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Studies of mutant p53-targeting small molecules

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Our knowledge is a little island in a great ocean of non-knowledge

Isaac Bashevis Singer

To my parents and Johan

ABSTRACT

Multiple cellular stresses, such as DNA damage, oncogene activation, hypoxia, and telomere erosion induce p53 protein levels leading to an array of biological responses, including cell cycle arrest and apoptosis. p53 exerts its function mainly through transcriptional regulation of specific target genes, but is also able to induce transcriptional-independent apoptosis.

The high frequency of p53 mutations in human tumors, the expression of high levels of mutant p53 protein, and the fact that the mutant p53-harbouring tumors frequently show increased resistance to conventional chemotherapy makes p53 an attractive target for cancer therapy. In the past years, several small molecules that restore mutant p53 function have been identified, among them PRIMA-1 and MIRA-1. Both compounds induce mutant p53-dependent apoptosis and restore native conformation, DNA binding, and transcriptional transactivation to mutant p53. PRIMA-1 and its more potent analog PRIMA-1^{MET} inhibit tumor growth in SCID mice. The maleimide analog MIRA-3 shows anti-tumor activity in SCID mice, however, the therapeutic window is narrow. Moreover, we show that PRIMA-1^{MET} not only induces apoptosis in human mutant p53-carrying tumor cells, but also has potent growth inhibitory effects in mouse tumors containing mutant p53 in syngeneic mice.

Another approach for a novel strategy of cancer therapy is based on already existing cancer drugs in combination with p53-reactivating molecules. This may reduce the side-effects of the currently used anti-cancer therapy. Therefore, we treated human tumor cells with the commonly used chemotherapeutic drug cisplatin in combination with the mutant p53-rescuing molecule PRIMA-1^{MET}. We observed a synergistic apoptotic effect *in vitro* and *in vivo*. This effect was dependent on mutant p53. This synergistic effect may be due to the ability of cisplatin to promote elevated levels of mutant p53 in tumor cells, presumably enhancing their sensitivity to PRIMA-1^{MET}, thus suggesting that any agent inducing mutant p53 levels may synergize with PRIMA-1^{MET}.

STIMA-1, another small mutant p53-reactivating molecule has structural resemblance to the already identified CP-31398 compound. We show that both CP-31398 and STIMA-1 have similar chemical activity as traditional Michael acceptors and this activity is related to the observed mutant p53-dependent growth suppression. However, mutant p53-dependent growth suppression of tumor cells was more pronounced for STIMA-1 than that for CP-31398.

Although several mutant p53-reactivating drugs have been successfully identified, it is important to continue searching for new molecules by applying diverse screening techniques. Identification of different structural types of mutant p53-rescue molecules may provide a better understanding of the molecular mechanisms of mutant p53 reactivation. In addition, already identified lead molecules may not be suitable for clinical use due to non-specific toxicity or undesirable pharmacodynamic properties.

This thesis characterizes three structurally different small molecules that target mutant p53. Comparing all three different scaffolds will shed light on the possible molecular mechanisms of mutant p53 rescue. This should facilitate the design of more potent and selective mutant p53-targeting anti-cancer drugs.

LIST OF PUBLICATIONS

I. Bykov VJN, **Zache N**, Stridh H, Westman J, Bergman J, Selivanova G and Wiman KG.
PRIMA-1^{MET} synergizes with cisplatin to induce tumor cell apoptosis
Oncogene, (2005), 24, 3484-91

II. **Zache N**, Lambert JMR, Wiman KG and Bykov VJN.
PRIMA-1^{MET} inhibits growth of mouse tumors carrying mutant p53
Submitted

III. Bykov VJN, Issaeva N, **Zache N**, Shilov A, Hultcrantz M, Bergman J, Selivanova G and Wiman KG.
Reactivation of mutant p53 and induction of apoptosis in human tumor cells by maleimide analogs
JBC, (2005), 280, 30384-91

IV. **Zache N**, Rökaeus N, Lambert JMR, Shen J, Hainaut P, Bergman J, Wiman KG and Bykov VJN.
Mutant p53 targeting by the low molecular weight compound STIMA-1
Submitted

ABBREVIATIONS

3D	3 dimensional
APAF-1	Apoptosis protease-activating factor 1
ARF	Alternative reading frame
ASPP	Apoptosis stimulating proteins of p53
ATM	Ataxia telangiectasia mutated
Bak	Bcl-2 homologous antagonist/killer
Bax	B cell associated X protein
Bcl-2	B cell lymphoma 2
Bcl-X_L	Bcl-2-related protein X_L
BH3	Bcl-2 homology 3
Bid	Bcl-2 Interacting Domain
CBP	CREB-binding protein
CDB3	Core domain binding peptide number 3
Chk2	Checkpoint kinase 2
c-myc	Cellular myelocytomatosis oncogene
Cop1	Constitutively photomorphogenic 1
DR5/KILLER	Death receptor 5/KILLER
FADD	Fas-associated death domain
GADD45	Growth arrest and DNA damage-inducible 45
HAUSP	Herpesvirus-associated ubiquitin-specific protease
HIF-1α	Hypoxia-inducible factor 1α
HIPK2A	Homeodomain-interacting protein kinase 2A
His	Histidine
Hsp70	Heat shock protein 70
kDa	Kilodalton
Map4	Microtubule-associated protein 4
MDM2	Murine double minute 2
MIRA-1	Mutant p53-dependent induction of rapid apoptosis
NCI	National Cancer Institute
p53AIP	p53-regulated Apoptosis-Inducing-Protein 1
PI3K	Phosphatidylinositol-3-kinase
Pirh2	p53 induced RING-H2
PML	Pro myelocytic leukemia
PRIMA-1^{MET}	p53 reactivation and induction of massive apoptosis (methylated)
Pu	Purine
Puma	p53 upregulated modulator of apoptosis
PTEN	Phosphatase and tensin homolog deleted on chromosome 10
Py	Pyrimidine
Ras	Human homologue for rat sarcoma
Ref-1	Redox factor 1
RITA	Reactivation of p53 and induction of tumor cell apoptosis
SCID	Severe combined immunodeficiency
SH3	src homology 3
STIMA-1	SH group targeting and induction of apoptosis
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand

VEGF	Vascular endothelial growth factor

CONTENTS

INTRODUCTION

Cancer

Cancer is a genetic disease that can be regarded as a collection of more than a hundred diverse diseases, each affecting a distinct cell or tissue type in the body [1]. Cancer is characterized by populations of cells that divide and grow in an uncontrolled fashion, invading and destroying adjacent tissues, and even spreading to distant anatomical sites (metastasis). Each year 10.9 million people are diagnosed with cancer worldwide, and 6.7 million people die from the disease, making cancer the second largest cause of death next to cardiovascular diseases [2]. Over the past decades, intense research has led to the identification of genes and molecular pathways that are altered or disrupted in cancer cells. This progress raises hopes for the design of more efficient cancer therapy targeting specific defects in tumors.

The history of p53

Tumor viruses were studied intensively in the 1970s, since they were suspected to be the cause of many human tumors [1]. Experiments in 1979 showed that the T-antigen of simian virus 40 (SV40) was constitutively associated with a protein of 53 kDa in SV40 transformed cells. This protein was subsequently named p53. The observation that many tumor cells overexpressed p53 and that p53 could immortalize cells, or fully transform cells when co-expressed with the Ras oncogene [3-5], led to the classification of p53 as an oncogene. However subsequent molecular studies showed that the transforming ability of p53 was the result of mutation and that the SV40 T-antigen actually was inhibiting the normal activities of p53. It was shown that wild type p53 does not transform cells [6] and that the p53 protein can effectively inhibit oncogenic transformation of cells in culture [7]. Collectively these results showed that wild type p53 really functions a potent tumor suppressor. Subsequent genetic analysis of the p53 gene in human tumors showed the presence of point mutations in 50% of all human cancers, resulting in the inactivation of the p53 tumor suppressor pathway. It has become increasingly clear that in numerous cancers the tumor suppressive function of p53 is lost as a consequence of mutation, binding to viral proteins, or as a result of alterations in genes whose products interact with p53 or transmit information to or from p53 [8]. Thus, one decade after its initial categorization as an oncogene, p53 began a new journey as the most powerful tumor suppressor gene.

