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MODULATING THE ACTIVITY OF OGG1 USING SMALL MOLECULES TO TARGET THE OXIDATIVE DNA DAMAGE RESPONSE IN CANCER AND INFLAMMATION

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MODULATING THE ACTIVITY OF OGG1 USING SMALL MOLECULES TO TARGET THE OXIDATIVE DNA DAMAGE RESPONSE IN CANCER AND INFLAMMATION

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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This work is dedicated to Ireny, my precious wife and sunshine,
who always radiates laughter and cheerfulness,
and shines upon me with her light and warmth.
She also happens to write my dedications.

POPULAR SCIENCE SUMMARY OF THE THESIS

The diversity of life on Earth is staggering with thousands of species existing. Whether they are single-celled organisms or complex multicellular ones like us, humans, all species share a common structural and functional unit, the cell. Each cell has its hereditary information stored in DNA. Being a polymer, DNA is made of monomeric nucleotides. A nucleotide consists of a nitrogen-containing base attached to a sugar molecule and a phosphate group. Four bases build up the DNA: adenine, thymine, cytosine and guanine or in short A, T, C and G. The sequence of these monomers forms a unique code which stores the information needed to compose different specialized proteins, similar to how a string of letters drawn from the alphabet composes different words in this thesis. The encoded composition of one protein together with the regulation of its expression level give rise to different cellular populations and distinct organisms.

Previous studies have demonstrated that normal cellular processes produce reactive oxygen species (ROS) as a by-product. As their name implies, ROS are very reactive and can interact with DNA resulting in guanine (G) modification. Since modified guanines mess up with the correct sequence of the DNA code, they have to be efficiently eliminated to avoid genetic mutations. This is the role of a key DNA repair protein called OGG1. ROS and OGG1 have been found to be involved in inflammatory responses. In addition, the level of ROS has been reported to rise in cancer. This made us curious whether shutting down the activity of OGG1 can cause cancer cell death or protect against inflammation.

To address this question, we started by designing a compound that can bind to OGG1 and impairs its activity. Our efforts led to the development of TH5487 which we used to shut down the activity of OGG1 or in other words inhibit it. We studied the effect of TH5487 in cellular and mouse models of inflammation. Interestingly, we found that TH5487 had a notable anti-inflammatory effect by interfering with the role of OGG1 in inflammation. TH5487 dampened the inflammatory response by reducing the level of several proteins that typically exacerbate inflammation. Importantly, TH5487 was well-tolerated in mice and showed a protective effect against lung inflammation. This is a novel approach to treat inflammation by targeting a DNA repair enzyme. Thus, targeting OGG1 has the potential to be used as a new treatment strategy for different inflammatory conditions.

We next examined the role of OGG1 in cancer and whether its inhibition can have anti-cancer effects. Our findings suggest that OGG1 makes an attractive target for treating cancer. We

found that cells divide much slower when OGG1 is inhibited. More DNA damage is observed in those cells after TH5487 treatment. Notably, the division of normal cells was largely unaffected by TH5487. This selective action on cancer cells is desirable as it may lead to fewer side effects in normal healthy cells.

One potential application for TH5487 is using it in combination with other anti-cancer treatments. Current chemotherapies and radiotherapies exert their anti-cancer effects by causing DNA damage, part of which is reparable by OGG1. One may envision that inhibiting OGG1 with TH5487 can be used as a combination therapy with other chemotherapies or radiotherapies to potentiate their anti-cancer effect. This remains to be tested in future research.

Finally, we investigated the mode of action of another OGG1 binding compound, TH10785. We observed that TH10785 enhances the capacity of OGG1 to repair modified guanines acting as an activator. TH10785 introduces a novel activity not displayed by native OGG1. Since accumulation of modified guanines has been associated with aging, future research is recommended to examine if OGG1 activation can be beneficial in such scenario. Using compounds to introduce new enzymatic functions is a novel concept that can pave the way for many exciting therapeutic applications.

ABSTRACT

The production of reactive oxygen species (ROS) is increased in several pathological conditions including cancer and inflammation. Multiple lines of evidence suggest that ROS are involved in signaling events that promote tumorigenesis and inflammatory responses. If redox homeostasis is not maintained, high levels of ROS can induce oxidative DNA damage which is primarily repaired by base excision repair (BER). 8-oxoguanine DNA glycosylase 1 (OGG1) is a key DNA glycosylase that eliminates 8-oxo-7,8-dihydroxyguanine (8-oxoG) when present opposite to cytosine in duplex DNA to initiate BER. Recently, there has been a great interest in targeting the DNA damage response as an anti-cancer approach. In this respect, OGG1 has gathered particular attention for its established role in BER in addition to its newly identified functions in modulating gene transcription. This has motivated this thesis work aiming at studying the validity of OGG1 as a drug target in clinically relevant treatment strategies for cancer and inflammation.

In **Paper I**, we reported the development of TH5487, a potent pharmacologically active OGG1 inhibitor. In addition, we provided proof of concept that inhibiting OGG1 represents a novel anti-inflammatory strategy. We show that TH5487 engages with OGG1 reducing its activity and DNA binding capacity in *in vitro* assays. Notably, TH5487 impairs NF-κB binding to the promoter regions of proinflammatory cytokines. This results in suppression of proinflammatory gene expression in cells stimulated with tumor necrosis factor-alpha (TNF-α) or lipopolysaccharide (LPS). Importantly, we found that TH5487 is well-tolerated *in vivo*, where it reduces the expression of inflammatory mediators and perturbs neutrophil infiltration in mice lungs. Thus, targeting OGG1 can be a potential beneficial strategy to treat inflammatory conditions.

In **Paper II**, we sought to characterize TH5487 regarding its effect on genomic 8-oxoG accumulation. Moreover, we studied OGG1 recruitment kinetics to regions of DNA damage as well as OGG1-chromatin dynamics after TH5487 treatment. We show that TH5487 impairs the repair of potassium bromate induced 8-oxoG lesions and results in fewer incisions. The inhibitor treatment alters both OGG1 recruitment kinetics and OGG1-chromatin binding as evident by the results of laser microirradiation experiments and fluorescence recovery after photobleaching (FRAP) assays respectively indicating that TH5487 interferes with OGG1 recruitment and activity in cells.

Paper III validates OGG1 as a potential target for cancer therapy. We reported the crystal structure of human OGG1 in complex with TH5487 showing that the inhibitor targets the

active site of OGG1. We found that TH5487 treatment is selectively toxic to a large panel of cancers cells but not to normal immortalized cells. We show that TH5487 treatment induces replication stress as demonstrated by accumulation of phosphorylated γH2AX in S-phase cells. Furthermore, it significantly reduces the replication fork speed. Importantly, TH5487 treatment downregulates a set of DNA replication genes altering the cellular transcriptional profile which contributes to replication stress. TH5487 was not found to reduce tumor growth in xenograft mouse models, probably due to binding to serum albumin proteins. This warrants the development of new formulations with an improved pharmacokinetic profile.

In **Paper IV**, we show that NEIL1 and NEIL2 can potentially compensate for OGG1 inhibition. The recruitment of NEIL1—and to a lesser extent NEIL2—to sites of DNA damage is altered in TH5487-treated cells. In addition, NEIL1 and NEIL2 are more tightly bound to chromatin in oxidatively stressed cells after *OGG1* depletion and inhibition. Importantly, we observe a higher level of genomic 8-oxoG lesions in *NEIL1*- and *NEIL2*-siRNA depleted cells treated with TH5478 suggesting a potential backup function for NEIL1 and NEIL2 after OGG1 inhibition.

In **Paper V,** we elucidated the mechanism of action of a small-molecule OGG1 activator *in vitro* and *in cellulo*. We demonstrated that in the presence of TH10785, OGG1 efficiently processes abasic sites by a new activity not found in native OGG1. Cells treated with TH10785 become more dependent on PNKP to complete the repair process. This novel concept of small-molecule activation paves the way to potentially establish new enzymatic functions in DNA repair enzymes, potentiate weak functions or recover lost ones through chemical intervention.

LIST OF SCIENTIFIC PAPERS

I. Visnes, T.*, Cázares-Körner, A.*, Hao, W.*, Wallner, O.*, Masuyer, G., Loseva, O., Mortusewicz, O., Wiita, E., Sarno, A., Manoilov, A., Astorga-Wells, J., Jemth, A.S., Pan, L., Sanjiv, K., Karsten, S., Gokturk, C., Grube, M., Homan, E.J., **Hanna, B.M.F.**, Paulin, C.B.J., Pham, T., Rasti, A., Berglund, U.W., Von Nicolai, C., Benitez-Buelga, C., Koolmeister, T., Ivanic, D., Iliev, P., Scobie, M., Krokan, H.E., Baranczewski, P., Artursson, P., Altun, M., Jensen, A.J., Kalderén, C., Ba, X., Zubarev, R.A., Stenmark, P., Boldogh, I., Helleday, T. ***Small-molecule inhibitor of OGG1 suppresses proinflammatory gene expression and inflammation.*** Science (2018) 362, 834–839.

II. **Hanna, B.M.F.**, Helleday, T., Mortusewicz, O. ***OGG1 inhibitor TH5487 alters OGG1 chromatin dynamics and prevents incisions***. Biomolecules (2020) 10, 1483.

III. Visnes, T.*, Benítez-Buelga, C.*, Cázares-Körner, A., Sanjiv, K., **Hanna, B.M.F.**, Mortusewicz, O., Rajagopal, V., Albers, J.J., Hagey, D.W., Bekkhus, T., Eshtad, S., Baquero, J.M., Masuyer, G., Wallner, O., Müller, S., Pham, T., Göktürk, C., Rasti, A., Suman, S., Torres-Ruiz, R., Sarno, A., Wiita, E., Homan, E.J., Karsten, S., Marimuthu, K., Michel, M., Koolmeister, T., Scobie, M., Loseva, O., Almlöf, I., Unterlass, J.E., Pettke, A., Boström, J., Pandey, M., Gad, H., Herr, P., Jemth, A.S., El Andaloussi, S., Kalderén, C., Rodriguez-Perales, S., Benítez, J., Krokan, H.E., Altun, M., Stenmark, P., Berglund, U.W., Helleday, T. ***Targeting OGG1 arrests cancer cell proliferation by inducing replication stress***. Nucleic Acids Research (2020) 48, 12234–12251.

IV. **Hanna, B.M.F**., Michel, M., Helleday, T., Mortusewicz, O. ***NEIL1 and NEIL2 Are Recruited as Potential Backup for OGG1 upon OGG1 Depletion or Inhibition by TH5487***. International Journal of Molecular Sciences (2021) 22, 4542.

V. Michel, M.*, Benítez-Buelga, C.*, Calvo, P.†, **Hanna, B.M.F.**†, Mortusewicz, O.†, Masuyer, G.†, Davies, J.†, Calvete, O.†, Rajagopal, V.†, Wallner, O.†, Sanjiv, K., Zhenjun, Z., Danada, A.N., Castañeda-Zegarra, S., Albers, J.J., Müller, S., Homan, E.J., Marimuthu, K., Visnes, T., Jemth, A.S., Chi, C., Karsten, S., Sarno, A., Wiita, E., Komor, A., Hank, E.C., Varga, M., Scaletti, E.R., Martilla, P., Rasti, A., Mamonov, K., Pandey, M., Von Nicolai, C., Ortis, F., Schömberg, F., Loseva, O., Stewart, J., Koolmeister, T., Henriksson, M., Michel, D., de Ory, A., Sastre-Perona, A., Scobie, M., Hertweck, C., Vilotijevic, I., Kalderén, C., Osorio, A., Stolz, A., Perona, R., Stenmark, P., Berglund, U.W., De Vega, M., Helleday, T. ***Small-molecule activation of OGG1 increases base excision repair by gaining a new enzymatic function.*** Manuscript.

* / † Authors contributed equally to this work.

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I. Visnes, T., Grube, M., **Hanna, B.M.F**., Benítez-Buelga, C, Cázares-Körner, A., Helleday, T. ***Targeting BER enzymes in cancer therapy*.** DNA repair (2018) 71, 118–126.

II. Baquero, J.M.*, Benítez-Buelga, C.*, Rajagopal, V., Zhenjun, Z., Torres-Ruiz, R., Müller, S., **Hanna, B.M.F.**, Loseva, O., Wallner, O., Michel, M., Rodríguez-Perales, S., Gad, H., Visnes, T., Helleday, T., Benítez, J., Osorio, A., 2021. ***Small-molecule inhibitor of OGG1 blocks oxidative DNA damage repair at telomeres and potentiates methotrexate anticancer effects***. Scientific Reports (2021) 11, 3490.

III. Walter, M., Mayr, F., **Hanna, B.M.F.,** Cookson, V., Mortusewicz, O., Helleday, T., Herr, P. ***The p53-induced UDP-glucose hydrolase NUDT22 prevents replication stress through pyrimidine salvage.*** Manuscript.

* Authors contributed equally to this work.

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LIST OF ABBREVIATIONS

1-10	2-OH-dA	2-hydroxy-2'-deoxyadenosine
	2-OH-dATP	2-hydroxy-2'-deoxyadenosine -5'-triphosphate
	3-meA	3-methyladenine
	3-meG	3-methylguanine
	3′-PUA	3′-phospho-α,β-unsaturated aldehyde
	5-CHO-dU	5-formyldeoxyuridine
	5-FU	5-fluoruracil
	5-hC	5-hydroxycytosine
	5-hmU	5-hydroxymethyluracil
	5-hU	5-hydroxyuracil
	5-OH-dC	5-hydroxy-2'-deoxycytidine
	5-OH-dCTP	5-hydroxy-2'-deoxycytosine-5'-triphosphate
	7-meG	7-methylguanine
	8-oxoG	8-oxo-7,8-dihydroxyguanine
	8-oxo-dGMP	8-oxo-7,8-dihydroxy-2'-deoxyguanosine-5'-monophosphate
	8-oxo-dGTP	8-oxo-7,8-dihydroxy-2'-deoxyguanosine-5'-triphosphate
A	A	Adenine
	AAG	Alkyladenine DNA glycosylase
	ADME	Absorption, distribution, metabolism and excretion
	AID	Activation-induced deaminase
	AlkBH	Human AlkB homolog
	AP	Apurinic/apyrimidinic
	APE1	AP endonuclease 1
	APOBEC	Apolipoprotein B mRNA editing catalytic polypeptide-like
	ATM	Ataxia telangiectasia mutated
B	BER	Base excision repair
	BrdU	5-Bromo-2′-deoxyuridine
C	C	Cytosine
	CETSA	Cellular thermal shift assay
	cGAS	Cyclic GMP–AMP synthase
	COX2	Cyclo-oxygenase 2
	CSR	Class switch recombination
D	dATP	Deoxyadenosine triphosphate
	dCTP	Deoxycytidine triphosphate
	DDR	DNA damage response
	DHU	Dihydrouracil
	DMSO	Dimethyl sulfoxide
	DNA	Deoxyribonucleic acid
	DNA-PKcs	DNA-dependent protein kinase catalytic subunit
	dNTP	Deoxyribonucleotide triphosphate
	DSB	Double-strand breaks
	dsDNA	Double-stranded DNA
	DSF	Differential scanning fluorimetry
E	EC_{50}	Median effective concentration
	EMA	European Medicine Agency

F	Fapy	Formamidopyrimidine
	FapyA	4,6-diamino-5-formamidopyrimidine
	FapyG	2,6-diamino-4-hydroxy-5-formamidopyrimidine
	FEN1	Flap endonuclease 1
	FDA	Food and Drug Administration
	FRAP	Fluorescence recovery after photobleaching
G	G	Guanine
	G4	G-quadruplex
	GEF	Guanine exchange factor
	GFP	Green fluorescent protein
	Gh	Guanidinohydantoin
H	HIGM	Hyper-IgM
	hSAECs	Human small-airway epithelial cells
	Hx	Hypoxanthine
I	IC_{50}	Median inhibitory concentration
	IFN-γ	Interferon-gamma
	IL-1β	Interleukin-1β
	iNOS	Inducible nitric oxide synthase
	IR	Ionizing radiation
L	LC-MS/MS	Liquid chromatography–tandem mass spectrometry
	LPS	Lipopolysaccharide
M	MAP	MUTYH-associated polyposis
	MAPK	Mitogen-activated protein kinase
	MBD4	Methyl-CpG-binding domain protein 4
	MED1	Methyl-CpG binding endonuclease 1
	MLE 12	Murine Lung Epithelial-12 cells
	MMR	Mismatch repair
	MPG	N-methylpurine DNA glycosylase
	MSH	MutS homolog
	MTH1	MutT homolog 1
	MUTYH	MutY DNA glycosylase
N	NEIL	Endonuclease VIII-like
	NF-κB	Nuclear factor-kappa-light-chain enhancer of activated B cells
	NUDIX	Nucleoside diphosphate linked to moiety-X
	NTHL1	Endonuclease III-like protein 1
O	OGG1	8-oxoguanine DNA glycosylase 1
P	PARP1	Poly (ADP-ribose) polymerase 1
	PCNA	Proliferating cell nuclear antigen
	PNKP	Polynucleotide kinase 3′-phosphatase
	Pol β	DNA polymerase β
	PQS	Potential G-quadruplex–forming sequences

R	RFC	Replication factor C
	ROS	Reactive oxygen species
	RNS	Reactive nitrogen species
S	SHM	Somatic hypermutation
	shRNA	Short hairpin RNA
	siRNA	Small interfering RNA
	SMUG1	Single-strand selective monofunctional uracil DNA glycosylase 1
	Sp	Spiroiminodihydantoin
	Sp1	Specificity factor 1
	SSBs	Single-strand breaks
	ssDNA	Single-stranded DNA
	STING	Stimulator of interferon genes
T	T	Thymine
	T-ALL	T-cell Acute lymphoblastic leukemia
	TDG	Thymine DNA glycosylase
	Tg	Thymine glycol
	Tm	Melting temperature
	TNF-α	Tumor necrosis factor-alpha
U	U	Uracil
	UNG	Uracil DNA glycosylase
	UV	Ultraviolet
X	XRCC1	X-ray repair cross-complementing protein 1
ϵ	ϵA	1, N^6-ethenoadenine
	ϵC	3, N^4-ethenocytosine

1 INTRODUCTION

1.1 THREATS TO GENOMIC INTEGRITY

Despite being the carrier of genetic information, DNA is far from being chemically stable. Several exogenous and endogenous factors constitute a threat to genomic integrity. Exposure to these factors gives rise to several DNA lesions which can impact cellular phenotype and cause dysfunction or disease if left unrepaired[1] (Figure 1).

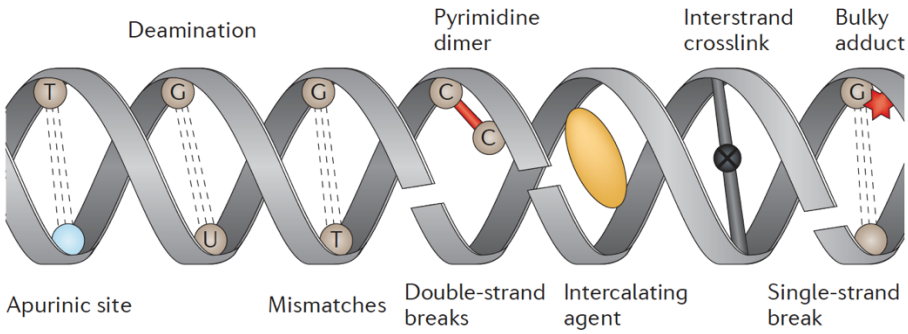

Figure 1: Types of DNA lesions resulting from exposure to endogenous and exogenous sources of damage. Spontaneous hydrolysis of the N-glycosidic bond in DNA creates abasic sites. Deamination, mismatches due to replication errors and reaction with reactive species are also among sources of endogenous DNA damage. Exposure to ionizing radiation generates single- and double-strand breaks, whereas non-ionizing ultraviolet radiation can lead to the formation of pyrimidine dimers. Exposure to platinum compounds results in the formation of interstrand, intrastrand crosslinks and bulky adducts. Genotoxic agents such as benzo[a]pyrene, daunorubicin and actinomycin-D act as DNA intercalating agents interfering with DNA replication and transcription. Reprinted with permission from Springer Nature, Helleday *at al.*, 2014[1].

