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ROLES OF NUDT5 AND NUDT15 BEYOND OXIDIZED NUCLEOTIDE SANITATION AND THEIR POTENTIAL AS THERAPEUTIC TARGETS

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Roles of NUDT5 and NUDT15 beyond oxidized nucleotide sanitation and their potential as therapeutic targets

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To family, friends, colleagues and my wonderful girlfriend, Simin, for their support and for accompanying me on this long and winding science odyssey.

ABSTRACT

The nucleotide precursor pool is readily susceptible to numerous sources of modification and damage, including alkylation, deamination and oxidation/nitrosylation, among others; most of which have deleterious effects on nucleic acid integrity and cellular fitness. In addition to DNA repair mechanisms, these metabolic byproducts are kept in check by so-called sanitation or “housekeeping” enzymes, chief among them the NUDIX hydrolase superfamily. Increased metabolic demand and strain in certain contexts, such as cancer, may require a greater reliance on these proteins; therefore, they are attractive drug targets.

The human NUDIX hydrolase, MTH1 (NUDT1), sanitizes the nucleotide pool of 8-oxo-guanine triphosphates, considered the most common oxidative lesion, thereby preventing mutagenesis of nucleic acids and preserving their integrity. Other NUDIX enzymes, namely NUDT15 (MTH2) and NUDT5, are proposed to perform similar functions as MTH1, and, therefore, may serve as resistance mechanisms for cells treated with MTH1 inhibitors. However, very little is known about their biological functions in human cells.

The focus of this thesis was to determine the biological roles of NUDT15 and NUDT5 and if they are desirable drug targets for treating cancer. Surprisingly, we found that neither of these proteins appeared to be important for oxidized nucleotide metabolism, but, rather, they had unexpected and diverse functions in nucleotide metabolism with cancer therapeutic implications. These findings should encourage further study of the human NUDIX family.

In **Paper I**, we compared NUDT15 biochemically, structurally and in a cellular context to MTH1. NUDT15 hydrolyzed 8-oxo-dGTP about 230-fold less efficiently than MTH1, and its depletion in cancer cells neither affected cell survival nor oxidized nucleotide content of DNA. The NUDT15 crystal structure explained this deviation from MTH1 and shows that 8-oxo-dGTP is poorly accommodated in the enzyme active site. We also identified 6-thio-(d)GTP, the active metabolites of thiopurine chemotherapeutics, as NUDT15 substrates.

In **Paper II**, we expounded upon the role of NUDT15 in thiopurine metabolism and why the R139C missense mutant causes thiopurine intolerance in patients. NUDT15 efficiently hydrolyzes 6-thio-(d)GTP, thus mediating the amount of the active thiopurine metabolites in cells. In addition, the R139C mutation does not impact catalytic ability of NUDT15, but rather causes destabilization of the protein structure and proteolytic degradation in cells, thus explaining why patients with this mutation are sensitive to thiopurine treatments.

Paper III presents further evidence that NUDT5 may not be an important contributor to sanitation of the oxidized nucleotide pool, describes the first small molecule NUDT5 inhibitors and confirms the nuclear ATP synthetic role for NUDT5 in breast cancer cells. Following an initial screening campaign and medicinal chemistry efforts, potent, cell-active NUDT5 inhibitors were identified using a CETSA-guided screening funnel. Lead compound, TH5427, abrogated progestin-dependent gene regulation and proliferation in breast cancer cells, thus, representing a bonafide probe to further study NUDT5 biology.

LIST OF SCIENTIFIC PUBLICATIONS

THESIS PUBLICATIONS

- I. Carter, M.*, Jemth, A. S.*, Hagenkort, A.*, Page, B. D. G.*, Gustafsson, R., Griese, J. J., Gad, H., **Valerie, N. C. K.**, Desroses, M., Boström, J., Warpman Berglund, U., *Helleday, T.*, and *Stenmark, P.* (2015) **Crystal structure, biochemical and cellular activities demonstrate separate functions of MTH1 and MTH2.**
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- II. **Valerie, N. C. K.***, Hagenkort, A.*, Page, B. D. G., Masuyer, G., Rehling, D., Carter, M., Bevc, L., Herr, P., Homan, E., Sheppard, N. G., *Stenmark, P.*, *Jemth, A. S.**, and *Helleday, T.* (2016) **NUDT15 Hydrolyzes 6-Thio-DeoxyGTP to Mediate the Anticancer Efficacy of 6-Thioguanine.**
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- III. *Page, B. D. G.**, **Valerie, N. C. K.***, Wright, R. H. G., Wallner, O., Isaksson, R., Carter, M., Rudd, S. G., Loseva, O., Jemth, A.-S., Almlöf, I., Font-Mateu, J., Llona-Minguez, S., Baranczewski, P., Jeppsson, F., Homan, E., Almqvist, H., Axelsson, H., Regmi, S., Gustavsson, A.-L., Lundbäck, T., Scobie, M., Strömberg, K., Stenmark, P., Beato, M., and *Helleday, T.* (2018) **Targeted NUDT5 inhibitors block hormone signaling in breast cancer cells.**
Nat Commun **9**, 250

OTHER PUBLICATIONS

- IV. *Llona-Minguez, S.**, Höglund, A.*, Jacques, S. A., Johansson, L., Calderón-Montaño, J. M., Claesson, M., Loseva, O., **Valerie, N. C. K.**, Lundbäck, T., Piedrafita, J., Maga, G., Crespan, E., Meijer, L., Burgos Morón, E., Baranczewski, P., Hagbjörk, A. L., Svensson, R., Wiita, E., Almlöf, I., Visnes, T., Jeppsson, F., Sigmundsson, K., Jensen, A. J., Artursson, P., Jemth, A. S., Stenmark, P., Warpman Berglund, U., Scobie, M., and *Helleday, T.* (2016) **Discovery of the First Potent and Selective Inhibitors of Human dCTP Pyrophosphatase 1.**
J Med Chem **59**, 1140-1148
- V. *Rudd, S. G.**, **Valerie, N. C. K.***, and *Helleday, T.* (2016) **Pathways controlling dNTP pools to maintain genome stability.**
DNA Repair (Amst) **44**, 193-204 (Review)
- VI. *Herold, N.**, Rudd, S. G.*, Ljungblad, L., Sanjiv, K., Myrberg, I. H., Paulin, C. B., Heshmati, Y., Hagenkort, A., Kutzner, J., Page, B. D. G., Calderón-Montaño, J. M., Loseva, O., Jemth, A. S., Bulli, L., Axelsson, H., Tesi, B., **Valerie, N. C. K.**, Höglund, A., Bladh, J., Wiita, E., Sundin, M., Uhlin, M., Rassidakis, G., Heyman, M., Tamm, K. P., Warpman-Berglund, U., Walfridsson, J., Lehmann, S., Grandér, D., Lundbäck, T., Kogner, P., Henter, J. I., Helleday, T., and *Schaller, T.* (2017) **Targeting SAMHD1 with the Vpx protein to improve cytarabine therapy for hematological malignancies.**
Nat Med **23**, 256-263

- VII. *Llona-Minguez, S.**, Höglund, A.*, Ghassemian, A., Desroses, M., Calderón-Montaño, J. M., Burgos Morón, E., **Valerie, N. C. K.**, Wiita, E., Almlöf, I., Koolmeister, T., Mateus, A., Cazares-Körner, C., Sanjiv, K., Homan, E., Loseva, O., Baranczewski, P., Darabi, M., Mehdizadeh, A., Fayezi, S., Jemth, A. S., Warpman Berglund, U., Sigmundsson, K., Lundbäck, T., Jenmalm Jensen, A., Artursson, P., Scobie, M., and *Helleday, T.* (2017) **Piperazin-1-ylpyridazine Derivatives Are a Novel Class of Human dCTP Pyrophosphatase 1 Inhibitors.** *J Med Chem* **60**, 4279-4292

Italics – corresponding authorship; * – equal contribution

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LIST OF ABBREVIATIONS

Genes and proteins

53BP1	Tumor protein p53 binding protein 1
AKT	Protein kinase B
ASCOM	ASC-2/NCOA6 complex
ASCT2	Amino acid transporter 2
BAF	SWI/SNF complex; Switch/Sucrose Non-Fermentable
CCND1	Cyclin D1
CDA	Cytidine deaminase
CDK2	Cyclin-dependent kinase 2
CDK4/6	Cyclin-dependent kinase 4/cyclin-dependent kinase 6
DCTPP1	Deoxycytosine triphosphate pyrophosphatase 1; dCTPase
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
EGFR	Epidermal growth factor receptor
Endo III	Endonuclease III
ER	Estrogen receptor
FPG	Formamidopyrimidine DNA glycosylase
GATA3	GATA binding protein 3
GEF	Guanine nucleotide exchange factor
GLS1	Glutaminase 1
GLUT1	Glucose transporter 1
GTPase	Guanosine triphosphatase
HMG1/2	High mobility group B 1/2
HP1 γ	Heterochromatin protein 1 gamma; CBX3
HPRT	Hypoxanthine phosphoribosyltransferase
IFN- γ	Interferon gamma
ITPase	Inosine triphosphate pyrophosphatase; ITPA
KDM5	Lysine demethylase 5; JARID1
Ku70	Thyroid autoantigen 70kDa; XRCC6
Ku86	Thyroid autoantigen 80kDa; XRCC5
MAP3K1	Mitogen-activated protein kinase kinase kinase 1
MAPK	Mitogen-activated protein kinase
MDM2	Mouse double minute 2, human homolog of
MMTV-luc	Mouse mammary tumor virus-luciferase
MSH2	MutS homolog 2
mTOR	Mammalian target of rapamycin
MUTYH	MutY homolog
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NMNAT1	Nicotinamide nucleotide adenyltransferase 1
NT5C2	5'-nucleotidase, cytosolic II
NTHL1	Endonuclease III-like protein 1
NucE	Nucleosome E
NUDIX	Nucleoside diphosphate linked to some other moiety, X
NUDT1/MTH1	NUDIX type 1; MutT homolog 1
NUDT15/MTH2	NUDIX type 15; MutT homolog 2
NUDT18/MTH3	NUDIX type 18; MutT homolog 3
NUDT5	NUDIX type 5; NUDIX5
NURF	Nucleosome remodeling factor complex
OGG1	Oxidized guanine glycosylase 1
PARG	PolyADP-ribose glycohydrolase
PARP1	PolyADP-ribose polymerase 1
PCAF	P300/CBP-associated factor; KAT2B; lysine acetyltransferase 2B
PCNA	Proliferating cell nuclear antigen
PI3K	Phosphoinositide-3-kinase
PIK3CA	Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic alpha
PPAT	Phosphoribosyl pyrophosphate amidotransferase

PR	Progesterone receptor
Rb	Retinoblastoma
RPA	Replication protein A2
RTK	Receptor tyrosine kinase
SAMHD1	SAM and HD domain-containing nucleoside triphosphohydrolase 1
SN2	System N transporter 2
SOD1	Superoxide dismutase 1
TNF- α	Tumor necrosis factor alpha
TOPOII β	DNA topoisomerase II beta
TP53	Tumor protein p53
TPMT	Thiopurine methyltransferase
TRPM2	Transient receptor potential cation channel subfamily M member 2
TS	Thymidylate synthase
UNG	Uracil DNA glycosylase
γ H2A.X	Histone H2A, variant X, phosphorylated at Ser139

Metabolites

(d)ITP	(2'-deoxy)inosine-5'-triphosphate
(d)XTP	(2'-deoxy)xanthosine-5'-triphosphate
2-OH-A	2-hydroxyadenine
2-OH-dADP	2-hydroxy-2'-deoxyadenosine-5'-diphosphate
2-OH-dATP	2-hydroxy-2'-deoxyadenosine-5'-triphosphate
2-thio-dTTP	2-thio-2'-deoxythymidine-5'-triphosphate
5-aza-dCTP	5-aza-2'-deoxycytosine-5'-triphosphate; decitabine triphosphate
5-COH-dC	5-formyl-2'-deoxycytosine
5-COH-dUDP	5-formyl-2'-deoxyuridine-5'-diphosphate
5-COH-U	5-formyluridine
5-Me-dCTP	5-methyl-2'-deoxycytosine-5'-triphosphate
5-OH-C	5-hydroxycytidine
5-OH-dCTP	5-hydroxy-2'-deoxycytosine-5'-triphosphate
5-OHMe-dC	5-hydroxymethyl-2'-deoxycytosine
6-MP	6-mercaptopurine; mercaptopurine
6-MTIMP	6-methylthioinosine-5'-monophosphate
6-MTITP	6-methylthioinosine-5'-triphosphate
6-TG	6-thioguanine; thioguanine
6-TGMP	6-thio-guanosine-5'-monophosphate
6-thio-(d)GTP	6-thio(-2'-deoxy)guanosine-5'-triphosphate
6-TIMP	6-thioinosine-5'-monophosphate
6-TITP	6-methylthio-inosine-5'-triphosphate
8-oxo-(d)GTP	8-oxo-7,8-dihydroxy(-2'-deoxy)guanosine-5'-triphosphate
8-oxo-dADP	8-oxo-7,8-dihydroxy-2'-deoxyadenosine-5'-diphosphate
8-oxo-dGDP	8-oxo-7,8-dihydroxy-2'-deoxyguanosine-5'-diphosphate
8-oxo-G	8-oxo-7,8-dihydroxyguanine; 8-oxo-guanine
ADPR	Adenosine diphosphate ribose; ADP-ribose
AMP	Adenosine-5'-monophosphate
araC	Cytosine arabinoside; cytarabine
ATP	Adenosine-5'-triphosphate
AZA-T	Azathioprine
BrdU	5-bromo-2'-deoxyuridine
dUMP	2'-deoxyuridine-5'-monophosphate
dUTP	2'-deoxyuridine-5'-triphosphate
FUdR	5-fluoro-2'-deoxyuridine; floxuridine
m ⁷ GDP	7-methylguanosine-5'-diphosphate
m ⁷ GMP	7-methylguanosine-5'-monophosphate
6-MT-dG	6-methylthio-2'-deoxyguanosine
NAD	Nicotinamide adenine dinucleotide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate

O ⁶ -Me-dG	O ⁶ -methyl-2'-deoxyguanosine
O ⁶ -Me-dGTP	O ⁶ -methyl-2'-deoxyguanosine-5'-triphosphate
OAADPR	<i>O</i> -acetyl-adenosine diphosphate ribose; <i>O</i> -acetyl-ADP-ribose
PAR	PolyADP-ribose
P _i	Inorganic phosphate
PP _i	Inorganic pyrophosphate
PRPP	Phosphoribosyl pyrophosphate
R5P	Ribose-5-phosphate
SAM	S-adenosylmethoinine
TGN	Thioguanine nucleotide

Other

ALL	Acute lymphoblastic leukemia
ALS	Alkali-labile site
AML	Acute myeloid leukemia
AP	Apurinic/aprimidinic site
BER	Base excision repair
CBCS	Chemical Biology Consortium Sweden
CETSA	Cellular thermal shift assay
ChIP	Chromatin immunoprecipitation
CRISPR	Clustered regularly interspaced short palindromic repeats
DARTS	Drug affinity responsive target stability
DCIS	Ductal carcinoma <i>in situ</i>
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSB	DNA double-strand break
DSF	Differential scanning fluorimetry
ECAVG	European Comet Assay Validation Group
ERE	Estrogen response element
ESCODD	European Standards Committee on Oxidative DNA Damage
GC-MS	Gas chromatography-mass spectrometry
gRNA	Guide ribonucleic acid
HPLC	High-performance liquid chromatography
HR	Hormone receptor
HSV	Herpes simplex virus
IR	Ionizing radiation
IBD	Inflammatory bowel disease
ITDRF	Isothermal dose-response fingerprint
LCBKI	Laboratories for Chemical Biology at Karolinska Institutet
MG assay	Malachite green assay
MMR	Mismatch repair
MMS	Methyl methanesulfonate
MNNG	<i>N</i> -methyl- <i>N'</i> -nito- <i>N</i> -nitrosoguanidine
mRNA	Messenger ribonucleic acid
NHEJ	Non-homologous end joining
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RNAi	Interfering ribonucleic acid
ROS	Reactive oxygen species
siRNA	Small interfering ribonucleic acid
SSB	DNA single-strand break
T _{agg}	Aggregation temperature
TBS	Tris-buffered saline
T _m	Melting temperature
TPP	Thermal proteome profiling
WT	Wild-type

1 INTRODUCTION

1.1 CANCER AS A METABOLIC DISEASE

1.1.1 *Oncogenes promote the cancer metabolism phenotype*

Cancer was first described as a metabolic disease by Otto Warburg nearly a century ago¹. Warburg and colleagues made the initial observation that cancer cells consumed far more glucose than cells originating from normal tissue, and the glucose consumed by cancer cells was predominantly converted to lactate by aerobic glycolysis², a vastly inefficient energetic process compared to oxidative phosphorylation. For this reason, the preference for aerobic glycolysis in cancer cells was puzzling for a number of years, until the realization that cancer cell proliferation depends on utilizing nutrients to generate biomass and the preference for aerobic glycolysis is a secondary consequence³. Today, it is generally accepted that carcinogenesis is initiated by activated oncogenes (and loss of tumor suppressors), which drive the development of many of the phenomena associated with the hallmarks of cancer⁴, including cancer cell energy metabolism⁵ (**Figure 1**). Nonetheless, the Warburg Effect, or the reliance of cancer cells on aerobic glycolysis, is a fundamentally important observation in our understanding of the metabolic reprogramming that occurs in cancer cells.

Proto-oncogenes, such as Myc, RAS and phosphoinositol-3-kinase (PI3K)⁶⁻⁸, drive the expression of genes that induce proliferation, invasion, metabolic re-circuiting and resistance to apoptosis^{9,10}. Due to their growth rate, cancer cells are highly dependent on the fundamental building blocks required for biomacromolecule synthesis: glucose (as mentioned above) and glutamine, which meets metabolic needs for nucleotide, protein, lipid, and nicotinamide adenine dinucleotide phosphate (NADPH) synthesis¹¹. To sustain this high level of proliferation, gain-of-function mutations within the receptor tyrosine kinase (RTK)/PI3K/protein kinase B (*AKT*)/mammalian target of rapamycin (*mTOR*) regulatory axis increase expression of glucose transporter 1 (*GLUT1*) on the cell membrane and, thus, directly increase the inward flux of glucose far in excess of ordinary cellular requirements¹²⁻¹⁵. As a result of this influx, oncogenically transformed cells have a reprogrammed metabolism that can favor glucose utilization *via* glycolysis¹⁶. While glucose uptake mechanisms in cancer cells are fairly well defined, the oncogenic mechanisms causing glutamine influx are less concrete. That being said, Myc has been shown to play a principal role in amino acid transporter 2 (*ASCT2*) and system N transporter 2 (*SN2*) expression, which directly regulate the amount of glutamine uptake by the cell¹⁷⁻¹⁹, and upregulate expression of glutamine-metabolizing enzymes, which encourages the flow of glutamine through the transporters^{5,9}. On the other hand, loss of the Retinoblastoma (*Rb*) tumor suppressor family of proteins causes glutamine influx *via* E2F-dependent expression of *ASCT2* and glutaminase 1 (*GLS1*), indicating that both activation of oncogenes and loss of tumor suppressors contribute to glutamine addiction in cancer²⁰. Altogether, these data suggest that to maintain viability in a proliferative state, proto-oncogenes are wired to increase nutrients available to generate biomass, but at what cost?

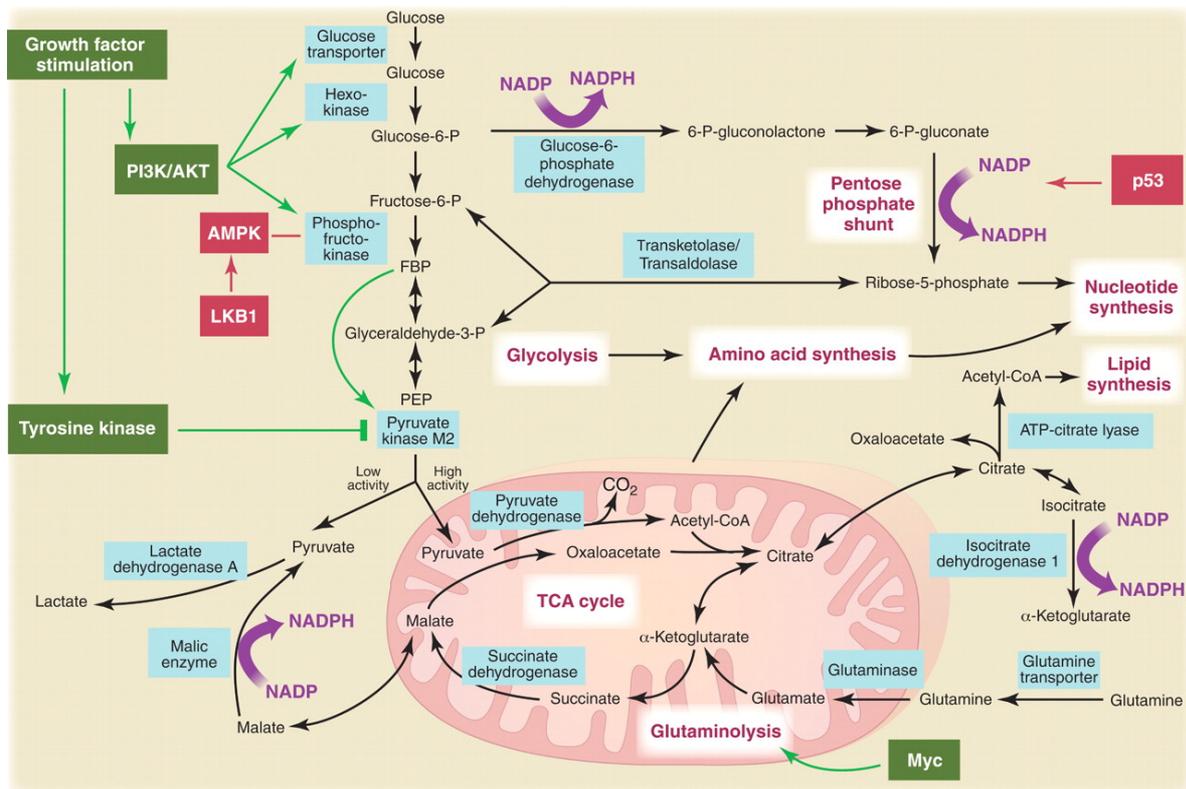
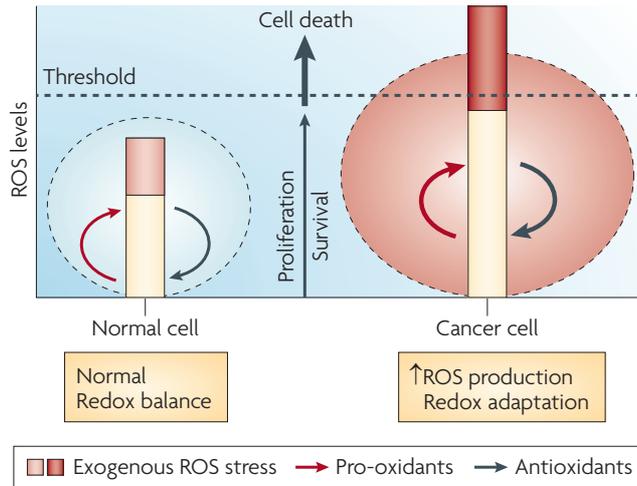


Figure 1: Oncogenes and tumor suppressors regulating metabolic pathways responsible for the Warburg effect. Oncogenes are shown in green, tumor suppressors in red, key metabolic pathways are labeled in purple with white boxes, and the enzymes controlling critical steps in these pathways are shown in blue. Reprinted with permission from the American Association for the Advancement of Science (AAAS), from Vander Heiden, Thompson and Cantley³.