The p53 family

Almost 20 years after the discovery of p53, two structural homologues named p73 [9] and p63 [10-13] were identified. The transactivation domain, the oligomerization domain and in particular the DNA binding domain are conserved in all three members. Both p53 homologues contain two promoters encoding two distinct classes of proteins, one containing the N-terminal transactivation domain (TAp63, TAp73) and the other lacking it (ΔNp63,ΔNp73). This picture was further complicated when recent studies discovered an alternative promoter in the p53 gene and identified multiple p53 splice variants [14], which may explain the difficulties in linking the p53 status to biological properties and drug sensitivity in human cancers. The existence of alternatively spliced forms of p63 and p73, and the different isoforms of p53, contribute to this complexity, resulting in a complicated network of proteins involved in the control of cell proliferation, apoptosis and development [15]. In comparison to their "big brother", p63 and p73 are rarely mutated in human tumors [9, 12, 16]. Although some tumors show loss of heterozygosity in the p73 gene, there is no conclusive evidence that the remaining allele is inactivated [17]. Furthermore, the p73 gene is reported to be transcriptionally silenced due to hypermethylation in several leukemias and lymphomas [18]. An additional study indicates that inactivation of p63 or p73 can contribute to tumor development in mice and the loss of both genes can cooperate with p53 in tumor suppression [19].

Taken together, the three family members take part in the regulation of cell cycle arrest and apoptosis, but available data suggests a stronger involvement of p63 and p73 in development and differentiation [20-22].

The guardian of the genome

p53 is mutated in around half of all human tumors, making it the most frequently mutated gene in human cancer so far. Since the paradigm shift from oncogene to tumor suppressor gene in the late 80s, the central role of p53's tumor suppression function has been further supported by studying genetically engineered p53 null mice. Although born normally, these mice showed significantly increased susceptibility to spontaneous development of tumors at a young age [23]. Additionally, oncogenic viruses, such as the human papilloma virus (HPV), can promote malignancies through HPV-E6 expression which binds p53 and promotes its destruction by the ubiquitin–proteasome pathway [24].

These studies provide evidence for p53 involvement in suppressing malignant transformation of cells and development of tumors.

p53 structure

The p53 gene is located on chromosome 17p13.1 and encodes a nuclear protein of 393 amino acid residues with several well characterized functional domains (Fig. 1). The transactivation domain (TAD) is located at the amino-terminus and mediates both transcriptional activity and MDM2-binding [25]**.** The adjacent proline-rich domain (PRD) has been shown to be essential for interaction with SH3-containing proteins [26], while the DNA-binding domain (DBD), located in the center of the protein is of significance for sequence-specific DNA binding. Furthermore, this domain is frequently mutated by missense mutations in human cancers. In conjunction with the PRD and DBD, a 21 amino acid sequence (aa 92-112) was identified as being crucial for p53 degradation by both MDM2 and HPV-E6 [27, 28]. Additionally, it was demonstrated that the proline-rich region is necessary for achieving full apoptotic p53 function in response to DNA-damage caused by chemotherapeutic agents [29].

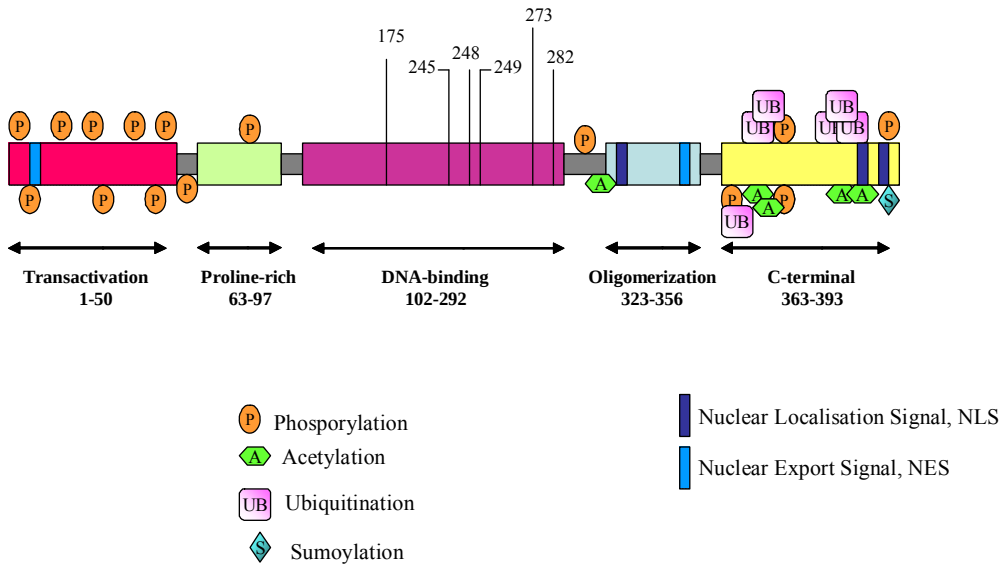

Figure 1. Schematic representation of the p53 protein with its functional domains and the positions of different posttranslational modifications. The majority of the p53 mutations are located within the DNA-binding domain, affecting in a high frequency six hotspots residues.

Stabilization of p53 induces the formation of a p53 tetramer composed of four p53 monomeric subunits linked via the oligomerization domain (OD). Tetramerization of p53 is required for optimal p53 transactivation and function [30]. The OD is located at the

carboxy terminus, which contains nuclear export signals (NES) and nuclear localization signals (NLS), as well as ubiquitination sites for p53 degradation. Additionally, other posttranslational modifications sites have been reported throughout the p53 protein and many of these have been shown to be critical for p53 tumor suppressor function. The last 30 C-terminal amino acids of p53 possess a negative regulatory function, which inhibits the specific DNA-binding and transcriptional activity of p53 [31, 32].

p53, the transcription factor

The p53 protein plays a major role in the maintenance of genome stability in mammalian cells. Since p53 is a potent inducer of apoptosis, the protein levels of p53 must be kept low under normal conditions. MDM2, a p53 target gene, targets p53 for ubiquitination and rapid proteasomal degradation, establishing a negative feedback loop. Upon cellular stress such as DNA damage, oncogene activation, telomere erosion or hypoxia p53 becomes functionally activated through a complex series of posttranslational modifications [8, 33]. Once activated p53 acts as a sequence specific transcription factor capable of inducing different biological responses (Fig. 2) such as cell cycle arrest, apoptosis, senescence and differentiation [33]. The choice of response is dependent on the intrinsic and extrinisic signals to the cell.

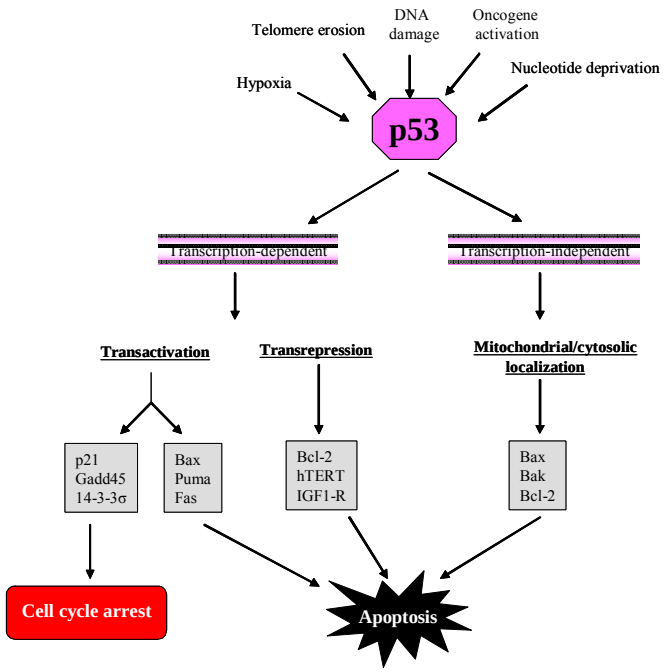

Figure 2. Various stress signals activate p53, resulting in transcription-dependent and independent responses.

p53 transactivates genes containing specific p53 binding sites in their regulatory domain. The consensus p53 binding site consists of two copies of the inverted pentameric sequence PuPuPuC(A/T)(T/A)GPyPyPy separated by 0-13 base pairs [34]. One copy of the motif is insufficient for binding and disparities within the motif decrease the affinity for p53. Many p53 responsive genes have been described [33, 35]. DNA microarray studies have indicated the upregulation of several hundred genes by wild type p53 [36, 37] and bioinformatic studies of the human genome sequence have identified 4428 genes with at least one putative p53-binding site [38]. However it remains unknown how many of these sites p53 will actually bind to. Interestingly p53 can also stimulate target genes that lack the canonical sequence such as p53-induced gene 3, PIG3 [39] and the pro-apoptotic phosphatase PAC1 gene [40]. A recent study using global proteome analysis identified 55 putative p53-regulated proteins, many of which had no previous connection to p53. These identified p53-regulated proteins fall into different functional categories, including mRNA processing, translation, redox regulation, and apoptosis [41]. This innovative approach may lead to the discovery of more novel targets that are important for improved cancer therapy. Nevertheless, the complexity of p53-dependent biological responses increases with the ever growing list of novel p53 target genes.

