



(51) International Patent Classification:

C07K 14/47 (2006.01) *A61K 39/395* (2006.01)
C07K 16/00 (2006.01) *G01N 33/53* (2006.01)
A61K 38/17 (2006.01)

(21) International Application Number:

PCT/US2016/048508

(22) International Filing Date:

25 August 2016 (25.08.2016)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

62/216,400 10 September 2015 (10.09.2015) US

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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

- with international search report (Art. 21(3))
- with sequence listing part of description (Rule 5.2(a))

(54) Title: SYNTHETIC ANTIBODIES TO BAX AND USES THEREOF

Clone	EC ₅₀ (nM)	Sequences			
		CDRL3	CDRH1	CDRH2	CDRH3
Kabat numbering	90....	30...	50..a.55..	94	
2B1	2.3	QYSGSGHYLI	IYSSSM	SISSSSYTS	RGYWWYAWWASAMD
3E8	2.6	QSSYSLI	LSYYSM	SISPYGYTY	RGGAYYFGYYGSGSYAMD
3G11	5.5	QWSFGPI	ISYYSM	SIYPYSSSTY	RSSAMD
2D9	7.6	QWSHYLI	LYYSM	SISPSYGYTS	RSSFYYALD
3G9	9.5	QHYYSWPPI	LYSYYI	SISPYSSSTY	RSSSYAGMD
2C11	10	QSYVSP	ISSYYI	SISYYSSSTY	RVSYGHAIVGYSSGMD
3H4	11	QSWYYSYPI	LSYSSM	SISYYSYTS	RYYGYGGLD
2A6	13	QSAGGYPLI	IYSSSM	SISPYSSYTS	RSFGYGWAFD
3H1	19	QHSYPI	ISYSSI	SIYSYSGSTY	RYGAMD
2A2	24	QYYYP	ISSSSI	SIYSYGYTY	RYSAMMD
3G3	26	QGAWSGGHII	LSYSSM	YISPYGYTY	RGWAYYYGYWGPGSLD
2D5	46	QWGYSHSLI	ISYSSI	SISPYGYSTY	RSHFGALD
2D2	66	QSYWVSPF	LYYSI	SIYPYSGSTY	RSYGYAMD
2A5	250	QYHYWYPI	ISYYSM	SISPYGYSTY	RAGAMD

FIG. 1A

(57) Abstract: Synthetic fragment antigen-binding (Fab) antibodies are disclosed that bind to an N- terminal activation site of BCL-2-associated X-protein (BAX) and inhibit BAX activation. Also disclosed are methods of using the Fabs for measuring inactive monomeric BAX levels, screening for small molecules that bind to an N-terminal activation site of BAX, inhibiting apoptotic cell death, and predicting the ability of a cancer therapy to promote apoptotic cell death.

SYNTHETIC ANTIBODIES TO BAX AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims the benefit of U.S. Provisional patent application No. 62/216,400, filed on September 10, 2015, the contents of which is herein incorporated by reference in its entirety.

STATEMENT OF GOVERNMENT INTEREST

[0002] This invention was made with government support under grant numbers HL095929, CA178394 and CA155472 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

[0003] Throughout this application various publications are referred to by number in parentheses. Full citations for these references may be found at the end of the specification. The disclosures of these publications, and all patents, patent application publications and books referred to herein, are hereby incorporated by reference in their entirety into the subject application to more fully describe the art to which the subject invention pertains.

[0004] Apoptosis plays a critical role in maintaining normal tissue homeostasis in multicellular organisms and its deregulation results in an imbalance of homeostasis contributing to several diseases (1,2) The BCL-2 protein family plays a central role in regulating the mitochondrial pathway of apoptosis (3,4) The mitochondrial outer membrane permeabilization (MOMP) is considered the key event that is regulated by the complex network of protein-protein interactions between pro-apoptotic and anti-apoptotic members of the BCL-2 family. Activation of pro-apoptotic members BAX and/or BAK is required for induction of MOMP, whereas the anti-apoptotic or survival proteins such as BCL-2, BCL-XL and MCL-1, inhibit the pro-apoptotic proteins and prevent MOMP. Activation of BAX and BAK or inhibition of anti-apoptotic BCL-2 proteins is regulated by direct interaction with the BH3-only proteins.

[0005] The activation pathway of BAX represents the gateway to apoptosis and understanding the function of BAX and its regulation mechanisms is an area of intensive investigation. BAX is predominantly found in the cytosolic compartment in an inactive conformation (5,6). Upon cellular stress, BAX is triggered and undergoes a series of conformational changes that enable its translocation to the mitochondrial membrane and

oligomerization leading to MOMP induction (7,8). The structure of the BAX monomer in the inactive conformation was previously determined by nuclear magnetic resonance (NMR) spectroscopy (9). The inactive BAX structure adopts a typical BCL-2 fold, consisting of nine α -helices linked with variable loops. In contrast to BAK and anti-apoptotic BCL-2 proteins that reside at the mitochondrial outer membrane, the structure of BAX was determined with its hydrophobic C-terminal helix $\alpha 9$ bound to the canonical hydrophobic groove. When the C-terminal $\alpha 9$ helix dissociates from the canonical hydrophobic groove, it binds to the mitochondrial outer membrane facilitating the mitochondrial translocation of BAX (9). Structural analysis of a hydrocarbon stapled BIM BH3 helix bound to monomeric BAX uncovered an activation site at the N-terminal surface of BAX (10). This activation site regulates the trigger mechanism for conformational activation of cytosolic BAX leading to the release of the hydrophobic $\alpha 9$ helix and exposure of the hydrophobic $\alpha 2$ helix (BH3 domain) (10-12). Mitochondrial translocated BAX undergoes further conformational changes on the membrane that induce BAX oligomerization and MOMP (13-16), or is inhibited by anti-apoptotic Bcl-2 proteins (17-20).

