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(54) Title: STABILIZED EZH2 PEPTIDES

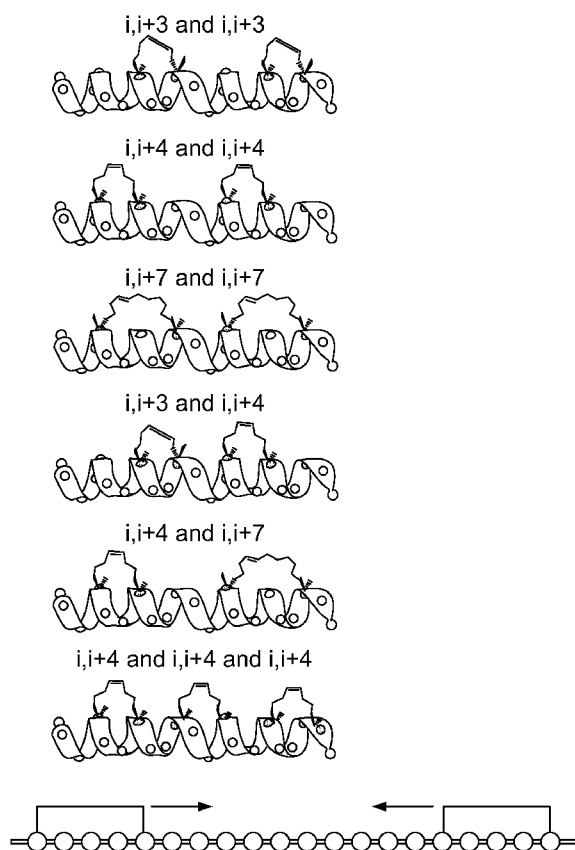


FIG. 1C

(57) Abstract: Provided herein are polypeptides containing stabilized therapeutic peptides related to enhancer of zeste homolog 2 (EZH2), histone lysine N-methyltransferase. Also provided are compositions containing these polypeptides and methods of using such peptides in the treatment of cancer that include administering to a subject one of the polypeptides.



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STABILIZED EZH2 PEPTIDES

CLAIM OF PRIORITY

This application claims the benefit of, and incorporates by reference, U.S. Provisional Patent Applications Nos. 61/790,023, filed on March 15, 2013.

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TECHNICAL FIELD

This disclosure relates to structurally stabilized therapeutic peptides related to enhancer of zeste homolog 2 (EZH2), histone lysine N-methyltransferase and methods of using such peptides in the treatment of cancer.

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SEQUENCE LISTING

The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on March 12, 2014, is named 00530-0309WO1_SL.txt and is 30,362 bytes in size.

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BACKGROUND

Enhancer of zeste homolog2 (EZH2) is a SET-domain-containing histone lysine N-methyltransferase that participates in the epigenetic regulation of cell lineage determination and homeostasis. EZH2 forms the Polycomb repressive complex2 (PRC2) with embryonic ectoderm development (EED) and suppressor of zeste 12 homolog (SUZ12) to catalyze histone H3 Lys27 trimethylation, and hyperactivity of this enzymatic function has been linked to aberrant repression of tumor suppressor genes in diverse cancers.

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Epigenetic modifications of DNA and histones by a variety of protein complexes produce combinatorial codes of chromatin modification, amplifying the

complexity of how genetically encoded information is employed. Such epigenetic information is decoded by "reader" proteins that, in conjunction with transcription factors, regulate the differential expression of genes during development and homeostasis. Two broad classes of protein complexes, Trithorax (trxG) and Polycomb (PcG), are responsible for the deposition of histone marks that dictate whether a gene is activated or repressed, respectively¹⁻⁵. TrxG generally maintains an 'on state' of gene expression by catalyzing methylation of Lys4 of Histone H3 (H3K4), while PcG maintains an 'off state' by catalyzing trimethylation of Lys27 of Histone H3 (H3K27). In mammals, there are two distinct PcG complexes, Polycomb repressive complex 1 (PRC1) and Polycomb repressive complex 2 (PRC2). PRC2 catalyzes trimethylation of H3K27 and, at certain sites, facilitates the recruitment of PRC1 to methylated histones to repress target genes^{3,4}. PRC2 is composed of three essential core components, enhancer of zeste homolog 2 (EZH2), suppressor of zeste 12 (SUZ12) and embryonic ectoderm development (EED). The conserved suppressor of variegation, enhancer of zeste, trithorax (SET) domain of EZH2 represents the active site for catalysis of H3K27 methylation⁶.

In addition to the established roles of the epigenetic machinery in cell homeostasis and development, recent studies have implicated discrete protein subcomponents, such as EZH2, in the pathogenesis of diverse cancers⁷⁻¹². EZH2 overexpression has been linked to repression of tumor suppressor genes and derepression of genes involved in metastasis¹³⁻¹⁶. In certain cancers, this EZH2 deregulation has been linked to pathologic alterations in microRNA levels¹⁷⁻²⁰. Somatic mutations that alter the substrate specificity and functional activity of EZH2 have also been found in B cell non-Hodgkin's lymphoma²¹⁻²⁴. Correspondingly, reduced expression of EZH2 by shRNA or siRNA induces proliferative arrest in cancer cell lines that overexpress EZH2^{25,26}. The genetic ablation of *Ezh2* alone prevents the development of a murine T cell lymphoma that results from inactivation of Snf5, a core component of the Swi/Snf remodeling complex²⁷. Collectively, these findings implicate EZH2 deregulation in the development and maintenance of cancer.

SUMMARY

The present disclosure provides structurally stabilized peptides related to (e.g., sharing sequence homology with) portions or fragments of Enhancer of zeste

homolog2 (EZH2), and methods for using such stabilized peptides as therapeutic and/or prophylactic agents.

In some aspects, the present disclosure provides internally cross-linked polypeptides comprising the amino acid sequence A₀B₀C₀D₀E₀F₀G₀A₁B₁C₁D₁E₁F₁G₁
 5 A₂B₂C₂D₂E₂F₂G₂A₃B₃C₃D₃E₃F₃G₃A₄ (SEQ ID NO:1) wherein A₀ is S or a conservative substitution; B₀ is M or a conservative substitution; C₀ is F or a conservative substitution; D₀ is S or a conservative substitution; E₀ is S or a conservative substitution; F₀ is N or a conservative substitution; G₀ is R or a conservative substitution; A₁ is Q or a conservative substitution or a staple to E₁; B₁
 10 is K or a conservative substitution; C₁ is I or a conservative substitution; D₁ is L or a conservative substitution; E₁ is E, Q or a conservative substitution or a staple to A₁; F₁ is R or a conservative substitution; G₁ is T or a conservative substitution; A₂ is E, Q or a conservative substitution; B₂ is I or a conservative substitution; C₂ is L or a conservative substitution; D₂ is N or a conservative substitution; E₂ is Q or a conservative substitution; F₂ is E, Q or a conservative substitution; G₂ is W or a conservative substitution; A₃ is K or a conservative substitution; B₃ is Q or a conservative substitution; C₃ is R or a conservative substitution; D₃ is R or a conservative substitution; E₃ is I or a conservative substitution; F₃ is Q or a conservative substitution; G₃ is P or a conservative substitution; A₄ is V or a conservative substitution; 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 internal staples and/or an internal stitch; the side chains of four amino acids are replaced by internal staples, internal stitches, or a combination of internal staples and stitches; or the side chains of at least four amino
 20 acids are replaced by internal staples, internal stitches, or a combination of internal staples and stitches. In some embodiments C₀, F₀, G₀, B₁, C₁, F₁, C₂, D₂, F₂, G₂, C₃, E₃, G₃ and A₄ are not substituted. Thus, in some embodiments, these residues are F, N, R, K, I, R, L, N, E, W, R, I, P and V, respectively.

In some embodiments, internally cross-linked polypeptides of the disclosure include
 30 the sequence SMFSSNRQKILERTEILNQEWKQRRIQPV (SEQ ID NO:2), wherein: none, one or more (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18) of the amino acids are replaced by a conservative amino acid substitution or, in some

cases, a conservative substitution that does not alter the binding face of the peptide. In some cases, the internally cross-linked peptide is at least 80% (e.g., 80%, 85%, 90%, 95%, 98%, 99%, 99.5%, or 100%) identical to the sequence of SEQ ID NO:2. The amino acid side chains that are replaced by a cross-link are consider non-identical
 5 in calculating percent identity.

In some embodiments, internally cross-linked polypeptides of the disclosure comprises:

SMFSSNRQKILERTEILNQEWKQRRIQPV (SEQ ID NO:2);
 FSSNRQKILERTEILNQEWKQRRIQPV (SEQ ID NO:40),
 10 FSSNRQKILERTQILNQEWKQRRIQPV (SEQ ID NO:41),
 RRSMFSSNRQKILERTEILNQEWKQRRIQPV (SEQ ID NO:42),
 RRFSSNRQKILERTEILNQEWKQRRIQPV (SEQ ID NO:43),
 RRFSSNRQKILERTQILNQEWKQRRIQPV (SEQ ID NO:44),
 RSMFSSNRQKILERTEILNQEWKQRRIQPV (SEQ ID NO:45),
 15 RFSSNRQKILERTEILNQEWKQRRIQPV (SEQ ID NO:46),
 RFSSNRQKILERTQILNQEWKQRRIQPV (SEQ ID NO:47),
 SMFSSNRQKILERTEILNQEWKQRRIQPVRR (SEQ ID NO:48),
 FSSNRQKILERTEILNQEWKQRRIQPVRR (SEQ ID NO:49),
 FSSNRQKILERTQILNQEWKQRRIQPVRR (SEQ ID NO:50),
 20 SMFSSNRQKILERTEILNQEWKQRRIQPVRR (SEQ ID NO:51),
 FSSNRQKILERTEILNQEWKQRRIQPVRR (SEQ ID NO:52), and
 FSSNRQKILERTQILNQEWKQRRIQPVRR (SEQ ID NO:53),

wherein the side chains of two amino acids separated by three or six amino acids comprises an internal staple selected from Table 1 (**FIG 43**). In addition, 1, 2, 3,
 25 4, 5, 6, 7, 8, 9, 10, 11, 12 or 13 of the amino acids can be substituted, preferably conservatively. Within the sequence:

S M F S S N R Q K I L E R T E I L N Q E W K Q R R I Q P V (SEQ ID NO:2) the underlined residues are preferably not substituted. In some embodiments there is an internal cross-link (staple) between 8th and 12th amino acids of SEQ ID NO:1 (i.e., the
 30 side chains of these residues is replaced by a staple. In SEQ ID NO:1, there is preferably an internal cross-link (staple) between A₁ and E₁. Some cases the E in SEQ ID NO:2

(or SEQ ID NO:1) are replaced by Q. In some cases the stabilized peptides do not include any E and in some cases have a get positive charge.

In some embodiments negatively charged residues in any of the stabilized peptides are replaced by Q or R.

5 In some embodiments an RR dipeptide is appended to the amino or carboxy terminus of the stapled peptide.

In preferred embodiments, the staple is an alkyl or alkynyl.

In some embodiments, internally cross-linked polypeptides of the disclosure include an internal staple replacing the side chains of two amino acids separated by
10 three or six amino acids comprises an internal staple selected from Table 1 (**FIG 43**). In some embodiments, internally cross-linked peptides are selected from the group consisting of SEQ ID NOs:2-39. In some embodiments, the internal staples and/or the internal stitch replacing the side chains of the three amino acids includes an internal stitch selected from Table 1 (**FIG 43**). In some embodiments, the internal
15 staples and/or the internal stitch comprises at least two internal staples (replacing the side chains of 4 amino acids, i.e., each staple is between two amino acids separated by 3 amino acids). In some embodiments, the internal staples and/or the internal stitch comprises 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
20 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
25 acids. In some embodiments, the internal stitch replacing the side chains of the three amino acids cross-links a pair of amino acids separated by two, three, or six 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
30 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 polypeptides of the disclosure are selected from the group consisting of SEQ ID NOs:3-41. In some embodiments, internally cross-linked polypeptides of the disclosure include internal staples, internal stitches, or a combination of internal staples and internal stitches replacing the side chains of at least four amino acids, such as at least one staple and at least one stitch. In some embodiments, the at least one staple cross-links a pair of amino acids separated by two, three, or six amino acids and the at least one stitch cross-links a first amino acid to a second amino acid and a third amino acid, 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, such staples are selected from Table 1 (**FIG 43**).

In some aspects, the disclosure provides pharmaceutical compositions that include one or more internally cross-linked polypeptides of the disclosure. In some embodiments, such pharmaceutical compositions can also include one or more medicaments for the treatment of cancer and/or the alleviation of one or more symptoms associated with cancer.

In some aspects, the disclosure provides kits for identifying agents that interact with a stabilized EZH2 peptide. Such kits can include one or more internally cross-linked polypeptides of the disclosure and EED or a portion of EED that binds EZH2.

In some aspects the disclosure provides a method for identifying compounds that disrupts the interaction of EZH2 with EED the method comprising contacting test compound with a stabilized peptide described herein (e.g., one of SEQ ID NOS: 3-41, preferably SEQ ID NO:27); and detecting the level of binding between the stabilized peptide and the EED peptide the presence of an test agent, wherein a change in the level of binding between the stabilized peptide and the EED peptide indicates that the agent is a candidate agent that binds to the EED and decreases binding to EZH2. In some embodiments, these methods can include selecting the candidate agent, and optionally, administering the candidate agent to an animal model of a cancer, e.g., to determine if the agent inhibits the cancer.

In some aspects, the disclosure provides methods for treating cancer in a subject. These methods can include selecting a subject suffering from cancer; and

administering to the subject an effective amount of the stabilized peptides of claims described herein. In some embodiments, methods include assessing a level of EZH2 in the subject before and after treatment; and continuing treatment until a decrease in the level EZH2 is observed.

5 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. Methods and materials are described herein for use in the present invention; other, suitable methods and materials known in the art can also be used. The materials, methods, and examples are illustrative only and not intended
10 to be limiting. All publications, patent applications, patents, sequences, database entries, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control.

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

DESCRIPTION OF THE DRAWINGS

FIG 1A | Examples of non-natural amino acids containing olefinic tethers that can be used to generated hydrocarbon stapled EZH2 peptides.

FIG 1B | Examples of single staple compositions spanning i, i+3; i, i+4, and i,
20 i+7 positions for singly stapled EZH2 peptides.

FIG 1C | Examples of staple compositions for multiply stapled EZH2 peptides.

FIG 1D | Examples of staple compositions for tandemly stapled EZH2 peptides.

25 **FIG 2** | The location of stapling position 'A' (47-51) is marked with red spheres and position 'C' (54-58) is marked with blue spheres. The alpha-helical EED-binding domain of EZH2 is colored gold and EED is colored gray. The native methionine of SAH-EZH2 was replaced with norleucine ('N_L') due to decreased efficiency of the metathesis reaction in the presence of sulfur. Insertion of single (i,
30 i+3), (i, i+4), or (i, i+7) staples, and multiple staples in combinations thereof, are also envisioned. Sequences "A" are disclosed as SEQ ID NOS 3, 26 and 19, respectively,

in order of appearance and Sequences "C" are disclosed as SEQ ID NOS 5, 33 and 20, respectively, in order of appearance.

FIG 3 | Circular dichroism analysis of EZH2(42-68), SAH-EZH2_A(42-68) and SAH-EZH2_C(42-68) peptides, demonstrating structural stabilization by hydrocarbon stapling.

FIG 4 | Binding affinity of SAH-EZH2 peptides for EED as measured by fluorescence polarization. Data represents mean $\pm$ s. e. m. for experiments performed in triplicate.

FIG 5 | Co-precipitation of purified HA-EED (40 nM final concentration) with FITC-labeled SAH-EZH2 peptides.

FIG 6 | Cellular penetrance of SAH-EZH2 peptides. Lysates of treated MLL-AF9 leukemia cells were electrophoresed and analyzed by fluorescence scan.

FIG 7 | Design of an optimized SAH-EZH2 peptide (SAH-EZH2_A[42-68]E54Q) and a negative control derivative (SAH-EZH2_A [42-68]E54Q/E59Q) by glutamate mutagenesis. Sequences are disclosed as SEQ ID NOS 40 and 26-28, respectively, in order of appearance.

FIG 8 | Enhanced cellular penetrance of E54Q and E54Q/E59Q mutants of SAH-EZH2_A(42-68), as measured by fluorescence scan of lysates from FITC-peptide treated MLL-AF9 leukemia cells.

FIG 9 | EED-binding affinity of SAH-EZH2_A E54Q (SAH-EZH2) and SAH-EZH2_A E54Q/E59Q (SAH-EZH2mut) peptides, as measured by fluorescence polarization. Data represent mean $\pm$ s.e.m. for experiments performed in triplicate.

FIG 10 | Comparative binding of SAH-EZH2 glutamate mutants to purified HA-EED (40 nM final concentration) as assessed by anti-FITC pull-down and EED western analysis.

FIG 11 | Localization of EED (red) and SAH-EZH2_A (42-68) E54Q and E54Q/E59Q peptides (green) to the nuclei (DAPI, blue) of COS-7 cells. Whereas the FITC-labeled stapled peptides demonstrate robust cellular uptake, the corresponding unmodified peptide shows no cellular penetrance.

FIG 12 | Binding of SAH-EZH2_A (42-68)E54Q (designated SAH-EZH2), but not SAH-EZH2_A (42-68)E54Q/E59Q (designated SAH-EZH2_{MUT}), to native EED, as

assessed by anti-FITC immunoprecipitation from peptide-treated MLL-AF9 cell culture and EED western analysis.

FIG 13 | Cellular uptake and intracellular stability of SAH-EZH2_A peptides.

(a) SAH-EZH2_A peptides manifest a markedly increased propensity for cellular uptake compared to the corresponding unmodified peptide, with E54Q and E54Q/E59Q mutagenesis further enhancing uptake as reflected by the increased cellular fluorescence. Appending the cell penetrating sequence TAT to the unmodified EZH2 peptide likewise enhances peptide uptake, as measured by cellular fluorescence at 8 hours. (b) Analysis of cellular lysates by electrophoresis and fluorescence scan over time reveals that SAH-EZH2 peptides (SEQ ID NOS 40, 26-28 and 54, respectively, in order of appearance) persist at high levels in the cell, whereas intact TAT-EZH2 is not detected by the 2 hour timepoint. These data indicate that SAH-EZH2 peptides achieve rapid cellular uptake and remain fully intact. Thus, in contrast to SAH-EZH2 peptides, the TAT-EZH2 signal observed by fluorescence at 8 hours (a) reflects the presence of FITC, but not the fully intact peptide.

FIG 14 | Co-immunoprecipitation of HA-EED with FLAG-EZH1/FLAG-EZH2 by anti-FLAG agarose (coomassie blue staining). HA-EED was confirmed by HA-antibody western analysis (data not shown).

FIG 15 | Dissociation of HA-EED/EZH1 and HA-EED/EZH2 complexes (40 nM final concentration) by SAH-EZH2, but not SAH-EZH2_{MUT} as assessed by anti-HA pull-down and EZH1/EZH2 western analysis.

FIG 16 | Selective reduction of H3K27Me3 by SAH-EZH2, as assessed by trimethylation mark western analysis. MLL-AF9 leukemia cells were treated with SAH-EZH2 or SAH-EZH2_{MUT} (10 μM final concentration) twice daily for 7 days before analysis.

FIG 17 | Flow cytometric analysis of SAH-EZH2-treated MLL-AF9 leukemia cells. SAH-EZH2 decreased the number of cells manifesting H3Lys27 trimethylation.

FIG 18 | SAH-EZH2 dose-responsively decreases H3K27Me3 levels in MLL-AF9 leukemia and Karpas422 lymphoma cells, as measured after 8 days of treatment.

FIG 19 | SAH-EZH2, but not its mutant control, inhibits the proliferation of MLL-AF9 cells. Data represent mean and s.e.m for experiments performed in

triplicate. MLL-AF9 leukemia cells were treated with 10 μ M final concentration of SAH-EZH2 or SAH-EZH2_{MUT} twice daily; water was used as vehicle.

FIG 20 | Anti-proliferative effects of SAH-EZH2 on B cell lymphoma cell lines with wild-type or the indicated mutant versions of EZH2 protein.

5 **FIG 21** | The anti-leukemic activity of SAH-EZH2 is not mediated by a pro-apoptotic effect, as assessed by FACS analysis and 7AAD/Annexin V staining after 8 days of treatment with SAH-EZH2 peptides. MLL-AF9 leukemia cells were treated with 10 μ M final concentration of SAH-EZH2 or SAH-EZH2_{MUT} twice daily; water was used as vehicle.

10 **FIG 22** | Apoptosis analysis of MLL-AF9 cells after extended incubation with SAH-EZH2 peptides (16 days)

FIG 23 | Cell cycle analysis of SAH-EZH2-treated cells. Cells were stained with PI and analyzed by flow cytometry after 8 days of treatment. Data was analyzed using ModFIT software and represents mean $\pm$ s.d. for experiments performed in duplicate. MLL-AF9 leukemia cells were treated with 10 μ M final concentration of SAH-EZH2 or SAH-EZH2_{MUT} twice daily; water was used as vehicle.

15 **FIG 24** | SAH-EZH2, but not its mutant control, upregulates the expression of p19ARF upon treatment of MLL-AF9 cells (7 days), as assessed by western analysis. MLL-AF9 leukemia cells were treated with 10 μ M final concentration of SAH-EZH2 or SAH-EZH2_{MUT} twice daily; water was used as vehicle.

20 **FIG 25** | Suppression of colony-forming potential by SAH-EZH2 as compared to vehicle and SAH-EZH2_{MUT}, $p < 0.02258$. Micrograph insets demonstrate representative colony morphologies.

FIG 26 | SAH-EZH2-treated MLL-AF9 cells exhibited decreased colony-forming capacity compared to vehicle and SAH-EZH2_{MUT}-treated cells, as evidenced by the low power images.

FIG 27 | Morphology of SAH-EZH2-treated MLL-AF9 leukemia cells. Whereas vehicle and SAH-EZH2_{MUT}-treated cells retain characteristic blastic features, SAH-EZH2-treated cells exhibit the morphology of monocyte/macrophage differentiation

30 **FIG 28** | Flow cytometric analysis of macrophage-specific cell surface marker expression (F4/80) of SAH-EZH2-treated (8 days) MLL-AF9 cells.

FIG 29A | Comparative changes in cell surface marker gene expression based on analysis of a 288 gene set in MLL-AF9 cells treated with vehicle (water), SAH-EZH2, or SAH-EZH2_{MUT} (10 μ M peptide, 8 days).

FIG 29B | Real Time qPCR analysis of macrophage/monocyte-specific marker expression. SAH-EZH2 treated (8 days) cells exhibit increased expression of macrophage/monocyte specific markers. pAdam<0.11, pFcer1a<0.14, pACE< 0.29.

FIG 30 | (a) Cells were harvested from mice and FACS-sorted to isolate undifferentiated MLL-AF9 L-GMP cells (Lin-Kit+Sca1-FcgR+CD34+) and differentiated leukemic blasts (Lin+Kit-Sca1-). (b) Decreased CD133 expression in SAH-EZH2-treated (8 days) MLL-AF9 leukemia cells (left, p<0.015) and differentiated leukemic Lin+/kit- blast cells (right, p<0.023).

FIG 31 | Effect of SAH-EZH2 peptides on HPC5, C1498 and M1 cell proliferation. Data represent mean $\pm$ s.e.m. for experiments performed in triplicate

FIG 32 | The non-leukemic HPC5 cell line showed no apparent morphologic differences in response to SAH-EZH2 peptides

FIG 33 | Comparative effect of SAH-EZH2 on the morphology of MLL-AF9 (differentiated) and HPC5 (undifferentiated) cells observed under high-magnification.

FIG. 34A | Dose-responsive effects of SAH-EZH2 and the small molecule EZH2 inhibitor GSK126 (McCabe et al. Nature, 492: 108-112, 2012) on the viability of EZH2-dependent (MLL-AF9) and EZH2-independent (32D) cell lines. Whereas GSK126 decreased the viability of both the EZH2-dependent and EZH2-independent (non-leukemic) cell lines, the reduction in viability by SAH-EZH2 was specific for the EZH2-dependent cell line, highlighting the selective mechanism of action of SAH-EZH2.

FIG. 34B | Dose-responsive effect of SAH-EZH2, but not GSK126, on the viability of MDA-MB231 breast cancer cells and DU145 prostate cancer cells, both of which are driven by pathologic EZH2 activity.

FIG 35 | Comparison of the effects of SAH-EZH2 and GSK126 on lineage specific cell surface marker expression of MLL-AF9 cells. Whereas the most notable effect of GSK126 was to increase expression of Fcer1a, SAH-EZH2 exhibited broader activity, with pronounced increases in the expression levels of Adam8, Fcer1a, and ACE. Whereas both GSK126 and SAH-EZH2 decreased the expression of cancer

stem cell marker CD133, as measured after 8 days of treatment, SAH-EZH2 manifested a comparatively more robust suppressive effect.

FIG 36 | Treatment of MLL-AF9 cells with SAH-EZH2 causes a dose-responsive decrease in EZH2 protein levels, whereas GSK126 has no such effect.

5 These data highlight a distinct mechanism of action for SAH-EZH2, reflected by its capacity to target EED and dissociate the components of the PRC2 complex, leading to overall reduction of EZH2 levels.

FIG 37 | Combination treatment of MLL-AF9 and Karpas422 cells with SAH-EZH2 and GSK126 manifests enhanced suppression of cellular viability compared to that of the respective compounds administered as single agents (top), with CalcuSyn analysis documenting the synergistic effect of the combination (bottom).

FIG 38 | Table showing the design of a doubly stapled SAH-EZH2 peptide and its improved binding activity compared to SAH-EZH2 (QKILERTQILNQEWKQRR (SEQ ID NO:55),
 15 FSSNRXXKILXRTQILNQEWKQRRIQPV (SEQ ID NO:27),
 FSSNRXXKILXRTXILNXEWKQRRIQPV (SEQ ID NO:56)

FIG 39 | Graphs demonstrating the anti-proliferative effect of a doubly stapled SAH-EZH2(42-68)_{AC} (FSSNRXXKILXRTXILNXEWKQRRIQPV (SEQ ID NO:56) peptide on the breast cancer cell line MDA-MB231 and the prostate cancer cell line DU145.

FIG 40 | Graphs demonstrating the anti-proliferative activity of a doubly stapled SAH-EZH2(42-68)_{AC} peptide on Ewing Sarcoma cell line TC-71 and neuroblastoma cell line Kelly.

FIG 41 | Graph demonstrating anti-proliferative effect of a doubly stapled SAH-EZH2(42-68)_{AC} peptide on the chronic myelogenous leukemia cell line K562.

FIG 42 | Graphs demonstrating the anti-proliferative effect of a doubly stapled SAH-EZH2(42-68)_{AC} peptide on prostate cancer cell lines.

FIG 43 | A table depicting various stapled peptides (Table 1). Sequence disclosed as SEQ ID NO: 2.

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DETAILED DESCRIPTION

Stabilized Peptides

The present disclosure provides structurally stabilized peptides related to portions or fragments of EZH2 (referred to at times as stabilized α -helices of EZH2 or SAH-EZH2) comprising 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 and/or stitched peptides.

Amino acids are the building blocks of the peptides herein. The term “amino acid” refers to a molecule containing both an amino group and a carboxyl group as well as a side chain. Amino acids suitable for inclusion in the peptides disclosed herein include, without limitation, natural alpha-amino acids such as D- and L-isomers of the 20 common naturally occurring alpha-amino acids found in peptides (e.g., Ala (A), Arg (R), Asn (N), Cys (C), Asp (D), Gln (Q), Glu (E), Gly (G), His (H), Ile (I), leu (L), Lys (K), Met (M), Phe (F), Pro (P), Ser (S), Thr (T), Trp (W), Tyr (Y), and Val (V), unnatural alpha-amino acids (including, but not limited to α,α -disubstituted and N-alkylated amino acids), natural beta-amino acids (e.g., beta-alanine), and unnnatural beta-amino acids. Amino acids used in the construction of peptides of the present invention can be prepared by organic synthesis, or obtained by other routes, such as, for example, degradation of or isolation from a natural source.

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-1-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 $-Q=O)C_6H_5$; $-CF_3$;

-CN; -halo; -NO₂; CH₃), and statine. Additionally, amino acids can be derivatized to include amino acid residues that are hydroxylated, phosphorylated, sulfonated, acylated, and glycosylated, to name a few.

A "peptide" or "polypeptide" comprises a polymer of amino acid residues linked together by peptide (amide) bonds. The term(s), as used herein, refers to proteins, polypeptides, and peptide of any size, structure, or function. Typically, a peptide or polypeptide will be at least three amino acids long. A peptide or polypeptide may refer to an individual protein or a collection of proteins. In some instances, peptides can include only natural amino acids, although non-natural amino acids (i.e., compounds that do not occur in nature but that can be incorporated into a polypeptide chain) and/or amino acid analogs as are known in the art may alternatively be employed. Also, one or more of the amino acids in a peptide or polypeptide may be modified, for example, by the addition of a chemical entity such as a carbohydrate group, a hydroxyl group, a phosphate group, a farnesyl group, an isofarnesyl group, a fatty acid group, a linker for conjugation, functionalization, or other modification, etc. A peptide or polypeptide may also be a single molecule or may be a multi- molecular complex, such as a protein. A peptide or polypeptide may be just a fragment of a naturally occurring protein or peptide. A peptide or polypeptide may be naturally occurring, recombinant, or synthetic, or any combination thereof. "Dipeptide" refers to two covalently linked amino acids.

In some instances, peptides can include (e.g., comprise, consist essentially of, or consist of) at least seven (e.g., 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28 or 29) contiguous amino acids of : A) the amino acid sequence SMFSSNRQKILERTEILNQEWKQRRIQPV (SEQ ID NO:2); B) the amino acid sequence FSSNRQKILERTEILNQEWKQRRIQPV (SEQ ID NO:40); C) the amino acid sequence FSSNRQKILERTQILNQEWKQRRIQPV (SEQ ID NO:41), or D) the amino acid sequence: A₀B₀C₀D₀E₀F₀G₀ A₁B₁C₁D₁E₁F₁G₁ A₂B₂C₂D₂E₂F₂G₂ A₃B₃C₃D₃E₃F₃G₃ A₄ (SEQ ID NO:1) wherein:

wherein A₀ is S or a conservative substitution; B₀ is M or a conservative substitution; C₀ is F or a conservative substitution; D₀ is S or a conservative substitution; E₀ is S or a conservative substitution; F₀ is N or a conservative substitution; G₀ is R or a conservative substitution; A₁ is Q or a conservative

substitution or a staple to E₁; B₁ is K or a conservative substitution; C₁ is I or a conservative substitution; D₁ is L or a conservative substitution; E₁ is E, Q or a conservative substitution or a staple to A₁; F₁ is R or a conservative substitution; G₁ is T or a conservative substitution; A₂ is E, Q or a conservative substitution; B₂ is I or a conservative substitution; C₂ is L or a conservative substitution; D₂ is N or a conservative substitution; E₂ is Q or a conservative substitution; F₂ is E, Q or a conservative substitution; G₂ is W or a conservative substitution; A₃ is K or a conservative substitution; B₃ is Q or a conservative substitution; C₃ is R or a conservative substitution; D₃ is R or a conservative substitution; E₃ is I or a conservative substitution; F₃ is Q or a conservative substitution; G₃ is P or a conservative substitution; A₄ is V or a conservative substitution; 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 internal staples and/or an internal stitch; the side chains of four amino acids are replaced by internal staples, internal stitches, or a combination of internal staples and stitches; or the side chains of at least four amino acids are replaced by internal staples, internal stitches, or a combination of internal staples and stitches. In some embodiments C₀, F₀, G₀, B₁, C₁, F₁, C₂, D₂, F₂, G₂, C₃, E₃, G₃ and A₄ are not substituted.