Mechanisms of regulation

Stabilization

Rapid degradation of p53 in normal cells is critical to efficiently dampen p53 activity. However stabilization of the p53 protein is necessary to induce biological responses upon different and diverse forms of stress. It is becoming clear that each of these stress signals is likely to inhibit MDM2-mediated degradation of p53, but is achieved through numerous independent pathways.

MDM2 (**m**urine **d**ouble **m**inute 2), an E3-ubiquitin ligase was first isolated from a spontaneously transformed mouse fibroblast cell line, in which the MDM2 gene was amplified [42]. MDM2 is considered oncogenic because of its amplification in tumors and its ability to inactivate p53. The human homolog, HDM2, is frequently overexpressed in human tumors, especially sarcomas [43]

MDM2's role as a major regulator of p53 was further supported, when MDM2 deficient mice showed very early embryonic lethality [44, 45]. The observation that the

lethality is entirely rescued by the simultaneously depletion of p53 strongly supports the model in which loss of MDM2 leads to uncontrolled p53 activity [45].

So how does MDM2 regulate p53? MDM2, a p53 target gene itself, regulates p53 in a negative auto-regulatory feedback loop through binding to p53's transactivation domain, thus catalyzing p53 ubiquitination and proteasomal degradation [46]. Due to this interference, the ability of p53 to activate gene expression is inhibited [47, 48].

Several reports have identified reduced binding between MDM2 and either phosphorylated p53 proteins or phosphorylated peptides representing the MDM2 binding region of p53. These studies suggest a potential role for serine (Ser)15 [49] and Ser20 [50]. Phosphorylation at these p53 sites certainly correlates with stabilization of p53 in response to some signals, although the observation that different patterns of phosphorylation occur in response to different stabilization signals indicates that no individual site is responsible for stabilization in response to all signals. Indeed murine studies using mutated residues equivalent for Ser15 [51, 52] and Ser20 [53] indicate that phosphorylation is not essential for all forms of DNA damaged induced stabilization of p53.

Other findings identified a p53 deubiquitinating enzyme, HAUSP, which rescues ubiquitinated p53 from degradation and serves as a potent p53 stabilizer [54, 55].

DNA damage can also stabilize p53 via phosphorylation of MDM2 by ATM [56], thereby reducing its ability to bind p53. Oncogene activation can block the p53-MDM2 interaction through $p14^{ARF}$ (mouse $p19^{ARF}$) binding to MDM2, thus inhibiting MDM2's E3 ubiquitin ligase activity, resulting in p53 stabilization [57, 58]. Anything that causes disruption between MDM2- p53 interactions is a candidate mechanism for DNA-damaged-induced p53 protein stabilization, but there are also some mechanisms, which do not involve MDM2. One alternative is the involvement of two newly identified ubiquitin ligases termed Pirh2 and Cop1. Both are p53 target genes and induce p53 ubiquitination and degradation [59, 60]. Furthermore, it has been shown that the Jun N-terminal kinase, JNK, is able to bind to the core domain of p53 and promote its ubiquitination and degradation in non- stressed cells [61].

p53 activation

The conversion of p53 from a latent to an active state can be modulated by different types of posttranslational modifications. Phosphorylation of p53 is intimately associated with the activation of p53 in response to cellular stress. Currently at least 15 phosphorylation sites have been reported and it has been well accepted that phosphorylation of p53 by numerous specific protein kinases contributes to its stabilization and transcriptional activity under stress conditions [62, 63]. However, none of the stimuli can induce all the phosphorylation pathways and the p53 phosphorylation patterns vary in response to different stimuli. In addition, different cell types respond differently to the same stimulus. It is equally noteworthy that dephosphorylation may play an important role in regulation of p53 function. In unstressed cells, Ser376,378 and threonine 55 are commonly phosphorylated [64, 65], but after irradiation it has been shown that Ser376 is dephosphorylated and thereby exposes a consensus binding site for the 14-3-3 protein [65]. Furthermore, it has been shown that the phosphorylation of some specific sites may affect p53 transcriptional target selection, for example phosphorylation at Ser46 by HIPK2A, which leads to selective transactivation of p53AIP1, a pro-apoptotic target gene [66, 67].

The peptidyl-prolyl isomerase Pin1 is able to bind phosphorylated p53 and induce conformational changes of p53, which increases its transcriptional activity [68, 69]. This suggests a new mechanism in controlling p53 stability and function after phosphorylation.

The C-terminal of p53 is not only modified by phosphorylation but also by acetylation, ubiquitination and other posttranslational modifications [70, 71]. Six lysine residues within the C-terminal are targeted for ubiquitination [72] and four of them even for acetylation [63] suggesting that acetylation of p53 may inhibit its ubiquitination [73].
DNA damaged-induced phosphorylation of the N-terminus of p53 leads to an increased association with the histone acetyltransferases p300 /CBP, p53 acetylation and increased p53 transactivation activity [74]. Acetylation of p53 contributes to p53 specific DNA-binding and transcriptional activity, but the exact physiological role of acetylation should be further investigated [63, 75].

Other p53 co-activators are ASPP1 and ASPP2, belonging to the ASPP family. These proteins interact with p53 through its DBD and specifically induce p53-dependent apoptosis [76].

Subcellular localization

Not only does MDM2 function as an ubiquitin-ligase but also as a shuttle for p53. MDM2 carries p53 from the nucleus into the cytoplasm, where degradation occurs through cytoplasmic proteasomes. MDM2 contains both nuclear import and export sequences and mutations within the nuclear export sequence inhibits the ability of MDM2 to promote the degradation of p53 [77]. However, p53 itself has two nuclear export signals (NES) that function in the absence of MDM2 [78], indicating that the two proteins could shuttle independently of each other. Nevertheless, degradation of p53 by MDM2 depends directly on the ability of MDM2 to shuttle from the nucleus to the cytoplasm [79]. Moreover it has been shown that the ubiquitin ligase activity of MDM2 is crucial for efficient p53 export, since mutated lysines residues in the C-terminus blocked the transport of p53 out of the nucleus [80]. In either case it is clear that regulation of subcellular localization is a potent mechanism to regulate p53 stability.

Redox modulation

A variety of cellular functions including apoptotic signalling are regulated by redox modulation. Studies have shown that the conformation of p53 and its DNA binding activity are also regulated by the redox state of the protein. p53 is susceptible to oxidation, resulting in inhibition of DNA binding whereas reduction favours DNA binding [81-83]. Furthermore, it has been shown that p53 is dependent on the coordination of zinc for both correct folding and specific DNA binding in intact cells [84]. Several cysteine residues in the core DNA-binding domain are involved in zinc coordination, and mutational analysis has shown that cysteines at positions 173, 235 and 239 participate in DNA binding and are also critical for transcriptional activation and suppression of transformation [83]. The redox state of p53 affects binding to target DNA in such a way that consensus DNA sequences are recognized by reduced p53 only and not by oxidized p53, whereas non-specific DNA is equally well recognized by both forms of p53 [85]. After genotoxic stress conditions, p53 might be regulated by both the presence of oxygen intermediates and the antioxidant defence mechanism of the cell [81, 82]. Antioxidant systems usually protect from cell death by scavenging reactive oxygen species (ROS). A recent report has shown that selenomethionine and Ref-1 can

maintain p53 in a reduced state in cultured cells [86]. In addition, others have suggested that redox-sensitive proteins such as HIF-1α [87] and Ref-1 [88] can interact with p53 and alternate its activity or level.

