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REVIEW

Translational approaches targeting the p53 pathway for anti-cancer therapy

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The p53 tumour suppressor blocks cancer development by triggering apoptosis or cellular senescence in response to oncogenic stress or DNA damage. Consequently, the p53 signalling pathway is virtually always inactivated in human cancer cells. This unifying feature has commenced tremendous efforts to develop p53-based anti-cancer therapies. Different strategies exist that are adapted to the mechanisms of p53 inactivation. In p53-mutated tumours, delivery of wild-type p53 by adenovirus-based gene therapy is now practised in China. Also, remarkable progress has been made in the development of p53-binding drugs that can rescue and reactivate the function of mutant or misfolded p53. Other biologic approaches include the development of oncolytic viruses that are designed to specifically replicate in and kill p53-defective cells. Inactivation of wt-p53 frequently results from dysregulation of MDM2, an E3 ligase that regulates p53 levels. Small-molecule drugs that inhibit the interaction of MDM2 and p53 and block p53 degradation are currently tested in clinical trials. This survey highlights the recent developments that attempt to modulate the function of p53 and outlines strategies that are being investigated for pharmacological intervention in the p53 pathway.

Abbreviations

aa, amino acid; CAS, Chemical Abstracts Service number; MDM, murine double minute; wt, wild-type

Introduction

The ubiquitously expressed tumour suppressor p53 is a multifunctional protein that regulates cellular stress responses such as cell cycle arrest, apoptosis and senescence (Zilfou and Lowe, 2009). Owing to the final nature of the two latter responses, the activity of p53 is tightly regulated. In healthy cells, p53 is present at low level that is maintained by its constant proteasomal degradation (Hock and Vousden, 2010). Upon cellular stresses, such as oncogene overexpression or DNA damage, the expression level of p53 is enhanced (Harris and Levine, 2005). The rise in p53 protein level results from reduced degradation rather than enhanced transcription or translation and allows for the rapid activation of p53-mediated stress responses.

The tumour-suppressive function of p53 predominantly relies on its function as a transcription factor, although various controversial transcription-independent pro-apoptotic activities of p53 have been described (Essmann *et al.*, 2005; Vaseva and Moll, 2009). As a transcription factor, p53 can positively or negatively regulate the expression of numerous target genes, encoding for instance pro-apoptotic Bcl-2 proteins (e.g. Bax, Bak, Puma, Noxa), caspases, death receptors (e.g. Fas), DNA repair proteins or the cell cycle inhibitor p21. The net result of p53 activation appears to be dictated by the balance of p53 target gene expression and manifests as transient cell cycle arrest, cell death by apoptosis or induction of senescence (Jänicke *et al.*, 2008; Qian and Chen, 2010). Each of these stress responses tends to prevent cellular transformation and tumorigenesis by removing damaged cells from the proliferative cycle. It is thus obvious that inactivation of p53 by either mutation or dysregulation is commonly observed in human cancer. Mutations in p53 that disturb its proper function are found in about 50% of human cancers, and the remaining half of tumours seem to have malfunctions of the p53 pathway (Soussi *et al.*, 2000).

Mice without functional p53 are prone to the development of spontaneous tumours. It could be demonstrated that even established tumours regress after re-expression of endogenous p53 (Martins *et al.*, 2006; Ventura *et al.*, 2007; Xue *et al.*, 2007). Tumour regression was mainly due to apoptosis, but, depending on cell type, also p53-mediated senescence and clearance of tumour cells by the immune system were observed. With the aim to restore p53 activity in human cancer, a number of anti-tumour therapies have recently been developed (Fischer and Schulze-Osthoff, 2005). A straightforward strategy is the re-expression of wild-type (wt)-p53 in tumour cells. To this end, viruses have been engineered that mediate expression of transgenic wt-p53. However, unselective infection of transformed and non-transformed cells appears to be critical and target-specific infection is more desirable. Additionally, the host immune response attenuates the success of virus-mediated gene therapy or oncolytic virotherapy (Ritter *et al.*, 2002). Owing to the high frequency of mutated and hence non-functional p53 in tumour cells, viruses have been engineered that specifically replicate in and finally lyse cells with mutant p53, while leaving healthy cells unharmed. Alternative strategies have been developed to reactivate mutant p53. Mutant p53 forms have usually a destabilized structure (Bullock *et al.*, 2000) but can astoundingly be activated by compounds promoting the native conformation of p53.

The low expression of wt-p53 in healthy unstressed cells is controlled by negative-regulatory proteins such as MDM2 and MDMX (MDM4). The E3 ligase MDM2 mediates ubiquitylation of p53, thereby marking it for nuclear export and proteasomal degradation. In contrast, MDMX, though homologous to MDM2, shows little ubiquitylation activity towards p53 but enhances the activity of MDM2 (Hock and Vousden, 2010). MDM2 has been verified as a p53 target gene, creating an autoregulatory feedback loop. This feedback mechanism is additionally influenced by another p53 target gene, $p14^{Arf}$, that counteracts MDM2-mediated p53 inactivation and thereby enhances p53 levels (Harris and Levine, 2005). Consequently, an insufficient p53 response can be caused by enhanced MDM2-mediated ubiquitylation and degradation of p53. Therefore, small molecule drugs have been developed and have entered clinical trials with the aim to specifically prevent the interaction of MDM2 with p53, thereby blocking the transfer of ubiquitin moieties to the tumour suppressor. These drugs effectively induce enhanced levels of p53 and the desired p53 response. We will first provide a short introduction to p53 regulation and the diverse mechanisms underlying its inactivation. We will then use this knowledge to review the recent achievements in p53-based anti-cancer therapy.

The p53 protein: structure, function and regulation

Since the discovery of p53 more than 30 years ago, great progress has been made in elucidating its structure, function and regulation. The protein p53 was initially identified as an oncogene because of its high expression in tumours. Soon after its discovery, it was recognized that the sequence of tumour-derived p53 differs from that originating from normal tissue. This led to the conclusion that the normal p53 protein acts as tumour suppressor rather than an oncogene. Meanwhile, p53 has become one of the most studied tumour suppressors, reflecting its central role in counteracting cellular transformation. Interestingly, only in 1997, two p53-related genes, p63 and p73, were identified (Kaghad *et al.*, 1997; Yang *et al.*, 1998). Both proteins share homology with p53 but differ in their biological functions from p53. In certain conditions, however, also p63 and p73 can function as tumour suppressors.

The p53 protein is a transcription factor that specifically binds to target sequences as a tetramer (Harms and Chen, 2006). The wt-p53 protein consists of 393 amino acids (aa) that constitute five functional domains, including two N-terminal transactivation domains (TAD1: aa 1–42, TAD2: aa 43–63) that are followed by a proline-rich domain (aa 64–92), the central DNA-binding domain (DBD: aa 102–292), the C-terminal tetramerization domain (aa 326–356) and basic domain (aa 364–393). Although studies on p53 mainly focus on the canonical α-isoform (herein called wt-p53), at least 10 different isoforms have been described (Bourdon *et al.*, 2005; Schwerk and Schulze-Osthoff, 2005). The N-terminally truncated Δ40p53 isoforms lack most of the first TAD and are generated by alternative splicing of intron 2 or alternative initiation of translation. The further truncated Δ133p53 isoforms arise from the usage of an alternative promoter in intron 4. The ΔN-isoforms have been studied in some detail and mostly negatively modulate wt-p53 (Bourdon *et al.*, 2005). Alternative splicing also produces C-terminal isoforms that lack the tetramerization domain and instead possess 10 or 15 alternative amino acids. Data on the function of these isoforms are scarce. Until now, p53β has been proposed to augment apoptosis induction by wt-p53 (Bourdon *et al.*, 2005) and was recently implicated in the modulation of cellular senescence of premalignant cells (Fujita *et al.*, 2009). In contrast, p53β and p53γ did not significantly impact on wt-p53-induced apoptosis or senescence upon DNA damage in cancer cell lines (Graupner *et al.*, 2009). In line with the latter results, the dominant-negative function of the N-terminally truncated and tetramerization-proficient isoform Δ133p53α on wt-p53 is not conserved in Δ133p53β and Δ133p53γ that lack the tetramerization domain (Jänicke *et al.*, 2009). Further research will reveal how the isoforms p53β and p53γ impact on wt-p53. This could eventually open up new ways to employ p53 isoforms for diagnostic and therapeutic applications.

