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MECHANISMS OF CANCER CELL DEATH BY MUTANT p53-REACTIVATING COMPOUND APR-246

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Cover illustration: *p53 is known as the "Guardian of the Genome" because it protects our DNA from damage and thereby prevents cancer formation. p53 mutation disrupts this protective capacity as illustrated by the broken shield. The plaster represents mutant p53-reactivating compound APR-246 that restores normal p53 function. Cancer cells have elevated antioxidants defense systems (pictured as jail bars) to capture oxidants. APR-246 also binds antioxidants, leading to increased oxidative stress contributing to cancer cell death.*
See http://www.ceder.graphics/

Mechanisms of Cancer Cell Death by Mutant p53-Reactivating Compound APR-246

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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This thesis and all my time, work and effort
spent in cancer research is dedicated to my family.

To my father, who fought so bravely and strongly,
but yet lost his battle against cancer.

To my mother and my two brothers,
for their infinite support, care and love.

POPULAR SCIENCE SUMMARY OF THE THESIS

We have approximately 22,000 genes in our genome. A gene functions as a template for production of a specific protein, for example an enzyme or a cell surface receptor. In principle, one gene encodes one protein, but in many cases several versions of a protein are produced by one specific gene, depending on how the genetic information is decoded and how the protein is modified after its production. All cells in our body are descendants of a fertilized egg and thus they all carry the same set of genes. However, different sets of genes are active in different cell types, endowing cells with their unique properties and functions. Different cell types form the various complex organ systems in our body (Weinberg, 2007).

All our cells have quality checks and are rigorously monitored in order for cells only to produce their assigned proteins. When a cell divides, its genome will be copied to the next daughter cells. Mistakes in this process will be identified through constant quality checks and repaired. However, if a mistake cannot be repaired, the cell may undergo a program called "apoptosis" which is a controlled form of cell suicide or cell death. This will eliminate cells with potentially dangerous mutations that could otherwise give rise to cancer. Thus, failure to initiate apoptosis to eliminate such cells can lead to tumor formation (Weinberg, 2007).

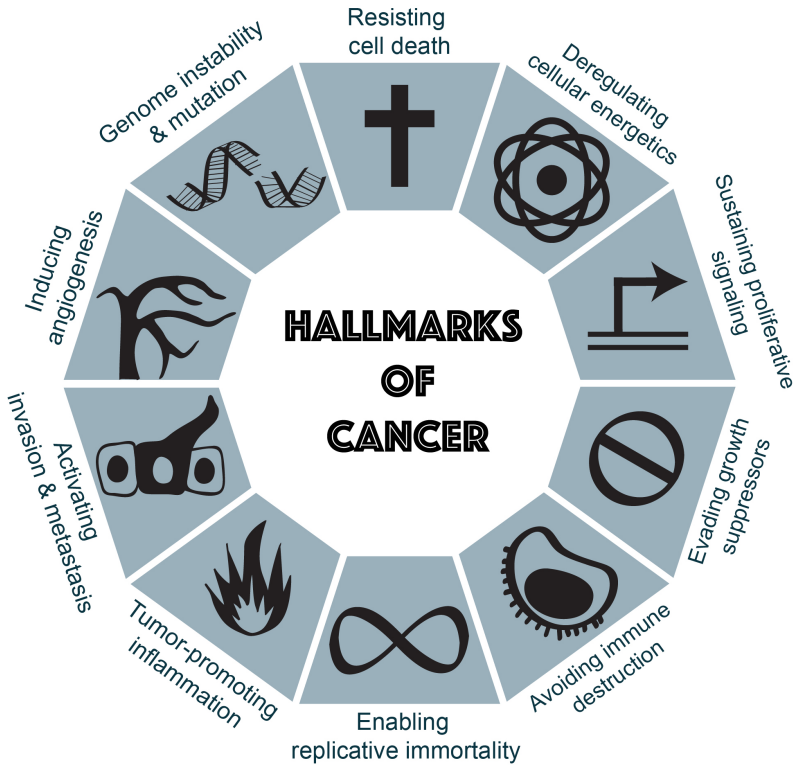

Figure 1 Classical "Hallmarks of Cancer" as proposed by Hanahan & Weinberg that enable tumor growth and spread. "Tumor-promoting inflammation" and "Genomic instability & mutation" are enabling characteristics that give cancer the tools to acquire the hallmarks. Figure is modified from Hanahan & Weinberg, 2011.

Cancer can start in more or less any cell type and organ in the body. Therefore, cancer is not one disease, but a collective name for more than 200 diseases depending on which organ and cell type the development of cancer initiates (Cancerfonden, 2018).

There are several important differences between normal cells and cancer cells. These are the so called "Hallmarks of cancer" (Hanahan & Weinberg, 2000, 2011) (Figure 1). One hallmark of cancer is the ability to avoid cell death or apoptosis. A protein called p53 has a key role in this process. p53 has been dubbed "Guardian of the genome" as this protein will be activated when there is damage to the genome. p53 can also be activated by radiation or different types of drugs. Activation of p53 triggers cell death by apoptosis to eliminate incipient cancer cells. p53 is therefore an important protector against cancer, a so called tumor suppressor. In fact, in around half of all cancers the *TP53* gene that codes for p53 is mutated and the normal (wild type) function of p53 is lost (Mello & Attardi, 2018; Soussi & Wiman, 2007), allowing cancer cells to avoid cell death by apoptosis and growing beyond control (Figure 2).

Some *TP53* mutations can give the p53 protein new functions which can actually stimulate cancer development (Brosh & Rotter, 2009; Mantovani *et al*, 2019). The compound that is the focus of this thesis, APR-246, can target mutant p53 and restore, or in other words "reactivate", its normal function to trigger cancer cell suicide by apoptosis (Bykov *et al*, 2002b). APR-246 is being tested in clinical trials (phase III) on patients with mutant *TP53* myelodysplastic syndrome (MDS), a type of blood cancer. APR-246 is the clinically most advanced compound to target mutant p53 (Bykov *et al*, 2018) (Figure 2).

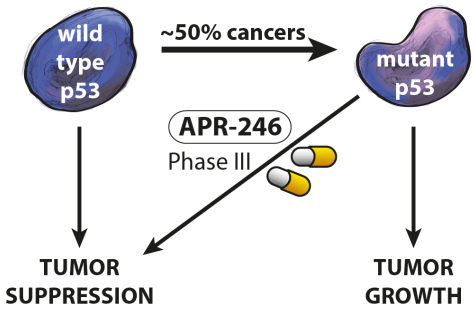

Figure 2 Mutant p53 reactivating compound APR-246 (Eprenetapopt). The tumor suppressor p53 is mutated in almost half of all cancers. Mutation of p53 by substitution of one amino acid (protein building blocks) leads to inactivation of its normal function resulting in new tumor promoting activities. APR-246 is currently undergoing Phase III clinical trials, based on the hypothesis that it may reactivate p53, thereby resulting in tumor suppression. Parts of figure is from the review Eriksson, Ceder *et al* 2019.

APR-246 is a prodrug which means that APR-246 itself is inactive. APR-246 is spontaneously converted to its active product called MQ (Lambert *et al.*, 2009) – thus MQ does all the action in the cell. In **Paper I**, we discovered that MQ is pumped out of cells by a protein called MRP1. We used an MRP1 inhibitor to block this pump which resulted in more MQ staying inside cancer cells. The additional treatment with the MRP1 inhibitor greatly increased the efficacy of APR-246 in cultured cancer cells. The combination treatment was highly effective in suppressing tumor growth in mice and it almost doubled their survival time. In **Paper II**, we

identified that MQ binds specific cysteines (i.e. a type of protein building block) in the p53 protein and that this binding differs between normal p53 and mutant p53.

In **Paper III**, we discovered that APR-246 may be an efficient treatment for patients with acute lymphoblastic leukemia (ALL), another type of blood cancer. ALL patients are usually children and with today's treatment 90% of childhood ALL patients will survive (Barncancerfonden, 2017a). However, sometimes ALL patients relapse and become resistant to treatment. These relapsed ALL patients often have mutated p53 but may also produce a high amount of a protein called ASNS (Hof *et al*, 2011; Lanvers-Kaminsky, 2017). ASNS makes ALL insensitive to one of the standard treatments used in the clinics called asparaginase (Aslanian *et al*, 2001). We discovered that APR-246 may target the function of ASNS, thereby increasing asparaginase efficiency and killing of ALL cancer cells.

Most mutations in the *TP53* gene are acquired, i.e. they are not present at birth and occur before or during cancer development – so called somatic mutations. However, sometimes *TP53* mutation is inherited from a parent, meaning that the mutation is present in all cells of the body. This is a so called germline *TP53* mutation. Such mutations occur in Li-Fraumeni Syndrome (LFS), where individuals develop cancer, even multiple cancers, with very high incidence during their life time (Malkin, 2011). Many of these develop tumors already during childhood or adolescence. Families with hereditary breast cancer may also have germline *TP53* mutations (without being classified as LFS) (Evans *et al*, 2020). However, not all family members develop cancer and therefore it is important to understand which types of germline *TP53* mutations increase the risk for cancer and which do not. In **Paper IV**, we studied ten newly identified germline *TP53* mutations found in Swedish families with LFS or hereditary breast cancer. We evaluated if the different *TP53* gene mutations produce a p53 protein that has lost its normal tumor suppressive function. This is important information so that families with these mutations may know if they have an increased risk of developing cancer. If so, they can undergo preventive measures in order to decrease their cancer risk or detect cancer early.

The first three projects are aimed at improving our understanding of mutant p53-reactivating compound APR-246. They suggest approaches for increasing treatment efficacy and novel combination strategies. The thesis has also addressed the role of mutant p53 in response to APR-246 and pathological properties in families with LFS or hereditary breast cancer. All in all, these studies provide novel preclinical understanding of the role of mutant p53 in cancer and response to treatment, both highly relevant in the combat against cancer.

ABSTRACT

Tumor suppressor *TP53* is the most frequently mutated gene in cancer. A majority of *TP53* mutations result in a mutant p53 that disrupts its DNA binding capabilities but may also acquire novel gain-of-function activities that contribute to tumor growth. The investigational drug APR-246 (Eprenetapopt) is the most clinically advanced compound to target mutant p53 and is being tested in a phase III clinical trial in mutant *TP53* myelodysplastic syndrome (MDS). APR-246 is converted to its active product methylene quinuclidinone (MQ). MQ binds to cysteines in p53 promoting a folded structure and DNA binding, leading to cancer cell death. MQ also targets thiols or selenols in e.g. glutathione (GSH) or various enzymes. Depletion of glutathione and inhibition of antioxidant enzymes increase oxidative stress contributing to APR-246-induced cancer cell death.

In **Project I**, combination treatment of APR-246 and multidrug resistance protein 1 (MRP1) inhibitor resulted in synergistic growth suppression *in vitro* in tumor cell lines, *in vivo* in esophageal cancer xenografts, and *ex vivo* in esophageal and colorectal cancer patient-derived organoids (PDO). We show that inhibition of MRP1 results in increased intracellular ^{14}C-content after ^{14}C-APR-246 treatment. This was attributed to retention of GSH-conjugated MQ (GS-MQ). We demonstrate that GS-MQ binding is reversible and that retention of GS-MQ creates an intracellular MQ pool that may target numerous thiols contributing to APR-246-induced growth suppression. In **Project II** we studied the spectrum of MQ-targeted cysteines in p53. This was enabled by first establishing a method utilizing the reducing agent $NaBH_4$ to lock the MQ cysteine adducts into a stable form, overcoming reversibility. Cys182, Cys229 and Cys277 in the p53 core domain showed most prominent MQ modification. Additional modification at Cys124 and Cys141 was found in mutant p53. The electrophilic properties of MQ enables targeting of multiple cellular thiols. In **Project III** we identified novel MQ targets using CEllular Thermal Shift Assay (CETSA). Asparaginase synthetase (ASNS) was stabilized upon MQ treatment and thus is a potential MQ target. In acute lymphoblastic leukemia (ALL), ASNS is associated with resistance to standard treatment asparaginase. Asparaginase depletes extracellular asparagine which renders asparagine-auxotrophic ALL cells sensitive and therefore ASNS expression allows ALL cell survival. We found that combination treatment of APR-246 and asparaginase leads to synergistic growth suppression in ALL cells and may offer a novel treatment strategy for ALL. Lastly, in **Project IV** we assessed the functional activity of novel germline *TP53* mutations identified in a Swedish cohort of families with Li-Fraumeni syndrome (LFS) or hereditary breast cancer (HrBC). Assessing the pathological outcome of *TP53* mutations is important for understanding the cancer risk of these families.

The first three projects are aimed at improving our understanding of mutant p53-reactivating compound APR-246. They suggest approaches for increasing treatment efficacy and novel combination strategies. The thesis has also addressed the role of mutant p53 in response to APR-246 and pathological properties in families with LFS or HrBC. All in all, these studies provide novel preclinical understanding of the role of mutant p53 in cancer and response to treatment, both highly relevant in the combat against cancer.

LIST OF SCIENTIFIC PAPERS

I. **Ceder, S.**, Eriksson, S. E., Cheteh, E. H., Dawar, S., Benitez, M. C., Bykov, V. J. N., Fujihara, K. M., Grandin, M., Li, X., Ramm, S., Behrenbruch, C., Simpson, K. J., Hollande, F., Abrahmsen, L., Clemons, N. J. and Wiman, K. G.
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EMBO Mol Med 2020: e10852

II. **Ceder, S.**, Bykov, V. J. N., Hagberg, L., Mermelekas, G., Jafari, R., Abrahmsen, L. and Wiman, K. G.
"Spectrum of p53 cysteines targeted by APR-246 active product MQ"
Manuscript

III. **Ceder, S.**, Eriksson, S. E., Yu, L. Y., Cheteh, E.H., Zhang, S. M., Fujihara, K. M., Bianchi, J., Bykov, V. J. N., Abrahmsen, L., Clemons, N. J., Nordlund, P., Rudd, S. and Wiman, K. G.
"Mutant p53-reactivating compound APR-246 synergizes with asparaginase in inducing growth suppression in acute lymphoblastic leukemia cells"
Manuscript

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CONTENTS

LIST OF ABBREVIATIONS

Abbreviation	Explaination
13q14	Chromosome 13, long (q) arm, region 1, band 4
^{14}C-APR-246	Carbon-14 (radioactive isotope)-labelled APR-246
17p13	Chromosome 17, short (p) arm, region 1, band 3
3BA	3-benzoylacrylic acid
5-FU	Fluorouracil
A	Adenine (nucleobase)
A or Ala	Alanine
Akt	Protein kinase B
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
APC	Adenomatous polyposis coli
ARE	Antioxidant response elements
ARF	Alternative Reading Frame
ARF-BP1	ARF binding protein 1
As	Arsenic
ASNS	Asparagine synthethase
ATM	Ataxia telangiectasia mutated
ATO	Arsenic trioxide
ATP	Adenosine triphosphate
ATR	ATM–Rad3-related protein
BCL-2	B-cell lymphoma 2
BCR	B cell receptor
BCR-ABL1	Fusion gene of BCR and ABL1 (Philadelphia chromosome)
BH3	BCL-2 Homology (3 BH domains)
BRCA1	Breast cancer type 1 susceptibility protein
BSO	Buthionine sulfoximide
BTK	Bruton's tyrosine kinase
C	Cytosine (nucleobase)
C or Cys	Cysteine
c-Met	Mesenchymal epithelial transition
CAF	Cancer-associated fibroblast
CAR-T	Chimeric antigen receptor T
Cas9	CRISPR associated protein 9
CBP	CREB-binding protein
CDK	Cyclin-dependent kinases
CETSA	CEllular Thermal Shift Assay
CHK1	Checkpoint kinase 1
CLL	Chronic lymphocytic leukemia
CML	Chronic myelomonocytic leukemia
COP1	Constitutively photomorphogenic 1
CRISPR	Clustered regularly interspaced short palindromic repeats
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4

Cys	Reduced cysteine
CySS	Oxidized cysteine (Cys-Cys)
D or Asp	Asparatic acid
DNA	Deoxyribonucleic acid
DUB	Deubiquitinating enzymes
E or Glu	Glutamic acid
e^-	Electron
EGFR	Epidermal growth factor receptor
EMA	European Medicines Agency
EMT	Epithelial-mesenchymal-transition
EPOR	Erythropoietin-receptor
FDA	U.S. Food and Drug Administration
FDXR	Ferredoxin reductase
G	Guanine (nucleobase)
G or Gly	Glycine
G6PD	Glucose-6-phosphate dehydrogenase
GADD45	Growth arrest and DNA damage-inducible protein
GCL	Glutamate cysteine ligase
Glu	Glutamate/Glutamic acid
GOF	Gain-of-function
GPI	Glucose phosphate isomerase
GR	Glutathione reductase
Grx	Glutaredoxin
GS-MQ	Glutathione-conjugated MQ
GSH	Reduced glutathione
GSSG	Oxidized glutathione
H or His	Histidine
HAT	Histone acetyl transferases
HAUSP	Herpesvirus-associated ubiquitin-specific protease
HDAC	Histone deacetylase
HER2	Human epidermal growth factor receptor-2
HGSOC	High Grade Serous Ovarian Cancer
HIF1	Hypoxia-inducible factor 1
HIPK2	Homeodomain interacting protein kinase 2
HK III	Hexokinase 3
HO-1	Heme oxygenase 1
iASPP	Inhibitory member of the ASPP family
IDH	Isocitrate dehydrogenase [NADP]
K or Lys	Lysine
KEAP1	Kelch-ECH-associated protein 1
KRAS	GTPase KRas
LFS	Li-Fraumeni syndrome
LMW	Low molecular weight
LOF	Loss of heterozygosity
LOH	Loss-of-function

MAPK	Mitogen activate protein kinase
MCL	Mantle cell lymphoma
MDM2	Mouse double minute 2
MDS	Myelodysplastic syndrome
MEK	Mitogen-activated extracellular signal-regulated kinase
MHC I	Major histocompatibility complex class 1
miR-15a	MicroRNA-15a
MQ	Methylene quinuclidinone
Mre11	Meiotic recombination 11
MRP1	Multidrug resistance protein 1
MS-CETSA	Mass spectrometry-based CETSA
mTOR	Mechanistic target of rapamycin
mut p53	Mutated p53
N or Asn	Asparagine
$NaBH_4$	Sodium borohydride
NADPH	Nicotinamide adenine dinucleotide phosphate
NK cells	Natural killer cells
NCI	US National Cancer Institute
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHL	Non Hodgkin Lymphoma
NIH	National Cancer Institute
NoLS	Nucleolar localization signals
NQO1	NAD(P)H:quinone oxireductase
NRF2	Nuclear factor erythroid 2-related factor 2
NSCLC	Non small cell lung cancer
ox	Oxidation (loss of electrons by an atom)
p53	Protein encoded by the gene *TP53*
p53AIP1	p53-regulated apoptosis-inducing protein 1
PARP	Poly (ADP-ribose) polymerase
PCAF	p300/CBP-associated factor
PD-1	Programmed cell death protein 1
PD-L1	Programmed death-ligand 1
PDO	Patient-derived organoid
PI3K	Phosphatidylinositol 3-kinase
PI3KCA	PI3K catalytic subunit alpha isoform
PIG3	p53 inducible gene 3
Pin1	Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1
PLD	Pegylated Liposomal Doxorubicin Hydrochloride
PML-RARα	Promyelocytic leukemia/retinoic acid receptor alpha
PPP	Pentose phosphate pathway
PRIMA-1	p53 reactivation and induction of massive apoptosis
PRIMA-1Met	Methylated PRIMA-1
PSMA2	Proteasome subunit alpha type-2
PSMC1	Proteasome 26S ATPase Subunit 1
PTEN	Phosphatase and tensin homologue deleted on chromosome 10

Pu	Purine
Py	Pyrimidine
Q or Gln	Glutamine
R or Arg	Arginine
R-S^- or R-SH	Thiol group (contains sulfur)
R-Se^- or R-SeH	Selenol group (contains selenium)
RB1	Retinoblastoma-associated protein
RE	Response elements
red	Reduction (gain of electrons by an atom)
Redox	Reduction and oxidation reactions
S o Ser	Serine
SAH-p53	Stabilized alpha-helix of p53
SCO2	Synthesis of cytochrome C oxidase 2
Se	Selenium
SESN1	Sestrin 1
siRNA	Small interfering RNA
SLC7A11	Solute carrier family 7 member 11
T	Thymine (nucleobase)
T or Thr	Threonine
TCGA	The Cancer Genome Atlas Program
TCR	T cell receptor
TERT	Telomerase reverse transcriptase
TGF-β	Transforming growth factor beta
TNFR1	Tumor necrosis factor receptor 1
TNFα	Tumor necrosis factor alpha
TP53	Gene encoding p53 protein
TP53INP1	Tumor protein p53-inducible nuclear protein 1
TRP14	Thioredoxin-related protein 14
Trx	Thioredoxin
TrxR1	Thioredoxin reductase 1
U or Sec	Selenocysteine
ULBP1	UL16-binding protein 1
V or Val	Valine
VEGF	Vascular endothelial growth factor
W or Trp	Tryptophan
WB-CETSA	Western blot-based CETSA
wt p53	Wild type p53
Y or Tyr	Tyrosine
YY1	Ying yang 1
ZMC-1	Zinc metallochaperone-1
Zn^{2+}	Zinc ion

1 INTRODUCTION

1.1 CANCER

It is estimated that at least every third person in Sweden will be diagnosed with cancer during her/his life time. In other words, cancer is common and everyone is affected in one way or another. Cancer could be seen as 200 different diseases, depending on which organ and cell type the tumor has arisen from (Cancerfonden, 2018). Cancer cells are characterized by certain traits or so called "Hallmarks of Cancer" as proposed by Hanahan and Weinberg in two classical review articles (Hanahan & Weinberg, 2000, 2011). The Hallmarks of Cancer consists of capabilities acquired during the multiple steps of cancer development from initiation to metastatic dissemination (Figure 1 and a modified version in Figure 3). Another important feature of cancer and important to therapy response is redox imbalance which is described in

Figure 3 Targeting Hallmarks of Cancer. The classical "Hallmarks of Cancer" in blue as proposed by Hanahan & Weinberg and a potential addition in green relevant for this thesis. In red is an updated version of mechanisms for therapeutic targeting of these hallmarks as was initially suggested by Hanahan & Weinberg. Figure is modified from Hanahan & Weinberg 2011.

section 1.6 and has been added as another Hallmark of Cancer in Figure 3. These hallmarks illustrate the complexity of the disease, the many ways therapy resistance can occur as well as the uniqueness of each tumor and patient. On top of that, a tumor does not only consist of tumor cells but is a highly complicated landscape with many other components such as immune cells, fibroblasts, endothelial cells, pericytes as well as the extracellular matrix which all may play a role in tumor initiation, growth and progression (Dunn *et al*, 2004; Pietras & Ostman, 2010).

One enabling characteristic that give cancer cells the tools to develop these capabilities is genomic instability that leads to random mutations (Hanahan & Weinberg, 2011). Mutations that inactivate tumor suppressors (genes that prevent growth or tumor formation) or activates oncogenes (genes that drive tumor formation) are selected in a Darwinian process that continues throughout the development of a tumor. Thus, cancer is also a highly dynamic disease and tumor heterogeneity increases with time (Dagogo-Jack & Shaw, 2018). Different cancer types carry different mutations that drive tumor formation and each patient will have their unique fingerprint of mutations. Several novel highly precise treatments that target specific pathways aberrant in a patient's tumor, many of which are described in the Hallmarks of Cancer, have been developed in recent years (red text in Figure 3). The future of cancer therapy moves towards a highly personalized and complex treatment strategy which will be determined based on an individual patient's genomic fingerprint containing mutations but importantly also its functional consequence (Letai, 2017).

1.2 TARGETING HALLMARKS OF CANCER

1.2.1 Resisting cell death

Cancer is an evolutionary process driven by mutations and leading to uncontrolled cell cycle progression. Fortunately, if proliferation becomes aberrant, cells have innate tumor-suppressive mechanisms that will trigger a highly regulated form of programmed cell death called apoptosis (Lowe *et al*, 2004). Thus, effective tumor suppression requires a highly controlled system with various abnormality sensors set in place to trigger apoptosis at the right moment in order to prevent uncontrolled proliferation and cancer development (Hanahan & Weinberg, 2011; Junttila & Evan, 2009). One example is the DNA damage response pathway, which upon DNA damage results in kinases (e.g. ATM, ATR, Chk1 and Chk2) that phosphorylate transcription factor p53. This leads to p53 stabilization which can unleash the highly regulated process of apoptosis (Junttila & Evan, 2009). There are several other sensors or signals that may trigger p53 stabilization (discussed in section1.3.1) which activate a cascade of chain reactions with various outcomes, including apoptosis, depending on the initial signal. p53 is considered the "Guardian of the Genome" as these outcomes ultimately act to preserve genome integrity and may for example trigger a DNA repair program. However, upon severe stress or oncogenic signals, p53 may be activated to induce apoptosis, and thus it is not unexpected that the *TP53* gene is mutated in a large fraction of human tumors (see Table 1). In other words, there is a strong selection against a functional p53 pathway during tumor development (Junttila & Evan, 2009) (discussed in section 1.4). Thus, p53 plays a major role

in the hallmark “Resisting cell death” (Figure 3) and targeting of mutated p53 is an interesting strategy for novel cancer therapy (discussed in section 1.5).

