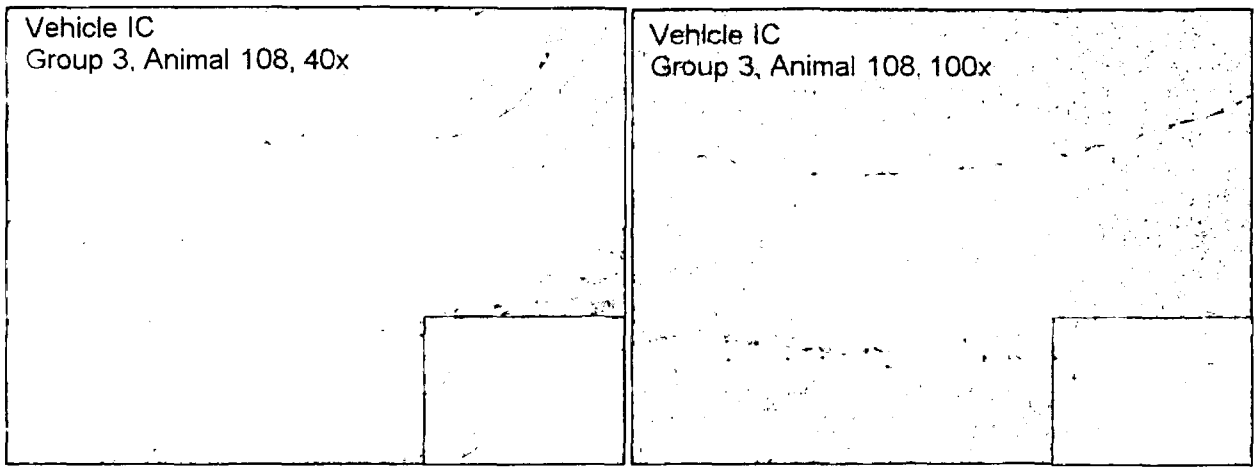
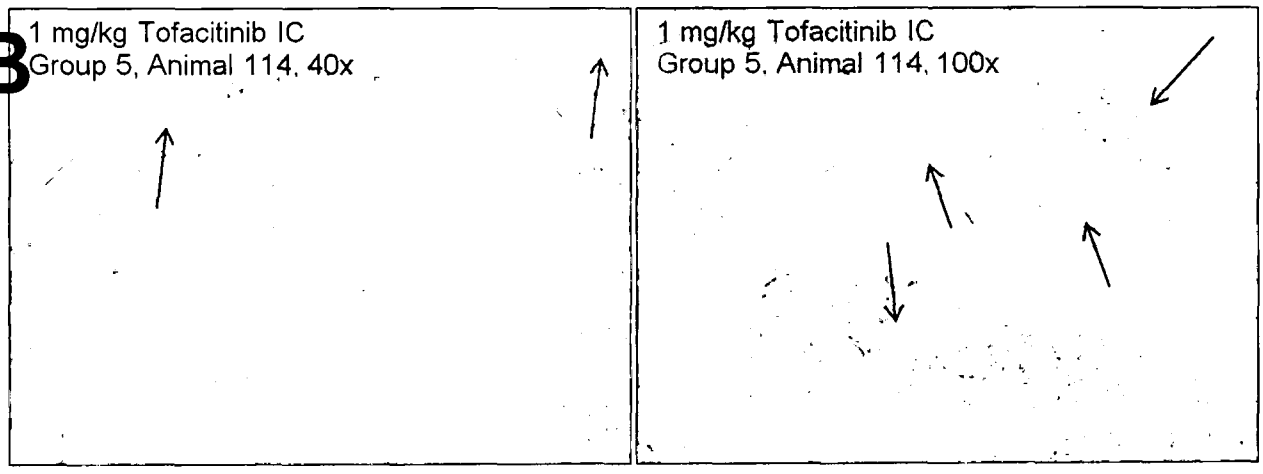

400 mg/kg SNA352 PO
Group 8, Animal 94, 40x
400 mg/kg SNA352 PO
Group 8, Animal 94, 100x

FIGURE 60

A



B



C

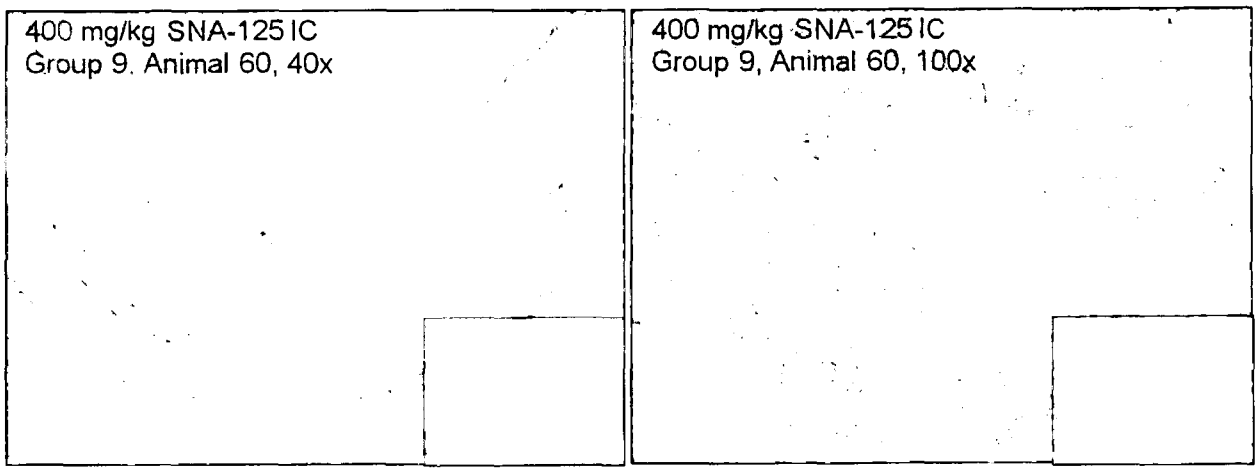


FIGURE 61

D

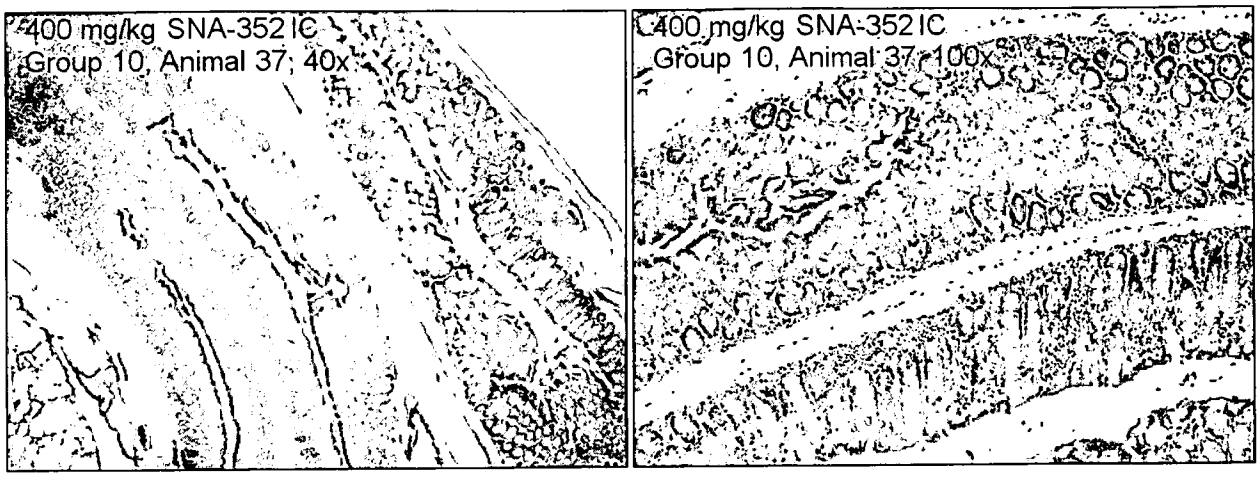

400 mg/kg SNA-352 IC
Group 10, Animal 37, 40x
400 mg/kg SNA-352 IC
Group 10, Animal 37, 100x

FIGURE 61

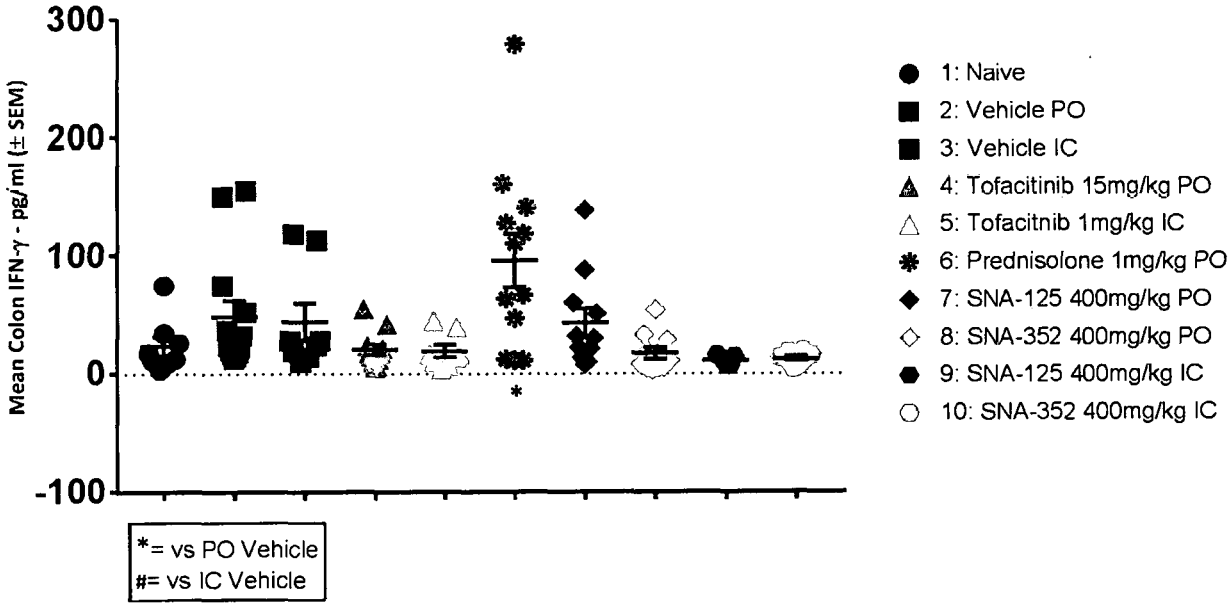
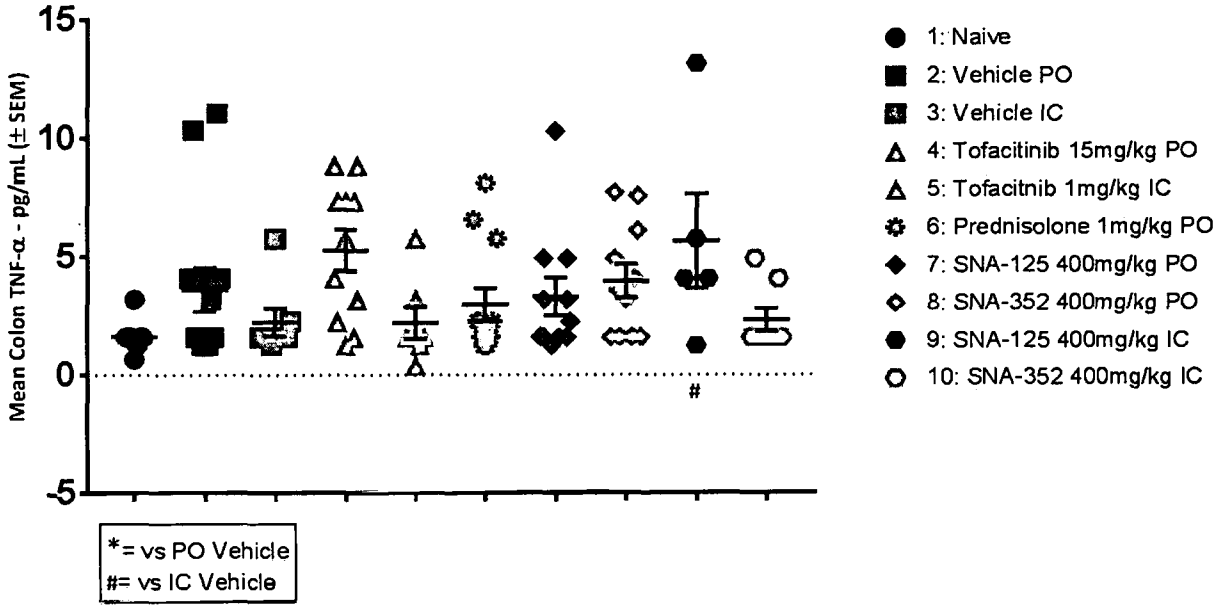

300
200
100
0
-100
Mean Colon IFN-γ - pg/ml (± SEM)
*= vs PO Vehicle
#= vs IC Vehicle
1: Naive
2: Vehicle PO
3: Vehicle IC
4: Tofacitinib 15mg/kg PO
5: Tofacitnib 1mg/kg IC
6: Prednisolone 1mg/kg PO
7: SNA-125 400mg/kg PO
8: SNA-352 400mg/kg PO
9: SNA-125 400mg/kg IC
10: SNA-352 400mg/kg IC

FIGURE 62

A


15
10
5
0
-5
Mean Colon TNF-α - pg/mL (± SEM)
#
*= vs PO Vehicle
#= vs IC Vehicle
1: Naive
2: Vehicle PO
3: Vehicle IC
4: Tofacitinib 15mg/kg PO
5: Tofacitnib 1mg/kg IC
6: Prednisolone 1mg/kg PO
7: SNA-125 400mg/kg PO
8: SNA-352 400mg/kg PO
9: SNA-125 400mg/kg IC
10: SNA-352 400mg/kg IC

B

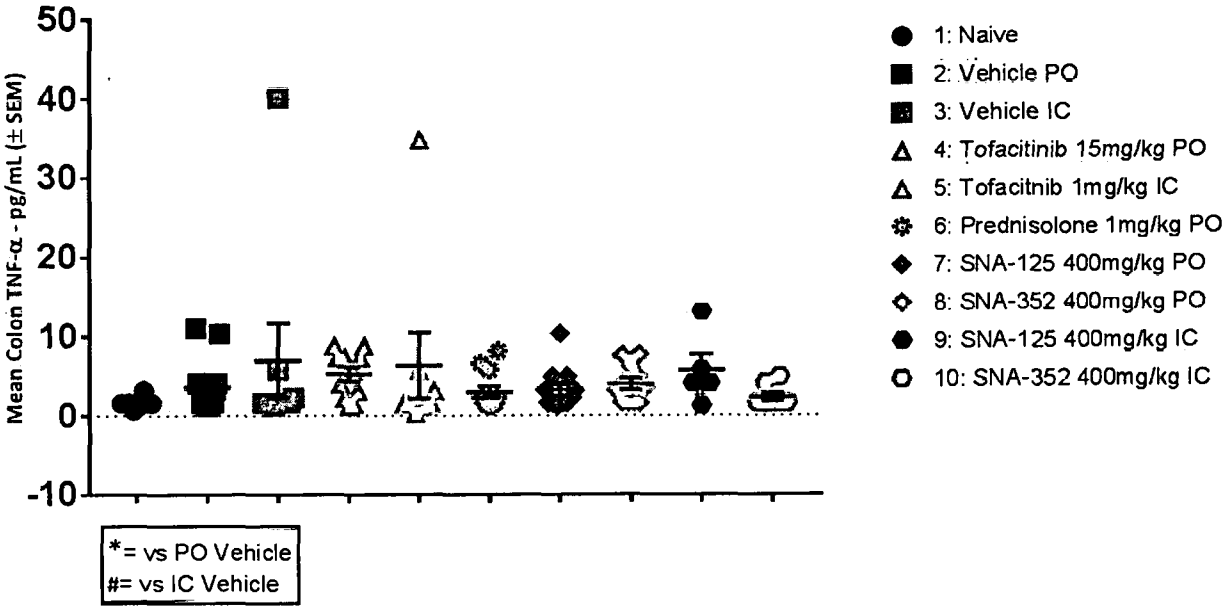
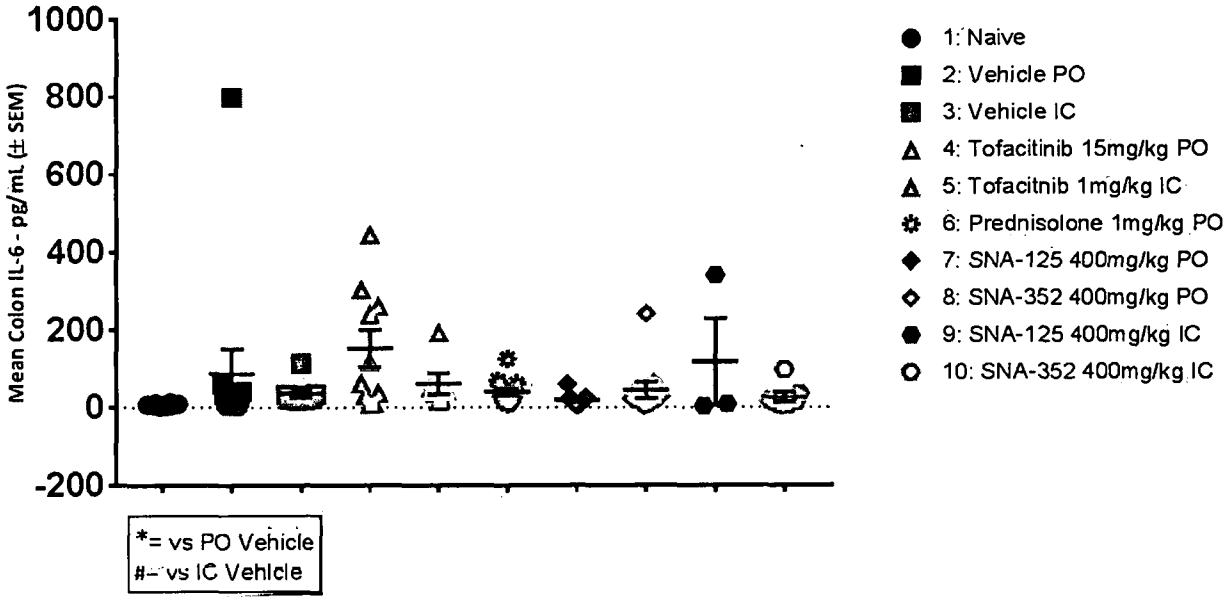

50
40
30
20
10
0
-10
Mean Colon TNF-α - pg/mL (± SEM)
*= vs PO Vehicle
#= vs IC Vehicle
1: Naive
2: Vehicle PO
3: Vehicle IC
4: Tofacitinib 15mg/kg PO
5: Tofacitnib 1mg/kg IC
6: Prednisolone 1mg/kg PO
7: SNA-125 400mg/kg PO
8: SNA-352 400mg/kg PO
9: SNA-125 400mg/kg IC
10: SNA-352 400mg/kg IC

FIGURE 63

A


1000
800
600
400
200
0
-200
Mean Colon IL-6 - pg/mL (± SEM)
*= vs PO Vehicle
#= vs IC Vehicle
1: Naive
2: Vehicle PO
3: Vehicle IC
4: Tofacitinib 15mg/kg PO
5: Tofacitnib 1mg/kg IC
6: Prednisolone 1mg/kg PO
7: SNA-125 400mg/kg PO
8: SNA-352 400mg/kg PO
9: SNA-125 400mg/kg IC
10: SNA-352 400mg/kg IC

B

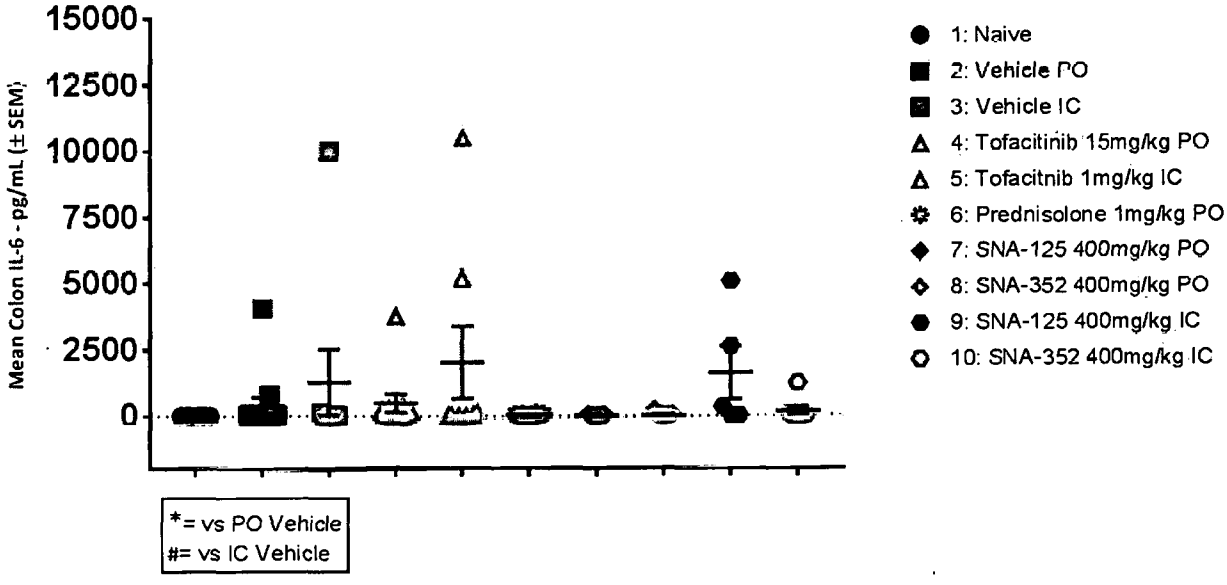
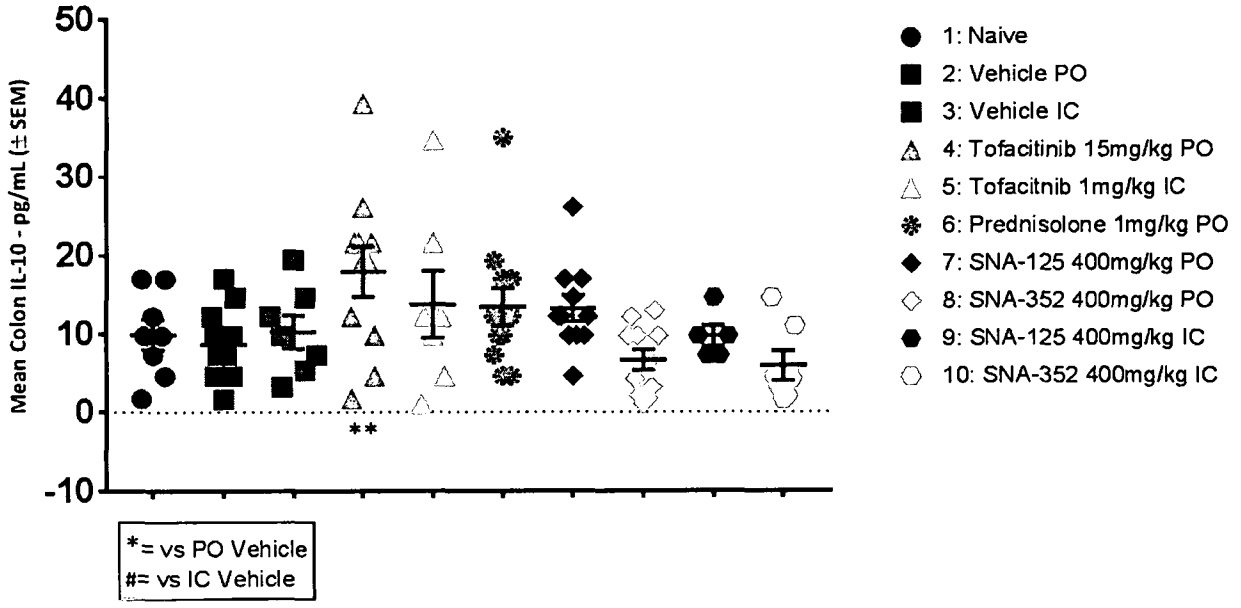

15000
12500
10000
7500
5000
2500
0
Mean Colon IL-6 - pg/mL (± SEM)
*= vs PO Vehicle
#= vs IC Vehicle
1: Naive
2: Vehicle PO
3: Vehicle IC
4: Tofacitinib 15mg/kg PO
5: Tofacitnib 1mg/kg IC
6: Prednisolone 1mg/kg PO
7: SNA-125 400mg/kg PO
8: SNA-352 400mg/kg PO
9: SNA-125 400mg/kg IC
10: SNA-352 400mg/kg IC

FIGURE 64

A


Mean Colon IL-10 - pg/mL (± SEM)
50
40
30
20
10
0
-10
**
*= vs PO Vehicle
#= vs IC Vehicle
1: Naive
2: Vehicle PO
3: Vehicle IC
4: Tofacitinib 15mg/kg PO
5: Tofacitnib 1mg/kg IC
6: Prednisolone 1mg/kg PO
7: SNA-125 400mg/kg PO
8: SNA-352 400mg/kg PO
9: SNA-125 400mg/kg IC
10: SNA-352 400mg/kg IC

B

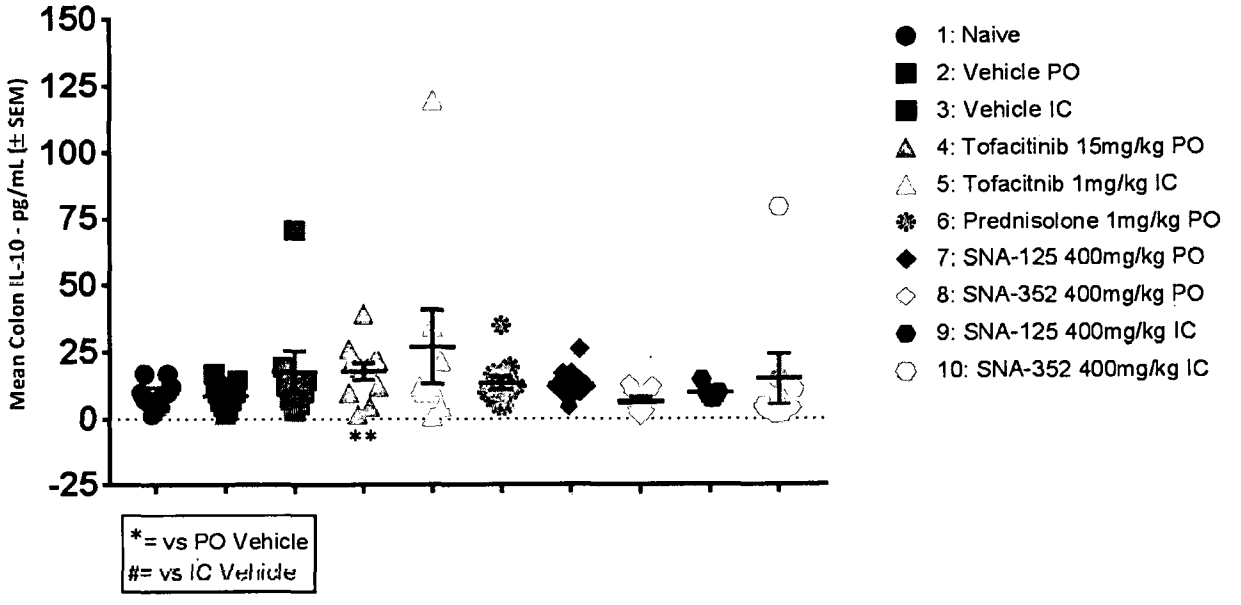

Mean Colon IL-10 - pg/mL (± SEM)
150
125
100
75
50
25
0
-25
**
*= vs PO Vehicle
#= vs IC Vehicle
1: Naive
2: Vehicle PO
3: Vehicle IC
4: Tofacitinib 15mg/kg PO
5: Tofacitnib 1mg/kg IC
6: Prednisolone 1mg/kg PO
7: SNA-125 400mg/kg PO
8: SNA-352 400mg/kg PO
9: SNA-125 400mg/kg IC
10: SNA-352 400mg/kg IC

FIGURE 65

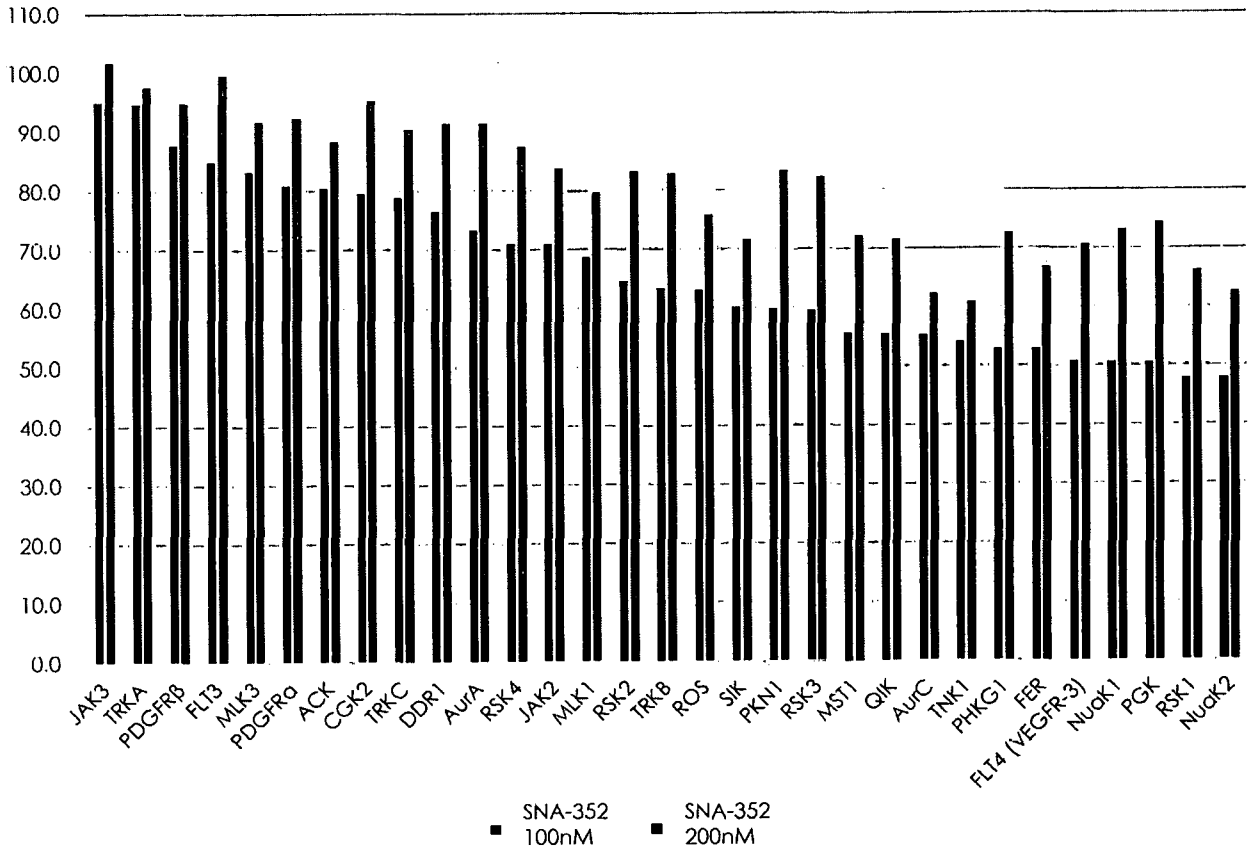
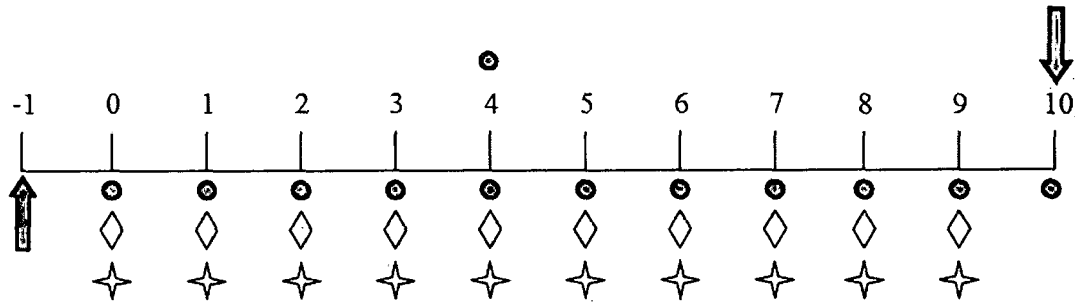

110.0
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
JAK3
TRKA
PDGFRβ
FLT3
MLK3
PDGFRα
ACK
CGK2
TRKC
DDR1
AurA
RSK4
JAK2
MLK1
RSK2
TRKB
ROS
SIK
PKN1
RSK3
MST1
QIK
AurC
TNK1
PHKG1
FER
FLT4 (VEGFR-3)
NuaK1
PGK
RSK1
NuaK2
SNA-352 100nM
SNA-352 200nM

FIGURE 66



Animal shaving

Treatment:
Test Items administered

IMQ Cream Application:
Daily topical application of IMQ on shaved backs and onto ears.