Mutation of p53 occurs in about 50% of human tumours, and most of these mutations are located in the DBD (Walker *et al.*, 1999), influencing the specific binding of p53 to target sequences. The most common mutations concentrate on only a few nucleotides. Several of these hot spot mutations result in the exchange of amino acids that make contact with the DNA (Figure 1). Therefore, exchange of these amino acids ablates or weakens binding of p53 to DNA or alters sequence specificity of the mutant p53. Altered sequence specificity in turn can even result in mutant p53 with oncogenic functions, thus turning the tumour suppressor to an oncogene. Mutations that cause structural destabilization of the DBD may not only result in its inactivation but also in deregulation of p53 by interfering with protein–protein interaction. Interestingly,

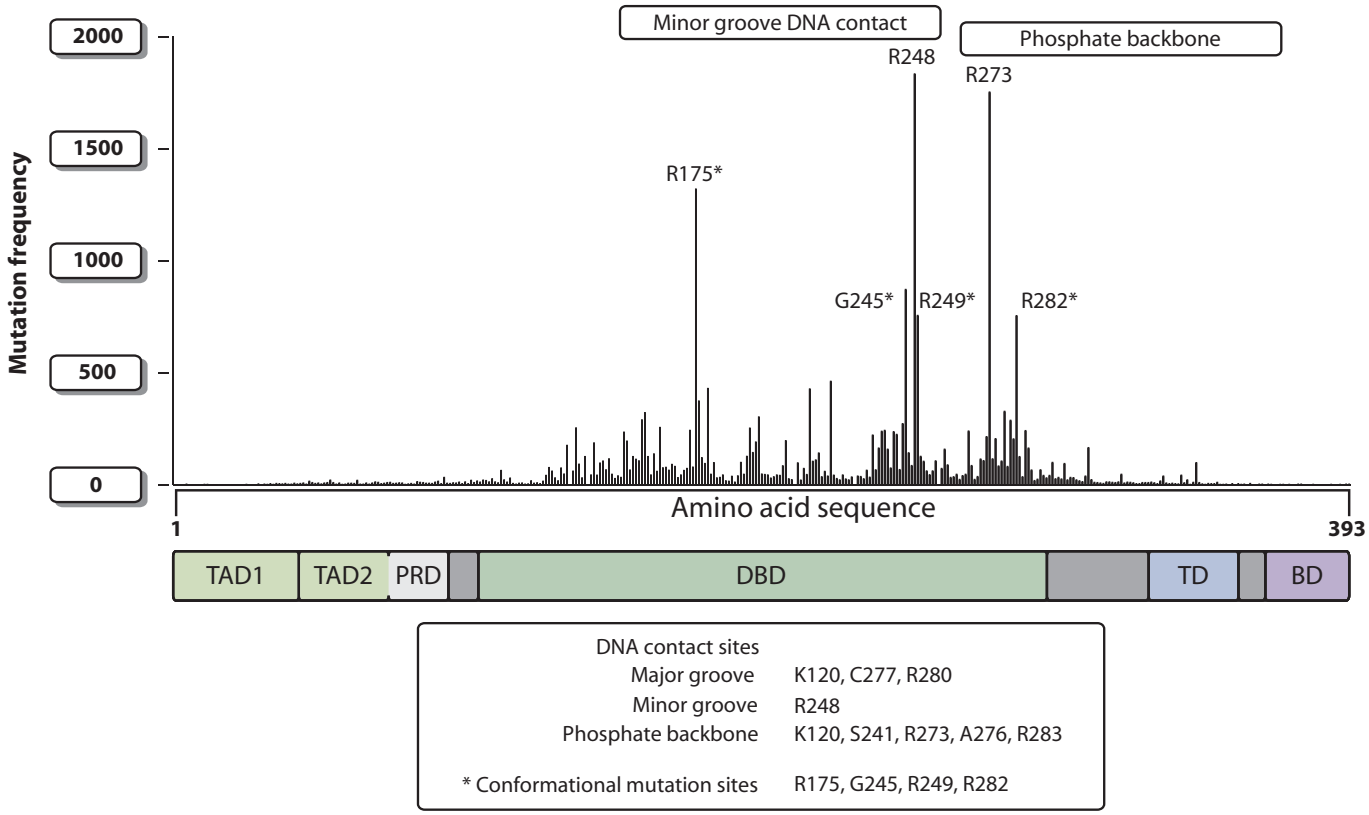


Figure 1

Spectrum of p53 mutations in human cancers. Mutation data from the curated UMD TP53 mutation database (http://p53.free.fr/database) including 25 905 human tumours were processed, and the mutation frequency of each amino acid in p53 is depicted. Specific hot spot mutations, the location of the mutation within the p53 domain structure and their impact on p53 are indicated. Point mutations can be categorized in contact and structural mutations. Contact mutations are located at the interface with the major and minor grooves of DNA, whereas structural mutations mostly result in destabilization of p53. TAD: transactivation domain, PRD: proline-rich domain, DBD: DNA-binding domain, TD: tetramerization domain, BD: basic domain.

mutations that interfere with p53 oligomerization as well as deletion of p53 are not common in human cancers. It is assumed that, once an inactivating mutation in one p53 allele has occurred, the resulting protein efficiently blocks the activity of the wt-p53 expressed from the second allele. Thus, diverse mechanisms exist that impede a proper p53 response, and hence, also diverse strategies have been developed to overcome them.

Not only the function but also the proper regulation of p53 abundance are premises for the initiation of an accurate p53 response. In unstressed cells, the expression level of p53 is kept low by a constant proteasomal degradation of p53 (Figure 2). The mark for proteasomal degradation (i.e. poly-ubiquitylation) is attached to C-terminal lysine residues of p53 by the E3 ligase MDM2 (Toledo and Wahl, 2006). Transfer of the ubiquitin moiety from an upstream E2 ligase depends on the RING (really interesting new gene) domain of MDM2 (Itahana *et al.*, 2007). Interestingly, although MDM2 is necessary and sufficient for ubiquitylation of p53, its homologue MDMX is required for efficient p53 regulation. MDMX also contains a RING domain but does itself not show significant ubiquitin ligase activity towards p53 (Linares *et al.*, 2003). Nevertheless, MDMX forms heteromers with MDM2 and enhances MDM2-mediated ubiquitylation of p53 (Linares *et al.*, 2003). The importance of both MDM2 and MDMX is underlined by the fact that single knockout of each gene confers embryonic lethality (Toledo and Wahl, 2006). MDM2 and also MDMX are frequently over-expressed in tumours that possess wt-p53.

