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(54) Title: STABILIZED P53 PEPTIDES AND USES THEREOF

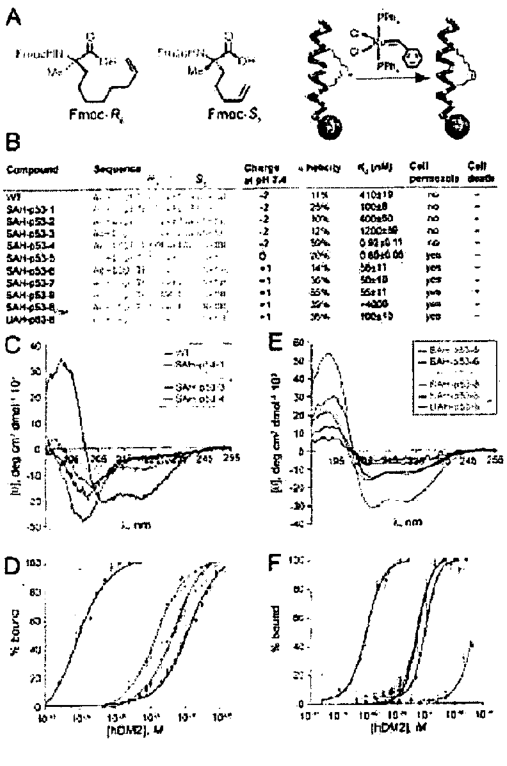

FIGURE 1

(57) Abstract: Cross-linked peptides related to human p53 and bind to HMD2 or a family member of HDM2 useful for promoting apoptosis, e.g., in the treatment of and identifying therapeutic agents that binding to HMD2 or a family member of HDM2.

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Stabilized p53 Peptides and Uses Thereof

BACKGROUND

[0001] The human p53 transcription factor induces cell cycle arrest and apoptosis in response to DNA damage[1] and cellular stress,[2] thereby playing a critical role in protecting cells from malignant transformation. The E3 ubiquitin ligase HDM2 controls p53 levels through a direct binding interaction that neutralizes p53 transactivation activity, exports nuclear p53, and targets it for degradation via the ubiquitylation-proteasomal pathway.[3,4] Loss of p53 activity, either by deletion, mutation, or HDM2 overexpression, is the most common defect in human cancer.[5] Tumors with preserved expression of wild type p53 are rendered vulnerable by pharmacologic approaches that stabilize native p53. In this context, HDM2 targeting has emerged as a validated approach to restore p53 activity and resensitize cancer cells to apoptosis *in vitro* and *in vivo*.[6] HDMX (HDM4) has also been identified as a regulator of p53. Moreover, studies have shown a similarity between the p53 binding interface of HDM2 and that of HDMX.[6a]

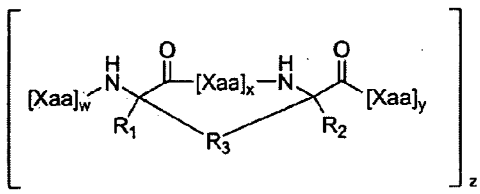
[0002] The p53-HDM2 protein interaction is mediated by the 15-residue α-helical transactivation domain of p53, which inserts into a hydrophobic cleft on the surface of HDM2.[7] Three residues within this domain (F19, W23, and L26) are essential for HDM2-binding.[8,9]

SUMMARY

[0003] Described below are stably cross-linked peptides related to a portion of human p53 ("stapled p53 peptides"). These cross-linked peptides contain at least two modified amino acids that together form an internal cross-link (also referred to as a tether) that can help to stabilize the alpha-helical secondary structure of a portion of p53 that is thought to be important for binding of p53 to HDM2. Accordingly, a cross-linked polypeptide described herein can have improved biological activity relative to a corresponding polypeptide that is not cross-linked. The stapled p53 peptides are thought to interfere with binding of p53 to HDM2 thereby inhibiting the destruction of p53. The stapled p53 peptide described herein

can be used therapeutically, e.g., to treat a variety of cancers in a subject. For example, cancers and other disorders characterized by an undesirably low level or a low activity of p53 and/or cancers and other disorders characterized by an undesirably high level of activity of HDM2. The modified peptides may also be useful for treatment of any disorder associated with disrupted regulation of the p53 transcriptional pathway, leading to conditions of excess cell survival and proliferation (e.g., cancer and autoimmunity), in addition to conditions of inappropriate cell cycle arrest and apoptosis (e.g., neurodegeneration and immune deficiency).

[0004] In one aspect, the invention features a modified polypeptide of Formula (I),



Formula (I)

or a pharmaceutically acceptable salt thereof,

wherein;

each R_1 and R_2 are independently H or a C_1 to C_{10} alkyl, alkenyl, alkynyl, arylalkyl, cycloalkylalkyl, heteroarylalkyl, or heterocyclylalkyl;

R_3 is alkylene, alkenylene or alkynylene, or $[R_4'\text{-K-}R_4]_n$; each of which is substituted with 0-6 R_5;

R_4 and R_4'are independently alkylene, alkenylene or alkynylene (e.g., each are independently a C1, C2, C3, C4, C5, C6, C7, C8, C9 or C10 alkylene, alkenylene or alkynylene);

R_5 is halo, alkyl, OR_6, $N(R_6)_2$, SR_6, SOR_6, SO_2R_6, CO_2R_6, R_6, a fluorescent moiety, or a radioisotope;

K is O, S, SO, SO_2, CO, CO_2, $CONR_6$, or  , aziridine, episulfide, diol, amino alcohol;

R_6 is H, alkyl, or a therapeutic agent;

n is 2, 3, 4 or 6;

x is an integer from 2-10;

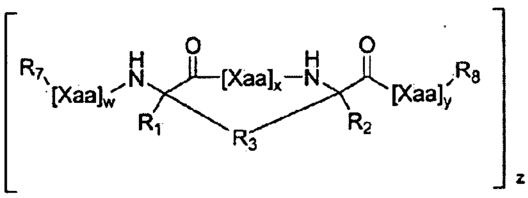
w and y are independently an integer from 0-100;

z is an integer from 1-10 (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10); and

each Xaa is independently an amino acid (e.g., one of the 20 naturally occurring amino acids or any naturally occurring non-naturally occurring amino acid);

wherein the polypeptide comprises at least 8 contiguous amino acids of SEQ ID NO:1 (human p53) or a variant thereof, SEQ ID NO:2 or a variant thereof, or another polypeptide sequence described herein except that: (a) within the 8 contiguous amino acids of SEQ ID NO:1 the side chains of at least one pair of amino acids separated by 3, 4 or 6 amino acids is replaced by the linking group, R_3, which connects the alpha carbons of the pair of amino acids as depicted in Formula I; and (b) the alpha carbon of the first of the pair of amino acids is substituted with R_1 as depicted in formula I and the alpha carbon of the second of the pair of amino acids is substituted with R_2 as depicted in Formula I.

[0005] In another aspect, the invention features a modified polypeptide of Formula (II),



Formula (II)

or a pharmaceutically acceptable salt thereof,

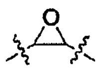
wherein;

each R_1 and R_2 are independently H or a C_1 to C_{10} alkyl, alkenyl, alkynyl, arylalkyl, cycloalkylalkyl, heteroarylalkyl, or heterocyclylalkyl;

R_3 is alkylene, alkenylene or alkynylene, or $[R_4'\text{-K-}R_4]_n$; each of which is substituted with 0-6 R_5;

R_4 and R_4'are independently alkylene, alkenylene or alkynylene (e.g., each are independently a C1, C2, C3, C4, C5, C6, C7, C8, C9 or C10 alkylene, alkenylene or alkynylene);

R_5 is halo, alkyl, OR_6, $N(R_6)_2$, SR_6, SOR_6, SO_2R_6, CO_2R_6, R_6, a fluorescent moiety, or a radioisotope;

K is O, S, SO, SO_2, CO, CO_2, $CONR_6$, or  , aziridine, episulfide, diol, amino alcohol;

R_6 is H, alkyl, or a therapeutic agent;

n is 2, 3, 4 or 6;

x is an integer from 2-10;

w and y are independently an integer from 0-100;

z is an integer from 1-10 (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10); and

each Xaa is independently an amino acid (e.g., one of the 20 naturally occurring amino acids or any naturally occurring non-naturally occurring amino acid);

R_7 is PEG, a tat protein, an affinity label, a targeting moiety, a fatty acid-derived acyl group, a biotin moiety, a fluorescent probe (e.g. fluorescein or rhodamine) linked via, e.g., a thiocarbamate or carbamate linkage;

R_8 is H, OH, NH_2, NHR_{8a}, $NR_{8a}R_{8b}$;

wherein the polypeptide comprises at least 8 contiguous amino acids of SEQ ID NO:1 (human p53) or a variant thereof, SEQ ID NO:2 or a variant thereof, or another polypeptide sequence described herein except that: (a) within the 8 contiguous amino acids of SEQ ID NO:1 the side chains of at least one pair of amino acids separated by 3, 4 or 6 amino acids is replaced by the linking group, R_3, which connects the alpha carbons of the pair of amino acids as depicted in formula I; and (b) the alpha carbon of the first of the pair of amino acids is substituted with R_1 as depicted in Formula II and the alpha carbon of the second of the pair of amino acids is substituted with R_2 as depicted in Formula II.

[0006] In the case of Formula I or Formula II, the following embodiments are among those disclosed.

[0007] In cases where x = 2 (i.e., i + 3 linkage), R_3 can be a C7 alkylene, alkenylene. Where it is a alkenylene there can one or more double bonds. In cases where x = 6 (i.e., i + 4 linkage), R_3 can be a C1, C12 or C13 alkylene or alkenylene. Where it is a alkenylene there can one or more double bonds. In cases where x = 3 (i.e., i + 4 linkage), R_3 can be a C8 alkylene, alkenylene. Where it is a alkenylene there can one or more double bonds.

[0008] SEQ ID NO:1 is the sequence of human p53. The stapled peptides can include the sequence Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Leu Leu Pro Glu Asn (SEQ ID NO:2; corresponds to amino acids 14 to 29 of SEQ ID NO:1). The stapled peptide can also include the sequence Phe Ser Asn Leu Trp Arg Leu Leu Pro Gln Asn (SEQ ID NO:5) or the sequence Gln Ser Gln Gln Thr Phe Ser Asn Leu Trp Arg Leu Leu Pro Gln Asn (SEQ ID NO:6). In these sequence as in SEQ ID NO:1, 2, 3 and 4), the side chains of two amino acids separated by 2, 3, 4 or 6 amino acids can be replaced by the linking group R_3. For example, in SEQ ID NO:5, the side chains of Ser and Pro can be replaced by the linking group R_3.

[0009] The stapled polypeptide can include all or part (e.g., at least 10, at least 11, at least 12, at least 13) of the following amino acid sequence: $Xaa_1Ser_2Gln_3Xaa_4Thr_5Phe_6Xaa_7Xaa_8Leu_9Trp_{10}Xaa_{11}Leu_{12}Leu_{13}Xaa_{14}Xaa_{15}Asn_{16}$. (SEQ ID NO:3) wherein each of Xaa_1, Xaa_4, Xaa_7, Xaa_8, Xaa_{11}, Xaa_{14}, Xaa_{15} are any amino acid (e.g., any of the 20 naturally occurring amino acids).

[0010] In some situations:

Xaa_1 = Leu or Gln or the linking group R_3

Xaa_4 = Glu or Gln or the linking group R_3

Xaa_7 = Ser or the linking group R_3

Xaa_8 = Asp or any amino acid other than Asp and Glu (preferably Asn; e.g., Xaa_8 can be Asp or Asn) or the linking group R_3

Xaa_{11} = Lys or a positively charged amino acid (preferably Arg) or the linking group R_3

Xaa_{14} = Pro or the linking group R_3

Xaa_{15} = Glu or any amino acid other than Asp and Glu (preferably Gln) or the linking group R_3.

In some situations, the peptide comprises SEQ ID NO:3 wherein Xaa_1 = Leu or Gln or the linking group R_3; Xaa_4 = Glu or Gln or the linking group R_3; Xaa_7 = Ser or the linking group R_3; Xaa_8 = Asp, Asn or the linking group R_3; Xaa_{11} = Lys, Arg or the linking group R_3; Xaa_{14} = Pro or the linking group R_3; Xaa_{15} = Glu, Gln or the linking group R_3. In the stapled peptides, any position occupied by Gln can be Glu instead and any position occupied by Glu can be Gln instead. Similarly, any position occupied by Asn can be Asp instead and any position occupied by Aps can be Asn instead. The choice of Asn or Arg and Gln or Glu will depend on the desired charge of the stapled peptide.

[0011] In some cases the peptide comprises a portion of SEQ ID NO:3 having the sequence: $Gln_3Xaa_4Thr_5Phe_6Xaa_7Xaa_8Leu_9Trp_{10}Xaa_{11}Leu_{12}Leu_{13}$ (SEQ ID NO:4).

[0012] Within SEQ ID NO:3, the pairs of amino acid that can be cross-linked include, but are not limited to: the 5th and 12th amino acids; 4th and 11th amino acids; 7th and 11th amino acids; and 7th and 14th amino acids

[0013] In some instances, the modified peptide binds to HDM2 (e.g., GenBank® Accession No.: 228952; GI:228952) and/or HDM4 (also referred to as HDMX; GenBank® Accession No.: 88702791; GI:88702791). In some instances it can be useful to create an inactive stapled peptide by replacing one or more (e.g., all three) of Phe_6, Trp_{10}, Leu_{13} with another amino acid, e.g., Ala.

[0014] Additional useful peptides include non-cross-linked peptides having the following amino acid sequence:

$Xaa_1Ser_2Gln_3Xaa_4Thr_5Phe_6Xaa_7Xaa_8Leu_9Trp_{10}Xaa_{11}Leu_{12}Leu_{13}Xaa_{14}Xaa_{15}Asn_{16}$. (SEQ ID NO:3) wherein each of Xaa_1, Xaa_4, Xaa_7, Xaa_8, Xaa_{11}, Xaa_{14}, Xaa_{15} are any amino acid (e.g., any of the 20 naturally occurring amino acids).

[0015] In some cases of such non-cross-linked peptides:

Xaa_1 = Leu or Gln or the linking group R_3

Xaa_4 = Glu or Gln or the linking group R_3

Xaa_7 = Ser or the linking group R_3

Xaa_8 = Asp or any amino acid other than Asp and Glu (preferably Asn) or the linking group R_3

Xaa_{11} = Lys or a positively charged amino acid (preferably Arg) or the linking group R_3

Xaa_{14} = Pro or the linking group R_3

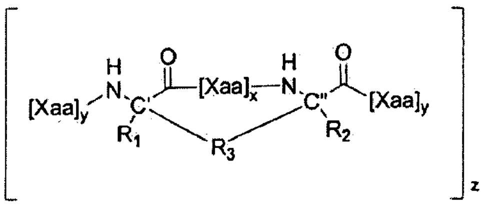
Xaa_{15} = Glu or any amino acid other than Asp and Glu (preferably Gln) or the linking group R_3

[0016] In some cases the non-cross-linked peptide comprises a portion of SEQ ID NO:3 having the sequence: $Gln_3Xaa_4Thr_5Phe_6Xaa_7Xaa_8Leu_9Trp_{10}Xaa_{11}Leu_{12}Leu_{13}$ (SEQ ID NO:4).

[0017] In some instance the modified peptide further comprises, for example: PEG, a fatty acid, or an antibody (e.g., an antibody that targets the modified peptide to a cell expressing p53, HDM2 or HDM4).

[0018] In some instances, each w is independently an integer between 3 and 15. In some instances each y is independently an integer between 1 and 15. In some instances, R_1 and R_2 are each independently H or C_1-C_6 alkyl. In some instances, R_1 and R_2 are each independently C_1-C_3 alkyl. In some instances, at least one of R_1 and R_2 are methyl. For example R_1 and R_2 are both methyl. In some instances R_3 is alkyl (e.g., C_8 alkyl) and x is 3. In some instances, R_3 is C_{11} alkyl and x is 6. In some instances, R_3 is alkenyl (e.g., C_8 alkenyl) and x is 3. In some instances x is 6 and R_3 is C_{11} alkenyl. In some instances, R_3 is a straight chain alkyl, alkenyl, or alkynyl. In some instances R_3 is $-CH_2-CH_2-CH_2-CH=CH-CH_2-CH_2-CH_2-$. In some instances R_3 is $-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-CH=CH-CH_2-CH_2-CH_2-$. In some instances R_3 is $-CH_2-CH_2-CH_2-CH=CH-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-$.

[0019] In certain instances, the two alpha, alpha disubstituted stereocenters (alpha carbons) are both in the R configuration or S configuration (e.g., i, i+4 cross-link), or one stereocenter is R and the other is S (e.g., i, i+7 cross-link). Thus, where Formula I is depicted as



the C' and C'' disubstituted stereocenters can both be in the R configuration or they can both be in the S configuration, for example when x is 3. When x is 6, the C' disubstituted stereocenter is in the R configuration and the C" disubstituted stereocenter is in the S configuration. The R_3 double bond may be in the E or Z stereochemical configuration. Similar configurations are possible for the carbons in Formula II corresponding to C' and C" in the formula depicted immediately above.

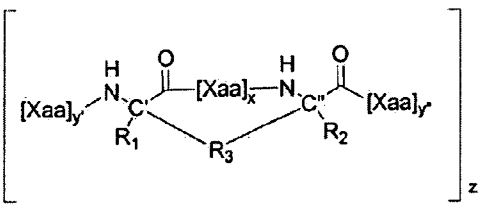
[0020] In some instances R_3 is $[R_4\text{-K-}R_4']_n$; and R_4 and R_4'are independently alkylene, alkenylene or alkynylene (e.g., each are independently a C1, C2, C3, C4, C5, C6, C7, C8, C9 or C10 alkylene, alkenylene or alkynylene

[0021] In some instances, the polypeptide includes an amino acid sequence which, in addition to the amino acids side chains that are replaced by a cross-link, have 1, 2, 3, 4 or 5 amino acid changes in any of SEQ ID NOs:1-4.

[0022] The tether can include an alkyl, alkenyl, or alkynyl moiety (e.g., C_5, C_8 or C_{11} alkyl or a C_5, C_8 or C_{11} alkenyl, or C_5, C_8 or C_{11} alkynyl). The tethered amino acid can be alpha disubstituted (e.g., C_1-C_3 or methyl). $[Xaa]_y$ and $[Xaa]_w$ are peptides that can independently comprise at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25 or more contiguous amino acids (preferably 2 or 5 contiguous amino acids) of a p53 polypeptide (e.g., any of SEQ ID NOs:1-4) and

$[Xaa]_x$ is a peptide that can comprise 3 or 6 contiguous amino acids of acids of a p53 peptide.

[0023] The peptide can comprise 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50 amino acids of p53 polypeptide. The amino acids are contiguous except that one or more pairs of amino acids separated by 3 or 6 amino acids are replaced by amino acid substitutes that form a cross-link, e.g., via R_3. Thus, at least two amino acids can be replaced by tethered amino acids or tethered amino acid substitutes. Thus, where formula I is depicted as



$[Xaa]_{y'}$, $[Xaa]_x$ and $[Xaa]_{y''}$ can each comprise contiguous polypeptide sequences from the same or different p53 peptides. The same is true for Formula II.

[0024] The peptides can include 10 (11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50 or more) contiguous amino acids of a p53 polypeptide wherein the alpha carbons of two amino acids that are separated by three amino acids (or six amino acids) are linked via R_3, one of the two alpha carbons is substituted by R_1 and the other is substituted by R_2 and each is linked via peptide bonds to additional amino acids.

[0025] In some instances the polypeptide acts as dominant negative inhibitor p53 degradation. In some instances, the polypeptide also includes a fluorescent moiety or radioisotope or a moiety that can chelate a radioisotope (e.g., mercaptoacetyltriglycine or 1, 4, 7, 10-tetraazacyclododecane-N,N',N'', N'''-tetraacetic acid (DOTA)) chelated to a radioactive isotope of Re, In or Y). In some instances, R_1 and R_2 are methyl; R_3 is C_8 alkyl, C_{11} alkyl, C_8 alkenyl, C_{11} alkenyl, C_8 alkynyl, or C_{11} alkynyl; and x is 2, 3, or 6. In some instances, the polypeptide includes a PEG linker, a tat protein, an affinity label, a targeting moiety, a fatty acid-derived acyl group, a biotin moiety, a fluorescent probe (e.g. fluorescein or rhodamine).

[0026] Also described herein is a method of treating a subject including administering to the subject any of the compounds described herein. In some instances, the method also includes administering an additional therapeutic agent, e.g., a chemotherapeutic agent.

[0027] The peptides may contain one or more asymmetric centers and thus occur as racemates and racemic mixtures, single enantiomers, individual diastereomers and diastereomeric mixtures and geometric isomers (e.g. *Z* or *cis* and *E* or *trans*) of any olefins present. All such isomeric forms of these compounds are expressly included in the present invention. The compounds may also be represented in multiple tautomeric forms, in such instances, the invention expressly includes all tautomeric forms of the compounds described herein (e.g., alkylation of a ring system may result in alkylation at multiple sites, the invention expressly includes all such reaction products). All such isomeric forms of such compounds are included as are all crystal forms.