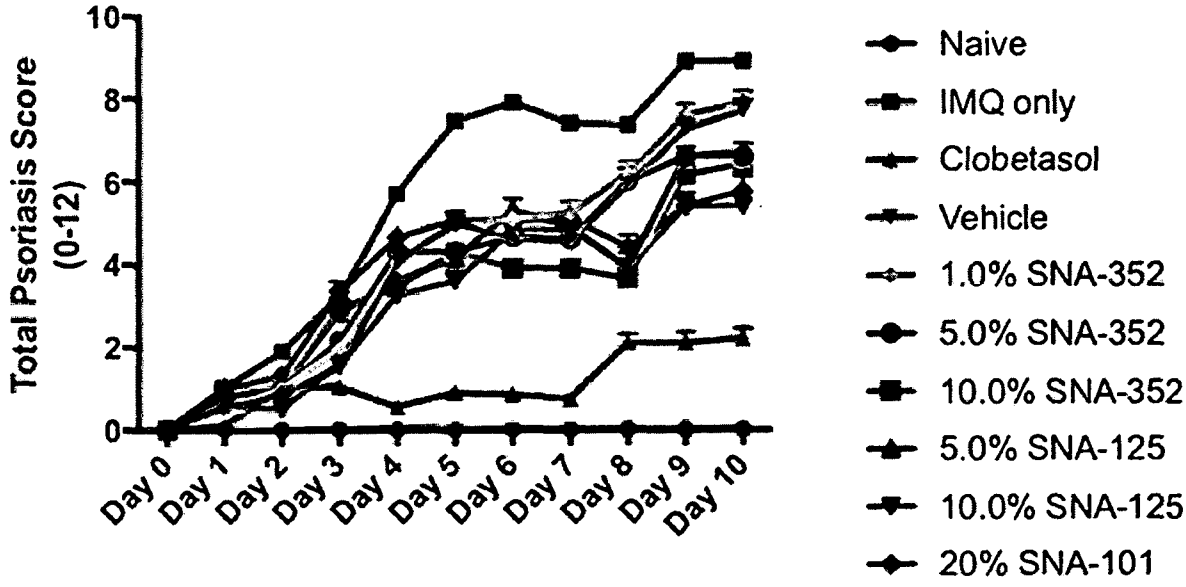
Left Ear Biopsy Punch

Body weight, Ear thickness, and Psoriasis clinical score
- Ear thickness only days 0, 4, 6, 8, and 10.

Termination:
Animals will be terminated on Day 10. Spleen and back skin will be collected.

FIGURE 67

A


Total Psoriasis Score (0-12)
10
8
6
4
2
0
Day 0
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
Day 8
Day 9
Day 10
Naive
IMQ only
Clobetasol
Vehicle
1.0% SNA-352
5.0% SNA-352
10.0% SNA-352
5.0% SNA-125
10.0% SNA-125
20% SNA-101

B

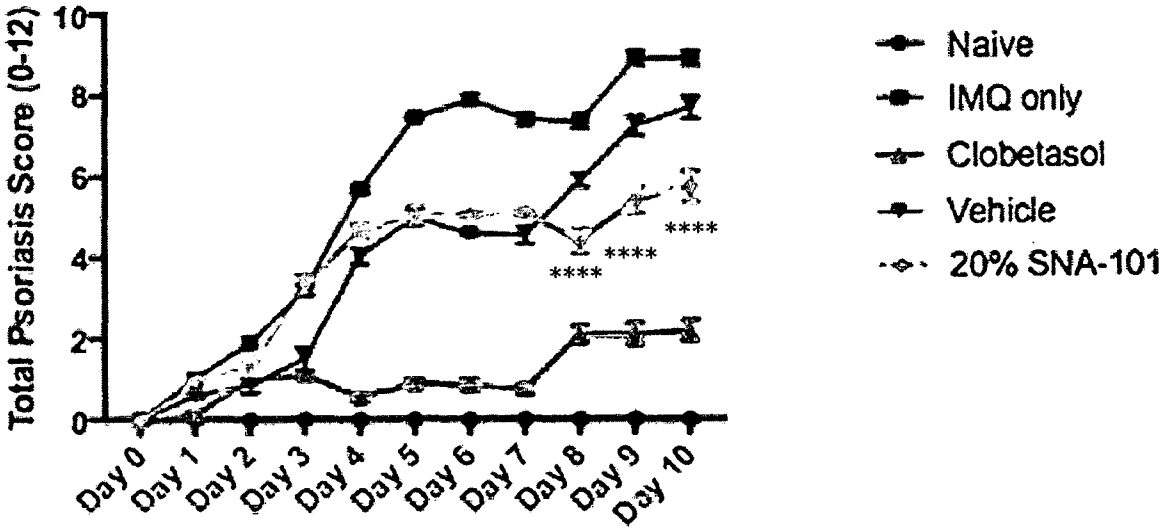
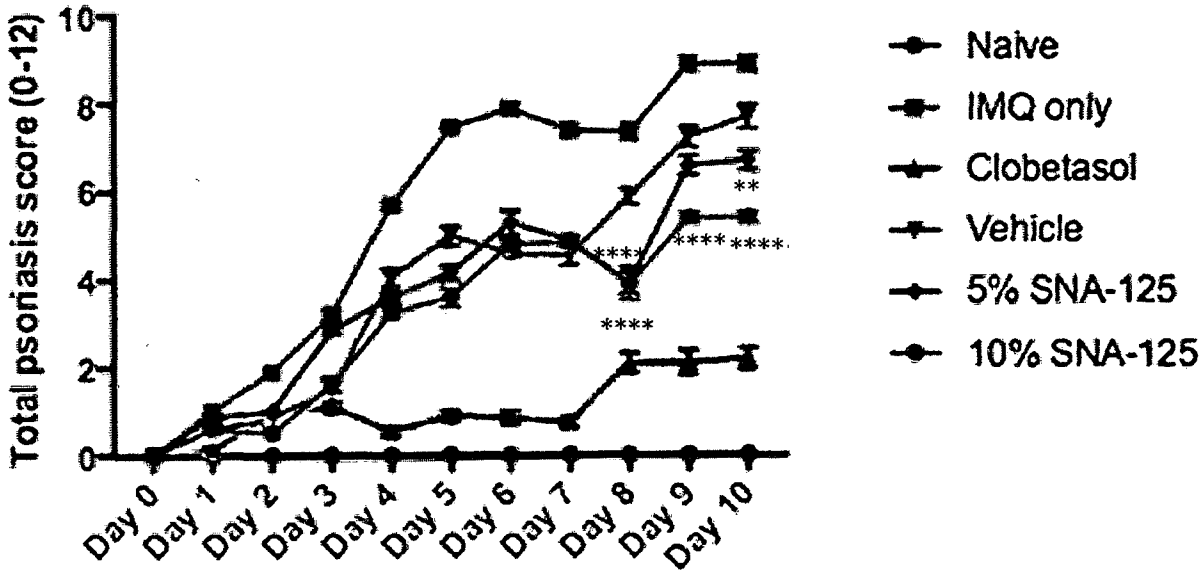

Total Psoriasis Score (0-12)
10
8
6
4
2
0
Day 0
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
Day 8
Day 9
Day 10
****
****
****
Naive
IMQ only
Clobetasol
Vehicle
20% SNA-101

FIGURE 68

C


Total psoriasis score (0-12)
10
8
6
4
2
0
Day 0
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
Day 8
Day 9
Day 10
**
****
****
****
****
Naive
IMQ only
Clobetasol
Vehicle
5% SNA-125
10% SNA-125

D

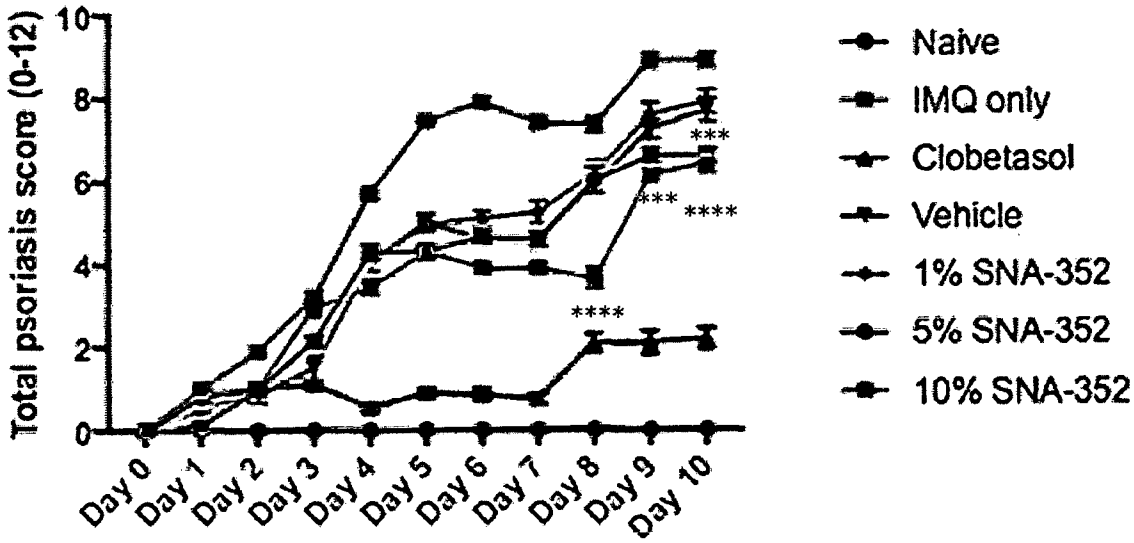
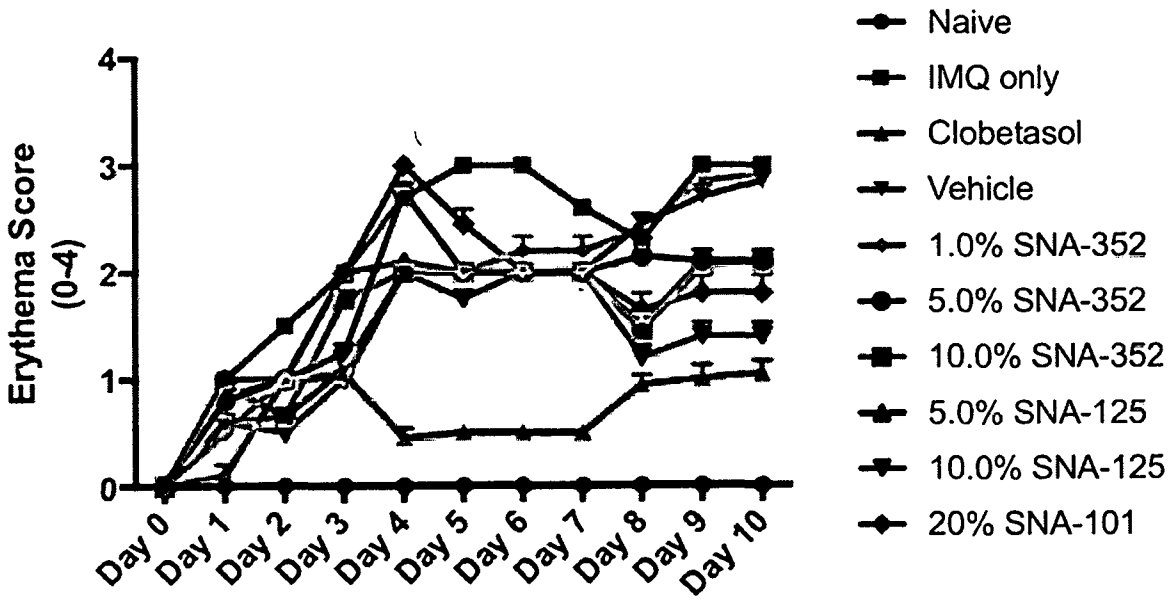

Total psoriasis score (0-12)
10
8
6
4
2
0
Day 0
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
Day 8
Day 9
Day 10
***
***
****
****
Naive
IMQ only
Clobetasol
Vehicle
1% SNA-352
5% SNA-352
10% SNA-352

FIGURE 68

A


Erythema Score (0-4)
0
1
2
3
4
Day 0
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
Day 8
Day 9
Day 10
Naive
IMQ only
Clobetasol
Vehicle
1.0% SNA-352
5.0% SNA-352
10.0% SNA-352
5.0% SNA-125
10.0% SNA-125
20% SNA-101

B

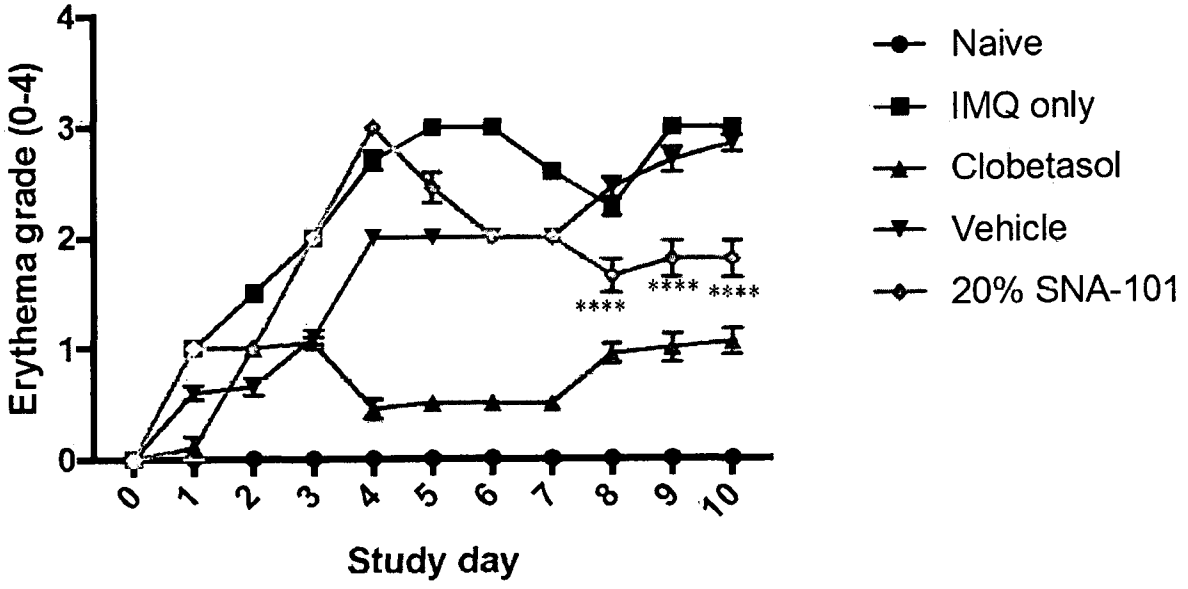
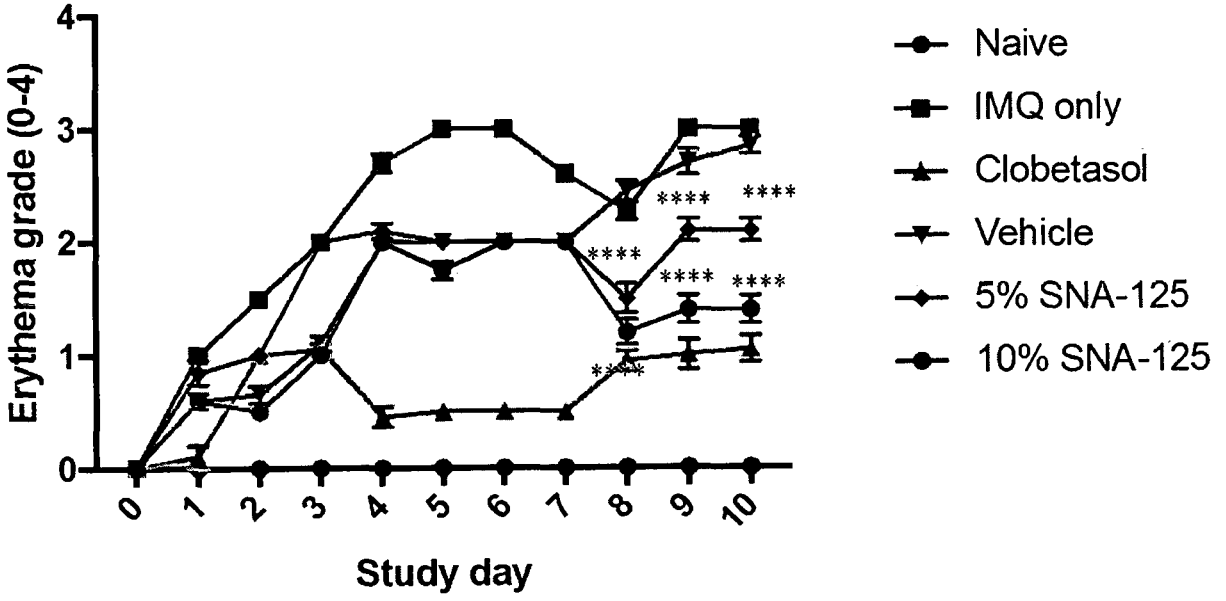

Erythema grade (0-4)
0
1
2
3
4
0 1 2 3 4 5 6 7 8 9 10
Study day
****
****
****
Naive
IMQ only
Clobetasol
Vehicle
20% SNA-101

FIGURE 69

C


Erythema grade (0-4)
Study day
Naive
IMQ only
Clobetasol
Vehicle
5% SNA-125
10% SNA-125

D

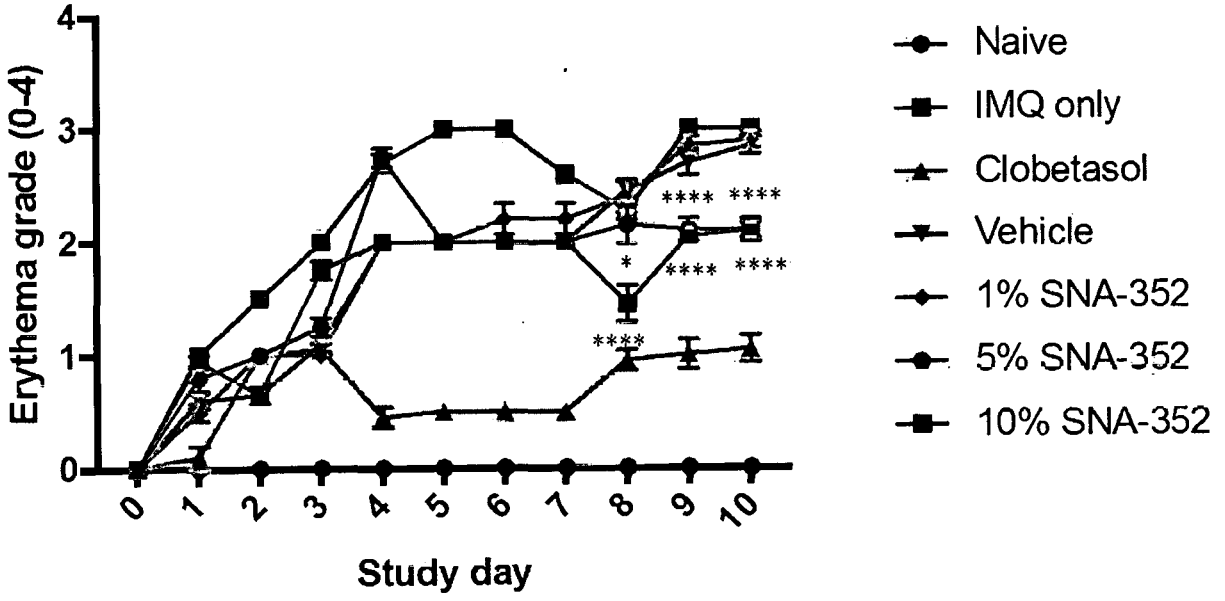
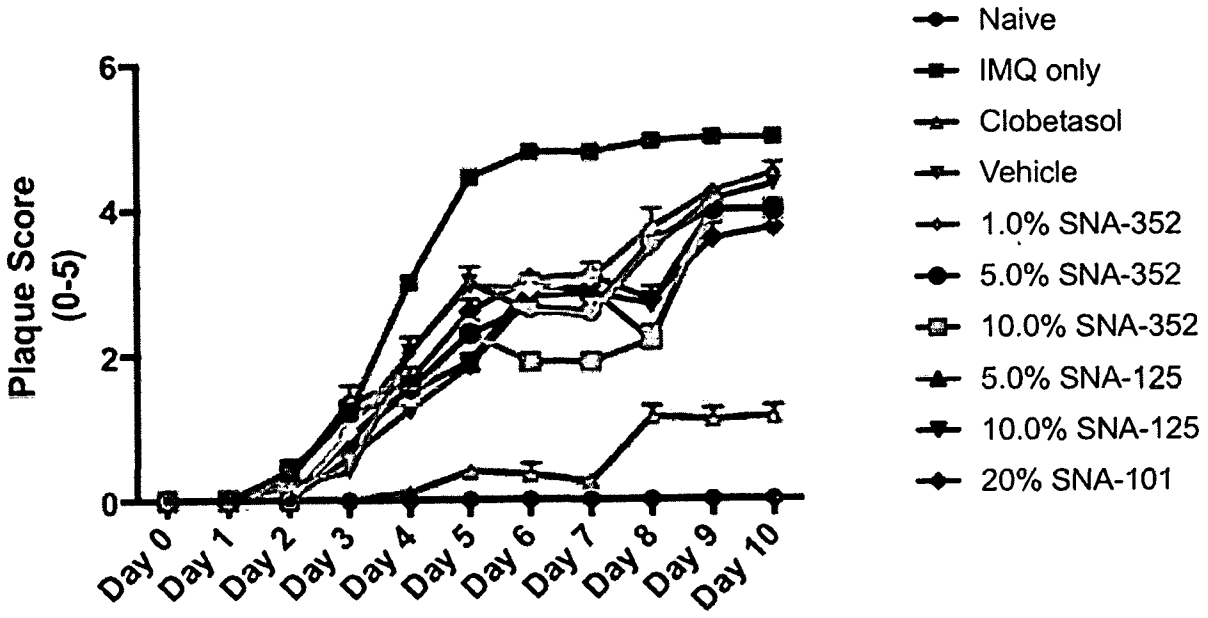

Erythema grade (0-4)
Study day
Naive
IMQ only
Clobetasol
Vehicle
1% SNA-352
5% SNA-352
10% SNA-352

FIGURE 69

A


Plaque Score (0-5)
0
2
4
6
Day 0
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
Day 8
Day 9
Day 10
Naive
IMQ only
Clobetasol
Vehicle
1.0% SNA-352
5.0% SNA-352
10.0% SNA-352
5.0% SNA-125
10.0% SNA-125
20% SNA-101

B

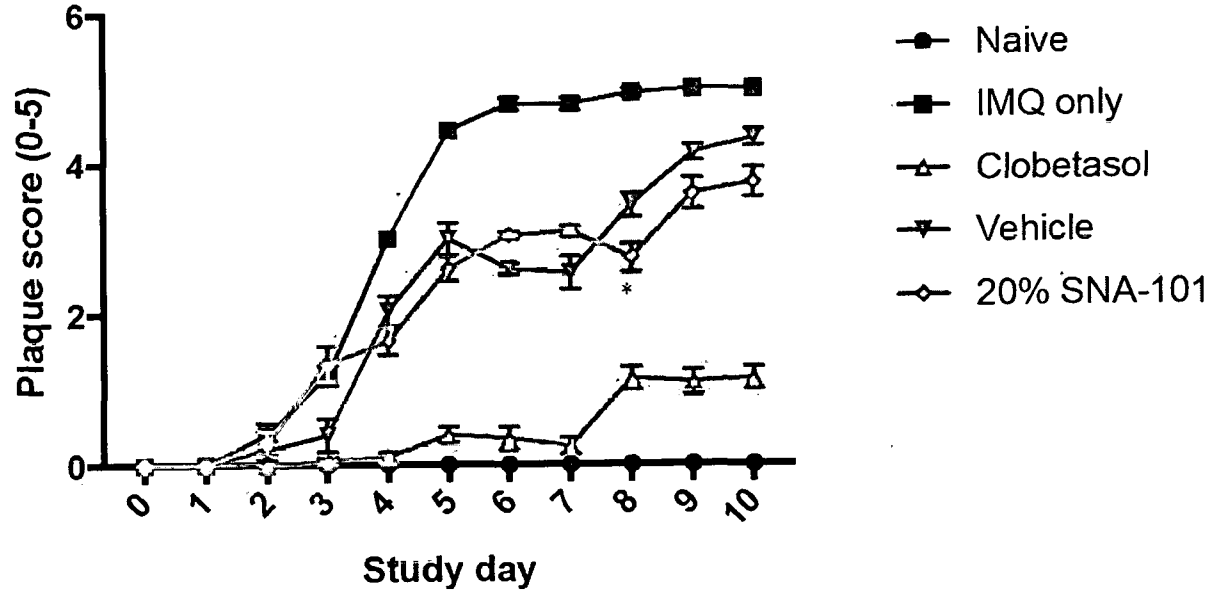
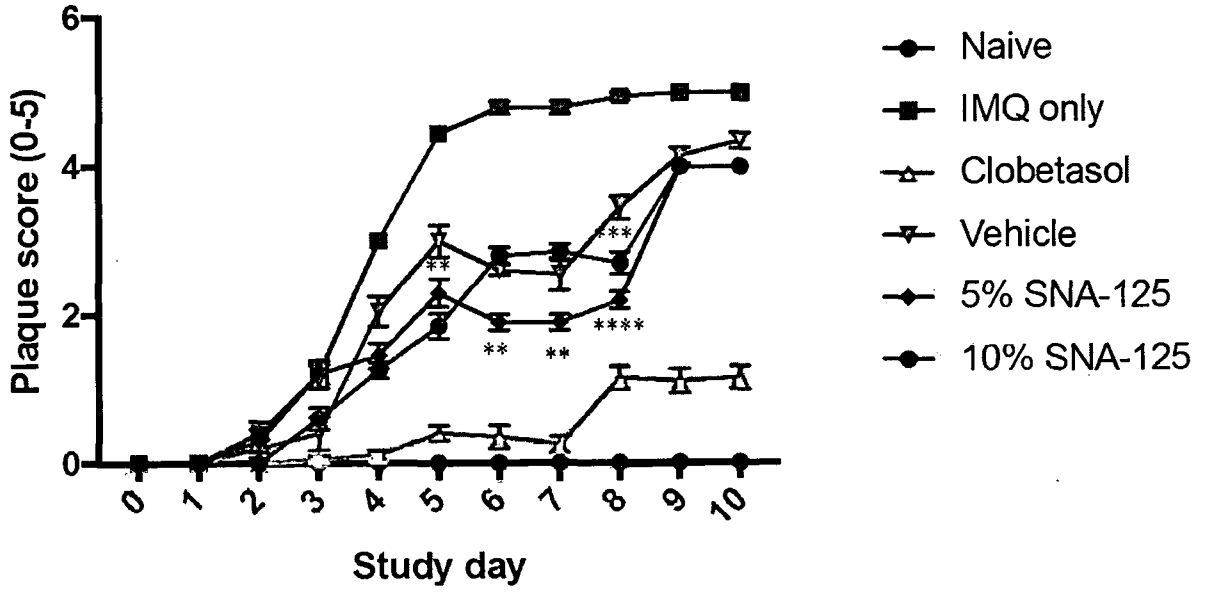

Plaque score (0-5)
0
2
4
6
0
1
2
3
4
5
6
7
8
9
10
Study day
*
Naive
IMQ only
Clobetasol
Vehicle
20% SNA-101

FIGURE 70

C


Plaque score (0-5)
Study day
Naive
IMQ only
Clobetasol
Vehicle
5% SNA-125
10% SNA-125

