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(54) **Titre : PEPTIDES ALPHA-HELICOIDAUX STABILISES ET LEURS APPLICATIONS**
(54) **Title: STABILIZED ALPHA HELICAL PEPTIDES AND USES THEREOF**

(57) **Abrégé/Abstract:**

Novel polypeptides and methods of making and using the same are described herein. The polypeptides include cross-linking (~hydrocarbon stapling~) moieties to provide a tether between two amino acid moieties, which constrains the secondary structure of the polypeptide. The polypeptides described herein can be used to treat diseases characterized by excessive or inadequate cellular death.

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(54) Title: STABILIZED ALPHA HELICAL PEPTIDES AND USES THEREOF

(57) Abstract: Novel polypeptides and methods of making and using the same are described herein. The polypeptides include cross-linking ("hydrocarbon stapling") moieties to provide a tether between two amino acid moieties, which constrains the secondary structure of the polypeptide. The polypeptides described herein can be used to treat diseases characterized by excessive or inadequate cellular death.



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Stabilized Alpha Helical Peptides and Uses Thereof

CLAIM OF PRIORITY

This application claims priority under 35 USC §119(e) to U.S. Patent Application Serial No. 60/517,848, filed on November 5, 2003, and U.S. Patent Application Serial No. 60/591,548, filed on July 27, 2004.

BACKGROUND

Apoptosis, or programmed cell death, plays a critical role in the development and maintenance of homeostasis in all multicellular organisms. Susceptibility to apoptosis varies markedly among cells and is influenced by both external and internal cellular events. Positive and negative regulator proteins that mediate cell fate have been defined, and dysregulation of these protein signaling networks has been documented in the pathogenesis of a wide spectrum of human diseases, including a variety of cancers. BCL-2 is the founding member of this family of apoptotic proteins and was first identified at the chromosomal breakpoint of t(14;18)(q32;q21) lymphomas (Bakhshi et al. 1985 *Cell* 41:899; Cleary et al. 1985 *Proc. Nat'l. Acad. Sci. USA* 82:7439).

Gene rearrangement places BCL-2 under the transcriptional control of the immunoglobulin heavy chain locus, generating inappropriately high levels of BCL-2 and resultant pathologic cell survival. Such aberrations in apoptosis have been identified in lymphocytic and myelogenous leukemias and a host of other malignancies, and have been linked to tumor progression and acquired resistance to chemotherapy-induced apoptosis. The BCL-2 family of proteins has expanded significantly and includes both pro- and anti-apoptotic molecules that provide the checks and balances that govern susceptibility to cell death (FIG. 1). Not surprisingly, apoptotic proteins have become key targets for the development of therapeutics to both prevent precipitous cell death in diseases of cell loss and activate cell death pathways in malignancy.

The BCL-2 family is defined by the presence of up to four conserved "BCL-2 homology" (BH) domains designated BH1, BH2, BH3, and BH4, all of which include α -helical segments (Chittenden et al. 1995 *EMBO* 14:5589; Wang et al. 1996 *Genes Dev.* 10:2859). Anti-apoptotic

proteins, such as BCL-2 and BCL-X_L, display sequence conservation in all BH domains. Pro-apoptotic proteins are divided into “multidomain” members (e.g. BAK, BAX), which possess homology in the BH1, BH2, and BH3 domains, and the “BH3-domain only” members (e.g. BID, BAD, BIM, BIK, NOXA, PUMA), that contain sequence homology exclusively in the BH3 amphipathic α -helical segment. BCL-2 family members have the capacity to form homo- and heterodimers, suggesting that competitive binding and the ratio between pro- and anti-apoptotic protein levels dictates susceptibility to death stimuli. Anti-apoptotic proteins function to protect cells from pro-apoptotic excess, i.e., excessive programmed cell death. Additional “security” measures include regulating transcription of pro-apoptotic proteins and maintaining them as inactive conformers, requiring either proteolytic activation, dephosphorylation, or ligand-induced conformational change to activate pro-death functions. In certain cell types, death signals received at the plasma membrane trigger apoptosis via a mitochondrial pathway (FIG. 2). The mitochondria can serve as a gatekeeper of cell death by sequestering cytochrome c, a critical component of a cytosolic complex which activates caspase 9, leading to fatal downstream proteolytic events. Multidomain proteins such as BCL-2/BCL-X_L and BAK/BAX play dueling roles of guardian and executioner at the mitochondrial membrane, with their activities further regulated by upstream BH3-only members of the BCL-2 family. For example, BID is a member of the “BH3-domain only” subset of pro-apoptotic proteins, and transmits death signals received at the plasma membrane to effector pro-apoptotic proteins at the mitochondrial membrane. BID has the unique capability of interacting with both pro- and anti-apoptotic proteins, and upon activation by caspase 8, triggers cytochrome c release and mitochondrial apoptosis. Deletion and mutagenesis studies determined that the amphipathic α -helical BH3 segment of pro-apoptotic family members functions as a death domain and thus represents a critical structural motif for interacting with multidomain apoptotic proteins. Structural studies have demonstrated that the BH3 helix interacts with anti-apoptotic proteins by inserting into a hydrophobic groove formed by the interface of BH1, 2 and 3 domains. Activated BID can be bound and sequestered by anti-apoptotic proteins (e.g., BCL-2 and BCL-X_L) and can trigger activation of the pro-apoptotic proteins BAX and BAK, leading to cytochrome c release and a mitochondrial apoptosis program.

BAD is also a “BH3-domain only” pro-apoptotic family member whose expression likewise triggers the activation of BAX/BAK. In contrast to BID, however, BAD displays preferential binding to anti-apoptotic members, BCL-2 and BCL-X_L. Whereas the BAD BH3

domain exhibits high affinity binding to BCL-2, BAD BH3 peptide is unable to activate cytochrome c release from mitochondria *in vitro*, suggesting that BAD is not a direct activator of BAX/BAK. Mitochondria that overexpress BCL-2 are resistant to BID-induced cytochrome c release, but co-treatment with BAD can restore BID sensitivity. Induction of mitochondrial apoptosis by BAD appears to result from either: (1) displacement of BAX/BAK activators, such as BID and BID-like proteins, from the BCL-2/BCL-X_L binding pocket, or (2) selective occupation of the BCL-2/BCL-X_L binding pocket by BAD to prevent sequestration of BID-like proteins by anti-apoptotic proteins. Thus, two classes of "BH3-domain only" proteins have emerged, BID-like proteins that directly activate mitochondrial apoptosis, and BAD-like proteins, that have the capacity to sensitize mitochondria to BID-like pro-apoptotics by occupying the binding pockets of multidomain anti-apoptotic proteins.

The objective of identifying or generating small molecules to probe apoptotic protein functions *in vitro* and specifically manipulate apoptotic pathways *in vivo* has been challenging. High throughput screening has identified several molecules that inhibit the interaction of the BAK BH3 domain with BCL-X_L at micromolar affinities. In addition to the potential drawback of identifying low affinity compounds, the technique is limited in its ability to generate panels of compounds tailored to the subtle binding specificities of individual members of protein families. Alternate approaches to manipulating apoptosis pathways have derived from peptide engineering, a technique that uses non-specific peptide sequence to generate compounds with desired three-dimensional structures. One application of this technique involved the generation of "pro-apoptotic" α -helices comprised of nonspecific peptide sequence used to induce cell death by disrupting mitochondrial membranes.

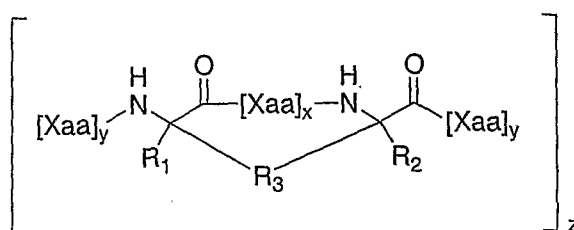
The alpha-helix is one of the major structural components of proteins and is often found at the interface of protein contacts, participating in a wide variety of intermolecular biological recognition events. Theoretically, helical peptides, such as the BH3 helix, could be used to selectively interfere with or stabilize protein-protein interactions, and thereby manipulate physiologic processes. However, biologically active helical motifs within proteins typically have little structure when taken out of the context of the full-length protein and placed in solution. Thus, the efficacy of peptide fragments of proteins as *in vivo* reagents has been compromised by loss of helical secondary structure, susceptibility to proteolytic degradation, and inability to penetrate intact cells. Whereas several approaches to covalent helix stabilization have been

reported, most methodologies involve polar and/or labile crosslinks (Phelan et al. 1997 *J. Am. Chem. Soc.* 119:455; Leuc et al. 2003 *Proc. Nat'l. Acad. Sci. USA* 100:11273; Bracken et al., 1994 *J. Am. Chem. Soc.* 116:6432; Yan et al. 2004 *Bioorg. Med. Chem.* 14:1403). Subsequently, Verdine and colleagues developed an alternate metathesis-based approach, which employed α,α -disubstituted non-natural amino acids containing alkyl tethers (Schafmeister et al., 2000 *J. Am. Chem. Soc.* 122:5891; Blackwell et al. 1994 *Angew Chem. Int. Ed.* 37:3281).

SUMMARY

This invention is based, in part, on the discovery that stably cross-linking a polypeptide having at least two modified amino acids (a process termed "hydrocarbon stapling") can help to conformationally bestow the native secondary structure of that polypeptide. For example, cross-linking a polypeptide predisposed to have an alpha-helical secondary structure can constrain the polypeptide to its native alpha-helical conformation. The constrained secondary structure can increase resistance of the polypeptide to proteolytic cleavage and also increase hydrophobicity. Surprisingly, in some instances, the polypeptides can penetrate the cell membrane (e.g., through an energy-dependent transport mechanism, e.g., pinocytosis). Accordingly, the crosslinked polypeptides described herein can have improved biological activity relative to a corresponding uncrosslinked polypeptide. For example the cross-linked polypeptide can include an alpha-helical domain of a BCL-2 family member polypeptide (e.g., BID-BH3 domain), which can bind to BAK/BAX and/or BCL-2/BCL-X_L to promote apoptosis in a subject. In some instances, the crosslinked polypeptide can be used to inhibit apoptosis. The cross-linked polypeptides described herein can be used therapeutically, e.g., to treat cancer in a subject.

In one aspect, the invention features polypeptide of formula (I),



formula (I)

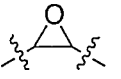
wherein;

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

R_3 is alkyl, alkenyl, alkynyl; $[R_4-K-R_4]_n$; each of which is substituted with 0-6 R_5 ;

R_4 is alkyl, alkenyl, or alkynyl;

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  ;

R_6 is H, alkyl, or a therapeutic agent;

n is an integer from 1-4;

x is an integer from 2-10;

each y is 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.

In some instances, the polypeptide binds to a BCL-2 family protein. The polypeptide can bind to an anti-apoptotic protein. The polypeptide can bind to a pro-apoptotic protein. The polypeptide can bind and activate BAX or BAK. In some instances, the polypeptide binds to a BH1, BH2 and/or BH3 domain.

In some instances, the polypeptide activates cell death, for example the polypeptide can trigger cytochrome c release and activate mitochondrial cell death.

In other instances, the polypeptide can inhibit cell death.

In some instances, the polypeptide includes a BH3 domain.

In some instances, x is 2, 3, or 6.

In some instances, each y 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.

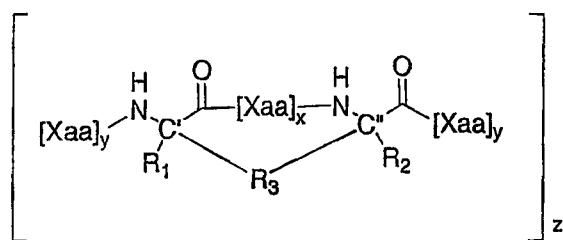
In some instances, R_3 is alkenyl (e.g., C_8 alkenyl) and x is 3.

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 $-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}=\text{CH}-\text{CH}_2-\text{CH}_2-\text{CH}_2-$.

In certain embodiments the two alpha, alpha disubstituted stereocenters 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.

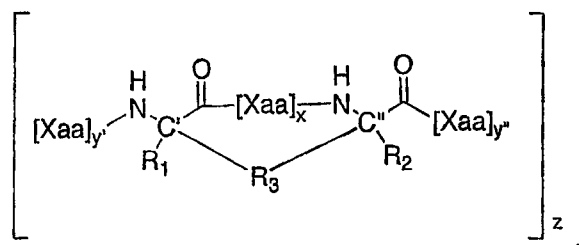
In some instances R_3 is $[\text{R}_4-\text{K}-\text{R}_4]_n$; and R_4 is a straight chain alkyl, alkenyl, or alkynyl.

In some instances, the polypeptide includes an amino acid sequence which is at least about 60% (70%, 80%, 85%, 90%, 95% or 98%) identical to the amino acid sequence of EDIIRNI*RHL*QVGDSN_LDRSIW (SEQ ID NO:99), wherein * is a tethered amino acid. For example, there can be 1, 2, 3, 4, 5 or more amino acid changes, e.g., conservative changes.

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). In some instances, the polypeptide can include an amino acid sequence which is at least about 60% (70%, 80%, 85%, 90%, 95% or 98%) identical to the amino acid sequence of EDIIRNIARHLA*VGD*N_LDRSIW (SEQ ID NO:92), wherein * is a tethered amino acid. For example, there can be 1, 2, 3, 4, 5 or more amino acid changes, e.g., conservative changes. In some instances, the polypeptide is transported through the cell membrane (e.g., through an active transport or endocytotic mechanism or by passive transport). In certain embodiments the polypeptide does not include a Cys or Met.

In some embodiments the polypeptide comprises at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 50, or more contiguous amino acids of a BCL-2 or BCL-2 like domain, e.g., a BH3 domain or BH3-like domain, e.g., a polypeptide depicted in any of FIGs. 5a, 5b, and 28a-28h. Each $[Xaa]_y$ is a peptide that can independently comprise at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25 or more contiguous amino acids of a BCL-2 or BCL-2 like domain, e.g., a BH3 domain or BH3-like domain, e.g., a polypeptide depicted in any of FIGs. 5a, 5b, and 28a-28h. $[Xaa]_x$ is a peptide that can comprise 3 or 6 contiguous amino acids of acids of a BCL-2 or BCL-2 like domain, e.g., a BH3 domain or BH3-like domain, e.g., a polypeptide depicted in any of FIGs. 5a, 5b, and 28a-28h.

The polypeptide can comprise 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50 contiguous amino acids of acids of a BCL-2 or BCL-2 like domain, e.g., a BH3 domain or BH3-like domain, e.g., a polypeptide depicted in any of FIGs. 5a, 5b, and 28a-28h wherein two amino acids that are separated by three amino acids (or six amino acids) are replaced by amino acid substitutes that are linked 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



$[Xaa]_y$ and $[Xaa]_y$ can each comprise contiguous polypeptide sequences from the same or different BCL-2 or BCL-2 like domains.

The invention features cross-linked polypeptides comprising 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 BCL-2 or BCL-2 like domain, e.g., a BH3 domain or BH3-like domain, e.g., a polypeptide depicted in any of Figs. 5a, 5b (SEQ ID Nos:92-117), and 28a-28h (SEQ ID Nos:1-91) 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.

In some embodiments the polypeptide has apoptotic activity.

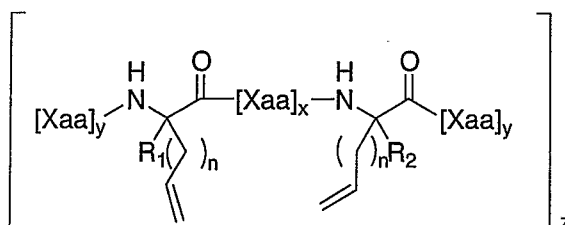
In some instances, the polypeptide also includes a fluorescent moiety or radioisotope.

In some instances, the polypeptide includes 23 amino acids; 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 an affinity label, a targeting moiety, and/or a biotin moiety.

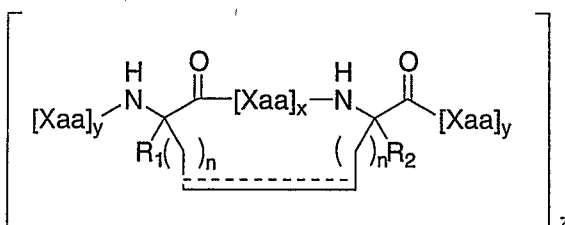
In some instances, the polypeptide is a polypeptide selected from the group consisting of the polypeptides depicted in and of FIGs. 28a-h (SEQ ID NOS: 1-83) and 5a-b (SEQ ID NOS: 84-114). In another aspect, the invention features a method of making a polypeptide of formula (III), including

providing a polypeptide of formula (II); and



formula (II)

treating the compound of formula (II) with a catalyst to promote a ring closing metathesis, thereby providing a compound of formula (III)



formula (III)

wherein

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

each n is independently an integer from 1-15;

x is 2, 3, or 6

each y is 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;

In some instances, the polypeptide binds to a BCL-2 family member protein.

In some instances, the catalyst is a ruthenium catalyst.

In some instances, the method also includes providing a reducing or oxidizing agent subsequent to the ring closing metathesis.

In some instances, the reducing agent is H₂ or the oxidizing agent is osmium tetroxide

In some instances, the invention features 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.

In some instances, the invention features a method of treating cancer in 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.

In some instances, the invention features a library of the compounds described herein.

In some instances, the invention features a method of identifying a candidate compound for the promotion of apoptosis, including;

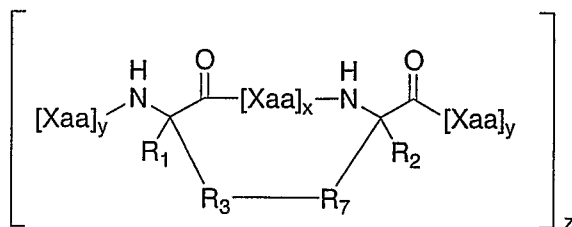
providing mitochondria;

contacting the mitochondria with any of the compounds described herein;

measuring cytochrome c release; and

comparing the cytochrome c release in the presence of the compound to the cytochrome c release in the absence of the compound, wherein an increase in cytochrome c release in the presence of the compound of formula 1 identifies the compound as a candidate compound for the promotion of apoptosis.

In some instances, the invention features a polypeptide of the formula (IV),



wherein;

each R₁ and R₂ are independently H, alkyl, alkenyl, alkynyl, arylalkyl, cycloalkylalkyl, heteroarylalkyl, or heterocyclalkyl;

R_3 is alkyl, alkenyl, alkynyl; $[R_4-K-R_4]_n$ or a naturally occurring amino acid side chain; each of which is substituted with 0-6 R_5 ;

R_4 is alkyl, alkenyl, or alkynyl;

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  ;

R_6 is H, alkyl, or a therapeutic agent;

R_7 is alkyl, alkenyl, alkynyl; $[R_4-K-R_4]_n$ or a naturally occurring amino acid side chain; each of which is substituted with 0-6 R_5 ;

n is an integer from 1-4;

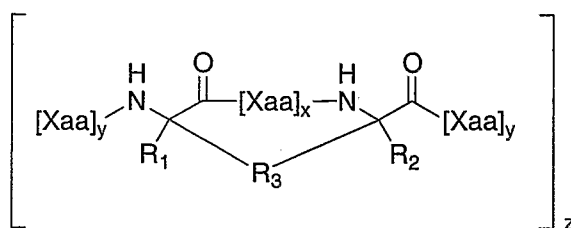
x is an integer from 2-10;

each y is 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;

In some instances, the invention features a polypeptide of formula (I)



formula (I)

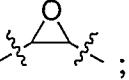
wherein;

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

R_3 is alkyl, alkenyl, alkynyl; $[R_4-K-R_4]_n$; each of which is substituted with 0-6 R_5 ;

R_4 is alkyl, alkynyl, or alkynyl;

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  ;

R_6 is H, alkyl, or a therapeutic agent;

n is an integer from 1-4;

x is an integer from 2-10;

each y is independently an integer from 0-100;

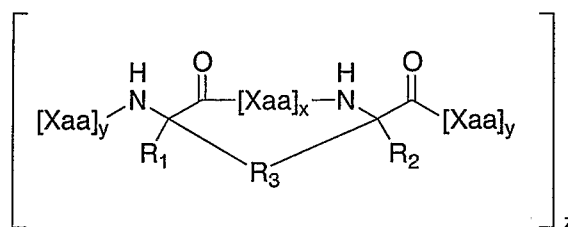
z is an integer from 1-10 (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10); and

each Xaa is independently an amino acid;

wherein the polypeptide has at least 5% alpha helicity in aqueous solution as determined by circular dichroism.

In some instances, polypeptide has at least 15%, at least 35%, at least 50%, at least 60%, at least 70%, at least 80% or at least 90% alpha helicity as determined by circular dichroism.

In some instances, the invention features a polypeptide of formula (I),



formula (I)

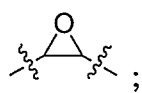
wherein;

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

R_3 is alkyl, alkenyl, alkynyl; $[R_4-K-R_4]_n$; each of which is substituted with 0-6 R_5 ;

R_4 is alkyl, alkynyl, or alkynyl;

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 ;

R_6 is H, alkyl, or a therapeutic agent;

n is an integer from 1-4;

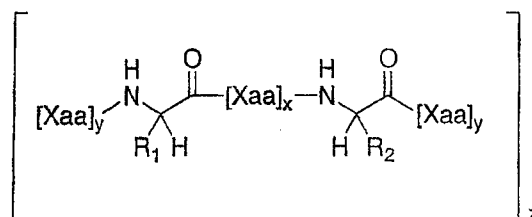
x is an integer from 2-10;

each y is 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;

wherein the polypeptide has at least a 1.25-fold increase in alpha helicity as determined by circular dichroism compared to the polypeptide of formula (V)



formula (V)

wherein R₁, R₂, Xaa, x, y, and z are all as defined for formula (I) above.

In some instances, the polypeptide has at least a 1.5-fold, at least 1.75 fold, at least 2.0-fold, at least 2.5-fold, at least 3-fold, or at least 4-fold increase in alpha helicity as determined by circular dichroism compared to the polypeptide of formula (V).

In some instances, the invention features a method of identifying a candidate compound for the inhibition of apoptosis, including;

providing mitochondria;

contacting the mitochondria with a compound described herein;

measuring cytochrome c release; and

comparing the cytochrome c release in the presence the compound described herein to the cytochrome c release in the absence of the compound described herein, wherein a decrease in cytochrome c release in the presence of the compound described herein identifies the compound described herein as a candidate compound for the inhibition of apoptosis.

Combinations of substituents and variables envisioned by this invention are only those that result in the formation of stable compounds. The term "stable", as used herein, refers to compounds which possess stability sufficient to allow manufacture and which maintains the integrity of the compound for a sufficient period of time to be useful for the purposes detailed herein (e.g., therapeutic administration to a subject or generation of reagents to study or discover a biological pathway either *in vitro* or *in vivo*).

The compounds of this invention may contain one or more asymmetric centers and thus occur as racemates and racemic mixtures, single enantiomers, individual diastereomers and diastereomeric mixtures. All such isomeric forms of these compounds are expressly included in the present invention. The compounds of this invention 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 expressly included in the present invention. All crystal forms of the compounds described herein are expressly included in the present invention.

The term "amino acid" refers to a molecule containing both an amino group and a carboxyl group. 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.

A "non-essential" amino acid residue is a residue that can be altered from the wild-type sequence of a polypeptide (e.g., a BH3 domain) 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.

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). Thus, a predicted nonessential amino acid residue in a BH3 polypeptide, for example, is preferably replaced with another amino acid residue from the same side chain family.

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

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).

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).

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, C₁-C₁₀ 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-).

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. The alkenyl moiety contains the indicated number of carbon atoms. For example, C₂-C₁₀ indicates that the group may have from 2 to 10 (inclusive) carbon atoms in it. The term "lower alkenyl" refers to a C₂-C₈ 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.

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, C₂-C₁₀ indicates that the group may have from 2 to 10 (inclusive) carbon atoms in it. The term "lower alkynyl" refers to a C₂-C₈ 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.

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.

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.

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, benzimidazolyl, pyrimidinyl, thiophenyl or thienyl, quinoliny, 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.

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, morpholinyl, tetrahydrofuranyl, and the like.

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, and cyano groups.

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

FIG. 1 depicts BCL-2 family members having one or more conserved BCL-2 homology (BH) domains.

FIG. 2 depicts a model of BID-mediated mitochondrial apoptosis. TNF-RI/Fas induces cleavage of BID, which translocates to the mitochondria and trigger apoptosis.

FIG. 3 depicts a synthetic strategy for the generation of chiral α,α -disubstituted non-natural amino acids containing olefinic side chains.

FIG. 4a depicts chemical structures of certain non-natural amino acids.

FIG. 4b depicts the crosslinking of synthetic amino acids at positions *i* and *i*+4 and *i* and *i*+7 by olefin metathesis.

FIG. 5a depicts SAHB3 compounds generated by non-natural amino acid substitution and olefin metathesis (SEQ ID Nos:92-96, 118, 119, 97-108, 120, 121, 110, 111, 122 and 123, respectively).

Fig. 5b depicts certain crosslinked peptides used in the studies described herein (SEQ ID NOs:112-117, respectively).
selected BCL-2 family members.

FIG. 7 depicts the results of a study showing that chemical crosslinking enhances the alpha helicity of SAHB3_{BID} compounds compared to the unmodified BID BH3 peptide.

FIG. 8 depicts the results of a study showing that a gly→glu mutant of SAHB3_{BID}A polypeptide displays similar helical contact to the corresponding gly containing polypeptide.

FIG. 9 depicts the results of a study showing that truncation of the 23-mer SABH3_{BID}B ("SAHB3b") to a 16-mer results in loss of α -helicity.

FIG. 10a depicts the results of a study showing that the kinetics of *in vitro* trypsin proteolysis is retarded 3.5-fold by the SABH3_{BID}A crosslink.

FIG. 10b depicts the results of a study of *ex vivo* serum stability of peptides, demonstrating a 10-fold increase in half-life of the cross-linked peptide compared to the unmodified peptide.

FIG 10c depicts the results of an *in vivo* study showing that SAHB3_{BID}A is maintained at higher serum concentrations over time compared to BID BH3 peptide.

FIG. 11a depicts the results of a study showing that SAHB3_{BID} peptides display high affinity binding to GST-BCL2 in a fluorescence polarization competitive binding assay.

FIG. 11b depicts the results of a study showing that the negative control Gly to Glu point mutants of SAHB3_{BID}A and B are relatively poor binders.

FIG. 11c depicts the results of a study showing that truncation of SAHB3_{BID}B from a 23-mer to a 16-mer results in a more than 6-fold drop in K_i , coincident with a significant decrease in percent helicity of the truncated compound.

FIG. 11d depicts the results of a BCL-2 fluorescence polarization direct binding assay demonstrating a more than 6-fold enhancement in binding affinity of SAHB3_{BID}A compared to unmodified BID BH3.

FIG. 11e depicts the results of a BAX fluorescence polarization direct binding assay demonstrating that incorporation of a crosslink results in measurable binding of SAHB3_{BID}A and SAHB3_{BID(G→E)}A to a multidomain pro-apoptotic BCL-2 family member. The unmodified BID BH3 peptide shows no binding.

FIG. 11f depicts HSQC spectra that demonstrate a conformational change in ¹⁵N-labeled BCL-X_L upon SAHB3_{BID}A binding, which is similar to that seen upon BID BH3 binding, confirming that SAHB3_{BID}A binds to the defined hydrophobic pocket of BCL-X_L.

FIGs. 12a and 12b depict the results of studies showing the percent of cytochrome c released by SAHB3_{BID} compounds from purified mouse liver mitochondria.

FIGs. 13a and 13b depict the results of a study showing that SAHB3_{BID}A- and SAHB3_{BID}B- induced cytochrome c release is faster and more potent than that of unmodified peptide.

FIG. 14 depicts the results of a study showing that the Gly to Glu mutation of SAHB3_{BID}A selectively eliminates Bak-dependent cytochrome release, underscoring the specificity of action of SAHB3_{BID}A-induced cytochrome c release shown in FIG. 13.

FIG. 15 depicts the results of a study showing that Jurkat T-cell leukemia cells, upon exposure to FITC-BID BH3 and FITC-BID helix 6, lack fluorescent labeling whereas Jurkat T-cell leukemia cells, upon exposure to FITC-SAHB3_{BID} demonstrate a positive FITC signal, and that these results are not significantly altered by trypsin-treatment of the cells.

FIG. 16a depicts the results of a study showing that Jurkat T- cells exposed to cross-linked peptides FITC-SAHB3_{BID}A and SAHB3_{BID(G→E)}A demonstrated fluorescent labeling, whereas Jurkat T- cells exposed to unmodified BH3 peptides FITC-BID and FITC-BID_(G→S) did not.

FIG 16b depicts the results of a study showing that cellular import of FITC-SAHB3_{BID}A is time-dependent at 37 °C, as assessed by FACS analysis.

FIGs. 17a and 17b depict the results of a study showing Jurkat T- cells treated with FITC-peptides at 4 °C and 37 °C. FIG. 17a shows that FITC-BID BH3 did not label the cells at either temperature, and FITC-SAHB3_{BID}A labeled the cells at 37 °C but not 4 °C. FIG. 17b shows that FITC-BID helix 6 labels but also permeabilizes the cells in a temperature-independent manner. However, in contrast, FITC-SAHB3_{BID}A only labels the cells at 37 °C and does so without cellular permeabilization, consistent with active transport of SAHB3_{BID}A via an endocytic pathway.

FIG. 17c depicts the results of a study showing that Jurkat T- cells, when preincubated with or without sodium azide and 2-deoxyglucose followed by treatment with FITC-peptides, showed no labeling for either condition with the FITC-BID BH3 polypeptide. The cells showed reduced labeling for FITC-SAHB3_{BID}A under sodium azide and 2-deoxyglucose conditions, and showed labeling with FITC-BID helix 6 under both conditions. These results are consistent with an ATP-dependant cellular uptake (e.g., endocytosis pathway) for SAHB3_{BID} import.

FIG 18 depicts the results of a study showing that FITC-SAHB3_{BID}A uptake is not inhibited by cellular treatment with the glycosaminoglycan heparin, indicating that there are distinctions between the mechanism of binding and uptake of FITC-SAHB3_{BID}A compared to other cell penetrating peptides (CPPs), such as HIV TAT and Antennapedia peptides.

FIG. 19 depicts the results of a study showing that FITC-SAHB3_{BID}A compounds display cytoplasmic labeling with a vesicular distribution in Jurkat T-cells, whereas plasma membrane fluorescence is not evident. On the other hand, FITC-BID BH3 displays no cellular labeling of cells and FITC-BID helix 6 labels the cells diffusely and causes significant architectural destruction.

FIG. 20 depicts the results of a study showing that FITC-SAHB3_{BID}A co-localizes with a mitochondrial membrane marker in Jurkat T-cells.

FIG 21a and FIG 21b depicts the results of a study showing that FITC-SAHB3_{BID}A colocalizes in live BCL-2 overexpressing Jurkat T-cells with dextran-labeled endosomes but not transferring-labeled endosomes, indicating that FITC-SAHB3_{BID}A is imported into cells by fluid-phase pinocytosis.

FIG 21c depicts the results of a study showing that by 24 hours after treatment, FITC-SAHB3_{BID}A colocalizes in the live cells with mitochondria labeled by MitoTracker.

FIGs. 22a, 22b, and 22c depict the results of a study showing that SAHB3_{BID}A triggers metabolic arrest in a dose responsive fashion in the leukemia cell lines tested, whereas BID BH3 and SAHB3_{BID(G→E)}A had essentially no effect in this dose range.

