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MUTANT p53 REACTIVATION BY PRIMA-1

MOLECULAR MECHANISM AND BIOLOGICAL EFFECTS

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Stockholm 2008

Published by Karolinska Institutet.
Printed by Larserics Digital Print AB, Stockholm.

Doctoral Thesis
Mutant p53 reactivation by PRIMA-1, Molecular mechanism and biological effects

ISBN 978-91-7409-206-6

To my parents

"You do not really understand something unless you can explain
it to your grandmother"

Albert Einstein

ABSTRACT

Cancer has become one of the leading causes of death worldwide. It is now clear that tumors arise due to the malfunction of a number of genes, thus emphasising the importance of targeted therapies directed to crucial types of genetic alterations. The tumor suppressor gene, p53, is such a target as it is a key regulator of life and death for cells exposed to diverse forms of stress such as oncogene activation, hypoxia and DNA damaging agents. Moreover it is inactivated by mutation in about half of all human cancers, or by alternative mechanisms in the remaining cases. Loss of p53 tumor suppressor function often counteracts effective cancer treatment, since most of anti-cancer drugs attack tumors by activating p53-dependent apoptosis. Mutant p53 rescue has been demonstrated with low molecular weight compounds including substances found in our group: MIRA-1, STIMA-1 and PRIMA-1. Previous studies have shown that PRIMA-1 (p53 reactivation and induction of massive apoptosis) and its more potent analogue PRIMA-1^{MET} restore wild type properties to mutant p53, transactivation of several p53 target genes and induction of apoptosis in a mutant p53-dependent manner.

In papers I and II we investigated the *in vitro* and *in vivo* effect of PRIMA-1 on mouse models. At first we showed the ability of PRIMA-1 to trigger apoptosis by targeting the p53 mutant ser249 often found in hepatocellular carcinoma patients exposed to aflatoxin B1. Mice receiving i.v. injections of PRIMA-1 developed tumors more slowly than the control group. Next we demonstrated that PRIMA-1-induced apoptosis *in situ* in tumors grafted into immunocompetent mice.

In our transcriptomics study, paper III, we confirmed the impact of PRIMA-1 on mRNA upregulation of several genes involved in cell cycle and cell death, including p53 target genes. Furthermore it revealed a new insight into PRIMA-1-induced apoptosis by indicating the participation of the endoplasmic reticulum stress response following PRIMA-1 treatment in a mutant p53-dependent manner. This newly identified pathway not only reconciliates previous divergent studies with regards to the involvment of the Bax-dependent or JNK-dependent pathways, but also gives a strong advantage to PRIMA-1 as a candidate drug capable of targeting multiple pathways, thus increasing the odds to avoid drug resistance.

As shown in paper IV, PRIMA-1 is rapidly degraded, both *in vitro* and *in vivo*, into active compounds which are prompt to react with thiol groups. While PRIMA-1 covalently binds to cysteines in the p53 core domain, it is not excluded that it also binds to several other proteins with exposed thiol groups. However, as shown by transfer of PRIMA-1-treated recombinant mutant p53 into null p53 cells, the modification of p53 *per se* is sufficient to induce p53-dependent transactivation of target genes and trigger apoptosis. The same experiments with PRIMA-1-treated BSA failed to induce apoptosis. The preference for binding to mutant p53 can be due to the unstable structure of mutant p53 that enhances accessability to thiol groups. Moreover thiol-alkylation has probably a stronger impact for mutant p53 than for other proteins due to p53's central role in determining cell fate.

In conclusion, this thesis sheds new light on the mechanism of mutant p53 reactivation by PRIMA-1, as well as the downstream cascade of activated pathways which converge towards the induction of apoptosis. This work validates the principle of reactivation of mutant p53 as a working strategy for anticancer drug development.

SUMMARY IN POPULAR SCIENCE

Le cancer, qui se décline sous plus de 200 types, est l'une des premières causes de mortalité dans le monde, après les maladies cardio-vasculaires. Dans l'année 2002, près de 25 millions de personnes vivaient avec le cancer, 10,9 millions de nouveaux cas étaient diagnostiqués et 6,7 millions sont mortes des suites d'un cancer. Les causes sont multiples : la consommation de tabac, notre régime alimentaire, l'exposition à divers facteurs de notre environnement et une certaine prédisposition génétique, mais le résultat est le même, une maladie génétique d'une complexité extrême.

Le cancer de façon simplifiée se résume à une accumulation d'altérations génétiques permettant à une cellule d'échapper aux contrôles et signaux de son milieu. La carcinogenèse (développement d'un cancer) s'étend sur plusieurs années voire décennies. Par différents types d'altérations, telles que des mutations, la cellule acquiert des capacités qui lui permettent entre autre d'échapper de la mort cellulaire programmée (apoptose), l'autosuffisance en facteurs de croissance, la non-réponse aux facteurs anti-prolifératifs, le développement de nouveaux vaisseaux sanguins (angiogenèse), ou encore la capacité à envahir les tissus voisins (métastases). Chaque capacité peut être attribuée à une altération d'un ou plusieurs gènes. Les nouvelles thérapies ont donc pour but de nos jours de cibler de façon spécifique ces gènes afin d'obtenir une meilleure réponse et de moindre effets secondaires.

p53, surnommé le gardien du génome, joue un rôle essentiel pour la maintenance de l'information génétique. p53 est impliqué dans un grand nombre de voies de signalisation dont celles pour la régulation de la progression cellulaire, l'induction de l'apoptose ou bien encore la réponse à la réparation à l'ADN. Sous la pression d'un stress oncogénique, d'irradiation UV, d'hypoxie, p53 est stabilisée et transactive des gènes cibles tels que p21, GADD45, 14-3-3σ créant un arrêt cellulaire permettant à la cellule de réparer les éventuelles altérations de l'ADN, ou tels que Bax, Puma, Noxa initiant l'apoptose.

Les fonctions anti-tumorales de p53 sont inhibées dans la majorité des cancers. Dans plus de 50% des cancers la perte d'activité sauvage de p53 est associée à une mutation du gène. L'inactivation conséquente de p53 permet à la cellule d'échapper à la mort cellulaire et ainsi de continuer de proliférer tout en accumulant de nouvelles altérations génétiques.

Le sujet de cette thèse est centré sur l'approche thérapeutique de réactivation des mutants p53 dans les cancers en utilisant des composés à faible poids moléculaire. L'idée théorique de base est d'utiliser une drogue qui cible de façon spécifique les mutants p53 et leur rend leurs propriétés sauvages, initiales. En pratique, la molécule PRIMA-1 (p53 reactivation and induction of massive apoptosis) a été retenue pour ses capacités à répondre aux attentes décrites ci-dessus. Le traitement par PRIMA-1 de cellules portant un mutant p53 a pour conséquence la mort de ces cellules alors que des cellules soit sans p53 soit avec p53 sauvage ne sont guère affectées. L'arrêt cellulaire observé dans un premier temps suivi de l'apoptose observée dans un second temps contiennent les signatures d'une mort dépendante de p53, surexpression de Bax, p21, Puma.

Cette thèse a pour but de comprendre le ou les mécanismes possibles par lesquels PRIMA-1 induit l'apoptose des cellules exprimant un mutant p53, et d'appliquer le traitement par PRIMA-1 *in vivo* dans deux modèles de souris.

Les principaux résultats, au travers des quatre articles soutenus dans cette thèse, sont:

- PRIMA-1 se lie à p53 dans les cellules *in vitro* et *in vivo.* La dégradation de PRIMA-1 est essentielle à son activité spécifique. De plus la modification par PRIMA-1 de p53 est nécessaire et suffisante pour induire la transactivation des gènes cibles de p53, 14-3-3 lié à l'arrêt cellulaire et Bax, Puma et Noxa liés à l'apoptose.

- L'efficacité à induire la mort cellulaire par PRIMA-1, étudiée essentiellement par les changements d'expression des protéines, est également dépendante des régulations au niveau des ARN messagers de certains gènes telles que Noxa et Bax. De cette étude nous avons proposé un modèle attribuant un rôle important au stress du réticulum endoplamisque dans

- L’efficacité anti-tumorale de PRIMA-1 est démontrée dans deux modèles de souris, un premier modèle utilisant une xénogreffe, une inoculation de cellules humaines dans une souris, du mutant p53ser249 fréquemment observé dans les carcinomes hépatocellulaires (cancer du foie), et un second, un modèle syngéneique, qui consiste à inoculer des cellules de la même espèce que l’hôte, dans ce cas particuliers des cellules cancéreuses de souris dans une souris, obtenant ainsi un meilleur modèle ou le système immunitaire de l’animal n’est pas déficient.

De façon générale, les propriétés anti-tumorales de PRIMA-1 sont observées dans plusieurs modèles *in vivo* et soutenues par les maintes études *in vitro*, dont celles présentées dans cette thèse. Ces données font de cette molécule innovante un candidat plein d’espoir de réussite pour un essai clinique, et pour le développement de nouvelles drogues encore plus efficaces.

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III. **Lambert JMR**, Moshfegh A, Hainaut P, Wiman KG and Bykov VJN.
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Submitted

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Submitted

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Contribution of the p53ser249 mutant to the transformed phenotype of human hepatocellular carcinoma cell lines.
Submitted

LIST OF ABBREVIATIONS

ARF	Alternative reading frame
ASPP	Apoptosis stimulating proteins of p53
Bax	Bcl2 associated X protein
Bcl2	B cell lymphoma 2
BSA	Bovine serum albumin
DNA	Deoxyribonucleic acid
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
GADD45	Growth arrest and DNA damage-inducible 45
GST	Glutathione-S-Transferase
i.p.	intraperitoneal
i.v.	intravenous
MDM2	Murine double minute 2
MEF	Mouse embryo fibroblasts
MIRA-1	Mutant p53-dependent induction of rapid apoptosis
mRNA	messenger ribonucleic acid
NCI	National Cancer Institute, USA
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PRIMA-1^{MET}	p53 reactivation and induction of massive apoptosis (methylated)
PTEN	Phosphatase and tensin homolog deleted on chromosome 10
PUMA	p53 upregulated modulator of apoptosis
Rb	Retinoblastoma protein
ROS	Reactive oxygen species
SCID	Severe combined immunodeficiency
STIMA-1	SH group targeting and induction of massive apoptosis
TUNEL	Terminal Transferase dUTP Nick End Labeling
UV	Ultra violet
VEGF	Vascular endothelial growth factor

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INTRODUCTION

Cancer

Definition

The history of cancer brings us back some thousands years B.C. to Egypt where the first descriptions of what we today call cancer, were recorded on papyri. About 1-2000 years later the name of carcinoma from karkinos (the Greek name for crab) was implemented by Hippocrates, (460-370 BC) after his observations where he noticed that blood vessels around a malignant tumor looked like the claws of a crab.

Today, cancer is a general term whose definition can be shortened to the pathology related to abnormal and uncontrolled cell growth. The disease is divided in four major groups, carcinomas, sarcomas, lymphomas and leukemias which are themselves subdivided into subgroups leading to a total of over 200 types of cancers. Even if they all share the same definition, there is a wide range of variations between them in their origins and causes, their symptoms and their treatments.

Cancer has become one of the leading causes of death in the world after cardio-vascular related diseases. In the year 2002, there were 10.9 million new cases, 6.7 million deaths and 24.8 million persons living with cancer [1]. Although epidemiological evidence has shown a causative link between genetic predisposition, tobacco consumption, diet, sexual habits, environmental exposure or viral infection, cancer can affect all human beings independently of social rank, ethnic background, education level or geographic location as illustrated in figure 1.

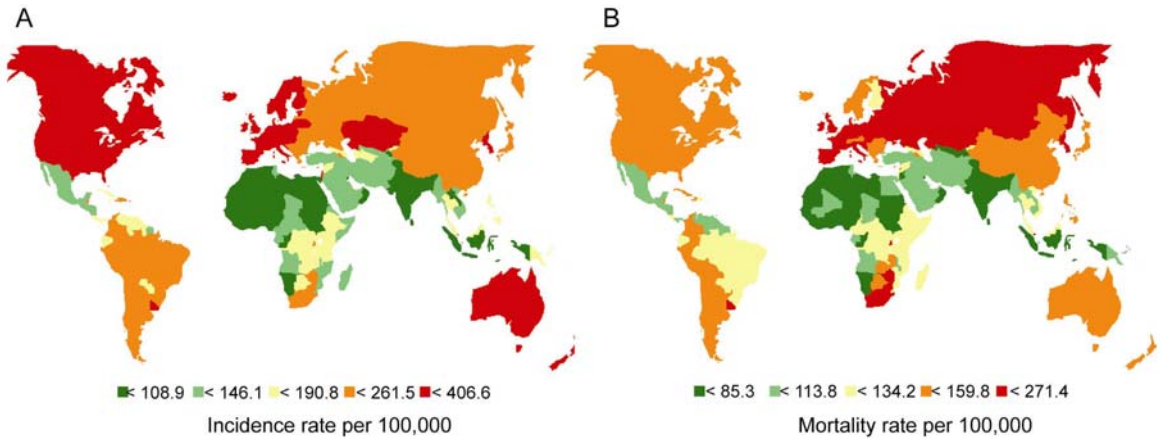

Figure 1. World map of the age standardized (A) incidence and (B) mortality rate per 100,000 males of all cancer sites but non-melanoma skin, the GLOBOCAN database 2002, WHO, IARC (http://www-dep.iarc.fr/)

Carcinogenesis, a multistep process

The development of cancer, carcinogenesis, is a long and complex accumulation of genetic and epigenetic alterations. It can take years to decades from the first hit to the development of a tumor. There are six major cellular functions governing cell proliferation and homeostasis that need to be acquired by the cells in order to become malignant. These acquired capabilities are insensitivity to growth-inhibitory signals, autosufficiency with growth signals, evasion from apoptosis, limitless replication, sustained angiogenesis and tissue invasion [2]. Alterations leading to these phenotypes are genetic, including point mutation, deletion, chromosomal translocation and gene amplification, as well as epigenetic including DNA hypermethylation and histone modification. They generally lead to the activation of proto-oncogenes to oncogenes (K-ras, src, c-myc) and/or to the inactivation of tumor suppressor genes (p53, Rb, APC).

As genetic alterations of these genes occurs, it confers to the cells a growth or survival advantage. They can then expand as they proliferate more than their normal neighbors. Cells which have become altered in this way are more likely to accumulate secondary genetic “hits” which may further enhance growth or survival if mutation occurs in critical control genes. This succession of accumulation of alterations in cells and clonal expansions, referred as the Darwinian evolution, is one model for the development of cancer [3]. But lately a new model involving cancer stem cells has been suggested. Mutations would occur in the less differentiated cells also called stem cells, and can then be transmitted to descendant differentiated cells which then expand. When stem cells are genetically altered they become what is called cancer stem cells. After one stem cells enter the mitosis, two daughter cells are created, one becomes a stem cell while the other one differentiates and goes through a limited number of divisions. Similarly cancer stem cells have the ability of selfrenewal, and thus are prone to accumulate further genetic alterations [4].

