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DIHYDROOROTATE DEHYDROGENASE – NEW INSIGHTS INTO AN OLD TARGET

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Dihydroorotate Dehydrogenase – New Insights Into an Old Target
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By

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To my family

ABSTRACT

Purine and pyrimidine nucleotides are important for both biosynthetic and regulatory pathways in cells. As constituents of the DNA and RNA molecules, the requirements for nucleotides in actively dividing cells are increased. Therefore, the enzymes from the purine and pyrimidine nucleotide synthesis pathways are considered attractive targets for treatment of diseases associated with deregulated cell proliferation, such as cancer. Dihydroorotate dehydrogenase (DHODH), an enzyme from the *de novo* pyrimidine ribonucleotide synthesis pathway located on the inner mitochondrial membrane, has been intensively studied in the past few years as a target for anti-cancer therapy. As a result, several inhibitors of DHODH are currently in clinical trials for treatment of myeloid malignancies.

In a screen for p53 activators, we discovered a novel class of DHODH inhibitors with a tetrahydroindazole core structure (**Paper I**). The hit compound from the screen, the chiral compound named HZ00, increased p53 translation without increasing p53 mRNA levels or the stability of the protein. Furthermore, treatment with HZ00 accumulated cancer cells in the S-phase of the cell cycle while it led to cell cycle arrest in normal fibroblasts. After screening of commercially available HZ analogues, we discovered the more potent compound HZ05. The active enantiomer of the chiral HZ compound was the (*R*)-enantiomer, confirmed by the co-crystallization of DHODH with HZ05 where only (*R*)-HZ05 was bound to the quinone tunnel of DHODH (membrane binding domain). Additionally, both compounds acted synergistically with the MDM2 inhibitor nutlin-3 (which protects p53 from degradation) in different tumor cell lines and in a melanoma xenograft model.

Following the discovery of the HZ compounds as inhibitors of DHODH, we investigated the structure and function of DHODH using mass spectrometry and molecular dynamics simulations (**Paper II**) and afterwards optimized the HZ compounds in a structure activity relationship study (SAR, **Paper III**). Using non-denaturing mass spectrometry, we established that the interactions between DHODH and its co-factor FMN, the lipids present in the mitochondrial membrane, as well as ligands such as the DHODH inhibitor brequinar, have stabilizing effect on the protein. Furthermore, molecular dynamic stimulations demonstrated the flexibility of the membrane binding domain and the transmembrane helix. We also illustrated that the lipid interactions that stabilize the membrane binding domain may affect the binding of longer ligands, which is important for the design of new inhibitors. The optimization of the HZ-compounds was based on a metabolic soft spot study and the crystal structure of DHODH with (*R*)-HZ05 obtained in **Paper I**. The activity of many of the new HZ inhibitors was improved in the *in vitro* enzymatic assay as well as in cellular assays using a melanoma cell line. Based on *in vitro* metabolic stability analysis we identified a lead compound in the series, which demonstrated high specificity towards DHODH. The new active HZ-analogues were also able to trigger p53 transcription factor function and affected the cell growth/viability of a melanoma cell line with only a small effect on a normal fibroblast, suggesting a reasonably wide therapeutic window. Supplementation with an excess of uridine ameliorated both, the activation of p53 by the HZ compounds and their effect on cell growth/viability. Under our experimental conditions, the new HZ analogues did not activate DNA damage markers. Additionally, we demonstrated that the ability of

DHODH inhibitors to induce apoptosis related γ-H2AX was prevented by co-treatment with the pan-caspase inhibitor Z-VAD-FMK.

Due to the re-emerging interest in DHODH as a drug target, we investigated the early events triggered after inhibition of this enzyme (**Paper IV**). For that purpose, we performed RNA sequencing analysis of cells treated with two DHODH inhibitors for up to 4 h. One of the earliest significant changes was the fast downregulation of PNUTS mRNA. This event was observed at the protein level as well. The PNUTS decrease was accompanied by an increase of p53 protein, although the correlation between the two changes requires further investigation. Nevertheless, due to the early decrease in PNUTS we propose that the downregulation of PNUTS mRNA may be a marker for DHODH inhibition.

LIST OF SCIENTIFIC PAPERS

I. Ladds[§], M. J. G. W., van Leeuwen[§], I.M.M., Drummond[§], C. J., Chu, S., Healy, A. R., **Popova, G.**, Pastor-Fernández, A., Mollick, T., Darekar, S., Sedimbi, S. K., Nekulova, M., Sachweh, M. C. C., Campbell, J., Higgins, M., Tuck, C., Popa, M., Safont, M. M., Gelebart, P., Fandalyuk, Z., Thompson, A. M., Svensson, R., Gustavsson, A., Johansson, L., Färnegårdh, K., Yngve, U., Saleh, A., Haraldsson, M., D'Hollander, A. C. A., Franco, M., Zhao, Y., Håkansson, M., Walse, B., Larsson, K., Peat, E. M., Pelechano, V., Lunec, J., Vojtesek, B., Carmena, M., Earnshaw, W. C., McCarthy, A. R., Westwood, N., Arsenian Henriksson, M., Lane, D. P., Bhatia, R., McCormack, E., and Laín[*], S.
A DHODH inhibitor increases p53 synthesis and enhances tumor cell killing by p53 degradation blockage.
Nature Communications 9, 1107 (2018).

II. Costeira-Paulo, J., Gault, J., **Popova, G.**, Ladds, M., J. G. W., van Leeuwen, I. M. M., Sarr, M., Olsson, A., Lane[*], D. P., Laín[*], S., Marklund[*], E. G., and Landreh[*], M.
Lipids shape the electron acceptor-binding site of the peripheral membrane protein dihydroorotate dehydrogenase.
Cell Chemical Biology 25, p 1 – 9 (2018)

III. **Popova[*], G.**, Ladds, M., J. G. W., Johansson, L., Saleh, A., Larsson, J., Sandberg, L., Sahlberg, S. H., Qian, W., Gullberg, H., Garg, N., Gustavsson, A., Haraldsson, M., Lane, D. P., Yngve, U., and Laín, S.
Optimization of tetrahydroindazoles as inhibitors of human dihydroorotate dehydrogenase and evaluation of their activity and in vitro metabolic stability.
Submitted manuscript

IV. **Popova[§*], G.**, Sanchez[§], Y.P., Lane, D., Pelechano, V., Laín[*], S.
Protein phosphatase 1 nuclear targeting subunit, PNUTS, an early marker of DHODH inhibition.
Manuscript

[§] These authors contributed equally
[*] Corresponding author

PAPERS NOT INCLUDED IN THIS THESIS

Ladds, M. J. G. W., Pastor-Fernández, A., **Popova, G.**, van Leeuwen, I.M.M., Eng, K. E., Drummond, C. J., Johansson, L., Svensson, R., Westwood, N., McCarthy, A. R., Tholander, F., Popa, M., Lane, D. P., McCormack, E., McInerney, G. M., Bhatia, R., and Laín, S.
Autophagic flux blockage by accumulation of weakly basic tenovins leads to elimination of B-Raf mutant tumour cells that survive vemurafenib.
PLOS ONE 13(4): e0195956 (2018).

CONTENTS

LIST OF ABBREVIATIONS

A	Adenine
ADP	Adenosine diphosphate
ADSL	Adenylosuccinate lyase
AIR	Aminoimidazole ribonucleotide
AKT	AKT8 virus oncogene cellular homolog
AML	Acute myeloid leukaemia
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
AP-1	Activator protein 1
APAF-1	Apoptotic protease activating factor 1
APRT	Adenine phosphoribosyltransferase
ARF, $p14^{ARF}$	Alternative reading frame protein
ATIC	Aminoimidazole carboxamide ribonucleotide formyltransferase / IMP cyclohydrolase
ATM	Ataxia telangiectasia-mutated protein kinase
ATP	Adenosine triphosphate
ATR	ATM and Rad3-related protein kinase
BAK	BCL-2 homologous antagonist/killer
BAX	BCL-2–associated X protein
BCL-2	B-cell lymphoma
BH-3	Bcl-2 homology domain
BIM	BCL-2 interacting mediator of cell death
BKVN	BK virus nephropathy
BrdU	Brome deoxyuridine
C	Cytosine
c-ABL	Ableson leukemia oncogene cellular homolog
CA	Carbonyc anhydrase
CAD	Carbamoyl phosphate synthetase, Aspartate transcarbamylase, dihydroorotase
CAFs	Cancer associated fibroblasts
cAMP	Cyclic adenosine monophosphate
CAT	Catalytic domain of DHODH
CBP	CREB binding protein
cCMP	Cyclic cytidine monophosphate
cdc42	Small GTPase of Rho family

CDK	Cyclin-dependent kinase
CDL	Cardiolipin
CETSA	Cellular thermal shift assay
cGMP	Cyclic guanosine monophosphate
ChIP-PET	Chromatin immunoprecipitation coupled with pair-ends ditag sequencing analysis
CHK-1	Checkpoint kinase 1
CHK-2	Checkpoint kinase 2
CKI	Cyclin-dependent kinase inhibitor
CMV	Cytomegalovirus
cNMPs	Cyclic nucleotide monophosphates
CP	Creatinine phosphate
CPS1	Carbamoyl phosphate synthetase 1
CPT	Carnitine palmitoyltransferase
CT	C-terminal domain of 53
CTP	Cytidine triphosphate
CTPS	CTP synthase
cUMP	Cyclic uridine monophosphate
DAXX	Death domain-associated protein
DBD	DNA binding domain of 53
DCIP	2,3-dichlorophenolindophenol
DDR	DNA damage response
DEPTOR	DEP domain-containing mTOR-interacting protein
DHO	Dihydroorotate
DHODH	Dihydroorotate dehydrogenase
DMAR	Disease-modifying antirheumatic drug
DNA	Deoxyribonucleic acids
DREAM complex	Dimerization partner, RB-like, E2F and multi-vulval class B complex
dTMP	Deoxythymidine monophosphate
dUMP	Deoxyuridine monophosphate
EMA	European Medicines Agency
ERK	Extracellular signal-regulated kinase
FAD	Flavin adenine dinucleotide
FASN	Fatty acid synthase gene
FDA	Food and Drug Administration
FGAR	*N*-Formylglycinamide ribonucleotide

FMN	Flavin mononucleotide
G	Guanine
GADD45	Growth arrest and DNA damage-inducible 45
GAR	Glycinamide ribonucleotide
GART	Glycinamide ribonucleotide transformylase
GDP	Guanosine diphosphate
GEFs	Guanine exchange factors
GLUT1	Glucose transporter 1
GMP	Guanosine monophosphate
GO	Gene ontology
GOF	Gain of function mutation
GSH	Glutathione
GTP	Guanosine triphosphate
GTPases	Guanosine triphosphate hydrolases
HAUSP	Herpesvirus-associated ubiquitin-specific protease
HBV	Hepatitis B virus
HIF-1α	Hypoxia-inducible factor 1-alpha
HIV	Human immunodeficiency virus
HK	Hexokinase
HLM	Human liver microsomes
HNDF	Human normal dermal fibroblasts
HoxA9	Homeobox protein Hox-A9
HPRT	Hypoxanthine guanine phosphoribosyltransferase
HSV	Herpes simplex virus
HZ	Tetrahydroindazole
IC_{50}	Inhibitory concentration
IMP	Inosine monophosphate
ISCU	Iron-sulphur cluster assembly enzyme
JAK3	Janus kinase 3
L11	Ribosomal protein L11
L23	Ribosomal protein L23
L5	Ribosomal protein L5
LA	Lactic acid
LAT1	L-type amino acid transporter
LDAO	lauryldimethylamine *N*-oxide
LDH	Lactate dehydrogenase

LDHA	LDH isoform A
LFS	Li-Fraumeni syndrome
LKB1	Liver kinase 1
LOF	Loss of function mutation
K_{sol}	Solubility constant
Lys	Lysine
MAPK	Mitogen-activated protein kinase
MCT	Monocarboxylate transporter
MD	Molecular dynamics
MDM2	Mouse double minute 2 homologue
MDMX	Double minute X human homolog; also known as MDM4
MLH1	MutL homolog 1
MLM	Mouse liver microsomes
mRNA	messenger RNA
MS	Multiple sclerosis
MS analysis	Mass spectrometry analysis
MSH2	MutS homolog 2
MTHFD2	Methylenetetrahydrofolate dehydrogenase 2
mTORC	Mammalian target of rapamycin complex
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromidefor
MYC	Myelocytomatosis oncogene cellular homolog
NA	Not analyzed
NAD^+	Nicotinamide adenine dinucleotide
NC	Not calculated
NDP	Nucleotide diphosphate
NEDD8	Neural precursor cell expressed developmentally down-regulated protein 8
nESI-MS	Non-denaturing nano-electrospray ionisation mass spectrometry
NF-κB	Nuclear factor kappa B
NMP	Nucleotide mono phosphate
NOXA	Phorbol-12-myristate-13-acetate-induced protein 1
NTP	Nucleotide triphosphate
OA	Orotic acid
OXPHOS	Oxidative phosphorylation
p300	A histone acetyltransferase

PAICS	Phosphoribosyl aminoimidazole carboxylase / Phosphoribosyl aminoimidazole succinocarboxamide synthase
PAINS	Pan assay interference compound
PARP	Poly (ADP)-ribose polymerase
PC	Phosphatidyl choline
PCNA	Proliferating cell nuclear antigen
PDH	Pyruvate dehydrogenase
PDK	Pyruvate dehydrogenase kinase
PDK2	Pyruvate dehydrogenase kinase-2
PDX	Patient derived xenograft
PE	Phosphatidyl ethanolalmine
PFAS	Phosphoribosyl formylglycinamide synthase
PFK	Phosphofructokinase
PGM	Phosphoglycerate mutase
PI	Propidium iodide
PI3K	Phosphoinositide 3 kinase
PIP2	Phosphatidylinositol 2-phosphate
PIP3	Phosphatidylinositol 3-phosphate
PKA	Protein kinase A
PKB	Protein kinase B
PKM2	Pyruvate kinase isoform M2
PNUTS/ PPP1R10	Protein phosphatase 1 nuclear targeting subunit
PPAT	Phosphoribosyl pyrophosphate amidotransferase
PPP	Pentose phosphate pathway
PRPP	Phosphoribosyl pyrophosphate
PRR	Proline rich region of p53
PsA	Psoriatic arthritis
PTEN	Phosphatase and tensin homolog
PUMA	p53 upregulated modulator of apoptosis
qRT-PCR	Quantitative real time polymerase chain reaction
RA	Rheumatoid arthritis
RAS	Rat sarcoma
RASSF1A	RAS association domain family protein 1A
RB1	Retinoblastoma protein
RING	Really interesting new gene
RNA	Ribonucleic acids

RNA Pol II	RNA polymerase II
RRM2B	Ribonucleotide-diphosphate reductase subunit M2 B
S6K	Ribosomal protein S6 kinase
SAR	Structure-activity relationship
SD	Standard deviation
SirT1	Silent mating type information regulation 2 homolog 1 (sirtuin)
SMAD	Contraction of Sma and Mad (Mothers against decapentaplegic)
SRB	Sulforhodamine B
STAT6	Signal transducer and activator of transcription 6
T	Thymine
TAD	Transactivation domain of p53
TCA cycle	Tricarboxylic acid cycle
TET	Tetramerization domain of 53
TKT	Transketolase
TLK	Transketolase
TM helix	Trans membrane helix
TP53	tumor protein 53; p53
TS	Thymidylate synthase
U	Uracil
U2OS	Human bone osteosarcoma epithelial cells
UDP-sugars	Uridine diphosphate sugars
UMP	Uridine monophosphate
UMPS	Uridine monophosphate synthetase
UTP	Uridine triphosphate
VDAC	Voltage-dependent anion channel
VEGFR2	Vascular endothelial growth factor receptor 2
YY1	Yin yang
Z-VAD-FMK	*N*-Benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone
γ-H2AX	Histone H2AX phosphorylated at Ser 139

1 INTRODUCTION

1.1 NUCLEOTIDE METABOLISM

1.1.1 Nucleotides

Nucleotides are molecules comprised of a base (a nitrogen containing ring), a pentose (a five-carbon sugar) and one to three phosphate groups (**Figure 1a**). Depending on the pentose, the nucleotides are divided into ribonucleotide (RNA) or deoxyribonucleotide acids (DNA). The bases are separated into two groups: the bicyclic purines adenine (A) and guanine (G) and the monocyclic pyrimidines cytosine (C), thymine (T), and uracil (U) (Figure 1b). The number of phosphate groups determines whether the nucleotides are mono- (NMP), di- (NDP), or triphosphates (NTP) respectively [1].

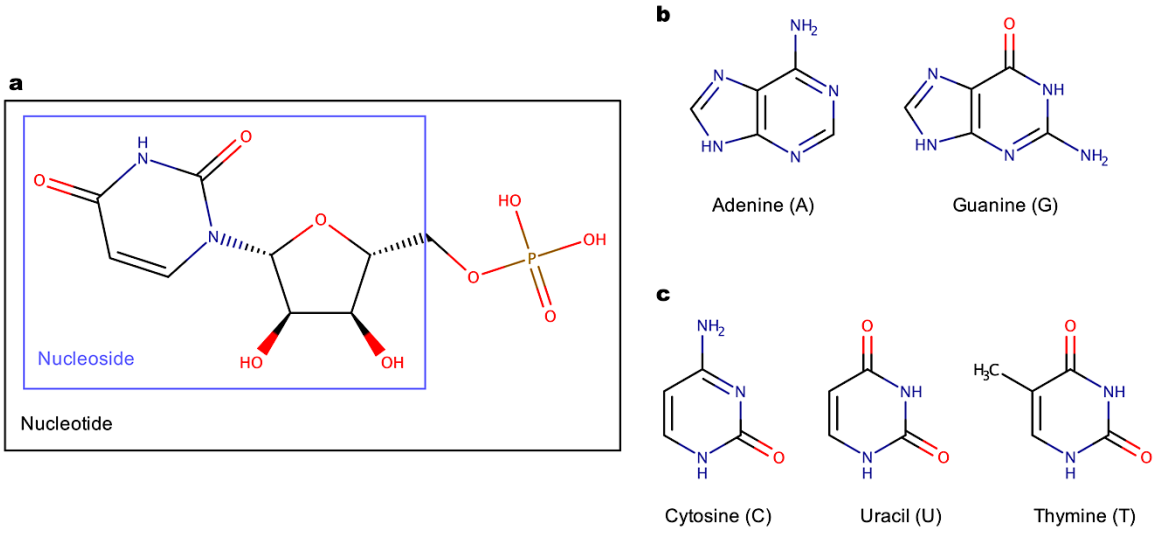


Figure 1. Structures of nucleotides and nuclear bases. A structure of uridine monophosphate (UMP) as an example of a nucleotide structure. (a) UMP contains the pentose ribose, the nuclear base uracil and one phosphate. The base and pentose are forming a nucleoside. Structures of the (b) purine and (c) pyrimidine bases.

1.1.2 The role of nucleotides and nucleosides in cells

Nucleotides have various functions in the cell, with the most prominent one being their role as subunits of the deoxyribonucleic acid (DNA) or ribonucleic acids (RNA) [1]. Containing the genetic information of a cell, however, is not the only role of the nucleotides. The covalent bonds between the phosphate groups of the NTPs contain high energy which is used in various cellular processes and makes these molecules convenient temporary energy carriers [1, 2]. Among the nucleotides, ATP (adenosine 5'-triphosphate) is considered the main energy source in cells. The hydrolysis of ATP to ADP is used to fuel different cellular processes, such as the phosphorylation of creatinine to creatinine phosphate (CP) in cells with high energy demands, the initial stages of glycolysis, fast axonal transport, and ion transport through active transporters [2-5]. The hydrolysis of GTP (guanosine 5'-triphosphate) by

GTPases also provides energy for numerous cellular processes such as regulation of signal transduction pathways, protein translation, proliferation, differentiation, and vesicular traffic [2, 6, 7]. Protein glycosylation is another process that requires hydrolysis of nucleotides. The formation of UDP-sugars, or sugar activation, requires the hydrolysis of UTP [8]. Similar to the synthesis of UDP-sugars, the synthesis of membrane phospholipids depends upon hydrolysis of CTP [9, 10]. Furthermore, cyclic NMP (cNMPs) cAMP, cGMP, cUMP, and cCMP function as second messengers [11, 12], and extracellular NTPs can activate different cellular functions through binding to purino- or pyrimidinoreceptors [13]. In addition to the numerous functions of the nucleotides, a recent study has shown that ATP can function as a hydrotrope and thus stabilizes proteins [14]. The diverse functions of nucleotides in cells require continuous regulations of their cellular levels through coordinated synthesis by the *de novo* and salvage synthesis pathways.

