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Review

Wild type p53 reactivation: From lab bench to clinic

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ABSTRACT

The p53 tumor suppressor is the most frequently inactivated gene in cancer. Several mouse models have demonstrated that the reconstitution of the p53 function suppresses the growth of established tumors. These facts, taken together, promote the idea of p53 reactivation as a strategy to combat cancer. This review will focus on recent advances in the development of small molecules which restore the function of wild type p53 by blocking its inhibitors Mdm2 and MdmX or their upstream regulators and discuss the impact of different p53 functions for tumor prevention and tumor eradication. Finally, the recent progress in p53 research will be analyzed concerning the role of p53 cofactors and cellular environment in the biological response upon p53 reactivation and how this can be applied in clinic.

1. Introduction

Major efforts for the development of novel anti-cancer drugs are currently focused on targeting oncogene addiction, whereas strategies aimed to restore the function of the tumor suppressor p53 have been much less popular so far, perhaps due to the fact that it was unclear whether the loss of p53 function is required for the maintenance of established tumors. Elegant in vivo studies using three different approaches to engineer mice with "switchable" p53, demonstrated that restoration of p53 leads to an impressive regression of already developed tumors–lymphomas, soft tissue sarcomas and hepatocellular carcinomas [1–3]. The important conclusion from these studies is that tumors remain susceptible to the p53-mediated tumor suppression. The absence of toxic effects in normal tissues upon p53 reconstitution suggests that normal cells are not significantly affected upon p53 re-establishment achieved by non-genotoxic treatment [4]. In contrast, it appears that tumor cells carry the signals which support the growth suppression invoked by p53.

p53 is a potent tumor suppressor and is a subject of intensive studies for more than 30 years (for a review, see [5]). It is well established that p53 is a transcriptional factor activated by different types of stresses which regulates the expression of genes involved in control of cell cycle and cell death (reviewed in [6]). Activated p53 can prevent the propagation of cells carrying oncogenic lesions

via a multitude of pathways, i.e.: induction of growth arrest, senescence or apoptosis, modulation of tumor stroma, angiogenesis and metabolism, as well as the block of invasion and metastasis [5]. This explains why loss of p53 function is selected for during tumor development, resulting in p53 inactivation in the majority of human tumors. Indeed, the unbiased sequencing studies of thousands of cancer genomes recognize the *TP53* as the most frequently mutated gene [7,8]. This fact, taken together with the conclusions from the mouse in vivo experiments firmly support the notion that restoration of p53 function in tumors could be an attractive and tumor-cell specific strategy for treating cancer.

Reactivation of p53 appears to be feasible, since the p53 protein, in contrast to many other tumor suppressors such as Rb or PTEN, is usually expressed in tumors, although functionally inert. Different strategies for the reconstitution of p53 function could be envisioned, depending on the type of p53 inactivation. Re-folding of mutant p53 in tumors carrying TP53 point mutations appears to be an attractive strategy [9,10]. In tumors expressing wild type p53 the promising approach is to block major p53 inhibitors, Mdm2 and MdmX, or viral E6 oncogene in HPV-driven cervical cancers. This review is focused on p53 reactivation via inhibition of Mdm2 and MdmX.

2. Mdm2 and MdmX – major inhibitors of wild type p53 in tumors

In the absence of TP53 mutations, the tumor suppressor function of p53 is frequently impaired due to a diverse alterations

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which converge on two p53 inhibitors, Mdm2 and MdmX (encoded by Mdm4 gene). Mouse models provided a compelling evidence that Mdm2 and closely related MdmX are the major negative regulators of p53. Genetically engineered mice lacking either Mdm2 or MdmX die in utero. Importantly, in the p53-null background the embryonic lethality is completely rescued, underscoring the fundamental role of Mdm2 and MdmX in p53 regulation [11–13].

Mdm2 can inhibit p53 via a number of mechanisms (reviewed in [14]). The most studied is Mdm2 binding to the N-terminal transactivation domain of p53 which blocks its transcription function [15]. Mdm2 also functions as a E3 ubiquitin ligase which promotes either monoubiquitination of p53 leading to enhanced nuclear export [16], or polyubiquitination of p53 that targets p53 for proteasomal degradation [17,18]. In addition, Mdm2 negatively affects p53 mRNA translation [19]. While MdmX also binds the p53 N-terminus and blocks its transcriptional function, it does not possess an intrinsic E3 ligase activity [20] and is unable to target p53 for degradation. Mdm2 forms oligomers with itself or MdmX through RING-finger domains; hetero-oligomerization of Mdm2 and MdmX renders a more efficient E3 ligase activity towards p53 [21]. This fact helps to explain the functional non-redundancy of Mdm2 and MdmX observed in mouse models.

Human cancers frequently have elevated levels of Mdm2 leading to the inhibition of p53 function. This phenomenon has been reported in sarcomas, gliomas, hematological malignancies, melanomas, and carcinomas (reviewed in [22]).

Alterations leading to Mdm2 overexpression involve gene amplification (in 30% of sarcomas), elevated transcription, increased mRNA stability, enhanced translation, and altered post-translational modifications (reviewed in [23]). A single nucleotide polymorphism at position 309 (SNP309) in the human Mdm2 P2 promoter, which creates a novel binding site for the transcriptional activator Sp1, leads to an increased Mdm2 expression and attenuates p53 function. This has been shown to accelerate tumor development in humans [24].

In addition, a number of factors which control Mdm2/X activity are deregulated in cancers. Loss of expression of the negative regulator of Mdm2 p14ARF, or enhanced activity of positive regulators of Mdm2 Akt or Wip1, as well as other mechanisms, increase the inhibitory function of Mdm2/X towards p53 [25].