[0028] Amino acids containing both an amino group and a carboxyl group bonded to a carbon referred to as the alpha carbon. Also bonded to the alpha carbon is a hydrogen and a side-chain. Suitable amino acids include, without limitation, both the D- and L- isomers of the 20 common naturally occurring amino acids found in peptides (e.g., A, R, N, C, D, Q, E, G, H, I, L, K, M, F, P, S, T, W, Y, V (as known by the one letter abbreviations)) as well as the naturally occurring and unnaturally occurring amino acids prepared by organic synthesis or other metabolic routes. The table below provides the structures of the side chains for each of the 20 common naturally-occurring amino acids. In this table the "–" at right side of each structure is the bond to the alpha carbon.

Amino acid	Single Letter	Three Letter	Structure of side chain
Alanine	A	Ala	CH_3-
Arginine	R	Arg	HN=C(NH_2)-NH-(CH_2)$_3$-
Asparagine	N	Asn	H_2N-C(O)-CH_2-
Aspartic acid	D	Asp	HO(O)C-CH_2-
Cysteine	C	Cys	HS-CH_2-
Glutamine	Q	Gln	H_2N-C(O)-(CH_2)$_2$-
Glutamic acid	E	Glu	HO(O)C-(CH_2)$_2$-
Glycine	G	Gly	H-
Histidine	H	His	N=CH-NH-CH=C-CH_2-
Isoleucine	I	Ile	CH_3-CH_2-CH(CH_3)-
Leucine	L	Leu	(CH_3)$_2$-CH-CH_2-
Lysine	K	Lys	H_2N-(CH_2)$_4$-
Methionine	M	Met	CH_3-S-(CH_2)$_2$-
Phenylalanine	F	Phe	Phenyl-CH_2-
Proline	P	Pro	-N-(CH_2)$_3$-CH-
Serine	S	Ser	HO-CH_2-
Threonine	T	Thr	CH_3-CH(OH)-
Tryptophan	W	Trp	Phenyl-NH-CH=C-CH_2-
Tyrosine	Y	Tyr	4-OH-Phenyl-CH_2-
Valine	V	Val	CH_3-CH(CH_2)-

[0029] A "non-essential" amino acid residue is a residue that can be altered from the wild-type sequence of a polypeptide (without abolishing or substantially altering its activity. An "essential" amino acid residue is a residue that, when altered from the wild-type sequence of the polypeptide, results in abolishing or substantially abolishing the polypeptide activity.

[0030] A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art.

These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine).

[0031] The symbol "⟋⟋ "when used as part of a molecular structure refers to a single bond or a *trans* or *cis* double bond.

[0032] The term "amino acid side chain" refers to a moiety attached to the α-carbon in an amino acids. For example, the amino acid side chain for alanine is methyl, the amino acid side chain for phenylalanine is phenylmethyl, the amino acid side chain for cysteine is thiomethyl, the amino acid side chain for aspartate is carboxymethyl, the amino acid side chain for tyrosine is 4-hydroxyphenylmethyl, etc. Other non-naturally occurring amino acid side chains are also included, for example, those that occur in nature (e.g., an amino acid metabolite) or those that are made synthetically (e.g., an alpha di-substituted amino acid).

[0033] The term polypeptide encompasses two or more naturally occurring or synthetic amino acids linked by a covalent bond (e.g., a amide bond). Polypeptides as described herein include full length proteins (e.g., fully processed proteins) as well as shorter amino acids sequences (e.g., fragments of naturally occurring proteins or synthetic polypeptide fragments).

[0034] The term "halo" refers to any radical of fluorine, chlorine, bromine or iodine. The term "alkyl" refers to a hydrocarbon chain that may be a straight chain or branched chain, containing the indicated number of carbon atoms. For example, C_1-C_{10} indicates that the group may have from 1 to 10 (inclusive) carbon atoms in it. In the absence of any numerical designation, "alkyl" is a chain (straight or branched) having 1 to 20 (inclusive) carbon atoms in it. The term "alkylene" refers to a divalent alkyl (i.e., -R-).

[0035] The term "alkenyl" refers to a hydrocarbon chain that may be a straight chain or branched chain having one or more carbon-carbon double bonds in either *Z* or *E* geometric configurations.. The alkenyl moiety contains the indicated number of carbon atoms. For example, C_2-C_{10} indicates that the group may have from 2 to 10 (inclusive) carbon atoms in it. The term "lower alkenyl" refers to a C_2-C_8 alkenyl chain. In the absence of any numerical designation, "alkenyl" is a chain (straight or branched) having 2 to 20 (inclusive) carbon atoms in it.

[0036] The term "alkynyl" refers to a hydrocarbon chain that may be a straight chain or branched chain having one or more carbon-carbon triple bonds. The alkynyl moiety contains the indicated number of carbon atoms. For example, C_2-C_{10} indicates that the group may have from 2 to 10 (inclusive) carbon atoms in it. The term "lower alkynyl" refers to a C_2-C_8 alkynyl chain. In the absence of any numerical designation, "alkynyl" is a chain (straight or branched) having 2 to 20 (inclusive) carbon atoms in it.

[0037] The term "aryl" refers to a 6-carbon monocyclic or 10-carbon bicyclic aromatic ring system wherein 0, 1, 2, 3, or 4 atoms of each ring may be substituted by a substituent. Examples of aryl groups include phenyl, naphthyl and the like. The term "arylalkyl" or the term "aralkyl" refers to alkyl substituted with an aryl. The term "arylalkoxy" refers to an alkoxy substituted with aryl.

[0038] The term "cycloalkyl" as employed herein includes saturated and partially unsaturated cyclic hydrocarbon groups having 3 to 12 carbons, preferably 3 to 8 carbons, and more preferably 3 to 6 carbons, wherein the cycloalkyl group additionally may be optionally substituted. Preferred cycloalkyl groups include, without limitation, cyclopropyl, cyclobutyl, cyclopentyl, cyclopentenyl, cyclohexyl, cyclohexenyl, cycloheptyl, and cyclooctyl.

[0039] The term "heteroaryl" refers to an aromatic 5-8 membered monocyclic, 8-12 membered bicyclic, or 11-14 membered tricyclic ring system having 1-3 heteroatoms if monocyclic, 1-6 heteroatoms if bicyclic, or 1-9 heteroatoms if tricyclic, said heteroatoms selected from O, N, or S (e.g., carbon atoms and 1-3, 1-6, or 1-9 heteroatoms of N, O, or S if monocyclic, bicyclic, or tricyclic, respectively), wherein 0, 1, 2, 3, or 4 atoms of each ring may be substituted by a substituent. Examples of heteroaryl groups include pyridyl, furyl or furanyl, imidazolyl, 1,2,3-triazolyl, 1,2,4-triazolyl, benzimidazolyl, pyrimidinyl, thiophenyl or thienyl, quinolinyl, indolyl, thiazolyl, and the like. The term "heteroarylalkyl" or the term "heteroaralkyl" refers to an alkyl substituted with a heteroaryl. The term "heteroarylalkoxy" refers to an alkoxy substituted with heteroaryl.

[0040] The term "heterocyclyl" refers to a nonaromatic 5-8 membered monocyclic, 8-12 membered bicyclic, or 11-14 membered tricyclic ring system having 1-3 heteroatoms if monocyclic, 1-6 heteroatoms if bicyclic, or 1-9 heteroatoms if tricyclic, said heteroatoms selected from O, N, or S (e.g., carbon atoms and 1-3, 1-6, or 1-9 heteroatoms of N, O, or S if monocyclic, bicyclic, or tricyclic, respectively), wherein 0, 1, 2 or 3 atoms of each ring may be substituted by a substituent. Examples of heterocyclyl groups include piperazinyl, pyrrolidinyl, dioxanyl, aziridinyl, oxiryl, thiiryl, morpholinyl, tetrahydrofuranyl, and the like.

[0041] The term "substituents" refers to a group "substituted" on an alkyl, cycloalkyl, aryl, heterocyclyl, or heteroaryl group at any atom of that group. Suitable substituents include, without limitation, halo, hydroxy, mercapto, oxo, nitro, haloalkyl, alkyl, alkaryl, aryl, aralkyl, alkoxy, thioalkoxy, aryloxy, amino, alkoxycarbonyl, amido, carboxy, alkanesulfonyl, alkylcarbonyl, azido, and cyano groups.

[0042] The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

DESCRIPTION OF DRAWINGS

[0043] **FIG 1** Synthesis, Sequence, and Biochemical Analysis of SAH-p53 Peptides. (A) Non-natural amino acids were synthesized as described and cross-linked by ruthenium-catalyzed ring-closing olefin metathesis. (B) SAH-p53 compounds were generated by stapling the $p53_{14\text{-}29}$ sequence at the indicated positions. Charge, α-helicity, HDM2 binding affinity, cell permeability, and cell viability are indicated for each compound. (C, E) Circular dichroism spectra revealed enhancement of alpha-helicity for SAH-p53 compounds. (D, F) Fluorescence polarization assays using FITC-peptides and $HDM2_{17\text{-}125}$ demonstrated subnanomolar HDM2-binding affinities for select SAH-p53 peptides. Note: UAH-p53-8 is the "unstapled" form of SAH-p53-8.

[0044] **FIG 2** SAH-p53-8 Reactivates the p53 Transcriptional Pathway. HDM2 overexpressing SJSA-1 cells were exposed to the indicated peptides and Western analyses for p53, HDM2 and p21 were performed at 8-30 h of treatment.

[0045] **FIG 3** Reactivation of Apoptosis in SAH-p53-8-treated SJSA-1 Cells. SAH-p53-8 demonstrated specific, dose-dependent cytotoxicity and apoptosis induction. Cell viability assay of SJSA-1 cells treated with SAH-p53 peptides (A). Caspase-3 activation assay of SJSA-1 cells treated with SAH-p53 peptides (B). Comparison of caspase-3 activation in SJSA-1, HCT-116 $p53^{+/+}$, and HCT-116 $p53^{-/-}$ cells treated with SAH-p53-peptides (25 μM) (C).

[0046] **FIG. 4** Electrospray mass spectrum (positive ion mode) of peptide SAH-p53-4.

[0047] **FIG. 5** To determine whether SAH-p53 peptides have increased proteolytic stability, the wild type $p53_{14\text{-}29}$ peptide and SAH-p53-4 were exposed to serum *ex vivo*. SAH-p53-4 displayed a serum half-life ($t_{1/2}$) almost four times longer than that of the unmodified wild type peptide.

[0048] **FIG. 6** To determine if SAH-p53 peptides 1-4 were cell permeable, Jurkat T-cell leukemia cells were incubated with fluoresceinated p53 peptides for 4 hours followed by washing, trypsinization, and FACS analysis to evaluate cellular fluorescence. None of the peptides tested produced cellular fluorescence.

[0049] **FIG. 7** (A) SJSA-1 cells were treated with FITC-SAH-p53-5 and 4.4 kDa TRITC-dextran for 4 hours. Confocal microscopy revealed co-localization of FITC-SAH-p53-5 peptide with TRITC-dextran in pinosomes. (B) To assess whether the permeability of FITC-SAH-p53-5 was temperature-dependent, Jurkat T-cell leukemia cells were incubated with fluoresceinated p53 peptides for 4 hours at either 4 °C or 37 °C followed by washing, trypsinization, and FACS analysis to evaluate cellular fluorescence. (C) To determine the kinetics of cell permeability, Jurkat T-cell leukemia cells were exposed to FITC-SAH-p53-5 peptide and cellular fluorescence was evaluated by FACS analysis at successive time points. FITC-SAH-p53-5-treated cells displayed a time-dependent increase in cellular fluorescence. (D) SJSA-1 cells were treated with FITC-wild type, SAH-p53-8, and SAH-p53-8_{F19A} peptides for 4 hours followed by FACS and confocal microscopy analyses. Cellular fluorescence was observed after treatment with FITC-SAH-p53 peptides, but not with FITC-wild type p53 peptide.

[0050] **FIG. 8** SJSA-1 cells were incubated with FITC-peptides followed by lysis and anti-FITC pull down. Native HDM2 co-immunoprecipitated with FITC-SAH-p53-8 but not with wild-type or mutant SAH-p53-8_{F19A} peptides. Left: silver stained gel; right: Western Blots.

[0051] **FIG. 9** Annexin V binding as an indicator of apoptosis. RKO cells were treated with peptides at different doses for 24 hours followed by staining with propidium iodide and FITC-tagged annexin V. Apoptosis induction was quantified by FACS and the data analyzed with FloJo software.

[0052] **FIG. 10** Fluorescence polarization binding assay of stapled peptides. Fluoresceinated peptides (5 n*M*) were incubated with recombinant $HDM2_{17-125}$ (25 p*M* – 10 μ*M*) at room temperature. Binding activity was measured by fluorescence polarization, and *K*d values were obtained by linear regression.

[0053] **FIG. 11** Cell viability assay. SJSA-1 osteosarcoma cells were treated with different concentrations of SAH-p53-8 alone or in combination with the chemotherapeutic agent doxorubicin (20 μ*M*) for 24 h. Cell viability was assayed by addition of CellTiter-Glo™ bioluminescence reagent and reading on a plate reader.

[0054] **FIG. 12** Competition by fluorescence polarization. Fluoresceinated, wild type $p53_{14\text{-}29}$ (25 n*M*) was incubated with recombinant $HDM2_{17\text{-}125}$. Unlabeled SAH-p53s were titrated into the mixture, and displacement of the labeled ligand was measured by fluorescence polarization.

[0055] **FIG. 13** Caspase-3 activation assay. SJSA-1 osteosarcoma cells were treated with different concentrations of SAH-p53s for 24 h. The cells were then exposed to a caspase-3 specific substrate (Ac-DEVD-AMC). Fluorescence as a result of cleavage was measured in a microplate reader. To determine the specificity of the activity, certain peptides were incubated alongside DEVD-CHO, a substrate known to inhibit caspase-3 specifically.

[0056] **FIG. 14** Immunohistochemistry on mouse tumor xenografts. Two mice each containing three SJSA-1-derived tumor xenografts were treated with 10 mg kg^{-1} SAH-p53-8 (A) or vehicle (B) every 12 hours for two days. Paraffin sections were obtained from the tumor xenografts and were stained using an α-p53 antibody. p53 deficiency due to HDM2 amplification is evidenced in the untreated control (B), while p53 accumulation near capillaries is seen in the sample treated with SAH-p53-8 (A).

[0057] **FIG. 15** Amino acid sequence of human p53 (GenBank® Accession No. CAA42627; gi:50637).

[0058] **FIG. 16** Sequences of various stapled peptides.

DETAILED DESCRIPTION

[0059] Described herein are internally cross-linked alpha helical domain polypeptides related to human p53. The polypeptides include a tether (also called a cross-link) between two non-natural amino acids that significantly enhance the alpha helical secondary structure of the polypeptide. Generally, the tether or cross-link (sometimes referred to as staple) extends across the length of one or two helical turns (i.e., about 3.4 or about 7 amino acids). Accordingly, amino acids positioned at i and i+3; i and i+4; or i and i+7 are ideal candidates for chemical modification and cross-linking. Thus, for example, where a peptide has the sequence ...Xaa_1, Xaa_2, Xaa_3, Xaa_4, Xaa_5, Xaa_6, Xaa_7, Xaa_8, Xaa_9... (wherein "..." indicates the optional presence of additional amino acids), cross-links between Xaa_1 and Xaa_4, or between Xaa_1 and Xaa_5, or between Xaa_1 and Xaa_8 are useful as are cross-links between Xaa_2 and Xaa_5, or between Xaa_2 and Xaa_6, or between Xaa_2 and Xaa_9, etc. The polypeptides can include more than one crosslink within the polypeptide sequence to either further stabilize the sequence or facilitate the stabilization of longer polypeptide stretches. If the polypeptides are too long to be readily synthesized in one part, independently synthesized, cross-linked peptides can be conjoined by a technique called native chemical ligation (Bang, et al., *J. Am. Chem Soc.* 126:1377).

[0060] Described herein are stabilized alpha-helix of p53 (SAH-p53) peptides that exhibit high affinity for HDM2, and, in contrast to the corresponding unmodified p53 peptide, readily enter cells through an active uptake mechanism. As described below, SAH-p53 treatment reactivated the p53 tumor suppressor cascade by inducing the transcription of p53-responsive genes, providing the first example of a stapled peptide that kills cancer cells by targeting a transcriptional pathway.

[0061] To design SAH-p53 compounds, we placed synthetic olefinic derivatives at positions that avoid critical HDM2-binding residues. Hydrocarbon staples spanning the *i*, *i+7* positions were generated by olefin metathesis (FIG 1A). An initial panel of four SAH-p53 peptides was synthesized, each containing a distinctively localized cross-link (FIG 1B). To evaluate the structural impact of installing an all-hydrocarbon *i*, *i*+7 staple, we conducted circular dichroism (CD) experiments to determine α-helicity. While the wild type p53 peptide displayed 11% α-helical content in water at pH 7.0, SAH-p53s 1-4 demonstrated 10-59% α-helicity (FIGs. 1B and 1C). Fluorescence polarization binding assays using $HDM2_{17\text{-}125}$ and FITC-labeled derivatives of SAH-p53s 1-4 identified SAH-p53-4 as a subnanomolar interactor, having an affinity for HDM2 almost three orders of magnitude greater than that of the wild type peptide (FIGs. 1B and 1D). SAH-p53-4 also demonstrated improved proteolytic stability (FIG. 5).

[0062] We found that the initial SAH-p53 compounds generated were incapable of penetrating intact Jurkat T-cells (FIG. 1B and FIG. 6). We noted that SAH-p53s 1-4 were negatively charged (-2) at physiological pH. Positive charge is a characteristic feature of certain classes of cell penetrating peptides.[11] In developing a second generation of compounds, we replaced aspartic and glutamic acids with asparagines and glutamines to adjust peptide charge and mutated select amino acids previously reported to participate in p53 nuclear export (L14Q) and ubiquitylation (K24R)[4, 12] (FIG. 1B). SAH-p53s **5-8** exhibited a 2-8.5 fold enhancement in α-helical content, retained high binding affinity for HDM2, and demonstrated time- and temperature-dependent cellular uptake by FACS and confocal microscopy (FIGs. 1B, 1E, 1F and 7). Cell viability assays using RKO or SJSA-1 cancer cells exposed to SAH-p53 peptides indicated that SAH-p53-**8**, which contained point mutations in both nuclear export and ubiquitylation sites, was the only structurally-stabilized, cell-permeable, and high affinity HDM2 binder that adversely affected cell viability (FIGs. 1B and 4A).

[0063] To determine if HDM2-targeting by SAH-p53-8 could specifically restore native p53 levels, we treated SJSA-1 cells with wild-type, 8, and 8_{F19A} peptides for 8-30 hours and monitored p53 protein levels by Western analysis (FIG. 2). Cells exposed to SAH-p53-8 demonstrated increased p53 proteins levels that peaked at 18 hours post-treatment. p53 resuppression by 24 hours correlated with the time-dependent upregulation of HDM2 by p53, consistent with an intact p53-HDM2 counter-regulatory mechanism.[13] SAH-p53-8 likewise induced upregulation of the cyclin-dependent kinase inhibitor p21.[14] p21 upregulation in cells treated with 8 was detected at 12 hours, reaching peak levels at 18 hours. Baseline levels were restored by 30 hours, consistent with resuppression of native p53. HDM2 and p21 levels were unchanged in SJSA-1 cells treated with wild-type or 8_{F19A}, highlighting the specificity of SAH-p53-8 modulation of the p53 signaling pathway.

[0064] To examine whether SAH-p53-**8**-mediated stabilization of native p53 could inhibit cancer cells by reactivating the apoptotic pathway, we conducted viability and caspase-3 assays using SJSA-1 cells exposed to wild-type, 8, and 8_{F19A} for 24 hours (FIG. 3). Whereas the wild-type and 8_{F19A} peptides had no effect on cell viability, SAH-p53-8 exhibited dose-dependent inhibition of SJSA-1 cell viability (EC_{50}=8.8 *μM*) (FIG. 3A). Caspase-3 activation by fluorescence monitoring of the cleaved caspase-3 substrate Ac-DEVD-AMC[15] showed that neither the wild-type nor the 8_{F19A} peptides had any effect; however, 8 triggered dose-dependent caspase-3 activation (EC_{50}=5.8 *μM*) that was blocked by DEVD-CHO, a specific caspase-3 inhibitor, demonstrating that SAH-p53-8 specifically inhibited cell viability by activating apoptosis in HDM2-overexpressing SJSA-1 cells (FIG. 3B). As can be seen from FIG. 3C, the SAH-p53-8-meidated inhibition of cell viability observed in SJSA-1 cells was also observed in HCT 116 cells, a colon cancer cell line, but not in an HCT 116 cell line variant lacking p53 (HCT 116 $p53^{-/-}$.

[0065] The identification of multiple organic compounds and p53 peptidomimetics with anti-HDM2 activity[8, 16] holds promise for achieving clinical benefit from manipulating the p53 pathway. By generating a stapled peptide-based HDM2 inhibitor, we have documented an *in situ* interaction between SAH-p53-**8** and HDM2 (FIG. 8), confirming that its pro-apoptotic activity derives from restoration of the p53 pathway.

[0066] RKO cells were treated with peptides at different doses for 24 hours followed by staining with propidium iodide and FITC-tagged annexin V. Apoptosis induction was quantified by FACS and the data analyzed with FloJo software. As shown in FIG. 9, p53-SAH-p53-6 caused significant apoptosis.

