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(71) Applicant: **DANA-FARBER CANCER INSTITUTE, INC.** [US/US]; 450 Brookline Avenue, Boston, Massachusetts 02215-5450 (US).

(72) Inventors: **WALENSKY, Loren D.**; Dana-Farber Cancer Institute, Inc., 450 Brookline Avenue, Boston, Massachusetts 02215-5450 (US). **BIRD, Gregory H.**; Dana-Farber Cancer Institute, Inc., 450 Brookline Avenue, Boston, Massachusetts 02215-5450 (US). **GUERRA, Rachel**; Dana-Farber Cancer Institue, Inc., 450 Brookline Avenue, Boston, Massachusetts 02215-5450 (US). **HARVEY, Edward**; Dana-Farber Cancer Institute, Inc., 450 Brookline Avenue, Boston, Massachusetts 02215-5450 (US).

(74) Agent: **KOIPALLY, Joseph J.**; Fish & Richardson P.C., P.O. Box 1022, Minneapolis, Minnesota 55440-1022 (US).

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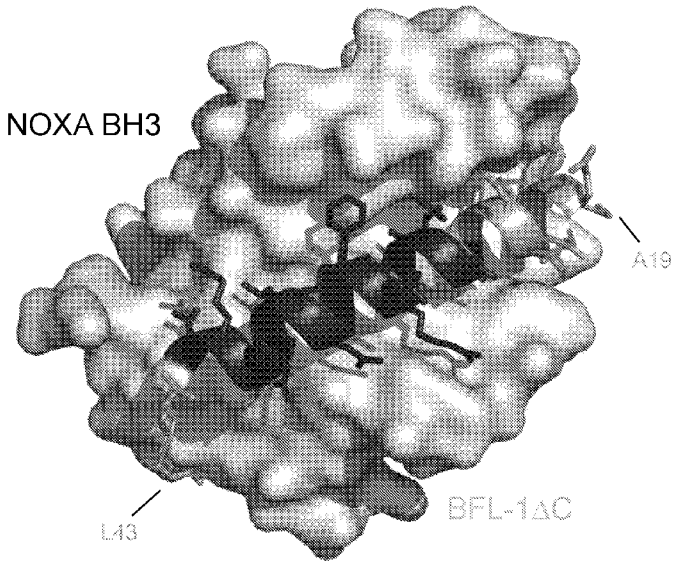

FIG. 1

(57) Abstract: This disclosure features structurally-stabilized and/or cysteine-reactive peptide inhibitors for selective targeting of BFL-1, or dual targeting of BFL-1 and MCL-1. Also disclosed are methods of using such structurally-stabilized and cysteine-reactive peptides in the treatment of BFL-1- and/or MCL-1-expressing or -dependent cancers or diseases of cellular excess (e.g., autoimmune or inflammatory conditions). Also provided are combination therapies comprising such structurally-stabilized and/or cysteine-reactive peptides and inhibitors of the DNA damage response pathway, such as an ATM kinase inhibitor, ATR kinase inhibitor, CHKl/2 inhibitor, or PARP inhibitor; or an inhibitor of MCL-1, or a selective inhibitor of BCL-2, or an inhibitor of BCL-2/BCL-XL, for the treatment of BFL-1-expressing or -dependent cancers (e.g., AML), BFL-1 and MCL-1-expressing or -dependent cancers, or diseases of cellular excess (e.g., autoimmune or inflammatory conditions).

WO 2019/118719 A1

[Continued on next page]

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SELECTIVE TARGETING OF APOPTOSIS PROTEINS BY STRUCTURALLY-STABILIZED AND/OR CYSTEINE-REACTIVE NOXA PEPTIDES

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application No. 62/599,229, filed December 15, 2017, which is incorporated by reference herein in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

This invention was made with government support under grant numbers 5R35CA197583 and R50CA211399 awarded by the National Institutes of Health. The government has certain rights in the invention.

TECHNICAL FIELD

This disclosure relates to structurally-stabilized and/or cysteine-reactive NOXA peptides and methods for using such peptides alone or in combination with an inhibitor of a DNA damage response pathway member (e.g., ATM kinase inhibitor, ATR kinase inhibitor, CHK1/2 inhibitor, PARP inhibitor), or an inhibitor of MCL-1, or a selective inhibitor of BCL-2, or an inhibitor of BCL-2/BCL-XL in the treatment of cancer and diseases of cellular excess such as autoimmune and inflammatory conditions.

BACKGROUND

The BCL-2 protein family, which includes both pro-apoptotic and anti-apoptotic members, forms a complex network of checks and balances that dictate cell fate. The family is structurally defined by the presence of up to four conserved “BCL-2 homology” (BH) domains, all of which include alpha-helical portions. Anti-apoptotic proteins such as BFL-l and MCL-1 display sequence conservation in all BH domains, whereas pro-apoptotic proteins are divided into “multi-BH domain” members (e.g., BAX and BAK) and “BH3-only” members (e.g., BIM and NOXA) that display sequence similarity only in the alpha-helical BH3 domain. The BH3-only subgroup is diverse and transmits pro-death signals from disparate stimuli to the apoptotic machinery located at the mitochondria. The BH3-only protein's death signal will either be neutralized by anti-apoptotic proteins or delivered, directly or indirectly, to the mitochondrial executioners BAX and BAK. When activated, BAX/BAK induce outer mitochondrial membrane permeabilization, enabling released mitochondrial factors to induce caspases, which irreversibly execute the death program.

Human cancers invariably suppress mitochondrial apoptosis to resist treatment and ensure cell survival. The most common strategy involves overexpression of one or more anti-apoptotic BCL-2 family proteins, such as BCL-2, BCL-X_L, MCL-1, or BFL-1. These proteins share a common mechanism of mitochondrial apoptosis suppression, involving entrapment of the BH3 helices of pro-apoptotic members, including the multidomain proteins BAX and BAK, and the BH3-only stress-sensor proteins, such as BIM, BID, BAD, and NOXA. Given the overlap in binding specificity among anti-apoptotic proteins, molecular targeting of one member often leads to upregulation of another – resulting in the oncologic version of "whack-a-mole." For example, small molecule targeting of BCL-2 by the selective small molecule inhibitor ABT-737 has been shown to trigger upregulation of MCL-1 or BFL-1 in lymphoma cell lines. With a selective small molecule inhibitor of BCL-2 now FDA-approved and selective compounds for MCL-1 recently advanced to clinical trials, BFL-1 remains a high priority, undrugged cancer target. Indeed, in addition to its capacity to confer resistance to selective BCL-2 and MCL-1 inhibitors, BFL-1 is an independent oncogenic driver in a host of human cancers, including melanoma, leukemia, and lymphoma.

BCL-2-related protein BFL-1/A1 has been implicated in suppressing the mitochondrial apoptotic pathway in a wide variety of liquid and solid tumors. For example, when overexpressed or mutated to resist ubiquitin-mediated degradation, BFL-1 induces chemoresistance in discrete lymphomas, including the BCR-dependent/elevated NFκB subclass of germinal center lymphomas and diffuse large B-cell lymphomas. BFL-1 was also identified as a pathologic survival factor in approximately 30% of human melanomas, including those with clinically relevant BRAF V600E resistance mutations.

Myeloid Cell Leukemia-1 (MCL-1), an anti-apoptotic BCL-2 family survival protein, has been implicated in the development, maintenance, and chemoresistance of a broad range of cancers and is one of the top ten most widely expressed pathologic factors in human cancers (*see, e.g.,* Beroukhim, *Nature*, 463(7283):899-905 (2010)). Highly overexpressed in human cancers, MCL-1 mounts formidable apoptotic resistance by binding and sequestering the essential BH3 domain helices of pro-apoptotic BCL-2 family members.

Thus, compounds that interfere with BFL-1 and/or MCL-1 activity could be useful in treating a variety of cancers and diseases of cellular excess such as autoimmune and inflammatory conditions.

SUMMARY

This disclosure relates to structurally-stabilized and/or cysteine-reactive NOXA BH3 peptides that can covalently bind to BFL-1 and also engage BFL-1 and/or MCL-1 through non-covalent interactions. This disclosure also features methods for using such peptides alone or in combination with other therapeutic agents (*e.g.,* an inhibitor of a DNA damage response pathway member (*e.g.,* ATM kinase inhibitor, ATR kinase inhibitor, CHK1/2 inhibitor, PARP inhibitor), a selective inhibitor of MCL-1, a selective inhibitor of BCL-2, an inhibitor of BCL-2/BCL-XL) in the treatment of BFL-1- and/or MCL-1-dependent or expressing cancers (*e.g.,* hematologic malignancies, melanoma).

In a first aspect, this disclosure provides a peptide that includes an electrophilic warhead; and a modified amino acid sequence of the sequence set forth in SEQ ID NO:39. The modified amino acid sequence is at least five amino acids in length; comprises at least one peptide structure stabilizing modification, and one or more (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14) amino acids of SEQ ID NO:39 are substituted by another amino acid. The peptide binds BFL-1/A1 or binds both BFL-1/A1 and MCL-1. The at least five amino acids can be a variant of, *e.g.,* ATQLR (**SEQ ID NO:110**), RFGDK (**SEQ ID NO:143**), LNFRQ (**SEQ ID NO:124**), TQLRR (**SEQ ID NO:145**), FGDKL (**SEQ ID NO:146**), QLRRF (**SEQ ID NO:147**), GDKLN (**SEQ ID NO:148**), LRRFG (**SEQ ID NO:149**), DKLNF (**SEQ ID NO:150**), RRFGD (**SEQ ID NO:142**), and KLNFR (**SEQ ID NO:144**). For example, at least two of the five amino acids are modified for structure stabilization (*e.g.,* by substituting with non-natural amino acids that can form a hydrocarbon staple) and one or more (e.g., 1, 2, 3) amino acids are substituted with other amino acids. In certain instances, the substitution(s) are on the non-interacting face of the helix. In certain instances, the substitution(s) are on the non-interacting face of the helix. In some cases, the substitutions are conservative amino acid substitutions. In certain embodiments, the one or more amino acids of the at least five amino acids are substituted by an amino acid or amino acids selected from the group consisting of L-alanine, D-alanine, α-aminoisobutyric acid, N-methyl glycine, serine, a substituted alanine, and a glycine derivative.

In some embodiments, the modified amino acid sequence is 5 to 35 amino acids in length (*e.g.,* 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 25, 30, 32, 35). In some embodiments, the modified amino acid sequence is 8 to 18 (e.g., 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18) amino acids in length.

In some embodiments, the peptide structure stabilizing modification is a staple and/or stitch. In some instances, the staple is at one or more of position *i* and *i + 3*; *i* and *i +4*; or *i* and *i+ 7*. In some embodiments, the peptide structure stabilizing modification comprises substitution of at least two (*e.g.,* 2, 3, 4, 5, 6) amino acids of SEQ ID NO:39 with non-natural amino acids with olefinic side chains. In some instances, the non-natural amino acids with olefinic side chains are selected from the group consisting of: S-pentenyl alanine, R-octenyl alanine; R-propenylalanine, S-pentenylalanine; R- pentenylalanine, S-pentenylalanine; Bis-pentenylglycine, S-pentenylalanine, R-octenylalanine; and Bis-pentenylglycine, S-octenylalanine, R-octenylalanine. In certain embodiments, the staple and/or stitch involves positions: 1 and 5; 2 and 6; 3 and 7; 6 and 10; 10 and 14; 11 and 15; 12 and 16; 1 and 8; 3 and 10; 6 and 13; 7 and 14; or 8 and 15 (wherein the positions are based on SEQ ID NO:39).

In certain embodiments, the peptide structure stabilizing modification is a hydrocarbon staple/stitch, a lactam staple/stitch; a UV-cycloaddition staple/stitch; an oxime staple/stitch; a thioether staple/stitch; a double-click staple/stitch; a bis-lactam staple/stitch; a bis-arylation staple/stitch; or a combination of any two or more thereof.

In certain embodiments, the one or more (e.g., 1, 2, 3, 4, 5, 6) amino acids of SEQ ID NO:39 that are substituted by another amino acid are on the BFL-1/A1 non-interacting face of the helix of SEQ ID NO:39. In some embodiments, 0 to 5 amino acids in SEQ ID NO:39 are removed from the C-terminal or are removed and replaced with 1 to 6 amino acids from the group consisting of alanine, D-alanine, α-aminoisobutyric acid, N-methyl glycine, serine, a substituted alanine, and a glycine derivative. In some embodiments, 0 to 6 amino acids on the non-interacting face in SEQ ID NO:39 are substituted with an amino acid selected from the group consisting of alanine, D-alanine, α-aminoisobutyric acid, N-methyl glycine, serine, a substituted alanine, and a glycine derivative. In some embodiments, one or more of the following amino acids A1, L4, R5, F7, G8, L11, N12, and Q15 of SEQ ID NO:39 are substituted with an alpha-methylated or alpha-ethylated natural amino acids. In some embodiments, one or more of the following amino acids A1, L4, R5, F7, G8, L11, N12, and Q15 of SEQ ID NO:39 are substituted with an amino acid selected from the group consisting of L-alanine, D-alanine, α-aminoisobutyric acid, N-methyl glycine, serine, a substituted alanine, and a glycine derivative. In some embodiments, one or more of the following amino acids T2, Q3, R6, K10, F13, and R14 of SEQ ID NO:39 are substituted with an alpha-methylated or alpha-ethylated natural amino acids. In certain embodiments, one or more of the following amino acids T2, Q3, R6, K10, F13, and R14 of SEQ ID NO:39 are substituted

with an amino acid selected from the group consisting of L-alanine, D-alanine, α-aminoisobutyric acid, N-methyl glycine, serine, a substituted alanine, and a glycine derivative. In certain embodiments, one or more of the following amino acids A1, T2, Q3, R5, R6, F7, G8, K10, L11, N12, F13, R14, and Q15 of SEQ ID NO:39 are modified with an alpha methyl or ethyl, or are substituted with an amino acid selected from the group consisting of L-alanine, D-alanine, α-aminoisobutyric acid, N-methyl glycine, serine, a substituted alanine, and a glycine derivative. In certain instances, the one or more amino acids of SEQ ID NO:39 that are substituted by another amino acid are on the BFL-1/A1 interacting face of the helix of SEQ ID NO:39. In other instances, the one or more amino acids of SEQ ID NO:39 that are substituted by another amino acid are on the BFL-1/A1 non-interacting and interacting faces of the helix of SEQ ID NO:39. In certain embodiments, the one or more amino acids of SEQ ID NO:39 are substituted by an amino acid or amino acids selected from the group consisting of L-alanine, D-alanine, α-aminoisobutyric acid, N-methyl glycine, serine, a substituted alanine, and a glycine derivative.

In some embodiments, one or more of the following (numbering based on SEQ ID NO: 39) are not substituted, stapled, or stitched: L4 and G8 together; R5 and D9 together; F7 and L11 together; G8 and N12 together; D9 and F13 together; T2 and D9 together; L4 and L11 together; R5 and N12 together; A1; L4; G8; or D9.

In some embodiments, overall hydrophobicity of the peptide is reduced relative to a peptide of SEQ ID NO:39 or 16. In some embodiments, overall positive charge of the peptide is reduced relative to a peptide of SEQ ID NO:39 or 16. In certain embodiments, overall hydrophobicity and overall positive charge of the peptide is reduced relative to a peptide of SEQ ID NO:39 or 16.

In some embodiments, the electrophilic warhead is a non-natural amino acid bearing an electrophilic group. In certain instances, the non-natural amino acid bearing an electrophilic group has an electrophilic acrylamide or substituted acrylamide linked to the polypeptide backbone. In some instances, the non-natural amino acid bearing an electrophilic group is selected from the group consisting of: (S)-1-acryloylpyrrolidine-3-carboxamide; 1-acrylopiperidine-4-carboxamide, (R)-1 acryloylpiperidine-3-carboxamide; (S)-1-acryloylpiperidine-3-carboxamide; (S)-1-acryloylpyyrolidine-2-carboxamide; (R)-1-acryloylpyrrolidine-2-carboxamide; (E)-4-(dimethylamino)but-2-enamide; acrylamide; aziridine, diaziridine, azetidine, pyrrolidine, imidazolidine, pyrazolidine, oxazolidine,

isoxazolidine, thiazolidine, isothiazolidine, piperidine, piperazine, morpholine, thiomorpholine, azepaneazirine, diazirine, azete, pyrrole, imidazole, pyrazole, oxazole, isoxazole, thiazole, isothiazole, pyridine, diazines, oxazine, thiazine, azepine phenyl (aniline); naphthalene, anthracene, phenanthrene, indole, isoindole, indolizine, quinolone, isoquinoline, quinoxaline, phthalzine, quinazoline, purine, carbazole, indazole, benzimidazole, azaindole, α-cyanoacrylamide, propiolamide, trans 4-dimethylamino-2-butenamide, trans 4-piperidinyl-2-butenamide, a substituted acrylamide, and a vinyl-sulfonamide. In one embodiment, the electrophilic warhead is a cysteine-reactive D-nipecotic acid moiety. In another embodiment, the electrophilic warhead is a cysteine-reactive moiety. In certain instances, the electrophilic warhead is at the N-terminus of the peptide. In other instances, the electrophilic warhead is not at the N-terminus of the peptide.

In a second aspect, the disclosure features a peptide comprising an electrophilic warhead and five or more amino acids of: A0B0C0D0E0A1B1C1D1E1A2B2C2D2E2 (SEQ ID NO:93), wherein:

(a) A0 is absent, A, D-alanine, α-aminoisobutyric acid, or a staple/stitch position;
(b) B0 is absent, T, A, D-alanine, α-aminoisobutyric acid, I, M, B (norleucine), or a staple/stitch position;
(c) C0 is absent, Q, A, D-alanine, α-aminoisobutyric acid, or a staple/stitch position;
(d) D0 is absent, L, A, D-alanine, α-aminoisobutyric acid, I, or F;
(e) E0 is R, A, D-alanine, α-aminoisobutyric acid, K, T, or a staple/stitch position;
(f) A1 is R, A, D-alanine, α-aminoisobutyric acid, E, or a staple/stitch position;
(g) B1 is F, A, D-alanine, α-aminoisobutyric acid, I, L, V, or a staple/stitch position;
(h) C1 is G, A, D-alanine, α-aminoisobutyric acid, or a staple/stitch position;
(i) D1 is D;
(j) E1 is absent, K, A, D-alanine, α-aminoisobutyric acid, or a staple/stitch position;
(k) A2 is absent, L, A, D-alanine, α-aminoisobutyric acid, W, V, or a staple/stitch position;
(l) B2 is absent, N, A, D-alanine, α-aminoisobutyric acid, S, D, or a staple/stitch position;
(m) C2 is absent, F, A, L, D-alanine, α-aminoisobutyric acid, or a staple/stitch position;
(n) D2 is absent, R, A, D-alanine, α-aminoisobutyric acid, E, or a staple/stitch position; and

(o) E2 is absent, Q, A, D-alanine, α-aminoisobutyric acid, L, or a staple/stitch position.

The peptide binds BFL-1/A1 (covalently and/or non-covalently); or the peptide binds BFL-1/A1 (covalently) and MCL-1 (non-covalently).

In certain embodiments, the peptide is 5 to 35 (e.g., 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 22, 25, 30, 35) amino acids in length. In certain embodiments, the peptide is 8 to 18 (e.g., 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18) amino acids in length.

In some embodiments, overall hydrophobicity of the peptide is reduced relative to a peptide of SEQ ID NO:39 or 16. In some embodiments, overall positive charge of the peptide is reduced relative to a peptide of SEQ ID NO:39 or 16. In certain embodiments, overall hydrophobicity and overall positive charge of the peptide is reduced relative to a peptide of SEQ ID NO:39 or 16.

In some embodiments, the electrophilic warhead is a non-natural amino acid bearing an electrophilic group. In certain instances, the non-natural amino acid bearing an electrophilic group has an electrophilic acrylamide or substituted acrylamide linked to the polypeptide backbone. In some instances, the non-natural amino acid bearing an electrophilic group is selected from the group consisting of: (S)-1-acryloylpyrrolidine-3-carboxamide; 1-acrylopiperidine-4-carboxamide, (R)-1 acryloylpiperidine-3-carboxamide; (S)-1-acryloylpiperidine-3-carboxamide; (S)-1-acryloylpyyrolidine-2-carboxamide; (R)-1-acryloylpyrrolidine-2-carboxamide; (E)-4-(dimethylamino)but-2-enamide; acrylamide; aziridine, diaziridine, azetidine, pyrrolidine, imidazolidine, pyrazolidine, oxazolidine, isoxazolidine, thiazolidine, isothiazolidine, piperidine, piperazine, morpholine, thiomorpholine, azepaneazirine, diazirine, azete, pyrrole, imidazole, pyrazole, oxazole, isoxazole, thiazole, isothiazole, pyridine, diazines, oxazine, thiazine, azepine phenyl (aniline); naphthalene, anthracene, phenanthrene, indole, isoindole, indolizine, quinolone, isoquinoline, quinoxaline, phthalzine, quinazoline, purine, carbazole, indazole, benzimidazole, azaindole, α-cyanoacrylamide, propiolamide, trans 4-dimethylamino-2-butenamide, trans 4-piperidinyl-2-butenamide, a substituted acrylamide, and a vinyl-sulfonamide. In one embodiment, the electrophilic warhead is a cysteine-reactive D-nipecotic acid moiety. In another embodiment, the electrophilic warhead is a cysteine-reactive moiety. In certain instances, the electrophilic warhead is at the N-terminus of the peptide. In other instances, the electrophilic warhead is not at the N-terminus of the peptide.

In some embodiments, the peptide contains a staple and/or stitch. In some instances, the staple, if present, is at one or more of positions *i* and *i + 3*; *i* and *i +4*; or *i* and *i+ 7*. In some embodiments, the peptide structure stabilizing modification comprises substitution of at least two (*e.g.,* 2, 3, 4, 5, 6) amino acids of SEQ ID NO:93 with non-natural amino acids with olefinic side chains. In some instances, the non-natural amino acids with olefinic side chains are selected from the group consisting of: S-pentenyl alanine, R-octenyl alanine; R-propenylalanine, S-pentenylalanine; R- pentenylalanine, S-pentenylalanine; Bis-pentenylglycine, S-pentenylalanine, R-octenylalanine; and Bis-pentenylglycine, S-octenylalanine, R-octenylalanine. In certain embodiments, the peptide comprises a hydrocarbon staple/stitch, a lactam staple/stitch; a UV-cycloaddition staple/stitch; an oxime staple/stitch; a thioether staple/stitch; a double-click staple/stitch; a bis-lactam staple/stitch; a bis-arylation staple/stitch; or a combination of any two or more thereof. In certain embodiments any two or more of the following positions in SEQ ID NO:93 are involved in a staple or stitch: A0, B0, C0, E0, A1, B1, C1, E1, A2, B2, C2, D2, E2. In certain instances, a staple position can be introduced after position E2.

In a third aspect, the disclosure provides a peptide that covalently binds to BFL-1/A1 (*e.g.,* to C55). The peptide comprising an electrophilic warhead and a sequence that is at least 14% identical to an amino acid sequence set forth in any one of SEQ ID NOs.: 94-109. The peptide selectively binds BFL-1/A1 over MCL-1. In some embodiments, the peptide is 8 to 18 (e.g., 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18) amino acids in length.

In certain embodiments, the electrophilic warhead is at the N-terminus of the peptide. In other embodiments, the electrophilic warhead is not at the N-terminus of the peptide. In some embodiments, the electrophilic warhead is a non-natural amino acid bearing an electrophilic group. In certain instances, the non-natural amino acid bearing an electrophilic group has an electrophilic acrylamide or substituted acrylamide linked to the polypeptide backbone. In some cases, the linker is a nitrogen containing heterocycle, a nitrogen containing heterocyclic amino acid, an amino-functionalized benzene ring, a carbocycle, a polycycle, or a heterocycle. In some instances, the non-natural amino acid bearing an electrophilic group is selected from the group consisting of: (S)-1-acryloylpyrrolidine-3-carboxamide; 1-acrylopiperidine-4-carboxamide, (R)-1 acryloylpiperidine-3-carboxamide; (S)-1-acryloylpiperidine-3-carboxamide; (S)-1-acryloylpyyrolidine-2-carboxamide; (R)-1-acryloylpyrrolidine-2-carboxamide; (E)-4-(dimethylamino)but-2-enamide; acrylamide; aziridine, diaziridine, azetidine, pyrrolidine, imidazolidine, pyrazolidine, oxazolidine,

isoxazolidine, thiazolidine, isothiazolidine, piperidine, piperazine, morpholine, thiomorpholine, azepaneazirine, diazirine, azete, pyrrole, imidazole, pyrazole, oxazole, isoxazole, thiazole, isothiazole, pyridine, diazines, oxazine, thiazine, azepine phenyl (aniline); naphthalene, anthracene, phenanthrene, indole, isoindole, indolizine, quinolone, isoquinoline, quinoxaline, phthalzine, quinazoline, purine, carbazole, indazole, benzimidazole, azaindole, α-cyanoacrylamide, propiolamide, trans 4-dimethylamino-2-butenamide, trans 4-piperidinyl-2-butenamide, a substituted acrylamide, and a vinyl-sulfonamide. In one embodiment, the electrophilic warhead is a cysteine-reactive D-nipecotic acid moiety.

In certain instances, the peptide comprises a hydrocarbon staple/stitch, a lactam staple/stitch; a UV-cycloaddition staple/stitch; an oxime staple/stitch; a thioether staple/stitch; a double-click staple/stitch; a bis-lactam staple/stitch; a bis-arylation staple/stitch; or a combination of any two or more thereof.

In some instances, wherein the peptide comprises a sequence that is at least 15%, 20%, 27%, 34%, 40%, 47%, 50%, 53%, 60%, 65% 70%, 75%, 80%, 85%, 90%, 95%, or 97%, or 100% identical to the amino acid sequence. In some instances, the variation from the amino acid sequence is on the BFL-1 non-interacting alpha-helical face of the amino acid sequence. In certain instances, the amino acid sequence is modified to contain an alpha-methyl or alpha-ethyl natural amino acid, and/or is substituted with one or more amino acids selected from the group consisting of alanine, D-alanine, α-aminoisobutyric acid, N-methyl glycine, serine, a substituted alanine, and a glycine derivative.

In a fourth aspect, this disclosure features a peptide that covalently binds to BFL-1/A1 (*e.g.,* to C55 of BFL-1/A1), wherein the peptide comprises a sequence that is at least 14% identical to an amino acid sequence set forth in SEQ ID NOs.: 60-75. In certain instances, the peptide selectively binds BFL-1/A1 over MCL-1. In some instances, the peptide covalently binds BFL-1/A1 and non-covalently binds MCL-1.

In certain embodiments, the electrophilic warhead is a non-natural amino acid bearing an electrophilic group.

In some embodiments, the peptide comprises a sequence that is at least 15%, 20%, 27%, 34%, 40%, 47%, 50%, 53%, 60%, 65% 70%, 75%, 80%, 85%, 90%, 95%, 97%, or 100% identical to the amino acid sequence. In certain instances, the variation from the amino acid sequence is on the BFL-1 non-interacting alpha-helical face of the amino acid sequence. In some instances, the amino acid at one or more of positions 2, 3, 6, 7, 8, 9, 12, 13, 14, 15, or

16 (wherein position 1 is "J") of the recited sequences can be substituted to another amino acid. In some instances, one or more of the amino acids at these positions is substituted to one or more of alanine, D-alanine, or α-aminoisobutyric acid. In some instances, the amino acid at positions 7 and/or 15 is substituted with a glutamic acid, and/or the amino acid at position 13 is substituted with an aspartic acid, and/or the amino acid at position 16 is substituted with a leucine, and/or the amino acids at one or more of positions 2, 3, 6, 8, 9, 12, 14, and 15 is substituted with alanine, D-alanine, or α-aminoisobutyric acid (wherein position 1 is "J"). In some instances, the amino acids at positions 8 and/or 12 is/are substituted with alanine, D-alanine, or α-aminoisobutyric acid (wherein position 1 is "J").

In some instances, the peptide comprises or consists of the sequence JAT**X_1**LREFGD**X_2**LNFRQ (**SEQ ID NO:62**), wherein the peptide selectively binds BFL-1/A1 over MCL-1, and wherein the peptide covalently binds BFL-1/A1 and non-covalently binds MCL-1; and wherein J is an electrophilic warhead; and X_1 and X_2 are non-natural amino acids.

In some instances, the peptide comprises or consists of the sequence JAT**X_1**LRRAGD**X_2**LNFRQ (**SEQ ID NO:60**), wherein the peptide selectively binds BFL-1/A1 over MCL-1, and wherein the peptide covalently binds BFL-1/A1 and non-covalently binds MCL-1; and wherein J is an electrophilic warhead; and X_1 and X_2 are non-natural amino acids.

In some instances, the electrophilic warhead has an electrophilic moiety linked to the polypeptide backbone. In some cases, the linker is a nitrogen containing heterocycle, a nitrogen containing heterocyclic amino acid, an amino-functionalized benzene ring, a carbocycle, a polycycle, or a heterocycle. In some embodiments, the electrophilic moiety is an electrophilic acrylamide, substituted acrylamide, a vinyl-sulfonamide, or an α, β unsaturated amide. In some embodiments, the electrophilic warhead is a non-natural amino acid bearing an electrophilic group. In some instances, the non-natural amino acid bearing an electrophilic group is selected from the group consisting of: (S)-1-acryloylpyrrolidine-3-carboxamide; 1-acrylopiperidine-4-carboxamide, (R)-1 acryloylpiperidine-3-carboxamide; (S)-1-acryloylpiperidine-3-carboxamide; (S)-1-acryloylpyyrolidine-2-carboxamide; (R)-1-acryloylpyrrolidine-2-carboxamide; (E)-4-(dimethylamino)but-2-enamide; acrylamide; aziridine, diaziridine, azetidine, pyrrolidine, imidazolidine, pyrazolidine, oxazolidine, isoxazolidine, thiazolidine, isothiazolidine, piperidine, piperazine, morpholine,

thiomorpholine, azepaneazirine, diazirine, azete, pyrrole, imidazole, pyrazole, oxazole, isoxazole, thiazole, isothiazole, pyridine, diazines, oxazine, thiazine, azepine phenyl (aniline); naphthalene, anthracene, phenanthrene, indole, isoindole, indolizine, quinolone, isoquinoline, quinoxaline, phthalzine, quinazoline, purine, carbazole, indazole, benzimidazole, azaindole, α-cyanoacrylamide, propiolamide, trans 4-dimethylamino-2-butenamide, trans 4-piperidinyl-2-butenamide, a substituted acrylamide, and a vinyl-sulfonamide. In one embodiment, the electrophilic warhead is a cysteine-reactive D-nipecotic acid moiety.

In certain embodiments, the peptide is 15 to 35 (*e.g.,* 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35) amino acids in length. In some embodiments, the modified amino acid sequence is 8 to 18 (e.g., 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18) amino acids in length.

In a fifth aspect, the disclosure provides a peptide that covalently binds to BFL-1/A1 (e.g., to C55 of BFL-1/A1), wherein the peptide comprises at least two (e.g., 2, 3, 4, 5, 6, 7, 8, 9) amino acids of the BFL-1/A1 interacting face of the alpha helix of an amino acid sequence set forth in any one of SEQ ID NOs.:60-75.

In certain embodiments, the peptide is 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 amino acids in length.

In certain embodiments, the peptide includes at least five (e.g., 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25) amino acids of SEQ ID NOs.:60-75, of which three, two, or one amino acid are substituted. In some instances, the substitution(s) is to alanine, D-alanine, or α-aminoisobutyric acid.

In some embodiments, the electrophilic warhead is a cysteine-reactive D-nipecotic acid moiety. In other embodiments, the electrophilic warhead is a cysteine-reactive moiety.

In certain instances, the non-natural amino acids are non-natural amino acids with olefinic side chains. In some instances, the non-natural amino acids with olefinic side chains are S-pentenyl alanine and/or R-octenyl alanine.

In a sixth aspect, the disclosure features a peptide that covalently binds to BFL-1/A1, the peptide comprising an amino acid sequence set forth in any one of SEQ ID NOs.:60-75. In some embodiments, the peptide comprises or consists of the amino acid sequence set forth in SEQ ID NO: 62. In some embodiments, the peptide comprises or consists of the amino acid sequence set forth in SEQ ID NO: 60.

In some embodiments, X_1 is R-octenyl alanine and X_2 is S-pentenyl alanine. In some embodiments, X_1 is S-pentenyl alanine and X_2 is R-octenyl alanine. In certain embodiments, X_1 is S-pentenyl alanine and X_2 is S-pentenyl alanine. In other embodiments, X_1 is R-propenylalanine and X_2 is S-pentenyl alanine. In yet other embodiments, X_1 is R-pentenylalanine and X_2 is S-pentenyl alanine.

In certain embodiments, the electrophilic warhead comprises an electrophilic moiety linked to the polypeptide backbone. In some embodiments, the electrophilic moiety is a non-natural amino acid bearing an electrophilic group. In certain instances, the non-natural amino acid bearing an electrophilic group comprises an electrophilic acrylamide, substituted acrylamide, a vinyl-sulfonamide, or an α, β unsaturated amide. In some instances, the non-natural amino acid bearing an electrophilic group is selected from the group consisting of: (S)-1-acryloylpyrrolidine-3-carboxamide; 1-acrylopiperidine-4-carboxamide, (R)-1 acryloylpiperidine-3-carboxamide; (S)-1-acryloylpiperidine-3-carboxamide; (S)-1-acryloylpyyrolidine-2-carboxamide; (R)-1-acryloylpyrrolidine-2-carboxamide; (E)-4-(dimethylamino)but-2-enamide; acrylamide; aziridine, diaziridine, azetidine, pyrrolidine, imidazolidine, pyrazolidine, oxazolidine, isoxazolidine, thiazolidine, isothiazolidine, piperidine, piperazine, morpholine, thiomorpholine, azepaneazirine, diazirine, azete, pyrrole, imidazole, pyrazole, oxazole, isoxazole, thiazole, isothiazole, pyridine, diazines, oxazine, thiazine, azepine phenyl (aniline); naphthalene, anthracene, phenanthrene, indole, isoindole, indolizine, quinolone, isoquinoline, quinoxaline, phthalzine, quinazoline, purine, carbazole, indazole, benzimidazole, azaindole, α-cyanoacrylamide, propiolamide, trans 4-dimethylamino-2-butenamide, trans 4-piperidinyl-2-butenamide, a substituted acrylamide, and a vinyl-sulfonamide. In certain instances, the electrophilic warhead is a cysteine-reactive D-nipecotic acid moiety. In other instances, the electrophilic warhead is a cysteine-reactive moiety.

In some embodiments, the peptide is 15 to 35 (*e.g.,* 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35) amino acids in length. In some embodiments, the peptide is 8 to 18 (e.g., 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18) amino acids in length.

In a seventh aspect the disclosure relates to a pharmaceutical composition comprising a NOXA peptide described herein and a pharmaceutically acceptable carrier.

In an eighth aspect, the disclosure features a method of treating a BFL-1/A1-expressing or BFL-1/A1-dependent disease in a human subject in need thereof. The method

involves administering to the human subject a therapeutically effective amount of a NOXA peptide described herein, or a pharmaceutical composition described herein.

In some embodiments, the BFL-1/A1-expressing or BFL-1/A1-dependent disease is selected from the group consisting of a hematologic malignancy, a solid tumor, an autoimmune disease, and an inflammatory disease. In certain instances, the hematologic malignancy is a leukemia or a lymphoma. In certain instances, the solid tumor that is a melanoma, a breast cancer, or a lung cancer. In certain instances, the autoimmune disease that is autoimmune colitis, thyroiditis, arthritis, nephritis, dermatitis, vasculitis, system lupus erythematosus, diabetes, or Sjogren's disease. In certain instances, the inflammatory disease that is asthma, psoriasis, inflammatory colitis, thyroiditis, arthritis, nephritis, dermatitis, or vasculitis.

In some embodiments, the BFL-1/A1-expressing or BFL-1/A1-dependent disease is a cancer. In some embodiments, the cancer is a solid tumor or a liquid tumor. In some embodiments, the cancer is a breast cancer, an autonomic ganglia cancer, a pancreatic cancer, a skin cancer, a CNS cancer, a hematopoietic or lymphoid cancer, a lung cancer, a large intestine cancer, a stomach cancer, a soft tissue sarcoma, or a bone cancer. In some embodiments, the hematologic malignancy is acute myeloid leukemia, acute lymphoblastic leukemia, chronic myeloid leukemia, chronic lymphocytic leukemia, myelodysplastic syndrome, or multiple myeloma.

In some embodiments, the NOXA peptide is selected from SEQ ID NOs.: 60-79, 151, and 153. In some embodiments, the NOXA peptide is SEQ ID NO: 62. In some embodiments, the NOXA peptide is SEQ ID NO: 60.

In some embodiments, the method involves further administering an inhibitor of a DNA damage response pathway member. In some embodiments, the DNA damage response pathway member inhibitor is an ataxia-telangiectasia mutated (ATM) kinase inhibitor, an ataxia telangiectasia and rad3-related (ATR) kinase inhibitor, a checkpoint kinase 1 and/or a checkpoint kinase 2 (CHK1/2) inhibitor, and/or a poly(ADP-ribose) polymerase (PARP) inhibitor. In some instances, the ATM inhibitor is selected from the group consisting of KU-559403, KU-55933, KU-60019, CP-466722, chloroquine phosphate, CGK733, and AZD0156. In certain instances, the ATR inhibitor is selected from the group consisting of schisandrin B, NU6027, NVP-BEZ235, VE-821, VE-822/VX-970, AZ20, and AZD6738. In certain instances, the CHK1/2 inhibitor is selected from the group consisting of AZD7762,

CCT 241533, LY 2603618, LY 2606368, NSC 109555 ditosylate, PD 407824, PF47736, SB 218078, TCS 2312, and SRA737. In certain instances, the PARP inhibitor is selected from the group consisting of olaparib, A-966492, veliparib, rucaparib, AG-14361, iniparib, INO-1001, niraparib, talazoparib, AZD2461, 2X-121, BMN 673, and E7449.

In a ninth aspect, the disclosure features a method of treating a MCL-1-expressing or MCL-1-dependent disease in a human subject in need thereof. The method involves administering to the human subject a therapeutically effective amount of a NOXA peptide described herein, or a pharmaceutical composition described herein.

In some embodiments, the NOXA peptide is selected from SEQ ID NOs.: 60-79, 151, and 153 or a structurally-stabilized version of a sequence set forth in SEQ ID NOs.: 126-141.

In some embodiments, the method further involves administering an inhibitor of a DNA damage response pathway member. In some embodiments, the DNA damage response pathway member inhibitor is an ATM kinase inhibitor, an ATR kinase inhibitor, a CHK1/2 inhibitor, and/or a PARP inhibitor. In some instances, the ATM inhibitor is selected from the group consisting of KU-559403, KU-55933, KU-60019, CP-466722, chloroquine phosphate, CGK733, and AZD0156. In certain instances, the ATR inhibitor is selected from the group consisting of schisandrin B, NU6027, NVP-BEZ235, VE-821, VE-822/VX-970, AZ20, and AZD6738. In certain instances, the CHK1/2 inhibitor is selected from the group consisting of AZD7762, CCT 241533, LY 2603618, LY 2606368, NSC 109555 ditosylate, PD 407824, PF47736, SB 218078, TCS 2312, and SRA737. In certain instances, the PARP inhibitor is selected from the group consisting of olaparib, A-966492, veliparib, rucaparib, AG-14361, iniparib, INO-1001, niraparib, talazoparib, AZD2461, 2X-121, BMN 673, and E7449.

In a tenth aspect, the disclosure features a method of treating a BFL-1/A1-expressing or BFL-1/A1-dependent disease and an MCL-1-expressing or MCL-1-dependent disease in a human subject in need thereof. The method involves administering to the human subject a therapeutically effective amount of a NOXA peptide described herein, or a pharmaceutical composition described herein.

In some embodiments, the NOXA peptide is selected from SEQ ID NOs.: 60-79, 151, and 153 or a structurally-stabilized version of a sequence set forth in SEQ ID NOs.: 126-141. In some embodiments, the NOXA peptide is selected from SEQ ID NOs.: 60 and 62 or a structurally-stabilized version of a sequence set forth in SEQ ID NOs.: 126 and 128.

In some embodiments, the method further involves administering an inhibitor of a DNA damage response pathway member. In some embodiments, the DNA damage response pathway member inhibitor is an ATM kinase inhibitor, an ATR kinase inhibitor, a CHK1/2 inhibitor, and/or a PARP inhibitor. In some instances, the ATM inhibitor is selected from the group consisting of KU-559403, KU-55933, KU-60019, CP-466722, chloroquine phosphate, CGK733, and AZD0156. In certain instances, the ATR inhibitor is selected from the group consisting of schisandrin B, NU6027, NVP-BEZ235, VE-821, VE-822/VX-970, AZ20, and AZD6738. In certain instances, the CHK1/2 inhibitor is selected from the group consisting of AZD7762, CCT 241533, LY 2603618, LY 2606368, NSC 109555 ditosylate, PD 407824, PF47736, SB 218078, TCS 2312, and SRA737. In certain instances, the PARP inhibitor is selected from the group consisting of olaparib, A-966492, veliparib, rucaparib, AG-14361, iniparib, INO-1001, niraparib, talazoparib, AZD2461, 2X-121, BMN 673, and E7449.

In an eleventh aspect, the disclosure provides a combination therapy for treating a BFL-1/A1-expressing or BFL-1/A1-dependent disease (*e.g.,* hematologic malignancy, a solid tumor, an autoimmune disease, and an inflammatory disease) comprising a NOXA peptide described herein and an inhibitor of a DNA damage response pathway member. In some embodiments, the DNA damage response pathway member inhibitor is an ATM kinase inhibitor, an ATR kinase inhibitor, a CHK1/2 inhibitor, and/or a PARP inhibitor.

In a twelfth aspect, the disclosure provides a combination therapy for treating a MCL-1 expressing or MCL-1-dependent disease (*e.g.,* hematologic malignancy, a solid tumor, an autoimmune disease, and an inflammatory disease) comprising a NOXA peptide described herein and an inhibitor of a DNA damage response pathway member. In some embodiments, the DNA damage response pathway member inhibitor is an ATM kinase inhibitor, an ATR kinase inhibitor, a CHK1/2 inhibitor, and/or a PARP inhibitor.

In a thirteenth aspect, the disclosure provides a combination therapy for treating a BFL-1/A1-expressing or BFL-1/A1-dependent disease and MCL-1 expressing or MCL-1-dependent disease (*e.g.,* hematologic malignancy, a solid tumor, an autoimmune disease, and an inflammatory disease) comprising a NOXA peptide described herein and an inhibitor of a DNA damage response pathway member. In some embodiments, the DNA damage response pathway member inhibitor is an ATM kinase inhibitor, an ATR kinase inhibitor, a CHK1/2 inhibitor, and/or a PARP inhibitor.

These embodiments apply to the eleventh to thirteenth aspects. In some embodiments, the ATM inhibitor is selected from the group consisting of KU-559403, KU-55933, KU-60019, CP-466722, chloroquine phosphate, CGK733, and AZD0156. In certain embodiments, the ATR inhibitor is selected from the group consisting of schisandrin B, NU6027, NVP-BEZ235, VE-821, VE-822/VX-970, AZ20, and AZD6738. In certain instances, the CHK1/2 inhibitor is selected from the group consisting of AZD7762, CCT 241533, LY 2603618, LY 2606368, NSC 109555 ditosylate, PD 407824, PF47736, SB 218078, TCS 2312, and SRA737. In certain instances, the PARP inhibitor is selected from the group consisting of olaparib, A-966492, veliparib, rucaparib, AG-14361, iniparib, INO-1001, niraparib, talazoparib, AZD2461, 2X-121, BMN 673, and E7449. In some embodiments, the NOXA peptide is selected from SEQ ID NOs.: 60-79, 151, and 153 or a structurally-stabilized version of a sequence set forth in SEQ ID NOs.: 126-141. In some embodiments, the NOXA peptide is selected from SEQ ID NOs.: 60 and 62 or a structurally-stabilized version of a sequence set forth in SEQ ID NOs.: 126 and 128.

In a fourteenth aspect, the disclosure provides a stapled peptide comprising an amino acid sequence that is at least 50% identical (*e.g.,* at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% identical) to the amino acid sequence set forth in SEQ ID NO:44. In certain instances, the stapled peptide comprises an amino acid sequence set forth in SEQ ID NO:44 except that it has one to ten (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10) amino acid substitutions. In certain instances, the substitution(s) is to an amino acid or amino acids selected from the group consisting of L-alanine, D-alanine, α-aminoisobutyric acid, N-methyl glycine, serine, a substituted alanine, and a glycine derivative. The stapled peptide binds BFL-1 and/or MCL-1.

In a fourteenth aspect, the disclosure provides a stapled peptide comprising an amino acid sequence that is at least 50% identical (*e.g.,* at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% identical) to the amino acid sequence set forth in SEQ ID NO:46. In certain instances, the stapled peptide comprises an amino acid sequence set forth in SEQ ID NO:46 except that it has one to ten (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10) amino acid substitutions. In certain instances, the substitution(s) is to an amino acid or amino acids selected from the group consisting of L-alanine, D-alanine, α-aminoisobutyric acid, N-methyl glycine, serine, a substituted alanine, and a glycine derivative. The stapled peptide binds BFL-1 and/or MCL-1.

These embodiments apply to the fourteenth aspect. In some embodiments, the stapled peptide further comprises a cysteine-reactive moiety. In some embodiments, the stapled peptide further comprises a cysteine-reactive D-nipecotic acid moiety. These moieties can be attached or included at the N-terminus of the stapled peptide. In some embodiments, the stapled peptide is combined with a DNA damage response pathway inhibitor. In some embodiments, the DNA damage response pathway inhibitor is an ATM kinase inhibitor. In some embodiments, the DNA damage response pathway inhibitor is an ATR kinase inhibitor. In some embodiments, the DNA damage response pathway inhibitor is a CHK1/2 inhibitor. In some embodiments, the DNA damage response pathway inhibitor is a PARP inhibitor. In some embodiments, the ATM inhibitor is selected from the group consisting of KU-559403, KU-55933, KU-60019, CP-466722, chloroquine phosphate, CGK733, and AZD0156. In certain embodiments, the ATR inhibitor is selected from the group consisting of schisandrin B, NU6027, NVP-BEZ235, VE-821, VE-822/VX-970, AZ20, and AZD6738. In certain embodiments, the CHK1/2 inhibitor is selected from the group consisting of AZD7762, CCT 241533, LY 2603618, LY 2606368, NSC 109555 ditosylate, PD 407824, PF47736, SB 218078, TCS 2312, and SRA737. In certain embodiments, the PARP inhibitor is selected from the group consisting of olaparib, A-966492, veliparib, rucaparib, AG-14361, iniparib, INO-1001, niraparib, talazoparib, AZD2461, 2X-121, BMN 673, and E7449. The stapled peptides disclosed herein can be used to treat a BFL-1-expressing or –dependent condition (*e.g.,* cancer, autoimmune disease, inflammatory disease). The stapled peptides disclosed herein can be used to treat a MCL-1-expressing or –dependent condition (*e.g.,* cancer, autoimmune disease, inflammatory disease).

In a fifteenth aspect, the disclosure provides a combination therapy for treating a MCL-1-expressing and a BFL-1/A1-expressing, or MCL-1-dependent and BFL-1/A1-dependent, disease in a human subject in need thereof, comprising a NOXA stabilized peptide described herein and an inhibitor of MCL-1. In certain instances, the MCL-1 inhibitor is selected from the group consisting of S64315, S63845, BAY 1000394, MIK665, AMG397, and AMG176.

In a sixteenth aspect, the disclosure provides a combination therapy for treating a BCL-2-expressing and a BFL-1/A1-expressing, or BCL-2-dependent and BFL-1/A1-dependent, disease in a human subject in need thereof, comprising a NOXA stabilized peptide described herein and an inhibitor of BCL-2. In certain instances, the BCL-2 inhibitor is venetoclax.

In a seventeenth aspect, the disclosure provides a combination therapy for treating a BCL-XL-expressing and a BFL-1/A1-expressing, or BCL-XL-dependent and BFL-1/A1-dependent, disease in a human subject in need thereof, comprising a NOXA stabilized peptide described herein and an inhibitor of BCL-XL. In certain instances, the BCL-XL inhibitor is navitoclax.

In an eighteenth aspect, the disclosure provides a combination therapy for treating a BCL-XL-, BCL-2-, and/or MCL-1-expressing and a BFL-1/A1-expressing, or BCL-XL-, BCL-2-, and MCL-1-dependent and BFL-1/A1-dependent, disease in a human subject in need thereof, comprising a NOXA stabilized peptide described herein and an inhibitor of BCL-XL, BCL-2, and/or MCL-1. In certain instances, the MCL-1 inhibitor is selected from the group consisting of S64315, S63845, BAY 1000394, MIK665, AMG397, and AMG176 In certain instances, the BCL-2 inhibitor is venetoclax. In certain instances, the BCL-XL inhibitor is navitoclax.

These embodiments apply to each of the fifteenth to eighteenth aspects. In some embodiments, the NOXA peptide is selected from SEQ ID NOs.: 60-79, 151, and 153 or a structurally-stabilized version of a sequence set forth in SEQ ID NOs.: 126-141. In some embodiments, the NOXA peptide is selected from SEQ ID NOs.: 60 and 62 or a structurally-stabilized version of a sequence set forth in SEQ ID NOs.: 126 and 128.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the exemplary methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present application, including definitions, will control. The materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the structure of the NOXA BH3/BFL-1ΔC complex (PDB: 3MQP), highlighting in dark gray the NOXA residues selected for the shortened NOXA peptide staple scan library.

Figure 2 provides the sequences of a NOXA BH3 staple scan peptide library, with all-hydrocarbon (*i, i+4*) or (*i, i+7*) staples placed sequentially across the length of the peptide template bearing an N-terminal, cysteine-reactive, D-nipecotic acid moiety. The sequences (*i.e.,* with the D-nipecotic acid derivatization placed at the stapled peptide N-terminus) are assigned **SEQ ID NOs.: 1-21**, from top to bottom.

Figure 3 shows the reactivity of D-nipecotic acid (D-NA)-bearing NOXA SAHBs with BFL-1ΔC C4S/C19S, which only retains the native Cys55.

Figure 4 shows cell permeability and reactivity of D-NA-NOXA SAHBs toward BFL-1 in 293T cells expressing HA-BFL-1ΔC C4S/C19S.

Figure 5 shows the quantification of LDH release upon treatment of A375P cells with NOXA-15 for 30 min, demonstrating the induction of non-specific cell lysis by NOXA-15.

Figure 6 provides sequences of a NOXA-15 SAHB alanine scan library bearing an N-terminal, cysteine-reactive, D-nipecotic acid moiety. The sequences (*i.e.,* with the D-nipecotic acid derivatization placed at the stapled peptide N-terminus) are assigned **SEQ ID NOs.: 22-34**, from top to bottom.

Figure 7 is a bar graph LDH release of a D-NA-NOXA-15 SAHB alanine scan library demonstrating three peptides that lack lytic activity as a result of alanine mutation.

Figure 8 is a Coomassie-stained gel showing reactivity of a D-NA-NOXA-15 SAHB alanine scan library with recombinant BFL-1ΔC C4S/C19S, emphasizing the broad tolerance for alanine mutation except at select positions, such as L29A and D34A, which show reduced or partial reactivity due to focal alanine mutagenesis.

Figure 9 is a western blot showing the cell permeability and reactivity of non-lytic, D-NA-NOXA-15 alanine-mutant SAHBs toward BFL-1 in 293T cells expressing HA-BFL-1ΔC C4S/C19S.

Figure 10 lists the sequences of NOXA-15 SAHB mutants bearing N-terminal, cysteine-reactive, D-nipecotic acid moiety. The sequences (*i.e.,* with the D-nipecotic acid

derivatization placed at the stapled peptide N-terminus) are assigned **SEQ ID NOs.: 35-38**, from top to bottom.

Figure 11 is a bar graph depicting LDH release of D-NA-NOXA-15 SAHB Arg to Glu charge reversal mutant peptides demonstrating marked reduction or elimination of lytic activity.

Figure 12 is a Coomassie-stained gel showing the reactivity of D-NA-NOXA-15 SAHB Arg to Glu charge reversal mutants with recombinant BFL-1ΔC C4S/C19S.

Figure 13 are blots showing cell permeability and reactivity of non-lytic, D-NA-NOXA-15 SAHBs Arg to Glu charge reversal mutants toward BFL-1 in 293T cells expressing HA-BFL-1ΔC C4S/C19S.

Figure 14 is a silver-stained gel showing that D-NA-NOXA-15 F32A exhibits a striking competitive advantage for BFL-1 targeting over its acetylated counterpart as demonstrated by streptavidin pulldown of an equimolar mixture of recombinant GST-MCL-ΔNΔC, BCL-X_LΔC (tagless), His-BFL-1ΔC, and biotinylated SAHB.

Figure 15 is a silver-stained gel showing that D-NA-NOXA-15 R31E exhibits a striking competitive advantage for BFL-1 targeting over its acetylated counterpart as demonstrated by streptavidin pulldown of an equimolar mixture of recombinant GST-MCL-ΔNΔC, BCL-X_LΔC (tagless), His-BFL-1ΔC, and biotinylated SAHB.

Figure 16 is a blot showing that D-NA-NOXA-15 F32A displays enhanced targeting of native BFL-1 compare to the corresponding acetylated construct in A375P lysates, as monitored by streptavidin pulldown and BFL-1 western analysis. Both constructs similarly engage MCL-1, as demonstrated by MCL-1 western analysis of the streptavidin pull down.

Figure 17 is a blot showing that D-NA-NOXA-15 R31E displays enhanced targeting of native BFL-1 compare to the corresponding acetylated construct in A375P lysates, as monitored by streptavidin pulldown and BFL-1 western analysis. Both constructs similarly engage MCL-1, as demonstrated by MCL-1 western analysis of the streptavidin pull down.

Figure 18 includes blots showing that D-NA-NOXA15 F32A, but not its acetylated counterpart, effectively competes with tBID for HA-BFL-1ΔC C4S/C19S interaction in 293T lysates and achieves robust covalent conjugation, as measured by immunoprecipitation and

western analysis (left). In contrast, neither D-NA- nor Ac-NOXA15 F32A is able to dissociate the interaction of FLAG-MCL-1 and tBID (right).

Figure 19 shows blots that indicate that D-NA-NOXA15 R31E, but not its acetylated counterpart, effectively competes with tBID for HA-BFL-1ΔC C4S/C19S interaction in 293T lysates and achieves robust covalent conjugation, as measured by immunoprecipitation and western analysis (left). In contrast, neither D-NA- nor Ac-NOXA15 R31E is able to dissociate the interaction of FLAG-MCL-1 and tBID (right).

Figure 20 is a graph showing that D-NA-NOXA-15 R31E, but not its acetylated counterpart, displays the ability to disrupt the interaction of BFL-1ΔC C4S/C19S and FITC-BID BH3 in a fluorescence polarization competitive binding assay.

Figure 21 is a graph showing that neither D-NA- nor Ac-NOXA15 R31E competes for FITC-BID BH3 binding to BFL-1ΔC C4S/C19S/C55S, demonstrating the necessity of C55 in the BH3 binding pocket of BFL-1 for selective covalent targeting

Figure 22 is a graph depicting that both Ac- and D-NA-NOXA-15 R31E display a similar and relatively limited-to-moderate capacity to compete with FITC-BID BH3 binding to MCL-1ΔNΔC.

Figure 23 is a graph showing that neither Ac- nor D-NA-NOXA-15 R31E is able to compete for FITC-BID BH3 binding to BCL-X_LΔC.

Figure 24 is a plot of AML cell lines that display varying levels of dependency on BFL-1 for cell survival as determined by genome-wide CRISPR screening.

Figure 25 is a table showing genetic dependencies that are positively correlated with BFL-1 dependency in AML cell lines as determined by genome-wide CRISPR screening.

Figure 26 are graphs showing that treatment of BFL-1-dependent U937 cells with the combination of the cysteine-reactive NOXA-15 R31E and the ATM inhibitor KU-55933 caused a synergistic decrease in cell viability (left), as reflected by Calcusyn analysis and the plotted dose-effect curves (right).

Figure 27 is a bar graph showing that U937 cells treated with the cysteine-reactive NOXA-15 R31E and KU-55933 singly and in combination exhibit significantly increased caspase-3/7 activation over time upon combination treatment compared to single agent treatments.

Figure 28 are graphs showing that co-treatment of the MV4-11 cell line, which is not dependent on BFL-1, resulted in little to no cytotoxic synergy of cysteine-reactive NOXA-15 R31E and the ATM inhibitor.

Figure 29 is a bar graph showing that MV4-11 cells treated with a combination of the cysteine-reactive NOXA-15 R31E and KU-55933 demonstrate no increase in caspase-3/7 activation compared to the single agent treatments.

Figure 30 is a table providing crystallization conditions and data collection and refinement statistics for the crystal structures of apo BFL-1 and its complex with a cysteine-reactive NOXA SAHB inhibitor.

Figure 31A provides the amino acid sequence of BFL-1ΔC (**SEQ ID NO:83**) with α-helices shaded to correspond with the structure shown in Figure 31B. BH1-3 domains are underlined.

Figure 31B shows ribbon and surface views of apo BFL-1ΔC (PDB ID: 5WHI) demonstrating the relative disposition of α-helices and the BH3-binding surface groove, which is formed by α-helices 2-4. A surface accessible cysteine located within the groove is noted.

Figure 31C shows ribbon diagram overlay of the apo structures of BFL-1 (PDB ID: 5WHI), MCL-1 (PDB ID: 4WMS), and BCL-X_L (PDB ID: 1R2D) demonstrating the similarities (above) and differences (below) among the orientations of their α-helices.

Figure 31D tabulates the comparative α-carbon distance measurements between apical and basal residues of the BH3-binding grooves of apo BFL-1, MCL-1, and BCL-X_L.

Figure 31E shows an alignment of amino acid sequences of the α2- α5 regions of BCL-2 family proteins, shaded to correspond with the structures in Figure 31D. BFL-1 is the only protein with a cysteine located at the indicated position within the BH3-binding groove (see C in α3). The sequences from top to bottom are assigned **SEQ ID NOs.: 84-91**.

Figure 31F shows a structural comparison of the BH3-binding grooves of BCL-2 family proteins, revealing that only BFL-1 contains a surface accessible cysteine residue.

Figure 32A provides the results of *in vitro* crosslinking analysis demonstrating the selective derivatization of C55-bearing BFL-1 constructs by an *i, i+7*-stapled NOXA BH3 peptide (aa 26-40) containing an N-terminal, cysteine-reactive, D-nipecotic acid (D-NA).

*AT**8**LRRFGD**X**LNFRQ, wherein * = Ac or D-Nipecotic Acid; 8 = R-octenyl alanine; and X = S-pentenyl alanine. (**SEQ ID NO:92**)

Figure 32B provides intact mass spectrometry analysis demonstrating the gain in molecular weight of BFL-1ΔC C4S/C19S upon covalent reaction with D-NA-NOXA SAHB.

Figure 32C shows the results of incubation of the indicated C-terminally biotinylated-NOXA SAHBs with a mixture of recombinant MCL-1, BCL-X_L, and BFL-1 proteins demonstrates selective covalent modification of BFL-1 by D-NA-NOXA SAHB (lane 3). Streptavidin pull downs revealed no interaction between NOXA SAHBs and BCL-X_L, and relatively minimal, equivalent engagement of MCL-1 (lanes 5-6). In contrast, D-NA-NOXA SAHB, but not Ac-NOXA SAHB, showed robust BFL-1 pull down, as demonstrated by silver stain (lane 6).

Figure 32D is a blot showing lysates from BFL-1-expressing A375P melanoma cells that were subjected to the identical conditions described in **Figure 32C**, followed by BFL-1 western blot of electrophoresed eluates from the streptavidin pull downs. Incubation with D-NA-NOXA SAHB resulted in markedly enhanced BFL-1 pull down compared to Ac-NOXA SAHB.

Figure 33A shows the crystal structure of the D-NA-NOXA SAHB/BFL-1ΔC complex (PDB ID: 5WHH) demonstrating the complementary hydrophilic interactions between the amphipathic BH3 helix and BFL-1, with the *i*, *i*+7 staple observed in cis orientation and located on the non-interacting surface.

Figure 33B shows a stereoview of the BFL-1/D-NA-NOXA SAHB covalent complex**.**

Figure 33C shows the crystal structure of the D-NA-NOXA SAHB/BFL-1ΔC complex demonstrating the complementary hydrophobic interactions between the amphipathic BH3 helix and BFL-1, with the *i*, *i*+7 staple observed in cis orientation and located on the non-interacting surface.

Figure 33D identifies the covalent bond between C55 of BFL-1 and the D-NA moiety.

Figure 33E shows the noncovalent NOXA BH3 (PDB ID: 3MQP) complexes with BFL-1/A1.

Figure 33F shows the covalent D-NA-NOXA SAHB complex with BFL-1.

Figure 33G demonstrates how the D-NA moiety engages in supplementary contacts with residues of a hydrophobic cavity located subjacent to C55.

Figure 34A is a ribbon overlay of the apo and D-NA-NOXA SAHB-bound structures of BFL-1, demonstrating the relative upward displacement of α2 and α3, and downward displacement of α4, upon covalent ligand interaction.

Figure 34B tabulates the comparative α-carbon distance measurements between apical and basal residues of the unliganded and NOXA BH3-bound grooves of BFL-1 and MCL-1.

Figure 34C shows focal changes that are evident in the corresponding difference distance matrix plot (see black arrowheads).

Figure 34D shows a plot of the α-carbon distance differences upon covalent engagement of BFL-1 by D-NA-NOXA SAHB quantitatively demonstrates the displacement of residues within the α2-α4 region of the BH3-binding groove.

Figure 34E shows the apo form of BFL-1ΔC.

Figure 34F shows that the liganded form of BFL-1ΔC results in a larger interaction surface, which includes the formation of a discrete hydrophobic cavity subjacent to C55 (see white arrow).

Figure 35A shows a difference distance matrix plot for NOXA BH3 non-covalent engagement with BFL-1, revealing focal changes in the α2-α4 region (see black arrowheads) similar to that observed upon covalent BH3 targeting.

Figure 35B shows a difference distance matrix plot for murine NOXA BH3 non-covalent engagement with MCL-1, notably lacking the focal α2-α4 region changes observed for the NOXA BH3 interactions (covalent and non-covalent) with BFL-1.

Figure 36 shows the chemical structures of exemplary unnatural amino acids used to generate various kinds of staples (top). The middle panel illustrates peptides with staples of various lengths. The bottom panel illustrates a staple walk along a peptide sequence.

Figure 37 is a schematic showing representations of various kinds of double and triple stapling strategies along with exemplary staple walks.

Figure 38 is a schematic showing exemplary staple walks using various lengths of branched double staple moieties.

Figure 39 is a schematic showing exemplary chemical alterations that are employed to generate stapled peptide derivatives.

Figure 40 are graphs showing that treatment of BFL-1-dependent U937 cells with the combination of cysteine-reactive NOXA-15 R31E and the ATM inhibitor AZD0156 caused a synergistic decrease in cell viability (left), as reflected by Calcusyn analysis dose-effect plot (right). Left panel: for each concentration in the left panel, the bars are (from left to right): (i) D-NA-NOXA-15 R31E, (ii) AZD0156, and (iii) 8:1 D-NA-NOXA-15 R31E:AZD0156. Right panel: each dot in the plot is classified as "Strong synergy".

Figure 41 is a bar graph showing that U937 cells treated with cysteine-reactive NOXA-15 R31E and AZD0156 singly and in combination exhibit significantly increased caspase-3/7 activation over time upon combination treatment compared to single agent treatment. For each time point, the bars are (from left to right): (i) 40 μM D-NA-NOXA-15 R31E, (ii) 5 μM AZD0156, and (ii) 40 μM D-NA-NOXA-15 R31E + 5 μM AZD0156.

Figure 42 are graphs showing that co-treatment of the MV4;11 cell line, which is not dependent on BFL-1, resulted in little to no cytotoxic synergy of cysteine-reactive NOXA-15 R31E and the ATM inhibitor AZD0156. Left panel: for each concentration in the left panel, the bars are (from left to right): (i) D-NA-NOXA-15 R31E, (ii) AZD0156, and (iii) 8:1 D-NA-NOXA-15 R31E:AZD0156. Right panel: the dots are (from left to right): (i) strong antagonism, (ii) strong synergy, (iii) synergy, (iv) additivity, (v) additivity, and (vi) additivity.

Figure 43 is a bar graph showing that MV4;11 cells treated with cysteine-reactive NOXA-15 R31E and AZD0156 singly and in combination exhibit no increase in caspase-3/7 activation compared to single agent treatments. For each time point, the bars are (from left to right): (i) 40 μM D-NA-NOXA-15 R31E, (ii) 5 μM AZD0156, and (ii) 40 μM D-NA-NOXA-15 R31E + 5 μM AZD0156.

Figure 44 are graphs showing that treatment of BFL-1-dependent OCI-AML-3 cells with the combination of cysteine-reactive NOXA-15 R31E and the ATM inhibitor AZD0156 caused a synergistic decrease in cell viability (left), as reflected by Calcusyn analysis dose-effect plot (right). Left panel: for each concentration in the left panel, the bars are (from left

to right): (i) D-NA-NOXA-15 R31E, (ii) Indicated Drug, and (iii) NOXA:Drug Combination. Right panel: each dot in the plot is classified as "Strong synergy".

Figure 45 are graphs showing that treatment of BFL-1-dependent Jurkat cells with the combination of cysteine-reactive NOXA-15 R31E and the ATM inhibitor AZD0156 caused a synergistic decrease in cell viability (left), as reflected by Calcusyn analysis dose-effect plot (right). Left panel: for each concentration in the left panel, the bars are (from left to right): (i) D-NA-NOXA-15 R31E, (ii) Indicated Drug, and (iii) NOXA:Drug Combination. Right panel: the dots are (from left to right): (i) strong antagonism, (ii) antagonism, (iii) strong synergy, (iv) strong synergy, (v) strong synergy, and (vi) strong synergy.

Figure 46 are graphs showing that treatment of BFL-1-dependent U937 cells with the combination of cysteine-reactive NOXA-15 R31E and the ATM inhibitor Ku-60019 caused a synergistic decrease in cell viability (left), as reflected by Calcusyn analysis dose-effect plot (right). Left panel: for each concentration in the left panel, the bars are (from left to right): (i) D-NA-NOXA-15 R31E, (ii) Indicated Drug, and (iii) NOXA:Drug Combination. Right panel: the dots are (from left to right): (i) additivity, (ii) strong synergy, (iii) strong synergy, (iv) strong synergy, (v) strong synergy, and (vi) strong synergy.

Figure 47 are graphs showing that treatment of BFL-1-dependent U937 cells with the combination of cysteine-reactive NOXA-15 R31E and the ATM inhibitor Ku-55933 caused a synergistic decrease in cell viability (left), as reflected by Calcusyn analysis dose-effect plot (right). Left panel: for each concentration in the left panel, the bars are (from left to right): (i) D-NA-NOXA-15 R31E, (ii) Indicated Drug, and (iii) NOXA:Drug Combination. Right panel: the dots are (from left to right): (i) strong antagonism, (ii) strong synergy, (iii) strong synergy, (iv) strong synergy, and (v) strong synergy.

Figure 48 are graphs showing that co-treatment of the MV4;11 cell line, which is not dependent on BFL-1, resulted in little to no cytotoxic synergy of cysteine-reactive NOXA-15 R31E and the ATM inhibitor Ku-60019. Left panel: for each concentration in the left panel, the bars are (from left to right): (i) D-NA-NOXA-15 R31E, (ii) Indicated Drug, and (iii) NOXA:Drug Combination. Right panel: the dots are (from left to right): (i) additivity, (ii) strong synergy, (iii) additivity, (iv) synergy, (v) additivity, and (vi) additivity.

Figure 49 are graphs showing that co-treatment of the MV4;11 cell line, which is not dependent on BFL-1, resulted in little to no cytotoxic synergy of cysteine-reactive NOXA-15 R31E and the ATM inhibitor Ku-55933. Left panel: for each concentration in the left panel,

the bars are (from left to right): (i) D-NA-NOXA-15 R31E, (ii) Indicated Drug, and (iii) NOXA:Drug Combination. Right panel: the dots are (from left to right): (i) synergy, (ii) synergy, (iii) additivity, (iv) antagonism, (v) antagonism, and (vi) strong synergy.

Figure 50 are graphs showing that treatment of BFL-1-dependent U937 cells with the combination of cysteine-reactive NOXA-15 R31E and the CHK1/2 inhibitor AZD7762 caused a synergistic decrease in cell viability (left), as reflected by Calcusyn analysis dose-effect plot (right). Left panel: for each concentration in the left panel, the bars are (from left to right): (i) D-NA-NOXA-15 R31E, (ii) Indicated Drug, and (iii) NOXA:Drug Combination. Right panel: the dots are (from left to right): (i) additivity, (ii) antagonism, (iii) strong synergy, (iv) strong synergy, (v) strong synergy, and (vi) strong synergy.

Figure 51 are graphs showing that treatment of BFL-1-dependent U937 cells with the combination of cysteine-reactive NOXA-15 R31E and the PARP inhibitor Olaparib caused a synergistic decrease in cell viability (left), as reflected by Calcusyn analysis dose-effect plot (right). Left panel: for each concentration in the left panel, the bars are (from left to right): (i) D-NA-NOXA-15 R31E, (ii) Indicated Drug, and (iii) NOXA:Drug Combination. Right panel: the dots are (from left to right): (i) strong antagonism, (ii) strong synergy, (iii) strong synergy, (iv) strong synergy, (v) strong synergy, and (vi) synergy.

