Title: INHIBITORS OF THE BINDING BETWEEN HDM2 AND THE PROTEASOME

Abstract: This invention provides methods for determining whether a test compound interacts with the binding between HDM2 and the proteasome. It further provides compounds, pharmaceutical compositions comprising said test compound, and the use of said compounds and compositions, in particular as anti-cancer agent, even more particular for treating cell proliferative disorders in a subject.
INHIBITORS OF THE BINDING BETWEEN HDM2 AND THE PROTEASOME

This invention is based on the characterization of the interaction of HDM2 or related proteins with the proteasome and how disturbance of this interaction can affect e.g. the ubiquitin-(Ub)-proteasome proteolysis (UPS) pathway. It accordingly provides compound-HDM2 binding sites. In a further aspect this invention also provides related nucleic acids, amino acids, vectors, host cells, pharmaceutical compositions and articles of manufacture. This invention further provides methods for determining whether a test compound interacts with the binding between HDM2 and the proteasome, as well as pharmaceutical compositions comprising said test compound, in particular as anti-cancer agent, even more particular for treating cell proliferative disorders in a subject.

BACKGROUND OF THE INVENTION

Hdm2 is a key oncogene, which is activated in a large number of cancer patients through various mechanisms including hdm2 gene amplifications, and deletion of upstream tumor suppressors such as p14ARF and PTEN. Hdm2 is overexpressed in several types of malignancies including osteosarcomas, soft tissue sarcomas and gliomas and high levels of hdm2 are associated with poor prognosis. Interestingly, a single nucleotide polymorphism in the hdm2 promoter which increases hdm2 expression has been associated with accelerated tumor formation in both hereditary and sporadic cancers in humans.

HDM2 promotes tumorigenesis by associating with cell cycle regulatory proteins, modulating their activity and stability. The number of HDM2 substrates is rapidly expanding, key examples include the tumor suppressor p53 and its family members p63 and p73, E2F1, HIF1α and p21^{waf1,cip1}. Most extensively studied is p53. HDM2 binds and ubiquitinates the p53 protein which results in a rapid degradation of p53 by the proteasome. Abrogation of HDM2-p53 complex degradation causes p53 stabilization and subsequent transcriptional activation of p53 downstream genes (reviewed in Brooks and Gu). In addition to the ubiquitin ligase function, other activities of HDM2 are also required for p53 degradation, as evidenced by the accumulation of ubiquitylated p53 when phosphorylation in the central domain of HDM2 is abrogated (Blattner et al., 2002). The association of HDM2 with different subunits of the 26S proteasome such as S4, S5a, S6a and S6b (3rd Mdm2 workshop, Sept. 2005 in Constance, Germany) might play a key role in this process. Consistent
with the key role of HDM2 tumorigenesis, antagonists of the hdm2 oncogene, i.e., peptides and small molecules, inhibit tumor cell proliferation in vitro and the growth of human xenografts in immunodeficient mice in vivo. This positions the HDM2 protein as an attractive target for the development of anti-cancer therapy.

Given the central role of HDM2 in the Ub-proteasome pathway it is to be expected that blocking of the HDM2-proteasome interaction could provide a novel mechanism for the development of new chemotherapeutics for the treatment of cancer.

It is an object of the present invention to characterize protein and proteasome binding sites within HDM2 or related proteins and binding sites within the different proteasome subunits, that allow to identify compounds capable to interfere with e.g. the HDM2 - proteasome interaction and accordingly useful in the treatment of cell proliferative disorders through protection against proteolysis.


References


SUMMARY OF THE INVENTION

The present invention makes use of assays that determine the interaction of HDM2 or related proteins or protein binding fragments thereof with proteins or small molecules, as well as of HDM2-proteasome binding sites, in particular interactions comprising a proteasome subunit selected from the group consisting of S2, S4, S5a, S6a or S6b or a fragment thereof.

The assays are useful to identify whether a test compound can alter the interaction of HDM2 with the proteasome or a proteasome subunit. The assays are also useful to determine whether the test compound protects a protein against proteolysis e.g. proteolysis by the UPS-pathway or by another proteolytic system e.g. proteolysis by trypsin.

In a further embodiment the present invention relates to pharmaceutical compositions comprising compounds identified in the herein mentioned assays and the therapeutic use thereof to inhibit proliferative conditions, such as cancer and psoriasis. This invention provides a method for inhibiting the abnormal growth of cells, including transformed cells, by administering an effective amount of a compound of the invention. Abnormal growth of cells refers to cell growth independent of normal regulatory mechanisms (e.g. loss of contact inhibition). This includes the inhibition of tumour growth both directly by causing growth arrest, terminal differentiation and/or apoptosis of cancer cells, and indirectly, by inhibiting neovascularization of tumours.

This and further aspects of the present invention will be discussed in more detail hereinafter.

BRIEF DESCRIPTION OF THE DRAWING

Figure 1: JNJ-#1 binds to HDM2.
GST-HDM2, GST-HDMX were incubated with 10μM of JNJ-#1 or with DMSO for control. Then 40 ng trypsin was added and the mixture was incubated for 15 min on ice. Samples were separated by a 12 or 15% SDS-PAGE gel and blotted onto Immobilon-P membrane. HDM2 was detected by incubating the membrane with the HDM2 antibodies 4B2, SMP14 and C18. Western blots were developed by ECL.

Figure 2: Dose dependency of the inhibition of proteolysis of HDM2 by JNJ-#1. GST-HDM2 was incubated with the indicated doses of JNJ-#1 or with DMSO, 10 μM of active and inactive nutlin or MG132 for control. 40 ng of trypsin were added and the mixture was incubated for 15 min on ice. Samples were separated by 10% SDS-PAGE gel and blotted onto Immobilon P membrane. The membrane was incubated with the anti-HDM2 antibody 4B2. The Western blot was developed by ECL.

Figure 3: JNJ-#1 Induces p53 and Downstream Effectors.
U-87 MG glioblastoma cells were incubated with the indicated concentrations of JNJ-#1 for 24 h. JNJ-#1 was dissolved as 5 mM stock solution in dimethylsulfoxide (DMSO) and subsequently diluted into tissue culture media to result in the final concentrations indicated. Total cell lysates were prepared and analyzed by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS/PAGE). Protein expression was detected using specific antibodies. Actin protein levels were revealed as a control for equal loading.

Figure 4: JNJ-#1 enhances HDM2-p53 association.
JAR choriocarcinoma cells were incubated with the indicated concentrations of JNJ-#1, Nutlin-3 or the inactive enantiomer of Nutlin-3 for 1.5 hours. HDM2/p53 complexes were co-immunoprecipitated from cell lysates, and protein expression was detected using specific antibodies as indicated in the methods section. Immunoprecipitated HDM2 proteins were revealed using SMP-14 antibody (sc-965) and p53 protein was revealed as specified under Western Blot analysis.

Figure 5 JNJ-#1 does not inhibit p53 ubiquitination in cells.
U2OS cells were transfected with His-tagged ubiquitin. and incubated with 10 μM of JNJ-#1 or Nutlin-3 for 2 hours. After incubation ubiquitylated proteins were purified by adsorption to Ni2+-agarose and separated by SDS-PAGE. P53 was detected by Western blotting. (TCL: Total cell lysate)
Figure 6: Dose dependency of the inhibition of the interaction of HDM2 and the proteasome.
GST-HDM2 was expressed in bacteria. 100 ng of the protein were incubated with proteasomes in the presence of the indicated doses of JNJ-#1 or in the presence of DMSO for control. For Input control, 10 µl of the mixture were separated by an 10% SDS-PAGE gel and blotted onto an Immobilon membrane. HDM2 was immunoprecipitated with the anti-HDM2 antibody C18 and loaded onto a 10% SDS-PAGE gel. The proteins were blotted onto Immobilon-P membranes. The top part of the membranes were hybridized with the anti-HDM2 antibody 4B2, the lower part with an antibody directed against the proteasomal subunit S8. Western blots were developed by ECL.

Figure 7: JNJ-#1 prevents the association of HDM2 with the proteasome in living cells.
A) 293T cells were incubated for 1.5 hours with 10 µM nutlin or JNJ-#1, or with DMSO for control. Cells were lysed and HDM2 was immunoprecipitated using the C-18 antibody. The immunoprecipitates were separated by a 10% SDS-PAGE gel and blotted onto Immobilon-P membrane. For expression control, 50 µg of total cell lysate (TCL) were separated by SDS-PAGE and blotted onto Immobilon-P membrane. Both membranes were hybridized with antibodies directed against S6b and HDM2. Western blots were developed by ECL. B) 293T cells were transfected with Myc-MDM2. 36 hours after transfection, 10 µM JNJ-#1 DMSO, for control, were added. Cells were lysed after 1.5 hours and MDM2 was immunoprecipitated with the anti-Myc antibody 9E10 and processed as described in part A.

Figure 8: JNJ-#1 prevents p53 degradation in vitro.
p53 and MDM2 expressed in baculoviruses were purified and incubated for 5 hours with ubiquitin; E1 and E2 enzymes, 26S proteasomes and where indicated with the indicated doses of JNJ-#1 or with DMSO, 10 µM of active and inactive nutlin or MG132 for control. The mixture was separated by a 10% SDS-PAGE gel and blotted onto an Immobilon membrane. The membrane was hybridized with the anti-p53 antibody DO-1. The Western Blot was developed by ECL.

Figure 9: Alignment of sequences containing the ED(X)Y-motif of different proteasomal subunits and of HDM2.

Figure 10: HDM2 binds with the EDY sequence of the S6b protein of the 26S proteasome.
100 ng of MDM2 expressed from Baculoviruses were incubated with 100 ng of a GST-fusion protein containing the EDY sequence of the S6b protein. 10% of the sample was loaded onto a 10% SDS-PAGE gel for input control (Input). To the remaining lysate, glutathione-sepharose was added, the GST-fusion proteins were collected by centrifugation, loaded onto a 10% SDS-PAGE gel and transferred onto Immobilone P blotting membrane. The membrane was hybridized with antibodies against MDM2 and GST (Pulldown).

Figure 11: Overexpression of the EDY sequence prevents p53 degradation.
A) H1299 cells were transfected with cDNAs encoding p53 and thioredoxin (lane 1), with cDNAs encoding p53, thioredoxin and MDM2 (lane 2), with cDNAs encoding p53, thioredoxin with a sequence from the S6b protein containing the EDY motif inserted and MDM2 (lane 3) or with cDNAs encoding p53, thioredoxin with a sequence from the HDM2 protein containing the EDY motif inserted and MDM2 (lane 4). B) U2OS cells were transfected with a cDNA encoding thioredoxin (lane 1), with a cDNA encoding thioredoxin with a sequence from the HDM2 protein containing the EDY motif inserted (lane 2) or with a cDNA encoding thioredoxin with a sequence from the S6b protein containing the EDY motif inserted (lane 3).

Cells were lysed 48 hours after transfection and p53 protein levels (and PCNA levels for loading control) were determined by Western blotting.

Figure 12: Full protein sequences containing the ED(X)Y-motif of different proteasomal subunits and of HDM2 and the polynucleotide sequences of the ED(X)Y-motifs.

Figure 13: The central domain of HDM2 reduces the association of MDM2 with S6b.
293 cells were transfected with plasmids encoding Myc-tagged wild type (wt) or mutant MDM2 harboring the indicated deletions together with a plasmid encoding V5-tagged S6b. 24 hours after transfection, cells were lysed and MDM2 was precipitated using the antibody 9E10 coupled to protein A Agarose. The beads were washed and loaded onto a 10% SDS-PAGE gel. Proteins were transferred onto Immobilone membrane and probed for the presence of MDM2 and S6b (IP: a-Myc). TCL: 50 μg of cellular protein were separated by SDS-PAGE, blotted onto Immobilone membrane and probed for S6b and PCNA, for loading control.
Figure 14A and B: Photoaffinity labeling of HDM2 proteins and detection of the tritium label.

Equal molar amounts of proteins were mixed with photoaffinity and tritium labeled JNJ-#1 in a microtiterplate on ice. Final JNJ-#1 concentrations are indicated. Mixture was UV irradiated, on ice, during 10 minutes. Reaction mixture was run through a column and the eluates were denaturized, followed by PAGE gel separation. Proteins were than transferred to a PVDF membrane by Western blotting. Blots were exposed to a phosphor imigar screen. After two weeks exposure, the phosphor imigar screen was analyzed on a Fujix Bas 2000 Bio-Imaging Analyzer (figure 14A). Bands were quantified, BLU (Biochemical Light Unit) values of the quantification were normalized versus the aspecific binding to BSA. The mean value of 3 experiments is shown in figure 14B.

DETAILED DESCRIPTION

Definitions

As used in this application, except as otherwise expressly provided herein, each of the following terms shall have the meaning set forth below.