p53 inactivation in tumors

p53 function in tumors can be lost by numerous mechanisms including lesions in the p53 upstream signalling cascade resulting in prevention of p53 activation, p53 mutations or mutations of downstream mediators of p53 function. Around 50 % of all human tumors carry a p53 mutation, of which 95 % are clustered within the DNA binding domain. The tumor-associated mutations are predominantly missense mutations that result in a single amino-acid substitution leading to a mutational spectrum which is different from that seen in other tumor suppressor genes, in which large deletions or frameshift mutations tend to result in a complete loss of protein expression [33]. p53, on the other hand, shows a high frequency of point mutations of certain codons with 28 % of the mutations affecting only six residues, R175, R245, R248, R249, R273 and R282, also known as "hot spot" mutations (Fig. 1) (http://www-p53.iarc.fr/). p53 mutants can be divided in two main types, contact mutants that carry substitutions of residues that bind DNA directly, and structural mutants that affect protein folding [89]. Since these mutants are unable to bind and transactivate p53 target genes, the expression of MDM2, the major regulator of p53 levels, is abrogated, resulting in the elevated expression of mutant p53 protein levels in many tumor cells. Since mutant p53 proteins are often more stable than wild type p53, selection of these mutants can lead to a dominant-negative inhibition of wild type p53 via the formation of wild type and mutant p53 tetramers [90]. Mutation of p53 typically occurs in one allele whereas the other one is often lost [91], indicating that the efficiency of dominant negative inhibition might not be complete and almost certainly depends on the nature of the initial point mutation. However, partial inactivation of wild type p53 function by mutant p53 might allow for some selective advantage for tumor progression.

Other mutants may acquire oncogenic properties that contribute to tumor development, the so called gain-of-function (GOF) [92, 93]. Mutant p53 is able to bind to p63 and p73, thereby inhibiting their activities [94]. However the mechanism underlying the p53 gain-of-function is not clear. This type of interaction is not seen between the wild type p53 and its family members. Nevertheless, it appears to be affected by a p53 polymorphism at codon 72 which influences whether or not the mutant p53 protein can bind and

inactivate p73 [95]. Another possible gain-of-function activity of mutant p53 is promiscuous DNA binding and illegitimate transactivation of genes such as VEGF and c-myc [96].

Interruption of the p53-MDM2 interaction is mediated by the ARF tumor suppressor protein upon oncogene activation. $p14^{ARF}$ is encoded by the INK4a locus, which also encodes the p16 tumor suppressor protein. $p14^{ARF}$ is inactivated in a variety of cancers [97]. For example epigenetic silencing or loss of the ARF locus results in the loss of p53 function in response to oncogene activation. This type of mutation is commonly seen in tumors which retain wild type p53.

The inability of some tumor cells to induce a p53 response can be a result from defects in components of the apoptotic cascades including APAF-1 or caspase 9 [98]. The inactivation of APAF-1 and caspase 9 can substitute for the loss of p53.

An alternative mechanism to impair the p53 tumor suppressor pathway is inactivation of upstream checkpoint kinases such as Chk2 and ATM, which contribute to p53 activation. Chk2 mutations have been reported in breast cancers [99].

Germline mutation of p53 occur in the hereditary Li-Fraumeni syndrome that predisposes individuals to sarcomas, lymphomas, breast and brain tumors at a relatively young age [100].

p53 and cell cycle arrest

Regulation of the cell cycle involves several crucial steps, including detecting and repairing DNA damage. Checkpoint controls operating throughout the cell cycle are vital for the cell to ensure cell cycle progression only when the preceding step is properly completed. The arrest of the cell cycle is a reversible process that can take place at the G1/S or G2/M transition.

The p53 target p21 is a key inhibitor of several cyclin dependent kinases (CDK), including cyclinE/CDK2 (G1 arrest) and cyclinB/CDK1 (G2 arrest) [101, 102]. Two independent studies have shown that the requirement for p21 is different in establishing growth arrest upon DNA damage. Embryonic fibroblasts obtained from p21 null mice are only partially defective in their ability to undergo G1 arrest [103, 104], suggesting that

another p53 gene product contributes to the complete response. In contrast, the G1 arrest response was completely abrogated in a human tumor cell line expressing wild type p53 in the absence of p21 [105].

Another p53-regulated target called Gadd45, whose protein product is expressed in response to a wide variety of DNA damaging agents [106], has been shown to induce G2 growth arrest by inhibiting the cyclin B/CDK1 [107]. Furthermore, quantitative analysis of gene expression patterns have shown that the p53 target 14-3-3σ is strongly induced by ionizing irradiation, suggesting that cytoplasmic 14-3-3σ binds and inhibits the translocation of cyclinB/CDK1 to the nucleus, a process required for the initiation of mitosis [108], thereby maintaining the G2 checkpoint.

p53 and apoptosis

Analysis of cells and tissues of p53 null mice have shown that functional p53 is necessary for DNA damage-induced apoptosis [109-111]. The significance of p53-mediated apoptosis to protect cells from transformation has been shown in tissue culture, where p53 null cells failed to undergo cell death after subsequent introduction of a variety of oncogenes [112]. Two distinct signalling pathways, the intrinsic and the extrinsic pathway can trigger apoptosis.

The intrinsic response

The intrinsic pathway is activated by signals that originate from within the cell and involves cytochrome c release from the mitochondria. This pathway can be regulated by pro-and anti-apoptotic proteins of the Bcl-2 family [113]. The family consists of pro-survival proteins including Bcl-X_L, pro-apoptotic proteins including Bax and Bak, and the "BH3-only" pro-apoptotic factors including Puma and Noxa. The BH3 domain is required for the pro-apoptotic activity in these proteins [114, 115]. p53 transactivates Bax [116, 117] and Puma [118] and downregulates Bcl-2 [117, 119]. Induction of the pro-apoptotic Bcl-2 family members will trigger cytochrome c release followed by apoptosome formation and activation of a caspase cascade resulting in cell death.

APAF-1 and p53AIP1 are factors of the p53-induced apoptotic program. APAF-1, an effector downstream of the mitochondria, binds to cytochrome c and forms the apoptosome, and then initiates caspase 9 cleavage [120], whereas p53AIP1 appears to affect the mitochondrial membrane potential leading to apoptosis.

p53 can also activate expression of genes that inhibit survival signals, for example PTEN [121], a negative regulator of the PI3K pathway.

The extrinsic response

The extrinsic response is initiated outside the cell and involves the activation of pro-apoptotic cell surface receptors including Fas and DR5/ KILLER. These receptors are transmembrane proteins termed death receptors, referring to their ability to trigger the apoptotic program. After ligand binding, the cytoplasmic tail of the receptor acts via the FADD protein to assemble a **d**eath receptor-**i**nducing **s**ignalling **c**omplex (DISC) to induce caspase 8, thereby triggering an effector cascade leading to an apoptotic response. The Fas receptor is a p53 target, which is transcriptionally upregulated upon DNA damage [122]. However, Fas induction by γ-irradiation is tissue-specific and occurs in lung, thymus, spleen but not in heart or liver [123]. The DR5/ KILLER receptor of the TRAIL family is also a target of p53 that is induced upon DNA damage and triggers apoptosis specifically in some tissues [124, 125].

Caspase 8 is activated in the death receptor pathway, but is also able to induce the cleavage of the BH3 only protein Bid to truncated Bid, which then is able to activate Bax [126], thus establishing a link between the extrinsic and the intrinsic pathway.

p53 transrepression

In addition to transactivating specific genes, p53 is also able to repress genes that are involved in apoptosis and tumor suppression [127]. One of the first identified p53-repressed targets was the anti-apoptotic Bcl-2 gene [128]. Subsequently, p53 has been shown to repress Bcl-X_L [129], and several other genes implicated in apoptosis, including Map4 [130], hTERT/telomerase [131], and survivin [132]. In addition, p53-mediated repression of the insulin-like growth factor 1 gene (IGF1-R) [133] can block survival signalling and thus contribute to the induction of apoptosis, and downregulation of the multi-drug-resistant gene 1 (MDR1) can counteract drug resistance [134].