According to both theories, cancer appears as a multistep process of accumulation of alterations on specific genes leading to clonal expansions and tumor formation.

Cancer treatment

The total eradication of the tumor is the optimal solution to cure cancer. In the majority of the cases the first option is surgical removal of the tumor mass. But in order to achieve better results and ensure a partial or in the best case complete remission there is a need for adjuvant therapies. Although both radiotherapy and chemiotherapy are broadly used, they are associated with two major drawbacks, the unspecificity and the severity of the side effects. Over the last decades the interest for the development of targeting drug therapies has become an important field of research. The aim with these emerging therapies is to specifically target one or several of the hallmarks described above, thus inhibiting tumor growth and/or tumor vascularization and inducing apoptosis and cell death.

Several of these targeted drugs are already in clinical use and have shown promising results as a cancer treatment. A first example is Bevacizumab or Avastin (Roche), which is a monoclonal antibody targeting VEGF. By binding to VEGF, it blocks angiogenesis, the formation of new blood vessels. It is used as a part of a first line treatment for metastatic colorectal cancer [5].

A second example of targeted therapy is Erlotinib or Tarceva (Roche) which belongs to the tyrosine kinase inhibitors family. It targets the EGF receptor on the cell surface by preventing receptor autophosphorylation and subsequent activation of downstream signalling cascades which govern cell proliferation and survival. It has been in routine clinical use since 2005 as a second line treatment for non-small cell lung cancer [6].

However many drugs have only a transient effect and recurrence is observed often for certain types of cancer especially lung cancers. As progresses in the understanding of carcinogenesis progresses, new potential targets are identified enhancing the scope for the development of novel treatment specifically targeted to eliminate cancer cells.

p53

Discovery of the Guardian of the genome

Already in the 1950's it was known that some viruses such as SV40, adenoviruses or papillomaviruses could induce cancer when inoculated into an animal host. The oncogenic potentiql of these tumor viruses was attributed to the expression of specific viral oncogenic proteins, the large T-antigen and the small t-antigen in the case of SV40, E1A and E1B in adenoviruses, and E6 and E7 in HPV. In the late 1970's two independent groups reported that SV40 T-antigen coimmunoprecipitated with a protein of molecular weight 53,000 Da [7,8]. This novel protein was further named p53. Its levels were much greater in infected cells than in normal cells. These findings together with the fact that a p53 cDNA clone was able to immortalize and transform cells when added with the Ras oncogene to the culture attributed an oncogenic role to p53 [9-11].

Later in the 1980's, new molecular studies showed that the previous cDNA clone used carried a mutation at the codon 135. Interestingly when using a wild type cDNA clone the previous results, which showed the oncogenic effect of p53, could not be reproduced. Several new conclusions rapidly emerged following these observations and the results from new experiments changing the way to look at p53. Firstly, wild type p53 was not able to transform cells while the mutant protein could [12,13]. Secondly, wild type p53 could inhibit oncogene-mediated transformation of cell cultures [14]. Thirdly, p53 mutations were commonly found in cells kept in culture as it gives them growth advantages [15]. Lastly, loss of heterozygosity of p53 was often observed in cancer where one allele is mutated and the second allele is lost by alternative mechanisms [16,17]. Taken all together with the observation that mutant p53 is often mutated in human cancer had for consequence that the role of p53 was reconsidered and p53 was moved to the tumor suppressor genes family with the retinoblastoma gene.

Structure

The p53 gene is located on the short arm of the chromosome 17 at the locus 17p13. The genomic sequence is transcripted into a mRNA transcript composed of 11 exons but whose first exon is non-coding. mRNA is then processed and translated into a 393 amino acid nuclear protein conserved throughout evolution.

Based on structural and functionnal analysis, p53 is subdivided in 5 domains, the transactivation domain at the N-terminus, the proline rich domain, the DNA binding domain, also called core domain, the oligomerization domain followed by the nuclear localization signals at the C-terminus (Fig 2). Each domain has a specific function.

The transactivation domain, located between amino acids 20-60, is split into two smaller sub-domains both required for full p53 transactivation activity [18,19]. p53 negative regulator, MDM2 or viral proteins such as E1B 55K can bind this domain repressing p53 transcription activity [20,21].

Next the proline-rich domain made of the five repeats "PXXP" has been shown to be implicated in the p53-dependent apoptosis [22].

In the central part of the protein, the DNA binding domain stretched between amino acids 100-300. This domain binds to the p53 consensus DNA sequences which consist of two PuPuPuC(A/T)(A/T)GPyPyPy palindromes separated by a 0 to 21 base pair spacer, where Pu is a purine and Py is a pyrimidine [23]. Through its binding p53 can transactivate or transrepress specific genes containing a consensus p53 response element. The structure of the core domain is made of 3 loops. The first loop binds the major groove of the DNA strand and the second loop binds its minor groove. The third loop stabilizes the second loop [24]. This structure is maintained by a zinc ion which binds to the core cysteine residues Cys176, Cys 238 and Cys242, and the histidine His179 [25]. A total of 10 cysteines residues are localized in the DNA binding domain [24]. Interestingly the majority of p53 mutations tend to be clustered within the DNA binding domain and tend to result in a loss of p53 DNA binding and transactivation potential.

The oligomerization domain is located at the C-terminus, between amino acids 320-360. It is involved in the formation of dimers and tetramers which are dimers of dimers. Upon stabilization p53 monomers form tetramers leading consequently to a higher p53 affinity to bind DNA and thus transactivation of downstream target genes [26].

The last 30 amino acids, also called the basic domain contains two nuclear localization signals. This last part of the C-terminus functions as a negative regulator of DNA binding. Post translational modifications of this terminus increase p53 stability and transactivation activity. Interestingly in response to DNA damage, Ser315 and Ser392 are phosphorylated, Lys320, 373 and 382 are acetylated, and Lys386 is sumoylated. Overall following stress, both termini are highly post-translationnaly modified as reviewed by Appella & Anderson [27]. While the N-terminus is more prone to phosphorylation, the C-terminus is subject to more diversed modifications.

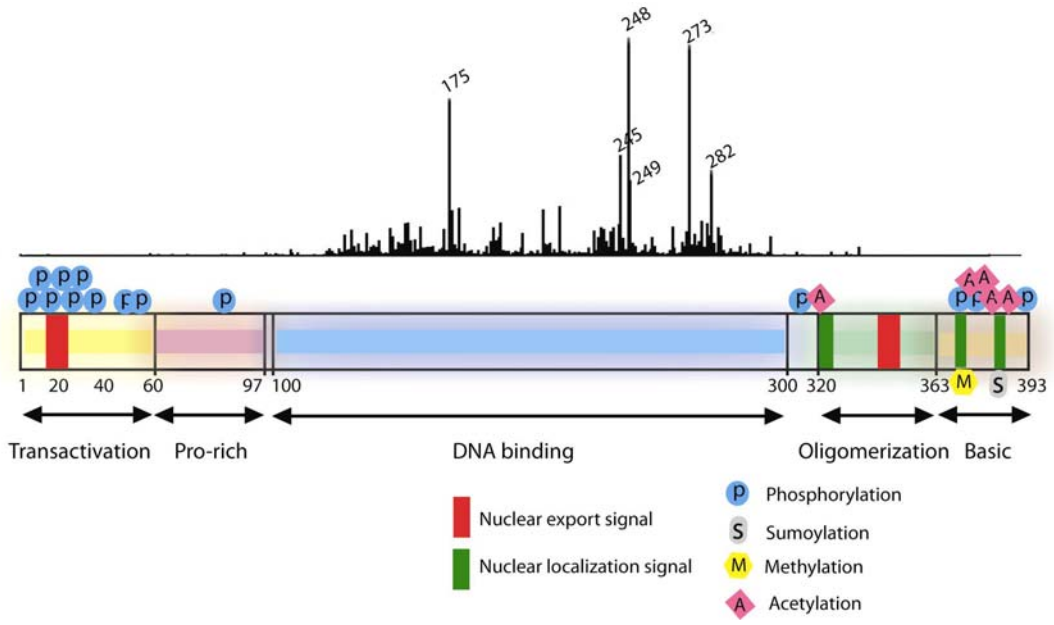

Figure 2. Schematic representation of the p53 protein. The protein is made of 5 domains, both C- and N-termini are prone to post translationnal modifications as indicated. The majority of p53 mutations are clustered in the DNA binding domain. The so called hot spot mutants are illustrated with bar indicating the frequency of reported mutations.

The *p53 family*

Twenty years after the discovery of p53, two p53 homologs were discovered p63 [28-30] and p73 [31] creating the p53 family. Evolution analysis suggests that p63 is the ancestor of the p53 family, and p53 is the youngest member.

All three members have similar genomic sequence and the proteins share stuctural similarities. All full length proteins have a transactivation domain, a DNA binding domain and an oligomerization domain. All members of the family have two separate promoters giving rise to two groups of isoforms, the one containing the transactivation domain (TA isoforms), the other one lacking the N-terminus (ΔN isoforms) [32]. An alternative form of p53 lacking only the first 40 amino acids has also been described [33]. Both p63 and p73 have a wide range of isoforms due to alternative splicing of the C-terminus [32]. Recently a similar feature has been described for p53 where three different splicings of the C-terminus were identified [34]. A regulation between different isoform is taking place, as it has been shown for ΔNp73 which inhibits by complex formation its TA counter part and even p53 activity[35].

When overexpressed both p63 and p73 have similar functions to p53 and can transactivate p53-target genes and induce apoptosis [30,31,36]. However the ability of p63 and p73 to bind DNA

varies between different isoforms, and no proof of interaction has been seen with endogenous p63 and p73. Interestingly TAp73/63 are required for p53 to activate the promoters of some proapoptotic genes such as Noxa, Perp and Bax [37]. However p63 and p73 are seldom found to be mutated, and mutations are not associated with cancer development, mitigating their suggested role as tumor suppressor genes.

Both p63 and p73 are involved in development and differentiation. p63 is required for the differentiation and the maintenance of epithelial stem cells. Indeed p63 knock-out mice fail to develop an epidermis and are not viable [38]. p73 is essential for the neurogenesis as illustrated by p73 knock-out mice which show neuronal defects such as hippocampal dysgenesis, hydrocephalus, chronic infections and inflammation, as well as loss of pheromone response [39].

Overall p63 and p73 even if they participate in the regulation of cell cycle progression and apoptosis, play a major role in development and differentiation.

p53 regulation

Considering its important role in cell fate due to its potential to eliminate a cell or to prevent it from replicating, p53 expression is tightly regulated. p53 is usually expressed at low level in normal non-stressed cells. The protein half time is about 30 minutes [40]. Upon activation, due to oncogenic stress, hypoxia or UV irradiation, p53 is stabilized mainly via post-translational modifications, such as phosphorylation. One of the major players protein in p53 regulation is MDM2, however other proteins can also, directly or indirectly, affect p53 stability and thus activity. It includes MDMX, JNK1, ARF, and the more recently identified Pirh2 and COP1.

Stabilisation

While p53 is rapidly degraded in normal conditions to hamper its activity, it needs to be stabilized when cells are exposed to diverse forms of stress in order to fulfil its biological functions.

MDM2 (Mouse double minute 2) is a ring finger E3 ubiquitin ligase. It was initially identified as amplified DNA sequences in spontaneously transformed derivative of mouse 3T3 cells [41]. MDM2 is found to be amplified in some tumors, especially sarcomas, expressing wild type p53 [42]. MDM2 overexpression inhibits p53-induced cell cycle arrest and apoptosis [43]. Deletion of the MDM2 gene leads to early embryonyc lethality in mice. However mice with deletion of both p53 and MDM2 are viable and develop normally [44]. This indicated a major role for MDM2 as a negative regulator of p53 function.

MDM2 is a p53 transcription target gene. In turn MDM2 can bind to p53 transactivation domain and thus inhibit p53 functions [45]. Following DNA damage the level of p53 is increased following by the upregulation of MDM2. High MDM2 level correlated with decrease of p53 protein level. MDM-2 targets p53 for proteosomal degradation [46,47]. Mutations of the 6 lysines residues located at the C-terminus (positions 370, 372, 373, 381, 382 and 386) prompt to be ubiquitinated did not fully abolished MDM2-dependent ubiquitination of p53 suggesting the involvement of other lysine residues in the other domains of p53 [48]. Overall the interaction between p53 and MDM2 creates an autoregulatroy negative feedback loop.

As mentionned above other proteins play some role in p53 stability. ARF, an alternative reading frame protein encoded by the INK4A locus [49], can bind to both MDM2 and p53 [50]. ARF binding to MDM2 prevents p53 ubiquitination and degradation, resulting in an increase of p53 stability [51,52]. ARF is also able to sequester MDM2 in nucleoli preventing its interaction with p53 which remains in the nucleoplasm [53].

MDMX deficient mice are embrionically lethal, but as for MDM2, loss of p53 rescued these animals [54]. MDMX is an analogous protein of MDM2, which also binds to p53 and acts as a negative regulator of p53 functions. However it does not target p53 for degradation. MDMX stabilizes MDM2 and increases its ability to degrade p53 [55]. Both MDMX and MDM2 are required to maximize p53 inhibition (Fig 3).

More recently two other ubiquitin ligases were identified, Pirh2 and COP1. As in the case of

MDM2 they are induced by p53. They can both promote ubiquitination and degradation of p53 in a MDM2-independent manner [56,57].

Activation

Several forms of stress can lead to DNA damage and consequently p53 activation such as genotoxic chemicals such as cisplatin, UV radiation, shortening of telomeres, ROS, ionizing radiation. p53 can also be activated by non enotoxic stress such as oncogenic stress caused by over activiy of a proto-oncogene as for example the amplification of the proto-oncogene c-myc which leads to DNA damage by inducing ROS [58] (Fig 3).

Following stress p53 is stabilized and protein accumulates due to a longer halt life and to an increase rate of translation while mRNA level does not change [59,60]. The activation of p53 is also modulated by phosphorylation events mediated by effector protein kinases.

DNA-PK belongs to PI3-kinase family and is activated following DNA strand breaks as the ones induced by ionizing radiation [61]. Moreover DNA-PK phosphorylate Ser15 in the N-terminus of p53 [62]. As expected exposing cells to ionizing radiation leads not only to an increase of p53 level but also to Ser15 phosphorylation [63]. The stabilisation of p53 can be explained by the phosphorylation which disrupts p53-MDM2 interaction, as the amount of MDM2 protein coimmunoprecipitated with p53 protein strongly decreased upon phosphorylation by DNA-PK [63]. However DNA-PK is not required for p53 response following DNA damage as shown in DNA-PK deficient MEF cells which upon ionizing radiation still had p53 accumulation, Ser15 phosphorylation and p53 DNA binding [64].

