Mdm2 binds to p53 in cells in which mdm2 is not overexpressed, i.e. in cells in which mdm2 is expressed at normal or low levels, and this interaction targets p53 for degradation. The present invention exploits this mechanism of p53 degradation to stabilise a substance comprising a mdm2 binding domain linked to a coupling partner in cells in which this mdm2 mediated degradation pathway does not operate efficiently. In contrast, in normal cells expressing functional mdm2, the substance will tend to be unstable as it will be marked for degradation through the interaction of the endogenous mdm2 with the mdm2 binding domain of the substance. Accordingly, the substances can be used to deliver the coupling partner to such cells, e.g. for use in the diagnosis and/or treatment of cancer, viral infections or other conditions associated with non functional p53 or mdm2.
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Materials and Methods Relating to Stabilising Substances in Cells

Field of the Invention

The present invention relates materials and methods relating to stabilising substances in cells, and in particular in cells in which a mdm2 mediated degradation pathway does not operate efficiently, using substances comprising a mdm2 binding domain linked to a coupling partner.

Background of the Invention

The tumour suppressor protein p53 is activated upon genotoxic insult to cells and acts as a transcription factor to induce cell cycle arrest or apoptosis in cells after DNA damage. Previous reports have suggested that cell proliferation might depend on a fine balance between expression of the oncogene mdm2 and the tumour suppressor p53 (Chen et al., 1994; Finlay, 1993; Otto and Deppert, 1993). This is due to an autoregulatory feedback loop for p53 activity involving mdm2, as mdm2 is transcriptionally activated by binding of p53 to an internal promoter within the mdm2 gene (Juven et al., 1993; Wu et al., 1993). It then binds the N-terminus of p53 thereby preventing p53 from interacting with the transcriptional machinery (Momand et al., 1992; Oliner et al., 1993).

The most impressive support for the importance of this autoregulatory loop in maintaining cell survival stems from experiments with transgenic mice. Mdm2 -/- mice are only viable in the absence of p53 (Jones et al., 1995; Montes de Oca Luna et al., 1995). This argues that the level of p53 activity has to be tightly controlled in early development and that mdm2 is involved in this control pathway.

The cDNA sequence of human mdm2 is set out in WO 93/20238, which discloses that excess levels of mdm2 are present in some tumour cells such as certain types of sarcoma. This application discloses that the overexpression of mdm2 interferes with the normal feedback loop between mdm2 and p53, allowing cells overexpressing
mdm2 to escape from p53-regulated growth control by binding p53. WO 93/20238 therefore suggests that molecules that inhibit the binding of p53 to mdm2 could be used as therapeutics for conditions in which mdm2 is overexpressed by alleviating this sequestration of p53, and thereby re-establishing normal p53 control. WO 93/20238 maps the domains of p53 that are necessary for mdm2 binding to amino acid residues 13-41, as well as additional residues on either the carboxy or the amino terminal side of this peptide.

WO 96/02642 describes experiments to refine the peptide motif of p53 responsible for binding to mdm2, and shows that the motif is less extensive than disclosed in WO 93/20238. WO 96/02642 discloses that a FxxLW motif between amino acid residues 18-23 of p53 (where x is any amino acid) is sufficient to bind to mdm2. This motif can be used to screen for therapeutic compounds capable of disrupting the interaction so that the transcriptional activity of p53 in cells overexpressing mdm2 can be restored.

Using phage display, a set of peptides that act as highly potent inhibitors in p53-mdm2 binding assays have been found based that contain mutations in the above mdm2 binding motif (Böttger et al, 1996).

However, a significant drawback in pursuing therapies based on this model of the role of mdm2 is that overexpression of mdm2 only occurs a small group of sarcomas, limiting the therapeutic applications of compounds found to be capable of disrupting the binding of p53 and mdm2.

**Summary of the Invention**

The present invention is based on the finding that mdm2 binds to p53 in cells in which mdm2 is not overexpressed, i.e. in cells in which mdm2 is expressed at normal or low levels, and that in these cells, this interaction targets p53 for degradation.
More particularly, the present invention concerns materials and methods that exploit this mechanism of p53 degradation to stabilise a substance comprising a mdm2 binding domain linked to a coupling partner in cells in which this mdm2 mediated degradation pathway does not operate efficiently. Examples of this are cells which are p53 null, or contain lower than normal amounts of endogenous p53, or cells in which mutant p53 accumulates and is not marked for degradation by mdm2, e.g. because the p53 is not active for DNA specific binding and so does not induce mdm2 production. In contrast, in normal cells expressing functional mdm2, the substance will tend to be unstable as it will be marked for degradation through the interaction of the endogenous mdm2 with the mdm2 binding domain of the substance.

These results arose from experiments using highly potent peptide inhibitors of the interaction between mdm2 and p53 which were expressed as peptide aptamers on the surface of bacterial thioredoxin. Conventional peptides can have a very low potential to function in vivo because of their poor uptake and susceptibility to degradation. Accordingly, the experiments described herein are based on the construction of peptide aptamers presenting the peptide sequence of inhibitory peptides on the active site loop of Escherichia coli thioredoxin (LaVallie et al, 1993). These aptamers are characterised in mdm2 binding assays in vitro.

Microinjecting plasmids coding for these aptamers into cells containing p53 responsive reporter elements led to striking activation of the β-galactosidase reporter gene. Cells with normal low levels of mdm2 responded even more dramatically than tumour cells which have accumulated high levels of mdm2.

The most potent aptamer, TIP 12/1, showed a similar binding affinity for mdm2 as bacterial full length wt p53. This made it a powerful inhibitor which could be expressed in mammalian cells.

Accordingly, in a first aspect, the present invention
provides a substance which comprises a mdm2 binding domain linked to a coupling partner for use in a method of treatment, wherein the substance is stabilised in cells in which a mdm2 mediated degradation pathway does not operate efficiently, e.g. cells which do not express normal levels of mdm2.

In a further aspect, the present invention provides the use of a substance which comprises a mdm2 binding domain linked to a coupling partner in the preparation of a medicament wherein the substance is stabilised in cells in which a mdm2 mediated degradation pathway does not operate efficiently.

In a further aspect, the present invention provides a method of selectively stabilising a substance in cells in which a mdm2 mediated degradation pathway does not operate efficiently, the method comprising exposing the cells a substance which comprises a mdm2 binding domain linked to a coupling partner so that the substance is marked for degradation in cells containing functional mdm2. In this aspect of the invention, the method can be used to treat cells in vitro or to treat patients having conditions that respond to the coupling partner or a product derivable from the coupling partner.