"HDM2" also known as "MDM2" shall mean the "mouse double minute 2 homolog" (SwissProt entry Q00987) and is not restricted to the human protein but includes related proteins such as the mouse protein (SwissProt entry P23804), the dog protein (SwissProt entry P56950), the horse protein (SwissProt entry P56951), the cat protein (SwissProt entry Q7YRZ8) or a protein having at least 70, 80, 90, 95, 97 or 99% sequence identity to the human sequence (SwissProt entry Q00987). HDM2, also known as p53-binding protein MDM2 was originally cloned by Oliner et al. (Nature, 1992; 358:80-83). With "related proteins" is meant proteins having at least 40, 60, or 69% sequence identity to the human sequence (SwissProt entry Q00987).

"Proteasome" shall mean a large-multisubunit complex that targets the degradation of ubiquitinilated proteins.

"Proteasome subunit" shall mean proteasome subunit 6A, S6A or PSMC3; proteasome subunit 6B, S6B or PSMC4; proteasome subunit 5A, S5A or PSMD4; proteasome subunit 2, S2 or PSMD2; or proteasome subunit 4, S4 or PSMC1. It is not restricted to the human proteins but includes related proteins such as the mouse S6A
(SwissProt entry Q88685), the rat S6A (SwissProt entry Q63569), the mouse S6B (SwissProt entry P54775), the rat S6B (SwissProt entry Q63570), the bovine S6B (SwissProt entry Q3T030), the macaque S6B (SwissProt entry Q4R7L3), the mouse S5A (SwissProt entry Q35226), the bovine S5A (SwissProt entry Q58DA0), the mouse S2 (SwissProt entry Q8VDM4), the bovine S2 (SwissProt entry P56701), the mouse S4 (SwissProt entry P62192), or the rat S4 (SwissProt entry P62193). It also includes a protein having at least 70, 80, 90, 95, 97 or 99% sequence identity to the human sequences S6A (SwissProt entry P17980), S6B (SwissProt entry P43686), S5A (SwissProt entry P55036), S2 (SwissProt entry Q13200), or S4 (SwissProt entry P62191).

"Administering" shall mean delivering in a manner, which is effected or performed using any of the various methods and delivery systems known to those skilled in the art. Administering can be performed, for example, topically, intravenously, pericardially, orally, via implant, transmucosally, transdermally, intramuscularly, subcutaneously, intraperitoneally, intrathecally, intralymphatically, intraleSIONALLY, or epidurally. Administering can also be performed, for example, once, a plurality of times, and/or over one or more extended periods.

The terms "polypeptide," "peptide" and "protein" are used interchangeably herein, and each means a polymer of amino acid residues. The amino acid residues can be naturally occurring or chemical analogues thereof. Polypeptides, peptides and proteins can also include modifications such as glycosylation, lipid attachment, sulfation, hydroxylation, and ADP-ribosylation.

"Subject" shall mean any animal, such as a mammal or a bird, including, without limitation, a cow, a horse, a sheep, a pig, a dog, a cat, a rodent such as a mouse or rat, a turkey, a chicken and a primate. In the preferred embodiment, the subject is a human being.

"Treating" shall include, without limitation, eliminating, reversing the course of, slowing the progression of, reducing the symptoms of, or otherwise ameliorating, a disease in a subject.

30 As used herein, a "compound" is an organic or inorganic assembly of atoms of any size, and includes small molecules (less than about 2500 Daltons) or larger molecules, e.g. peptides, polypeptides, whole proteins and polynucleotides.
The terms "candidate substance" and "test compound" are used interchangeably and refer to a substance that is believed to interact with the binding of HDM2, related proteins or fragments thereof, with the proteasome, proteasome subunits or fragments thereof. Exemplary candidate substances that can be investigated using the methods of the present invention include, but are not restricted to peptides, enzymes, enzyme substrates, co-factors, sugars, oligonucleotides, chemical compounds small molecules and monoclonal antibodies.

"Modulate" shall mean an increase, decrease or other alteration of any or all chemical and biological activities or properties of a wild type or mutant HDM2, proteasome, proteasome subunit or related proteins.

"Interact" shall mean detectable direct and indirect interactions between molecules, including "binding" interactions between molecules. Interactions can, for example, be protein-protein or protein-nucleic acid in nature. Such interactions can be detected using known procedures, for example, yeast two-hybrid assay, immunoprecipitation, SPA-assay or filter binding assays.

With "proteasome binding domain" or "proteasome binding fragment" is meant part of the HDM2 protein or related protein that can bind to the proteasome or a proteasome subunit.

With "protein binding fragments of HDM2" is meant fragments comprising at least 10 amino acids that are contiguous in the parent protein, but may desirably contain at least 11, 12, 13, 14, 15, 20, 30, 40, 60, 80, 100, 150, 200, 250, 300, or 350 amino acids that are contiguous in the parent protein and wherein said fragments are capable of binding other proteins such as but not limited to another part of the HDM2 protein, the proteasome or a proteasome subunit.

With "protein binding fragments of HDM2 related proteins" is meant at least 10 amino acids that are contiguous in the parent protein, but may desirably contain at least 11, 12, 13, 14, 15, 20, 30, 40, 60, 80, 100, 150, 200, 250, 300, or 350 amino acids that are contiguous in the parent protein and wherein said fragments are capable of binding other proteins.

With protein binding fragments of a proteasome or proteasome subunit" is meant at least 10 amino acids that are contiguous in the parent protein, but may desirably contain at least 11, 12, 13, 14, 15, 20, 30, 40, 60, 80, 100, 150, 200, 250, 300, or 350 amino acids that are contiguous in the parent protein and wherein said fragments are
capable of binding proteins such as e.g. HDM2, or a proteasome binding fragment thereof.

Embodiments of the Invention

5 Assays

Assays can be designed in many formats generally known in the art of screening compounds for biological activity or for binding proteins.

10 The assays can exploit the fact that disturbance of the interaction between HDM2 and the proteasome, proteasome subunits and the binding fragments thereof affect the downstream-targeted degradation of ubiquitinilated proteins.

Furthermore, the present invention makes use of methods for identifying compounds that specifically bind to the HDM2 proteins, wherein said compounds may affect the interaction between HDM2 and the proteasome or its subunits.

The binding of a compound to HDM2 can only require a simple linear stretch of amino acids, can comprise for example a modified (e.g. phosphorylation or hydroxylation) or conformationally sensitive motif or can require several amino acid sequences distributed in a specific way along a protein.

Thus the present invention encompass the use of a compound in an assay that incorporates at least one step wherein the interaction of HDM2, a related protein or the protein binding fragments thereof with said compound, protects HDM2, a related protein or a HDM2 binding protein against proteolysis. In particular the compound protects HDM2, a related protein or a HDM2 binding protein against proteolysis by the UPS-pathway. In another particular embodiment the compound protects HDM2, a related protein or a HDM2 binding protein against proteolysis by a proteolytic enzyme such as trypsin.

Thus, the present invention encompass the use of a compound in an assay, said assay comprising:

a) contacting the compound to be tested with a HDM2 protein, a related protein or a protein binding fragment thereof and
b) determining whether said compound affects the proteolysis of a HDM2 protein, a related protein, a protein binding fragment thereof or of a HDM2 binding protein.

In an embodiment of the invention, proteolysis of a HDM2 protein, a related protein, a protein binding fragment thereof or of a HDM2 binding protein can be effected in cells, cell lysates, in vitro UPS systems or in vivo systems.

In another embodiment the HDM2 binding protein is p53.

In another embodiment the HDM2 binding protein is other than p53.

In another embodiment, the invention encompass the use of a compound in an assay wherein said assay simply measures the binding of a candidate compound to the polypeptide (e.g. HDM2, a related protein a protein binding fragment thereof, a HDM2 binding protein, a proteasome subunit or a protein binding fragment thereof) or to cells or membranes bearing the polypeptide, or a fusion protein comprising said polypeptide by means of a label directly or indirectly associated with the candidate compound.

The compounds can be labeled with labeling agents such as radioisotopes, enzymes, fluorescent substances, luminous substances, photoaffinitive substances etc. Examples of the radioisotopes include $^{125}$I, $^{131}$I, $^{3}$H and $^{14}$C. Enzymes are usually made detectable by conjugation of an appropriate substrate which, in turn catalyses a detectable reaction. Examples thereof include, for example, beta-galactosidase, beta-glucosidase, alkaline phosphatase, peroxidase and malate dehydrogenase, preferably horseradish peroxidase. The luminous substances include, for example, luminol, luminol derivatives, luciferin, aequorin and luciferase.

The present invention also encompass the photoaffinity labeled compounds of formula (II) and the photoaffinity and tritium labeled compounds of formula (III) and (IV).
Alternatively, the screening method may involve competition with a labeled competitor. In a preferred embodiment, this labeled competitor is a ligand known to bind to the above described proteasome subunits or fragments thereof or to the above described HDM2 protein, related proteins or fragments thereof.

It will be readily appreciated by the skilled artisan that the discovery of the interaction of HDM2 or the proteasome binding fragments thereof with the compounds of formula (I) may also be used in a method for the structure-based or rational design of an antagonist of the UPS-pathway, by:

a) identifying contacting atoms in the binding site of the compound of formula (I) that interact with HDM2, related proteins or fragments thereof,

b) design test compounds that interact with the atoms identified in (a) to modulate the activity of the proteasome, and
contact said designed test compound with HDM2, a related protein or a protein binding fragment thereof to measure the capability of said compound to modulate the UPS activity.

It will be further appreciated that this will normally be an iterative process. This invention further provides a method for evaluating the potential of a test compound to interact with binding domains of e.g. HDM2 or related proteins said method comprising:

(a) using molecular modeling techniques to formulate a three dimensional structure of the binding domain,

(b) employing computational means to perform a fitting operation between the test compound and the three-dimensional structure of the binding domain, and

(c) analyzing the results of said fitting operation to quantify the association of the test compound with the three dimensional structure of the binding domain.

Molecular modeling techniques are known in the art, including both hardware and software appropriate for creating and utilizing models of receptors and enzyme conformations. Numerous computer programs are available and suitable for the processes of computer modeling, model building and computationally identifying, selecting and evaluating potential interacting compounds in the methods described herein. These include for example, GRID (available from Oxford University, UK), MCGSS (available from Accelrys, Inc., San Diego, CA), AUTODOCK (available from Oxford Molecular Group), FLEX X (available from Tripos, St. Louis. MO), DOCK (available from University of California, San Francisco, CA), CAVEAT (available from University of California, Berkeley), HOOK (available from Accelrys, Inc., San Diego, CA) and 3D database systems such as MACCS-3D (available from MDL Information Systems, San Leandro, CA), UNITY (available from Tripos, St. Louis.MO) and CATALYST (available from Accelrys, Inc., San Diego, CA). Potential candidate substances may also be computationally designed "de novo" using software packages as LUDI (available from Biosym Technologies, San Diego, CA), LEGEND (available from Accelrys, Inc, San Diego, CA) and LEAPFROG (available from Tripos, St. Louis.MO). Compound deformation energy and electrostatic repulsion, may be analyzed using programs such as GAUSSIAN 92, AMBER, QUANTA/CHARMM and INSIGHT II/DISCOVER. These computer evaluation and modeling techniques may be performed on any suitable hardware including for example, workstations available from Silicon Graphics, Sun Microsystems and others. These modeling
techniques, methods, hardware and software packages are representative and are not intended to be a comprehensive listing. Other modeling techniques known in the art may also be employed in accordance with this invention. See for example, N.C. Cohen, *Molecular Modeling in Drug Design*, Academic Press (1996).

In this screening, the quality of fit of such compounds to the binding site may be judged either by shape complementarity or by estimated interaction energy (Meng, E.C. et al., *J. Comma.Chem* 13:505-524 (1992)).


Once a compound has been designed or selected by the above methods, the affinity with which that compound may bind or associate with a binding domain may be tested and optimized by computational evaluation and/or by testing biological activity after synthesizing the compound. Inhibitors or compounds may interact with the binding domain in more than one conformation that is similar in overall binding energy. In those cases, the deformation energy of binding is taken to be the difference between the energy of the free compound and the average energy of the conformations observed when the compound binds to the binding domain.

A compound designed or selected as binding or associating with a binding domain may be further computationally optimized so that in its bound state it would preferably lack repulsive electrostatic interaction with the binding domains. Such non-complementary (e.g., electrostatic) interactions include repulsive charge-charge, dipole-dipole and charge-dipole interactions. Specifically, the sum of all electrostatic interactions between the inhibitor and a binding domain when the inhibitor is bound, preferably make a neutral or favorable contribution to the enthalpy of binding. Weak binding compounds will also be designed by these methods so as to determine SAR. See, for example, U. S. Appl. Nos. 60/275,629; 60/331,235; 60/379,617; and 10/097,249.