1.1.3 Nucleotide synthesis pathways

The cellular nucleotide pools are maintained by two nucleotide synthesis pathways: the *de novo* and the salvage pathways. The *de novo* pathway uses simple molecules, such as amino acids and sugars, for the synthesis of the final NMP products. The salvage pathways rely on the degradation of DNA and RNA, the conversion between products from the pathway, as well as the import of intermediates or final products from the pathway [15].

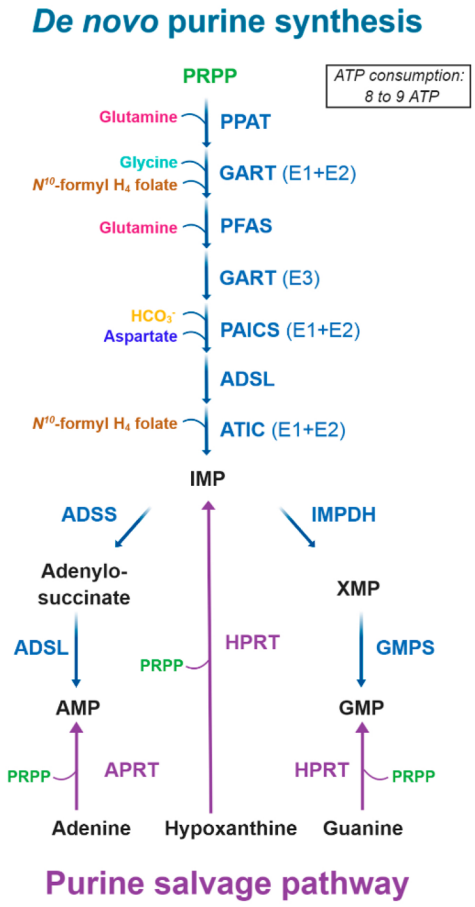


Figure 2. Schematic representation of the *de novo* and salvage purine synthesis pathways. The abbreviations used in the scheme are: phosphoribosyl pyrophosphate amidotransferase (PPAT); Glycinamide Ribonucleotide Transformylase (GART); Phosphoribosyl formylglycinamidine Synthase (PFAS); Phosphoribosyl aminoimidazole Carboxylase And Phosphoribosyl amino-imidazolesuccinocarboxamide Synthase (PAICS); Adenylosuccinate Lyase (ADSL); 5-Aminoimidazole-4-Carboxamide Ribonucleotide Formyltransferase (ATIC); Inosine Monophosphate Dehydrogenase (IMPDH); Guanine Monophosphate Synthase (GMPS); Adenylosuccinate Synthase (ADSS); hypoxanthine phosphoribosyltransferase (HPRT); adenine phosphoribosyltransferase (APRT). The figure was modified from Villa *et al*. [16]. All copyrights MDPI (2019).

1.1.3.1 Purine synthesis pathways

AMP and GMP are synthesized from inosine monophosphate (IMP), a central molecule in the *de novo* purine synthesis pathway. The synthesis of IMP consists of 10 steps carried out

by 6 enzymes (**Figure 2**) [17]. The first step of the pathway is catalyzed by phosphoribosyl pyrophosphate amidotransferase (PPAT), which transfers an amide group from glutamine to phosphoribosyl pyrophosphate (PRPP) forming 5-phosphoribosylamine, glutamate and inorganic phosphate [18]. The multifunctional enzyme glycinamide ribonucleotide transformylase (GART) catalyzes three non-sequential steps from the pathway – the formation of glycinamide ribonucleotide (GAR; step 2); the formation of *N*-formylglycinamide ribonucleotide (FGAR, step 3); and the formation of aminoimidazole ribonucleotide (AIR, step 5) [19]. The fourth step of the pathway is catalyzed by phosphoribosyl formylglycinamide synthese (PFAS) [17]. The next steps are catalyzed by phosphoribosyl aminoimidazole carboxylase / phosphoribosyl aminoimidazole succinocarboxamide synthase (PAICS; steps 6 and 7), adenylosuccinate lyase (ADSL; step 8), and aminoimidazole carboxamide ribonucleotide formyltransferase / IMP cyclohydrolase (ATIC; steps 9 and 10) [17, 20]. The salvage pathway includes the enzymes hypoxanthine-guanine phosphoribosyltransferase (HPRT) and adenine phosphoribosyltransferase (APRT), which convert hypoxanthine, guanine and adenine to IMP, GMP, and AMP, respectively [17].

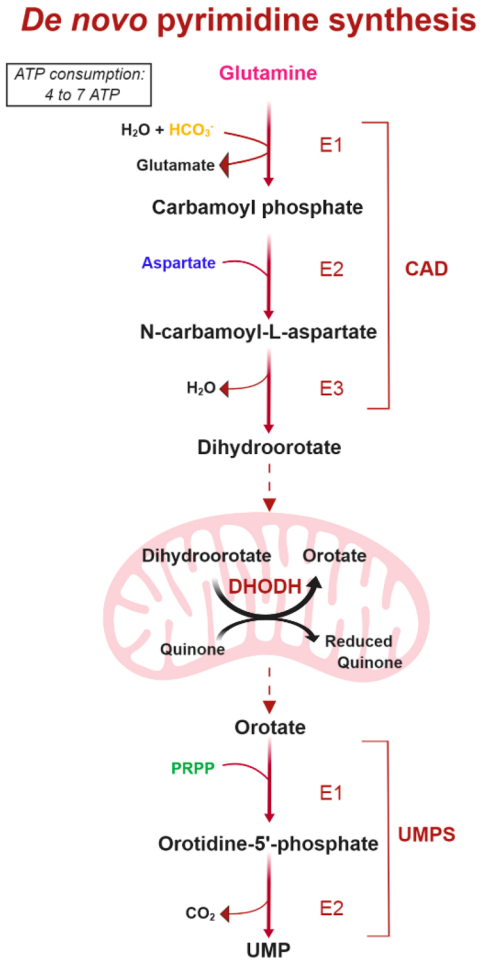


Figure 3. Schematic representation of the *de novo* pyrimidine synthesis pathways. The abbreviations used in the scheme are: Carbamoyl-Phosphate Synthetase 2, Aspartate Transcarbamylase, And Dihydroorotase (CAD); Dihydroorotate Dehydrogenase (DHODH); Uridine Monophosphate Synthetase (UMPS). The figure was modified from Villa *et al.* [16]. All copyrights MDPI (2019).

1.1.3.2 *Pyrimidine synthesis pathways*

Uridine monophosphate (UMP) has a central role in both the *de novo* and the salvage pyrimidine nucleotide synthesis pathways as a precursor for all pyrimidine nucleotides [21, 22]. The production of UMP in the *de novo* synthesis pathway consists of 6 catalytic reactions (**Figure 3**). The first three steps of the pathway are catalyzed by the multifunctional enzyme carbamoyl phosphate synthase, aspartate transcarbamylase, dihydroorotase (CAD). Through the three consecutive reactions CAD converts glutamine and aspartate to dihydroorotate [21]. Dihydroorotate is oxidized to orotic acid (OA) in the only redox reaction by the enzyme dihydroorotate dehydrogenase (DHODH), the only monofunctional enzyme in the *de novo* synthesis pathway. The last two reactions in the pathway are catalyzed by the bifunctional uridine monophosphate synthase (UMPS), which has transferase and

decarboxylase functions [15, 21, 22]. The remaining pyrimidine nucleotides - CTP and deoxythymidine monophosphate (dTMP) are synthesized by the enzymes CTP synthetase (CTPS) and thymidylate synthase (TS) from UTP and deoxyuridine monophosphate (dUMP) respectively [22, 23]. The enzymes from the salvage pyrimidine pathway use extra- and intracellular uridine or cytidine, and pyrimidine nucleotides as substrates to produce their respective nucleosides [24].

1.1.4 Regulation of nucleotide synthesis

The cellular nucleotide levels are regulated by several mechanisms - allosteric regulation of the enzymes, regulation of gene expression, metabolic regulation, and growth-state dependent regulation.

1.1.4.1 Allosteric regulation

The activity of several of the enzymes from the nucleotide synthesis pathways are controlled allosterically through feedback regulation by intermediates or end products. The first step of the *de novo* purine synthesis pathway is regulated allosterically by the levels of the final products AMP and GMP. The feedback regulation of this step regulates not only the *de novo* synthesis, but also the purine salvage pathway, as well as the rate of DNA synthesis [25]. The first reaction from the *de novo* pyrimidine synthesis pathway is also allosterically regulated by the levels of the final products of the pathway. The activity of the carbamoyl phosphate synthase domain of CAD is inhibited by increased levels of UTP that acts as a competitive inhibitor of the enzyme substrate ATP [26].

1.1.4.2 Regulation of gene expression

The enzymes from the purine and pyrimidine synthesis pathway are regulated at the transcriptional level as well. One of the main regulators for both *de novo* pathways is the transcription factor c-MYC [27-29]. In the study of Liu *et al.* (2008), the expression of several genes from the *de novo* synthesis pathways were increased following MYC upregulation, however only *PPAT* and *DHODH* were identified as direct target genes. Additionally, a ChIP-PET sequencing indicated that the *CAD* gene contains c-MYC binding site [28]. Other transcription factors, such as HIF-1 alpha, have also been associated with regulation of nucleotide synthesis [30]. Furthermore, predictions based on DNA binding motifs have identified numerous transcription factors as potential regulators of the expression of nucleotide synthesis related genes [15].

1.1.4.3 Signaling pathways regulating nucleotide synthesis

The intricate regulation of nucleotide synthesis is also carried out by several signaling pathways (**Figure 4**). Generally, these pathways regulate cell growth and proliferation, such as the MAPK/ERK and the mTORC pathways [16].

The MAPK cascade, which includes the RAS/MEK/ERK axis, is activated by cell surface receptors in response to different extracellular signals. The activation of the cascade leads to

activation of different transcription factors, including c-MYC, which results in sustained cell proliferation and evading of apoptosis [31]. Activation of RAS and the MAPK cascade maintains the nucleotide pools through upregulation of the *de novo* synthesis pathways by supplying intermediates into it [32-34], or by activation of the enzymes such as CAD [35, 36].

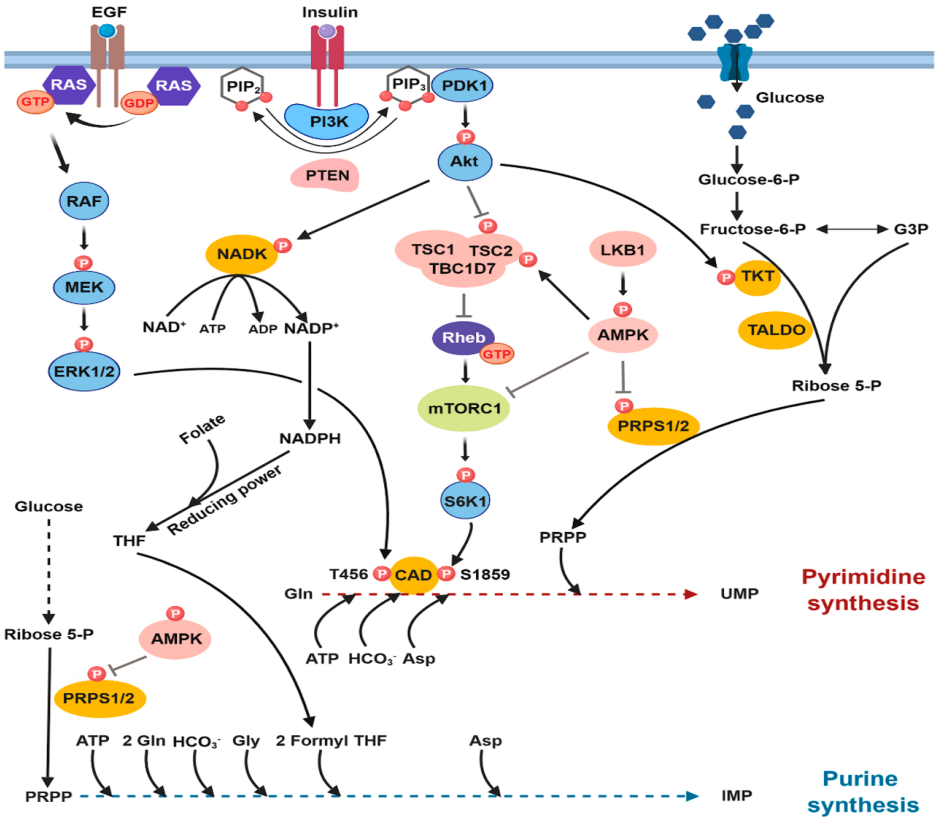


Figure 4. Signaling pathways in nucleotide metabolism. Growth signals can activate RAS/ERK and mTORC1pathways that can activate CAD and the *de novo* pyrimidine synthesis. AKT activation enhances and AMPK activation decreases PRPP availability for nucleotide synthesis. Tumor suppressors are shown in pink, key signaling kinases are shown in light blue, metabolic enzymes are shown in orange, and small GTPases in dark blue. 5'phosphoribosyl-pyrophosphate (PRPP); phosphoribosyl pyrophosphate synthetase (PRPS); transketolase (TKT); AMP-activated protein kinase (AMPK). The figure is from Villa *et al*. [16]. All copyrights MDPI (2019).

Another signaling pathway that leads to an increase in the *de novo* nucleotide synthesis is the mTORC pathway. Similarly to the MAPK cascade, mTORC activation is associated with cell growth and proliferation [37]. Activation of mTORC1 signaling increases the *de novo* purine synthesis through induction of methylenetetrahydrofolate dehydrogenase 2 (MTHFD2), an enzyme from the folate cycle [38]. Additionally, activation of the mTORC1 signaling increases the *de novo* pyrimidine synthesis as well. This increase is facilitated by Ribosomal protein S6 kinase (S6K), which phosphorylates and activates CAD [39, 40].

The PI3K/AKT signaling pathway, which is upstream of mTORC, regulates both, the *de novo* and salvage purine nucleotide synthesis pathways [41, 42]. PI3K/AKT pathway is activated by growth factors that ultimately lead to cell growth and survival, as well as regulation of the cell cycle [43]. The activation of AKT kinase by PI3K affects the *de novo* purine synthesis pathway indirectly through regulation of the activity of the pentose phosphate pathway, and

directly by increasing the activity of ATIC. Furthermore, AKT modulates PRPP levels, thus affecting the salvage purine synthesis pathway as well [41].

Activation of signaling pathways can also lead to downregulation of nucleotide synthesis. Depending on the growth state of the cells, protein kinase A (PKA) can phosphorylate CAD, and thus decrease the rate of the *de novo* pyrimidine synthesis [44]. In a model proposed by Sigoillot *et al.* (2002), PKA activation is correlated with the MAPK decrease of activity. Another protein that down-regulates nucleotide synthesis is the Phosphatase and Tensin homolog (PTEN). As antagonist of PI3K, activation of PTEN inhibits the phosphorylation of AKT and the following downstream pathways, thus decreasing the purine synthesis [45]. Moreover, Mathur *at al.* (2017) demonstrated that PTEN mutant cells and xenograft models are sensitized to treatment with DHODH inhibitors [46]. Another pathway that indirectly decreases nucleotide synthesis is the Hippo signaling. The Hippo pathway is responsible for the regulation of tissue homeostasis and organ development [47], as well as regulating the expression of glucose transporters. The decreased glucose uptake reduces the biosynthesis of nucleotides [48].

The importance of balanced nucleotide levels in cells is exemplified by genetic disorders caused by alterations in nucleotide metabolism due to mutations in the nucleotide biosynthesis genes or in the genes from the regulatory pathways.

1.2 ALTERATIONS IN NUCLEOTIDE METABOLISM

1.2.1 Inborn errors of nucleotide metabolism

Genetic disorders in the nucleotide metabolism genes are rare, but they can lead to disabilities and can even be lethal. Treatment options at present are limited, and the disorders are often misdiagnosed. The number of known defects in enzymes from the nucleotide synthesis pathways and enzymes from the nucleotide catabolism pathway is more than 35, and about 1/3 of these have severe clinical outcomes. The diseases are developed due to the depletion of nucleotides or because of toxicity of intermediate products [49-51].

1.2.1.1 Inborn errors in purine metabolism

The inborn errors in purine metabolism are caused mainly by autosomal recessive mutations [52], however, mutations in *IMPDH1* are autosomal dominant [53]. Defect in several other genes, like *HPRT* and *PRPS* lead to deficiencies or deficiency/superactivity, respectively [54, 55]. The symptoms that manifested are heterogeneous, but they could be defined in several distinct groups: neurological, immunological, hematological, and renal [49].

1.2.1.2 Inborn errors in pyrimidine metabolism

All genetic disorders in the pyrimidine metabolism discovered so far are autosomal disorders. Symptoms are diverse, and include immunological, neurological, hematological, renal, gastro-intestinal problems, and congenital anomalies [50].

1.2.2 Cancer

1.2.2.1 What is cancer?

The term cancer is used to describe different malignancies that have a common feature - deregulation of cell proliferation caused by genetic mutations that leads to perpetual proliferation [56]. In addition to deregulation of cell proliferation, Hanahan and Weinberg attributed five more characteristic features of the cancer cells in their article "The Hallmarks of Cancer": evading apoptosis, self-sufficiency in growth signals, insensitivity to anti-growth signals, tissue invasion and metastasis, and sustained angiogenesis [57]. Eleven years later, these cancer hallmarks were supplemented by four more: deregulation of cellular energetics, avoiding immune destruction, genome instability and mutation, and tumor-promoting inflammation [58].

1.2.2.2 Cancer is a genetic disease

The development of cancer is being widely viewed as a genetic disorder caused by somatic or germline mutations. According to the clonal evolution model for tumor formation, transformed cells arise from a single mutation that confers a growth advantage (**Figure 5**). Due to their genetic instability, the transformed cells accumulate additional mutations and form a heterogeneous cell population. Selective pressure from the local environment allows populations with higher variability to circumvent the cell death pathways allowing the transformed cells to acquire even more mutations until a tumor is formed [59, 60].

Mutations that occur in genes important for the growth and proliferation of cells can be separated into two categories: proto-oncogenes and tumor suppressor genes. Somatic mutations in the proto-oncogenes, like *RAS* or *MYC*, have dominant effect that lead to increases of their activity and turn them into oncogenes. In contrast, mutations in tumor suppressor genes such as *TP53*, *RB1* or *PTEN*, which regulate cell growth and proliferation in normal cells, are usually recessive and require mutations in both alleles [1, 61]. Another class of genes with high impact for cancer formation are the genes involved in DNA-repair. For example, mutations in *MSH2* (MutS homolog 2) or *MLH1* (MutL homolog 1) genes can lead to genetic instability and result in cancer cells heterogenicity [60].

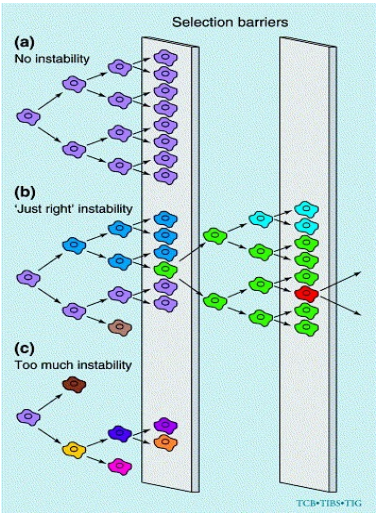


Figure 5. Genetic instability and heterogeneity drive cancer formation. (a) Normal cells have homogenous cell population with low or no genomic instability. (b) Tumor precursor cells with mutations that increase their genetic instability can form diverse cell populations. Selective pressure allows some of these cells to continue to proliferate by overcoming the selection barrier. The genetic diversity of these cells permits accumulation of additional mutations, higher heterogeneity, and ultimately tumor formation. (c) Cells with extensive genome damage activate the cell death pathways or the immune system. The cell diversity is decreased due to increased cell death or cell cycle arrest; therefore, the tumor precursor cells cannot pass the selective barrier. The figure is taken from Cahill *et al.* [60] and re-printed with permission of publisher. Copyright Elsevier (1999).

