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Exploiting Nucleotide Metabolism to Improve Cancer Therapy

by targeting dUTPase, dCTPase and NUDT15

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To everyone who has supported me during this journey!

Without you no page would have been possible!

ABSTRACT

Synthetic nucleobase- and nucleoside-analogs have stood the test of time and remain a cornerstone in the treatment regimen against various forms of cancer. Due to their resemblance to endogenous nucleotides these antimetabolites interfere with cellular pathways, including nucleotide metabolism, as well as DNA and RNA synthesis. However, the treatment efficacy of nucleobase- and nucleoside-analogs can be hampered by "house-cleaning" enzymes involved in sanitation and balance of the nucleotide pool. In this work, we validated whether targeting nucleoside triphosphate hydrolases, involved in sanitation of the nucleotide pool, is a promising strategy to improve the efficacy of commonly used nucleobase- and nucleoside-analogs. These include:

1. **dUTPase** to potentiate **5-fluorouracil** treatment
2. **dCTPase** to potentiate **decitabine** treatment
3. **NUDT15** to potentiate **6-thioguanine** treatment

We characterized dUTPase, dCTPase and NUDT15 by various biochemical and biophysical techniques and assessed their role in intracellular nucleotide homeostasis using RNA interference. Through the development and use of small molecule inhibitors targeting these hydrolases, we highlighted the benefit of inhibiting nucleotide pool sanitization to improve nucleobase- and nucleoside-analog therapy.

In **Paper I** we demonstrated that dUTPase inhibition reinforces 5-fluorouracil-induced replication defects and cytotoxicity. With this study, we contributed to the characterization of dUTPase inhibitors and increased our understanding of the mode of action of this combination treatment.

In the second study (**Paper II**), we developed small molecule inhibitors against dCTPase to explore the biological function of this nucleoside triphosphate hydrolase in the context of endogenous nucleotide homeostasis and decitabine treatment. We showed that chronic inhibition of dCTPase has a cytostatic effect on cancer cells and potentiates cellular effects of decitabine therapy.

Targeting enzymes involved in sanitation of the oxidized nucleotide pool is a novel treatment strategy that exploits the dysregulated reduction-oxidation environment of tumors. Based on the increasing attention to MTH1 as a prime example for this approach, we validated whether

the sequence homolog NUDT15 (also known as MTH2) fulfills comparable enzymatic functions, making it a potential target for cancer therapy. With extensive biochemical and cellular experiments we demonstrated that NUDT15 possesses only minimal activity with oxidized nucleotides and is non-essential for cancer cell survival (**Paper III**). While assessing the cellular function of NUDT15, we discovered activity with the thiopurine effector metabolites, 6-thio-dGTP and 6-thio-GTP.

In light of several pharmacogenetic studies, which link thiopurine hypersensitivity to the NUDT15 R139C variant, we further elucidated the role of NUDT15 (wild type and mutants) in thiopurine metabolism (**Paper IV**). We combined biochemical analyses with cellular experiments on genetically modified cell lines to demonstrate that NUDT15 has a strong preference for thiolated guanine substrates and that this activity counteracts thiopurine efficacy in cancer cells. Furthermore, we propose that the observed hypersensitivity of NUDT15 R139C positive patients is not caused by impaired enzymatic activity, but is a result of untenable protein stability.

Inspired by these findings, we developed first-in-class NUDT15 inhibitors and validated whether pharmacological inhibition of NUDT15 is a promising strategy to sensitize leukemia cells to thiopurine treatment (**Paper V**). NUDT15 inhibition significantly increased the availability of thiopurine nucleoside triphosphates, leading to potentiation of DNA incorporation, DNA damage and cytotoxicity.

Overall, these studies demonstrated that preventing the sanitation of nucleotide-analogs, by inhibiting nucleoside triphosphate hydrolases, is a promising strategy to improve the efficacy of nucleobase- and nucleoside-analog treatments.

LIST OF SCIENTIFIC PUBLICATIONS

This thesis is based on the following publications, which are referred to by their Roman numerals in the text:

Paper I. **dUTPase inhibition augments replication defects of 5-fluorouracil**. **A Hagenkort***, CBJ Paulin*, M Desroses*, T Koolmeister, O Loseva, A-S Jemth, E Wiita, E Homan, T Lundbäck, A-L Gustavsson, M Scobie and T Helleday. *Manuscript*.

Paper II. **TH1217, a chemical probe to explore dCTPase pharmacology**. A Höglund*, S Llona-Minguez*, JM Calderón-Montaño, M Claesson, E Wiita, O Loseva, A Ghassemian, Y Heshmati, SA Jacques, C Cazares-Körner, L Johansson, M Desroses, **A Hagenkort**, NCK Valerie, T Lundbäck, ML Orta, E Burgos Morón, TK Våtsveen, P Baranczewski, BDG Page, K Sanjiv, NG Sheppard, P Rouhi, H Gad, C Göktürk, I Almlöf, M Häggblad, U Martens, I Mavrommati, F Jeppsson, EK Dolatabadi, FZ Gaugaz, A Mateus, T Koolmeister, M Carter, K Sigmundsson, A Jenmalm Jensen, B Lundgren, P Artursson, S Lehmann, E Hellström-Lindberg, U Warpman Berglund, M Scobie, V D'Angiolella, A Sarno, M Oksvold, J Walfridsson, A-S Jemth, P Stenmark and T Helleday. *Manuscript*.

Paper III. **Crystal structure, biochemical and cellular activities demonstrate separate functions of NUDT15 and MTH1**. M Carter*, **A Hagenkort***, A-S Jemth*, BDG Page*, R Gustafsson, J Griese, H Gad, NCK Valerie, M Desroses, J Boström, U Warpman Berglund, T Helleday, P Stenmark. *Nature Communications*. 2015; 6:7871.

Paper IV. **NUDT15 mediates the cellular efficacy of 6-thioguanine by hydrolyzing 6-thio-(d)GTP**. NCK Valerie*, **A Hagenkort***, BDG Page, G Masuyer, D Rehling, M Carter, L Bevc, P Herr, E Homan, NG Sheppard, P Stenmark, A-S Jemth*, T Helleday. *Cancer Research.* 2016; 76(18):5501-11.

Paper V. **NUDT15 inhibitors increase the efficacy of thiopurine treatment**. **A Hagenkort***, M Desroses*, A Cázares-Körner, R Krimpenfort, A Throup, T Koolmeister, O Wallner, O Loseva, I Almlöf, A Sarno, L Bevc, T Lundbäck, H Axelsson, S Regmi, P Baranczewski, C Kalderén, M Carter, D Rehling, BDG Page, NCK Valerie, P Stenmark, M Scobie, U Warpman Berglund, E Homan, A-S Jemth and T Helleday. *Manuscript*.

*These authors contributed equally to this work.

Additional publications, not included in this thesis:

Paper VI. **Targeting MTH1 nucleotide triphosphatase prevents sanitation of oxidised dNTP pools and kills cancer cells**. H Gad*, T Koolmeister*, A-S Jemth*, S Eshtad*, SA Jacques, CE Ström, LM Svensson, B Einarsdottir, N Schultz, T Lundbäck, A Saleh, C Gokturk, P Baranczewski, R Svensson, K Strömberg, L Bräutigam, MC Jacques-Cordonnier, M Desroses, AL Gustavsson, RPA Berntsson, R Gustafsson, R Olofsson, S Vikingsson, F Johansson, O Loseva, K Sanjiv, L Johansson, A Höglund, **A Hagenkort**, T Pham, M Altun, FZ Gaugaz, B Evers, M Henriksson, K Vallin, O Wallner, LGJ Hammarström , E Wiita, I Almlöf, H Axelsson, M Häggblad, F Jeppsson, U Martens, C Lundin, B Lundgren, I Granelli, A Jenmalm Jensen, P Artursson, JA Nilsson, P Stenmark, M Scobie, U Warpman Berglund, T Helleday. *Nature*. 2014; 508(7495):215-21. *These authors contributed equally to this work.

Paper VII. **Cancer-specific synthetic lethality between ATR and CHK1 kinase activities**. K Sanjiv, **A Hagenkort**, J Calderón-Montaño,T Koolmeister, P Reaper, O Mortusewicz, S Jacques, N Schultz, M Scobie, P Charlton, J Pollard, U Warpman Berglund and T Helleday. *Cell Reports*. 2016; 14(2):298-309.

Paper VIII. **CHK1 activity is required for continuous replication fork elongation but not stabilization of post-replicative gaps after UV irradiation**. I Elvers, **A Hagenkort**, F Johansson, T Djureinovic, A Lagerqvist, N Schultz, I Stoimenov, K Erixon, T Helleday. *Nucleic Acids Research*. 2012; 40(17):8440-8.

LIST OF ABBREVIATIONS

(Anti) metabolites	NTP	ribonucleoside triphosphate
	dNTP	deoxyribonucleoside triphosphate
	A	adenine
	C	cytosine
	G	guanine
	T	thymine
	U	uracil
	dUMP	2´-deoxyuridine-5'-monophosphate
	dUTP	2´-deoxyuridine-5'-triphosphate
	dTMP	2´-deoxythimidine-5'-monophosphate
	dTTP	2´-deoxythimidine-5'-triphosphate
	5-FU	5-fluorouracil
	FdUrd	5-fluoro-2'-deoxyuridine
	5-fluoro-dUMP	5-fluoro-2'-deoxyuridine-5'-monophosphate
	5-fluoro-dUTP	5-fluoro-2'-deoxyuridine-5'-triphosphate
	dCMP	2´-deoxycytidine-5'-monophosphate
	dCTP	2´-deoxycytidine-5'-triphosphate
	5-aza-C	5-aza-cytidine
	5-aza-dC	5-aza-deoxycytidine (decitabine)
	5-aza-dCTP	5-aza-2‘-deoxycytidine-5‘-triphosphate
	5-aza-dCMP	5-aza-2‘-deoxycytidine-5‘-monophosphate
	8-oxo-dGMP	8-oxo-2'-deoxyguanosine-5'-monophosphate
	8-oxo-dGTP	8-oxo-2'-deoxyguanosine-5'-triphosphate
	8-oxo-GMP	8-oxo-guanosine-5'-monohosphate
	8-oxo-GTP	8-oxo-guanosine-5'-triphosphate
	AZA-T	azathioprine
	6-MP	6-mercaptopurine
	6-TG	6-thioguanine
	6-thio-dGMP	6-thio-2'-deoxyguanosine-5'-monophosphate
	6-thio-dGTP	6-thio-2'-deoxyguanosine-5'-triphosphate
	6-thio-GMP	6-thio-guanosine-5'-monoposphate
	6-thio-GTP	6-thio-guanosine-5'-triphosphate

Enzymes	NTPase	nucleoside triphosphate phosphatase
	dNTPase	deoxynucleoside triphosphate phosphatase
	dCTPase	deoxycytidine triphosphatase
	dUTPase	deoxyuridine triphosphatase
	Nudix	nucleoside diphosphate linked to some other moiety X
	MTH1	MutT Homolog 1
	MTH2	MutT Homolog 2 (NUDT15)
	TPMT	thiopurine S-methyltransferase
	TS	thymidylate synthase
	RNR	ribonucleoside diphosphate reductase
Diseases	ALL	acute lymphoblastic leukemia
	AML	acute myeloid leukemia
	IBD	inflammatory bowel disease
	MDS	myelodysplastic syndrome
Other	DNA	deoxyribonucleic acid
	RNA	ribonucleic acid
	MMR	mismatch repair
	BER	base excision repair
	HR	homologous recombination
	ROS	reactive oxygen species
	redox	Reduction-oxidation
	SAM	S-adenosyl-L-methionine

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INTRODUCTION

The genetic composition and the epigenetic make-up are important factors influencing the blueprint and function of a cell. To guarantee correct cell performance and avoid disease it is of vital importance to keep the genetic and epigenetic information of a cell intact. Our DNA and the molecular DNA precursors are constantly exposed to various exogenous and endogenous hazards that can irrevocably damage the hereditary information [1]. Accumulation of genetic mutations is one of the major causes for neoplastic diseases, which today are a major cause of death in the western world [2]. Along with surgery and radiation therapy intervention with pharmaceutical agents is a component of almost every anti-cancer treatment regimen. One of the oldest cancer treatment strategies is the use of synthetic nucleobase- and nucleoside-analogs (antimetabolites) to disturb nucleotide homeostasis and genome integrity, which even today remains a cornerstone treatment against many forms of cancer [3]. Due to their resemblance to endogenous molecular building blocks, these agents interfere with various cellular pathways, including nucleotide metabolism, as well as DNA and RNA synthesis [3]. Among the oldest and most commonly used nucleobase- and nucleoside-analogs are 5-fluorouracil (5-FU), decitabine (5-aza-dC) and 6-thioguanine (6-TG) [4-6]. However, dose-limiting side effects due to poor cancer cell selectivity, long-term toxicity and often occurring drug resistance are major disadvantages associated with these treatments [3]. To be able to increase the therapeutic success it is important to understand the complexity of endogenous and synthetic (d)NTP metabolism. The treatment efficacy of nucleobase- and nucleoside-analogs can be hampered by the activity of "house-cleaning" enzymes involved in sanitation and balance of the nucleotide pool. This work elucidates the importance of the nucleoside triphosphate hydrolases dUTPase, dCTPase and NUDT15 for cellular integrity, both alone and in the context of nucleobase- and nucleoside-analog treatment.

1.1 The importance of genome integrity

Life on Earth appears immensely diverse, ranging from single cell organisms to complex multicellular compositions like the human body. Even though they all seem extraordinarily unalike, in fact all living organisms are composed of a common single unit, the cell [7]. The hereditary blueprint of each cell is stored in the form of a complementary nucleotide double helix, better known as deoxyribonucleic acid or DNA. The sequence of the bases along the sugar-phosphate backbone creates a unique code that stores the information for protein composition, much like the alphabet is a code that creates each word in this thesis. In a process termed *transcription*, the code of a DNA segment (gene) is copied to a complementary ribonucleic acid (RNA) strand. Whereas both, DNA and RNA, are nucleotide polymers, RNA differs from DNA by 1) the sugar; ribose instead of deoxyribose and 2) the composition of the nucleobases; replacing thymine with uracil. Guided by the three letter code of the RNA, ribosomes align one out of 20 amino acids to form proteins, in a process called *translation*. Besides the sequence of the nucleobases in the DNA, which determines the protein composition, it is the regulation of gene expression that creates distinct cell populations and unique organisms.

Chemical reactions, including oxidation, methylation or deamination, caused by exogenous and endogenous sources are a constant threat to the fundamental genetic information [1]. These include reactive oxygen and nitrogen species (ROS, RNS), UV radiation and chemicals. Even though the 3 billion base pair long macromolecule is supercoiled and highly protected by the nuclear membrane it is estimated that a human cell is confronted with 10^5 DNA lesions on a daily basis [8]. During replication DNA polymerases insert, guided by the base-pairing rules, free nucleotide triphosphates along the parent strand to create a complementary daughter strand. While DNA polymerases are highly selective against ribonucleotides, they often possess limited selectivity towards the nitrogenous base [9-11]. The high chemical liability of the free nucleotide pool, which is needed for DNA synthesis and repair, is therefore a significant hazard for the integrity of the genetic information [12, 13].

In order to preserve the genetic information and maintain cellular fitness the cell has developed numerous protective mechanisms that 1) prevent utilization of erroneous nucleotides and 2) repair damaged DNA lesions. The first group includes a variety of “house-cleaning” enzymes that sanitize and balance the nucleotide pool of potentially

deleterious metabolites, e.g. by hydrolysis of the triphosphate chain. If the amount of hazardous precursors exceeds the "house-cleaning" performance of these enzymes, incorporation of damaged nucleotides into nascent DNA is inevitable. For this scenario the cell has developed a complex network of biochemical pathways that detect and repair erroneous DNA lesions. These include the action of the base-excision repair (BER) and the mismatch repair (MMR) machinery. With the help of damage-specific DNA glycosylases the BER machinery removes small, non-helix-distorting base lesions, like oxidized, alkylated or deaminated nucleotides or the non-canonical base U [14]. The MMR system recognizes and repairs erroneous base-base mismatches, as well as insertions and deletions occurring during DNA replication and recombination [15]. In addition to these complex machineries DNA damage stimulates a network of signaling events which, depending on the damage status, can induce cell cycle arrest, cellular senescence or even cell death.

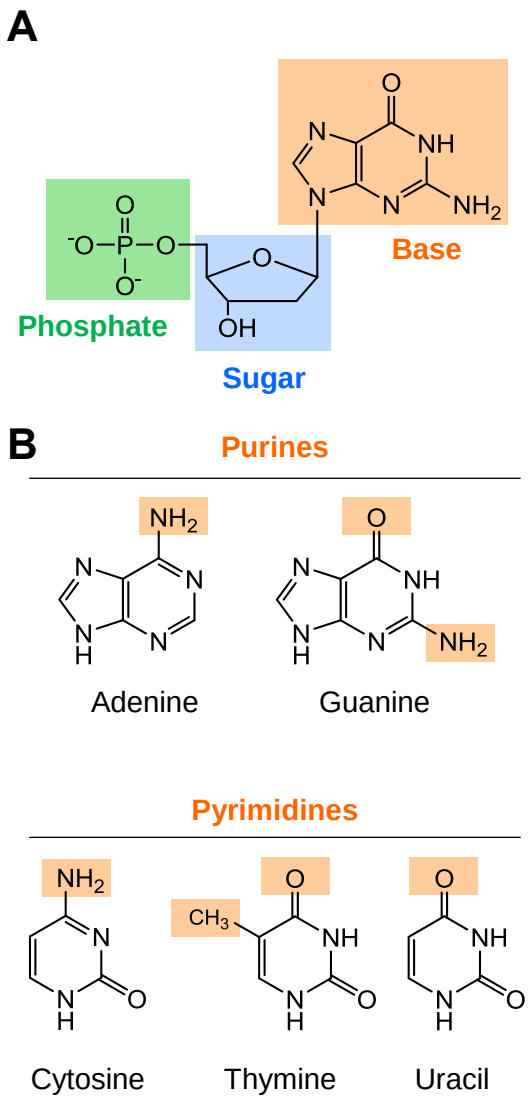
Despite the interplay of protective and reparative mechanisms changes in the genetic information are common [1]. Irretrievable alterations in the DNA, including deletions, insertions, substitutions and frameshift mutations, can cause expression of defective and redundant proteins or prevent proteins from being synthesized. Disturbed expression of oncogenes and tumor suppressor genes can have severe consequences for the cell including: self-sufficiency in growth signals, resistance to growth suppressors and cell death, replicative immortality, induction of angiogenesis, capability to metastasise, alterations in energy metabolism and evasion of immune detection [16]. If accumulated, the interplay of these cancer hallmarks can lead to the transformation of a healthy cell into a cancerous cell.