3. Pharmacological rescue of wtp53 by prevention of Mdm2/X-mediated inhibition

Several classes of small molecules which interfere with the inhibition of p53 by Mdm2/X have been reported. Development and properties of inhibitors of p53/Mdm2/X interaction have been extensively reviewed in [26–28]. In brief, these molecules can act via targeting Mdm2 or/and MdmX, or their upstream regulators, or p53 itself (Fig. 1).

3.1. Blocking the p53 binding to Mdm2

The majority of small molecules developed so far which target the p53/Mdm2 complex mimic p53 peptide which interacts with Mdm2 and bind to the hydrophobic cleft in Mdm2, thus acting by steric hindrance (Fig. 1). The first discovered molecules of this class are nutlins (cis-imidasoline compounds, developed by Hoffman-La Roche), followed by the development of spiro-oxindole compounds (MI series, including MI-63, MI-219), and benzodiazepinediones [29–31]. p53 reactivation by nutlin has been intensively studied by a number of labs. In different types of cancer cells p53 becomes stabilized and activated by nutlin, leading to the expression of p53 target genes, such as p21 and PUMA, followed by the induction of growth arrest or apoptosis in cultured cells and tumor suppression in human cancer xenografts in mice

(reviewed in [26]). These studies provide the proof-of-concept of p53 rescue by inhibiting Mdm2 and a strong evidence for the feasibility of this strategy.

In fact, MDM2 inhibitors, along with Bcl-2 inhibitors, such as ABT 263/Navitoclax developed by Abbott/Genentech, are currently regarded as the most successful examples of pharmacological inhibitors of protein–protein interaction (PPIs) [32]. The initial success of nutlins encouraged many companies and academic labs to design novel compounds blocking the p53 binding site in Mdm2. A new potent compound of MI series, MI-888, has been recently reported to induce a complete and sustainable regression of osteosarcoma xenograft tumors with amplified Mdm2 without obvious toxic effects [33].

Orally available nutlin analog RG7112 is the first Mdm2 inhibitor which has been tested in clinic in patients with liposarcoma and acute leukemia. In a proof-of-mechanism study of RG7112 in liposarcoma patients, the majority of which had Mdm2 amplification, administration of RG7112 for 10 days induced p53 and its target p21 and lead to decreased tumor cell proliferation. These molecular events have been associated with a stable disease in 14 out of 20 patients and a partial response in one patient. It has to be noted though, that all patients experienced adverse effects, some of which were serious, including neutropenia and thrombocytopenia [34].

Second generation Mdm2 inhibitor developed by Hoffman-La Roche, RG7388, has even higher potency and selectivity [35]. We are currently witnessing an explosion of discoveries of compounds blocking the p53-interacting pocket of Mdm2. Big pharmaceutical companies, such as Novartis and Sanofi, have several Mdm2 inhibitors in clinical development.

3.2. Targeting MdmX

The pro-apoptotic activity of Mdm2 inhibitors has been observed only in a subset of cancer cell lines, which can limit their application in clinic [36]. One of the factors which compromises the efficiency of Mdm2 inhibitors is the overexpression of another negative p53 regulator, MdmX. Mdm2 and MdmX regulate p53 in a non-redundant fashion and act synergistically, as mentioned above. Due to structural differences between the p53 binding pockets in MdmX and Mdm2, molecules which inhibit Mdm2 have low affinity to MdmX. For example, nutlin has ~400-fold lower potency against MdmX than Mdm2 [37]. High levels of MdmX in cancer cells make Mdm2 antagonists ineffective, therefore the inhibition of both Mdm2 and MdmX is required for the full-scale p53 activation [38,39]. Moreover, MdmX is upregulated in many different cancers, such as retinoblastoma and tumors in breast, lung and stomach [40,41]. MdmX plays a crucial role in p53 inactivation in melanoma, cancer which is characterized by surprisingly low incidence of p53 mutations [42]. This makes MdmX an important target for cancer therapy.

Small molecule targeting p53 binding site in MdmX, SJ-172550, has been discovered using high throughput biochemical screen [43]. SJ-172550 does not kill cancer cells on its own, but has an additive effect when combined with Mdm2 inhibitors.

Alternative approaches to inhibit MdmX, such as blocking its mRNA, have been also developed. XI-100 blocks MdmX transcription and acts synergistically with nutlin in cancer cell lines overexpressing MdmX [44].

3.3. Blocking Mdm2 and MdmX simultaneously

Recent efforts have been focused on the identification of dual Mdm2/MdmX antagonists, i.e., 'two in one' inhibitors which can offer an effective therapy for a more broad range of tumors. This idea have been pursued by Vassilev and colleagues who identified
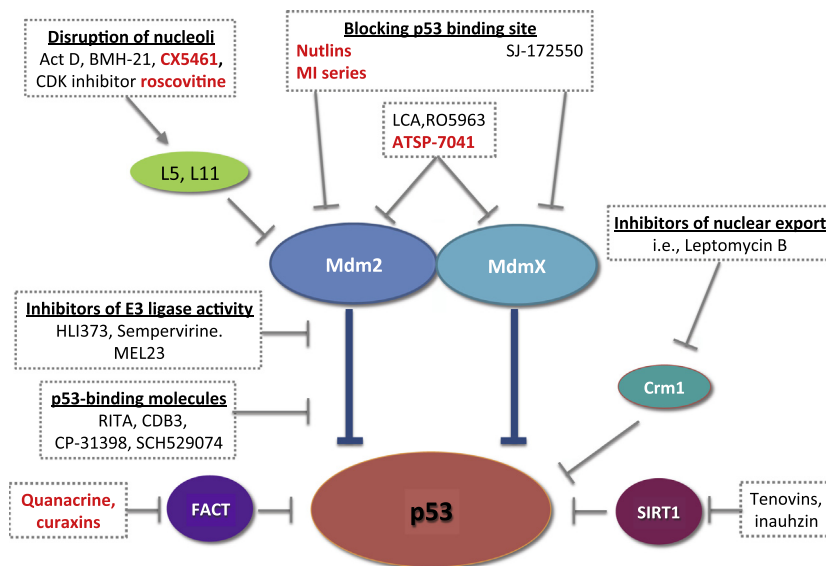
Fig. 1. Different mechanism of action of small molecules activating wild type p53. Compounds indicated in red are in clinical development.

small molecule RO-5963 that induces the formation of Mdm2 and MdmX homo- or heterodimers which can not bind p53. This leads to p53 activation, resulting in cell cycle arrest and apoptosis [45]. Novel pyrrolopyrimidine-based α-helix mimetics acting as dual Mdm2/MdmX inhibitors in vitro have been developed which can induce p53 and p21 in lung cancer cell line [46].