1.1.2 Reactive oxygen species (ROS) and redox balance in cancer

A topic of debate for many years has revolved around the subject of reactive oxygen species (ROS) and why, or if, there are generally higher levels in cancer cells. ROS, in the form of free radical, ionic or non-radical oxygen species, are formed *via* mitochondria and peroxisomes as a byproduct of proliferation and are counterbalanced by ROS scavengers found predominantly in the cytoplasm²¹. Typically, ROS perform essential biological functions within cells (signaling related to growth, differentiation, transcription, etc.) when present at tolerable levels; however, if ROS leak into the mitochondrial periphery, their intrinsic reactivity causes detrimental oxidation of proteins, lipids and ribonucleic/deoxyribonucleic acids (RNA/DNA)^{21,22}. Several oncogenes are capable of inducing ROS in transformed cells²³⁻²⁶ and, in some instances, the ROS produced is required for the tumorigenic potential of the oncogene²⁷. However, proto-oncogenes, such as RAS, can control expression of antioxidants that can suppress high ROS in cancer cells²⁸, thereby permitting tumorigenesis and suggesting that a majority of cancer cells do have high intrinsic oxidative stress that is kept in check by ROS scavengers^{21,22,24}. As such, the redox balancing point in cancer cells is likely shifted upwards, due to increased ROS production and subsequent elimination by scavengers²² (**Figure 2a**), implying that abrupt shifts may cause selective cell death. Therefore, specific targeting of ROS production²⁹ and metabolism^{30,31} is a current area of intense research that has yielded promising results (**Figure 2b**).

a



b

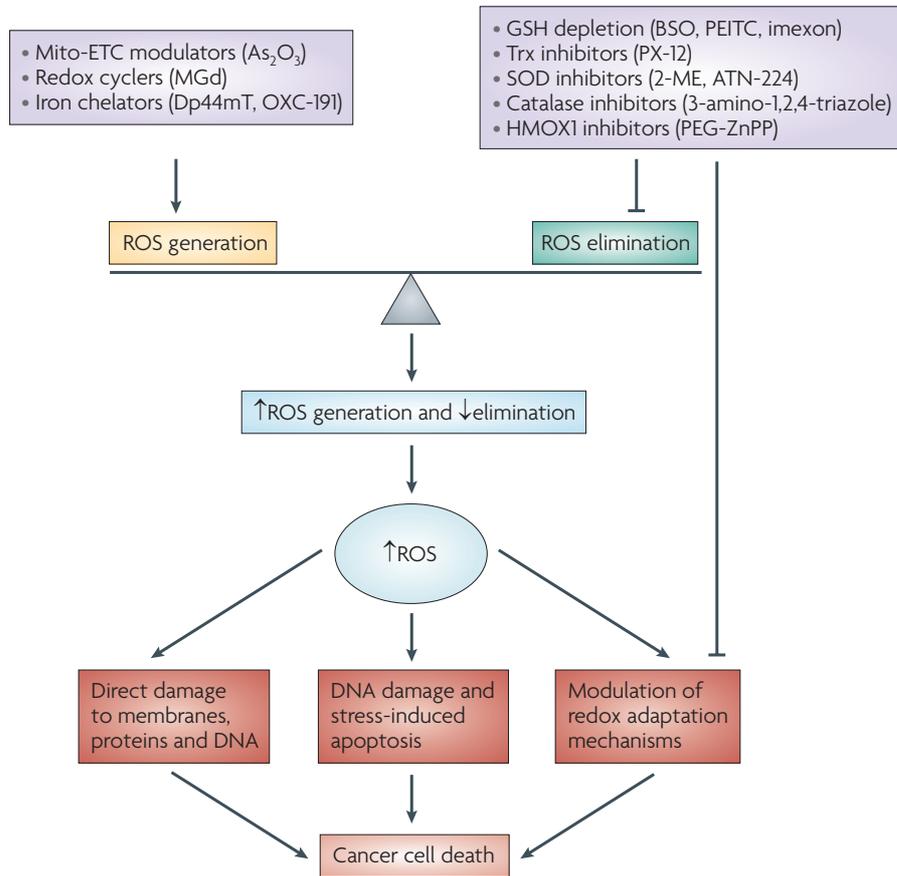


Figure 2: The higher redox balance point in cancer cells may provide redox-targeted therapeutic opportunities. **a**, Due to the tension between higher ROS production and greater redox buffering capacity, cancer cells may be susceptible to redox modulation and selective cell killing. **b**, Stimulating ROS generation or targeting mechanisms of ROS elimination with small molecules can induce selective cancer cell killing *via* redox imbalance. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Drug Discovery²², copyright (2009).

1.2 OXIDIZED NUCLEOTIDE-INDUCED MUTAGENESIS AND DNA REPAIR

1.2.1 The nucleotide pool is readily susceptible to oxidation

The innate reactivity of ROS exposes biomacromolecules to oxidative modifications, and, in relation to carcinogenesis, the DNA is a major source of reactive damage that can cause mutations and tumorigenesis³². In fact, the free nucleotide pool is orders of magnitude more susceptible to modifications, such as methylation³³ and oxidation³⁴, than nucleic acids. Upon subjection to ROS, free nucleotides, particularly purines, are able to undergo a number of modifications due to their inherent chemical lability^{35,36}. Oxidation of guanine, in particular, is favorable due to its chemical structure³⁷. 8-oxo-7,8-dihydroxyguanine (8-oxo-guanine; 8-oxo-G) is one of the most abundant oxidized species in cells^{38,39} and, thus, the most studied oxidized metabolite. Other notable nucleobase oxidation products *in vitro* include 2-hydroxyadenine (2-OH-A), 5-formyluridine (5-COH-U) and 5-hydroxycytidine (5-OH-C)³⁶. These modifications can occur in any combination of nucleobase, ribose and phosphate(s)³⁵.

1.2.2 Incorporation of oxidized nucleotides into nucleic acids

Oxidized nucleotides can readily be incorporated into DNA⁴⁰⁻⁴³ or RNA⁴⁴ by DNA and RNA polymerases, respectively. In DNA, 8-oxo-dGTP is indiscriminately paired equally opposite adenine or cytidine, depending on its conformation (**Figure 3**), resulting in A:T → C:G transversion mutations^{34,41,45-50}, while 8-oxo-GTP negatively affects transcriptional fidelity upon incorporation into RNA⁴⁴. Similarly, 2-hydroxy-2'-deoxyadenosine-5'-triphosphate (2-OH-dATP) and 5-hydroxy-2'-deoxycytosine-5'-triphosphate (5-OH-dCTP) also cause transversion mutations in mammalian or bacterial genomes⁵¹⁻⁵³. Overall, the incorporation of oxidized nucleobases is mutagenic and highly correlated with carcinogenesis^{39,54,55}.

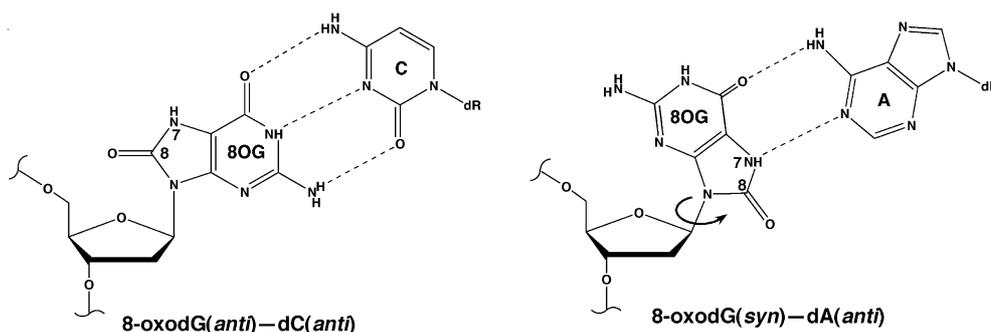


Figure 3: Mispairing of 8-oxo-guanine with adenine results in transversion mutations. 8-oxo-guanine pairs with cytosine in an anti-anti conformation, while it is rotated around the ribose bond to mispair with adenine in the syn-anti conformation. Reprinted with permission from Elsevier, from Krahn, *et al.*⁵⁶.

1.2.3 Repair and toxicity of oxidized nucleotides incorporated into DNA

1.2.3.1 Base excision repair (BER)

To maintain genomic integrity, prokaryotic and eukaryotic cells possess a multitude of DNA damage surveillance and repair pathways, which can also effectively recognize and remove oxidized nucleotides. Once incorporated into DNA, oxidized nucleotides can be excised by either the base excision repair (BER) or mismatch repair (MMR) pathways^{57,58} (**Figure 4**). In the case of BER, lesion-specific DNA glycosylases identify the error and then excise either the modified base or the opposing base⁵⁹. Specific for 8-oxo-dG are oxidized guanine glycosylase (OGG1), which targets 8-oxo-dG:dC mis-pairs⁶⁰⁻⁶³, and MutY homolog (MUTYH), which can recognize mis-paired 8-oxo-dG:dA following DNA replication⁶⁴⁻⁶⁶. MUTYH also recognizes and excises 2-OH-dA, making it a broader detector of oxidized nucleotide mis-pairs^{67,68}. Recently, however, it was found that MUTYH was able to resolve 8-oxo-dG:dA, but not 2-OH-dA:dG and :dC mis-pairs, in human carcinoma cell extracts, suggesting that the base excision activity of 2-OH-dA by MUTYH may be less active than originally thought⁶⁹.

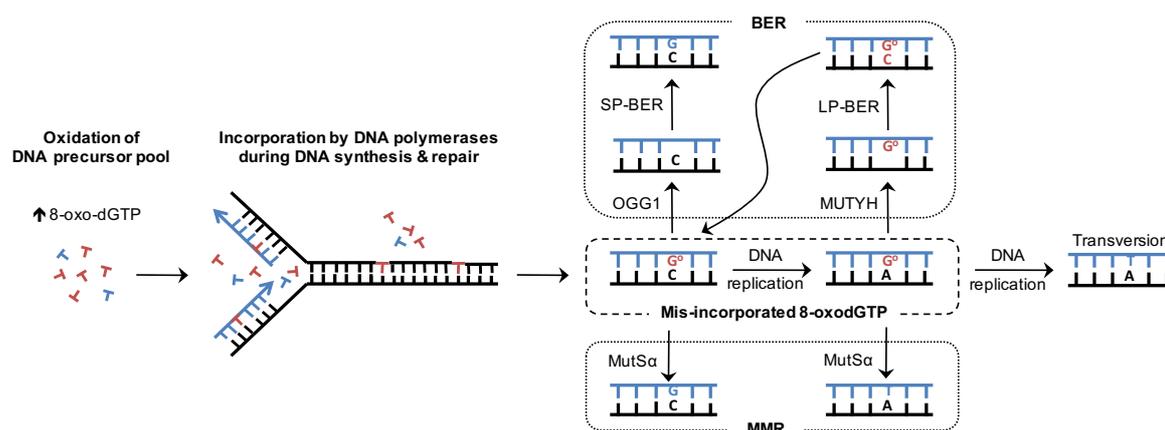


Figure 4: Repair of 8-oxo-dG lesions in DNA by BER or MMR pathways. 8-oxo-dG can be generated from oxidation of the nucleotide pool or direct oxidation of DNA (red). Nascent DNA synthesis and nucleotides are depicted in blue. SP-BER – short patch-base excision repair; LP-BER – long patch-base excision repair. Reprinted with permission from Elsevier, from Rudd, Valerie and Helleday⁷⁰.

1.2.3.2 Mismatch repair (MMR)

MMR corrects base mis-pairing on nascent DNA following replication, and, thus, also has a role in removing oxidized bases from DNA, although the evidence is less clear than with BER⁵⁹ (**Figure 4**). MutS Homolog 2 (MSH2), a key MMR component, has been shown to reduce the presence of 8-oxo-dG in genomic DNA⁵⁷, while over-expression of MTH1, an 8-oxo-dGTP hydrolase (described in greater detail below), attenuates the mutagenesis rate of MMR-deficient tumors⁷¹. Additionally, MutS α , a heterodimer comprised of MSH2 and MSH6, can recognize 2-OH-dATP mis-pairs and MSH2-deficient mice have increased genomic levels of 2-OH-dA^{72,73}, suggesting the importance of MMR in recognition and excision of this lesion. On the other hand, *in vitro* studies have shown that MMR can poorly

recognize 8-oxo-dG mis-pairs and does not efficiently correct these lesions^{69,74}, however, these discrepancies may be sequence-specific⁷⁵.

1.2.3.3 Toxicity of oxidized nucleotide-induced DNA repair

Repair of 8-oxo-dG and 2-OH-dA, but also 5-OH-dCTP⁷⁶, lesions in DNA can have dire consequences on cell viability, depending on the context⁷⁷. The first evidence that 8-oxo-dG may be toxic arose from the fact that while *Mth1*- or *Ogg1*-knockout mice developed spontaneous tumors in the liver and lungs, *Mth1/Ogg1* double knockout mice did not develop lung tumors, yet high levels of 8-oxo-dG were detected in the nuclear DNA of lung cells⁷⁸. This would insinuate that the increase in DNA 8-oxo-dG content caused by depletion of both factors imposes a barrier on tumorigenesis instead of exacerbating it. Indeed, accumulation of 8-oxo-dG in nuclear DNA can result in apoptotic cell death by futile BER cycling, which induces single strand DNA breaks (SSBs) and ATP exhaustion *via* polyADP-ribose polymerase 1 (PARP1) hyperactivation^{79,80}. A similar phenomenon occurs in mitochondria but leads to loss of mitochondrial DNA and necrosis⁷⁹. Thus, in cancer cells, which may have higher loads of oxidative stress, there may be a greater reliance on oxidized nucleotide sanitation enzymes to permit cell viability.

1.2.4 Other roles for oxidized nucleotides in cells

Besides the mutagenesis angle that is well established in the field, oxidized nucleotides, particularly 8-oxo-guanine, appear to serve other necessary functions in cells that complicate our originally straightforward understanding of their purpose. It would seem that not all incorporation of 8-oxo-guanine into DNA is detrimental to the cell, as the positioning of these lesions in different regions of the DNA can have distinctive outcomes. The oxidized guanine DNA glycosylase, OGG1, primarily known for BER of 8-oxo-guanine from DNA, is heavily involved in modulating 8-oxo-guanine-mediated cellular functions on the basis of: 1) binding 8-oxo-guanine lesions in DNA or 2) remaining in a complex with the 8-oxo-guanine excision product⁸¹. Thus, this may indicate that a balance exists between oxidized nucleotide sanitation and incorporation, and importantly, there is still much to learn about the role of 8-oxo-guanine in cells.

1.2.4.1 Signaling roles of the OGG1:8-oxo-guanine excision complex

The potential roles of 8-oxo-guanine in cellular signaling were not recognized until the discovery that the OGG1:8-oxo-guanine excision complex can behave as a guanine nucleotide exchange factor (GEF) and activate RAS small guanosine triphosphatases (GTPases; **Figure 5a**)⁸². Rather than binding the OGG1 active site^{83,84}, 8-oxo-guanine may stimulate its glycosylase activity as a potential cofactor, implying a feed-forward response that encourages OGG1 activity⁸². However, this alternative binding site for 8-oxo-guanine has not yet been elucidated. Activation of RAS proteins by OGG1:8-oxo-guanine then initiates a phosphorylation cascade beginning with Raf1 and spreading to MAP (mitogen-activated protein) kinases⁸⁵. Further work illustrated that the OGG1:8-oxo-guanine complex can also activate the Rho family kinase, Rac1⁸⁶, and that OGG1 is required for Rho-GTPase

activation upon challenge with oxidative stress⁸⁷. Another intriguing finding was that exposure of mouse lungs to exogenous 8-oxo-guanine, that presumably forms the complex with OGG1 *in cellulo*, also activated RAS GTPase and the transcription of multiple pro-inflammatory genes⁸⁸. Therefore, 8-oxo-guanine, with the help of OGG1-mediated BER, appears to facilitate numerous cell signaling functions in response to oxidative stress^{88,89}.

1.2.4.2 8-oxo-guanine-mediated transcriptional regulation via OGG1

In assessing the physiological ramifications of 8-oxo-guanine, OGG1 knockout (*Ogg1*^{-/-}) mice, which lack a functional gene product, were found to have decreased inflammation following bacterial infection⁹⁰ or exposure to pro-inflammatory agents⁹¹, which first alluded to a role in regulating the inflammatory response⁸⁹. These findings suggested that activation of RAS by OGG1:8-oxo-guanine⁸² and induction of pro-inflammatory cytokine expression by 8-oxo-guanine⁸⁸ held systemic physiological importance.

An obvious starting point to consider was the effect of 8-oxo-guanine lesions on binding of transcription factors to guanine-rich promoters that regulate inflammatory responses. This is typically mediated by NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) or Sp1. However, conflicting evidence proved this hypothesis inconclusive⁹²⁻⁹⁴. Instead, there was proof suggesting that DNA repair factors and transcription factors compete for binding to oxidatively damaged promoter regions^{94,95}, with occupancy of the promoter by OGG1 physically obstructing the binding of transcription factors⁹⁵. More recently, however, it was determined that OGG1 bound upstream of the NF- κ B consensus binding site facilitates recruitment of NF- κ B to oxidized promoters and enhances transcription of pro-inflammatory genes in response to TNF- α (tumor necrosis factor alpha) exposure^{96,97} (**Figure 5b**). Hence, this likely explains the dampened inflammatory response seen in *Ogg1*^{-/-} mice^{90,91}.

Along similar lines, oxidized promoter regions have recently been found to enhance transcription by facilitating G-quadruplex formation⁹⁸. G-quadruplex formation in promoters is known to stimulate transcription⁹⁹, such as is the case with the vascular endothelial growth factor (*VEGF*) gene¹⁰⁰, which is also known to contain 8-oxo-guanine lesions in its promoter region¹⁰¹. Fleming, Ding and Burrows illustrated that apurinic (AP) sites, generated from OGG1 BER repair intermediates, enhance G-quadruplex formation on the *VEGF* or endonuclease III-like protein 1 (*NTHL1*) promoters. This, in turn, increases gene transcription⁹⁸ (**Figure 5c**), depending on if the 8-oxo-guanine resides in the template or coding DNA strands¹⁰². In contrast, 8-oxo-guanine present in telomeric repeats can block formation of G-quadruplexes and restore telomerase activity⁵⁰, which illustrates the importance of oxidized guanine in regulating G-quadruplex formation (**Figure 5c**). Taken together, these findings delineate exciting new roles for 8-oxo-guanine and OGG1 in epigenetic regulation of transcription^{81,103}.

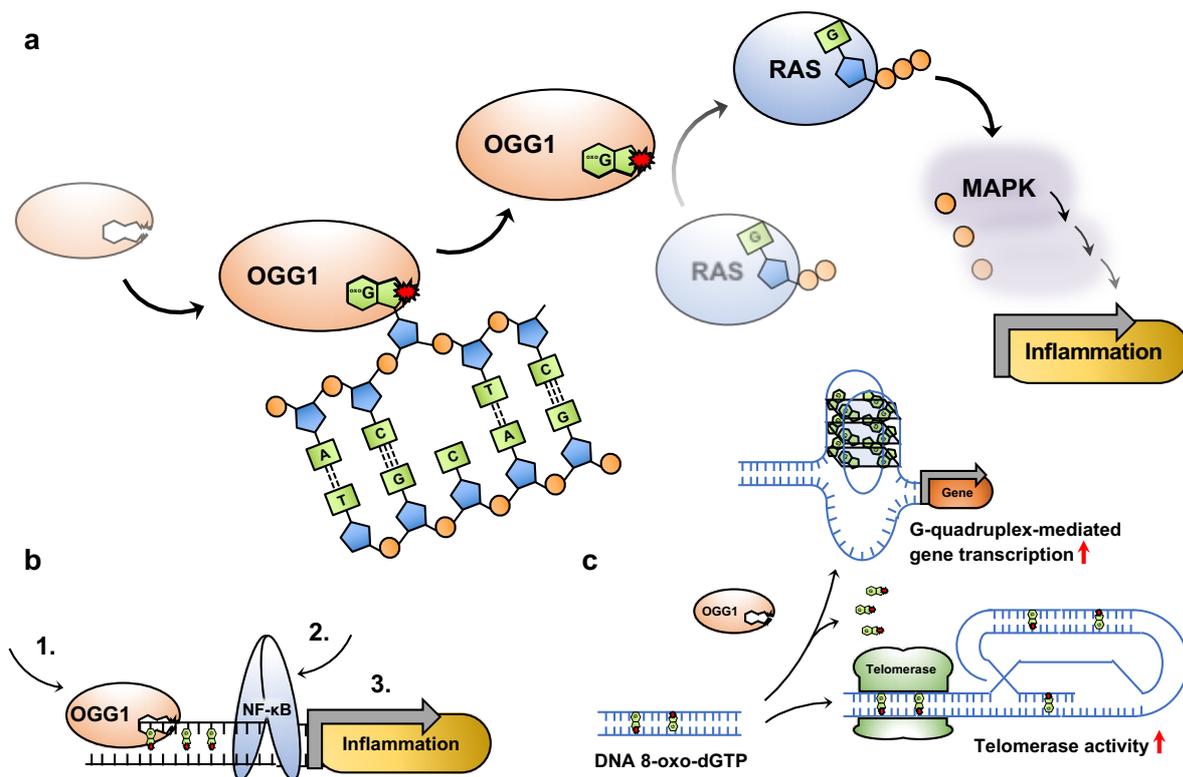


Figure 5: Roles for 8-oxo-guanine beyond mutagenesis. a, Excised 8-oxo-G in complex with OGG1 can function as a GEF and activate RAS GTPases that promote transcription of inflammatory genes *via* MAP kinases. b, 8-oxo-guanine in gene promoters recruits OGG1, which facilitates adjacent binding of the NF-κB transcription factor downstream and expression of inflammatory response genes. c, DNA:8-oxo-guanine disrupts G-quadruplex formation, which enables telomerase activation in telomeric regions, but excision by OGG1 in promoter regions permits G-quadruplex formation and gene transcription.

1.3 “PREVENTATIVE DNA REPAIR” BY NUCLEOTIDE PRECURSOR POOL SANITATION ENZYMES

(*excerpts taken with permission from Elsevier, from Rudd, Valerie and Helleday*⁷⁰)

1.3.1 Sanitation enzymes police the nucleotide precursor pool

The DNA precursor pool is readily susceptible to modification from a myriad of sources within the cellular environment. To counteract the potential toxicity of modified nucleotide precursors, cells have evolved various mechanisms to maintain cellular fitness. While a number of retrospective solutions (i.e., after incorporation into nucleic acids), such as DNA repair, have garnered more attention, nucleotide pool sanitation enzymes offer a more favorable alternative: preventative maintenance (reviewed specifically by Galperin¹⁰⁴ and Nagy¹⁰⁵). As numerous modified dNTPs are efficient DNA polymerase substrates^{42,43,53,106,107}, sanitation enzymes help eliminate them from the nucleotide pool and work in concert with DNA repair machinery to maintain genome integrity¹⁰⁸ (**Figure 6a**). Modified nucleoside monophosphates may then be poor substrates for their respective nucleoside kinases, as is the case with 8-oxo-(d)GMP and guanylate kinase (**Figure 6b**)^{34,106}.

