The p53-Mdm2 Pathway: Targets for the Development of New Anticancer Therapeutics

Daniella I. Zheleva, David P. Lane and Peter M. Fischer*

Cyclacel Limited, James Lindsay Place, Dundee DD1 5JJ, Scotland, UK

Abstract. The tumour suppressor p53 is at the centre of a network of regulatory pathways that guard over the continued integrity of the living cell and its progeny after exposure to different forms of stress, particularly those capable of inducing DNA damage. Tumour cells very frequently circumvent this control by disabling the function of p53, or other proteins in the p53 network, through mutation. Here we review the different therapeutic strategies that have been adopted to exploit common neoplastic aberrations in the p53 pathways. We emphasise in particular those approaches where modulation with pharmaceutical agents has already shown some promise, including pharmacological rescue of mutant p53, modulation of the protein-protein interaction between p53 and one of its negative regulators, Mdm2, as well as interference with downstream targets.

Keywords: p53, Mdm2, anticancer drug, tumour suppressor, oncogene

1. INTRODUCTION

Human cancer can be viewed as a disease characterised by loss of cell-cycle control and increased genetic instability. Two classes of genes, oncogenes and tumour suppressors, play a major role in tumour formation, growth, and progression. Activation of proto-oncogenes that promote cell proliferation, in combination with the inactivation of tumour suppressor genes that inhibit cell proliferation by means of cell cycle arrest and induction of apoptosis, leads to malignancy and tumour progression. Mutational inactivation of the p53 gene product is one of the most common genetic events that occur in human cancers, highlighting the central role of p53 as a tumour suppressor [1,2]. The p53 protein acts as a transcription factor that binds to as many as 300 different promotor elements in the genome, broadly altering patterns of specific gene expression. The role of p53 as a coordinator of the DNA damage-induced cell cycle checkpoint pathway prevents the propagation of permanently damaged cells [3]. The p53 gene thus plays a critical role as guardian of the genome in preventing human neoplasia [4]. Over half of all human tumours contain mutations in p53 that inactivate its function. Germ-line mutations are responsible for the majority of cases of the familial Li-Fraumeni cancer syndrome; individuals inheriting only a single active allele of the gene are very tumour-prone due to the dominant negative effect of mutant p53. Furthermore, transgenic mice in which the p53 locus has been inactivated are extraordinarily cancer-prone [5]. Perhaps most striking is the high frequency of p53 mutations that occur somatically. Numerous mutations have been discovered, and more than half of human tumours completely lack functional p53 through point mutation of one allele and complete loss of the other allele. This state is often associated with the high-level accumulation of the mutant p53 protein in the nucleus of the tumour cells [6].

*Address correspondence to this author at Cyclacel Limited, James Lindsay Place, Dundee DD1 5JJ, Scotland, UK; Tel: +44 (0)1382 206062; Fax: +44 (0)1382 206067; e-mail: pfischer@cyclacel.com

In the 22 years since the first identification of p53 as a host protein that bound the SV40 virus large T-antigen, investigation of the p53 gene and the p53 protein has become a major focus in cancer research [7]. The first clinical applications of this enormous body of work are now becoming apparent and provide a powerful illustration of both the difficulty and excitement of using genetic knowledge of cancer to derive new treatments [6]. In the past decade, the genetic and biochemical analysis of the p53 pathway that leads from cellular stress to cell cycle arrest and apoptosis has identified many targets for therapeutic intervention. It has also led to a growing realisation that the toxicity and efficacy of many of the current treatments are also profoundly affected by the activity of the p53 pathway. Thus most cytotoxic drugs induce the p53 response in normal tissues, hence contributing to their toxicity, whereas tumours that retain the normal p53 gene function are in many cases more responsive [6].

2. THE p53 TUMOUR SUPPRESSOR PROTEIN

2.1 Structure and Function

The human p53 gene encodes a 393-residue phosphoprotein that functions as a tetrameric nuclear transcription factor to activate genes involved in cell cycle arrest and apoptosis in response to various forms of cellular stress (reviewed in [8]) such as DNA damage [9], faulty spindle formation [10], depletion of oxygen [11], ribonucleotide depletion [12], transcriptional abnormalities [13], and teratogens [14]. Studies with p53 knock-out animals have shown that this pathway is a key component of the normal apoptotic response to ionising radiation and, in the absence of p53, animals become very prone to the development of neoplasias.

The biochemical activity of p53 most tightly linked to its biological function as a damage-responsive effector

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protein involves its ability to bind to DNA sequence-specifically [15] and to function as a transcription factor [16]. There are several candidate target genes possibly responsible for p53-dependent cell cycle arrest and apoptosis (reviewed in [17]). They include the gene encoding $p21^{WAF1/CIP1}$, which participates both in G1 and G2 arrests by inhibition of CDKs, the GADD45 gene involved in DNA repair, and the gene encoding 14-3-3σ, which causes G2 arrest by sequestration of the mitotic Cdc25C phosphatase [18-20]. The Bax and Fas/APO1 genes are also transactivated by p53 and are, in part, responsible for p53 pro-apoptotic activity [21]. Studying the mechanism underlying the development of p53-dependent apoptosis, Polyak *et al.* [22] suggest a three-step process: (i) the transcriptional induction of redox-related genes, (ii) the formation of reactive oxygen species, and; (iii) the oxidative degradation of mitochondrial components, culminating in cell death. The crucial factors determining whether a cell's fate will be cell cycle arrest or death are not yet completely understood. However, they probably involve the integration of a number of environmental and cellular cues [23].

p53 is composed of at least four functional domains that regulate its activity as a transcription factor: (i) an N-terminal trans-activation domain, which is required for interaction with components of the transcriptional machinery [24-26], (ii) a central conserved core DNA-binding domain containing most of the inactivating mutations found in human tumours [27-30], (iii) a tetramerisation domain that facilitates sequence-specific DNA binding [31], and (iv) a C-terminal negative regulatory domain whose phosphorylation primes the latent sequence-specific DNA-binding function of p53 for activation [32].

The stress-regulated transactivation function of p53 is co-ordinated by specific protein-protein interactions that can in turn be modulated by covalent and noncovalent modifications [33]. The N- and C-terminal regulatory domains contain heterologous protein docking sites and phosphorylation, SUMO-lation, and acetylation sites implicated in the modulation of p53 protein-protein interactions. The transactivation domain contains the binding site for one of the main negative cellular regulators of the p53 tumour suppressor protein, the product of the Mdm2 oncogene (reviewed in [34]).