Another member of the PI3-kinase family, ATM can also phosphorylate Ser15 following ionizing radiation [65]. However phosphorylation of this residue following UV radiation was not induced by ATM, but by ATR [66]. Moreover ATM activates downstream Chk2 kinase [67], and ATR activates downstream Chk1 kinase [68], in both cases resulting in Ser20 phosphorylation and p53 stabilization [69,70]. However when mutating the murine Ser23 to Ala23 (equivalent to human Ser20), it did not prevent p53 accumulation nor p21 and MDM2 upregulation, suggesting that Ser20 is not required for p53 stabilization [71].

Phosphorylation of Thr18 is also induced by DNA damage, and is known to be responsible for the disruption of the MDM2-p53 interaction [72]. However the responsible kinase is not known, but casein kinase I has been suggested as a candidate by *in vitro* study using recombinant proteins. However inhibing phosphorylation of Ser15 prevent phosphorylation of Thr18 by casein kinase I [73].

Stabilisation of p53 may be achieved via modifications of other proteins as well. Phosphorylation of MDM2 at Ser395 by ATM inhibits the binding of MDM2 to p53 [74].

Beside phosphorylation, dephosphorylation can happen also in a ATM-dependent manner. Indeed Ser376 is dephosphorylated after ionizing radiation, creating a binding site for 14-3-3 which has been shown to activate p53 DNA binding activity [75].

As discussed later, activity of p53 can also be regulated by its interaction with other proteins such as the ASPP1 and 2, p300, and the two other members of the p53 family p63 and p73.

Cellular localisation

p53 which functions as a transcription factor, is mostly localised in the nucleus and expressed at low level. Its localisation in the nucleus is essential for its proper function [76]. Interestingly some tumors have developed mechanism to sequester p53 in cytoplasm, and thus impaired its functions [77]. The shuttling of p53 between nuclear and cytoplasm is mediated by the nuclear localization signals, NLS, and the nuclear export signals, NES (Fig 2).

The NES at the C-terminus is exposed in monomers or dimers, but masked in tetramers. Thus formation of tetramers which can efficiently bind DNA and transactivate p53 targets genes is then linked to p53 nuclear localization [78]. The other NES at the N-terminus has been shown to be

inactivated by ser15 and ser37 phosphorylation after DNA damage allowing rapid p53 nuclear accumulation [79]. This NES overlaps with the MDM2 binding sequence [63,80]. Thus phosphorylation of the NES might affect p53-MDM2 interaction, and allow p53 stabilization, tetramerization and then masking of the NES at the C-terminus.

Monoubiquitination of p53 by MDM2 leads to p53 nuclear export, and polyubiquitination to its degradation [81]. However it has been shown that p53 can be ubiquitinated and degraded by proteasome both in cytoplasm and in nucleus, as long as both MDM2 and p53 colocalised [82,83].

Redox modulation

Several studies have shown that the activity of p53 and its ability to bind DNA was influenced by its redox status. Indeed oxidation of p53 leads to the loss of the wild type conformation and consequently to the loss of DNA binding activity, while reduction promotes wild type p53 conformation and binding to DNA [84]. Redox status of p53 influences its binding to the DNA consensus sequence, however binding to unspecific sequences remains unaffected [85].

Besides it redox status, the correct folding of p53 and its ability to bind DNA is strongly dependent on the zinc atom which binds to Cys176, 238, 242 and His179 residues in the core domain [86,87]. The presence of zinc, but not other divalent metals as copper or cadmium, is required for proper folding of p53 in vitro. Exposure of recombinant p53 to metal chelators such as EDTA results in lost of wild type conformation [86,88].

Redox factors such as Ref1 and thioredoxin can also affect p53 DNA binding. Ref1 binds to p53 and enhances its DNA binding in a both redox dependent and independent manner [89]. It has been shown that selenium has some cancer protection properties. Interestingly in the form of selenomethionine, it activates p53 in a redox dependent manner which involved Ref1 [90]. Overexpression of thioredoxin increased p53 DNA binding and transcriptional activity in cooperation with Ref1 [91,92].

p53 functions

As mentioned earlier p53 in the absence of cellular stress is kept at low levels and remains inactive. Upon stress stimuli p53 becomes activated and as a consequence cells can undergo phenotypical changes, such as increased DNA repair, cell cycle arrest, senescence and apoptosis (Fig 3). p53 functions as a transcription factor which has the ability to bind, in a sequence-specific manner, to over several hundred promoters in the genome to transactivate downstream target genes involved in many cellular networks. More over in particular cases, such as in the apoptotic response, p53 can also transrepress anti-apoptotic genes, and can through protein-protein interactions activate a transcription-independent response.

Overall there are complex interactions between p53 and central regulatory signalling networks, which allows a cell either to arrest and take care of an alteration by augmented DNA repair activity or to be eliminated by apoptosis. The different pathways, where p53 plays an essential role, are described below.

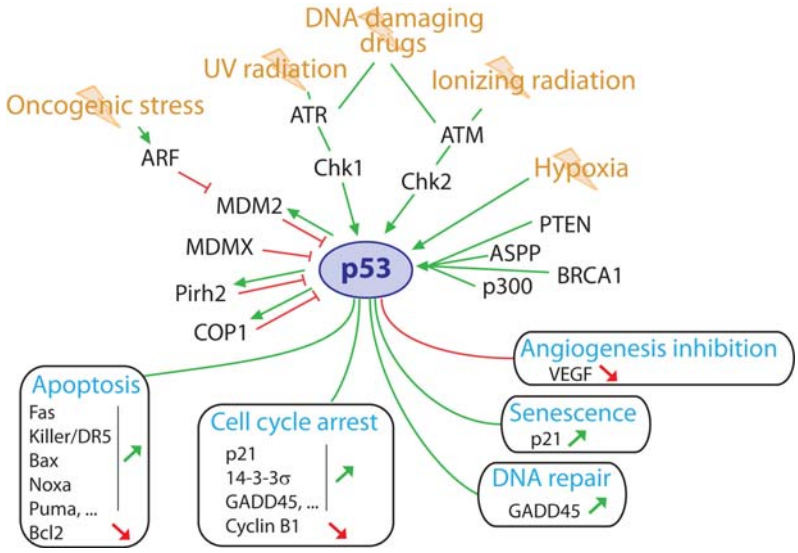

Figure 3. p53 pathways. Upon stress stimuli such as UV radiation, hypoxia, oncogenic stress, p53 is stabilized and activated through different mechanisms involving phosphorylation by ATM/Chk2 or ATM/Chk1 or protein-protein interaction. Activated p53 can lead to different cellular responses including apoptosis, cell cycle arrest and DNA repair. p53 levels are tightly regulated by several negative feedback loops such as the one with MDM2.

Cell cycle arrest

Normal cells as they are proliferating and renewing are going through different phases, in a process referred as the cell cycle. From the G1 phase where the cell has a single copy of its genome, it goes through the DNA-Synthesis, S-phase phase, over to the G2 phase where its DNA content is doubled. Finally the cell enters the mitosis, M-phase to give two daughter cells with identical genomes. During its life a cell can be exposed to various DNA damaging agents such as UV radiations. Interestingly it has been observed that following DNA damage the cell is able to arrest either at the G1/S transition or at the G2/M in order to initiate DNA repair before the cell goes on [93]. The role of these checkpoints is to avoid the propagation of a mutagenic lesions to the daughter cells.

Kastan et al. showed that primary murine fibroblasts lacking both p53 alleles were not able to induce cell cycle arrest following ionizing radiation (IR). They also found that the expression of the gene GADD45 following IR was dependent on the presence of wild type p53, which more over bound to a conserved region in the GADD45 gene. Here they identified the first evidence of the involvement of p53 in the cell cycle arrest [94].

Similarly induction of p21, a cyclin-dependent kinase inhibitor, also called waf1 or cip1, was shown to lead to G1 arrest in a wild type p53-dependent manner. A p53 binding site was identified 2.4 kb upstream of p21 coding sequence [95]. Each phase of the cell cycle are controlled by different cyclin dependent kinases, CDK, which in complex with cyclins can phosphorylate and regulate downstream substrates. p21 binds strongly to cyclin A/Cdk2, cyclin E/Cdk2, cyclin D1/Cdk4, and cyclin D2/Cdk4 complexes, inhibiting downstream phosphorylation of the tumor suppressor Rb [96]. Hypophosphorylated Rb then binds the transcription factors E2F which cannot longer transcribe cell cycle genes [97].

Cdc2, also called CDK1, when bound to cyclin B1 allows the cell to enter mitosis. Inhibition of the activation or the formation of this complex leads to G2 arrest [98]. The mechanisms of this inhibition could be due to direct effect of p53 which represses cyclin B1 [99]. G2 arrest can also be the consequence of the induction of p53 targets genes, GADD45, which dissociates the complex cdc2/cyclin B1 [100,101], 14-3-3σ, which anchors Cdc2 in the cytoplasm [102], and p21, a direct inhibitor of cdc2 [103,104].

Apoptosis

Within the wide field of research of p53, apoptosis, programmed cell death, is one of the most, if not the most, studied subject. The implication of p53 in the apoptotic machinery was first shown by the group of M. Oren in 1991. They used a murine myeloid leukaemia cell line lacking p53 and transfected it with a temperature-sensitive p53 mutant allowing them to express either a wild type or a mutant protein by simply shifting the temperature between 32 or 37°C. Interestingly only the introduction of the wild type protein induced apoptosis and the effect could be counteracted by the pro-survival factor interleukin-6 [105]. However murine immature thymocytes lacking p53 die normally when exposed to glucocorticoids but are resistant to the lethal effects of ionizing radiation. The same cells but this time expressing p53 could undergo apoptosis after ionizing radiation. These results demonstrate that p53 is not required for all forms of apoptosis [106].

p53-dependent apoptosis involves transactivation and transrepression of target genes, but also transcription independent activation of pro-apoptotic targets. There are two main pathways, the death receptor pathway (extrinsic) and the mitochondrial pathway (intrinsic), and the newly identified one, endoplasmic reticulum pathway.

The extrinsic pathway

In the extrinsic pathway, signal molecules known as ligands are released by other cells, as the natural killer cells from the immune system, bind to transmembrane death receptors on the target cell. These activated receptors will first oligomerize, and then recruit and bind Fas-associated death protein (FADD) to form a death-inducing signal complex (DISC). In turn the DISC recruits caspase-8, an initiator caspase which activates caspase-3, an effector caspase leading to the induction of apoptosis [107].

p53 can induce the transcription of several transmembrane death receptors such as Fas, and Killer/DR5 which bind respectively Fas ligand (FasL), and the tumor-necrosis-factor-related apoptosis inducing ligand (TRAIL) [108,109].

A third death receptor, Perp, has been shown to be a transcriptional target of p53, and in collaboration with E2F-1, it can induce apoptosis, however the mechanism remains under investigation [110].

Death receptors can also activate the intrinsic pathway through the caspase-8-mediated cleavage of Bid, a proapoptotic member of the Bcl2 family. Truncated Bid translocates to the mitochondria leading to the release of cytochrome c and the activation of the caspase-9 and -3 [111].

The intrinsic pathway

This pathway is regulated by the members of the Bcl2 family and results in the release of cytochrome c from the mitochondria, followed by caspase activation.

The Bcl2 family is composed of both anti-apoptotic proteins Bcl-x_L, Bcl2, Mcl1, and pro-apoptotic proteins such as Bax, Bak and the BH3-only proteins including Bid, Noxa and Puma. The BH3 domain of these latter proteins is required for their pro-apoptotic activity [112]. BH3 proteins when activated are responsible for antagonizing the antideath Bcl2 members, and for inducing Bax/Bak relocalization and oligomerization at the mitochondrial outer membrane [113,114]. In normal conditions Bax is located in the cytoplasm and Bak is bound to the mitochondrial outer membrane. Both Bax and Bak are then in inactive forms. The conformational changes and oligomerization of Bax and Bak lead to the formation of pores in the mitochondrial membrane [115]. Consequently cytochrome c and several other proteins as Smac and Diablo, are released from the mitochondria inner space to the cytosol. On one hand cytochrome c promotes the oligomerization of Apaf1 in a ADP/ATP dependent manner forming a complex called the apoptosome which can activate the caspase-9 which in turn cleave and activate downstream caspase-3 to initiate the destruction of the cell [116]. On the other hand Smac and Diablo binds to IAP and antagonize the caspase inhibitory activity of inhibitor of apoptosis proteins (IAP) [117,118].

Several proteins, essentially within the Bcl2 members, are regulated by p53. Following DNA damage both Noxa [119] and Puma [120] are transcriptionaly upregulated by p53. Bax is also a known p53 transcription target, however Bax can also induce apoptosis in a transcription-independent but p53-dependent manner as it was shown in staurosporine-treated cytoplasts [121].

The ER pathway

Following severe and prolonged ER stress, unfolded protein response can activate several pathways to induce apoptosis, the CHOP pathway, the JNK pathway and the mitochondrial pathway. It is only recently that the involvment of p53 in ER stress-dependent apoptosis has been reported.

Li et al., showed that in mouse embryo fibroblasts (MEF) deficient for Noxa and Puma the ER stress-dependent apoptosis was reduced. Both Puma and Noxa are proapoptotic members of the Bcl2 family and are known p53 targets. Interestingly $p53^{-/-}$ MEF not only partially inhibited apoptosis, but also reduced strongly the levels of Puma and Noxa at the transcription levels [122].

Bourdon et al., discovered Scotin, a new proapoptotic p53 protein target, in a screening where they compared expression of RNA from $p53^{+/+}$ and $p53^{-/-}$ irradiated mice. They showed that Scotin is localized to the ER and at the nuclear membrane, and that Scotin induces apoptosis in a caspase-dependent manner [123].

Overall p53 seems to play a certain role in the induction of apoptosis linked to the sustained ER-stress.

Transcription independent apoptosis

Besides inducing transcription-dependent apoptosis p53 can also induce transcription independent apoptosis. The first proof came from a study using a temperature-sensitive p53 mutant Val135 grown at the permissive temperature. Following DNA damage p53 could transcriptionally upregulated its targets and induced apoptosis. When the same cells were treated with drugs that block transcription or translation, upregulation of p53 targets was abolished while apoptosis was maintained [124]. Several mechanisms have since then been described including the one previously mentioned for Bax activation.

After only one hour, a fraction of stress-induced p53 translocates to the mitochondria. p53 is then able to bind and form a complex with Bcl2 and Bcl-x_L, releasing Bax which can lead to the permeabilization of the mitochondrial membrane and consequently the release of cytochrome c [125]. The observation was confirmed in vivo in gamma-irradiated mice. Accumulation of mitochondrial p53 leading to caspase-3 activation and cell death was seen in some organs including brain, spleen, thymus, after only half an hour whereas p53 targets genes were only seen after 2 hours (puma) or later for Noxa and Bax [126].