In silico screen lead to the identification of lithocholic acid (LCA), an endogenous steroidal bile acid. LCA binds to the p53 binding sites of both Mdm2 and MdmX with a fivefold preference for MdmX. These findings raise an interesting possibility that LCA could be a natural ligand for Mdm proteins [47].

Our phenotypic screen led to the identification of the thiophen-derived small molecule RITA, which prevents p53 inhibition by both Mdm2 and MdmX and induces apoptosis in a variety of cell lines bearing wild-type p53 [48–50], see below for further details.

3.4. Inhibition of E3 ligase activity of Mdm2

Inhibitors of E3 ligase activity of Mdm2 towards p53 have been discovered in biochemical screens, using decreased Mdm2 auto-ubiquitination as a readout. First molecule of this class is HLI98C [51], followed by a less genotoxic, more soluble and potent analog HLI373 [52] and by the identification of a natural compound sempervirine [53]. These compounds stabilize p53, induce p53-dependent transcription and selectively kill transformed cells expressing p53.

Cell-based screen allowed to identify MEL23 and MEL24 compounds which inhibit the E3 ligase activity of Mdm2/MdmX complex, reduce the viability of cancer cells with wild type p53 and synergise with DNA-damaging agents [54].

JNJ-26854165 (serdemetan) developed by Johnson&Johnson is suggested to block p53 association with the proteasome, but the exact mechanism has not been reported [55]. It demonstrated a modest clinical activity and the ability to induce p53 in a phase I clinical trial, although its administration has been associated with toxic effects [56].

3.5. Stapled peptides

Early studies have identified potent peptides which sterically block p53/Mdm2 complex, although they were not very promising for clinical applications [57,58]. A new chemistry – such as

hydrocarbon stapling of peptides – helps to resolve the major limitations of peptides as drugs, i.e., unstable secondary structure and proneness to proteolytic degradation. Stabilized alpha-helix-8 (SAH-8), modeled after Mdm2-binding peptide of p53, can prevent Mdm2/p53 interaction and activate p53 in osteosarcoma cells [59]. Recently a high affinity stapled peptide ATSP-7041 has been developed which blocks p53 complexes with both Mdm2 and MdmX in vitro and in cells [60]. ATSP-7041 inhibits growth and induces apoptosis in Mdm2-amplified osteosarcoma cells and in breast cancer cells with high expression of MdmX and can suppress xenograft tumor growth in mice. In addition, it has a favorable pharmacokinetics. This study provide a proof-of-concept that stapled peptides can serve as potent and selective inhibitors of p53/Mdm2/MdmX complex.

3.6. Compounds affecting upstream regulators of Mdm2/X

A number of factors acting upstream of Mdm2 could be targeted in order to impair its stability, expression, or activity toward p53. Mdm2 is negatively regulated by ribosomal proteins L5, L11 [61] and several others which are released from nucleoli upon nucleolar stress. Thus, compounds which can disrupt nucleoli, could inhibit Mdm2 [62]. Indeed, low (nanomolar) doses of Actinomycin D (Act D), compound which binds to GC-rich regions in DNA and disrupts RNA synthesis, in particular ribosomal RNA biosynthesis [63], induce p53 without significant genotoxic effect. Strikingly, the genome-wide analysis of gene expression profiles show a high degree of similarity between the patterns of genes induced by p53 in colon cancer cells upon treatment with Act D and nutlin [64]. However, it should be kept in mind that Act D activates p53 in normal cells as well. For example, this highly potent compound can cause p53-dependent reversible cell cycle arrest in normal keratinocytes [64].

Interestingly, a recent chemical library screen lead to the discovery of several molecules which activate the p53 pathway and kill cancer cells via interference with the DNA topology [65]. One of the compounds, BMH-21, binds to GC-rich sequences, in particular in ribosomal DNA genes, leading to the inhibition of RNA Pol I via proteasome-dependent degradation of Pol I subunit RPA194 and segregation of nucleolar proteins [66].

Small molecule CX5461 blocks rRNA biosynthesis by inhibiting PolI transcription initiation step, and induces p53-dependent apoptosis selectively in cancer cells [67].

CDK inhibitor roscovitine can also disrupt the nucleolus and thereby inhibit p53/Mdm2 interaction [62]. In addition, roscovitine can downregulate Mdm2 expression [68].

Inhibitors of nuclear export, such as a blocker of nuclear export protein Crm1 Leptomycin B, are also candidate p53 activators. Although Leptomycin B has been shown to induce apoptosis in wild type p53-expressing cancer cells and has been tested in Phase I clinical trial, its high toxicity precludes further clinical development. Less toxic Crm1 inhibitor, compound 3, has been shown to be more efficient than Leptomycin B; it can inhibit cervical cancer cell growth in vitro and in vivo [69].

Several posttranslational modifications have been shown to be important for p53 activity, including acetylation (reviewed by [70]). This opens a possibility to target enzymes involved in p53 modifications. One of such enzymes is deacetylase SirT1, a member of sirtuin family, which removes activating acetyl groups from p53, leading to its de-stabilization and loss of transcriptional activity [71]. Sirtuin inhibitors have been identified which are able to stabilize p53 and trigger its activation in cancer cells and inhibition of tumor xenograft growth in vivo, including tenovins [72] and inauhzin [73].