2.2 Mutant p53

About 95 % of the > 10,000 somatic tumourigenic p53 mutations occur in the core DNA-binding domain [2]. The majority are single missense mutations and oncogenic p53 is therefore generally a full-length protein with a single amino acid substitution in the core domain. The most frequent cancer-associated mutations occur at the hot spots R^{175}, G^{245}, R^{248}, R^{249}, R^{273}, and R^{282}, all of which are situated in the DNA-binding core domain of p53. These mutations can be divided into two categories: (i) DNA-contact

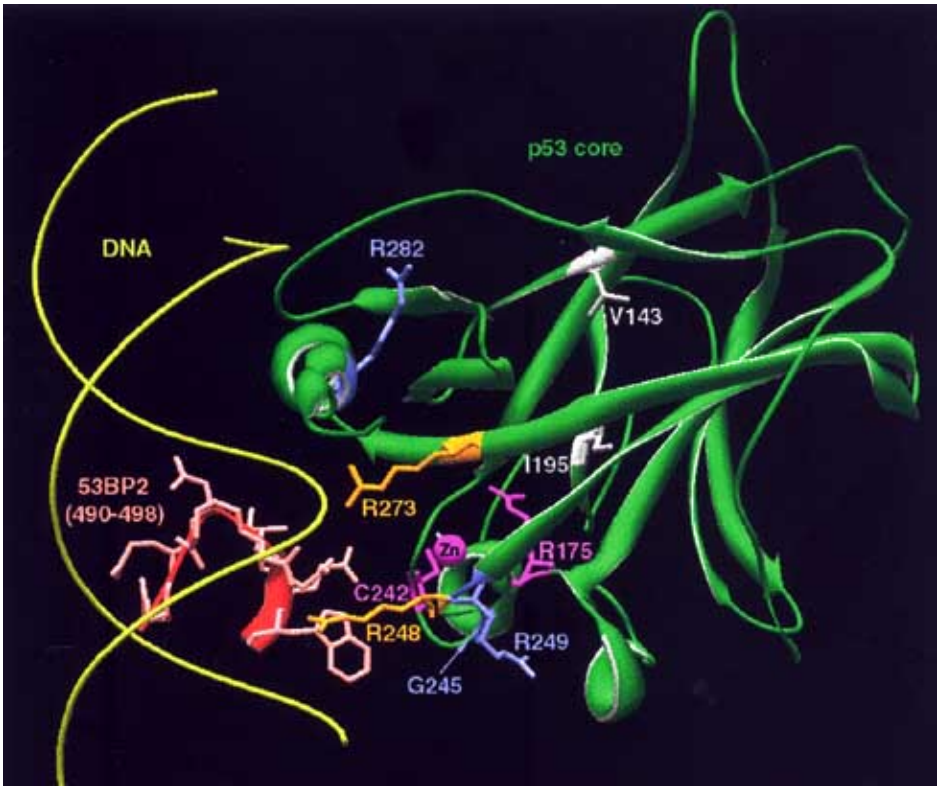


Fig. (1). Composite illustration of p53 core domain interactions with DNA (PDB code 1TSR, [36]) and 53BP2 (code 1YCS, [183]); only the sequence fragment corresponding to the p53-stabilisation peptide CDB3 [129] is shown). Positions of common p53 mutations are indicated; the colour coding is as follows: DNA contacts (orange), DNA-binding region (blue), β-sandwich (gray), and zinc region (pink).

mutations (R^{248} and R^{273}), which retain native core domain structure but lead to loss of DNA-binding residues, and (ii) mutations that result in structural changes in the p53 DNA-binding domain [35,36]. Such structural changes can occur as a result of mutations that cause a local distortion, mainly in proximity to the DNA-binding site (*e.g.* $R^{249}S$), or mutations that can cause global unfolding (*e.g.* $R^{282}W$) [37] (Fig. **1**). The majority of mutant p53 forms have lost the ability to bind consensus target DNA [15,38]. The p53 core domain has a comparatively low thermal stability (3 kcal/mol at 37 ºC, compared to 5 – 15 kcal/mol for most proteins) and zinc binding is essential for folding. Thus reduction of stability by < 3 kcal/mol through a destabilising mutation will result in protein denaturation under physiological conditions [39]. This explains the extraordinary sensitivity of the p53 gene to mutation.

The dominant-negative effect of mutant p53 stems from the facts that generally the tetramerisation domain in mutated p53 proteins is structurally intact and that upon oligomerisation, wt and mutant p53 heterotetramers assume an inactive mutant-like conformation [40]. There is evidence that any structural changes leading to reduction in the DNA-binding affinity of a p53 tetramer are sufficient for dominance [41].

3. THE Mdm2 ONCOGENE

The Mdm2 oncogene was first cloned as an amplified gene on a murine double-minute chromosome in the 3T3DM cell line, a spontaneously transformed derivative of BALB/c3T3 [42]. NIH3T3 or Rat2 cells overexpressing the gene were tumourigenic in mice, indicating that Mdm2 by itself possesses transforming activity. The Mdm2 gene can immortalise rat embryo fibroblasts and co-operates with the activated ras oncogene to transform these cells [43]. It is amplified or overexpressed in about 40 – 60 % of human osteogenic sarcomas and in about 30 % of soft tissue sarcomas [44,45], implicating it in the development of these malignancies. The human homologue of the Mdm2 gene (sometimes referred to as Hdm2) encodes a 491-amino acid polypeptide that contains a p53-binding domain, an acidic region, a zinc-finger, and a ring-finger domain.

An important function of Mdm2 is to bind to p53, inhibiting the latter's ability to act as a transcription factor [46] and stimulating its degradation through the ubiquitin pathway [47-50]. p53 also activates Mdm2 expression at the level of transcription [51,52], suggesting that Mdm2 can function as a negative feedback regulator of p53. Mouse embryos with inactivated Mdm2 alleles die shortly after implantation. However, mice carrying inactivated Mdm2 and p53 are viable [53-55], suggesting an important function of Mdm2 in down-regulating p53. In cell culture experiments, Mdm2 overexpression abrogates the ability of p53 to induce cell cycle arrest and apoptosis [56,57]. Tumour suppression by ARF^{INK4A} involves neutralisation of Mdm2-mediated inhibition of p53 [58,59].

However, Mdm2 has p53-independent functions in cell cycle control and differentiation. It interacts physically and functionally with several regulators of the cell cycle, such as pRb and E2F1 [60-62], p107 [61], as well as TAF250/CCG1 [63]. In transgenic animals and in the absence of p53, Mdm2 overexpression in the mammary gland leads to uncontrolled entry into S phase and polyploidy [64]; overexpression in the skin results in hyperproliferation and inhibition of differentiation [65]. In cell culture, Mdm2 inhibits muscle cell differentiation and MyoD-dependent transactivation [66]. Mdm2 also interacts with ribosomal protein L5 [67] and RNA [68], suggesting that it may regulate protein translation. These activities may also be responsible for or contribute to the transforming properties of Mdm2.

3.1 Mdm2 as a Target for Cancer Therapy

One of the strategies used to stabilise p53 has been to knock down the expression in tumour cells of its negative regulator, Mdm2, using antisense technology. It has been successfully demonstrated that an Mdm2-directed phosphorothioate AS-ON effectively inhibits Mdm2 expression in tumour cells containing Mdm2 amplifications [69]. Inhibition of Mdm2 expression in human cancer cells *in vitro* results in activation of p53 and induces apoptosis or cell death [70]. The ability of AS-ONs to activate p53 has been shown in cell lines containing wt p53 with various levels of Mdm2 expression. There was no effect, however, on levels of mutant p53, although Mdm2 expression is inhibited in cells with mutant p53. In addition, AS-ON gave an increase in E2F1 levels following microinjection [71].