Figure 52 are graphs showing that co-treatment of the MV4;11 cell line, which is not dependent on BFL-1, resulted in little to no cytotoxic synergy of cysteine-reactive NOXA-15 R31E and the CHK1/2 inhibitor AZD7762. Left panel: for each concentration in the left panel, the bars are (from left to right): (i) D-NA-NOXA-15 R31E, (ii) Indicated Drug, and (iii) NOXA:Drug Combination. Right panel: the dots are (from left to right): (i) strong synergy, (ii) synergy, (iii) additivity, (iv) antagonism, (v) antagonism, and (vi) additivity.

Figure 53 are graphs showing that co-treatment of the MV4;11 cell line, which is not dependent on BFL-1, resulted in no cytotoxic synergy of cysteine-reactive NOXA-15 R31E and the PARP inhibitor Olaparib. Left panel: for each concentration in the left panel, the bars are (from left to right): (i) D-NA-NOXA-15 R31E, (ii) Indicated Drug, and (iii) NOXA:Drug Combination. Right panel: each of the six dots are strong antagonism.

Figure 54 shows the cell viability (A), Calcusyn dose-effect plots (B), and caspase-3/7 activation (C) results for KG-1 cells treated with D-NA-NOXA SAHB-15 R31E, ATM inhibitor AZD0156, or the combination at the indicated doses. A: for each concentration, the bars are (from left to right): (i) D-NA-NOXA-15 R31E, (ii) Indicated Drug, and (iii)

NOXA:Drug Combination. B: the dots are (from left to right): (i) strong antagonism; (ii)-(vi) additivity. C: for each time point, the bars are (from left to right): (i) 40 μM D-NA-NOXA-15 R31E, (ii) 5 μM AZD0156, and (ii) 40 μM D-NA-NOXA-15 R31E + 5 μM AZD0156.

Figure 55 shows the cell viability of MV4;11 (A) and U937 (B) cells treated with the selective MCL-1 inhibitor S63845, ATM inhibitor AZD0156, or the combination at the indicated doses. For both panels, for each concentration, the bars are (from left to right): (i) 0 nM S63845; (ii) 1.25 nM S63845; (iii) 2.5 nM S63845; (iv) 5 nM S63845; and (v) 10 nM S63845.

Figure 56 shows (A) the covalent reactivity of D-NA-NOXA SAHB-15 F32A and R31E peptides with recombinant BFL-1ΔC (top) and full-length BFL-1 (bottom), as reflected by the shift in BFL-1 molecular weight (denoted by *) and detected by reducing and denaturing gel electrophoresis; (B) Covalent reactivity of D-NA-NOXA SAHB-15 F32A and R31E peptides with HA-BFL-1ΔC (top) and full length HA-BFL1 (bottom) expressed in 293T cells, as reflected by the shift in HA-BFL-1 molecular weight (denoted by *) upon treatment with NOXA SAHBs (20 μM) for 12 hours and detected by reducing and denaturing gel electrophoresis of lysates, and anti-HA western analysis; and (C) cysteine-reactive NOXA SAHB-15 R31E and NOXA SAHB-15 F32A peptide targeting of native BFL-1 as assessed by streptavidin pulldown of U937 lysates and anti-BFL-1 western analysis. Of note, native BFL-1 runs at a higher molecular weight than recombinant or expressed BFL-1.

Figure 57 shows the loss of mitochondrial membrane potential in response to treatment of U937 cells for 24 hours with the indicated doses of D-NA-NOXA SAHB-15 R31E and AZD0156, as single agents and in combination. FCCP is a mitochondrial uncoupler that serves as a positive control for loss of mitochondrial membrane potential.

Figure 58 shows the effects of treatment of U937 cells with the indicated doses of AZD0156 for 2 hours on the phosphorylation status of ATM S1981 and CHK2 S33/35, as monitored by western blot.

Figure 59 shows the effect of AZD0156 treatment (5 μM) on the level of BAX protein in U937 cells by BAX western analysis of lysates prepared at the indicated time points.

Figure 60 shows that treatment of U937 cells with the indicated doses of D-NA-NOXA SAHB-15 R31E for 30 min (A) and 4 hours (B) causes no LDH release.

Figure 61 shows the capacity of select cysteine-reactive NOXA SAHB constructs to induce unwanted, non-specific cytotoxicity by membrane perturbation. **SEQ ID NOs: 78** and **150-153** (from top to bottom).

Figure 62 shows (A) *BFL-1* dependency Z-scores and *BFL-1* scaled rank values across AML cell lines evaluated in a genome-scale CRISPR-Cas9 screen, (B) *BFL-1* dependency Z-scores and *BFL-1* scaled rank values for top-scoring BFL-1 dependent human cancer cell lines among those evaluated in a genome-scale CRISPR-Cas9 screen, and (C) Pearson and Spearman correlations for *BFL-1* co-dependencies in AML cell lines from a genome-scale CRISPR-Cas9 screen and identified based on the following criteria: (1) gene scored as a potential dependency in at least 2 AML cell lines (score < -0.1), (2) gene is expressed in at least 2 AML cell lines (TPM >1), (3) gene is not predicted to be a pan-lethal gene, and (4) Pearson and Spearman correlations are both > 0.9.

DETAILED DESCRIPTION

There are currently no Food and Drug Administration approved drugs available for targeting the apoptotic resistance driven by MCL-1 and BFL-1/A1. In particular, no selective BFL-1 targeting agents, dual BFL-1/MCL-1-targeting agents, nor the combination of a covalent and non-covalent dual inhibitor have been advanced to clinical testing. This disclosure provides structurally-stabilized and cysteine-reactive NOXA BH3 peptides that demonstrate the properties of selective covalent BFL-1 targeting agents and dual BFL-1/MCL-1-targeting agents that can covalently bind to BFL-1 and non-covalently bind to MCL-1. This disclosure also features methods for using such stabilized peptides alone or in combination with other therapeutic agents (*e.g.,* ATM kinase /ATR kinase inhibitors) in the treatment of BFL-1 and/or MCL-1 -dependent and/or -expressing cancers (*e.g.,* hematologic malignancies, melanoma or other solid tumors, diseases of cellular excess such as autoimmune or inflammatory diseases, or other cancers described herein below). Such stapled peptides are useful to reactivate apoptosis, singly and in combination, in cancers or diseases of cellular excess that express and/or are dependent on MCL-1 and/or BFL-1.

<u>Proteins</u>

The amino acid sequence of human MCL-1 is provided below (UniProtKB - Q07820):

```
        10         20         30         40         50
MFGLKRNAVI GLNLYCGGAG LGAGSGGATR PGGRLLATEK EASARREIGG
```

```
        60         70         80         90        100
GEAGAVIGGS AGASPPSTLT PDSRRVARPP PIGAEVPDVT ATPARLLFFA
       110        120        130        140        150
PTRRAAPLEE MEAPAADAIM SPEEELDGYE PEPLGKRPAV LPLLELVGES
       160        170        180        190        200
GNNTSTDGSL PSTPPPAEEE EDELYRQSLE IISRYLREQA TGAKDTKPMG
       210        220        230        240        250
RSGATSRKAL ETLRRVGDGV QRNHETAFQG MLRKLDIKNE DDVKSLSRVM
       260        270        280        290        300
IHVFSDGVTN WGRIVTLISF GAFVAKHLKT INQESCIEPL AESITDVLVR
       310        320        330        340        350
TKRDWLVKQR GWDGFVEFFH VEDLEGGIRN VLLAFAGVAG VGAGLAYLIR (SEQ ID
NO:80)
```

The amino acid sequence of human BFL-1 is provided below (UniProtKB – Q16548):

```
        10         20         30         40         50
MTDCEFGYIY RLAQDYLQCV LQIPQPGSGP SKTSRVLQNV AFSVQKEVEK
        60         70         80         90        100
NLKSCLDNVN VVSVDTARTL FNQVMEKEFE DGIINWGRIV TIFAFEGILI
       110        120        130        140        150
KKLLRQQIAP DVDTYKEISY FVAEFIMNNT GEWIRQNGGW ENGFVKKFEP
       160        170
KSGWMTFLEV TGKICEMLSL LKQYC  (SEQ ID NO:81)
```

The amino acid sequence of human NOXA is provided below (UniProtKB – Q13794):

```
        10         20         30         40         50
MPGKKARKNA QPSPARAPAE LEVECATQLR RFGDKLNFRQ KLLNLISKLF

CSGT     (SEQ ID NO:82)
```

NOXA Peptides

An exemplary NOXA peptide of this disclosure is the BH3 peptide starting from amino acid 26 to amino acid 40 of SEQ ID NO:82. This peptide has the amino acid sequence: ATQLRRFGDKLNFRQ (**SEQ ID NO:39**) and interacts with BFL-1/A1. The numbering used for this peptide is provided below.

Position	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
Amino Acid	A	T	Q	**L**	R	R	F	G	**D**	K	L	N	F	R	Q

The BFL-1 interacting residues (*i.e.,* the residues of the NOXA BH3 alpha helix that interact with BFL-1) for NOXA are: Leu-21, Glu-22, Val-23, Glu-24, Cys-25, Ala-26, Leu-29, Arg-30, Phe-32, Gly-33, Asp-34, Leu-36, Asn-37, and Gln-40 (wherein the amino acid

numbering is based on SEQ ID NO:82). In the context of the position numbering for SEQ ID NO:39 provided above, the BFL-1 interacting residues are: Ala-1, Leu-4, Arg-5, Phe-7, Gly-8, Asp-9, Leu-11, Asn-12, and Gln-15. Alanine-1, Leucine-4, Phenylalanine-7, Glycine-8, and Leucine-11 are involved in hydrophobic interactions; while Arginine-5, Aspartate-9, and Asparagine-12 are involved in hydrogen bond interactions.

The residues of NOXA that do not interact with BFL-1 (*i.e.,* the residues of the NOXA BH3 alpha helix that are on the non-interacting side of the helix) are: Thr-27, Gln-28, Arg-31, Lys-35, Phe-38, and Arg-39 (wherein the amino acid numbering is based on SEQ ID NO:82). In the context of the position numbering for SEQ ID NO:39 provided above, the residues that are non on the interacting face of the NOXA peptide alpha helix are residues: Thr-2, Gln-3, Arg-6, Lys-10, Phe-13, and Arg-14.

The MCL-1 interacting residues (*i.e.,* the residues of the NOXA BH3 alpha helix that interact with MCL-1) of the NOXA alpha-helix are: Gln-28, Leu-29, Arg-30, Phe-32, Gly-33, Leu-36, Asn-37, Gln-40 (wherein the amino acid numbering is based on SEQ ID NO:82).

The residues of NOXA that do not interact with MCL-1 (*i.e.,* the residues of the NOXA BH3 alpha helix that are on the non-interacting side of the helix) are: Ala-26, Thr-27, Arg-31, Asp-34, Lys-35, Phe-38, and Arg-39 (wherein the amino acid numbering is based on SEQ ID NO:82).

In certain embodiments, the NOXA BH3 peptides of this disclosure can have 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or 13 amino acid substitutions in SEQ ID NO:39 (*e.g.,* 1, 2, 3, 4, 5, 6, 7, , 9, 10, 11, 12, or 13 amino acids are conservatively or non-conservatively substituted). In some instances, one to three amino acids of SEQ ID NO:39 are substituted. The amino acid substitutions in SEQ ID NO:39 can be on one or both the interacting and non-interacting side of the alpha helix. Much greater variability is permitted in the non-interacting side of the alpha helix of the NOXA peptide that does not interact with BFL-1 than on the interacting side. In fact, just about every one of those amino acids (e.g., 5, 4, 3, 2, or 1 amino acid of the non-interacting face of the helix) can be substituted (e.g., conservative or non-conservative amino acid substitutions or alanine). In certain embodiments, the interacting face of the helix of these peptides have 1 to 9, 1 to 8, 1 to 7, 1 to 6, 1 to 5, 1 to 4, 1 to 3, 1 to 2, or 1 amino acid substitution(s). In some instances, the substitution(s) is/are a conservative amino acid substitution. In other instances, the substitution(s) is/are a non-conservative amino acid substitution. In some instances, where there are more than one amino acid substitution, the

substitutions are both conservative and non-conservative amino acid substitutions. In some cases, where one to three amino acids (e.g., 1, 2, or 3) of SEQ ID NO:39 are substituted, the substitutions are all on the non-interacting face of the NOXA helix. In some cases, where one to three amino acids (e.g., 1, 2, or 3) of SEQ ID NO:39 are substituted, the substitutions are all on the interacting face of the NOXA helix. In some cases, where one to three amino acids (e.g., 1, 2, or 3) of SEQ ID NO:39 are substituted, the substitutions are both on the interacting and non-interacting face of the NOXA helix. In certain instances, the substituted amino acid(s) are selected from the group consisting of L-Ala, D-Ala, Aib, Sar, Ser, a substituted alanine, or a substituted glycine derivative. In certain instances, one or more of A26, T27, Q28, R30, R31, F32, G33, K35, L36, N37, F38, R39, or Q40 (numbering according to SEQ ID NO:82) are substituted. In certain instances, the substituted amino acid(s) are selected from the group consisting of L-Ala, D-Ala, Aib, Sar, Ser, a substituted alanine, or a substituted glycine derivative. In certain instances, one or both of L29 and D34 (numbering according to SEQ ID NO:82) of SEQ ID NO:39 are not substituted. In some instances L29 (numbering according to SEQ ID NO:82) of SEQ ID NO:39 is substituted with isoleucine, phenylalanine, L-Ala, D-Ala, Aib, Sar, Ser, a substituted alanine, or a substituted glycine derivative.

In certain embodiments, the NOXA BH3 peptides of this disclosure can have 1, 2, 3, 4, 5, amino acid removed/deleted from the C-terminus of the sequence set forth in SEQ ID NO:39. In certain embodiments, the NOXA BH3 peptides of this disclosure can have 1, 2, 3, 4, 5, amino acid removed/deleted from the N-terminus of the sequence set forth in SEQ ID NO:39. In certain embodiments, the NOXA BH3 peptides of this disclosure can have 1, 2, 3, 4, 5, amino acid removed/deleted from both the N-terminus and C-terminus of the sequence set forth in SEQ ID NO:39. In certain instances, these removed amino acids can be replaced with 1-6 (1, 2, 3, 4, 5, or 6) amino acids selected from the group consisting of L-Ala, D-Ala, Aib, Sar, Ser, a substituted alanine, or a substituted glycine derivative.

Non-limiting examples of modifications to the sequence of SEQ ID NO:39 are provided below:

Position (numbered according to SEQ ID NO:82)	**Exemplary Modifications**
A26	D-Ala, Aib, Sar, Ser, a substituted alanine, or a substituted glycine derivative; deletion
T27	I, M, B; L-Ala, D-Ala, Aib, Sar, Ser, a substituted alanine, or a substituted glycine derivative; deletion
Q28	L-Ala, D-Ala, Aib, Sar, Ser, a substituted alanine, or a substituted glycine derivative; deletion
L29	I, F, L-Ala, D-Ala, Aib, Sar, Ser, a substituted alanine, or a substituted glycine derivative
R30	K, T; L-Ala, D-Ala, Aib, Sar, Ser, a substituted alanine, or a substituted glycine derivative; deletion
R31	E, L-Ala, D-Ala, Aib, Sar, Ser, a substituted alanine, or a substituted glycine derivative
F32	I, L, V, L-Ala, D-Ala, Aib, Sar, Ser, a substituted alanine, or a substituted glycine derivative
G33	L-Ala, D-Ala, Aib, Sar, Ser, a substituted alanine, or a substituted glycine derivative
D34	-
K35	L-Ala, D-Ala, Aib, Sar, Ser, a substituted alanine, or a substituted glycine derivative
L36	W; V; L-Ala, D-Ala, Aib, Sar, Ser, a substituted alanine, or a substituted glycine derivative; deletion
N37	D, S, L-Ala, D-Ala, Aib, Sar, Ser, a substituted alanine, or a substituted glycine derivative; deletion
F38	A, L, L-Ala, D-Ala, Aib, Sar, Ser, a substituted alanine, or a substituted glycine derivative; deletion
R39	E, L-Ala, D-Ala, Aib, Sar, Ser, a substituted alanine, or a substituted glycine derivative; deletion
Q40	L, L-Ala or poly-L-Ala, D-Ala or poly-D-Ala, Aib, Sar, Ser, a substituted alanine, or a substituted glycine derivative; deletion

The disclosure also encompasses NOXA peptides that are at least 14% (e.g., at least 14 to 50%, at least 14 to 45%, at least 14 to 40%, at least 14 to 35%, at least 14 to 30%, at least 14 to 25%, at least 14 to 20%, at least 20% to 50%, at least 20% to 45%, at least 20% to 40%, at least 20% to 35%, at least 20% to 30%, at least 20% to 25%, at least 15%, at least 20%, at least 27%, at least 34%, at least 40% at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%) identical to SEQ ID NO:39. The variability in amino acid sequence of SEQ ID NO:39 can be on one or both the interacting and non-

interacting side of the alpha helix. Just about every one of the amino acids on the non-interacting face of the NOXA BH3 helix can be varied. The amino acids on the interacting face of the helix can also be varied.

In some embodiments, the disclosure features variants of SEQ ID NO:39, wherein the NOXA peptide variants non-covalently bind to BFL-1. This disclosure also features variants of SEQ ID NO:39, wherein the NOXA peptide variants non-covalently bind to MCL-1. In some instances, the variants of SEQ ID NO:39 described herein non-covalently bind to both BFL-1 and MCL-1.

In certain instances, the NOXA peptide has an amino acid sequence set out below:

ATQLRRAGDKLNFRQ (**SEQ ID NO:94**);

ATQLRRFGDKANFRQ (**SEQ ID NO:95**);

ATQLREFGDKLNFRQ (**SEQ ID NO:96**);

ATQLRRFGDKLNFEQ (**SEQ ID NO:97**);

AAQLRAFGAKLNAA (**SEQ ID NO:98**);

AAQLRAFGAKLNAAAA (**SEQ ID NO:99**);

AAQLRAFGAKLNAE (**SEQ ID NO:100**);

AAQLRAFGAKLNAEAA (**SEQ ID NO:101**);

AAQLREFGAKLNAA (**SEQ ID NO:102**);

AAQLREFGAKLNAAAA (**SEQ ID NO:103**);

AAQLRAFGDKLNAA (**SEQ ID NO:104**);

AAQLRAFGDKLNAAAA (**SEQ ID NO:105**);

AAQLRAFGDKLNAE (**SEQ ID NO:106**);

AAQLRAFGDKLNAEAA (**SEQ ID NO:107**);

AAQLREFGDKLNAA (**SEQ ID NO:108**); or

AAQLREFGDKLNAAAA (**SEQ ID NO:109**).

The NOXA peptides described herein can be optimized for therapeutic use. For example, if any of the above-described NOXA peptides cause membrane disruption (cell lysis), the peptides can be optimized by lowering the overall peptide hydrophobicity. This can for example be achieved by substituting especially hydrophobic residues with an amino acid with lower hydrophobicity (e.g., alanine). Membrane disruption can also be lowered by reducing the overall positive charge of the peptide. This can be accomplished by substituting basic residues with uncharged or acidic residues. In certain instances, both the overall peptide hydrophobicity and the overall positive charge of the peptide are lowered.

In certain embodiments, the NOXA peptides described herein are between 5 and 35 amino acids in length. In certain embodiments, the NOXA peptides described herein are between 5 and 25 amino acids in length. In certain embodiments, the NOXA peptides described herein are between 5 and 20 amino acids in length. In certain embodiments, the NOXA peptides described herein are between 5 and 18 amino acids in length. In certain embodiments, the NOXA peptides described herein are between 10 and 35 amino acids in length. In certain embodiments, the NOXA peptides described herein are between 10 and 25 amino acids in length. In certain embodiments, the NOXA peptides described herein are between 10 and 20 amino acids in length. In certain embodiments, the NOXA peptides described herein are between 10 and 18 amino acids in length. In certain embodiments, the NOXA peptides described herein are between 15 and 26 amino acids in length. In certain embodiments, the NOXA peptides described herein are between 15 and 18 amino acids in length.

In certain instances, the NOXA peptides are structurally stabilized. In order to stabilize these NOXA peptides, the peptides can include two or more substitutions to replace, *e.g.,* an amino acid of the NOXA peptide with a non-natural amino acid, so that the peptide can be stapled and/or stitched.

Stabilized Peptides

A peptide helix is an important mediator of key protein-protein interactions that regulate many important biological processes such as apoptosis; however, when such a helix is taken out of its context within a protein and prepared in isolation, it usually adopts a random coil conformation, leading to a drastic reduction in biological activity and thus diminished therapeutic potential. The present disclosure provides structurally stabilized

peptides of NOXA. The present disclosure includes structurally stabilized NOXA peptides (such as those described above) comprising at least two modified amino acids joined by an internal (intramolecular) cross-link (or staple) and, in certain instances, having a reactive group (" a warhead" such as a non-natural amino acid bearing an electrophilic group) that can form a covalent bond with a cysteine (Cys) residue within a target protein to which the structurally stabilized peptide binds (e.g., BFL-1). Stabilized peptides as described herein include stapled peptides and stitched peptides as well as peptides containing multiple stitches, multiple staples or a mix or staples and stitches, or other chemical strategies for structural reinforcement (*see. e.g.,* Balaram P. *Cur. Opin. Struct. Biol.* 1992;2:845; Kemp DS, et al., J. *Am. Chem. Soc.* 1996;118:4240; Orner BP, et al., *J. Am. Chem. Soc.* 2001;123:5382; Chin JW, et al., *Int. Ed.* 2001;40:3806; Chapman RN, et al., *J. Am. Chem. Soc.* 2004;126:12252; Horne WS, et al., *Chem., Int. Ed.* 2008;47:2853; Madden et al., *Chem Commun* (Camb). 2009 Oct 7; (37): 5588–5590; Lau et al., *Chem. Soc. Rev.*, 2015,44:91-102; and Gunnoo et al., *Org. Biomol. Chem.*, 2016,14:8002-8013; all of which are incorporated by reference herein in its entirety).

In certain embodiments, one or more of the NOXA polypeptides described herein can be stabilized by peptide stapling (*see, e.g.,* Walensky, *J. Med. Chem.*, 57:6275-6288 (2014), the contents of which are incorporated by reference herein in its entirety). A peptide is "stabilized" in that it maintains its native secondary structure. For example, stapling allows a polypeptide, predisposed to have an α-helical secondary structure, to maintain its native α-helical conformation. This secondary structure increases resistance of the polypeptide to proteolytic cleavage and heat, and also may increase target binding affinity, hydrophobicity, and cell permeability. Accordingly, the stapled (cross-linked) polypeptides described herein have improved biological activity relative to a corresponding non-stapled (un-cross-linked) polypeptide.

"Peptide stapling" is a term coined from a synthetic methodology wherein two olefin-containing side-chains (e.g., cross-linkable side chains) present in a polypeptide chain are covalently joined (e.g., "stapled together") using a ring-closing metathesis (RCM) reaction to form a cross-linked ring (see, e.g., Blackwell et al., *J. Org. Chem.*, 66: 5291-5302, 2001; Angew et al., *Chem. Int. Ed.* 37:3281, 1994). As used herein, the term "peptide stapling" includes the joining of two (e.g., at least one pair of) double bond-containing side-chains, triple bond-containing side-chains, or double bond-containing and triple bond-containing side chain, which may be present in a polypeptide chain, using any number of reaction conditions

and/or catalysts to facilitate such a reaction, to provide a singly “stapled” polypeptide. The term “multiply stapled” polypeptides refers to those polypeptides containing more than one individual staple, and may contain two, three, or more independent staples of various spacing. Additionally, the term “peptide stitching,” as used herein, refers to multiple and tandem "stapling" events in a single polypeptide chain to provide a “stitched” (e.g., tandem or multiply stapled) polypeptide, in which two staples, for example, are linked to a common residue. Peptide stitching is disclosed, e.g., in WO 2008/121767 and WO 2010/068684, which are both hereby incorporated by reference in their entirety. In some instances, staples, as used herein, can retain the unsaturated bond or can be reduced.

In certain embodiments, one or more of the polypeptides described herein can be stabilized by, *e.g.,* hydrocarbon stapling. In some embodiments, the stapled peptide is a polypeptide comprising or consisting of amino acids 26-40 of NOXA, or comprising 1 to 13 (*e.g.,* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or 13) amino acid substitutions, deletions and/or insertions therein). In certain instances, the stapled peptide includes at least two (*e.g.,* 2, 3, 4, 5, 6) amino acid substitutions, wherein the substituted amino acids are separated by two, three, or six amino acids, and wherein the substituted amino acids are non-natural amino acids with olefinic side chains. There are many known non-natural or unnatural amino acids any of which may be included in the peptides of the present disclosure. Some examples of unnatural amino acids are 4-hydroxyproline, desmosine, gamma-aminobutyric acid, beta-cyanoalanine, norvaline, 4-(E)-butenyl-4(R)-methyl-N- methyl-L-threonine, N-methyl-L-leucine, 1-amino-cyclopropanecarboxylic acid, 1- amino-2-phenyl-cyclopropanecarboxylic acid, 1-amino-cyclobutanecarboxylic acid, 4- amino-cyclopentenecarboxylic acid, 3-amino-cyclohexanecarboxylic acid, 4-piperidylacetic acid, 4-amino-l-methylpyrrole-2-carboxylic acid, 2,4-diaminobutyric acid, 2,3- diaminopropionic acid, 2,4-diaminobutyric acid, 2-aminoheptanedioic acid, 4- (aminomethyl)benzoic acid, 4-aminobenzoic acid, ortho-, meta- and /para-substituted phenylalanines (e.g., substituted with $-C(=O)C_6H_5$; $-CF_3$; -CN; -halo; $-NO_2$; CH_3), disubstituted phenylalanines, substituted tyrosines (e.g., further substituted with $-C=O)C_6H_5$; $-CF_3$; -CN; -halo; $-NO_2$; CH_3), and statine. Additionally, amino acids can be derivatized to include amino acid residues that are hydroxylated, phosphorylated, sulfonated, acylated, or glycosylated.

Hydrocarbon stapled polypeptides include one or more tethers (linkages) between two non-natural amino acids, which tether significantly enhances the α-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 cross-linking. Thus, for example, where a peptide has the sequence . . . X1, X2, X3, X4, X5, X6, X7, X8, X9 . . . , cross-links between X1 and X4, or between X1 and X5, or between X1 and X8 are useful hydrocarbon stapled forms of that peptide, as are cross-links between X2 and X5, or between X2 and X6, or between X2 and X9, etc. The use of multiple cross-links (e.g., 2, 3, 4, or more) is also contemplated. The use of multiple cross-links is very effective at stabilizing and optimizing the peptide, especially with increasing peptide length. Thus, the disclosure encompasses the incorporation of more than one cross-link within the polypeptide sequence to either further stabilize the sequence or facilitate the structural stabilization, proteolytic resistance, acid stability, thermal stability, cellular permeability, and/or biological activity enhancement of longer polypeptide stretches. Additional description regarding making and use of hydrocarbon stapled polypeptides can be found, e.g., in U.S. Patent Publication Nos. 2012/0172285, 2010/0286057, and 2005/0250680, the contents of all of which are incorporated by reference herein in their entireties.

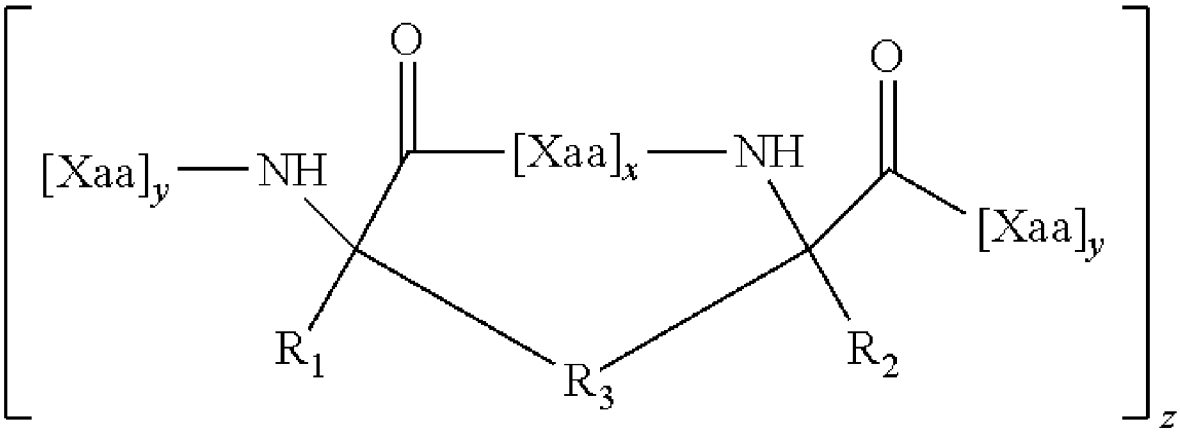
In certain embodiments when a staple is at the *i* and *i+3* residues, R-propenylalanine and S-pentenylalanine; or R- pentenylalanine and S-pentenylalanine are substituted for the amino acids at those positions. In certain embodiments when a staple is at the *i* and *i+4* residues, S-pentenyl alanine is substituted for the amino acids at those positions. In certain embodiments when a staple is at the *i* and *i+7* residues, S-pentenyl alanine and R-octenyl alanine are substituted for the amino acids at those positions. In some instances, when the peptide is stitched, the amino acids of the peptide to be involved in the "stitch" are substituted with Bis-pentenylglycine, S-pentenylalanine, and R-octenylalanine; or Bis-pentenylglycine, S- octenylalanine, and R-octenylalanine.

In certain embodiments, the staple and/or stitch is made at positions 1 and 5; 2 and 6; 3 and 7; 6 and 10; 10 and 14; 11 and 15;12 and 16; 1 and 8; 3 and 10; 6 and 13; 7 and 14; or 8 and 15, wherein the positions provided are based on SEQ ID NO:39; or a modified version of SEQ ID NO:39 as described above (*e.g.,* any one of SEQ ID NOs.:94 through 109). Staple positions can be varied by testing different staple locations in a staple walk.

Figure 36 top panel shows exemplary chemical structures of non-natural amino acids that can be used to generate various crosslinked compounds. **Fig. 36 middle panel** illustrates peptides with hydrocarbon cross-links between positions *i* and *i+3*; *i* and *i+4*; and *i* and *i+7*

residues. **Fig. 36 bottom panel** illustrates a staple walk along a peptide sequence. **Fig. 37** shows various peptide sequences with double and triple stapling strategies, and exemplary staple walks. **Fig. 38** illustrates exemplary staple walks using various lengths of branched stitched moieties.

In one aspect, a NOXA SAHB polypeptide has the formula (I),



wherein:

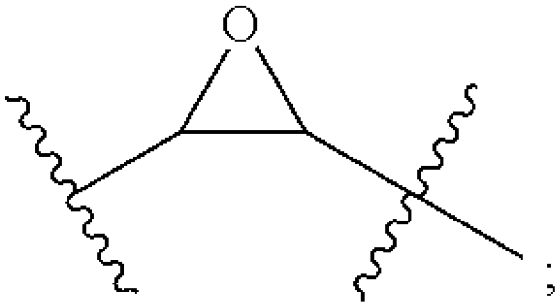
each R_1 and R_2 are independently H or a C_1 to C_{10} alkyl, alkenyl, alkynyl, arylalkyl, cycloalkylalkyl, heteroarylalkyl, or heterocyclylalkyl;

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;

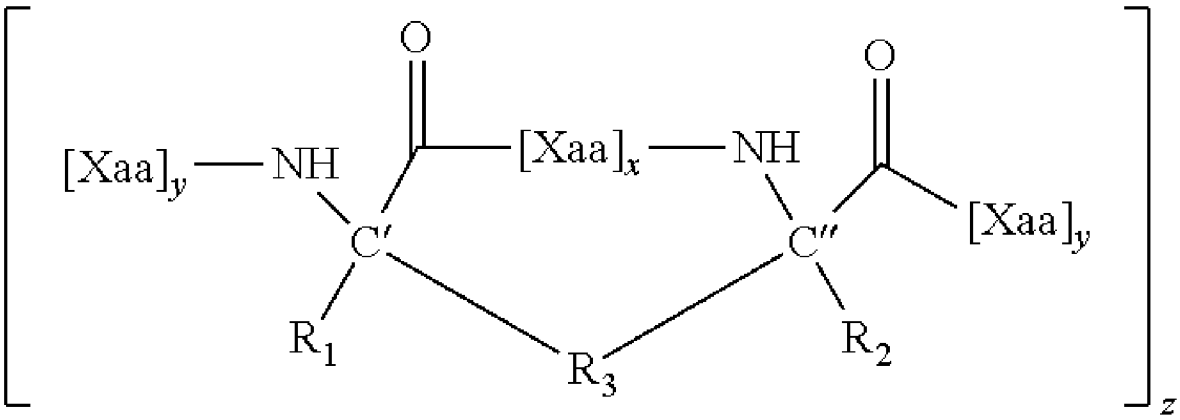
each y is independently an integer from 0-100;
z is an integer from 1-10 (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10);

and each Xaa is independently an amino acid. In some embodiments, the N-terminal $[Xaa]_y$ of formula (I) is not present, A, AT, ATQLR (**SEQ ID NO:110**), ATQLRR (**SEQ ID NO:111**), ATQLRRF (**SEQ ID NO:112**), ATQLRRFGD (**SEQ ID NO:113**), ATQLRRFGDK (**SEQ ID NO:114**); or ATQLRRFGDKL (**SEQ ID NO:115**). In some embodiments, $[Xaa]_x$ is TQL, QLR, LRR, FGD, LNF, NFR, FRQ, TQLRRF (**SEQ ID NO:116**), LRRFGD (**SEQ ID NO:117**), FGDKLN (**SEQ ID NO:118**), GDKLNF (**SEQ ID NO:119**), or DKLNFR (**SEQ ID NO:120**). In some embodiments, the C-terminal $[Xaa]_y$ of formula (I) is RFGDKLNFRQ (**SEQ ID NO:121**); FGDKLNFRQ (**SEQ ID NO:122**); GDKLNFRQ (**SEQ ID NO:123**); LNFRQ (**SEQ ID NO:124**); Q; DKLNFRQ (**SEQ ID NO:125**); or RQ. In certain instances, the sequences set forth above and in SEQ ID NOs.:110-125 can have at least one (e.g., 1, 2, 3, 4, 5, 6) amino acid substitution or deletion. The NOXA SAHB polypeptides can include any amino acid sequence described herein.

The tether can include an alkyl, alkenyl, or alkynyl moiety (e.g., C_5, C_8, or C_{11} alkyl, 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, x is 2, 3, or 6. In some instances, each y is independently an integer between 1 and 15, or 3 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 can both be methyl. In some instances, R_3 is alkyl (e.g., C_8 alkyl) and x is 3. In some instances, R_3 is C_{11} alkyl and x is 6. In some instances, R_3 is alkenyl (e.g., C_8 alkenyl) and x is 3. In some instances, x is 6 and R_3 is C_{11} alkenyl. In some instances, R_3 is a straight chain alkyl, alkenyl, or alkynyl. In some instances, R_3 is $—CH_2—CH_2—CH_2—CH{=}CH—CH_2—CH_2—CH_2—$.

In another aspect, 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, e.g., 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 can be in the E or Z stereochemical configuration.

In some instances, R_3 is $[R_4—K—R_4]_n$; and R_4 is a straight chain alkyl, alkenyl, or alkynyl.

In some embodiments, the disclosure features internally cross-linked ("stapled" or "stitched") peptides comprising the amino acid sequence ATQLRRFGDKLNFRQ (**SEQ ID NO:39**), wherein the side chains of two amino acids separated by two, three, or six amino acids are replaced by an internal staple; the side chains of three amino acids are replaced by an internal stitch; the side chains of four amino acids are replaced by two internal staples, or the side chains of five amino acids are replaced by the combination of an internal staple and an internal stitch. In certain instances, the amino acids at one or more of positions 4, 8, and 9 of SEQ ID NO: 39 are not replaced with a staple or stitch. In certain instances, the amino acids at positions 4 and/or 9 of SEQ ID NO: 39 are not replaced with a staple or stitch. The stapled/stitched peptide can be 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 amino acids in length. In a specific embodiment, the stapled/stitched peptide is 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 amino acids in length. In a specific embodiment, the stapled/stitched peptide is 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, or 18 amino acids in length. Exemplary NOXA stapled peptides are shown in Figure 2. In certain embodiments, the stapled polypeptide comprises or consists of the amino acid sequence set forth in any one of SEQ ID NOs:2 to 21. In one embodiment, the NOXA stapled peptide has the amino acid sequence set forth in SEQ ID NO:16. In certain embodiments, the stapled polypeptide

comprises or consists of the amino acid sequence set forth in any one of SEQ ID NOs: 22 to 34. In certain embodiments, the stapled polypeptide comprises or consists of the amino acid sequence set forth in any one of SEQ ID NOs: 35 to 38. In certain embodiments, the stapled polypeptide comprises or consists of the amino acid sequence set forth in any one of SEQ ID NOs:94 to 109, wherein at least two amino acids separated by 2, 3 or 6 amino acids are modified to structurally stabilize the peptide (*e.g.,* by substituting them with non-natural amino acids to permit hydrocarbon stapling).

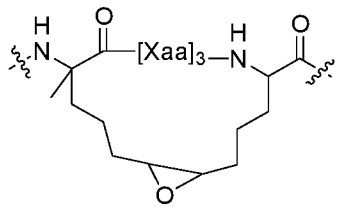
While hydrocarbon tethers are common, other tethers can also be employed in the NOXA BH3 peptides described herein. For example, the tether can include one or more of an ether, thioether, ester, amine, or amide, or triazole moiety. In some cases, a naturally occurring amino acid side chain can be incorporated 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. Triazole-containing (e.g., 1, 4 triazole or 1, 5 triazole) crosslinks can be used (*see, e.g.,* Kawamoto et al. 2012 *Journal of Medicinal Chemistry* 55:1137; WO 2010/060112). In addition, other methods of performing different types of stapling are well known in the art and can be employed with the NOXA BH3 peptides described herein (see, e.g., *Lactam stapling*: Shepherd et al., *J. Am. Chem. Soc.*, 127:2974–2983 (2005); *UV-cycloaddition stapling*: Madden et al., *Bioorg. Med. Chem.* Lett., 21:1472–1475 (2011); *Disulfide stapling*: Jackson et al., A*m. Chem. Soc.*,113:9391–9392 (1991); *Oxime stapling*: Haney et al., *Chem. Commun.*, 47:10915–10917 (2011); *Thioether stapling*: Brunel and Dawson, *Chem. Commun.*, 552–2554 (2005); *Photoswitchable stapling*: J. R. Kumita et al., *Proc. Natl. Acad. Sci. U. S. A.*, 97:3803–3808 (2000); *Double-click stapling*: Lau et al., *Chem. Sci.*, 5:1804–1809 (2014); *Bis-lactam stapling*: J. C. Phelan et al.,, *J. Am. Chem. Soc.*, 119:455–460 (1997); and *Bis-arylation stapling*: A. M. Spokoyny et al., *J. Am. Chem. Soc.*, 135:5946–5949 (2013)).

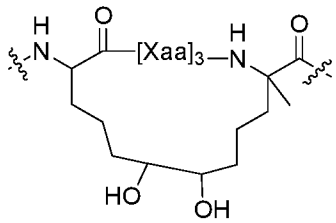
It is further envisioned that the length of the tether can be varied. For instance, a shorter length of tether can be used where it is desirable to provide a relatively high degree of constraint on the secondary alpha-helical structure, whereas, in some instances, it is desirable

to provide less constraint on the secondary alpha-helical structure, and thus a longer tether may be desired.

Additionally, while tethers spanning from amino acids *i* to *i+3*, *i* to *i+4*, and *i* to *i+7* are common 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 and also used in combination to install multiple tethers.

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, aminohydroxylation 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 therapeutic agent. Such derivatization can alternatively be achieved by synthetic manipulation of the amino or carboxy-terminus of the polypeptide or via the amino acid side chain. Other agents can be attached to the functionalized tether, e.g., an agent that facilitates entry of the polypeptide into cells.

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.

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

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

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

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

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

The stapled peptides can also be modified, e.g., to further facilitate cellular uptake or increase *in vivo* stability, in some embodiments. For example, acylating or PEGylating a peptidomimetic macrocycle facilitates cellular uptake, increases bioavailability, increases

blood circulation, alters pharmacokinetics, decreases immunogenicity and/or decreases the needed frequency of administration.

In some embodiments, the stapled peptides disclosed herein have an enhanced ability to penetrate cell membranes (e.g., relative to non-stapled peptides).

Methods of synthesizing the stabilized peptides described herein are known in the art. Nevertheless, the following exemplary method may be used. It will be appreciated that the various steps may be performed in an alternate sequence or order to give the desired compounds. Synthetic chemistry transformations and protecting group methodologies (protection and deprotection) useful in synthesizing the compounds described herein are known in the art and include, for example, those such as described in R. Larock, Comprehensive Organic Transformations, VCH Publishers (1989); T.W. Greene and P.G.M. Wuts, Protective Groups in Organic Synthesis, 3d. Ed., John Wiley and Sons (1999); L. Fieser and M. Fieser, Fieser and Fieser's Reagents for Organic Synthesis, John Wiley and Sons (1994); and L. Paquette, ed., Encyclopedia of Reagents for Organic Synthesis, John Wiley and Sons (1995), and subsequent editions thereof.

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

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

Longer peptides could be made by conjoining individual synthetic peptides using native chemical ligation. Alternatively, the longer synthetic peptides can be synthesized by well-known recombinant DNA techniques. Such techniques are provided in well-known standard manuals with detailed protocols. To construct a gene encoding a peptide of this

invention, the amino acid sequence is reverse translated to obtain a nucleic acid sequence encoding the amino acid sequence, preferably with codons that are optimum for the organism in which the gene is to be expressed. Next, a synthetic gene is made, typically by synthesizing oligonucleotides which encode the peptide and any regulatory elements, if necessary. The synthetic gene is inserted in a suitable cloning vector and transfected into a host cell. The peptide is then expressed under suitable conditions appropriate for the selected expression system and host. The peptide is purified and characterized by standard methods.

The peptides can be made in a high-throughput, combinatorial fashion, e.g., using a high-throughput multiple channel combinatorial synthesizer available from Advanced Chemtech. Peptide bonds can be replaced, e.g., to increase physiological stability of the peptide, by: a retro-inverso bonds (C(O)-NH); a reduced amide bond (NH-CH_2); a thiomethylene bond (S-CH_2 or CH_2-S); an oxomethylene bond (O-CH_2 or CH_2-O); an ethylene bond (CH_2-CH_2); a thioamide bond (C(S)-NH); a trans-olefin bond (CH=CH); a fluoro substituted trans-olefin bond (CF=CH); a ketomethylene bond (C(O)-CHR) or CHR-C(O) wherein R is H or CH_3; and a fluoro-ketomethylene bond (C(O)-CFR or CFR-C(O) wherein R is H or F or CH_3.

The polypeptides can be further modified by: acetylation, amidation, biotinylation, cinnamoylation, farnesylation, fluoresceination, formylation, myristoylation, palmitoylation, phosphorylation (Ser, Tyr or Thr), stearoylation, succinylation and sulfurylation. As indicated above, peptides can be conjugated to, for example, polyethylene glycol (PEG); alkyl groups (e.g., C1-C20 straight or branched alkyl groups); fatty acid radicals; and combinations thereof. α, α-Disubstituted non-natural amino acids containing olefinic side chains of varying length can be synthesized by known methods (Williams et al. *J. Am. Chem. Soc.*, 113:9276, 1991; Schafmeister et al., *J. Am. Chem Soc.*, 122:5891, 2000; and Bird et al., *Methods Enzymol.*, 446:369, 2008; Bird et al, *Current Protocols in Chemical Biology*, 2011). For peptides where an *i* linked to *i*+7 staple is used (two turns of the helix stabilized) either: a) one S5 amino acid and one R8 is used; or b) one S8 amino acid and one R5 amino acid is used. R8 is synthesized using the same route, except that the starting chiral auxillary confers the R-alkyl-stereoisomer. Also, 8-iodooctene is used in place of 5-iodopentene. Inhibitors are synthesized on a solid support using solid-phase peptide synthesis (SPPS) on MBHA resin (*see, e.g.,* WO 2010/148335).

Fmoc-protected α-amino acids (other than the olefinic amino acids Fmoc-S_5-OH, Fmoc-R_8-OH , Fmoc-R_8-OH, Fmoc-S_8-OH and Fmoc-R_5-OH), 2-(6-chloro-1-H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU), and Rink Amide MBHA are commercially available from, e.g., Novabiochem (San Diego, CA). Dimethylformamide (DMF), N-methyl-2-pyrrolidinone (NMP), N,N-diisopropylethylamine (DIEA), trifluoroacetic acid (TFA), 1,2-dichloroethane (DCE), fluorescein isothiocyanate (FITC), and piperidine are commercially available from, e.g., Sigma-Aldrich. Olefinic amino acid synthesis is reported in the art (Williams et al., *Org. Synth.*, 80:31, 2003).

Again, methods suitable for obtaining (e.g., synthesizing), stapling, and purifying the peptides disclosed herein are also known in the art (see, e.g., Bird et. al., *Methods in Enzymol.,* 446:369-386 (2008); Bird et al, *Current Protocols in Chemical Biology*, 2011; Walensky et al., *Science*, 305:1466-1470 (2004); Schafmeister et al., *J. Am. Chem. Soc.*, 122:5891-5892 (2000); U.S. Patent Application No. 12/525,123, filed March 18, 2010; and U.S. Patent No. 7,723,468, issued May 25, 2010, each of which are hereby incorporated by reference in their entirety).

In some embodiments, the peptides are substantially free of non-stapled peptide contaminants or are isolated. Methods for purifying peptides include, for example, synthesizing the peptide on a solid-phase support. Following cyclization, the solid-phase support may be isolated and suspended in a solution of a solvent such as DMSO, DMSO/dichloromethane mixture, or DMSO/NMP mixture. The DMSO/dichloromethane or DMSO/NMP mixture may comprise about 30%, 40%, 50% or 60% DMSO. In a specific embodiment, a 50%/50% DMSO/NMP solution is used. The solution may be incubated for a period of 1, 6, 12 or 24 hours, following which the resin may be washed, for example with dichloromethane or NMP. In one embodiment, the resin is washed with NMP. Shaking and bubbling an inert gas into the solution may be performed.

Properties of the stapled (cross-linked) polypeptides of the invention can be assayed, for example, using the methods described below.

Assays to Determine α-Helicity: Compounds are dissolved in an aqueous solution (e.g. 5 mM potassium phosphate solution at pH 7, or distilled H_2O, to concentrations of 25-50 μM). Circular dichroism (CD) spectra are obtained on a spectropolarimeter (e.g., Jasco J-710, Aviv) using standard measurement parameters (e.g. temperature, 20°C; 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 is calculated by dividing the mean residue ellipticity by the reported value for a model helical decapeptide (Yang et al., *Methods Enzymol.* 130:208 (1986)).

Assays to Determine Melting Temperature (Tm): Cross-linked or the unmodified template peptides are dissolved in distilled H_2O or other buffer or solvent (e.g. at a final concentration of 50 μM) and Tm is determined by measuring the change in ellipticity over a temperature range (e.g. 4 to 95 °C) on a spectropolarimeter (e.g., Jasco J-710, Aviv) using standard parameters (e.g. wavelength 222 nm; step resolution, 0.5 nm; speed, 20 nm/sec; accumulations, 10; response, 1 sec; bandwidth, 1 nm; temperature increase rate: 1°C/min; path length, 0.1 cm).

In Vitro Protease Resistance Assays: 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, typically buries and/or twists and/or shields the amide backbone and therefore may prevent or substantially retard proteolytic cleavage. The peptidomimetic macrocycles of the present invention may be subjected to in vitro enzymatic proteolysis (e.g. trypsin, chymotrypsin, pepsin) to assess for any change in degradation rate compared to a corresponding uncrosslinked or alternatively stapled polypeptide. For example, the peptidomimetic macrocycle and a corresponding uncrosslinked polypeptide are 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, the peptidomimetic macrocycle and peptidomimetic precursor (5 mcg) are incubated with trypsin agarose (Pierce) (S/E ~125) for 0, 10, 20, 90, and 180 minutes. Reactions are quenched by tabletop centrifugation at high speed; remaining substrate in the isolated supernatant is quantified by HPLC-based peak detection at 280 nm. The proteolytic reaction displays first order kinetics and the rate constant, k, is determined from a plot of ln[S] versus time.

Peptidomimetic macrocycles and/or a corresponding uncrosslinked polypeptide can be each incubated with fresh mouse, rat and/or human serum (e.g. 1-2 mL) at 37°C for, e.g., 0, 1, 2, 4, 8, and 24 hours. Samples of differing macrocycle concentration may be prepared by serial dilution with serum. To determine the level of intact compound, the following procedure may be used: The samples are extracted, for example, by transferring 100 μL of sera to 2 ml centrifuge tubes followed by the addition of 10 μL of 50% formic acid and 500

μL acetonitrile and centrifugation at 14,000 RPM for 10 min at 4+/-2°C. The supernatants are then transferred to fresh 2 ml tubes and evaporated on Turbovap under N_2<10 psi, 37°C. The samples are reconstituted in 100 μL of 50:50 acetonitrile:water and submitted to LC-MS/MS analysis. Equivalent or similar procedures for testing ex vivo stability are known and may be used to determine stability of macrocycles in serum.

In Vivo Protease Resistance Assays: A key benefit of peptide stapling is the translation of in vitro protease resistance into markedly improved pharmacokinetics in vivo.

In vitro Binding Assays: To assess the binding and affinity of peptidomimetic macrocycles and peptidomimetic precursors to acceptor proteins, a fluorescence polarization assay (FPA) can be used, for example. The FPA technique measures the molecular orientation and mobility using polarized light and fluorescent tracer. When excited with polarized light, fluorescent tracers (e.g., FITC) attached to molecules with high apparent molecular weights (e.g. FITC-labeled peptides bound to a large protein) emit higher levels of polarized fluorescence due to their slower rates of rotation as compared to fluorescent tracers attached to smaller molecules (e.g. FITC-labeled peptides that are free in solution).

Stabilized NOXA Peptide Variants

The disclosure also provides NOXA SAHB peptide variants. These variants can differ by one or more amino acids from the amino acid sequence set forth in SEQ ID NO:39. In addition to amino acid substitutions, C- and/or N-terminal deletions are also envisioned.

In some embodiments, internally cross-linked peptides can be made by modifying (e.g., by amino acid substitution) a polypeptide of SEQ ID NO:39. Such NOXA SAHB variants can include peptides with reduced hydrophobicity and/or overall positive charge relative to the peptide of SEQ ID NO:39; or relative to the stapled counterpart of the variant peptide (*i.e.,* a stapled version of the peptide without the variation from SEQ ID NO:39). In some embodiments, an internal staple replaces the side chains of 2 amino acids, i.e., each staple is between two amino acids separated by, for example, 3, 4, or 6 amino acids. In some embodiments, an internal stitch replaces the side chains of 3 amino acids, *i.e.,* the stitch is a pair of crosslinks between three amino acids separated by, for example, 3 and 6 amino acids. In some embodiments, the internal staples and/or the internal stitch comprise at least two internal staples (replacing the side chains of 4 amino acids, *i.e.,* each staple is between two amino acids separated by, for example, 3 amino acids). In some embodiments, the internal staples and/or the internal stitch comprise a combination of at least one internal staple and an

internal stitch. In some embodiments, the internal stitch replaces the side chain of a first amino acid and a second and a third amino acid thereby cross-linking the first amino acid (which lies between the second and third amino acids) to the second and third amino acid via an internal cross-link, wherein the first and second amino acid are separated by two, three, or six amino acids, the first and the third amino acids are separated by two, three, or six amino acids, and the second and third amino acids are distinct amino acids. In some embodiments, the side chains of the four amino acids of the internally cross-linked polypeptides of the disclosure are replaced by two distinct internal staples. In some embodiments, a first of the two distinct internal staples cross-links a first pair of amino acids separated by two, three, or six amino acids, and a second of the at least two distinct internal staples cross-links a second pair of amino acids separated by two, three, or six amino acids. In some embodiments, internally cross-linked NOXA polypeptide variants of the disclosure are prepared from a polypeptide of SEQ ID NO: 39 and having *e.g.,* 1, 2, 3, 4, 5, 6, 7, 8, or 9 amino acid substitutions (e.g., 1, 2, 3, 4, 5, 6, 7, 8, or 9 amino acids are conservatively or non-conservatively substituted). Exemplary NOXA stapled peptide variants are provided in Figure 6 and Figure 10.

In some instances, stabilized peptides can have 1 to 8 amino acid substitutions (e.g., 1, 2, 3, 4, 5, 6, 7, or 8) on the non-interacting face of the helix of the stabilized NOXA peptide (i.e., the part of the NOXA BH3 helix that does not interact with BFL-1). The "interacting face" of the polypeptides described herein includes those amino acid residues of the alpha helix that interact (e.g., interact specifically or bind specifically) with a BFL-1 protein. In certain instances, the peptides have 1 to 9, 1 to 8, 1 to 7, 1 to 6, 1 to 5, 1 to 4, 1 to 3, or 1 to 2 or 1 amino substitution on the interacting face of the helix of the peptide. In some instances, it can be useful to replace an amino acid on the interacting face of any one of the peptides of SEQ ID NOS: 2-38 with another amino acid, *e.g.,* Ala or a derivative such as D-Ala, or α-aminoisobutyric acid. In certain embodiments, the amino acid substitutions are conservative amino acid substitution(s). In some instances, the peptides described herein can include one of SEQ ID NOs:2-38 with one or more (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 1 to 2, 1 to 3, 1 to 4, 1 to 5, 1 to 6, 1 to 7, 1 to 8, 1 to 9, 1 to 10, 1 to 11, or 1 to 12) conservative amino acid substitutions. Significant variability is permitted in the amino acids that are not on the interacting face of the helix of the peptides described herein. For example, almost all, if not all, of these amino acids can be substituted (e.g., with a conservative amino acid). A "conservative amino acid substitution" means that the substitution replaces one amino acid

with another 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).

In some instances, the peptides of this disclosure include sequences with at least 14%, 15%, 20%, 27%, 34%, 40%, 47%, 50%, 53%, 60%, 65% 70%, 75%, 80%, 85%, 90%, 95%, or or 97% identity to an amino acid sequence of SEQ ID NOs:2-38. In certain cases, the variability in the amino acid sequence is on the non-interacting face of the helix. In some cases, the variability in the amino acid sequence is on the interacting face of the helix. In certain cases, the variability in the amino acid sequence is on both the non-interacting and interacting face of the helix. In certain instances one or more of A-1, T-2, Q-3, R-5, R-6, F-7, G-8, K-10, L-11, N-12, F-13, R-14, and Q-15 (numbering according to SEQ ID NO:39) are substituted. In some instances, the substitution is to L-alanine, D-alanine, or α-aminoisobutyric acid. In some instances, 1 to 5 (*e.g.,* 1, 2, 3, 4 or 5) amino acids at the C- and/or N-terminus are deleted. Methods for determining percent identity between amino acid sequences are known in the art. For example, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). In a preferred embodiment, the length of a reference sequence aligned for comparison purposes is at least 30%, preferably at least 40%, more preferably at least 50%, even more preferably at least 60%, and even more preferably at least 70%, 80%, 90%, or 100% of the length of the reference sequence. The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The determination of percent identity between two amino acid sequences is accomplished using the BLAST 2.0 program. Sequence comparison is performed using an ungapped alignment and using the default parameters (Blossom 62 matrix, gap existence cost of 11, per residue gapped cost of 1, and a lambda ratio of 0.85).

The mathematical algorithm used in BLAST programs is described in Altschul et al. (Nucleic Acids Res. 25:3389-3402, 1997).

The stapled polypeptide comprises at least two modified amino acids joined by an internal intramolecular cross-link (or "staple"), wherein the at least two amino acids are separated by 2, 3, or 6 amino acids. Stabilized peptides herein include stapled peptides, including peptides having two staples and/or stitched peptides. The at least two modified amino acids can be unnatural alpha-amino acids (including, but not limited to α,α-disubstituted and N-alkylated amino acids). There are many known unnatural amino acids any of which may be included in the peptides of the present invention. Some examples of unnatural amino acids are 4-hydroxyproline, desmosine, gamma-aminobutyric acid, beta-cyanoalanine, norvaline, 4-(E)-butenyl-4(R)-methyl-N- methyl-L-threonine, N-methyl-L-leucine, 1-amino-cyclopropanecarboxylic acid, 1- amino-2-phenyl-cyclopropanecarboxylic acid, 1-amino-cyclobutanecarboxylic acid, 4- amino-cyclopentenecarboxylic acid, 3-amino-cyclohexanecarboxylic acid, 4-piperidylacetic acid, 4-amino-l-methylpyrrole-2-carboxylic acid, 2,4-diaminobutyric acid, 2,3- diaminopropionic acid, 2,4-diaminobutyric acid, 2-aminoheptanedioic acid, 4- (aminomethyl)benzoic acid, 4-aminobenzoic acid, ortho-, meta- and /para-substituted phenylalanines (e.g., substituted with -C(=O)C_6H_5; -CF_3; -CN; -halo; -NO2; CH_3), disubstituted phenylalanines, substituted tyrosines (e.g., further substituted with -Q=O)C_6H_5; -CF_3; -CN; -halo; -NO_2; CH_3), and statine.

In certain embodiments, the stapled NOXA polypeptide variant comprises or consists of the amino acid sequence set forth in any one of **SEQ ID NOs:22 to 34** (without including the N-terminal nipecotic acid).

In certain embodiments, the stapled NOXA polypeptide variant comprises or consists of the amino acid sequence set forth in any one of **SEQ ID NOs:35 to 38** (without including the N-terminal nipecotic acid).

In certain embodiments, the stapled NOXA polypeptide variant comprises or consists of a structurally stabilized versions of the amino acid sequences set forth in any one of **SEQ ID NOs:94 to 109**. A non-limiting example of structural stabilization of these peptides is achieved by hydrocarbon stapling by introducing non-natural amino acids at positions separated by 2, 3, or 6 amino acids in these sequences.

In certain embodiments, the stapled polypeptide comprises or consists of an amino acid sequence selected from the group consisting of:

AT**X_1**LRR<u>A</u>GD**X_2**LNFRQ (**SEQ ID NO:40**);

AT**X_1**LRRFGD**X_2**<u>A</u>NFRQ (**SEQ ID NO:41**);

AT**X_1**LR<u>E</u>FGD**X_2**LNFRQ (**SEQ ID NO:42**); and

AT**X_1**LRRFGD**X_2**LNF<u>E</u>Q (**SEQ ID NO:43**),

wherein X_1 and X_2 in the above sequences are non-natural amino acids which can be covalently joined ("stapled together") using a ring-closing metathesis (RCM) reaction to form a cross-linked ring.

In some embodiments, the stapled polypeptide variant comprises or consists of an amino acid sequence selected from the group consisting of:

AT**8**LRR<u>A</u>GD**X**LNFRQ (**SEQ ID NO:44**);

AT**8**LRRFGDX<u>A</u>NFRQ (**SEQ ID NO:45**);

AT**8**LR<u>E</u>FGDXLNFRQ (**SEQ ID NO:46**);

AT**8**LRRFGDXLNF<u>E</u>Q (**SEQ ID NO:47**),

AA**8**LRAFGAXLNAA (**SEQ ID NO:48);**

AA**8**LRAFGAXLNAAAA (**SEQ ID NO:49**);

AA**8**LRAFGAXLNAE (**SEQ ID NO:50**);

AA**8**LRAFGAXLNAEAA (**SEQ ID NO:51**);

AA**8**LREFGAXLNAA (**SEQ ID NO:52);**

AA**8**LREFGAXLNAAAA (**SEQ ID NO:53);**

AA**8**LRAFGDXLNAA (**SEQ ID NO:54);**

AA**8**LRAFGDXLNAAAA (**SEQ ID NO:55);**

AA**8**LRAFGDXLNAE (**SEQ ID NO:56);**

AA**8**LRAFGDXLNAEAA (**SEQ ID NO:57);**

AA**8**LREFGDXLNAA (**SEQ ID NO:58**); and

AA**8**LREFGDXLNAAAA (**SEQ ID NO:59),**

wherein "8" = R-octenyl alanine: and "X" = S-pentenyl alanine.

Warhead Bearing Stabilized NOXA Peptides

This disclosure features stabilized NOXA peptides that include a warhead – *i.e.,* a reactive group such as a non-natural amino acid bearing an electrophilic group. Importantly, these warhead containing peptides allow for significant variability in the amino acid sequence of the associated NOXA peptide (much more than in the absence of the warhead) as the interaction between the NOXA peptide and BFL-1 is cemented by the covalent bond between the electrophile and the cysteine at position 55 of BFL-1. Thus, even if there are substitutions and/or deletions in SEQ ID NO:39, including substitutions on the BFL-1 interacting face of the NOXA helix, these warhead bearing peptides are likely to be effective in binding BFL-1. Furthermore, with the warhead present, the size of the NOXA peptide (e.g., any one of SEQ ID NOs:39-59) can be reduced in length (e.g., from 15 to 14, 13, 12, 11, 10, 9, 8, 7, 6, or 5 amino acids).

The electrophile can be installed not only in the context of a non-natural amino acid, but also as a chemical cap to the N or C terminus of the stabilized (e.g., cross-linked) NOXA polypeptide. In some instances, the electrophile can be installed within the stabilized peptide. Such warhead bearing stabilized peptides are able to form covalent bonds with at least some of the proteins they interact with. For example, such warhead bearing NOXA peptides can covalently modify BFL-1 (*e.g.,* by covalent bonding with C55 of BFL-1), noncovalently interact with BFL-1 and/or non-covalently interact with MCL-1.

The warheads may be at the N-terminus, C-terminus, or within the polypeptide sequence. In some cases, the warhead is a non-natural electrophile bearing amino acid. In certain embodiments, the warhead is selected from the group consisting of: 3S-1-pyrrolidine-3-carboxylic acid terminating in acrylamide; D-homoproline terminating in acrylamide; L-homoproline terminating in acrylamide; isonipecotic acid terminating in acrylamide; D-nipecotic acid terminating in acrylamide; L-nipecotic acid terminating in acrylamide; D-proline terminating in acrylamide; L-proline terminating in acrylamide; trans-4-dimethylaminocrotonic acid; and acrylic acid.

In some embodiments, the electrophilic warhead is a cysteine-reactive D-nipecotic acid moiety. In other embodiments, the electrophilic warhead is a cysteine-reactive moiety.

In certain embodiments, the warhead is a non-natural amino acid bearing an electrophilic group that is selected from the group consisting of: (S)-1-acryloylpyrrolidine-3-carboxamide; 1-acrylopiperidine-4-carboxamide, (R)-1 acryloylpiperidine-3-carboxamide; (S)-1-acryloylpiperidine-3-carboxamide; (S)-1-acryloylpyyrolidine-2-carboxamide; (R)-1-acryloylpyrrolidine-2-carboxamide; (E)-4-(dimethylamino)but-2-enamide; and acrylamide. In other embodiments, the warhead is not an amino acid. For example, the electrophilic moiety and peptide are linked via a nitrogen containing heterocycle, either saturated (aziridine, diaziridine, azetidine, pyrrolidine, imidazolidine, pyrazolidine, oxazolidine, isoxazolidine, thiazolidine, isothiazolidine, piperidine, piperazine, morpholine, thiomorpholine, azepane) or unsaturateed (azirine, diazirine, azete, pyrrole, imidazole, pyrazole, oxazole, isoxazole, thiazole, isothiazole, pyridine, diazines, oxazine, thiazine, azepine). The peptide and electrophile can also be linked by a substituted amino-functionalized ring (e.g. N-arylacrylamide) such as phenyl (aniline) or by more complex bicyclic or polycyclic rings, for instance, naphthalene, anthracene, phenanthrene, indole, isoindole, indolizine, quinolone, isoquinoline, quinoxaline, phthalzine, quinazoline, purine, carbazole, indazole, benzimidazole, azaindole. The electrophilic warhead in some embodiments is an acrylamide, or more generally defined as an α,β-unsaturated carbonyl, such as α-cyanoacrylamide, propiolamide, trans 4-dimethylamino-2-butenamide, or trans 4-piperidinyl-2-butenamide, or any other substituted acrylamide, or N-functionalized vinylsulfonyl, alpha-fluoro acetyl, alpha-chloro acetyl, alpha-bromo acetyl, and alpha-iodo acetyl or other electrophilic moiety. The electrophile can not only be installed in the context of a non-natural amino acid, but also as a chemical cap to the N or C terminus of the cross-linked (e.g., stapled, stitched) polypeptide.

In one aspect, the BFL-1 binding warhead bearing NOXA peptide has an amino acid sequence set out in any one of SEQ ID NOs.: 94-109. In another aspect, the BFL-1 binding warhead bearing NOXA peptide has an amino acid sequence that contains 5 or more (*e.g.,* 5, 6, 7, 8,. 10, 11, 12, 13, 14) amino acids of a sequence set out in any one of SEQ ID NOs.: 94-109. In another aspect, the BFL-1 binding warhead bearing NOXA peptide has an amino acid sequence that contains 1-5 deletions (e.g., 1, 2, 3, 4, 5) at the C- or N-terminus of a sequence set out in any one of SEQ ID NOs.: 94-109. In some instances, the electrophilic warhead is at the N-terminus of the peptide. In other instances, the electrophilic warhead is within the peptide.

In one embodiment, the BFL-1 binding warhead-bearing NOXA peptide has a sequence selected from the sequences set out below:

JATQLRRAGDKLNFRQ (**SEQ ID NO:126**);

JATQLRRFGDKANFRQ (**SEQ ID NO:127**);

JATQLREFGDKLNFRQ (**SEQ ID NO:128**);

JATQLRRFGDKLNFEQ (**SEQ ID NO:129**);

JAAQLRAFGAKLNAA (**SEQ ID NO:130**);

JAAQLRAFGAKLNAAAA (**SEQ ID NO:131**);

JAAQLRAFGAKLNAE (**SEQ ID NO:132**);

JAAQLRAFGAKLNAEAA (**SEQ ID NO:133**);

JAAQLREFGAKLNAA (**SEQ ID NO:134**);

JAAQLREFGAKLNAAAA (**SEQ ID NO:135**);

JAAQLRAFGDKLNAA (**SEQ ID NO:136**);

JAAQLRAFGDKLNAAAA (**SEQ ID NO:137**);

JAAQLRAFGDKLNAE (**SEQ ID NO:138**);

JAAQLRAFGDKLNAEAA (**SEQ ID NO:139**);

JAAQLREFGDKLNAA (**SEQ ID NO:140**); or

JAAQLREFGDKLNAAAA (**SEQ ID NO:141**).

In certain instances the above warhead-bearing sequences can be structurally stabilized by any method known in the art. For example, at least two amino acids (e.g., 2, 3, 4, 5) of the sequence separated by 2, 3, or 6 amino acids can be replaced with non-natural amino acids that can form a staple and/or stitch. In some embodiments, the electrophilic warhead (J) is a cysteine-reactive D-nipecotic acid moiety. In other embodiments, the electrophilic warhead (J) is a cysteine-reactive moiety.

In some embodiments, the stapled polypeptide comprises or consists of an amino acid sequence selected from the group consisting of:

JAT**X**$_1$LRRAGD**X**$_2$LNFRQ (**SEQ ID NO:60**);

JAT**X**$_1$LRRFGD**X**$_2$ANFRQ (**SEQ ID NO:61**);

JAT**X**$_1$LREFGD**X**$_2$LNFRQ (**SEQ ID NO:62**); and

JAT**X**$_1$LRRFGD**X**$_2$LNFEQ (**SEQ ID NO:63**),

JAA**X**$_1$LRAFGA**X**$_2$LNAA (**SEQ ID NO:64**);

JAA**X**$_1$LRAFGA**X**$_2$LNAAAA (**SEQ ID NO:65**);

JAA**X**$_1$LRAFGA**X**$_2$LNAE (**SEQ ID NO:66**);

JAA**X**$_1$LRAFGA**X**$_2$LNAEAA (**SEQ ID NO:67**);

JAA**X**$_1$LREFGA**X**$_2$LNAA (**SEQ ID NO:68**);

JAA**X**$_1$LREFGA**X**$_2$LNAAAA (**SEQ ID NO:69**);

JAA**X**$_1$LRAFGD**X**$_2$LNAA (**SEQ ID NO:70**);

JAA**X**$_1$LRAFGD**X**$_2$LNAAAA (**SEQ ID NO:71**);

JAA**X**$_1$LRAFGD**X**$_2$LNAE (**SEQ ID NO:72**);

JAA**X**$_1$LRAFGD**X**$_2$LNAEAA (**SEQ ID NO:73**);

JAA**X**$_1$LREFGD**X**$_2$LNAA (**SEQ ID NO:74**); and

JAA**X**$_1$LREFGD**X**$_2$LNAAAA (**SEQ ID NO:75**),

wherein "X_1" and "X_2" in the above sequences are non-natural amino acids which can be covalently joined ("stapled together") using a ring-closing metathesis (RCM) reaction to form a cross-linked ring and "J" is a non-natural electrophile containing amino acid or an electrophilic warhead presented in the context of a moiety that is not an amino acid (the electrophile can serve as a chemical cap)). In a particular embodiment, "J" is a cysteine-reactive D-nipecotic acid moiety. In another particular embodiment, "J" is a cysteine-reactive moiety.

In some embodiments, the stapled polypeptide comprises or consists of an amino acid sequence listed above, or in **SEQ ID NOs.: 2-21** where multiple residues (e.g., 1, 2, 3, 4, 5, 6) on the non-interacting face can be replaced with residues selected from the group consisting of Ala (alanine), D-Ala (D-alanine), Aib (α-aminoisobutyric acid), Sar (N-methyl

glycine), and Ser (serine), other substituted alanine, and a glycine derivative. Additionally, one or more of these residues (e.g., 1, 2, 3, 4, 5, 6) can be appended to the C-terminus of the peptide. In some instances, 0 to 5 amino at the C-terminus of the above peptides (SEQ ID NOs:60-75) or those of SEQ ID NOs.: 2-21 are replaced with a residue or residues selected from the group consisting of Ala (alanine), D-Ala (D-alanine), Aib (α-aminoisobutyric acid), Sar (N-methyl glycine), and Ser (serine), other substituted alanine, and a glycine derivative.