[0067] A fluorescence polarization binding assay was used to assess binding of peptides to $HDM2_{17-125}$. Fluoresceinated peptides (5 n*M*) were incubated with recombinant $HDM2_{17-125}$ (25 p*M* – 10 μ*M*) at room temperature. Binding activity was measured by fluorescence polarization, and K_D values were obtained by linear regression. The results of this analysis are shown in FIG. 10.

[0068] The effect of SAH-p53-8 alone or in combination with doxorubicin was examined as follows. SJSA-1 osteosarcoma cells were treated with different concentrations of SAH-p53-8 alone or in combination with the chemotherapeutic agent doxorubicin (20 μ*M*) for 24 h. Cell viability was assayed by addition of CellTiter-Glo™ bioluminescence reagent and reading on a plate reader. The results of this analysis are shown in FIG. 11.

[0069] The ability of various SAH-p53s to compete with wild-type $p53_{14-29}$ for binding to $HDM2_{17-125}$ was assessed as follows. Fluoresceinated, wild type $p53_{14-29}$ (25 nM) was incubated with recombinant $HDM2_{17-125}$. Unlabeled SAH-p53s were titrated into the mixture, and displacement of the labeled ligand was measured by fluorescence polarization. The results of this analysis are shown in FIG. 12.

[0070] The effect of various peptides on caspase-3 activation was examined as follows. SJSA-1 osteosarcoma cells were treated with different concentrations of SAH-p53s for 24 h. The cells were then exposed to a caspase-3 specific substrate. Fluorescence as a result of cleavage was measured in a microplate reader. To determine the specificity of the activity, certain peptides were incubated alongside DEVD-CHO, a substrate known to inhibit caspase-3 specifically. The results of this analysis are shown in FIG. 13.

[0071] α,α-Disubstituted non-natural amino acids containing olefinic side chains of varying length can synthesized by known methods (Williams et al. 1991 *J. Am.*

Chem. Soc. 113:9276; Schafmeister et al. 2000 *J. Am. Chem Soc.* 122:5891). For peptides where an i linked to i+7 staple is used (two turns of the helix stabilized) either one S5 amino acid and one R8 is used or one S8 amino acid and one R5 amino acid is used. R8 is synthesized using the same route, except that the starting chiral auxillary confers the R-alkyl-stereoisomer. Also, 8-iodooctene is used in place of 5-iodopentene. Inhibitors are synthesized on a solid support using solid-phase peptide synthesis (SPPS) on MBHA resin.

[0072] <u>Amino acid and peptide synthesis</u>
In the studies described above, Fmoc-protected α-amino acids (other than the olefinic amino acids Fmoc-S_5-OH, Fmoc-R_8-OH , Fmoc-R_8-OH, Fmoc-S_8-OH and Fmoc-R_5-OH), 2-(6-chloro-1-*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU), and Rink Amide MBHA resin were purchased from Novabiochem (San Diego, CA). Dimethylformamide (DMF), *N*-methyl-2-pyrrolidinone (NMP), *N,N*-diisopropylethylamine (DIEA), trifluoroacetic acid (TFA), 1,2-dichloroethane (DCE), fluorescein isothiocyanate (FITC), and piperidine were purchased from Sigma-Aldrich and used as supplied. The synthesis of the olefinic amino acids has been described elsewhere.[1,2]

[0073] The polypeptides in the studies described above were synthesized manually using Fmoc solid phase peptide chemistry on Rink amide MBHA resin with loading levels of 0.4-0.6 mmol/g resin. The following protocol was used:

1. The Fmoc protective group was removed with 20% piperidine in NMP for 30 min.
2. The resin was washed with NMP five times.
3. The subsequent Fmoc-protected amino acid was coupled for 30 min (60 min for a cross-linker) using Fmoc-AA (10 equiv., 4 equiv. for a cross-linker), HCTU (9.9 equiv., 3.9 equiv. for a cross-linker), and DIEA (20 equiv., 7.8 equiv. for a cross-linker).
4. The resin was washed with NMP five times.
5. Repeat from step 1.

All peptides were capped with a β-alanine residue at the *N*-terminus. CD experiments make use of peptides that have been acetylated at the *N*-terminus. The acetylation reaction consisted of deprotection of the Fmoc group as outlined above, followed by reaction with acetic anhydride and DIEA. All other experiments shown make use of fluoresceinated peptides at the *N*-terminus. To this end, the peptides with the deprotected *N*-terminus were exposed to fluorescein isothiocyanate in DMF overnight in the presence of DIEA.

[0074] The ring-closing metathesis reaction was performed on the *N*-terminal capped peptide while still on the solid support in a disposable fritted reaction vessel. The resin was exposed to a 10 m*M* solution of bis(tricyclohexylphosphine)benzylidine ruthenium (IV) dichloride (Grubbs First Generation Catalyst) in 1,2-dichloroethane or dichloromethane for 2 hours. The catalyst addition and 2 hour metathesis reaction was repeated once. The resin-bound peptide was washed with CH_2Cl_2 three times and dried under a stream of nitrogen.

[0075] The peptide was cleaved from the resin and deprotected by exposure to Reagent K (82.5% TFA, 5% thioanisole, 5% phenol, 5% water, 2.5% 1,2-ethanedithiol) and precipitated with methyl-*tert*-butyl ether at 4°C and lyophilized.

[0076] The lyophilized peptides were purified by reverse phase HPLC using a C_{18} column (Agilent). The peptides were characterized by LC-MS and amino acid analysis. Mass spectra were obtained either by electrospray in positive ion mode or by MALDI-TOF. A representative LC trace and mass spectrum are shown below (FIGs. 4-A and 4-B) and the mass spectral data for all the compounds are likewise shown below in Table 2.

Table 2: Mass spectral data for various polypeptides

Compound	Calculated Mass	Found Mass	Method
WT p53$_{14\text{-}29}$	2033.26	2033.12 [M + H]	MALDI-TOF
SAH-p53-1	2097.41	2097.14 [M + H]	MALDI-TOF
SAH-p53-2	2132.40	2132.84 [M + Na]	MALDI-TOF
SAH-p53-3	2089.37	2089.18 [M + Na]	MALDI-TOF
SAH-p53-4	2140.48	2140.70 [M + H]	MALDI-TOF
SAH-p53-5	2138.5	2139.0 [M + H]	ESI
SAH-p53-6	2165.5	1083.2 [M/2 + H]	ESI
SAH-p53-7	2152.4	1077.2 [M/2 + H]	ESI
SAH-p53-8	2180.5	1112.9 [M/2 + Na]	ESI
SAH-p53-8$_{F19A}$	2104.4	1052.9 [M + H]	ESI
unstapled SAH-p53-8	2208.5	2209.1 [M + H]	ESI
FITC-WT p53$_{14\text{-}29}$	2401.59	2402.94 [M + Na]	MALDI-TOF
FITC-SAH-p53-1	2466.74	2467.29 [M + Na]	MALDI-TOF
FITC-SAH-p53-2	2479.74	2479.27 [M + Na]	MALDI-TOF
FITC-SAH-p53-3	2437.72	2437.31 [M + Na]	MALDI-TOF
FITC-SAH-p53-4	2509.81	2509.10 [M + Na]	MALDI-TOF
FITC-SAH-p53-5	2401.59	2402.94 [M + Na]	MALDI-TOF
FITC-SAH-p53-6	2512.8	1257.2 [M/2 + H]	ESI
FITC-SAH-p53-7	2499.8	1250.6 [M/2 + H]	ESI
FITC-SAH-p53-8	2527.8	1286.3 [M/2 + Na]	ESI

FITC-SAH-p53-8$_{F19A}$	2451.7	1248.5 [M/2 + Na]	ESI
unstapled FITC-SAH-p53-8	2555.9	1278.5 [M/2 + Na]	ESI

[0077] <u>Circular Dichroism (CD) Spectroscopy</u>

For circular dichroism (CD) spectroscopy compounds were dissolved in H_2O to concentrations ranging from 10-50 μM. The spectra were obtained on a Jasco J-715 spectropolarimeter at 20°C. The spectra were collected using a 0.1 cm pathlength quartz cuvette with the following measurement parameters: wavelength, 185-255 nm; step resolution 0.1 nm; speed, 20 nm min^{-1}; accumulations, 6; bandwidth, 1 nm. The helical content of each peptide was calculated as reported previously.[3]

[0078] <u>Ex vivo Protease Stability</u>

To assess the protease stability of the peptides, fluoresceinated peptides (2.5 μg) were incubated with fresh mouse serum (20 μL) at 37°C for 0-24 hours. The level of intact fluoresceinated compound was determined by flash freezing the serum specimens in liquid nitrogen, lyophilization, extraction in 1:1 CH_3CN:H_2O containing 0.1% TFA, followed by HPLC-based quantitation using fluorescence detection at excitation/emission settings of 495/530 nm.

[0079] <u>Protein Production and Fluorescence Polarization</u>

Purified HDM2$_{17-125}$ was prepared as follows. *Escherichia coli* BL21 (DE3) containing the plasmid encoding HDM2$_{17-125}$ with an *N*-terminal hexahistidine tag and a thrombin cleavage site were cultured in kanamycin- and chloramphenicol-containing Luria Broth and induced with 0.1 mM isopropyl β-D-thiogalactoside (IPTG). The cells were harvested after 4 hours by centrifugation for 20 min at 3200 rpm, resuspended in buffer A (20 m*M* Tris pH 7.4, 0.5 M NaCl) and lysed by sonication. Cellular debris was pelleted by centrifugation for 30 minutes at 15,000 rpm, and the supernatant was incubated with Ni-NTA agarose (QIAGEN) for 2 h. The resin was washed with buffer A and eluted with a gradient of imidazole ranging from 5 m*M* to 500 m*M*. The fractions containing the eluted protein were concentrated and diluted 1:1 with thrombin cleavage buffer (5 mM $CaCl_2$, 20 m*M*

Tris pH 7.4, 1 μL mL^{-1} β-mercaptoethanol, and 0.8 U mL^{-1} thrombin). The cleavage reaction was incubated overnight at 4°C. The reaction was concentrated to 2 mL and purified by gel filtration using a G75 column. Purity of the protein was assessed by SDS-PAGE, FPLC and MALDI-TOF and determined to be >90%. Its identity was further confirmed by digestion followed by mass spectrometry of the resulting peptide fragments.

[0080] Fluoresceinated compounds (L_T = 5-25 n*M*) were incubated with $HDM2_{17\text{-}125}$ in binding assay buffer (140 mM NaCl, 50 mM, Tris pH 8.0) at room temperature. Binding activity was measured by fluorescence polarization on a Perkin-Elmer LS50B luminescence spectrophotometer using a cuvette containing a stirbar or a Spectramax M5 Microplate Reader (Molecular Devices). K_d values were determined by nonlinear regression analysis of dose response curves using Prism software 4.0 Graphpad. In the case of compounds where $L_T < K_d$ and under the assumption that $L_T \approx L_{free}$, binding isotherms were fitted to the equation

$$P = P_f + \left[(P_b - P_f) \times \frac{R_T}{K_D + R_T} \right] \quad (1)$$

where P is the measured polarization value, P_f is the polarization of the free fluorescent ligand, P_b is the polarization of the bound ligand, and R_T is the receptor/protein concentration.

[0081] With compounds where $L_T > K_d$, the assumption that $L_T \approx L_{free}$ does not hold due to ligand depletion. As such, binding isotherms were fitted to the more explicit equation

$$P = P_f + (P_b - P_f) \left[\frac{(L_T + K_D + R_T) - \sqrt{(L_T + K_D + R_T)^2 - 4L_T R_T}}{2L_T} \right] \quad (2)$$

where P is the measured polarization value, P_f is the polarization of the free fluorescent ligand, P_b is the polarization of the bound ligand, L_T is the total concentration of fluorescent ligand and R_T is the receptor/protein concentration.[4] Each data point represents the average of an experimental condition performed in at least triplicate.

[0082] Flow Cytometry

Jurkat T-cell leukemia cells were grown in RPMI-1640 (Gibco) medium with 10% fetal bovine serum, 100 U mL^{-1} penicillin, 100 μg mL^{-1}, 2 mM glutamine, 50 mM Hepes pH 7, and 50 μM β-mercaptoethanol. SJSA-1 cells were cultured in McCoy's 5A media (ATCC) supplemented with 10% fetal bovine serum and 100 U mL^{-1} penicillin. Jurkat cells (50,000 cells per well) were treated with fluoresceinated peptides (10 μM) for up to 4 hours at 37°C. After washing with media, the cells were exposed to trypsin (0.25%; Gibco) digestion (30 min, 37°C), washed with PBS, and resuspended in PBS containing 0.5 mg mL^{-1} propidium iodide (BD Biosciences). Cellular fluorescence and propidium iodide positivity were analyzed using a FACSCalibur flow cytometer (Becton Dickinson) and FlowJo software (TreeStar). The identical experiment was performed with 30 min pre-incubation of cells at 4°C followed by 4 hour incubation with fluoresceinated peptides at 4°C to assess temperature-dependence of fluorescent labeling.

[0083] Confocal Microscopy

Jurkat T-cell leukemia cells were incubated with fluoresceinated compounds for 24 hours at 37°C. After washing with PBS, the cells were cytospun at 600 rpm for 5 minutes onto Superfrost plus glass slides (Fisher Scientific). The cells were fixed in 4% paraformaldehyde, washed with PBS, incubated with TOPRO-3 iodide (100 nM; Molecular Probes) to conterstain nuclei, treated with Vectashield mounting medium (Vector), and imaged by confocal microscopy (BioRad 1024 or Nikon E800).

[0084] In a similar fashion, SJSA-1 osteosarcoma cells (1 x 10^5 cells) were incubated in with fluoresceinated compounds for 24 hours at 37°C in Lab-Tek™-CC2 Chamber Slides (Nunc). After washing with PBS, the cells were fixed in 4% paraformaldehyde, washed with PBS, and treated with DAPI-containing (nuclear counterstain) Vectashield mounting medium (Vector), coverslipped and imaged by confocal microscopy (BioRad 1024 or Nikon E800).

[0085] Western Blotting

SJSA-1 osteosarcoma cells (1 x 10^6) incubated at 37 °C were treated with p53 peptides (20 μ*M*) in serum-free media for 4 hours, followed by serum replacement and additional incubation for 4-26 additional hours. The cells were lysed (20 m*M* Tris-HCl pH 8.0, 0.8% SDS, 1 m*M* PMSF, 1 U mL^{-1} benzonase nuclease) and the crude lysates were clarified by brief centrifugation and total protein concentration was determined by using the Pierce BCA protein assay. Aliquots containing 5 μg of total protein were run on 4-12% Bis-Tris polyacrylamide gels (Invitrogen). Proteins were detected by chemiluminescence reagent (Perkin Elmer) using antibodies specific for p53 (DO-1 clone; Calbiochem), HDM2 (IF2 clone; EMD Biosciences), p21 (EA10 clone; Calbiochem), and β-actin (Sigma-Aldrich).

[0086] Cell Viability and Apoptosis High-Throughput Assays

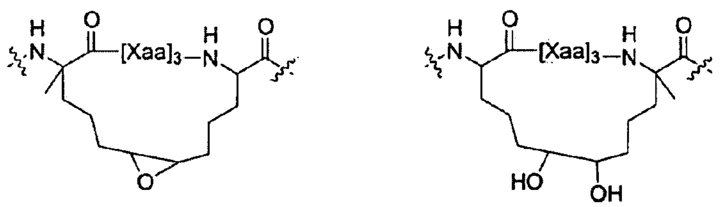
SJSA-1 osteosarcoma cells (4 x 10^5 cells per well) were incubated in 96-well plates and treated with p53 peptides in serum-free media for 4 hours, followed by serum replacement and additional incubation for 20 hours. Cell viability was assayed by addition of CellTiter-Glo™ bioluminescence reagent (Promega) and reading luminescence in a Spectramax M5 microplate reader (Molecular Devices). The extent of apoptosis was measured through the detection of caspase-3 activity by exposing the cells to a caspase-3-specific substrate (Oncogene). Fluorescence as a result of substrate cleavage was measured in a Spectramax M5 microplate reader (Molecular Devices).

[0087] Co-Immunoprecipitation of FITC-SAH-p53 Peptides and Endogenous HDM2

SJSA-1 osteosarcoma cells (1 x 10^6) were treated with FITC-p53 peptides (15 μM) in serum-free media for 4 hours, followed by serum replacement and additional 8 hour incubation. The cells were thoroughly washed with serum-containing media and PBS and exposed to lysis buffer (50 mM Tris pH 7.6, 150 mM NaCl, 1% Triton-X100, 1 mM PMSF, 1 U mL^{-1} benzonase nuclease [EMD Biosciences] and complete protease inhibitor tablet [Roche]) at room temperature. All subsequent steps were all performed at 4 °C. The extracts were centrifuged, and the supernatants were incubated with protein A/G sepharose (50 μL 50% bead slurry per 0.5 mL lysates; Santa Cruz Biotechnology). The pre-cleared supernatants (500 μL) were collected after centrifugation, incubated with 10 μL of goat-anti-FITC antibody (AbCam) for 1.5 h followed by protein A/G sepharose for an additional 1.5 hours. The immunoprecipitation reactions were pelleted and washed three times with lysis buffer. The precipitated proteins were suspended in SDS-containing loading buffer, boiled, and the supernatants were processed by SDS-PAGE on 4-12% Bis-Tris gels (Invitrogen). The proteins were blotted into Immobilon-P membranes (Millipore). After blocking, the blots were incubated with either a 1:100 dilution of mouse anti-human HDM2 antibody (IF2 clone; EMD Biosciences) or a 1:200 dilution rabbit anti-FITC antibody (Zymed) in 3% BSA in PBS followed by anti-mouse or anti-rabbit horseradish peroxidase-conjugated IgG (Pharmingen). The HDM2 protein and FITC peptides were visualized using the Western Lightning™ chemiluminescence reagent (Perkin Elmer) and exposing to film. The gels were stained using a silver stain kit (Bio-Rad) following manufacturer's instructions.

[0088] Polypeptides

In some instances, the hydrocarbon tethers (i.e., cross links) described herein can be further manipulated. In one instance, a double bond of a hydrocarbon alkenyl tether, (e.g., as synthesized using a ruthenium-catalyzed ring closing metathesis (RCM)) can be oxidized (e.g., via epoxidation or dihydroxylation) to provide one of compounds below.



Either the epoxide moiety or one of the free hydroxyl moieties can be further functionalized. For example, the epoxide can be treated with a nucleophile, which provides additional functionality that can be used, for example, to attach a tag (e.g., a radioisotope or fluorescent tag). The tag can be used to help direct the compound to a desired location in the body or track the location of the compound in the body.

Alternatively, an additional therapeutic agent can be chemically attached to the functionalized tether (e.g., an anti-cancer agent such as rapamycin, vinblastine, taxol, etc.). Such derivitization can alternatively be achieved by synthetic manipulation of the amino or carboxy terminus of the polypeptide or via the amino acid side chain. Other agents can be attached to the functionalized tether, e.g., an agent that facilitates entry of the polypeptide into cells.

[0089] While hydrocarbon tethers have been described, other tethers are also envisioned. For example, the tether can include one or more of an ether, thioether, ester, amine, or amide moiety. In some cases, a naturally occurring amino acid side chain can be incorporated into the tether. For example, a tether can be coupled with a functional group such as the hydroxyl in serine, the thiol in cysteine, the primary amine in lysine, the acid in aspartate or glutamate, or the amide in asparagine or glutamine. Accordingly, it is possible to create a tether using naturally occurring amino acids rather than using a tether that is made by coupling two non-naturally occurring amino acids. It is also possible to use a single non-naturally occurring amino acid together with a naturally occurring amino acid.

[0090] It is further envisioned that the length of the tether can be varied. For instance, a shorter length of tether can be used where it is desirable to provide a relatively high degree of constraint on the secondary alpha-helical structure, whereas, in some instances, it is desirable to provide less constraint on the secondary alpha-helical structure, and thus a longer tether may be desired.

Additionally, while examples of tethers spanning from amino acids i to i+3, i to i+4; and i to i+7 have been described in order to provide a tether that is primarily on a single face of the alpha helix, the tethers can be synthesized to span any combinations of numbers of amino acids.

[0091] In some instances, alpha disubstituted amino acids are used in the polypeptide to improve the stability of the alpha helical secondary structure.

However, alpha disubstituted amino acids are not required, and instances using mono-alpha substituents (e.g., in the tethered amino acids) are also envisioned.

[0092] As can be appreciated by the skilled artisan, methods of synthesizing the compounds of the described herein will be evident to those of ordinary skill in the art. Additionally, the various synthetic steps may be performed in an alternate sequence or order to give the desired compounds. Synthetic chemistry transformations and protecting group methodologies (protection and deprotection) useful in synthesizing the compounds described herein are known in the art and include, for example, those such as described in R. Larock, Comprehensive Organic Transformations, VCH Publishers (1989); T.W. Greene and P.G.M. Wuts, Protective Groups in Organic Synthesis, 3d. Ed., John Wiley and Sons (1999); L. Fieser and M. Fieser, Fieser and Fieser's Reagents for Organic Synthesis, John Wiley and Sons (1994); and L. Paquette, ed., Encyclopedia of Reagents for Organic Synthesis, John Wiley and Sons (1995), and subsequent editions thereof.