Soft tissue sarcomas are a group of tumours that possess a very high frequency (20 – 30 %) of Mdm2 gene amplification and Mdm2 protein overexpression is correlated with poor prognosis of sarcoma patients [34]. It was shown very recently that treatment of xenografted clinically relevant soft tissue sarcomas with an Mdm2 AS-ON resulted in significant reduction of tumour growth [72]. Interestingly, this effect was observed regardless of the p53 status of the sarcomas. Despite the fact that wt p53 may be prerequisite for effective Mdm2 inhibition in certain cell types [73], there are in fact several lines of evidence suggesting that Mdm2 inhibition may be effective not only against a wt p53 background, but also when p53 is mutant [72,74] (see following section on E2F1).

Another interesting observation made with Mdm2 AS-ONs is the synergy between Mdm2 inhibition and DNA-damaging chemotherapy agents [69,75-77]. The negative regulation of p53 by Mdm2 may limit the magnitude of p53 activation by DNA-damaging agents currently used (chemotherapy and radiotherapy), thereby limiting their therapeutic effectiveness. Thus if the Mdm2 feedback inhibition of p53 is interrupted, an increase in functional p53 levels will increase the therapeutic effectiveness of such agents by restoring the wt p53 function that leads to apoptosis and/or by reversing p53-associated drug resistance

The product of the E2F1 gene, initially identified as a growth-promoting transcription factor, has recently been described as an inducer of apoptosis in different cell lines [78-82]. Moreover, E2F1 induction of apoptosis does not require functional p53, at least in human breast and ovarian

carcinoma cells [81]. The interplay between Mdm2 and E2F1 has been analysed recently in several reports. Initially, it was shown that Mdm2 promotes E2F1 transactivation through a mechanism involving a physical contact of both proteins [62]. Supporting this observation are several reports showing that overexpression of Mdm2 in cells devoid of p53 has a direct effect on E2F1 transcriptional activity and induction of apoptosis [83,84].

4. THERAPEUTIC STRATEGIES

According to our current knowledge, the status of p53 in tumour cells represents an important tumour marker and could be used as an indicator for tumour prognosis and determination of appropriate therapeutic strategies. When p53 is mutant the following strategies can be used to restore its tumor suppressor activity: (i) gene therapy with wt p53, (ii) mimicking downstream genes (*e.g.* p21, CDK inhibitors), and (iii) pharmacological rescue of mutant p53. On the other hand, when p53 is wt, efforts should be focused on p53 protein stabilisation leading to an increase in its transcriptional activity responsible for the tumour suppressor function. The therapeutic strategies in this case include: (i) disruption of the Mdm2-p53 interaction; (ii) inhibition of Mdm2 ubiquitin ligase activity; (iii) inhibition of nuclear export. As p53 mediates some of the cytotoxic effects of DNA-damaging cancer treatments (*e.g.* DNA damaging agents and ionising irradiation) the strategies used to stabilise wt p53 should result in chemo- and radiosensitisation of tumour cells when used in combination with such agents. Here we will review the drug discovery efforts made to date towards developing therapeutics designed to restore the tumour suppressor activity of p53 and/or to inhibit the oncogenic properties of Mdm2 in tumor cells.

4.1 Gene Therapy

Gene therapy, in which the normal p53 gene is re-introduced into tumour cells using either physical or viral vectors, has been evaluated extensively both in preclinical and clinical models (reviewed in [6,85-87]). In order to administer the p53 cDNA sequences, replication-defective adenoviruses driven by strong viral promotores have been used [88]. Efforts have been made to improve the vectors and to alter the p53 sequence in order to prevent the oligomerisation of the protein with its endogenous dominant-negative counterpart, so as to increase its resistance to degradation, improve the thermodynamic stability and folding of the molecule, and to increase its DNA-binding activity (reviewed in [6]). A fusion of p53 with herpesvirus VP22 protein has been described, which results in an active fusion protein that can spread to non-infected neighbouring cells [89]. A construct has been developed in which the expression of the therapeutic gene takes place exclusively in cells lacking wt p53 [90].

Not only is mutated p53 generally inactive as a transcription factor, but its ubiquitination and degradation are also impaired, leading to accumulation in cancer cells [91]. Such elevated levels of mutant p53 in neoplastic cells provide an intriguing target for the design of antiproliferative agents. An elegant approach was taken to develop an adenovirus hybrid (known as ONYX-015), which targets and kills only cells containing mutant p53 (reviewed in [92]). The adenovirus E1B gene product that normally binds to wt p53 was inactivated through mutation so that the virus cannot replicate in cells with a functional p53 protein. Preliminary *in vivo* evaluation of ONYX-015 looks promising and synergistic efficacy in combination with common anticancer treatments provides a hopeful precedent for manipulating the p53 pathway successfully in the treatment of specific cancer types.

4.2 Mimicking Downstream Genes

The first physiologically relevant gene product shown to be induced by p53 was the p21 CDK regulator that appears to mediate in part p53's tumour suppressor function [19]. p21 is a key regulator of cell division involved in mediation of negative growth signals, in differentiation and senescence, and modulation of the apoptotic response [93]. Originally p21 was identified as a CDK- and PCNA-binding protein that is able to inhibit CDK catalytic activity [94]. Structure-function analysis has shown that p21 contains at least three distinct regulatory or interaction sites that can mediate its biochemical function in cells. The N-terminal half has been shown to be sufficient for both cyclin-CDK binding and inhibition, since it contains a cyclin-binding motif and a CDK interaction site [95,96]. A second cyclin-binding motif lies at the extreme C-terminus, which is sufficient to bind and inhibit certain cyclin-CDK complexes [96-98]. Furthermore, the C-terminus also contains a region which interacts with the replication and repair protein PCNA [99,100]. The interaction of p21 with PCNA blocks the latter's ability to act as a processivity factor for DNA polymerases, modulating the primer-template recognition complex and inhibiting DNA replication in *vitro*. Although there is clear evidence that p21-PCNA complexes form in response to DNA damage, it has proven difficult to show a significant effect of p21 on DNA replication in cells mediated by its interaction with PCNA.

Although it is not necessarily evident that any one p53-dependent gene product alone can replace the tumour suppressor function of p53, it has been shown that p21 is as effective as p53 in some experimental models of cancer treatment [101]. For example, p21 gene dosage plays a role in modulating the rate of tumourigenesis in breast cancer in an $ATM^{-/-}$ background [102].

The concept of mimicking p53-downstream genes has been explored further by using short peptides derived from N-and C-terminal domains of p21. When introduced into cells through conjugation with a membrane-penetrating peptide, the p21(141-160) sequence was shown to inhibit pRb phosphorylation and to induce G1/S growth arrest [96]. Antiproliferative effects consistent with cellular CDK2 and CDK4 inhibition in human tumour cell lines have been observed by several groups using similar membrane-permeable peptides [103,104]. Furthermore, peptidomimetic conversion of such peptide leads into pharmaceutically useful leads has been initiated [97,105].

