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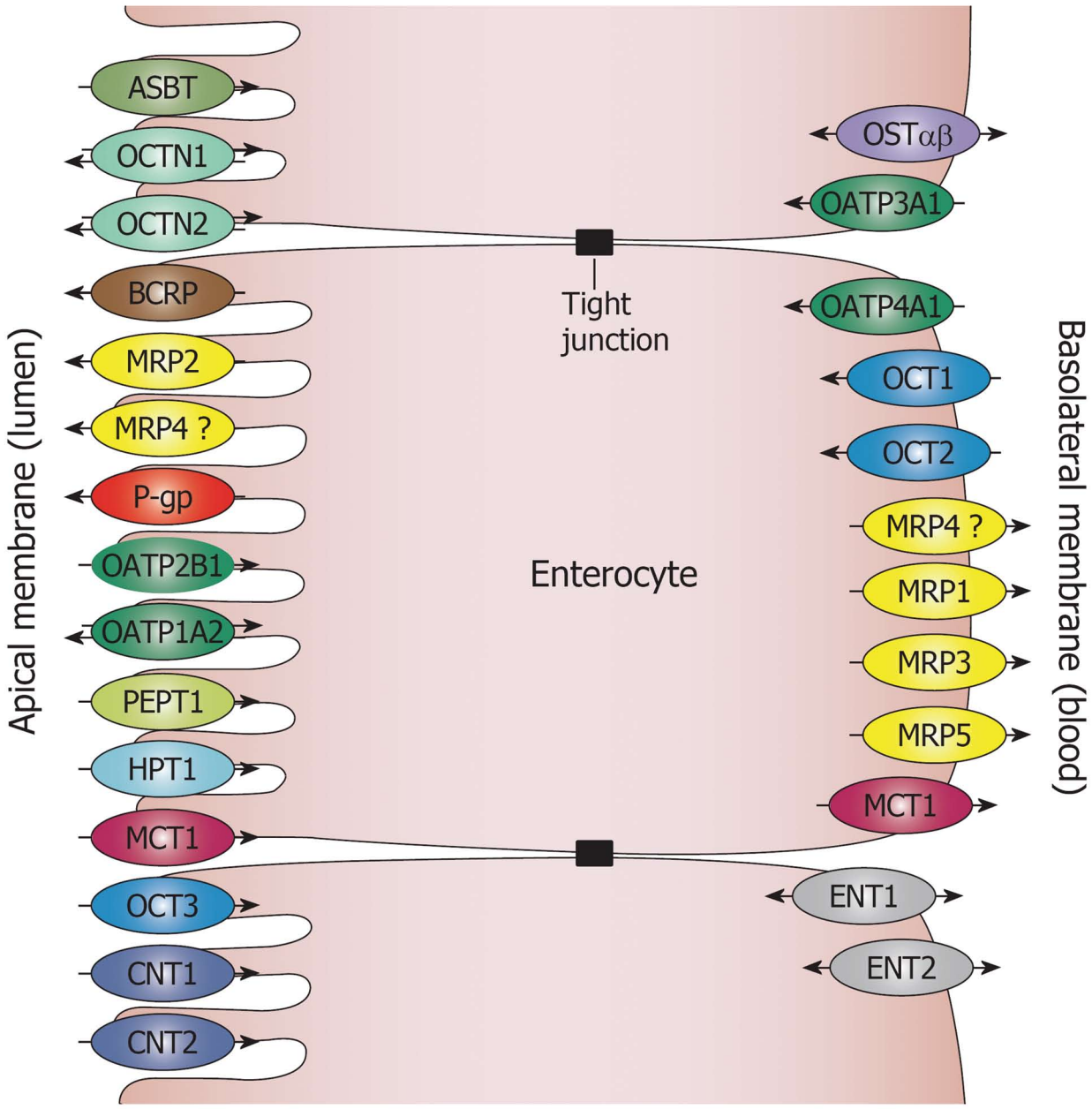

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TOPIC HIGHLIGHT

Ashim K Mitra, Professor, *Series Editor*

Utility of transporter/receptor(s) in drug delivery to the eye

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Abstract

The eye is a highly protected organ, and designing an effective therapy is often considered a challenging task. The anatomical and physiological barriers result in low ocular bioavailability of drugs. Due to these constraints, less than 5% of the administered dose is absorbed from the conventional ophthalmic dosage forms. Further, physicochemical properties such as lipophilicity, molecular weight and charge modulate the permeability of drug molecules. Vision-threatening diseases such as glaucoma, diabetic macular edema, cataract, wet and dry age-related macular degeneration, proliferative vitreoretinopathy, uveitis, and cytomegalovirus retinitis alter the pathophysiological and molecular mechanisms. Understanding these mechanisms may result in the development of novel treatment modalities. Recently, transporter/receptor targeted prodrug approach has generated significant interest in ocular drug delivery. These transporters and receptors are involved in the transport of essential nutrients, vitamins, and xenobiotics across biological membranes. Several influx transporters (peptides, amino acids, glucose, lactate and nucleosides/nucleobases) and receptors (folate and biotin) have been identified on conjunctiva, cornea, and retina. Structural and functional delineation of these transporters will enable more drugs targeting the posterior segment to be successfully delivered topically. Prodrug derivatization targeting transporters and receptors expressed on ocular tissues has been the subject of intense research. Several prodrugs have been designed to target these transporters and enhance the absorption of poorly permeating parent drug. Moreover, this approach might be used in gene delivery to modify cellular function and membrane receptors. This review provides comprehensive information on ocular drug delivery, with special emphasis on the use of transporters and receptors to improve drug bioavailability.

Key words: Anterior segment; Posterior segment; Transporter; Receptor; Eye; Ocular diseases

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DRUG DELIVERY TO THE EYE

The eye is one of the most complex organs in the human body. The eye may be described as being comprised of three distinguishable regions: the outer cornea and sclera; the middle layer, which consists of the iris, ciliary body, and the choroid; and the inner region, or retina (Figure 1). Drug delivery to the eye presents unique challenges due to the complexity of this organ. Based on the route of administration, ocular drug delivery is classified into three types: (1) topical; (2) systemic; and (3) intraocular delivery. Dosage forms such as eye drops, suspensions and ointments are used for topical delivery. Eye drops account for approximately 90% of ophthalmologic market formulations[1,2] and are widely used in the delivery of anesthetics,

antihistamines, β-receptor blockers, non-steroidal anti-inflammatory drugs, parasympatholytics, parasympathomimetics, prostaglandins, steroids, and sympathomimetics[3]. In some cases, eye drops devoid of medications are used for lubricating and tear-replacing solutions.

Ocular bioavailability of drugs following topical administration is significantly less (1%-5%) and hence this route is predominantly used for treatment of anterior segment disorders[4,5]. Most drugs administered topically are washed away rapidly by the nasolachrymal drainage and high tear fluid turnover[6]. Regardless of the low ocular bioavailability, eye drops are widely used because of their affordability and ease in scale up and manufacturing processes.

Systemic administration of drugs is preferred for posterior segment disorders affecting the retina[4]. This involves the administration of drugs as tablets, capsules or intravenous injections. The presence of the blood retinal barrier, which is selectively permeable to more lipophilic molecules, limits the entry of drug molecules into the eye and hence only 1%-2% of administered drug reaches the vitreous cavity[7]. For example, lipid-soluble drugs such as chloramphenicol and minocycline penetrate the blood retinal barrier, while aminoglycosides (amikacin) and β-lactams (cefazolin) used in the treatment of endophthalmitis, do not reach the vitreous in adequate concentrations[8]. This demands frequent administration of high doses, resulting in non-specific absorption and systemic toxicity[9,10]. Intraocular delivery in the form of intravitreal and periocular injections is becoming a popular approach for treatment of posterior segment diseases. Intravitreal administration involves the injection of drug solution/suspension directly into vitreous humor *via* pars plana using a 30 G needle[11]. Unlike topical and systemic routes, intravitreal injection offers high concentrations of drug in the choroid and retina. Nevertheless, intravitreal injections are very painful and are associated with several side effects such as cataract, endophthalmitis and retinal detachment[12]. Periocular injection involves administration of drug *via* peribulbar, posterior juxtascleral, retrobulbar, sub-tenon and subconjunctival routes (Figure 2)[13]. Periocular refers to the region surrounding the eye, and drugs that are placed close to sclera reach the posterior segment by three routes: transcleral (sclera→ choroid→ retina); transcorneal route (tear film → cornea → aqueous humor → lens → vitreous humor); and systemic circulation through the conjunctival and choroidal capillaries[13] (Figure 3). Lee *et al*[14] studied the permeation of radio-labeled mannitol following subconjunctival injection in rabbits. They concluded that direct penetration through the sclera is the primary pathway to the posterior segment, followed by recirculation pathway and transcorneal pathways.

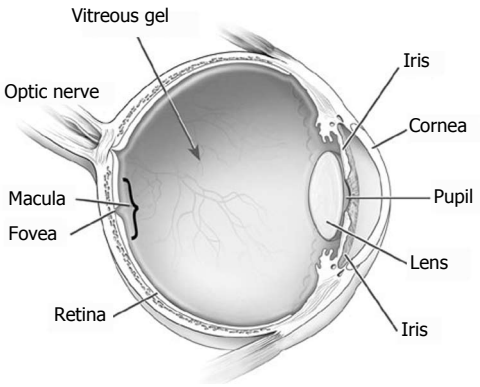

Figure 1 Structure of the eye. Credit: National Eye Institute, National Institutes of Health.

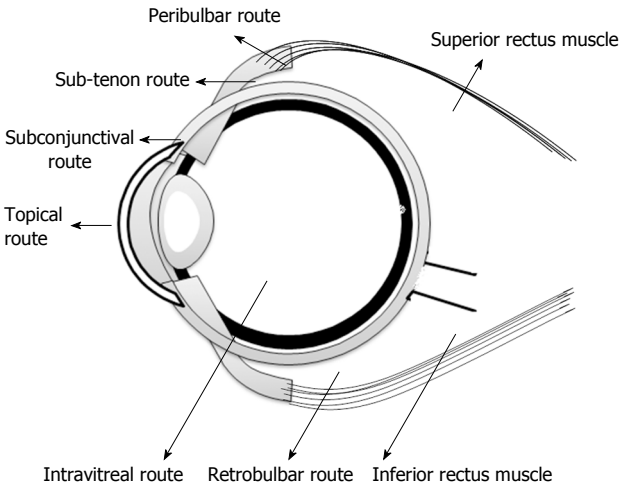

Figure 2 Routes of ocular drug delivery.

CHALLENGES TO OCULAR DRUG DELIVERY

The unique anatomic and physiologic properties of the eye make it a complex organ, offering numerous challenges in developing ocular drug delivery strategies. Due to these constraints, less than 1% of the administered dose is absorbed when conventional ophthalmic forms such as solutions, suspensions, and ointments are applied to the eye[15], and up to 90% of marketed ophthalmic products may be identified as a type of conventional delivery system. This apparent disparity is quite significant and drives the translational research in the area of ocular drug delivery to overcome the unmet needs regarding the treatment of both anterior and posterior segment eye diseases[16]. Poor bioavailability of drugs from ocular dosage forms to the anterior segment is attributed to factors such as solution drainage, lacrimation, tear dilution, tear turnover, nonproductive absorption, poor residence time, and the permeability barrier of the corneal epithelial membrane[17]. Drugs applied topically to the eye can reach the intraocular tissues *via* the corneal and/or non-corneal (conjunctival-scleral) routes[18,19]. Tight junctions present in the apical side of the conjunctival epithelium impede the paracellular transport of hydrophilic substrates through the conjunctiva[20,21]. Thus, a healthy conjunctiva is impervious and impermeable to toxins, microbes, and allergens. However, several hydrophilic molecules have been shown to possess greater permeability through the

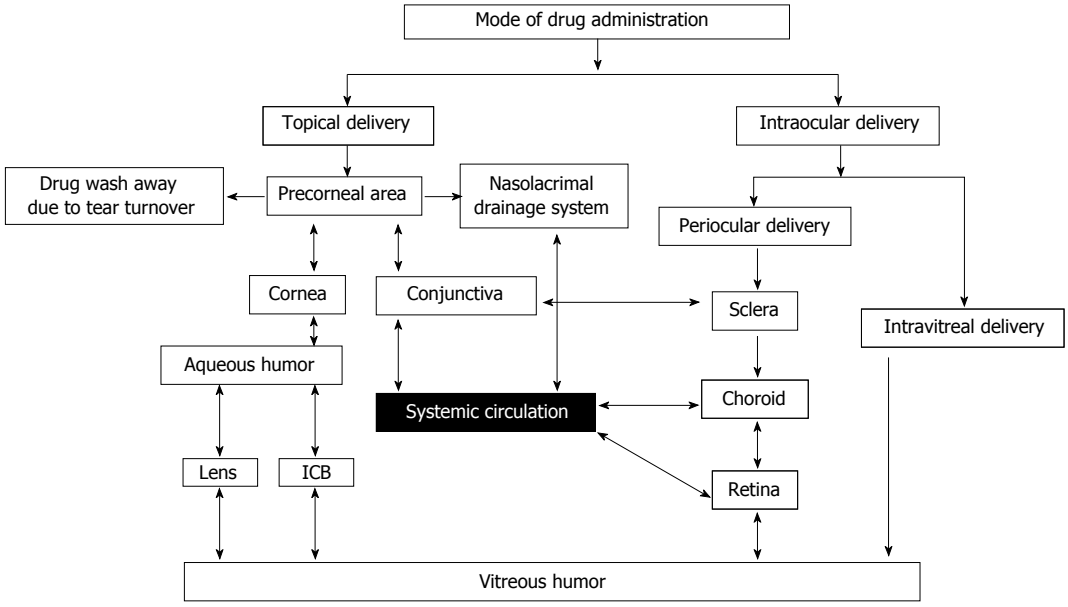

Figure 3 Disposition of drugs in the eye.

non-corneal route than through the corneal route[22-27]. Conversely, a variety of lipophilic molecules were found to preferentially traverse the cornea rather than the non-corneal region[22,28,29]. However, the presence of hydrous stroma in the cornea may hamper permeation of highly lipophilic molecules through the cornea. The passage of molecules through the cornea depends on their lipophilicity, molecular weight, charge, and degree of ionization[18,30,31].

The blood-retinal barrier (BRB) restricts penetration of drugs into the posterior segment when administered systemically or periocularly[32]. Anatomically, two BRB's may be differentiated: the outer BRB, presented by the retinal pigment epithelial (RPE), and the inner BRB, presented by the endothelium of the retinal vasculature[33]. Molecules with optimum membrane permeability characteristics and substrates of one of the membrane transporters can cross the BRB[33-37]. Specialized membrane transporters such as amino acid, dicarboxylate, monocarboxylic acid, nucleoside, organic ion, and peptide transporters channel nutrients, metabolites, and xenobiotics to the retina. Structural and functional delineation of these transporters will enable more drugs targeting the posterior segment to be successfully delivered topically.