Specific computer software is available in the art to evaluate compound deformation energy and electrostatic interaction. Examples of programs designed for such uses include: Gaussian 92, revision C (M. J. Frisch, Gaussian, Inc., Pittsburgh, Pa.,
COPYRGT 1992); AMBER, version 4.0 (P. A. Kollman, University of California at San Francisco, COPYRGT 1994); QUANTA/CHARMM (Molecular Simulations, Inc., Burlington, Mass. COPYRGT 1994); and Insight II/Discover (Biosysm Technologies Inc., San Diego, Calif. COPYRGT 1994). Other hardware systems and software packages will be known to those skilled in the art.

Once a compound that associates with a binding domain has been optimally selected or designed, as described above, substitutions may then be made in some of its atoms or side groups in order to improve or modify its binding properties. Generally, initial substitutions are conservative, i.e., the replacement group will have approximately the same size, shape, hydrophobicity and charge as the original group. It should, of course, be understood that components known in the art to alter conformation may be avoided. Such substituted chemical compounds may then be analyzed for efficiency of fit to a proteasome binding domain or a protein binding domain by the same computer methods described in detail, above.

Use of the compounds according to the invention.

EP1809622 concerns the preparation, formulation and pharmaceutical properties of inhibitors of the interaction between HDM2 and p53 wherein said inhibitor is a compound of formula (I),

![Chemical Structure](image)

(I)

a N-oxide form, an addition salt or a stereochemically isomeric form thereof, wherein

- m is 0, 1, or 2 and when m is 0 then a direct bond is intended;
- n is 0, 1, 2, or 3 and when n is 0 then a direct bond is intended;
- p is 0, or 1 and when p is 0 then a direct bond is intended;
- s is 0, or 1 and when s is 0 then a direct bond is intended;
t is 0 or 1 and when t is 0 then a direct bond is intended;

X is C(=O) or CHR^8; wherein

R^2 is hydrogen, C_{1-6}alkyl, C_{3-7}cycloalkyl, -C(=O)-NR^{17}R^{18}, hydroxycarbonyl, arylC_{1-6}alkyloxy-carbonyl, heteroaryl, heteroaryl-carbonyl, heteroarylC_{1-6}alkyloxy-carbonyl, piperazinyI-carbonyl, pyrrolidinyl, piperidinyl-carbonyl, C_{1-6}alkyloxy-carbonyl, C_{1-6}alkyl substituted with a substituent selected from hydroxy, amino, aryl, and heteroaryl; C_{3-7}cycloalkyl substituted with a substituent selected from hydroxy, amino, aryl, and heteroaryl; piperazinyl-carbonyl substituted with hydroxy, hydroxyC_{1-6}alkyl, hydroxyC_{1-6}alkyloxyC_{1-6}alkyl; pyrrolidinyl substituted with hydroxyC_{1-6}alkyl; or piperidinyl-carbonyl substituted with one or two substituents selected from hydroxy, C_{1-6}alkyl, hydroxyC_{1-6}alkyl, C_{1-6}alkyloxyC_{1-6}alkyl, C_{1-6}alkyl(dihydroxy)C_{1-6}alkyl or C_{1-6}alkyloxy(hydroxy)C_{1-6}alkyl;

R^{17} and R^{18} are each independently selected from hydrogen, C_{1-6}alkyl, di(C_{1-6}alkyl)aminoC_{1-6}alkyl, arylC_{1-6}alkyl, C_{1-6}alkyloxyC_{1-6}alkyl, hydroxyC_{1-6}alkyl, hydroxyC_{1-6}alkyl(C_{1-6}alkyl) or hydroxyC_{1-6}alkyl(arylC_{1-6}alkyl);

\[
\text{\textbf{Q}} \quad \text{\textbf{-CRI=CRJ}} \quad \text{\textbf{C}} \quad \text{\textbf{and then the dotted line is a bond, -C(=O)-CH<, -C(=O)-N<,}}
\]
\[
\text{\textbf{-CHR^9-CH< or -CHR^9-N<, wherein}}
\]
\[
\text{\textbf{each R^9 is independently hydrogen or C_{1-6}alkyl;}}
\]

R^1 is hydrogen, aryl, heteroaryl, C_{1-6}alkyloxy-carbonyl, C_{1-12}alkyl, or C_{1-12}alkyl substituted with one or two substituents independently selected from hydroxy, aryl, heteroaryl, amino, C_{1-6}alkyloxy, mono- or di(C_{1-6}alkyl)amino, morpholinyl, piperidinyl, pyrrolidinyl, piperazinyl, C_{1-6}alkylpiperazinyl, arylC_{1-6}alkylpiperazinyl,

\[
\text{\textbf{heteroarylC_{1-6}alkylpiperazinyl, C_{3-7}cycloalkylpiperazinyl and}}
\]
\[
\text{\textbf{C_{3-7}cycloalkylC_{1-6}alkylpiperazinyl;}}
\]

R^2 is hydrogen, halo, C_{1-6}alkyl, C_{1-6}alkyloxy, arylC_{1-6}alkyloxy, heteroarylC_{1-6}alkyloxy, phenylthio, hydroxyC_{1-6}alkylcarbonyl, C_{1-6}alkyl substituted with a substituent selected from amino, aryl and heteroaryl; or

C_{3-7}cycloalkyl substituted with a substituent selected from amino, aryl and heteroaryl;
R³ is hydrogen, C₁₋₆alkyl, heteroaryl, C₃₋₇cycloalkyl, C₁₋₆alkyl substituted with a substituent selected from hydroxy, amino, aryl and heteroaryl; or C₃₋₇cycloalkyl substituted with a substituent selected from hydroxy, amino, aryl and heteroaryl;

R⁴ and R⁵ are each independently hydrogen, halo, C₁₋₆alkyl, polyhaloC₁₋₆alkyl, cyano, cyanoC₁₋₆alkyl, hydroxy, amino or C₁₋₆alkyloxy; or R⁴ and R⁵ together can optionally form a bivalent radical selected from methylenedioxy or ethylenedioxy;

R⁶ is hydrogen, C₁₋₆alkyloxycarbonyl or C₁₋₆alkyl;

when p is 1 then R² is hydrogen, arylC₁₋₆alkyl, hydroxy or heteroarylC₁₋₆alkyl;

Z is a radical selected from

![Chemical structures]

wherein each R¹⁰ or R¹¹ are each independently selected from hydrogen, halo, hydroxy, amino, C₁₋₆alkyl, nitro, polyhaloC₁₋₆alkyl, cyano, cyanoC₁₋₆alkyl, tetrazoloC₁₋₆alkyl, aryl, heteroaryl, arylC₁₋₆alkyl, heteroarylC₁₋₆alkyl, aryl(hydroxy)C₁₋₆alkyl, heteroaryl(hydroxy)C₁₋₆alkyl, arylethoxycarbonyl, heteroarylcarbonyl, C₁₋₆alkylcarbonyl, arylC₁₋₆alkylcarbonyl, heteroarylC₁₋₆alkylcarbonyl, C₁₋₆alkyloxy, C₃₋₇cycloalkylcarbonyl, C₃₋₇cycloalkyl(hydroxy)C₁₋₆alkyl,
arylC₆₋₈alkyloxyC₆₋₈alkyl, C₆₋₈alkyloxyC₆₋₈alkyloxyC₆₋₈alkyl,
C₆₋₈alkylcarbonyloxyC₆₋₈alkyl, C₆₋₈alkyloxy carbonylC₆₋₈alkyloxyC₆₋₈alkyl,
hydroxyC₆₋₈alkyloxyC₆₋₈alkyl, C₆₋₈alkyloxy carbonylC₂₋₈alkenyl
C₆₋₈alkyloxyC₆₋₈alkyl, C₆₋₈alkyloxy carbonyl, C₆₋₈alkylcarbonyloxy,
aminocarbonyl, hydroxyC₆₋₈alkyl, aminoc₆₋₈alkyl, hydroxycarbonyl,
hydroxycarbonylC₆₋₈alkyl and -(CH₂)ᵣ-(C(=O)ᵢ)-(CHR¹⁹)ᵣ-NR¹⁰R¹⁴; wherein

v is 0, 1, 2, 3, 4, 5, or 6 and when v is 0 then a direct bond is intended;

r is 0, or 1 and when r is 0 then a direct bond is intended;

u is 0, 1, 2, 3, 4, 5, or 6 and when u is 0 then a direct bond is intended;

R¹⁰ is hydrogen or C₆₋₈alkyl;

R¹² is hydrogen, C₆₋₈alkyl, C₃₋₇cycloalkyl, C₆₋₈alkyl substituted with a substituent
selected from hydroxy, amino, C₆₋₈alkyloxy and aryl; or C₃₋₇cycloalkyl substituted
with a substituent selected from hydroxy, amino, aryl and C₆₋₈alkyloxy;

R¹³ and R¹⁴ are each independently selected from hydrogen, C₁₋₁₂alkyl,
C₆₋₈alkylcarbonyl, C₆₋₈alkylsulfonyl, arylC₆₋₈alkylcarbonyl,
C₃₋₇cycloalkyl, C₃₋₇cycloalkylcarbonyl, -(CH₂)ᵣ-NR¹⁵R¹⁶, C₁₋₁₂alkyl
substituted with a substituent selected from hydroxy, hydroxycarbonyl,
cyano, C₆₋₈alkyloxy carbonyl, C₆₋₈alkyloxy, aryl or heteroaryl; or
C₃₋₇cycloalkyl substituted with a substituent selected from hydroxy,
C₆₋₈alkyloxy, aryl, amino, arylC₆₋₈alkyl, heteroaryl or heteroarylC₆₋₈alkyl;
or
R¹³ and R¹⁴ together with the nitrogen to which they are attached can
optionally form a morpholinyl, piperidinyl, pyrrolidinyl, piperazinyl, or
piperazinyl substituted with a substituent selected from C₁₋₆alkyl, arylC₁₋₆alkyl,
arylC₆₋₈alkyloxy carbonyl, heteroarylC₁₋₆alkyl, C₃₋₇cycloalkyl and
C₃₋₇cycloalkylC₁₋₆alkyl; wherein

k is 0, 1, 2, 3, 4, 5, or 6 and when k is 0 then a direct bond is intended;

R¹⁵ and R¹⁶ are each independently selected from hydrogen, C₁₋₆alkyl,
arylC₁₋₆alkyloxy carbonyl, C₃₋₇cycloalkyl, C₁₋₁₂alkyl substituted with
a substituent selected from hydroxy, C\textsubscript{1-6}alkyloxy, aryl, and heteroaryl; and C\textsubscript{3-7}cycloalkyl substituted with a substituent selected from hydroxy, C\textsubscript{1-6}alkyloxy, aryl, arylC\textsubscript{1-6}alkyl, heteroaryl, and heteroarylC\textsubscript{1-6}alkyl; or

\begin{align*}
\text{R}^{15} \text{ and R}^{16} \text{ together with the nitrogen to which they are attached can optionally form a morpholinyl, a piperazinyl or a piperazinyl substituted with C\textsubscript{1-6}alkyloxy carbonyl;}
\end{align*}

aryl is phenyl or naphthalenyl;

each phenyl or naphthalenyl can optionally be substituted with one, two or three substituents each independently selected from halo, hydroxy, C\textsubscript{1-6}alkyl, amino, polyhaloC\textsubscript{1-6}alkyl and C\textsubscript{1-6}alkyloxy; and each phenyl or naphthalenyl can optionally be substituted with a bivalent radical selected from methylenedioxy and ethylenedioxy;

heteroaryl is pyridinyl, indolyl, quinolinyl, imidazolyl, furanyl, thiienyl, oxadiazolyl, tetrazolyl, benzofuranyl or tetrahydrofuranyl;

each pyridinyl, indolyl, quinolinyl, imidazolyl, furanyl, thiienyl, oxadiazolyl, tetrazolyl, benzofuranyl, or tetrahydrofuranyl can optionally be substituted with one, two or three substituents each independently selected from halo, hydroxy, C\textsubscript{1-6}alkyl, amino, polyhaloC\textsubscript{1-6}alkyl, aryl, arylC\textsubscript{1-6}alkyl or C\textsubscript{1-6}alkyloxy; and each pyridinyl, indolyl, quinolinyl, imidazolyl, furanyl, thiienyl, benzofuranyl, or tetrahydrofuranyl can optionally be substituted with a bivalent radical selected from methylenedioxy or ethylenedioxy;

with the proviso that when m is 1; the substituents on the phenyl ring other than R\textsuperscript{2} are in the meta position; s is 0; and t is 0; then

\begin{align*}
Z \text{ is a radical selected from (a-1), (a-3), (a-4), (a-5), (a-6), (a-7), (a-8) or (a-9).}
\end{align*}

A particular group of compounds are those compounds of formula (I) wherein Z is a radical selected from (a-1), (a-2), (a-3) or (a-4).