Overall p53 plays an important role throughout the different signalling pathways leading to apoptosis with or without transactivation activity via the mitochondria or not.

p53 transrepression

p53-mediated response to diverse type of stress stimuli does not only involve transactivation of some genes, but also transrepression of other genes as it was already mentioned in the regulation of cell cycle arrest with cyclin B1.

Indeed both cyclin B1 and Cdc2 expression are decreased at both protein level and RNA level following p53 induction. Repression of cyclin B1 by p53 requires one region of the cyclin B1 promoter which contains binding sites for other proteins including E2F, SP1 and USF, but not for p53. Thus the downregulation of cyclin B1 might be indirect and involve upregulation of p21 by p53 which in turn can inhibit the phosphorylation of Rb, which in turn inhibit E2F activity, and consequently E2F ability to induce cyclinB1 [99].

The first reported anti-apoptotic gene whose expression is repressed upon p53 activation is Bcl2 [127]. In a panel of breast tumor cell lines, Bcl2 and p53 expressions were found to be inversely correlated [128]. Moreover overexpression of mutant p53 could induce down-regulation of bcl-2 both at protein and mRNA levels [128]. Miyashita et al. reported the presence of a p53 negative responsive element, 195 bp segment from the 5' UTR of Bcl2, which p53 could bind to downregulate Bcl2 transcription [129]. It has also been reported that Bcl2 has a p53 binding site overlapping the one of another more potent activator, Brn-3a. Thus when p53 binds to this site, it is less active than the other activator, and it results in decrease of Bcl2 transcription [130]. Thus

several mechanism can be involved in p53-dependent gene repression.

Additional anti-apoptotic genes including BclX and survivin can be transrepressed by p53 [131,132].

MDR1 (multi drug resistance) gene can be transrepressed by p53 as shown by experiments where MDR1 was upregulated when cells were transfected with mutant p53 but not with wild type p53[133]. The repression is due to p53 binding to a novel DNA sequence in MDR1 promoter. This sequence is very similar to the described p53 consensus sequence at the difference of the head to tail orientation of the four consensus quarter-sites instead of head to head. Interestingly changing the head to tail to the head to head results in activation of MDR1 [134].

VEGF, one of the main mediators in the promotion of angiogenesis, is another gene which expression inversly correlates with wild type p53. Introduction of wild type p53 results in decrease of VEGF mRNA, and VEGF activity in a dose dependent manner, while no changes are observed when using mutant p53 [135,136]. p53 can thus mediate angiogenesis, however the mechanism for p53 to maintain low levels of VEGF is unknown.

A newly described mechanism in p53-mediated transcription came along the discovery of microRNA (miRNA) which can silence gene expression as siRNA does *in vitro*. miRNA34a, b and c, were identified to be regulated by p53 following DNA damage or oncogenic stress. miRNA34 are involved in cell cycle arrest [137]. miRNA34 overexpression leads to downregulation of genes associated with G1/S transition such as the CDK4 and 6, Cyclin E2 and E2F3 and E2F5 transcription factors, and genes associated with G2/M phase including Cyclin A2 and Aurora kinase B [138].

Senescence

p53 also participates in the process of senescence, a permanent form of cell cycle arrest resulting from different form of stress such as DNA damage, oxidative stress, or from the lost of replicative capacity due to shortened telomeres after the cells have reached a certain number of doublings. Cells entering senescence undergo morphological changes including nuclei enlargement, flattening and granulation [139,140].

It is experimentally clear that senescence is linked to the presence of p53. For example overexpression of p53 in the EJ human bladder carcinoma cell line leads to morphological changes and positive β-galactosidase staining, marker of senescence, whereas no changes or staining can be detected when p53 expression is turned off [141].

The activation of both Rb and p53 pathways are involved in the signalling network leading to senescence. The mechanism involves overexpression of Rb upstream p16 which keeps Rb hypophosphorylated as observed in senescent cells and p21 induction which can also lead to the inhibition of pRb phosphorylation [142,143]. Interestingly p21 is a p53 target making the link between p53 and Rb pathways.

DNA repair

Exposure to UV light, reactive oxygen species or to some drugs can lead to DNA damage such as modification of the bases, DNA strand breaks or adducts formation. Depending on the damage the cells arrest at different stage of the cell cycle, and activate different response to repair the damage. Interestingly p53 is involved in the cell cycle arrest as already described and also in several types of DNA repair including nucleotide excision repair (NER), base exclusion repair (BER) and in homologous recombination after double strand breaks (DSB).

In NER whose aim is to remove bulky adducts and UV photoproducts, p53 works as a chromatin relaxation factor by recruiting histone acetyltransferases to the chromatin [144].

In BER, which consist in the removal of a chemically modified base leaving an abasic (apurinic or apyrimidinic, AP) nucleotide. The AP site is then removed by an endonuclease, APE, and the gap is filled by a DNA polymerase and a ligase. p53 promotes BER by interacting with both APE and DNA polymerase β and by stabilizing interaction between polymerase β and the abasic DNA [145].

p53, Choice of functions

As described above, p53 upon activation can lead to several biological response, cell cycle arrest, senescence or apoptosis. But how a cell chooses which pathway to take? How a cell can determine whether it should arrest or die? It is important to understand the mechanism behind this choice, especially when developing anticancer drugs, as the aim is to kill the tumor cells, and not simply to arrest them and take the risk that they may start proliferating again later [146,147].

The reason why cancer cells are more prompt to undergo p53-mediated apoptosis than normal cells can be explained by the high level of stress pressing on a tumor cell, hypoxia and nutrient deprivation, as well as defect in DNA repair and oncogene deregulation [148].

A first explanation came from a study which showed that the two p53 point mutants, Pro175 and Leu181, retained the ability to induce G1/S arrest but failed to suppress the transformation of cells by oncogenes, such as the human papillomavirus E7 protein. The studied mutants showed a loss of apoptotic activity and no expression of apoptotic p53 target genes, which fully correlate with their inability to suppress transformation [149,150]. p53 with slight conformational modification can still bind to high affinity sites to the promoters of cell cycle arrest genes as p21, but not to low affinity sites that are present in the promoters of apoptotic target genes as Bax [151,152]. This theory is further supported by the fact that low amount of p53 results in cell cycle arrest, while high amount of the protein results in the induction of apoptosis [153]. However it cannot be the only explanation as the affinity for the binding site to the promoter of the proapoptotic p53 target Puma, is similar to the one of p21 and MDM2 [151].

Post-translational modifications can also influence p53 response as it is the case for the proapoptotic p53 target genes p53AIP1 (p53-regulated apoptosis-inducing protein 1). Upon DNA damage induced by UV radiation or adriamycin, Ser246 is phosphorylated and apoptosis is induced. When Ser246 was substituted with Ala246, apoptosis was blocked as well as induction of p53AIP1 [154]. Thus secondary modifications are important for p53 functions.

Another parameter which influences p53's choice to induce apoptosis or cell cycle arrest is the formation of complex with other protein partners. As mentioned before at least one of the p53 family member p73 or p63 are required for p53 to activate promoters of proapoptotic genes Noxa, Perp and Bax [37]. But other proteins may also influence p53, including p300, JMY, ASSP1 and 2 and BRCA1.

p53-dependent transcription of p21 requires the recruitment of the histone acetyltransferases p300/CBP which allows the acetylation of histones surrounding the p53-binding site and opens up the surrounding chromatin to allow access of the basal transcriptional machinery [155]. Interestingly p300 deficient cells failed to induce p21 and consequently to enter G1 arrest after exposure to DNA damaging agent, but instead went into apoptosis [156]. The picture becomes a bit more complex when adding JMY, a transcriptional cofactor which cooperates with p300 to enhance p53-dependent transcription of Bax, but without any effect on the level of p21 [157]. In agreement with previous findings, introduction of exogenous p300, in the absence of JMY, failed to significantly increase the level of apoptosis [157]. These results suggest a key regulator role for p300 in p53 response to DNA damaging agent.

Another group of proteins, ASPP proteins interact with p53 protein enhancing its apoptotic activity but not cell cycle arrest. ASPP by binding to p53 enhances affinity of p53 to the promoters of Bax and PIG-3, two apoptotic genes, but not to the promoters of p21 or MDM2. Supporting the involvement of ASPP in p53-mediated apoptosis, silencing of ASPP by siRNA suppresses the apoptotic function of endogenous p53 in response to cisplatin [158]. Furthermore ASPP1 and 2 are both found to be downregulated in a subset of human tumors and cell lines carrying wild type p53 [158,159].

BRCA1, another tumor suppressor gene, is also able to interact and stabilize p53, affecting only the transactivation of the subset of genes involved in DNA repair and growth arrest such as p21, GADD45 and p53R2 [160]. If DNA damages are too intensive, and DNA repair lasts for too long

period of time, p53 itself can then downregulate BRCA1 and finally be able to trigger apoptosis [161]. Silencing of BRCA1 in wild type p53 expressing cells abolished the induction of DNA repair genes, but not of the apoptotic PIG3 [160]. Introduction of a constitutively expressed BRCA1 construct into wild type p53 expressing cells protected them from undergoing apoptosis induced by adriamycin treatment, compared to control cells which were injected with a GFP-construct [160].

Finally the choice for p53 to start the apoptotic machinery can also depend on the survival signals that bind to cell surface receptors. Activation of the Akt signalling pathway was able to interfere and delay p53-dependent apoptosis [162]. However PTEN, which is negative regulator of the PI3′K/PKB/Akt signalling pathway by its ability to dephosphorylate $PI(3,4,5)P_3$ (phosphatidylinositol (3,4,5) trisphosphate), is induced by p53 [163,164]. Increased level of PTEN enables the induction of p53-dependent apoptosis [163].

p53 in cancer

Inactivation of the p53 gene is very common in all type of tumors. The first alteration related to human cancer was found in 1989, with mutations of the p53 gene in colon cancer [16]. Today the proportion of human tumors carrying a mutation in the p53 gene is estimated to around 50% of all human tumors [165] (Fig 4). In some cancers such as cervical cancers and melanomas, p53 mutations are rare, however p53 remains inactivated in those tumors due to others factors, respectively increase of MDM2 level or viral infection.

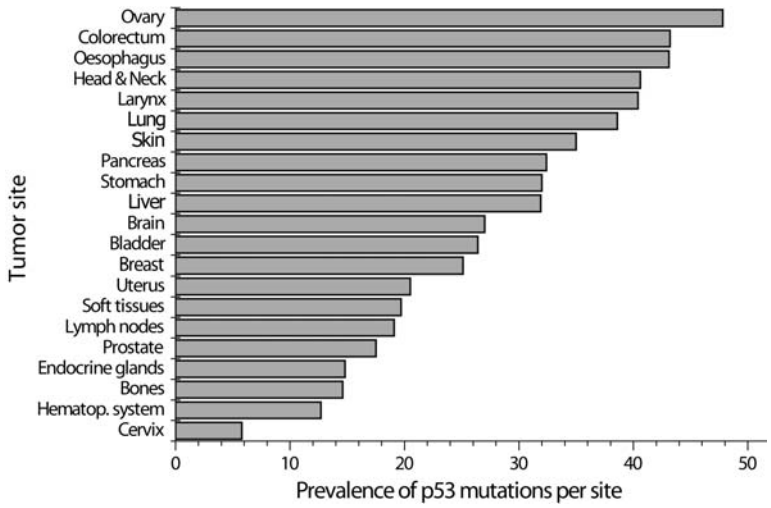

Figure 4. Frequency of p53 mutations in human tumors. Prevalence is calculated by comparing number of samples mutated versus samples analyzed, IARC p53 database R12 release, November 2007, WHO, IARC (www-p53.iarc.fr)

As mentioned earlier, some viruses produce viral oncoproteins which bind to p53 and fully inhibit its functions. HPV (Human Papilloma Virus) is strongly associated with development of cervical cancer [166]. HPV produces a protein called E6 which by specifically binding to wild type p53 induces its degradation [167].

Another mechanism to inactivate p53 involves the accumulation of MDM2 that can be due to gene amplification, enhanced transcription or upstream alterations. In melanoma p53 is inactivated rarely through p53 mutations but rather through $p14^{arf}$ mutations or hypermethylation. Normally following oncogenic stress $p14^{arf}$ expression is increased, $p14^{arf}$ then binds MDM2 and sequesters it in the nucleoli allowing p53 activation. Decrease of $p14^{arf}$ expression results to free shuttling of MDM2 between nucleus and cytosol. MDM2 can then binds to p53 and target it for degradation, thus leading to a reduced p53 activity [52].

In the majority of other cases, the most common alterations leading to p53 inactivation are

mutations (see IARC TP53 mutation database, http://www-p53.iarc.fr). Mostly missense, they are essentially scattered over the DNA binding domain, affecting p53 binding to DNA and transactivation of downstream target genes. Some codons, such as 175, 248, 273, also called hot spots, are mutated at a much higher frequency (Fig 2). Some mutations are specifically linked to certain exposure. This is the case for the mutation Arg to Ser at the codon 249 observed in some hepatocellular carcinoma linked to exposure to aflatoxin B1 a mycotoxin produced by *Apergillus* [168]. Similarly tobacco smoke is related to a certain p53 mutational pattern including an prevalence of G to T transversions at some codons [169]. In most of the case the mutation on one allele is associated with the lost of the wild type gene on the other allele, a classical hallmark of the inactivation of a tumor suppressor gene [170].

Mutant p53

Wild type p53 exerts its anti-tumor effect upon stress stimulation by activating or repressing a large group of target genes involved in many pathways including the above mentioned cell cycle arrest, DNA repair, apoptosis. Mutant p53 not only lose these functions, but also gain new ones, so called gain of functions which are cancer promoting functions. Moreover mutant p53 have the ability to inhibit wild type members of the p53 family, p63 and p73 through oligomerization [171].

Mutants p53 and wild type p53 both have 383 amino acids. They differ only by one amino acid substitution, which is sufficient to make the mutant protein unable to bind to the wild type p53 consensus sequence [24]. Moreover mutant p53 has a half life in cells 4 to 20 times longer than the one for wild type [172].

Mutant p53 has its own set of targets. Microarray study showed that a same set of genes was upregulated in H1299 transfected with His175, His273 or Gln281 mutant p53 vector. Most of the genes were related to cellular growth such as Cyclin B2, angiopoietin 1, Integrinα6 and MCL1 [173]. None of these genes contains a wild type p53 binding site. Interestingly upregulation was totally abrogated by transfection with Gln281 mutant p53 vector that contained to additional mutation in the N-terminus at codons 22 and 23, supporting the requirement of the N-terminus for the gain of function of mutant p53, shown by several others studies [173,174].

MDR1 was shown to be upregulated by mutant p53 [133]. In association with mutant p53-dependent upregulation of Bag-1, an anti-apoptotic gene [175], it contributes to the development of drug resistance of tumors carrying mutant p53.

Others genes are found to be regulated by mutant p53, it includes the oncogene c-myc. Mutant p53 transactivates c-myc promoter. The mutant p53 response element is localized near the junction exon 1/intron 1, but the exact sequence remains unknown. The N-terminus part of mutant p53 is required for the transactivation of c-myc, as it is the case for the other targets [176].