Most of commonly used anticancer chemotherapeutics are DNA damaging agents, whose potential is limited by severe side effects and increased risk of secondary malignancies. However, several DNA-targeting compounds such as low doses of Act D or BMH-21 mentioned above are non-genotoxic, but still are the potent killers of cancer cells, often engaging p53. Other examples of such molecules are well-known anti-malaria drug quinacrine and its optimized follow up derivatives curaxins, which activate p53, inhibit NF-kB and kill renal cell carcinoma cells in vitro and in vivo [74]. Further studies have shown that curaxins are non-genotoxic DNA intercalators which act by affecting the chromatin remodeling complex FACT (FAcilitates Chromatin Transcription), commonly deregulated in cancer [75,76]. The proposed mechanism of action involves "chromatin trapping" of FACT which results in the inhibition of NF-kB, as well as activation of p53 by FACT-associated casein kinase 2 [75]. Potent anticancer activity of quinacrine and curaxines in human tumor xenografts grown in mice promote the idea of developing DNA intercalating/chromatin modifying compounds as effective and safe anticancer drugs. This view is supported by recent testing of CBL0102, or quinacrine, in advanced cancer patients with liver metastases performed by Cleveland Biolabs, Inc and Incuron, LLC. Phase I clinical trial demonstrated a favorable pharmacological properties and mild side effects. Curaxin CBL0137 is currently being tested in Phase I trials as well.

3.7. p53-Targeting compounds

We have identified a small molecule RITA (reactivation of p53 and induction of tumor cell apoptosis), which prevents p53/Mdm2 interaction, induces p53 accumulation and transcriptional activity and triggers p53-dependent apoptosis in tumor cells of a different origin in vitro and in mice [48,77–79]. RITA was the first compound shown to bind the p53 N-terminus (residues 1-63). Our studies provided proof-of-principle that a small molecule can bind p53 N-terminus without hindering the transcriptional function of p53. RITA triggers a conformational shift preventing p53's interaction with its negative regulators such as Mdm2 and others [48]. Notably, RITA induces the proteasome-dependent degradation of MdmX protein via downregulation of Wip1 phosphatase, which makes it highly efficient in killing cells with overexpressed MdmX [50].

Our finding that RITA induces a conformational change in p53 encouraged us to test whether it can restore the activity of mutant p53. We found that RITA suppresses the growth and induces apoptosis in a p53-dependent manner, along with the induction of p53

target genes, in a variety of mutant p53 carrying cell lines of different origin [77,79]. These findings promote the idea of developing compounds capable of simultaneously targeting wild type and mutant p53.

Notably, at least some mutant p53-reactivating compounds which interact with the p53 DNA-binding domain (DBD), such as a synthetic peptide CDB3 and small molecules CP-31398 and SCH529074, can also reactivate wild type p53 by interfering with Mdm2-mediated degradation [80,81]. It is possible that they prevent the docking of the central domain of Mdm2 to p53 DBD, which is required for the efficient ubiquitination of p53 by Mdm2 [82]. Alternatively, these compounds might block other facets of Mdm2-mediated inhibition of p53. The mechanisms of activation of wild type p53 by two other mutant p53-reactivating molecules, PRIMA-1^{Met} (APR-246) [9] and p53R3 [83], are currently less clear. Intriguingly, PRIMA-1^{MET}/APR-246 activates wild type p53 transcriptional activity and induces p53-dependent apoptosis in melanoma cells in vitro and in vivo without apparent impact on p53 level or stability [84] nor on Mdm2 or E6-mediated ubiquitination of p53 [80].

Dual targeting of mutant and wild type p53 appears to be highly beneficial, since recent studies demonstrate that treatment with nutlin creates a selective pressure for p53 mutations in different types of cancer leading to resistant clones, which in some cases contributes to multidrug resistance [85,86]. The mechanism of simultaneous reactivation of mutant and wild type p53 by small molecules remains elusive in most cases. Further elucidation of the structural features of the full length p53 might help to understand their mode of action. p53 has proven to be a challenge for structural biologists due to its high conformational flexibility. However, several important achievements have been reported [87,88].

4. Tumor suppression by p53: prevention vs eradication

Induction of the apoptosis, growth arrest or senescence upon p53 activation by stress results in the elimination of mutated or damaged cells [5]. p53 binds to its consensus sequences in the promoters of target genes and acts as a transcriptional activator of expression of multiple pro-apoptotic factors (Puma, Noxa, Bax, Fas, etc.), or cell cycle arrest genes (p21, BTG2, etc.). In addition, p53 can repress transcription of anti-apoptotic and survival factors (i.e., BCL-2), as well as cell cycle-promoting genes (Cdc25A, Cyclin B). Interaction of p53 with Bcl-2 family proteins in cytoplasm can trigger apoptosis in a transcription-independent way (reviewed in [5]. It is widely accepted that these activities represent a clear mechanism of p53-mediated tumor suppression.

Reconstitution of p53 using several elegant in vivo models have proven that restoration of p53 confers increased survival of mice with established tumors in Eμ-myc lymphoma model and leads to the regression of established tumors due to the induction of apoptosis or senescence in autochthonous lymphoma and sarcoma model and liver carcinoma model [1–3].

Yet, the prevailing view that the induction of apoptosis or growth arrest is the core of p53 tumor suppressor function has been recently challenged by in vivo studies. Several groups have shown that the transgenic mice expressing mutants compromised for cell cycle arrest, senescence and apoptosis are still able to function as tumor suppressors.