In a further aspect, the present invention provides a pharmaceutical composition comprising one or more of the above substances in combination with a pharmaceutically acceptable carrier.

In a further aspect, the present invention provides a pharmaceutical composition comprising an expression vector comprising a nucleic acid sequence encoding a substance which comprises a mdm2 binding domain linked to a coupling partner, the nucleic acid being under the control of sequences to direct its expression, in combination with a pharmaceutically acceptable carrier. These compositions can be used in methods of gene therapy to deliver the substance to cells, wherein the substance is selectively stabilised in those cells in the population in which the
mdm2 mediated degradation pathway does not operate efficiently.

In a further aspect, the present invention provides an expression vector comprising a nucleic acid sequence encoding a substance which comprises a mdm2 binding domain linked to an effector, wherein the effector is a drug, a prodrug, a toxin, a label and/or a transport molecule.

Preferably, the mdm2 binding domain is or is based on one or more of the mdm2 binding domains of p53 disclosed in WO 93/20238 or WO 96/02642, or variants of these peptides which retain at least some binding affinity for mdm2. Preferably, the binding affinity of the mdm2 binding domain is substantially the same as or greater than the binding affinity of wild type p53 for mdm2. Examples of variant mdm2 binding sequences are the TIP peptides described below and in Böttger et al (1996).

The present invention allows a wide variety of coupling partners to be linked to the mdm2 binding domain including effectors, drugs, prodrugs, toxins, peptides, labels, and transport molecules. The synthesis and use of coupling partners are discussed further below.

The above methods can be used to obtain preferential stabilisation of the substance in populations of cells in which the mdm2 mediated degradation pathway does not operate efficiently. This is the case in p53 null cells in which mdm2 is not present or present at very low levels as mdm2 production is induced by p53. A further example is cells in which endogenous p53 tends to accumulate, e.g. because the p53 is mutated and is not active for DNA specific binding, and which therefore do not induce mdm2 production, or because those cells express a viral or cellular function that inactivates the transcription function of p53. In these cases, preferably the mdm2 binding domain of p53 in the substance does not include a domain of p53 associated with the DNA specific binding property of p53, so that the substance does not induce mdm2 production in the cells in which it is stable.
Brief Description of the Figures

Embodiments of the present invention will now be described by way of example with reference to the accompanying figures. Further aspects of the present invention will be apparent to those skilled in the art.

Figure 1: Schematic representation of the aptamers TIP and TIP 12/1 showing the peptide sequences inserted between G$^{33}$ and P$^{34}$ of E. coli thioredoxin. Deviations from the p53 wt sequence in TIP 12/1 are in bold with the non exchangeable amino acids underlined. The 3D structure for thioredoxin was obtained from the Protein Data Bank (PDB), Brookhaven National Laboratory and displayed using the public domain program RasMol.

Figure 2: Immunoprecipitation from cellular lysates of U2-OS, MCF-7 and OSA cells using anti-p53 Pab 421 (lanes 2, 5 and 7), anti-mdm2 Mab 4B2 (lanes 3, 6 and 8) or no antibody for controls (lanes 1 and 4). Precipitated proteins were separated by SDS PAGE and Western blotted. In (a) Western blots were stained with a mixture of anti-mdm2 monoclonal antibodies (3G5, 4B2 and SMP 14). In (b) they were stained with anti p53 rabbit antiserum CM1(1/1000). The position of mdm2 in comparison with the heavy (HC) and light chain (LC) of mouse immunoglobulins are marked on the left hand side of the blot, also the position of p53. In lanes 1-8 on both blots, aliquots of the same samples were analysed.

Figure 3: Soluble β-galactosidase assays of cell lysates transfected with RGCAlac and TIP 12/1 (black bars) or Trx (white bars) encoding DNA. The highest activity was measured in MCF-7 cells transfected with TIP 12/1 and set 100%.

Figure 4: Western blot of SAOS 2 cell lysates 48 hrs after transfecting of control plasmid (lane 1), wt p 53 alone (land 2) or together with mdm2 (lane 3) and F$^{19}$-A mutant p53 alone (lane 4) and in combination with mdm2 (lane 5).
Detailed Description

Cell types

The above methods can be used to obtain preferential stabilisation of the substance in populations of cells in which the mdm2 mediated degradation pathway does not operate efficiently. This is the case in p53 null cells in which mdm2 is not present or present at very low levels as mdm2 production is induced by p53. Another example is cells in which endogenous p53 tends to accumulate, e.g. because the p53 is mutated and is not active for DNA specific binding, and which therefore do not induce mdm2 production or because those cells express a viral or cellular function that inactivates the transcription function of p53. In these cases, preferably the mdm2 binding domain of p53 in the substance does not include a domain of p53 associated with the DNA specific binding property of p53, so that the substance does not induce mdm2 production in the cells in which it is stable.

Substances

The substances mentioned above comprise a mdm2 binding domain and one or more coupling partners.

In the present invention, "a mdm2 binding domain" means a substance that bind to mdm2 so that it is marked for degradation. Preferably, the mdm2 binding domain a peptide that is or is based on one or more p53 domains that interact with mdm2. Examples of mdm2 binding domains of p53 are disclosed in WO 93/20238 and WO 96/02642. Such peptides tend to be small molecules, preferably less than 25 amino acids, more preferably less than 20 amino acids, more preferably less than 15 amino acids, and more preferably less than 10 amino acids in length. However, in some instances, it may be preferable to employ larger polypeptides, e.g. to try and ensure that the mdm2 binding domain is displayed in a suitable conformation. The present invention also encompasses peptides which are sequence variants of mdm2 binding domains based on wild
type p53 sequence.

Variant peptides have an amino acid sequence which differs from wt p53 sequence, e.g. in the mdm2 binding motif between amino acids 13-41 described in WO96/02642, by one or more of addition, substitution, deletion and insertion of one or more amino acids, but which retains the activity of binding to mdm2. Such variants preferably include the motif FxxxW, where x is any amino acid, and will typically share at least about 70%, more preferably at least about 80%, more preferably at least about 90%, or more preferably at least about 95% amino acid sequence identity with the corresponding portion of human p53. Examples of variant mdm2 binding domains are the thioredoxin insert peptides (TIPs) disclosed in Böttger et al, 1996, and in the examples below, see especially peptide TIP 12/1.