Interaction with p73 was first demonstrated in 1999. In H1299 cotransfected with p73 and either wild type or mutant p53 (His175 or Trp248), p73 coimminoprecipated only with the mutants and not with the wild type p53. In the mean time expression of mutant p53 inhibited p73-dependent apoptosis [177]. Later on mutant p53 was shown to interact also with p63 inhibiting its activity *in vitro* and *in vivo* [178]. Thus binding of mutant p53 to p63 and p73 is a potential mechanism lying behind the inhibition of apoptosis.

Overall mutant p53 promotes cancer by promoting cell growth, inhibiting cell death and increasing drug resistance. Thus bad prognosis is often associated with the presence of mutant p53 [179,180]. However there are two types of mutants, the one that keep a wild type-like conformation but carry a mutation in a residue involved in DNA binding, such as codon 248, and the other type harbouring a mutation which leads to the loss of wild type conformation such as His175 [181]. Drugs sensitivity or resistance can be different from one type to the other type. As for example cells expressing His175 and His179 are more resistant to etoposide than His273 and Trp248, but when treated with cisplatin at low doses, both His175 and His273 have similar sensitivity [182].

Targeting p53 for treatment of cancer

As mentioned before, p53 is involved in many pathways including apoptosis, cell cycle regulation, senescence, DNA repair, which altogether give to p53 a unique central role in the life of a cell. In cancer cells p53 is often inactivated leading to the abrogation of the wild type functions. Interestingly the introduction of wild type p53 in cells lacking that gene leads to a massive apoptotic response [105].

Moreover the efficiency of radiotherapy as well as of many current anticancer drugs, including cisplatin, etoposide, adriamycin and 5-fluoracyl relies on the presence of wild type p53 [183,184]. Indeed wild type p53 upon DNA damage induced by these treatments can trigger cell cycle arrest or apoptosis to eliminate the damaged cells. The suppression of p53 activity that can be due to, for example, the absence of p53 expression or the presence of mutant p53 has been associated with resistance to some chemo- and radiotherapies [184-186].

Therefore therapies specifically targeting mutant p53 and restoring its functions in cancer cell to trigger apoptosis are a challenging field with promising results. Several approaches have been tested, and others are still under development, to deal with the different situations leading to the suppression of wild type p53 activity. It can be in a null, wild type of mutant p53 background. The different strategies and the most promising discovered compounds are described in the following paragraphs.

Wild type p53 dependent apoptosis

Gene therapy

Retrovirus

This type of virus possesses a RNA genome, which can be integrated in the genome of the infected cells after being reverse transcribed to DNA. Virus can then replicate with the cell's DNA. The transduction of wild type p53 in human lung cancer cell lines either lacking p53, H358a, or with mutant p53, H322a, resulted in growth suppression. However infection of cells expressing endogenous wild type p53, H460a, did not have any effect [187]. Similar results were found when growing cells as spheroids, apoptosis was only seen in transduced cells expressing mutant p53, H322a, but not in the one carrying wild type endogenous p53, WT226b [188]. The transfer of the gene was confirmed by in situ PCR.

In a phase I clinical trial patients with non-small cell lung cancer had injections of the retroviral vector containing wild type p53 gene directly at the tumor site [189]. No toxicity was observed up to five months after the treatment. Out of 9 patients who received the injections, 3 had tumor regression, 3 had tumor growth stabilized, 2 were invaluable as they died before the end of the evaluation period due to other reasons than the treatment and 1 did not respond. Transfer of the gene was also confirmed in the posttreatment tumor biopsies by in situ PCR, but transgene expression could not be detected. Still increased TUNEL staining indicating apoptosis was observed supporting indirectly gene expression [189].

There are two major drawbacks with retroviruses, they can damage the host genome when inserted and the efficiency of transduction is low.

Adenovirus Ad5CMV-p53

Adenoviruses are double stranded DNA viruses with higher transduction efficiency than retroviruses and which do not integrate into the host genome. Ad5CMV-p53 (Advexin®) is a recombinant E1-deleted serotype 5 adenoviral vector encoding wild type p53 [190]. Treatment with this vector of different cancer cell lines resulted in high expression level of p53 and p21, and consequenlty inhibition of cell proliferation and induction of apoptosis [191,192]. More than 95% of the cells were infected with multiple copies of the viral vector. Interestingly infection with the Ad5CMV-p53 enhances the cytotoxicity of anti-cancer drugs, including cisplatin, etoposide, doxorubicin and docetaxel [193]. Next in vivo studies also showed tumor growth inhibitory and prolonged survival following intratumor injections of Ad5CMV-p53 [192].

Clinical trials showed that single intratumor injection of Ad5CMV-p53 in patients with various tumor types such as non-small cell lung cancer or breast cancer, resulted in cancer cells killing

[194,195]. No evidence of toxicity was noticed.

Overall the low toxicity and the proven efficiency to inhibit tumor growth make it a good candidate for clinical use in combination with conventional chemotherapeutic agents. Phase III clinical trials are now ongoing (Introgen-T302 and Introgen-T301).

Modified adenovirus ONYX-015

Normally adenoviruses infect cells and force them to enter S phase by producing two proteins E1A and E1B which bind and inhibit the functions of respectively Rb and p53. ONYX-015, dl1520, belongs to the adenovirus family, but it has the particularity not to express the E1B 55K protein due to a 827 base pair deletion in the E1B region and an additional stop mutation at codon 2022, preventing the generation of truncated proteins. Thus in theory when ONYX-015 would infect normal cells, it would not replicate due to the suppressive effect of wild type p53. However when infection would take place in a cell lacking functional p53, the virus can replicate and lyse the cell. Indeed it has been shown by several groups that ONYX-015 was able to kill different type of cancer cells, all sharing the common feature of lacking functional p53 [196-198].

Similarly *in vivo* experiments showed a reduction of the tumor size of human p53-deficient xenograft in nude mice following ONYX-015 injection [198]. During Phase II clinical trial, patients with recurrent head and neck carcinoma received series of ONYX-015 injections. Tumors with mutant p53 were more likely to undergo ONYX-015-dependent necrosis (7 of 12), than tumors with wild type p53 (0 of 7) [199]. Other studies in other cancers demonstrated similar activity [200,201].

ONYX-015 can affect also some wild type p53 expressing cells, but it can be due to others mutations affecting genes involved in p53 downstream pathway [202].

Activation of p53

Most of the compounds, that are activating p53, are based on the disruption of the interaction between p53 and MDM2 leading to accumulation of wild type p53 in a cell.

SuperTIP

SuperTIP (thioredoxin insert protein), is a 12-amino acid recombinant protein which binds to the p53-binding pocket on the MDM2 protein. It was identified from a phage display library, when looking for peptide which could interfere with the interaction between p53 and MDM2 [203]. This recombinant gene was made so that the peptide would be displayed at the surface of the active loop of thioredoxin as an in-frame insert.

Expression of SuperTIP in living cells leads to a strong accumulation of p53 and downstream activation of p53-dependent transcription according to reporter assay experiments. Cell cycle arrest could also be observed 24 hours after addition of SuperTIP [204].

This first approach to block p53-MDM2 interaction has proved its efficiency to activate p53.

Nutlins

Inspired by previous findings, Vassilev et al. identified the Nutlins in a screen of a library of synthetic chemicals while searching compounds that could inhibit p53-MDM2 binding [205]. Crystallization of the complex MDM2-Nutlin showed that Nutlins bind to the p53-binding pocket of the MDM2 protein [205]. Treatment with Nutlin-1 of cells expressing wild type functional p53 (HCT116, RKO, SJSA-1) resulted in increased levels of p21 mRNA and protein as well as p53 stabilization leading to cell cycle arrest and apoptosis. None of these observations were seen in mutant p53 expressing cells (SW480, MDA-MB-435). Finally Nutlin-3, a more potent analog, suppressed tumor growth by 90% in nude mice inoculated subcutaneously with SJSA-1 osteosarcoma cells, compared to control mice treated with vehicle only [205,206].

RITA

When screening the NCI library for compounds inhibiting cell proliferation in HCT116 expressing wild type p53, but not in HCT116 p53-/-, 2,5-bis(5-hydroxymethyl-2-thienyl)furan was identified. It was later referred as RITA (reactivation of p53 and induction of tumor cell apoptosis)

[207]. RITA binds p53 N-terminus in vitro resulting to an increase level of p53 in the cells due to the inhibition of the formation of p53-MDM2 complex but also p53-p300. However, whether RITA binds to p53 or to MDM2 or both remains under debate [207-209].

RITA treatments of cells lead to the induction of p53 target genes MDM-2, GADD45α and Puma. RITA triggered apoptosis in HCT116 cells expressing wild type p53, but not in the p53 negative cells or in the human diploid fibroblasts. *In vivo* experiments in tumor xenograft models showed the same effects [207].

Reactivation of mutant p53

As mentioned previously p53 is frequently mutated in human cancers, allowing the cell to evade apoptosis and to continue proliferating. Moreover p53 mutations increase the resistance to anticancer therapy. Thus mutant p53 is a prime target for therapeutical intervention. The overall strategy, illustrated in figure 5, consists in finding a drug which would specifically target the mutant p53, restore its wild type functions and thus would lead to the induction of massive apoptosis. In the mean time wild type p53 and null p53 cells should not be affected.

Several approaches are described in the following paragraphs, including small peptides, chaperones and the low molecular weight compounds.

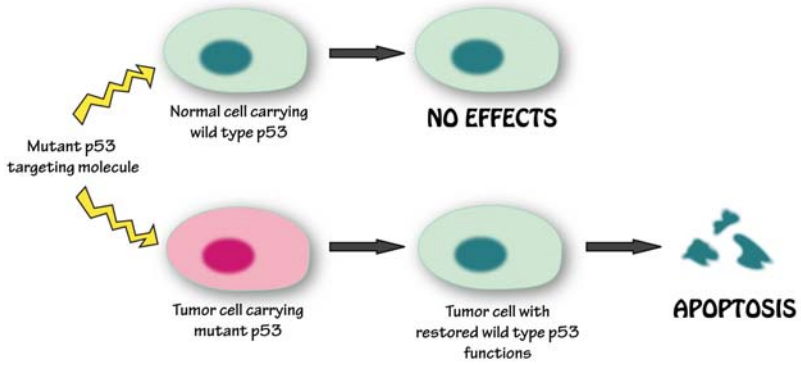

Figure 5. Strategy for mutant p53 targeted therapy

Short peptides

The C-terminus of p53 plays a central role for the ability of the protein to undergo conformational changes and to bind DNA. This is supported by the fact that deletion of the carboxy-terminus leads to the constitutive activation of p53 and introduction of antibodies whose epitopes are within the C-terminus can either activate or inactivate p53 [210]. For example microinjections of the antibody PAb421 which binds at the C-terminus resulted in the transcriptional activition of wild type p53 [211]. Interestingly the same antibody could also restore DNA binding activity to some mutant p53 proteins expressed in cells [212]. Short synthetic peptides mimicking regions of the C-terminus were also able to activate p53 [213]. Selivanova et al. identified a peptide, called peptide 46, corresponding to the residues 361-382, which could bind p53 core domain, and not only able to stimulate DNA binding, but also to induce apoptosis in cells carrying mutant p53 or overexpressing wild type p53, while leaving p53 null and normal nonmalignant cells unaffected [213,214]. Whether the peptide stabilizes p53 or creates new DNA contacts has not been elucidated yet.

Another peptide, CDB3, was identified by Fersht's group to stabilize p53 and thus restore p53 DNA binding [215]. This nine-residue peptide is derived from the p53 binding protein ASPP (residues 490-498), which is known to bind p53 core domain, to stabilize it and consequently to increase p53 transcriptional capacity [158]. CDB3 binds also to stabilize mutant p53, including I195T, G245S mutants. However CDB3 cannot rescue DNA contact mutant [215].

In vivo trials proved the efficiency of this approach. Treatment with p53-activating peptide of mice, previously inoculated either with tumor cells expressing wild type p53 (TA3/St $p53^{+/+}$ cells)

or R248Q mutant p53 (human Namalwa lymphoma), resulted in extended survival and disease-free animals [216].

Chemical chaperones

Due to hydrophobic and other interactions some low molecular weight compounds such as polyols, including glycerol, and methylamines, are efficient factors to stabilize proteins *in vitro* and protect them from unfolding induced by high temperature or chemical agents [217].

Interestingly when temperature-sensitive mutant tsp53 expressing cells were cultured in the presence of glycerol, trimethylamine N-oxide or deuterated water, at the nonpermissive temperature, 39.5°C, they behaved like the cells which were incubated at the permissive temperature, 32.5°C, that have then a wild type phenotype. It indicates that this chaperones are able to induce a conformational change to mutant p53 resulting in the restoration of the wild type phenotype [218].

Further studies were done using glycerol. Glycerol treatment induced the transcription of p53 targets, such as p21/Waf1, and results in growth suppression and apoptosis in a mutant p53 dependent manner. Both wild type p53 and null p53 cells remains insensitive to glycerol [219,220].

However the high concentrations, 0.6 M, used to rescue mutant p53 might be a limitation factor to apply that treatment in the clinic.

Reactivation of mutant p53 by low molecular weight compounds

Over the last ten years a serie of low molecular weight compounds with the ability to reactivate mutant p53 have been discovered. The most promising ones are presented in figure 6 and are described below.

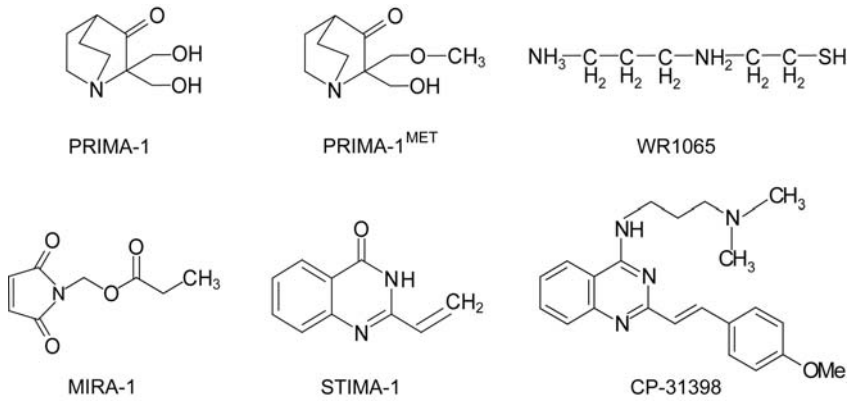

Figure 6. Chemical structure of some mutant p53 reactivating drugs

CP-31398

CP-31398, a styrylquinazoline compound, was identified by Foster et al in a screening of a chemical library for compounds that could stabilize wild type p53 active conformation upon heating according to the conformation specific antibody mAb1620 [221]. mAb1620 binds the correctly folded form of p53 in contrast to mAb240 which binds the denaturated form of p53. CP-31398 increased not only the stability of wild type p53 but also that of Ala173 and His273 mutant p53 core domains *in vitro*, and protect them from denaturation after heating at 37°C for 30 min. Similarly in living cells expressing Ala173, CP-31398 increased the fraction of 1620-positive cells. It also restored transcription activity to p53 as shown by reporter assays and by the induction of p21. In the same study it was shown that CP-31398 could inhibit tumor growth *in vivo* in nude mice inoculated either with A375.S2 melanoma cell line or DLD-1 colon carcinoma cell line, both carrying variants of mutant p53 at codon 249 and 241 respectively [221].