5. Involvement of non-canonical p53 functions in tumor suppression by p53

Despite the inability of transactivation-deficient mutant p53[25,26] (L25Q; W26S) to activate classical p53 target genes p21, Noxa, and Puma and to trigger cell cycle arrest, apoptosis and

senescence in response to DNA damage, p53[25,26] still shows a partial tumor suppressor activity when expressed in mice, as found by Attardi and her colleagues. Interestingly, p53[25,26] can regulate the expression of a subset of 'non-classical' genes, suggesting that other mechanisms can contribute to the tumor suppression by p53 [89]. Moreover, these data support the notion that the induction of Puma, Noxa and p21 culminating in apoptosis or growth arrest is important for the response to acute DNA damage, but not to non-genotoxic oncogenic stress, thus distinguishing between these two different p53 functions. Data obtained earlier in other mouse models, also points out to the idea that the response to acute DNA damage by the induction of apoptosis might differ from the tumor suppression pathway evoked by p53 in response to oncogene activation [90].

It should be noted, however, that the transcriptional transactivation function of p53 is necessary for tumor suppression, since transactivation-dead mutant p53[25,26,53,54] (carrying mutations in transactivation domain 1, L25Q;W26S, as well as inactivating mutations in transactivation domain 2, F53Q;F54S) was completely unable to suppress carcinogenesis in several models [91].

Expression of another p53 mutant defective in the induction of pro-apoptotic target genes and apoptosis – p53[RR] mutated at residue E177 (corresponding to human E180 and preventing cooperative DNA binding by p53) – lead to an increased tumor incidence in mice compared to control wild type mice. However, despite the inability to eliminate cancerous cells by apoptosis, this mutant p53 was still able to suppress the development of T-cell lymphomas. It is possible that the ability of p53[RR] to limit glycolysis and ROS accumulation via induction of expression of *Gls* and *Dram* underlies its partial tumor suppression [92].

Furthermore, in spite of the abrogation of p53-mediated cell cycle arrest, apoptosis and senescence, mice carrying p53[KR] mutated at K117R, K161R, K162R which abolishes its acetylation, do not succumb to the formation of early-onset spontaneous tumors. Taking into account that p53[KR] can activate the expression of metabolic target genes *Gls2* and *Tigar*, it is possible that the regulation of energy metabolism and ROS production could play a crucial role in the tumor suppressor function by p53[3KR] [93].

To test the involvement of growth arrest and apoptosis in p53-mediated tumor suppression, Strasser and his colleagues generated the triple knockout mice lacking p21, Puma and Noxa. Cells derived from these mice are resistant to p53-dependent apoptosis, cell cycle arrest and senescence upon DNA damage. Notably, these mice are not predisposed to the early onset of spontaneous tumors, in contrast to p53-null mice. The authors suggest that the ability of p53 to regulate DNA repair might be critical for the tumor prevention by p53 [94].

In summary, several recent in vivo studies suggest that the canonical p53 responses do not fully explain p53-mediated tumor suppression. However, one important distinction should be made between these models, which are investigating the development of tumors and the ones which address the tumor eradication upon p53 reconstitution in already developed tumors. Restoration of p53 in established tumors suppresses them by induction of apoptosis or senescence, as clearly demonstrated in seminal papers mentioned above. In contrast, in those transgenic mouse models which investigate the development of tumors in the absence of p53-dependent apoptosis and senescence, the question of tumor prevention by p53 has been addressed. Thus, the two branches of p53-mediated tumor suppression, tumor prevention and tumor eradication, probably require different, albeit partially overlapping, set of p53 functions (Fig. 2).

Indeed, anti-oxidant function of p53 is particularly important for the prevention of lymphomas [95]. Treatment of p53-null embryos in utero with anti-oxidants significantly decreased genomic instability, thus diminishing the chance for oncogenic

mutations [95]. The ability of p53 to induce anti-oxidant genes, such as sestrins, together with well documented plethora of p53-mediated responses that facilitate DNA repair [96] appears to be essential for the control of genomic integrity by p53, serving to prevent oncogenic alterations.

Recent discovery that a whole set of genes involved in autophagy is regulated by p53 [97], in addition to previously identified DRAM [98], suggests that this facet of p53 activity might be involved in tumor suppression. However, the role of autophagy in tumor suppression appears to be very much dependent on a context. Several recent studies suggest that the development of breast carcinoma, non-small-cell lung cancer and pancreatic carcinomas in mouse models is more efficiently impeded by p53 in the absence of essential autophagy factors, such as Becn-1, Atg7, or Atg5 [99–101]. It will be critical to elucidate more thoroughly the role of autophagy in p53-dependent tumor suppression, since it might contribute to cancer cell survival and promote resistance to anti-cancer therapies in some contexts.

Emerging is another non-canonical function p53 – facilitation of immune surveillance of cancer. p53-Deficiency in intestinal epithelial cells leads to an increasing inflammatory response, associated with enhanced EMT and tumor invasion [102]. Tumor regression upon p53 restoration in liver carcinoma has been found to be promoted by the infiltration of innate immune cells [3]. This is due to the production of cytokines by senescent tumor cells, which recruit predominantly tumor suppressor M1 macrophages. In contrast, cytokines produced by p53-deficient tumor cells induce tumor-promoting M2 macrophages [103]. Moreover, induction of expression of some p53 target genes can enhance the susceptibility of tumor cells to immune system. Two independent studies, including ours, identify ULPB2, encoding a ligand for NK cell activating receptor NKG2D, as a new p53 target gene induced upon the pharmacological reactivation of p53 in tumor cells. Expression of ULBP2 enhances the NK cell-mediated killing of tumor cells of a different origin [104,105]. Toll-like receptor 3 (TLR3), a crucial innate immune receptor, has been identified as a target gene of p53, suggesting that p53 can potentiate interferon-alpha-induced apoptosis [106]. p53 can also promote cell surface expression of major histocompatibility complex I via regulation of endoplasmic reticulum aminopeptidase 1 [107].