1.1.1 Exogenous sources of DNA damage

DNA is vulnerable to attack by several exogenous DNA damaging agents such as ultraviolet (UV) or ionizing radiation (IR), environmental chemicals and chemotherapeutic drugs. While exposure to non-ionizing ultraviolet radiation can lead to the formation of pyrimidine dimers, IR generates both DNA single-strand breaks (SSBs) and double-strand breaks (DSBs)[2–5]. Moreover, IR can indirectly induce DNA damage by generating reactive oxygen species (ROS) and reactive nitrogen species (RNS)[6]. Besides, several environmental mutagens contribute to DNA damage. For instance, Benzo[*a*]pyrene present in tobacco smoke and coal tar, is reported to induce DNA mutations. Its electrophilic diol epoxide metabolite intercalates in DNA and interacts with guanines eventually leading to G:C → T:A transversions[7,8]. Thus smoking can induce somatic mutations increasing the risk of lung cancer[9]. In addition, chemotherapeutics induce DNA damage via various modes of action. For example, exposure

to platinum compounds generates both interstrand and intrastrand crosslinks as well as bulky DNA-protein adducts[10,11]. Daunorubicin and actinomycin-D act as DNA intercalating agents interfering with both DNA replication and transcription[12]. On the other hand, temozolomide, a chemotherapeutic agent used to treat glioblastoma multiforme, methylates guanines and adenines generating O6-methylguanine as a primary lesion in addition to N7-methylguanine and N3-methyladenine[13].

1.1.2 Endogenous sources of DNA damage

Intriguingly, DNA is susceptible to damage caused by numerous endogenous events. DNA can undergo spontaneous decay. Spontaneous hydrolysis of the N-glycosidic bond in DNA gives rise to apurinic or apyrimidinic sites with depurination being more common than depyrimidation. It is estimated that this results in 10,000 abasic sites per human cell each day[14,15]. Such abasic sites can block DNA replication and transcription and generate single-strand breaks (SSBs)[16]. Moreover, DNA is prone to spontaneous hydrolytic deamination. For example, deamination of cytosine in DNA generates uracil (U) which can result in mutagenic U:G mispairs[15,17]. Cytosine is also prone to enzymatic deamination by members of the apolipoprotein B mRNA editing catalytic polypeptide-like (APOBEC) family and activation-induced deaminase (AID)[18–20]. Replicative DNA polymerases exhibit remarkable fidelity during DNA synthesis, nevertheless replication errors can occur resulting in mismatches[21,22] or misincorporation of uracil or ribonucleotides in DNA[17,23,24]. Furthermore, formaldehyde produced during metabolic processes such as amino acid metabolism and lipid peroxidation has been reported to react with adenine, guanine and cytosine generating N-hydroxymethyl DNA monoadducts in addition to inducing N-methylene crosslinks between adjacent purines[25–27]. DNA alkylation can occur endogenously due to the reaction of DNA with intracellular alkylating agents such as methyl donor S-adenosylmethionine[28,29]. Another notable cause of intrinsic DNA damage arises from interaction of DNA with reactive oxygen species (ROS) which will be further discussed in the next section.

1.2 OXIDATIVE STRESS

1.2.1 Reactive oxygen species

Reactive oxygen species (ROS) represent a family of highly reactive species derived from oxygen. Various endogenous sources account for ROS production in cells such as mitochondria[30], endoplasmic reticulum[31], peroxisomes[32], NADPH oxidase complexes of activated phagocytic cells[33] (Figure 2). Exposure to a number of exogenous agents can also contribute to ROS production such as ultraviolet light, ionizing radiation, alcohol, smoking,

environmental pollutants and some drugs[34]. Generally, ROS can be classified into radical species and non-radical ones. Radicals include superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$) and nitric oxide ($NO^{\cdot}$), whereas non-radicals include hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), hypochlorus acid (HOCl), hypobromous acid (HOBr) and peroxynitrite ($ONOO^-$) among others. Non-radicals can be involved in chemical reactions leading to the production of free radicals. Having an unpaired valence shell electron, free radicals tend to be highly reactive where they readily react with lipids, proteins and DNA[35,36]. ROS-mediated oxidation of DNA results in several oxidized DNA lesions, Apurinic/apyrimidinic sites (AP) in addition to strand breaks. This can block replication or result in miscoding events[37,38].

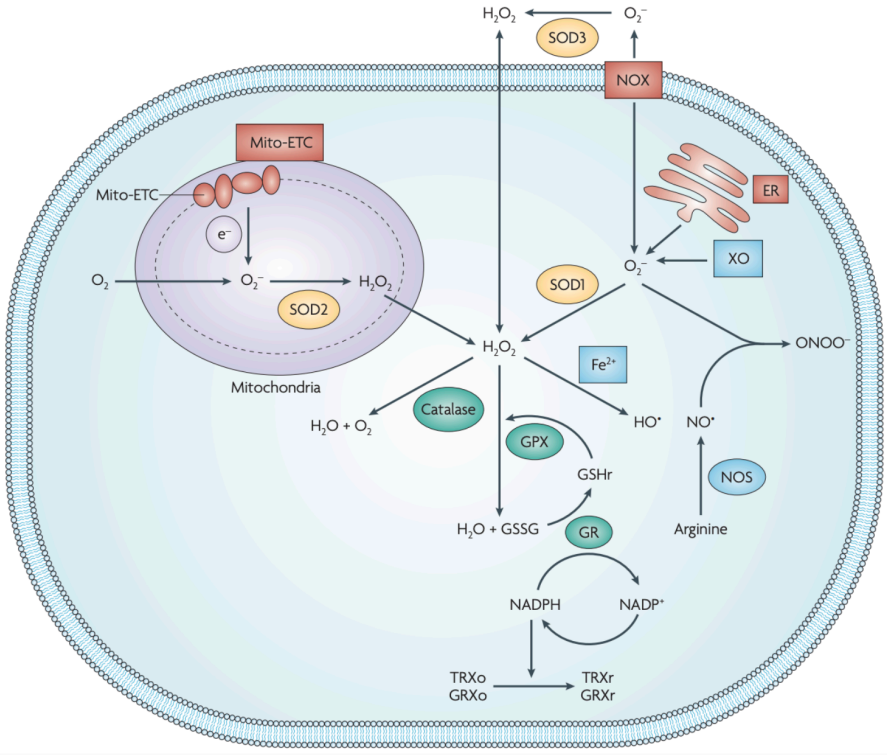

Figure 2: Schematic illustration of cellular ROS producers and scavengers. Reactive oxygen species (ROS) are produced from the mitochondrial electron transport chain (Mito-ETC), membrane-bound NAPDH oxidase enzymatic complexes (NOX) and endoplasmic reticulum (ER), shown in red. Superoxide ($O_2^{\cdot-}$) is the primary ROS produced by the mitochondria due to one-electron reduction of molecular oxygen by electrons escaping the mitochondrial respiratory chain. Superoxide dismutases (SOD, shown in yellow) can quickly convert superoxide into hydrogen peroxide (H_2O_2). Catalase enzyme catalyzes the breakdown of hydrogen peroxide into oxygen and water. H_2O_2 can also be converted into hydroxyl radical ($OH^{\cdot}$) in the presence of transition metals in what is referred to as Fenton reactions. Nitric oxide ($NO^{\cdot}$) is another radical species produced by nitric oxide synthase (NOS). $NO^{\cdot}$ can react with superoxide anion generating a reactive non-radical species, peroxynitrite ($ONOO^-$). To overcome the deleterious effects of ROS, cells maintain redox homeostasis by regulating the process of ROS production and elimination. Several ROS scavengers (shown in green) are responsible for ROS elimination. GPX, glutathione peroxidase; GR, glutathione reductase; GRXo, oxidized glutaredoxin; GRXr, reduced glutaredoxin; GSHr, reduced glutathione; GSSG, oxidized glutathione; TRXo, oxidized thioredoxin; TRXr, reduced thioredoxin; XO, xanthine oxidase. Reprinted with permission from Springer Nature, Trachootham *et al.*, 2009[39].

1.2.2 Oxidative stress and redox balance in cancer

Under normal physiological conditions, ROS levels are maintained in homeostasis by keeping a balance between ROS producers and ROS scavengers[39] (Figure 3). Multiple lines of evidence show that ROS are involved in several signaling pathways[40]. Loss of redox homeostasis has been implicated in several pathologies. Inflammation for instance is accompanied with high levels of ROS and has been associated with cancer progression[41]. Furthermore, oncogene activation, metabolism alteration and mitochondrial dysfunction generate high levels of ROS in cancer cells[42]. ROS elevation has been reported to promote proliferation and metastasis of tumor cells[43]. However, this is thought to come at the cost of creating oxidative stress and causing oxidative damage to cellular macromolecules including DNA, lipids and proteins[44].

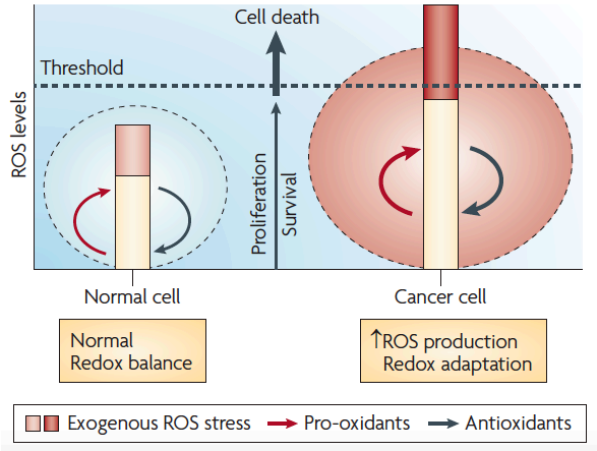

Figure 3: Redox balance in cancerous versus normal cells. Cells under normal physiological conditions maintain redox homeostasis by keeping a balance between generating and eliminating ROS. Normal cells can withstand some degree of exogenous ROS stress by using their antioxidant defence systems in order to keep the level of ROS below a certain toxic threshold (dotted line). Cancer cells adapt to their intrinsic high ROS levels by upregulating their antioxidant capacity to maintain the levels of ROS below the toxic threshold. Reprinted with permission from Springer Nature, Trachootham *et al.*, 2009[39].

In cancer, a vicious self-amplifying cycle of oxidative stress seems to exist. ROS generated from endogenous or exogenous sources can lead to oxidative DNA damage and genetic mutations. This in turn can drive genomic instability promoting tumorigenesis and resulting in oncogene activation, disruption of normal mitochondrial function and aberrant metabolism. Such events are known to further contribute to ROS production and subsequently induce more DNA damage (Figure 4).

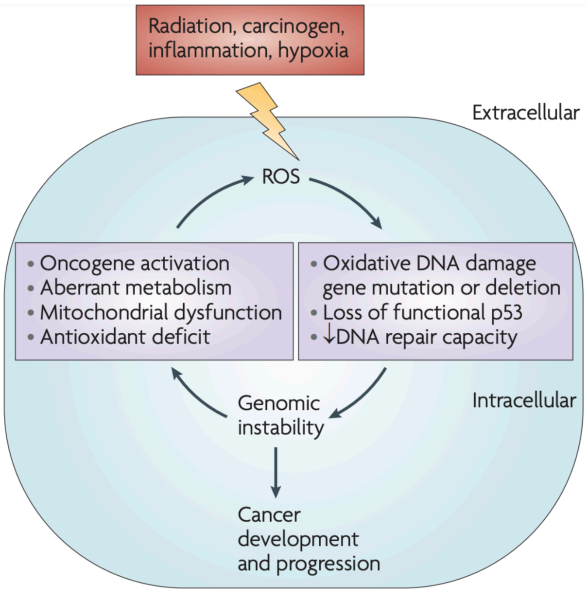

Figure 4: Oxidative stress in cancer. Numerous factors contribute to ROS generation in cancer such as inflammation and hypoxia. ROS react with DNA resulting in oxidative DNA damage. Accumulation of oxidative lesions in cancer cells with compromised DNA repair capacity can result in genomic instability leading to oncogene activation, disruption of normal mitochondrial function and aberrant metabolism. Such events contribute to more ROS generation amplifying the oxidative stress in cancer. Reprinted with permission from Springer Nature, Trachootham *et al.*, 2009[39].

In order to survive this intrinsically high level of oxidative stress, cancer cells adapt and upregulate their antioxidant capacity[39,43,45]. Consequently, the overall redox balancing point is shifted upwards in cancer cells compared to normal ones (Figure 3). This implies that cancer cells may be selectively vulnerable to killing via inducing redox imbalance. This can be achieved either by inducing ROS production or by interfering with ROS elimination mechanisms[39] (Figure 5). Indeed, targeting antioxidants or other non-oncogene addiction enzymes has yielded promising preclinical results suggesting that this strategy may offer therapeutic selectivity in fighting cancer[46–50].

Although the notion that a diet rich in antioxidants fights cancer has deep roots in the public, numerous studies have indicated that antioxidants accelerate the tumor progression rather than inhibit it.[51–54] This suggests that excessive accumulation of ROS in cancer cells can induce damage beyond repair to DNA and other cellular macromolecules and constitute a barrier to metastasis. As such, it is proposed that cancer cells might be dependent on pathways that repair oxidative DNA lesions or prevent their accumulation. This makes such pathways promising anti-cancer targets.

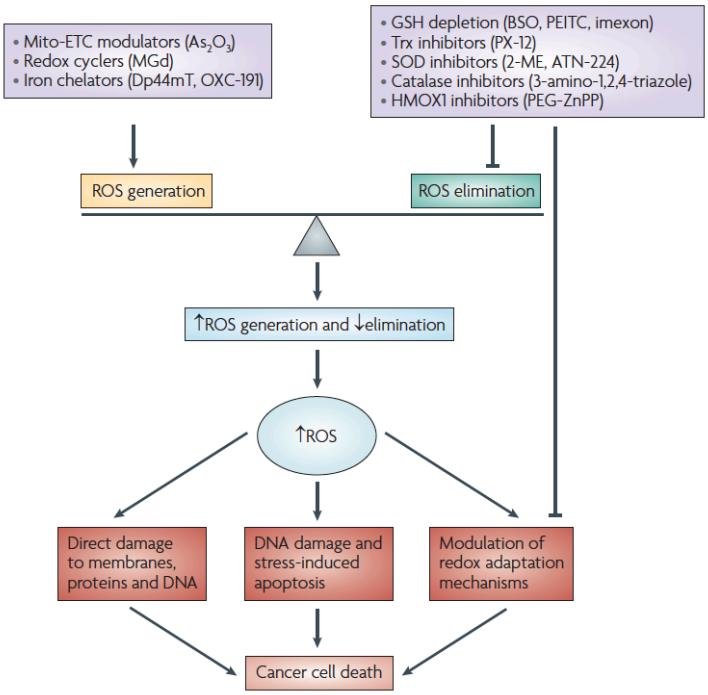

Figure 5: Strategies to therapeutically exploit oxidative stress in cancer. Since the redox dynamics are shifted in cancer cells towards more ROS generation and elimination, cancer cells are more dependent on their antioxidant buffering system. Accordingly, cancer cells are more vulnerable to oxidative stress-inducing agents which can either act by generating more ROS or by interfering with the cellular ROS elimination systems. This may provide a strategy to selectively target cancer cells. 2-ME, 2-methoxyestradiol; As_2O_3, arsenic trioxide; BSO, buthionine sulphoximine; GSH, glutathione; HMOX1, haem-oxygenase 1; MGd, motexafin gadolinium; Mito-ETC, mitochondrial electron transport chain; PEG-ZnPP, pegylated zinc protoporphyrin; PEITC, phenethyl isothiocyanate; SOD, superoxide dismutase; Trx, thioredoxin. Reprinted with permission from Springer Nature, Trachootham *et al.*, 2009[39].

1.3 MUTAGENESIS CAUSED BY NUCLEOTIDE OXIDATION

1.3.1 Oxidation of the free nucleotide pool

The high reactivity of ROS results in oxidative DNA modifications which can drive mutagenesis and tumorigenesis[15]. Compared to chromatin-bound DNA, the vulnerability of the free nucleotide pool to oxidative damage is orders of magnitude higher[55,56]. Guanine in particular is readily oxidizable due to its low redox potential[57,58]. Numerous oxidation products are generated upon oxidation of guanine. Among these oxidation products, 8-oxo-7,8-dihydroxyguanine (8-oxoG) is thought to be the most abundant and thus has been the one most extensively studied[38,59–61]. Besides, *in vitro* analysis has reported the formation of additional free nucleoside oxidation products upon reaction with ROS including 2-hydroxydeoxyadenosine (2-OH-dA), 5-hydroxydeoxycytidine (5-OH-dC) in addition to 5-formyldeoxyuridine (5-CHO-dU)[62]. Modified nucleotides can have mutagenic consequences if incorporated into DNA.

1.3.2 Incorporation of oxidized nucleotides into nucleic acid chains

DNA polymerases can readily incorporate oxidized nucleotides into DNA during replication and repair[63–65]. Unmodified deoxyguanines adopt an *anti* confirmation allowing them to form a Watson-Crick base pair with cytidine. However, 8-oxoG favours adopting a *syn* confirmation to avoid steric repulsion between its carbonyl oxygen O8 and the deoxyribose sugar. In its *syn* conformation, 8-oxoG can form a Hoogsteen base pair with adenine (Figure 6). This mispairing of 8-oxoG with adenine can result in A:T → C:G transversion mutations if left unrepaired[64,66,67]. Comparably, incorporation of 2-hydroxy-2'-deoxyadenosine -5'-triphosphate (2-OH-dATP) and 5-hydroxy-2'-deoxycytosine-5'-triphosphate (5-OH-dCTP) in both bacterial and human DNA can have mutagenic consequences by causing genetic transversions[68–71]. Moreover, oxidized nucleotides can be inserted into the growing mRNA by RNA polymerases leading to transcriptional errors[72].

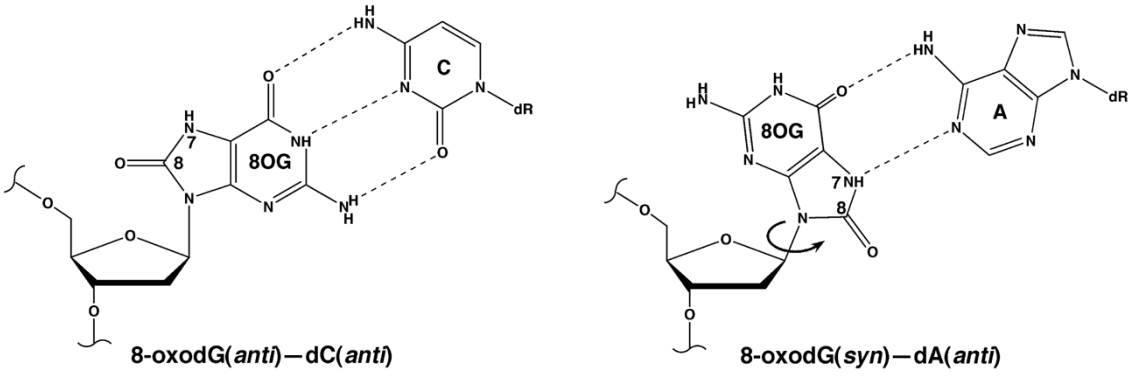

Figure 6: 8-oxoguanine and its mutagenic base-pairing potential. 8-oxoguanine when adopting an *anti* conformation, forms a Watson-Crick base pair with cytidine. However, in its *syn* conformation, 8-oxoguanine forms a Hoogsteen base pair with adenine resulting in a genetic transversion if left unrepaired. Reprinted with permission from Elsevier, Krahn *et al.*, 2003[73].

1.3.3 Nucleotide incorporation opposite to oxidized counterparts

Although oxidation of free nucleotide pool is more prevalent than oxidation of nucleotides that have been already incorporated into DNA, the latter should also be considered as they can drive mutagenesis. dNTP incorporation opposite to 8-oxoG has been studied *in vitro* using eukaryotic DNA polymerases. Such process has been shown to be polymerase dependent. For instance, the human DNA polymerase β (Pol β) preferentially incorporates dCTP pairing it with 8-oxoG at a ratio of 4:1 relative to dATP incorporation, however human DNA polymerase α preferentially incorporates dATP opposite to 8-oxoG at a ratio of 200:1 compared to dCTP incorporation[74]. dATP is also preferentially incorporated by DNA

polymerase δ opposite to 8-oxoG resulting in 8-oxoG:A mispair[75]. MUTYH and DNA polymerase λ have been shown to be involved in repairing this mispair[75].

From an evolutionary point of view, the lower promiscuity of DNA polymerases during nucleotide incorporation can be advantageous as it allows the cell to acquire new mutations and thus help it adapt to its environment[67]. Nevertheless, nucleotide misincorporation can drive mutagenesis and contribute to genomic instability.