1.2.2.3 Cancer as a metabolic disease

Cancer has also been considered a metabolic disease due to the changes in cellular energetics (**Figure 6**). This notion is not a new one, as Otto Warburg's theory from 1956 on tumor formation was based on the irreversible changes in the cellular respiration and the following substitution with fermentation (glycolysis) as the cells' energy source [62]. Recently, the interest towards cancer metabolism has increased and deregulated cellular energetics was listed as one of the hallmarks of cancer [58, 63].

Some of the metabolic changes induced in cancer cells are the overexpression of the enzymes from the glycolytic pathway, as well as an increase in the amount of glucose importers, such as GLUT1 [64, 65]. Furthermore, tumor cells have increased activity and levels of lactate dehydrogenase (LDH), and increased secretion of lactic acid (LA) [66]. Other metabolic changes include truncated tricarboxylic acid (TCA) cycle, which provides intermediates for anabolic pathways, and changes in cell signaling pathways linked to cellular metabolism [67-69].

In addition to the changes in metabolism of the tumor cells, the cells that surround the tumor (tumor stroma) also display metabolic alterations. In some tumors, the cancer associated fibroblast (CAFs) may adopt glycolysis as a main energy source and provide the tumor cells with intermediate metabolites. This metabolic change is referred to as reverse Warburg effect [70, 71].

The metabolic changes in cancer cells resulting from overexpression of metabolic enzymes, are induced by deregulation of tumor suppressors and oncoproteins, which regulate the nucleotide synthesis pathways as well.

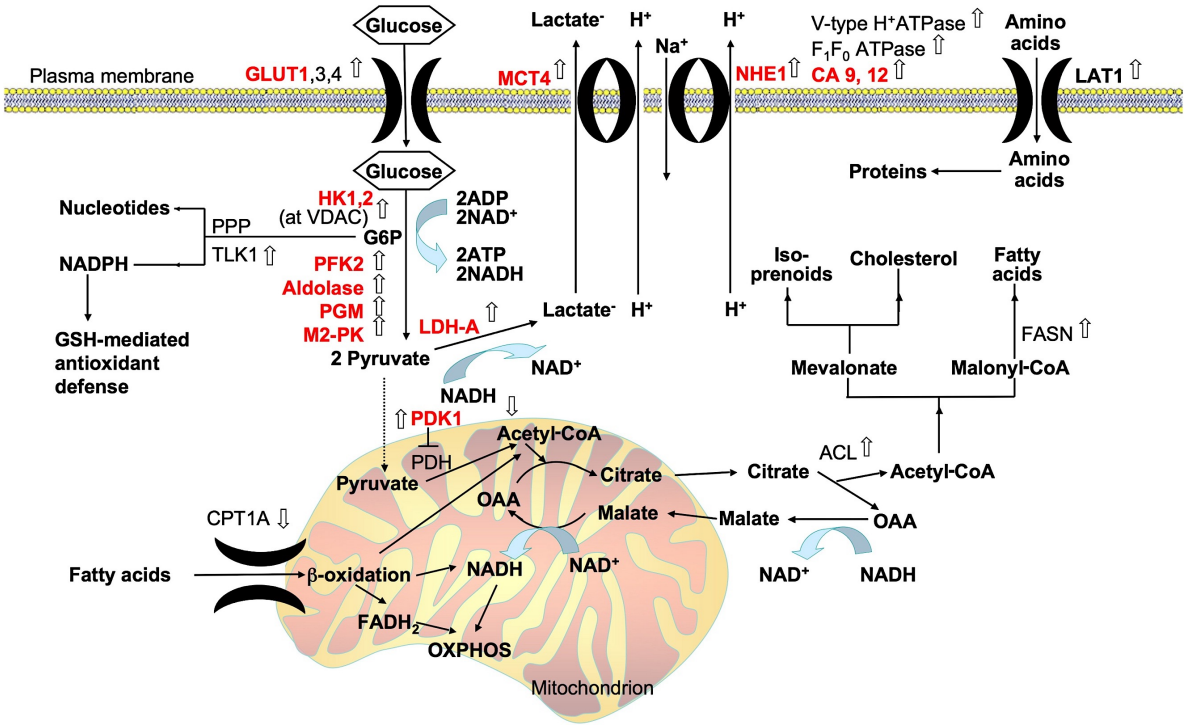


Figure 6. Changes in metabolic pathways in cancer cells. Glycolysis and tricarboxylic (TCA) cycle are altered in cancer cells. The pyruvate produced during glycolysis is converted by lactate dehydrogenase (LDH) to Lactic acid (LA) and exported from the cells. Intermediates from glycolysis pathway are used for nucleotide

synthesis. Intermediates from the TCA cycle are used to fuel anabolic processes associated with cell growth and proliferation. Changes of enzyme activity in cancer cells are indicated with arrows pointing up (activation) or down (inhibition). Metabolic changes induced upon activation of the hypoxia-inducible factor 1 (HIF-1α) are shown in red. Abbreviations used in the figure: carbonyc anhydrase (CA); carnitine palmitoyltransferase (CPT); glucose transporter (GLUT); glutathione (GSH); hexokinase (HK); oxidative phosphorylation (OXPHOS); L-type amino acid transporter (LAT1); LDH isoform A (LDHA); monocarboxylate transporter (MCT); pyruvate dehydrogenase (PDH); pyruvate dehydrogenase kinase (PDK); phosphofructokinase (PFK); phosphoglycerate mutase (PGM); pyruvate kinase isoform M2 (PKM2); pentose phosphate pathway (PPP); transketolase (TLK); voltage-dependent anion channel (VDAC). The figure is taken from Kroemer and Pouyssegur [67] and re-printed with permission of publisher. Copyright Elsevier (2008).

1.2.3 Deregulation of nucleotide biosynthesis in cancer cells

As described in 1.2., cancer cells proliferate continuously due to mutations or epigenetic changes that keep the proliferation pathways active. Therefore, tumor cells require constant supply of nucleotides, which is achieved by stimulation of the nucleotide synthesis pathways by oncoprotein activation or tumor suppressor inactivation.

1.2.3.1 Oncogenic activation of the RAS signaling pathway

Rat sarcoma gene (RAS), a GTPase that transduces signals from the cell surface membrane receptors to the effector cascades in the cytoplasm, is encoded by three genes in the human genome: *HRAS*, *NRAS* and *KRAS* [72]. The oncogenic activation of RAS is due to mutations in *RAS* genes, which prevent the hydrolysis of GTP or reduce the affinity for GTP and GDP [73]; however, through digital genome scanning, *RAS* amplifications were discovered in colorectal cancer cells as well [74].

Upon oncogenic activation, RAS can trigger several signaling pathways, including PI3K and the MAPK pathways resulting in cell proliferation. Furthermore, through activation of the transcription factor HIF-1α and mTOR pathway, RAS activation can indirectly increase nucleotide biosynthesis [72]. Additionally, the oncogenic activation of *KRAS* associated with inactivation of the tumor suppressor *LKB1* (liver kinase 1) increased the pyrimidine synthesis pathway through activation of the mitochondrial Carbamoyl-Phosphate Synthetase (CPS1), which indicates that KRAS can supply intermediates for the *de novo* pyrimidine pathway [75].

1.2.3.2 Oncogenic activation of PI3K/AKT/mTORC pathway

The PI3K/AKT/mTORC pathway regulates various cellular processes associated with increased cellular proliferation and enhanced cellular metabolism, including protein synthesis, nucleotide synthesis, as well as expression and translocation of glucose transporters [76, 77]. Oncogenic activation of the pathway can be due to mutations, amplification, or deregulation of any of the proteins in the signaling cascade.

Phosphoinositide 3-kinase (PI3K) phosphorylates phosphatidylinositol 2-phosphate (PIP2) to phosphatidylinositol 3-phosphate (PIP3) upon activation by RAS-related proteins, receptor-coupled tyrosine kinases or heterotrimeric G-proteins [76]. In different cancers, PI3K is activated by gene amplification [78] or by gain of function mutations in the catalytic

p110α domain or in two hotspot regions that prevent inhibition or activate the kinase without stimuli, respectively [76, 79-81]. Upon activation PI3K can trigger several effector proteins simultaneously, such as AKT/PKB (Protein Kinase B), Guanine exchange factors (GEFs) for the Rho/Rac/cdc42 GTPases, and TEC tyrosine kinase family, thus affecting cell morphology and motility, glycolysis, or B-cell development [76]. Downstream of PI3K, the effector protein AKT/PKB can trigger different biological processes that ultimately lead to upregulation of cell growth and proliferation and enhanced cell metabolism [77]. The oncogene activation of AKT is usually due to gene amplification or overexpression [82], however, activation mutations have also been reported [83]. A critical hub in the PI3K/AKT signaling pathway is the protein kinase mTOR1/2. Mutant forms of this kinase can lead to hyperactivation of the pathway due to preferential substrate phosphorylation, *e.g.* S6K1, or due to disruption of the interaction between mTOR and its inhibitor DEPTOR [84].

Since mTOR regulates both *de novo* nucleotide synthesis pathways, though through different mechanisms, the oncogenic upregulation of PI3K/AKT/mTORC will result in increased synthesis of nucleotides [38, 40, 85, 86]. Furthermore, the increased expression of glucose transporters and their translocation to the cell membrane [87] affect the nucleotide synthesis pathways as well by providing intermediate metabolites.

1.2.3.3 Oncogenic activation of transcription factor MYC

The transcription factor MYC is an oncoprotein activated by signals from various signaling pathways like MAPK or SMAD [88]. The protein is encoded by *MYC*, one of the most highly amplified genes in cancers [89], however, other modifications such as chromosomal translocations [90], or mutations that lead to protein stabilization can also result in neoplastic transformation [88].

Oncogenic activation of MYC regulates the pyrimidine synthesis pathway through the transcription of *CAD* [91], enhances glutamine metabolism [92], and coordinates the biosynthesis of nucleotides, and proteins through the translation of phosphoribosyl-pyrophosphate synthase 2 (PRPS2) [93]. Furthermore, all genes from both *de novo* purine and pyrimidine synthesis pathways have been found to be regulated by MYC, though not all of them directly [27]. By regulating both cell growth and nucleotide synthesis, MYC oncogenic activation supports cell proliferation.

1.3 P53 – A REGULATOR OF CELL FATE

The tumor suppressor p53, now referred to as “a guardian of the genome” [94], was initially regarded as an oncoprotein due to its association with Ras and ability to transform cells [95]. Further studies of p53 functions, however, indicated that deficiency, inactivation, or mutations of the protein result in tumor formation, while the wild type protein is a tumor suppressor [96, 97]. The p53 protein belongs to a family of closely related transcription factors that includes p63 and p73 [98].

1.3.1 Structure, function, and regulation of p53

1.3.1.1 Structure of p53 protein

The active form of p53 is a homotetramer. In humans, each subunit consists of 393 residues divided into 5 domains (**Figure 7**). The N-terminus of p53 consists of two domains: transactivation domain (TAD) and a proline rich region (PRR). The central region of the protein consists of the DNA binding domain, and the C-terminus consists of the tetramerization domain (TET) and a regulatory domain (CT) [99].

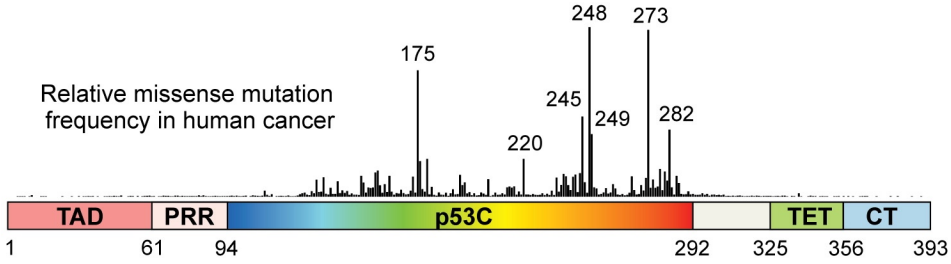


Figure 7. Structure of p53 domain organization and location of the most common missense mutations. The transactivation domain (TAD) and the proline-rich region (PRR) are located within the N-terminus of p53; The central domain of the protein contains the DNA binding domain (p53C, DBD); The C-terminus contains the tetramerization domain (TET) and C-terminus domain (CD). The most frequent missense mutations found in the DNA binding domain in human tumors as indicated above the domain organization diagram. The figure is taken from Joerger and Fersht [99] and re-printed with permission of publisher. Copyright Annual Reviews (2008).

The unstructured N-terminus of p53 contains the TAD and the PRR [100]. The TAD is a regulatory domain where transcription factors and p53 regulators bind [101, 102]. Notably, the p53 negative regulator MDM2 binds to this domain [103]. In contrast, the function of the PRR, which is important for protein-protein interactions, is largely unknown [104]. The core domain of the protein is the DNA binding domain that recognizes the target DNA sequences. Furthermore, this is the domain that is most frequently mutated in tumors [99, 105]. The active form of p53 is as a tetramer, and the formation of the tetramer is mediated by the TET [99]. The C-terminal (CT) domain of p53 has regulatory functions and it is an area that is modified by different post-translational modifications [106]. This domain has a loose structure, but upon binding to DNA it can adopt a helical structure and bind unspecifically to DNA [100].

1.3.1.2 p53 is a transcription factor

The main function of p53 in cells is as a transcription factor. The protein is activated by different stress stimuli, such as DNA damage, hypoxia, or nucleotide depletion. The stress signal is transmitted to mediator proteins, which activate p53 (**Figure 8**). Upon activation, p53 is stabilized and transported to the nucleus where it initiates the transcription of genes that amongst other events stop cell proliferation or activate cell death pathways [107].

One of the main effector proteins induced by p53 is the cyclin-dependent kinase inhibitor (CKI) $p21^{WAF1}$. This protein inhibits the cyclin dependent kinases (CDK2, CDK1, CDK4/6),

and thus prevents the G1/ S phase progression of the cells. Furthermore, p21 can induce cell cycle arrest by inhibition of the protein proliferating cell nuclear antigen (PCNA), which prevents S-phase entry. Different stress signals induce the expression of p21, including DNA damage, oxidative stress, and anticancer agents [108]. Recently, another CDK inhibitor, $p27^{KIP1}$, was identified as a p53 downstream effector as well [109]. $p27^{KIP1}$ inhibits the complexes between CDK2 with Cyclin A and Cyclin E, thus blocking the progress towards S phase [110, 111].

As a "guardian of the genome", p53 is activated by the DNA damage response (DDR) mediator proteins that mediate the effects of different DNA damage signals. Depending on the extent of the damage and the time necessary for repair, p53 initiates the expression of either DNA repair and cell cycle arrest proteins, or proteins involved in the apoptosis pathway [112]. The DNA repair effectors include the proteins from the Growth Arrest and DNA Damage-inducible 45 (GADD45) family [113] and 14-3-3σ family [114]. Proteins from both families also sustain the p53 signal through a feedback activation of p53 [113, 114].

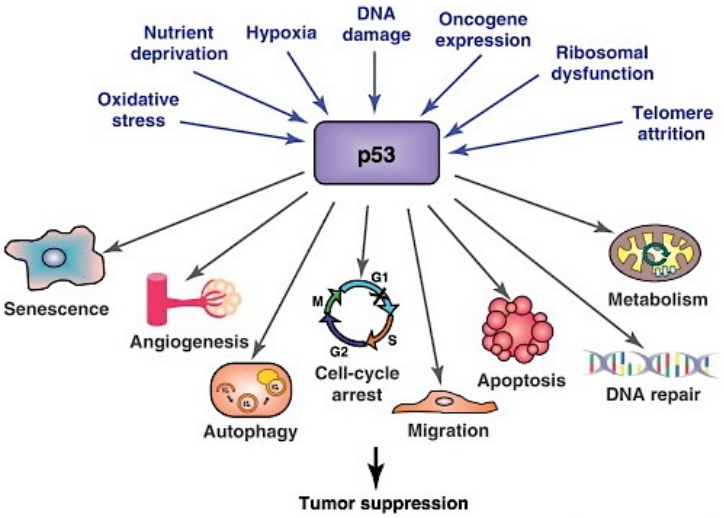


Figure 8. p53 signaling pathway. Upon activation, p53 initiates the transcription of genes that can activate cell death or cell survival genes, depending on the stress stimuli. The figure is taken from Bieging and Attardi [115] and re-printed with permission of publisher. Copyright Elsevier (2012).

If the DNA damage is extensive and cannot be repaired by the cell, p53 activates the cell death pathway by enhancing the expression of pro-apoptotic proteins and the expression of inhibitors of pro-survival proteins. The induction of p53-dependent apoptosis is associated with transcriptional upregulation of BH-3 proteins NOXA (Phorbol-12-myristate-13-acetate-induced protein 1) [116], PUMA (p53 upregulated modulator of apoptosis) [117], and the BCL-2 (B-cell lymphoma) family members BIM (BCL-2 interacting mediator of cell death) [118], BAX (BCL-2–associated X protein) [119] and APAF-1 (Apoptotic protease activating factor 1) [120]. Furthermore, p53 can interact directly with BAX/BAK (BCL-2 homologous antagonist/killer), which results in the permeabilization of the mitochondrial membrane, release of cytochrome-c and activation of the caspase cascade [121]. The role of p53 in other types of cell death pathways has not been studied extensively, although, p53 induced necroptosis [122] and ferroptosis have been reported [123]. The complexity of the p53 pathway, and the multitude of effector proteins regulated by p53, requires intricate regulation.

1.3.1.3 Regulation of p53

The main regulator of p53 is the E3-ubiquitin ligase mouse double minute 2 (MDM2). Under normal cellular conditions, MDM2 (HDM2 in humans) polyubiquitinates p53 and marks it for degradation in the proteasome (**Figure 9**) [124]. MDM2 mainly targets the C-terminal Lys residues of p53 [125], however, Lys residues within the DBD were also found to be ubiquitinated by the E3-ligase [126, 127]. The MDM2 protein contains a RING domain, which is important for the ubiquitin ligase activity towards p53, as well as for MDM2 autoubiquitination [128]. In addition to the role as a negative regulator of p53, low levels of MDM2 are associated with monoubiquitination of the transcription factor, which leads to translocation of p53 from the nucleus to the cytoplasm and activation of the apoptosis pathway [129]. The interaction between p53 and MDM2 is regulated by another RING domain protein, MDMX or MDM4 and by the tumor suppressor $p14^{ARF}$. *MDMX* is an oncogene that facilitates the ubiquitination of p53 by MDM2, as well as the autoubiquitination of MDM2 [130]. Unlike MDMX, $p14^{ARF}$ is a negative regulator of MDM2, and by binding to the E3-ligase, it activates p53 [131].