[0093] The peptides of this invention can be made by chemical synthesis methods, which are well known to the ordinarily skilled artisan. See, for example, Fields et al., Chapter 3 in Synthetic Peptides: A User's Guide, ed. Grant, W. H. Freeman & Co., New York, N.Y., 1992, p. 77. Hence, peptides can be synthesized using the automated Merrifield techniques of solid phase synthesis with the α-NH_2 protected by either t-Boc or Fmoc chemistry using side chain protected amino acids on, for example, an Applied Biosystems Peptide Synthesizer Model 430A or 431.

[0094] One manner of making of the peptides described herein is using solid phase peptide synthesis (SPPS). The C-terminal amino acid is attached to a cross-linked polystyrene resin via an acid labile bond with a linker molecule. This resin is insoluble in the solvents used for synthesis, making it relatively simple and fast to wash away excess reagents and by-products. The N-terminus is protected with the Fmoc group, which is stable in acid, but removable by base. Any side chain functional groups are protected with base stable, acid labile groups.

Longer peptides could be made by conjoining individual synthetic peptides using native chemical ligation. Alternatively, the longer synthetic peptides can be synthesized by well known recombinant DNA techniques. Such techniques are provided in well-known standard manuals with detailed protocols. To construct a gene encoding a peptide of this invention, the amino acid sequence is reverse translated to obtain a nucleic acid sequence encoding the amino acid sequence, preferably with codons that are optimum for the organism in which the gene is to be expressed. Next, a synthetic gene is made, typically by synthesizing oligonucleotides which encode the peptide and any regulatory elements, if necessary. The synthetic gene is inserted in a suitable cloning vector and transfected into a host cell. The peptide is then expressed under suitable conditions appropriate for the selected expression system and host. The peptide is purified and characterized by standard methods.

The peptides can be made in a high-throughput, combinatorial fashion, e.g., using a high-throughput multiple channel combinatorial synthesizer available from Advanced Chemtech.

[0095] In the modified polypeptides one or more conventional peptide bonds replaced by a different bond that may increase the stability of the polypeptide in the body. Peptide bonds can be replaced by: a retro-inverso bonds (C(O)-NH); a reduced amide bond ($NH-CH_2$); a thiomethylene bond ($S-CH_2$ or CH_2-S); an oxomethylene bond ($O-CH_2$ or CH_2-O); an ethylene bond (CH_2-CH_2); a thioamide bond (C(S)-NH); a trans-olefin bond (CH=CH); a fluoro substituted trans-olefin bond (CF=CH); a ketomethylene bond (C(O)-CHR) or CHR-C(O) wherein R is H or CH_3; and a fluoro-ketomethylene bond (C(O)-CFR or CFR-C(O) wherein R is H or F or CH_3.

[0096] The polypeptides can be further modified by: acetylation, amidation, biotinylation, cinnamoylation, farnesylation, fluoresceination, formylation, myristoylation, palmitoylation, phosphorylation (Ser, Tyr or Thr), stearoylation, succinylation and sulfurylation. The polypeptides of the invention may also be conjugated to, for example, polyethylene glycol (PEG); alkyl groups (e.g., C1-C20 straight or branched alkyl groups); fatty acid radicals; and combinations thereof.

[0097] Methods of Treatment

The present invention provides for both prophylactic and therapeutic methods of treating a subject at risk of (or susceptible to) a disorder or having a disorder associated with reduced p53 activity. This is because the polypeptides are expected to act as inhibitors of p53 binding to HDM2 and/or HDMX. As used herein, the term "treatment" is defined as the application or administration of a therapeutic agent to a patient, or application or administration of a therapeutic agent to an isolated tissue or cell line from a patient, who has a disease, a symptom of disease or a predisposition toward a disease, with the purpose to cure, heal, alleviate, relieve, alter, remedy, ameliorate, improve or affect the disease, the symptoms of disease or the predisposition toward disease. A therapeutic agent includes, but is not limited to, small molecules, peptides, antibodies, ribozymes and antisense oligonucleotides.

[0098] The polypeptides described herein can be used to treat, prevent, and/or diagnose cancers and neoplastic conditions. As used herein, the terms "cancer", "hyperproliferative" and "neoplastic" refer to cells having the capacity for autonomous growth, i.e., an abnormal state or condition characterized by rapidly proliferating cell growth. Hyperproliferative and neoplastic disease states may be categorized as pathologic, i.e., characterizing or constituting a disease state, or may be categorized as non-pathologic, i.e., a deviation from normal but not associated with a disease state. The term is meant to include all types of cancerous growths or oncogenic processes, metastatic tissues or malignantly transformed cells, tissues, or organs, irrespective of histopathologic type or stage of invasiveness. "Pathologic hyperproliferative" cells occur in disease states characterized by malignant tumor growth. Examples of non-pathologic hyperproliferative cells include proliferation of cells associated with wound repair.

[0099] Examples of cellular proliferative and/or differentiative disorders include cancer, e.g., carcinoma, sarcoma, or metastatic disorders. The compounds (i.e., polypeptides) can act as novel therapeutic agents for controlling osteosarcomas, colon cancer, breast cancer, T cell cancers and B cell cancer. The polypeptides may also be useful for treating mucoepidermoid carcinoma, retinoblastoma and medulloblastoma. The compounds can be used to treat disorders associated with unwanted proliferation of cells having reduced activity and/or expression of p53, particularly where the cells produce at least some active p53.

[0100] Examples of proliferative disorders include hematopoietic neoplastic disorders. As used herein, the term "hematopoietic neoplastic disorders" includes diseases involving hyperplastic/neoplastic cells of hematopoietic origin, e.g., arising from myeloid, lymphoid or erythroid lineages, or precursor cells thereof. Exemplary disorders include: acute leukemias, e.g., erythroblastic leukemia and acute megakaryoblastic leukemia. Additional exemplary myeloid disorders include, but are not limited to, acute promyeloid leukemia (APML), acute myelogenous leukemia (AML) and chronic myelogenous leukemia (CML) (reviewed in Vaickus, L. (1991) Crit Rev. in Oncol./Hemotol. 11:267-97); lymphoid malignancies include, but are not limited to acute lymphoblastic leukemia (ALL) which includes B-lineage ALL and T-lineage ALL, chronic lymphocytic leukemia (CLL), prolymphocytic leukemia (PLL), multiple mylenoma, hairy cell leukemia (HLL) and Waldenstrom's macroglobulinemia (WM). Additional forms of malignant lymphomas include, but are not limited to non-Hodgkin lymphoma and variants thereof, peripheral T cell lymphomas, adult T cell leukemia/lymphoma (ATL), cutaneous T-cell lymphoma (CTCL), large granular lymphocytic leukemia (LGF), Hodgkin's disease and Reed-Sternberg disease.

[0101] Examples of cellular proliferative and/or differentiative disorders of the breast include, but are not limited to, proliferative breast disease including, e.g., epithelial hyperplasia, sclerosing adenosis, and small duct papillomas; tumors, e.g., stromal tumors such as fibroadenoma, phyllodes tumor, and sarcomas, and epithelial tumors such as large duct papilloma; carcinoma of the breast including in situ (noninvasive) carcinoma that includes ductal carcinoma in situ (including Paget's disease) and lobular carcinoma in situ, and invasive (infiltrating) carcinoma including, but not limited to, invasive ductal carcinoma, invasive lobular carcinoma, medullary carcinoma, colloid (mucinous) carcinoma, tubular carcinoma, and invasive papillary carcinoma, and miscellaneous malignant neoplasms. Disorders in the male breast include, but are not limited to, gynecomastia and carcinoma.

[0102] Pharmaceutical Compositions and Routes of Administration
As used herein, the compounds of this invention, including the compounds of formulae described herein, are defined to include pharmaceutically acceptable derivatives or prodrugs thereof. A "pharmaceutically acceptable derivative or prodrug" means any pharmaceutically acceptable salt, ester, salt of an ester, or other derivative of a compound of this invention which, upon administration to a recipient, is capable of providing (directly or indirectly) a compound of this invention. Particularly favored derivatives and prodrugs are those that increase the bioavailability of the compounds of this invention when such compounds are administered to a mammal (e.g., by allowing an orally administered compound to be more readily absorbed into the blood) or which enhance delivery of the parent compound to a biological compartment (e.g., the brain or lymphatic system) relative to the parent species. Preferred prodrugs include derivatives where a group which enhances aqueous solubility or active transport through the gut membrane is appended to the structure of formulae described herein.

[0103] The compounds of this invention may be modified by appending appropriate functionalities to enhance selective biological properties. Such modifications are known in the art and include those which increase biological penetration into a given biological compartment (e.g., blood, lymphatic system, central nervous system), increase oral availability, increase solubility to allow administration by injection, alter metabolism and alter rate of excretion.

[0104] Pharmaceutically acceptable salts of the compounds of this invention include those derived from pharmaceutically acceptable inorganic and organic acids and bases. Examples of suitable acid salts include acetate, adipate, benzoate, benzenesulfonate, butyrate, citrate, digluconate, dodecylsulfate, formate, fumarate, glycolate, hemisulfate, heptanoate, hexanoate, hydrochloride, hydrobromide, hydroiodide, lactate, maleate, malonate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, nitrate, palmoate, phosphate, picrate, pivalate, propionate, salicylate, succinate, sulfate, tartrate, tosylate, trifluoromethylsulfonate, and undecanoate. Salts derived from appropriate bases include alkali metal (e.g., sodium), alkaline earth metal (e.g., magnesium), ammonium and N-$(alkyl)_4^+$ salts. This invention also envisions the quaternization of any basic nitrogen-containing groups of the compounds disclosed herein. Water or oil-soluble or dispersible products may be obtained by such quaternization.

[0105] The compounds of the formulae described herein can, for example, be administered by injection, intravenously, intraarterially, subdermally, intraperitoneally, intramuscularly, or subcutaneously; or orally, buccally, nasally, transmucosally, topically, in an ophthalmic preparation, or by inhalation, with a dosage ranging from about 0.001 to about 100 mg/kg of body weight, or according to the requirements of the particular drug. The methods herein contemplate administration of an effective amount of compound or compound composition to achieve the desired or stated effect. Typically, the pharmaceutical compositions of this invention will be administered from about 1 to about 6 times per day or alternatively, as a continuous infusion. Such administration can be used as a chronic or acute therapy. The amount of active ingredient that may be combined with the carrier materials to produce a single dosage form will vary depending upon the host treated and the particular mode of administration. A typical preparation will contain from about 5% to about 95% active compound (w/w). Alternatively, such preparations contain from about 20% to about 80% active compound.

[0106] Lower or higher doses than those recited above may be required. Specific dosage and treatment regimens for any particular patient will depend upon a variety of factors, including the activity of the specific compound employed, the age, body weight, general health status, sex, diet, time of administration, rate of excretion, drug combination, the severity and course of the disease, condition or symptoms, the patient's disposition to the disease, condition or symptoms, and the judgment of the treating physician.

[0107] Upon improvement of a patient's condition, a maintenance dose of a compound, composition or combination of this invention may be administered, if necessary. Subsequently, the dosage or frequency of administration, or both, may be reduced, as a function of the symptoms, to a level at which the improved condition is retained. Patients may, however, require intermittent treatment on a long-term basis upon any recurrence of disease symptoms.

[0108] Pharmaceutical compositions of this invention comprise a compound of the formulae described herein or a pharmaceutically acceptable salt thereof; an additional agent including for example, morphine or codeine; and any pharmaceutically acceptable carrier, adjuvant or vehicle. Alternate compositions of this invention comprise a compound of the formulae described herein or a pharmaceutically acceptable salt thereof; and a pharmaceutically acceptable carrier, adjuvant or vehicle. The compositions delineated herein include the compounds of the formulae delineated herein, as well as additional therapeutic agents if present, in amounts effective for achieving a modulation of disease or disease symptoms. The term "pharmaceutically acceptable carrier or adjuvant" refers to a carrier or adjuvant that may be administered to a patient, together with a compound of this invention, and which does not destroy the pharmacological activity thereof and is nontoxic when administered in doses sufficient to deliver a therapeutic amount of the compound.

[0109] Pharmaceutically acceptable carriers, adjuvants and vehicles that may be used in the pharmaceutical compositions of this invention include, but are not limited to, ion exchangers, alumina, aluminum stearate, lecithin, self-emulsifying drug delivery systems (SEDDS) such as d-α-tocopherol polyethyleneglycol 1000 succinate, surfactants used in pharmaceutical dosage forms such as Tweens or other similar polymeric delivery matrices, serum proteins, such as human serum albumin, buffer substances such as phosphates, glycine, sorbic acid, potassium sorbate, partial glyceride mixtures of saturated vegetable fatty acids, water, salts or electrolytes, such as protamine sulfate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride, zinc salts, colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone, cellulose-based substances, polyethylene glycol, sodium carboxymethylcellulose, polyacrylates, waxes, polyethylene-polyoxypropylene-block polymers, polyethylene glycol and wool fat. Cyclodextrins such as α-, β-, and γ-cyclodextrin, may also be advantageously used to enhance delivery of compounds of the formulae described herein.

[0110] The pharmaceutical compositions of this invention may be administered orally, parenterally, by inhalation spray, topically, rectally, nasally, buccally, vaginally or via an implanted reservoir, preferably by oral administration or administration by injection. The pharmaceutical compositions of this invention may contain any conventional non-toxic pharmaceutically-acceptable carriers, adjuvants or vehicles. In some cases, the pH of the formulation may be adjusted with pharmaceutically acceptable acids, bases or buffers to enhance the stability of the formulated compound or its delivery form. The term parenteral as used herein includes subcutaneous, intracutaneous, intravenous, intramuscular, intraarticular, intraarterial, intrasynovial, intrasternal, intrathecal, intralesional and intracranial injection or infusion techniques.

[0111] The pharmaceutical compositions may be in the form of a sterile injectable preparation, for example, as a sterile injectable aqueous or oleaginous suspension. This suspension may be formulated according to techniques known in the art using suitable dispersing or wetting agents (such as, for example, Tween 80) and suspending agents. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally acceptable diluent or solvent, for example, as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are mannitol, water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose, any bland fixed oil may be employed including synthetic mono- or diglycerides. Fatty acids, such as oleic acid and its glyceride derivatives are useful in the preparation of injectables, as are natural pharmaceutically-acceptable oils, such as olive oil or castor oil, especially in their polyoxyethylated versions. These oil solutions or suspensions may also contain a long-chain alcohol diluent or dispersant, or carboxymethyl cellulose or similar dispersing agents which are commonly used in the formulation of pharmaceutically acceptable dosage forms such as emulsions and or suspensions. Other commonly used surfactants such as Tweens or Spans and/or other similar emulsifying agents or bioavailability enhancers which are commonly used in the manufacture of pharmaceutically acceptable solid, liquid, or other dosage forms may also be used for the purposes of formulation.

[0112] The pharmaceutical compositions of this invention may be orally administered in any orally acceptable dosage form including, but not limited to, capsules, tablets, emulsions and aqueous suspensions, dispersions and solutions. In the case of tablets for oral use, carriers which are commonly used include lactose and corn starch. Lubricating agents, such as magnesium stearate, are also typically added. For oral administration in a capsule form, useful diluents include lactose and dried corn starch. When aqueous suspensions and/or emulsions are administered orally, the active ingredient may be suspended or dissolved in an oily phase is combined with emulsifying and/or suspending agents. If desired, certain sweetening and/or flavoring and/or coloring agents may be added.

[0113] The pharmaceutical compositions of this invention may also be administered in the form of suppositories for rectal administration. These compositions can be prepared by mixing a compound of this invention with a suitable non-irritating excipient which is solid at room temperature but liquid at the rectal temperature and therefore will melt in the rectum to release the active components. Such materials include, but are not limited to, cocoa butter, beeswax and polyethylene glycols.

[0114] The pharmaceutical compositions of this invention may be administered by nasal aerosol or inhalation. Such compositions are prepared according to techniques well-known in the art of pharmaceutical formulation and may be prepared as solutions in saline, employing benzyl alcohol or other suitable preservatives, absorption promoters to enhance bioavailability, fluorocarbons, and/or other solubilizing or dispersing agents known in the art.

When the compositions of this invention comprise a combination of a compound of the formulae described herein and one or more additional therapeutic or prophylactic agents, both the compound and the additional agent should be present at dosage levels of between about 1 to 100%, and more preferably between about 5 to 95% of the dosage normally administered in a monotherapy regimen. The additional agents may be administered separately, as part of a multiple dose regimen, from the compounds of this invention. Alternatively, those agents may be part of a single dosage form, mixed together with the compounds of this invention in a single composition.

[0115] Modification of Polypeptides

The stapled polypeptides can include a drug, a toxin, a derivative of polyethylene glycol; a second polypeptide; a carbohydrate, etc. Where a polymer or other agent is linked to the stapled polypeptide is can be desirable for the composition to be substantially homogeneous.

[0116] The addition of polyethelene glycol (PEG) molecules can improve the pharmacokinetic and pharmacodynamic properties of the polypeptide. For example, PEGylation can reduce renal clearance and can result in a more stable plasma concentration. PEG is a water soluble polymer and can be represented as linked to the polypeptide as formula:

$XO\text{--}(CH_2CH_2O)_n\text{--}CH_2CH_2\text{--}Y$ where n is 2 to 10,000 and X is H or a terminal modification, e.g., a $C_{1\text{-}4}$ alkyl; and Y is an amide, carbamate or urea linkage to an amine group (including but not limited to, the epsilon amine of lysine or the N-terminus) of the polypeptide. Y may also be a maleimide linkage to a thiol group (including but not limited to, the thiol group of cysteine). Other methods for linking PEG to a polypeptide, directly or indirectly, are known to those of ordinary skill in the art. The PEG can be linear or branched. Various forms of PEG including various functionalized derivatives are commercially available.

[0117] PEG having degradable linkages in the backbone can be used. For example, PEG can be prepared with ester linkages that are subject to hydrolysis. Conjugates having degradable PEG linkages are described in WO 99/34833; WO 99/14259, and U.S. 6,348,558.

[0118] In certain embodiments, macromolecular polymer (e.g., PEG) is attached to an agent described herein through an intermediate linker. In certain embodiments, the linker is made up of from 1 to 20 amino acids linked by peptide bonds, wherein the amino acids are selected from the 20 naturally occurring amino acids. Some of these amino acids may be glycosylated, as is well understood by those in the art. In other embodiments, the 1 to 20 amino acids are selected from glycine, alanine, proline, asparagine, glutamine, and lysine. In other embodiments, a linker is made up of a majority of amino acids that are sterically unhindered, such as glycine and alanine. Non-peptide linkers are also possible. For example, alkyl linkers such as $-NH(CH_2)_nC(O)-$, wherein n = 2-20 can be used. These alkyl linkers may further be substituted by any non-sterically hindering group such as lower alkyl (e.g., C_1-C_6) lower acyl, halogen (e.g., Cl, Br), CN, NH_2, phenyl, etc. U.S. Pat. No. 5,446,090 describes a bifunctional PEG linker and its use in forming conjugates having a peptide at each of the PEG linker termini.

[0119] Screening Assays

The invention provides methods (also referred to herein as "screening assays") for identifying polypeptides, small molecules, or bifunctional derivatives which bind to HDM2 and/or HDMX.

[0120] The binding affinity of polypeptides that bind HDM2 and/or HDMX can be measured using the methods described herein, for example, by using a titration binding assay. HDM2 and/or HDMX can be exposed to varying concentrations of a candidate compound (i.e., polypeptide) (e.g., 1 nM, 10 nM, 100 nM, 1 μM, 10 μM, 100 μM, 1 mM, and 10 mM) and binding can be measured using surface plasmon resonance to determine the Kd for binding. Additionally, the binding interactions of fluorescently-labeled SAH-p53 peptides to HDM2 and/or HDMX can be used in a competitive binding assay to screen for and identify peptides, small molecules, or bifunctional derivatives thereof that compete with FITC-SAH-p53 peptides, and further calculate Ki values for binding competition. Candidate compounds could also be screened for biological activity *in vivo*. Cell permeability screening assays in which fluorescently labeled candidate compounds are applied to intact cells, which are then assayed for cellular fluorescence by microscopy or high-throughput cellular fluorescence detection can also be used.

[0121] The assays described herein can be performed with individual candidate compounds or can be performed with a plurality of candidate compounds. Where the assays are performed with a plurality of candidate compounds, the assays can be performed using mixtures of candidate compounds or can be run in parallel reactions with each reaction having a single candidate compound. The test compounds or agents can be obtained using any of the numerous approaches in combinatorial library methods known in the art.

[0122] Thus, one can expose HDM2 (e.g., purified MDM2) or HDMX (e.g., purified MDM2) purified to a test compound in the presence of a stapled p53 peptide and determining whether the test compound reduces (inhibits) binding of the stapled p53 peptide to MDM2 or MDMX. A test compound that inhibits binding is a candidate inhibitor of the interaction between p53 and MDM2 or MDMX (or both). Test compounds can be tested for their ability to inhibit binding to MDM2 and MDMX in order to identify compounds that are relatively selective for inhibit p53 binding. In some cases, nutlin-3 (CAS 548472-68-0) can be used as a control since nutlin-3 is a selective inhibitor of p53 binding to HMD2

[0123] <u>Other applications</u>

A number of embodiments of the invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention. Accordingly, other embodiments are within the scope of the following claims.