Since genetic integrity is essential for cellular function and survival many chemotherapeutic agents have been developed that induce their cytotoxic effect through excessive DNA damage [17]. One of the major problems during anti-cancer therapy is the high heterogeneity within tumor cells [18]. A high mutator phenotype in cancer cells makes it possible for a part of the population to acquire compensatory mechanisms and escape the selection pressure. Tumor-relapse of more aggressive and treatment-resistant cells is often the devastating consequence. In addition to acquired drug resistance, intrinsic resistance, poor cancer selectivity and long-term toxicity are major problems. Understanding the mode of action of chemotherapeutic agents and cellular resistance mechanisms is therefore essential to achieve better tumor response.

1.2 The role of (d)NTPases in nucleotide homeostasis

Nucleotides contain three moieties: a five carbon sugar, a phosphate (-chain) and a nitrogenous base (**Figure 1A**) [7]. Nucleotides composed of deoxyribose ($C_5H_{10}O_4$) are polymerized to form DNA, whereas ribose ($C_5H_{10}O_5$) containing nucleotides are utilized for RNA synthesis. On the first carbon of the pentose a nucleobase is attached through an N-glycosidic linkage. Nucleobases are heterocyclic compounds with double (purines) or single (pyrimidines) rings containing nitrogen and carbon atoms. In eukaryotic DNA the canonical nucleobases are guanine (G), cytosine (C), adenine (A) and thymine (T) (**Figure 1B**) In RNA uracil (U) replaces the thymine code. Addition of (deoxy)ribose to the base changes the nomenclature from G, C, A, T and U to guanosine, cytidine, adenosine, thymidine and uridine, respectively. Creation of a phosphoester bond between phosphoric acid and the 5' carbon of the sugar transforms the nucleoside to a nucleotide. Sequential extension of additional phosphoric acids via phosphoanhydride bonds creates high energy nucleoside di- and triphosphates [7].

When one thinks of the function of nucleotides, the first thought often pertains to their role as building blocks for DNA and RNA synthesis, both of which are essential for cellular viability [7]. However, phosphoryl transfer from nucleoside triphosphates plays a key role in various cellular functions, including signaling and energy transduction [19]. ATP, for example, is the main energy carrier of the cell and additionally an essential intra- and extracellularly signaling molecule [20, 21]. In addition, GTPases rely on hydrolysis of GTP for signal transduction, protein biosynthesis and translocation of proteins [19]. Furthermore, nucleotides make up coenzymes in oxidation-reduction reactions (NAD^+, $NADP^+$, FMN, FAD), are important for the synthesis of polysaccharides (UDP-glucose and ADP-glucose) and phospholipids (CDP and CTP) [7, 22].



C

Nomenclature

1. Indication of the sugar component:
   - **d**=deoxyribose
   - no prefix= ribose
2. Abbreviation for the base:
   - adenine=**A**
   - guanine=**G**
   - cytosine=**C**
   - thymine=**T**
   - uracil=**U**
3. Indication of the number of phosphates:
   - monophosphate=**MP**
   - diphosphate=**DP**
   - triphosphate=**TP**

Examples:

dGTP=2'-deoxyguanosine-5'-triphosphate

GTP=guanosine-5'-triphosphate

dUMP=2'-deoxyuridine-5'-monophosphate

Figure 1: Nucleotides are important for cellular function, including DNA and RNA synthesis, metabolism and signaling. *A) Nucleotides consist of three moieties: a nitrogenous base, a sugar (ribose in RNA, deoxyribose in DNA) and a phosphate (-chain). B) The bases used for DNA and RNA synthesis can be divided into purines (adenine and guanine) and pyrimidines (cytosine, thymine (DNA) and uracil (RNA)). C) Nomenclature and abbreviations used in this thesis.*

Deoxynucleoside triphosphate phosphatases (dNTPases)

A balanced and clean nucleotide pool is essential to guarantee correct DNA and RNA synthesis, as well as cellular metabolism and signaling. The cell controls nucleotide homeostasis through a complex, interconnected network of biosynthetic and catabolic pathways (**Figure 2**) [13, 23]. *De novo* biosynthesis of ribonucleotides involves the stepwise assembly of the nitrogenous purine and pyrimidine rings. In addition, degraded nucleobases and nucleosides can be salvaged through the action of phosphoribosyltransferases and nucleoside kinases, respectively [24]. Deoxyribonucleotide formation is regulated by ribonucleoside diphosphate reductase (RNR), which reduces ribonucleoside diphosphates (NDPs) to deoxyribonucleoside diphosphates (dNDPs) [25]. Multiple kinases and phosphatases work in concert to precisely regulate the balance and availability of (d)NTPs. The nuclear dNTP pool is kept in a tightly regulated metastable state, in which dGTP is often limited to 5% and dATP and dTTP are most abundant [26]. Compared to the physiochemically protected DNA molecule, the free nucleotide pool is orders of magnitude more susceptible to undesired chemical reactions, such as oxidation, alkylation and deamination [12, 13].

One mechanism to control (d)NTP availability and nucleotide homeostasis is by cleaving the phosphoanhydride or phosphoester bond by (deoxy)nucleoside triphosphate phosphatases ((d)NTPases) (**Figure 2**). (d)NTPases can be classified into four, structurally different superfamilies: trimeric dUTPases, all-α NTP pyrophosphatases, inosine triphosphate pyrophosphatases and the Nudix superfamily [27, 28]. In this thesis the importance of the hydrolases dUTPase, dCTPase and NUDT15 for nucleotide homeostasis and cellular integrity is validated, both alone and in the aspect of therapeutic nucleobase- and nucleoside-analog treatment.

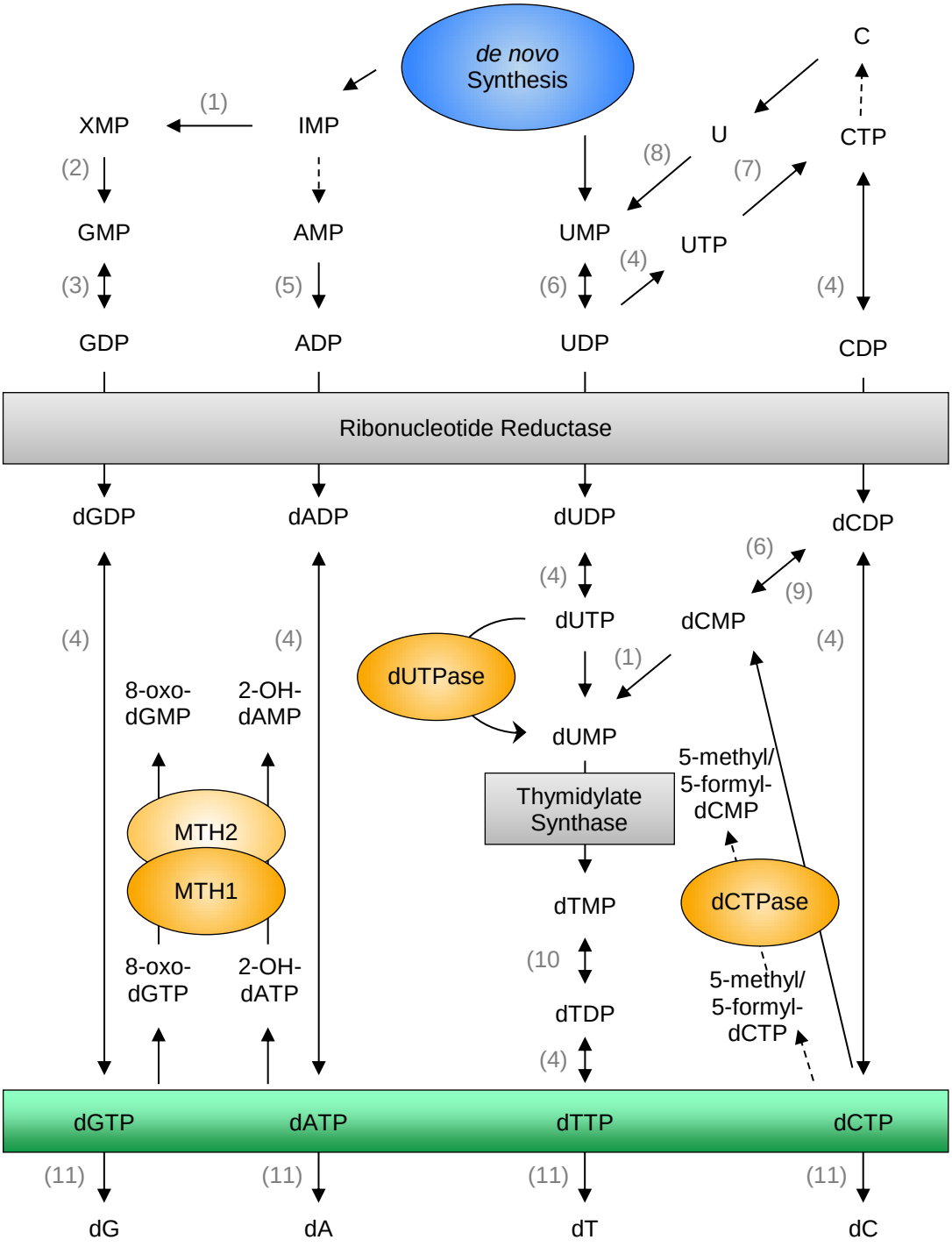


Figure 2: Overview of the complex network regulating nucleotide homeostasis as suggested before the work of this thesis. *Nucleotides are synthesized de novo or salvaged from existing building blocks. A precise balance between mono-, di- and triphosphates is essential for DNA and RNA synthesis, as well as cellular metabolism and signaling. (d)NTPases (orange) balance the availability of nucleoside triphosphate species by hydrolyzing the phosphate chain. (1) Inosine monophosphate dehydrogenase; IMPDH, (2) guanine monophosphate synthase; GMPS, (3) guanylate kinase; GUK, (4) nucleoside diphosphate kinase; NDPK, (5) adenylate kinase; AK, (6) uridine monophosphate-cytidine monophosphate kinase; UMP-CMPK, (7) cytidine triphosphate synthase; CTPS, (8) uridine kinase; UK, (9) deoxycytidine triphosphatase; dCTPase, (10) deoxythymidine monophosphate kinase; dTMPK, (11) SAM domain and HD domain-containing protein 1; SAMHD1. Graphical depiction adapted from reference [23] and [13].*

Deoxyuridine triphosphatase (dUTPase)

One major difference between DNA and RNA is the distinct composition of the bases, with thymine replacing uracil in DNA. Interestingly, the subtle distinction between thymine and uracil is a methyl group on the 5-position of the former (**Figure 1B**). It is believed that this refined difference evolved by the more stable nature of thymine and its enhanced base-pairing properties. Despite the structural similarity, incorporation of uracil instead of thymine in DNA causes mutations and cellular toxicity, both in prokaryotic and eukaryotic organisms [29, 30]. For this reason, the cell has developed highly efficient mechanisms to both prevent uracil from getting into DNA and to remove uracil from DNA if it is erroneously incorporated.

Since DNA polymerases incorporate dUTP and dTTP with similar efficiency, a high dTTP/dUTP ratio is necessary to guarantee thymine over uracil incorporation into DNA [31]. This balance is complicated by the fact that *de novo* thymidine synthesis is dependent on dUMP to be methylated to dTMP by the enzyme thymidylate synthase (TS) [23]. If dUMP levels accumulate mono- and di-phosphate kinases sequentially phosphorylate dUMP to dUTP, rendering the non-canonical deoxynucleotide available for DNA synthesis. Mis-incorporation of uracil into DNA is recognized by the BER machinery, leading to base-excision by uracil-DNA glycosylases [32, 33].

In order to prevent accumulation of deoxyuridine triphosphates and mis-incorporation during DNA synthesis, deoxyuridine triphosphatase (dUTPase) hydrolyzes dUTP back to dUMP and pyrophosphate (**Figure 3A**) [34]. This reaction not only limits dUTP accumulation, but additionally supplies TS with its substrate dUMP, ensuring the availability of the thymidine precursor.

By hydrolyzing dUTP to dUMP dUTPase is considered a main regulator of the uracil pool. Accordingly, basically every bacterial, archaeal or eukaryotic genome encodes a protein with dUTPase activity, even though they do not exhibit structural homology [28]. Human dUTPase is a propeller-shaped homotrimer, with each protomer folding into an eight-stranded jelly-roll β barrel (**Figure 3B**) [35]. The C-terminal β strand is stretched to the adjacent domain, tightly locking the molecules. Each enzymatic homotrimer contains three identical active sites, which are formed by the interplay of all three subunits (**Figure 3B**) [35]. High specificity for uracil and deoxyribose is reached by a tight hydrogen-bonding network

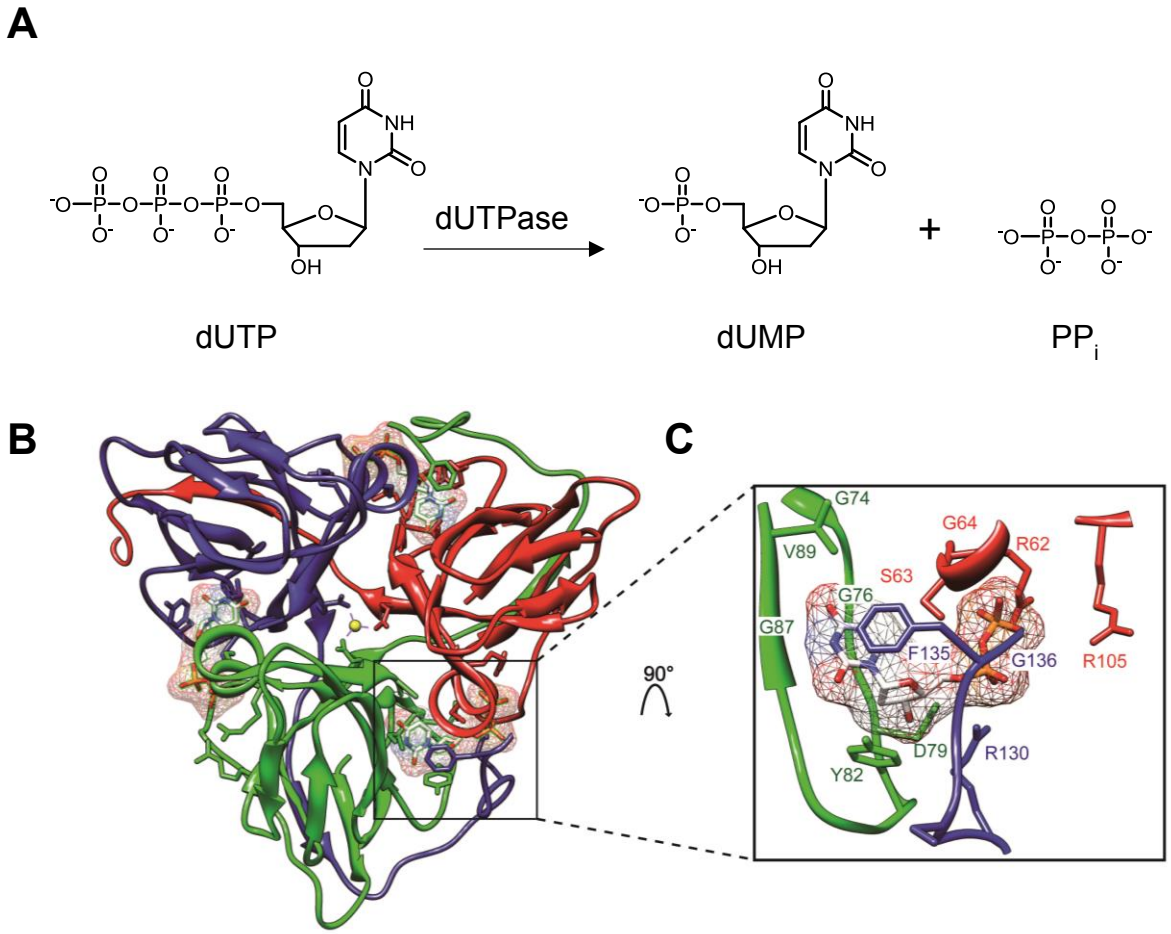


Figure 3: The dUTPase homotrimer hydrolyzes dUTP to dUMP and pyrophosphate. *A) dUTP is hydrolyzed to dUMP and pyrophosphate (PP_i) by the enzymatic activity of dUTPase. B) The human dUTPase trimer consists of three identical subunits (blue, red, green), which interconnect to form three identical active sites. C) A specific hydrogen bonding network built by all three protomers, ensures dUTP selectivity. Graphical depiction adapted from reference [35] (PDB accession code: 1Q5H).*

between dUTP and dUTPase [35]. The active site is capped by the flexible, glycine-rich C-terminal tail, which forms specific interactions with the bound dUTP.

Due to the fact that mitochondria contain a separate genome it is not surprising that both a mitochondrial and a nuclear dUTPase isoform exist in humans [36, 37]. The two isoforms are encoded by a single gene on chromosome 15. Two different promoters and two distinct 5' exons give rise to the two isoforms, which differ in the N-terminal mitochondrial target sequence. In addition, the nuclear isoform features a consensus target sequence for the cyclin-dependent protein kinase p34 (cdc2), suggesting cyclin-dependent regulation by phosphorylation of Serine 11 [38].

In healthy cells, expression of the nuclear dUTPase isoform is regulated in a cell cycle-dependent manner, both on mRNA and protein level [39-41]. Possible regulatory mechanisms for the S-phase specific expression pattern are the consensus E2F binding sequence in the putative promoter region and the observed phosphorylation at Serine 11 [32, 42]. On the contrary, the mitochondrial dUTPase isoform is constitutively expressed, likely resulting from the distinct replication pattern of mitochondrial DNA [39].