D

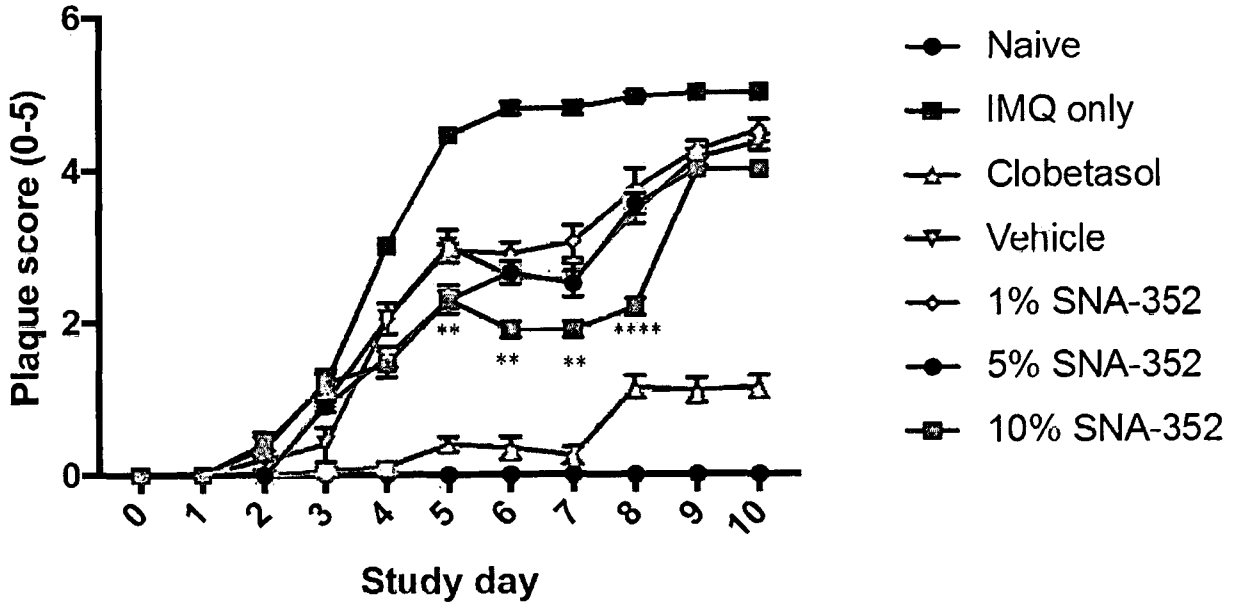
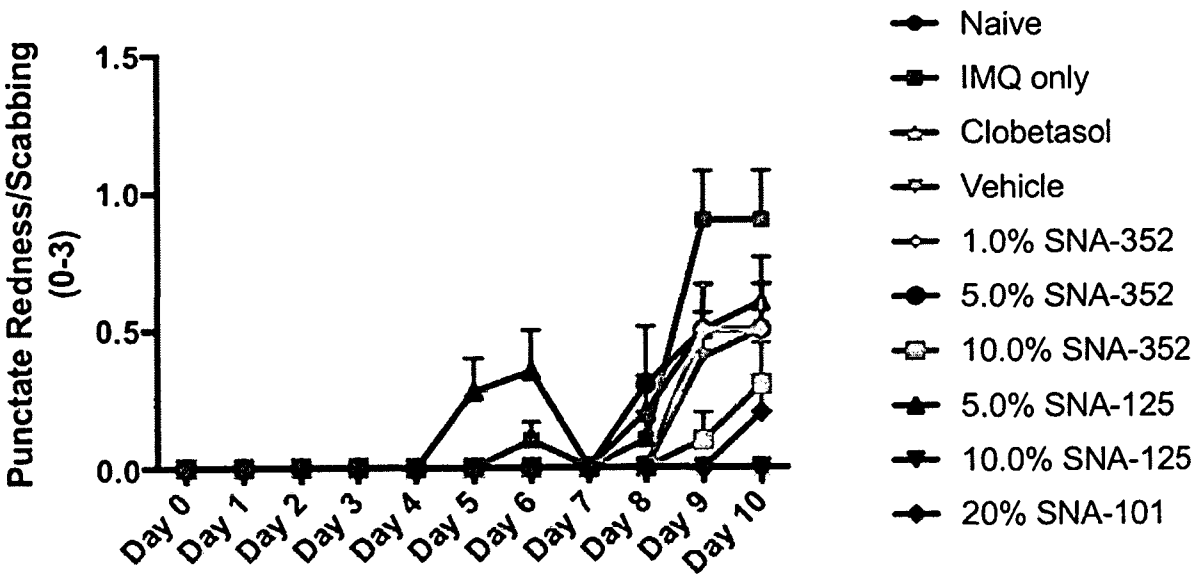

Plaque score (0-5)
Study day
Naive
IMQ only
Clobetasol
Vehicle
1% SNA-352
5% SNA-352
10% SNA-352

FIGURE 70

A


Punctate Redness/Scabbing (0-3)
1.5
1.0
0.5
0.0
Day 0
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
Day 8
Day 9
Day 10
Naive
IMQ only
Clobetasol
Vehicle
1.0% SNA-352
5.0% SNA-352
10.0% SNA-352
5.0% SNA-125
10.0% SNA-125
20% SNA-101

B

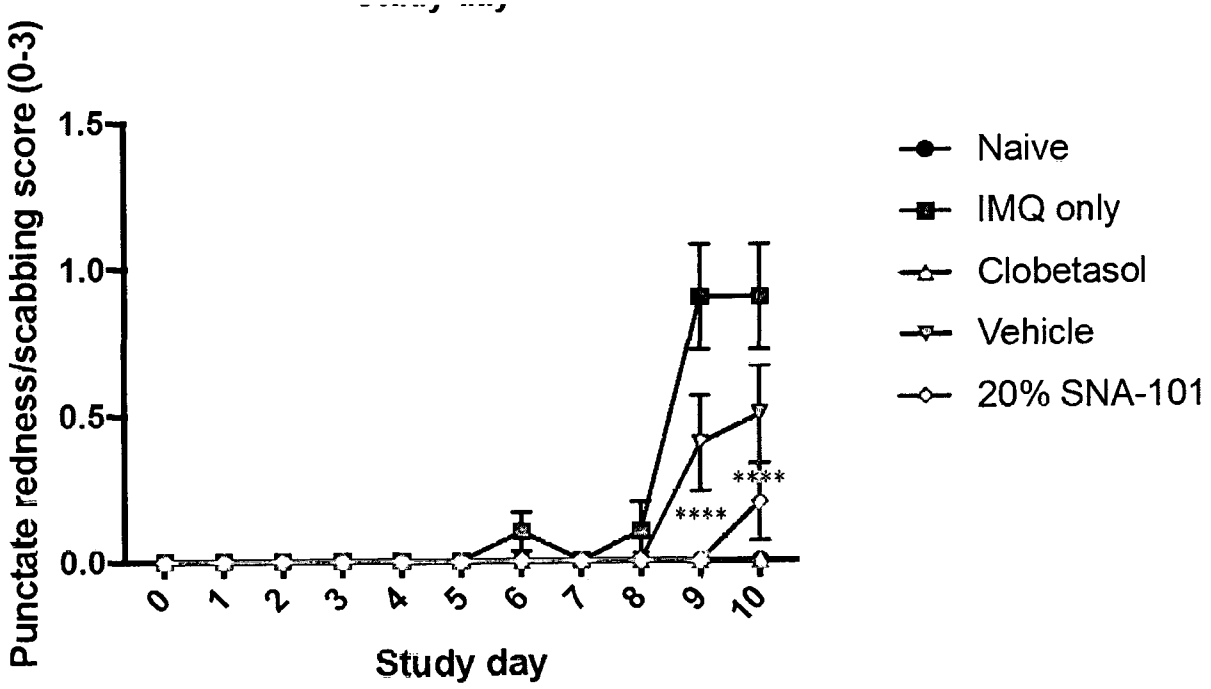
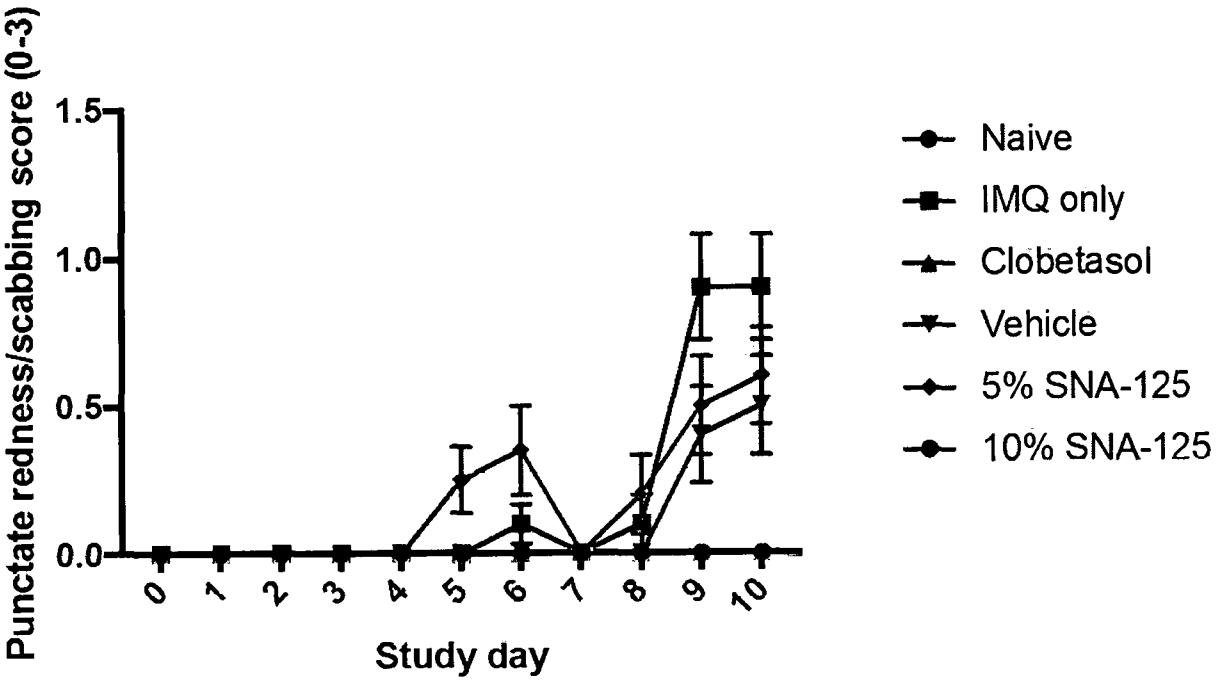

Punctate redness/scabbing score (0-3)
1.5
1.0
0.5
0.0
0
1
2
3
4
5
6
7
8
9
10
Study day
****
****
Naive
IMQ only
Clobetasol
Vehicle
20% SNA-101

FIGURE 71

C


Punctate redness/scabbing score (0-3)
1.5
1.0
0.5
0.0
0 1 2 3 4 5 6 7 8 9 10
Study day
Naive
IMQ only
Clobetasol
Vehicle
5% SNA-125
10% SNA-125

D

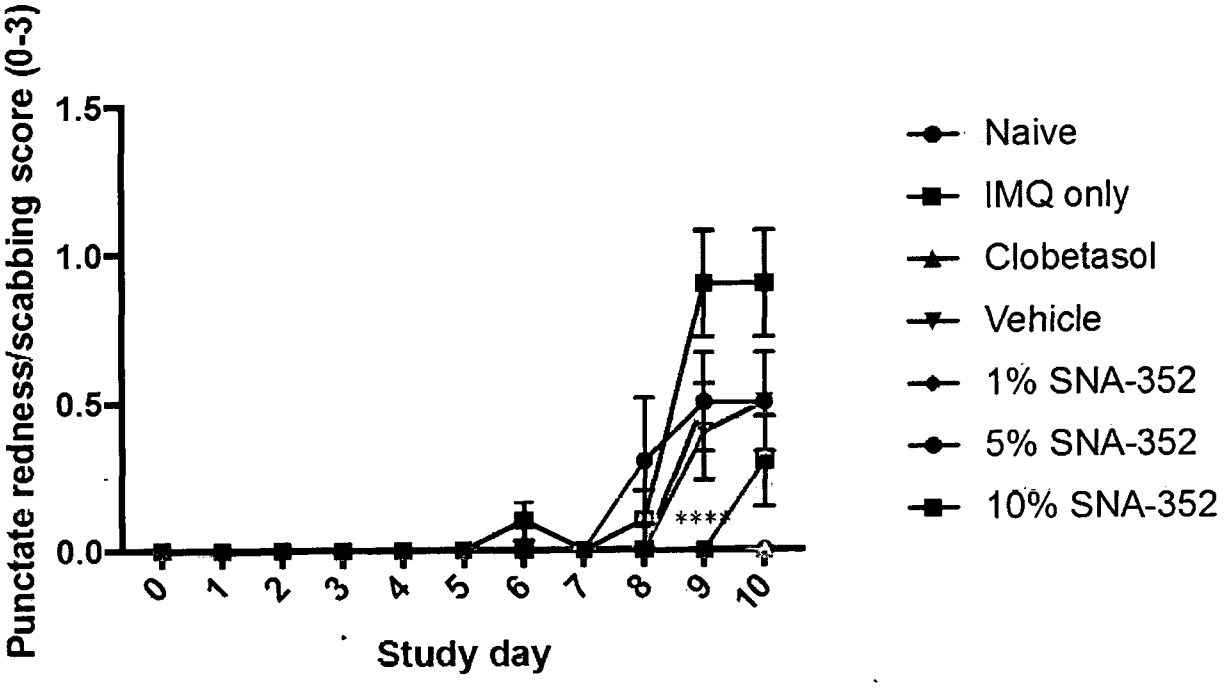

Punctate redness/scabbing score (0-3)
1.5
1.0
0.5
0.0
0 1 2 3 4 5 6 7 8 9 10
Study day
****
Naive
IMQ only
Clobetasol
Vehicle
1% SNA-352
5% SNA-352
10% SNA-352

FIGURE 71

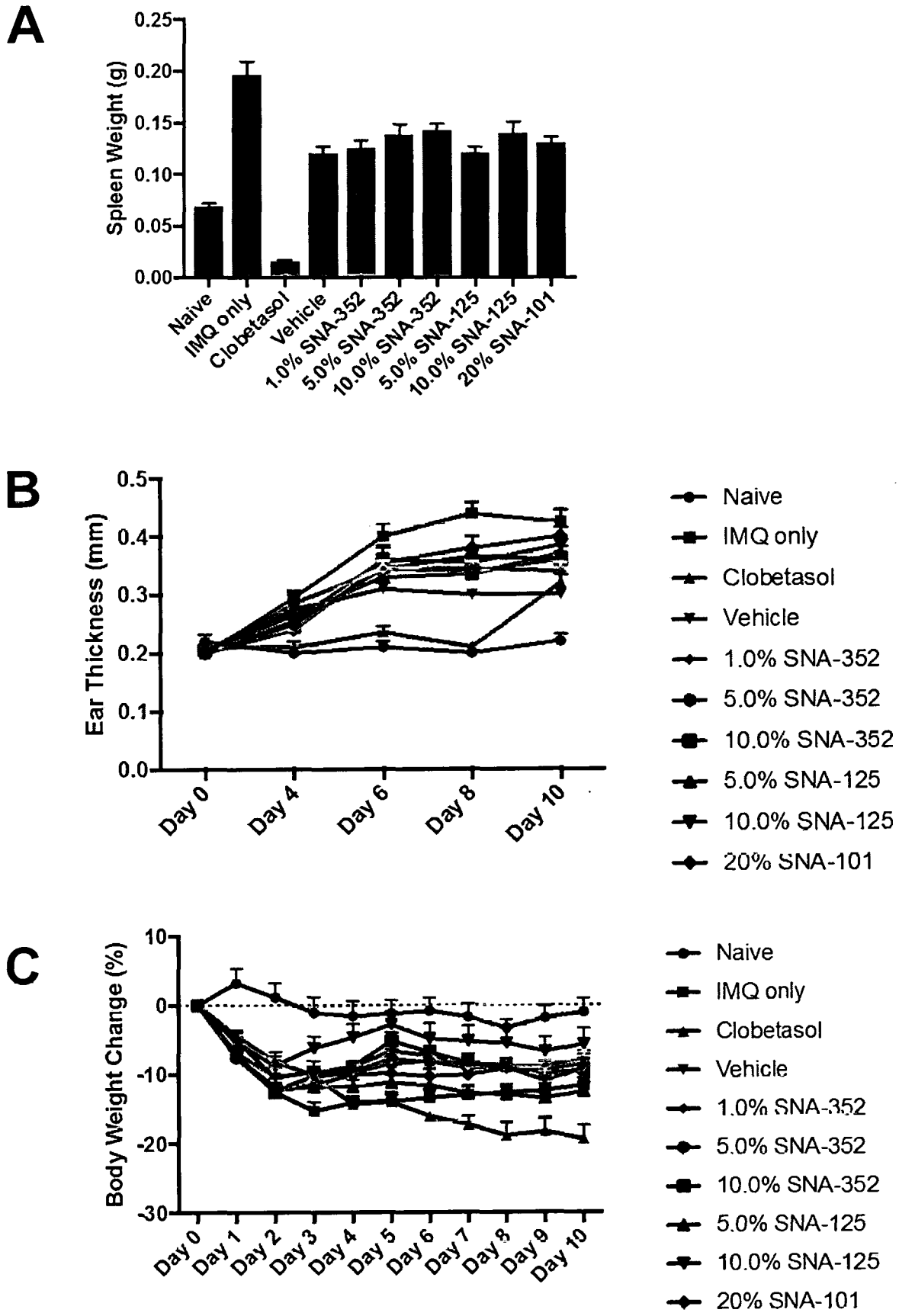

A
Spleen Weight (g)
0.25
0.20
0.15
0.10
0.05
0.00
Naive
IMQ only
Clobetasol
Vehicle
1.0% SNA-352
5.0% SNA-352
10.0% SNA-352
5.0% SNA-125
10.0% SNA-125
20% SNA-101
B
Ear Thickness (mm)
0.5
0.4
0.3
0.2
0.1
0.0
Day 0
Day 4
Day 6
Day 8
Day 10
Naive
IMQ only
Clobetasol
Vehicle
1.0% SNA-352
5.0% SNA-352
10.0% SNA-352
5.0% SNA-125
10.0% SNA-125
20% SNA-101
C
Body Weight Change (%)
10
0
-10
-20
-30
Day 0
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
Day 8
Day 9
Day 10
Naive
IMQ only
Clobetasol
Vehicle
1.0% SNA-352
5.0% SNA-352
10.0% SNA-352
5.0% SNA-125
10.0% SNA-125
20% SNA-101

FIGURE 72

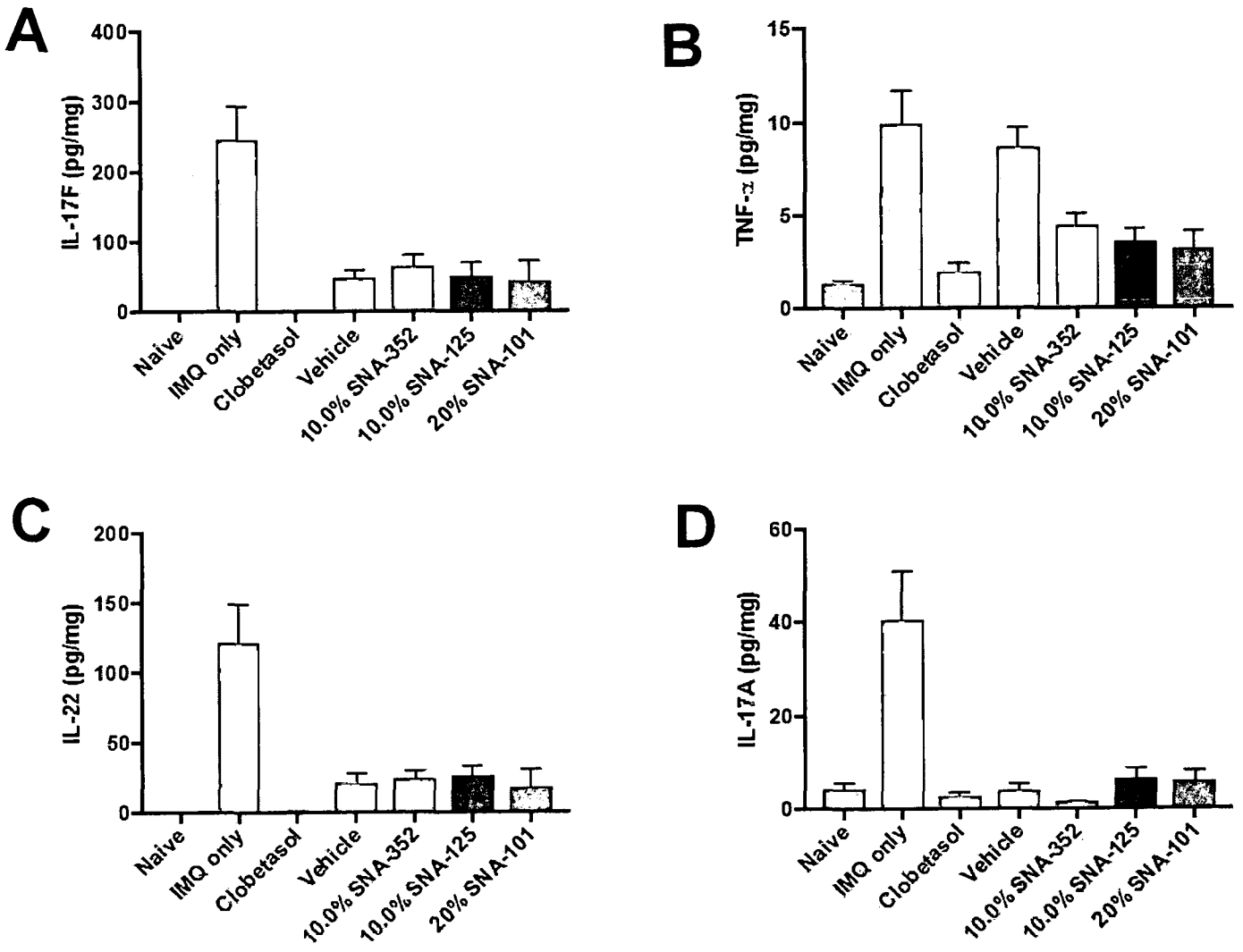
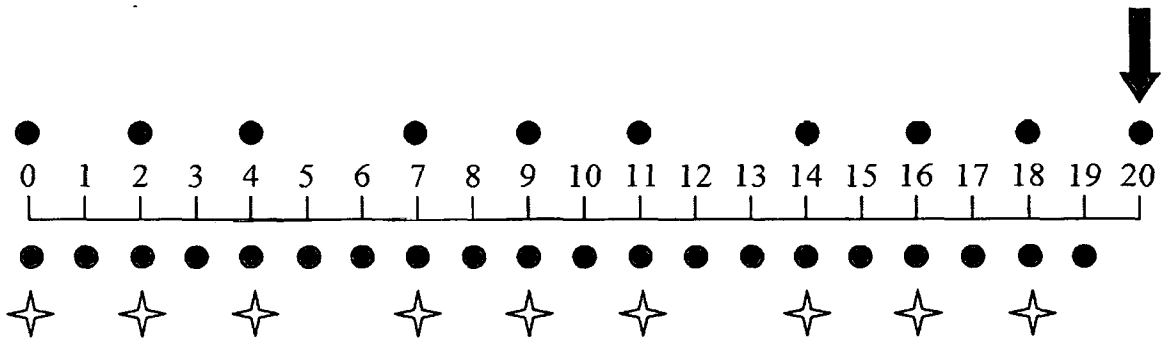

A
IL-17F (pg/mg)
400
300
200
100
0
Naive
IMQ only
Clobetasol
Vehicle
10.0% SNA-352
10.0% SNA-125
20% SNA-101
B
TNF-α (pg/mg)
15
10
5
0
Naive
IMQ only
Clobetasol
Vehicle
10.0% SNA-352
10.0% SNA-125
20% SNA-101
C
IL-22 (pg/mg)
200
150
100
50
0
Naive
IMQ only
Clobetasol
Vehicle
10.0% SNA-352
10.0% SNA-125
20% SNA-101
D
IL-17A (pg/mg)
60
40
20
0
Naive
IMQ only
Clobetasol
Vehicle
10.0% SNA-352
10.0% SNA-125
20% SNA-101

FIGURE 73



● **Body weight, Clinical Score and Ear Thickness**

✧ **IL23 Administration:** IL23 or PBS i.d. on ears

● **Treatment:** TIs administration. Positive control clobetasol was administered topically daily.

▼ **Termination:** Animals were terminated and ears were collected.

FIGURE 74

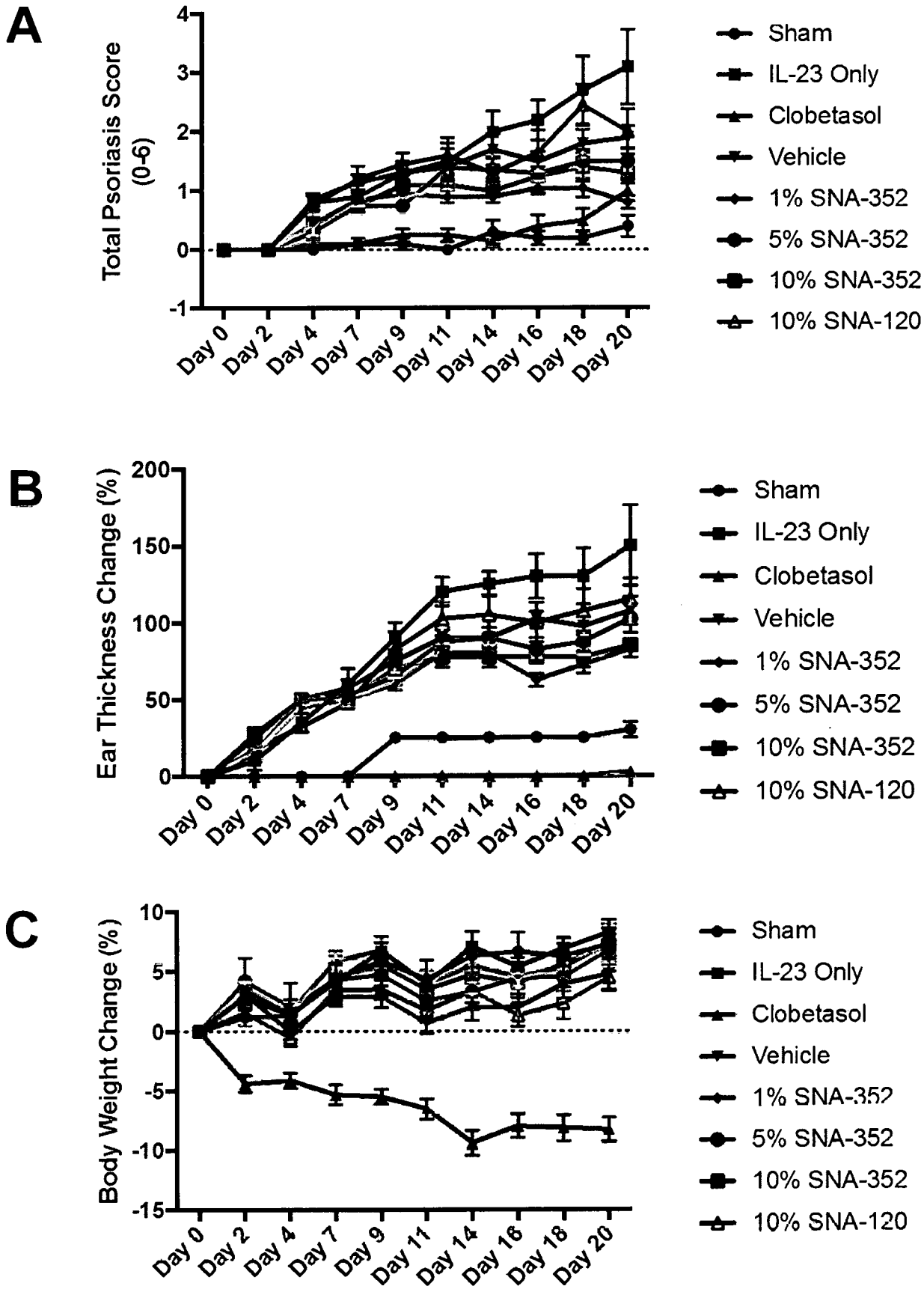

A
Total Psoriasis Score (0-6)
4
3
2
1
0
-1
Day 0
Day 2
Day 4
Day 7
Day 9
Day 11
Day 14
Day 16
Day 18
Day 20
Sham
IL-23 Only
Clobetasol
Vehicle
1% SNA-352
5% SNA-352
10% SNA-352
10% SNA-120
B
Ear Thickness Change (%)
200
150
100
50
0
Day 0
Day 2
Day 4
Day 7
Day 9
Day 11
Day 14
Day 16
Day 18
Day 20
Sham
IL-23 Only
Clobetasol
Vehicle
1% SNA-352
5% SNA-352
10% SNA-352
10% SNA-120
C
Body Weight Change (%)
10
5
0
-5
-10
-15
Day 0
Day 2
Day 4
Day 7
Day 9
Day 11
Day 14
Day 16
Day 18
Day 20
Sham
IL-23 Only
Clobetasol
Vehicle
1% SNA-352
5% SNA-352
10% SNA-352
10% SNA-120