1.2.1.1 BCL-2 inhibitors

Apoptosis serves as a natural barrier to tumor formation, as any potential unrestrained cell will be eliminated, and thus “Resisting cell death” is one of the cancer hallmarks (Hanahan & Weinberg, 2000) (Figure 3). In brief, there are two programs of apoptosis: intrinsic, via the mitochondria, and extrinsic, via the activation of death receptors such as Fas/CD95, TNFR and DF-5 and their ligands FasL, TNFα and TRAIL. The intrinsic pathway depends on a balance of proapoptotic Bax/Bak, located on the mitochondrial membrane, and anti-apoptotic BCL-2/BclXL proteins that inhibit Bax/Bak activity. Both Bax/Bak and BCL-2/BclXL are positively or negatively regulated, respectively, by BH3-only members. When the pro-apoptotic proteins outweigh the anti-apoptotic proteins the mitochondrial membrane is permeabilized resulting in the release of pro-apoptotic proteins such as cytochrome c. This will initiate a proteolytic cascade of caspase cleavage which will cleave other proteins and cause an ordered cell death and engulfment process (Hanahan & Weinberg, 2011; Lowe *et al.*, 2004). Apoptosis is a highly regulated pathway and genes involved are frequently mutated in cancer in order to defect cell death. Chronic lymphocytic leukemia (CLL) is the most common leukemia and almost all patients have overexpression of the oncogene BCL-2. This may be caused by inhibition of its negative regulators miR-15a and miR16-1 through 13q14 loss, or due to translocation of the *BCL-2* gene to the immunoglobulin heavy chain locus (Cimmino *et al*, 2005; Pekarsky *et al*, 2018). Venetoclax is the first U.S. Food and Drug Administration (FDA)-approved (in 2016) selective BCL-2 antagonist for the treatment of an aggressive form of CLL. Relapsed/refractory CLL patients or chemoresistant patients with 17p13 deletion (loss of *TP53*) had nearly 80% response rate after being treated with Venetoclax alone (Croce & Reed, 2016).

1.2.2 Sustaining proliferative signaling and evading growth suppressors

1.2.2.1 HER2 and PI3K inhibitors

It is generally considered easier to therapeutically target oncogenes, as mutations result in hyperactive protein variants, compared to tumor suppressors which usually are inactivated or deleted (Soussi & Wiman, 2015). There are many success stories in targeting oncogenes and several inhibitors have also been approved for clinical use. One example of a routinely targeted oncogene is human epidermal growth factor receptor-2 (HER2) that has diverse biological effects e.g. signaling via phosphatidylinositol 3-kinase (PI3K) and downstream protein kinase B (Akt) (Alzahrani, 2019; Moasser, 2007) which regulate many pathways including proliferation (Ellis & Ma, 2019). HER2 is overexpressed in breast cancer (15-20%) (Loibl & Gianni, 2017), ovarian cancer and some other solid tumors (Moasser, 2007). For years trastuzumab (HER2 inhibitor) has been standard treatment of care for HER2 positive breast cancer patients (Loibl & Gianni, 2017). In breast cancer, PI3K pathway is the most frequently mutated pathway (40% in hormone receptor positive breast cancer) (Ellis & Ma, 2019) and PI3K catalytic subunit alpha isoform (*PI3KCA*) (17.8%) is second most commonly mutated

gene (after *TP53*) in a study of 12 common tumor types (Kandoth *et al*, 2013). Due to its frequent aberrant activation, large efforts are made in targeting PI3K and many inhibitors have reached clinical trials. Some of these have been approved, including alpelisib in breast cancer, as well as idelalisib, copanlisib and duvelisib for hematologic malignancies (Alzahrani, 2019; Ellis & Ma, 2019). PI3K's downstream target Akt seems more difficult to target but several inhibitors are in clinical trials (Alzahrani, 2019). Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) negatively regulates PI3K and is one of the most frequently mutated tumor suppressors, with *PTEN* mutations in almost 10% in 12 common tumor types or over 60% in uterine corpus endometrial carcinoma (Kandoth *et al.*, 2013). Since many of these mutations result in loss of expression (Dillon & Miller, 2014), PTEN is a challenging therapeutic target.

1.2.2.2 Braf inhibitors

Another example of an activated oncogene is the serine/threonine-protein kinase B-raf (*BRAF*). Around 40-50% of metastatic melanoma patients have *BRAF* mutations, specifically in residue V600 (Sullivan *et al*, 2015) resulting in a hyperactivated BRAF kinase and constitutive signaling through the mitogen activate protein kinase (MAPK) pathway. Both the constitutive active BRAF kinase and PI3K/Akt pathway contribute to the cancer hallmark of "Sustaining proliferative signaling" (Hanahan & Weinberg, 2011; Proietti *et al*, 2020) Figure 3. In 2011 FDA (and 2012 European Medicines Agency [EMA]) approved $BRAF^{V600}$ inhibitor vemurafenib as a monotherapy for $BRAF^{V600}$ mutated melanoma patients. Vemurafenib rapidly suppresses melanoma growth in patients. Two more $BRAF^{V600}$ inhibitors, dabrafenib and encorafenib, were later approved (2013 and 2018 respectively) for treating $BRAF^{V600}$ melanoma (Proietti *et al.*, 2020). Also inhibitors targeting other components of the MAPK pathway have been approved such as the MEK inhibitor trametinib (Sullivan *et al.*, 2015). These novel molecularly targeted therapies together with novel immunotherapies have revolutionized treatment of metastatic melanoma over the past decade (Sullivan *et al.*, 2015).

1.2.2.3 CDK4/6 inhibitors

Besides "Sustained proliferative signaling", cancer cells have also acquired the capability of evading signals that suppress growth, another hallmark (Figure 3). This hallmark is tightly regulated by the two tumor suppressors p53 and pRb, the retinoblastoma protein. p53 may upon its activation halt progression of the cell cycle, and if the damage is beyond repair, trigger apoptosis (Hanahan & Weinberg, 2011). pRb acts as a gatekeeper of the G1-checkpoint and a negative regulator of cell cycle progression by repressing gene transcription needed for cell cycle transition but also remodels chromatin structure. In cancer, the *RB1* gene is often functionally inactivated by mutation or deletion (Giacinti & Giordano, 2006) while positive regulators of cell cycle progression are often amplified, such as cyclin D1 (2^{nd} most amplified locus in cancer), and cyclin-dependent kinases (CDK) such as CDK4 or CDK6 (Otto & Sicinski, 2017). These aberrantly regulated cell cycle regulators are attractive targets as exemplified by the many CDK4/6 inhibitors in clinical trial and approval of palbociclib, ribociclib and abemaciclib in breast cancer (Otto & Sicinski, 2017).

1.2.3 Deregulating energetics

Cancer cells have altered metabolic requirements in order to sustain their high proliferation rates. They rely on aerobic glycolysis, the so called "Warburg effect" (Hanahan & Weinberg, 2011). It is not fully understood why cancer cells switch to a much less efficient pathway for generating adenosine 5′-triphosphate (ATP) (Hanahan & Weinberg, 2011), but one reason may be the need to increase availability of nutrients for building biomass e.g. amino acids, nucleotides and lipids, but also reductive power in form of nicotinamide adenine dinucleotide phosphate (NADPH) (Vander Heiden *et al*, 2009). NADPH is important for the antioxidant system as will be discussed further in section 1.6.

1.2.3.1 Asparaginase

One successful example of how to specifically target the altered metabolism of cancer cells is asparaginase which has been standard treatment of care for acute lymphoblastic leukemia (ALL) patients for several decades (Hoelzer *et al*, 2016; Lanvers-Kaminsky, 2017). Asparaginase depletes extracellular asparagine, and as ALL cells are asparagine auxotrophs, the leukemic cells are highly sensitive to this treatment. Asparaginase treatment on its own may induce complete remission in up to 40-60% of patients. Many tumor cells are also highly dependent on glutamine for the production of NADPH (DeBerardinis *et al*, 2007), and thus asparaginase's glutaminase activity contributes to its anti-tumor activities (Emadi *et al*, 2014; Lanvers-Kaminsky, 2017; Parmentier *et al*, 2015). Asparaginase treatment will be further discussed in Project III.

1.3 TUMOR SUPPRESSOR p53

Inactivation of the p53 transcription factor, denoted the "Guardian of the Genome", is the most common anti-apoptotic lesion in cancer (Vousden & Lu, 2002) and p53 is thus a major player in the hallmark of "Resisting cell death" (Figure 3). Its role as a critical brake of tumor development is well established (Vogelstein *et al*, 2000). Although discovered more than four decades ago p53 (Lane & Crawford, 1979; Linzer & Levine, 1979), novel and sometimes bewildering roles of this tumor suppressor are still revealed. p53 was originally considered an oncogene, in part because tumors often express high levels of p53 protein (Soussi & Wiman, 2015) However, the findings that transfection of wild type p53 cDNA can suppress tumor cell growth and that the *TP53* gene is frequently mutated in common types of cancer made it clear that p53 actually is a tumor suppressor (Baker *et al*, 1989; Finlay *et al*, 1989; Nigro *et al*, 1989). Years later, two p53-related genes, p63 (Yang *et al*, 1998) and p73 (Kaghad *et al*, 1997), with overlapping DNA binding domain sequences and thus shared capacity to transactivate p53-responsive genes were identified (Bourdon, 2007b). Despite their ability to transactivate many of p53's downstream targets, the family members p63 and p73 are not redundant to p53, and loss of either gene will cause distinct phenotypes (Bourdon, 2007b). Furthermore, all three family members express different protein domains, so called isoforms, that may have distinct functions (Bourdon, 2007b) and are abnormally expressed in cancer (Bourdon, 2007a).

1.3.1 Triggers for p53 stabilization

1.3.1.1 Posttranslational modifications of p53

At basal or normal condition p53 levels are low and in a latent, inactive form, but upon certain triggers or stress stimuli p53 rapidly stabilizes which will lead to an outcome depending on the trigger and the cellular context (Giaccia & Kastan, 1998; Lavin & Gueven, 2006). Stabilization of p53 is achieved by inducing a cascade of posttranslational modifications of p53 protein resulting in an increased protein activity as well as inducing *TP53* transcription (Giaccia & Kastan, 1998). However, regulation of *TP53* transcription mainly occurs during development of certain tissues (Giaccia & Kastan, 1998) while for example oncogenic stress and DNA damage induce p53 protein activity via posttranslational modifications (Junttila & Evan, 2009). Depending on the initial trigger p53 has many sites that are targeted by various enzymes for posttranslational modifications such as phosphorylation, acetylation, methylation, ubiquitination and sumoylation (Figure 4). Posttranslational modifications mainly occur in the N-terminal transactivation and C-terminal oligomerization domains (Lavin & Gueven, 2006; Xu, 2003). The N-terminus is important for interaction with its negative regulator mouse double minute 2 (MDM2) and its transactivation capacities. Thus, depending on the initial signal and the position and type of posttranslational modification the effect on p53 may result in for example nuclear retention, disruption of MDM2 binding, enhanced DNA binding or additional posttranslational modifications (Lavin & Gueven, 2006; Xu, 2003). For example, DNA damage-induced Ser15, Thr18 and Ser20 phosphorylation on p53 may recruit other co-activators such as histone acetyl transferases (HAT), e.g. p300/CREB-binding protein (CBP) and its associated factor PCAF, leading to acetylation of the p53 C-terminal region and potentiation of p53 transcriptional activity (Gu & Roeder, 1997; Li *et al*, 2002b; Sakaguchi *et al*, 1998).

Another posttranslational modification includes a prolyl isomerase called Pin1 (Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1) which switches the bond between a proline and another amino acid from *cis* to *trans* conformation and vice versa (Mantovani *et al*, 2004). Upon DNA damage and phosphorylation of specific sites (Ser33, Thr81 and Ser315) on p53, Pin1 can bind and cause a confirmational change important for p53 transactivation function (Wulf *et al*, 2002; Zacchi *et al*, 2002; Zheng *et al*, 2002). Besides favoring p53 binding to target promotors, Pin1 is also important for p53 acetylation of specific sites and its interaction with oncoprotein and p53 negative regulator called iASPP (inhibitory member of the ASPP family) (Bergamaschi *et al*, 2003; Mantovani *et al*, 2007).

1.3.1.2 DNA damage

We are continuously exposed to chemicals that may be carcinogenic or mutagenic via food, water or in the air (Wogan *et al*, 2004). A clear example of a life-style exposure is smoking which is related to 90% of lung cancer risk in men, 70-80% in women (Walser *et al*, 2008) and other cancers (Jackson & Bartek, 2009), as tobacco carcinogens cause DNA adducts (Wogan *et al.*, 2004). Besides these adducts, smoking also causes chronic inflammation, another

hallmark (Figure 3) that is known to promote cancer (Hanahan & Weinberg, 2011; Walser *et al.*, 2008). DNA adducts were thought to occur mainly due to exposure of exogenous chemical carcinogens (Swenberg *et al*, 2011). However, DNA adducts may also occur as a result of several endogenous processes such as during disturbances in DNA replication (Jackson & Bartek, 2009) or by for example hydrolytic reactions and non-enzymatic methylations. Oxidative stress generated from oxidative respiration or redox cycling (discussed in section 1.6) also contribute to DNA damage. It has been estimated that 50,000 endogenous DNA lesions occur daily in every cell, and this number is likely to be higher under oxidative stress conditions (Swenberg *et al.*, 2011). Thus, having DNA damage sensors and repair systems set in place is vital in order to prevent mutations and maintain genome integrity for efficient tumor suppression (Jackson & Bartek, 2009).

Cells have evolved many mechanisms to deal with DNA damage, with different types of repair programs depending on the type of damage (Jackson & Bartek, 2009). The DNA damage sensors Ataxia telangiectasia mutated (ATM) and ATM–Rad3-related protein (ATR) become activated upon DNA damage or replication stress and orchestrates the DNA damage response signaling pathway (Marechal & Zou, 2013). Upon DNA damage ATM (Banin *et al*, 1998) or ATR (Tibbetts *et al*, 1999) phosphorylate Ser15 on p53, which in turn leads to Ser20 phosphorylation by Chk1 or Chk2, rendering MDM2 unable to bind p53 (Mantovani *et al.*, 2004). p53's negative regulators MDM2 and MDM4 can also be phosphorylated by ATM (Khosravi *et al*, 1999) or ATR (Shinozaki *et al*, 2003) inhibiting MDM2-p53 interaction and thus prevents p53 degradation (Junttila & Evan, 2009). DNA damage leads to a severalfold increase of the short 5-20 minutes half-life of p53 upon ultraviolet (UV) radiation (Giaccia & Kastan, 1998; Maltzman & Czyzyk, 1984). UV is the most pervasive environmental DNA-damaging agent and despite that the ozone layer absorbs most of the damaging UV spectrum, exposure passed through during strong sunlight can cause around 100,000 lesions per cell per hour (Jackson & Bartek, 2009). Besides various types of radiations, chemotherapy and oxidative stress can cause DNA damage and p53 activation (Christophorou *et al*, 2006; Jackson & Bartek, 2009; Junttila & Evan, 2009). The various fine-tuned effects that may occur upon different triggers is illustrated by the observation that both ionizing radiation (IR) and UV radiation lead to DNA damage but may activate different kinases to phosphorylate p53 on Ser15 (Ser18 in mice) (Giaccia & Kastan, 1998; Kastan *et al*, 1991). Double stranded DNA breaks activate ATM that phosphorylates p53 at various sites (Ser6, 9, 15, 20, 46 and Thr18) while for example phosphorylation at Thr81 occurs upon UV and H_2O_2 treatment (Lavin & Gueven, 2006). There are also sites that may undergo dephosphorylation upon radiation (Waterman *et al*, 1998). All these posttranslational modifications induced by DNA damage are important triggers for p53 in its role for arresting cells to enable DNA repair programs, or if repair is not possible induce apoptosis.

1.3.1.3 Oncogenic signaling

Acute p53-activating stress such as DNA damage may be transient and result in cell cycle arrest and DNA repair. In other cases, severe stress may trigger p53-dependent apoptosis (Junttila &

Evan, 2009). Activated oncogenes (e.g. *ras*, *myc*, *cyclin E*) can cause double stranded DNA breaks due to stalling and collapse of DNA replication forks (Halazonetis *et al*, 2008). This type of DNA damage activates p53 which will induce senescence or apoptosis to eliminate these damaged cells. p53-induced apoptosis upon DNA damage is a highly important mechanism of tumor suppression. If p53 is inactivated by for example mutation, DNA damage may contribute to genomic instability, an enabling hallmark of cancer (Figure 3) (Hanahan & Weinberg, 2011), and cause progression of a pre-cancerous lesion into cancer (Gorgoulis *et al*, 2005; Halazonetis *et al.*, 2008).

Oncogenic signaling is a persistent signal in tumor cells and may stabilize p53, not only by inducing DNA damage (Di Micco *et al*, 2006; Halazonetis *et al.*, 2008; Junttila & Evan, 2009) but also by the ARF protein (Alternative Reading Frame; $p19^{ARF}$ in mice and $p14^{ARF}$ in human) (Christophorou *et al.*, 2006). Mutated *TP53* and loss of ARF are often found to be mutually exclusive in tumors (Abraham & O'Neill, 2014). The *Ink4a-Arf* (*CDKN2A*) locus codes for two unrelated tumor suppressors, $p19^{ARF}$ (ARF) and $p16^{lnk4a}$ (Zindy *et al*, 2003). $p16^{lnk4a}$ antagonizes CDKs, thereby maintaining active unphosphorylated pRb protein and blocking cell cycle progression. $p19^{ARF}$ is activated upon oncogenic signaling but not DNA damage (Christophorou *et al.*, 2006) and antagonizes MDM2, resulting in either cell cycle arrest or apoptosis (Zindy *et al.*, 2003). ARF binds nucleolar localization signals (NoLS) on MDM2 and sequesters MDM2 in the nucleoli which inhibits its interaction with p53 (Lohrum *et al*, 2000b; Weber *et al*, 2000; Weber *et al*, 1999). ARF also binds the C-terminus of MDM2 and promotes rapid degradation of MDM2 and thus stabilization of p53 (Zhang *et al*, 1998). The importance of two proteins encoded by the *lnk4a-Arf* locus is demonstrated by the fact that loss of either gene predisposes to tumor development (Zindy *et al.*, 2003).

1.3.1.4 Other triggers

Many types of stress signals may trigger posttranslational modifications of p53 that result in its stabilization (Harris & Levine, 2005). These stress conditions do not need to involve DNA damage. One example is hypoxia, which induces HIF1α that can bind to and stabilize p53 (An *et al*, 1998). Hypoxia may also lead to p53 stabilization independently of HIFα via ATR kinase activation (Hammond *et al*, 2002). Nutrient deprivation via AMP-activated protein kinase (AMPK) may also induce p53 phosphorylation and thereby stabilize p53 (Lavin & Gueven, 2006). Furthermore, p53 may be activated if ribonucleoside triphosphates or ribosomes are limiting for cell cycle progression. Heat and cold chock conditions leading to protein denaturation and aggregation can also stabilize p53 (Harris & Levine, 2005).

1.3.1.5 Basal level p53

Besides the various tumor suppressive functions of p53 upon stabilization of acute cellular stress, p53 has many physiological roles at low or basal levels of expression, including the regulation of fertility, cell metabolism, mitochondrial respiration, autophagy, cell adhesion and stem cell maintenance and development (Junttila & Evan, 2009).

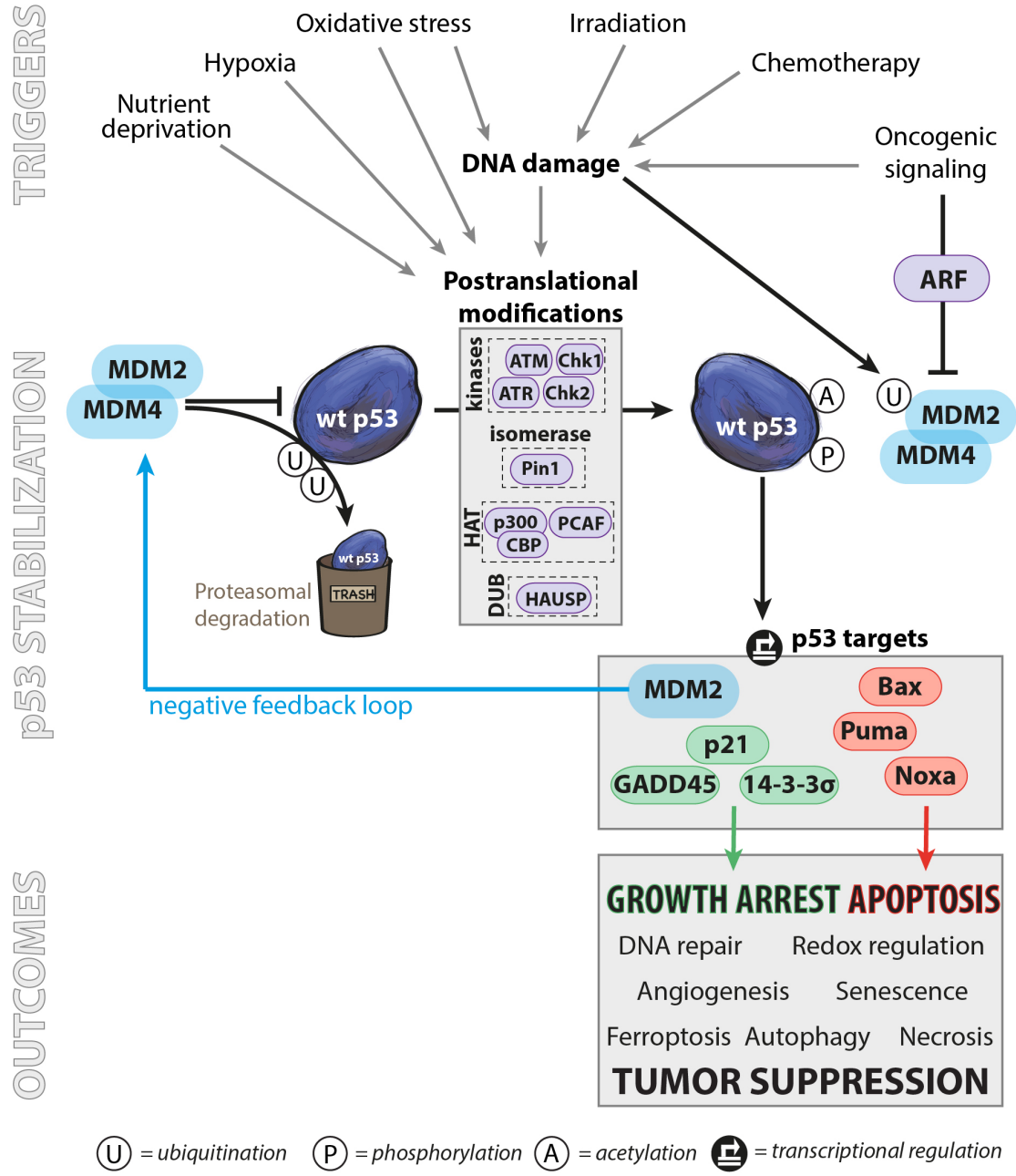

Figure 4 Triggers, regulation and outcomes of wild type p53 stabilization. Wild type (wt) p53 is stabilized by a range of different triggers and stresses at various levels. Physiological roles of p53 may not necessary need a trigger for p53 activation. Triggers induce p53 stabilization via posttranslational modifications for example phosphorylation, confirmational changes, acetylation or deubiquitination. Upon stabilization, p53 engages in transcriptional activation of downstream targets including its negative regulator MDM2 thereby forming a negative feedback loop. The various outcomes that may occur upon p53 stabilization are important for its role as a tumor suppressor. HAT = histone acetyl transferases, DUB = deubiquitinating enzymes. Parts of figure is from the review Eriksson, Ceder *et al* 2019.

1.3.2 Regulation of wild type p53

1.3.2.1 Negative regulation of p53

A key negative regulator of p53 is MDM2 and its close homolog MDMX (MDM4) which directly regulate p53 levels through several mechanisms (Hock & Vousden, 2014). MDM2 negatively regulates p53 in three ways: 1) inhibiting p53-dependent transactivation activity, 2)

exporting p53 from the nucleus and 3) ubiquitinating p53 for proteasomal degradation (Shangary & Wang, 2009). MDM2 knockout mice are embryonically lethal and will be rescued upon p53 inactivation (Jones *et al*, 1995; Montes de Oca Luna *et al*, 1995). MDMX mice show the same phenotype and are also rescued by loss of p53 (Migliorini *et al*, 2002; Parant *et al*, 2001), and thus MDMX is just as important as MDM2 in regulating p53 (Brooks & Gu, 2006).

As described in section 1.3.1, DNA damage induces posttranslational modifications of p53 that decrease p53-MDM2 binding, thereby stabilizing p53. Thus, regulation of MDM2 activity is critical for controlling p53 stabilization and consequently an attractive therapeutic target (described in section 1.5.1). MDM2 binds to hydrophobic residues at the p53 N-terminus (Shangary & Wang, 2009; Vassilev *et al*, 2004). These residues are also important for the transactivation activities of p53, supporting the notion that MDM2 binding can directly block transactivation of downstream p53 target genes (Kussie *et al*, 1996). MDM2 can also bind to the oligomerization domain on the C-terminal region which contributes to efficient MDM2 binding as well as degradation of p53 (Kubbutat *et al*, 1998). Even the DNA-binding domain has been reported to provide a binding site for MDM2 (Shimizu *et al*, 2002; Wallace *et al*, 2006).