FIG. 23 depicts the results of a study showing that SAHB3_{BID}A and SAHB3_{BID}B induced apoptosis in up to 50% of intact Jurkat cells at 10 μ M, an effect specifically inhibited by BCL-2 overexpression (black bars). Unmodified BID BH3 peptide and the gly to glu mutants had no effect based on comparison with the no treatment control.

FIG. 24 depicts the results of a study showing the dose response of Jurkat BCL-2 overexpressing cells treated with SAHB3_{BID}A, SAHB3_{BID(G→E)}A and SAHB3_{BID(G→S)}A. Whereas SAHB3_{BID}A and SAHB3_{BID(G→S)}A can overcome BCL-2 inhibition of apoptosis in this dose range, the gly to glu point mutant has no effect.

FIG. 25 depicts the results of a study showing that SAHB3_{BID}A treated leukemia cell lines REH, MV4;11, and SEMK2 underwent specific apoptosis induction, whereas the gly to glu point mutant SAHB3_{BID(G→E)}A had no effect on the cells.

FIGs. 26a and 26b depict the results of a study showing that both SAHB3_{BID}A and SAHB3_{BID(G→S)}A suppressed the growth of SEMK2 leukemia in NOD-SCID mice, with SAHB3_{BID(G→S)}A demonstrating a greater potency than SAHB3_{BID}A.

FIG. 27a and FIG. 27b depicts the results of a study showing that SAHB3_{BID}A blunts the progression of SEMK2 leukemia relative to vehicle in NOD-SCID mice. A dose responsive effect is noted in FIG 27a.

FIGs. 27c, 27d, 27e depict the results of an animal study showing that SAHB3_{BID}A inhibits the growth of RS4;11 leukemia relative to vehicle in SCID beige mice, with statistically significant prolongation of survival in SAHB3_{BID}A-treated mice compared to vehicle controls.

FIG 27f depicts the results of an animal study again showing that SAHB3_{BID}A causes regression of RS4;11 leukemia in SCID beige mice, in contrast with SAHB3_{BID(G→E)}A- and vehicle-treated mice which demonstrate leukemia progression.

Figs 28a-28h depict examples of various alpha helical domains of BCL-2 family member proteins (Fig. 28A-1 SEQ ID NOs:1 and 2; Fig 28A-2 SEQ ID NOs:3-9, respectively; Fig. 28B-1 SEQ ID NOs:10-15, respectively; Fig. 28C-2 SEQ ID NOs:16-16, respectively; Fig. 28D-1 SEQ ID NOs:27-30, respectively; Fig 28D-2 SEQ ID NOs:31-39, respectively; Fig. 28E-2 SEQ ID NOs:40-46, respectively; Fig. 28F-2 SEQ ID NO:47, respectively; Fig. 28F-3 SEQ ID NOs:48-59, respectively; Fig. 28G-1 SEQ ID NOs:60-61, respectively; Fig. 28G-2 SEQ ID NOs:62-69, respectively; and Fig. 28H SEQ ID NOs:70-91, respectively) amenable to crosslinking.

DETAILED DESCRIPTION

The invention is based, in part, on the discovery that cross-linked alpha helical domain polypeptides of BCL-2 family proteins have improved pharmacological properties over their uncrosslinked counterparts (e.g., increased hydrophobicity, resistance to proteolytic cleavage, binding affinity, *in vitro* and *in vivo* biological activity). Moreover, it has been surprisingly discovered that the cross-linked polypeptides can penetrate the cell membrane via a temperature- and energy-dependent transport mechanism (e.g., endocytosis, specifically fluid-phase pinocytosis). The polypeptides include a tether between two non-natural amino acids, which tether significantly enhances the alpha helical secondary structure of the polypeptide. Generally, the tether 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 crosslinking. Thus, for example, where a peptide has the sequence ...Xaa₁, Xaa₂, Xaa₃, Xaa₄, Xaa₅, Xaa₆, Xaa₇, Xaa₈, Xaa₉..., crosslinks between Xaa₁ and Xaa₄, or between Xaa₁ and Xaa₅, or between Xaa₁ and Xaa₈ are useful as are crosslinks between Xaa₂ and Xaa₅, or between Xaa₂ and Xaa₆, or between Xaa₂ and Xaa₉, etc. In addition, a model polypeptide was prepared incorporating two sets of crosslinks with one located between Xaa₁ and Xaa₅ and another between Xaa₉ and Xaa₁₃. The double crosslink was achieved by careful stereochemical control of the double bond metathesis reactions. Thus, the invention encompasses the incorporation of 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 crosslinked peptides can be conjoined by a technique called native chemical ligation (Bang, et al., *J. Am. Chem Soc.* 126:1377).

The novel cross-linked polypeptides are useful, for example, to mimic or study proteins or polypeptides having one or more alpha-helical domains. One family of proteins where family members have at least one alpha helical domain is the BCL-2 family of proteins. These proteins are involved in cellular apoptotic pathways. Some BCL-2 family members have a pro-apoptotic function, others have an anti-apoptotic function, and still others change functions with a change in cellular conditions. Accordingly, it is desirable to make stabilized polypeptides that would

mimic one or more motifs of the BCL-2 family members, thus modulating a variety of BCL-2 related activities.

Chemical Synthesis of a Panel of SAHB3_{BID} Compounds

α,α -Disubstituted non-natural amino acids containing olefinic side chains of varying length were synthesized according to the schema in FIG. 3 (Williams et al. 1991 *J. Am. Chem. Soc.* 113:9276; Schafmeister et al. 2000 *J. Am. Chem. Soc.* 122:5891). Chemically crosslinked BID BH3 peptides were designed by replacing two or four naturally occurring amino acids with the corresponding synthetic amino acids (FIG. 4a). Substitutions were made at discrete locations, namely the "i, and i+4 positions" or the "i, and i+7 positions", which facilitate crosslinking chemistry by placing reactive residues on the same face of the α -helix (FIG. 4b). Highly conserved amino acids among apoptotic proteins, in addition to those sequences found to be important in protein-protein interactions based on X-ray crystallographic and NMR studies (Muchmore et al. 1996 *Nature* 381:335; Sattler et al. 1997 *Science* 275:983), were specifically not replaced in certain circumstances, conserved amino acids could be replaced by other amino acids (e.g., synthetic non-naturally occurring amino acids) to enhance activity (this effect can be seen in the SAHB3_{BID} mutants described herein). SAHB3_{BID} compounds were generated by solid phase peptide synthesis followed by olefin metathesis-based crosslinking of the synthetic amino acids via their olefin-containing side chains. The variations of SAHB3_{BID} compounds generated are illustrated in FIG. 5a. SAHB3_{BID} (SAHB_A) variants incorporating specific mutations known to alter BID function (Wang et al. 1996 *Genes Dev.* 10:2859) were also constructed to serve as negative controls in biological experiments (FIG. 5a). The amino termini of selected compounds were further derivatized with fluorescein isothiocyanate (FITC) or biotin conjugated-lysine to generate labeled SAHB3_{BID} compounds for cell permeability studies and biochemical assays, respectively (FIG. 5a). In several syntheses, a C-terminal tryptophan was added to the sequence to serve as a UV label for purification and concentration determination purposes; the N-terminal glutamic acid was eliminated in several peptides in order to increase the overall pI of the compound to potentially facilitate cell penetration (see below). The metathesis approach was readily applied to the generation of alternate SAHB3s, including SAHB3_{BAD} and SAHB3_{BIM} (FIG. 5a).

The non-natural amino acids (R and S enantiomers of the 5-carbon olefinic amino acid and the S enantiomer of the 8-carbon olefinic amino acid) were characterized by nuclear

magnetic resonance (NMR) spectroscopy (Varian Mercury 400) and mass spectrometry (Micromass LCT). Peptide synthesis was performed either manually or on an automated peptide synthesizer (Applied Biosystems, model 433A), using solid phase conditions, rink amide AM resin (Novabiochem), and Fmoc main-chain protecting group chemistry. For the coupling of natural Fmoc-protected amino acids (Novabiochem), 10 equivalents of amino acid and a 1:1:2 molar ratio of coupling reagents HBTU/HOBt (Novabiochem)/DIEA were employed. Non-natural amino acids (4 equiv) were coupled with a 1:1:2 molar ratio of HATU (Applied Biosystems)/HOBt/DIEA. Olefin metathesis was performed in the solid phase using 10 mM Grubbs catalyst (Blackwell et al. 1994 *supra*) (Strem Chemicals) dissolved in degassed dichloromethane and reacted for 2 hours at room temperature. The amino termini of selected compounds were further derivatized with β -alanine and fluorescein isothiocyanate (FITC [Sigma]/DMF/DIEA) to generate fluorescently labeled compounds. A C-terminal tryptophan was incorporated to serve as a UV label for purification and concentration determination purposes; SAHBA compounds were also synthesized without the C-terminal tryptophan and N-terminal glutamic acid, the latter modification performed to increase the overall pI of the molecules. Isolation of metathesized compounds was achieved by trifluoroacetic acid-mediated deprotection and cleavage, ether precipitation to yield the crude product, and high performance liquid chromatography (HPLC) (Varian ProStar) on a reverse phase C18 column (Varian) to yield the pure compounds. Chemical composition of the pure products was confirmed by LC/MS mass spectrometry (Micromass LCT interfaced with Agilent 1100 HPLC system) and amino acid analysis (Applied Biosystems, model 420A).

FIG. 5b schematically depicts a subset of the peptides in FIG. 5a, including the stereochemistry of the olefinic amino acids (R and S enantiomers of the 5-carbon olefinic amino acid and the S enantiomer of the 8-carbon olefinic amino acid).

SAHB3_{BID} Compounds Display Enhanced α -helicity

We examined the percent helicity of pro-apoptotic BH3 domains, and found that these unmodified peptides were predominantly random coils in solution, with α -helical content all under 25% (FIG. 6). Briefly, compounds were dissolved in aqueous 50 mM potassium phosphate solution pH 7 to concentrations of 25-50 mM. CD spectra were obtained on a Jasco J-710 spectropolarimeter at 20°C using the following standard measurement parameters:

wavelength, 190-260 nm; step resolution, 0.5 nm; speed, 20 nm/sec; accumulations, 10; response, 1 sec; bandwidth, 1 nm; path length, 0.1 cm. The α -helical content of each peptide was calculated by dividing the mean residue ellipticity $[\phi]_{222\text{obs}}$ by the reported $[\phi]_{222\text{obs}}$ for a model helical decapeptide (Yang et al. 1986 *Methods Enzymol.* 130:208)).

In each case, the chemical crosslink(s) increased the percent α -helicity of BID's BH3 domain, with SAHB3_{BID}A and B achieving more than 5-fold enhancement (FIG. 7). SAHB3_{BID(G→E)}A, a negative control Gly to Glu point mutant of SAHB3_{BID}A, displays similar helical content to SAHB3_{BID}A (FIG. 8). Thus, the all-hydrocarbon crosslink can transform an apoptotic peptide that is essentially a random coil in aqueous solution into one that is predominantly α -helical in structure. Interestingly, the importance of the fourth helical turn in stabilizing BID BH3 peptides is underscored by the decrease in helicity observed when the SAHB3_{BID}B 23-mer is truncated to the 16-mer, SAHB3_{BID(tr)}B (FIG. 9).

The All-Hydrocarbon Crosslink Increases Protease Resistance of SAHB3_{BID} Compounds

The amide bond of the peptide backbone is susceptible to hydrolysis by proteases, thereby rendering peptidic compounds vulnerable to rapid degradation *in vivo*. Peptide helix formation, however, buries the amide backbone and therefore shields it from proteolytic cleavage. SAHB3_{BID}A was subjected to *in vitro* trypsin proteolysis to assess for any change in degradation rate compared to the unmodified BID BH3 peptide. SAHB3_{BID}A and unmodified peptide were incubated with trypsin agarose and the reactions quenched at various time points by centrifugation and subsequent HPLC injection to quantitate the residual substrate by ultraviolet absorption at 280 nm. Briefly, BID BH3 and SAHB3_{BID}A compounds (5 mcg) were incubated with trypsin agarose (Pierce) (S/E ~125) for 0, 10, 20, 90, and 180 minutes. Reactions were quenched by tabletop centrifugation at high speed; remaining substrate in the isolated supernatant was quantified by HPLC-based peak detection at 220 nm. The proteolytic reaction displayed first order kinetics and the rate constant, k , determined from a plot of $\ln[S]$ versus time ($k = -1 \times \text{slope}$) (FIG. 10a). The experiment, performed in triplicate, demonstrated a 3.5-fold enhancement in trypsin resistance of SAHB3_{BID}A compared to the unmodified peptide. Thus, enhanced protection of trypsin-sensitive amide bonds by burying them at the core of the α -helix affords a more stable peptidic compound, and may therefore render such compounds particularly stable in serum.

For *ex vivo* serum stability studies, FITC-conjugated peptides BID BH3 and SAHB3_{BID}A (2.5 mcg) were incubated with fresh mouse serum (20 mL) at 37°C for 0, 1, 2, 4, 8, and 24 hours. The level of intact FITC-compound was determined by flash freezing the serum specimens in liquid nitrogen, lyophilization, extraction in 50:50 acetonitrile/water containing 0.1% trifluoroacetic acid, followed by HPLC-based quantitation using fluorescence detection at excitation/emission settings of 495/530 nm. The results of this analysis are shown in FIG. 10b.

To investigate the *in vivo* stability of SAHB3_{BID}A, 10 mg/kg of FITC-conjugated BID BH3 peptide and SAHB3_{BID}A were injected into NOD-SCID mice and blood specimens withdrawn at 0, 1, 4 and 22 hours post-injection. Levels of intact FITC-compound in 25 µL of fresh serum were then measured. The results of this analysis, depicted in FIG. 10c, show that SAHB3_{BID}A was readily detectable over a 22 hour period, with 13% of the input still measurable a 22 hours. In contrast, only 12% of BID BH3 was detectable one hour after injection.

SAHB3_{BID} Compounds Retain High Affinity Anti-Apoptotic Binding

The all-hydrocarbon crosslinks were selectively placed on the charged face of the BID BH3 amphipathic helix in order to avoid interference with critical interactions between the binding pocket of multidomain apoptotic proteins and the hydrophobic residues of the BID BH3 helix. Fluorescence polarization competitive binding experiments were performed to evaluate the efficacy of SAHB3_{BID} compounds in competing with FITC-labeled unmodified BID BH3 peptide for GST-BCL-2 binding. All SAHB3_{BID} compounds demonstrate high affinity binding to GST-BCL2, with SAHB3_{BID}A and B, the two compounds with the greatest percent helicity, likewise displaying the highest affinity binding (Fig 11a). Of note, Gly to Glu mutation of SAHB3_{BID}A and B eliminates high affinity binding, as would be predicted from previous studies (FIG. 11b). We additionally determined that Gly to Ser mutation of SAHB3_{BID}A abolishes BCL-2 binding in this assay (data not shown). Truncation of the 23-mer SAHB3_{BID}B to a 16-mer results in loss of BCL-2 binding affinity, coincident with the decrement in α -helicity described above (FIG.11c).

FITC-labeled BID BH3 peptide binds to BCL-2 with a K_D of 220 nM, and once bound, displacement of this interaction by unlabeled BID BH3 occurs at an IC_{50} of 838 nM. This supports a model whereby BH3 binding to BCL-2 triggers an overall conformational change favoring the interaction, resulting in the need for excess amounts of unlabeled peptide to displace

prebound FITC-labeled BID BH3. We have further shown that the BAD BH3 domain has an enhanced K_D of 41 nM for BCL-2 binding, and that it can displace prebound FITC-BID BH3 with an IC_{50} of 173 nM. In a similar experiment, SAHB3_{BID}A was found to displace FITC-BID BH3 from BCL-2 with an IC_{50} of 62 nM, reflecting a more than 13-fold increase in displacement potency compared to unmodified BID BH3 peptide. These data confirm that SAHB3_{BID}A binds with enhanced affinity to BCL-2 compared to unmodified BH3 peptides, and suggest that preorganization of α -helical structure by chemical crosslinking provides a kinetic advantage for target binding.

Direct binding assays by fluorescence polarization demonstrated that incorporation of the crosslink into BID BH3 peptide resulted in enhanced binding affinity of SAHB3_{BID}A for both BCL-2, an anti-apoptotic multidomain protein, and BAX, a pro-apoptotic multidomain protein, compared to unmodified BID BH3 peptide (FIGs 11d and 11e). A direct BCL-2 fluorescence polarization binding assay demonstrated a 6-fold enhancement in BCL-2 binding affinity of SAHB3_{BID}A (K_D , 38.8 nM) compared to unmodified BID BH3 peptide (K_D , 269 nM) (FIG 11d). A Gly to Glu mutation, SAHB_{A(G→E)} (K_D , 483 nM), eliminates high affinity binding and serves as a useful control (FIG. 11d). Briefly, Escherichia coli BL21 (DE3) containing the plasmid encoding C-terminal deleted GST-BCL-2 were cultured in ampicillin-containing Luria Broth and induced with 0.1 mM IPTG. The bacterial pellets were resuspended in lysis buffer (1 mg/ml lysozyme, 1% Triton X-100, 0.1 mg/ml PMSF, 2 μ g/ml aprotinin, 2 μ g/ml leupeptine, 1 μ g/ml pepstatin A in PBS) and sonicated. After centrifugation at 20,000 $\times$ g for 20 min, the supernatant was applied to a column of glutathione-agarose beads (Sigma). The beads were washed with PBS and treated with 50 mM glutathione, 50 mM Tris-HCl (pH 8.0) to elute the protein, which was then dialyzed against binding assay buffer (140 mM NaCl, 50 mM Tris-HCl [pH 7.4]). Fluorescent compounds (25 nM) were incubated with GST-BCL2 (25 nM-1000nM) in binding buffer at room temperature. Binding activity was measured by fluorescence polarization on a Perkin-Elmer LS50B luminescence spectrophotometer. K_D values were determined by nonlinear regression analysis using Prism software (Graphpad). Full-length BAX protein was prepared as previously described (Suzuki et al, *Cell*, 103:645) and fluorescence polarization assay performed as described above.

SAHB3_{BID}A Binds to BCL-X_L

To determine if SAHB3_{BID}A specifically interacts with the defined binding groove of an anti-apoptotic multidomain protein, a two-dimensional $^{15}\text{N}/^1\text{H}$ heteronuclear single-quantum correlation (HSQC) spectrum of ^{15}N -labeled BCL-X_L before and after the addition of SAHB3_{BID}A was recorded and compared with the corresponding BID BH3/ ^{15}N -BCL-X_L spectrum. Briefly, Escherichia coli BL21 (DE3) containing the plasmid encoding C-terminal deleted BCL-X_L were cultured in M9-minimal medium containing $^{15}\text{NH}_4\text{Cl}$ (Cambridge Isotope Laboratories) to generate uniformly ^{15}N -labeled protein. Recombinant proteins were isolated from bacteria. Unlabeled SAHB3_{BID}A and BID BH3 peptides were generated and purified as described above. The following 1:1 complexes were prepared at 0.1 mM in 50 mM potassium phosphate (pH 7), 50 mM sodium chloride, 5% DMSO in D₂O or H₂O/D₂O (95:5): ^{15}N -BCL-X_L/unlabeled BID BH3, ^{15}N -BCL-X_L/unlabeled SAHB3_{BID}A. Two dimensional $^{15}\text{N}/^1\text{H}$ heteronuclear single-quantum spectra were recorded for the two complexes and analyzed for changes in resonance upon ligand binding.

The overall similarity of the HSQC spectra indicates that the structural changes occurring in BCL-X_L after addition of SAHB3_{BID}A are nearly identical to those observed with BID BH3 peptide (FIG. 11f).

SAHB3_{BID} Compounds Trigger Rapid and Specific Release of Mitochondrial Cytochrome C

In order to assess the biological activity of SAHB3_{BID} compounds *in vitro*, cytochrome c release assays were performed using purified mouse liver mitochondria. Mitochondria (0.5 mg/mL) were incubated for 40 minutes with 1 μM and 100 nM of SAHB3_{BID} compounds and then supernatants and mitochondrial fractions isolated and subjected to cytochrome c ELISA assay. Background cytochrome c release (10-15%) was subtracted from total release for each sample, and the percent actual cytochrome c release was determined (FIG. 12). The identical experiment was performed concurrently on mouse liver mitochondria isolated from *Bak*^{-/-} mice, which do not release mitochondrial cytochrome c in response to BID-BH3 activation; data from the BAK^{-/-} mitochondria therefore serve as a negative control for BAK-mediated cytochrome c release in response to SAHB3_{BID} treatments. In each case, except for the double cross-linked SAHB3_{BID}E (which may lack critical amino acids for biological activity or, in this case, be overly constrained by the dual cross-links), there is approximately a doubling of cytochrome c

release in response to 1 μ M SAHB3_{BID} compounds compared to the unmodified peptide (FIG. 12a). BAK-independent cytochrome c release is observed at this dose with SAHB3_{BID}A, B, and, in particular, D. Whereas this cytochrome c release may represent a nonspecific membrane perturbing effect of the α -helices, the role of a SAHB3_{BID}-induced, BAK-independent component of cytochrome c release is worthy of further exploration. Interestingly, the SAHB3_{BID} compound that induces the most significant level of BAK-independent cytochrome c release, SAHB3_{BID}D, is also the most hydrophobic of the SAHB3_{BID} compounds; SAHB3_{BID}D elutes from the reverse phase C18 column at 95% acetonitrile/5% water, compared to the other SAHB3_{BID} compounds that elute at 50-75% acetonitrile. BID mutants with defective BH3 domains can promote BAK-independent cytochrome c mobilization (Scorrano et al, *Dev Cell*, 2:55), and the highly hydrophobic BID helix 6 has been implicated in this activity (L. Scorrano, S.J. Korsmeyer, unpublished results). It is plausible that SAHB3_{BID}D displays both BAK dependent and independent cytochrome c release by mimicking features of BID helices 3 and 6. At ten-fold lower dosing SAHB3_{BID}A and B retain selective BAK-dependent cytochrome c release activity (FIG. 12b). The potency of SAHB3_{BID}B, in particular, compares favorably with maximally activated myristolated BID protein, which releases approximately 65% cytochrome c under these conditions at doses of 30 nM.

The most active SAHB3_{BID} compounds, A and B, were subjected to further kinetic studies to determine if helical preorganization can trigger more rapid cytochrome c release compared to the unmodified peptide. Similar to the above experiment, mouse liver mitochondria from wild-type and *Bak*^{-/-} mice were exposed to the compounds at various concentrations and assayed for cytochrome c release at 10 and 40 minute intervals. Whereas at 10 minutes the unmodified peptide causes less than 10% release at the highest dose tested (1 μ M), SAHB3_{BID}B has an EC50 for release at this timepoint of just under 400 nM, with almost maximal cytochrome release at 1 μ M (Fig 13a). Likewise, SAHB3_{BID}A triggers significant cytochrome c release at the 10 minute time interval. The EC50 for cytochrome c release at 40 minutes is 2.9 μ M for the unmodified peptide and 310 and 110 nM for SAHB3_{BID} A and B, respectively (Fig 13b). Thus, SAHB3_{BID}A and B display a 10-25 fold enhancement in cytochrome c release activity at the 40 minute time point. Whereas the BAK-dependent cytochrome c release increases over time, the BAK-independent release does not change between the 10 and 40 minute timepoints, suggesting that this distinct release occurs early and is maximally achieved within 10 minutes. Of note, the

negative control Gly to Glu point mutant of SAHB3_{BID}A, SAHB3_{BID(G→E)}A, generates only Bak-independent cytochrome c release, confirming that SAHB3_{BID}A functions via the Bak-dependent mitochondrial apoptosis pathway (Fig 14). Taken together, these cytochrome c release data indicate that SAHB3_{BID}A and B are capable of specifically inducing BAK-dependent cytochrome c release with markedly enhanced potency and kinetics compared to the unmodified peptide.

SAHB3_{BID} Compounds Penetrate Intact Cells

Fluorescein-derivatized SAHB3_{BID} compounds, BID BH3 peptides, and a BID helix 6 peptide were incubated with Jurkat T-cell leukemia cells in culture for 4-24 hours and subsequently FACS sorted to determine percent labeling of leukemia cells. In order to avoid confounding results from cell-surface bound compounds, the Jurkat cells were washed thoroughly and subjected to trypsin overdigestion, in accordance with recent reports. For each compound tested, there was no significant change in the FITC signal profile after trypsin digestion, suggesting that in the case of these peptides, little to no FITC-labeled compound is surface bound (FIG. 15). Whereas BID BH3-treated cells were FITC-negative, both FITC-SAHB3_{BID}A- and FITC-SAHB3_{BID(G→E)}A-treated cells were FITC-positive, as indicated by a rightward shift of the FITC signal (FIG. 16a). The similar profile of FITC-SAHB3_{BID}A and FITC-SAHB3_{BID(G→E)}A in these cell permeability studies is particularly important, given the use of the point mutant compound as a negative control in biological experiments. BID helix 6, a cell permeable and membrane perturbing peptide, was used as a positive control for FITC-labeling in this experiment.

Surprisingly, it was discovered that FITC-SAHB3_{BID}A appears to enter the cell via endocytosis, a temperature- and energy-dependent transport pathway. Cellular import of FITC-SAHB3_{BID}A occurred in a time-dependent manner (FIG. 16b). When cellular endocytosis was inhibited by performing the experiment at 4°C (FIG 17a, 17b) or by treatment with the energy poisons sodium azide and 2-deoxyglucose (FIG 17c), cell labeling was inhibited or markedly diminished, respectively. Of note, Jurkat cells labeled by FITC-SAHB3_{BID}A at 37°C are propidium iodide (PI) negative, confirming that the crosslinked peptide does not merely function as a permeabilizing agent (Fig 17b); in contrast, FITC-BID helix 6 readily penetrates at both temperatures, effectively permeabilizing the cells, as evidenced by the degree of PI positivity

(FIG. 17b). These data support an endocytic mechanism of entry for the SAHB3_{BID} compounds, consistent with recent reports citing cell-surface adherence followed by endocytosis as the mechanism of entry for other cell-penetrating peptides (CPPs), such as the HIV transactivator of transcription (TAT). Whereas highly basic CPPs, such as TAT and Antennapedia, are believed to be concentrated at the cell surface by adherence to negatively charged glycosaminoglycans SAHB3_{BID}A import was not inhibited in a dose-responsive manner by heparin (FIG 18) The biophysical properties of the SAHB3_{BID} amphipathic α -helix may facilitate distinct cell contacts via electrostatic and/or lipid membrane interactions.

Confocal microscopy experiments were employed in order to determine the intracellular localization of SAHB3_{BID}A. Jurkat T-cell leukemia cells were incubated with FITC-labeled compounds as described above or with serum replacement at 4 hours followed by additional 16 hours incubation at 37°C, and after washing twice with PBS, were cytopspun at 600 RPM for 5 minutes onto superfrost plus glass slides (Fisher). Cells were then fixed in 4% paraformaldehyde, washed with PBS, incubated with TO-PRO-3 iodide (100 nM) (Molecular Probes) to counterstain nuclei, treated with Vectashield mounting medium (Vector), and then imaged by confocal microscopy (BioRad 1024). For double labeling experiments, fixed cells were additionally incubated with primary antibody to TOM20, and rhodamine-conjugated secondary antibody prior to TOPRO-3 counterstaining. For live confocal microscopy, double labeling of Jurkat cells was performed with FITC-SAHB_A (10 μ M) and MitoTracker (100 nM, Molecular Probes), tetramethylrhodamine isothiocyanate (TRITC)-Dextran 4.4 kD or 70 kD (25 mcg/mL, Molecular Probes), or Alexa Fluor 594-transferrin (25 mcg/mL, Molecular Probes) for 4 hours (dextran and transferrin) or 24 hours (MitoTracker). Due to limitations of photobleaching, BCL-2 overexpressing Jurkat cells were used for live confocal microscopy in order to optimize FITC imaging. FITC-SAHB_A labeling of mitochondria was brighter in BCL-2 overexpressing Jurkats (consistent with the mechanism for SAHB activity), and thus image capture was facilitated using these cells. Treated Jurkats were washed twice and then resuspended in PBS and wet mount preparations analyzed with a BioRad 1024 (Beth Israel/Deaconess Center for Advanced Microscopy) or Zeiss LSM510 laser scanning confocal microscope (Children's Hospital Boston Imaging Core).

In fixed sections, SAHB3_{BID}A compounds localized to the cytoplasmic rim of the leukemic cells, with no plasma membrane or surface fluorescence evident; the vesicular pattern

of fluorescence suggested an organelle-specific localization (FIGs. 19a and 19b). Consistent with the FACS data, Jurkat cells treated with FITC-BID BH3 showed no fluorescent labeling (FIG. 19c). Whereas FITC-SAHB3_{BID}A-treated cells display selective intracellular fluorescence and maintain their cellular architecture (FIG 19a), FITC-BID helix 6-treated cells are diffusely labeled and demonstrate disrupted cellular morphology (FIGs. 19d). Colocalization studies using FITC-SAHB3_{BID}A and an antibody to mitochondrial membrane protein Tom20, demonstrated extensive overlap of SAHB3_{BID}A fluorescence with mitochondria, the expected site of SAHB3_{BID}'s molecular targets (FIG. 20).

Live cell imaging performed 4 hr after SAHB treatment demonstrated an initial colocalization of FITC-SAHB_A with dextran (4.4 kD or 70 kD)-labeled endosomes (Fig 21a), but not transferrin-labeled endosomes (Fig 21b), consistent with cellular uptake by fluid-phase pinocytosis (manuscript ref 27), the endocytic pathway determined for TAT and Antp peptides (manuscript ref 28). At a 24 hr time point, intracellular FITC-SAHB_A showed increased colocalization with MitoTracker-labeled mitochondria in live cells (Fig. 21c) consistent with the mitochondrial colocalization observed in fixed cells using an antibody to Tom20, a mitochondrial outer membrane protein (Fig. 20). Taken together, the FACS data and confocal imaging demonstrate that the all-hydrocarbon crosslink enables SAHB3_{BID}A compounds to be imported by intact cells (e.g., through an endocytotic mechanism).

SAHB3_{BID} Compounds Trigger Apoptosis of B-, T-, and Mixed-Lineage Leukemia (MLL) Cells

In order to assess whether SAHB3_{BID} compounds could arrest the growth of proliferating leukemia cells in culture, 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide, MTT assays using serial dilutions of SAHB3_{BID}A were performed on T-cell (Jurkat), B-cell (REH), and Mixed Lineage Leukemia (MLL)-cells (MV4;11, SEMK2, RS4;11) in culture. SAHB3_{BID}A inhibited the leukemic cells at IC₅₀s of 2.2 (Jurkat), 10.2 (REH), 4.7 (MV4;11), 1.6 (SEMK2), and 2.7 (RS4;11) μ M (FIG. 22a). Neither the BID BH3 peptide nor the SAHB_{A(G→E)} point mutant had an effect in this dose range (FIG. 22b, 22c).