An even more particular compound is compound JNJ\#1 described as compound 36 in EP1809622.
The invention concerns the use of a compound of formula (I) for the manufacture of a medicament for the treatment of a disorder mediated through the binding of HDM2 with the proteasome.

More in particular the invention concerns the use of a compound of formula (I) for the manufacture of a medicament for the treatment of a disorder mediated through proteolysis of HDM2, of a related protein or of a HDM2 binding protein.

Even more in particular the present invention concerns the use of a compound of formula (I) for the manufacture of a medicament for the treatment of a disorder mediated through the binding of HDM2 with the proteasome and through proteolysis of HDM2, of a related protein or of a HDM2 binding protein.

Thus, the invention concerns the use of a compound of formula (I) for the manufacture of a medicament for the treatment of a disorder mediated through proteolysis of HDM2, a related protein or a HDM2 binding protein, in particular a disorder mediated through proteolysis of HDM2, a related protein or a HDM2 binding protein by the UPS-pathway, also in particular a disorder mediated through proteolysis of HDM2, a related protein or a HDM2 binding protein by a proteolytic enzyme such as e.g. trypsin.

This invention also provides a method for treating a disorder mediated through the binding of HDM2 with the proteasome or through proteolysis of HDM2, a related protein or a HDM2 binding protein, by administering an effective amount of a compound of the present invention, to a subject, e.g. a mammal (and more particularly a human) in need of such treatment.

The compounds of the invention can have antiproliferative effects in tumour cells, even if such cells are devoid of functional p53. More in particular, the compounds of
the invention can have antiproliferative effects in tumours with wild-type p53 and/or in tumours overexpressing MDM2.

Thus, this invention also provides a method for inhibiting tumour growth by administering an effective amount of a compound of the present invention, to a subject, e.g. a mammal (and more particularly a human) in need of such treatment.

Examples of tumours which may be inhibited, but are not limited to, lung cancer (e.g. adenocarcinoma and including non-small cell lung cancer), pancreatic cancers (e.g. pancreatic carcinoma such as, for example exocrine pancreatic carcinoma), colon cancers (e.g. colorectal carcinomas, such as, for example, colon adenocarcinoma and colon adenoma), prostate cancer including the advanced disease, hematopoietic tumours of lymphoid lineage (e.g. acute lymphocytic leukemia, B-cell lymphoma, Burkitt's lymphoma), myeloid leukemias (for example, acute myelogenous leukemia (AML)), thyroid follicular cancer, myelodysplastic syndrome (MDS), tumours of mesenchymal origin (e.g. fibrosarcomas and rhabdomyosarcomas), melanomas, teratocarcinomas, neuroblastomas, gliomas, benign tumour of the skin (e.g. keratoacanthomas), breast carcinoma (e.g. advanced breast cancer), kidney carcinoma, ovary carcinoma, bladder carcinoma and epidermal carcinoma.

The compounds of formula (I) can prevent binding of HDM2 to the proteasome without preventing the binding between HDM2 and HDM2-binding proteins such as e.g. p53.

The compounds of formula (I) can prevent binding of HDM2 to the proteasome without preventing the ubiquitination of HDM2-binding proteins such as e.g. p53.

The compounds of formula (I) can prevent proteolysis of HDM2, related proteins or HDM2-binding proteins without preventing the binding between HDM2 and HDM2-binding proteins such as e.g. p53.

The compounds of formula (I) can prevent proteolysis of HDM2, related proteins or HDM2-binding proteins without preventing the ubiquitination of HDM2-binding proteins such as e.g. p53.

This invention will be better understood by reference to the Experimental Details that follow, but those skilled in the art will readily appreciate that these are only illustrative of the invention as described more fully in the claims that follow thereafter. Additionally,
throughout this application, various publications are cited. The disclosure of these publications is hereby incorporated by reference into this application to describe more fully the state of the art to which this invention pertains.

EXPERIMENTAL

Materials and Methods

Plasmids

The plasmids pDWM659 encoding Myc-MDM2 as well as the plasmids pcDNA3-Mdm2 and pcDNA3-p53 have been described previously (Blattner et al., 2002). Full length GST-HDM2, GST-HDM2 fragments GST-HDM2-1-206, GST-HDM2-293-493, GST-S6b-EDY peptide and GST-HDM2-EDY peptide were created by amplifying the respective sequences by PCR using primers containing appropriate restriction sites. GST-HDM2-100-200 MTAP protein was ordered with Abnova Corporation – Catalog number H00004507-P02. The PCR fragments were digested with EcoRI and NotI and cloned into the pGex-4T-2 vector. GST-HdmX was created by amplifying HdmX by PCR using reversely transcribed RNA as a template and primers encoding appropriate restriction sites. The PCR fragment was ligated into the pGEX-4T-2 vector. Expression and purification of proteins were performed according to the recommendation of the supplier of the pGEX-4T-2 vector (Amersham). HIS-p53 was created by amplifying p53 by PCR using cDNA as a template and primers encoding appropriate restriction sites. The PCR fragment was ligated into the pRSETA vector. Expression and purification of proteins were performed according to the recommendation of the supplier of the pRSETA vector (Invitrogen). Baculoviruses expressing E1 and the plasmid for UbcH5 were kindly provided by Martin Scheffner, Konstanz. Baculoviruses encoding Flag-Mdm2 and Flag-p53 have been described previously (Brignone et al., 2004).

Antibodies

The following antibodies were used: the anti-myc antibody 9E10 (Santa Cruz), the anti-proliferating nuclear cell antigen (PCNA) monoclonal antibody PC10 (Santa Cruz), the anti-S8 antibody clone P45-110 (Biomol), an anti-S6b rabbit polyclonal antibody (Biomol), the anti-HDM2 antibodies C18, SMP14 (Santa Cruz) and 4B2 (Oncogene Sciences), the anti-p53 antibody DO-1 (Santa Cruz), and the HRP-coupled anti-mouse (P0161) and anti-rabbit (P0448) antibody (DAKO) and True-blotted anti-
rabbit antibody (eBiosciences). The HRP-coupled antibody directed against V5 (Invitrogen) and the anti-GST antibody (Rockland).

**Cell lines and their treatments**

293T, H1299 and U2OS cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% foetal calf serum (FCS) and 100 units/ml penicillin/streptomycin at 37°C and 5% CO₂ in a humidified atmosphere. H1299 and 293T cells were transiently transfected by calcium-phosphate, U2OS cells were transfected with jetPEI (Biomol) according to the manufacturers recommendations.

**Immunoprecipitation and Western blotting**

Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in NP-40 buffer (150 mM NaCl, 50 mM Tris pH 8, 5 mM EDTA, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride). The protein extract was cleared by centrifugation at 13000 g at 4°C for 15 min and the protein concentration was determined by the method of Bradford. 3 µl of the 9E10 anti-myc antibody, pre-coupled to Protein A-Agarose (Pierce), were added to 300 µg of the lysate and the mixture was incubated on a rotating wheel at 4°C for 2 hours. The agarose was washed three times with NP-40 lysis buffer and resuspended in 1 x SDS sample buffer (2% sodium dodecyl sulfate, 0.08 M Tris pH 6.8, 10% glycerol, 2% β-mercaptoethanol, 0.001% bromophenol blue).

For Western blotting, 50 µg of protein were mixed with an equal volume of 2 x SDS sample buffer, heat denatured and loaded onto a SDS-10% PAGE gel. The proteins were transferred onto Immobilon-P blotting membrane (Millipore). Immunodetection was performed as described (Blattner et al., 2002).

**Coimmunoprecipitation**

**Coimmunoprecipitation of HDM2 and p53 from Cells:**

JAR choriocarcinoma cells were seeded in 10 cm dishes at 3.6 x 10⁶ cells/plate and were incubated the next day with the indicated concentrations of JNJ#1, Nutlin-3 or the inactive enantiomer of Nutlin-3 for 1.5 hours.

Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in Triton-X buffer (50 mM NaCl, 10 mM Tris pH 7.2, 5 mM EDTA, 1% Triton X-100). The protein extract was sonicated (for HDM2/p53 coIP), cleared by centrifugation at 13000 g at 4°C for 15 min and the protein concentration was determined by the method of BCA/Pierce. The lysate (1 mg) was pre-cleared by adding 20 µl mouse IgG serum and 30µl protein A-Agarose and incubating the
mixture on a rotating wheel at 4°C for 2 hours. Subsequently 10 μl of the 2A 10 anti-HDM2 antibody was added to the cleared lysate and rotated for 2 hours, and next 30μl protein A-Agarose was added followed by rotating an additional 16 hours at 4°C. Immunoprecipitates were washed three times using Co-IP wash buffer (100 mM NaCl, 50 mM Tris pH 7.5, 1 mM EDTA, 0.1% Triton X-100, 5% glycerol).

Immunoprecipitated HDM2 proteins were revealed using SMP-14 antibody (sc-965) and p53 protein was revealed as specified under Western Blot analysis.

**Coimmunoprecipitation of HDM2 and proteasomes from Cells:**

Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in NP-40 buffer (150 mM NaCl, 50 mM Tris pH 8, 5 mM EDTA, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride). The protein extract was cleared by centrifugation at 13000 g at 4°C for 15 min and the protein concentration was determined by the method of Bradford. 3 μl of the 9E10 anti-myc or anti-HDM2 antibody, pre-coupled to Protein A-Agarose (Pierce), were added to 1 mg of the lysate and the mixture was incubated on a rotating wheel at 4°C for 1.5 hours. The agarose was washed three times with NP-40 lysis buffer. 40 μl 1 x SDS sample buffer (2% sodium dodecyl sulfate, 0.08 M Tris pH 6.8, 10% glycerol, 2% β-mercaptoethanol, 0.001% bromophenol blue) were added and the samples were heat denatured before loading onto a SDS-10% PAGE gel. The proteins were transferred onto Immobilon-P blotting membrane (Millipore). Immunodetection was performed as described (Blattner et al., 2002).

**Coimmunoprecipitation of HDM2 and proteasomes in vitro:**

100 ng GST-Hdm2 expressed in bacteria and 2 μl proteasomes (USbio) were preincubated with JNJ#1 in 150 μl PWB buffer (50 mM Tris pH 7.5; 150 mM NaCl; 10% glycerol; 5 mM MgCl2; 2 mM ATP). Then HDM2 and proteasomes were combined and incubated for 30 min at room temperature. 20 μl of the mixture were taken for input control and 3 μl of anti-C18 pre-coupled to 20 μl protein-A Agarose were added. The mixture was incubated for 1.5 hours on a rotating wheel at 4°C, washed 3 x with PWB buffer and separated on a 10% SDS-PAGE gel.

**Western blotting of the lysates**

U87 glioblastoma cells were incubated with the indicated concentrations of JNJ#1 for 24 h. Total cell lysates were prepared and analyzed by SDS/PAGE. Levels of protein were detected using specific antibodies for p53 (DO-1, and pAb1801, Santa Cruz), p21 waf1/cip (BD Pharmingen), HDM2 (2A10, OP115 Calbiochem), E2F1 (sc-193, Santa Cruz), Rb (sc-102, Santa Cruz), pRb_Ser780 (9307L, Cell Signalling), anti-p73
(ab22045 Abcam), anti-p63 (sc-8431, Santa Cruz), cyclin G1 (sc-320, Santa Cruz), PIG3 (PC268, Calbiochem), MIC-1 (sc-10606, Santa Cruz). Actin protein levels (Ab-1, Oncogene Research products) were revealed as a control for equal loading. Protein-antibody complexes were visualized by chemiluminescence (Super Signal West Dura reagent, Pierce Chemical) and fluorescence (Odyssey) according to manufacturer's instructions.

**In vitro p53 degradation assay**
Flag-MDM2/p53 complexes were purified from High5 insect cells by a Flag-Agarose purification kit according to the recommendation of the supplier (Sigma). 0.2 μl partially purified E1 enzyme expressed in insect cells, 2 μl bacterial lysate of BL21 cells expressing UbCh5, 2 μl ubiquitin (5 μg/μl Sigma), 5 μl purified MDM2/p53 complexes, 1 μl Mg-ATP (100 mM) in 30 μl ubiquitin reaction buffer (25 mM Hapes pH 7.4; 10 mM NaCl; 3 mM MgCl2; 0.05% Triton X-100; 0.5 mM DTT).

After 30 min reaction time, JNJ#1 was added to a final concentration of 10 μM and incubated for 5 min at room temperature. 1 μl 26S proteasome (USbio) and 1 μl ATP (100 mM) were added and the reaction was incubated for 2.5 hours at 37°C. 1 μl of ATP (100 mM) was added and the reaction was incubated for further 2.5 hours. An equal volume of 2 x sample buffer was added, the reaction was separated by a 8% SDS-PAGE gel and blotted onto Immobilon-P membrane.