The abundance, subcellular localization and activity of p53 are regulated by post-translational modifications such as phosphorylation, acetylation, ubiquitylation and attachment of ubiquitin-like moieties such as SUMO and Nedd8 on certain residues mainly in the N- or C-terminal part of p53 (Toledo and Wahl, 2006). Phosphorylation primarily occurs at N-terminal Ser and Thr residues and affects p53 stability as well as the decision of whether p53 induces apoptosis or senescence. The phosphorylation at N-terminal residues influences binding of MDM2 and thereby modulates ubiquitylation of p53 at C-terminal lysine residues (Toledo and Wahl, 2006). Acetylation of these C-terminal residues also prevents ubiquitylation (Bode and Dong, 2004). Hence, both mechanisms stabilize p53 and enhance transactivation of

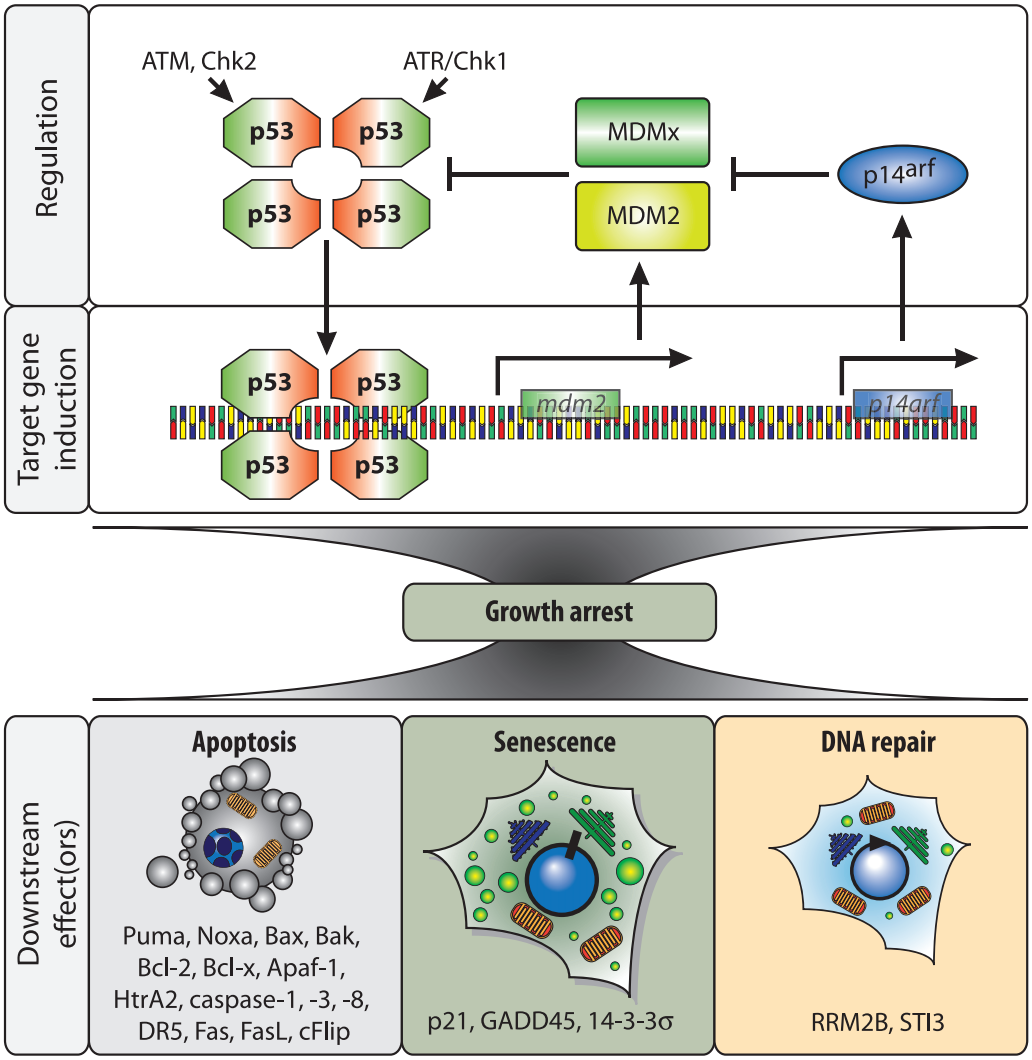


Figure 2

Tumour suppressor p53 regulation and signalling. Activation of the transcription factor p53 is predominantly mediated by stabilization, mostly due to phosphorylation by upstream kinases such as ATM/ATR or Chk1/2. As a tetramer the stabilized p53 binds to DNA consensus sequences and induces transcription of huge number of different target genes involved apoptosis, senescence or DNA repair. One of the target genes is the ubiquitin E3 ligase MDM2 that regulates the proteolytic degradation of p53 in a negative feedback loop. This negative regulation is facilitated by MDMX and attenuated by $p14^{Arf}$ that interferes with MDM2-mediated down-regulation of p53.

target genes that induce DNA repair, transient cell cycle arrest, senescence or apoptosis.

Strategies for tumours expressing mutant p53

As outlined above, inactivation of p53 most frequently results from mutation. In order to enable a p53 response in cells harbouring mutant p53, two basically different routes have been followed that both aim to restore p53 functions in tumour cells. These strategies include either the ectopic expression of functional wt-53 or, more specifically, the reactivation of mutant p53 in tumour cells. The rationale to re-express wt-p53 in tumours with mutant p53 is based on the observation that p53-deficient cells undergo apoptosis or senescence in response to wt-p53 expression. Although re-expression of wt-p53 in tumour cells can enable tumour control, one has to keep in mind that expression of mutant p53 is not exactly the same as the absence of p53. This is especially true in cases of p53 mutations within the DBD, which affect protein folding and sequence-specific DNA binding but do not necessarily interfere with oligomerization of p53. Transgenically expressed functional wt-p53 may form oligomers that incorporate endogenous mutant p53, thereby dampening or inactivating the p53 response (Liu *et al.*, 2000). Therefore, the expression level of wt-p53 supposedly needs to be higher than that of mutant p53 in order to overcome the inactivating effect of the endogenous mutant p53 (Chan

et al., 2004). Corroborating this assumption, wt-p53 suppresses tumour growth in a mouse model harbouring a p53 missense mutation (Wang *et al.*, 2011), whereas full tumour regression was seen in a p53-negative tumour model (Ventura *et al.*, 2007).

As p53 inactivation is common in human cancers, it can be assumed that reactivation of p53 is detrimental for established tumour entities. Indeed, substantial data have been accumulated, showing that inactivation of p53 promotes immortalization of cells in culture (Carnero *et al.*, 2000). Genetic knockout of p53 in mice results in a high frequency of cancer, especially lymphomas and sarcomas. To study the effect of re-expression of p53 in tumours, genetic animal models have been developed in which p53 expression is blocked either by a removable stop codon in the p53 gene locus or by suppressible RNA interference of p53 (Xue *et al.*, 2007). As anticipated, in the absence of p53, these animals like p53-null mice develop spontaneous tumours with a high frequency at early age. Interestingly, when endogenous p53 is genetically reintroduced in both models, p53 expression results in regression of the established tumours without side effects on normal tissue, thus proving that induction of p53 in p53-negative tumours is a valid anti-cancer strategy. Hence, several therapeutic strategies aim at expressing functional wt-p53 in tumours with a disturbed p53 pathway.

Gene therapeutic and viral approaches

Several companies and institutions have evaluated the restoration of p53 function by gene therapeutic approaches. Delivery of a functional wt-p53 gene into tumour cells by injection of an engineered retrovirus has been tested in a clinical trial for the treatment of patients with non-small cell lung carcinoma (Roth *et al.*, 1996). Unspecific infection of normal cells is a minor concern because normal cells recover from p53 expression, whereas especially cancer cells are sensitive to functional p53 (Lane *et al.*, 2010). As a vehicle for the delivery of functional p53 retroviral vectors have first been employed (Roth *et al.*, 1996). Although retrovirus integration into the host genome is desirable, it is considered a risk factor for tumorigenesis. In fact, retroviral transduction can transform cells and itself cause tumour development (Bishop, 1982). In contrast, adenoviruses do not integrate into the host genome, can be produced in GMP quality in large quantities and, unlike retroviruses, infect also non-proliferating cells. Consequently, several clinical trials have been conducted with replication-deficient adenoviruses that mediate the expression of functional wt-p53 in target cells. Despite remarkable examples of therapeutic efficacy in patients with Li-Fraumeni syndrome, an autosomal-dominant mutation of the p53 gene, the Food and Drug Administration (FDA) did not approve Advexin, the western version of a wt-p53-encoding adenovirus developed by Introgen Therapeutics. Nevertheless, the equivalent Gendicine, in combination with radiotherapy, is being applied in the treatment of head and neck cancer in China since several years and also shows efficacy in the combination treatment of hepatocellular carcinoma (Shi and Zheng, 2009; Yang *et al.*, 2010).