To assess whether this metabolic arrest represented apoptosis induction, Jurkat leukemia cells were treated with 10 μ M SAHB3_{BID}A and B, SAHB3_{BID(G→E)}A and B, and unmodified BID BH3 peptide, in serum-free media for 4 hours followed by a 16 hour incubation in serum-

containing media (ie. final peptide concentrations of 5 μ M), and then assayed for apoptosis by flow cytometric detection of annexin V-treated cells. SAHB3_{BID}A and B demonstrated between 40-60% annexin V positivity by 20 hours post treatment, whereas the unmodified peptide and SAHB3_{BID} point mutants had no effect (FIGs. 23a and 23b). Comparable studies that use either unmodified BH3 peptides with carrier reagents or engineered helices with nonspecific mitochondrial perturbing effects, required doses of 200-300 μ M to activate apoptosis. An additional control experiment using Jurkat cells engineered to overexpress BCL-2 was subsequently undertaken to assess whether SAHB3_{BID}-induced apoptosis could be decreased by excess BCL-2, which would suggest that the compounds specifically function within cells through the mitochondrial apoptosis pathway. Indeed, the pro-apoptotic effect of 10 μ M SAHB3_{BID}A and B on "wild-type" Jurkats was abolished in the BCL-2 overexpressing cells. This protective effect, however, can be overcome by dose escalation of SAHB3_{BID}A but not of SAHB3_{BID(G $\rightarrow$ E)}A (FIG. 24); in addition, a gly to ser point mutant of SAHB3_{BID}A (SAHB3_{BID(G $\rightarrow$ S)}A), which does not exhibit BCL-2 binding affinity (see above), is equally effective as a pro-apoptotic in "wild-type" and BCL-2 overexpressing Jurkat cells (FIG. 24). Apoptosis induction assays using SAHB3_{BID}A and SAHB3_{BID(G $\rightarrow$ E)}A were additionally performed in the REH, MV4;11, and SEMK2 cell lines with similar results (FIG. 25). Taken together, these data indicate that SAHB3_{BID} compounds can penetrate and kill proliferating leukemia cells. The observed pro-apoptotic effects are selectively abolished by gly to glu mutation of SAHB3_{BID}A and cellular overexpression of BCL-2, findings which underscore that SAHB3_{BID} compounds function via the defined mitochondrial apoptosis pathway.

SAHB3_{BID}A and SAHB3_{BIDG $\rightarrow$ S}A demonstrate leukemic suppression in vivo

NOD-SCID mice were subjected to 300 cGy total body irradiation followed by intravenous injection of 4×10^6 SEMK2-M1 leukemia cells exhibiting stable luciferase expression. The mice were monitored weekly for leukemia engraftment using the In Vivo Imaging System (IVIS, Xenogen), which quantitates total body luminescence after intraperitoneal injection of D-luciferin. On day 0, the leukemic mice were imaged and then treated intravenously with 10 mg/kg of SAHB3_{BID}A, SAHB3_{BIDG $\rightarrow$ S}A, or no injection on days 1, 2, 3, 5, 6. Total body luminescence was measured on days 4 and 7. Referring to FIG. 26a, analysis of tumor burden among the groups demonstrates leukemic suppression by SAHB3_{BID}A

and SAHB3_{BID}(G→S)A compared to untreated control mice. Referring to FIG. 26b, total body luminescence images demonstrate more advanced leukemia in the untreated group by day 7 (red density, representing high level leukemia, is seen throughout the skeletal system) compared to the SAHB3_{BID}A-treated mice, which demonstrate lower level and more localized disease. Interestingly, the G→S mutant, which cannot be sequestered by BCL-2 appears to be more potent than the parent compound, SAHB3_{BID}A, in suppressing leukemic growth.

In further animal experiments, leukemic mice (generated as above) were imaged on day 0 and then treated intravenously with 10 mg/kg SAHB3_{BID}A, 5 mg/kg SAHB3_{BID}A, or vehicle control (5% DMSO in D5W) on days 1, 2, 3, 6, and 7. Total body luminescence was measured on days 4 and 8. Referring to FIG. 27a, analysis of tumor burden among the groups demonstrates leukemic suppression by SAHB3_{BID}A in a dose-dependent manner compared to untreated control mice. Referring to FIG. 27b, total body luminescence images demonstrate more advanced leukemia in the untreated group by day 8 (red density represents high level leukemia) compared to the SAHB3_{BID}A-treated mice, whose leukemic progression is noticeably blunted.

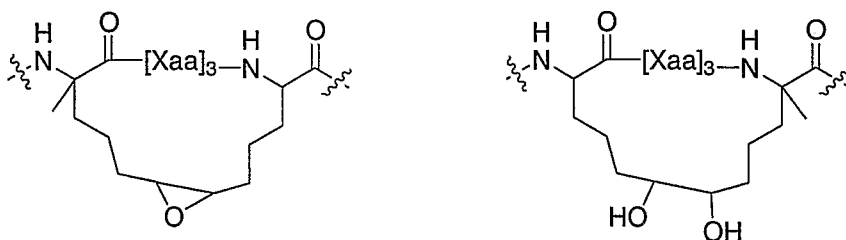
In additional animal experiments that instead employed SCID beige mice and RS4;11 leukemia cells, SAHB3_{BID}A treatment consistently suppressed leukemia growth *in vivo*. For *in vivo* leukemia imaging, mice were anesthetized with inhaled isoflurane (Abbott Laboratories) and treated concomitantly with intraperitoneal injection of D-luciferin (60 mg/kg) (Promega). Photonic emission was imaged (2 min exposure) using the In Vivo Imaging System (Xenogen) and total body bioluminescence quantified by integration of photonic flux (photons/sec) (Living Image Software, Xenogen). Starting on experimental day 1, mice received a daily tail vein injection of SAHB3_{BID}A (10 mg/kg) or vehicle (5% DMSO in D5W) for seven days. Mice were imaged on days 1, 3, and 5 and survival monitored daily for the duration of the experiment. The survival distributions of SAHB3_{BID}A and vehicle-treated mice were determined using the Kaplan-Meier method and compared using the log-rank test. The Fisher's Exact test was used to compare the proportion of mice who failed treatment between days 3 and 5, where treatment failure was defined as progression or death, and success as stable disease or regression. Expired mice were subjected to necropsy (Rodent Histopathology Core, DF/HCC).

Control mice demonstrated progressive acceleration of leukemic growth as quantitated by increased bioluminescent flux from days 1-5 (FIG. 27c). SAHB3_{BID}A treatment suppressed the leukemic expansion after day 3, with tumor regression observed by day 5. Representative mouse

images demonstrate the progressive leukemic infiltration of spleen and liver in mice, but regression of disease at these anatomical sites in SAHB_A-treated mice by day 5 of treatment (FIG 27d). The median time to death in this cohort was 5 days for control animals, whereas none of the SAHB_A-treated animals dies during the seven day treatment period, and instead survived for a median of 11 days (FIG 27e). Histologic examination of SAHB_A-treated mice showed no obvious toxicity of the compound to normal tissue. In an additional study comparing SAHB_{3BID}A- and SAHB_{3BID(G→E)}A-treated mice, animals receiving the point mutant SAHB did not exhibit tumor regression (FIG. 27f), highlighting the *in vivo* specificity of SAHB_{3BID}A's anti-leukemic activity.

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 (e.g., directing the compound to the thyroid when using an Iodine tag) 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.

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.

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.

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.

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*, 2d. Ed., John Wiley and Sons (1991); 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.

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 -NH₂ protected by either t-Boc or F-moc chemistry using side chain protected amino acids on, for example, an Applied Biosystems Peptide Synthesizer Model 430A or 431.

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 polychannel combinatorial synthesizer available from Advanced Chemtech.

Figures 28a-28f depict various peptides that include domains that are useful for creating cross-linked peptides.

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 aberrant (e.g., insufficient or excessive) BCL-2 family member expression or activity (e.g., extrinsic or intrinsic apoptotic pathway abnormalities). 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.

It is possible that some BCL-2 type disorders can be caused, at least in part, by an abnormal level of one or more BCL-2 family members (e.g., over or under expression), or by the presence of one or more BCL-2 family members exhibiting abnormal activity. As such, the reduction in the level and/or activity of the BCL-2 family member or the enhancement of the level and/or activity of the BCL-2 family member, which would bring about the amelioration of disorder symptoms.

The polypeptides of the invention 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.

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 breast cancer, ovarian cancer, colon cancer, lung cancer, metastasis of such cancers and the like. A metastatic tumor can arise from a multitude of primary tumor types, including but not limited to those of breast, lung, liver, colon and ovarian origin.

Examples of cancers or neoplastic conditions include, but are not limited to, a fibrosarcoma, myosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma,

angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, gastric cancer, esophageal cancer, rectal cancer, pancreatic cancer, ovarian cancer, prostate cancer, uterine cancer, cancer of the head and neck, skin cancer, brain cancer, squamous cell carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinoma, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilm's tumor, cervical cancer, testicular cancer, small cell lung carcinoma, non-small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma, retinoblastoma, leukemia, lymphoma, or Kaposi sarcoma.

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. Preferably, the diseases arise from poorly differentiated 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./Hematol. 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), 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.

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.

Examples of cellular proliferative and/or differentiative disorders of the lung include, but are not limited to, bronchogenic carcinoma, including paraneoplastic syndromes, bronchioloalveolar carcinoma, neuroendocrine tumors, such as bronchial carcinoid, miscellaneous tumors, and metastatic tumors; pathologies of the pleura, including inflammatory pleural effusions, noninflammatory pleural effusions, pneumothorax, and pleural tumors, including solitary fibrous tumors (pleural fibroma) and malignant mesothelioma.

Examples of cellular proliferative and/or differentiative disorders of the colon include, but are not limited to, non-neoplastic polyps, adenomas, familial syndromes, colorectal carcinogenesis, colorectal carcinoma, and carcinoid tumors.

Examples of cellular proliferative and/or differentiative disorders of the liver include, but are not limited to, nodular hyperplasias, adenomas, and malignant tumors, including primary carcinoma of the liver and metastatic tumors.

Examples of cellular proliferative and/or differentiative disorders of the ovary include, but are not limited to, ovarian tumors such as, tumors of coelomic epithelium, serous tumors, mucinous tumors, endometrioid tumors, clear cell adenocarcinoma, cystadenofibroma, brenner tumor, surface epithelial tumors; germ cell tumors such as mature (benign) teratomas, monodermal teratomas, immature malignant teratomas, dysgerminoma, endodermal sinus tumor, choriocarcinoma; sex cord-stomal tumors such as, granulosa-theca cell tumors, thecoma-fibromas, androblastomas, hill cell tumors, and gonadoblastoma; and metastatic tumors such as Krukenberg tumors.

The polypeptides described herein can also be used to treat, prevent or diagnose conditions characterised by overactive cell death or cellular death due to physiologic insult etc. Some examples of conditions characterized by premature or unwanted cell death are or alternatively unwanted or excessive cellular proliferation include, but are not limited to hypocellular/hypoplastic, acellular/aplastic, or hypercellular/hyperplastic conditions. Some

examples include hematologic disorders including but not limited to fanconi anemia, aplastic anemia, thalassemia, congenital neutropenia, myelodysplasia.

The polypeptides of the invention that act to decrease apoptosis can be used to treat disorders associated with an undesirable level of cell death. Thus, the anti-apoptotic peptides of the invention can be used to treat disorders such as those that lead to cell death associated with viral infection, e.g., infection associated with infection with human immunodeficiency virus (HIV). A wide variety of neurological diseases are characterized by the gradual loss of specific sets of neurons, and the anti-apoptotic peptides of the invention can be used in the treatment of these disorders. Such disorders include Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS) retinitis pigmentosa, spinal muscular atrophy, and various forms of cerebellar degeneration. The cell loss in these diseases does not induce an inflammatory response, and apoptosis appears to be the mechanism of cell death. In addition, a number of hematologic diseases are associated with a decreased production of blood cells. These disorders include anemia associated with chronic disease, aplastic anemia, chronic neutropenia, and the myelodysplastic syndromes. Disorders of blood cell production, such as myelodysplastic syndrome and some forms of aplastic anemia, are associated with increased apoptotic cell death within the bone marrow. These disorders could result from the activation of genes that promote apoptosis, acquired deficiencies in stromal cells or hematopoietic survival factors, or the direct effects of toxins and mediators of immune responses. Two common disorders associated with cell death are myocardial infarctions and stroke. In both disorders, cells within the central area of ischemia, which is produced in the event of acute loss of blood flow, appear to die rapidly as a result of necrosis. However, outside the central ischemic zone, cells die over a more protracted time period and morphologically appear to die by apoptosis. The anti-apoptotic peptides of the invention can be used to treat all such disorders associated with undesirable cell death.

Some examples of immunologic disorders that can be treated with the polypeptides described herein include but are not limited to organ transplant rejection, arthritis, lupus, IBD, crone's disease, asthma, multiple sclerosis, diabetes etc.

Some examples of neurologic disorders that can be treated with the polypeptides described herein include but are not limited to Alzheimer's Disease, Down's Syndrome, Dutch Type Hereditary Cerebral Hemorrhage Amyloidosis, Reactive Amyloidosis, Familial Amyloid Nephropathy with Urticaria and Deafness, Muckle-Wells Syndrome, Idiopathic Myeloma;

Macroglobulinemia-Associated Myeloma, Familial Amyloid Polyneuropathy, Familial Amyloid Cardiomyopathy, Isolated Cardiac Amyloid, Systemic Senile Amyloidosis, Adult Onset Diabetes, Insulinoma, Isolated Atrial Amyloid, Medullary Carcinoma of the Thyroid, Familial Amyloidosis, Hereditary Cerebral Hemorrhage With Amyloidosis, Familial Amyloidotic Polyneuropathy, Scrapie, Creutzfeldt-Jacob Disease, Gerstmann Straussler-Scheinker Syndrome, Bovine Spongiform Encephalitis, a Prion-mediated disease, and Huntington's Disease.

Some examples of endocrinologic disorders that can be treated with the polypeptides described herein include but are not limited to diabetes, hypothyroidism, hypopituitarism, hypoparathyroidism, hypogonadism, etc.

Examples of cardiovascular disorders (e.g., inflammatory disorders) that can be treated or prevented with the compounds and methods of the invention include, but are not limited to, atherosclerosis, myocardial infarction, stroke, thrombosis, aneurism, heart failure, ischemic heart disease, angina pectoris, sudden cardiac death, hypertensive heart disease; non-coronary vessel disease, such as arteriolosclerosis, small vessel disease, nephropathy, hypertriglyceridemia, hypercholesterolemia, hyperlipidemia, xanthomatosis, asthma, hypertension, emphysema and chronic pulmonary disease; or a cardiovascular condition associated with interventional procedures ("procedural vascular trauma"), such as restenosis following angioplasty, placement of a shunt, stent, synthetic or natural excision grafts, indwelling catheter, valve or other implantable devices. Preferred cardiovascular disorders include atherosclerosis, myocardial infarction, aneurism, and stroke.

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.

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.

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 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.

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.

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.

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.

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, including BCL-2 family member mediated disorders or symptoms thereof.

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.

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.

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.

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.

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.

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.

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.

Screening Assays

The invention provides methods (also referred to herein as "screening assays") for identifying polypeptides which modulate the activity of one or more BCL-2 family proteins or which bind to one or more BCL-2 family proteins (e.g., a polypeptide having at least one BH homology domain).

The binding affinity of polypeptides described herein can be determined using, for example, a titration binding assay. A BCL-2 family polypeptide or polypeptide comprising a BH domain (e.g., BID, BAK, BAX, etc.) can be exposed to varying concentrations of a candidate compound (i.e., polypeptide) (e.g., 1 nM, 10 nM, 100 nM, 1 μ M, 10 μ M, 100 μ M, 1 mM, and 10 mM) in the presence of a substrate such as a fluorescently labeled BH3 containing polypeptide or a fragment thereof (e.g., BID, BAD, BAK, BAX, etc.). The effect of each concentration of candidate compound is then analyzed to determine the effect of the candidate compound on BCL-2 family binding activity at varying concentrations, which can be used to calculate the K_i of the candidate compound. The candidate compound can modulate BCL-2 type activity in a competitive or non-competitive manner. Direct binding assays can also be performed between BCL-2 family proteins and fluorescently labeled candidate compounds to determine the K_d for the binding interaction. Candidate compounds could also be screened for biological activity in vitro, for example, by measuring their dose-responsive efficacy in triggering cytochrome c from purified mitochondria. Cell permeability screening assays are also envisioned, 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.

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.

In one embodiment, an assay is a cell-based assay in which a cell that expresses a BCL-2 family protein or biologically active portion thereof is contacted with a candidate polypeptide, and the ability of the test compound to modulate BCL-2 type activity is determined (e.g., in some instances increase in apoptosis and in other instances decrease apoptosis, via intrinsic or extrinsic cell death pathways). Determining the ability of the test compound to modulate BCL-2 type activity within cells can be accomplished by monitoring, for example, release of cytochrome c from the mitochondria or other relevant physiologic readout (e.g., annexin V staining, MTT assay, caspase activity assay, TUNEL assay).

In one embodiment, an assay is a biochemical assay, whereby crosslinked polypeptides can be linked to affinity resin in order to purify or identify new or known interactive partners in the apoptotic pathway.

Other applications

Biologically relevant applications for the peptides described herein are numerous and readily apparent, as indicated by the following cell compartment-based examples:

- (1) Cell surface – Natural peptides representing key helical regions of the HIV-1 protein gp41 (eg. C-peptide, T-20 peptide) have been shown to prevent viral fusion, and therefore, HIV infectivity. Helical peptides participate in fusion mechanisms essential to many virus-host cell infection paradigms (eg. Dengue, Hepatitis C, Influenza), and therefore, hydrocarbon-stapled analogues of these critical helical regions may function as effective antibiotics by inhibiting viral fusion. In general, ligands that interact with cell surface receptors, using helical interfaces to activate or inhibit signaling pathways, represent additional applications for the polypeptides described herein.
- (2) Intramembrane – Receptor dimerization and oligomerization are cardinal features of ligand-induced receptor activation and signaling. Transmembrane helical domains widely participate in such essential oligomerization reactions (eg. Epidermal Growth Factor Receptor [EGFR] family), and specific peptide sequences have been defined that facilitate these tight intramembrane helical associations. Aberrant activation of such receptors through

oligomerization are implicated in disease pathogenesis (eg. erbB and cancer). Therefore, in the appropriate setting, activation or inhibition of transmembrane inter-helical interactions would have therapeutic benefit.

(3) Cytosolic – Cytosolic targets include soluble protein targets and those associated with specific intracytosolic organelles, including the mitochondria, endoplasmic reticulum, Golgi network, lysosome, and peroxisome. Within the field of apoptosis, there are multiple cytosolic and mitochondrial apoptotic protein targets for hydrocarbon-stapled BCL-2 family domains. Within the BH3-only subgroup of pro-apoptotic proteins, two major subsets of BH3 domains have been identified: (1) BID-like BH3s (e.g., BIM) which are apoptosis “activators,” inducing BAK oligomerization and cytochrome c release at the mitochondrion and (2) BAD-like BH3s which are apoptosis “sensitizers” that selectively target anti-apoptotic multidomain proteins, enabling subliminal levels of activating domains to be maximally effective. In addition to distinct binding of BH3-only proteins to pro- vs. anti-apoptotic multidomain family members, BH3 domains display differential binding among anti-apoptotic proteins. For example, it has been demonstrated that BAD preferentially binds to the anti-apoptotic BCL-2, whereas BIM targets the anti-apoptotic MCL-1. Identifying and exploring these selective interactions are critically important because different BCL-2 family members are implicated in different types of cancer. For example, BCL-2 overexpression is responsible for the development of follicular lymphoma and chemotherapy resistance in general, whereas MCL-1 is believed to play an important role in the pathogenesis of multiple myeloma. The ability to transform the many BH3 domains into structurally stable and cell permeable reagents would provide an important opportunity to explore and differentially manipulate apoptotic pathways in cancer cells. Targeting further helix-dependent interactions in the cytosol or at cytosolic organelles is envisioned.

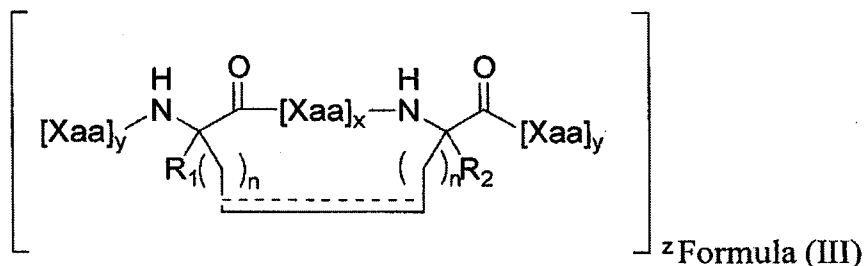
(4) Nuclear – Nuclear transcription factors and their modulatory proteins drive a host of physiologic processes based upon peptide helical interactions with nuclear proteins and nucleic acids. The feasibility of generating hydrocarbon-stapled peptides to engage in nuclear interactions has recently been demonstrated by our synthesis of a panel of hydrocarbon-stapled p53 peptides, which interact with MDM2 at picomolar affinities. In addition to modulating protein-protein interactions within the nucleus, protein-nucleic acid interactions are also apparent targets. Multiple transcription factor families, such as homeodomain, basic helix-loop-helix,

nuclear receptor, and zinc finger-containing proteins, directly interact with DNA via their peptide helices to activate or inhibit gene transcription. As an example, homeodomain proteins are a family of essential transcription factors that regulate genetic programs of growth and differentiation in all multicellular organisms. These proteins share a conserved DNA-binding motif, called the homeodomain, which contains a 60 amino acid long peptide that forms three α -helices, the third of which makes direct contact with the major groove of DNA. Like the BH3 domain of apoptotic proteins, the homeodomain is a critical effector motif with sufficient variation among homologs to facilitate differential binding specificities and physiologic activities. Protein-DNA interactions can be complex and extensive, and thereby present a challenge to small molecule development for the purpose of studying and selectively modulating transcriptional events. In higher organisms, homeodomain proteins are highly expressed during development, specifying the body plan and dictating tissue differentiation. Overexpression of specific homeoproteins (eg. CDX4) can activate tissue-specific differentiation programs resulting in, for example, blood formation from mouse embryonic stem cells. Deregulation of homeotic gene expression, such as aberrant upregulation of homeodomain proteins typically expressed in undifferentiated cells or inappropriate downregulation of such proteins normally expressed in differentiated cells, can contribute to the development and maintenance of cancer. For example, in pediatric alveolar rhabdomyosarcoma, fusion of the PAX3 or PAX 7 DNA binding domain to the transactivating domain of forkhead has been implicated in cellular transformation; translocations involving the DNA-binding domains of several HOX genes have been linked to the pathogenesis of leukemia. Thus, the ability to chemically-stabilize transcription factor helices, such as homeodomain peptides, for cellular delivery has the potential to yield a chemical toolbox for the investigation and modulation of diverse transcription programs responsible for a multitude of biological process in health and disease.

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.

WHAT IS CLAIMED IS:

1. A pro-apoptotic α -helix containing polypeptide comprising a BH3 domain, wherein said polypeptide is of Formula (III):



wherein;

each R_1 and R_2 is independently H, C_1 - C_{20} alkyl, C_2 - C_{20} alkenyl, C_2 - C_{20} alkynyl, arylalkyl, cycloalkylalkyl, heteroarylalkyl, or heterocyclalkyl;

each n is independently an integer from 1-15;

x is 2, 3 or 6;

each y is independently an integer from 3-15;

z is an integer from 1-10; and

each Xaa is independently an alpha amino acid and is the same amino acid as in an α -helix containing pro-apoptotic polypeptide.

2. The polypeptide of claim 1, wherein $-(CH_2)_n - (CH_2)_n-$ is C_8 alkyl or C_{11} alkyl.

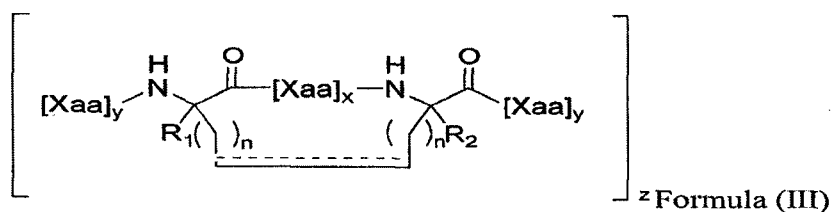
3. The polypeptide of claim 1, wherein $-(CH_2)_n - (CH_2)_n-$ is C_8 alkenyl or C_{11} alkenyl.

4. The polypeptide of claim 1, wherein R_1 and R_2 are H or C_{1-6} alkyl.

5. The polypeptide of claim 1, wherein x is 3 or 6 and z is 1.

6. The polypeptide of claim 1, wherein R_1 and R_2 are each independently C_1 - C_3 alkyl.

7. The polypeptide of claim 6, wherein at least one of R₁ and R₂ are methyl.
8. The polypeptide of claim 1, wherein x is 3 or 6 and R₁ and R₂ are methyl.
9. The polypeptide of claim 1, the polypeptide comprising an amino acid sequence which is at least about 60% identical to the amino acid sequence of SEQ ID NO:1, or comprising an amino acid sequence which is at least about 80% identical to the amino acid sequence of SEQ ID NO:2.
10. The polypeptide of claim 9, wherein at least one of R₁ and R₂ is C₁₋₆ alkyl.
11. The polypeptide of claim 1, wherein each R₁ and R₂ is independently H or C₁₋₃ alkyl.
12. The polypeptide of claim 1, further comprising a fluorescent moiety, a radioisotope, an affinity label, a targeting moiety or a biotin moiety.
13. The polypeptide of claim 1, wherein the polypeptide is a polypeptide selected from the polypeptides of SEQ ID NOs: 92-108, 110, 111 or 118-123.
14. An anti-apoptotic α -helix containing polypeptide comprising a BH3 domain, wherein said polypeptide is of Formula (III):



wherein;

each R₁ and R₂ is independently H, C₁-C₂₀ alkyl, C₂-C₂₀ alkenyl, C₂-C₂₀ alkynyl, arylalkyl, cycloalkylalkyl, heteroarylalkyl, or heterocyclalkyl;

each n is independently an integer from 1-15;

x is 2, 3 or 6;

each y is independently an integer from 3-15;
 z is an integer from 1-10; and
 each Xaa is independently an alpha amino acid and is the same amino acid as in an α -helix containing anti-apoptotic polypeptide.

15. The polypeptide of claim 14, wherein $-(CH_2)_n \text{ --- } (CH_2)_n-$ is C₈ alkyl or C₁₁ alkyl.
16. The polypeptide of claim 14, wherein $-(CH_2)_n \text{ --- } (CH_2)_n-$ is C₈ alkenyl or C₁₁ alkenyl.
17. The polypeptide of claim 14 wherein R₁ and R₂ are H or C₁₋₆ alkyl.
18. The polypeptide of claim 14, wherein x is 3 or 6 and z is 1.
19. The polypeptide of claim 14, wherein R₁ and R₂ are each independently C₁-C₃ alkyl.
20. The polypeptide of claim 19, wherein at least one R₁ and R₂ are methyl.
21. The polypeptide of claim 14, wherein x is 3 or 6 and R₁ and R₂ are methyl.
22. The polypeptide of claim 14, the polypeptide comprising an amino acid sequence which is at least about 60% identical to the amino acid sequence of SEQ ID NO:1, or comprising an amino acid sequence which is at least about 80% identical to the amino acid sequence SEQ ID NO:2.
23. The polypeptide of claim 22, wherein at least one of R₁ and R₂ is C₁₋₆ alkyl.
24. The polypeptide of claim 14, wherein each R₁ and R₂ is independently H or C₁-C₃ alkyl.
25. The polypeptide of claim 14, further comprising a fluorescent moiety, a radioisotope, an affinity label, a targeting moiety or a biotin moiety.