1.4 INFLAMMATION AND GENOMIC INSTABILITY

Genomic instability is widely perceived as a key characteristic of cancer[76,77]. A growing body of evidence suggests that inflammation contributes to mutagenesis and thus to cancer development[78–80]. Both inflammation and genomic instability are regarded as enabling hallmarks that promote tumor development and facilitate the acquisition of core and emerging hallmarks of cancer[80] (Figure 7). Inflammation is associated with the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) aiming at pathogen elimination. Being highly reactive, ROS and RNS can react with and inflict damage upon DNA. For instance, neutrophils and macrophages produce a range of ROS and RNS including superoxide, hydroxyl and nitrogen dioxide radicals, hydrogen peroxide, peroxynitrite anions and hypohalous acids[81–83]. In addition, proinflammatory cytokines released during inflammatory responses such as tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) induce intracellular production of ROS[84–86]. Furthermore, inflammation is involved in tumor initiation, growth and metastasis[78–80]. Numerous studies indicate that inflammation plays pro-tumorigenic roles by enriching the tumor microenvironment with (1) growth factors that promote tumor proliferation, (2) survival factors to resist cell death, (3) proangiogenic factors and extracellular matrix-modifying enzymes which promote angiogenesis and facilitate metastasis[87–90].

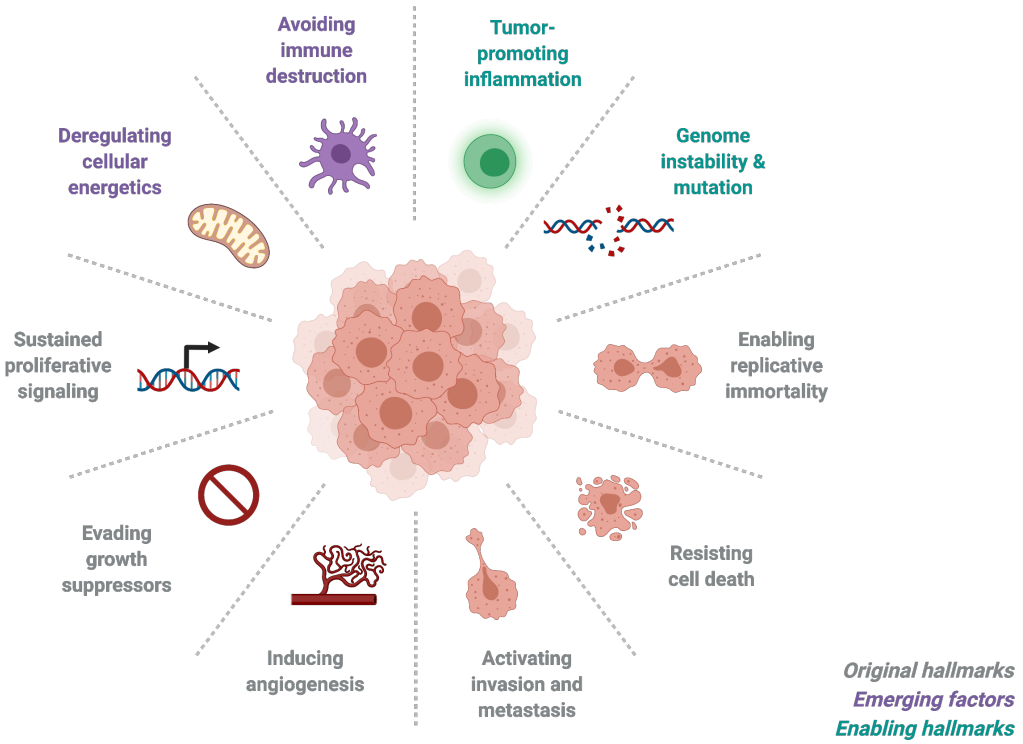

Figure 7: Schematic illustration of the hallmarks of cancer. Inflammation and genomic instability can promote tumor development and are thus classified as enabling hallmarks[80]. Created with BioRender.com

A complex relationship exists between inflammation and DNA damage. On one hand, inflammation contributes to DNA damage via the production of ROS and RNS. On the other hand, DNA damage aggravates inflammation in a positive feedback loop manner[91] (Figure 8). Poly (ADP-ribose) polymerase 1 (PARP1) represents one example for this relationship between inflammation and DNA damage. PARP1 is one of the first recruited factors that are involved in the DNA damage response (DDR) where it plays a key role in detecting DNA breaks and serves to recruit downstream DNA repair proteins[92–95]. Interestingly, PARP1 activity has been shown to be implicated in inflammatory responses. PARP1 inhibition has been shown to attenuate inflammation in pancreas, brain, intestines and liver disease models[96–99]. Inhibiting PARP1 leads to lower infiltration of immune cells[100,101,96] as well as lower expression of inflammatory cytokines[101,96,102] and adhesion molecules[96,102,103]. Moreover, PARP1 inhibition has been demonstrated to downregulate the expression of inducible nitric oxide synthase (iNOS) whose activity is associated with exacerbating inflammation[103,104]. Through PARylation, PARP1 is able to modulate the activity of nuclear factor-kappa-light-chain enhancer of activated B cells (NF-κB), a major regulator of inflammatory responses[105–108].

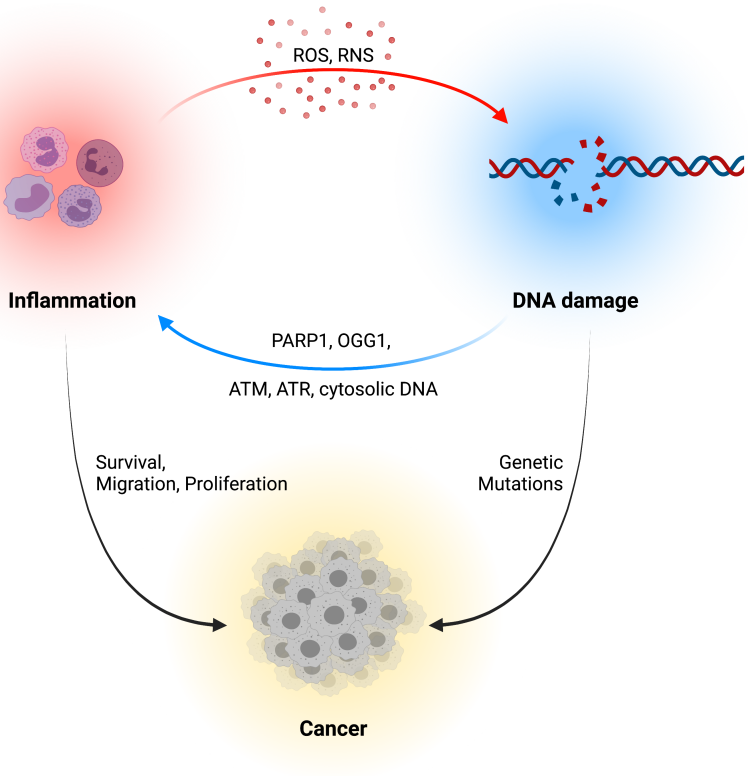

Figure 8: Schematic illustration of the intricate relationship between inflammation and DNA damage. Inflammation induces DNA damage via the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS). On the other hand, DNA damage aggravates inflammation in a positive feedback loop manner[91]. Created with BioRender.com

Other than the role of PARP1, DNA damage can augment inflammation in several ways. 8-oxoguanine DNA glycosylase 1 (OGG1), a key base excision repair enzyme facilitates the binding of NF-κB to its target DNA promoter sequences thereby enhancing the expression of proinflammatory cytokines[109–111]. Interestingly, ATM and ATR, vital players in the DNA damage response (DDR), have been demonstrated to promote NF-κB signaling resulting in increased cytokine expression[112–116]. Furthermore, genomic instability is often associated with the formation of micronuclei, which encapsulate lagging whole or fragmented chromosomes. Upon disruption of their nuclear envelope, micronuclei release their content DNA into the cytosol which can induce the expression of IFN-γ and IL-1β triggering an inflammatory response[117–120]. Cytosolic DNA activates the cyclic GMP–AMP synthase (cGAS)–stimulator of interferon genes (STING) pathway. Activation of the cGAS-STING pathway induces the expression of type I interferons triggering inflammation[121,122]. Due to the complex crosstalk between inflammation, DNA damage and repair processes, these pathways need to be well-regulated to maintain genomic integrity.

1.5 REPAIR OF 8-OXOGUANINE LESIONS

Thousands of 8-oxoG lesions are estimated to be generated in a normal cell per every day[123–125]. This figure is estimated to be 100-fold higher in cancer cells[41,126]. To overcome the mutagenic potential of oxidized guanines, cells have evolved to efficiently recognize such lesions and remove them. Three repair pathways act in concert to restrict the accumulation of 8-oxoG in human genome: preventative repair by MutT homolog 1 (MTH1), mismatch repair (MMR) and base excision repair (BER). While MTH1 targets oxidized guanines present in the nucleotide pool, 8-oxoguanine DNA glycosylase 1 (OGG1) and MutY homolog (MUTYH) act on nucleotides that have been incorporated into the DNA. OGG1 targets 8-oxoG:C mispairs in DNA, whereas MUTYH recognizes and excises adenine in mispaired 8-oxoG:A[127] (Figure 9).

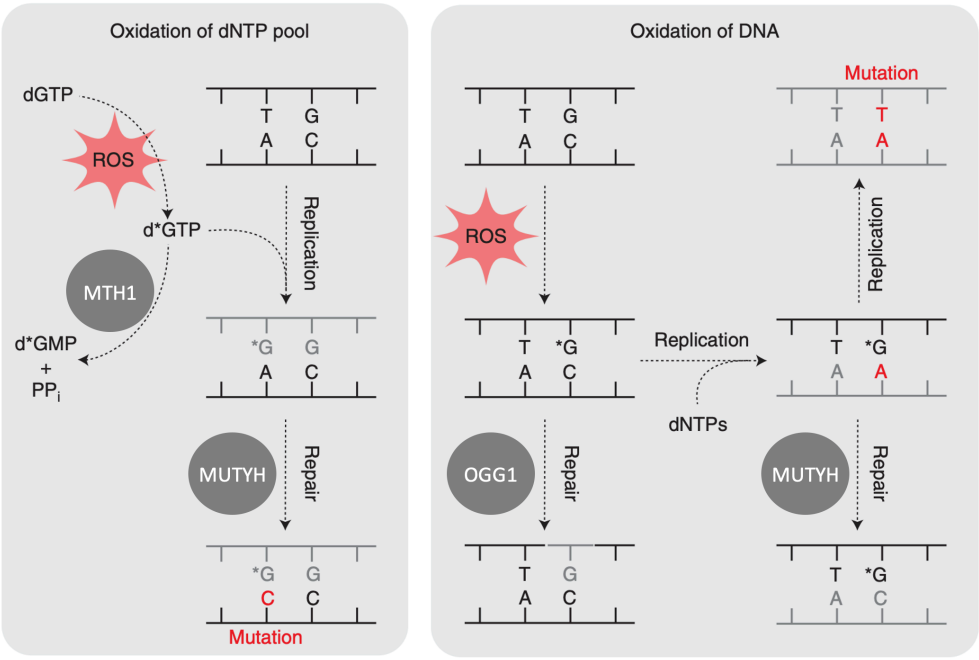

Figure 9: **Different cellular repair mechanisms help the cells overcome the mutagenic potential of 8-oxoguanine when present in the free nucleotide pool (left) and in duplex DNA (right).** 8-oxo-(d)GTP present in the free nucleotide pool (left) is hydrolyzed by MTH1 into the monophosphate form which prevents its incorporation into DNA. If 8-oxo-(d)GTP is erroneously incorporated into the nascent DNA strand opposite to adenine (A), MUTYH can recognize and excise the mis-incorporated A. A genetic mutation will arise from this attempted repair process. Otherwise, the correct base pair can be installed via the mismatch repair pathway (MMR). Excision of 8-oxoguanine in duplex DNA (right): OGG1 recognizes and excises 8-oxoG from duplex DNA when present opposite to cytosine initiating base excision repair (BER). If DNA replication proceeds before 8-oxoG excision, Adenine can be incorporated opposite to it by replicative DNA polymerases δ and ε. This mis-incorporated adenine can be excised by MUTYH allowing the correct base, cytosine, to be incorporated. If the cell fails to repair the 8-oxoG:A base pair before a second cycle of DNA replication, a G:C → T:A genetic transversion arises. Adapted from Krokan *et al. 2013*[128]. Reprinted with permission from Cold Spring Harbor Laboratory Press, Krokan and Bjørås, *2013*[128].

1.5.1 Preventative repair by MTH1

The human mutT homolog 1 (MTH1), also known as NUDT1 (NUDIX type 1) is a member of the NUDIX (nucleoside diphosphate linked to moiety-X) superfamily of hydrolases. The human NUDIX family encompasses 22 members sharing a NUDIX box, a common motif of 23-amino acids. NUDIX hydrolases catalyze the hydrolysis of nucleoside-like di- or triphosphates into their respective monophosphate form. MTH1 acts as a sanitation enzyme catalyzing the hydrolysis of 8-oxo-(d)GTP and 2-OH-(d)ATP into their respective monophosphate form thereby preventing their incorporation into DNA. In doing so, MTH1 prevents potential downstream genetic transversions and helps maintain genomic integrity[69,129–131].

1.5.2 Mismatch repair

The mismatch repair pathway (MMR) is involved in repairing DNA oxidative damage to a certain degree. Following replication, MMR factors recognize base mispairs on nascent DNA strand. MutSα, a heterodimer consisting of MSH2 and MSH6, corrects 2-OH-dATP mispairs[132]. Importantly, MutS Homolog 2 (MSH2), a key MMR factor, excises incorporated 8-oxo-(d)GMP. MSH2 deficient cells show higher 8-oxoG levels in their DNA[133]. Furthermore, mice deficient in MSH2 are reported to exhibit high levels of oxidized bases in their genomic DNA highlighting the role of MMR in protecting the genome against oxidative damage[134].

1.5.3 Base excision repair

DNA is not only vulnerable to oxidation, but to deamination and alkylation as well generating a wide array of lesions[14,15] (Figure 10). Although the generated DNA lesions do not significantly distort the DNA double helix, they need to be efficiently eliminated to maintain genomic integrity. Here comes the vital role of the base excision repair (BER) pathway, a conserved DNA repair pathway among the three domains of life: archaea, bacteria, and eukaryotes[136,137]. In fact, BER was first discovered in 1974 in *Escherichia coli* when Tomas Lindahl succeeded in purifying uracil DNA glycosylase (UNG) and described its role in excising deaminated cytosine, uracil, from DNA by cleaving the N-glycosidic bond between uracil and the deoxyribose moiety[138]. Enzymes that catalyze the cleavage of the N-glycosidic bond between a modified DNA base and the deoxyribose sugar are referred to as DNA glycosylases. DNA glycosylases are responsible for carrying out the first step in the BER pathway to repair a variety of DNA base lesions where each lesion is recognized by one or more glycosylases possessing overlapping substrate specificity[128].

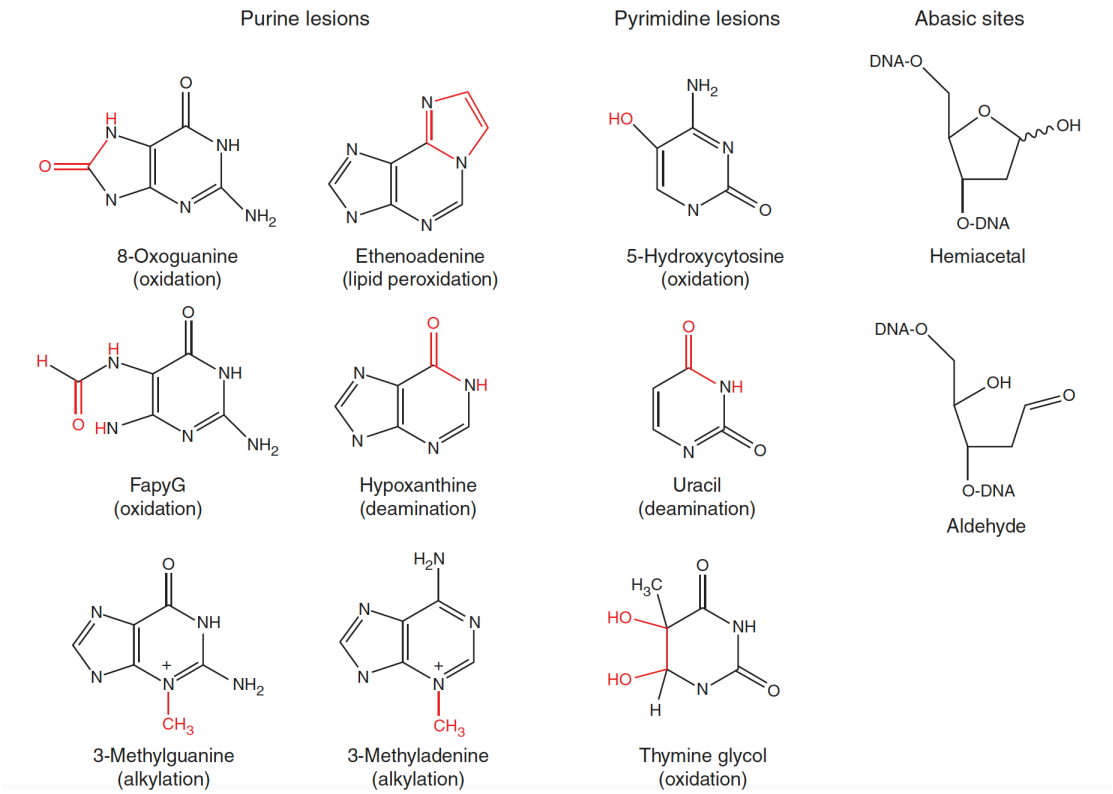

Figure 10: DNA base lesions and abasic sites commonly arising from oxidation, deamination and methylation. Reprinted with permission from Cold Spring Harbor Laboratory Press, Krokan and Bjørås, *2013*[128].

So far eleven DNA glycosylases have been identified in humans. Six of them are responsible for repairing DNA oxidative damage, four repair mispaired thymine and uracil lesions and one is devoted for alkylated DNA lesions in addition to deaminated purines (Table 1). DNA glycosylases can be classified according to their enzymatic activity as monofunctional or bifunctional. Glycosylases recognizing genomic uracil, thymine and alkylated DNA lesions belong to the monofunctional group where they cleave the N-glycosidic bond resulting in an apurinic/apyrimidinic site (AP-site), an abasic site. AP endonuclease (APE1) recognizes abasic sites and cleaves the DNA strand 5′ of the AP site resulting in a sugar moiety on the 5′ end of the nick and hydroxyl group on the 3′ end. The latter forms a substrate for DNA polymerase β (Pol β). Pol β removes the sugar moiety through its phosphodiestrase activity and fills in the gap. Subsequently, ligation takes place by DNA ligase 1 or DNA ligase 3/ X-ray repair cross-complementing protein 1 (XRCC1) scaffolding protein to seal the nick (Figure 11)[128,139–142].

Oxidative DNA lesions on the other hand are recognized and cleaved by bifunctional glycosylases which have an AP lyase activity in addition to its glycosylase activity. This lyase activity enables them to cleave the phosphodiester bond of the DNA backbone yielding

at the 3′ end of the nick either an α,β-unsaturatedaldehyde via β-elimination or a phosphate group via β,δ-elimination. β-elimination generates 5′-phosphate and a 3′-phospho-α,β-unsaturated aldehyde (3′-PUA). APE1 efficiently removes the 3′-PUA generating a 3′-hydroxyl group and enabling the polymerase to fill the nucleotide gap. On the other hand, β,δ-elimination generates a nucleotide gap with a 3′-phosphate group which can be removed by the phosphatase activity of polynucleotide kinase 3'-phosphatase (PNKP). The remaining steps of gap filling and ligation are the same as those outlined in the pathway initiated by monofunctional glycosylases[128,141,143].This process of repairing a single nucleotide is referred to as short-patch BER and takes place in both proliferating and non-proliferating cells.

Alternatively, long-patch BER occurs primarily in proliferating cells where a gap of at least 2 nucleotides is generated and filled in. It often occurs due to inefficient removal of the sugar at the 5' end of the nick. In long-patch BER, DNA polymerase δ/ε, proliferating cell nuclear antigen (PCNA) and flap endonuclease 1 (FEN1) are involved in the repair process in addition to the core factors of BER. Ligase 1 eventually carries out the ligation step[128,144]. Special focus will be given to OGG1 and NEIL glycosylases in the next section given their role in initiating the repair of a variety of oxidized DNA bases.

At least two characteristic mutational signatures have been associated with defective BER suggesting a crucial role for BER in maintaining genomic integrity. Mouse embryonic fibroblasts with defective single-strand selective monofunctional uracil DNA glycosylase 1 (SMUG1) display C:G → T:A transitions[145]. In addition, defective OGG1 has been associated with G:C → T:A transversions[146] highlighting the role of BER in preventing mutagenesis.