[0124] References

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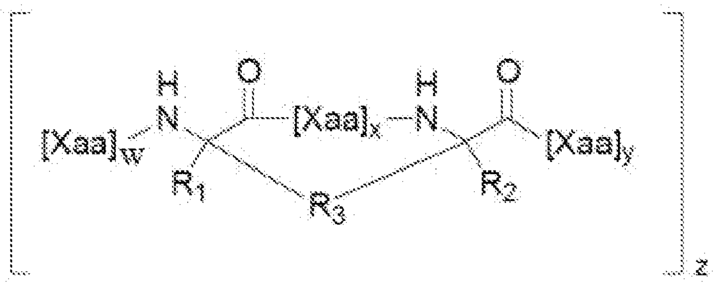
CLAIMS

1. A modified polypeptide of Formula (I) that binds to HDM2 or HDMX,



Formula (I)

or a pharmaceutically acceptable salt thereof,

wherein:

each R_1 and R_2 are independently H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkylalkyl, heteroarylalkyl, or heterocyclylalkyl;

each R_3 is independently alkylene, alkenylene, alkynylene, or $[R_4\text{-K-}R_4']_n$; each of which is substituted with 0-6 R_5;

each R_4 and R_4'are independently alkylene, alkenylene or alkynylene;

each R_5 is independently halo, alkyl, OR_6, $N(R_6)_2$, SR_6, SOR_6, SO_2R_6, CO_2R_6, R_6, a fluorescent moiety, or a radioisotope;

each K is independently O, S, SO, SO_2, CO, CO_2, $CONR_6$, or  ;

each R_6 is independently H, alkyl, or a therapeutic agent;

n is an integer from 1-4;

x is 6;

y and w are independently integers from 0-100;

z is an integer from 1-10; and

each Xaa is independently an amino acid;

wherein the modified polypeptide or the pharmaceutically acceptable salt thereof comprises at least 8 contiguous amino acids of (a) Leu_1 Ser_2 Gln_3 Glu_4 Thr_5 Phe_6 Ser_7 Asp_8 Leu_9 Trp_{10} Lys_{11} Leu_{12} Leu_{13} Pro_{14} Glu_{15} Asn_{16} or (b) Gln_1 Ser_2 Gln_3 Gln_4 Thr_5 Phe_6 Ser_7 Asn_8 Leu_9 Trp_{10} Arg_{11} Leu_{12} Leu_{13} Pro_{14} $G1n_{15}$ Asn_{16}, wherein the side chains of Ser_7 and Pro_{14} are replaced by the linking group R_3 and none or up to 6 amino acids other than Phe_6, Trp_{10} and Leu_{13} are independently replaced by any other amino acid.

2. The modified polypeptide or the pharmaceutically acceptable salt thereof of claim 1, wherein the modified polypeptide or the pharmaceutically acceptable salt thereof binds to HDM2.

3. The modified polypeptide or the pharmaceutically acceptable salt thereof of claim 1, wherein R_3 is an alkenylene containing a single double bond, and both R_1 and R_2 are H.

4. The modified polypeptide or the pharmaceutically acceptable salt thereof of claim 1, wherein y and w are independently integers from 1 to 15.

5. The modified polypeptide or the pharmaceutically acceptable salt thereof of claim 1, comprising the at least 8 contiguous amino acids of Leu_1 Ser_2 Gln_3 Glu_4 Thr_5 Phe_6 Ser_7 Asp_8 Leu_9 Trp_{10} Lys_{11} Leu_{12} Leu_{13} Pro_{14} Glu_{15} Asn_{16}, wherein none or up to 6 amino acids other than Phe_6, Trp_{10} and Leu_{13} are independently replaced by any other amino acid.

6. The modified polypeptide or the pharmaceutically acceptable salt thereof of claim 1, comprising the at least 8 contiguous amino acids of Gln_1 Ser_2 Gln_3 Gln_4 Thr_5 Phe_6 Ser_7 Asn_8 Leu_9 Trp_{10} Arg_{11} Leu_{12} Leu_{13} Pro_{14} Gln_{15} Asn_{16}, wherein none or up to 6 amino acids other than Phe_6, Trp_{10} and Leu_{13} are independently replaced by any other amino acid.

7. The modified polypeptide or the pharmaceutically acceptable salt thereof of claim 1, wherein the modified polypeptide or the pharmaceutically acceptable salt thereof does not have a net negative charge at pH 7.

8. The modified polypeptide or the pharmaceutically acceptable salt thereof of claim 7, wherein the modified polypeptide or the pharmaceutically acceptable salt thereof comprises at least one amino acid that has a positive charge at pH 7 either: (a) amino terminal to Leu_1 or the amino acid substituted for Leu_1 or (b) carboxy terminal to Asn_{16} or the amino acid substituted for Asn_{16}.

9. The modified polypeptide or the pharmaceutically acceptable salt thereof of claim 1, wherein R_1 and R_2 are each independently H or C_1-C_6 alkyl.

10. The modified polypeptide or the pharmaceutically acceptable salt thereof of claim 9, wherein R_1 and R_2 are each independently C_1-C_3 alkyl.

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11. The modified polypeptide or the pharmaceutically acceptable salt thereof of claim 10, wherein R_1 and R_2 are methyl.

12. The modified polypeptide or the pharmaceutically acceptable salt thereof of claim 1, wherein R_3 is C_{11} alkylene.

13. The modified polypeptide or the pharmaceutically acceptable salt thereof of claim 1, wherein R_3 is alkenylene.

14. The modified polypeptide or the pharmaceutically acceptable salt thereof of claim 13, wherein R_3 is C_8 alkenylene.

15. The modified polypeptide or the pharmaceutically acceptable salt thereof of claim 13, wherein R_3 is C_{11} alkenylene.

16. The modified polypeptide or the pharmaceutically acceptable salt thereof of claim 1, wherein R_3 is a straight chain alkylene, alkenylene, or alkynylene.

17. The modified polypeptide or the pharmaceutically acceptable salt thereof of claim 1, further comprising an amino-terminal fatty acid.

18. The modified polypeptide or the pharmaceutically acceptable salt thereof of claim 1, further comprising a targeting moiety.

19. The modified polypeptide or the pharmaceutically acceptable salt thereof of claim 1, wherein the modified polypeptide or the pharmaceutically acceptable salt thereof binds to HDMX.

20. The modified polypeptide or the pharmaceutically acceptable salt thereof of claim 1, wherein the modified polypeptide or the pharmaceutically acceptable salt thereof binds to HDMX and HDM2.

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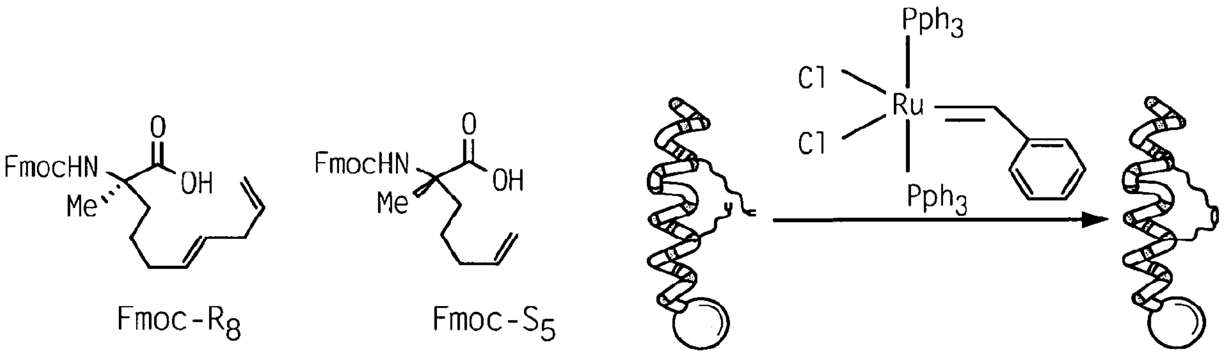

FIG. 1A

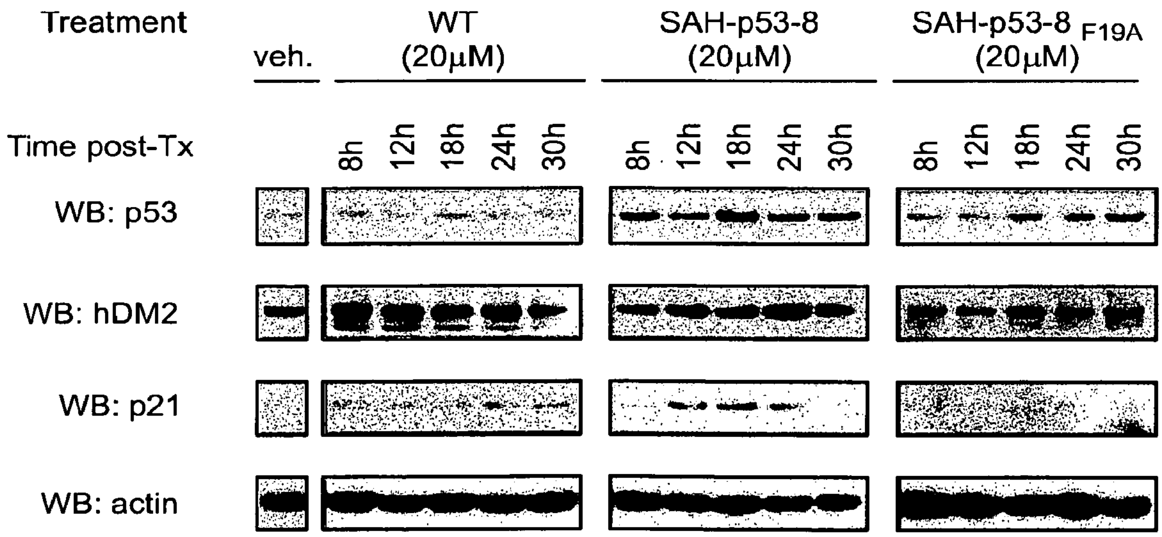

FIG. 2

compound	sequence * = R_8 * = S_5	charge at pH 7.4	α helicity	K_d (nM)	cell permeable	cell death
WT	Ac-LSQETFSDLWKLLPEN-NH_2	-2	11%	410±19	no	-
SAH-p53-1	Ac-LSQETFSD*WKLLPE*-NH_2	-2	25%	100±8	no	-
SAH-p53-2	Ac-LSQE*FSDLWK*LPEN-NH_2	-2	10%	400±50	no	-
SAH-p53-3	Ac-LSQ*TFSDLW*LLPEN-NH_2	-2	12%	1200±89	no	-
SAH-p53-4	Ac-LSQETF*DLWKLL*EN-NH_2	-2	59%	0.92±0.11	no	-
SAH-p53-5	Ac-LSQETF*NLWKLL*QN-NH_2	0	20%	0.80±0.05	yes	-
SAH-p53-6	Ac-LSQQTF*NLWRLL*QN-NH_2	+1	14%	56±11	yes	-
SAH-p53-7	Ac-QSQQTF*NLWKLL*QN-NH_2	+1	36%	50±10	yes	-
SAH-p53-8	Ac-QSQQTF*NLWRLL*QN-NH_2	+1	85%	55±11	yes	+
SAH-p53-8_{F19A}	Ac-QSQQTA*NLWRLL*QN-NH_2	+1	39%	>4000	yes	-
UAH-p53-8	Ac-QSQQTF*NLWRKK*QN-NH_2	+1	36%	100±10	yes	-

FIG. 1B

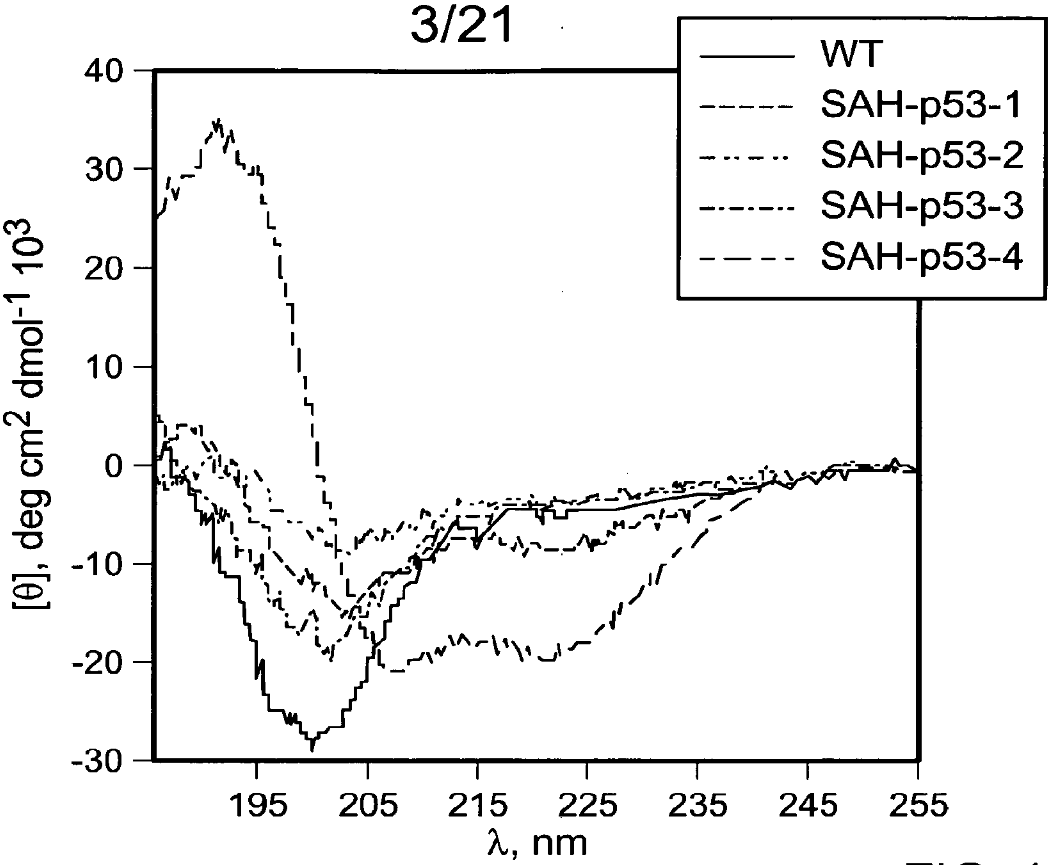
WT
SAH-p53-1
SAH-p53-2
SAH-p53-3
SAH-p53-4
[θ], deg cm2 dmol-1 103
λ, nm
40
30
20
10
0
-10
-20
-30
195
205
215
225
235
245
255

FIG. 1C

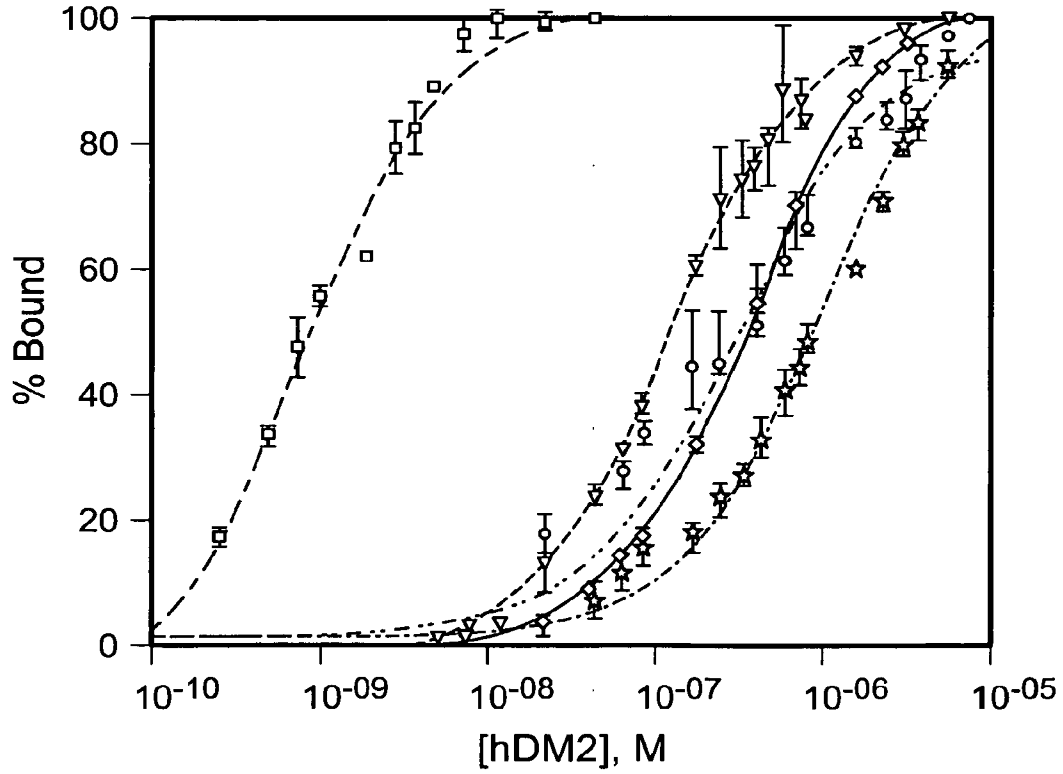
% Bound
[hDM2], M
100
80
60
40
20
0
10-10
10-09
10-08
10-07
10-06
10-05

FIG. 1D

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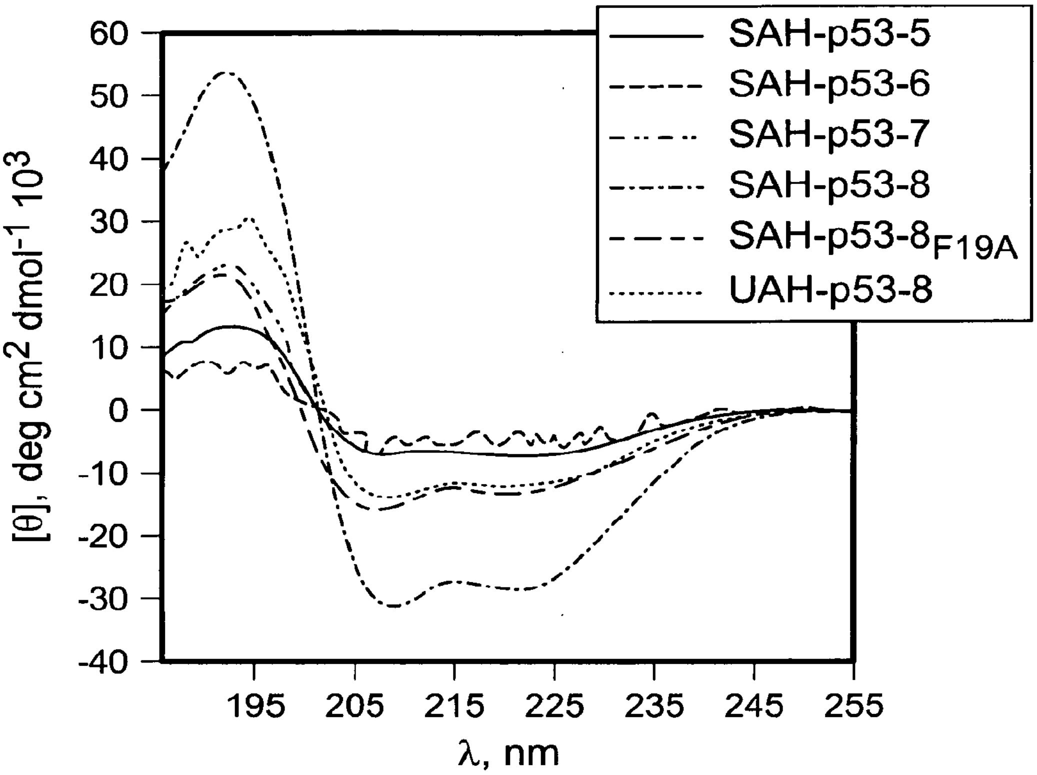