In contrast to gene therapy, oncolytic virotherapy relies on a different concept and aims to design viruses that specifically lyse tumour cells (Crompton and Kirn, 2007). The specific lysis of tumour cells is thought to be achieved by basically different strategies. One method is the deletion of p53-inhibitory proteins from the viral genome. These modified viruses still effectively replicate in p53-defective cells, whereas normal wt-p53-expressing cells mount an antiviral response. Alternatively, the adenoviral genome is modified in a way that viral proteins that are crucial for replication are expressed under the control of oncogenes (Bazan-Peregrino *et al.*, 2008). This results in effective viral replication in oncogene-expressing transformed cells and their lysis, whereas replication in normal cells is abrogated because the viral proteins necessary for replication are not expressed. In the adenovirus Onyx-015 (Onyx Pharmaceuticals), the p53-inhibitory protein E1B has been deleted, resulting in the specific replication in tumour cells with an inactivated p53 pathway (Bischoff *et al.*, 1996; Heise *et al.*, 1997). The therapeutic potential of Onyx-015 in combination with cisplatin and 5-FU has been investigated in phase II/III clinical trials for patients with recurrent squamous cell carcinomas. The success of this and similar approaches is, however, limited by the as yet low efficiencies of gene delivery. Furthermore, systemic application of adenoviral vectors is associated with hepatotoxicity. Thus, despite extensive clinical trials, Onyx-015 has not achieved FDA approval.

Small molecules rescuing mutant p53 function

The greatly increased knowledge about the structure and regulation of p53 has instigated highly sophisticated approaches to re-activate mutant p53. To this end, compounds have been developed that specifically act on mutant-p53 and restore wt-p53 function of the mutant protein (Table 1). Naturally, due to the diversity of p53 mutations, these compounds are relatively specific for a certain mutation and in turn mostly ineffective in tumours expressing a different p53 mutant. Most remarkable among small molecules for the rescue of mutant p53 are compounds such as PRIMA-1, CP31398 and PhiKan083 that have been proven effective in pre-clinical trials.

In a functional screen for compounds that specifically inhibit the growth of tumour cells with mutant p53, PRIMA-1 (p53 reactivation and induction of massive apoptosis; CAS 5608-24-2) was identified and later optimized to yield PRIMA-1^{Met} (Bykov *et al.*, 2002; 2005a). PRIMA-1 restored DNA binding not only of the R273H mutant but also of p53 versions with other mutations in the DNA-binding domain, except for the mutation C176F. This finding implicated that cysteine modification is involved in the mechanism of PRIMA-1 action. Indeed, the PRIMA-1 decomposition product methylene quinuclidinone can induce a covalent thiol modification in p53 (Lambert *et al.*, 2009). Also, other p53-reactivating molecules, such as MIRA-3 (Bykov *et al.*, 2005b) and STIMA-1 (Zache *et al.*, 2008), both of which are unsuitable for *in vivo* studies, have the potential to alkylate cysteine residues. Because the PRIMA-1 decomposition product as well as MIRA-3 and STIMA-1 are α β-unsaturated compounds, they can act as Michael acceptors (Lambert *et al.*, 2009). Alkylation of mutant p53 by another Michael acceptor, 3-benzoyl-acrylic acid, was recently analysed in detail and shown to stabilize p53 (Kaar *et al.*, 2010). Incubation of p53 mutant cells with PRIMA-1 enhanced binding of mutant p53 to the promoters of pro-apoptotic Bax and Puma, thereby

Table 1

Compounds targeting tumours with mutant p53

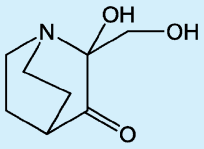
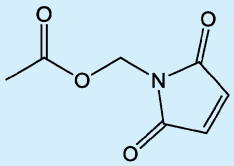
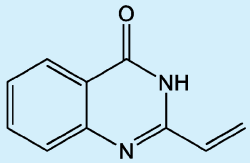
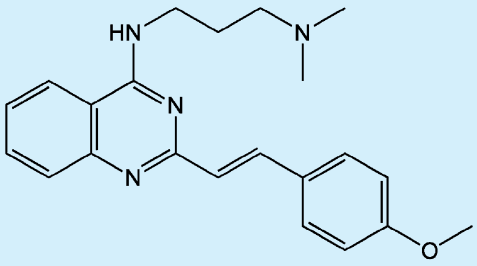
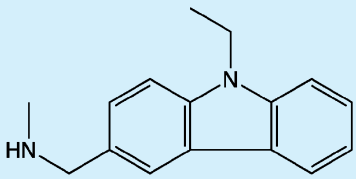
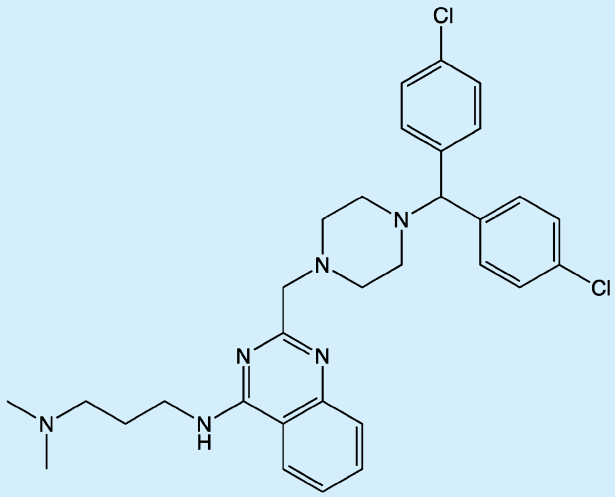
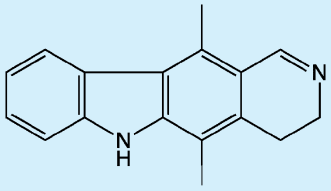
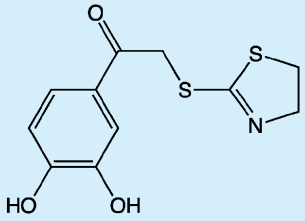
Compound	Structure	CAS No., Reference
PRIMA-1		5608-24-2 Bykov *et al.*, 2005a
Mira-3		7450-68-2 Bykov *et al.*, 2005b
STIMA-1		91634-12-7 Zache *et al.*, 2008
CP31398		259199-65-0 Foster *et al.*, 1999
PhiKan083		880813-36-5 Boeckler *et al.*, 2008
SCH529074		922150-11-6 Demma *et al.*, 2004

Table 1

Continued

Compound	Structure	CAS No., Reference
Ellipticine		519-23-3 Shi *et al.*, 1998
RETRA		1036069-26-7 Kravchenko *et al.*, 2008

The chemical compounds that have been developed for the therapy of human cancers expressing mutant p53. The table includes the chemical abstract service number (CAS; http://www.cas.org) of the compound and the relevant reference.

activating the mitochondrial apoptosis pathway (Shen *et al.*, 2008). PRIMA-1 and PRIMA-1^{Met} inhibit the growth of various transplanted human tumours and synergize with cisplatin-induced apoptosis in xenograft models (Bykov *et al.*, 2002; 2005a). Due to this potent anti-tumour activity in animal models, PRIMA-1 has entered phase I clinical trials.