[0006] A number of diseases and disorder are associated with premature or unwanted cell death and characterized by abnormal activation of BAX. The present invention addresses the need for inhibitors of BAX activation for therapeutic treatments.

SUMMARY OF THE INVENTION

[0007] The present invention discloses synthetic fragment antigen-binding (Fab) antibodies that bind to an N-terminal activation site of BCL-2-associated X-protein (BAX) and inhibit BAX activation. Also disclosed are methods of using the Fabs for measuring inactive monomeric BAX levels, screening for small molecules that bind to an N-terminal activation site of BAX, inhibiting apoptotic cell death, and predicting the ability of a cancer therapy to promote apoptotic cell death.

BRIEF DESCRIPTION OF THE DRAWINGS

[0008] Fig. 1A-1D. Discovery of synthetic antibody fragments that bind with high affinity to BAX at overlapping binding sites. (A) Synthetic antibody fragments (Fabs) that bind to BAX with the corresponding variable sequences of complementarity determining regions (CDRs) and half-maximal binding titers (EC_{50}) as determined by ELISA. (B) Top panel: Binding affinity (K_D), association rate constant (k_a) and dissociation rate constant (k_d) for select synthetic Fabs that bind to BAX as determined by biolayer interferometry. ELISA binding EC_{50} s are shown for comparison. Bottom panel: A representative biolayer

interferometry experiment using immobilized 3G11 and three different BAX concentrations (C) Synthetic Fabs, except 2A5, compete biotinylated 3G11 (b3G11) from binding to BAX using a competitive ELISA. (D) Binding of selected phage-bound expressed Fabs (Φ) to BAX as competitively inhibited by the corresponding free synthetic Fab proteins (100 nM). All synthetic Fabs, except 2A5, show at least 40% competition efficacy to different phage-bound expressed Fabs. Data shown in (B-D) represent mean $\pm$ SD from at least three independent experiments. In (A), SEQ ID NOs for CDRL3, CDRH1, CDRH2, and CDRH3 are respectively, SEQ ID NO:1-4 for Fab 2B1, SEQ ID NO:5-8 for 3E8, SEQ ID NO:9-12 for 3G11, SEQ ID NO:13-16 for 2D9, SEQ ID NO:17-20 for 3G9, SEQ ID NO:21-24 for 2C11, SEQ ID NO:25-28 for 3H4, SEQ ID NO:29-32 for 2A6, SEQ ID NO:33-36 for 3H1, SEQ ID NO:37-40 for 2A2, SEQ ID NO:41-44 for 3G3, SEQ ID NO:45-48 for 2D5, SEQ ID NO:49-52 for 2D2, and SEQ ID NO:53-56 for 2A5.

[0009] Fig. 2A-2F. Synthetic Fabs inhibit BAX activation induced by pro-apoptotic tBID in liposomal assay. (A) Synthetic BAX-binding Fabs or a VEGF specific Fab (YADS1) at 2 μ M have no capacity to induce BAX-mediated liposomal ANT/DPX release while tBID induced potent BAX-mediated liposomal release. (B) BAX-binding Fabs at 2 μ M, except 2A5 and YADS1, inhibit tBID-induced BAX-mediated liposomal ANT/DPX release. Bars represent mean $\pm$ SD value at 90 min that is normalized to tBID-induced maximum ANT/DPX release at 90 min (C-F). Representative liposomal ANT/DPX release experiments in kinetic representation showing the inhibitory activity of Fabs 3G11, 2B1 and 3E8 at 0.5 μ M, 1 μ M and 2 μ M Fab whereas 2A5 had no inhibitory effect. Experiments performed with 400 nM BAX, 30 nM tBID and up to 2 μ M Fabs (A-F). Data shown in (A-F) represent mean $\pm$ SD from triplicates normalized using either tBid-induced BAX mediated ANT/DPX release as 100% (A-B) or 1% triton release as 100% (C-F). Data shown are representative of at least three independent experiments.

[0010] Fig. 3A-3B. Synthetic Fab 3G11 inhibit BAX-mediated cytochrome c release and BAX mitochondrial translocation induced by pro-apoptotic tBID in isolated mitochondria. (A) Fab 3G11 inhibits dose-responsively tBID-induced BAX-mediated cytochrome c release from isolated BAK^{-/-} mitochondria. (B) 3G11 Fab inhibits dose-responsively tBID-induced BAX mitochondrial translocation in isolated BAK^{-/-} mitochondria. Data shown are representative of at least three independent experiments.

[0011] Fig. 4. 3G11 binds to the N-terminal surface of BAX. % deuterium incorporation of unbound BAX conformation was determined in solution. HXMS analysis suggests increased rates of deuterium exchange in the N-terminal region of BAX, the α 1- α 2 loop and

helices $\alpha 2$, part of $\alpha 5$, $\alpha 8$, and $\alpha 9$. The relative difference of % deuterium incorporation of BAX conformation bound to 3G11 minus the % deuterium incorporation of BAX conformation alone was also determined. HXMS analysis suggests increased protection from deuterium incorporation in residues of helices $\alpha 1$, $\alpha 6$, part of $\alpha 5$ and the $\alpha 1$ - $\alpha 2$ loop compared to the unbound BAX. The regions of significant protection from deuterium incorporation ($> 20\%$) are highlighted in dark on the sequence of BAX (SEQ ID NO:57). The ribbon representation of the full length BAX structure (PDB ID: 16F6) is shown in the bottom portion of the figure.