ROLE OF EFFLUX PUMPS IN OCULAR DRUG DELIVERY

Yet another barrier that can affect the ocular bioavailability is the presence of efflux transporters such as P-glycoprotein (P-gp) and multidrug resistance-associated protein (MRP)[38-42]. Several substrates have been identified for the efflux transporters expressed in *in vitro* cell culture models[40,43-46]. Drug resistance mediated by efflux transporters is quite common in the area of cancer research. Efflux transporters such as P-gp and MRP are members of the ATP binding cassette (ABC) transporter family that utilizes ATP for translocation of various substrates across membranes[47]. Efflux transporters prevent the entry of toxic substances into the cells and aid in the healthy state of cells. Earlier investigation by Dano in 1973 described the evidence of efflux transporter resulting in drug resistance in Ehrlich astrocytes[48]. Later, P-gp was identified in the multidrug resistant cells and found to be responsible in the efflux of various cancer drugs like paclitaxel and doxorubicin[49]. P-gp, a transmembrane glycoprotein (approximately 170 kDa) with 10-15 kDa of N-terminal glycosylation, binds to the drug molecules and transports them out of the cell utilizing ATP hydrolysis. P-gp has wide substrate specificity for several drug classes including steroids, cardian glycosides, glucocorticoids, non-nucleoside reverse transcriptase inhibitors, protease inhibitors and immunosuppressive drugs[50]. Ocular drug resistance is a relatively new science, and presence of efflux transporters on various ocular tissues like cornea, conjunctiva, iris and retina was not known until recently. Efflux transporters have been identified extensively in major organs like the small intestine, kidney and liver, and their implication in drug delivery is well known[51]. However, the knowledge and relative expression of efflux pumps in ocular tissues is very limited, and the data published so far is limited to cell lines and lower species. These efflux transporters prevent the entry of several drug molecules into the eye (Figure 4). P-gp is expressed on the corneal epithelium[48,50], conjunctival epithelial cells[52], iris and ciliary muscle cells[53], retinal capillary endothelial cells[54], retinal pigmented epithelial cells[55,56], and ciliary non-pigmented epithelium[57]. The expression of P-gp on cornea can significantly modulate the absorption of topically administered drugs. Dey *et al*[58] studied the ocular absorption of $^{[14C]}$ erythromycin in the presence and absence of P-gp inhibitors. In the presence of P-gp inhibitors such as testosterone, verapamil, quinidine, and

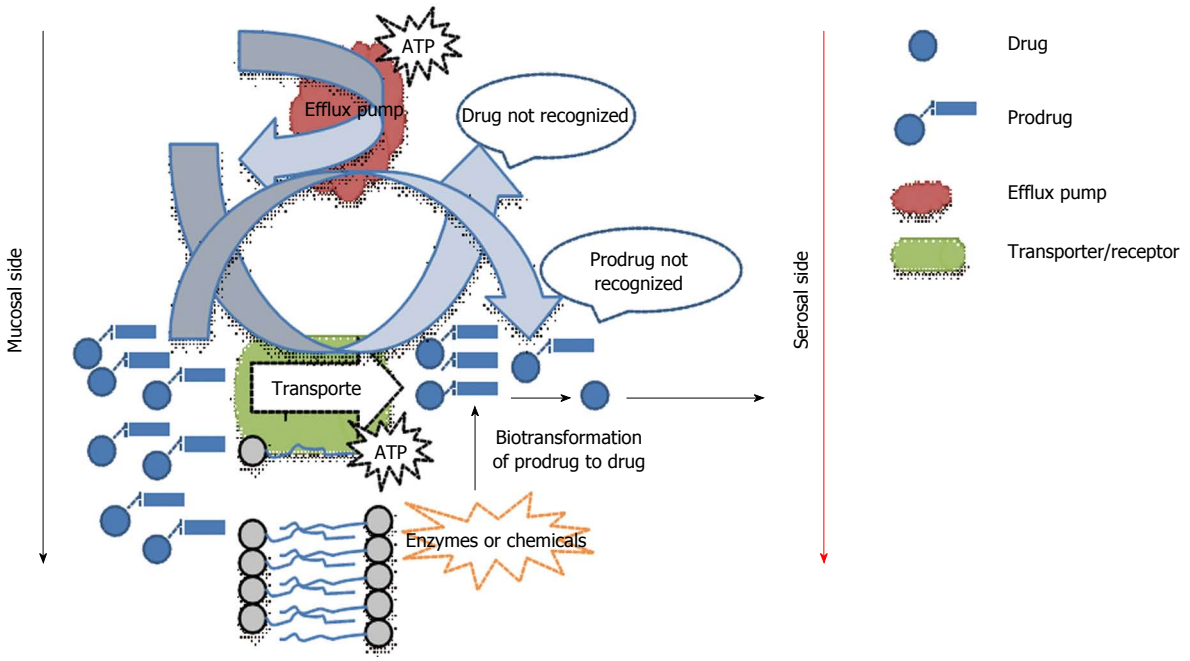

Figure 4 Role of efflux and influx transporters in ocular absorption.

cyclosporine A, the ocular bioavailability of [14C] erythromycin was significantly enhanced, indicating the role of P-gp in ocular absorption of topically applied drugs. MRP is another major class of ABCC efflux transporter leading to drug resistance, and the MRP family has nine members (MRP 1-9) with varying substrate specificity[59]. MRPs are organic anion transporters and they play a vital role in the transport of anionic and neutral drugs conjugated to acidic ligands. So far, isoforms of the MRP family have been identified on ocular tissues. MRP1 expression was identified in rabbit conjunctival epithelial cells[60] and RPE cells[61], while MRP2 and MRP5 expression was identified in corneal epithelium[62,63]. In a recent study, Vellonen *et al*[64] compared expression of efflux proteins [MDR1 (*ABCB1*), MRP1-6 (*ABCC1-6*), and BCRP (*ABCG2*)] in normal human corneal epithelial tissue, primary human corneal epithelial cells (HCEpiC), and corneal epithelial cell culture model (HCE model) based on human immortal cell line. They concluded that BCRP, MRP1, and MRP5 are expressed in the corneal epithelium, while MDR1, MRP2, MRP3, MRP4, and MRP6 are not significantly expressed. Conflicting results have been observed with the expression profile of the efflux transporters in various ocular tissues, especially the human corneal epithelium. Nevertheless, a wide array of ocular drugs including antibiotics, sulfated steroids, macrolides (azithromycin and erythromycin), and quinolones (ciprofloxacin and grepafloxacin) has been proven to be substrates for these efflux pumps, which deter their ocular bioavailability[65]. To a large extent, the role of efflux pumps in ocular drug resistance remains to be explored. Although the functional significance of efflux pumps in the eye have not been elaborated completely, one may reasonably assume that they present another strategy for defending the eye from potential harm due to toxic metabolites and other external harmful molecules. P-gp and MRP have been found to be expressed on several ocular tissues[66-68]. As more research evolves in this area, formulations that contain substrates of these efflux pumps may offer opportunities to enhance the ocular bioavailability by co-administration of efflux pump inhibitors[41,69-72].

TRANSPORTER/RECEPTOR MEDIATED DRUG DELIVERY

The eye is a highly compartmentalized organ with several anatomical and physiological barriers. The partial barriers that isolate the eye from the rest of the body impede the effective passage of many drugs[73]. Over the past two decades, several efforts have been made to increase the ocular bioavailability of drugs by enhancing the contact time of drugs with the target tissue, without compromising patient compliance[74,75]. Ophthalmic drug molecules should possess optimum hydrophilicity and lipophilicity for two reasons: (1) to facilitate the formulation of eye drops/injections; and (2) to allow sufficient permeability across the anatomical barriers such as corneal epithelium, choroid, retinal pigment epithelium. Drugs with an octanol/buffer distribution coefficient in the range of 100-1000 are considered to be optimum for corneal absorption[76,77]. Unfortunately, the buffer distribution coefficient of most drugs does not fall within this range, requiring the development of novel drug delivery strategies such as bioadhesive hydrogels, micro- and nanoparticles, liposomes and collagen shields. The prodrug approach is a more traditional, promising, and less expensive method for achieving the desired solubility and lipophilicity. This approach involves chemical modification of drug molecules using promoieties to improve their physicochemical properties[78].

The selection and linkage of pro-moieties depend on the metabolic enzymes, and after absorption, the prodrugs are subject to enzymatic hydrolysis resulting in the active parent drug. The bioreversion rate of the prodrug depends on affinity of prodrug linkage towards hydrolyzing enzyme(s), mainly esterases/peptidases and the turnover rate of the enzyme. Lipophilic chemical modification has been used successfully to improve their ocular bioavailability of various hydrophilic drugs[79,80]. For example, the bioavailability of ganciclovir (hydrophilic drug) after oral administration is 6%. This necessitates the use of high systemic doses of ganciclovir for attaining therapeutic concentrations in the eye, which gradually results in systemic toxicity. Intravitreal injections (0.2-0.4 mg) minimize systemic toxicity and increase the vitreal concentrations of ganciclovir; however, they are associated with patient noncompliance, and rapid elimination of vitreal ganciclovir (elimination $t_{1/2}$: approximately 13 h in humans) requires repeated intravitreal injections, leading to side effects like retinal detachment, endophthalmitis, and vitreal hemorrhage. Short-chain carboxylic mono- and di-esters of ganciclovir, especially aminobutyrate ester of ganciclovir, exhibited maximum stability, optimum lipophilicity and sufficient solution stability at neutral or slightly acidic pH (4.0-7.0) and excellent activity against various herpes viruses such as HSV-2 and VZV[81]. This study highlights the use of the prodrug approach in enhancing the ocular bioavailability of ganciclovir without compromising the antiviral activity. More recently, the progress in molecular cloning of transporter genes led to the identification of membrane transporters/receptors that play an important role in transferring exo- and endogenous nutrients[82]. Despite the high vascularity of the retina, blood retinal barriers regulate the movement of nutrients between circulation and neural retina[83]. Therefore, most nutrients are transported into the retinal cells by specific transport/receptor systems[84]. Identification of such membrane transporters/receptors including peptide, amino acids, nucleoside and nucleobase, glucose, monocarboxylic acid, organic anion and organic cation transporters, led to the development of prodrugs for poorly permeating drug molecules[85]. Transporter-targeted prodrugs offer several advantages including: (1) improving the stability of parent drug molecule; (2) altering the physicochemical properties such as solubility and lipophilicity; (3) improving the pharmacokinetics properties; and (4) improving the permeability of drugs as the prodrugs become substrates for the influx transporters and simultaneously evade the efflux pumps (Figure 4). Table 1 presents the list of transporter/receptor(s)in the eye.

Peptide transporter

Peptide transporters are among the most versatile membrane carrier systems with a wide range of substrate specificity. They are classified into three types: PepT1, PepT2 and peptide/histidine transporters (PHT1 and PHT2), with difference in their substrate specificity, transport capacity and affinity[86]. PepT1 belongs to solute carrier family 15 member 1 (SLC15A1) that is encoded in humans by the SLC15A1 gene. PepT1 is a low-affinity proton coupled transporter responsible for the translocation of di- and tri-peptides[87,88]. PepT2, another proton-coupled oligopeptide transporter belonging to the same family,is a high-affinity transporter that is responsible for the translocation of small peptides, β-lactam antibiotics and other peptidomimetic drugs[89]. PepT1 is predominantly expressed on the intestine and helps in the absorption of protein digestion products, while PepT2 is mainly expressed on the brain and kidney. The peptide/histidine transporters PHT1 and PHT2 are expressed on the lysosomal membrane of cells and are responsible for the efflux of histidine and small peptides from the lysosomes into the cytoplasm. The presence of an oligopeptide transport system on the corneal epithelium was identified by Anand *et al*[90] by studying the transport mechanism of L-valyl ester of acyclovir (L-val-ACV) across rabbit cornea in the presence of competitive inhibitors for human peptide transporter (hPepT1). Transcorneal permeation of L-val-ACV was approximately threefold higher across the intact rabbit cornea than ACV. Substrates of hPepT1 such as dipeptides, angiotensin converting enzyme inhibitors, and β-lactam antibiotics significantly inhibited the transport of L-val-ACV, indicating the presence of a carrier-mediated transport system specific for peptide. The oligopeptide transporter on the rabbit cornea opened up new avenues for the development of transporter-targeted prodrugs. Later, the same group evaluated the antiviral efficacy of val-val-ACV against herpetic epithelial and stromal keratitis. They concluded that val-val-ACV demonstrated higher water-solubility than ACV and lower cytotoxicity than trifluorothymidine. Val-val-ACV also showed excellent activity against HSV-1 in the stromal keratitis models and rabbit epithelia[91]. Peptide transporters are also expressed on the basolateral side of retina and neural retina[92,93]. The role of peptide transporters in the vitreal clearance of cephalexin, a peptide transporter substrate, was investigated using a dual probe microdialysis technique in the presence of glycyl-proline[92]. Co-administration of gly-pro increased the vitreal half-life and AUC of cephalexin, suggesting the involvement of peptide transporters in the clearance of cephalexin from the posterior chamber. Later studies performed by Majumdar *et al*[94] investigated the expression of peptide transporters on the retina. *Ex-vivo* uptake in excised rabbit retina/choroid tissues and *in vivo* retinal uptake using [^{3}H] gly-sar and peptidomimetics demonstrated the functional presence of peptide transporter on the retina. Berger *et al*[93] studied the distribution of peptide transporter (PepT2) in the retinal Müller glial cells of the rat nervous system. Peptide transporter facing the vitreous humor can be targeted following intravitreal administration of prodrugs to achieve higher drug levels in the retina. Identification and characterization of transporters on the basolateral side of RPE is relatively difficult. Some researchers have tried to identify these transporters following systemic administration of peptide substrates and measuring the vitreous

humor concentrations in the presence and absence of competitive inhibitors. For example, Dias *et al*[95] studied the ocular penetration of ACV and its peptide prodrugs val-ACV and val-val-ACV following systemic administration in rabbits using microdialysis. The anterior segment area under curve values of ACV, val-ACV and val-val-ACV were 53.70 (± 35.58), 139.85 (± 9.43) and 291.05 (± 88.13) min × μmol/L, respectively. However, the drug concentration in vitreous humor was below the detection limit. The same group studied the mechanism of a dipeptide ([^{3}H] glycylsarcosine) transport into vitreous humor, retina and aqueous humor, following systemic administration in the presence and absence of inhibitors. In the presence of inhibitors, the transport of glycylsarcosine into the aqueous, vitreous, and retina was significantly inhibited. These results indicate the expression of a peptide transporter on the blood-aqueous and blood-retinal barriers that can be exploited for the targeted delivery following systemic administration[96].

Amino acid transporter

Amino acid transporters are responsible for translocation of amino acids from blood to various organs. Amino acids are responsible for protein synthesis and play a significant role in maintenance of structural and functional integrity of conjunctiva and retina/RPE. Amino acid transporters are ubiquitous in nature, with overlapping substrate specificity; hence, they are heavily exploited for targeted delivery of drugs. Amino acid transporters can be classified on the basis of sodium dependence, charge, and substrate specificity[97]. A sodium-dependent transporter binds amino acids after binding to sodium ions and undergoes a conformational change that allows the dumping of sodium ions and amino acids into the cytoplasm. System B, $B^{0,+}$, IMINO, system X-(anionic), ASC (cationic, anionic, and neutral forms), and $ATB^{0,+}$, belong to the sodium-dependent transporter category, while system y^+ (cationic), $b^{0,+}$, and system L (large) do not depend on sodium for transporting amino acids. Large amino acid transporter (system L) is expressed in two isoforms, LAT1 and LAT2, which are involved in the uptake of large aromatic or branched amino acids from extracellular fluids. LAT1 transports large neutral amino acids such as Leu, Phe, Ile, Trp, Val, Tyr, His and Met, while LAT2 transports large and small neutral amino acids[7]. Amino acid transport systems have been characterized on corneal epithelium and endothelium. The presence of various amino acid transporters such as ASCT1, LAT1 and ATB^{0+} has been characterized on the cornea. These transporters are involved in the transport of several amino acids such as L arginine, L-phenylalanine and L-alanine across the cornea[98]. The presence and function of amino acid transporters on human retina are heavily published in literature[99,100]. Gandhi *et al*[101] investigated the presence of a LAT2 on the ARPE-19 cell line. The same group also reported the presence of sodium-dependent, $B^{0,+}$ amino acid transporter on rabbit corneal epithelium and human cornea and its interaction with the amino acid ester prodrugs of ACV (γ-glutamate-ACV and phenylalanine-ACV)[102]. Katragadda *et al*[103] studied the *in vivo* corneal absorption of the amino acid prodrugs ACV (L-alanine-ACV, L-serine-ACV, L-serine-succinate-ACV and L-cysteine-ACV) using a topical well model and microdialysis in rabbits. They concluded that L-serine-ACV seems to be a promising candidate for the treatment of ocular HSV infections due to its enhanced stability, comparable AUC, and high concentration at the last time point (C_{last}). Further studies also revealed higher antiviral activity against varicella-zoster and herpes simplex virus, and in comparison to ACV. $ATB^{0,+}$ is a broad substrate-specific transporter that recognizes neutral and cationic amino acids. Studies have the shown the potential of $ATB^{0,+}$ in delivery of antiviral drugs such as ACV and ganciclovir, which are covalently coupled to anionic amino acids[104]. Retinal cells have a basal requirement of amino acids for protein synthesis. Several amino acid neurotransmitters (glutamate, GABA and glycine) and neuroactive amino acids (aspartate, homocysteic acid, and taurine) have been identified in the retina[100,105-107]. High affinity, sodium-dependent glycine transporter (Glyt-1) is cloned on retinal neurons[100,108]. Glty-1 plays an important role in maintaining the glycine homeostasis in the retina of all vertebrate species. Glutamate, a major excitatory neurotransmitter, is mainly localized on the bipolar cells, retinal ganglion cells and slightly ischemic photoreceptors[109]. The vitreal levels of glutamate are mildly elevated with diabetic retinopathy and rhegmatogenous retinal detachments. This may be attributed to the high-affinity excitatory glutamate transport proteins that can be utilized in drug delivery[110]. Recently, Yamamoto *et al*[35] studied the gene expression level of *LAT1* and *LAT2* in ARPE-19 cells and concluded that both LAT1 and LAT2 are involved in L-leucine transport. These amino acid transport systems could help in the design of prodrugs that are likely to be transported across the retina for better ocular delivery and bioavailability.