Peptides can also include at least seven (e.g., 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28 or 29) contiguous amino acids of: A) the amino acid sequence: SMFSSNRQKILERTEILNQEWKQRRIQPV (SEQ ID NO:2); B) the amino acid sequence FSSNRQKILERTEILNQEWKQRRIQPV (SEQ ID NO:40); C) the amino acid sequence FSSNRQKILERTQILNQEWKQRRIQPV (SEQ ID NO:41),

wherein the peptide has a reinforced or stabilized alpha helical secondary structure (e.g., wherein the peptide includes at least one internal crosslink) and wherein the percent identity calculation includes the cross-linked amino acids and the cross-linked amino acids are considered non-conservative substitutions. In some cases the internal cross-link replaces the side chains of two amino acids separated by 3 amino acids. In some cases the internal cross-link replaces the side chains of two amino acids separated by 6 amino acids. In some cases there are two internal cross-links, each replacing the side chains of a pair of amino acids separated by 3 amino

acids and each cross-link being on essentially the same face of the resulting essentially alpha-helical peptide. In the peptides of SEQ ID NOs:2, 40 and 41, the amino acid residues corresponding to the underlined residues in the sequence S M E S S N R Q K I L E R T E I L N Q E W K Q R R I Q P V (SEQ ID NO:2) are not substituted, wherein the peptide has a reinforced or stabilized alpha helical secondary structure (e.g., wherein the peptide includes at least one internal crosslink).

In some instances, the peptide has or can be induced to have alpha helical secondary structure.

In some instances, SEQ ID NO:1 corresponds to or is (SEQ ID NO:2). In some instances, stabilized peptides can have at least 80% (e.g., 80%, 85%, 90%, 95%, 98%, 99%, 99.5%, or 100%) identity to SEQ ID NO: 2 or can include SEQ ID NO:1 or SEQ ID NO:2 with one or more (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, preferably 1-2, 1-3, 1-4 or 1-5) conservative amino acid substitutions. In some cases, the stabilized peptide has the sequence of SEQ ID NO:1 with one or two staples (e.g., one staple between two amino acids separated by 3 (or 6) amino acids or two staples each between two amino acids that are separated by 3 (or 6) amino acids). In addition, 1, 2, 3, 4 or 5 of the amino acids (whose side chains are not replaced with a staple) in this stabilized peptide can be replaced by a conservative substitution.

In some cases the staple is between A₁ and E₁, of SEQ ID NO:1.

Methods for detecting any reduction in binding can include comparing binding affinity following conservative amino acid substitution, wherein any amino acid substitution that reduces (e.g., substantially reduces) binding are not conservative amino acid substitutions. In some embodiments, substantially reduced binding can include binding that is 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 98%, 99%, or 100% less than binding of the unmodified stabilized peptide to EED.

Methods for assessing interaction between a stabilized EZH2 peptide and EED are disclosed herein. Methods for identifying the interactive face of a peptide are known in the art.

In some instances, a “conservative amino acid substitution” can include substitutions in which one amino acid residue is replaced 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
 5 side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine).

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
 10 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
 15 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
 20 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).

25 As disclosed above, peptides herein include at least two modified amino acids that together form an internal (intramolecular) cross-link (or staple), wherein the at least two modified amino acids are separated by: (A) two amino acids (i.e., i, i+3, shown in Table 1 (**FIG 43**) as $\diamond$), (B) three amino acid (i.e., i, i+4, shown in Table 1 (**FIG 43**) as $\circ$), or (C) six amino acids (i.e., i, i+7, shown in Table 1 (**FIG 43**) as $\dagger$).

30 In the case of a cross-link between i and i + 3 the cross-link can be a C7 alkylene or alkenylene. In the case of a cross- between i and i + 4 the cross-link can be a C8 alkylene or alkenylene. In the case of a cross-link between i and i + 7 the

cross-link can be a C11, C12 or C13 alkylene or alkenylene. When the cross-link is an alkenylene there can one or more double bonds.

In the case of a cross-link between i and $i + 3$ the cross-link can be a C6, C7, or C8 alkyl or alkene (e.g., a C6 alkene having a single double bond). In the case of a cross-link between i and $i + 4$ the cross-link can be a C8 alkyl or alkene. In the case of a cross-link between i and $i + 7$ the cross-link can be a C11, C12 or C13 alkyl or alkene (e.g., a C11 alkene having a single double bond). When the cross-link is an alkene there can be one or more double bonds.

"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 (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 spacings and compositions. 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 in WO 2008121767 and in WO 2010/068684, which are both hereby incorporated by reference. In some instances, staples, as used herein, can retain the unsaturated bond or can be reduced (e.g., as mentioned below in the stitching paragraph description).

While many peptide staples have all hydrocarbon cross-links, other type of cross-links or staples can be used. For example, triazole-containing (e.g., 1, 4 triazole or 1, 5 triazole) crosslinks can be used (Kawamoto et al. 2012 Journal of Medicinal Chemistry 55:1137; WO 2010/060112).

Stapling of a peptide using all-hydrocarbon cross-link has been shown to help maintain its native conformation and/or secondary structure, particularly under physiologically relevant conditions (Schafmiester et al., J. Am. Chem. Soc., 122:5891-5892, 2000; Walensky et al., Science, 305:1466-1470, 2004).

5 Stapling the polypeptide herein by an all-hydrocarbon crosslink predisposed to have an alpha-helical secondary structure can constrain the polypeptide to its native alpha-helical conformation. The constrained secondary structure may, for example, increase the peptide's resistance to proteolytic cleavage, may increase the peptide's thermal stability, may increase the peptide's hydrophobicity, may allow for better
10 penetration of the peptide into the target cell's membrane (e.g., through an energy-dependent transport mechanism such as pinocytosis), and/or may lead to an improvement in the peptide's biological activity relative to the corresponding uncrosslinked (e.g., "unstitched" or "unstapled") peptide.

 Stabilized peptides herein include at least two internally cross-linked or
15 stapled amino acids, wherein the at least two amino acids are separated by two (i.e., i, i+3, shown in Table 1 (**FIG 43**)), three (i.e., i, i+4, shown in Table 1 (**FIG 43**)), or six (i.e., i, i+7, shown in Table 1 (**FIG 43**)) amino acids. While at least two amino acids are required to support an internal cross-link (e.g., a staple), additional pairs of internally cross-linked amino acids can be included in a peptide, e.g., to support
20 additional internal cross-links (e.g., staples). For example peptides can include 1, 2, 3, 4, 5, or more staples. Examples of peptide staples are illustrated in the figures. Cross-linked peptides (e.g., stapled and/or stitched peptides) are generally referred to herein as SAH peptides.

 Alternatively or in addition, peptides can include three internally cross-linked
25 or stitched amino acids, e.g., yielding two staples arising from a common origin. A peptide stitch includes at least three internally cross-linked amino acids, wherein the middle of the three amino acids (referred to here as the core or central amino acid and shown in Table 1 (**FIG 43**) as "i") forms an internal cross-link (between alpha
30 carbons) with each of the two flanking modified amino acids. The alpha carbon of the core amino acid has side chains that are internal cross-links to the alpha carbons of other amino acids in the peptide, which can be saturated or not saturated. Amino acids cross-linked to the core amino acid can be separated from the core amino acid in

either direction by 2, 3, or 6 amino acids (e.g., i, i-3, i, i-4, i, i-7 (shown in Table 1 (FIG 43)), i, i+3, i, i+4, i, i+7 (shown in Table 1 (FIG 43)), where “i” is the core amino acid). The number of amino acids on either side of the core (e.g., between the core amino acid and an amino acid cross-linked to the core) can be the same or
 5 different. Examples of such three amino acid containing peptide stitches are illustrated in FIG 1D. In some instances, a stitch can include 3, 4, 5, or more internally cross-linked amino acids. In some instances, peptides can include 1, 2, 3, 4, 5, or more stitches.

In some embodiments, peptides herein can include a combination of at least
 10 one (e.g., 1, 2, 3, 4, or 5) staple and at least one (e.g., 1, 2, 3, 4, or 5) stitch.

Cross-linked peptides (e.g., stapled and/or stitched peptides) are generally referred to herein as SAHF peptides. Peptides can include cross-linked amino acids at one or more of the positions illustrated in Table 1.

In FIG 43 (Table 1) positions of cross-links are indicated by symbols and the
 15 letter “i”. For example, i_{10} (C1) can be linked via a i+3 staple to F_1 or G_0 (also called i-3) or a i+4 staple to G_1 or F_0 (also called i-4) or a i+7 staple to C_2 or C_0 (also called i-7). Of course, i_{10} (C1) could be stitched to, for example F_1 (i+3) and C_0 (i-7). In Table 1 (FIG. 43), the first row shows SEQ ID NO:1 and the second row shows an exemplary embodiment of SEQ ID NO: 1, SEQ ID NO:2.

Internal cross-links (e.g., staples and/or stitches) can be positioned on amino
 20 acids within a peptide to conserve the structural relationship of amino acids in the binding or interacting face of the peptide (e.g., to preserve the binding interface of a peptide). Alternatively, staples can be placed on the interacting face as long as binding affinity or activity is not altered. Exemplary cross-linked peptides include SEQ ID
 25 NOs: 3-39.

In some instances, peptides herein do not include an internal cross-link that disrupts the binding interface of SEQ ID NO:2. For examples, in some instances, peptides do not include an internal cross-link between two or more (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) amino acids on the interacting face of SEQ ID NO:2.

Selection of amino acids for modification (e.g., to support an internal cross-
 30 link) can also be facilitated by staple scanning. The term “staple scan” refers to the synthesis of a library of stapled peptides whereby the location of the i and i+3; i and

i+4; and i and i+7 single and multiple staple, or stitches, are positioned sequentially down the length of the peptide sequence, sampling all possible positions, to identify desired or optimal properties and activities for the stapled or stitched constructs.

Examples of staple scanning methods are illustrated in the figures.

5 Suitable tethers are described herein and in US 2005/0250680, PCT/US2008/058575, WO 2009/108261, and WO 2010/148335.

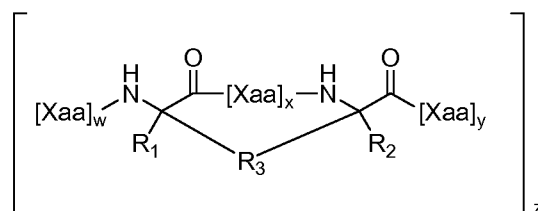
 Amino acid side chains suitable for use in the peptides disclosed herein are known in the art. For example, suitable amino acid side chains include methyl (as the alpha- amino acid side chain for alanine is methyl), 4-hydroxyphenylmethyl (as the
10 alpha-amino acid side chain for tyrosine is 4-hydroxyphenylmethyl) and thiomethyl (as the alpha-amino acid side chain for cysteine is thiomethyl), etc. A "terminally unsaturated amino acid side chain" refers to an amino acid side chain bearing a terminal unsaturated moiety, such as a substituted or unsubstituted, double bond (e.g., olefinic) or a triple bond (e.g., acetylenic), that participates in crosslinking reaction
15 with other terminal unsaturated moieties in the polypeptide chain. In certain embodiments, a "terminally unsaturated amino acid side chain" is a terminal olefinic amino acid side chain. In certain embodiments, a "terminally unsaturated amino acid side chain" is a terminal acetylenic amino acid side chain. In certain embodiments, the terminal moiety of a "terminally unsaturated amino acid side chain" is not further
20 substituted.

 As noted above an internal tether or cross-link can extend across the length of one helical turn (i.e., about 3.4 amino acids (i.e., i, i+3, or i, i+4) or two helical turns (i.e., about 7 amino acids (i.e., i, i+7). 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-
25 linking (see Table 1). Thus, for example, where a peptide has the sequence ...Xaa₁, Xaa₂, Xaa₃, Xaa₄, Xaa₅, Xaa₆, Xaa₇, Xaa₈, Xaa₉... (wherein, "..." indicates the optional presence of additional amino acids), cross-links between Xaa₁ and Xaa₄, or between Xaa₁ and Xaa₅, or between Xaa₁ and Xaa₈ are useful as are cross-links between Xaa₂ and Xaa₅, or between Xaa₂ and Xaa₆, or between Xaa₂ and Xaa₉, etc.

30 Polypeptides can include more than one crosslink within the polypeptide sequence to either further stabilize the sequence or facilitate the stabilization of longer polypeptide stretches. If the polypeptides are too long to be readily synthesized in

one part, independently synthesized, cross-linked peptides can be conjoined by a technique called native chemical ligation (Bang, et al., J. Am. Chem. Soc. 126:1377). Alternately, large peptides are routinely synthesized using a convergent approach whereby fully protected fragments are specifically and sequentially reacted to form the full length desired product, after final deprotection, such as in the industrial synthesis of Fuzeon.

The invention features a modified polypeptide of Formula (I),



Formula (I)

or a pharmaceutically acceptable salt thereof,

wherein;

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

R_3 is alkylene, alkenylene or alkynylene (e.g., a C_6 , C_7 , C_8 , C_{11} , C_{12} or C_{13} alkylene), or $[R_4'-K-R_4]_n$; each of which is substituted with 0-6 R_5 ;

R_4 and R_4' are independently alkylene, alkenylene or alkynylene (e.g., each are independently a C_1 , C_2 , C_3 , C_4 , C_5 , C_6 , C_7 , C_8 , C_9 or C_{10} alkylene, alkenylene or alkynylene);

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

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

R_6 is H, alkyl, or a therapeutic agent;

n is 2, 3, 4 or 6;

x is an integer from 2-10;

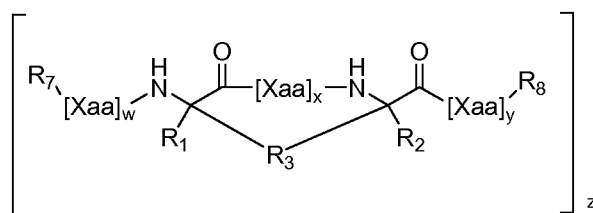
w and y are independently an integer from 0-100;

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

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

wherein the polypeptide comprises at least 8 contiguous amino acids of SEQ ID NO:1, 2, 40, 41 or a variant thereof, or another polypeptide sequence described herein except that: (a) within the 8 contiguous (e.g., 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34 or 35) amino acids of SEQ ID NO: 1, 2, 40, or 41 the side chains of at least one pair (e.g., one or two pairs) of amino acids separated by 2, 3 or 6 amino acids is replaced by the linking group, R₃, which connects the alpha carbons of the pair of amino acids as depicted in Formula I; and (b) the alpha carbon of the first of the pair of amino acids is substituted with R₁ as depicted in formula I and the alpha carbon of the second of the pair of amino acids is substituted with R₂ as depicted in Formula I.

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



Formula (II)

or a pharmaceutically acceptable salt thereof,

wherein;

each R₁ and R₂ are independently H or a C₁ to C₁₀ alkyl, alkenyl, alkynyl, arylalkyl, cycloalkylalkyl, heteroarylalkyl, or heterocyclalkyl;

R₃ is alkylene, alkenylene or alkynylene (e.g., a C₆, C₇, C₈, C₁₁, C₁₂ or C₁₃ alkylene) or [R₄'-K-R₄]_n; each of which is substituted with 0-6 R₅;

R₄ and R₄' are independently alkylene, alkenylene or alkynylene (e.g., each are independently a C₁, C₂, C₃, C₄, C₅, C₆, C₇, C₈, C₉ or C₁₀ alkylene, alkenylene or alkynylene);

R₅ is halo, alkyl, OR₆, NHR₆, N(R₆)₂, SR₆, SOR₆, SO₂R₆, CO₂R₆, R₆, a fluorescent moiety, or a radioisotope;

K is O, S, SO, SO₂, CO, CO₂, CONR₆, or , aziridine, episulfide, diol, amino alcohol, diamine;

R₆ is H, alkyl, or a therapeutic agent;

n is 2, 3, 4, 5, or 6;

5 x is an integer from 2-10;

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

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

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

10 R₇ is PEG, a tat protein, an affinity label, a targeting moiety, a fatty acid-derived acyl group, a biotin moiety, a fluorescent probe (e.g. fluorescein or rhodamine) linked via, e.g., a thiocarbamate or carbamate linkage;

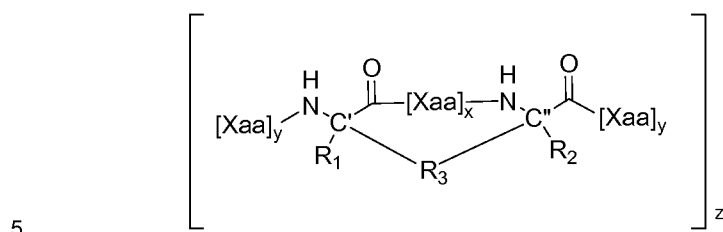
R₈ is H, OH, NH₂, NHR_{8a}, NR_{8a}R_{8b};

15 wherein the polypeptide comprises at least 8 contiguous amino acids (e.g., 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34 or 35) of SEQ ID NO: 1, 2, 40, or 41, or another polypeptide sequence described herein except that: (a) within the 8 contiguous amino acids of SEQ ID NO: 1, 2, 40, 41 or another polypeptide sequence describe the side chains of at least one pair of amino acids separated by 2, 4 or 6 amino acids is replaced by the linking group, R₃, which connects the alpha carbons of the pair of amino acids as depicted in formula I; and (b) the alpha carbon of the first of the pair of amino acids is substituted with R₁ as depicted in Formula II and the alpha carbon of the second of the pair of amino acids is substituted with R₂ as depicted in Formula II.

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

25 In cases where x = 2 (i.e., i + 3 linkage), R₃ can be a C7 alkylene, alkenylene. Where it is an alkenylene there can one or more double bonds. In cases where x = 6 (i.e., i + 4 linkage), R₃ can be a C11, C12 or C13 alkylene or alkenylene. Where it is an alkenylene there can one or more double bonds. In cases where x = 3 (i.e., i + 4 linkage), R₃ can be a C8 alkylene, alkenylene. Where it is an alkenylene there can one or more double bonds.

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



the C' and C'' disubstituted stereocenters can both be in the R configuration or they can both be in the S configuration, for example when x is 3. When x is 6, the C' disubstituted stereocenter is in the R configuration and the C'' disubstituted stereocenter is in the S configuration or the C' disubstituted stereocenter is in the S configuration and the C'' disubstituted stereocenter is in the R configuration. The R₃ double bond may be in the E or Z stereochemical configuration. Similar configurations are possible for the carbons in Formula II corresponding to C' and C'' in the formula depicted immediately above.

15 In some instances R₃ is [R₄-K-R₄']_n; and R₄ and R₄' are independently alkylene, alkenylene or alkynylene (e.g., each are independently a C₁, C₂, C₃, C₄, C₅, C₆, C₇, C₈, C₉ or C₁₀ alkylene, alkenylene or alkynylene).

In some instances, the polypeptide includes an amino acid sequence which, in addition to the amino acids side chains that are replaced by a cross-link, have 1, 2, 3, 4 or 5, 6, 7, 8, 9, 10, 11, 12 amino acid changes (e.g., conservative amino acid changes) in any of SEQ ID NOs: 1, 2, 40 or 41.

The tether can include an alkyl, alkenyl, or alkynyl moiety (e.g., C₆, C₈ or C₁₁ alkyl or a C₆, C₈ or C₁₁ alkenyl, or C₅, C₈ or C₁₁ alkynyl). The tethered amino acid can be alpha disubstituted (e.g., C₁-C₃ or methyl). [Xaa]_y and [Xaa]_w are peptides that can independently comprise at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, or 35 contiguous amino acids of SEQ ID NOs: 1 or 2 and [Xaa]_x is a peptide that can comprise 2, 3 or 6 contiguous amino acids of acids of SEQ ID NO: 1, 2, 40 or 41.

Peptides can contain one or more asymmetric centers and thus occur as racemates and racemic mixtures, single enantiomers, individual diastereomers and diastereomeric mixtures and geometric isomers (e.g. *Z* or *cis* and *E* or *trans*) of any olefins present. For example, peptides disclosed herein can exist in particular
5 geometric or stereoisomeric forms, including, for example, *cis*- and *trans*-isomers, R- and S-enantiomers, diastereomers, (D)-isomers, (L)-isomers, the racemic mixtures thereof, and other mixtures thereof. Enantiomers can be free (e.g., substantially free) of their corresponding enantiomer, and/or may also be optically enriched. "Optically enriched," as used herein, means that the compound is made up of a significantly
10 greater proportion of one enantiomer. In certain embodiments substantially free means that a composition contains at least about 90% by weight of a preferred enantiomer. In other embodiments the compound is made up of at least about 95%, 98%, or 99% by weight of a preferred enantiomer. Preferred enantiomers may be isolated from racemic mixtures using techniques known in the art, including, but not
15 limited to, for example, chiral high pressure liquid chromatography (HPLC) and the formation and crystallization of chiral salts or prepared by asymmetric syntheses (see, e.g., Jacques, et al, Enantiomers, Racemates and Resolutions (Wiley Interscience, New York, 1981); Wilen, S. H., et al., Tetrahedron 33:2725 (1977); Eliel, EX. Stereochemistry of Carbon Compounds (McGraw- Hill, NY, 1962); Wilen, S.H. Tables of Resolving Agents and Optical Resolutions p. 268 (EX. Eliel, Ed., Univ. of
20 Notre Dame Press, Notre Dame, IN 1972). All such isomeric forms of these compounds are expressly included in the present invention.

Peptides can also be represented in multiple tautomeric forms, in such instances, the invention expressly includes all tautomeric forms of the compounds
25 described herein (e.g., isomers in equilibrium (e.g., keto-enol), wherein alkylation at multiple sites can yield regioisomers), regioisomers, and oxidation products of the compounds disclosed herein (the invention expressly includes all such reaction products). All such isomeric forms of such compounds are included as are all crystal forms.

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

The term “halo” refers to any radical of fluorine, chlorine, bromine or iodine. The term “alkyl” refers to a hydrocarbon chain that may be a straight chain or branched chain, containing the indicated number of carbon atoms. For example, C₁-C₁₀ indicates that the group may have from 1 to 10 (inclusive) carbon atoms in it. In the absence of any numerical designation, “alkyl” is a chain (straight or branched) having 1 to 20 (inclusive) carbon atoms in it. The term “alkylene” refers to a divalent alkyl (i.e., -R-).

The term “alkenyl” refers to a hydrocarbon chain that may be a straight chain or branched chain having one or more carbon-carbon double bonds in either Z or E geometric configurations. The alkenyl moiety contains the indicated number of carbon atoms. For example, C₂-C₁₀ indicates that the group may have from 2 to 10 (inclusive) carbon atoms in it. The term “lower alkenyl” refers to a C₂-C₈ alkenyl chain. In the absence of any numerical designation, “alkenyl” is a chain (straight or branched) having 2 to 20 (inclusive) carbon atoms in it.

The term “alkynyl” refers to a hydrocarbon chain that may be a straight chain or branched chain having one or more carbon-carbon triple bonds. The alkynyl moiety contains the indicated number of carbon atoms. For example, C₂-C₁₀ indicates that the group may have from 2 to 10 (inclusive) carbon atoms in it. The term “lower alkynyl” refers to a C₂-C₈ alkynyl chain. In the absence of any numerical designation, “alkynyl” is a chain (straight or branched) having 2 to 20 (inclusive) carbon atoms in it.

The term “aryl” refers to a 6-carbon monocyclic or 10-carbon bicyclic aromatic ring system wherein 0, 1, 2, 3, 4, or 5 atoms of each ring may be substituted by a substituent. Examples of aryl groups include phenyl, naphthyl and the like. The term “arylalkyl” or the term “aralkyl” refers to alkyl substituted with an aryl. The term “arylalkoxy” refers to an alkoxy substituted with aryl.

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

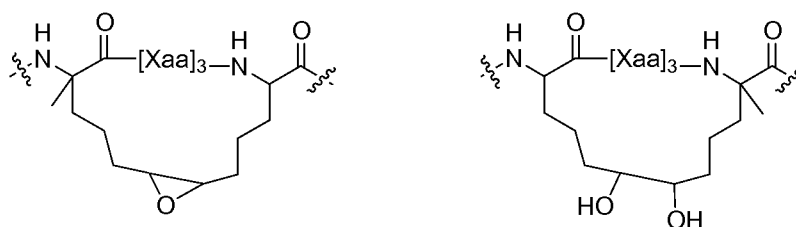
cyclohexenyl, cyclohexadienyl, cycloheptyl, cycloheptadienyl, cycloheptatrienyl, cyclooctyl, cyclooctenyl, cyclooctadienyl, cyclooctatrienyl, and cyclooctynyl.

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

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

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

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



Either the epoxide moiety or one of the free hydroxyl moieties can be further functionalized. For example, the epoxide can be treated with a nucleophile, which provides additional functionality that can be used, for example, to attach a tag (e.g., a radioisotope or fluorescent tag). The tag can be used to help direct the compound to a desired location in the body or track the location of the compound in the body. Alternatively, an additional therapeutic agent can be chemically attached to the functionalized tether (e.g., an anti-cancer agent such as rapamycin, vinblastine, taxol, etc.). Such 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.

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

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

Additionally, while examples of tethers spanning from amino acids i to $i+3$, i to $i+4$; and i to $i+7$ have been described in order to provide a tether that is primarily

on a single face of the alpha helix, the tethers can be synthesized to span any combinations of numbers of amino acids.

In some instances, alpha disubstituted amino acids are used in the polypeptide to improve the stability of the alpha helical secondary structure. However, alpha
5 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
10 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
15 polypeptide as formula:

$\text{XO}-(\text{CH}_2\text{CH}_2\text{O})_n-\text{CH}_2\text{CH}_2-\text{Y}$ 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
20 (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,
25 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
30 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
5 $-\text{NH}(\text{CH}_2)_n\text{C}(\text{O})-$, 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., $\text{C}_1\text{-C}_6$) 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.

10 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.

15 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 compounds of the 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
20 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.
25 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.

30 The peptides of this invention can be made by chemical synthesis methods, which are well known to the ordinarily skilled artisan. See, for example, Fields et al., Chapter 3 in *Synthetic Peptides: A User's Guide*, ed. Grant, W. H. Freeman & Co., New York, N.Y., 1992, p. 77. Hence, peptides can be synthesized using the automated

Merrifield techniques of solid phase synthesis with the α -NH₂ protected by either t-Boc or 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-CH₂); a thiomethylene bond (S-CH₂ or CH₂-S); an oxomethylene bond (O-CH₂ or CH₂-O); an ethylene bond (CH₂-CH₂); 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 CH₃; and a fluoro-ketomethylene bond (C(O)-CFR or CFR-C(O) wherein R is H or F or CH₃.

The polypeptides can be further modified by: acetylation, amidation, biotinylation, cinnamoylation, farnesylation, fluoresceination, formylation, myristoylation, palmitoylation, phosphorylation (Ser, Tyr or Thr), stearylation, succinylation and sulfurylation. As indicated above, peptides can be conjugated to,
5 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
10 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 one S5 amino acid and one R8 is used or one S8 amino acid and one R5 amino acid is used. R8 is synthesized using the same route, except that the starting chiral auxiliary confers the R-alkyl-stereoisomer. Also, 8-
15 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-S₅-OH, Fmoc-R₈-OH, Fmoc-R₈-OH, Fmoc-S₈-OH and Fmoc-R₅-OH), 2-(6-chloro-1-H-benzotriazole-1-yl)-1,1,3,3-tetramethylammonium hexafluorophosphate (HCTU), and
20 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
25 from, e.g., Sigma-Aldrich. Olefinic amino acid synthesis is reported in the art (Williams et al., Org. Synth., 80:31, 2003).

In some instances, peptides can include a detectable label. As used herein, a "label" refers to a moiety that has at least one element, isotope, or functional group incorporated into the moiety which enables detection of the peptide to which the label
30 is attached. Labels can be directly attached (ie, via a bond) or can be attached by a linker (e.g., such as, for example, a cyclic or acyclic, branched or unbranched, substituted or unsubstituted alkylene; cyclic or acyclic, branched or unbranched,

substituted or unsubstituted alkenylene; cyclic or acyclic, branched or unbranched, substituted or unsubstituted alkynylene; cyclic or acyclic, branched or unbranched, substituted or unsubstituted heteroalkylene; cyclic or acyclic, branched or unbranched, substituted or unsubstituted heteroalkenylene; cyclic or acyclic, branched or unbranched, substituted or unsubstituted heteroalkynylene; substituted or unsubstituted arylene; substituted or unsubstituted heteroarylene; or substituted or unsubstituted acylene, or any combination thereof, which can make up a linker).

Labels can be attached to a peptide at any position that does not interfere with the biological activity or characteristic of the inventive polypeptide that is being detected.

Labels can include: labels that contain isotopic moieties, which may be radioactive or heavy isotopes, including, but not limited to, ^2H , ^3H , ^{13}C , ^{14}C , ^{15}N , ^{31}P , ^{32}P , ^{35}S , ^{67}Ga , $^{99\text{m}}\text{Tc}$ (Tc-99m), ^{111}In , ^{123}I , ^{125}I , ^{169}Yb , and ^{186}Re ; labels that include immune or immunoreactive moieties, which may be antibodies or antigens, which may be bound to enzymes {e.g., such as horseradish peroxidase}; labels that are colored, luminescent, phosphorescent, or include fluorescent moieties (e.g., such as the fluorescent label FITC); labels that have one or more photoaffinity moieties; labels that have ligand moieties with one or more known binding partners (such as biotin-streptavidin, FK506-FKBP, etc.).

In some instances, labels can include one or more photoaffinity moieties for the direct elucidation of intermolecular interactions in biological systems. A variety of known photophores can be employed, most relying on photoconversion of diazo compounds, azides, or diazirines to nitrenes or carbenes (see, e.g., Bayley, H., *Photogenerated Reagents in Biochemistry and Molecular Biology* (1983), Elsevier, Amsterdam, the entire contents of which are incorporated herein by reference). In certain embodiments of the invention, the photoaffinity labels employed are o-, m- and p-azidobenzoyls, substituted with one or more halogen moieties, including, but not limited to 4-azido-2,3,5,6-tetrafluorobenzoic acid.

Labels can also be or can serve as imaging agents. Exemplary imaging agents include, but are not limited to, those used in positron emissions tomography (PET), computer assisted tomography (CAT), single photon emission computerized tomography, x-ray, fluoroscopy, and magnetic resonance imaging (MRI); anti-emetics; and contrast agents. Exemplary diagnostic agents include but are not limited

to, fluorescent moieties, luminescent moieties, magnetic moieties; gadolinium chelates (e.g., gadolinium chelates with DTPA, DTPA-BMA, DOTA and HP-DO3A), iron chelates, magnesium chelates, manganese chelates, copper chelates, chromium chelates, iodine -based materials useful for CAT and x-ray imaging, and

5 radionuclides. Suitable radionuclides include, but are not limited to, ^{123}I , ^{125}I , ^{130}I , ^{131}I , ^{133}I , ^{135}I , ^{47}Sc , ^{72}As , ^{72}Se , ^{90}Y , ^{88}Y , ^{97}Ru , ^{100}Pd , ^{101}mRh , ^{119}Sb , ^{128}Ba , ^{197}Hg , ^{211}At , ^{212}Bi , ^{212}Pb , ^{109}Pd , ^{111}In , ^{67}Ga , ^{68}Ga , ^{67}Cu , ^{75}Br , ^{77}Br , ^{99}mTc , ^{14}C , ^{13}N , ^{15}O , ^{32}P , ^{33}P , and ^{18}F .

Fluorescent and luminescent moieties include, but are not limited to, a variety of different organic or inorganic small molecules commonly referred to as "dyes,"
10 "labels," or "indicators." Examples include, but are not limited to, fluorescein, rhodamine, acridine dyes, Alexa dyes, cyanine dyes, etc. Fluorescent and luminescent moieties may include a variety of naturally occurring proteins and derivatives thereof, e.g., genetically engineered variants. For example, fluorescent proteins include green fluorescent protein (GFP), enhanced GFP, red, blue, yellow, cyan, and sapphire
15 fluorescent proteins, reef coral fluorescent protein, etc. Luminescent proteins include luciferase, aequorin and derivatives thereof. Numerous fluorescent and luminescent dyes and proteins are known in the art (see, e.g., U.S. Patent Publication 2004/0067503; Valeur, B., "Molecular Fluorescence: Principles and Applications," John Wiley and Sons, 2002; and Handbook of Fluorescent Probes and Research
20 Products, Molecular Probes, 9th edition, 2002).

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.
25 Am. Chem. Soc., 122:5891-5892 (2000); U.S. Patent Application Serial 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) and are described herein (see, e.g., Example 1).

In some embodiments, the peptides are substantially free of non-stapled
30 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 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 H₂O, to concentrations of 25-50 μ M). 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 (T_m): Cross-linked or the unmodified template peptides are dissolved in distilled H₂O or other buffer or solvent (e.g. at a final concentration of 50 μ M) and T_m 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 μ L of sera to 2 ml centrifuge tubes followed by the addition of 10 μ L of 50% formic acid and 500 μ L acetonitrile and centrifugation at 14,000 RPM for 10 min at 4 $\pm$ 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 μ L 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).