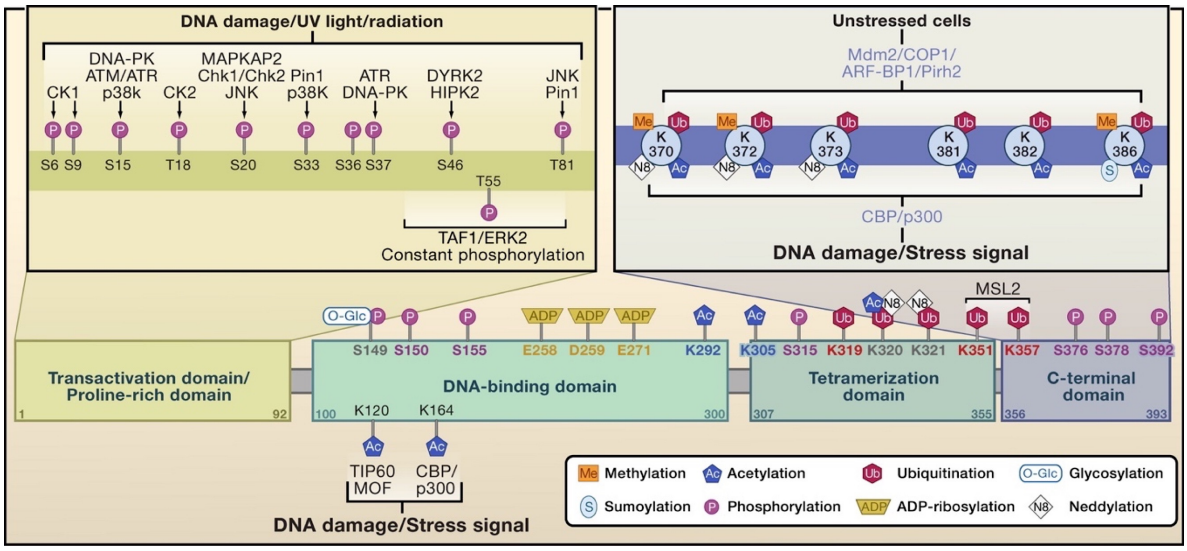


Figure 9. Posttranslational modifications of p53. The activity of p53 is regulated by different posttranslational modification: Ubiquitination (Ub); Phosphorylation (P); Acetylation (Ac); Sumoylation (S); Methylation (Me); Neddylation (N8); Glycosylation (O-Glc); ADP-ribosylation (ADP). The figure is taken from Kruse and Gu [132] and re-printed with permission of publisher. Copyrights Elsevier (2009).

The interactions between p53 and MDM2 are regulated by other proteins as well. The transcription factor Yin Yang (YY1) acts as a protein co-factor, which facilitates the MDM2-p53 interaction [133]. Another MDM2 regulator is the death domain-associated protein (DAXX) that together with the deubiquitinase HAUSP stabilizes MDM2 and prevents its auto-ubiquitination and degradation [134]. The ability of MDM2 to interact with and ubiquitinate p53 can also be inhibited. The tumor suppressor RASSF1A disrupts the interaction between MDM2, DAXX, and HAUSP, thus increasing the autoubiquitination of MDM2 and the activity of p53 [135]. Other proteins that lead to inhibition of MDM2 and the subsequent p53 increase are the ribosomal proteins L5, L11, and L23 upon ribosomal stress [136, 137].

Different modifications, such as phosphorylation or acetylation, are also important for the regulation of p53 (**Figure 9**). ATM, ATR, and other kinases activated upon DNA damage, phosphorylate the N-terminus of p53. This phosphorylation inhibits the binding of MDM2 [138]. Furthermore, ATM, using c-Abl as a mediator, can phosphorylate MDM2 leading to destabilization of the ligase and its subsequent degradation [139]. Acetylation of the C-terminal domain of p53 by p300/CBP stimulates the sequence-specific binding to DNA [140]. p300/CBP can also regulate the activity of p53 by acetylation of the RING domain of MDM2, which prevents p53 ubiquitination [141, 142]. Conversely, deacetylation of p53 by the histone deacetylase SirT1, inhibits the sequence-specific binding to DNA [143, 144].

The activity of p53 is regulated by other post-translational modifications as well (**Figure 9**). The C-terminus Lys386 is sumoylated, which enhances the transcription factor activity of p53 [145, 146]. Another modification at the C-terminus is neddylation of Lys370, -372, -373. The Nedd8 conjugation results in suppression of the activity of p53 [147]. The C-terminal Lys can be methylated as well. Depending on the methyltransferases and the modified Lys residue, the modification can either activate or inhibit the activity of p53 [148-150].

1.3.2 p53 mutations in cancer

The *TP53* gene is mutated in about 50% of human cancers. The frequent mutations reveal the significance of p53 for maintaining genome integrity. Furthermore, the function of the protein is impaired indirectly [107]. The majority of the mutations in p53 are point mutations that occur in the DNA binding domain, including the hotspot mutations (**Figure 7**) [105]. Mutations in *TP53* can result both in loss of function (LOF) as well as in gain of function (GOF).

1.3.2.1 Loss of function mutations (LOF)

LOF mutations in *TP53* may lead to initiation of tumor formation. The mutations can occur in the DNA binding domain, which interferes with the tumor suppression functions of p53; The mutations can also interfere with the conformation of the protein [151, 152]. Germline LOF mutations cause Li-Fraumeni syndrome (LFS), a genetic disease related to early onset of cancer [153, 154].

1.3.2.2 Gain of function mutations (GOF)

Not all missense mutations of *TP53* lead to loss of p53 activity, instead some p53 mutants can function as oncoproteins. Therefore, p53 GOF mutations have been associated with increased cell proliferation and metastasis formation, higher genomic instability, and promoting cancer metabolism [155]. An example of GOF mutations are LFS families with mutant p53, which have cancer incidence at earlier age compared to the LFS families with loss of function p53 mutation [156].

The oncoprotein function of mutant p53 is achieved through different mechanisms. Some p53 mutants, such as R175H, inhibit the activity of MDM2 and the p53 family member p73, while also restoring the activity of p63 [157]. Furthermore, mutant p53 can interact with DNA motifs that affect chromatin structure and promote DNA unwinding [158]. The

unspecific binding could also explain the ability of mutant p53 to promote the transcription of cell growth promoting proteins like vascular endothelial growth factor receptor 2 (VEGFR2) [159]. Among the expanded functions of mutant p53 is the effect on cellular metabolism through translocation of the glucose transporter GLUT1 to the cellular membrane [160].

1.3.3 Role of p53 in regulation of cellular metabolism

Cancer cells have enhanced glucose metabolism, and mutations in p53 can result in translocation of glucose transporter and induction of glycolysis, as discussed in 1.2.2.3. and 1.3.2.2., respectively [64, 160]. These effects are in contrast with the functions of the wild type protein, which represses glycolysis directly by inhibition of glucose transporters expression [161], or indirectly by affecting the activity of Parkin [162] or NF-κB [163]. Additionally, p53 promotes oxidative phosphorylation in the mitochondria [164], and it reduces the levels of pyruvate dehydrogenase kinase-2 (PDK2) [165], thus affecting the TCA cycle.

p53 is involved in the regulation of other metabolic pathways. For example, p53 downregulates lipid synthesis through inhibition of *FASN* [166, 167], and iron homeostasis by regulation of the iron-sulphur cluster assembly enzyme (ISCU) expression [168]. Furthermore, p53 is involved in the regulation of the serine metabolism and the associated nucleotide metabolism [169, 170]. The transcription factor can also repress the expression of GMPS through the activation of p21 [171]. In response to DNA damage, however, p53 can orchestrate the metabolic switch necessary for synthesis of new nucleotides via upregulation of the pentose phosphate pathway [172] and RRM2B [173].

GOF p53 mutations lead to changes in the cellular metabolism. In addition to the changes in glucose metabolism [160], mutant p53 promotes glycolysis and lipid biosynthesis [174, 175]. Mutant p53 also promotes nucleotide synthesis, by stimulating the expression of nucleotide metabolism genes and upregulation of the *de novo* and the salvage pathways [176].

1.4 DIHYDROOROTATE DEHYDROGENASE AS A DRUG TARGET

1.4.1 Structure and function of DHODH enzymes

1.4.1.1 Classification of DHODH enzymes

Dihydroorotate dehydrogenase (DHODH) enzymes are expressed in different organisms and grouped into two classes depending on several criteria: enzyme sequence, subcellular localization, substrate, and co-factor [177]. Class 1, expressed mainly in Gram positive bacteria, includes cytoplasmic enzymes. Based on the structure and the reaction substrate, Class 1 is divided into two subclasses: 1A and 1B [177]. The enzymes from Class 1A are homodimers that use FMN and flavin adenine dinucleotide (FAD) as co-factor [178, 179]. DHODH enzymes from Class 1B are heterotetramers, and the electron acceptor for the reaction is nicotinamide adenine dinucleotide (NAD^+) [180, 181]. The enzymes from Class 2 DHODH enzymes are found in prokaryotes as well as in eukaryotes. The proteins are

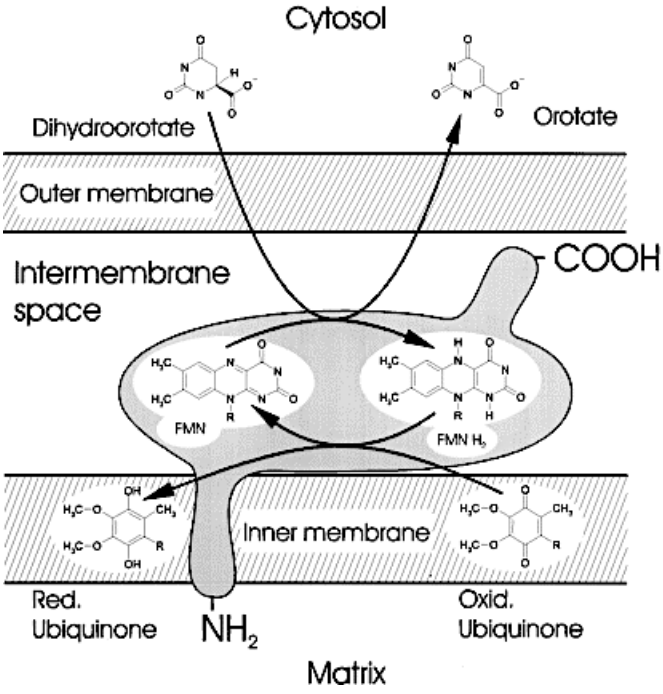
membrane bound monomers or homodimers. In eukaryotes, DHODH is bound to the inner mitochondrial membrane, and in prokaryotes the protein is bound to the cytosolic membrane [177, 182]. Human DHODH belongs to the Class 2 enzyme family and it is located on the outer surface of inner mitochondrial membrane. Substrates and products pass freely through the more permeable outer mitochondrial membrane to and from the cytoplasm [183].

1.4.1.2 Structure of human DHODH

Human DHODH consists of two domains: a catalytic domain (CAT) and a membrane-bound domain. The catalytic domain has α/β-barrel structure, and it contains the active site of the enzyme. The membrane bound α-helical domain that forms a tunnel towards the active site is also referred to as a quinone tunnel. The two domains are connected through a loop [184]. The N-terminus of the protein holds the mitochondrial localization signal, which is followed by a hydrophobic sequence that anchors the protein to the membrane [185]. The α-helical membrane domain is the binding region for ubiquinone (coenzyme Q10), which is reduced during the catalytic reaction. This region is also the binding site for numerous inhibitors [184, 186]. Several conserved residues are associated with the interactions with ubiquinone: Arg136 and Tyr356. These residues are also observed to interact with several known inhibitors of the enzyme [184, 187].

1.4.1.3 Function of human DHODH

DHODH catalyzes the fourth and only redox step in the *de novo* pyrimidine synthesis pathway - the oxidation of dihydroorotate to orotate, which requires the co-factor FMN as an electron acceptor (**Figure 10**) [21, 22]. The reaction follows a double displacement mechanism, where the first product is released before the second reaction starts. The oxidation of DHO to OA is accompanied by the reduction of the co-factor FMN. The oxidized state of FMN is restored with the second reaction where the substrate is ubiquinone (oxidized form) and the product is ubiquinol (reduced form) [177].



The function of DHODH is linked to the mitochondrial respiratory chain through the oxidation of the ubiquinol released from DHODH by complex III [21, 177]. In addition, DHODH is physically associated with complex II and complex III of the mitochondrial respiratory chain, and inhibition of DHODH leads to partial inhibition of complex III [188].

Figure 10. Schematic representation of the DHODH catalyzed reaction. The figure is taken from Rawls *et al.* [185] and re-printed with permission of publisher. Copyrights John Wiley and Sons (2001).

1.4.2 DHODH as a drug target

Human DHODH, similarly to other enzymes from the *de novo* pyrimidine biosynthesis pathway, has been considered as a drug target for diseases associated with increased cell proliferation. Numerous inhibitors have been described in the literature so far [24, 189-191], and as shown in **Paper I**, DHODH is a frequent target for small molecules [192]. Several inhibitors displayed promising results in cell culture and *in vivo* as anti-proliferative agents for the treatment of rheumatoid arthritis (RA), multiple sclerosis (MS) and different types of cancer. As synthesis of nucleotides is essential for viral replication, targeting DHODH is also an attractive approach to dampen viral infections [193-195]. At present, only leflunomide (Arava®) and its active metabolite teriflunomide (Aubagio®) are clinically approved as immune modulating drugs [196, 197]. DHODH inhibitors that are in clinical trials for the treatment of myeloid malignancies include BAY2402234 (Clinical trial identifier NCT03404726) and ASLAN003 (Clinical trial identifier NCT03451084).

1.4.2.1 Leflunomide and teriflunomide

Leflunomide is an isoxazole compound that is rapidly metabolized *in vivo* to the active metabolite teriflunomide (**Figure 11**) [198]. The effect of leflunomide/teriflunomide on immune cell proliferation [198, 199] was discovered before DHODH was identified as the compound's target [200, 201]. Leflunomide by itself does not inhibit DHODH *in vitro*, therefore, the observed effect *in vivo* or in cell extracts is due to the presence of its active metabolite [202]. The effect of leflunomide as an immunomodulator was studied in rat and mouse animal models [203-205]. The promising results from the animal studies resulted in clinical trials of leflunomide as a disease-modifying antirheumatic drug (DMARD) in Europe and USA [206-208]. Following the clinical trials, leflunomide was approved for use in the clinic for treatment of RA in 1999 under the trade name Arava® [196]. The adverse effects observed during the clinical trials were comparable to market available DMARDs such as methotrexate or sulphasalazine, however, the following post-market surveillance allowed identification of rare and unexpected adverse events [209]. Though some of the adverse effects depend on the treatment length and dosage [196, 207-209], animal experiments indicated that leflunomide has a teratogenic effect in rats [209, 210]. The adverse side effects may be due to the long half-life of the compound (15 – 18 days). Therefore, colestyramine or activated charcoals are used for its clearance [209]. Recent studies, however, did not show a connection between increased anomalies in women and exposure to leflunomide [211]. The active metabolite of leflunomide, teriflunomide, is also used in the clinic for treatment of relapsing multiple sclerosis (MS) [212, 213]. In 2012 and 2013 teriflunomide was approved for treatment of relapsing remitting MS in adult patients by the Food and Drug Administration (FDA) and the European Medicines Agency respectively (EMA) [214].

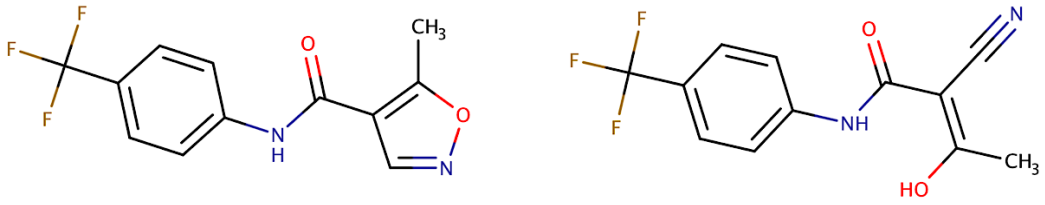


Figure 11. Structures of leflunomide and teriflunomide.

The effect of leflunomide as an immunosuppressive drug in animal models and the approval for RA treatment in the clinic resulted in extensive research on its potential use for treatment of other diseases. Several clinical trials have associated the efficacy of leflunomide for treatment of psoriasis and psoriatic arthritis (PsA). Due to the adverse effects, however, leflunomide is currently considered a third line therapy for PsA [215]. Leflunomide has also been tested for treatment of viral infections in pre-clinical and case reports studies after organ transplantation. The effect of leflunomide in clearing cytomegalovirus (CMV) and BK virus nephropathy (BKVN) infections, however, is slower compared to other available anti-viral treatments. Therefore, leflunomide is usually administered after an initial treatment with approved anti-viral drugs or in combination with drugs that achieve a rapid decrease of viral load. To date no double-blind clinical trials have been reported [216-218]. Leflunomide has also shown dose dependent results for treatment of herpes simplex virus (HSV) in Vero cells [219] as well as regression of HSV-1 and HSV-2 lesions in two HIV infected patients [220, 221]. The immunosuppressive effect of leflunomide, however, could lead to reactivation of other virus infections, such as hepatitis B (HBV) [222].

The effect of leflunomide as an anti-cancer drug has been investigated as well. In several neuroblastoma cell lines, treatment with leflunomide induces S-phase arrest and apoptosis, and reduced tumor growth in a xenograft mouse model [223]. These effects, however, could be due to inhibition of tyrosine kinases, since higher concentrations of leflunomide inhibit kinases such as JAK3 or STAT6 [224, 225]. Additionally, leflunomide inhibits the growth of melanoma cell lines and melanoma xenograft models [226]. This effect could also be attributed to the inhibition of aryl hydrocarbon receptor since uridine could not rescue the effect of the drug [227]. Reduction of cell growth after leflunomide treatment in medullary thyroid cancer cells *in vitro* has also been observed [228].

1.4.2.2 Brequinar

Brequinar (**Figure 12**) was discovered through a structure-activity relationship (SAR) study as an analogue of quinoline carboxylic acid. The compound showed antitumor activity against several cancer cell lines and inhibited the growth of different tumor xenograft models [229]. The target of the compound was identified as DHODH several years later, after the compound was already approved for Phase I clinical trials [230]. The pharmacokinetic properties and efficacy of brequinar in solid tumors was analyzed in several Phase I clinical trials. The treatments, however, did not meet the expected results. Due to the small cohort of patients and the short duration of the treatment, the results were deemed as insufficient for conclusions regarding the clinical effectiveness of brequinar [231-233].

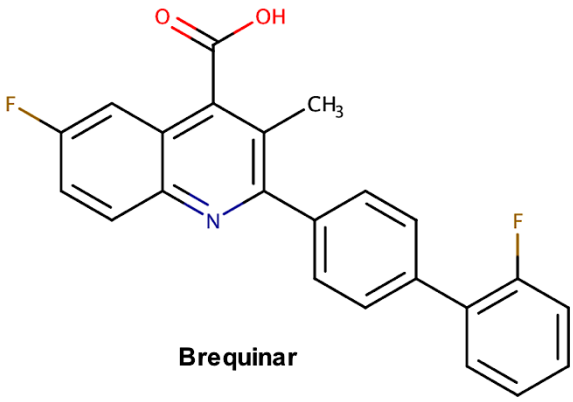


Figure 12. Structure of brequinar.

The efficacy of brequinar was tested in several Phase II clinical trials for treatment of different solid tumors as well. Patients with colorectal cancer exhibited high level of adverse events and the treatment had not effect [234]. Clinical trials with patients with melanoma and squamous-cell carcinoma of the head and neck were not responsive either. The adverse events, however, were moderate [235, 236]. Additionally, a moderate effect was observed in breast cancer patients [237], however, trials in gastrointestinal and lung cancer patients showed tumor regression in less than 10% of the patients [238, 239].

Despite the poor results of the clinical trials, several studies have investigated the effect of brequinar in cancer cell lines and *in vivo*. Brequinar sensitized breast and lung cancer cell lines to TRAIL induced apoptosis after co-treatment with doxorubicin [240]. A study by Sykes *et al*. showed that acute myeloid leukemia (AML) cells with constitutive HoxA9 expression differentiate after treatment with brequinar. Furthermore, brequinar had effect on an *in vivo* xenograft model, where the treatment reduced the tumor load [193]. The results of this study indicate that hematological tumors may be a better target for inhibitors of DHODH than solid tumors. The effect of brequinar, and leflunomide, have been tested on melanoma, myeloma, and lymphoma cells, with the aim to elucidate the mechanism of DHODH inhibitors. The study showed a link between c-MYC decrease and accumulation of cells in S-phase in cells sensitive to DHODH inhibition [241].

The activity of brequinar as an immunosuppressant has been investigated *in vitro* and *in vivo*. The observed effects, however, were only partially dependent on DHODH inhibition, since supplementation with uridine did not result in full recovery [242]. Brequinar also had an effect on CMV *in vitro* but the effect of the compound has not been tested *in vivo* [243]. Additionally, studies with influenza virus showed that brequinar treatment prevents nuclear export of mRNA by the virulence factors [244].