This anti-tumor effect *in vivo* is supported by a recent study where CP-31398 was able to block

UVB induced skin carcinogenesis in SKH-1 mice, by restoring wild type p53 functions to mutant p53. Upregulation of MDM2, p21, Pumaα, and downregulation of Bcl2 and cyclin D1 were observed, as well as activation of caspase-3 and PARP cleavage. No effects were seen in the $p53^{-/-}$ SKH-1 mice [222]. CP-31398 induces cell death through the intrinsic mitochondrial pathway in mutant p53 expressing cells, and also in wild type p53 cells as seen in HCT116 $p53^{+/+}$, but not in null p53 cells HCT116 $p53^{-/-}$ [223].

A drawback is the specificity of CP-31398 to target mutant p53 which remains under discussion as illustrated by several studies [224-226]. While mutant p53 carrying cells are effectively killed by CP-31398, some p53-independent cell death in also observed. In glioma cell lines p53-dependent apoptosis are seen within a few hours while p53-independent apoptosis occurs at a later stage [226]. The general cytotoxic effects may be explained by the capacity of CP-31398 to intercalate into DNA [224,227] and may be a limiting factor for its use in the clinic.

STIMA-1

Continuing on the same track, several compounds chemically related to CP-31398 were synthesized and tested in a WST-1 cellular proliferation assay to find compounds capable of suppressing cell growth in a mutant p53-dependent manner. 2-vinylquinazolin-4-(3H)-one, or STIMA-1 (SH group Targeting and Induction of Massive Apoptosis) was identified as the most potent candidate molecule [228]. Further study in living cells showed that low doses of STIMA-1 could induce apoptosis in mutant p53 expressing cells while higher doses were required to induce apoptosis in null p53 and wild type p53 cancer cells, as well as in human diploid fibroblasts. STIMA-1 treatment resulted in caspase activation and upregulation of p53 target genes Bax, Puma and p21. STIMA-1-induced apoptosis could be suppressed by addition of N-acetylcysteine suggesting its ability to alkylate thiol groups [228]. Interestingly the same finding was found with other molecules, CP-31398, MIRA-1 and PRIMA-1, all sharing the same ability to reactivate mutant p53 suggesting a common molecular mechanism for mutant p53 reactivation.

The use of STIMA-1 for further experiments including *in vivo* trials was limited due to its very poor solubility. Anyhow identification of this molecule gives important molecular clues to understand the mechanism behind p53 rescue by low molecular weight compounds.

WR1065

Amifostine (Ethyol®) is a pro-drug which is converted to its active dephosphorylated form, WR1065. Amifostine is used in the clinics to minimize the side-effects of radiotherapy and chemotherapy on the normal tissue [229]. Indeed it preferentially accumulates in normal tissue compared to tumor tissue [230].

Treatment of MCF-7 cells, carrying wild type p53, with either Amifostine or WR1065 lead to the stabilization of p53, induction of p21 and slowed down the progression through the G1/S phase [231]. It was further shown that WR1065 was able to trigger a similar response in TE-1 cells expressing the temperature sensitive $p53^{V272M}$ where it could restore the wild type conformation and induce transcription of some p53 targets, including p21, GADD45 and MDM2 [232]. The accumulation of p53 is the consequence of WR1065 activating JNK, thus decreasing the inhibition of p53 by inactive JNK. Consequently p53 is phosphorylated at Thr-81 and thus escapes proteasomal degradation [233]. The absence of post-translational modifications at Ser-15, -20, or -37 residues, generally induced by DNA damaging agents [233], associated with the absence of DNA fragmentation and of double strand breaks as measured by the comet assay [234], indicate that WR1065 acts through DNA-damage-independent mechanism. WR1065 was able to reduce p53 with a higher effectivity than the reducing agent DTT and therefore WR1065 is believed to enhance p53 activity through a redox-dependent mechanism [234].

MIRA-1

MIRA-1 (mutant p53-dependent induction of rapid apoptosis) belongs to another group of chemicals, the maleimide-derived molecules. It was identified in the Diversity Set of low molecular weight compounds from the NCI, while screening for compounds that could suppress cellular

growth in a mutant p53-dependent manner.

Similarly to previously described compounds, MIRA-1 can also trigger apoptosis involving the caspase activation in mutant p53 expressing cells, enhance the DNA binding activity of some p53 mutants, and induce p53 target genes such as MDM2 and Puma [235]. MIRA-1-mediated cell death is induced after only 6-12 hours after treatment. *In vivo* experiments with SCID mice treated i.p. with MIRA-3, a more potent analog of MIRA-1, showed a significant antitumoral activity at the lowest dose used. Systemic administration of higher doses had toxic consequences including weight loss and diarrhea [235]. Thus MIRA has a narrow therapeutic window.

Interestingly when testing different MIRA-1 analogs, all of them were not efficient at killing mutant p53 expressing cells. A look at their chemical structure revealed that all active compounds have a chemically active double bound which rapidly react with free thiol and amino groups by nucleophilic addition, as also seen in STIMA-1. Inactive compounds lack that double bound.

PRIMA-1

In a screen of a chemical library from the NCI, 2,2-bis(hydroxymethyl)-1-azabicyclo[2,2,2]octan-3-one, latter called PRIMA-1 (p53 reactivation and induction of massive apoptosis), was identified for its ability to inhibit cell growth in a mutant p53-dependent manner when using the Saos-2-His273 (Tet-off) system, which allows the conditional expression of mutant p53 when cells are cultured in the absence or presence of doxycycline [236]. The preference for PRIMA-1 to inhibit cellular growth in a mutant p53-dependent manner was confirmed by screening a broad panel of cell lines with different status (null, wild type or mutant) [236,237]. PRIMA-1 was distinguished from other anticancer drugs, including paclitaxel, cisplatin, adriamycin, 5-fluorouracil. PRIMA-1 had a very strong correlation with mutant p53 expression in all types of cancer cell lines, while cisplatin and 5-fluorouracil were associated with expression of wild type p53, adriamycin did not show any preference, as well as paclitaxel with the exception of renal and ovarian cancer lines were paclitaxel had a selective effect for mutant p53 lines [237].

Treatment with PRIMA-1 of mutant p53 expressing cells resulted in the restoration of wild type conformation according to conformation specific antibodies pAb1620 and pAb240, the rescue of wild type p53 DNA binding acitivity and consequently transcriptional transactivation of target genes including MDM2, p21, Bax and Puma [236,238]. Finally PRIMA-1 treatment resulted in the induction of massive apoptosis. I.v. injections of PRIMA-1 in SCID mice inoculated with Saos-2-His273 inhibited tumor growth as compared to the tumors from the mice which were treated with PBS. Upregulation of MDM2 and p21 was also found in tumor xenografts following PRIMA-1 treatment [236]. Combination of PRIMA-1 with DNA-damaging chemotherapeutic drugs, including cisplatin, adriamycin and camptothecin, lead to a significant synergistic effect [238,239]. One explanation could be due to enhanced level of mutant p53 induced by the DNA-damaging drugs. Alternatively it may be that restoring wild type p53 properties with PRIMA-1 enhances the activity of the other drugs eliminating the cells in a p53-dependent manner.

How PRIMA-1 induces apoptosis in a mutant p53-dependent manner has not been fully elucidated. While all reports confirm the specificity for PRIMA-1 to induce apoptosis in cells carrying mutant p53 but not in cells carrying wild type, there are still contradictory reports when explaining which pathways are involved. On one hand in DLD-1 colon cancer cell lines and in H1299 stably transfected with temperature sensitive p53, no upregulation of Bax, Bcl-XL or FAS were detected. Still in DLD-1 cells, JNK inhibitor decreased PRIMA-1-induced apoptosis by more than 2 fold suggesting the involvment of JNK in the induction of the programmed cell death by PRIMA-1 [240]. On the other hand in human breast cancer cells, MDA-31 and GI-101A, PRIMA-1 stimulated p53 binding to Bax and Puma promoters according to chromatin immunoprecipitation-PCR, but not to the promoter of JNK upstream activator, MAP4K4 [241]. While the latter study involves transcription-dependent activity, it has been shown that PRIMA-1 induces Bax-dependent apoptosis in a transcription-independent manner involving caspases [121]. The involvment of the activation of the caspases (2, 3 and 9) and the mitochondria intrinsic pathway in PRIMA-1-mediated apoptosis was further confirmed by two studies [242,243].

In chronic lymphocytic leukaemia, CLL, cells isolated from patient material, PRIMA-1 efficiently induced apoptosis in both hemizygous p53 deleted (2 patients) and non-deleted (2 patients), but

remained more selective in the hemizygous cells [244]. In the similar experiments with cells isolated from AML (acute myeloid leukaemia) patients, PRIMA-1 was more effective in the samples with hemizygous deletion of p53 [245]. While one p53 allele is deleted the other one is believed to be mutated. However in both studies the status of p53 has not been confirmed by sequencing. These results were encouraging and PRIMA-1 will enter clinical trial in early 2009 for the treatment of AML patients.

AIMS OF THE THESIS

The general aims of this thesis are to investigate the mechanisms of action behind PRIMA-1-induced apoptosis which is happening in a mutant p53 dependent manner, and to apply our *in vitro* knowledge in *in vivo* models and thus get a step closer to the clinical application.

Specific aims

- ***Paper I*** : To assess both *in vitro* and *in vivo* the efficiency of PRIMA-1 to reactivate p53Ser249, a hot spot mutation found in up to 50% of hepatocellular carcinomas cases in high risk regions around the globe.

- ***Paper II*** : To confirm the anti-tumor efficiency of PRIMA-1^{MET} *in vivo* in a syngeneic mouse model.

- ***Paper III*** : To analyze the impact of PRIMA-1^{MET} on the transcriptom using a microarray approach, and to obtain new clues about the mechanism of action.

- ***Paper IV*** : To investigate the mechanism of mutant p53 reactivation and to determine if PRIMA-1 directly binds mutant p53, and whether this binding *per se* is enough to induce the apoptotic response.

RESULTS AND DISCUSSION

Paper I

In vitro and in vivo cytotoxic effects of PRIMA-1 on Hepatocellular Carcinoma cells expressing mutant p53ser249.

As previously mentioned, PRIMA-1 is able to reactivate several p53 mutants, including His273 and His175, and consequently to induce massive apoptosis *in vitro* and to inhibit tumor growth *in vivo*.

In this paper we evaluated the potential of PRIMA-1 to target p53 mutant Ser249. This hot spot mutation is a hallmark of aflatoxin B_1 exposure. It is found in about fifty percent of hepatocellular carcinoma (HCC) in high incidence regions such as sub-Saharan Africa. Very few patients can benefit from the existing therapeutic regimes due to different factors such as late stage at the time of diagnosis or reduced hepatic function. Therefore there is an urgent need for the development of new drugs in HCC treatment.

We assessed the cytotoxicological effects of PRIMA-1 on the p53 null human hepatocellular carcinoma cell line Hep3B which was stably transfected with expression vectors encoding p53 mutants Ser249 or Gln248 or an empty vector construct as a control. We found that cells expressing one of the p53 mutants were more sensitive to PRIMA-1 as assessed by the increased subG1 population, an indicator of cell death, as detected by flow cytometry. Cell density was an important factor and modulated the sensitivity of the cells irrespectively of their p53 status with less confluent cells proving to be more sensitive to PRIMA-1. However when control and mutant p53 cells were grown at the same density mutant p53 expressing cells remain more sensitive.

Next we inoculated SCID mice with either Hep3B-Ser249 or Hep3B-Gln248, and treated them daily for ten days with either PBS or with 100mg/kg PRIMA-1. Mice inoculated with Hep3B-Gln248 developed tumors independently of treatment, however in mice inoculated with Hep3B-Ser249, PRIMA-1 treatment resulted in an inhibition of tumor growth. Since Hep3B-Gln248 tumors grew much faster than Hep3B-Ser249 treatment assessment of PRIMA-1 could not be completed.

In vitro we could not determine any restoration of binding to wild type p53 consensus DNA or restoration of p53-mediated transactivation in the two mutants following PRIMA-1 treatment. Furthermore we could not detect any changes in the expression of pro-apoptotic p53 targets Noxa and Puma in Mahlavu and PLC/PRF/5 cells which constitutively express Ser249. Thus we conclude that PRIMA-1 does not reactivate the transcriptionnal activity to mutants Ser249 or Gln248, and as such the PRIMA-1-induced apoptosis in these cells is taking place through a transcription independent mechanism, such as the one described in a study by Chipuk et al [121].

Finally we used PLC/PRF/5 cell line and knocked down the expression of the endogenous Ser249 by siRNA. Interestingly knocking down the expression of Ser249 made these cells more sensitive to PRIMA-1 treatment.

The main findings in this paper are that stably transfected cells with Ser249 mutant p53 have an increased sensitivity to PRIMA-1 treatment both in *in vitro* cytotoxic assays and *in vivo* in xenograft SCID mice. However the induction of apoptosis seen *in vitro* cannot be explained by the restoration of DNA binding or of transcriptional activity as they were not observed following PRIMA-1 treatment in these Hep3B cells. We also found that knocking down Ser249 in the endogenous expressing cell line PLC/PRF/5 led to an increased sensitivity to PRIMA-1 treatment. These contradictory results can be reconciled by the hypothesis that Ser249 confers a gain-of-function to the cancer cells by contributing as a prosurvival factor. Thus targeting mutant Ser249 by PRIMA-1, as well as suppressing its expression by siRNA knockdown abrogates this gain-of-function, thus cells become more sensitive to PRIMA-1. The gain-of-function theory of mutant p53 is supported by the high expression often observed in human tumors during their progression.

Paper II

PRIMA-1^{MET} inhibits growth of mouse tumors carrying mutant p53.

The anti-tumor efficiency of PRIMA-1 *in vivo* has been proven in several mouse models, as demonstrated in paper I and shown in at least two independent studies [236,238]. However all these studies used the SCID xenograft tumor model, consisting of inoculating human tumor cells into immunocompromised mice. This model has severe limitations such as the difference between the species of the host and inoculated cells, as well as the fact that the immunological response in the mouse is absent.

In order to overcome these limitations, in paper II we have used mouse tumor cells inoculated in a syngeneic host, a mouse, and we have addressed the question whether PRIMA-1^{MET} could inhibit tumor growth in a syngeneic mouse model.