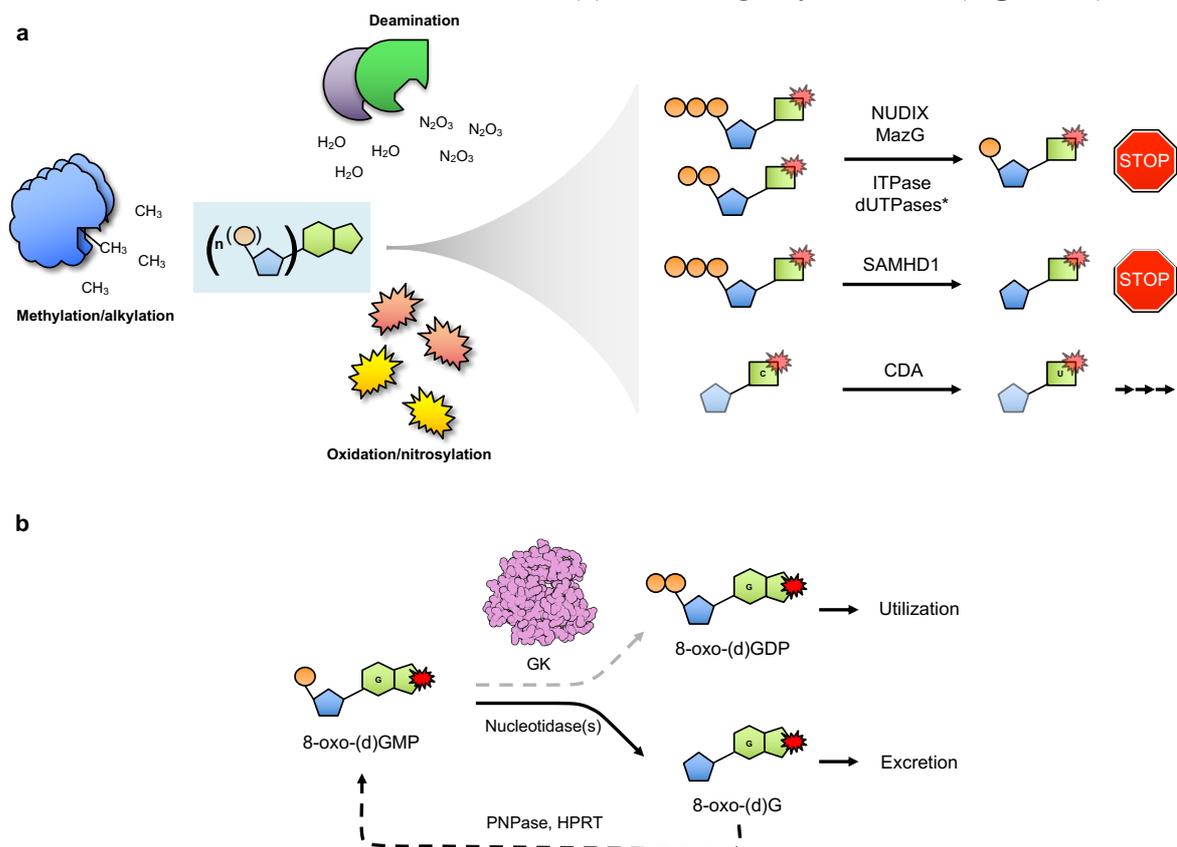


Figure 6: Sanitation enzymes prevent nucleic acid incorporation of modified or damaged nucleotides. a, Modification from multiple sources can alter the nucleobase or ribose sugar, which can cause deleterious effects once incorporated into nucleic acids. Sanitation enzymes are generally phosphohydrolases that continually cleanse the nucleotide pool of these polymerase substrates. **b,** 8-oxo-(d)GMP is a poor substrate of guanylate kinase (GK), thus preventing direct utilization of 8-oxo-(d)G. Cellular nucleotidases can convert 8-oxo-(d)GMP to 8-oxo-(d)G, which is excreted from the cell or can potentially be recycled back to 8-oxo-(d)GMP by a chain of reactions mediated by PNPase, among others^{109,110}. Mouse GK structure from Sekulic *et al.*¹¹¹.

1.3.2 NUDIX hydrolases – not one and the same

Perhaps the most common sanitation enzymes are those of the nucleoside diphosphate linked to some other moiety, X, (NUDIX) hydrolase family, which was a term first coined by Bessman and colleagues in the mid-90s¹¹². Family members, of which there are at least 22 described thus far in humans (**Figure 7**), typically catalyze the hydrolysis of nucleoside-like di- or triphosphates to their respective monophosphates and are related by a consensus 23-amino acid NUDIX box motif (Gx₅Ex₂[UA]xREx₂EE₂xGU, where “U” is an aliphatic, hydrophobic residue and “X” is any amino acid) that facilitates phosphohydrolase activity^{113,114}. Interestingly, the core residues of the catalytic domain are required for divalent cation coordination (usually Mg²⁺), which is essential for catalysis, but do not discriminate or direct the nucleobase substrates, *per se*, implying plasticity may exist within the superfamily^{104,115}. NUDIX enzymes were originally called MutT family proteins, after the antimutagenic *E. coli* 8-oxo-(d)GTPase^{44,45,48,116,117}, but the diversity of substrate preferences has suggested that a number of them have little or no role in preventing mutations to DNA, thus, prompting the change in nomenclature¹¹².

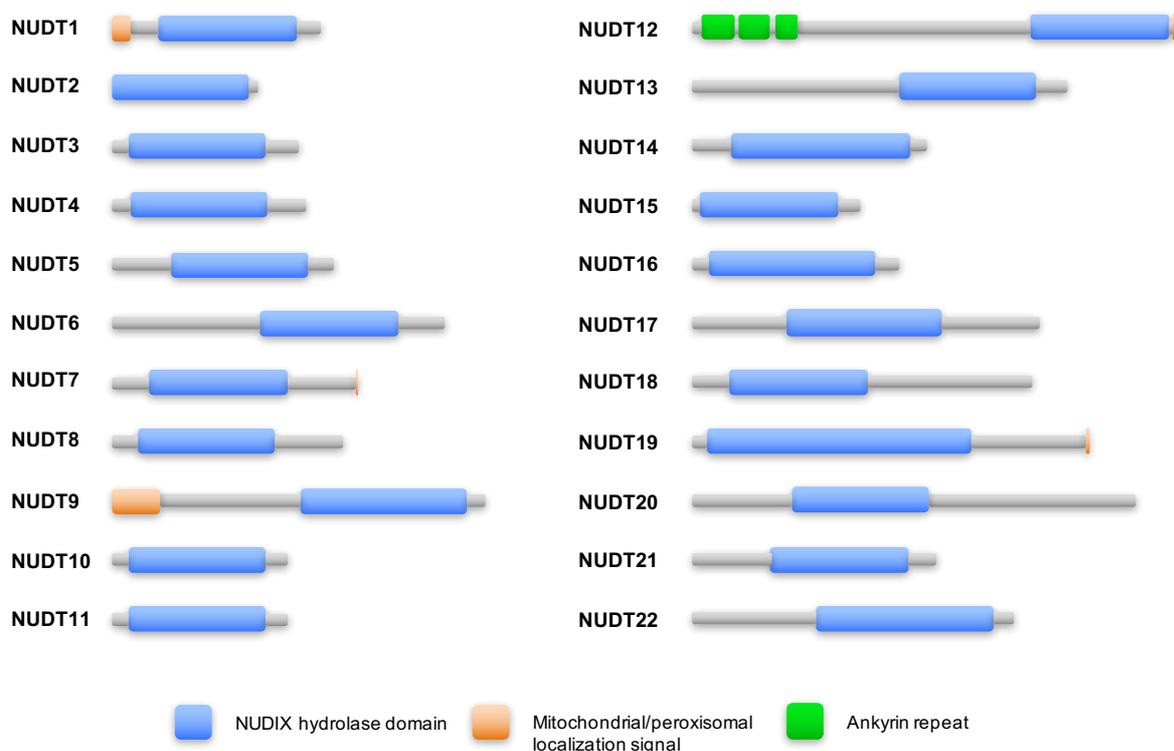


Figure 7: The 22 canonical human NUDIX proteins drawn to relative scale. NUDIX domains are depicted in blue, mitochondrial or peroxisomal localization signals in orange, and ankyrin repeats in green.

1.3.2.1 MTH1 sanitizes the oxidized nucleotide pool

Despite the variety in substrate preferences for NUDIX enzymes, by far the most studied class of precursor to date are the oxidized nucleotides and their sanitation by human NUDT1 (NUDIX type 1), also known as MutT homologue 1 (MTH1). MTH1, like its *E. coli* counterpart, MutT, hydrolyzes oxidized nucleotides, including 8-oxo-(d)GTP and 2-OH-(d)ATP, thereby preventing downstream transversion mutations in DNA and

RNA^{49,51,55,118,119}. The importance of MTH1 in maintaining genomic integrity and replicative potential has been shown extensively in cultured fibroblasts and oncogenic RAS-transformed cells¹²⁰⁻¹²³. Depletion of MTH1 in these studies triggered characteristic senescence phenotypes, including the presence of β -galactosidase, irreparable DNA damage, and induction of p53, p21 and p16INK4A tumor suppressor expression¹²³. Importantly, these cells did not undergo cell death and culturing MTH1-depleted cells in hypoxic conditions rescued the senescence phenotype^{121,123}.

More recently, we and others have demonstrated that MTH1 may be a potent drug target in cancer cells¹²⁴⁻¹³⁰, where a dysfunctional redox environment can impose a reliance on oxidized nucleotide sanitation (**Figure 8**). The mechanistic link between initially described inhibitors and the dependence on MTH1 for their cell killing effect has been controversial. Subsequent reports using MTH1 inhibitors, TH287 and TH588, indicated that cancer-specific cell killing may be independent of MTH1 inhibition (i.e., through an off-target effect)¹³¹⁻¹³³, while other, independent groups have also synthesized potent MTH1 inhibitors and saw lack of effect in cancer cells¹³⁴⁻¹³⁶. These recent reports have cast doubt on whether MTH1 is a bonafide cancer therapeutic target. That being said, depletion of MTH1 was recently shown to selectively increase telomere dysfunction and cell death in telomerase-positive cancer cells with shortened telomeres, thus representing a new potential avenue of inhibitor utilization⁵⁰.

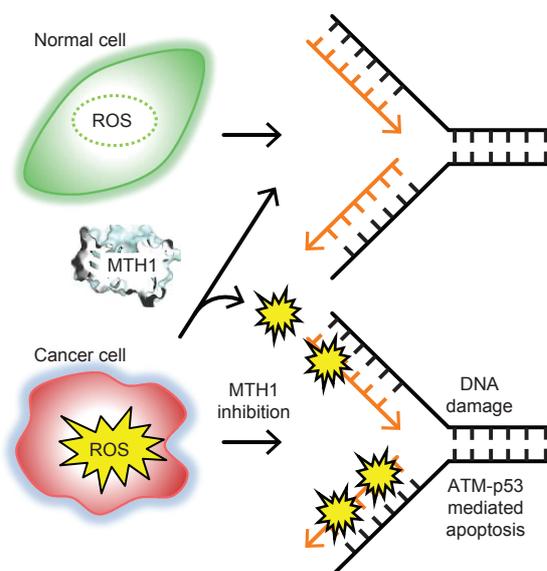


Figure 8: The original MTH1 inhibitor mechanism proposed by Gad *et al*¹²⁴. In cancer cells, treatment with MTH1 inhibitors causes DNA damage and ATM-p53-mediated apoptosis due to incorporation of oxidized nucleotides. Reprinted by permission from Macmillan Publishers Ltd: Nature¹²⁴, copyright (2014).

1.3.2.2 Back-up enzymes to MTH1?

Potential MTH1 back-ups include other members of the NUDIX family: NUDT15 (MTH2), NUDT18 (MTH3) and NUDT5. *In vitro*, each of these NUDIX enzymes is capable of hydrolyzing various oxidized nucleotides¹³⁷⁻¹³⁹ and knockdown in human cells was reported to increase 8-oxo-dGTP-induced mutations on a reporter plasmid¹⁴⁰; however, compared to MTH1, the enzyme kinetics for these proteins are relatively underwhelming. In light of this, it

is fair to question if these proteins confer the same physiological importance to mammalian cells, and, indeed, this issue was raised in a recent review¹⁴¹. As the author rationalizes, the fact that a number of human NUDIX enzymes are capable of hydrolyzing oxidized substrates, albeit poorly, suggests an evolutionary link to a more primitive NUDIX hydrolase (e.g., MutT). As such, bias towards mutagenic oxidized bases may be obscuring the physiological roles of NUDIX enzymes that hydrolyze other substrates far more efficiently.

1.3.2.3 *NUDT15 (MTH2)*

NUDT15 (MTH2), originally described to hydrolyze 8-oxo-dGTP in mice¹³⁷ and 8-oxo-dGTP/dGDP in humans^{139,140}, also has a previously overlooked dGTP activity¹³⁷. Like MTH1, NUDT15 was proposed to sanitize the nucleotide pool of these oxidation products based on *in vitro* characterization^{137,139,140}. It had the ability to revert mutagenesis on a bacterial plasmid when expressed in MutT-deficient *E. coli*¹³⁷ or increase mutagenesis of the same plasmid when depleted in HEK293T cells¹⁴⁰. Furthermore, Nudt15 expression was upregulated in mouse kidney following intestinal ischemia and reperfusion injury, which produces a rapid burst of oxidative stress, suggesting NUDT15 transcription is triggered by acute oxidative damage¹⁴². Intriguingly, ethanol toxicity, due to ROS production during its metabolism, decreased expression of Nudt15 in adolescent mice but showed no difference in adult mice¹⁴³. Meanwhile, longer-term depletion of MTH1 also resulted in a significant increase of NUDT15 messenger RNA (mRNA) expression¹²⁴. Despite this evidence, investigations with human NUDT15 concluded that it had about 40-fold less enzymatic activity towards 8-oxo-dGTP compared to MTH1¹³⁹. Thus, the role of NUDT15 in oxidized nucleotide metabolism, especially in humans, is far from clear.

Expanding on potential functions of NUDT15, a more recent study demonstrated that multiple mouse NUDIX proteins, including Nudt15, could decap mRNA¹⁴⁴, which is an important determinant of gene expression *via* regulation of mRNA half-life. Specifically, Nudt15 could cleave 7-methylguanosine-5'-monophosphate (m⁷GMP) and 7-methylguanosine-5'-diphosphate (m⁷GDP) from methylated, capped RNAs¹⁴⁴, signifying a potential alternative role for NUDT15 in mammalian cells. Another curious finding regarding NUDT15 is its interaction with the polymerase clamp PCNA (proliferating cell nuclear antigen)¹⁴⁵, suggesting that sanitation of modified nucleotides could occur at distinct subcellular locations, such as at the replication fork. Binding of NUDT15 to PCNA appeared to protect the latter from degradation, whereas ultraviolet irradiation, but not other insults, caused dissociation of the NUDT15-PCNA complex and PCNA degradation¹⁴⁵. Lastly, it was recently discovered that a missense mutant of NUDT15, R139C, is significantly correlated with thiopurine intolerance in acute lymphoblastic leukemia (ALL) and inflammatory bowel disease (IBD) patients of Asian descent¹⁴⁶⁻¹⁴⁸. This finding opens the enticing possibility that NUDT15 may play an important role in modulating the effectiveness of thiopurines and, potentially, other nucleoside analog therapeutics used to treat cancer and viral infections. As of this time, the physiological roles of NUDT15 have not been clearly elucidated, but these findings may hold important clues to reach that end.

1.3.2.4 *NUDT18 (MTH3)*

First described in 2012 by Takagi and colleagues¹³⁹, NUDT18 (MTH3) is able to hydrolyze 8-oxo-dGDP and 8-oxo-GDP to their respective monophosphates at physiological pH (and with similar efficiency) but has no activity towards the relevant triphosphate species. This is particularly interesting given that MTH1 and NUDT15 have negligible hydrolysis activity towards the diphosphate forms, which are readily converted to triphosphates in cells^{34,139}. Recent work by our group also suggests there is activity towards 8-oxo-dGDP, but also 8-oxo-(d)GTP, albeit at low efficiency, and depletion of NUDT18 had negligible effects on cell viability and proliferation¹¹⁴. Until recently, there were no other reports describing biological roles for NUDT18 in human cells. Knockout of NUDT18 by CRISPR/Cas9 appears to cause a proliferation defect in HeLa cells but for reasons yet unknown¹⁴⁹.

1.3.2.5 *NUDT5*

NUDT5, originally named YSA1H – after its yeast homolog, was first described as a homodimeric ADP-sugar hydrolase, with particular activity towards adenosine diphosphate ribose (ADP-ribose, ADPR) and ADP-mannose¹⁵⁰⁻¹⁵². ADPR is an important component of nicotinamide adenine dinucleotide (NAD) and polyADP-ribose (PAR) metabolism¹⁵³⁻¹⁵⁵. Analysis of the NUDT5-ADPR co-crystal structure indicated that Glu166 functions as a catalytic base to deprotonate a water molecule, which then hydrolyzes between the α and β phosphates of ADPR^{156,157}. It was also reported that several NUDIX hydrolases, including yeast Ysa1 and mouse Nudt5, but not human NUDT9, were able to efficiently hydrolyze *O*-acetyl-ADP-ribose (OAADPR), the product of NAD-dependent sirtuin deacetylases^{158,159}. NUDT9 is another NUDIX ADPR hydrolase that is localized to the mitochondria and shares sequence homology with the ADPR binding domain of the TRPM2 calcium channel^{160,161}. Interestingly, Δ *Ysa1* yeast are highly resistant to exogenous ROS due to accumulation of ADPR and OAADPR, which inhibited the mitochondrial electron transport chain and upregulated production of the ROS scavenger, reduced glutathione¹⁶².

In addition to ADPR, human NUDT5 has been proposed to hydrolyze 8-oxo-dGDP to 8-oxo-dGMP^{138,140,163-165}, utilizing the same techniques as for MTH1, NUDT15 and MTH3 analyses. 8-oxo-7,8-dihydroxy-2'-deoxyadenosine-5'-diphosphate (8-oxo-dADP), 2-hydroxy-2'-deoxyadenosine-5'-diphosphate (2-OH-dADP) and 5-formyl-2'-deoxyuridine-5'-diphosphate (5-COH-dUDP) may also be NUDT5 substrates^{164,165}. However, further analyses and comparison have demonstrated that ADPR has a 400-fold higher enzymatic efficiency than 8-oxo-dGDP and optimal 8-oxo-dGDP turnover occurs at pH 10.5^{141,165}, suggesting that ADPR is the biologically relevant substrate. Nonetheless, NUDT5 is capable of hydrolyzing 8-oxo-dGDP and, intriguingly, the modality of binding in the active site is noticeably different compared to the NUDT5-ADPR co-crystal structures – with the phosphates of 8-oxo-dGDP being completely inverted compared to ADPR¹⁶⁶. In this case, hydrolysis occurs at the β phosphate as opposed to the α phosphate during ADPR hydrolysis. Thus, as the co-crystal and substrate competition studies imply, NUDT5 may be able to accommodate diverse substrates by different binding conformations in its active site¹⁶⁵.

A limited number of cellular experiments have, thus far, supported a role for NUDT5 in ADPR, but not 8-oxo-guanine, metabolism. Reports of NUDT5 depletion in HeLa or IMR-90 fibroblast cells causing a p53-mediated G1 cell cycle arrest were loosely linked to increases in 8-oxo-guanine, but, in combination with what is known from biochemical analyses, these conclusions were not convincing^{141,167,168}. In addition, depletion of NUDT5 in HEK293T cells could increase the mutation frequency of a shuttle plasmid by about 1.5-fold¹⁴⁰. While statistically significant, it is reasonable to express concern that this difference is not biologically important.

In line with its originally described function, multiple lines of evidence suggest NUDT5 is intimately involved in ADPR metabolism. Besides the functions already described for yeast Ysa1, human NUDT5 is activated by ADP-ribosylation of its cysteine residues in response to both exogenously- and endogenously-generated nitric oxide in macrophage cells¹⁶⁹, while similar experiments with ADPRase-I, a rat homolog of human NUDT9, indicated an inhibitory effect of ADP-ribosylation¹⁷⁰. This might suggest that NUDT5 is selectively activated by nitric oxide, which, for example, is produced in macrophages that are stimulated by TNF- α /IFN- γ (interferon gamma) during an inflammatory response. Likewise, there are indications that NUDT5 (as well as NUDT9) is biologically active in the maintenance of ATP levels and mitochondrial energy homeostasis in response to PARP1 hyperactivation by alkylating agents¹⁵⁴. The authors also demonstrated that interfering RNA (RNAi) knockdown of NUDT5/9 (or small molecule inhibition of NUDIX activity) in HeLa lysates blocked ATP exhaustion and AMP generation in response to treatment, implying that NUDIX ADPR hydrolases contribute to energetic failure and cell death in response to PARP activation. More recently, NUDT5 was implicated in the regulation of hormone-responsive genes and proliferation of breast cancer cells following stimulation with progesterin or estradiol¹⁷¹. Interestingly, NUDT5 was found to synthesize nuclear ATP from PARP1- and polyADP-ribose glycohydrolase (PARG)-derived ADPR, rather than hydrolyze it to AMP, in the presence of inorganic pyrophosphate (PP_i). NUDT5 depletion caused a blockade of nuclear ATP generation, which is required by ATP-dependent chromatin remodeling complexes for gene regulation¹⁷²⁻¹⁷⁶, thus inhibiting proliferative responses from hormones in the breast cancer cells¹⁷¹.

An additional thought-provoking finding, which was a side note in two recent studies, is that NUDT5 may also play a role in 6-thioguanine metabolism^{177,178}. Utilizing clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 screening in cancer cells¹⁷⁷ or haploid embryonic stem cells¹⁷⁸, the researchers identified multiple guide RNAs (gRNAs) targeting *NUDT5* that caused resistance to thioguanine selection, on par with disruption of the hypoxanthine phosphoribosyltransferase (*HPRT*) gene¹⁷⁷. The authors proposed a model where NUDT5 ablation may cause depletion of phosphoribosyl pyrophosphate (PRPP), a required cofactor for HPRT to activate thioguanine, by decreasing cellular ribose-5-phosphate (R5P) production. This would imply that R5P generated *via* NUDT5 activity is more important than originally thought. While early, the expanding roles of NUDT5 in human cells represent exciting advances that should foster further exploration of its functions.