1.4.2.3 BAY 2402234 and ASLAN003

BAY2402234 is a novel and selective DHODH inhibitor, which is currently in a Phase I clinical trial for treatment of myeloid malignancies (Clinical Trials Identifier: NCT03404726). The compound caused differentiation of several AML cell lines, as well as in *in vivo* AML xenograft and patient derived xenograft (PDX) models. Furthermore, BAY2402234 treatment reduced the tumor burden as a monotherapy in the *in vivo* models.

Transcriptional and proteomics analysis after 24 h and 48 h revealed induction of genes and proteins associated with differentiation, apoptosis, and genes from the p53 pathway. At the same time, there was a negative regulation of MYC target gene expression. Furthermore, the proteomics analysis showed that the phosphorylation of several kinases from the MAPK pathway, important for AML development, are decreased [245].

ASLAN003, another compound that is currently in clinical trial (Clinical Trials Identifier: NCT03451084) for the treatment of myeloid malignancies, also induces differentiation of AML cells *in vivo* and *in vitro*. Treatment with ASLAN003 also triggers apoptosis and induces differentiation through activation of the transcription factor AP-1 and reduction of protein synthesis. In *in vivo* xenograft models and AML and PDX cell lines, ASLAN003 increased the survival and prevented the tumor infiltration [246].

1.4.2.4 Perspectives in the field

Despite numerous studies and clinical trials, the underlying mechanism of the cellular response to DHODH inhibition are still largely unknown. The various mechanisms employed by the cells to control nucleotide synthesis, and their dysregulation in cancer, could provide information about suitable drug combinations depending on the type of tumor. Furthermore, elucidation of the cellular response triggered by inhibition of DHODH could contribute to identification of tumors susceptible to monotherapy with DHODH inhibitors.

2 AIMS

The aims of this thesis were to (i) identify novel small molecules, which are activators of the tumour suppressor of p53, elucidate their target and mechanism of action, and optimize the molecules; (ii) investigate DHODH as a drug target.

The specific aims of each paper were:

Paper I: Identification of novel small molecule activators of p53 through a phenotypic screen as alternative to currently available p53 activators and discovery of their target.

Paper II: Exploring the interactions between DHODH and inner mitochondrial membrane lipids, their significance for DHODH function and for the development of novel inhibitors of the enzyme.

Paper III: Optimization of the small molecules discovered in **Paper I** and development of a lead compound with potential to be developed as a therapeutic.

Paper IV: Exploring the early cellular responses triggered by inhibition of DHODH by small molecules. The identification of these changes may allow to identify cells particularly susceptible to treatment with DHODH inhibitors and/or to identify markers of DHODH inhibition.

3 RESULTS AND DISCUSSION

3.1 DISCOVERY OF NOVEL DHODH INHIBITORS WHILE IN SEARCH FOR P53 ACTIVATORS (PAPER I)

3.1.1 Screening for p53 activators and characterization of HZ00

A treatment strategy for tumors with wild type p53 is to activate this tumor suppressor with small molecules that either inhibit p53 negative regulators or affect the localization of the protein [247]. Aiming to identify novel small molecules activators of p53, we conducted a phenotypic screen with two commercially available small molecule libraries. We used two reporter cell lines stably transfected with the p53 reporter plasmid pRGC-ΔFosLacZ: a murine fibroblast cell line T22 and the melanoma cell line ARN8, derived from A375 cell line. Based on the ability of the compounds to activate p53 in the tumor cells, but not in the fibroblasts, we selected a compound with a tetrahydroindazole (HZ) structure that we called HZ00 for further characterization and target identification.

The hit compound HZ00 contains a chiral center. We showed that the active enantiomer in our assays was (*R*)-HZ00. Furthermore, the compound did not have characteristics of a pan assay interference compound (PAINS) [248], and showed promising *in vitro* pharmacokinetic properties. Additionally, HZ00 did not increase the levels of several DNA damage markers, such as phosphorylated ATM, ATR, CHK-1, or CHK-2 at the timepoints tested.

HZ00 increased p53 levels in ARN8 cells in a dose-dependent manner, but had little effect on a human normal dermal fibroblast (HNDF) or on the expression of p53 target genes. Furthermore, HZ00 treatment caused a slight increase of p21, in contrast to the MDM2 inhibitor nutlin-3 [249]. Through a cellular thermal shift assay (CETSA), we discovered that MDM2 or MDMX are not a direct target of HZ00, nor did HZ00 stabilize p53 in a cycloheximide chase experiment (**Figure 13a**). Since the p53 mRNA was not significantly increased, we analyzed the levels of newly synthesized p53 protein by examining the incorporation of ^{35}S-labelled methionine/cysteine, and saw an increase after HZ00 treatment (**Figure 13b**).

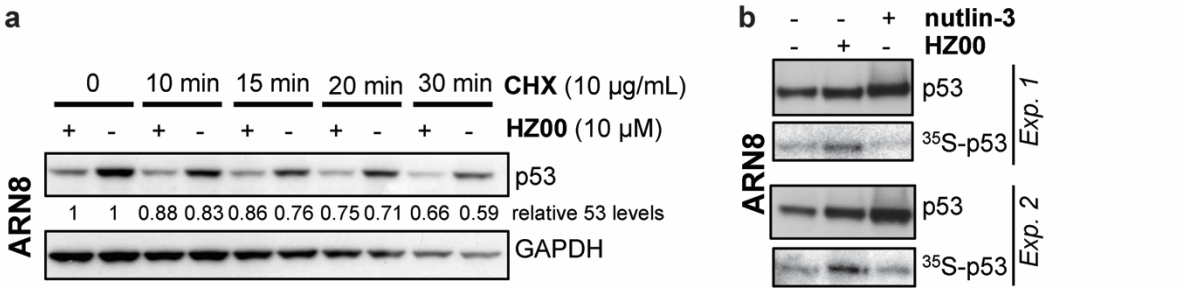


Figure 13. Effect of HZ00 on p53 protein levels. (a) ARN8 cells were treated with HZ00 or vehicle control (DMSO) for 7 h prior addition of cycloheximide for the indicated time. Protein levels were analyzed by western blot. Protein levels decrease was quantified using Adobe Photoshop. GAPDG was used as loading control. (b) ARN8 cells were treated 20 µM HZ00, 5 µM nutlin-3, or vehicle control for 5 h 50 min prior labeling with ^{35}S-Met-Cys for 30 min. Levels of newly synthesized p53 was isolated by immunoprecipitation and detected by western blot followed by autoradiography. The figure shows two independent biological experiments.

The selectivity of the compounds towards ARN8 cells compared to HNDF cells was also shown in an MTT proliferation assay, and a propidium iodide flow cytometry analysis revealed that HZ00 treatment of ARN8 cells increased the sub-G1 population. The differences in the mechanism of p53 activation between HZ00 and nutlin-3a, increased expression of p53 or increased stability respectively, prompted us to test whether the compounds will increase the cell death. The effect of the co-treatment in ARN8 xenograft models was indeed synergistic.

3.1.2 Target identification

After the characterization of the hit compound, we focused on the identification of the HZ00 target. We started with analysis of the effect of the compound on the cell cycle. A time course BrdU/PI flow cytometry analysis revealed that treatment with HZ00 leads to accumulation of ARN8 cells in S-phase. Additionally, the HZ00 treatment resulted in reduction of total RNA levels and changes in the nucleoli structure.

Increased p53 and accumulation of cells in S-phase has been associated with nucleotide depletion [250]. Therefore, we tested whether supplementation with different purine and pyrimidine nucleosides could ameliorate the effect of the HZ00 treatment. Since supplementation with uridine rescued the cells, we hypothesized that the target is an enzyme from the pyrimidine synthesis pathway. We continued with the target identification by supplementation of HZ00 treated cells with intermediate products from the pathway. We identified DHODH as the likely target of the compound, since supplementation with orotic acid rescued cell growth but dihydroorotate did not.

To confirm the target, we compared the effect of HZ00 with two known DHODH inhibitors, teriflunomide and brequinar, in a cell growth/viability assay as well as their effect on p53 transcription factor function activation. In both experiments the assays were performed with and without supplementation with excess of uridine, which rescued the effect of the compounds.

3.1.3 DHODH as a drug target

3.1.3.1 DHODH is a frequent small molecule target

After DHODH was identified as the target of HZ00, we re-tested the compounds from the screen, which activated p53, in an enzymatic assay with purified enzyme. Despite the diverse structures, we identified many of the compounds as inhibitors of DHODH. These results indicated that DHODH is a frequent small molecule target.

3.1.3.2 In search of more potent HZ compounds

We also tested other commercially available HZ analogues. Among the tested HZ compounds, HZ05 was the most potent inhibitor of DHODH. Like HZ00, HZ05 also possessed a chiral center, and the active enantiomer in the DHODH enzymatic assay, the cell growth/viability assay and p53 transcription factor activation assay was (*R*)-HZ05 (**Figure 14**). The co-crystal structure of DHODH with HZ05 confirmed that the bound enantiomer

was indeed the (*R*)-HZ05. Furthermore, we discovered that (*R*)-HZ05 is bound to the transmembrane domain (quinone tunnel) of DHODH, as teriflunomide and brequinar [184], thus preventing the electron transfer to ubiquinone. Similar to HZ00, ARN8 cells treated with HZ05 accumulated in S-phase. Co-treatment with nutlin-3 in ARN8 cells increased the population of cells in sub-G1 and the *in vivo* anticancer effect. The activity of the compound in xenograft models, however, was low compared to HZ00, which could be due to the short half-life of the compound.

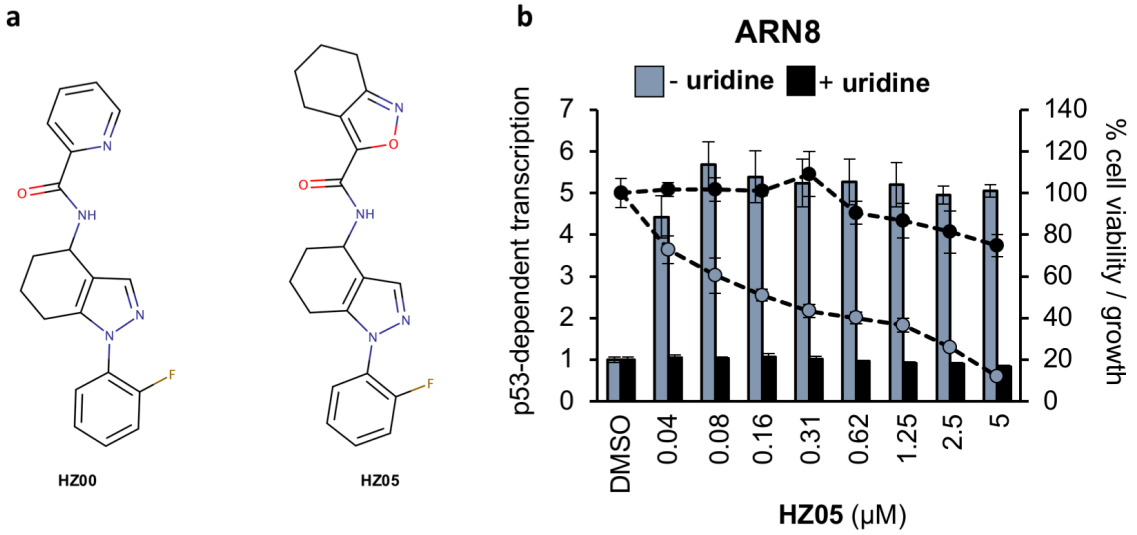


Figure 14. Characterization of HZ05. (a) Chemical structures of the hit compound HZ00 and its analogue HZ05. (b) Effect of HZ05 on p53 transcription factor activity (bars) and cell growth/viability (lines) in ARN8 cells with or without uridine supplementation. The values correspond to the mean of three technical replicates ± SD.

An RNA sequencing analysis of ARN8 cells treated with (*R*)-HZ00 and HZ05 displayed the similarities between the compounds from the HZ series after 5 h treatment. Furthermore, the sequencing analysis revealed upregulation of p53 target genes, and repression of genes from the p53-DREAM [251] complex.

3.1.4 DHODH/MDM2 inhibition - model of action

The activity of the DHODH inhibitors form the HZ series was tested in several cancer cell lines. Among the most responsive ones were the osteosarcoma line U2OS, and two leukemia cell lines, MV-4-11 and SIG-M5, where S-phase accumulation and increase of Sub-G1 population were observed. After assessing the effect of single agent treatments, we analyzed the effect of the co-treatment with the MDM2 inhibitor nutlin-3. This combination increased the percent of death cancer cells, as previously observed with ARN8 cells, when cells were pre-treated with the HZ compound before the addition of nutlin-3. Furthermore, single agent treatment and co-treatment had little effect on the cell cycle of HNDF cells used as normal cells control.

Aside from the staggered treatment (pre-treatment with DHODH inhibitors followed by addition of MDM2 inhibitor), we also performed experiments where both compounds were added simultaneously. From the results of these treatments we hypothesized that inhibition

of DHODH leads to accumulation of cells in S-phase with increased levels of p53 (**Figure 15**). Treatment of cells at this vulnerable stage of the cell cycle with high level of p53 with inhibitors of MDM2 may push cells towards cell death pathways. To test the model, we used U2OS cells and first analyzed the levels of p53 in the S-phase population after treatment with HZ05 detecting a significant increase of p53. Afterwards, we confirmed the model by annexin V/PI staining, which was increased after staggered treatment with HZ05 and nutlin-3.

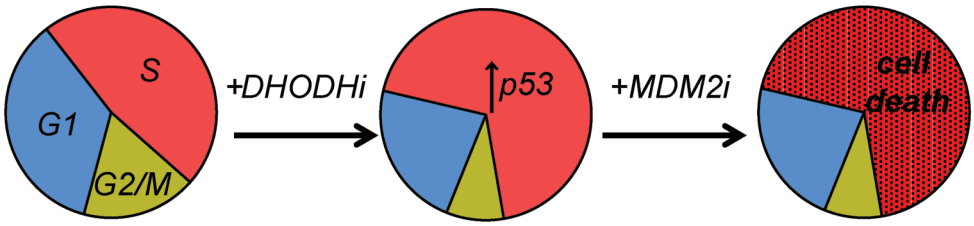


Figure 15. Model of action of DHODH/MDM2 inhibitors combination. Treatment with inhibitors of DHODH leads to accumulation of cell in S-phase with increased p53 protein levels. Consecutive treatment with MDM2 inhibitors leads to cell death of the population in S-phase.

After the discovery of a new class of DHODH inhibitors, and the identification of a new therapeutic model, we focused on studying DHODH as a drug target and the improvement of the HZ-series as anti-cancer therapeutics.

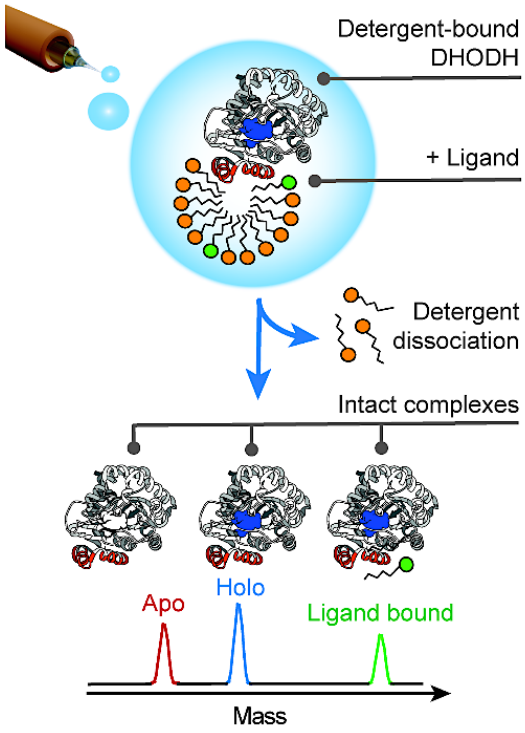
3.2 THE INTERACTION OF DHODH WITH THE LIPIDS FROM THE MITOCHONDRIAL MEMBRANE ARE IMPORTANT FOR THE ENZYME FUNCTION (PAPER II)

DHODH is a membrane bound protein [184, 185] but the significance of the membrane interactions for its function or its pharmacological inhibition have not been investigated. Partly, this could be due to the difficulties associated with structural studies of membrane bound proteins, such as low stability in detergents [252]. Improvements of the analytical methods, however, have provided new tools for investigation of membrane bound proteins, for example, native mass spectrometry has been developed for studying membrane proteins [253].

3.2.1 Employing mass spectrometry for investigation of DHODH interactions

3.2.1.1 FMN stabilizes DHODH

To investigate the interactions between DHODH and the mitochondrial membrane proteins we adapted non-denaturing nano-electrospray ionisation mass spectrometry (nESI-MS) method, previously used for studying membrane proteins. Employing this method, we initially analysed the complex between DHODH and its co-factor FMN. The interactions between DHODH and FMN were preserved in the gas phase in the solution containing



lauryldimethylamine N-oxide (LDAO) for micelle formation. Through this method, we were able to distinguish the apo-, holo- and ligand bound state of the protein. Furthermore, we established that the co-factor stabilises the protein conformation.

Figure 16. Strategy for studying DHODH - ligand interactions. The interactions between DHODH and its co-factor FMN, or ligands such as the DHODH inhibitor brequinar, are preserved upon release from detergent after collisional activation. This MS method allows discrimination between apo-, holo-, and ligand bound forms of DHODH.

3.2.1.2 Lipids - DHODH interactions: effect on protein stability

Prior the MS analysis of the DHODH-lipid interactions, we tested whether phospholipids from the mitochondrial membrane will affect the activity of DHODH in an enzymatic assay. The three tested lipids, phosphatidyl ethanolamine (PE), phosphatidyl choline (PC), and cardiolipin (CDL), increased the reaction rate. The observed increase with all three phospholipids, was comparable, which indicated that there is no specific recognition between the enzyme and the different types of lipids. In contrast, the nESI-MS analysis showed that PE forms fewer complexes compared to CDL and PC. Since the difference between the three lipids is in their charged head groups, we concluded that the lipid-DHODH interactions are through charged phospholipid head groups and DHODH. We also explored the lipid-protein interactions in conditions with increasing PE concentration. The MS peak was observed after binding of three PE molecules, pointing to limited interactions between DHODH and the lipids from the mitochondrial membrane.

Finally, we studied the lipid-DHODH binding effect on the protein stability. The thermal stability analysis showed that membrane association does not provide additional stability. In contrast, increasing concentrations of DMSO (chemical destabilization) revealed that the complex between DHODH and PE was more stable compared to the complexes with the enzyme co-factor FMN.

3.2.1.3 Effect of ligand binding on DHODH stability

Similarly to FMN, the binding of ligands to DHODH leads to stabilization of the protein. For this analysis, we performed molecular dynamics stimulations (MD) based on the crystal structure of brequinar bound to DHODH in the absence of lipids or detergents [184, 187]. The all-atom MD stimulations showed that the membrane binding site is flexible, and binding

of a ligand result in stabilization of this domain. Furthermore, through the MD stimulations, a second flexible, lipid interacting region was identified.

3.2.2 Understanding the interactions between DHODH and the lipids from the inner mitochondrial membrane through molecular dynamics

All-atom MD stimulations were also used for analysis of the lipid - DHODH interaction. Using PE bilayer model, we studied the position of DHODH within the mitochondrial membrane. We discovered that in addition to the interactions between the membrane-binding domain with the lipids, an additional flexible region from the protein interacts with the membrane. This trans membrane (TM) helix anchors the protein to the lipid bilayer and maintains correct orientation of DHODH.

The charged limited interactions between the lipids and DHODH, and the dynamic lipid bilayer structure, provide a potential regulatory mechanism based on the orientation of the enzyme. Similar regulatory mechanism is described for other membrane proteins, such as the GTPase K-Ras [254].