In vitro Displacement Assays to Characterize Antagonists of Peptide-Protein Interactions: To assess the binding and affinity of compounds that antagonize the interaction between a peptide and an acceptor protein, a fluorescence polarization assay (FPA) utilizing a fluoresceinated peptidomimetic macrocycle derived from a peptidomimetic precursor sequence is 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). A compound that antagonizes the interaction between the fluoresceinated peptidomimetic macrocycle and an acceptor protein will be detected in a competitive binding FPA experiment.

Binding Assays in Intact Cells: It is possible to measure binding of peptides or crosslinked polypeptides to their natural acceptors on or in intact cells by immunoprecipitation experiments.

Cellular Penetrability Assays: To measure the cell penetrability of peptides or crosslinked polypeptides, intact cells are incubated with fluoresceinated crosslinked polypeptides (10 μ M) for 4 hrs in serum-free media or in media supplemented with human serum at 37°C, washed twice with media and incubated with trypsin (0.25%) for 10 min at 37°C. The cells are washed again and resuspended in PBS. Cellular fluorescence is analyzed, for example, by using either a FACSCalibur flow cytometer or Cellomics' KineticScan.RTM. HCS Reader.

Pharmaceutical Compositions

One or more of the stabilized peptides disclosed herein (e.g., one or more of SEQ ID NOs: 2-41) can be formulated for use as or in pharmaceutical compositions. 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 administration, 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 cancer).

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.

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, intracutaneous, intra-venous, intra-muscular, intra-articular, intra-arterial, intra-synovial, intra-sternal, intra-theccal, 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 embodiments, the present disclosure provides methods for using any one or more of the peptides or pharmaceutical compositions (indicated below as 'X') disclosed herein in the following methods:

Substance X for use as a medicament in the treatment of one or more diseases or conditions disclosed herein (e.g., cancer, referred to in the following examples as 'Y'). Use of substance X for the manufacture of a medicament for the treatment of Y; and substance X for use in the treatment of Y.

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.

Methods of Treatment

The disclosure includes methods of using the peptides herein for the prophylaxis and/or treatment of cancer. The terms "treat" or "treating," as used herein, refers to partially or completely alleviating, inhibiting, ameliorating, and/or relieving the disease or condition from which the subject is suffering.

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.

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.

Treatment Methods

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.

5 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

10 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.

EXAMPLES

15 Described below are internally cross-linked peptides related to EZH2 (SAH-EZH2) peptides that directly target EED, dissociate the EZH2/EED complex, and thereby reduce both EZH2 protein levels and H3Lys27 trimethylation. MLL-AF9 leukemia cells, which are dependent on the catalytic activity of PRC2, undergo

20 growth arrest and monocyte/macrophage differentiation upon treatment with SAH-EZH2.

Example 1: Synthesis and EED-Binding Activity of SAH-EZH2 Peptides

Hydrocarbon stapling has been applied to recapitulate the natural structure of

25 alpha-helical binding motifs, yielding bioactive agents to dissect and target intracellular protein interactions³⁵⁻³⁸. To disrupt the PRC2 complex, we designed stapled peptides of variable length based on the alpha-helical, EED-binding domain of EZH2 that comprises amino acids 40-68³² (**FIG 2**). Two staple positions were

sampled owing to their location at the opposite side of the key alpha-helical binding

30 interface (**FIG 2**), and insertion of the hydrocarbon staple enhanced alpha-helical structure, with the “A” staple conferring the most significant structural stabilization as assessed by circular dichroism (**FIG 3**). The binding affinity of FITC-labeled SAH-

EZH2 peptides for EED was measured by fluorescence polarization (FP) assay (**FIG 4**) and co-immunoprecipitation (**FIG 5**), documenting effective engagement of the EED target. However, significant differences emerged when comparing SAH-EZH2 peptides of different length. Whereas the longest constructs, SAH-EZH2_A(40-68) and SAH-EZH2_B(40-68) displayed the most potent dose-responsive EED-binding activities, followed by SAH-EZH2_A(42-68) and SAH-EZH2_B(42-68), the foreshortened SAH-EZH2_A(42-64) and SAH-EZH2_B(42-64) peptides lacking the IQPV motif exhibited no appreciable engagement of recombinant EED by FP or of expressed HA-EED by anti-FITC pull down (**FIG 5**). These data are consistent with structure-function studies that documented the critical role of I65, P67 and V68 in the EZH2/EED interaction³²⁻³⁴. Thus, we identified SAH-EZH2 constructs comprising amino acids 40-68 and 42-68 as submicromolar EED binders.

In preparation for phenotypic studies in treated cells, we evaluated the cellular uptake of SAH-EZH2 peptides in MLL-AF9 leukemia cells exposed to FITC-labeled derivatives, followed by fluorescence analysis of electrophoresed cellular extracts (**FIG 6**). SAH-EZH2_A peptides generally displayed enhanced cellular uptake in comparison with the corresponding constructs bearing the B-positioned staple, with the intermediate and shortest peptides exhibiting the most robust dose-responsive uptake. Based upon EED-binding potency and observed cellular penetrance, SAH-EZH2_A(42-68) emerged as the lead construct for optimization and biological testing.

SAH-EZH2 peptides were designed by replacing two naturally occurring amino acids with the non-natural S5 amino acids at i, i+4 positions (i.e. flanking 3 amino acids) to generate a staple spanning one α -helical turn, or a combination of R8 and S5 at i, i+7 positions, respectively, to generate a staple spanning two α -helical turns.

Asymmetric syntheses of α , α -disubstituted amino acids were performed as previously described in detail (Schafmeister et al., J. Am. Chem. Soc., 122:5891-5892, 2000; Walensky et al., Science, 305:1466-1470, 2004; Bird et al. Current Protocols in Chemical Biology, 2011).

Alanine scanning and “staple scanning” was performed to respectively identify residues and binding surfaces critical for interaction, which dictates the design of optimized constructs and negative control mutants (*see* **FIG 1**). The N-

termini of SAHs are capped with acetyl or a fluorophore (e.g. FITC, rhodamine), depending upon the experimental application.

Doubly stapled peptides are generated by installing two-S5-S5, two -R8-S5, or other combinations of crosslinking non-natural amino acids (see **FIG 1**). Multiply
5 stapled or stitched peptides are generated using similar principles.

Synthesis of the SAH-EZH2 peptides shown in Table 2 was performed using solid phase Fmoc chemistry and ruthenium-catalyzed olefin metathesis, followed by peptide deprotection and cleavage, purification by reverse phase high performance
10 liquid chromatography/mass spectrometry (LC/MS), and quantification by amino acid analysis (AAA) (Bird et al., Methods Enzymol., 446:369-386, 2008).

TABLE 2: EZH2 Peptides

(B = norleucine; J = Aib; X = stapling amino acid; sc = scrambled sequence)

15

EZH2(40-68)	SMFSSNRQKILERTEILNQEWKQRRIQPV (SEQ ID NO:2)
SAH-EZH2(40-68) _A	SBFSSNRXKILXRTEILNQEWKQRRIQPV (SEQ ID NO:3)
SAH-EZH2(40-68) _B	SBFSSNRQKILXRTEILNQEWKQRRIQPV (SEQ ID NO:4)
SAH-EZH2(40-68) _C	SBFSSNRQKILERTXILNQEWEKQRRIQPV (SEQ ID NO:5)
SAH-EZH2(40-68) _D	SBFSSNRQKILERTXILNQXWKQRRIQPV (SEQ ID NO:6)
SAH-EZH2(40-68) _E	SBFSSNRQKILERTEILNQEWEKXRRIQPV (SEQ ID NO:7)
EZH2(40-66)-sc	SBFSSNRQKILERTEILNQEBWQRRIQ (SEQ ID NO:8)
SAH-EZH2(40-66) _C -sc	SBFSSNRQKILERTXILNQEWEKQRRIQ (SEQ ID NO:9)
SAH-EZH2(40-66) _E -sc	SBFSSNRQKILERTEILNQEWEKXRRIQ (SEQ ID NO:10)
EZH2(47-64)	QKILERTEILNQEWKQRR (SEQ ID NO:11)
EZH2(47-64)-sc	QKILERTEILNQEBWQRR (SEQ ID NO:12)
SAH-EZH2(47-64) _C -sc	QKILERTXILNQEWEKQRR (SEQ ID NO:13)
SAH-EZH2(47-64) _E -sc	QKILERTEILNQEWEKXRR (SEQ ID NO:14)
SAH-EZH2(45-64) _A	NRXKILXRTEILNQEWKQRR (SEQ ID NO:15)
SAH-EZH2(45-64) _C	NRQKILERTXILNQEWEKQRR (SEQ ID NO:16)
SAH-EZH2(45-64) _A -MUT	NRXKALXRTEIANQEAKQRR (SEQ ID NO:17)
SAH-EZH2(45-64) _C -MUT	NRQKALERTXIANXEAKQRR (SEQ ID NO:18)
SAH-EZH2(42-64) _A	FSSNRXKILXRTEILNQEWKQRR (SEQ ID NO:19)
SAH-EZH2(42-64) _C	FSSNRQKILERTXILNQEWEKQRR (SEQ ID NO:20)
SAH-EZH2(42-64) _A -S41J/E54Q	FJSNRXKILXRTEILNQEWKQRR (SEQ ID NO:21)
SAH-EZH2(42-64) _A -S42J/E54Q	FSJNRXKILXRTEILNQEWKQRR (SEQ ID NO:22)
SAH-EZH2(42-64) _C -S41J/E51Q	FJSNRQKILQRTXILNQEWEKQRR (SEQ ID NO:23)
SAH-EZH2(42-64) _C -S41J/E51Q	FSJNRQKILQRTXILNQEWEKQRR (SEQ ID NO:24)
SAH-EZH2(42-64) _C -S41J/E51Q-MUT	FJSNRQKALQRTXIANXEAKQRR (SEQ ID NO:25)
SAH-EZH2(42-68) _A	FSSNRXKILXRTEILNQEWKQRRIQPV (SEQ ID NO:26)
SAH-EZH2(42-68) _A -E54Q	FSSNRXKILXRTEILNQEWKQRRIQPV (SEQ ID NO:27)
SAH-EZH2(42-68) _A -E54,59Q	FSSNRXKILXRTEILNQEWKQRRIQPV (SEQ ID NO:28)
SAH-EZH2(42-68) _A -K48QE54QE59K	FSSNRXKILXRTEILNQEWKQRRIQPV (SEQ ID NO:29)
SAH-EZH2(42-68) _B	FSSNRQKILXRTEILNQEWKQRRIQPV (SEQ ID NO:30)
SAH-EZH2(42-68) _B -E54Q	FSSNRQKILXRTEILNQEWKQRRIQPV (SEQ ID NO:31)
SAH-EZH2(42-68) _B -E54,59Q	FSSNRQKILXRTEILNQEWKQRRIQPV (SEQ ID NO:32)
SAH-EZH2(42-68) _C	FSSNRQKILERTXILNQEWEKQRRIQPV (SEQ ID NO:33)
SAH-EZH2(42-68) _C -E51Q	FSSNRQKILQRTXILNQEWEKQRRIQPV (SEQ ID NO:34)
SAH-EZH2(42-68) _C -E51,59Q	FSSNRQKILQRTXILNQXWKQRRIQPV (SEQ ID NO:35)
SAH-EZH2(42-68) _D	FSSNRQKILERTXILNQXWKQRRIQPV (SEQ ID NO:36)
SAH-EZH2(42-68) _D -E51Q	FSSNRQKILQRTXILNQXWKQRRIQPV (SEQ ID NO:37)
SAH-EZH2(42-68) _D -E54Q	FSSNRQKILERTXILNQXWKQRRIQPV (SEQ ID NO:38)
SAH-EZH2(42-68) _D -E51,54Q	FSSNRQKILQRTXILNQXWKQRRIQPV (SEQ ID NO:39)
EZH2(42-68)	FSSNRQKILERTEILNQEWKQRRIQPV (SEQ ID NO:40)
EZH2(42-68) E54Q	FSSNRQKILERTXILNQEWKQRRIQPV (SEQ ID NO:41)

Example 2: Targeted Disruption of the EZH2/EED Complex and Suppression of H3K27 Methylation

We previously observed that neutralization of negatively charged residues, such as Asp and Glu, can enhance the permeability of stapled peptides^{35-38,39}. Sequence comparison of EZH2 homologs indicates that Glu54 is not conserved, suggesting that this residue is dispensable for binding to EED^{32,32}. Therefore, we mutated the non-interacting Glu54 residue of SAH-EZH2_A(42-68) to Gln (**FIG 7**) and indeed observed further enhancement of cellular uptake (**FIG 8**) without compromising *in vitro* binding activity (**FIG 9, 10**). To generate a negative control peptide for cellular studies, we further mutated Glu59 - a highly conserved residue

among EZH2 homologs - to Gln in an effort to disrupt the salt bridge between Arg200 of EED and Glu59 of EZH2 at the binding interface (**FIG 7**)^{32,32}. We found this doubly mutant construct to manifest similar cellular penetrance (**FIG 8**) but markedly reduced in vitro EED binding activity, as assessed by fluorescence polarization assay (**FIG 9**) and FITC-SAH pull down of HA-EED (**FIG 10**). Consistent with these data, confocal microscopy analyses documented robust cellular uptake and nuclear localization of both SAH-EZH2_A(42-68)E54Q and SAH-EZH2_A(42-68)E54Q/E59Q (hereafter referred to as SAH-EZH2 and SAH-EZH2_{MUT}, respectively) (**FIG 11**), but only SAH-EZH2 effectively co-precipitated native EED from MLL-AF9 cells treated with the corresponding FITC-labeled peptides (**FIG 12**). Whereas SAH-EZH2 peptides manifested marked cellular uptake compared to the corresponding unmodified peptides, appending a cell penetrating TAT moiety to the unmodified peptide (42-68) conferred uptake (**FIG 13a**). However, time course analysis of intracellular full-length peptide by electrophoresis of lysates and fluorescence scan detection, documented that the SAH-EZH2 peptides persisted in full-length form over time, whereas the TAT-EZH2 peptide was undetectable by 2 hours (**FIG 13b**). These data highlight that hydrocarbon stapling of the EZH2 peptide sequence not only confers robust cellular uptake but enables intracellular persistence of the peptide in full-length form (**FIG 13**).

Since the catalytic activity of PRC2 depends on the interaction between EZH2 and EED, we explored the capacity of SAH-EZH2 to disrupt EED engagement with EZH2, and its homolog EZH1 (**FIG 14, 15**). Of note, EZH1 shares 86% amino acid identity with the EED-binding domain of EZH2, and is believed to form an alternate, catalytically active PRC2 complex with EED and Suz12³⁸⁻⁴². In *Ezh2*^{-/-} murine embryonic stem cells, EZH1 partially compensates for loss of EZH2 and maintains trimethylation of a subset of developmental genes^{32,40}. Indeed, HA-EED co-immunoprecipitated Flag-EZH1 and Flag-EZH2 upon co-expression in sf21 cells (**FIG 14**). When SAH-EZH2 peptides were incubated with these purified EZH1/EED and EZH2/EED complexes, dose-responsive dissociation of the protein interactions was observed for SAH-EZH2 but, importantly, not for the mutant control (**FIG 15**). To determine if the capacity of SAH-EZH2 to target and dissociate these complexes functionally impaired PRC2 methyltransferase activity, MLL-AF9 cells were treated

with SAH-EZH2 and histone modification was followed by western blot and flow cytometry (**FIG 16, FIG 17**). Of note, an extended treatment course (7 days) was employed owing to the prolonged half-life of the trimethyl mark.^{32,43,44} Both experimental read-outs documented that SAH-EZH2 treatment decreased trimethylation of H3K27 (**FIG 16, FIG 17**). The selectivity of SAH-EZH2 activity was highlighted by the inactivity of SAH-EZH2_{MUT} and the lack of a SAH-EZH2 effect on the H3K4, H3K9 and H3K36 methyl marks (**FIG 16**). Importantly, inhibition of H3K27 methylation in both MLL-AF9 and Karpas422 cells was dose-responsive (**FIG 18**). Thus, SAH-EZH2 targets native EED, dissociates its interactions with EZH1 and EZH2, and selectively decreases the trimethylation of H3K27.

Example 3: SAH-EZH2 Arrests Proliferation of MLL-AF9 Leukemia Cells

Since MLL-AF9 cells are dependent on EZH2 and the PRC2 complex for growth^{33,34,40-42}, we explored whether the biochemical activity of SAH-EZH2 translated into an anti-leukemic effect. We treated MLL-AF9 cells for an extended incubation period and noted significantly slowed growth by eight days for SAH-EZH2- but not mutant control-exposed cells (**FIG 19**). These data are consistent with prior studies in which knockdown or knockout of EZH2 or EED impaired proliferation of MLL-AF9 cells^{33,34,40}. SAH-EZH2 also had a selective impact on the viability of mutant EZH2-driven B cell lymphoma cells bearing activating mutations Y641N or A677G, but not an EZH2-independent lymphoma cell bearing wild-type EZH2, further highlighting the mechanistic specificity of SAH-EZH2 activity (**FIG. 20**). To investigate whether the decreased growth rate of MLL-AF9 cells was associated with increased apoptosis, we stained cells with Annexin V5-PE and 7-AAD after 2, 4 and 8 day incubations with SAH-EZH2 or its mutant control peptide, followed by flow cytometric analysis. Little to no increase in apoptotic cells was observed upon treatment with SAH-EZH2 peptides for 8 (**FIG 21**) or even 16 days (**FIG 22**), whereas exposure to the chemotherapeutic agent, camptothecin, triggered a robust apoptotic effect. Given the lack of an apoptotic effect, we investigated whether the observed decreased growth of MLL-AF9 cells upon exposure to SAH-EZH2 was associated with modulation of the cell cycle. Whereas

propidium iodide staining and flow cytometric analysis of SAH-EZH2-treated MLL-AF9 cells showed no cell cycle effects at day 4 of treatment (data not shown), by day 8, an increased percentage of cells in S phases and a corresponding decrease in G2/M was observed (**FIG 23**). Compared to cells treated with the mutant control stapled peptide, SAH-EZH2 induced an increase of cells in S phase from 25.02% to 33.94%, and a decrease in G2/M phase from 20.26% to 10.20%. These data suggest that the effect of SAH-EZH2 on MLL-AF9 cell proliferation results from reduction of the fraction of cells in the G2/M phase. Interestingly, knockdown or genetic ablation of *Ezh2* was previously reported to derepress the cell cycle inhibitory Ink4A/Arf locus^{16,43-45}. Consistent with this finding, SAH-EZH2 treatment of MLL-AF9 cells increased p19ARF levels whereas cells treated with SAH-EZH2_{MUT} or no peptide showed little to no p19ARF expression (**FIG 24**). Taken together, these data indicate that the observed proliferative arrest of cells treated with SAH-EZH2 derives from a cell cycle blockade that coincides with increased expression of p19ARF.

Example 4: SAH-EZH2 Induces Monocytic/Macrophage Differentiation of MLL-AF9 Leukemia Cells

As MLL-AF9 cells are EZH2-dependent and manifest differentiation blockade due to epigenetic deregulation⁴⁶, we monitored the impact of SAH-EZH2-mediated disruption of PRC2 activity on the morphology of treated cells. MLL-AF9 cells were exposed to SAH-EZH2 for 14 days, followed by plating on methylcellulose with continued compound incubation. By day 7 after plating, SAH-EZH2 treatment decreased colony-forming capacity by 70% (**FIG 25, FIG 26**) compared to vehicle control, with SAH-EZH2_{MUT} demonstrating no effect. Whereas vehicle- and SAH-EZH2_{MUT}-treated cells retained the morphology of immature leukemic blasts, exposure to SAH-EZH2 triggered myeloid cell maturation, with treated cells manifesting a monocyte/macrophage phenotype (**FIG 27**). The apparent differentiation of MLL-AF9 leukemia was supported by the positive staining of SAH-EZH2-treated cells with the macrophage specific antibody F4/80 (**FIG 28**). These observations phenocopy the induced differentiation of MLL-AF9 cells upon shRNA knockdown or genetic ablation of *Ezh2* or *Eed*^{33,33,34,34}. The sequence-based specificity of SAH-EZH2 modulation of PRC2 transcriptional targets was further

confirmed by analysis of a cell surface marker 288 gene set in treated cells (FIG 29A). Induced expression of the macrophage lineage-specific markers *Ace1*, *Adam8* and *Fcer1a*, as detected by real time qPCR, exemplified the differentiation effect of SAH-EZH2 (FIG 29B). Conversely, the expression of CD133, a marker associated with hematopoietic stem cells and populations of cancer stem cells^{33,34,47-50}, decreased significantly upon treatment with SAH-EZH2 (FIG 30). Reduction in CD133 expression is likewise observed when monocyte/ granulocyte progenitors differentiate to mature monocytes/granulocytes⁵¹, and when comparing leukemic stem cell enriched MLL-AF9 leukemic granulocyte monocyte progenitor (L-GMP) cells to the more differentiated leukemic Lin+/kit- blast cells harvested from mice (FIG 30).

To probe the selectivity of the SAH-EZH2 anti-leukemic effect, we evaluated proliferative and morphologic responses in the immortalized, but non-tumorigenic, primitive hematopoietic progenitor cell line (HPC5), and two additional mouse myeloid leukemia cell lines (M1 and C1492). Of note, HPC5 cells were originally derived from adult bone marrow and then immortalized by expression of the LIM homeobox gene, *lhx2*⁵²; these cells are unique in that they partially reconstitute hematopoiesis in lethally irradiated mice without causing leukemia. As observed for MLL-AF9 cells, SAH-EZH2, but not its mutant derivative, inhibited the proliferation of C1498 and M1 myeloid leukemia cells, but the non-tumorigenic HPC5 cells showed no effect (FIG 31). Correspondingly, the undifferentiated appearance of HPC5 cells was unaffected by treatment with vehicle, SAH-EZH2_{MUT}, or SAH-EZH2 (FIG 32, FIG 33). These data are consistent with the previous observation that shRNA knockdown of PRC2 components EED and Suz12 significantly decreased the proliferation of MLL-AF9;Nras^{G12D} but had minimal impact on the proliferation of the non-leukemic transformed cell line 32D³⁴. Thus, we find that the SAH-EZH2 treatment induces proliferation arrest and differentiation of leukemia cells but not untransformed hematopoietic cells.

Example 5: Comparative anti-leukemic activity of SAH-EZH2 and the small molecule EZH2 inhibitor GSK126

To compare the functional and mechanism-based cellular activities of SAH-EZH2 and GSK126, a small molecule inhibitor of EZH2 enzymatic activity (McCabe

et al. *Nature*, 492: 108-112, 2012), we conducted viability assays on the EZH2-dependent cell line MLL-AF9 and the EZH2-independent (non-leukemic) cell line 32D. Whereas GSK126 decreased the viability of both the EZH2-dependent and EZH2-independent (non-leukemic) cell lines, the reduction in viability by SAH-EZH2 was specific for the EZH2-dependent cell line, highlighting the selective mechanism of action of SAH-EZH2 (**FIG 34A**). We further demonstrated the dose-responsive effect of SAH-EZH2, but not GSK126, on the viability of MDA-MB231 breast cancer cells and DU145 prostate cancer cells, both of which are driven by pathologic EZH2 activity (**FIG 34B**). We next compared the effects of SAH-EZH2 and GSK126 on lineage specific surface marker expression in MLL-AF9 cells. Whereas the most notable effect of GSK126 was to increase expression of Fcrla, SAH-EZH2 exhibited broader activity, increasing the expression levels of Adam8, Fcrla, and ACE (**FIG 35**). Whereas both GSK126 and SAH-EZH2 decreased the expression of cancer stem cell marker CD133, as measured after 8 days of treatment, SAH-EZH2 manifested a comparatively more robust suppressive effect (**FIG 35**). As GSK126 has been reported to directly inhibit the enzymatic activity of EZH2 whereas SAH-EZH2 targets EED to dissociate the enzymatic complex, we explored the potential impact of these differential mechanisms of action on EZH2 protein levels in cells. We find that SAH-EZH2, but not GSK126, treatment induces a dose-responsive reduction in EZH2 protein levels (**FIG 36**), consistent with SAH-EZH2 mediated destabilization of the EZH2 protein upon dissociation of the EZH2/EED complex. Given the distinct mechanisms of action of SAH-EZH2 and GSK126, we explored whether co-administration of the two compounds would enhance the anti-leukemic effects. Indeed, we observe that combination treatment of MLL-AF9 and Karpas422 cells with SAH-EZH2 and GSK126 dose-responsively enhances anti-proliferative activity compared to that of the respective compounds administered as single agents (**FIG 37**, top). Calcsyn analysis (Chou TC, *Pharm Rev*, 58:621-681, 2006) revealed ED₅₀ combination indices of 0.11 and 0.74 for MLL-AF9 and Karpas422 cells, respectively, indicating a synergistic effect of the combination (i.e. a combination index <1 reflects synergy) (**FIG 37**, bottom). Thus, SAH-EZH2 manifests a unique mechanism of action by targeting EED, dissociating the EZH2/EED complex, and thereby reduces EZH2 protein levels. As a result, the H3K27Me3 mark is reduced over time and

PRC2 transcriptional effects are inhibited, leading to anti-proliferative and induced-differentiation effects on EZH2-dependent leukemia cells.

Example 6: Enhanced EED binding affinity of a doubly stapled SAH-EZH2 peptide.

The EED binding affinities of SAH-EZH2 and SAH-EZH2(42-68)_{AC} were assessed by their capacities to displace FITC-labeled EZH2(42-68) peptide from EED. Serially diluted acetyl-SAHEZH2 and the doubly stapled acetyl-SAHEZH2(42-68)_{AC} (dose range from 2.5 nM to 10 μM) were incubated with purified EED (200 nM) and FITC-labeled EZH2(42-68) peptide (20 nM) for 30 min, followed by measurement of fluorescence polarization. N-terminal acetylated SAH-EZH2(42-68)_{AC} displaced FITC-labeled EZH2(42-68) peptide from EED two times more efficiently than SAH-EZH2. The non-binding EZH2(47-64) construct was used as a negative control.

Example 7: Anti-proliferative activity of the doubly stapled SAH-EZH2(42-68)_{AC} peptide on a diverse spectrum of cancer cell lines.

To test the anti-proliferative effect of SAH-EZH2(42-68)_{AC}, breast (MDA-MB231) and prostate (DU145) cancer cell lines were treated with SAH-EZH2(42-68)_{AC} at the indicated doses for 8 days and measured for viability by Cell Titer Glo®. SAH-EZH2(42-68)_{AC} was added to the culture medium once every other day. SAH-EZH2(42-68)_{AC} demonstrated enhanced dose-dependent anti-proliferative effects compared to SAH-EZH2 treatment, which was administered twice daily (Figure 34A) compared to the every other day schedule of SAH-EZH2(42-68)_{AC} (Figure 39). We further tested the anti-proliferative effects of SAH-EZH2(42-68)_{AC} on a Ewing Sarcoma cell line (TC71) and neuroblastoma cell line (Kelly). The cells were treated with SAH-EZH2(42-68)_{AC} at the indicated doses for 6 days on an every other day schedule and then measured for viability using Cell Titer Glo®. SAH-EZH2(42-68)_{AC} exhibits noticeable anti-proliferative effects on both TC71 and Kelly in a dose-dependent manner (Figure 40). To test the anti-proliferative effect on chronic myelogenous leukemia, SAH-EZH2(42-68)_{AC} was added to K562 cells at 10 μM every other day and viability was measured at days 2, 4, 6, and 10 using Cell Titer

Glo®. SAH-EZH2(42-68)_{AC} decreased proliferation of K562 cells in a dose-responsive manner (Figure 41). To assess the anti-proliferative effects of SAH-EZH2(42-68)_{AC} on androgen-dependent and advanced androgen-independent prostate cancer, SAH-EZH2(42-68)_{AC} was added to cell culture every other day at the indicated doses and viability measured using Cell Titer Glo®. Whereas SAH-EZH2(42-68)_{AC} was observed to anti-proliferative activity in each case, advanced androgen independent cancer cells (Abl, 22Rv1) were more sensitive to SAH-EZH2(42-68)_{AC} treatment (IC₅₀s for growth < 1 µM) compared to the androgen-dependent LNCaP cells (Figure 42).

METHODS USED IN THE EXAMPLES

Synthesis of Stabilized Alpha-Helix of EZH2 Peptides: SAH-EZH2 peptides were designed based on the EED-binding domain of EZH2, with replacement of natural amino acids with olefinic residues at sites on the non-interacting face of the EZH2 helix, as defined by X-ray crystallography³². Hydrocarbon stapling, peptide purification and quantitation, and characterization by circular dichroism were performed as previously described in detail^{35,36}.

Cell Lines and Tissue Culture: MLL-AF9 leukemia cells were generated as described in Krivtsov et al.⁴⁶ and cultured in AF9 media, containing IMDM (Invitrogen) supplemented with 5% fetal calf serum (FCS), 5 ng/µl mouse recombinant IL3 (R&D systems), and 1% penicillin/streptomycin (Invitrogen). COS7 cells (ATCC) were cultured in DMEM media supplemented with 10% FCS and 1% penicillin/streptomycin. Sf21 cells (Invitrogen) were cultured in Grace insect media (Invitrogen) supplemented with 10% FCS and 1% penicillin/streptomycin.

SAH-EZH2/EED Binding Analyses: For fluorescence polarization binding analysis, C-terminal His-tagged EED (His-EED) was expressed in *Escherichia coli* BL21(DE3) using 0.3 mM IPTG for 6 h at room temperature. Bacterial pellet lysate was loaded onto a Ni-NTA column (Novagen) for purification of His-EED. Fluorescinated stapled peptides (final concentration, 25 nM) were incubated with His-EED (serial dilution range, 25 nM-8 µM) at room temperature until equilibrium was reached (~15 min). Binding activity was measured by fluorescence polarization on a Perkin-Elmer LS50B luminescence 5 spectrophotometer. K_D values were determined by following equation,

$$y = AF + \left(\frac{AB - AF}{L} \right) \times \frac{L + K_D + X - \sqrt{(L + K_D + X)^2 - 4 \times L \times X}}{2}$$

y: Fluorescence polarization, X: Receptor concentration, L: Ligand concentration

AF: Minimum value of y, AB: Maximum value of y

5 For anti-FITC pull down-based binding assays, HA-EED was produced in Sf21 cells as described in Shen et al.^{29,40}. FITC-SAH-EZH2 peptides (final concentration, 50, 10, and 2 nM) were mixed with 2 µl, 0.5 µl and 0.2 µl of anti-FITC antibody (Abcam, 1 mg/ml) respectively, and incubated with 30 µl of protein G agarose (Roche) in 400 µl binding buffer (pH 8.0, 20 mM Tris-HCl, 150 mM NaCl, 0.3% NP40, 10% glycerol).
10 After successive washes, HA-EED (final concentration of 40 nM each tube) was added and incubated for 3 h, followed by detection of HA-EED bound to FITC-SAH-EZH2 peptides by anti-HA (Santa Cruz Biotechnology) western analysis. Detection of FITC-SAH-EZH2 peptides was accomplished by fluorescence scan (GE Healthcare Life Sciences, Typhoon 9200).

15 Cellular Uptake of SAH-EZH2 Peptides: MLL-AF9 leukemia cells were seeded in 48-well plates at densities of 1.0×10^6 cells per well in 200 µl of AF9 media. FITC-SAH-EZH2 peptides (final concentrations, 4, 2 and 1 µM) were added to the cells and incubated for 8 h. Cells were then washed three times with phosphate buffer saline (PBS, Sigma) and then trypsinized (0.05% Trypsin-EDTA, Invitrogen) at 37°C
20 for 5 min to remove any residual peptide attached to the cell surface. The cells were lysed with SDS loading buffer (Biorad) and brief sonication. Lysates were electrophoresed on SDS-PAGE gels (Biorad, Criterion tris-tricine 10-20%) and subjected to fluorescence scan (GE healthcare, Typhoon imager 9400)

Confocal Microscopy: COS7 cells were plated in 8-well CC²™ Chamber
25 Slides™ (Nunc) for 1-2 days prior to experimentation. Slides were washed twice with serum-free DMEM media (Invitrogen) and FITC-SAH-EZH2 peptides were added in serum-free DMEM media at a final concentration of 4 µM. After 3 h incubation, an equal volume of DMEM media supplemented with 20% FCS was added, and the cells subjected to an additional 5 h incubation. Slides were washed three times with PBS
30 and fixed with 4% paraformaldehyde (Electron Microscopy) for 12 min. After blocking with 5% goat serum (Vector) and staining with EED antibody (Millipore,

produced in rabbit), the slides were stained with Alexa Fluor 647 donkey anti-rabbit IgG (Invitrogen). Nuclei were stained upon application of mounting medium containing DAPI (Vector). Confocal images were acquired on a Zeiss LSM700 microscopy and analyzed with ZEN 2011 software (Zeiss).

