Novel Activators of the Tumour Suppressor p53

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The investigation of new possibilities for the therapy of malignant diseases has been the focus of scientific interest for many years. During the last decades numerous drugs have been discovered, and cancer treatment is not imaginable without them. They include DNA-damaging agents, mitosis inhibitors, antimetabolites, topoisomerase inhibitors and, more recently, inhibitors of signal transduction. Furthermore protein–protein interactions play a vital role in cellular metabolism and cell proliferation.[1–3] Hence, inhibitors of some carefully selected protein–protein interactions are considered as novel cytostatic agents.

The tumour-suppressor protein p53 plays a paramount role in the development of malignant tumours. Approximately half of all human tumours contain a mutated form of the corresponding gene. p53 is a transcription factor that ensures cell-cycle arrest or apoptosis when DNA damage occurs. If the function of p53 is completely disabled or even restrained, the cell lacks this important ability of cell-cycle control. This can lead to uncontrolled proliferation and ultimately to cancer.

The inactivation of p53 is often caused by a mutation of the gene itself. However, a study showed that in about 7% of examined tumour tissues, the function of p53 is compromised by an increased activity of its main negative regulator, the mouse double minute 2 (Mdm2) protein.[4] Mdm2 is characterised by E3-ubiquitin-ligase activity towards p53.[5] Depending on the degree of ubiquitination, p53 is either immediately degraded by proteasomes or transported out of the nucleus into the cytoplasm, where it is degraded proteasomally.[6] Moreover, Mdm2 binds to the transactivation site of p53 and thus inhibits its ability to activate transcription. With the aid of these mechanisms, p53 activity is maintained on low levels under unstressed conditions. Furthermore transcription of the Mdm2 gene is stimulated by p53, leading to an elevated Mdm2 level. The result is a feedback loop that adjusts the p53 concentration according to requirements.[5]

Kussie et al. first described the crystal structure of the p53–Mdm2 complex. For this purpose the p53-binding domain of Mdm2 was expressed and incubated with a 15-residue transactivation-domain peptide of p53. Kussie found that protein–protein binding primarily occurs through interaction between a deep hydrophobic cleft of Mdm2 and an amphipathic α-helix of the p53-derived peptide. The three amino acids Phe19, Trp23 and Leu26 protrude particularly deeply into the hydrophobic cleft. These very groups are involved in the transactivation; this supports the hypothesis that Mdm2 inactivates p53 by concealing the transactivation domain.[7,8]

As already shown, Mdm2 is overexpressed in many malignancies.[4] Inhibition of the interaction between Mdm2 and p53 has therefore been proposed as a novel strategy for tumour therapy.[9] Several studies have proven that the disintegration of the p53–Mdm2 interaction or the suppression of Mdm2 expression leads to activation of p53 and thus to reduced tumour growth.[10] The fact that only a limited number of amino acid residues are involved in the p53–Mdm2 interaction has given rise to speculation that small molecules should be capable of occupying this cleft and lead to an inhibition of the binding of p53 to the Mdm2 protein.

The first attempts to inhibit these interactions were performed with short peptides, derived from the primary structure of p53 and optimised with non-ribosomal amino acids. The IC_{50} values determined with an ELISA competition assay were at 5 nM for the most potent peptide (Scheme 1 A).[11]

The cyclopeptide chlorofusin (Scheme 1 B), a metabolite of *Microdochium caespitosum*,[12] was identified as an inhibitor of the Mdm2–p53 interaction in a screening of more than 53 000 microbial extracts, and showed an IC_{50} value of 4.6 μM in an in vitro assay.[13]

Recently Robinson et al. synthesised a series of protein epitope mimetics (PEMs) that showed IC_{50} values well below 1 μM.[14]

In order to overcome the known problems of peptidic drugs, such as poor bioavailability and a short half-life, the search for non-peptide inhibitors was intensified. Meanwhile, a number of small molecules that inhibit the interaction between Mdm2 and p53 have been identified. In 2001, Holak et al. presented a number of chalcone derivatives capable of blocking the Mdm2–p53 interaction (e.g., Scheme 1 C).[15] However, the IC_{50} values for these inhibitors were high, ranging from 50 to more than 250 μM. In 2002, similar results were obtained with some molecules designed and synthesised on the basis of the crystal structure of the p53–Mdm2 complex (Scheme 1 D).[16]

Recently Vassilev et al. presented a number of *cis*-imidazoline analogues with IC_{50} values for the release of recombinant p53 from the complex with Mdm2 in the range of 100 to 300 nM.[10] The authors obviously used the results of earlier works. Incorporation of 6-chlorotryptophan (corresponding to Trp23 of p53) in a synthetic heptapeptide lead to a 60-fold increase in affinity of the mentioned peptide (Scheme 1 A);[11] this suggests the use of halogen-substituted aromatic rings as a structural motif. These optimisations finally lead to the structures shown in Scheme 2 and named "Nutlins" by the authors. In fact, examination of the binding mode by X-ray structure analysis revealed that one of