**Limited proteolysis**
100 ng of GST fused to HdmX or fragments of HDM2 were incubated for 5 minutes with JNJ#1, active or inactive nutlin, MG132 or DMSO, for control. Then 40 ng trypsin was added and the mixture was incubated for 15 min on ice. Proteolysis was stopped by addition of 2x sample buffer. Samples were loaded onto an 12 or 15% SDS-PAGE gel and HDM2 or HDMX were detected by Western blotting.

**Photoaffinity Labeling of the proteins: Material**
The baculovirus expressed proteins, Flag HDM2FL and Flag HDM2 (AA200-491) were kindly provided by Christine Blattner (Forschungszentrum Karlsruhe, Institute of Genetics & Toxicology, Germany).
Recombinant protein p53HIS was isolated from an E.Coli expression system. BSA was obtained from Pierce (23209).
To determine the concentration, proteins were subjected to SDS-PAGE together with a concentration curve of BSA. Gels were stained with Coomassie Blue, intensity of
the bands was measured and concentration of the protein was determined via the BSA curve.

**Photoaffinity Labeling of the proteins: Procedure**

Equal amounts of proteins (21 pmol) were diluted in a microtiterplate to a total volume of 100 µl binding buffer (10 mM HEPES pH 7.6, 150 mM NaCl, 0.1% tween 20) containing photoaffinity labeled and tritium labeled JNJ-#1. The samples were incubated on ice for 15 minutes, followed by 10 min of UV irradiation at 4 °C (365 nm ultraviolet hand held lamp EN-280L from Spectroline). The UV irradiation experiments were carried out by maintaining the distance of 5 cm between the sample and light source.

After irradiation, the mixture was run through a column (microspin G25 from Pharmacia 27-5325-01) to remove access of compound. The eluates were denaturized with Nupage denaturating agent (Invitrogen, NP0009) and Nupage LDS sample buffer (Invitrogen, NP007), followed by PAGE gel separation (Nupage Bis-Tris 4-12% gels from Invitrogen with MOPS running buffer).

To make the tritium signal more available for detection, proteins were transferred to PVDF membrane by Western blotting (using the XCell II™ Blot Module). After two weeks exposure the phosphor imager screens were analyzed on a Fujix Bas 200, the amount of radioactivity linked to the proteins. Bands were quantified.

**Process for making the photoaffinity labeled and/or tritium labeled JNJ-#1**

Hereinafter, “DCM” is defined as dichloromethane, “DMA” is defined as N,N-dimethylacetamide, “EtOAc” is defined as ethyl acetate, “THF” is defined as tetrahydrofuran, “DIPEA” is defined as diisopropylethylamine and “MeOH” is defined as methanol.

**A. Preparation of the intermediate compounds**

Example A1

a) Preparation of intermediate 1

Methanesulfonyl chloride (1ml, 9.2mmol) was added dropwise to a solution of 3-methyl-3H-diazirine-3-propanol (1g, 8.76mmol), triethylamine (1.8ml, 13.1mmol) in DCM (20ml) at 5°C. The mixture was stirred at 5°C for 2 hours. Potassium carbonate 10% and DCM were added, the mixture was extracted, the organic layer was
separated, dried over MgSO₄, filtered and the solvent was evaporated, yielding 1.6g (100%) of intermediate 1.
NMR (DMSO) : 3H (s, 1.02ppm), 2H (m, 1.45ppm), 2H (m, 1.6ppm), 3H (s, 3.18ppm), 2H (t, J = 5.6Hz, 4.18ppm)
b) Preparation of intermediate 2

Sodium hydride (60% in oil, 0.3g, 7.52mmol) was added portionwise at 5°C under N₂ flow to a solution of 4-chloro- 2-pyridinemethanol (0.8g, 4.14mmol) in THF (10ml). The mixture was stirred at room temperature for 1 hour. A solution of intermediate 1 (0.54g, 3.76mmol) in THF (5ml) was added dropwise at room temperature. The reaction mixture was refluxed overnight. Water and EtOAc were added. The mixture was extracted, the organic layer was separated, dried over MgSO₄, filtered and evaporated. The residue (0.8g) was purified by column chromatography over silica gel (eluent 25/95 MeOH/CO). The pure fractions were collected and the solvent was evaporated, yielding 0.5g (50%) of intermediate 2.
NMR (DMSO) : 3H (s, 1ppm), 4H (m, 1.42ppm), 2H (t, J = 5.6Hz, 3.48ppm), 2H (s, 4.52ppm), 2H (m, 7.46ppm), 1H (d, J =5.3Hz , 8.5ppm)

Example A2
a) Preparation of intermediate 3

A mixture of 3-[[2-[(1,1-dimethylethoxy)carbonyl][4-nitrophenyl]amino]ethyl]- 1H-indole-1-carboxylic acid, 1,1-dimethylethyl ester (0.042 mol) and Raney Nickel (20g) in MeOH (200ml) was hydrogenated at room temperature for 3 hours under a 3 bar pressure, then filtered over celite. Celite was washed with DCM/MeOH. The filtrate was evaporated. The crude oil was dissolved in DCM. The organic layer was washed with potassium carbonate 10%, dried (MgSO₄), filtered and the solvent was evaporated. The residue (18g) was purified by column chromatography over silica gel (15-40µm) (eluent: DCM/MeOH 99.5/0.5). The pure fractions were collected and the solvent was evaporated, yielding intermediate 3.
b) Preparation of intermediate 4

A mixture of intermediate 2 (0.0016 mol), intermediate 3 (0.0016 mol) and HCl/isopropanol (0.2 eq.) in acetonitrile (10ml) was stirred at 65°C overnight. Potassium carbonate 10% was added. The mixture was extracted with EtOAc. The organic layer was separated, dried (MgSO₄), filtered and the solvent was evaporated. The residue (0.28g) was purified by column chromatography over silica gel (eluent: DCM/MeOH/NH₄OH 95/5/0.1). The pure fractions were collected and the solvent was evaporated, yielding 0.16g (17%) of intermediate 4.

Example A3
a) Preparation of intermediate 5

\[
\text{N-(2-(6-bromo-1H-indol-3-yl)ethyl)-4-nitroaniline}
\]

A mixture of 6-bromotryptamine (0.0048 mol), 1-fluoro-4-nitro- benzene (0.0053 mol) and DIPEA (0.0122 mol) was stirred at 210°C overnight, then brought to room temperature, taken up in water/acetonitrile (50/50) and extracted with DCM/MeOH. The organic layer was separated, dried (MgSO₄), filtered and the solvent was evaporated. The insoluble residue was recovered. The residue was purified by column chromatography over silicagel (70-200µm) (eluent: DCM/MeOH 98/2). The pure fractions were collected and the solvent was evaporated. The residue (1.227g) was purified by column chromatography over silica gel (15-40µm) (eluent: DCM 100). The pure fractions were collected and the solvent was evaporated, yielding 0.657g (45%) of intermediate 5.

b) Preparation of intermediate 6

\[
\text{N¹-(2-(6-bromo-1H-indol-3-yl)ethyl)benzene-1,4-diamine}
\]

To a mixture of intermediate 5, (5.50 mg), Platinum on Carbon (5 %, 5.045 mg) and vanadium pentoxide (0.598 mg) was added 100 µl of a preprepared solution of THF
(1.00 ml, distilled from sodium) and thiophene 4 % in diisopropyl ether (8 μl). The mixture was degassed and then stirred for 4 hours at room temperature under hydrogen atmosphere. The mixture was filtered over an Acrodisk filter which was rinsed with THF. The filtrate was depleted with THF to a total volume of 1.0 ml, giving a product concentration of approximately 0.55 mg/ml of intermediate 6. Structure was confirmed via LC-MS.

c) Preparation of intermediate 7

\[
\text{N}^1-(2-(6-	ext{tritio}-1H-	ext{indol-3-yl})\text{ethyl})\text{benzene-1,4-diamine}
\]

Diisopropyl ethylamine (6.75 μl) was added to intermediate 6 (500 μl). Part of this solution (241 μl, containing approx. 1.20 mg of intermediate 6 was added to Palladium on Carbon (10 %, 1.20 mg) which was brought into a small reaction vial. The mixture was degassed and tritium gas (103.6 GBq, 2.8 Ci) was brought onto the reaction mixture, which was stirred for 45 minutes. The mixture was then frozen with liquid nitrogen and tritium gas was removed. Labile tritium was removed via lyophilization of the solvent. The residue was taken up in THF (250 μl) and filtered over a GHP Acrodisk® filter, which was then rinsed with THF (2 x 125 μl), yielding intermediate 7. This solution was used as such in the next reaction step.

Example A4

a) Preparation of intermediate 8

\[
\text{N}-(2-(1H-	ext{indol-3-yl})\text{ethyl)-2-bromo-4-nitroaniline}
\]

A mixture of 2-bromo-1-fluoro-4-nitrobenzene (1.09 g, 5 mmol), tryptamine (0.80 g, 5 mmol) and potassium carbonate (1.04 g, 7.5 mmol) in N,N-dimethyl acetamide (5.0 ml) was stirred for 24 hours at 75°C. It was then allowed to cool to room temperature and diethyl ether (100 ml) was added. The mixture was washed with water (3 x 30 ml) and with brine (3 x 30 ml). The solution was dried on magnesium sulphate, filtered and concentrated, yielding 1.73 g (96 %) of intermediate 8 (100 % TLC purity (Rf: 0.125; hexane-ethyl acetate 80:20; v/v)).

b) Preparation of intermediate 9

\[
\text{H}_2\text{N-Br}
\]
-30-

\[ N^1-(2-(1H\text{-}indol\text{-}3\text{-}yl})\text{ethyl}\text{-}2\text{-bromobenzene\text{-}1,4-diamine} \]

To a mixture of intermediate 8 (11.3 mg), Platinum on Carbon (5%, 11.0 mg) and vanadium pentoxide (1.12 mg) was added THF (1.00 ml, distilled from sodium) and thiophene 4% in diisopropyl ether (8 μl). The mixture was degassed and then stirred for 30 minutes at room temperature under hydrogen atmosphere. The mixture was filtered over an Acrodisk filter which was rinsed with THF. The filtrate was depleted with THF to a total volume of 1.1 ml, giving a product concentration of approx. 10.0 mg/ml, yielding intermediate 9. Structure was confirmed via LC-MS.

c) Preparation of intermediate 10

\[ N^1-(2-(1H\text{-}indol\text{-}3\text{-}yl})\text{ethyl}\text{-}2\text{-tritiobenzene\text{-}1,4-diamine} \]

Diisopropyl ethylamine (13.5 μl) was added to intermediate 9 (1.00 ml). Part of this solution (140 μl, containing approx. 1.40 mg of intermediate 9 was added to Palladium on Carbon (10%, 1.40 mg) which was brought into a small reaction vial. The mixture was degassed and tritium gas (103.6 GBq, 2.8 Ci) was brought onto the reaction mixture, which was stirred for 20 minutes. The mixture was then frozen with liquid nitrogen and tritium gas was removed. Labile tritium was removed via lyophilization of the solvent. The residue was taken up in THF (250 μl) and filtered over a GHP Acrodisk® filter, which was then rinsed with THF (2 x 125 μl), yielding intermediate 10. This solution was used as such in the next reaction step.