5 Immunoprecipitation Analyses: To assess the interaction between SAH-EZH2 peptides and native EED, MLL-AF9 leukemia cells (2×10^7) were cultured in 10 ml AF9 media using 10 cm dishes (BD Biosciences) and FITC-SAH-EZH2 peptides added to final concentration of 20 μ M, followed by 8-12 h incubation. Treated cells were washed and trypsinized as above, lysed and then incubated with 50 μ l of FITC-
10 antibody (1 mg/ml) and 100 μ l of protein G agarose (Roche) for 3 h. The amount of EED bound to FITC-SAH-EZH2 peptides was assessed by anti-EED western analysis (Millipore). To monitor for dissociation of native EED-EZH1/EZH2 complexes by SAH-EZH2, Sf21 cells were co-transfected with HA-EED and FLAG-EZH1 or FLAG-EZH2 baculovirus. EED-EZH1 and EED-EZH2 complexes were purified by
15 anti-FLAG agarose (Sigma). EED-EZH1 and EED-EZH2 complexes (40 nM final concentration) were incubated with FITC-SAH-EZH2 peptides (final concentrations, 10, 3 and 1 μ M) in 200 μ l binding buffer for 2 h. After successive washes, the mixtures were incubated with anti-HA agarose (Sigma) and the level of HA-EED-bound EZH1 and EZH2 assessed by anti-HA western analysis.

20 Analysis of Trimethylation Status: MLL-AF9 leukemia cells were seeded in 48-well plates at densities of 2×10^5 cells per well in 200 μ l of AF9 media and then treated with SAH-EZH2 peptides (10 μ M) twice daily for 7 days. Cells were then lysed in loading buffer (Biorad) with brief sonication and electrophoresed on SDS-PAGE gels (Thermoscientific, 4-20%). Levels of Lys4, Lys9, Lys27 (07-449,
25 Millipore) and Lys36 trimethylation were analyzed by the corresponding trimethyl mark-specific antibodies (Millipore). For flow cytometric analysis of H3 Lys27 trimethylation, MLL-AF9 cells were treated with SAH-EZH2 peptides (10 μ M) twice daily for 7 days and then fixed in 70% cold ethanol for overnight at -20 °C. Cells were washed twice in PBS, stained with anti-Lys27-Me3 antibody (Cell Signaling,
30 #9733S) for 20 min and then anti-rabbit IgG Alexafluoro-647 (Invitrogen) for 20 min, followed by FACS analysis using a BD FACSCalibur.

Cell Proliferation, Apoptosis, and Cell Cycle Analyses: To monitor the effect of SAH-EZH2 peptides on cell proliferation, MLL-AF9 cells were seeded in 48 well plates at densities of 2×10^5 cells per well in 150 μ l of AF9 media and treated with SAH-EZH2 peptides (10 μ M) twice daily. Cells were counted every other day using a
5 hematocytometer. To evaluate apoptosis induction, the cells were stained with Annexin V5-PE and 7-AAD after 8 or 16 days of treatment with SAH-EZH2 peptides. Camptothecin (Biovision) was used as a positive control (2 μ M, 48 h). Cells were analyzed using a BD FACScaliber and data was processed using Flowjo software. For cell cycle analysis, cells were stained with propidium iodide (BD Biosciences)
10 after 4 or 8 days of treatment with SAH-EZH2 peptides. Cells were analyzed using a BD FACScalibur and data processed using Modfit software.

Cellular Differentiation Analyses: To monitor the morphology of treated cells, MLL-AF9 leukemia cells were exposed to SAH-EZH2 peptides in AF9 media for 14 days, as described above. Cells were counted and then diluted into Methocult
15 (STEMCELL technologies) supplemented with 10 ng/ml IL3, 10 ng/ml IL6, 10 ng/ml SCF, 5% FCS, and 1% penicillin/streptomycin at a density of 1000 cells/ml with continued exposure to SAH-EZH2 peptides (20 μ M). Aliquots of methocult (1 ml) were plated in 3 cm dishes (BD Biosciences) and colonies counted after 7 day incubation. Cells were washed with PBS (Sigma), cytopun, and then stained using
20 modified Giemsa stain (Sigma). Morphology was assessed by light microscopy (Nikon Eclipse E800 microscope). HPC5 cells were treated as above except that a higher concentration of SCF (100 ng/ml) was used in the culture media. To detect macrophage-specific cell surface marker expression, MLL-AF9 cells were treated with SAH-EZH2 peptides for 7 d followed by successive washes in PBS and
25 immunostaining with Rat Anti Mouse F4/80 Antigen Alexa 647 (AbD Serotec) and PE Anti-Mouse Neutrophils Monoclonal antibody (CEDARLANE) antibodies. Cells were analyzed by BD FACS Calibur and data processed using Flowjo software.

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OTHER EMBODIMENTS

It is to be understood that 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. An internally cross-linked polypeptide comprising the amino acid sequence
 $A_0B_0C_0D_0E_0F_0G_0A_1B_1C_1D_1E_1F_1G_1A_2B_2C_2D_2E_2F_2G_2A_3B_3C_3D_3E_3F_3G_3A_4$ (SEQ ID
 NO:1) wherein:

5 wherein A_0 is S or a conservative substitution; B_0 is M or a conservative
 substitution; C_0 is F or a conservative substitution; D_0 is S or a conservative
 substitution; E_0 is S or a conservative substitution; F_0 is N or a conservative
 substitution; G_0 is R or a conservative substitution; A_1 is Q or a conservative
 substitution or a staple to E_1 ; B_1 is K or a conservative substitution; C_1 is I or a
 10 conservative substitution; D_1 is L or a conservative substitution; E_1 is E, Q or a
 conservative substitution or a staple to A_1 ; F_1 is R or a conservative substitution; G_1 is
 T or a conservative substitution; A_2 is E, Q or a conservative substitution; B_2 is I or a
 conservative substitution; C_2 is L or a conservative substitution; D_2 is N or a
 conservative substitution; E_2 is Q or a conservative substitution; F_2 is E, Q or a
 15 conservative substitution; G_2 is W or a conservative substitution; A_3 is K or a
 conservative substitution; B_3 is Q or a conservative substitution; C_3 is R or a
 conservative substitution; D_3 is R or a conservative substitution; E_3 is I or a
 conservative substitution; F_3 is Q or a conservative substitution; G_3 is P or a
 conservative substitution; A_4 is V or a conservative substitution,

20 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 internal staples and/or an
 internal stitch;

25 the side chains of four amino acids are replaced by internal staples, internal
 stiches, or a combination of internal staples and stiches; or

the side chains of at least four amino acids are replaced by internal staples,
 internal stiches, or a combination of internal staples and stiches.

2. The internally cross-linked peptide of claim 1, selected from:

SMFSSNRQKILERTEILNQEWKQRRIQPV (SEQ ID NO:2),
 FSSNRQKILERTEILNQEWKQRRIQPV (SEQ ID NO:40),
 5 FSSNRQKILERTQILNQEWKQRRIQPV (SEQ ID NO:41),
 RSMFSSNRQKILERTEILNQEWKQRRIQPV (SEQ ID NO:42),
 RRFSSNRQKILERTEILNQEWKQRRIQPV (SEQ ID NO:43),
 RRFSSNRQKILERTQILNQEWKQRRIQPV (SEQ ID NO:44),
 RSMFSSNRQKILERTEILNQEWKQRRIQPV (SEQ ID NO:45),
 10 RFSNRQKILERTEILNQEWKQRRIQPV (SEQ ID NO:46),
 RFSNRQKILERTQILNQEWKQRRIQPV (SEQ ID NO:47),
 SMFSSNRQKILERTEILNQEWKQRRIQPVRR (SEQ ID NO:48),
 FSSNRQKILERTEILNQEWKQRRIQPVRR (SEQ ID NO:49),
 FSSNRQKILERTQILNQEWKQRRIQPVRR (SEQ ID NO:50),
 15 SMFSSNRQKILERTEILNQEWKQRRIQPVR (SEQ ID NO:51),
 FSSNRQKILERTEILNQEWKQRRIQPVR (SEQ ID NO:52), and
 FSSNRQKILERTQILNQEWKQRRIQPVR (SEQ ID NO:53),

wherein

the side chains of two amino acids separated by two, three or six amino acids
 20 are replaced by an internal staple;

the side chains of three amino acids are replaced by internal staples and/or an
 internal stitch;

the side chains of four amino acids are replaced by internal staples, internal
 stiches, or a combination of internal staples and stiches; or

25 the side chains of at least four amino acids are replaced by internal staples,
 internal stiches, or a combination of internal staples and stiches.

3. The cross-linked peptide of claims 1 or 2, wherein the amino acid sequence
 is (SEQ ID NO:2).

30

4. The cross-linked peptide of claims 1-3, wherein the internal staple replacing the side chains of the two amino acids separated by two or six amino acids comprises an internal staple selected from Table 1.

5 5. The cross-linked peptide of claim 4, wherein the internally cross-linked peptide is selected from the group consisting of SEQ ID NOs: 2-39

6. The cross-linked peptide of claims 1-3, wherein the internal staples and/or the internal stitch replacing the side chains of the three amino acids comprise an
10 internal stitch selected from Table 1.

7. The cross-linked peptide of claims 1-3, wherein the internal staples and/or the internal stitch replacing the side chains of the three amino acids comprises at least two internal staples.

15 8. The cross-linked peptide of claims 1-3, wherein the internal staples and/or the internal stitch replacing the side chains of the three amino acids comprises a combination of at least one internal staple and an internal stitch.

20 9. The cross-linked peptide of claims 6-8, wherein 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 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
25 acids, and the second and third amino acids are distinct amino acids.

10. The cross-linked peptide of claims 6-8, wherein the internal stitch replacing the side chains of the three amino acids cross-links a pair of amino acids separated by two, three, or six amino acids.

30 11. The cross-linked peptide of claims 1-3, wherein the side chains of the four amino acids are replaced by two distinct internal staples.

12. The cross-linked peptide of claim 11, wherein 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
 5 second pair of amino acids separated by two, three, or six amino acids.

13. The cross-linked peptide of claims 11-12, wherein the internally cross-linked peptide comprises SEQ ID NO:1, wherein C₀, F₀, G₀, B₁, C₁, F₁, C₂, D₂, F₂, G₂, C₃, E₃, G₃ and A₄ are not substituted.

10

14. The cross-linked peptide of claim 13 wherein the peptide comprises
 SMFSSNRQKILERTEILNQEWKQRRIQPV (SEQ ID NO:2),
 FSSNRQKILERTEILNQEWKQRRIQPV (SEQ ID NO:40),
 FSSNRQKILERTQILNQEWKQRRIQPV (SEQ ID NO:41),
 15 RRSMFSSNRQKILERTEILNQEWKQRRIQPV (SEQ ID NO:42),
 RRFSSNRQKILERTEILNQEWKQRRIQPV (SEQ ID NO:43),
 RRFSSNRQKILERTQILNQEWKQRRIQPV (SEQ ID NO:44),
 RSMFSSNRQKILERTEILNQEWKQRRIQPV (SEQ ID NO:45),
 RFSSNRQKILERTEILNQEWKQRRIQPV (SEQ ID NO:46),
 20 RFSSNRQKILERTQILNQEWKQRRIQPV (SEQ ID NO:47),
 SMFSSNRQKILERTEILNQEWKQRRIQPVRR (SEQ ID NO:48),
 FSSNRQKILERTEILNQEWKQRRIQPVRR (SEQ ID NO:49),
 FSSNRQKILERTQILNQEWKQRRIQPVRR (SEQ ID NO:50),
 SMFSSNRQKILERTEILNQEWKQRRIQPVRR (SEQ ID NO:51),
 25 FSSNRQKILERTEILNQEWKQRRIQPVRR (SEQ ID NO:52), or
 FSSNRQKILERTQILNQEWKQRRIQPVRR (SEQ ID NO:53),

wherein the side chains of two amino acids separated by three or six amino acids comprises an internal staple selected from Table 1 (**FIG 43**) and wherein 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 or 13 of the amino acids can be substituted, preferably
 30 conservatively.

15. The cross-linked peptide of claim 14 wherein the first cross-link is a C11 alkylene between the alpha carbons of 8' and X' and the second cross-link is a C11 alkylene between the alpha carbons of 8'' and X''.

5 16. The cross-linked peptide of claim 14 wherein the alpha carbons of 8', X', 8'' and X'' are substituted with a methyl group.

17. The cross-linked peptide of claims 1-3, wherein the internal staples, internal stitches, or the combination of internal staples and internal stitches replacing
10 the side chains of the at least four amino acids comprises at least one staple and at least one stitch.

18. The cross-linked peptide of claim 17, wherein the at least one staple cross-links a pair of amino acids separated by two, three, or six amino acids and the at least
15 one stitch cross-links a first amino acid to a second amino acid and a third amino acid, 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.

20 19. The cross-linked peptide of claims 17-18, wherein the at least one staple is selected from Table 1.

20. A pharmaceutical composition comprising the cross-linked peptides of claims 1-19.

25 21. The pharmaceutical composition of claim 20, comprising a medicament for the treatment of cancer.

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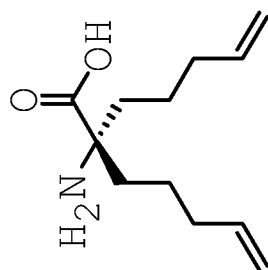


FIG. 1A

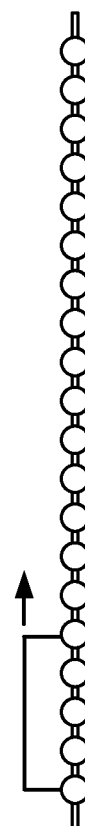
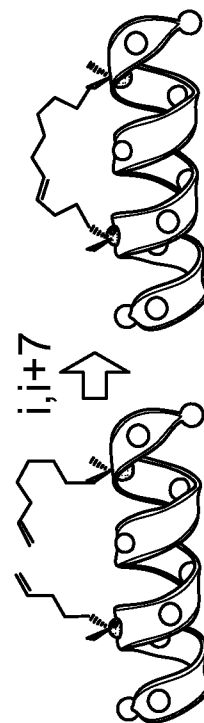
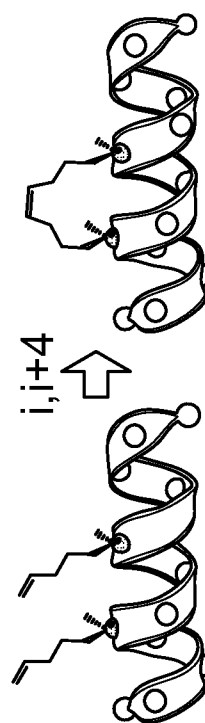
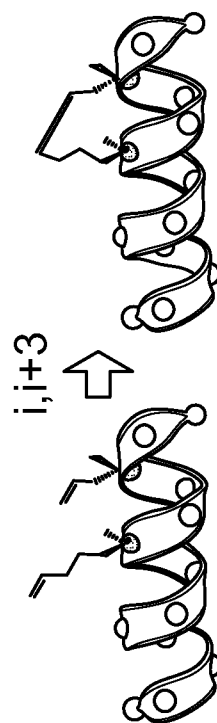
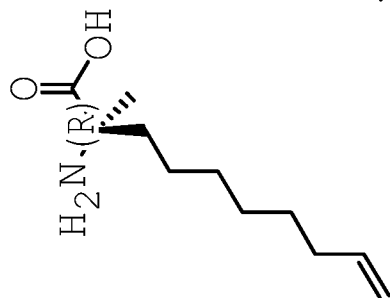
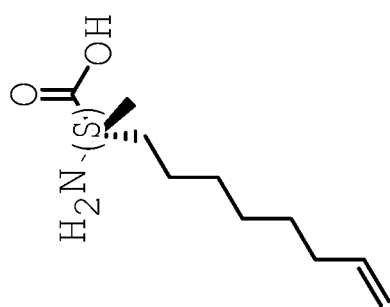
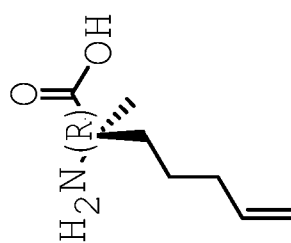
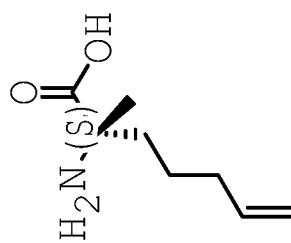
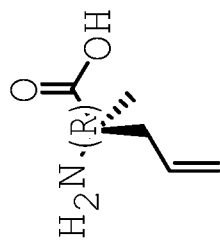


FIG. 1B

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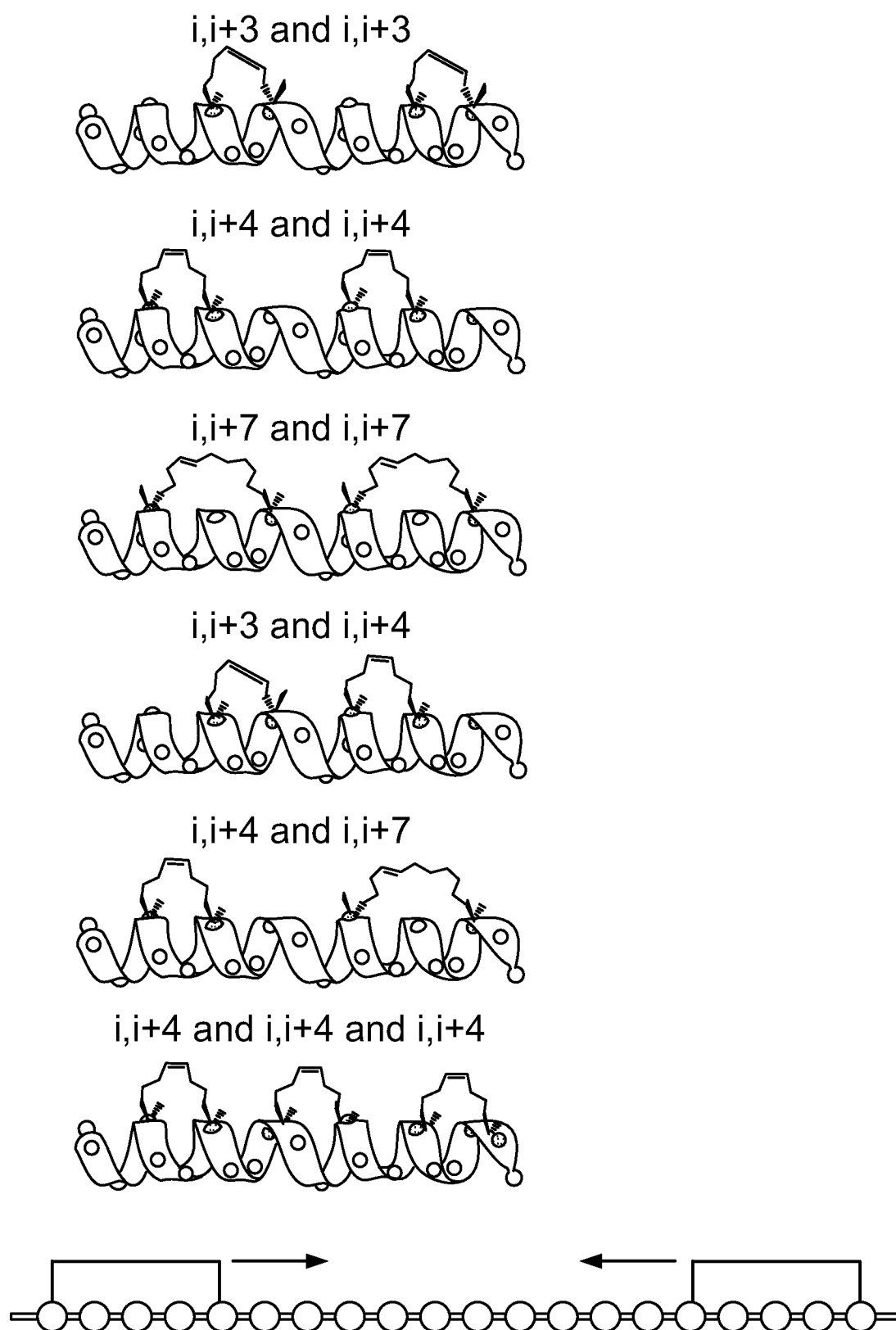


FIG. 1C

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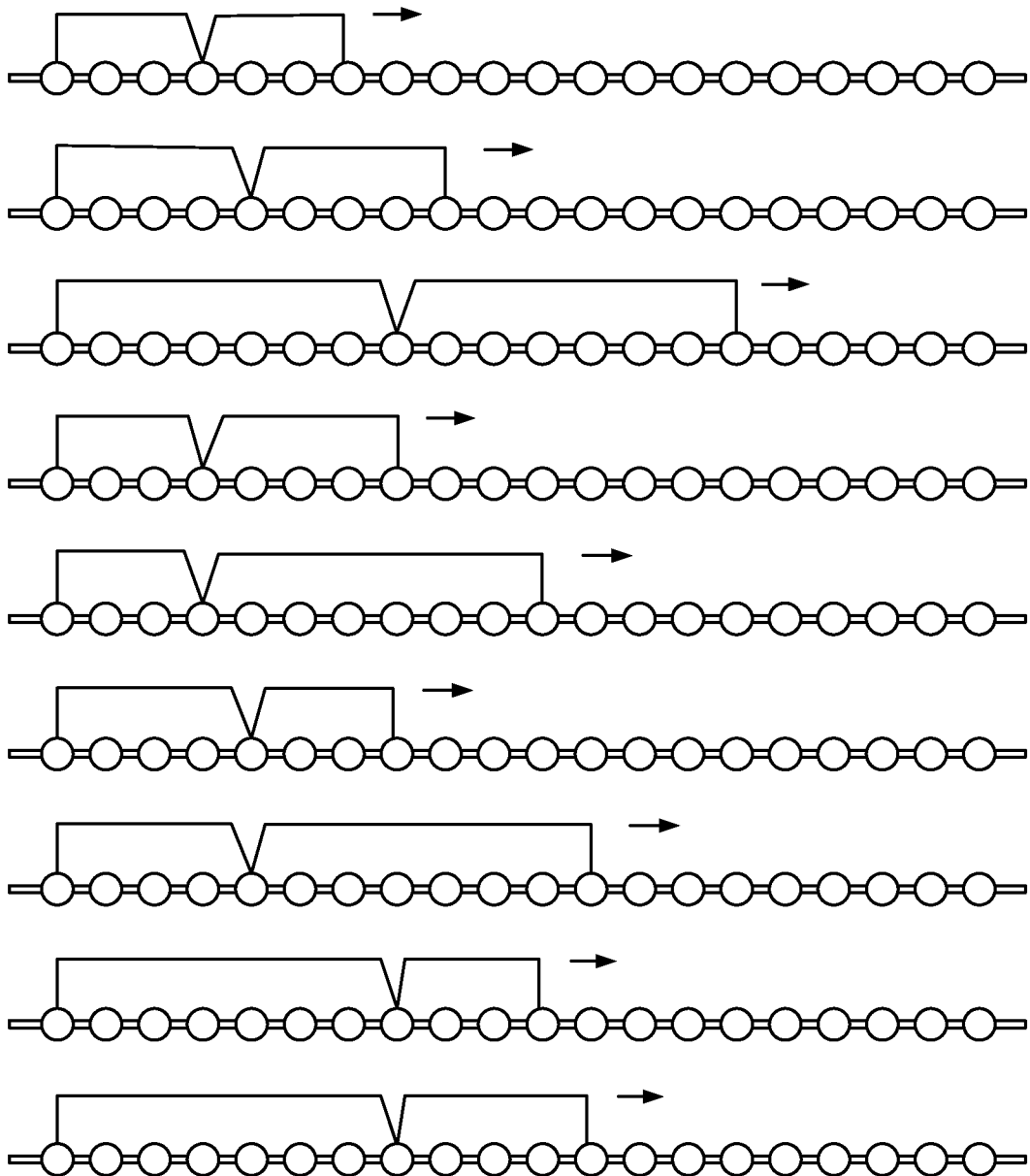
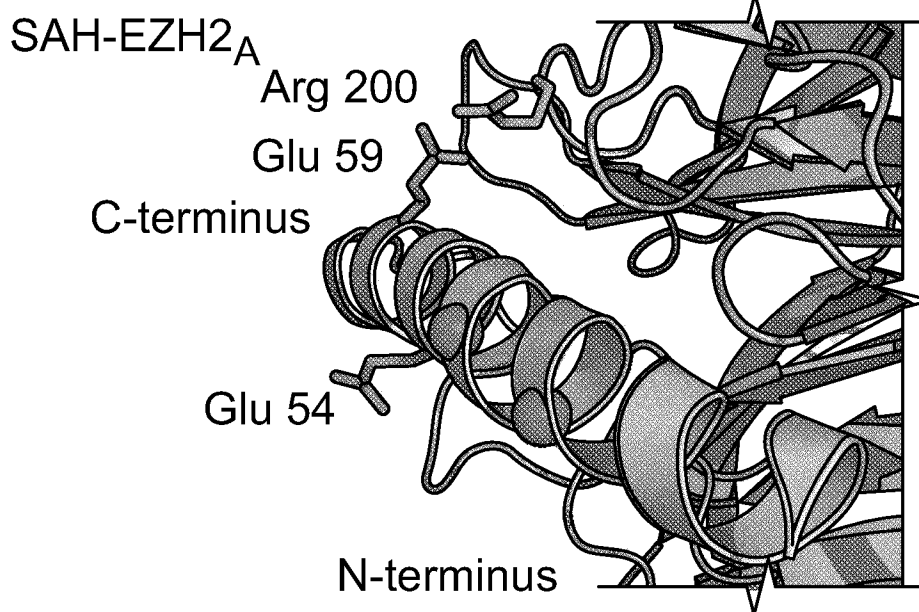


FIG. 1D

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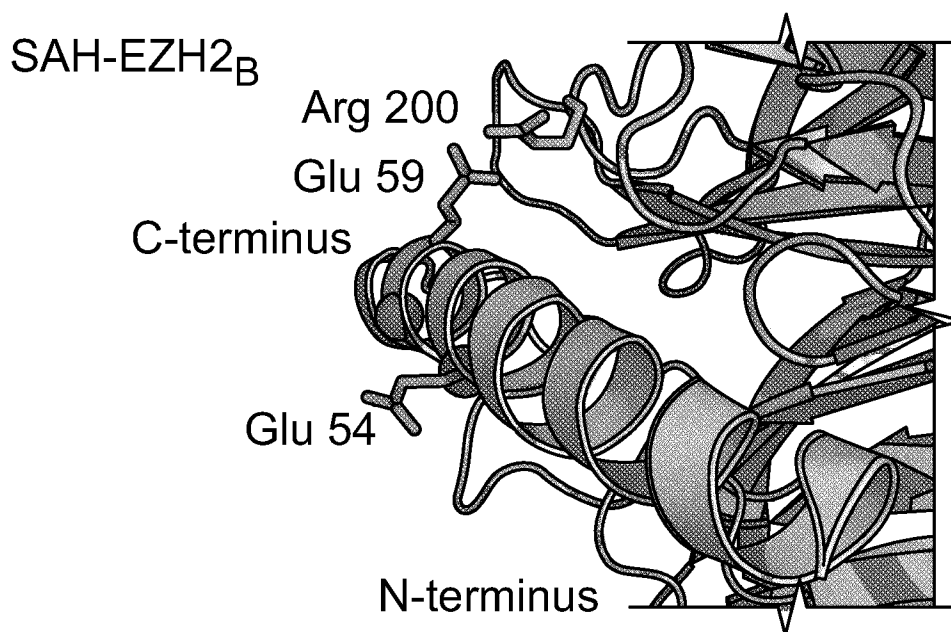
a



SAH-EZH2_A(40-68) SN_LFSSNRXKILXRTEILNQEWKQRRIQPV

SAH-EZH2_A(42-68) FSSNRXKILXRTEILNQEWKQRRIQPV

SAH-EZH2_A(42-64) FSSNRXKILXRTEILNQEWKQRR



SAH-EZH2_B(40-68) SN_LFSSNRQKILERTXILNXEWKQRRIQPV

SAH-EZH2_B(42-68) FSSNRQKILERTXILNXEWKQRRIQPV

SAH-EZH2_B(42-64) FSSNRQKILERTXILNXEWKQRR

FIG. 2

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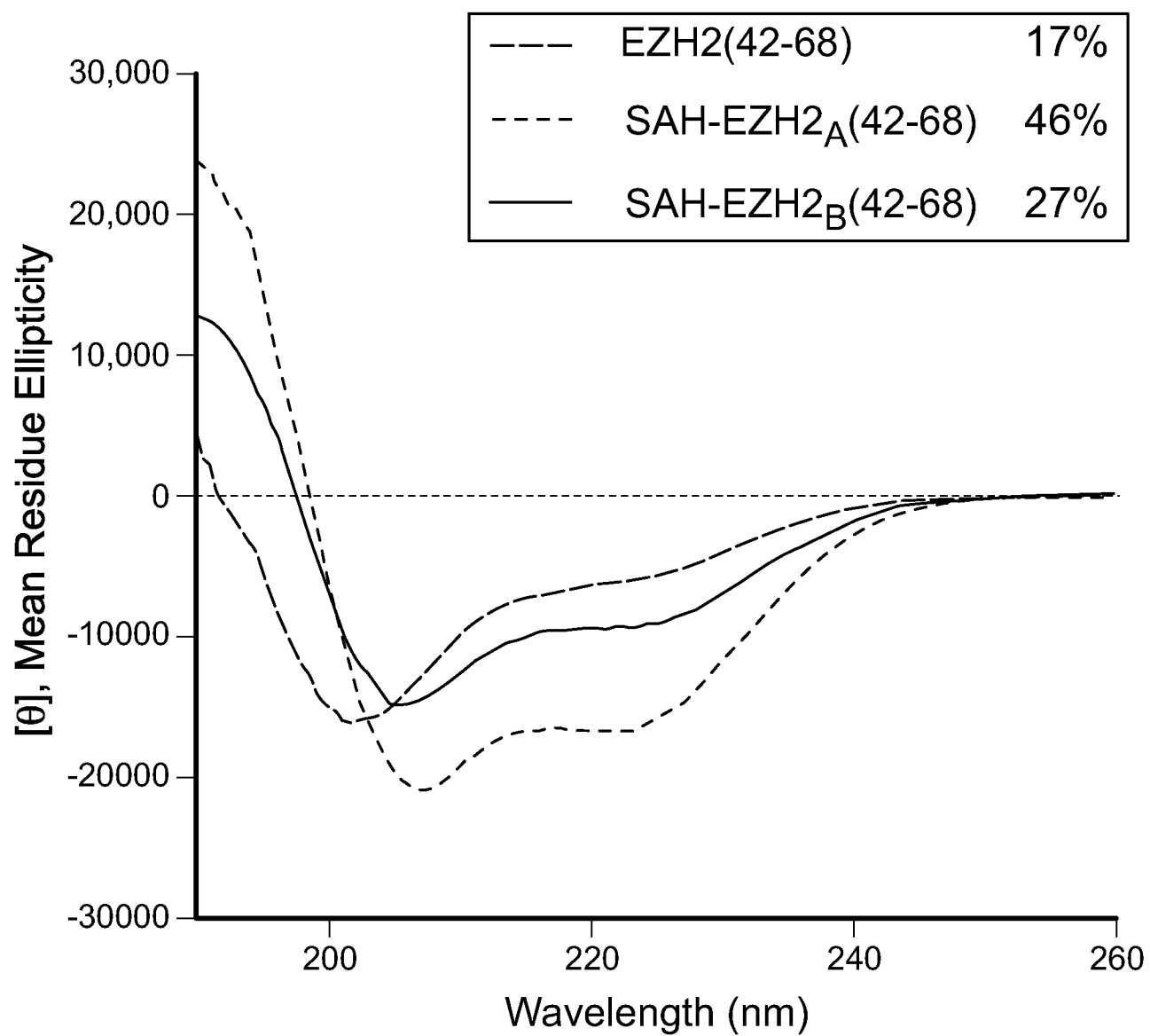