The coupling partner can be one or more compound(s) whose delivery to the target population of cells is desired for direct or indirect diagnostic or therapeutic effect. Preferred coupling partners include (a) therapeutic reagents, such as drugs, toxins (e.g. ricin) or prodrugs which are converted to active form by reaction with a second reagent such as an enzyme, (b) diagnostic reagents such as labels or precursors of compounds that can produce a detectable result by reaction with a second reagent, and/or (c) transport molecules such as the Penetratin peptide described in WO 91/19981 to provide delivery of the substance to cells.

The skilled person can use the techniques described herein and others well known in the art to produce large amounts of the substances disclosed herein. In preferred embodiments, the substances are peptides and can be produced using recombinant techniques and/or chemically synthesised.

Thus, where the substance or a part of it is peptidyl, a convenient way of producing it is to express nucleic acid encoding it in a suitable expression system. The use of
expression system has reached an advanced degree of sophistication today.

Thus, the present invention also encompasses a method of making the substances disclosed herein, the method including expression from nucleic acid encoding the substance. This can conveniently be achieved by growing a host cell in culture, containing a vector comprising the nucleic acid under the control of sequences to direct its expression, under appropriate conditions which cause or allow expression of the peptide. Peptides may also be expressed in *in vitro* systems, such as reticulocyte lysate.

Systems for cloning and expression of a peptide in a variety of different host cells are well known. Suitable host cells include bacteria, eukaryotic cells such as mammalian and yeast, and baculovirus systems. Mammalian cell lines available in the art for expression of a heterologous peptide include Chinese hamster ovary cells, HeLa cells, baby hamster kidney cells, COS cells and many others. A common, preferred bacterial host is *E. coli*.

Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. Vectors may be plasmids, viral e.g. 'phage, or phagemid, as appropriate. For further details see, for example, Molecular Cloning: a Laboratory Manual: 2nd edition, Sambrook et al., 1989, Cold Spring Harbor Laboratory Press. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in Current Protocols in Molecular Biology, Ausubel et al. eds., John Wiley & Sons, 1992.

A still further aspect provides a method which includes introducing the nucleic acid into a host cell. The introduction, which may (particularly for *in vitro*
introduction) be generally referred to without limitation as "transformation", may employ any available technique. For eukaryotic cells, suitable techniques may include calcium phosphate transfection, DEAE-Dextran, electroporation, liposome-mediated transfection and transduction using retrovirus or other virus, e.g. vaccinia or, for insect cells, baculovirus. For bacterial cells, suitable techniques may include calcium chloride transformation, electroporation and transfection using bacteriophage. As an alternative, direct injection of the nucleic acid could be employed.

Marker genes such as antibiotic resistance or sensitivity genes may be used in identifying clones containing nucleic acid of interest, as is well known in the art.

The introduction may be followed by causing or allowing expression from the nucleic acid, e.g. by culturing host cells (which may include cells actually transformed although more likely the cells will be descendants of the transformed cells) under conditions for expression of the gene, so that the encoded peptide is produced. If the peptide is expressed coupled to an appropriate signal leader peptide it may be secreted from the cell into the culture medium. Following production by expression, a polypeptide may be isolated and/or purified from the host cell and/or culture medium, as the case may be, and subsequently used as desired, e.g. in the formulation of a composition which may include one or more additional components, such as a pharmaceutical composition which includes one or more pharmaceutically acceptable excipients, vehicles or carriers (e.g. see below).

Introduction of nucleic acid may take place in vivo by way of gene therapy, as discussed below.

The peptides can also be generated wholly or partly by chemical synthesis. The compounds of the present invention can be readily prepared according to well-established, standard liquid or, preferably, solid-phase peptide
synthesis methods, general descriptions of which are broadly available (see, for example, in J.M. Stewart and J.D. Young, Solid Phase Peptide Synthesis, 2nd edition, Pierce Chemical Company, Rockford, Illinois (1984), in M. Bodansky and A. Bodansky, The Practice of Peptide Synthesis, Springer Verlag, New York (1984); and Applied Biosystems 430A Users Manual, ABI Inc., Foster City, California), or they may be prepared in solution, by the liquid phase method or by any combination of solid-phase, liquid phase and solution chemistry, e.g. by first completing the respective peptide portion and then, if desired and appropriate, after removal of any protecting groups being present, by introduction of the residue X by reaction of the respective carbonic or sulfonic acid or a reactive derivative thereof.

Pharmaceutical compositions

The agents of the invention can be formulated in pharmaceutical compositions, e.g. as medicaments for activating p53 in cells that do not overexpress mdm2. These compositions may comprise, in addition to one of the above substances, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material may depend on the route of administration, e.g. oral, intravenous, cutaneous or subcutaneous, nasal, intramuscular, intraperitoneal routes.

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may include a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene
glycol or polyethylene glycol may be included.

For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer’s Injection, Lactated Ringer’s Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

Whether it is a polypeptide, antibody, peptide, nucleic acid molecule, small molecule or other pharmaceutically useful compound according to the present invention that is to be given to an individual, administration is preferably in a “prophylactically effective amount” or a “therapeutically effective amount” (as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors, and typically takes account of the disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners. Examples of the techniques and protocols mentioned above can be found in Remington’s Pharmaceutical Sciences, 16th edition, Osol, A. (ed), 1980.

Alternatively, targeting therapies may be used to deliver the active agent more specifically to certain types of cell, by the use of targeting systems such as antibody or cell specific ligands. Targeting may be desirable for a variety of reasons; for example if the agent is unacceptably toxic, or if it would otherwise require too high a dosage, or if it would not otherwise be able to
enter the target cells.

Instead of administering these agents directly, they could be produced in the target cells by expression from an encoding gene introduced into the cells, eg in a viral vector (a variant of the VDEPT technique - see below). The vector could be targeted to the specific cells to be treated, or it could contain regulatory elements which are switched on more or less selectively by the target cells.

Alternatively, the agent could be administered in a precursor form, for conversion to the active form by an activating agent produced in, or targeted to, the cells to be treated. This type of approach is sometimes known as ADEPT or VDEPT; the former involving targeting the activating agent to the cells by conjugation to a cell-specific antibody, while the latter involves producing the activating agent, e.g. an enzyme, in a vector by expression from encoding DNA in a viral vector (see for example, EP-A-415731 and WO 90/07936).

A composition may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated. The composition can be used to treat conditions such as cancer, virus infections or any other condition in which p53 or mdm2 is not functioning.