The negative regulation by p53 appears to be mediated by different mechanisms. One proposed mechanism does not require the binding to specific p53 sequences, but rather involves the recruitment of p53 into a large repressor complex via interaction with the

co-repressor mSin3a and histone deacetylases (HDAC) [135]. p53-mSin3a-mediated transrepression has been shown to induce apoptosis [135, 136].

p53-induced transcription-independent apoptosis

Mutant forms of p53 fail to activate transcription, but some retain the ability to induce apoptosis, suggesting that p53 transcriptional activity, in some situations, is dispensable for the induction of apoptosis [88, 137].

In γ-irradiated primary thymocytes, a fraction of activated p53 was able to translocate to the mitochondria where it can interact through its DBD with the anti-apoptotic proteins Bcl-2 and Bcl-X_L, resulting in disruption of the stabilization of the mitochondrial membrane mediated by these Bcl-2 family members [138]. Furthermore, cytosolic p53 can directly activate pro-apoptotic Bax, and p53-dependent Bax activation is not impaired by blocking transcription nor by using an endogenous transcription-inactive mutant of p53, further supporting the idea of transcription-independent p53-mediated induction of apoptosis [139, 140].

To summarize, p53 is an executioner of apoptosis that acts via both transactivation of death-promoting genes and simultaneous transrepression of pro-survival genes (Fig. 2).

Choice of response

p53 is considered the most powerful tumor suppressor, but how does it decide between cell cycle arrest and apoptosis?

Several factors, including cell type, presence or absence of survival factors in the external environment, extent of DNA damage, and p53 protein levels, play a role in deciding whether p53 will induce cell cycle arrest or apoptosis [88]. Although the mechanism governing the decision of the cell is not elucidated, deletion of p21 can cause cells that normally undergo p53-dependent cell cycle arrest to undergo apoptosis instead.

Analysis of p53 mutants has shown that the cellular response partially depends on which p53 target genes become transcriptionally active [141, 142]. Some tumor-derived mutants, for example the p53A143 mutant, retain the capacity to activate genes involved in G1 arrest, but show a reduced ability to bind p53 specific sequences in promoters of pro-apoptotic genes. It has been proposed that p53 binding affinity differs

in various target promoters. Mutants with minor conformational changes are still able to bind high affinity sites in promoters of cell cycle arrest genes, but are unable to bind low affinity sites that are present in promoters of apoptotic target genes. This could explain the observation that low levels of p53 protein induces cell cycle arrest, whereas higher levels of p53 activate the apoptotic machinery [88]. *In vivo* studies investigating the binding of p53 to promoters have to some extent supported this model [143, 144] by confirming the presence of high-and low-affinity p53-binding sites in cell cycle arrest and apoptotic promoters. However, not all known pro-apoptotic target genes are regulated by low-affinity p53-binding sites. The binding affinity of p53 to the Puma promoter which regulates a potent pro-apoptotic gene was found to be similar to that of the p21 and MDM2 promoters.

Several other studies suggest that covalent modifications including phosphorylation may play a vital role in the decision making of what p53 responsive gene to choose. Phosphorylation of Ser46 is specifically required for the efficient transactivation of the pro-apoptotic p53AIP1 gene [67], suggesting that this modification might be crucial for conformational changes altering the p53 DNA-binding specificity directly (Fig.3).

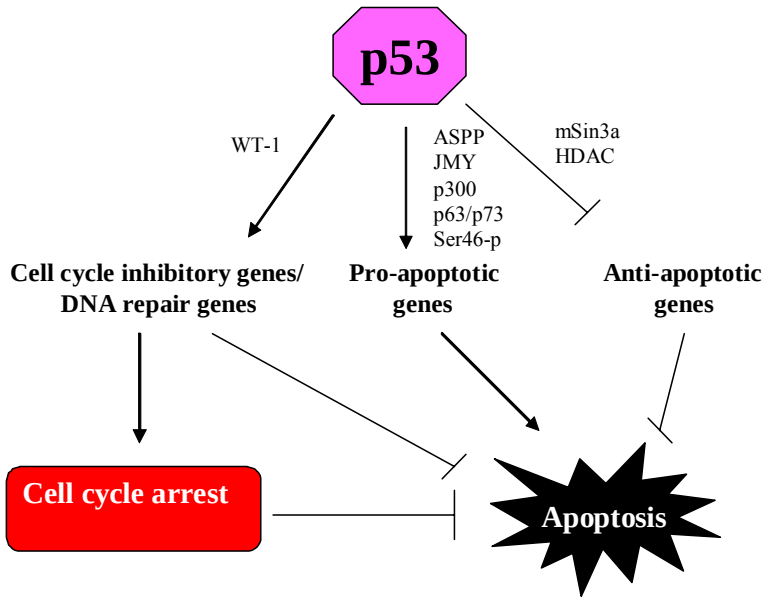

Figure 3. Different co-activators, co-repressors or posttranslational modifications of p53 determine its choice of response

Furthermore, p53 forms complexes with other transcriptional regulators, including acetyltransferases such as p300/CBP, to function efficiently as an activator of gene

expression. This type of interaction allows the acetylation of histones, which opens up the chromatin and thus gives access to the basal transcriptional machinery [145]. JMY, a transcriptional co-factor, can stimulate p53-dependent gene expression from the Bax promoter without changes in p53 protein levels, suggesting that JMY stimulation of p53 is due to transcriptional co-activation with p300 and not p53 protein accumulation. Interestingly, JMY can induce the Bax protein, but not the p21 protein [146]. A study in colorectal cancer suggests that p300 levels are critical in determining p53's response after DNA damage. At high levels of p300, the p53 activation is transient, leading to the transactivation of p21, with minimal activation of pro-apoptotic pathways. However if the levels of p300 are low or absent, p53 stability is increased and results in reduced p21 activation, but augmented Puma activation, thus favouring apoptosis over cell cycle arrest[147].

Additionally, ASPP proteins have been shown to selectively enhance the DNA binding of p53 to the Bax promoter *in vivo* and to stimulate the promoters of the pro-apoptotic responsive genes and not other targets such as p21 [76]. In agreement with this notion, inhibition of the ASPP expression was shown to selectively block the apoptotic response to p53 [148].

More evidence pertinent to p53's choice of response came from experiments revealing that the induction of apoptosis by p53 requires the presence of at least one other p53 family member, p63 or p73 [149]. This is consistent with the finding that p53 does not bind promoters of pro-apoptotic target genes in p63/p73 double knockouts. The significance of this finding is still unknown, and since wild type p53 does not interact with its family members, an indirect contribution of p63 and p73 is suggested.

Moreover it has been shown that the Wilms tumor suppressor gene product, WT1, binds p53 and inhibits p53-dependent apoptosis, without affecting p53-dependent growth arrest [150].

The growing family of co-factors that interact with p53 and are required for p53-dependent induction of apoptosis emphasizes the complexity of the network by which the apoptotic response can be regulated.

Therapeutic strategies

Around half of all human tumors carry mutated p53. However, p53 is most likely non-functional in the remaining wild type p53-carrying tumors. In the clinic, the functional status of p53 has been related to prognosis, progression and therapeutic response of tumors [151, 152] with tumor cells containing wild type p53 are usually more sensitive than those bearing mutant p53. All these characteristics make p53 an ideal molecular target for cancer therapy [151, 153].

Restoration or imitation of p53 function in p53-deficient tumors, will result in either a direct (tumor growth inhibition) or indirect (sensitization to treatment) therapeutic benefit. Although activation of p53 is generally viewed as the most direct and promising anti-cancer strategy, it is not a favourable event for normal tissues. p53 reactivation as a result of genotoxic stress associated with chemo-or radiation therapy was found to be responsible for massive apoptosis in several normal tissues known to be sensitive to genotoxic stress, possibly contributing to the severe side-effects of cancer treatment [154, 155]. That is why attempts to target human tumors without damaging the surrounding healthy cells have been explored. Thus, a chemical inhibitor of p53 named pifithrin-α (PFT-α) has been identified [156]. The compound was successfully used in *in vitro* models to protect normal cells from otherwise lethal doses of chemo- and radiotherapy [157]. PFT-α was also shown to protect mice from lethal genotoxic stress associated with an anti-cancer treatment without promoting tumor formation [156]. Therefore it has been proposed that this compound could be used in tumors that lack functional p53 in order to avoid damaging of the healthy adjacent tissues [157, 158].