1.3.3 Other (d)NTP pool sanitizers and their clinical implications

1.3.3.1 All- β dUTPases

The all- β dUTPases, with remarkable specificity, prevent pooling of dUTP in cells (**Figure 9**)¹⁷⁹. dUTP is a non-canonical NTP resultant from thymidine biosynthesis and is a detriment to DNA integrity *via* the potential double-strand breaks formed following excision by uracil-DNA glycosylases (UNG)¹⁸⁰⁻¹⁸². This outcome was purported by the discovery that dUTPase function is essential for viability in both bacteria and yeast¹⁸³⁻¹⁸⁵. While human dUTPase is suggested to maintain DNA replication fidelity¹⁷⁹, its singular importance in cell viability has not been explored extensively. For cancer cells, however, dUTPase appears to be crucial for survival in the presence of thymidylate synthase (TS) inhibitors, such as non-canonical fluorodeoxyuridine (FUdR), by producing dUMP for thymidine synthesis¹⁸⁶. This has suggested that there is a window for anti-neoplastic therapies modulating the thymidine pool¹⁸⁷⁻¹⁸⁹. Indeed, dUTPase inhibitors, such as TAS-114, have been developed and are in clinical trials to potentiate the anticancer effects of TS inhibitors^{190,191}.

1.3.3.2 All- α NTP pyrophosphatases

The all- α NTP pyrophosphatase superfamily includes MazG, the MazG-like hydrolases and dimeric dUTPases (**Figure 9**)¹⁹². Like the all- β dUTPases, all- α dUTPases hydrolyze dUTP but also can hydrolyse dUDP due to significant deviations in protein structure¹⁹³. All- α dUTPases are expressed in select prokaryotic and eukaryotic organisms (usually where there are no all- β dUTPases present in the genome) and demonstrates the essential nature of this function. MazG and MazG-like enzymes are ubiquitously expressed and were believed to selectively hydrolyze canonical NTPs to their respective monophosphates^{192,194}. This has changed with the recent discovery that mycobacterial MazG sanitizes cells of the oxidized pyrimidine, 5-OH-dCTP^{76,195}, and that human DCTPP1 (XTP3-transactivated protein A, dCTPase) prefers modified cytidine triphosphates, including fluorinated or formylated species¹⁹⁶ and 5-methyl-2'-deoxycytosine-5'-triphosphate (5-methyl-dCTP), which can affect global methylation and epigenetic signatures that promote breast cancer¹⁹⁷. For this reason, dCTPase may have clinical significance and can be targeted with small molecule inhibitors¹⁹⁸⁻²⁰⁰.

1.3.3.3 ITPases

ITPases efficiently hydrolyze the non-canonical purines, (d)ITP and (d)XTP, and prevent their incorporation into DNA and RNA (**Figure 9**)^{201,202}. Resulting from deamination reactions in the cell and defects in purine synthesis²⁰³, misincorporation of these nucleotides does not cause mutations in *E. coli*²⁰⁴, but in higher organisms their incorporation is likely mutagenic and affects transcription of nascent mRNA, possibly by including ribonucleotide incorporation into DNA and vice versa²⁰³. This is suggested by the fact that ITPase null mice are embryonic lethal²⁰⁵. Additionally, one of the NUDIX enzymes, NUDT16, preferentially hydrolyses (d)IDP, which can otherwise cause incorporation-induced DNA damage²⁰⁶. Clearly, the redundancy of sanitation enzymes targeting deaminated purine species suggests

an important role in genome maintenance; however, the detailed physiological roles and ramifications of these nucleotide species in cells require further study.

1.3.3.4 SAMHD1

Beyond the pyrophosphatase sanitizing enzymes, particular hydrolases, such as SAMHD1, possess unique NTP triphosphohydrolase activity, reducing the nucleotide substrate to its nucleoside core (**Figure 9**)²⁰⁷. A member of the HD domain-containing superfamily²⁰⁸, SAMHD1 has broad substrate accommodation for canonical dNTPs, suggesting that it may oppose ribonucleotide reductase and tightly control dNTP pools²⁰⁹. However, it can hydrolyze modified dNTPs as well, such as O⁶-methyl-2'-deoxyguanosine-5'-triphosphate (O⁶-Me-dGTP), 5-Me-dCTP and 2-thio-2'-deoxythymidine-5'-triphosphate (2-Thio-dTTP)²¹⁰, but also the anti-cancer agent, clofarabine-5'-triphosphate (clofarabine)²¹¹. More recently, SAMHD1 has been implicated as a major barrier to cytarabine (cytosine arabinoside, araC) efficacy in acute myeloid leukemia (AML) patients by preventing accumulation of ara-CTP, the active species incorporated into DNA^{212,213}. Subsequently, it was further confirmed to control the response of several other antimetabolite-based therapies for treating cancer or viral infections, including fludarabine, decitabine, trifluridine, vidarabine, nelarabine, aciclovir, and ganciclovir²¹⁴⁻²¹⁶. SAMHD1 regulates these responses in several manners. Some may be substrates of SAMHD1 (such as 5-aza-dCTP, decitabine triphosphate²¹⁶), while others directly (allosterically) or indirectly (influence dNTP pools) regulate its triphosphohydrolase activity. Thus, at multiple levels, SAMHD1 is a critical determinant of efficacy with regard to nucleoside analog-based therapeutics and represents an immensely attractive drug target²¹⁷.

As the efficiency of hydrolysis between canonical and modified nucleotides is similar²¹⁰, it remains an open question whether SAMHD1 has a physiological role in nucleotide pool sanitation. How this impacts upon its apparent tumor suppressor functions will also be an interesting avenue of study. These are outstanding questions that will need to be addressed by in-depth biochemical and cell-based characterization, but the importance of SAMHD1 in cancer and preventing viral propagation has clearly emerged in recent years^{217,218}.

1.3.3.5 Cytidine deaminase

Cytidine deaminase (CDA) is an important component of the pyrimidine salvage pathway by irreversibly deaminating cytidine and deoxycytidine to uridine and deoxyuridine, respectively – the building blocks for nascent pyrimidines²¹⁹. It has been typically viewed in a negative light as a resistance mechanism to cytidine analogue therapies²²⁰. Interestingly, while it is not a sanitation enzyme in the traditional sense, CDA may also provide cancer therapeutic opportunities with *specific* cytidine analogues (**Figure 9**). It was recently demonstrated that CDA deaminates 5-hydroxymethyl-2'-deoxycytosine (5-hydroxymethyl-dC, 5-OHMe-dC) and 5-formyl-2'-deoxycytosine (5-formyl-dC, 5-COH-dC) to their uridine counterparts, which are then phosphorylated and incorporated into DNA, causing cell death²²¹. As 5-OHMe-dU and 5-COH-dU are not substrates for dUTPase and CDA is expressed lower in

normal tissues, this appears a promising cancer therapeutic strategy; however, combining these with traditional cytidine analogues may provide the most effective strategy²²².

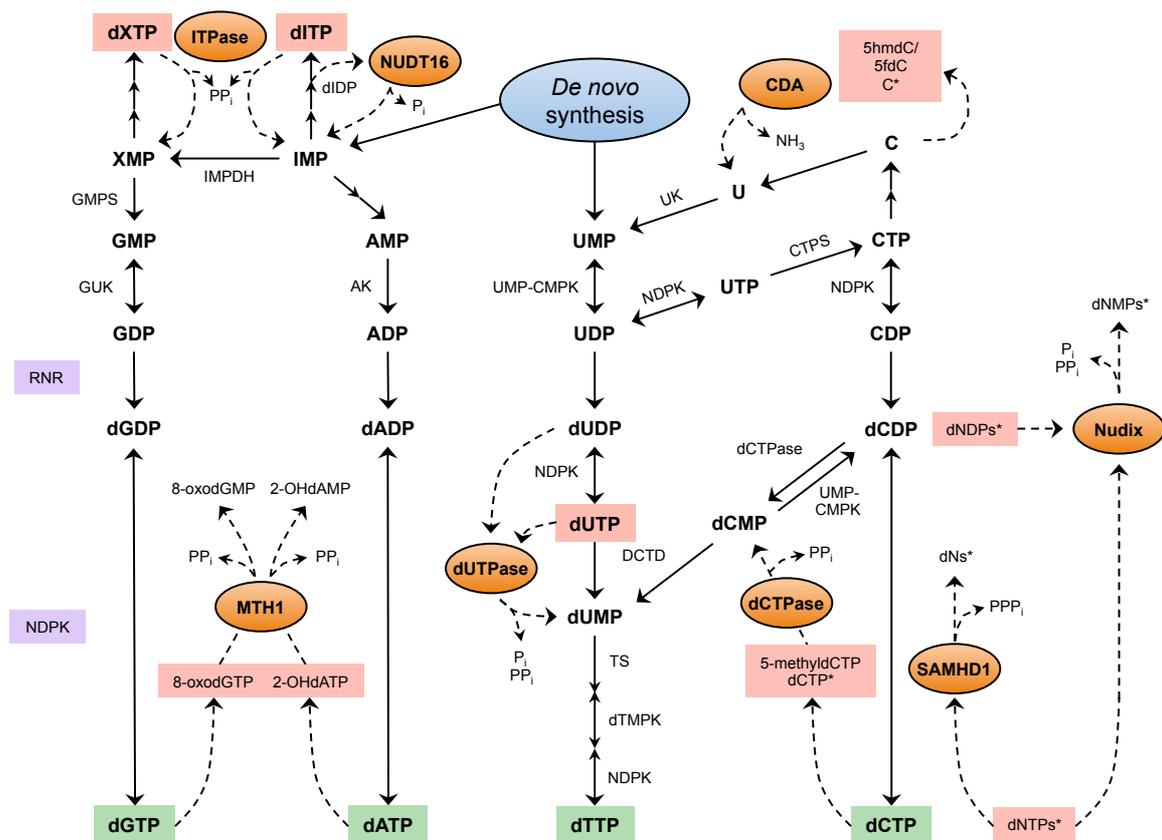


Figure 9: Nucleotide pool sanitation enzymes in the context of *de novo* nucleotide synthesis Sanitation enzymes depicted with orange ovals, generalized nucleotide biosynthesis enzymes with purple squares, canonical deoxynucleotides with green boxes, and potentially deleterious deoxynucleotides with red boxes. Generic modified species are marked with an asterisk (*). Reprinted with permission from Elsevier, from Rudd, Valerie and Helleday⁷⁰. IMPDH—inosine monophosphate dehydrogenase; GMPS—guanine monophosphate synthase; GUK—guanylate kinase; RNR—ribonucleotide reductase; NDPK—nucleoside diphosphate kinase; AK—adenylate kinase; UK—uridine kinase; UMP-CMPK—uridinemonophosphate-cytidine monophosphate kinase; CTPS—cytidine triphosphate synthase; DCTD—deoxycytidine monophosphate deaminase; TS—thymidine synthase; dTMPK—deoxythymidine monophosphate kinase; P_i—inorganic phosphate; PP_i—inorganic pyrophosphate; PPP_i—inorganic triphosphate.

1.3.4 Future and therapeutic perspectives

Nucleotide pool sanitation enzymes are clearly an important aspect of maintaining genome integrity. Recent studies have generated substantial progress in this field, yet as mentioned above, many outstanding questions remain. Detailed interrogation by an interdisciplinary approach may yield the most beneficial results. For example, substrates identified in biochemical screens can be related to phenotypes elicited from loss-of-function studies in cells, thus linking metabolism and novel biology. This is important to keep in mind for translational applications, especially when considering the number of nucleoside analogues used in the clinic²²³. In the context of cancer or combating viral infection, then, it is likely that sanitation enzymes may be therapeutically relevant as drug targets²¹⁷.

1.4 THIOPURINES AS ANTI-METABOLITE THERAPEUTICS

1.4.1 Discovery and clinical uses

Nucleoside analogs (also known as antimetabolites) are a class of drugs that target proliferating cells by interfering with the synthesis of human or viral nucleic acids²²³. In the 1950s, Elion and Hitchings initiated ground-breaking research into the synthesis and clinical utility of nucleoside analogs, including the discovery of the anti-herpes simplex virus (HSV) analog, acyclovir, and allopurinol for treatment of hyperuricemia and gout²²⁴⁻²²⁶. But it was the discovery of a new class of anti-cancer drugs, known as thiopurines, that would have the most therapeutic impact. Since this time, thiopurines have been a mainstay in clinical practice for treating various ailments, such as inflammation²²⁷ and cancer (ALL)^{228,229} but also functioning as immunosuppressants for organ transplants^{227,229}.

1.4.2 Metabolism and mechanisms of action

Three variations of thiopurine prodrugs are routinely available in the clinic: azathioprine (AZA-T), 6-mercaptopurine (6-MP, mercaptopurine) and 6-thioguanine (6-TG, thioguanine). Metabolic conversion of thiopurine analogs is a relatively complex process that includes many purine salvage pathway components and reactive intermediates that can simultaneously improve or adversely affect treatment (**Figure 10**)²³⁰. AZA-T, 6-MP and 6-TG are metabolized slightly differently in cells; where thioguanine conversion is most straightforward and azathioprine is readily converted to mercaptopurine in cells by a non-enzymatic reaction²³¹.

Although 6-methylthioinosine-5'-monophosphate (6-MTIMP), resultant from thiopurine S-methyltransferase (TPMT) conversion of 6-thioinosine-5'-monophosphate (6-TIMP), is a potent inhibitor of phosphoribosyl pyrophosphate amidotransferase (PPAT), the early and rate-determining step of *de novo* purine biosynthesis²³², it is a common misconception that thiopurines exert their effects through blocking production of purine nucleotides. This is in spite of evidence in existence since the 1970s suggesting the contrary²³³. More recent clinical evidence demonstrated that *de novo* purine synthesis was unchanged in mercaptopurine-treated patients, as compared to untreated individuals, and that methotrexate, a potent inhibitor of *de novo* purine biosynthesis, greatly potentiated thiopurine efficacy²³⁴. In fact, the accumulation of the thioguanine nucleotides (TGN), 6-thio-GTP and 6-thio-dGTP, not 6-MTIMP, is highly correlated with cytotoxicity^{235,236}.

6-thio-dGTP is an efficient substrate for cellular DNA polymerases, replacing the occurrence of guanine nucleotides by up to 0.1%²³⁷⁻²⁴⁰; however, the incorporation itself is not particularly toxic or mutagenic^{229,238,241,242}. Incorporation opposite thymidine and cytidine is roughly equal, but the problem arises when some of the 6-thio-dGTP is methylated by S-adenosylmethionine (SAM), which, during the following DNA replication cycle, can create a 6-MeThio-dG:dT mispair^{243,244}. Highly similar to repair of O⁶-Me-dG mispaired lesions²⁰⁵, the cellular MMR system recognizes the misincorporation of thymidine opposite 6-MeThio-dG and attempts to correct the pairing on the nascent DNA strand²⁴⁴; however, the correct match for this lesion cannot be made and the cell gets stuck in a futile repair cycle (**Figure 11**)²⁴⁵⁻²⁴⁸. This results in unrepaired gaps left opposite of 6-MeThio-dG lesions and catastrophic DNA damage ensues during the next S-phase, when recombination events create lethal DNA structures^{229,247,249}. Compared with most antimetabolites, cytotoxicity by thiopurines is noticeably delayed, requiring approximately three rounds of DNA replication for toxic effect²²⁹. Cytotoxicity is predominantly mediated by MMR²⁴⁵, although there is also evidence that 6-thio-GTP can complicate mRNA transcription²⁵⁰ and inhibit Rac1 GTPase activity²⁵¹, which may explain residual toxicity in MMR-deficient cells. This multifaceted mechanism of action requires constant monitoring in the clinic to limit adverse side effects^{227,229,252}.

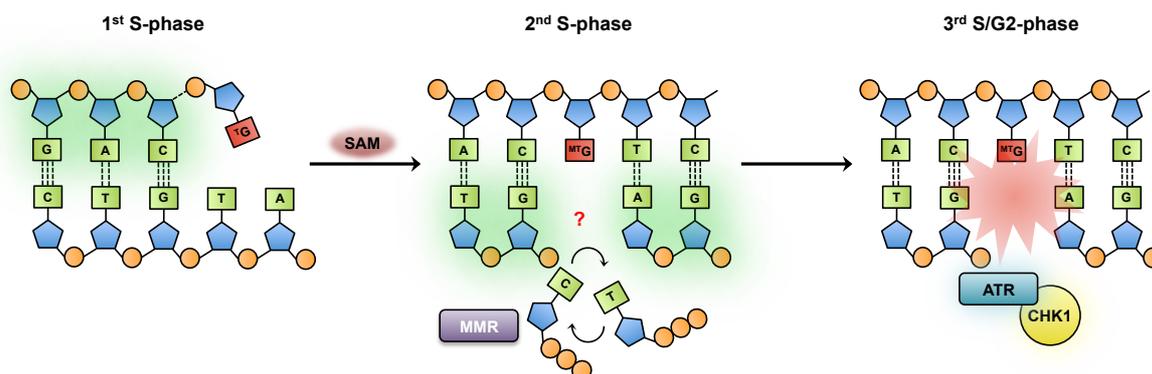


Figure 11: Mechanism of thiopurine-induced toxicity by futile mismatch repair. 6-thio-dGTP can be incorporated opposite thymidine and, upon methylation by SAM, cannot be repaired by MMR during the following S-phase. Gaps left from the unrepaired lesions result in irreparable DNA damage, G2 delay *via* ATR and Chk1 activation, and, inevitably, cell death by the third S/G2-phase. Green highlights indicate the nascent DNA strand.

1.4.3 Pharmacogenetics and clinical thiopurine response

Over time, key genetic determinants of thiopurine resistance and sensitivity have emerged that influence clinical regimens (**Figure 11**). Inactivating mutations to *HPRT1*, the gene that encodes the committal step in *de novo* purine biosynthesis and is required for active thiopurine metabolite conversion, are frequently found *in vitro*²⁵³, as there is only a single copy of the X-linked gene in cells. However, despite its utility to evaluate mutagenesis potential²⁵⁴, HPRT activity has little bearing on response to thiopurines in patients²⁵⁵.

TPMT can convert 6-TIMP, 6-TGMP, mercaptopurine and thioguanine to their inactive, methylated counterparts, thus, effectively removing them from the nucleotide pool^{256,257}. The physiological function of TPMT is unknown, but loss-of-function mutations to the *TPMT* gene, particularly homozygous mutations, can be deadly to patients treated with thiopurines^{235,236}. Although rare (approximately 1 in 300 patients or 0.3% incidence), patients homozygous for these mutations suffer from leukopenia and subsequent infections due to dangerously high TGN levels^{235,236}. Therefore, many clinics now screen patients for *TPMT* mutations prior to administering thiopurine treatments for ALL or IBD^{258,259}.

Mutations to ITPase (*ITPA* or inosine triphosphate pyrophosphatase), particularly 94C>A (P32T), have also been associated with thiopurine intolerance for treatment of ALL and IBD^{260,261}. This mutation is known to negatively affect the catalytic rate of ITPase, which causes two of its thiopurine-based substrates, 6-thioinosine-5'-triphosphate (6-TITP) and 6-methylthioinosine-5'-triphosphate (6-MTITP), to accumulate in erythrocytes and cause toxicity in patients²⁶²⁻²⁶⁵. Interestingly, an additional contributing factor to the incidence of thiopurine sensitivity in patients may relate to reduced protein stability of the ITPase P32T mutant²⁶⁴.

Mutations to *TMPT* and *ITPA* alone, however, cannot totally account for sensitivity to thiopurines, as only a quarter of European patients (and even fewer patients of Asian descent) suffering from thiopurine intolerance carry *TPMT* mutations²⁶⁶⁻²⁶⁸. More recently, missense mutations of the *NUDT15* gene (R139C) correlated strongly with thiopurine sensitivity in leukemia and IBD patients¹⁴⁶⁻¹⁴⁸. The incidence rate of this mutation, particularly in East Asians (9.8%) and Hispanics (3.9%), is relatively high, and patients homozygous for this alteration can only tolerate 10% of a typical thiopurine dosing¹⁴⁷. Combined, these studies have suggested that *NUDT15* also plays a major role in thiopurine metabolism.

Another particularly influential alteration involves 5'-nucleotidase, cytosolic II (*NT5C2*), which removes the phosphate from purine nucleoside monophosphates and permits their excretion from cells^{269,270}. *NT5C2* is known to hydrolyze the thiopurine intermediates, 6-TIMP and 6-TGMP, and, thus, is integral to thiopurine metabolism²⁷¹. Missense mutations to *NT5C2*, encoding R367Q, R238W or K359Q, further activate enzyme activity and are strongly associated with relapse in ALL patients treated with thiopurines^{272,273}. A recent publication demonstrating the clonal evolution of the *NT5C2* mutations in response to thiopurines also identified that these relapsed leukemias can be treated with inhibitors of inosine monophosphate dehydrogenase (IMPDH), due to their increased reliance on purine biogenesis²⁷⁴.

1.5 TARGETING HORMONE RECEPTOR POSITIVE BREAST CANCER

1.5.1 Hormone receptors drive breast carcinogenesis

It is commonly assumed that breast cancer originates from deactivating mutations to tumor suppressors or activating mutations to oncogenes, such as *PIK3CA*²⁷⁵, *TP53*²⁷⁶, *MAP3K1*²⁷⁷, *GATA3*²⁷⁸ or *AKT1*²⁷⁹; however, this may be an oversimplification of the disease²⁸⁰. Studies from approximately 30 years ago have indicated that around 30% of women aged 40 or older have ductal carcinoma *in vitro* (DCIS), a precancerous lesion, although roughly 1 in 8 women are actually diagnosed with breast cancer²⁸¹. Already by this stage, cells in DCISs are laden with many of the same mutations and genetic rearrangements that are found in invasive breast cancer²⁸², indicating that mutational load may not be sufficient for breast carcinogenesis²⁸⁰.

Meanwhile, evidence implicating the steroid hormones, estrogen and progesterone, and their nuclear receptors as drivers of this disease can be inferred from the fact that the prevalence of breast cancer is on par with colon cancer until a woman reaches menopause, when incidence rate drops significantly²⁸³. Furthermore, supplementation of estrogens in combination with progestins (progesterone analogs), which is typical for hormone replacement therapies, increases breast cancer risk²⁸⁴, possibly due to cell proliferation in the breast epithelium²⁸⁵. This may be directly related to the increased stability of progestins²⁸⁰ and their ability to activate other nuclear receptors²⁸⁶, as supplementation with estrogens alone apparently poses no additional cancer risk²⁸⁷.

Exposure of breast epithelial cells to estrogen or progesterone can induce expression of *CCND1* (cyclin D1)^{288,289}, which regulates G1-S cell cycle progression *via* cyclin-dependent kinase (CDK)-dependent and -independent functions^{290,291}. Cyclin D1 is an established oncogene and is overexpressed in up to 50% of breast cancers, with high correlation to estrogen receptor (ER)-positive cancer cells^{291,292}. Thus, in an intricate relationship with ER, cyclin D1 overexpression is believed to foster breast cancer proliferation by increasingly diverse mechanisms²⁹².