The most well-known mechanism for regulation of p53 is ubiquitination by MDM2's E3 ligase activity that targets p53 for proteasomal degradation (Haupt *et al*, 1997; Honda *et al*, 1997; Kubbutat *et al*, 1997). However, MDM2 ubiquitination must not necessarily induce p53 degradation, but depending on ubiquitination chain length it can result in different outcomes. Low levels of MDM2 leads to mono-ubiquitination and nuclear export of p53 while high MDM2 levels leads to poly-ubiquitination of p53 and nuclear degradation by the proteosome (Li *et al*, 2003). MDMX, on the other hand, lacks E3 ligase activity and cannot ubiquitinate p53. But like MDM2, MDMX also interacts directly with p53's N-terminal transactivation domain and efficiently blocks p53 transcription activity. (Brooks & Gu, 2006; Burgess *et al*, 2016). MDMX and MDM2 form heterodimers by binding each others C-termini and the heterodimers efficiently ubiquitinate p53 (Leslie *et al*, 2015). MDMX mutations that disable heterodimerization with MDM2 are also embryonically lethal, and so its heterodimer formation seems essential for p53 degradation, at least during embryonic development (Kruse & Gu, 2009; Leslie *et al.*, 2015).

Other mechanisms by which MDM2 blocks p53 function include blocking co-activators such as p300 (Kobet *et al*, 2000) and recruiting repressors such as histone deacetylases (Ito *et al*, 2002) which both results in inhibition of p53s transcriptional activity. MDM2 binding has also been reported to induce a conformational shift in p53, making it unable to bind DNA (Sasaki *et al*, 2007). Lastly, it should be mentioned that other E3-ligases can target p53 for proteasomal degradation including constitutively photomorphogenic 1 (COP1) (Dornan *et al*, 2004), Pirh2 (Leng *et al*, 2003) and ARF binding protein 1(Arf-BP1) (Chen *et al*, 2005).

1.3.2.2 Regulation of MDM2

Under normal conditions the p53 network is "off" and only activated upon stress or damage (Vogelstein *et al.*, 2000). p53 activation result in high p53 levels and therefore a rapid negative regulatory mechanism is essential to terminate the p53 response when the problem is resolved. This function is carried out by MDM2, which consequently is upregulated as it is an important downstream target of p53. Additionally, p53 also upregulates E3 ligases PirH2 (Leng *et al.*, 2003) and Cop1 (Dornan *et al.*, 2004), as well as the phosphatase Wip1 (Lu *et al*, 2007) which dephosphorylates and stabilizes MDM2. Together with MDM2 they form a complex feedback loop, regulating wild type p53 and ensuring high p53 turnover allowing cells to return to an unstressed state once the p53 induction is removed (Hock & Vousden, 2014). Other positive regulators of MDM2 include transcription factor ying yang 1 (YY1) which works as a cofactor to promote MDM2 interaction with p53 while also being compromised by ARF1 (Sui *et al*, 2004). Akt has been reported to phosphorylate MDM2 at Ser166 resulting in the translocation of MDM2 to the nucleus where it can exert its negative regulation on p53 (Gottlieb *et al*, 2002; Mayo & Donner, 2001; Ogawara *et al*, 2002; Zhou *et al*, 2001).

The regulation of p53 by MDM2 is complicated and as mentioned previously many factors may affect stability of p53 or MDM2 and their interaction. Like p53, MDM2 is also regulated by phosphorylation and acetylation of various sites. For example, phosphorylation of Ser395 by ATM inhibits MDM2 function, while phosphorylation of other sites (Ser166 and Ser186) increases its E3 ligase activity . Other proteins may bind and inhibit MDM2 function, for example ribosomal proteins L5, L11 and L23, thereby regulating p53 activation during ribosomal stress (Kruse & Gu, 2009). Furthermore, MDM2 is an unstable protein as it ubiquitinates itself or is ubiquitinated by other E3 ligases (Fang *et al*, 2000; Kruse & Gu, 2009). For example, upon DNA damage MDM2 is autoubiquitinated resulting in increased p53 activity. Thus, controlling MDM2 degradation is another mean in regulating p53 activity (Brooks & Gu, 2006; Stommel & Wahl, 2004). The discovery of deubiquitinating enzymes (DUBs) challenged the existing concept of the monodirectional destiny of a ubiquitinated substrate and showed that ubiquitination is a highly dynamic process (Brooks & Gu, 2006). A DUB called herpesvirus-associated ubiquitin-specific protease (HAUSP) can remove the ubiquitination on p53 (Li *et al*, 2002a) but also on MDM2 (Cummins & Vogelstein, 2004), and thereby controlling p53 stability. HAUSP's capacity to remove ubiquitination on p53 while at the same regulating autoubiquitination of MDM2 triggered by DNA damage works as a "switch" that allows a quick p53 stabilization in response to stress (Brooks & Gu, 2006).

1.3.3 Downstream targets and outcomes of p53 activation

p53 is a transcription factor and is biologically active as a homotetramer that binds DNA (Joerger & Fersht, 2010; Raj & Attardi, 2017). p53 binds to a DNA binding motif or response elements (RE) with the consensus sequence 5'-Pu-Pu-Pu-C-A/T-A/T-G-Py-Py-Py-3' (where Pu is purine and Py is pyrimidine), located in the promotor of its target genes and thereby activates transcription of these specific genes (el-Deiry *et al*, 1992; Farmer *et al*, 1992; Funk *et al*, 1992). More than one hundred genes are transcriptionally activated by p53 (Andrysik *et al*, 2017;

Donehower *et al*, 2019) and the list continues to grow (Lane & Levine, 2010). p53 also mediates transcription-independent activities by directly interacting with other proteins or enzymes (Kruiswijk *et al*, 2015).

The pro-apoptotic genes of Bax, Puma and Noxa have p53 binding sites and induce apoptosis upon transactivation by p53 (Shaw *et al*, 1992). Another important role is p53's ability to inhibit cell proliferation and growth (Vousden & Prives, 2009). Induction of p21 (as well as GADD45 [Growth Arrest And DNA Damage-Inducible Protein] and 14-3-3 sigma) (el-Deiry, 1998; el-Deiry *et al.*, 1992) results in G1-arrest and is very sensitive as already low levels of p53 will induce p21. This allows cells to survive safely once the stress is removed, demonstrating that p53 activation must not necessarily trigger cell death. p21 induction can also lead to senescence (Brown *et al*, 1997), an irreversible cell cycle arrest, which prevents malignant progressions and in this way most likely holds back many abundant precancerous lesions that we all carry (Vousden & Prives, 2009). Although cell death and cell cycle arrest seem to be the major roles of p53 there are also many other cellular outcomes of p53 stabilization. For example, p53 mediates antiangiogenic activities (Teodoro *et al*, 2007) and antioxidant activities by reducing intracellular reactive oxygen species (Liu *et al*, 2008; Sablina *et al*, 2005) (described in section 1.3.3.1). Additionally, p53 engages factors involved in DNA repair such as inducing RAD51-dependent homologous repair as well as repressing the aberrant processing of replication forks if lesions are not repaired (Gatz & Wiesmuller, 2006). During starvation p53 can regulate autophagy by inducing lysosomal proteins DRAM or through the negative regulator mTOR (Crighton *et al*, 2006; Mathew *et al*, 2007). p53 may also regulate other tumor suppressors, for example PTEN (Stambolic *et al*, 2001) which negatively regulates the PI3K/Akt survival pathway (Song *et al*, 2012). Thus, p53 activation may result in a whole range of various outcomes, including proliferation, differentiation, stem cell reprogramming, metabolism and migration (Kruiswijk *et al.*, 2015).

In many cases it is not straight forward if p53 activation will lead to positive or negative regulation of outcomes as reflected in its dual roles in cell fate where it can induce apoptosis but also has the capability to promote cell survival (Kruiswijk *et al.*, 2015). Likewise, p53 activity may both increase and decrease oxidative stress or both inhibit and induce autophagy. The various activities of p53 are part of its tumor suppressive function. Thus, perturbation of p53 may provide tumors with survival advantages (Kruiswijk *et al.*, 2015). But how does p53 decide on the outcome of its activation? It has been suggested that p53's activities depend on the amount of stress, in that basal or low stress leads to roles in mediating homeostasis while at irreparable damage p53-activated outcomes will eliminate the damaged cell (Kruiswijk *et al.*, 2015; Li *et al*, 2011b). Although hundreds of genes are regulated by p53, a conserved core program of around 100 genes is activated, independently of cell type of cell response, and these genes cooperate to promote tumor suppression (Andrysik *et al.*, 2017).

1.3.3.1 Oxidative stress

As mentioned, wild type p53 can both increase and decrease oxidative stress while p53 itself is affected by redox homeostasis (Eriksson *et al*, 2019; Kruiswijk *et al.*, 2015) (Figure 5).

Redox regulation is introduced in section 1.6. Due to the important roles of the cysteines located in the DNA binding core domain (discussed in section 1.5.2.5), p53 is highly redox sensitive and dependent on reduction by antioxidants systems e.g. TrxR-Trx (Thioredoxin [Trx] reductase) and glutathione (GSH) (Eriksson *et al.*, 2019). Additionally, the master antioxidant regulator Nuclear factor erythroid 2-related factor 2 (NRF2) can regulate p53 activity by inducing MDM2 transcription (Todoric *et al*, 2017; You *et al*, 2011). Many of the p53 target genes with antioxidant capacities seem to be sensitive to low levels of p53, while p53 targets that display prooxidant and apoptotic capabilities are activated upon higher p53 levels (Polyak *et al*, 1997; Sablina *et al.*, 2005; Wu *et al*, 2017). Examples of targets with antioxidant capacities and part of the mentioned conserved core program (Andrysik *et al.*, 2017) include: p21 (by competing with NRF2 antagonist Keap1 for NRF2 binding (Chen *et al*, 2009), TIGAR (by promoting NADPH-generating pentose phosphate pathway [PPP] (Lee *et al*, 2014)) and Sestrins 1/2 (by activating NRF2(Bae *et al*, 2013)). Several of the pro-apoptotic genes in the conserved core program (Andrysik *et al.*, 2017) have mitochondrial functions and are associated with mitochondrial leakage of oxidant species e.g. Bax, Bak, Puma and Noxa (Eriksson *et al.*, 2019). p53 protein may also directly interact with and inhibit PPP-rate-limiting G6PD (glucose-6-phosphate dehydrogenase), thereby resulting in decreased NAPDH production and thus reductive power(Jiang *et al*, 2011).

Ferroptosis is a type of a cell death that is characterized by iron and lipid hydroperoxide accumulation and is considered important for p53 tumor suppression (Jiang *et al*, 2015; Maiorino *et al*, 2018; Stockwell *et al*, 2017; Tarangelo *et al*, 2018). Sensitivity to ferroptosis is associated with availability of GSH, cysteine and NADPH as well as iron homeostasis and fatty acid metabolism (Stockwell *et al.*, 2017). Both NRF2 and p53 transactivation may prevent ferroptosis, although p53 may also stimulate ferroptosis for example by its negative regulation of cystine/glutamate antiporter SLC7A11 (Jiang *et al.*, 2015; Maiorino *et al.*, 2018; Tarangelo *et al.*, 2018). p53 and its relationship to redox homeostasis is complicated. Although the negative regulation of SLC7A11 results in a decreased import of glutathione building blocks, many other p53 target genes act to increase GSH production such as TIGAR (Lee *et al.*, 2014), GLS2 (Hu *et al*, 2010; Suzuki *et al*, 2010), Sestrins ½ (Bae *et al.*, 2013) and p21-dependent NRF2 activation (Chen *et al.*, 2009). Furthermore, the observed increase in oxygen species and DNA oxidation upon p53 downregulation is a reflection of the antioxidant capacities of p53 (Sablina *et al.*, 2005). In this context it is also noteworthy that the tumor incidence of p53 null mice can be lowered upon dietary supplementation of N-acetylcysteine (NAC [which can be used for GSH production]).

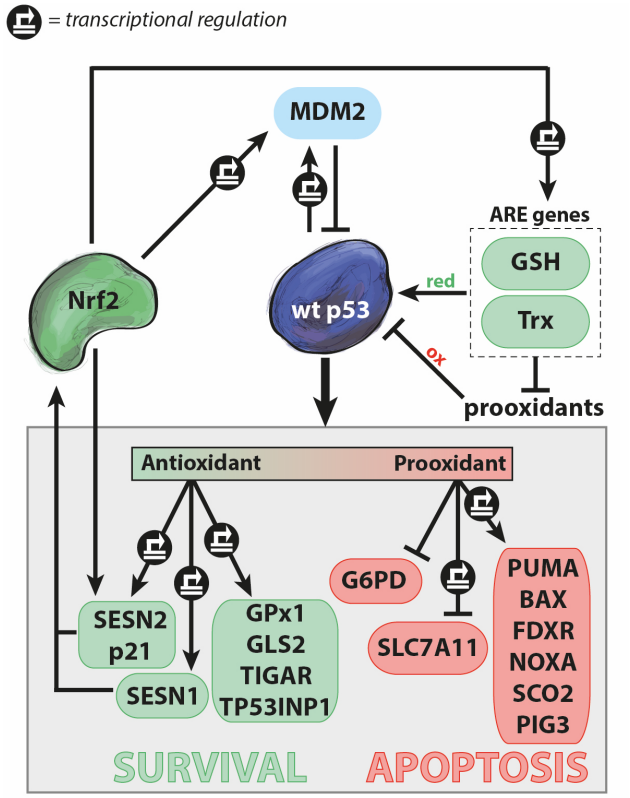

Figure 5 Regulation of redox balance by wild type p53 and vice versa. Wild type p53 function is dependent on a reduced environment while its function is compromised in oxidative conditions. p53 may induce targets that have antioxidant capacities but can also result in activities that generate oxidative stress. p53 may induce activities that lead to NRF2 activation while NRF2 can negatively regulate p53 by inducing MDM2. Red and green indicate prooxidant and antioxidant activities, respectively. Figure is from the review Eriksson, Ceder *et al* 2019.

1.4 MUTANT p53

Since many processes regulated by wild type p53 are integrated in its tumor suppressive activity, perturbation of some of these processes by *TP53* mutation provides tumors with survival advantages (Kruiswijk *et al.*, 2015). Due to a large fraction of tumors harboring *TP53* mutations resulting in high expression of a mutant form of the protein (Soussi & Wiman, 2015), there is both a clinical need and a high interest in targeting mutant p53. The focus of this thesis, is the compound APR-246 which targets mutant p53, increases oxidative stress and induces cancer cell death (Bykov *et al.*, 2002b; Lambert *et al.*, 2009). APR-246 will be discussed in detail in section 1.5.3.

1.4.1 Mutations in *TP53* gene

Despite intensive efforts to understand the various roles of p53, many questions are still unanswered and so p53 remains a truly dynamic and exciting field to unwind (Mello & Attardi, 2018). *TP53* is the most frequently mutated gene in cancer with a mutation frequency in at least 42% of the cases in 12 common tumor types (Kandoth *et al.*, 2013). As shown in Table 1, mutation frequency varies depending on cancer type, for example from rare cases (2.2%) in kidney renal clear cell carcinoma to almost all cases (95%) in high-grade serous ovarian cancer.

Advanced stages of cancer are often associated with a higher frequency of mutated *TP53*, for example metastatic or advanced prostate cancer (29% [in the MSK-IMPACT cohort]) which has a 4-fold higher frequency of mutated *TP53* compared to untreated primary prostate cancer (7% [in the TCGA dataset]) (Zehir *et al*, 2017). Also, acute lymphoblastic leukemia (ALL) rarely (5%) has *TP53* mutations while such mutations are more often (10-30%) found in relapsed patients (Blau *et al*, 1997; Comeaux & Mullighan, 2017; Diccianni *et al*, 1994; Gump *et al*, 2001; Ma *et al*, 2015; van Leeuwen, 2020). *TP53* mutation status and its association with prognosis is debated, as *TP53* mutation has been associated with poor survival, but there are also studies indicating no such association (Donehower *et al.*, 2019; Robles & Harris, 2010; Roth, 1999). Thus, the impact of *TP53* mutation may depend on multiple factors, e.g. cancer type and clinical stage. Moreover, mutant p53 associated RNA expression signatures may be a more valuable prognostic tools than *TP53* status alone (Donehower *et al.*, 2019). Further highlighting the important role of p53 as a tumor suppressor is the observation that tumors that do not carry *TP53* mutation often have mutation in other components of the p53 pathway, for example mutations leading to increased expression of p53's negative regulator MDM2 (Bond *et al*, 2004). p53 may also be inactivated by the human papilloma virus (HPV) protein E6 that target p53 for degradation (Leroy *et al*, 2017). This is clinically relevant in cervical cancer which may explain why cervical cancer rarely (5%) has *TP53* mutations (Olivier *et al*, 2010).

As tumor suppressors prevent cancer development, mutations in tumor suppressor genes are usually nonsense or out-of-frame mutations resulting in loss of protein expression as commonly seen in the adenomatous polyposis coli (APC) or retinoblastoma 1 (RB1) genes (Soussi & Wiman, 2015). Oncogenes, on the other hand, are almost exclusively altered by missense mutations of specific residues leading to constitutive activation. This applies to for example BRAF, KRAS, and PIK3CA (Vogelstein *et al*, 2013). However, the majority of the mutations in *TP53* are missense point mutations in the DNA binding domain (80%), that results in expression of mutant p53 protein that fails to bind DNA (Soussi & Wiman, 2015).

Tumor type (TCGA study)	**Frequency of mutation in *TP53* (%)**
Bladder urothelial carcinoma (BLCA)	50.0
Breast adenocarcinoma (BRCA)	32.9
Colon and rectal carcinoma (COAD/READ)	58.6
Glioblastoma multiforme (GBM)	28.3
Head and neck squamous cell carcinoma (HNSC)	69.8
Kidney renal clear cell carcinoma (KIRC)	2.2
Acute myeloid leukemia (LAML)	7.5
Lung adenocarcinoma (LUAD)	51.8
Lung squamous cell carcinoma (LUSC)	79.3
Ovarian serous carcinoma (OV)	94.6
Uterine corpus endometrial carcinoma (UCEC)	27.8
Pan-Cancer	**42.0**

Table 1 Percentage of mutated *TP53* in 3281 tumors across twelve individual tumor types and Pan-Cancer according to Kandoth, *et al* 2013 based on data from The Cancer Genome Atlas Program (TCGA) by the National Cancer Institute (NIH).

1.4.1.1 Mutation spectrum

When a novel cancer gene is discovered, a unique three-phase pattern is observed as described by Soussi and colleagues (Leroy *et al.*, 2017). These phases are the discovery, validation and the clinical relevance of the gene, and for several genes these phases can be rapid. *BRAF*, for example was first described in 2002 and is now a well established oncogene and known to carry one specific mutation V600E in melanoma and many other cancer types. To put this in perspective, since the discovery of *TP53* more than 40 years ago, 60,000 variants have been reported in the *TP53* variant database. Around 1500 different missense *TP53* variants have been described, most of which (85%) were reported early in the discovery phase. This is a reflection of the increased use of NGS which has led to the discovery of rare novel germline *TP53* variants with unknown significance. It raises the question concerning the pathogenicity of the many novel and rare variants reported (Leroy *et al.*, 2017) and therefore functional characterization of reported variants is important as described in Project IV.

In cancer, the majority of *TP53* mutations are missense mutations that give rise to single amino acid substitutions in the p53 protein (Vousden & Lu, 2002). Most of the *TP53* mutations occur in the central conserved DNA-binding domain (around 80%) (Donehower *et al.*, 2019; Muller & Vousden, 2013; Sabapathy & Lane, 2018). Also frameshifts (deletions and insertions) and nonsense mutations may occur (Sabapathy & Lane, 2018). Ten so called "hot spot" residues account for around half of all *TP53* mutations (Lane, 2019), six of these represent 28% of all mutations (Vousden & Lu, 2002) and include R175, G245, R248, R249, R273 and R282 (Bullock & Fersht, 2001; Freed-Pastor & Prives, 2012; Vousden & Lu, 2002). Mutations in p53 have been divided into two categories; structural mutants (for example R175H and R249S) that reduces the stability of the folded protein, and DNA-contacts mutants (for example R248Q and R273H) that affect residues that are essential for DNA binding (Muller & Vousden, 2013; Sabapathy & Lane, 2018). Since missense mutant p53 is unable to transactivate its own feedback regulator MDM2 many tumors express high levels of mutant p53 (Donehower *et al.*, 2019). For this reason, high expression of p53 has often been used as a surrogate marker to indicate mutant p53 (Kobel *et al*, 2016; Robles & Harris, 2010). Often *TP53* mutation occurs in one of the two alleles (Sabapathy & Lane, 2018). In almost all (>91%) of *TP53* mutant tumors, the second wild type p53 allele is lost by mutation, chromosomal (17p) deletion, or copy-neutral loss of heterozygosity (LOH) (Donehower *et al.*, 2019).

1.4.1.2 Germline TP53 mutations

Germline *TP53* mutations are found in highly cancer-prone Li-Fraumeni syndrome (LFS) (Li & Fraumeni, 1969) and also in families with hereditary breast cancer (Leroy *et al*, 2014). p53's important tumor suppressor role becomes evident in LFS patients where 75% of the patients carry germline p53 mutations (Guha & Malkin, 2017). LFS is a complex hereditary cancer predisposition disorder associated with early-onset cancer in various organs (Malkin *et al*, 2016). Tumors that typically occur in LFS are soft-tissue sarcoma, osteosarcoma, adrenocortical carcinomas, central nervous system tumors and early onset (< 31 years of age) breast cancer in women (Evans *et al.*, 2020). Early onset breast cancer may also occur without

family history of cancer, which is attributed to *de novo TP53* variants. Germline carriers of *TP53* mutation have a lifetime risk of developing cancer with 75% for male and 100% for females (Malkin *et al.*, 2016). The most common germline *TP53* mutations are the same as the somatic *TP53* mutations found in tumors i.e. hot spot mutations at codons 175, 245, 248, 273 and 282 (Malkin, 2011). Due to the early onset of cancer, it is suggested that other genetic modifiers also have roles in LFS, for example various polymorphism in the p53 network. One such polymorphism exists in exon four in *TP53*, which causes an increased affinity to MDM2 resulting in increased p53 degradation and earlier onset of tumor formation (Bougeard *et al*, 2006). Another polymorphism with similar results is located in the MDM2 promoter and lead to increased affinity of the Sp1 transcription factor, elevated levels of MDM2 expression and as a consequence enhanced degradation of p53 (Bond *et al.*, 2004). Due to their high lifetime risk of developing cancer, symptom-free members of LFS families undergo periodic surveillance. However due to the large phenotypic heterogeneity, disease management of these individuals is difficult (Malkin *et al.*, 2016). There are limited preventive options available for LFS patients, except prophylactic mastectomy for women to reduce the risk of developing breast cancer (Guha & Malkin, 2017). Unfortunately, it is nearly impossible to predict age of onset, likelihood and cancer type or prevent cancer in LFS (Malkin *et al.*, 2016).

1.4.2 Mutation outcome

1.4.2.1 Loss-of-function and gain-of-function

The finding that missense mutant p53 proteins in general are unable to transactivate target genes and that they also may have oncogenic activities that are advantageous for tumors has led to the notion that tumors may be addicted to mutant p53 (Leroy *et al.*, 2017). This suggests a strong selection for maintained expression of mutant p53 proteins that have a positive role for tumor development either by a loss-of-function (LOF) or gain-of-function (GOF) (Mantovani *et al.*, 2019). The observation that tumors with missense *TP53* mutations have higher oncogenic potential than *TP53* null tumors may be explained by the GOF activity of missense mutants (Brosh & Rotter, 2009; Dittmer *et al*, 1993; Olive *et al*, 2004). Mutant p53 may also exert dominant negative effects that lead to LOF or impairment of wild type p53 activity (Boettcher *et al*, 2019; de Vries *et al*, 2002; Hegi *et al*, 2000; Srivastava *et al*, 1993). This has recently been thoroughly demonstrated in acute myeloid leukemia (AML) by CRISPR/Cas9 of common missense *TP53* mutations (Boettcher *et al.*, 2019). Functional DNA binding and transcriptional analyses revealed that the various missense p53 mutants did not lead to GOF, but rather LOF by dominant negative effects. Neither did AML patients harboring missense *TP53* mutations have a more aggressive disease or difference in survival compared to AML patients with truncating mutations. As wild type p53 functions as a tetramer (Joerger & Fersht, 2010; Raj & Attardi, 2017), a dominant negative effect may occur by heterodimerization of mutant p53 with the wild type protein leading to a mixed dimer that is transcriptionally deficient (Milner & Medcalf, 1991; Shaulian *et al*, 1992). Furthermore, all of the subunits in this mixed tetramer are positive for mutation-confirmation specific antibody PAb240 while negative for wild type-confirmation specific antibody PAb1620 (Milner &

Medcalf, 1991). Due to the many important roles of p53 as the Guardian of the Genome (section 1.3), depriving cells of its tumor suppressive functions provides an advantage for cancer development (Mantovani *et al.*, 2019). Many studies have convincingly shown both LOF and GOF activities of mutant p53 and it is possible that the outcome of *TP53* mutation is context and cancer type dependent, where for instance LOF may be driving progression of myeloid malignancies and GOF drive epithelial malignancies in promoting invasion and metastasis (Boettcher *et al.*, 2019; Lane, 2019).