Nucleoside transporters

Nucleosides are transported *via* two carrier-mediated mechanisms, namely, facilitated diffusion, also referred to as equilibrative (sodium-independent) transport system and energy-dependent transporters also referred to as concentrative (sodium-dependent) transport system[111]. These transporters have been found in the epithelium of kidneys, intestine, conjunctiva, and choroid plexus[112-114]. Two types of equilibrative (labeled hENT1 and hENT2) and five types of concentrative transporters (labeled N1 through N5) have been reported so far[114-118]. The equilibrative nucleoside transporters (ENT) are differentiated by their relative sensitivities to nitrobenzylthioinosine (NBT). hENT1 is sensitive to NBT, whereas hENT2 is not[119,120]. The differences between concentrative transporters are in their substrate specificities. The N1 transporter is specific to purines and uridine; N2 is selective to pyrimidines and adenosine; N3 has broad specificity for purines and pyrimidines; N4 is pyrimidine selective,

Table 1 List of transporter/receptor(s) present in the eye

Tissue	Transporter/receptor	Subtypes	Ref.
Cornea	Amino acid	LAT1, LAT2, Phenylalanine, tyrosine	[213,214]
	Glucose	GLUT1	[215]
	Nucleoside		[216]
	Peptide	hPEPT1	[90]
	Folate		[180]
	Biotin		[175]
Conjunctiva	Acid-base	NKCC, HE1	[217]
	Amino acid	$B^{o,+}$	[218]
	Glucose	GLUT1	[157,219]
	Peptide	Dipeptide	[220]
	Monocarboxylate		[221]
	Nucleoside		[113]
Lens	Amino acid	System A, L, Gly, Ly+, β, ASC	[222]
	Ascorbic acid	SVCT2	[223]
	Glucose	GLUT1, GLUT3	[224]
	Glutathione	R-GSHT	[225]
Iris-ciliary body	Glucose	GLUT1, GLUT4	[226]
	Nucleoside		[227]
Retina	Amino acid	Glycine, glutamine, arginine, proline, taurine	[99,228,229]
	Glucose	GLUT1, GLUT3	[230]
	Monocarboxylic acid	MCT1, MCT3	[231,232]
	Nucleoside		[121,233]
	Peptide	PEPT1, PEPT2, PHT1, PHT2	[92,93,95,96]
	Vitamins(ascorbic acid, biotin, folic acid, riboflavin)	SVCT2, RFT, FR-α, SMVT	[85,183]

but transports adenosine and guanosine as well; and the N5 transporter is NBT sensitive and preferentially transports guanosine[114,118,120-122]. In the eye, sodium-dependent transporters have been found in the retina[121] and conjunctiva[123]. Transport of guanosine and adenosine investigated in retinal cell cultures indicated strong temperature dependency with maximal uptake of both substrates occurring at 37 ℃[121]. The transport was found to be significantly decreased when calcium and sodium ions containing electrolytes were substituted with other salts in the buffers used in the experiments. Substrate specificity testing revealed that adenosine inhibited guanosine uptake and not vice versa, indicating that separate processes exist for the uptake of each substrate. Moreover, L-N^6-phenyl isopropyladenosine, N^6-dimethyladenosine, 8-bromo adenosine, 5'-deoxy-5'-methylthioadenosine, and inosine significantly reduced the transport of adenosine and guanosine. The conjunctival mechanisms involved with nucleoside transport were first elucidated by Hosoya *et al*[113]. They reported mucosal presence of both sodium-dependent and sodium-independent hENT2 on excised rat conjunctiva. Uridine transport across the conjunctiva follows a strong mucosal to serosal directionality, temperature sensitivity, and phlorizin sensitivity. A structural feature necessary for coupling of the substrate and the transporter is the 3'-hydroxyl group of the D-ribose present in the nucleoside.

Glucose transporter

The energy for metabolic and electrochemical activity in the eye comes largely from oxidative breakdown of glucose[124]. The most prevalent and classical view regarding energy metabolism in the eye is that glucose is the primary substrate and that the highest rate of glycolysis and respiration manifests in the photoreceptor cells[125-128]. However, an entirely different hypothesis was suggested by Jones *et al*[129], Tsacopoulos *et al*[130-132] and Poitry-Yamate *et al*[133,134] based on their research on honeybee drone retina and guinea pig retina. Their research led to the proposal that glycolysis occurs in glial cells and that Müller cells predominate as the sole aerobic producers of lactate, serving as the primary fuel in the photoreceptors and other retinal neurons. Extensive research to establish metabolic processes occurring in the eye led to the conclusion that under normal conditions, when ambient glucose supply to the eye is adequate, glucose serves as the primary source of energy in the retina, rather than glial-generated lactate[135-137]. It has also been shown that lactate production does occur in Müller cells *via* aerobic metabolism of glucose[138-140]. Changes in metabolism and metabolic rate have profound implications in the progression of various ocular diseases[141-150]. Seven isoforms of the glucose transporter (GLUT1 through GLUT7) have been identified so far[151]. The facilitative glucose transporter, GLUT1, was found to be expressed in the cornea, iris-ciliary body, lens, and retina[152-156]. In addition to these glucose transporters, Na^+-D-glucose transporter (SGLT1) has been found in the mucosal side of the conjunctiva[157]. Although a wealth of information is available regarding glucose transporters, their utility in ocular drug delivery still remains an elusive goal, likely due to the high substrate specificity associated with these transporters.

VITAMIN TRANSPORTERS

Ascorbic acid transporter

Ascorbic acid, also known as vitamin C, is a water soluble vitamin responsible for several metabolic and physiological functions due to its antioxidant property. Ascorbic acid protects the cornea and other intraocular tissues by absorbing the UV radiations between 280-310 nm. Higher levels of ascorbic acid in the eye prevent lens cataracts and inhibit peroxidase activity. Human ocular tissues contain significantly higher amounts of ascorbic acid due to their protective role. The concentration of ascorbic acid in tear fluid, corneal epithelium and aqueous humor are 23 ± 9.6 μmol/L, 1.33 ± 0.48 mg/g and 0.20 ± 0.1 mg/mL, respectively. The concentration of ascorbic acid in aqueous humor is approximately 20-fold higher than the plasma concentrations[158]. These figures intrigued the researcher to study the presence of ascorbic acid transporter and its role in the transport of ascorbic acid. Cellular transport of ascorbic acid is mediated by hexose transporters (GLUT) and sodium-dependent vitamin C transporters (SVCT1 and SVCT2). GLUT1 is a low affinity and high capacity transporter that facilitates

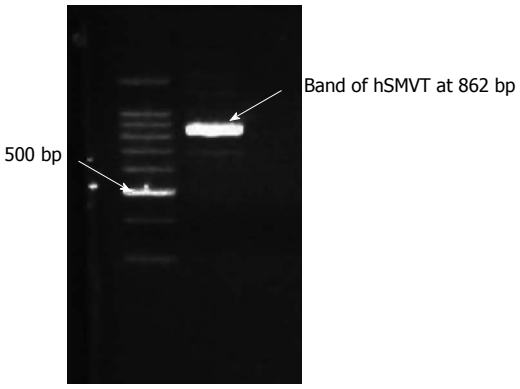

Figure 5 hSMVT cDNA was generated by reverse transcription polymerase chain reaction amplification of total RNA from ARPE-19 cells (lane 2). Aliquots of polymerase chain reaction products were analyzed by gel electrophoresis on 0.8% agarose. Ethidium bromide staining of the gel showed a approximately 862 bp band corresponding. Reproduced with permission from[176].

the transport of the oxidized form of ascorbic acid (dehydroascorbic acid), while SVCT1 and SVCT2 are high affinity and low capacity sodium-dependent transporters that transport the reduced form, L-ascorbic acid. Interestingly, the ascorbic acid concentrations are higher in diurnal animals as compared to nocturnal animals. Neither SVCT1 nor SVCT2 was observed in the ciliary body of rat (nocturnal animal), while albino rabbit (diurnal animal) SVCT2 was expressed abundantly in pigmented epithelium of the ciliary body and expressed moderately in the deeper layers of the corneal epithelium[159]. SVCT2 is widely expressed in several ocular tissues such as ciliary body, cornea, lachrymal gland, and retina[160].

The presence of ascorbic acid on bovine corneal endothelial cells and its role in the transport of ascorbic acid to the stroma was reported by Bode *et al*[158]. Talluri *et al*[161] studied the uptake mechanism of L-ascorbic acid by rabbit corneal epithelial cells and characterized the specific transporter involved in this translocation. They concluded that SVCT2 is responsible for the uptake of L-ascorbic acid. Further, the uptake was found to be sodium-dependent and saturable at higher concentrations. Ascorbic acid transporter is utilized to some extent in drug delivery, especially in the transport of glucosamine by the facilitative glucose transporter, GLUT1. Glucosamine is an essential sugar derivative and a widely used nutraceutical agent that helps in the synthesis of glycoproteins and glycosaminoglycans[162]. Glucosamine has significant modulatory effects on insulin resistance and diabetes-associated complications[160]. Recently, SVCT2 transporter has been used in the delivery of neurotropic agents to the central nervous system (CNS). Information available in the literature supports the use of ascorbic acid-conjugated prodrugs nipecotic, kynurenic and diclofenamic acids for brain delivery[163,164]. Luo *et al*[165] demonstrated that amino acid-conjugated prodrugs of saquinavir improved its solubility, metabolic stability and absorptive permeability. Hence, SVCT targeted prodrug approach can be utilized as an attractive strategy to enhance the ocular absorption of drugs.

Biotin carrier system

Biotin, also known as B-complex vitamin (vitamin B_7), is a water soluble vitamin essential for normal cellular growth, function, and development. Biotin is a cofactor for the carboxylases that catalyze various metabolic reactions such as gluconeogenesis, fatty acid biosynthesis, and catabolism of several branched chain amino acids[166,167]. Biotin is primarily absorbed and metabolized in the intestine, liver and placenta[168-170]. The involvement of sodium-dependent multivitamin transporter (SMVT) in the uptake of biotin, pantothenate and lipoate from human placenta was first report by Grassel[171]. Studies by Said *et al*[169,172] and several other groups concluded that SMVT is the primary transport system responsible for the uptake of biotin uptake[170]. SMVT plays an important role in the transport of vitamins and cofactors essential for the normal functioning of the eye. Moreover, adequate biotin concentrations are required for the development of retina and correct ocular morphogenesis. So far, no study has been published relating to the biotin concentrations in mammalian retina[173]. The circulating blood is responsible for maintaining biotin concentrations in the retina. Nevertheless, the biotin transport from the circulating blood is regulated by the blood-retinal barrier, comprised of retinal capillary endothelial cells (inner BRB) and retinal pigment epithelial cells (outer BRB). Ohkura *et al*[174] examined the biotin transport mechanism at the inner BRB and concluded that SMVT is involved in the transport of biotin from the circulating blood to the retina, across the inner BRB.

SMVT expressed on the inner BRB could be exploited in drug delivery into the retina due to its excellent capacity (K_m) and broad substrate specificity. Biotin prodrugs and polymeric conjugates utilize SMVT to increase the permeability of drugs. Janoria *et al*[175] studied the presence of SMVT on rabbit corneal epithelial cells. From *in vitro* and *ex vivo* studies they concluded that SMVT is expressed on corneal epithelial cells and is responsible for the uptake of biotin, pantothenic acid and lipoic acid. The presence of biotin in tears further substantiates the physiological significance of this transporter. The same research group[176] characterized the presence of SMVT in human retinal pigmented epithelium cell line (ARPE-19) cells and studied the role of SMVT on the uptake of biotin-ganciclovir in both ARPE-19 and rabbit retina. Molecular identification of SMVT was conducted with reverse transcriptase polymerase chain reaction (RT-PCR) in ARPE-19 cells. The band between 800 and 900 bp in gel electrophoresis confirmed the presence of hSMVT (Figure 5). They concluded that biotin-ganciclovir prodrug is recognized by the SMVT transport system in ARPE-19 cell line and rabbit retina. Further, biotin-ganciclovir exhibited a better, therapeutically desirable pharmacological profile in the vitreous fluid, compared to ganciclovir (Table 2). These findings would be of great interest in exploring the potential of SMVT to deliver biotin conjugates.

Folate carrier system

Folate, also known as vitamin B_9, is a water soluble es-

Table 2 Vitreous pharmacokinetic parameters of ganciclovir and biotin-ganciclovir following intravitreal administration (mean ± SD)

Parameters	GCV	(Biotin-GCV)	
		Biotin-GCV	Regenerated GCV
AUC (mg/mL per minute)	10.6 ± 1.27	17.5 ± 1.38[a]	1.85 ± 0.744
λ_z ($\times 10^{-3}$/min)	2.58 ± 0.124	3.19 ± 0.536	
$T_{1/2}$ (min)	270 ± 15.7	222 ± 40.5	
V_{ss} (mL)	1.56 ± 0.100	1.47 ± 0.106	
Cl (μL/min)	4.39 ± 0.603	5.45 ± 0.673	
MRT last (min)	197 ± 22.2	175 ± 17.6	264 ± 9.26
Clast (μg/mL)	7.06 ± 1.38	8.28 ± 2.27	
C_{max} (μg/mL)			5.37 ± 0.435
T_{max} (min)			66.7 ± 23.1

[a]$P < 0.05$ *vs* the control. GCV: Ganciclovir; AUC: Area under the vitreous time concentration curve; λ_z: Elimination rate constant; $T_{1/2}$: Vitreal elimination half-life; V_{ss}: Volume of distribution at steady; Cl: Clearance state; MRT: Mean residence time. Reproduced with permission from[176].

sential vitamin that enters the cells through a membrane-associated folate binding protein in addition to classical high affinity/low capacity carrier system[177,178]. Folic acid is a synthetic form of folate that plays an important role in maintaining numerous bodily function,s including the development of visual system. Folic acid deficiency results in retinal edema, retinal dysfunction, damage of photoreceptor cells, nutritional amblyopia, and optic neuropathy, leading to loss of visual function[179,180]. The hydrophilic nature of folic acid prevents it from entering the lipoidal cell membrane. Transport of folate across the cell membrane occurs predominantly *via* three pathways: folate receptors (FR), reduced folate carrier (RFC), and proton-coupled folate transporter (PCFT)[181]. FRs are coded by two specific genes: FR-α and FR-β, with differential tissue expression[181]. FR-α is distributed throughout the retina, including the basolateral membrane of retinal pigment epithelium[182], while RFT-1 is present only on the apical surface of retinal pigment epithelium[183]. Folate from the choroidal blood vessels is taken by the FR-α located on the basolateral side of RPE and is transferred to the apical membrane of the RPE. RFT-1, present on the apical surface, transports the folate to adjacent metabolically active photoreceptor cells[184]. Tumor cells overexpress FR, and hence folate has been widely used for targeting anti-cancer drugs in the form of prodrugs and delivery systems (folate conjugated nanoparticles and micelles)[185,186]. Kansara *et al*[85] investigated the expression of FR-α in human-derived retinoblastoma cell line (Y-79). These studies have also demonstrated the mechanism and intracellular regulation of folic acid uptake using various membrane transport inhibitors. Later, the same group developed and characterized folate conjugated polymeric micelles for retinoblastoma cells using doxorubicin as a model drug. Uptake of doxorubicin in Y-79 cells over-expressing FRs was approximately four times higher with folate-conjugated polymeric micelles than with pure

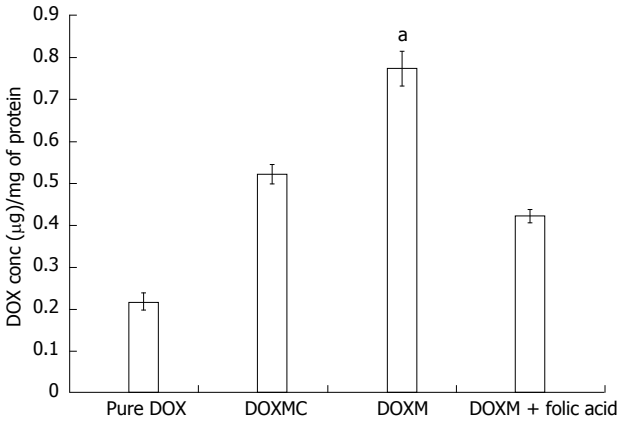

Figure 6 Quantitative uptake of doxorubicin in Y-79 cells using doxorubicin, doxorubicin-loaded in polymeric micelles, folate conjugated polymeric micelles and folate conjugated polymeric micelles in presence of folic acid. [a]$P < 0.05$. Reproduced with permission from[186].