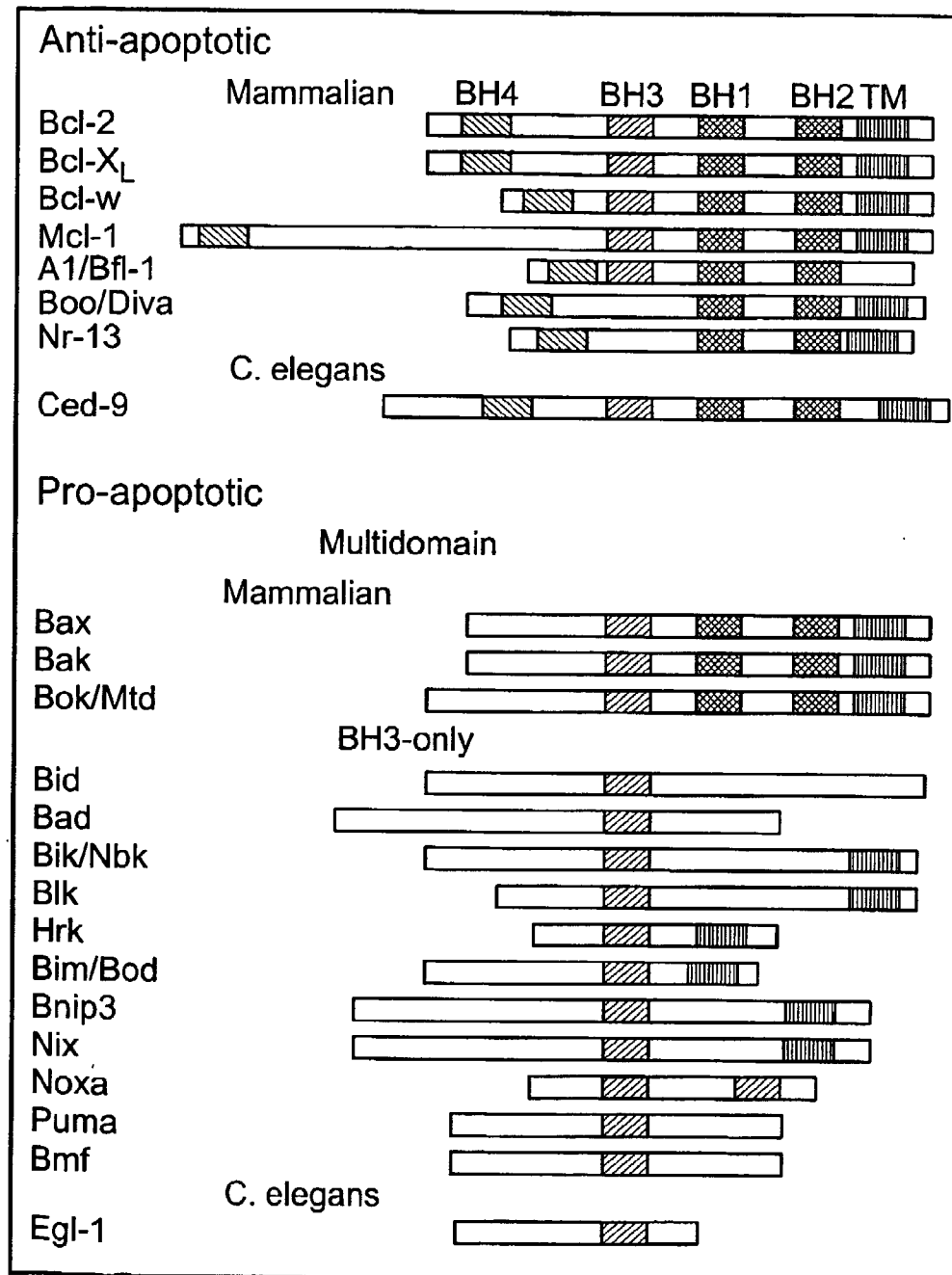


FIG. 1

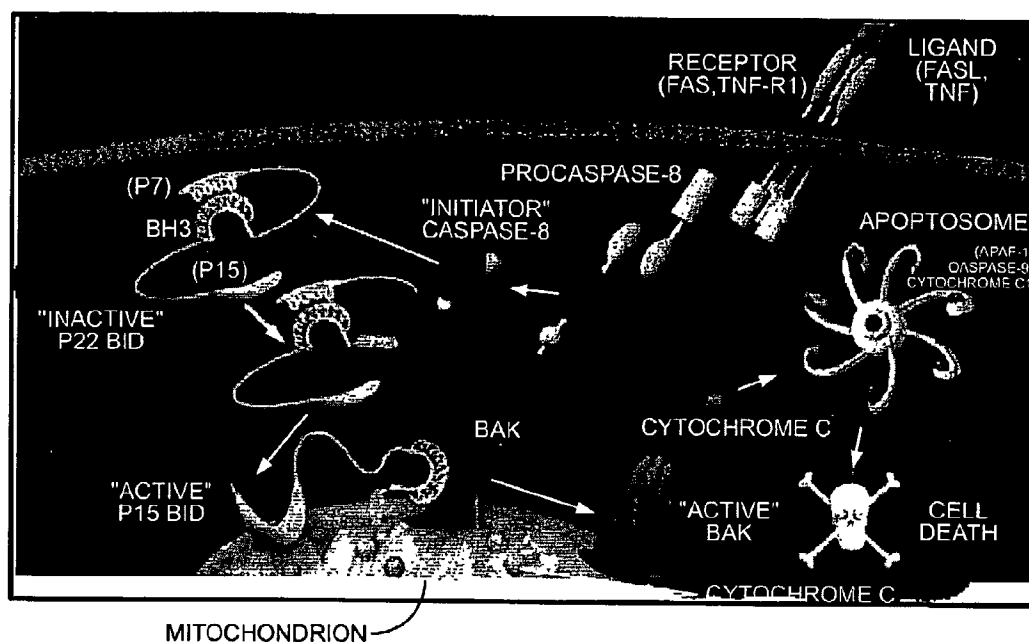


FIG. 2

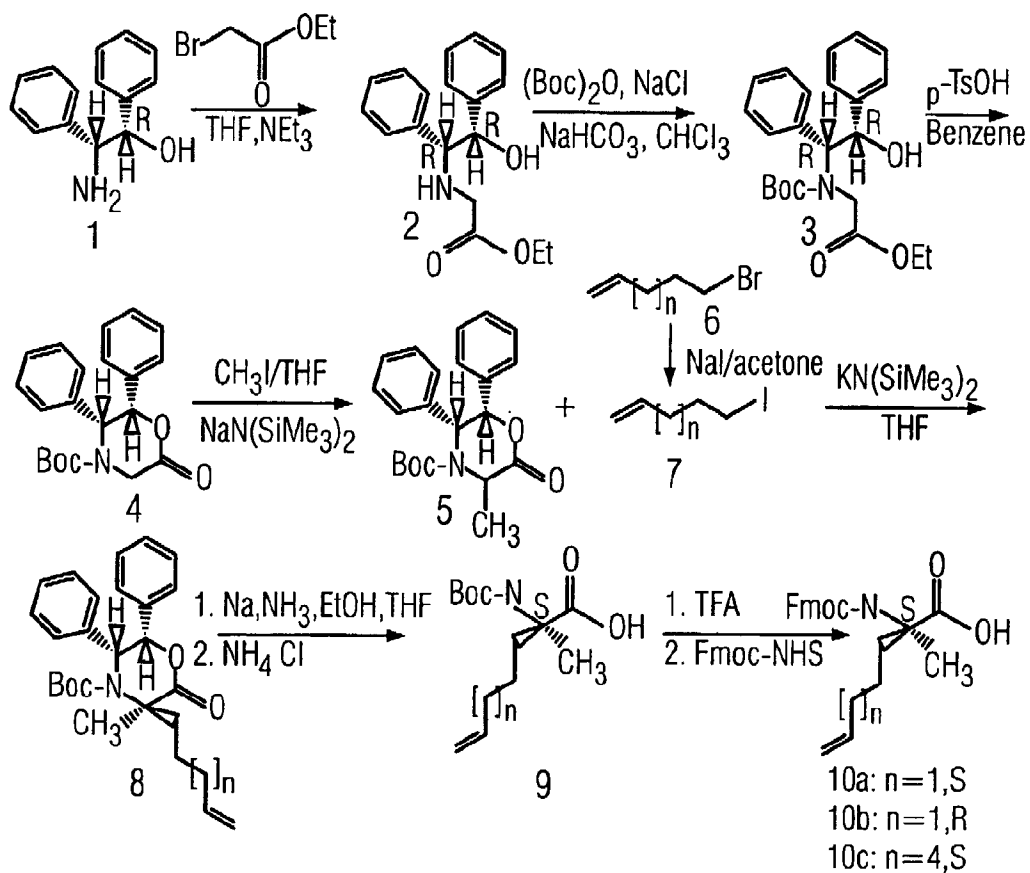


FIG. 3

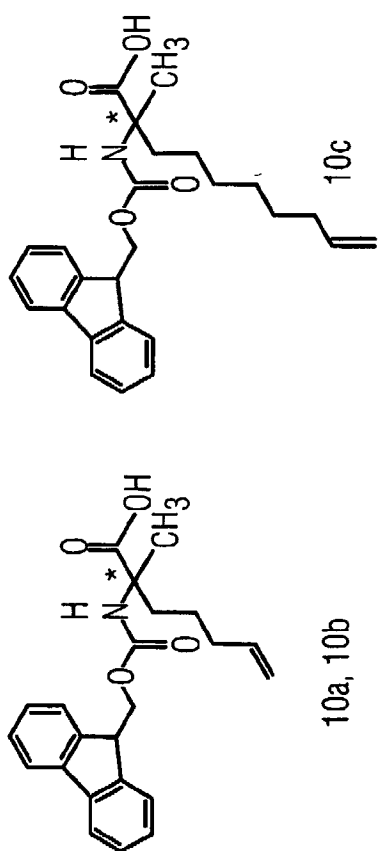


FIG. 4A

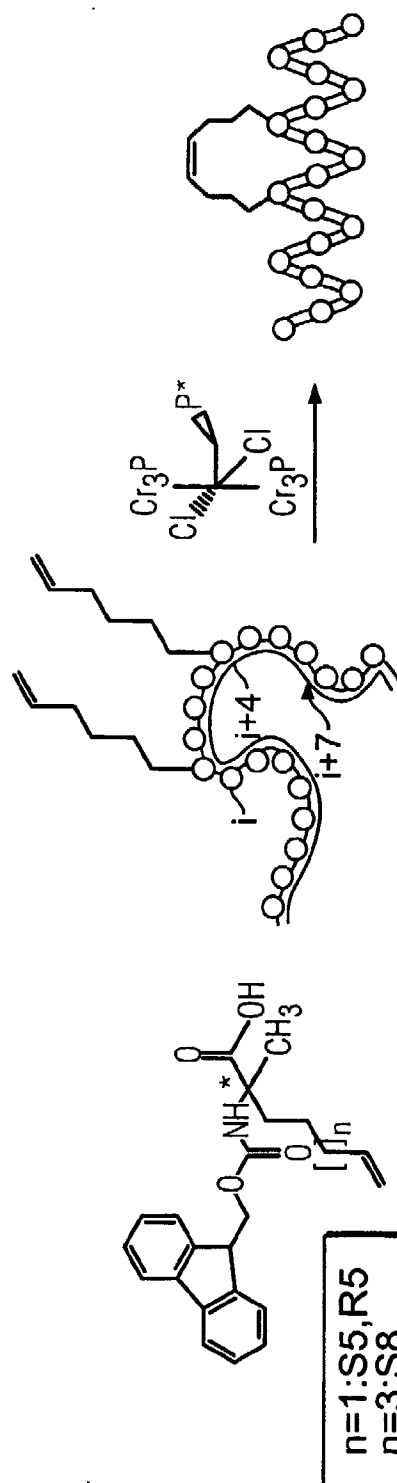


FIG. 4B

<u>Compound</u>	<u>Sequence</u>	<u>HRMS</u>
SAHB3BIDA	EDIIRNIARHLA*VGD*N _L DRSIW	2752.8
SAHB3BID(-E,W)A	DIIRNIARHLA*VGD*N _L DRSI	2436.6
SAHB3BID(G→E)A	EDIIRNIARHLA*VED*N _L DRSIW	2824.8
SAHB3BID(-E,W,G→E)A	DIIRNIARHLA*VED*N _L DRSI	2507
SAHB3BID(G→S)A	DIIRNIARHLA*VSD*N _L DRSIW	2654.7
FITC-SAHB3BIDA	FITC-Ap-EDIIRNIARHLA*VGD*N _L DRSIW	3169.6
FITC-SAHB3BID(G→E)A	FITC-Ap-EDIIRNIARHLA*VED*N _L DRSIW	3242.8 * = 10a
Biot-SAHB3BIDA	K(Biot)-DIIRNIARHLA*VGD*N _L DRSIW	3108.9 # = 10b
Biot-SAHB3BID(G→E)A	K(Biot)-DIIRNIARHLA*VED*N _L DRSIW	3179.1 ^ = 10c
SAHB3BIDB	EDIIRNI*RHL*QVGDSN _L DRSIW	2825.7
SAHB3BID(-E,W)B	DIIRNI*RHL*QVGDSN _L DRSI	2511.6
SAHB3BID(tr)B	RNI*RHL*QVGDSN _L DRW	2155.8
SAHB3BID(G→E)B	EDIIRNI*RHL*QVEDSN _L DRSIW	2710.8
Biot-SAHB3BIDB	K(Biot)-DIIRNI*RHL*QVGDSN _L DRSIW	3179.73
Biot-SAHB3BID(G→E)B	K(Biot)-DIIRNI*RHL*QVEDSN _L DRSI	3066.7
SAHB3BIDC	EDIIRNIA*HLA*VGDSN _L DRSIW	2683.5
SAHB3BIDD	EDIIRNIAR#LAQVGD^N _L DRSIW	2785.8
SAHB3BIDE	EDII#NIA#HLA*VGD*N _L DRSIW	2691.8
SAHB3BADA	NLWAAQRYGRELR*N _L SD*FVDSFKK	3090.8
FITC-SAHB3BID(-EW)A	FITC-A _β DIIRNIARHAA*VGA*N _L DRSI	2855.4
FITC-SAHB3BID(-EW,G→E)A	FITC-A _β DIIRNIARHAA*VEA*N _L DRSI	2927.5
SAHB3BID(-EW,L→A,D→A)A	DIIRNIARHAA*VGA*N _L DRSI	2352.4
SAHB3BIMA	IWIAQELR*IGD*FNAYYARR	2645.4
FITC-SAHB3BIMA	FITC-A _β IWIAQELR*IGD*FNAYYARR	3063.5
FITC-SAHB3BADA	FITC-A _β NLWAAQRYGRELR*N _L SD*FVDSFKK	3507.7

FIG. 5A

Compound	Sequence
BID BH3	EDIIRNIARHLAQVGSN _L DRSIW
SAHB _A	EDIIRNIARHLA*VGD*N _L DRSIW
SAHB _A (G→E)	EDIIRNIARHLA*VED*N _L DRSIW
SAHB _B	EDIIRNI*RHL*QVGSN _L DRSIW
SAHB _C	EDIIRNIA*HLA*VGSN _L DRSIW
SAHB _D	EDIIRNIAR⊗LAQVGD*N _L DRSIW