In neoplastic tissue, dUTPase expression was found to be deregulated and profoundly variable, both in quantity and intracellular localization [40]. Expression of nuclear dUTPase did not correlate with the proliferation marker Ki67 in human tumors [40, 41]. The intracellular distribution of dUTPase was highly variable, covering solely cytoplasmic, as well as exclusively nuclear staining. Moreover, Strahler *et al.* measured low levels of dUTPase in breast, lung and colon cancers and increased expression in neuroblastomas and hematopoietic malignancies [43]. Depending on the genetic background of the cell, both conditions could be considered beneficial for the tumor. High dUTPase expression could protect fast replicating cells from deoxyuridine mis-incorporation-induced toxicity. On the other hand, tumors with low dUTPase levels could benefit from non-toxic genetic instability, induced by uracil substituted DNA [37].

In summary, to prevent accumulation of the deoxyuridine pool the hydrolysis of dUTP to dUMP by dUTPase is of vital importance for both prokaryotic and eukaryotic organisms [29, 30].

Deoxycytidine triphosphatase (dCTPase)

The enzyme deoxycytidine triphosphatase (dCTPase, dCTP pyrophosphatase 1, DCTPP1, XTP3-transactivated protein A or XTP3-TPA) has recently been suggested to be involved in nuclear and mitochondrial genome integrity, by possessing hydrolase activity with deoxycytidine and 5-modified deoxycytidine triphosphate species (**Figure 4**) [27]. A role for dCTPase in carcinogenesis is supported by the finding that overexpression of the enzyme in breast, gastric, lung, liver, cervical and esophagus cancer tissue was associated with cancer growth and poor clinical prognosis [44-46]. The suggested role of dCTPase in nucleotide homeostasis and carcinogenesis makes it of high interest to further characterize the cellular function of this all-α NTP pyrophosphatase family member.

As the name suggests, dCTP is the preferred canonical substrate for dCTPase (dCTP k_{cat}/K_M = 119,000 versus 28,000, 25,000 and no detectable activity, for dTTP, dATP and dGTP, respectively) [27]. Accordingly, transient depletion of dCTPase resulted in accumulation of the intracellular dCTP levels [27]. However, dCTPase knockdown had no effect on dTTP levels, even though deamination of dCMP (dUMP) provides the immediate precursor for *de novo* thymidine synthesis (**Figure 2**) [27]. The slight increase in relative dCTP levels associated with dCTPase-depletion, were not found to affect proliferation or viability of MRC-5 and HeLa cells, over a seven day period [27].

Interestingly, modifications at the 5-position of deoxycytidine, e.g. halogenation to 5-iodo-dCTP or 5-bromo-dCTP, significantly increased the enzymatic activity *in vitro* [47, 48]. Halogenated cytidine species have been observed in chronically inflamed tissue and herpes simplex virus (HSV)-infected cells [49]. Salvage of these non-canonical (deoxy)cytidine-species could disturb the epigenetic make-up of cells, which has been suggested to link inflammation and tumorigenesis [50-52]. The hydrolysis of halogenated nucleoside triphosphates by dCTPase could act as a protective mechanism to minimize inflammation-induced mutagenesis.

Besides halogenated species, 5-methyl-dCTP and 5-formyl-dCTP have been identified as dCTPase substrates [27, 46]. Whereas 5-methyl-C is a well characterized mediator of epigenetic gene silencing, little is known about the recently identified oxidized forms 5-hydroxymethyl-C, 5-formyl-C and 5-carboxy-C [53-56]. Hydrolysis of 5-methyl-dCTP and 5-formyl-dCTP by dCTPase could counteract usage of the damaged cytosine-species for nuclear and mitochondrial DNA synthesis and thereby preserve epigenome fidelity. Cellular data on the importance of dCTPase for epigenetic integrity are limited and contradicting. Song *et al.* demonstrated that dCTPase levels influence global methylation levels and associated stemness [46]. On the other hand, Requena *et al.* found no alterations of 5-methyl-dC levels in DNA after depletion of dCTPase alone, but after stimulation with additional 5-methyl-dC [27].

In addition, further research is needed to understand the metabolism and the occurrence of 5-modified deoxycytidine triphosphates. It has been suggested that the substrate specificity of deoxycytidine kinase (DCK) and cytidine monophosphate kinase 1 (CMPK1) provides a barrier for salvage of 5-hydroxymethyl-dC, 5-formyl-dC and 5-carboxy-dC, limiting the formation of their triphosphate derivatives [57].

Overall, these findings suggest not only a role for dCTPase in balancing dCTP levels, but also in sanitization of 5-modified deoxycytidine analogs, such as halogenated nucleotides during inflammation and epigenetically involved 5-methyl-dCTP and 5-formyl-dCTP. By balancing and sanitizing the dNTP pool dCTPase activity might play a critical role in maintaining nucleotide homeostasis and epigenome fidelity.

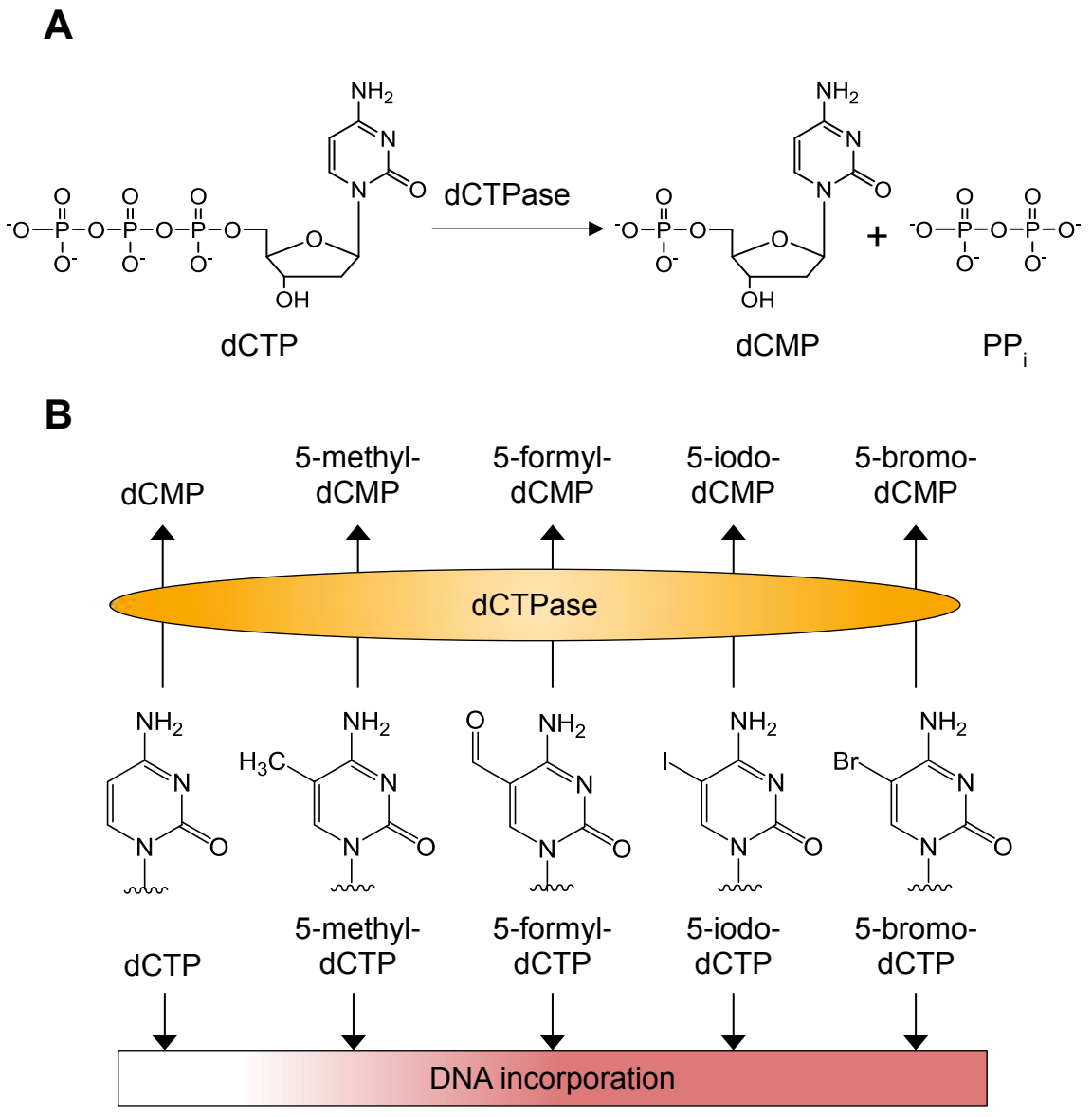


Figure 4: dCTPase hydrolyzes deoxycytidine and 5-modified deoxycytidine triphosphates to the corresponding monophosphates and pyrophosphate (PP_i). *A) dCTP is converted to dCMP and PP_i by the activity of dCTPase. B) 5-modifications of deoxycytidine increase the enzymatic activity of dCTPase. Substrate examples are: dCTP, 5-methyl-dCTP, 5-formyl-dCTP, 5-iodo-dCTP and 5-bromo-dCTP.*

The Nudix hydrolase family

The nucleoside diphosphate linked to some other moiety, X, (Nudix) hydrolase family comprises in humans at least 24 enzymes and 5 pseudogenes, from which various members have been linked to nucleotide homeostasis and cell viability [58, 59]. The protein family is categorized by their collective 23-amino acid Nudix box motif (Gx_5Ex_2[UA]xREx_2EExGU), where "U" is an aliphatic, hydrophobic residue and "x" is any amino acid [59]. While these core residues are essential for divalent cation coordination (in most cases Mg^{2+}), needed for the enzymatic activity, the nucleobase selectivity is achieved by adjacent side chains forming the active site [28, 60]. Thus, the highly conserved sequence of the Nudix box leads to similar enzymatic activity and the heterogeneity of the active site generates the broad substrate specificity within the Nudix superfamily.

As the name indicates, the Nudix family members hydrolyze nucleoside diphosphates that are linked to a diverse array of moieties. Substrates for Nudix enzymes include (deoxy)nucleoside tri- and di-phosphates (canonical, alkylated, oxidized and halogenated derivatives), dinucleotide coenzymes, nucleoside diphosphate sugars and alcohols, dinucleoside polyphosphates (Np_nN) and capped RNAs [58]. Due to the substrate diversity, Nudix enzymes have been linked to various cellular processes, including sanitation of modified or hazardous materials, balancing the (deoxy)nucleoside triphosphate pool and mediating signaling pathways. However, for many Nudix family members, the preferred physiological substrate and function remains to be elucidated.

Several members of the Nudix family were originally classified as MutT Homologs (MTH), deriving from the *Escherichia coli (E. coli)* 8-oxo-dGTP sanitizing enzyme MutT. Even though this nomenclature frequently remains, in cases of distinct substrate preference it can be misleading and should be revised to the Nudix-classification in these cases [58].

MutT homolog 1 (MTH1)

The increased cell growth, metabolic activity and oncogene activation of many cancers often create a dysregulated reduction-oxidation (redox) environment [61, 62]. In addition to direct damage to the DNA molecule, oxidation of the DNA precursor pool is a major hazard for genome integrity. In fact, while the DNA bases are protected by helix formation and the nuclear membrane, the free dNTP pool is exposed to a highly reactive environment and located physically closer to mitochondria, the main source of ROS [12, 13]. To date, more

than 20 oxidatievely damaged base lesions have been identified [63]. Due to its high abundance, stability and mutagenicity oxidized guanine (8-oxo-G) is one of the best characterized lesions. Guided by base pairing properties 8-oxo-dGTP can be mis-inserted by DNA polymerases opposite adenine and cytosine in the coding strand [64]. If these substitutions remain undetected and unrepaired by the BER machinery (including the DNA glycosylases OGG1 and MUTYH) 8-oxo-G can cause A:T → C:G and G:C → T:A transversion mutations [64, 65].

To prevent oxidative damage to cellular components, including proteins, lipids, DNA and the nucleotide pool, cancer cells often adapt by upregulating redox protective mechanisms [66]. The high mutagenicity of 8-oxo-dGTP highlights the importance for mechanisms that prevent accumulation of this DNA precursor [67]. The MutT Homolog 1 (MTH1 or Nudix-type 1; NUDT1) was the first identified human homolog of the *E. coli* 8-oxo-dGTPase MutT [68-70]. Upregulation of MTH1 has been observed in many cancer types and is considered as a possible protective mechanism to prevent oxidative damage [71-73].

In addition to 8-oxo-dGTP, the human MTH1 protein sanitizes the nucleotide pool from the oxidized purines 2-OH-dATP, 8-OH-dATP and the corresponding ribonucleotides 2-OH-ATP, 8-OH-ATP and 8-oxo-GTP [74-76]. By hydrolyzing these oxidatively damaged purines, MTH1 prevents their accumulation and hazardous incorporation into DNA and RNA. RNA interference-mediated silencing of MTH1 has been shown to result in accumulation of 8-oxo-G in DNA, which was associated with increased DNA damage and cell death [71, 77-80]. MTH1 knockout mice are fertile and have the same life span as wild type mice, but possess an increased risk for spontaneous tumorigenesis. Isolated fibroblasts of these mice have been shown to be hypersensitive to H_2O_2 [81, 82]. Whereas MTH1 has been shown to be an important sanitizer of the oxidized nucleotide pool, other consequences of MTH1 inhibition are only just now beginning to be uncovered. Besides being DNA building blocks, (d)GTP and (d)ATP are involved in various cellular functions, including RNA synthesis, metabolism and cell signaling. Disturbing (d)GTP and (d)ATP homeostasis could therefore have additional consequences for cellular integrity.

High hopes lie on exploiting the function of MTH1 for cancer specific treatment and several potent small molecule inhibitors have been presented in the scientific literature [71, 77, 83, 84]. However, the validity of MTH1's role in cancer and as a drug target has recently been questioned. Several small molecule compounds have been published that inhibit the

8-oxo-dGTPase activity of MTH1 *in vitro*, but do not induce cancer cell death [84-86]. Interestingly, these compounds were found to not affect 8-oxo-G levels in DNA, which may explain the lack of cellular toxicity (unpublished data) [85]. Whereas additional research is needed to clarify the controversy and complexity of MTH1 biology, exploiting the oxidative stress levels of cancer remains a promising strategy for anti-cancer therapy.

NUDT15 and other potential sanitizers of the oxidized nucleotide pool

The increasing interest to exploit the dysregulated redox environment of cancer cells for novel anti-cancer treatments raised the interest for additional sanitizers of the oxidized nucleotide pool. Several Nudix family members, including NUDT15 (MTH2), NUDT18 (MTH3) and NUDT5, have been suggested to function as hydrolases of oxidatively damaged nucleotides [87-89].

The closest MTH1 sequence homolog MutT Homolog 2 (MTH2, Nudix-type 15 or NUDT15) has been shown to hydrolyze 8-oxo-dGTP as well as 8-oxo-dGDP to 8-oxo-dGMP *in vitro* [65, 87, 88], although to a considerably lesser extent than MTH1. In line with this, overexpression of mouse NUDT15 protein reduced the mutation frequency of *E. coli* cells depleted of the 8-oxo-dGTPase MutT [87]. Furthermore, siRNA-mediated knockdown of NUDT15, as well as NUDT5, increased 8-oxo-dGTP-induced A:T → C:G substitution mutations on a reporter plasmid transfected into human 293T cells [65].

Interestingly, NUDT15 was found to physically interact with its C-terminal region to the N-terminus of proliferating cell nuclear antigen (PCNA), despite lacking a consensus motif for PCNA-binding, like the PIP-box or KA-box motif [90]. The authors suggested that this binding stabilizes and protects PCNA from UV-irradiation-induced acetylation and degradation by the proteasome. These findings suggest that the 8-oxo-dGTPase activity of NUDT15 could be of particular importance during exceptional situations and at distinct subcellular locations, as for example the replication fork. To complicate the picture, NUDT15 has been suggested to possess mRNA decapping activity, both on mono-methylated and un-methylated capped RNAs [91, 92].

Overall, the low enzymatic activity of NUDT15, NUDT18 and NUDT5 with oxidized nucleotides raised doubt about the physiological importance of these activities in a cellular context. Additional cellular experiments are needed to understand the contribution of these hydrolases for sanitation of the oxidized nucleotide pool. In addition, the 8-oxo-(d)GTPase

activity of the Nudix family member NUDT17, which structurally clusters together with NUDT1, NUDT15 and NUDT18, has received no experimental attention before the start of this thesis [88].

1.3 Antimetabolites: disrupting nucleotide homeostasis

Since a balanced and undamaged nucleotide pool is critical for cellular survival, interfering with nucleotide homeostasis has been a long exploited strategy to treat cancer. Synthetic nucleobase- and nucleoside-analogs (antimetabolites) have been developed that resemble endogenous metabolites and thereby interfere with various cellular processes. Besides for cancer therapy, nucleobase- and nucleoside-analogs are commonly used as anti-viral agents and as immunosuppressants [3].

Upon treatment, nucleobase- and nucleoside-analogs undergo the same metabolic processing as endogenous nucleotides. Cellular uptake, of the often hydrophilic compounds, is mediated by specific nucleoside transporters, including equilibrative uniporters, antiporters, concentrative transporters and ABC transporters [93-96]. Inside the cell, the analogs are substrates for the endogenous purine and pyrimidine salvage pathways. These involve various nucleoside kinases and nucleoside monophosphate kinases that lead to phosphorylation of the antimetabolites [3]. Subsequent metabolic activity of a nucleoside diphosphate kinase, creatine kinase or 3-phosphoglycerate kinase leads to the formation of nucleoside triphosphate analogs [3]. The phosphorylated non-canonical derivatives compete with physiological nucleotides and thereby disturb various cellular processes, including nucleotide homeostasis, DNA and RNA synthesis, enzyme function, signaling and metabolism. Due to this multifaceted attack, the physiological consequences of nucleobase- and nucleoside-analogs are often complex and dependent on the molecular background of the cell.

In this section, the metabolism and molecular mode of action for the antimetabolites 5-FU, decitabine and 6-TG will be discussed in more detail. A summary of their drug class, active metabolites, primary target(s) and molecular mechanism can be found in **Figure 5**.