1.4.2.2 Interaction with other family members, p63 and p73

The molecular mechanisms by which mutant p53 may exert GOF effects can be divided into alteration of the DNA-binding ability, enhancement or repression transcription factors, as well as interaction with proteins to change their functions (Muller & Vousden, 2013). Most of the mutations in *TP53* occur in the DNA-binding domain and disrupt DNA binding. Mutant p53 may retain some DNA-binding capacity and show promiscuous transactivation of genes that allows it to function as an oncogenic transcription factor, while the usual wild type p53 response is impaired. Mutant p53 can also interact with other transcription factors to either enhance or repress them, one of the best examples of this being the interaction with its family members p63 and p73. p63 and p73 encode numerous different isoforms that to some extent mimic wild type p53 by activating some targets genes shared by wild type p53. Although they also have their own distinct biological roles, mutant p53 can engage in interaction with some of these isoforms to repress or enhance their function (Oren & Rotter, 2010). Mutant p53 exert dominant negative effects on p63 and p73 by preventing their binding to DNA but also tethers p63 to DNA sites not normally bound by p63 (Muller & Vousden, 2013; Murphy *et al*, 2000). Inactivation of p63/p73 seems to play a pivotal role in the GOF activity by p53 as ablation of p63/p73 often mimics the effects of mutant p53 while overexpression of p63/p73 can counteract mutant p53 activity (Oren & Rotter, 2010). For example, co-expression of mutant p53 (R175H) and p73a results in decreased transcriptional activity of pro-apoptotic *Bax* (1.2.1.1) and thus reduced p73-mediated apoptosis (Murphy *et al.*, 2000). Besides interacting with transcription factors, mutant p53 can also interact with other proteins and modulate their function (Muller & Vousden, 2013), for example master antioxidant regulator NRF2 (Lisek *et al.*, 2018; Liu *et al.*, 2017; Walerych *et al.*, 2016) (section 1.4.2.4) or Mre11, an important factor of homologous recombination-mediated DNA repair (Song *et al*, 2007) (section 1.4.2.3).

1.4.2.3 Hallmarks of cancer

Considering the accumulating and intense p53 research literature for the past four decades and the many different variants of mutant p53 that exist, mutant p53 seems to contribute to most or even all of the Hallmarks of cancer in one way or another (Freed-Pastor & Prives, 2012; Solomon *et al*, 2011) as illustrated in Figure 6. As mentioned, these contributions may be attributed to LOF and/or GOF activities of mutant p53. Eliminating wild type p53 tumor suppression contributes to both evading growth suppression and resisting apoptosis as p53 is a major regulator of these pathways (Solomon *et al.*, 2011). Mutant p53 fails to induce p21 and thus p21 cannot inhibit cyclin-dependent kinases resulting in evasion of growth suppression

(Elbendary *et al*, 1996). *TP53* mutation leads to loss of wild type p53-mediated apoptosis, but mutant p53 also protects cells from other apoptosis inducers. Mutant p53 increases induction of the NFκB pathway in response to TNFα, while loss of mutant p53 results in sensitization to TNFα-induced apoptosis (Weisz *et al*, 2007). TNFα is a ligand of death receptor TNFR1 (section 1.2.1.1) and thus induces extrinsic apoptosis (Weinberg, 2007) but can also activate the NFκB pathway (Weisz *et al.*, 2007). The transcription factor NFκB is often implicated in cancer, as it can both inhibit apoptosis and support cell proliferation (Karin, 2006). As constitutive activation of NFκB promotes tumor development by chronic inflammation, and due to the fact that wild type p53 and NFκB normally antagonize each other, activation of the NFκB pathway by mutant p53 contributes to several Hallmark of cancer (Gudkov & Komarova, 2016; Solomon *et al.*, 2011; Weisz *et al.*, 2007).

Wild type p53 is dubbed Guardian of the Genome due to its ability trigger to growth arrest and induce DNA repair upon DNA damage (Solomon *et al.*, 2011). In the presence of mutant p53 many normal responses are lost. For example, genotoxic agents that normally trigger growth suppression or apoptosis through wild type p53 stabilization will no longer induce these responses. Mice carrying *TP53* R248W mutations were reported to have interchromsomal translocation resulting in genomic instability which was not observed in *TP53* null mice (Song *et al.*, 2007). The interaction of mutant p53 with the nuclease Mre11 (Meiotic Recombination 11), was shown to hamper DNA repair by homologous recombination, resulting in genomic instability. Mutant p53 therefore prevented the recruitment of the Mre11-Rad50-NBS1 (MRN) complex and DNA double-stranded breaks, consequently leading to failed ATM recruitment (Song *et al.*, 2007). Moreover, DNA damage induces mutant p53 phosphorylation on Ser15 via ATM, which results in mutant p53 accumulation (Frum *et al*, 2016). As wild type p53 directly interacts with RAD51, BRCA1 and BRCA2, factors important for repair of double stranded DNA breaks, mutation or loss of wild type *TP53* results in inappropriate chromosomal rearrangements and further genomic instability (Murphy *et al.*, 2000). This allows cells with mutant p53 to accumulate more DNA damage, resulting in increased genomic instability and emergence of cells that drive tumor progression (Blagih *et al*, 2020; Sabapathy & Lane, 2018). Indeed, an analysis of >10,000 tumors from the TCGA dataset showed that *TP53* mutation is associated with enhanced chromosomal instability, including deletion of tumor suppressor genes and amplification of oncogenes (Donehower *et al.*, 2019). Telomeres protect chromosome ends and are shortened during cell propagation to prevent immortality (Hanahan & Weinberg, 2011). Importantly, telomere dysfunction leads to DNA damage signals that activate wild type p53, a tumor suppressive function that is lost upon *TP53* mutation (Roake & Artandi, 2017). Telomerases can counteract telomere erosion by extending telomeric DNA and enables unlimited replication (Hanahan & Weinberg, 2011). One study has shown that p53 mutants can transactivate the telomerase reverse transcriptase (TERT) promotor (Scian *et al*, 2004).

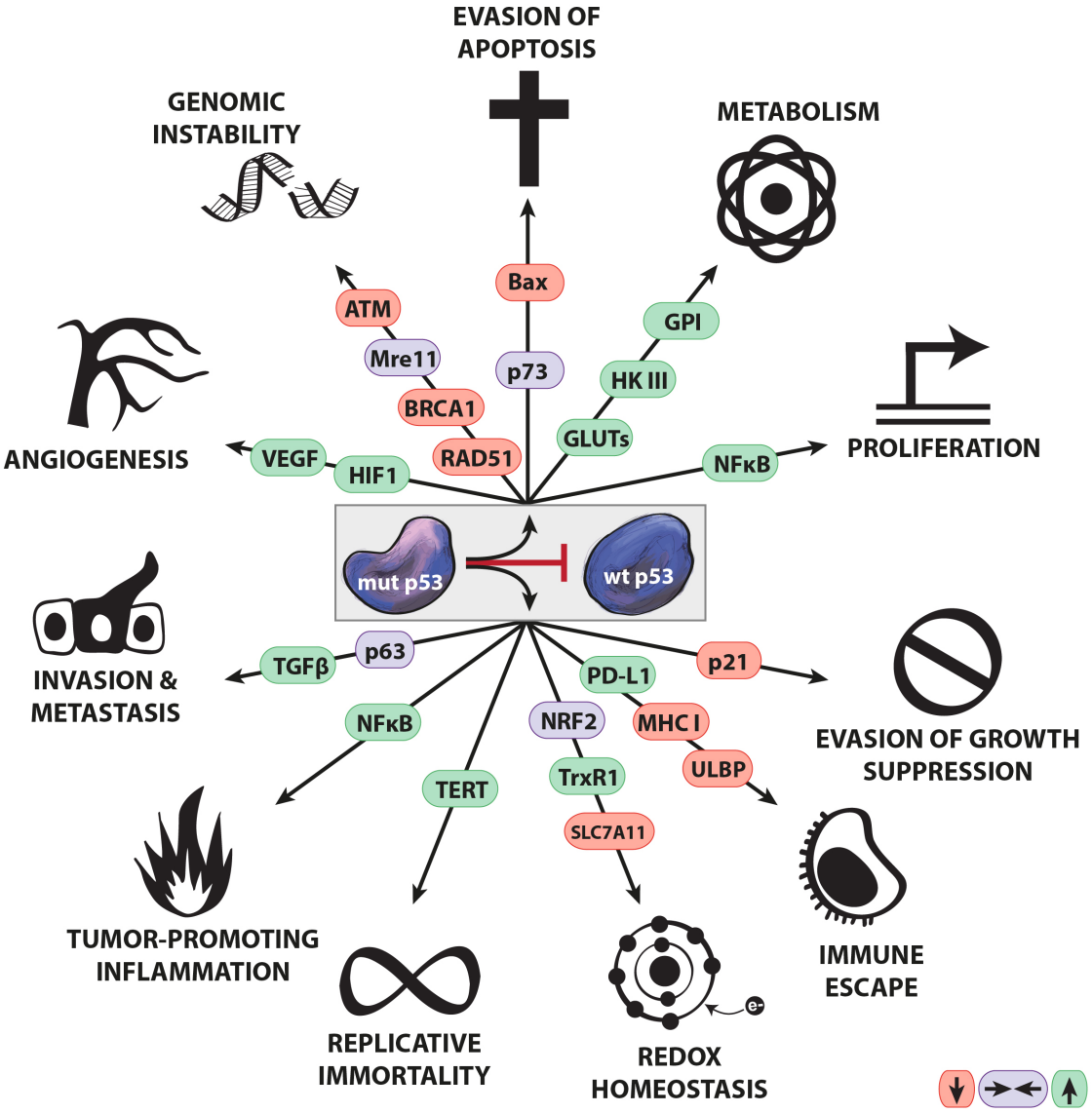

Figure 6 Mutant p53 in cancer. In tumor cells that carry one wild type and one mutant TP53 allele, mutant (mut) p53 may exert a dominant negative effect (⊣) on the co-expressed wild type (wt) p53, resulting in inhibition of wt p53 function. Mutant p53 may also, at least in some cellular contexts, have gain-of-function (GOF) activities that favor tumor progression. Such activities may include illegitimate transactivation of transcription and promiscuous binding to cellular proteins. Genes and proteins whose transcription and/or function are inhibited by p53 are indicated in red, and genes and proteins whose transcription and/or function are stimulated by mutant p53 are indicated in green. Novel binding partners for mutant p53 are indicated in purple. Figure is inspired and modified from Hallmarks of Cancer by Hanahan & Weinberg 2011.

Furthermore, mutant p53 may also support the Hallmark "Avoid Immune Destruction" as wild type p53 regulates antigen presentation pathways which are important for immune recognition (Blagih *et al.*, 2020). For example, wild type p53 can promote endogenous antigen presentation by increased surface MHC class I-peptide complexes and peptide loading, which are both functions that may be lost in p53 mutant and null cells (Wang *et al*, 2013; Zhu *et al*, 1999). Since recognition of natural killer (NK) cells and T cells may be modulated by wild type p53, perturbation of wild type p53 function may lead to immune escape (Blagih *et al.*, 2020). Wild type p53 may regulate expression of UL16-binding protein 1 (ULBP1) and ULBP2 recognized

by NK cells (Li *et al*, 2011a; Textor *et al*, 2011), another regulation lost upon mutation. Cancer cells often overexpress PD-L1 which is an inhibitor signals for T cells, and loss of p53 function is correlated to increased PD-L1 expression (Biton *et al*, 2018; Blagih *et al.*, 2020; Cortez *et al*, 2016).

Many studies have provided evidence that mutant p53 can drive and enhance invasion and motility by increasing signaling through the cytokine TGF-β (Transforming growth factor beta) (Adorno *et al*, 2009), receptor EGFR (Epidermal growth factor receptor) (Sauer *et al*, 2010) or the receptor tyrosine kinase MET (Grugan *et al*, 2013; Muller *et al*, 2013) that are often associated with increased metastatic capacity. Additionally, by interacting with the p63 isoform TAp63, which does not interact with wild type p53, mutant p53 can regulate a pro-invasive transcription factor program (Muller & Vousden, 2014). Supporting this notion, a study using a mouse model of pancreatic cancer with mutant p53 showed that loss of TAp63 resulted in less potent induction of metastasis (Tan *et al*, 2014). This also supports the idea that the p63 and p73 family plays a pivotal role in mutant p53 GOF. As wild type p53 regulates a wide array of pathways to counteract metastasis, for example by preventing EMT (epithelial-mesenchymal-transition) (Muller *et al*, 2011), mutant p53 can promote invasion and metastasis in many more ways than mentioned here.

As wild type p53 participates in almost every facet of cell behavior (Kruiswijk *et al.*, 2015), the loss of its function by mutations will likely lead to the perturbation of many of these pathways. Furthermore, the presence of high level mutant p53 gives cancer cells the capacity to withstand not only DNA damage signals but also other types of stresses such as oxidative and proteotoxic stress, nutrient fluctuations, physical constraints and anti-tumor immune responses (Mantovani *et al.*, 2019).

1.4.2.4 Oxidative stress

Wild type p53 regulates an ocean of processes including metabolic activities and redox homeostasis that both contribute in antioxidant and prooxidant responses (Kruiswijk *et al.*, 2015). Since mutant p53 may exert dominant negative effects on co-expressed wild type p53, it presumably disrupts wild type p53-mediated regulation in redox homeostasis in this setting (Boettcher *et al.*, 2019; de Vries *et al.*, 2002). The relationship between mutant p53 and redox homeostasis is complicated (Eriksson *et al.*, 2019). Mutant p53 also been shown to interact with the master antioxidant regulator NRF2 (Walerych *et al.*, 2016) (Figure 7).NRF2 is further described in section 1.6. In brief, NRF2 is a transcription factor that transactivates antioxidant response element (ARE) genes upon oxidative stress (Rojo de la Vega *et al*, 2018). However, its interaction with mutant p53 perturbs this regulation and some ARE genes are activated (*TrxR1*, *Trx*) while others are repressed (*HO-1*, *NQO1*, *SLC7A11*) (Lisek *et al.*, 2018; Liu *et al.*, 2017). Mutant p53 is associated with upregulation of glucose transporters and changes in metabolism may also affect redox homeostasis (Eriksson *et al.*, 2019; Gomes *et al*, 2018). Furthermore, several p53 mutants are found to induce HIF α/VEGF signaling (Khromova *et al*, 2009). Since VEGF can activate NRF2, angiogenesis may also affect redox regulation (Eriksson *et al.*, 2019; Rojo de la Vega *et al.*, 2018).

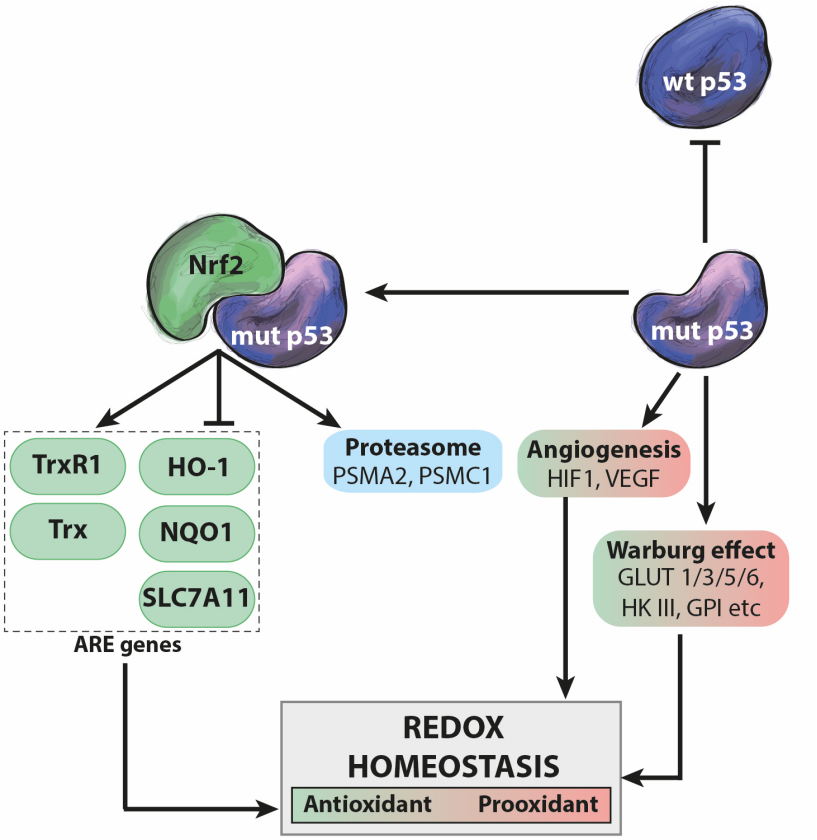

Figure 7 Interaction of mutant p53 and the of redox homeostasis. Mutant p53 may exert dominant negative effects on wild type (wt) p53 thereby interfere with wt p53 redox regulations. Mutant p53 may also interact with master antioxidant regulator NRF2 which leads to aberrant regulation of ARE genes or genes of the proteosome machinery. Furthermore, mutant p53 affects both angiogenesis and metabolic activities that can contribute to both antioxidant and prooxidant effects. Figure is from the review Eriksson, Ceder *et al* 2019.

1.5 THERAPEUTIC TARGETING OF P53

1.5.1 Reactivating wild type p53

1.5.1.1 p53-MDM2 interaction

In cancers harboring wild type p53, radiation and traditional chemotherapy will induce DNA damage and subsequent p53 accumulation due to posttranslational modifications of p53 (Shangary & Wang, 2009). Nonetheless, great efforts have been made design strategies to induce a p53 response by directly and specifically targeting the interaction of p53 with its negative regulator MDM2 (Kruse & Gu, 2009; Shangary & Wang, 2009) or by inhibiting MDM2 expression and E3 ligase activity as well as its intracellular localization (Lohrum *et al*, 2000a; Tisato *et al*, 2017). This is based on the observation that MDM2 is often dysregulated in wild type p53 tumors by amplification or deletion of its negative regulator ARF (Wiman, 2006). MDM2 amplification occurs in up to 4% in all tumors (Meric-Bernstam *et al*, 2019) and with even higher frequency in some specific cancer types. For example, around 20% of sarcomas and 15% of breast cancers overexpress MDM2 and/or MDMX (Burgess *et al.*, 2016). The MDM2-p53 interaction site is located in the transactivation domain at the p53 N-terminus and in an N-terminal 106-amino acid long region on MDM2 (Chen *et al*, 1993; Picksley *et al*,

1994). Crystal structures of MDM2's N-terminal domain together with short peptides of p53 (residues 15-29) have enabled detailed studies of this interaction. p53 forms a hydrophobic α-helix, in which three residues Phe19, Trp23 and Leu26, insert into a deep pocket in MDM2. These three residues are also important for the transcriptional transactivation activity of p53 (Kussie *et al.*, 1996). p53 binds to a similar pocket in MDMX although the binding site in MDMX is shallower and more narrow (Popowicz *et al*, 2008).

1.5.1.2 Nutlins and its derivatives

The first small molecules to inhibit MDM2-p53 interaction were a series of cis-imidazoline analogues named Nutlins (Vassilev *et al.*, 2004). In a nanomolar concentration range these compounds displaced recombinant p53 from MDM2, with Nutlin-3 displaying the most potent binding activity. Nutlin-3 and as well as many of the other MDM2 inhibitors mimic the helical p53 peptide by binding into the hydrophobic pocket in MDM2 (Shangary & Wang, 2009; Vassilev *et al.*, 2004). Also other helix-binding proteins can be targeted by nutlins e.g. members of BCL-2 family, although to lower affinity (Shangary & Wang, 2009). Nutlins induce p53-dependent cell cycle arrest or cell death in cancer cell lines, while normal cells only undergo transient growth arrest (Shangary & Wang, 2009; Wiman, 2006). RG7112, a Nutlin derivative, was the first MDM2 inhibitor to be tested in clinical trials (Tisato *et al.*, 2017). A Phase I study in patients with MDM2-amplified liposarcoma demonstrated proof-of-concept effect with an increased p53 and p21 expression by immunohistochemistry compared to baseline (Ray-Coquard *et al*, 2012) as was also seen in mRNA expression levels in circulating leukemic cells from AML and CLL patients (Andreeff *et al*, 2016). However, high doses were needed for clinical efficacy, and toxicity and several clinical adverse events related to the treatment were reported. Idasanutlin (RG7388), another Nutlin derivate, is a second generation MDM2 inhibitor with even higher potency than RG7112 *in vitro* and *in vivo* (Ding *et al*, 2013; Tisato *et al.*, 2017). A Phase I/Ib study showed tolerable safety (Montesinos *et al*, 2020) and analysis of leukemic blasts from the Idasanutlin-treated patients showed that MDM2 protein expression in leukemic blasts was correlated to clinical response (Reis *et al*, 2016). Idasanutlin was further tested in a Phase III clinical trial in AML patients (regardless of *TP53* status) in combination with cytarabine (Montesinos *et al.*, 2020; Tisato *et al.*, 2017). The Phase III study had an integrated Phase II safety and efficacy criteria with a blinded interim analysis for futility (Montesinos *et al.*, 2020). Unfortunately the study has been terminated for futility based on efficacy results (according to clinicaltrials.gov, NCT02545283).

1.5.1.3 Other small molecules targeting p53-MDM2 interaction

Besides Nutlin and Nutlin-derivatives, other classes of compounds have been identified that inhibit the p53-MDM2 interaction, such as benzodiazepines that have three benzene rings mimicking the three residues of p53, and spiro-oxindole compounds where the oxindole ring mimics the side chain of one of the residues in p53 (Trp23) and the spiro-pyrrolidine ring mimics the other two residues (Liu *et al*, 2019; Tisato *et al.*, 2017). The spiro-oxide compound SAR405838 (MI-77303) also mimics the three residues while additional interactions are formed with the residues 10-18 of MDM2, achieving a high degree of binding. SAR405838

has completed two phase I clinical trials with acceptable safety profile (de Jonge *et al*, 2017; de Weger *et al*, 2019; Liu *et al.*, 2019) and limited activity in patients with solid tumors out of which 89% had MDM2 amplifications (de Jonge *et al.*, 2017). Most small molecules that target MDM2 work in similar way as Nutlins-3a i.e. they target the pocket of MDM2 and also bind its homolog MDMX, but with a lower affinity, (Liu *et al.*, 2019) even though MDM2 and MDMX binding pockets are structurally similar (Shangary & Wang, 2009). There has also been interest in targeting the NoLS of ARF and MDM2 that localize the proteins to the nucleolus and thereby prevent p53 degradation by MDM2 (Lohrum *et al.*, 2000a).

Up until 2019, seven compounds targeting MDM2 have or are being evaluated in clinical trials. Idasanutlin has progressed the furthest and several compounds are in Phase I studies, including those mentioned above as well as AMG-232, APG-115 and HDM201 (Burgess *et al.*, 2016; Liu *et al.*, 2019). Although major efforts are being made, clinical efficacy has not fulfilled expectations and currently there is no p53-MDM2/MDMZ interacting inhibitor approved for clinical use.

1.5.1.4 Stapled peptides targeting p53-MDM2/MDM4 interaction

A different class of therapeutics are stapled peptides that mimic the p53 amino acid sequence bound by MDM2 and MDMX (Burgess *et al.*, 2016). The peptides are generated by a chemical method called peptide stapling (Bernal *et al*, 2007; Brown *et al*, 2013) where introduction of hydrocarbon linkers between non-adjacent amino acids causes a turn of the α-helix resulting in better stability and affinity (Brown *et al.*, 2013; Mortensen *et al*, 2019). Unmodified wild type p53 peptide is poorly taken up by cells (Bernal *et al.*, 2007) and as the wild type p53 peptide is an amino acid sequence that is also bound by other proteins, it has low affinity for MDM2 and MDM4 (Brown *et al.*, 2013). Initial developed stabilized alpha-helix of p53 (SAH-p53) peptides do not enter cells due to its negative charge at physiological pH. However, replacing certain amino acids to generate positively charged stapled peptide (SAH-p53-8) allowed active uptake by cells (Bernal *et al.*, 2007). The uptake can further be improved by for example nanocarriers such as lipid bilayer disks (lipodiscs) as shown with EGFR-targeted lipodiscs loaded with VIP116 stapled peptide targeting p53-MDM2/MDM4 (Lundsten *et al*, 2020). Thus, rational design of stapled peptides can generate stable products that are readily taken up by cells (Bernal *et al.*, 2007; Brown *et al.*, 2013) and tumor as shown by the radiolabeled ^{125}I-PM2 stapled peptide (Spiegelberg *et al*, 2018). Treatment with PM2 improved median survival by 50% in mice carrying colorectal cancer xenografts harboring wild type p53, while treatment of mice with mutant *TP53* or null tumor xenografts had negligible effect (Spiegelberg *et al.*, 2018). There are many stapled peptides that are being tested preclinically and first to be tested in a clinical setting is ALRN-6924 (Burgess *et al.*, 2016). ALRN-6924 activates p53-dependent transcription with anti-leukemic effect *in vitro* and *in vivo* in mice (Carvajal *et al*, 2018). It was considered well tolerated in patients with solid tumors and lymphomas (Meric-Bernstam *et al*, 2017; Meric-Bernstam *et al.*, 2019) and is being tested in several Phase I and II clinical trials in combination with chemotherapies or molecular targeted treatments in solid tumors and hematological malignancies (Burgess *et al.*, 2016).