*=S5, ⊗=R5, ⚡=S8

FIG. 5B

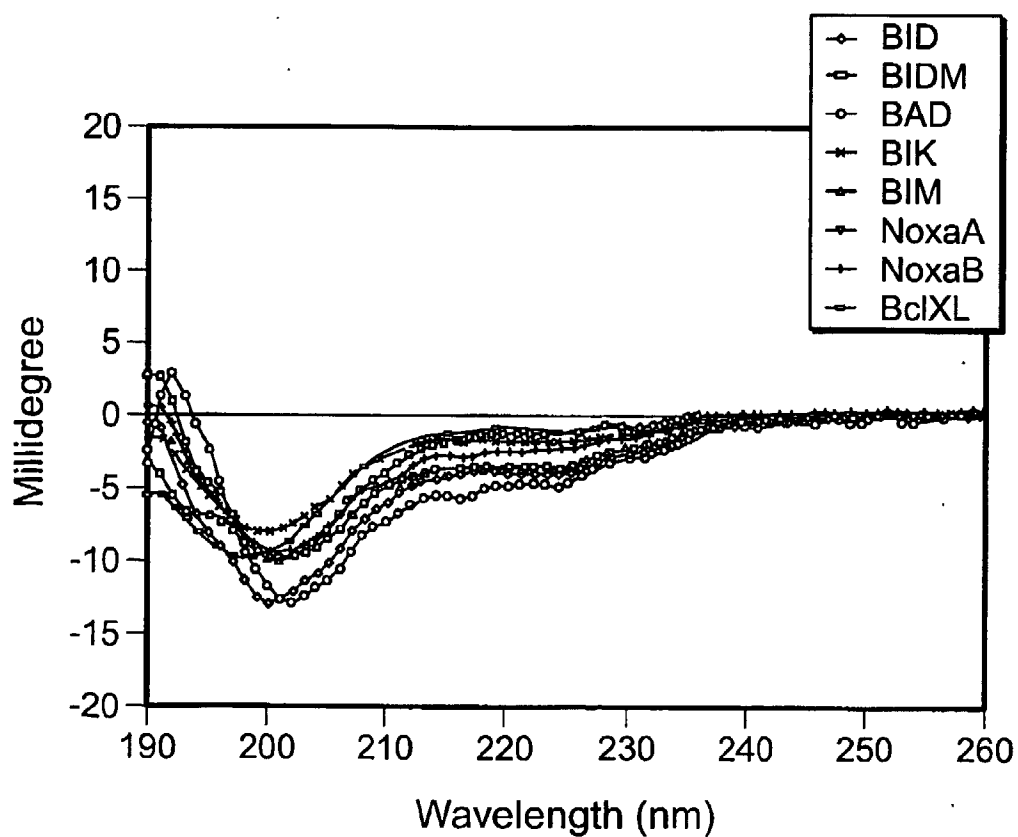


FIG. 6A

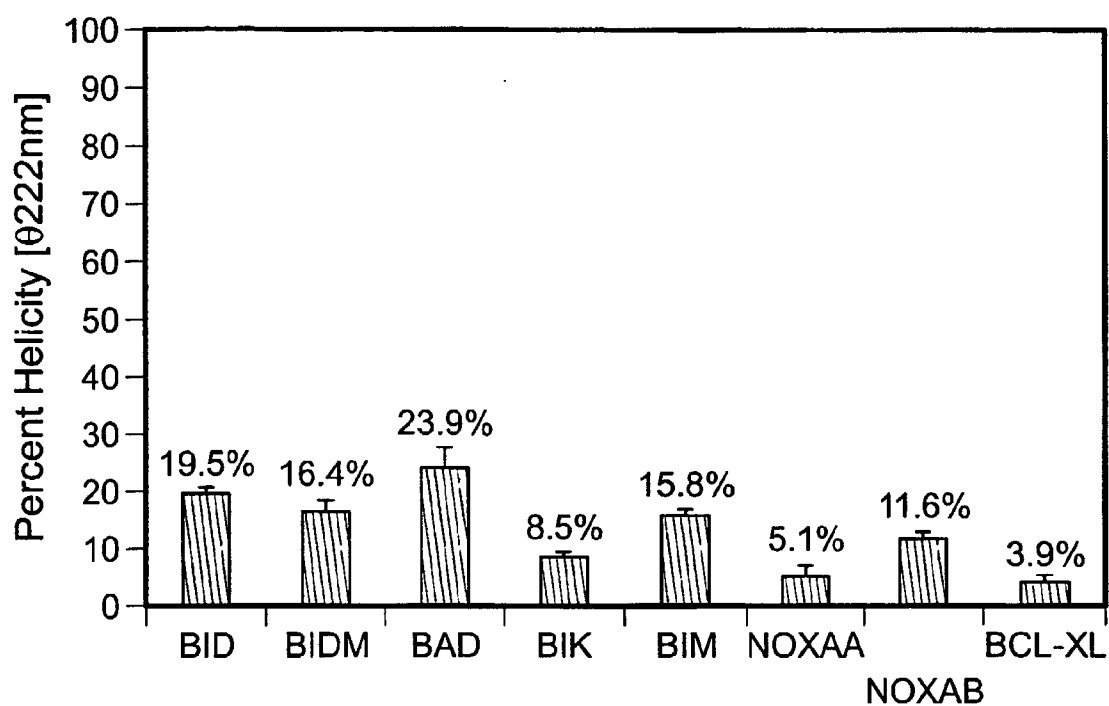


FIG. 6B

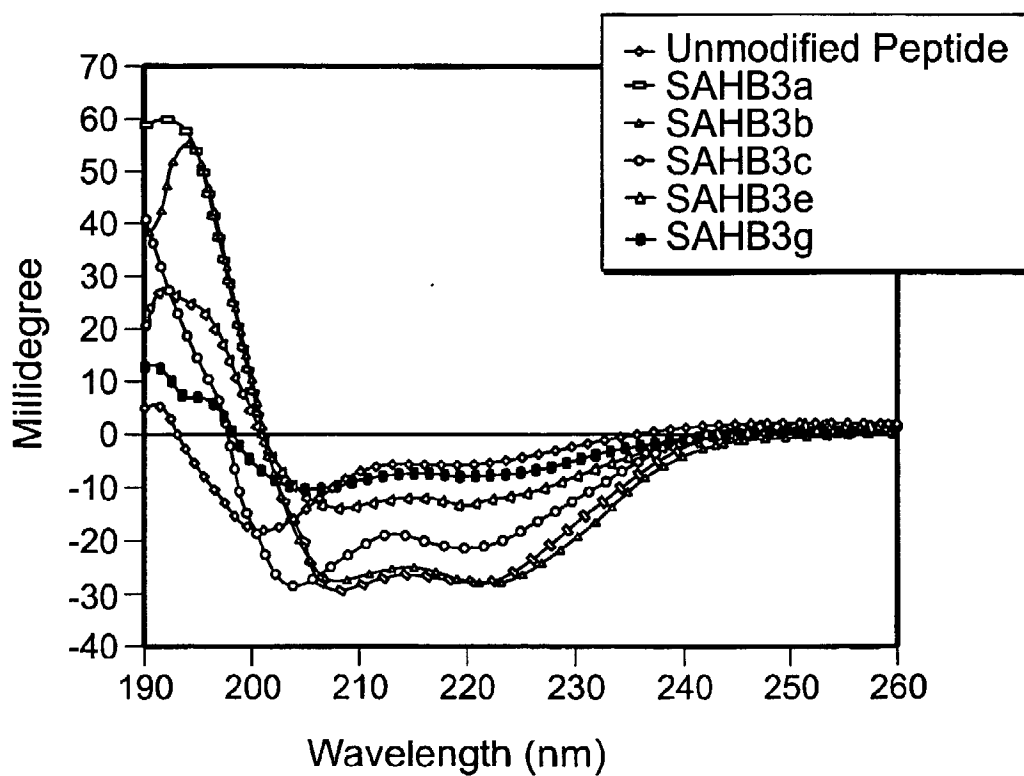


FIG. 7A

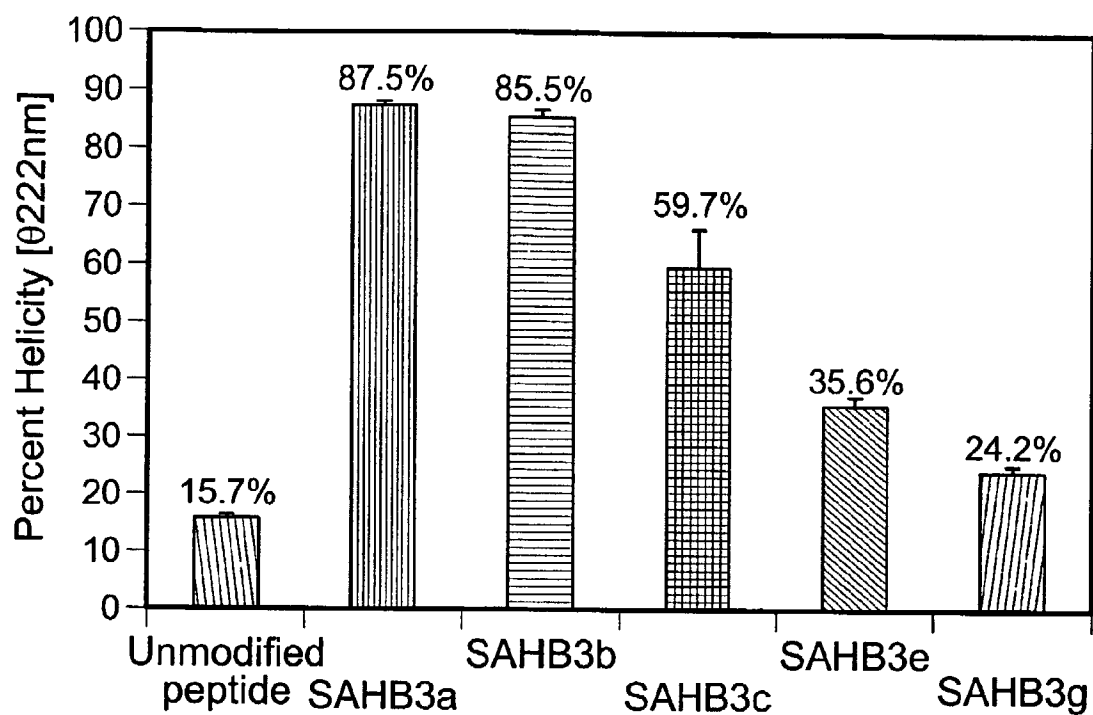


FIG. 7B

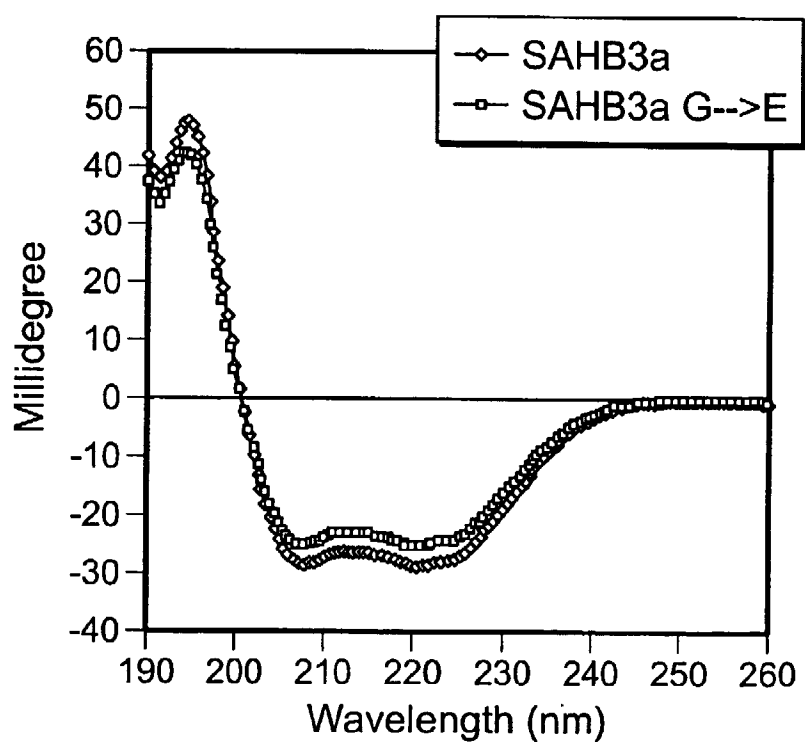


FIG. 8A

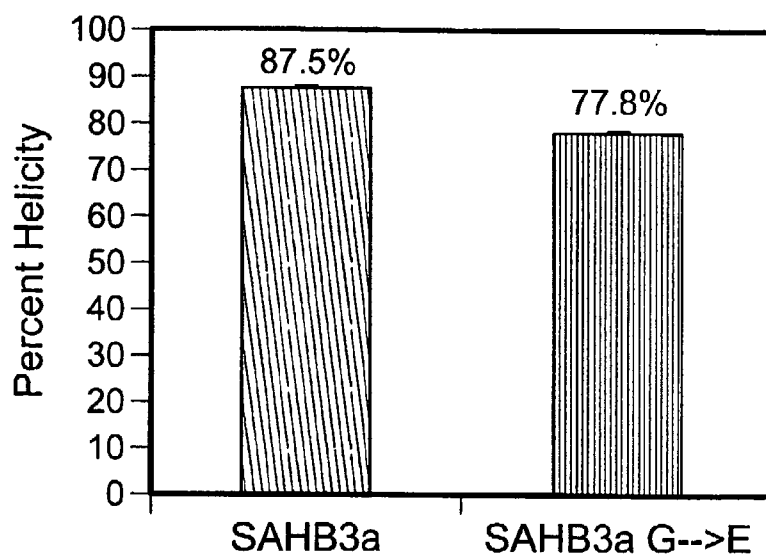


FIG. 8B

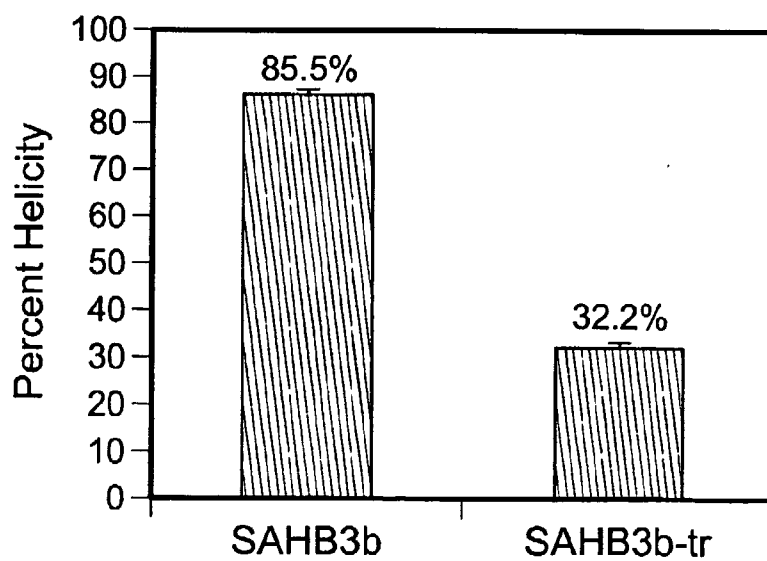


FIG. 9

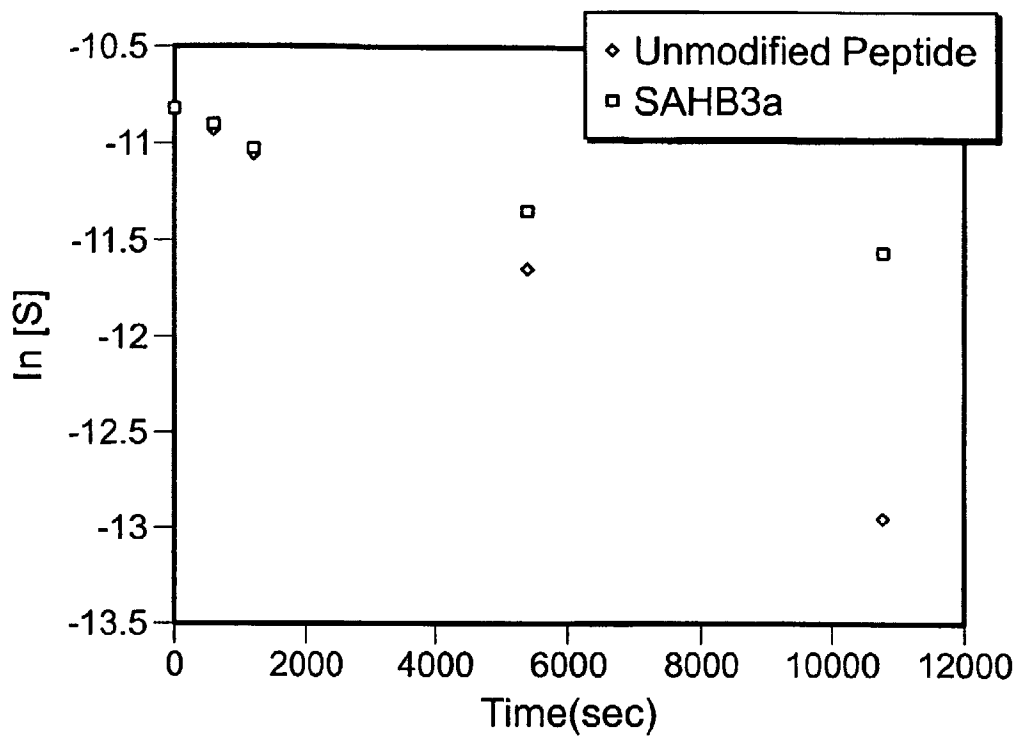


FIG. 10A

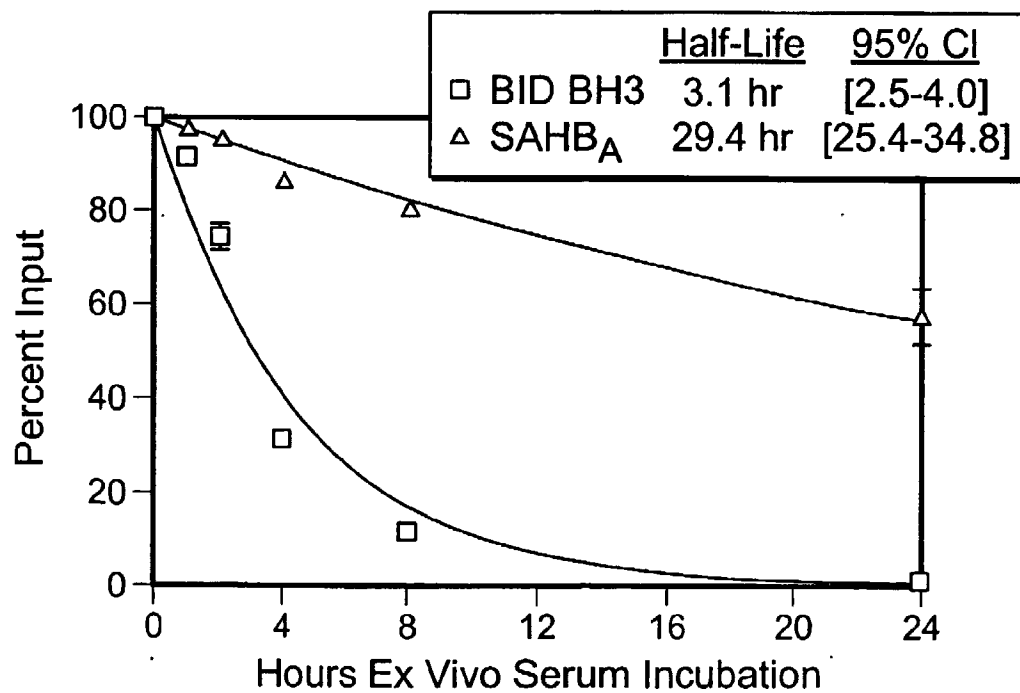


FIG. 10B

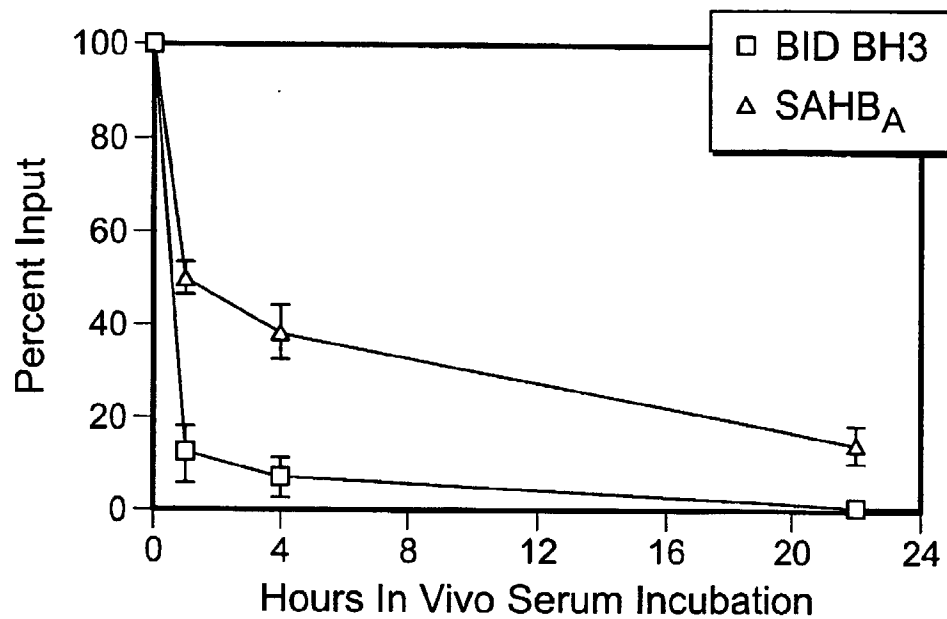


FIG. 10C

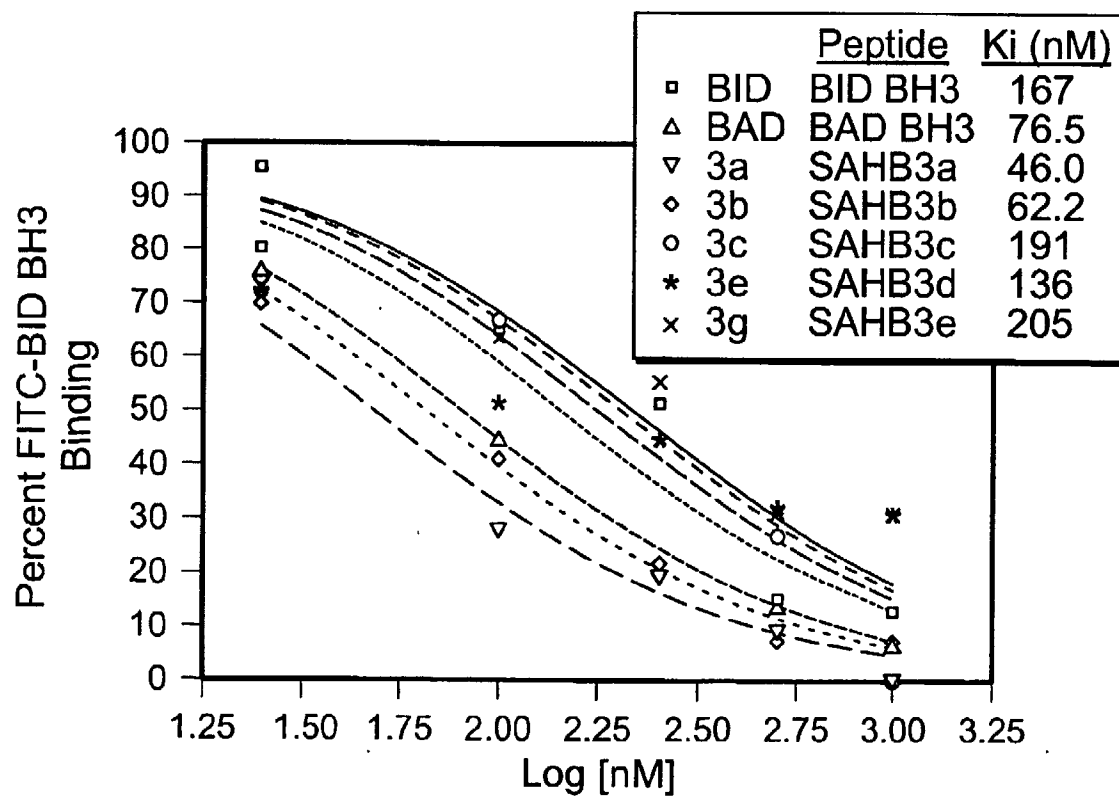


FIG. 11A

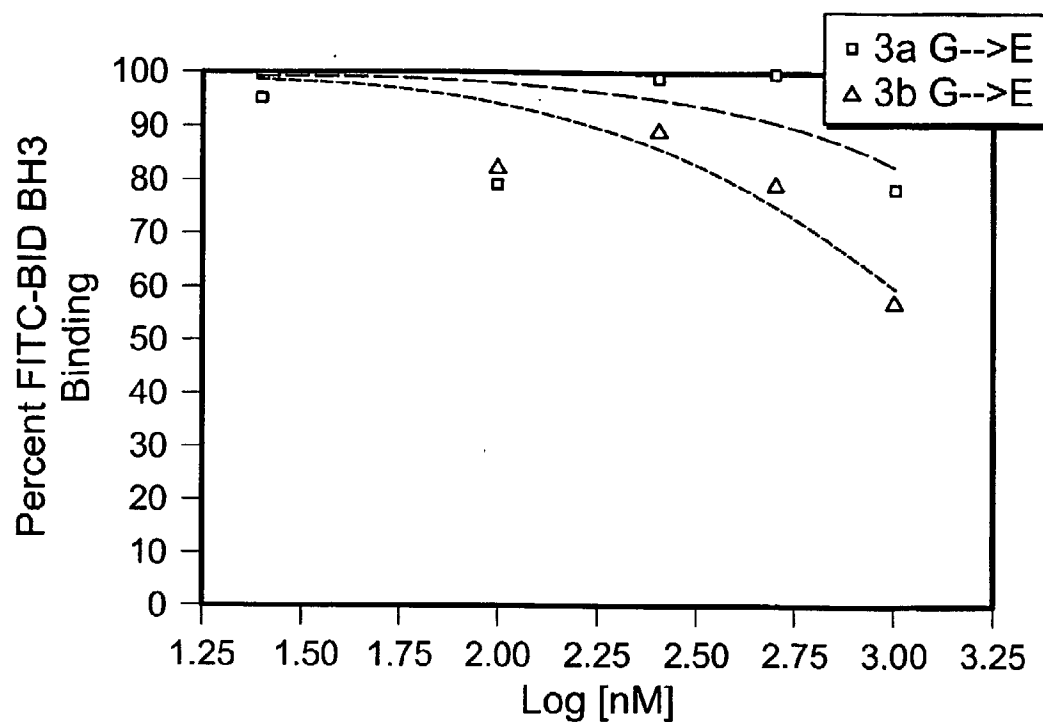


FIG. 11B

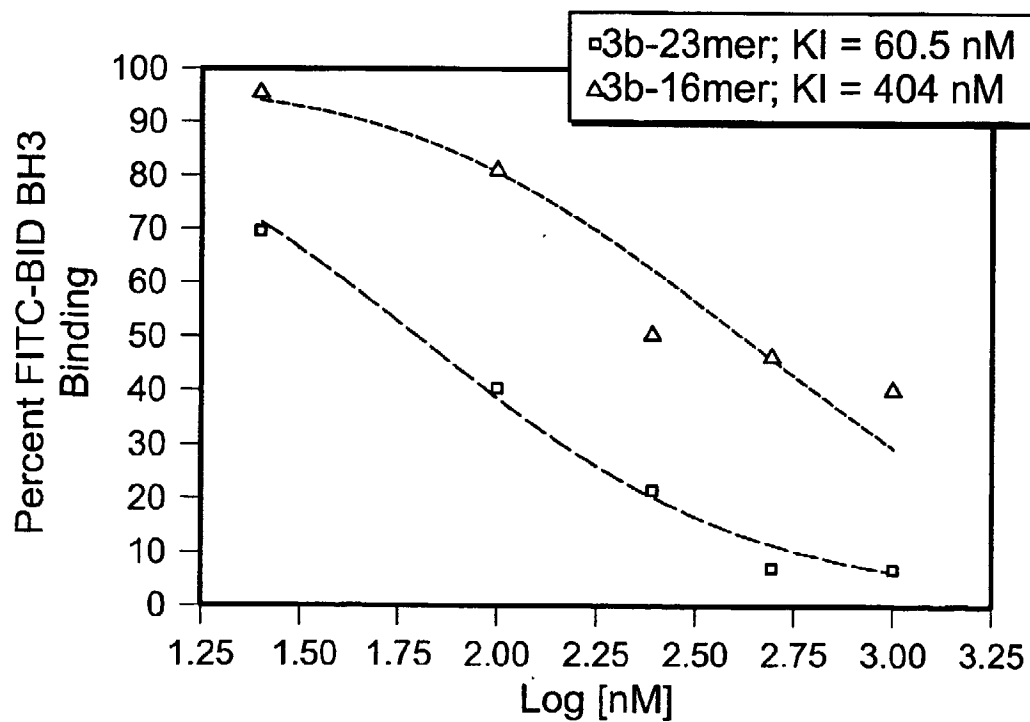


FIG. 11C

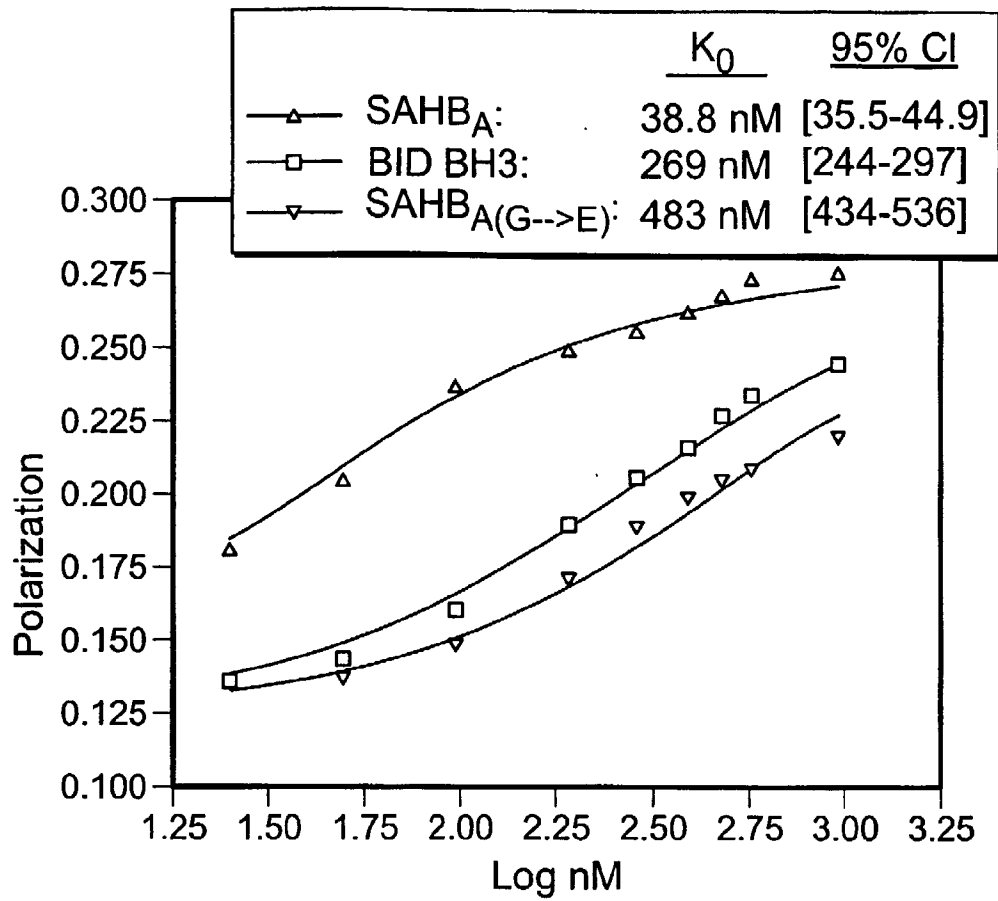


FIG. 11D

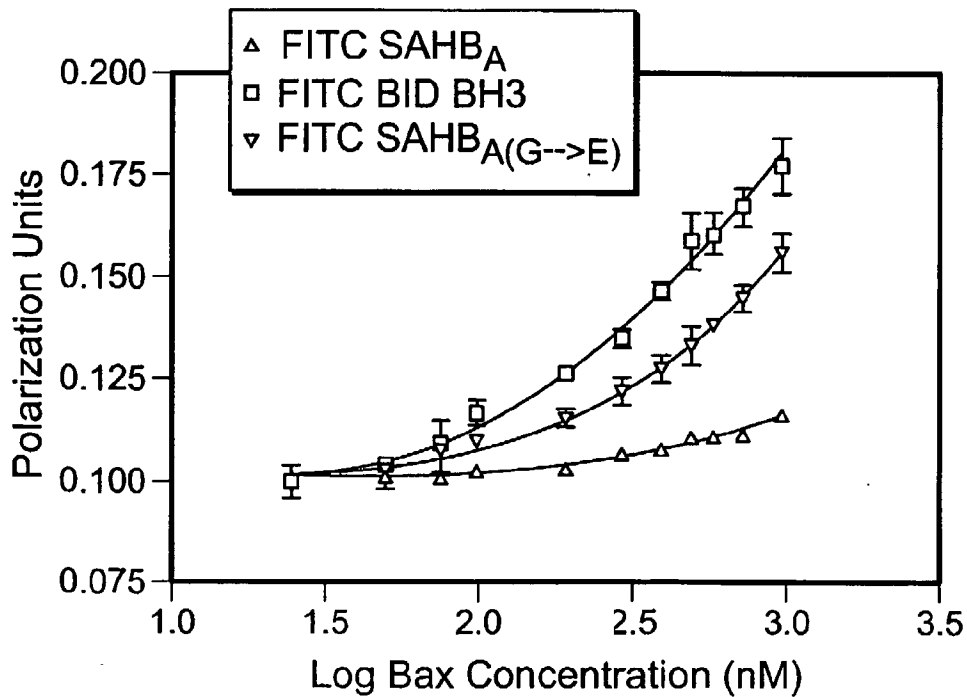


FIG. 11E

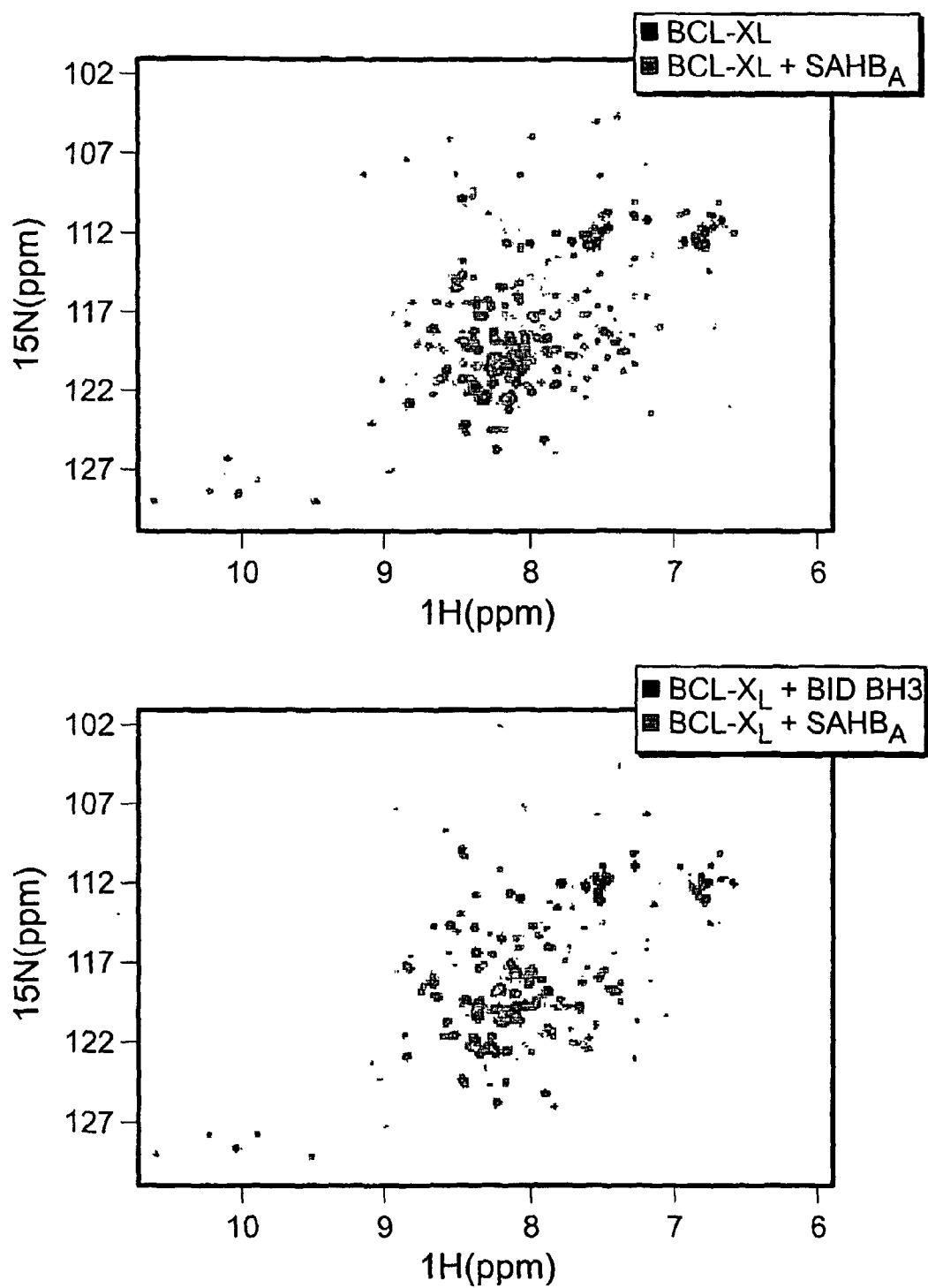


FIG. 11F

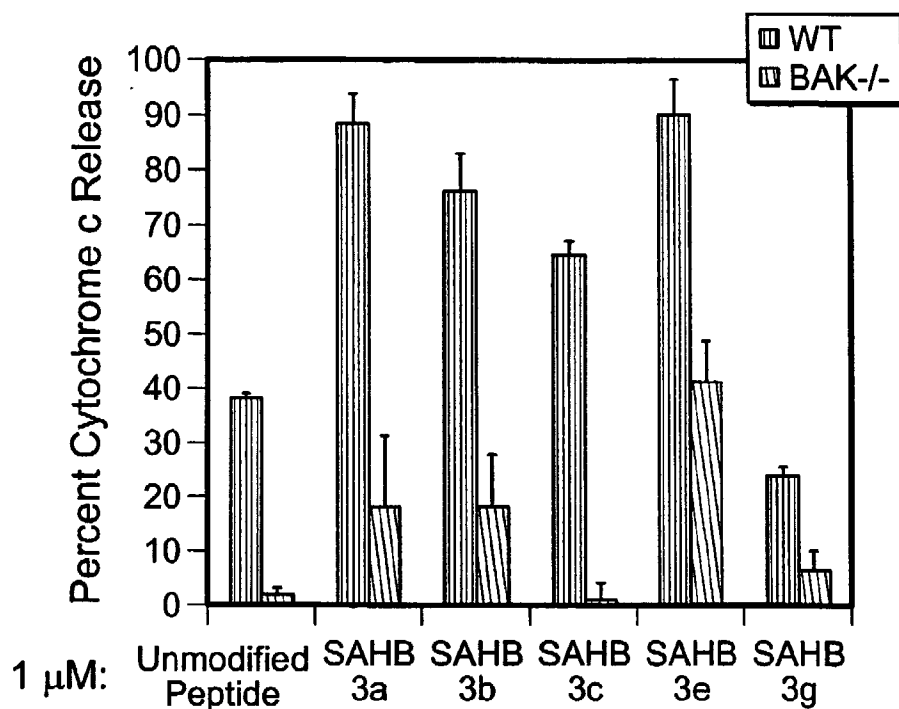


FIG. 12A

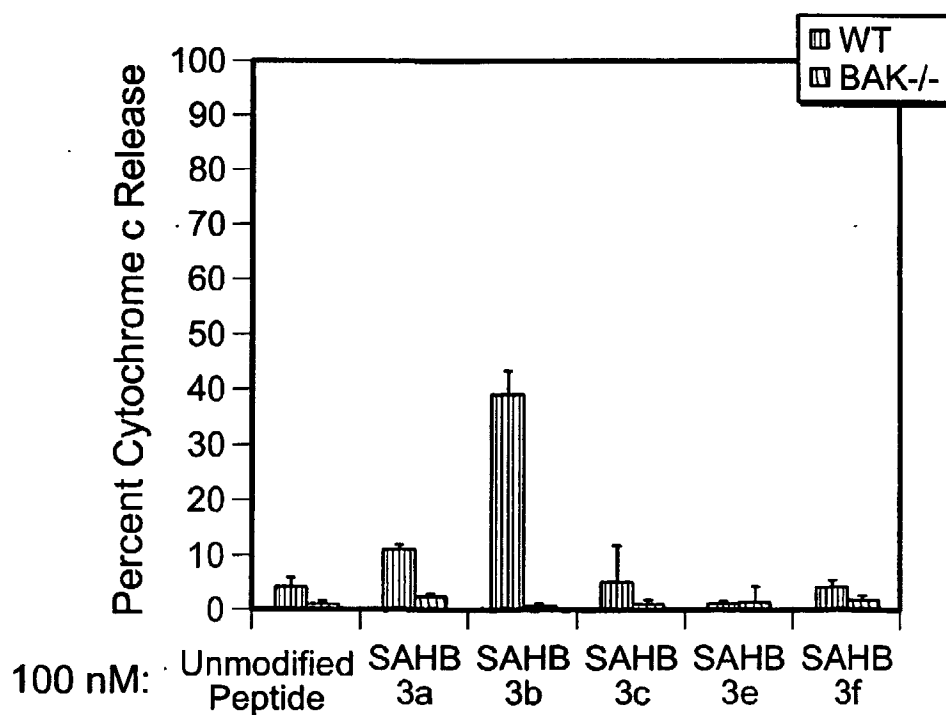


FIG. 12B

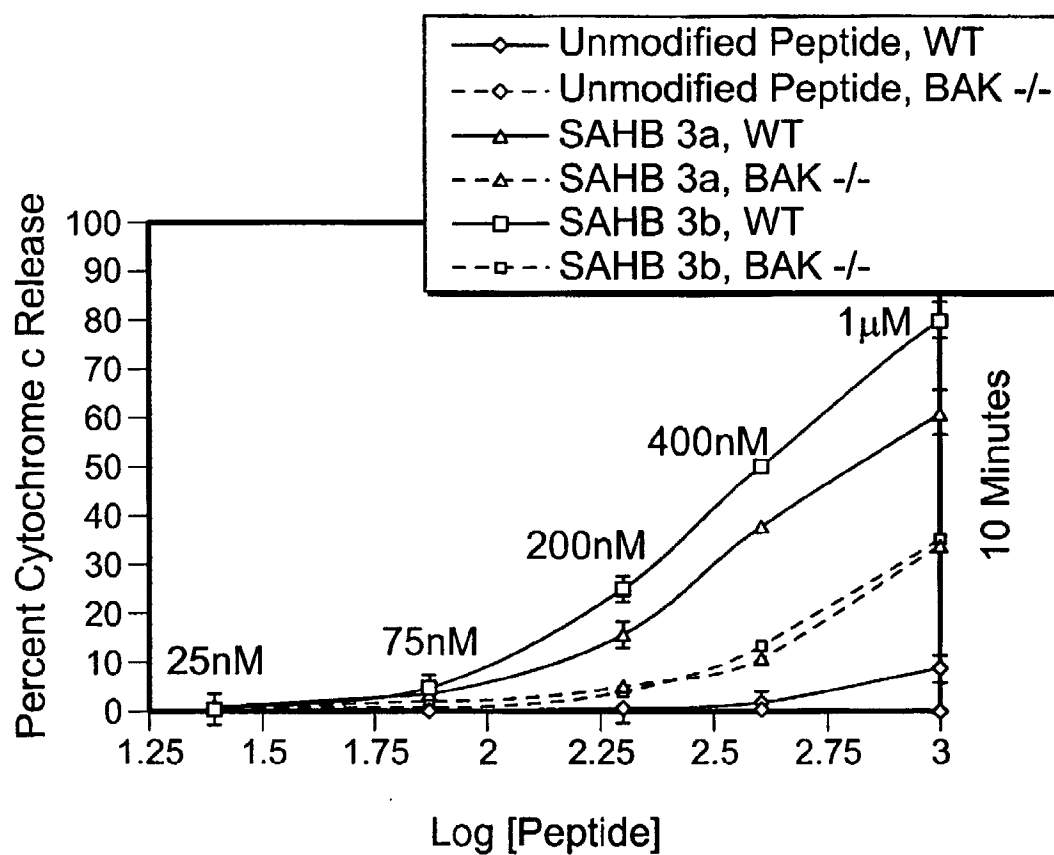


FIG. 13A

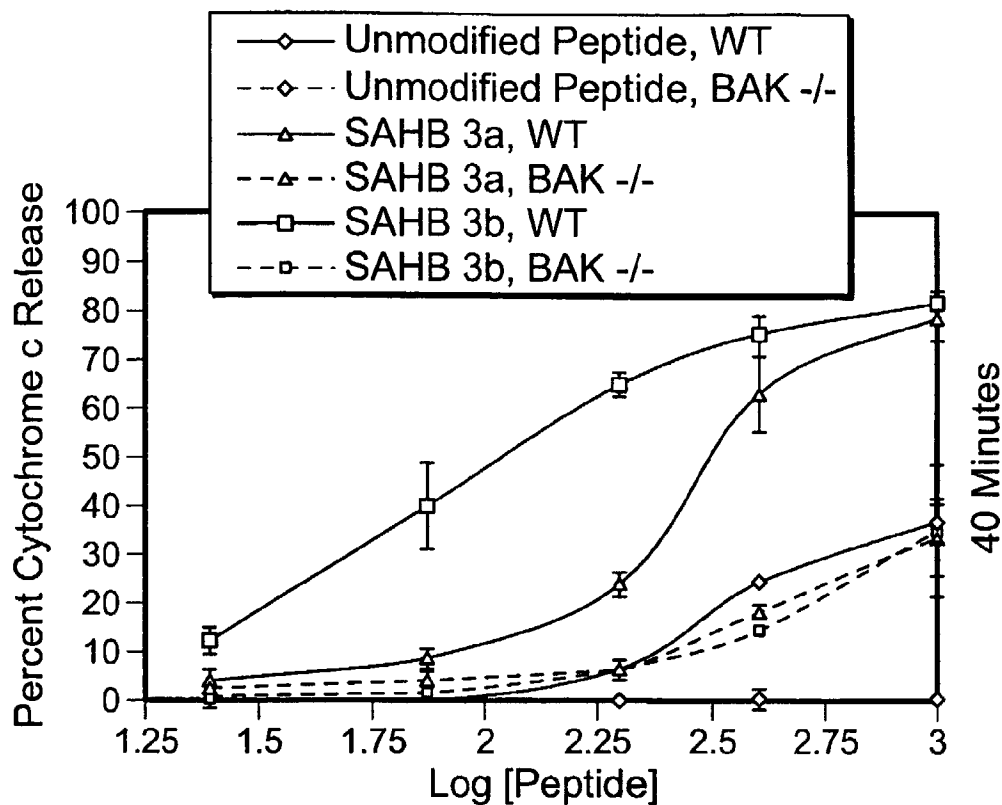


FIG. 13B

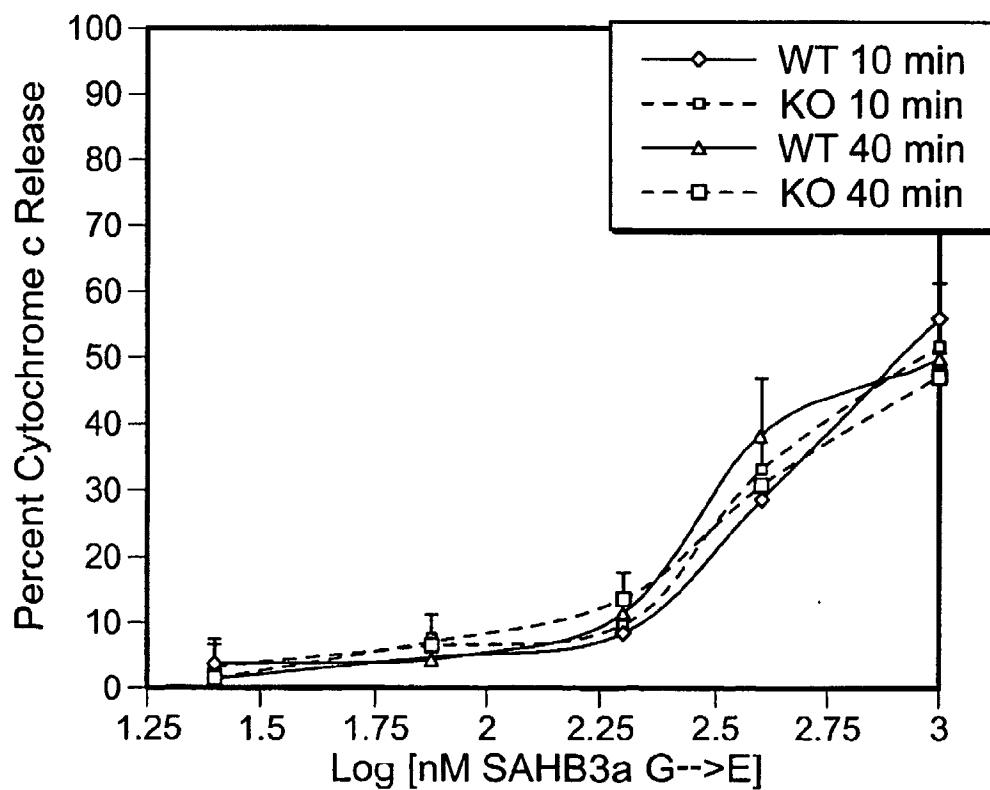