Table 1: Human DNA glycosylases, their substrates and the phenotype resulting from glycosylase deficiency or mutation. 3-meA, 3-methyladenine; 3-meG, 3-methylguanine; 5-FU, 5-fluoruracil; 5-hC, 5-hydroxycytosine; 5-hmU, 5-hydroxymethyluracil; 5-hU, 5-hydroxyuracil; 7-meG, 7-methylguanine; 8-oxoG, 8-oxo-7,8-dihydroxyguanine; A, adenine; AAG, alkyladenine DNA glycosylase; AlkBH, human AlkB homolog; CSR, class switch recombination; DHU, dihydrouracil; ds, double-stranded; Fapy, formamidopyrimidine; FapyA, 4,6-diamino-5-formamidopyrimidine; FapyG, 2,6-diamino-4-hydroxy-5-formamidopyrimidine; G, guanine; Gh, guanidinohydantoin; HIGM, hyper-IgM; Hx, hypoxanthine; MAP, MUTYH-associated polyposis; MBD4, methyl-CpG-binding domain protein 4; MED1, methyl-CpG binding endonuclease 1; MPG, N-methylpurine DNA glycosylase; MUTYH, MutY homolog; NEIL, endonuclease VIII-like; NTHL1, endonuclease III-like protein 1; OGG1, 8-oxoguanine DNA glycosylase 1; SMUG1, single-strand selective monofunctional uracil DNA glycosylase 1; SHM, somatic hypermutation; Sp, spiroiminodihydantoin; ss, single-stranded; T, thymine; TDG, thymine DNA glycosylase; Tg, thymine glycol; U, uracil; UNG2, uracil DNA glycosylase 2; ϵA, 1, N6-ethenoadenine; ϵC, 3, N4-ethenocytosine. Adapted from Krokan *et al. 2013*[128]. Reprinted with permission from Cold Spring Harbor Laboratory Press, Krokan and Bjørås, *2013*[128].

Enzyme	Substrates and (minor substrates)	Mouse knockout	Human disease
UNG2	U, 5-FU in ss and dsDNA, U:A and U:G context (alloxan, 5-hydroxyuracil, isodialuric acid)	Partial defect in CSR, skewed SHM, B-cell lymphomas.	Complete defect in CSR, HIGM syndrome, infections, lymphoid hyperplasia.
SMUG1	5-hmU, U:G > U:A > ssU, 5-FU, εC in ss and dsDNA	Viable and fertile. *SMUG1*/*UNG*/*MSH* triple knockouts show reduced longevity.	Low SMUG1 expression is associated with poor prognosis of aggressive breast cancer[147].
TDG	U:G > T:G (5-hmU in dsDNA, 5-FU)	Embryonic lethal. TDG has an epigenetic role in development.	Unknown
MBD4 (MED1)	U:G and T:G, 5-hmU in CpG context (εC, 5-FU in dsDNA)	Viable and fertile, C to T transitions, intestinal neoplasia	Mutated in carcinomas with microsatellite instability.
MPG (AAG)	3-meA, 7-meG, 3-meG, Hx, εA	Viable and fertile. Triple knockouts of *MPG*/*AlkBH2*/*AlkBH3* are hypersensitive to inflammatory bowel disease.	Unknown
OGG1	8-oxoG:C, Fapy:C	Viable and fertile, *OGG1*/*MUTYH* double knockouts are cancer prone. OGG1 activity is associated with CAG repeat expansion in Huntington's disease mice models[148].	*OGG1* R46Q variant exhibits a lower activity in renal cancers[149,150]. *OGG1* S326C variant shows reduced enzymatic activity[151,152]. Conflicting results have been reported regarding its association with different cancer types[153–161]. *OGG1* A53T and A288V variants display reduced catalytic activity and have been identified in brain tissues of Alzheimer's disease patients and are thought to be related to the high level of oxidative DNA damage present in Alzheimer's disease[162].

Table 1: Continued

Enzyme	Substrates and (minor substrates)	Mouse knockout	Human disease
MUTYH	A opposite to 8-oxoG/C/G	*OGG1*/*MUTYH* double knockouts are cancer prone.	MUTYH variants are associated with colon polyposis in what is referred to as MUTYH-associated polyposis (MAP) syndrome. Colorectal cancer cells with defective MUTYH display a characteristic mutatoinal signature with prevalent G:C→T:A transversions [163,164].
NTHL1	Tg, FapyG, 5-hC, 5-hU in dsDNA	Viable and fertile. *NTHL1*/*NEIL1* double knockouts are cancer prone.	NTHL1 deficiency is associated with NTHL1-tumor syndrome characterized by a higher risk for colorectal polyposis, colorectral cancer and breast cancer. NTHL1 deficiency results in a unique NTHL1-associated mutational signature[165,166].
NEIL1	Tg, FapyG, FapyA, 8-oxoG, 5-hU, DHU, Sp and Gh in ss and dsDNA	Viable and normal at birth, but develop obesity after 7 months. *NEIL1* knockout mice develop a metabolic syndrome characterized by obesity, fatty liver disease as well as dyslipidemia and a higher tendency for developing hyperinsulinemia[167]. *NTHL1*/*NEIL1* double knockouts are cancer prone.	Unknown
NEIL2	Similar to NEIL1	*NEIL2* null mice accumulate DNA damage in actively transcribed DNA regions and elicit a stronger inflammatory response compared to wild-type counterparts[168]. Double *NEIL1* and *NEIL2* knockout mice models do not dsiplay a high mutation frequency nor cancer predisposition[169].	Unknown
NEIL3	FapyG, FapyA, Sp and Gh in ssDNA	Viable and fertile, but shows memory and learning deficit. Triple knockout mice models of *NEIL1*, *NEIL2* and *NEIL3* do not dsiplay high a mutation frequency nor are predisposed to cancer[169].	Unknown

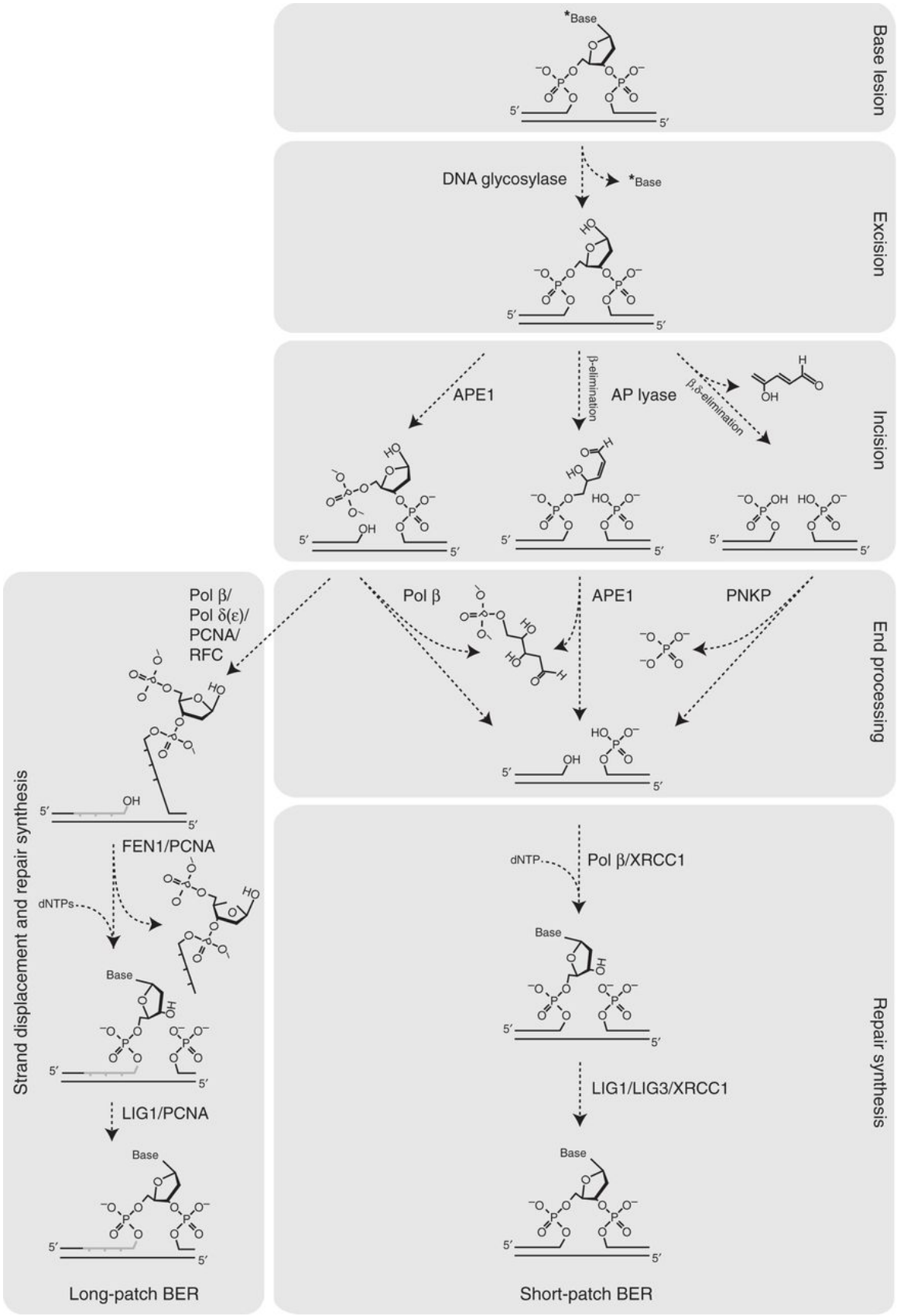

Figure 11: Schematic illustration of the steps of the base excision repair pathway (BER) showing the involved DNA repair factors in short-patch and long-patch BER. Reprinted with permission from Cold Spring Harbor Laboratory Press, Krokan and Bjørås, *2013*[128].

1.6 OGG1

The human 8-oxoguanine DNA glycosylase 1 (OGG1) gene lies on the short arm of chromosome 3 (3p26). Alternative splicing produces eight isoforms of human OGG1 according to the National Center for Biotechnology Information (NCBI) with α-OGG1 (OGG1 type 1a), and β-OGG1 (OGG1 type 2a) being the two major ones. The α-isoform (39 kDa) is present mainly in the nucleus and consists of 345 amino acid residues, whereas β-Ogg1 (47 kDa) localizes to the mitochondria and consists of 424 amino acids. Both isoforms share the first 316 amino acids which carry a mitochondrial targeting signal at amino acid residues 9–26. However, they significantly differ in their C-termini. α-OGG1 carries a dominant nuclear localization signal, whereas the β-isoform has an acidic/hydrophobic region at its C-terminus[170] (Figure 12).

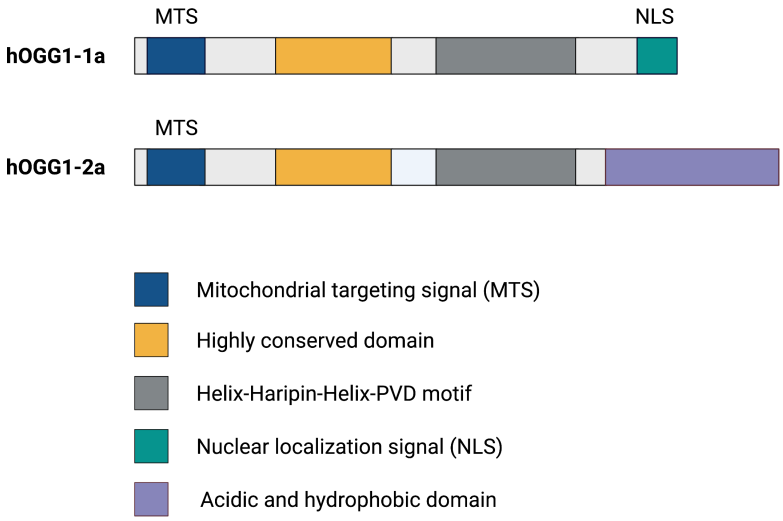

Figure 12: Schematic illustration of the difference between the structure of the two major isoforms of human OGG1 (hOGG1). hOGG1-1a is a nuclear isoform that has a C-terminal nuclear localization signal (NLS). The main mitochondrial isoform is hOGG1-2a has an acidic and hydrophobic domain at its C-terminus. MTS, mitochondrial targeting signal, Oka *et al.*, 2008[171]. Created with BioRender.com

1.6.1 OGG1's role in BER

Being a bifunctional BER enzyme, OGG1 possesses a weak β-elimination-mediated AP lyase activity in addition to its glycosylase activity[172]. Following base excision with its glycosylase activity, OGG1 dissociates at a slow rate from its tightly bound abasic site product. This dissociation step is considered the rate limiting step in OGG1-initiated BER. OGG1 turnover is stimulated by the downstream APE1 which competes for abasic sites to which it has higher affinity than OGG1[173].

By sliding on DNA in a random bi-directional pattern scanning for its substrate, OGG1 recognizes and repairs mutagenic 8-oxoguanine (8-oxoG) and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG) when paired with cytosine in duplex DNA[174–176]. OGG1 displays a characteristic helix–hairpin–helix structural motif followed by an extended loop rich in Glycine and Proline residues. The glycosylase catalytic activity requires two conserved residues: Asp 268 and Lys 249[177,178]. Lys 249 is essential for the nucleophilic attack on the C1´ of the deoxyribose sugar in order to excise the 8-oxoG base, whereas Asp 268 stabilizes the ribose oxocarbenium cation intermediate formed during the initial base-excision step[179] (Figure 13). Upon OGG1 binding to the DNA, the DNA duplex is sharply bent in a ~70° kink where the oxidatively modified base, 8–oxoG, is flipped out and becomes stalked between Phe 319 and Cys 253 bringing it in close proximity to the catalytic amino acid residues. An Arginine residue, Arg 154, recognizes the cytosine base lying opposite to the 8-oxoG adding to substrate specificity[149,177].

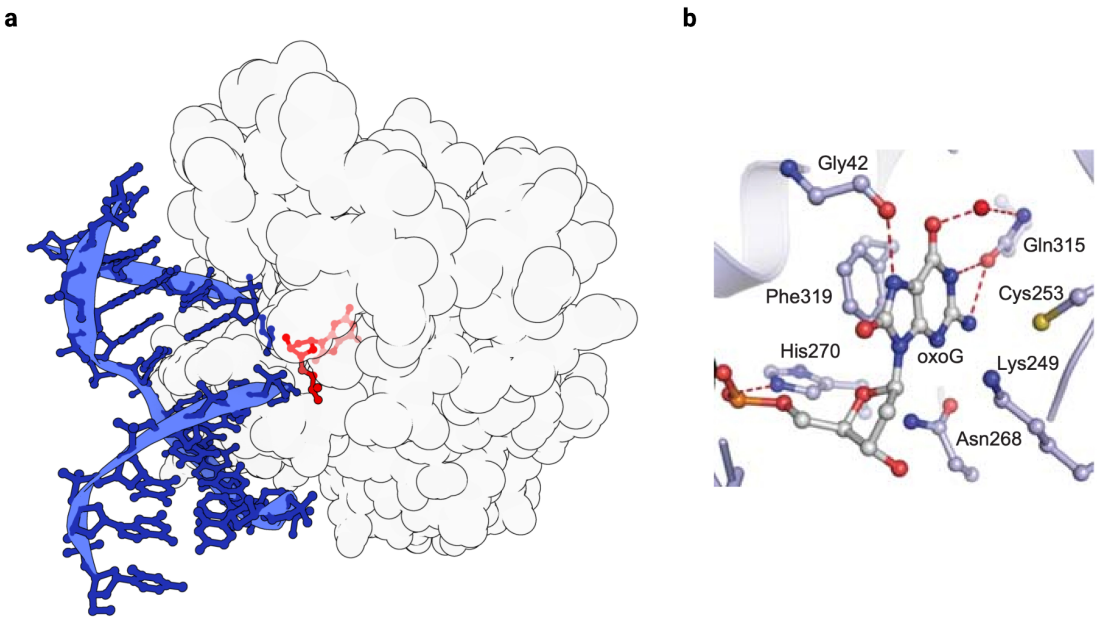

Figure 13: Interaction of OGG1 glycosylase with 8-oxoguanine. (**a**) Overall structure of human OGG1 (grey) in complex with DNA (blue). The 8-oxoguanine substrate lesion (red) is flipped out into OGG1's active site. Illustration prepared using 3D protein imaging[180]. (**b**) Close-up view of the active site of OGG1 with extrahelically flipped 8-oxoG lesion. PDB, 2NOZ, Radom *et al.*, 2007[181]. Adapted from Cold Spring Harbor Laboratory Press, Krokan and Bjørås, *2013*[128].

Interestingly, mice lacking OGG1 are viable and fertile. Analysis of nuclear DNA extracted from the liver of 13–15-week-old OGG1 deficient mice shows that steady-state level of genomic 8-oxoG is 1.7–7-folds higher[182,183]. Initial studies showed that OGG1 null mice do not develop a pathological phenotype despite the higher load of genomic 8-oxoG[183], however longer monitoring of *OGG1* knockout mice indicated that lung tumors develop in those mice

1.5 years after birth[182]. Intriguingly, a dampened inflammatory response is observed OGG1 null mice following *Helicobacter pylori* infection[184] or upon treatment with proinflammatory agents[185].

A number of OGG1 polymorphic variants have been identified[186]. In renal cancers, *OGG1* R46Q exhibits a lower enzymatic activity[150,187]. In addition, *OGG1* variants A53T and A288V have been identified in brain tissues of Alzheimer's disease patients showing a reduced catalytic activity. Those variants are thought to be related to the high level of oxidative DNA damage observed in Alzheimer's disease[162]. *OGG1* S326C has also been characterized in numerous studies. This variant is associated with lower enzymatic activity where the extra cysteine residue is involved in disulfide bond formation and promotes OGG1 dimerization[151,152]. Epidemiological studies have reported conflicting results regarding whether *OGG1* S326C is associated with a higher cancer risk. Several studies have highlighted an association between this variant and different cancer types[153–157] however, others have reported the absence of such association[158–161]. These contradicting results prompt further investigation.

1.6.2 Regulation of OGG1's activity

OGG1's enzymatic activity is regulated via protein–protein interactions. For instance, APE1 stimulates OGG1's lyase activity in *in vitro* assays[173,188]. In addition, it stimulates the release of OGG1 from AP sites bypassing the AP lyase activity of OGG1 which enables the cells to avoid a potentially rate limiting step[189]. XRCC1 is another interacting partner that stimulates OGG1[190,191]. Moreover, PARP1 interacts with OGG1 where OGG1 stimulates the PARylation activity of PARP1. However, this leads to a reduction in the BER activity of OGG1[192]. Furthermore, OGG1 is subject to a number of posttranslational modifications that modulates its activity such acetylation[193–195], phosphorylation[196,197] and O-GlcNAcylation[198].

Paradoxically, OGG1's BER activity is reported to be impaired under conditions of oxidative stress but restored after normalizing the cellular redox status. This was attributed to the oxidation and S-nitrosylation of OGG1's cysteine residues[199–202]. This reversible reduction in OGG1's activity was associated with an increase in ROS levels intracellularly and accumulation of 8-oxoG at the promoter regions of several genes suggesting a new role for 8-oxoG and OGG1 in transcriptional regulation.

1.6.3 Roles of OGG1 beyond BER

1.6.3.1 OGG1's role in transcription regulation

Mounting evidence suggests that OGG1 contributes to transcription regulation. Recent findings suggest that 8-oxoG is not only a premutagenic lesion but serves as an epigenetic mark to regulate gene expression[203,204]. In line with this, several proinflammatory genes have promoter regions rich in GC bases which are readily oxidizable under conditions of oxidative stress[109]. Although transcription factors and DNA repair enzymes are suggested to compete on binding to promoter regions harbouring oxidative damage[205], it has been shown that OGG1 contributes to transcription factors recruitment and transcription machinery assembly. For instance, OGG1-DNA interaction upstream of the consensus motif of NF-κB facilitates NF-κB binding. This in turn enhances the expression of NF-κB target proinflammatory genes upon inducing inflammation with tumor necrosis factor-alpha (TNF-α)[109,111,110]. This finding likely accounts for the reduced inflammatory response seen in OGG1 null mice[184,185].