**Gene Therapy**

As a further alternative, the nucleic acid encoding the substance can be used in a method of gene therapy, to treat a patient with the coupling partner or a compound derivable from the coupling partner. This is particularly useful in the present invention for the stabilisation of the coupling partner in cells which a mdm2 mediated degradation pathway does not operate efficiently, as is the case in many tumour cells. As selective delivery has been a considerable problem facing those skilled in the art using gene therapy, this feature of the invention makes it particularly useful to improve selective delivery to these
classes of cells; the agent being unstable in normal cells but stable in cells lacking p53 or mdm2 function.

Vectors such as viral vectors have been used in the prior art to introduce genes into a wide variety of different target cells. Typically, the vectors are exposed to the target cells so that transfection can take place in a sufficient proportion of the cells to provide a useful therapeutic or prophylactic effect from the expression of the desired polypeptide. The transfected nucleic acid may be permanently incorporated into the genome of each of the targeted tumour cells, providing long lasting effect, or alternatively the treatment may have to be repeated periodically.

A variety of vectors, both viral vectors and plasmid vectors, are known in the art, see US Patent No. 5,252,479 and WO 93/07282. In particular, a number of viruses have been used as gene transfer vectors, including papovaviruses, such as SV40, vaccinia virus, herpesviruses, including HSV and EBV, and retroviruses. Many gene therapy protocols in the prior art have used disabled murine retroviruses.

As an alternative to the use of viral vectors other known methods of introducing nucleic acid into cells includes electroporation, calcium phosphate co-precipitation, mechanical techniques such as microinjection, transfer mediated by liposomes and direct DNA uptake and receptor-mediated DNA transfer.

**Experimental Procedures**

**Cloning and expression of peptide aptamers**

pTrx (Invitrogen) was cleaved with RsrII. The following oligomers were phosphorylated, annealed and then ligated into the cleaved vector:

For TIP wt: 5'-'3'

GTCCGCTCTGACTAGGAAACATTTTCAGACCTATGGAAACTACTTCCTGAAAACG

and 5'-'3'
GACCGTTTTTCAGGAAGTATGTTTCCATAGGTCTGAAAAATGTTTTCTGACTCAGAGGC
For TIP 12/1: 5' - 3'
GTCCGCTCTGTGATGATCGCTGGTTATGATTATGGGAGGTCTTAATGAAAACG
and 5' - 3'
GACCGTTTTCCATTAAGACCCTCCCATAATCCCATAAAACGAGGGATACCTTCAGAGGC

The resulting peptide inserts are illustrated in Figure 1.

E. coli 1724 cells were transformed with the resulting plasmids. They were grown in RM medium at 30°C overnight, then inoculated into fresh induction medium and grown to OD 0.5. The cultures were transferred to 37°C, induced with L-tryptophan at a final concentration of 100 μg/ml and grown for three to four hours. Soluble extracts were obtained by resuspending pellets in ice cold 20 mM Tris/HC1, pH 8, 2.5 mM EDTA with protease inhibitors (1 mM PMSF, 1mM benzamidine, leupeptin, approtinin and pepstatin at 10 μg/ml each) and three times shock freezing, thawing and sonicating, followed by centrifugation for 20 min at 10,000 g. Heat shock lysates were obtained by resuspending pellets to an OD of 100 and then treating at 80°C for 10 min followed by centrifugation at 10,000g for 20 min.

Purification of soluble extracts was carried out by loading clear soluble lysates onto an ion exchange Q 50 column (BioRad) and eluting with a linear gradient of 0.05M-1M KCl in 50 mM Tris/HC1 pH 7.8, 0.1% Triton X-100, 10% glycerol and 50 mM KCl. Active fractions were identified with anti-thioredoxin antibody (Invitrogen) on dot blots, concentrated using Centriprep 3 filters (Amicon) and loaded onto a G100 column (Pharmacia), preequilibrated with 30 mM HEPES, pH 8.0, 500 mM KCl, 0.1% Triton X100, 10% glycerol. Active fractions after elution were pooled, concentrated and dialysed against PBS.

For cloning of TIP 12/1, TIP wt and Trx into pcDNA3 for expression in mammalian cells, the thioredoxin coding region complete with the peptide insertions, was amplified from pTrx, pTrx 12/1 and pTrx wt using the following
primers:
5′-3′: CCGGATCCATTGGGCGATAAATTATTCACCTG and 5′-3′
CTCGAGCTAACCTGCTAGGGAATTCC.
The resulting PCR products were cleaved with BamHI and
Eco RI and ligated into BamHI, EcoRI cleaved pcDNA3. The
TIP 12/1 sequence in pcDNA3 was verified by sequencing.

ELISA assays
ELISAs were carried out as previously described
(Böttger et al, 1996). Briefly, plates were coated with
1µg/ml p53 or dilutions of p53 overnight at 4°C. They were
blocked and a preincubated mixture of GST-hdm2 (1.3 µg/ml)
and synthetic peptides inhibition ELISA) or hdm2 alone
(direct binding ELISA) was added for 1 hr. Binding was
established with the anti mdm2 monoclonal antibody SMP 14
(Picksley et al, 1994) and HRP conjugated anti-mouse IgG.

Cell culture and microinjection
All cells were grown in Dulbecco’s modified Eagle
medium (DMEM) supplemented with 10% FCS, for T22 cells 1
mg/ml G418 was added.
For microinjection, cells were seeded onto tissue
culture dishes and grown to 60-70% confluence.
Microinjection was performed using an Eppendorf
microinjection system (Microinjector 5242, Micromanipulator
5170) mounted to an Axiovert 35 M with heated stage.
Antibody injections were intranuclear or cytoplasmic.
Plasmid injections were intranuclear. Purified mouse
monoclonal antibodies 3G5 and 4B2 were injected in PBS at
a concentration of ca 1.3 mg/ml. Plasmid DNA encoding for
TIP 12/1, TIP and Trx was purified using Quiagen
purification system or phenol/chloroform precipitation and
injected at a concentration of 0.25 mg/ml in water.
Following microinjection fresh medium was added to the cell
cultures and they were incubated for 24 hours.

Detection of β-galactosidase activity
Cells were washed with PBS and fixed with 2% formaldehyde/0.2% glutaraldehyde in PBS for 5 min on ice. They were again washed with PBS and overlaid with 0.25 mg/ml X-gal in reaction mix (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM magnesium chloride in PBS). Cells were incubated at 37°C for 16 hours. Blue stained cells were observed.