The development of small-molecule ATP-antagonistic inhibitors of CDKs as antiproliferative agents has been a widely-used approach in the search for new, nongenotoxic anticancer therapeutics. We have reviewed this subject elsewhere [106,107].

4.3 Pharmacological Rescue of Mutant p53 Function

More than half of human cancers harbour missense mutations in the p53 gene, resulting in inactivation of p53's tumour suppressor functions [2]. Such cancers are aggressive and often refractive to chemo- and radiotherapy. Mutant p53 thus represents one of the most important targets for therapeutic intervention in oncology. Two questions are central to the potential success or failure of pharmacological rescue of mutant p53. Firstly, owing to the different effects of known mutations on protein structure leading to inactivation, it appears likely *a priori* that individual mutants or groups of mutants (*e.g.* globally denatured, distorted native structure, and contact mutants) will require different approaches. The second question concerns the likelihood of small-molecule agents with pharmaceutically useful properties as drugs being able to stabilise effectively the bioactive structure of mutant p53 proteins. Progress has recently been achieved in answering both of these questions. Structure-based design [108] and genetic screening [109] methods have been applied successfully in identifying second-site suppressor mutations in p53 that can counteract numerous natural deactivating mutations. Since enhancement of overall thermodynamic core domain stability, correction of local structural perturbations, as well as addition of compensating DNA-binding residues could be achieved, in each case with some degree of generality, a good approach will be to try and mimic such suppressor mutations with pharmaceutical agents. The fact that generic solutions may be feasible is also indicated by the fact that many transcriptionally inactive p53 mutants nevertheless retain residual specific DNA-binding capability at 37 ^{o}C or at lower temperatures. Therefore, if agents can be found which bind exclusively to the native p53 structure without interfering with DNA binding, then the protein folding equilibrium should be shifted towards the unique bioactive structure, regardless of the potential multitudes of inactive mutant structures. In fact DNA binding itself is an example of this phenomenon, as it increases the apparent melting temperature of the p53 core domain by 7 ^{o}C [110].

The p53 tetramer normally adopts a latent and inactive quaternary conformation [111]. The C-terminal 30-residue regulatory domain is clearly involved in activation, since its deletion results in a constitutively active form of p53 [112]. Activation of p53 can involve a host of both N- and C-terminal modifications, as well as protein-protein interactions (reviewed in [113]). An allosteric mechanism of activation is indicated by the fact that synthetic peptides corresponding to the C-terminal p53 sequence, or antibodies recognising an epitope in that region, can activate latent p53 [111,114]. Studies using p53 variants with selected deletions showed that binding of the free p53(363-393) peptide to p53 requires the presence of both C- (363-393) and N-terminal (80-93) sequences in the p53 protein [115]. The latent state of tetrameric p53 may thus involve an architecture in which these N- and C-terminal segments associate inter- or intramolecularly [116]. It can be imagined that interruption of this association, either through post-synthetic modification or binding of molecules with affinity for either segment, will relieve the tense latent state and produce a relaxed active state (Fig. **2**). Surprisingly, it was demonstrated that synthetic peptides from the p53 C-terminus were not only capable of activating latent p53, but that they could re-activate many mutant forms of p53 both *in vitro* and when introduced into cells [117,118]. The p53 mutants amenable to such rescue encompassed both protein forms where DNA contact residues (*e.g.* $R^{273}H$, $R^{248}Q$) or structural residues (*e.g.* $R^{249}S$, $G^{245}S$) were mutated (reviewed in [113]). These findings suggest that there is a mechanistic link between activation of latent p53 and re-activation of mutant p53. However, since *e.g.* C-terminal deletion or phosphorylation does not reactivate the majority of mutant p53 forms [108,119], the situation is clearly more complicated. It has been speculated [113] that the rescue p53 C-terminal peptides may form stable bioactive complexes with mutant p53 in which the peptide participates directly in DNA binding. Very recently, however, the above allosteric model has been questioned [120], since structural analysis of full-length and C-terminally truncated p53 proteins that are latent or active for DNA-binding *in vitro* has not provided any evidence for conformational differences in the two states [121]. Alternatively, it has been suggested that the C-terminal region of p53 may bind to DNA directly, thus preventing DNA-binding by the p53 core [122,123], or interaction of the C-terminal region with another unidentified factor inhibiting p53 transcriptional activity [124].

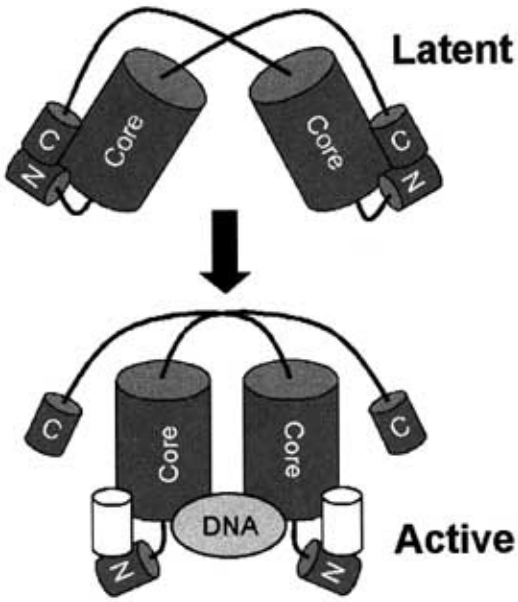


Fig. (2). Model for allosteric activation of p53. In the latent state inter- and/or intramolecular interactions involving the N- and C-terminal, as well as the core domains of the p53 monomers in the tetramer unit (dimer shown for the sake of simplicity), prevent effective and sequence-specific DNA binding. Binding of allosteric activators (white cylinders) relieve the tension of the latent state allowing the core domains to adopt relative positions permitting DNA binding and thus transactivation. (Adapted from [113]).