In some embodiments, the stapled polypeptide comprises or consists of an amino acid sequence selected from the group consisting of:

JAT**8**LRRAGD**X**LNFRQ (**SEQ ID NO:76**);

JAT**8**LRRFGDXANFRQ (**SEQ ID NO:77**);

JAT**8**LREFGDXLNFRQ (**SEQ ID NO:78**);

JAT**8**LRRFGDXLNFEQ (**SEQ ID NO:79**)

JAA**8**LREFGA**X**LNAA (SEQ ID NO:151); and

JAA**8**LREFGA**X**LNAAAA (SEQ ID NO:153),

wherein "8" = R-octenyl alanine, "X" = S-pentenyl alanine, and "J" = a non-natural electrophile containing amino acid or an electrophilic warhead presented in the context of a moiety that is not an amino acid. In a particular embodiment, "J" is a cysteine-reactive D-nipecotic acid moiety. In another particular embodiment, "J" is a cysteine-reactive moiety.

In some embodiments, the disclosure features a warhead-bearing NOXA stapled peptide that is at least 14%, 15%, 20%, 27%, 34%, 40%, 47%, 50%, 53%, 60%, 65% 70%, 75%, 80%, 85%, 90%, 95%, or 97%,identical to the amino acid sequence set forth in any one of SEQ ID NOs.: 60-79, and wherein the modified peptide covalently binds BFL-1. In some embodiments, these modified peptides also non-covalently bind to BFL-1 and/or MCL-1. In some embodiments, these modified peptides have reduced hydrophobicity and/or overall positive charge relative to the stapled NOXA peptide prior to the amino acid variation. In some embodiments, hydrophobicity or positive charge are independently enhanced to optimize cell penetrance. In some embodiments, these modified peptides have reduced hydrophobicity and/or overall positive charge relative to the peptide of SEQ ID NO:16.

In certain embodiments, the disclosure features a warhead-bearing NOXA stapled peptide that has 1-10 (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10) amino acid substitutions relative to

the amino acid sequence set forth in any one of SEQ ID NOs.: 60-79, and wherein the modified peptide covalently binds BFL-1. In some embodiments, these modified peptides also non-covalently bind to BFL-1 and/or MCL-1. In some embodiments, these modified peptides have reduced hydrophobicity and/or overall positive charge relative to the stapled NOXA peptide prior to the amino acid substitution. In some embodiments, these modified peptides have reduced hydrophobicity and/or overall positive charge relative to the peptide of SEQ ID NO:16. In some embodiments, hydrophobicity or positive charge are independently enhanced to optimize cell penetrance.

Exemplary Stabilized NOXA Peptide Variants and Warhead Bearing Stabilized NOXA Peptides

In a specific embodiment, the stapled peptide comprises the amino acid sequence AT8LREFGDXLNFRQ (SEQ ID NO:46) with 0 to 6 (*i.e.,* 0, 1, 2, 3, 4, 5, 6) amino acid substitutions, insertions, and/or deletions, wherein "8" = R-octenyl alanine: and "X" = S-pentenyl alanine. In a particular embodiment, the stapled peptide further comprises one or more of the modifications described in the sections "NOXA Peptides" and "Stabilized Peptides" above. In another particular embodiment, the stapled peptide consists of the amino acid sequence AT8LREFGDXLNFRQ (SEQ ID NO:46), wherein "8" = R-octenyl alanine: and "X" = S-pentenyl alanine. In certain embodiments, the zero to six (*i.e.,* 0, 1, 2, 3, 4, 5, 6) amino acids of SEQ ID NO:46 that are substituted by another amino acid are on the BFL-1/A1 non-interacting face of the helix of SEQ ID NO:46. In some embodiments, 0 to 3 amino acids in SEQ ID NO:46 are removed from the C-terminal or are removed and replaced with 1 to 6 amino acids from the group consisting of alanine, D-alanine, α-aminoisobutyric acid, N-methyl glycine, serine, a substituted alanine, and a glycine derivative. In some embodiments, 0 to 6 amino acids on the non-interacting face in SEQ ID NO:46 are substituted with an amino acid selected from the group consisting of alanine, D-alanine, α-aminoisobutyric acid, N-methyl glycine, serine, a substituted alanine, and a glycine derivative. In some embodiments, one or more of the following amino acids A1, L4, R5, F7, G8, L11, N12, and Q15 of SEQ ID NO:46 are substituted with an alpha-methylated or alpha-ethylated natural amino acids. In some embodiments, one or more of the following amino acids A1, L4, R5, F7, G8, L11, N12, and Q15 of SEQ ID NO:46 are substituted with an amino acid selected from the group consisting of L-alanine, D-alanine, α-aminoisobutyric acid, N-methyl glycine, serine, a substituted alanine, and a glycine derivative. In some embodiments, one or more of the following amino acids T2, R6, F13, and R14 of SEQ ID

NO:46 are substituted with an alpha-methylated or alpha-ethylated natural amino acids. In certain embodiments, one or more of the following amino acids T2, R6, F13, and R14 of SEQ ID NO:46 are substituted with an amino acid selected from the group consisting of L-alanine, D-alanine, α-aminoisobutyric acid, N-methyl glycine, serine, a substituted alanine, and a glycine derivative. In certain embodiments, one or more of the following amino acids A1, T2, R5, R6, F7, G8, L11, N12, F13, R14, and Q15 of SEQ ID NO:46 are modified with an alpha methyl or ethyl, or are substituted with an amino acid selected from the group consisting of L-alanine, D-alanine, α-aminoisobutyric acid, N-methyl glycine, serine, a substituted alanine, and a glycine derivative. In certain instances, the one to six amino acids of SEQ ID NO:46 that are substituted by another amino acid are on the BFL-1/A1 interacting face of the helix of SEQ ID NO:46. In other instances, the one to six amino acids of SEQ ID NO:46 that are substituted by another amino acid are on the BFL-1/A1 non-interacting and interacting faces of the helix of SEQ ID NO:46. In certain embodiments, the one to six amino acids of SEQ ID NO:46 are substituted by an amino acid or amino acids selected from the group consisting of L-alanine, D-alanine, α-aminoisobutyric acid, N-methyl glycine, serine, a substituted alanine, and a glycine derivative.

In another specific embodiment, the stapled peptide comprises the amino acid sequence AT8LRRAGDXLNFRQ (SEQ ID NO:44) with 0 to 6 (i.e., 0, 1, 2, 3, 4, 5, 6) amino acid substitutions, insertions, and/or deletions, wherein "8" = R-octenyl alanine: and "X" = S-pentenyl alanine. In a particular embodiment, the stapled peptide further comprises one or more of the modifications described in the sections "NOXA Peptides" and "Stabilized Peptides" above. In another particular embodiment, the stapled peptide consists of the amino acid sequence AT8LRRAGDXLNFRQ (SEQ ID NO:44), wherein "8" = R-octenyl alanine: and "X" = S-pentenyl alanine. In certain embodiments, the zero to six (i.e., 0, 1, 2, 3, 4, 5, 6) amino acids of SEQ ID NO:44 that are substituted by another amino acid are on the BFL-1/A1 non-interacting face of the helix of SEQ ID NO:44. In some embodiments, 0 to 3 amino acids in SEQ ID NO:44 are removed from the C-terminal or are removed and replaced with 1 to 6 amino acids from the group consisting of alanine, D-alanine, α-aminoisobutyric acid, N-methyl glycine, serine, a substituted alanine, and a glycine derivative. In some embodiments, 0 to 6 amino acids on the non-interacting face in SEQ ID NO:44 are substituted with an amino acid selected from the group consisting of alanine, D-alanine, α-aminoisobutyric acid, N-methyl glycine, serine, a substituted alanine, and a glycine derivative. In some embodiments, one or more of the following amino acids A1, L4, R5, G8,

L11, N12, and Q15 of SEQ ID NO:44 are substituted with an alpha-methylated or alpha-ethylated natural amino acids. In some embodiments, one or more of the following amino acids A1, L4, R5, G8, L11, N12, and Q15 of SEQ ID NO:44 are substituted with an amino acid selected from the group consisting of L-alanine, D-alanine, α-aminoisobutyric acid, N-methyl glycine, serine, a substituted alanine, and a glycine derivative. In some embodiments, one or more of the following amino acids T2, R6, F13, and R14 of SEQ ID NO:44 are substituted with an alpha-methylated or alpha-ethylated natural amino acids. In certain embodiments, one or more of the following amino acids T2, R6, F13, and R14 of SEQ ID NO:44 are substituted with an amino acid selected from the group consisting of L-alanine, D-alanine, α-aminoisobutyric acid, N-methyl glycine, serine, a substituted alanine, and a glycine derivative. In certain embodiments, one or more of the following amino acids A1, T2, R5, R6, G8, L11, N12, F13, R14, and Q15 of SEQ ID NO:44 are modified with an alpha methyl or ethyl, or are substituted with an amino acid selected from the group consisting of L-alanine, D-alanine, α-aminoisobutyric acid, N-methyl glycine, serine, a substituted alanine, and a glycine derivative. In certain instances, the one to six amino acids of SEQ ID NO:44 that are substituted by another amino acid are on the BFL-1/A1 interacting face of the helix of SEQ ID NO:44. In other instances, the one to six amino acids of SEQ ID NO:44 that are substituted by another amino acid are on the BFL-1/A1 non-interacting and interacting faces of the helix of SEQ ID NO:44. In certain embodiments, the one to six amino acids of SEQ ID NO:44 are substituted by an amino acid or amino acids selected from the group consisting of L-alanine, D-alanine, α-aminoisobutyric acid, N-methyl glycine, serine, a substituted alanine, and a glycine derivative.

In another specific embodiment, the peptide is a BFL-1-binding warhead-bearing NOXA peptide described herein. For example, the peptides of SEQ ID NO:46, 44, or their variants described above, are modified to include a non-natural electrophile containing amino acid or an electrophilic warhead presented in the context of a moiety that is not an amino acid (the electrophile can serve as a chemical cap) (*e.g.,* a cysteine-reactive moiety; a cysteine-reactive D-nipecotic acid moiety).

In a specific embodiment, the BFL-1-binding warhead bearing NOXA peptide comprises the sequence JATQLREFGDKLNFRQ (SEQ ID NO:128) with 0 to 6 (*i.e.*, 0, 1, 2, 3, 4, 5, 6) amino acid substitutions, insertions, and/or deletions (as described above), wherein the peptide is structurally stabilized by any method known in the art, wherein the electrophilic warhead (J) is a cysteine-reactive moiety, (e.g., a D-nipecotic acid moiety). In a

particular embodiment, the BFL-1-binding warhead-bearing NOXA peptide comprises the sequence JATX$_1$LREFGDX$_2$LNFRQ (SEQ ID NO:62) with 0 to 6 (*i.e.*, 0, 1, 2, 3, 4, 5, 6) amino acid substitutions, insertions, and/or deletions (as described above), wherein "X$_1$" and "X$_2$" are non-natural amino acids which can be covalently joined ("stapled together") using a ring-closing metathesis (RCM) reaction to form a cross-linked ring and "J" is a non-natural electrophile containing amino acid or an electrophilic warhead presented in the context of a moiety that is not an amino acid (the electrophile can serve as a chemical cap) (e.g., wherein "J" is a cysteine-reactive moiety, e.g., wherein "J" is a cysteine-reactive D-nipecotic acid moiety). In another particular embodiment, the BFL-1-binding warhead-bearing NOXA peptide comprises the sequence JAT8LREFGDXLNFRQ (SEQ ID NO:78) with 0 to 6 (i.e., 0, 1, 2, 3, 4, 5, 6) amino acid substitutions, insertions, and/or deletions (as described above), wherein "8" = R-octenyl alanine, "X" = S-pentenyl alanine, and "J" = a non-natural electrophile containing amino acid or an electrophilic warhead presented in the context of a moiety that is not an amino acid (e.g., wherein "J is a cysteine-reactive moiety, e.g., wherein "J" is a cysteine-reactive D-nipecotic acid moiety). In a particular embodiment, the BFL-1-binding warhead-bearing NOXA peptide (e.g., SEQ ID NO:62, or 78, or 128) further comprises one or more of the modifications described in the sections "NOXA Peptides" and "Stabilized Peptides" above.

In a specific embodiment, the BFL-1-binding warhead bearing NOXA peptide consists of the sequence JATQLREFGDKLNFRQ (SEQ ID NO:128) with 0 to 6 (i.e., 0, 1, 2, 3, 4, 5, 6) amino acid substitutions, insertions, and/or deletions (as described above), wherein the peptide is structurally stabilized by any method known in the art, wherein the electrophilic warhead (J) is a cysteine-reactive moiety, (e.g., a D-nipecotic acid moiety). In a particular embodiment, the BFL-1-binding warhead-bearing NOXA peptide consists of the sequence JATX$_1$LREFGDX$_2$LNFRQ (SEQ ID NO:62) with 0 to 6 (i.e., 0, 1, 2, 3, 4, 5, 6) amino acid substitutions, insertions, and/or deletions (as described above), wherein "X$_1$" and "X$_2$" are non-natural amino acids which can be covalently joined ("stapled together") using a ring-closing metathesis (RCM) reaction to form a cross-linked ring and "J" is a non-natural electrophile containing amino acid or an electrophilic warhead presented in the context of a moiety that is not an amino acid (the electrophile can serve as a chemical cap) (e.g., wherein "J" is a cysteine-reactive moiety, e.g., wherein "J" is a cysteine-reactive D-nipecotic acid moiety). In another particular embodiment, the BFL-1-binding warhead-bearing NOXA peptide consists of the sequence JAT8LREFGDXLNFRQ (SEQ ID NO:78) with 0 to 6 (i.e.,

0, 1, 2, 3, 4, 5, 6) amino acid substitutions, insertions, and/or deletions (as described above), wherein "8" = R-octenyl alanine, "X" = S-pentenyl alanine, and "J" = a non-natural electrophile containing amino acid or an electrophilic warhead presented in the context of a moiety that is not an amino acid (e.g., wherein "J is a cysteine-reactive moiety, e.g., wherein "J" is a cysteine-reactive D-nipecotic acid moiety).

In another specific embodiment, the BFL-1-binding warhead-bearing NOXA peptide comprises the sequence JATQLRRAGDKLNFRQ (SEQ ID NO:126), wherein the peptide is structurally stabilized by any method known in the art, wherein the electrophilic warhead (J) is a cysteine-reactive moiety, (e.g., a D-nipecotic acid moiety). In a particular embodiment, the BFL-1-binding warhead-bearing NOXA peptide comprises the JATX_1LRRAGDX_2LNFRQ (SEQ ID NO:60), wherein "X_1" and "X_2" are non-natural amino acids which can be covalently joined ("stapled together") using a ring-closing metathesis (RCM) reaction to form a cross-linked ring and "J" is a non-natural electrophile containing amino acid or an electrophilic warhead presented in the context of a moiety that is not an amino acid (the electrophile can serve as a chemical cap) (e.g., wherein "J" is a cysteine-reactive moiety, e.g., wherein "J" is a cysteine-reactive D-nipecotic acid moiety). In another particular embodiment, the BFL-1-binding warhead-bearing NOXA peptide comprises the sequence JAT8LRRAGDXLNFRQ (SEQ ID NO:76), wherein wherein "8" = R-octenyl alanine, "X" = S-pentenyl alanine, and "J" = a non-natural electrophile containing amino acid or an electrophilic warhead presented in the context of a moiety that is not an amino acid (e.g., wherein "J is a cysteine-reactive moiety, e.g., wherein "J" is a cysteine-reactive D-nipecotic acid moiety). In a particular embodiment, the BFL-1-binding warhead-bearing NOXA peptide (e.g., SEQ ID NO:60, or 76, or 126) further comprises one or more of the modifications described in the sections "NOXA Peptides" and "Stabilized Peptides" above. In another specific embodiment, the BFL-1-binding warhead-bearing NOXA peptide consists of the sequence JATQLRRAGDKLNFRQ (SEQ ID NO:126), wherein the peptide is structurally stabilized by any method known in the art, wherein the electrophilic warhead (J) is a cysteine-reactive moiety, (e.g., a D-nipecotic acid moiety). In a particular embodiment, the BFL-1-binding warhead-bearing NOXA peptide consists of the JATX_1LRRAGDX_2LNFRQ (SEQ ID NO:60), wherein "X_1" and "X_2" are non-natural amino acids which can be covalently joined ("stapled together") using a ring-closing metathesis (RCM) reaction to form a cross-linked ring and "J" is a non-natural electrophile containing amino acid or an electrophilic warhead presented in the context of a moiety that is not an

amino acid (the electrophile can serve as a chemical cap) (e.g., wherein “J” is a cysteine-reactive moiety, e.g., wherein “J” is a cysteine-reactive D-nipecotic acid moiety). In another particular embodiment, the BFL-1-binding warhead-bearing NOXA peptide consists of the sequence JAT8LRRAGDXLNFRQ (SEQ ID NO:76), wherein wherein “8” = R-octenyl alanine, “X” = S-pentenyl alanine, and “J” = a non-natural electrophile containing amino acid or an electrophilic warhead presented in the context of a moiety that is not an amino acid (e.g., wherein “J is a cysteine-reactive moiety, e.g., wherein “J” is a cysteine-reactive D-nipecotic acid moiety).

Methods of Treatment

The disclosure features methods of using any of the stabilized peptides (or pharmaceutical compositions comprising said stabilized peptides) described herein for the prophylaxis and/or treatment of a cancer, an autoimmune disease, or an inflammatory disease. The terms "treat" or "treating," as used herein, refers to alleviating, inhibiting, or ameliorating the disease or condition from which the subject is suffering.

The peptides described herein can be useful for treating a human subject with a BFL-1-expressing cancer. The peptides described herein can also be useful for treating a human subject with a BFL-1-dependent cancer. In certain embodiments, the cancer is a solid tumor or a liquid tumor. In certain embodiments, the BFL-1-dependent cancer is a melanoma, a leukemia, lymphoma, or other hematologic malignancy or solid tumor. In certain embodiments, the hematologic malignancy is acute myeloid leukemia, acute lymphoblastic leukemia, chronic myeloid leukemia, chronic lymphocytic leukemia, myelodysplastic syndrome, or multiple myeloma. In certain embodiments, the BFL-1-dependent cancer is a breast cancer, an autonomic ganglia cancer, a pancreatic cancer, a skin cancer, a CNS cancer, a hematopoietic or lymphoid cancer, a lung cancer, a large intestine cancer, a stomach cancer, a soft tissue sarcoma, or a bone cancer. In certain instances, the solid tumor is a melanoma, a breast cancer or a lung cancer. In some embodiments, the BFL-1-expressing or -dependent cell causes autoimmune disease or other inflammatory condition characteristic of a disease of cellular excess. In certain instances, the autoimmune disease is autoimmune colitis, thyroiditis, arthritis, nephritis, dermatitis, vasculitis, system lupus erythematosus, diabetes, or Sjogren's disease. In some instances, the inflammatory disease, asthma, psoriasis, inflammatory colitis, thyroiditis, arthritis, nephritis, dermatitis, or vasculitis. In a specific embodiment, the BFL-1-dependent cancer is acute myeloid leukemia, acute lymphoblastic

leukemia, chronic myeloid leukemia, chronic lymphocytic leukemia, myelodysplastic syndrome, or multiple myeloma.

The peptides described herein can be useful for treating a human subject with an MCL-1-expressing cancer. The peptides described herein can also be useful for treating a human subject with a MCL-1-dependent cancer. In certain embodiments, the MCL-1-dependent cancer is a melanoma, a leukemia, lymphoma, or other hematologic malignancy or solid tumor. In certain embodiments, the hematologic malignancy is acute myeloid leukemia, acute lymphoblastic leukemia, chronic myeloid leukemia, chronic lymphocytic leukemia, myelodysplastic syndrome, or multiple myeloma. In certain instances, the solid tumor is a melanoma, a breast cancer or a lung cancer. In some embodiments, the BFL-1 expressing or dependent cell causes autoimmune disease or other inflammatory condition characteristic of a disease of cellular excess. In certain instances, the autoimmune disease is autoimmune colitis, thyroiditis, arthritis, nephritis, dermatitis, vasculitis, system lupus erythematosus, diabetes, or Sjogren's disease. In some instances, the inflammatory disease, asthma, psoriasis, inflammatory colitis, thyroiditis, arthritis, nephritis, dermatitis, or vasculitis. In a specific embodiment, the MCL-1-dependent cancer is acute myeloid leukemia.

The peptides described herein can be useful for treating a human subject with a BFL-1- and MCL-1 coexpressing cancer. The peptides described herein can also be useful for treating a human subject with a BFL-1 and MCL-1-dependent cancer. In certain embodiments, the BFL-1 and MCL-1-dependent cancer is a melanoma, a leukemia, lymphoma, or other hematologic malignancy or solid tumor. In certain embodiments, the hematologic malignancy is acute myeloid leukemia, acute lymphoblastic leukemia, chronic myeloid leukemia, chronic lymphocytic leukemia, myelodysplastic syndrome, or multiple myeloma. In certain instances, the solid tumor is a melanoma, a breast cancer or a lung cancer. In some embodiments, the BFL-1 expressing or dependent cell causes autoimmune disease or other inflammatory condition characteristic of a disease of cellular excess. In certain instances, the autoimmune disease is autoimmune colitis, thyroiditis, arthritis, nephritis, dermatitis, vasculitis, system lupus erythematosus, diabetes, or Sjogren's disease. In some instances, the inflammatory disease, asthma, psoriasis, inflammatory colitis, thyroiditis, arthritis, nephritis, dermatitis, or vasculitis. In a specific embodiment, the BFL-1-and MCL-1-dependent cancer is acute myeloid leukemia.

In certain embodiments, the human subject in need thereof is administered a peptide selected from the group consisting of SEQ ID NO:16 and SEQ ID NOs.: 60-79. In certain embodiments, the human subject in need thereof is administered the peptide of SEQ ID NO: 62. In certain embodiments, the human subject in need thereof is administered the peptide of SEQ ID NO:60. In certain embodiments, the human subject in need thereof is administered a peptide that is at least 14%, 15%, 20%, 27%, 34%, 40%, 47%, 50%, 53%, 60%, 65% 70%, 75%, 80%, 85%, 90%, 95%, or 97% identical to an amino acid sequence of selected from the group consisting of SEQ ID NO:16 and SEQ ID NOs.: 60-79.

In a specific embodiment, the human subject in need thereof is administered the peptide AT8LREFGDXLNFRQ (SEQ ID NO:46), wherein "8" = R-octenyl alanine: and "X" = S-pentenyl alanine. In a specific embodiment, the human subject in need thereof is administered the peptide AT8LRRAGDXLNFRQ (SEQ ID NO:44), wherein "8" = R-octenyl alanine: and "X" = S-pentenyl alanine. In a specific embodiment, the human subject in need thereof is administered a BFL-1-binding warhead-bearing NOXA peptide described herein. In a specific embodiment, the BFL-1-binding warhead bearing NOXA peptide has the sequence JATQLREFGDKLNFRQ (SEQ ID NO:128), wherein the peptide is structurally stabilized by any method known in the art, wherein the electrophilic warhead (J) is a cysteine-reactive moiety, (e.g., a D-nipecotic acid moiety). In a particular embodiment, the BFL-1-binding warhead-bearing NOXA peptide has the sequence JATX_1LREFGDX_2LNFRQ (SEQ ID NO:62), wherein "X_1" and "X_2" are non-natural amino acids which can be covalently joined ("stapled together") using a ring-closing metathesis (RCM) reaction to form a cross-linked ring and "J" is a non-natural electrophile containing amino acid or an electrophilic warhead presented in the context of a moiety that is not an amino acid (the electrophile can serve as a chemical cap) (e.g., wherein "J" is a cysteine-reactive moiety, e.g., wherein "J" is a cysteine-reactive D-nipecotic acid moiety). In another particular embodiment, the BFL-1-binding warhead-bearing NOXA peptide has the sequence JAT8LREFGDXLNFRQ (SEQ ID NO:78), wherein "8" = R-octenyl alanine, "X" = S-pentenyl alanine, and "J" = a non-natural electrophile containing amino acid or an electrophilic warhead presented in the context of a moiety that is not an amino acid (e.g., wherein "J is a cysteine-reactive moiety, e.g., wherein "J" is a cysteine-reactive D-nipecotic acid moiety). In another specific embodiment, the BFL-1-binding warhead-bearing NOXA peptide has the sequence JATQLRRAGDKLNFRQ (SEQ ID NO:126), wherein the peptide is structurally stabilized by any method known in the art, wherein the electrophilic warhead (J)

is a cysteine-reactive moiety, (e.g., a D-nipecotic acid moiety). In a particular embodiment, the BFL-1-binding warhead-bearing NOXA peptide has the JATX$_1$LRRAGDX$_2$LNFRQ (SEQ ID NO:60), wherein "X$_1$" and "X$_2$" are non-natural amino acids which can be covalently joined ("stapled together") using a ring-closing metathesis (RCM) reaction to form a cross-linked ring and "J" is a non-natural electrophile containing amino acid or an electrophilic warhead presented in the context of a moiety that is not an amino acid (the electrophile can serve as a chemical cap) (e.g., wherein "J" is a cysteine-reactive moiety, e.g., wherein "J" is a cysteine-reactive D-nipecotic acid moiety). In another particular embodiment, the BFL-1-binding warhead-bearing NOXA peptide has the sequence JAT8LRRAGDXLNFRQ (SEQ ID NO:76), wherein wherein "8" = R-octenyl alanine, "X" = S-pentenyl alanine, and "J" = a non-natural electrophile containing amino acid or an electrophilic warhead presented in the context of a moiety that is not an amino acid (e.g., wherein "J is a cysteine-reactive moiety, e.g., wherein "J" is a cysteine-reactive D-nipecotic acid moiety). The skilled artisan will understand that, for peptides comprising two non-natural amino acids that can be covalently joined ("stapled together") using a ring-closing metathesis reaction to form a cross-linked ring, the ring-closing metathesis reaction will have been performed on the peptide prior to administering the peptide (i.e., the stabilized peptide) to the subject in need of treatment. In other words, a cross-linked ring produced from covalent joining of the two non-natural amino acids will be present in the stabilized peptide prior to administering the stabilized peptide to the subject.

In some instances, the human subject in need thereof is co-administered with a NOXA stabilized peptide disclosed herein an inhibitor of a DNA damage response pathway member. Non-limiting examples of DNA damage response pathway members include Ataxia-telangiectasia mutated (ATM) kinase, ataxia-telangiectasia and rad3-related (ATR) kinase, checkpoint kinase 1 (CHK1), checkpoint kinase 2 (CHK2), and poly(ADP-ribose polymerase (PARP). In some instances, the human subject in need thereof is co-administered with a NOXA stabilized peptide disclosed herein an ATM kinase or ATR kinase inhibitor. ATM and ATR kinase inhibitors are known in the art (*see, e.g.,* Weber et al., *Pharmacology & Therapeutics*, 149:124-0138 (2015); Awasthi et al., *J Cell Sci* 2015 128: 4255-4262). Non-limiting examples of ATM inhibitors include KU-559403, KU-55933, KU-60019, CP-466722, CGK733, and AZD0156. In some instances, the human subject in need thereof is co-administered with a NOXA stabilized peptide disclosed herein a checkpoint kinase 1/checkpoint kinase 2 (CHK1/2) inhibitor. CHK1/2 inhibitors are known in the art (*see, e.g.,*

Zabludoff et al., 2008; Anderson, V. E. et al. Cancer Res 71, 463-472, doi:10.1158/0008-5472.CAN-10-1252 (2011); Scagliotti, G. et al. Invest New Drugs 34, 625-635, doi:10.1007/s10637-016-0368-1 (2016); Hong, D. et al. J Clin Oncol 34, 1764-1771, doi:10.1200/JCO.2015.64.5788 (2016); Jobson, A. G. et al. Pharmacol 72, 876-884, doi:10.1124/mol.107.035832 (2007); Smaill, J. B. et al. Eur J Med Chem 43, 1276-1296, doi:10.1016/j.ejmech.2007.07.016 (2008); Jackson, J. R. et al. Cancer Res 60, 566-572 (2000); Teng, M. et al. J Med Chem 50, 5253 (2007); Booth, L. et al. 786-796 (2018)). Non-limiting examples of CHK1/2 inhibitors include AZD7762 (CHK1/2), CCT 241533 (CHK2), LY 2603618, (CHK1), LY 2606368 (CHK1/2), NSC 109555 ditosylate (CHK2), PD 407824 (CHKE1), PF47736 (CHEK1), SB 218078 (CHK1), TCS 2312 (CHK1), and SRA737 (CHK1). In some instances, the human subject in need thereof is co-administered with a NOXA stabilized peptide disclosed herein a PARP inhibitor. PARP inhibitors are known in the art (see, e.g., Menear et al., 2008; Penning, T. D. et al. J Med Chem 53, 3142-3153, doi:10.1021/jm901775y (2010); Donawho, C. K. et al. Clin Cancer Res 13, 2728-2737, doi:10.1158/1078-0432.CCR-06-3039 (2007); Daniel, R. A. et al. Clin Cancer Res 15, 1241-1249, doi:10.1158/1078-0432.CCR-08-1095 (2009); Curtin, N. J. e. a. Clin Cancer Res 10, 881-889 (2004); Liu, X. et al. Clin Cancer Res 18, 510-523, doi:10.1158/1078-0432.CCR-11-1973 (2012); Brock, W. A. et al. Cancer Lett 205, 155-160, doi:10.1016/j.canlet.2003.10.029 (2004); Bridges, B. A. et al. Oncotarget 5, 5076-5086 (2014); Laird, J. H. et al. Clin Cancer Res 24, 5143-5152, doi:10.1158/1078-0432.CCR-18-0401 (2018); Oplustil O'Connor, L. et al. Cancer Res 76, 6084-6094, doi:10.1158/0008-5472.CAN-15-3240 (2016); McGonigle, S. et al. Oncotarget 6, 41307-41323 (2015)). Non-limiting examples of PARP inhibitors include olaparib, A-966492, veliparib, rucaparib, AG-14361, iniparib, INO-1001, niraparib, talazoparib, AZD2461, 2X-121, BMN 673 (talazoparib), and E7449.

In certain embodiments, the human subject in need thereof is administered a peptide selected from the group consisting of SEQ ID NO:16 and SEQ ID NOs.: 60-79, 151, and 153 and a DNA damage response pathway member inhibitor. In certain embodiments, the human subject in need thereof is administered a peptide that is at least 14%, 15%, 20%, 27%, 34%, 40%, 47%, 50%, 53%, 60%, 65% 70%, 75%, 80%, 85%, 90%, 95%, or 97% identical to an amino acid sequence of selected from the group consisting of SEQ ID NO:16 and SEQ ID NOs.: 60-79, 151, and 153 and a DNA damage response pathway member inhibitor. In certain embodiments, the human subject in need thereof is administered a peptide selected from the group consisting of SEQ ID NO:16 and SEQ ID NOs.: 60-79, 151, and 153 and an

ATM inhibitor or an ATR kinase inhibitor. In certain embodiments, the human subject in need thereof is administered a peptide that is at least 14%, 15%, 20%, 27%, 34%, 40%, 47%, 50%, 53%, 60%, 65% 70%, 75%, 80%, 85%, 90%, 95%, or 97% identical to an amino acid sequence of selected from the group consisting of SEQ ID NO:16 and SEQ ID NOs.: 60-79, 151, and 153 and an ATM kinase or ATR kinase inhibitor. In certain embodiments, the human subject in need thereof is administered a peptide selected from the group consisting of SEQ ID NO:16 and SEQ ID NOs.: 60-79, 151, and 153 and CHK1/2 inhibitor or PARP inhibitor. In certain embodiments, the human subject in need thereof is administered a peptide that is at least 14%, 15%, 20%, 27%, 34%, 40%, 47%, 50%, 53%, 60%, 65% 70%, 75%, 80%, 85%, 90%, 95%, or 97% identical to an amino acid sequence of selected from the group consisting of SEQ ID NO:16 and SEQ ID NOs.: 60-79, 151, and 153 and a CHK1/2 inhibitor or a PARP inhibitor.

In certain embodiments, the peptide co-administered to the human subject in need thereof with a DNA damage response pathway member inhibitor (e.g., an ATM inhibitor, an ATR kinase inhibitor, a CHK1/2 inhibitor, or a PARP inhibitor) is AT8LREFGDXLNFRQ (SEQ ID NO:46), wherein “8” = R-octenyl alanine: and “X” = S-pentenyl alanine. In a specific embodiment, the peptide co-administered to the human subject in need thereof with a DNA damage response pathway member inhibitor (e.g., an ATM inhibitor, an ATR kinase inhibitor, a CHK1/2 inhibitor, or a PARP inhibitor) is AT8LRRAGDXLNFRQ (SEQ ID NO:44), wherein “8” = R-octenyl alanine: and “X” = S-pentenyl alanine. In a specific embodiment, the peptide co-administered to the human subject in need thereof with a DNA damage response pathway member inhibitor (e.g., an ATM inhibitor, an ATR kinase inhibitor, a CHK1/2 inhibitor, or a PARP inhibitor) is a BFL-1-binding warhead-bearing NOXA peptide described herein. In a specific embodiment, the BFL-1-binding warhead bearing NOXA peptide has the sequence JATQLREFGDKLNFRQ (SEQ ID NO:128), wherein the peptide is structurally stabilized by any method known in the art, wherein the electrophilic warhead (J) is a cysteine-reactive moiety, (e.g., a D-nipecotic acid moiety). In a particular embodiment, the BFL-1-binding warhead-bearing NOXA peptide has the sequence JATX1LREFGDX2LNFRQ (SEQ ID NO:62), wherein “X1” and “X2” are non-natural amino acids which can be covalently joined (“stapled together”) using a ring-closing metathesis (RCM) reaction to form a cross-linked ring and “J” is a non-natural electrophile containing amino acid or an electrophilic warhead presented in the context of a moiety that is not an amino acid (the electrophile can serve as a chemical cap) (e.g., wherein “J” is a cysteine-

reactive moiety, e.g., wherein "J" is a cysteine-reactive D-nipecotic acid moiety). In another particular embodiment, the BFL-1-binding warhead-bearing NOXA peptide has the sequence JAT8LREFGDXLNFRQ (SEQ ID NO:78), wherein "8" = R-octenyl alanine, "X" = S-pentenyl alanine, and "J" = a non-natural electrophile containing amino acid or an electrophilic warhead presented in the context of a moiety that is not an amino acid (e.g., wherein "J is a cysteine-reactive moiety, e.g., wherein "J" is a cysteine-reactive D-nipecotic acid moiety). In another specific embodiment, the BFL-1-binding warhead-bearing NOXA peptide has the sequence JATQLRRAGDKLNFRQ (SEQ ID NO:126), wherein the peptide is structurally stabilized by any method known in the art, wherein the electrophilic warhead (J) is a cysteine-reactive moiety, (e.g., a D-nipecotic acid moiety). In a particular embodiment, the BFL-1-binding warhead-bearing NOXA peptide has the JATX1LRRAGDX2LNFRQ (SEQ ID NO:60), wherein "X1" and "X2" are non-natural amino acids which can be covalently joined ("stapled together") using a ring-closing metathesis (RCM) reaction to form a cross-linked ring and "J" is a non-natural electrophile containing amino acid or an electrophilic warhead presented in the context of a moiety that is not an amino acid (the electrophile can serve as a chemical cap) (e.g., wherein "J" is a cysteine-reactive moiety, e.g., wherein "J" is a cysteine-reactive D-nipecotic acid moiety). In another particular embodiment, the BFL-1-binding warhead-bearing NOXA peptide has the sequence JAT8LRRAGDXLNFRQ (SEQ ID NO:76), wherein wherein "8" = R-octenyl alanine, "X" = S-pentenyl alanine, and "J" = a non-natural electrophile containing amino acid or an electrophilic warhead presented in the context of a moiety that is not an amino acid (e.g., wherein "J is a cysteine-reactive moiety, e.g., wherein "J" is a cysteine-reactive D-nipecotic acid moiety). The skilled artisan will understand that, for peptides comprising two non-natural amino acids that can be covalently joined ("stapled together") using a ring-closing metathesis reaction to form a cross-linked ring, the ring-closing metathesis reaction will have been performed on the peptide prior to administering the peptide (i.e., the stabilized peptide) to the subject in need of treatment. In other words, a cross-linked ring produced from covalent joining of the two non-natural amino acids will be present in the stabilized peptide prior to administering the stabilized peptide to the subject.

In some instances, the human subject in need thereof (e.g., a human subject having a cancer that expresses, or is dependent on, both BFL-1 and MCL-1) is co-administered with a NOXA stabilized peptide disclosed herein an inhibitor of MCL-1. MCL-1 inhibitors are known in the art (see, e.g., Clinical Trial ID NCT02979366; Kotschy et al. Nature,

538(7626):477-482 (2016); Ayaz et al. ACS Chem Biol, 11(6):1710-1719 (2016); Clinical Trial ID NCT02992483; Clinical Trial ID NCT03465540; Caenepeel et al.,Proceedings: AACR Annual Meeting 2017, DOI: 10.1158/1538-7445.AM2017-2027). Non-limiting examples of MCL-1 inhibitors include S64315, S63845, BAY 1000394, MIK665, AMG397, and AMG176. In certain embodiments, the human subject in need thereof is administered a peptide selected from the group consisting of SEQ ID NO:16 and SEQ ID NOs.: 60-79, 151, and 153 and an MCL-1 inhibitor. In certain embodiments, the human subject in need thereof is administered a peptide that is at least 14%, 15%, 20%, 27%, 34%, 40%, 47%, 50%, 53%, 60%, 65% 70%, 75%, 80%, 85%, 90%, 95%, or 97% identical to an amino acid sequence of selected from the group consisting of SEQ ID NO:16 and SEQ ID NOs.: 60-79, 151, and 153 and an MCL-1 inhibitor. In certain embodiments, the peptide co-administered to the human subject in need thereof with an MCL-1 inhibitor is AT8LREFGDXLNFRQ (SEQ ID NO:46), wherein "8" = R-octenyl alanine: and "X" = S-pentenyl alanine. In a specific embodiment, the peptide co-administered to the human subject in need thereof with an MCL-1 inhibitor is AT8LRRAGDXLNFRQ (SEQ ID NO:44), wherein "8" = R-octenyl alanine: and "X" = S-pentenyl alanine. In a specific embodiment, the peptide co-administered to the human subject in need thereof with an MCL-1 inhibitor is a BFL-1-binding warhead-bearing NOXA peptide described herein. In a specific embodiment, the BFL-1-binding warhead bearing NOXA peptide has the sequence JATQLREFGDKLNFRQ (SEQ ID NO:128), wherein the peptide is structurally stabilized by any method known in the art, wherein the electrophilic warhead (J) is a cysteine-reactive moiety, (e.g., a D-nipecotic acid moiety). In a particular embodiment, the BFL-1-binding warhead-bearing NOXA peptide has the sequence JATX_1LREFGDX_2LNFRQ (SEQ ID NO:62), wherein "X_1" and "X_2" are non-natural amino acids which can be covalently joined ("stapled together") using a ring-closing metathesis (RCM) reaction to form a cross-linked ring and "J" is a non-natural electrophile containing amino acid or an electrophilic warhead presented in the context of a moiety that is not an amino acid (the electrophile can serve as a chemical cap) (e.g., wherein "J" is a cysteine-reactive moiety, e.g., wherein "J" is a cysteine-reactive D-nipecotic acid moiety). In another particular embodiment, the BFL-1-binding warhead-bearing NOXA peptide has the sequence JAT8LREFGDXLNFRQ (SEQ ID NO:78), wherein "8" = R-octenyl alanine, "X" = S-pentenyl alanine, and "J" = a non-natural electrophile containing amino acid or an electrophilic warhead presented in the context of a moiety that is not an amino acid (e.g., wherein "J is a cysteine-reactive moiety, e.g., wherein "J" is a cysteine-reactive D-nipecotic acid moiety). In another specific embodiment, the BFL-1-binding warhead-bearing NOXA

peptide has the sequence JATQLRRAGDKLNFRQ (SEQ ID NO:126), wherein the peptide is structurally stabilized by any method known in the art, wherein the electrophilic warhead (J) is a cysteine-reactive moiety, (e.g., a D-nipecotic acid moiety). In a particular embodiment, the BFL-1-binding warhead-bearing NOXA peptide has the JATX_1LRRAGDX_2LNFRQ (SEQ ID NO:60), wherein "X_1" and "X_2" are non-natural amino acids which can be covalently joined ("stapled together") using a ring-closing metathesis (RCM) reaction to form a cross-linked ring and "J" is a non-natural electrophile containing amino acid or an electrophilic warhead presented in the context of a moiety that is not an amino acid (the electrophile can serve as a chemical cap) (e.g., wherein "J" is a cysteine-reactive moiety, e.g., wherein "J" is a cysteine-reactive D-nipecotic acid moiety). In another particular embodiment, the BFL-1-binding warhead-bearing NOXA peptide has the sequence JAT8LRRAGDXLNFRQ (SEQ ID NO:76), wherein wherein "8" = R-octenyl alanine, "X" = S-pentenyl alanine, and "J" = a non-natural electrophile containing amino acid or an electrophilic warhead presented in the context of a moiety that is not an amino acid (e.g., wherein "J is a cysteine-reactive moiety, e.g., wherein "J" is a cysteine-reactive D-nipecotic acid moiety). The skilled artisan will understand that, for peptides comprising two non-natural amino acids that can be covalently joined ("stapled together") using a ring-closing metathesis reaction to form a cross-linked ring, the ring-closing metathesis reaction will have been performed on the peptide prior to administering the peptide (i.e., the stabilized peptide) to the subject in need of treatment. In other words, a cross-linked ring produced from covalent joining of the two non-natural amino acids will be present in the stabilized peptide prior to administering the stabilized peptide to the subject.

In some instances, the human subject in need thereof (e.g., a human subject having a cancer that expresses, or is dependent on, both BFL-1 and BCL-2) is co-administered with a NOXA stabilized peptide disclosed herein an inhibitor of BCL-2. BCL-2 inhibitors are known in the art (see, e.g., Roberts et al. N Engl J Med 374(4):311-322 (2016)). An example of a BCL-2 inhibitor is venetoclax. In certain embodiments, the human subject in need thereof is administered a peptide selected from the group consisting of SEQ ID NO:16 and SEQ ID NOs.: 60-79, 151, and 153 and a BCL-2 inhibitor. In certain embodiments, the human subject in need thereof is administered a peptide that is at least 14%, 15%, 20%, 27%, 34%, 40%, 47%, 50%, 53%, 60%, 65% 70%, 75%, 80%, 85%, 90%, 95%, or 97% identical to an amino acid sequence of selected from the group consisting of SEQ ID NO:16 and SEQ ID NOs.: 60-79, 151, and 153 and a BCL-2 inhibitor. In certain embodiments, the peptide

co-administered to the human subject in need thereof with a BCL-2 inhibitor is AT8LREFGDXLNFRQ (SEQ ID NO:46), wherein "8" = R-octenyl alanine: and "X" = S-pentenyl alanine. In a specific embodiment, the peptide co-administered to the human subject in need thereof with a BCL-2 inhibitor is AT8LRRAGDXLNFRQ (SEQ ID NO:44), wherein "8" = R-octenyl alanine: and "X" = S-pentenyl alanine. In a specific embodiment, the peptide co-administered to the human subject in need thereof with a BCL-2 inhibitor is a BFL-1-binding warhead-bearing NOXA peptide described herein. In a specific embodiment, the BFL-1-binding warhead bearing NOXA peptide has the sequence JATQLREFGDKLNFRQ (SEQ ID NO:128), wherein the peptide is structurally stabilized by any method known in the art, wherein the electrophilic warhead (J) is a cysteine-reactive moiety, (e.g., a D-nipecotic acid moiety). In a particular embodiment, the BFL-1-binding warhead-bearing NOXA peptide has the sequence JATX_1LREFGDX_2LNFRQ (SEQ ID NO:62), wherein "X_1" and "X_2" are non-natural amino acids which can be covalently joined ("stapled together") using a ring-closing metathesis (RCM) reaction to form a cross-linked ring and "J" is a non-natural electrophile containing amino acid or an electrophilic warhead presented in the context of a moiety that is not an amino acid (the electrophile can serve as a chemical cap) (e.g., wherein "J" is a cysteine-reactive moiety, e.g., wherein "J" is a cysteine-reactive D-nipecotic acid moiety). In another particular embodiment, the BFL-1-binding warhead-bearing NOXA peptide has the sequence JAT8LREFGDXLNFRQ (SEQ ID NO:78), wherein "8" = R-octenyl alanine, "X" = S-pentenyl alanine, and "J" = a non-natural electrophile containing amino acid or an electrophilic warhead presented in the context of a moiety that is not an amino acid (e.g., wherein "J is a cysteine-reactive moiety, e.g., wherein "J" is a cysteine-reactive D-nipecotic acid moiety). In another specific embodiment, the BFL-1-binding warhead-bearing NOXA peptide has the sequence JATQLRRAGDKLNFRQ (SEQ ID NO:126), wherein the peptide is structurally stabilized by any method known in the art, wherein the electrophilic warhead (J) is a cysteine-reactive moiety, (e.g., a D-nipecotic acid moiety). In a particular embodiment, the BFL-1-binding warhead-bearing NOXA peptide has the JATX_1LRRAGDX_2LNFRQ (SEQ ID NO:60), wherein "X_1" and "X_2" are non-natural amino acids which can be covalently joined ("stapled together") using a ring-closing metathesis (RCM) reaction to form a cross-linked ring and "J" is a non-natural electrophile containing amino acid or an electrophilic warhead presented in the context of a moiety that is not an amino acid (the electrophile can serve as a chemical cap) (e.g., wherein "J" is a cysteine-reactive moiety, e.g., wherein "J" is a cysteine-reactive D-nipecotic acid moiety). In another particular embodiment, the BFL-1-binding warhead-bearing NOXA

peptide has the sequence JAT8LRRAGDXLNFRQ (SEQ ID NO:76), wherein wherein "8" = R-octenyl alanine, "X" = S-pentenyl alanine, and "J" = a non-natural electrophile containing amino acid or an electrophilic warhead presented in the context of a moiety that is not an amino acid (e.g., wherein "J is a cysteine-reactive moiety, e.g., wherein "J" is a cysteine-reactive D-nipecotic acid moiety). The skilled artisan will understand that, for peptides comprising two non-natural amino acids that can be covalently joined ("stapled together") using a ring-closing metathesis reaction to form a cross-linked ring, the ring-closing metathesis reaction will have been performed on the peptide prior to administering the peptide (i.e., the stabilized peptide) to the subject in need of treatment. In other words, a cross-linked ring produced from covalent joining of the two non-natural amino acids will be present in the stabilized peptide prior to administering the stabilized peptide to the subject.

In some instances, the human subject in need thereof (e.g., a human subject having a cancer that expresses, or is dependent on, both BFL-1 and BCL-XL) is co-administered with a NOXA stabilized peptide disclosed herein an inhibitor of BCL-XL. BCL-XL inhibitors are known in the art (see, e.g., Zhu et al. Aging Cell; 15(3):428-35 (2016)). An example of a BCL-XL inhibitor is navitoclax. In certain embodiments, the human subject in need thereof is administered a peptide selected from the group consisting of SEQ ID NO:16 and SEQ ID NOs.: 60-79, 151, and 153 and a BCL-XL inhibitor. In certain embodiments, the human subject in need thereof is administered a peptide that is at least 14%, 15%, 20%, 27%, 34%, 40%, 47%, 50%, 53%, 60%, 65% 70%, 75%, 80%, 85%, 90%, 95%, or 97% identical to an amino acid sequence of selected from the group consisting of SEQ ID NO:16 and SEQ ID NOs.: 60-79, 151, and 153 and a BCL-XL inhibitor. In certain embodiments, the peptide co-administered to the human subject in need thereof with a BCL-XL inhibitor is AT8LREFGDXLNFRQ (SEQ ID NO:46), wherein "8" = R-octenyl alanine: and "X" = S-pentenyl alanine. In a specific embodiment, the peptide co-administered to the human subject in need thereof with a BCL-XL inhibitor is AT8LRRAGDXLNFRQ (SEQ ID NO:44), wherein "8" = R-octenyl alanine: and "X" = S-pentenyl alanine. In a specific embodiment, the peptide co-administered to the human subject in need thereof with a BCL-XL inhibitor is a BFL-1-binding warhead-bearing NOXA peptide described herein. In a specific embodiment, the BFL-1-binding warhead bearing NOXA peptide has the sequence JATQLREFGDKLNFRQ (SEQ ID NO:128), wherein the peptide is structurally stabilized by any method known in the art, wherein the electrophilic warhead (J) is a cysteine-reactive moiety, (e.g., a D-nipecotic acid moiety). In a particular embodiment, the BFL-1-binding

warhead-bearing NOXA peptide has the sequence JATX$_1$LREFGDX$_2$LNFRQ (SEQ ID NO:62), wherein "X$_1$" and "X$_2$" are non-natural amino acids which can be covalently joined ("stapled together") using a ring-closing metathesis (RCM) reaction to form a cross-linked ring and "J" is a non-natural electrophile containing amino acid or an electrophilic warhead presented in the context of a moiety that is not an amino acid (the electrophile can serve as a chemical cap) (e.g., wherein "J" is a cysteine-reactive moiety, e.g., wherein "J" is a cysteine-reactive D-nipecotic acid moiety). In another particular embodiment, the BFL-1-binding warhead-bearing NOXA peptide has the sequence JAT8LREFGDXLNFRQ (SEQ ID NO:78), wherein "8" = R-octenyl alanine, "X" = S-pentenyl alanine, and "J" = a non-natural electrophile containing amino acid or an electrophilic warhead presented in the context of a moiety that is not an amino acid (e.g., wherein "J is a cysteine-reactive moiety, e.g., wherein "J" is a cysteine-reactive D-nipecotic acid moiety). In another specific embodiment, the BFL-1-binding warhead-bearing NOXA peptide has the sequence JATQLRRAGDKLNFRQ (SEQ ID NO:126), wherein the peptide is structurally stabilized by any method known in the art, wherein the electrophilic warhead (J) is a cysteine-reactive moiety, (e.g., a D-nipecotic acid moiety). In a particular embodiment, the BFL-1-binding warhead-bearing NOXA peptide has the JATX$_1$LRRAGDX$_2$LNFRQ (SEQ ID NO:60), wherein "X$_1$" and "X$_2$" are non-natural amino acids which can be covalently joined ("stapled together") using a ring-closing metathesis (RCM) reaction to form a cross-linked ring and "J" is a non-natural electrophile containing amino acid or an electrophilic warhead presented in the context of a moiety that is not an amino acid (the electrophile can serve as a chemical cap) (e.g., wherein "J" is a cysteine-reactive moiety, e.g., wherein "J" is a cysteine-reactive D-nipecotic acid moiety). In another particular embodiment, the BFL-1-binding warhead-bearing NOXA peptide has the sequence JAT8LRRAGDXLNFRQ (SEQ ID NO:76), wherein wherein "8" = R-octenyl alanine, "X" = S-pentenyl alanine, and "J" = a non-natural electrophile containing amino acid or an electrophilic warhead presented in the context of a moiety that is not an amino acid (e.g., wherein "J is a cysteine-reactive moiety, e.g., wherein "J" is a cysteine-reactive D-nipecotic acid moiety). The skilled artisan will understand that, for peptides comprising two non-natural amino acids that can be covalently joined ("stapled together") using a ring-closing metathesis reaction to form a cross-linked ring, the ring-closing metathesis reaction will have been performed on the peptide prior to administering the peptide (i.e., the stabilized peptide) to the subject in need of treatment. In other words, a cross-linked ring produced from covalent joining of the two non-natural amino acids will be present in the stabilized peptide prior to administering the stabilized peptide to the subject.

In some instances, the human subject in need thereof (e.g., a human subject having a cancer that expresses, or is dependent on, BFL-1 and BCL-XL, BCL-2, and/or MCL-1) is co-administered with a NOXA stabilized peptide disclosed herein an inhibitor of BCL-XL, BCL-2, and/or MCL-1. In some instances, the human subject in need thereof (e.g., a human subject having a cancer that expresses, or is dependent on, each of BFL-1, BCL-XL, BCL-2, and MCL-1) is co-administered with a NOXA stabilized peptide disclosed herein an inhibitor of BCL-XL, BCL-2, and MCL-1. In some instances, the human subject in need thereof (e.g., a human subject having a cancer that expresses, or is dependent on, each of BFL-1, BCL-XL, and BCL-2) is co-administered with a NOXA stabilized peptide disclosed herein an inhibitor of BCL-XL and BCL-2. In some instances, the human subject in need thereof (e.g., a human subject having a cancer that expresses, or is dependent on, each of BFL-1, BCL-XL, and MCL-1) is co-administered with a NOXA stabilized peptide disclosed herein an inhibitor of BCL-XL and MCL-1. In some instances, the human subject in need thereof (e.g., a human subject having a cancer that expresses, or is dependent on, each of BFL-1, BCL-2, and MCL-1) is co-administered with a NOXA stabilized peptide disclosed herein an inhibitor of BCL-2 and MCL-1. BCL-XL inhibitors are known in the art (see, e.g., Zhu et al. Aging Cell; 15(3):428-35 (2016)). An example of a BCL-XL inhibitor is navitoclax. BCL-2 inhibitors are known in the art (see, e.g., Roberts et al. N Engl J Med 374(4):311-322 (2016)). An example of a BCL-2 inhibitor is venetoclax. MCL-1 inhibitors are known in the art (see, e.g., Clinical Trial ID NCT02979366; Kotschy et al. Nature, 538(7626):477-482 (2016); Ayaz et al. ACS Chem Biol, 11(6):1710-1719 (2016); Clinical Trial ID NCT02992483; Clinical Trial ID NCT03465540; Caenepeel et al.,Proceedings: AACR Annual Meeting 2017, DOI: 10.1158/1538-7445.AM2017-2027). Non-limiting examples of MCL-1 inhibitors include S64315, S63845, BAY 1000394, MIK665, AMG397, and AMG176. In certain embodiments, the human subject in need thereof is administered a peptide selected from the group consisting of SEQ ID NO:16 and SEQ ID NOs.: 60-79, 151, and 153 and a BCL-XL inhibitor, a BCL-2 inhibitor, and/or a MCL-1 inhibitor. In certain embodiments, the human subject in need thereof is administered a peptide that is at least 14%, 15%, 20%, 27%, 34%, 40%, 47%, 50%, 53%, 60%, 65% 70%, 75%, 80%, 85%, 90%, 95%, or 97% identical to an amino acid sequence of selected from the group consisting of SEQ ID NO:16 and SEQ ID NOs.: 60-79, 151, and 153 and a BCL-XL inhibitor, a BCL-2 inhibitor, and/or a MCL-1 inhibitor. In certain embodiments, the peptide co-administered to the human subject in need thereof with a BCL-XL inhibitor, a BCL-2 inhibitor, and/or a MCL-1 inhibitor is AT8LREFGDXLNFRQ (SEQ ID NO:46), wherein “8” = R-octenyl alanine: and “X” = S-

pentenyl alanine. In a specific embodiment, the peptide co-administered to the human subject in need thereof with a BCL-XL inhibitor, a BCL-2 inhibitor, and/or a MCL-1 inhibitor is AT8LRRAGDXLNFRQ (SEQ ID NO:44), wherein "8" = R-octenyl alanine: and "X" = S-pentenyl alanine. In a specific embodiment, the peptide co-administered to the human subject in need thereof with a BCL-XL inhibitor, a BCL-2 inhibitor, and/or a MCL-1 inhibitor is a BFL-1-binding warhead-bearing NOXA peptide described herein. In a specific embodiment, the BFL-1-binding warhead bearing NOXA peptide has the sequence JATQLREFGDKLNFRQ (SEQ ID NO:128), wherein the peptide is structurally stabilized by any method known in the art, wherein the electrophilic warhead (J) is a cysteine-reactive moiety, (e.g., a D-nipecotic acid moiety). In a particular embodiment, the BFL-1-binding warhead-bearing NOXA peptide has the sequence JATX$_1$LREFGDX$_2$LNFRQ (SEQ ID NO:62), wherein "X$_1$" and "X$_2$" are non-natural amino acids which can be covalently joined ("stapled together") using a ring-closing metathesis (RCM) reaction to form a cross-linked ring and "J" is a non-natural electrophile containing amino acid or an electrophilic warhead presented in the context of a moiety that is not an amino acid (the electrophile can serve as a chemical cap) (e.g., wherein "J" is a cysteine-reactive moiety, e.g., wherein "J" is a cysteine-reactive D-nipecotic acid moiety). In another particular embodiment, the BFL-1-binding warhead-bearing NOXA peptide has the sequence JAT8LREFGDXLNFRQ (SEQ ID NO:78), wherein "8" = R-octenyl alanine, "X" = S-pentenyl alanine, and "J" = a non-natural electrophile containing amino acid or an electrophilic warhead presented in the context of a moiety that is not an amino acid (e.g., wherein "J is a cysteine-reactive moiety, e.g., wherein "J" is a cysteine-reactive D-nipecotic acid moiety). In another specific embodiment, the BFL-1-binding warhead-bearing NOXA peptide has the sequence JATQLRRAGDKLNFRQ (SEQ ID NO:126), wherein the peptide is structurally stabilized by any method known in the art, wherein the electrophilic warhead (J) is a cysteine-reactive moiety, (e.g., a D-nipecotic acid moiety). In a particular embodiment, the BFL-1-binding warhead-bearing NOXA peptide has the JATX$_1$LRRAGDX$_2$LNFRQ (SEQ ID NO:60), wherein "X$_1$" and "X$_2$" are non-natural amino acids which can be covalently joined ("stapled together") using a ring-closing metathesis (RCM) reaction to form a cross-linked ring and "J" is a non-natural electrophile containing amino acid or an electrophilic warhead presented in the context of a moiety that is not an amino acid (the electrophile can serve as a chemical cap) (e.g., wherein "J" is a cysteine-reactive moiety, e.g., wherein "J" is a cysteine-reactive D-nipecotic acid moiety). In another particular embodiment, the BFL-1-binding warhead-bearing NOXA peptide has the sequence JAT8LRRAGDXLNFRQ (SEQ ID NO:76), wherein wherein "8" =

R-octenyl alanine, “X” = S-pentenyl alanine, and “J” = a non-natural electrophile containing amino acid or an electrophilic warhead presented in the context of a moiety that is not an amino acid (e.g., wherein “J is a cysteine-reactive moiety, e.g., wherein “J” is a cysteine-reactive D-nipecotic acid moiety). The skilled artisan will understand that, for peptides comprising two non-natural amino acids that can be covalently joined (“stapled together”) using a ring-closing metathesis reaction to form a cross-linked ring, the ring-closing metathesis reaction will have been performed on the peptide prior to administering the peptide (i.e., the stabilized peptide) to the subject in need of treatment. In other words, a cross-linked ring produced from covalent joining of the two non-natural amino acids will be present in the stabilized peptide prior to administering the stabilized peptide to the subject.

In some embodiments, the human subject has a MCL-1 and/or BFL-1 expression/dependent-cancer (*e.g.,* melanoma, a leukemia, lymphoma, or other hematologic malignancy or solid tumor, or another cancer described herein. In some cases, the solid tumor is a melanoma, a breast cancer or lung cancer. In some embodiments, the BFL-1 expressing or dependent cell causes autoimmune disease or other inflammatory condition characteristic of a disease of cellular excess.). In certain instances, the autoimmune disease is autoimmune colitis, thyroiditis, arthritis, nephritis, dermatitis, vasculitis, system lupus erythematosus, diabetes, or Sjogren's disease. In some instances, the inflammatory disease, asthma, psoriasis, inflammatory colitis, thyroiditis, arthritis, nephritis, dermatitis, or vasculitis. In a specific embodiment, the human subject has AML.

In general, methods include selecting a subject and administering to the subject an effective amount of one or more of the peptides herein, e.g., in or as a pharmaceutical composition, and optionally repeating administration as required for the prophylaxis or treatment of a cancer, e.g., melanoma or lymphoma, and can be administered orally, intravenously or topically. A subject can be selected for treatment based on, e.g., determining that the subject has a cancer that expresses BFL-1; determining that the subject has a cancer that expresses MCL-1; or determining that the subject has a cancer that expresses both BFL-1 and MCL-1. The peptides of this disclosure can be used to determine if a subject’s cancer expresses BFL-1, or if a subject’s cancer is dependent on BFL-1.

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.

An effective amount can be administered in one or more administrations, applications or dosages. A therapeutically effective amount of a therapeutic compound (i.e., an effective dosage) depends on the therapeutic compounds selected. The compositions can be administered one from one or more times per day to one or more times per week; including once every other day. The skilled artisan will appreciate that certain factors may influence the dosage and timing required to effectively treat a subject, including but not limited to the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of the therapeutic compounds described herein can include a single treatment or a series of treatments. For example, effective amounts can be administered at least once.

Pharmaceutical Compositions

One or more of any of the stabilized peptides described herein (*e.g.,* one or more of SEQ ID NOs: 2-38 and 60-79, 151, and 153) can be formulated for use as or in pharmaceutical compositions. The pharmaceutical compositions may be used in the methods of treatment described herein (see above). In certain embodiments, the pharmaceutical composition comprises an amino acid sequence that is identical to an amino acid sequence set forth in SEQ ID NOs: 2-38 and 60-79, except for 1 to 10, 1 to 9, 1 to 8, 1 to 7, 1 to 6, 1 to 5, 1 to 4, 1 to 3, 1 to 2, or 1 amino acid substitution, insertion, or deletion. These changes to the amino acid sequences can be made on the BFL-1 non-interacting alpha-helical face of these peptides and/or on the BFL-1 interacting alpha-helical face. Such compositions can be formulated or adapted for administration to a subject via any route, *e.g.,* any route approved by the Food and Drug Administration (FDA). Exemplary methods are described in the FDA's CDER Data Standards Manual, version number 004 (which is available at fda.give/cder/dsm/DRG/drg00301.htm). For example, compositions can be formulated or adapted for administration by inhalation (*e.g.,* oral and/or nasal inhalation (*e.g.,* via nebulizer or spray)), injection (*e.g.,* intravenously, intra-arterial, subdermally, intraperitoneally, intramuscularly, and/or subcutaneously); and/or for oral administration, transmucosal

adminstration, and/or topical administration (including topical (*e.g.,* nasal) sprays and/or solutions).

In some instances, pharmaceutical compositions can include an effective amount of one or more stabilized peptides. The terms "effective amount" and "effective to treat," as used herein, refer to an amount or a concentration of one or more compounds or a pharmaceutical composition described herein utilized for a period of time (including acute or chronic administration and periodic or continuous administration) that is effective within the context of its administration for causing an intended effect or physiological outcome (e.g., treatment of infection).

Pharmaceutical compositions of this invention can include one or more peptides and any pharmaceutically acceptable carrier and/or vehicle. In some instances, pharmaceuticals can further include one or more additional therapeutic agents in amounts effective for achieving a modulation of disease or disease symptoms. For example, the pharmaceutical composition may include an ATM or ATR inhibitor. Non-limiting examples of ATM kinase inhibitors include KU-559403, KU-55933, KU-60019, CP-466722, CGK733, and AZD0156. Non-limiting examples of ATR kinase inhibitors include schisandrin B, NU6027, NVP-BEZ235, VE-821, VE-822/VX-970, AZ20, and AZD6738.

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 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, intra-cutaneous, intra-venous, intra-muscular, intra-articular, intra-arterial, intra-synovial, intra-sternal, intra-thecal, intra-lesional and intra-cranial injection or infusion techniques.

Pharmaceutical compositions can be in the form of a solution or powder for inhalation and/or nasal administration. Such compositions 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.

Pharmaceutical compositions can 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.

Alternatively or in addition, pharmaceutical compositions can 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.

In some instances, one or more peptides disclosed herein can be conjugated, for example, to a carrier protein. Such conjugated compositions can be monovalent or multivalent. For example, conjugated compositions can include one peptide disclosed herein conjugated to a carrier protein. Alternatively, conjugated compositions can include two or more peptides disclosed herein conjugated to a carrier.

As used herein, when two entities are "conjugated" to one another they are linked by a direct or indirect covalent or non-covalent interaction. In certain embodiments, the association is covalent. In other embodiments, the association is non-covalent. Non- covalent interactions include hydrogen bonding, van der Waals interactions, hydrophobic interactions, magnetic interactions, electrostatic interactions, etc. An indirect covalent interaction is when two entities are covalently connected, optionally through a linker group.

Carrier proteins can include any protein that increases or enhances immunogenicity in a subject. Exemplary carrier proteins are described in the art (see, e.g., Fattom et al., *Infect. Immun.*, 58:2309-2312, 1990; Devi et al., *Proc. Natl. Acad. Sci. USA* 88:7175-7179, 1991; Li et al., Infect. Immun. 57:3823-3827, 1989; Szu et al., *Infect. Immun.* 59:4555-4561,1991; Szu et al., *J. Exp. Med.* 166:1510-1524, 1987; and Szu et al., *Infect. Immun.* 62:4440-4444, 1994). Polymeric carriers can be a natural or a synthetic material containing one or more primary and/or secondary amino groups, azido groups, or carboxyl groups. Carriers can be water soluble.

EXAMPLES

The following examples are provided to better illustrate the claimed invention and are not to be interpreted as limiting the scope of the invention. To the extent that specific materials are mentioned, it is merely for purposes of illustration and is not intended to limit the invention. One skilled in the art can develop equivalent means or reactants without the exercise of inventive capacity and without departing from the scope of the invention.

Example 1: Development of Potent, Selective, Cell Permeable, Cysteine-Reactive Stapled NOXA BH3 Inhibitors of BFL-1

We previously developed selective, covalent stapled peptide inhibitors of BFL-1 based on the sequence of BIM BH3 , combining the high-affinity non-covalent interactions of a natural BH3 domain helix with the irreversible blockade resulting from covalent reaction of an electrophilic warhead. A covalent BIM SAHB that selectively neutralized the anti-apoptotic activity of BFL-1 *in vitro* and effectively triggered apoptosis in the context of BFL-1-driven melanoma was successfully designed and characterized (Huhn et al., *Cell Chem Biol.*, 23(9):1123-1134 (2016)). Although this cysteine-reactive BIM SAHB only targets BFL-1 covalently, it retains the ability to form non-covalent interactions with a broad spectrum of BCL-2 family proteins, including BCL-X_L, MCL-1, and potentially BAX. The BH3-only protein NOXA, however, has a narrower spectrum of BCL-2 protein binding activity, exhibiting dual selectivity for MCL-1 and BFL-1 (Stewart et al., *Nature Chemical Biology*, 6, 595–601 (2010)). Here, the NOXA BH3 template was used to tune the covalent and non-covalent selectivity of a warhead-bearing SAHB for BFL-1 so that such stapled peptides are sufficiently potent or cell permeable for ultimate therapeutic use. These new constructs are unique in that they either only target BFL-1 (by covalent and/or non-covalent means) or exhibit exclusive dual-targeting of BFL-1 (by covalent and/or non-covalent means) and MCL-1 (by non-covalent means).

To generate constructs with particular binding selectivities, cell penetrance properties, *in vivo* stability, and biological activity to enable the development of a drug for clinical use, staple scanning methodology was first used to generate a library of cysteine-reactive NOXA SAHBs to optimize binding potency, selectivity, and cellular penetrance. Importantly, a novel, shortened template NOXA sequence was designed lacking N- and C-terminal residues of the classic BH3 domain sequence that did not appear to make critical contacts with the protein target based on structural data (**Figure 1**). Staple scanning was achieved by sequential

placement of (*i, i+4*) and (*i, i+7*) staples across the length of the peptide template, while keeping the N-terminal D-nipecotic acid (D-NA) reactive group constant (**Figure 2**). We measured the reactivity of this NOXA library by incubating each peptide with BFL-1ΔC and detecting a higher molecular weight conjugate upon reducing and denaturing gel electrophoresis, and Coomassie staining (**Figure 3**). Those peptides capable of covalent reaction with BFL-1 were advanced to cellular testing for peptide uptake. 293T cells transfected with HA-BFL-1ΔC C4S/C19S were treated with peptide (20 μM, 6 hr), and then harvested and lysates analyzed by anti-HA western blotting (**Figure 4**). The presence of a BFL-1 doublet indicated that the warhead-bearing peptide was both capable of gaining intracellular access and binding to BFL-1 covalently. The NOXA peptide with (*i, i+7*) staple position 15 exhibited a dramatic increase in covalent targeting efficiency compared to BIM $SAHB_A$-3. However, upon LDH release testing in A375P cells, this peptide was unexpectedly found to cause membrane disruption (**Figure 5**).

To remedy this shortcoming, the NOXA 15 construct was subjected to alanine scanning mutagenesis in an effort to identify changes in composition that could mitigate lytic behavior (**Figure 6**). Especially hydrophobic residues were converted to alanine as one design strategy for eliminating lytic properties by decreasing hydrophobic content. Indeed, the sequential mutation of alanine across the length of the NOXA sequence yielded three warhead-bearing NOXA SAHBs with little to no lytic activity (**Figure 7**). Notably, each of the mutations that reduced lysis lowered the overall peptide hydrophobicity. Surprisingly, the majority of mutations along the sequence of NOXA had no negative influence on BFL-1 covalent binding activity, except for Leu29 and Asp34 (**Figure 8**), which are two residues of the "LXXXGDE" minimal consensus BH3 domain binding motif (Day et al., *J. Mol. Biol.*, 380(5):958-971 (2008)). These data indicate that stapled peptides bearing an electrophilic warhead for C55 targeting of BFL-1 are strikingly less sensitive to alteration of particular amino acid residues, even if they reside on the BFL-1 interaction surface of the NOXA helix. The cysteine-reactive NOXA-15 F32A and NOXA-15 L36A SAHBs were both cell permeable and covalently targeted BFL-1 (**Figure 9**).