Lastly, we examined the ubiquinone binding site of the protein, which is in the membrane-binding domain, and is the binding site of small molecule inhibitors of DHODH. The MD stimulations showed that the lipids around the membrane-binding site assisted in stabilizing the quinone binding site. Due to the size of the ubiquinone molecule, we suggest that parts of the molecule interact with the lipids from the membrane. Similar architecture is observed in the binding site of complex I from the mitochondrial respiratory chain [255]. Therefore, these interactions should be considered in drug design, since they can affect the activity of the small molecules.

3.3 OPTIMIZATION OF THE HZ-COMPOUNDS SERIES (PAPER III)

The DHODH inhibitors discovered in **Paper I** showed promising results in several cell lines, and HZ00 had good *in vitro* pharmacokinetic properties. Nevertheless, the progress of this class of molecules towards *in vivo* studies requires further medicinal chemistry optimization.

3.3.1.1 Design of new HZ00 and HZ05 analogues

We based the design of new HZ analogues on a metabolic soft spot identification study with (*R*)-HZ05, and the crystal structure of (*R*)-HZ05 with DHODH obtained in **Paper I**. The metabolic soft spot analysis suggested that modifications in the Ar^1 moiety of the molecules could improve the metabolic profile. The co-crystal structure data, on the other hand, indicated the major interactions between the HZ-compound and the amino acids from the quinone tunnel of DHODH. Therefore, we decided that small Ar^1 substituents may be less likely to be susceptible to oxidative metabolism. We also aimed to introduce an interaction with Tyr356, which is present in several known inhibitors [184, 245]. The modifications at

the Ar^2 were focused on testing the extension towards the solvent, and potentially the introduction of larger substituents.

3.3.1.2 Activity of the new inhibitors from the HZ-series

The newly synthesized analogues were tested in *in vitro* kinetic enzymatic assay that detects the colorimetric change of the final electron acceptor 2,3-dichlorophenolindophenol (DCIP). The activity of many of the new inhibitors was improved compared to the originally described HZ00 and HZ05. The strongest inhibitors among the HZ00 substituents were compounds **30** with an IC_{50} = 15 nM, and **38** with an IC_{50} = 17 nM. The most active HZ05 analogues were able to inhibit DHODH at concentrations lower than 5 nM. Compounds **43** (IC_{50} = 4 nM); **45** (IC_{50} = 3 nM); **46** (IC_{50} = 1.2 nM), and **51** (IC_{50} = 2.3 nM) were the most active analogues from the series.

3.3.1.3 Solubility, in vitro metabolic stability, and selectivity

The most active analogues from both HZ-analogue series were selected for analysis of their solubility and *in vitro* metabolic stability. The solubility and human liver microsomes (HLM) *in vitro* metabolic stability of Compounds **30** and **51** were improved compared to their respective analogues (*R*)-HZ00 and (*R*)-HZ05. The *in vitro* metabolic stability in mouse liver microsomes (MLM), however, was only marginally improved in **51**.

The results suggested that compound **51** have the highest potential for *in vivo* mouse studies. Furthermore, the main candidate for pre-clinical testing demonstrated low activity in a kinase screen, thus showing high specificity towards DHODH.

3.3.1.4 Activity of the compounds in cellular assays

The ability of the new analogues to induce p53 dependent transcription correlated to their activity in the *in vitro* enzymatic assay. As in **paper I**, we assessed the activation of p53 through a CPRG reporter assay. Furthermore, we supplemented the reactions with excess of uridine to distinguish between DHODH-related p53 activation and activation of p53 through other mechanisms. Some of the highly active compounds caused a decrease in p53 induced transcription, however, we expected this decrease to be related to an impairment of cell growth/viability.

The effect of the HZ analogues on the growth and viability of cells was assessed by sulforhodamine B assays (SRB) using two cell lines: the melanoma cell line ARN8 and human fibroblast cells (HNDFs). The specificity of the compounds was assessed again by supplementation with excess of uridine. The effect on the melanoma cell line was stronger compared to the fibroblast cells, similarly to the originally described tetrahidroindazoles in **Paper I**. Furthermore, the activity of many of the compounds was improved or similar compared to HZ00 and HZ05. The analogues with the strongest effect on the growth/viability of ARN8 cells from the HZ00 series were **30** and **38**, while the most active analogues from the HZ05 series were compounds **43**, **45**, **46**, and **51**. These results correspond to the inhibitory activity in the *in vitro* enzymatic assay. The effect of several of the compounds, however, was not recovered at high compounds concentrations, which may be caused by off target effects.

3.3.1.5 Inhibition of DHODH increases apoptosis induced γ-H2AX

After the analysis of p53 transcription factor activity, and the overall effect of the HZ-analogues on cell growth/viability, we evaluated the protein level changes by western blot analysis. We checked the changes in the levels of p53, p-Ser15, H2AX, as well as γ-H2AX after supplementation with uridine after 24 h treatment with several of the HZ05 analogues and brequinar, used as a reference compound. The treatments increased p53, phosphorylated p53, and γ-H2AX, however, the uridine supplementation restored the protein levels to those of the vehicle control. γ-H2AX increase could be due to DNA damage [256, 257] or due to induction of apoptosis [258, 259]. Furthermore, γ-H2AX increase after treatment with DHODH inhibitors have been shown in two studies, and it has been related to both DNA damage [46] and apoptosis [260]. However, uridine supplementation was not tested in neither of these studies.

To determine which alternative is prevailing in our assays, we tested two DNA damage markers, p-CHK-1 and p-CHK-2. Neither of these markers were affected by inhibition of DHODH. To analyze whether γ-H2AX is increased due to induction of apoptosis, we examined the protein levels after addition of the pan caspase inhibitor Z-VAD-FMK [261]. Addition of Z-VAD-FMK reduced the induction of γ-H2AX, as well as cleaved PARP, but had no effect on p53, p-Ser p53, Bax, or Bcl-2, which are located upstream of the caspase cascade. We confirmed that inhibition of DHODH triggers the apoptosis response through live cell imaging assays, using a fluorescent caspase 3/7 substrate.

Taken together, the lead compound from this structure activity relationship study, **51,** showed suitable solubility and *in vitro* HLM metabolic stability. The half-life in MLM, however, indicates that the series might need additional optimization for development of a clinical candidate.

3.4 MECHANISM OF DHODH INHIBITION (PAPER IV)

Numerous potent and specific DHODH inhibitors were discovered in the last few years [192, 245, 246], however, the mechanism that triggers the cellular response is still not well understood. With the aim to identify the early events triggered by the inhibition of DHODH that ultimately result in cell cycle arrest [250], apoptosis [223], or cell differentiation [193], we performed a time course RNA sequencing.

3.4.1.1 DHODH inhibition downregulates genes regulating chromatin dynamics

The RNA sequencing analysis of the melanoma cell line ARN8 revealed that the early cellular response after treatment with two DHODH inhibitors, SLL391 (compound **51** in **Paper III**) and brequinar, is downregulation of the differentially expressed genes. The gene expression changes caused by both compounds were consistent, which indicated that the observed differences were not due to off target effects of the compounds. The initial changes were mainly in histone mRNAs. This indicates that the treatments lead to modification of chromatin dynamics and drive additional alterations in gene expression, which are either a consequence or a cause for changes in the cell cycle profiles. Treatment with DHODH

inhibitors leads to accumulation of ARN8 cells in S-phase, therefore, these changes are expected [192, 250]. Consistent with these results, the gene ontology (GO) analysis of molecular functions showed downregulation of RNA polymerase function, chromatin binding, and DNA binding, as well as downregulation of processes such as gene silencing.

In the later time points (3 and 4 h) the number of upregulated genes started to increase as well. Among the biological processes identified in the GO analysis were positive regulation of the intrinsic apoptotic pathway and cellular response to chemical stimuli. The upregulated differentially expressed genes between the two inhibitors had lower similarity compared to the downregulated genes.

3.4.1.2 Inhibition of DHODH downregulate PNUTS expression

Of the genes significantly downregulated at an early time point (2 h after treatment with SLL391, and 3 h after treatment with brequinar), was the protein phosphatase 1 nuclear targeting subunit (PNUTS, PPP1R10). This protein is a negative regulator of the activity of protein phosphatase 1 [262, 263]. Therefore, PNUTS regulates important regulators of the cell cycle and cellular stress response [264-266].

The sequencing data was initially validated at mRNA levels by qRT-PCR, and the fold change reduction in gene expression was consistent with the sequencing data. Afterwards, we checked whether the mRNA changes correspond to protein changes by western blot analysis. The PNUTS protein levels were reduced, however, the decrease was observed after longer treatments. The decrease of PNUTS coincided with an increase in p53 protein levels, both changes were time-dependent. Furthermore, the levels of p53 raised after increasing the compound concentration. Changes of compound concentration, however, did not affect the level of PNUTS in our experimental conditions.

In contrast to our data, positive correlation between p53 and PNUTS have been reported under hypoxic conditions [265]. However, DHODH inhibition can induce cell death in p53 deficient cell lines as well, which indicates that the correlation observed under our experimental conditions may be cell line specific [245]. Additionally, PNUTS downregulation has been associated with apoptosis in a p53-independent manner [265], with modulation of the cell cycle progression through Rb1 phosphorylation [264, 267], with c-MYC downregulation [266], and with control of transcription by RNA Pol II [268]. Due to the various cellular processes regulated by PNUTS and the association of some of PNUTS-modulated proteins with nucleotide synthesis regulation [16], the significance of this change requires further investigation. In any case, the fast and significant change in *PNUTS* mRNA levels, may be a hallmark of DHODH inhibition and a useful biomarker of drug activity.

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5 REFERENCES

1. Alberts, B., *Molecular biology of the cell*. 5th ed. 2008, New York: Garland Science.
2. Zala, D., et al., *The advantage of channeling nucleotides for very processive functions.* F1000Res, 2017. **6**: p. 724.
3. Schlattner, U., et al., *Cellular compartmentation of energy metabolism: creatine kinase microcompartments and recruitment of B-type creatine kinase to specific subcellular sites.* Amino Acids, 2016. **48**(8): p. 1751-74.
4. Gennerich, A. and R.D. Vale, *Walking the walk: how kinesin and dynein coordinate their steps.* Curr Opin Cell Biol, 2009. **21**(1): p. 59-67.
5. Faller, L.D., *Mechanistic studies of sodium pump.* Arch Biochem Biophys, 2008. **476**(1): p. 12-21.
6. Bourne, H.R., D.A. Sanders, and F. McCormick, *The GTPase superfamily: a conserved switch for diverse cell functions.* Nature, 1990. **348**(6297): p. 125-32.
7. Colicelli, J., *Human RAS superfamily proteins and related GTPases.* Sci STKE, 2004. **2004**(250): p. RE13.
8. Kleczkowski, L.A., D. Decker, and M. Wilczynska, *UDP-sugar pyrophosphorylase: a new old mechanism for sugar activation.* Plant Physiol, 2011. **156**(1): p. 3-10.
9. Kennedy, E.P. and S.B. Weiss, *The function of cytidine coenzymes in the biosynthesis of phospholipides.* J Biol Chem, 1956. **222**(1): p. 193-214.
10. Bladergroen, B.A. and L.M. van Golde, *CTP:phosphoethanolamine cytidylyltransferase.* Biochim Biophys Acta, 1997. **1348**(1-2): p. 91-9.
11. Hartwig, C., et al., *cAMP, cGMP, cCMP and cUMP concentrations across the tree of life: High cCMP and cUMP levels in astrocytes.* Neurosci Lett, 2014. **579**: p. 183-7.
12. Seifert, R., *cCMP and cUMP: emerging second messengers.* Trends Biochem Sci, 2015. **40**(1): p. 8-15.
13. Seifert, R. and G. Schultz, *Involvement of pyrimidinoceptors in the regulation of cell functions by uridine and by uracil nucleotides.* Trends Pharmacol Sci, 1989. **10**(9): p. 365-9.
14. Patel, A., et al., *ATP as a biological hydrotrope.* Science, 2017. **356**(6339): p. 753-756.
15. Lane, A.N. and T.W. Fan, *Regulation of mammalian nucleotide metabolism and biosynthesis.* Nucleic Acids Res, 2015. **43**(4): p. 2466-85.
16. Villa, E., et al., *Cancer Cells Tune the Signaling Pathways to Empower de Novo Synthesis of Nucleotides.* Cancers, 2019. **11**(5): p. 688.
17. Pedley, A.M. and S.J. Benkovic, *A New View into the Regulation of Purine Metabolism: The Purinosome.* Trends Biochem Sci, 2017. **42**(2): p. 141-154.
18. Hartman, S.C., *Phosphoribosyl Pyrophosphate Amidotransferase. Purification and General Catalytic Properties.* J Biol Chem, 1963. **238**: p. 3024-35.
19. Welin, M., et al., *Structural studies of tri-functional human GART.* Nucleic Acids Res, 2010. **38**(20): p. 7308-19.
20. An, S., et al., *Reversible compartmentalization of de novo purine biosynthetic complexes in living cells.* Science, 2008. **320**(5872): p. 103-6.
21. Jones, M.E., *Pyrimidine nucleotide biosynthesis in animals: genes, enzymes, and regulation of UMP biosynthesis.* Annu Rev Biochem, 1980. **49**: p. 253-79.
22. Evans, D.R. and H.I. Guy, *Mammalian pyrimidine biosynthesis: fresh insights into an ancient pathway.* J Biol Chem, 2004. **279**(32): p. 33035-8.

23. Longley, D.B., D.P. Harkin, and P.G. Johnston, *5-fluorouracil: mechanisms of action and clinical strategies.* Nat Rev Cancer, 2003. **3**(5): p. 330-8.

24. Loffler, M., et al., *Pyrimidine pathways in health and disease.* Trends Mol Med, 2005. **11**(9): p. 430-7.

25. Yamaoka, T., et al., *Feedback inhibition of amidophosphoribosyltransferase regulates the rate of cell growth via purine nucleotide, DNA, and protein syntheses.* J Biol Chem, 2001. **276**(24): p. 21285-91.

26. Levine, R.L., N.J. Hoogenraad, and N. Kretchmer, *Regulation of activity of carbamoyl phosphate synthetase from mouse spleen.* Biochemistry, 1971. **10**(20): p. 3694-9.

27. Liu, Y.C., et al., *Global regulation of nucleotide biosynthetic genes by c-Myc.* PLoS One, 2008. **3**(7): p. e2722.

28. Zeller, K.I., et al., *Global mapping of c-Myc binding sites and target gene networks in human B cells.* Proc Natl Acad Sci U S A, 2006. **103**(47): p. 17834-9.

29. Mannava, S., et al., *Direct role of nucleotide metabolism in C-MYC-dependent proliferation of melanoma cells.* Cell Cycle, 2008. **7**(15): p. 2392-400.

30. Shukla, S.K., et al., *MUC1 and HIF-1alpha Signaling Crosstalk Induces Anabolic Glucose Metabolism to Impart Gemcitabine Resistance to Pancreatic Cancer.* Cancer Cell, 2017. **32**(3): p. 392.

31. Chang, F., et al., *Signal transduction mediated by the Ras/Raf/MEK/ERK pathway from cytokine receptors to transcription factors: potential targeting for therapeutic intervention.* Leukemia, 2003. **17**(7): p. 1263-1293.

32. Santana-Codina, N., et al., *Oncogenic KRAS supports pancreatic cancer through regulation of nucleotide synthesis.* Nature Communications, 2018. **9**: p. 4945.

33. Gaglio, D., et al., *Oncogenic K-Ras decouples glucose and glutamine metabolism to support cancer cell growth.* Molecular Systems Biology, 2011. **7**: p. 523.

34. Ying, H., et al., *Oncogenic Kras maintains pancreatic tumors through regulation of anabolic glucose metabolism.* Cell, 2012. **149**(3): p. 656-70.

35. Graves, L.M., et al., *Regulation of carbamoyl phosphate synthetase by MAP kinase.* Nature, 2000. **403**(6767): p. 328-332.

36. Sigoillot, F.D., S.M. Sigoillot, and H.I. Guy, *Breakdown of the regulatory control of pyrimidine biosynthesis in human breast cancer cells.* Int J Cancer, 2004. **109**(4): p. 491-8.

37. Saxton, R.A. and D.M. Sabatini, *mTOR Signaling in Growth, Metabolism, and Disease.* Cell, 2017. **169**(2): p. 361-371.

38. Ben-Sahra, I., et al., *mTORC1 induces purine synthesis through control of the mitochondrial tetrahydrofolate cycle.* Science, 2016. **351**(6274): p. 728-733.

39. Robitaille, A.M., et al., *Quantitative phosphoproteomics reveal mTORC1 activates de novo pyrimidine synthesis.* Science, 2013. **339**(6125): p. 1320-3.

40. Ben-Sahra, I., et al., *Stimulation of de novo pyrimidine synthesis by growth signaling through mTOR and S6K1.* Science, 2013. **339**(6125): p. 1323-8.

41. Wang, W., et al., *The phosphatidylinositol 3-kinase/akt cassette regulates purine nucleotide synthesis.* J Biol Chem, 2009. **284**(6): p. 3521-8.

42. Saha, A., et al., *Akt phosphorylation and regulation of transketolase is a nodal point for amino acid control of purine synthesis.* Mol Cell, 2014. **55**(2): p. 264-76.

43. Franke, T.F., *PI3K/Akt: getting it right matters.* Oncogene, 2008. **27**(50): p. 6473-88.

44. Sigoillot, F.D., D.R. Evans, and H.I. Guy, *Growth-dependent regulation of mammalian pyrimidine biosynthesis by the protein kinase A and MAPK signaling cascades.* J Biol Chem, 2002. **277**(18): p. 15745-51.

45. Hopkins, B.D., et al., *PTEN function: the long and the short of it.* Trends Biochem Sci, 2014. **39**(4): p. 183-90.

46. Mathur, D., et al., *PTEN Regulates Glutamine Flux to Pyrimidine Synthesis and Sensitivity to Dihydroorotate Dehydrogenase Inhibition.* Cancer Discov, 2017. **7**(4): p. 380-390.

47. Piccolo, S., S. Dupont, and M. Cordenonsi, *The biology of YAP/TAZ: hippo signaling and beyond.* Physiol Rev, 2014. **94**(4): p. 1287-312.

48. Cox, A.G., et al., *Yap regulates glucose utilization and sustains nucleotide synthesis to enable organ growth.* EMBO J, 2018. **37**(22).

49. Balasubramaniam, S., J.A. Duley, and J. Christodoulou, *Inborn errors of purine metabolism: clinical update and therapies.* J Inherit Metab Dis, 2014. **37**(5): p. 669-86.

50. Balasubramaniam, S., J.A. Duley, and J. Christodoulou, *Inborn errors of pyrimidine metabolism: clinical update and therapy.* J Inherit Metab Dis, 2014. **37**(5): p. 687-98.

51. Nyhan, W.L., *Disorders of purine and pyrimidine metabolism.* Mol Genet Metab, 2005. **86**(1-2): p. 25-33.

52. van Gennip, A.H., *Defects in metabolism of purines and pyrimidines.* Ned Tijdschr Klin Chem, 1999. **24**: p. 171–175.

53. Kennan, A., et al., *Identification of an IMPDH1 mutation in autosomal dominant retinitis pigmentosa (RP10) revealed following comparative microarray analysis of transcripts derived from retinas of wild-type and Rho(-/-) mice.* Hum Mol Genet, 2002. **11**(5): p. 547-57.

54. Kelley, W.N., et al., *A specific enzyme defect in gout associated with overproduction of uric acid.* Proc Natl Acad Sci U S A, 1967. **57**(6): p. 1735-9.

55. de Brouwer, A.P.M.v.B.H.N.S.B.A.W.F.C.J.D.J., *PRPS1 mutations: four distinct syndromes and potential treatment.* Am J Hum Genet, 2010. **86**: p. 506–518.

56. Weinberg, R.A., *How cancer arises.* Scientific American, 1996. **275**(3): p. 62-70.

57. Hanahan, D. and R.A. Weinberg, *The hallmarks of cancer.* Cell, 2000. **100**(1): p. 57-70.

58. Hanahan, D. and R.A. Weinberg, *Hallmarks of Cancer: The Next Generation.* Cell, 2011. **144**(5): p. 646-674.

59. Nowell, P.C., *The clonal evolution of tumor cell populations.* Science, 1976. **194**(4260): p. 23-8.

60. Cahill, D.P., et al., *Genetic instability and darwinian selection in tumours.* Trends Cell Biol, 1999. **9**(12): p. M57-60.