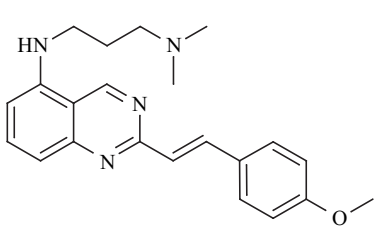
Using antibodies that can discriminate between native and denatured forms of p53 (pAb1620 and pAb240, respectively [125,126]) on the basis of recognising discontinuous epitopes unique to either folding state, high through-put assays have been set up to screen for nonpeptidic compounds which stabilise the temperature-sensitive active conformation of wt p53 and can restore the active conformation and function of mutant p53 [127,128] (Fig. **3**). Foster *et al.* [128] screened in excess of 100,000 compounds and apparently found multiple classes of compounds that scored in these screens. The SARs of the active compounds (*e.g.* CP-31398 & 257042 in Fig. **3**) observed were suggestive of a bidentate ligand, *i.e.* the type of compound one could imagine to stabilise a protein structure by insertion between two spatially adjacent secondary structure elements: all active compounds contained a hydrophobic tri- or tetracyclic (hetero)aromatic group and a small aliphatic or alicyclic group with an ionisable 3º amine function; these two groups were linked through a 3-C saturated alkyl chain. The prototype compound CP-31398 was capable of rescueing p53 mutants that are severely structurally compromised, *e.g.* R^{273} (DNA contact), R^{249} (DNA binding region), and R^{175} (Zn region), both *in vitro* and *in vivo*. Using a p53-null cell line transfected with mutant p53, it was shown that CP-31398 induced time-dependent accumulation of transcriptionally active p53. This result indicates that the compound binds exclusively to newly synthesised mutant p53 that has not yet lost the active conformation. Similarly the compound did not rescue heat-denatured wt p53 DNA-binding domain *in vitro*. It should be noted, however, that recent application of various biophysical measurements was unable to detect direct binding and stabilisation of p53 core domain by CP-31398 [129].

A somewhat different approach was taken by Bykov *et al.* [127], who used a cell-based proliferation assay as their screen. A cell line with a tetracycline-repressable H^{273}-mutant p53 was employed and the assay endpoint was selective test compound-mediated growth inhibition in the absence of tetracycline. The compound PRIMA-1 (Fig. **3**) was identified in this manner and was also shown to induce apoptotic cell death in a manner dependent on transcriptional transactivation by p53. Again both structural and DNA contact mutants were rescued effectively, as assessed in cells and biochemically by restoration of specific DNA-binding to mutant p53 from appropriate cell extracts. Of a total of 14 mutants tested, only one (C^{176} involved in Zn chelation mutated to Phe) was refractory to reactivation by PRIMA-1. Mechanistically PRIMA-1 appears to work differently to CP-31398: the former apparently forces accumulated misfolded mutant p53 into the active conformation, whereas the latter's effects depend on the presence of newly synthesised and still properly folded mutant p53. Antitumour activity in a xenograft model was also demonstrated with PRIMA-1.

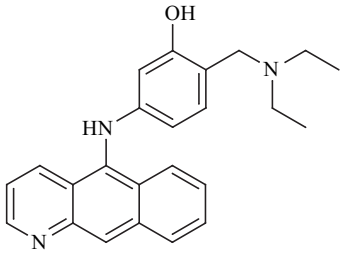
Yet another mechanism of p53 core structure stabilisation was demonstrated very recently with peptides derived from a p53-binding protein, 53BP2 [129]. The peptide ^{490}REDEDEIEW^{498} (CDB3) was shown by NMR to bind to the p53 core domain at the edge of the DNA-binding domain, presumably in a pose somewhat similar to native 53BP2 (Fig. **1**). The peptide was capable of inducing the re-folding of denatured p53 core domain, as well as restoring sequence-specific DNA binding to various p53 mutants, including the highly destabilised I^{195}T mutant. A chaperone mechanism was invoked by the authors: CDB3 may maintain existing or newly synthesised mutant p53 in a bioactive conformation, CDB3 then being exchanged for the tighter-binding cognate DNA, thus leading to an active p53-DNA complex.

4.4 Interference with p53-Specific Chaperones

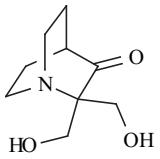
It has been shown that p53 binds to certain molecular chaperones, such as Hsp40, Hsp70, and Hsp90 [130], which are implicated in the folding of nascent polypeptide chains, in partial unfolding of polypeptide during intracellular transport, and in the repair or degradation of damaged polypeptides [131]. Chaperones are over-expressed in tumour



CP-31398

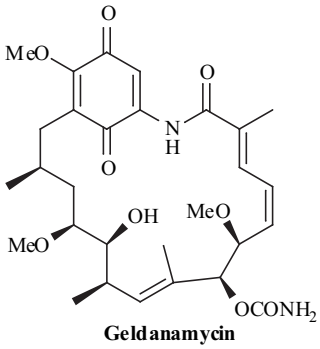


CP-257042

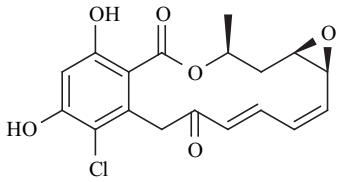


PRIMA-1

Fig. (3). Small-molecule allosteric stabilisers of the bioactive p53 structure.



Geldanamycin



Radicicol

Fig. (4). Examples of Hsp90 inhibitors.

cells and play a role in their survival, functioning as anti-apoptotic effectors in cells exposed to toxic levels of DNA-damaging agents. It has been suggested that at least in part the anti-apoptotic function of heat-shock proteins may be related to the control of the conformation and inactivation of p53 [132]. Several antitumour fungal antibiotics (Fig. **4**) have been shown to inhibit the Hsp90-dependent unfolding of p53 and these findings form a precedent for developing therapeutically relevant agents that modulate chaperone-dependent anti-apoptotic pathways [133]. The structural basis for Hsp90 inhibition with such agents is well understood [134] and this renders feasible the discovery of simpler and more selective inhibitors. In fact Hsp90-active compounds are already undergoing clinical trials [135].

4.5 Inhibition of the p53-Mdm2 Interaction

4.5.1 Peptide and Small-molecule Inhibitors

Many efforts have been devoted to the characterisation of the region of Mdm2 that binds to p53. This domain was mapped to residues 19 – 102 by co-immunoprecipitation experiments and functional assays [136,137]. The Mdm2-binding domain of p53, on the other hand, was narrowed down to a 15-residue peptide from the transactivation domain. Using synthetic peptides, the sequence ^{18}TFSDLW^{23} was identified as essential for binding to Mdm2 [138]. Mutating residues L^{14}, F^{19}, L^{22}, and W^{23} confirmed their importance for binding to Mdm2 [139]. Residues L^{22} and W^{23} are also required for transcriptional activation by p53 or binding to TAFII31 (transcription cofactor), demonstrating an overlap between the transactivation and Mdm2-binding domains [139,140]. The 3D structure of a 109-residue N-terminal domain of *Xenopus laevis* Mdm2 bound to the 15-residue transactivation domain peptide of p53 revealed that Mdm2 possesses a deep hydrophobic cleft into which the p53 peptide binds as an amphipathic α-helix [141] (Fig. **5**). Residues F^{19}, W^{23} and L^{26} of p53 insert deeply into the Mdm2 cleft, which contains 14 conserved hydrophobic and aromatic amino acids that make multiple van der Waals contacts to p53. This rather unique protein-protein interaction appears to be widely conserved during evolution [142]. MdmX, a new