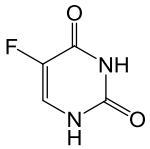
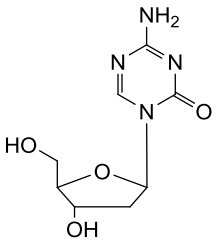
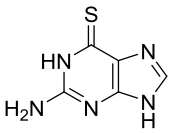
Structure			
Therapeutic agent	5-fluorouracil	decitabine	6-thioguanine
Abbreviation	5-FU	5-aza-dC	6-TG
Drug class	fluoropyrimidine	cytidine analog	thiopurine
Active metabolite	5-fluoro-dUMP 5-fluoro-dUTP	5-aza-dCTP	6-thio-dGTP 6-thio-GTP
Primary target(s) and mechanism	TS inhibition, nucleotide imbalance, DNA and RNA mis-incorporation, DNA damage	DNA incorporation, hypomethylation, DNMT1 trapping, DNA damage	DNA and RNA mis-incorporation, DNA damage

Figure 5: Nucleobase- and nucleoside-analogs discussed in this thesis.

Fluoropyrimidines

Fluoropyrimidines belong to the oldest and most-effective chemotherapeutics that are used today. Even though the precise mechanism of action was not known during the development of the first fluoropyrimidine in 1957 the invention is a great example of rational drug discovery [4]. Heidelberger *et al.* based the synthesis of fluorinated pyrimidine analogs on four observed phenomena 1) replacing molecular hydrogen by fluorine leads to profound alterations in biological function, 2) nucleic acid precursor-analogs have anti-tumor activity, 3) rat hepatomas mis-incorporate uracil into DNA to a greater extent than healthy cells, 4) uracil-based synthetic compounds possess anti-tumor activity [97-101]. Today, almost 60 years after the first application, 5-FU remains a cornerstone in the treatment regimen against a wide array of solid tumors, including colorectal cancer, pancreas, breast, gastric, and ovarian cancers [101]. However, despite improved treatment regimens, side effects and drug resistance remain major drawbacks of fluoropyrimidine therapy.

Mode of action of fluoropyrimidines

Even though 5-FU belongs to the oldest chemotherapeutics used today the exact mechanism of toxicity remains debated. 5-FU treatment results in a multifaceted phenotype due to its intertwined mode of action involving 1) inhibition of the enzyme thymidylate synthase (TS) and subsequent nucleotide pool imbalance 2) mis-incorporation of dUTP and 5-fluoro-dUTP into DNA and 3) disturbance of RNA synthesis by 5-fluoro-UTP.

One of the main consequences of 5-FU treatment is inhibition of TS, an essential enzyme for thymidine production. TS catalyzes the reductive methylation of dUMP to dTMP, utilizing 5,10-methylenetetrahydrofolate (CH_2THF) as the methyl donor (**Figure 6**) [102]. The substrate dUMP can originate from two main sources: deamination of dCMP by deoxycytidylate deaminase (DCD) and reduction of UDP by RNR [32]. The oxidized by-product dihydrofolate (DHF) is recycled by the combined action of dihydrofolate reductase (DHFR) and serine hydroxymethyltransferase (SHMT). Since the TS reaction product dTMP is the sole source of *de novo* thymidine, TS is considered one of the few metabolic bottlenecks in the biosynthesis of DNA precursors [101]. The necessity of CH_2THF as a cofactor highlights the importance of adequate folate metabolism for thymidine production.

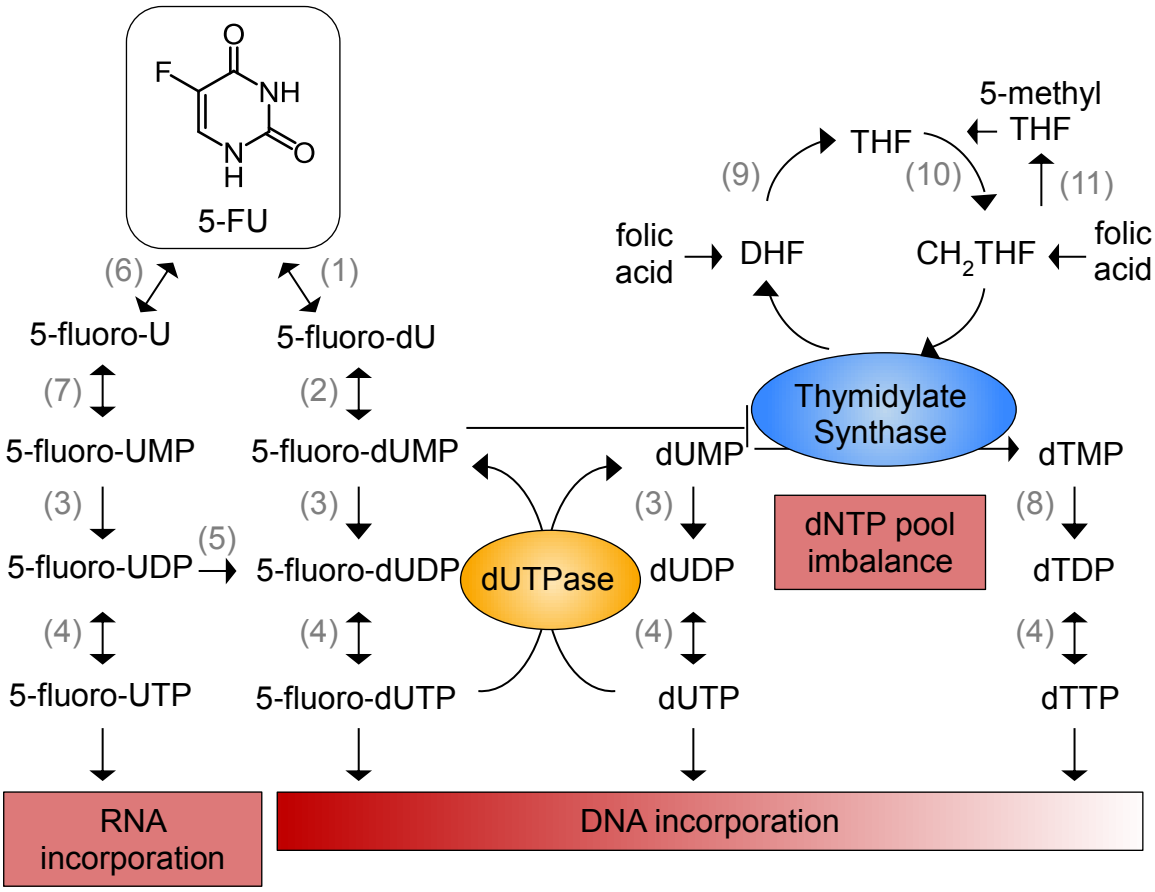


Figure 6: Metabolism and mode of action of 5-fluorouracil (5-FU). *The non-canonical base 5-FU is converted by uracil metabolizing enzymes into the main active components 5-fluoro-dUMP, 5-fluoro-UTP and 5-fluoro-dUTP. Whereas 5-fluoro-dUMP inhibits the enzyme thymidylate synthase (TS), inducing dNTP pool imbalance, 5-fluoro-UTP and 5-fluoro-dUTP get mis-incorporated into RNA and DNA, respectively. (1) thymidine phosphorylase; TP, (2) thymidine kinase; TK, (3) pyrimidine monophosphate kinase; NMPK, (4) pyrimidine diphosphate kinase; NDPK, (5) ribonucleoside diphosphate reductase; RNR, (6) uridine phosphorylase; UP, (7) uridine kinase; UK, (8) deoxythymidine monophosphate kinase; dTMPK, (9) dihydrofolate reductase; DHFR, (10) serine hydroxymethyltransferase; SHMT1, (11) methylentetrahydrofolate-reductase; MTHFR. Graphical depiction adapted from Reference [101].*

The presence of two distinct, cofactor and substrate, binding pockets provides the possibility to impede the enzymatic function of TS with two structurally distinct classes of antimetabolites: antipyrimidines and antifolates. Anti-pyrimidines that inhibit TS function include among others the fluoropyrimidines 5-FU, 5-fluoro-deoxyuridine (FdUrd) and the pro-drug capecitabine. The therapeutic fluoropyrimidine bases are metabolized to the main effector metabolite 5-fluoro-2'-deoxyuridine-5'-monophosphate (5-fluoro-dUMP) by enzymes of the pyrimidine nucleotide synthesis pathway. 5-fluoro-dUMP competes with the natural substrate dUMP for covalent binding to the substrate pocket of TS and thereby inhibits the enzyme [103, 104]. Antifolates, on the other hand, can exhibit TS inhibition either directly by binding to the enzymatic co-factor pocket (e.g. by ZD1694 and ZD9331) or indirectly through depletion of the CH_2THF pool as a consequence of DHFR inhibition (e.g. by methotrexate and metoprine) [101].

Targeting TS, directly or indirectly, leads to depletion of the product dTMP while the substrate dUMP accumulates before the metabolic blockage. It was originally assumed that the extreme depletion of dTMP and subsequent shortage of the DNA precursor dTTP are the main causes of the observed DNA synthesis arrest and cytotoxicity; a condition termed "thymineless death" [105]. However, it has been suggested that, besides dTTP deficiency, expansion of the dUTP pool and subsequent uracil mis-incorporation into DNA are important components of TS-induced toxicity [106]. Moreover, treatment with fluoropyrimidines leads to accumulation of 5-fluoro-dUTP, which further raises the uracil pool. Since DNA polymerases incorporate dTTP, dUTP and 5-fluoro-dUTP with similar efficacy, the nucleotide imbalance can result in DNA mis-incorporation of uracil and fluoro-uracil [31]. These DNA lesions are subject for the base excision repair (BER) and mis-match repair (MMR) machinery, which try to resolve the DNA damage. However, if the nucleotide imbalance persists, futile excision and re-incorporation of erroneous (fluoro-)uracil creates numerous abasic sites, single- and double-strand breaks [31, 106-111]. Activation of homologous recombination (HR) is a last attempt to repair these lesion and prevent cellular death [112].

Various research groups have demonstrated the importance of BER and HR for fluoropyrimidine toxicity, by depletion of major repair proteins [112-115]. A more discrepant picture has developed over the importance of the different DNA-glycosylases for uracil and 5-FU excision and fluoropyrimidine-induced cell death, resulting from the use of different model systems and TS-inhibitors. It has been shown that knockout of the major

replication-associated uracil-DNA glycosylase, UNG, in mouse embryonic fibroblasts does not affect the sensitivity to TS inhibitors [116]. In line with this, FdUrd or 5-FU efficacy was not altered by expression of Ugi, a protein inhibitor of UNG family members [117]. However, higher vertebrates, but not lower eukaryotes such as *Saccharomyces cerevisiae*, express a second glycosylase named SMUG1. Qian An *et al.* suggests that SMUG1 expression, contrary to UNG, influences 5-FU toxicity through excision of 5-FU lesions from DNA [118]. This study was disputed by a study from Pettersen *et al.* who showed that even though UNG-initiated BER is mainly responsible for repairing 5-FU in DNA, this mechanism does not contribute to cytotoxicity of 5-FU, but of FdUrd [119]. In addition, the activity of the thymine DNA glycosylase (TDG) has been linked to 5-FU-induced DNA strand breaks, cell cycle alterations and DNA damage [110].

Besides the incorporation of 5-fluoro-dUTP into DNA several studies have suggested that mis-incorporation of 5-fluoro-UTP into RNA contributes to fluoropyrimidine-induced toxicity and might even be the prevalent cause [119-124]. RNA mis-incorporation of the fluoro-substituted nucleotide has been shown to disturb among others: maturation of ribosomal RNA, activity of small nuclear RNA-protein complexes, RNA exosome function and modification of transfer RNAs [125-129]. Successful rescue experiments with uridine highlight the importance of RNA-disturbances for TS inhibitor-induced toxicity [120, 130, 131].

These data demonstrate that the working mechanism of fluoropyrimidines is complex and the exact cause of toxicity not fully understood. Generally, one can say that the 5-FU-induced toxicity results from a complex molecular interplay involving: dTTP depletion, accumulation and incorporation of (5-fluoro-) dUTP into DNA, as well as disturbance of RNA synthesis by 5-fluoro-UTP. The contribution of each part to cell toxicity is most likely dependent on the type of TS-inhibitor and the genetic make-up of the cell.

In the clinic, administration of 5-FU as a single agent achieves response rates of only ~10% [132]. During 6 decades of clinical application various combination therapies and treatment regimens have been established to improve the therapeutic success. Combining 5-FU with modulating agents like leucovorin, levamisole or methotrexate or administration by continuous venous infusion increased the objective response rate in patients [133]. However, response rates merely reach 50% and drug resistance is an inevitable problem.

Intrinsic or acquired drug resistance can result from alterations in each step of the molecular mechanism, including cellular uptake and efflux, drug metabolism and catabolism, target expression and alterations in the cellular response. Several causes for 5-FU resistance have been described, among others upregulation of TS [134, 135], Bcl-2 [136], Bcl-XL [136, 137], and Mcl-1 [138], and methylation of the MLH1 gene [139], highlighting the importance of DNA repair pathways for toxicity. In addition, expression of the nucleotide hydrolase dUTPase has been found to negatively correlate with 5-FU treatment success [40].

Link between dUTPase and 5-fluorouracil metabolism

The physiological function of dUTPase is to protect cells from dUTP pool expansion and thereby prevent toxic consequences of uracil mis-incorporation into DNA (**Figure 6**) [140]. However, from a treatment perspective dUTPase activity could hamper accumulation of dUTP and the active 5-FU metabolite 5-fluoro-dUTP and thereby reduce intended cytotoxicity.

Evidence for an influential role of dUTPase in TS inhibitor-induced toxicity has been presented in prokaryotic and eukaryotic systems. In *E. coli,* ectopic overexpression of dUTPase caused resistance to FdUrd-induced DNA strand breaks and toxicity [141]. In the human colorectal adenocarcinoma cell line HT29 overexpression of both human and *E. coli* dUTPase reduced FdUrd-associated dUTP levels, DNA damage and cytotoxicity [141]. Interestingly, a study by Leslie *et al.* suggested that the observed protective effects of dUTPase overexpression on TS-inhibitor toxicity were a time dependent phenomenon [142]. Whereas dUTPase overexpressing cells were less sensitive in the first 24 hours of the treatment, similar toxicity was demonstrated after 48 hours. In addition, the influence of dUTPase depletion on TS inhibitor effectiveness has been validated in several studies using siRNA-mediated protein knockdown. Overall, depletion of dUTPase negatively reflected the phenotype observed after overexpression of the nucleotide triphosphatase. If TS-targeted therapies were combined with dUTPase depletion intracellular dUTP pools were found to be increased, which potentiated DNA damage and cell death. This has been studied in non-small cell lung cancer (A549, H358, H1299, and H460), colon cancer (SW620), cervix (HeLa) and breast cancer (MCF-7) cell lines [143-145].

All these studies confirm that overexpression and knockdown of dUTPase significantly influence the efficacy of TS inhibitors. In addition, endogenous dUTPase levels were found to be predictive for response to TS inhibitors in various cancer cell lines [146, 147]. In line

with this, in colorectal cancer patients treated with 5-FU, high intra-tumoral dUTPase expression was found to be significantly associated with reduced treatment response, shorter time to progression and shorter survival [40]. Furthermore, primary tumors with metastasis were found to express higher levels of dUTPase, compared to non-metastatic tumors [41, 148]. Due to these clinical observations, dUTPase was suggested as a biomarker for both prognostic TS inhibitor effectiveness and metastatic potential.

Decitabine

Whereas protein structure and function is determined by the genetic sequence, the expression level is influenced among others by epigenetic alterations, including DNA methylation and histone modifications [149]. Methylation of cytosine residues in CpG promoter islands induces gene silencing through chromosome condensation [150, 151]. This epigenetic information is induced and maintained by the methylation activity of the protein family of DNA methyltransferases (DNMTs) [152]. During a post-replicative process DNMTs covalently bind to the cytosine ring and subsequently transfer a methyl group from S-adenosyl-L-methionine (SAM) to the 5-carbon of the base [151]. Fulfillment of the complete reaction is necessary to release the methyltransferase from the DNA strand. While in healthy cells 60-90% of cytosine residues in CpG sequences are methylated, cancers often utilize an abnormal methylation status to silence critical tumor suppressor genes, like Rb1, VHL or *h*MLH1 [153-156]. Reversing epigenetic alterations is a promising strategy to treat cancer, since the underlying genetic information remains intact.

In 1964, Pliml and Sorm synthesized decitabine (5-aza-2'-deoxycytidine or 5-aza-dC), a cytosine-analog in which nitrogen (N) substitutes the 5-carbon (**Figure 7**) [5]. Following intracellular uptake, the synthetic base gets phosphorylated by deoxycytidine kinases forming 5-aza-dCTP, which gets inserted by DNA polymerases into nascent DNA [93, 157, 158]. The paired 5-aza-C:G dinucleotide is recognized as endogenous CpG sequence by DNMT. A subsequent nucleophilic attack forms a covalent bond between the 6-carbon of the base and the methyltransferase [159-161]. The nitrogen at the 5-position of the analog prevents the beta-elimination normally executed by the 5-carbon of cytosine, covalently trapping the DNMT molecule to the DNA [161].