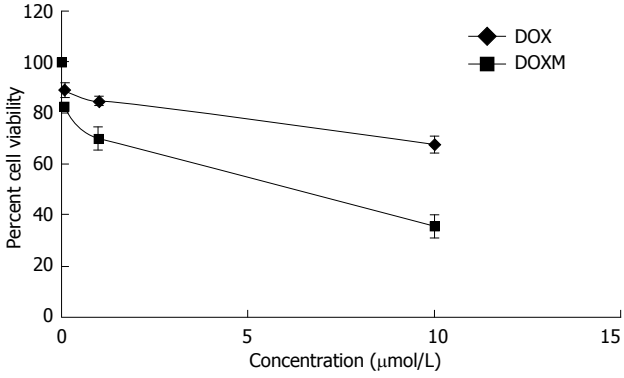

Figure 7 Cell viability studies of doxorubicin in ARPE-19 cells following treatment with doxorubicin and folate conjugated polymeric micelles. Reproduced with permission from[186].

drug (Figure 6). Moreover, folate-conjugated polymeric micelles of doxorubicin exhibited higher cytotoxicity in retinoblastoma cell line (Y-79 cells) when compared with pure doxorubicin (Figure 7)[186]. Such systems can provide sustained and targeted delivery of drugs to retinoblastoma cells following intravitreal administration. Jwala *et al*[180] characterized the expression of folate transport proteins in Staten's Seruminstitut rabbit corneal (SIRC) epithelial cell line. They observed a linear increase in the uptake of [^{3}H] Folic acid over 30 min, and the uptake process followed saturation kinetics with apparent K_m of 14.2 nmol/L, V_{max} of 1.5×10^{-5} μmol/min per milligram protein and K_d of 2.1×10^{-6}/min. Molecular evidence of FR-α and PCFT was established in SIRC epithelial cell line using RT-PCR and Western blotting analysis (Figures 8 and 9). Permeability studies have further confirmed the existence of the folate carrier-mediated system across the rabbit cornea. Drug targeting *via* FRs is an effective method for cell-selective drug delivery, since this process allows a satisfactory transport rate and ligand-dependent cell specificity. Targetability of various delivery systems such as liposomes, polymer conjugates, polymeric mi-

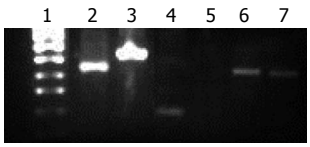

Figure 8 Reverse transcription polymerase chain reaction analysis of folate receptor-α, reduce folate carrier, proton coupled folate transporter. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase. Reproduced with permission from[180]. FR: Folate receptors; RFC: Reduced folate carrier; PCFT: Proton-coupled folate transporter.

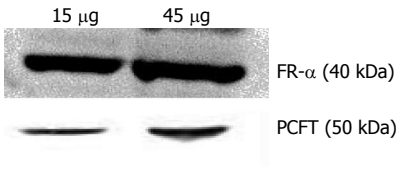

Figure 9 Western blotting analysis of folate receptor-α and proton coupled folate transporter. Reproduced with permission from[180]. FR: Folate receptors; PCFT: Proton-coupled folate transporter.

celles and nanoparticulates has been achieved with a covalently attached folate on the surface[187].

Riboflavin

Riboflavin, or vitamin B2, is water soluble and highly photosensitive. In its active forms, flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) function as critical cofactors involved in the transfer of electrons during several biological redox reactions[188,189]. Since the primary source of riboflavin is dietary intake, lack of this vitamin in food, particularly during pregnancy and adolescence can lead to developmental abnormalities and other well documented clinical manifestations[189-193]. Riboflavin is found in almost all parts of the eye, including corneal epithelium and substantia propria, conjunctiva, lens, iris-ciliary body, aqueous and vitreous humors, choroid, and retina[194]. Riboflavin deficiency produces corneal vascularization, lenticular cataracts, changes in conjunctiva and lachrymal glands, and eye lesions[195-200]. Three riboflavin transporters (RFT) have been reported so far: RFT1, RFT2, and RFT3[201-203]. Structural elucidation of RFTs occurred recently, and mechanisms involving riboflavin transport *via* RFTs is still being researched rigorously. Kansara *et al*[204] investigated the uptake mechanism and intracellular transport of riboflavin in human-derived Y-79 cells, which are a model for neural retina. They were the first to establish functional evidence for the presence of a high affinity riboflavin transporter in this *in vitro* cell model. The carrier-mediated active transport system was found to be energy- and temperature-dependent, but sodium- and pH-independent, in nature. Several studies have been done to further the understanding of the transporter and its function in brain[205], intestine and nutrition[206,207], diseases[208,209], and microbes[210-212]. However, studies on the transporter do not seem to have caught the interest of scientists in eye research.

CONCLUSION

Drug delivery to the eye remained a major obstacle for scientists in the field. Better understating of the anatomical and physiological barriers, including the drug efflux mechanisms, is crucial to optimizing the drug delivery to the eye. Identification of nutrient transporter/receptor(s) and understanding their roles in targeted delivery of drugs to various ocular tissues has gained a lot of attention recently. This strategy can successfully evade efflux mechanism and simultaneously overcome the tight junctions that hinder the permeability of most drug molecules. Receptors can be utilized for targeted delivery of nanocarriers, which is yet another exciting and promising approach that allows sustained delivery of drugs for diseases affecting the back of the eye. On the whole, the field of ocular drug delivery holds a great future for the development of less invasive, targeted, and controlled release formulations, especially for the treatment of posterior segment diseases.

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REVIEW

Phytochemicals in ocular health: Therapeutic potential and delivery challenges

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Abstract

Diabetic retinopathy (DR) and age-related macular degeneration (AMD) are the leading causes of blindness in adults. The impact of these conditions on the quality of life is increasing in significance with a rise in life expectancy. The role of hyperglycemia, oxidative stress and inflammatory responses in the development and/or progression of DR and AMD, and several other sight threatening ocular diseases, is well established. In proliferative retinopathy, signals sent by the retina for nourishment, triggers the growth of fragile and abnormal blood vessels. Changes in ocular pressure may lead to rupture of these blood vessels causing severe vision problems. Recent *in vitro* and preclinical studies demonstrate that certain phytochemicals possessing potent antioxidant and anti-inflammatory activity and ocular blood flow enhancing properties may be very useful in the treatment of, or as a prophylactic measure for, DR and AMD. Apart from these properties they have also been investigated for their anti-bacterial, hormonal, enzyme stimulation, and anti-angiogenic activities. The attractive aspect of these potential therapeutic candidates is that they can act on multiple pathways identified in the etiology of DR, AMD, cataract and other ocular diseases. However, results from clinical trials have been somewhat ambiguous, raising questions about the concentrations of these bioflavonoids achieved in the neural retina following oral administration. Unfortunately, as of date, an efficient noninvasive means to deliver therapeutic agents/candidates to the back-of-the eye is still not available. This review examines some of these promising natural agents and discusses the challenges encountered in delivering them to the posterior segment ocular tissues through the oral route.

Key words: Diabetic retinopathy; Age-related macular degeneration; Phytochemicals; Ocular drug delivery

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INTRODUCTION

A report from the World Health Organization (WHO) in 2011 estimated that approximately 285 million people were suffering from visual impairment worldwide out of which 39 million face blindness while 246 million suffer from moderate to severe vision impairment. With an increase in the average survival age and the percentage of diabetics, diabetes related retinopathies are rapidly gaining in significance. It has been predicted that, without additional steps, these numbers will increase to 75 million blind and 200 million visually impaired by the year 2020 (WHO, 2010). Age seems to be a causative factor in blindness as 90% and 58% of the population with blindness are aged above 45 years and 60 years respectively.

Oxidative stress, inflammatory mechanisms and decreased antioxidant capacity in the ocular tissues are all thought to play an active role in the development and progression of these ocular diseases. The following sections briefly highlight the etiology of these ocular disorders, followed by discussions on the therapeutic potential of various phytochemicals and challenges encountered in their delivery to the ocular tissues.

Diabetic retinopathy

Diabetic retinopathy (DR) is the most common diabetes associated eye disease and is the leading cause of blindness in American adults[1,2]. The WHO estimates that over 360 million worldwide will suffer from diabetes by the year 2030. Currently, 10% of the diabetics suffer from type 1 diabetes whereas 90% suffer from type 2 diabetes. DR in type 1 diabetics approaches 80% and 90 % prevalence rate after 10 years and 20 years of diabetes, respectively. Up to 21% of patients with type 2 diabetes have recently been found to have retinopathy at the time of first diagnosis of diabetes, and most develop some degree of retinopathy over subsequent decades[3].

DR can be broadly categorized into three stages; background DR, pre-proliferative DR and proliferative DR (PDR)[4-6]. In background DR, hyperglycemia is considered to induce thickening of capillary basement membranes and death of pericytes, which support the vessel wall, and endothelial cells of retinal blood vessels. Microaneurysms and vascular leakage follow, and blockage of retinal capillaries take place. In pre-proliferative DR, loss of vascular patency leads to areas of increasing retinal hypoxia and multiple hemorrhages. Increased areas of tissue non-perfusion stimulate the production of angiogenic factors leading to the proliferation of vessels, which is a typical feature of PDR. The newly formed blood vessels by themselves do not lead to vision loss, but leakage of blood through their weak walls can result in severe vision loss and can ultimately lead to complete loss of sight.

Hyperglycemia and tissue hypoxia, are considered to be principal factors in the DR pathology described above. Multiple studies demonstrate the relationship between high blood glucose, oxidative stress and initiation of DR[6-11] as shown in Figure 1. The retina is highly susceptible to oxidative damage since: (1) it has an abundance of the polyunsaturated fatty acids in its membrane bilayers, whose double bonds are prime targets for peroxidation reactions; (2) it is periodically subjected to continuous light which, by photoexcitation, can initiate free radical formation and peroxidation reactions; and (3) the retina is a highly metabolic tissue that requires a high rate of blood flow in order to receive an adequate oxygen supply. Oxidative damage in biological systems occurs when endogenous antioxidant mechanisms are overwhelmed by free oxygen radicals or reactive oxygen species (ROS). These radicals are extremely unstable and can cause cytotoxicity and cellular damage by reacting with plasma membrane lipids, DNA, RNA and metal-containing compounds[12].

Multiple pathways have been suggested to be activated during hyperglycemia associated oxidative stress, which subsequently leads to damage of retinal blood vessels. These include nitric oxide (NO) synthesis[13,14], nuclear factor-kappa B (NF-κB) expression, secretion of cytokines such as interleukin (IL)-1β, lipid peroxide generation, activation of retinal caspase-3, protein kinase C (PKC) stimulation and alterations of retinal glutamate levels[12,15-20].

In advanced stages, *i.e.*, PDR, reduced retinal blood flow induces retinal ischemia which leads to hypoxic conditions in the retina. Hypoxia stimulates production of a variety of local agents including vascular endothelial growth factor (VEGF)[6,21-25], prostaglandins (PGs), cyclooxygenase-2 (COX-2) and NO, all of which participates in increasing vascular permeability and angiogenesis[6]. The VEGF family plays a key role in the regulation of vascular patency and is involved in both physiological and pathological angiogenesis, stimulating endothelial cells to migrate, proliferate and form tubes[6,26-29]. VEGF is also a potent vascular bed permeability enhancer[30,31]. Hypoxia is a stimulant for COX-2 transcription also in various tissues[6,32-34], including human vascular endothelium[6,32] and neural cells[35]. NF-κB, an oxygen sensitive transcription factor[6,36,37] mediates the induction of COX-2 in hypoxic conditions. The angiogenic properties of COX-2 are likely to directly involve VEGF, as COX-2 has been shown to up-regulate VEGF synthesis which can be inhibited with selective or non-selective COX inhibitors[6,38,39].

In the context of DR, the actions of PGs E2 and I2 are also considered to be important in the development of angiogenesis, breakdown of the blood-retinal barrier and alterations in retinal blood flow[6,40-45]. These PGs are produced *via* the COX-2 pathway[6,46-48]. Prostaglandins, and in particular PGE2, are also strong inducers of VEGF in cell types such as synovial fibroblasts monocytes and lung and retinal Müller cells. There is evidence that the vascular events prior to angiogenesis may involve the induction of COX-2 followed by VEGF. It has thus been suggested that angiogenesis may be mediated by dual interdependent gene expression pathways that involve *COX-2* and *VEGF*[6,49].

NO is known to participate in vascular permeability and angiogenesis *via* interactions with VEGF and COX-2. NO reacts with superoxide anions to form peroxynitrite, a highly reactive oxidant. Excess production of peroxynitrite in diabetes may exhibit cytotoxic effects by increasing DNA damage, stimulating lipid peroxidation and depleting glutathione levels[50-52]. Peroxynitrite alters tyrosine in proteins to form nitrotyrosine and nitration of proteins can inactivate mitochondrial and cytosolic proteins and damage cellular elements leading to nitrative stress[53]. PDR is the most damaging stage of DR as it leads to the generation of abnormally located retinal blood vessels with weak capillary walls. Microvascular leakage from these newly formed blood vessels can lead

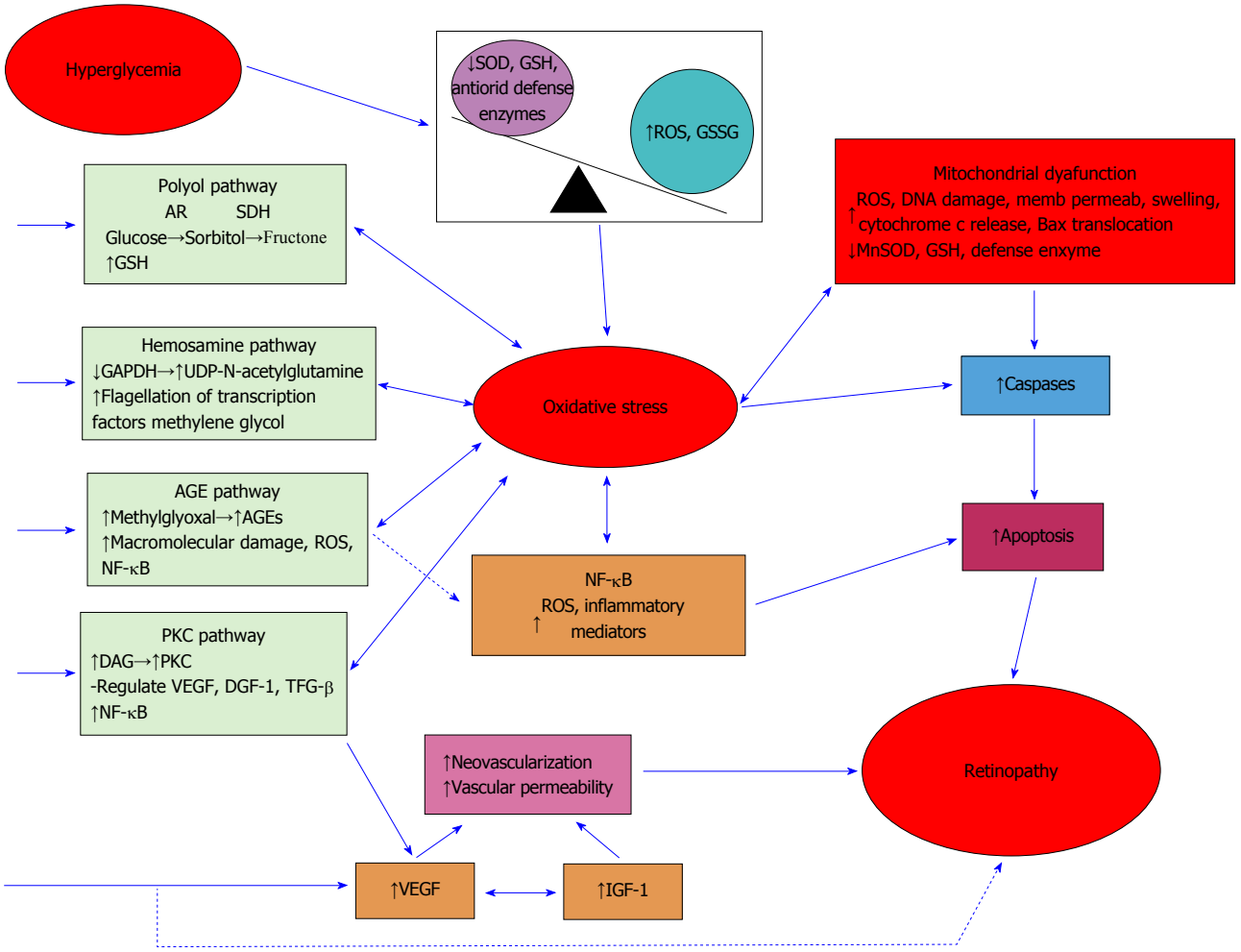

Figure 1 Oxidative stress mediated dysmetabolisms in diabetic retinopathy. AR: Aldose reductase; SDH: Sorbitol dehydrogenase; GSH: Glutathione; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; AGEs: Advanced glycation end product; ROS: Reactive oxygen species; NF-κB: Nuclear factor κB; PKC: Protein kinase C; DAE: Diacylglycerol; VEGF: Vascular endothelial growth factor; DGF-1: Dispersed gene family-1; TFG-β: Transforming growth factor-β; SOD: Superoxide dismutase; GSSG: Oxidant glutathione; IGF-1: Insulin-like growth factor-1; MnSOD: Manganese superoxide dismutase. Reproduced with permission from[200].

to total blindness through a variety of mechanisms.