1.5.2 Therapeutic options for HR+ breast cancer

In the clinic, breast cancers expressing one or both of these receptors are known as hormone receptor (HR)-positive and account for 60-75% of breast cancers (ER+ and 65% of these are also progesterone receptor [PR]+)²⁹³. Additionally, stratifying patients based on combined ER/PR status instead of expression of either alone may be a better discriminator of prognosis²⁹⁴. The current standard-of-care for HR-positive breast cancers includes surgery, followed by radiation and endocrine therapy²⁹³. Endocrine therapies target the ER: tamoxifen and raloxifene are competitive ER antagonists, while fulvestrant binding causes ER degradation. Alternatively, aromatase inhibitors block estrogen biosynthesis. However, it is known that these treatments are prone to resistance development²⁹⁵⁻²⁹⁷. Inhibitors of CDK4/6, which are regulated by cyclin D1, are also showing promising activity as breast cancer treatments, although resistance mechanisms to this novel therapy are already emerging²⁹⁸. All of these treatment options target the cell proliferation spurred by active hormone receptors.

1.5.3 Regulation of hormone-dependent transcription

To transcribe genetic material, transcriptional machinery must be able to access genomic DNA. For this to occur, chromatin needs to be rearranged by direct modification of histones and the help of chromatin remodeling enzymes^{175,299}. PARylation (polyADP-ribosylation) is one such modification regulating chromatin structure³⁰⁰, predominantly *via* PARP1^{301,302}. PARP1 activity is crucial for gene regulation³⁰³, especially during development³⁰⁴, neurogenesis³⁰⁵ and by activated nuclear receptors³⁰⁶, such as the ER³⁰⁶ and PR^{171,174}.

Transcription of estrogen-dependent genes appears to involve the concerted efforts of a DNA topoisomerase II β (TOPOII β):PARP1 complex (**Figure 12**)³⁰⁶. Interestingly, in order to transcribe the estrogen-responsive *pS2* gene, a transient DNA double strand break (DSB) formed by TOPOII β within the estrogen responsive element (ERE) of the *pS2* promoter on nucleosome E (NucE) activates PARP1 to PARylate histone H1. Modification of histone H1 by PARylation facilitates exchange with high mobility group B 1/2 (HMG1/2). Another possible explanation for exchange of histone H1 with HMG1/2 is that PARP1 competes with histone H1 for binding to nucleosomes and causes its exclusion³⁰³. Consequently, replacement with HMG1/2 permits repair of the DSB by the DNA-PKcs:Ku70/86 canonical non-homologous end joining (NHEJ) repair complex and access of ER α (along with its transcriptional coactivators) to transcribe the *pS2* gene product³⁰⁶.

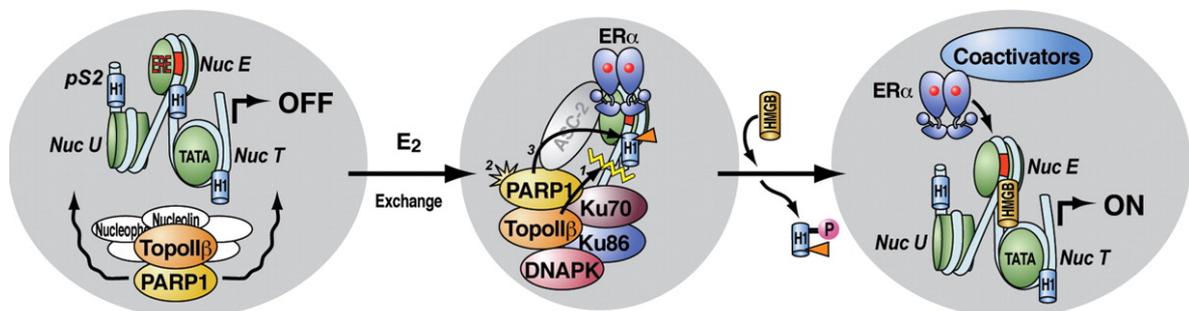


Figure 12: PARP1 and TOPOII β cooperate to enable transcription of estrogen-responsive genes. Transient DSBs induced by TOPOII β activate PARP1, which facilitates replacement of linker histone H1 with HMG1/2 and access by ER α transcriptional machinery. Reprinted with permission from the American Association for the Advancement of Science, from Ju *et al.*³⁰⁶.

Roughly 85% of progestin-responsive genes depend on functional PARP1 for their transcription¹⁷⁴. The remodeling of chromatin in response to the progestin, R5020, is transient (occurring over approximately 30 minutes) and mediated by a cohort of remodeling complexes (**Figure 13**)^{174,307}. Initially, phosphorylated PR is recruited along with the nucleosome remodeling factor (NURF) and ASC-2/NCOA6 (ASCOM) complexes to chromatin, where they facilitate removal of the heterochromatin protein 1 gamma (HP1 γ)/lysine demethylase 5 (KDM5) repressive complex. Simultaneously, the PR:CDK2 complex binds to cyclin A and PARP1, and CDK2 phosphorylates PARP1 on Serine 785 and 786, which, in turn, activates its PARylation activity. PARP1:CDK2 then phosphorylates/PARylates histone H1, preceding its displacement from chromatin³⁰⁸. Histone H2A/H2B is then displaced by ATP-dependent chromatin remodeling *via* the P300/CBP-

associated factor (PCAF)/PR-SWI/SNF (BAF) complex. Following additional PARylation of other chromatin targets by PARP1, PAR chains are degraded and chromatin is returned to a repressive state following transcription^{174,307}.

The involvement of PARP1 in these diverse modes of transcriptional regulation and the varied mechanisms resulting in its activation highlights the essential nature of PARP1 catalytic activity for these processes. For example, PARP1 is activated by transient DNA damage for ER-mediated transcription, whereas it is activated by phosphorylation *via* CDK2 in response to progestins^{171,306}. A central theme is the PARylation of linker histone H1, which after PARP1 itself, is one of the most heavily PARylated proteins in cells^{300,309}. PARylation of histones causes loss of affinity for DNA and initial relaxation of chromatin structure^{300,310}. This, in turn, facilitates further rearrangements by ATP-dependent chromatin remodeling complexes and completes access to DNA by transcriptional machinery³⁰⁷.

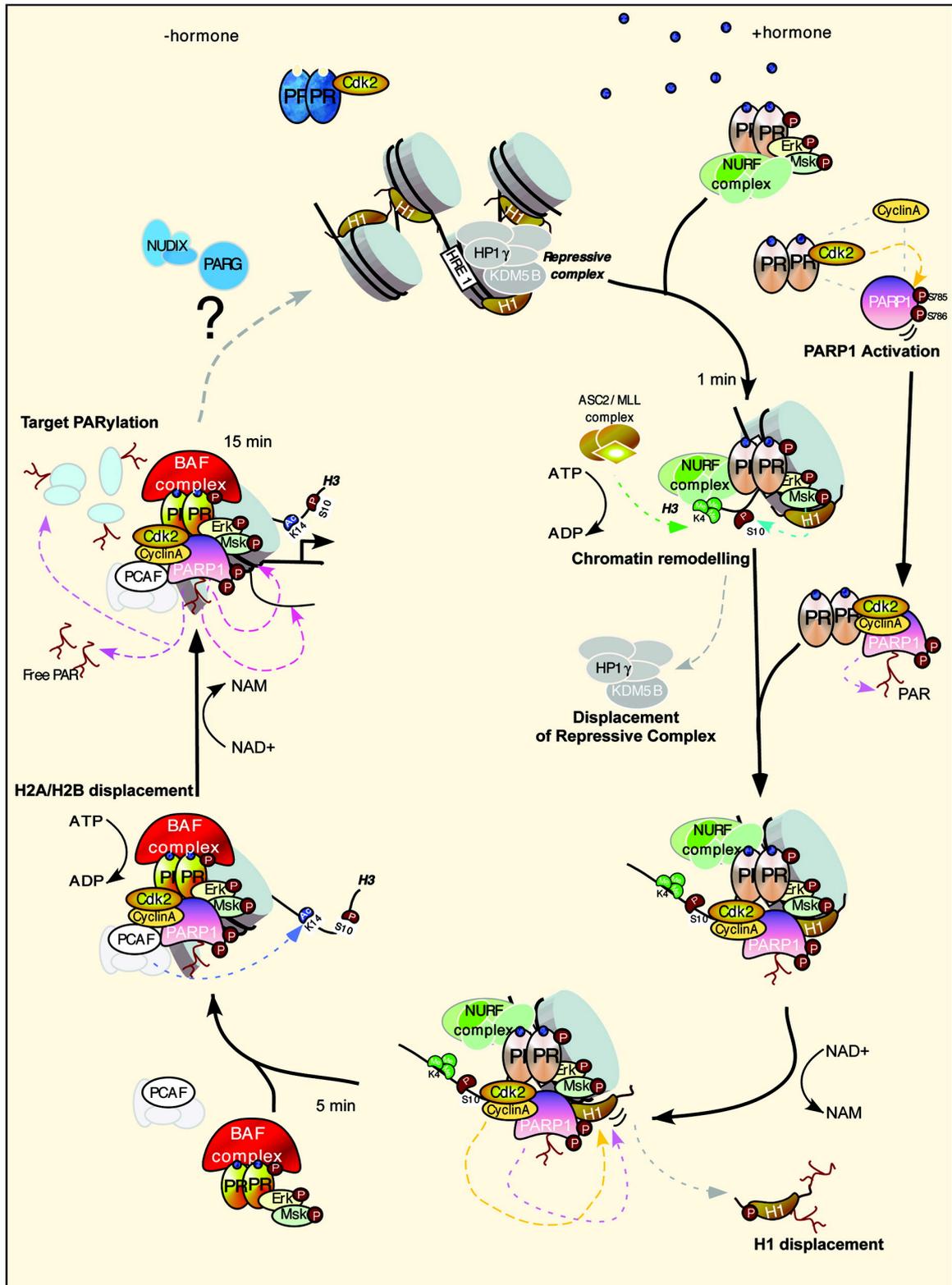


Figure 13: Progestin-mediated transcriptional events are dependent on PARP1 and CDK2. Following initial removal of the HP1 γ /KDM5B repressive complex, activation of PARP1 by CDK2:cyclin A results in histone H1 displacement, facilitating histone H2A/H2B displacement by chromatin remodeling complexes and progestin-dependent gene transcription. Reprinted with permission from Taylor & Francis Online, from Wright and Beato³⁰⁷.

1.6 NUCLEAR ATP SYNTHESIS

For decades, it has been a foregone conclusion that the ATP that fuels all of the cell's activities arises from oxidative phosphorylation in mitochondria, the powerhouse of the cell. However, nuclear ATP synthesis is a phenomenon that was first described in 1955 by Allfrey and Mirsky during their studies of protein synthesis in isolated thymus nuclei^{311,312}. Nuclei were found to “possess a capacity for aerobic ATP synthesis”³¹¹ that could be inhibited by anaerobic conditions, similar to mitochondrial oxidative phosphorylation, but not by other agents that also blocked ATP synthesis in mitochondria³¹². Similarly, work by Betel suggested that the ribose-phosphate metabolized from nucleotides or nucleic acids may be responsible for this occurrence³¹³. Interestingly, when the nuclei were treated with DNase they were unable to synthesize ATP, but this could be rescued by the addition of polynucleotides³¹², thus indicating that nucleic acids were the source of nuclear ATP generation that could be used to power nuclear functions.

Years later, Sei-ichi Tanuma found that, in the presence of PP_i and magnesium, degradation of PAR in HeLa S3 cell nuclei could produce ATP and R5P, which was catalyzed by a mysterious ADP-ribose pyrophosphorylase³¹⁴. The author proposed that the catabolism of PAR to ADPR by PARG propelled the synthesis of ATP from ADPR and PP_i . Intriguingly, addition of exogenous ATP, AMP, fluoride (phosphatase inhibitor) or R5P blocked the ATP synthesis reaction. It was hypothesized that, in addition to modifying chromatin structure, PAR functions as a reservoir for ATP, which may be utilized for ATP-dependent processes, such as DNA repair, replication and transcription.

Subsequently, the same group reported that the ATP produced from PAR by the concerted efforts of PARG and ADPR pyrophosphorylase was required for DNA repair synthesis in isolated HeLa S3 nuclei³¹⁵. Cells synchronized in G1-phase and treated with alkylating agent, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), were found to generate ATP following PAR catabolism. This ATP was utilized for repair-mediated DNA synthesis within minutes of MNNG treatment, as measured by ³H-thymidine incorporation. In the absence of PP_i or with the PARG inhibitor, Oen B, ATP synthesis was blocked, as was previously described³¹⁴, but so was DNA synthesis for repair. Roughly 8% of the PAR was converted to ATP, which was maximal at around 20 minutes. A similar follow-up report demonstrated that nuclei supplemented with ATP in the presence of PP_i could synthesize DNA normally, while loss of DNA synthesis occurred if a PARG inhibitor was used or if PP_i was excluded from the reaction mixture³¹⁶. This would collectively suggest that ATP specifically generated from PAR catabolism and PP_i is required to maintain DNA replication.

A direct requirement for PAR-derived ATP synthesis was similarly shown for DNA ligation following BER³¹⁷. The findings were generally analogous to those presented before but with a few noteworthy differences: 1) the production of ATP from PAR required active DNA synthesis, which can generate copious amounts of PP_i (although significantly less ATP could also be synthesized from exogenous addition of PP_i), 2) only ADPR formed from PAR catabolism could create ATP (addition of exogenous ADPR could not generate ATP), 3) the

ATP generated during nick-induced DNA synthesis is specifically utilized for DNA ligation, which is ATP-dependent and rate-limiting for completion of BER. Thus, in replicating cells, PAR can act as an energetic store that is tapped under shortage of cellular ATP to complete DNA repair.

More recently, work elucidating a mechanism for nuclear ATP synthesis from PAR-derived ADPR and PP_i was described¹⁷¹. Catabolism of PAR formed from the stimulation of quiescent breast cancer cells by estradiol or progestin could generate nuclear ATP, which was required for ATP-dependent chromatin remodeling and gene regulation. The enzyme responsible in this phenomenon was NUDT5, previously known only to hydrolyze ADPR to AMP^{150,151}. In response to hormone, NUDT5 formed a complex with NMNAT1, PARP1 and PARG, thus completing a self-sufficient unit that can singlehandedly process NAD^+ , PAR, ADPR and ATP. Therefore, PAR-derived ATP catalyzed by NUDT5 may be formed at local sites of chromatin, where immediate influx of ATP is required to expose transcriptionally accessible DNA.

2 DOCTORAL THESIS

2.1 PURPOSE/AIMS

Many of the human NUDIX enzymes outside of MTH1 are poorly understood, but some have been implicated in oxidized nucleotide sanitation *in vitro*, including NUDT5 and NUDT15. **Therefore, the overall aim of this thesis is to elucidate the biological function(s) of NUDT5 and NUDT15 in human cells and to evaluate their potential as therapeutic targets for small molecule inhibitors.**

The specific aims are as follows:

- Identify the biological function(s) of NUDT5/NUDT15 with specific focus on their involvement in oxidized nucleotide metabolism, as well as NAD/nucleotide and thiopurine metabolism, respectively
 - Identify substrates with biochemical substrate screening
 - Discern the effects of NUDT5/NUDT15 depletion on proliferation, cell cycle, DNA damage, cell death and oxidized nucleotide metabolism in cells
 - Relate biochemical and cellular data
- Evaluate the potential involvement of NUDT5/NUDT15 in human disease(s) and as therapeutic targets, particularly for cancer
- Assess and develop potent, specific small molecule inhibitors for NUDT5/NUDT15 to further study their biological functions and potential for therapeutic targeting

The above-mentioned aims were addressed in the component papers of this thesis and clarified by answering specific research questions, as follows:

Paper I

- Does NUDT15 (MTH2) possess similar substrate preferences to MTH1 *in vitro*?
- Does the NUDT15 crystal structure explain the observed substrate preferences and why it is similar or different from MTH1?
- Does depletion of NUDT15 by RNAi in cells phenocopy or enhance MTH1 depletion?
- Do the collective data suggest that NUDT15 is important for oxidized nucleotide sanitation, similar to MTH1?
- Are there substrates for NUDT15 that indicate its physiological importance in cells?

Paper II

- What is the catalytic efficiency of NUDT15 towards 6-thio-GTP and 6-thio-dGTP compared to a known, endogenous substrate (dGTP)?
- Can the co-crystal structure of 6-thio-GMP in the NUDT15 active site explain the preference for thionylated guanosine triphosphates?

- Does the NUDT15 R139C missense variant affect catalysis of substrate hydrolysis *in vitro*?
- Does the NUDT15 R139C mutant express normally in cells? How does it compare to wild-type protein expression?
- What is the cause of lower basal expression of the R139C mutant in cells?
- How does NUDT15 expression affect cellular sensitivity to 6-thioguanine? Can depletion of endogenous, wild-type NUDT15 mimic the enhanced toxicity to thiopurines seen in NUDT15 mutant patients?

Paper III

- What are the preferred substrates for NUDT5 *in vitro* and how does the profile compare to MTH1?
- Do RNAi experiments support the biochemical substrate profiles, particularly with regard to oxidized nucleotide sanitation and ADP-ribose metabolism?
- Following medicinal chemistry optimization of hits from high-throughput screening, can cellular target engagement techniques be used to identify potent and cell-active molecules in lieu of phenotypic assays?
- How can the structure of NUDT5 improve the design of our NUDT5 inhibitors?
- Can top NUDT5 inhibitors identified by target engagement profiling confirm the emerging role of NUDT5 in hormonal gene regulation in breast cancer cells? Do the results match potency rankings from the target engagement analyses?

2.1 RESEARCH APPROACH

The approaches to address the research questions of the doctoral thesis were truly multidisciplinary in nature and were comprised of multiple biochemical, biophysical/structural, molecular and cell biology and chemical biology methodologies. In this way, research questions could be addressed by several orthogonal methods and explored more in detail. Generally, the design of the research approach began from biochemical and structural understanding of NUDT5 and NUDT15, which then was related to phenotypic observations in cells by RNAi and with potent, small molecule inhibitors synthesized in the Helleday Laboratory. This process is outlined below:

1. Biochemical evaluation with purified NUDT5 and NUDT15
 - a. Substrate analyses by coupled enzymatic assay (malachite green) or high-performance liquid chromatography (HPLC)
 - b. Structural insight to substrate preference by *in silico* docking and co-crystallography with substrates or products
2. RNAi-mediated ablation in cells – relate back to biochemical results
 - a. Investigate general effects on cell proliferation, survival, etc.
 - b. Discern influence on oxidized nucleotide sanitation by analyzing markers of DNA damage and by modified alkaline comet assay
 - c. Confirm phenotypic changes relate to substrate preferences *in vitro* – or not
 - d. Cytostatic or cytotoxic effects on cancer cell lines?
3. Compromise enzymatic activity with small molecule inhibitors – relate back to biochemical and RNAi data
 - a. Confirm inhibition of enzyme by biochemical assays
 - b. In addition to the RNAi points listed above, confirm intracellular target engagement and specificity
 - c. Explore utility of inhibitors in combination with other cytostatic or cytotoxic agents
4. Follow-up studies for confirmation of clinical relevance in animal models of disease (future work)

2.2 KEY METHODOLOGIES

2.2.1 The OGG1 modified alkaline comet assay

2.2.1.1 Background

The comet assay has had many different variations published over the years but was first described by Östling and Johanson in 1984 to measure repair of DNA breaks following ionizing radiation (IR)³¹⁸. The assay was based on the premise of the nucleoid structure observed upon loss of DNA supercoiling, which can be measured using electrophoresis³¹⁹. Although the pH 9.5 buffer used was clearly basic, DNA is not denatured at this pH, so it is often referred to as a “neutral” comet assay³²⁰. Singh *et al.* then first described the alkaline comet assay (pH>13) in 1988³²¹, which is capable of converting more lesions to DNA breaks. Thus, the alkaline comet assay is suitable to detect a wider range of DNA lesions, namely, alkali-labile sites (ALS), which includes apurinic/apyrimidinic (AP) sites, and DNA-DNA/DNA-protein cross-links³²⁰. In this way, the alkaline comet assay can provide more information regarding genotoxic treatments and is typically preferred³²².

That being said, there are many perceived misconceptions with regard to the variations of the comet assay, the most common of which stipulates that the neutral comet assay only detects DNA DSBs and the high pH of the alkaline comet assay is required to detect DNA SSBs^{322,323}. IR, the insult used to exemplify both the neutral and alkaline comet assay^{318,321}, induces many more SSBs than DSBs³²⁴ and give reproducible comets by either assay. Similarly, this misconception has been further put to rest by other studies with hydrogen peroxide and methyl methane sulfonate (MMS) that demonstrated the same result³²⁵.

Other variants of the alkaline comet assay emerged that included incubation steps with DNA glycosylases^{320,322,323}, including those associated with oxidation damage surveillance, such as formamidopyrimidine DNA glycosylase (Fpg)³²⁶, OGG1³²⁷ and endonuclease III (Endo III)³²⁸; repair of UV-induced damage, such as T4 endonuclease V^{329,330}; and repair of uracil incorporation in DNA, such as UNG³³¹. These inclusions have given added value to the comet assay, particularly with Fpg being utilized for human biomonitoring of oxidative damage to DNA³²², but also to mechanistic studies affecting metabolism or repair of specific glycosylase substrates.

2.2.1.2 General protocol (**Figure 14**)

Cells are treated in culture and then harvested. Following washes with PBS, the cells are embedded in low melting point (LMP) agarose. Prior to this point, one can briefly treat cells on ice with hydrogen peroxide, potassium bromate, or the photosensitizer, Ro19-8022, and light as positive controls for the assay^{322,327,332,333}. An initial layer of agarose is applied to a microscope slide, followed by the layer containing the cells of interest. The cells are then lysed with buffer containing Triton X-100 for at least 1-2 hours, but typically overnight, although lysis time appears to bear little effect on assay performance³²². The cells are then incubated with enzyme buffer as a control or human OGG1 to assay for oxidized nucleotide

lesions in the DNA³²⁷. Next, the slides are submerged in alkaline buffer (pH>13) prior to electrophoresis in a comet assay tank. The incubation time prior to electrophoresis, as well as voltage and the duration of electrophoresis, has the greatest effect on assay variability^{322,334,335}; therefore, incubation times were kept at 30 minutes and electrophoresis was constant at 25V for 30 minutes in all experiments. The cells are then incubated in a Tris-buffered neutralization solution to stop the alkaline reaction. At this point, the slides can be kept in a humidified chamber at 4°C for several weeks; however, drying the slides prior to analysis may improve visualization on the microscope³²². Addition of a fluorescent DNA dye, such as SYBR Gold or YOYO-1, prior to microscopy will permit visualization of the DNA. Analysis of the comets is performed with the aid of software, such as Comet Assay IV or OpenComet. Supercoiled DNA is found in the head and freed DNA loops and fragments comprise the comet “tail”³³⁶. Several metrics have been utilized to measure comets, including tail moment, Olive moment, and percent DNA in the tail^{337,338}, but use of percent DNA in the tail is preferred since it is proportional to DNA break frequency^{322,338}. Detailed protocols for the OGG1 modified comet assay used in the thesis work are found in **Papers I and III**.