61. Knudson, A.G., Jr., *Genetics of human cancer.* Annu Rev Genet, 1986. **20**: p. 231-51.

62. Warburg, O., *On the origin of cancer cells.* Science, 1956. **123**(3191): p. 309-14.

63. Coller, H.A., *Is Cancer a Metabolic Disease?* American Journal of Pathology, 2014. **184**(1): p. 4-17.

64. Altenberg, B. and K.O. Greulich, *Genes of glycolysis are ubiquitously overexpressed in 24 cancer classes.* Genomics, 2004. **84**(6): p. 1014-20.

65. Rastogi, S., et al., *Glut-1 antibodies induce growth arrest and apoptosis in human cancer cell lines.* Cancer Lett, 2007. **257**(2): p. 244-51.

66. Magrath, I., et al., *Prognostic factors in Burkitt's lymphoma: importance of total tumor burden.* Cancer, 1980. **45**(6): p. 1507-15.

67. Kroemer, G. and J. Pouyssegur, *Tumor cell metabolism: cancer's Achilles' heel.* Cancer Cell, 2008. **13**(6): p. 472-82.

68. Christofk, H.R., et al., *Pyruvate kinase M2 is a phosphotyrosine-binding protein.* Nature, 2008. **452**(7184): p. 181-6.

69. Wang, H.Q., et al., *Positive feedback regulation between AKT activation and fatty acid synthase expression in ovarian carcinoma cells.* Oncogene, 2005. **24**(22): p. 3574-3582.

70. Pavlides, S., et al., *The reverse Warburg effect Aerobic glycolysis in cancer associated fibroblasts and the tumor stroma.* Cell Cycle, 2009. **8**(23): p. 3984-4001.

71. Martinez-Outschoorn, U.E., et al., *Oxidative stress in cancer associated fibroblasts drives tumor-stroma co-evolution A new paradigm for understanding tumor metabolism, the field effect and genomic instability in cancer cells.* Cell Cycle, 2010. **9**(16): p. 3256-3276.

72. Pylayeva-Gupta, Y., E. Grabocka, and D. Bar-Sagi, *RAS oncogenes: weaving a tumorigenic web.* Nat Rev Cancer, 2011. **11**(11): p. 761-74.

73. Bos, J.L., *The ras gene family and human carcinogenesis.* Mutat Res, 1988. **195**(3): p. 255-71.

74. Mita, H., et al., *A novel method, digital genome scanning detects KRAS gene amplification in gastric cancers: involvement of overexpressed wild-type KRAS in downstream signaling and cancer cell growth.* BMC Cancer, 2009. **9**: p. 198.

75. Kim, J., et al., *CPS1 maintains pyrimidine pools and DNA synthesis in KRAS/LKB1-mutant lung cancer cells.* Nature, 2017. **546**(7656): p. 168-172.

76. Fruman, D.A., et al., *The PI3K Pathway in Human Disease.* Cell, 2017. **170**(4): p. 605-635.

77. Manning, B.D. and L.C. Cantley, *AKT/PKB signaling: navigating downstream.* Cell, 2007. **129**(7): p. 1261-74.

78. Woenckhaus, J., et al., *Genomic gain of PIK3CA and increased expression of p110alpha are associated with progression of dysplasia into invasive squamous cell carcinoma.* J Pathol, 2002. **198**(3): p. 335-42.

79. Rudd, M.L., et al., *A unique spectrum of somatic PIK3CA (p110alpha) mutations within primary endometrial carcinomas.* Clin Cancer Res, 2011. **17**(6): p. 1331-40.

80. Kang, S., et al., *Oncogenic transformation induced by the p110beta, -gamma, and -delta isoforms of class I phosphoinositide 3-kinase.* Proc Natl Acad Sci U S A, 2006. **103**(5): p. 1289-94.

81. Burke, J.E., et al., *Oncogenic mutations mimic and enhance dynamic events in the natural activation of phosphoinositide 3-kinase p110alpha (PIK3CA).* Proc Natl Acad Sci U S A, 2012. **109**(38): p. 15259-64.

82. Altomare, D.A. and J.R. Testa, *Perturbations of the AKT signaling pathway in human cancer.* Oncogene, 2005. **24**(50): p. 7455-64.

83. Stemke-Hale, K., et al., *An integrative genomic and proteomic analysis of PIK3CA, PTEN, and AKT mutations in breast cancer.* Cancer Res, 2008. **68**(15): p. 6084-91.

84. Grabiner, B.C., et al., *A diverse array of cancer-associated MTOR mutations are hyperactivating and can predict rapamycin sensitivity.* Cancer Discov, 2014. **4**(5): p. 554-63.

85. Rabinovich, S., et al., *Diversion of aspartate in ASS1-deficient tumours fosters de novo pyrimidine synthesis.* Nature, 2015. **527**(7578): p. 379-383.

86. Lee, J.S., et al., *Urea Cycle Dysregulation Generates Clinically Relevant Genomic and Biochemical Signatures.* Cell, 2018. **174**(6): p. 1559-1570 e22.

87. Makinoshima, H., et al., *Signaling through the Phosphatidylinositol 3-Kinase (PI3K)/Mammalian Target of Rapamycin (mTOR) Axis Is Responsible for Aerobic Glycolysis mediated by Glucose Transporter in Epidermal Growth Factor Receptor (EGFR)-mutated Lung Adenocarcinoma.* Journal of Biological Chemistry, 2015. **290**(28): p. 17495-17504.

88. Dang, C.V., *MYC on the path to cancer.* Cell, 2012. **149**(1): p. 22-35.

89. Beroukhim, R., et al., *The landscape of somatic copy-number alteration across human cancers.* Nature, 2010. **463**(7283): p. 899-905.

90. Dalla-Favera, R., et al., *Human c-myc onc gene is located on the region of chromosome 8 that is translocated in Burkitt lymphoma cells.* Proc Natl Acad Sci U S A, 1982. **79**(24): p. 7824-7.

91. Boyd, K.E. and P.J. Farnham, *Myc versus USF: discrimination at the cad gene is determined by core promoter elements.* Mol Cell Biol, 1997. **17**(5): p. 2529-37.

92. Gao, P., et al., *c-Myc suppression of miR-23a/b enhances mitochondrial glutaminase expression and glutamine metabolism.* Nature, 2009. **458**(7239): p. 762-5.

93. Cunningham, J.T., et al., *Protein and nucleotide biosynthesis are coupled by a single rate-limiting enzyme, PRPS2, to drive cancer.* Cell, 2014. **157**(5): p. 1088-103.

94. Lane, D.P., *Cancer. p53, guardian of the genome.* Nature, 1992. **358**(6381): p. 15-6.

95. Eliyahu, D., et al., *Participation of p53 cellular tumour antigen in transformation of normal embryonic cells.* Nature, 1984. **312**(5995): p. 646-9.

96. Donehower, L.A., et al., *Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours.* Nature, 1992. **356**(6366): p. 215-21.

97. Bartek, J., et al., *Aberrant expression of the p53 oncoprotein is a common feature of a wide spectrum of human malignancies.* Oncogene, 1991. **6**(9): p. 1699-703.

98. Belyi, V.A., et al., *The origins and evolution of the p53 family of genes.* Cold Spring Harb Perspect Biol, 2010. **2**(6): p. a001198.

99. Joerger, A.C. and A.R. Fersht, *Structural biology of the tumor suppressor p53.* Annu Rev Biochem, 2008. **77**: p. 557-82.

100. Bell, S., et al., *p53 contains large unstructured regions in its native state.* J Mol Biol, 2002. **322**(5): p. 917-27.

101. Thut, C.J., et al., *p53 transcriptional activation mediated by coactivators TAFII40 and TAFII60.* Science, 1995. **267**(5194): p. 100-4.

102. Gu, W., X.L. Shi, and R.G. Roeder, *Synergistic activation of transcription by CBP and p53.* Nature, 1997. **387**(6635): p. 819-23.

103. Kussie, P.H., et al., *Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain.* Science, 1996. **274**(5289): p. 948-53.

104. Toledo, F., et al., *Mouse mutants reveal that putative protein interaction sites in the p53 proline-rich domain are dispensable for tumor suppression.* Mol Cell Biol, 2007. **27**(4): p. 1425-32.

105. Cho, Y., et al., *Crystal structure of a p53 tumor suppressor-DNA complex: understanding tumorigenic mutations.* Science, 1994. **265**(5170): p. 346-55.

106. Bode, A.M. and Z. Dong, *Post-translational modification of p53 in tumorigenesis.* Nat Rev Cancer, 2004. **4**(10): p. 793-805.

107. Vogelstein, B., D. Lane, and A.J. Levine, *Surfing the p53 network.* Nature, 2000. **408**(6810): p. 307-10.

108. Abbas, T. and A. Dutta, *p21 in cancer: intricate networks and multiple activities.* Nat Rev Cancer, 2009. **9**(6): p. 400-14.

109. Hsu, S.P., et al., *Progesterone up-regulates p27 through an increased binding of the progesterone receptor-A-p53 protein complex onto the non-canonical p53 binding motif in HUVEC.* J Steroid Biochem Mol Biol, 2019. **185**: p. 163-171.

110. Dubravka, D. and D.W. Scott, *Regulation of the G1 phase of the mammalian cell cycle.* Cell Research, 2000. **10**(1): p. 1-16.

111. Chu, I.M., L. Hengst, and J.M. Slingerland, *The Cdk inhibitor p27 in human cancer: prognostic potential and relevance to anticancer therapy.* Nature Reviews Cancer, 2008. **8**(4): p. 253-267.

112. Zhang, X.P., et al., *Cell fate decision mediated by p53 pulses.* Proc Natl Acad Sci U S A, 2009. **106**(30): p. 12245-50.

113. Tamura, R.E., et al., *GADD45 proteins: central players in tumorigenesis.* Curr Mol Med, 2012. **12**(5): p. 634-51.

114. Pennington, K.L., et al., *The dynamic and stress-adaptive signaling hub of 14-3-3: emerging mechanisms of regulation and context-dependent protein-protein interactions.* Oncogene, 2018. **37**(42): p. 5587-5604.

115. Bieging, K.T. and L.D. Attardi, *Deconstructing p53 transcriptional networks in tumor suppression.* Trends Cell Biol, 2012. **22**(2): p. 97-106.

116. Oda, E., et al., *Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis.* Science, 2000. **288**(5468): p. 1053-8.

117. Nakano, K. and K.H. Vousden, *PUMA, a novel proapoptotic gene, is induced by p53.* Mol Cell, 2001. **7**(3): p. 683-94.

118. O'Connor, L., et al., *Bim: a novel member of the Bcl-2 family that promotes apoptosis.* EMBO J, 1998. **17**(2): p. 384-95.

119. Thornborrow, E.C., et al., *A conserved intronic response element mediates direct p53-dependent transcriptional activation of both the human and murine bax genes.* Oncogene, 2002. **21**(7): p. 990-9.

120. Robles, A.I., et al., *APAF-1 is a transcriptional target of p53 in DNA damage-induced apoptosis.* Cancer Res, 2001. **61**(18): p. 6660-4.

121. Speidel, D., *Transcription-independent p53 apoptosis: an alternative route to death.* Trends Cell Biol, 2010. **20**(1): p. 14-24.

122. Vaseva, A.V., et al., *p53 opens the mitochondrial permeability transition pore to trigger necrosis.* Cell, 2012. **149**(7): p. 1536-48.

123. Jiang, L., et al., *Ferroptosis as a p53-mediated activity during tumour suppression.* Nature, 2015. **520**(7545): p. 57-62.

124. Haupt, Y., et al., *Mdm2 promotes the rapid degradation of p53.* Nature, 1997. **387**(6630): p. 296-9.

125. Rodriguez, M.S., et al., *Multiple C-terminal lysine residues target p53 for ubiquitin-proteasome-mediated degradation.* Mol Cell Biol, 2000. **20**(22): p. 8458-67.

126. Chan, W.M., et al., *Ubiquitination of p53 at multiple sites in the DNA-binding domain.* Mol Cancer Res, 2006. **4**(1): p. 15-25.

127. Michael, D. and M. Oren, *The p53-Mdm2 module and the ubiquitin system.* Semin Cancer Biol, 2003. **13**(1): p. 49-58.

128. Fang, S., et al., *Mdm2 is a RING finger-dependent ubiquitin protein ligase for itself and p53.* J Biol Chem, 2000. **275**(12): p. 8945-51.

129. Li, M., et al., *Mono- versus polyubiquitination: differential control of p53 fate by Mdm2.* Science, 2003. **302**(5652): p. 1972-5.

130. Linares, L.K., et al., *HdmX stimulates Hdm2-mediated ubiquitination and degradation of p53.* Proc Natl Acad Sci U S A, 2003. **100**(21): p. 12009-14.

131. Lowe, S.W. and C.J. Sherr, *Tumor suppression by Ink4a-Arf: progress and puzzles.* Curr Opin Genet Dev, 2003. **13**(1): p. 77-83.

132. Kruse, J.P. and W. Gu, *Modes of p53 regulation.* Cell, 2009. **137**(4): p. 609-22.

133. Sui, G., et al., *Yin Yang 1 is a negative regulator of p53.* Cell, 2004. **117**(7): p. 859-72.

134. Tang, J., et al., *Critical role for Daxx in regulating Mdm2.* Nat Cell Biol, 2006. **8**(8): p. 855-62.

135. Song, M.S., et al., *The tumour suppressor RASSF1A promotes MDM2 self-ubiquitination by disrupting the MDM2-DAXX-HAUSP complex.* EMBO J, 2008. **27**(13): p. 1863-74.

136. Dai, M.S., et al., *Ribosomal protein L23 activates p53 by inhibiting MDM2 function in response to ribosomal perturbation but not to translation inhibition.* Mol Cell Biol, 2004. **24**(17): p. 7654-68.

137. Zhang, Y., et al., *Ribosomal protein L11 negatively regulates oncoprotein MDM2 and mediates a p53-dependent ribosomal-stress checkpoint pathway.* Mol Cell Biol, 2003. **23**(23): p. 8902-12.

138. Meek, D.W., *The p53 response to DNA damage.* DNA Repair (Amst), 2004. **3**(8-9): p. 1049-56.

139. Meulmeester, E., et al., *ATM-mediated phosphorylations inhibit Mdmx/Mdm2 stabilization by HAUSP in favor of p53 activation.* Cell Cycle, 2005. **4**(9): p. 1166-70.

140. Gu, W. and R.G. Roeder, *Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain.* Cell, 1997. **90**(4): p. 595-606.

141. Wang, X., et al., *Inhibition of p53 degradation by Mdm2 acetylation.* FEBS Lett, 2004. **561**(1-3): p. 195-201.

142. Tang, Y., et al., *Acetylation is indispensable for p53 activation.* Cell, 2008. **133**(4): p. 612-26.

143. Brooks, C.L. and W. Gu, *The impact of acetylation and deacetylation on the p53 pathway.* Protein Cell, 2011. **2**(6): p. 456-62.

144. Vaziri, H., et al., *hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase.* Cell, 2001. **107**(2): p. 149-59.

145. Gostissa, M., et al., *Activation of p53 by conjugation to the ubiquitin-like protein SUMO-1.* EMBO J, 1999. **18**(22): p. 6462-71.

146. Rodriguez, M.S., et al., *SUMO-1 modification activates the transcriptional response of p53.* EMBO J, 1999. **18**(22): p. 6455-61.

147. Abida, W.M., et al., *FBXO11 promotes the Neddylation of p53 and inhibits its transcriptional activity.* J Biol Chem, 2007. **282**(3): p. 1797-804.

148. West, L.E. and O. Gozani, *Regulation of p53 function by lysine methylation.* Epigenomics, 2011. **3**(3): p. 361-9.

149. Chuikov, S., et al., *Regulation of p53 activity through lysine methylation.* Nature, 2004. **432**(7015): p. 353-60.

150. Shi, X., et al., *Modulation of p53 function by SET8-mediated methylation at lysine 382.* Mol Cell, 2007. **27**(4): p. 636-46.

151. Bullock, A.N. and A.R. Fersht, *Rescuing the function of mutant p53.* Nat Rev Cancer, 2001. **1**(1): p. 68-76.

152. Rivlin, N., et al., *Mutations in the p53 Tumor Suppressor Gene: Important Milestones at the Various Steps of Tumorigenesis.* Genes Cancer, 2011. **2**(4): p. 466-74.

153. Malkin, D., *Li-fraumeni syndrome.* Genes Cancer, 2011. **2**(4): p. 475-84.

154. Jacks, T., et al., *Tumor spectrum analysis in p53-mutant mice.* Curr Biol, 1994. **4**(1): p. 1-7.

155. Muller, P.A. and K.H. Vousden, *p53 mutations in cancer.* Nat Cell Biol, 2013. **15**(1): p. 2-8.

156. Bougeard, G., et al., *Molecular basis of the Li-Fraumeni syndrome: an update from the French LFS families.* J Med Genet, 2008. **45**(8): p. 535-8.

157. Stindt, M.H., et al., *Functional interplay between MDM2, p63/p73 and mutant p53.* Oncogene, 2015. **34**(33): p. 4300-10.

158. Will, K., et al., *Specific interaction of mutant p53 with regions of matrix attachment region DNA elements (MARs) with a high potential for base-unpairing.* Proc Natl Acad Sci U S A, 1998. **95**(23): p. 13681-6.

159. Pfister, N.T., et al., *Mutant p53 cooperates with the SWI/SNF chromatin remodeling complex to regulate VEGFR2 in breast cancer cells.* Genes Dev, 2015. **29**(12): p. 1298-315.

160. Zhang, C., et al., *Tumour-associated mutant p53 drives the Warburg effect.* Nat Commun, 2013. **4**: p. 2935.

161. Schwartzenberg-Bar-Yoseph, F., M. Armoni, and E. Karnieli, *The tumor suppressor p53 down-regulates glucose transporters GLUT1 and GLUT4 gene expression.* Cancer Res, 2004. **64**(7): p. 2627-33.

162. Zhang, C., et al., *Parkin, a p53 target gene, mediates the role of p53 in glucose metabolism and the Warburg effect.* Proc Natl Acad Sci U S A, 2011. **108**(39): p. 16259-64.

163. Kawauchi, K., et al., *p53 regulates glucose metabolism through an IKK-NF-kappaB pathway and inhibits cell transformation.* Nat Cell Biol, 2008. **10**(5): p. 611-8.

164. Matoba, S., et al., *p53 regulates mitochondrial respiration.* Science, 2006. **312**(5780): p. 1650-3.

165. Contractor, T. and C.R. Harris, *p53 negatively regulates transcription of the pyruvate dehydrogenase kinase Pdk2.* Cancer Res, 2012. **72**(2): p. 560-7.

166. Menendez, J.A. and R. Lupu, *Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis.* Nat Rev Cancer, 2007. **7**(10): p. 763-77.

167. Li, J.N., et al., *Pharmacological inhibition of fatty acid synthase activity produces both cytostatic and cytotoxic effects modulated by p53.* Cancer Res, 2001. **61**(4): p. 1493-9.

168. Funauchi, Y., et al., *Regulation of iron homeostasis by the p53-ISCU pathway.* Sci Rep, 2015. **5**: p. 16497.

169. Amelio, I., et al., *Serine and glycine metabolism in cancer.* Trends Biochem Sci, 2014. **39**(4): p. 191-8.

170. Mattaini, K.R., M.R. Sullivan, and M.G. Vander Heiden, *The importance of serine metabolism in cancer.* J Cell Biol, 2016. **214**(3): p. 249-57.

171. Holzer, K., et al., *Proteomic Analysis Reveals GMP Synthetase as p53 Repression Target in Liver Cancer.* Am J Pathol, 2017. **187**(2): p. 228-235.

172. Franklin, D.A., et al., *p53 coordinates DNA repair with nucleotide synthesis by suppressing PFKFB3 expression and promoting the pentose phosphate pathway.* Sci Rep, 2016. **6**: p. 38067.