FIG. 3

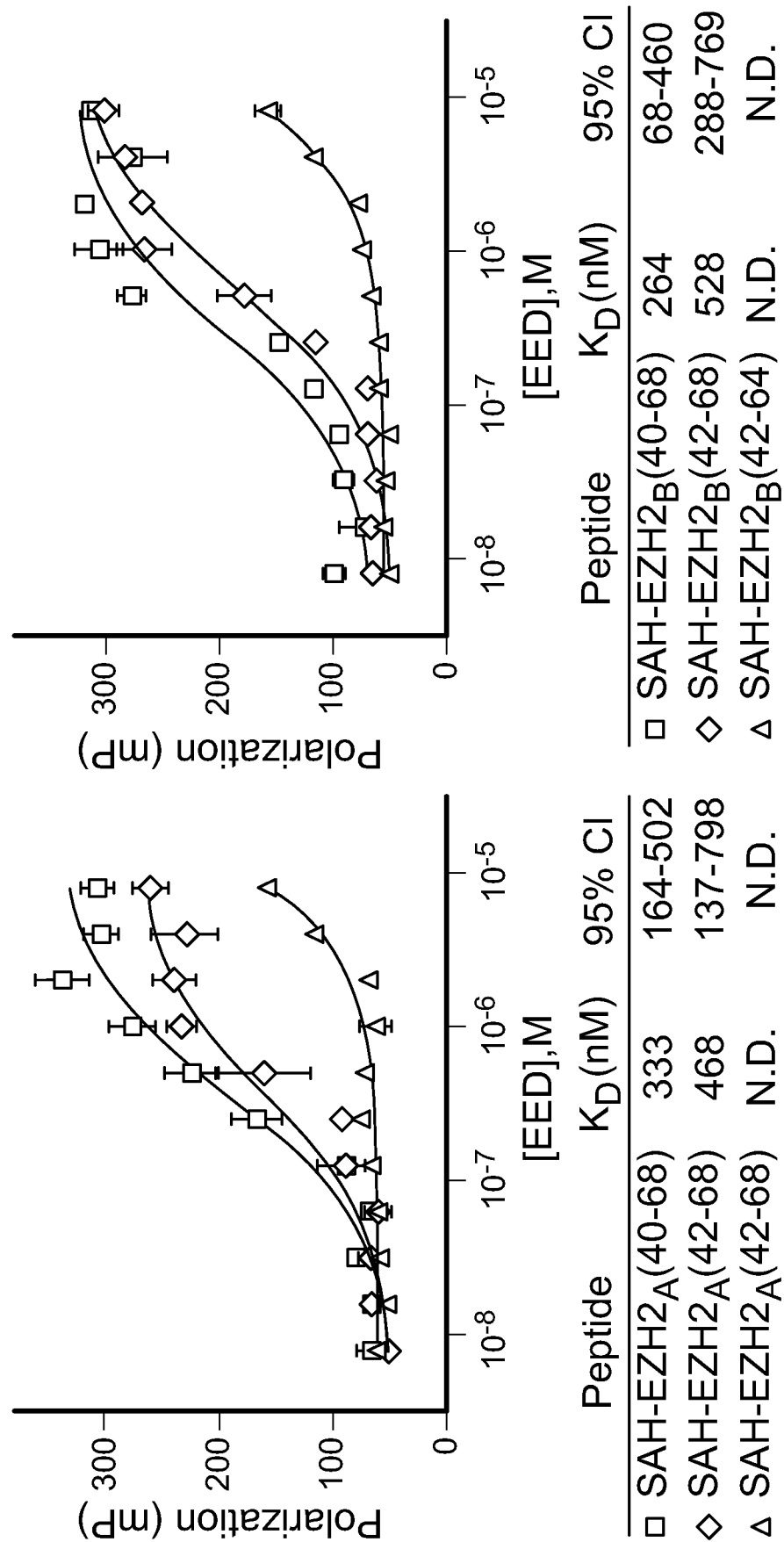


FIG. 4

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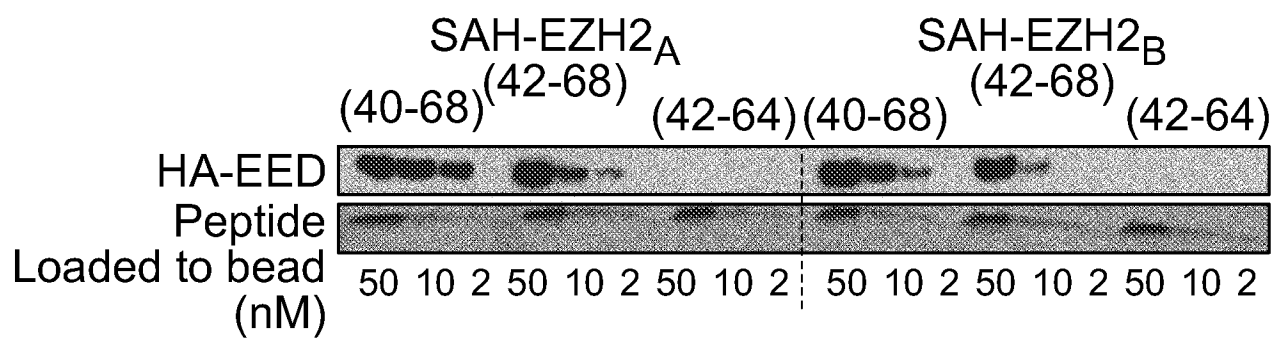


FIG. 5

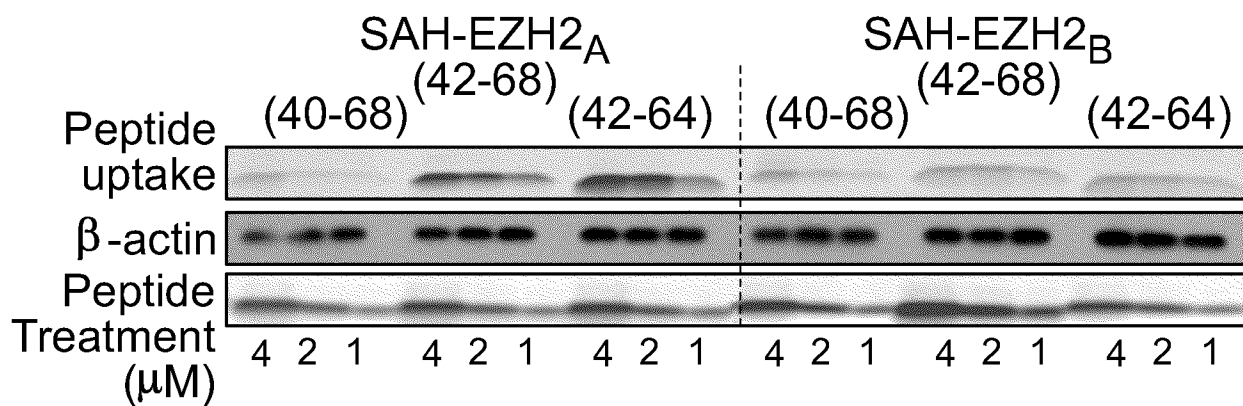


FIG. 6

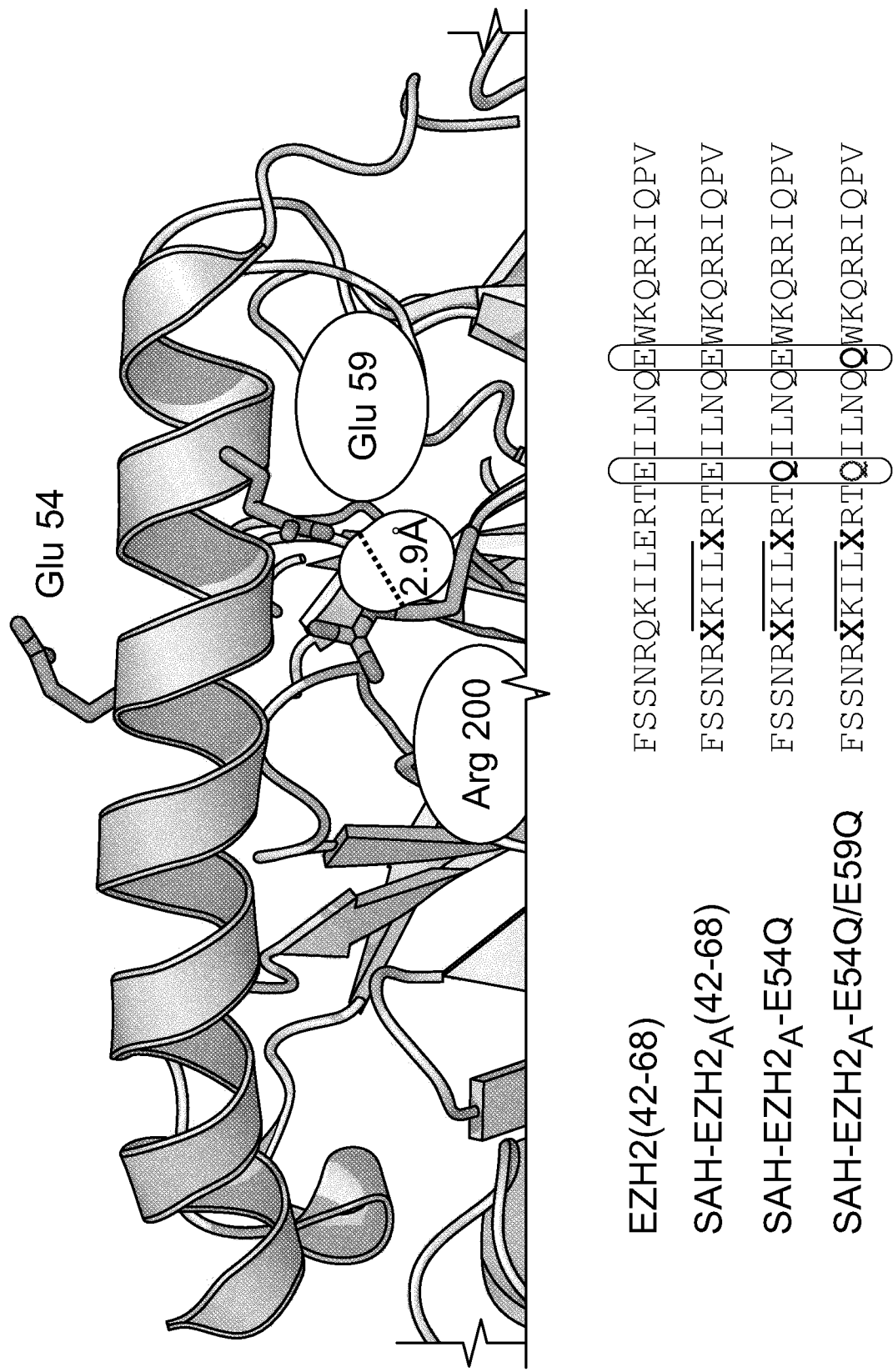


FIG. 7

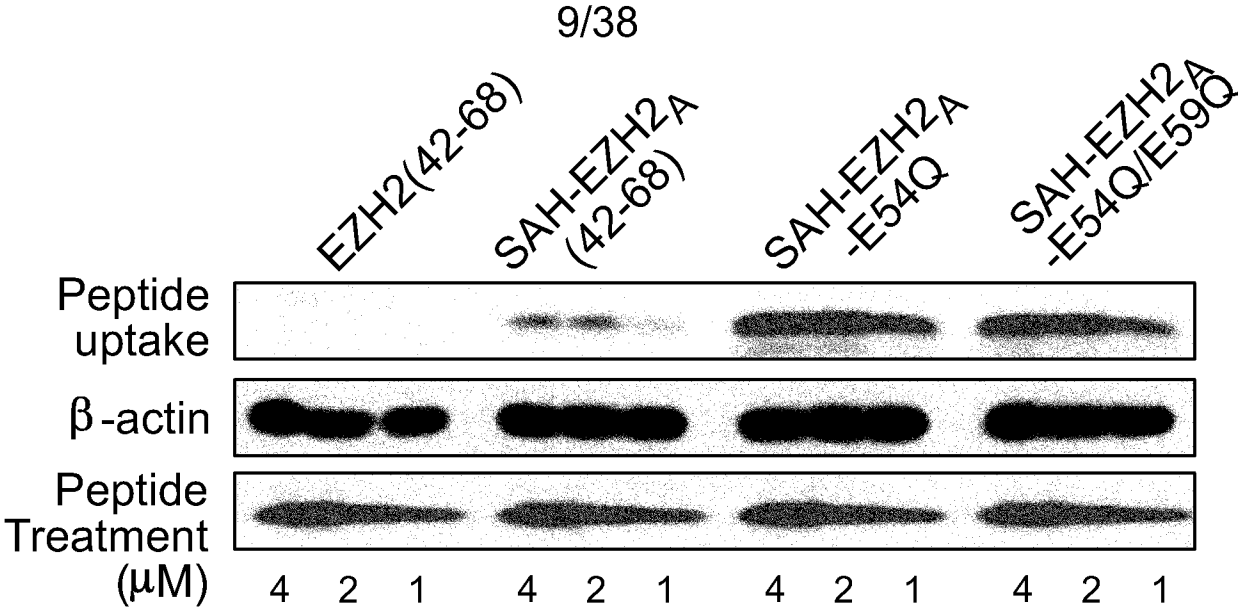
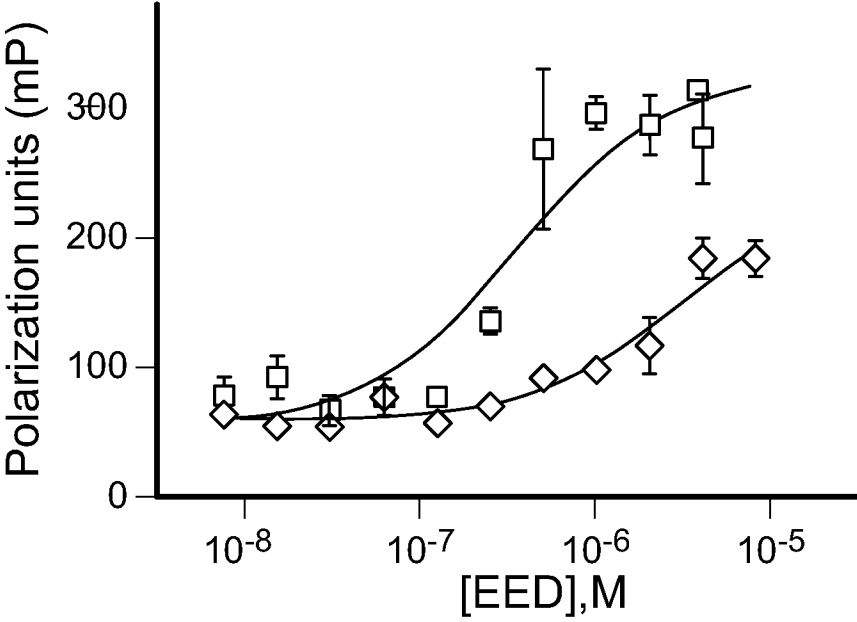


FIG. 8



Peptide	K _D	95% CI
□ SAH-EZH2	320 nM	52-588
◇ SAH-EZH2 _{MUT}	>10 μ M	-

FIG. 9

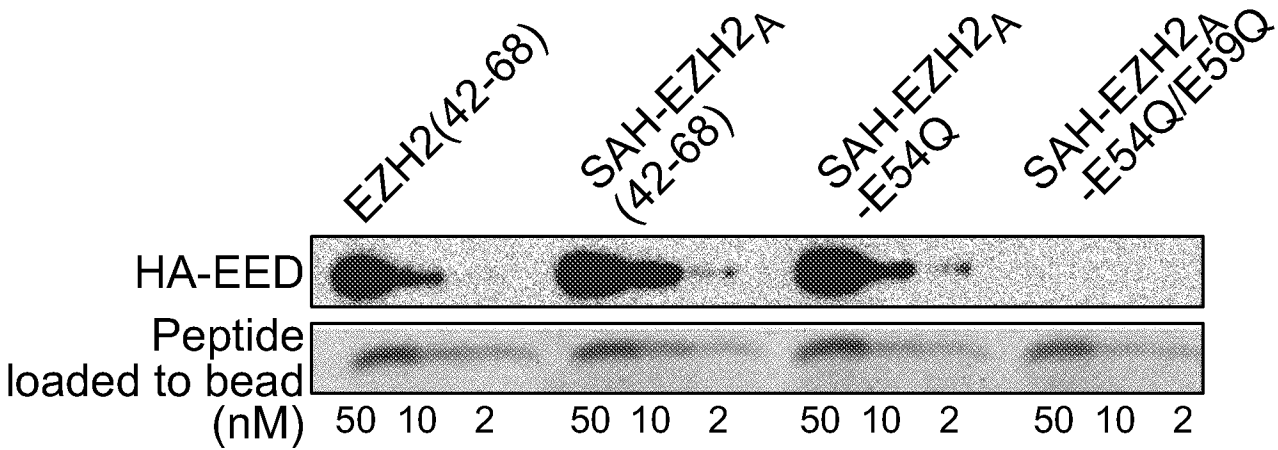


FIG.10

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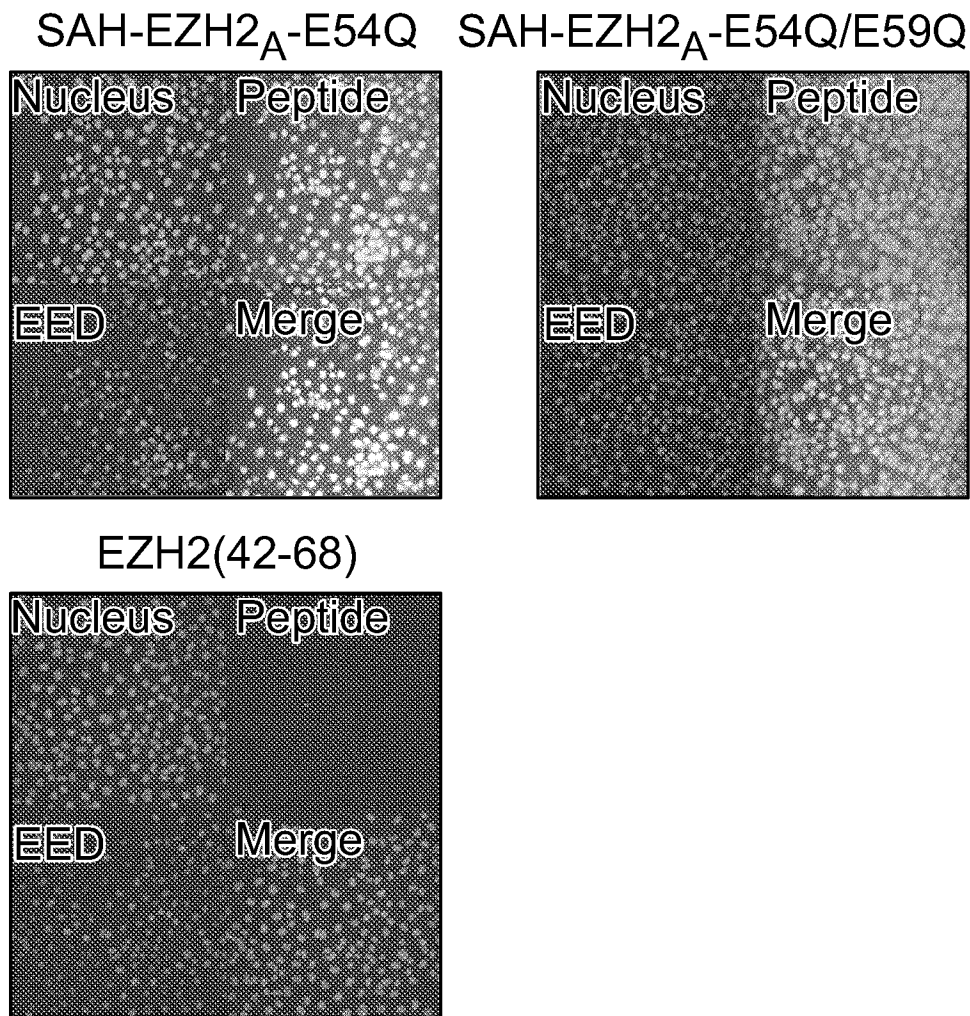


FIG. 11

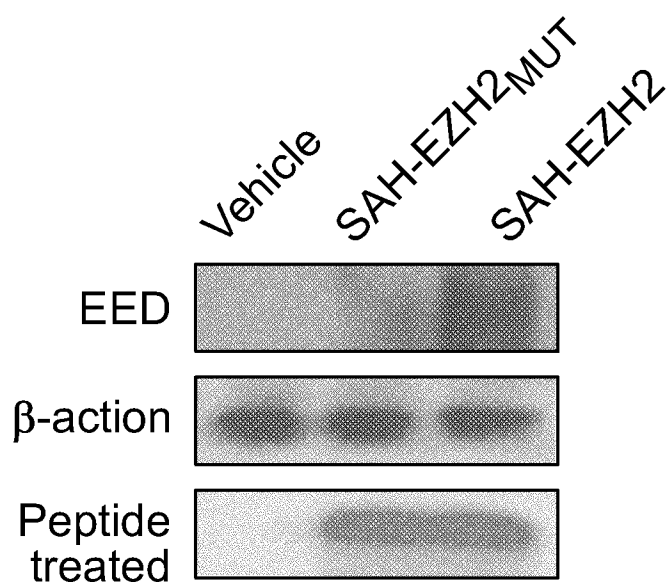


FIG. 12

Cellular uptake (COS7) of peptide after
8 hour incubation

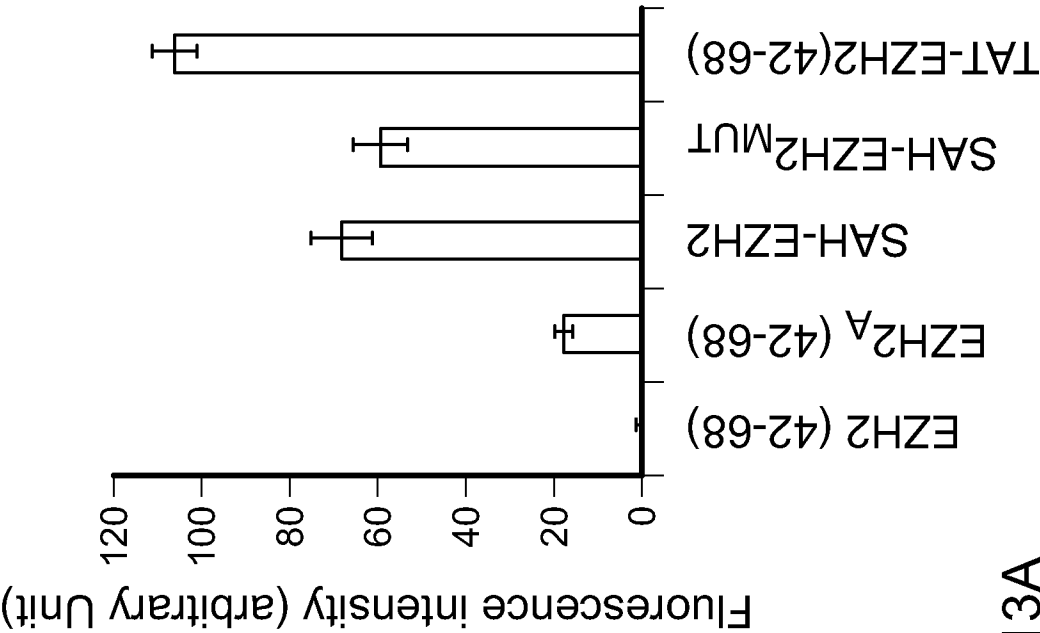
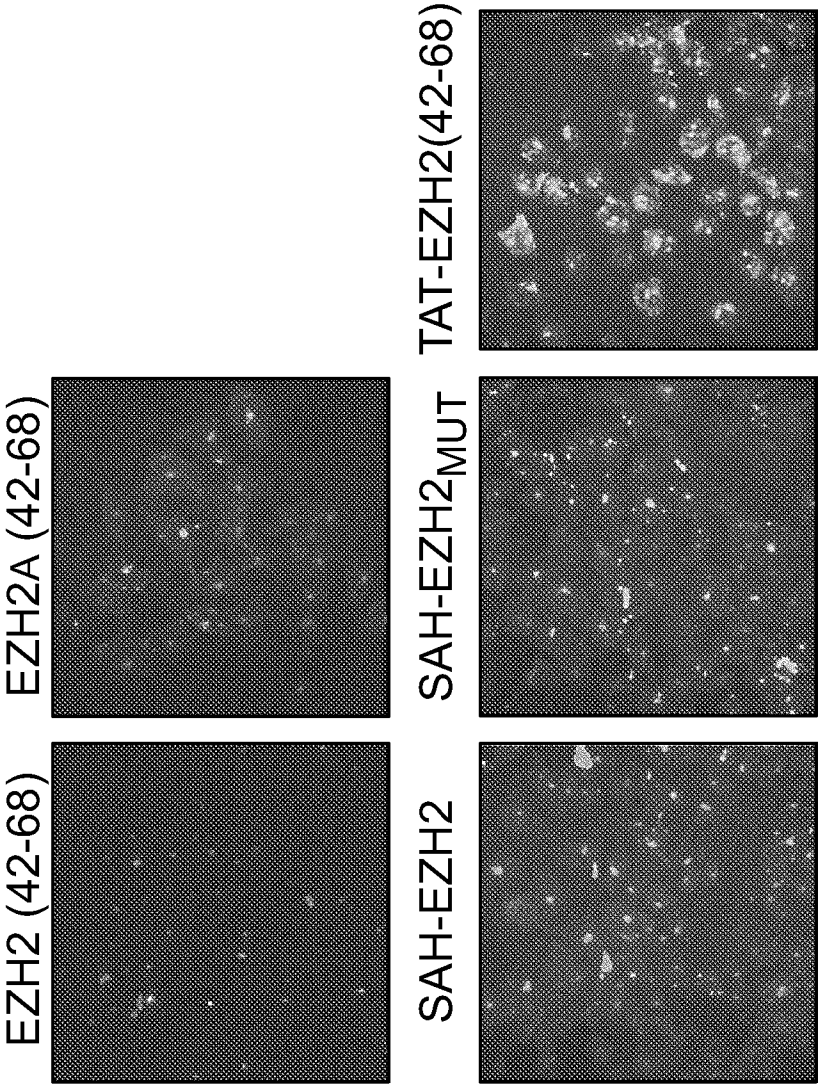


FIG. 13A

Cell lysate (MLL-AF9) analysis for peptide uptake and proteolysis

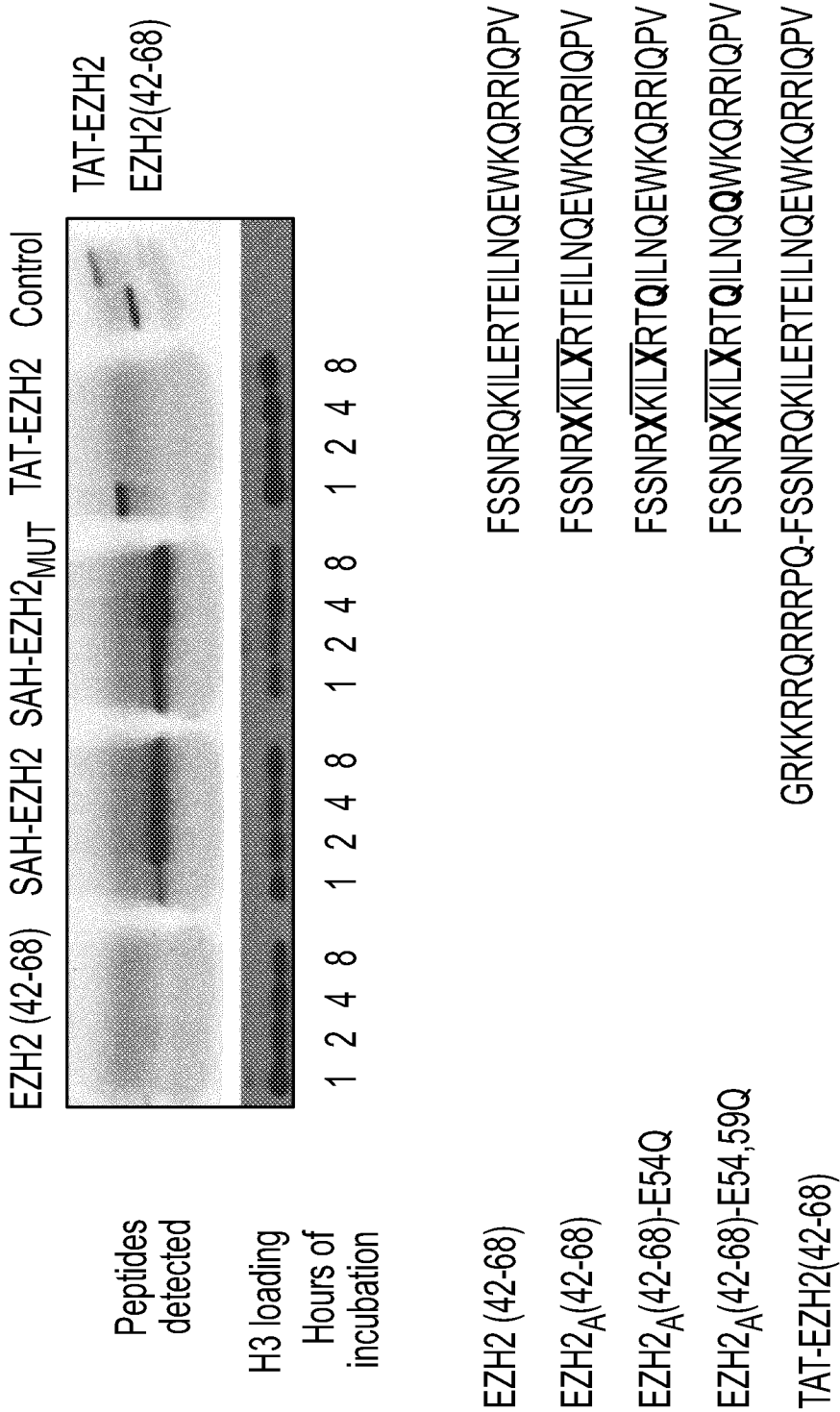
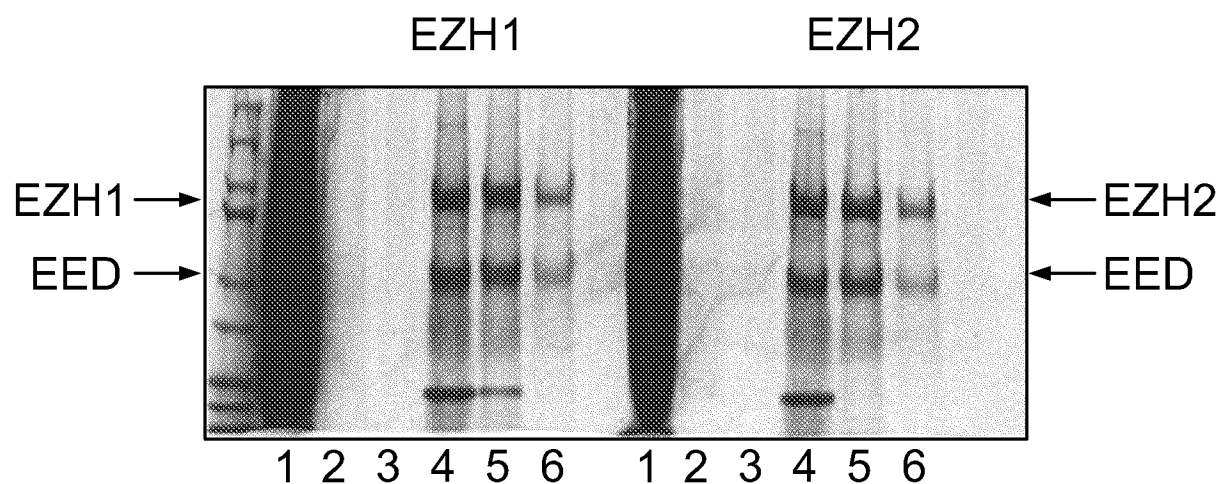