[0012] Fig. 5A-5F. Select BAX mutants inhibit functional inhibition and binding to BAX by 3G11. (A-D) Representative liposomal ANTS/DPX release experiments in kinetic representation showing the inhibitory activity of 3G11 (2 μ M) with BAX WT that is weakened by the K21E mutation and completely abolished by the R134E or the double mutation R134E/K21E. Experiments performed with 400 nM BAX WT or BAX mutants, 30 nM tBID and 2 μ M 3G11. (E) % Inhibition based on the maximum tBID-induced BAX activation for BAX WT and mutants at 90 min in presence or absence of 3G11 Fab. (F) ELISA binding profiles for 3G11 binding to BAX WT and mutants. Half-maximal binding titers (EC_{50}) were determined as follows: 8 ± 2 nM for BAX WT, 29 ± 4 for K21E, 89 ± 2 nM for R134E mutant and 91 ± 2 nM for the R134E/K21E double mutant. Data shown in (A-D) represent mean $\pm$ SD from triplicates and at least two independent experiments.

DETAILED DESCRIPTION OF THE INVENTION

[0013] The invention provides a synthetic fragment antigen-binding (Fab) antibody that binds to an N-terminal activation site of BCL-2-associated X-protein (BAX) and inhibits BAX activation. Preferably, the antibody maintains BAX in its inactive, monomeric form. The antibody can bind to residues of helices $\alpha 1$ and $\alpha 6$, and to residues of $\alpha 1$ - $\alpha 2$ loop of BAX. Preferably, the antibody binds to BAX with a half-maximal binding (EC_{50}) affinity of 2nM-70nM. Preferably, the antibody blocks the interaction of BAX with a BAX activating partner, such as, for example, tBID, PUMA, BIM and/or NOXA.

[0014] In different embodiments, the Fab is selected from the group consisting of

2B1 Fab, having a CDRL3 region comprising the amino acid sequence QYSGSGHYLI (SEQ ID NO:1), a CDRH1 region comprising the amino sequence IYSSSM (SEQ ID NO:2), a CDRH2 region comprising the amino acid sequence SSSSSSYTS (SEQ ID NO:3) and a CDRH3 region comprising the amino acid sequence RGYWYYWAWWASAMD (SEQ ID NO:4);

3E8 Fab, having a CDRL3 region comprising the amino acid sequence QSSYSLI (SEQ ID NO:5), a CDRH1 region comprising the amino sequence LSYYSM (SEQ ID NO:6), a CDRH2 region comprising the amino acid sequence SISPYGYTY (SEQ ID NO:7) and a CDRH3 region comprising the amino acid sequence RGGAYYFGYYGSGSYAMD (SEQ ID NO:8);

3G11 Fab, having a CDRL3 region comprising the amino acid sequence QWSFGPI (SEQ ID NO:9), a CDRH1 region comprising the amino sequence ISYYSM (SEQ ID NO:10), a CDRH2 region comprising the amino acid sequence SIYPYSSSTY (SEQ ID NO:11) and a CDRH3 region comprising the amino acid sequence RSSAMD (SEQ ID NO:12);

2D9 Fab, having a CDRL3 region comprising the amino acid sequence QWSHYLI (SEQ ID NO:13), a CDRH1 region comprising the amino sequence LYYYSM (SEQ ID NO:14), a CDRH2 region comprising the amino acid sequence SISPSYGYTS (SEQ ID NO:15) and a CDRH3 region comprising the amino acid sequence RSSFYYYALD (SEQ ID NO:16);

3G9 Fab, having a CDRL3 region comprising the amino acid sequence QHYYYSPWPI (SEQ ID NO:17), a CDRH1 region comprising the amino sequence LYSYYI (SEQ ID NO:18), a CDRH2 region comprising the amino acid sequence SISPYYSSTY (SEQ ID NO:19) and a CDRH3 region comprising the amino acid sequence RSSYSYAGMD (SEQ ID NO:20);

2C11 Fab, having a CDRL3 region comprising the amino acid sequence QSYVSPI (SEQ ID NO:21), a CDRH1 region comprising the amino sequence ISSYYI (SEQ ID NO:22), a CDRH2 region comprising the amino acid sequence SISSYYSSTY (SEQ ID NO:23) and a CDRH3 region comprising the amino acid sequence RVSYGHAYVGYSSGMD (SEQ ID NO:24);

3H4 Fab, having a CDRL3 region comprising the amino acid sequence QSWYYSYPI (SEQ ID NO:25), a CDRH1 region comprising the amino sequence LSYSSM (SEQ ID NO:26), a CDRH2 region comprising the amino acid sequence SISSYYSYTS (SEQ ID NO:27) and a CDRH3 region comprising the amino acid sequence RYYGYGGGID (SEQ ID NO:28);

2A6 Fab, having a CDRL3 region comprising the amino acid sequence QSAGGYPLI (SEQ ID NO:29), a CDRH1 region comprising the amino sequence IYYSSM (SEQ ID NO:30), a CDRH2 region comprising the amino acid sequence SISPYSSYTS (SEQ ID

NO:31) and a CDRH3 region comprising the amino acid sequence RSFGYGWAFD (SEQ ID NO:32);

3H1 Fab, having a CDRL3 region comprising the amino acid sequence QHSYPI (SEQ ID NO:33), a CDRH1 region comprising the amino sequence ISYSSI (SEQ ID NO:34), a CDRH2 region comprising the amino acid sequence SIYSYSGSTY (SEQ ID NO:35) and a CDRH3 region comprising the amino acid sequence RYGAMD (SEQ ID NO:36);