In summary, it appears that some of p53 biochemical activities might be required for the prevention of tumor formation, whereas others are more important for the eradication of already formed
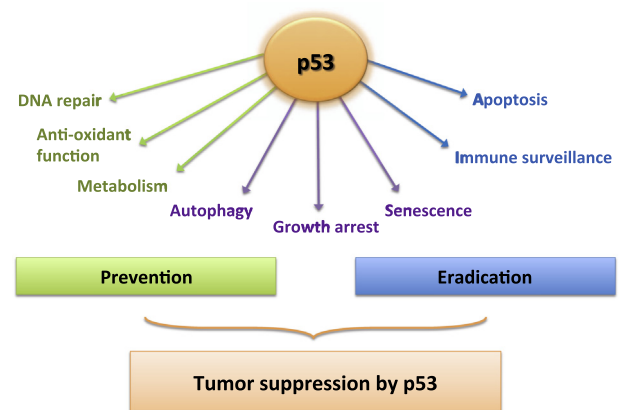

Fig. 2. Different activities contribute to tumor prevention and tumor eradication by p53. Regulation of DNA repair and metabolism as well as antioxidant response are more important for tumors prevention (indicated in green) whereas the induction of apoptosis and immune surveillance are more important for tumor eradication (indicated in blue). Induction of senescence, autophagy and growth arrest might have opposite effects on p53 tumor suppression, depending on the context (indicated in purple).

tumors (Fig. 2). It is tempting to speculate that for the prevention of tumors and, perhaps ageing, small molecules could be developed which specifically enhance the "prevention" function of p53, whereas to eliminate already established tumors we should apply small molecules activating "eradication " function of p53 .

6. p53: To kill or not to kill

Rigorous efforts that have been dedicated to development of Mdm2/MdmX inhibitors for cancer therapy are starting to be translated into clinic; a number of clinical trials are being performed, as mentioned above (Fig. 1). It is therefore of utmost importance to understand which functional aspects of p53 these compounds activate. Reactivation of p53 tumor eradication function – i.e., apoptosis – in established cancers is the goal of p53-based therapies. However, p53-mediated apoptosis in normal tissues is the cause of pathological loss of cells upon chemotherapy, neurodegenerative diseases, stroke and ischemia; it might also contribute to ageing [5]. Although mouse models have demonstrated that tumor cells have higher propensity to die upon p53 restoration, the molecular basis for the phenomenon needs to be elucidated (see below for more discussion). We need to find out how to manipulate p53 to promote death selectively in tumor, but not in normal cells.

Recent study points out that the induction of senescence by p53 might impair the response to chemotherapy in clinic in breast cancer patients [108]. However, in some types of tumors, as for example in acute promyelocytic leukemia (APL) driven by the pro-myelocytic leukemia (PML)–retinoic acid receptor-α (PML–RARA) fusion protein, induction of senescence by p53 is beneficial for patients [109]. Thus, it appears that the induction of senescence can affect anti-cancer therapy in quite opposite ways. The outcome probably depends on the type of factors secreted by senescent cells, which in turn determines the type of immune cells which are recruited to tumor. It needs to be investigated how the secretome of senescent cells is regulated. Further, triggering p53-mediated growth arrest, DNA repair, and/or antioxidant response in established cancers is not desirable since it will counteract chemo- or radiotherapy. Yet, these functions of p53 can prevent tumor formation. Thus, we need to rigorously determine the mechanisms of p53 life/death decision if we are to clinically exploit drugs activating p53 in different types of cancer.

7. Mechanisms of p53-mediated cell fate decisions

In order to harness p53 activities, it is crucial to meticulously investigate the mechanisms by which p53 induces its vast range of responses. p53 could be envisioned as a complex protein assembly that remodels itself in a different way depending on a type of stimulus and cellular environment, leading to different biological outcomes. In essence, to understand p53 is to understand how its interaction with proteins and, thus DNA, is controlled.

There are a lot of questions to address concerning the mechanisms of differential transcription programs elicited by p53 for triggering distinct biological outcomes. p53 response could be determined by a number of factors, such as p53 posttranslational modifications, cooperativity of binding to DNA, levels and duration of its induction, and binding to its cofactors. In particular, it is not clear whether it is p53 in vivo promoter selectivity that sets off different transcription programs. Our analysis of genome-wide chromatin occupancy by p53 using ChIP-seq revealed the "p53 default program", i.e., the pattern of major p53-bound sites that is similar upon p53 activation by nutlin3a, RITA or 5-FU in breast cancer cells, despite of different transcription programs and biological outcomes triggered by these compounds [110]. Thus, our study suggests that p53 cofactors, but not the selective binding of p53

to promoters, play a key role in the differential regulation of target genes by p53. Therefore, the grand challenge of p53 research is to identify cellular cofactors as well as small molecules that can switch p53 transcriptional and biological responses in a desired way (Fig. 3).

In spite of numerous studies, it still remains elusive, which factors direct p53 to a certain transcriptional program. We are just beginning to get a deeper understanding of at least some mechanisms governing different p53 activities. p53 is regulated by numerous posttranslational modifications, such as phosphorylation, acetylation, ubiquitination, neddylation, sumoylation and methylation (reviewed in [111]). For example, phosphorylation at Ser46 by HIPK2, as well as acetylation at Lys120 by Tip60 facilitates the transcriptional activation of pro-apoptotic targets by p53 [112,113]. Posttranslational modifications affect p53's interactions with its numerous binding partners which, in turn, differentially modulate p53-induced transcriptional programs and thus biological outcome. Studies that have looked into p53 interactions with its partners have been well reviewed [5,114]. Some examples of cofactors which cooperate with p53 in induction of cell cycle arrest genes include Hzf1, Brn3A, and hnRNP K [5,49,115]. BRCA1, as well as Ref-1 upon induction by selenium methionine, promote the induction of DNA repair genes [116,117]. In contrast, ASPP cooperates with p53 in activation of pro-apoptotic targets [118]. We have recently identified Sp1 as an important cofactor for the apoptosis induction by p53 [110].