1.5.2 Reactivating mutant p53

p53 is a tumor suppressor, and as discussed above (section 1.2.2), targeting tumor suppressors is challenging since the aim is to restore a normal function as opposed to inhibiting the oncogenic activity of an activated oncogene. In many cases, tumor suppressor proteins are not expressed in tumor cells, due to for example large deletions in tumor suppressor genes. However, p53 is unique among the tumor suppressor genes, considering its high frequency of missense mutation and high levels of expression in tumors (Soussi & Wiman, 2015). Also, missense mutant p53 protein can exert dominant negative effect on co-expressed wild type p53 (Boettcher *et al.*, 2019; de Vries *et al.*, 2002; Hegi *et al.*, 2000; Srivastava *et al.*, 1993) and at least some missense p53 mutants have probably acquired GOF (Brosh & Rotter, 2009; Mantovani *et al.*, 2019). The high expression of missense mutant p53 protein and the fact that the *TP53* gene is by far the most frequently mutated gene in cancer, makes mutant p53 a highly attractive anti-cancer target (Bykov *et al.*, 2018). Several studies have shown that restoration of wild type p53 in mice causes tumor regression (Martins *et al*, 2006; Ventura *et al*, 2007; Xue *et al*, 2007) and so if the abundant missense mutant p53 were to be reactivated to a wild type-like protein it should elicit a robust anti-tumor response (Bykov *et al.*, 2018). That the function of missense mutated p53 proteins can be restored has been demonstrated by studies introducing second-site suppressor mutations (described below) that add novel DNA contacts or increase the stability of the folded state of the core domain (Brachmann *et al*, 1998; Nikolova *et al*, 2000; Wieczorek *et al*, 1996). Intensive efforts have been and are being made using rational drug design, molecular modelling and chemical library screening in order to identify compounds that target missense mutant p53, reactivate it and induce tumor suppression (Bykov *et al.*, 2018). There are also preclinical efforts to target the 10% of *TP53* mutations that are nonsense mutations (i.e. have premature stop codons), which result in non-functional truncated p53 protein (Bykov *et al.*, 2018; Zhang *et al*, 2017).

1.5.2.1 p53 protein structure

The 393 amino acid-containing p53 protein contains several well defined domains: an amino-terminal (N-terminal) transactivation domain (TA), a proline-rich (PR) SH3 ligand domain, central sequence specific DNA-binding core domain (DBD), a tetramerization domain and a carboxy (COOH)-terminal (C-terminal) regulatory region (Bullock & Fersht, 2001; Vousden & Lu, 2002). There are several regions in the p53 protein that are conserved across species, and four out of five of these regions are located in the DNA-binding core domain (Cho *et al*, 1994). Nuclear-localization signals are located in the C-terminal region while nuclear export signals are located in both the N- and C-termini (Vousden & Lu, 2002). At least 36 residues are reported to be modified by for example phosphorylation, methylations, acetylation, ubiquitination, glycosylation, sumoylation, ADP-ribosylation and neddylation (Kruse & Gu, 2009). Many of the DNA damage-induced phosphorylations occur at residues in the transactivation domain, for example S15 and S20 by ATM/ATR and Chk1/2, respectively. MDM2 and histone acetyltransferases CBP/p300 bind to the p53 N-terminus and ubiquitinate or acetylate sites in the C-terminus (Vousden & Lu, 2002). The C-terminal oligomerization

domain residues 312-365) mediate the formation of p53 tetramers that bind DNA containing four copies of consensus sequence (Cho *et al.*, 1994; el-Deiry *et al.*, 1992).

Almost all *TP53* missense mutations occur in the DNA-binding domain (Vousden & Lu, 2002). Thus, the first published crystal structure of p53's DNA binding core domain (residues 102-292) with a consensus DNA binding site in 1994 (Cho *et al.*, 1994) provided good understanding of mutant p53 nature (Bullock & Fersht, 2001). The core domain consists of a β sandwich that serves as a scaffold for two large loops (L2 and L3 loop) and a loop-sheet-helix (LSH) motif (L1, S2, S2′, S10, H2) (Cho *et al.*, 1994). The β sandwich is a barrel-like structure of several β sheets. p53 contains a zinc atom that is tightly bound and important for the DNA binding activity. The zinc atom is coordinated by the two large loops: C176 and H179 in the L2 loop and C238 and C242 in the L3 loop. DNA-p53 interaction involves three parts: 1) LSH (H2 helix and L1 loop) contact with the major DNA groove 2) L3 loop contact with the minor DNA groove and, 3) phosphate contacts to the DNA backbone flanked by the major and minor groove contacts. Residues K120, C277 and R280 in the LSH make contact with the major DNA groove. R248 in the L3 loop contacts the minor DNA groove (Cho *et al.*, 1994). Contact with the DNA backbone involves the phosphate groups on DNA and several residues including K120, S241, R248, R273, A276, R280, D281 and R283 (Cho *et al.*, 1994; Wieczorek *et al.*, 1996).

The six hot spot mutants R175, G245, R248, R249, R273 and R282 (Bullock & Fersht, 2001; Freed-Pastor & Prives, 2012; Vousden & Lu, 2002) that account for almost a third of *TP53* mutations (Vousden & Lu, 2002) are all located in the DNA core domain (Cho *et al.*, 1994). The two most frequently mutated of these R248 (9.6%) and R273 (8.8%) have direct contact with DNA (Cho *et al.*, 1994). The other four mutants are critical for stabilizing p53 structure for example R175 which is close to the zinc binding site. R249 is adjacent to DNA binding R248 on the L3 loops and surrounded by parts of L2, L3, S3 of the β sandwich. R282 on the H2 helix is important for LSH structure. G245 on the L3 loop is important for the L3 confirmation to form contact between G245, C247 (in contact with zinc) and R249 (previous mentioned hot spot). Most mutations occur at residues that are closest to DNA i.e. L2 and L3 loops and LSH as these locations will have the most detrimental effects on DNA binding.

1.5.2.2 The concept: reactivation of DNA contact mutants

As described (section 1.4.1.1), missense mutations of p53 can be divided into two types of mutations based on the wild type p53 crystal structure (Cho *et al.*, 1994): DNA contact and structural mutations (Nikolova *et al.*, 2000; Wieczorek *et al.*, 1996). The arginines at position 248 and 273 make contact with the DNA backbone (Cho *et al.*, 1994) and so mutations of these sites result in loss of DNA binding while the native structure of the core domain is maintained. The residue T284 is located in the α-helix of p53's DNA binding domain that lies in the DNA's major groove. Substituting threonine (T) with arginine (R) allows contact with the DNA backbone due to the long basic side chain or arginine. Thus, introducing a second mutation at T284R of some DNA contact mutants (R273H, R273C and R248Q) resulted in novel protein-

DNA backbone contacts and enhanced binding to DNA motifs in p53 downstream targets p21 and GADD45 (Wieczorek *et al.*, 1996). In the R273 mutant introduction of T284R restored transcriptional activity of a p53-responsive reporter plasmid to a comparable level of wild type p53 activity and inhibited proliferation of Saos-2 osteosarcoma cells. This indicates that mutations of some of the residues that bind the DNA backbone (i.e. S241, R282, R273 and R283 (Cho *et al.*, 1994)) do not completely abolish sequence specific binding to p53 motifs in downstream target genes (e.g. p21 and GADD45) and may possibly be restored by pharmacological intervention (Wieczorek *et al.*, 1996). Other residues that are in contact with DNA bases, such as K120, C277 and R280, seem more important for DNA binding specificity and such mutants are therefore presumably more difficult to restore.

1.5.2.3 The concept: reactivation of structural mutants

A major fraction of *TP53* mutations affect the structural integrity and stability of the DNA binding domain of p53, so called structural mutants (Brachmann *et al.*, 1998). These mutations result in destabilization of local confirmation or a global denaturation of the entire protein (Bullock *et al*, 2000). Introduction of certain second-site suppressor mutation into structural mutants increased stabilization of parts of the core domain and restored p53 function (Brachmann *et al.*, 1998; Nikolova *et al.*, 2000). The second-site suppressor mutation N268D is located in the β-sandwich and mutation likely increases p53 core domain stability by forming new contacts between the two β-sandwich sheets. In contrast, the second-site suppressor mutations N239Y and S240N are both located in the L3 loop and form new interactions with the DNA backbone or β-sandwich sheet respectively. The individual second-site suppressor mutations did not induce global stabilization but local stabilizations were observed, suggesting that compounds targeting specific regions may have activity in specific tumorigenic mutations depending on the location of the mutation (Brachmann *et al.*, 1998). However, double second-site mutations N268 and N239Y resulted in global stabilization and recovery of sequence specific DNA binding of tumor mutations G245S and V143A (Nikolova *et al.*, 2000). G245S locates to the L3 loop of the DNA-binding region and is only weakly destabilized (Brachmann *et al.*, 1998; Bullock & Fersht, 2001). V143A is an example of mutations located in the β-sandwich sheet, a location that accounts for a quarter of all missense mutations, and leads to global denaturation of the protein (Bullock & Fersht, 2001). Thus, several structural p53 mutants may be rescued by amino acid substitution elsewhere in the core domain, suggesting that small molecule-mediated rescue may be feasible. However, mutations affecting residues that coordinate the zinc atom i.e. C176 (L2 loop), H179 (H1 helix in the L2 loop) and C238 and C242 (both L3 loop), will most likely be difficult to rescue as loss of zinc leads to structural collapse (Bullock & Fersht, 2001).

1.5.2.4 Temperature sensitive mutants

Many p53 mutants are temperature sensitive and retain native structure at lower temperatures while unfolded at 37°C (Friedlander *et al*, 1996; Kaar *et al*, 2010; Zhang *et al*, 1994). Hot spot mutant V143A has even stronger DNA binding and transcriptional abilities than wild type p53 at 32.5°C. However, at 37°C it looses its structure as shown by undetectable staining with

monoclonal antibody PAb1620 that recognizes wild type p53 confirmation (Zhang *et al.*, 1994). The V143A, R175H, R248W, R249S and R273H mutants that are not able to bind DNA at 37°C can actually bind DNA at lower temperatures (25-33°C), and R273H, R248W and V143A activate transcription of the MDM2 promotor at 26°C. Heating the mutant proteins to 37°C irreversibly abolished their DNA binding activity, although this destabilization could partly be rescued with the monoclonal anti-p53 antibody PAb1801. This illustrates that many hot spot mutants may have intrinsic capacity to bind DNA and can suggest that they potentially can be stabilized by small molecules (Friedlander *et al.*, 1996).

1.5.2.5 Cysteines - targets for electrophilic modifications

Electrophiles are electron-deficient molecules that react with other molecules that have unshared valence electron pairs i.e. nucleophiles. A covalent bond is formed upon the donation of an unshared electron pair from a nucleophile to an electrophile (Eriksson *et al.*, 2019; LoPachin *et al*, 2019). This type of reaction is important for many intracellular processes for instance enzyme activity and function (LoPachin *et al.*, 2019). Intracellularly, deprotonated cysteines or selenocysteines are the strongest nucleophiles (Pace & Weerapana, 2013) and thus prime targets of electrophilic compounds (Eriksson *et al.*, 2019). Although cysteine is the least abundant amino acid incorporated into proteins (2%) (Miseta & Csutora, 2000; Pace & Weerapana, 2013), its importance is reflected by the fact that it is one of the most frequently mutated amino acids associated with disease (Wu *et al*, 2007). The large atomic radius of the sulfur atom and the low dissociation energy of the S-H bond makes the thiol group of cysteines highly reactive. The thiol ionization state of the cysteine determines its nucleophilicity and reactivity, rendering it highly sensitive to quick (within minutes) changes in the protein environment. Besides reacting with electrophiles, cysteines may also bind metals, catalyze redox reactions and form disulfide bonds. Many of these processes are important for transcription factors and enzymes, such as kinases and protease, and thus many proteins have cysteines in sites important for catalytic activity, allosteric regulation or metal binding ligands (Pace & Weerapana, 2013).

p53 has ten cysteines that are all located in the DNA binding core domain and are important for p53 structure. The cysteines have different thiol reactivity depending on their nucleophilic character and solvent accessibility. Thus, cysteines that are strong nucleophiles and exposed to the surface of the protein are the most reactive (Eriksson *et al.*, 2019; Kaar *et al.*, 2010) (Figure 8). The wild type confirmation of p53 is important for its ability to bind DNA (Rainwater *et al*, 1995) and due to cysteine's importance in intracellular reactions it is not a surprise that redox modifications affects p53's ability to bind DNA (Hainaut & Milner, 1993a; Hupp *et al*, 1993; Rainwater *et al.*, 1995). Three cysteines (C176, C238 and C242) and H179 coordinate a zinc atom in the core domain (Bullock *et al*, 1997; Cho *et al.*, 1994), rendering p53 DNA binding dependent on a reducing environment (Hainaut & Milner, 1993a). The zinc atom binds with high affinity to these cysteines which results in a stable structure y bridging to the two loose L2 and L3 loops that bind DNA (Bykov *et al*, 2009; Cho *et al.*, 1994). Thus, the zinc atom is crucial for proper folding of p53 (Bullock *et al.*, 1997; Eriksson *et al.*, 2019). Indeed, the hot

spot R175H mutation close to the zinc binding site is characterized by global denaturation (Bullock & Fersht, 2001; Bykov *et al.*, 2009). Zinc is vital for the DNA binding ability of several other transcription factors, besides p53, e.g. NFκB (Hainaut & Milner, 1993a; Zabel *et al*, 1991). Furthermore, the zinc atom also protects the cysteines from oxidation, which would otherwise lead to disulfide-linked aggregation of p53 protein due to the formation of intramolecular or intermolecular disulfide bridges between p53 cysteines (Bykov *et al.*, 2009). Oxidation of p53 could also lead to disulfide crosslinks with cysteines on other redox-sensitive proteins. For example, the R175H mutation, adjacent to C176 perturbs zinc coordination leading to an oxidation-prone mutant p53 protein. To summarize, both redox status and zinc bioavailability regulates p53 folding and activity (Bykov *et al.*, 2009), which renders p53 highly sensitive to electrophilic assaults (Eriksson *et al.*, 2019) (Figure 8).

1.5.2.6 Soft electrophiles

Electrophilic ("electron lover") compounds have atoms that are electron-deficient, thus partially positive, and react with nucleophilic ("nucleus lover") groups that have unshared outer shell electron pairs (Fessenden *et al*, 1998). Many of the mutant p53-reactivating compounds identified so far share the property of targeting cysteines and are so called soft electrophiles (Bykov *et al.*, 2018; Eriksson *et al.*, 2019). Electrophiles can be divided based on their electronic disposition (softness or hardness) which determines the type of nucleophiles they will react with (LoPachin *et al.*, 2019). The softness or hardness is determined by the ease of electrons to delocalize. A covalent bond is formed when the two atoms share outer-shell (valence) electrons, for example a single bond between two atoms is the sharing of one pair of electrons (Fessenden *et al.*, 1998). As mentioned above, sulfur is a relatively large atom (Pace & Weerapana, 2013) and since the outer-shell electrons are far from the nucleus, electrons are easily distorted. This characterizes a so called soft nucleophile (LoPachin *et al.*, 2019). Since electrophiles preferentially react with nucleophiles that are of comparable softness or hardness (LoPachin *et al.*, 2019), soft electrophiles preferentially react with cysteines e.g. cysteines located in the core domain of p53(Bykov *et al.*, 2018). Hard nucleophiles, such as the amino groups on lysine or histidine, are therefore not preferentially bound by soft electrophiles (LoPachin *et al.*, 2019). For example the hard electrophilic group of cisplatin forms DNA adducts by binding to guanine residues which have hard nucleophilic groups. Besides the softness and hardness, also other factors, e.g. steric hindrance, will affect whether an electrophile reacts with a nucleophile (LoPachin *et al.*, 2019). Importantly, any electrophilic compound that targets protein thiols would also be expected to induce oxidative stress, for example by conjugating to low molecular weight molecules such as the tripeptide glutathione in which thiol binding is less restricted by steric hindrance than thiol binding in larger proteins (Bauer *et al*, 2016; Bykov *et al.*, 2018; Eriksson *et al.*, 2019) (Figure 8).

The first published mutant p53-reactivating compound was thiol binding CP-31398 (Foster *et al*, 1999). It was shown to stabilize wild type p53 binding and maintain active confirmation of newly synthesized mutant p53 (Foster *et al.*, 1999; Rippin *et al*, 2002). Furthermore, CP-31398 inhibited tumor growth of melanoma and colon carcinoma-derived xenografts (Foster *et al.*,

1999) and progression of bladder cancer growth in a transgenic mouse model (Madka *et al*, 2013). To date there are no ongoing clinical trials with CP-31398 (Bykov *et al.*, 2018).

The mutant p53-reactivating compound PRIMA-1 (p53 Reactivation and Induction of Massive Apoptosis) was identified by Bykov, Wiman and colleagues in a cellular screen of the US National Cancer Institute (NCI) Diversity set containing 2000 low molecular weight compounds with diversified structures (Bykov *et al.*, 2002b). p53 null Saos-2 osteosarcoma cells containing exogenous tetracycline-regulated mutant p53 R273H (Tet-off) were treated with the library compounds to asses mutant p53-dependent growth suppression. PRIMA-1 enhanced DNA binding of mutant p53, induced expression of p53 downstream targets such as p21, PUMA, BAX and MDM2 and exhibited mutant p53-dependent anti-tumor activity *in vivo* (Bykov *et al.*, 2002b; Bykov *et al.*, 2005b). PRIMA-1Met, now called APR-246 or Eprenetapopt, is a methylated form of PRIMA-1, and was shown to be more active than the original compound possibly due to increased lipophilicity and cell permeability (Bykov *et al.*, 2005b). APR-246 is the most clinically advanced mutant p53-targeting compound and results from phase Ib/II clinical trial in *TP53* mutant MDS/AML have recently been published (Sallman *et al.*, 2021). APR-246 is currently tested in a Phase III clinical trial in *TP53* mutant MDS. Mechanism of action and clinical trials will be further discussed in section 1.5.3.

STIMA-1 and MIRA-1 are two other soft electrophiles the preferentially target mutant p53 expressing cells and induce p53 target genes (Bykov *et al*, 2005a; Zache *et al*, 2008a). MIRA-1 was found in the same screen that identified PRIMA-1 as a mutant p53-reactivating compound (Bykov *et al.*, 2009). Fersht and colleagues identified the Michael acceptor 3-benzoylacrylic acid (3BA) and showed that it thermostabilizes the core domain of wild type p53 and several hot spot mutants (Kaar *et al.*, 2010). 3BA increased the melting temperature of hot spot mutants R175H, Y220C, G245S, R249S and R282W by up to 3°C through covalent binding of cysteines. Derivatives of 3BA that lacked the α,β-unsaturated double bond, characteristic for a Michael acceptor, were not able to react with p53, demonstrating that the Michael addition reaction is essential for targeting wild type and mutant p53. Analysis by mass spectrometry (MS) showed that C124 and C141 were first to react (Figure 8), followed by C135, C182 and C277, and lastly C176 and C275.

Fersht's group also identified another class of thiol-reactive mutant p53 reactivating-compounds that bind cysteines through nucleophilic aromatic substitution. These were electrophilic 2-sulfonylpyrimidines (SP) among which PK11007 showed anti-cancer activity both in a p53-dependent and independent manner (Bauer *et al.*, 2016). PK11007 reactivated mutant p53 and stabilized wild type p53 by binding the surface exposed cysteines C277 and C182. PK11007 also induced oxidative stress by depleting glutathione, which had a more pronounced effect on mutant p53-harboring cells.

Recently, a different type of compound, arsenic trioxide (ATO), has been shown to promote folded structure of several p53 mutants. Unlike the other molecules described, ATO does not contain carbons but has two cysteine-binding arsenic (As) atoms. Crystal structures of mutant p53 showed that the As atom covalently bound to a cryptic cysteine triad (C124, C135 and

C141) between the β sandwich and the LSH motif resulting in a confirmation shift of the cysteines, particularly C141. Specifically structural mutants were reactivated by ATO and increased in thermostability and capacity to bind p53 target genes *PUMA* and *CDKN1A* (p21). DNA binding p53 mutants were less affected by ATO. A cysteine triad can also be found in the oncogenic PML-RARα fusion protein in acute promyelocytic leukemia (APL) for which ATO is FDA-approved. ATO is being tested in phase I clinical trials in p53 mutated hematological diseases (Chen *et al*, 2020).

1.5.2.7 Zn^{2+} chelating compounds

As mentioned in section 1.5.2.5, the zinc atom in p53, coordinated by C176, H129, C238 and C242, is important for the structural integrity of the core domain (Bullock *et al.*, 1997; Cho *et al.*, 1994). Several studies have shown that manipulating zinc concentrations affects wild type p53 structure (Butler & Loh, 2003; Hainaut & Milner, 1993b; Meplan *et al*, 2000). This also has relevance for reactivation of mutant p53 (Bykov *et al.*, 2018) (Figure 8). An analysis of the NCI database for substances that preferentially target mutants p53 (R175, R248 and R273) compared to wild type p53 identified the thiosemicarbazone zinc metallochaperone-1 (ZMC1 [NSC319726]) (Yu *et al*, 2012). It is thought that ZMC1 is a synthetic metallochaperone by functioning as a Zn^{2+} ionophore i.e. a molecule that transports metal ions, in this case Zn^{2+} (Blanden *et al*, 2015; Loh, 2010; Yu *et al*, 2014). ZMC1 binds extracellular Zn^{2+} and diffuses it across the plasma membrane. TOV-112D ovarian cancer cells that harbor R175H mutant *TP53* showed an increased Zn^{2+} concentration upon treatment with ZMC-1. R175H is a common "hot spot" *TP53* mutation and due to the close proximity of the substituted residue to the zinc atom binding site, the R175H mutant is unable to bind zinc(Blanden *et al.*, 2015). ZMC1 was found to restore the zinc binding capacity of the R175H mutant which reactivated its wild type p53 function (Blanden *et al.*, 2015; Yu *et al.*, 2014). ZMC-1 treatment also depletes glutathione, chelates iron and induces oxidative stress (Yu *et al.*, 2012). However, in the presence of N-acetyl cysteine (NAC), ZMC-1 was still able to promote wild type confirmation and apoptosis in R175H harboring cells (Yu *et al.*, 2014).

COTI-2 is another thiosemicarbazone that has been reported to reactivate mutant p53 and have anti-tumor activity (Lindemann *et al*, 2019; Salim *et al*, 2016; Synnott *et al*, 2020). Its p53-dependent mechanisms of action are not clear and it also has p53-independent effects, including inhibition of the PI3K-AKT pathway (Bykov *et al.*, 2018). Nevertheless, it was shown to promote a folded structure of unfolded R175H mutant p53 to wild type confirmation as shown by PAb1620 staining in SKBR3 cells (Synnott *et al.*, 2020). COTI-1 has been tested in Phase I clinical studies in several solid cancers, but the current status is unknown according to clinicaltrials.gov.

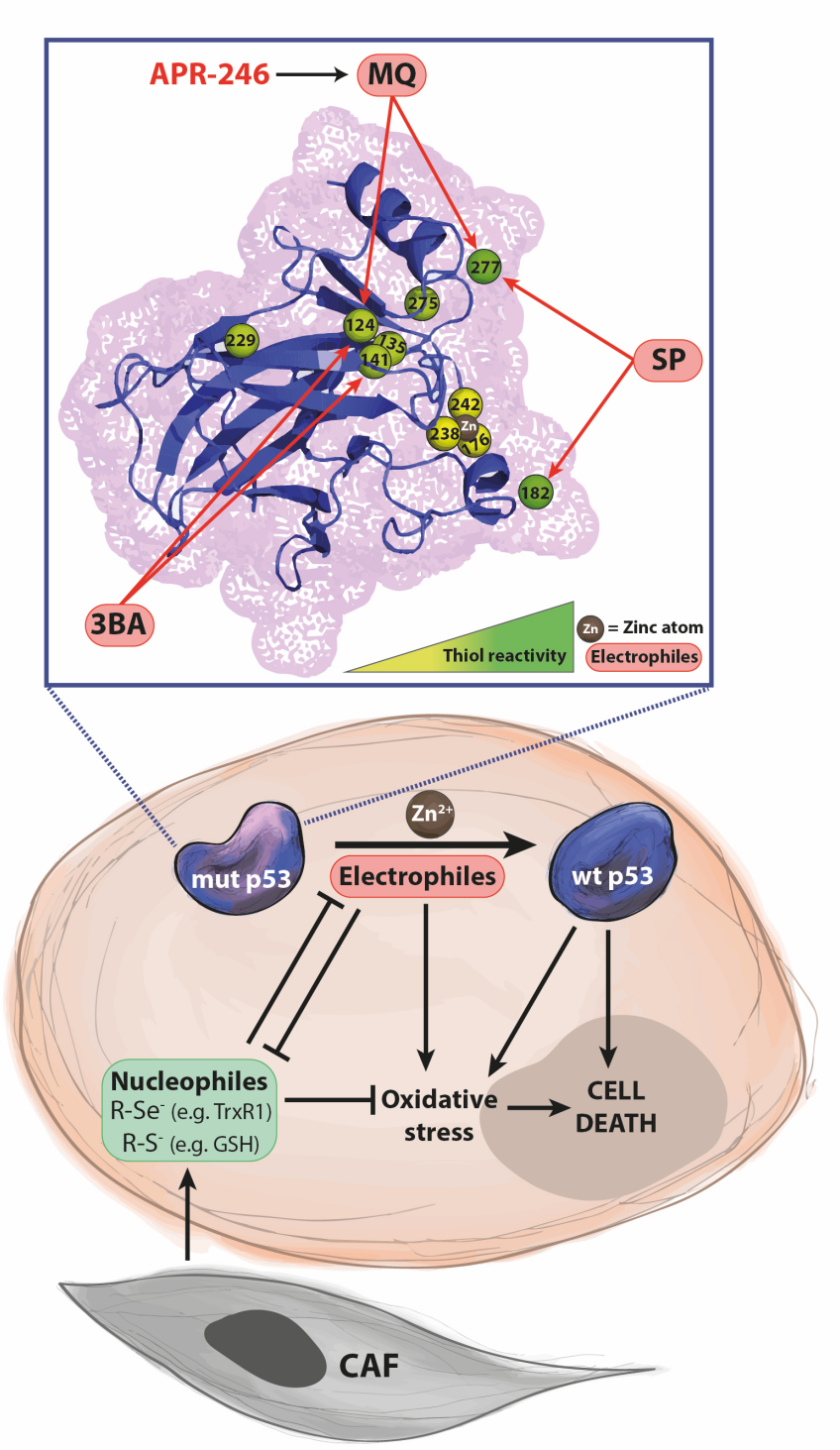

Figure 8 Mutant p53 rescue and induction of oxidative stress as mechanisms of cell death by mutant p53 reactivating compounds. Mutant p53 reactivating compounds are electrophiles that target cysteines in mutant p53 which results in stabilization of its protein structure. Electrophiles also target nucleophiles such as cysteines (R-S-) in low molecular weight molecules (e.g. glutathione [GSH]) or proteins, or selenocysteine (R-Se-) in selenoproteins (e.g. thioredoxin reductase 1 [TrxR1]) that are part of the antioxidant defense systems. Electrophiles induce oxidative stress contributing to its mechanism of action. Zinc chelation also reactivates mutant p53 and has effects on redox homeostasis. The crystal structure in the top of the figure shows the wild type p53 core domain (Cho *et al*, 1994) with cysteines colored according to their thiol reactivity (green most reactive, yellow least reactive) and the zinc atom in brown. Cysteines targeted by mutant p53 reactivating compounds have been indicated. MQ = methylene quinuclidinone, SP = sulfonylpyridines, 3BA = 3-benzoylacylic acid. Figure is from the review Eriksson, Ceder *et al* 2019.