We first assessed the efficiency of PRIMA-1^{MET} in inhibiting cellular growth of several mouse tumor cells from sarcoma, mammary carcinoma and fibrosarcoma origin, with different p53 status (wild type, null or endogenous mutant) using WST-1 proliferation assay. The IC_{50} value, the concentration which reduces the cell proliferation by fifty percent, showed that cells expressing mutant p53 are the most sensitive to PRIMA-1^{MET} while wild type and p53 null cells are more resistant. Indeed MC1M cells carrying the mutant Met213 were about three and a half times more sensitive than the TA3-Stockholm cells expressing wild type p53. Similarly MCO4 cells, carrying a triple mutant p53, were about two times more sensitive than the null p53 MCO1 cells. However not all mutant cells tested here were sensitive to PRIMA-1^{MET} as was the case for TA3-Hauschka cells carrying two mutations in the p53 genes whose IC_{50} value was the same than the TA3-Stockholm. These discrepancies may be explained by the fact that PRIMA-1^{MET} cannot reactivate all p53 mutants, a observation also reported in other studies [236].

We further confirmed the inhibitory effect of PRIMA-1^{MET} on MCO1 and MCO4 using several *in vitro* assays which showed that the growth inhibitory effect could be explained by the induction of apoptosis which occured in a mutant p53 dependent manner in the range of concentrations used in this study.

Next we continued with the *in vivo* experiments using doses of PRIMA-1^{MET} up to 100mg/kg. According to the toxicology analysis performed on NMRI mice, this dose does not lead to weight loss or any pathological changes on the major organs, including lungs, lever, spleen and kidneys, neither any variations in hematological parameters were observed as compared to PBS-treated animals.

In both experiments, when C3H/Hen mice were inoculated with MC1M grown as ascites tumors (in peritoneal cavity), or when Balb/c mice were inoculated with MCO4 cells, daily PRIMA-^{MET} administration, for ten days, resulted in a significant tumor growth suppression compared to the mice from the control group who received PBS injections. The administrations of PRIMA-1MET by i.p. and i.v. were equally efficient. We observed that PRIMA-1^{MET} induced massive apoptosis in MCO4 tumor tissues as determined by TUNEL staining, while no apoptosis were seen in the PBS-treated group. Using hematoxylin and eosin staining, we observed a decrease in mitotic figures and reduced vascularization in the PRIMA-1^{MET} treated group as compared to the control mice.

Alltogether our data demonstrate *in vitro* as well as *in vivo* in a syngeneic host the mutant p53-dependent anti-tumor properties of PRIMA-1^{MET}. These data further support the clinical potential for drugs targeting mutant p53 as a potential therapeutic treatment.

Paper III

Mutant p53 rescue by PRIMA-1^{MET} induces endoplasmic reticulum stress mediated apoptosis.

Previous studies on PRIMA-1 or its analog PRIMA-1^{MET} essentially focused on the biological effects, namely induction of apoptosis and tumor growth inhibition. While most of the work has been concentrated at the protein level, very little has been done at the mRNA level. We observed that PRIMA-1 treatment results in the upregulation of p21, Bax and MDM2 proteins in a mutant p53 dependent manner. Bax-dependent apoptosis induced by PRIMA-1 was shown to be p53 dependent but transcription independent.

In this paper we investigated the impact of PRIMA-1^{MET} on global gene expression in the presence or absence of mutant p53His273 in the Saos-2 cell line. We extracted RNA from the different samples after 6 and 12 hours incubation with 37 μM of PRIMA-1^{MET} and ran microarrays (Affymetrix Gene Chip Human Genome U133 Plus 2.0 arrays).

Out of our microarray study we found that more genes were affected in the mutant p53-expressing cells than in the p53 null cells. When choosing a 1.3 fold change cutoff, we obtained 185 genes that were either down or upregulated following PRIMA-1^{MET} treatment in the Saos-2-His273, and only 15 genes in the parental Saos-2. Interestingly in the Saos-2-His273 cells, we found several p53 targets such as Noxa, YY1, and a total of 23 genes with putative p53 binding site to be affected. We did not find any p53 target genes with altered transcription in the parental Saos-2 cells. We focused then the rest of our study on the genes specifically affected in the mutant expressing cell line. We selected several genes among the most up- or downregulated, involved in different pathways, and analyzed their expression by Real Time PCR to validate our microarray. We confirmed 11 genes including Noxa, VEGF, YY-1 and GADD45B. The microarray did not detect changes in the classical p53 targets. Since PRIMA-1^{MET}-induced apoptosis has been shown by several groups to involve Bax, we checked Bax mRNA by Real Time PCR, a more sensitive method, in the same samples that were used for our microarrays. We found that while the level of Bax mRNA was unaffected in the Saos-2 cells, it was upregulated by 2.43 $\pm$ 0.92 fold change in the Saos-2-His273.

Next we transferred our microarray data to the Ingenuity Pathway Analysis software to determine which signalling pathways were the most influenced by PRIMA-1^{MET} in the Saos-2-His273. When comparing untreated to 6 hours treated, cell cycle and proliferation pathways were the most affected, whereas at the latter time point of 12 hours, cell death pathways were the most affected. This was in full agreement with our observations of the cell population analyzed by flow cytometry after 48 hours of treatment. We observed a G2-arrest and an increase in the sub-G1 population, an indicator of cell death, in the mutant p53-expressing cells after treatment, but not in the p53 null cells. We observed upregulation of genes involved in cell cycle, such as GADD45B, 3.05 $\pm$ 0.65 fold change and 14-3-3γ, 1.80 $\pm$ 0.17 fold change and in apoptosis, such as NOXA, 3.18 $\pm$ 0.73 fold upregulation, according to the microarrays. We confirmed those genes by Real Time PCR except 14-3-3γ. However, we observed 14-3-3 upregulation by Western blotting in the mutant cells after PRIMA-1^{MET} treatment. The discrepancy between our microarray and our Real Time PCR data may be explained by a difference in the sensitivity between the methods, the efficiency of the probes, or the transient effect of mRNA up- or downregulation when compared with the protein level which might be more stable. We also confirmed NOXA upregulation at the protein level in Saos-2-His273 as well as other cell lines expressing mutant p53 including SW480, H1299-His175 and HCT116 p53$^{-/trp248}$.

Our next findings emerged from the observation that many genes, involved in the actincytoskeleton rearrangement were found in the list of genes affected in the mutant cells. It included genes such as WIRE which plays a role in actin redistribution and depolymerization, and Limk2 which induces membranes blebbing. Staining of the actincytoskeleton revealed that in null cells it was not affected in contrast to mutant cells where it was totally disrupted with 50 μM PRIMA-1^{MET}. At lower concentration, 25 μM, membrane blebbing and actin clumps were observed. These modifications are hallmarks of the cells going though apoptosis, and are most probably not a direct effect of PRIMA-1^{MET} but simply a consequence of the cell entering the

programmed cell death.

Finally XBP1 was among the most upregulated genes in Saos-2-His273 at 12 hours, 2.35 ± 0.39 fold induction on the microarray confirmed by Real Time PCR 2.36 ± 0.29 fold. XBP1 is associated with endoplasmic reticulum (ER) stress where upon stress its mRNA is cleaved and the spliced form induces the transactivation of chaperone genes. XBP1mRNA was cleaved after PRIMA1^{MET} treatment in a mutant p53-dependent manner. DNAJB2, a Hsp40-like protein, and VEGF were upregulated after PRIMA-1^{MET} treatment, which is in agreement with their reported induction in ER-stress. Noxa, also induced as mentioned earlier, has been shown to be involved in ER-stress-mediated apoptosis. Finally we immunoprecipitated GRP78, another protein involved in ER-stress which functions as an ER chaperone by binding to unfolded protein. We detected a ~20% increase in the fraction of total pulled down protein coimmunoprecipitating with GRP78 suggesting an increase in the amount of unfolded protein.

Overall we showed that PRIMA-1^{MET} has a mutant p53 dependent effect on the transcription of genes involved in cell cycle arrest and in apoptosis. Interestingly we propose a new model for PRIMA-1^{MET}-induced apoptosis involving the participation of the ER (Fig 7). As we report later in paper IV, reactivation of mutant p53 leads to the production of ROS, which in turn can induce ER-stress. Moreover reactivation of p53 leads to upregulation of Bax, which has been shown to interact with IRE-1 kinase leading to increase ER-stress. Reports have shown that PRIMA-1-induced apoptosis can go via the mitochondrial – Bax pathway, whereas another report showed the involvment of the JNK pathway. Interestingly ER-stress can trigger apoptosis through both pathways which is of great interest with respect to drug resistance.

Thus we conclude that PRIMA-1^{MET} induces ER-stress-mediated apoptosis in a mutant p53 dependent manner.

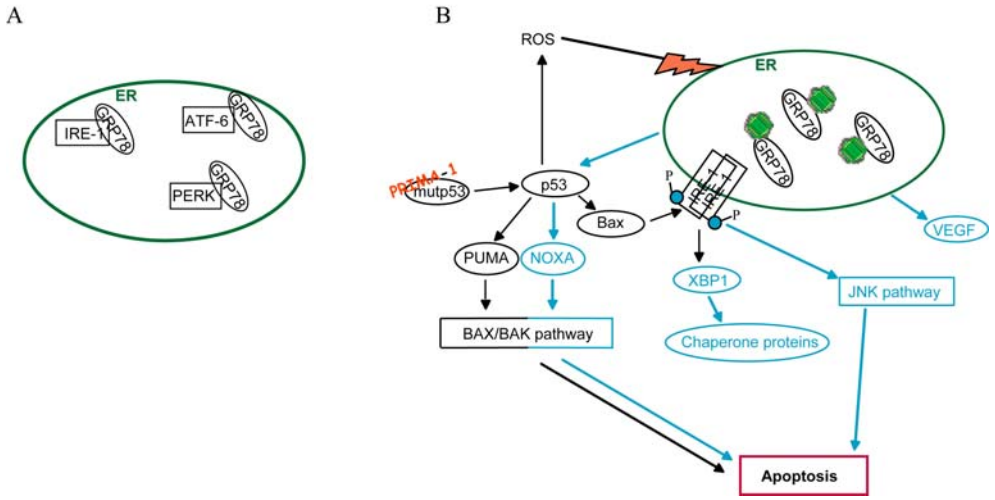

Figure 7. PRIMA-1^{MET} model for inducing ER stress. (A) In non stress conditions GRP78 is localized in the ER and bound to transcription factors including IRE-1. (B) PRIMA-1^{MET} reactivates mutant p53 inducing the mitochondrial pathway, via Puma and Bax, leading to apoptosis. In the mean time reactivation of mutant p53 is followed by the production of reactive oxygen species, ROS, which can induce the stress on the ER. Consequently it can then lead to unfolded proteins that accumulate in the ER. During ER stress GRP78 binds unfolded proteins and releases IRE-1 which is dimerized. Activated IRE-1 leads to the cleavage of XBP1 and the activation of the JNK pathway leading to apoptosis. Bax, which is directly upregulated followed PRIMA-1^{MET} treatment, can also interacts directly with IRE-1 and activate it. During sustained ER stress Noxa is induced in a p53-dependent manner. The consequently downstream effects of ER stress are highlighted in blue.

Paper IV

PRIMA-1 reactivates mutant p53 by covalent binding to the core domain.

Much is now known about the biological response following PRIMA-1 treatment, namely inhibition of tumor growth and induction of massive apoptosis in a mutant p53-dependent manner. Concerning the mechanism behind these effects, many studies have focused on the downstream part of p53 reactivation and primarily on the pathways related to apoptosis. In this study we investigated the molecular mechanism of mutant p53 protein reactivation by PRIMA-1.

First we incubated PRIMA-1 in PBS at physiological conditions for different periods of time and analyzed the decomposition profile by liquid chromatography. We observed PRIMA-1 degradation as indicated by the appearance of two new peaks on the chromatogram as the peak from the original compound was diminishing. Similar results were obtained from experiments in cell lines, and *in vivo* in mice where the degradation rate was more rapid. *In vitro* it took about 40 hours for half of the product to be degraded, whereas *in vivo* all PRIMA-1 was decomposed after 1 hour. Of note PRIMA-1 was rapidly excreted in the urine with low levels detected in the blood after 24 hours. Among the degradation products we identified methylene quinuclidinone (MQ), which contains a chemically active double bond. The decomposition of PRIMA-1 to MQ should lead to the generation of formaldehyde. Indeed we could detect formaldehyde after degradation of PRIMA-1. However formaldehyde at those concentrations was not responsible to the effects seen in cells. To confirm that MQ was the active compound, we used a PRIMA-1 analog that is not degraded in the same way. Interestingly no biological effects were detected, allowing us to conclude that PRIMA-1 works as a prodrug which is degraded into an active compound, MQ.

MQ contains a chemically active double bond prone to participate in reactions of nucleophilic addition, similarly to others substances MIRA-1 and STIMA-1. Thus we checked whether PRIMA-1 could react with thiol groups and whether the interaction with thiol groups is required for PRIMA-1 biological activity. Indeed when we incubated cells in the presence of N-acetylcysteine, it completely blocked PRIMA-1-induced apoptosis. Our in vitro assays have shown that the number of free thiol groups in recombinant mutant p53 proteins, GST-His175 and GST-Gln248, decreased after incubation with pre-warmed PRIMA-1 indicating that PRIMA-1 degradation products bind covalently to thiol groups in mutant p53. Further support came from mass spectrometry analysis where it was shown that molecular weight of the p53 core domain was increasing upon PRIMA-1 incubation with mass change corresponding to molecular weight of MQ. Binding to mutant p53 was also observed in living cells treated with radiolabeled PRIMA-1 and followed by p53 immunoprecipitation.

As PRIMA-1 reacts with thiol groups, it is also capable of reacting with proteins other than mutant p53. Therefore we have treated *in vitro* recombinant GST-His175 and GST-Gln248 with pre-warmed PRIMA-1, and transfered them into p53 null cells. Only p53 mutant proteins that had been treated with pre-warmed PRIMA-1 resulted in the upregulation of Bax, NOXA and PUMA mRNA, the increase of 14-3-3, Bax and PUMA protein levels, as well as caspase activation. It could not be observed when transfering untreated mutant p53, or PRIMA-1-treated BSA. We thus conclude that modification of mutant p53 *per se* is enough to trigger apoptosis.

The main conclusions from this paper are that p53 reactivation by PRIMA-1 requires its degradation into active compounds, which then can covalently bind to thiol groups. Moreover the modification of mutant p53 by PRIMA-1 degradation products *per se* is sufficient to restore wild type activity and trigger apoptosis in human tumor cells. This link between the modification of thiol groups and the reactivation of wild type activity to mutant p53 opens new possibilities to design novel more potent anticancer drugs based on similar mechanisms of action.