Immunofluorescence

VRn.6 cells were fixed with 4% paraformaldehyde in PBS for 10 min, washed and permeabilised with 1% NP40 in PBS. Primary antibodies rabbit anti β-galactosidase or monoclonal anti-thioredoxin antibody (Anti-Thio™ Antibody, Invitrogen) were applied 1/500, Texas red conjugated goat anti mouse Ig or FITC conjugated goat and rabbit Ig F(ab’)_2 fragments (Jackson) were applied 1/500 as second antibodies. Incubations were carried out for 1 hour or 45 min at RT. Washes were carried out in PBS.

T22 cells were fixed with ice cold acetone/methanol (½) for 8 min at 4°C. Primary antibodies, protein A purified CM5 at 2μg/ml or anti-Thio™ 1/500 were added for 1 hour at RT, after PBS washes FITC conjugated anti mouse Ig at 1/80 or Texas red anti rabbit Ig at 1/400 were added for 30 min at RT.

Transient transfections for reporter induction

Cells were seeded into 6 well plates at 1.5 x 10^6 cells per well. They were grown to a density of 80% confluence and transfected using different lipophilic reagents (lipofectin and lipofectam, Promega, DOSPER and DOTAP, Boehringer). 2.5 μg TIP encoding plasmid DNA and 1 μg RGCAFosLacZ DNA and 5 - 10 μg of lipophilic reagent according to the instructions of the manufacturers were mixed in serum free medium and applied to the cells. 2-4 hours after transfection complete medium was added. 48 hours after transfection β-galactosidase activity was measured using CPRG (Boehringer) as a substrate. Cells
were scraped into PBS and centrifuged. Pellets from each well were dissolved in 50 µl Reporter Lysis buffer (Promega) and incubated on ice for 15 min. Soluble lysates were incubated with CPRG in 100 mM phosphate buffer, pH 7.0. OD at 595 nm was measured 1 - 24 hours later. To measure transfection efficiencies in each experiment, cells in a separate well were transfected with 2.5 µg pG3 DNA, encoding firefly luciferase. Luciferase activity was measured using Promegas luciferase assay system. The same lysates served as control for endogenous β-galactosidase activity.

**Immunoprecipitations**

Cells were grown to > 90% confluence on 14 cm dishes. They were scraped into PBS and lysed in 50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% NP 40 and protease inhibitors (Boehringer Complete™). To 100 µl lysate 1 µg PAb 421 in 400 µl DMEM/10% FCS or 400 µl Mab 4B2 supernatant was added and the lysates incubated overnight at 4°C. Protein G sepharose beads were added and incubation carried out for 2 hours. Beads were washed 5 times with PBS, 0.2% Tween and then boiled in SDS sample buffer. Proteins were separated on PAGE gels, western blotted and blots stained with rabbit polyclonal anti-p53 antibodies CM5 and CM1 followed by HRP anti rabbit IgG or a mixture of anti-mdm2 monoclonal antibodies 3G5, SMP14 and 4B2 followed by HRP anti-mouse IgG (DAKO). HRP activity was established by ECL (Amersham).

**Transient transfections for establishing p53 levels in the presence and absence of mdm2**

SAOS 2 cells were seeded 24 hrs before transfection to 80% confluence in 10 cm dishes. Calcium phosphate mediated transfections were performed as previously described (Lin and Green, 1989). 5 µg of p53 wt and mutant encoding plasmids (pcDNA3) or control vector and 5 µg mdm2 encoding plasmid (X2, Haupt et al., 1996) were cotransfected per 10
cm dish. 48 hrs after transfection cell lysates were fractionated by 12% SDS-PAGE and transferred to nitrocellulose membranes. Blots were stained with rabbit anti p53 antiserum CM5 (1/8000) and HRP conjugated anti rabbit Ig. Peroxidase activity was established using ECL.

Site directed mutagenesis

Construction of F\textsuperscript{19}\textsuperscript{A} was accomplished by site directed mutagenesis using the Transformer\textsuperscript{TM} site directed mutagenesis kit (Clonetech). The sequence of the selection primer was: 5′-3′ GACTCTGGGATCGATGACCGACC, the sequence of the mutagenic primer was: 5′-3′ GAGCCAGGAGACGCCTCAGGCTATG. The sequence of the p53 mutant F\textsuperscript{19}\textsuperscript{A} was confirmed by sequencing.

Results

Peptide inserts into Thioredoxin create potent inhibitors of the p53- mdm2 interaction.

Figure 1 shows a schematic representation of the three aptamers we constructed by inserting additional peptide sequences into the active site of E.coli thioredoxin. TIP 12/1 (Thioredoxin Insert Protein) contains the sequence we identified previously by phage display as the most potent inhibitor of the mdm2-p53 interaction in in vitro assays (Böttger et al, 1996). TIP wt contains the sequence corresponding to p53 wild type sequence F\textsuperscript{13} to N. As controls we expressed thioredoxin lacking a peptide insertion (Trx) in bacteria.

All these proteins were easily expressed in E. coli. They could be purified from soluble bacterial lysates by heat shock to almost 80% homogeneity without loss of mdm2 binding activity. We also purified the TIPs to near homogeneity from soluble bacterial lysates which were not heat shocked by ion exchange chromatography and gel filtration. Results obtained with TIPs purified both ways were identical.

We tested the aptamers for their capacity to inhibit
the p53-mdm2 interaction in ELISA assays. Binding of GST-
mdm2 (1-188) to p53 coated ELISA plates was inhibited by
increasing amounts of bacterially expressed TIPs. Table 1
shows IC\textsubscript{50} values obtained in this assay in comparison with
the IC\textsubscript{50} for the corresponding peptides and bacterially
expressed full length p53 (Midgley et al, 1992). The
following conclusions can be drawn.

1. TIP 12/1 inhibits the interaction between p53 and mdm2
in this assay with the same strength as full length
p53. This should make it a suitable agent to be
tested in cellular systems for effects on the
interaction between p53 and mdm2 \textit{in vivo}.

2. TIP wt inhibits the interaction 20 times less than TIP
12/1. This has to be attributed to the 50 times less
potent inhibition achieved by the wt peptide when
compared with peptide 12/1 in peptide competition
assays (Böttger et al, 1997).

3. Trx does not show inhibition in our assay, making it
a suitable negative control for \textit{in vivo} experiments.

In these \textit{in vitro} assays, TIP 12/1 exhibits strong
enough inhibitory potential to compete against endogenous
levels of wt p53 in tumour cells for binding to mdm2. It
therefore offers an agent that should be capable to
function inside mammalian cells.