In addition to hyperglycemia associated increased ROS generation, it has recently been demonstrated that the total antioxidant capacity (TAC) of the vitreous humor and aqueous humor is lowered in DR. Mancino *et al*[54] determined the antioxidant capacity of blood, aqueous humor and vitreous of controls (non-diabetic) and of patients with non-proliferative DR (NPDR) and with PDR. The authors observed that the control group displayed significantly higher TAC levels than the diabetic sub-groups in both the vitreous and aqueous humor. PDR patients had decreased TACs in the vitreous and aqueous humor as compared with control subjects and with the NPDR patient subgroup, pointing to the role of oxidative stress in the progression of NPDR to PDR[54]. The results strongly support the need for increased antioxidant levels in the retina, aqueous humor and vitreous humor.

Age-related macular degeneration

Age-related macular degeneration (AMD), which can be categorized into dry and wet AMD, is the leading cause of irreversible vision loss in the developed world. As per the statistical data published by the National Eye Institute, AMD affected 1.75 million persons in the United States, in 2004, a number which is expected to rise to nearly 3 million by 2020 due to the aging of the population[55]. Like DR, progression of AMD is linked to the activation of inflammatory and immunological pathways[56]. Presence of excess ROS and decreased antioxidant capacity in the ocular tissues is also considered to play a significant role in the initiation and progression of AMD[57,58]. Ding *et al*[59] provides a summary of the molecular pathways involving inflammation, angiogenesis and oxidative stress, considered to play a role in the development of AMD.

Cataract

Oxidative stress induced damage to the lens fibers has also been well documented. It is thought that these free radicals accelerate and aggravate cataract development. Additionally, diabetic lenses show an impaired antioxidant capacity that increases their susceptibility to oxidative stress. The loss of antioxidants is exacerbated by glycation and inactivation of lens antioxidant enzymes

like superoxide dismutase[60].

CURRENT TREATMENT STRATEGIES

During the first two stages of DR blood sugar, pressure, and cholesterol control is recommended. Additionally, antioxidant therapy has shown reasonable promise. Free radical scavenging activity of the antioxidants protects the retinal blood vessel endothelial cells and pericytes from apoptosis in a high glucose and oxygen rich environment, as in the retina[61-67]. PDR is treated with laser surgery. This procedure is called scatter laser treatment. Although some loss of peripheral vision may be noticed, scatter laser treatment can save the remaining sight. Scatter laser treatment works better before the fragile, new blood vessels have started to bleed. Even if bleeding has started, scatter laser treatment may still be possible, depending on the amount of bleeding. If the bleeding is severe, a surgical procedure called a vitrectomy may be needed. Recent studies have demonstrated that VEGF, PKC and COX-2 inhibitors, antibodies and proteins may be effective in controlling PDR[61-66,68-77]. Anti-VEGF injection therapy, photodynamic therapy using intravenous verteporfin and sometimes laser surgery are used to treat and control the progression of AMD. High doses of antioxidants and zinc have also been shown to be useful in AMD[78].

A major shortcoming of the current therapeutic options is that no one candidate appears to be capable of acting on the multiple pathways involved in the initiation and progression of these ocular diseases. Another major challenge is achieving therapeutic concentrations of the active ingredients in the neural retina, where significant damage to the retinal blood vessels and neuronal cells occur. Identification and targeted delivery of compounds that can act on multiple pathways would be a significant advancement in the prevention and treatment of these sight-threatening ocular diseases.

POTENTIAL OF NATURAL COMPOUNDS

Bioflavonoids

Over the century, flavonoids or bioflavonoids have been identified as the most common group of plant polyphenols that give color and flavor to fruits and vegetables. Till now more than 8000 polyphenolic compounds have been identified and these flavonoids can be classified into different subclasses which include flavones, flavonols, flavanones, flavanols, anthocyanins and isoflavones[79]. Flavonoids have gained prominence in the pharmaceutical arena by virtue of their therapeutically beneficial properties. Bioflavonoids possess antioxidant, anti-angiogenic, and/or anti-inflammatory activities and are also capable of reducing fluid retention and strengthening capillary walls. Interestingly, the etiology of most ocular diseases involve free radical mediated oxidative damage, hypoxia, decreased blood supply to ocular tissues and, in certain conditions, angiogenesis, increased vascular permeability and leakage of vascular contents[80,81]. Thus, select bioflavonoids may be effective in the prevention or treatment of ocular diseases (*e.g.*, DR and macular degeneration) that lead to vision loss if left untreated.

The following section briefly describes various pharmacological activities of a few bioflavonoids that may be useful in the prevention or treatment of DR and AMD and other ocular diseases.

Hesperidin

Hesperidin is a flavanone glycoside consisting of the flavanone hesperitin and the disaccharide rutinose. Hesperidin is classified as a citrus flavonoid and is the predominant flavonoid in lemons and oranges. The peel and membranous parts of these fruits have the highest hesperidin concentrations. Therefore, orange juice containing pulp is richer in the flavonoid than that without pulp. Sweet oranges (*Citrus sinensis*) and tangelos are the richest dietary sources of hesperidin. Hesperidin is metabolized to its aglycone, hesperetin. Figure 2 depicts the structures of hesperidin and hesperetin.

Like some of the other flavonoids, hesperidin and its aglycone hesperetin, has been reported to possess significant radical scavenging, antioxidant and neuroprotective properties[82]. These compounds have been demonstrated to prevent lipopolysaccharide, peroxynitrite, and various other free radical, *e.g.*, azobisisobutyronitrile and benzoylperoxide, mediated cytotoxic effect[83-90]. Additionally, both hesperidin and hesperetin have been observed to exhibit anti-inflammatory activity mediated through the inhibition of the COX-2 pathway, synthesis of PGE2 and nitrogen dioxide, metabolism of arachidonic acid as well as histamine release[84,87,91-94].

What makes hesperidin and hesperetin particularly attractive is their effect on ocular blood flow and vascular permeability. Chiou *et al*[95] demonstrated that hesperidin, and especially hesperetin, produces marked increase in ocular blood flow and retinal function recovery following retinal ischemia. Additionally, these compounds have been demonstrated to be effective in the treatment of chronic venous insufficiency[96,97]. Reports also suggest that hesperidin can prevent microvascular leakage through their capillary wall strengthening action: hesperidin methyl chalcone, given intravenously significantly inhibited the macromolecular permeability-increasing effect of bradykinin, LTB4, and histamine[98]. Furthermore, hesperidin and hesperitin can reduce platelet aggregation, a factor involved in the blockage of retinal blood vessels[99,100].

Thus, hesperidin and its aglycone hesperetin appear to be capable of modulating multiple pathways involved in the generation and progression of DR and AMD. These compounds can protect against free-radical induced damage to the retinal neuronal cells, protect the health of retinal vascular cells, inhibit inflammatory mechanisms that can lead to the induction of angiogenesis and also prevent microvascular leakage of the retinal blood

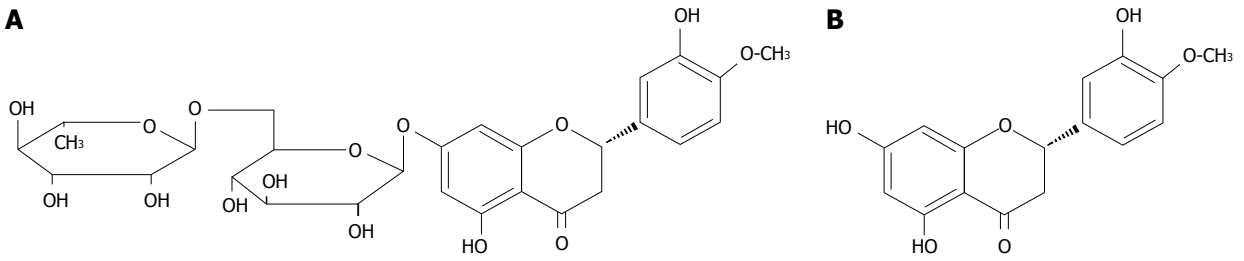

Figure 2 Chemical structure of hesperidin (A) and hesperetin (B).

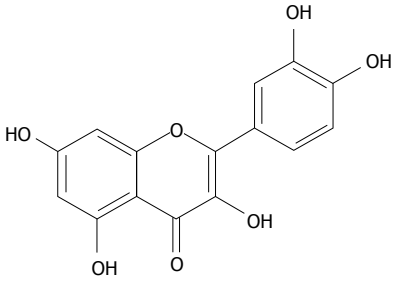

Figure 3 Chemical structure of quercetin [2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one].

vessels. Taken together, hesperidin may prove to be a very important therapeutic candidate in the treatment of DR as well as cataract, AMD and ocular tumors through similar mechanisms.

Quercetin

Quercetin, abundantly found in red wine, grapes and other fruits, is one of the most studied flavonoid for its beneficial effects. Quercetin is the aglycone (Figure 3) form of a number of other flavonoid glycosides, such as rutin and quercitrin, found in citrus fruit, buckwheat and onions. Quercetin forms the glycosides quercitrin and rutin with rhamnose and rutinose, respectively.

An extensive amount of *in vitro* and *in vivo* animal research has focused on the antioxidant potential of quercetin[101-106]. Recently, its antioxidant activity was studied on retinal cell lines; Hanneken *et al*[107] investigated quercetin's ability to protect ARPE-19 and human retinal pigment epithelium (RPE) cells from oxidative stress induced death *in vitro*. It was found that quercetin exhibited good efficacy, high potency and low toxicity in RPE cells and importantly, it was observed to be effective even after the RPE cells were exposed to oxidative stress, but before cell death occurred. There are a few other investigations that demonstrate the protective effect of quercetin on retinal cell lines[108-110].

Quercetin is also capable of increasing the choroidal blood flow and possesses anti-angiogenic activity. Zhuang *et al*[111] reported that quercetin inhibited the formation of choroidal neovascularization both *in vivo* and *in vitro* and increased choroidal blood flow. Quercetin was studied for its anti-angiogenic activity and was found to inhibit retinal and choroidal angiogenesis in the rhesus choroid-retina endothelial cell line, RF/6A. Quercetin prevented endothelial cell proliferation, migration, and tube formation in a dose dependent manner[112]. Quercetin's anti-angiogenic activity was thought to be mediated through the inhibition of matrix metalloproteinases (MMP)-2 activation[113]. Other reports also substantiate the anti-angiogenic activity of quercetin[114,115]. However, it has also been reported that one of the metabolites of quercetin has an opposite effect. Quercetin and quercetin-3'-glucuronide were found to inhibit the VEGF receptor-2 but quercetin-3'-sulphate stimulated the VEGF receptor-2[116].

A number of reports also indicate anti-inflammatory activity of quercetin *in vivo* in animal models[106,117-119]. It's anti-inflammatory activity was reported to be through the inhibition of COX-2[120,121], iNOS expression[122,123], tumor necrosis factor (TNF)-α, IL-1β, IL-6 and IL-8[124]. Quercetin has also been studied for its positive effect on cataract[125-128].

Baicalein

Both baicalin (7-glucuronic acid 5,6-dihydroxyflavone) and its aglycone, baicalein, are known for its strong antioxidant properties, anti-inflammatory properties, antiviral properties, anti-cancer properties, and scavenging potential (Figure 4)[129].

Some recently published work suggests the usefulness of baicalin in countering both diabetes-related complications and metabolic disorders[130,131]. It possesses antioxidant, anti-angiogenic and anti-inflammatory activities. Ling *et al*[132] reported marked inhibition of angiogenesis, decreased migration of human umbilical vein endothelial cells and reduced VEGF induced new blood vessel growth by baicalein. In another study involving retinal vessels; baicalein treatment ameliorated inflammatory mediators in the retina like IL-18, TNF-α, and IL-1β in the rats with DR, and reduced the glial fibrillary acidic protein and VEGF expression by Muller cells, and significantly reduced vascular abnormality and ganglion cell loss within the retina[133]. Reports also suggest that baicalein is capable of suppressing IL-6 and IL-8 expression in ARPE-19 cell lines[134] and to protect the retinal ganglion cells (RGC) from oxidative stress[109] and ischemia induced[110] cell death. Similar results were observed with ARPE-19 and human RPE cells[107]. Protection of retinal cells against oxidative stress and ischemia/reperfusion (I/R) *in vivo* were reported for baicalein[135]. Liu *et al*[105] reported the antioxidant activity of baicalein on human

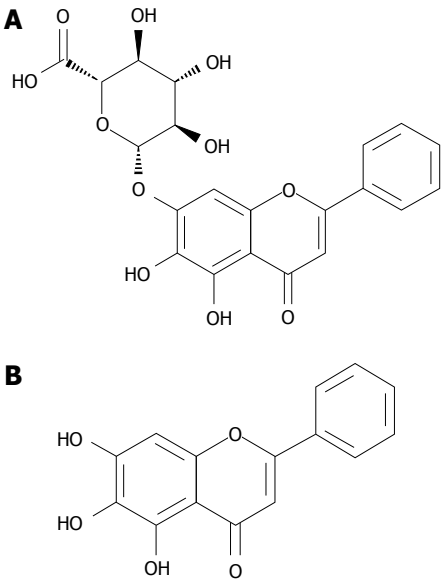

Figure 4 Chemical structure of (A) Baicalein (Baicalein 7-O-glucuronide; 5,6-Dihydroxy-4-oxygen-2-phenyl-4H-1-benzopyran-7-beta-D-glucopyranose acid) (B) baicalein (5,6,7-Trihydroxy-2-phenyl-chromen-4-one).

retinal pigment epithelium cells, it was found to down regulate the levels of VEGF and MMP-9.

Epigallocatechin gallate

Epigallocatechin gallate (EGCG), also known as epigallocatechin 3-gallate, is the ester of epigallocatechin and gallic acid (Figure 5), and is a type of catechin. EGCG is the most abundant catechin in tea; it is found in green- but not black-tea and is a potent antioxidant that may have therapeutic applications in the treatment of many disorders.

Hanneken *et al*[107] evaluated the ability of specific dietary and synthetic flavonoids to protect ARPE-19 and human RPE cells from oxidative stress induced death *in vitro*. Oxidative stress was induced by treatment with t-BOOH or H_2O_2. It was found that EGCG exhibited good activity and low toxicity in RPE cells. The authors suggested that the flavonoids were probably acting through the inhibition of ROS accumulation and through induction of transcription factor, nuclear erythroid 2 p45-related factor 2, and its downstream phase-2 gene, heme-oxygenase 1, in human RPE cells[107].

EGCG is also an inhibitor of angiogenesis. EGCG was reported to inhibit angiogenesis by inhibiting hypoxia-inducible factor-1α protein expression[136] and in turn VEGF expression[136,137]. Jung *et al*[138] observed that treatment with EGCG (intraperitoneal administration) in nude mice decreased tumor growth, microvessel density and tumor cell proliferation. However, the authors reported that other tea catechins such as (-)-epigallocatechin, (-)-epicatechin gallate, and (-)-epicatechin were ineffective *in vitro* against Erk1/2 (extracellularly-regulated kinase-1 and -2; important mediators in the up-regulation of VEGF expression) activation, whereas EGCG inhibited Erk1/2 activation in a dose dependent manner[138].