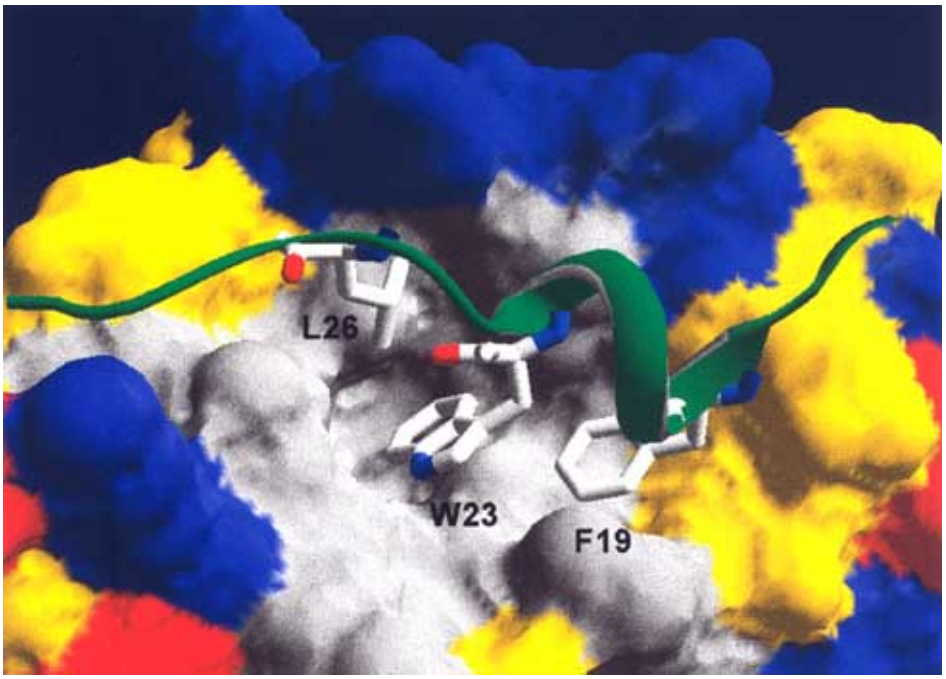


Fig. (5). Interface between p53 E^{17}-N^{29} peptide (backbone ribbon in green) and the Mdm2 hydrophobic p53-binding cleft (acidic, basic, polar, and non-polar surface shown in red, blue, yellow, and grey, respectively). The side chains of the three predominant p53 residues involved in intermolecular contacts are shown with labels.

	p53 Sequence Position	16				20					25		27	IC_{50} (μM)[a]
Peptide Number	1	Gln	Glu	Thr	Phe	Ser	Asp	Leu	Trp	Lys	Leu	Leu	Pro	8.7
	2			Thr	Phe	Ser	Asp	Leu	Trp					700
	3	Met	Pro	Arg	Phe	Met	Asp	Tyr	Trp	Glu	Gly	Leu	Asn	0.3
	4				Phe	Met	Asp	Tyr	Trp	Glu	Gly	Leu		8.9
	5				Phe	Met	Aib	Tyr	Trp	Glu	Ac_3c	Leu		2.2
	6				Phe	Met	Aib	Pmp	Trp	Glu	Ac_3C	Leu		0.3
	7				Phe	Met	Aib	Pmp	6-Cl-Trp	Glu	Ac_3c	Leu		0.005

[a]Inhibition of wild-type p53 binding to GST-Hdm2

Structure of peptide 7:

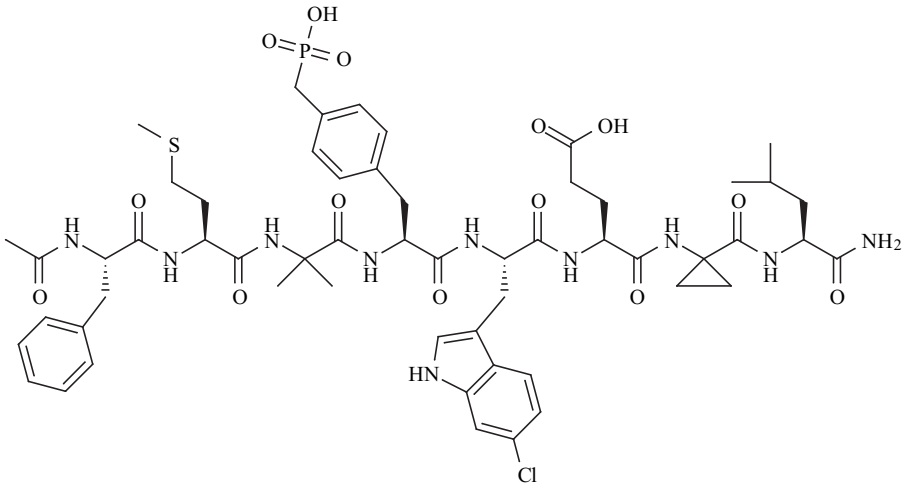


Fig. (6). p53-derived peptide inhibitors of Mdm2.

member of the Mdm2 family, also binds to p53 in a similar manner [143,144]. The molecular mass of the F^{19}, W^{23}, and L^{26} sidechains amounts to *ca.* 300 Da, suggesting that modulation of the p53-Hdm2 protein-protein interaction with drug-like small molecules may in fact be feasible.

The Mdm2-binding site on p53 was identified with the aid of a set of overlapping synthetic peptides [138] and was mapped to the sequence ^{18}TFSDLW^{23}. Although longer peptides encompassing this sequence were potent inhibitors of p53/Mdm2 complex formation, the hexapeptide p53(18-23) itself had little affinity [145]. Screening of phage-displayed peptide libraries also revealed sequences containing the Mdm2-binding motif [146]. Here the starting 12mer peptide MPRFMDYWEGLN (Fig. **6**) had submicromolar affinity and was 28-fold more potent than the corresponding wt p53-derived peptide ^{16}QETFSDLWKLLF^{27}. Substitution and truncation studies revealed that the 8mer peptide FMDYWEGL was the minimal active sequence retaining micromolar affinity for Mdm2 [145]. Based on the known binding mode of the corresponding p53 sequence [141], the helical structure of this peptide was stabilised by introduction of α,α-disubstituted amino acid residues α-aminoisobutyric (Aib) acid and 1-aminocyclopropanecarboxylic acid (Ac_3c) in place of the Asp and Gly residues, respectively. Molecular modelling suggested proximity of the Tyr side chain to the ε-amino group of the Mdm2 Lys^{94} residue and a phosphonomethylphenylalanine (Pmp) residue was used to replace Tyr. The resulting peptide was about 7-fold more potent, suggesting that the hypothetical stabilising salt bridge between the phosphonate and amino groups was in fact operating. Finally, inspection of the binding pocket for Trp^{23} showed incomplete occupancy, suggesting substituents at the indole 6-position would improve binding. This was the case and substantial potency gain was obtained. Thus starting with the wild-type p53 12mer sequence the affinity was increased by > 1,700-fold [147].