FIG. 14

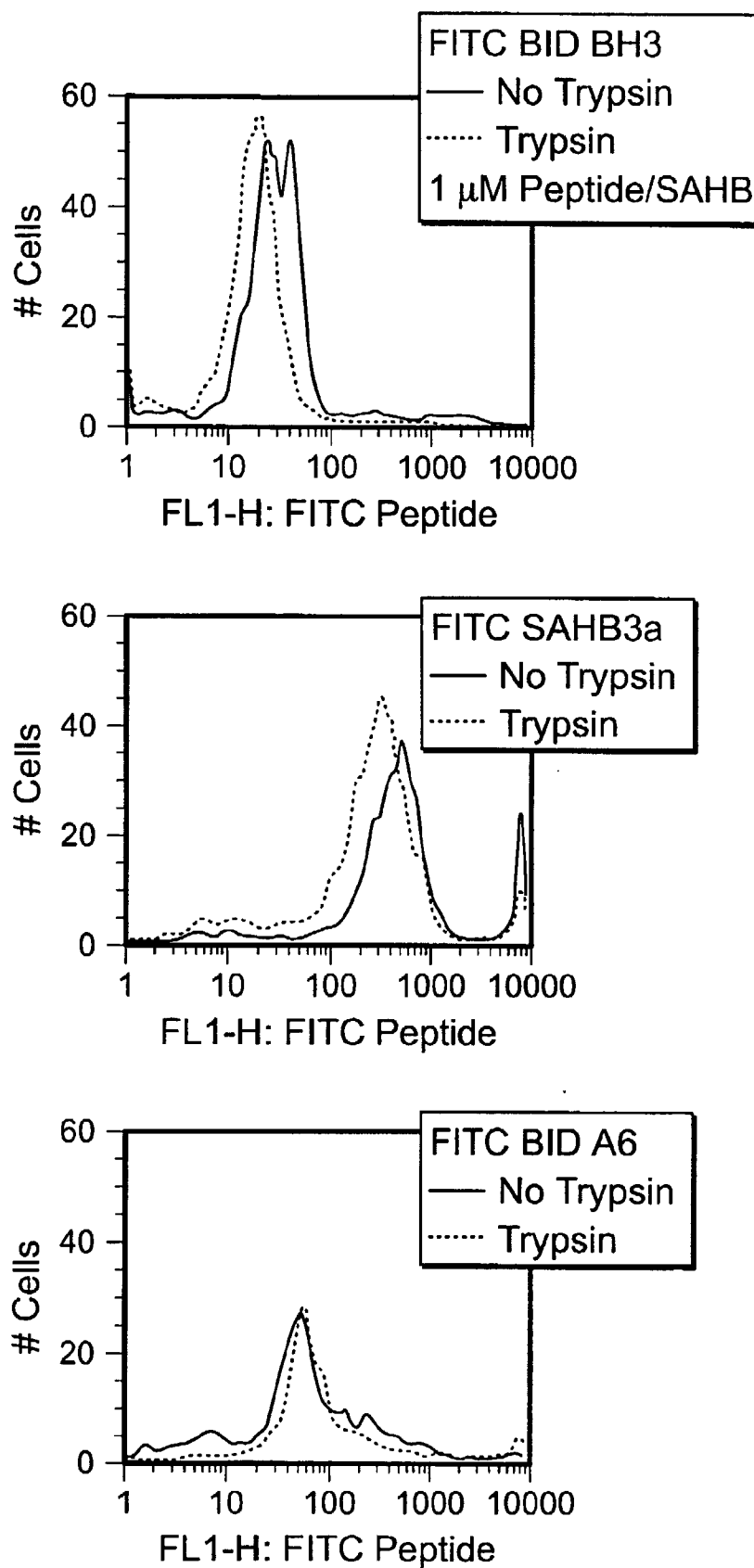


FIG. 15

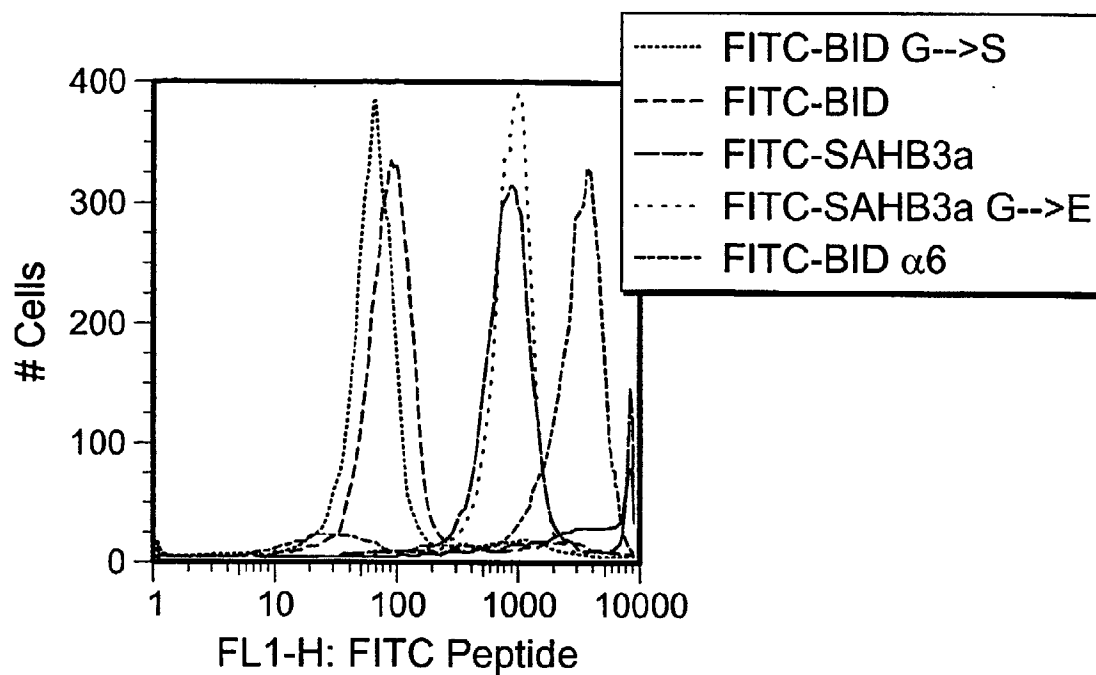


FIG. 16A

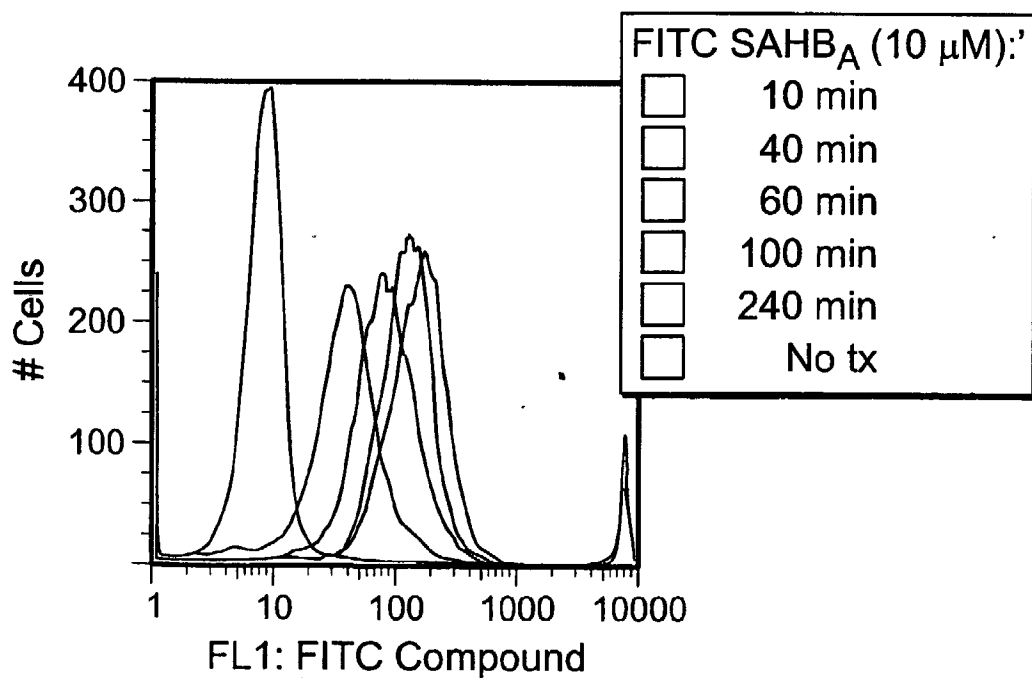


FIG. 16B

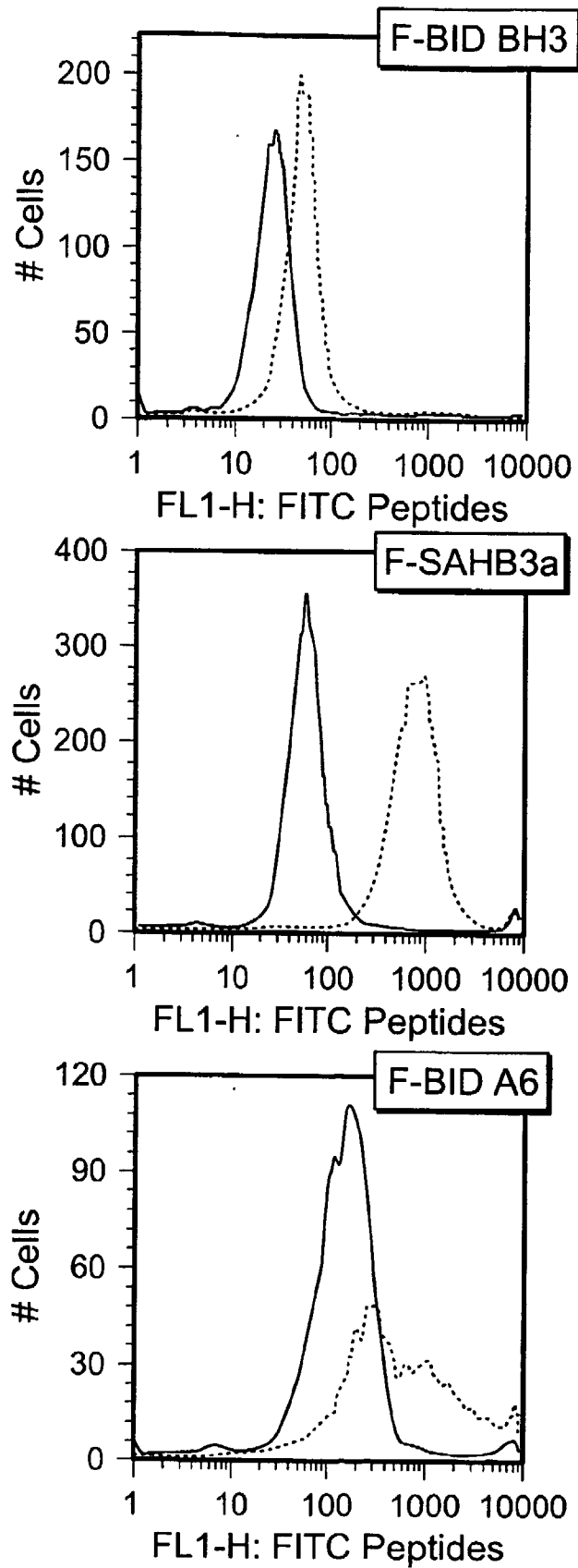


FIG. 17A

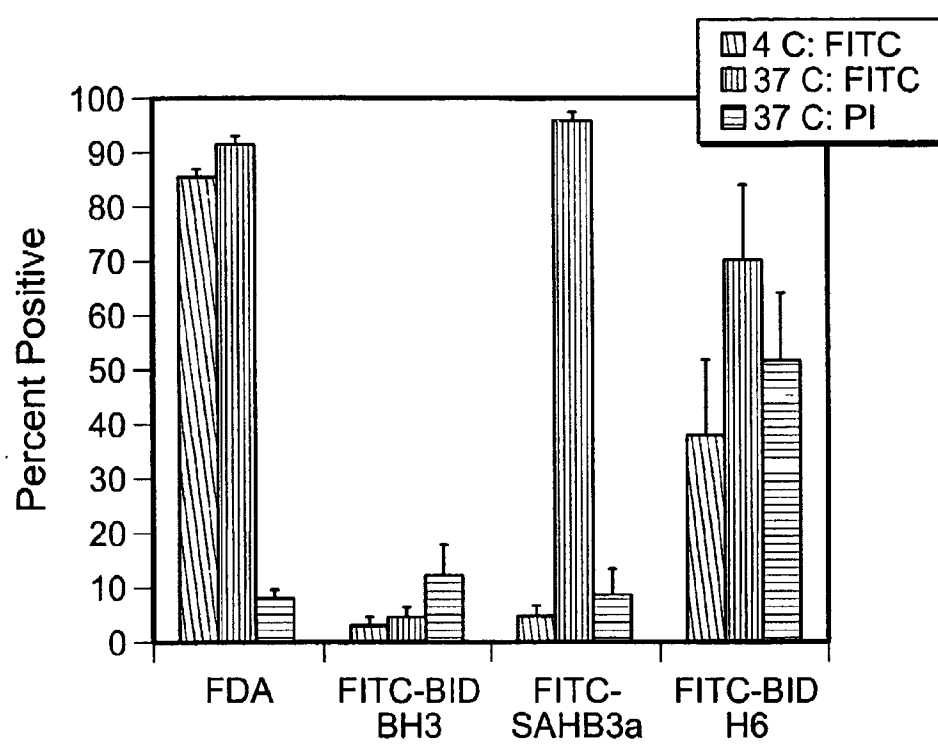


FIG. 17B

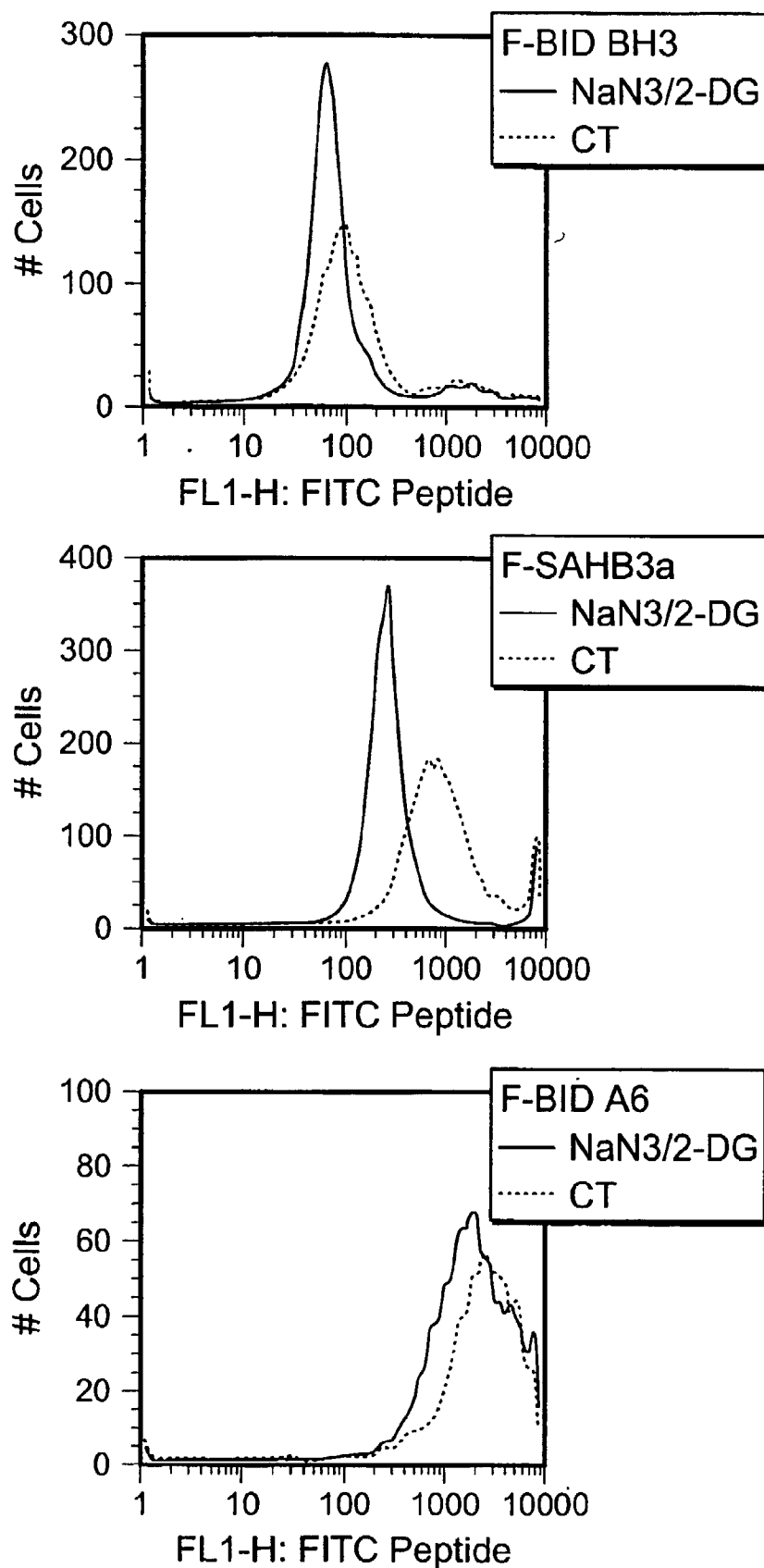


FIG. 17C

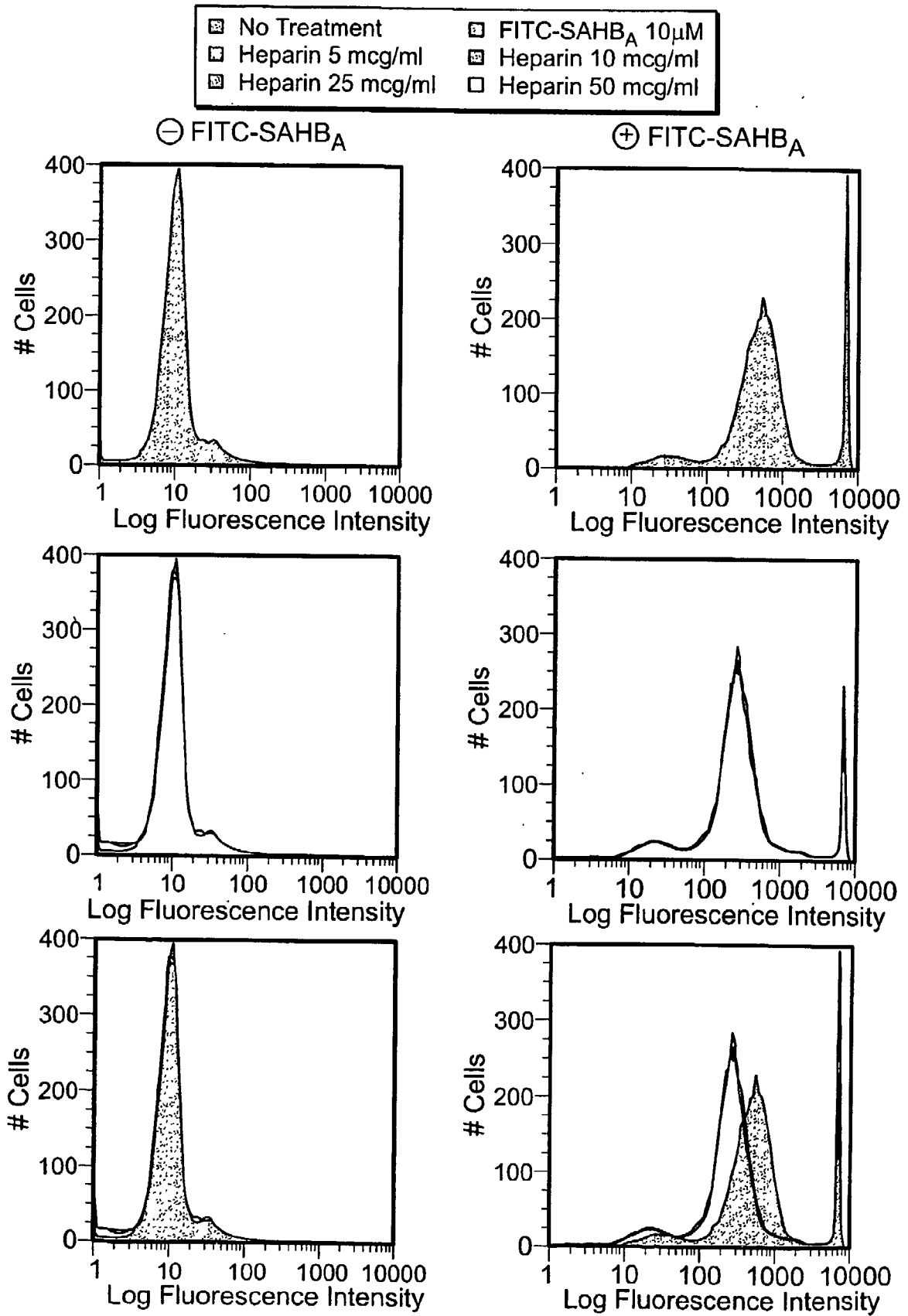
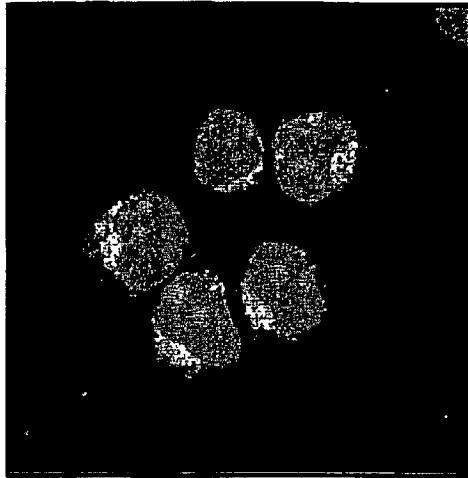
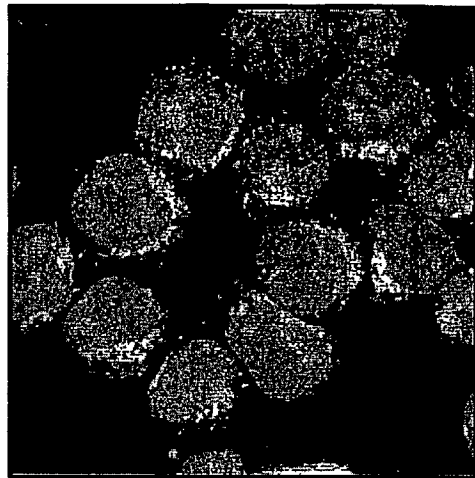


FIG. 18



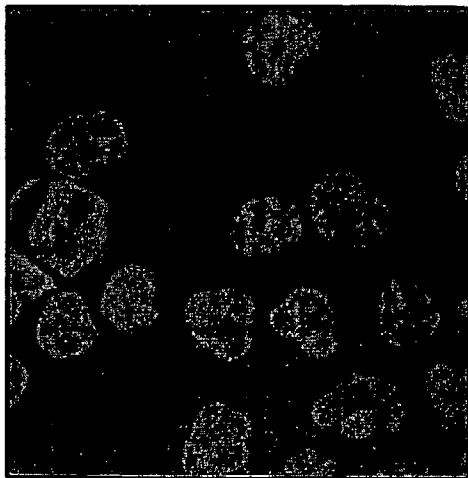
SAHB3a

FIG. 19A



SAHB3aG-->E

FIG. 19B



BID BH3

FIG. 19C

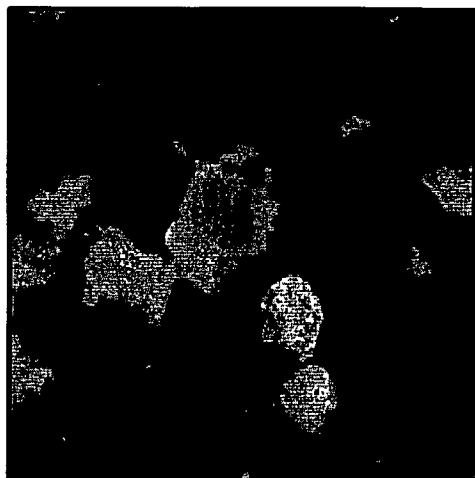
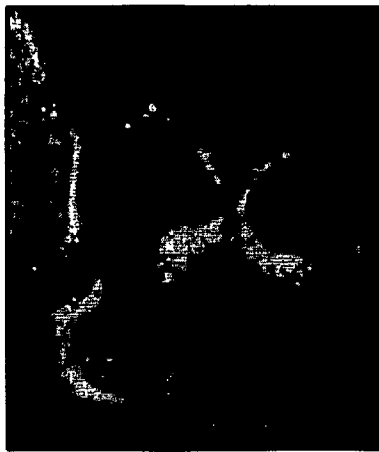
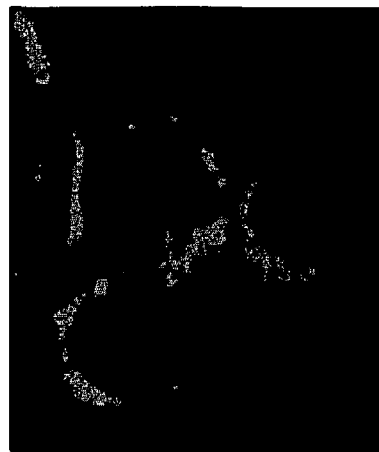
BID α 6

FIG. 19D



SAHB3a

FIG. 20A



Tom20

FIG. 20B



Overlay

FIG. 20C

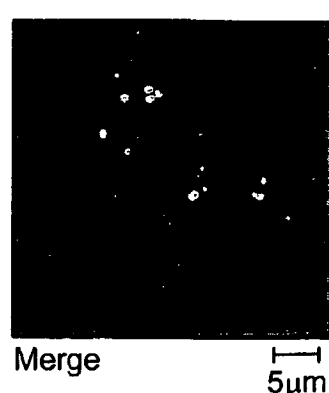
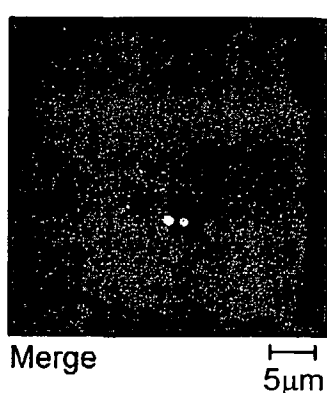
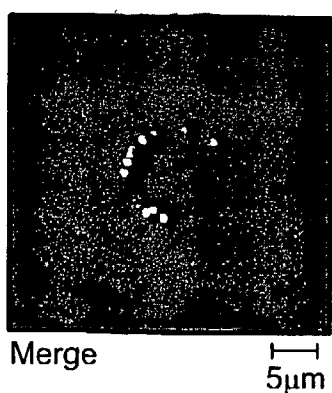
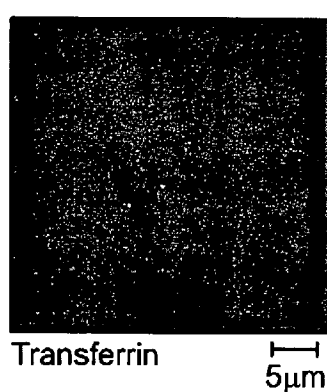
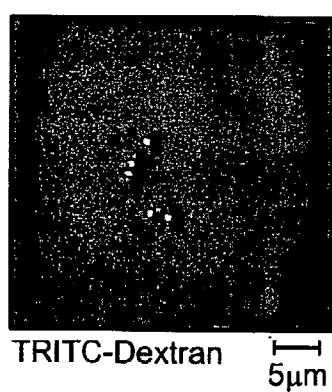
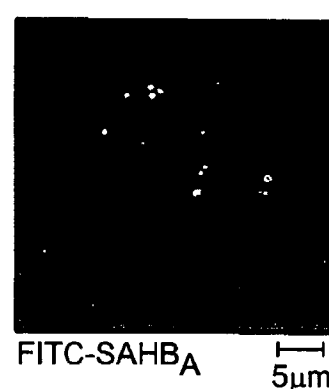
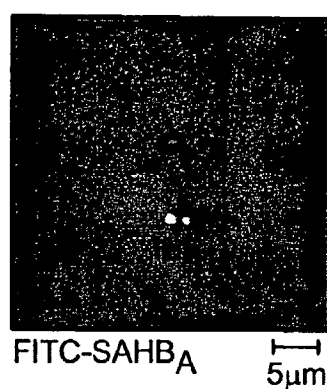
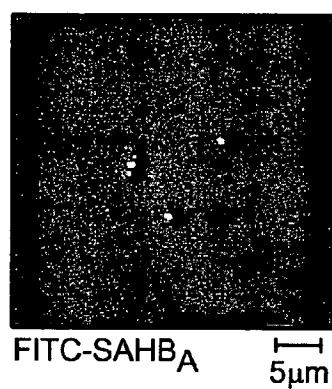