2A2 Fab, having a CDRL3 region comprising the amino acid sequence QYYYPPI (SEQ ID NO:37), a CDRH1 region comprising the amino sequence ISSSSI (SEQ ID NO:38), a CDRH2 region comprising the amino acid sequence SIYSYYGYTY (SEQ ID NO:39) and a CDRH3 region comprising the amino acid sequence RYSAMD (SEQ ID NO:40);

3G3 Fab, having a CDRL3 region comprising the amino acid sequence QGAWSGGHLI (SEQ ID NO:41), a CDRH1 region comprising the amino sequence LSYSSM (SEQ ID NO:42), a CDRH2 region comprising the amino acid sequence YISPYYGYTY (SEQ ID NO:43) and a CDRH3 region comprising the amino acid sequence RGWAYYYYGYWGPSGLD (SEQ ID NO:44);

2D5 Fab, having a CDRL3 region comprising the amino acid sequence QWGYSHSHLI (SEQ ID NO:45), a CDRH1 region comprising the amino sequence ISYSS (SEQ ID NO:46), a CDRH2 region comprising the amino acid sequence SISPYYGSTY (SEQ ID NO:47) and a CDRH3 region comprising the amino acid sequence RSHFGALD (SEQ ID NO:48);

2D2 Fab, having a CDRL3 region comprising the amino acid sequence QSYYWVSPF (SEQ ID NO:49), a CDRH1 region comprising the amino sequence LYYSSI (SEQ ID NO:50), a CDRH2 region comprising the amino acid sequence SIYPYSGSTY (SEQ ID NO:51) and a CDRH3 region comprising the amino acid sequence RSYGYAMD (SEQ ID NO:52); and

2A5 Fab, having a CDRL3 region comprising the amino acid sequence QYHYWYYPI (SEQ ID NO:53), a CDRH1 region comprising the amino sequence ISYYSM (SEQ ID NO:54), a CDRH2 region comprising the amino acid sequence SISPYYGSTY (SEQ ID NO:55) and a CDRH3 region comprising the amino acid sequence RAGAMD (SEQ ID NO:56).

[0015] The complete heavy and light chain variable domain amino acid sequences for the 14 Fabs are shown below.

1) 3E8

-7-

heavy chain, 242 residues

EVQLVESGGGLVQPGGSLRLSCAASGFNLSYYSMHWVRQAPGKGLEWVASISPYYG
 YTTYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCARGGAYYFGYYGSGS
 YAMDYWGQGTLLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVS
 WNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKK
 VEPKSCDKTHTCHHHHHH (SEQ ID NO:58)

light chain, 225 residues

DIQMTQSPSSLSASVGDRVTITCRASQSVSSAVAWYQQKPGKAPKLLIYSASSLYSGV
 PSRFGSRSGTDFTLTISLQPEDFATYYCQQSSYS~~~LITFGQGTKVEIKRTVAAPSV
 FIFPPSDSQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDS
 YLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGECGGSDYKDDDDK
 (SEQ ID NO:59)

2) 3G3

heavy chain, 240 residues

EVQLVESGGGLVQPGGSLRLSCAASGFNLSYSSMHWVRQAPGKGLEWVAYISPYYG
 YTTYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCARGWAYYYYGYWGPS~
 ~GLDYWGQGTLLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSW
 NSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVE
 PKSCDKTHTCHHHHHH (SEQ ID NO:60)

light chain, 228 residues

DIQMTQSPSSLSASVGDRVTITCRASQSVSSAVAWYQQKPGKAPKLLIYSASSLYSGV
 PSRFGSRSGTDFTLTISLQPEDFATYYCQQGAWSGGHLITFGQGTKVEIKRTVAAPS
 VFIFPPSDSQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDS
 TYLSSTLTLSKADYEKHKVYACEVTHXGLSSPVTKSFNRGECGGSDYKDDDDK
 (SEQ ID NO:61)

3) 3G9

heavy chain, 234 residues

EVQLVESGGGLVQPGGSLRLSCAASGFNLSYYSIHWVRQAPGKGLEWVASISPYYS
 TYYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCARSSSYA~~~~~GM
 DYWGQGTLLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSG
 ALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKS
 CDKTHTC HHHHHH (SEQ ID NO:62)

light chain, 228 residues

DIQMTQSPSSLSASVGDRVTITCRASQSVSSAVAWYQQKPGKAPKLLIYSASSLYSGV
 PSRFGSRSGTDFTLTISLQPEDFATYYCQQHYYYSPWPITFGQGTKVEIKRTVAAPS
 VFIFPPSDSQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDS
 TYLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGECGGSDYKDDDDK
 (SEQ ID NO:63)

4) 3G11

heavy chain, 230 residues

EVQLVESGGGLVQPGGSLRLSCAASGFNISYYSMHWVRQAPGKGLEWVASIYPYSSS
 TYYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCARSS~~~~~AMD
 YWGQGTLLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGA

LTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHNKPSNTKVDKKVEPKSC
DKTHTCHHHHHH (SEQ ID NO:64)

light chain, 225 residues

DIQMTQSPSSLSASVGDRVITTCRASQSVSSAVAWYQQKPGKAPKLLIYSASSLYSGV
PSRFGSGRSGTDFTLTISLQPEDFATYYCQQWSFG~~~PITFGQGTKVEIKRTVAAPSV
FIFPPSDSQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDST
YLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGECGGSDYKDDDDK
(SEQ ID NO:65)