The identification of p53-targeting compounds has been the focus of intense research. Since this signalling pathway is very well studied one can take advantage of it and develop tools affecting individual components or effectors within the p53 pathway. p53 function is inactivated in tumors either by mutations/deletions in the gene itself, by viral p53-inactivating proteins, or through the deregulation of other members of the pathway. Modulation of the p53 pathway may target any of these factors.

Gene therapy

Reconstitution of p53 function in cancer cells by introduction of exogenous wild type p53 genes has been investigated as a strategy for novel cancer therapy. Overexpression of p53 is sufficient to induce apoptosis in most cancer cells with somewhat reduced

efficacy in tumor cells containing wild type p53 [159]. Various p53 gene therapy protocols have been proposed, and among them the replication-deficient adenovirus-mediated p53 gene delivery protocols represents a common approach, due to high amount virus generation and a broad spectrum of targets cells [160].

ONYX-015 is an adenovirus with the 55KD E1B coding sequence disrupted, thus making the virus unable to replicate in wild type p53-carrying cells, but allowing replication in p53 deficient cells due to p53 mutation, p53 depletion, loss of $p14^{ARF}$ or MDM2 overexpression, representing the majority of tumor cells. Clinical trials using ONYX-015 have shown that administration of this virus is safe and well tolerated, and that it has a therapeutic effect in at least some patients [161, 162].

Targeting p53 by small molecules

Small molecules have several advantages as therapeutic agents, including the possibility of systemic administration, which will potentially allow treatment of patients with disseminated malignant disease. Compounds that target p53 for anti-cancer drug development can be achieved via two major approaches: random screening or rational drug design. Both strategies have their advantages and disadvantages.

Rational design

Unlike the classical method of drug discovery by trial-and error testing of chemical substances, rational drug design begins with the knowledge of specific chemical responses. Current drug discovery efforts are based on rational identification of chemical compounds that will bind to specific target molecules. These efforts are based on the 3D structure of the target molecules and use of a variety of computer-based molecular modelling techniques to exploit the 3D structural information. The interacting surface of MDM2 and p53 as well as the structures of both proteins are well studied and they are therefore suitable targets for the rational design of anti-cancer drugs. The discovery of nutlins [163] that activate p53 by inhibiting the binding of MDM2 to p53 is a notable achievement and an example of successful rational drug design. The structure-based drug design operates against a relatively narrow target defined by structural analysis of interacting molecular cell surfaces, verifying the specificity of small molecules emerging from such screenings *in vitro*. However, the designed compounds could still affect other proteins in the cellular environment, thus leading to unexpected and undesired biological effects.

CDB3, a short peptide derived from the p53-interacting protein ASPP [76], has been specifically developed to bind to the p53 core domain. The peptide has been shown to reactivate mutant p53 *in vitro* [164]. However, CDB3 binds p53 and restores its native conformation in living cells, it only has a weak biological response [165], suggesting that the peptide has unknown functions and may interfere with the p53 tumor suppressor function, thus consequently resulting in the prevention of the expected biological outcome of the p53 reactivation. The lack of potent apoptosis response may be due to the fact that CDB3 inhibits the interaction between p53 and the ASPP protein.

Random cell-based screening

The advantage of a random cell-based screening is the usage of a functional assay, thus bringing selection conditions closer to the final application of the compounds. This allows testing for a specific required activity of compounds by filtering out the cytotoxic ones. This strategy permits a much broader approach of drug identification than rational drug design.

The primary screening involves individual testing of numerous compounds from chemical libraries, greatly reducing the complexity of the library by selecting a set of "hits" that include molecules with the desired properties without losing any prospective candidate compounds. The newly selected "hits" are forming a sublibrary, which then will be subjected to more accurate selection by passing it through additional assays to raise the probability of secluding desired compounds. The selection of the assays is significant for the detection of the desired drug. The most common argument raised against the cell-based readout systems is the difficulty of verifying the specificity of the small molecules emerging from such screenings. However, one also could benefit from this problem, since further studies investigating the molecules mechanism of action might lead to the discovery of new targets and maybe even to new regulatory pathways in the cell.

Tumors with wild-type p53

Therapeutic strategies targeting tumors harbouring wild type p53 focus on activating the function of the endogenous p53 gene in the tumor. Members of the p53 pathway such as MDM2 and $p14^{ARF}$ might be targets for screening molecules modulating p53

activity. Small molecules that inhibit MDM2-p53 interaction might be able to reactivate the p53 pathway in cancers that overexpress MDM2 [166].

Recently, the first potent and selective small-molecule antagonists of the p53-MDM2 interaction, the nutlins, were identified [163]. The nutlins bind tightly into the p53 pocket of MDM2 and displace p53 from its complex with MDM2. They penetrate cell membranes and inhibit the interaction of p53-MDM2, leading to stabilization and activation of p53 target genes [163, 167]**.** Their anti-tumor effect was only observed in cells carrying wild type p53 but not in cells with mutant or deleted p53, suggesting that the activity of nutlins is derived from activation of the p53 pathway. RITA, another small molecule has been shown to disrupt the interaction between p53 and MDM2 and induce apoptosis in wild type p53 carrying cells [165].

The use of small peptides derived from $p14^{ARF}$, which map at the $p14^{ARF}$/MDM2 interface, can activate p53, providing an additional target for modulating the MDM2-degradation pathway [168]. Unfortunately, practical application of all these approaches, despite the high specificity, is restricted by the problem of *in vivo* delivery of peptides.

Reactivation of mutant p53 by small molecules

Due to their potency and specificity, small peptides have been widely used for interruption of protein function. Both amino-and carboxy- terminal peptides have been developed for the purpose of enhancing or restoring p53 function. A series of carboxy - terminal peptides were used to interfere with the carboxy-terminal negative transcriptional regulatory element of p53. Among them, a 22 amino acid peptide, corresponding to a p53 fragment from amino acids 361 to 382, manifested high potential for restoring DNA-binding and transcriptional activity in some mutant p53-harbouring cell lines [169, 170]. The effect was dependent on the expression of mutant p53 and the peptide was not toxic to wild type p53 or p53 null cells. These findings provide evidence of mutant p53 rescue by peptides, raising hope for the development of anti-cancer drugs targeting mutant p53-carrying tumors (Fig.4).

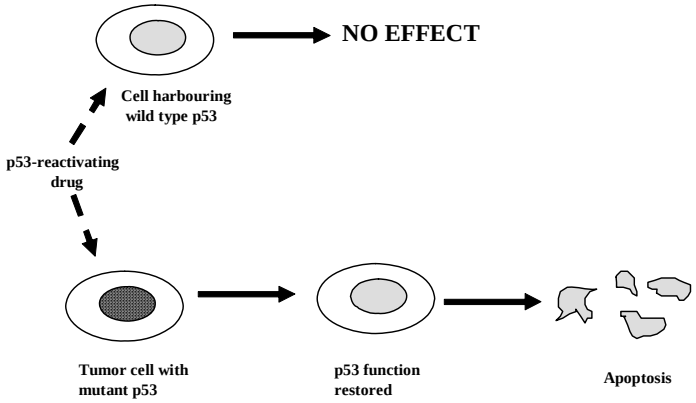

Figure 4. A strategy for mutant p53 reactivation. Introduction of the mutant p53-reactivating drug will induce apoptosis, while normal tissue will be unharmed.

CP-31398

CP-31398, a styrylquinazoline was identified in the screening of multiple classes of small molecules with the ability to stabilize wild type-associated epitope (mAb1620) of the p53 binding domain *in vitro* [171, 172]. This was the first compound reported with the ability to alter mutant p53 to wild type conformation and rescue p53 function in some tumor cell lines and xenografts. Administration of CP-31398 suppressed the growth of p53 mutated tumor xenografts including the DLD-1 colon carcinoma cells (mutation at 241) and the A375.S2 melanoma cells (mutation at 249) in mice without obvious toxicity [171]. It has been reported that in some cell systems the ability of CP-31398 to promote the expression of the mAb1620 epitopes does not correlate with enhancement of p53-dependent transcription [173]. Since even wild type p53 requires appropriate activation signals such as hypoxia, telomere shortening or DNA damage to become transcriptionally active, and lack of such signals or the inability to sense them may prevent the transcriptional activity of p53 even if the conformation and function of mutant p53 is restored by CP-31398. Indeed a study demonstrated that continues exposure to CP-31398 does not induce p21 induction and cell death in Li-Fraumeni cells until late in the crisis period when telomere shortening occurred [174]. It has been suggested that CP-31398 stabilizes only newly synthesized p53 that is in the active conformation [171], but yet to date there is no physical evidence for direct interaction between CP-31398 and p53. Evidence of some p53-independent effects suggest that

p53 may not be its only target. The global alteration of gene expression profile rather than merely p53 targets following treatment of CP-31398 suggests other pathways may exist in CP-31398-induced cell cycle arrest and apoptosis [175]. There is also evidence that CP-31398 can stabilize p53 family members, suggesting that this may be part of its mechanism of action in mutant p53-expressing tumors [176].