Based on the finding that mutation of the p53 DBD is associated with destabilization, Foster *et al.* (1999) searched a library of >100 000 chemical compounds for their ability to stabilize the DBD of p53. Stabilization of the DBD was evaluated by binding of the pAb1620 antibody that is specific for the intact conformation of p53. In this screen CP31398 (CAS 259199-65-0) was identified and shown to increase DNA binding of both wt and mutant p53. Notably, CP31398 significantly up-regulated the cell cycle inhibitor p21 in cells expressing mutant p53 (Foster *et al.*, 1999). The stabilization of p53 by CP31398 was independent of MDM2 and proteasomal degradation (Wang *et al.*, 2003) and associated with enhanced p53 DNA binding (Demma *et al.*, 2004). However, NMR studies failed to detect a direct interaction of CP31398 with p53 (Rippin *et al.*, 2002). Because CP31398 contains a reactive group similar to PRIMA-1, it might also stabilize p53 by alkylation of cysteine residues (Lambert *et al.*, 2009). In APC$^{min/+}$ mice that spontaneously develop intestinal tumours CP31398 induced elevated p53 levels, expression of p21 and apoptosis (Rao *et al.*, 2008). Reduced tumour incidence was also reported in a rat model of azoxymethane-induced colon carcinoma (Rao *et al.*, 2009). Furthermore, CP31398 was shown to be effective in the treatment of non-melanoma skin cancers and even reduced UV-induced photocarcinogenesis (Tang *et al.*, 2007). Apoptosis induction by CP31398 was reported in different cell lines regardless of their p53 status (Takimoto *et al.*, 2002; Wischhusen *et al.*, 2003). Hence, in addition to restoring the active conformation of p53, CP-31398 presumably possesses additional activities that affect tumour cells.

The crystal structure of a series of p53 mutants formed the basis for an *in silico* screen performed by the Fersht group (Boeckler *et al.*, 2008). Several p53 mutants reduce the melting temperature of p53, leading to its rapid degradation. The Y220C mutation, which is among the 10 most frequent p53 mutations (Petitjean *et al.*, 2007), destabilizes the p53 protein and creates a crevice on the p53 surface that is opposite to the DBD (Joerger *et al.*, 2006). Virtual screening and rational drug design with more than 2.5 million compound structures were applied to identify molecules that could interact with the crevice created by Y220C mutation. The serial application of sophisticated filters resulted in 421 hit compounds. From these 80 were selected and analysed for their potential to increase the melting temperature of the p53 core domain. One of them, the carbazole PhiKan059 (CAS 880813-36-5) bound to the Y220C-induced cleft and stabilized the mutant p53 protein. Further optimization resulted in the identification of PhiKan083 (1-(9-ethyl-9*H*-carbazole-3-yl)-*N*-methylmethanamine), which bound to the oncogenic Y220C mutant with reasonable affinity. Although biological activities of PhiKan083 or related compounds have not yet been disclosed, the Y220C mutation might constitute an interesting target for p53-stabilizing drugs.

By the use of recombinantly expressed DBD from the R273H p53 mutant in a DNA-binding assay, the piperazinyl-quinazoline SCH529074 (CAS 922150-11-6) was found to enhance DNA binding activity of mutant p53 (Demma *et al.*, 2004). This small molecule reactivates mutant p53 presumably by acting as a chaperone and can restore DNA-binding activity to mutant p53. In addition to reactivating mutant p53, SCH529074 inhibited ubiquitylation of p53 by MDM2. Furthermore, SCH529074 triggered apoptosis in different tumour cell lines and reduced tumour growth in a xenotransplant model (Demma *et al.*, 2010).

The alkaloid ellipticine (CAS 519-23-3) was identified by screening the NCI60 panel of tumour cell lines for

compounds that showed cytotoxic or anti-proliferative activity on a subset of cell lines harbouring mutant p53 (Shi *et al.*, 1998). Subsequently, the analogue 9-hydroxy-ellipticine was shown to induce expression of the p53 target genes Bax and p21 (Sugikawa *et al.*, 1999). Incubation of $p53^{R175H}$ expressing tumour cells with ellipticine was associated with reporter gene induction, enhanced binding of the wt-conformation-specific pAb1620 antibody and increased binding of mutant p53 to the MDM2 promoter (Peng *et al.*, 2003). However, ellipticine was also shown to act by DNA intercalation and by inhibiting topoisomerase II (Auclair, 1987; Froelich-Ammon *et al.*, 1995; Stiborova *et al.*, 2001), suggesting that several mechanisms could account for its anti-tumour effects.

Kravchenko *et al.* (2008) screened a drug library for induction of p53-dependent reporter gene activity in A431 cells bearing a p53 mutation (R273H) in the DBD. Using this approach, the small molecule RETRA (reactivation of transcriptional reporter activity, CAS 1036069-26-7) was selected and shown to cause tumour regression in mouse xenograft models. At low micromolar concentrations, RETRA specifically induced p53-like activity in cells with mutant but not wt p53. Although its exact mechanism of action is unclear, RETRA presumably targets not p53 itself but its homologue p73. RETRA is assumed to release p73 from an inhibitory complex with mutant p53. Thus, these data demonstrate that targeting of other p53 family members might be another promising strategy for treating tumours with mutant p53.

Strategies for tumours expressing wt-p53

Attenuation of p53 expression due to excessive degradation can arise from over-expression of p53-inhibitory proteins. In animal models, the over-expression of MDM2 has been shown to increase tumour formation, whereas attenuated MDM2 expression resulted in reduced tumour formation (Mendrysa *et al.*, 2006). Two mechanisms have been identified that are causative for enhanced expression of MDM2, namely a single nucleotide polymorphism at nucleotide 309 (SNP309, T-to-G) in the promoter region of MDM2 (Bond *et al.*, 2004) and amplification of the MDM2 gene (Oliner *et al.*, 1992; Momand *et al.*, 1998). SNP309 causes enhanced binding of the transcriptional activator Sp1 (Bond *et al.*, 2004) and is associated with enhanced cancer risk. In line, mice carrying the G-allele of SNP309 are more tumour-prone (Post *et al.*, 2010). Another single nucleotide polymorphism in the MDM2 promoter, SNP285C (G-to-C), has the opposite effect and almost reverses the effect of SNP309G (Knappskog *et al.*, 2011). Also the oestrogen receptor mediates transcription of MDM2 via the region in the MDM2 promoter that contains SNP309 (Kinyamu and Archer, 2003). Consequently, earlier diagnosis of cancer (e.g. invasive ductal breast cancer, colorectal cancer or soft tissue sarcoma) correlates with the presence of SNP309 G-allele in female individuals (Atwal *et al.*, 2008). Regardless of the underlying mechanisms that lead to elevated expression of MDM2, the activation of the p53 pathway is attenuated. The increased MDM2-mediated proteasomal degradation prevents sufficient induction of a p53 response by transactivation of p53 target genes.

Degradation of p53 is mediated by the proteasome, while inhibition of the proteasome enhances the expression level of p53. This in part mediates the therapeutic activity of the proteasome inhibitor PS-341 (bortezomib, Velcade) that is used in the treatment of multiple melanoma (Yang *et al.*, 2009). More specific strategies, however, are intended to act further upstream by preventing ubiquitylation of p53, thereby blocking its nuclear export and proteasomal degradation. The basis for this strategy was laid by the detailed elucidation of the amino acids of p53 interacting with MDM2 (Picksley *et al.*, 1994). X-ray crystallography showed that the p53:MDM2 interaction does not involve large surface regions (Kussie *et al.*, 1996), as it is often the case for protein-protein interactions. Rather four hydrophobic key residues (Phe^{19}, Leu^{22}, Trp^{23}, Leu^{26}) of an amphipatic α-helix in the N-terminus of p53 reach into cavities on the surface of MDM2 and constitute the p53:MDM2 interface.