5) 3H1

heavy chain, 230 residues

EVQLVESGGGLVQPGGSLRLSCAASGFNISYSSIHWRQAPGKGLEWVASIYSYSGS
TYYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCARYG~~~~~AM
DYWGQGTLLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSG
ALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHNKPSNTKVDKKVEPKS
CDKHTCHHHHHH (SEQ ID NO:66)

light chain, 224 residues

DIQMTQSPSSLSASVGDRVITTCRASQSVSSAVAWYQQKPGKAPKLLIYSASSLYSGV
PSRFGSGRSGTDFTLTISLQPEDFATYYCQQHSY~~~~PITFGQGTKVEIKRTVAAPSV
FIFPPSDSQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDST
YLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGECGGSDYKDDDDK
(SEQ ID NO:67)

6) 3H4

heavy chain, 234 residues

EVQLVESGGGLVQPGGSLRLSCAASGFNLSYSSMHWVRQAPGKGLEWVASISSYYS
YTSYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCARYYGYGG~~~~~G
IDYWGQGTLLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNS
GALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHNKPSNTKVDKKVEPK
SCDKHTCHHHHHH (SEQ ID NO:68)

light chain, 227 residues

DIQMTQSPSSLSASVGDRVITTCRASQSVSSAVAWYQQKPGKAPKLLIYSASSLYSGV
PSRFGSGRSGTDFTLTISLQPEDFATYYCQQSWYYSY~PITFGQGTKVEIKRTVAAPS
VFIFPPSDSQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDS
TYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGECGGSDYKDDDDK
(SEQ ID NO:69)

7) 2A2

heavy chain, 230 residues

EVQLVESGGGLVQPGGSLRLSCAASGFNISSSSIHWVRQAPGKGLEWVASIYSYYGY
TYYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCARYS~~~~~AMD
YWGQGTLLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGA
LTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHNKPSNTKVDKKVEPKSC
DKTHTCHHHHHH (SEQ ID NO:70)

light chain, 224 residues

DIQMTQSPSSLSASVGDRVITITCRASQSVSSAVAWYQQKPGKAPKLLIYSASSLYSGV
PSRFGSGRSGTDFTLTISSLQPEDFATYYCQQYYY~~~~PITFGQGTKVEIKRTVAAPSV
FIFPPSDSQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDS
TYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGECGGSDYKDDDDK
(SEQ ID NO:71)

8) 2A5

heavy chain, 230 residues

EVQLVESGGGLVQPGGSLRLSCAASGFNIYSSSMHWVRQAPGKGLEWVASISSSSSY
TSYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCAR~AG~~~AMDYWGQG
TLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVH
TFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTC
HHHHHH (SEQ ID NO:72)

light chain, 227 residues

DIQMTQSPSSLSASVGDRVITITCRASQSVSSAVAWYQQKPGKAPKLLIYSASSLYSGV
PSRFGSGRSGTDFTLTISSLQPEDFATYYCQQYHYWYY~PITFGQGTKVEIKRTVAAPS
VFIFPPSDSQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDS
TYSLSSTLTLSKADYEKHKVYACEVTHXGLSSPVTKSFNRGECGGSDYKDDDDK
(SEQ ID NO:73)

9) 2A6

heavy chain, 234 residues

EVQLVESGGGLVQPGGSLRLSCAASGFNIYSSSMHWVRQAPGKGLEWVASISPYSSY
TSYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCARSFGYGW~~~~~AF
DYWGQGTTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSG
ALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKS
CDKTHTC HHHHHH (SEQ ID NO:74)

light chain, 227 residues

DIQMTQSPSSLSASVGDRVITITCRASQSVSSAVAWYQQKPGKAPKLLIYSASSLYSGV
PSRFGSGRSGTDFTLTISSLQPEDFATYYCQQSAGGYP~LITFGQGTKVEIKRTVAAPS
VFIFPPSDSQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDS
TYSLSSTLTLSKADYEKHKVYACEVTHXGLSSPVTKSFNRGECGGSDYKDDDDK
(SEQ ID NO:75)

10) 2B1

heavy chain, 239 residues

EVQLVESGGGLVQPGGSLRLSCAASGFNIYSSSMHWVRQAPGKGLEWVASISSSSSY
TSYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCARGYWYYWAWWAS~~~
AMDYWGQGTTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSW
NSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVE
PKSCDKTHTC HHHHHH (SEQ ID NO:76)

light chain, 228 residues

DIQMTQSPSSLSASVGDRVITITCRASQSVSSAVAWYQQKPGKAPKLLIYSASSLYSGV
PSRFGSGRSGTDFTLTISSLQPEDFATYYCQQYSGSGHYLITFGQGTKVEIKRTVAAPS
VFIFPPSDSQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDS
TYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGECGGSDYKDDDDK
(SEQ ID NO:77)

11) 2C11

heavy chain, 240 residues

EVQLVESGGGLVQPGGSLRLSCAASGFNISSYYIHWVRQAPGKGLEWVASISSYYSS
TYYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCARVSYGHAYVGYSS~G
MDYWGGQGLTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN
SGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEP
KSCDKTHTC HHHHHH (SEQ ID NO:78)

light chain, 225 residues

DIQMTQSPSSLSASVGDRVTITCRASQSVSSAVAWYQQKPGKAPKLLIYSASSLYSGV
PSRFGSGRSGTDFTLTISLQPEDFATYYCQQSYVS~~~PITFGQGTKVEIKRTVAAPSV
FIFPPSDSQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEVDSKDS
TYSLSSTLTLSKADYEKHKVYACEVTHXGLSSPVTKSFNRGECGGSDYKDDDDK
(SEQ ID NO:79)