FIG. 1E

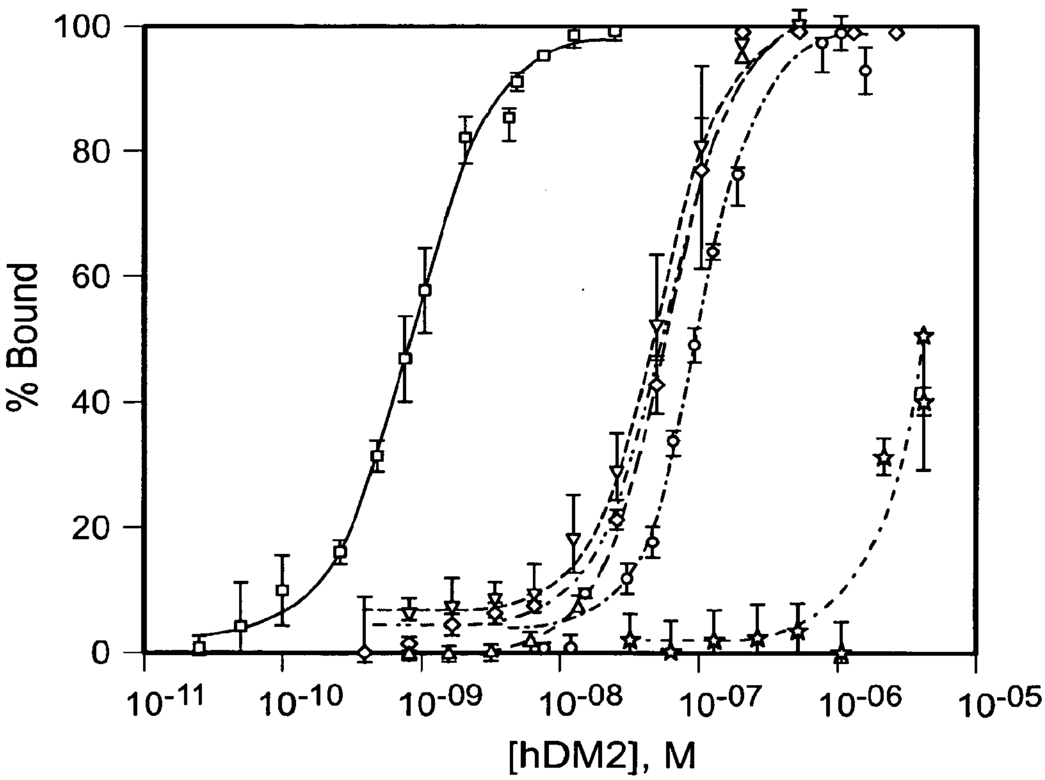

FIG. 1F

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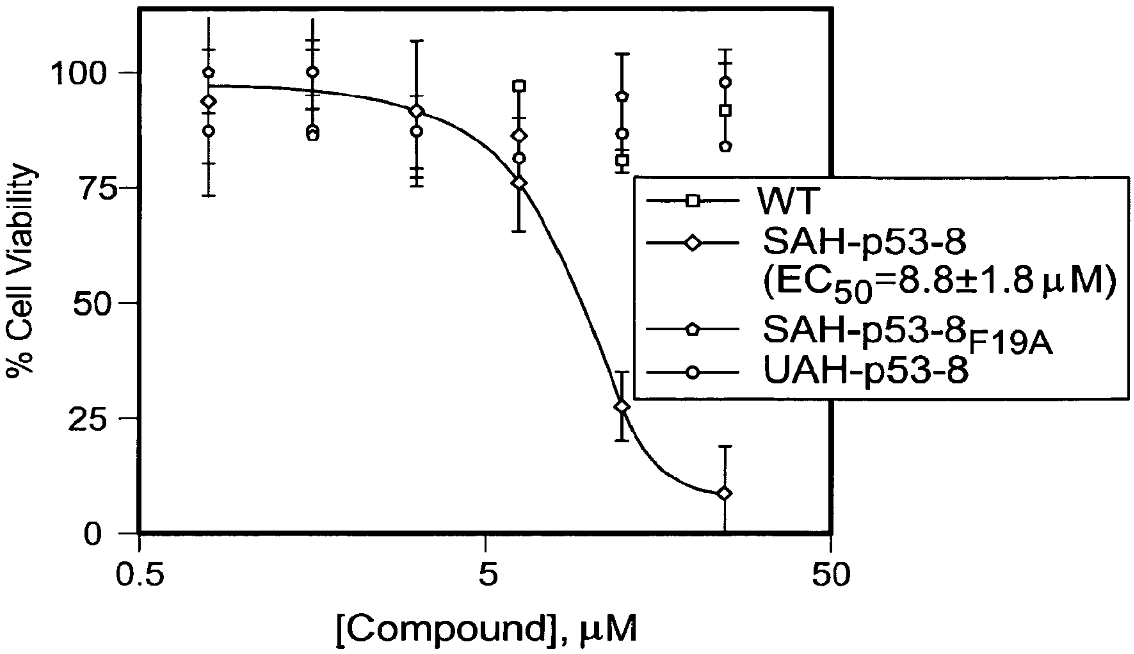

FIG. 3A

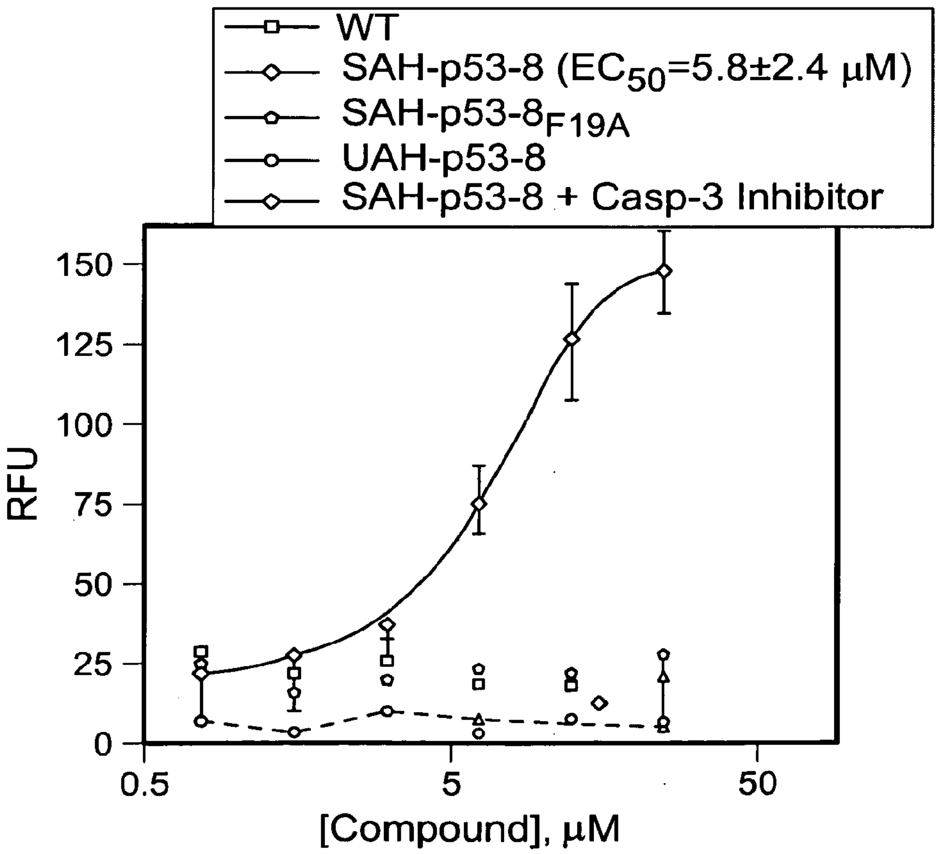

FIG. 3B

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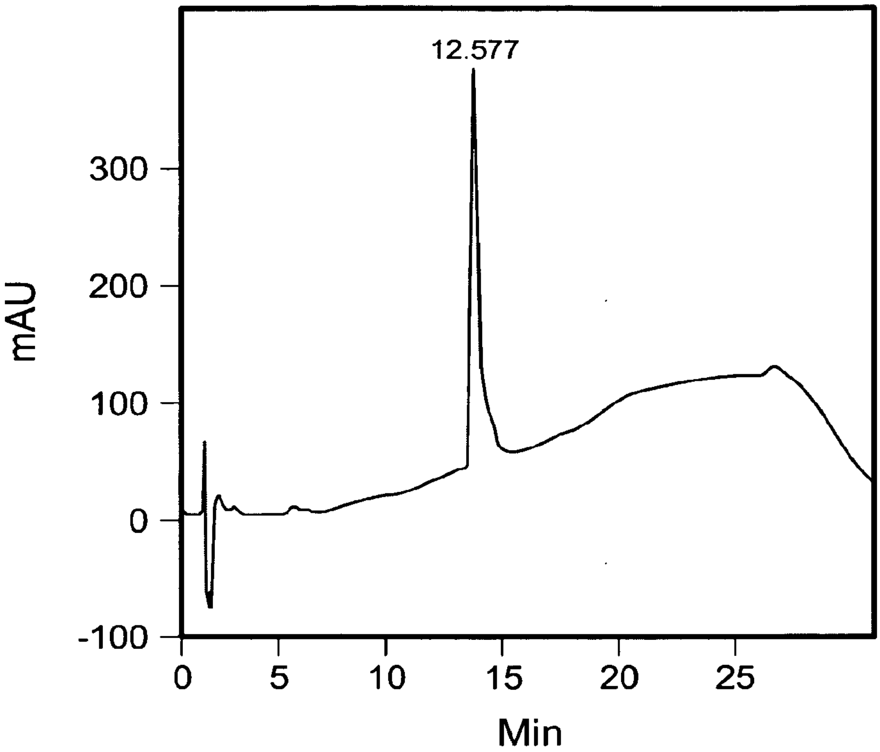

FIG. 4A

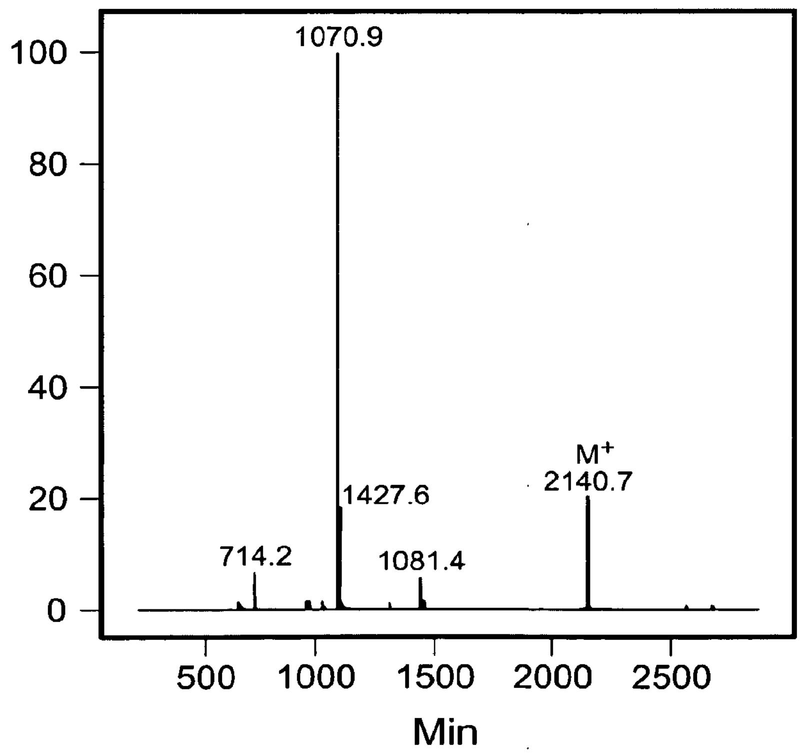

FIG. 4B

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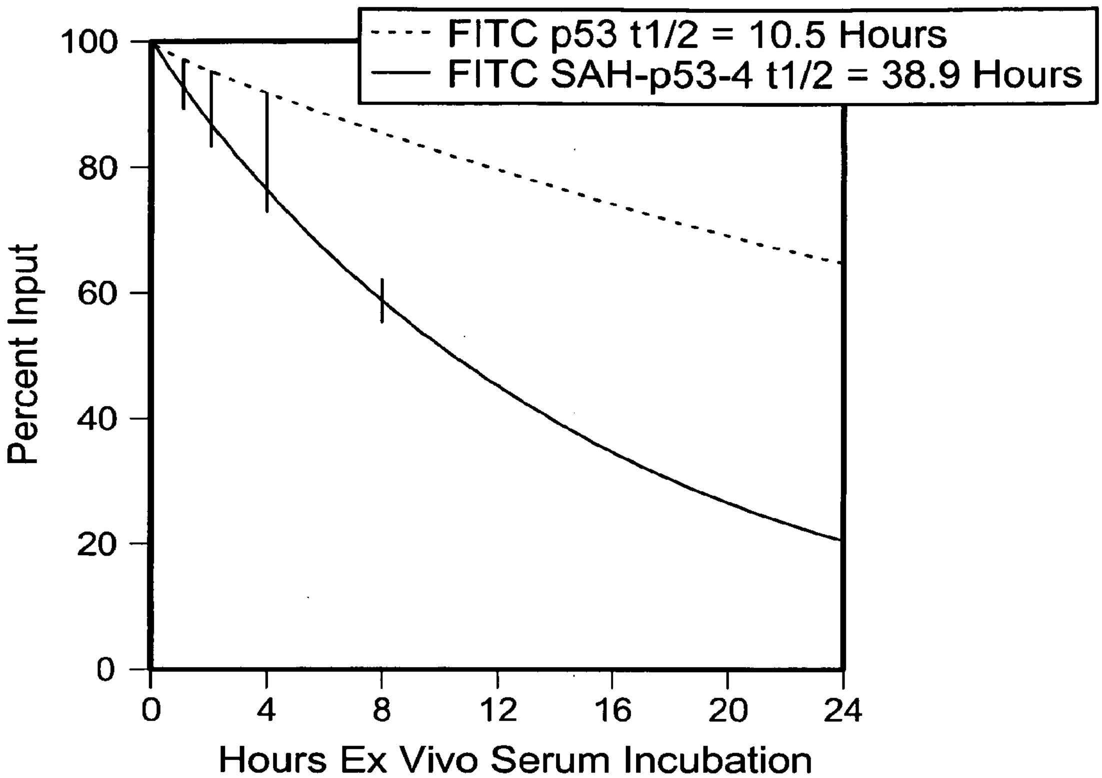


FIG. 5

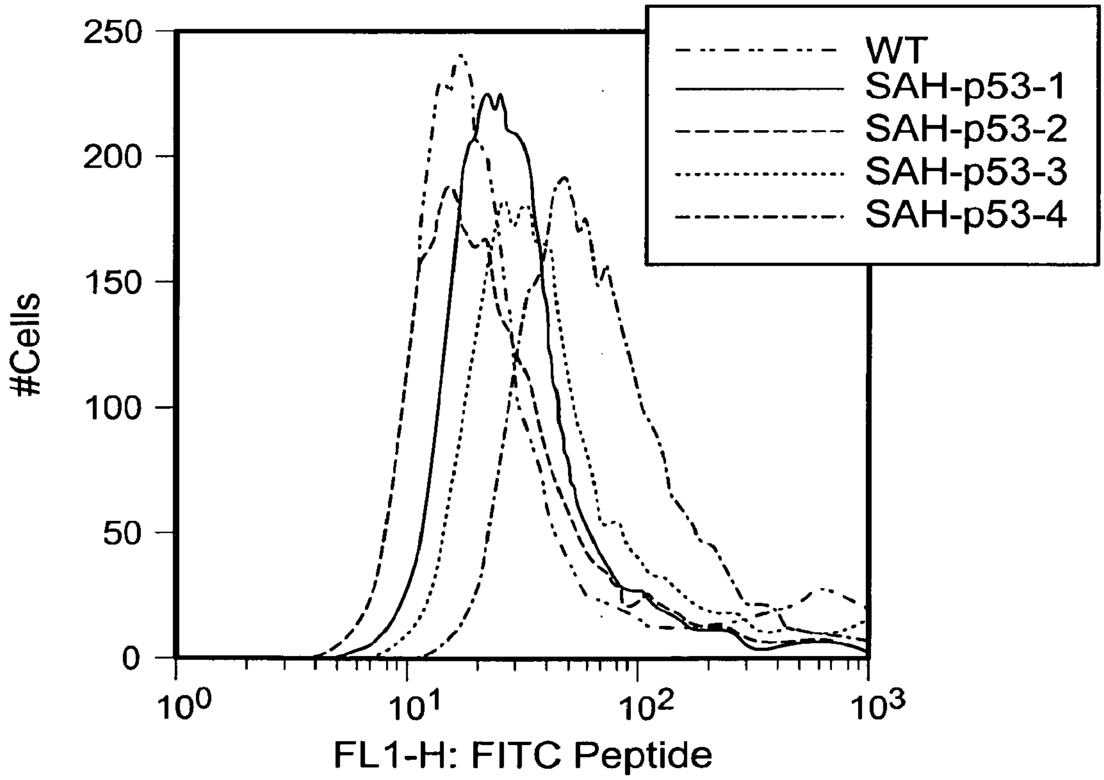


FIG. 6

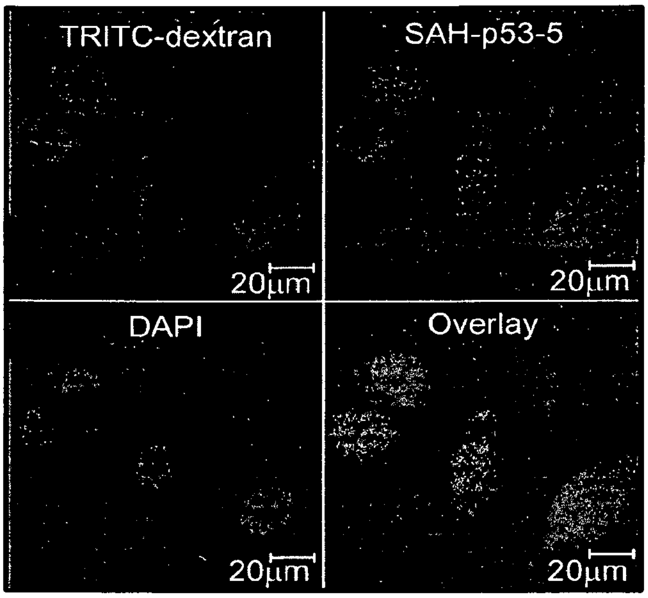

TRITC-dextran
SAH-p53-5
DAPI
Overlay
20μm
20μm
20μm
20μm

FIG. 7A

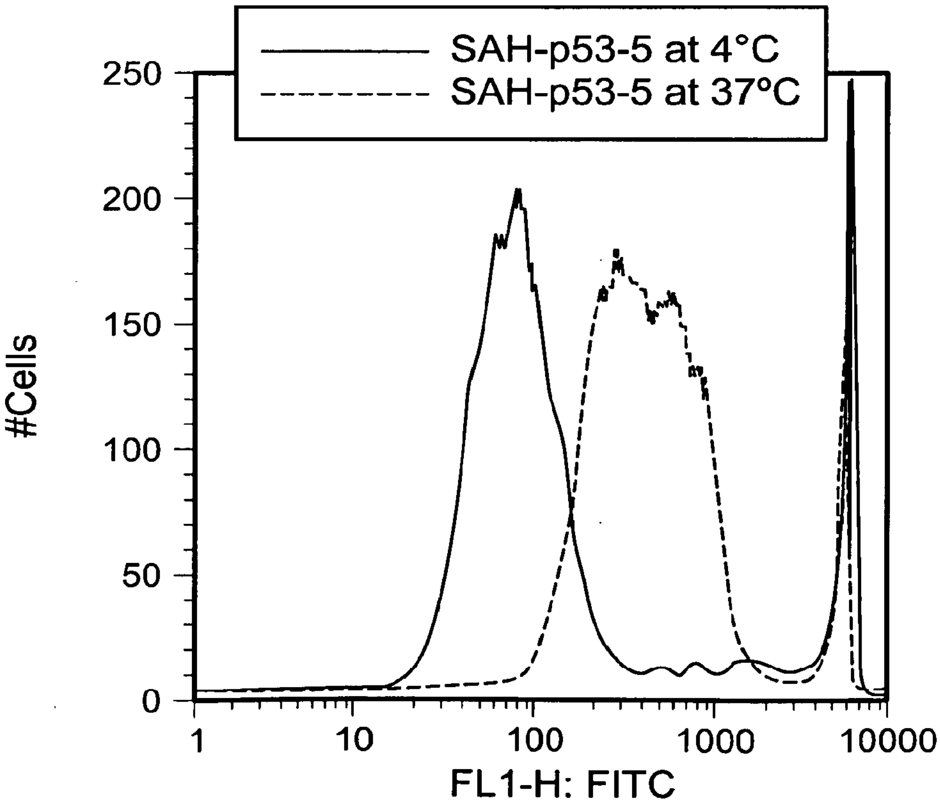

SAH-p53-5 at 4°C
SAH-p53-5 at 37°C
250
200
150
100
50
0
#Cells
1
10
100
1000
10000
FL1-H: FITC