1.5.3 APR-246

1.5.3.1 Mutant p53 reactivation

Both prodrugs PRIMA-1 and APR-246 (Eprenetapopt/PRIMA-1Met) are spontaneously converted to the active product methylene quinuclidinone (MQ) (Lambert *et al.*, 2009) (Figure 9). Of note, this also generates formaldehyde which does not seem to contribute to the growth suppression, as shown by treating cells with up to 50μM formaldehyde. MQ is a Michael acceptor due to its chemically active double bond which is prone to participate in reaction of nucleophilic addition (described in 1.5.3.5). PRIMA-D (APR-320), an analog of PRIMA-1 that cannot be converted to MQ, or MQ-H (lacks Michael acceptor activity) are completely inactive (Lambert *et al.*, 2009; Mohell *et al.*, 2015; Zhang *et al*, 2018b). In cells, the most common nucleophilic targets will be thiol (-SH) groups, which are often found in proteins, for example p53 (Cho *et al.*, 1994), but the most predominant cellular thiol is found in the antioxidant glutathione which is present at millimolar concentration in cells (Berg *et al*, 2007). The covalent binding of MQ to the mutant p53 core domain promotes wild type p53 conformation and apoptosis (Lambert *et al.*, 2009). PRIMA-1 and APR-246 have demonstrated anti-cancer efficacy in several cancer types *in vitro* in cultured cells, *in vivo* in tumor xenograft mouse models as well as *ex vivo* in primary cultures and patient-derived organoids (Bykov *et al.*, 2002b; Ceder *et al.*, 2020; Demir *et al.*, 2020; Liu *et al.*, 2015; Synnott *et al.*, 2017; Zache *et al*, 2008b; Zandi *et al*, 2011) while their growth-inhibitory effect is less pronounced in non-cancerous fibroblasts (Ceder *et al.*, 2020; Hang *et al.*, 2018; Mlakar *et al*, 2019) or keratinocytes (Mlakar *et al.*, 2019).

APR-246/MQ fulfills several criteria for a mutant p53 reactivating compound suggested by Fersht and colleagues. These include that the compound should bind and thermostabilize mutant p53, restore wild type p53 confirmation, induce p53 dependent transactivation of target genes and show p53-dependent synergy with MDM2 inhibitors that induce wild type p53 (Liu *et al*, 2013). MQ binding to wild type and mutant p53 has been demonstrated by mass spectrometry in Project II but also previously (Lambert *et al.*, 2009; Zhang *et al.*, 2018b). Thermostabilization has been observed using differential scanning fluorimetry (DSF, [measures protein melting temperature]) and circular dichroism spectroscopy (CD, [assesses α-helix and β-sheet structure]) of MQ-treated wild type and mutant (R273H and R175H) p53 recombinant core domains (Zhang *et al.*, 2018b). This study identified C277, which is the most reactive cysteine in p53 and located on the DNA binding surface (Cho *et al.*, 1994; Kaar *et al.*, 2010), to be essential for MQ-mediated thermostabilization (Zhang *et al.*, 2018b) in line with the findings in Project II. Also C124, that lies in the center of the flexible L1/S3 pocket (Cho *et al.*, 1994), was targeted by MQ (Zhang *et al.*, 2018b). R175H mutant p53 with Cys to Ala replacements at C124 and C277 could not be reactivated by APR-246, to induce cell death and activation of downstream targets in transiently transfected H1299 lung adenocarcinoma cells (*TP53* null).

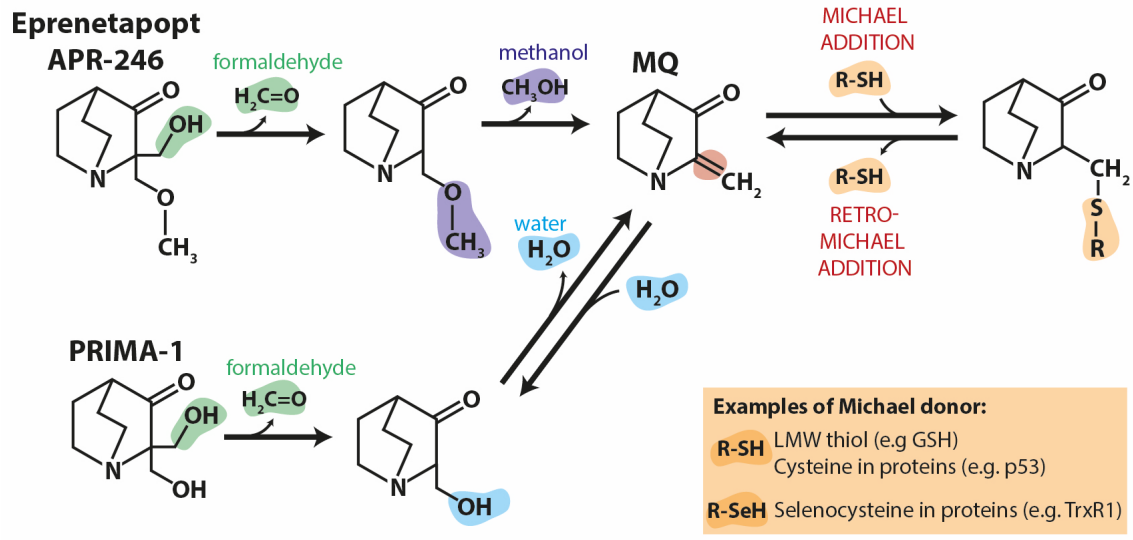

Figure 9 APR-246 (Eprenetapopt) and PRIMA-1 form active product MQ. APR-246 (PRIMA-1Met/Eprenetapopt) and PRIMA-1 both convert to active product methylene quinuclidinone (MQ) (Lambert *et al*, 2009). MQ is an Michael acceptor, a soft electrophile with an α,β-unsaturated bond (red) that is prone to nucleophilic addition with a Michael donor for example low molecular weight (LMW) thiols e.g. glutathione (GSH) or a thiol group (R-SH) in proteins e.g. p53. Another example of Michael donors are selenocysteine (Se)-containing proteins e.g. thioredoxin reductase 1 (TrxR1). MQ covalently conjugates to cysteine or selenocysteine in a Michael addition. The MQ binding is reversible (Ceder *et al*, 2020) and may undergo retro-Michael addition, resulting in MQ travelling between thiols.

PRIMA-1/APR-246 also promote wild type p53 confirmation as shown by positive wild type-confirmation specific PAb1620 antibody immunostaining (Bykov *et al.*, 2002b; Liang *et al*, 2011; Zhang *et al.*, 2018b) and immunoprecipitation (Demir *et al.*, 2020) as well as loss of immunostaining using the antibody PAb240 (Bykov *et al.*, 2002b; Liang *et al.*, 2011) or HO3.5 (Zhang *et al.*, 2018b) binding to epitopes exposed in mutated p53. PRIMA-1/APR-246 induced expression of p53 targets such as p21 (Bykov *et al.*, 2002b; Demir *et al.*, 2020; Liu *et al.*, 2015). Preferential growth suppression in mutant p53 cells have been shown in various cell systems including isogenic cell lines and upon p53 knockdown (Ali *et al.*, 2011; Bykov *et al.*, 2002b; Bykov *et al.*, 2005b; Ceder *et al.*, 2020; Demir *et al.*, 2020; Liu *et al.*, 2015; Roh *et al.*, 2011; Shi *et al*, 2008). Furthermore, synergistic effects have been reported upon combination treatment with PRIMA-1 and Nutlin-3 in pancreatic cancer cells harboring mutant *TP53* (Izetti *et al.*, 2014). Synergy with APR-246 has also been observed with Nutlin-3, Idasanutlin and MI-773 (SAR405838) in mutant *TP53*-carrying ovarian cancer cells (Ceder et al, unpublished data).

1.5.3.2 Redox effects

Apart from reactivating mutant p53, PRIMA-1/APR-246 can also disrupt redox homeostasis due to the electrophilic properties of MQ, contributing to PRIMA-1/APR-246-induced cancer cell death (Ceder *et al.*, 2020; Haffo *et al*, 2018; Liu *et al.*, 2017; Mlakar *et al.*, 2019; Peng *et al*, 2013; Synnott *et al*, 2018; Tessoulin *et al.*, 2014)

Glutathione is a low molecular weight molecule consisting of just three amino acids: γ-glutamate, cysteine and glycine, and is highly abundant inside cells (Berg *et al.*, 2007). Compared to thiols in protein, the thiol in glutathione is less sterically hindered and therefore readily conjugated by MQ (Lambert *et al.*, 2009). Cells are highly adaptable to counteract oxidative stress through various mechanisms (section 1.6). However, any treatment with APR-246 is accompanied by glutathione depletion and oxidative stress, which may contribute to cell death. Since also the function of wild type p53 is dependent on a reduced environment (Hainaut & Milner, 1993a), and since all activities are due to the reactivity of MQ it becomes difficult to separate the oxidative stress-induced mechanisms from the mutant p53-reactivating mechanisms. One of the building blocks, cysteine, is imported by xCT in its oxidized form (cystine). Both high expression of xCT and high glutathione levels are correlated with decreased sensitivity to APR-246 (Ceder *et al.*, 2020; Liu *et al.*, 2017). Also, inhibition of glutathione production by for example buthionine sulfoximide (BSO) drastically sensitizes cells to APR-246 (Lambert *et al.*, 2009; Tessoulin *et al.*, 2019; Tessoulin *et al.*, 2014). Glutathione-conjugated MQ (GS-MQ) is exported via the multidrug resistance protein 1 (MRP1) and upon inhibition leads to pronounced synergy with APR-246 as described in Project I (Ceder *et al.*, 2020). Thus, APR-246 sensitivity is dependent on mutant p53, cellular thiol status as well as drug accumulation, but neither factor alone can fully explain the sensitivity to APR-246 .

Glutathione is intracellularly highly abundant and nucleophilic due to its cysteine, however, the most potent cellular nucleophile is selenocysteine (Sec or U) (Ralston, 2018). Selenocysteine is a structural analogue of cysteine but with a selenium (Se) instead of the sulfur atom (Arner, 2010). Out of the 25 selenoprotein genes in human, around half are enzymes (Ralston, 2018). Thioredoxin reductase 1 (TrxR1) is one such enzyme and uses NADPH for the reduction of oxidized thioredoxin (Trx) and other substrates. Both TrxR1 and Trx are important for redox regulation (Arner, 2009). TrxR1 forms a homodimer with another TrxR1 protein subunit. The selenium located in the selenylsulfide-motif (-GCUC-) at the C-terminal active site is reduced by the transfer of two electron from NADPH by the dithiol in the N-terminal site (of the other TrxR1 subunit) and further transferred to the substrate (e.g. oxidized Trx). Thus, both subunits are required for its normal function (Arner, 2009). APR-246 targets the selenocysteine in TrxR1, resulting in inhibition of TrxR1 activity which decreases the antioxidant defense capacity of cells (Peng *et al.*, 2013). In addition, modification of the selenocysteine residue by MQ converts TrxR1 to a dedicated NADPH oxidase (Peng *et al.*, 2013), as has previously been seen with other compounds that modulate TrxR1's selenocysteine (Anestal & Arner, 2003; Anestal *et al*, 2008). Cisplatin inactivates TrxR1 in a similar way, and it has been proposed that this mechanism is important for its anti-tumor activity. Thus, APR-246 treatment does not just interrupt the antioxidant capacities of TrxR1, it also converts it to a NADPH oxidase with prooxidant properties which results in elevated oxidative stress contributing to APR-246-mediated cell death (Peng *et al.*, 2013). Furthermore, MQ also inhibits Trx and glutaredoxin (Grx) which both are important antioxidants for

maintaining redox homeostasis, probably contributing to the oxidative stress observed in cells treated with APR-246 (Haffo *et al.*, 2018).

1.5.3.3 Mutant p63 and p73 reactivation

p63 and p73, the other two members of the p53 family, share extensive sequence homology with p53, especially within the DNA binding domain. Therefore, it is not surprising that mutant versions of p63 and p73 could be reactivated by APR-246, as shown by upregulation of downstream targets p21 and Noxa (Rokaeus *et al*, 2010). p63 is important for regulating skin development and mice that have deleted p63 lack stratified epithelia in several organs (Barbieri & Pietenpol, 2006; Senoo *et al*, 2007). Mutation of p63 is associated with several rare disorders, including the ectodermal dysplasia syndrome (ED) (Aberdam *et al*, 2020; Rinne *et al*, 2007). ED results in abnormal development of tissues (e.g. skin, hair, teeth, nails and exocrine glands) that develop from the embryonal ectoderm (outer) layer (Rinne *et al.*, 2007). Two major phenotypes are presented in ED patients: ectrodactyly, ectodermal dysplasia, and cleft lip/palate (EEC) and 1/ankyloblepharon-ectodermal dysplasia-clefting syndrome (AEC) (Aberdam *et al.*, 2020). Besides the abnormal development of tissues around 40% of EEC patients also have clefts lips. AEC patients may be born with eyelids fusion (ankyloblepharon) and typically have extreme dry skin and sometimes patches that erode and do not heal . EEC syndrome is mainly caused by point mutations in the DNA binding domain while AEC syndrome-associated mutations occur in the C-terminal steril α-motif (SAM) domain (Rinne *et al.*, 2007). There is no curative treatment for AEC and EEC patients (Aberdam *et al.*, 2020). APR-246 rescued the mutant p63-associated phenotype of primary skin keratinocytes derived from EEC (Shen *et al*, 2013) and AEC syndrome patients (Aberdam *et al.*, 2020). Fibroblasts from EEC patients that were reprogrammed to induced pluripotent stem cell (iPSC) lines and had impaired ability to differentiate into epidermal and corneal epithelial cells were rescued by APR-246. Treatment with APR-246 reverted the lineage commitment and restored normal p63 signaling (Shalom-Feuerstein *et al*, 2013). Moreover, APR-246 was reformulated in a cream and applied topically on the eroded skin of two AEC patients (hand and scalp) (Aberdam *et al.*, 2020). In both patients the area treated with the cream showed re-epithelization of the eroded skin and decreased pain. These data suggest that APR-246 may be used for the treatment of local AEC erosions to decrease pain, leading to increased quality of life for these patients.

1.5.3.4 Synergies with other compounds

Combination treatment of APR-246 with several chemotherapeutic drugs and other targeted treatments have been reported to result in synergistic cell death or growth suppression (Bykov *et al*, 2016). There may be several reasons for why a combination treatment with APR-246 leads to a synergistic anti-tumor outcome (Bykov *et al.*, 2018). Mutant p53 reactivation to a wild type p53-like protein might increase sensitivity to DNA-damaging chemotherapeutic drugs that trigger the p53 activation cascade. Indeed, several studies have shown that cisplatin synergizes with APR-246 in inducing cancer cell death (Bykov *et al.*, 2005b; Fransson *et al.*, 2016; Kobayashi *et al.*, 2013; Liu *et al.*, 2015; Mohell *et al.*, 2015; Roh *et al.*, 2011). The observed synergy may also be due to that cells are inactivating cisplatin by glutathione

conjugation followed by efflux of the conjugate (Ghosh, 2019; Ishikawa *et al*, 1994). Therefore, depletion of glutathione by MQ will most likely inhibit cisplatin export (Bykov *et al.*, 2018). Synergy between APR-246 and compounds may also occur due to the compromised antioxidant capacity and increased oxidative stress induction attributed to APR-246 (section 1.5.3.2) (Bykov *et al.*, 2016). For example, cisplatin also induces oxidative stress, shown by increase in reactive oxygen species (ROS) such as hydroxyl radicals (Brozovic *et al*, 2010), which may sensitize the cells to the redox effects of MQ. Other chemotherapeutic agents having shown synergistic growth suppression or cell death in combination with APR-246 are listed in Table 2. Some of these combinations are being explored in the clinical trials including the combination with 5-azacitidine in *TP53* mutant MDS patients (Sallman *et al.*, 2021) (section 1.5.3.7).

The finding that mutant p53 binds master antioxidant regulator NRF2 (Lisek *et al.*, 2018; Liu *et al.*, 2017; Walerych *et al.*, 2016) described in section 1.4.2.4 suggests interesting possible combination treatments with APR-246. Several proteosome inhibitors are currently used in the clinic for the treatment of multiple myeloma and mantle cell lymphoma (Sherman & Li, 2020). Bortezomib was the first to obtain FDA approval (2003) as a third of relapsed, refractory multiple myeloma patients responded to the treatment in Phase II clinical trials (Fricker, 2020). Bortezomib has recently become frontline treatment for multiple myeloma (Sherman & Li, 2020). Carfilzomib and ixazomib were FDA-approved 2012 and 2015, respectively. It is thought that proteosome inhibitors are effective in hematological tumors due to the high secretory load making these cells dependent on proteostasis mechanisms (Sherman & Li, 2020). Although these inhibitors are effective at inhibiting the proteosome, the downstream mechanisms that ultimately lead to cell death remain uncertain (Fricker, 2020). The proteosome machinery was identified as a major common target by mutant p53 via its interaction with NRF2 (Walerych *et al.*, 2016) (Figure 7). In the presence of NRF2, mutant p53 binds to the promotors of proteosome genes (*PSMA2* and *PSMC1*) and upregulates NRF2-dependent transcriptional activity which may be a resistance mechanism against proteosome inhibitors (Figure 7). This interaction was abolished upon treatment with PRIMA-1, and PRIMA-1 in combination with carfilzomib resulted in synergistic reduction of cell viability and proteosome activity in breast cancer cell lines. The combination treatment also resulted in synergistic tumor growth inhibition and significantly reduced frequency of lymph node and lung metastases in an orthotopic breast cancer xenograft model (Walerych *et al.*, 2016).

Mutant p53, through entrapment of NRF2, also negatively regulates transcription of the *SLC7A11* gene, and hence production of the cystine/glutamate antiporter (Liu *et al.*, 2017). The cystine/glutamate antiporter system x_c^- is made up of two subunits, xCT (*SLC7A11*) and 4F2 (*SLC3A2*), and imports cystine (oxidized cysteine) while exporting glutamate (Lewerenz *et al*, 2013). Cysteine is one of the key building blocks for the antioxidant glutathione (Lu, 2013) and a rate limiting substrate for glutathione synthesis (Lewerenz *et al.*, 2013). Mutant p53 and NRF2 binding to the *SLC7A11* promotor results in limited intracellular cysteine availability and therefore diminished glutathione synthesis, rendering mutant p53 harboring cells sensitive to oxidative stress (Liu *et al.*, 2017). *SLC7A11* expression was demonstrated to be a robust

	Type of therapy	Name of therapy	Reference
Chemotherapeutics	Alkylating agents	Cisplatin, carboplatin	(Bykov *et al*, 2005b; Fransson *et al*, 2016; Kobayashi *et al*, 2013; Liu *et al*, 2015; Mohell *et al*, 2015; Roh *et al*, 2011)
	Anthracyclines	Doxorubicin, daunorubicin	(Ali *et al*, 2011; Demir *et al*, 2020; Fransson *et al.*, 2016; Magrini *et al*, 2008; Mohell *et al.*, 2015)
	Antimetabolites	5-Aza, 5-FU, fludarabine, gemcitabine	(Ali *et al.*, 2011; Liu *et al.*, 2015; Maslah *et al*, 2020; Mohell *et al.*, 2015)
	Microtubule inhibitor	Eribulin	(Synnott *et al*, 2017)
Other therapies	p53-MDM2 inhibitor	Nutlin-3	(Izetti *et al*, 2014)
	Radiation		(Krayem *et al*, 2019; Nikolaev *et al*, 2020; Supiot *et al*, 2008)
	xCT inhibitors	Sulfasalazine, erastin	(Ceder *et al*, 2020; Liu *et al*, 2017)
	MRP1 inhibitors	MK-571, reversan	(Ceder *et al.*, 2020)
	GCL inhibitor	BSO	(Lambert *et al*, 2009; Tessoulin *et al*, 2019; Tessoulin *et al*, 2014)
	TrxR1 inhibitor	Auranofin	(Lisek *et al*, 2018)
	Proteosome inhibitor	Carfilzomib	(Walerych *et al*, 2016)
	BRAF inhibitor	Vemurafenib	(Krayem *et al*, 2016)
	BRAF inhibitor + radiation	Vemurafenib	(Krayem *et al.*, 2019)
	MEK inhibitor	Pimasertib	(Najem *et al*, 2017)
	PI3K inhibitors	BKM120, wortmannin, PHEN	(Ali *et al*, 2016; Li *et al*, 2018)
	mTOR inhibitor	Rapamycin	(Ali *et al.*, 2016)
	Histone methylation inhibitor	DZNep	(Cui *et al*, 2014)
	PARP inhibitors	Olaparib, PHEN	(Deben *et al*, 2016; Synnott *et al.*, 2017; Yin *et al*, 2018)
	Prooxidant	Piperlongumine	(Hang *et al*, 2018)

Table 2 Promising combination treatments with APR-246. The table summarize various types of compounds that have been published to lead to increased sensitivity or synergistic cell death / growth suppression in combination treatment with APR-246 or PRIMA-1. 5-FU = Fluorouracil, 5-Aza = 5-azacitidine, DZNep = 3-Deazaneplanocin A, PHEN = 6(5H)-phenanthridinone

predictive biomarker for APR-246 sensitivity. Furthermore, the combination of the xCT inhibitor sulfasalazine and APR-246 resulted in synergistic tumor suppression in esophageal cancer patient-derived xenografts and decreased GSH levels in the tumors (Liu *et al.*, 2017). Later data suggests that the decreased glutathione levels led to increased retention of APR-246's active product MQ (Ceder *et al.*, 2020). Glutathione-conjugated MQ (GS-MQ) is exported via efflux pump multidrug resistance protein 1 (MRP1). GS-MQ export can effectively be blocked by MRP1 inhibitors such as MK-571 and reversan, as well as by MRP1 knockdown using siRNA (small interfering RNA), which were shown to result in pronounced synergistic growth suppression *in vitro*, *in vivo* and *ex vivo* (Ceder *et al.*, 2020) (Project I). Both increasing drug accumulation and limiting the antioxidant capacity of cells are effective means to cause synergistic cell death in combination with APR-246. Synergy by limiting antioxidant capacity has also been demonstrated using glutathione synthesis inhibitor BSO (Lambert *et al.*,

2009; Tessoulin *et al.*, 2019; Tessoulin *et al.*, 2014) and TrxR1 inhibitor auranofin (Lisek *et al.*, 2018).

1.5.3.5 The active product MQ, a Michael acceptor

A common feature of the mutant p53-reactivating compounds MQ (the active product of APR-246 and PRIMA-1), CP31398, MIRA-1, STIMA-1, 3-benzoylacrylic acid and KSS-9 is their electrophilic character and ability to perform Michael addition reactions. In other words, their chemical structures contain a carbon-carbon double bond (C=C) in close proximity to an electron withdrawing group (Bykov *et al.*, 2009). An analysis of the NCI database comparing sensitivity of wild type p53 and mutant p53 expressing cells against various types of thiol-reactive compounds (and a group of randomly selected compounds), showed that Michael acceptors were the most selectively active on mutant p53-expressing cells (Zhang *et al*, 2018a). Thus, Michael addition reactivity seem to be important for mutant p53 reactivation.

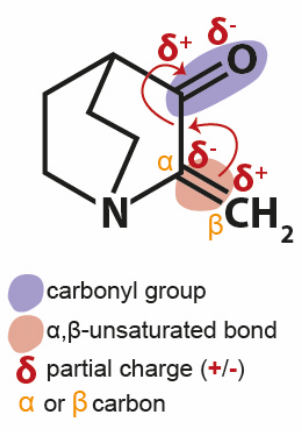

Figure 10 Michael acceptor MQ (APR-246's active product). MQ has an α,β-unsaturated double bond conjugated to an electron-withdrawing carbonyl group which makes it polar and susceptible to nucleophilic addition with e.g. a thiol group.