FIG. 21A

FIG. 21B

FIG. 21C

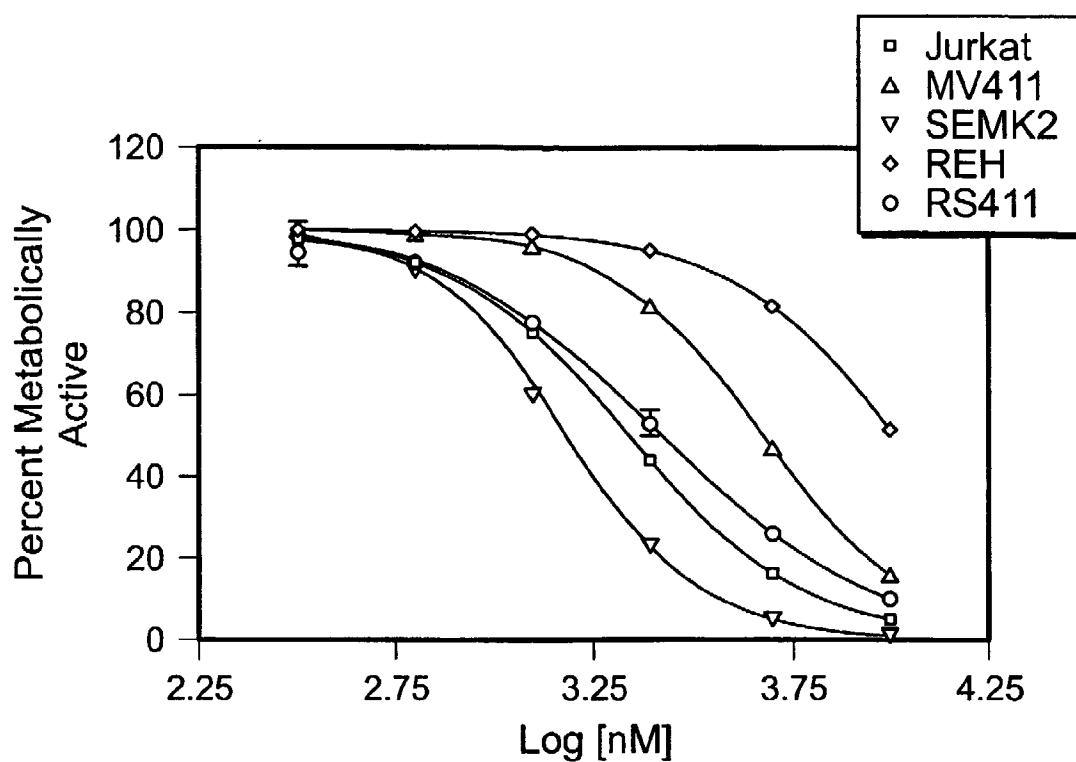


FIG. 22A

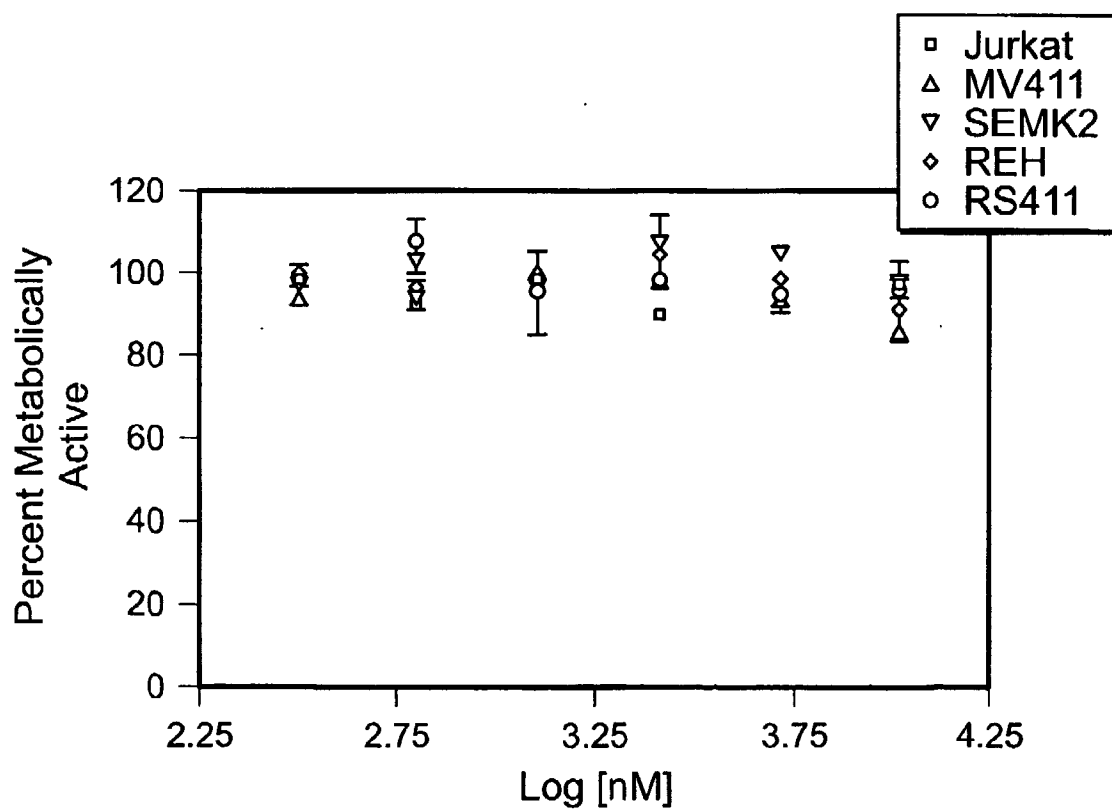


FIG. 22B

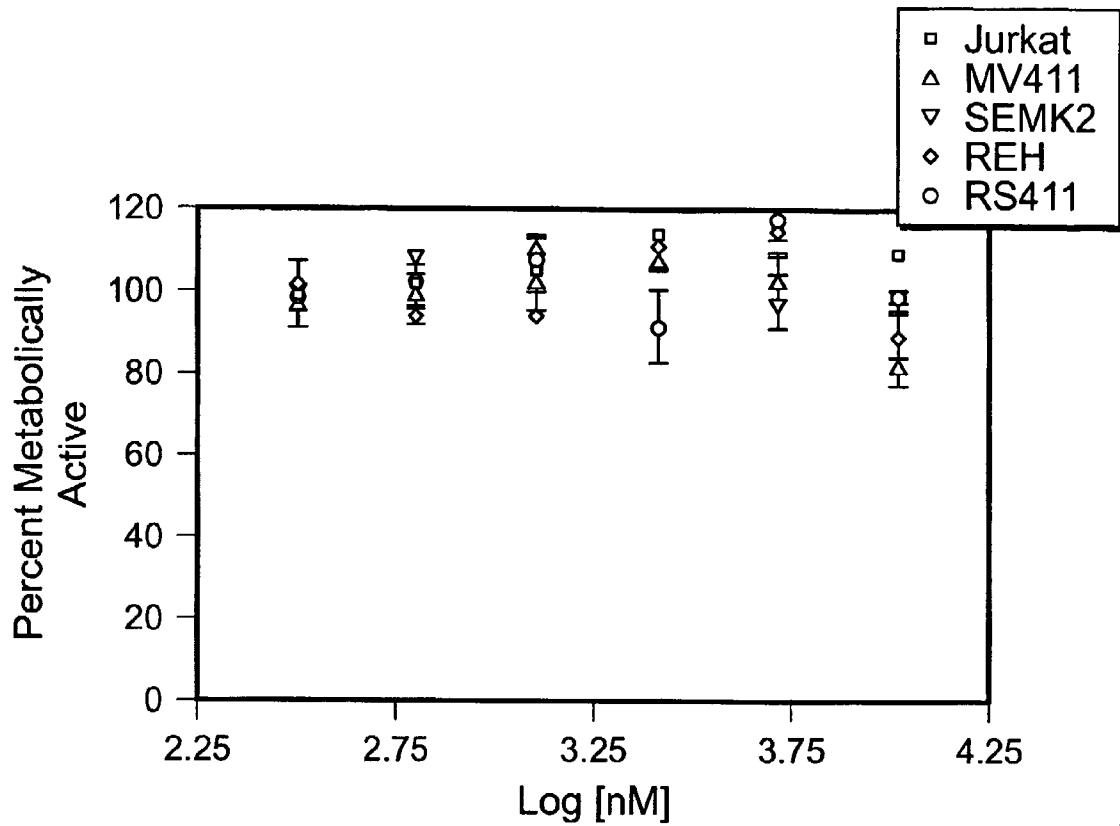


FIG. 22C

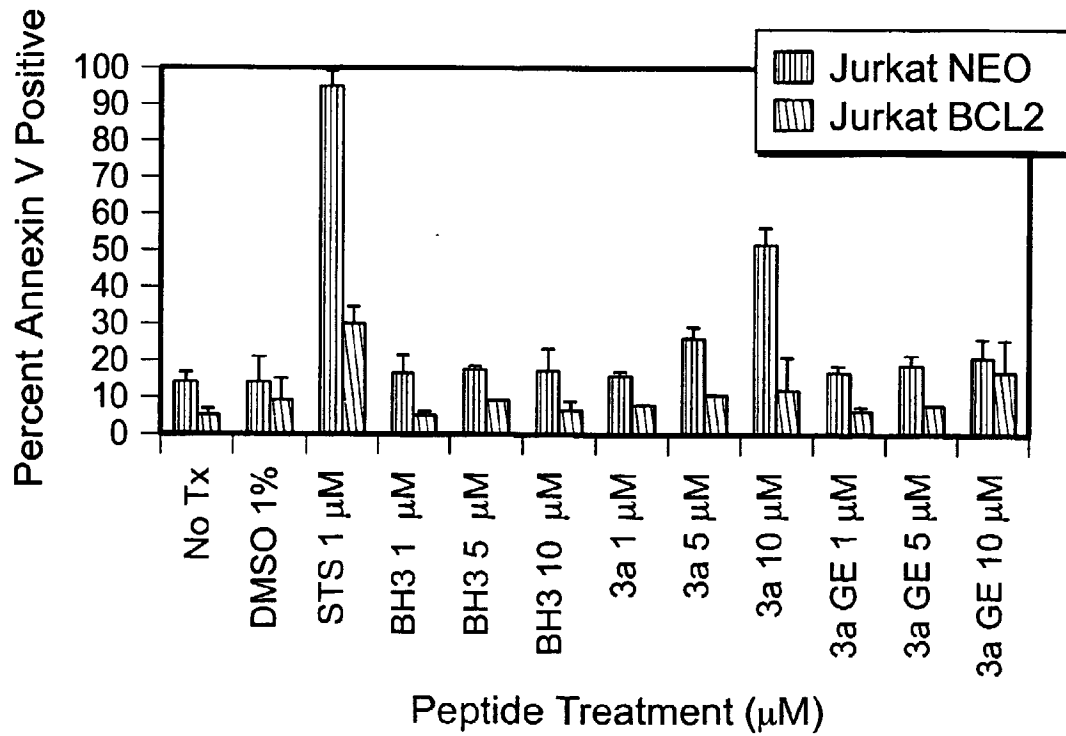


FIG. 23A

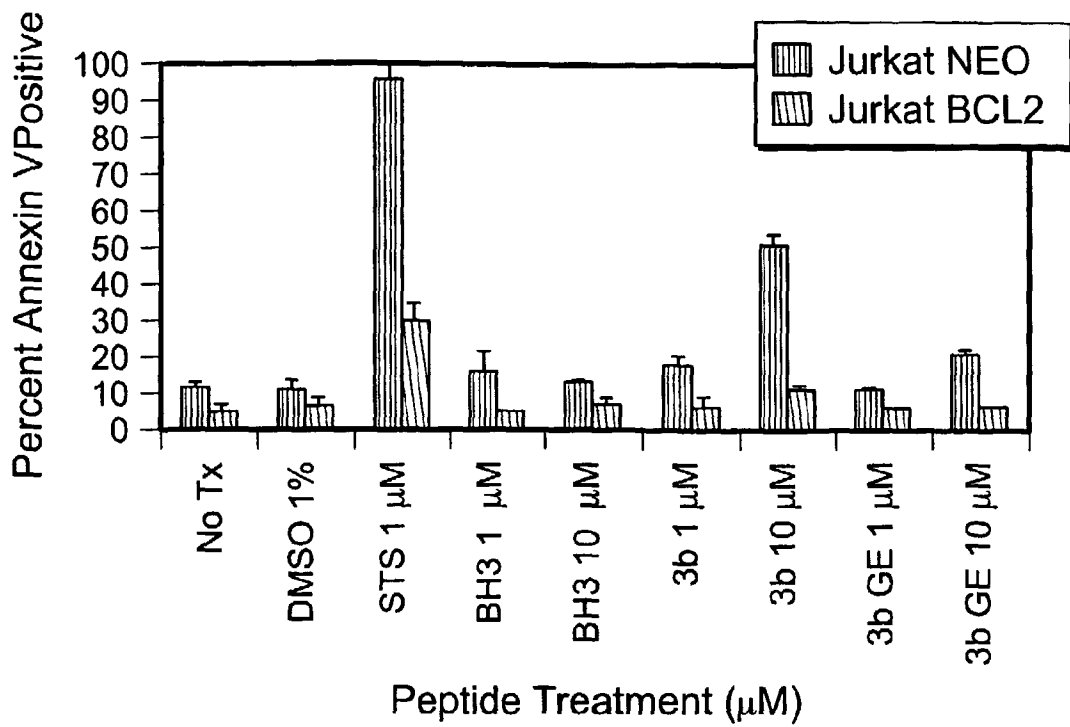


FIG. 23B

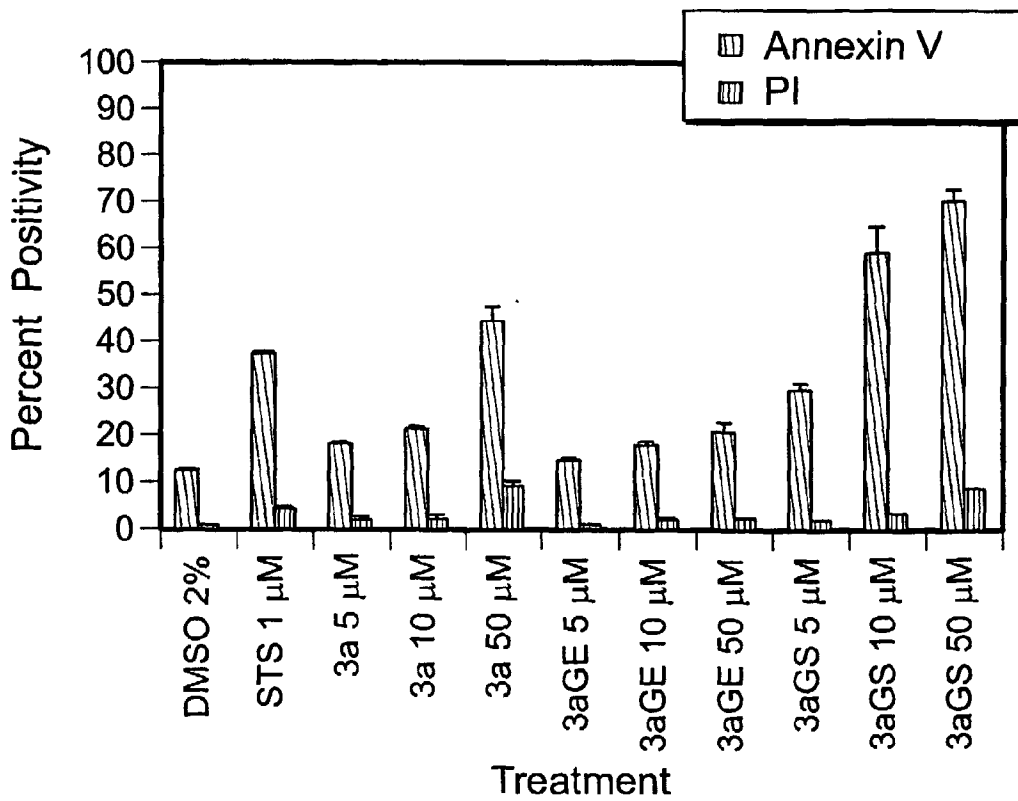


FIG. 24

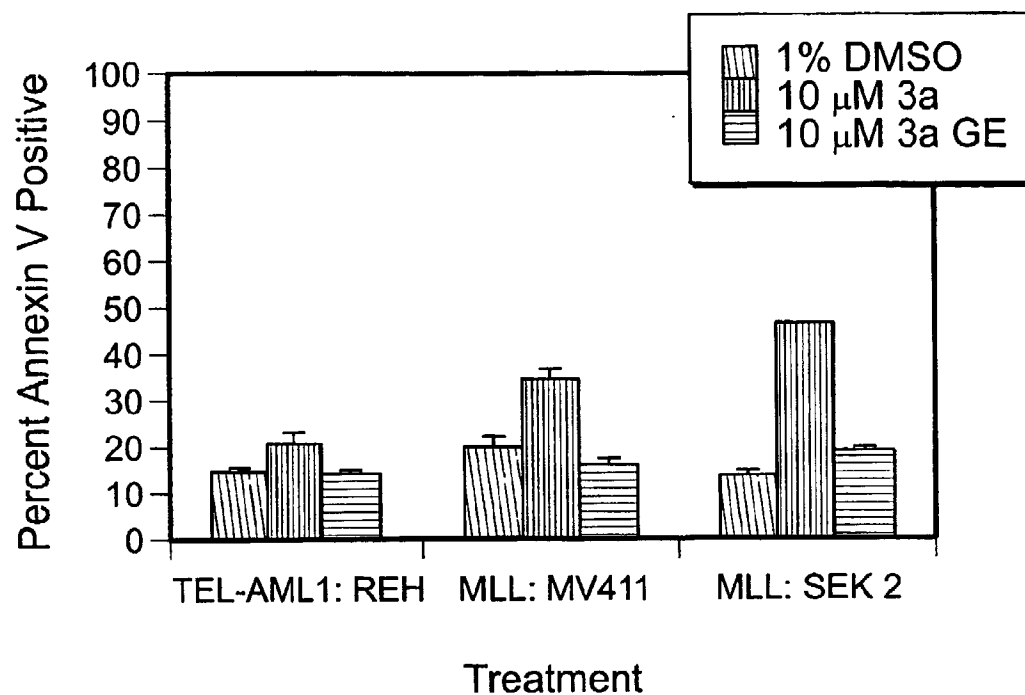


FIG. 25

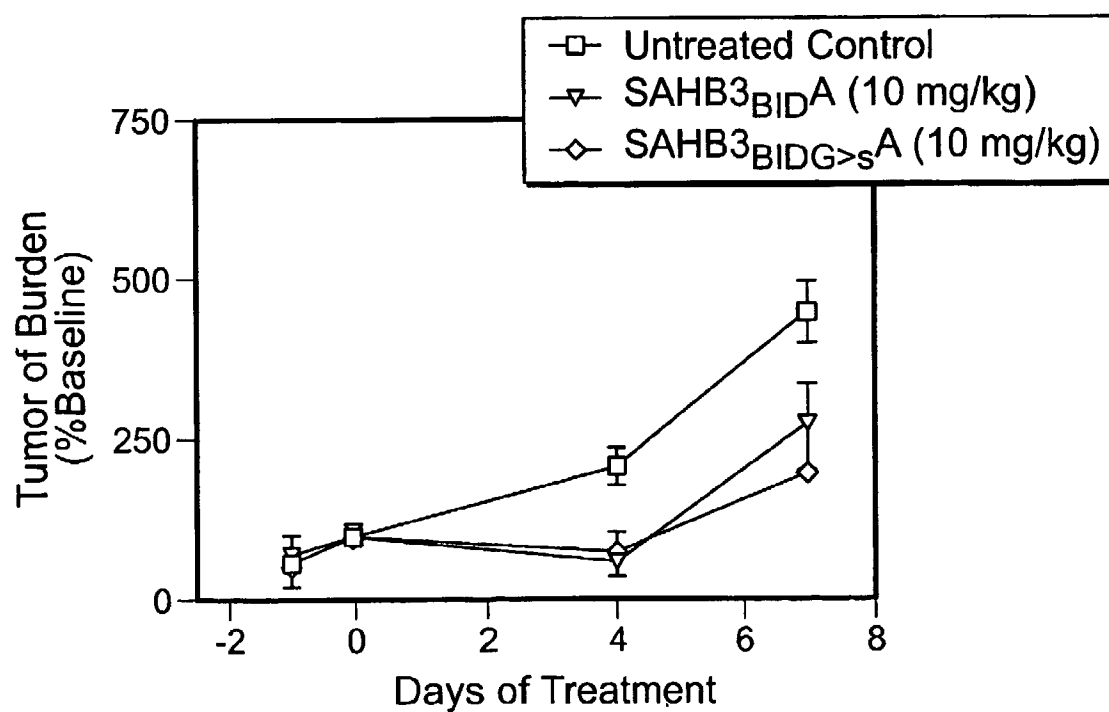


FIG. 26A

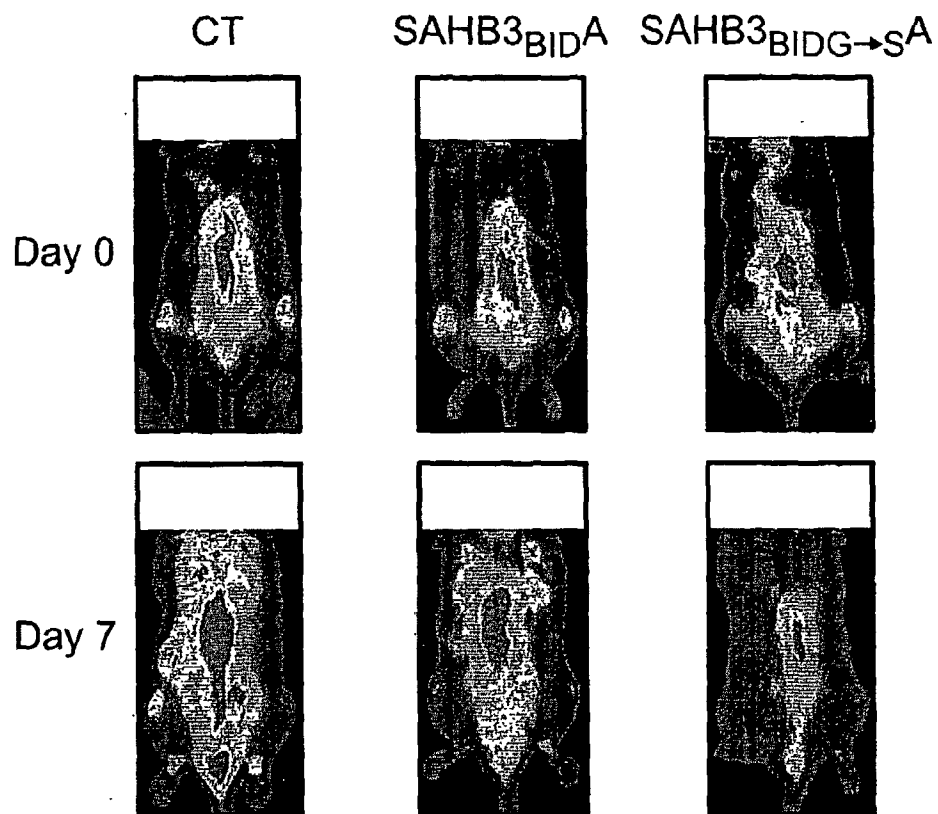


FIG. 26B

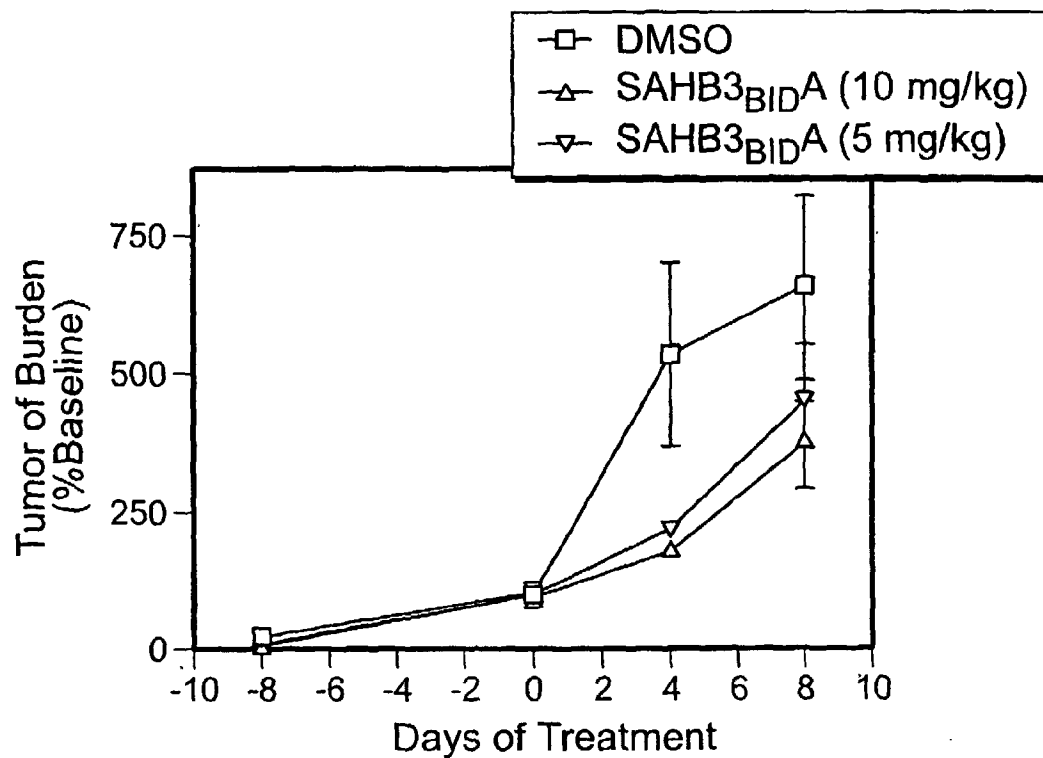


FIG. 27A

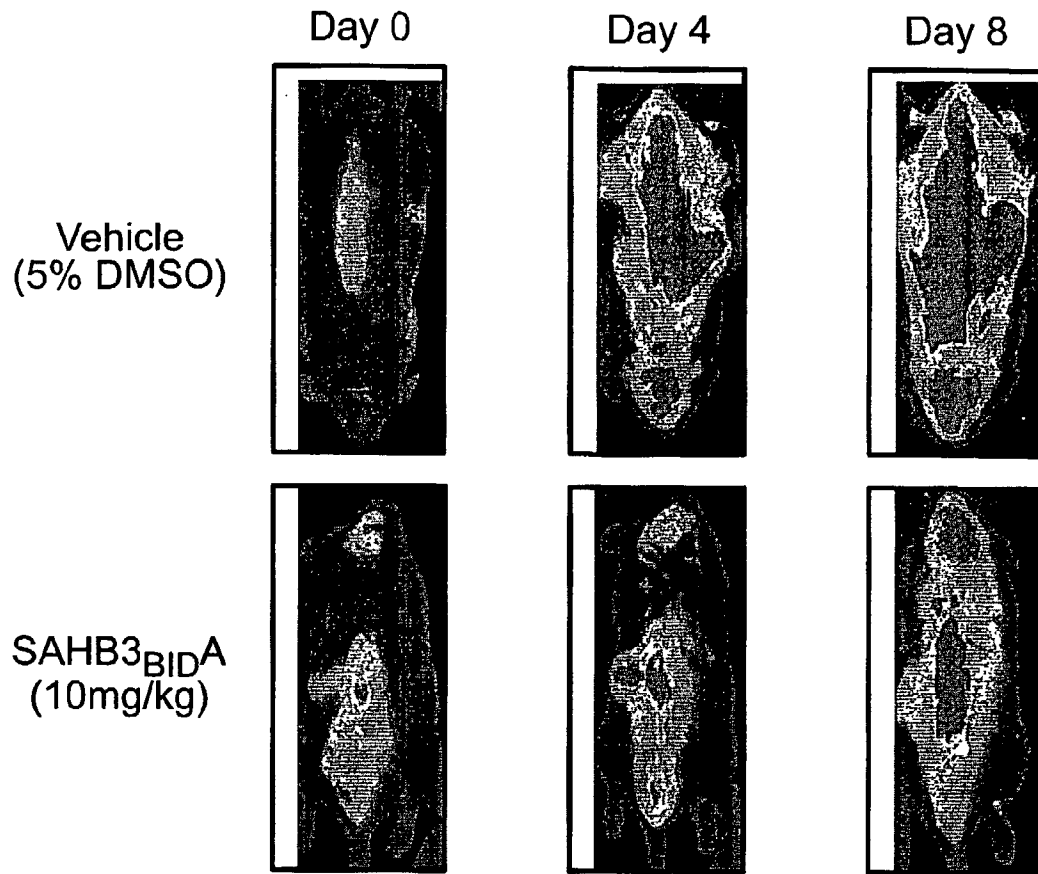


FIG. 27B

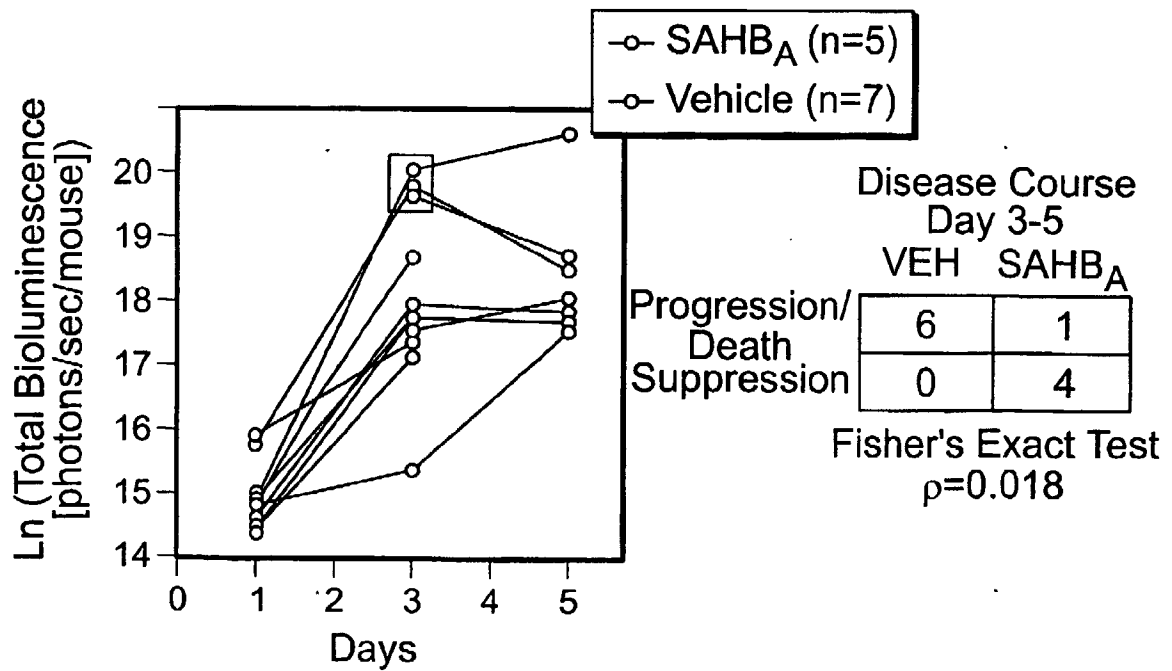


FIG. 27C

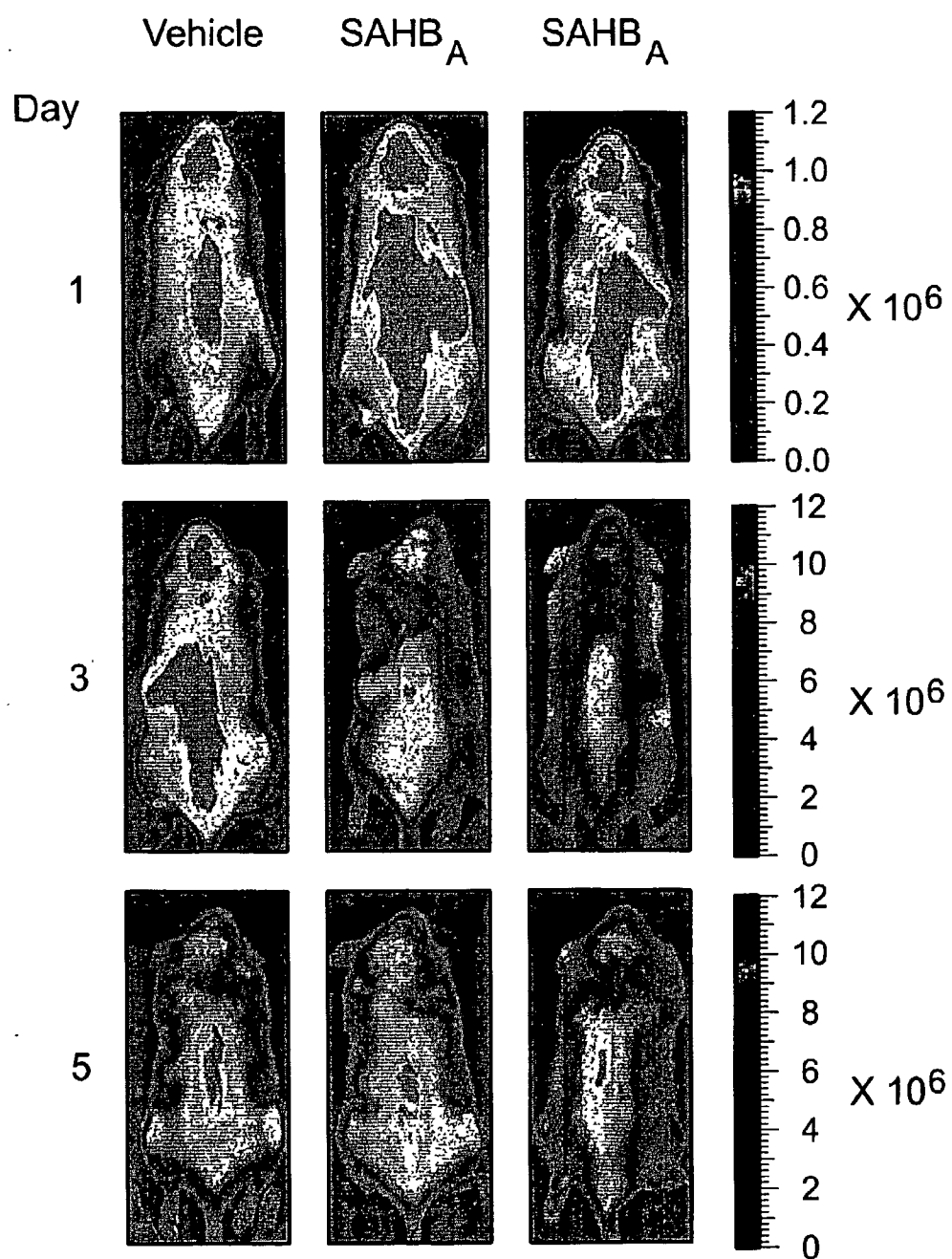


FIG. 27D

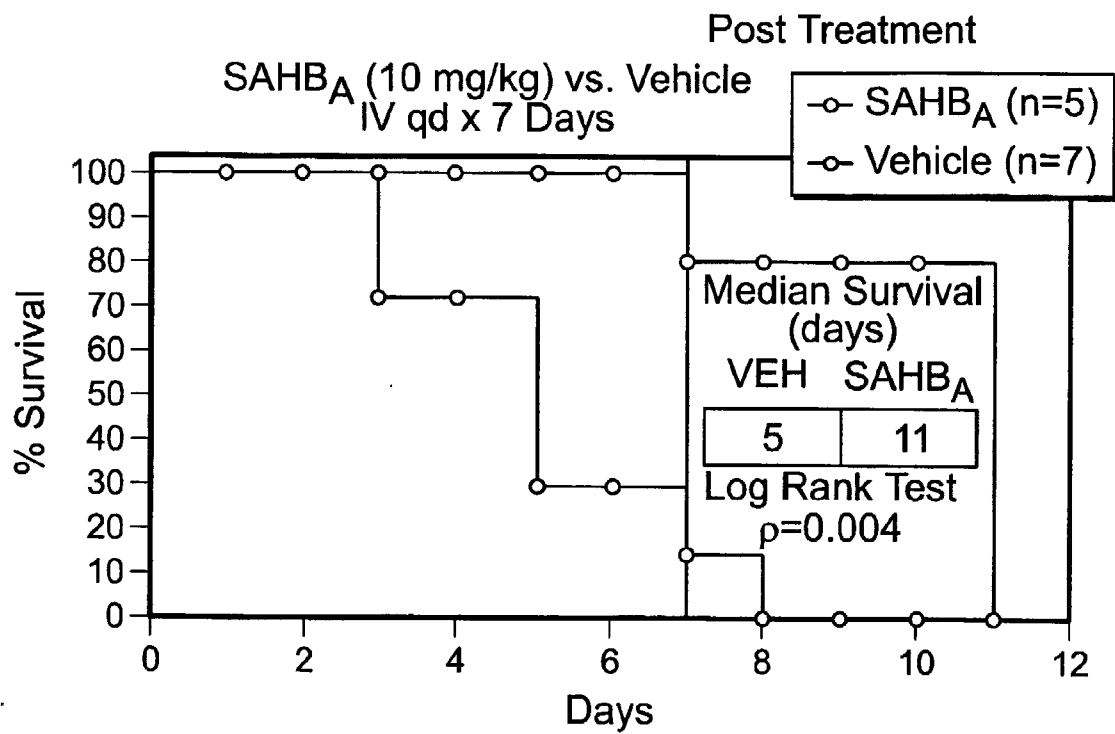


FIG. 27E

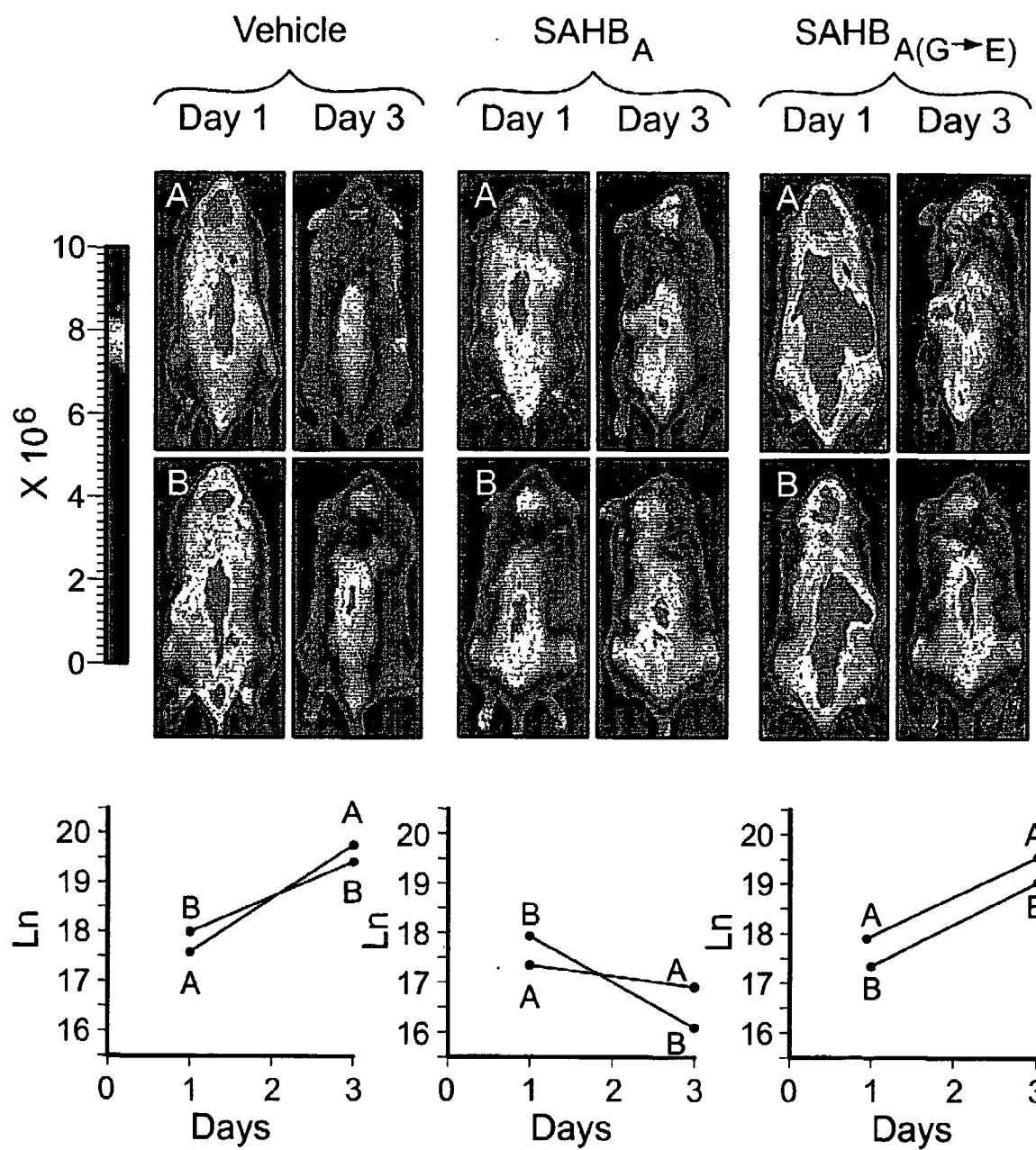


FIG. 27F

h8bc3	MARARQEGSSPEVEGLARDGPRPFPLGRLVPSAVSCGLCSPGLAAAPAA	50
m8bc3	MARARQEGSSPEVEGLARDSPRPFLGRLMP SAVSCSLCSPGLPAAPAA	50
h8bc3	PTLLPAAYLCAPTAPPAVTAALGGSRWPGGPRSRPRGPRPDGPQPSLSLA	100
m8bc3	PALLPAAYLCAPTAPPAVTAALGGPRWPGGHRSPRGPRPDGPQPSLSPA	100
h8bc3	EQHLESPVPSAPGALAGGPTQAAPGVGEEEEQWAREIGAQ	150
m8bc3	QQHLESPVPSAPEALAGGPTQAAPGVVVEEEWAREIGAQ	150
h8bc3	QYERRRQEEQQRHRPSPWRVLYNLIMQLLPLPRGHRAPEMEPN	193
m8bc3	QYERRRQEEQHRHRPSPWRVMYNLFMQLLPLPRQPGAPEMEPN	193

FIG. 28A-1

Bbc3	133	WAREIGAQLRRMADDLNAQY
EgI -1	50	IGYEIGSKLAAMCDDFDAQM
Bsd	106	AAQRYGRELRRMSDEFVDSF
BIm	84	PEIWI IAQELRRIGDEFNAYY
BId	82	IIRNIARHLAQVGD SMDRSI
BIk	53	GSDALALRLACIGDEM DVSL
Hrk	29	AAQLTAARLKALGDELHQRT

FIG. 28A-2

BIK	56	ALALRLACIGDEMDVSLR
Bim1	87	WIAQELRRIGDEFNAYYA
Noxa	24	ECATQLRRFGDKLNFRQK
Bid	85	NIARHLAQVGDSMDRSIP
Bad	109	RYGRELRRMSDEFVDSFK
EGL-1	53	EIGSKLAAMCDDFDAQMM

FIG. 28B-1

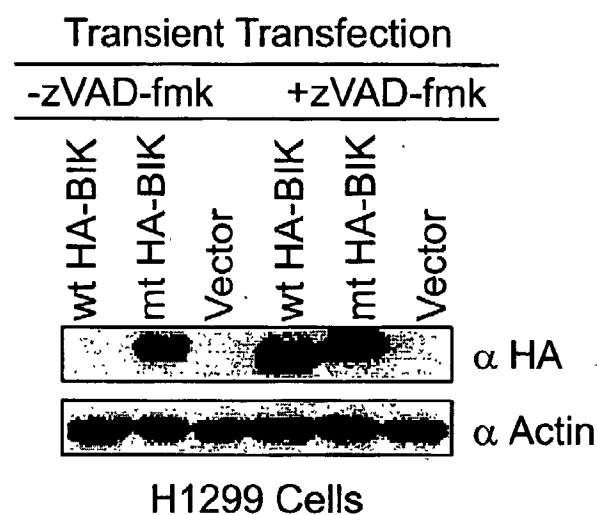


FIG. 28B-2

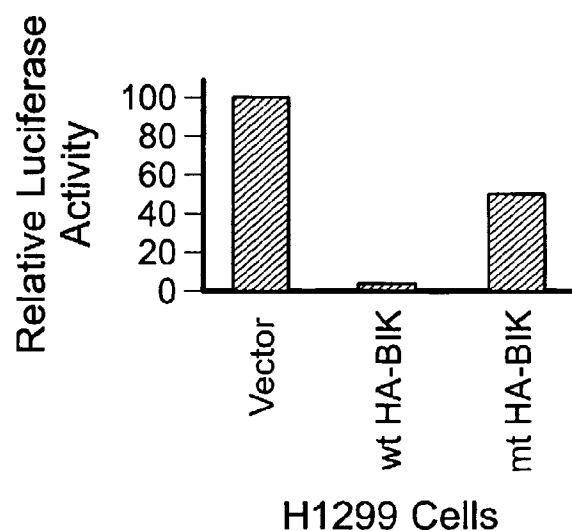


FIG. 28B-3

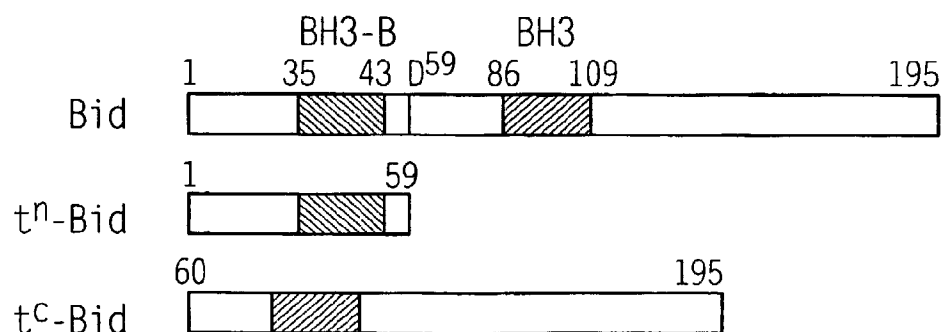


FIG. 28C-1

hBid	37	LDA LGHEL P	45	} BH3-B
mBid	35	LEV LGREL P	43	
hBid	90	LAQVGDSMD	98	} BH3
mBid	90	LAQIGDEMD	98	
hBik	61	LACIGDEMD	69	
hBimL	92	LRRIGDEFN	100	
hHrk	37	LKALGDELH	45	
hBad	113	LRRMSDEFV	121	
hBak	78	LAIIGDDIN	86	
hBax	63	LKRIGDELD	71	
mMtd	71	LLRLGDELE	79	

FIG. 28C-2

1	MTLRRLLEDWCRGMDMNP RKALLIAGISQSCSVAEIEEAL	hMAP-1
1	MTLRRLLEDWCRGMDMNP RKALLVAGIPPTCGVADIEEAL	mMAP-1
1	MAMTLLEDWCRGMDVNSQRALLVWGIPVNCDEAEIEETL	hMa1
1	MALALLLEDWCRITMSVDEQKSLMVTGIPADFEEAEIQEVL	hMa2
40	QAGLAPLGEYRLLGRMFRRDENRKVALVGLTAETSHALV	hMAP-1
40	QAGLAPLGEYRLLGRMFRRDENKNVALIGLTVETGSALV	mMAP-1
40	QAAMPQVS-YRMLGRMFWRTEENAKAALLELTGAVDYAAI	hMa1
40	QETLKS LGRYRLLGKIFRRQENANAVLLELLEDTDVSAI	hMa2
79	PKEIPGKGGIWRVIFKPPDPDNTFLSRLNEFLAGEGMTV	hMAP-1
79	PKEIPAKGGVWRVIFKPPDTSDFLCRLNEFLKGEGMTM	mMAP-1
78	PREMPGKGGVWKVLFKPPTSDAEFLERLHLFLAREGWTV	hMa1
79	PSEVQGKGGVWKVIFKTPNQDTEFLERLNLFLKEGQTV	hMa2
118	GELSRALGHENGSLDPEQG-MIPEMMAPMLAQAL-EALQ	hMAP-1
118	GELTRVLGNRNDPLGLDPGIMIPEIRAPMLAQALNEALK	mMAP-1