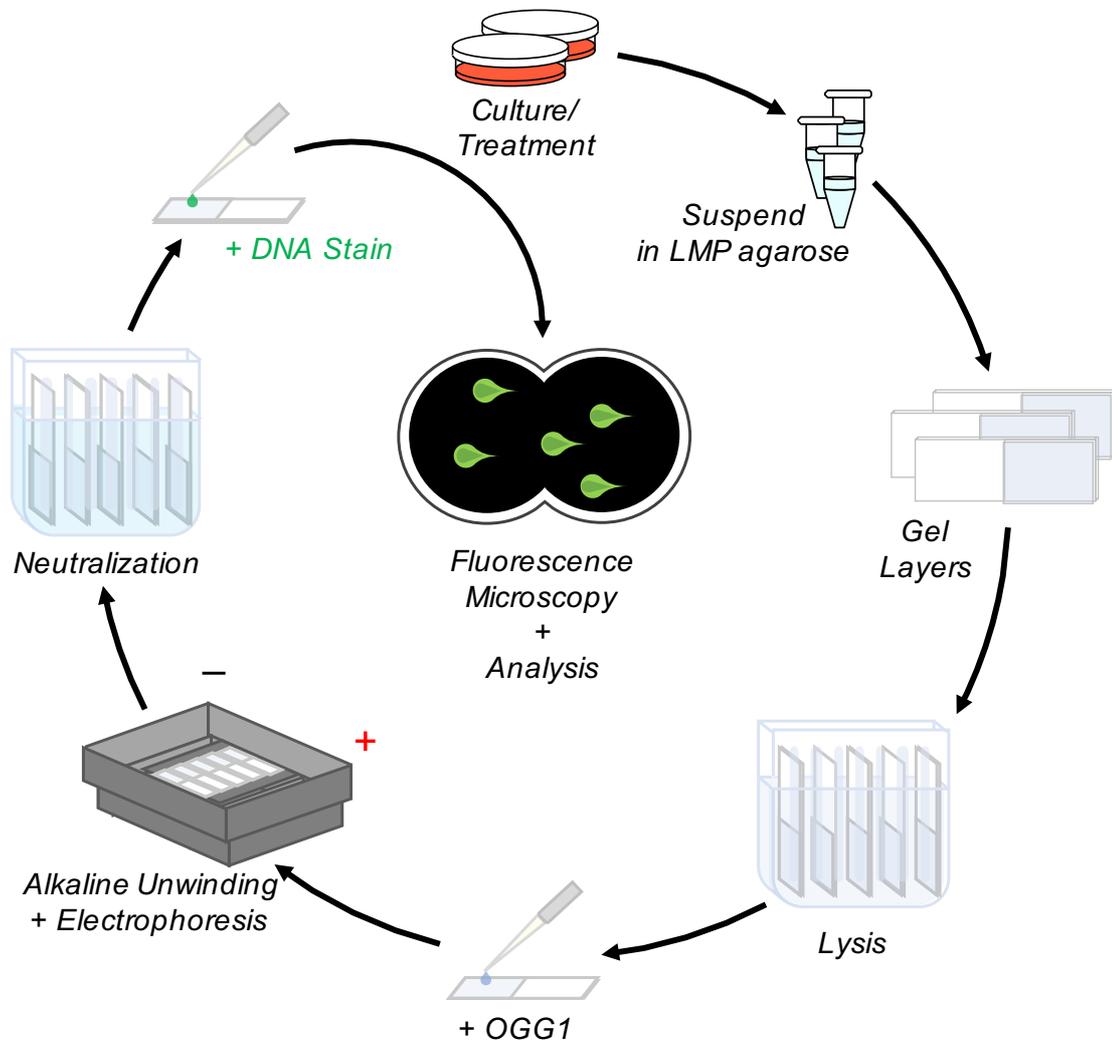


Figure 14: Graphical procedure of the modified (+ OGG1) alkaline comet assay. All points from the lysis step onwards are performed in the dark to limit background exogenous DNA damage.

2.2.1.3 *Utility in the current studies*

The issue of accurately measuring 8-oxo-guanine is well known and is why the European Standards Committee on Oxidative DNA Damage (ESCODD) was established in the 1990s^{320,322,325}. Depending on the assay used, chromatographic methods could detect orders of magnitude higher 8-oxo-guanine than enzyme-based methods, such as the comet assay³²⁵. As 8-oxo-guanine is among the most common byproducts of oxidative damage and is useful as a biomarker for human diseases, it was vital to identify the most accurate method of detection, especially for endogenous, background levels. Chromatographic methodologies, such as HPLC or gas chromatography-mass spectrometry (GC-MS), are expected to be most accurate, due to their detection limits. These methods were compared with the alkaline elution, alkaline unwinding and comet assays for the ability and sensitivity in detecting 8-oxo-guanine from multiple sources³³⁹⁻³⁴². Antibody staining for 8-oxo-guanine, however, was not included in the evaluation. The results indicated that chromatographic methods, particularly HPLC, were more sensitive for detecting differences in 8-oxo-guanine in a dose-dependent manner, especially for samples treated with exogenous oxidation^{320,322,340}. However, the methods were unsuitable for measuring endogenous 8-oxo-guanine due to oxidation artifacts introduced during sample preparation³⁴⁰. Thus, while the comet assay is less sensitive than chromatographic means, it is very effective for measuring background 8-oxo-guanine lesions³²².

The OGG1 modified alkaline comet assay had the most utility in the studies conducted for this thesis work due to its selectivity for 8-oxo-guanine³²⁷ and the tightknit relationship of MTH1, NUDT5 and NUDT15 to this nucleobase. From past studies, it was known that MTH1 depletion or inhibitors can induce selective comet tails in OGG1-treated samples^{124,129}, suggesting an increased presence of 8-oxo-guanine in DNA, thus, they were used as controls.

2.2.1.4 *Issues/Complications*

One of the overarching issues with the comet assay, which has been touched upon in numerous reviews and commentaries, is the fact that there is very little standardization of the protocol³²⁰. Studies conducted to evaluate inter-laboratory variability of the comet assay showed that there was very little variability within the same lab, but, perhaps not surprisingly, there were major disparities among different labs³⁴³⁻³⁴⁷. The conclusions on the source(s) of discrepancy ranged from image analysis, staining, or protocol used^{343,344} to the duration of enzyme incubation³⁴⁸. Scoring of comets can also be time-consuming and subjective, especially if automated software is not used³³⁷. In light of the number of variations and modifications to the comet assay that have been introduced over the years, it is no surprise that this problem becomes magnified. The ESCODD and European Comet Assay Validation Group (ECAVG) consortia have highlighted these issues and have laid forth a framework for assay standardization. In principle, standardization should be extended to all variations of the comet assay to minimize problems with reproducibility among independent groups.

The second major problem arises from technical complications while performing the comet assay³³⁷. Among the most frequent issues are gels detaching from microscope slides and unexplainable variations in comet appearance, such as random highly damaged cells, large tails detected in unexposed control samples and comet tails extending in different directions³³⁷. Prolonged lysis times are known to increase the propensity of gels detaching from microscope slides, so care should be taken when extending lysis for longer than a day³²². In some cases, especially when monitoring endogenous 8-oxo-guanine in DNA, we found that cells that had been in active culture for more than 3 or 4 weeks gave high background signals that masked the effects of adding OGG1 in the modified alkaline comet assay. Another important source of technical variability can appear in modified forms of the comet assay, where differences in purity or activity of glycosylase purification can have lasting impacts on comet measurement and inter-experimental variability³²⁰.

2.2.2 The cellular thermal shift assay (CETSA)

2.2.2.1 Background

In general, the efficacy of a candidate drug molecule can be directly related to its ability to bind specific target(s) inside cells³⁴⁹⁻³⁵². Adverse toxicity may arise when target proteins become saturated by a ligand in certain cell types or may result from non-specific binding to other proteins or macromolecules. Thus, information regarding the potency and specificity of target engagement within cells is invaluable to drug development and clinicians³⁴⁹⁻³⁵².

The cellular thermal shift assay (CETSA) was born from the biophysical principle that ligands bound to a protein of interest can result in thermal stabilization³⁵³⁻³⁵⁷. As a particular protein is heated, the energy infusion will eventually cause unfolding and aggregation as the intramolecular bonds are broken. The temperature at which 50% of protein is aggregated is known as the aggregation temperature (T_{agg})^{356,357}. The thermal shift of proteins by a ligand is likely entropically-driven, as increasing ligand concentration raises the probability that the protein of interest is not unfolded^{358,359}. Therefore, data from CETSA can complement thermal shift assays performed *in vitro*. Detection of a specific protein is possible with antibodies, and the typical readouts are western blotting (for lower-throughput studies) and AlphaScreen® technology, which utilizes antibody-conjugated donor and acceptor fluorescent beads and is best suited for higher-throughput screening applications³⁵⁷. The recent demonstration of its utility as a primary screening assay was reported in the search for stabilizers of TS³⁶⁰, whereas its use for monitoring target engagement *in situ* was also recently described³⁶¹. Importantly, CETSA, like other cellular target engagement methodologies, permits indirect relation of target occupancy to relevant phenotypic events³⁵⁷.

More recently, the principles of CETSA have been combined with the power of mass spectrometry to create a new technique known as thermal proteome profiling (TPP or mass spectrometry CETSA [MS-CETSA])^{362,363}. This advancement has increased capacity for target engagement analysis from one protein of interest to nearly the entire proteome (roughly 7000 proteins) in a single experiment. While the traditional CETSA assay is great for monitoring target engagement of a particular protein, TPP can provide information regarding off-target interactions of a given ligand and identify previously unknown targets of drugs currently used in the clinic^{362,363}. Along these lines, phenotypic alterations and responses to an inhibitor or ligand may be inferred from clustering and mapping of the thermal proteomic profile³⁶³. Altogether, this showcases the potential power of CETSA when applied to the cellular proteome.

Therefore, in many shapes and instances, the development of CETSA has transformed the way academia and industry approach and prioritize confirmation of cellular target engagement. The core methodology itself is straightforward and can be performed in virtually any laboratory that has a ligand for testing, high affinity antibodies for particular target(s) of interest and a gradient polymerase chain reaction (PCR) machine. As further applications of

the CETSA assay evolve and alternative assays spurred by its success arise, the scientific community as a whole will reap the benefits.

2.2.2.2 General protocol (*Figure 15*)

The basic principle of CETSA implies that two essential steps must occur: 1) Heating of cell samples in the absence or presence of a ligand to denature protein that is not bound by the ligand and 2) separation/clarification of aggregated protein from soluble, stabilized protein that is detected by an antibody³⁵⁷. Outside of these two basic tenets, there are many steps within the protocol that can be altered and require empirical optimization, as the biophysical properties of proteins and potential ligands vary tremendously³⁵⁷. In addition, there are several flavors of the CETSA assay that will yield different information regarding a ligand's potential affinity for a protein of interest. First off, CETSA can be performed with intact cells or cell lysates, which, due to gross changes in environmental complexity, usually results in different melting temperatures for a given protein^{356,357,362,363}. Traditional CETSA is predicated on a dose-dependent shift in a protein's melt curve (T_{agg}) by a given ligand, whereas relative stabilizing potential and affinity may be evaluated by isothermal dose-response fingerprint CETSA (ITDRF_{CETSA})^{356,357}. ITDRF_{CETSA} permits comparisons of ligand potency by monitoring protein stability at a single temperature.

For the sake of simplicity, the protocols employed in **Paper III** used the originally-described settings for heating to investigate NUDT5 inhibitors, namely a 3-minute heating duration^{356,357}. CETSA with cell lysates was performed by collecting HL-60 leukemic cells, washing them with PBS, and resuspending them in Tris-buffered saline (TBS) complemented with protease inhibitors. The addition of protease inhibitors ensures degradation of proteins does not impact protein levels following cell lysis. In some cases, where other post-translational modifications may influence protein stability, phosphatase or deubiquitination inhibitors may also be added to the buffer to ensure their integrity³⁶⁴. Next, the cells were aliquoted in PCR tubes and lysed by freeze-thaw cycles with a dry ice/ethanol bath and 37°C water bath. To clarify the cellular debris from the released proteins, centrifugation was performed and the lysates were transferred to new PCR tubes. Clarified lysates were then incubated with NUDT5 inhibitors of interest or the equivalent volume of dimethyl sulfoxide (DMSO) for 30 minutes at room temperature to allow for inhibitor binding equilibration. Following incubation, the samples were heated in a gradient PCR machine at the given temperatures for 3 minutes, followed by a 3-minute temperature equilibration step at room temperature. Another high-speed centrifugation step pellets the aggregated proteins, and the resulting lysate is used for western blotting with anti-NUDT5 antibody and anti-superoxide dismutase 1 (SOD1) antibody, as a loading control due to its high thermostability³⁶⁵.

Experiments with intact HL-60 cells, including the ITDRF_{CETSA} studies, followed many of the same steps as above. Particular differences included the treatment of cells in culture for 3 hours with DMSO or NUDT5 inhibitor. When collecting for analysis, the cells were washed twice with PBS to remove excess compound. This was a necessary step because cell membranes are ruptured at higher temperatures, including the range where NUDT5

stabilization is seen^{356,357}. The cells were prepared in TBS buffer with protease inhibitors as before, heated for 3 minutes, cooled, and immediately freeze-thawed to lyse the cells. Following centrifugation, the clarified protein lysates were analyzed by western blotting. The ITDRF_{CETSA} experiments were performed at 83°C, as most of the NUDT5 protein had aggregated at this temperature.

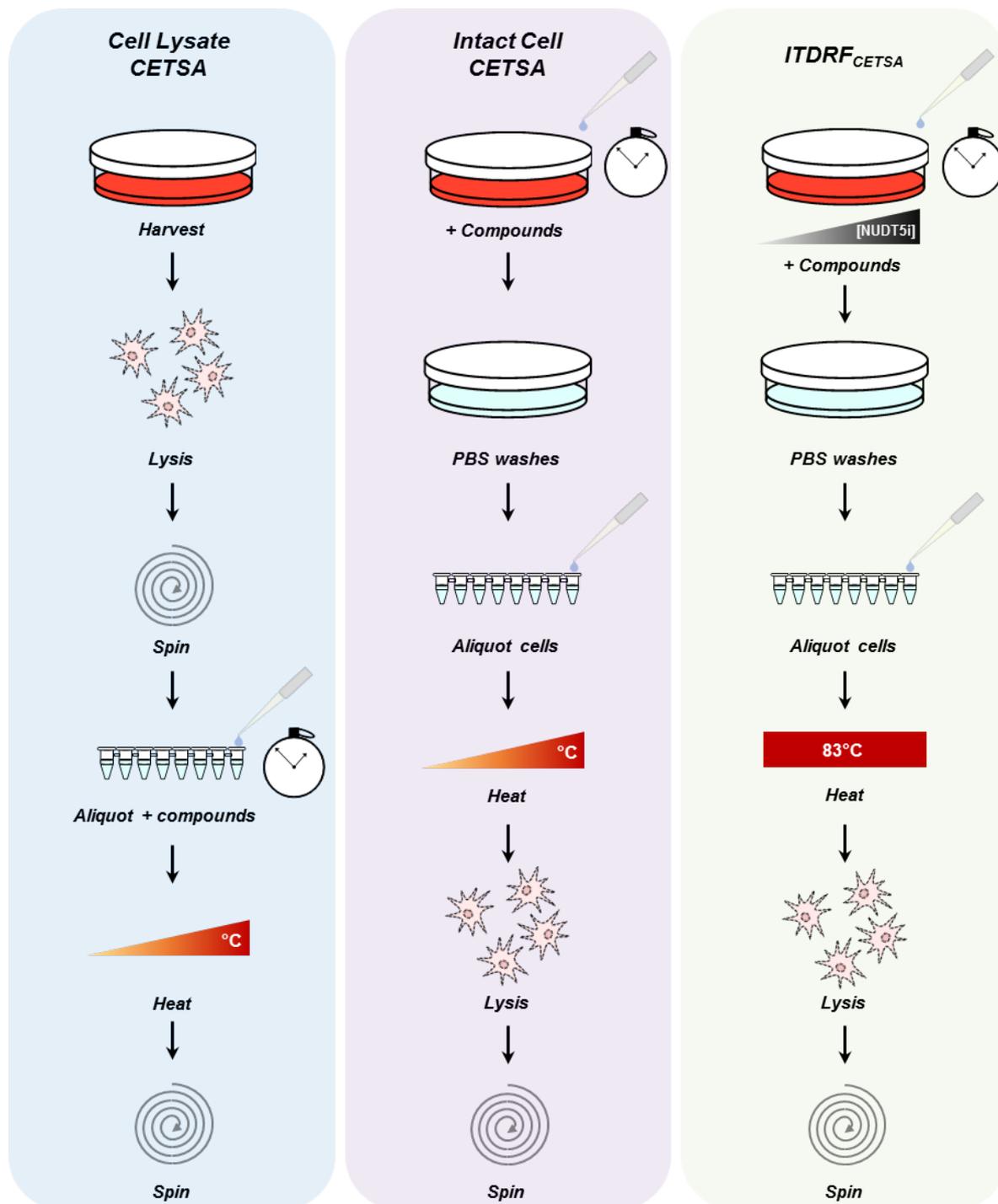


Figure 15: Graphical procedure for CETSA experiments performed during the thesis work. Prior to lysis or heating, all cells were resuspended in TBS complemented with protease inhibitors to minimize proteolysis during sampler preparation.

2.2.2.3 *Utility for the current studies*

CETSA ended up being a crucial assay for evaluation of NUDT5 inhibitors. Due to the lack of information regarding its biological functions at the time, functional or phenotypic screening assays were simply not possible. However, CETSA gave us the opportunity to evaluate the NUDT5 inhibitors in cells without the need of understanding its function.

We realized early on that gradation of the CETSA assay was possible and could be useful to screen our NUDT5 inhibitor library to distinguish the most active compounds. The screening funnel we devised started with NUDT5 stabilization in cell lysates, followed by stabilization in intact cells, and, lastly, qualitatively ranking inhibitors that cleared the first two phases by their ability to stabilize NUDT5 at the lowest treatment dose with ITDRF. To facilitate more rapid screening of our inhibitor library, we utilized an isothermal temperature of 83°C to evaluate NUDT5 inhibitors at a single 20 µM dose and compared NUDT5 protein levels to a 37°C control and 83°C DMSO-treated control. This, in effect, gave relative stabilizing ability without requiring full melt curve analyses for every compound. Importantly, however, one cannot accurately determine relative affinity from these single points, as the aggregation curves may differ. Absolute affinity can be derived from dose-dependent shifts in the melting curves (not magnitude of shifts alone), whereas relative comparison is possible with ITDRF^{353,356}. As a result of this CETSA-guided approach, we identified our lead inhibitor, TH5427, and confirmed its ability to dose-dependently shift the T_{agg} of NUDT5.

2.2.2.4 *Issues/Complications*

Despite all of the abilities that CETSA has introduced, there are still drawbacks inherent to the fundamentals of the concept or that may be improved upon with newer iterations of the assay. The key for CETSA to work as advertised is for the user to have access to a high affinity antibody to probe for a native protein³⁵⁷. In many cases, as with proteins that are not commonly studied or in high demand, obtaining good antibodies may prove difficult. Using a less-than-acceptable antibody will complicate all iterations of the CETSA assay and convolute interpretation of results. One way of getting around this is to express a recombinant protein that is fused to an epitope tag. Antibodies recognizing epitope tags are commonplace and alleviate affinity concerns, as we have experienced in our lab with other projects. Nonetheless, the user would have to engineer cell lines in order to achieve this workaround.

The number of variables required for optimization of a CETSA protocol for a particular protein may be very complex. These areas include ligand exposure times, heating temperature, and duration of heating, among others^{357,359}. Furthermore, while CETSA is quite suitable for soluble proteins, there may be further optimization required to extract chromatin-bound or membrane-bound proteins, although measures to incorporate these classes of proteins in CETSA analyses, including addition of detergents, have been recently described^{366,367}.

2.3 SUMMARY OF RESEARCH PAPERS

2.3.1 *Crystal structure, biochemical and cellular activities demonstrate separate functions of MTH1 and MTH2*

NUDT15 (MTH2) had been proposed as a “back-up” enzyme to MTH1 that sanitizes oxidized nucleotides from the dNTP pool^{137,139,140}. The incorporation of oxidized bases into nucleic acids causes transversion mutations and, potentially, cell death^{34,41,44-49,79}. In this study, we reported that NUDT15 is very different from MTH1 and has a minimal role in 8-oxo-dGTP sanitation, which can be primarily attributed to poor fit of the 8-oxo-guanine nucleobase in the enzyme active site. This data was further verified by biochemical and cell biology experimentation to assess NUDT15 functions.

First, we explored the substrate activities of NUDT15, MTH1 and closely related NUDIX enzymes, NUDT17 and NUDT18, by the malachite green assay (MG assay) and HPLC (**Figure 16a, b**). The MG assay measures inorganic phosphate (P_i) levels in aqueous solution and was employed by coupling a NUDIX hydrolase and an additional phosphatase to generate free P_i. Compared to MTH1, NUDT15 (and the others) had virtually no hydrolysis activity towards 8-oxo-dGTP or 2-OH-dATP. Substrate saturation and HPLC experiments further confirmed that both MTH1 and NUDT15 hydrolyzed dGTP at similar efficiencies but MTH1 is approximately 230-fold more efficient at hydrolyzing 8-oxo-dGTP.

To understand if structural differences could account for the distinct substrate preferences, we next solved the crystal structure of NUDT15 to 1.8 Å by X-ray crystallography (**Figure 16c, d**). Despite high sequence similarity to MTH1, NUDT15 was confirmed to be a homodimer by both crystallography and size-exclusion chromatography. The overall structure of NUDT15 was highly similar to MTH1, but major differences in the substrate binding site of NUDT15 (namely, the orientation of Gln44 and differences in key residues forming the base of the active site) suggested that 8-oxo-dGMP would be a poor fit.

We then wanted to determine if the limited activity towards 8-oxo-dGTP *in vitro* was reflected in cells when NUDT15 was depleted by siRNA. NUDT15 depletion had no effect on overall survival in multiple cancer cell lines by clonogenic survival assay, but also did not further decrease the survival of MTH1 depleted cells. In addition, NUDT15 knockdown had no effect on cell cycle progression or proliferation (flow cytometry), and NUDT15 protein expression did not fluctuate over the cell cycle (double thymidine block and western blot). We then tested the effect of NUDT15, MTH1 or combined knockdown on induction of the DNA damage response by immunostaining for RPA and 53BP1 foci. While MTH1 knockdown increased these markers, NUDT15 knockdown had no effect alone or in combination with MTH1 siRNA. Similarly, OGG1-specific lesions were quantified using the modified alkaline comet assay, and as before, MTH1 depletion induced a significant increase in comet tail moment with OGG1 treatment¹²⁴ but NUDT15 depletion had no effect (**Figure 16e**). Cumulatively, these data suggest NUDT15 has a very different role from MTH1 in cells, as there there appears to be no effect on oxidized nucleotide metabolism.

In an attempt to identify true substrates for NUDT15, we screened a larger panel of NUDIX-like substrates by malachite green assay and HPLC (**Figure 16f**). Interestingly, NUDT15 showed moderate activity hydrolyzing the canonical nucleotides, dGTP, dTTP and dUTP, but very low activity against 8-oxo-dGTP and other oxidized nucleosides, especially when compared to MTH1. However, we also saw that NUDT15 had activity in hydrolyzing 6-thio-(d)GTP, the active species in thiopurine treatments, in line with recent reports that patients possessing NUDT15 missense mutations were hypersensitive to thiopurine treatments¹⁴⁶⁻¹⁴⁸. The crystal structure also indicated that the primary point mutation, Arg139Cys (R139C), was located within alpha helix 2 of the protein. Thus, altogether, this work demonstrated that NUDT15 is likely not an oxidized nucleotide pool sanitation enzyme like MTH1.