Small molecule inhibitors of the p53-Hdm2 interaction have also been discovered. It has been shown that certain chalcones (1,3-diphenyl-2-propen-1-ones), compounds that have long been known to possess antitumor activitities [148], *e.g.* compound B-1 (Fig. **7**), inhibit the interaction between p53 and Mdm2 *in vitro* with high micromolar potency [149]. Using multidimensional NMR techniques [150], evidence for direct binding of the chalcone derivatives to the W^{23} binding pocket subsite of the p53 binding cleft of Hdm2 (involving residues L^{54}, G^{58}, Y^{67}, F^{91}, S^{92}, and V^{93}) was presented. Another class of small-molecule inhibitors of the p53-Mdm2 interaction are acyltryptophanylpiperazides [151] (Fig. **7**). They were developed as a result of peptidomimetic design starting from p53-derived peptides and antagonise the p53-Mdm2 complex with low micromolar potency. Screening microbial extracts for the presence of inhibitors of the p53-Hdm2 interaction, a fungal metabolite known as chlorofusin (Fig. **7**) was identified [152]. Although the ability of these small

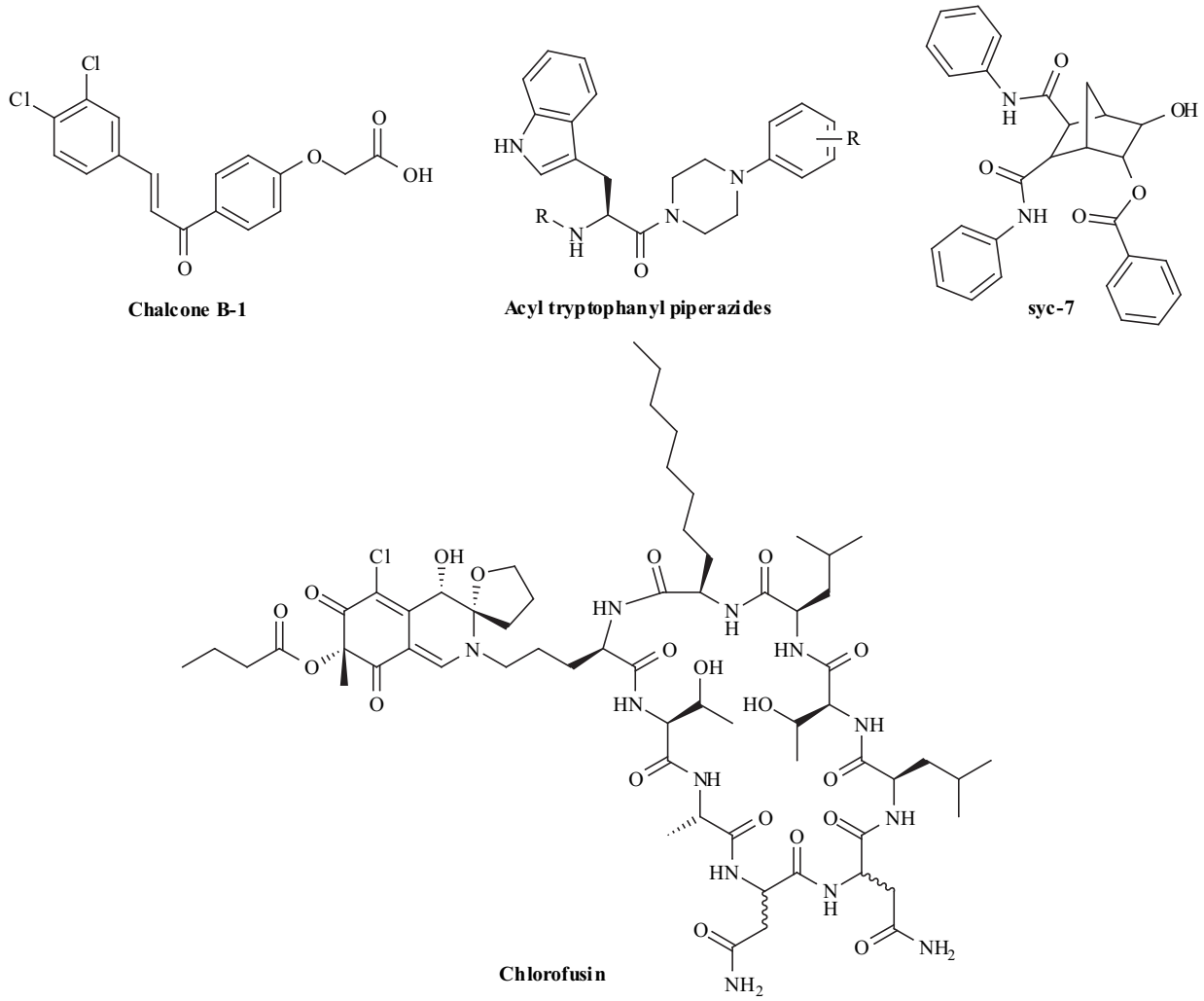


Fig. (7). Inhibitors of the p53-Mdm2 interaction.

molecules to disrupt the interaction between Hdm2 and p53 has been demonstrated and confirmed using structural methods *in vitro*, little has been reported concerning their biological activity in tumour cells. Very recently Zhao and colleagues reported small-molecule inhibitors of the p53-Mdm2 interaction, which were selected using virtual screening based on the crystal structure of a p53-Mdm2 complex [153]. One of the inhibitors, syc-7 (Fig. **7**), stimulated p53 accumulation and transcriptional activity. It induced apoptosis via caspase-3 activation in cells with wt p53. The cytotoxic effect of the compound did not, however, depend on the cellular status of p53 and /or Mdm2.

The attractiveness of p53-Mdm2 antagonism as a strategy for anticancer drug development stems in part from the detailed structural understanding of this compact protein-protein interaction [154]. The first published small-molecule inhibitors of p53-Mdm2 are evidence of the feasibility of this approach and the future will show their usefulness as anticancer therapeutics.

4.5.2 Antiproliferative Activities of Agents Disrupting the Mdm2-p53 Interaction

Inhibition of the interaction between p53 and Mdm2 in tumour cells should lead to the accumulation and activation of p53, and this in turn would induce tumour cell death by apoptosis. That this is the case has been demonstrated convincingly by several groups. Lane *et al.* [145] have shown that the expression of a modified thioredoxin protein, which displays on its surface a peptide inhibitor of the p53-Mdm2 interaction, induces p53 accumulation and activity, as well as apoptosis. Disruption of p53-Mdm2 complex formation in a transformed rat thyroid epithelial cell line (Vh1) by microinjection of an antibody to the p53-binding domain of Mdm2 (3G5) resulted in a dramatic increase in p53-dependent transcription [155]. Vh1 cells overexpress wt p53 and the Mdm2 feedback loop is the mechanism allowing the transformed cells to tolerate this phenomenon. Similarly, different tumour types, including well-differentiated thyroid carcinomas [156], testicular cancers [157], and a significant proportion of breast cancers [158,159] can tolerate overexpression of wt p53 and one could predict that they will be hypersensitive to the disruption of the p53-Hdm2 autoregulatory feedback loop [155]. Wasylyk and co-workers [160] have shown that expression of a peptide homologue of p53 that binds to Mdm2 leads to increased p53 levels and transcriptional activity. The consequences are increased expression of the downstream effectors Mdm2 and p21, inhibition of colony formation, cell cycle arrest, and cell death. There is also a decrease in E2F activity that might have been due to the