FIGURE 75

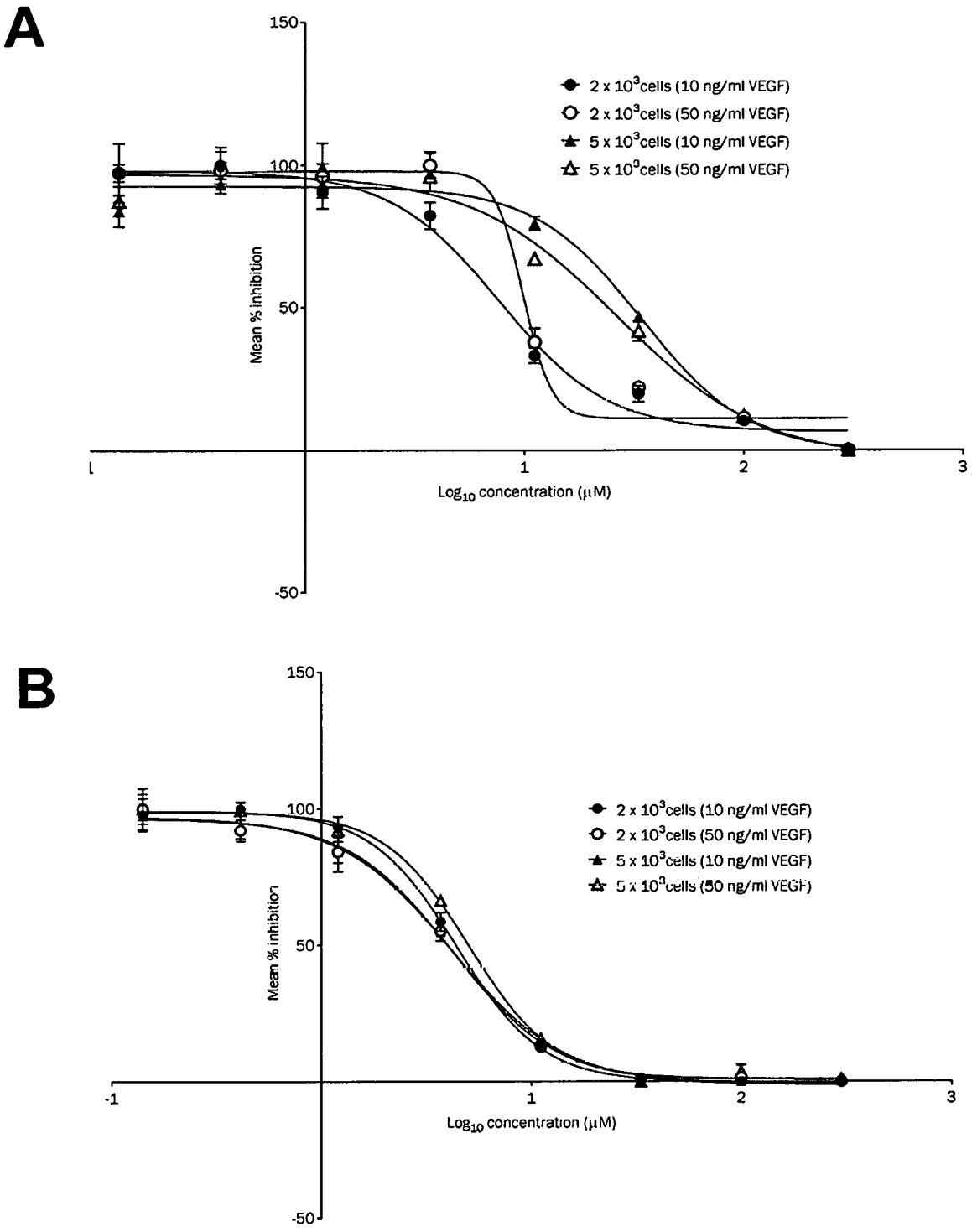
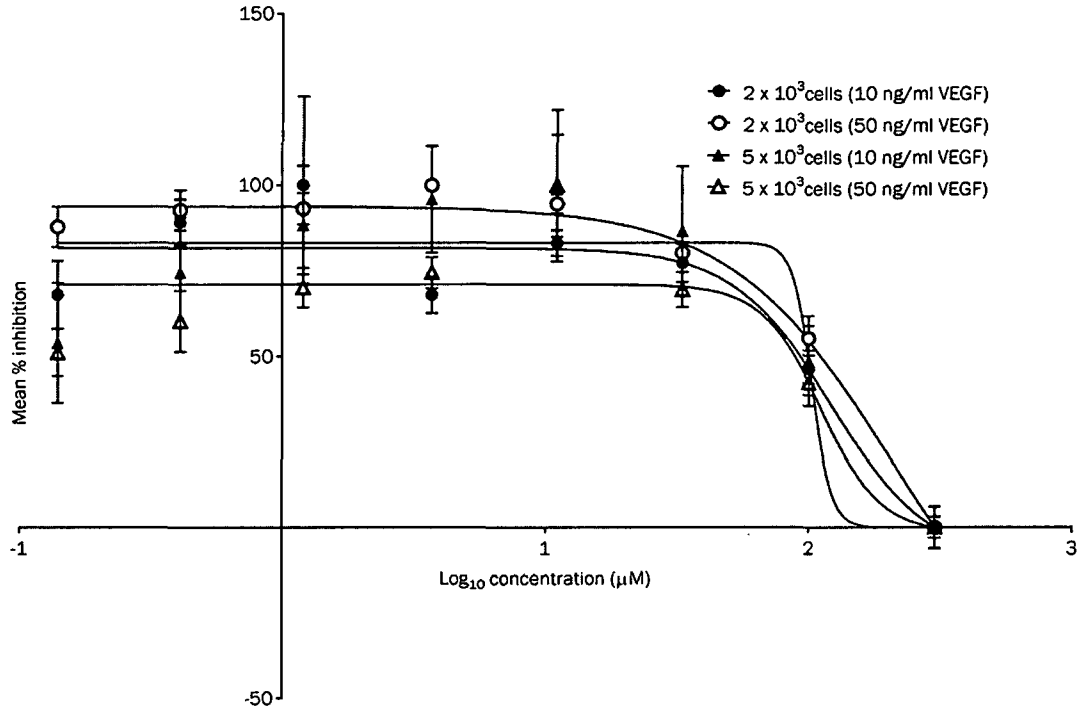

A
150
100
50
-50
Mean % inhibition
2 x 10³cells (10 ng/ml VEGF)
2 x 10³cells (50 ng/ml VEGF)
5 x 10³cells (10 ng/ml VEGF)
5 x 10³cells (50 ng/ml VEGF)
1
2
3
Log10 concentration (μM)
B
150
100
50
-50
Mean % inhibition
2 x 10³cells (10 ng/ml VEGF)
2 x 10³cells (50 ng/ml VEGF)
5 x 10³cells (10 ng/ml VEGF)
5 x 10³cells (50 ng/ml VEGF)
-1
1
2
3
Log10 concentration (μM)

FIGURE 76

C


150
100
50
-50
-1
1
2
3
Mean % inhibition
Log10 concentration (μM)
2 x 10^3cells (10 ng/ml VEGF)
2 x 10^3cells (50 ng/ml VEGF)
5 x 10^3cells (10 ng/ml VEGF)
5 x 10^3cells (50 ng/ml VEGF)

D

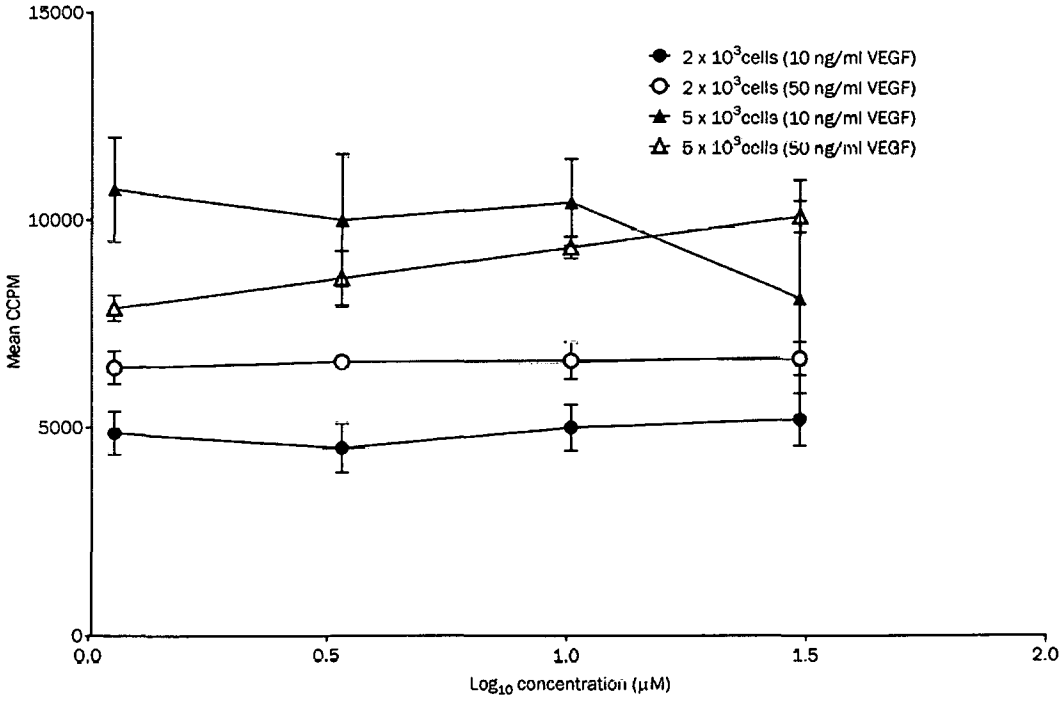
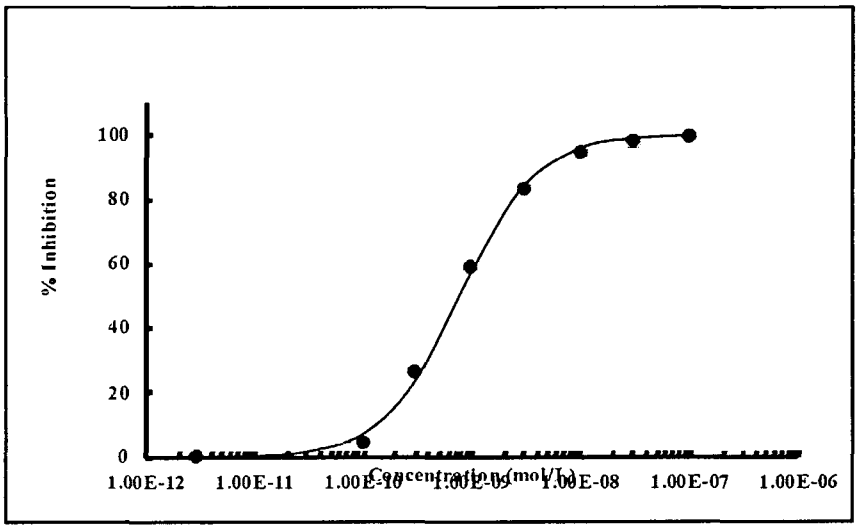

15000
10000
5000
0
0.0
0.5
1.0
1.5
2.0
Mean CCPM
Log10 concentration (μM)
2 x 10^3cells (10 ng/ml VEGF)
2 x 10^3cells (50 ng/ml VEGF)
5 x 10^3cells (10 ng/ml VEGF)
5 x 10^3cells (50 ng/ml VEGF)

FIGURE 76

A

IC50(M)	7.68E-10
Slope	1.23



B

IC50(M)	1.49E-07
Slope	1.40

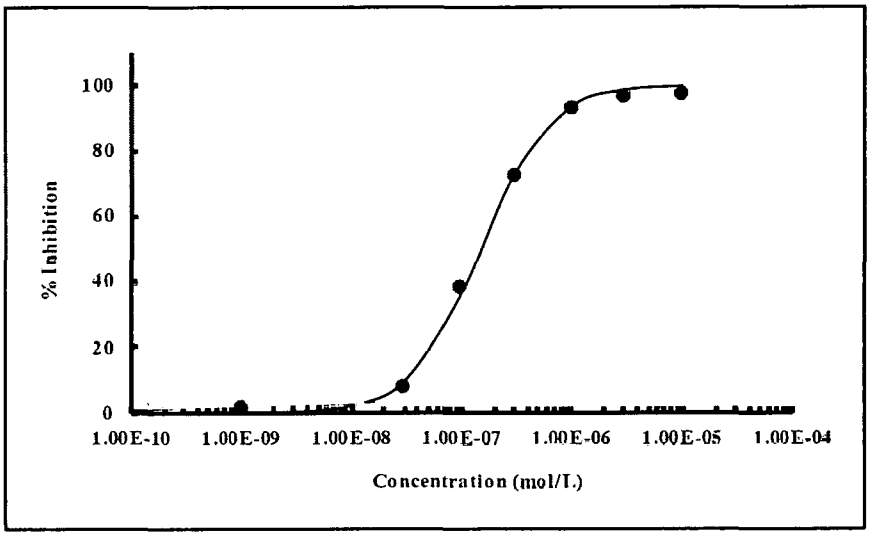
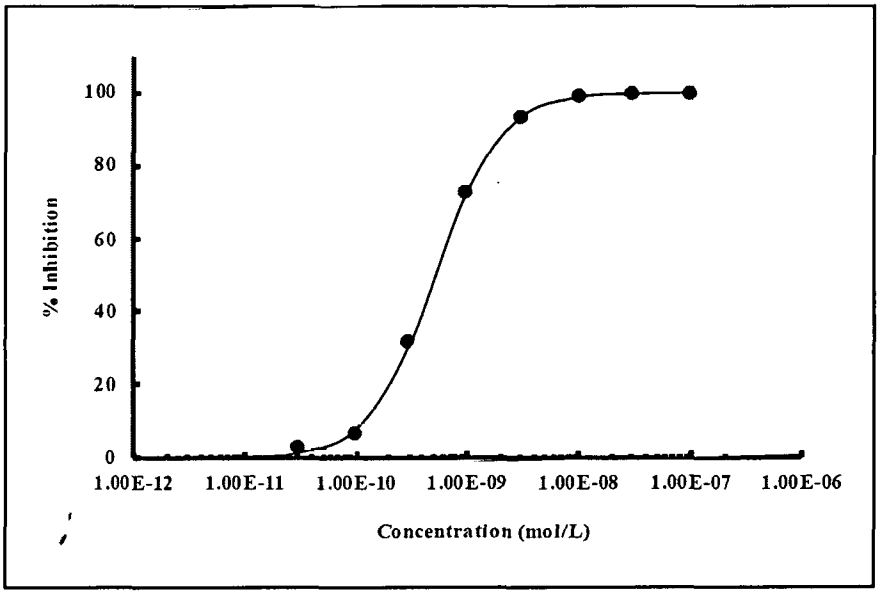


FIGURE 77

A

IC50(M)	5.20E-10
Slope	1.50



B

IC50(M)	2.61E-07
Slope	1.54

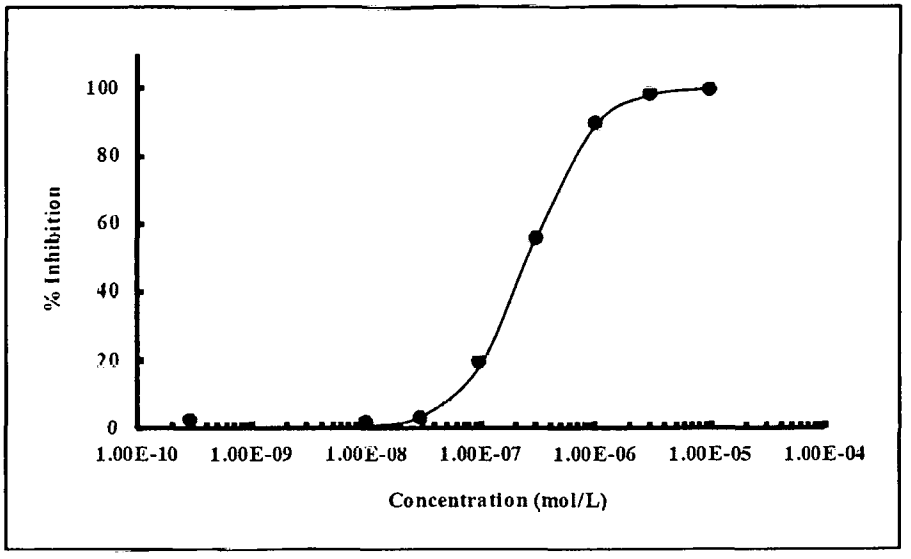
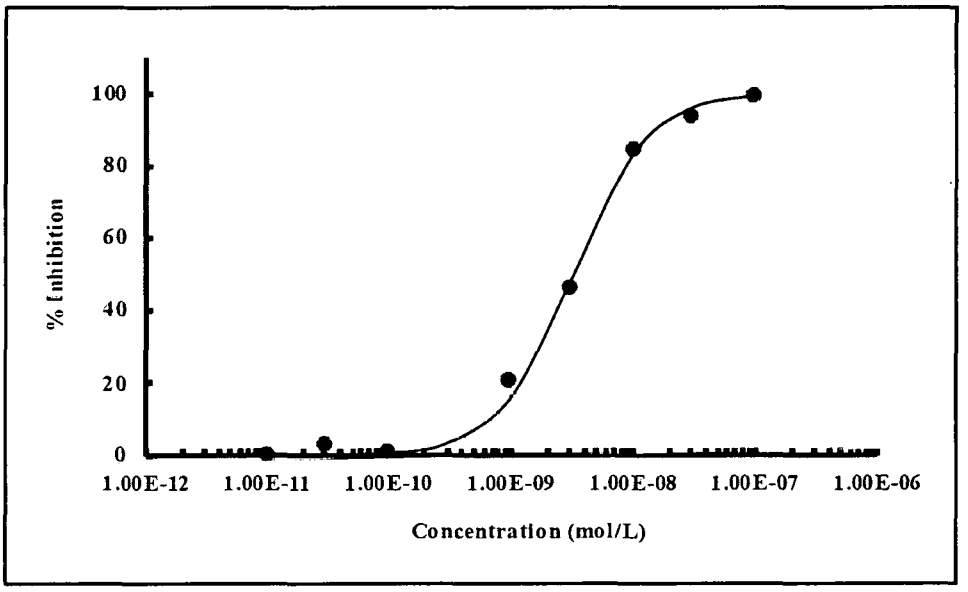


FIGURE 78

A

IC50(M)	3.22E-09
Slope	1.42



B

IC50(M)	3.52E-08
Slope	0.98

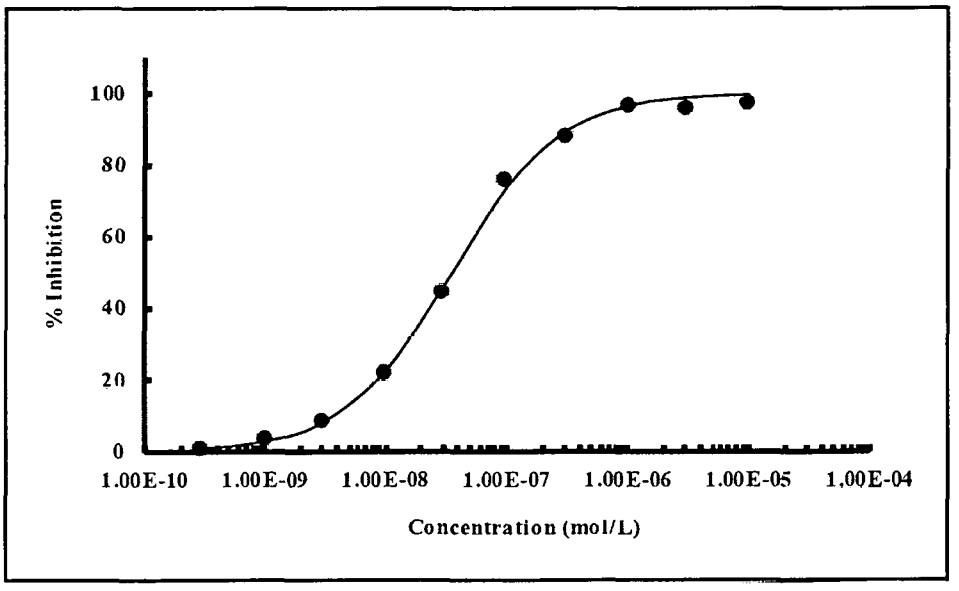
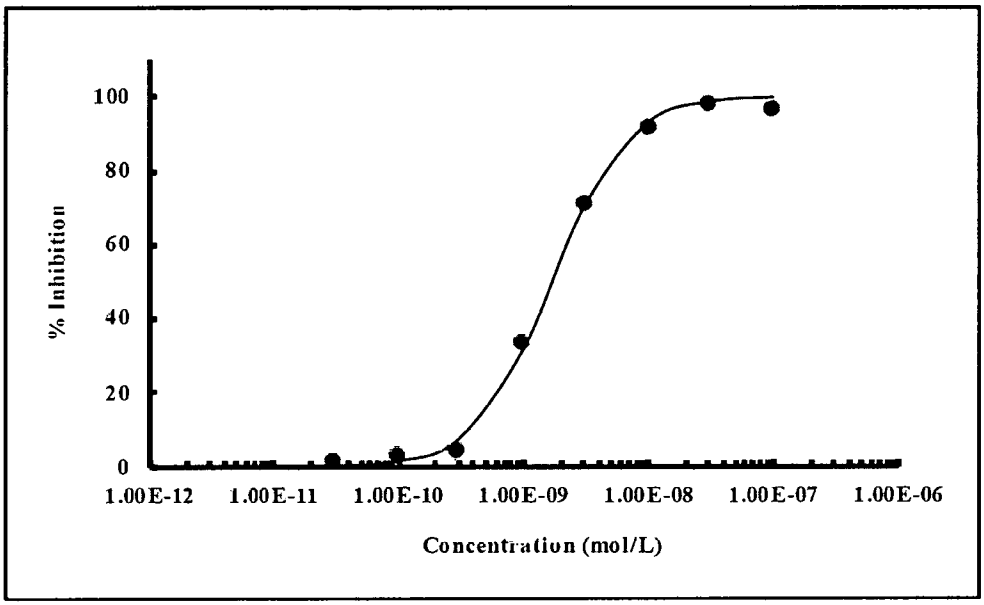


FIGURE 79

A

IC50(M)	1.68E-09
Slope	1.46



B

IC50(M)	2.04E-08
Slope	1.10

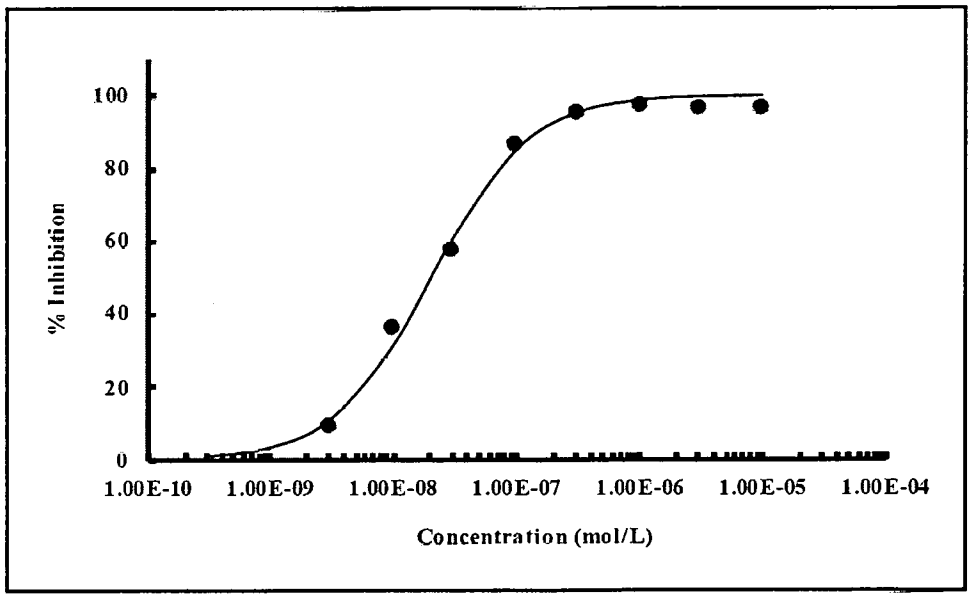
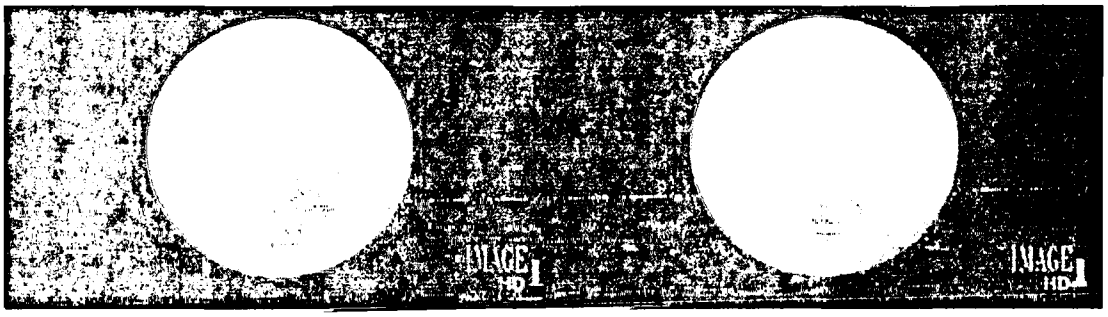
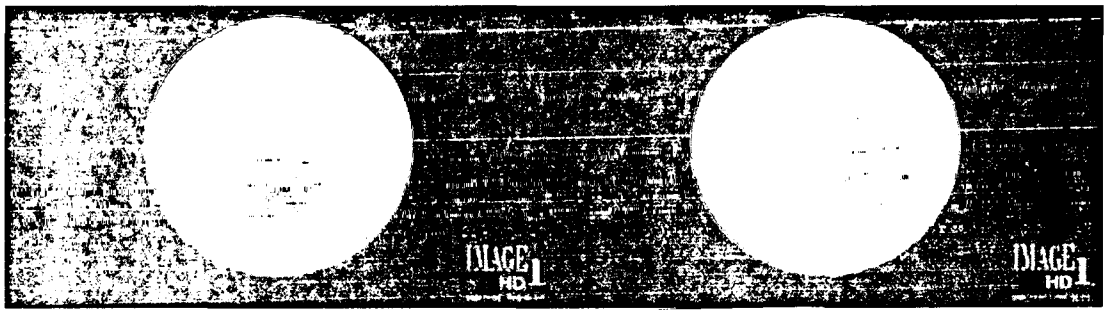


FIGURE 80

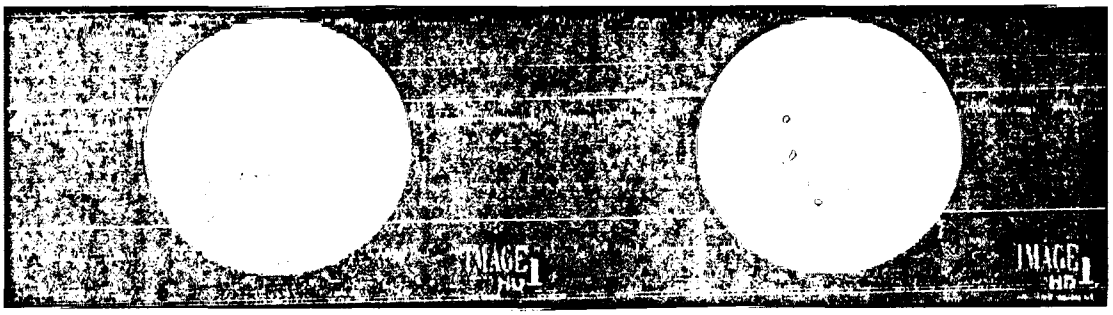
SIB-01: Oxazolone-Induced Colitis Endoscopy Images Day 2



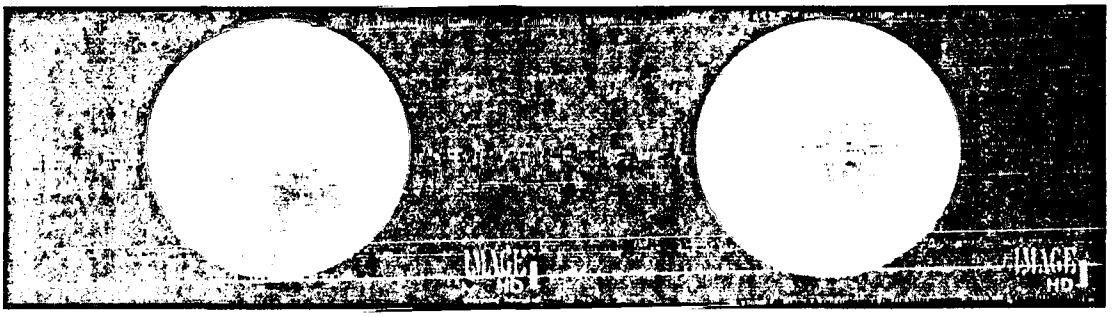
Naïve Control (Average Score = 0.00)



Vehicle Control P.O. (Average Score = 2.13)



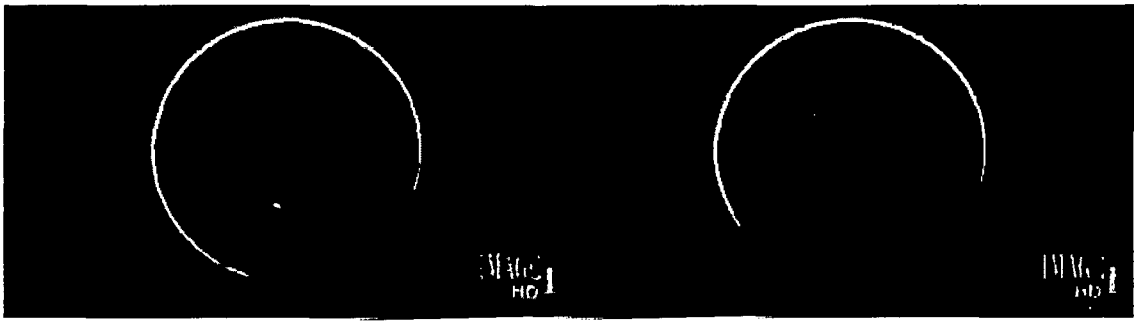
Vehicle Control I.C. (Average Score = 1.92)



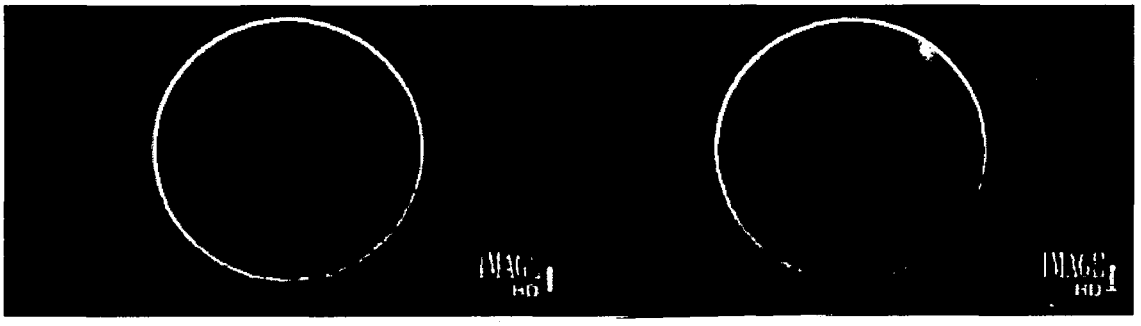
Tofacitnib 15mg/kg P.O. (Average Score= 1.92)

FIGURE 81

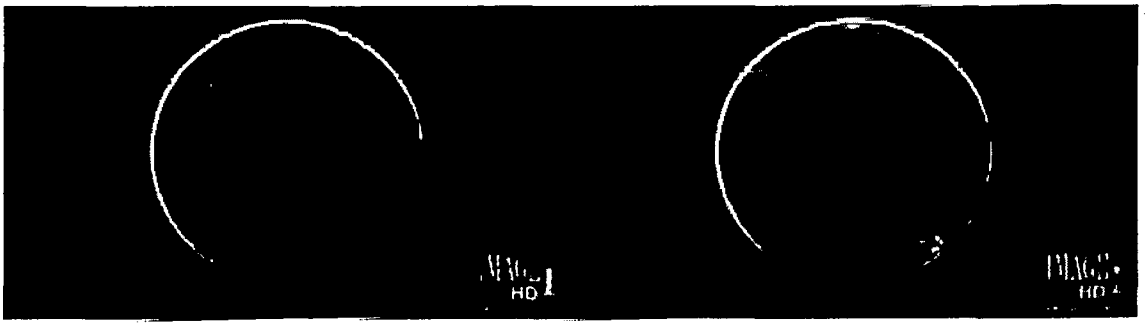
SIB-01: Oxazolone-Induced Colitis Endoscopy Images Day 2



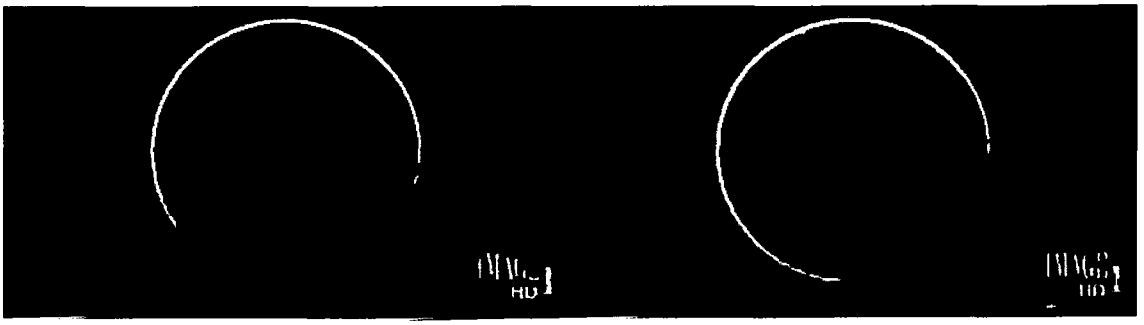
Tofacitnib 15mg/kg I.C. (Average Score = 1.73)