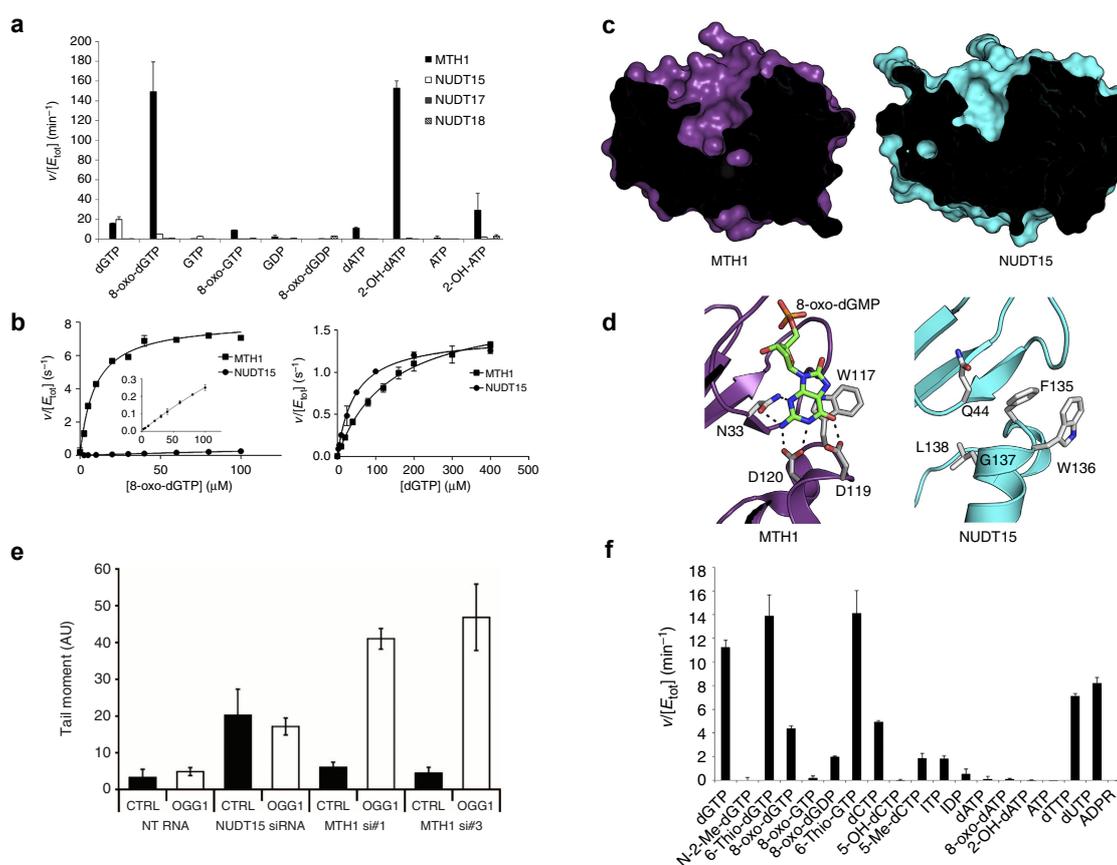


Figure 16: NUDT15 is biochemically, structurally and biologically distinct from MTH1. **a**, Enzymatic activities of MTH1, NUDT15, NUDT17 and NUDT18 against canonical and oxidized nucleoside substrates by malachite green assay. **b**, Substrate saturation curves compare the enzyme kinetics of MTH1 and NUDT15 in hydrolyzing 8-oxo-dGTP and dGTP. **c**, Comparison of the enzyme binding pockets of MTH1 (purple) and NUDT15 (cyan). **d**, Comparing 8-oxo-dGMP bound in the MTH1 active site with key binding interactions (purple) and the structural dissimilarity of the same residues in the NUDT15 active site (cyan). **e**, OGG1-specific lesions identified by modified alkaline comet assay with knockdown of NUDT15 or MTH1 in U-2 OS cells. **f**, Broadened screening of potential NUDT15 substrates by malachite green assay.

2.3.2 NUDT15 hydrolyzes 6-thio-deoxyGTP to mediate the anticancer efficacy of 6-thioguanine

From **Paper I**, we knew that NUDT15 does not have a substantive role in hydrolyzing oxidized nucleotides but could catalyze hydrolysis of thiopurine triphosphates. The ramifications of this activity, and of the recently-identified NUDT15 missense variant, R139C, on thiopurine toxicity in cells required further investigation.

In **Paper II**, using wild-type (WT) NUDT15 purified protein, we confirmed that NUDT15 hydrolyzed 6-thio-(d)GTP to 6-thio-(d)GMP by HPLC (**Figure 17a**). We then showed that the R139C mutant still possessed catalytic activity and could hydrolyze 6-thio-(d)GTP, as well as the canonical nucleotide, (d)GTP, with little difference in activity when compared to NUDT15 WT protein by substrate saturation kinetics analyses (**Figure 17b**). This suggested that the R139C mutation did not directly affect NUDT15 catalytic activity. We then confirmed that 6-thio-GTP was a NUDT15 substrate by solving the co-crystal structure of the 6-thio-GTP hydrolysis product, 6-thio-GMP, in complex with NUDT15. Overall, the binding was very similar to that of dGTP except that the preference for 6-thionylated substituents can be explained by the greater accommodation of this group by a hydrophobic pocket in the NUDT15 active site, comprised of the residues Phe135, Leu138 and Gln44³⁶⁸.

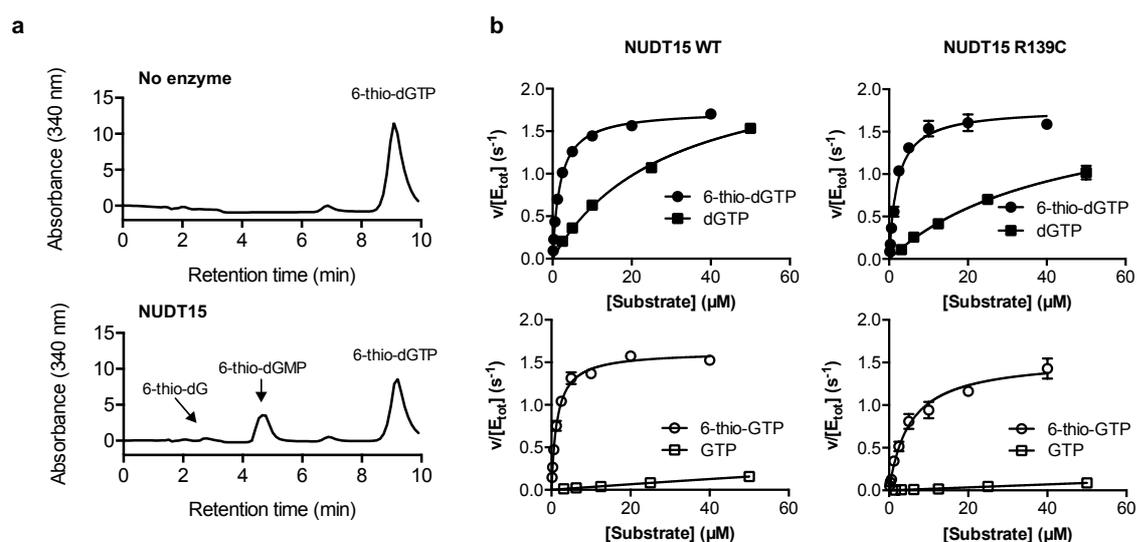


Figure 17: NUDT15 preferably hydrolyzes 6-thio-(d)GTP and the R139C missense mutant maintains catalytic function. **a**, Catalyzed hydrolysis of 6-thio-dGTP to 6-thio-dGMP and 6-thio-dG by NUDT15 with HPLC. **b**, Substrate saturation curves comparing wild-type and R139C NUDT15-catalyzed hydrolysis of 6-thio-(d)GTP compared to (d)GTP.

Next, we wanted to understand how the R139C mutation sensitized patients to thiopurines, since it is still capable of hydrolyzing 6-thio-(d)GTP *in vitro*. We then over-expressed doxycycline (DOX)-inducible HA-tagged NUDT15 WT and R139C in HCT116 colon carcinoma cells. Upon DOX treatment, WT NUDT15 protein was robustly expressed but the R139C mutant was barely detectable by western blot (**Figure 18a**). mRNA expression of WT and R139C transcripts was virtually identical, so we then considered that changes in protein turnover of R139C may be the cause. Indeed, when we treated the HCT116 cells expressing

NUDT15 WT and R139C with MG-132, a proteasome inhibitor³⁶⁹, we could rescue the protein levels as early as 3 hours following addition (**Figure 18b**). p53 protein, which is also rapidly turned over in cells by MDM2³⁷⁰, was blotted as a control. Thus, we concluded that the R139C mutant is expressed in cells but rapidly degraded by the proteasome.

Due to the fact that R139C had activity *in vitro* but was degraded when expressed in cells, we considered that the mutation, which replaces an arginine within helix $\alpha 2$ with a cysteine, may be destabilizing the protein structure. When we studied the thermal stability of NUDT15 WT and R139C by differential scanning fluorimetry (DSF), we saw that WT NUDT15 had a T_m of 58°C, while the R139C mutant had a melting temperature (T_m) of 46°C (**Figure 18c**). We also noticed that the basal fluorescence for NUDT15 R139C was drastically higher than for the WT protein. Sypro orange dye preferentially binds to hydrophobic surfaces of proteins as they unfold³⁷¹. Thus, the increased basal fluorescence from the R139C mutant suggests that the structure may be more loose and open than the WT counterpart.

Mutagenesis of Arg139 to serine or alanine had the same effect as mutation to cysteine, while mutation to lysine could mostly restore the thermal stability of NUDT15 (**Figure 18c**). Arg139 is important for several intramolecular interactions in the protein structure, most notably, an ionic interaction with Asp132, which is on an adjacent alpha helix (**Figure 18d**). Arginine and lysine are able to make this interaction while the other mutants cannot. Thus, the R139C mutation destabilizes the NUDT15 protein structure likely due to loss of stabilizing intramolecular bonding networks.

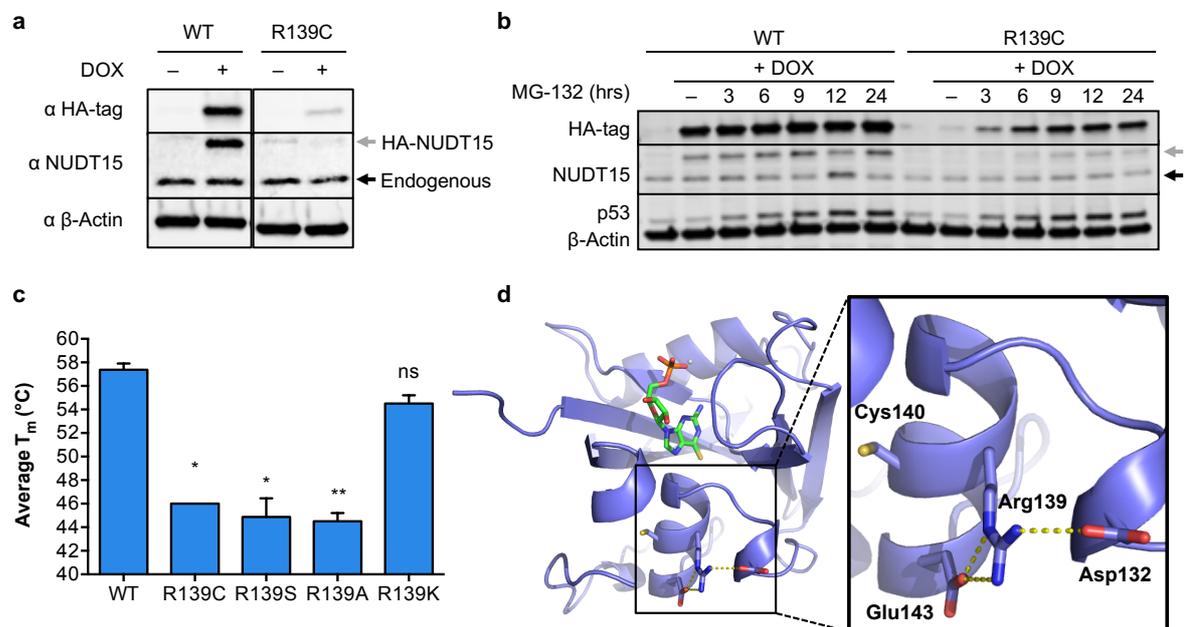


Figure 18: NUDT15 R139C is unstable and rapidly degraded in cells due to loss of key intramolecular bonding. **a**, HA-tagged NUDT15 WT and R139C expression in HCT116 cells. **b**, HCT116 cells expressing HA-tagged WT or R139C mutant NUDT15 were subjected to MG-132 treatment. p53 protein was blotted as a control. **c**, DSF with WT NUDT15 and various Arg139 mutants. **d**, Arg139 of NUDT15 makes a key intrahelical bond with Glu143 and interhelical ionic interaction with Asp132 of the adjacent alpha helix (bonds in yellow).

We next sought to understand how loss of NUDT15 would affect 6-thioguanine toxicity. Mismatch repair (MMR)-deficient and -proficient HCT116 and HCT116 3-6 cells, respectively, have been used extensively for studying 6-thioguanine toxicity^{244,247,372-374} and were transduced with Control or NUDT15 shRNA. Toxicity of thioguanine was assessed in both cell lines by clonogenic survival assay (**Figure 19a**). As expected, shControl-expressing HCT116 3-6 cells were more sensitive to thioguanine than MMR-deficient HCT116 cells. Depletion of NUDT15 rendered the HCT116 3-6 cells extremely sensitive to thioguanine, while even the parental HCT116 cells displayed some increased sensitivity.

Futile repair cycling and prolonged G2 checkpoint activation are signatures of thiopurine DNA incorporation in MMR-proficient cells²⁴⁵⁻²⁴⁸. We then performed a time course to see if a very low dose of thioguanine (150 nM) could selectively affect the G2 DNA damage checkpoint response in NUDT15 knockdown cells (**Figure 19b**). Thioguanine addition robustly induced phosphorylation of Chk1 and then Chk2, as well as subsequent G2 phase accumulation (indicated by inhibitory CDK2 phosphorylation)³⁷⁵, in a time-dependent manner in the NUDT15-depleted cells but less so in control cells.

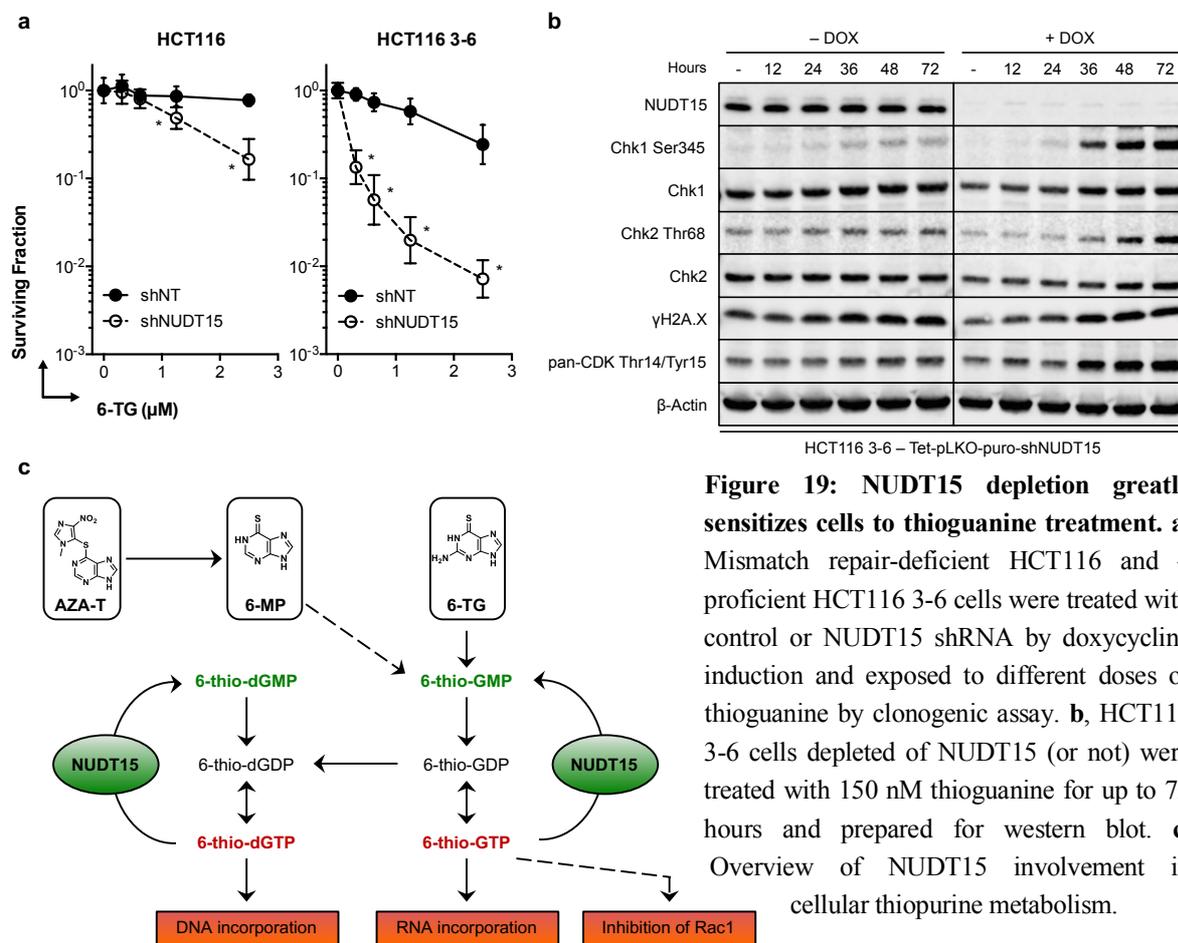


Figure 19: NUDT15 depletion greatly sensitizes cells to thioguanine treatment. **a**, Mismatch repair-deficient HCT116 and -proficient HCT116 3-6 cells were treated with control or NUDT15 shRNA by doxycycline induction and exposed to different doses of thioguanine by clonogenic assay. **b**, HCT116 3-6 cells depleted of NUDT15 (or not) were treated with 150 nM thioguanine for up to 72 hours and prepared for western blot. **c**, Overview of NUDT15 involvement in cellular thiopurine metabolism.

Therefore, NUDT15 appears to be a barrier to the anti-cancer efficacy of thioguanine by hydrolyzing the cell-active metabolites, 6-thio-GTP and 6-thio-dGTP, to inactive monophosphates (**Figure 19c**). Our data suggests that patients homozygous for the NUDT15 R139C mutation are hypersensitive to thiopurine treatments due to inherent protein instability from loss of key intramolecular bonding interactions and subsequent protein degradation.

2.3.3 Targeted NUDT5 inhibitors block hormone signaling in breast cancer cells

NUDT5 is another member of the NUDIX family implicated in oxidized nucleotide pool sanitation^{138,140,163-165}; however, the physiological relevance of this activity, and ambiguity surrounding its substrate preferences¹⁴¹, has not been extensively evaluated.

In **Paper III**, we first assessed the substrate specificity of NUDT5 by MG assay and HPLC. Distinct from MTH1, NUDT5 was unable to hydrolyze any of the oxidized or canonical nucleoside substrates tested but efficiently hydrolyzed ADP-ribose (**Figure 20a**). Similarly, we compared NUDT5-mediated hydrolysis of the two proposed substrates, ADP-ribose and 8-oxo-dGDP, and identified hydrolysis products by HPLC. Matching with the MG data, NUDT5 hydrolyzed ADP-ribose to AMP but had no activity towards 8-oxo-dGDP.

NUDT5 was then depleted in U-2 OS cells before assessing DNA damage markers by immunofluorescence microscopy and OGG1-specific lesions in DNA by the modified alkaline comet assay. Knockdown of NUDT5 with two specific siRNAs caused no increases in γ H2A.X, RPA or 53BP1 foci, nor was there an increase in OGG1-specific DNA lesions by modified alkaline comet assay when compared to MTH1 inhibitor, TH1579 (karonudib; **Figure 20b**)¹²⁹.

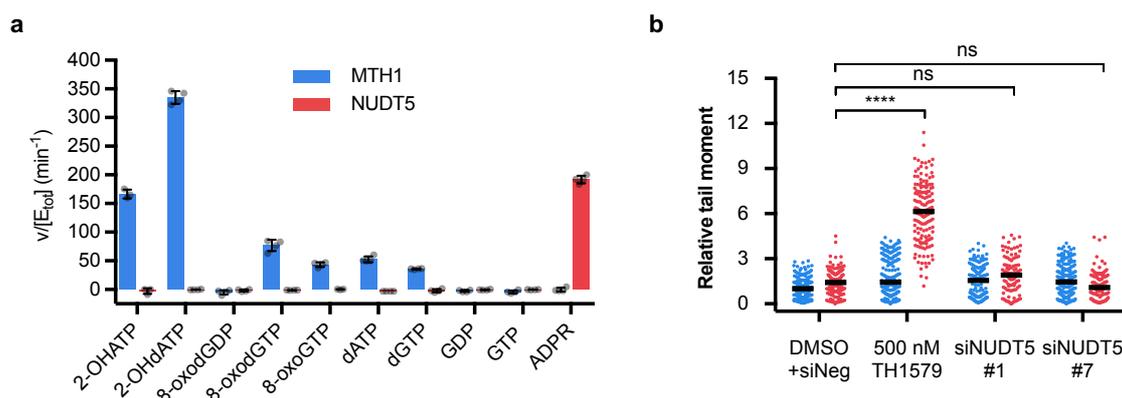


Figure 20: NUDT5 preferentially hydrolyzes ADPR. **a**, Relative enzymatic activities of NUDIX substrates by MTH1 (blue) and NUDT5 (red) by *in vitro* malachite green assay. **b**, U-2 OS cells treated with MTH1 inhibitor, TH1579 (karonudib), or depleted of NUDT5 were analyzed by the modified alkaline comet assay, which includes an hOGG1 incubation step (red) and is compared to buffer-treated samples (blue).

To assess NUDT5 function with regard to ADP-ribose hydrolysis in a cellular context, we depleted NUDT5 by siRNA, prepared cell lysates for enzymatic reactions with spiked ADP-ribose and analyzed the hydrolysis by HPLC. As we expected, NUDT5-depleted U-2 OS cells hydrolyzed ADP-ribose less efficiently than control cells and this effect could be completely rescued by addition of purified NUDT5 protein to the reaction mixture. Altogether, the data suggest that NUDT5 is minimally involved in oxidized nucleotide metabolism and that ADP-ribose is a primary substrate.

In conjunction with Laboratory for Chemical Biology Karolinska Institutet/Chemical Biology Consortium Stockholm (LCBKI/CBCS), we performed a small molecule high-throughput

screen of approximately 72,000 compounds utilizing a screening-compatible, enzyme-coupled MG assay. From hit compound, TH1167, medicinal chemistry efforts to improve biochemical potency were focused on diversification at the 8-position of the theophylline core and the far tail-end of the molecule. We obtained a co-crystal structure of TH1713 in the active site of the NUDT5 homodimer, and the theophylline ring (present in all compounds within this series) was firmly anchored in place by pi-stacking and hydrogen bonding interactions. The theophylline 8-position, meanwhile, was directed towards the solvent space.