B. Preparation of the final compounds

Example B1

Preparation of compound 1

\[ \text{HN} \]

A mixture of intermediate 4 (0.0002 mol) and HCl 3N (0.001 mol) in acetonitrile (5ml) was stirred at 65°C for 3 hours. Potassium carbonate 10% was added. The mixture was extracted with EtOAc. The organic layer was separated, dried (MgSO₄), filtered and the solvent was evaporated. The residue (0.08g) was dissolved in 2-propanone/oxalic acid (degradation). The residue (0.007g) was then purified by column chromatography over silica gel (3.5μm). The pure fractions were collected and the solvent was evaporated, yielding 0.05g (45%) of compound 1.
Example B2
Preparation of compound 2

\[
N^1-(2-(6-tritio-1H-indol-3-yl)ethyl)-N^4-(2-(((3-methyl-3H-diazirin-3-yl)methoxy)methyl)pyridin-4-yl)benzene-1,4-diamine
\]

To the solution of intermediate 7 was added a solution of intermediate 2 (1.2 mg) in acetonitrile (120 μl). The solvent was removed at aspirator pressure and the residue was covered with argon gas, protected from light, and kept for 30 minutes on an oil bath warmed to 130°C. The reaction mixture was allowed to cool to room temperature and was then taken up in THF (5.0 ml). The total radioactivity of the solution was 2.30 GBq (62 mCi), it contained the desired compound 2 in a 30.3 % radiochemical purity. Purification was performed via preparative HPLC. All manipulations were carried out with exclusion of light. The crude material was concentrated at aspirator pressure on a 30°C water bath and the residue was dissolved in \(N,N\)-dimethyl formamide (40 μl) and injected on an Xbridge 5μ RP18 column (4.6 mm ID x 250 mm) at a flow rate of 2.0 ml/min and with UV detection at 260 nm. A 12 minutes isocratic run with 0.05 M aqueous ammonium acetate brought to pH 9.0 – acetonitrile (52:48; v/v) was used and the product fraction, having a retention time of 10.2 minutes, was collected in 2 fractions. Fraction 1 from the beginning of the peak until its maximum and fraction 2 from its maximum onward. Fraction 1 had a purity of 75 % and fraction 2 had a HPLC purity of 95 %. The organic part of fraction 1 was largely removed at aspirator pressure and the aqueous residue was extracted with DCM (4 x 1.0 ml). The combined extracts were concentrated at aspirator pressure and the residue was again purified via HPLC as before giving fractions 3 and 4. Fractions 2 and 4 were combined, the organic part was largely removed at aspirator pressure and the aqueous residue (3 ml) was extracted with DCM (4 x 1.5 ml). The DCM extracts were combined, concentrated at aspirator pressure and the residue was dissolved again in DCM (5.5 ml) and stored as compound 2. The purified material (total radioactivity: 66.5 MBq or 1.8 mCi) was dissolved in DCM (5.50 ml) and had a radiochemical purity of 95 % and a specific activity of 189 GBq/mmole (5.10 Ci/mmole). Structure confirmation was obtained via HPLC co-elution with reference material and via LC-MS.
Example B3
Preparation of compound 3

\[
N^1-(2-(1H-indol-3-yl)ethyl)-2-tritio-N^4-(2-((3-methyl-3H-diazirin-3-yl)methoxy)methyl)pyridin-4-yl)benzene-1,4-diamine
\]

To the solution of intermediate 10 was added a solution of intermediate 2 (1.2 mg) in acetonitrile (120 μl). The solvent was removed at aspirator pressure and the residue was covered with argon gas, protected from light, and kept for 30 minutes on an oil bath warmed to 130°C. The reaction mixture was allowed to cool to room temperature and was then taken up in THF (5.0 ml). The total radioactivity of the solution was 703 MBq (19 mCi), it contained the desired compound 3 in a 54.5% radiochemical purity. Purification was performed via preparative HPLC. All manipulations were carried out with exclusion of light. Half of the crude material was concentrated at aspirator pressure on a 30°C water bath and the residue was dissolved in \(N,N\)-dimethyl formamide (100 μl) and injected on an X Terra 5 μ RP18 column (4.6 mm ID x 250 mm) at a flow rate of 2.0 ml/min and with UV detection at 265 nm. A 12 minutes isocratic run with 0.05 M aqueous ammonium acetate at neutral pH – acetonitrile (50:50; v/v) was used and the product fraction, having a retention time of 5.9 min, was collected. From the solvent, the organic part was largely removed at aspirator pressure and the aqueous residue was extracted with DCM (4 x 1.0 ml). The combined extracts were concentrated at aspirator pressure and the residue was dissolved in acetonitrile (20.0 ml), yielding compound 3. The compound had a total radioactivity of 284 MBq (7.7 mCi), a radiochemical purity of 98.1% and a specific activity of 308 GBq/mmol (8.3 Ci/mmol). Structure confirmation was obtained via HPLC co-elution with reference material and via LC-MS.

Results

**JNJ-#1 binds to HDM2.**

To investigate binding of JNJ-#1, one of the compounds shown to be an HDM2 antagonists in PCT publication WO2006/032631, to HDM2, we determined its influence on proteolysis of HDM2 by the proteolytic enzyme Trypsin. We therefore incubated bacterially expressed HDM2, fused to GST, in the presence or absence of JNJ-#1 with Trypsin under conditions were proteolysis of HDM2 was incomplete (limited proteolysis).
As we show in figure 1, JNJ-#1 strongly reduced proteolysis of full length HDM2, but not proteolysis of the HDM2 family member HDMX, which is a close homologue of HDM2.

**JNJ-#1 interferes with HDM2 proteolysis at doses as low as 100 nM.**

We next determined the dose of JNJ-#1 that is required for the interference with HDM2 proteolysis. We incubated bacterially expressed HDM2 fused to GST in the presence of increasing concentrations of JNJ-#1 with Trypsin. To investigate the specificity of the JNJ-#1 compound, we also incubated GST-HDM2 with the active and inactive nutlin-3 isomers as well as with the proteasome inhibitor MG132. As we show in figure 2, proteolysis of HDM2 was inhibited by JNJ-#1 already at doses as low as 100 nM. Increasing the dose of JNJ-#1 up to 1 μM further reduced proteolysis of HDM2, and no further reduction was observed at 3 μM. In contrast, incubation of GST-HDM2 with a dose of 10 μM active or inactive nutlin-3 isomer affected proteolysis only weakly and incubation of GST-HDM2 with 10 μM of MG132 had no affect.

**JNJ-#1 induces p53 and activates downstream signaling in tumor cells.**

JNJ-#1 has been identified to bind and change confirmation of HDM2. JNJ-#1 was first investigated as to whether the compound affects the expression of HDM2 binding partners such as p53 and E2F1 and their downstream signaling molecules. U-87 MG glioblastoma cells were incubated with JNJ-#1 for 24 hours, and as shown in Figure 3 JNJ-#1 induced p53 starting at 1 μM, further increasing up to 10 μM. The expression of E2F-1, which is essential for S-phase progression was dramatically decreased. In parallel to the p53 induction, a clear increase in the downstream genes p21<sup>waf1</sup>, cip1, MIC-1, and PIG3 was observed after 24 hours of incubation, starting at 1 μM, but reaching its maximal induction at 10 μM. HDM2 itself, which is also transcriptionally induced by p53, was not detectable under control conditions, but clearly induced by JNJ-#1 at 1 to 10 μM. Induction of the cyclin dependent kinase (cdk) inhibitor p21<sup>waf1</sup>, cip1 is expected to result in decrease in cdk mediated phosphorylation of the Retinoblastoma (Rb) tumor suppressor protein, which results in a block of E2F-1 activity and thereby G1 cell cycle arrest. As shown in Figure 3, indeed a potent decrease in Ser780-Rb phosphorylation was observed already at 1 μM, which parallels p21<sup>waf1</sup>, cip1 induction.

**JNJ-#1 does not prevent binding of HDM2 to p53 in JAR choriocarcinoma cells**

Since JNJ-#1 was identified as an HDM2 antagonist, and induces p53 protein levels, we subsequently investigated whether the compound displaces p53 from HDM2,
thereby preventing p53 degradation. For this purpose we utilized JAR choriocarcinoma cells, which have high HD2M expression levels due to a gene amplification. As illustrated in Figure 4, when endogenous HD2M was immunoprecipitated from JAR cells, the amount of p53 attached was increased in the presence of JNJ-#1. The positive control HD2M antagonist Nutlin-3, which is known to bind the N-terminal pocket of HD2M efficiently displaced p53 from the HD2M protein, while the inactive enantiomer of Nutlin-3 had no effect. These data indicate that JNJ-#1 affect HD2M function through a novel mechanism of action.

**JNJ-#1 does not inhibit p53 ubiquitination in U2OS cells**

JNJ-#1 leads to the accumulation of p53. One very likely mechanism would be that JNJ-#1 prevents p53 ubiquitylation by affecting HD2M ubiquitin ligase activity. To test this option, we performed an cellular ubiquitylation assay in the presence of the compound. As shown in figure 5, ubiquitylation of p53 was strongly reduced in the presence of Nutlin-3 while it was not affected at all by JNJ-#1.

**JNJ-#1 prevents binding of HD2M to the proteasome in a dose dependent manner.**

We previously observed that HD2M associates with the proteasome and we speculated that this interaction might impact on p53 degradation. To investigate the impact of JNJ-#1 on the interaction of HD2M with the proteasome, we incubated bacterially expressed HD2M fused to GST and proteasomes with increasing doses of JNJ-#1. We then mixed HD2M and the proteasomes and incubated the mixture for 30 min at room temperature prior to immune-precipitation of HD2M. We separated the complexes by SDS-PAGE and determined the relative amount of proteasomes associated with HD2M by Western blotting.

As we show in figure 6, GST alone bound only weakly to proteasomes, as visualized by the S8 subunit. When we included HD2M in the assay, a significantly higher amount of proteasomes co-precipitated with GST-HD2M than with GST-alone. The addition of JNJ-#1 at a concentration as low as 50 nM did not affect this association. However, when we raised the concentration of JNJ-#1 to 500 nM, the association of HD2M with proteasomes was strongly reduced. Increasing the dose of JNJ-#1 to 5 μM further reduced the association of HD2M with the proteasome.

**JNJ-#1 prevents binding of HD2M to the proteasome in cells.**

We next determined whether JNJ-#1 also prevented the association of HD2M with the proteasome in cells. We incubated cells with 10 μM JNJ-#1, with 10 μM nutlin-3 or with DMSO for control, immunoprecipitated HD2M with an anti-HD2M antibody and determined the relative amount of associated S6b by Western blotting. The
proteasome subunit S6b co-precipitated with HDM2 in the absence of JNJ-#1. However, in the presence of 10 μM JNJ-#1 or in the presence of nutlin-3, the association of HDM2 with S6b was no longer detectable (data not shown). To confirm the result, we repeated the experiment with overexpressed Myc-tagged MDM2. We transfected Myc-MDM2 in 293 T cells. Twenty-four hours after transfection, 10 μM JNJ-#1 or DMSO for control were added to the cells. After 1.5 hour incubation time, Myc-MDM2 was precipitated using the anti-Myc-antibody 9E10. Associating proteasomes were determined by Western blotting. Like with endogenous HDM2, the proteasome co-precipitated with Myc-tagged MDM2. JNJ-#1 again prevented the interaction completely (figure 7).

**JNJ-#1 prevents degradation of p53 in vitro.**

To determine whether the reduction in the association of HDM2 with the proteasome in the presence of JNJ-#1 influences p53 degradation, we employed an in vitro degradation assay. We mixed p53 and HDM2 that were expressed in baculoviruses with ubiquitin, 26S proteasomes and E1 and E2 enzymes. In the absence of JNJ-#1, p53 was quickly degraded. However, when we performed the reaction in the presence of JNJ-#1, degradation of p53 was completely abrogated (figure 8).

**HDM2/MDM2 share a sequence motif comprising the amino acids EDY**

To determine the interaction site with the proteasome on MDM2, we transfected 293T cells with a series of MDM2 deletion mutants together with a plasmid encoding the S6b protein of the proteasome. After co-transfection of S6b with a mutant of MDM2 lacking the central domain, binding of MDM2 to S6b was significantly enhanced in comparison to the binding of wild type MDM2 to S6b (figure 13). From this result, we concluded that a sequence motif in the central domain interferes with the binding of MDM2 to the proteasome. Therefore, the central domain might reduce the interaction of MDM2 with the proteasome by binding to a sequence of MDM2 that is also able to associate with the proteasome, so a sequence motif should be common to MDM2 and the proteasome. In search for such a sequence, we aligned the central domain of MDM2 with several subunits of the proteasome. By this, we identified a three amino acid sequence, the EDY motif, that is present in MDM2 and in several subunits of the 26S proteasome (figure 9, figure 12).

**A peptide containing the EDY motif associates with MDM2**

We determined whether MDM2 is able to associate with the EDY motif of the proteasome by performing a GST-pulldown assay using a GST-fusion protein
comprising a peptide from the S6b protein encompassing the EDY sequence and MDM2 expressed from baculovirus. As we show in figure 10, to our surprise MDM2 clearly associated with the GST-fusion protein encompassing the S6b-derived peptide, but not with GST alone.

Overexpression of the EDY motif of HDM2/MDM2 or S6b interferes with p53 degradation
If the EDY-motif is mediating the association of HDM2 with the proteasome, a peptide containing the EDY sequence should compete with the proteasome for binding to HDM2. Moreover, if the association of HDM2 with the proteasome is important for p53 degradation, p53 should accumulate after overexpression of such a peptide. In figure 11, we show that this is indeed the case. When we transfected a thioredoxin-insertion construct where the sequence encompassing the EDY motif of S6b or HDM2 was expressed in an outer loop of the thioredoxin protein into H1299 cells, p53 degradation by HDM2 was strongly reduced. Likewise, when we expressed the Thioredoxin-insertion proteins in U2OS cells, degradation of endogenous p53 was almost completely abrogated.

JNJ-#1 binds to the N-terminal domain of HDM2.