12) 2D2

heavy chain, 232 residues

EVQLVESGGGLVQPGGSLRLSCAASGFNLYSSIIHWVRQAPGKGLEWVASIYPYSGS
TYYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCARSYGY~~~~~AM
DYWGQGLTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSG
ALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKS
CDKTHTC HHHHHH (SEQ ID NO:80)

light chain, 227 residues

DIQMTQSPSSLSASVGDRVTITCRASQSVSSAVAWYQQKPGKAPKLLIYSASSLYSGV
PSRFGSGRSGTDFTLTISLQPEDFATYYCQQSYWVS~PFTFGQGTKVEIKRTVAAPS
VFIFPPSDSQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDS
TYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGECGGSDYKDDDDK
(SEQ ID NO:81)

13) 2D5

heavy chain, 232 residues

EVQLVESGGGLVQPGGSLRLSCAASGFNISYSSIIHWVRQAPGKGLEWVASISPYYS
TYYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCARSHFG~~~~~ALD
YWGGQGLTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSG
LTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC
DKTHTC HHHHHH (SEQ ID NO:82)

light chain, 228 residues

DIQMTQSPSSLSASVGDRVTITCRASQSVSSAVAWYQQKPGKAPKLLIYSASSLYSGV
PSRFGSGRSGTDFTLTISLQPEDFATYYCQQWGYSHSLITFGQGTKVEIKRTVAAPS
VFIFPPSDSQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDS
TYSLSSTLTLSKADYEKHKVYACEVTHXGLSSPVTKSFNRGECGGSDYKDDDDK
(SEQ ID NO:83)

14) 2D9

heavy chain, 234 residues

EVQLVESGGGLVQPGGSLRLSCAASGFNLYYYSMHWVRQAPGKGLEWVASISPSYG
YTSYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCARSSFYFY~~~~~~AL

DYWGQGTLLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSG
 ALTSGVHTFPAVLQSSGLYSLSSVTVTPSSSLGTQTYICNVNHKPSNTKVD
 KKVEPKSCDKTHTC HHHHHH (SEQ ID NO:84)

light chain, 225residues

DIQMTQSPSSLSASVGDRVITTCRASQSVSSAVAWYQQKPGKAPKLLIYSASSLYSGV
 PSRFGSGRSGTDFTLTISSLQPEDFATYYCQQWSHY~~~LITFGQGTKVEIKRTVAAPS
 VFIFPPSDSQLKSGTASVVCLLNFPYPREAKVQWKVDNALQSGNSQESVTEQDSKDS
 TYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGECGGSDYKDDDDK
 (SEQ ID NO:85).

[0016] The Fabs bind to and inhibit BAX.

[0017] The Fabs can be produced reproducibly in large amounts using bacterial expression or mammalian expression systems.

[0018] The invention further provides a method of inhibiting apoptotic cell death by contacting BCL-2-associated X-protein (BAX) with any of the synthetic Fab antibodies disclosed herein in an amount effective to inhibit activation of BAX and apoptotic cell death. The synthetic Fab antibody can be conjugated to a therapeutic agent and/or to an agent that facilitates transport across a cell membrane. The synthetic Fab antibody can administered to a subject using any known method effective to administer an amount of the antibody that inhibits activation of BAX, including, for example, lentiviral or adenoviral expression, a plasmid, nanoparticles or liposomes.

[0019] The synthetic Fab antibody can be administered to a subject having, for example, a disease or disorder selected from the group consisting of a cardiovascular disease or disorder (e.g., arteriosclerosis, heart failure, heart transplantation, aneurism, chronic pulmonary disease, ischemic heart disease, hypertension, thrombosis, and/or cardiomyopathies), a neurodegenerative or neurological disease or disorder (e.g., Alzheimer's disease, Parkinson's disease, Huntington's disease, retinitis pigmentosa, spinal muscular atrophy, various forms of cerebellar degeneration, and/or amyotrophic lateral sclerosis), a liver disease or disorder, a kidney disease or disorder, a metabolic disease or disorder, an immunological disorder (e.g., organ transplant rejection, arthritis, lupus, IBD, Crohn's disease, asthma, multiple sclerosis, and/or diabetes), ischemia (e.g., stroke, myocardial infarction and/or reperfusion injury), infertility, a blood disorder (e.g., fanconi anemia, aplastic anemia, thalassemia, congenital neutropenia, and/or myelodysplasia), renal hypoxia, diabetes, hepatitis, asthma and AIDS.

[0020] Also provided is a method for measuring inactive monomeric BAX levels in a tissue sample from a subject comprising contacting the sample with any of the synthetic Fab

antibodies disclosed herein and measuring the amount of antibody bound to the sample, wherein the amount of bound antibody is indicative of the level of inactive monomeric BAX in the tissue sample.

[0021] Further provided is a method for screening for small molecules that bind to an N-terminal activation site of BAX comprising contacting BAX with any of the synthetic Fab antibodies disclosed herein in the presence and in the absence of a small molecule and measuring the amount of antibody that binds to BAX, wherein a decrease in the amount of antibody binding to BAX in the presence of the small molecule indicates that the small molecule is a candidate for binding to the N-terminal activation site of BAX. It is also possible that the small molecule binds to a site on BAX that causes allosteric conformational changes that cause a decrease in the amount of antibody binding to BAX.