known physical and functional interactions of Mdm2 with E2F1/DP1. An octamer synthetic peptide derived from p53, which is a very potent inhibitor of the p53-Mdm2 interaction *in vitro*, and is capable of penetrating tumour cells, induces the accumulation and activation of p53 [161]. This peptidic inhibitor induces the death of cells overexpressing Mdm2 (SJSA-1 osteosarcoma) through a mechanism distinct from that of DNA-damaging agents. In all of the above-mentioned studies the cellular effects of Mdm2-p53 inhibitors were dependent on the presence of wt p53. A report from Kanovsky and collaborators, however [162], showed that three synthetic peptides from the Mdm2-binding domain of human p53 (residues 12-26, 12-20, and 17-26), linked to a cellular delivery sequence, were as effective in causing cell death in p53-null cancer cells as in those having mutant or normal p53. The same three cytotoxic peptides had no effect on the growth of normal cells, including human blood-derived cells. Peptide-induced cell death was not accompanied by expression of apoptosis-associated proteins such as Bax and p21, based on which the authors concluded that the antiproliferative effects of these p53-derived peptides are not completely dependent on p53 activity and may prove useful as general anticancer agents.

Although the Mdm2-p53 interaction offers an ideal potential target for novel cancer therapies, the therapeutic specificity may depend on the extent to which this p53-inhibitory action of Mmd2 is also required by normal cells. Transgenic data have established that Mdm2 and the related protein MdmX are needed to prevent embryonic lethality [54,55,163]. Microinjection of normal human fibroblasts with 3G5 antibody (directed against the p53-binding domain of Mdm2) induces expression of p53-responsive genes, and furthermore results in p53-dependent growth arrest [164]. This shows that normal cell proliferation can be dependent on negative regulation of p53 by Mdm2, a finding that raises an important note of caution for Mdm2-directed cancer therapies. Although activation of p53 in normal tissue will be unavoidable, there is a hope that the sensitisation of malignant cells to p53-induced apoptosis will allow selective killing of the tumour [87].

4.6 Stabilisation of p53 by Inhibition of Mdm2 Ubiquitin Ligase Activity

The INK4a/ARF gene locus encodes both $p16^{INK4a}$, a CDK inhibitor which promotes pRb-dependent cell cycle arrest, and the alternative reading-frame protein ARF [165]. The ARF protein is a recently discovered physiological regulator of p53. ARF is induced by oncogenes and protects against their inappropriate expression by inducing p53-dependent cell cycle arrest / apoptosis [166]. ARF targets p53 by interacting with the Mdm2 protein and prevents Mdm2-mediated degradation of p53 [58,59]. It has also been reported to inhibit the ubiquitin ligase activity of Mdm2 [167] and also to sequester Mdm2 in the nucleolus [168]. In any case the inhibition of Mdm2-mediated p53 degradation appears to be associated with a decrease in the levels of polyubiquitinated p53 [169]. Therapeutic agents that mimic the effect of ARF are an attractive approach to cancer therapy for tumours expressing wt p53. If the Mdm2 pathway were operating in a tumour cell to keep the level of p53 low, the use of an ARF mimetic agent would activate p53 in the absence of DNA damage.

Using overlapping peptides based on the human $p14^{ARF}$ sequence, Midgley *et al.* [170] mapped the Mdm2-binding site to the N-terminal 20mer sequence. A similar approach based on mouse $p19^{ARF}$ also showed the presence of an N-terminal binding site, although here it extended to the first 40 residues. In both cases an N-terminal 15mer was sufficient for Mdm2-binding. Weber *et al.* [168] came to similar conclusions, *i.e.* the presence of Mdm2-binding sites in the N-terminal 15 residues of both human and mouse ARF. The sites within ARF and Mdm2 that interact with each other were recently mapped to a resolution of 5 residues using surface plasmon resonance [171]. Consistent with earlier studies it was found that ARF interacts with Mdm2 through two short motifs present in the N-terminus. The ARF-interacting region of Mdm2 is also composed of two short sequences located in the central acidic domain, between residues 235-264 and 270-289. The novel molecular mechanism of interaction and the limited size of the protein domains involved thus provide opportunities for the peptidomimetic development of anti-cancer therapeutics [172].

4.7 Inhibition of Nuclear Export

The activities of both p53 and Mdm2 are determined in part by nuclear-cytoplasmic shuttling. Both proteins contain nuclear export sequences and co-compartmentalisation of p53 and Mdm2 is a major determinant for Mdm2-mediated degradation of p53 [173]. Furthermore, the conformational phenotype of p53 also appears to be linked to nuclear translocation [174]. The antitumour antibiotic leptomycin B [175,176] (Fig. **8**) is an inhibitor of the CRM1 export receptor [177] which is capable of killing neuroblastoma cells in a p53-dependent manner while only inducing a reversible growth arrest in normal cells (reviewed in [178]). Thus selective activation of p53 in cervical carcinoma cells, in over 90 % of which the p53 suppressor pathway is disrupted by human papillomavirus, could be achieved with leptomycin B [179]. Leptomycin B (under the name elactocin) underwent earlier clinical development on the basis of its antitumour activities but development was discontinued due to insufficient therapeutic index [180]. Given the new insights into its mode of action, a reassessment of leptomycin B as an inducer of the p53 response in tumours expressing wt p53 has been advocated, as well as a wider search for nuclear export inhibitors that specifically promote p53 function [6,178].

5. CONCLUSION

In this review we have shown how the rapidly increasing knowledge about the aberrant p53-governed pathways in transformed cells can be used to devise novel therapeutic strategies that might permit selective tumour therapy. The groundwork for the effective deployment of medicinal chemistry, aimed at translating the structure-function understanding of relevant enzymes and protein-protein interactions into effective mechanism-based medicines, is

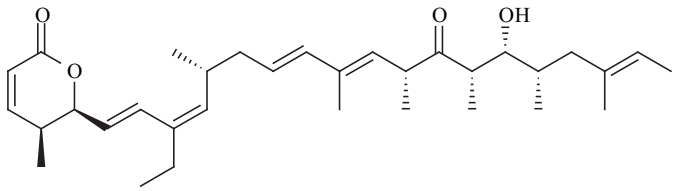


Fig. (8). Leptomycin B.

now being laid in several areas. Finally we must not omit to mention that transcriptional activation of p53-responsive genes can also present problems, particularly because many of the severe side effects limiting the therapeutic effectiveness of conventional chemo- and radiotherapy are in part due to p53-mediated apoptosis. For this reason, strategies aimed at temporary suppression of p53 are also being pursued [181,182].

ABBREVIATIONS

AS-ON	=	Antisense oligonucleotide
CDK	=	Cyclin-dependent protein kinase
PCNA	=	Proliferating cell nuclear antigen
pRb		Retinoblastoma protein
wt	=	Wild type

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