173. Nakano, K., et al., *A ribonucleotide reductase gene is a transcriptional target of p53 and p73.* Oncogene, 2000. **19**(37): p. 4283-4289.

174. Dando, I., M. Cordani, and M. Donadelli, *Mutant p53 and mTOR/PKM2 regulation in cancer cells.* IUBMB Life, 2016. **68**(9): p. 722-6.

175. Freed-Pastor, W.A., et al., *Mutant p53 disrupts mammary tissue architecture via the mevalonate pathway.* Cell, 2012. **148**(1-2): p. 244-58.

176. Kollareddy, M., et al., *Regulation of nucleotide metabolism by mutant p53 contributes to its gain-of-function activities.* Nat Commun, 2015. **6**: p. 7389.

177. Reis, R.A.G., et al., *The dihydroorotate dehydrogenases: Past and present.* Arch Biochem Biophys, 2017. **632**: p. 175-191.

178. Fagan, R.L., et al., *Mechanism of flavin reduction in the class 1A dihydroorotate dehydrogenase from Lactococcus lactis.* Biochemistry, 2007. **46**(13): p. 4028-36.

179. Marcinkeviciene, J., et al., *A second dihydroorotate dehydrogenase (Type A) of the human pathogen Enterococcus faecalis: expression, purification, and steady-state kinetic mechanism.* Arch Biochem Biophys, 2000. **377**(1): p. 178-86.

180. Argyrou, A., M.W. Washabaugh, and C.M. Pickart, *Dihydroorotate dehydrogenase from Clostridium oroticum is a class 1B enzyme and utilizes a concerted mechanism of catalysis.* Biochemistry, 2000. **39**(34): p. 10373-84.

181. Nielsen, F.S., P.S. Andersen, and K.F. Jensen, *The B form of dihydroorotate dehydrogenase from Lactococcus lactis consists of two different subunits, encoded by the pyrDb and pyrK genes, and contains FMN, FAD, and [FeS] redox centers.* J Biol Chem, 1996. **271**(46): p. 29359-65.

182. Alves, C.N., J.R. Silva, and A.E. Roitberg, *Insights into the mechanism of oxidation of dihydroorotate to orotate catalysed by human class 2 dihydroorotate dehydrogenase: a QM/MM free energy study.* Phys Chem Chem Phys, 2015. **17**(27): p. 17790-6.

183. Chen, J.J. and M.E. Jones, *The cellular location of dihydroorotate dehydrogenase: relation to de novo biosynthesis of pyrimidines.* Arch Biochem Biophys, 1976. **176**(1): p. 82-90.

184. Liu, S., et al., *Structures of human dihydroorotate dehydrogenase in complex with antiproliferative agents.* Structure, 2000. **8**(1): p. 25-33.

185. Rawls, J., et al., *Requirements for the mitochondrial import and localization of dihydroorotate dehydrogenase.* Eur J Biochem, 2001. **267**(7): p. 2079-87.

186. Costeira-Paulo, J., et al., *Lipids Shape the Electron Acceptor-Binding Site of the Peripheral Membrane Protein Dihydroorotate Dehydrogenase.* Cell Chem Biol, 2018. **25**(3): p. 309-317 e4.

187. Walse, B., et al., *The structures of human dihydroorotate dehydrogenase with and without inhibitor reveal conformational flexibility in the inhibitor and substrate binding sites.* Biochemistry, 2008. **47**(34): p. 8929-36.

188. Fang, J., et al., *Dihydro-orotate dehydrogenase is physically associated with the respiratory complex and its loss leads to mitochondrial dysfunction.* Biosci Rep, 2013. **33**(2): p. e00021.

189. Christopherson, R.I., S.D. Lyons, and P.K. Wilson, *Inhibitors of de novo nucleotide biosynthesis as drugs.* Acc Chem Res, 2002. **35**(11): p. 961-71.

190. Munier-Lehmann, H., et al., *On dihydroorotate dehydrogenases and their inhibitors and uses.* J Med Chem, 2013. **56**(8): p. 3148-67.

191. Vyas, V.K. and M. Ghate, *Recent developments in the medicinal chemistry and therapeutic potential of dihydroorotate dehydrogenase (DHODH) inhibitors.* Mini Rev Med Chem, 2011. **11**(12): p. 1039-55.

192. Ladds, M., et al., *A DHODH inhibitor increases p53 synthesis and enhances tumor cell killing by p53 degradation blockage.* Nat Commun, 2018. **9**(1): p. 1107.

193. Sykes, D.B., et al., *Inhibition of Dihydroorotate Dehydrogenase Overcomes Differentiation Blockade in Acute Myeloid Leukemia.* Cell, 2016. **167**(1): p. 171-186.e15.

194. Kulkarni, O.P., et al., *4SC-101, a novel small molecule dihydroorotate dehydrogenase inhibitor, suppresses systemic lupus erythematosus in MRL-(Fas)lpr mice.* Am J Pathol, 2010. **176**(6): p. 2840-7.

195. Cheung, N.N., et al., *Broad-spectrum inhibition of common respiratory RNA viruses by a pyrimidine synthesis inhibitor with involvement of the host antiviral response.* J Gen Virol, 2017. **98**(5): p. 946-954.

196. Schattenkirchner, M., *The use of leflunomide in the treatment of rheumatoid arthritis: an experimental and clinical review.* Immunopharmacology, 2000. **47**(2-3): p. 291-8.

197. Haghikia, A. and R. Gold, *Multiple sclerosis: TOWER confirms the efficacy of oral teriflunomide in MS.* Nat Rev Neurol, 2014. **10**(4): p. 183-4.

198. Bartlett, R.R., et al., *Leflunomide (HWA 486), a novel immunomodulating compound for the treatment of autoimmune disorders and reactions leading to transplantation rejection.* Agents Actions, 1991. **32**(1-2): p. 10-21.

199. Popovic, S. and R.R. Bartlett, *Disease modifying activity of HWA 486 on the development of SLE in MRL/1-mice.* Agents Actions, 1986. **19**(5-6): p. 313-4.

200. Greene, S., et al., *Inhibition of dihydroorotate dehydrogenase by the immunosuppressive agent leflunomide.* Biochem Pharmacol, 1995. **50**(6): p. 861-7.

201. Cherwinski, H.M., et al., *The immunosuppressant leflunomide inhibits lymphocyte proliferation by inhibiting pyrimidine biosynthesis.* J Pharmacol Exp Ther, 1995. **275**(2): p. 1043-9.

202. Davis, J.P., et al., *The immunosuppressive metabolite of leflunomide is a potent inhibitor of human dihydroorotate dehydrogenase.* Biochemistry, 1996. **35**(4): p. 1270-3.

203. Pasternak, R.D., et al., *Disease modifying activity of HWA 486 in rat adjuvant-induced arthritis.* Agents Actions, 1987. **21**(3-4): p. 241-3.

204. Thoss, K., et al., *Immunomodulation of rat antigen-induced arthritis by leflunomide alone and in combination with cyclosporin A.* Inflamm Res, 1996. **45**(2): p. 103-7.

205. Glant, T.T., et al., *Immunomodulation of proteoglycan-induced progressive polyarthritis by leflunomide.* Immunopharmacology, 1992. **23**(2): p. 105-16.

206. Mladenovic, V., et al., *Safety and effectiveness of leflunomide in the treatment of patients with active rheumatoid arthritis. Results of a randomized, placebo-controlled, phase II study.* Arthritis Rheum, 1995. **38**(11): p. 1595-603.

207. Strand, V., et al., *Treatment of active rheumatoid arthritis with leflunomide compared with placebo and methotrexate. Leflunomide Rheumatoid Arthritis Investigators Group.* Arch Intern Med, 1999. **159**(21): p. 2542-50.

208. Smolen, J.S., et al., *Efficacy and safety of leflunomide compared with placebo and sulphasalazine in active rheumatoid arthritis: a double-blind, randomised, multicentre trial. European Leflunomide Study Group.* Lancet, 1999. **353**(9149): p. 259-66.

209. Alcorn, N., S. Saunders, and R. Madhok, *Benefit-risk assessment of leflunomide: an appraisal of leflunomide in rheumatoid arthritis 10 years after licensing.* Drug Saf, 2009. **32**(12): p. 1123-34.

210. Brent, R.L., *Teratogen update: reproductive risks of leflunomide (Arava); a pyrimidine synthesis inhibitor: counseling women taking leflunomide before or during pregnancy and men taking leflunomide who are contemplating fathering a child.* Teratology, 2001. **63**(2): p. 106-12.

211. Pfaller, B., et al., *A critical review of the reproductive safety of Leflunomide.* Clin Rheumatol, 2019.

212. O'Connor, P.W., et al., *A Phase II study of the safety and efficacy of teriflunomide in multiple sclerosis with relapses.* Neurology, 2006. **66**(6): p. 894-900.

213. Confavreux, C., et al., *Oral teriflunomide for patients with relapsing multiple sclerosis (TOWER): a randomised, double-blind, placebo-controlled, phase 3 trial.* Lancet Neurol, 2014. **13**(3): p. 247-56.

214. Bayas, A. and M. Maurer, *Teriflunomide for the treatment of relapsing-remitting multiple sclerosis: patient preference and adherence.* Patient Prefer Adherence, 2015. **9**: p. 265-74.

215. Boyd, A.S., *Leflunomide in dermatology.* J Am Acad Dermatol, 2012. **66**(4): p. 673-9.

216. Chon, W.J. and M.A. Josephson, *Leflunomide in renal transplantation.* Expert Rev Clin Immunol, 2011. **7**(3): p. 273-81.

217. Chacko, B. and G.T. John, *Leflunomide for cytomegalovirus: bench to bedside.* Transpl Infect Dis, 2012. **14**(2): p. 111-20.

218. Chon, W.J., et al., *Use of leflunomide in renal transplant recipients with ganciclovir-resistant/refractory cytomegalovirus infection: a case series from the University of Chicago.* Case Rep Nephrol Dial, 2015. **5**(1): p. 96-105.

219. Knight, D.A., et al., *Inhibition of herpes simplex virus type 1 by the experimental immunosuppressive agent leflunomide.* Transplantation, 2001. **71**(1): p. 170-4.

220. Roger, M.R. and G.M. Anstead, *Leflunomide in the Treatment of a Pseudotumoral Genital Herpes Simplex Virus Infection in an HIV Patient.* Case Rep Infect Dis, 2017. **2017**: p. 1589356.

221. Henao-Martinez, A.F., et al., *Successful treatment of acyclovir-resistant herpes simplex virus type 2 proctitis with leflunomide in an HIV-infected man.* J Clin Virol, 2012. **54**(3): p. 276-8.

222. Durmus, O., et al., *Hepatitis B virus reactivation in a juvenile rheumatoid arthritis patient under treatment and its successful management: a complicated case.* Rheumatol Int, 2013. **33**(5): p. 1345-9.

223. Zhu, S., et al., *Leflunomide reduces proliferation and induces apoptosis in neuroblastoma cells in vitro and in vivo.* PLoS One, 2013. **8**(8): p. e71555.

224. Xu, X., et al., *Inhibition of protein tyrosine phosphorylation in T cells by a novel immunosuppressive agent, leflunomide.* J Biol Chem, 1995. **270**(21): p. 12398-403.

225. Siemasko, K., et al., *Inhibition of JAK3 and STAT6 tyrosine phosphorylation by the immunosuppressive drug leflunomide leads to a block in IgG1 production.* J Immunol, 1998. **160**(4): p. 1581-8.

226. White, R.M., et al., *DHODH modulates transcriptional elongation in the neural crest and melanoma.* Nature, 2011. **471**(7339): p. 518-22.

227. O'Donnell, E.F., et al., *The aryl hydrocarbon receptor mediates leflunomide-induced growth inhibition of melanoma cells.* PLoS One, 2012. **7**(7): p. e40926.

228. Alhefdhi, A., et al., *Leflunomide suppresses growth in human medullary thyroid cancer cells.* J Surg Res, 2013. **185**(1): p. 212-6.

229. Dexter, D.L., et al., *Activity of a novel 4-quinolinecarboxylic acid, NSC 368390 [6-fluoro-2-(2'-fluoro-1,1'-biphenyl-4-yl)-3-methyl-4-quinolinecarb oxylic acid sodium salt], against experimental tumors.* Cancer Res, 1985. **45**(11 Pt 1): p. 5563-8.

230. Peters, G.J., et al., *Inhibition of pyrimidine de novo synthesis by DUP-785 (NSC 368390).* Invest New Drugs, 1987. **5**(3): p. 235-44.

231. Arteaga, C.L., et al., *Phase I clinical and pharmacokinetic trial of Brequinar sodium (DuP 785; NSC 368390).* Cancer Res, 1989. **49**(16): p. 4648-53.

232. Noe, D.A., et al., *Phase I and pharmacokinetic study of brequinar sodium (NSC 368390).* Cancer Res, 1990. **50**(15): p. 4595-9.

233. de Forni, M., et al., *Phase I and pharmacokinetic study of brequinar (DUP 785; NSC 368390) in cancer patients.* Eur J Cancer, 1993. **29A**(7): p. 983-8.

234. Dodion, P.F., et al., *Phase II trial with Brequinar (DUP-785, NSC 368390) in patients with metastatic colorectal cancer: a study of the Early Clinical Trials Group of the EORTC.* Ann Oncol, 1990. **1**(1): p. 79-80.

235. Natale, R., et al., *Multicenter phase II trial of brequinar sodium in patients with advanced melanoma.* Ann Oncol, 1992. **3**(8): p. 659-60.

236. Urba, S., et al., *Multicenter phase II trial of brequinar sodium in patients with advanced squamous-cell carcinoma of the head and neck.* Cancer Chemother Pharmacol, 1992. **31**(2): p. 167-9.

237. Cody, R., et al., *Multicenter phase II study of brequinar sodium in patients with advanced breast cancer.* Am J Clin Oncol, 1993. **16**(6): p. 526-8.

238. Moore, M., et al., *Multicenter phase II study of brequinar sodium in patients with advanced gastrointestinal cancer.* Invest New Drugs, 1993. **11**(1): p. 61-5.

239. Maroun, J., et al., *Multicenter phase II study of brequinar sodium in patients with advanced lung cancer.* Cancer Chemother Pharmacol, 1993. **32**(1): p. 64-6.

240. He, T., et al., *Inhibition of the mitochondrial pyrimidine biosynthesis enzyme dihydroorotate dehydrogenase by doxorubicin and brequinar sensitizes cancer cells to TRAIL-induced apoptosis.* Oncogene, 2014. **33**(27): p. 3538-49.

241. Dorasamy, M.S., et al., *Dihydroorotate dehydrogenase Inhibitors Target c-Myc and Arrest Melanoma, Myeloma and Lymphoma cells at S-phase.* J Cancer, 2017. **8**(15): p. 3086-3098.

242. Xu, X., et al., *In vitro and in vivo mechanisms of action of the antiproliferative and immunosuppressive agent, brequinar sodium.* J Immunol, 1998. **160**(2): p. 846-53.

243. Wachsman, M., et al., *Antiviral activity of inhibitors of pyrimidine de-novo biosynthesis.* Antiviral Chemistry & Chemotherapy, 1996. **7**(1): p. 7-13.

244. Zhang, L., et al., *Inhibition of pyrimidine synthesis reverses viral virulence factor-mediated block of mRNA nuclear export.* Journal of Cell Biology, 2012. **196**(3): p. 315-326.

245. Christian, S., et al., *The novel dihydroorotate dehydrogenase (DHODH) inhibitor BAY 2402234 triggers differentiation and is effective in the treatment of myeloid malignancies.* Leukemia, 2019. **33**(10): p. 2403-2415.

246. Zhou, J., et al., *ASLAN003, a potent dihydroorotate dehydrogenase inhibitor for differentiation of acute myeloid leukemia.* Haematologica, 2019.

247. Ladds, M. and S. Lain, *Small molecule activators of the p53 response.* J Mol Cell Biol, 2019. **11**(3): p. 245-254.

248. Baell, J. and M.A. Walters, *Chemical con artists foil drug discovery.* Nature, 2014. **513**(7519): p. 481-483.

249. Vassilev, L.T., et al., *In vivo activation of the p53 pathway by small-molecule antagonists of MDM2.* Science, 2004. **303**(5659): p. 844-8.

250. Fairus, A.K.M., et al., *Dihydroorotate dehydrogenase (DHODH) inhibitors affect ATP depletion, endogenous ROS and mediate S-phase arrest in breast cancer cells.* Biochimie, 2017. **135**: p. 154-163.

251. Fischer, M., et al., *The p53-p21-DREAM-CDE/CHR pathway regulates G2/M cell cycle genes.* Nucleic Acids Res, 2016. **44**(1): p. 164-74.

252. Carpenter, E.P., et al., *Overcoming the challenges of membrane protein crystallography.* Curr Opin Struct Biol, 2008. **18**(5): p. 581-6.

253. Konijnenberg, A., et al., *Extending native mass spectrometry approaches to integral membrane proteins.* Biol Chem, 2015. **396**(9-10): p. 991-1002.

254. Li, Z.L. and M. Buck, *Computational Modeling Reveals that Signaling Lipids Modulate the Orientation of K-Ras4A at the Membrane Reflecting Protein Topology.* Structure, 2017. **25**(4): p. 679-689 e2.

255. Zhu, J., K.R. Vinothkumar, and J. Hirst, *Structure of mammalian respiratory complex I.* Nature, 2016. **536**(7616): p. 354-358.

256. Rogakou, E.P., et al., *DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139.* J Biol Chem, 1998. **273**(10): p. 5858-68.

257. Paull, T.T., et al., *A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage.* Curr Biol, 2000. **10**(15): p. 886-95.

258. Rogakou, E.P., et al., *Initiation of DNA fragmentation during apoptosis induces phosphorylation of H2AX histone at serine 139.* J Biol Chem, 2000. **275**(13): p. 9390-5.

259. Solier, S. and Y. Pommier, *The apoptotic ring: a novel entity with phosphorylated histones H2AX and H2B and activated DNA damage response kinases.* Cell Cycle, 2009. **8**(12): p. 1853-9.

260. Arnould, S., et al., *Checkpoint kinase 1 inhibition sensitises transformed cells to dihydroorotate dehydrogenase inhibition.* Oncotarget, 2017. **8**(56): p. 95206-95222.

261. Sarin, A., M.L. Wu, and P.A. Henkart, *Different interleukin-1 beta converting enzyme (ICE) family protease requirements for the apoptotic death of T lymphocytes triggered by diverse stimuli.* J Exp Med, 1996. **184**(6): p. 2445-50.

262. Jagiello, I., et al., *Subunit structure and regulation of protein phosphatase-1 in rat liver nuclei.* J Biol Chem, 1995. **270**(29): p. 17257-63.

263. Kreivi, J.P., et al., *Purification and characterisation of p99, a nuclear modulator of protein phosphatase 1 activity.* Febs Letters, 1997. **420**(1): p. 57-62.

264. De Leon, G., T.C. Sherry, and N.A. Krucher, *Reduced expression of PNUTS leads to activation of Rb-phosphatase and caspase-mediated apoptosis.* Cancer Biol Ther, 2008. **7**(6): p. 833-41.

265. Lee, S.J., et al., *Protein phosphatase 1 nuclear targeting subunit is a hypoxia inducible gene: its role in post-translational modification of p53 and MDM2.* Cell Death Differ, 2007. **14**(6): p. 1106-16.

266. Dingar, D., et al., *MYC dephosphorylation by the PP1/PNUTS phosphatase complex regulates chromatin binding and protein stability.* Nat Commun, 2018. **9**(1): p. 3502.

267. Choy, M.S., et al., *Understanding the antagonism of retinoblastoma protein dephosphorylation by PNUTS provides insights into the PP1 regulatory code.* Proc Natl Acad Sci U S A, 2014. **111**(11): p. 4097-102.

268. Cortazar, M.A., et al., *Control of RNA Pol II Speed by PNUTS-PP1 and Spt5 Dephosphorylation Facilitates Termination by a "Sitting Duck Torpedo" Mechanism.* Mol Cell, 2019. **76**(6): p. 896-908 e4.

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