With limited biological roles for NUDT5 established in the literature, we utilized a compound screening funnel that prioritized cellular target engagement by CETSA^{356,357} to identify potent, cell-active NUDT5 probes (**Figure 21a**). We selected several compounds with $IC_{50} \leq 100$ nM by the MG assay. These compounds were analyzed by CETSA with HL-60 cell lysates and tested for their ability to stabilize intracellular NUDT5 at 83°C and 20 μ M. Notably, three compounds, TH5423, TH5424 and TH5427, stabilized NUDT5 to greater than 50% of 37°C NUDT5 protein levels. These three compounds, and TH1659, were then tested for their ability to stabilize NUDT5 with intact HL-60 cells at 20 μ M, and, again, TH5423-5427 stood out from TH1659. Each of these compounds was then tested by ITDRF_{CETSA} to determine which compound was able to stabilize intracellular NUDT5 at the lowest concentration (**Figure 21b**). Indeed, TH5427 was clearly the most potent and stabilized NUDT5 at < 1 μ M, both by CETSA and by DARTS, a protease degradation-based cellular target engagement assay³⁷⁶ (**Figure 21c**).

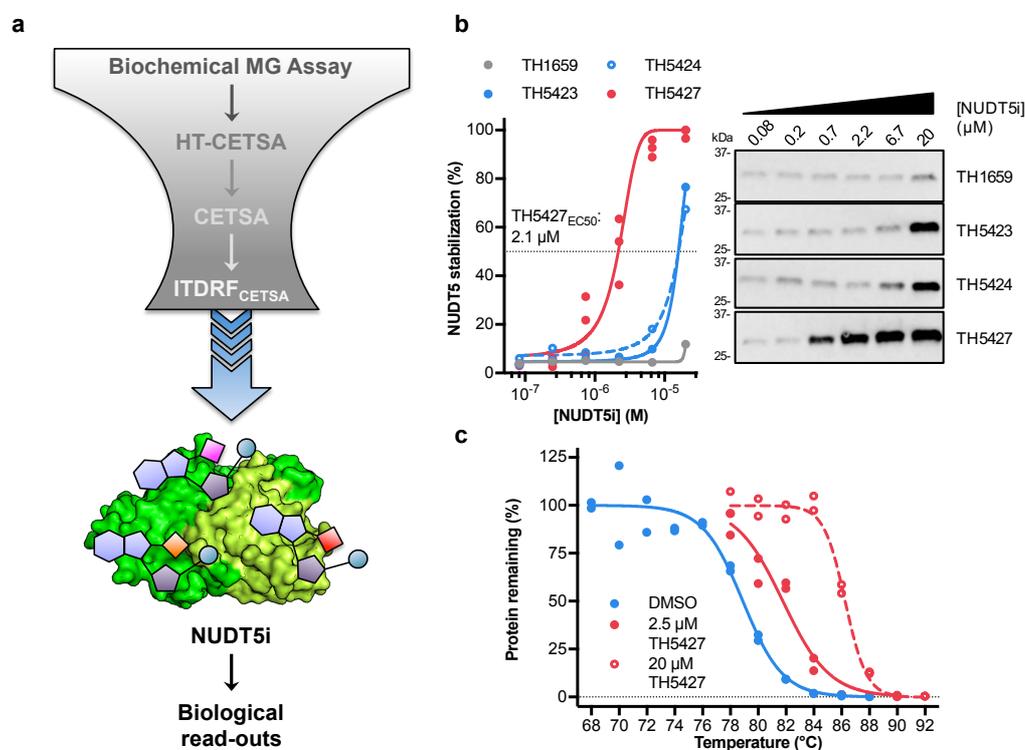


Figure 21: CETSA-guided screening of top NUDT5 inhibitors identifies TH5427 as a lead agent. a, Screening funnel for top NUDT5 inhibitors with successive CETSA. **b**, ITDRF_{CETSA} for NUDT5 inhibitors with intact HL-60 cells. **c**, CETSA with TH5427 at 2.5 or 20 μ M in intact HL-60 cells.

Having now identified potent, cell-active NUDT5 inhibitors, we needed to test them in a biological context. To this end, we established a collaboration with the Miguel Beato lab at the Center for Genomic Regulation in Barcelona. They recently discovered that NUDT5 can synthesize ATP in the nucleus following hormone stimulation of breast cancer cells, and this PAR-derived nuclear ATP is required to power ATP-dependent chromatin remodeling and, thus, hormone-dependent gene regulation and cell proliferation¹⁷¹ (**Figure 22**).

NUDT5 is able to synthesize ATP from ADP-ribose in the presence of PP_i *in vitro*. Thus, we tested if TH5427 could block the formation of AMP and ATP from the ADP-ribose formed from ³²P-PAR and bovine PARG by thin layer chromatography and radiography. Addition of TH5427 completely abrogated NUDT5 activity and reinforced the fundamental biological role for NUDT5 in producing AMP and ATP from ADP-ribose. With a nuclear-targeted luciferase reporter³⁷⁷, we showed that TH5427 blocked nuclear ATP formation following R5020 stimulation at just 1.5 μM in T47D breast cancer cells. Additionally, it impeded the displacement of histone H1 from DNA 30 minutes following hormone addition, as assayed by ChIP with 5 different histone H1-interacting regions¹⁷³. Then 6 hours following R5020 addition, TH5427 blocked the transcription of the progesterone-dependent genes, *EGFR* and *MMTV-luc*. Finally, TH5427 was able to completely inhibit progesterone-dependent cell proliferation by BrdU incorporation 24 hours post-R5020 treatment.

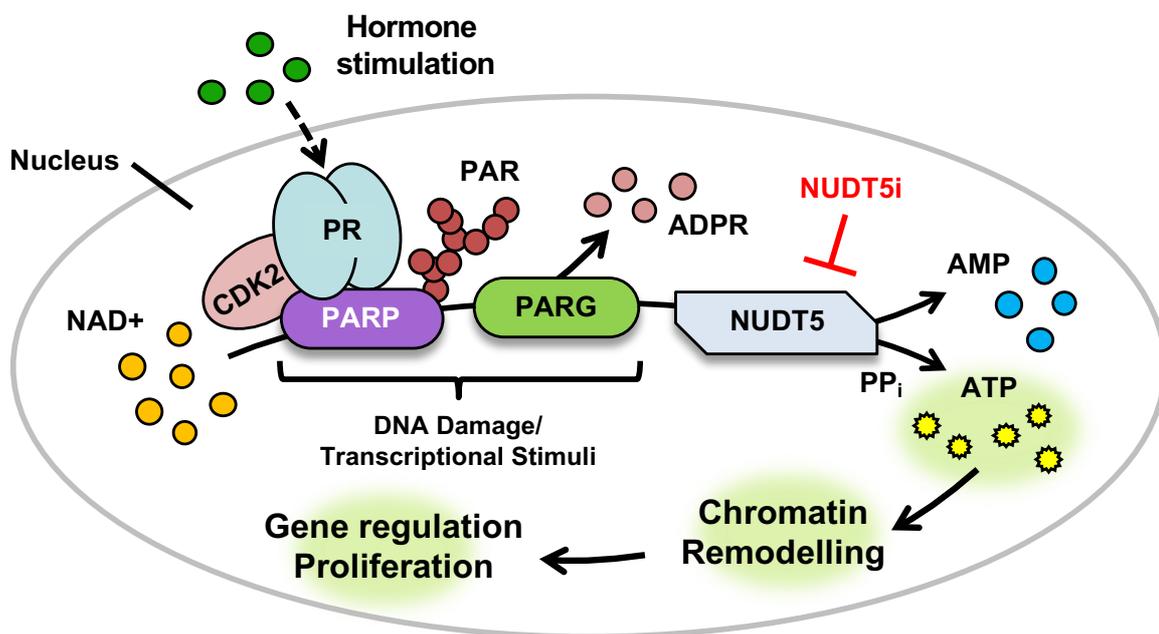


Figure 22: NUDT5 is required for hormone-dependent, PAR-derived nuclear ATP synthesis and gene regulation in breast cancer cells. In response to hormone, NUDT5 synthesizes ATP from ADPR, which is required for ATP-dependent chromatin remodelling and subsequent gene regulation and proliferation.

Altogether, the data suggest that TH5427 is a potent, selective and cell-active NUDT5 inhibitor that can be used to probe PAR and ADP-ribose metabolism, novel NUDT5 biology and uncover novel therapeutic strategies for treating human diseases.

2.4 DISCUSSION AND IMPLICATIONS

At the time this thesis work commenced, the best-characterized function of human NUDIX hydrolases was the sanitation of the oxidized nucleotide pool by hydrolysis of mutagenic 8-oxo-(d)GTP and 2-OH-(d)ATP to their respective monophosphate species^{44,45,48,49,64,112,113,119,124,138,141,378}, thus preventing their incorporation into nucleic acids by DNA and RNA polymerases. This makes sense because the first described NUDIX protein, *E. coli* MutT^{45,64,116}, performed this function and, prior to renaming to NUDIX by Bessman and colleagues¹¹², they were called the MutT family of proteins. Thus, human NUDIX proteins were identified by sequential and structural homology and initially tested for their ability to hydrolyze oxidatively-damaged nucleotides like MutT. In turn, this led to the discovery of MTH1 (MutT homology 1; NUDT1)¹¹⁹ and, later, other NUDIX hydrolases that could also facilitate hydrolysis of 8-oxo-guanine species, including NUDT15 (MTH2)^{137,139,140} and NUDT5^{138,140,157,163-166} – work pioneered by Sekiguchi, Kamiya, and Nakabeppu, among others.

It should be no surprise, then, that functional analyses of NUDIX proteins were biased towards oxidized nucleotide pool sanitation and 8-oxo-G-centric in nature. This inclination for a particular phenotype likely obscured the relevant functions of several of the human NUDIX proteins, as was recently alluded to in a review by Alex McLennan¹⁴¹, who has contributed immensely to our understanding of NUDIX enzyme biochemistry and function. Nonetheless, this phase served as an important starting point and generated tremendous interest in establishing the relevance of the NUDIX superfamily, particularly with respect to cancer.

It was also this perspective that generated our lab's interest in studying MTH1 and, soon thereafter, NUDT15 and NUDT5, as potential anticancer targets for small molecule inhibitors synthesized in-house. The early success in the lab regarding the potential of small molecule inhibitors targeting MTH1^{124,129} led us to pursue the possibility that NUDT15 and NUDT5 may be MTH1 redundancy factors that could serve as a resistance mechanism to MTH1 inhibitors. Instead, as was addressed in the component papers of this thesis, NUDT15 and NUDT5 appear to be important for diverse functions that are unrelated to oxidized nucleotide metabolism, at least, under the tested conditions and cell lines.

2.4.1 NUDT15 (MTH2)

Papers I and II highlighted that NUDT15 has a prominent role in regulating thiopurine efficacy in cells, namely by hydrolyzing the active metabolites, 6-thio-GTP and 6-thio-dGTP, to their respective monophosphates^{368,379}. This was in line with initial papers identifying the NUDT15 R139C mutation as a highly correlated predisposition factor for thiopurine-induced leukopenia¹⁴⁶⁻¹⁴⁸ and is now further substantiated by numerous follow-up studies with patients of different ethnic backgrounds. Like other factors influencing thiopurine toxicity, such as *TPMT* or *ITPA*, screening patients for *NUDT15* missense mutations will likely become common clinical practice.

Just prior to publishing **Paper II**, another group published a highly similar story³⁸⁰. In most respects, their findings agreed with our own, except with regard to the reason for sensitivity seen in NUDT15 R139C patients. Their conclusions were that R139C was not enzymatically active due to thermoinstability, but this did not affect protein expression in cells. The reasons for this disparity are likely due to key differences in experimental approaches. First of all, the *in vitro* enzymatic assays were performed at 37°C and not room temperature (20-21°C), as was done in our studies³⁷⁹. Given the lowered protein stability of R139C, it would make sense that we saw activity and the other group did not. A logical conclusion, then, is that the R139C mutation does not directly affect enzyme catalysis, but, at physiological temperature, unfolding of the protein would compromise enzyme function. It was puzzling, therefore, that the other group did not see degradation of R139C when expressed in cells, as the proteasome system should flag the protein for destruction³⁸¹. One reason for this could be that they utilized a transient transfection approach or, perhaps, the choice of protein fusion tag. In any case, greater perspective will be gained if these studies are reproduced by a third, independent group.

One of the other objectives within the NUDT15 project was the development and evaluation of NUDT15 inhibitors, which was alluded to in another thesis from our research group³⁸² and is not included as part of this thesis. Identical to methods with RNAi, NUDT15 inhibitors can potentiate the effects of thiopurines by increasing the active triphosphates, with further sensitization seen in MMR-proficient leukemias than in MMR-deficient cells³⁸². An interesting question becomes then – if NUDT15 inhibitors were developed to a pre-clinical stage, would there be any interest in using them? The answer is likely complicated on multiple levels. For one, thiopurines are a cytotoxic therapy that will also affect rapidly proliferating normal cells, such as in the gut and bone marrow, which is the reason doses have to be lowered or treatment completely halted, in some cases. Additionally, patients with nonfunctional NUDT15 are deathly sensitive to thiopurines¹⁴⁶⁻¹⁴⁸. The only way this would be practical is if expression of NUDT15 was higher in cancer cells than in normal cells; however, this has yet to be properly studied. Furthermore, long-term side effects, such as the photoreactivity of thiopurines and their effects on DNA, proteins, and lipids, have to be considered, especially since children are among those typically treated with the drug^{229,383,384}. Another possibility is that thiopurine dosing regimens could be titrated down if combined with NUDT15 inhibitors, which may curb some of the negative side effects. However, thiopurines are used as maintenance therapies for ALL, not primary treatments, and 90+% of childhood ALL cases are cured with the current standard-of-care^{228,229}. Thus, there is very little incentive to experiment further with dosing and risk losing an already-great cure rate.

A logical follow-up question following the role in thiopurine metabolism is: Does NUDT15 influence the efficacy of other nucleoside analogs? This question is readily apparent when considering the multiple nucleoside analog drugs that are directly or indirectly regulated by SAMHD1^{211-216,385}. Interestingly, thioguanine triphosphates are not substrates of SAMHD1²¹³, which may explain why NUDT15 is relevant in this circumstance. Extensive screening of both clinical and generic nucleoside analogs may yield further direct substrates

of, not only NUDT15, but other NUDIX proteins as well, which may be informative regarding clinical utility or for determining endogenous substrates.

Quite frustratingly, very little progress was made on the physiological function(s) of NUDT15 during the course of this thesis, as thiopurines are not endogenously-occurring metabolites. Based on experiments performed by myself and others, blockade of NUDT15 function by RNAi^{368,379} or potent NUDT15 inhibitors³⁸² had no discernable effects on cell cycle, proliferation, or cell survival for up to several weeks with numerous cell types^{368,379,382}. This by no means excludes the possibility that NUDT15 has important functions in cells but suggests that whatever changes occur following loss of NUDT15 are not significantly impacting normal cellular processes under tested conditions. Still, though, the linkage to oxidative damage metabolism is ever-present^{137,139,140,142}, most recently exemplified by NUDT15 CRISPR/Cas9 knockout cells being sensitized to hydrogen peroxide¹⁴⁹. As was the case with our experiences, NUDT15 knockout cells had no changes to their proliferation rate¹⁴⁹. Clues to NUDT15's cellular function(s) may be uncovered by substrate profiling, which has thus far suggested that dGTP, dTTP, and dUTP are endogenous NUDT15 substrates³⁶⁸; however, the relative importance of NUDT15 in modulating dNTP pools is still unclear. Nonetheless, the work from this thesis has cast doubt on the role for NUDT15 in metabolizing oxidized nucleotides and defined how NUDT15 affects thiopurine treatments in patients.

2.4.2 NUDT5

In **Paper III**, we reported the first small molecule inhibitors of NUDT5 and utilized them to confirm its role in generating nuclear ATP for chromatin remodeling in response to progestin in breast cancer cells³⁸⁶. We used an unorthodox approach to evaluate our compound library: a CETSA-guided screening funnel. Initially, this was chosen out of necessity because there were no known NUDT5-linked phenotypic assays that could reliably inform about the cellular activity of our inhibitors. However, we later realized that this approach could alleviate any potential biases that might arise from performing a phenotypic screen. Why try and define what we think NUDT5 inhibitors are doing in cells, when we can reliably select out the compounds that bind NUDT5 in cells with the most potency? Our goal was to identify the best, cell-active NUDT5 inhibitor, and then use it to further interrogate NUDT5 cellular functions.

To our knowledge, no one has reported utilizing CETSA prioritization as a primary screening method in cells for a focused library of small molecule inhibitors. From the initial publication in 2013, one of the recognized advantages of CETSA was the ability to rank inhibitors by using the ITDRF method to determine relative target engagement potency^{356,357,359,364}. More recently, CETSA with AlphaScreen® was used as a primary screen to identify ligands that stabilized TS³⁶⁰, and the authors were able to identify mM inhibitors. What this implies is that one can screen any set of molecules against a protein of interest in cells and not just identify, but also rank, inhibitors without knowing anything about the protein's function. It cannot be understated just how powerful that is. On that note, it is well established that targeted

therapies are only as good as their ability to bind the target in cells, which is why prioritizing target engagement, not just confirming it, should be a goal for inhibitor and drug development programs³⁴⁹⁻³⁵².

By identifying potent, cell-active NUDT5 inhibitors, we were able to confirm a new function for NUDT5 that was recently reported¹⁷¹. The ability for cells to synthesize ATP in the nucleus has been conveyed over several decades but has never gained momentum as a bonafide source of ATP production, likely because it was assumed that this ATP was from mitochondrial contamination or passive diffusion. However, looking back at the series of manuscripts on the topic, one can see a clear pattern that has developed and led to the point we are today. Allfrey and Mirsky first saw that HeLa nuclei could generate their own ATP from DNA³¹² and Betel added that it was dependent on ribose-phosphate³¹³. Tanuma and colleagues identified that ATP could be formed in nuclei from PAR catabolism *via* an enzyme called ADPR pyrophosphorylase, which catalyzes the formation of ATP from ADPR and PP_i³¹⁴. This ATP appeared to be important for facilitating repair synthesis of DNA^{315,316}. Oei and Ziegler demonstrated that PAR-derived ATP was dependent on active DNA replication and required for ligation following DNA repair³¹⁷. Now, we have helped confirm that PAR catabolism and PP_i can make ATP *via* NUDT5^{171,386}, bringing the process full circle.

The ability of NUDT5 to synthesize ATP from ADPR and PP_i is certainly unexpected, not just because NUDT5 is a hydrolase, but also because the reaction is energetically unfavorable¹⁷¹. For hydrolysis, approximately 22 kJ/mol of energy is released but for ATP synthesis, 12 kJ/mol is required for the reaction to occur¹⁷¹. Wright and colleagues concluded that loss of a phosphorylation on Thr45 may result in a NUDT5 conformational change that can facilitate PP_i binding and ATP synthesis^{171,387}. However, in light of the high melting temperature and resistance to proteolysis seen in **Paper III**, it is difficult to envision that a single phosphorylation event will cause inversion of the NUDT5 dimer^{171,387}. In fact, the model proposed by Wright and colleagues would completely disrupt the NUDT5 active sites, as they are composed of residues from both monomers at the dimer interface^{156,157,165,166,386}. As these conclusions were based on *in silico* modeling, crystallographic structure determination studies would be immensely insightful. Another possibility is that energetic coupling during PAR hydrolysis by PARG propels the unfavorable ATP synthesis reaction; since NUDT5 can form a complex with NMNAT1, PARP and PARG¹⁷¹ and PAR degradation is absolutely required to produce nuclear ATP³¹⁴⁻³¹⁶ (addition of exogenous ADPR is not enough³¹⁷).

An interesting scientific question revolves around the purpose of PAR formation. It is well known that PARylation orchestrates chromatin dynamics and recruitment of DNA modifying enzymes, but could it also be an emergency energy store, as Tanuma, Oei and Ziegler proposed^{314,317}? During chromatin remodeling, which occurs during transcription, replication, and repair processes, among others; the cell likely requires large quantities of ATP in close proximity to the dependent enzymes at a moment's notice^{171,387}. As PAR is involved in

coordinating most, if not all, of these processes, could it be the source of ATP (*via* NUDT5) for all of them? In the presence of excess ATP, ATP synthesis from PAR catabolism does not occur, which could indicate that this process is only activated when local ATP concentrations are low^{314,316,317}. Further supporting this possibility is the fact that ATP, at physiologically-relevant concentrations of 1-10 mM, can inhibit PARP1 catalysis of PAR^{303,388}, likely by both indirect and direct binding to the enzyme active site³⁸⁸. Kim *et al.* also proposed that PARP1 was a sensitive metabolic switch that could modulate transcriptional activity of chromatin depending on local ATP concentrations³⁰³. Collectively, this may indicate an unappreciated purpose of PAR formation.

With the tools we currently have available, such as the ATP³⁸⁹ and NAD⁺³⁹⁰ FRET probes, it will be interesting to see what other PARP-related functions require ATP production from NUDT5. PARP1, for example, which is known to complex with NUDT5, is well characterized for its role in repairing DNA damage, but also regulating chromatin structure, transcription, proteasomal degradation and mitochondrial function³⁹¹. PAR itself is also implicated in numerous other functions – including spindle assembly during mitosis, translational elongation, macromolecular complex assembly, and regulation of RNA splicing³⁹¹⁻³⁹³. Most, if not all, of these processes also require large amounts of ATP, so it is conceivable that NUDT5, or some other unknown protein, may also produce ATP from PAR in these circumstances. Even if we restrict the scope of NUDT5 and PAR-derived ATP synthesis to the process of transcription, it is entirely possible that transcriptional programs of several nuclear receptors, like the androgen, retinoic acid, thyroid, and AP-1 receptors³⁰⁶ – are activated in the same fashion as estrogen-responsive gene regulation^{171,306,387}. It is tempting to speculate whether NUDT5-mediated ATP synthesis is required for chromatin remodeling, ligation of the transient DSB formed prior to transcription (as suggested for BER³¹⁷), or possibly both, during this process.

As for the development and future of NUDT5 inhibitors, we are currently working towards suitable pre-clinical candidates. Early *in vivo* characterization of TH5427 is promising, but further experiments are required, both for pre-clinical efficacy and for selectivity profiling. Of immediate interest is the confirmation that NUDT5 is an important determinant for breast cancer proliferation, which has been previously suggested in animal studies³⁹⁴, and if the ATP synthesis mechanism is responsible for this effect. Should this be the case, one argument may be that PARP inhibitors should be just as effective at blocking hormone-dependent breast cancer growth. The counterargument is that PARP1/2 are involved in several processes beyond hormone-dependent transcriptional regulation³⁹¹⁻³⁹³ (see above) and clinical-grade PARP inhibitors trap PARP to DNA as part of their effective mechanism^{395,396}, both of which would entail unwanted side effects. Thus, a NUDT5 inhibitor will likely be a better option in this regard. Nonetheless, TH5427 represents a selective NUDT5 probe molecule that can be used to uncover the intricacies of NUDT5, ADPR and PAR biology.

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