The discovery of CP-31398 reveals a unique pathway different from the well-known DNA-damage induced p53 pathway with no obvious phosphorylation at the amino-terminus of p53 [176]. Further understanding of the mechanism may lead to novel strategies for p53 stabilization and tumor suppression in cancers.

PRIMA-1

The colorimetric cell proliferation assay WST-1 was used as a readout system to identify mutant p53-reactivating molecules. The assay was based on the human osteosarcoma cell line Saos-2-His273. This cell line carries tetracycline-regulated mutant p53 (Tet-Off) and treatment with doxycycline resulted in downregulation of mutant p53 expression levels in those cells (Fig. 5). Using this approach, we identified PRIMA-1 and MIRA-1, two mutant p53-reactivating molecules [177, 178].

Figure 5. Cellular screening to identify compounds that inhibit cell growth and/or induce apoptosis only in the presence of mutant p53.

PRIMA-1 is the second class of compounds to have the capability of restoring tumor suppressor function to mutant p53. PRIMA-1 has been shown to rescue both transcription-dependent and transcription-independent p53-mediated apoptosis in human tumor cells [179, 180]. Furthermore it was shown that intravenous administration of PRIMA-1 caused inhibition of human tumor xenograft growth in mice, without any obvious signs of toxicity [177]. PRIMA-1^{MET} is a methylated form of PRIMA-1 that is even more potent in inducing mutant p53-dependent apoptosis than PRIMA-1 itself [181]

and also acts synergistically with cisplatin to inhibit tumor xenograft growth in SCID mice[181]. Moreover nucleolar translocation seems to be critical for mutant p53 reactivation since PRIMA-1^{MET} treatment results in a striking redistribution of mutant p53 to the nucleoli, together with the PML bodies-associated proteins PML, CBP and Hsp70[182]. However, the exact molecular mechanism underlying PRIMA-1 and PRIMA-1^{MET}-mediated reactivation of mutant p53 remains to be elucidated.

AIMS OF THE THESIS

The general aim of the thesis was to characterize different mutant p53-reactivating small molecules. The novel structural scaffolds with mutant p53-targeting capacity may help understand the molecular mechanisms of mutant p53 rescue and thus facilitate the design of more potent and selective mutant p53-targeting anti-cancer drugs.

SPECIFIC AIMS:

- **Paper I:** To study if combined treatment with PRIMA-1^{MET} and commonly used anti-cancer drugs could result in a synergistic apoptotic effect in human tumor cells.

- **Paper II:** To examine the effect of PRIMA-1^{MET} on mouse tumors carrying mutant p53

- **Paper III:** To characterize maleimide analogs, a novel class of mutant p53 targeting compounds

- **Paper IV:** To characterize STIMA-1, a novel mutant p53 targeting compound

RESULTS AND DISCUSSION

PAPER I: PRIMA-1^{MET} synergizes with cisplatin to induce tumor cell apoptosis

The aim of this study was to examine if PRIMA-1^{MET} could act synergistically with cisplatin *in vitro* and *in vivo*. If PRIMA-1^{MET} restores wild type p53 function to mutant p53, it might increase the sensitivity of tumor cells to conventional chemotherapeutic drugs that show selectivity for wild type p53-carrying tumors. Synergy is defined as the combination of two drugs to create a significant effect larger than the sum of their individual effects.

At first we performed a cell proliferation assay using p53 null H1299 human lung carcinoma cells and the same cells transfected with a vector expressing His175 mutant p53. Since the vector is under the control of a tetracycline promoter, treatment with doxycycline will turn off p53 expression in these cells. The assay demonstrated a strong synergistic growth-suppressing effect of the combined treatment with PRIMA-1^{MET} and cisplatin, camptothecin, CP-31398 and adriamycin. The synergy was observed only in mutant p53-expressing cells.
Testing the sensitivity of wild type p53-carrying HCT116 and the isogenic p53 null HCT116 cell line to the same panel of chemotherapeutic drugs revealed that PRIMA-1^{MET} did not sensitize the wild type p53-expressing cells to anti-cancer drugs.

Further on we only investigated PRIMA-1^{MET} in combination with cisplatin treatment. As a next step we ran FACS-PI staining and a caspase activation assay. Both techniques showed that PRIMA-1^{MET} in combination with cisplatin induces mutant p53-dependent apoptosis in a synergistic manner. Moreover, FACS profiling of cells stained with anti-Bax antibodies revealed a mutant p53-dependent synergistic induction of Bax in H1299-His175 cells upon combined treatment.

Furthermore, we inoculated SCID mice with H1299-His175 tumor xenografts and tested the effect of PRIMA-1^{MET} alone or in combination with cisplatin. The average tumor size was 840 mm^3 and 992 mm^3 for groups treated with PRIMA-1^{MET} and cisplatin, respectively. In the PBS-treated group the average size of tumors was of 1130 mm^3. Strikingly, the average tumor size of mice that received the combined treatment

was only 277 mm^3. In addition, a heterogeneous pattern of mutant p53 expression was revealed in untreated H1299-His175 cells by immunostaining and FACS analysis. PRIMA-1^{MET} treatment eliminated selectively cells containing high levels of mutant p53, while cells harbouring low levels of mutant p53 survived. The synergistic effect is likely to result from an increase of mutant p53 levels induced by cisplatin which renders the tumor cells more sensitive to PRIMA-1^{MET}. This suggests that PRIMA-1^{MET} may synergize with any drug that enhances mutant p53 levels.

However, a colony formation assay has shown that PRIMA-1^{MET} acted synergistically with cisplatin also in p53 null H1299 cells. This could be due to the fact that both compounds have p53-independent effects. However in spite of this, the p53-independent synergy was less significant than the mutant p53-dependent synergistic effect.

Thus, the combination of PRIMA-1^{MET} and currently used chemotherapeutic drugs may represent a novel and more efficient therapeutic strategy for the treatment of mutant p53-carrying tumors.

PAPER II: PRIMA-1^{MET} inhibits growth of mouse tumors carrying mutant p53

In this study, our aim was to test the effect of PRIMA-1^{MET} on mouse tumor cells carrying endogenous mutant p53, and use a better *in vivo* model than SCID mice. Previously we have shown that the low molecular weight compound PRIMA-1 can reactivate mutant p53 in different *in vitro* assays and inhibit growth of human tumor xenografts in SCID mice in a mutant p53-dependent manner. The SCID model has its limitations, including the interspecies difference between the host and the graft. This together with the mitigated immune defence may compromise the results obtained from testing anti-cancer drugs in SCID mice. Here we tested the effect of PRIMA-1^{MET} on a mouse sarcoma, mammary carcinomas and on chemically induced mouse fibrosarcomas.

First we tested PRIMA-1^{MET} on the tumors and tumor lines in a WST-1 proliferation assay. The mutant p53-carrying MC1M sarcoma was most sensitive to PRIMA-1^{MET} treatment, whereas the wild type p53-carrying TA3-Stockholm, TA3-Hauschka (wild type/mutant p53) and p53 null Ehrlich/ELD tumors were less sensitive. Moreover, the mutant p53-carrying MCO4 cell line was more sensitive than the p53 null MCO1 cells, demonstrating that PRIMA-1^{MET} treatment not only affects human mutant p53-harbouring tumor cells, but also several mouse mutant p53-containing tumors and cell lines.