In summary, p53-induced biological outcomes are dictated by a number of collaborating or antagonizing factors and their combinations, making it a daunting task to predict the p53 response in different settings. Compounds activating p53 could serve as an excellent molecular tools to address these crucial questions of p53 biology.

8. Small molecules as research tools to address the p53 choice of transcriptional programs

To investigate the molecular mechanisms affecting p53 outcomes we have used two small molecules, nultin and RITA, which induce growth arrest or apoptosis, respectively, in several cancer cell lines, such as HCT116, MCF7 and U2OS. Both compounds inhibit the p53/Mdm2 interaction, albeit targeting different proteins: whereas nutlin, as described above, blocks the p53-binding pocket in Mdm2, RITA binds to p53 and induces a conformational shift making p53 recognition by Mdm2 inefficient [29,48]. Further, it's important to mention that the levels of both Mdm2 and MdmX eventually subside after RITA treatment [119–121], which is essential, since the level of MdmX determine the sensitivity of cells to nutlin [122,123]. Although the mechanisms underlying the decreased levels of Mdm2 and MdmX are only partially understood, these studies suggest that certain branches of the negative feedback loop induced by p53 are relieved after RITA. In contrast, the binding of nutlin to Mdm2 stabilizes the protein
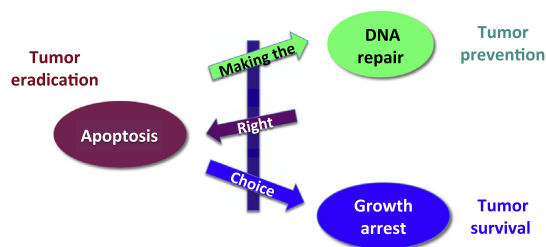

Fig. 3. p53 can trigger different responses, leading to different biological outcomes. However, the mechanisms which control p53's choice of life/death decisions are still unclear.

and protects Mdm2 from degradation. This, together with a strong induction of Mdm2 mRNA (as a consequence of Mdm2 transactivation by p53) it leads to a fast decrease of p53 levels upon removal of the compound [124].

Data mentioned above suggest that the disruption of p53/Mdm2 complex is not the sole effect of small molecules: along with the prevention of Mdm2/p53 complex, they might also differently affect other events. Nutlin has been shown to act as an allosteric agonist of Mdm2 by inducing a conformational change in the acidic domain of Mdm2 which facilitates p53 ubiquitination [82]. Further, proteomics screen identified nucleophosmin (NPM) as a predominant partner of nutlin-bound Mdm2 [125]. In line with the notion that nutlin affects Mdm2 interactome is the finding that Mdm2 bound by nultin targets for the degradation HIPK2, kinase facilitating p53-mediated induction of pro-apoptotic genes [121]. While binding of nultin changes the repertoire of Mdm2-binding partners, RITA affects the p53 interactome (for a review, see [25]). We found that Mdm2 released from p53 upon RITA can target for degradation itself and several of its substrates, including those important for p53-mediated growth arrest: p53 cofactor hRNP K and cdk inhibitor p21, but not HIPK2, thus contributing to the switch from growth arrest to cell death [49].

9. Transcriptional repression vs transcriptional activation

Expression of survival factors blocking apoptosis downstream of p53, such as anti-apoptotic Mcl-1 or Bcl-2 can block death-inducing signals, resulting in growth arrest upon p53 activation [126,127]. We found that p53 can confer the transcriptional repression of major proliferative and survival factors upon RITA resulting in rapid decline of the key components of the IGFR-PI3K-Akt pathway, c-Myc, Mcl-1 and survivin [128]. This ablates pro-proliferative and survival pathways, reducing the cancer cell's ability to counteract pro-apoptotic signals. The same degree of repressed survival factors was not observed upon nutlin; however, the combination of nutlin with depletion of Mcl-1 or c-Myc acted synergistically to induce apoptotic response.

Notably, our data suggest that the induction of pro-apoptotic genes and the repression of survival genes may require distinct cofactors and/or posttranslational modifications of p53 [128]. While p53 transactivation function is relatively well understood, there is still much to learn about the molecular mechanisms of p53-mediated transcriptional repression [129]. p53 can repress genes indirectly, as for example via long non-coding RNAs [130] or p21 [131]. However, p21 is depleted upon p53 activation by RITA [49]. Moreover, the repression of a number of genes by p53 upon RITA coincides with p53 binding to its consensus sites in the promoters of these genes, suggesting a direct regulation by p53 [110,128].

Our search for factors which facilitate the transcriptional repression by p53 has implicated JNK in repression of pro-survival genes *Mcl-1*, *eIF4E*, *PIK3CA* and *PIK3CB* [132]. Interestingly, we found JNK to be a crucial factor which can convert p53 from a transactivator to a repressor of some genes involved in negative regulation of p53, such as *PPM1D*, encoding phosphatase Wip1 (wild type p53 induced protein1) and MdmX. Establishment of JNK-p53 positive feedback loop and inhibition of p53-Wip1 negative feedback loop result in enhanced and sustained p53 activation, which produces a robust apoptotic outcome [132].

10. Possible mechanisms of tumor-selective growth suppression by p53

The risk of inhibiting Mdm2 comes from the fact that a sudden activation of p53 in normal tissues might have undesirable toxic effects in the Mdm2-null background [133]. However, studies using xenograft models in mice demonstrated that tumor suppressing doses of nutlin, RITA, MI-219 and stapled peptide ATSP-7041 do not cause weight loss and are well tolerated [29,48,60,134]. A number of studies point towards the ability of p53 to kill cancer cells without detrimental effects in normal cells in vitro and in vivo [135]. However, the molecular pathways by which reconstituted p53 becomes pro-apoptotic selectively in malignant tumors have not been defined yet and are a subject of intensive studies.