In an alternative iterative approach to neutralizing the lytic activity of the cysteine-reactive NOXA-15 peptide, two different arginines were mutated to glutamic acid residues within the NOXA sequence, with the goal of reducing the overall positive charge of the peptide (**Figure 10**). Indeed both peptides, whose overall charge was reduced from +2 to 0,

retained covalent activity for BFL-1 (**Figure 12**), demonstrated little-to-no cell lysis (**Figure 11**), and achieved improved cellular uptake (**Figure 13**).

Thus, using two distinct approaches, non-lytic, cell-permeable, cysteine-reactive NOXA SAHBs were inventively generated for advancement to biochemical characterization and cellular studies.

Example 2: Biochemical Characterization of the Selectivity of a Cell-Permeable, Cysteine-Reactive Stapled NOXA Peptides Reveals Decreased Non-Covalent Affinity for MCL-1 with Retained Covalent Selectivity for BFL-1

Having made significant changes to the native NOXA BH3 sequence, including peptide length, staple placement, and mutagenesis, it was next sought to determine the effect of these modifications on the selectivity of two lead cell permeable peptides, the cysteine-reactive D-NA-NOXA-15 F32A and R31E. It was first examined how these changes coupled with the addition of a cysteine-reactive electrophile influenced the balance between non-covalent and covalent SAHB interactions in the context of an anti-apoptotic protein mixture. The biotinylated D-NA-NOXA-15 F32A SAHB or corresponding acetylated construct was incubated in an equimolar mixture with recombinant BFL-1, BCL-X_L, and MCL-1, and then subjected to streptavidin pull-down. The acetylated (Ac-) NOXA-15 F32A SAHB demonstrated no binding to BFL-1, BCL-X_L, and detectable but minimal direct interaction with MCL-1 (**Figure 14**), consistent with the new design feature of significant truncation of the native BH3 sequence. However, D-NA-NOXA-15 F32A SAHB displayed a shift in the interaction propensity toward BFL-1, with notably more BFL-1 engagement as a result of covalent BFL-1ΔC conjugation (**Figure 14**). The same covalent versus non-covalent interactions were observed for Ac- versus D-NA-NOXA-15 R31E in the same experimental context (**Figure 15**). Additionally, the preferential interactions of these peptides were assessed with endogenous protein in lysates from the A375P melanoma cell line. When subjected to streptavidin pulldown, the biotinylated Ac- and D-NA-NOXA-15 F32A or R31E SAHBs demonstrated approximately equivalent binding to MCL-1, whereas the warhead-bearing constructs again showed markedly increased engagement of BFL-1 (**Figures 16 and 17**).

In addition to studying the direct binding of the lead D-NA-NOXA-15 SAHBs to anti-apoptotic proteins, their capacity to competitively disrupt inhibitory BFL-1 complexes were also evaluated. For clinically useful biological activity, the capacity to inhibit unoccupied anti-apoptotic protein(s) to lower the apoptotic threshold and to competitively displace pro-

apoptotic proteins from anti-apoptotic complexes to induce apoptosis are two distinct and desirable functionalities. For the competition experiment, tBID was added to lysates from 293T cells transiently transfected with HA-BFL-1ΔC C4S/C19S, the mixture was incubated with biotinylated Ac- or D-NA-NOXA-15 F32A SAHBs, anti-HA immunoprecipitation was performed, and blotted for HA and tBID. Acetylated NOXA-15 F32A was incapable of competing with tBID for HA-BFL-1 binding, whereas the warhead-bearing D-NA-NOXA-15 F32A construct covalently trapped HA-BFL-1, as exemplified by complete conversion of the protein to the higher molecular weight species and near total inhibition of tBID co-immunoprecipitation (**Figure 18, left**). Strikingly, when the experiment was repeated using lysates from 293T cells transiently expressing FLAG-MCL-1, neither NOXA-15 F32A peptides were able to non-covalently disrupt the tBID/FLAG-MCL-1 co-immunoprecipitation (**Figure 18, right**), demonstrating a distinct shift in the binding selectivity of NOXA-15 F32A SAHB toward BFL-1. The results were identical when performed with Ac- versus D-NA-NOXA-15 R31E (**Figure 19**), establishing the same functional binding preferences for this construct.

Fluorescence polarization experiments comparing the capacity of acetylated versus cysteine-reactive NOXA-15 R31E SAHBs to compete with FITC-BID BH3 peptide for anti-apoptotic protein binding demonstrated a similar effect (**Figures 20-23**). The warhead-bearing D-NA-NOXA-15 R31E SAHB was only able to compete with FITC-BID BH3 for binding to BFL-1ΔC C4S/C19S (**Figure 20**), and this activity was dependent on the presence of Cys55 in the BH3-binding pocket, as this effect was not observed with BFL-1ΔC C4S/C19S/C55S (**Figure 21**). Neither Ac- nor D-NA-NOXA-15 R31E peptides were capable of significantly dissociating the interaction of FITC-BID BH3 with either MCL-1ΔNΔC (**Figure 22**) or BCL-XLΔC (**Figure 23**). These biochemical experiments demonstrated that the various modifications made to the NOXA BH3 sequence to impart cellular uptake and reduce lytic activity resulted in a stapled peptide with exquisite covalent selectivity for BFL-1, and diminished non-covalent affinity for MCL-1, in a competitive binding context.

Example 3: Cysteine-Reactive NOXA SAHB Synergizes with ATM Inhibition in BFL-1-Dependent AML Cell Lines

Given the selectivity of the cysteine-reactive NOXA-15 R31E SAHB for BFL-1, it was postulated that the ideal setting to test its cellular activity would be in cancer cell lines that are especially dependent on the expression of BFL-1 for survival. Large genome-wide RNAi screening efforts have been undertaken to map genetic dependencies across hundreds

of cancer cell lines (Tsherniak, *Cell*, 170(3):564-576 (2017)). A next-generation CRISPR-Cas9 screening data set for cancer cell lines, specifically focusing on AML, was queried and examined for which cells exhibit dependency on BFL-1 (**Figure 24**). Cell lines with more positive dependency Z-score values exhibit little-to-no dependency on BFL-1 (*e.g.,* MV4-11, top right), whereas more negative Z-score values represent increasing dependency on BFL-1 (*e.g.,* U937, bottom left). Because most cancer cell lines express a spectrum of anti-apoptotic proteins, it was further hypothesized that combining a cysteine-reactive NOXA-15 R31E SAHB with a second dependency could potentially enhance cytotoxicity, particularly if the BFL-1 dependency was in the moderate range (Z-score values between -4 and -2). To inform this effort, genes were evaluated that showed significant co-dependency with BFL-1 dependency. ATM, a serine/threonine kinase activated by DNA double-strand breaks, was the most highly correlated co-dependent gene in AML (**Figure 25**). Co-treatment of the AML cell line U937, which exhibited the highest dependency on BFL-1, with the cysteine-reactive NOXA-15 R31E SAHB and a small molecule ATM inhibitor KU-55933 resulted in highly synergistic cytotoxicity (CI<0.3, strong synergism), as analyzed by the CalcuSyn software (**Figure 26**). Additionally, when treated with the cysteine-reactive NOXA-15 R31E and KU-55933 singly and in combination, the U937 cells exhibited significantly increased caspase-3/7 activation over time upon combination treatment compared to single agent treatments (**Figure 27**), suggesting synergism in apoptosis induction as causal of the cytotoxic effects. Correspondingly, the AML cell line that was least dependent on BFL-1, MV4-11, is essentially insensitive to the cysteine-reactive NOXA-15 R31E SAHB alone over the same dose range as applied above, and displays little to no cytotoxic synergy when combined with the ATM inhibitor (**Figure 28**), and no increase in caspase-3/7 activation for combination treatment compared to the single agent treatments (**Figure 29**).

Example 4: Crystal Structures of Anti-Apoptotic BFL-1 and its Complex with a Covalent Stapled Peptide Inhibitor

The structures of anti-apoptotic targets in complex with BH3 helices and their mimetics can provide a roadmap for molecular refinement and therapeutic development, so it was sought to solve the structures of BFL-1, alone and in complex with a covalent stapled peptide inhibitor, for the first time. The structure of apo BFL-1 provides the opportunity to compare unliganded anti-apoptotic protein structures, and also reveals the conformational consequences and molecular features of covalent targeting of BFL-1 with a cysteine-reactive stapled peptide inhibitor.

Structure of Apo BFL-1

The structure of unliganded BFL-1 was solved by X-ray crystallography at 1.69 Å resolution (**Figure 30,** PDB ID: 5WHI) and revealed a globular protein comprised of a series of α-helices joined by loops, which typically contain helix-breaking glycines or prolines (**Figure 31A-B**). BFL-1 shares all key elements of the conserved structure of multidomain BCL-2 family proteins, including a core α5-α6 hairpin that is surrounded by α-helices 1-4 and 7-8. At the site of α9 truncation, which is performed to facilitate anti-apoptotic protein expression and crystallization, lies the canonical surface groove lined by residues from α-helices 2-4. Specifically with respect to the only other apo structures of anti-apoptotic proteins, MCL-1 and BCL-X_L the relative position, accessibility and conformation of the grooves show differences. An overlay of all three structures demonstrates near complete overlap of α-helices 1, 5, 6, 7, and 8, but notably less conservation of the orientation of α3 and the adjacent distal portion of α2 and proximal portion of α4 (**Figure 31C**). The latter results in a variable pitch of the canonical groove, with that of BCL-X_L displaced most downward, followed by MCL-1 and BFL-1, which progressively shift toward the horizontal. In these unbound structures, the width of the binding pockets, as assessed by measuring the distances between conserved residues at the roof and floor of the groove, are also distinct. Whereas the width of the empty BH3-binding pocket is essentially the same for MCL-1 and BFL-1 across multiple measurements, the BCL-X_L groove is narrower, particularly between the midpoints of α-helices 3 and 4 (**Figure 31D**). The latter derives from a notable difference in the relative disposition of these α-helices, which are oriented anti-parallel to one another in BCL-X_L but progressively more V-shaped in MCL-1 and BFL-1. Whereas structure-based design of anti-apoptotic inhibitors have largely derived from BH3-bound protein complexes, such conformational distinctions among the apo structures inform alternative topographies for molecular targeting.

One of the most intriguing differences among the structures of unliganded anti-apoptotic proteins is the presence of a solvent-exposed cysteine within the BH3-binding groove of apo BFL-1 (**Figure 31B**). No such cysteine is found in apo MCL-1 or BCL-X_L, nor in any other BCL-2 family protein structure (**Figure 31E-F**). This cysteine can indeed be exploited for stapled BIM and NOXA BH3-based covalent targeting of BFL-1 *in vitro* and in cells. Incorporation of cysteine-reactive warheads into small molecules resembling the BCL-2 or MCL-1 inhibitors can enable a switch in target specificity that favors BFL-1 inhibition. To provide a structural blueprint for such small molecule development and also analyze the

conformational consequences of covalent BH3 engagement, the crystal structure of a cysteine-reactive stapled BH3 peptide in complex with BFL-1 was pursued next.

Covalent Targeting of BFL-1 C55 by D-NA-NOXA SAHB

A series of D-nipecotic acid (D-NA)-derivatized stabilized alpha-helices of BCL-2 domains (SAHBs) modeled after the BH3-domain of human NOXA BH3 were generated - altering sequence composition, staple type and location - and the constructs were subjected to crystallization screening. Promising, reproducible crystals formed when BFL-1ΔC was complexed with a NOXA SAHB comprising amino acids 26-40 and an inserted *i, i+7* staple at the 28,35 position (hereafter called D-NA-NOXA SAHB) (**Figure 32A**). To verify the efficacy of site-selective derivatization of BFL-1 by D-NA-NOXA SAHB, a series of BFL-1 constructs (WT, C55S, C4S/C19S, C4S/C19S/C55S) were incubated with either an acetyl- or D-NA-capped NOXA SAHB, and BFL-1 crosslinking was monitored by electrophoresis and Coomassie stain. The only combinations of peptide and protein that led to a discrete increase in molecular weight was D-NA-NOXA SAHB with those BFL-1 constructs bearing C55, namely wild-type and C4S/C19S (**Figure 32A**). Indeed, intact mass spectrometry documented complete conversion of BFL-1 C4S/C19S to the appropriately-sized D-NA-NOXA SAHB adduct upon peptide incubation (**Figure 32B**). The BFL-1 targeting capability of biotinylated D-NA-NOXA SAHB was validated in a competitive streptavidin pull-down assay using a mixture of recombinant MCL-1, BCL-X_L, and BFL-1 proteins, all of which contain cysteines. Whereas both Ac-NOXA SAHB and D-NA-NOXA SAHB pulled down a similarly small amount of MCL-1, only D-NA-NOXA SAHB caused both robust derivatization of BFL-1, as seen in the input lane, and substantial pull-down of BFL-1 (**Figure 32C**). Finally, to validate this activity in the context of a complex protein lysate, Ac- and D-NA-derivatized NOXA SAHBs were incubated with lysates from BFL-1-expressing A375P melanoma cells, and again markedly increased pull-down of native BFL-1 protein was noted for the warhead-bearing NOXA SAHB (**Figure 32D**).

Crystal Structure of the /BFL-1/D-NA-NOXA SAHB Complex

Having documented the robust BFL-1-targeting capacity of D-NA-NOXA SAHB, the crystal structure of its complex with BFL-1ΔC (C4S/C19S) was solved at 2.38 Å resolution (**Figure 33A**-B**, Figure 30**, PDB ID: 5WHH). All ligand atoms generated excellent electron density, showing the stapled peptide bound in the groove defined by α2-α4, with a calculated interface area per molecule of 771 $Å^2$. The hydrophilic residues R30, D34, and N37 of the

amphipathic α-helix make complementary electrostatic and hydrogen-bonding interactions with E80/D81, R88, and K147 of the BFL-1 surface, respectively (**Figure 33A**). Residues L29, F32, and L36 of the hydrophobic face of D-NA-NOXA SAHB directly engage the respective residues F95, V44/V48, and V40/V90 of the canonical hydrophobic groove (**Figure 33C**). The *i, i+7* staple is observed in the *cis* configuration and is predominantly solvent-exposed (**Figure 33A-C**). In addition, the covalent bond between C55 and the acrylamide moiety of D-NA is readily visualized (**Figure 33D**). Whereas the acrylamide moiety maintains a similar orientation as NOXA BH3 C25 with respect to BFL-1 C55 (**Figure 33E-F**), the piperidine group of D-NA fortuitously engages a complementary hydrophobic pocket formed by residues L52, L56, V74, and F95 at the BFL-1 surface (**Figure 33F-3G**). Thus, the chemical structure of the installed acrylamide-bearing moiety itself provides an opportunity to facilitate further BFL-1 interaction at the hydrophobic groove.

Solving both the apo and SAHB-derivatized structures of BFL-1 afforded a comparison of the unliganded and liganded conformations. Upon BFL-1 interaction, D-NA-NOXA SAHB induces a conformational "opening" of the groove, such that α2/α3 and α4 are displaced away from each other by 2.8 Å, when comparing, for example, the distances between the α-carbons of residues 55 and 70 of the apo and BH3-bound forms (**Figure 34A-B**). This discrete structural change is demonstrated quantitatively by a difference distance matrix plot of BFL-1/D-NA-NOXA SAHB versus apo BFL-1 (**Figure 34C-D**). An RMSD value of 0.897 for the comparison between all conserved α-carbons of the two BFL-1 structures underscores the significance of the conformational change.

The observed SAHB-induced remodeling of the BFL-1 canonical groove is notable for the formation of a new surface pocket subjacent to BFL-1 C55 (**Figure 34E**). This surface change is also evident upon BFL-1 interaction with an unstapled NOXA BH3 peptide, underscoring the general relevance of this finding beyond conformational accommodation to the hydrocarbon staple or the acrylamide moiety (**Figure 35A**). Importantly, this change in groove topography upon NOXA BH3 interaction is unique to BFL-1, in that structural comparison of unliganded and NOXA BH3-bound MCL-1 shows no such remodeling, and distance measurements between residues at the top and bottom of the groove are largely unchanged (**Figure 34B, 35B**). Indeed, the similarity of the MCL-1 groove between unbound and bound structures, a reflection of BH3-binding accessibility, may explain the relatively high affinity of MCL-1 for diverse BH3 domain helices. This

conformational difference between MCL-1 and BFL-1 may likewise explain why both the Ac- and D-NA-derivatized forms of NOXA SAHB can engage MCL-1 non-covalently, whereas the foreshortened Ac-NOXA SAHB has little to no interaction with BFL-1 unless equipped with the cysteine-reactive warhead. Given the potential energetic requirements associated with pocket opening, the irreversible nature of covalent reaction with the surface accessible C55 residue of BFL-1 may provide a substantial binding advantage. Ultimately, compounds that mimic NOXA BH3 in the C25 region and induce formation of this distinctive pocket (**Figure 33F-G, 34E**) could yield novel agents with preferential selectivity for BFL-1 over MCL-1, as exemplified by D-NA-NOXA SAHB (**Figure 32C**).

Conclusion

Solving the structure of apo BFL-1 provided the first opportunity to compare the BH3-liganded and unliganded forms of BFL-1, revealing structural differences in its surface groove. Importantly, the distinct topography of the unliganded groove can be exploited for small molecule discovery by *in silico* screening, and the structures of putative hits in complex with BFL-1 obtained by soaking a molecule of interest into the apo crystal. The unoccupied BFL-1 groove likewise demonstrates the surface accessibility of a uniquely-positioned cysteine residue, available for selective covalent targeting by cysteine-reactive stapled peptides and potentially by small molecule mimetics thereof. Indeed, the structure of BFL-1 in complex with a D-nipecotic acid-derivatized NOXA BH3 α-helix, reinforced by an i, $i+7$ staple, reveals both ligand-induced opening of the surface pocket and the creation of a small cavity subjacent to the disulfide bond amenable to supplementary hydrophobic interactions.

In this first structure of a covalent complex between BFL-1 and a stapled BH3 peptide, it was demonstrated that an installed i, $i+7$ staple localizes to the non-interacting face of the helix, preserves the native interactions between NOXA BH3 and BFL-1, and remodels groove topography to maximize ligand-target engagement. By installing the acrylamide-bearing moiety, which irreversibly reacts with C55 located within the canonical groove of BFL-1, the non-covalent MCL-1-binding preference of this shortened, i, $i+7$-stapled NOXA BH3 construct was transformed into a robust, covalent inhibitor of BFL-1. Taken together, these structural and biochemical results inform new opportunities to develop highly selective BFL-1-targeting agents and dual BFL-1/MCL-1 inhibitors for clinical use.

Example 5: Further Validation of the Selective Synergy of Cysteine-Reactive NOXA SAHBs and targeted inhibition of the DNA Damage Response Pathway in Reactivating Cell Death in BFL-1-Dependent AML Cell Lines

To investigate the ability of NOXA-15 R31E SAHB to synergize with a DNA damage response pathway member, U937 cells or MV4;11 cells were treated with D-NA-NOXA SAHB and/or the ATM inhibitor AZD0156 and cell viability and caspase-3/7 activation was measured. NOXA-15 R31E SAHB demonstrated selective cytotoxicity and synergy upon combination with AZD0156 in U937 cells (which are BFL-1-dependent), but not in MV4;11 cells (which are not BFL-1-dependent) (**Figures 40-43**).

To further validate the selective cytotoxicity and synergy of NOXA-15 R31E SAHB upon combination with AZD0156 in BFL-1 dependent U937 but not MV4;11 cell lines (**Figures 40-43**), we performed a series of confirmatory studies. We conducted the above-described cytotoxicity studies on additional cancer cell lines that exhibit dependency on BFL-1, such as OCI-AML-3 AML and Jurkat T cell leukemia cells (Kim et al., 2005, Meyers et al., 2017), and observed pharmacologic synergy similar to that seen in U937 cells (**Figures 44-45**). Next, we tested two additional ATM inhibitors, Ku-60019 (Golding et al., 2009) and Ku-55933 (Hickson et al., 2004), and noted the same pattern of synergy in U937 cells (**Figures 46-47**) but not in MV4;11 cells (**Figures 48-49**) as observed for AZD0156. We found that alternative inhibitors of the DNA damage response (DDR) pathway, including the CHK1/2 inhibitor AZD7762 (Zabludoff et al., 2008) and the PARP inhibitor olaparib (Menear et al., 2008), behaved similarly to the ATM inhibitors in the U937 (**Figures 50-51**) versus MV4;11 (**Figures 52-53**) synergy analyses. As a further measure of specificity, we tested the compounds on KG-1 AML cells, which were previously shown to be relatively unresponsive to the addition of an ATM inhibitor to a pro-apoptotic stimulus (Boehrer et al., 2009). We observed dose-responsive cytotoxicity in response to single agent D-NA-NOXA SAHB-15 R31E treatment, consistent with BFL-1 dependency, but no synergistic effect of AZD0156 when applied in combination (**Figure 54**). Finally, we found that the MCL-1 selective inhibitor S63845 exhibits both single agent activity and synergy in combination with ATM inhibition in MV4;11 cells, which are dependent on MCL-1 (Kotschy et al. 2016), but no activity in U937 cells, which are BFL-1 dependent and instead responsive to D-NA-NOXA SAHB-15 R31E (**Figure 55**). Indeed, the contrasting activities of S63845 and D-NA-NOXA SAHB-15 R31E in MV4;11 vs. U937 cells underscores the exquisite functional specificities of the two anti-apoptotic inhibitors for MCL-1 and BFL-1, respectively.

Example 6: Intracellular and Mechanistic Activity of BFL-1 and DNA Damage Response Pathway Inhibitors in Treated Cells.

We confirmed that both of our lead constructs could covalently crosslink to full-length BFL-1 protein, which bears a C-terminal helix that could potentially compete with D-NA-NOXA SAHBs for interaction at the canonical BH3-binding pocket in cancer cells. We generated recombinant full-length BFL-1 and expressed HA-tagged full-length BFL-1 in 293T cells, and then conducted crosslinking analyses *in vitro* and in treated cells, respectively. We consistently observed covalent crosslinking to recombinant and expressed full-length BFL-1 for both the F32A and R31E analogs of D-NA-NOXA SAHB-15, albeit at somewhat reduced efficiency compared to treatment of the corresponding C-terminally truncated species (**Figure 56A-B**). Thus, to confirm effective targeting of native BFL-1 in AML and identify the superior compound for our battery of planned cytotoxicity analyses, we treated lysates from the AML cell line that demonstrated the strongest BFL-1 dependency score, U937, with biotinylated NOXA SAHB-15 F32A and R31E peptides followed by streptavidin pull-down and BFL-1 western analysis. Consistent with our crosslinking results demonstrated above (e.g. Examples 1-3), incorporation of the acrylamide moiety into the NOXA SAHB-15 peptides markedly enhanced BFL-1 targeting, and in this case, of native BFL-1 protein from U937 AML cells (**Figure 56C**). This experiment further identified D-NA-NOXA SAHB-15 R31E as the more effective binder of native BFL-1 protein between the two constructs and was therefore selected for the battery of cancer cell testing.

From a mechanistic standpoint, we further confirmed that the cytotoxic effect, synergy, and observed caspase 3/7 activation upon treatment of U937 cells with D-NA-NOXA SAHB-15 R31E and the AZD0156 combination likewise tracked with mitochondrial membrane depolarization, as measured by TMRE staining (**Figure 57**). To demonstrate that AZD0156 was effectively targeting ATM at the applied doses, we treated U937 cells with the ATM inhibitor for 2 hr and monitored ATM auto-phosphorylation at S1981 and downstream CHK2 phosphorylation at S33/35 by western blot. Indeed, we observed dose-responsive inhibition of S1981 auto-phosphorylation, which was otherwise constitutively elevated in U937 cells, and potent blockade of CHK2 phosphorylation even at the lowest treatment dose (**Figure 58**). In evaluating the potential mechanistic basis for the observed synergy, we noted a time-dependent increase in the protein level of pro-apoptotic BAX in response to AZD0156 treatment (**Figure 59**), suggesting that BAX upregulation combined with pharmacologic blockade of the relevant anti-apoptotic inhibitor of BAX-mediated apoptosis in U937 cells,

BFL-1, could account for the enhanced cytotoxicity. Finally, we conducted LDH release assays upon treating U937 cells with a serial dilution of D-NA-NOXA SAHB-15 R31E used in single agent and combination treatments, and observed no non-specific cell membrane-lytic activity, as measured at 30 min and 4 hr (**Figure 60**). The above-described battery of experiments required for optimizing stapled peptides for selective target protein binding (e.g. BFL-1), cellular penetrance without membrane disruption, and on-mechanism cytotoxic activity highlights the non-obviousness of the design of stapled peptides, such as cysteine-reactive NOXA SAHBs, for therapeutic development. As a further example, a series of D-nipecotic warhead-bearing constructs exhibited varying propensities for non-specific membrane lysis, ranging from 0 to ~60% in HeLa cells treated with 20 μM peptide for 2 hours (**Figure 61**). Overall, the BFL-1 genetic dependency and co-dependency analyses (**Figure 62**) informed the selection of cancer cell subtypes to effectively apply and validate the selective BFL-1 targeting activity of cysteine-reactive NOXA SAHBs and their synergy upon combination with inhibitors of the DNA Damage Response pathway.

Materials and Methods Used in Examples

Stapled Peptide Synthesis

Hydrocarbon-stapled peptides corresponding to the BH3 domains of BCL-2 family proteins, and either N-terminally derivatized with acetyl, FITC-βAla, or electrophilic warheads, or C-terminally derivatized with Lys-biotin or Lys-FITC, were synthesized, purified, and quantitated using our previously reported methods (Bird et al., *Methods Enzymol.*, 446:369-86 (2008); Bird et al., *Curr. Protoc. Chem. Biol.*, 3(3):99-117 (2011)). Acrylamide-bearing peptides were synthesized by either coupling acrylic acid or trans-crotonic acid to the peptide N terminus, or by first coupling the Fmoc-protected cyclic amino acids (Chem-Impex International) followed by Fmoc deprotection and acylation with acrylic acid, using standard Fmoc coupling and depro- tection methods (Huhn et al., *Cell Chem Biol.*, 23(9):1123-1134 (2016)).

Recombinant Protein Expression and Purification

Recombinant anti-apoptotic BFL-1ΔC (aa 1-151) and its cysteine to serine mutants were cloned into PET17b (Novagen, N-terminal hexahistidine tag), expressed in *Escherichia coli* LOBSTR BL21(DE3) (Kerafast), and purified by sequential Ni-affinity and size-

exclusion chromatography as described (Pitter et al., *Methods Enzymol.*, 446:387-408 (2008)). Recombinant anti-apoptotic BCL-XLΔC (aa 1-212) and MCL-1ΔNΔC (aa 172-329) were cloned into the PGEX-4T-1 (GE Healthcare, N-terminal GST tag) expression vector, expressed in BL21(DE3) *Escherichia coli* (Sigma Aldrich), and purified as described previously (Huhn et al., 2016 (*supra*), Pitter et al., 2008 (*supra*)). In purifying BCL-XLΔC, the GST tag was cleaved using thrombin (12-15 units) to provide a size difference between BCL-XLΔC and GST-MCL-1ΔNΔC for facile protein identification by silver stain in streptavidin pull-down experiments.

Recombinant full-length BFL-1 (aa-175) containing an N-terminal hexahistidine tag was cloned into the PTYB1 vector (New England Biolabs) and purified as above except for the addition of a chitin affinity chromatography and DTT elution step. Protein purity and identity was confirmed by Coomassie staining and western blot analysis using a mouse monoclonal anti-His_6 tag antibody (Abcam).

In Vitro Covalent Conjugation Assay

His-BFL-1ΔC C4S/C19S protein (5 μM) was pre-treated with 10 mM DTT in 50 mM Tris (pH 8.0) and 100 mM NaCl for 30 min at room temperature (final volume 9.5 μL) and then combined with a 10:1 molar ratio of cysteine-reactive NOXA SAHB for an additional 1-hr incubation at room temperature. The samples were then boiled in 3X loading buffer with DTT and electrophoresed on 12% Bis-Tris gel, then subjected to Coomassie staining.

Cellular Uptake

Cells were cultured using their standard culture medium containing 10% fetal bovine serum (FBS) and penicillin-streptomycin (293T, A375P: DMEM; U937, MV4-11; RMP1). For cysteine-reactive peptide uptake, 293T cells were transfected with 2 μg pCMV plasmid containing HA-BFL-1ΔC C4S/C19S using Lipofectamine LTX Plus (Thermo Scientific). 24 hr post-transfection, cells were treated with 20 μM of the indicated SAHB in DMEM containing 5% FBS for 8 hr. Cells were harvested and lysed by incubation with 1% CHAPS lysis buffer (150 mM NaCl, 50 mM Tris pH 7.4, 100 mM DTT). Protein concentration of the soluble fraction was measured using a BCA kit according to manufacturer's instructions (Thermo Scientific). Samples were then boiled in LDS buffer and subjected to western analysis using 1:1000 dilutions of anti-HA (Sigma Aldrich, #12CA5) and anti-actin (Sigma Aldrich, #A1978) antibodies.

Streptavidin Pulldown

WT His-BFL-1ΔC, BCL-XLΔC (tagless), and GST-MCL-1ΔNΔC (1 μM each) were combined and incubated with 3 mM DTT in PBS for thirty minutes at room temperature. Mixtures were treated with vehicle or the indicated C-terminally biotinylated NOXA SAHB (1 μM) for four hours and were then added to 30 μL PBS-washed high-capacity streptavidin (SA) agarose beads (Thermo Fisher Pierce) and incubated with rotation at room temperature for two hours. The beads were then washed three times with NP-40 lysis buffer (1% NP-40, 50 mM Tris pH 8, 100 mM NaCl, 2.5 mM MgCl2) and three times with PBS. To elute bound protein, beads were boiled for ten minutes with 10% SDS containing 10 mg/mL biotin. After elution, samples were boiled with 3x LDS and 2M DTT for twenty minutes. Gel electrophoresis using a 12% Bis-Tris gel was conducted on inputs (2%) and eluates (5 μL) and gels were developed using the Pierce silver staining kit (Thermo Fisher).

BFL-1 Targeting in Cancer Cell Lysates

Cultured A375P and U937 cells were trypsinized or harvested, respectively, washed with PBS, and lysed by incubating with 1% CHAPS lysis buffer (1% CHAPS, 150 mM NaCl, 50 mM Tris pH 7.4, 100 μM DTT). The protein concentrations of the soluble fraction were measured using a BCA kit according to the manufacturer's instructions (Thermo Scientific). Lysate samples (1 mg) were incubated with vehicle or C-terminally biotinylated NOXA SAHBs (30 μM) overnight in 1% CHAPS lysis buffer at 4ºC. Biotin capture was accomplished by incubating the mixture with high-capacity SA agarose (Thermo Scientific) for 2 hours at 4ºC, followed by centrifugation and washing the pelleted beads three times with lysis buffer (1 mL). Bead-bound proteins were eluted by boiling in 10% SDS containing 10 mg/mL biotin for 10 minutes and then subjected to electrophoresis and western blotting using BFL-1 (Abcam, #125259) and MCL-1 (Rockland #600-401-394S) antibodies.

BFL-1/BH3 Complex Disruption

To evaluate the capacity of biotinylated SAHBs to compete with tBID for interactions with BFL-1 and MCL-1, 293T cells were transfected with either HA-BFL-1ΔC C4S/C19S or FLAG-MCL-1 in the p3XFLAG-CMV-10 vector (Sigma) as above. After 24 hr, cells were trypsinized, washed with PBS, lysed in 1% CHAPS buffer, and the supernatant collected for protein concentration determination by BCA kit. Lysate samples (0.5 mg) were incubated with 0.25 μM recombinant tBID (R&D Systems) and 5 μM biotinylated NOXA SAHBs for 6 h at RT. The mixtures were then subjected to HA or FLAG immunoprecipitation, followed by

western analysis using 1:1000 dilutions of HA (Sigma Aldrich, #12CA5), FLAG (Sigma-Aldrich, F7425), and BID (Santa Cruz sc-11423) antibodies.

Competitive Fluorescence Polarization Binding Assay

Fluorescence polarization (FP) assays were performed as previously described (Pitter et al., 2008 (*supra*)). Briefly, direct binding curves were first generated by FITC-BID BH3 (25 nM) with serial dilutions of anti-apoptotic protein, and FP was measured at 5 min on a SpectraMax M5 microplate reader (Molecular Devices). For competition assays, a serial dilution of acetylated or warhead-bearing NOXA SAHB was added to the indicated recombinant protein at ~EC75 concentration, as determined by the direct binding assay. FITC-BID BH3 (25 nM) was then added and fluorescence polarization was measured at equilibrium. IC50 values were calculated by nonlinear regression analysis of competitive binding curves using PRISM software (GraphPad).

LDH Release Assay.

A375P and U937 cancer cells were cultured in DMEM and RPMI, respectively, containing 10% fetal bovine serum (FBS) and penicillin-streptomycin, and plated in 96-well plates (5 x 10^3 cells per well). After overnight incubation for A375P cells, or immediately upon plating for U937 cells, the cells were treated with the indicated concentrations of D-NA-NOXA SAHBs in DMEM or RPMI supplemented with 5% FBS for the indicated time period (30 min, 4 h). After plate centrifugation at 1,500 rpm (Thermo Scientific Sorvall Four-Place Swinging Bucket Rotor [75006445]; 478 x g) for 5 min at 4°C, LDH release was quantified by transferring 100 μL of cell-culture medium to a clear plate (Corning), incubating with 100 μL of LDH reagent (Roche) for 30 min while shaking, and then measuring absorbance at 490 nm on a microplate reader (Spectramax M5, Molecular Devices).

Cell Viability and Caspase-3/7 Activation Assays.

U937, MV4;11, OCI-AML-3, Jurkat, and KG-1 cells cultured in RPMI containing 10% FBS and penicillin-streptomycin were plated in 96-well plates (5 x 10^3 cells per well) and, after overnight incubation, treated with the indicated concentrations of D-NA-NOXA SAHB and/or AZD0156, Ku-60019, Ku-55933, AZD7762, olaparib, or S63845 (all from Selleck Chemicals) in RPMI supplemented with 5% FBS for the indicated durations. Cell viability and caspase-3/7 activation was measured using CellTiter-Glo and Caspase-Glo 3/7

chemiluminescence reagents (Promega), respectively, and luminescence was detected by a Spectramax M5 microplate reader. Synergy of drug interactions was calculated using the CalcuSyn software (Biosoft).

Preparation of SAHB-conjugated BFL-1

BFL-1ΔC/D-NA-NOXA SAHB conjugates were prepared for structural analysis by treating BFL-1ΔC (30 μM) with DTT (20 mM) for 30 min at 4^0C, followed by sequential incubation with D-NA-NOXA SAHB peptide at peptide:protein molar ratios of 1.2x, 1.2x, and 1x for 1 hour each at 4^0C. The resulting conjugate was then purified using size-exclusion chromatography and conjugation efficiency assessed by SDS-PAGE gel electrophoresis and intact mass spectrometry.

X-ray Crystallography

Apo His-BFL-1ΔC C4S/C19S was expressed and purified as described above and buffer exchanged into 20 mM HEPES pH 7.5, 300 mM NaCl, 5% glycerol, 50 mM arginine, and 1 mM DTT. An equal volume (100 nL) of 6.3 mg/mL (340 μM) apo BFL-1ΔC was mixed with reservoir solution (35% PEG1500 and 0.1 M sodium cacodylate pH 6), and crystals were prepared in hanging drops at 20^0C. The crystals were transferred briefly into crystallization buffer containing 25% glycerol prior to flash-freezing in liquid nitrogen. Diffraction data from apo BFL-1ΔC crystals were collected at beamline 24ID-C of the NE-CAT at the Advanced Photon Source (Argonne National Laboratory), and data sets were integrated and scaled using XIA2 package (Evans, *Acta Crystallogr D Biol Crystallogr* 62, 72-82 (2006); Kabsch, *Acta Crystallogr D Biol Crystallogr* 66, 133-144 (2010); Winter, *J Appl Crystallogr* 43, 186-190 (2010)). The structure was solved by molecular replacement using the program Phaser (McCoy et al., *J Appl Crystallogr* 40, 658-674 (2007)) and the search model PDB entry 1NB8. Iterative manual model building and refinement using Phenix (Adams et al., *Acta Crystallogr D Biol Crystallogr* 66, 213-221 (2010)) and Coot (Emsley and Cowtan, *Acta Crystallogr D Biol Crystallogr 60, 2126-2132*, (2004)) led to a model (PDB: 5WHI) with excellent statistics, including maximum diffraction of 1.69Å.

His-BFL-1ΔC C4S/C19S in complex with D-NA-NOXA SAHB was generated as described above and buffer exchanged into 20 mM HEPES pH 7, 100 mM NaCl, and 1 mM DTT. An equal volume (200 nL) of (400 μM) His-BFL-1ΔC/D-NA-NOXA SAHB complex was mixed with reservoir solution (20% PEG3350 and 0.2 M sodium malonate), and crystals were formed in hanging drops at 20^0C. The crystals were transferred briefly into

crystallization buffer containing 25% glycerol prior to flash-freezing in liquid nitrogen. Diffraction data from His-BFL-1ΔC/D-NA-NOXA SAHB crystals were collected at beamline 24ID-C of the NE-CAT at the Advanced Photon Source (Argonne National Laboratory). After initial molecular replacement, the ligand was positioned and preliminarily refined using Buster and Rhofit (Smart et al., 2012). A model of the crystallographic data (PDB: 5WHH, maximum diffraction of 2.38 Å) was generated as described above.

Difference Distance Matrix Plots

To generate heat maps, alpha carbon coordinates conserved between structures were extracted from PDB files. Difference distance matrix plots were produced using the DDMP program from the Center for Structural Biology at Yale University (New Haven, CT).

Quantification and Statistical Analysis

For the structural analyses, bond length and difference distance matrix plot quantifications were conducted using pymol and the DDMP program. Refinement statistics for the X-ray structures were generated by the Phenix software package. Biochemical and cellular data are mean ± SD for experiments performed in technical triplicate and repeated at least two times with similar results. p values less than 0.05 were considered statistically significant by unpaired, two-tailed Student's t test, and indicated by an asterisk. Data were analyzed using Prism Software 7.0 (GraphPad) and CalcuSyn (Biosoft).

Data and Software Availability

X-ray crystallography data was deposited to the PDB under accession numbers 5WHI (apo BFL-1) and 5WHH (BFL-1/D-NA NOXA SAHB).

Mitochondrial Membrane Potential Assay

U937 cells were plated in 6-well plates (2 x 10^6 cells per well) and treated with the indicated concentrations of D-NA-NOXA SAHB and/or AZD0156. After 24 hr, cells were incubated with TMRE (1 μM) for 30 min according to the manufacturer's instructions (TMRE-Mitochondrial Membrane Potential Assay Kit, Abcam). Cells were then resuspended in 0.2% BSA in PBS and seeded at 2 x 10^5 cells per well in black 96-well plates (Corning). TMRE-mitochondrial membrane potential was read by measuring fluorescence (Ex/Em = 549/575 nm) on a Spectramax M5 microplate reader.

Phospho-signaling Analysis.

U937 cells cultured in RPMI containing 10% FBS and penicillin-streptomycin were plated in 6-well plates (2 x 10^6 cells per well) and treated with the indicated concentrations of AZD0156 for 2 hr. Cells were then washed with PBS, lysed in 1% CHAPS buffer, and the supernatant collected for protein concentration determination by BCA kit. Samples were subjected to electrophoresis and western analysis using 1:1000 dilutions of phospho-ATM (S1981, Cell Signaling, #4526), ATM (Abcam, #ab78), phospho-CHK2 (Ser33/35, Cell Signaling, #2665), CHK2 (Cell Signaling, #2662), and actin (Sigma #A1978) antibodies.

Dynamic Assessment of BAX Protein Levels.

U937 cells were plated in 6-well plates (2 x 10^6 cells per well), treated with AZD0156 (5 μM), and the cells harvested and lysed at the indicated time points. Cell lysates were then subjected to electrophoresis and western blotting with BAX (Santa Cruz sc-493) and actin (Sigma # A1978) antibodies.

OTHER EMBODIMENTS

While the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

WHAT IS CLAIMED IS:

1. A peptide comprising:

(i) an electrophilic warhead; and

(ii) a modified amino acid sequence of the sequence set forth in SEQ ID NO:39, wherein the modified amino acid sequence is at least five amino acids in length; comprises at least one peptide structure stabilizing modification, and one or more amino acids of SEQ ID NO:39 are substituted by another amino acid, and

wherein the peptide:

(a) binds BFL-1/A1; or

(b) binds BFL-1/A1 and MCL-1.

2. The peptide of claim 1, wherein the modified amino acid sequence is 5 to 35 amino acids in length.

3. The peptide in claim 1, wherein the modified amino acid sequence is 8 to 18 amino acids in length.

4. The peptide of claim 1, 2, or 3, wherein the peptide structure stabilizing modification is a staple and/or stitch.

5. The peptide of claim 4, wherein the staple is at one or more of position i and $i + 3$; i and $i + 4$; or i and $i + 7$.

6. The peptide of any one of claims 1 to 5, wherein the peptide structure stabilizing modification comprises substitution of at least two amino acids of SEQ ID NO:39 with non-natural amino acids with olefinic side chains.

7. The peptide of claim 6, wherein the non-natural amino acids with olefinic side chains are selected from the group consisting of: S-pentenyl alanine, R-octenyl alanine; R-propenylalanine, S-pentenylalanine; R- pentenylalanine, S-pentenylalanine; Bis-pentenylglycine, S-pentenylalanine, R-octenylalanine; and Bis-pentenylglycine, S-octenylalanine, R-octenylalanine.

8. The peptide of any one of claims 1 to 5, wherein the peptide structure stabilizing modification is a hydrocarbon staple/stitch, a lactam staple/stitch; a UV-cycloaddition staple/stitch; an oxime staple/stitch; a thioether staple/stitch; a double-click staple/stitch; a bis-lactam staple/stitch; a bis-arylation staple/stitch; or a combination of any two or more thereof.

9. The peptide of any one of claims 1 to 8, wherein the one or more amino acids of SEQ ID NO:39 that are substituted by another amino acid are on the BFL-1/A1 non-interacting face of the helix of SEQ ID NO:39.

10. The peptide of any one of claims 1 to 9, wherein 0 to 5 amino acids in SEQ ID NO:39 are removed from the C-terminal or are removed and replaced with 1 to 6 amino acids from the group consisting of alanine, D-alanine, α-aminoisobutyric acid, N-methyl glycine, serine, a substituted alanine, and a glycine derivative.

11. The peptide of any one of claims 1 to 10, wherein 0 to 6 amino acids on the non-interacting face in SEQ ID NO:39 are substituted with an amino acid selected from the group consisting of alanine, D-alanine, α-aminoisobutyric acid, N-methyl glycine, serine, a substituted alanine, and a glycine derivative.

12. The peptide of any one of claims 1 to 11, wherein one or more of the following amino acids A1, L4, R5, F7, G8, L11, N12, and Q15 of SEQ ID NO:39 are substituted with an alpha-methylated or alpha-ethylated natural amino acids.

13. The peptide of any one of claims 1 to 11, wherein one or more of the following amino acids A1, L4, R5, F7, G8, L11, N12, and Q15 of SEQ ID NO:39 are substituted with an amino acid selected from the group consisting of L-alanine, D-alanine, α-aminoisobutyric acid, N-methyl glycine, serine, a substituted alanine, and a glycine derivative.

14. The peptide of any one of claims 1 to 12, wherein one or more of the following amino acids T2, Q3, R6, K10, F13, and R14 of SEQ ID NO:39 are substituted with an alpha-methylated or alpha-ethylated natural amino acids.

15. The peptide of any one of claims 1 to 12, wherein one or more of the following amino acids T2, Q3, R6, K10, F13, and R14 of SEQ ID NO:39 are substituted with an amino acid selected from the group consisting of L-alanine, D-alanine, α-aminoisobutyric acid, N-methyl glycine, serine, a substituted alanine, and a glycine derivative.

16. The peptide of any one of claims 1 to 9, wherein the one or more amino acids of SEQ ID NO:39 are substituted by an amino acid or amino acids selected from the group consisting of L-alanine, D-alanine, α-aminoisobutyric acid, N-methyl glycine, serine, a substituted alanine, and a glycine derivative.

17. The peptide of any one of claims 1 to 16, wherein:

(i) overall hydrophobicity of the peptide is reduced relative to a peptide of SEQ ID NO:39 or 16;

(ii) overall positive charge of the peptide is reduced relative to a peptide of SEQ ID NO:39 or 16; or

(iii) a combination of (i) and (ii).

18. The peptide of any one of claims 1 to 17, wherein the electrophilic warhead is a non-natural amino acid bearing an electrophilic group.

19. The peptide of claim 18, wherein the non-natural amino acid bearing an electrophilic group has an electrophilic acrylamide or substituted acrylamide linked to the polypeptide backbone.

20. The peptide of claim 19, wherein the non-natural amino acid bearing an electrophilic group is selected from the group consisting of: (S)-1-acryloylpyrrolidine-3-carboxamide; 1-acrylopiperidine-4-carboxamide, (R)-1 acryloylpiperidine-3-carboxamide; (S)-1-acryloylpiperidine-3-carboxamide; (S)-1-acryloylpyyrolidine-2-carboxamide; (R)-1-acryloylpyrrolidine-2-carboxamide; (E)-4-(dimethylamino)but-2-enamide; acrylamide; aziridine, diaziridine, azetidine, pyrrolidine, imidazolidine, pyrazolidine, oxazolidine, isoxazolidine, thiazolidine, isothiazolidine, piperidine, piperazine, morpholine, thiomorpholine, azepaneazirine, diazirine, azete, pyrrole, imidazole, pyrazole, oxazole, isoxazole, thiazole, isothiazole, pyridine, diazines, oxazine, thiazine, azepine phenyl (aniline); naphthalene, anthracene, phenanthrene, indole, isoindole, indolizine, quinolone, isoquinoline, quinoxaline, phthalzine, quinazoline, purine, carbazole, indazole, benzimidazole, azaindole, α-cyanoacrylamide, propiolamide, trans 4-dimethylamino-2-butenamide, trans 4-piperidinyl-2-butenamide, a substituted acrylamide, and a vinyl-sulfonamide.

21. The peptide of any one of claims 1 to 17, wherein the electrophilic warhead is a cysteine-reactive D-nipecotic acid moiety.

22. The peptide of any one of claims 1 to 17, wherein the electrophilic warhead is a cysteine-reactive moiety.

23. The peptide of any one of claims 1 to 22, wherein the electrophilic warhead is at the N-terminus of the peptide.

24. The peptide of any one of claims 1 to 22, wherein the electrophilic warhead is not at the N-terminus of the peptide.

25. A peptide comprising:

(i) an electrophilic warhead; and

(ii) five or more amino acids of: A0B0C0D0E0A1B1C1D1E1A2B2C2D2E2 (SEQ ID NO:93), wherein:

(a) A0 is absent, A, D-alanine, α-aminoisobutyric acid, or a staple/stitch position;
(b) B0 is absent, T, A, D-alanine, α-aminoisobutyric acid, I, M, B (norleucine), or a staple/stitch position;
(c) C0 is absent, Q, A, D-alanine, α-aminoisobutyric acid, or a staple/stitch position;
(d) D0 is absent, L, A, D-alanine, α-aminoisobutyric acid, I, or F;
(e) E0 is R, A, D-alanine, α-aminoisobutyric acid, K, T, or a staple/stitch position;
(f) A1 is R, A, D-alanine, α-aminoisobutyric acid, E, or a staple/stitch position;
(g) B1 is F, A, D-alanine, α-aminoisobutyric acid, I, L, V, or a staple/stitch position;
(h) C1 is G, A, D-alanine, α-aminoisobutyric acid, or a staple/stitch position;
(i) D1 is D;
(j) E1 is absent, K, A, D-alanine, α-aminoisobutyric acid, or a staple/stitch position;
(k) A2 is absent, L, A, D-alanine, α-aminoisobutyric acid, W, V, or a staple/stitch position;
(l) B2 is absent, N, A, D-alanine, α-aminoisobutyric acid, S, D, or a staple/stitch position;
(m) C2 is absent, F, A, L, D-alanine, α-aminoisobutyric acid, or a staple/stitch position;
(n) D2 is absent, R, A, D-alanine, α-aminoisobutyric acid, E, or a staple/stitch position; and
(o) E2 is absent, Q, A, D-alanine, α-aminoisobutyric acid, L, or a staple/stitch position,

wherein the peptide:

(a) binds BFL-1/A1; or

(b) binds BFL-1/A1 and MCL-1.

26. The peptide of claim 25, wherein the peptide is 5 to 35 amino acids in length.

27. The peptide of claim 25, wherein the peptide is 8 to 18 amino acids in length.

28. The peptide of claim 25, 26, or 27, wherein:

(i) overall hydrophobicity of the peptide is reduced relative to a peptide of SEQ ID NO:39 or 16;

(ii) overall positive charge of the peptide is reduced relative to a peptide of SEQ ID NO:39 or 16; or

(iii) a combination of (i) and (ii).

29. The peptide of any one of claims 25 to 28, wherein the electrophilic warhead is a non-natural amino acid bearing an electrophilic group.

30. The peptide of claim 29, wherein the non-natural amino acid bearing an electrophilic group has an electrophilic acrylamide or substituted acrylamide linked to the polypeptide backbone.

31. The peptide of claim 30, wherein the non-natural amino acid bearing an electrophilic group is selected from the group consisting of: (S)-1-acryloylpyrrolidine-3-carboxamide; 1-acrylopiperidine-4-carboxamide, (R)-1 acryloylpiperidine-3-carboxamide; (S)-1-acryloylpiperidine-3-carboxamide; (S)-1-acryloylpyyrolidine-2-carboxamide; (R)-1-acryloylpyrrolidine-2-carboxamide; (E)-4-(dimethylamino)but-2-enamide; acrylamide;

aziridine, diaziridine, azetidine, pyrrolidine, imidazolidine, pyrazolidine, oxazolidine, isoxazolidine, thiazolidine, isothiazolidine, piperidine, piperazine, morpholine, thiomorpholine, azepaneazirine, diazirine, azete, pyrrole, imidazole, pyrazole, oxazole, isoxazole, thiazole, isothiazole, pyridine, diazines, oxazine, thiazine, azepine phenyl (aniline); naphthalene, anthracene, phenanthrene, indole, isoindole, indolizine, quinolone, isoquinoline, quinoxaline, phthalzine, quinazoline, purine, carbazole, indazole, benzimidazole, azaindole, α-cyanoacrylamide, propiolamide, trans 4-dimethylamino-2-butenamide, trans 4-piperidinyl-2-butenamide, a substituted acrylamide, and a vinyl-sulfonamide.

32. The peptide of any one of claims 25 to 29, wherein the electrophilic warhead is a cysteine-reactive D-nipecotic acid moiety.

33. The peptide of any one of claims 25 to 29, wherein the electrophilic warhead is a cysteine-reactive moiety.

34. The peptide of any one of claims 25 to 33, wherein the electrophilic warhead is at the N-terminus of the peptide.

35. The peptide of any one of claims 25 to 33, wherein the electrophilic warhead is not at the N-terminus of the peptide.

36. The peptide of any one of claims 25 to 33, wherein the staple, if present, is between amino acids at position i and $i + 3$; i and $i + 4$; or i and $i + 7$.

37. The peptide of any one of claims 25 to 36, wherein the peptide comprises substitution of at least two amino acids of SEQ ID NO:93 with non-natural amino acids with olefinic side chains.

38. The peptide of claim 37, wherein the non-natural amino acids with olefinic side chains are selected from the group consisting of: S-pentenyl alanine, R-octenyl alanine; R-propenylalanine, S-pentenylalanine; R- pentenylalanine, S-pentenylalanine; Bis-pentenylglycine, S-pentenylalanine, R-octenylalanine; and Bis-pentenylglycine, S-octenylalanine, R-octenylalanine.

39. The peptide of any one of claims 25 to 36, wherein the peptide comprises a hydrocarbon staple/stitch, a lactam staple/stitch; a UV-cycloaddition staple/stitch; an oxime staple/stitch; a thioether staple/stitch; a double-click staple/stitch; a bis-lactam staple/stitch; a bis-arylation staple/stitch; or a combination of any two or more thereof.

40. A peptide that covalently binds to BFL-1/A1, the peptide comprising an electrophilic warhead and a sequence that is at least 14% identical to an amino acid sequence set forth below:

ATQLRRAGDKLNFRQ (**SEQ ID NO:94**);

ATQLRRFGDKANFRQ (**SEQ ID NO:95**);

ATQLREFGDKLNFRQ (**SEQ ID NO:96**);

ATQLRRFGDKLNFEQ (**SEQ ID NO:97**);

AAQLRAFGAKLNAA (**SEQ ID NO:98**);

AAQLRAFGAKLNAAAA (**SEQ ID NO:99**);

AAQLRAFGAKLNAE (**SEQ ID NO:100**);

AAQLRAFGAKLNAEAA (**SEQ ID NO:101**);

AAQLREFGAKLNAA (**SEQ ID NO:102**);

AAQLREFGAKLNAAAA (**SEQ ID NO:103**);

AAQLRAFGDKLNAA (**SEQ ID NO:104**);

AAQLRAFGDKLNAAAA (**SEQ ID NO:105**);

AAQLRAFGDKLNAE (**SEQ ID NO:106**);

AAQLRAFGDKLNAEAA (**SEQ ID NO:107**);

AAQLREFGDKLNAA (**SEQ ID NO:108**); or

AAQLREFGDKLNAAAA (**SEQ ID NO:109),**

wherein the peptide selectively binds BFL-1/A1 over MCL-1.

41. The peptide of claim 40, wherein the peptide is 8 to 18 amino acids in length.

42. The peptide of claim 40 or 41, wherein the electrophilic warhead is at the N-terminus of the peptide.

43. The peptide of claim 40 or 41, wherein the electrophilic warhead is not at the N-terminus of the peptide.

44. The peptide of any one of claims 40 to 43, wherein the electrophilic warhead is a non-natural amino acid bearing an electrophilic group.

45. The peptide of claim 44, wherein the non-natural amino acid bearing an electrophilic group has an electrophilic acrylamide or substituted acrylamide linked to the polypeptide backbone.

46. The peptide of claim 45, wherein the non-natural amino acid bearing an electrophilic group is selected from the group consisting of: (S)-1-acryloylpyrrolidine-3-carboxamide; 1-acrylopiperidine-4-carboxamide, (R)-1 acryloylpiperidine-3-carboxamide; (S)-1-acryloylpiperidine-3-carboxamide; (S)-1-acryloylpyyrolidine-2-carboxamide; (R)-1-acryloylpyrrolidine-2-carboxamide; (E)-4-(dimethylamino)but-2-enamide; acrylamide; aziridine, diaziridine, azetidine, pyrrolidine, imidazolidine, pyrazolidine, oxazolidine, isoxazolidine, thiazolidine, isothiazolidine, piperidine, piperazine, morpholine, thiomorpholine, azepaneazirine, diazirine, azete, pyrrole, imidazole, pyrazole, oxazole,

isoxazole, thiazole, isothiazole, pyridine, diazines, oxazine, thiazine, azepine phenyl (aniline); naphthalene, anthracene, phenanthrene, indole, isoindole, indolizine, quinolone, isoquinoline, quinoxaline, phthalzine, quinazoline, purine, carbazole, indazole, benzimidazole, azaindole, α-cyanoacrylamide, propiolamide, trans 4-dimethylamino-2-butenamide, trans 4-piperidinyl-2-butenamide, a substituted acrylamide, and a vinyl-sulfonamide.

47. The peptide of any one of claims 40 to 44, wherein the electrophilic warhead is a cysteine-reactive D-nipecotic acid moiety.

48. The peptide of any one of claims 40 to 44, wherein the electrophilic warhead is a cysteine-reactive moiety.

49. The peptide of any one of claims 40 to 48, wherein the peptide comprises a hydrocarbon staple/stitch, a lactam staple/stitch; a UV-cycloaddition staple/stitch; an oxime staple/stitch; a thioether staple/stitch; a double-click staple/stitch; a bis-lactam staple/stitch; a bis-arylation staple/stitch; or a combination of any two or more thereof.

50. The peptide of any one of claims 40 to 49, wherein the peptide comprises a sequence that is at least 15%, 20%, 27%, 34%, 40%, 47%, 50%, 53%, 60%, 65% 70%, 75%, 80%, 85%, 90%, 95%, or 97%, or 100% identical to the amino acid sequence.

51. The peptide of claim 40 or 41, wherein the 86% variation from the amino acid sequence is on the BFL-1 non-interacting alpha-helical face of the amino acid sequence.

52. A peptide that covalently binds to BFL-1/A1, the peptide comprising a sequence that is at least 14% identical to an amino acid sequence set forth below:

JAT**X_1**LRRAGD**X_2**LNFRQ (**SEQ ID NO:60**);

JAT**X_1**LRRFGD**X_2**ANFRQ (**SEQ ID NO:61**);

JAT**X_1**LREFGD**X_2**LNFRQ (**SEQ ID NO:62**);

JAT**X_1**LRRFGD**X_2**LNFEQ (**SEQ ID NO:63**);

JAA**X_1**LRAFGA**X_2**LNAA (**SEQ ID NO:64**);

JAA**X_1**LRAFGA**X_2**LNAAAA (**SEQ ID NO:65**);

JAA**X_1**LRAFGA**X_2**LNAE (**SEQ ID NO:66**);

JAA**X_1**LRAFGA**X_2**LNAEAA (**SEQ ID NO:67**);

JAA**X_1**LREFGA**X_2**LNAA (**SEQ ID NO:68**);

JAA**X_1**LREFGA**X_2**LNAAAA (**SEQ ID NO:69**);

JAA**X_1**LRAFGD**X_2**LNAA (**SEQ ID NO:70**);

JAA**X_1**LRAFGD**X_2**LNAAAA (**SEQ ID NO:71**);

JAA**X_1**LRAFGD**X_2**LNAE (**SEQ ID NO:72**);

JAA**X_1**LRAFGD**X_2**LNAEAA (**SEQ ID NO:73**);

JAA**X_1**LREFGD**X_2**LNAA (**SEQ ID NO:74**); or

JAA**X_1**LREFGD**X_2**LNAAAA (**SEQ ID NO:75**),

wherein the peptide selectively binds BFL-1/A1 over MCL-1, and wherein the peptide covalently binds BFL-1/A1 and non-covalently binds MCL-1; and

wherein J is an electrophilic warhead; and X_1 and X_2 are non-natural amino acids.

53. The peptide of claim 52, wherein the peptide is 8 to 18 amino acids in length.

54. The peptide of claim 52 or 53, wherein the electrophilic warhead is a non-natural amino acid bearing an electrophilic group.

55. The peptide of claim 52, 53, or 54, where the peptide comprises a sequence that is at least 15%, 20%, 27%, 34%, 40%, 47%, 50%, 53%, 60%, 65% 70%, 75%, 80%, 85%, 90%, 95%, 97%, or 100% identical to the amino acid sequence.

56. The peptide of claim 52 or 53, wherein the 86% variation from the amino acid sequence is on the BFL-1 non-interacting alpha-helical face of the amino acid sequence.

57. The peptide of any one of claims 52 to 56, wherein the amino acid at one or more of positions 2, 3, 6, 7, 8, 9, 12, 13, 14, 15, or 16 (wherein position 1 is "J") of the recited sequences can be substituted to another amino acid.

58. The peptide of any one of claims 52 to 56, wherein the amino acid at one or more of positions 2, 3, 6, 7, 8, 9, 12, 13, 14, 15, or 16 (wherein position 1 is "J") of the recited sequences is substituted to an alanine, D-alanine, or α-aminoisobutyric acid.

59. The peptide of claim 52 or 53, wherein the amino acid at positions 7 and/or 15 is substituted with a glutamic acid, and/or wherein the amino acid at position 13 is substituted with an aspartic acid, and/or wherein the amino acid at position 16 is substituted with a leucine, and/or wherein the amino acids at one or more of positions 2, 3, 6, 8, 9, 12, 14, and 15 is substituted with alanine, D-alanine, or α-aminoisobutyric acid (wherein position 1 is "J").

60. The peptide of claim 59, wherein the amino acids at positions 8 and/or 12 is/are substituted with alanine, D-alanine, or α-aminoisobutyric acid.

61. The peptide of claim 52, 53, or 54, wherein the peptide comprises or consists of the sequence JAT**X_1**LREFGD**X_2**LNFRQ **(SEQ ID NO:62)**,

wherein the peptide selectively binds BFL-1/A1 over MCL-1, and wherein the peptide covalently binds BFL-1/A1 and non-covalently binds MCL-1; and

wherein J is an electrophilic warhead; and X_1 and X_2 are non-natural amino acids.

62. The peptide of claim 52, 53, or 54, wherein the peptide comprises or consists of the sequence JAT**X_1**LRRAGD**X_2**LNFRQ (**SEQ ID NO:60**),

wherein the peptide selectively binds BFL-1/A1 over MCL-1, and wherein the peptide covalently binds BFL-1/A1 and non-covalently binds MCL-1; and

wherein J is an electrophilic warhead; and X_1 and X_2 are non-natural amino acids.

63. The peptide of any one of claims 52 to 62, wherein the electrophilic warhead has an electrophilic moiety linked to the polypeptide backbone where the linker is a nitrogen containing heterocycle nitrogen containing heterocyclic amino acid or amino-functionalized benzene ring, carbocycle, polycycle or heterocycle.

64. The peptide of claim 63, wherein the electrophilic moiety is an electrophilic acrylamide, substituted acrylamide, a vinyl-sulfonamide, or an α, β unsaturated amide.

65. The peptide of any one of claims 52 to 62, wherein the electrophilic warhead is a non-natural amino acid bearing an electrophilic group.

66. The peptide of claim 65, wherein the non-natural amino acid bearing an electrophilic group is selected from the group consisting of: (S)-1-acryloylpyrrolidine-3-carboxamide; 1-acrylopiperidine-4-carboxamide, (R)-1 acryloylpiperidine-3-carboxamide; (S)-1-acryloylpiperidine-3-carboxamide; (S)-1-acryloylpyyrolidine-2-carboxamide; (R)-1-acryloylpyrrolidine-2-carboxamide; (E)-4-(dimethylamino)but-2-enamide; acrylamide; aziridine, diaziridine, azetidine, pyrrolidine, imidazolidine, pyrazolidine, oxazolidine, isoxazolidine, thiazolidine, isothiazolidine, piperidine, piperazine, morpholine, thiomorpholine, azepaneazirine, diazirine, azete, pyrrole, imidazole, pyrazole, oxazole, isoxazole, thiazole, isothiazole, pyridine, diazines, oxazine, thiazine, azepine phenyl (aniline), naphthalene, anthracene, phenanthrene, indole, isoindole, indolizine, quinolone, isoquinoline,

quinoxaline, phthalzine, quinazoline, purine, carbazole, indazole, benzimidazole, azaindole; α-cyanoacrylamide, propiolamide, trans 4-dimethylamino-2-butenamide, trans 4-piperidinyl-2-butenamide, a substituted acrylamide, and a vinyl-sulfonamide.

67. The peptide of any one of claims 52 to 62, wherein the electrophilic warhead is a cysteine-reactive D-nipecotic acid moiety.

68. The peptide of any one of claims 52 to 62, wherein the electrophilic warhead is a cysteine-reactive moiety.

69. The peptide of any one of claims 52 to 68, wherein the peptide is 15 to 35 amino acids in length.

70. A peptide that covalently binds to BFL-1/A1, the peptide comprising at least two amino acids of the BFL-1/A1 interacting face of the alpha helix of an amino acid sequence set forth below:

JAT**X_1**LRRAGD**X_2**LNFRQ (**SEQ ID NO:60**);

JAT**X_1**LRRFGD**X_2**ANFRQ (**SEQ ID NO:61**);

JAT**X_1**LREFGD**X_2**LNFRQ (**SEQ ID NO:62**);

JAT**X_1**LRRFGD**X_2**LNFEQ (**SEQ ID NO:63**);

JAA**X_1**LRAFGA**X_2**LNAA (**SEQ ID NO:64**);

JAA**X_1**LRAFGA**X_2**LNAAAA (**SEQ ID NO:65**);

JAA**X_1**LRAFGA**X_2**LNAE (**SEQ ID NO:66**);

JAA**X_1**LRAFGA**X_2**LNAEAA (**SEQ ID NO:67**);

JAA**X_1**LREFGA**X_2**LNAA (**SEQ ID NO:68**);

JAA**X_1**LREFGA**X_2**LNAAAA (**SEQ ID NO:69**);

JAA**X_1**LRAFGD**X_2**LNAA (**SEQ ID NO:70**);

JAA**X_1**LRAFGD**X_2**LNAAAA (**SEQ ID NO:71**);

JAA**X_1**LRAFGD**X_2**LNAE (**SEQ ID NO:72**);

JAA**X_1**LRAFGD**X_2**LNAEAA (**SEQ ID NO:73**);

JAA**X_1**LREFGD**X_2**LNAA (**SEQ ID NO:74**); or

JAA**X_1**LREFGD**X_2**LNAAAA (**SEQ ID NO:75),**

wherein J is an electrophilic warhead; and wherein X_1 and X_2 are non-natural amino acids.

71. The peptide of claim 70, wherein the peptide is 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 amino acids in length.

72. The peptide of claim 70 or 71, wherein the electrophilic warhead is a cysteine-reactive D-nipecotic acid moiety.

73. The peptide of claim 70 or 71, wherein the electrophilic warhead is a cysteine-reactive moiety.

74. The peptide of any one of claims 70 to 73, wherein the non-natural amino acids are non-natural amino acids with olefinic side chains.

75. The peptide of claim 74, wherein the non-natural amino acids with olefinic side chains are S-pentenyl alanine and/or R-octenyl alanine.

76. A peptide that covalently binds to BFL-1/A1, the peptide comprising an amino acid sequence set forth below:

JAT**X_1**LRRAGD**X_2**LNFRQ (**SEQ ID NO:60**);

JAT**X_1**LRRFGD**X_2**ANFRQ (**SEQ ID NO:61**);

JAT**X_1**LREFGD**X_2**LNFRQ (**SEQ ID NO:62**);

JAT**X_1**LRRFGD**X_2**LNFEQ (**SEQ ID NO:63**);

JAA**X_1**LRAFGA**X_2**LNAA (**SEQ ID NO:64**);

JAA**X_1**LRAFGA**X_2**LNAAAA (**SEQ ID NO:65**);

JAA**X_1**LRAFGA**X_2**LNAE (**SEQ ID NO:66**);

JAA**X_1**LRAFGA**X_2**LNAEAA (**SEQ ID NO:67**);

JAA**X_1**LREFGA**X_2**LNAA (**SEQ ID NO:68**);

JAA**X_1**LREFGA**X_2**LNAAAA (**SEQ ID NO:69**);

JAA**X_1**LRAFGD**X_2**LNAA (**SEQ ID NO:70**);

JAA**X_1**LRAFGD**X_2**LNAAAA (**SEQ ID NO:71**);

JAA**X_1**LRAFGD**X_2**LNAE (**SEQ ID NO:72**);

JAA**X_1**LRAFGD**X_2**LNAEAA (**SEQ ID NO:73**);

JAA**X_1**LREFGD**X_2**LNAA (**SEQ ID NO:74**); or

JAA**X_1**LREFGD**X_2**LNAAAA (**SEQ ID NO:75),**

wherein J is an electrophilic warhead; and wherein X_1 and X_2 are non-natural amino acids.

77. The peptide of claim 76, wherein the peptide comprises or consists of the sequence JAT**X_1**LREFGD**X_2**LNFRQ (SEQ ID NO:62), wherein J is an electrophilic warhead; and wherein X_1 and X_2 are non-natural amino acids.

78. The peptide of claim 76, wherein the peptide comprises or consists of the sequence JAT**X_1**LRRAGD**X_2**LNFRQ (SEQ ID NO:60), wherein J is an electrophilic warhead; and wherein X_1 and X_2 are non-natural amino acids.

79. The peptide of any one of claims 76 to 78, wherein:

X_1 is R-octenyl alanine and X_2 is S-pentenyl alanine;

X_1 is S-pentenyl alanine and X_2 is R-octenyl alanine;

X_1 is S-pentenyl alanine and X_2 is S-pentenyl alanine;

X_1 is R-propenylalanine and X_2 is S-pentenyl alanine; or

X_1 is R- pentenylalanine and X_2 is S-pentenyl alanine.

80. The peptide of any one of claims 76 to 79, wherein the electrophilic warhead comprises an electrophilic moiety linked to the polypeptide backbone.

81. The peptide of claim 80, wherein the electrophilic moiety is a non-natural amino acid bearing an electrophilic group.

82. The peptide of claim 81, wherein the non-natural amino acid bearing an electrophilic group comprises an electrophilic acrylamide, substituted acrylamide, a vinyl-sulfonamide, or an α, β unsaturated amide.

83. The peptide of any one of claims 81 or 82, wherein the non-natural amino acid bearing an electrophilic group is selected from the group consisting of: (S)-1-acryloylpyrrolidine-3-carboxamide; 1-acrylopiperidine-4-carboxamide, (R)-1 acryloylpiperidine-3-carboxamide; (S)-1-acryloylpiperidine-3-carboxamide; (S)-1-acryloylpyyrolidine-2-carboxamide; (R)-1-acryloylpyrrolidine-2-carboxamide; (E)-4-(dimethylamino)but-2-enamide; acrylamide; aziridine, diaziridine, azetidine, pyrrolidine, imidazolidine, pyrazolidine, oxazolidine, isoxazolidine, thiazolidine, isothiazolidine, piperidine, piperazine, morpholine, thiomorpholine, azepaneazirine, diazirine, azete, pyrrole, imidazole, pyrazole, oxazole, isoxazole, thiazole, isothiazole, pyridine, diazines, oxazine, thiazine, azepine phenyl (aniline); naphthalene, anthracene, phenanthrene, indole, isoindole, indolizine, quinolone, isoquinoline, quinoxaline, phthalzine, quinazoline, purine, carbazole, indazole, benzimidazole, azaindole, α-cyanoacrylamide, propiolamide, trans 4-

dimethylamino-2-butenamide, trans 4-piperidinyl-2-butenamide, a substituted acrylamide, and a vinyl-sulfonamide.