Besides, promoter regions of highly transcribed genes are rich in G-quadruplex (G4) structural motifs. G-quadruplex formation in promoters has been associated with enhanced transcription[206]. One example is the vascular endothelial growth factor (*VEGF*) gene, whose promoter region is known to harbour G4[207] and 8-oxoG lesions[208]. OGG1-mediated base excision of 8-oxoG located in potential G-quadruplex–forming sequences (PQS) in promoter-coding strands of *VEGF* unmasks the PQS. This enables DNA to change its conformation and adopt a G4 fold resulting in higher transcription[204]. A similar effect has also been reported for *NTHL1* gene[204]. On the contrary, 8-oxoG lesions present in telomeres have been observed to destabilize telomeric G4 structures. This enhances telomerase accessibility promoting telomere elongation[209] and suggesting a role for 8-oxoG in telomere maintenance.

1.6.3.2 OGG1's role in signaling

In addition to its role in modulating transcription, OGG1 is reported to be involved in cellular signaling pathways. It has been shown that the excised 8-oxoG base in complex with OGG1 activates Ras signaling by acting as a guanine exchange factor (GEF). Ras activation consequently induces gene expression via mitogen-activated protein kinase (MAPK) signaling[210]. OGG1 in complex with 8-oxoG has also been reported to physically interact with and activate small GTPases Rac1[211] and Rho[212] which then activate different downstream cellular pathways. This widens the scope of OGG1's functions beyond its conventional role in BER and opens up new exciting applications for targeting OGG1.

1.7 NEIL GLYCOSYLASES

The redox potential of 8-oxoG is lower than that of guanine making 8-oxoG markedly sensitive to further oxidation generating hydantoin lesions[213–215]. Such lesions are recognized and excised by the Nei endonuclease VIII–like family of DNA glycosylases (NEIL)[216–219]. Three human NEIL enzymes have been discovered: NEIL1, NEIL2 and NEIL3 displaying a broad substrate specificity against oxidized pyrimidines (Table 1). After base excision, NEIL3 incises the resulting AP site with a low efficiency via β-elimination[216,220]. On the other hand, NEIL1 and NEIL2 glycosylases robustly incise the DNA backbone via β,δ-elimination giving rise to a nucleotide gap flanked by 5′-phosphate and a 3′-phospho-α,β-unsaturated aldehyde (3′-PUA). The 3′-phosphate group of the 3′-PUA is removed by the phosphatase activity of the bifunctional enzyme polynucleotide kinase 3′-phosphatase (PNKP). After the nucleotide gap is processed by PNKP, a DNA polymerase incorporates a new nucleotide to fill in the gap and finally the nick is sealed by a DNA ligase[221,222,128].

1.7.1 NEIL1 glycosylase

Although 8-oxoG is not a major substrate for NEIL glycosylases, its further oxidation products, spiroiminodihydantoin (Sp) and guanidinohydantoin (Gh) are efficiently targeted by all three NEIL enzymes[216–219]. Nevertheless, *in vitro* assays show that NEIL1 weakly excises 8-oxoG in models for clustered lesions when 8-oxoG is present as the third or fourth nucleotide 5'-upstream of a single-strand break[223]. In addition to Sp and Gh, NEIL1 excises FapyA, FapyG, thymine glycol and 5-hydroxyuracil[224,225]. DNA lesions present in bubble, bulge, and single-stranded DNA (ssDNA) can be targeted by NEIL1 but at a lower rate compared to double-stranded DNA (dsDNA)[224,226].

NEIL1 expression is induced during the S-phase of cell cycle suggesting that it plays a role in repairing replication-associated DNA damage[227]. In accordance with this, NEIL1 was reported to contribute to pre-replicative BER of oxidized DNA bases located on ssDNA template at the replication fork[228]. In such context, NEIL1 was observed to only bind to the lesion but not to excise it, which prevents generating toxic double-strand breaks (DSBs). This in turn obstructs the replication fork progression. The fork would then regress to allow the lesion repair[228,229].

Beside its role in pre-replicative BER, NEIL1 excises DNA inter-strand crosslinks[230–232] as well as hydantoin lesions in G-quadruplex motifs in promoter and telomeric sequences, and hence can potentially contribute to gene regulation and telomere maintenance[233,234]. Interestingly, acetylated NEIL1 preferentially binds to actively transcribed genomic

sequences in addition to transcription start sites of weakly-transcribed genes whose overexpression is associated with poor prognosis in cancer. This enrichment was found to correlate with a low mutation rate suggesting that NEIL1 protects against oxidative damage of transcription start sites[235].

NEIL1 depletion sensitizes cells to ionizing radiation (IR) indicating that it is involved in repairing IR-induced DNA damage[236]. Interestingly, homozygous and heterozygous NEIL1 deficient mice are viable but develop fatty liver disease, obesity, dyslipidemia and a higher tendency for hyperinsulinemia suggesting a potential metabolic function for NEIL1[167].

1.7.2 NEIL2 glycosylase

NEIL1 and NEIL2 share a common set of substrate DNA lesions. Both can recognize and excise FapyA, FapyG, thymine glycol and 5-hydroxyuracil in single-stranded, duplex and bubble-structure oligonucleotides as well as Gh and Sp, the oxidation products of 8-oxoG. In addition, NEIL2 has been reported to possess some activity towards 8-oxoG in *in vitro* assays[221]. Similar to NEIL1 and NEIL3, NEIL2 excises damaged bases in promoter quadruplex structures which may facilitate transcription initiation[233]. NEIL2 seems to act as a backup for NEIL1 where it has been shown to contribute to pre-replicative BER repair of oxidized DNA lesions in *NEIL1*-depleted cells[228].

Notably, NEIL2 is thought to play a role in transcription-coupled BER. It has been shown to associate with RNA polymerase II on actively transcribed genes but not on silent ones. Consistently, cells deficient in NEIL2 exhibited more DNA damage in active genes relative to silent ones[237]. In agreement with this finding, NEIL2 null mice accumulate DNA oxidative damage in actively transcribed genomic regions. Besides, mouse embryonic fibroblasts derived from NEIL2 null mice show more chromosomal abnormalities and telomere loss than those derived from wild type mice (WT). Intriguingly, NEIL2 deficiency resulted in a stronger inflammatory response in that mouse model suggesting that NEIL2 potentially plays a protective role against inflammation[168].

1.7.3 NEIL3 glycosylase

NEIL3 possesses a glycosylase activity towards Sp, Gh, thymine glycol, FapyA and FapyG[216,219]. Moreover, it contributes to unhooking inter-strand crosslinks induced by psoralen or generated from abasic sites[232,238,239]. NEIL3 displays a tightly regulated expression pattern with high levels detected in highly proliferative cells such as embryonic stem cells, neural progenitor cells, murine hematopoietic cells in addition to cancer cells[240–

[243]. NEIL3 has been shown to maintain telomere integrity by repairing oxidative damage in telomeric DNA during the S-phase of cell cycle[244]. In addition, NEIL3 can recognize its substrate lesions in promoter and telomeric G-quadruplex DNA structure[233,234].

NEIL3 appears to play a role in preventing autoimmunity since NEIL3 deficiency is associated with high levels of autoantibodies[245]. NEIL3 null mice show reduced proliferation capacity of neural stem/progenitor cells resulting in learning and memory impairment[246]. Accordingly, NEIL3 is thought to play a role in protecting proliferating cells and neural progenitor cells against oxidative DNA damage.

1.8 OVERLAPPING ROLES OF BER FACTORS

Mice knockout studies have shed the light on the overlapping functions of BER proteins. In general, mice lacking a single DNA glycosylase show remarkable resilience against the loss of the glycosylase activity[247]. With the exception of thymine DNA glycosylase whose loss is embryonically lethal[248], mice deficient in a single glycosylase are viable and only display a slightly elevated mutation frequency without a clear pathological phenotype[249]. This may be explained in light of the overlapping substrate specificity of BER factors (Table 1) in addition to overlap between BER and other repair pathways. In contrast to single knockouts, double or triple knockout mouse models usually display a stronger phenotype. For example, mice lacking both OGG1 and MUTYH are noticed to be highly prone to cancer with shortened life spans[250]. Similarly, double *NTHL1* and *NEIL1* knockout mice develop lung and liver tumors at a higher incidence than each of the single knockout[251]. Surprisingly *NEIL1* and *NEIL2* double knockout mice as well as *NEIL1*, *NEIL2* and *NEIL3* triple knockout mice display no elevated mutation frequency nor higher cancer predisposition[169].

Despite the apparent overlapping substrate specificity in *in vitro* assays, BER glycosylases have been well-conserved in eukaryotes during evolution. This selective pressure to conserve them suggests that they may have specialized distinguishable activities in cells. For instance, such activity might be specific to certain phases of the cell cycle[252], or during embryonic development[248] or might be specific to certain genomic regions such as actively transcribed or silent genes[237], replication forks[228,229] or telomeres[244]. Accordingly, targeting one BER factor might still have a pharmacological effect despite the presence of other apparently redundant BER factors as the latter might repair the DNA damage with suboptimal efficiency or at a suboptimal time or can lead to an inefficient downstream processing of the BER intermediates[253]. For instance, four BER glycosylases have been reported to eliminate 5-fluoruracil (5-FU) from DNA in addition to MMR[254]. However, cells are sufficiently

sensitized to fluoropyrimidines after targeting only one factor[255–257]. Nevertheless, the overlapping functions of BER glycosylases need to be carefully considered on attempting to pharmacologically target a single BER glycosylase.

1.9 TARGETING BER, A PROMISING APPROACH FOR CANCER THERAPY

Given the vital role BER plays in maintaining genomic integrity by eliminating DNA oxidative damage and considering the high levels of ROS in cancer cells, targeting BER is viewed as a promising potential anti-cancer approach. Targeting BER is anticipated to be of relevance to cancer therapy via three different approaches: (a) synthetic lethality approach in cancers cells where additional DNA repair pathways are impaired[253] (b) sensitization to endogenous high oxidative stress in cancer cells, or (c) sensitization to exogenously administered chemotherapy and radiotherapy (Figure 14).

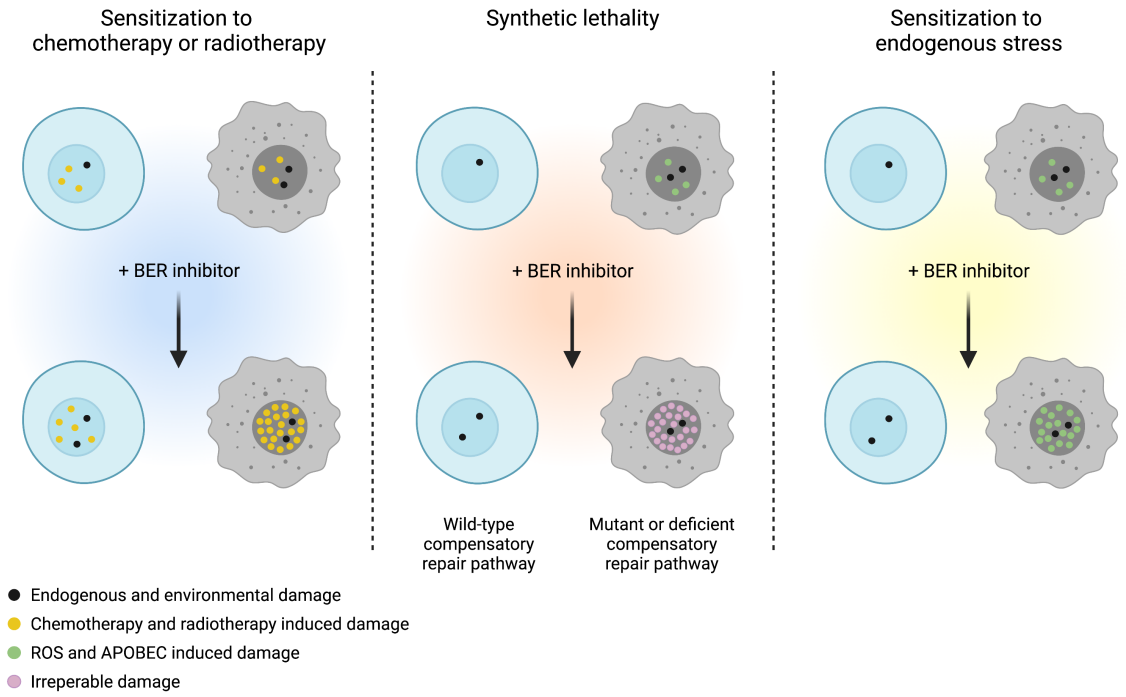

Figure 14: Schematic illustration of potential strategies of targeting BER as an anti-cancer approach. Three strategies can potentially result in different outcomes in normal cells (blue) versus cancer cells (grey) upon targeting base excision repair (BER), Visnes *et al.*, 2018[253]. Created with BioRender.com

1.9.1 Sensitization to co-administered chemotherapy or radiotherapy

Another strategy where targeting BER might prove beneficial is to sensitize cancer cells to chemotherapy or radiotherapy. Irradiation as well as classical chemotherapeutics work by inducing DNA damage. However, they tend to affect all dividing cells leading to a broad range of side effects. Decreasing the administered dose of radio- or chemotherapy may spare the patients from these toxic side effects but will be accompanied by a lower efficacy. This issue can potentially be addressed by concomitant BER targeting. Several studies have demonstrated that knocking down some BER components strongly sensitize cancer cells to chemotherapy and radiotherapy. BER inhibitors are thought to have a similar effect. Surprisingly, overexpressing some BER factors have also resulted in a similar hypersensitive phenotype[258]. This might seem paradoxical but can be explained when considering the nature of the DNA intermediates formed when these BER factors are overexpressed. For example, overexpressing a certain BER factor might generate a lot of abasic sites. The resultant high load of AP sites might overwhelm the BER capacity and generate toxic DNA strand breaks resulting in a hypersensitive phenotype[258].

It is well-established that irradiation results in higher levels of ROS and induces a multitude of DNA lesions including oxidative DNA lesions and DNA strand breaks[259–261]. Since the former are typically eliminated by BER, it becomes evident that manipulating the status of BER in irradiated cells can affect the outcome of irradiation. For example, OGG1 protects against radiation-induced DNA damage in human leukemia cells. Cells expressing a mutant OGG1 version exhibit a severe G2/M arrest and eventually more apoptosis after irradiation compared to cell expressing wild type OGG1[262].

Several BER components can also affect the cellular response to DNA crosslinking agents psoralen and cisplatin. On one hand, psoralen-induced monoadducts are recognized and repaired by NEIL1. HeLa cells become hypersensitive to psoralen after depleting *NEIL1* and/or *APE1*[263]. In addition, NEIL1 and NEIL3 are reported to contribute to repairing bulky psoralen-induced inter-strand cross-links[238,264,265]. On the other hand, cisplatin treatment is known to generate ROS as well as DNA mono-adducts, intra-strand and inter-strand cross-links[266]. Accordingly, the status of UNG, Pol β and APE1 influence cisplatin cytotoxicity[267,268,269]. NTHL1 is another promising candidate to potentiate cisplatin cytotoxicity since its depletion re-sensitizes the otherwise cisplatin-resistant tumor cells[270]. This motivates developing BER inhibitors considering their promising potential applications.

1.9.2 Synthetic lethality approach

Synthetic lethality is a term that refers to cell death upon simultaneous perturbation of two genes, whereas each of which alone does not result in loss of viability (Figure 15). Such perturbation can occur via a genetic mutation, RNA-interference or via pharmacologically inhibiting the protein product of the gene[271]. There is some degree of overlap in DNA repair pathways where some pathways can compensate for others. This concept can be exploited to specifically target cancer cells deficient in a certain repair pathway by inhibiting a compensatory one. Normal cells are not affected since the initial repair pathway in question is functional there[271]. PARP1 inhibition in homologous recombination deficient cells is an extensively studied example of synthetic lethality[272,273]. Although it is not a core component of BER[274], PARP1 is a key player in single-strand break repair and prevents the formation of excessive SSBs during BER[275–279]. PARP inhibitors (e.g., Olaparib, Niraparib, Rucaparib and Talazoparib) are approved by the Food and Drug Administration (FDA) and European Medicine Agency (EMA) for the treatment of ovarian, breast or pancreatic cancer patients with somatic and/or germline *BRCA1* or *BRCA2* gene mutation. Moreover, clinical trials to evaluate combination therapies with other anti-cancer drugs are ongoing[280–282].

Advances made to RNA interference and the CRISPR–Cas9 technology has facilitated large-scale screening efforts aiming at determining synthetic lethal interaction partners. APE1 is another promising candidate to induce synthetic lethality in cancer cells. Synthetic lethality was observed upon APE1 inhibition in cells deficient in Ataxia telangiectasia mutated (ATM) or concomitantly treated with inhibitors of ATM or DNA-dependent protein kinase catalytic subunit (DNA-PKcs)[283].

In addition to exploiting tumor specific mutations, synthetic lethal interactions might rely on intrinsic conditions of the tumors such as metabolic alterations, hypoxia or elevation in ROS in what is referred to as conditional synthetic lethality[271]. This broadens the concept of synthetic lethality and opens new avenues for specifically targeting cancer cells.

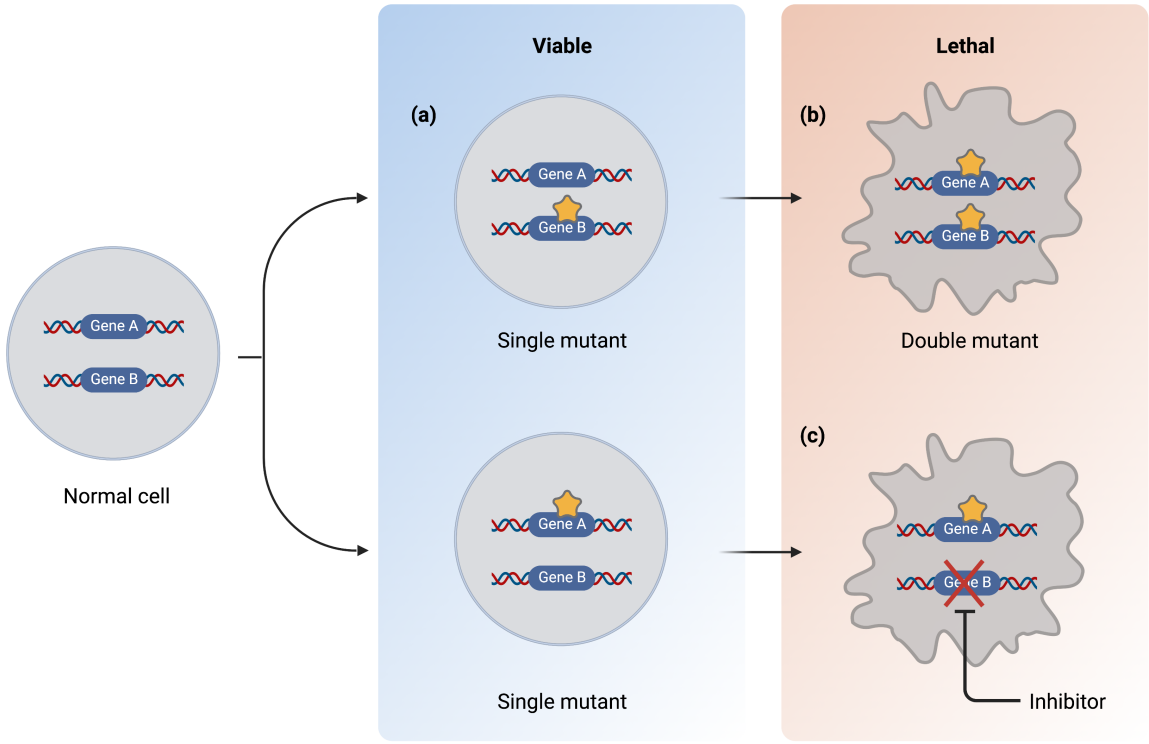

Figure 15: Schematic illustration for the principle of synthetic lethality. Cells can tolerate pharmacological inhibition or mutation of either gene A or gene B alone (**a**). Concomitant loss of gene A and B results in cell death whether it is caused by double mutation (**b**) or by inhibiting the protein product of gene B in cells with mutant gene A (**c**). Mutation is depicted as a star shape. Viable cells are represented by circles whereas inviable cells are represented by irregular shaped ones, O'Neil *et al.*, 2017[271]. Created with BioRender.com

1.9.3 Sensitization to endogenous stress in cancer cells

Cancer cells exhibit high levels of ROS owing to a number of factors such as mitochondrial dysfunction, aberrant metabolism, oncogene expression, cross-talk with tumor-infiltrating immune cells among others[43]. This might be exploited in a conditional synthetic lethality approach. Being one of the most abundant DNA oxidative lesions[60,61,125], MTH1, MUTYH and OGG1 function in concert to cope with the high load of 8-oxoG[253].