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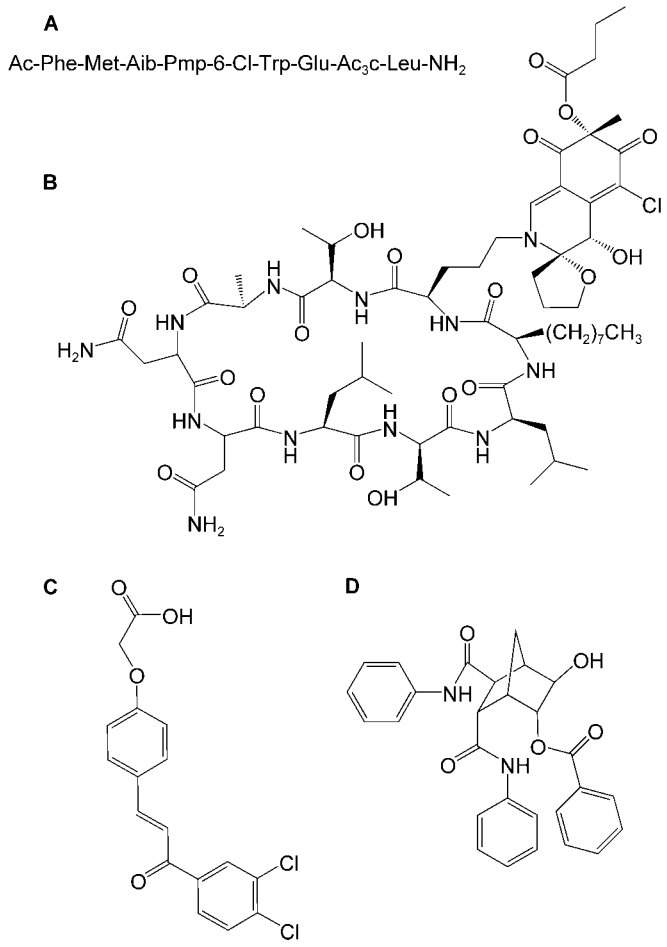
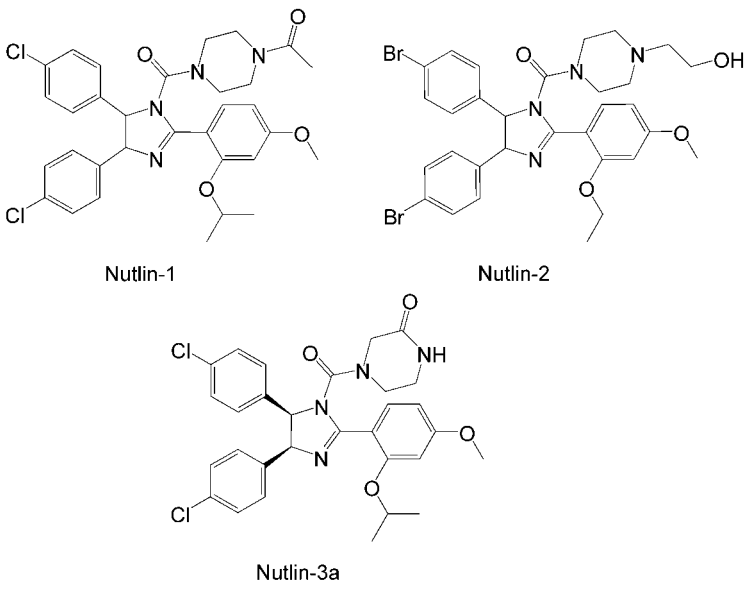


Scheme 1. *Known inhibitors of the p53–Mdm2 interaction. A) Oligopeptide containing non-ribosomal amino acids. (Aib: α-aminoisobutyric acid, Pmp: phosphonomethylphenylalanine, 6-Cl-Trp: 6-chlorotryptophan, Ac_3c: 1-amino-cyclopropanecarboxylic acid); B) chlorofusin; C) chalcone derivative; D) polycyclic derivative syc-7. See text for more details.*



Scheme 2. *Imidazoline derivatives Nutlin-1, Nutlin-2, Nutlin-3a.*

the bromophenyl rings points into the Trp23 binding pocket. The other bromophenyl ring replaces Leu26, and the ethyl ether side chain takes the position occupied by Phe19.

In the case of Nutlin-3, the racemic mixture that had been generated during the synthesis was purified by chromatography on a chiral column. One enantiomer (Nutlin-3a) turned out to be 150 times more active than the other. Nutlin-1 and Nutlin-2 were always used as racemic mixtures. All Nutlins possess *cis*-configuration.