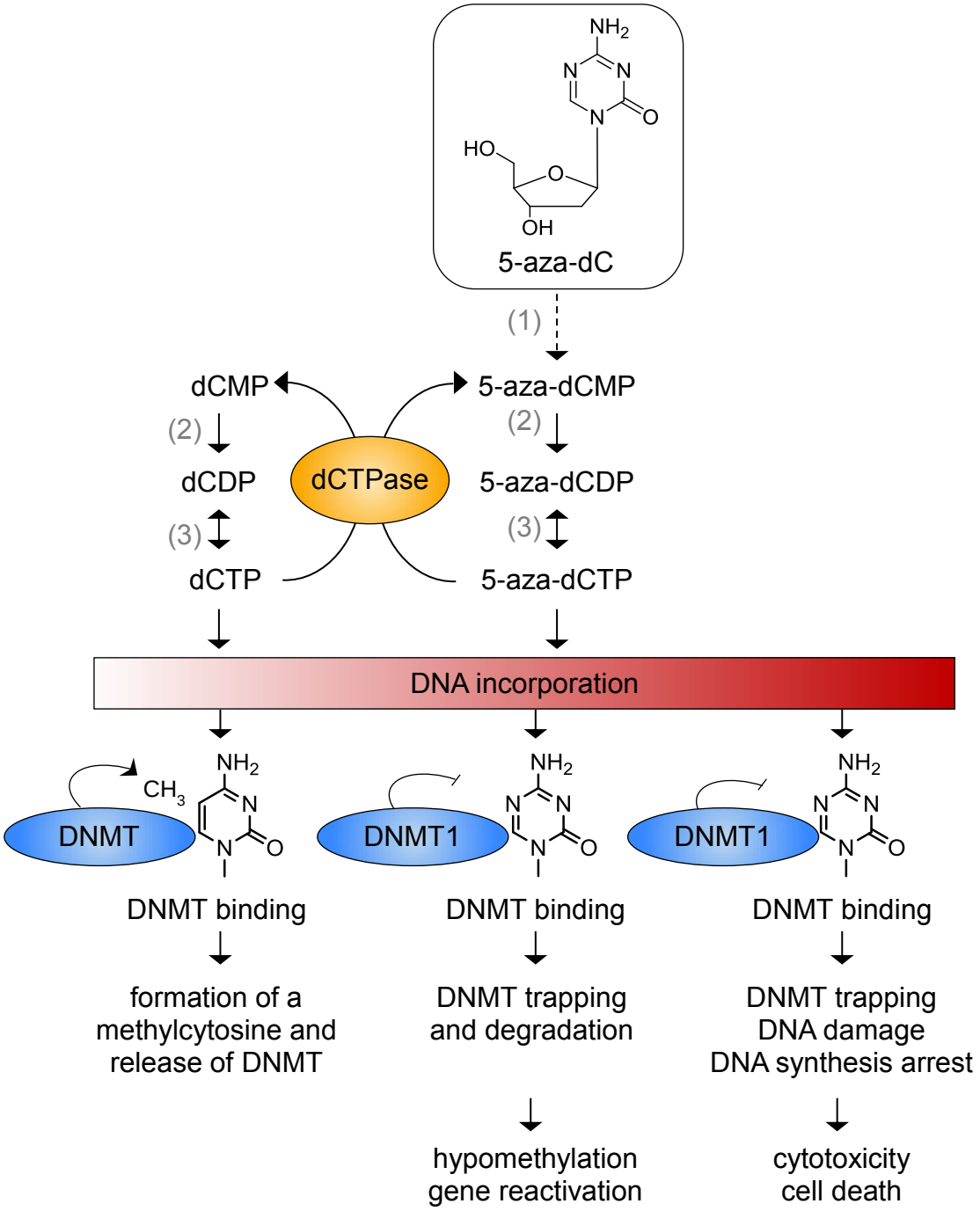


Figure 7: DNMT1 activity and mode of action of the deoxycytidine analog decitabine (5-aza-dC). *To maintain the epigenetic signature DNMTs covalently bind cytosine and transfer a methyl-group from SAM to the 5-carbon of the base (left panel).The synthetic cytosine analog 5-aza-dC enters the cell and gets phosphorylated by (1) deoxycytidine kinase (2) deoxycytidine monophosphate kinase and (3) nucleoside diphosphate kinase. If accumulated the triphosphate species can get mis-incorporated into DNA by DNA polymerases. In an attempt to methylate the cytosine analog DNMT covalently binds the synthetic base. Due to the 5-nitrogen DNMTs cannot proceed with the methylation reaction, which traps the enzyme to the base. Whereas low levels of decitabine (middle panel) result in hypomethylation and gene reactivation, high levels (right panel) cause DNA damage and DNA synthesis arrest. Graphical depiction adopted from [151].*

Dependent on the amount of incorporated 5-aza-dC into DNA two distinct physiological outcomes are the consequence. In the case of low 5-aza-dC levels, DNMTs are degraded, resulting in demethylation and restoration of gene expression [162]. On the other hand, increased levels of 5-aza-dC cause disturbances in DNA synthesis and cell cycle progression which, if accumulated, induce DNA damage and cytotoxicity [163, 164]. Decitabine has been proven effective in the treatment against hematologic malignancies, including acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL) and myelodysplastic syndrome (MDS). However, the therapeutic success is limited to a response rate of only 50% and cancer relapse is a common problem [165, 166].

Recently, the activity of the nucleotide hydrolases dCTPase and dUTPase has been suggested to influence decitabine efficacy [48]. Knowing from previous studies that dCTPase hydrolyzes 5-modified deoxycytidine species Requena *et al.* demonstrated activity with the active decitabine metabolite 5-aza-dCTP [27]. In line with this, dCTPase depletion potentiated decitabine-induced global DNA demethylation and cell death. Interestingly, the authors of this study suggest, that besides 5-aza-dCTP incorporation, the metabolism to 5-aza-dUMP and subsequent TS inhibition is a component of the mode of action of decitabine. They support this hypothesis by dUTPase and dCTPase knockdown experiments, that demonstrated increased levels of uracil mis-incorporation and double-strand break formation when dUTPase and dCTPase were depleted during decitabine treatment. Decitabine treatment additionally up-regulated several enzymes involved in pyrimidine metabolism, including dCTPase and dUTPase [48]. This study suggests that targeting dUTPase and dCTPase activity could be a novel strategy to improve the treatment response to decitabine.

Thiopurines

Historically, the development of medicine was based on a trial-and-error process, which was often subjected to chance. During the 1940's George Hitchings and Gertrude Elion questioned this traditional way of drug development and rather believed in a process today known as "rational drug design". At the time, the success of sulfur containing drugs raised their attention and led them to the hypothesis that this principal could be applied to interfere with cellular proliferation. Based on this assumption they synthesized the first synthetic guanine analog, in which they substituted the 6-oxygen of the purine ring with sulfur: 6-TG was discovered [48]. In addition, the development of the thiopurine pro-drugs 6-mercaptopurine (6-MP) and azathioprine (AZA-T) can be accredited to the research of Hitchings and Elion, who in 1988 received the Nobel Prize in Physiology or Medicine for their pioneering work on the *"important principles for drug treatment"*. Still today, 6-TG, 6-MP and AZA-T are commonly used anti-cancer drugs, which are additionally repurposed as anti-inflammatory and immunosuppressive agents. However, treatment response to thiopurines is highly variable, reaching from intrinsic or acquired drug resistance to cases of hypersensitivity.

Metabolism of thiopurines

6-TG, 6-MP and AZA-T are pro-drugs, which need to undergo extensive metabolism to exert their cytotoxic effects (**Figure 8**) [167, 168]. AZA-T is the most distinct pro-drug, which through a non-enzymatic reaction is converted to 6-MP and an imidazole group. Both 6-TG and 6-MP are transported into the cell where they enter the purine salvage pathway. In a first step, hypoxanthine phosphoribosyltransferase (HPRT) adds ribose-5-phosphate to the base and thereby creates the nucleoside monophosphates 6-thio-IMP and 6-thio-GMP. Additional metabolism by inosine monophosphate dehydrogenase (IMPDH) and guanine monophosphate synthetase (GMPS) converts 6-thio-IMP to 6-thio-GMP. The sequential activity of deoxynucleotide kinases and reductases creates the active metabolites 6-thio-dGTP and 6-thio-GTP, which are accountable for the majority of cytotoxic effects of this drug-class.

The efficiency of thiopurine activity is not only determined by conversion of the pro-drugs to the active metabolites, but also by competing reactions that inactivate various derivatives [167, 168]. Thiopurine S-methyltransferase (TPMT) methylates 6-MP, 6-TG, as well as their

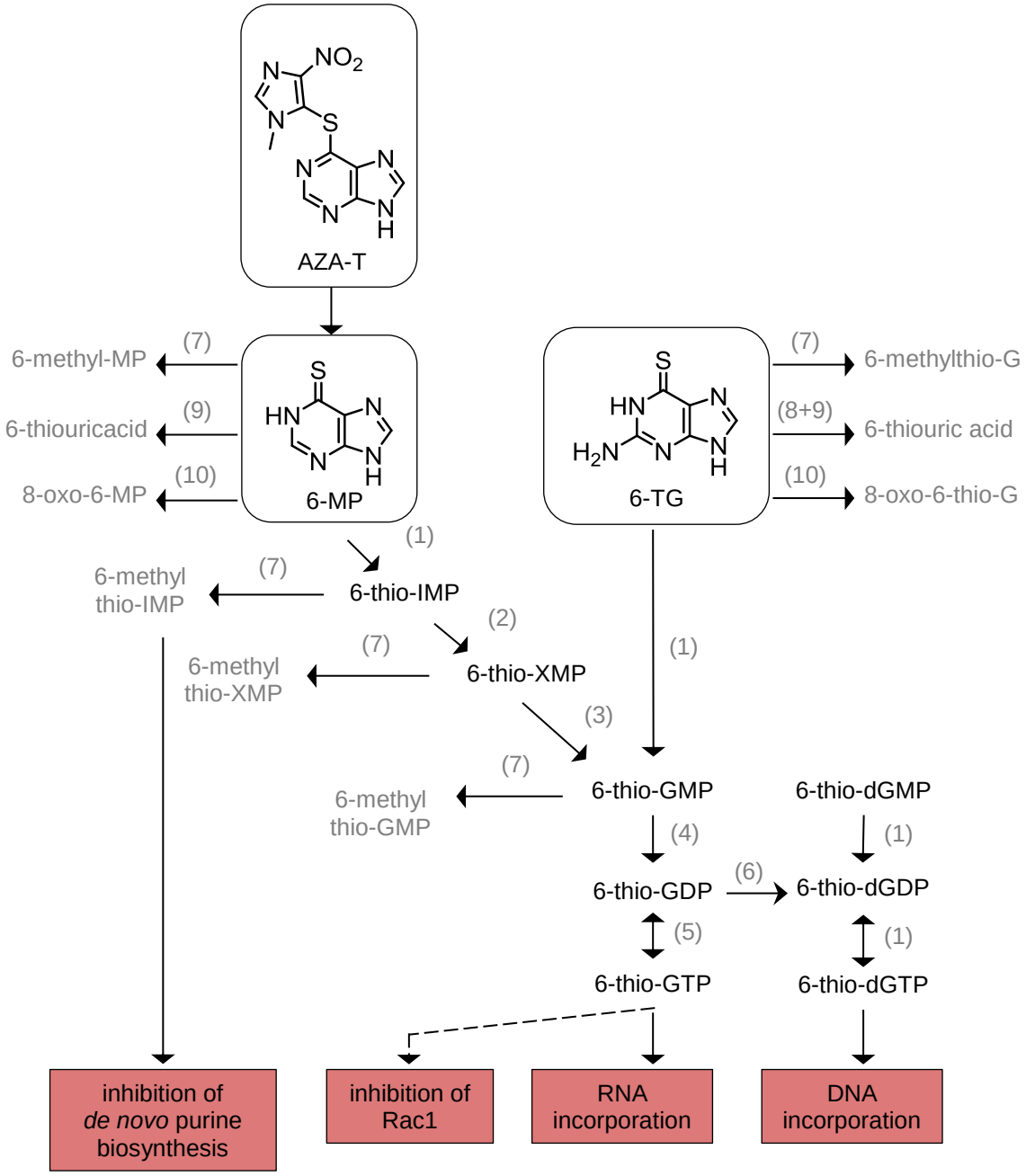


Figure 8: Metabolism and mode of action of thiopurines. *AZA-T, 6-MP and 6-TG are metabolized to the active derivatives 6-thio-dGTP and 6-thio-GTP, which are incorporated into DNA and RNA respectively. (1) hypoxanthine–guanine phosphoribosyltransferase; HPRT, (2) inosine monophosphate dehydrogenase; IMPDH, (3) guanine monophosphate synthetase; GMPS, (4) monophosphate kinase; NMPK, (5) diphosphate kinase; NDPK, (6) ribonucleoside reductase; RNR, (7) TPMT: thiopurine S-methyltransferase, (8) guanine deaminase: GAH, (9) xanthine oxidase: XO, (10) aldehyde oxidase: AO. Abbreviations: AZA-T: azathioprine, 6-MP: 6-mercaptopurine, 6-TG: 6-thioguanine. Graphical depiction adapted from references [167, 168].*

corresponding nucleoside monophosphates 6-thio-IMP and 6-thio-GMP and thereby prevents their progression in the biosynthetic pathway. Besides detoxification of AZA-T, 6-MP and 6-TG by TPMT, xanthine oxidase (XO) and aldehyde oxidase (AO) contribute to the catabolism of the thiopurines [167, 168].

Mechanism of thiopurine-induced toxicity

The mode of action of thiopurines is complex and even after six decades of clinical application not completely understood. The main active metabolites of all three thiopurine pro-drugs are 6-thio-dGTP and 6-thio-GTP (**Figure 8**). By the close resemblance to dGTP and GTP these synthetic analogs interfere with nucleotide metabolism, as well as DNA and RNA synthesis.

With a similar K_m to unmodified dGTP DNA polymerases incorporate 6-thio-dGTP into DNA, replacing between 0.01 and 0.1% of canonical guanine with the sulfur containing analog (**Figure 9**) [169-171]. However, the presence of 6-TG in DNA is neither particularly toxic nor mutagenic [167]. 6-TG incorporated into DNA is non-enzymatically methylated, most likely by SAM, creating 6-methylthio-G. During the next round of replication, the non-canonical base ambiguously pairs with cytosine and thymine [172]. These mis-pairs essentially activate the MMR machinery, which attempts to find a correct daughter-strand partner for the 6-methylthio-G in the template DNA strand [173, 174]. However, since the erroneous base is present in the template DNA strand, processing of this mis-pair is inevitably futile [15]. Subsequently, the DNA damage and the MMR machinery trigger checkpoint activation, which over the ATR/CHK1 axes results in a strong G_2 cell cycle arrest [175-177]. Since the mis-incorporation of thiopurines is usually unproblematic until the second round of replication, cytotoxicity often does not manifest until day two or three of drug treatment. Eventually, too high levels of incorporated 6-thio-dGTP and resulting futile cycles of DNA repair induce cellular death.

Besides incorporation of 6-thio-dGTP into DNA, accumulating evidence has been presented that incorporation of the ribonucleotide 6-thio-GTP into newly synthesized RNA is a contributing component of thiopurine-induced toxicity [178, 179]. In addition, the 6-MP and AZA-T derivative 6-methylthio-IMP has been shown to inhibit *de novo* purine biosynthesis, by inhibition of the enzyme phosphoribosylpyrophosphate amidotransferase (PPAT) [180].

It is less well understood how thioguanine species influence the activity of GTP-utilizing enzymes, e.g. GTPases. Evidence has been presented, that the immunosuppressive effects of 6-TG are caused through 6-thio-GTP-induced inhibition of Rac1 (a GTPase), which induces T-cell apoptosis [181, 182]. Overall, the exact contribution of these mechanisms to the pharmacological effects of thiopurines is poorly understood and clearly dependent on the genetic make-up of the cell [183, 184].

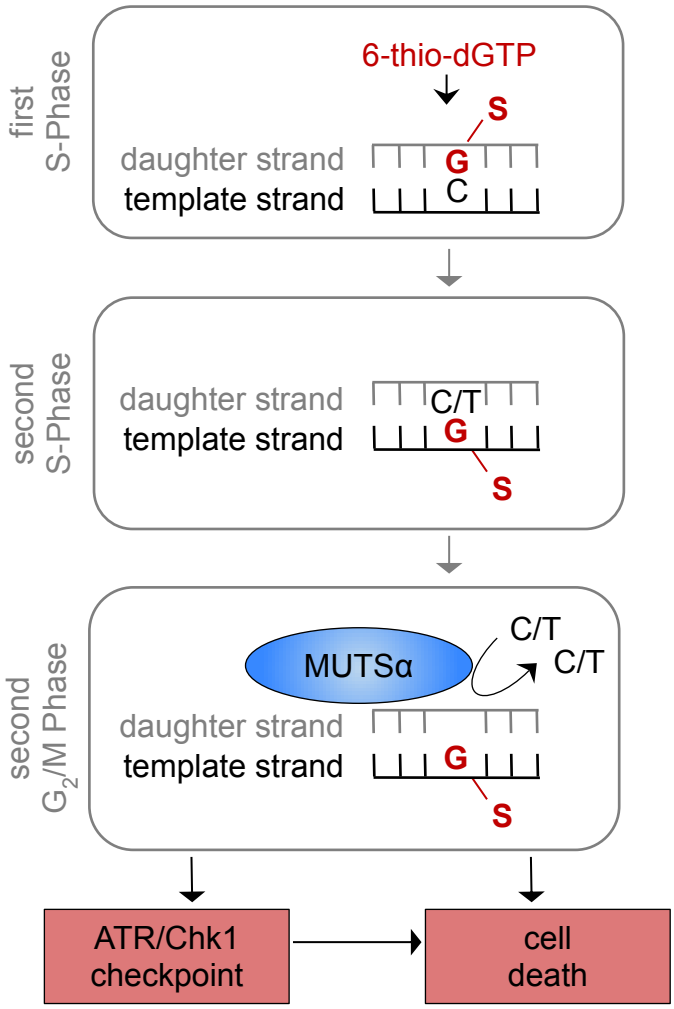


Figure 9: Mechanism of cell toxicity induced by incorporation of 6-thio-dGTP into DNA. *In the first round of replication DNA polymerases mis-incorporate 6-thio-dGTP, which gets methylated by SAM. During the second round of replication, 6-methylthio-G (depicted as G-SM) pairs preferentially with cytosine or thymine. The presence of 6-methylthio-G in the template strand is recognized by the MMR machinery (involving the dimer MUTSα (MSH2/MSH6), MLH1 and PMS2), but inevitable futile repair leads to accumulation of abasic sites that, if accumulated, cause cell death.*

Link between NUDT15 and thiopurine treatment

Clinically, sensitivity to all three thiopurine drugs is highly variable, making it necessary to carefully monitor treatment effects and adapt the dosing regimen. In an attempt to understand this deviation and to prevent over-dose-induced side effects, several pharmacogenetic studies have been performed to link genetic alterations to treatment sensitivity. During these studies single nucleotide polymorphisms in the TPMT gene, which cause enzymatic inactivity, have been identified as major risk factor for thiopurine hypersensitivity [185-188]. In fact, it has been advised by the FDA to screen for TPMT inactivating mutations prior to thiopurine therapy to be able to adjust the treatment dose and prevent side-effects, such as leukopenia and hair loss. Even though pre-treatment screenings for TPMT mutations has been beneficial, a high number of hypersensitive cases cannot be explained by this mechanism. This observation has been especially prevalent in the Asian population, where TPMT mutations are less common [189-192]. These patients with thiopurine hypersensitivity evoked the need for additional research, to explain and prevent the observed side-effects in these patients.