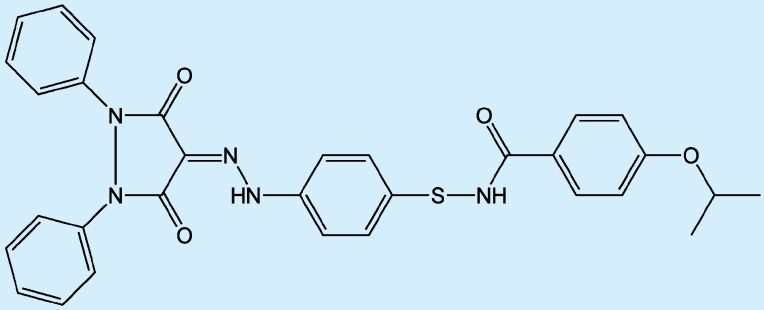
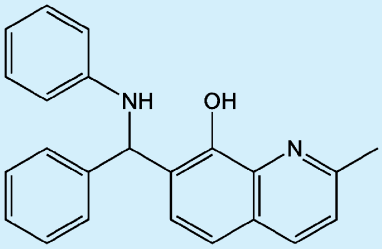
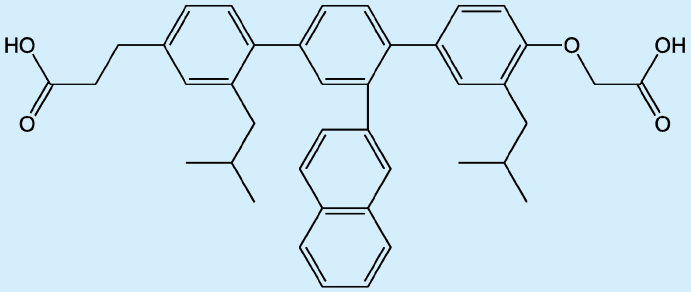
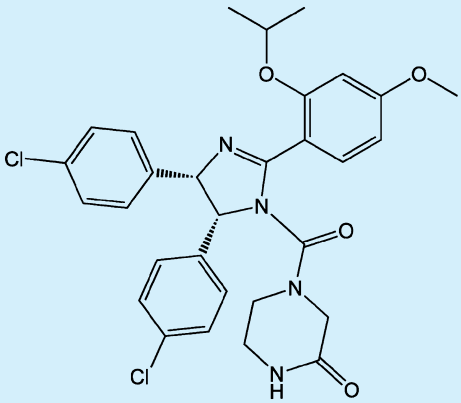
The small binding interface as well as the characteristics of the involved amino acids of p53 indicated that the p53:MDM2 interaction could be a suitable drug target. This assumption was supported by experiments showing that a 6-mer peptide of p53 reflecting the amino acid sequence (^{18}TFSDLW^{23}) specifically binds to MDM2 (Picksley *et al.*, 1994). Phage display experiments and screening of peptide libraries identified an identical peptide sequence to interfere with the p53:MDM2 interaction (Bottger *et al.*, 1996). Functional analyses confirmed the ability of the p53 peptide to induce elevated levels of p53 and activation of a p53 response (Bottger *et al.*, 1997). However, the molecular properties of peptides generally prevent their efficient application as drugs due to poor cell permeability, bioavailability and stability. Bernal *et al.* (2007) therefore stabilized the active peptides by generating so-called stapled peptides. Stapled peptides contain non-natural amino acids that allow the formation of inter-peptide bonds resulting in increased resistance to degradation. Analysis of these stapled peptides showed that also the cell permeability is enhanced as compared with natural peptides. The stapled peptides retained MDM2 binding and induced increased levels of p53 in cultured cells. Instigated by these promising results, further structure-based design as well as screening of drug libraries finally culminated in the identification of small molecule drugs that specifically interfered with the p53:MDM2 interaction and at the same time demonstrated sufficient stability, bioavailability and cell permeability. The identified small molecules belong to different classes of compounds, such as sulphonamides, quiloninoles, terphenyls, *cis*-imidazolines (nutlins), benzodiazepinediones, spiro-oxindoles, pyrrolidine-2-ones and isoindolinones (Table 2). The compounds with the highest specificity and lowest K_i values are benzodiazepinediones, spiro-oxindoles and the nutlins.

MDM2 inhibitors of the sulphonamide, quinolinole and terphenyl classes

A first step in the design of small molecule inhibitors targeting the p53:MDM2 interaction was the identification of sulphonamide I (NSC279287, CAS 59541-35-4) in the NCBI database using a pharmacophore model. In a subsequent MDM2 binding assay, sulphonamide I showed an IC_{50} of approximately 32 μM as compared with 13 μM for the p53

Table 2

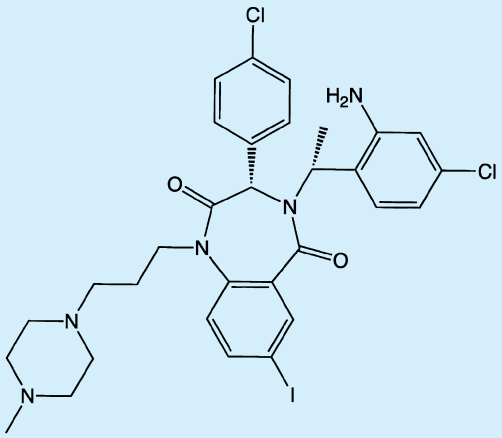
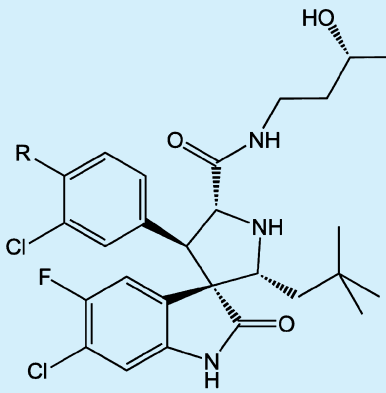
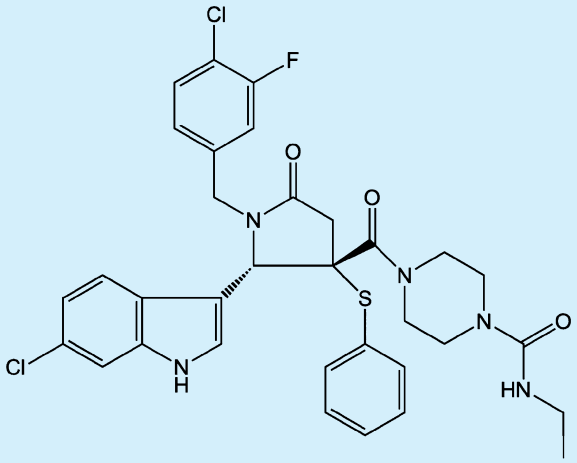
Compounds targeting tumours with wild-type p53

Compound	Structure	CAS No. Reference
Sulfonamide I		59541-35-4 Galatin and Abraham, 2004
NSC66811		6964-62-1 Lu *et al.*, 2006
Terphenyl compound 14		1239876-90-4 Yin *et al.*, 2005
Nutlin-3a		675576-98-4 Vassilev *et al.*, 2004

peptide ([16]QETFSDLWKLLP[27]). The efficacy of NSC279287 to induce a p53 response was verified in a p53 reporter gene assay using MDM2-over-expressing osteosarcoma cells (Galatin and Abraham, 2004). Another combined approach that used computational database screening of a subset of the NCI database together with a structure-based screening identified 354 potential MDM2 inhibitors. In a fluorescence-based binding assay, the quinolinole NSC66811 (CAS 6964-62-1)

Table 2

Continued

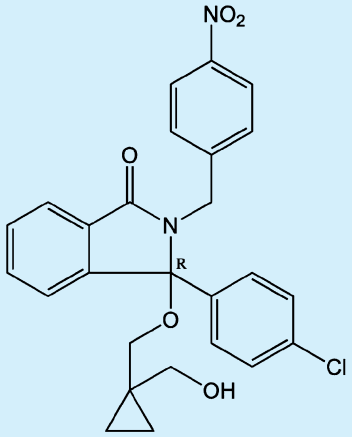
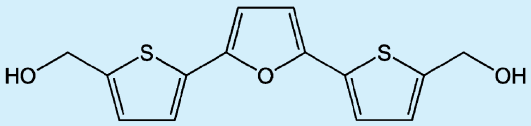
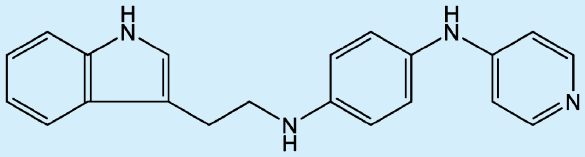
Compound	Structure	CAS No. Reference
TDP665759		787632-66-0 Koblish *et al.*, 2006
MI-219 (R = H) MI-319 (R = F)		1201143-87-4 1015232-46-8 Mohammad *et al.*, 2009
PXN822		1216938-86-1 Priaxon AG

was identified and shown to inhibit MDM2 with a significantly lower K_i (120 nM) than the natural p53 peptide (Lu *et al.*, 2006).