Several lines of evidence highlights MTH1's role in protecting cancer cells against oncogene-induced oxidative stress[284–287]. A number of small-molecule MTH1 inhibitors have been reported with varying degrees of cytotoxic efficacy. While some MTH1 inhibitors display a potent anti-cancer profile[288–290], others were reported to have no anti-cancer effect despite strong target engagement[291]. The effect of MTH1 inhibitors remains to be further mechanistically characterized, however reported data suggest that the cytotoxic effect of some MTH1 inhibitors is associated with inducing mitotic arrest[292] and 8-oxoG accumulation in DNA as detected by immunostaining and modified comet assays[290]. MTH1 inhibitors

which fail to trigger mitotic arrest and subsequent accumulation of genomic 8-oxoG lesions, did not display an anti-cancer effect[290]. Since 8-oxoG is a major substrate for BER, one might rationalize that inhibiting BER can enhance MTH1 inhibitors' cancer killing ability. Accordingly, inhibitors of OGG1 or MUTYH, which act downstream of MTH1, can potentially be used to kill oxidatively stressed cancer cells alone or together with MTH1 inhibitors. In support of this hypothesis, cell proliferation was found to be impaired in pancreatic cancer cells after knocking down *MUTYH*[293]. In addition, depleting *MTH1* and *MUTYH*, alone or in combination induced apoptosis in a mismatch repair defective T-cell Acute lymphoblastic leukemia (T-ALL) cell line[294]. Moreover, OGG1 overexpression prevents senescence in Ras-transformed cells[295] suggesting that targeting OGG1 might interfere with cancer cell proliferation.

Taken together, these findings imply that targeting BER glycosylases may be a promising strategy to therapeutically exploit the augmented ROS production in cancer. This prompted us to seek developing small molecules that can modulate OGG1's activity.

2 DOCTORAL THESIS

2.1 RESEARCH AIMS

The base excision repair pathway is an evolutionary conserved pathway responsible for eliminating oxidized, alkylated and deaminated bases to maintain genomic integrity. OGG1 is the major glycosylase responsible for the recognition and excision of 8-oxoG, one of the most frequent oxidative DNA lesions[140,296]. OGG1's functions are not confined to DNA repair, but extend to transcription regulation and signaling[297]. The overall aim of this thesis is to evaluate the therapeutic potential of modulating the activity of OGG1 in cancer and inflammation models via small-molecule intervention.

The specific aims of the thesis are:

- Characterize TH5487, an in-house developed small-molecule inhibitor that targets OGG1's active site.
- Study whether inhibiting OGG1 alleviate inflammation by suppressing proinflammatory cytokine expression.
- Examine whether NEIL1 or NEIL2 glycosylases compensate for OGG1 loss of function after OGG1 inhibtion.
- Evaluate OGG1's potential as an anti-cancer target and describe the effect of OGG1 inhibitor on cell proliferation and accumulation of DNA damage.
- Characterize a small-molecule OGG1 activator that allows OGG1 to acquire a novel enzymatic function not shown by native OGG1.

The component papers of this thesis addressed those specific aims by tackling the following posed research questions:

Paper I:

- Can biochemical, target engagement and cellular profiling identify potent small-molecule inhibitors for OGG1 glycosylase?
- Does TH5487, the hit molecule engage with OGG1 impairing its ability to bind to its DNA substrate lesions?
- Can TH5487 treatment impair NF-κB binding to guanine-rich proinflammatory gene promoter regions?
- Does OGG1 inhibition with TH5487 downregulate the expression of proinflammatory genes *in cellulo* and *in vivo*?

- Does OGG1 inhibition affect the immune cell infiltration in mouse models of inflammation?

Paper II:

- Does OGG1 inhibition result in accumulation of genomic 8-oxoG after inducing oxidative stress?
- How does TH5487 affect OGG1 free nuclear mobility and OGG1-chromatin binding?
- Does TH5487 treatment alter OGG1 recruitment kinetics to regions of DNA damage?
- What is the effect of OGG1 inhibition on generating DNA strand breaks?

Paper III:

- What is the therapeutic potential of OGG1 as an anti-cancer target?
- Does TH5487 selectively suppress cancer cell growth?
- Does TH5487 treatment induce replication stress?
- What is the mechanism behind the TH5487-induced proliferation arrest?
- Is TH5487 active *in vivo*? Can TH5487 treatment inhibit the growth of xenografts tumors in mice?

Paper IV:

- Does TH5487 target NEIL glycosylases?
- Does TH5487 alter the recruitment kinetics of NEIL1 or NEIL2 to laser-induced DNA damage regions?
- Is NEIL1- or NEIL2 -chromatin binding affected in oxidatively stressed cells after depleting or inhibiting OGG1?
- Do more genomic 8-oxoG lesions accumulate in *NEIL1*-or *NEIL2*-depleted cells after TH5487 treatment?

Paper V:

- How does TH10785 affect the activtiy of OGG1 in *in vitro* biochemical assays?
- What is the effect of TH10785 on OGG1's AP lyase activity?
- What are the reaction products generated after TH10785 treatment?
- Does TH10785 target OGG1's active site?
- Does TH10785 affect OGG1-chomatin binding?
- Do cells become more dependent on PNKP after TH10785 treatment to complete the BER pathway?

2.2 METHODOLOGY

Multidisciplinary methods were used in the component papers of this doctoral thesis. Those methods allowed us to address our research questions from different angles including, medicinal chemistry, biochemistry, structural biology, molecular biology and pharmacology.

A thorough description of all the methods used in this doctoral project is available in the individual component publications and manuscript of this thesis. Special focus will be given to discussing key methods and ethical considerations here.

2.2.1 Differential scanning fluorimetry (DSF)

Studying protein-inhibitor interactions is a fundamental objective in preclinical drug development projects. Thermal shift assays (TSA) such as differential scanning fluorimetry (DSF) and cellular thermal shift assays (CETSA) are widely used methods to evaluate target engagement. Thermal shift assays are based on the principle that ligand binding can enhance the protein's thermal stability against denaturation and are thus used to assess whether the ligand targets the protein of interest[298].

In DSF, protein unfolding is monitored over a temperature gradient in the presence of specialized fluorescent dyes such as SYPRO orange which nonspecifically bind to hydrophobic residues of the protein emitting fluorescence. By increasing the temperature, the protein starts to unfold exposing its hydrophobic interior. Being accessible to the dye, SYPRO orange binds to the protein's hydrophobic core and emits detectable fluorescence. Further elevation in the temperature leads to protein aggregation and dye dissociation. Plotting changes in fluorescence as a function of increasing temperature gives a sigmoidal curve from which information about the melting temperature (Tm) can be extracted (Figure 16). By comparing the thermal stability of a native protein to that incubated with a putative ligand, one can assess the shift in the melting temperatures (ΔTm) and subsequently evaluate target engagement[299].

Among the virtues of DSF is its simplicity, short assay running time and compatibility with high-throughput screening of chemical compound libraries. However, it comes with some limitations. First, it requires the purification of large amounts of the protein of interest. This might be difficult or expensive to achieve. Moreover, since DSF assay is performed using purified proteins, it is inherently artificial in nature and lacks the relevant cellular environment[298]. One also needs to check for autofluorescence of the tested compounds. In addition, if the investigated compound can bind to both the native and unfolded form of the

protein, the calculated ΔTm might be small resulting in underestimating the compound's target engagement ability[300].

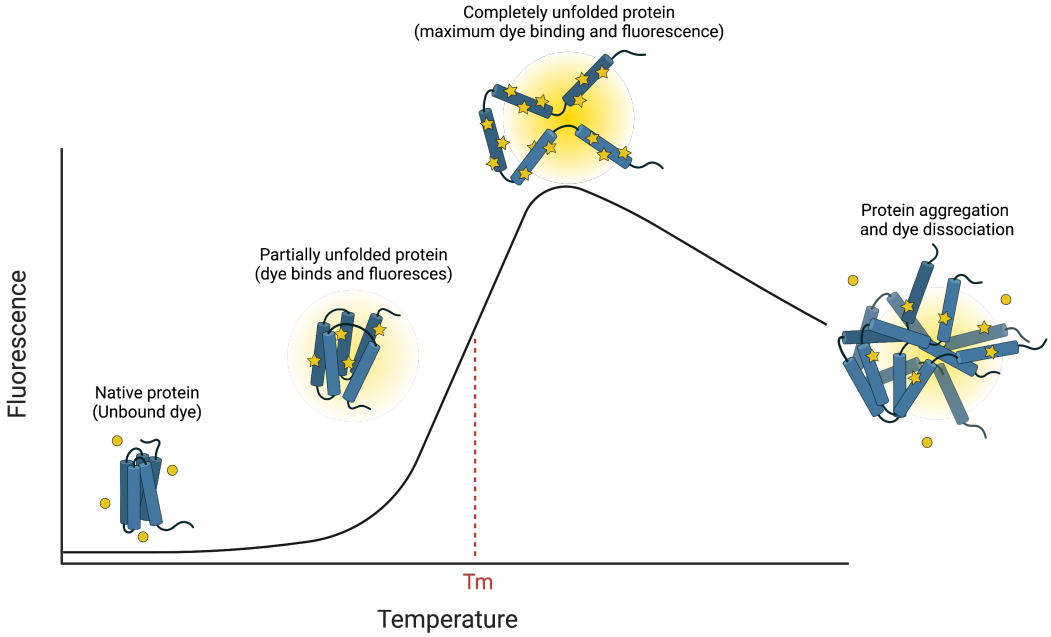

Figure 16: Schematic illustration for the principle of differential scanning fluorimetry (DSF). DSF is used to evaluate target engagement by monitoring protein unfolding over a temperature gradient in the presence of a specialized dye that binds to the protein's hydrophobic residues. Ligand binding stabilizes the protein against thermal denaturation resulting in a higher melting temperature (Tm). Created with BioRender.com

2.2.2 Laser microirradiation and live cell imaging

A multitude of DNA repair factors are involved in the DNA damage response (DDR) to signal the presence of DNA damage and to initiate the relevant repair pathway. Fluorescence microscopy has provided significant insights into how different DNA repair factors are recruited to DNA damage regions to initiate the DDR after inducing global DNA damage using ionizing radiation or chemotherapeutics. However, using such global DNA damaging agents may not be optimum if the recruitment kinetics of DNA repair factors are to be evaluated. Furthermore, examining fixed cells does not allow to study the dynamic changes that occurs in live cells over time. Thanks to advancements made in confocal microscopy, better understanding of DNA damage inducing laser systems and fluorescent labelling, laser microirradiation has become a powerful tool to study DNA repair pathways in live cells.

For live cell imaging, one needs to generate a cell line expressing the DNA repair factor of interest fused to a fluorescent tag. Laser microirradiation is performed using a confocal microscope equipped with an integrated laser source and an environmental chamber to

control the CO_2% and temperature. Targeted DNA damage is induced at sub-micron pre-defined regions in the cell nucleus using laser microirradiation (Figure 17). This makes it possible to analyze the coordinated steps of DNA repair temporally and spatially at single-cell resolution providing valuable insights into the kinetics of DNA repair factor recruitment and dissociation at sites of DNA damage.

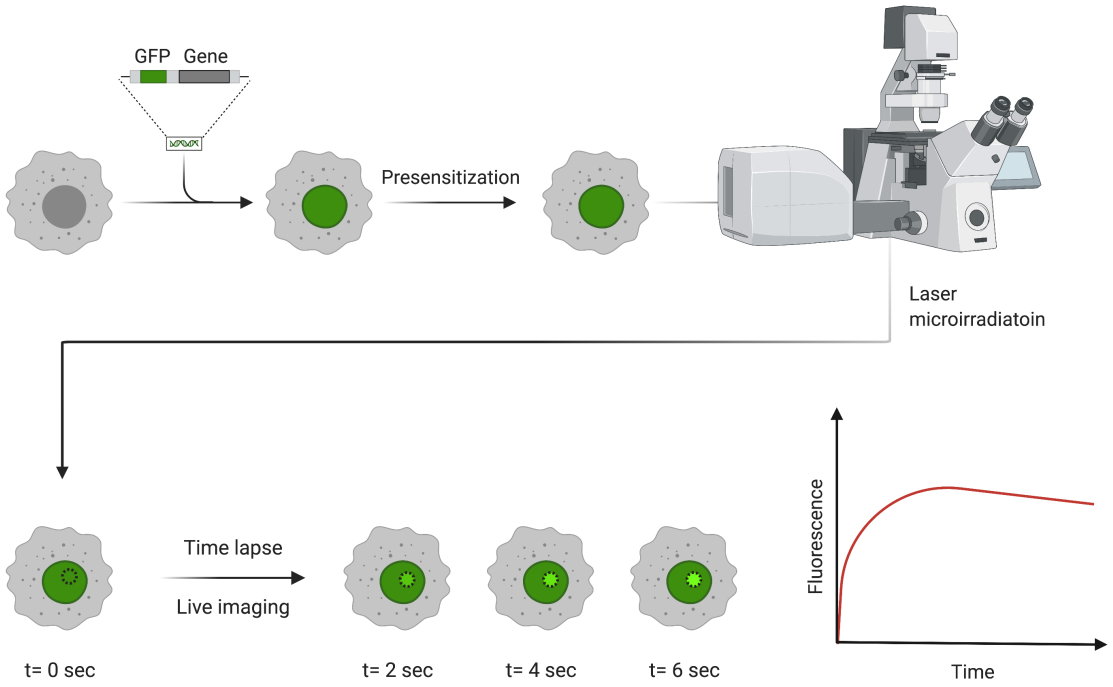

Figure 17: Schematic illustration for the principle of laser microirradiation. A cell line expressing the protein of interest fused with a fluorescent tag (e.g., GFP, green fluorescent protein) is generated. Sensitizing agents such as Hoechst 33342 or 5-Bromo-2′-deoxyuridine (BrdU) may be employed. Laser microirradiation is performed using a confocal microscope equipped with an integrated laser source and an environmental chamber to control the CO_2% and temperature. Targeted DNA damage is induced at sub-micron pre-defined nuclear regions. Fluorescence at the irradiated region is recorded and plotted against time to study the recruitment kinetics of the protein of interest to regions of laser-induced DNA damage. Created with BioRender.com

Fusing the fluorescent tag at the N-or C-terminus might affect the activity of the protein of interest especially if the active site is located close to one of these ends. Accordingly, one needs to carefully plan the plasmid construction and assess the activity of the tagged protein. Using laser to induce DNA damage may result in a variety of DNA damage lesion types rather than a single one including UV-induced photolesions, oxidative DNA damage, single strand breaks and double strand breaks among others[301]. Thus, several parameters must be controlled for laser microirradiation experiments. The laser wavelength being used can influence the type of DNA lesions being generated. Moreover, the laser source input power affects the complexity of the induced DNA damage. One also needs to consider if sensitizing

agents such as Hoechst 33342, or 5-Bromo-2′-deoxyuridine (BrdU) are to be used or not. Using such photosensitizers may allow DNA damage induction at lower laser powers resulting in a lower cellular phototoxicity. However, photosensitizers may result in DNA damage on their own affecting the cell cycle and the chromatin structure. This can lead to undesired effects that need to be taken into account when planning the experiment[301–303].

2.2.3 Fluorescence recovery after photobleaching (FRAP)

Fluorescence recovery after photobleaching (FRAP) is an advanced microscopy technique used to study the mobility of fluorescently tagged proteins in single living cells. FRAP experiments can provide information about the diffusion kinetics of the DNA repair factor of interest and whether it is freely mobile, or chromatin bound. Since it entitles live imaging, a cell line expressing the fluorescently tagged protein of interest needs to be established.

A FRAP experiment typically begins with irreversibly photobleaching a pre-defined nuclear region using a focused laser beam of high intensity. This creates a relatively darker region in the otherwise fluorescent specimen. Subsequently, fluorescent molecules diffuse from the surrounding non-bleached region into the bleached area resulting in fluorescence recovery (Figure 18). By plotting the fluorescence changes that occur over time in the bleached area, one can determine how much the mobile fraction is based on the initial and final fluorescence intensities. From this plot, more information can be extracted to describe the speed of fluorescence recovery such as the time needed to reach the maximal fluorescence intensity (T_{max}) and the time needed to recover half the maximal plateau fluorescence intensity ($T_{1/2}$ or half-life)[304].

FRAP is more suitable for adhesive cell lines. Suspension cell lines can be used after cell attachment to the surface of culture dishes coated with poly-L-lysine for instance. Living cells often move during the course of the experiment. Thus, it is recommended to compensate for this by using an appropriate alignment algorithm. In addition, bleaching may occur over time during the experiment. Accordingly, the overall loss in fluorescence must be accounted for when analyzing the FRAP data[304].

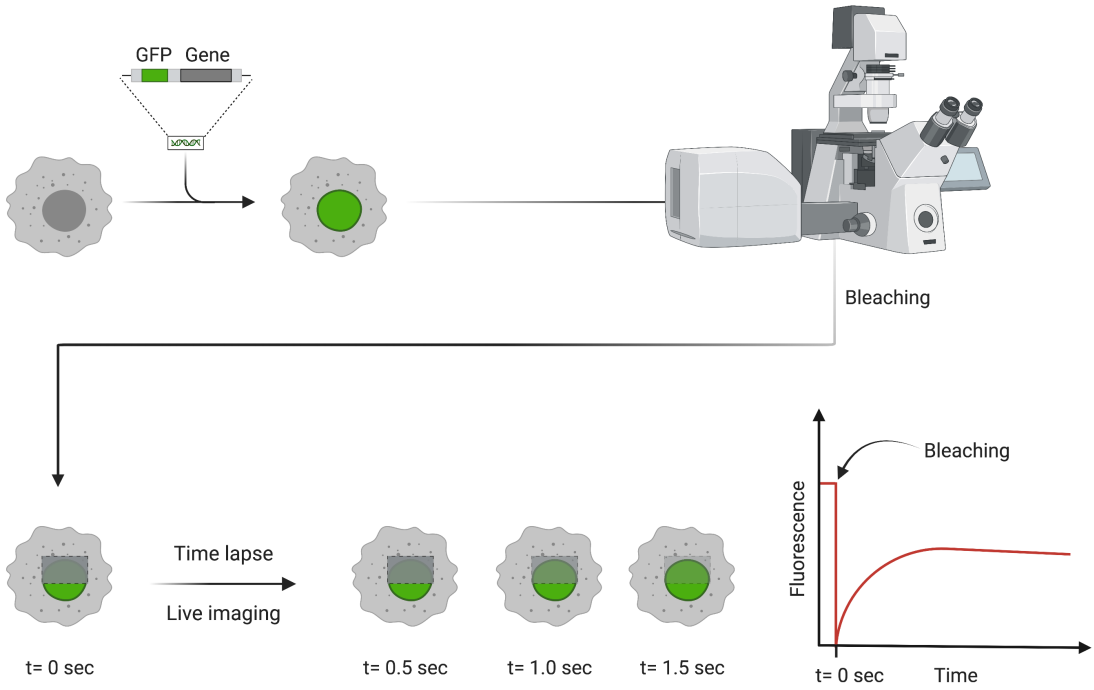

Figure 18: Schematic illustration for the principle of fluorescence recovery after photobleaching (FRAP). A cell line expressing the protein of interest fused with a fluorescent tag (e.g., GFP, green fluorescent protein) is generated. FRAP is performed using a confocal microscope equipped with an integrated laser source and an environmental chamber to control the CO_2% and temperature. A pre-defined nuclear region is irreversibly photobleached using a focused laser beam of high intensity. Fluorescent molecules diffuse from the surrounding non-bleached region into the bleached area resulting in fluorescence recovery. Monitoring the fluorescence intensity of the bleached region over time provides insights into how mobile the protein of interest is. Created with BioRender.com

2.2.4 Ethical considerations

In vivo animal experiments described in papers **I** and **III** were done in compliance with the ethical guidelines and approved by the regional Ethical Review Committee in Stockholm (ethical permit N89/14). *In vivo* inflammation experiments described in paper **I** were done at the University of Texas Medical Branch (UTMB) in accordance with ethical guidelines of the National Institute of Health (NIH), as well as NIH Guide for Care and Use of Experimental Animals and approved by UTMB's Animal Care and Use Committee (approval no. 0807044C –2013-2018). In paper **III**, we included experiments that were done using blood samples obtained from healthy donors after signing an appropriate informed consent. This was approved by the Ethical Review Committee at the Fuenlabrada University Hospital, Madrid, Spain. No ethical permits were required for papers **II**, **IV** and **V** since experiments reported there were done using established cancer cell lines.