In order to verify the supposed mode of action of the inhibitors, subsequent experiments were based on the following assumptions:

Firstly, inhibition of p53–Mdm2 binding should lead to stabilisation and accumulation of p53 as its nuclear export, and degradation should be blocked. Secondly, Mdm2 concentration should also increase because an elevated p53 level stimulates Mdm2 expression. Finally, other genes along the p53 pathway should be activated. At the cellular level, these events should lead to cell-cycle arrest in G1 or G2 phase or to apoptosis. However, when using a cell with a p53 mutant that lacks transactivation activity, none of these events should occur.

The first test was carried out to see whether the inhibition of p53–Mdm2 binding would activate the p53 pathway. For this purpose, different cancer-cell lines (possessing wild-type p53 as well as p53 that is incapable of binding DNA because of mutation or deletion) were incubated with the inhibitors. The levels of p53, Mdm2 and $p21^{Waf1/Cip1}$ were determined by Western blot. p21 is an important transcription product of p53 that arrests the cell cycle by inhibiting cyclin-dependent kinases (CDKs). As one would expect, the wild-type p53 cancer cells showed a dose-dependent increase in all three proteins after eight hours. In contrast, the cells containing inactive p53 only showed increased levels of p53, but not of Mdm2 and $p21^{Waf1/Cip1}$. The observed p53 accumulation the mutants displayed can be ascribed to the inability of inactivated p53 to upregulate the Mdm2 protein.

To affirm that the elevated p53 level is due to decreased degradation rather

than increased expression of the p53 gene, different wild-type cancer cells were treated with Nutlin-1, and the expression of p21 and its transcription activator p53 was monitored by means of real-time RT-PCR. Indeed, the concentration of p21 increased according to p53 accumulation, whereas p53 transcription itself remained unchanged. Therefore, the mechanism by which Nutlin-1 upregulates p53 is post-translational.

p53 arrests the cell cycle in the G1 as well as in the G2 phase. This is accomplished in different ways, for example, with the expression of p21. By labelling cells with BrdU it was shown that, in the case of wild-type-p53, Nutlin-1 caused cells to remain in the G1 and G2 phases, and virtually no cells reached the S or M phases. This phenomenon is not observed in cells with mutated p53. Hence, activation of the p53 pathway by Nutlin-1 is evident. Furthermore, treatment of cancer cells with Nutlin-3 caused apoptosis. Only one enantiomer was active in this respect.

The Nutlins had to be shown not to activate p53 independently from the binding to Mdm2. For this purpose, Vassilev et al. benefited from the fact that many genotoxic compounds indirectly produce Ser15 phosphorylation of p53, a residue that lies close to the binding site of Mdm2. Different cancer cells were treated with Nutlin-1 as well as with doxorubic in and etoposide, two genotoxic drugs. The subsequent Western blot of the cell lysates showed that all three substances caused accumulation of p53, however, only doxorubicin and etoposide caused phosphorylation of Ser15. Hence, the activation of p53 by Nutlin-1 apparently has no genotoxic reason.

Eventually, different in vivo tests were carried out. Human osteosarcoma cells were implanted into nude mice. Subsequent oral administration of Nutlin-3 in high doses (200 mg per kg body mass twice a day for 20 days) was well tolerated. Tumour growth decreased by 90% compared to the untreated control group. The mice did not show significant side effects. Dose-related studies were not performed. According to these studies, the Nutlins could be employed in the treatment of tumours that depend on the Mdm2–p53 system. However it remains unclear why the biological assays in the studies of Vassilev et al. were not all performed with both enantiomers of all three Nutlins or at least, as one would expect, with only one derivative. In order to examine the ability to induce apoptosis it would be helpful to use cancer cells whose apoptosis is mainly dependent on p53. The mentioned inhibitors, either alone or in combination with conventional cytostatic agents, could then be expected to selectively cause apoptosis in cancer cells.[17]

The outstanding significance of p53 in cancer development and the resulting interest is reflected by the large number of publications dealing with this matter. The p53-targeting strategies in the therapy of malignant diseases are manifold. Activating p53 by blocking the interaction with its regulator Mdm2 is an interesting approach and represents a good example of the application of inhibitors of protein–protein interactions. Just a few years ago, the mere possibility of inhibiting protein–protein interactions with small molecules was questioned. By now there are a number of such inhibitors either in clinical trials or even on the market.[3] Inhibitors for the interaction between p53 and Mdm2 have also existed for several years, although it is only now that the development of the Nutlins has provided drug-like molecules with IC_{50} values on the nanomolar scale.[18] The concept of activating p53, the most important tumour suppressor protein, by inactivating the interaction with its negative regulator Mdm2 is promising—although studies have yet to prove whether the Nutlin molecules are able to serve as valuable drugs in cancer treatment.

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