Recent pharmacogenetic screens have identified a link between thiopurine hypersensitivity and the NUDT15 variant R139C. Both in patients with inflammatory bowel disease (IBD) [190, 193, 194] and childhood leukemia [195-201] the NUDT15 genotype significantly influenced the treatment sensitivity to thiopurines. During all these studies mutations in locus rs116855232, resulting in an arginine (Arg; R) to cysteine (Cys; C) substitution at position 139 of NUDT15, were a risk factor for thiopurine hypersensitivity. The described NUDT15 variants were most common in East Asians, as well as Hispanics, but rare in Europeans and were not detected in African patients [195]. These studies suggested a first link between the Nudix enzyme NUDT15 and thiopurine treatment sensitivity.

PRESENT INVESTIGATION

2.1 Objective of this work

The aim of this study was to validate the hydrolases dUTPase, dCTPase and NUDT15 as drug targets to disturb nucleotide homeostasis and cellular integrity, both as mono-therapy and in the context of antimetabolite treatment. The following questions were addressed:

Paper I

- How does 5-FU treatment affect DNA replication?
- How does dUTPase inhibition influence the 5-FU-induced phenotype?
- Is inhibition of dUTPase a promising strategy to increase 5-FU sensitivity?

Paper II

- What are the biochemical properties of the dCTPase inhibitor TH1217?
- What is the cellular phenotype evoked by pharmacological inhibition of dCTPase?
- Is inhibition of dCTPase a promising strategy to increase decitabine sensitivity?

Paper III

- Do the Nudix enzymes NUDT15, NUDT17 and NUDT18 possess enzymatic activity with 8-oxo-(d)GTP or 2-OH-(d)ATP?
- Does NUDT15 depletion affect incorporation of 8-oxo-dGTP into DNA, DNA integrity and cancer cell survival?
- What substrates are hydrolyzed by NUDT15?

Paper IV

- Does NUDT15 show enzymatic activity and preference towards 6-thio-(d)GTP?
- What causes thiopurine hypersensitivity in patients with the NUDT15 mutations leading to expression of the variant R139C?
- Do the biochemical and biophysical properties of NUDT15 R139C differ from the wild type protein?
- Does depletion of NUDT15 affect sensitivity to 6-TG?

Paper V

- Is NUDT15 a possible target to increase thiopurine sensitivity of leukemia cells?
- Can we develop potent and selective small molecule inhibitors against NUDT15?
- Do NUDT15 inhibitors increase thiopurine sensitivity of leukemia cells?
- What is the mechanism of action of the NUDT15 inhibitor and thiopurine combination-treatment?

2.2 Research design

To investigate the importance of dUTPase, dCTPase and NUDT15 in nucleotide homeostasis and to validate these (d)NTPases as possible therapeutic targets to increase antimetabolite efficacy we used the following study design.

1. *In vitro* assessment of substrate specificity
2. Characterization of cellular phenotypes induced by protein knockdown
3. Validation of knockdown-induced antimetabolite sensitization
4. Development of small molecule inhibitors against the (d)NTPases
5. Characterization of the molecular mechanisms of action underlying the co-treatment strategies

2.3 Results

Paper I: dUTPase inhibition augments replication defects of 5-fluorouracil

TS-inhibitors, including fluoropyrimidines and antifolates, remain a medication of choice in the treatment regimens against a variety of solid tumors. However, despite six decades of clinical application the exact working mechanism is still debated. The high incidence of intrinsic and acquired drug resistance highlights the necessity to fully understand the mode of action of TS-directed chemotherapies. Accumulating evidence points to the fact that dUTPase activity is an important factor influencing the efficacy of TS inhibitors. By hydrolyzing dUTP and 5-fluoro-dUTP to the corresponding monophosphate, dUTPase activity could significantly hamper the potency of TS inhibitors (**Figure 10A**).

In this study, we unraveled DNA replication defects evoked by 5-FU treatment and assessed the role of dUTPase activity for treatment sensitivity. We showed, by extensive cellular experiments, that 5-FU treatment decreases replication fork progression and this leads to accumulation of cells in S-phase, DNA damage and eventually cell death (**Figure 10B-D**). These effects could be amplified by either depletion of dUTPase protein or inhibition of dUTPase activity with small molecule inhibitors. We demonstrated that dUTPase inhibition alone does not cause replication or viability defects, but significantly potentiates the cellular effect of 5-FU therapy. The influence of dUTPase activity on 5-FU-sensitivity underlines the importance of 5-fluoro-dUTP and dUTP accumulation and mis-incorporation into DNA for 5-FU-induced cytotoxicity.

With this study, we not only extended the current knowledge about the 5-FU mechanism of action, but we also contributed to the characterization of small molecule dUTPase inhibitors [202].

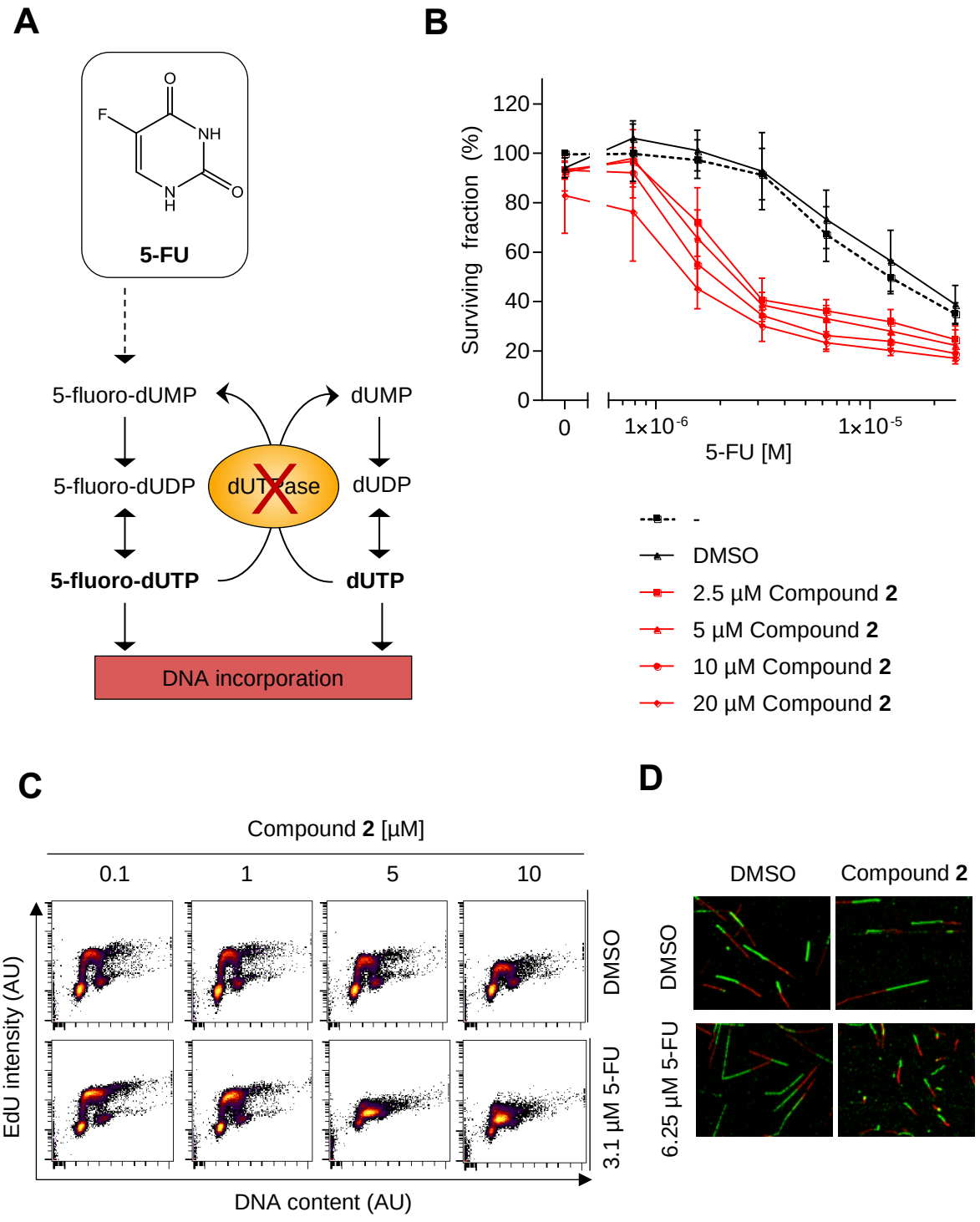


Figure 10: Main results of Paper I "dUTPase inhibition augments replication defects of 5-fluorouracil". *A) dUTPase activity hampers 5-FU toxicity by hydrolyzing endogenous dUTP and the active 5-FU derivative 5-fluoro-dUTP. B) dUTPase inhibition sensitized colorectal cancer cells to 5-FU treatment. C) Inhibition of dUTPase potentiated the replication defects of 5-FU, as analyzed by measuring EdU incorporation by FACS. D) 5-FU treatment reduced the speed of single replication forks, which was augmented when dUTPase was inhibited.*

Paper II: TH1217, a chemical probe to explore dCTPase pharmacology

Increasing evidence has been presented that the fairly uncharacterized dCTPase enzyme is, through its hydrolyzing activity with deoxycytidine and 5-modified deoxycytidine triphosphates, involved in deoxycytidine homeostasis and carcinogenesis (**Figure 11A**). Here, we characterized the small molecule dCTPase inhibitor TH1217 by various biochemical, biophysical and cellular assays and demonstrate its value for investigating dCTPase biology *in vivo* (**Figure 11B**).

We validated how TH1217 binds to the active site of dCTPase by solving the co-crystal structure and confirmed target engagement inside cancer cells by assessing thermal and proteolytic degradation. Furthermore, we demonstrated that TH1217 displays exquisite selectivity over related hydrolases and a wide panel of relevant pharmacological targets.

In a cellular setting, inhibition of dCTPase by TH1217 led to an increase of intracellular dCTP levels, supporting "effect-through-target". Short-term inhibition of dCTPase did not affect replication speed or cellular survival of HL-60 cells (**Figure 11C-D**). However, chronic exposure (15 days) of HL-60, DOHH-2 and WILL-2 cells to sub-micromolar concentrations of TH1217 impaired cell proliferation (**Figure 11E**). This was not a result of changes in the global methylation status, either after TH1217 treatment or dCTPase protein depletion, despite substrate preference of dCTPase towards 5-methyl-dCTP.

In addition, we demonstrated that the activity of dCTPase with the active decitabine metabolite 5-aza-dCTP counteracts the treatment efficacy in AML cells. Inhibition of dCTPase by TH1217 potentiated the cellular effects of decitabine treatment in AML cells, which was supported by knockdown experiments. TH1217 treatment increased decitabine-induced accumulation of DNMT1 crosslinking to chromatin, augmented DNA and cell death.

Overall, we demonstrated that TH1217 is a valuable tool compound to unravel the cellular function of dCTPase both, in endogenous nucleotide homeostasis and in the context of pharmacological intervention with deoxycytidine analogs.

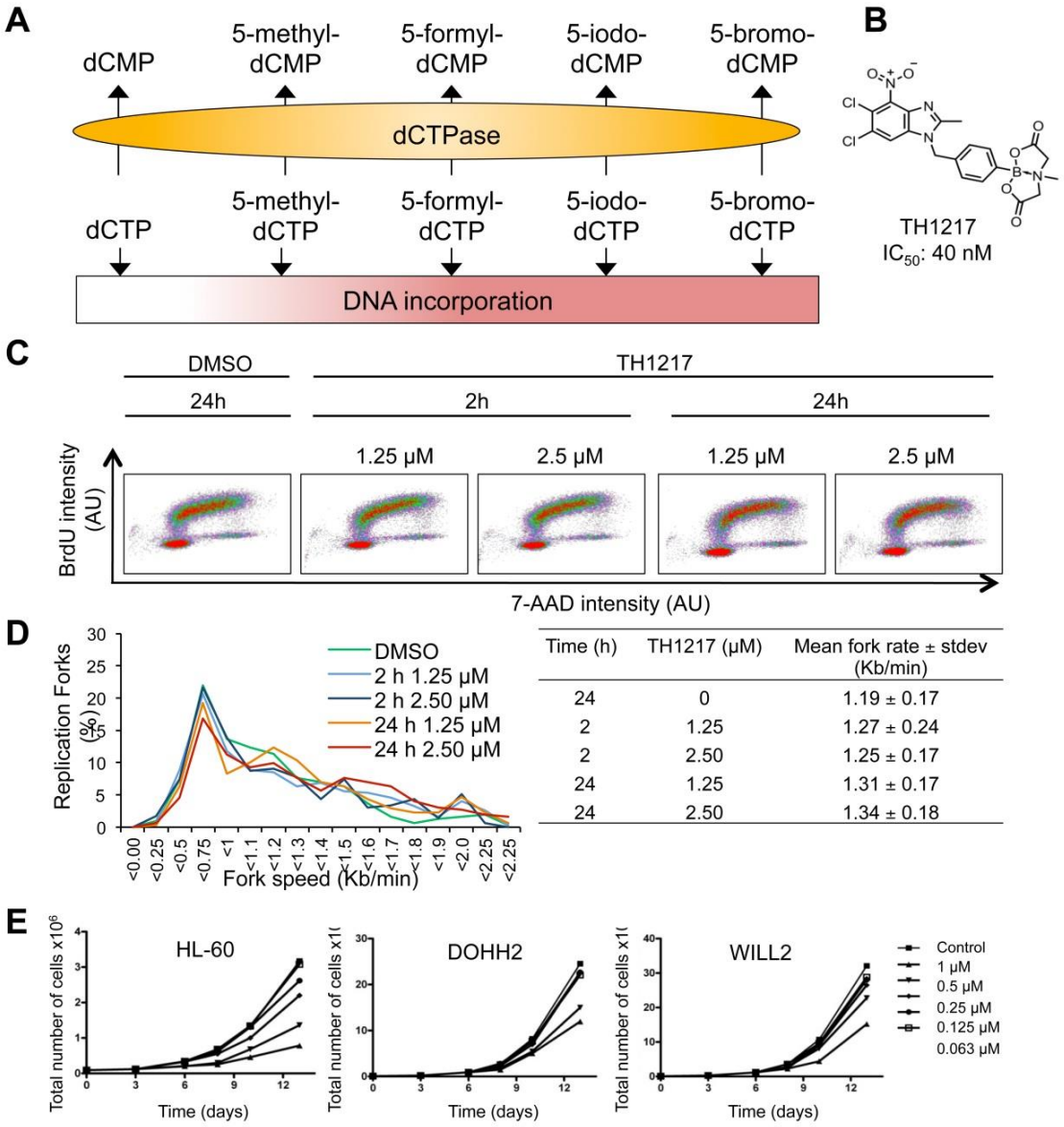


Figure 11: Main results of Paper II "TH1217, a chemical probe to explore dCTPase pharmacology". *A) dCTPase is involved in homeostasis of the canonical and non-canonical deoxycytidine pool by hydrolyzing the triphosphate to the monophosphate species. B) Chemical structure of TH1217 C) TH1217 did not influence replication speed, assessed by BrdU incorporation. D) Speed of single replication forks was not influenced by dCTPase inhibition. E) Chronic exposure to TH1217 influenced cellular proliferation.*

Paper III: Crystal structure, biochemical and cellular activities demonstrate separate functions of MTH1 and MTH2

Many cancer cells acquire a deregulated redox environment that causes oxidative damage to cellular components, including the free dNTP pool. Exploiting this phenotype by targeting sanitizers of the nucleotide pool is a novel strategy to induce cancer cell-specific toxicity. Here, we assessed the activity of the Nudix enzymes NUDT15 (MTH2), NUDT17 and NUDT18 with oxidized guanine and adenine species, to validate their role as sanitizers of the oxidized nucleotide pool.

We demonstrated that, of the tested Nudix enzymes, NUDT15 possesses the second highest 8-oxo-(d)GTPase activity, after MTH1 (**Figure 12A**). To characterize this activity in more detail we performed a thorough kinetic comparison of MTH1 and NUDT15 with canonical and oxidized (d)GTP. Whereas MTH1 had a 40 times higher k_{cat}/K_M for 8-oxo-dGTP compared to dGTP, NUDT15 displayed a 9 times higher k_{cat}/K_M for dGTP compared to 8-oxo-dGTP (**Figure 12B**).

To understand the different substrate specificity of these two sequence homologs, we determined the first NUDT15 crystal structure. Noticeably, even though MTH1 and NUDT15 share an overall similar fold, a shift in the NUDT15 α2 helix results in a distinct substrate binding pocket (**Figure 12C**). Whereas MTH1 is able to form a tight hydrogen bonding network with 8-oxo-dGTP involving Asp119 and Asp120, NUDT15 lacks residues with hydrogen bonding abilities at these positions (**Figure 12D**). As such, NUDT15 is unable to potently bind 8-oxo-dGTP.

Especially in a biological setting, where only a fraction of the total dGTP pool is oxidized, the importance of NUDT15 for 8-oxo-(d)GTP sanitization is questionable [67]. In order to validate the physiological importance of NUDT15 activity, we analyzed the phenotype evoked by NUDT15 and MTH1 protein depletion. As described previously, MTH1 knockdown increased the 8-oxo-dG level in DNA, leading to DNA damage and cell death (**Figure 12E-F**). In contrast, depletion of NUDT15 had no effect on these parameters. Simultaneous knockdown of both, MTH1 and NUDT15, did not reinforce the phenotype induced by MTH1 depletion.

Altogether, our data suggest that NUDT15 has negligible activity towards 8-oxo-(d)GTP and is not a biologically relevant sanitizer of the oxidized dNTP pool, at least in the tested experimental set-up. Even less 8-oxo-(d)GTPase and 2-OH-(d)ATPase activity of the

structurally related NUDT17 and NUDT18 suggests that MTH1 is the most prominent sanitizer of these oxidized nucleotides known today.

To understand the cellular function of NUDT15, we performed a substrate screen with various endogenous and synthetic nucleotide species. While NUDT15 possessed low enzymatic activity with dTTP, dCTP and 8-oxo-dGTP, slightly higher activity was observed with dGTP. Interestingly, this substrate screen unraveled that the active thiopurine metabolites 6-thio-dGTP and 6-thio-GTP are good substrates for NUDT15 (**Figure 12G**). These data offered the first biochemical explanation for the observed thiopurine hypersensitivity in patients with NUDT15 mutations.