[0022] The method can comprise a competitive ELISA screening assay. In an embodiment of the methods described herein, the methods are useful for identifying therapeutic cell death inhibitors. In an embodiment of the methods described herein, the methods are useful for identifying therapeutic cell death activators.

[0023] In an embodiment of the methods described herein, the small molecule has a molecular weight of 2000 daltons or less. In an embodiment of the methods described herein, the small molecule has a molecular weight of 1500 daltons or less. In an embodiment of the methods described herein, the small molecule has a molecular weight of 1000 daltons or less. In an embodiment of the methods described herein, the small molecule has a molecular weight of 800 daltons or less. In an embodiment of the methods described herein, the small molecule has a molecular weight of either 2000, 1500, 1000, 800, 700, 600, 500 or 400 daltons or less. In an embodiment of the methods described herein, the small molecule is a small organic molecule.

[0024] Still further provided is a method of predicting the ability of a cancer therapy to promote apoptotic cell death comprising contacting a cancerous tissue sample with any of the synthetic Fab antibodies disclosed herein, with and without the cancer therapy, and measuring the amount of antibody that binds to the tissue sample, wherein a decrease in the amount of bound antibody with the cancer therapy indicates that the cancer therapy promotes activation of BAX and apoptotic cell death, and the amount of bound antibody without the cancer therapy can predict the capacity of the cancer therapy to promote activation of BAX and apoptotic cell death in the cancerous tissue.

[0025] In the methods disclosed herein, the synthetic Fab antibody can be labeled with a detectable label, such as a fluorescent label or a radioactive label. The antibody can be biotinylated.

[0026] As used herein, “BAX” is BCL-2-associated X-protein. In an embodiment, the BAX is mammalian. In a preferred embodiment, the BAX is a human BAX. In an embodiment, the BAX comprises consecutive amino acid residues having the following sequence:

MDGSGEQPRGGGPTSSEQIMKTGALLLQGFIQDRAGRMGGEAPELALDPVPQDASTKKLSECLKRIGD
ELDSNMELQRMIAAVDTDSPREVFFRVAADMFSNGNFWGRVVALFYFASKLVLKALCTKVPPELIRTI
MGWTLDFLRERLLGWIQDQGGWDGLLSYFGTPTWQTVTIFVAGVLTASLTIWKKMG (SEQ ID NO:57,
human).

[0027] All combinations of the various elements described herein are within the scope of the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

[0028] This invention will be better understood from the Experimental Details, which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims that follow thereafter.

EXPERIMENTAL DETAILS

Introduction

[0029] Synthetic antibody technology was applied to BCL-2 family proteins to probe novel conformations and functional regions on the surface of BAX. Synthetic antibody discovery utilizes diversity targeted to the antibody complementarity determining regions (CDRs) that is encoded by designed, synthetic oligonucleotides and screened by phage or yeast display (22,23). Since library construction and selection is performed entirely *in vitro*, the state of the target can be controlled through the antibody discovery process. Therefore, the specificity of the output antibodies can be tuned to user-specified stringency (24,25). Using a phage display screen, synthetic antibodies were identified that bind with high affinity and specificity to BAX. Although BAX can be expressed and purified readily, and studied by NMR and other biophysical techniques, it is prone to aggregation under some conditions (such as detergents). This aggregation phenomenon is presumably linked to its function of forming oligomeric pores in the mitochondrial membrane, but presents a formidable challenge in raising conformation-specific antibodies using techniques requiring immunization. Thus, there was a strong rationale for utilizing *in vitro* selection.

[0030] Antibodies were identified that can be used as structural and biochemical probes to dissect key regulatory mechanisms and conformations of BAX (26,27). Fourteen novel synthetic antibody fragments (Fabs) were identified that specifically target BAX. These synthetic Fabs have no significant homology in the CDR sequences suggesting a diversity of molecular interactions with BAX. They bind to BAX with nanomolar affinities and occupy overlapping binding sites on BAX. Additionally, the synthetic Fabs inhibit BAX in assays using liposomal membranes or isolated mitochondria. Further analysis using isolated mitochondria suggest that the Fabs bind to cytosolic BAX and inhibit its ability to translocate and insert onto the mitochondria outer membrane. Structural studies using NMR and hydrogen/deuterium exchange mass spectrometry showed that a representative synthetic Fab (3G11) forms a stoichiometric and stable complex with monomeric and inactive BAX, with a binding site that involves residues of helices $\alpha 1/\alpha 6$ and of the $\alpha 1$ - $\alpha 2$ loop. Therefore, binding of the synthetic Fabs overlaps with the N-terminal activation site of BAX suggesting a novel mechanism of BAX inhibition through direct competition with the BAX activation process. These Fabs provide new tools for probing BAX activity in an unparalleled manner and provide a strategy for therapeutic inhibition of BAX in disease.

Materials and Methods

[0031] *Production of Recombinant BAX* - Human full-length wild type BAX, truncated $\alpha 9$ BAX (BAX ΔC) and BAX mutants in pTYB1 vector (New England Biolabs) were expressed in BL21 CodonPlus (DE3)-RIPL + *E. coli* strain and purified as previously described (9, 10).