84. The peptide of any one of claims 76 to 79, wherein the electrophilic warhead is a cysteine-reactive D-nipecotic acid moiety.

85. The peptide of any one of claims 76 to 79, wherein the electrophilic warhead is a cysteine-reactive moiety.

86. The peptide of any one of claims 76 to 85, wherein the peptide is 15 to 35 amino acids in length.

87. The peptide of claim 76 to 85, wherein the peptide is 8 to 18 amino acids in length.

88. A pharmaceutical composition comprising the peptide of any one of claims 1 to 87, and a pharmaceutically acceptable carrier.

89. A method of treating a BFL-1/A1-expressing or BFL-1/A1-dependent disease in a human subject in need thereof, the method comprising administering to the human subject a therapeutically effective amount of the peptide of any one of claims 1 to 87, or the pharmaceutical composition of claim 88.

90. The method of claim 89, wherein the BFL-1/A1-expressing or BFL-1/A1-dependent disease is selected from the group consisting of a hematologic malignancy, a solid tumor, an autoimmune disease, and an inflammatory disease.

91. The method of claim 90, wherein the BFL-1/A1-expressing or BFL-1/A1-dependent disease is a hematologic malignancy that is a leukemia or a lymphoma.

92. The method of claim 90, wherein the BFL-1/A1-expressing or BFL-1/A1-dependent disease is a solid tumor that is a melanoma, a breast cancer, or a lung cancer.

93. The method of claim 90, wherein the BFL-1/A1-expressing or BFL-1/A1-dependent disease is an autoimmune disease that is autoimmune colitis, thyroiditis, arthritis, nephritis, dermatitis, vasculitis, system lupus erythematosus, diabetes, or Sjogren's disease.

94. The method of claim 90, wherein the BFL-1/A1-expressing or BFL-1/A1-dependent disease is an inflammatory disease that is asthma, psoriasis, inflammatory colitis, thyroiditis, arthritis, nephritis, dermatitis, or vasculitis.

95. The method of claim 89, wherein the BFL-1/A1-expressing or BFL-1/A1-dependent disease is a cancer.

96. The method of claim 95, wherein the cancer is a solid tumor or a liquid tumor.

97. The method of claim 95, wherein the cancer is a breast cancer, an autonomic ganglia cancer, a pancreatic cancer, a skin cancer, a CNS cancer, a hematopoietic or lymphoid cancer, a lung cancer, a large intestine cancer, a stomach cancer, a soft tissues sarcoma, or a bone cancer.

98. The method of claim 90, wherein the hematologic malignancy is acute myeloid leukemia, acute lymphoblastic leukemia, chronic myeloid leukemia, chronic lymphocytic leukemia, myelodysplastic syndrome, or multiple myeloma.

99. A method of treating a BFL-1/A1-expressing or BFL-1/A1-dependent disease in a human subject in need thereof, the method comprising administering to the human subject a therapeutically effective amount of the peptide of any one of claims 1 to 87, or the

pharmaceutical composition of claim 88, in combination with an inhibitor of a DNA damage response pathway member.

100. The method of claim 99, wherein the inhibitor is an ataxia-telangiectasia mutated (ATM) kinase inhibitor, an ataxia telangiectasia and rad3-related (ATR) kinase inhibitor, a checkpoint kinase 1 and/or a checkpoint kinase 2 (CHK1/2) inhibitor, and/or a poly(ADP-ribose) polymerase (PARP) inhibitor.

101. The method of claim 99, wherein the BFL-1/A1-expressing or BFL-1/A1-dependent disease is selected from the group consisting of a hematologic malignancy, a solid tumor, an autoimmune disease, and an inflammatory disease.

102. The method of claim 101, wherein:

the hematologic malignancy is a leukemia or a lymphoma;

the solid tumor is a melanoma, a breast cancer, or a lung cancer;

the autoimmune disease is autoimmune colitis, thyroiditis, arthritis, nephritis, dermatitis, vasculitis, system lupus erythematosus, diabetes, or Sjogren's disease; and

the inflammatory disease, asthma, psoriasis, inflammatory colitis, thyroiditis, arthritis, nephritis, dermatitis, or vasculitis.

103. The method of claim 101, wherein the hematologic malignancy is acute myeloid leukemia, acute lymphoblastic leukemia, chronic myeloid leukemia, chronic lymphocytic leukemia, myelodysplastic syndrome, or multiple myeloma.

104. The method of claim 99, wherein the BFL-1/A1-expressing or BFL-1/A1-dependent disease is a cancer.

105. The method of claim 104, wherein the cancer is a solid tumor or a liquid tumor.

106. The method of claim 104, wherein the cancer is a melanoma, a lymphoma, or a leukemia.

107. The method of claim 104, wherein the cancer is acute myeloid leukemia.

108. The method of claim 104, wherein the cancer is a breast cancer, an autonomic ganglia cancer, a pancreatic cancer, a skin cancer, a CNS cancer, a hematopoietic or lymphoid cancer, a lung cancer, a large intestine cancer, a stomach cancer, a soft tissues sarcoma, or a bone cancer.

109. The method of any one of claims 99 to 108, wherein the inhibitor is an ATM inhibitor is selected from the group consisting of KU-559403, KU-55933, KU-60019, CP-466722, chloroquine phosphate, CGK733, and AZD0156.

110. The method of any one of claims 99 to 108, wherein the inhibitor is an ATR inhibitor is selected from the group consisting of schisandrin B, NU6027, NVP-BEZ235, VE-821, VE-822/VX-970, AZ20, and AZD6738.

111. The method of any one of claims 99 to 108, wherein the CHK1/2 inhibitor is selected from the group consisting of AZD7762, CCT 241533, LY 2603618, LY 2606368, NSC 109555 ditosylate, PD 407824, PF47736, SB 218078, TCS 2312, and SRA737.

112. The method of any one of claims 99 to 108, wherein the PARP inhibitor is selected from the group consisting of olaparib, A-966492, veliparib, rucaparib, AG-14361, iniparib, INO-1001, niraparib, talazoparib, AZD2461, 2X-121, BMN 673, and E7449.

113. A combination therapy for treating a BFL-1/A1-expressing or BFL-1/A1-dependent disease comprising a peptide of any one of claims 1 to 87 and an inhibitor of the DNA damage response pathway.

114. The combination therapy of claim 113, wherein the inhibitor is an ATM kinase inhibitor, an ATR kinase inhibitor, a CHK1/2 inhibitor, or a PARP inhibitor.

115. A combination therapy for treating a MCL-1-expressing or MCL-1-dependent disease comprising a peptide of any one of claims 1 to 87 and an inhibitor of a DNA damage response pathway member.

116. The combination therapy of claim 115, wherein the inhibitor is an ATM kinase inhibitor, an ATR kinase inhibitor, a CHK1/2 inhibitor, or a PARP inhibitor.

117. The combination therapy of any one of claims 113 to 116, wherein the peptide is selected from the group consisting of:

JAT**X_1**LRRAGD**X_2**LNFRQ (**SEQ ID NO:60**);

JAT**X_1**LRRFGD**X_2**ANFRQ (**SEQ ID NO:61**);

JAT**X_1**LREFGD**X_2**LNFRQ (**SEQ ID NO:62**);

JAT**X_1**LRRFGD**X_2**LNFEQ (**SEQ ID NO:63**);

JAA**X_1**LRAFGA**X_2**LNAA (**SEQ ID NO:64**);

JAA**X_1**LRAFGA**X_2**LNAAAA (**SEQ ID NO:65**);

JAA**X_1**LRAFGA**X_2**LNAE (**SEQ ID NO:66**);

JAA**X_1**LRAFGA**X_2**LNAEAA (**SEQ ID NO:67**);

JAA**X_1**LREFGA**X_2**LNAA (**SEQ ID NO:68**);

JAA**X_1**LREFGA**X_2**LNAAAA (**SEQ ID NO:69**);

JAA**X_1**LRAFGD**X_2**LNAA (**SEQ ID NO:70**);

JAA**X_1**LRAFGD**X_2**LNAAAA (**SEQ ID NO:71**);

JAA**X_1**LRAFGD**X_2**LNAE (**SEQ ID NO:72**);

JAA**X_1**LRAFGD**X_2**LNAEAA (**SEQ ID NO:73**);

JAA**X_1**LREFGD**X_2**LNAA (**SEQ ID NO:74**); or

JAA**X_1**LREFGD**X_2**LNAAAA (**SEQ ID NO:75**),

wherein J is a cysteine-reactive acrylamido D-nipecotic acid moiety or other cysteine-reactive moiety, X_1 is R-octenyl alanine and X_2 is S-pentenyl alanine.

118. The combination therapy of any one of claims 113 to 116, wherein the peptide is JAT**X_1**LREFGD**X_2**LNFRQ (**SEQ ID NO:62**); wherein J is a cysteine-reactive acrylamido D-nipecotic acid moiety or other cysteine-reactive moiety, X_1 is R-octenyl alanine and X_2 is S-pentenyl alanine.

119. The combination therapy of any one of claims 113 to 116, wherein the peptide is JAT**X_1**LRRAGD**X_2**LNFRQ (**SEQ ID NO:60**); wherein J is a cysteine-reactive acrylamido D-nipecotic acid moiety or other cysteine-reactive moiety, X_1 is R-octenyl alanine and X_2 is S-pentenyl alanine.

120. The combination therapy of any one of claims 113 to 119, wherein the inhibitor is an ATM kinase inhibitor is selected from the group consisting of KU-559403, KU-55933, KU-60019, CP-466722, chloroquine phosphate, CGK733, and AZD0156.

121. The combination therapy of any one of claims 113 to 119, wherein the inhibitor is an ATR kinase inhibitor is selected from the group consisting of schisandrin B, NU6027, NVP-BEZ235, VE-821, VE-822/VX-970, AZ20, and AZD6738.

122. The combination therapy of any one of claims 113 to 119, wherein the inhibitor is a CHK1/2 inhibitor selected from the group consisting of AZD7762, CCT 241533, LY 2603618, LY 2606368, NSC 109555 ditosylate, PD 407824, PF47736, SB 218078, TCS 2312, and SRA737.

123. The combination therapy of any one of claims 113 to 119, wherein the inhibitor is a PARP inhibitor selected from the group consisting of olaparib, A-966492, veliparib, rucaparib, AG-14361, iniparib, INO-1001, niraparib, talazoparib, AZD2461, 2X-121, BMN 673, and E7449.

124. A method of treating a MCL-1-expressing and a BFL-1/A1-expressing, or MCL-1-dependent and BFL-1/A1-dependent, disease in a human subject in need thereof, the method comprising administering to the human subject a therapeutically effective amount of the peptide of any one of claims 1 to 87, or the pharmaceutical composition of claim 88.

125. The method of claim 124, further comprising administering to the subject an inhibitor of a DNA damage response pathway member.

126. The method of claim 125, wherein the inhibitor is an ATM kinase inhibitor, an ATR kinase inhibitor, a CHK1/2 inhibitor, or a PARP inhibitor.

127. The method of claim 124, 125, or 126, wherein the peptide is selected from the group consisting of:

JAT**X_1**LRRAGD**X_2**LNFRQ (**SEQ ID NO:60**);

JAT**X_1**LRRFGD**X_2**ANFRQ (**SEQ ID NO:61**);

JAT**X_1**LREFGD**X_2**LNFRQ (**SEQ ID NO:62**);

JAT**X_1**LRRFGD**X_2**LNFEQ (**SEQ ID NO:63**);

JAA**X_1**LRAFGA**X_2**LNAA (**SEQ ID NO:64**);

JAA**X_1**LRAFGA**X_2**LNAAAA (**SEQ ID NO:65**);

JAA**X_1**LRAFGA**X_2**LNAE (**SEQ ID NO:66**);

JAA**X_1**LRAFGA**X_2**LNAEAA (**SEQ ID NO:67**);

JAA**X_1**LREFGA**X_2**LNAA (**SEQ ID NO:68**);

JAA**X_1**LREFGA**X_2**LNAAAA (**SEQ ID NO:69**);

JAA**X_1**LRAFGD**X_2**LNAA (**SEQ ID NO:70**);

JAA**X_1**LRAFGD**X_2**LNAAAA (**SEQ ID NO:71**);

JAA**X_1**LRAFGD**X_2**LNAE (**SEQ ID NO:72**);

JAA**X_1**LRAFGD**X_2**LNAEAA (**SEQ ID NO:73**);

JAA**X_1**LREFGD**X_2**LNAA (**SEQ ID NO:74**); or

JAA**X_1**LREFGD**X_2**LNAAAA (**SEQ ID NO:75**),

wherein J is a cysteine-reactive acrylamido D-nipecotic acid moiety or other cysteine-reactive moiety, X_1 is R-octenyl alanine and X_2 is S-pentenyl alanine.

128. The method of claim 124, 125, or 126, wherein the peptide is JAT**X_1**LREFGD**X_2**LNFRQ (**SEQ ID NO:62**); wherein J is a cysteine-reactive acrylamido D-nipecotic acid moiety or other cysteine-reactive moiety, X_1 is R-octenyl alanine and X_2 is S-pentenyl alanine.

129. The method of claim 124, 125, or 126, wherein the peptide is JAT**X_1**LRRAGD**X_2**LNFRQ (**SEQ ID NO:60**); wherein J is a cysteine-reactive acrylamido D-nipecotic acid moiety or other cysteine-reactive moiety, X_1 is R-octenyl alanine and X_2 is S-pentenyl alanine.

130. The combination therapy of any one of claims 125 to 129, wherein the inhibitor is an ATM kinase inhibitor is selected from the group consisting of KU-559403, KU-55933, KU-60019, CP-466722, chloroquine phosphate, CGK733, and AZD0156.

131. The combination therapy of any one of claims 125 to 129, wherein the inhibitor is an ATR kinase inhibitor is selected from the group consisting of schisandrin B, NU6027, NVP-BEZ235, VE-821, VE-822/VX-970, AZ20, and AZD6738.

132. The combination therapy of any one of claims 125 to 129, wherein the inhibitor is a CHK1/2 inhibitor selected from the group consisting of AZD7762, CCT 241533, LY 2603618, LY 2606368, NSC 109555 ditosylate, PD 407824, PF47736, SB 218078, TCS 2312, and SRA737.

133. The combination therapy of any one of claims 125 to 129, wherein the inhibitor is a PARP inhibitor selected from the group consisting of olaparib, A-966492, veliparib, rucaparib, AG-14361, iniparib, INO-1001, niraparib, talazoparib, AZD2461, 2X-121, BMN 673, and E7449.

134. A stabilized peptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs.: 40 to 43.

135. The stabilized peptide of claim 134, wherein the peptide comprises the amino acid sequence set forth in SEQ ID NO: 40.

136. The stabilized peptide of claim 134, wherein the peptide comprises the amino acid sequence set forth in SEQ ID NO: 42.

137. The stabilized peptide of any one of claims 134 to 136, further comprising an electrophilic warhead.

138. The stabilized peptide of claim 137, wherein the electrophilic warhead is at the N-terminus of the stabilized peptide.

139. The stabilized peptide of claim 137 or 138, wherein the electrophilic warhead is a cysteine-reactive D-nipecotic acid moiety.

140. The stabilized peptide of claim 137 or 138, wherein the electrophilic warhead is a cysteine-reactive moiety.

141. A pharmaceutical composition comprising a stabilized peptide of any one of claims 137 to 139, and a pharmaceutically acceptable carrier.

142. A combination therapy for treating a BFL-1-expressing or BFL-1-dependent disease or MCL-1-expressing or MCL-1-dependent disease comprising a stabilized peptide of any one of claims 137 to 140 and an inhibitor of a DNA damage response pathway member.

143. The combination therapy of claim 142, wherein the inhibitor is an ATM kinase inhibitor, an ATR kinase inhibitor, a CHK1/2 inhibitor, or a PARP inhibitor.

144. A method of treating a MCL-1-expressing and a BFL-1/A1-expressing, or MCL-1-dependent and BFL-1/A1-dependent, disease in a human subject in need thereof, the method comprising administering to the human subject a therapeutically effective amount of the stabilized peptide of any one of claims 137 to 140, or the pharmaceutical composition of claim 141.

145. A stabilized peptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs.: 44 to 53.

146. The stabilized peptide of claim 145, wherein the peptide comprises the amino acid sequence set forth in SEQ ID NO: 44.

147. The stabilized peptide of claim 145, wherein the peptide comprises the amino acid sequence set forth in SEQ ID NO: 46.

148. The stabilized peptide of any one of claims 145 to 147, further comprising an electrophilic warhead.

149. The stabilized peptide of claim 148, wherein the electrophilic warhead is at the N-terminus of the stabilized peptide.

150. The stabilized peptide of claim 148 or 149, wherein the electrophilic warhead is a cysteine-reactive D-nipecotic acid moiety.

151. The stabilized peptide of claim 148 or 149, wherein the electrophilic warhead is a cysteine-reactive moiety.

152. A pharmaceutical composition comprising a stabilized peptide of any one of claims 148 to 151, and a pharmaceutically acceptable carrier.

153. A combination therapy for treating a BFL-1-expressing or BFL-1-dependent disease or MCL-1-expressing or MCL-1-dependent disease comprising a stabilized peptide of any one of claims 148 to 151 and an inhibitor of a DNA damage response pathway member.

154. The combination therapy of claim 153, wherein the inhibitor is an ATM kinase inhibitor, an ATR kinase inhibitor, a CHK1/2 inhibitor, or a PARP inhibitor.

155. A method of treating a MCL-1-expressing and a BFL-1/A1-expressing, or MCL-1-dependent and BFL-1/A1-dependent, disease in a human subject in need thereof, the method comprising administering to the human subject a therapeutically effective amount of the stabilized peptide of any one of claims 148 to 151, or the pharmaceutical composition of claim 152.

156. A combination therapy for treating a MCL-1-expressing and a BFL-1/A1-expressing, or MCL-1-dependent and BFL-1/A1-dependent, disease in a human subject in need thereof, comprising a peptide of any one of claims 1 to 87 and an inhibitor of MCL-1.

157. The combination therapy of claim 156, wherein the MCL-1 inhibitor is selected from the group consisting of S64315, S63845, BAY 1000394, MIK665, AMG397, and AMG176.

158. A combination therapy for treating a BCL-2-expressing and a BFL-1/A1-expressing, or BCL-2-dependent and BFL-1/A1-dependent, disease in a human subject in need thereof, comprising a peptide of any one of claims 1 to 87 and an inhibitor of BCL-2.

159. The combination therapy of claim 158, wherein the BCL-2 inhibitor is venetoclax.

160. A combination therapy for treating a BCL-XL-expressing and a BFL-1/A1-expressing, or BCL-XL-dependent and BFL-1/A1-dependent, disease in a human subject in need thereof, comprising a peptide of any one of claims 1 to 87 and an inhibitor of BCL-XL.

161. The combination therapy of claim 160, wherein the BCL-XL inhibitor is navitoclax.

162. A combination therapy for treating a BCL-XL-, BCL-2-, and/or MCL-1 expressing and a BFL-1/A1-expressing, or a BCL-XL-, BCL-2, and/or MCL-1-dependent and BFL-

1/A1-dependent, disease in a human subject in need thereof, comprising a peptide of any one of claims 1 to 87 and an inhibitor of BCL-XL, an inhibitor of BCL-2, and/or an inhibitor of MCL-1.

163. The combination therapy of claim 162, wherein the MCL-1 inhibitor is selected from the group consisting of S64315, S63845, BAY 1000394, MIK665, AMG397, and AMG176.

164. The combination therapy of claim 162, wherein the BCL-2 inhibitor is venetoclax.

165. The combination therapy of claim 162, wherein the BCL-XL inhibitor is navitoclax.

166. The combination therapy of any one of claims 156 to 165, wherein the peptide is selected from the group consisting of:

JAT**X_1**LRRAGD**X_2**LNFRQ (**SEQ ID NO:60**);

JAT**X_1**LRRFGD**X_2**ANFRQ (**SEQ ID NO:61**);

JAT**X_1**LREFGD**X_2**LNFRQ (**SEQ ID NO:62**);

JAT**X_1**LRRFGD**X_2**LNFEQ (**SEQ ID NO:63**);

JAA**X_1**LRAFGA**X_2**LNAA (**SEQ ID NO:64**);

JAA**X_1**LRAFGA**X_2**LNAAAA (**SEQ ID NO:65**);

JAA**X_1**LRAFGA**X_2**LNAE (**SEQ ID NO:66**);

JAA**X_1**LRAFGA**X_2**LNAEAA (**SEQ ID NO:67**);

JAA**X_1**LREFGA**X_2**LNAA (**SEQ ID NO:68**);

JAA**X_1**LREFGA**X_2**LNAAAA (**SEQ ID NO:69**);

JAA**X_1**LRAFGD**X_2**LNAA (**SEQ ID NO:70**);

JAA**X_1**LRAFGD**X_2**LNAAAA (**SEQ ID NO:71**);

JAA**X_1**LRAFGD**X_2**LNAE (**SEQ ID NO:72**);

JAA**X_1**LRAFGD**X_2**LNAEAA (**SEQ ID NO:73**);

JAA**X_1**LREFGD**X_2**LNAA (**SEQ ID NO:74**); or

JAA**X_1**LREFGD**X_2**LNAAAA (**SEQ ID NO:75**),

wherein J is a cysteine-reactive acrylamido D-nipecotic acid moiety or other cysteine-reactive moiety, X_1 is R-octenyl alanine and X_2 is S-pentenyl alanine.

167. The combination therapy of any one of claims 156 to 165, wherein the peptide is JAT**X1**LREFGD**X2**LNFRQ (SEQ ID NO:62); wherein J is a cysteine-reactive acrylamido D-nipecotic acid moiety or other cysteine-reactive moiety, X_1 is R-octenyl alanine and X_2 is S-pentenyl alanine.

168. The combination therapy of any one of claims 156 to 165, wherein the peptide is JAT**X_1**LRRAGD**X_2**LNFRQ (SEQ ID NO:60); wherein J is a cysteine-reactive acrylamido D-nipecotic acid moiety or other cysteine-reactive moiety, X_1 is R-octenyl alanine and X_2 is S-pentenyl alanine.

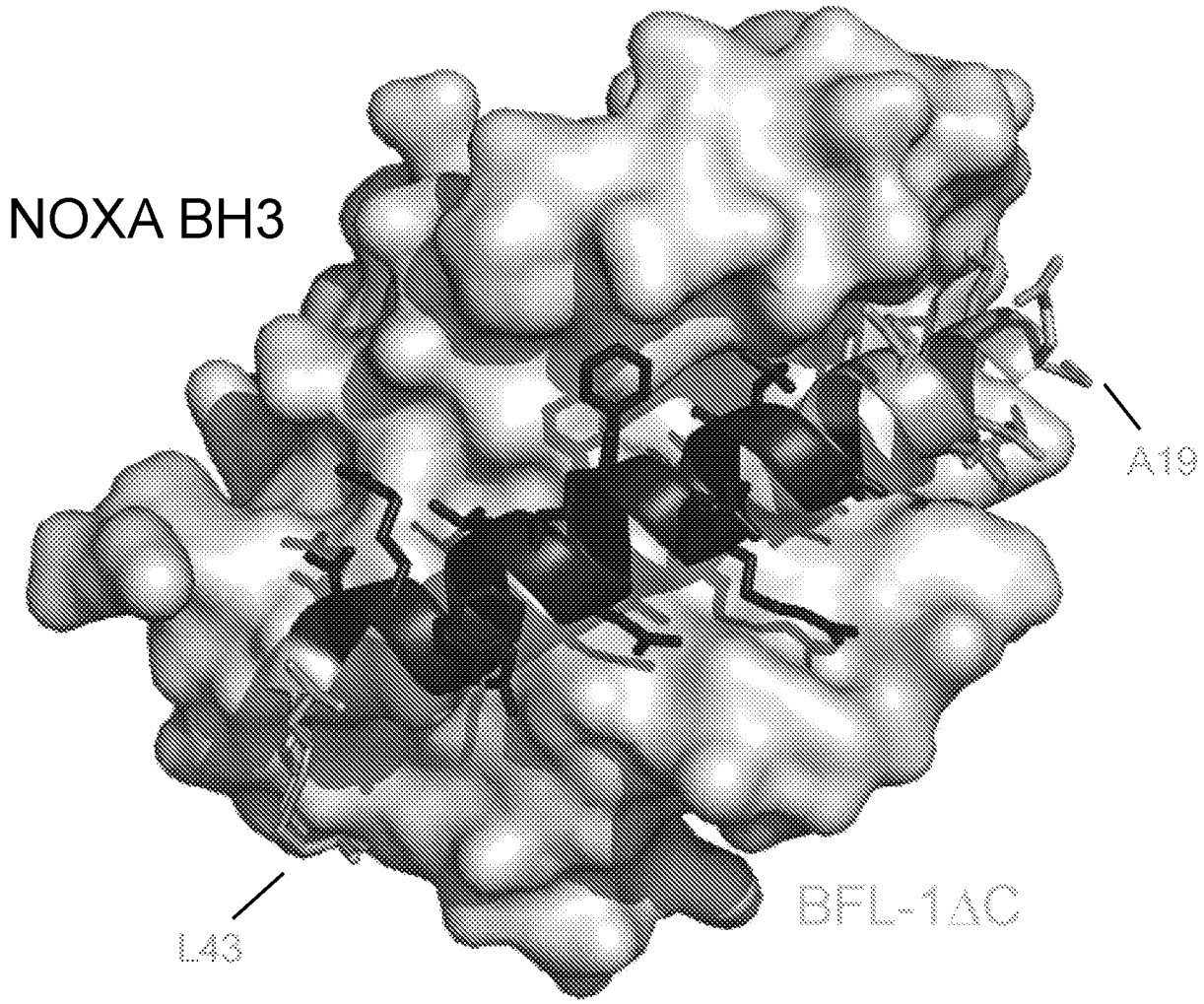


FIG. 1

NOXA SAHB$_{26\text{-}40}$	ATQLRRFGDKLNFRQ
NOXA SAHB-1	XTQLXRFGDKLNFRQ
NOXA SAHB-2	AXQLRXFGDKLNFRQ
NOXA SAHB-3	ATXLRRXGDKLNFRQ
NOXA SAHB-4	ATQXRRFXDKLNFRQ
NOXA SAHB-5	ATQLXRFGXKLNFRQ
NOXA SAHB-6	ATQLRXFGDXLNFRQ
NOXA SAHB-7	ATQLRRXGDKXNFRQ
NOXA SAHB-8	ATQLRRFXDKLXFRQ
NOXA SAHB-9	ATQLRRFGXKLNXRQ
NOXA SAHB-10	ATQLRRFGDXLNFXQ
NOXA SAHB-11	ATQLRRFGDKXNFRX
NOXA SAHB-12	ATQLRRFGDKLXFRQX
NOXA SAHB-13	8TQLRRFXDKLNFRQ
NOXA SAHB-14	A8QLRRFGXKLNFRQ
NOXA SAHB-15	AT8LRRFGDXLNFRQ
NOXA SAHB-16	ATQ8RRFGDKXNFRQ
NOXA SAHB-17	ATQL8RFGDKLXFRQ
NOXA SAHB-18	ATQLR8FGDKLNXRQ
NOXA SAHB-19	ATQLRR8GDKLNFXQ
NOXA SAHB-20	ATQLRRF8DKLNFRX

Note: D-nipecotic acid derivatization placed at the stapled peptide N-terminus

FIG. 2

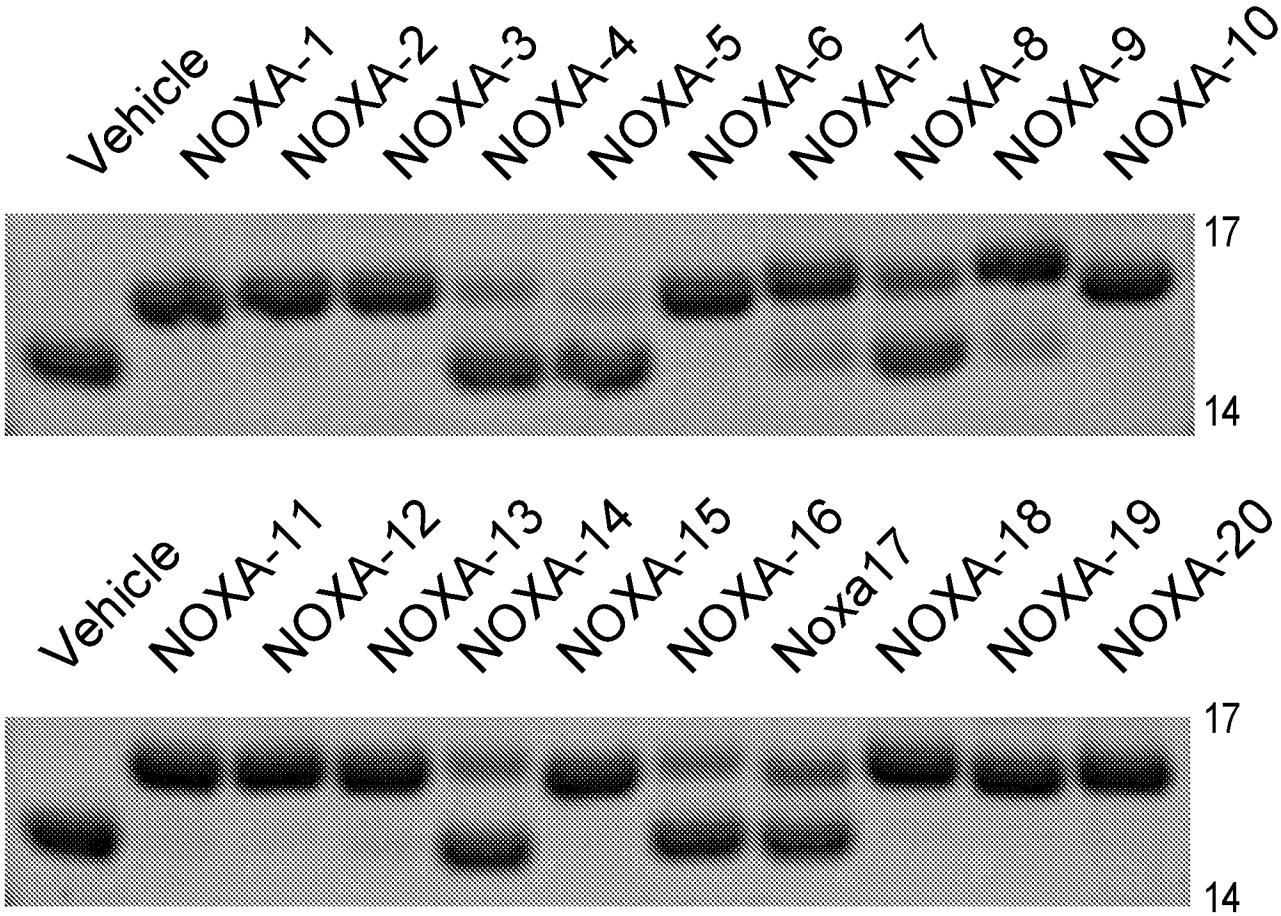

Vehicle
NOXA-1
NOXA-2
NOXA-3
NOXA-4
NOXA-5
NOXA-6
NOXA-7
NOXA-8
NOXA-9
NOXA-10
17
14
Vehicle
NOXA-11
NOXA-12
NOXA-13
NOXA-14
NOXA-15
NOXA-16
Noxa17
NOXA-18
NOXA-19
NOXA-20
17
14

FIG. 3

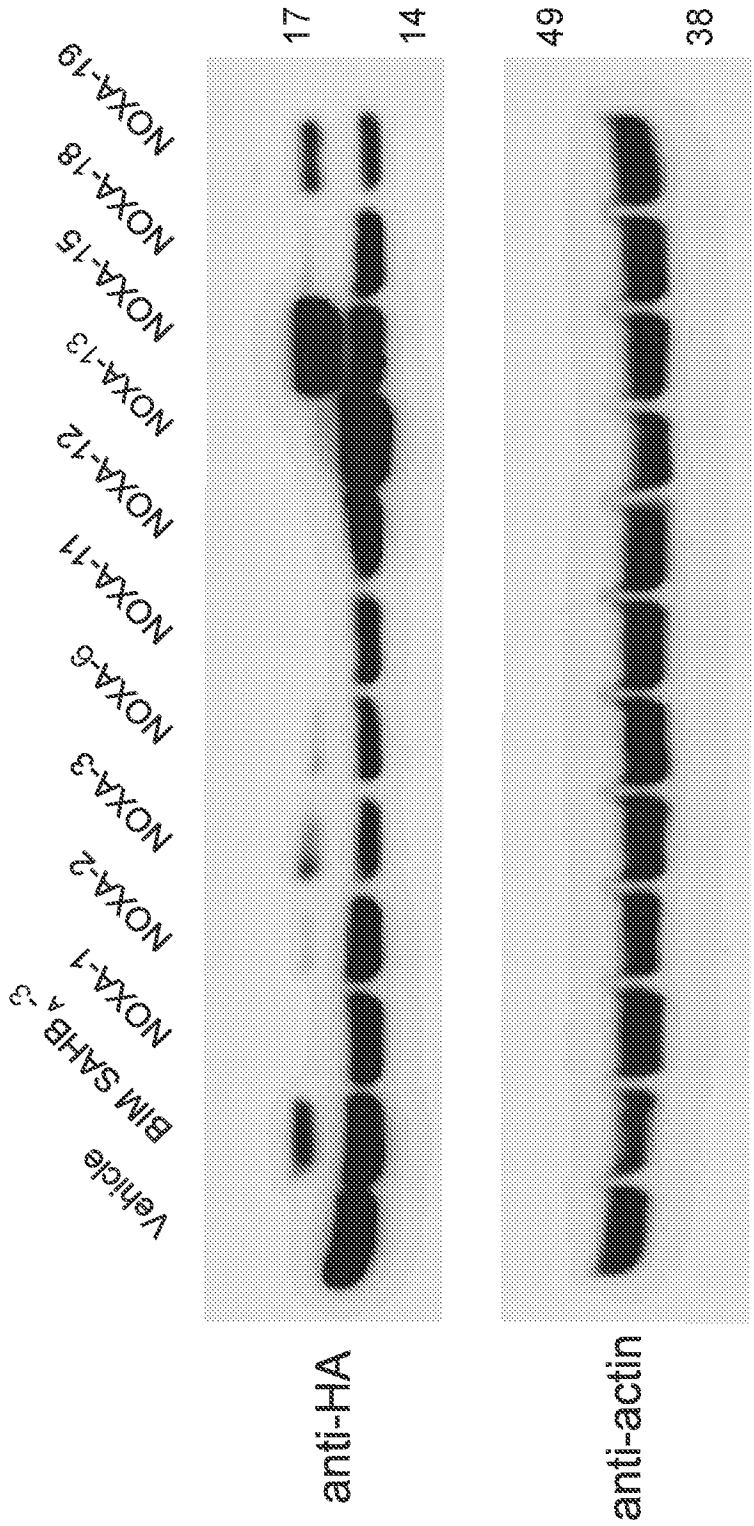

Vehicle
BIM SAHB A -3
NOXA-1
NOXA-2
NOXA-3
NOXA-6
NOXA-11
NOXA-12
NOXA-13
NOXA-15
NOXA-18
NOXA-19
anti-HA
17
14
anti-actin
49
38

FIG. 4

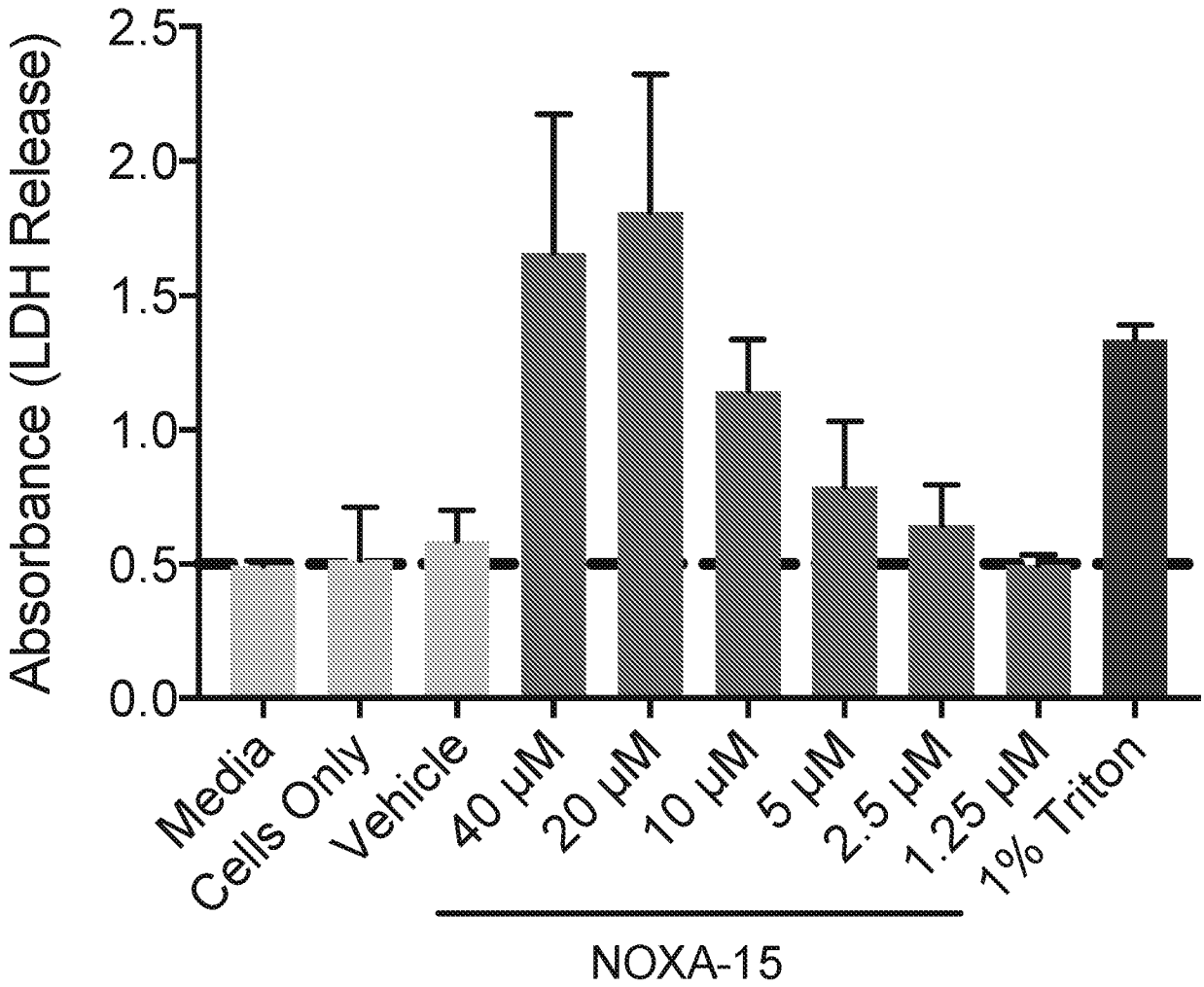

Absorbance (LDH Release)
2.5
2.0
1.5
1.0
0.5
0.0
Media
Cells Only
Vehicle
40 μM
20 μM
10 μM
5 μM
2.5 μM
1.25 μM
1% Triton
NOXA-15

FIG. 5

NOXA SAHB-15 A26E	ET8LRRFGDXLNFRQ
NOXA SAHB-15 T27A	AA8LRRFGDXLNFRQ
NOXA SAHB-15 L29A	AT8ARRFGDXLNFRQ
NOXA SAHB-15 R30A	AT8LARFGDXLNFRQ
NOXA SAHB-15 R31A	AT8LRAFGDXLNFRQ
NOXA SAHB-15 F32A	AT8LRRAGDXLNFRQ
NOXA SAHB-15 G33A	AT8LRRFADXLNFRQ
NOXA SAHB-15 D34A	AT8LRRFGAXLNFRQ
NOXA SAHB-15 L36A	AT8LRRFGDXANFRQ
NOXA SAHB-15 N37A	AT8LRRFGDXLAFRQ
NOXA SAHB-15 F38A	AT8LRRFGDXLNARQ
NOXA SAHB-15 R39A	AT8LRRFGDXLNFAQ
NOXA SAHB-15 Q40A	AT8LRRFGDXLNFRA

Note: D-nipecotic acid derivatization placed at the stapled peptide N-terminus

FIG. 6

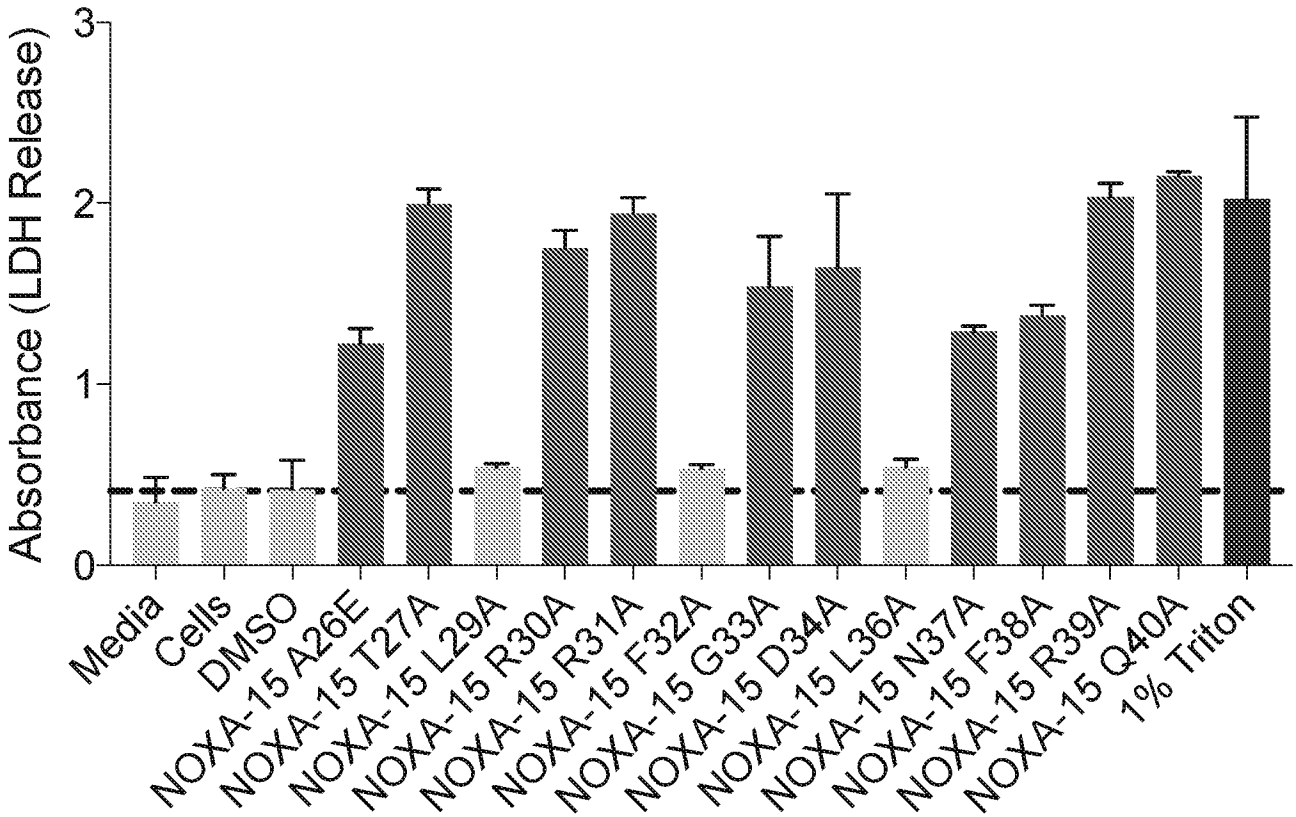

Absorbance (LDH Release)
3
2
1
0
Media
Cells
DMSO
NOXA-15 A26E
NOXA-15 T27A
NOXA-15 L29A
NOXA-15 R30A
NOXA-15 R31A
NOXA-15 F32A
NOXA-15 G33A
NOXA-15 D34A
NOXA-15 L36A
NOXA-15 N37A
NOXA-15 F38A
NOXA-15 R39A
NOXA-15 Q40A
1% Triton

FIG. 7

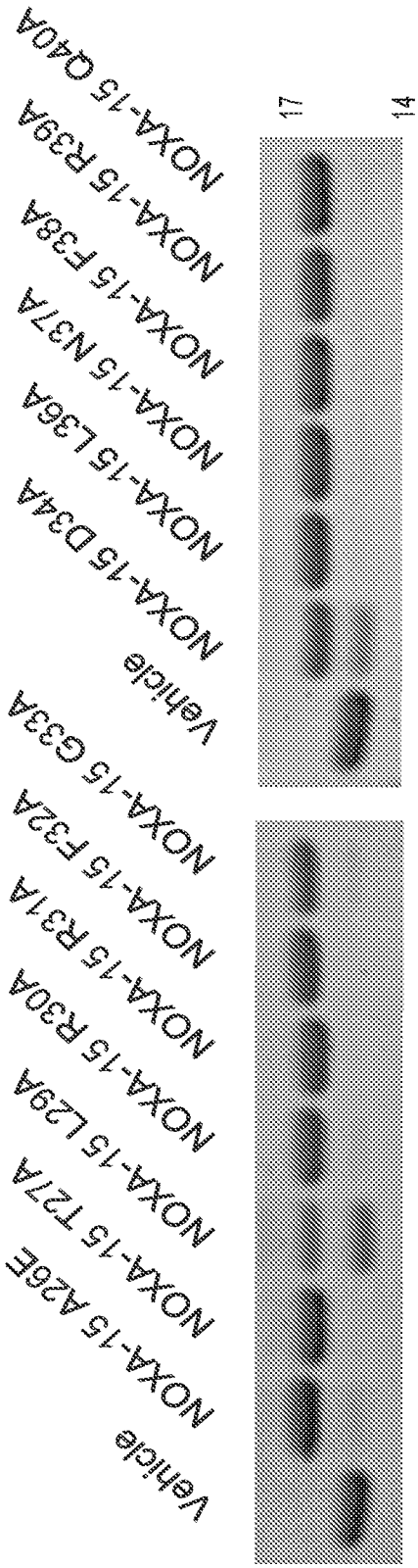

Vehicle
NOXA-15 A26E
NOXA-15 T27A
NOXA-15 L29A
NOXA-15 R30A
NOXA-15 R31A
NOXA-15 F32A
NOXA-15 G33A
Vehicle
NOXA-15 D34A
NOXA-15 L36A
NOXA-15 N37A
NOXA-15 F38A
NOXA-15 R39A
NOXA-15 Q40A
17
14

FIG. 8

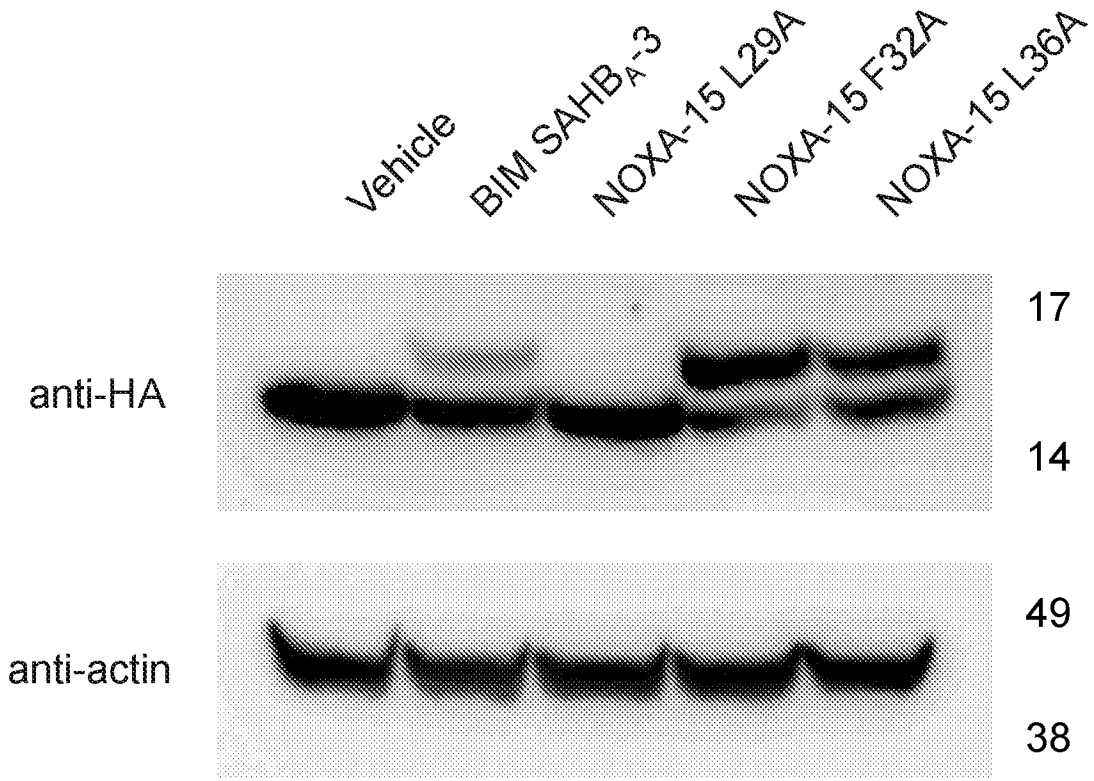

Vehicle
BIM SAHB$_A$-3
NOXA-15 L29A
NOXA-15 F32A
NOXA-15 L36A
anti-HA
17
14
anti-actin
49
38

FIG. 9

NOXA SAHB-15 R31E	AT8LREFGDXLNFRQ
NOXA SAHB-15 R39E	AT8LRRFGDXLNFEQ
NOXA SAHB-15 N37D	AT8LRRFGDXLDFRQ
NOXA SAHB-15 Q40L	AT8LRRFGDXLNFRL

Note: D-nipecotic acid derivatization placed at the stapled peptide N-terminus

FIG. 10

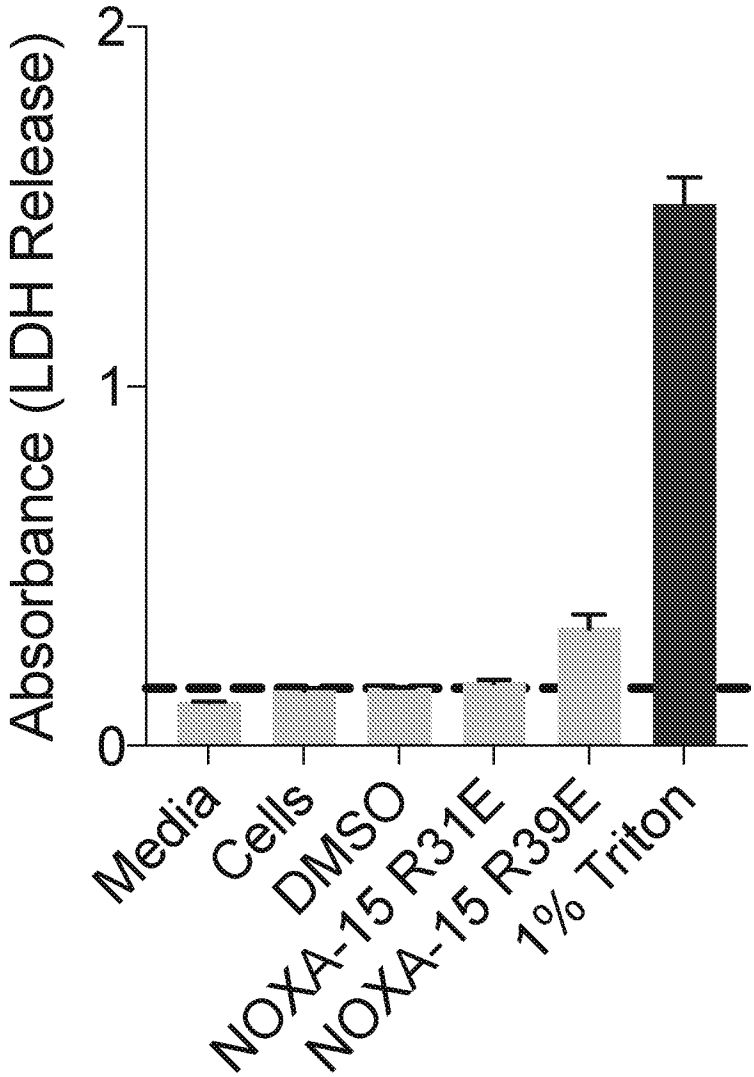

Absorbance (LDH Release)
2
1
0
Media
Cells
DMSO
NOXA-15 R31E
NOXA-15 R39E
1% Triton

FIG. 11

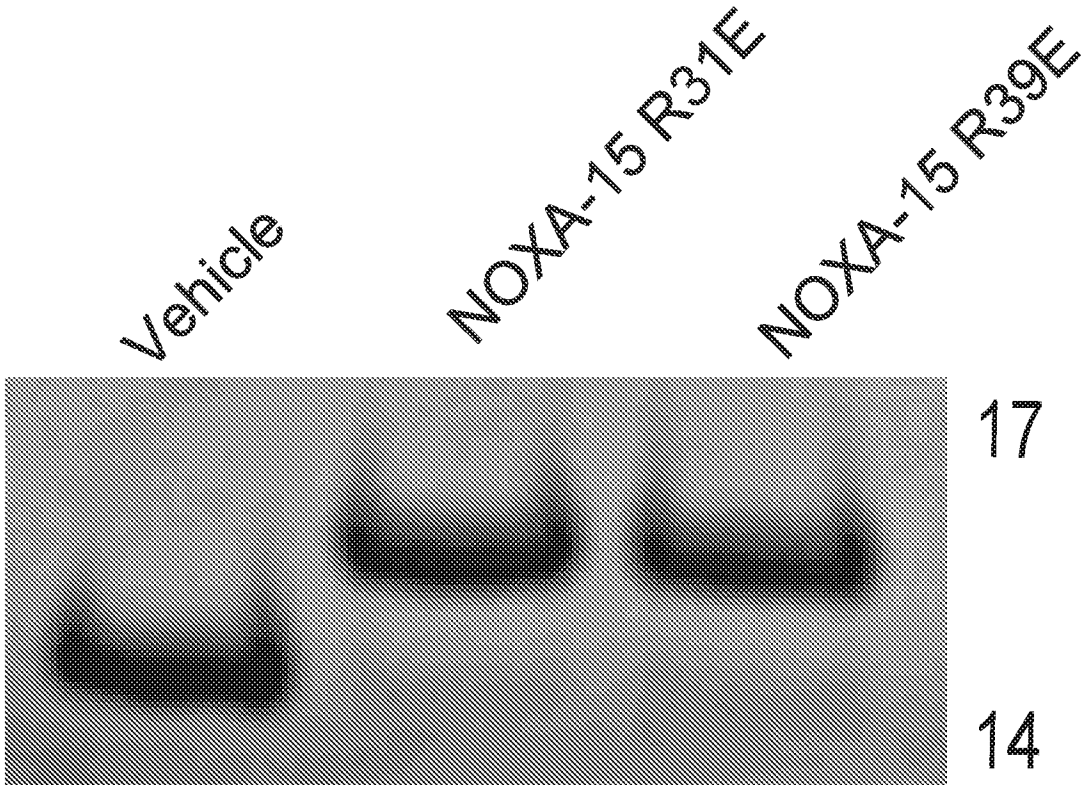


FIG. 12

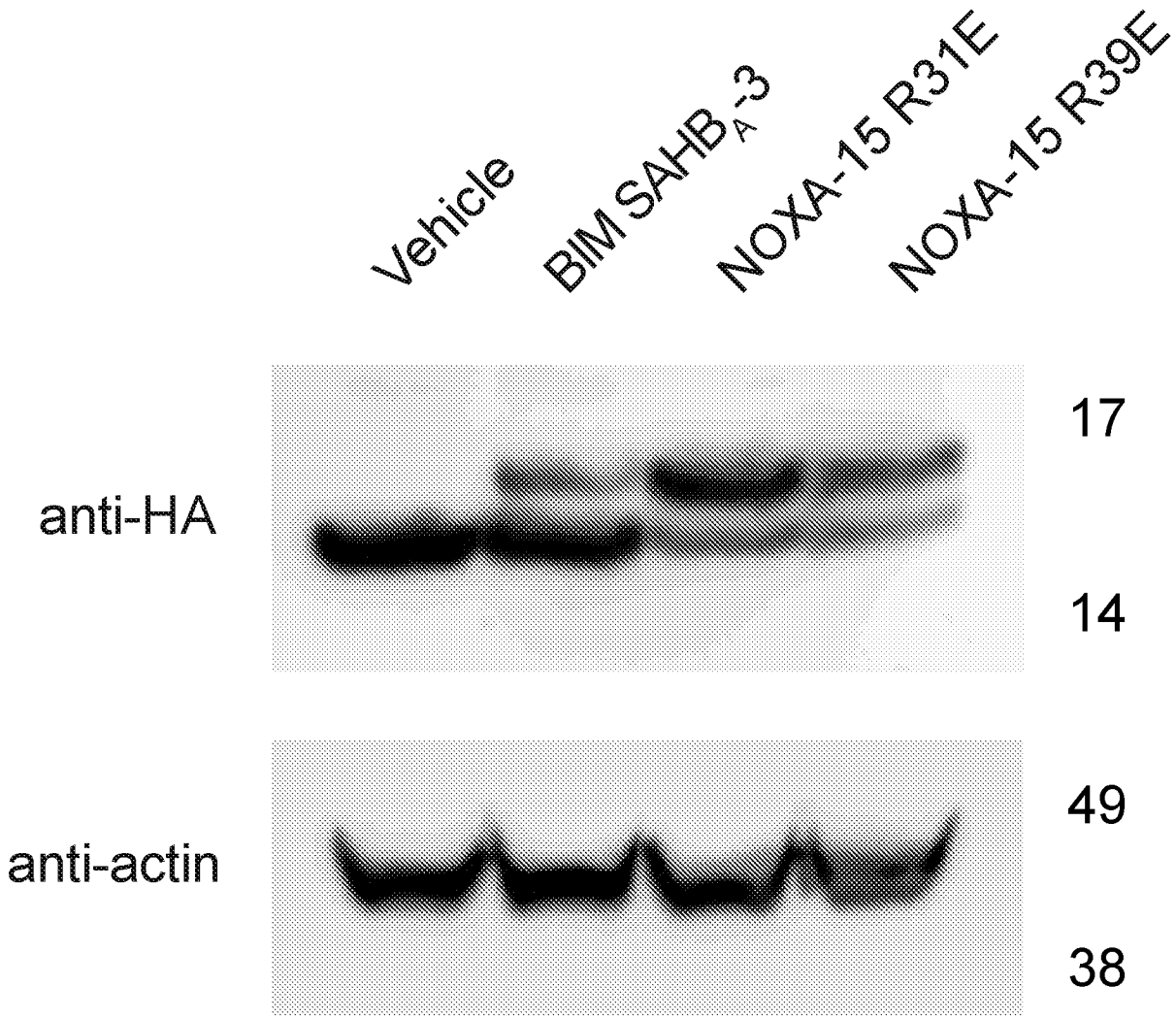

Vehicle
BIM SAHB$_A$-3
NOXA-15 R31E
NOXA-15 R39E
anti-HA
17
14
anti-actin
49
38

FIG. 13

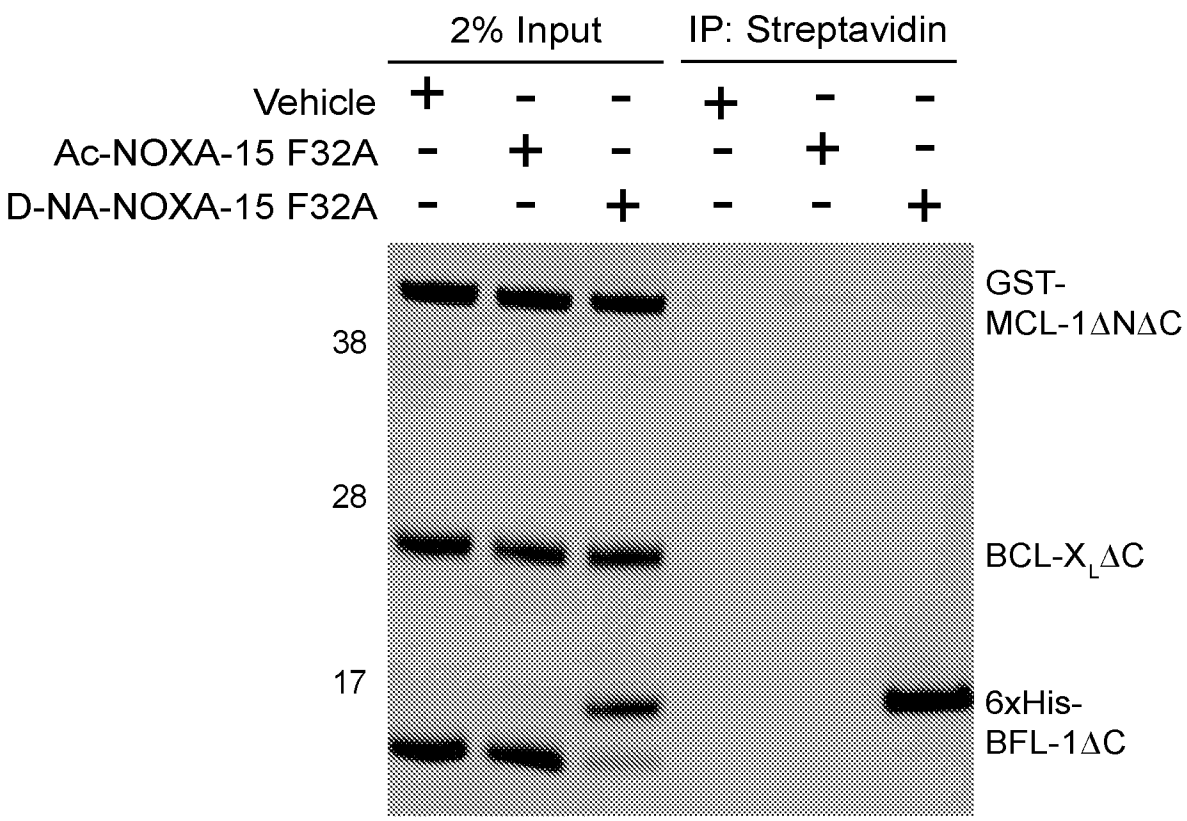

2% Input
IP: Streptavidin
Vehicle + - - + - -
Ac-NOXA-15 F32A - + - - + -
D-NA-NOXA-15 F32A - - + - - +
38
28
17
GST-
MCL-1ΔNΔC
BCL-XLΔC
6xHis-
BFL-1ΔC

FIG. 14

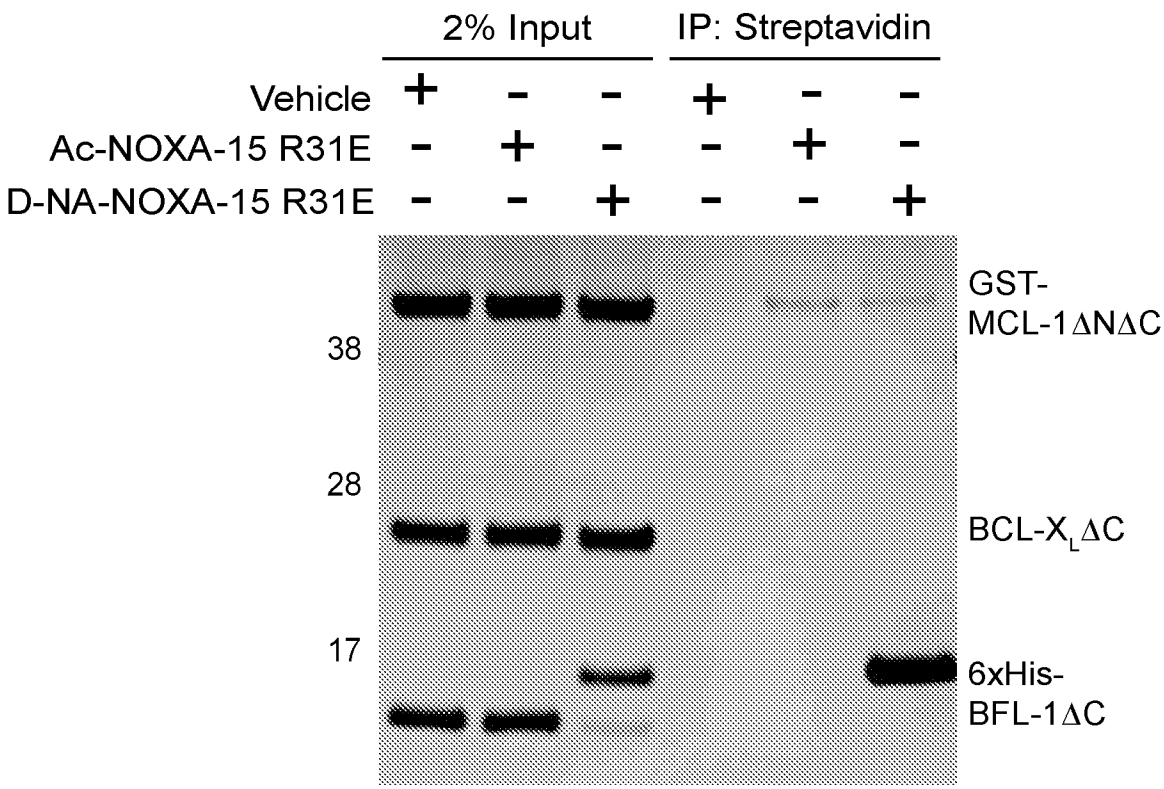

2% Input
IP: Streptavidin
Vehicle + - - + - -
Ac-NOXA-15 R31E - + - - + -
D-NA-NOXA-15 R31E - - + - - +
38
28
17
GST-
MCL-1ΔNΔC
BCL-XLΔC
6xHis-
BFL-1ΔC

FIG. 15

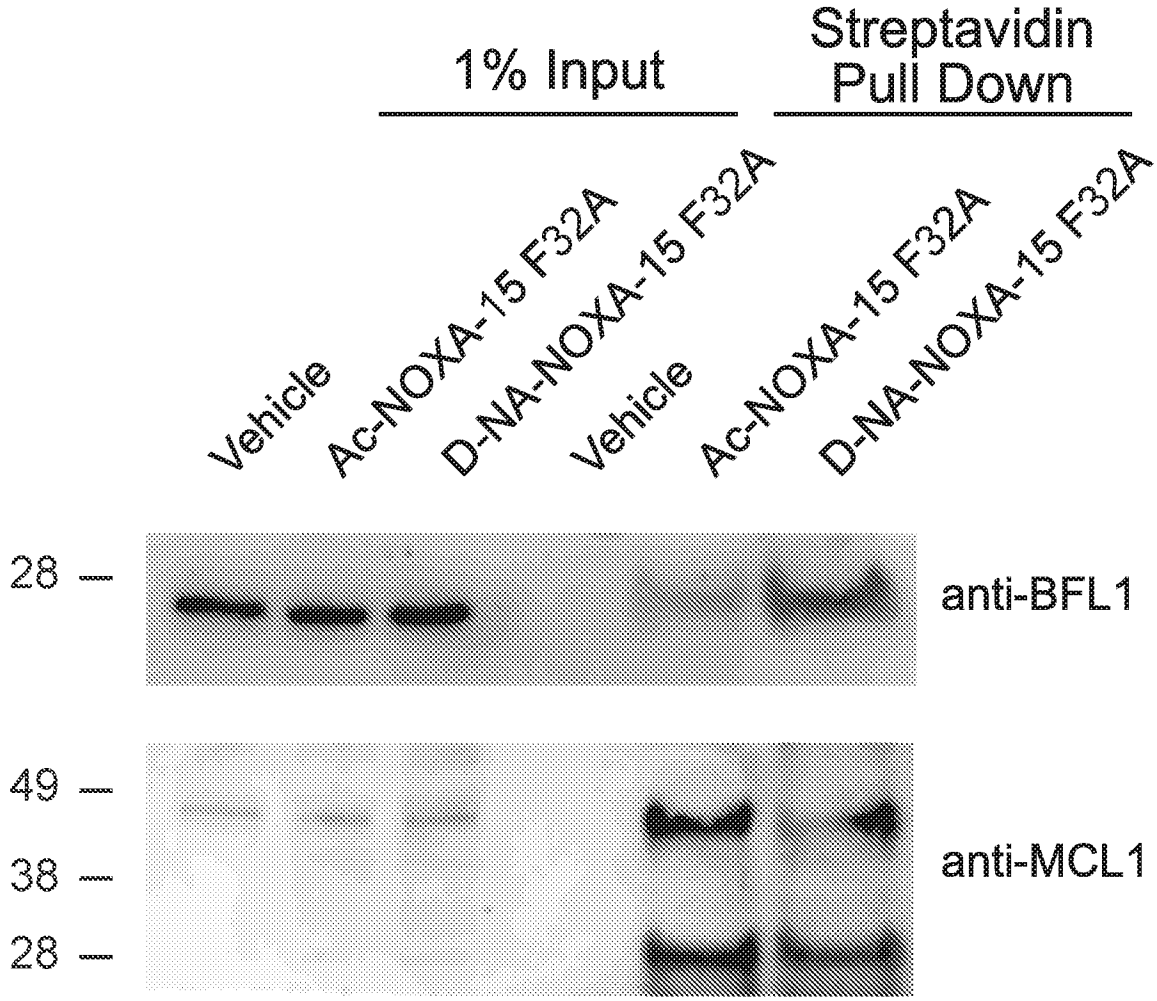

1% Input
Streptavidin Pull Down
Vehicle
Ac-NOXA-15 F32A
D-NA-NOXA-15 F32A
Vehicle
Ac-NOXA-15 F32A
D-NA-NOXA-15 F32A
28
anti-BFL1
49
38
28
anti-MCL1