CONCLUSIONS AND FUTURE PERSPECTIVES

While in the first two articles we have focused on the validation of the mutant p53-dependent anti-tumor activity of PRIMA-1. In the two last articles we have investigated and uncovered the mechanism of mutant p53 reactivation and brought a new insight of the induction of apoptosis.

p53 is mutated in more than half of hepatocellular carcinomas in high incidence regions, with the particularity that in over 90% of the cases it is a transversion resulting in amino acid change Arg to Ser at codon 249. Added to the lack of efficient therapeutic drugs, PRIMA-1 was presented as an ideal candidate to treat HCC in those regions. We showed that stably transfected cells with Ser249 have increased sensitivity to PRIMA-1 treatment *in vitro* as compared to cells transfected with an empty vector construct. Anti-tumor effect was also seen *in vivo* in xenograft SCID mice. PRIMA-1-induced apoptosis was shown to be transcription-independent in these cells. Silencing Ser249 in endogenous expressing cells PLC/PRF/5 increased sensitivity to PRIMA-1 treatment. However this was explained by the plausible gain-of-function attributed to Ser249. Even if not investigated in this study we could postulate that any drug treatment would give similar results. Thus overall PRIMA-1 seems to be a good candidate for the treatment of HCC patients carrying Ser249 mutation. As a next step it would be interesting to isolate primary tumor cells from patients and treat them with PRIMA-1 and follow the response, and assess whether PRIMA-1 can force them to enter apoptosis in a mutant p53-dependent manner.

As all *in vivo* experiments in previous PRIMA-1 studies were performed using human xenografts in SCID mice, our new data in a syngeneic host confirms and validates the mutant p53-dependent anti-tumor properties of PRIMA-1^{MET}. The next logical step to give even stronger support to the efficiency of PRIMA-1 to inhibit growth of tumors expressing mutant p53 would have been to repeat these experiments in another murine model, where mice develop spontaneous tumors. In the mean time the efficiency of PRIMA-1 has been confirmed in human primary cells from AML and CLL patients.

Our transcriptomics study confirms the mutant p53-dependent effect of PRIMA-1^{MET} on the transcription of genes involved in cell cycle arrest and in apoptosis. Most interestingly we completed the complex picture of PRIMA-1^{MET}-induced apoptosis by introducing the participation of the ER stress response since we see cleavage of XBP1 and the induction of VEGF, Noxa and Hsp proteins following PRIMA-1 treatment. ER stress is known to induce apoptosis by activating several pathways. Two of these pathways, JNK and Bax, have been previously implicated in PRIMA-1-induced cell death. Since PRIMA-1 can interact with thiol groups it might lead to general missfolding of proteins. DTT, an antioxidant molecule is known to interfere with protein folding and to induce ER stress. However in our experiment we did not see any increase of missfolding protein following PRIMA-1 treatment in p53 null cells. Therefore we concluded that PRIMA-1 does not act directly on the ER and instead that ER stress is activated by ROS induction following mutant p53 reactivation and also by activation of IRE-1 by Bax which is upregulated after PRIMA-1 treatment. Whether PRIMA-1 response is transcription-dependent or independent seems to be dependent on cell lines and duration of treatment. In the light of our study transcription activation of target genes is involved in PRIMA-1 response and strongly correlates with cell death induction. But as shown by other studies including the first one presented in this paper, transcription of apoptotic genes does not seems to be essential to induce cell death, which happens in a mutant p53 dependent manner.

PRIMA-1 has been shown to work as a prodrug, as it needs to be degraded into new metabolites to be active. It is interesting to see that even if starting with compounds that at first glance have very distinct chemical structure, STIMA-1, MIRA-1 and PRIMA-1, we could find that they all share one common property to interact with thiol groups through an active double bond present in their structure or appearing in one of their degradation products. It shows that even if a drug does not proceed to clinical trials or even to *in vivo* experiments, as it is the case for STIMA-1, it gives important clues to mechanisms of action and can participate in the design and development of more potent and specific drugs, maybe more stable and with a larger therapeutic window.

A major controversy is around the fact that p53 reactivation is mediated by the ability of PRIMA-1 to alkylate thiol groups. Since thiol groups are present in most if not all proteins, it is hard to believe that PRIMA-1 can specifically target mutant p53. We do not deny that PRIMA-1 binds to other proteins, as was shown by PRIMA-1 binding to BSA for example. However PRIMA-1 cannot access all the cysteines due to steric convenience. Cysteine residues in mutant p53 proteins which dose not have the wild type conformation might be more exposed to external interactions, and thus react with PRIMA-1. Moreover not all modified proteins will have an impact on cell survival, as we have shown by modifying BSA with PRIMA-1. At the same time PRIMA-1-modified mutant p53 induced massive apoptosis in tumor cells. So not all proteins can be targeted by PRIMA-1 and those which are targeted may not gain pro-apoptotic properties. However we cannot exclude that it does not account for the toxicity related to high doses of PRIMA-1. *In vivo* studies showed that PRIMA-1 did not show any sign of toxicity at the working doses which gives a mutant p53-dependent anti-tumor effects.

Finally the fact that modification of p53 *per se* is enough to restore wild type activity and trigger apoptosis in human tumor cells supports the potential of the type of drug like PRIMA-1, and opens new possibilities to design novel more potent anticancer drugs based on similar mechanisms.

Altogether our *in vitro* as well as *in vivo* data further support the clinical potential for PRIMA-1 as a mutant p53 targeting drug. The knowledge of the mechanism of PRIMA-1 activity opens a novel way for future development of new more potent and specific drugs against tumors resistant to current therapies.

ACKNOWLEDGEMENTS

Since the beginning of my PhD I have received support from many people in both my professional and my personal life. I would like to express my sincere gratitude to my colleagues, my friends and my family, who all in a direct or indirect way contributed to this thesis. Particularly to:

my Swedish supervisor, **Klas Wiman**, for accepting me into the group at CCK without even knowing me. You gave me not only a desk and bench space to work, but also exciting projects to work on. Thank you for providing everything needed to complete the projects properly to reach the highest quality outcome. Thank you, in particular, for trusting me and encouraging me all these years. I am also very grateful for the opportunities you gave me to travel around the globe to present my work and create contacts.

my Belgian supervisor, **Pierre Hainaut**, for letting me do a training period in your group and subsequently encouraging me to pursue a PhD. I would also like to thank you for your mainted support despite the distance. Whenever I was back in Lyon and at IARC, you made time for a discussion with me and I always left with great new ideas from you. Thank you also for being supportive when I needed help with my mum.

Vladimir, even if not my supervisor on the papers, you have been acting in that role. You are such a great person with so many ideas and such an amazingly broad knowledge. Thank you for always being supportive, present, ready to answer all my questions, to solve some issues, to give me advice and feedback, and to discuss my projects. I admire your calmness and your optimism. Thank you for pushing me to never give up whatever happened in my professional and private life.

all former and present member of the group at CCK, KI, who helped me in one way or another. **Nicole**, thank you for your constant support in all situations and for the long chats on the phone talking about science, p53, STIMA, the lab, crêpes and football. I am grateful that you introduced me to kickboxing to evacuate the stress and the pressure. But I am still waiting for you to kick my butt... **Jin,** the sunshine in the lab, always smiling and telling something to make us laugh. I liked the way you compared people with food. Thank you for your help with the experiments and for the scientific discussions. **Salah**, بابا, the happy guy sitting behind me and always joking, **Ruby**, the gossip girl, always here to give good advice, and **Anna**, the singing star, always overflowing with energy, a lot of thanks not only for your precious help and strong support in the lab with experiments and discussions, but also your help outside the lab. Without you and the rest of the party crew, I would never have known that not more than 5 people could sit on my chair at the same time. You all contributed to create a pleasant atmosphere on the fourth floor, and showed me how to party properly in Sweden. Next Friday, party at my place? **Marianne**, you are a perfect model of a successfull, willing and hard working person who remains extremly nice and friendly. Thank for being here and encouraging me. **Susanne,** thank you for the morning chats, you have become an essential piece in the lab. Simply remember that French cars are not always made for Swedish winters. **Nina**, thank you for your laughs and your company to eat ice cream, there is always room for a magnum.
I would like to thank other present and former members from the group **Cheya**, always up for some fun and a great DJ during our Friday parties, **Sofia** and **Cinzia**, the last two great members who joined the group, **Lena**, for simply being a good friend, **Magda**, amazing chef doing “kämpegott” food, **Masoud,** knowing more about French football than I, **Nader**, **Helene**, **Tao** and **Petr** for the variety of help and support in the lab.
Many thanks to **Ann Britt** for her precious help with administrative matters.

all former and present members of the group at IARC for their help and their support despite the 2250km which separated us.
Mounia, your driving force and your broad knowledge will make you reach the stars. Thank you for the long discussions dealing about everything from basic science to foolish dreams. So much laughing, so many tears! **Magali O,** thank you for your scientific support. It is always a pleasure to meet you at IARC or somewhere else around the world. **Virginie,** always early in the lab, always

with a smile, and ready to come up with a joke at anytime. It has been an honour to work with you on the organization of the meeting between the two groups. **Alexis**, I always look forward to meeting you to talk over a beer or a croque-monsieur. Thank you for being a comfort to me while my mum was in your service. **Ghyslaine**, you are so active in the lab, a central pillar. I really enjoyed seeing you and discussing with you. It was always a good time working with you. **Florence**, thank you for supervising me during my master thesis, I learned much from you, and I guess that if I went for a PhD it is also thanks to you. **Michelle**, thank you for the continuous huge help with all the administrative work.
I would also like to thank the other present and former members of the group for helping me and making my stays at IARC so successful. **Audrey**, **Iris**, for a good collaboration, **Helen, Carole,** les papiches **Sophie** and **Hind, Séverine**, **Dominique**, **Chiara**, **Aurélia**, **Agnès**, **Elodie**, **Brigitte**, **Emilie**, **Amélie T,** keep a VIP sit for me at your defense, **Ewy**, **Sabine**, **Dominique** and **Claude**. **Rita**, thank you for your constant warm welcome as I entered the building.

Many thanks to the friends and colleagues from the 4th floor at CCK, for making it a great place to work, **Olle Sangfeld**'s, **Olle Larsson**'s, **Anders Zetterberg**'s and **Bertha Brodin**'s groups and the histo lab. Special thanks to **Diana**, for all the fun and teasing during parties, **Bertha**, who gave me the opportunity to be an amanuens for biomedicin students, **Pádraig**, for the early morning discussions and your expertise in science and in wine, **Elin**, **Wessen**, **Linda**, **Bita**, **Sandra, Inger** and **Liss**.

Many thanks to the friends and colleagues from the 3rd floor at CCK, **Lars Holmgren**'s, **Arne Östman**'s and **Dan Grandér**'s groups, especially to the party member **Mahdi**, alias Player for coming up with the nicknames "Frenchy", "French frieees" and for being such an amazingly good friend, **Jacob**, linje 14 (h)an-kommer, and also to **Janna**, for sharing the organization of Journal Club, **Mira**, **Christina**, **Marcus**, **Åsa**, **Maja**, **Jeroen, Staffan**, **Micke** and **Masako**.

I would also like to thank other friends and colleagues from CCK, **Ali**, for all the work and time spent on the microarray study, **Chiara**, **Rona**, **Inga**, **Tomadher**, **Dara**, **Hanna** and **Thierry Soussy.**

Lots of thanks to the different actors who make CCK a great working place, and allow it to run its activity properly, **Elle**, **Elisabeth** and **Emily**, for making every visit in the shop the opportunity to laugh, **Joe**, for your help and advice with technical issues, **Sören**, **Juan**, **Eva-Lena**, **Marie**, **Anders** for all the IT support, **Anki** for your great help with PhD-related matters and **Anne Jensen** for your kind help at diverse occasions.

Thanks to **Tina Dalianis**, chairman of the department, for making CCK a friendly and creative research environment.

Many thanks to the CDPs (Cell Death People) **Bertrand**, **Linn** and **Aris** for letting me join the network and participate in this very good experience. I'll show up anytime you need, I am ready to set up a course!

Many thanks to other friends and colleagues from other department at KI, **Galina Selivanova**'s group, especially **Ying**, **Elisabeth** and **Clemens**, and many thanks to **Margareta** and **Kent** from the MTC animal house for their big help with *in vivo* experiments.

Next, I would like to thank some friends who in one way or another contributed to the completion of this work:
Olivier, as you know I consider you like a brother. Thanks for the long conversations on the phone and in the car nearby the roint-point in front of your parent's. Thanks for the great moments we spent together, casino, belote, grill,... **Eddy**, you are so unique, and it is what makes you such a great friend. You always find a free spot in your overbooked schedule to meet and come up with a joke or something to make me laugh, but you also can be serious at times, or can you? **Pierre**, even if we didn't meet often, as we both spent more time out of France, I always look forward to seeing you and enjoying a raclette. **Nadège**, you are the one who has known me the longest, soon 30 years, hehe. May our friendship keep on going on the same track. **Amélie**,

thanks for all the fun, I know I can count on you for anything, from partying to more serious aspects of life. Thank you for suggesting that I apply to IARC for my training period. Guys, if you were not there I would have had to clone you.
Many thanks to the KTH gang, **Fadia**, **Lorena** and **Reefat** for warmly welcoming me in Sweden. The largest part of my will to keep on studying in Stockholm was due to your friendship.
I would like also to thank all my other friends in Sweden, France, and in many other countries, especially **Jinlong**, **Rolando** & **Agneta**, **Julien**, **Karine**, **Delphine** & **Manu**, **Marine**, **Johan**, **Micke**, **Suru**, **Nishu**, **Nare**, **Erik**, **Elise** and **Nadja**.

To finish I would like to thank my family and relatives for their huge help and deepest love.
Mum & **Dad**, **Micheline** & **Bernard** as I call you despite everyone's comments, I am so proud of having you as parents. You have given me such a great environment to grow, you always trusted me, and supported me in everything I started. Mum even if you scared me when you got cancer, you gave me an even stronger will to fight cancer.
Maud and **Magali**, my wonderful sisters, for the great time. We are a strong team always here for each other, and I think we have now reached the level where we don't really know if you are the big sisters or if I am the big brother. Thank you for making me with **Lionnel** and **Guillaume**, such a proud uncle of the little devils: **Marine**, **Baptiste**, **Cyrielle** and **Morgane**.
Gilbert & **Jacqueline**, thank you so much for everything, I would not have gone so far if you had not pushed me to keep on going with my studies. I always look forward to seeing you, to talk and enjoy life.
Of course many thanks to my wonderful family in the US, especially **Christina** and **Kathleen**.
Finally I would like to thank **Brigitte** & **Jacques**, as well as **Roger** & **Véronique**, even if you are not family, I consider you as such. You have known me since my first day, and have always been supportive, I always enjoy seeing you.

This work was supported by the Swedish Cancer Society (Cancerfonden), the Cancer Society of Stockholm (Cancerföreningen), the EU 6th Framework Program, the Karolinska Institute, and the International Agency for Research on Cancer.

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death-inducing, and dominant-negative activities. *Mol Cell* **2**, 305-316 (1998).
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