The carbonyl group in MQ i.e. the carbon atom connected by a double bond to an oxygen atom [C=O], is shown by purple shading in Figure 10. The oxygen (O) is more electronegative than carbon (C) which means that the bonding electrons are attracted towards oxygen (Fessenden *et al.*, 1998). This results in a polar bond with an uneven distribution of electron density towards the oxygen, causing a negative partial charge (δ-) at the oxygen and a positive partial charge (δ+) at the carbon. A carbon-carbon double bond (C=C) (red shading in Figure 10) conjugated to an electron-withdrawing carbonyl group is polarized, and therefore prone to nucleophilic attack at the partially positive (δ+) carbon (Fessenden *et al.*, 1998). Michael acceptors, such as MQ (Lambert *et al.*, 2009), have an α,β-unsaturated C=C double bond coupled to an (electronegative) C=O group (Fessenden *et al.*, 1998). Michael acceptors react with Michael donors (nucleophiles) in a Michael addition reaction forming a Michael adduct (Mather *et al*, 2006; Michael, 1887) (Figure 9).

In the cellular environment Michael donors are largely represented by thiol groups found on highly abundant glutathione (GSH) or cysteines in proteins, for example in p53. As described, these cysteines are important for p53 function (Bykov *et al.*, 2018). Selenocysteine, found for example in TrxR1, although much less abundant is much more reactive with Michael acceptors (Poole, 2015). MQ is a soft electrophile that preferentially reacts with soft nucleophiles such as thiols and selenols – in cysteine and selenocysteine, respectively (LoPachin *et al.*, 2019; Ralston, 2018). Inside cells, MQ undergoes Michael additions forming a reversible adduct (Figure 9) on the partially positively charged β-carbon of MQ (as indicated in orange in Figure 10) (Lambert *et al.*, 2009). The Michael addition reaction rate with thiols is affected by pH (-

log[H^+], hydrogen/proton concentration) (Jackson *et al*, 2017; Mather *et al.*, 2006). The thiol group (-SH) in cysteine is ionizable. Thus, at increasing pH (i.e. lower proton concentration) the thiol group is deprotonated, generating a thiolate anion (-S^- [negatively charged ion]) which is much more reactive (Poole, 2015). The thiol Michael addition reaction rate increases with pH due to increased availability of the thiolate anion (Mather *et al.*, 2006). Therefore, the reactivity of various cysteines with MQ is pH-dependent and also vary depending on the local environment affecting their pKa and hence the fraction deprotonated thiol (thiolate anion) at a given pH.

The binding of MQ and thiols is reversible (Ceder *et al.*, 2020) (Figure 9) which is discussed in Project I. This reversibility allows MQ to transfer between cellular thiol targets, for example between glutathione and p53, which presumably is an important feature of its mechanism of action. But the reversibility also makes the study of MQ-bound targets, such as those evaluated in Project III, difficult as MQ adducts may be lost or new MQ adducts may be formed during sample preparation. Since MQ's reactivity derives from the electronegative oxygen, reducing the carbonyl group renders MQ inactive. A metal hydride reduction method, as for example sodium borohydride ($NaBH_4$), is required to reduce the carbonyl group of a ketone (or aldehyde) while leaving a carbon-carbon double bond intact (Fessenden *et al.*, 1998). Alternatively if there is an adduct at this bond, $NaBH_4$ would prevent the reversible reaction, the retro-Michael addition. If MQ is bound to a p53 cysteine, as described in Project II, the MQ-thiol adduct becomes stable upon $NaBH_4$ treatment. Therefore, reducing MQ enables the study of MQ-adducts by preventing any adducts from being lost, and new to be formed during sample preparation. This could be an important approach to study specific p53 cysteines bound by MQ, as described in project II, but would also enable pharmacodynamic studies (Abrahmsén & Hagberg personal communication) or the identification of novel MQ targets.

1.5.3.6 Michael acceptors in clinical use

Electrophilic functional groups and Michael acceptors have often been excluded or ignored in drug discovery programs due to their presumed indiscriminate reactivity (Barf & Kaptein, 2012; Bauer, 2015; Jackson *et al.*, 2017). MQ binding to thiol targets is reversible (Ceder *et al.*, 2020) and reversible covalent inhibitors are considered to have lower risk of toxicities since levels of drug-protein adducts may not be sufficient to trigger an immune response (Bauer, 2015). Furthermore, unlike the other mutant p53 reactivating compounds (Bykov *et al.*, 2018), APR-246 is a prodrug and not instantly reactive, as the Michael acceptor MQ first needs to be formed (Lambert *et al.*, 2009). These reasons could in part account for the benign safety profile described in APR-246-treated patients in the clinical trials (Ceder *et al.*, 2020; Lehmann *et al.*, 2012)

Nevertheless, several Michael acceptors have received FDA-approval for example afatanib, ibrutinib and osimertinib. These Michael acceptors undergo Michael addition with specific cysteines located in or close to the ATP-binding pockets of their protein targets (Barf & Kaptein, 2012; Jackson *et al.*, 2017). Ibrutinib was FDA-approved 2013 for CLL and mantle cell lymphoma (MCL). It binds covalently to C481 close to the ATP-binding site of Bruton's

tyrosine kinase (BTK) a key player in the B cell receptor (BCR) pathway and upregulated in CLL (Honigberg *et al*, 2010; Pan *et al*, 2007). This cysteine is conserved in nine other tyrosine kinases and indeed, ibrutinib also targets cysteines in for example EGFR (C797), HER2 (C805) and HER4 (C803) (Chen *et al*, 2016; Davids & Brown, 2014; Grabinski & Ewald, 2014).

Activating mutations of epidermal growth factor receptor (EGFR) are major drivers in non-small cell lung cancer (NSCLC) (Tsakonas & Ekman, 2018). The clinical efficacy of the first generation EGFR inhibitors was decreased due to a second-site mutation T790M which occurs in around 50-60% of the resistant cases of NSCLC (Engel *et al*, 2016; Thress *et al*, 2015). This fueled the development of second and third generation EGFR inhibitors and Michael acceptors afatinib and osimertinib that were approved for metastatic NSCLC harboring *EGFR* mutation in 2013 and 2015, respectively (Jackson *et al.*, 2017). Both target C797 by Michael addition and osimertinib selectively targets this cysteine in the EGFR T790M mutant protein (Jackson *et al.*, 2017) but may also show efficacy in patients without this mutation (Eide *et al*, 2020). The treatment-acquired *EGFR* mutation C797S substitutes the cysteine for the much less nucleophilic serine (Jackson *et al.*, 2017) and is associated with osimertinib resistance (Thress *et al.*, 2015). This demonstrates that thiol Michael addition reaction is important for the efficacy of these types of covalent inhibitors and that Michael acceptors that target thiols may indeed be very promising molecules for drug development and novel treatment strategies in patients.

1.5.3.7 APR-246 in clinical trials

The First-in-Human study of APR-246 in patients with hematological malignancies or prostate cancer concluded that APR-246 is safe with a favorable pharmacokinetic profile (Deneberg *et al.*, 2016; Lehmann *et al.*, 2012). APR-246 (Eprenetapopt) is currently being evaluated in several clinical trials in hematological malignancies and solid tumors as listed in Table 3. A phase III study is ongoing in *TP53* mutant myelodysplastic syndrome (MDS) in combination with azacitidine. This combination was shown to be well-tolerated in a Phase Ib/II study with high rates of clinical response and molecular remission (Sallman *et al.*, 2021). In 2020, Aprea Therapeutics received FDA Breakthrough Therapy Designation for APR-246 in the combination with azacitidine for the treatment of MDS with *TP53* mutation. More recently, Aprea Therapeutics also received FDA Fast Track Designation for APR-246 in the treatment of *TP53* mutant acute myeloid leukemia (AML) (see https://www.aprea.com).

Clinical Trial phase	Target indication	Treatment line	Combination treatment with APR-246 /Eprenetapopt	n*	Estimated study completion and NCT identifier
Phase III	*TP53* mutant MDS	First line	Azacitidine	154	Nov. 2020 NCT03745716
Phase I/II	*TP53* mutant MDS/AML/CML/ myeloproliferative neoplams	First line	Azacitidine	53	May 2021 NCT03588078
Phase I/II	*TP53* mutant MDS/AML/CML/ myeloproliferative neoplams	First line	Azacitidine	56	Jun. 2021 Published data (Sallman *et al*, 2021) NCT03072043
Phase II	*TP53* mutant MDS/AML	Post-transplant, maintenance	Azacitidine	31	Sep. 2021 NCT03931291
Phase I	*TP53* mutant AML	First line and relapsed/ refractory	Venetoclax and Azacitidine	80	Dec. 2021 NCT04214860
Phase I/II	Bladder cancer, gastric cancer, NSCLC, urothelial carcinoma, advanced solid tumors	Relapsed/ refractory	Pembrolizumab	118	Jun. 2022 NCT04383938
Phase I/II	*TP53* mutant NHL/CLL/MCL	Relapsed/ refractory	Ibrutinib +/- Venetoclax and Rituximab	116	Jun. 2023 NCT04419389
Phase I/II	*TP53* mutant HGSOC	Platinum - sensitive recurrent	Carboplatin and PLD	200	Completed NCT02098343
Phase II	*TP53* mutant HGSOC	Platinum-resistant recurrent	Carboplatin and PLD	36	Completed NCT03268382
Phase I	Hematological neoplasms/Prostate carcinoma	Refractory		36	Completed Published data (Deneberg *et al*, 2016; Lehmann *et al*, 2012) NCT00900614
Phase I/II	*BRAF* V600 mutant Melanoma	Unresectable and/or metastatic	Dabrafenib	3	Terminated NCT03391050
Phase I/II	Platinum resistant oesophageal cancer	Relapsed/ refractory	Cisplatin and 5-FU	38	Study suspended Pre-print available (Fujihara *et al*, 2020) NCT02999893

Table 3 Clinical trials of mutant p53 reactivating APR-246 (Eprenetapopt) registered at ClinicalTrials.gov. Drug mechanisms: **APR-246/Eprenetapopt**: mutant p53 reactivation, **Azacitidine**: cytidine analog (interferes with DNA replication), **Venetoclax**: BCL2 antagonist, **Pembrolizumab**: anti-PD-1antibody, **Ibrutinib**: Bruton's tyrosine kinase (BTK) inhibitor, **Rituximab**: anti-CD20 antibody, **Cisplatin**: DNA alkylating agent, **5-FU** (Fluorouracil): fluorinated uracil analogue (interferes with DNA replication), **Carboplatin**: DNA alkylating agent, **PLD** (Pegylated Liposomal Doxorubicin Hydrochloride): DNA intercalating drug in a liposome-encapsulated form, **Dabrafenib**: B-Raf inhibitor. *Estimated patient enrollment or actual enrollment.

1.6 OXIDATIVE STRESS

Oxidative stress may be viewed as an imbalance of when the oxidants outweigh the antioxidant capacity (Reuter *et al*, 2010). Exogenous sources resulting in oxidative stress include radiation or electrophilic compounds as described in earlier sections. But oxygen species may also be produced during endogenous processes e.g. oxidative phosphorylation in the mitochondria. Reactive oxygen species can cause DNA damage contributing to genomic instability (Holmstrom & Finkel, 2014). As described in section 1.3.1 DNA damage leads to wild type p53 stabilization and induction of for example growth suppression or apoptosis. Cells have an incorporated redox sensing system and NRF2 is considered as the master antioxidant regulator (Rojo de la Vega *et al.*, 2018) (Figure 11). In a way, NRF2 is similar to p53: both are transcription factors that are kept at low levels during unstressed situations while stabilized in response to certain stress triggers. NRF2 is kept at low levels by an E3 ligase complex containing Kelch-ECH-associated protein 1 (KEAP1) which forms a dimer with NRF2 and ubiquitinates it, resulting in proteasomal degradation. KEAP1 has sensor cysteines (especially C151) that upon reacting with electrophiles and reactive oxygen species leads to a confirmation change so it that KEAP1 no longer ubiquitinates NRF2. Thus, upon oxidative stress newly synthesized NRF2 translocates to the nucleus where it can transactivate the over 200 genes

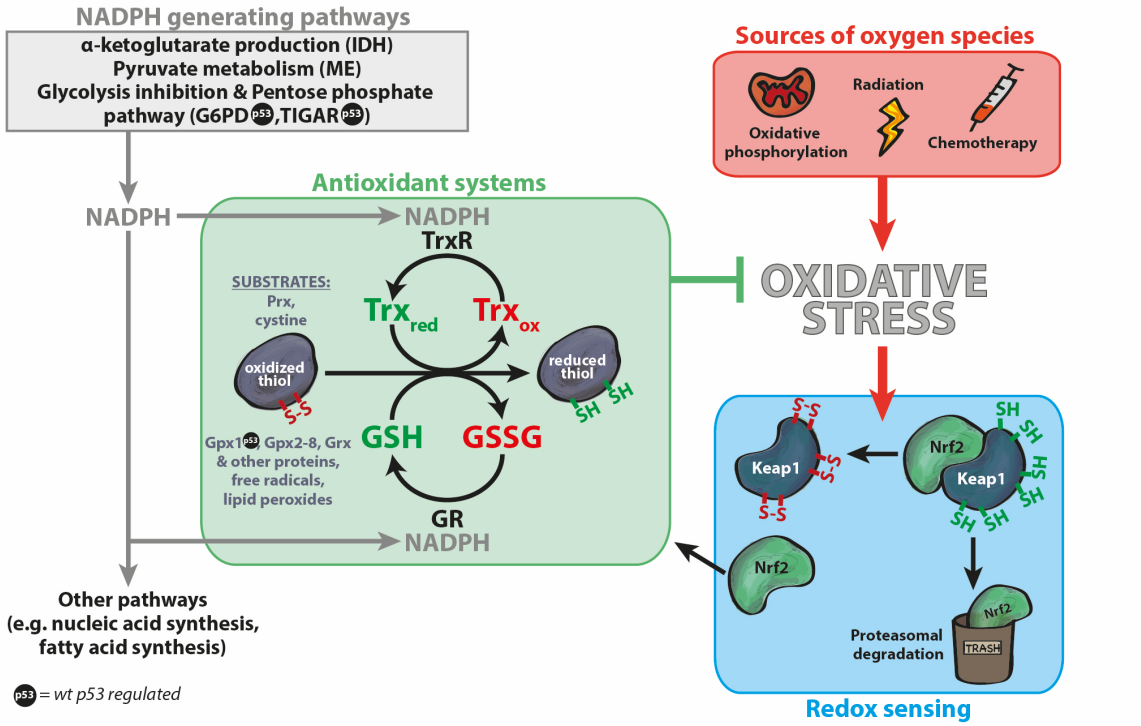

Figure 11 Overview of redox homeostasis and indicated wild type p53 regulated pathways. Oxidative stress may be increased from exogenous and endogenous sources as indicated in the red box. This leads to oxidation of cysteines in Keap1 which thereby no longer ubiquitinates NRF2 for proteasomal degradation. Newly synthesized NRF2 can then transactivate ARE-containing genes which are part of the antioxidant defense system. The two major antioxidant systems are Trx and GSH. When Trx or GSH are oxidized they may be NADPH-dependently reduced by TrxR and GR, respectively. NADPH generating pathways are indicated in the grey box and may also be consumed by other pathways besides antioxidant defense systems. IDH = isocitrate dehydrogenases, ME = malic enzymes, G6PD = glucose-6-phosphate dehydrogenase, Prx = peroxiredoxins, Gpx = glutathione peroxidases, Grx = glutaredoxin, other abbreivations are mentioned in the text. Figure is from the review Eriksson, Ceder *et al* 2019.

containing antioxidant response elements (ARE). In cells, thiol-containing proteins and low molecular weight (LMW) thiols have important biochemical roles in maintaining the redox homeostasis as they can easily be oxidized and regenerated. Two such entity with antioxidant activity are glutathione (GSH) and thioredoxin (Forman & Dickinson, 2003). When Trx or GSH reduce oxidized thiols their oxidized forms may be NADPH-dependently reduced by TrxR or glutathione reductase (GR) respectively (Eriksson *et al.*, 2019). Thus, NADPH is an important reductive power in cells and can be generated by the pentose phosphate pathway (PPP), α-ketoglutarate production or pyruvate metabolism.

The role of oxidative stress in cancer is complicated. Oxidative stress may initiate cancer development and support proliferation, while oxidative stress can also cause cancer cell death (Hayes *et al*, 2020). Although antioxidants can protect against oxidative stress-induced DNA damage and therefore be cancer-preventive, they have a different effect once a tumor is already formed (Holmstrom & Finkel, 2014). Studies have shown that treatment with antioxidants in tumor carrying-mice accelerates tumor progression and increase metastasis (Le Gal *et al*, 2015; Sayin *et al*, 2014). Likewise, epidemiological studies that evaluated antioxidant supplements in cancer patients showed no effects or even accelerated cancer incidence (Alpha-Tocopherol, 1994; Holmstrom & Finkel, 2014). Aberrant proliferation of cancer cells may generate oxidative stress. In order to cope with the increased oxidative burden cancer cells upregulate antioxidant systems and adapt their metabolic activity (Hayes *et al.*, 2020; Holmstrom & Finkel, 2014). One way to increase antioxidant defense systems is upregulating NRF2 activation which has been show to promote tumor growth, metastasis and therapy resistance (Rojo de la Vega *et al.*, 2018). The "Warburg effect" refers to the increased use of aerobic glycolysis by cancer cells, which provides glucose for the NADPH-generating PPP. This gives cells reductive power to sustain their high need of antioxidants (Holmstrom & Finkel, 2014). Both reactive oxygen species and NRF2 have been shown to play roles in many or in all of the Hallmarks of Cancer (Rojo de la Vega *et al.*, 2018; Trachootham *et al*, 2009). Considering the central role of redox imbalance in cancer and its important role in response to electrophilic compounds it has in this thesis been awarded its own Hallmark of cancer as marked in green in Figure 3.

1.6.1 Glutathione

As mentioned, the tripeptide glutathione is present at millimolar concentration (1-10mM) in mammalian cells (Berg *et al.*, 2007; Cole, 2014b; Lu, 2013). Glutathione is synthesized *de novo* in the cytosol in a highly regulated process (Lu, 2013) (Figure 13). Cysteine (Cys) availability is a key determinant for glutathione synthesis(Lu, 2013). Cysteine is imported in its oxidized from, cystine (CySS), via the antiporter xCT (*SLC7A11*) (Lewerenz *et al.*, 2013). Extracellularly, cysteine is readily autoxidized resulting in the formation of a disulfide bond between two cysteine molecules (CySS) (Lu, 2013). In addition, cysteine may be derived from methionine via the transulfuration pathway. The imported CySS is NADPH-dependently reduced by enzymes Trx and thioredoxin-related protein 14 (TRP14) (Eriksson *et al.*, 2019) into two cysteine molecules which may be used for the synthesis of γ-glutamylcysteine in an

ATP-dependent reaction catalyzed by rate limiting GCL (glutamate-cysteine ligase) (Lu, 2013). GCL comprises of two subunits, GCL-catalytic subunit (GCLC) and GCL-modifier subunit (GCLM), on of which (GCLC) is negatively feedback-regulated by GSH (Seelig *et al*, 1984). The last step of GSH synthesis in which glycine is added is catalyzed by glutathione synthetase (GS) (Lu, 2013). Besides being incorporated in glutathione, cysteine itself is also potent antioxidant .

One of the most important roles of glutathione is to protect from oxidative damage by serving as a sulfhydryl buffer, for example by reacting with hydrogen peroxide and organic peroxides, harmful byproducts generated from aerobic metabolism. Glutathione cycles between a reduced thiol form (GSH) and an oxidized form (GSSG) where two tripeptides are connected by a disulfide bond. Glutathione reductase (GR) can reduce GSSG back to GSH using NADPH as the electron donor (Berg *et al.*, 2007). The oxidized form makes up less than 1% (Forman & Dickinson, 2003) of the total glutathione pool. In other words, most cells have a ratio GSH to GSSG greater than 500 (Berg *et al.*, 2007), as GSSG has deleterious prooxidant activities and often accumulate upon oxidative stress (Cole, 2014b). The GSH to GSSG ratio (GSH/GSSG) is an important determinant of the intracellular redox potential(Lu, 2013). Therefore, to maintain this ratio, GSSG will rapidly be reduced by GR (Forman & Dickinson, 2003), or exported through for example MRP1 (Cole, 2014b) upon oxidative stress and the accumulation of GSSG. When GSH and GSSG are released from cells, activities of γ-glutamyltranspeptidase (GGT) and dipeptidase will lead to degradation of GSH to its building blocks that can be salvaged and used for GSH synthesis, thereby forming the γ-glutamyl cycle (Lu, 2013).

The millimolar concentration of glutathione reflects its many essential roles in the cell, not only in protecting from oxidative damage, but also in processes such as cell differentiation, proliferation and apoptosis (Cole, 2014b). Importantly, glutathione also plays a role in drug and free radical detoxification since it can conjugate to electrophilic compounds nonenzymatically or through the action of glutathione S-transferases (GST) (Forman & Dickinson, 2003). In cells, endogenous oxygen species are the major source of DNA damage and thus counteracting this oxidative damage is essential to prevent cancer, as DNA damage is a substantial contributor to chromosome instability and accumulation of mutations and deletions (Sablina *et al.*, 2005).

1.6.2 Efflux pump MRP1

1.6.2.1 ABC-family

ATP-binding cassette (ABC) transporters play a major role in exporting solutes across a membrane against a concentration gradient. Their evolutionary importance becomes evident as all eukaryotes, including bacteria and Achaea express these membrane proteins (Cole, 2014a). The ABC super family consists of 48 members divided into seven subfamilies (A-G). One of the major causes of multidrug resistance in cancer is failure of chemotherapy, and one of the primary reasons for this is overexpression are some of the members of the ABC-family. Not all members of the ABC family mediate drug resistance, but members from the ABC

subfamilies B, C and G contain known multidrug transporters (Bush & Li, 2002). The ABCB1 (MDR1) was first described and is now known to transport a wide variety of molecules including drugs and dyes.

1.6.2.2 Structure and function

Multidrug resistance protein 1 (MRP1 or *ABCC1*) was discovered in 1992 and was the first identified member of the C subfamily. The ABCC1 gene was amplified at least 100-fold in the multidrug resistant lung cancer cell line from where the mRNA first was isolated (Cole *et al*, 1992). MRP1 is a 190kDa protein and, unlike many of the other ABC proteins that contain a 4-domain structure, MRP1 has a 5-domain structure, with three membrane-spanning domains (MSD) forming a pore which allows transportation powered by ATP hydrolysis at the two nucleotide-binding domains (NBD) (Cole, 2014a, b). Although MRP1 was identified in a multidrug resistant cancer cell line, it has several important physiological roles as it also exports endogenous substrates. Endogenous substrates can be exported either unconjugated such as folic acid, vitamin B12 or bilirubin, but also conjugated to either GSH (e.g. proinflammatory leukotriene C4), glucuronide (e.g. the steroid hormone 17β-estradiol) or sulfate (e.g. the steroid estrone 3-sulfate). MRP1 also exports byproducts from other processes that might be damaging such as the product and mediator of oxidative stress 4-hydroxy-2,3-*trans*-nonenal (4-HNE) generated from peroxidation of arachidonic acid in membrane phospholipids (Cole, 2014b). The relationship between glutathione and MRP1 is interesting, as some substrates need to be conjugated to GSH, while others are exported in the presence of GSH such as vincristine, etoposide and some anthracyclines. Some substances like Verapamil can even cause export of glutathione itself. Importantly, and as mentioned, GSSG, which can accumulate intracellularly upon oxidative stress can be exported through MRP1. Thus, MRP1 is a critical contributor to the thiol-redox homeostasis in cells (Ballatori *et al*, 2009; Bush & Li, 2002).

1.6.2.3 Drug resistance

Elevated MRP1 levels (mRNA and protein) can be found in most solid tumors and has been correlated with a negative clinical outcome as data indicate a role in drug resistance (Bush & Li, 2002). Due to its associated with drug resistance, targeting MRP1 could have therapeutic benefits. However, as MRP1 has important functions in normal cells as well, needs to be carefully modulated. Furthermore, MRP1 is found at pharmacologically sanctuary sites where it likely serves a protective role. For example, at the blood-testis barrier, MRP1 protects the testicular tubules against xenobiotic induced damage (Wijnholds *et al*, 1997; Wijnholds *et al*, 1998). The quinolein derivative MK-571 is the most commonly used MRP1 inhibitor, but it can also inhibit other MRPs (Csandl *et al*, 2016), and was originally developed as a cysteinyl leukotriene receptor (CysLTR1) antagonist (Jones *et al*, 1989) for the purpose of treating asthma as it completely inhibits MRP1-mediated transport of leukotriene C4 (LTC4) (Cole, 2014b; Li, 2006). Previous studies have combined MK-571 with chemotherapeutics, for instance with vincristine and demonstrated that MK-571 can revert resistance (Gekeler *et al*, 1995). Interestingly, the MRP1/*ABCC1* promoter contains p53 binding motifs (Bush & Li,

2002) and studies show that while wild type p53 represses MRP1 (Wang & Beck, 1998), mutant p53 is associated with MRP1 accumulation (Sullivan *et al*, 2000).

1.7 ACUTE LYMPHOBLASTIC LEUKEMIA (ALL)

A child is diagnosed with cancer almost every day in Sweden. Children rarely develop cancer and pediatric cancers are less than 1% of all cancer cases (Barncancerfonden, 2017b). One third of childhood tumors are leukemias, predominantly (90%) acute lymphoblastic leukemia (ALL) (Barncancerfonden, 2017a). Around half of the ALL cases occur in children and adolescents (Tran & Hunger, 2020) with the peak incidence at 2-5 years of age (Hein *et al*, 2020; Hunger & Mullighan, 2015). ALL arises in the bone marrow and are mainly of B cell precursor subtype or have T cell lineage (around 15%) (Hein *et al.*, 2020), but subtype is also defined by differentiation status and genetics (Greaves, 2018). Symptoms of ALL include pallor, tiredness, pain in legs, bruises and wounds that do not disappear or heal, increased infection sensitivity and the continuous worsening of symptoms (Barncancerfonden, 2017a). Treatment of pediatric ALL has been a success story in oncology; from a deadly disease with survival rate below 10% in the 1960s to over 90% surviving patients today (Greaves, 2018; Hunger & Mullighan, 2015). Nonetheless, ALL remains the most frequent cause of death from cancer under the age of 20 in the United States (Hunger & Mullighan, 2015).