Expression of peptide aptamers in mammalian cells activates
p53 dependent transcription.

We therefore went on to clone TIP 12/1, TIP wt and Trx
into pcDNA3 (Promega), a vector where these proteins would
be expressed under the control of the strong CMV promoter
in mammalian cells.

At first we wanted to see if a stably transfected p53
responsive \(\beta\)-galactosidase reporter could be switched on by
microinjecting the plasmids encoding for TIPs. A recently
established transformed rat thyroid epithelial cell line stably transfected with pRGCA fos-lac Z (Blaydes et al, 1997), VRn.6 cells, seemed to provide a suitable model. It was shown before that these cells express wt p53 and also overexpress mdm2 at the protein level. The β-galactosidase reporter is strongly responsive to UV induction of p53 in VRn.6 cells. Microinjection of the monoclonal antibody 3G5 into the nuclei of these cells could also switch on the p53 reporter (Blaydes et al, 1997). We have shown before that 3G5 binds mdm2 exactly within the p53 binding pocket and blocks p53 mdm2 association (Böttger et al, 1997). If the effect of 3G5 injection on the induction of p53 dependent transcription was due to interrupting the p53-mdm2 interaction, our inhibiting aptamers should exert a similar effect.

In these experiments we found the strong induction of β-galactosidase activity after microinjection of 3G5 into the nuclei of VRn.6 cells. To be certain that the cells which responded with β-galactosidase activity were the injected ones we carried out dual immunofluorescence studies. We stained cells after injections with anti β-galactosidase antiserum and with anti mouse immunoglobulin. This showed that the cells expressing the reporter enzyme (left hand panel, blue) are also positive for the injected antibody (right hand panel, green). Injecting TIP 12/1 encoding plasmid into the nuclei of VRn.6 cells also has a strong effect on induction of the reporter enzyme. There is no induction after microinjection of the control thioredoxin encoding plasmid, although it clearly is expressed.

The remarkable strength of this response lead us to test whether this was due to the relatively high levels of p53 and mdm2 present in the VRn.6 line. To do this the microinjection experiments were then carried out in T22 cells, a mouse prostate derived cell line, also stably transfected with the same reporter plasmid. These cells normally contain very low levels of p53 and mdm2. On
treatment with DNA damaging agents the p53 protein accumulates and the cells show remarkable p53-dependent β-galactosidase induction (Hupp et al., 1995; Lu and Lane, 1993). On microinjection of 3G5 and TIPs encoding plasmids into T22 cells, we again detect immense induction of β-galactosidase with 3G5, but no induction with 4B2, an anti mdm2 antibody that targets an epitope outside the p53 binding pocket on mdm2. A remarkable reporter induction was caused by our strongest aptamer, TIP 12/1. Lower levels of β-galactosidase activity are observed with TIP and no activity with the control thioredoxin. This reflects exactly the capacity of the three aptamers to inhibit the mdm2-p53 interaction in vitro. Staining of the injected cells with anti-thioredoxin antibody confirmed that the differences in reporter enzyme activity were not due to differential expression of the TIP proteins.

The strong induction of reporter enzyme in T22 cells, which do not overexpress mdm2, lead us to believe, that activation of p53 by release from mdm2 complexes must be a very strong stimulus for p53 activation, similar to induction of p53 by UV and much stronger than the previously observed effect of allosteric activation of latent p53 in T22 cells by PAb 421 (Hupp et al., 1995).

We sought to analyse more cell lines, preferentially not dependent on a stably integrated reporter plasmid. We therefore transiently transfected cells containing wt p53 with p53 responsive reporter plasmids (RGCAFosLacZ) and TIP 12/1 or Trx control. We choose OSA cells, a human osteosarcoma cell line (Florenes et al, 1994) as an example for a cell line with highly elevated mdm2 levels due to gene amplification. We also used U2-OS cells, another osteosarcoma cell line, which has no gene amplification for mdm2 but elevated levels of mdm2-mRNA (Florenes et al., 1994) and MCF-7 cells, a breast cancer cell line with heterogeneously expressed low levels of wt p53 and no reported mdm2 elevation. First we tried to analyse the protein levels expressed in these cells and the degree of
complex formation with mdm2, using immunoprecipitations. We precipitated from cell lysates with the monoclonal anti-
mdm2 antibody 4B2 and the monoclonal anti-p53 antibody 421. Figure 2 shows the results.

In OSA cells, 4B2 coprecipitates p53 in similar amounts to PAb 421 (Fig 2b, lane 7 for PAb 421 and lane 8 for 4B2). 4B2 precipitates mdm2 (Fig 2a, lane 7), suggesting an excess of mdm2 that does not bind p53 whilst all p53 is in complex with mdm2. In U2-OS and MCF-7 cells neither 4B2 nor PAb 421 precipitate readily detectable amounts of mdm2 (Fig 2a, lanes 2 and 5 for PAb 421, lanes 3 and 6 for 4B2, lanes 1 and 4 represent controls for precipitations without antibodies). After superexposure of ECL film for 60 min, a very faint band at the size of mdm2 appears in U2-OS cell precipitates (not shown). In MCF-7 cells PAb 421 precipitates p53 (Fig 2b, lane 5), but no coprecipitation of p53 by anti-mdm2 antibody 4B2 is observed (Fig 2b, lane 6, signal is the same as in control precipitation, lane 4). In U2-OS cells PAb 421 precipitates p53 (Figure 2b, lane 3) and 4B2 precipitates a large percentage of p53 (Figure 2b, lane 3), suggesting a high degree of hdm2-p53 complex formation.

We then cotransfected (RGCAFosLacZ) reporter plasmid and TIP 12/1 or Trx encoding DNA into all three cell lines to compare the levels of reporter enzyme induction directly. Different lipophilic transfection agents were used and the transfection efficiency was monitored on separate plates by transfecting pG3, a plasmid encoding firefly luciferase under a constitutive promoter, and measuring luciferase activity. This allowed us to exclude the possibility that the differences in p53 dependent transcriptional activation of the reporter are related to transfection efficiency (data not shown). Figure 3 shows an average of four experiments of induction of β-
galactosidase activity by TIP 12/1, compared to Trx in each cell line.