Terphenyl compounds that inhibit the p53:MDM2 interaction by occupying the binding pocket in MDM2 have been identified by screening a small terphenyl library (Yin *et al.*, 2005). Terphenyl 14, carrying two *iso*-butyl and one naphtyl moiety that reach in the hydrophobic groove on MDM2, showed the lowest K_i (182 nM) in a fluorescence polarization-based binding assay. Chen *et al.* (2005) also found that the

Table 2

Continued

Compound	Structure	CAS No. Reference
Isoindolinone compound 74a		1202803-74-4 Hardcastle *et al.*, 2011
RITA		213261-59-7 Issaeva *et al.*, 2004
JNJ26852165 (Serdemetan)		881202-45-5 Arts *et al.*, 2008

The chemical compounds that have been developed for the therapy of human cancers expressing wild-type p53. The table includes the chemical abstract service number (CAS; http://www.cas.org) of the compound and the relevant reference.

linear terphenyl structure is a suitable scaffold for hydrophobic moieties that reach into the p53-binding pocket of MDM2. Compounds 1 and 6 of their set of terphenyl compounds induced p53-dependent reporter gene expression and triggered p53 and p21 protein expression in HCT116 cells.

Nutlins

So far, non-peptidic small molecule drugs like the so-called nutlins, developed by Hoffmann La Roche and named after their research site in Nutley (NJ), belong to the most promising MDM2 inhibitors. These imidazoline compounds bind into the p53 pocket of MDM2 and increase p53 activity with nanomolar potency. The three reported compounds nutlin-1, -2 and -3 differ slightly in their substituting moieties but share a common core structure. The active enantiomers of the nutlins were shown to displace p53 from MDM2 with IC_{50} values of 90 to 260 nM (Vassilev *et al.*, 2004). Furthermore, X-ray crystallography verified that nutlins bind to the p53-binding pocket of MDM2 by mimicking the key p53 amino acids. Cell culture experiments proved that nutlins induce p53 stabilization, induction of p21 target gene expression as well as cell cycle arrest or apoptosis (Vassilev *et al.*, 2004). The anti-proliferative and pro-apoptotic function of nutlin-3 has been verified in several tumour cell lines as well as in primary samples from acute myeloid leukaemia and neuroblastoma (Kojima *et al.*, 2005; Tovar *et al.*, 2006; Van Maerken *et al.*, 2009). Finally, the anti-tumour activity of nutlin-3 has been shown to be comparable with doxorubicin in a xenograft model of SJSA-1 osteosarcoma cells without apparent side effects (Vassilev *et al.*, 2004). All these experiments revealed that the efficacy of nutlins depends on the presence of wt-p53, whereas no cytotoxic effect is observed in samples with mutant p53. Interestingly, induction of apoptosis by nutlin-3 seems to correlate with MDM2 gene amplification. In addition, nutlin-3 synergistically enhances the anti-tumour activity of various chemotherapeutic drugs, suggesting that nutlins might be a promising agent together with existing therapies. An oral formulation of nutlin-3 (RO50455337) has entered early clinical trials for the treatment of patients with solid tumours and haematological malignancies.

Benzodiazepinediones and spiro-oxindoles

MDM2 inhibitors that belong to the class of benzodiazepinediones were discovered by high-throughput screening

of chemical libraries using an assay that detects the influence of inhibitor binding on protein stability. Parks *et al.* (2005) reported IC_{50} values of 420 nM and 490 nM for the compounds 20 and 44 respectively. The crystal structure of the active enantiomer of compound 20 (i.e. compound 1) confirmed an interaction with the p53-binding pocket of MDM2, similar to the p53 peptide. Compound 1 stabilized wt-p53 and induced p21 expression and growth inhibition in tumour cells (Grasberger *et al.*, 2005). The pharmacokinetic properties of the lead compound 1 were optimized by the introduction of a pentanoic acid (Parks *et al.*, 2006) or a 3-(4-methylpiperazin-1-yl)propyl moiety, resulting in TDP521252 and TDP665759 (Koblish *et al.*, 2006). Both compounds are active as MDM2 inhibitors, and TDP665759 (CAS 548472-68-0) synergizes with doxorubicine treatment in a xenograft model (Koblish *et al.*, 2006).

Spiro-oxindoles were originally developed by a structure-based *de novo* design approach. As mentioned above, the MDM2 interaction is mediated by four key hydrophobic residues of p53, including most importantly Trp^{23} as well as Phe^{19}, Leu^{22} and Leu^{26}. The drug design started with a search for chemical moieties that mimic the binding of Trp^{23} to MDM2 and identified that oxindole could closely match the interaction made by Trp^{23} (Ding *et al.*, 2005). This finding inspired the search for natural oxindole-containing compounds resulting in the selection of spiro-oxindole–pyrrolidine as a structural scaffold. In a subsequent optimization procedure, additional hydrophobic moieties were added, and the final compound 1d was shown to bind to MDM2 with a K_i of 86 nM. The potency of compound 1d is underlined by inhibition of cell growth in wt-p53 expressing cells with an IC_{50} of 830 nM (Ding *et al.*, 2005). However, due to a weaker binding than the p53 peptide, the spiro-oxindole 1d was further optimized. To better mimic the Leu^{22} and Glu^{17} interaction of p53 with MDM2, an additional fluor atom was introduced into the Phe^{19}-mimicking chlorophenyl ring (Ding *et al.*, 2006). The resulting MI-63 had an improved K_i value of 3 nM, an IC_{50} of 280 nM and induced p53 and p21 expression in prostate carcinoma and rhabdomyosarcoma cells but was still unsuitable for *in vivo* evaluations (Canner *et al.*, 2009). MI-43, in which the Cl in the chlorophenyl moiety was exchanged for a Br atom, activated a p53 response in wt-p53 expressing lung cancer cell lines and synergized with etoposide-induced apoptosis (Sun *et al.*, 2008). Improvement of pharmacological properties of MI-63 by the exchange of the 4-ethyl-morpholino group for a butanediolyl moiety and addition of a fluor atom produced MI-219. This compound was shown to inhibit tumour growth in mouse xenograft models without being toxic to normal tissue (Shangary *et al.*, 2008). Another analogue, MI-319, which like MI-63 contains a fluor atom in the Phe^{19}-mimicking chlorophenyl moiety in combination with the butanediolyl moiety, was tested site by site with MI-219 and nutlin-3. All three compounds demonstrated efficient growth inhibition of wt-p53-expressing primary lymphoma cells and p53-dependent induction of apoptosis (Mohammad *et al.*, 2009). To date, the MI-series of compounds, generated by Ascenta and the University of Michigan, belong to the most potent tryptophan-based inhibitors, which bind to Mdm2 with remarkable affinity.

Other MDM2 inhibitors

In the PXN series of MDM2 inhibitors a pyrrolidine-2-one serves as scaffold to attach hydrophobic moieties that block the p53-binding pocket of MDM2. Remarkably, the developing company (Priaxon, Munich, Germany; http://www.priaxon.de) reports PXN727 and PXN822 to show a lower binding K_d than MI-219 and nutlin-3. Furthermore, PXN822 induced p53 target gene expression of p21 and Puma in ovarian teratocarcinoma cells, accompanied by subsequent apoptosis. PXN822 showed inhibition of tumour growth in the LNCaP mouse xenograft model after oral administration.