Prednisolone 1.0mg/kg P.O. (Average Score = 1.83)



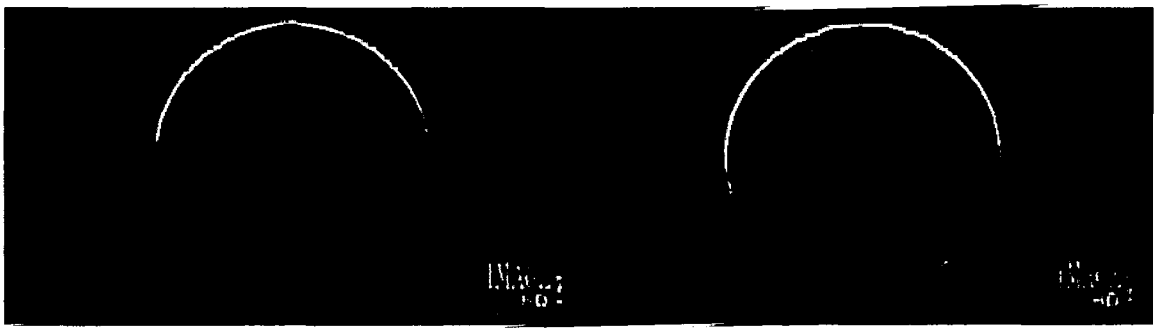
SNA-125 400mg/kg P.O (Average Score = 1.67)



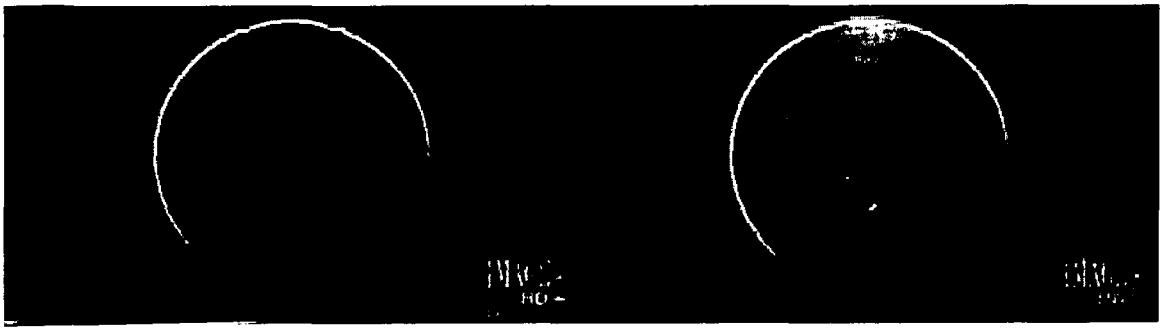
SNA-352 400mg/kg P.O. (Average Score= 1.82)

FIGURE 82

SIB-01: Oxazolone-Induced Colitis Endoscopy Images Day 2



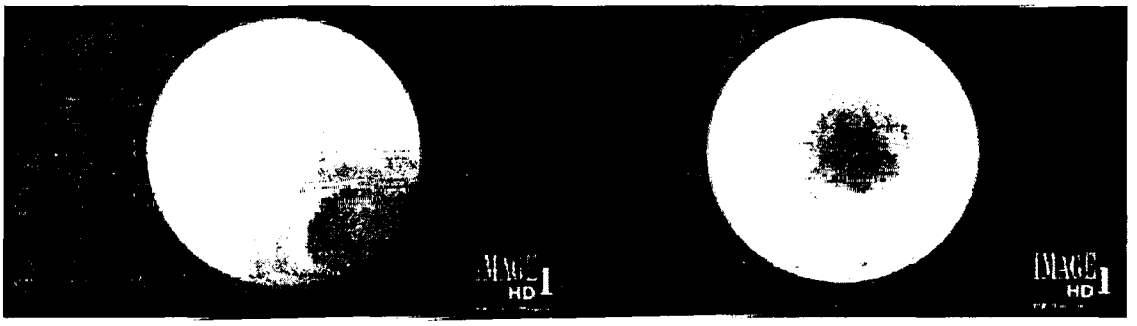
SNA-125 400mg/kg I.C. (Average Score = 2.10)



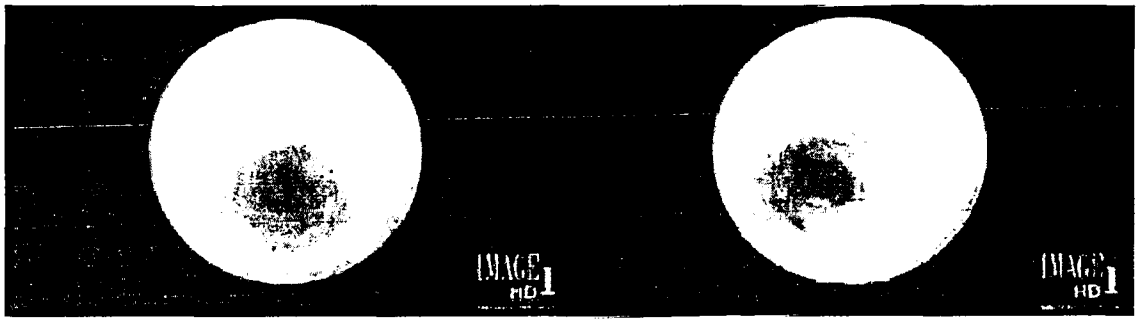
SNA-352 400mg/kg I.C (Average Score = 2.00)

FIGURE 83

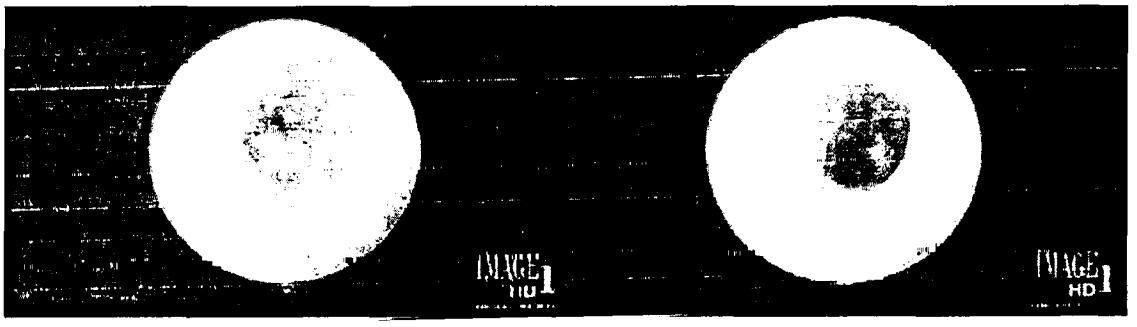
SIB-01: Oxazolone-Induced Colitis Endoscopy Images Day 4



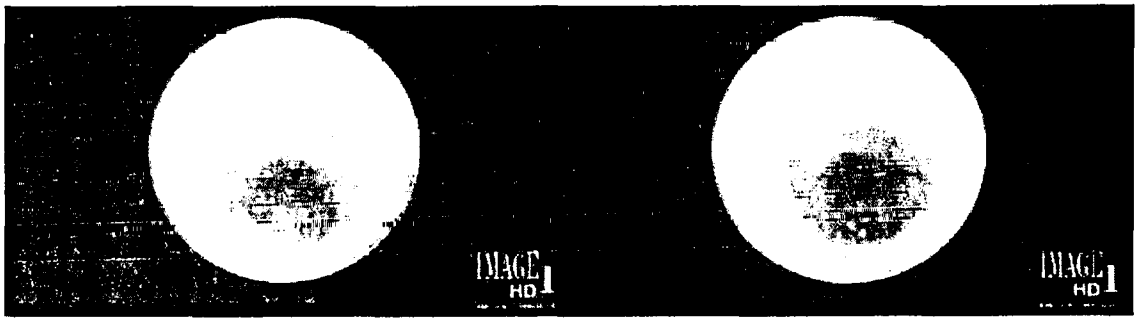
Naïve Control (Average Score = 0.00)



Vehicle Control P.O. (Average Score = 1.92)



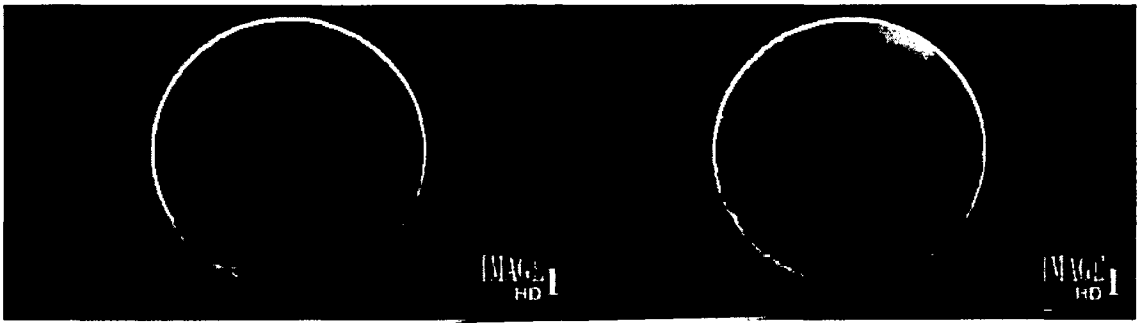
Vehicle Control I.C. (Average Score = 2.00)



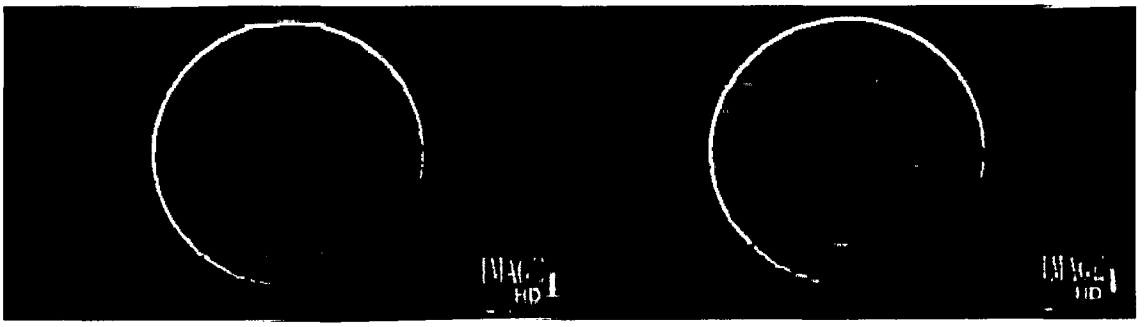
Tofacitnib 15mg/kg P.O. (Average Score= 1.82)

FIGURE 84

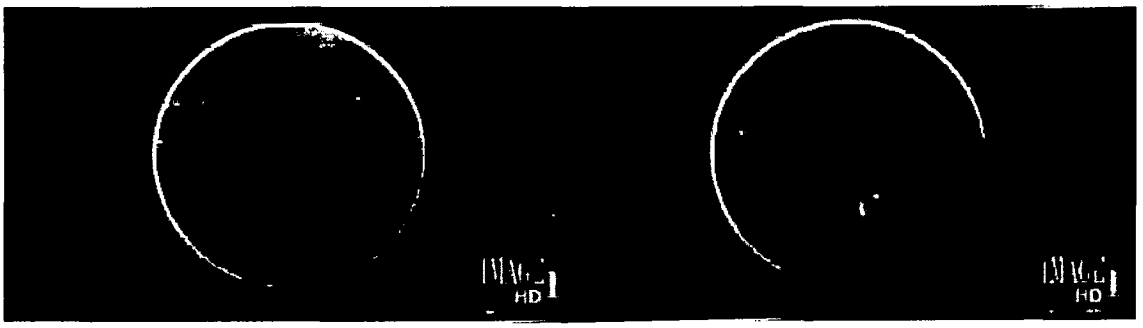
SIB-01: Oxazolone-Induced Colitis Endoscopy Images Day 4



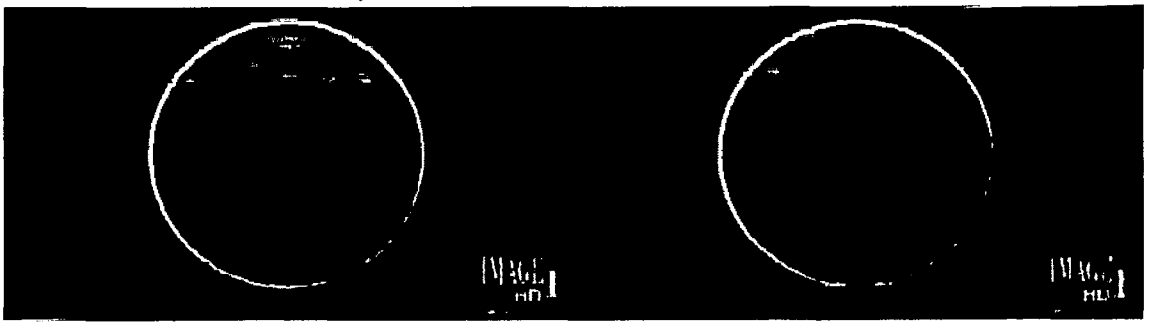
Tofacitnib 15mg/kg I.C. (Average Score = 1.63)



Prednisolone 1.0mg/kg P.O. (Average Score = 1.83)



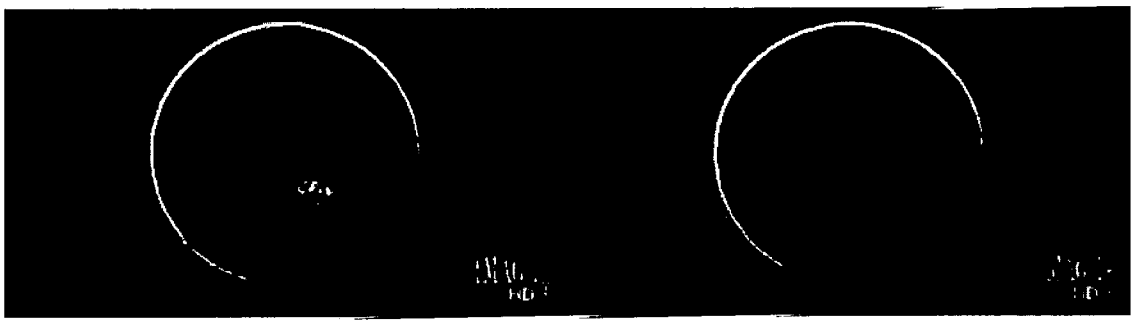
SNA-125 400mg/kg P.O (Average Score = 1.64)



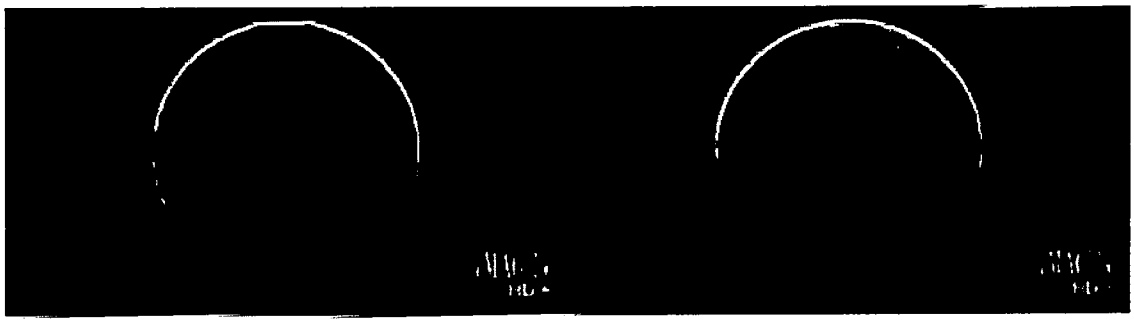
SNA-352 400mg/kg P.O. (Average Score= 1.55)

FIGURE 85

SIB-01: Oxazolone-Induced Colitis Endoscopy Images Day 4



SNA-125 400mg/kg I.C. (Average Score = 1.40)



SNA-352 400mg/kg I.C (Average Score = 2.13)

FIGURE 86

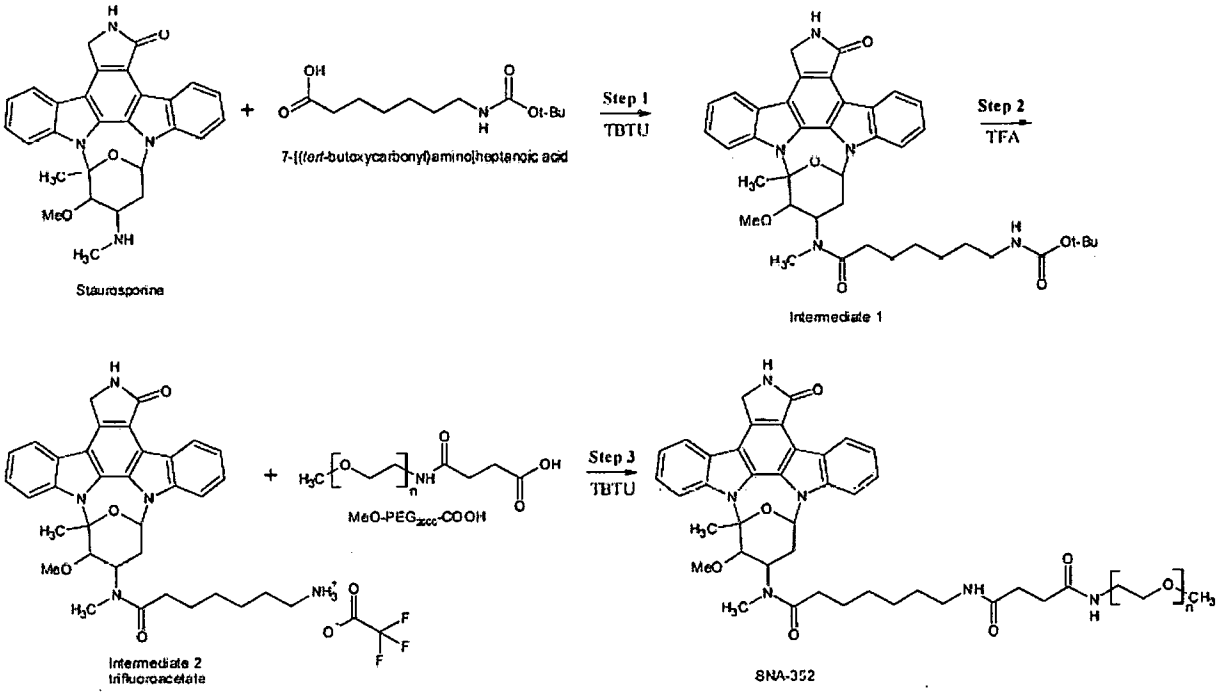
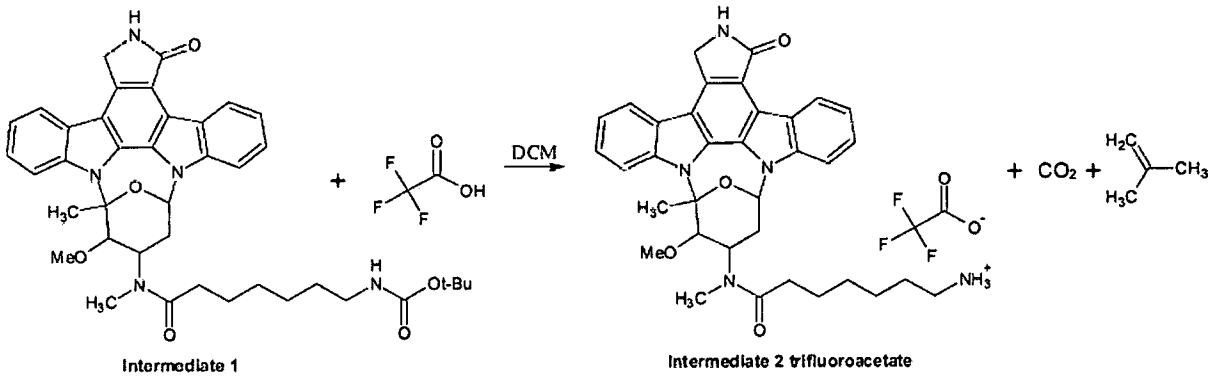

Staurosporine
7-[(tert-butoxycarbonyl)amino]heptanoic acid
Step 1
TBTU
Intermediate 1
Step 2
TFA
Intermediate 2
trifluoroacetate
MeO-PEG2000-COOH
Step 3
TBTU
SNA-352

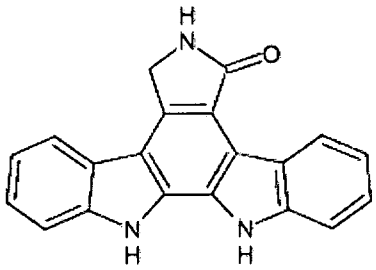
FIGURE 87



Intermediate 1

Intermediate 2 trifluoroacetate

FIGURE 88



Nominal Mass = 311 Da

FIGURE 89

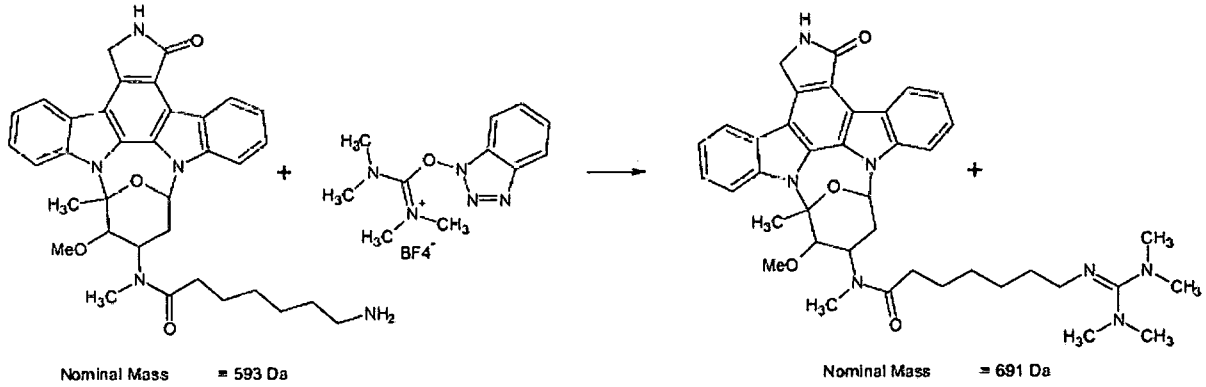
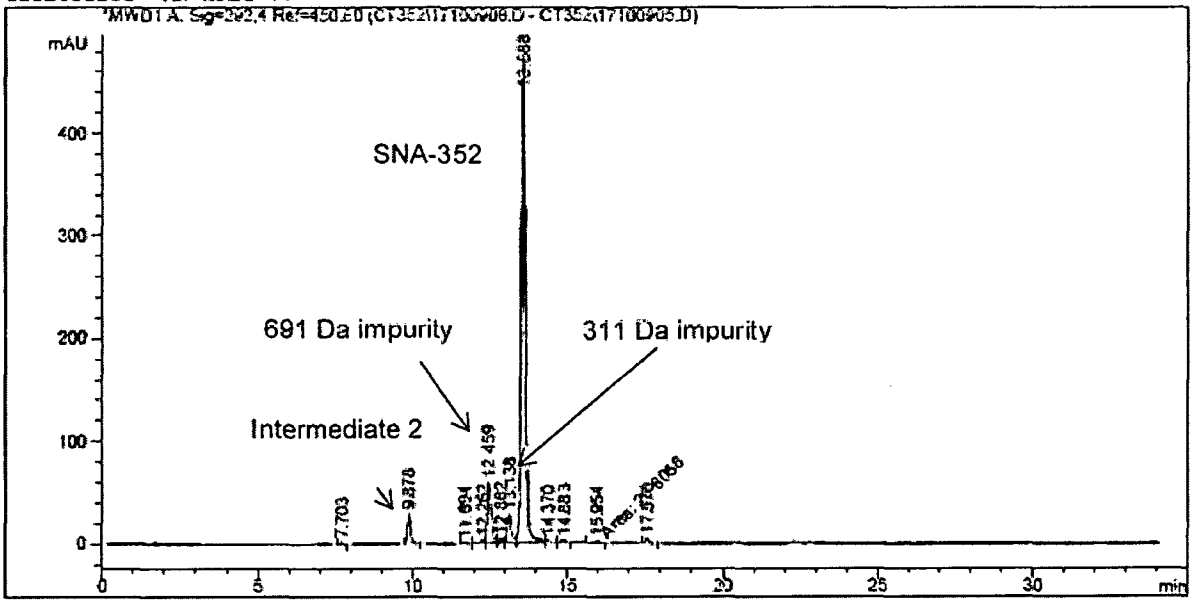

Nominal Mass = 593 Da
Intermediate 2
Nominal Mass = 691 Da
Guanidine side product

FIGURE 90

Sintesi di CT352, S12
Fase Org
ca. 3 gocce, ev sotto N2, dil a 1.5 ml di 0.1% HCO2H H2
O/ACN 1/1

```
Sample Name      : CT352 S12 F. Org                Seq. Line :   9
                                                    Location : Vial 28
                                                        Inj :   1
Acq. Instrument  : hplc25                         Inj Volume : 5 µl
Method           : C:\OLDPC-1\DISCOC-1\1METHO-1\CT352.M
```

CT352 Colonna Zorbax Eclipse XDB-C18 Solvent Saver 150 x 3.0 mm 5 micron P.N. 993967-302 S.N. USTD002295 AN-HPLC-77



Area Percent Report

```
Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs
```

Signal 1: MWD1 A, Sig=292,4 Ref=450,80
Signal has been modified after loading from rawdata file!

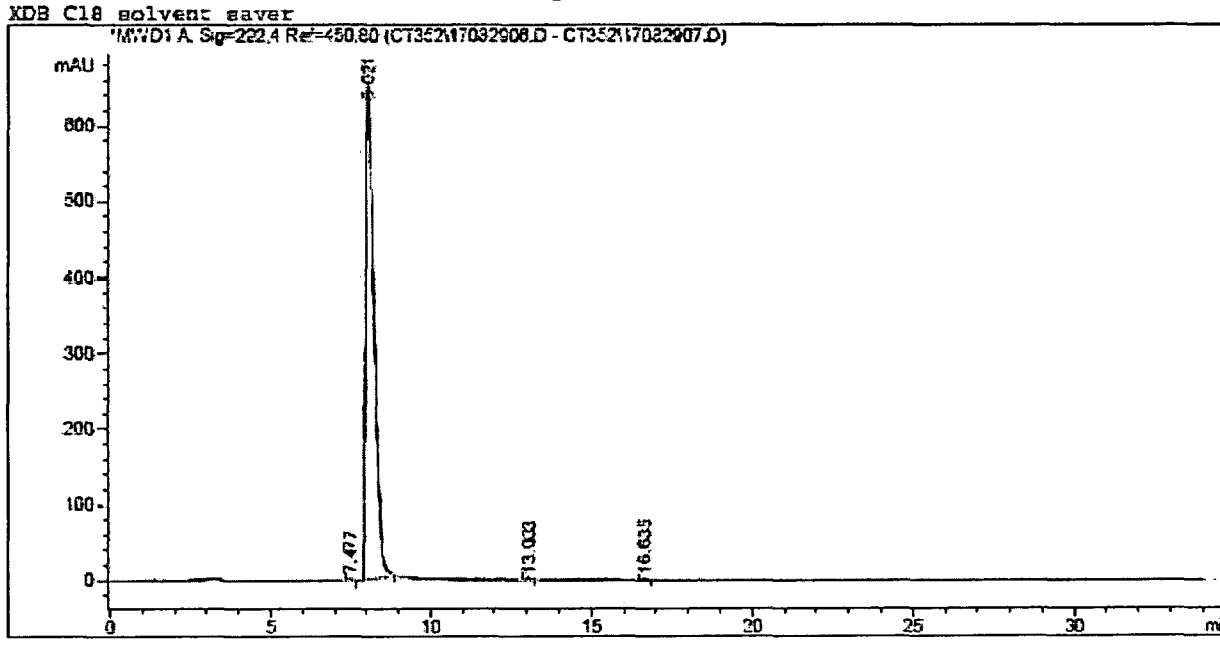
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	7.703	BB	0.1057	7.72623	1.07740	0.1327
2	9.878	BB	0.1040	196.30214	28.63175	3.3711
3	11.694	PV	0.1162	8.91902	1.20757	0.1532
4	12.262	VV	0.1166	25.74119	3.24191	0.4421
5	12.459	VV	0.1493	579.18414	60.69742	9.9464
6	12.862	VV	0.1468	47.41964	4.90317	0.8143
7	13.138	VV	0.1273	248.74199	28.64723	4.2717
8	13.588	VV	0.1510	4577.17041	472.47000	78.6042
9	14.370	VV	0.1984	36.74153	2.48700	0.6310
10	14.883	VV	0.1975	38.13782	2.68926	0.6549
11	15.954	MM	0.2642	20.80558	1.31226	0.3573
12	17.570	BB	0.1263	36.17314	4.38036	0.6212
Totals :				5823.06284	611.74534	

FIGURE 91

Staurosporina, Iris, Lot ST018B
0.15 mg/ml in 0.1% HCO2H H2O/ACN 20/80

Sample Name	: Staurosporina	Seq. Line : Location : Inj :	7 Vial 3 1
Acq. Instrument	: hplc25	Inj Volume :	5 µl
Acq. Method	: C:\OLDPC-1\DISCOC-1\1METHO-1\CT352.M		
Analysis Method	: C:\OLDPC-1\DISCOC-1\1METHO-1\CT352LAV.M		

XDB C18 solvent saver



Area Percent Report

Sorted By	:	Signal	
Multiplier	:	1.0000	
Dilution	:	1.0000	

Use Multiplier & Dilution Factor with ISTDs

Signal 1: MWD1 A, Sig=292,4 Ref=450,80
Signal has been modified after loading from rawdata file!