Surprisingly, most induction of the p53 reporter is
achieved by TIP 12/1 in MCF-7 cells and in U2-OS cells, where the level of mdm2 is below the detection limit. A ca. 100 times lower effect of reporter enzyme induction TIP 12/1 is observed in OSA cells. Figure 3 also shows, that transfection of control plasmid alone induces a low level response of p53 dependent transcriptional activation in MCF-7 and U2-OS cells. This effect has been reported before (Renzing and Lane, 1993). It is, however, almost completely absent in OSA cells.

This confirms the concept, that in cells overexpressing mdm2 by gene amplification, p53 dependent transcriptional activation is repressed. This can be overcome by inhibiting the interaction between mdm2 and p53. Much more striking, however, is the observation that in cells with undetectable levels of mdm2, specific inhibitors of the p53-mdm2 interaction can induce p53 dependent transcriptional activation to an extent that is comparable with induction by UV.

Disruption of the p53-mdm2 interaction leads to the accumulation of p53

The dramatic induction of the p53 response by UV and other genotoxic agents is accompanied by the accumulation of high levels of p53 protein due at least in part to its extended half life in treated cells. However, it is not clear that this is the sole mechanism by which the p53 response is activated an other mechanisms such as allosteric activation of DNA binding function have also been proposed to play a role (Hupp et al, 1995). The very strong induction of the p53 response by the TIP 12/1 encoding plasmid and MAb 3G5 injections lead us to ask if this induction occurred independently of the accumulation of p53 protein. To test this hypothesis, Mabs 3G5 and 4B2 and TIP 12/1 and Trx encoding plasmids were injected into T22 cells and the levels of p53 analysed in immunofluorescence. We found that p53 accumulates to high levels in the cells injected with the interaction
25 disrupting 3G5 antibody, but not with the control anti-mdm2 antibody. In a similar way, cells injected with the TIP 12/1 expression plasmid accumulate high levels of p53, whereas those injected with control plasmid do not. These striking results demonstrate that disruption of the p53-mdm2 interaction mirrors the genotoxic response not only by activating p53 dependent transcription, but also by leading to the accumulation of p53 protein. The implication of these results was that in normal cells mdm2 targets p53 for destruction. When we transfected wild type p53 into p53 negative SAOS 2 cells we found that cotransfection of an mdm2 expression plasmid greatly reduced the level of p53 that accumulated (Figure 4, lanes 2 and 3), supporting the idea that mdm2 could target p53 for degradation. To further test this hypothesis we constructed a point mutant F19-->A in murine p53 that mutated one of the key contact residues of the p53-mdm2 interface identified in the crystal structure and in our phage display analysis (Böttger et al, 1997; Kussie et al, 1996). We confirmed that this mutant p53 was unable to bind to mdm2 but was transcriptionally active (data not shown). When this mutant p53 was transfected into the SAOS 2 cells its accumulation unlike that of wild type p53 was not affected by cotransfection of the mdm2 expression plasmid (Figure 4, lanes 4 and 5).

Discussion

Peptide aptamers to disrupt protein-protein interactions

The above results show that peptide aptamers able to block the binding of p53 to mdm2 in cellular assays. To do this peptide sequences capable of binding tightly to mdm2 that had been identified from phage peptide libraries were displayed on the active site loop of thioredoxin. The aptamer proteins (TIPs) were readily expressed in E.coli and easily purified from soluble lysates. In in vitro assays the peptides inserted into the active site loop of thioredoxin showed the same greatly enhanced binding
compared to the wild type p53 peptide sequence that we had seen in our earlier analysis of the free peptides, demonstrating the successful transfer of the optimised sequence from phage display to insert protein. Critically, the inhibitory potential of the 15 kD TIP 12/1 protein was the same as that of tetrameric wt p53 and greatly exceeded that achieved by the simple transplantation of the wild type p53 sequence into thioredoxin. When expressed in mammalian cells both aptamers, TIP 12/1 and TIP wt were able to induce p53 dependent transcriptional activation of a reporter gene. The intensity of the effect in microinjection experiments exerted by TIP 12/1 in comparison with TIP wt was proportional to their in vitro inhibitory potential. With these mdm2 binding aptamers we therefore have developed powerful tools to study the biological consequences of disrupting the interaction between mdm2 and p53 in tumour cells. This precise approach of aptamer selection and design describes a road leading from the identification of peptides that are able to disrupt a very specific protein-protein interaction by combinatorial library approaches towards verifying the expected biological effect in vivo. This concept in combination with the use of peptide aptamer libraries, for instance expressed and screened the yeast two hybrid system (Colas et al, 1996) should have a great potential for studying networks of protein-protein interactions in eucaryotic cells on the biochemical as well as on the functional level. It certainly now enables us to draw some interesting conclusions about the significance of inhibiting the interaction between mdm2 and p53 in tumour cells.

Activation of p53 by blocking binding to mdm2

It had been assumed that disrupting the interaction between the two proteins would potentially release transcriptionally active p53 in tumour cells that overexpress mdm2 due to gene amplification. Although our
experiments with OSA cells suggest that this is true, these cells do not seem to be the most susceptible. In contrast, MCF-7 cells and U2-OS cells as well as T22 cells, all of which have hardly detectable mdm2 levels, respond with an immense transcriptional activation of p53 after applying mdm2 inhibitors.

It appears that the interaction of p53 with mdm2 is the prime restraint for p53 activity in all analysed tumour cells. Comparison of the response in different cell lines argues that the release of transcriptionally active p53 is reciprocal to the mdm2 content of the cells. Higher affinity inhibitors or specific targeting of our TIP aptamers to the nucleus could be expected to overcome mdm2 repression of p53 to a much higher degree in cells with amplified mdm2 levels, like OSA cells.

This result also demonstrates that inhibitors of the p53-mdm2 interaction do not interfere with the capacity of p53 to interact with the transcription apparatus. It had been feared that the close similarity between the target site for mdm2 and for the transcription complex on the N-terminus of p53 might mean that any inhibitor of mdm2 binding also blocks the transcriptional apparatus from binding to p53.