FIG. 13B

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1. Flow through



2. 1st Wash



3. 2nd Wash



4. 1st Elute



5. 2nd Elute

6. Proteins left bound to resin after elution

FIG. 14

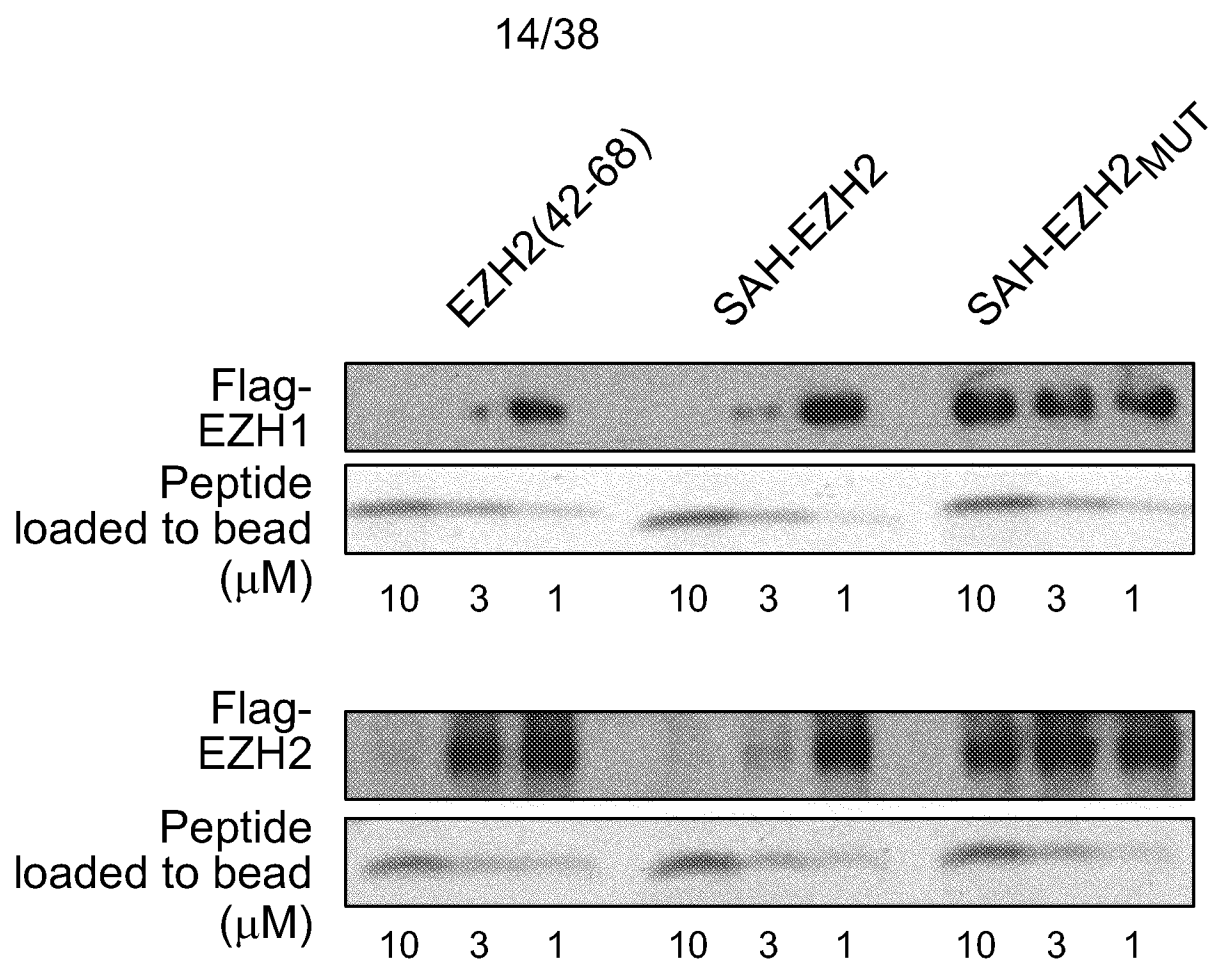


FIG. 15

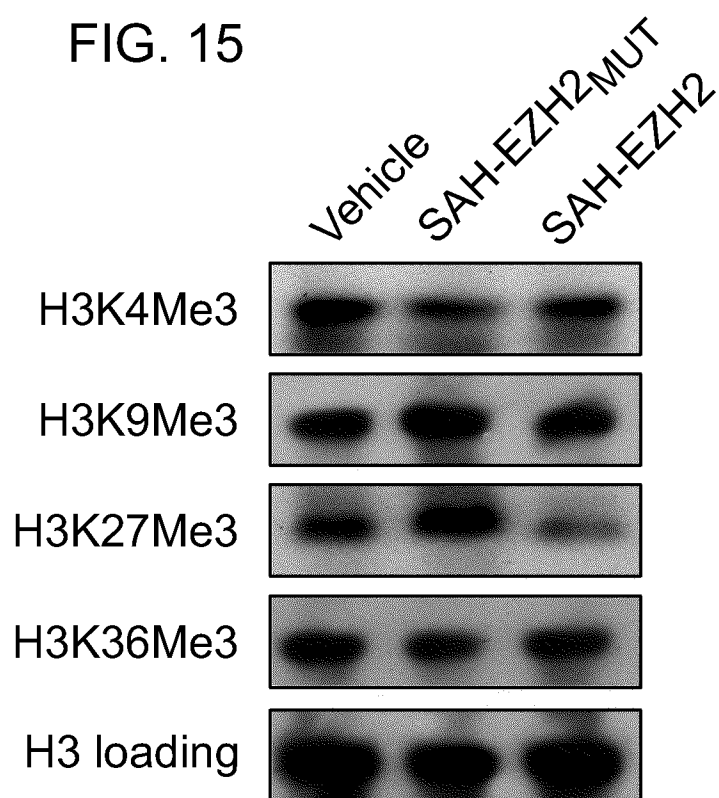


FIG. 16

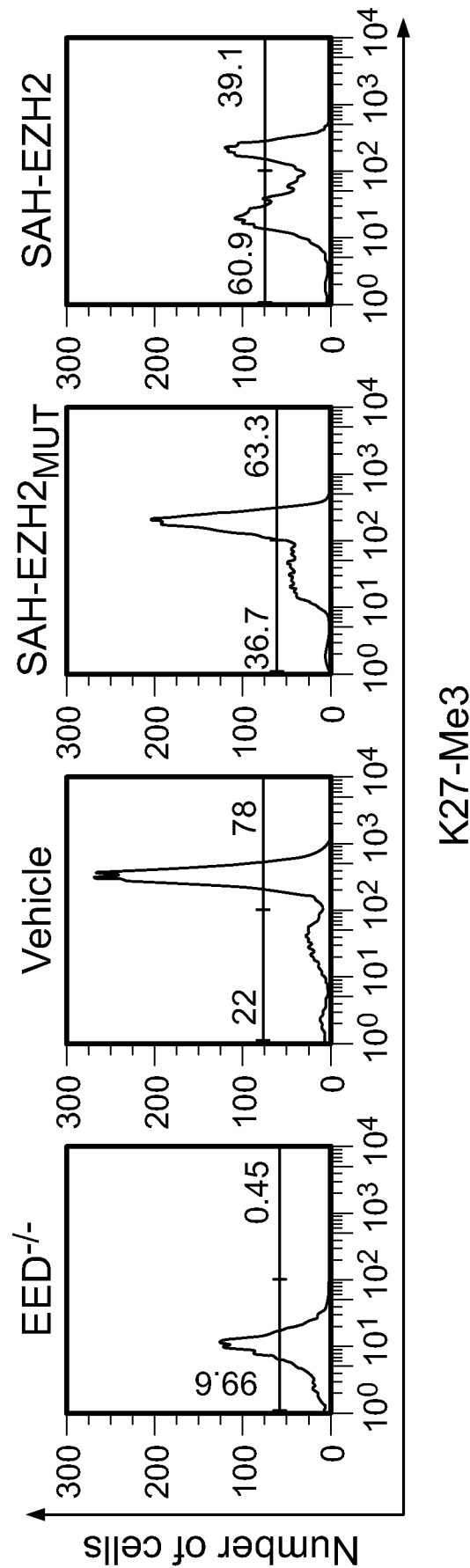


FIG. 17

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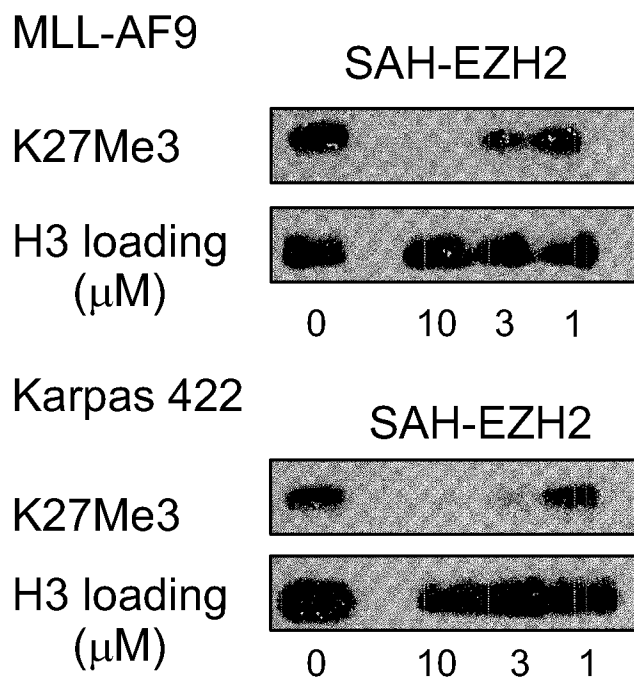


FIG. 18

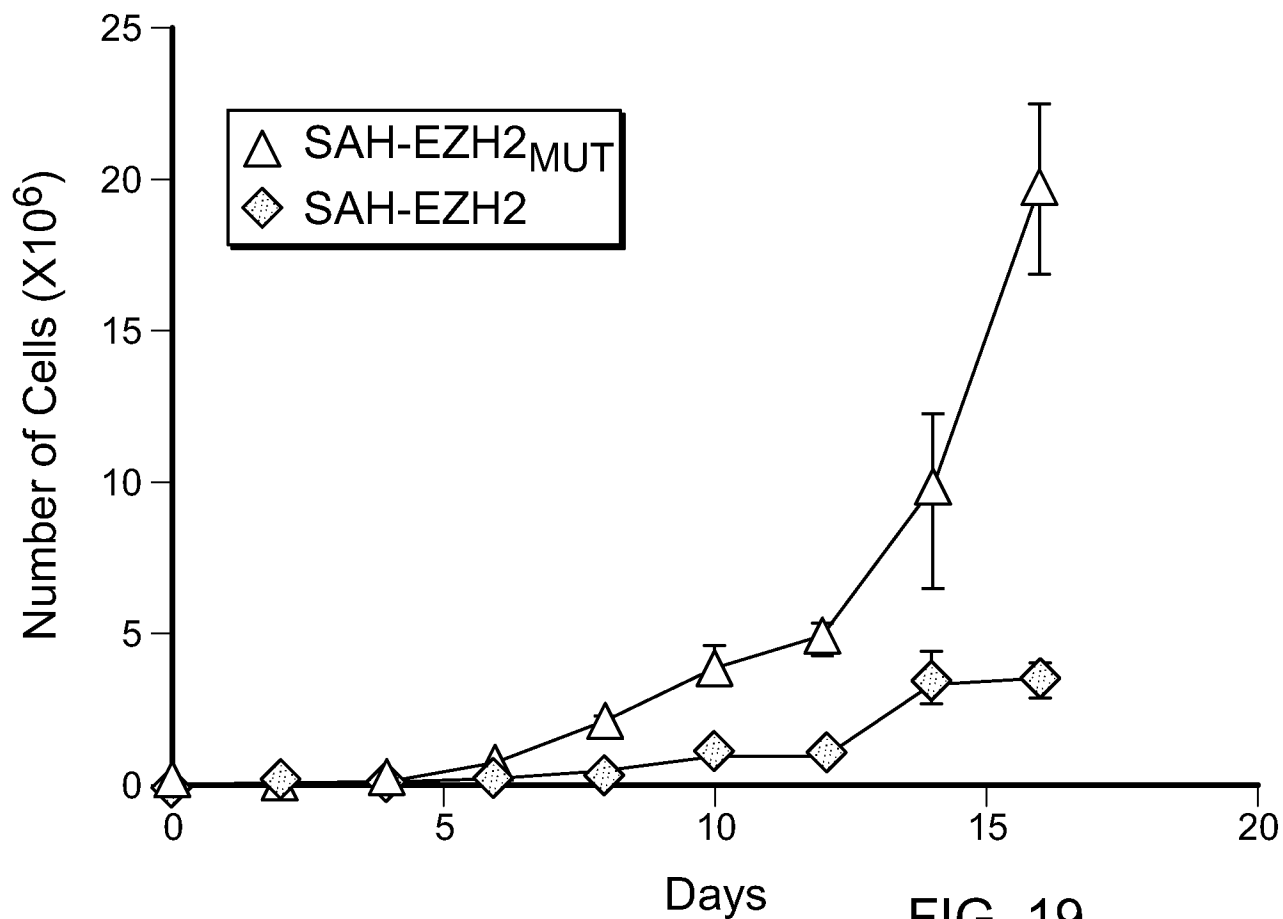


FIG. 19

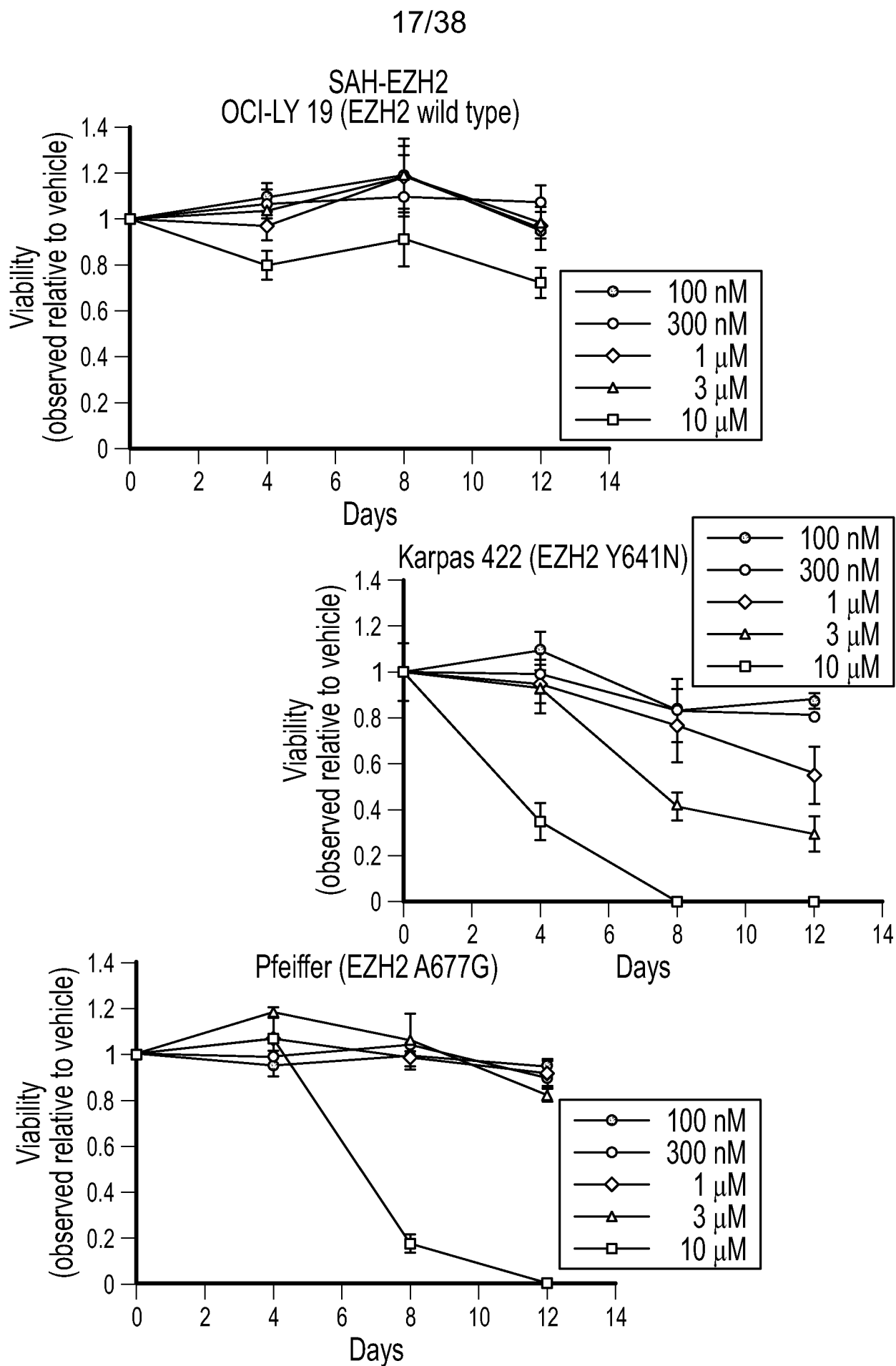


FIG. 20

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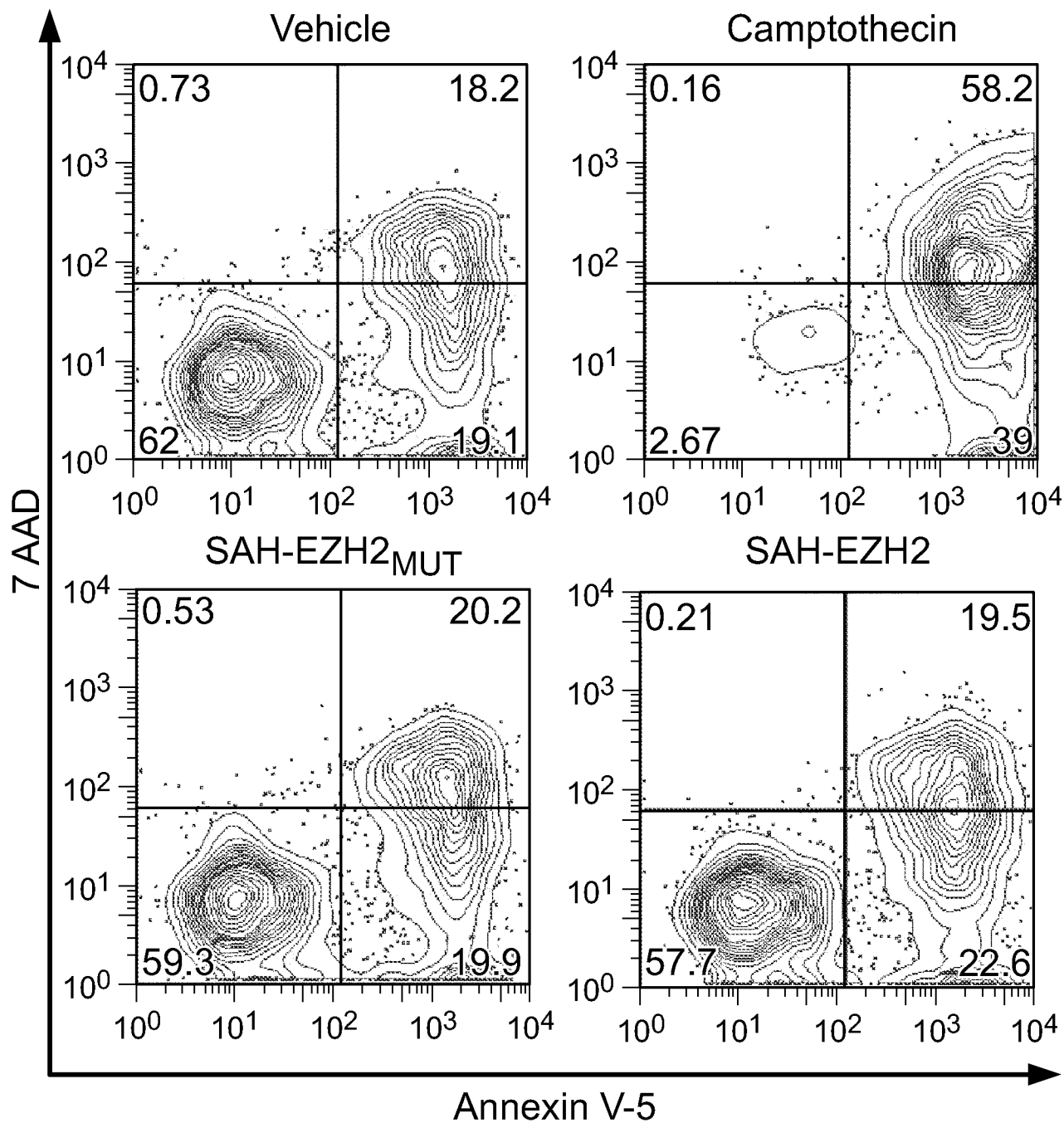
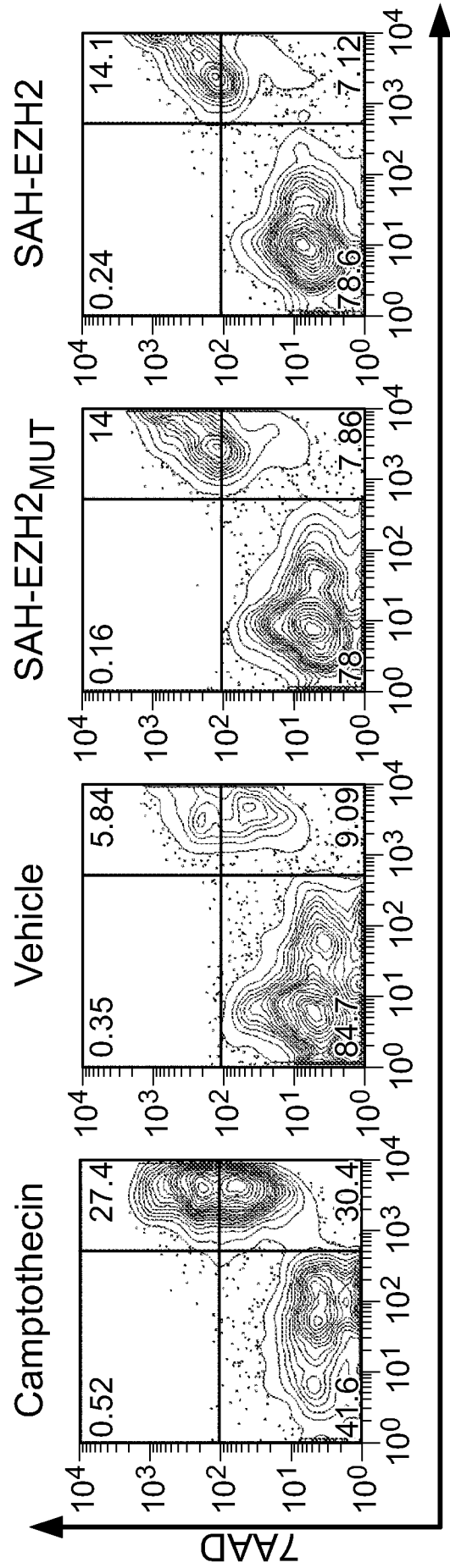


FIG. 21



Annexin V-5

FIG. 22

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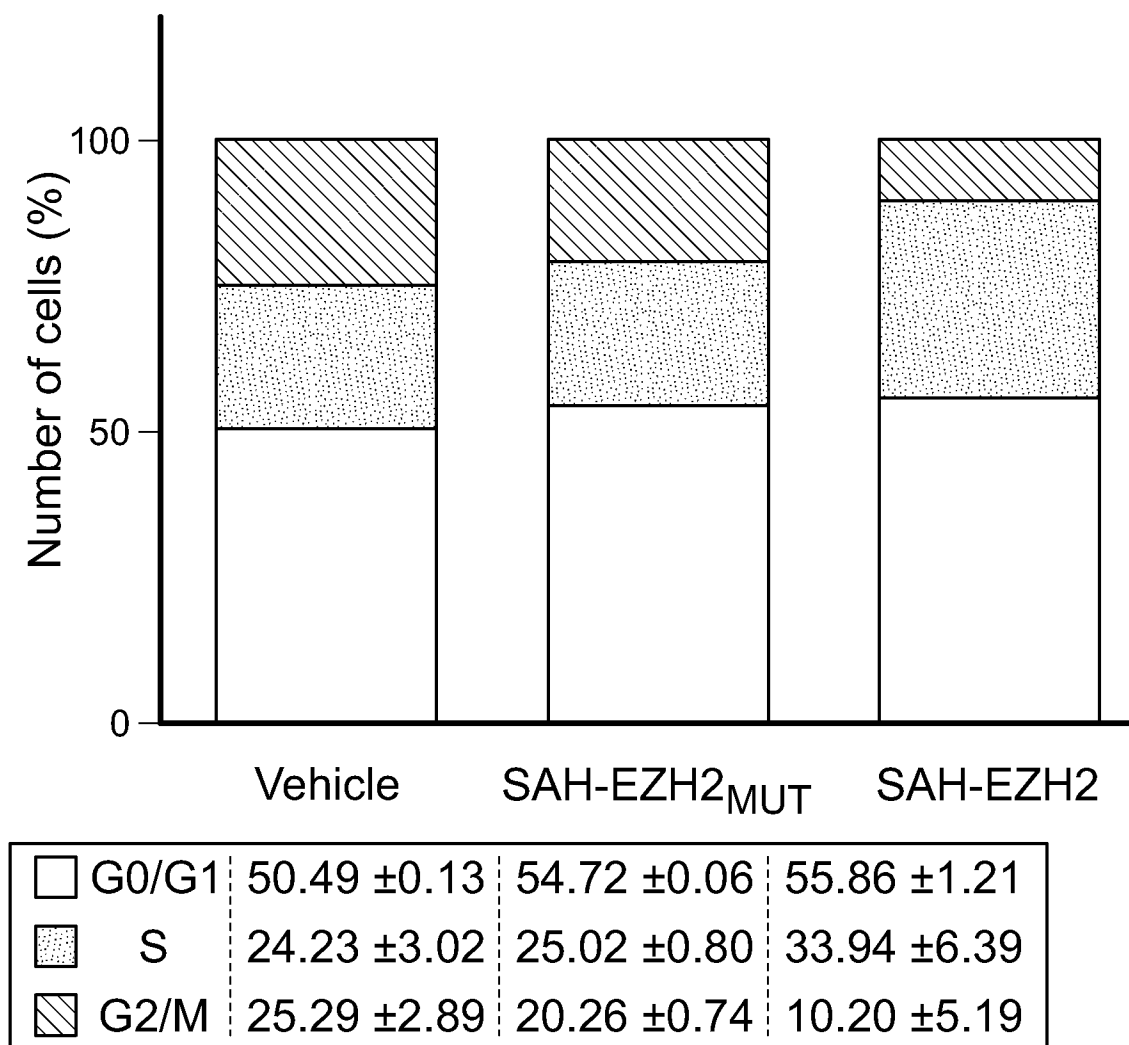


FIG. 23

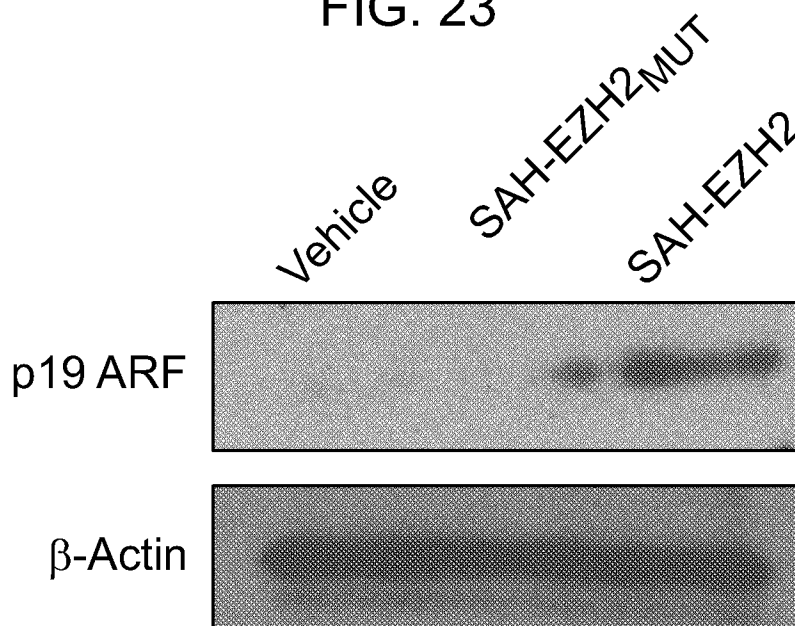


FIG. 24

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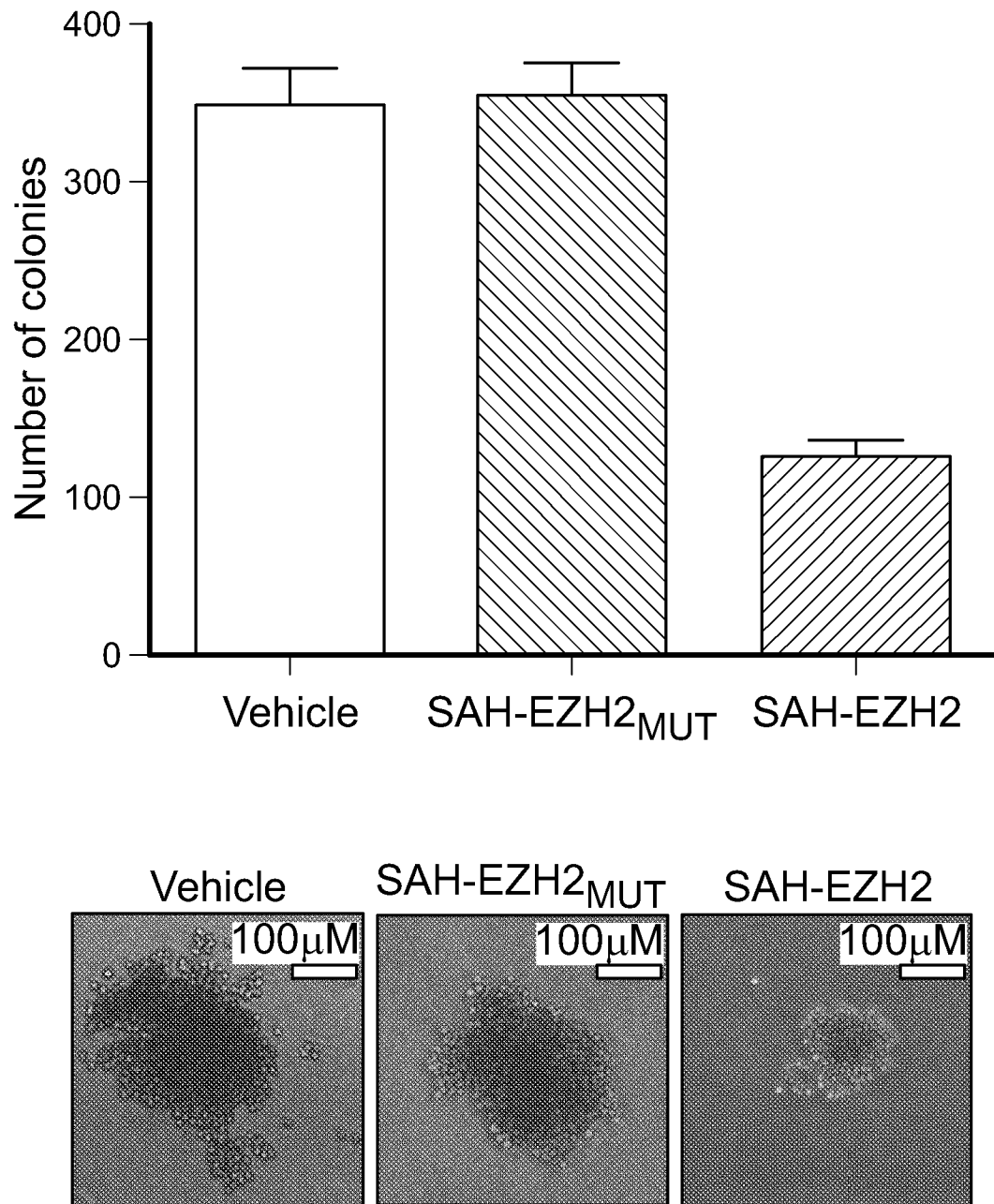
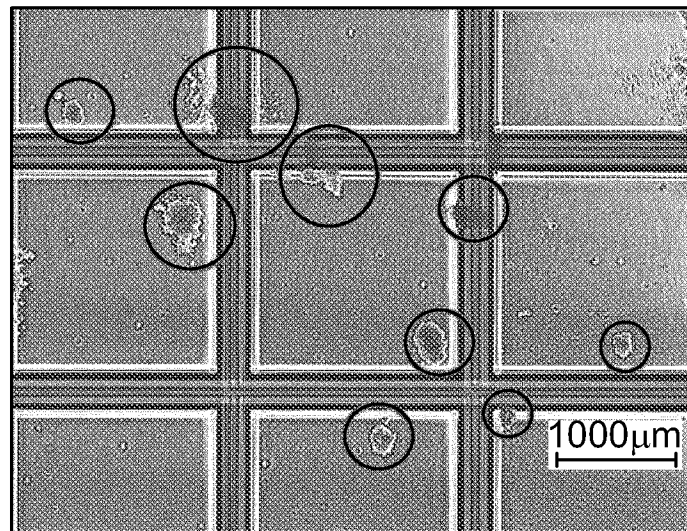