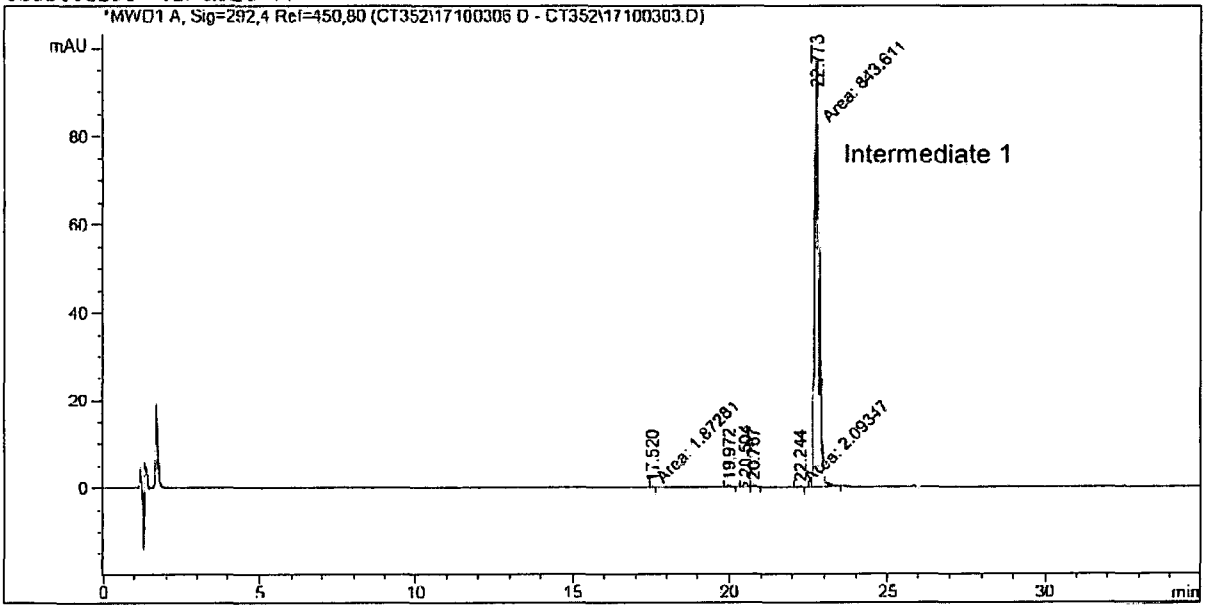
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	7.477	BP	0.1111	15.84782	2.14637	0.1308
2	8.021	PB	0.2849	1.19042e4	658.52509	99.5212
3	13.033	PB	0.1180	16.88188	2.18817	0.1411
4	16.635	BB	0.1237	24.74549	3.01599	0.2069
Totals :				1.19615e4	665.87561	

FIGURE 92

Sintesi di Intermedio 1, S10
P2, ca. 24h a ta
ca. 0.5 goccia di mix sciolta in 0.5 ml di DMF e dil a
2.0 ml di 0.1% HCO2H H2O/ACN 1/1

```
==================================================================
                                                Seq. Line :   7
Sample Name      : CT352 S10-P2                  Location : Vial 10
                                                     Inj :   1
Acq. Instrument : hplc25                      Inj Volume : 5 µl
Acq. Method     : C:\OLDPC-1\DISCOC-1\1METHO-1\CT352.M

Analysis Method : C:\OLDPC~1\DISCOC~1\1METHO~1\CT352.M
```

CT352 Colonna Zorbax Eclipse XDB-C18 Solvent Saver 150 X 3.0 mm 5 micron P.N. 993967-302 S.N. USTD002295 AN-HPLC-77



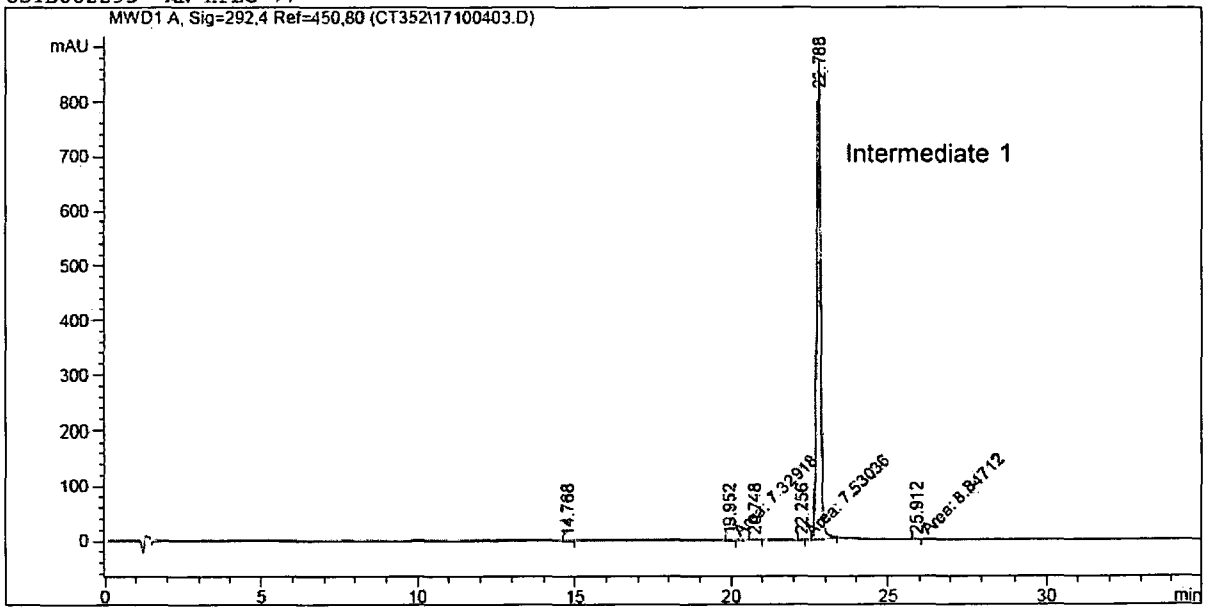

```
==================================================================
                        Area Percent Report
==================================================================

Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: MWD1 A, Sig=292,4 Ref=450,80
 Signal has been modified after loading from rawdata file!

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]   [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1  17.520 MM    0.1178    1.87281 2.64970e-1   0.2166
   2  19.972 BB    0.1167    4.20729 4.97458e-1   0.4866
   3  20.504 VV    0.1239    8.81514   1.09514    1.0196
   4  20.767 VB    0.1167    4.00739 4.73635e-1   0.4635
   5  22.244 MM    0.1479    2.09347 2.35916e-1   0.2421
   6  22.773 MM    0.1442  843.61127   97.52409  97.5716

Totals :                   864.60737  100.09120
```

FIGURE 93

Sintesi di Intermedio 1, S10
Solido finale secco
ca. 0.3 mg sciolti in 0.5 ml di DMF e dil a 2.0 ml con
H2O ac/ACN 0.5 ml/1 ml
conc. fin ca. 0.15 mg/ml

```
==================================================================
                                                Seq. Line :   4
Sample Name      : S10-solido fin                Location : Vial 16
                                                     Inj :   1
Acq. Instrument : hplc25                      Inj Volume : 5 µl
Acq. Method     : C:\OLDPC~1\DISCOC~1\1METHO~1\CT352.M

Analysis Method : C:\OLDPC~1\DISCOC~1\1METHO~1\CT352.M
```

CT352 Colonna Zorbax Eclipse XDB-C18 Solvent Saver 150 x 3.0 mm 5 micron P.N. 993967-302 S.N USTD002295 AN-HPLC-77



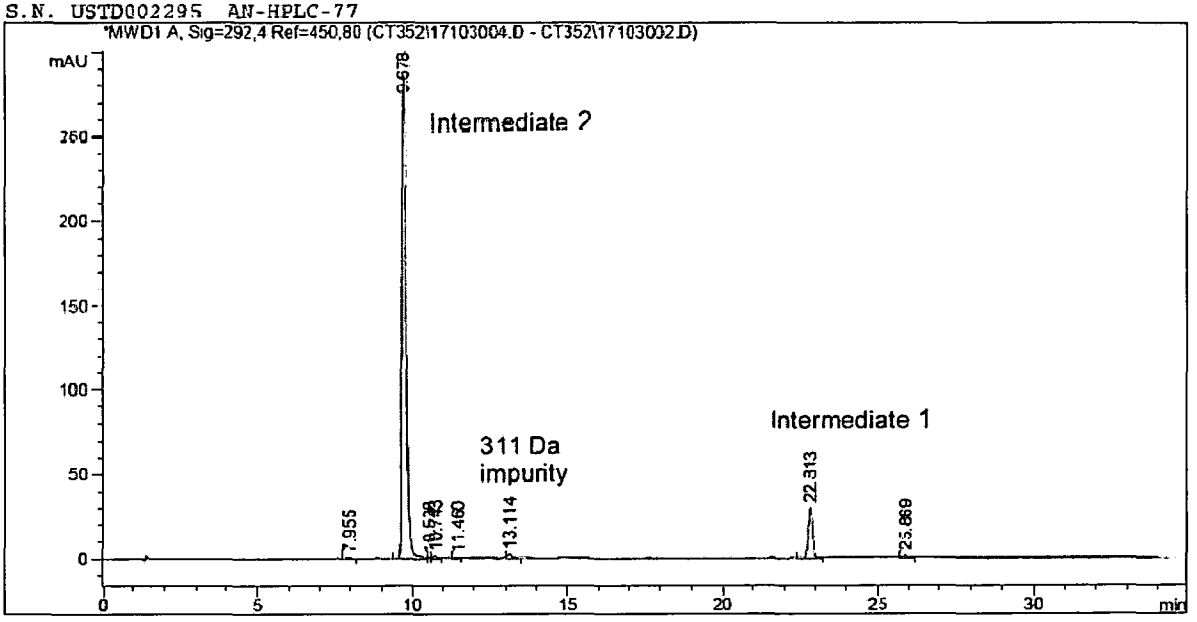

```
==================================================================
                        Area Percent Report
==================================================================

Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: MWD1 A, Sig=292,4 Ref=450,80

Peak RetTime Type  Width     Area      Height      Area
  #   [min]      [min]    [mAU*s]     [mAU]        %
----|-------|----|-------|----------|----------|--------|
   1  14.768 PB   0.1143   5.80665 7.04966e-1   0.0752
   2  19.952 MM   0.1419   7.32918 8.61002e-1   0.0949
   3  20.748 BB   0.1222   8.05828   1.06618    0.1043
   4  22.256 MM   0.1389   7.53036 9.03862e-1   0.0975
   5  22.788 PB   0.1347 7689.13330  873.71881  99.5137
   6  25.912 MM   0.1487   8.84712 9.91542e-1   0.1145

Totals :                 7726.70480  878.32606
```

FIGURE 94

Sintesi di Intermedio 2, S15
P2, ca. 3h 1-24°C
ca. 1 goccia in 8 ml di 0.1% HCO2H H2O/ACN 1/1

```
==========================================================================
                                                    Seq. Line :   5
Sample Name      : S15-P2                           Location : Vial 4
                                                        Inj :   1
Acq. Instrument : hplc25                          Inj Volume : 5 ul
Acq. Method      : C:\OLDPC~1\DISCOC~1\1METHO~1\CT352.M

Analysis Method : C:\OLDPC~1\DISCOC~1\1METHO~1\CT352ON.M
```

CT352 cond Colonna Zorbax Eclipse XDB-C18 Solvent Saver 150 x 3.0 mm 5 micron P.N. 993967-302 S.N. USTD002295 AN-HPLC-77

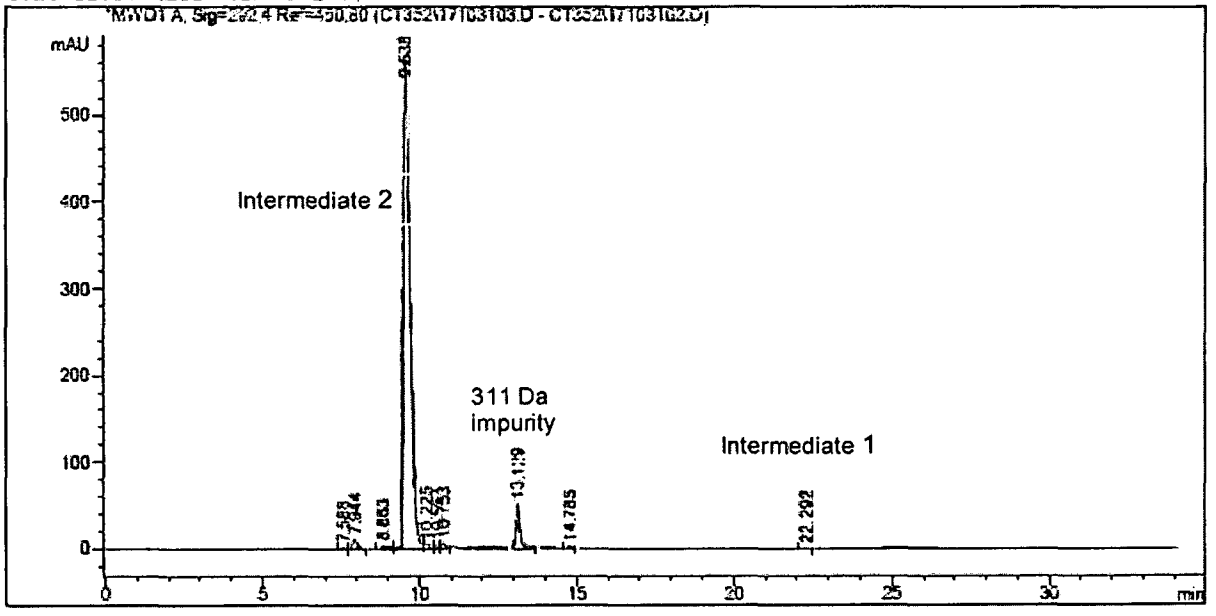



```
==========================================================================
                        Area Percent Report
--------------------------------------------------------------------------

Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: MWD1 A, Sig=292,4 Ref=450,80
 Signal has been modified after loading from rawdata file!

Peak RetTime Type  Width     Area      Height     Area
  #   [min]      [min]    [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1   7.955 VB    0.1381   8.71636 9.40454e-1   0.2569
   2   9.670 VV    0.1616 3030.93555  286.02844  89.3284
   3  10.528 VV    0.1154   8.58063 9.32189e-1   0.2529
   4  10.743 VV    0.1423  18.07328    1.75416   0.5327
   5  11.460 BV    0.1582  11.15381 9.55668e-1   0.3287
   6  13.114 VB    0.1468  29.79571    2.83181   0.8781
   7  22.813 VV    0.1371 266.79166   29.60991   7.8629
   8  25.869 VV    0.1924  18.97751    1.31480   0.5593

Totals :                 3393.02451  324.36744
```

FIGURE 95

Sintesi di Intermedio 2, S15
P4, dopo una notte in frigo
ca. 1 goccia in 8 ml di 0.1% HCO2H H2O/ACN 1/1

```
==========================================================================
                                                    Seq. Line :   4
Sample Name      : S15-P4                            Location : Vial 6
                                                         Inj :   1
Acq. Instrument : hplc25                           Inj Volume : 5 µl
Acq. Method      : C:\OLDPC-1\DISCOC-1\1METHO-1\CT352.M

Analysis Method : C:\OLDPC-1\DISCOC-1\1METHO-1\CT352ON.M

CT352 cond Colonna Zorbax Eclipse XDB-C18 Solvent Saver 150 x 3.0 mm 5 micron P.N. 993967-302
S.N. USTD002295  AN-HPLC-77
```



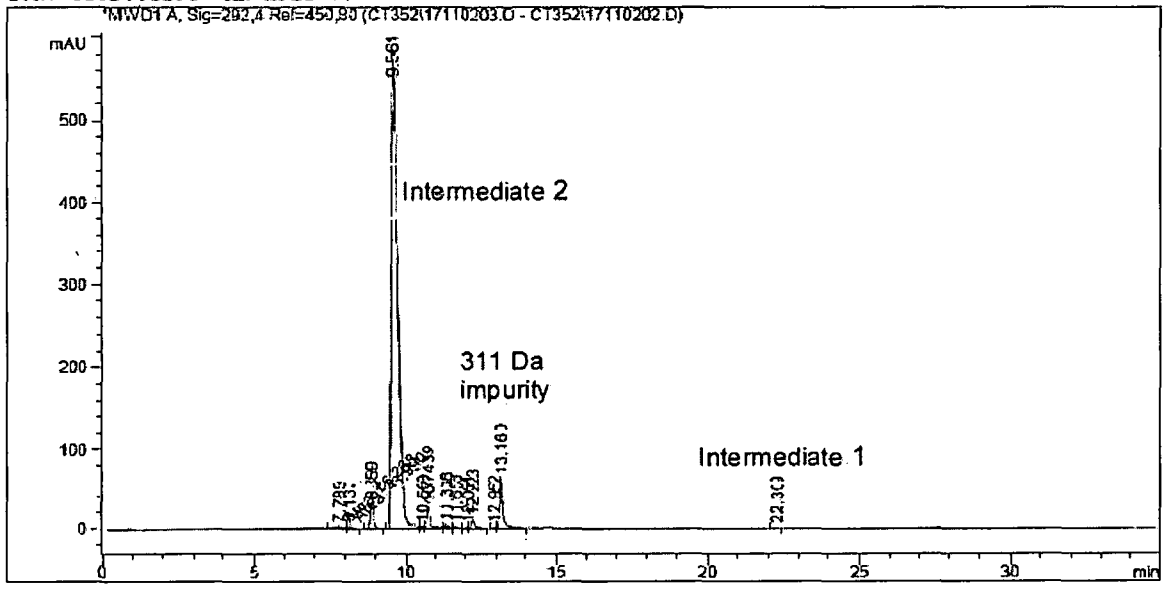

```
==========================================================================
                          Area Percent Report
==========================================================================

Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: MWD1 A, Sig=292,4 Ref=450,80
 Signal has been modified after loading from rawdata file!

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]    [mAU*s]    [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1   7.588 VV    0.1111   12.72750    1.66657   0.1375
   2   7.944 VV    0.1444  110.85382   11.50391   1.1976
   3   8.803 VV    0.1996   51.37194    3.41256   0.5550
   4   9.538 VV    0.2263 8426.00488  563.90912  91.0302
   5  10.225 VV    0.1854   73.37234    5.43923   0.7927
   6  10.563 VV    0.1255   33.84338    3.89016   0.3656
   7  10.753 VV    0.1284   57.00337    6.25205   0.6158
   8  13.129 VV    0.3312  461.64999   51.21633   4.9874
   9  14.785 VV    0.1563   22.38738    1.97364   0.2419
  10  22.292 PV    0.1233    7.06296 8.30015e-1   0.0763

Totals :                  9256.27758  650.09359
```

FIGURE 96

Sintesi di Intermedio 2, S15
Solido finale
ca. 0.27 mg/ml in 0.1% HCO2H H2O/ACN 1/1

```
==========================================================================
                                                  Seq. Line :   4
Sample Name      : S15-solido fin                  Location : Vial 9
Acq. Operator    : Carla Greco                        Inj :   1
Acq. Instrument  : hplc25                      Inj Volume : 5 µl
Acq. Method      : C:\OLDPC-1\DISCOC-1\1METHO~1\CT352.M

Analysis Method : C:\OLDPC-1\DISCOC-1\1METHO~1\CT352ON.M
```

CT352 cond Colonna Zorbax Eclipse XDB-C18 Solvent Saver 150 x 3.0 mm 5 micron P.N. 993967-302
S.N. USTD002295 AN-HPLC-77



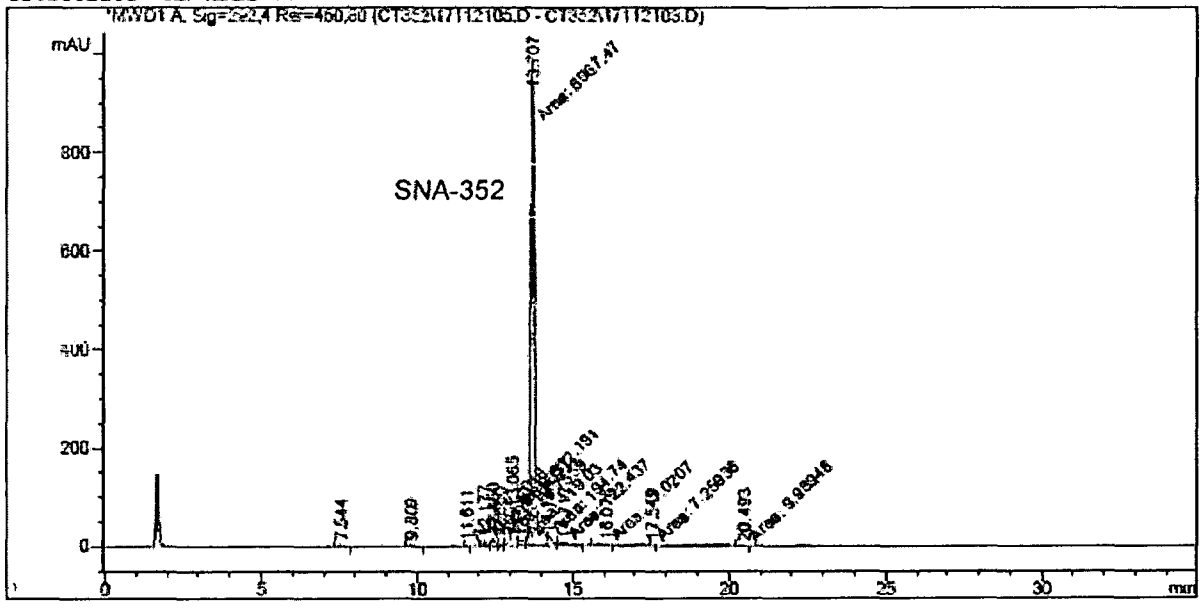

```
==========================================================================
                          Area Percent Report
==========================================================================

Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: MWD1 A, Sig=292,4 Ref=450,80
 Signal has been modified after loading from rawdata file!

Peak RetTime Type  Width     Area      Height     Area
  #   [min]      [min]    [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1   7.789 MF    0.3615   66.26802    3.05505   0.6679
   2   8.137 FM    0.1599   43.99094    4.58628   0.4434
   3   8.859 VB    0.1252  247.46057   29.71457   2.4941
   4   9.561 PV    0.2241 8571.78711  574.25092  86.3919
   5  10.560 VV    0.1096   14.43958    1.96818   0.1455
   6  10.743 VV    0.1162  202.23616   25.59935   2.0383
   7  11.338 VV    0.1225   38.76874    4.50040   0.3907
   8  11.655 VV    0.1621   21.55056    1.82067   0.2172
   9  12.051 VV    0.1496   17.18001    1.54653   0.1732
  10  12.223 VV    0.1407  100.35847   10.03129   1.0115
  11  12.952 VV    0.1016   10.98888    1.65297   0.1108
  12  13.160 VV    0.1315  579.64526   64.09421   5.8420
  13  22.309 BV    0.1291    7.30861 8.11225e-1   0.0737

Totals :                  9921.98291  723.62165
```

FIGURE 97

Sintesi di CT352, S19
P2, 3h45 a ta
ca. 1 goccia di mix in 6 ml di 0.1% HCO2H H2O/ACN 1/1

		Seq. Line :	6
Sample Name	: S1922	Location :	Vial 7
		Inj :	1
Acq. Instrument	: hplc25	Inj Volume :	5 µl
Method	: C:\OLDPC-1\DISCOC-1\1METHO-1\CT352.M		

CT352 Colonna Zorbax Eclipse XDB-C18 Solvent Saver 150 x 3.0 mm 5 micron P.N. 993967-302 S.N. USTD002295 AN-HPLC-77



Area Percent Report

Sorted By	:	Signal
Multiplier	:	1.0000
Dilution	:	1.0000

Use Multiplier & Dilution Factor with ISTDs

Signal 1: MWD1 A, Sig=292,4 Ref=450,80
Signal has been modified after loading from rawdata file!

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	7.544	VV	0.1289	15.37591	1.81349	0.1489
2	9.809	BB	0.1056	29.40324	4.10357	0.2848
3	11.611	MM	0.1133	9.40480	1.38386	0.0911
4	12.177	MF	0.1172	66.98373	9.52835	0.6488
5	12.450	MF	0.1218	103.20093	14.12242	0.9996
6	12.660	MF	0.1502	49.75585	5.24172	0.4819
7	13.065	MF	0.1483	612.19104	68.79339	5.9296
8	13.361	MF	0.1907	119.03000	10.40470	1.1529
9	13.707	MF	0.1512	8967.46680	988.44519	86.8582
10	14.194	MF	0.1339	194.73990	17.61823	1.8862
11	14.717	FM	0.3852	122.43675	5.29801	1.1859
12	16.076	MM	0.3954	17.02065	7.17426e-1	0.1649
13	17.549	MM	0.1020	7.25936	9.92077e-1	0.0703
14	20.493	MM	0.2258	9.98948	7.37431e-1	0.0968
Totals :				1.03243e4	1129.19984	

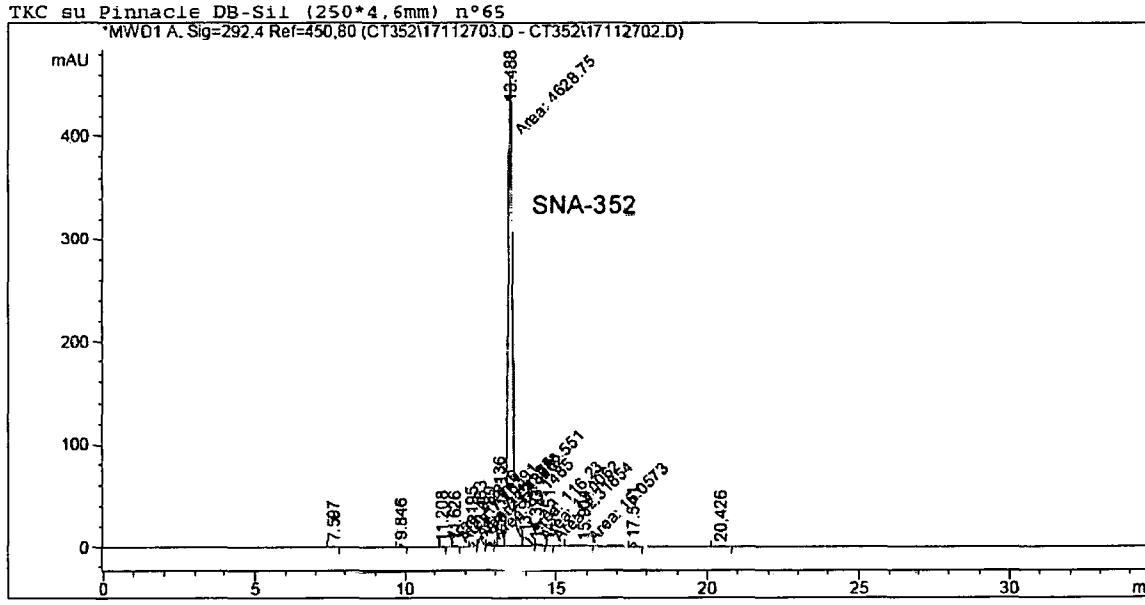
FIGURE 98

Sintesi di CT352, S19
prodotto grezzo
1 goccia in 5 ml in 0.1% HCO2H H2O/ACN 1/1

		Seq. Line :	4
Sample Name	: S19 grezzo	Location :	Vial 10
		Inj :	1
Acq. Instrument	: hplc25	Inj Volume :	5 µl
Acq. Method	: C:\OLDPC~1\DISCOC~1\1METHO~1\CT352.M		

Analysis Method : C:\OLDPC~1\DISCOC~1\1METHO~1\TKC.M

TKC su Pinnacle DB-Si1 (250*4,6mm) n°65



Area Percent Report

Sorted By	:	Signal
Multiplier	:	1.0000
Dilution	:	1.0000

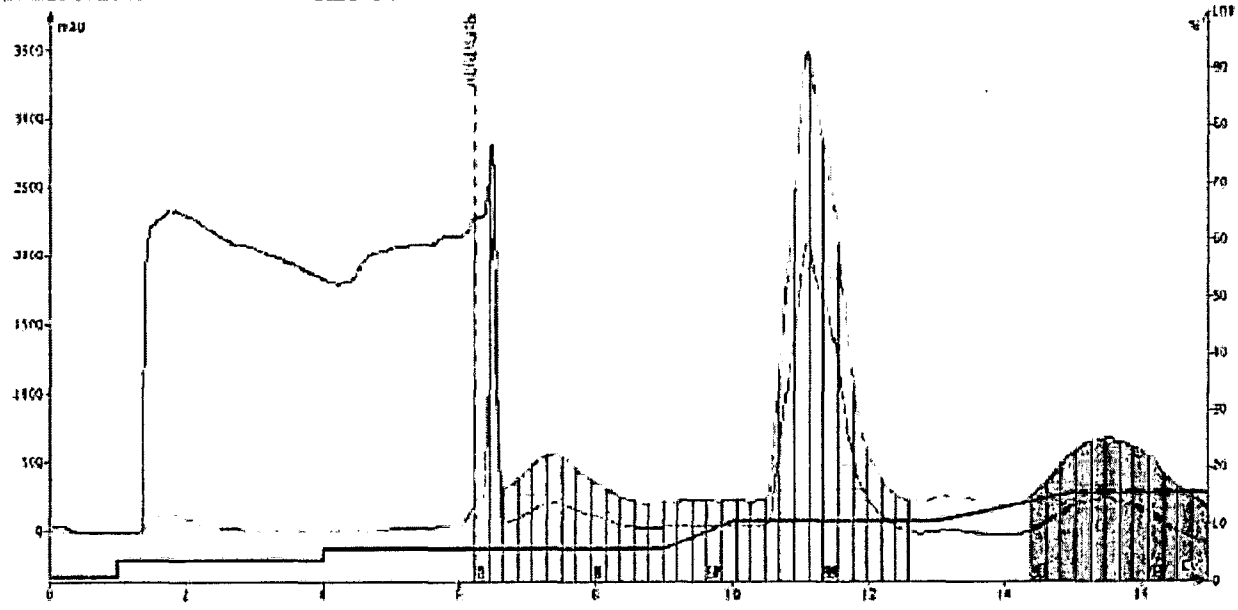
Use Multiplier & Dilution Factor with ISTDs

Signal 1: MWD1 A, Sig=292,4 Ref=450,80
Signal has been modified after loading from rawdata file!