Figure 14 demonstrates that JNJ-#1 binds strongly to full length HDM2 but not to the C terminal part (AA200-491) of HDM2. The binding of JNJ-#1 to the C-terminal part of hdm2 is comparable with its binding to p53, and BSA (aspecific binding control). In the experiment, proteins were mixed with photoaffinity and tritium labeled JNJ-#1 followed by UV irradiation. Reaction mixture was separated on a PAGE gel and proteins were transferred to a PVDF membrane by Western blotting. Blots were exposed to a phosphor imager screen. After two weeks exposure to phosphor imager screens were analyzed on a Fujix Bas 2000 Bio-Imaging Analyzer (figure 14A). Bands were quantified, BLU (Biochemical Light Unit) values of the quantification were normalized versus the aspecific binding to BSA. The mean value of 3 experiments is shown in figure 14B.
WHAT IS CLAIMED IS:

1. Use of a compound for the manufacture of a medicament for the treatment of a disorder mediated through the binding of HDM2 with the proteasome.

2. Use as claimed in claim 1 wherein the compound can prevent binding of HDM2 to the proteasome without preventing the binding between HDM2 and a HDM2-binding protein.

3. Use as claimed in claim 1 wherein the compound can prevent binding of HDM2 to the proteasome without preventing the ubiquitination of a HDM2-binding protein.

4. Use as claimed in any of claims 1 to 3, wherein said disorder is further mediated through proteolysis of HDM2, of a related protein or of a HDM2 binding protein.

5. Use as claimed in claim 4, wherein said proteolysis is mediated by the UPS pathway.

6. Use as claimed in claim 4, wherein said proteolysis is mediated through a proteolytic enzyme.

7. Use as claimed in any of claims 2 to 6 wherein the HDM2 binding protein is p53.

8. Use as claimed in any of claims 1 to 7 wherein the compound is a compound of formula (I)
a N-oxide form, an addition salt or a stereochemically isomeric form thereof, wherein

m is 0, 1, or 2 and when m is 0 then a direct bond is intended;

n is 0, 1, 2, or 3 and when n is 0 then a direct bond is intended;

p is 0, 1 and when p is 0 then a direct bond is intended;

s is 0, 1 and when s is 0 then a direct bond is intended;

t is 0 or 1 and when t is 0 then a direct bond is intended;

X is C(=O) or CHR⁵; wherein

R⁵ is hydrogen, C₁₋₆alkyl, C₃₋₇cycloalkyl, -C(=O)-NR¹⁷R¹⁸, hydroxycarbonyl, arylC₁₋₆alkyloxy carbonyl, heteroaryl, heteroarylcarbonyl, heteroarylc₁₋₆alkyloxy carbonyl, piperazinylcarbonyl, pyrrolidinyl, piperidinylcarbonyl, C₁₋₆alkyloxy carbonyl, C₁₋₆alkyl substituted with a substituent selected from hydroxy, amino, aryl, and heteroaryl; C₃₋₇cycloalkyl substituted with a substituent selected from hydroxy, amino, aryl, and heteroaryl; piperazinylcarbonyl substituted with hydroxy, hydroxyC₁₋₆alkyl, hydroxyC₁₋₆alkyloxyC₁₋₆alkyl; pyrrolidinyl substituted with hydroxyC₁₋₆alkyl; or piperidinylcarbonyl substituted with one or two substituents selected from hydroxy, C₁₋₆alkyl, hydroxyC₁₋₆alkyl, C₁₋₆alkyloxyC₁₋₆alkyl, C₁₋₆alkyl(di hydroxy)C₁₋₆alkyl or C₁₋₆alkyloxy(hydroxy)C₁₋₆alkyl;

R¹⁷ and R¹⁸ are each independently selected from hydrogen, C₁₋₆alkyl, di(C₁₋₆alkyl)aminoC₁₋₆alkyl, arylC₁₋₆alkyl, C₁₋₆alkyloxyC₁₋₆alkyl, hydroxyC₁₋₆alkyl, hydroxyC₁₋₆alkyl(C₁₋₆alkyl) or hydroxyC₁₋₆alkyl(arylC₁₋₆alkyl);

-Q⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻˓
each $R^9$ is independently hydrogen or $C_{1-6}$alkyl;

$R^1$ is hydrogen, aryl, heteroaryl, $C_{1-6}$alkyloxycarbonyl, $C_{1-12}$alkyl, or $C_{1-12}$alkyl substituted with one or two substituents independently selected from hydroxy, aryl, heteroaryl, amino, $C_{1-6}$alkyloxy, mono- or di($C_{1-6}$alkyl)amino, morpholinyl, piperidinyl, pyrrolidinyl, piperazinyl, $C_{1-6}$alkylpiperazinyl, aryl$C_1$.

$S$alkylpiperazinyl, heteroaryl$C_{1-6}$alkylpiperazinyl, $C_{3-7}$cycloalkylpiperazinyl and $C_{3-7}$cycloalkyl$C_{1-6}$alkylpiperazinyl;

$R^2$ is hydrogen, halo, $C_{1-6}$alkyl, $C_{1-6}$alkyloxy, aryl$C_{1-6}$alkyloxy, heteroaryl$C_{1-6}$alkyloxy, phenylthio, hydroxy$C_{1-6}$alkyloxycarbonyl, $C_{1-6}$alkyl substituted with a substituent selected from amino, aryl and heteroaryl; or $C_{3-7}$cycloalkyl substituted with a substituent selected from amino, aryl and heteroaryl;

$R^3$ is hydrogen, $C_{1-6}$alkyl, heteroaryl, $C_{3-7}$cycloalkyl, $C_{1-6}$alkyl substituted with a substituent selected from hydroxy, amino, aryl and heteroaryl; or $C_{3-7}$cycloalkyl substituted with a substituent selected from hydroxy, amino, aryl and heteroaryl;

$R^4$ and $R^5$ are each independently hydrogen, halo, $C_{1-6}$alkyl, polyhalo$C_{1-6}$alkyl, cyano, cyano$C_{1-6}$alkyl, hydroxy, amino or $C_{1-6}$alkyloxy; or $R^4$ and $R^5$ together can optionally form a bivalent radical selected from methylenedioxy or ethylenedioxy;

$R^6$ is hydrogen, $C_{1-6}$alkyloxycarbonyl or $C_{1-6}$alkyl;

when $p$ is 1 then $R^7$ is hydrogen, aryl$C_{1-6}$alkyl, hydroxy or heteroaryl$C_{1-6}$alkyl;

$Z$ is a radical selected from

(a-1)  
(a-2)  
(a-3)  
(a-4)
wherein

each $R^{10}$ or $R^{11}$ are each independently selected from hydrogen, halo, hydroxy, amino, C$_{1-6}$alkyl, nitro, polyhaloC$_{1-6}$alkyl, cyano, cyanoC$_{1-6}$alkyl, tetrazoloC$_{1-6}$alkyl, aryl, heteroaryl, arylC$_{1-6}$alkyl, heteroarylc$_{1-6}$alkyl, aryl(hydroxy)C$_{1-6}$alkyl, heteroaryl(hydroxy)C$_{1-6}$alkyl, arylcarbonyl, heteroarylcarbonyl, C$_{1-6}$alkylcarbonyl, arylC$_{1-6}$alkylcarbonyl, heteroarylc$_{1-6}$alkylcarbonyl, C$_{1-6}$alkoxy,

C$_{3-7}$cycloalkylcarbonyl, C$_{3-7}$cycloalkyl(hydroxy)C$_{1-6}$alkyl, arylC$_{1-6}$alkyloxyC$_{1-6}$alkyl, C$_{1-6}$alkyloxyC$_{1-6}$alkyloxyC$_{1-6}$alkyl, C$_{1-6}$alkylcarbonyloxyC$_{1-6}$alkyl, C$_{1-6}$alkyloxy carbonylC$_{1-6}$alkyloxyC$_{1-6}$alkyl, hydroxyC$_{1-6}$alkyloxyC$_{1-6}$alkyl, C$_{1-6}$alkyloxy carbonylC$_{2-6}$alkenyl C$_{1-6}$alkyloxyC$_{1-6}$alkyl, C$_{1-6}$alkyloxy carbonyl, C$_{1-6}$alkylcarbonyloxy,

aminocarbonyl, hydroxyC$_{1-6}$alkyl, aminoC$_{1-6}$alkyl, hydroxycarbonyl, hydroxycarbonylC$_{1-6}$alkyl and -($CH_2$)$_r$-(C(=O)$_v$)-(CHR$_2$)$_u$-NR$_{12}$R$_{14}$, wherein

$v$ is 0, 1, 2, 3, 4, 5, or 6 and when $v$ is 0 then a direct bond is intended;

$r$ is 0, or 1 and when $r$ is 0 then a direct bond is intended;

$u$ is 0, 1, 2, 3, 4, 5, or 6 and when $u$ is 0 then a direct bond is intended;

$R^{19}$ is hydrogen or C$_{1-6}$alkyl;

$R^{12}$ is hydrogen, C$_{1-6}$alkyl, C$_{3-7}$cycloalkyl, C$_{1-6}$alkyl substituted with a substituent selected from hydroxy, amino, C$_{1-6}$alkyloxy and aryl; or C$_{3-7}$cycloalkyl substituted with a substituent selected from hydroxy, amino, aryl and C$_{1-6}$alkyloxy;

$R^{13}$ and $R^{14}$ are each independently selected from hydrogen, C$_{1-12}$alkyl, C$_{1-6}$alkylcarbonyl, C$_{1-6}$alkylsulfonyl, arylC$_{1-6}$alkyl carbonyl,
C₃₋₇cycloalkyl, C₃₋₇cycloalkylcarbonyl, -(CH₂)ₙ-NR¹⁵R¹⁶, C₁₋₁₂alkyl substituted with a substituent selected from hydroxy, hydroxycarbonyl, cyano, C₁₋₆alkyloxy carbonyl, C₁₋₆alkyloxy, aryl or heteroaryl; or C₃₋₇cycloalkyl substituted with a substituent selected from hydroxy, C₁₋₆alkyloxy, aryl, amino, arylC₁₋₆alkyl, heteroaryl or heteroarylC₁₋₆alkyl; or R¹³ and R¹⁴ together with the nitrogen to which they are attached can optionally form a morpholinyl, piperidinyl, pyrrolidinyl, piperazinyl, or piperazinyl substituted with a substituent selected from C₁₋₆alkyl, arylC₁₋₆alkyl, arylC₁₋₆alkyloxy carbonyl, heteroarylC₁₋₆alkyl, C₃₋₇cycloalkyl and C₃₋₇cycloalkylC₁₋₆alkyl; wherein

k is 0, 1, 2, 3, 4, 5, or 6 and when k is 0 then a direct bond is intended;

R¹⁵ and R¹⁶ are each independently selected from hydrogen, C₁₋₆alkyl, arylC₁₋₆alkyloxy carbonyl, C₃₋₇cycloalkyl, C₁₋₁₂alkyl substituted with a substituent selected from hydroxy, C₁₋₆alkyloxy, aryl, and heteroaryl; and C₃₋₇cycloalkyl substituted with a substituent selected from hydroxy, C₁₋₆alkyloxy, aryl, arylC₁₋₆alkyl, heteroaryl, and heteroarylC₁₋₆alkyl; or R¹⁵ and R¹⁶ together with the nitrogen to which they are attached can optionally form a morpholinyl, a piperazinyl or a piperazinyl substituted with C₁₋₆alkyloxy carbonyl;

aryl is phenyl or naphthalenyl;

each phenyl or naphthalenyl can optionally be substituted with one, two or three substituents each independently selected from halo, hydroxy, C₁₋₆alkyl, amino, polyhaloC₁₋₆alkyl and C₁₋₆alkyloxy; and
each phenyl or naphthalenyl can optionally be substituted with a bivalent radical selected from methylenedioxy and ethylenedioxy;

heteroaryl is pyridinyl, indolyl, quinolinyl, imidazolyl, furanyl, thiienyl, oxadiazolyl, tetrazolyl, benzofurany or tetrahydrofuranyl;
each pyridinyl, indolyl, quinolinyl, imidazolyl, furanyl, thiienyl, oxadiazolyl, tetrazolyl, benzofurany, or tetrahydrofuranyl can optionally be substituted with one, two or three substituents each independently selected from halo, hydroxy, C₁₋₆alkyl, amino,
polyhaloC₃₋₆alkyl, aryl, arylC₃₋₆alkyl or C₃₋₆alkyloxy; and
each pyridinyl, indolyl, quinolinyl, imidazolyl, furanyl, thieryl, benzofuranyl, or
tetrahydrofuranyl can optionally be substituted with a bivalent radical selected
from methylenedioxy or ethylenedioxy;

with the proviso that
when m is 1; the substituents on the phenyl ring other than R² are in the meta position;
s is 0; and t is 0; then
Z is a radical selected from (a-1), (a-3), (a-4), (a-5), (a-6), (a-7), (a-8) or (a-9).