It has been reported that amplified MAPK signaling in aggressive lesions supports the suppression of malignant tumors by p53 upon its reconstitution [136,137]. Recent in vivo studies identify MKK7-JNK signaling as a crucial component that senses oncogene activation and links the oncogenic stress to p53-mediated tumor suppression [138]. The question remained though, how oncogenic stress is sensed by MKK7-JNK and how JNK modulates the p53 function.

Deregulation of tumor suppressor and oncogenic pathways in cancer frequently leads to different types of stresses experienced by cancer cells, including replication stress, proteotoxic stress, metabolic stress and an increased accumulation of reactive oxygen species (ROS) (for review, see [139]). Our recent study suggest that elevated ROS in malignant tumors might provide an activating signal to p53 via JNK, which in turn facilitates p53 transcriptional activity, in particular, the transcriptional repression of survival genes [132]. It is tempting to speculate that the enhanced and sustained p53 activity achieved due to high level of ROS and JNK activation may constitute one of the reasons for the selective elimination of advanced cancers by reinstated p53, observed in mouse models. The relative contribution of the ROS/MAPK pathway in oncogenic signaling and preferential suppression of malignant tumors by p53 is an interesting subject for future studies.

11. Combination therapy

The multi-gene nature of cancer suggests that the ablation of a single target is unlikely to produce a sustained effect. Targeting two or more unique alterations in cancer cells to produce synthetic lethality appears to be a more feasible approach to achieve therapeutic activity and selectivity and to prevent the development of drug resistance. One of the important biochemical differences between normal and cancer cells is a decreased capability of cancer cells to buffer high ROS levels. While increased ROS production contributes to tumorigenesis, it must be restrained even in cancer cells if they are to avoid the damaging effects of ROS on vital intracellular macromolecules [140]. Therefore, cancer cells with increased burden of oxidative stress are likely to be more vulnerable to damage by further ROS insults [141,142].

We have previously shown that in addition to inhibiting p53/Mdm2 interaction, small molecule RITA confers inhibition of TrxR1, one of the key enzymes which keeps ROS balance [143]. Our study suggests that the dual targeting of p53 and TrxR1 by compounds such as RITA leading to ROS induction, or a combination of p53 reactivating compounds with inhibitors of cellular anti-oxidant systems confers synthetic lethality [132]. Indeed, we showed that growth arrest/senescence by nutlin could be switched to apoptosis upon low dose of TrxR inhibitor auranofin [132]. Simultaneous targeting of p53 and cellular antioxidant system might allow to maximally exploit the p53-mediated tumor suppression as a therapeutic strategy.

Nutlin has been shown to synergize with a number of chemotherapeutic drugs including those targeting tubulin (vinblastine), DNA-damaging agents (doxorubicin), S-phase agents

(fludarabine and gemcitabine), and radiation [28]. The major disadvantage of combining p53 reactivation with chemotherapy is the augmentation of p53 activation both in cancer and in normal tissues which can produce side effects. In fact, the contribution of p53 to tissue toxicity upon chemotherapy prompted researchers to develop small molecules inhibiting p53 to minimize side effects [144,145].

Combination therapy might help to avoid the development of resistance, such as for example, mutations in Mdm2 [146]. As mentioned above, it was found in several studies that treatment with nutlin leads to selection of p53 mutants, which could accumulate while Mdm2 is inhibited and in addition to be a cause of resistance. Mutant p53 might even display gain-of-function activities promoting tumor growth. Therefore it could be a promising strategy to combine wild type reactivating compounds with those restoring mutant p53 function, such as PRIMA-1^{MET}/Apr246.

Search for the rational combinations of drugs which can produce synergy when combined with Mdm2 inhibitors, revealed that low doses of CDK inhibitors promote apoptosis induction when combined with nutlin [147]. siRNA screen against known human kinases pinpointed to several pathways, including the MAP kinase pathway and the sphingosine kinase pathway whose inhibition may synergize with nutlin [148]. Genome-wide short hairpin RNA screen for genes that are lethal in combination with p53 activation by nutlin has identified the inhibition of ATM and MET kinases as means to convert the cellular response by nutlin from cell cycle arrest into apoptosis in diverse cancer cell types [149].

Cyclotherapy, i.e., induction of growth arrest in normal tissues thus protecting them from the effects of chemotherapeutics while unshielding cancer cells is an emerging strategy to minimize side effects. Since normal cells have an intact p53 pathway, low doses of non-genotoxic p53 activators, such as nutlin or actinomycin D result in a reversible cell cycle arrest, making them immune to drugs targeting actively proliferating cells, such as Aurora kinase inhibitor. In contrast, cancer cells carrying mutant p53 continue to proliferate and are selectively killed [150,151]. In vivo study supports this idea by showing that nutlin-3 pretreatment protects mice from neutropaenia induced by the Polo-Like-Kinase-1 (PLK-1) inhibitor BI2536 [152]. Thus, cyclotherapy is a promising strategy to improve the therapeutic window of classic chemotherapeutic agents by non-genotoxic p53 activators that can induce a mild 'cytostatic' effect in normal tissues.

12. Concluding remarks

Reactivation of p53 is a very promising anti-cancer strategy which is currently being tested in clinic. There are a number of critical issues which remain to be addressed in order to apply p53-reactivating drugs for the benefit of patients. We still have to find out which biomarkers can predict the response to p53-targeted therapy, the mechanisms of intrinsic and acquired resistance and possible side effects. The efficient implementation of p53-targeting treatments into clinical practice requires thorough understanding of mechanisms governing p53 response in cancer cells. Small molecules could serve as valuable tools to address these burning questions of p53 biology.

Acknowledgments

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