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	7.597	VV	0.1349	8.72940	9.52284e-1	0.1660
2	9.846	BB	0.1011	16.71797	2.52992	0.3178
3	11.208	MM	0.1330	3.23260	4.05101e-1	0.0615
4	11.626	MM	0.1145	7.16391	1.04246	0.1362
5	12.195	MF	0.1155	27.43985	3.95909	0.5217
6	12.463	MF	0.1272	72.89617	9.55417	1.3859
7	12.780	FM	0.1193	33.14960	3.96553	0.6302
8	13.136	MF	0.1391	276.55096	33.13208	5.2578
9	13.488	MF	0.1675	4628.74609	460.56250	88.0018
10	13.965	MF	0.2536	116.22983	7.63789	2.2098
11	14.323	FM	0.1908	13.00619	1.13601	0.2473
12	14.751	MM	0.1058	2.31854	3.65362e-1	0.0441
13	15.904	MM	0.5770	15.05732	4.34939e-1	0.2863
14	17.541	BV	0.1253	32.87804	4.07411	0.6251
15	20.426	PP	0.2127	5.71728	3.77493e-1	0.1087

FIGURE 99

Sample Name	2017-Nov-27 11.39		
Date	2017-Nov-27 11.54		
Method	SNA -352		
Project	SNA_352		
Comment	ca. 91 g di SNA-352 grz lot S19		
Cartridge	SNAP 750g	Detection Mode	UV1+UV2
Flowrate	200 ml/min	UV1 (Collection)	292 nm
Solvent A	Dichloromethane	UV2 (Collection)	210 nm
Solvent B	Methanol	Collect All	On
Rack Type	240 ml		
Max Fraction Volume	210 ml		
Dispense Order	S		
Initial Waste	6.23 CV		



Gradient

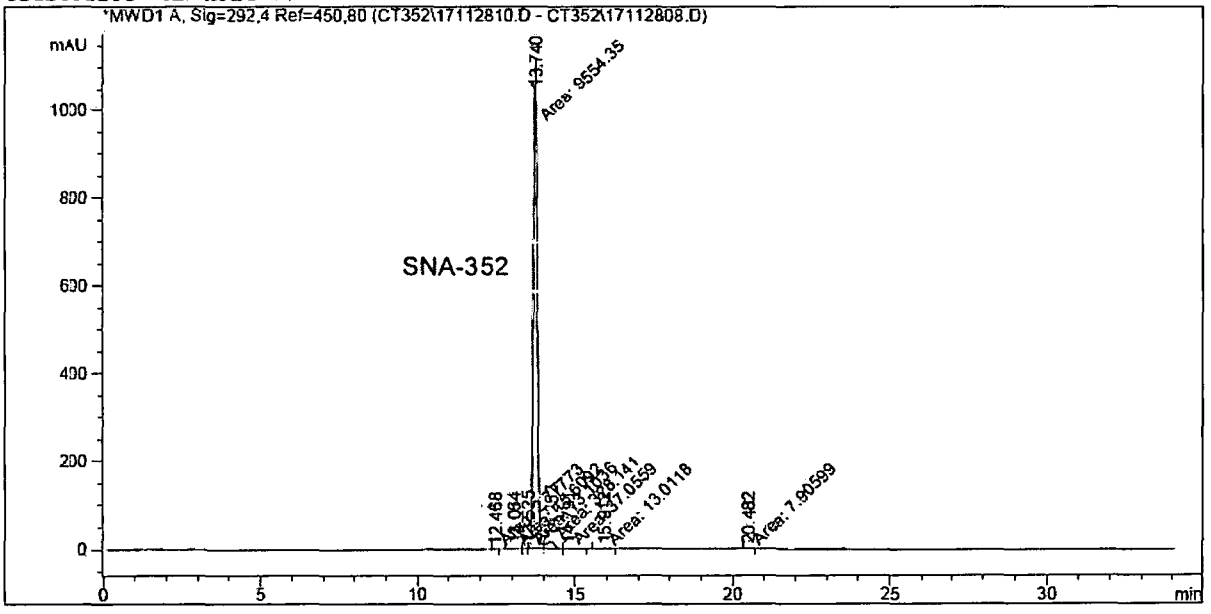
	Solvents	Mix	Length (CV)	
Equil.	A/B	0%	1.5	flowrate 200 ml/min
1	A/B	0%	1.0	
2	A/B	3%	3.0	
3	A/B	5%	5.0	
4	A/B	5% - 10%	1.0	
5	A/B	10%	3.0	
6	A/B	10% - 15%	2.0	
7	A/B	15%	1.9	

FIGURE 100

Purificazione di CT352 su fase diretta, S19
Purl, Lot A
punta di pasteur in 2 ml di 0.1% HCO2H H2O/ACN 1/1

```
==========================================================================
                                                    Seq. Line :  11
Sample Name      : S19Purl Lot A di                  Location : Vial 34
                                                        Inj :   1
Acq. Instrument : hplc25                           Inj Volume : 5 µl
Acq. Method     : C:\OLDPC-1\DISCOC-1\1METHO-1\CT352.M

Analysis Method : C:\OLDPC-1\DISCOC-1\1METHO-1\CT352.M
```

CT352 Colonna Zorbax Eclipse XDB-C18 Solvent Saver 150 x 3.0 mm 5 micron P.N. 993967-302 S.t
USTD002295 AN-HPLC-77



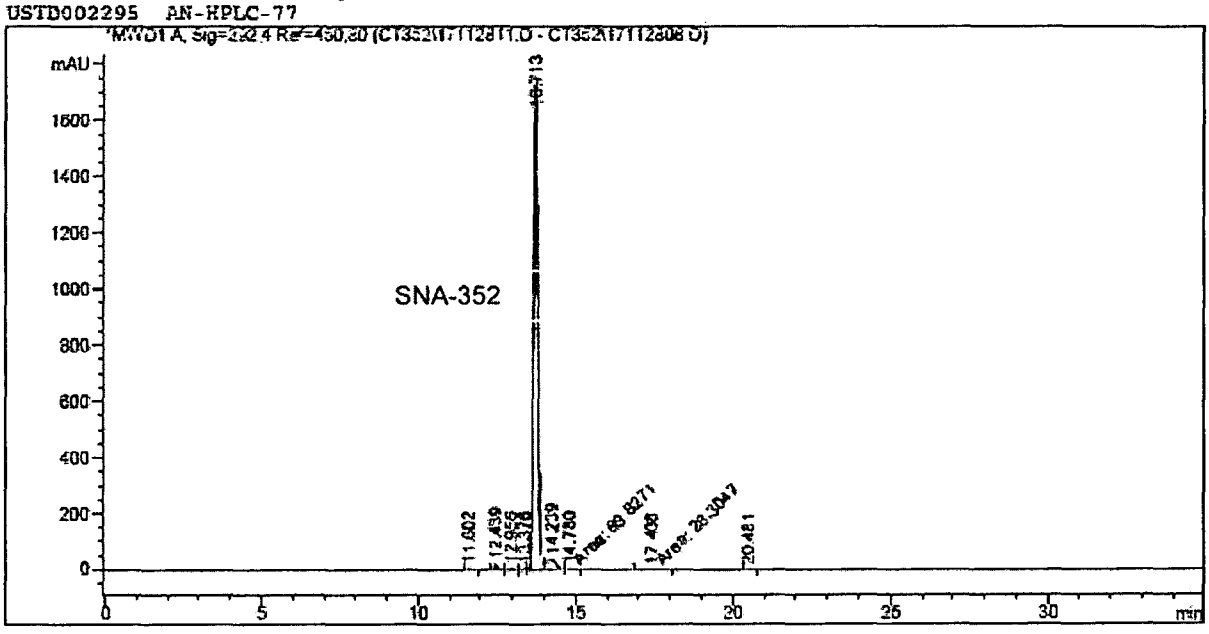

```
==========================================================================
                        Area Percent Report
--------------------------------------------------------------------------

Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: MWD1 A, Sig=292,4 Ref=450,80
 Signal has been modified after loading from rawdata file!

Peak RetTime Type  Width     Area      Height      Area
  #   [min]       [min]   [mAU*s]      [mAU]       %
 ---|-------|----|-------|----------|-----------|--------|
   1  12.468 MM    0.1083    2.77773 4.27378e-1   0.0279
   2  13.064 MF    0.2499   15.60923    1.04102   0.1565
   3  13.525 MF    0.0926   13.10265    2.35040   0.1314
   4  13.740 MF    0.1424 9554.34863 1118.56030  95.8122
   5  14.252 FM    0.3263  328.14099   16.75898   3.2906
   6  14.791 FM    0.3743   37.05594    1.65013   0.3716
   7  15.912 MM    0.4367   13.01178 4.96588e-1   0.1305
   8  20.482 MM    0.1718    7.90599 7.66865e-1   0.0793

Totals :                  9971.95395 1142.05975
```

FIGURE 101

Purificazione di CT352 su fase diretta, S19
Purl, Lot B
punta di pasteur in 3 ml di 0.1% HCO2H H2O/ACN 1/1

```
Sample Name      : S19Purl Lot B                Seq. Line :  12
                                                Location : Vial 35
                                                    Inj :   1
Acq. Instrument  : hplc25                     Inj Volume : 5 µl
Acq. Method      : C:\OLDPC-1\DISCOC-1\1METHO-1\CT352.M

Analysis Method  : C:\OLDPC-1\DISCOC-1\1METHO-1\CT352.M
```

CT352 Colonna Zorbax Eclipse XDB-C18 Solvent Saver 150 x 3.0 mm 5 micron P.N. 993967-302 S.N. USTD002295 AN-HPLC-77



Area Percent Report

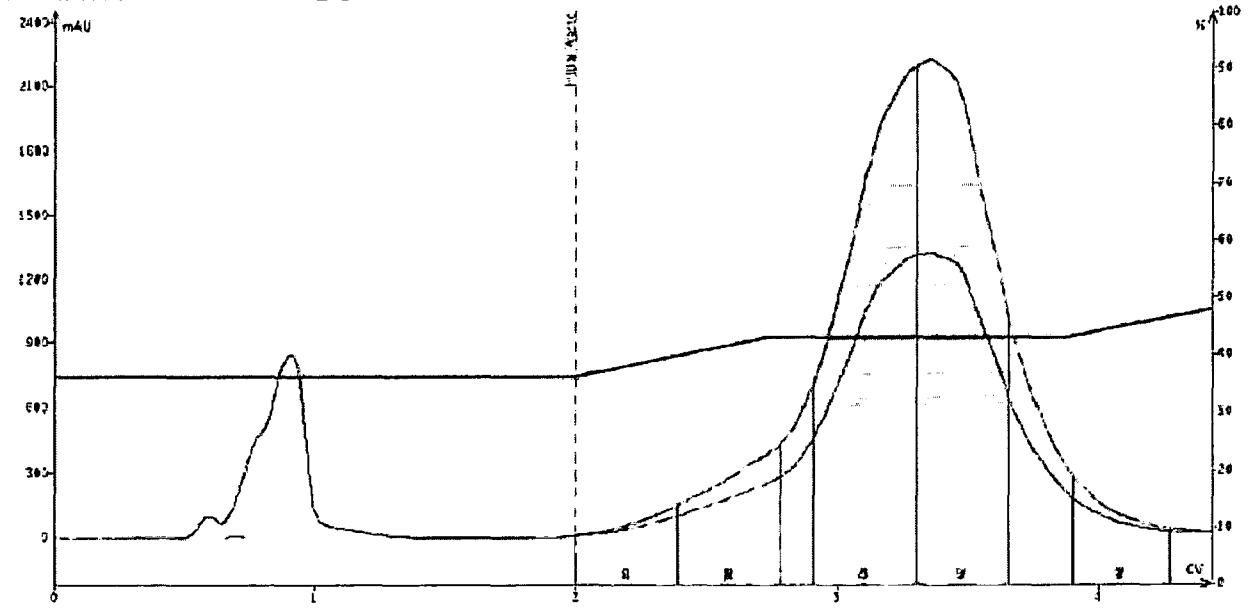
```
Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs
```

Signal 1: MWD1 A, Sig=292,4 Ref=450,80
Signal has been modified after loading from rawdata file!

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	11.602	VB	0.1088	40.44542	5.56237	0.2341
2	12.439	VV	0.1148	147.75142	19.41193	0.8552
3	12.956	VV	0.1823	72.25388	6.09630	0.4182
4	13.376	VV	0.1333	50.38086	5.37846	0.2916
5	13.713	VV	0.1473	1.60720e4	1746.81531	93.0237
6	14.239	VV	0.3162	784.47662	34.38376	4.5405
7	14.780	MF	0.3631	69.82712	3.20482	0.4042
8	17.408	MM	0.6829	28.30468	6.90753e-1	0.1638
9	20.481	BB	0.1813	11.87339	9.06647e-1	0.0687
Totals :				1.72773e4	1822.51035	

FIGURE 102

Sample Name	2017CG14_S19Pur6		
Method	SNA-352 SNAP C18 400 g		
Project			
Comment	ca. 3.6 g di LotA in 9 g H20		
Cartridge	SNAP C18 400g	**Detection Mode**	UV1+UV2
Flowrate	100 ml/min	**UV1 (Collection)**	292 nm
Solvent A	Water	**UV2 (Collection)**	210 nm
Solvent B	Acetonitrile		
Rack Type	240 ml		
Max Fraction Volume	200 ml		
Dispense Order	S		
Initial Waste	2 CV		

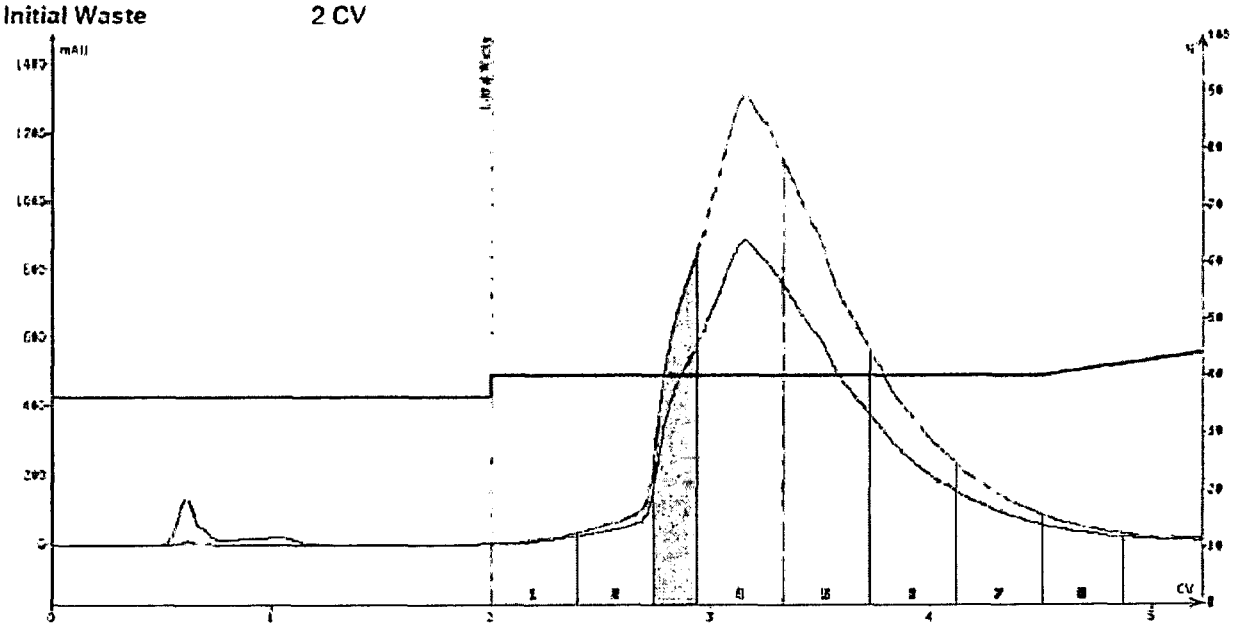


Gradient

	Solvents	Mix	Length (CV)	
Equil.	A/B	36%	1.5	flowrate 100 ml/min
1	A/B	36%	2.0	
2	A/B	36% - 43%	0.7	
3	A/B	43%	1.1	Isocratic Hold Enabled
4	A/B	43% - 48%	0.5	Isocratic Hold Disabled

FIGURE 103

Sample Name	2017CG14-S19Pur10		
Method	SNA-352 SNAP C18 400 g		
Project			
Comment	ca. 3 g lotB in ca. 7ml di H2O		
Cartridge	SNAP C18 400g	**Detection Mode**	UV1+UV2
Flowrate	100 ml/min	**UV1 (Collection)**	292 nm
Solvent A	Water	**UV2 (Collection)**	210 nm
Solvent B	Acetonitrile		
Rack Type	240 ml		
Max Fraction Volume	200 ml		
Dispense Order	S		
Initial Waste	2 CV		



Gradient

	Solvents	Mix	Length (CV)	
Equil.	A/B	36%	1.5	flowrate 100 ml/min
1	A/B	36%	2.0	
2	A/B	40%	2.5	
3	A/B	40% - 44%	0.7	

Log

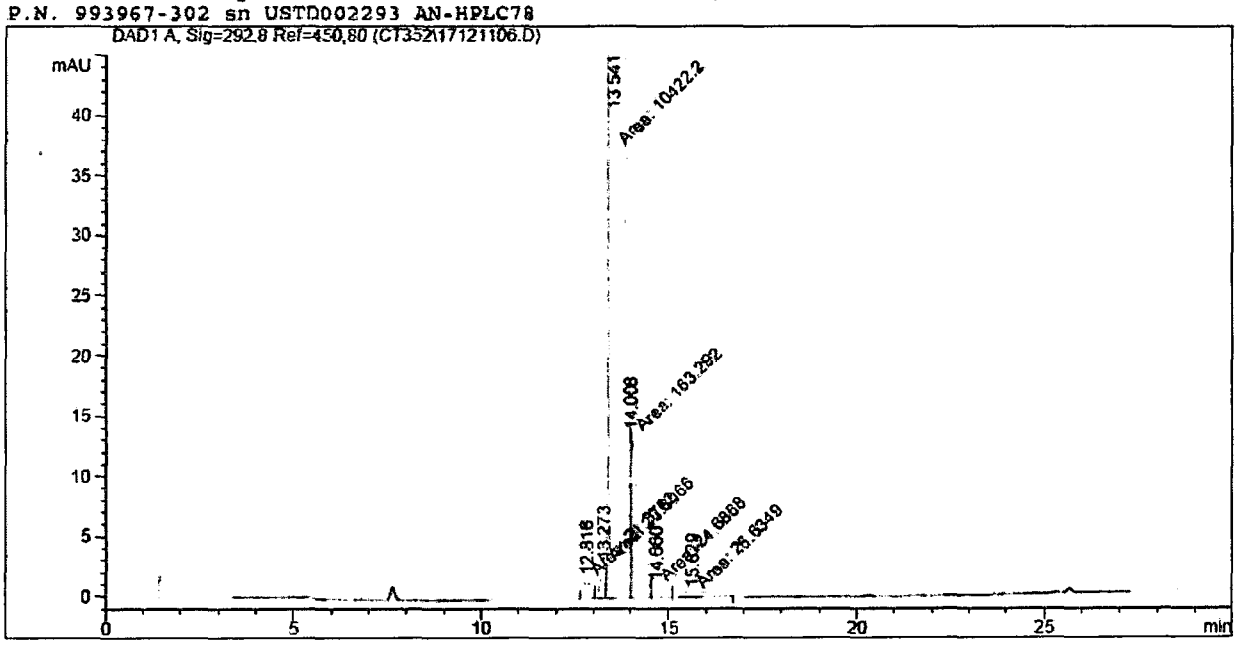
Occurred At	Message
4.9 CV	Collection changed. From : UV1+UV2, Collect All To : UV1+UV2

FIGURE 104

Purezza SNA-352 (Serichim lot 2017CG14/S19) conc. 0.86 mg/mL

Sample Name	: SNA 352 sz.2	Seq. Line	: 4
		Location	: Vial 43
		Inj	: 1
Acq. Instrument	: hplc dad	Inj Volume	: 5 µl
Acq. Method	: C:\HPCHEM\1\METHODS\CT352.M		
Analysis Method	: C:\HPCHEM\1\METHODS\CT352.M		

CT352 Zorbax Eclipse XDB-C18 Solvent Saver 3x150mmx5µm
P.N. 993967-302 sn USTD002293 AN-HPLC78



Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: DAD1 A, Sig=292,8 Ref=450,80

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	12.816	MF	0.2133	21.37618	1.67006	0.2001
2	13.273	FM	0.1589	26.80664	2.81130	0.2509
3	13.541	FM	0.1780	1.04222e4	975.89691	97.5405
4	14.008	MF	0.1500	163.29196	13.56484	1.5282
5	14.660	MF	0.3832	24.68675	1.07369	0.2310
6	15.629	FM	1.0402	26.63487	4.26779e-1	0.2493

Totals : 1.06850e4 995.44357

FIGURE 105

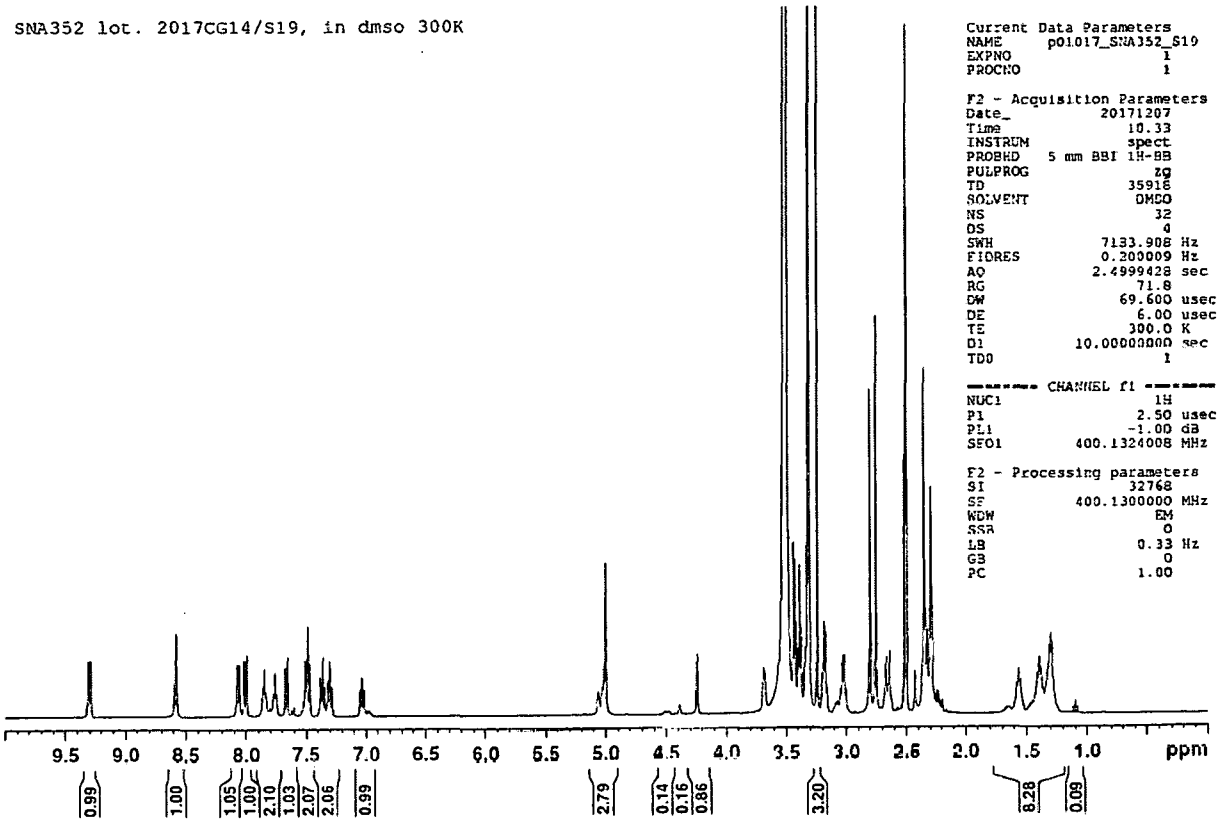

SNA352 lot. 2017CG14/S19, in dmso 300K
Current Data Parameters
NAME p01017_SNA352_S19
EXPNO 1
PROCNO 1
F2 - Acquisition Parameters
Date_ 20171207
Time 10.33
INSTRUM spect
PROBHD 5 mm BBI 1H-BB
PULPROG zg
TD 35918
SOLVENT DMSO
NS 32
DS 4
SWH 7183.908 Hz
FIDRES 0.200009 Hz
AQ 2.4999428 sec
RG 71.8
DW 69.600 usec
DE 6.00 usec
TE 300.0 K
D1 10.00000000 sec
TD0 1
CHANNEL f1
NUC1 1H
P1 2.50 usec
PL1 -1.00 dB
SFO1 400.1324008 MHz
F2 - Processing parameters
SI 32768
SF 400.1300000 MHz
WDW EM
SSB 0
LB 0.33 Hz
GB 0
PC 1.00
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 ppm
0.99 1.00 1.05 1.00 2.10 1.03 2.07 2.06 0.99 2.79 0.14 0.16 0.86 3.20 8.28 0.09

FIGURE 106

TEST REPORT n. 2017/059

SNA-352

Product : *(N-{7-[N-[(9S,10R,11R,13R)-2,3,10,11,12,13-hexahydro-10-methoxy-9-methyl-1-oxo-9, 13-epoxy-1H,9H-diindolo[1,2,3-gh:3',2',1'-lm]pyrrolo[3,4-j][1,7]benzodiazonin-11-yl] (methyl)amino]-7-oxoheptyl}-N'-mPEG$_{2000}$butanediamide)*

Formula : mPEG-$C_{39}H_{43}N_6O_6$ Mol. Weight: 2556.7 (*)

CAS n°: ***** Quantity: 11.2 g

Lot number : 2017CG14/S19

Parameter	Unit	Results	Anal. Meth.
Aspect		Off-white powder	visual
Purity *(HPLC, 292 nm)*	*% area*	97.5	MI CT352 001
Impurities *(HPLC, 292 nm)*			MI CT352 001
Unknown Rrt 0.95	*% area*	0.20	
Unknown Rrt 0.98	*% area*	0.25	
Unknown Rrt 1.03	*% area*	1.53	
Unknown Rrt 1.08	*% area*	0.23	
Unknown Rrt 1.15	*% area*	0.25	

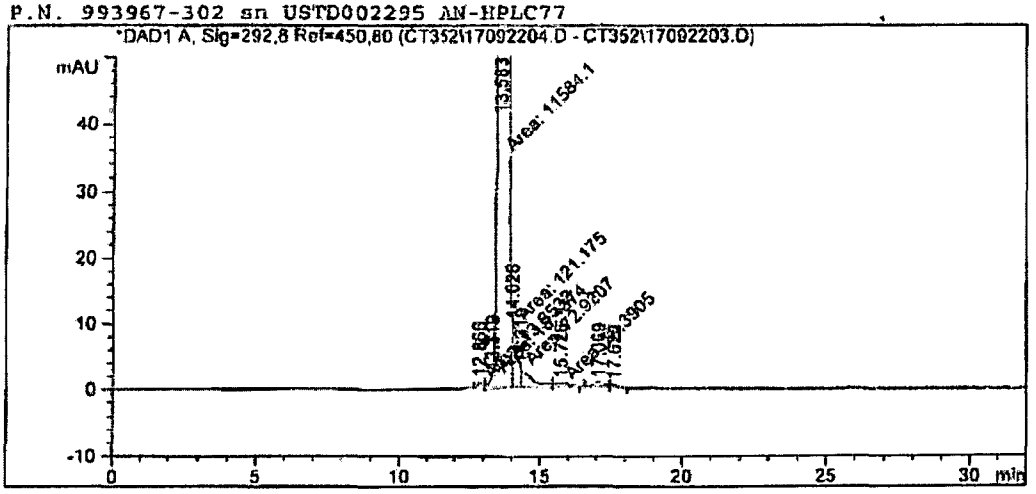
(*) Based on M_w = 1981 Da of MeO-PEG-NH-CO-$(CH_2)_2$-COOH
(**) For the product stored in a freezer at -20°C. Retest date is limited to the parameters reported in this COA. The product is not qualified for clinical testing on humans.

FIGURE 107

Purezza SNA-352 (Serichim lot 2017CG14/S7) conc. 0.93 m g/mL

		Seq. Line :	3
Sample Name	: SNA 352 sz.1	Location :	Vial 2
		Inj :	1
Acq. Instrument	: hplc dad	Inj Volume :	5 µl
Acq. Method	: C:\HPCHEM\1\METHODS\CT352.M		
Analysis Method	: C:\HPCHEM\1\METHODS\CT352.M		

CT352 Zorbax Eclipse XDB-C18 Solvent Saver 3x150mmx5µm
P.N. 993967-302 sn USTD002295 AN-HPLC77



Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: DAD1 A, Sig=292,8 Ref=450,80
Signal has been modified after loading from rawdata file!

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	12.866	MF	0.2216	13.85318	1.04180	0.1168
2	13.319	MF	0.1627	18.43740	1.88866	0.1555
3	13.583	FM	0.1852	1.15841e4	1042.58472	97.7010
4	14.026	MF	0.1568	121.17472	9.73946	1.0220
5	14.319	FM	0.4259	72.92074	3.85378	0.6150
6	15.726	FM	0.6061	20.39047	5.60701e-1	0.1720
7	17.069	PV	0.3597	18.20313	5.97039e-1	0.1535
8	17.626	VB	0.2297	7.60889	4.44517e-1	0.0642
Totals :				1.18567e4	1059.71067	

Results obtained with enhanced integrator!