Isoindolinones have also been tested as a scaffold for MDM2 inhibitors. The initially identified isoindolinones showed IC_{50} values of 5–15 μM in growth inhibition and p53-dependent reporter gene assays (Hardcastle *et al.*, 2006). Further optimization resulted in identification of the active isoindolinone enantiomer 74a, which potently induced stabilization of p53 and p21 protein expression in SJSA-1 cells. The IC_{50} of compound 74a was 170 nM as compared with 61 nM for nutlin-3 in an MDM2-p53 binding assay and similar for both compounds in growth inhibition assays (Hardcastle *et al.*, 2011). As with other MDM2 inhibitors, the cytotoxic activity of isoindolinones appears to be strictly dependent on wt-p53.

MDMX inhibitors

A unifying feature of the aforementioned inhibitors is their binding to the p53-binding pocket in MDM2. The three most prominent examples of small molecule MDM2 inhibitors belong to *cis*-imidazolines (nutlin-3a), spiro-oxindoles (e.g. MI-219) and benzodiazepinediones (TDP665759). Their high specificity, however, renders them less effective in settings, where augmented p53 degradation is not caused by increased MDM2 expression, but by enhanced levels of MDMX. More than threefold increased levels of MDMX expression have been found in almost 20% of colon, breast and lung cancers (Danovi *et al.*, 2004). Furthermore, an amplification of the MDMX gene is found in about 65% of retinoblastoma (Laurie *et al.*, 2006), indicating that MDMX is an interesting therapeutic target. Heterodimerization of MDMX with MDM2 stimulates ubiquitin ligase activity of MDM2, although MDMX does show little intrinsic E3 ligase activity towards p53 *in vitro* (Hock and Vousden, 2010). The p53-binding pockets of MDMX and MDM2 are structurally different and MDM2 inhibitors such as nutlins only weakly interact with the p53-binding pocket of MDMX (Popowicz *et al.*, 2007). Interestingly, high-throughput screening of drug libraries recently identified SJ-172550 (CAS 431979-47-4) as a lead compound that interferes with p53:MDMX interaction and showed p53-dependent cytotoxic effects in tumour cell lines (Reed *et al.*, 2010). Also, the benzofuroxan XI-006 (NSC207895; CAS 58131-57-0) was shown to activate the p53 pathway by modulating MDMX, albeit in this case by inhibiting MDMX transcription (Wang *et al.*, 2010).

Compounds directly interacting with p53

Another interesting drug is RITA, a furan derivative identified from the NCI diversity set of compounds (Issaeva *et al.*, 2004). RITA (reactivation of p53 and induction of tumour cell apoptosis, CAS 213261-59-7) was selected for its ability to kill

HCT116 colon cancer cells with wt-p53 more efficiently than p53-deficient HCT116 cells. In addition, this compound was shown to inhibit colon cancer growth *in vivo*. As mechanism for the stabilization of p53, it was proposed that RITA binds to p53 rather than MDM2 and induces a conformational change in p53 that prevents docking of MDM2 (Issaeva *et al.*, 2004). This mechanism was challenged to some degree because RITA was not found to interfere with p53:MDM2 binding *in vitro* (Krajewski *et al.*, 2005). Whether binding to p53 is the only mechanism by which RITA increases p53 activity in cells is a matter of debate. RITA is known to inhibit the expression of survival molecules and to trigger a DNA damage response.

The tryptamine derivative JNJ-26854165 (Serdemetan, CAS 881202-45-5) has been shown to block the proteasomal degradation of p53 (Arts *et al.*, 2008). JNJ-26854165 reduces proliferation of colon, lung, breast, ovarian and prostate cancer cell lines. Rapid induction of apoptosis was seen in wt-p53-expressing leukaemia cells lines, but at later time points also in cell lines expressing mutant-p53 (Kojima *et al.*, 2010). An anti-tumour activity of JNJ-26854165 was also established in non-small cell lung, breast, colon and glioblastoma tumour xenograft models (Arts *et al.*, 2008). Due to its broad anti-tumour activity, JNJ-26854165 is being assessed in a phase I trial as an oral agent for advanced solid tumours.

p53 not one of a kind

Since the discovery of p53 in 1979, substantial progress has been made in understanding the function and regulation of this tumour suppressor. Indeed, not many other proteins can compete with p53's fame in cancer science, which is reflected by an enormous number of publications about the tumour suppressor that has been also dubbed the 'guardian of the genome'. The vast amount of information has instigated diverse strategies to restore the function of the p53 pathway in cancer. Still, in stark contrast to the knowledge about p53 biology is its translation into effective anti-cancer therapies. Although more than 150 clinical trials involving p53 are listed in the NCI clinical trial database, two-thirds of them are concerned with classification of the p53 status in tumour patients (Cheok *et al.*, 2011). Of course, classification of tumour entities is indispensable given the variable reasons for dysfunctional p53 signalling and the high selectivity of drugs developed for certain defects of the p53 pathway.

Because efforts to exploit p53 in cancer therapies have not yet really paid off, some researchers are now looking to p73 and p63 as alternative targets for tumour treatment. Although p63 and p73 do not share several relevant activities with p53, at least in certain conditions both proteins can act as tumour suppressors. P53, p63 and p73 show overlapping specificity regarding their DNA-binding elements and also induce the expression of the pro-apoptotic p53 target genes Puma and Noxa (Lozano and Zambetti, 2005; Rocco *et al.*, 2006). Similarly, expression of the pro-apoptotic protein Bak is induced not only by p53 but also by p73 (Graupner *et al.*, 2011).

Hence, screening for small molecules that activate p73 in the presence of defective p53 signalling is an interesting approach in the search for alternative cancer therapeutics. There is evidence that p53-targeting drugs can also affect p73. For instance, despite the selectivity of MDM2 inhibitors for wt-p53, recent reports suggest that nutlin-3 can induce apoptosis in some p53-deficient tumour cells. Furthermore, nutlin-3 stabilizes p73 in p53-deficient cells and transactivates target genes that partially overlap with p53. Another example is the above mentioned compound RETRA, which was originally discovered as a small molecule that may reactivate p53-like functions in cells bearing mutations in p53. Although the exact molecular mechanisms remain unclear, it is meanwhile believed that RETRA can release p73 from an inactive complex with mutant p53 (Kravchenko *et al.*, 2008). Thus, at least in certain conditions, targeting of p73 or p63 might be an interesting approach to interfere with alternative tumour suppressor pathways.

Future prospects of p53-targeted cancer therapies

The importance of a dysfunctional p53 pathway as a cause for the initiation, progression and therapy resistance of cancer has become increasingly evident in the past decade. Many promising candidate compounds have been developed that restore or activate p53-dependent apoptosis in tumours. As p53 also influences metastatic behaviour and angiogenesis, p53-based therapies might provide additional therapeutic benefits. Targeting the p53:MDM2 interaction using small molecules to reactivate a p53 response represents an attractive therapeutic strategy for the treatment of cancers with wt-p53. Moreover, intense research has been performed to reactivate mutant p53. Many new drugs are currently being designed, and most of them will certainly remain in the preclinical state. However, many compounds bring the hope that, with sufficient modification by tools of structural biology and combinatorial chemistry, it might be possible to derive sufficiently potent drugs to induce apoptosis. A number of small molecule drugs have already progressed to advanced preclinical development or early-phase clinical trials. Although convincing data has partially been presented on their cancer selectivity, side effects on normal tissues have to be carefully examined. Considerable evidence supports the view that tumour cells are more apoptosis-prone than normal cells because of their aberrant genotypes and chromosomal instability. Eventually, the success of p53-based therapies will depend on the identification of suitable tumour entities and biological contexts. Exploiting the various mechanisms at which the p53 pathway can be targeted offers hope that p53-targeting tumour treatment may be not far from realization. Indeed, drug combinations involving traditional genotoxic agents have been found to be synergistic in inducing p53 accumulation and activation of apoptosis. P53-based therapies will certainly provide useful tools in combating cancer, either as single agents or more likely in combination with classical therapeutic regimens.

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Conflict of interest

The authors state no conflict of interest.

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