Next MC1M ascites cells were inoculated intraperitonealy in C3H/Hen mice and treated with either PBS or with 100 mg/kg PRIMA-1^{MET} intravenously (i.v.) or intraperitonealy (i.p) for 10 days. PRIMA-1^{MET} treatment either i.v. or i.p. inhibited tumor growth as compared to control treatment with PBS. Furthermore we counted viable tumor cells in each group by trypan blue staining. PRIMA-1^{MET} treatment, both i.v. and i.p., significantly reduced the number of viable tumor cells compared to PBS ($p < 0.05$, according to the independent t-test). Moreover, mutant p53-harbouring MCO4 cells were inoculated subcutaneously in Balb/c mice and treated i.v. with PBS, 25 mg/kg or 100 mg/kg PRIMA-1^{MET} for 10 days. Only mice that received 100 mg/kg PRIMA-1^{MET} exhibited a significant decrease in tumor size compared to the control group. The difference in average tumor size between the PBS and the 100 mg/kg PRIMA-1^{MET}–treated animals was statistically significant from day 9 of the treatment.

In summary, our *in vivo* studies show that systemic administration of PRIMA-1^{MET} inhibits growth of mouse tumors carrying mutant p53 in mice with intact immune system without any signs of toxicity.

PAPER III: Reactivation of mutant p53 and induction of apoptosis in human tumor cells by maleimide analogs

Our screening of the NCI library of low molecular weight compounds for mutant p53-dependent growth suppression in human cancer cells led to the identification of MIRA-1.

In this article we describe the characteristics of MIRA-1 as a mutant p53-targeting compound. MIRA-1 suppressed growth of mutant p53-expressing Saos-2-His273 cells, but did not significantly affect growth of the same cells in the presence of doxycycline, which shuts off mutant p53 expression. Two structural analogs, named MIRA-2 and -3, showed mutant p53-dependent activity similar to that of MIRA-1 in a cell proliferation assay. DNA fragmentation analysis (FACS–PI) revealed that MIRA-1 increased the fraction of cells with a sub-G1 DNA content in the presence of mutant p53. Moreover, MIRA-3 induced significant caspase activation in the mutant p53-expressing Saos-2 cells, indicating induction of mutant p53-dependent apoptosis. p53-mediated transcriptional transactivation is critical for MIRA-1-induced cell death since cells could be rescued from MIRA-1 by cycloheximide, an inhibitor of protein biosynthesis. Moreover, DNA band shift assays (EMSA) showed that MIRA-1 enhances the specific DNA binding of several p53 mutants including Trp282 and His175.

Subsequently the effect of MIRA-3 (intraperitoneal injection) was tested in SCID mice inoculated with H1299-His175 xenografts. MIRA-3 had an anti-tumor effect upon systemic administration but also exhibited toxic effects at high doses suggesting a narrow therapeutic window for treatment.

The 3-4 carbon double bond in the maleimide group is vital for the mutant p53-dependent activity, since analogs lacking this double bond were inactive *in vitro* and failed to preserve the native conformation of wild type p53 or mutant proteins.

In conclusion, MIRA-1 and its active analogs could serve as lead compounds for the development of novel anti-cancer agents that rescue wild type conformation and function of mutant p53. Further optimization of these compounds is needed to increase their potency and reduce their general toxicity. The fact that active MIRA analogs can

potentially react with thiol groups suggests that thiol modification may have a role in mutant p53 reactivation.

PAPER IV: Mutant p53 targeting by the low molecular weight compound STIMA-1

Some derivatives of the 2-styrylquinazolin-4(3H)-one compound are known to possess biological activity against cancer cells [183]. To explore the anti-tumor effect of this class of compounds, we synthesized a series of 2-styrylquinazolin-4(3H)-one related derivatives [184] and tested them for mutant p53-dependent inhibition of cellular growth. Thus we identified a novel low molecular weight compound called STIMA-1 (**S**H group **T**argeting and **I**nduction of **M**assive **A**poptosis) which suppresses growth of mutant p53-expressing cells but not their corresponding p53 null counterparts. STIMA-1 has a structural resemblance to the recently identified CP-31398 compound.

We assessed the effect of STIMA-1 on H1299 lung adenocarcinoma and Saos-2 osteosarcoma cells carrying exogenous mutant p53 (His175, His273) and the corresponding parental p53 null H1299 and Saos-2 cells. According to cell proliferation (WST-1), DNA fragmentation (FACS-PI) and caspase-activation assays, STIMA-1 induced growth inhibition and apoptosis in human tumor cells in a mutant p53-dependent manner. STIMA-1 also stimulated DNA binding of His175 mutant p53 in H1299-His175 cells, as shown by an ELISA assay with an immobilized DNA oligonucleotide containing a p53 binding site. Western blot analysis revealed that STIMA-1 induces expression of p53 target proteins such as MDM2, p21 and Puma.

Further evaluation of cisplatin versus STIMA-1 in a cell proliferation assay comparing wild type p53 HCT116 cells and the isogenic p53 null HCT116 cells confirmed opposite patterns of activity. Cisplatin inhibited growth of tumor cell lines in a wild type p53-dependent manner, whereas STIMA-1 preferentially killed mutant p53-harbouring tumors.

Moreover, a HPLC assay showed that 90% of STIMA-1 and only 20% of CP-31398 formed adducts with N-acetylcysteine, a cysteine analog containing an acetyl group instead of an amino group. Furthermore we showed that STIMA-1-induced growth suppression in H1299-His175 cells was completely blocked by N-acetylcysteine, while the effect of CP-31398 was only partially blocked. Additionally, the number of free thiol groups in a GST-His175 mutant recombinant protein was examined after treatment. It was shown that STIMA-1 was much more efficient in binding thiol groups

than CP-31398. Thus, we found that STIMA-1 can modify thiol groups in proteins and that inhibition of this thiol-modifying activity rescues cells from STIMA-1-induced apoptosis.

In conclusion, we have identified STIMA-1, a low molecular weight compound that selectively targets mutant p53-carrying tumor cells and may restore tumor suppressor activity to mutant p53 by affecting its redox status. This raises the possibility that thiol group modification plays a role in mutant p53 reactivation in tumor cells and might help understand the molecular mechanisms of mutant p53 rescue and thus facilitate the design of more potent and selective mutant p53-targeting anti-cancer drugs.

CONCLUSIONS

PAPER I: PRIMA-1^{MET} synergizes with cisplatin to induce tumor cell apoptosis

- PRIMA-1^{MET} acts synergistically with cisplatin and several other chemotherapeutic drugs *in vitro*

- The synergistic effect was dependent on mutant p53

- Restoration of wild type p53 function by PRIMA-1^{MET} may increase sensitivity to chemotherapeutic drugs that preferentially target wild type p53-carrying tumor cells

- Cisplatin caused elevated levels of mutant p53 in tumor cells, presumably enhancing their sensitivity to PRIMA-1^{MET}

- Synergy was observed between PRIMA-1^{MET} and cisplatin *in vivo* upon systemic administration of suboptimal concentrations of both drugs

PAPER II: PRIMA-1^{MET} inhibits growth of mouse tumors carrying mutant p53

- A cell proliferation assay demonstrated that PRIMA-1^{MET} treatment results in a mutant p53-dependent growth suppression in mouse tumors and tumor cell lines

- PRIMA-1^{MET} inhibits growth of mutant p53-carrying MC1M and MCO4 tumors *in vivo* in syngeneic mice without any signs of toxicity

PAPER III: Reactivation of mutant p53 and induction of apoptosis in human tumor cells by maleimide analogs

- The maleimides MIRA-1 and MIRA-3 inhibit cell growth and induce cell death in a mutant p53-dependent manner
- MIRA-1 preserves native conformation of wild-type and mutant p53 upon heating
- MIRA-1 enhances the DNA-binding of His175 and Trp282 mutant
- MIRA-1 and MIRA-3 induce p53 target genes, such as MDM2, p21 and Puma
- MIRA-3 shows anti-tumor activity *in vivo,* but the therapeutic window is narrow

PAPER IV: Mutant p53 targeting by the low molecular weight compound STIMA-1

- The low molecular weight compound STIMA-1, which is structurally unrelated to PRIMA-1^{MET} and MIRA-1 inhibits cell growth and apoptosis in a mutant p53-dependent manner
- STIMA-1 upregulates p53 target genes, such as MDM2, p21 and Puma
- STIMA-1 selectively targets mutant p53-carrying tumors cells, while cisplatin preferentially inhibits growth of the wild type p53-carrying or p53 null cells
- STIMA-1 enhances p53 DNA-binding in mutant p53 carrying cells
- STIMA-1 is able to modify thiol groups in proteins and the inhibition of this thiol-modifying activity rescues cells from STIMA-1-induced apoptosis.

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