EGCG exhibited a protective effect against cell death

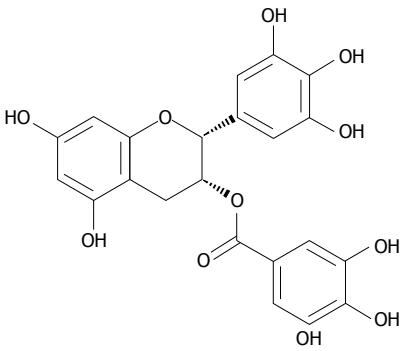

Figure 5 Chemical structure of epigallocatechin gallate [(2R,3R)-5,7-dihydroxy-2-(3,4,5-trihydroxyphenyl)chroman-3-yl] 3,4,5-trihydroxybenzoate.

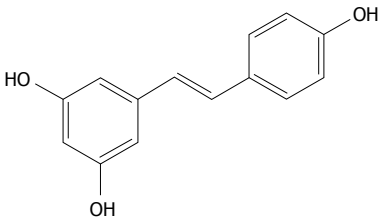

Figure 6 Chemical structure of Resveratrol is 3,5,4'-trihydroxystilbene.

by H_2O_2 in HLEB-3 cells[139] and ultraviolet irradiation in ARPE-19 cells[140]. In another study by Zhang *et al*[141] EGCG provided protection to retinal neurons from oxidative stress in RGC-5 cell line and to the retina against I/R in rats. Interestingly, intravenous administration of epigallocatechin and EGCG was found to reduce the intraocular pressure in normotensive rabbits suggesting a possible role in glaucoma therapy[142].

STILBENE DERIVATIVES: RESVERATROL

Resveratrol (RES) is a stilbene derivative (Figure 6) found in the skin of grapes and some other fruits. It is a phytoalexin, produced by plants during pathogenic infections. The pharmacological activity of RES was not noticed until 1992 when Baur *et al*[143] proved its cardio protective action in rodent models.

Most of the ophthalmic diseases such as AMD, PDR, proliferative vitreoretinopathy and cataract are the direct consequences of oxidative stress at a molecular level on various ocular tissues[144,145]. King *et al*[146] reported that RES prevented oxidative stress in cultured human RPE cells. They observed a 20% decrease in oxidation, compared to the control group, when treated with 100 μmol/Lol per liter RES. Concentration dependent anti-proliferative activity of RES was observed with 25%, 49% and 80% reduction of cell proliferation at 100, 200 and 400 μmol/L RES, respectively. Pintea *et al*[147] performed similar experiments with cultured human RPE cells and observed reduced levels of ROS following exposure to 500 μmol/L hydrogen peroxide in the presence of 25, 50 and 100 μmol/L RES. They also reported that RES at concentrations of 25-100 μmol/L

showed protective effects without any cytotoxicity and increased superoxide dismutase, glutathione peroxidase, and catalase activity in a dose dependent manner. Zheng *et al*[148] also demonstrated the anti-oxidative activity of RES against oxidative stress induced by hydrogen peroxide in human lens epithelial cells (LEC). The authors observed that pretreatment with 20 μmol/L RES for 12 h is optimum for anti-oxidant activity.

RES has been reported to have both pro-apoptotic and anti-apoptotic activities[149]. Gurong *et al* reported that these activities of RES depend on the forkhead box O genes in porcine LEC. They observed that when this gene is silenced by specific small interference RNAs the protective activity of RES was lost. The authors also reported a reduction of cell senescence markers in RES treated LEC.

Alex *et al*[150] performed experiments on anti-proliferative activity of RES, EPCG and curcumin using ARPE-19 and human RPE culture cells at various concentrations. After 72 h RES was found to be the most effective in inhibiting cell growth and cell division. Oxidative stress and over expression of factors such as VEGF, angiotensin-converting enzyme, MMP-9, and endothelial nitric oxide synthase are responsible for ocular neovascularization. Yar *et al*[151] investigated the effect of RES on ocular neovascularization and found decreased mRNA levels of these factors.

Increased glucose levels in diabetes causes accumulation of inflammatory mediators in RPE[152-157]. As discussed earlier, inflammatory molecules such as cytokines, IL-6, TNF-α, arachidonic acid and COX, PG, intercellular adhesion molecules (ICAM), monocyte chemotactic protein (MCP) are responsible for ocular inflammation and pathological conditions such as DR[158] and AM[159]. Kubota *et al*[160] used oral doses of 5, 50, 100, 200 mg/kg of bodyweight in endotoxin induced uveitis (EIU) mice model with RES. They found a significant dose dependent reduction in leukocyte adhesion molecules, ICAM. The levels of ICAM and MCP were also reduced in the retina and RPE-choroid on administration of 50 mg/kg of RES in the EIU mice model. All other inflammatory mediator levels were also diminished.

Zhou *et al*[161] observed increased levels of the glaucoma biomarker, endothelial leukocyte adhesion molecule-1, when acutely treated with H_2O_2. Chronic treatment resulted in sustained stress response activation[162]. Luna *et al*[163] observed a significant inhibition of these markers on administration of RES. They also noted that at high concentrations (200 μmol/L and 400 μmol/L) RES is cytotoxic and leads to cell death in less than 48 h. At concentrations of 50-100 μmol/L RES exerts its anti-inflammatory activity.

CURCUMIN

Curcumin is a natural phenol obtained from Curcuma longa of the Zingiberaceae family. It is the major of the three curcuminoids. Curcumin is used as an anti-inflammatory and anti-oxidant in various pathological conditions.

Oxidative stress is one of the major reasons for age related atherogenesis[164-169]. Awasthi *et al*[170] used an *in vitro* rat model to establish the activity of curcumin as an antioxidant in treating cataract. When treated with curcumin, 75 mg/kg, there was a significant increase in the glutathione S-transferase isozyme, rGST8-8, which uses 4-hydroxy-2-nonenal/L, a highly electrophilic product of lipid peroxidation, as a substrate and reduces it. It was observed that naphthalene, through an oxidative stress mediated pathway, causes cataract in rat and rabbit models. When treated with 0.005% w/w curcumin, there was significantly less opacification and apoptosis of LEC[171]. Suryanarayana *et al*[172] also studied the effect of curcumin in galactose induced cataract in rats. They observed that at 0.002% curcumin inhibited the onset and maturity of cataract by inhibiting oxidative species, and at 0.01% delayed the onset but accelerated the maturity of cataract formation indicating the increased oxidative stress conditions in hyperglycemic conditions. The results were supported by the report from Renu and Mamta who used 0.05% of curcumin and observed an increase in oxidative stress factors VGEF, IL-1β and nitrotyrosine and decreased GSH[173]. Manikandan *et al*[174] reported the protective action of curcumin in selenium induced cataract in Wister rats by inhibiting inducible nitric oxide synthase (iNOS) expression. Kumar *et al*[175] reported the reduced expression of α A and α B crystallins, produced during oxidative stress conditions in ocular tissues, in the presence of curcumin. Kimura *et al*[176] reported the inhibition of NF-κB and TNF-α by curcumin in human corneal epithelial cells.

Gupta *et al*[177] reported the anti-inflammatory effect of curcumin in diabetic rats, investigating the inflammatory mediators VGEF and TNF-α. They observed a significant reduction of these mediators in DR. Chen *et al*[178] studied the anti-inflammatory activity of curcumin in human corneal cells using sodium chloride in the medium which served as an *in vitro* dry eye syndrome model. Curcumin, at 5 μmol/L, prevented an increase in IL-1β, p38 mitogen-activated protein (MAP) kinase, JNK MAP kinase and NF-κB. Mandal *et al* confirmed the anti-inflammatory effect of curcumin in retinal cells. They suggested that curcumin modifies the activity of the inflammatory mediators such as NF-κB, phosphatidylinositol-3-kinase, nuclear factor erythroid 2-related factor 2 and prevents AMD.

An *et al*[179] evaluated anti-proliferative activity of curcumin in cultured rabbit RPE and reported a dose dependent and time dependent inhibition of RPE cells by increased Ca^{2+}, which causes a decrease in DNA content. Lu *et al*[180] used N18 mouse-rat hybrid retina ganglion cells for the determination of the anti-proliferative effect of curcumin. In this study, arrest of N18 cells in the G2/M phase induces endoplasmic reticulum stress leading to apoptosis of retinal ganglion based on caspase-3-dependent and independent pathways. Huang *et al*[181]

Table 1 Route of administration dependent physiological barriers encountered in the diffusion path to the neural retina

Physiological barriers	Route of administration			
	Topical	Peri-ocular	Systemic	Oral
Precorneal loss	√			
Corneal ultrastructure	√			
Corneal efflux proteins	√			
Corneal tight junctions	√			
Conjunctival efflux proteins	√			
Conjunctival tight-junctions	√			
Conjunctival vasculature	√			
Aqueous humor outflow	√			
Anterior chamber metabolism	√			
Scleral vasculature	√	√	√	√
Choroidal vasculature	√	√	√	√
Bruch's membrane	√	√	√	√
Outer BRB				
RPE tight junctions	√	√	√	√
RPE efflux pumps	√	√	√	√
Inner BRB				
Tight-junctions			√	√
Efflux proteins			√	√
Low concentration gradient			√	√
First pass effect				√
Absorption limitations				√
Metabolism in the GIT				√
Systemic metabolism				√

RPE: Retinal pigment epithelium; BRB: Blood retinal barrier; GIT: Gastrointestinal tract.

reported that curcumin induced apoptosis in bovine LEC by lowering the cell DNA levels. Curcumin was also found to inhibit human fetal retinal pigment epithelium cell proliferation by arresting the cell in G2/M phase of cell cycle[182].

Anti-angiogenic activity of curcumin was investigated in corneal endothelial cells of transgenic mouse model[183]. Curcumin was found to have angiostatic activity, inhibiting endogenous gelatinase B expression induced by fibroblast growth factor-2 which is responsible for angiogenesis. Mrudula *et al*[184] also investigated for anti angiogenic activity of *Curcumin* in streptozotocin-induced diabetic rat retina at 0.002% or 0.01% curcumin or 0.5% turmeric for a period of 8 wk. They observed inhibition of VGEF expression in treated rats. I/R injury leads to the neuron loss, glial activation and vascular degeneration. When this condition was treated with 0.01%-0.25% Curcumin, there was inhibition of injury induced NF-κB and STAT-3 which leads to inhibition of I/R injury and 0.05% Curcumin lead to vaso-protective effect[185]. Vasireddy *et al*[186] reported anti-protein aggregation of Curcumin in case of *P23H* mutation where rhodopsin molecules forms misfolding and forms aggregates leading to loss of activity.

Thus, curcumin seems to be a promising candidate for as antioxidant, anti-inflammatory, anti-proliferative, and anti-angiogenic activity.

OCULAR DRUG DELIVERY

Although the potential of the phytochemicals as therapeutic agents for oxidative stress associated ocular diseases has been demonstrated, their delivery to the ocular tissues and physiological diffusion barriers encountered has not been investigated. In order to limit/prevent oxidative damage it is imperative that the bioflavonoids reach the deeper ocular tissues such as the neural retina and lens, the sites of free radical induced damage, in effective concentrations. Unfortunately, drug delivery to the ocular tissues, especially the posterior segment ocular tissues, is a very challenging task.

The eye is a secluded organ, protected by various physiological barriers that restrict entry of xenobiotics into the ocular tissues from the external environment (topical or periocular) or systemic circulation (Table 1). The choroidal blood vessels possess many large fenestrations and pinocytic vesicles that allow free exchange of endogenous/exogenous substances between the choroidal stroma and choroidal vasculature.

Thus, systemically administered therapeutic agents can easily diffuse out into the choroidal stroma from the systemic circulation[187]. Bruch's membrane, separating the choroid from the RPE, acts as a barrier to the diffusion of only macromolecules, like proteins and genes, and thus does not pose much of a diffusional barrier to small molecules. The RPE, on the other hand, presents a formidable permeation barrier to small drug molecules in their diffusion into the neural retina and vitreous humor from the choroidal stroma[187-189]. Epithelial cells of the RPE are joined together by tight junctions[190-194] similar to those observed in the blood-brain barrier, severely restricting paracellular diffusion of hydrophilic molecules. The RPE cells also express the efflux proteins P-gp and multidrug resistance-associated protein (MRP) on the basolateral membrane[195-198]. As a result, transretinal permeation of compounds that are substrates of these efflux proteins, from the systemic circulation (choroidal side) into the neural retina/vitreous humor, is strongly modulated by RPE P-gp/MRP mediated efflux. The RPE, thus, presents a major barrier to the exchange of therapeutic agents between the choroidal stroma and the neural retina/vitreous humor, and is referred to as the outer BRB.

Similar to the RPE, the endothelial cells of the blood vessels perfusing the neural retina express efflux proteins and tight-junction proteins[189,199]. The efflux proteins are polarized on the apical membrane of the endothelial cells (facing the lumen of blood vessels) and prevent entry of xenobiotics from the systemic circulation into the neural retina. Like in the outer BRB, tight junction proteins expressed on the inner BRB also severely limits paracellular diffusion of hydrophilic compounds. The barrier properties of the retinal blood vessel endothelial cells are commonly referred to as the inner BRB. Additionally, because of extensive intestinal and hepatic metabolism, the hydrophilic metabolites of the phytochemicals are seen in the systemic circulation and are available for penetration into the ocular tissues. The ambiguity in the results obtained from the limited number of clinical studies that have been carried out with the phytochemi-

cals could be because of the inadequate delivery of the compounds into the tissues of the eye[200].

Periocularly administered agents also encounter the RPE barrier. Thus, the inner and outer BRB acts as a considerable physiological barrier to the ocular delivery of therapeutic agents through the systemic and transcleral routes of administration. Additionally, transcleral permeation is also challenged by the scleral and choroidal blood flow and lymphatics.

Topical administration is the most favored route of administration for ocular disorders. Although significant advances have been made with respect to drug delivery into the front of the eye through the topical route, back-of the eye delivery remains a significant challenge. It is thought that following topical application diffusion into the cornea, followed by lateral migration into the sclera and then diffusion across the choroid and RPE into the vitreous, is the major pathway for topically administered agents[201]. Thus, scleral and choroidal vascular and lymphatic systems as well as the RPE present significant barriers in the diffusional path. Additionally, precorneal loss, corneal ultrastructure and efflux proteins expressed on the corneal membrane present additional physiological barriers to topically administered agents.

OCULAR DELIVERY OF THE PHYTOCHEMICALS

The important bioflavonoids are well-established substrates of P-gp/MRP[202-210], and their glucuronides are extremely hydrophilic in nature as well as probable substrates of efflux proteins[211-213]. In view of the ocular barriers, very little, if any, of the parent compound and their metabolites can permeate into the neural retina from the systemic circulation. Considering that the majority of the free radical induced cellular damage is in the neural retina, achieving therapeutic drug concentrations in this tissue is absolutely necessary. It is noted, however, that oral administration may be beneficial in some aspects of the overall disease pathogenesis, *e.g.*, in increasing retinal circulation and in decreasing microvascular leakage. There is some evidence that the hydrophilic metabolites are converted into the parent forms at the site of action[214]. However penetration of these metabolites across the retina is doubtful. Approaches based on direct intravitreal injections and transscleral applications have proven to be effective in delivering therapeutic levels of drug candidates to the back-of-the eye. Although, the intravitreal route is invasive in nature and is associated with various risk factors such as endophthalmitis and retinal detachment, they remain the most effective approach to date since this route avoids all the blood-ocular barriers. Intravitreal nanoparticulate systems can be tailored through judicial formulation approaches to achieve sustained release of the therapeutic agents for prolonged periods of time[215,216] thus minimizing the frequency of injection. Bourges *et al*[217] injected nanoparticles of different particle sizes and charge into vitreous humor of rabbit eye. They observed the migration of these particles from vitreous humor to retinal layers. They also observed the presence of the nanoparticles even after 4 mo of the single injection. Transscleral approaches, such as subconjunctival delivery, helps in achieving high local concentration gradient of therapeutic agents. However, the challenges posed by the scleral and choroidal lymphatics and vasculature have to be taken into account. Misra *et al*[218] investigated subconjunctivally biodegradable hydrogel implants for sustained release of insulin to treat DR. Based on hematoxylin and eosin stain, these implants proved to deliver drug to retina for very long time without causing any harmful effects on eye.