Mdm2 regulates p53 levels

We further show that inhibition of the interaction of p53 with mdm2 leads to a rise in p53 protein levels. This strongly implies that binding of mdm2 to p53 is a requirement for p53 degradation or destabilisation. This conclusion is strongly supported by the finding that the P^{19,20}A mutation blocks mdm2 dependent reductions in p53 levels in transfection studies. Marston et al found that an in frame deletion of conserved box 1 resulted in a mutant p53 that was very stable but retained transcriptional activity (Marston et al, 1994) whilst it is well established that larger N-terminal deletions also stabilise p53. The model that p53 drives the transcription
of the key regulator of its own degradation also provides an intensely attractive model for the stability of the p53 protein in cells in which the protein is inactive transcriptionally. This includes cells that contain only mutant p53 such as the majority of human tumour cells and cells infected or transformed by DNA tumour viruses whose products bind an inactivate the transcriptional function of p53. The model argues that these cells lack sufficient functional mdm2 expression to target p53 degradation. The model is completely consistent with our earlier finding that point mutant p53 proteins, inactive in transcription assays, are stable when transfected into p53 null cells but unstable in cells that contain wild type p53 activity (Vojtesek and Lane, 1993; Midgley et al in preparation).

If mdm2 targets p53 for degradation, inhibition of this interaction could prevent newly synthesised p53 from being rapidly turned over and so lead to the observed increase in transcriptionally active p53. It would be of great interest to explore if the rise of p53 after UV or other genotoxic damage indeed involves release of the protein from mdm2. This model therefore provides much scope for further study. It contains striking parallels to the model for HPV E6 dependent degradation of p53 and it is a valid speculation that mdm2 may target p53 for ubiquitin dependent degradation. It becomes possible to target other proteins for degradation in a p53 like manner so that they are unstable in normal cells, but stabilised by DNA damage or in cells lacking mdm2 activity by splicing onto them an mdm2 recognition peptide and it is possible to design super active p53 proteins that cannot be targeted for degradation but are transcriptionally active.
Table 1
In vitro inhibitory potential of TIPs compared to free peptides and full length p53

<table>
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<tr>
<th>Inhibitor</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; in nM</th>
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<tr>
<td>TIP wt</td>
<td>15 000</td>
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<tr>
<td>TIP 12/1</td>
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References:

The references cited herein are incorporated by reference in their entirety.


Claims:

1. A substance which comprises a mdm2 binding domain linked to a coupling partner for use in a method of medical treatment, wherein the substance is stabilised in cells in which a mdm2 mediated degradation pathway does not operate efficiently.

2. The substance of claim 1 wherein the cells are p53 null cells, cells in which the level of p53 is reduced as compared to normal cells, and/or cells in which endogenous p53 tends to accumulate.

3. The substance of claim 1 or claim 2 wherein the mdm2 binding domain is a peptide having an amino acid sequence corresponding to human p53 which has the property of binding to mdm2.

4. The substance of claim 3 wherein the mdm2 binding domain does not include a domain of p53 associated with the DNA specific binding property of p53 so that the substance does not induce mdm2 production.

5. The substance of any one of the preceding claims wherein the mdm2 binding domain is a peptide having less than 25 amino acids in length and having an amino acid sequence having at least 70% amino acid sequence identity with a corresponding portion of human p53.

6. The substance of any one of the preceding claims wherein the mdm2 binding domain includes the peptide motif FxxxW, where x is any amino acid.

7. The substance of any one of the preceding claims wherein the coupling partner is an effector, a drug, a prodrug, a toxin, a peptide, a label or a transport molecule.
8. Use of a substance of any one of claims 1 to 7 in the preparation of a medicament wherein the substance is stabilised in cells in which a mdm2 mediated degradation pathway does not operate efficiently.

9. The use of claim 8 wherein the medicament is for the treatment of cancer, viral infections or other condition associated with non functional p53 or mdm2.

10. A method of selectively stabilising a substance in cells in which a mdm2 mediated degradation pathway does not operate efficiently, the method comprising exposing the cells to a substance of any one of claims 1 to 7 so that the substance is marked for degradation in cells containing functional mdm2 and is stabilised in cells in which a mdm2 mediated degradation pathway does not operate efficiently.

11. A pharmaceutical composition comprising one or more of the substances of any one of claims 1 to 7 in combination with a pharmaceutically acceptable carrier.

12. A pharmaceutical composition comprising an expression vector comprising a nucleic acid sequence encoding a substance of any one of claims 1 to 7, the nucleic acid being under the control of sequences to direct its expression, in combination with a pharmaceutically acceptable carrier.

13. Use of a pharmaceutical composition of claim 12 in the preparation of a medicament for use in gene therapy to deliver the substance to cells, wherein the substance is selectively stabilised in those cells in the population in which the mdm2 mediated degradation pathway does not operate efficiently.

14. The use of claim 13 wherein the medicament is for the treatment of cancer, viral infections or other condition
associated with non functional p53 or mdm2.

15. An expression vector comprising a nucleic acid sequence encoding a substance which comprises a mdm2 binding domain linked to an effector, wherein the effector is a drug, a prodrug, a toxin, a label and/or a transport molecule.

16. The expression vector of claim 15 wherein the mdm2 binding domain is a peptide having an amino acid sequence corresponding to human p53 which has the property of binding to mdm2.

17. The substance of claim 16 wherein the mdm2 binding domain does not include a domain of p53 associated with the DNA specific binding property of p53 so that the substance does not induce mdm2 production.

18. The substance of any one of claims 15 to 17 wherein the mdm2 binding domain is a peptide having less than 25 amino acids in length and having an amino acid sequence having at least 70% amino acid sequence identity with a corresponding portion of human p53.

19. The substance of any one of claims 15 to 18 wherein the mdm2 binding domain includes the peptide motif FxxxW, where x is any amino acid.
TIP
PPLSQETPSDLWKLLPENG

TIP 12/1
PPLSMPRFMDYWEGNLNENG

FIGURE 1
FIGURE 2
FIGURE 4
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C07K14/47 C07K14/00 C12N15/12 A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database consulted during the international search (name of database and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
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</table>

Further documents are listed in the continuation of box C. Patent family members are listed in annex.

Date of the actual completion of the international search

27 August 1998

Date of mailing of the international search report

09/09/1998

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Authorized officer

Cervigni, S
## INTERNATIONAL SEARCH REPORT

**Page dimensions:** 595.0x842.0

### DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
</table>
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           vol. 387, 15 May 1997, pages 299-303,  
           XP002075660  
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           11-14,  
           18,19 |
| X        | WO 96 02642 A (UNIV DUNDEE ; PICKSLEY  
           STEVEN MICHAEL (GB); LANE DAVID PHILIP  
           (GB)) 1 February 1996  
           cited in the application  
           see abstract; claims | 12-19 |
| Y        | WILLS K N ET AL: "DEVELOPMENT AND CHARACTERIZATION OF RECOMBINANT ADENO-VIRUSES ENCODING HUMAN P53 FOR GENE THERAPY OF CANCER"  
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