1.7.1 Genetic alterations in ALL

Childhood ALL arises due to a combination of genetic predisposition or susceptibility at birth that results in a preleukemic state and secondary alterations that leads to ALL (Figure 12) (Hein *et al.*, 2020; Zelent *et al*, 2004). Many of the chromosomal rearrangements in ALL (e.g. *ETV6-RUNX1*, *TCF3-PBX1*, *BCR-ABL*) and hyperdiploidy (more than the usual diploid chromosomes) have been shown to occur *in utero* or prenatally and result in a preleukemic clone. A second alteration or factor is needed to trigger the transformation into ALL, which occurs in a small fraction (0.2-1%) of these children (Ford *et al*, 1993; Hein *et al.*, 2020). This also means that a large fraction of children that have the genetic predisposition and a preleukemic population actually never progress to clinical ALL (Greaves, 2018). For example, the *ETV6-RUNX1* rearrangement occurs in 1-5% (Hein *et al*, 2019; Mori *et al*, 2002) of healthy newborns and the *TCF3-PBX1* gene fusion in around 0.6% (Hein *et al.*, 2019). These prenatal alterations are not sufficient for development of ALL and a second alteration is required to transform preleukemia to ALL. The frequency of *ETV6-RUNX1* in healthy newborns is 100-500-fold higher than the risk of developing leukemia supporting (Hein *et al.*, 2020; Mori *et al.*, 2002), supporting the idea that secondary mutations are required for developing of ALL. This is also evident from the long latency of disease onset which may range between 1 to 15 years of age depending on which prenatal alteration is predominant (Hein *et al.*, 2020; Hein *et al.*, 2019). It still remains unclear what causes the prenatal or initiating mutations and there is no pregnancy exposure associated with the genetic predisposition leading to preleukemia (Greaves, 2018). It is also unclear exactly how preleukemia develops into postnatal ALL, although the type of cell (e.g. stage of hemapoietic differentiation) where the genetic alteration

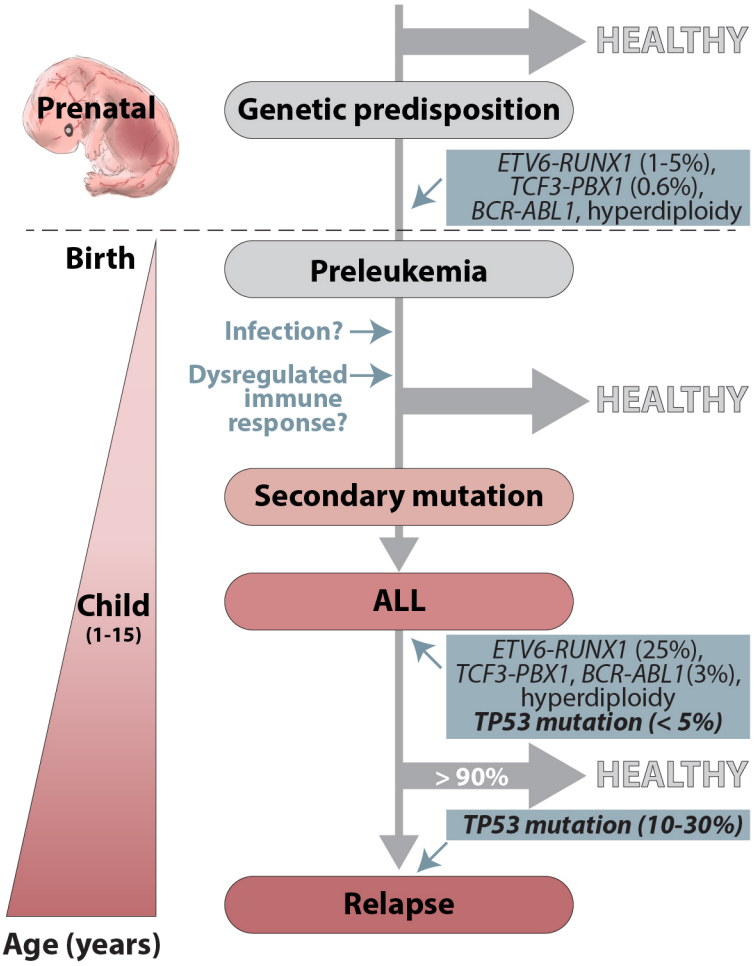

Figure 12 Timeline of childhood ALL development. Some mutations occur prenatally and results in a preleukemic clone which upon a secondary mutation may lead to development of ALL. *TP53* mutations are rare in ALL but occur more frequently in relapsed patients. Inspired and modified from Hein *et al*, 2020 licensed under CC BY 4.0 https://creativecommons.org/licenses/by/4.0/

occurs in appears to be important. Infection or dysregulated immune response have been suggested to contribute to the leukemic transformation (Hein *et al.*, 2020).

ALL have distinct somatic genetic alterations such as changes in chromosome number (aneuploidy) or chromosomal rearrangements that can lead to deletion or gains in DNA sequences or result in the expression of a fusion protein. Chromosomal translocation results in the juxtaposition of an oncogene to a regulatory region of a gene that is actively transcribed, consequently leading to dysregulated and high transcription of the oncogene. Translocation may involve the juxtaposition of two genes in a manner that results in the expression of a chimeric fusion protein that has novel oncogenic functions (Hunger & Mullighan, 2015). The most common translocation in pediatric ALL (25%) results in the fusion of the two transcription factor genes *ETV6* and *RUNX1* (*TEL-AML1*) (Hein *et al.*, 2020; Hunger & Mullighan, 2015). Fusion gene formation may also lead to constitutive activation of kinases such as c-Abl in *BCR-ABL1* translocation (also known as the Philadelphia [Ph] chromosome) (Zelent *et al.*, 2004). This is rare in childhood ALL (3%) but more common in adults (25%) (Hein *et al.*, 2020). This translocation results in a protein distinctly expressed in the leukemic

cells and thus offers the possibility for therapeutic targeting. Twenty years ago, STI-571 (Gleevec) became the first FDA-approved drug to specifically target an oncogenic protein (BCR-ABL) only expressed in cancer (Druker *et al*, 2001).

1.7.1.1 TP53 mutations

Although *TP53* is the most frequently mutated gene in cancer, it is very rarely mutated in ALL with an incidence less than 5% at diagnosis (van Leeuwen, 2020). In ALL, genetic alterations involving tumor suppressors or cell cycle regulators are common, but for unknown reasons *TP53* is spared (Comeaux & Mullighan, 2017). An exception is a rare subset of low hypodiploid B cell precursor ALL which is strongly associated with inherited *TP53* mutations or the Li-Fraumeni syndrome (Greaves, 2018). Already 30 years ago, it was observed that 28% of the relapsed T-ALL patients, had acquired *TP53* mutation which were absent at the first diagnosis (Hsiao *et al*, 1994). This observation has been confirmed in several studies, identifying mutations, deletion or 17p chromosomal aberrations in 10-30% of relapsed ALL cases (Blau *et al.*, 1997; Comeaux & Mullighan, 2017; Diccianni *et al.*, 1994; Gump *et al.*, 2001; Ma *et al.*, 2015; van Leeuwen, 2020). Relapsed childhood ALL with *TP53* mutation predict poor response to therapy and poor prognosis (Hof *et al.*, 2011).

2 RESEARCH AIMS

I. Investigate the role of MRP1 in APR-246/MQ drug export and redox balance for APR-246-mediated cancer cell death and how to target this mechansism for improved anti-cancer efficacy of APR-246.

II. Determine the spectrum of cysteines in the p53 core domain targeted by APR-246's active product MQ by locking its reversible binding.

III. Explore ASNS as a putative target of APR-246 to improve asparaginase standard-treatment-of-care for ALL.

IV. Functionally characterize potential novel germline *TP53* variants from Swedish families with hereditary breast cancer or Li-Fraumeni Syndrome.

3 RESULTS AND DISCUSSION

3.1 PAPER I

A thiol-bound reservoir enhances APR-246-induced mutant p53 tumor cell death

The efflux pump MRP1 plays an important role in GSH-conjugated drug export but also in redox homeostasis by regulating the export of both GSH and GSSG (Cole, 2014a) (Figure 13). Since APR-246's active product MQ is conjugated to GSH, and since MQ also induces oxidative stress (Lambert *et al.*, 2009), we hypothesized that MRP1 may play a role in APR-246-mediated cell death.

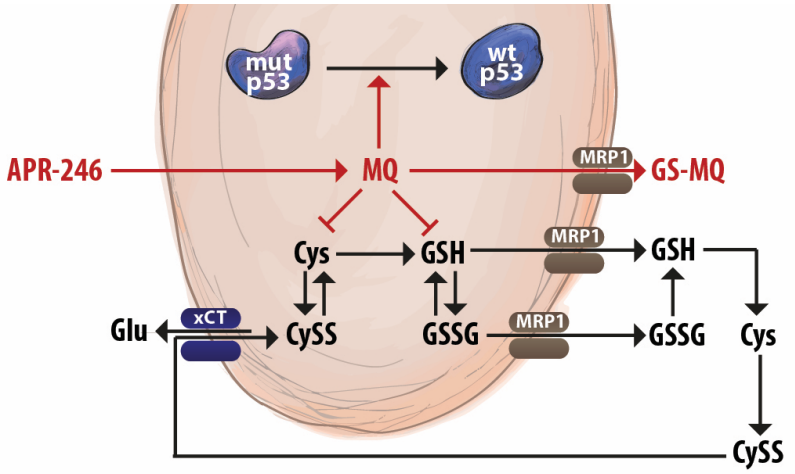

Figure 13 Overview of MRP1's and xCT's role in GSH and Cys cycling and APR-246 mechanism. Antiporter xCT exports glutamate (Glu) and imports cystine (CySS [oxidized Cys]), CySS is reduced into cysteines (Cys) which can be used for glutathione production (GSH). GSH and oxidized GSH (GSSG) are exported by MRP1. Outside cells GSSG is reduced to GSH which is cleaved by peptidases to form Cys which is oxidized to CySS and can again be taken up by xCT. APR-246's active product MQ can reversibly bind to GSH and Cys, as well as thiols in mutant p53 thereby reactivate p53 and induce cell death. The GSH-conjugated MQ (GS-MQ) is exported by MRP1. Depletion of antioxidants GSH and Cys and accumulation of prooxidants GSSG and CySS lead to oxidative stress and contribute to cell death. Part of figure is from Eriksson, Ceder *et al*, 2019.

Our analysis of data from the Cancer Dependency Map (DepMap) of 37 ovarian cancer cell lines identified MRP1 (*ABCC1*) mRNA as the gene whose expression showed the strongest correlation to PRIMA-1 resistance, in accordance with a previous analysis of the NCI database (Bykov *et al*, 2002a). Indeed, the combination treatment of APR-246 and MRP1 inhibitor MK-571 resulted in synergistic growth suppression in 20 cancer cell lines and *ex vivo* in esophageal and colorectal cancer patient-derived organoids (PDO). Another MRP1 inhibitor, reversan, and MRP1 knockdown confirmed synergistic growth suppression, while overexpression of MRP1 resulted in decreased APR-246 sensitivity. Using an esophageal cancer xenograft model in mice we showed that the combination treatment effectively suppressed tumor growth and increased survival. Inhibition of MRP1 with either of the two inhibitors or knockdown by siRNA resulted in increased ^{14}C-content after ^{14}C-APR-246 treatment. The increased intracellular level of ^{14}C could be attributed to retention of GSH-conjugated MQ (GS-MQ), but

not prodrug APR-246, as demonstrated by mass spectrometry. Furthermore, we showed that GS-MQ binding is reversible since addition of N-acetylcysteine (NAC) resulted in NAC-MQ formation. Cells harboring mutant p53 are the most sensitive to single APR-246 treatment and exhibited the strongest synergy upon combination treatment with APR-246 and MK-571. Furthermore, high glutathione (GSH + GSSG) and low ^{14}C-content after ^{14}C-APR-246 treatment correlated with low APR-246 sensitivity. However, neither mutant p53, thiol status nor drug accumulation alone could fully explain APR-246 sensitivity.

Antiporter xCT imports cystine (CySS [oxidized cysteine]) and exports glutamate (Lewerenz *et al.*, 2013). Upon import, CySS is reduced to cysteine (Cys) which may be used for GSH synthesis (Lu, 2013) (Figure 13). A previous study demonstrated pronounced synergistic growth suppression upon combination treatment with xCT inhibitors and APR-246 (Liu *et al.*, 2017). This was partly explained by the depletion of glutathione due to limited cystine/cysteine availability. Surprisingly, upon MRP1 inhibition with MK-571 we also detected a drop in total glutathione (GSH+GSSG) which was accompanied by increased expression of NRF2-regulated xCT (Rojo de la Vega *et al.*, 2018) and increased intracellular Cys and CySS concentrations. However, upon the combination treatment with APR-246, the MK-571-induced Cys level dropped suggesting that intracellular Cys was consumed, modified or produced to a lower rate upon APR-246 treatment. We then compared the effect of MRP1 that limits drug export and xCT inhibition that limits cystine/cysteine availability. At concentrations of inhibitors that decreased total glutathione (GSH + GSSG) to similar extent, we saw an even more striking increase in intracellular ^{14}C content upon ^{14}C-APR-246 treatment in the xCT-inhibited cells, compared to MRP1-inhibited cells. This suggests that cysteine/cystine availability is an important factor for MQ retention as well as APR-246-induced growth suppression.

The retention of GS-MQ upon efflux pump MRP1 inhibition allows the formation of an intracellular active drug pool from which MQ may target thiols (or selenols) in other low molecular weight molecules or high molecular weight molecules such as p53. This active drug retention in combination with GSH depletion results in pronounced synergistic growth arrest upon the combination treatment with APR-246 and MRP1 inhibitor. Since reversible covalent inhibitors are considered to have lower risk of toxicities (Bauer, 2015), the reversible nature of MQ binding may not only be important for the efficacy, but also account for the benign safety profile observed in the clinical trials (Lehmann *et al.*, 2012).

The main findings of **Project I** are:

- MRP1 inhibition increases GS-MQ retention in cells and shifts intracellular thiol status
- GS-MQ binding is reversible and allows formation of an intracellular drug pool that can target other thiols for example in p53
- MRP1 inhibtion results in pronounced synergistic growth suppression *in vitro*, *in vivo* and *ex vivo*.

3.2 PAPER II

Spectrum of p53 cysteines targeted by APR-246 active product MQ

The reversible binding of MQ complicates the study of MQ adducts, as adducts may be lost or new ones formed during sample preparation. For this reason we used the reducing agent $NaBH_4$ to reduce the ketone group of MQ, rendering MQ inactive or locked to the bound thiol. We analyzed MQ adducts on wild type p53 and R273H and R175H mutant recombinant p53 core domains (S94-K292). Samples were treated with APR-246's active product MQ with or without $NaBH_4$, trypsinized and then analyzed by mass spectrometry. Without $NaBH_4$ treatment, p53 cysteines were found to be modified at a frequency of <1.5% at MQ incubation concentrations up to 200 μM MQ but some up to 20-30% at 2 mM MQ. A much higher fraction of individual cysteines was found to be modified in samples treated with $NaBH_4$, with some cysteines being almost completely MQ-conjugated after incubation at 100 μM MQ.

The ten cysteines of p53 display different thiol reactivity based on chemical context and solvent accessibility in the folded protein (Kaar *et al.*, 2010). Kaar et al. concluded that C182 and C277 are the most solvent accessible cysteines in the p53 core domain. Previously, C277 has been identified as an MQ target by thermostability measurements and mass spectrometry (Zhang *et al.*, 2018b). Also other mutant p53-reactivating compounds with Michael acceptor activity, i.e. 3BA (Kaar *et al.*, 2010) and PK11007 (Bauer *et al.*, 2016) (described in 1.5.2.6), are known to target C182 and C277. The latter makes direct DNA contact (Cho *et al.*, 1994) but despite PK11007 binding to this residue, p53 DNA binding was not compromised and transactivation of p53 targets was restored (Bauer *et al.*, 2016). In agreement with these studies, we found that C182 and C277 are the most MQ-modified cysteines in wild type p53 and the two mutant proteins. Additionally, C229 was highly modified in all three recombinant proteins. Furthermore, Zhang et al. showed that C124 is important for mutant p53 reactivation by APR-246 in R175H mutant p53-transfected cells (Zhang *et al.*, 2018b). C124 is also targeted by the other two mutant p53-reactivating compounds (Bauer *et al.*, 2016; Kaar *et al.*, 2010). Indeed, we identified C124 as an MQ target in the mutants but only to a low extent in wild type protein. Similarly, C135 and C141 were modified to a greater extent in the mutants than in the wild type core domain. Mutation at R175H is structurally detrimental due to its proximity to the zinc atom coordinated by C176, H179, C238 and C242 (Cho *et al.*, 1994), and thus the unfolding temperature (melting point) is significantly lowered. Therefore, one might expect that more cysteines are exposed in the R175H core domain also at room temperature (Bykov *et al.*, 2018). However, we did not observe an overall higher degree of modification in the R175H mutant compared to the R273H mutant.

The main findings of **Project II** are:

- C182 and C277 in the p53 core domain are major targets of mutant p53 reactivating compound APR-246's active product MQ
- Reversible MQ adducts are locked upon $NaBH_4$ reduction, enabling studies of the degree of modifcation of individual cysteines in p53.

3.3 PAPER III

Mutant p53-reactivating compound APR-246 synergizes with asparaginase in inducing growth suppression in acute lymphoblastic leukemia cells

Given the reactive and reversible nature of APR-246's active product MQ adduct formation, it is likely that APR-246 targets additional proteins than what has been described. We applied mass spectrometry-based cellular thermal shift assay (MS-CETSA) to identify potential novel MQ targets. MS-CETSA identified asparagine synthetase (ASNS) as one of the most thermostabilized proteins upon MQ treatment. We validated thermostabilization of ASNS using Western blot-CETSA (WB-CETSA) in acute lymphoblastic leukemia (ALL) cells. Presence of mutant p53, low GSH level and low xCT level were factors that correlated with increased APR-246 sensitivity in solid tumors (Ceder *et al.*, 2020; Liu *et al.*, 2017), but seemed less relevant for APR-246 sensitivity in ALL cells.

Although we did not see a correlation of mutant p53 and APR-246 sensitivity in our small panel of cell lines, mutant p53 reactivation and APR-246 efficacy have been demonstrated in ALL cells (Demir *et al.*, 2020). *TP53* mutation is rare in ALL but occurs at higher frequency in relapsed patients (van Leeuwen, 2020). For decades, asparaginase has been used for treatment of ALL, based on the finding that ALL cells are asparagine-auxotrophs (Lanvers-Kaminsky, 2017). We confirmed the observation (Aslanian *et al.*, 2001) that ASNS-expressing ALL cells are less sensitive to asparaginase treatment. Since ASNS was identified as a potential MQ target, we combined APR-246 and asparaginase treatment and observed synergy in several of the tested ALL cell lines. Eight out of the ten tested ALL cell lines exhibited synergistic growth suppression.

The finding that APR-246's active product MQ target ASNS creates a novel therapeutic option for ALL patients as APR-246 is currently being tested in Phase III clinical trials. Also other solid tumors that are sensitive to both asparaginase and APR-246 may benefit from this combination treatment.

The main findings in **Project III** are:

- ASNS is a putative target of APR-246's active product MQ
- Combination treatment with APR-246 and standard-treatment-of-care asparaginase results in syneristic growth suppression in ALL cells.

3.4 PAPER IV

Functional characterization of novel germline *TP53* variants in Swedish families

In a Swedish cohort of Li-Fraumeni syndrome (LFS) or hereditary breast cancer (HrBC) patients we identified 24 different *TP53* variants. Ten of these had not been reported as germline mutations in the International Agency for Research on Cancer (IARC) nor in TCGA by the NIH. Of these ten we functionally characterized four frame-shift mutations, one deletion, one nonsense mutation and three missense mutations.

We determined wild type p53 activity using an eGFP reporter system containing several p53 consensus DNA-binding sites and expression of p53 targets e.g. MDM2 as assessed by Western blotting. We also evaluated the capacity of cells harboring the mutant variants to induce cleaved caspase 3 and cell death. None of the frameshift variants nor the deletion variant were able to induce GFP expression by binding the consensus sites or express p53 target MDM2. The nonsense mutant did show partial wild type p53 activity by GFP expression and also induction of MDM2. This could be related to the fact that its premature stop codon is situated relatively close to the C-terminus. Induction of GFP expression by the F134L and R110C missense mutant was also partial, while the P190S missense mutant induced GFP expression to similar levels as wild type p53. P190S was later determined not to be a germline mutation but a somatic mutation in a woman with breast cancer and family history of breast cancer. The two missense mutants, R110C and P190S, were able induce expression of MDM2, indicating that they retain p53 transcriptional transactivation activity. Annexin V/p53 co-staining by flow cytometry revealed that none of the mutants induced Annexin V in p53-positive cells to the same degree as wild type p53 transfected cells, although Annexin V, as marker of cell death, was stained to some extent.

As described in the previous section 1.4.1.1, a large number of mutations have been reported in the *TP53* variant database (Leroy *et al.*, 2017). Therefore it is of high importance to understand which mutations are pathogenic in order to offer genetic counselling to families with hereditary cancer and the presence of these mutations. In our functional assays, some of the mutants identified from LFS or HrBC families were found to be potentially pathogenic although to a varying degree.

The major findings in **Project IV** are:

- *TP53* variants that have not previously been reported as germline mutations were identified in families with Li-Fraumeni syndrome or hereditary breast cancer.
- Frameshift variants and a deletion variant completely lacked wild type p53 activity while the nonsense and missense mutants showed some activity.

3.5 ETHICAL CONSIDERATIONS

Project I includes experiments with material derived from colorectal or esophageal cancer patients. The patient-derived material was used for establishing patient-derived organoids (PDO) which were used for *in vitro* experiments. All experiments followed the principles in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report as described in the Material and Methods section in the paper. All experiments involving PDOs were approved by the local ethical committees and all patients gave individual informed consent. This project also involved experiments in mice. All of these experiments were approved by the local ethical committee and are further described in the Material and Method section (Ceder *et al.*, 2020).

The *TP53* mutants examined in Project IV are derived from patients, although no patient material was used in the study. All patient gave consent to participate in the clinical biobank used for diagnostic and technical development as described in the Material and Methods section (Kharaziha *et al*, 2019).

4 CONCLUSIONS AND FUTURE PERSPECTIVES

Most of the mutant p53 reactivating compounds, including APR-246's active product MQ, have electrophilic properties (Bykov *et al.*, 2018). Consequently, any study with these compounds also affects the redox environment in cells which subsequently affects mutant p53 (section 1.5.2.5). In the opposite direction, mutant p53 also affects redox homeostasis (section 1.4.2.4). In **Project I** we showed MQ adduct formation is reversible and in **Project II** we established a method involving a reducing agent to lock MQ adducts. Locking MQ to its bound thiol enables identification of novel targets but may also facilitate the use of MQ as a biomarker after APR-246 treatment. In Project I we also discovered that both intracellular GSH and Cys content determine APR-246 sensitivity. Both GSH and Cys content is regulated by antiporter xCT expression which has been identified as a predictive biomarker for APR-246 sensitivity (Liu *et al.*, 2017). Both xCT and the potential MQ target ASNS (identified in **Project III**) are regulated via NRF2 and another transcription factor ATF4 (Chen *et al*, 2004; Ishii & Mann, 2014). Mutant p53 interacts with NRF2 and affects its transactivation activities (Lisek *et al.*, 2018; Liu *et al.*, 2017; Walerych *et al.*, 2016). Thus, exploring the relationship between mutant p53, NRF2, xCT and ASNS would be highly interesting, especially since PRIMA-1 (APR-246 analogue) treatment is shown to disrupt mutant p53-NRF2 interaction (Walerych *et al.*, 2016). High activity of xCT may render tumor cells glutamine-dependent as glutamine is utilized for generating glutamate that is exported with the imported cystine (Koppula *et al*, 2020; Timmerman *et al*, 2013). Since asparaginase also has glutaminase activities (Parmentier *et al.*, 2015), APR-246-induced xCT upregulation (shown in Project I) may make ALL cells more sensitive to asparaginase. This would provide another explanation for the observed synergy in ALL cells, especially for those with low ASNS expression. Furthermore, tumor cells that express high levels of antiporter xCT have high NADPH consumption due to constant NADPH-dependent reduction of imported cystine. This renders these tumors dependent on the NADPH-generating phosphate pentose pathway (PPP) and glucose (Joly *et al*, 2020; Liu *et al*, 2020). Hence, using inhibitors to target PPP or glucose uptake in combination with APR-246 should be explored as combination treatments. Lastly, in **Project IV** we identified *TP53* germline mutations that have not been previously described. Due to the vast number of reported *TP53* mutations (section 1.4.1.1) it is highly important to identify which mutations may be pathological and which not.

In summary, the first three projects provide important new knowledge on the mechanisms for APR-246-induced cancer cell death. Our data establish a solid foundation for novel combination treatment strategies with APR-246, for example combination with compounds regulating redox homeostasis or metabolism. Furthermore, the projects also highlight the various roles of mutant p53 in response to treatment as well as its pathogenic role in families with hereditary cancer. Understanding both the drivers of tumor development and the players in treatment responses are important weapons in the combat against cancer.

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