CONCLUSION

Several phytochemicals are potential candidates that can be used as a prophylactic agent in DR and AMD. However, systemically they exist mainly as the hydrophilic metabolites whose permeation into the ocular tissues are questionable and needs investigation. Moreover, activity of these metabolites is also not well established. In view of the challenges faced in the ocular delivery of the phytochemicals through the oral or systemic routes, topical instillation appears to be the most promising mode of administration. Although the retinal barriers, specifically the outer BRB, is still encountered by this route, the high local concentrations achieved and the absence of significant metabolism of the aglycones in the ocular tissues presents a greater likelihood of achieving therapeutic concentrations in the back-of-the eye. Studies carried out in our laboratory suggests that while significant hesperetin levels can be achieved in the RPE-choroid tissue through the topical route, getting a compound into the vitreous humor is still a challenge. Vitreal concentrations reflect free drug concentrations in the neural retina since the diffusional barrier between these two ocular components is thought to be negligible. Concentrations in the RPE-choroid by itself cannot guarantee levels in the neural retina since the compound may be in the choroid or bound to the RPE or Bruch membrane proteins. Thus, penetration into the back-of-the eye is governed by the physicochemical characteristics of the molecules and the susceptibility to being effluxed. Moreover, the compounds are rapidly cleared from the ocular tissues. Thus, further investigations into the development of novel approaches for sustained delivery of the phytochemicals to the posterior segment ocular tissues, through direct intravitreal injections or trans-scleral delivery, are needed. For anterior segment diseases, the topical formulations prepared using currently available technologies, should be an effective means of testing the therapeutic effectiveness of these promising compounds.

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REVIEW

Drug-transporter interaction testing in drug discovery and development

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Abstract

The human body consists of several physiological barriers that express a number of membrane transporters. For an orally absorbed drug the intestinal, hepatic, renal and blood-brain barriers are of the greatest importance. The ATP-binding cassette (ABC) transporters that mediate cellular efflux and the solute carrier transporters that mostly mediate cellular uptake are the two superfamilies responsible for membrane transport of vast majority of drugs and drug metabolites. The total number of human transporters in the two superfamilies exceeds 400, and about 40-50 transporters have been characterized for drug transport. The latest Food and Drug Administration guidance focuses on P-glycoprotein, breast cancer resistance protein, organic anion transporting polypeptide 1B1 (OATP1B1), OATP1B3, organic cation transporter 2 (OCT2), and organic anion transporters 1 (OAT1) and OAT3. The European Medicines Agency's shortlist additionally contains the bile salt export pump, OCT1, and the multidrug and toxin extrusion transporters, multidrug and toxin extrusion protein 1 (MATE1) and MATE2/MATE2K. A variety of transporter assays are available to test drug-transporter interactions, transporter-mediated drug-drug interactions, and transporter-mediated toxicity. The drug binding site of ABC transporters is accessible from the cytoplasm or the inner leaflet of the plasma membrane. Therefore, vesicular transport assays utilizing inside-out vesicles are commonly used assays, where the directionality of transport results in drugs being transported into the vesicle. Monolayer assays utilizing polarized cells expressing efflux transporters are the test systems suggested by regulatory agencies. However, in some monolayers, uptake transporters must be coexpressed with efflux transporters to assure detectable transport of low passive permeability drugs. For uptake transporters mediating cellular drug uptake, utilization of stable transfectants have been suggested. *In vivo* animal models complete the testing battery. Some issues, such as *in vivo* relevance, gender difference, age and ontogeny issues can only be addressed using *in vivo* models. Transporter specificity is provided by using knock-out or mutant models. Alternatively, chemical knock-outs can be employed. Compensatory changes are less likely when using chemical knock-outs. On the other hand, specific inhibitors for some uptake transporters are not available, limiting the options to genetic knock-outs.

Key words: ATP-binding cassette transporter; Solute carrier; Drug efflux; Drug uptake; Absorption-distribution-metabolism-excretion-toxicity; Regulatory guidance; ATPase; Vesicular transport; Monolayer assay; *In vivo*

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IMPORTANT BARRIERS AND TRANSPORTERS

The human body harbors a number of physiological barriers. From an oral drug administration point of view the intestinal, hepatic, renal and blood-brain barriers are considered pivotal.

The intestinal barrier is the site of absorption of orally administered drugs. The main cellular components of the intestinal barrier are the enterocytes. Generally, the small intestine is considered of utmost importance. The large surface area and the stomach-proximal position make the small intestine the site of absorption of many oral drugs. With the development of controlled release formulations, more and more studies are concerned with absorption through the colon. The activity of several metabolic enzymes is lower in the colon than in the small intestine[1,2] making the colon an attractive site for absorption. The regional transporter expression data from several papers are inconclusive. The only consensus is that there is significantly higher expression of P-glycoprotein (P-gp)/multidrug resistance protein 1 (ABCB1) in the colon compared to the small intestine, and higher expression of multidrug resistance associated protein 2 (MRP2, ABCC2) in the small intestine compared to the colon[1,3]. Transporters that are expressed in the enterocytes are depicted in Figure 1A. The only transporters that are highly expressed in the intestine and are on the shortlists of both the Food and Drug Administration (FDA)[4] and the European Medicines Agency (EMA)[5] are the apically located P-gp[6] and breast cancer resistance protein (BCRP, ABCG2). These transporters are known to transport many xenobiotics and, therefore, constitute a barrier for drug absorption *via* the intestines.

Two major interfaces connecting the blood and brain compartments are the blood-brain barrier (BBB) and the blood-cerebrospinal fluid barrier (BCSFB). The BBB is by far the more important barrier, as the surface area of the human BBB is approximately 100-fold larger than the surface area of the BCSFB[7,8]. In addition, the distance between neurons and brain capillaries is less than 20 nm in the BBB while the distance between the brain ventricles and circumventricular organs is in millimeter or centimeter range in the BCSFB[9]. The barrier function in the BBB is provided by the microcapillary endothelial cells that contain no fenestrations. Transporters that are expressed in the brain microcapillary endothelial cells are depicted in Figure 1B. Similar to the intestinal barrier, the two transporters on the list of regulatory agencies are the luminally located P-gp and BCRP, indicating that, from a drug development point of view, the BBB mainly functions as a barrier for drug absorption.

The hepatic barrier is the major site of excretion of drugs and drug metabolites. The transporters that are expressed in the parenchymal cells (hepatocytes) are depicted in Figure 1C. The hepatic transporters on the FDA short list are uptake transporters of the organic anion transporting polypeptide (OATP)/Solute Carrier OATP (SLCO) family members rganic anion transporting polypeptide 1B1 (OATP1B1)/SLCO1B1 and OATP1B3 (SLCO1B3), and efflux transporters P-gp and BCRP. The EMA short list adds three additional hepatic transporters: organic cation transporter 1 (OCT1, SLC22A1), bile salt export pump (BSEP, ABCB11) and multidrug and toxin extrusion protein 1 (MATE1, SLC47A1). BSEP transports bile salts and, therefore, has toxicological significance. Noticeably, missing from both lists is MRP2 (ABCC2), a transporter on the canalicular membrane, which transports many drugs and phase II drug metabolites into the bile. The vectorial summation of the activity of the sinusoidal/basolateral uptake transporters and canalicular/apical efflux transporters drives the secretory function of this barrier.

The renal barrier is the other major site of excretion. The main cellular components of the renal secretory transport are the proximal tubule epithelial cells (PTC). The transporters that are expressed in the PTC are shown in Figure 1D. The renal transporters on the FDA short list are basolateral uptake transporters OCT2 (SLC22A2), OAT1 (SLC22A6), OAT3 (SLC22A8) and apical efflux transporters P-gp and BCRP. The EMA guidance also refers to MATE1 and MATE2/MATE2K (SLC47A2) as transporters that should be considered. This arrangement is similar to the hepatocyte, suggesting that the PTC mainly work in a secretory fashion as well. It should be noted that although significant xenobiotic reuptake occurs through PTC, literature data mainly focus on reuptake of physiological substrates.

In general, the transporters listed above have been shown to play a role in ADMET (Absorption-Distribution-Metabolism-Excretion-Toxicity) of drugs. However, regulatory guidances[4] note that additional transporters (*e.g.*, MRPs) should be considered when relevant for the therapeutic class of drug being studied.

TRANSPORTER-MEDIATED PERMEATION *VS* PASSIVE PERMEABILITY/DIFFUSION

In the pharmaceutical industry transcellular permeation of drugs has been viewed as the combination of passive and/or transporter-mediated processes[10]. Sequencing of the human genome yielded 883 putative transporter genes[11]. The suggested number of two main superfamilies of human membrane transporters, the ATP-binding cassette (ABC) transporters, mediating mainly cellular efflux, and the solute carriers (SLC), mediating mainly cellular uptake of their substrates, is well over 400[12]. It is likely that any particular cell may express dozens of transporters. Because of the large number of transporters and the broad substrate specificity of many of transporters, as well as the energetically unfavorable transbilayer permeation of small charged molecules, it has been suggested that drug transport is essentially carrier mediated[13]. It has been hypothesized that lack of saturation of transcellular permeation of some drugs, which is considered by many as the proof of passive diffusion[14],

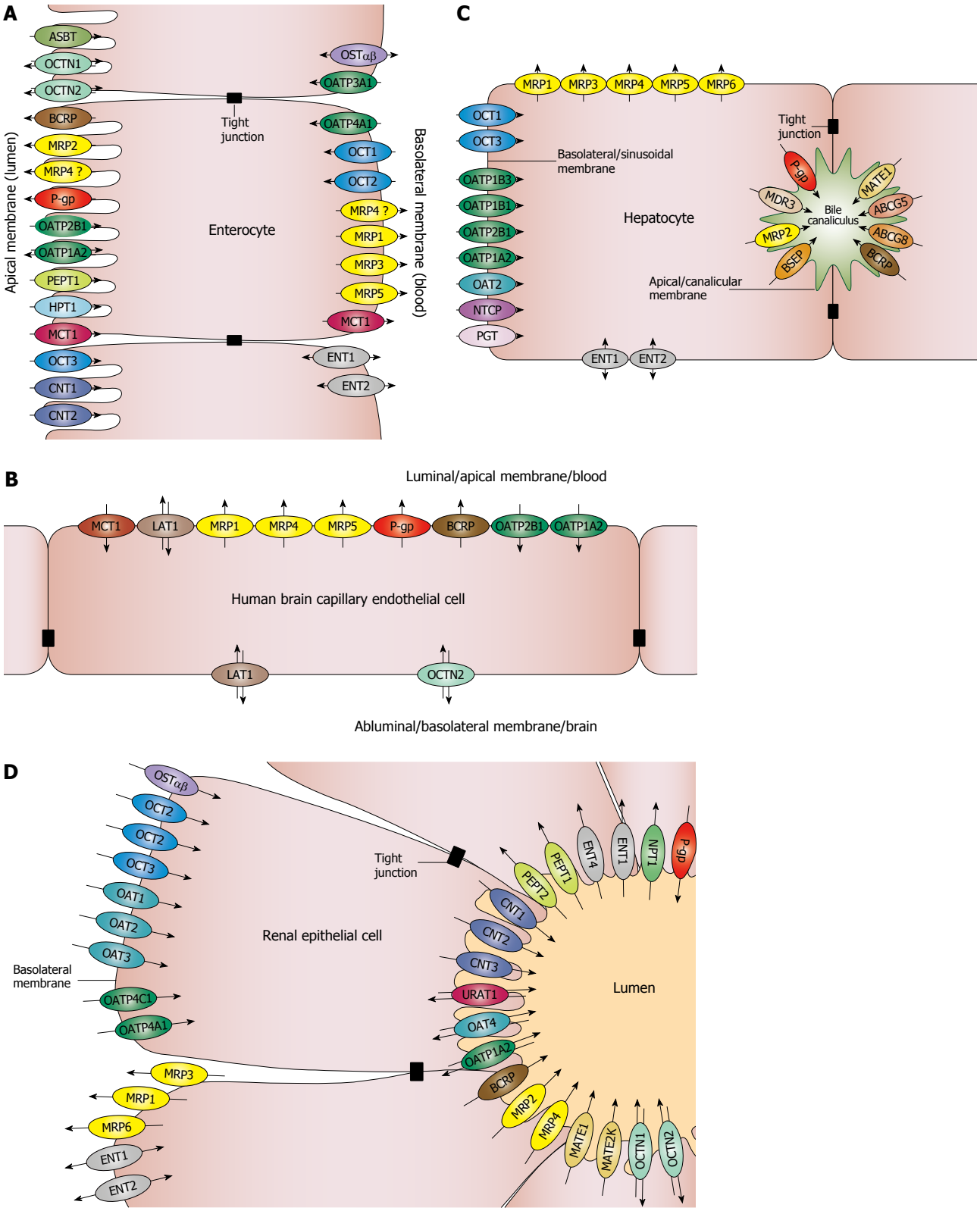

Figure 1 Expression of transporters in human enterocytes (A), brain microcapillary endothelial cells (B), hepatocytes (C) and renal proximal tubule epithelial cells (D). OCTN: Organic cation transporter novel; BCRP: Breast cancer resistance protein; MRP: Multidrug resistance associated protein; OATP: Organic anion transporting polypeptide; ENT: Equilibrative nucleoside transporter; PEPT: Peptide transporter; P-gp: P-glycoprotein; MATE: Multidrug and toxin extrusion protein; BSEP: Bile salt export pump.

is the result of transport by a series of transporters with different affinities[13]. It has also been argued that lack of stereospecificity in permeability of some drugs can be attributed to the broad substrate specificity of transporters[15]. Correlation of apparent permeability coefficients (P_{app}) for the same drug across different cell lines is a

focus of the debate[10,13]. However, multiple drugs show identical P_{app} values in A > B and B > A direction when the known transporters are blocked[16-18]. As in polarized monolayers the transporter expression and activity on the basolateral and apical membranes are likely different these observations require explanation. Even the simplest models used to extract kinetic parameters of transcellular transport of drugs require extensive computation[16,19-23]. Therefore, the consideration of multiple transporters may be a challenging concept to develop into a generally accepted model for use by the pharmaceutical industry.

IN VITRO TESTING

The vast majority of drugs are effluxed by ABC transporters. Other important transporters include members of the MATE/SLC47 and the equilibrative nucleoside transporter (SLC29) families. In addition, efflux action by SLCO[24,25] and OCT novel (SLC22A4-5) family members[26,27] has been suggested. From a pharmacological point of view the main function of MATE transporters is drug efflux. However, based on their classification as an SLC, as well as the predominant assay format (cellular uptake), these transporters will be discussed among uptake transporters. The list of the ABC and SLC transporters identified by the regulatory agencies as of special importance is shown in Table 1.

Efflux transporters

Both membrane-based assays and cellular assays are widely used to test drug transport and drug-drug interactions by ABC efflux transporters. Membrane assays include ATPase and vesicular transport (VT) assays[28]. ATPase assays are based on coupling of ATPase activity to transport and can be considered as surrogate transport assays. VT assays utilize inside-out vesicles and measure accumulation of substrates into the vesicles. Cell-based assays include monolayer efflux assays, cytotoxicity assays, cellular accumulation and efflux assays as well as dye efflux assays. Monolayer efflux assays monitor transcellular transport of substrates and measure the vectorial contribution of transporters. Monolayer efflux assays can be performed in a bidirectional mode or in a unidirectional mode in the presence and absence of an inhibitor. Cytotoxicity assays are mostly used to measure efflux transporter mediated drug resistance[29,30] which can be reversed by a transporter specific inhibitor. It is assumed that efflux transporters inhibit accumulation, hence, efficacy of substrate chemotherapeutics. Thus, the assay is a surrogate transport assay. Cell accumulation and efflux assays are performed in cells overexpressing the transporter. The most common setup involves accumulation in the presence and absence of a specific inhibitor. In cellular efflux assays, after the initial loading, substrate efflux is measured in the presence and absence of specific inhibitors and cell associated drug content is plotted as the percentage of drug remaining in the cells *vs* time[31]. With the exception of reversal agent development neither cellular accumulation nor cellular efflux assays are commonly performed in drug ADME studies. Dye efflux assays monitor efflux activity of transporters using fluorescent probe substrates or non-fluorescent precursor probes[29]. The Calcein assay is the prototype of dye efflux assays which use non-fluorescent dyes as probes[29]. The non-fluorescent calcein-AM, which is a substrate for both P-gp[32] and MRP1[33], is cleaved by intracellular esterases to yield the fluorescent calcein, which is a substrate for MRP1, but not for P-gp[34]. Calcein is hydrophilic and will not diffuse out of the cells, therefore it accumulates at a slower rate in P-gp or MRP1 overexpressing cells compared to control cells, unless the transporters are inhibited. The advantage of using a non-fluorescent substrate is that it can be conveniently performed in high throughput without the need of a fluorescence activated cell sorter or extensive washing. Dye efflux assays are commonly performed as inhibition assays[35] applicable to various cell types and, therefore, can be done in a tissue/cell type specific manner[36].