FIG. 16

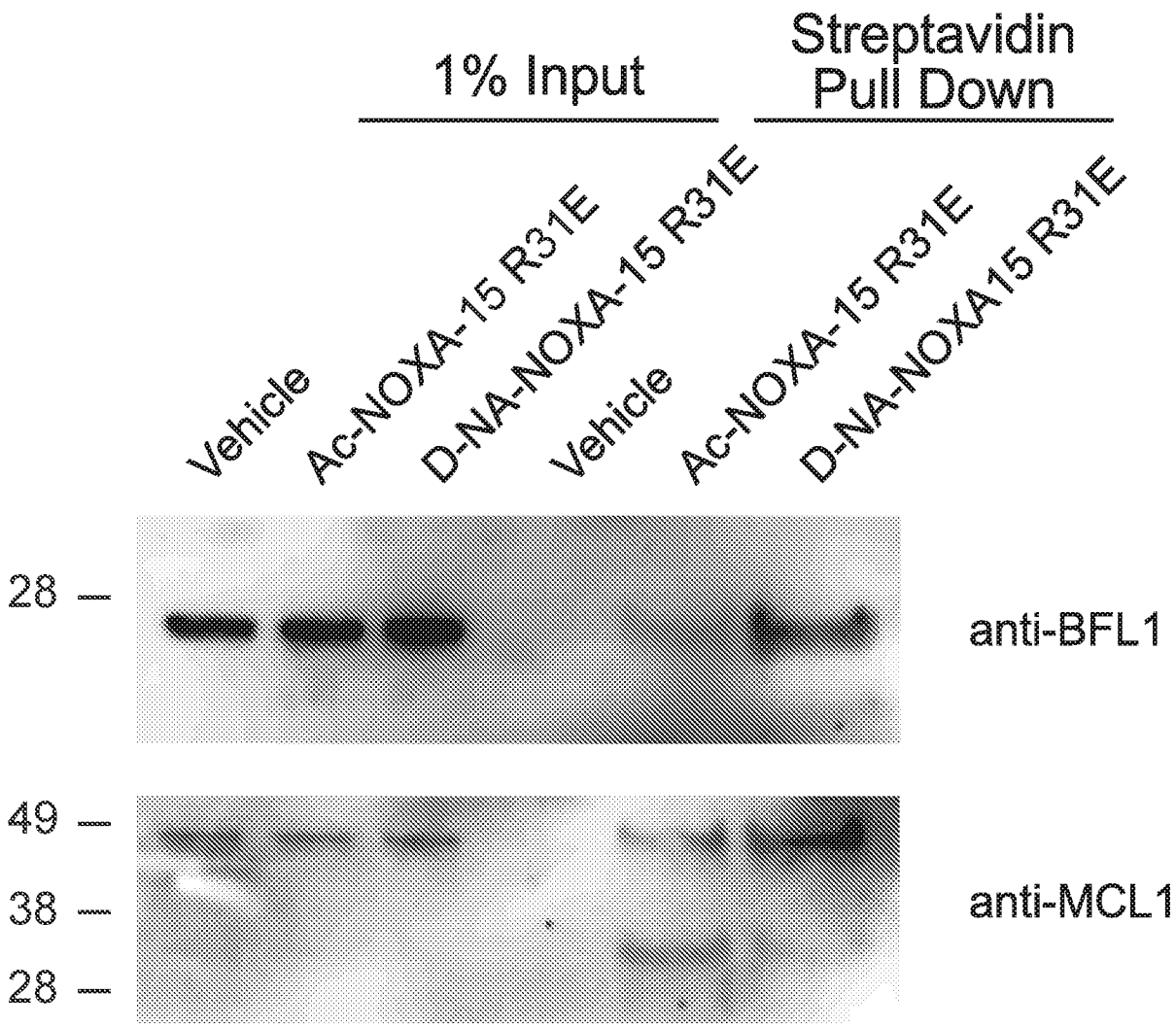

1% Input
Streptavidin Pull Down
Vehicle
Ac-NOXA-15 R31E
D-NA-NOXA-15 R31E
Vehicle
Ac-NOXA-15 R31E
D-NA-NOXA15 R31E
28
anti-BFL1
49
38
28
anti-MCL1

FIG. 17

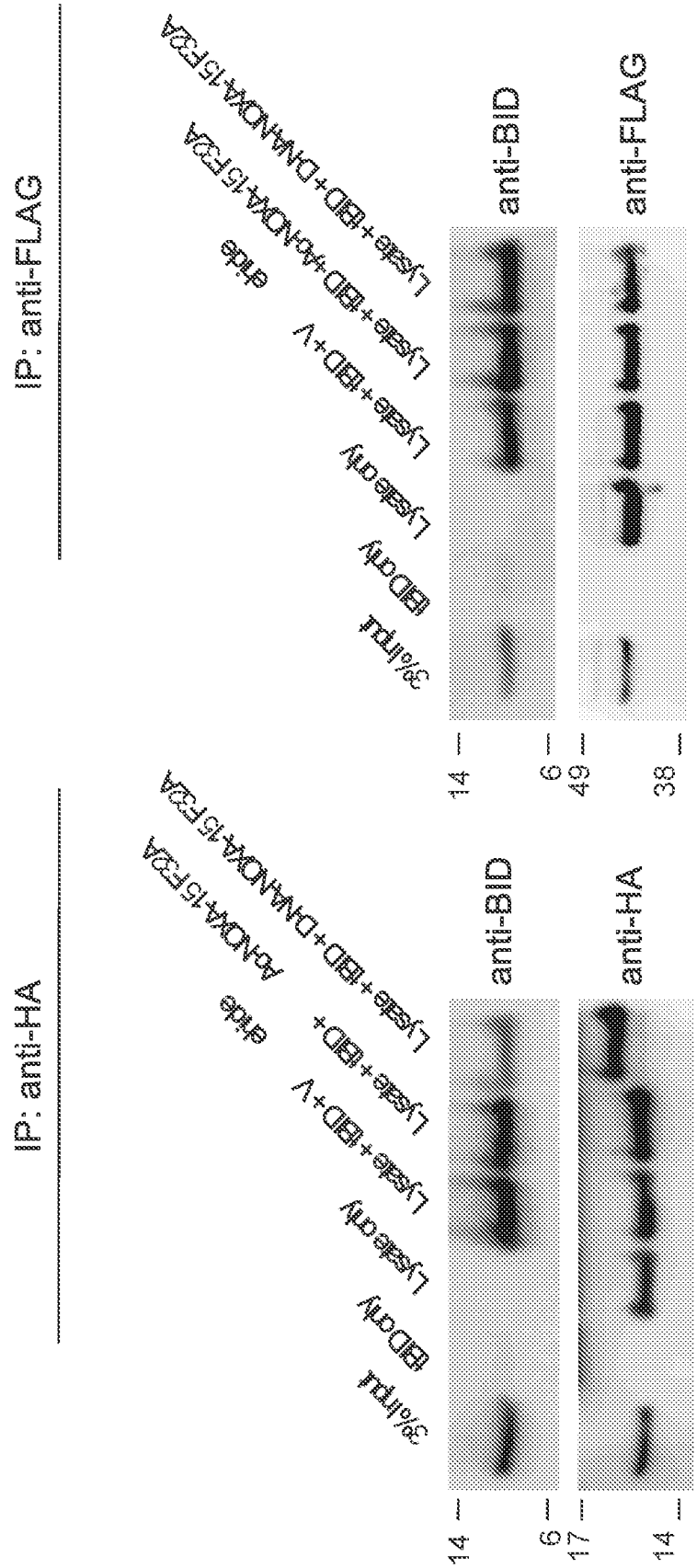

IP: anti-HA
3% input
tBID only
Lysate only
Lysate + tBID + V
Lysate + tBID + eride
Lysate + tBID + DNA-NOXA-15F32A
AcNOXA-15F32A
anti-BID
anti-HA
14
6
17
14
IP: anti-FLAG
3% input
tBID only
Lysate only
Lysate + tBID + V
Lysate + tBID + AcNOXA-15F32A
eride
Lysate + tBID + DNA-NOXA-15F32A
anti-BID
anti-FLAG
14
6
49
38

FIG. 18

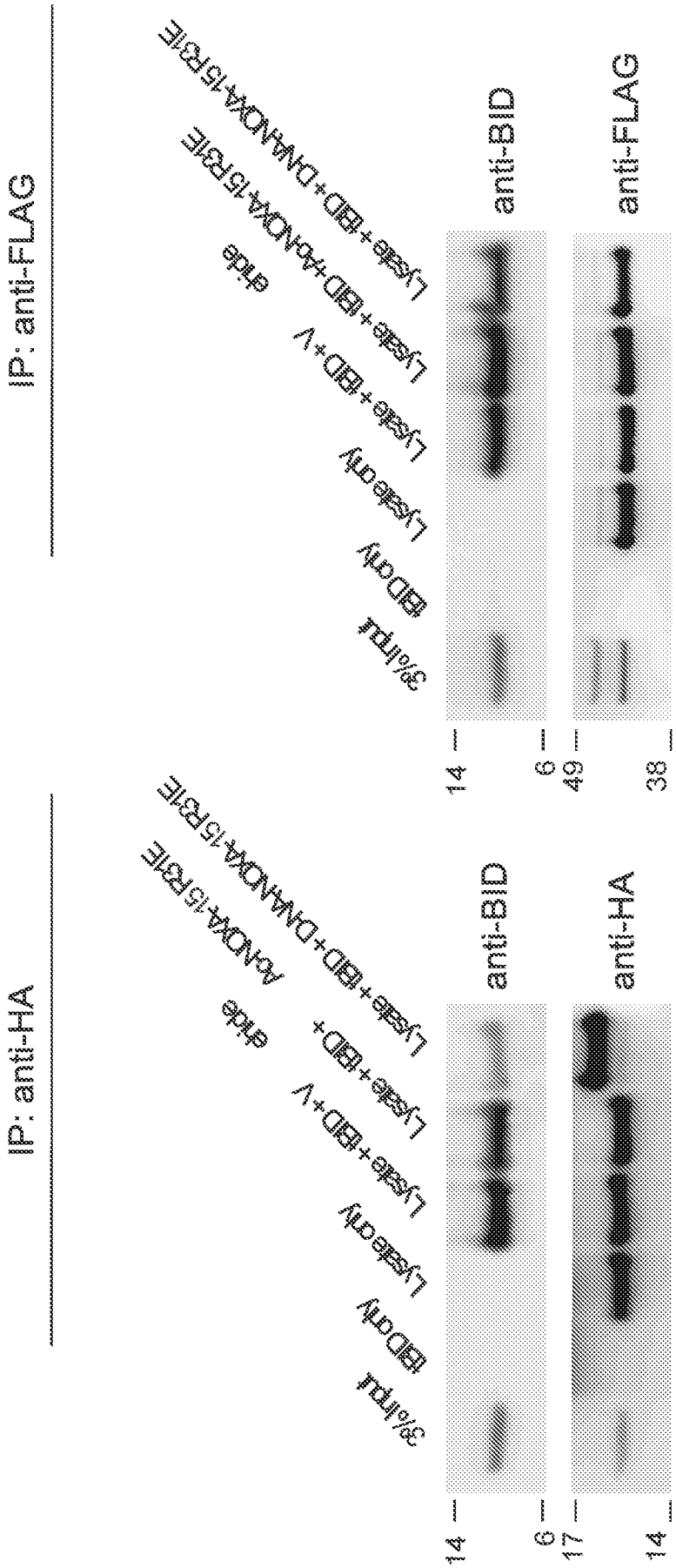

IP: anti-HA
3% Input
tBID only
Lysate only
Lysate + tBID + V
Lysate + tBID +
Lysate + tBID + DNANOXA-15R31E
AoNOXA-15R31E
ehide
anti-BID
anti-HA
14
6
17
14
IP: anti-FLAG
3% Input
tBID only
Lysate only
Lysate + tBID + V
Lysate + tBID + AoNOXA-15R31E
ehide
Lysate + tBID + DNANOXA-15R31E
anti-BID
anti-FLAG
14
6
49
38

FIG. 19

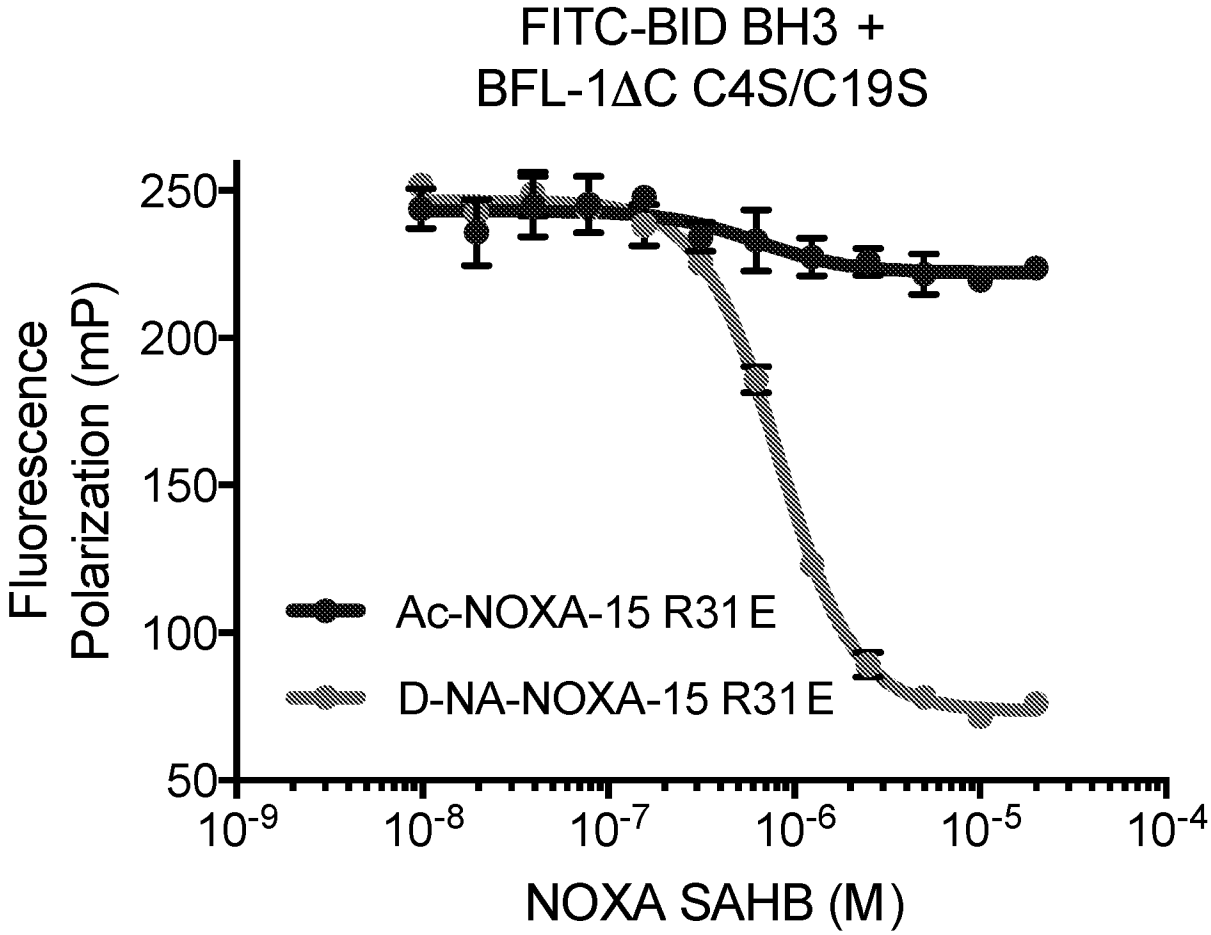

FITC-BID BH3 +
BFL-1ΔC C4S/C19S
Fluorescence Polarization (mP)
250
200
150
100
50
Ac-NOXA-15 R31E
D-NA-NOXA-15 R31E
10-9
10-8
10-7
10-6
10-5
10-4
NOXA SAHB (M)

FIG. 20

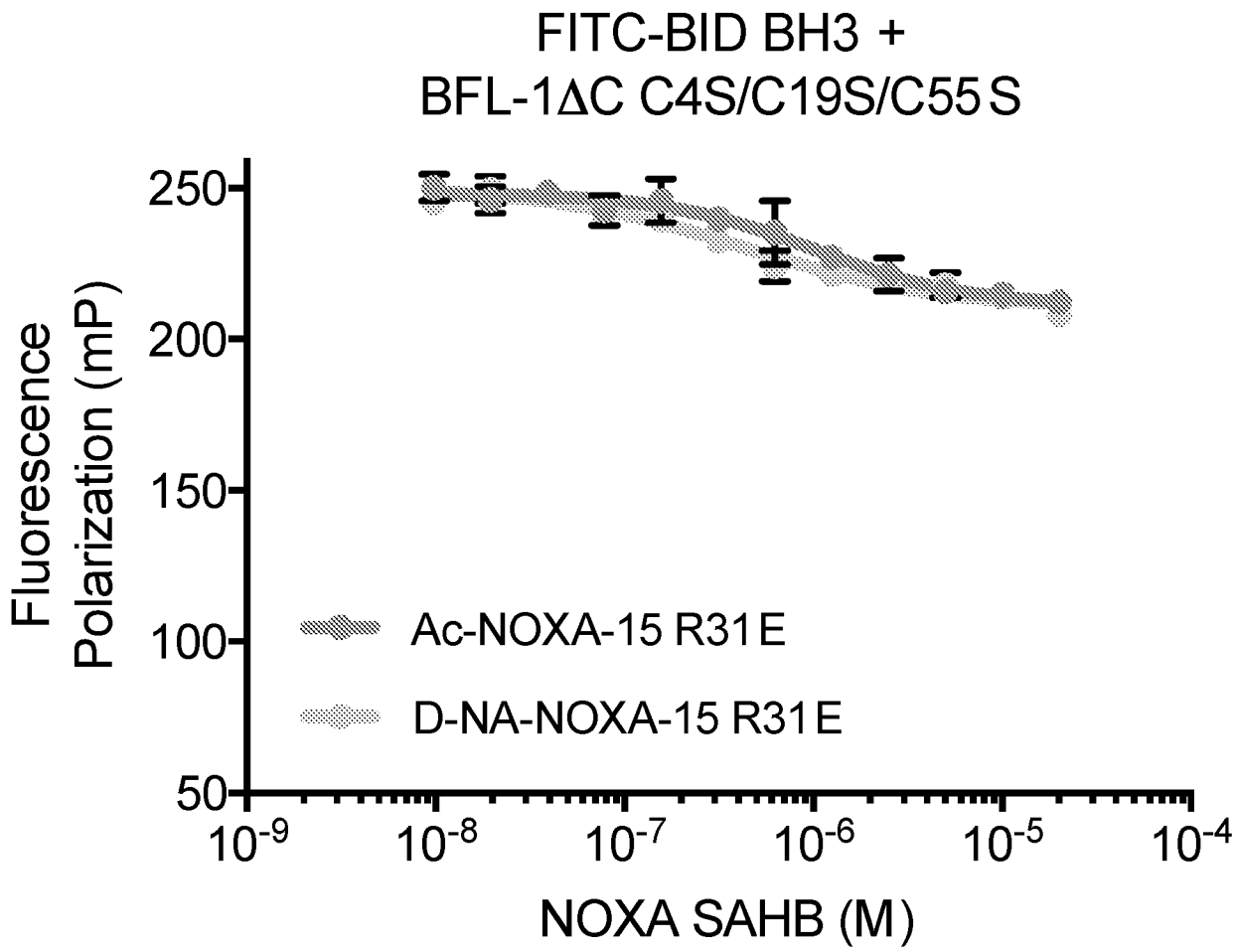

FITC-BID BH3 +
BFL-1ΔC C4S/C19S/C55S
Fluorescence Polarization (mP)
250
200
150
100
50
Ac-NOXA-15 R31E
D-NA-NOXA-15 R31E
10-9
10-8
10-7
10-6
10-5
10-4
NOXA SAHB (M)

FIG. 21

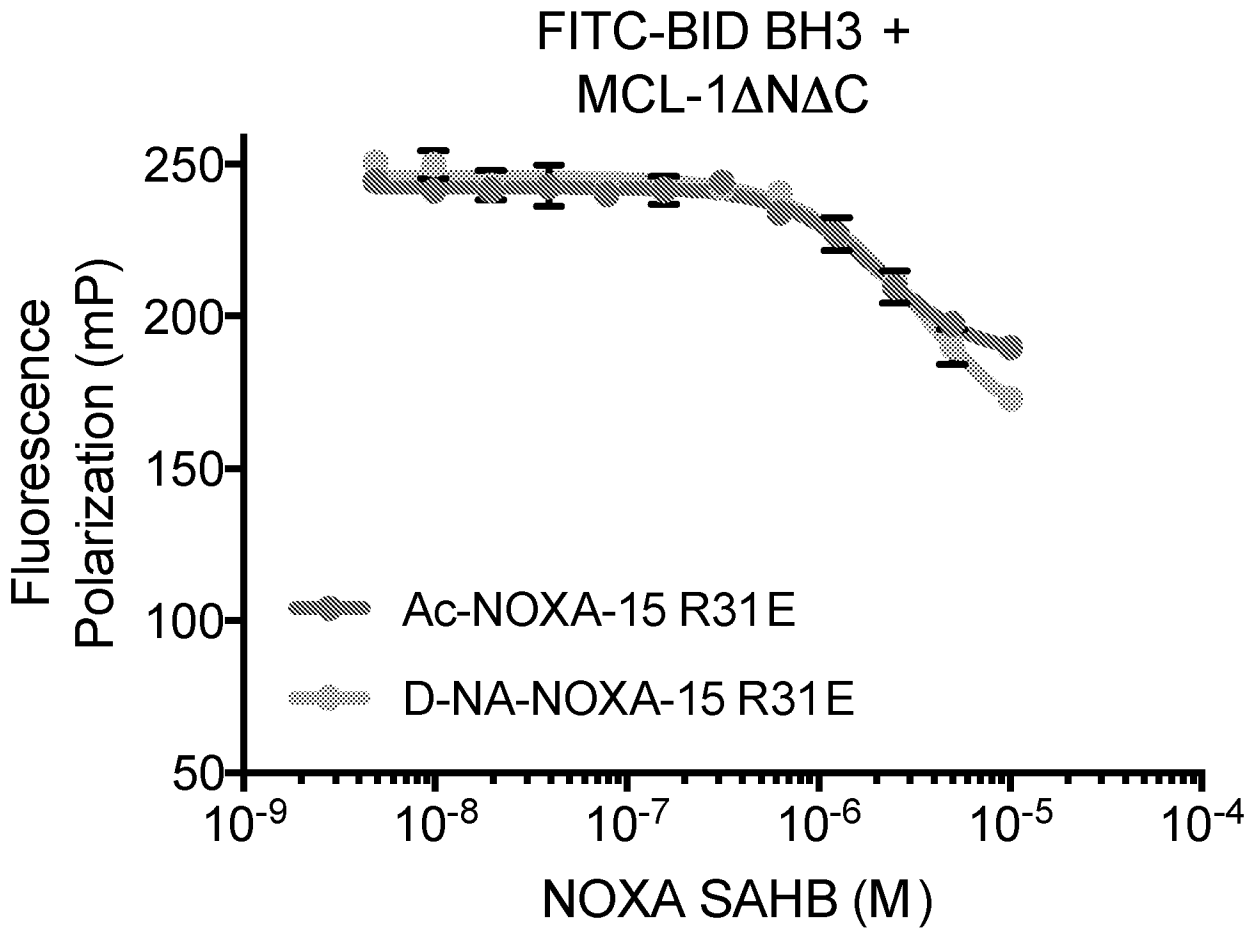

FITC-BID BH3 +
MCL-1ΔNΔC
Fluorescence
Polarization (mP)
250
200
150
100
50
Ac-NOXA-15 R31E
D-NA-NOXA-15 R31E
10-9
10-8
10-7
10-6
10-5
10-4
NOXA SAHB (M)

FIG. 22

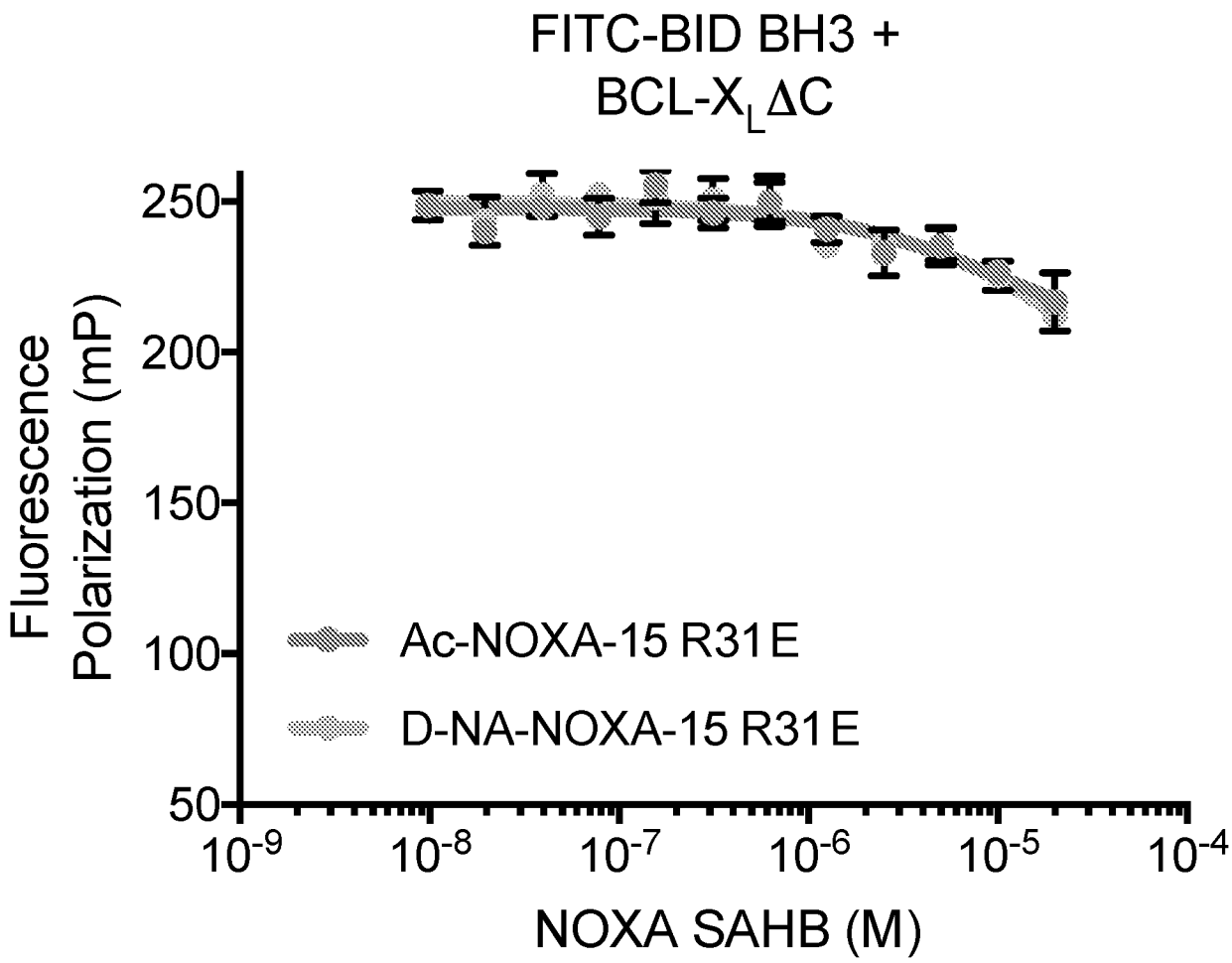

FITC-BID BH3 +
BCL-X$_L$ΔC
Fluorescence Polarization (mP)
250
200
150
100
50
Ac-NOXA-15 R31E
D-NA-NOXA-15 R31E
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
NOXA SAHB (M)

FIG. 23

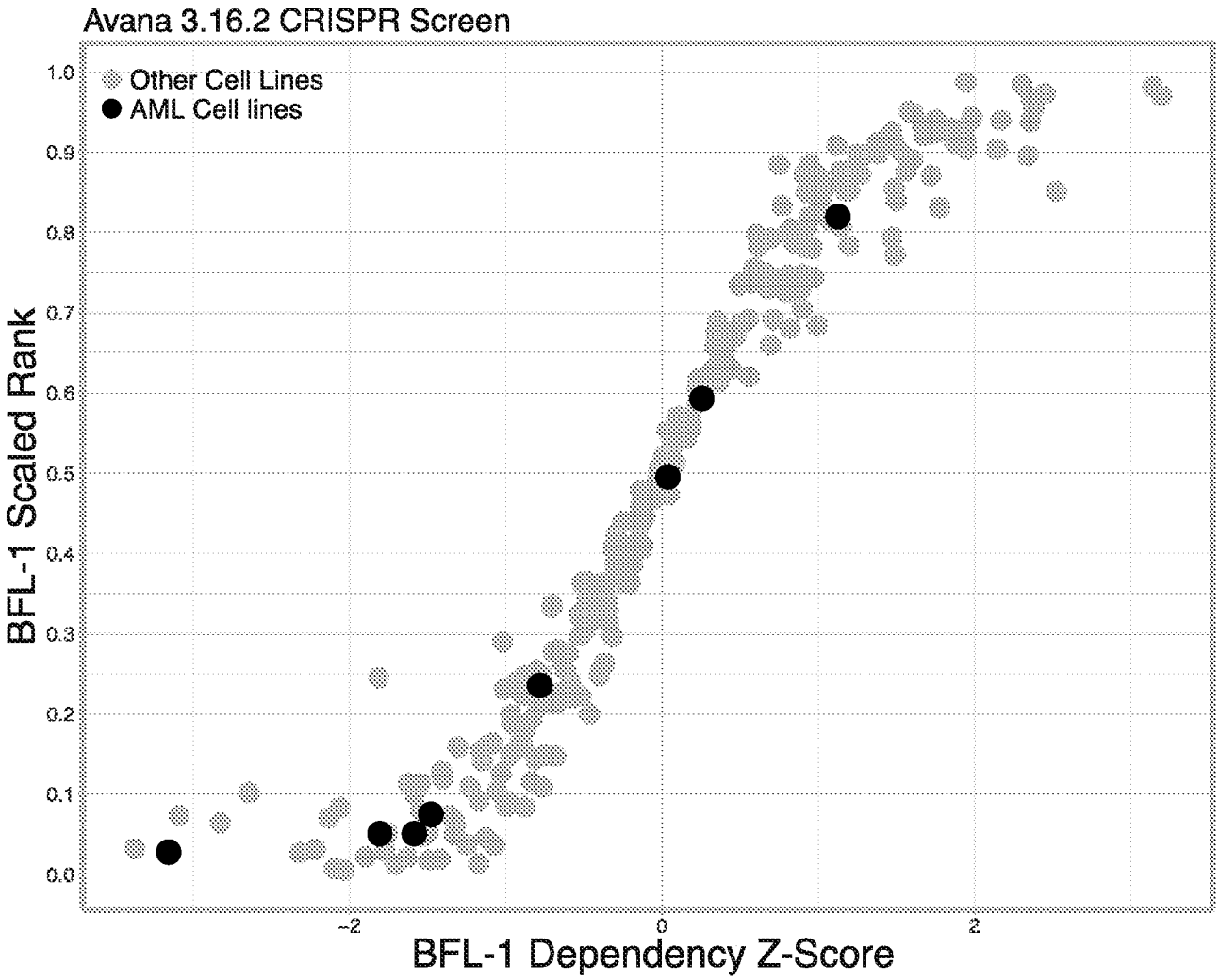

Avana 3.16.2 CRISPR Screen
Other Cell Lines
AML Cell lines
BFL-1 Scaled Rank
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-2
0
2
BFL-1 Dependency Z-Score

FIG. 24

	Pearson Correlation Combined CRISPR	Pearson Correlation CERES Avana	Spearman Correlation CERES Avana
BCL2A1	1	1	1
ATM	0.986	0.857	0.881
SLFNL1	0.983	0.694	0.762
RFTN2	0.98	0.51	0.667
EDC4	0.975	0.89	0.881
DCST2	0.963	-0.073	-0.071
SULT1C4	0.95	0.868	0.857
TR1QK	0.946	0.749	0.905
TAS2R50	0.945	0.887	0.857
IQCA1	0.943	0.818	0.905

FIG. 25

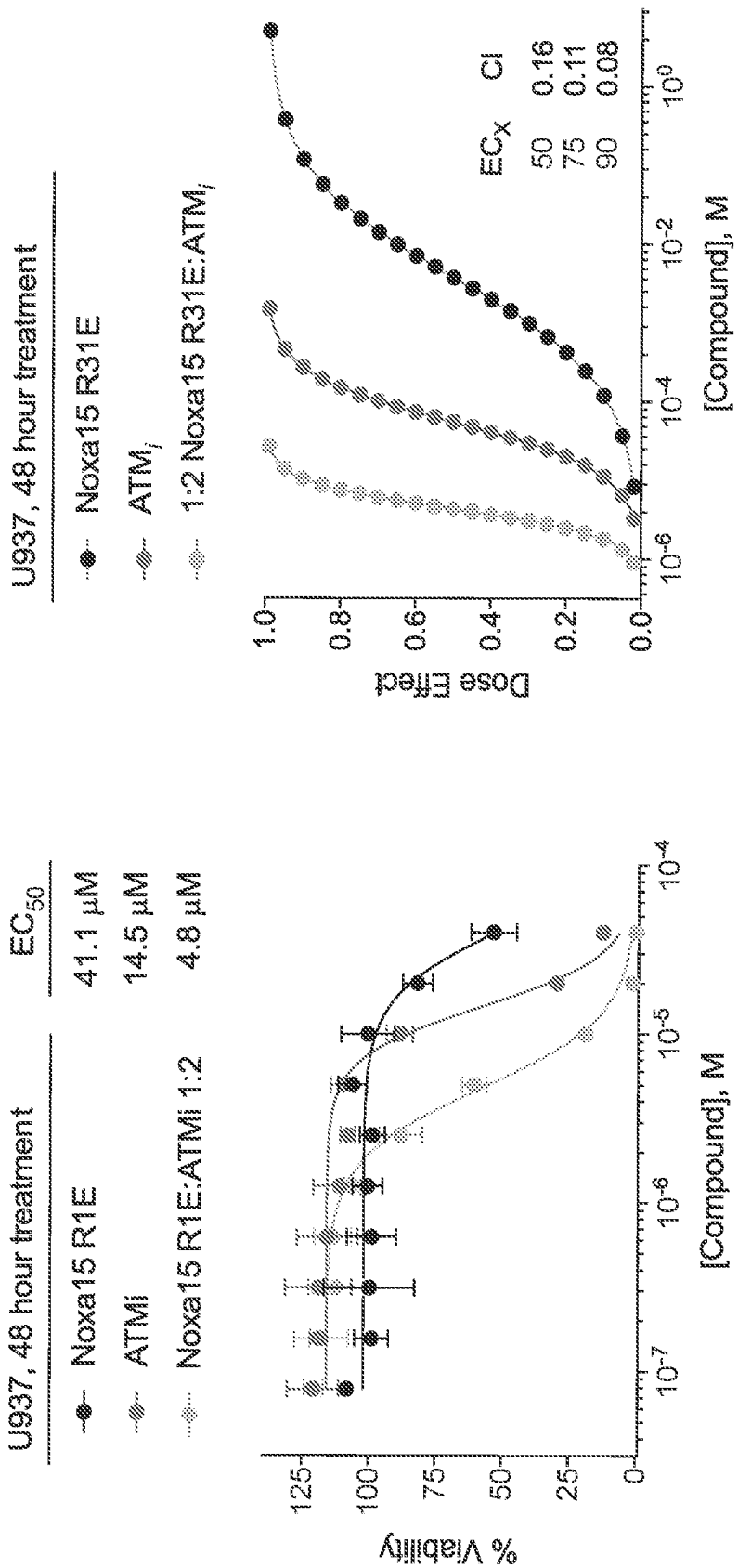

U937, 48 hour treatment
Noxa15 R1E
ATMi
Noxa15 R1E:ATMi 1:2
EC50
41.1 µM
14.5 µM
4.8 µM
% Viability
125
100
75
50
25
0
10-7
10-6
10-5
10-4
[Compound], M
U937, 48 hour treatment
Noxa15 R31E
ATMi
1:2 Noxa15 R31E:ATMi
Dose Effect
1.0
0.8
0.6
0.4
0.2
0.0
10-6
10-4
10-2
100
[Compound], M
ECx CI
50 0.16
75 0.11
90 0.08

FIG. 26

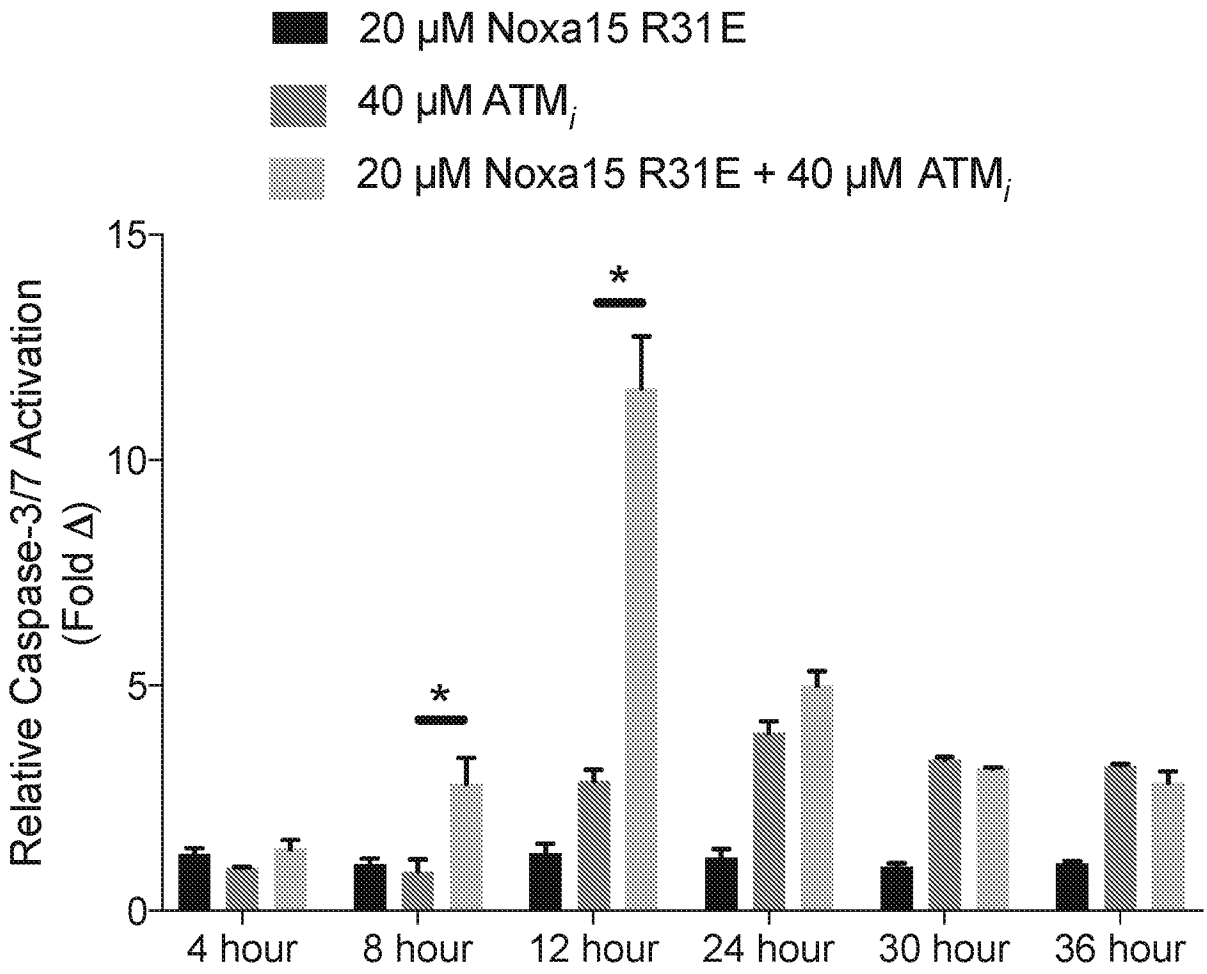
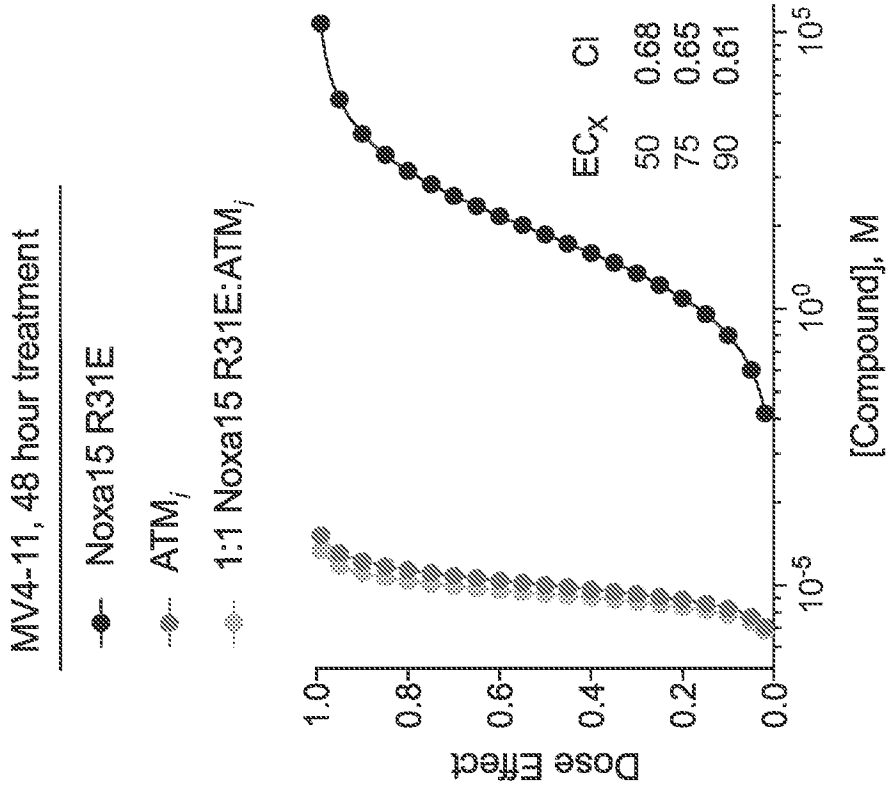

20 μM Noxa15 R31E
40 μM ATM$_i$
20 μM Noxa15 R31E + 40 μM ATM$_i$
Relative Caspase-3/7 Activation (Fold Δ)
15
10
5
0
*
*
4 hour
8 hour
12 hour
24 hour
30 hour
36 hour

FIG. 27


MV4-11, 48 hour treatment
Noxa15 R31E
ATMi
1:1 Noxa15 R31E:ATMi
Dose Effect
1.0
0.8
0.6
0.4
0.2
0.0
10^{-5}
10^{0}
10^{5}
[Compound], M
EC_x CI
50 0.68
75 0.65
90 0.61

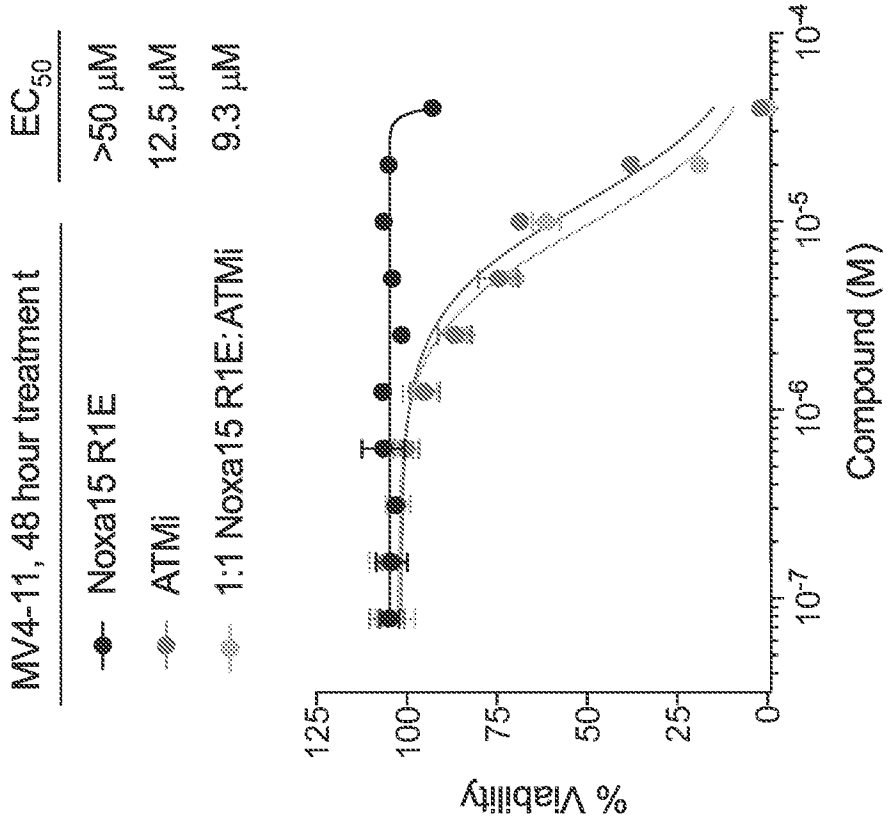

MV4-11, 48 hour treatment
EC_{50}
Noxa15 R1E >50 μM
ATMi 12.5 μM
1:1 Noxa15 R1E:ATMi 9.3 μM
% Viability
125
100
75
50
25
0
10^{-7}
10^{-6}
10^{-5}
10^{-4}
Compound (M)

FIG. 28

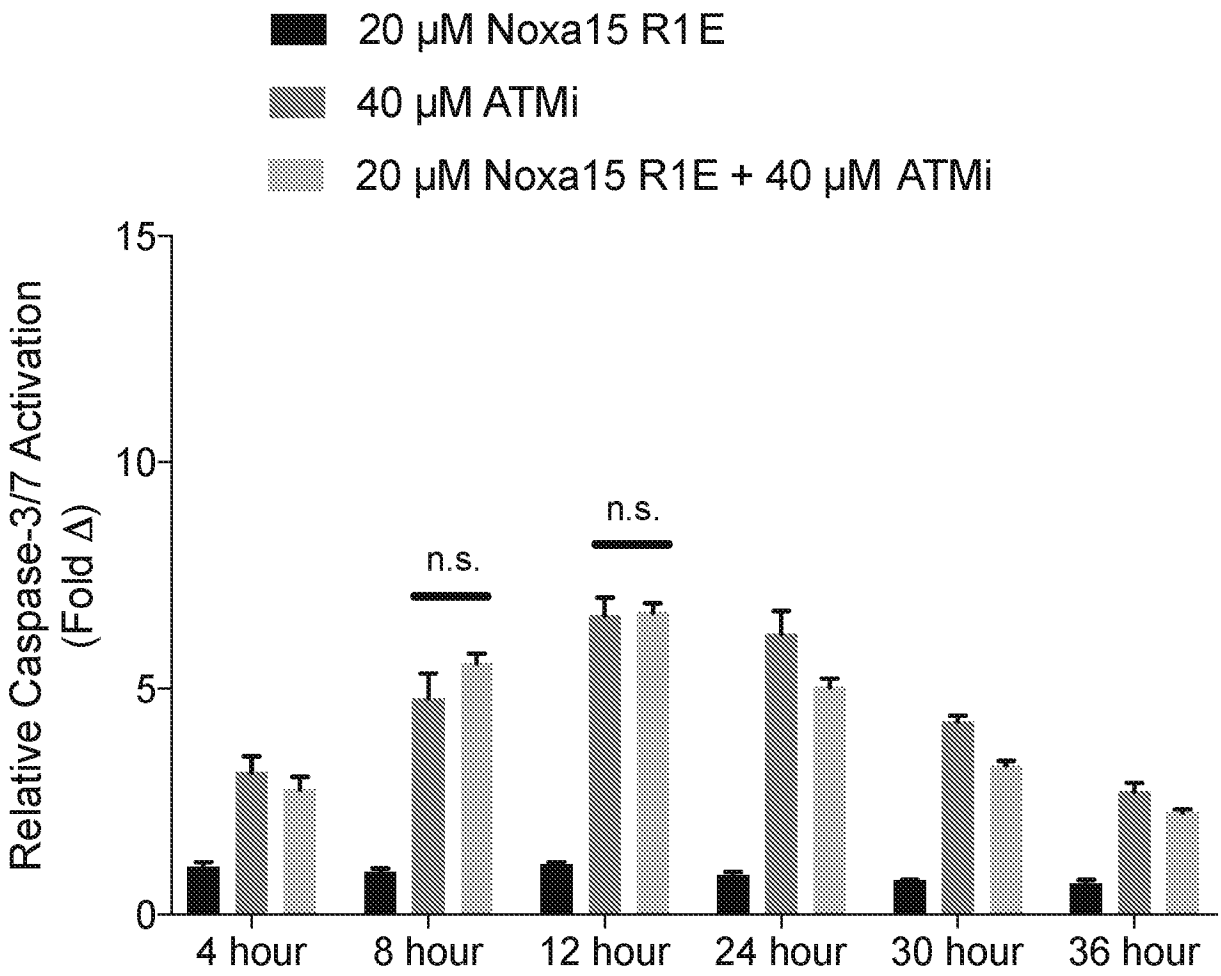

20 μM Noxa15 R1E
40 μM ATMi
20 μM Noxa15 R1E + 40 μM ATMi
Relative Caspase-3/7 Activation (Fold Δ)
15
10
5
0
n.s.
n.s.
4 hour
8 hour
12 hour
24 hour
30 hour
36 hour

FIG. 29

Table 1 Crystallization conditions and data collection and refinement statistics for crystal structures

Structure Name	BFL-1 apo	BFL-1/Peptide
Ligand	-	Peptide
RCSB accession code	5WHI	5WHH
Data collection [a]		
Space group	$P2_1$	$P\ 3_2 21$
Cell dimensions		
a, *b*, *c* (Å)	39.51 43.09 43.44	86.71 86.71 40.36
a, b, g (°)	90 104.15 90	90 90 120
Resolution (Å)	28.63 - 1.69 (1.75 - 1.69) [b]	43.35 - 2.38 (2.46 - 2.38)
R_{pim}	0.027 (0.677)	0.056 (0.683)
I / sI	13.56 (1.14)	11.23 (1.09)
Completeness (%)	97.98 (98.12)	99.03 (92.60)
Redundancy	3.3 (3.3)	9.8 (6.1)
Structure solution		
PDB entries used for molecular replacement	3I1H	3I1H
Refinement		
Resolution (Å)	28.63 - 1.69	43.35 - 2.38
No. reflections, unique	15702	7157
R_{work} / R_{free}	0.1955/0.2340	0.2102/0.2882
No. atoms	1245	1326
Protein	1186	1272
Ligand/ion	5	1
Water	54	53
B-factors		
Protein	47.3	45.4
Ligand/ion	157.5	32.0
Water	50.4	44.8
R.m.s. deviations		
Bond lengths (Å)	0.008	0.007
Bond angles (°)	0.86	0.96
Ramachandran Plot		
Preferred	97.9%	92.7%
Allowed	1.4%	7.3%
Not Allowed	0.7%	0.0%

[a] A single crystal was used to collect data for each of the structures reported here.
[b] Values in parentheses are for highest-resolution shell.

FIG. 30

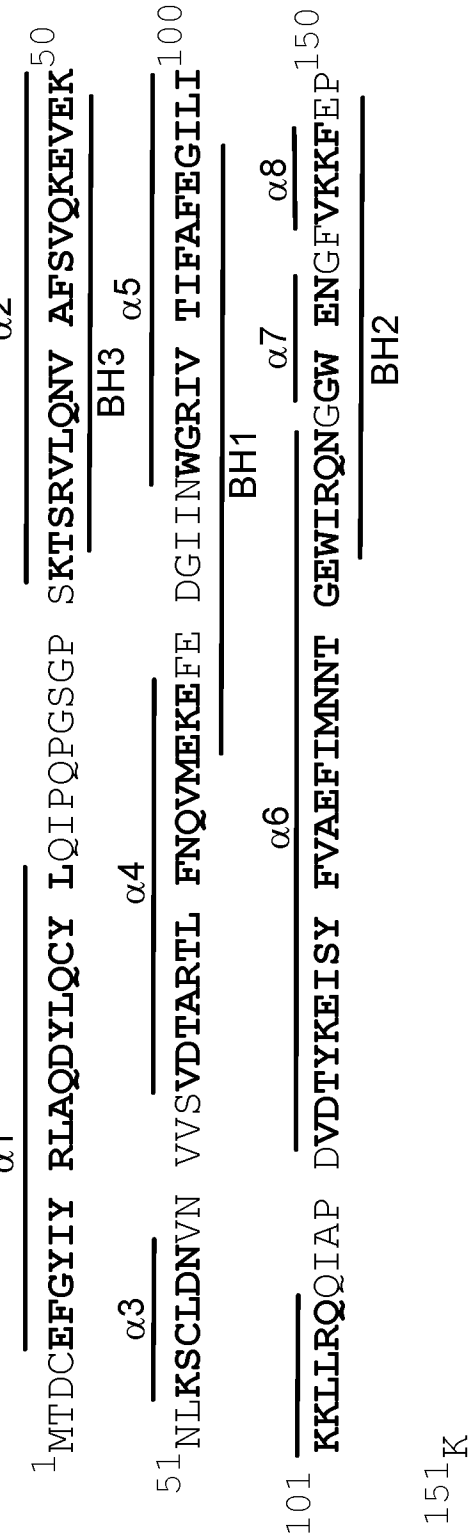

α1
α2
α3
α4
α5
α6
α7
α8
BH1
BH2
BH3
1 MTDCEFGYIY RLAQDYLQCY LQIPQPGSGP SKTSRVLQNV AFSVQKEVEK 50
51 NLKSCLDNVN VVSVDTARTL FNQVMEKEFE DGIINWGRIV TIFAFEGILI 100
101 KKLLRQQIAP DVDTYKEISY FVAEFIMNNT GEWIRQNGGW ENGFVKKFEP 150
151 K

FIG. 31A

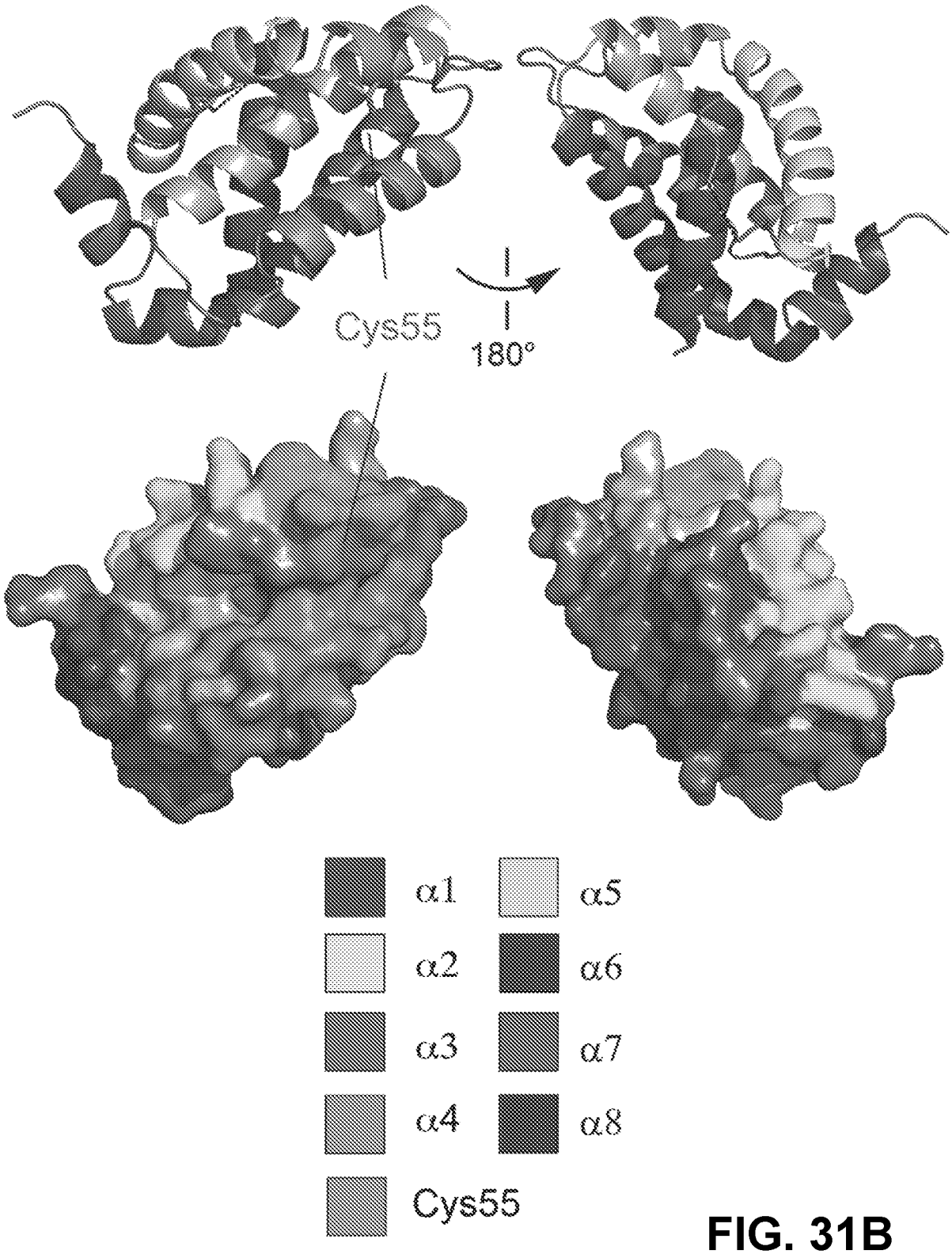


FIG. 31B

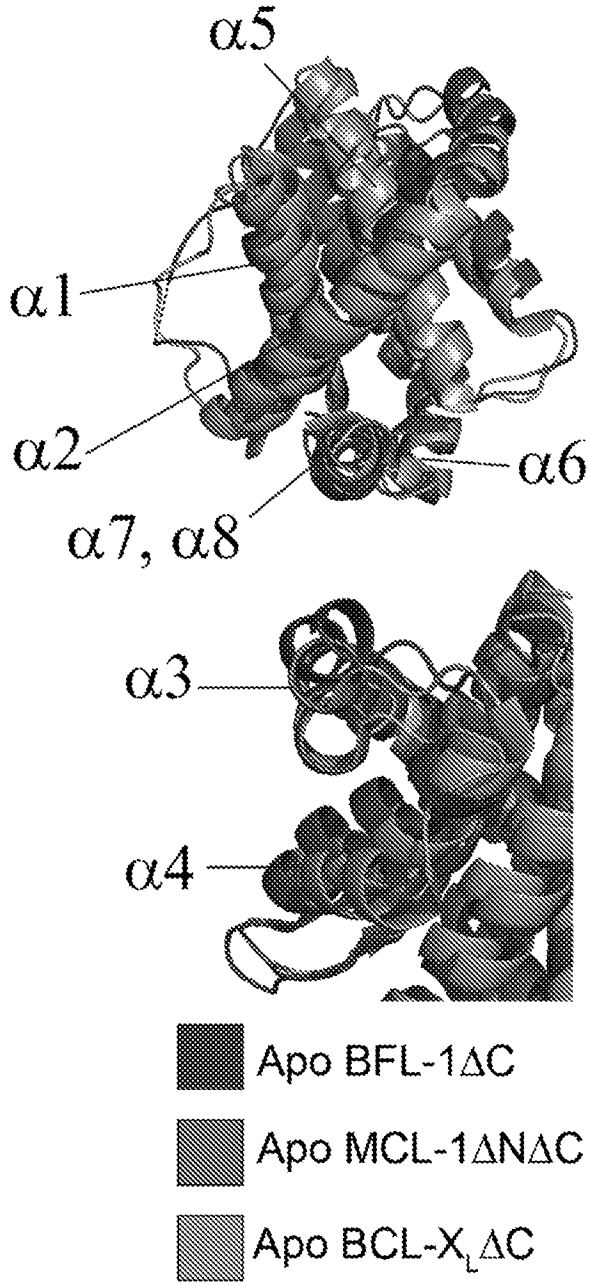


FIG. 31C

Amino Acids	Apo Distance (Å)
BFL-1 Cys 55 to Leu 70	10.2
MCL-1 Met 231 to Leu 246	10.9
BCL-X_L Leu 108 to Ser 122	10.6
BFL-1 Cys 55 to Val 74	11.3
MCL-1 Met 231 to Val 249	10.7
BCL-X_L Leu 108 to Val 126	6.5
BFL-1 Val 59 to Leu 70	8.3
MCL-1 Leu 235 to Leu 246	8.0
BCL-X_L Leu 112 to Ser 122	7.0

FIG. 31D

```
                 α2          α3          α4                α5
BFL-1     KTSRVLQNVAFSVQKEVEVEKNLKSCLDNVNVVSVDTARTLFNQVMEKEFED--GIINWGRIVTIFAFEGILIKKLLRQ
MCL-1     TSRKALETLRRVGDGVQRNHETAFQGMLRK-LDIKNEDDVKLSRVMIHVFSD--GVTNWGRIVTLISFGAFVAKHLKT
BCL-2       VVHKTLRQAGDDFSRRYRRDFAEMSSQ-LHLTPFTARGRFATVVEELFRD—GV-NWGRIVAFFEFGGVMCVESVN
BCL-XL    MAAVKQALREAGDEFELRYRRAFSDLTSQ-LHITPGTAYQSFEQVVNELFRD—GV NWGRIVAFFSFGGALCVESVD
BCL-W       PLHQAMRAAGDEFETRFRRTFSDLAAQ-LLHVTPGSAQQRFTQVSDELQG—GP-NWGRLVAFFVFGAALCAESVN
BCL-B       PEAAVLRSAAARLRQIHRSFFSAYLG-----YPGNRFELVALMADSVLSDSPGP-TWGRVVTLTFAGTL
BAX     ASTKKLLSECLKRIGDELDSNME—LQRMIAA---VDTDSPREVFFRVAADMFSD--GNFNWGRVVALFYFASKLVLKALC
BAK       TMGQVRQLAIIGDDINRRYDSEFQTMLQH-LQPTAENAYEYETKIATSLFES—GI-NWGRVVALLGFGYRLALHVVQ
```

FIG. 31E

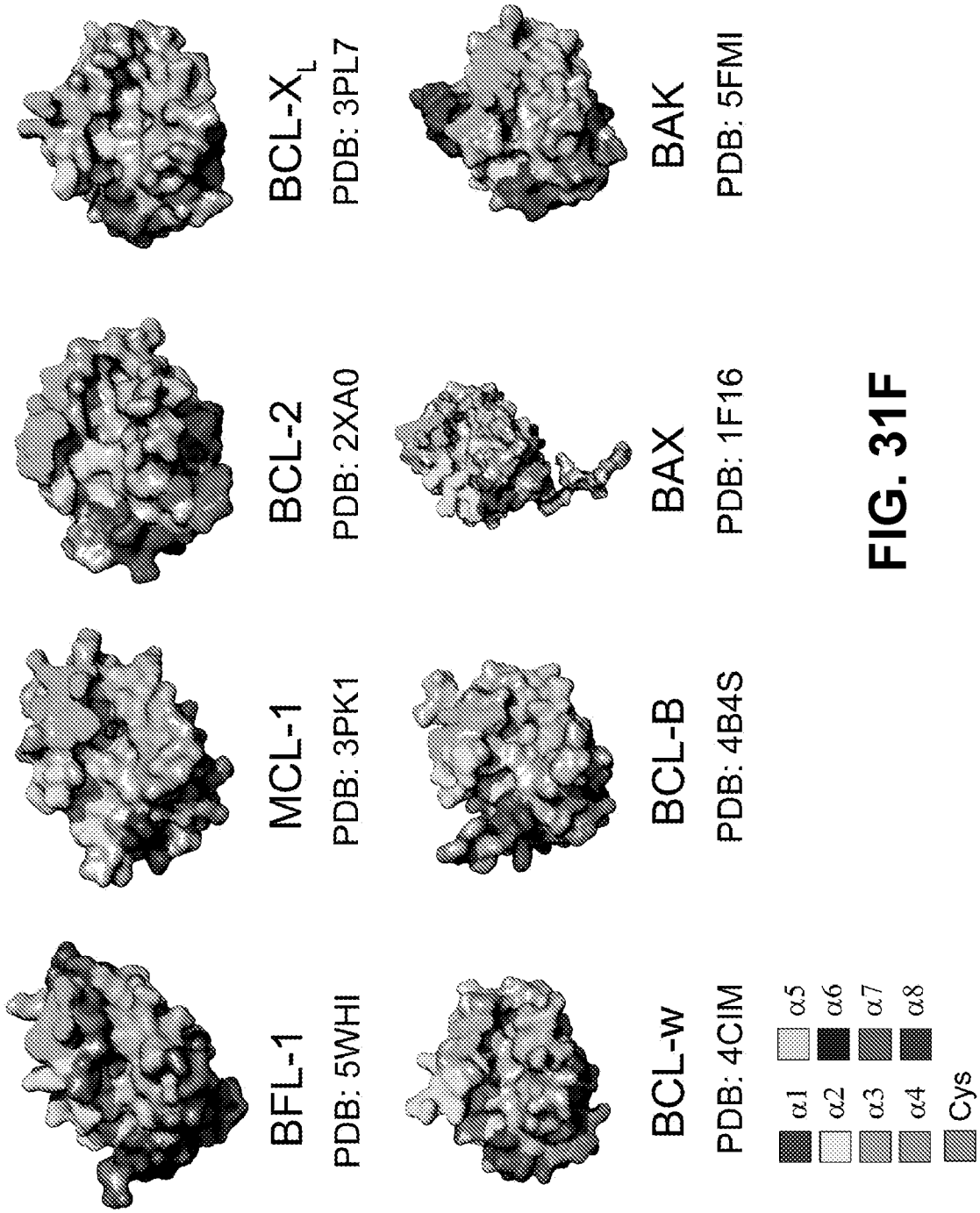
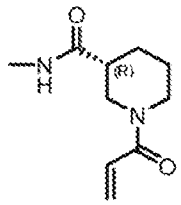

BFL-1
PDB: 5WHI
MCL-1
PDB: 3PK1
BCL-2
PDB: 2XA0
BCL-X_L
PDB: 3PL7
BCL-w
PDB: 4CIM
BCL-B
PDB: 4B4S
BAX
PDB: 1F16
BAK
PDB: 5FMI
α1
α2
α3
α4
α5
α6
α7
α8
Cys

FIG. 31F

NOXA SAHB: ^{26}*AT8LRRFGDXLNFRQ^{40}

* = Ac or



D-Nipecotic Acid

	Ac-NOXA SAHB					D-NA-NOXA SAHB			
BFL-1 WT	+	+	–	–	–	+	–	–	–
BFL-1 C55S	–	–	+	–	–	–	+	–	–
BFL-1 C4S/C19S	–	–	–	+	–	–	–	+	–
BFL-1 C4S/C19S/C55S	–	–	–	–	+	–	–	–	+

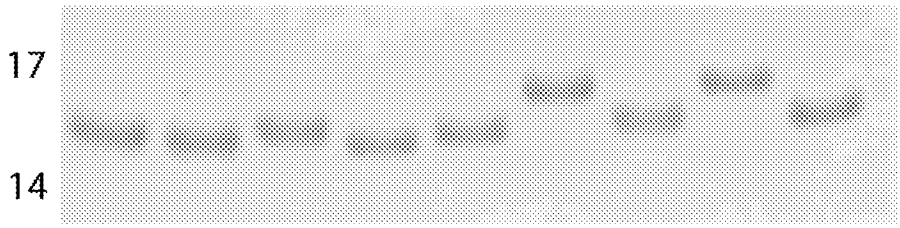


FIG. 32A

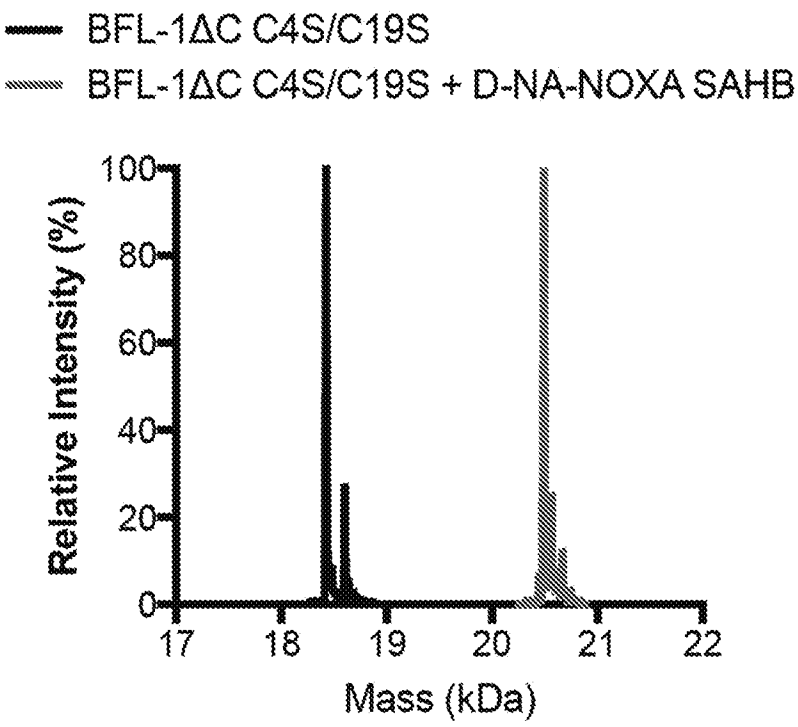

BFL-1ΔC C4S/C19S
BFL-1ΔC C4S/C19S + D-NA-NOXA SAHB
Relative Intensity (%)
100
80
60
40
20
0
17
18
19
20
21
22
Mass (kDa)