*** End of Report ***

FIGURE 108

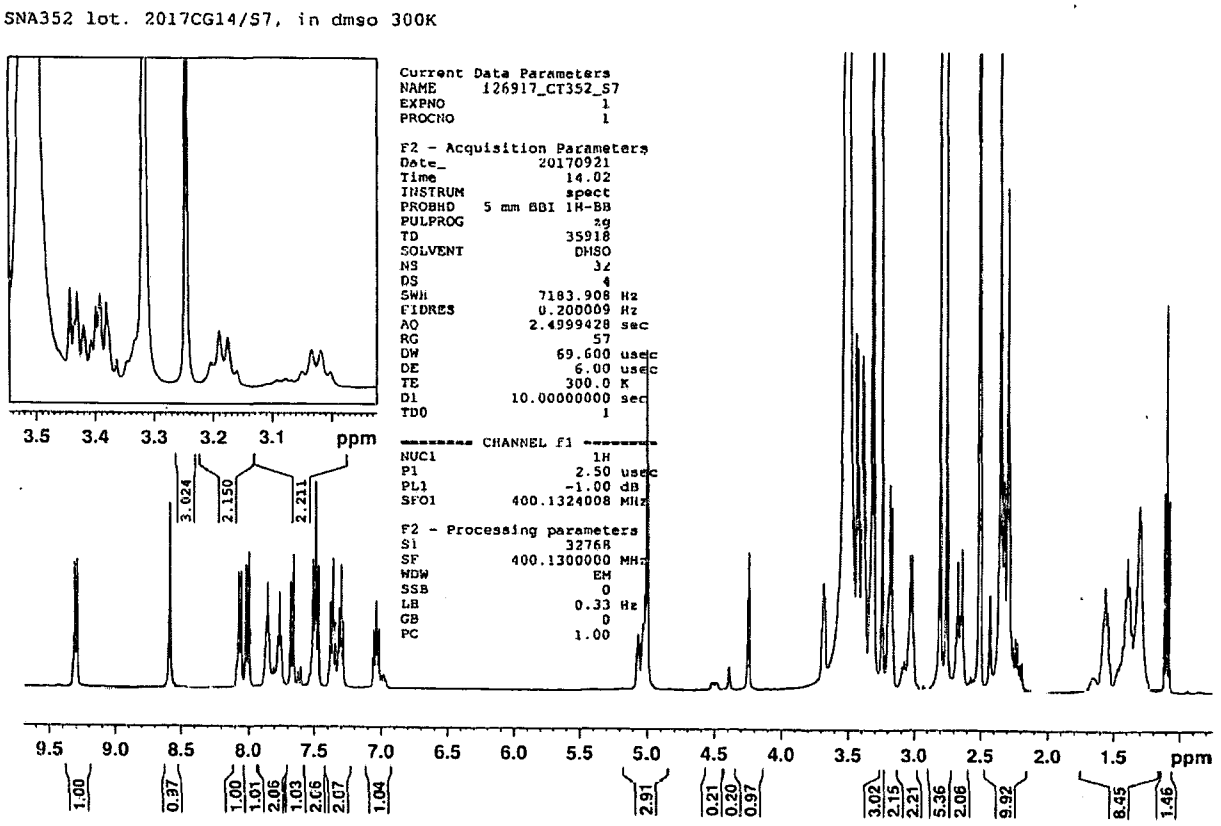

SNA352 lot. 2017CG14/S7, in dmso 300K
Current Data Parameters
NAME 126917_CT352_S7
EXPNO 1
PROCNO 1
F2 - Acquisition Parameters
Date_ 20170921
Time 14.02
INSTRUM spect
PROBHD 5 mm BBI 1H-BB
PULPROG zg
TD 35918
SOLVENT DMSO
NS 32
DS 4
SWH 7183.908 Hz
FIDRES 0.200009 Hz
AQ 2.4999428 sec
RG 57
DW 69.600 usec
DE 6.00 usec
TE 300.0 K
D1 10.00000000 sec
TD0 1
CHANNEL f1
NUC1 1H
P1 2.50 usec
PL1 -1.00 dB
SFO1 400.1324008 MHz
F2 - Processing parameters
SI 32768
SF 400.1300000 MHz
WDW EM
SSB 0
LB 0.33 Hz
GB 0
PC 1.00
3.5 3.4 3.3 3.2 3.1 ppm
3.024
2.150
2.211
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 ppm
1.00
0.97
1.00
1.01
2.06
1.03
2.66
2.07
1.04
2.91
0.21
0.20
0.97
3.02
2.15
2.21
5.36
2.06
9.92
8.45
1.46

FIGURE 109

TEST REPORT n. 2017/044

SNA-352

Product : *(N-{7-[N-[(9S,10R,11R,13R)-2,3,10,11,12,13-hexahydro-10-methoxy-9-methyl-1-oxo-9, 13-epoxy-1H,9H-diindolo[1,2,3-gh:3',2',1'-lm]pyrrolo[3,4-j][1,7]benzodiazonin-11-yl] (methyl)amino]-7-oxoheptyl}-N'-mPEG$_{2000}$butanediamide)*

Formula : mPEG-$C_{39}H_{43}N_6O_6$ Mol. Weight: 2556.7 (*)

CAS n°: ***** Quantity: 0.900 g

Lot number : 2017GC14/S7

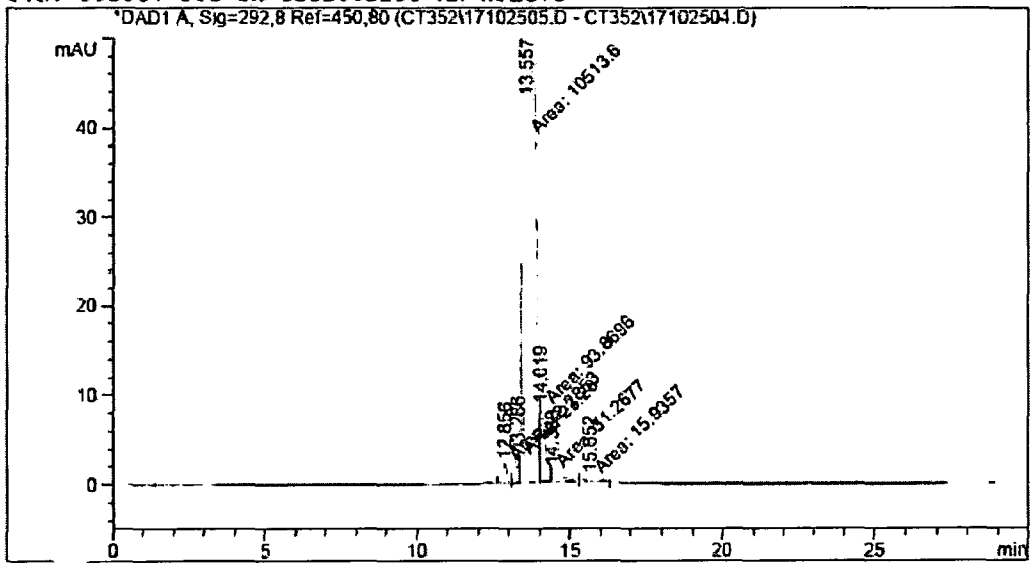
Parameter	Unit	Results	Anal. Meth.
Aspect		Off-white powder	visual
Purity *(HPLC, 292 nm)*	*% area*	97.7	MI CT352 001
Impurities *(HPLC, 292 nm)*			MI CT352 001
Unknown Rrt 0.95	*% area*	0.12	
Unknown Rrt 0.98	*% area*	0.16	
Unknown Rrt 1.03	*% area*	1.02	
Unknown Rrt 1.05	*% area*	0.62	
Unknown Rrt 1.16	*% area*	0.17	
Unknown Rrt 1.26	*% area*	0.15	
Unknown Rrt 1.30	*% area*	0.06	

(*) Based on M_w = 1981 Da of MeO-PEG-NH-CO-$(CH_2)_2$-COOH
(**) For the product stored in a freezer at -20°C. Retest date is limited to the parameters reported in this COA. The product is not qualified for clinical testing on humans.

FIGURE 110

Purezza SNA-352 (Serichim lot 2017CG14/S14) conc.0.90 m
g/mL

```
================================================================================
                                                        Seq. Line :   4
Sample Name      : SNA 352 sz.2                          Location : Vial 13
                                                            Inj :   1
Acq. Instrument : hplc dad                           Inj Volume : 5 µl
Acq. Method     : C:\HPCHEM\1\METHODS\CT352.M

Analysis Method : C:\HPCHEM\1\METHODS\CT352.M

CT352 Zorbax Eclipse XDB-C18 Solvent Saver 3x150mmx5µm
P.N. 993967-302 sn USTD002293 AN-HPLC78
```




```
================================================================================
                            Area Percent Report
================================================================================

Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: DAD1 A, Sig=292,8 Ref=450,80
 Signal has been modified after loading from rawdata file!

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]    [mAU*s]    [mAU]      %
----|-------|----|-------|----------|----------|--------|
   1  12.856 MF    0.1786   22.28531    2.08019   0.2083
   2  13.286 MF    0.1369   23.27996    2.83470   0.2176
   3  13.557 MF    0.1685 1.0513604   929.38947  98.2558
   4  14.019 FM    0.1866   93.86961    8.38213   0.8773
   5  14.389 FM    0.4178   31.26773    1.24717   0.2922
   6  15.652 FM    0.5479   15.93574 4.84723e-1   0.1489

Totals :                  1.07003e4   944.41839

 Results obtained with enhanced integrator!
================================================================================
                            *** End of Report ***
```

FIGURE 111

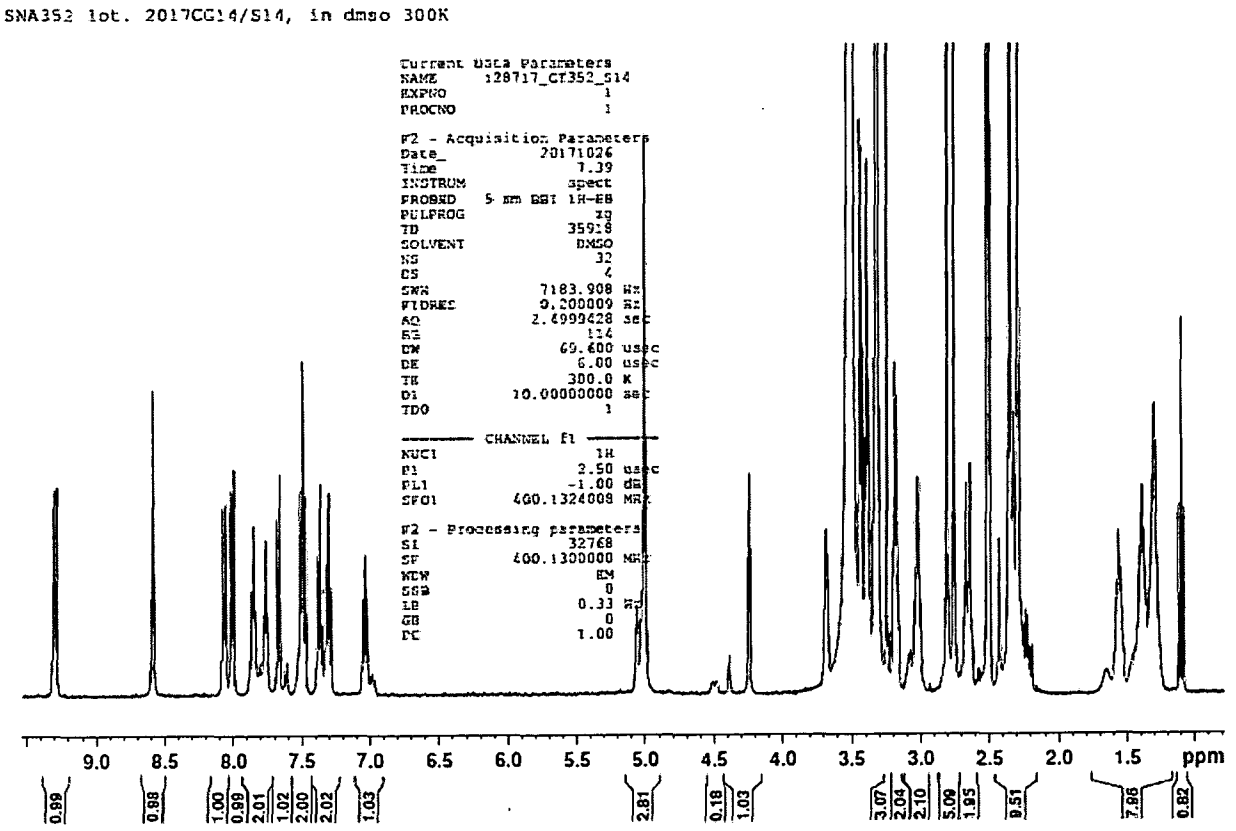

SNA352 lot. 2017CG14/S14, in dmso 300K
Current Data Parameters
NAME 128717_CT352_S14
EXPNO 1
PROCNO 1
F2 - Acquisition Parameters
Date_ 20171026
Time 7.39
INSTRUM spect
PROBHD 5 mm BBI 1H-BB
PULPROG zg
TD 35918
SOLVENT DMSO
NS 32
DS 4
SWH 7183.908 Hz
FIDRES 0.200009 Hz
AQ 2.4999428 sec
RG 114
DW 69.600 usec
DE 6.00 usec
TE 300.0 K
D1 10.00000000 sec
TD0 1
CHANNEL f1
NUC1 1H
P1 2.50 usec
PL1 -1.00 dB
SFO1 400.1324009 MHz
F2 - Processing parameters
SI 32768
SF 400.1300000 MHz
WDW EM
SSB 0
LB 0.33 Hz
GB 0
PC 1.00
9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 ppm
0.99 0.88 1.00 0.99 2.01 1.02 2.00 2.02 1.03 2.81 0.18 1.03 3.07 2.04 2.10 5.09 1.95 9.51 7.86 0.82

FIGURE 112

TEST REPORT n. 2017/053

SNA-352

Product : *(N-{7-[N-[(9S,10R,11R,13R)-2,3,10,11,12,13-hexahydro-10-methoxy-9-methyl-1-oxo-9, 13-epoxy-1H,9H-diindolo[1,2,3-gh:3',2',1'-lm]pyrrolo[3,4-j][1,7]benzodiazonin-11-yl] (methyl)amino]-7-oxoheptyl}-N'-mPEG$_{2000}$butanediamide)*

Formula : **mPEG-$C_{39}H_{43}N_6O_6$** Mol. Weight: **2556.7 (*)**

CAS n°: ***** Quantity: **2.5 g**

Lot number : **2017CG14/S14**

Parameter	Unit	Results	Anal. Meth.
Aspect		Off-white powder	visual
Purity *(HPLC, 292 nm)*	*% area*	98.2	MI CT352 001
Impurities *(HPLC, 292 nm)*			MI CT352 001
Unknown Rrt 0.95	*% area*	0.21	
Unknown Rrt 0.98	*% area*	0.22	
Unknown Rrt 1.03	*% area*	0.88	
Unknown Rrt 1.06	*% area*	0.29	
Unknown Rrt 1.15	*% area*	0.15	

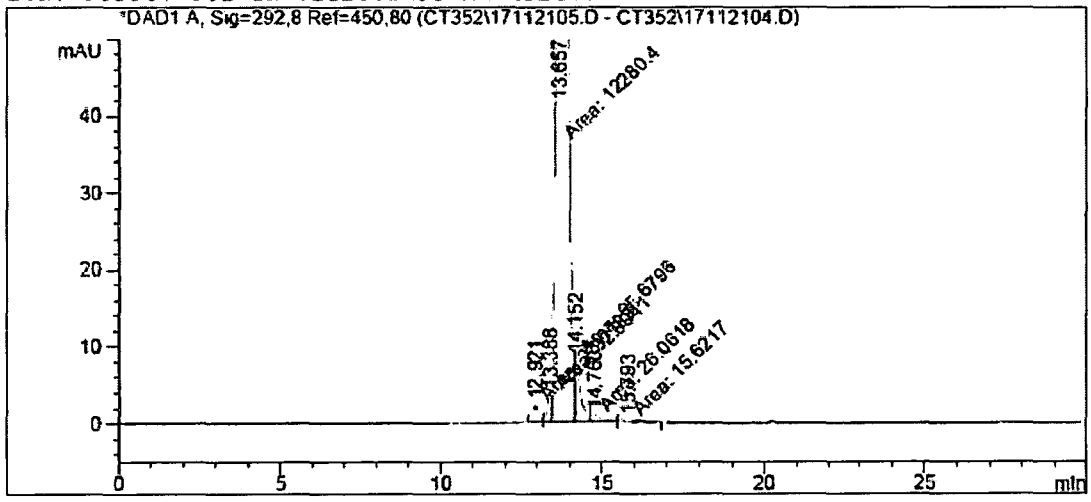
(*) Based on M_w = 1981 Da of MeO-PEG-NH-CO-$(CH_2)_2$-COOH
(**) For the product stored in a freezer at -20°C. Retest date is limited to the parameters reported in this COA. The product is not qualified for clinical testing on humans.

FIGURE 113

Purezza SNA-352 (Serichim lot 2017CG14/S18) conc. 0.90 mg/mL

```
Seq. Line :   4
Sample Name      : SNA 352 sz.2                    Location : Vial 43
                                                       Inj :   1
Acq. Instrument  : hplc dad                     Inj Volume : 5 µl
Acq. Method      : C:\HPCHEM\1\METHODS\CT352.M

Analysis Method  : C:\HPCHEM\1\METHODS\CT352.M
```

CT352 Zorbax Eclipse XDB-C18 Solvent Saver 3x150mmx5µm
P.N. 993967-302 sn USTD002293 AN-HPLC78



Area Percent Report

```
Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs
```

Signal 1: DAD1 A, Sig=292,8 Ref=450,80
Signal has been modified after loading from rawdata file!

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	12.921	MF	0.1989	28.01832	2.34828	0.2245
2	13.388	MF	0.1465	32.99406	3.75284	0.2644
3	13.657	MF	0.1818	1.22804e4	1125.68201	98.4103
4	14.152	FM	0.1895	95.67960	8.41643	0.7667
5	14.760	MF	0.4579	26.06182	9.48622e-1	0.2088
6	15.793	FM	0.5821	15.62168	4.47263e-1	0.1252
Totals :				1.24788e4	1141.59544	

Results obtained with enhanced integrator!

*** End of Report ***

FIGURE 114

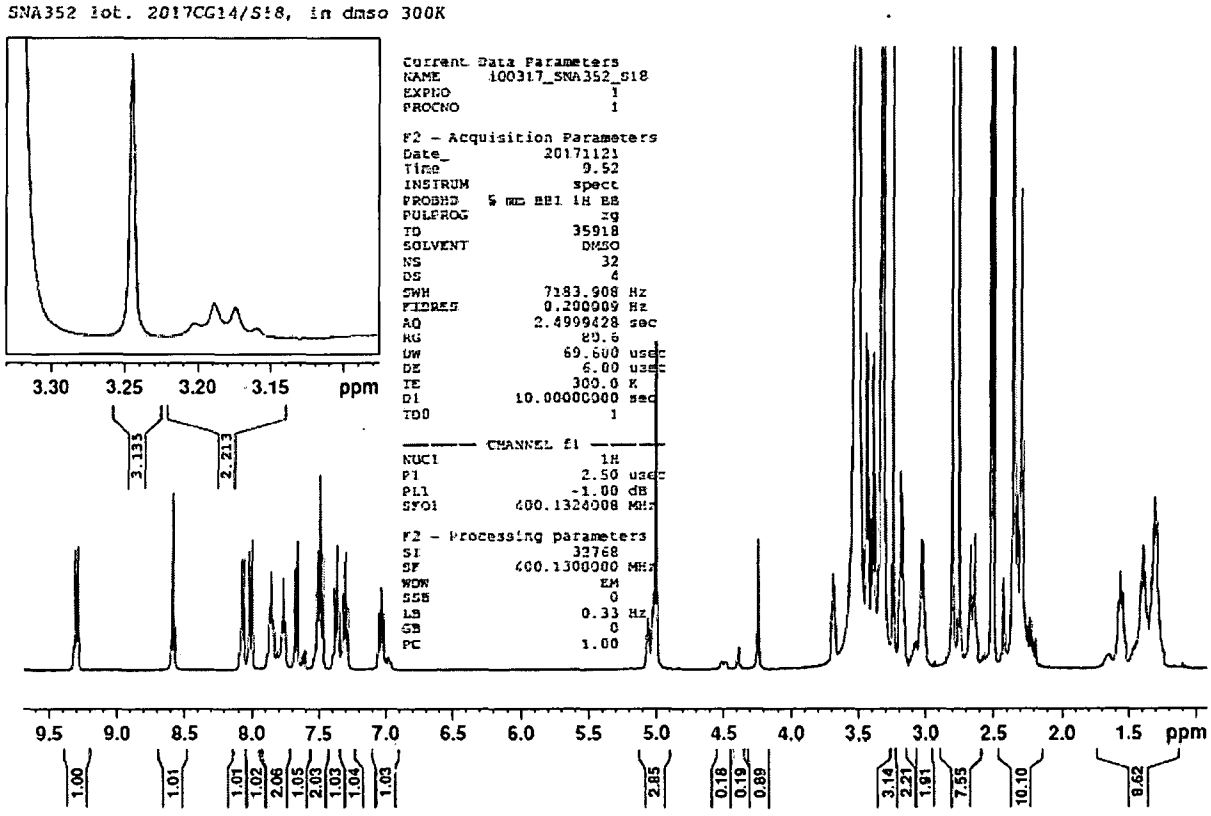

SNA352 lot. 2017CG14/S18, in dmso 300K
Current Data Parameters
NAME 100317_SNA352_S18
EXPNO 1
PROCNO 1
F2 - Acquisition Parameters
Date_ 20171121
Time 9.52
INSTRUM spect
PROBHD 5 mm BB1 1H BB
PULPROG zg
TD 35918
SOLVENT DMSO
NS 32
DS 4
SWH 7183.908 Hz
FIDRES 0.200009 Hz
AQ 2.4999428 sec
RG 20.6
DW 69.600 usec
DE 6.00 usec
TE 300.0 K
D1 10.00000000 sec
TD0 1
CHANNEL f1
NUC1 1H
P1 2.50 usec
PL1 -1.00 dB
SFO1 400.1324008 MHz
F2 - Processing parameters
SI 32768
SF 400.1300000 MHz
WDW EM
SSB 0
LB 0.33 Hz
GB 0
PC 1.00
3.30 3.25 3.20 3.15 ppm
3.135
2.213
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 ppm
1.00
1.01
1.01
1.02
2.06
1.05
2.03
1.03
1.04
1.03
2.85
0.18
0.19
0.89
3.14
2.21
1.91
7.55
10.10
8.62

FIGURE 115

TEST REPORT n. 2017/054

SNA-352

Product : *(N-{7-[N-[(9S,10R,11R,13R)-2,3,10,11,12,13-hexahydro-10-methoxy-9-methyl-1-oxo-9, 13-epoxy-1H,9H-diindolo[1,2,3-gh:3',2',1'-lm]pyrrolo[3,4-j][1,7]benzodiazonin-11-yl] (methyl)amino]-7-oxoheptyl}-N'-mPEG$_{2000}$butanediamide)*

Formula : **mPEG-$C_{39}H_{43}N_6O_6$** Mol. Weight: **2556.7 (*)**

CAS n°: ***** Quantity: **9.6 g**

Lot number : **2017CG14/S18**

Parameter	Unit	Results	Anal. Meth.
Aspect		Off-white powder	visual
Purity *(HPLC, 292 nm)*	*% area*	98.4	MI CT352 001
Impurities *(HPLC, 292 nm)*			MI CT352 001
Unknown Rrt 0.95	*% area*	0.22	
Unknown Rrt 0.98	*% area*	0.26	
Unknown Rrt 1.03	*% area*	0.77	
Unknown Rrt 1.08	*% area*	0.21	
Unknown Rrt 1.15	*% area*	0.13	

(*) Based on M_w = 1981 Da of MeO-PEG-NH-CO-$(CH_2)_2$-COOH
(**) For the product stored in a freezer at -20°C. Retest date is limited to the parameters reported in this COA. The product is not qualified for clinical testing on humans.

FIGURE 116

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2018/023133

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1, 2, 5, 6(completely); 3, 4, 7-67(partially)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2018/023133

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K47/60 A61P11/00 A61P1/00 A61P17/00 A61P27/02
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PENG ET AL.: "STAUROSPORINE INDUCES MEGAKARYOCYTIC DIFFERENTIATION THROUGH THE UPREGULATION OF JAK/STAT3 SIGNALING PATHWAY", ANNALS OF HEMATOLOGY, vol. 90, 2011, pages 1017-1029, XP002781386,	1-40,48, 61,63, 65,67
A	abstract	41-47, 49,51, 53-60, 62,64,66
	----- -/--	

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 May 2018	02/08/2018

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Trifilieff-Riolo, S

3

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MUKTHAVARAM ET AL.: "HIGH-EFFICIENCY LIPOSOMAL ENCAPSULATION OF A TYROSINE KINASE INHIBITOR LEADS TO IMPROVED IN VIVO TOXICITY AND TUMOR RESPONSE PROFILE", INTERNATIONAL JOURNAL OF NANOMEDICINE, vol. 8, 2013, pages 3991-4006, XP002781387,	1-40,48, 50,61,63
A	abstract	41-47, 49,51, 53-60, 62,64,66

Y	TIAN ET AL.: "EFFECT OF STAUROSPORINE ON OUTFLOW FACILITY IN MONKEYS", INVESTIGATIVE OPHTHALMOLOGY AND VISUAL SCIENCE, vol. 40, no. 5, 1999, pages 1009-1011, XP002781388,	1-40,52, 65,67
A	the whole document	41-47, 49,51, 53-60, 62,64,66

Y	MISHRA ET AL: "PEGYLATION IN ANTI-CANCER THERAPY: AN OVERVIEW", ASIAN JOURNAL OF PHARMACEUTICAL SCIENCES,, vol. 11, no. 3, 1 January 2016 (2016-01-01), pages 337-348, XP002781148, the whole document	1-40,48, 50,52, 61,63, 65,67

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1, 2, 5, 6(completely); 3, 4, 7-67(partially)

   compound SNA-352 , its use ot treat following diseases: skin, lung, gastrointestinal system, eye, joint, autoimmune, inflammatory condition

   ---

2. claims: 3, 4, 7-67(all partially)

   conjugate comprising compound 2 as active entity to treat "a cell" or skin, lung, gastrointestinal, eye, joint, autoimmune or inflammatory diseases

   ---

3. claims: 3, 4, 7-67(all partially)

   conjugate comprising compound 3 as active entity to treat "a cell" or skin, lung, gastrointestinal, eye, joint, autoimmune or inflammatory diseases

   ---

4. claims: 3, 4, 7-67(all partially)

   conjugate comprising compound 4 as active entity to treat "a cell" or skin, lung, gastrointestinal, eye, joint, autoimmune or inflammatory diseases

   ---

5. claims: 3, 4, 7-67(all partially)

   conjugate comprising compound 5 as active entity to treat "a cell" or skin, lung, gastrointestinal, eye, joint, autoimmune or inflammatory diseases

   ---

6. claims: 3, 4, 7-67(all partially)

   conjugate comprising compound 6 as active entity to treat "a cell" or skin, lung, gastrointestinal, eye, joint, autoimmune or inflammatory diseases

   ---

7. claims: 3, 4, 7-67(all partially)

   conjugate comprising compound 7 as active entity to treat "a cell" or skin, lung, gastrointestinal, eye, joint, autoimmune or inflammatory diseases

   ---

8. claims: 3, 4, 7-67(all partially)

   conjugate comprising compound 8 as active entity to treat "a

cell" or skin, lung, gastrointestinal, eye, joint, autoimmune or inflammatory diseases

---

9. claims: 3, 4, 7-67(all partially)

conjugate comprising compound 9 as active entity to treat "a cell" or skin, lung, gastrointestinal, eye, joint, autoimmune or inflammatory diseases

---

10. claims: 3, 4, 7-67(all partially)

conjugate comprising compound 10 as active entity to treat "a cell" or skin, lung, gastrointestinal, eye, joint, autoimmune or inflammatory diseases

---

11. claims: 3, 4, 7-67(all partially)

conjugate comprising compound 11 as active entity to treat "a cell" or skin, lung, gastrointestinal, eye, joint, autoimmune or inflammatory diseases

---

12. claims: 3, 4, 7-67(all partially)

conjugate comprising compound 12 as active entity to treat "a cell" or skin, lung, gastrointestinal, eye, joint, autoimmune or inflammatory diseases

---

13. claims: 3, 4, 7-67(all partially)

conjugate comprising compound 13 as active entity to treat "a cell" or skin, lung, gastrointestinal, eye, joint, autoimmune or inflammatory diseases

---

14. claims: 3, 4, 7-67(all partially)

conjugate comprising compound 14 as active entity to treat "a cell" or skin, lung, gastrointestinal, eye, joint, autoimmune or inflammatory diseases

---

15. claims: 3, 4, 7-67(all partially)

conjugate comprising compound 15 as active entity to treat "a cell" or skin, lung, gastrointestinal, eye, joint, autoimmune or inflammatory diseases

---

16. claims: 3, 4, 7-67(all partially)

conjugate comprising compound 16 as active entity to treat "a cell" or skin, lung, gastrointestinal, eye, joint, autoimmune or inflammatory diseases

---

17. claims: 3, 4, 7-67(all partially)

conjugate comprising compound 17 as active entity to treat "a cell" or skin, lung, gastrointestinal, eye, joint, autoimmune or inflammatory diseases

---

18. claims: 3, 4, 7-67(all partially)

conjugate comprising compound 18 as active entity to treat "a cell" or skin, lung, gastrointestinal, eye, joint, autoimmune or inflammatory diseases

---

19. claims: 3, 4, 7-67(all partially)

conjugate comprising compound 19 as active entity to treat "a cell" or skin, lung, gastrointestinal, eye, joint, autoimmune or inflammatory diseases

---

20. claims: 3, 4, 7-67(all partially)

conjugate comprising compound 20 as active entity to treat "a cell" or skin, lung, gastrointestinal, eye, joint, autoimmune or inflammatory diseases

---

21. claims: 3, 4, 7-67(all partially)

conjugate comprising compound 21 as active entity to treat "a cell" or skin, lung, gastrointestinal, eye, joint, autoimmune or inflammatory diseases

---

22. claims: 3, 4, 7-67(all partially)

conjugate comprising compound 22 as active entity to treat "a cell" or skin, lung, gastrointestinal, eye, joint, autoimmune or inflammatory diseases

---

23. claims: 3, 4, 7-67(all partially)

conjugate comprising compound 23 as active entity to treat "a cell" or skin, lung, gastrointestinal, eye, joint, autoimmune or inflammatory diseases

---

24. claims: 3, 4, 7-67(all partially)

conjugate comprising compound 24 as active entity to treat "a cell" or skin, lung, gastrointestinal, eye, joint, autoimmune or inflammatory diseases

---

25. claims: 3, 4, 7-67(all partially)

conjugate comprising compound 25 as active entity to treat "a cell" or skin, lung, gastrointestinal, eye, joint, autoimmune or inflammatory diseases

---

26. claims: 3, 4, 7-67(all partially)

conjugate comprising compound 26 as active entity to treat "a cell" or skin, lung, gastrointestinal, eye, joint, autoimmune or inflammatory diseases

---

27. claims: 3, 4, 7-67(all partially)

conjugate comprising compound 27 as active entity to treat "a cell" or skin, lung, gastrointestinal, eye, joint, autoimmune or inflammatory diseases

---

28. claims: 3, 4, 7-67(all partially)

conjugate comprising compound 28 as active entity to treat "a cell" or skin, lung, gastrointestinal, eye, joint, autoimmune or inflammatory diseases

---

29. claims: 3, 4, 7-67(all partially)

conjugate comprising compound 29 as active entity to treat "a cell" or skin, lung, gastrointestinal, eye, joint, autoimmune or inflammatory diseases

---

30. claims: 3, 4, 7-67(all partially)

conjugate comprising compound 30 as active entity to treat "a cell" or skin, lung, gastrointestinal, eye, joint, autoimmune or inflammatory diseases

---

31. claims: 3, 4, 7-67(all partially)

conjugate comprising compound 31 as active entity to treat "a cell" or skin, lung, gastrointestinal, eye, joint, autoimmune or inflammatory diseases

---

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

32. claims: 3, 4, 7-67(all partially)

conjugate comprising compound 32 as active entity to treat "a cell" or skin, lung, gastrointestinal, eye, joint, autoimmune or inflammatory diseases

---

33. claims: 3, 4, 7-67(all partially)

conjugate comprising compound 33 as active entity to treat "a cell" or skin, lung, gastrointestinal, eye, joint, autoimmune or inflammatory diseases

---

34. claims: 3, 4, 7-67(all partially)

conjugate comprising compound 34 as active entity to treat "a cell" or skin, lung, gastrointestinal, eye, joint, autoimmune or inflammatory diseases

---

35. claims: 3, 4, 7-67(all partially)

conjugate comprising compound 35 as active entity to treat "a cell" or skin, lung, gastrointestinal, eye, joint, autoimmune or inflammatory diseases

---

36. claims: 3, 4, 7-67(all partially)

conjugate comprising compound 36 as active entity to treat "a cell" or skin, lung, gastrointestinal, eye, joint, autoimmune or inflammatory diseases

---

37. claims: 3, 4, 7-67(all partially)

conjugate comprising compound 37 as active entity to treat "a cell" or skin, lung, gastrointestinal, eye, joint, autoimmune or inflammatory diseases

---

38. claims: 3, 4, 7-67(all partially)

conjugate comprising compound 38 as active entity to treat "a cell" or skin, lung, gastrointestinal, eye, joint, autoimmune or inflammatory diseases

---

39. claims: 3, 4, 7-67(all partially)

conjugate comprising compound 39 as active entity to treat "a cell" or skin, lung, gastrointestinal, eye, joint, autoimmune or inflammatory diseases

---

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

40. claims: 3, 4, 7-67(all partially)

conjugate comprising compound 40 as active entity to treat "a cell" or skin, lung, gastrointestinal, eye, joint, autoimmune or inflammatory diseases

---