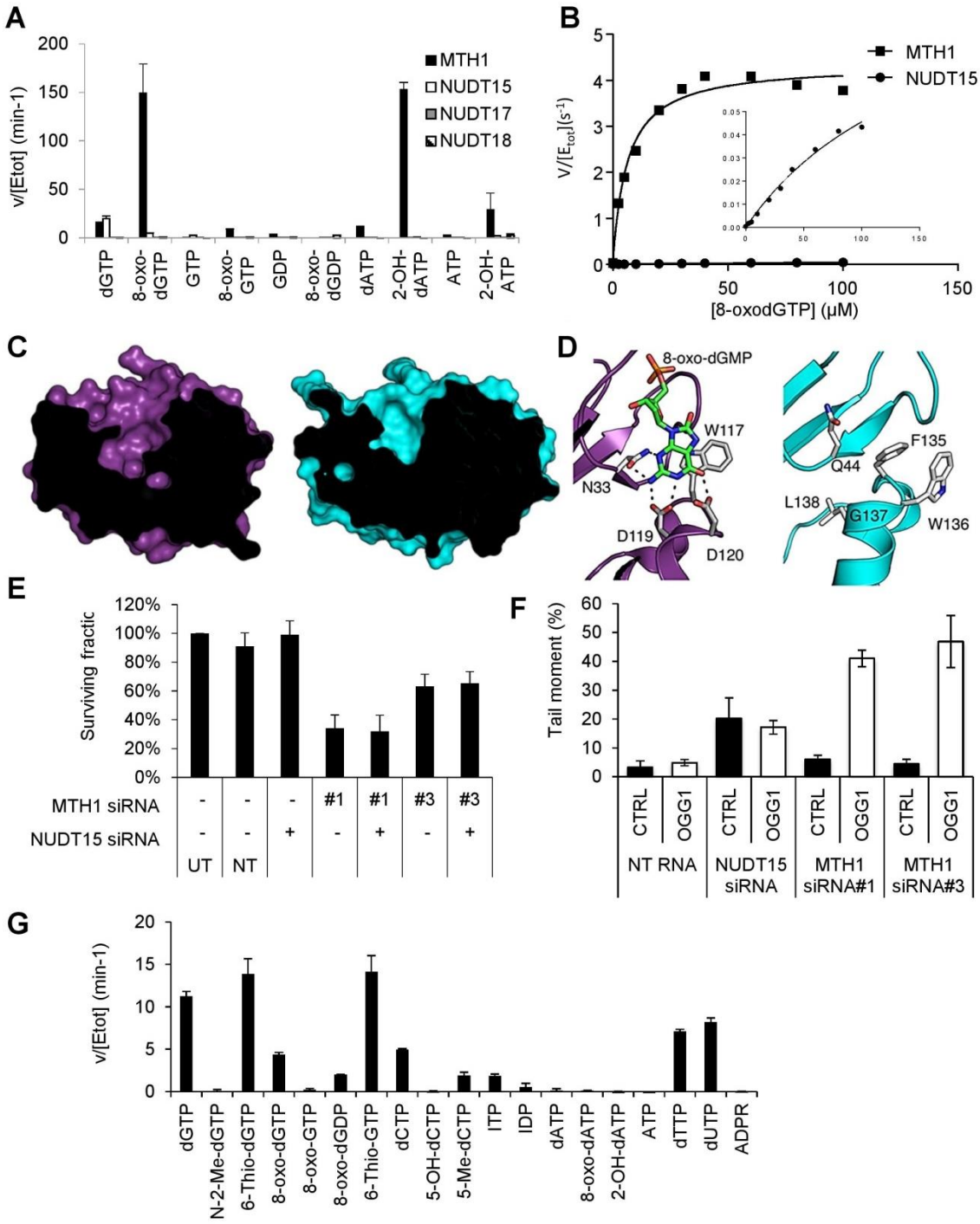


Figure 12: Main results of Paper III "Crystal structure, biochemical and cellular activities demonstrate separate functions of MTH1 and NUDT15". *A) NUDT15, NUDT17 and NUDT18 possess limited activity with (oxidized) purines, when compared to MTH1 B) NUDT15 shows limited activity with the oxidized nucleotide 8-oxo-dGTP. C) Despite an overall similar structure, a shift in the α2 helix of NUDT15 creates a considerably shallower putative binding pocket. D) MTH1 forms a strong hydrogen bonding network with 8-oxo-dGMP, involving the residues Asp119 and Asp120. Trp136 and Gly137, which occupy equivalent positions in the NUDT15 structure, do not possess hydrogen bond possibilities. E) Depletion of NUDT15 had no effect on cell survival and did not increase the phenotype of MTH1 depletion. F) In contrast to MTH1, NUDT15 depletion did not increase 8-oxo-d levels in DNA, as assessed by the modified comet assay. G) A substrate screen identified the thiopurine effector metabolites, 6-thio-dGTP and 6-thio-GTP, as novel NUDT15 substrates.*

Paper IV: NUDT15 mediates the cellular efficacy of 6-thioguanine by hydrolyzing 6-thio-(d)GTP

Recently, several pharmacogenetic studies have uncovered a link between thiopurine hypersensitivity and the NUDT15 variant R139C [190, 193-201]. While investigating in the cellular function of NUDT15 we identified 6-thio-dGTP and 6-thio-GTP as novel substrates for the Nudix enzyme, offering the first biochemical explanation for the clinical data (**Paper III**). In the light of these findings, we aimed to understand 1) the role of NUDT15 in thiopurine metabolism and 2) the consequence of the arginine to cysteine substitution at position 139 of NUDT15 for thiopurine sensitivity.

For this purpose, we performed extensive *in vitro* kinetic analyses to characterize the substrate preference of NUDT15. These studies confirmed that NUDT15 hydrolyzes both 6-thio-dGTP and 6-thio-GTP, and as a result of increased substrate affinity prefers the thiolated over the canonical nucleotides dGTP and GTP (**Figure 13A-B**). To understand this substrate preference we identified the co-crystal structure of NUDT15 in complex with the 6-thio-GTP hydrolysis product 6-thio-GMP. The steric fit and the accommodation of the 6-thio moiety in the hydrophobic pocket explain the lower K_M values of NUDT15 for the thiolated over canonical nucleotides.

We hypothesized that the observed thiopurine hypersensitivity of patients expressing the NUDT15 variant R139C might be a result of enzymatic inactivity. However, we found that substituting arginine at position 139 to cysteine does not impair the enzymatic activity of NUDT15, but instead negatively influences protein stability. Thermal stability experiments indicated that loss of the ionic interaction between Arg139 and Asp132 leads to reduced protein stability. In line with this, ectopic overexpression of NUDT15 wild type and R139C in cancer cells resulted in comparable mRNA expression, but reduced protein levels for the R139C variant. Addition of the proteasome inhibitor MG-132 increased the NUDT15 R139C protein levels (**Figure 13C**). These experiments suggest that proteasomal degradation of the unstable NUDT15 variant R139C leads to reduced NUDT15 protein levels, preventing sanitation of 6-thio-(d)GTP and thereby causing thiopurine hypersensitivity.

We next investigated whether the thiopurine hypersensitivity observed in patients expressing NUDT15 R139C, can be mimicked by shRNA-mediated depletion of NUDT15. Whereas knockdown of NUDT15 had no profound effect on cellular viability, it significantly potentiated 6-TG-induced cytotoxicity (**Figure 13D**).

The effect of NUDT15 depletion on 6-TG sensitivity was found in the MMR-proficient HCT-116 3/6 cells and in their MMR-deficient counterpart HCT-116, even though to a lesser extent.

Altogether, these data highlight that NUDT15 is an important mediator of thiopurine sensitivity due to the hydrolyzing activity with the effector metabolites 6-thio-dGTP and 6-thio-GTP (**Figure 13E**). Furthermore, we suggest that NUDT15 R139C is degraded under cellular conditions, rendering the patients expressing this variant hypersensitive to thiopurine therapy.

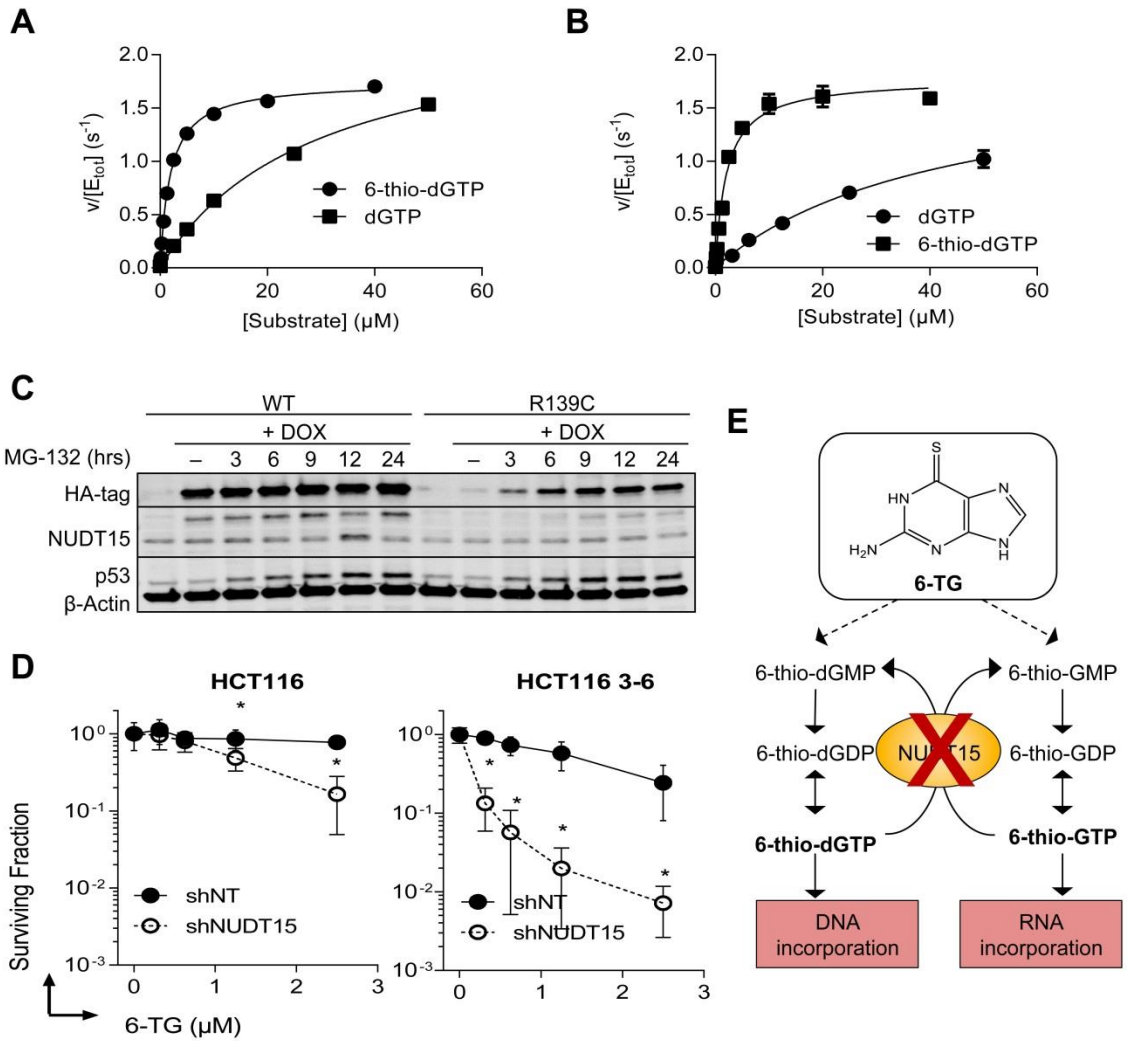


Figure 13: Main results of Paper IV "NUDT15 mediates the cellular efficacy of 6-thioguanine by hydrolyzing 6-thio-(d)GTP". *A) Kinetic analyses revealed that NUDT15 WT and B) NUDT15 R139C prefer thiolated guanine over the endogenous species dGTP. C) Overexpressed NUDT15 R139C was found to be unstable under cellular conditions, which could be rescued by addition of the proteasome inhibitor MG-132. D) Depletion of NUDT15 sensitized MMR-proficient (HCT116 3/6) and MMR-deficient (HCT116) cells to 6-TG treatment. E) NUDT15 is a major player in thiopurine metabolism due to its activity with the effector metabolites 6-thio-dGTP and 6-thio-GTP.*

Paper V: Targeting NUDT15 with small molecule inhibitors to increase thiopurine efficacy

Encouraged by the clinical and experimental data demonstrating that NUDT15 activity influences 6-TG sensitivity, we aimed to develop first-in-class inhibitors of NUDT15 to validate the Nudix enzyme as a potential drug target to increase thiopurine efficacy.

First, we confirmed that NUDT15 is an important mediator of thiopurine sensitivity in AML cells, by utilizing shRNA-mediated protein depletion. Importantly, overexpression of NUDT15 wild type, but not the catalytic dead (E67A) or unstable (R139C) protein variant, rescued the cytotoxicity induced by NUDT15 depletion, confirming on target effects of the shRNA.

Subsequently, 17.946 compounds were screened for their inhibitory activity of NUDT15 (**Figure 14A**). Extensive hit-to-lead optimization led to the development of several potent small molecule inhibitors of NUDT15, possessing desirable *in vitro* ADME properties. Whereas the developed NUDT15 inhibitors did not affect cellular viability alone, they significantly sensitized AML cells to 6-TG treatment, confirming data obtained by NUDT15 knockdown (**Figure 14B-C**). No effect of NUDT15 inhibition was found on the sensitivity of four ALL cell lines to 6-TG treatment. Genetic modifications in MMR-associated genes might be one explanation for the lack of sensitization, which was supported by the use of the MMR-deficient cell line HCT116 and their MMR-proficient isogenic counterpart HCT116 3/6.

We next investigated the mode of action of the combination treatment NUDT15 inhibitors and 6-TG. HPLC experiments demonstrated that inhibition of NUDT15 prevents hydrolysis of 6-thio-GppCp in cellular lysates. The accumulation of thiolated triphosphates upon NUDT15 inhibition caused increased mis-incorporation of 6-thio-dGTP and 6-thio-GTP into DNA and RNA, respectively (**Figure 14D-E**). This was associated with potentiation of 6-TG induced DNA damage and cytotoxicity (**Figure 14F**).

Overall, we here presented the development of the first-in-class small molecule inhibitors of NUDT15 and demonstrated that NUDT15 inhibition potentiates the cellular effect of 6-TG, including accumulation of thioguanosine triphosphate species, mis-incorporation into DNA and RNA, DNA damage and cell toxicity. Besides being valuable tools to unravel the physiological function of NUDT15, these compounds could improve the efficacy of thiopurines in patients.

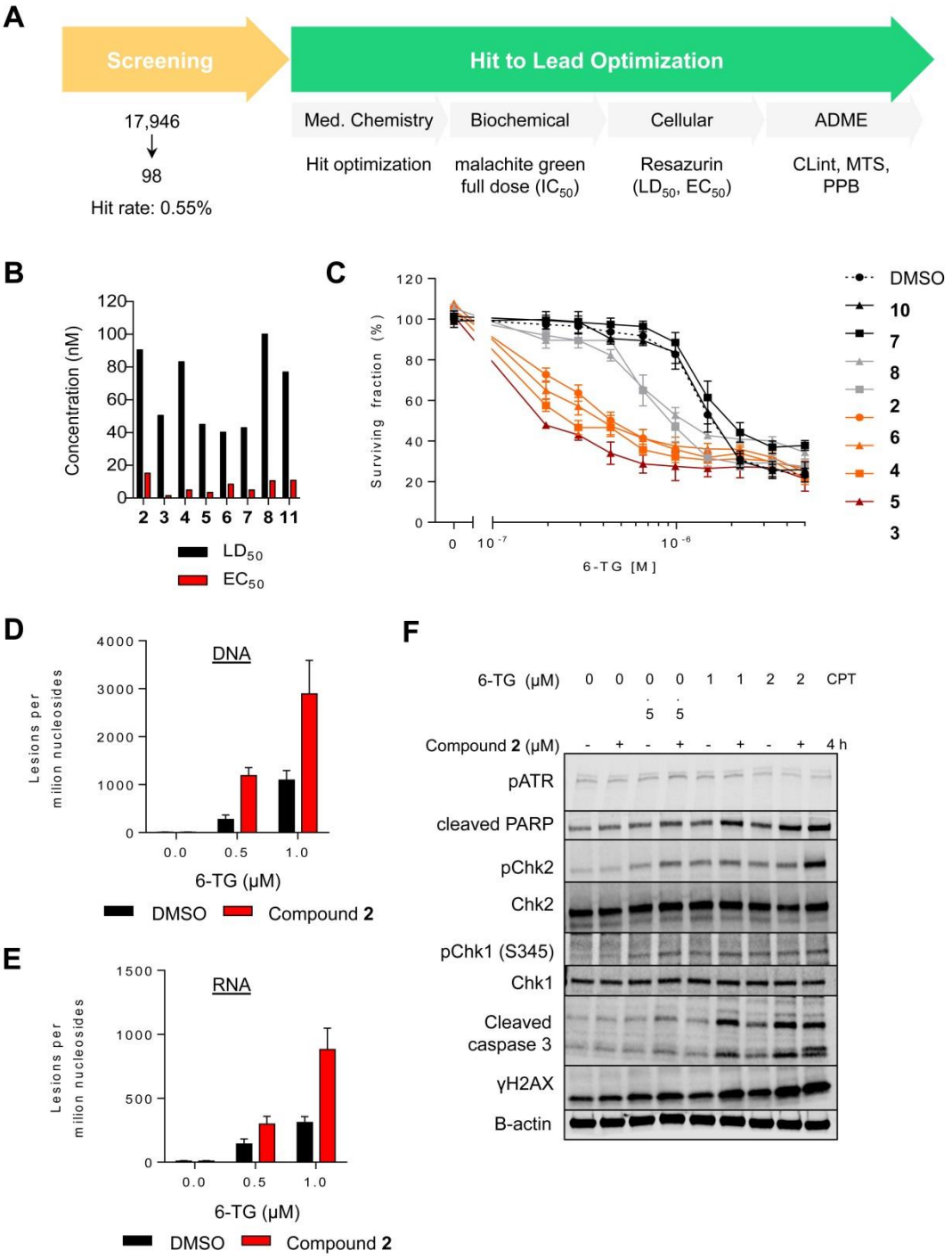


Figure 14: Main results of Paper V "Targeting NUDT15 with small molecule inhibitors to increase thiopurine efficacy". *A) A malachite green-based screen was performed to identify compounds with inhibitory potential towards NUDT15. Subsequent medicinal chemistry and lead optimization led to the development of first-in-class NUDT15 inhibitors. B) NUDT15 inhibition did not affect cell viability (LD_{50}) as a mono-treatment, but significantly sensitized cells to 6-TG treatment (EC_{50}). C) Combination treatment with NUDT15 inhibitors increased the cellular sensitivity to 6-TG. D) NUDT15 inhibition augmented incorporation of the active metabolites 6-thio-dGTP and 6-thio-GTP into DNA and E) RNA, respectively. F) Inhibition of NUDT15 enhanced 6-TG-induced DNA damage.*

2.4 Discussion and future perspectives

The therapeutic efficacy of many anti-cancer agents remains variable, unpredictable and often limited by debilitating side effects and drug resistance. To improve anti-cancer therapy it is, on the one hand, important to identify novel drug targets and treatment strategies. On the other hand, research should strive on validating key players involved in existing treatments, which can improve the efficacy of current state-of-the-art therapies.