FIG. 7B

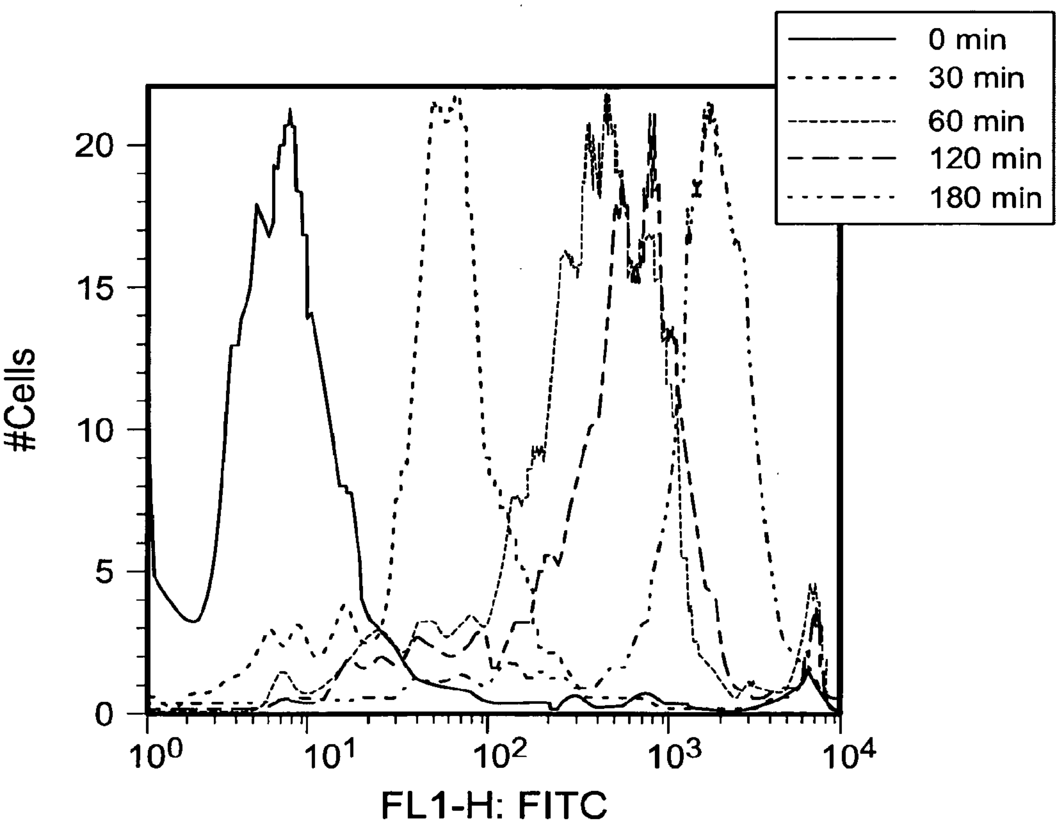
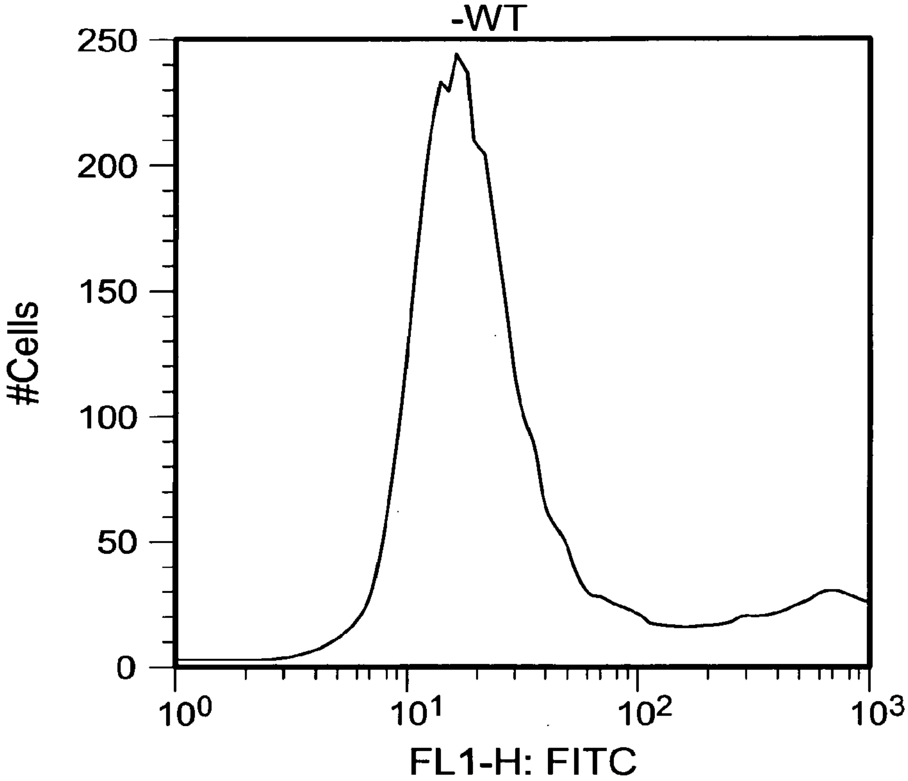

0 min
30 min
60 min
120 min
180 min
#Cells
20
15
10
5
0
10^0
10^1
10^2
10^3
10^4
FL1-H: FITC

FIG. 7C



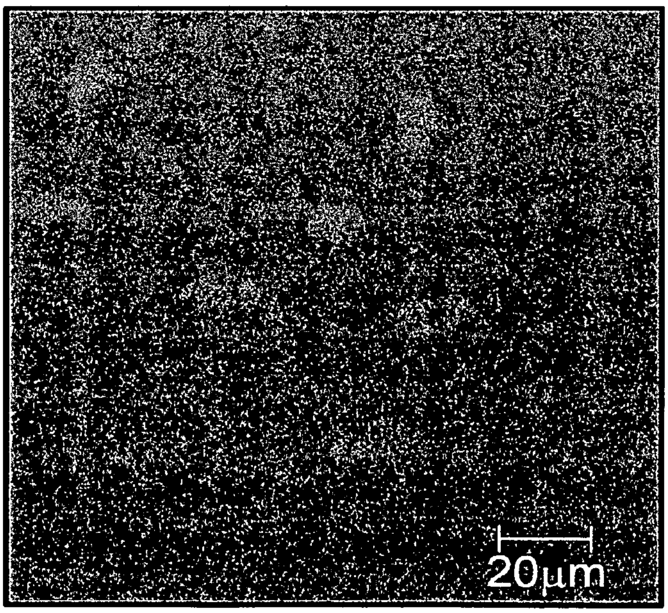


FIG. 7D-1

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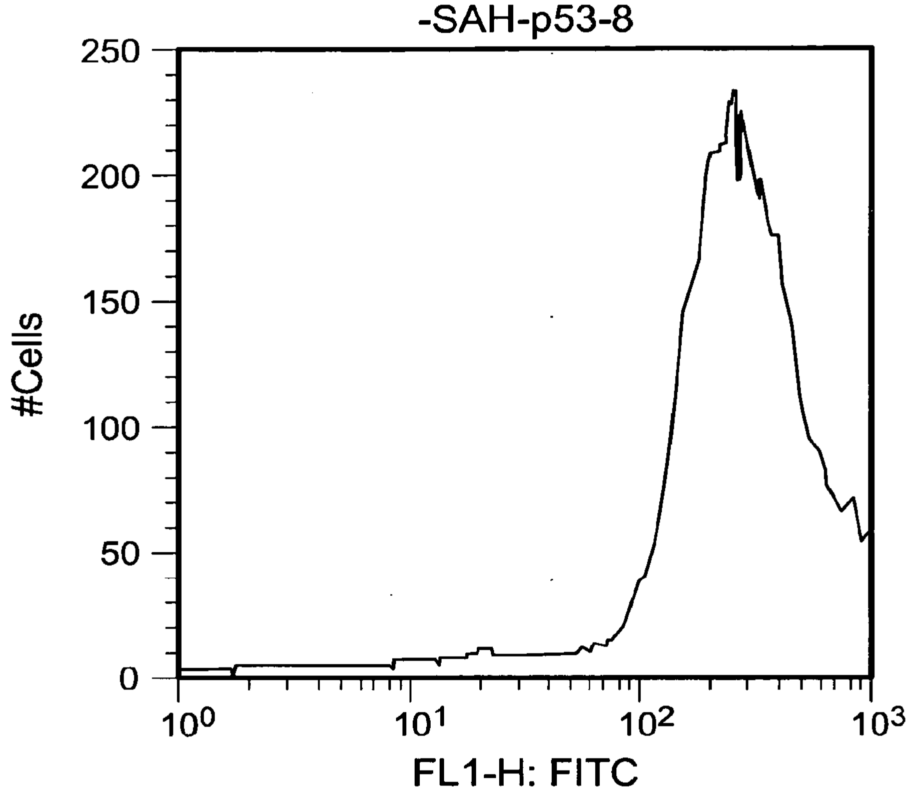

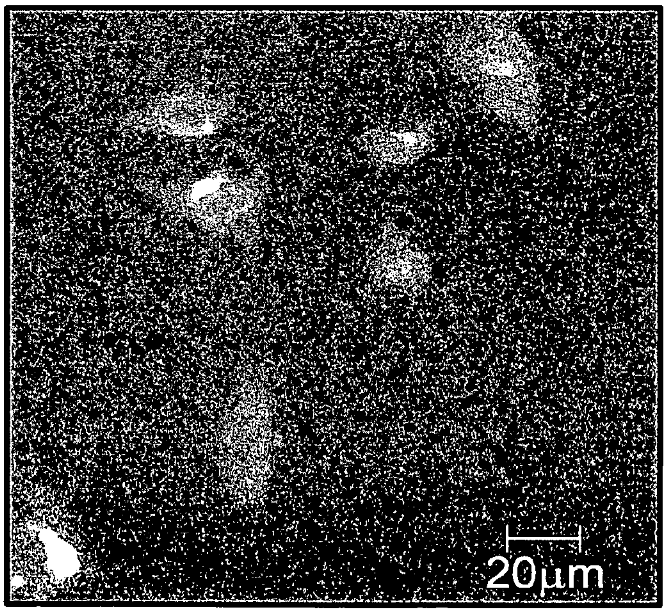

FIG. 7D-2

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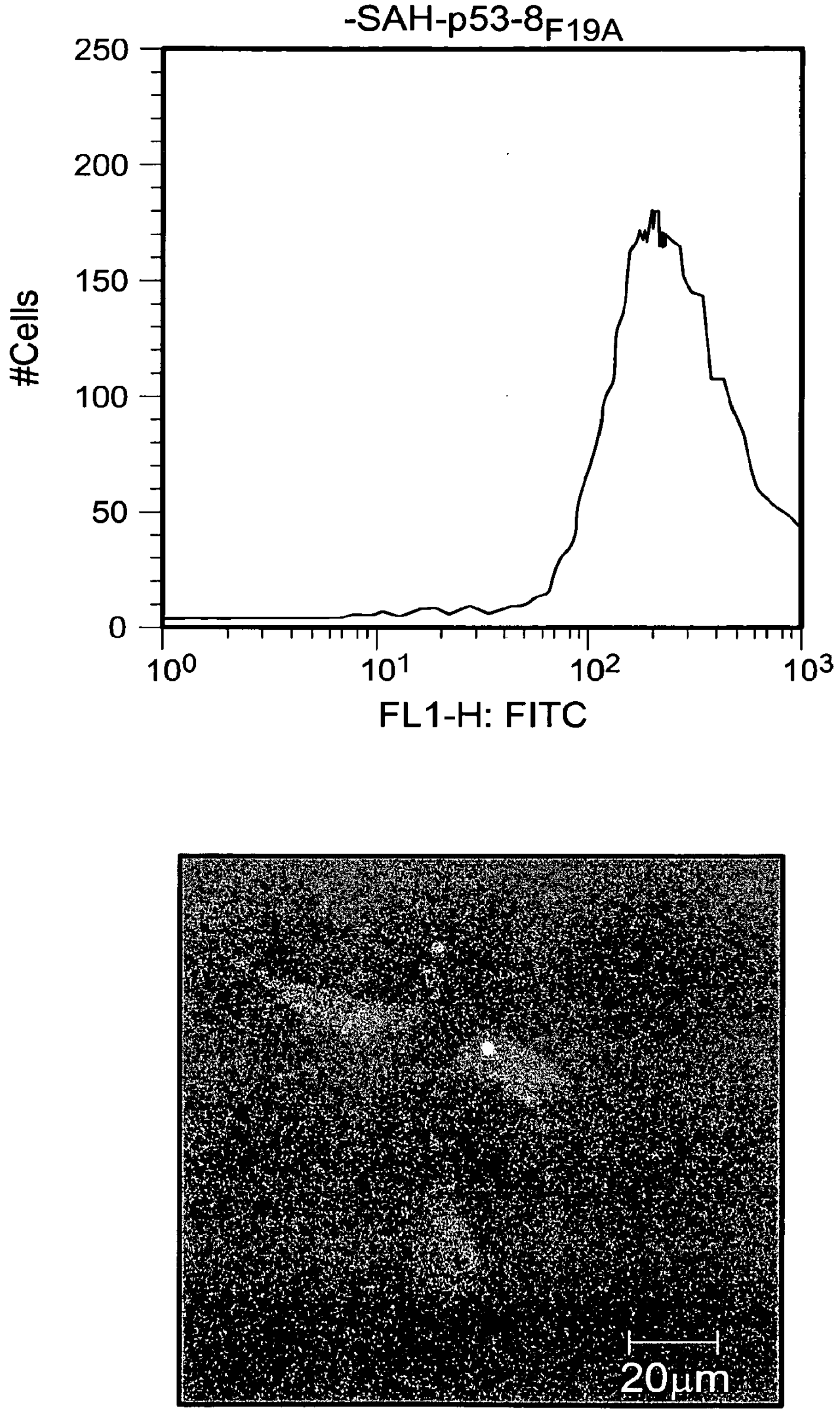


FIG. 7D-3

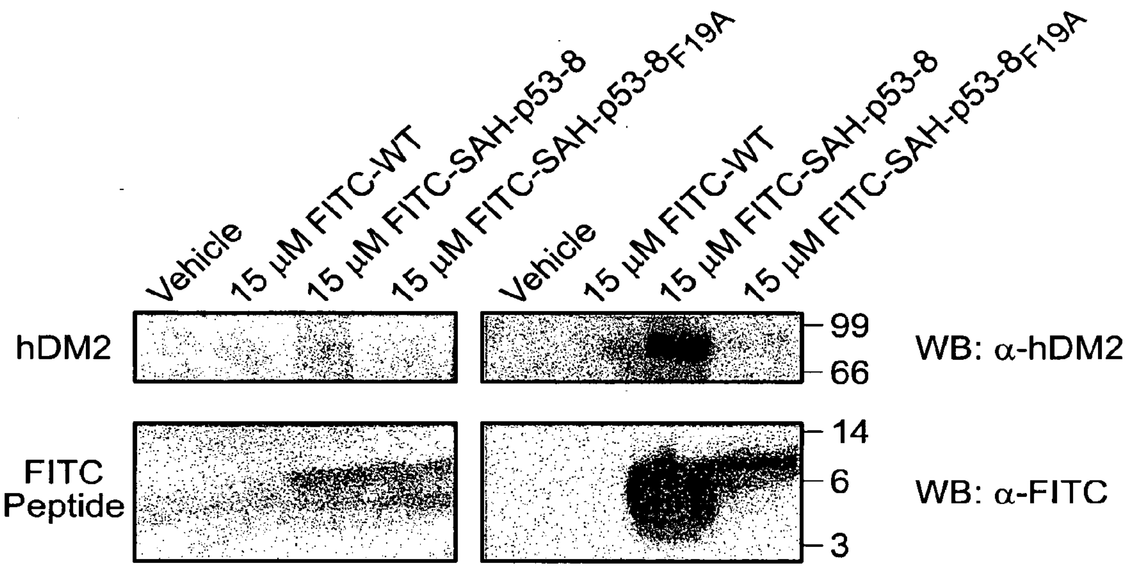
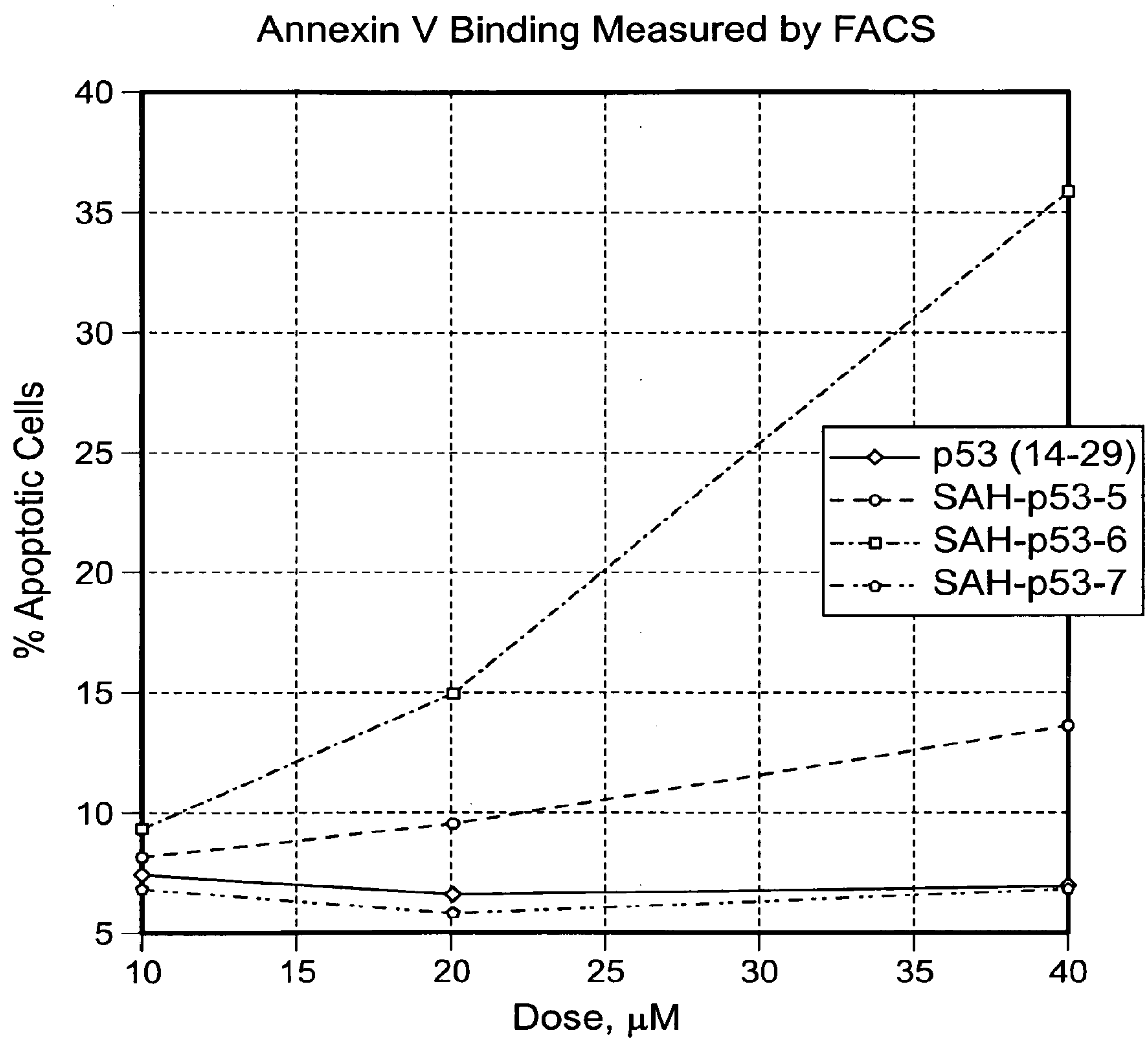
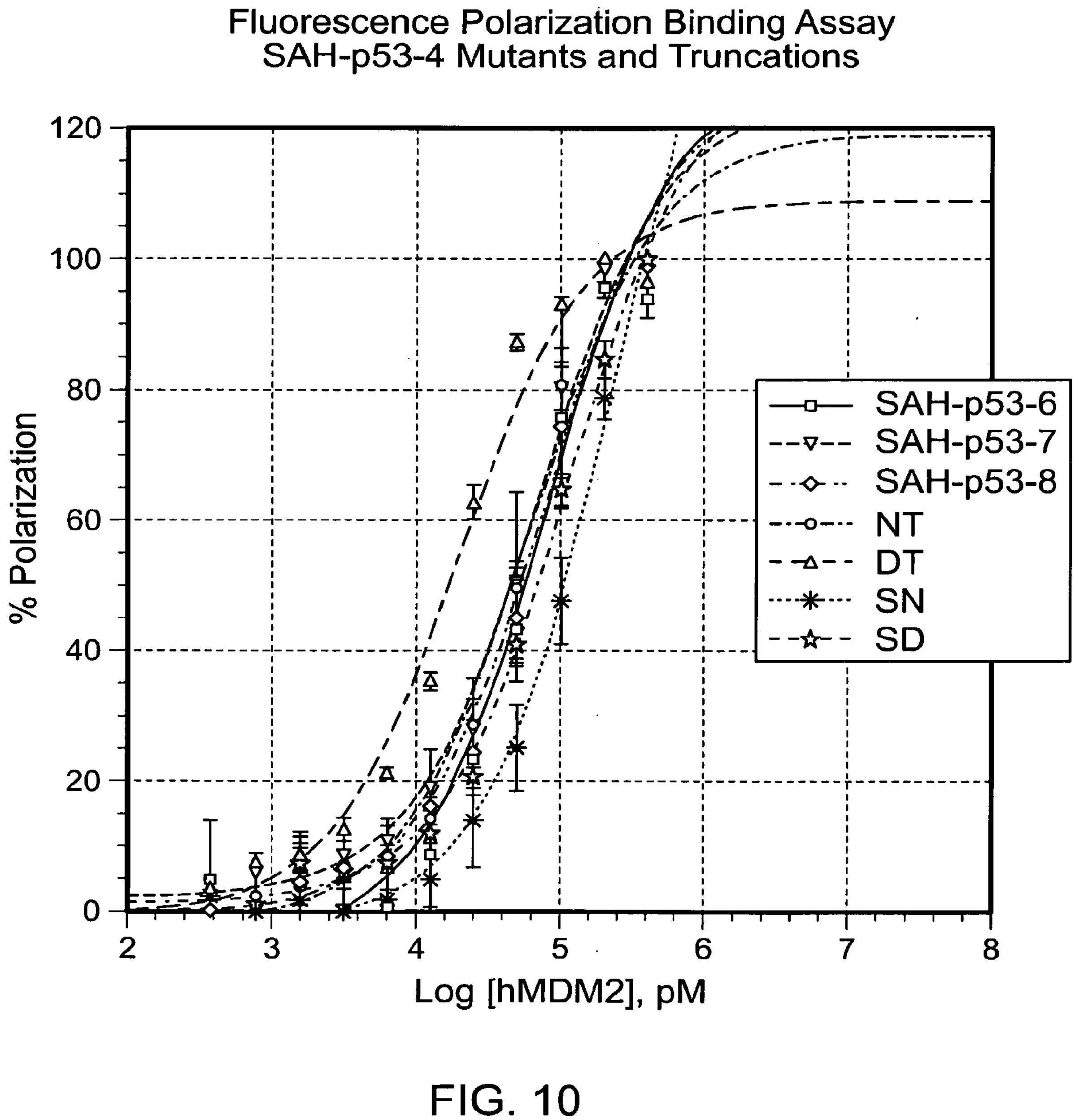