9. Use as claimed in any of claims 1 to 8 wherein the compound of formula (I) is
compound JNJ#1

10. Use of a compounds of formula (I) as defined in claim 8 and 9 in an assay said
assay comprising:
    a) contacting the compound to be tested with a HDM2 protein, a related
protein or a protein binding fragment thereof and
    b) determining whether said compound affects the proteolysis of a HDM2
protein, a related protein, a protein binding fragment thereof or of a HDM2
binding protein.

11. Use of a compound of formula (I) as defined in claim 8 and 9, in a
method for the structure-based or rational design of an inhibitor of the
UPS-pathway, said method comprising:
    a) identifying contacting atoms in the binding site of the compound of
formula (I) that interact with HDM2, related proteins or fragments
thereof,
    b) design test compounds that interact with the atoms identified in (a)
to modulate the activity of the proteasome, and
c) contact said designed test compound with HDM2, a related protein or a protein binding fragment thereof to measure the capability of said compound to modulate the UPS activity.

12. Use of a compounds of formula (I) as defined in claim 8 and 9 in an assay wherein said assay measure the binding of the compound to HDM2, a related protein a protein binding fragment thereof, a proteasome subunit or a protein binding fragment thereof.

13. Use according to claim 12 wherein the compound is a labeled compound.

14. The photoaffinity labeled compounds of formula (II) and the photoaffinity and tritium labeled compounds of formula (III) or (IV).
Fig 1

<table>
<thead>
<tr>
<th>GST-Hdm2 (full length)</th>
<th>GST-HdmX (full length)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
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<td>+</td>
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<tr>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

#1 (10 μM)  
Trypsin  
- Hdm2/HdmX (full length)

Fig 2

Trypsin

<table>
<thead>
<tr>
<th>J&amp;J #1</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
</tr>
<tr>
<td>100 nM</td>
</tr>
<tr>
<td>300 nM</td>
</tr>
<tr>
<td>1 μM</td>
</tr>
<tr>
<td>3 μM</td>
</tr>
<tr>
<td>Nutlin active, 10 μM</td>
</tr>
<tr>
<td>Nutlin inactive, 10 μM</td>
</tr>
<tr>
<td>MG132, 10 μM</td>
</tr>
</tbody>
</table>

- GST-Hdm2
Fig 3

<table>
<thead>
<tr>
<th>JNJ - #1</th>
<th>0</th>
<th>0.3</th>
<th>1</th>
<th>3</th>
<th>10 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>hdm2</td>
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<td>MIC-1</td>
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<td>PIG3</td>
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<tr>
<td>p21^{waf1,cip1}</td>
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<tr>
<td>Rb</td>
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<tr>
<td>Ser780-Rb</td>
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<tr>
<td>E2F1</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>actin</td>
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</tbody>
</table>

Fig 4

<table>
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<tr>
<th>Control</th>
<th>JNJ-#1</th>
<th>Nutlin active</th>
<th>Nutlin inactive</th>
<th>Conc (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3</td>
<td>10</td>
<td>10</td>
<td>0</td>
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<tr>
<td></td>
<td></td>
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<td></td>
<td>hdm2-CoIP</td>
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<td></td>
<td></td>
<td>hdm2</td>
</tr>
</tbody>
</table>

Total lysate
Fig 9

PRS6A_HUMAN : 418-420: telth ED-Y megil
PRS6B_HUMAN : 361-363: eevdl ED-Y varpd
PSMD4_HUMAN : 323-326: epake EDdX dvmqd
PSMD2_HUMAN : 437-439: ylyss ED-Y iksqa
MDM2_HUMAN : 257-259: eslds ED-Y slsee
MDM2_HUMAN : 392-394: qsges ED-Y sqpst
PRS4_HUMAN : 432-439: qegtp EglY l----

Fig 10

GST-pulldown

- GST-S6b peptide
- GST

input

- Hdm2
Fig 11

A  
-  +  +  +  Trx-peptide
+  +  +  +  Mdm2

B

- p53
PCNA

Fig 12

>PRS6A_HUMAN 26S protease regulatory subunit 6A - Homo sapiens (Human). S6A - clear EDY pattern
MNLLPNIESPVTQRQKMAETWDEABQDGIGERVLKMSBEBEIIRTRLLDS
EIKIMKSEVLRVTHELQMMDKIKNNSRKKINVKLTPLYPSNVIFLLDVD - 100
PNDQKRSDGANLDSQKRGKCAVIKSTMQRTYFPVIGLVDAEKLKPGDL
VGVNKDSYLILELTPYDSRVMKDRPERTEQYSD9GLKDQ10BLVE - 200
AVLPPMHHKEFLIGQPQPGKVIYMGGPTGKTLLARACAAQTKATFLK
LARGQPVQMPFIDGAKLVRDAAFAAKSAPFIIFDELDAIGTKRFDEK - 300
AGDREVFQRTMLELLNQLDFQNPQTVKVIATNVRDLDFALLRSGRDLR
KIEFPPMPNEARARIMQIHSRMRNVSPDNYEELRCRRDFNGAQCKAVC - 400
VEAGMIALRRAFELTHEIDMNGIEYFVQAKKANLQYYA

ACG GAG CTC ACC CAC GAG GAC TAC ATG GAA GGC ATC CTG (refSeq NM_002804)

>PRS6B_HUMAN 26S protease regulatory subunit 6B - Homo sapiens (Human). S6B - clear EDY pattern
MEEGILVERKAEIDEIPALSVSPQPTQGLEPGEEFEDLYSRYKQKQE
LJFLEQVQREYIKDEQNKLKCEFHLAQEPKRIQSIPLVQFPLAVDQNT - 100
AVGSGTSTNYVROLSTIDVERLKNFNASVHALKHSNALVDPBRASS
IMMTSLQDKPDVYADIGGMDYQVRVREAVLPLTHFELYKQIGIDPR - 200
GVLMGYPGPCKTMLAKGAVAHTTAAPFRVVGSEVFQKYLQEGPRMVRDV
FRLKTABLEIDIAAKRFDQATGADREVQRILLLELNNQMDGF - 300
QNVRVIMATNRAVLTFPDRILPLRDKRQKRLIPSTITS
KMNSGAGDIEVRLPDYVARIDIKSIDGADINSICIQESGMLAVRENYIVALKDFE - 400
KAYKTVIKKDQEBEFYK

GAG GAG GTT GAC TTG GAA GAC TAT GTG GCC CGG CCA GAT (refSeq NM_006503)
-7/9-

>FSMD4_HUMAN 26S proteasome non-ATPase regulatory subunit 4 - Homo sapiens (Human). SSA - clear EDY pattern
MVLESTMFCVDNENTSRYNGDFLPTRLQAOQQAVNVIVCHSKTSRNSPENNVG
LITLANCEVTQITLITPDTGRILSKLHTVQPKKIFITCGTIRVWLAIKLRHR - 100
QGHKNMRMRAP_FVGSFVDEKNDKVLKRLKKEKVVNDIFGEEEVNT
EKLHFVAVNLGKDTQGSLVTPVPGSPSLADALISSPILAGERGGMGLGLG
ASDPEFVIPSAPDLAMVLLAVSMEOQRQEBBEEARAAAAASAAEAGIAT
TGTEBDDALAMKMTLITQQEFGRGTGLPDSLMSMTETEEQIAYAMQSMLQAEF
GQARSADIDASASSMTEDPAKEDDDYEVQCBEPLQSVELNFLGVPNNAE
AIRNAMASLQATKGDKKEEDEKD

GAG CCA GCC AAG GAG GAG GAT TAC GAC GTG ATG CAG GAC (refSeq NM_002810)

>FSMD2_HUMAN 26S proteasome non-ATPase regulatory subunit 2 - Homo sapiens (Human). S2 - clear EDY pattern
MEBGCRDAPVQPSQFAPAGTDKEPQSDKERDADGDKKEQRESEEDK
QLQDELMFVRBGLKGDSTLYRPALEELRQRISSTTSMTSPKPFLKFLR - 100
PHYKLKIEYBNAPGFPRAPADDISSLVAMTSQGERBCLKLYRLVQSEBE
LASHGMYVRLAGEVAKQWELDDLADKEVQREPFLTLVKEIVYPMNAHNA
HEBRACDMLIEOQVMDKLEDENAYAKVCLYLTSCNVYSEPSENSALLR
CALGVFRFRRPFRLALLMNDMLMELVIDTPSDKVQQKMAFPLGR
HGVPFLELSDVEVYDLTEKMSVNQILSNFLALARELDIMEFKPVDYI
THLENRRFGSSQSQQVSAMRLHASSVFNGVNAVAFQDGLLTDQGKLYW
KNKDGMLSAASGMLDQWDDVGGLQTDVYLSLEYIKSAGALLACGI
VNSWGRNEDCPAALALLSDDVEVLHNSNTMLGRSFPGHLGLAYAGASNSNREDVLT
LLPVMDGKSSMEVEAQGTAALCMAGVSCGDVSTILTQDMKSETEL
KDTYARMNPKLGELNHLKGRATAILAALAVSEFRSPFANTLVDVCA
AGSNVLKVQLQHLCSHIFDSEKEEKKDKKDEKKEKAPDMGADAQHH
GVAHGLIALIAMGEBIGAENLARFTGHMLR YEPFLLRAVPVLALLISVS
NFRLNLIDTSLFKSHDAPVEPSYNSIFAMGMVSGTNARLAAMLRQLAQ
YHAKDPPNFLFMRVLAGQGTHLKGCTILTLCPYHSRGQLMOSQAVALGTLTV
VSPFGLDVRNITLGSYKSYVVLGLVAAMQPRMLVTFDEBLRPLFVSVRQVGVV
DVVQQAGKPKTTITGFQTHHTPVLLAHGERELATEEFLPVFTPILGFVIL
RKNPYNDL

TAC CTG TAC TCC TCT TAC ATT ATT AAG TCA GTA GTA TGT (refSeq NM_002808)

>MDM2_HUMAN Ubiquitin-protein ligase E3 B3 Mdm2 - Homo sapiens (Human). MDM2 - clear EDY pattern, present twice
MCNTNSVPTDAGVTTSQTIPASEQETLVRPKPLLKKLKSQGAKQKRTYTM
KEVLVFYQIYMTKRLYDKQHHVYCVSNDDLGLDFGVPFSVKEHKKLYI - 100
TMJRNLYVQTBGSQDSNGTSHKLEESDGKRDVQLEQKEIPSS
HLVRSPSTSSRSSRASERTSNSELSQERQKRHHSISLSDFSSLALC
VIREICCLSSLRSSGSSGTQGOSDPNLADVEHSEHGSQDSPQVFEBFE
VEHLSLQADISOEQQEILEDEDEVQVQYQAEBDTSESSEDIEFLA
DVWXKCANMCENMNPLSHCNRCWALREWNLFFBDKKGKKEIEKAKLENS
TQAEBQFGDVPCDKTIVNSDRESCVBEHDNITQASQSOSEDITQDSTS
SSIIYQSSQDVKVEPFRBQPDESQEKVESBBSSLPNAIRPCVICQGPRKNGCI
VHGTKHGLMACFTCAYKLARKNPCPCVRQPIQMYLTFYFP

GAA TCT CTC GAC TCA GAA GAT TAT AGC CTT AGT GAA GTA GAA (refSeq NM_002392)

CAA TCA CAA GAA AGT GAA GAC TAT TCT CAG CCA TCA ACT (refSeq NM_002392)
>PR54_HUMAN 26S protease regulatory subunit 4 - Homo sapiens (Human). S4 - NOT clear EDY pattern
MQQSQSGHGPGGKGDSDKKKKYYBPFPFTVRGKKKKTGGPDAAKSLP
LVTPTQQCLLLKLERIKDYLLMEMBELFIRNQEQMKPLEEKBEBERSKVD - 100
DLRGTMSVGTELEEEEIDNHAIVTSVGSBHYVSILSFVDDDLLEFGCSV
LLNHKHAVIGVLMDDTDPLVTVMKEKFBETYADIGGLDNQIEIKES - 200
VELPLTHPCRSMKPPGKPVLYGPFGTGTGGKTLAKAVANQTSATFLRVT
VGSEQLQKIDGQPLVORELFRVAEBHAPSVIFIDSEIAITKRYDSNNG - 300
GEREIQTMLBLLNQLDGFDGIDDVVKVIMATMRIETLDPALIRPGIDRKL
IEFPFPEDKTKRIFQIHTSRMTLADDVTLDDLIMKDDLSGADIAICT - 400
EALMLARRRGMKVINEDFKSKERNVLYKKQEGETPPGSLYL
CAG GAA GCC ACC CCT GAG GGG CTG TAT CTC TAA (refSeq NM_002802)

**Fig 13**

**IP: α-Myc**

- **WB: α-V5**

- **WB: α-Myc**

**TCL:**

- **WB: α-V5**

- **WB: α-PCNA**