Disturbing nucleotide homeostasis and genome integrity has been proven a valid point of attack, both in old and novel treatment approaches. Nucleobase- and nucleoside-analogs stand the test of time and remain an important cornerstone in various anti-cancer treatment regimens. Due to their resemblance to endogenous nucleotides, these antimetabolites enter the purine and pyrimidine salvage pathways and compete with canonical DNA and RNA building blocks. However, nucleotide metabolism is complex and the products are not only essential for DNA and RNA synthesis, but also for intracellular metabolism and signaling. It is therefore not surprising that the mechanism of action of the antimetabolites 5-FU, decitabine and 6-TG is multifaceted and even today not fully understood. A better understanding of their metabolism and mechanism of action could help to improve their therapeutic efficacy.

One component influencing the potency of nucleobase- and nucleoside-analogs is the inactivation of their active triphosphate derivatives by nucleotide hydrolases. In this study, we shed light on the metabolism and mode of action of 5-FU, decitabine and 6-TG by investigating the importance of their degradation by nucleotide hydrolases. We validated whether targeting the activity of dUTPase, dCTPase and NUDT15 is a promising strategy to improve the sensitivity to these commonly used chemotherapeutic agents. The development of small molecule inhibitors made it possible to characterize the importance of these (d)NTPases in nucleotide homeostasis and cellular integrity, both alone and in the aspect of antimetabolite therapy.

dUTPase

To shed light on the working mechanism of commonly used fluoropyrimidines we analyzed 5-FU-induced replication defects and the influence of dUTPase activity on these. We demonstrated that 5-FU treatment leads to a reduced replication fork speed. Inhibition of dUTPase accentuated the replication defects, as well as the cytotoxicity. These data support

that toxicity of 5-FU is, at least partially, attributable to intracellular accumulation of 5-fluoro-dUTP and dUTP, mis-incorporation into DNA and subsequent replication defects.

Despite extending the knowledge about the mode of action of fluoropyrimidines, we also highlight the benefit of dUTPase inhibitors to increase the therapeutic efficacy of 5-FU therapy. We have demonstrated that dUTPase inhibitors, as well as dUTPase depletion, significantly potentiate the replication defects and cellular toxicity of 5-FU. The notion that dUTPase is a good combination target for TS-directed therapies is supported by a number of studies that demonstrated a negative correlation between dUTPase expression and therapeutic outcome [41, 143-148]. The benefit of this combination therapy is therefore most likely dependent on the molecular make-up of the cell.

Furthermore, we investigated the importance of dUTPase activity for cellular integrity. dUTPase depletion resulted in a small increase in cell debris, but no difference in long-term survival could be detected by colony formation assay. In line with this, dUTPase inhibitors did not impair cell viability after a treatment period of 72 hours. These experiments indicate that inhibition of dUTPase activity does not induce severe cytotoxicity, at least at the time points and set-ups tested. Upregulation of thymidylate kinase and thymidine kinase, resulting from a feedback mechanism to counteract dUTPase inhibitor-induced dTTP depletion, could prevent profound consequences of dUTPase inhibitors alone [144]. The lack of toxicity induced by dTUPase inhibition is supported by successful clinical phase one trials with the dUTPase inhibitor TAS-144. These demonstrated both, a favorable safety profile and desirable pharmacokinetic parameters [203].

Several new implications for dUTPase inhibitors have recently been suggested. Chen *et al.* showed that the activity of dUTPase impacts ribonucleotide reductase-induced genome instability [204]. In addition, Requena *et al.* suggested that dUTPase reduces decitabine efficacy [48]. The development and characterization of dUTPase inhibitors will further facilitate research on the biological function and importance of dUTPase, both in endogenous and therapeutic nucleotide metabolism. This could open up novel implications for dUTPase inhibitors in the treatment of cancer and other diseases.

In summary, we demonstrated the importance of (5-fluoro-)dUTP and dUTPase activity for 5-FU treatment efficacy and thereby extend our current understanding of the mode of action. Especially with the development of several small molecule inhibitors of dUTPase and the first clinical trials, this treatment approach could soon improve the treatment response of

patients to TS-based therapy [203, 205, 206]. With this study, we support that targeting the hydrolase dUTPase is a promising strategy to increase 5-FU efficacy.

dCTPase

The nucleotide hydrolase dCTPase remains quite unstudied, but recent data suggest a role in nucleotide homeostasis and carcinogenesis. By hydrolyzing deoxycytidine and 5-modified deoxycytidine triphosphates dCTPase has been suggested to sanitize 1) halogenated nucleotides during inflammation 2) epigenetically involved 5-methyl-dCTP and 5-formyl-dCTP and 3) the active decitabine derivative 5-aza-dCTP. By developing and characterizing the potent dCTPase inhibitor, TH1217, we provide the possibility to further explore dCTPase biology in these contexts. We showed that inhibition of dCTPase increases intracellular dCTP levels. However, this imbalance had no impact on DNA replication and cellular viability. Chronic treatment with TH1217, on the other hand, had a cytostatic effect on various cancer cell lines. This could result from epigenetic changes induced by reduced sanitation of 5-methyl-dCTP and 5-formyl-dCTP when dCTPase is inhibited. However, no changes in 5-methyl-dC could be detected in DNA after 1 and 6 days of TH1217 treatment. Overall, the physiological function of dCTPase remains to be uncovered. The development of TH1217 will facilitate the research on the role of dCTPase in nucleotide homeostasis and in epigenome fidelity. In addition, we supported a current study that highlighted the importance of dCTPase in decitabine metabolism [48]. The dCTPase inhibitors could therefore open up novel opportunities to increase the efficacy of decitabine treatment.

NUDT15

Besides improving the efficacy of commonly used chemotherapeutic agents it is of great need to identify novel anti-cancer targets to remove cancer from the “major cause of death”-list. Exploiting the disturbed redox environment of many cancers, by targeting sanitation of endogenously damaged nucleotides, has gained increasing interest as novel anti-cancer strategy. We and others have shown that cancer cells rely on sanitation of the oxidized nucleotide pool and that targeting the 8-oxo-dGTPase MTH1 is a promising target for cancer therapy [71, 77-80].

Based on the increasing interest for enzymes involved in sanitation of the oxidized nucleotide pool, we investigated whether additional Nudix family members possess activity with oxidized adenine or guanine nucleotides (**Paper III**). We assessed the activity of the MTH1

sequence homologs NUDT15, NUDT17 and NUDT18 with 8-oxo-(d)GTP and 2-OH-(d)ATP. Among these Nudix enzymes, the closest MTH1 sequence homolog NUDT15 (MTH2) possessed the highest enzymatic activity with the tested substrates and was therefore further characterized. Detailed enzymatic characterization suggested that NUDT15 is a poor 8-oxo-dGTPase. Opposite to MTH1, NUDT15 knockdown did not increase 8-oxo-dG levels in DNA and was not relevant for cancer cell survival, under the conditions tested. These data suggest that NUDT15, despite its sequence homology to MTH1, is not involved in 8-oxo-(d)GTP sanitization in cells, making MTH1 the most prominent sanitizer of the oxidized nucleotide pool known to date.

Since NUDT15 displayed a 9 times greater k_{cat}/K_M for dGTP over 8-oxo-dGTP, we tested the activity of NUDT15 with the canonical nucleotide at a physiologically relevant concentration (5 µM) [207, 208]. In depth kinetic analysis could not confirm NUDT15-catalyzed hydrolysis at these concentrations. However, regional accumulation or the activity in a certain biological context should not be excluded at this time. In addition, nucleotide metabolism is complex and regulated by the tight interplay of multiple enzymes, which could back up or reshuffle nucleotide homeostasis upon NUDT15 depletion.

To unravel the cellular function and substrate of NUDT15 we screened NUDT15 activity with a panel of potential Nudix substrates (**Paper III)**. During this screen, we identified the active thiopurine derivatives, 6-thio-dGTP and 6-thio-GTP, as novel NUDT15 substrates. This finding was especially interesting in the light of recently published pharmacogenic studies, which linked thiopurine hypersensitivity to the NUDT15 variant R139C.

We therefore characterized the importance of NUDT15 in thiopurine metabolism and investigated the influence of NUDT15 mutations on this function (**Paper IV**). Our studies revealed that NUDT15 activity reduces treatment sensitivity, by hydrolyzing the effector metabolites 6-thio-dGTP and 6-thio-GTP. We furthermore suggest that the NUDT15 R139C variant is unstable in a cellular setting and subsequent accumulation of 6-thio-(d)GTP is the cause for thiopurine hypersensitivity. These findings support a recent study by Moriyama *et al.* that showed the involvement of NUDT15 (wild type and mutants) in thiopurine metabolism [209].

Our data and the pharmacogenetic studies strongly suggest introducing pre-treatment screenings into the routine of thiopurine therapy, to identify patients with unstable NUDT15 variants and subsequently adjust the treatment dose to prevent over-dose-induced side effects.

Right now, the unstable NUDT15 variants are considered risk factors for increased side effects, like leukopenia and hair loss. However, these observations could also be exploited to open the road for a novel treatment strategy: combining thiopurine treatment with NUDT15 inhibitors.

Encouraged by this idea we decided to develop inhibitors against NUDT15 to validate the approach of targeting NUDT15 to increase thiopurine efficacy. In **Paper V** we presented the development of the first-in-class NUDT15 inhibitors and demonstrated how these can be utilized to increase the sensitivity to thiopurine therapy. The developed compounds will be valuable to further unravel NUDT15 biology, both alone and in the context of nucleotide-analog treatment.

One has to note that thioguanine triphosphates are not endogenously occurring nucleotides and therefore, the physiological function and the natural substrate of NUDT15 remain to be elucidated. The cellular data in **Paper III, IV** and **V** highlighted that both knockdown and inhibition of NUDT15 does not lead to a discernable phenotype. Knockdown of NUDT15 using siRNA or shRNA did not affect cell cycle progression, DNA damage nor cell viability of the cancer cell lines tested (**Paper III** and **Paper IV**). Also the developed NUDT15 inhibitors did not alter cell cycle progression, replication or viability at the time-points and in the cell lines tested (**Paper V**). The lack of importance of NUDT15 for cellular integrity is supported by the fact that the patients positive for NUDT15 R139C possess no increased risk for disease and appear healthy.

Besides research on improving existing chemotherapeutic agents, an open mindset and basic research is needed to develop novel cancer-specific therapies. The research on the Nudix enzymes MTH1 and NUDT15 are good examples for this. Validating the importance of MTH1 for cancer cell survival has led to the development of new therapeutic strategies and novel, highly promising anti-cancer drugs. Characterization of the MTH1 sequence homolog NUDT15 could have provided an additional treatment strategy to prevent sanitation of the oxidized nucleotide pool. Instead, these studies revised our knowledge about the protein's function and unraveled a previously unknown player in thiopurine metabolism.

Summary

In summary, these studies emphasize that hydrolysis of the active triphosphate derivatives of nucleobase- and nucleoside-analogs significantly reduces their treatment efficacy. Inhibiting (d)NTPases involved in this degradation is an attractive strategy to increase treatment sensitivity.

For the studies present here one question remains: whether these combination approaches will increase the treatment efficacy in cancer cells and simultaneously prevent adverse effects. *In vivo* absorption, distribution, metabolism and excretion (ADME) of the small molecule inhibitors will influence the degree of exposure for healthy and cancer cells. Additional research is needed to assess whether the proposed combination therapies will increase the therapeutic index and provide a benefit for the patient. The upregulation or deregulation of dUTPase, dCTPase and NUDT15 in certain tissues and cancers suggest an important role for these hydrolases during carcinogenesis. In addition, this offers an opportunity to increase the therapeutic index for antimetabolite therapy by adding dUTPase, dCTPase and NUDT15 inhibitors to the treatment regimen.

Numerous additional antimetabolites have been developed that antagonize (deoxy)ribonucleotide metabolism. Many of which have successfully been used in the treatment of cancer and also as anti-viral or immunosuppressive agents. These include among others the pyrimdine-analogs cytarabine, gemcitabine, lamivudine and zidovudine and the purine-analogs fludarabine, cladribine, clofarabine, acyclovir and ganciclovir [23]. Identification of novel substrates and biological functions of these hydrolases could open up new avenues for therapeutic intervention, not only for anti-cancer treatment.

In addition, innovative research on endogenously occuring nucleotides and their potential to disturb cancer cell integrity is of great interest to identify novel, cancer-specific therapy. The recently discovered 5-formyl-dC and the connected hypersensitivity of CDA overexpressing tumors are good examples of novel anti-cancer strategies exploiting the damaged nucleotide pool [53-57].

Overall, we show that preventing the sanitation of nucleotide-analogs by inhibiting nucleoside triphosphate hydrolases is a promising strategy to improve the efficacy of nucleobase- and nucleoside-analog treatments. In addition, the developed dUTPase, dCTPase

and NUDT15 inhibitors are valuable tools to unravel the importance of these hydrolases in endogenous and therapeutic nucleotide homeostasis.

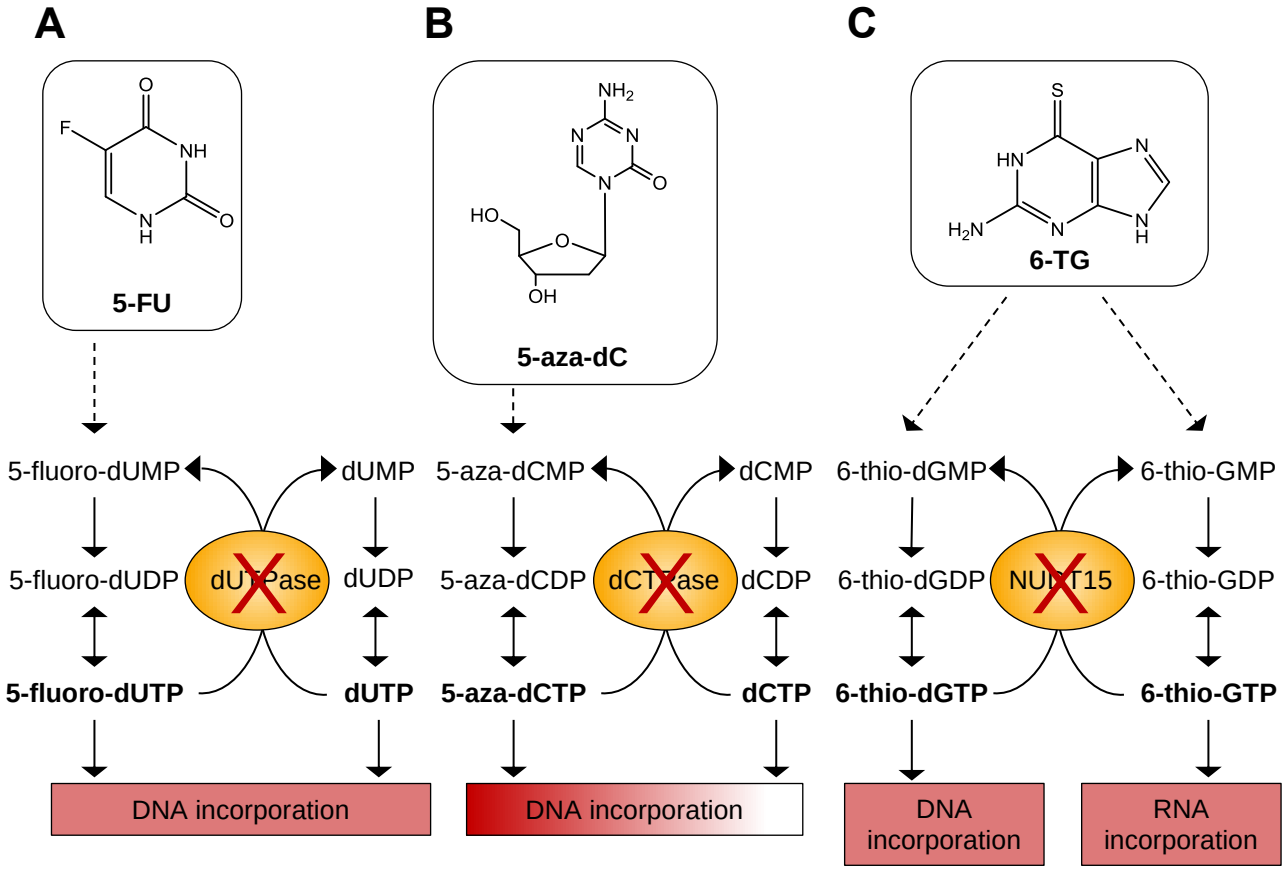


Figure 15: Examples of how (d)NTPases can be targeted to increase antimetabolite efficacy. *In this thesis were covered: A) targeting dUTPase to potentiate 5-fluorouracil treatment, B) targeting dCTPase to potentiate decitabine treatment and C) targeting NUDT15 to potentiate 6-thioguanine treatment. Inhibiting the hydrolases dUTPase, dCTPase and NUDT15 prevents the hydrolysis of the nucleoside triphosphate analogs derived from 5-FU, 5-aza-dC and 6-TG, respectively. Increased availability of the triphosphate species leads to mis-incorporation into DNA (and for certain species into RNA) causing overall DNA damage and cytotoxicity.*

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APPENDIX