Vehicle
15 μM FITC-WT
15 μM FITC-SAH-p53-8
15 μM FITC-SAH-p53-8F19A
Vehicle
15 μM FITC-WT
15 μM FITC-SAH-p53-8
15 μM FITC-SAH-p53-8F19A
hDM2
FITC Peptide
99
66
14
6
3
WB: α-hDM2
WB: α-FITC

FIG. 8



Annexin V Binding Measured by FACS
% Apoptotic Cells
Dose, μM
40
35
30
25
20
15
10
5
10
15
20
25
30
35
40
p53 (14-29)
SAH-p53-5
SAH-p53-6
SAH-p53-7

FIG. 9



Fluorescence Polarization Binding Assay
SAH-p53-4 Mutants and Truncations
% Polarization
Log [hMDM2], pM
0
20
40
60
80
100
120
2
3
4
5
6
7
8
SAH-p53-6
SAH-p53-7
SAH-p53-8
NT
DT
SN
SD

FIG. 10

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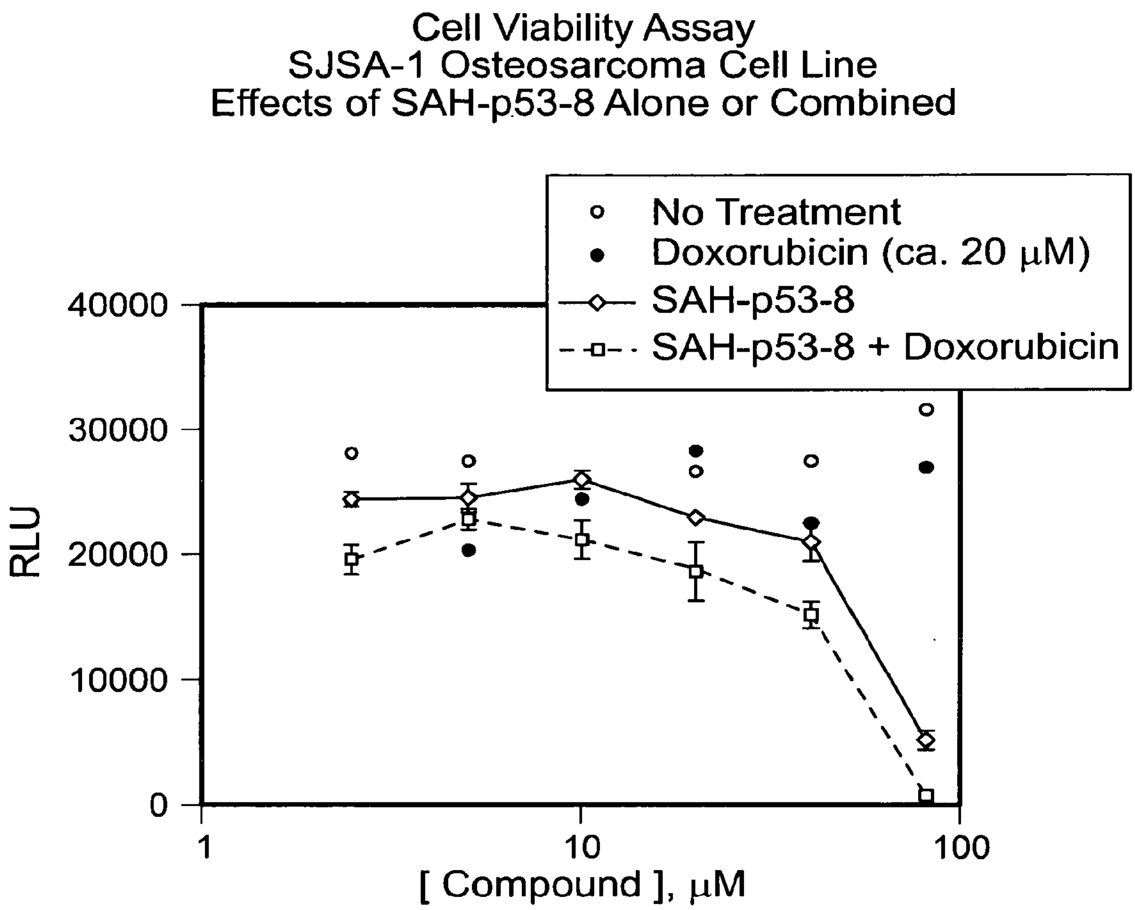

FIG. 11

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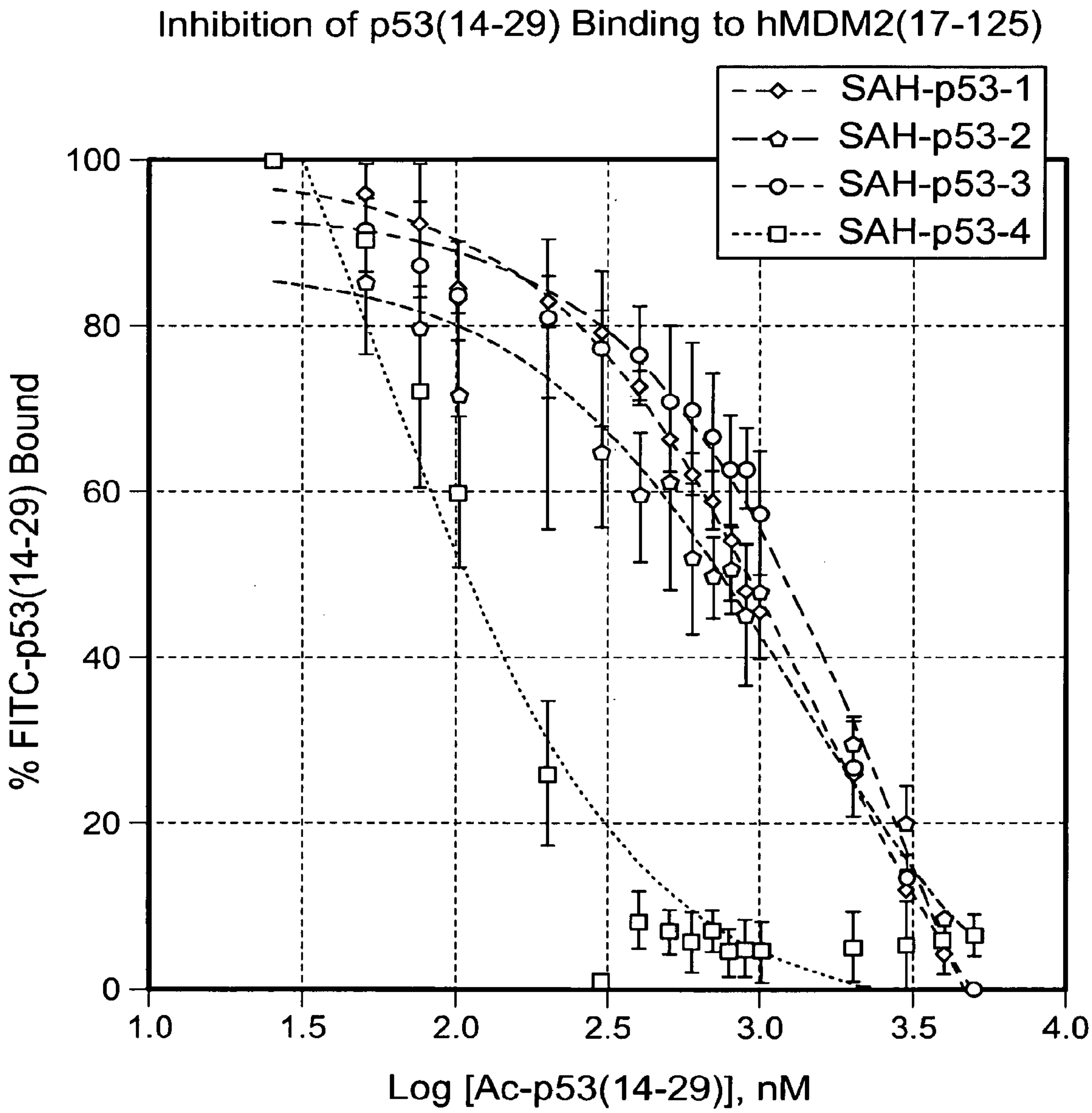

FIG. 12

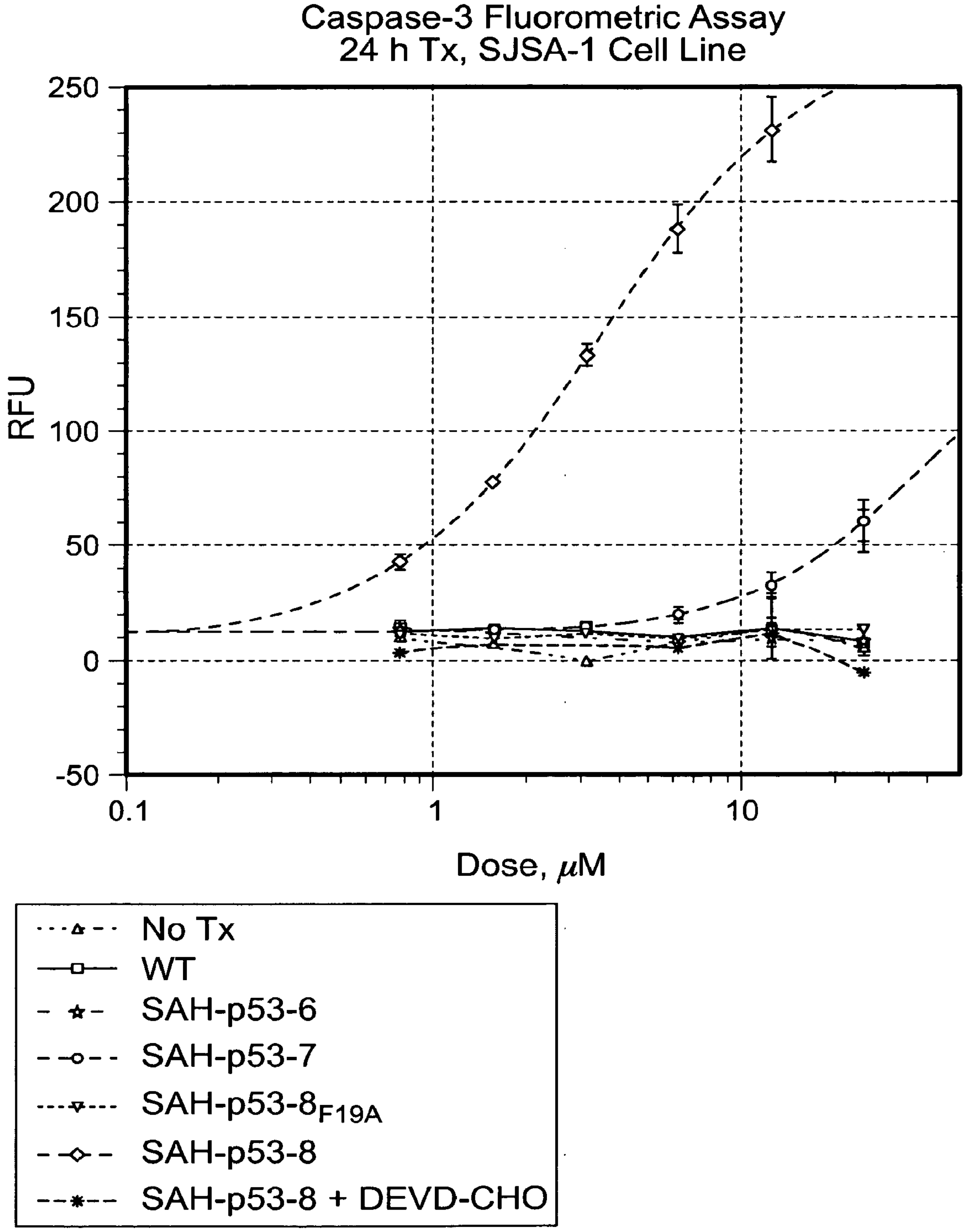
Caspase-3 Fluorometric Assay
24 h Tx, SJSA-1 Cell Line
250
200
150
100
50
0
-50
RFU
0.1
1
10
Dose, μM
No Tx
WT
SAH-p53-6
SAH-p53-7
SAH-p53-8F19A
SAH-p53-8
SAH-p53-8 + DEVD-CHO

FIG. 13

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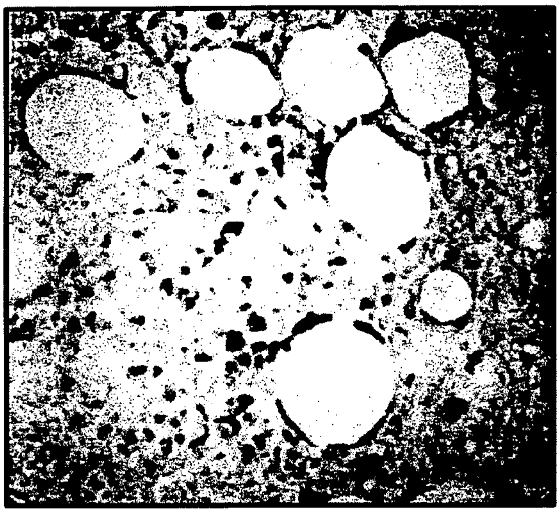

FIG. 14A

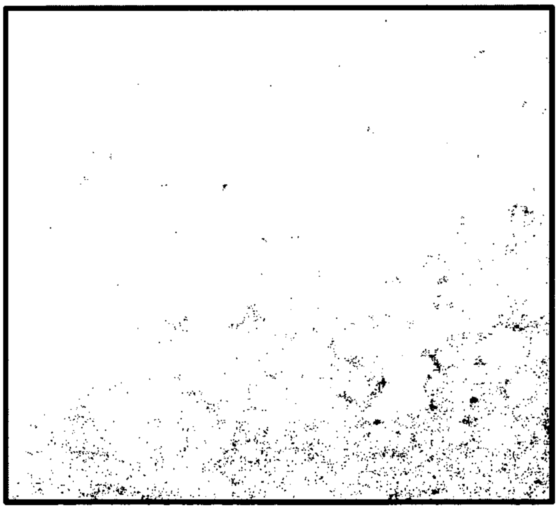

FIG. 14B

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  1 meepqsdpsv epplsqetfs dlwkllpenn vlsplpsqam ddlmlspddi eqwftedpgp
 61 deaprmpeaa prvapapaap tpaapapaps wplsssvpsq ktyqgsygfr lgflhsgtak
121 svtctyspal nkmfcqlakt cpvqlwvdst pppgtrvram aiykqsqhmt evvrrcphhe
181 rcsdsdglap pqhlirvegn lrveylddrn tfrhsvvvpy eppevgsdct tihynymcns
241 scmggmnrrp iltiitleds sgnllgrnsf evrvcacpgr drrteeenlr kkgephhelp
301 pgstkralpn ntssspqpkk kpldgeyftl qirgrerfem frelnealel kdaqagkepg
361 gsrahsshlk skkgqstsrh kklmfktegp dsd
```

FIG. 15

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1. Cap-Linker-L-*-Q-E-T-F-S-D-*-W-K-L-L-P-E-N-NH_2
2. Cap-Linker-L-S-Q-*-T-F-S-D-L-W-*-L-L-P-E-N-NH_2
3. Cap-Linker-L-S-Q-E-*-F-S-D-L-W-K-*-L-P-E-N-NH_2
4. Cap-Linker-L-S-Q-E-T-*-S-D-L-W-K-L-*-P-E-N-NH_2
5. Cap-Linker-L-S-Q-E-T-F-*-D-L-W-K-L-L-*-E-N-NH_2 (SAH-p53-4)
6. Cap-Linker-L-*-Q-E-T-F-S-*-L-W-K-L-L-P-*-N-NH_2
7. Cap-Linker-L-S-Q-E-T-F-S-D-*-W-K-L-L-P-E-*-NH_2

• "Cap" denotes Ac (acetyl) or FITC (fluorescein thiocarbomoyl); "Linker" denotes ß-alanine or no linker; "*" indicates the amino acid pairs R_5-S_8 Or R_8-S_5 in either uncross-linked (unstapled, unmetathesized) or cross-linked (stapled, metathesized) form

8. Cap-Linker-L-S-Q-Q-T-F-*-D-L-W-K-L-L-*-E-N-NH_2
9. Cap-Linker-L-S-Q-E-T-F-*-D-L-W-K-L-L-*-Q-N-NH_2
10. Cap-Linker-L-S-Q-Q-T-F-*-D-L-W-K-L-L-*-Q-N-NH_2
11. Cap-Linker-L-S-Q-E-T-F-*-N-L-W-K-L-L-*-Q-N-NH_2
12. Cap-Linker-L-S-Q-Q-T-F-*-N-L-W-K-L-L-*-Q-N-NH_2
13. Cap-Linker-L-S-Q-Q-T-F-*-N-L-W-R-L-L-*-Q-N-NH_2
14. Cap-Linker-Q-S-Q-Q-T-F-*-N-L-W-K-L-L-*-Q-N-NH_2
15. Cap-Linker-Q-S-Q-Q-T-F-*-N-L-W-R-L-L-*-Q-N-NH_2 (SAH-p53-8)
16. Cap-Linker-Q-S-Q-Q-T-A-*-N-L-W-R-L-L-*-Q-N-NH_2 (SAH-p53-8_{F19A})

• "Cap" denotes Ac (acetyl), FITC (fluorescein thiocarbamoyl), DOTA (cryptand capable of chelating radaoctive In), lauroyl, heptanoyl, and myristoyl; "Linker" denotes ß-alanine or no linker; "*" indicates the amino acid pairs R_5-S_8 or R_8-S_5 in either uncross-linked (unstapled, unmetathesized) or cross-linked (stapled, metathesized) form

17. Cap-Linker-Q-Q-T-F-*-D-L-W-R-L-L-*-E-N-NH_2
18. Cap-Linker-Q-Q-T-F-*-D-L-W-R-L-L-*-NH_2
19. Cap-Linker-L-S-Q-Q-T-F-*-D-L-W-*-L-L-NH_2
20. Cap-Linker-Q-Q-T-F-*-D-L-W-*-L-L-NH
21. Cap-Linker-Q-Q-T-A-*-D-L-W-R-L-L-*-E-N-NH_2

• "Cap" denotes Ac (acetyl), FITC (fluorescein thiocarbamoyl), lauroyl, heptanoyl, and myristoyl; "Linker" denotes ß-alanine or no linker; "*" indicates the amino acid pairs R_8-S_5 (peptides 17, 18, and 21) or S_5-S_5 (peptides 19 and 20) in either uncross-linked (unstapled, unmetathesized) or cross-linked (stapled, metathesized) forms

22. Cap-K(Myr)-Linker-Q-S-Q-Q-T-F-*-N-L-W-R-L-L-*-Q-N-NH_2
23. Cap-K(Biotin)-Linker-Q-S-Q-Q-T-F-*-N-L-W-R-L-L-*-Q-N-NH_2
24. Cap-K(PEG3)-Linker-Q-S-Q-Q-T-F-*-N-L-W-R-L-L-*-Q-N-NH_2
25. Cap-Linker-Q-S-Q-Q-T-F-*-N-L-W-R-L-L-*-Q-N-NH_2 diol
26. Cap-Linker-Q-S-Q-Q-T-A-*-N-L-W-R-L-L-*-Q-N-NH_2 diol

• "Cap" denotes Ac (acetyl) or FITC (fluorescein thiocarbamoyl); "Linker" denotes ß-alanine or no linker; "*" indicates the amino acid pair R_8-S_5 in either uncross-linked (unstapled, unmetathesized) or cross-linked (stapled, metathesized) form; "diol" indicates a dihydroxylated cross-link olefin

FIG. 16-1

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27. Cap-Linker-Q-S-Q-Q-T-F-*-D-L-W-R-L-L-*-Q-N-NH_2 (SAH-p53-10)
28. Cap-Linker-Q-T-F-*-N-L-W-R-L-L-*-NH_2 (SAH-p53-11)
29. Cap-Linker-Q-S-Q-Q-T-F-*-N-L-W-*-L-L-P-Q-N-NH_2 (SAH-p53-$8S_A$)
30. Cap-Linker-Q-S-*-Q-T-F-*-N-L-W-R-L-L-P-Q-N-NH_2 (SAH-p53-$8S_B$)
31. Cap-Linker-*-T-F-S-*-L-W-K-L-L-NH_2 (SAH-p53-12)
32. Cap-Linker-E-T-F-*-D-L-W-*-L-L-NH_2 (SAH-p53-13)
33. Cap-Linker-Q-T-F-*-N-L-W-*-L-L-NH_2 (SAH-p53-14)
34. Cap-Linker-*-S-Q-E-*-F-S-N-L-W-K-L-L-NH_2 (SAH-p53-15)

FIG. 16-2