2.3 RESULTS

2.3.1 Paper I: Development of a potent small-molecule inhibitor of OGG1 reveals that targeting OGG1 suppresses the expression of proinflammatory cytokines

Due to the polarity of their binding pocket, targeting DNA-binding proteins with small-molecule inhibitors has been considered a challenging task[305,306]. DNA glycosylases were often regarded as almost undruggable targets. This was true for OGG1 until potent small-molecule inhibitors for OGG1 were developed by our work in **Paper I** as well as by others[307] around the same time. In **Paper I**, we reported the development of TH5487, a potent pharmacologically active OGG1 inhibitor. In addition, we provided proof of concept that inhibiting OGG1 may represent a novel anti-inflammatory strategy.

In **Paper I**, a fluorescence-based high-throughput biochemical assay was used to screen a library of 17,940 compounds. A hit molecule was identified with a median inhibitory concentration (IC_{50}) in the low micromolar range (8.6 μM). Further hit expansion efforts led to the development of TH5487, a potent OGG1 inhibitor with an IC_{50} of 342 nM. To evaluate target engagement, we performed differential scanning fluorimetry (DSF) and cellular thermal shift assay (CETSA). TH5487 treatment was found to stabilize OGG1 against thermal denaturation *in vitro* and in cellular context as well. Structural biology experiments helped determine the binding site for the developed series of inhibitors. The X-ray crystal structure of murine OGG1 in complex with TH5675, a more soluble analog of TH5487, was resolved indicating that the molecule binds to OGG1's active site. Electrophoretic mobility shift assays showed that TH5487 hinders the binding of OGG1 to its substrate lesion *in vitro* in a dose-dependent manner validating OGG1 inhibition. In line with this, FRAP assays performed using Jurkat A3 acute lymphoblastic leukemia T-cells expressing OGG1-GFP showed that TH5487 treatment results in higher nuclear mobility of OGG1. This suggests that TH5487 treatment impairs OGG1 binding to its oxidized DNA substrate lesions *in cellulo*. Notably, more genomic 8-oxoG lesions accumulated in cells pre-challenged with potassium bromate ($KBrO_3$) upon treatment with TH5487, as detected by liquid chromatography–tandem mass spectrometry (LC-MS/MS) further confirming OGG1 inhibition.

Multiple lines of evidence suggest that OGG1 has a broader scope of functions than its originally identified role in BER. It has been reported that upon induction of an inflammatory response OGG1 plays a role in mediating transcription of inflammatory cytokines by binding to 8-oxoG in gene promoter regions and facilitating the binding of NF-κB, a major transcriptional

regulator of inflammatory responses[109–111]. This prompted us to examine whether OGG1 inhibition with TH5487 downregulates the expression of proinflammatory cytokines. To test this, the expression of a panel of proinflammatory cytokines, C-C and C-X-C chemokines was profiled in murine airway epithelial cells (MLE 12) and human small-airway epithelial cells (hSAECs) stimulated with tumor necrosis factor-alpha (TNF-α) and lipopolysaccharide (LPS). TH5487 treatment decreased the expression of proinflammatory cytokines and chemokines in a dose-dependent manner. Mechanistically, chromatin immunoprecipitation demonstrated that TH5487 reduces OGG1 binding to gene promoter regions of proinflammatory cytokines. The consequential downstream binding of NF-κB to the same regions was also reduced in cells stimulated with TNF-α and treated with TH5487.

To evaluate its potential anti-inflammatory role *in vivo*, mice were stimulated intranasally with TNF-α to induce an inflammatory response. TH5487 was administered intraperitoneally prior to the TNF-α challenge in a prophylactic setup as well as post the exposure to TNF-α. Long-term TH5487 administration was well-tolerated in mice as they showed no change in body weight nor in the level of different haematological and serum parameters after TH5487 injection. While the expression of several proinflammatory cytokines and chemokines was strongly induced by TNF-α, it was suppressed in the mice group treated with TH5487. This was translated into lower recruitment of neutrophils and suppressed lung inflammation indicating that TH5487 is active *in vivo* in both inhibiting inflammation and alleviating ongoing inflammatory responses.

In conclusion, we identified in **Paper I** TH5487, a potent OGG1 inhibitor. We show that TH5487 inhibits OGG1's activity and impairs OGG1-DNA binding in *in vitro* assays. TH5487 engages with OGG1 in thermal shift assays. Notably, TH5487 impairs NF-κB binding to the promoter regions of proinflammatory cytokines. This results in suppressing proinflammatory gene expression in TNF-α and LPS stimulated cells. Importantly, TH5487 is well-tolerated in mice. TH5487 shows anti-inflammatory effects *in vivo* such as reducing the expression of inflammatory mediators and perturbing neutrophil infiltration. Thus, targeting OGG1 can be a potential beneficial strategy to treat inflammatory conditions.

2.3.2 Paper II: Characterizing the effect of TH5487 on OGG1-chromatin dynamics, OGG1 recruitment kinetics and double strand breaks

In **Paper II**, our goal was to further characterize TH5487 regarding its effect on 8-oxoG accumulation, OGG1 recruitment kinetics to regions of DNA damage as well as on OGG1-chromatin dynamics. To this end, we employed U2OS osteosarcoma cell line as a solid tumor cell line in our experiments to complement our findings that have been reported in paper I using the hematological T lymphocyte leukemia cell line, Jurkat A3. In **Paper II**, DNA damage was induced globally using potassium bromate as well as locally in laser microirradiation experiments.

To assess the level of genomic 8-oxoG, we performed immunofluorescence experiments using an antibody against 8-oxoG. OGG1 inhibition resulted in more 8-oxoG accumulation in cells that have been pre-challenged with potassium bromate. We next established a U2OS cell line with stable expression of OGG1-GFP to be used in live imaging experiments. FRAP assays show that potassium bromate treatment induced a reduction in OGG1 nuclear mobility likely due to introducing oxidative DNA damage to which OGG1 bound. Interestingly, OGG1 regained its nuclear mobility in TH5487-treated cells that have been exposed to potassium bromate indicating that TH5487 impairs OGG1 binding to its substrate DNA lesions in living cells. In line with this observation, OGG1-GFP was found to be loosely bound to chromatin in an *in situ* extraction assay following TH5487 treatment.

To evaluate OGG1 recruitment kinetics, DNA damage was locally induced in laser microirradiation experiments and OGG1-GFP recruitment to the sites of laser-induced damage was monitored as a function of time. Inhibiting OGG1 with TH5487 led to lower recruitment of OGG1-GFP to sites of DNA damage in living cells providing additional evidence that TH5487 indeed interferes with OGG1-chromatin dynamics. Furthermore, less γH2AX was detected in cells concomitantly treated with TH5487 and potassium bromate or menadione as oxidizing agents. This effect was TH5487 concentration-dependent suggesting that TH5487 impairs OGG1's catalytic activity resulting in fewer incisions.

In summary, our findings in **Paper II** sheds light on the consequences of OGG1 inhibition using TH5487. We show that TH5487 impairs the repair of potassium bromate induced 8-oxoG lesions and results in fewer incisions using immunostaining assays. Furthermore, the inhibitor treatment alters both OGG1 recruitment kinetics and chromatin binding dynamics as evident by the results of laser microirradiation experiments and FRAP assays respectively.

2.3.3 Paper III: Investigating the potential of TH5487 for cancer therapy

Genomic instability is regarded as one of the hallmarks of cancer[76,80]. Multiple factors contribute to the inherent high level of DNA damage displayed in cancer cells. On one hand, oncogene expression induces replication stress and contributes to oxidative DNA damage by disrupting redox homeostasis[39,308]. On the other hand, cancer cells are characterized by impaired DNA repair capacity[309,310]. Consequently, targeting the DNA damage response (DDR) can be therapeutically exploited as an attractive strategy to target cancer cells[311]. In this study, we examined whether targeting OGG1 has an anti-cancer therapeutic potential.

Since Ras oncogene expression has been reported to generate ROS and oxidative DNA damage[312,313], we used in **Paper III** isogenic BJ fibroblasts immortalized by expressing human telomerase reverse transcriptase (hTERT) and transformed or not with SV40 large T antigen and HRAS G12V[314], as a model to validate OGG1 as an anti-cancer target. Knocking down *OGG1* with siRNA significantly reduced the cellular viability and clonogenic formation ability of the oncogene-expressing cells despite being well-tolerated in the immortalized non-transformed cells. Similarly, slower proliferation was observed upon depleting *OGG1* in A3 acute lymphoblastic leukemia T-cells using doxycycline-inducible small hairpin RNA (shRNA) constructs targeting *OGG1*. Importantly, *in vivo* experiments involving mouse xenografts of A3 cells harbouring the same shRNA construct and luciferase showed tumor size regression and enhanced survival after *OGG1* depletion. These data suggest that OGG1 plays a protective role against oncogene-induced stress in cancer cells grown both *in vitro* and *in vivo* validating OGG1 as a potential anti-cancer target.

Resolving the X-ray crystal structure of human OGG1 in complex with TH5487 confirmed that the molecule targets OGG1's active site. A π-stacking interaction was observed between the benzimidazolone core of TH5487 and His270 in addition to lipophilic interactions with the exo-site residues, Leu323 and Ile152. The amino group of the latter also forms a hydrogen bond with the carbonyl oxygen of TH5487. In addition, a water-mediated interaction was observed between the bromine atom of the inhibitor and Ser326. Notably, TH5487 binding induces a conformational change in OGG1. TH5487-bound OGG1 adopts a closed conformation which makes it inaccessible to its normal substrate DNA lesions.

We next evaluated the cellular viability of a large panel of cancer and normal cell lines. While TH5487 was well-tolerated by normal cells, it suppressed the growth of a broad range of cancer cells of different tissues of origin. The proliferative defect observed after OGG1 inhibition appears to be reversible since releasing TH5487 pre-treated cells into fresh medium

enables the cells to resume division suggesting that TH5487 induces a cytostatic effect rather than a cytotoxic one. Surprisingly, quantification of genomic 8-oxoG lesions by LC-MS/MS did not reveal a major increase in the lesion level after TH5487 treatment although OGG1 substrate lesions were detected in modified comet assay. OGG1 inhibition was found to induce replication stress as evident by accumulation of γH2AX during the S-phase in addition to a notable reduction in replication fork speed which likely accounts for the observed proliferation arrest.

To further investigate the mechanism behind TH5487-induced replication stress, RNA sequencing was performed for A3 cells after TH5487 or dimethyl sulfoxide (DMSO) treatment. Interestingly, a "DNA replication" signature was among the most downregulated gene sets as identified by enrichment analysis of the differential gene expression profile. This downregulation may not be explained by changes in the cell cycle distribution since replicating cells in S-phase were not excluded from the examined cell population. Among the downregulated genes are those encoding for the MCM2–7 complex. The promoter of *MCM4,* a DNA replication licensing factor contains one or more binding motifs for SP1 transcription factor according to the eukaryotic promoter database. It has been reported that OGG1 is involved in the recruitment of SP1 to its binding motifs in promoter regions[109]. This prompted us to assess the level of oxidative DNA damage at the SP1 binding motif in *MCM4* promoter after OGG1 inhibition. We observed more oxidative DNA damage in this region after TH5487 treatment. This was associated with a mild but significant reduction in the expression level of *MCM4* mRNA. These data suggest that TH5487 downregulates the expression of several DNA replication genes altering the cellular transcriptional profile which contributes to replication stress.

To test if TH5487 suppresses the proliferation of tumor cells *in vivo*, A3 tumor xenografts were monitored in mice after oral TH5487 administration. The tumor growth was not suppressed after TH5487 treatment. This is likely explained by the undetectable target engagement in CETSA assays on cells derived from the xenografts. Since serum protein binding affects drug bioavailability, we examined whether the TH5487 has a high affinity to albumin. Indeed, *in vitro* biochemical assay showed that TH5487 loses its efficacy after being incubated with bovine serum albumin suggesting that albumin strongly competes with OGG1 for the inhibitor molecule. This highlights the need for developing new formulations with a better pharmacokinetics profile to effectively target cancer *in vivo*.

In conclusion, our results in **Paper III** validates OGG1 a potential anti-cancer target. We reported structural insights on how TH5487 interacts with the active site of human OGG1. TH5487 treatment is selectively toxic to a large panel of cancer cells but not to normal immortalized cells. OGG1 inhibition induces replication stress as demonstrated by accumulation of γH2AX in S-phase cells. Importantly, TH5487 significantly reduces the replication fork speed. Moreover, TH5487 treatment downregulates a set of DNA replication genes altering the cellular transcriptional profile. Taken together, our findings suggest that OGG1 inhibition is a potential promising approach to target cancer cells.

2.3.4 Paper IV: Identifying potential backup repair pathways that may compensate for OGG1 inhibition

BER glycosylases show overlapping substrate specificity to some degree. This explains why knockout mice lacking a single glycosylase are viable and show no evident disease phenotype with exception to thymine DNA glycosylase whose deficiency results in embryonic lethality[128,249]. Since 8-oxoG lesions are readily oxidizable to spiroiminodihydantoin (Sp) and guanidinohydantoin (Gh) which are primarily recognized and excised by NEIL glycosylases, we were curious whether NEIL1 or NEIL2 can compensate for OGG1 inhibition. Although 8-oxoG is not one of their major substrates, both NEIL1 and NEIL2 have been reported to possess some activity towards this lesion in *in vitro* assays[221,223].

First, we excluded an off-target binding of TH5487 to NEIL1. No thermal stabilization of NEIL1 was detected in DSF assays upon incubation with TH5487. We then performed laser microirradiation experiments using U2OS cells stably expressing NEIL1-GFP or NEIL2-GFP to study NEIL1/2 recruitment kinetics. While an increased and prolonged recruitment of NEIL1-GFP to sites of laser-induced DNA damage was observed, NEIL-2 recruitment in OGG1 inhibitor treated cells was similar to that observed in control cells. However, NEIL2 accumulation at sites of DNA damage in TH5487-treated cells was more prolonged suggesting that NEIL1 and —to a lesser extent— NEIL2 are potentially recruited as backup for OGG1.

FRAP assays show that NEIL1-GFP nuclear mobility becomes lower after treating U2OS cells with TH5487 and menadione, an oxidizing agent known to induce the formation of 8-oxoG[171]. Consistently, NEIL1-chromatin binding increases after TH5487 treatment as shown by the results of an *in situ* extraction assay. Importantly, this chromatin retention occurs in a TH5487 dose-dependent manner.

To exclude the possibility that the observed phenotypes are due to an off-target effect of TH5487, we knocked down *OGG1* with siRNA in cells expressing NEIL1-GFP or NEIL2-GFP and challenged them with menadione to induce oxidative stress. Immunostaining results suggested that *OGG1* depletion in oxidatively stressed cells results in the accumulation of genomic 8-oxoG lesions. In addition, more retention of NEIL1-GFP and NEIL2-GFP at the chromatin was observed in those cells after *in situ* extraction suggesting that NEIL1 and NEIL2 may play a backup role to repair the accumulated oxidized guanine lesions following *OGG1* depletion.

Interestingly, the cellular viability of *NEIL1*- or *NEIL2*- siRNA knockdown cells after TH5487 treatment is slightly improved. This may be explained by the reduced incisions detected in those cells by staining for γH2AX which highlights that role of NEIL1 and NEIL2 in initiating BER of oxidized guanines. Notably, co-treatment of *NEIL1*- and *NEIL2*- depleted cells with menadione and TH5487 resulted in accumulation of more 8-oxoG lesions in DNA.

In summary, our work in **Paper IV** supports a potential backup role for NEIL1 and NEIL2 glycosylases in OGG1 inhibitor treated cells. This is supported by the altered recruitment of NEIL1—and to a lesser extent—NEIL2 in TH5487-treated cells. FRAP and *in situ* extraction assays reveal that NEIL1 and NEIL2 are more tightly bound to chromatin in oxidatively stressed cells after *OGG1* depletion and inhibition. Furthermore, the high level of genomic 8-oxoG lesions observed in siRNA *NEIL1*- and *NEIL2*-depleted cells after treatment with TH5478 and menadione suggests that NEIL1 and NEIL2 potentially compensate for OGG1 inhibition.

2.3.5 Paper V: Characterization of a small-molecule activator of OGG1

Having a fundamental role in repairing one of the most prevalent DNA lesions, OGG1 makes an attractive target for drug development. Small-molecule OGG1 inhibitors have been recently developed by us[315] and others[307] and showed promising potential applications in inflammation and cancer[315–318]. Interestingly, the scope of chemical entities targeting OGG1 is not confined to inhibitors. Recently, small-molecule activators of OGG1 have been reported further expanding the range of potential applications of modulating OGG1 activity[319,320]. In **Paper V**, we sought to investigate the mechanism of action of a class of small-molecule OGG1 activators.

Among a set of previously reported small-molecule OGG1 activators[319], TH10785 was found to strongly engage with OGG1 in DSF assay protecting it against thermal denaturation. To

assess the effect of TH10875 on OGG1 activity, we performed an *in vitro* fluorescence-based biochemical assay and followed the reaction progression. An enhancement in the reaction rate was observed in a TH10785 dose-dependent manner, with a maximal activity detected at 6.25 μM TH10785 corresponding to 4-folds of that obtained with DMSO. However, further increasing the concentration of the activator reduced the reaction rate progressively back to the control levels resulting in a bell-shaped activity profile.

Human OGG1 crystal structure in complex with TH10785 indicated that the molecule binds OGG1's active site. The activity of OGG1 active site mutants was not affected by TH10785 suggesting that it is critical for TH10785 to bind OGG1's active site to infleunce its biochemical activity. Molecular dynamic simulations based on the resolved crystal structure suggest a fast desorption of TH10785 from OGG1's surface. In line with this, studying TH10785 binding to OGG1 in a competitive fluorescence polarization assay supported a low affinity of TH10785 to OGG1. In living cells, CETSA confirmed that TH10785 modestly stabilizes OGG1 against thermal denaturation. Laser microirradiation experiments showed that TH10785 treatment resulted in an increased recruitment of OGG1-GFP to regions of DNA damage. Moreover, potassium bromate-induced oxidative damage in telomeric regions was repaired faster in cells treated with TH10785. Furthermore, FRAP assays showed that TH10785 treatment results in higher OGG1-GFP nuclear mobility in cells pre-challenged with potassium bromate, suggesting a faster dissociation of OGG1 from oxidized DNA and hence faster completion of BER.

Finally, we hypothesized that simultaneous treatment with TH10785 and PNKP inhibition will result in accumulation of a DNA 3′-phosphate intermediate. Since the nick lacks a 3′-OH, neither DNA polymerases nor ligases would further process this 3′-phosphate intermediate resulting in accumulation of DNA strand breaks. To test our hypothesis, we co-treated U2OS cells with TH10785 and PNKP inhibitor and assessed the levels of γH2AX and 53BP1 as markers for activated DNA damage response. Indeed, such co-treatment resulted in higher levels of γH2AX and 53BP1 as detected by immunofluorescence indicating that TH10785 treatment generates a DNA 3′-phosphate intermediate which requires the phosphatase activity of PNKP to be further processed.

In conclusion, we elucidated the mechanism of action of a small-molecule OGG1 activator in **Paper V** both *in vitro* and *in cellulo*. In the presence of TH10875, cells appear to be less dependent on APE1, and more dependent on PNKP to complete the repair process. OGG1 activation may have potential implications in conditions characterized by high levels of ROS

such as inflammation, cancer and aging among others. This novel concept of small-molecule activation opens new avenues to potentially establish new enzymatic functions in DNA repair enzymes, potentiate weak functions or recover lost ones through chemical intervention.

2.4 DISCUSSION AND FUTURE PERSPECTIVES

2.4.1 Drugging OGG1 glycosylase with small-molecule modulators

Each human cell is estimated to encounter approximately 70,000 DNA lesions per day[124]. 8-oxoG is among the most abundant lesions due to the low redox potential of guanine[60,61]. More oxidative DNA damage is generally induced in cancer due to oncogenic signaling, mitochondrial dysfunction, or aberrant metabolism. Oxidative stress is also involved in the pathogenesis of inflammation[41]. 8-oxoG has been the focus of extensive research efforts due to its abundancy and mutagenic properties. OGG1 is the major glycosylase that initiates BER of 8-oxoG. Having a well-characterized role in BER, OGG1 might make an attractive target for drug development.

No potent OGG1 inhibitors have been reported until very recently. DNA glycosylases were often viewed as almost undruggable targets. Targeting DNA-, RNA- and carbohydrate-binding proteins with small molecules has been described by Hajduk *et al*. as a challenging task because of the polar or charged nature of their binding pocket[305,306]. This turns out not to be valid for human DNA glycosylases since computational assessment of their druggability by Michel *et al.* reveals that those enzymes have high druggability scores and are indeed druggable[321].