117	QDVARVLGFQNPPT--PTPG--PEMPAEMLNYITLDNVIQ	hMa1
118	SGMFRALGQEGVSPAPVPCT-SPELLAHL LGAMAHAPQ	hMa2
155	PALQCLKYKKLRVFSGRISPEPGEEFGRWMFHTTQM IK	hMAP-1
157	PTLOYLRYKKLSVFSGRDPPGPGEEFESWMFHTSQVMK	mMAP-1
151	PLVESTIWKRLTLFSGKGHPRAWRGNFDPWLEHTMEVLE	hMa1
156	PLLP-MRYRKLRVFSGSAVPAPEEESFENVLEQATEIVK	hMa2
194	AWQVPDVEKRRRLIESLRGPALDVIRVLKINNPLITVDE	hMAP-1
196	TWQVSDVEKRRRLIESLRGPAFEIRVLKINNPFITVAE	mMAP-1
190	EWQVSDVEKRRRLMESLRGPAADVIRTLKSNNPATITAE	hMa1
194	EWFPVTEAEKKRWLAESLRGPALQLMHIVQADNPSTISVEE	hMa2
233	CLQALEEVFGVTDNPRELQVKYLTTHKDEEKL SAYVLR	hMAP-1
235	CLKTLETIFGIIDNPRLQVKYLTTHYQKDEEKL SAYVLR	mMAP-1
229	CLKALEQVFGSVESRDQIKFLNTYQNPGEKL SAYVIR	hMa1
233	CLFAFKQVFGSLESRRTAQVRYLKTYQEEGEKV SAYVLR	hMa2
272	LEPLLQKLVQRGAIERDAVNQARLDQVIAGAVHKIT-IRR	hMAP-1
274	LEPLLQKLVQKGAIEKEVVNQARLDQVIAGAVHKS-VRR	mMAP-1
268	LEPLLQKVVEKGAIDKDNVNQARLEQVIAGANHSGAIRR	hMa1
272	LETLLRRAVEKRAIPRRIADQVRLQVMAGATLNQMLWC	hMa2
310	ELN-LPEDGPAPGFLQLLVLIKDYEAEE--EEEALLQA	hMAP-1
312	ELG-LPEGSPAPGLQLLTLIKDKEA-E--EEEVLLQA	mMAP-1
307	QLW-LTGAGEGPGPKPL-----	hMa1
311	RLRELKDQGPSPSELELMKVITREEEEEFASFENESIEEP	hMa2
345	ILEGNFT	hMAP-1
346	ELEGYCT	mMAP-1
323	SVAGADP	hMa1
350	ERDGYGRWNHEGDD	hMa2

FIG. 28D-1

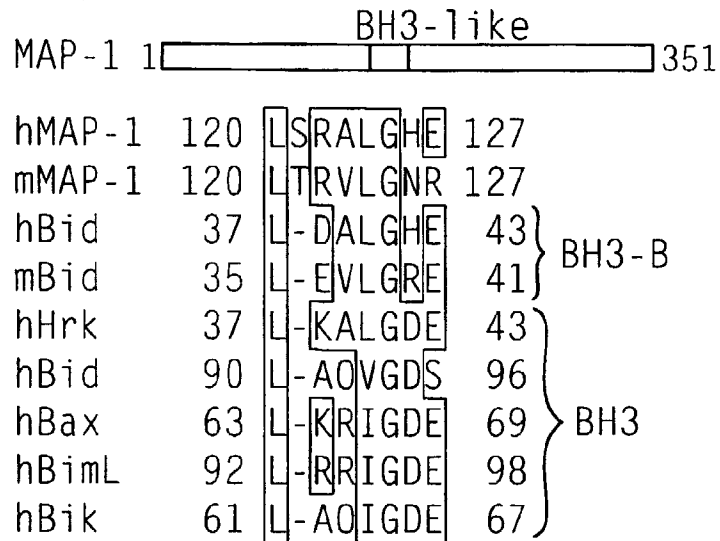


FIG. 28D-2

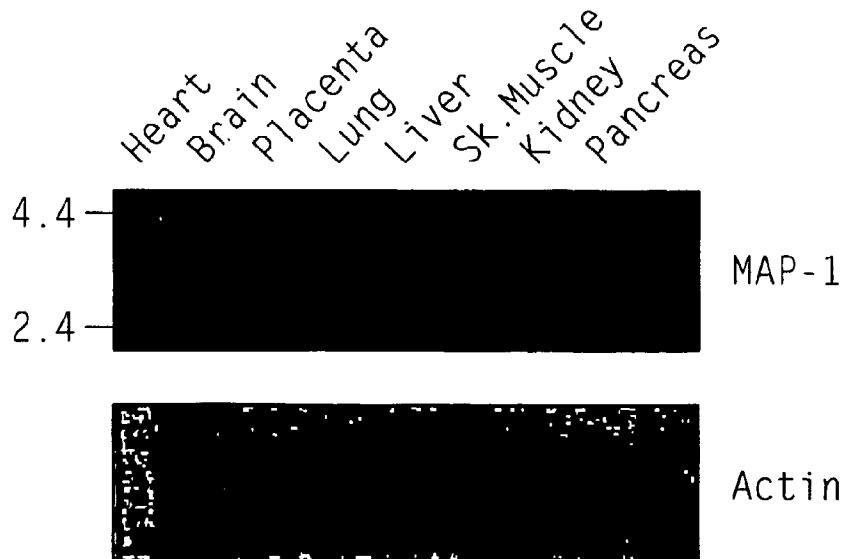


FIG. 28D-3

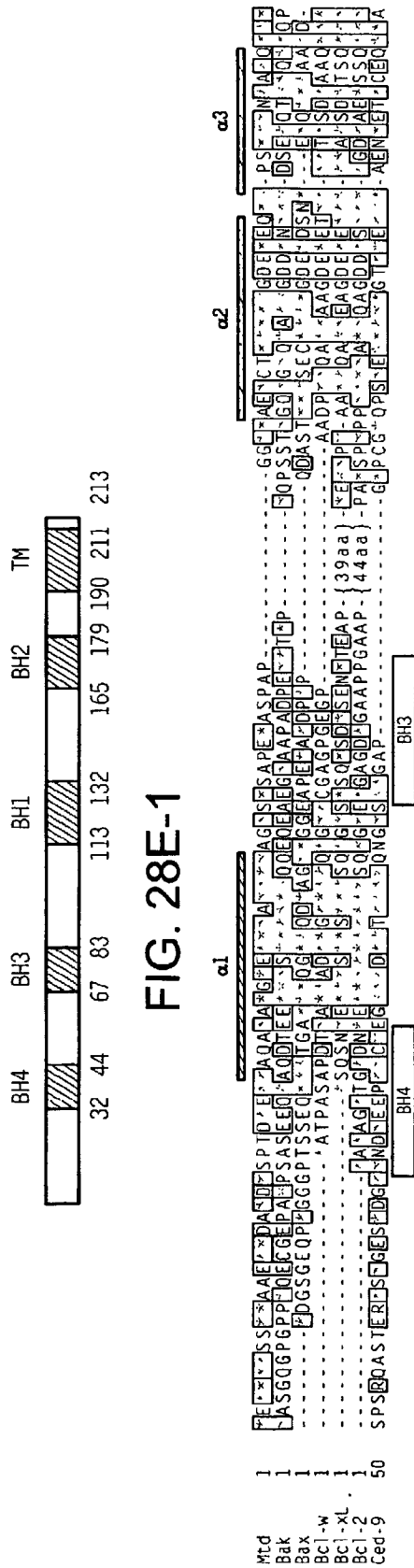


FIG. 28E-1

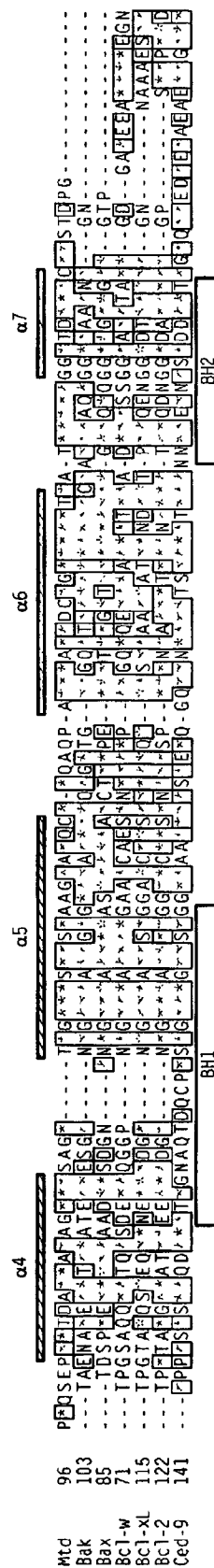
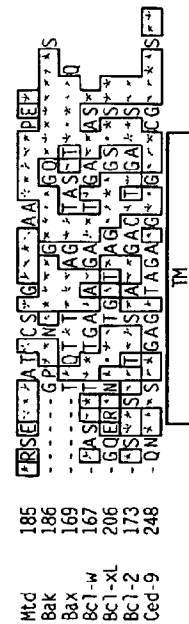


FIG. 28E-2



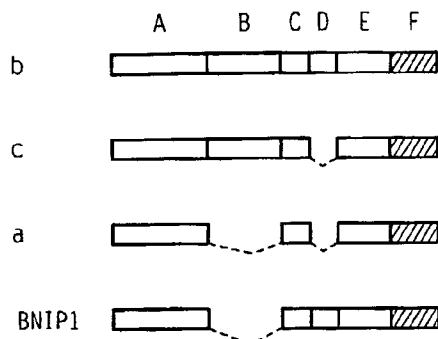


FIG. 28F-1

			← BH3 →	
BNIP1b	154	KAELLQGGDLLRQR		167
BIK	56	ALRLACIGDEMDVS		69
BLK	52	ALRLACIGDEMOLC		65
BID	87	ARHLAQVGDSMDRS		100
BAK	75	GRQLAIIGDDINRR		88
HRK	34	AARLKALGDELHQR		47
BAX	60	SECLRRIGDELDN		73
BIML	89	AQELRRIGDEFNAY		102
BOK	68	CTVLLRLGDELEQI		81
BCL2L1	87	KQALREAGDEFELR		100
BCL2	94	HLTLRQAGDDFSRR		107
MCL1	210	LETLLRRVGDGVQRN		223
		* **		

FIG. 28F-3



FIG. 28F-4

MAAPQDVNVRICNQZIVKTDLEVKALIQDI 30

RDCSGPLSALTRLNTKVKRKTQQLRNRIQP 60
I-

VLYQRATINZASTITTXLYELTDFSSTQN 90
-- Present only in BNIP1b & BNIP1e --

DFNSPTYFVTFSDLZQLAKZQDKXSEKQLL 120
-----I

LQXVENRKKQNLKNQASWRKANLTCKLAID 150
I----- Present only in

I-----BH3-----I
NIRKANLLQOODLLAQRXYKKSLAQYSST 180
BNIP1 & BNIP1b---I

I----- ANK1 -----
ITKSLNGISROKAQQVQQSEANQSLVTSS 210

-----I I----- ANK2 --
RTILDAKEETXSNSOTIQLORKLITKYMRR 240

----I I----- TM -----
ZLTDKLLIFLALRLTLATVLYIVKKRLFPF 271
-I
L*

FIG. 28F-2

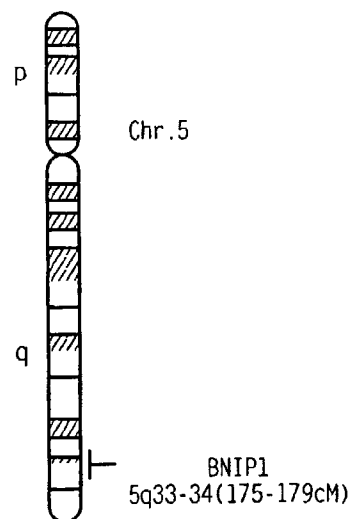


FIG. 28F-5

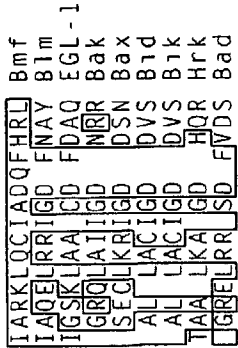


FIG. 28G-2

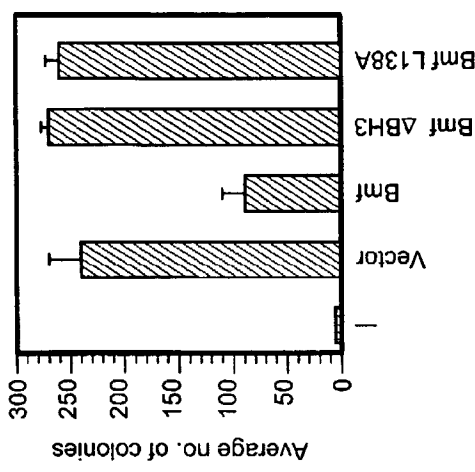


FIG. 28G-5

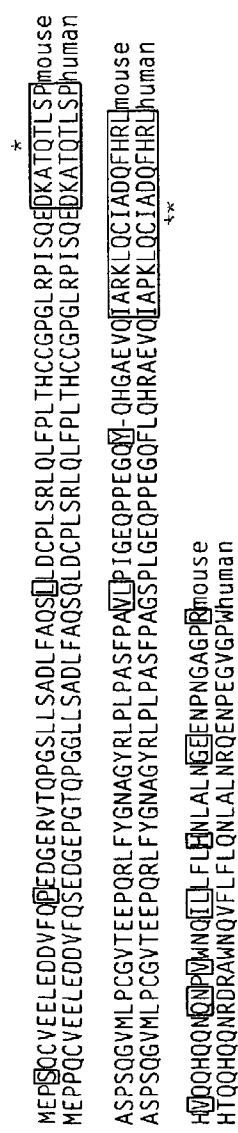


FIG. 28G-1

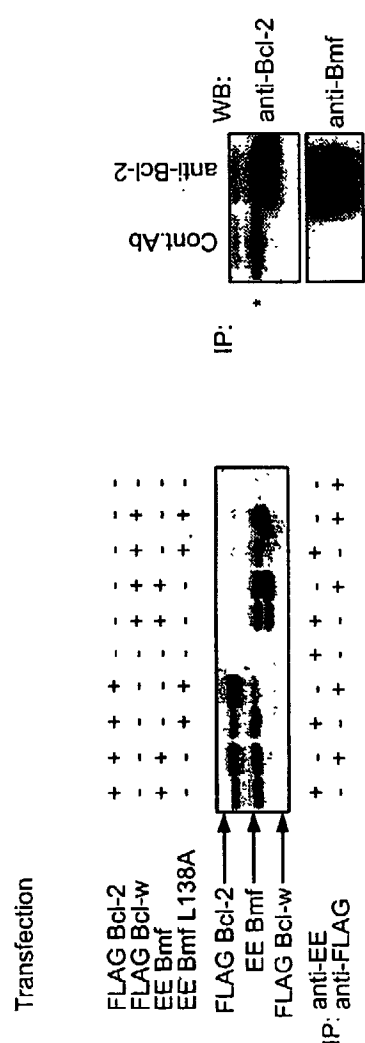


FIG. 28G-4

FIG. 28G-3

Alignment of BH3 Domains of Select BCL2-Family Members

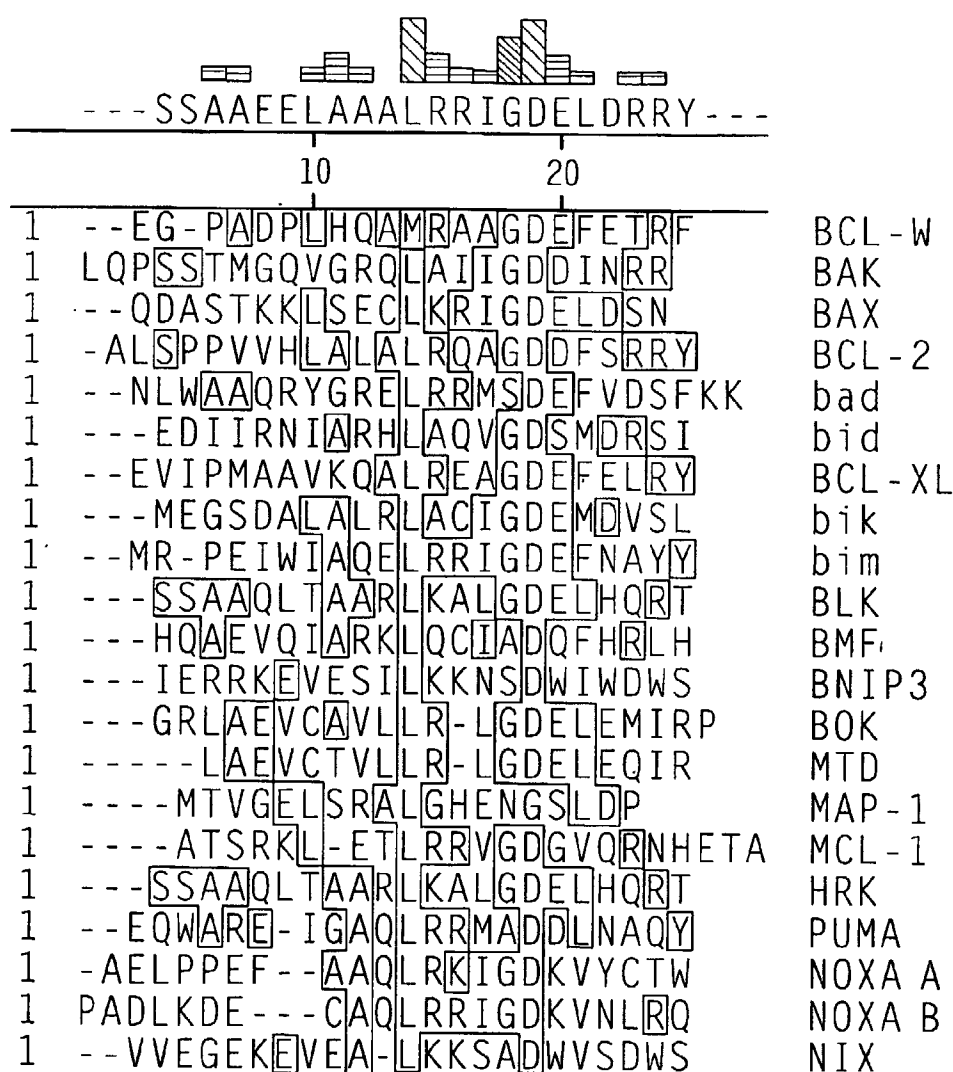


FIG. 28H