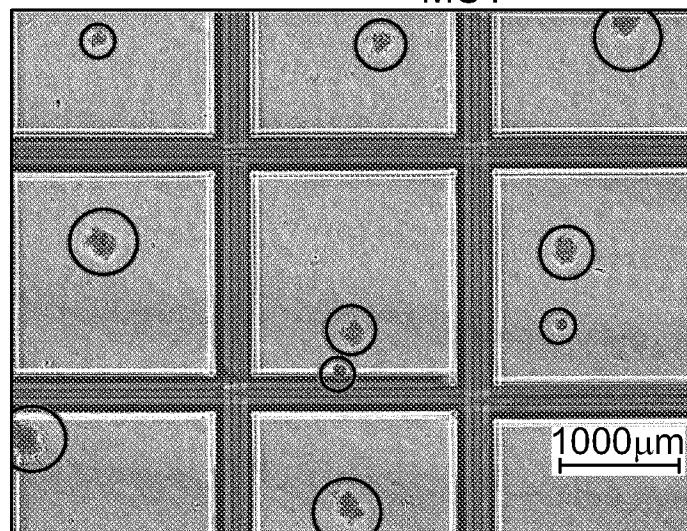
FIG. 25

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Vehicle



SAH-EZH2_{MUT}



SAH-EZH2

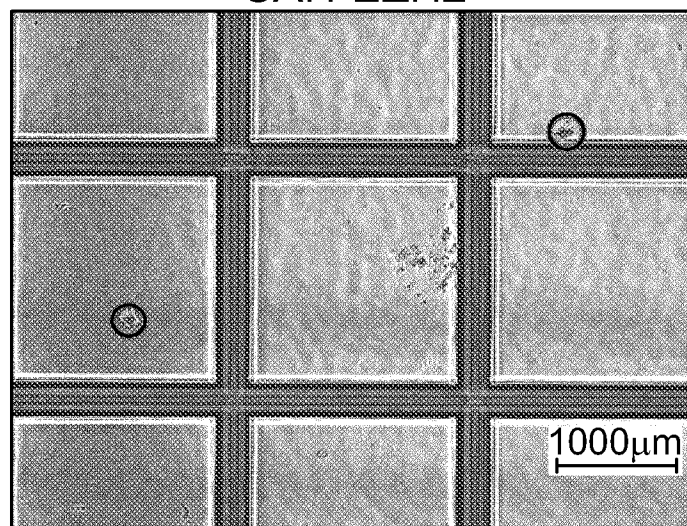


FIG. 26

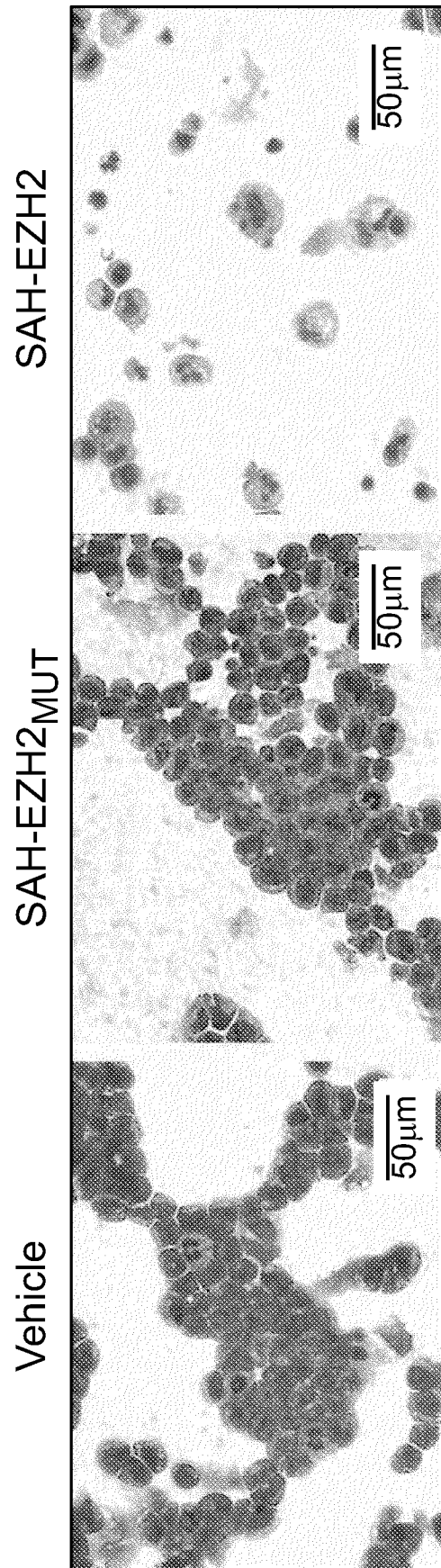


FIG. 27

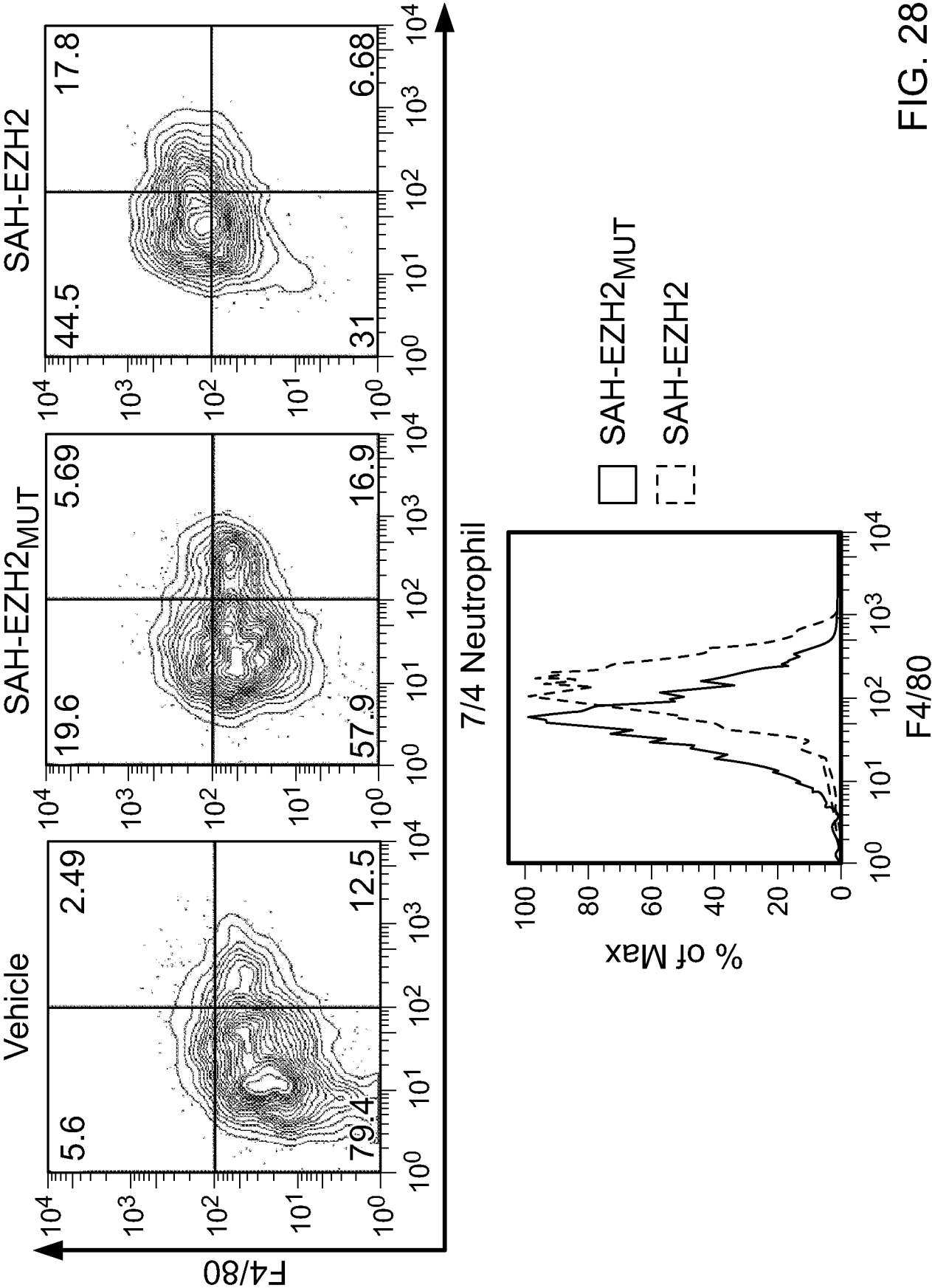


FIG. 28

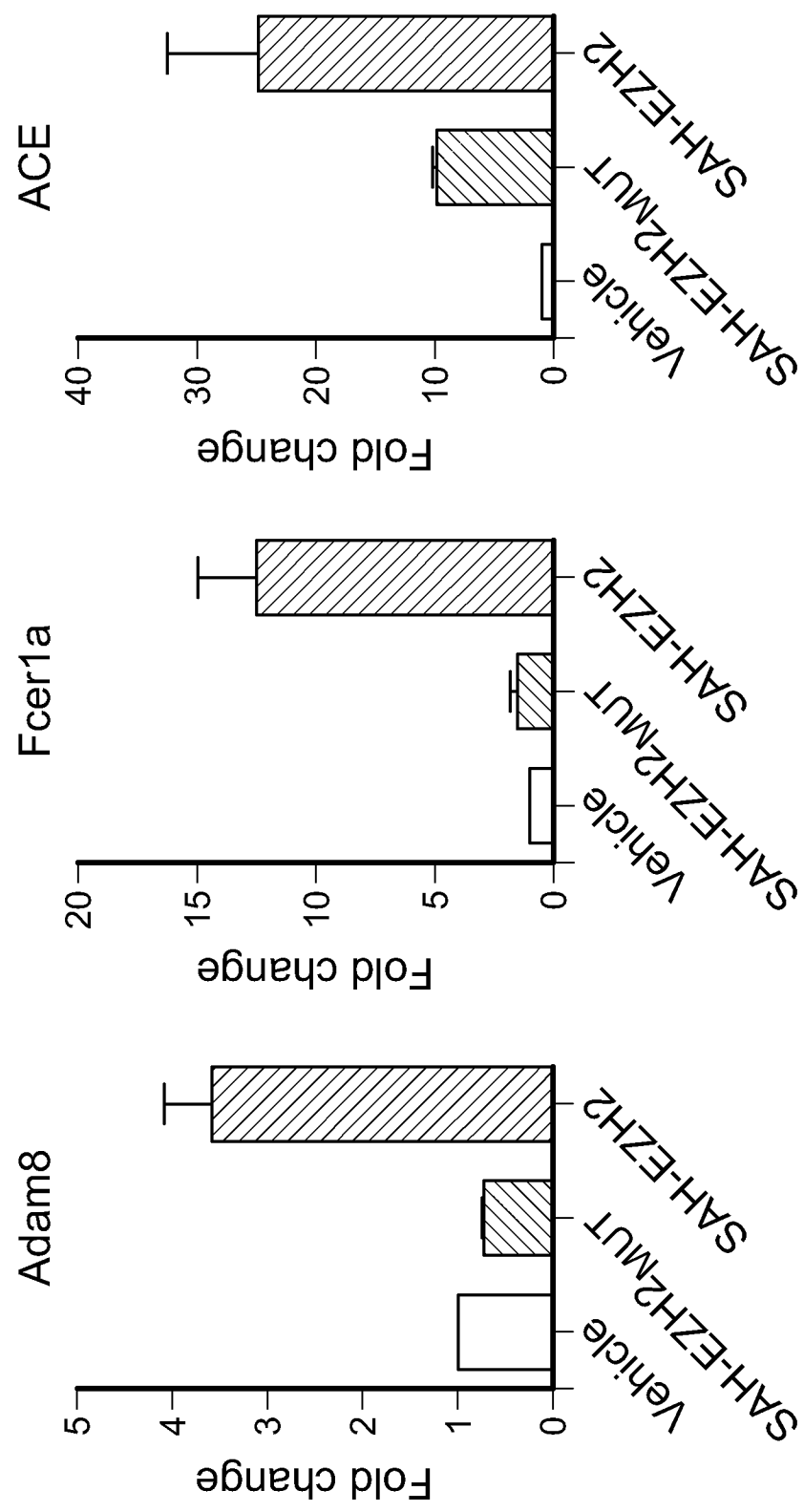


FIG. 29

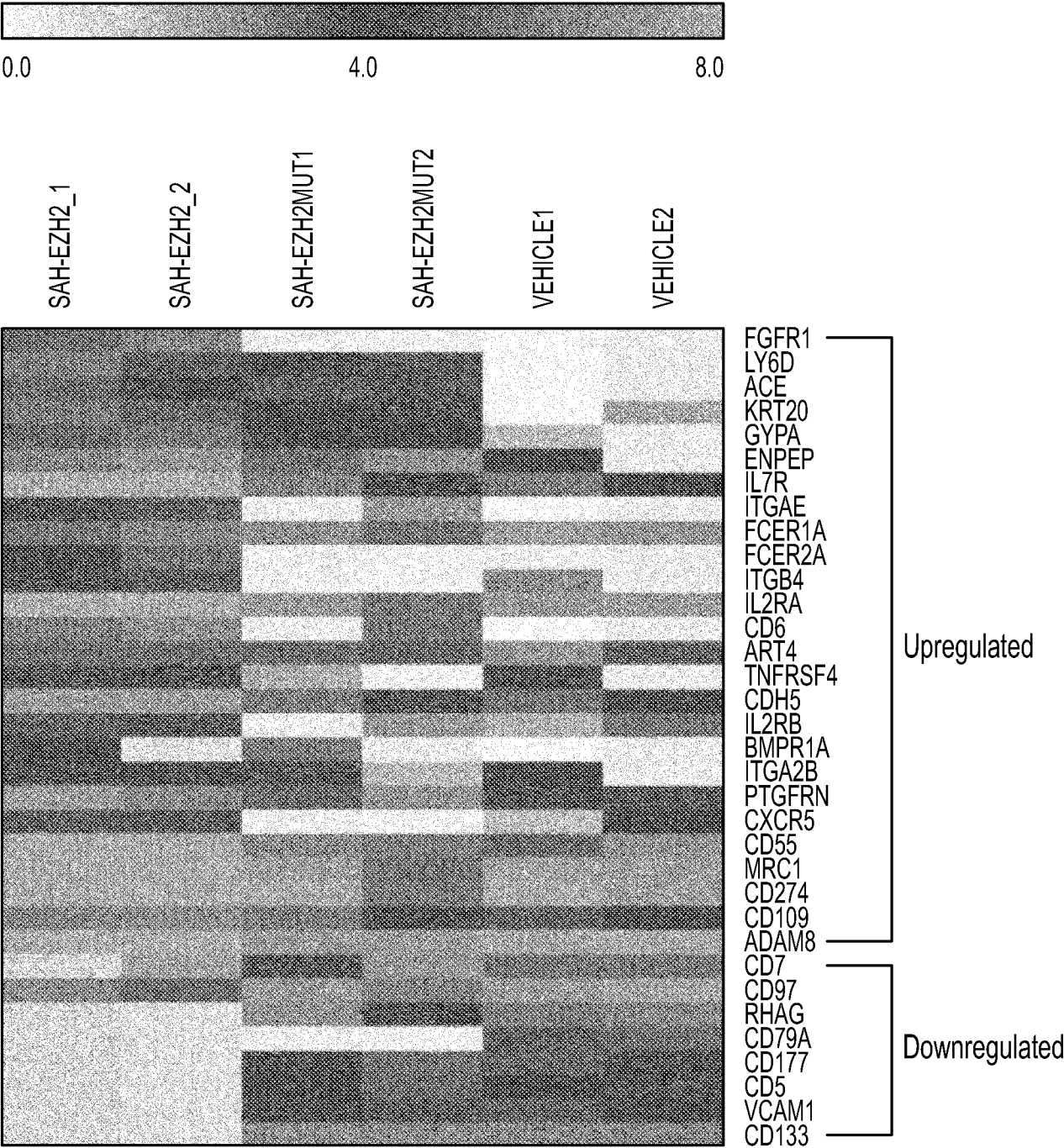


FIG. 29A

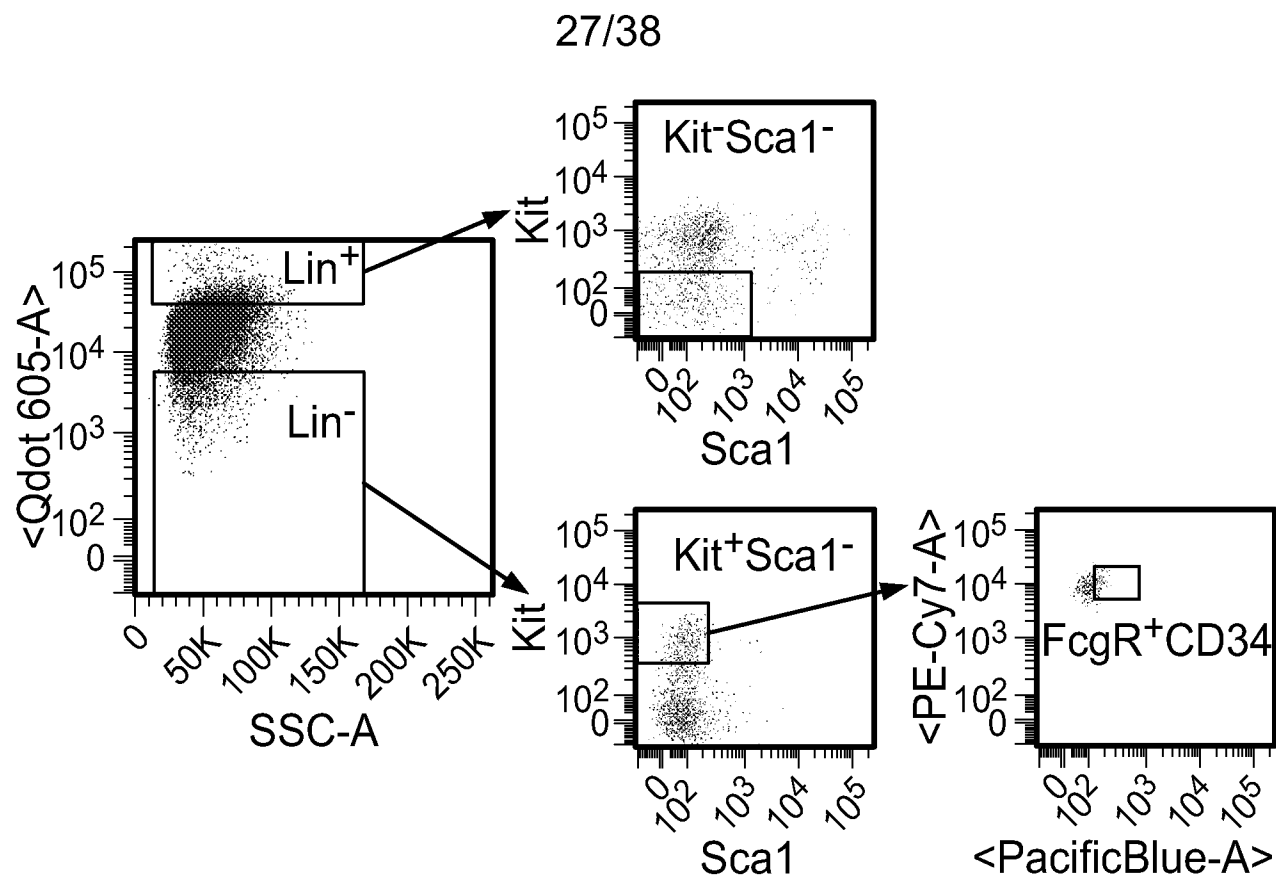


FIG. 30A

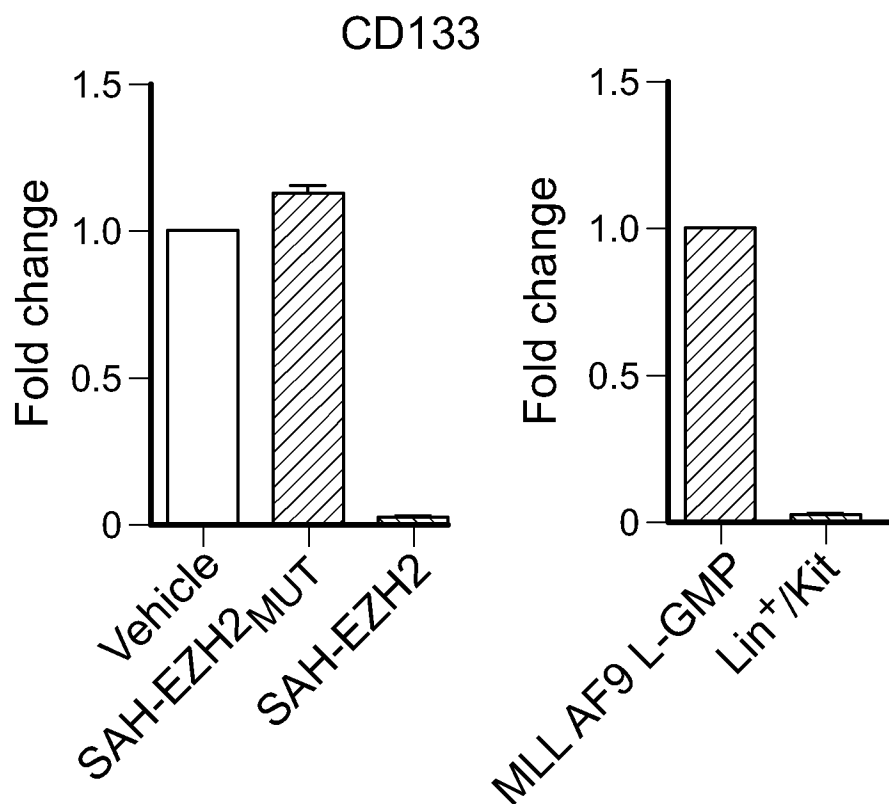


FIG. 30B

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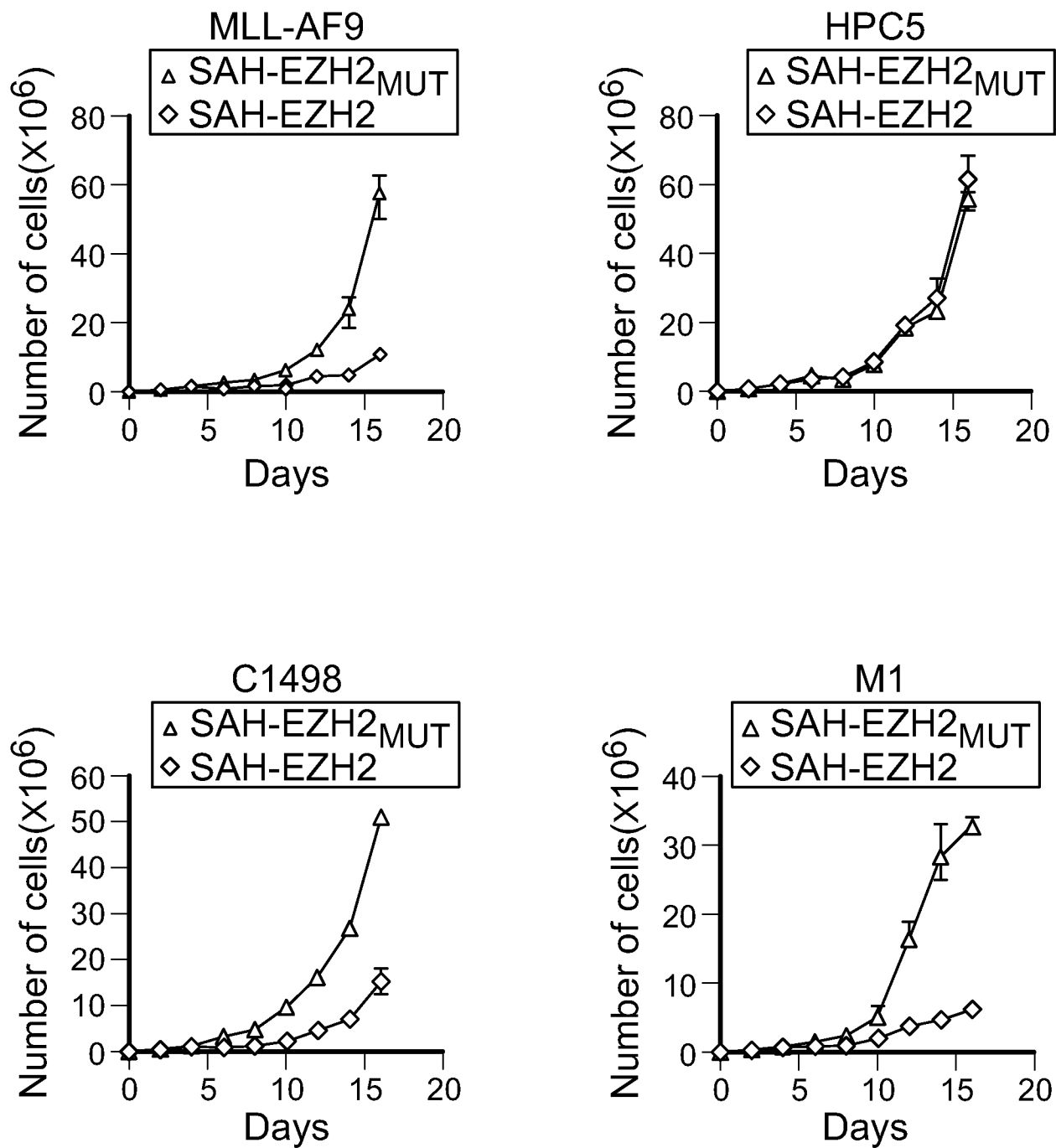