Two large studies correlated P-gp ATPase and P-gp monolayer efflux measurements[37,38]. Both studies found that a group of high passive permeability substrates that were efficacious ATPase activators did not appear to be P-gp substrates in the monolayer assay. The likely explanation is that the contribution of the transporter to the overall permeability of these compounds is negligible. These compounds were then termed as non-transported substrates[37]. However, several of these compounds such as verapamil[37], ketoconazole[37] and itraconazole (Fekete *et al*: manuscript in preparation) have shown P-gp dependent BBB permeability in humans[39] and mice[40,41]. Due to their high passive permeability, none of the cellular or other vesicular assays would work for these compounds. Therefore, for this group of ABC transporter substrates the ATPase assay is the only assay that predicts a P-gp limited penetration of the BBB.

Passive permeability is a key determinant in assay selection. For example, low passive permeability compounds may be false negatives in P-gp ATPase activation assays[37]. VT/uptake assays work best for low passive permeability compounds[28]. For low and intermediate passive permeability compounds monolayer assays work well, although, for some low passive permeability compounds, an uptake transporter is required for significant transcellular transport[42]. Passive permeability does not play a role in membrane assays when used in an inhibition format. However, monolayer assays will not necessarily work for low passive permeability inhibitors. The effect of passive permeability on assay selection is depicted in Figure 2.

Membrane lipid composition is also an important determinant of transporter activity. BCRP[43-45] and BSEP[46,47] activity is significantly greater in mammalian or cholesterol enriched insect cell membranes than in native insect cell membranes, which contain significantly lower amounts of cholesterol[44], and both BCRP[48] and BSEP[49] are localized in cholesterol rich microdomains. Interestingly, perhaps with the exception of the cyclosporin A-BSEP inter-

Table 1 Characteristics of transporters on the shortlists of regulatory

Transporter	Expression (tissue/cell type/localization)	Physiological substrates	Select drug substrates	Guidance
P-gp	Brain/endothelial cell/apical Kidney/epithelial cell/apical Liver/hepatocyte/canalicular Small intestine/enterocyte/apical (colon)	Phospholipids, cytokines, steroids	Aliskiren, ambrisentan, colchicine, dabigatran etexilate, digoxin, everolimus, fexofenadine, imatinib, indinavir, itraconazole, lapatinib, maraviroc, nilotinib, paclitaxel, posaconazole, ranolazine, saxagliptin, sirolimus, sitagliptin, talinolol, tolvaptan, topotecan, vinca alkaloids	FDA/EMA
BCRP	Brain/endothelial cell/apical Liver/hepatocyte/canalicular Small intestine/enterocyte/apical Kidney/epithelial cell/apical Placenta/syncytiotrophoblast/apical (maternal)	Vitamins (riboflavin, biotin), porphyrins, estrogen sulfate conjugates	Methotrexate, mitoxantrone, daunorubicin, doxorubicin, imatinib, irrinotecan, lapatinib, rosuvastatin, pitavastatin, provastatin, sulfasalazine, topotecan	FDA/EMA
BSEP	Liver/hepatocyte/canalicular	Taurocholate, glycocholate	Pravastatin, paclitaxel, vinblastine	EMA
OATP1B1	Liver/hepatocyte/basolateral	Bilirubin and its conjugates, thyroxin, triiodothyronine, bile acids, eicosanoids (thromboxane B2, prostaglandin E2, leukotriene C4), dehydroepiandrosterone sulfate, estradiol 17β-glucuronide, estrone 3-sulfate, glycocholate	Atrasentan, atorvastatin, bosentan, ezetimibe, fluvastatin, glyburide, methotrexate, olmesartan, pitavastatin, pravastatin, repaglinide, rifampin, rosuvastatin, simvastatin acid, SN-38 (active metabolite of irinotecan), valsartan	FDA/EMA
OATP1B3	Liver/hepatocyte/basolateral	Estradiol 17β-glucuronide, taurocholate, estrone 3-sulfate, dehydroepiandrosterone sulfate, thyoxin	Atorvastatin, bosentan, digoxin, methotrexate, olmesartan, paclitaxel, pitavastatin, rosuvastatin, telmisartan, valsartan	FDA/EMA
OAT1	Kidney/proximal tubular cell/basolateral	Para-aminohippuric acid, homocysteine, Cysteine,dicarboxylates, prostaglandine E2, urate, estrone-3-sulfate	Adefovir, captopril, cidofovir, furosemide, lamivudine, methotrexate, oseltamivir, tenofovir, zalcitabine, zidovudine	FDA/EMA
OAT3	Kidney/proximal tubular cell/basolateral	Estrone 3-sulfate, estradiol 17β-glucoronide, cAMP, taurocholate, cortisol, dehydroepiandrosterone sulfate, prostaglandine E2, urate, succinate, para-aminohippuric acid	Acyclovir, bumetanide, ciprofloxacin, famotidine, furosemide, methotrexate, oseltamivir acid, (the active metabolite of oseltamivir), penicillin G, pravastatin, rosuvastatin, sitagliptin, valacyclovir, zidovudine	FDA/EMA
1-Oct	Liver/hepatocyte/basolateral Small intestine/enterocyte/basolateral	Corticosterone, β-oestradiol, progesterone, testosterone, choline, creatinine, guanidine, L-carnitine, thiamine, thyramine, acetylcholine, dopamine	Acyclovir, amantadine, gancyclovir, imatinib, lamivudin, metformin, oxaliplatin, quinidine, quinine, ranitidine, zalcitabine	EMA
2-Oct	Kidney/epithelial cell/basolateral	β-oestradiol, progesterone, testosterone, choline, creatinine, guanidine, L-carnitine, acetylcholine, dopamine, epinephrine, norepinephrine, histamin, serotonin, choline, dopamine, prostagladine E2	Amantadine, amilorid, cimetidine, cisplatin, dofetilide, famotidine, lamivudin, metformin, oxaliplatin, pindolol, procainamide, ranitidine, zalcitabine	FDA/EMA
MATE1	Kidney/epithelial cell/apical Liver/hepatocyte/canalicular	Choline, creatinine, guanidine, corticosterone, estrone 3-sulfate, thiamine	Acyclovir, cimetidine, fexofenadine, gancyclovir, metformin, procainamide, topotecan	EMA
MATE2/MATE2K	Kidney/epithelial cell/apical	Choline, creatinine, guanidine, corticosterone, estrone 3-sulfate, thiamine	Acyclovir, cimetidine, gancyclovir, metformin, procainamide, topotecan	EMA

FDA: Food and Drug Administration; P-gp: P-glycoprotein; EMA: European Medicines Agency; BCRP: Breast cancer resistance protein; OAT: Organic anion transporter; OCT: Organic cation transporter; MATE1: Multidrug and toxin extrusion protein 1.

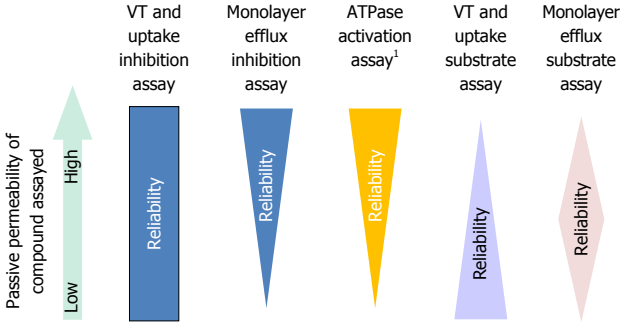

Figure 2 Application of different types of assays for low-high passive permeability compounds. [1]Shown for P-glycoprotein ATPase. VT: Vesicular transport.

action, cholesterol loading did not affect IC_{50} data[43,44,46]. All in all, these data show that the best and certainly the most relevant expression systems are the mammalian/human cells.

Transporter expression levels may affect apparent ADME parameters. Apparent K_M values generated in monolayer assays displayed a linear correlation with P-gp expression[50]. In contrast, the intrinsic K_M values that were based on intracellular concentrations showed independence from transporter expression[19,22]. Some IC_{50} values were also shown to depend on transporter expression with increasing values in higher expressers[36,51]. The phenomenon was predicted by simulations[21] and appears

to have *in vivo* relevance[52,53]. The simulation study also predicted that in a VT system steady-state is established in seconds, as no permeability barriers exist[21].

The monolayer assay system is the suggested assay format for efflux transporter substrate and inhibition assays[4,5]. The advantage of the system is that it shows if the contribution of an efflux transporter is comparable in magnitude to passive permeability and, thus, modulates transcellular permeability of substrate drugs. However, in some aspects VT substrate and inhibition assays offer advantages over the monolayer assays, as data obtained in VT assays are not confounded by permeability barriers. Along this line, in earlier publications VT inhibition assays have been suggested as drug-drug interaction assays for low passive permeability drugs[54].

Digoxin is the consensus substrate for P-gp[4] and PSC833 is a commercially available P-gp specific inhibitor[55]. Dabigatran etexilate[56-58] or fexofenadine[58-60] could also be considered as probes as these are lower bioavailability substrates and are potential probes for clinical drug-drug interaction studies. However, only fexofenadine has been extensively studied *in vitro*[59,60]. Quinidine is an acceptable alternative to digoxin in microdialysis experiments where application of digoxin is not feasible due to non-specific adherence to tubing as well as toxicity[55,60]. No consensus has been reached on the probe substrates and inhibitors for BCRP. Topotecan[4,61], rosuvastatin[4], prazosin[44,62] and sulfasalazine[63,64] have all been suggested. However, these compounds are substrates of multiple efflux transporters that are co-expressed with BCRP on apical membranes. On the contrary, chlorothiazide, a non-metabolized[65], low bioavailability drug[66] is a specific BCRP substrate[67] and a potential probe. Ko134 and Ko143 have been extensively used in preclinical studies as BCRP-specific inhibitors. For BSEP, taurocholate is the consensus probe[46,68,69] and cyclosporin A, a cholestatic drug[70] is the reference inhibitor used most often[46,68,69,71,72]. Potential ABC transporter probe substrates are listed in Table 1.

Uptake transporters

Cellular uptake of drugs and endobiotics is mediated *via* uptake transporters of the SLC superfamily. The list of the uptake transporters identified by the regulatory agencies as important is shown in Table 1. Mechanistically these transporters are uniporters (*e.g.*, OCT1), symporters [*e.g.*, sodium taurocholate cotransporting polypeptide (NTCP, SLC10A1), peptide transporter 1 (PEPT1, SLC15A1)] or antiporters (*e.g.*, OATP1B1, OAT1, MATE1).

Membrane assays are applicable to symporters where the driving force of the transport is known and the assay set-up is straightforward. Na^+-taurocholate cotransporting polypeptide (NTCP)-mediated taurocholate transport into right-side out (ROV) rat sinusoidal membrane vesicles has been shown[73]. Proton gradient driven dipeptide transport into ROV prepared from intestinal brush-border membranes has also been published[74]. For exchangers (*e.g.*, OATPs, OATs) a vesicular uptake assay would be cumbersome to perform even if the identity of the exchange ion was known.

The most common assay system for uptake transporters are primary cells [*e.g.*, hepatocytes, brain microcapillary endothelial cells (BME), proximal tubule cells (PTC) of the kidney], cancer cell lines (*e.g.*, Caco-2), immortalized cell lines (*e.g.*, human brain endothelial cell line, hCMEC/D3) or transfectants. Transfectants are the test systems recommended by regulatory agencies[4].

Oocytes microinjected with the mRNA or cDNA of the respective transporter have been used early on. Oocytes offer the option of electrophysiological measurements as the transport of many substrates is electrogenic. However, the system is transient, the quality of the oocytes display seasonal variations, the lipid composition of the plasma membrane is different from physiological and the throughput is low-to-intermediate[75,76].

For uptake transporters brain slices[77], liver slices[78,79] and kidney slices[80,81] are commonly used to compute clearance values.

Uptake transporters have highly overlapping substrate specificities and multiple family members are expressed in the same cell type. Quantification of contribution of the different transporters is a challenge. OATP1B1 and OATP1B3 have very similar substrate specificities and are both expressed in hepatocytes. Estrone-3 sulfate and cholecystokinine octapeptide (CCK-8) are selective substrates of OATP1B1 and OATP1B3, respectively, and can be used as reference substrates to determine activities of these transporters in a hepatocyte preparation[82]. The most notable non-statin drugs are bosentan, a substrate of OATP1B1[83] and OATP1B3[83], valsartan[84] or repaglinide[85], substrates of OATP1B1, and telmisartan[86] or nafcilin[87], substrates of OATP1B3. Fluo-3 is a highly sensitive fluorescent probe of OATP1B3[88]. Rifampin and cyclosporin A are the recommended reference inhibitors[4] however various statins are also commonly used[89]. For clinical drug-drug interaction studies the use of statins as victims/probes has been suggested[4]. OAT1 and OAT3 are co-expressed in the basolateral membrane of PTC. These transporters have overlapping substrate specificities, with OAT3 having a bias for amphiphilic, larger molecular weight compounds[90]. Adefovir can be used as a reference substrate for OAT1 and benzylpenicillin for OAT3[80]. Tenofovir[91], azydothimidine/zidovudine[92], para-aminohippurate[4] for OAT1 and methotrexate[93], cimetidine[94], furosemide[95], estrone-3-sulfate[4] for OAT3 are also applicable. Probenecid inhibits both transporters[4] but benzylpenicilline is considered an OAT3-specific inhibitor[80,96]. P-aminohippurate has been used as a specific OAT1 inhibitor[80] and also as inhibitor of both transporters[96]. For OCT1[97,98] and OCT2[98-100] metformin is an accepted drug substrate. Alternatively, 1-methyl-4-phenylpyridinium (MPP^+) and cimetidine can be used for both OCT1[101,102] and OCT2[4,103]. Cimetidine or verapamil can be used as an OCT1[98,104] and OCT2 inhibitor[4,98,104], although, clinical relevance of cimetidine mediated inhibition of OCT2

has been questioned lately[105]. Metformin is a relevant substrate for both MATE1[106] and MATE2/MATE2K[106] and cimetidine[105] and verapamil[104] are potent inhibitors. Importantly, pyrimethamine has been shown to selectively inhibit MATE1 and MATE2/MATE2K[105,107].

Uptake transporters play a major role in pharmakokinetics of substrate drugs. Inhibition of hepatic[108] and/or renal[109] clearance by co-administered drugs can lead to clinically significant drug-drug interactions. Interactions of the hepatic uptake transporters often result in > 5-fold increase in C_{max} values of victim drugs[110]. Nevertheless, most *in vitro* assays commonly employ either physiological substrates such as estrone-3-sulfate or estradiol-17β-glucuronide for anion transporters or synthetic non-drug substrates, such as tetraethyl-ammonium for cation transporters[89]. Broad-scale application of LC/MS/MS methodology in drug quantification will facilitate revalidation of uptake transporter assays using drug probes.

IN VIVO TESTING

In vivo studies using knock-out and mutant animals shows the paramount importance of transporters[61,76,111]. Obviously, *in vivo* significance of a transporter in clearance of a drug can only be addressed by *in vivo* studies[61]. Other important applications, such as gender difference, as well as age and ontogeny are also preferably studied *in vivo*[112-114]. With the availability of double and triple knockouts, transporter complementation[115] and transporter-enzyme interplay[116] can now be addressed. Nevertheless, utilization of knockouts is perhaps not as extensive as originally envisioned. Compensatory changes may mask the effect of transporter deletion. P-gp is upregulated in Bsep knockout mice and the metabolism of bile acids is altered as well[117,118]. Cytochrome P450 enzymes which share substrate specificity with P-gp are dramatically up-regulated in P-gp knockout mice in a gender specific manner[119]. Species specificity issues also limit utilization of these models by the pharmaceutical industry. In addition to differences in substrate specificities[120], significant differences have been observed in transporter expression between species. Canalicular expression of MRP2/Mrp2 is about 10-fold greater in rodents than in humans[121] and the ratio of BCRP/P-gp expression in the BBB is about 4-fold greater in humans than it is mice[122]. Chemical knockouts can circumvent the problems stemming from compensatory changes. However, species specificity issues can only be overcome by utilization of humanized models. As the availability of humanized models increases, the relevance of *in vivo* studies will certainly increase as well[123-125].

CONCLUSION

In the past decade utilization of transporter assays by the pharmaceutical industry has been rapidly growing. Lower activity pharmacogenomic variants such as BCRP 412G>A[126] and OATP1B1 521 >C[85] make it possible to show the impact of the wild type transporters on human pharmacokinetics of substrate drugs and clearly demonstrate clinical relevance of drug-transporter interactions.

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