FIG. 32B

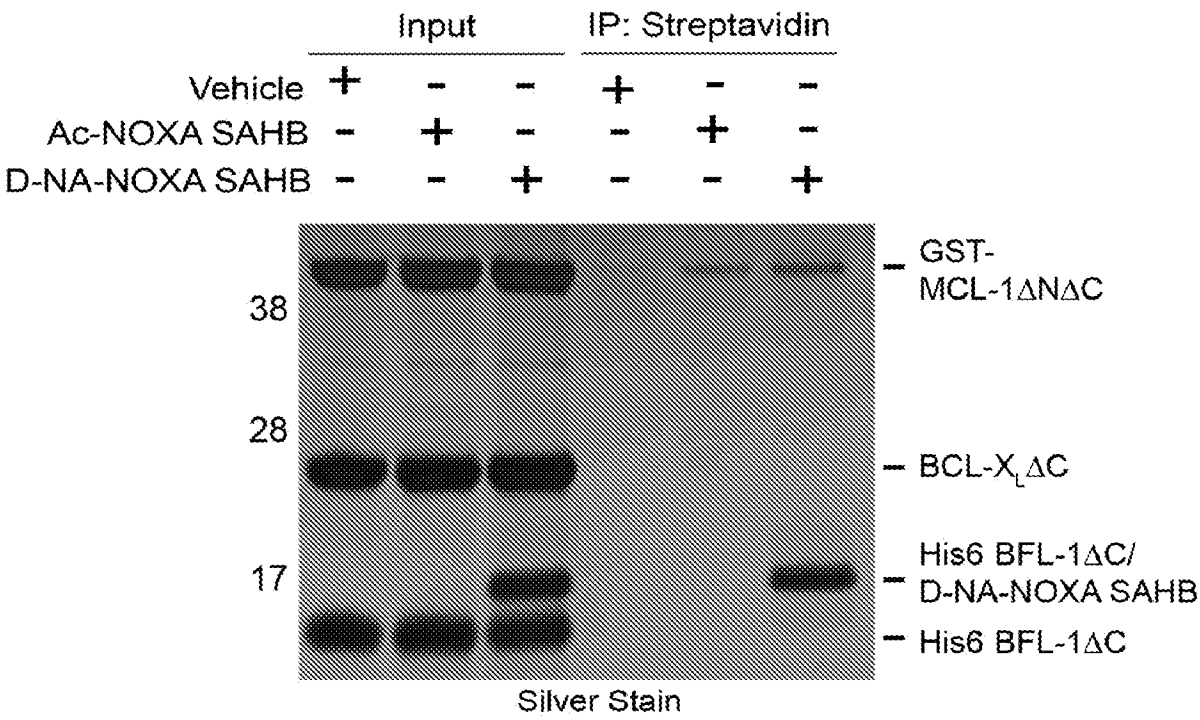

Input
IP: Streptavidin
Vehicle + - - + - -
Ac-NOXA SAHB - + - - + -
D-NA-NOXA SAHB - - + - - +
38
28
17
GST-
MCL-1ΔNΔC
BCL-XLΔC
His6 BFL-1ΔC/
D-NA-NOXA SAHB
His6 BFL-1ΔC
Silver Stain

FIG. 32C

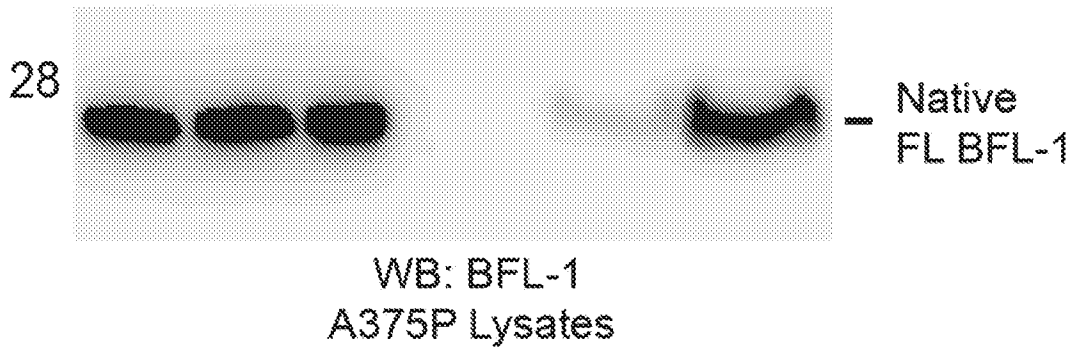


FIG. 32D

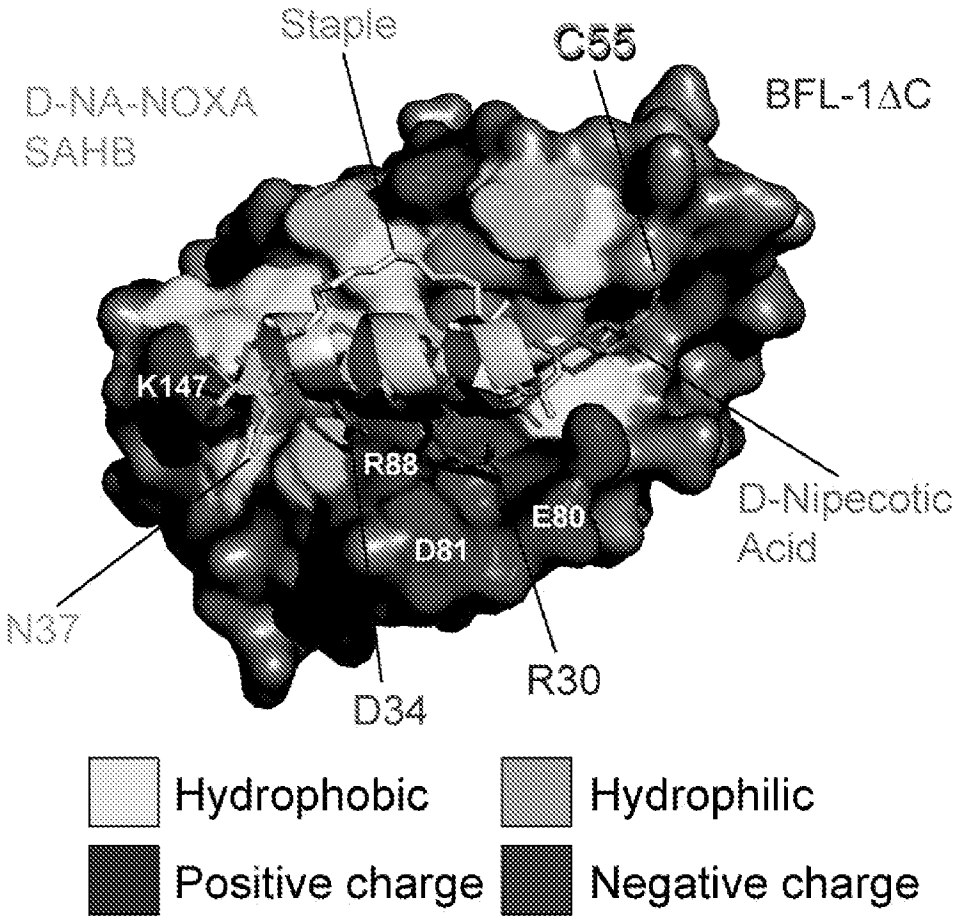

Staple
C55
D-NA-NOXA
SAHB
BFL-1ΔC
K147
R88
E80
D81
D-Nipecotic
Acid
N37
R30
D34
Hydrophobic
Hydrophilic
Positive charge
Negative charge

FIG. 33A

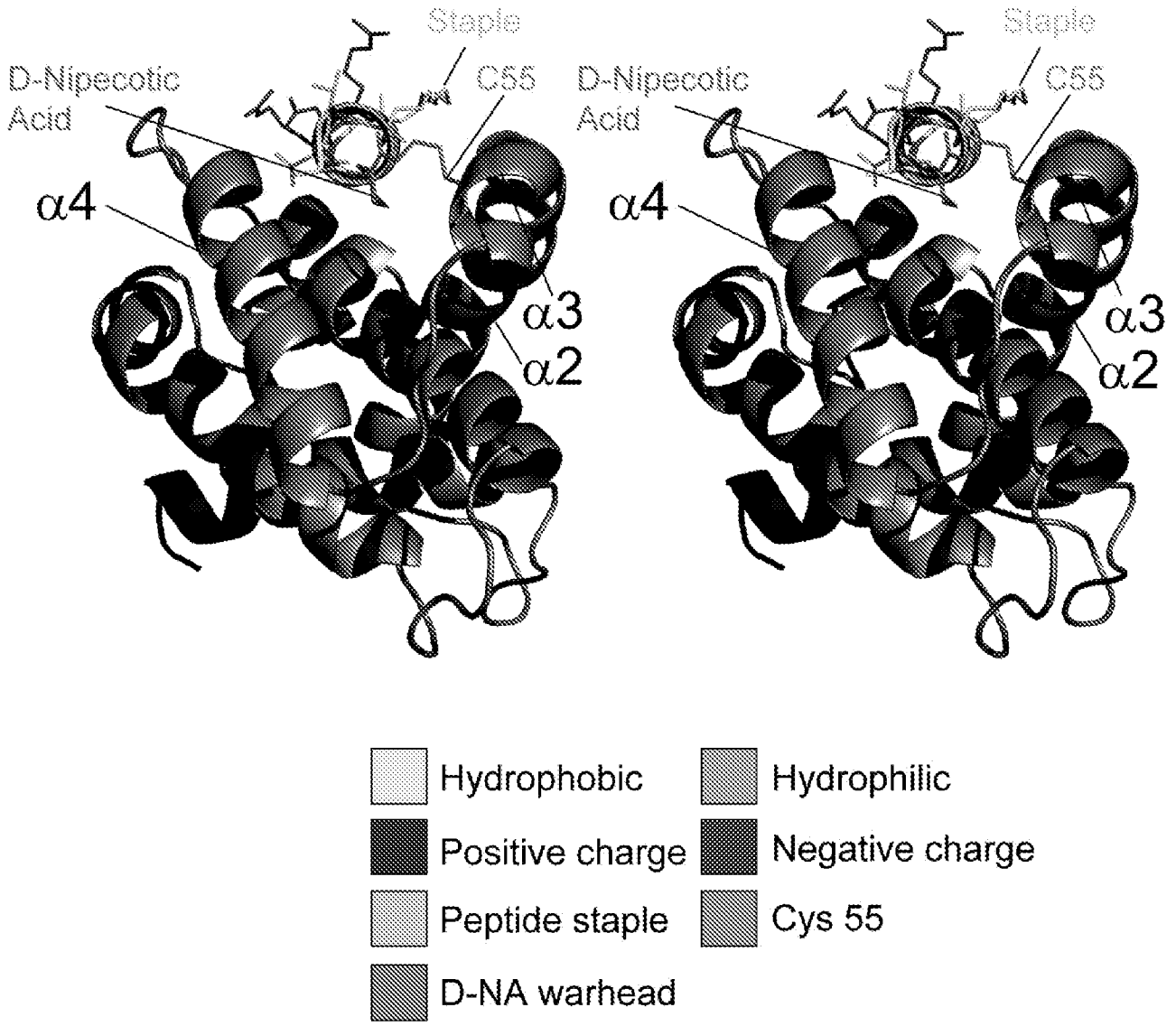

Staple
D-Nipecotic Acid
C55
α4
α3
α2
Staple
D-Nipecotic Acid
C55
α4
α3
α2
Hydrophobic
Hydrophilic
Positive charge
Negative charge
Peptide staple
Cys 55
D-NA warhead

FIG. 33B

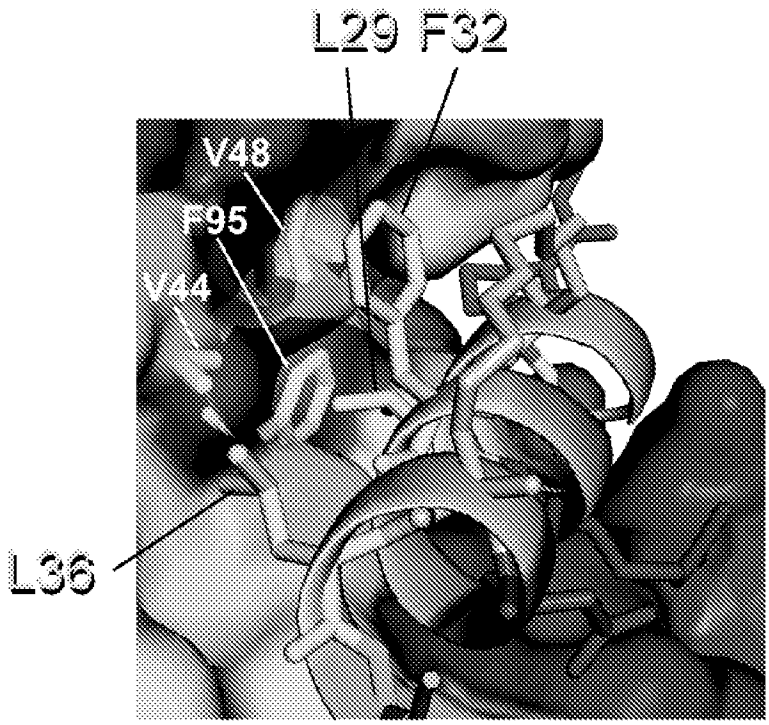


FIG. 33C

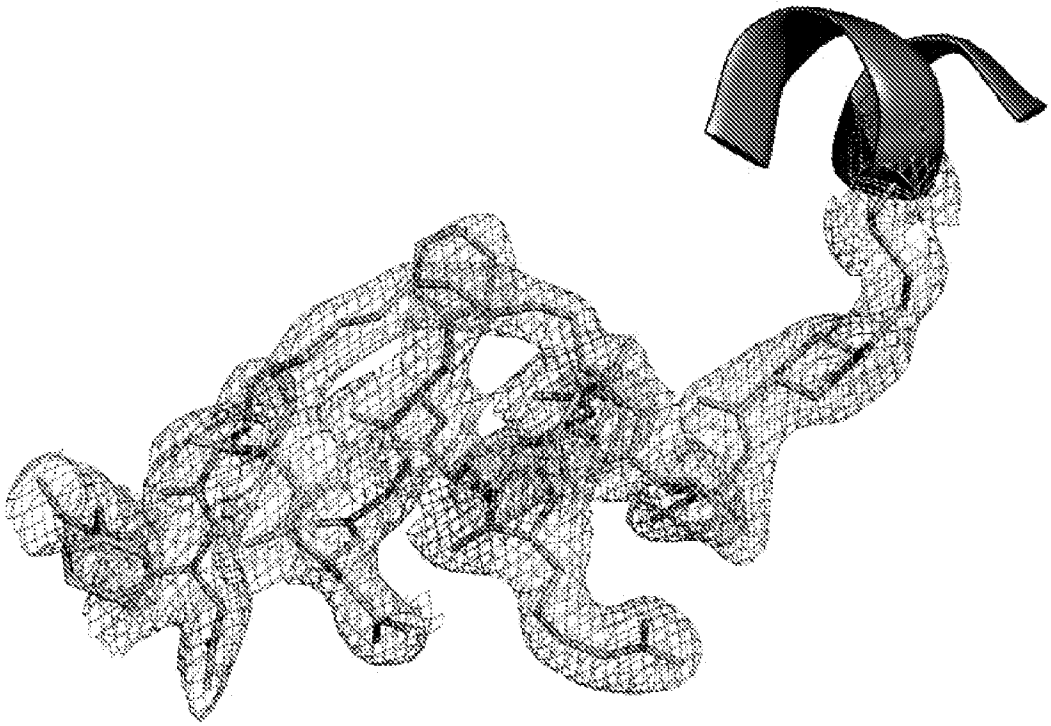


FIG. 33D

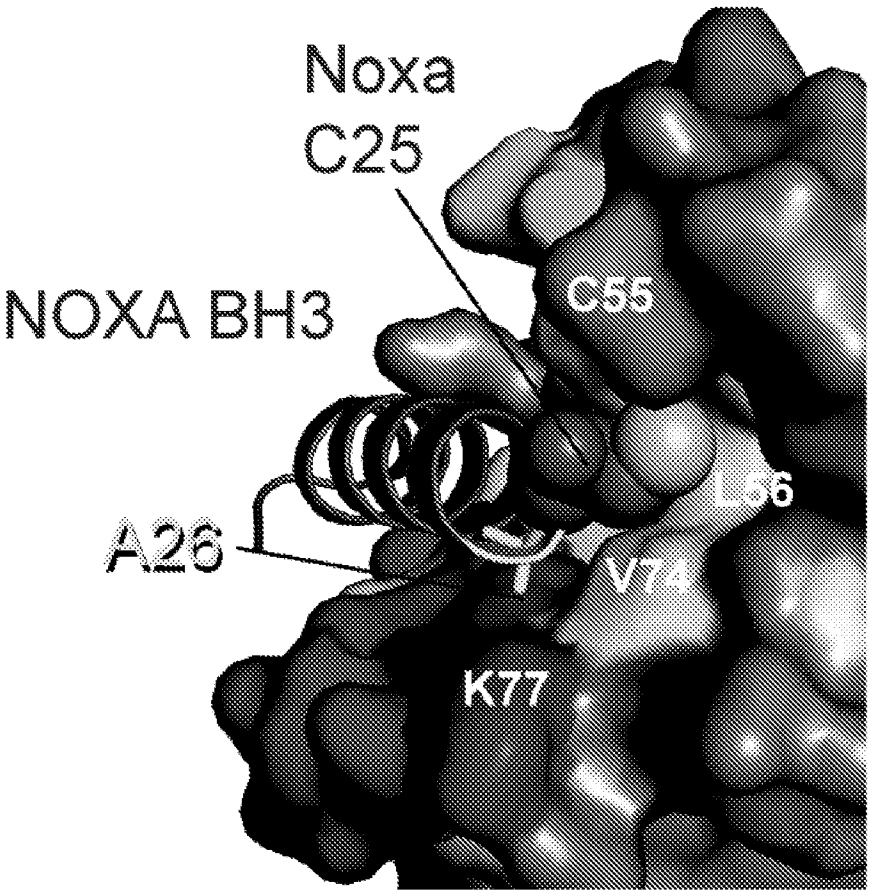


FIG. 33E

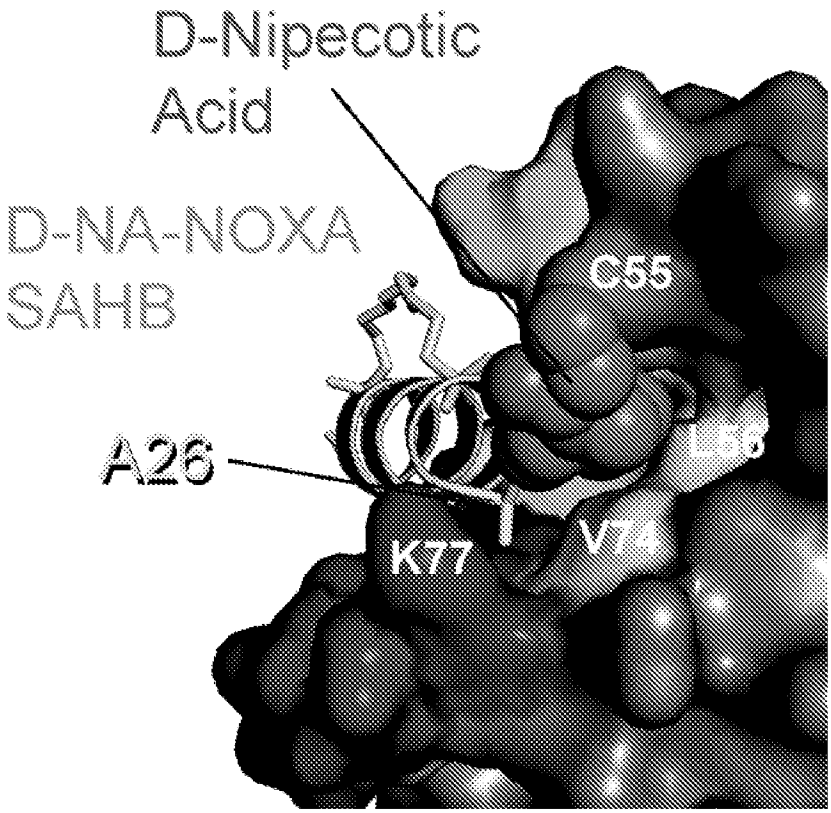


FIG. 33F

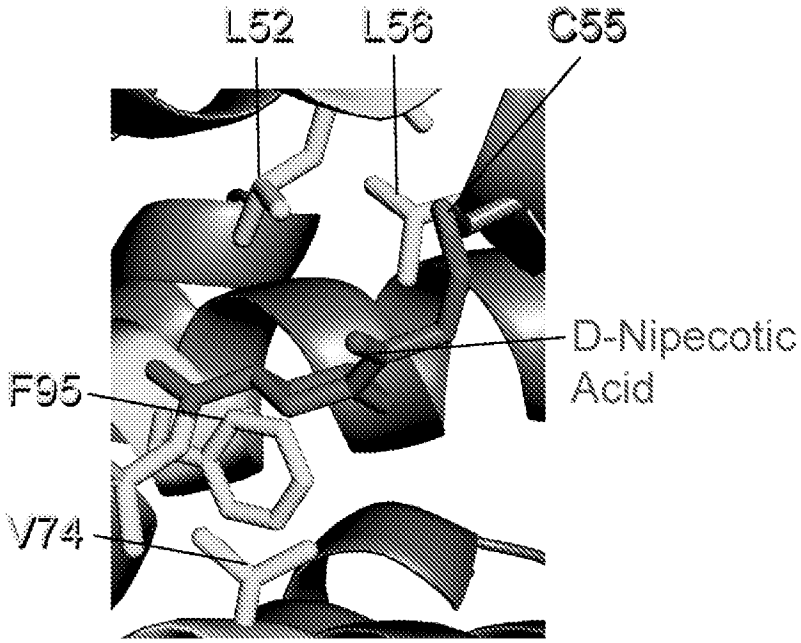


FIG. 33G

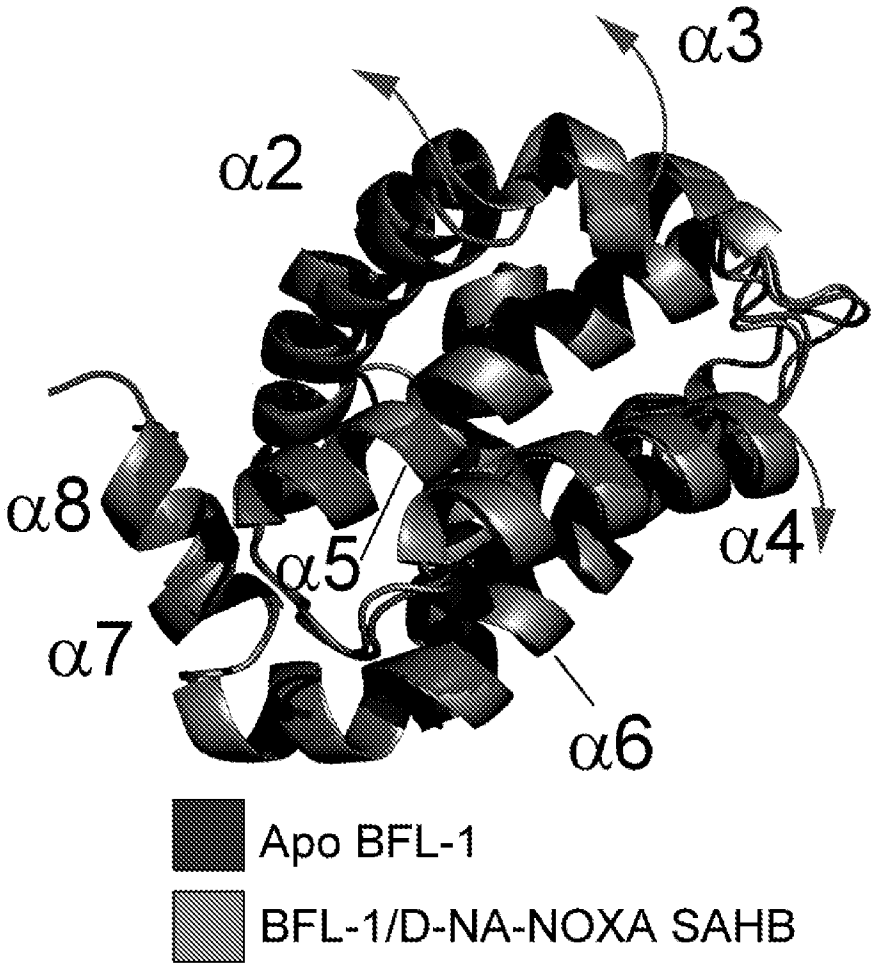


FIG. 34A

Amino Acids	Apo Distance (Å)	NOXA Bound Distance (Å)
BFL-1 Cys 55 to Leu 70	10.2	13.0
MCL-1 Met 231 to Leu 246	10.9	10.4
BFL-1 Cys 55 to Val 74	10.6	12.9
MCL-1 Met 231 to Val 249	10.7	10.6
BFL-1 Val 59 to Leu 70	8.3	9.6
MCL-1 Leu 235 to Leu 246	8.0	7.3

FIG. 34B

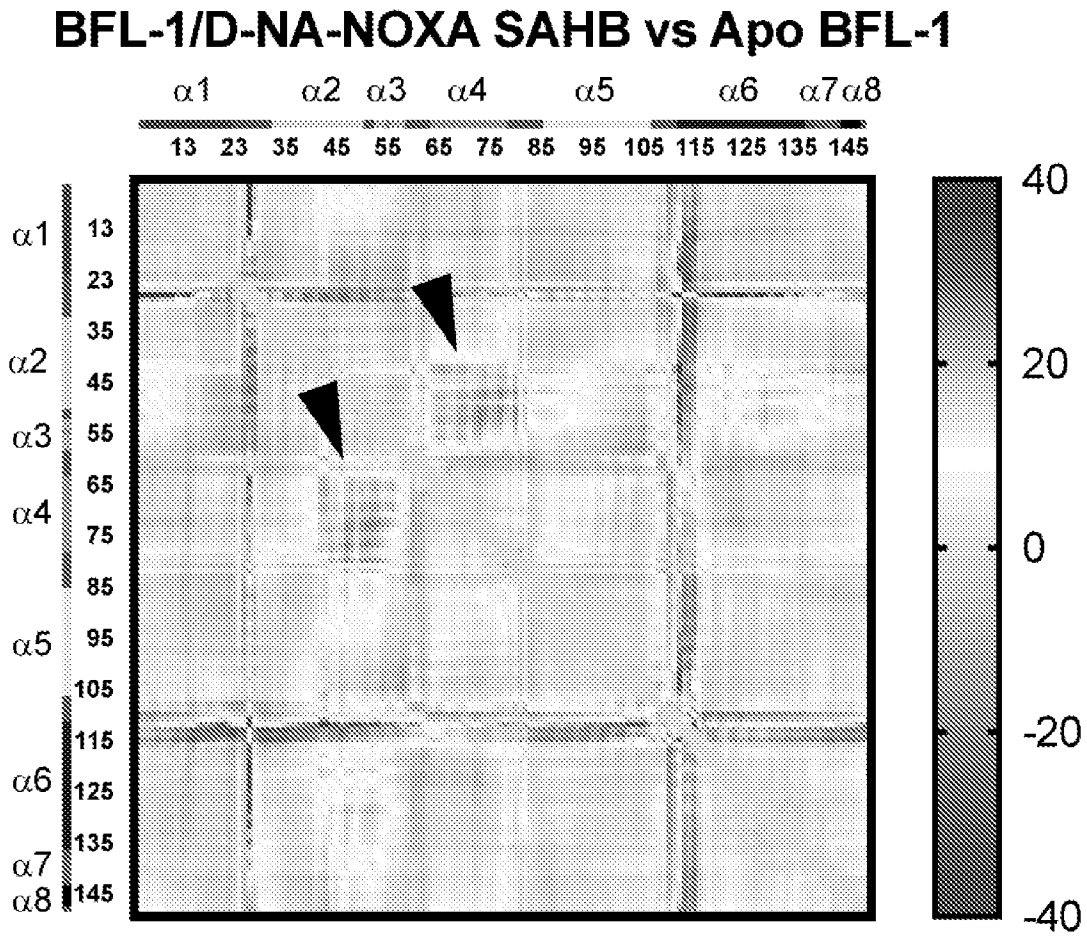

BFL-1/D-NA-NOXA SAHB vs Apo BFL-1
α1 α2 α3 α4 α5 α6 α7α8
13 23 35 45 55 65 75 85 95 105 115 125 135 145
α1 13
23
35
α2 45
α3 55
65
α4 75
85
α5 95
105
115
α6 125
135
α7
α8 145
40
20
0
-20
-40

FIG. 34C

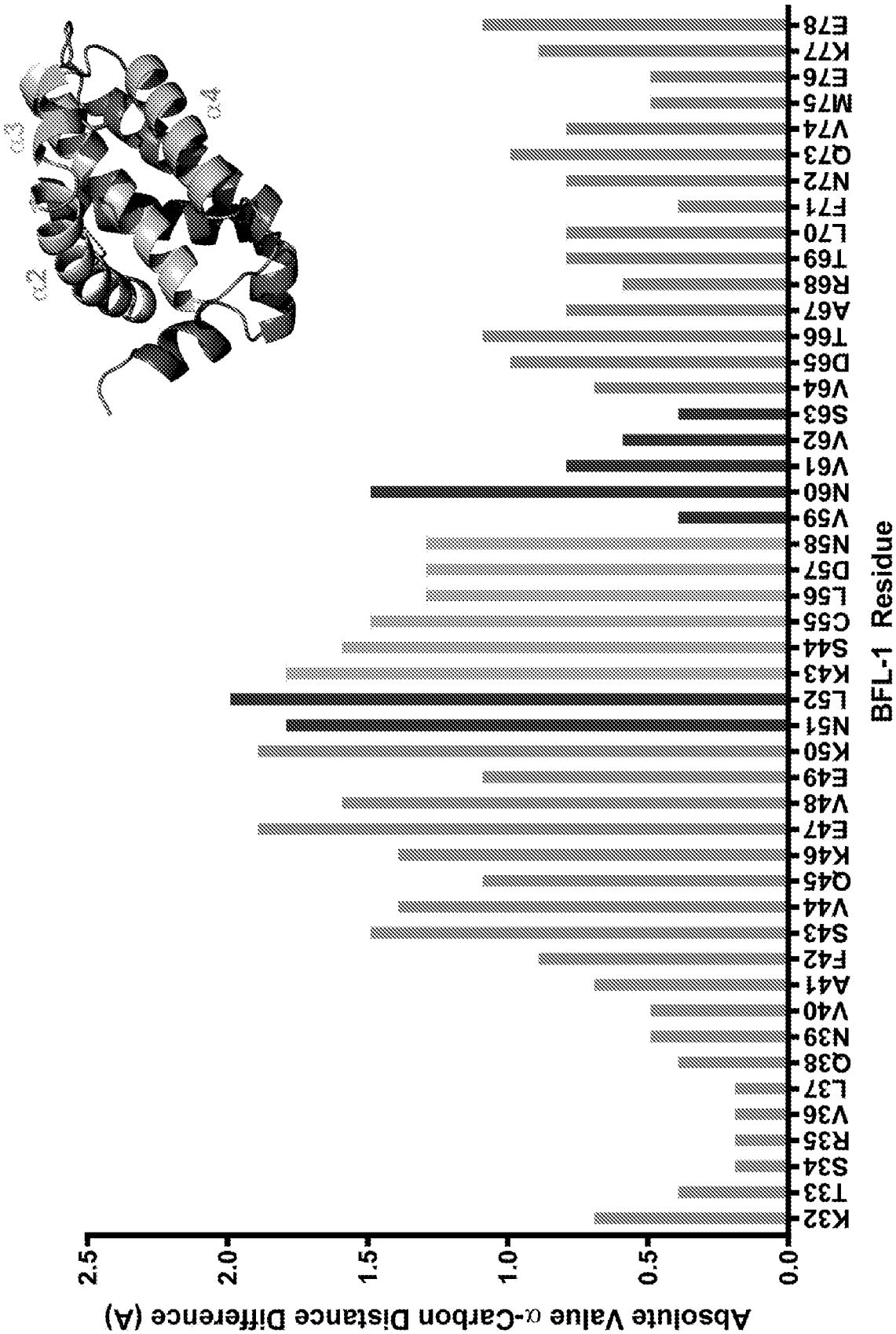
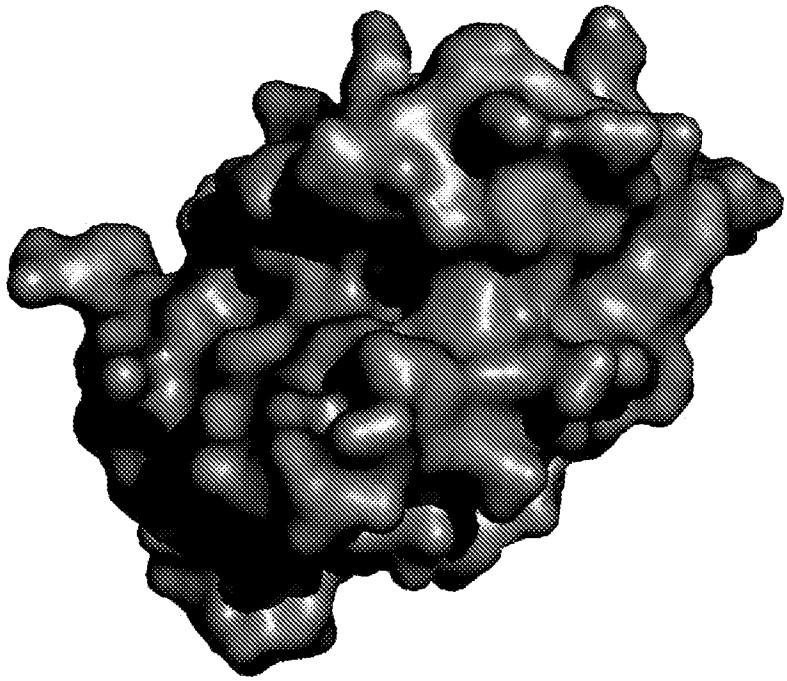
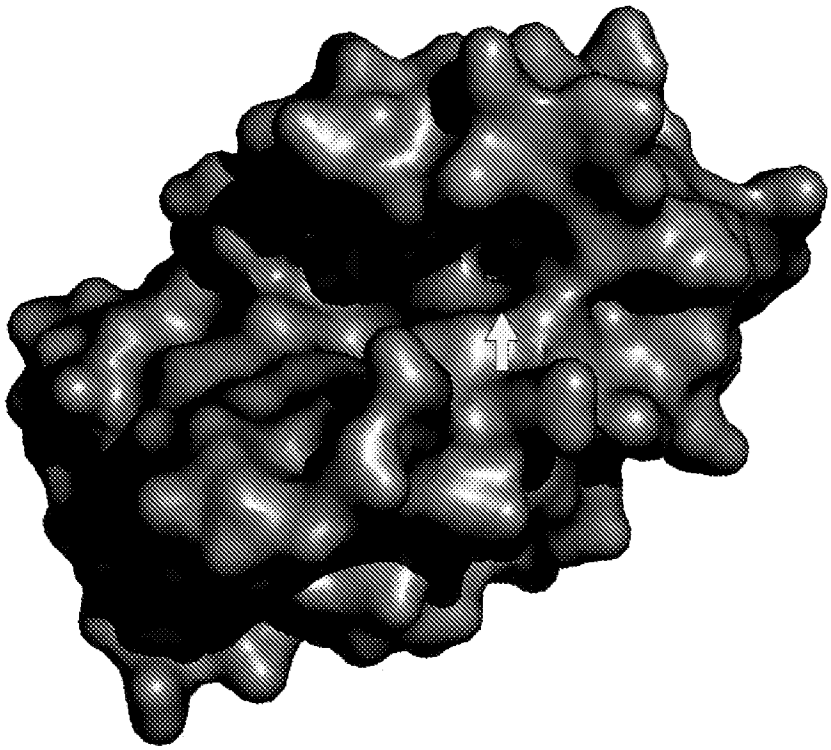

α2
α3
α4
Absolute Value α-Carbon Distance Difference (A)
2.5
2.0
1.5
1.0
0.5
0.0
K32
T33
S34
R35
V36
L37
Q38
N39
V40
A41
F42
S43
V44
Q45
K46
E47
V48
E49
K50
N51
L52
K43
S44
C55
L56
D57
N58
V59
N60
V61
V62
S63
V64
D65
T66
A67
R68
T69
L70
F71
N72
Q73
V74
M75
E76
K77
E78
BFL-1 Residue

FIG. 34D



Apo BFL-1ΔC

FIG. 34E



BFL-1ΔC/D-NA-NOXA SAHB

FIG. 34F

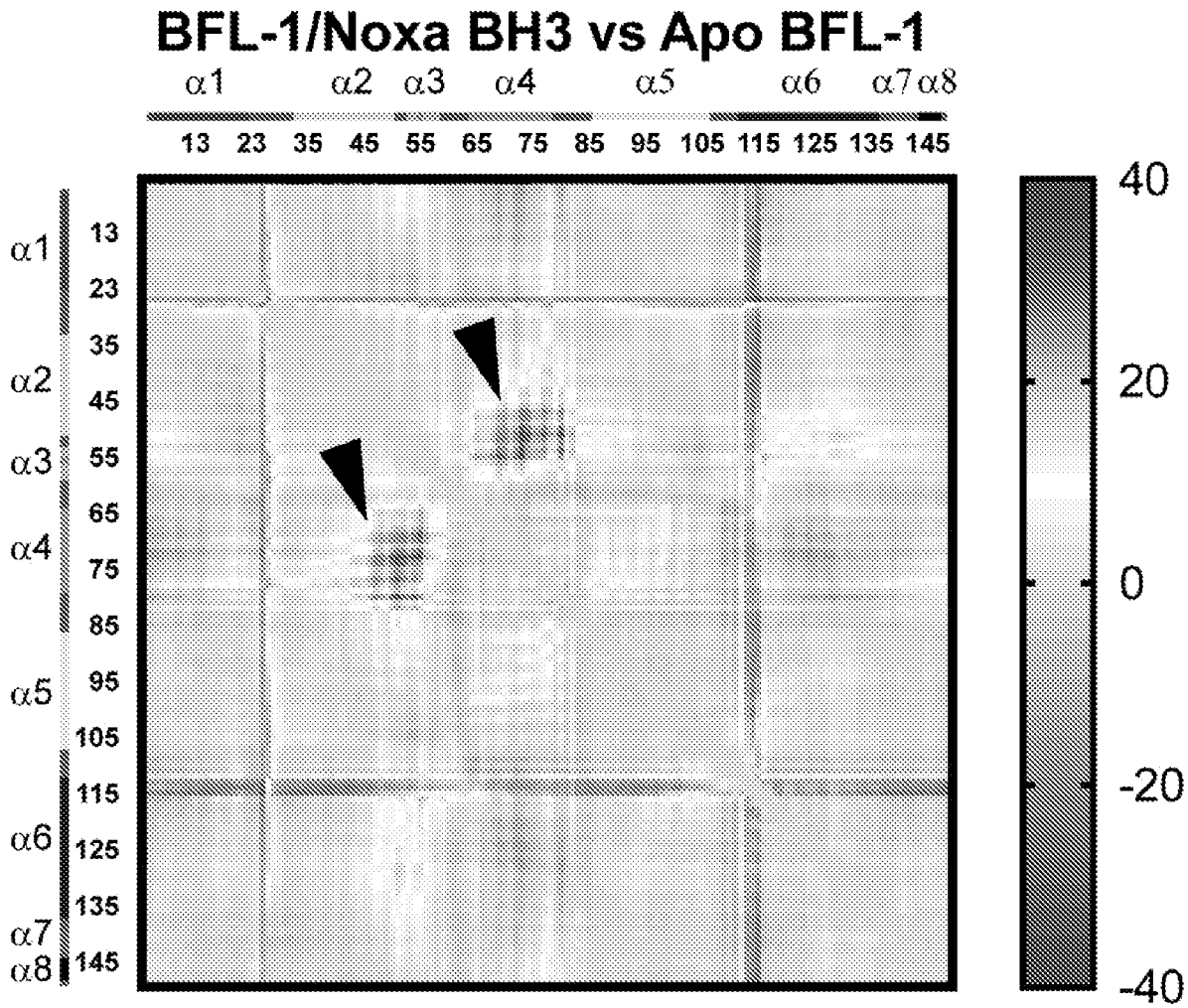

BFL-1/Noxa BH3 vs Apo BFL-1
α1 α2 α3 α4 α5 α6 α7 α8
13 23 35 45 55 65 75 85 95 105 115 125 135 145
α1 13
23
α2 35
45
α3 55
65
α4 75
85
α5 95
105
α6 115
125
α7 135
α8 145
40
20
0
-20
-40

FIG. 35A

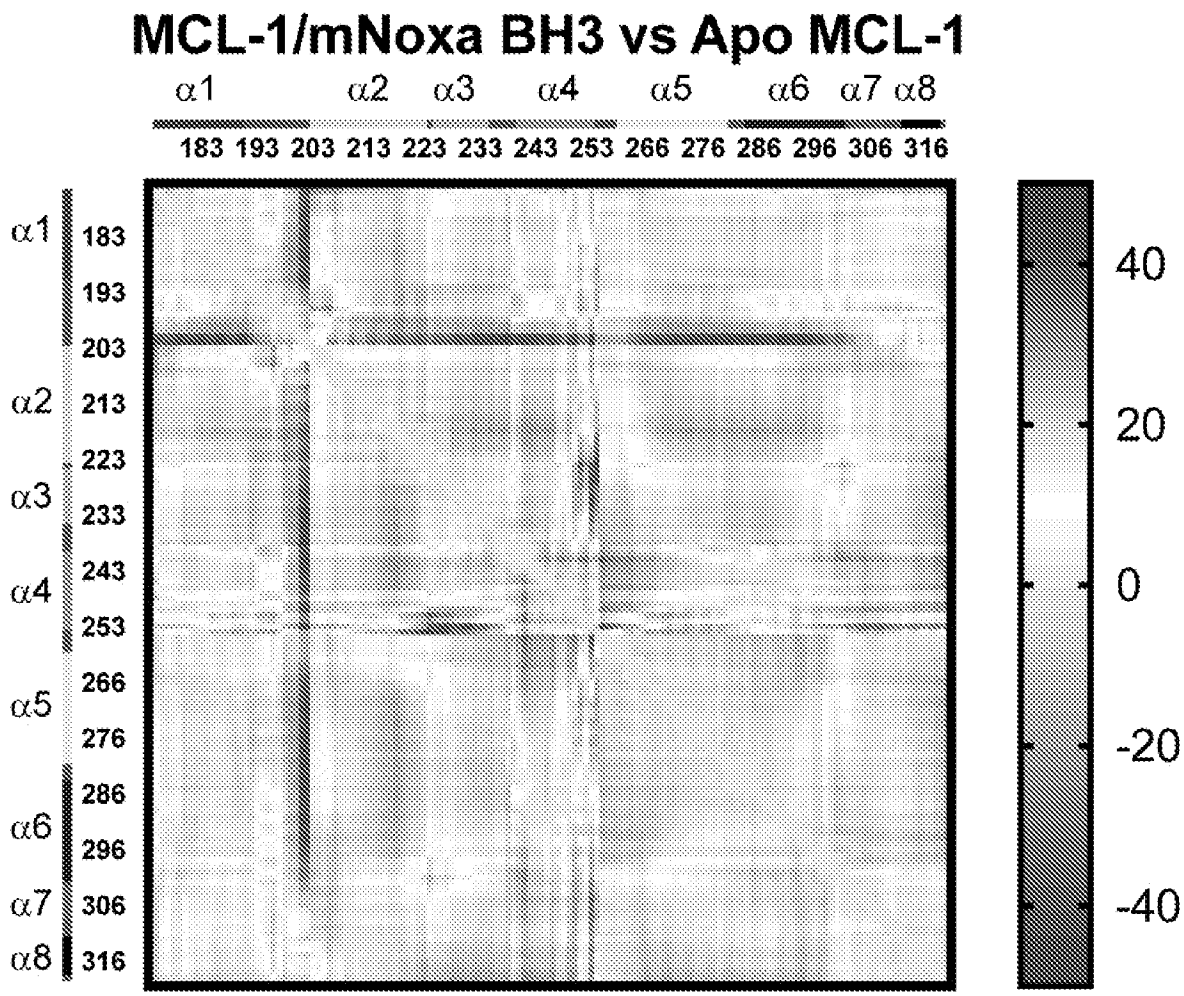

MCL-1/mNoxa BH3 vs Apo MCL-1
α1 α2 α3 α4 α5 α6 α7 α8
183 193 203 213 223 233 243 253 266 276 286 296 306 316
α1 183
193
203
α2 213
223
α3 233
α4 243
253
α5 266
276
α6 286
296
α7 306
α8 316
40
20
0
-20
-40

FIG. 35B

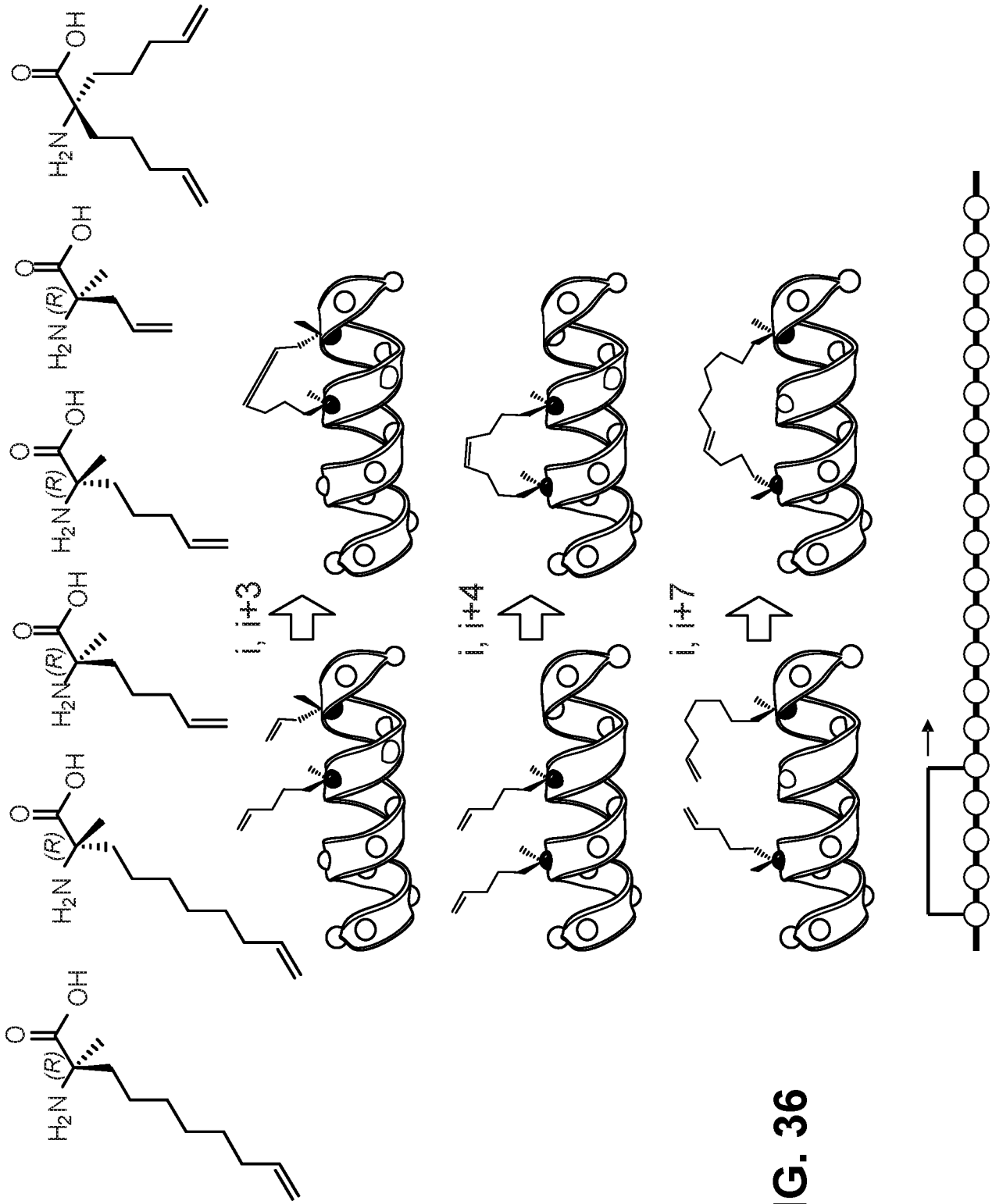


FIG. 36

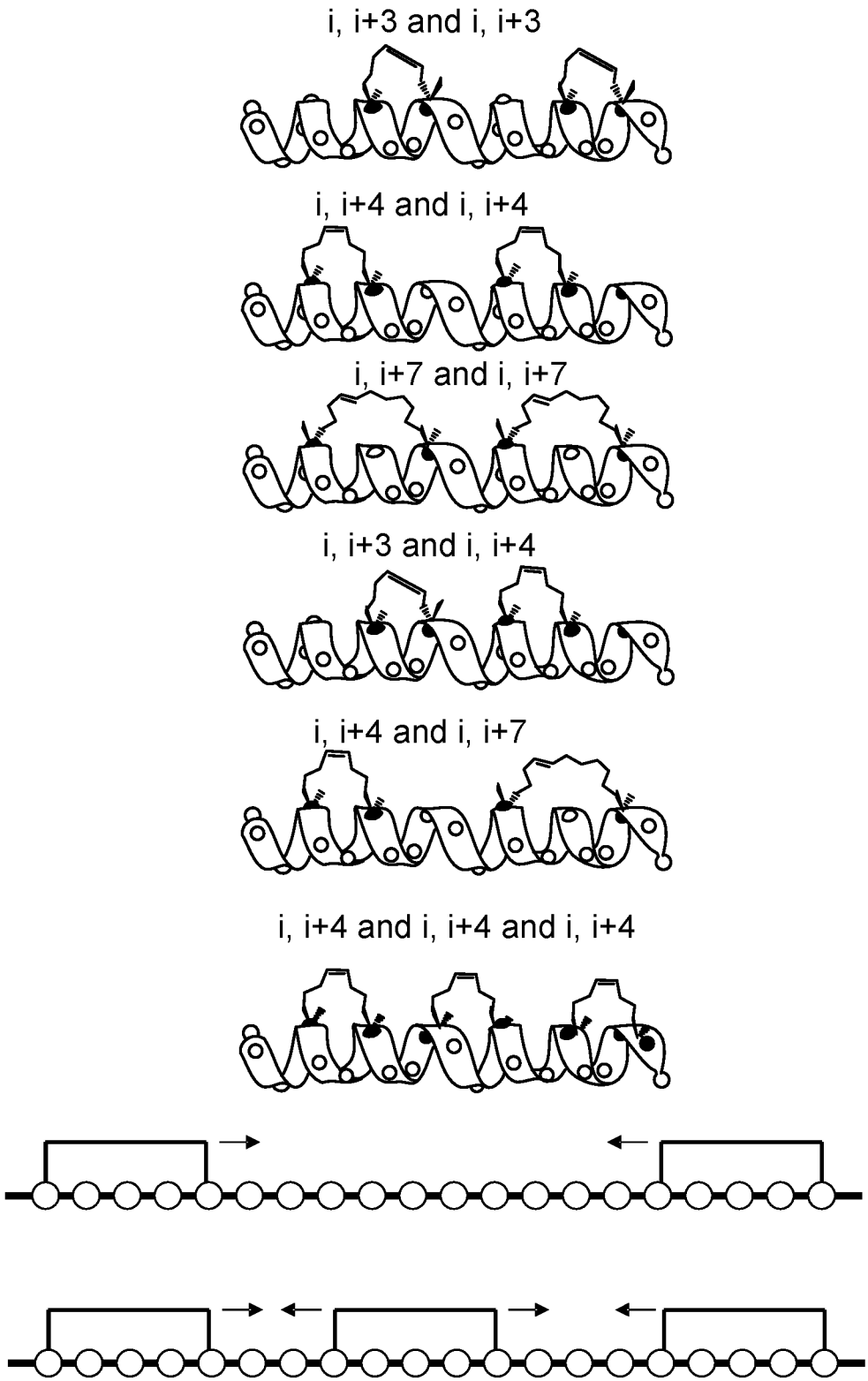

i, i+3 and i, i+3
i, i+4 and i, i+4
i, i+7 and i, i+7
i, i+3 and i, i+4
i, i+4 and i, i+7
i, i+4 and i, i+4 and i, i+4

FIG. 37

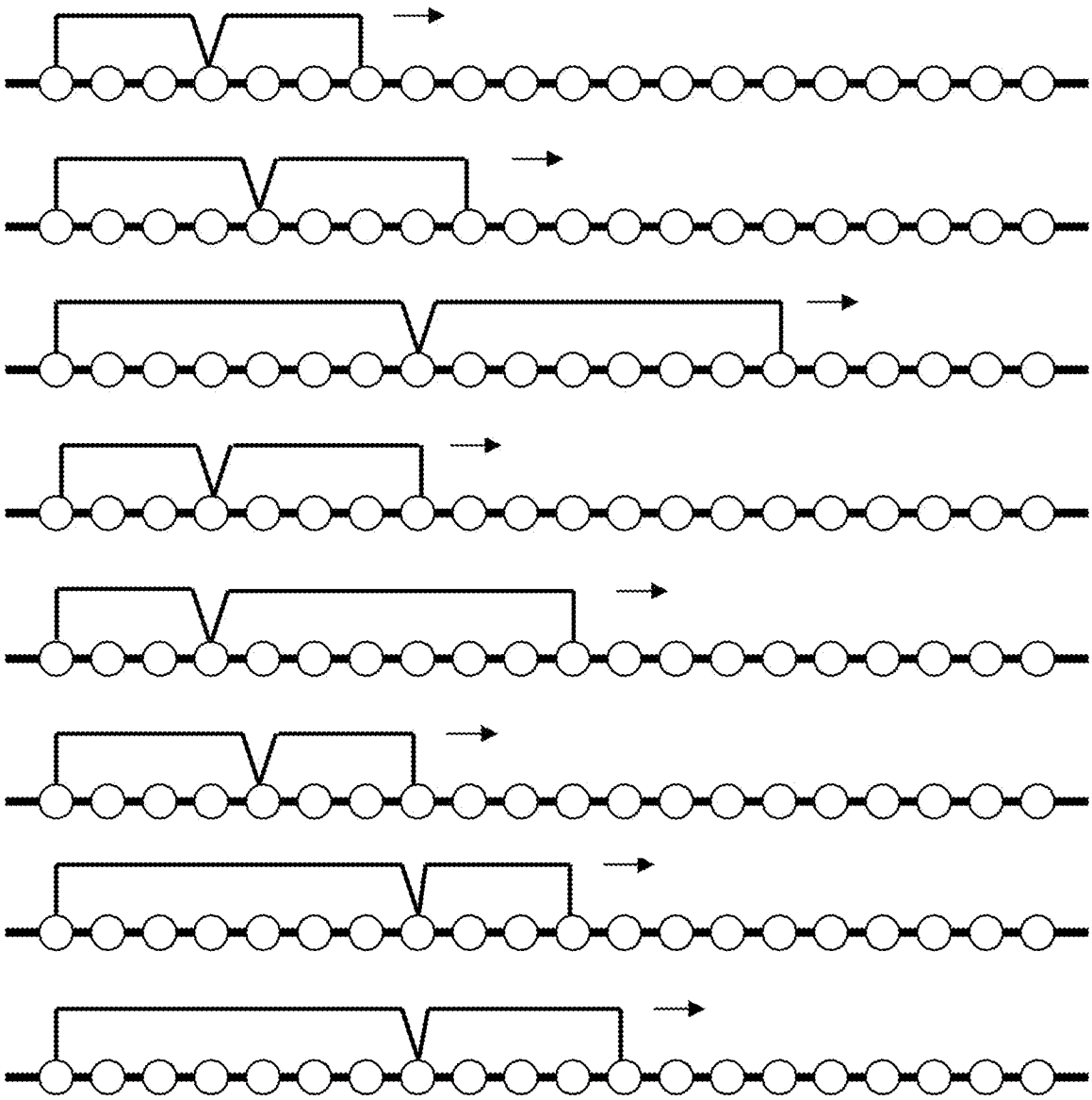


FIG. 38

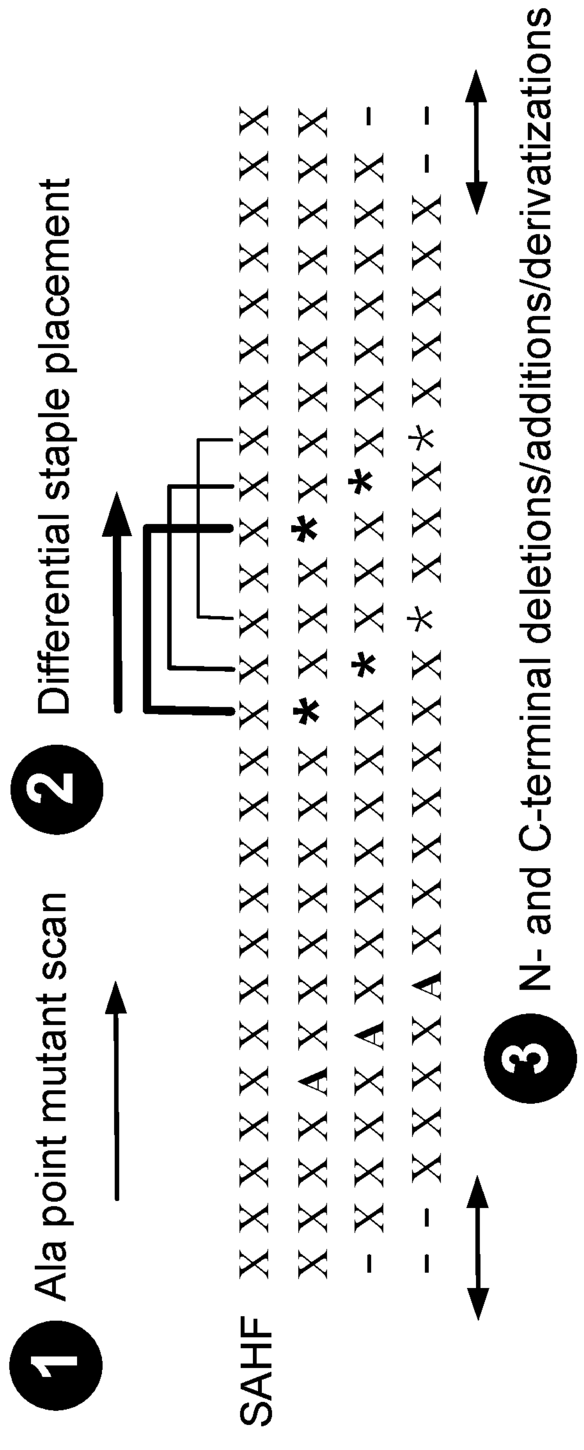

1 Ala point mutant scan
2 Differential staple placement
3 N- and C-terminal deletions/additions/derivatizations
SAHF

FIG. 39

FIG. 40

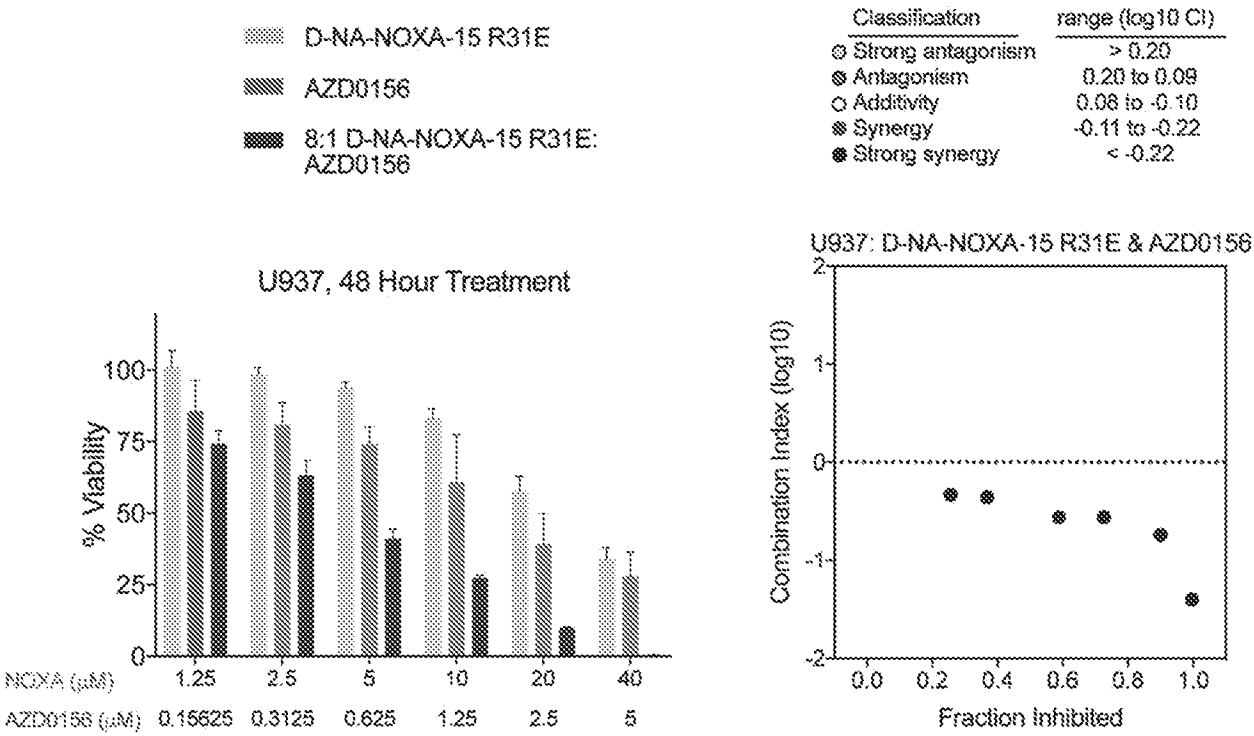

D-NA-NOXA-15 R31E
AZD0156
8:1 D-NA-NOXA-15 R31E: AZD0156
Classification
range (log10 CI)
Strong antagonism
> 0.20
Antagonism
0.20 to 0.09
Additivity
0.08 to -0.10
Synergy
-0.11 to -0.22
Strong synergy
< -0.22
U937, 48 Hour Treatment
% Viability
100
75
50
25
0
NOXA (μM)
1.25
2.5
5
10
20
40
AZD0156 (μM)
0.15625
0.3125
0.625
1.25
2.5
5
U937: D-NA-NOXA-15 R31E & AZD0156
Combination Index (log10)
2
1
0
-1
-2
0.0
0.2
0.4
0.6
0.8
1.0
Fraction Inhibited

FIG. 41

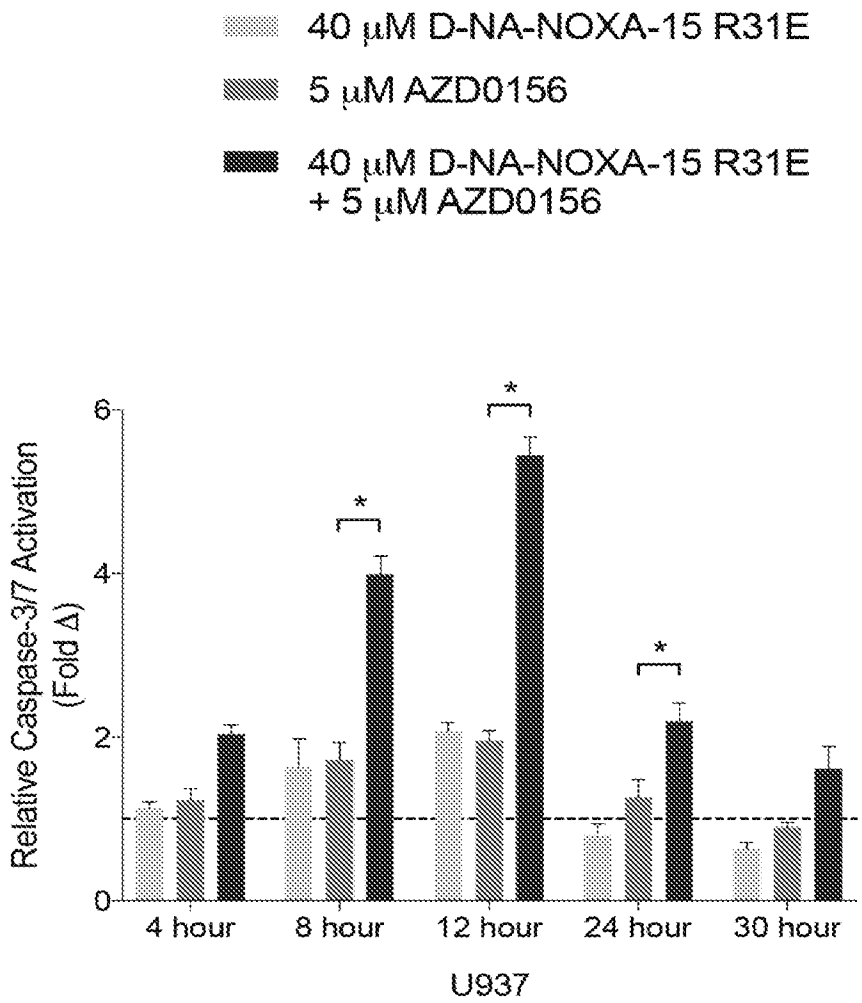
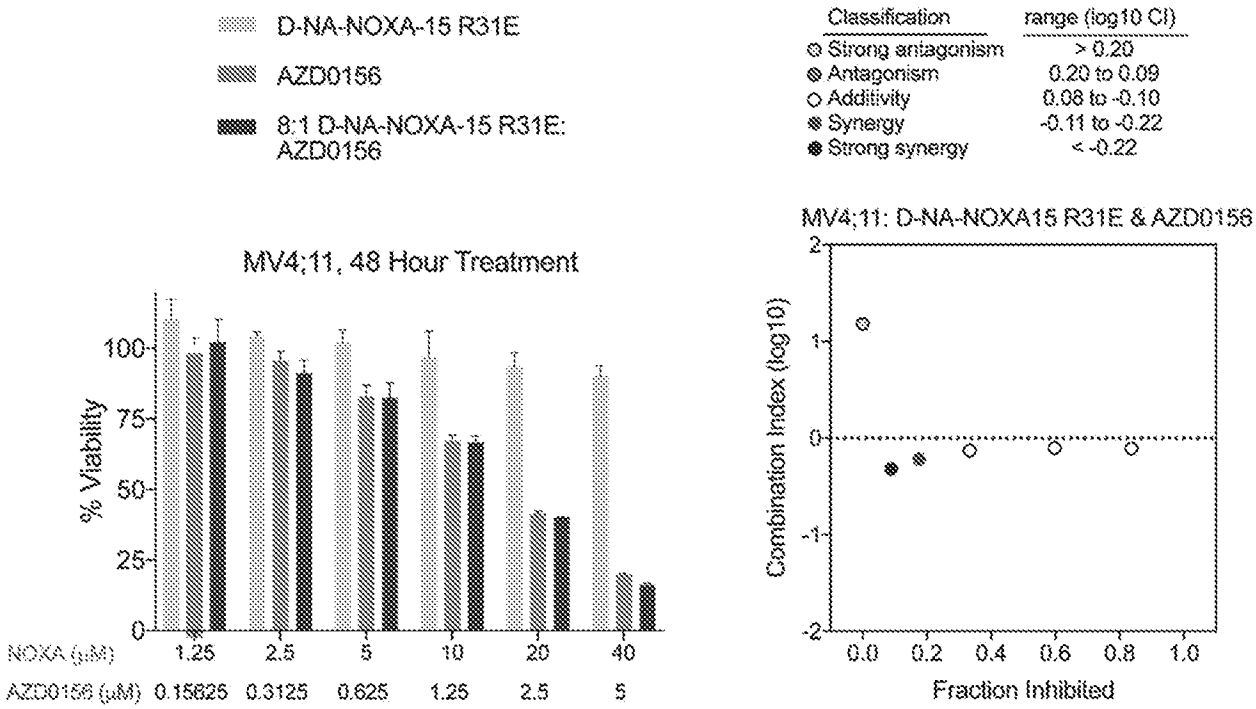

40 μM D-NA-NOXA-15 R31E
5 μM AZD0156
40 μM D-NA-NOXA-15 R31E
+ 5 μM AZD0156
Relative Caspase-3/7 Activation
(Fold Δ)
0
2
4
6
*
*
*
4 hour
8 hour
12 hour
24 hour
30 hour
U937

FIG. 42


D-NA-NOXA-15 R31E
AZD0156
8:1 D-NA-NOXA-15 R31E: AZD0156
MV4;11, 48 Hour Treatment
% Viability
100
75
50
25
0
NOXA (μM) 1.25 2.5 5 10 20 40
AZD0156 (μM) 0.15625 0.3125 0.625 1.25 2.5 5
Classification range (log10 CI)
Strong antagonism > 0.20
Antagonism 0.20 to 0.09
Additivity 0.08 to -0.10
Synergy -0.11 to -0.22
Strong synergy < -0.22
MV4;11: D-NA-NOXA15 R31E & AZD0156
Combination Index (log10)
2
1
0
-1
-2
0.0 0.2 0.4 0.6 0.8 1.0
Fraction Inhibited