FIG. 31

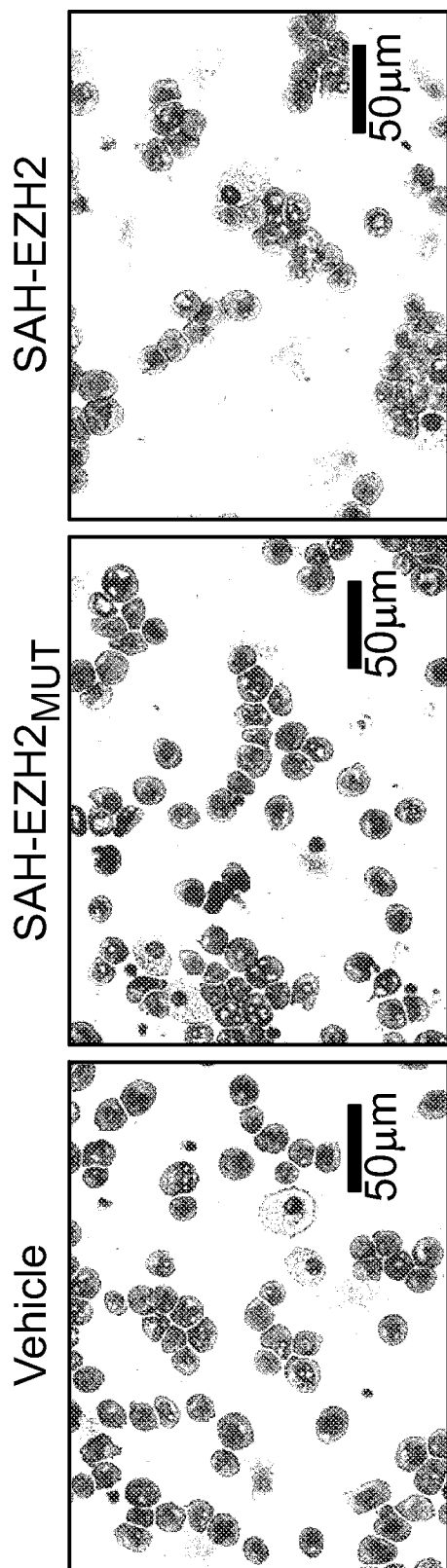


FIG. 32

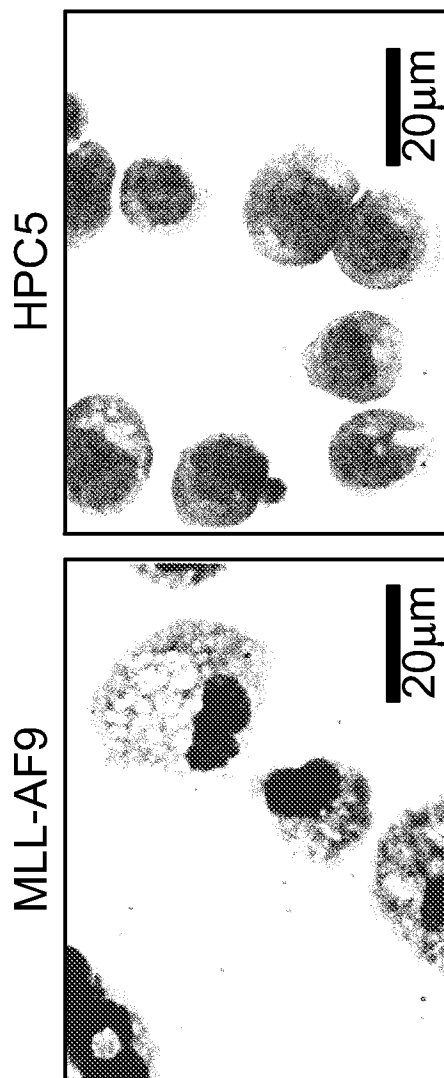


FIG. 33

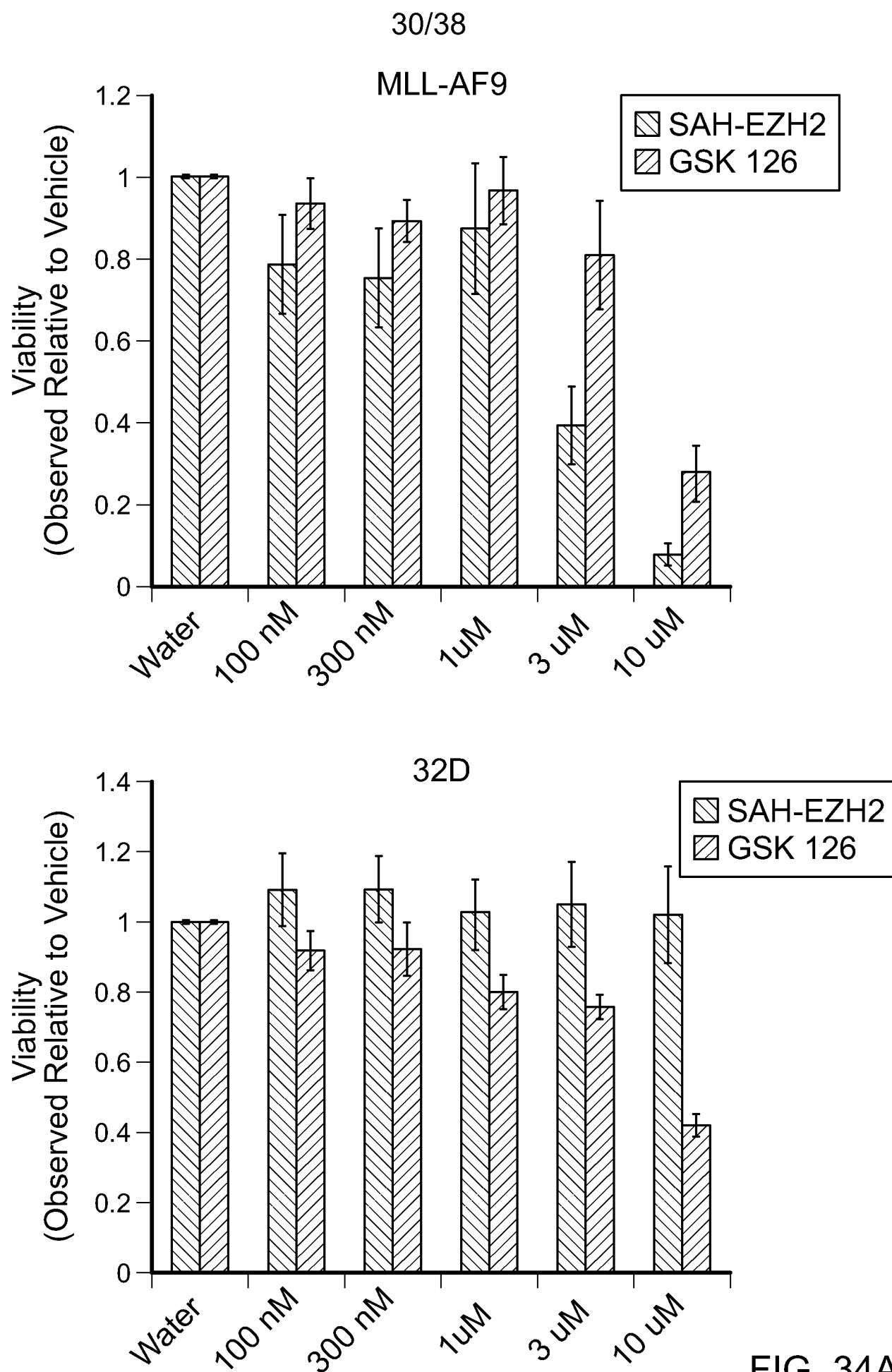


FIG. 34A

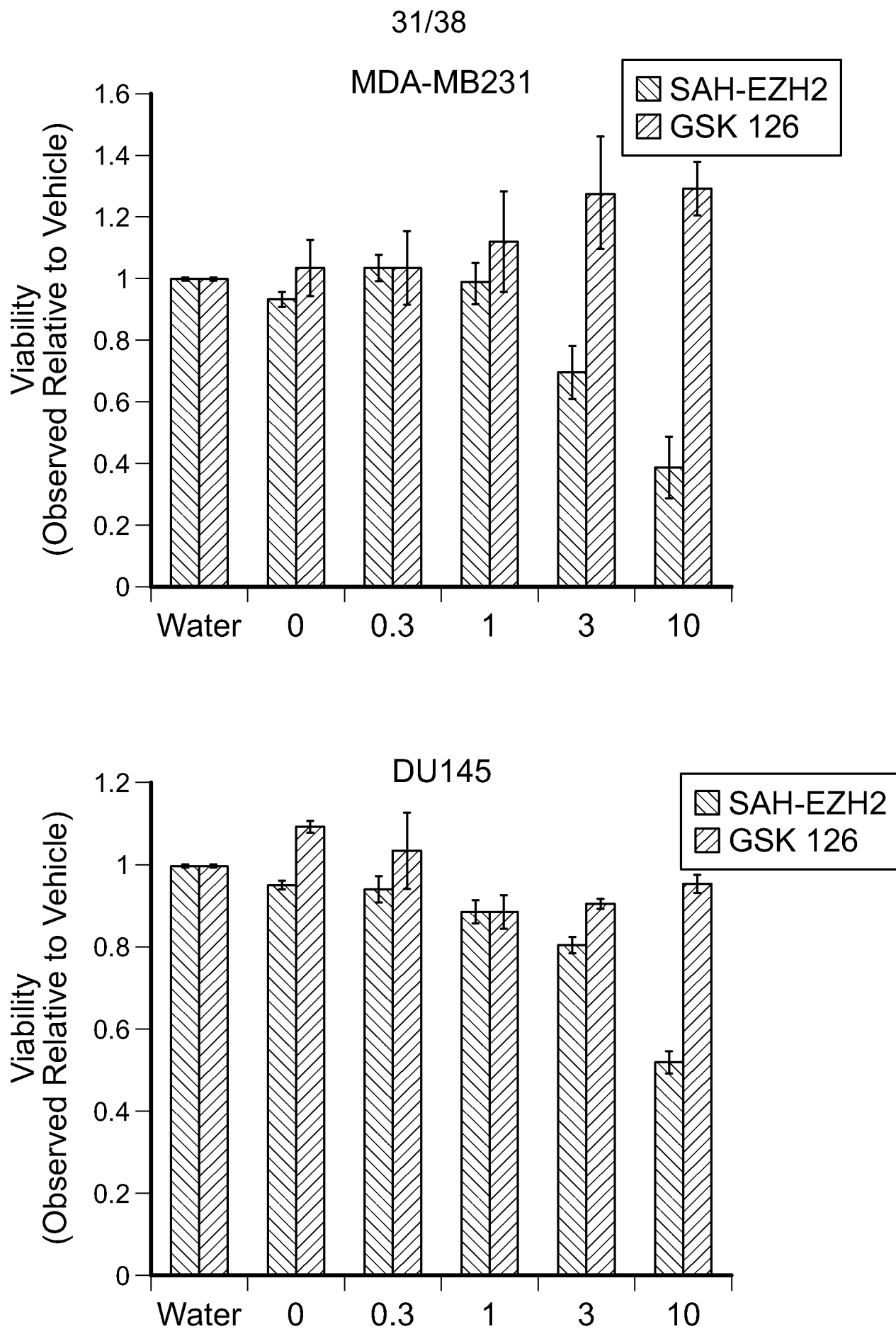


FIG. 34B

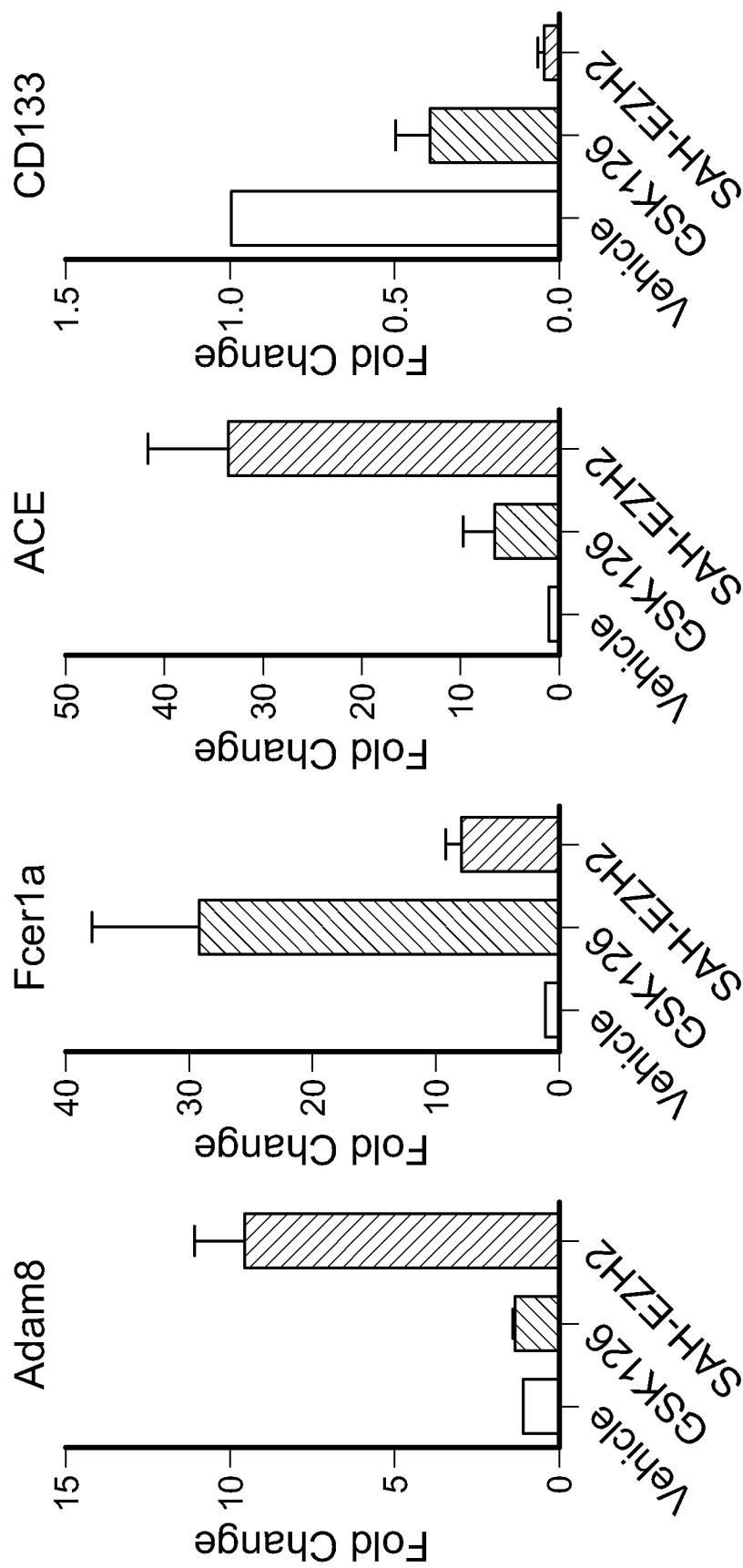


FIG. 35

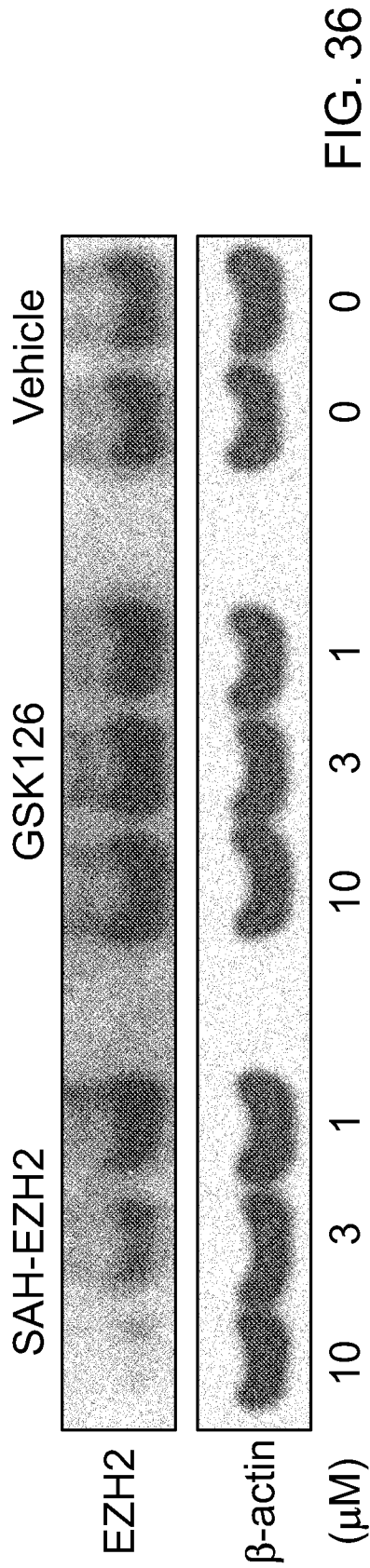


FIG. 36

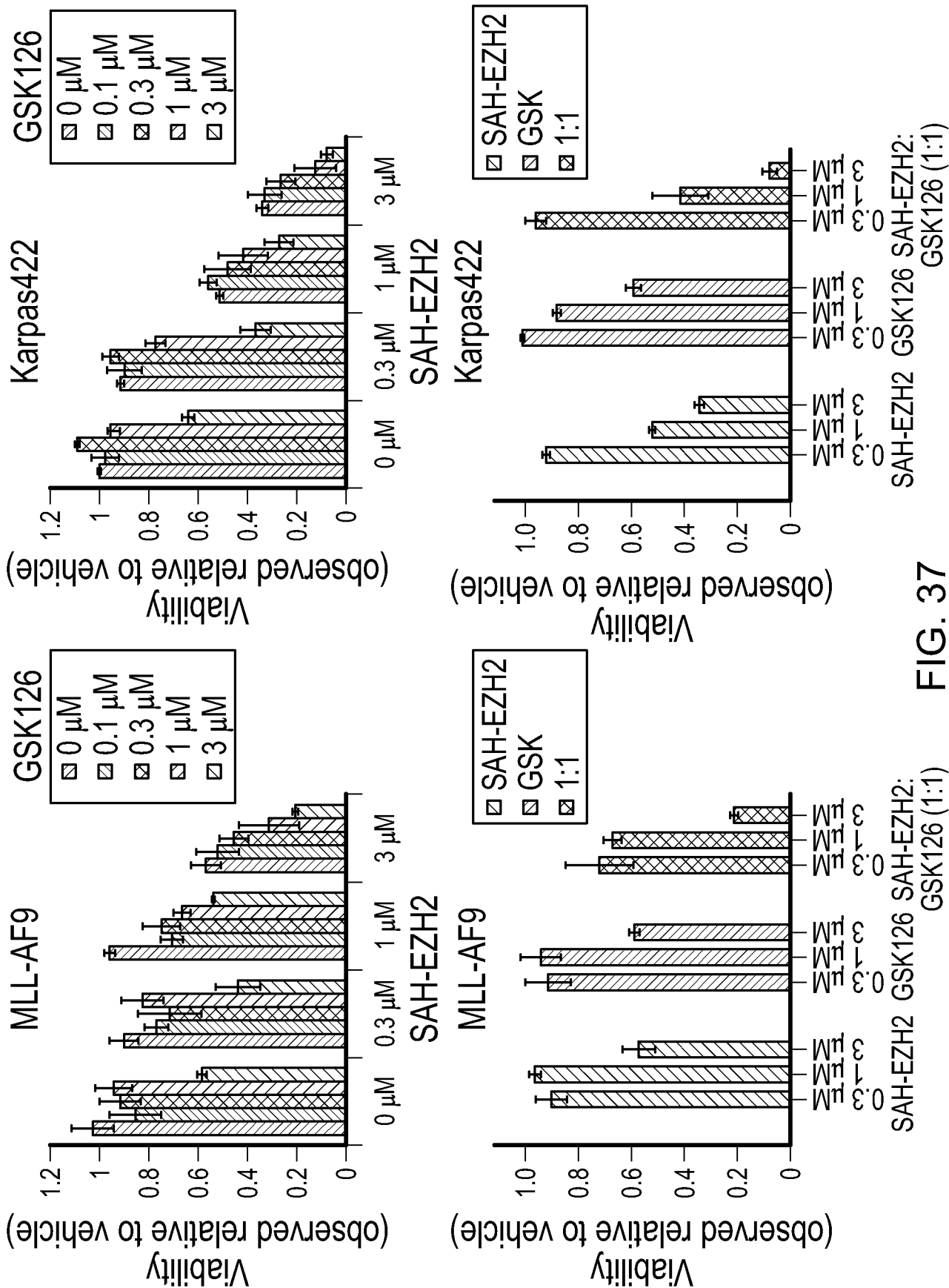


FIG. 37

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Competitor	K_D	95% CI	
◇ EZH2(47-64)	-	-	QKILERTQILNQEWKQRR
○ SAH-EZH2	1.13 μ M	0.51 μ M- 2.52 μ M	FSSNRXXKILXRTQILNQEWKQRRRIQPV
△ EZH2(42-68)AC	0.65 μ M	0.34 μ M- 1.25 μ M	FSSNRXXKILXRTXILNXEWKQRRRIQPV

FIG. 38

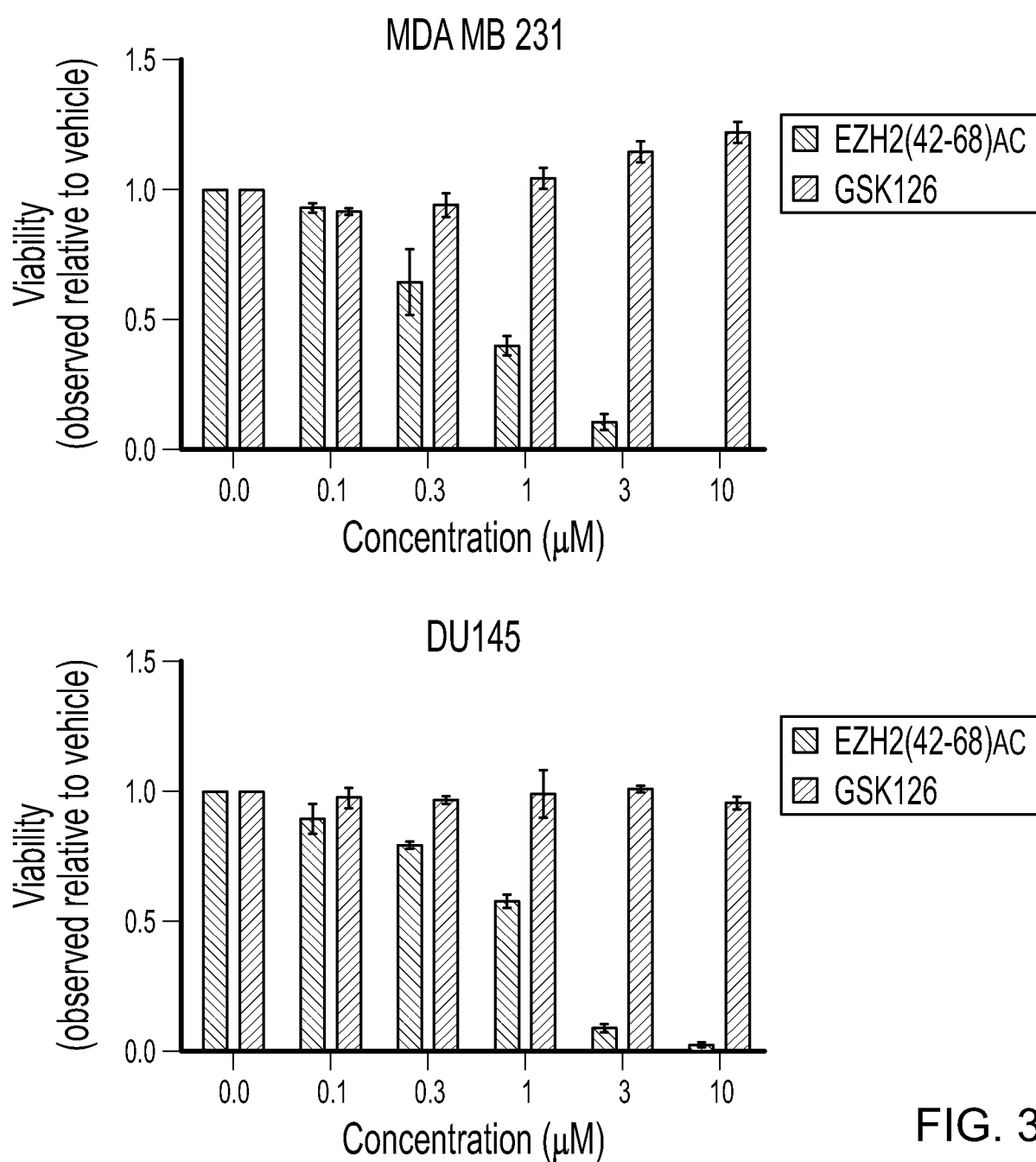
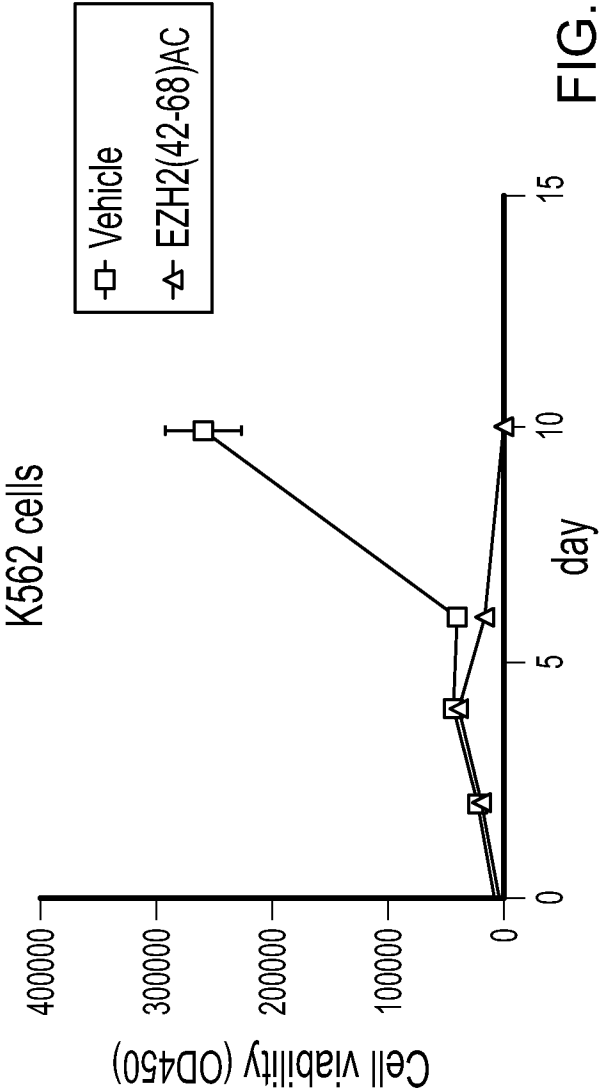
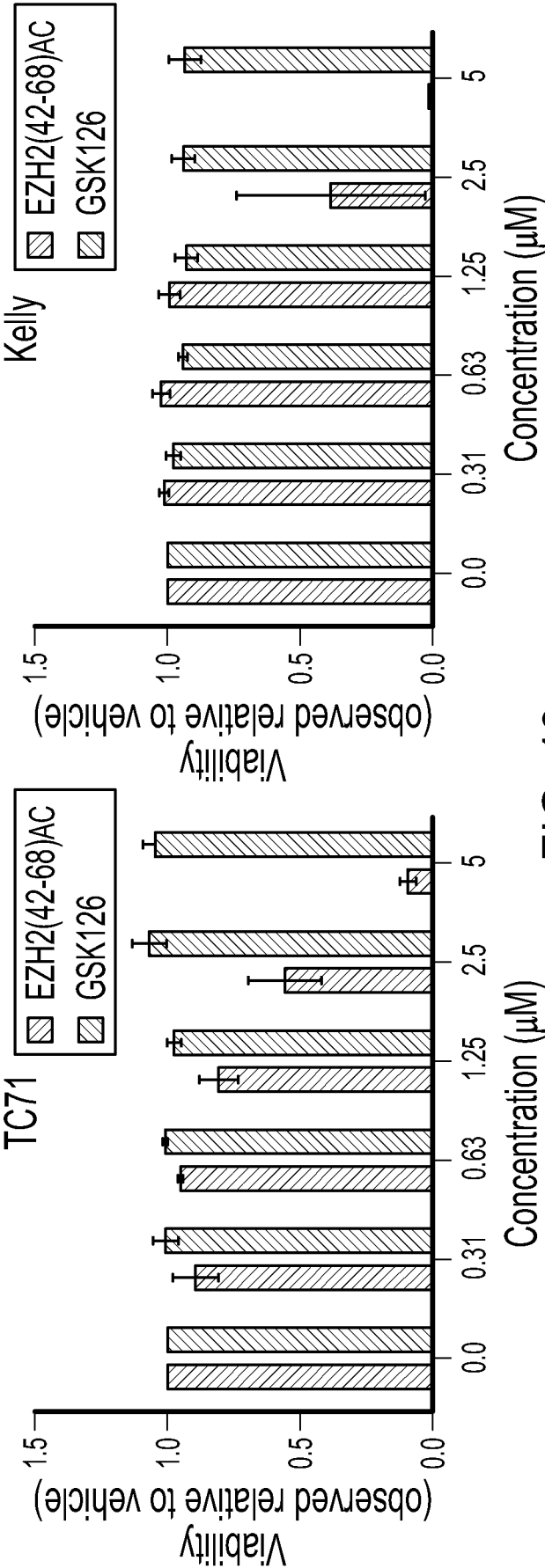


FIG. 39



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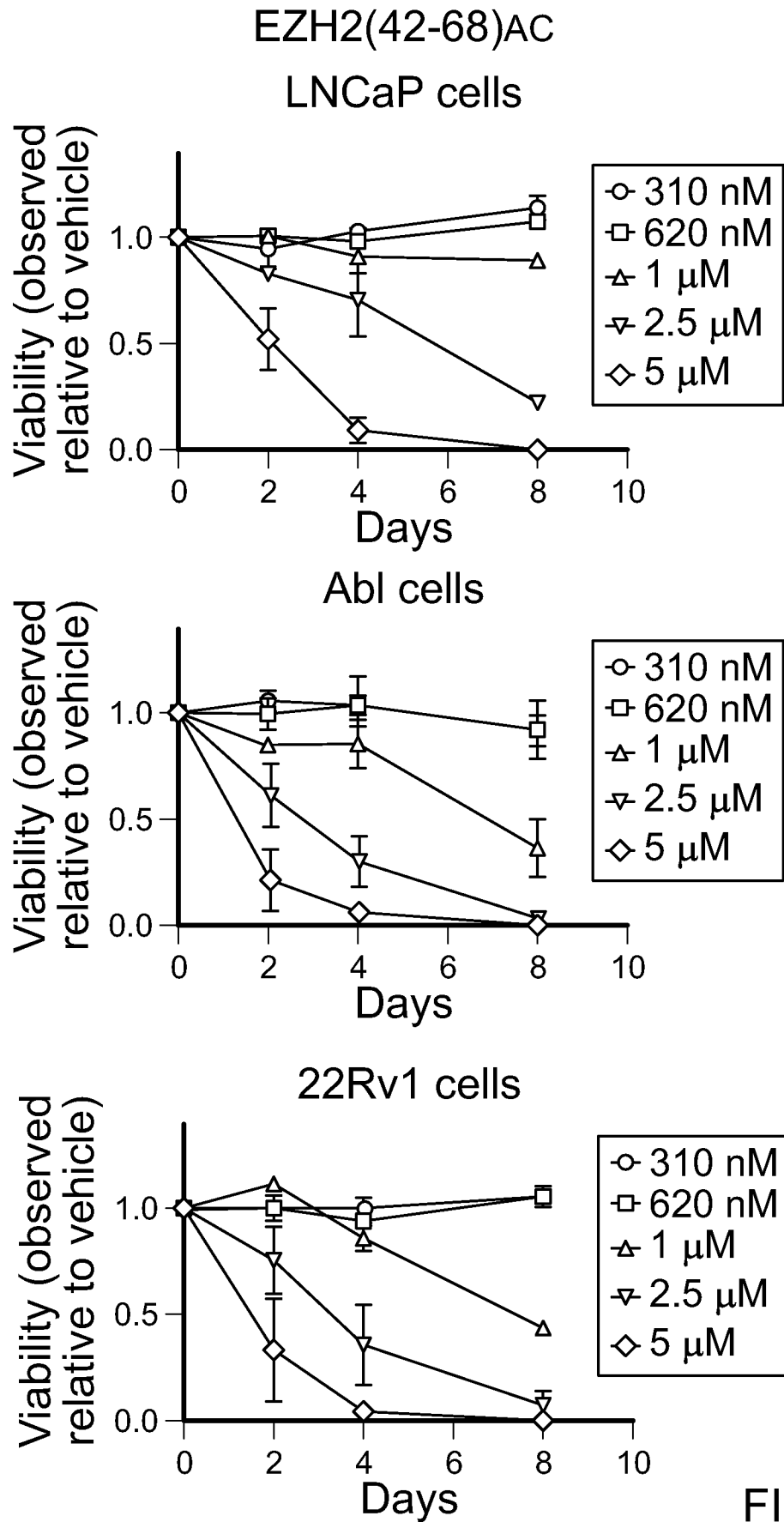


FIG. 42

A ₀	B ₀	C ₀	D ₀	E ₀	F ₀	G ₀	A ₁	B ₁	C ₁	D ₁	E ₁	F ₁	G ₁	A ₂	B ₂	C ₂	D ₂	E ₂	F ₂	G ₂	A ₃	B ₃	C ₃	D ₃	E ₃	F ₃	G ₃	A ₄
S	M	F	S	S	N	R	Q	K	I	L	E	R	T	E	I	L	N	Q	E	W	K	Q	R	R	I	Q	P	V
i ₁			◇	○			†																					
i ₂				◇	○			†																				
		i ₃			◇	○			†																			
□			i ₄			◇	○			†																		
○	□			i ₅			◇	○			†																	
	○				i ₆			◇	○			†																
		○	□			i ₇			◇	○			†															
‡			○	□			i ₈			◇	○			†														
	‡			○	□			i ₉			◇	○																
		‡			○	□			i ₁₀			◇	○				†											
			‡			○	□			i ₁₁			◇	○			†											
				‡			○	□			i ₁₂			◇	○			†										
					‡			○	□			i ₁₃			◇	○			†									
						‡			○	□			i ₁₄			◇	○				†							
							‡			○	□			i ₁₅			◇	○				†						
								‡			○	□						◇	○									
											○	□							◇	○								
											○	□										†						

FIG. 43

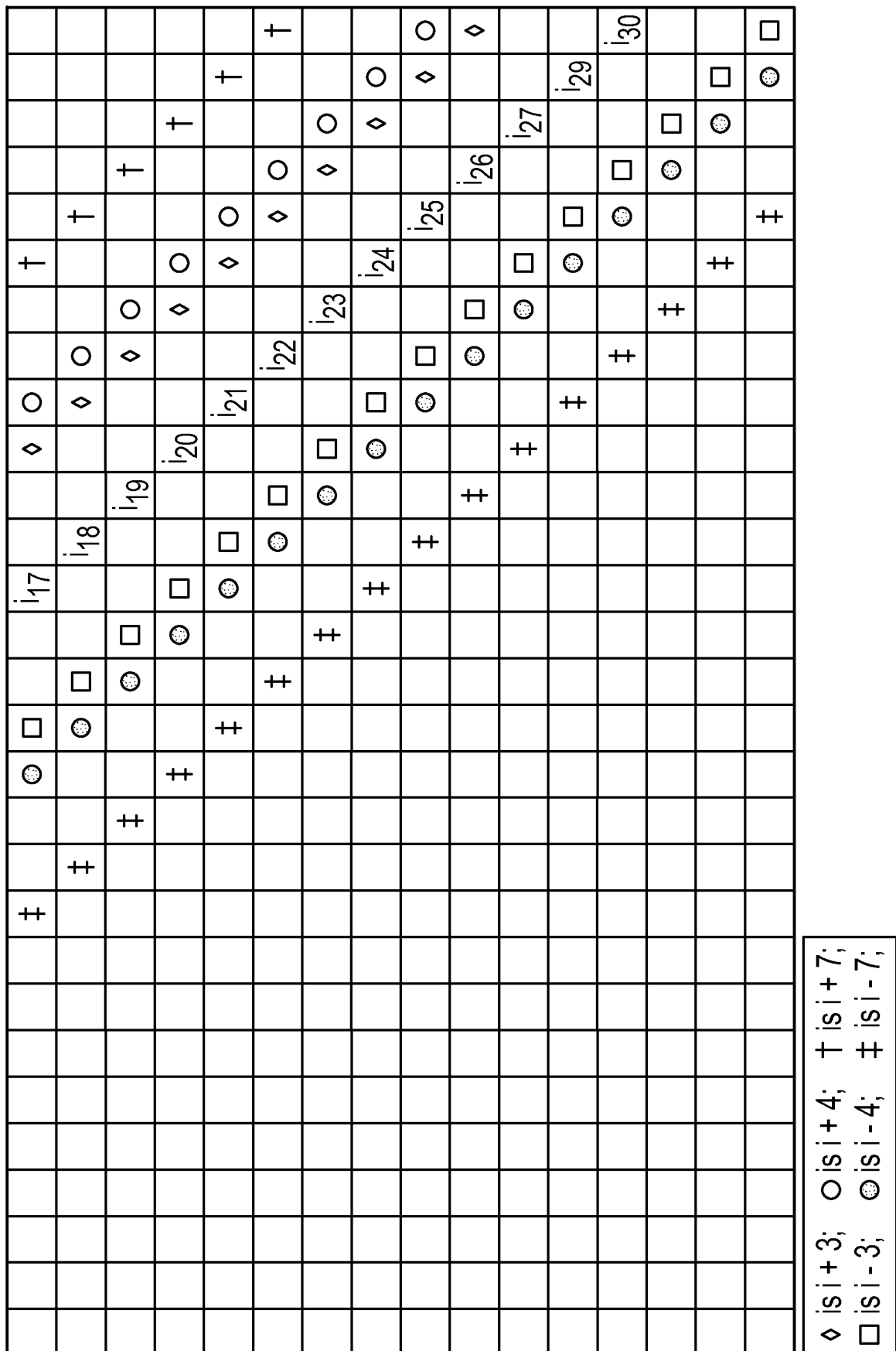


FIG. 43 (Cont.)