Previous attempts to develop OGG1 inhibitors showed moderate success. Donley *et al.* identified five inhibitors from a high-throughput screen[322]. The identified inhibitors were hydrazide-containing molecules. They impaired the formation of Schiff base during OGG1-mediated catalysis. However, the molecules did not hinder OGG1 binding to 8-oxoG-containing substrates. Results reported by Donley *et al.*[322] suggest that the developed molecules inhibit both the glycosylase and the AP lyase activity of OGG1. However, it is more likely that the hydrazide inhibitors interfere with the AP lyase activity only. This is supported by the clear reduction in OGG1-induced strand cleavage of AP-site containing substrate and Schiff base inhibition after the compound treatment[322]. In line with this, in **Paper I** we did not observe any target engagement in the DSF assay upon incubating OGG1 with O8, one of the hydrazide inhibitors developed by Donley *et al.*[322]. Moreover, a reduced APE1 activity on an AP-site containing substrate pre-treated with O8 was detected in **Paper**

I suggesting that OGG1 might not be the sole target of O8. Nevertheless, the work of Donley *et al.*[322] helped pave the way towards more potent OGG1 inhibitors by establishing relevant screening and validation assays.

Recently, drug development efforts succeeded in developing more potent small-molecule inhibitors targeting OGG1[307,315]. In **Paper I**, we reported the development of TH5487, a potent small-molecule active site inhibitor of OGG1. TH5487 binds OGG1 in *in vitro* electrophoretic mobility shift assays hindering its binding to its DNA substrate lesion. Differential scanning fluorimetry (DSF) assays validate target engagement demonstrating that TH5487 stabilizes OGG1 *in vitro* protecting it against thermal denaturation. Consistently, cellular thermal shift assays (CETSA) confirmed target engagement in cells. In line with this, hydrogen-deuterium exchange mass spectrometry suggests that TH5487 targets the active site pocket of OGG1.

More structural insights were obtained from the crystal structure of mouse OGG1 in complex with TH5675, a more soluble analogue of TH5487, reported in **Paper I**. X-ray crystallography demonstrates that the molecule indeed targets OGG1's active site. This was further confirmed by resolving the crystal structure of human OGG1 in complex with TH5487 in **Paper III**. The resolved crystal structure showed that TH5487 binds OGG1's active site albeit in a different orientation than that of the 8-oxoG substrate. Importantly, human OGG1 conformation changes upon binding to TH5487 where it adopts a closed conformation which makes OGG1 active site inaccessible to its DNA substrate lesions. This may explain the increased nuclear OGG1 mobility detected in FRAP assays in **Paper I** and **Paper II** as well as the reduced OGG1-chromatin binding observed in *in situ* extraction assays reported in **Paper II**. Interestingly, Tahara *et al.* have recently reported dual inhibitors of MTH1 and OGG1 expanding the available portfolio of OGG1 inhibitors and adding a new tool to explore the role of both MTH1 and OGG1 in maintaining genomic integrity[323].

In **Paper V**, we provided additional proof of the druggability of OGG1. In this paper, we studied the mechanism of action of TH10785, a small-molecule activator of OGG1. Target engagement was confirmed by DSF. Importantly, the crystal structures of both mouse and human OGG1 in complex with TH10785 were solved where the molecule was found to bind OGG1's active site. Some similarities were observed between TH5487 and TH10785 binding orientation. For instance, the cyclohexane ring of TH10785 occupies a deep hydrophobic site of OGG1 flanked by Cys253, Leu256 and Met257 in a position similar to that of TH5487. This suggests that TH10785 binds to the active site as TH5487, albeit the two molecules have

a different mechanism of action. OGG1 activation may have potential implications in telomere maintenance, protecting against senescence and aging.

In conclusion, our work validates OGG1 as a druggable glycosylase either by small-molecule inhibitors (**Paper I, Paper II** and **Paper III**) or by small-molecule activators (**Paper V**). This provides the research community with new pharmacological tools to study OGG1's role in BER and beyond. Modulating the activity of OGG1 with small molecules can have several potential applications in pathologies where OGG1 is involved such as cancer or inflammation.

2.4.2 OGG1 inhibition as a strategy for treating inflammation

Despite its mutagenicity, human promoter regions are rich in GC content. Mounting evidence suggests that 8-oxoG is not only a mutagenic lesion but can serve as an epigenetic mark to regulate gene transcription[203,324]. A genome-wide analysis reported by Saxonov *et al.* classifies 72% of human gene promoters as regions of high GC content[325]. Furthermore, genes located in genomic regions with high GC content tend to be actively transcribed[326]. In inflammatory conditions, ROS-mediated signaling is involved in regulating the expression of proinflammatory genes[327–329]. Notably, the consensus binding motifs of transcription factors NF-κB and specificity factor (Sp1), major orchestrators of inflammatory responses, are rich in guanines[330,331]. In addition, OGG1 deficient mice show a dampened inflammatory response and a reduced chemokine and cytokine expression after being challenged with *H. pylori* or lipopolysaccharide (LPS)[184,185]. These observations suggested that OGG1 may play an atypical role in regulating gene transcription. Indeed, mounting evidence indicates that OGG1 and 8-oxoG are involved in modulating gene expression[332,333,208,111,203,204].

In **Paper I**, we hypothesized that OGG1 inhibition with TH5487 can have anti-inflammatory effects. In accordance with our hypothesis, human embryonic kidney (HEK) 293T deficient in OGG1 displayed lower expression of chemokine C-X-C motif ligand 1 (CXCL1) after TNF-α stimulation. This is in line with a similar phenotype reported previously after siRNA-mediated depletion of *OGG1* in mice airway epithelium, where lower levels of proinflammatory cytokines were detected after stimulation with a pollen extract allergen or TNF-α[109,334]. Importantly, TH5487 reduced CXCL1 expression following exposure to TNF-α in wild-type HEK 293T cells but not in *OGG1* knockout cells suggesting that the observed phenotype is due to OGG1 inhibition and not the result of an off-target effect of TH5487.

Challenging murine airway epithelial cells (MLE 12) with TNF-α induced the expression of large panel of proinflammatory cytokines as well as C-C and C-X-C chemokines. OGG1 inhibition with TH5487 suppressed the expression of the same inflammatory cytokines and

chemokines. The effect was noted to be TH5487 dose-dependent. Besides, similar observations were made after inducing an inflammatory response with LPS. Consistently, SU268, a structurally different OGG1 inhibitor reported by Tahara *et al.*[307], dampened cytokine expression in MLE 12 cells supporting OGG1's role in regulating proinflammatory gene transcription. Furthermore, TH5487 had comparable effects in human small-airway epithelial cells (hSAECs) exposed to TNF-α and LPS, where it suppressed the expression of C-X-C and C-C chemokines, as well as TNF and interleukin 6.

Mechanistically, TH5487 treatment reduced OGG1-chromatin binding in A3 cells as shown in FRAP assays reported in **Paper I**. A comparable effect was observed in FRAP assays and *in situ* extraction assays in **Paper II** in U2OS cells treated with TH5487 suggesting that TH5487 impairs OGG1-DNA binding. In addition, lower recruitment of OGG1 and NF-κB to promoter regions of proinflammatory cytokines was observed in **Paper I** in TNF-α stimulated cells treated with TH5487. This is in consistent with previous studies showing that OGG1-DNA interactions facilitate NF-κB binding to its response elements promoting the expression of NF-κB target genes[109,111,110]. The observed lower expression of proinflammatory cytokines in **Paper I** is attributed to lower NF-κB binding to the regulatory regions of the cytokines as a consequence of lower OGG1 recruitment. This is further supported our data showing that O8, an OGG1 inhibitor previously reported by Donley *et al.*[322], neither impaired OGG1 binding to its substrate DNA lesion nor affected gene transcription. TH5487 treatment did not inhibit the phosphorylation of RelA subunit of NF-κB excluding a direct effect of the inhibitor on NF-κB activity.

In vivo experiments reported in **Paper I** show that TH5487 is well-tolerated in mice. Proinflammatory cytokine expression was suppressed in TNF-α intranasally challenged mice when TH5487 was administered prophylactically. This was translated into reduced lung inflammation and lower neutrophil infiltration. When TH5487 was administered after stimulation with TNF-α, lower neutrophil count was observed in mice airways suggesting that the inhibitor interrupts ongoing inflammatory responses. In line with our findings, OGG1 inhibitor SU268 has been reported to suppress inflammatory responses and improve survival of mice during *Pseudomonas aeruginosa* infection demonstrating that OGG1 inhibition can have favourable anti-inflammatory effects[335].

Numerous inflammatory conditions are currently treated with corticosteroids systemically and topically[336,337]. Using OGG1 inhibitors as an anti-inflammatory strategy can have some advantages over steroids. While topically administered steroids might cause skin atrophy and

mucocutaneous infections as side effects[338], long-term oral administration of steroids can result in osteoporosis, hyperglycaemia, adrenal suppression and immunosuppression[337,339]. Although long-term administration of TH5487 is yet to be examined, it is unlikely to cause similar side effects since it has a different mechanism of action. By suppressing the expression of a wide array of proinflammatory cytokines, OGG1 inhibition might show higher efficacy. Furthermore, OGG1 inhibitors may result in a favourable anti-inflammatory effect when administered prophylactically. One potential side effect of using OGG1 inhibitors might be inducing mutagenesis arising from G:C → T:A transversions. This needs to be carefully examined in future research.

Taken together, our findings in **Paper I** indicates that OGG1 plays a key role in regulating gene transcription in inflammatory conditions. We provided proof of concept that inhibiting OGG1 can have broader implications beyond BER. We introduced to the scientific community a novel class of potent and selective OGG1 inhibitors that possess a promising anti-inflammatory potential. Future research is recommended to further characterize the efficacy and safety of OGG1 inhibitors in different inflammatory disease models.

2.4.3 Targeting OGG1 as an anti-cancer approach

Production of reactive oxygen species (ROS) is inherently augmented in cancer cells due to a number of factors including oncogene expression such as *MYC* and *RAS*, increased metabolic rate and mitochondrial dysfunction[312,340,313,43,39]. Such oxidative stress results in accumulation of oxidative DNA lesions in cancer cells[341,342]. 8-oxoguanine lesions, the major substrate for OGG1, are among the most abundant oxidative lesions[343,124,125]. This prompted us to investigate whether OGG1 inhibitor, TH5487, exerts an anti-cancer effect.

In **Paper III**, we validated OGG1 as a potential anti-cancer target. Knocking down *OGG1* with siRNA affected cell viability and colony formation of oncogene-expressing cells but not immortalized ones. Similarly, shRNA-mediated depletion of *OGG1* affected A3 cancer cell proliferation *in vitro* and *in vivo.* Importantly, OGG1 inhibition with TH5487 impaired the cellular proliferation of a broad panel of cancer cells belonging to different tissues of origin but was well-tolerated by normal immortalized cells. This observed therapeutic window can have favourable implications as it may allow to specifically target cancer cells or reduce toxic side effects in normal healthy cells. The viability effects reported in **Paper III** might highlight a potential cancer cell addiction to a functional OGG1 which may be the reason why *OGG1* knockout mice are mostly cancer free[344]. This may also explain the relative low

frequency of C → A mutation signature in human tumor cells despite the role of oxidative stress in promoting tumorigenesis[345].

One method to evaluate the efficacy of OGG1 small-molecule modulators is to assess the level of 8-oxoG. The level of 8-oxoguanine has been suggested as a prognostic biomarker for cancer risk or as well as a predictive biomarker for sensitivity to cancer therapy[346–349]. However, absolute quantification of 8-oxoguanine proves challenging from a technical point of view[350–353]. To reach a more conclusive picture regarding the level of 8-oxoG, it is recommended to combine different detection methods. Accordingly, we examined the level 8-oxoG using distinct methods namely: LC-MS/MS (**Paper I** and **Paper III**), immunostaining (**Paper II**, **Paper IV** and **Paper V**), modified comet assay (**Paper III** and **Paper V**) and qPCR-based assay (**Paper III** and **Paper V**). Inducing oxidative stress with oxidizing agents such as potassium bromate[354,355] and menadione[171] has been reported to drive the formation of oxidized guanine lesions. Challenging cells with such agents prior to or together with TH5487 shows that the inhibitor treatment impairs the repair of genomic 8-oxoG lesions as detected by LC-MS/MS and immunostaining (**Paper I**, **Paper II**, **Paper IV**, and **Paper V)**. This is in agreement with the results of the *in vitro* biochemical activity assay reported in **Paper I** demonstrating a lower activity of OGG1 after TH5487 treatment. In addition, it is supported by the lower OGG1 recruitment to laser-induced DNA damage regions observed in **Paper II** after TH5487 treatment.

Unexpectedly, the absolute level of 8-oxoG remained close to the background level in A3 cells after treatment with TH5487 alone as detected by LC-MS/MS although modified comet assay did reveal more strand breaks after incubation with purified OGG1 (**Paper III**). This suggests that OGG1 substrates other than 8-oxoG might be accumulating and become detectable using the modified comet assay. Accordingly, TH5487-induced proliferation defect might be—at least partly—not a direct consequence of 8-oxoG accumulation in DNA questioning how applicable it is to employ the total level of 8-oxoG as a biomarker in cancer research. 8-oxoG has been reported to be enriched in certain genomic regions rather than having a random distribution in the genome. For instance, the promoter regions as well as 5′- and 3′-untranslated regions of murine embryonic fibroblasts (MEF) and human non-tumorigenic epithelial breast cells (MCF10A) are rich in 8-oxoG[356–358]. This enrichment was significantly increased after knocking out *OGG1*[356]. In line with this, more 8-oxoG lesions were detected in the SP1-binding motif at the promoter regions of *MCM4*, a DNA replication licensing factor. Since irrelevant or misleading results might be obtained after measuring the

level of 8-oxoG in the entire genome[359], studying of 8-oxoG enrichment at certain genomic regions using sensitive methods is recommended.

Several observations were reported in **Paper III** explaining the TH5487-induced proliferation arrest. Gene expression profiling of TH5487-treated A3 cells revealed downregulation of DNA replication genes. This contributed to replication stress, which manifested as γH2AX accumulation in S-phase cells. Furthermore, replication fork progression was markedly impaired in TH5487-treated cells suggesting that OGG1 inhibition induces replication stress. The proliferation arrest induced by OGG1 inhibition or depletion was observed to be reversible as the cells resumed normal division after replacing the cell culture medium with a fresh one. This suggests that TH5487 is cytostatic rather than cytotoxic.

OGG1 inhibitors might offer some degree of selectivity in targeting cancer cells since there was a large therapeutic window observed when determining the median effective concentration (EC_{50}) of TH5487 in cancer cell lines and non-transformed cells. This is likely due to the high level of replication and oxidative stress encountered in cancer cells[39,360]. This selectivity might offer an advantage over typical chemotherapeutics and thus can potentially lead to fewer adverse effects by sparing normal cells.

Cytostatic drugs might not be effective as monotherapy. Accordingly, TH5487 might be combined with other anti-cancer drugs. In this regard, a synergistic effect has been observed between TH5487 and methotrexate[318]. This is explained in light of the ability of methotrexate to induce ROS production[361–363]. Radiotherapy as well as some classical chemotherapeutics such as platinum drugs and anthracyclines are reported to induce oxidative stress and oxidative DNA lesions[364–367]. Consequently, one might rationalize that combining those therapies with TH5487 might have a favourable anti-cancer outcome. The same might be expected on combining TH5487 with MTH1 inhibitors as some inhibitors which belong to the latter group have been shown to induce mitotic arrest and 8-oxoG accumulation in DNA[49,290,292]. Whether such combinations are beneficial remains to be validated in future research. Combining OGG1 inhibitors with radio- or chemotherapeutics can have a potential synergistic outcome. By impairing the repair of the induced DNA damage, OGG1 inhibitors may potentiate the effect of the combined DNA damaging agent. However, one needs to carefully consider dose-limiting toxicities that may arise from such combinations.

BER glycosylases have some degree of overlapping substrate specificity[128,247]. While 8-oxoG is not the major substrate for NEIL1 and NEIL2 glycosylases, Parsons *et al.*[223] and Wallace

et al.[221] reported a weak activity for NEIL1 and NEIL2 respectively, towards this lesion. In **Paper IV**, we observed an increased recruitment of NEIL1 to laser-induced DNA damage regions as well as prolonged accumulation of NEIL2 at those sites in U2OS cells treated with TH5487. Besides, NEIL1- and NEIL2-chromatin binding increased after OGG1 inhibition or depletion. Notably, the level of genomic 8-oxoG increased in *NEIL1*- and *NEIL2*- depleted cells. Taken together, our results suggest a compensatory role for NEIL1 and NEIL2 upon functional loss of OGG1. This might have implications on the efficacy of OGG1 inhibitors especially in tumors with high expression levels of NEIL1 or NEIL2.

To our knowledge, no role for NEIL1 or NEIL2 glycosylases in transcription regulation have been identified so far. By acting on Sp and Gh lesions generated upon further oxidation of 8-oxoG, it is not likely that NEIL glycosylases may compensate for OGG1's role in mediating the transcription of proinflammatory mediators and hence the backup role of NEIL1/2 was not observed in **Paper I**. Furthermore, this backup role was not observed in **Paper III** which might reflect a suboptimal efficiency of NEIL1 or NEIL2 in repairing the accumulated damage. Alternatively, the compensatory function of NEIL glycosylases might be accompanied with inefficient processing of the downstream BER intermediates. Furthermore, the compensatory role of NEIL1 or NEIL2 might have been undetectable in **Paper III** owing to the low level of genomic 8-oxoG lesions detected by LC-MS/MS in A3 cells following TH5487 treatment.

Pharmacokinetic profiling of TH5487 in **Paper I** reveals that the molecule is well-tolerated in mice. However, oral administration of TH5487 in subcutaneous A3 xenograft mice in **Paper III** did not result in xenograft growth arrest. No target engagement was observed in the xenograft cells. Binding to serum album proteins can be—at least partly—the reason behind the non-detectable target engagement and hence the absent *in vivo* efficacy. The absorption, distribution, metabolism, and excretion (ADME) profile of TH5487 reported in **Paper I** reveals very high plasma protein binding. In line with this, OGG1 inhibition *in vitro* was significantly reduced after incubating TH5487 with bovine serum albumin proteins suggesting that the molecule has high affinity to albumin proteins. This was not observed in *in vivo* experiments reported in **Paper I** where the molecule was administered intraperitoneally. New formulations with improved pharmacokinetic properties are thus needed to be able to effectively target cancer cells via systemic administration. Alternatively, TH5487 *in vivo* efficacy should be evaluated preclinically in different mouse models using different routes of drug administration.

2.4.4 Concluding remarks

OGG1-initiated base excision repair has been the focus of a great number of studies, yet there is still a pressing need to improve our understanding of the function of OGG1, particularly regarding its role in tumor cells as well as how it modulates gene transcription. In this thesis, we evaluate the therapeutic potential of modulating the activity of OGG1 in cancer and inflammation models via small-molecule intervention. We report the development of TH5487, a potent active site inhibitor of OGG1. We show that OGG1 inhibition is a promising anti-inflammatory strategy that results in the downregulation of a broad panel of proinflammatory cytokines. Furthermore, we provide evidence that OGG1 inhibition induces replication stress and proliferation arrest in cancer cells and thus suggests OGG1 as a novel anti-cancer target. Moreover, we characterize TH10785, a small-molecule OGG1 activator that introduces a novel enzymatic function not reported in native OGG1.

As for the future of OGG1 inhibitors, characterization of the anti-inflammatory effect of TH5487 in preclinical models of inflammation other than LPS- and TNF-α induced lung inflammation is currently being sought. Importantly, we are currently working towards optimizing the ADME properties of OGG1 inhibitors particularly their solubility and affinity to plasma proteins to enhance their target engagement *in vivo*. This is to be followed up by evaluating the tolerability and efficacy of the new optimized compounds in *in vivo* cancer models. Identifying synergistic combination partners for OGG1 inhibitors may help bring them closer to the clinic.

Of particular interest is investigating the effect of the OGG1 activator in *in vivo* physiologically relevant models. For instance, enhancing the activity OGG1 after TH10785 treatment might prove beneficial in repairing aging-associated DNA damage. Ongoing medicinal chemistry efforts are directed towards developing more potent OGG1 activators. Using small molecules to introduce new biological functions in existing enzymes could potentially also be applicable to many other proteins and therefore could open up a whole new area of drug discovery.

TH5487 and TH10785 represent potent small-molecule OGG1 modulators that can be employed to manipulate OGG1 activity and uncover new OGG1 biology in physiologically relevant context.

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