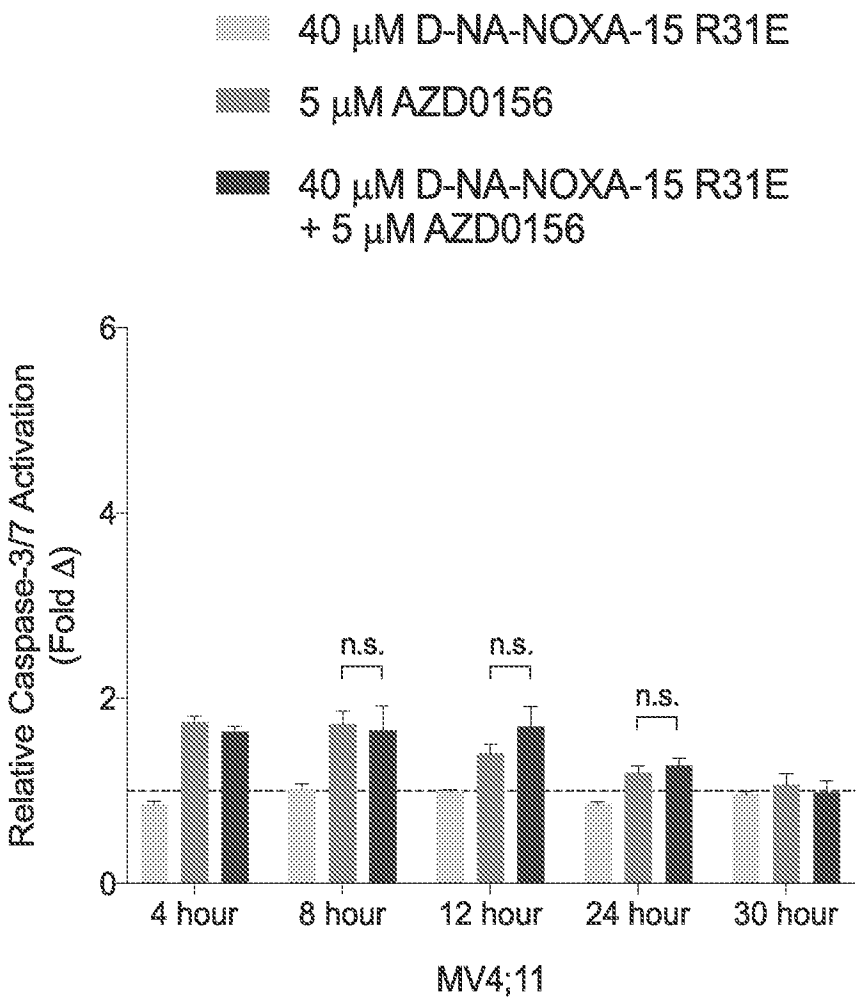

40 μM D-NA-NOXA-15 R31E
5 μM AZD0156
40 μM D-NA-NOXA-15 R31E
+ 5 μM AZD0156
Relative Caspase-3/7 Activation
(Fold Δ)
6
4
2
0
n.s.
n.s.
n.s.
4 hour
8 hour
12 hour
24 hour
30 hour
MV4;11

FIG. 43

FIG. 44

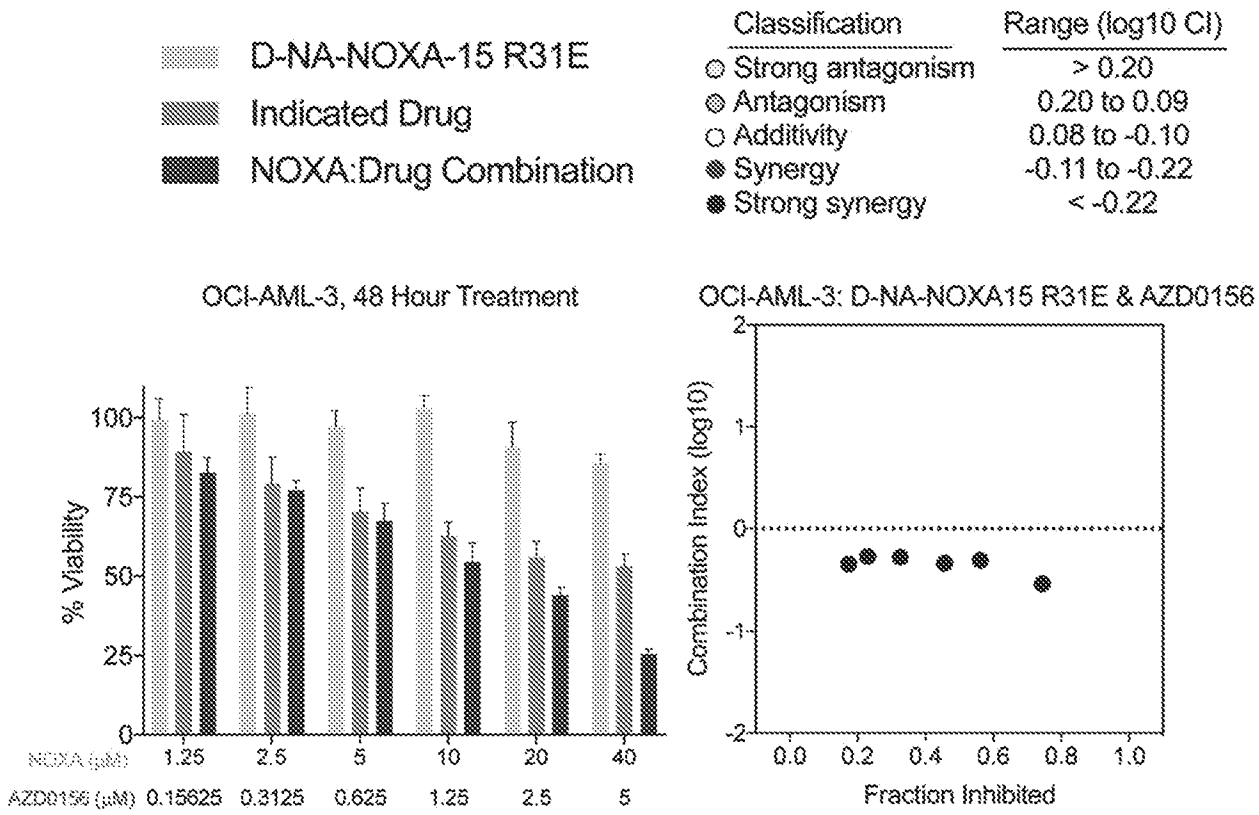

D-NA-NOXA-15 R31E
Indicated Drug
NOXA:Drug Combination
Classification
Range (log10 CI)
Strong antagonism
> 0.20
Antagonism
0.20 to 0.09
Additivity
0.08 to -0.10
Synergy
-0.11 to -0.22
Strong synergy
< -0.22
OCI-AML-3, 48 Hour Treatment
% Viability
100
75
50
25
0
NOXA (μM)
1.25
2.5
5
10
20
40
AZD0156 (μM)
0.15625
0.3125
0.625
1.25
2.5
5
OCI-AML-3: D-NA-NOXA15 R31E & AZD0156
Combination Index (log10)
2
1
0
-1
-2
0.0
0.2
0.4
0.6
0.8
1.0
Fraction Inhibited

FIG. 45

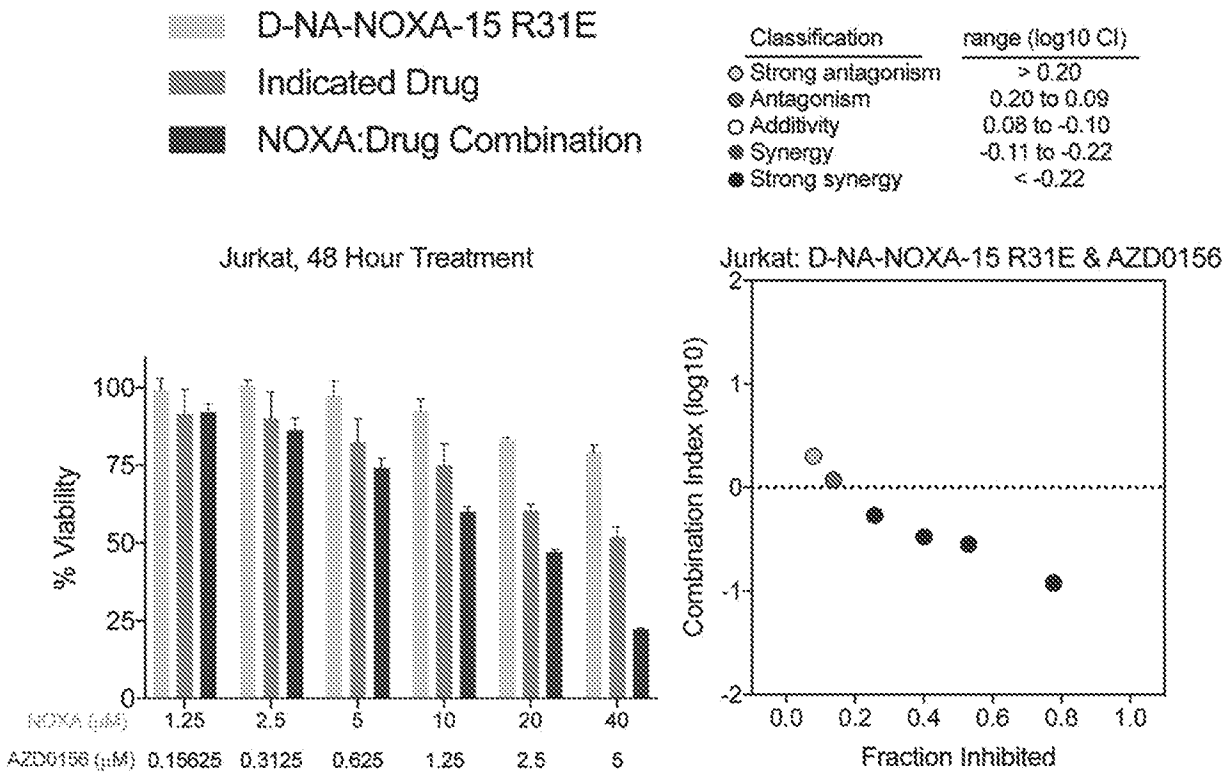

D-NA-NOXA-15 R31E
Indicated Drug
NOXA:Drug Combination
Classification
range (log10 CI)
Strong antagonism
> 0.20
Antagonism
0.20 to 0.09
Additivity
0.08 to -0.10
Synergy
-0.11 to -0.22
Strong synergy
< -0.22
Jurkat, 48 Hour Treatment
% Viability
100
75
50
25
0
NOXA (μM)
1.25
2.5
5
10
20
40
AZD0156 (μM)
0.15625
0.3125
0.625
1.25
2.5
5
Jurkat: D-NA-NOXA-15 R31E & AZD0156
Combination Index (log10)
2
1
0
-1
-2
0.0
0.2
0.4
0.6
0.8
1.0
Fraction Inhibited

FIG. 46

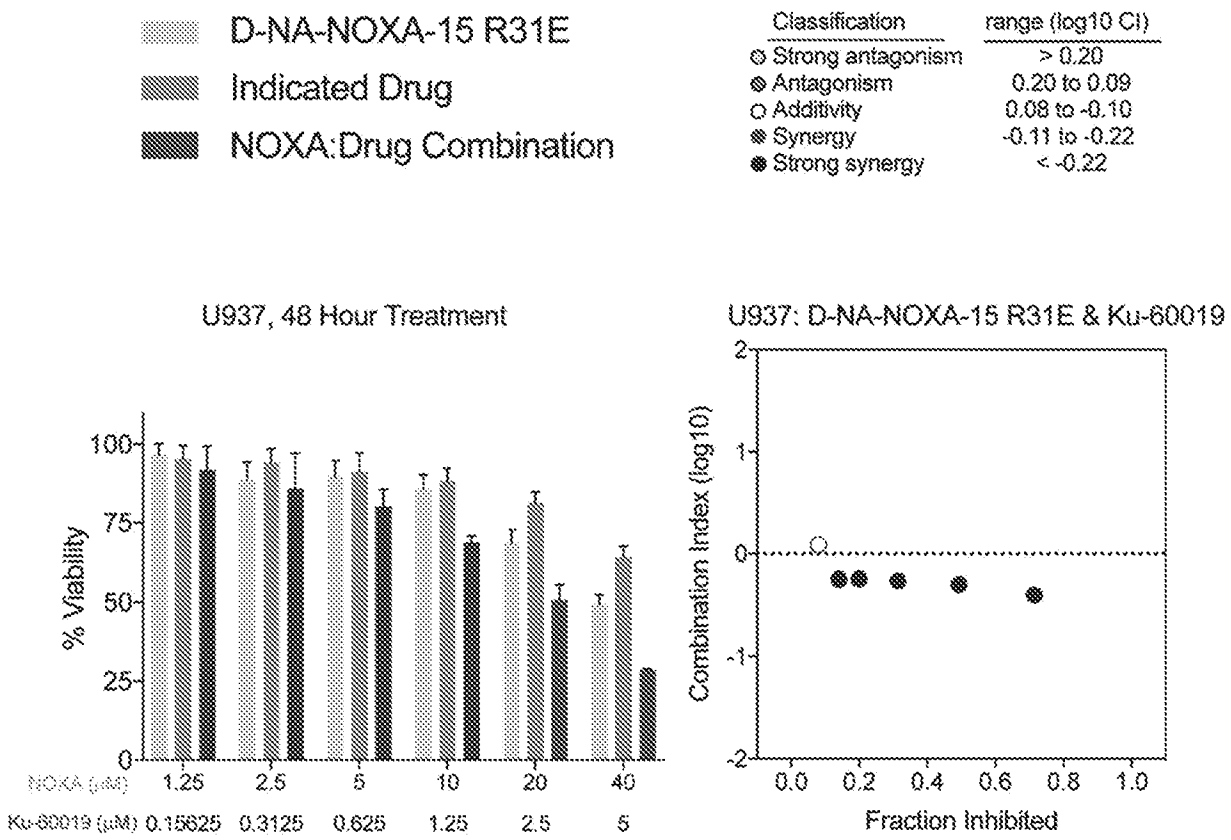

D-NA-NOXA-15 R31E
Indicated Drug
NOXA:Drug Combination
Classification
range (log10 CI)
Strong antagonism
> 0.20
Antagonism
0.20 to 0.09
Additivity
0.08 to -0.10
Synergy
-0.11 to -0.22
Strong synergy
< -0.22
U937, 48 Hour Treatment
% Viability
100
75
50
25
0
NOXA (μM)
1.25
2.5
5
10
20
40
Ku-60019 (μM)
0.15625
0.3125
0.625
1.25
2.5
5
U937: D-NA-NOXA-15 R31E & Ku-60019
Combination Index (log10)
2
1
0
-1
-2
0.0
0.2
0.4
0.6
0.8
1.0
Fraction Inhibited

FIG. 47

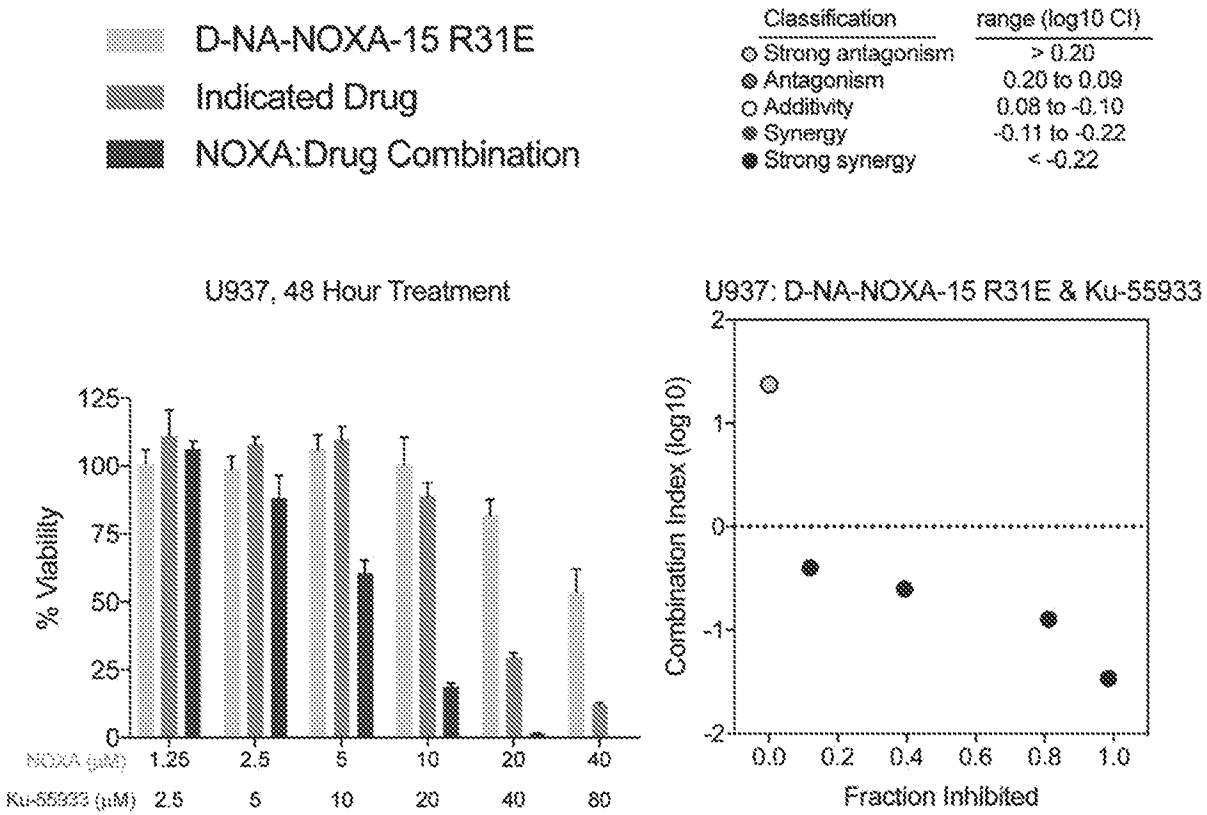

D-NA-NOXA-15 R31E
Indicated Drug
NOXA:Drug Combination
Classification
range (log10 CI)
Strong antagonism
> 0.20
Antagonism
0.20 to 0.09
Additivity
0.08 to -0.10
Synergy
-0.11 to -0.22
Strong synergy
< -0.22
U937, 48 Hour Treatment
% Viability
125
100
75
50
25
0
NOXA (μM)
1.25
2.5
5
10
20
40
Ku-55933 (μM)
2.5
5
10
20
40
80
U937: D-NA-NOXA-15 R31E & Ku-55933
Combination Index (log10)
2
1
0
-1
-2
0.0
0.2
0.4
0.6
0.8
1.0
Fraction Inhibited

FIG. 48

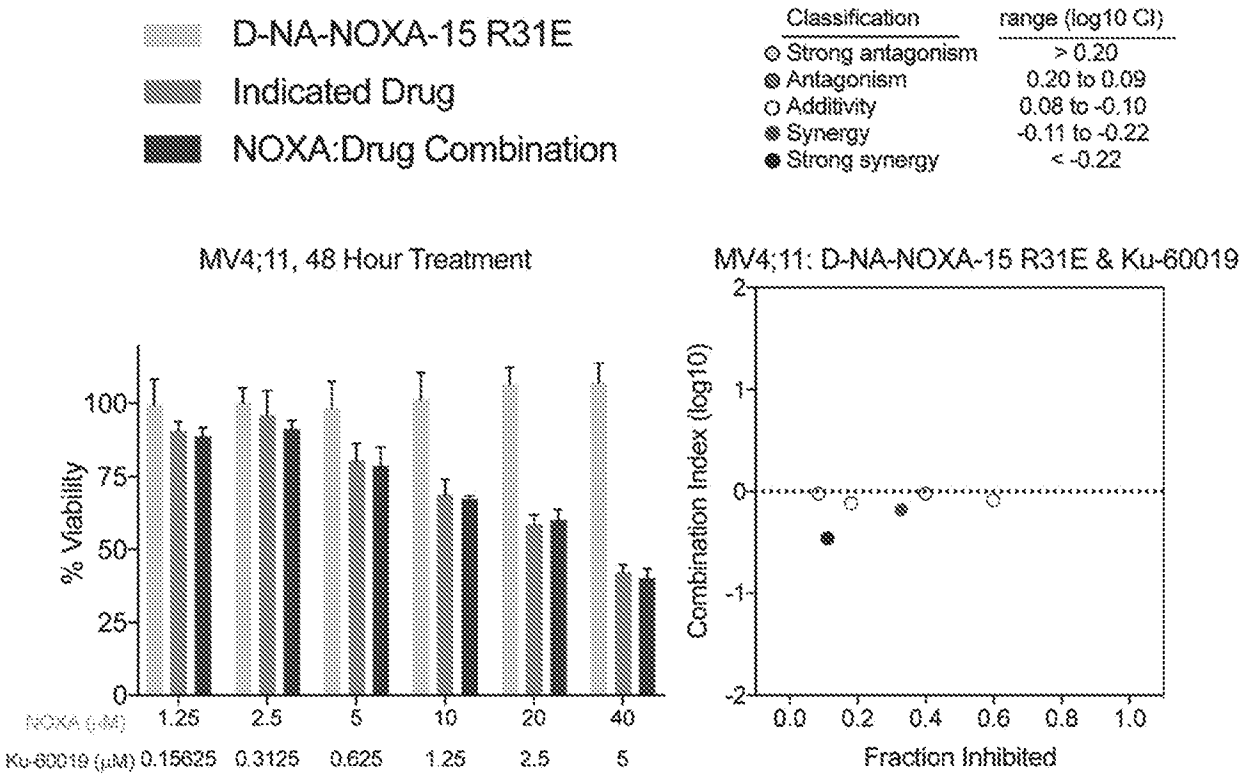

D-NA-NOXA-15 R31E
Indicated Drug
NOXA:Drug Combination
Classification
range (log10 CI)
Strong antagonism
> 0.20
Antagonism
0.20 to 0.09
Additivity
0.08 to -0.10
Synergy
-0.11 to -0.22
Strong synergy
< -0.22
MV4;11, 48 Hour Treatment
% Viability
100
75
50
25
0
NOXA (μM)
1.25
2.5
5
10
20
40
Ku-60019 (μM)
0.15625
0.3125
0.625
1.25
2.5
5
MV4;11: D-NA-NOXA-15 R31E & Ku-60019
Combination Index (log10)
2
1
0
-1
-2
0.0
0.2
0.4
0.6
0.8
1.0
Fraction Inhibited

FIG. 49

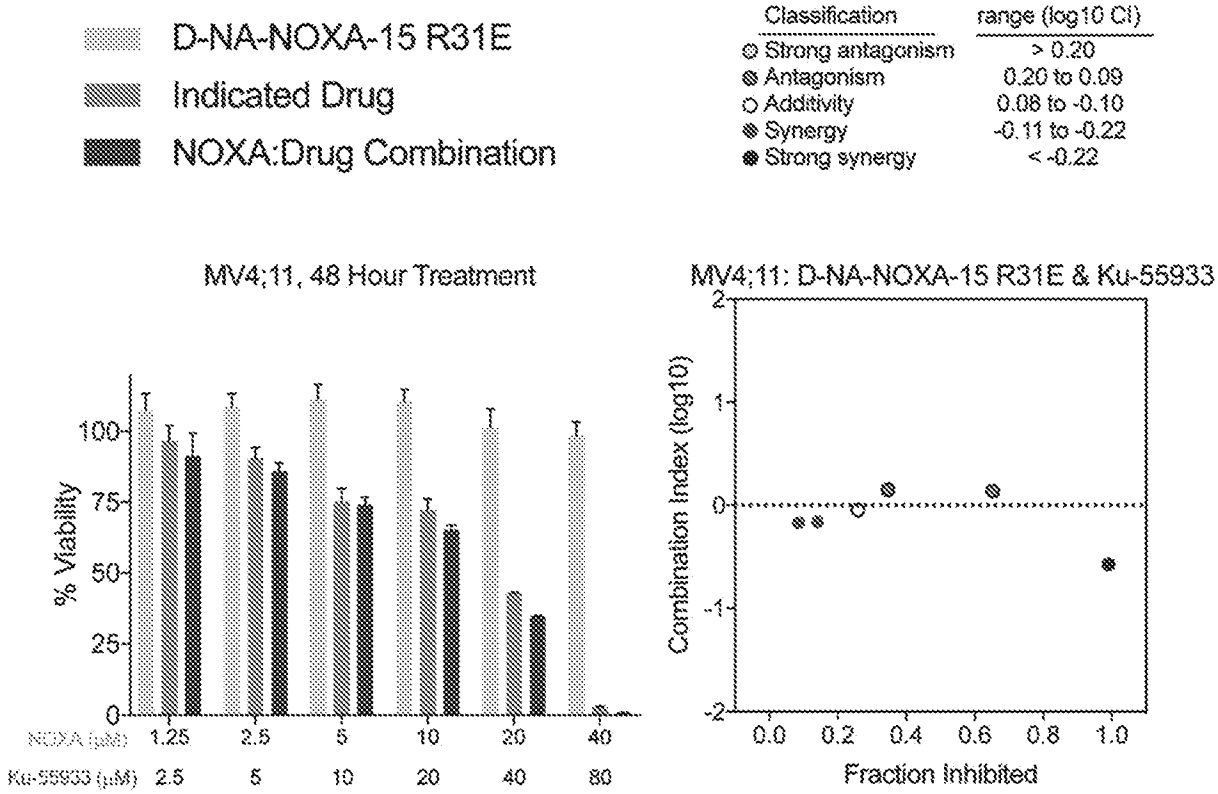

D-NA-NOXA-15 R31E
Indicated Drug
NOXA:Drug Combination
Classification
range (log10 CI)
Strong antagonism
> 0.20
Antagonism
0.20 to 0.09
Additivity
0.08 to -0.10
Synergy
-0.11 to -0.22
Strong synergy
< -0.22
MV4;11, 48 Hour Treatment
% Viability
100
75
50
25
0
NOXA (μM) 1.25 2.5 5 10 20 40
Ku-55933 (μM) 2.5 5 10 20 40 80
MV4;11: D-NA-NOXA-15 R31E & Ku-55933
Combination Index (log10)
2
1
0
-1
-2
0.0 0.2 0.4 0.6 0.8 1.0
Fraction Inhibited

FIG. 50

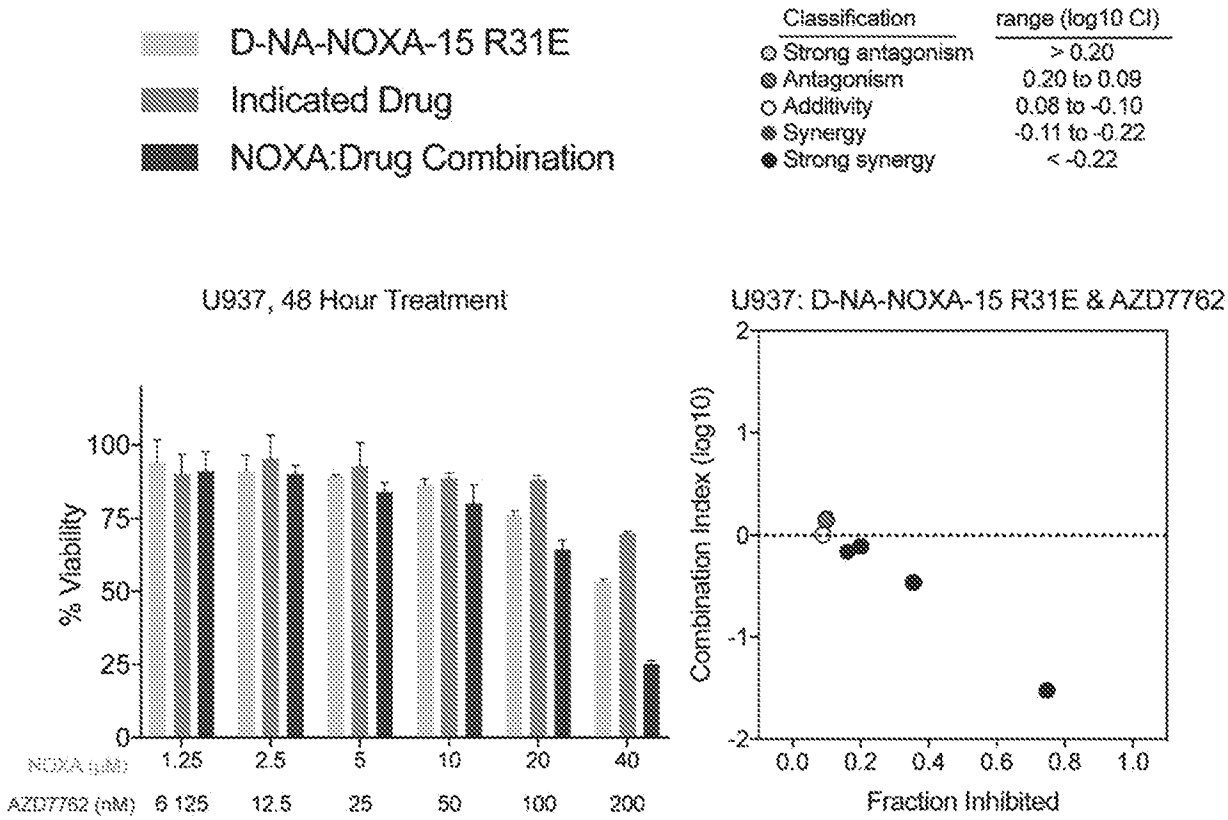

D-NA-NOXA-15 R31E
Indicated Drug
NOXA:Drug Combination
Classification
range (log10 CI)
Strong antagonism
> 0.20
Antagonism
0.20 to 0.09
Additivity
0.08 to -0.10
Synergy
-0.11 to -0.22
Strong synergy
< -0.22
U937, 48 Hour Treatment
% Viability
100
75
50
25
0
NOXA (μM)
1.25
2.5
5
10
20
40
AZD7762 (nM)
6.125
12.5
25
50
100
200
U937: D-NA-NOXA-15 R31E & AZD7762
Combination Index (log10)
2
1
0
-1
-2
0.0
0.2
0.4
0.6
0.8
1.0
Fraction Inhibited

FIG. 51

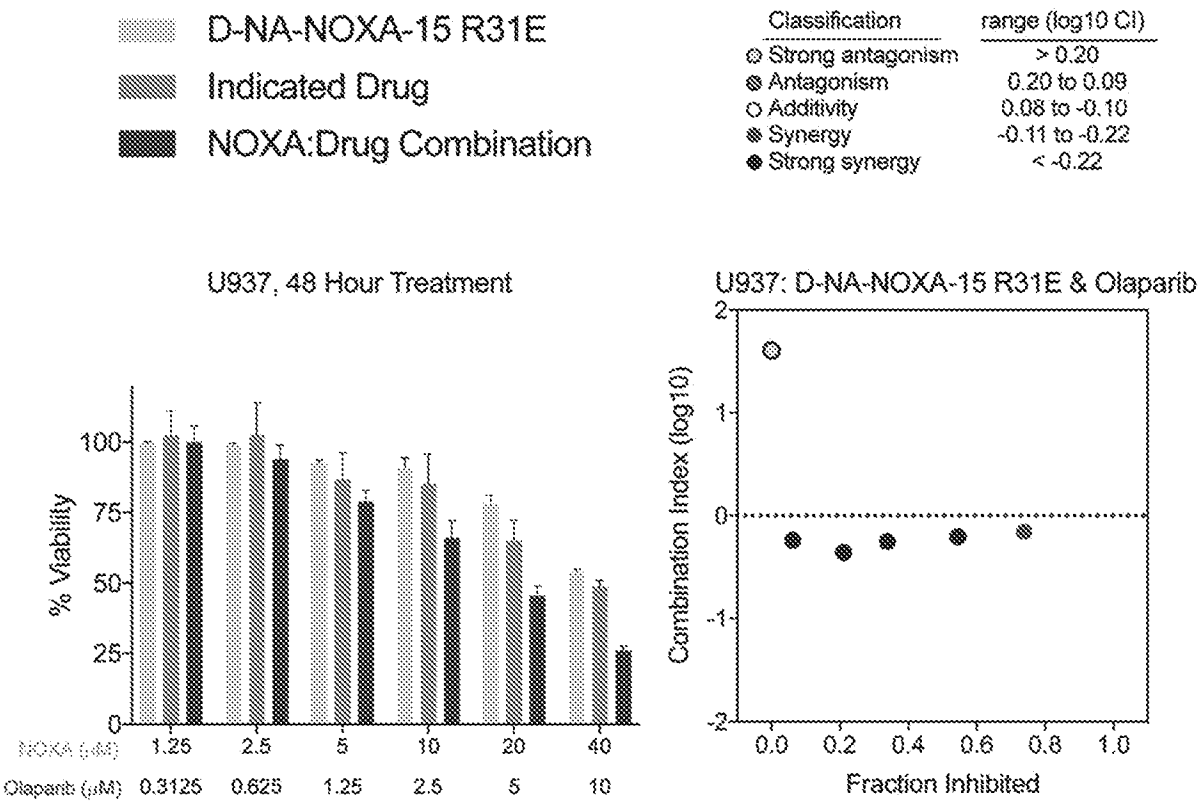

D-NA-NOXA-15 R31E
Indicated Drug
NOXA:Drug Combination
Classification
range (log10 CI)
Strong antagonism
> 0.20
Antagonism
0.20 to 0.09
Additivity
0.08 to -0.10
Synergy
-0.11 to -0.22
Strong synergy
< -0.22
U937, 48 Hour Treatment
% Viability
100
75
50
25
0
NOXA (μM)
1.25
2.5
5
10
20
40
Olaparib (μM)
0.3125
0.625
1.25
2.5
5
10
U937: D-NA-NOXA-15 R31E & Olaparib
Combination Index (log10)
2
1
0
-1
-2
0.0
0.2
0.4
0.6
0.8
1.0
Fraction Inhibited

FIG. 52

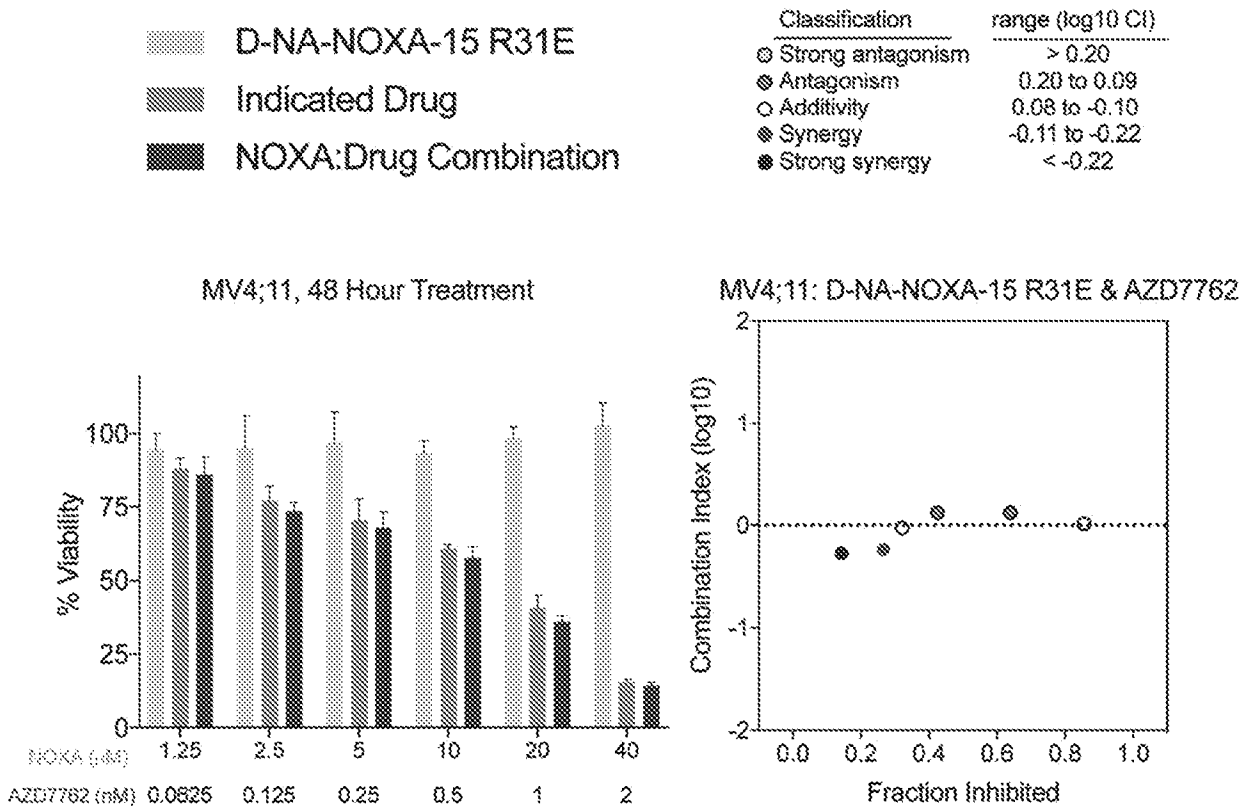
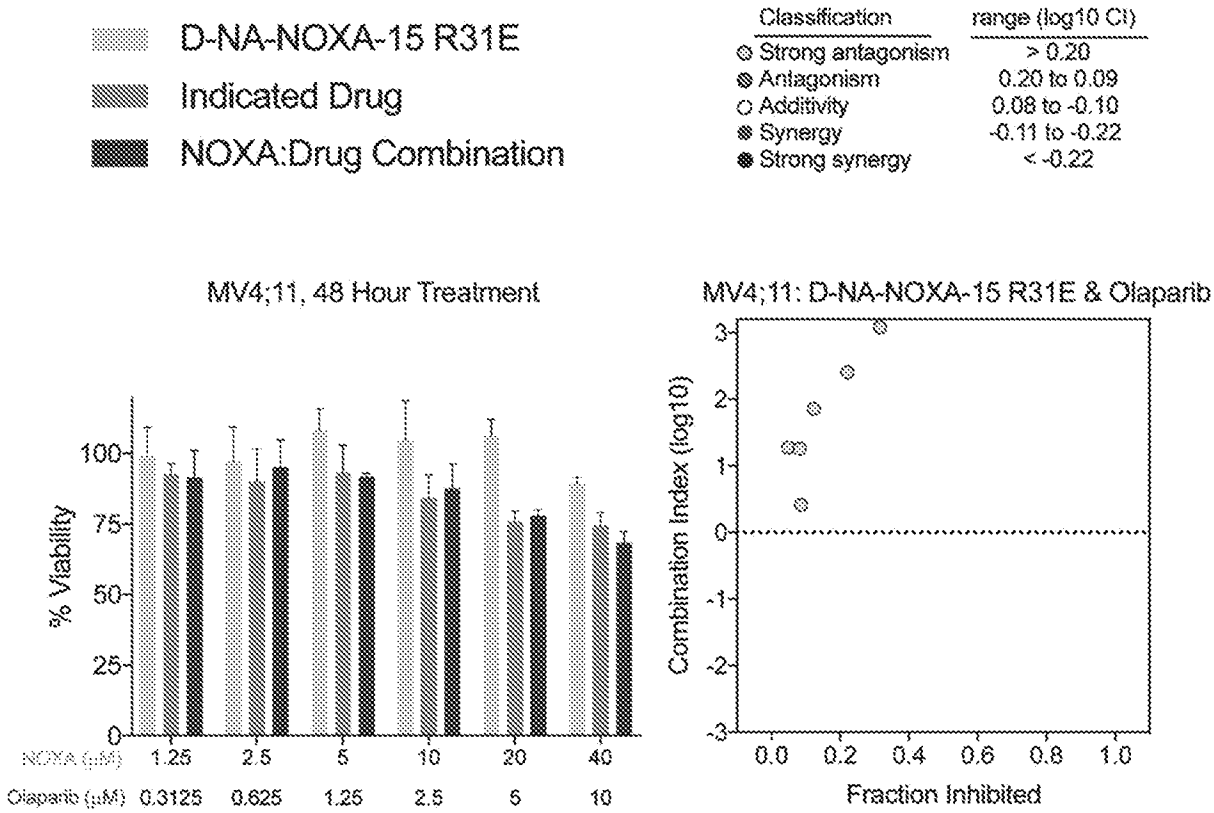

D-NA-NOXA-15 R31E
Indicated Drug
NOXA:Drug Combination
Classification
range (log10 CI)
Strong antagonism
> 0.20
Antagonism
0.20 to 0.09
Additivity
0.08 to -0.10
Synergy
-0.11 to -0.22
Strong synergy
< -0.22
MV4;11, 48 Hour Treatment
% Viability
100
75
50
25
0
NOXA (μM)
1.25
2.5
5
10
20
40
AZD7762 (nM)
0.0625
0.125
0.25
0.5
1
2
MV4;11: D-NA-NOXA-15 R31E & AZD7762
Combination Index (log10)
2
1
0
-1
-2
0.0
0.2
0.4
0.6
0.8
1.0
Fraction Inhibited

FIG. 53


D-NA-NOXA-15 R31E
Indicated Drug
NOXA:Drug Combination
Classification
range (log10 CI)
Strong antagonism
> 0.20
Antagonism
0.20 to 0.09
Additivity
0.08 to -0.10
Synergy
-0.11 to -0.22
Strong synergy
< -0.22
MV4;11, 48 Hour Treatment
% Viability
100
75
50
25
0
NOXA (μM)
1.25
2.5
5
10
20
40
Olaparib (μM)
0.3125
0.625
1.25
2.5
5
10
MV4;11: D-NA-NOXA-15 R31E & Olaparib
Combination Index (log10)
3
2
1
0
-1
-2
-3
0.0
0.2
0.4
0.6
0.8
1.0
Fraction Inhibited

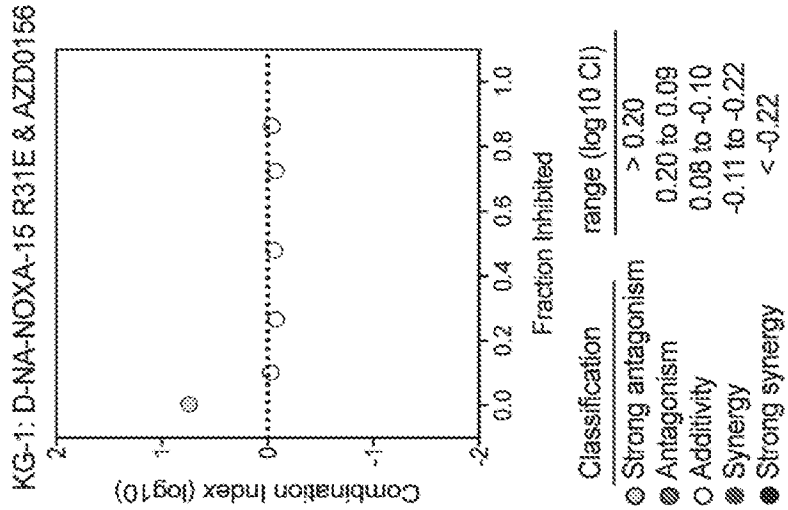


FIG. 54A

FIG. 54B

FIG. 54C

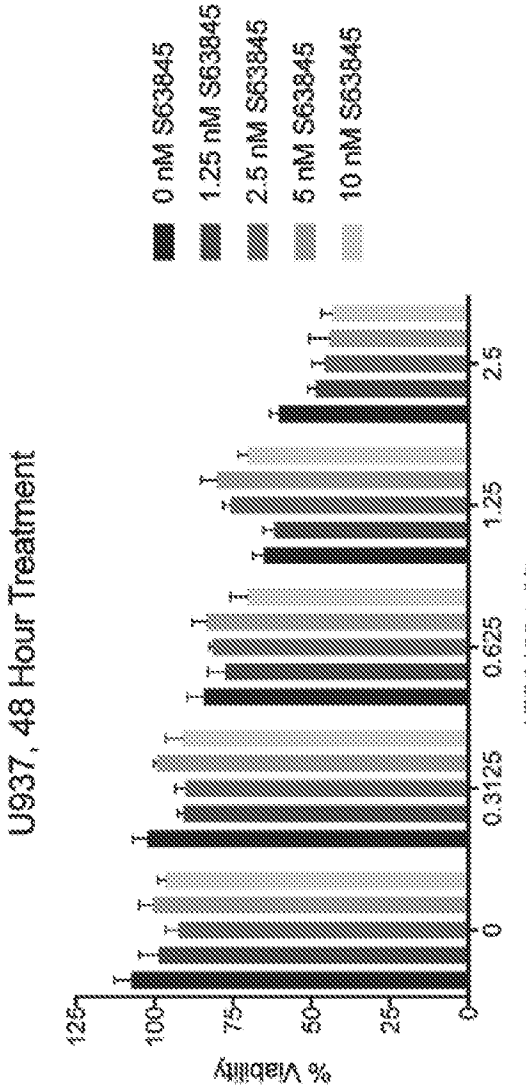


FIG. 55B

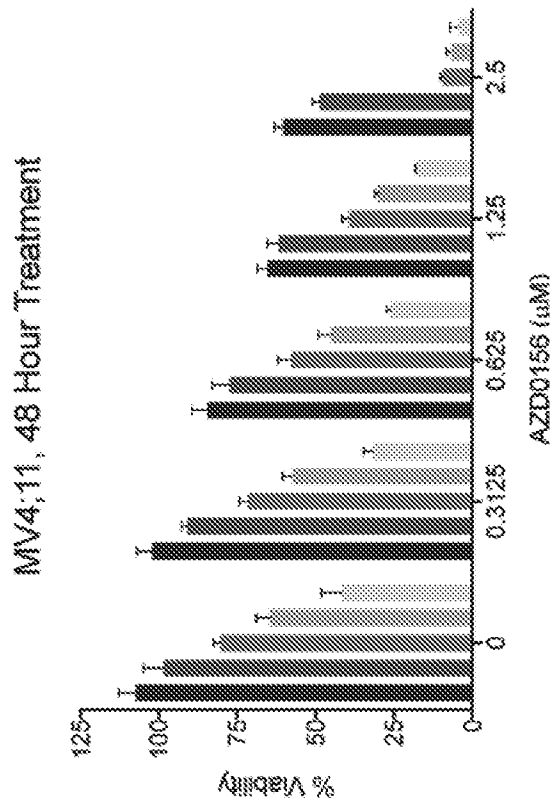


FIG. 55A

FIG. 56A

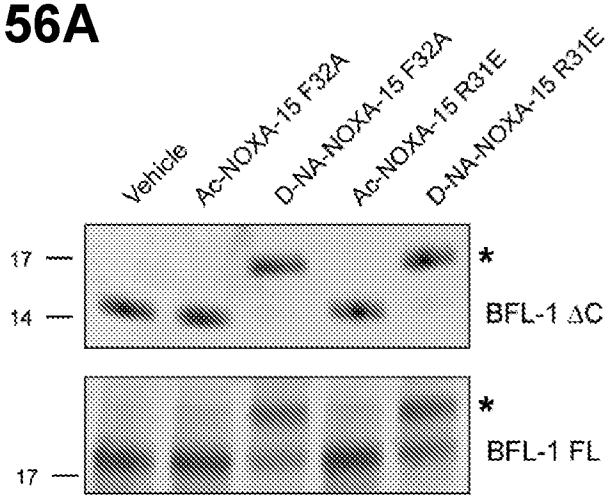


FIG. 56B

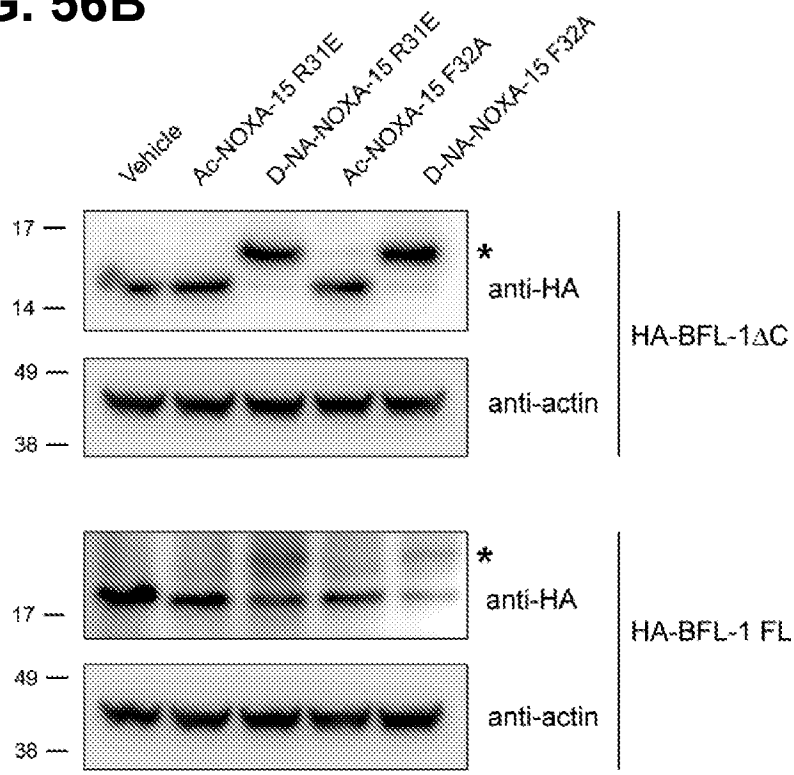
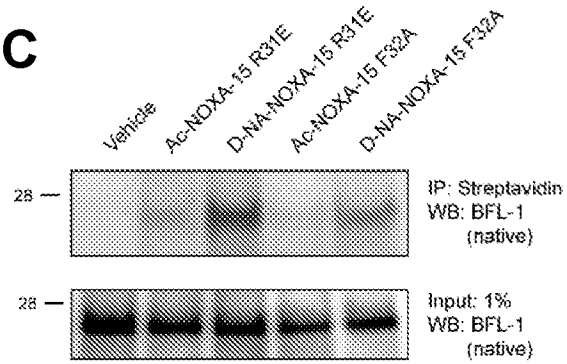


FIG. 56C



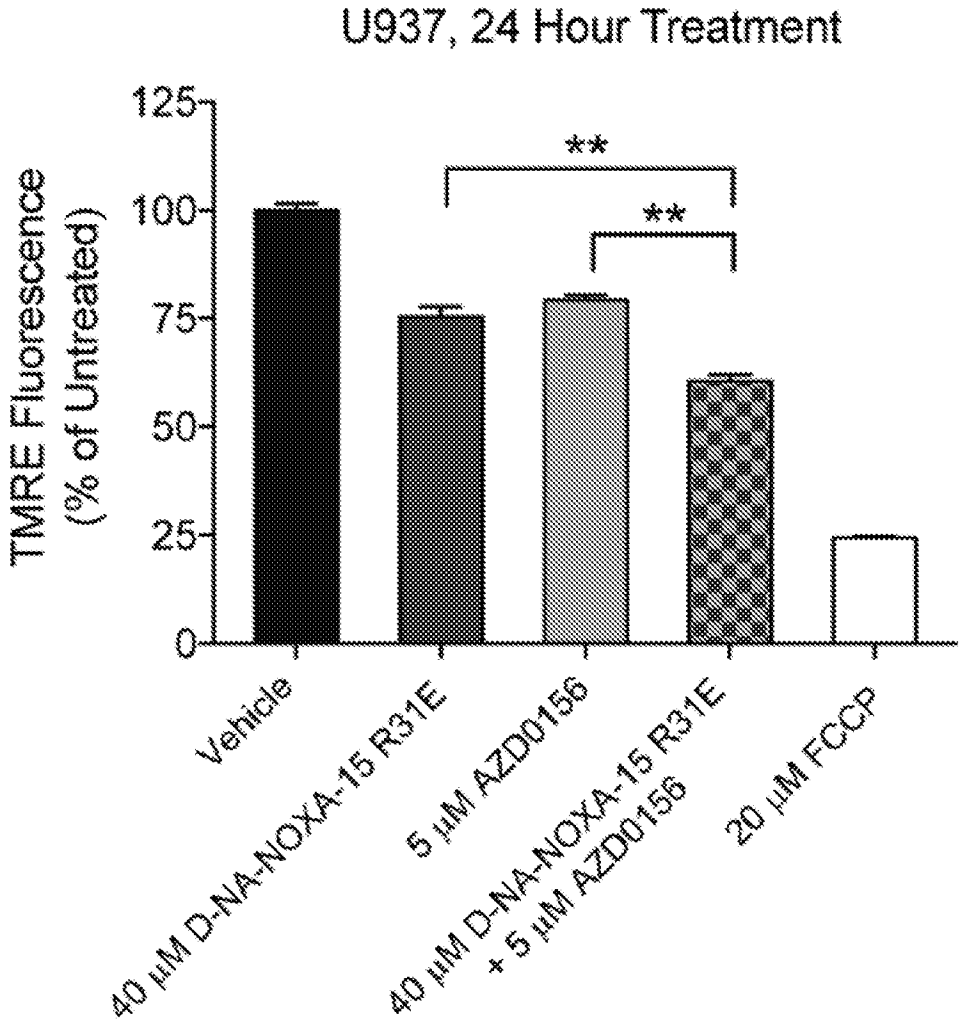

U937, 24 Hour Treatment
TMRE Fluorescence
(% of Untreated)
125
100
75
50
25
0
**
**
Vehicle
40 μM D-NA-NOXA-15 R31E
5 μM AZD0156
40 μM D-NA-NOXA-15 R31E
+ 5 μM AZD0156
20 μM FCCP

FIG. 57

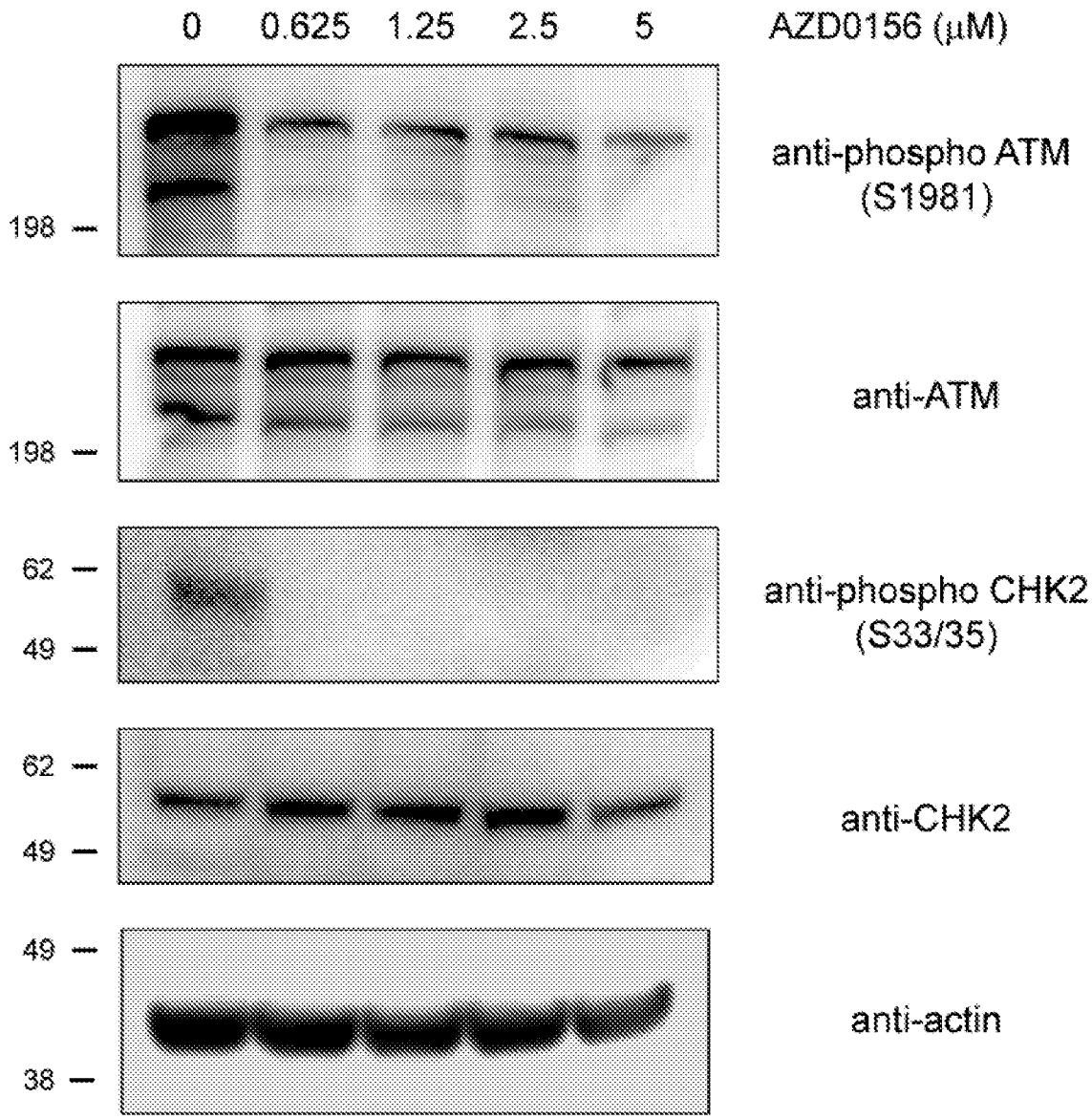

0
0.625
1.25
2.5
5
AZD0156 (μM)
anti-phospho ATM (S1981)
198
anti-ATM
198
62
49
anti-phospho CHK2 (S33/35)
62
49
anti-CHK2
49
38
anti-actin

FIG. 58

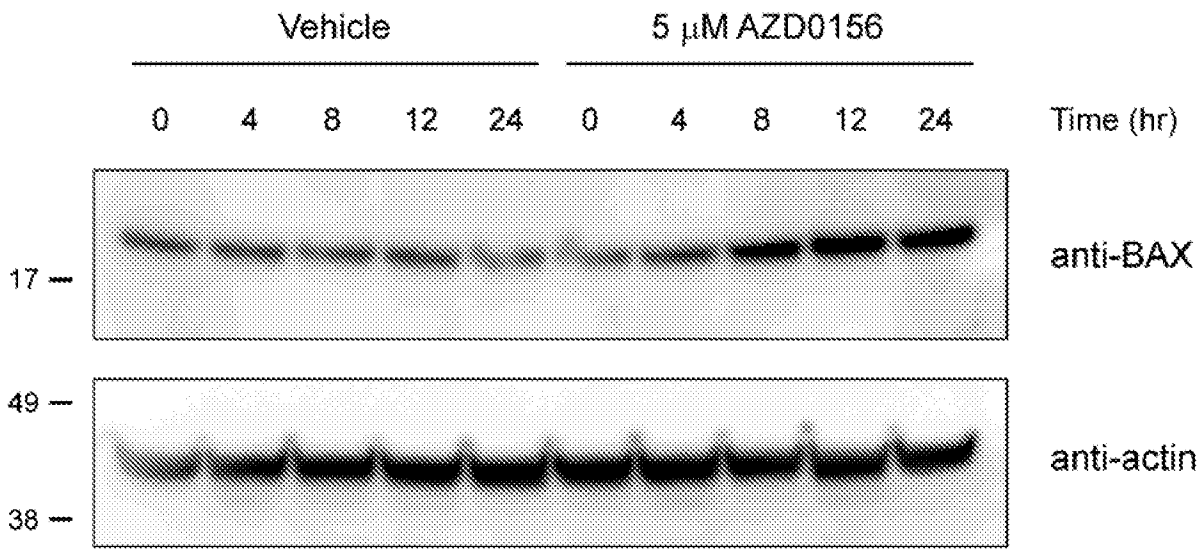
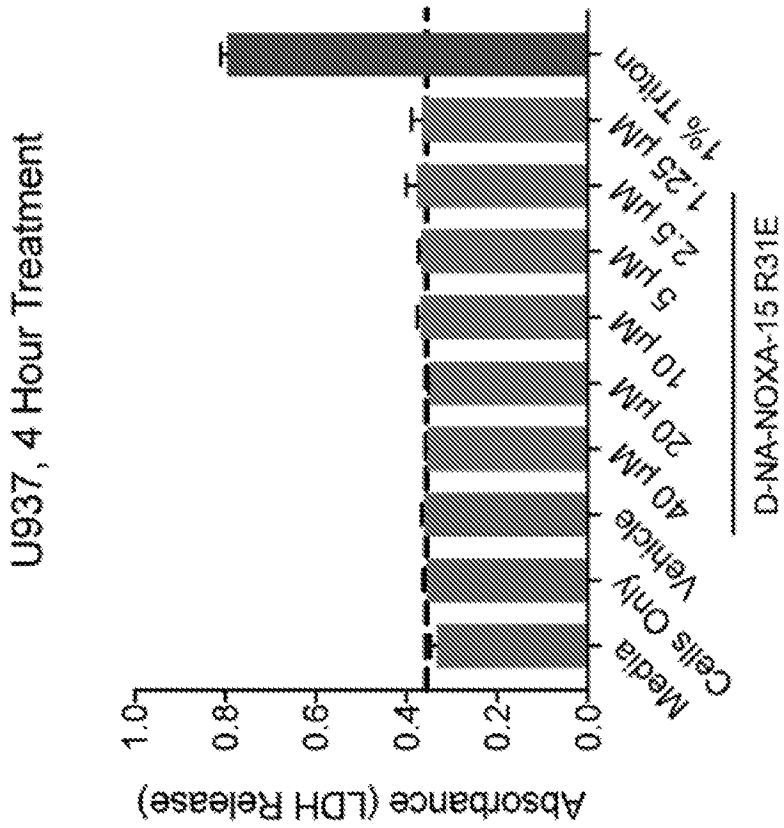

Vehicle
5 μM AZD0156
0 4 8 12 24 0 4 8 12 24
Time (hr)
17
anti-BAX
49
38
anti-actin

FIG. 59


U937, 4 Hour Treatment
Absorbance (LDH Release)
1.0
0.8
0.6
0.4
0.2
0.0
Media
Cells Only
Vehicle
40 µM
20 µM
10 µM
5 µM
2.5 µM
1.25 µM
1% Triton
D-NA-NOXA-15 R31E

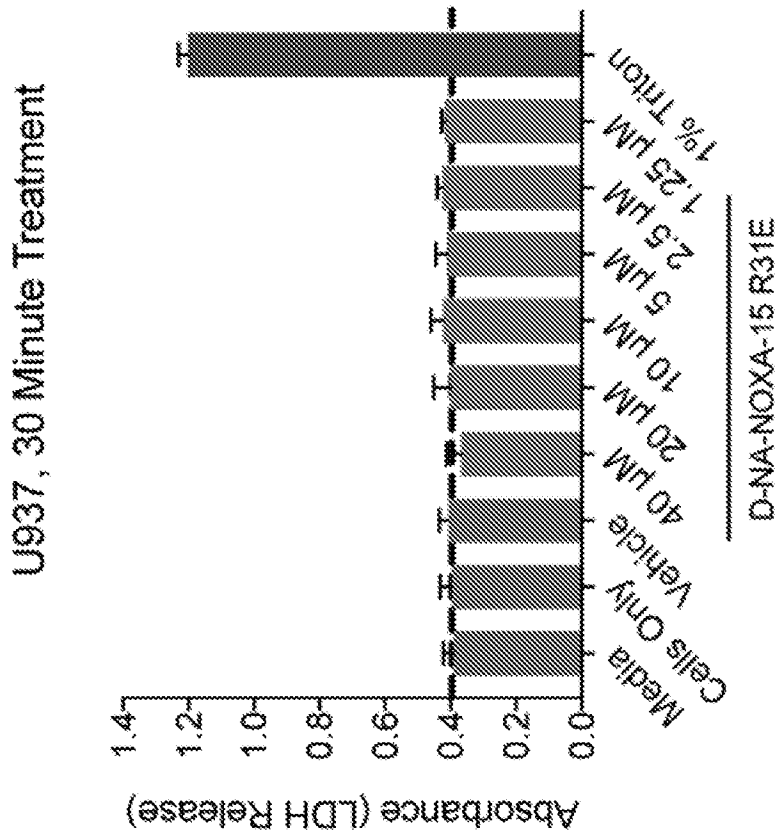

U937, 30 Minute Treatment
Absorbance (LDH Release)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Media
Cells Only
Vehicle
40 µM
20 µM
10 µM
5 µM
2.5 µM
1.25 µM
1% Triton
D-NA-NOXA-15 R31E

FIG. 60

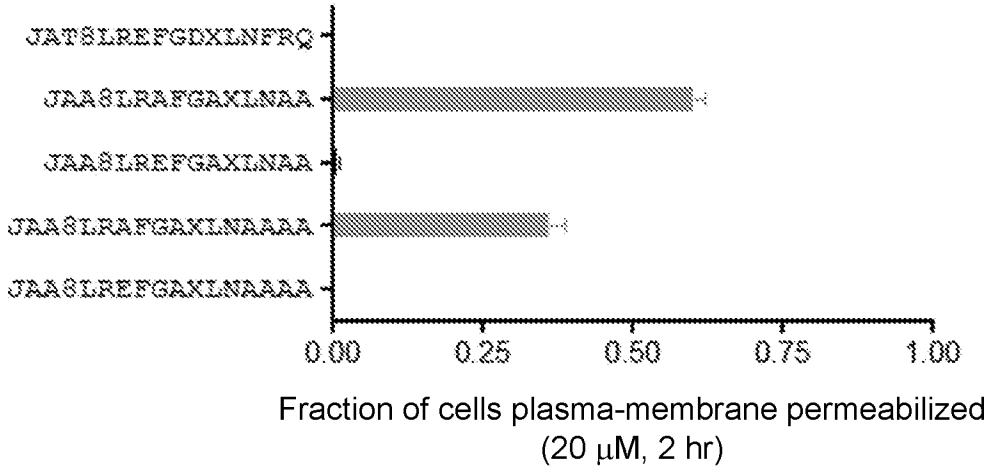

JATSLREFGDXLNFRQ
JAASLRAFGAXLNAA
JAASLREFGAXLNAA
JAASLRAFGAXLNAAAA
JAASLREFGAXLNAAAA
0.00
0.25
0.50
0.75
1.00
Fraction of cells plasma-membrane permeabilized
(20 μM, 2 hr)

FIG. 61

A

	BFL-1 Depedency Z-score	BFL-1 Scaled Rank
U937	-2.880572590	0.133559706
NB4	-2.137596737	0.187775891
THP1	-1.558836432	0.164799095
P31FUJ	-1.255901014	0.258064516
TF1	-0.960518814	0.304753820
MOLM13	-0.323784907	0.419524618
NOMO1	0.276081075	0.536672326
MV4;11	0.498330955	0.563950198

B

Cell Line	Cancer Type	BFL-1 Dependency Z-Score	BFL-1 Scaled Rank
DU4475	Breast	-5.145431813	0.090548953
IMR32	Autonomic Ganglia	-4.058526463	0.116525184
PSN1	Pancreas	-3.975630695	0.134352009
COLO679	Skin	-3.494531252	0.140350877
U178	CNS	-3.468540224	0.153027731
NB1	Autonomic Ganglia	-3.247641011	0.178494624
ASPC1	Pancreas	-3.112237399	0.147934352
U787MG	CNS	-2.963316233	0.163893605
U937	Hematopoetic/Lymphoid	-2.880572590	0.133559706
HT144	Skin	-2.796976029	0.200565931
SKMEL30	Skin	-2.667202852	0.160611205
NCIH2110	Lung	-2.516536028	0.182795699
MDST8	Large Intestine	-2.470023481	0.178720996
NCIH2122	Lung	-2.465532621	0.182229768
NCIN87	Stomach	-2.370858183	0.218222977
MORCPR	Lung	-2.161270828	0.210526316
NB4	Hematopoetic/Lymphoid	-2.137596737	0.187775891
UACC62	Skin	-2.111832692	0.171533673
KELLY	Autonomic Ganglia	-2.071612986	0.177589134
SKNMC	Bone	-2.043786655	0.213186191

C

	Pearson Correlation Coefficient	Spearman Correlation Coefficient
BFL1	1	1
DCTPP1	0.941102590	0.928571429
ATM	0.937773368	0.976190476
GEN1	0.936089024	0.904761905
ASCC1	0.934906261	0.928571429
NSUN6	0.916185332	0.904761905
FBXO42	0.911441405	0.904761905
DYNC2LI1	0.907170948	0.904761905
JRKL	0.904267905	0.928571429
C3orf62	0.902864788	0.904761905

FIG. 62

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2018/065438

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K38/17 A61M37/00 C07K14/47
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K A61M C07K

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS, Sequence Search, EMBASE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2017/040329 A2 (DANA-FARBER CANCER INST INC [US]) 9 March 2017 (2017-03-09) The query sequence SEQ ID NO:39 has 100.00 % identity (100.00 % similarity) over 15 positions in a common overlap (range (q:s): 1-15:8-22) with subject GSP:BD076622 (length: 25) from WO2017040323-A2 published on 2017-03-09.; figures 1-25	1-24

X	WO 2017/040323 A2 (DANA-FARBER CANCER INST INC [US]) 9 March 2017 (2017-03-09) figures 1-29	1-24

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 May 2019	22/05/2019

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Seranski, Peter

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2018/065438

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 25-168
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

Continuation of Box II.2

Claims Nos.: 25-168

The present application contains 168 claims, of which 23 are independent. There is no clear distinction between the independent claims because of overlapping scope. There are so many claims, and they are drafted in such a way that the claims as a whole are not in compliance with the provisions of clarity and conciseness of Article 6 PCT, as it is particularly burdensome for a skilled person to establish the subject-matter for which protection is sought.
The reasons are as
follows: The claims are directed in a first aspect to a multitude of peptides (see independent claims 1, 25, 40, 52, 70, 76, 134, 145), in asecond aspect to a multitude of different methods of treatment (independent claims 89, 99, 124, 144, 155) to a multitude of Pharmaceutical compositions (independent claims 88, 141, 152) and in a last aspect to a multitude of Combination Therapies (independent claims 113, 142, 153, 156, 158, 160, 162)
The non-compliance with the
substantive provisions is to such an extent that a meaningful search of the whole claimed subject-matter could not be carried out (Article 17(2) PCT and PCT Guidelines 9.30).

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guidelines C-IV, 7.2), should the problems which led to the Article 17(2) declaration be overcome.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2018/065438

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		EP 3341008 A2	04-07-2018
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