[0032] *Fab protein expression* - The phage display vectors for the 14 identified clones were converted to Fab protein expression vectors by insertion of a stop codon and a 6-Histidine tag upstream of the P3 gene fusion. Fab proteins were expressed periplasmically in *E. coli* BL21(DE3) (New England Biolabs, Ipswich, MA) by growth in low-phosphate media at 30°C for 18–22 h. The cells were harvested by centrifugation and lysed by using the BugBuster lysis reagent according to the manufacturer's instructions (Novagen, Madison, WI). The lysate was subjected to centrifugation, and the supernatant was applied to a nickel column (Ni-NTA resin, Qiagen, Valencia, CA) pre-equilibrated with Tris-buffered saline (TBS), pH 7. The Fab-bound Ni-NTA column was then washed with 20-column volume TBS buffer with 20 mM imidazole. Next, the protein was eluted with TBS buffer with 250 mM imidazole. The eluent was dialyzed into PBS at pH 8.0, and applied to a Protein A affinity column (beads from Pierce Thermo Scientific, Rockford, IL) for further purification. The

Fab-bound beads were washed with PBS, pH 8.0 (15 column volumes), and the Fab proteins were eluted with 100 mM glycine, pH 2.0. The eluted Fab proteins were immediately neutralized to ~ pH 7 using 1 M Tris buffer, pH 8.0. Fractions containing the Fab proteins were buffer-exchanged into PBS, pH 7.0 and were used directly in following binding and activity assays, or flash-frozen and stored at 80°C for future use. The Fab protein concentrations were determined by measuring the absorbance at 280 nm. The extinction coefficients at 280 nm were calculated from the Fab protein sequences using ExPASy.

[0033] *Origin of sequence of the CDR regions* - The antibody framework of all the BAX antibodies is a common scaffold based on Herceptin (mAb 4D5), which is a humanized variant of a mouse anti-Her2 monoclonal antibody developed by Genentech (South San Francisco, CA). The complementarity determining regions (CDRs), which bind the antigen and provide the mAb specificity, contain wholly "synthetic" (artificial) sequences. The nature of these synthetic CDR sequences reflects the engineered nature of the phage antibody library from which the antibodies were obtained: Koellhoffer et al., *Chembiochem*, 2012, 13, 2549.

[0034] *Sequences of antibodies expressed in cells* - The BAX antibodies were converted to single chain variable fragments (scFvs) and cloned into cell expression plasmids (discussed below). DNA sequences for the scFv open reading frames are indicated below for different Fabs:

3E8, SEQ ID NO:86

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GGATCCGACATCCAGATGACCCAGTCCCCAAGCTCCCTGAGCGCATCCGTGGGCGATAGGGTGACCATCA
CATGCAGGGCATCTCAGAGCGTGTCTAGCGCAGTGGCATGGTACCAGCAGAAGCCAGGCAAGGCCCTAA
GCTGCTGATCTACAGCGCTCCTCTCTGTATTCCGGAGTGCCTTCTCGGTTCTCCGGCAGCCGGAGCGGA
ACCGACTTTACCCCTGACAATCAGCTCCCTGCAGCCAGAGGATTTGCGCACATACTATTGCCAGCAGTCTA
GCTACTCCCTGATCACCTTTGGCCAGGGCACAAAGTGGAGATCAAGGGAGGAGGCAGCGGAGGAGGCTC
CGGAGGCGGCTCTGAGGTGCAGCTGGTGGAGAGCGGAGGAGGACTGGTGCAGCCTGGAGGCAGCCTGAGG
CTGTCTGTGCAGCATCTGGCTTCAACCTGTCTTACTATAGCATGCACTGGGTGCGCCAGGCACCAGGCA
AGGGCCTGGAGTGGGTGGCCTCCATCTCTCCCTACTATGGCTACACCTACTATGCCGACTCTGTGAAGGG
CCGGTTCACAATCAGCGCCGATACCTCCAAGAACACAGCCTATCTGCAGATGAATAGCCTGAGGGCAGAG
GACACCGCAGTGTACTATTGTGCCAGAGGCGGCGCTACTATTTGGCTACTATGGCAGCGGCTCCTACG
CCATGGATTATTGGGGCCAGGGCACCCCTGGTGACAGTGCCTCTTAATCTAGA
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3G11, SEQ ID NO:87

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GGATCCGACATCCAGATGACCCAGAGCCCAAGCTCCCTGAGCGCATCCGTGGGCGATAGGGTGACCATCA
CATGCAGGGCATCTCAGAGCGTGTCTAGCGCAGTGGCATGGTACCAGCAGAAGCCAGGCAAGGCCCTAA
GCTGCTGATCTACTCCGCTCCTCTCTGTATAGCGGCGTGCCTTCCCGTTCTCCGGCAGCCGGAGCGGA
ACCGACTTTACCCCTGACAATCAGCTCCCTGCAGCCTGAGGATTTGCGCACATACTATTGCCAGCAGTGGA
GCTTCGGCCCCAATCACCTTTGGCCAGGGCACAAAGTGGAGATCAAGGGAGGAGGCTCTGGAGGAGGCAG
CGGAGGCGGCTCCGAGGTGCAGCTGGTGGAGTCCGGCGGCGGCTGGTGCAGCCAGGAGGCTCTCTGAGG
CTGAGCTGTGCCGCTCCGGCTTCAACATCTCCTACTATTCTATGCACTGGGTGCGCCAGGCACCAGGCA
AGGGCCTGGAGTGGGTGGCTCCATCTACCCCTATTCTAGCTCCACCTACTATGCCGACTCTGTGAAGGG
CCGGTTTACAATCTCTGCCGATACCAGCAAGAACACAGCCTACCTGCAGATGAATAGCCTGAGGGCAGAG
GACACCGCAGTGTACTATTGTGCCAGATCTAGCGCCATGGATTATTGGGGCCAGGGCACCCCTGGTGACAG
TGTCTCTTAATCTAGA
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3H1, SEQ ID NO:88

GGATCCGACATCCAGATGACCCAGAGCCCAAGCTCCCTGAGCGCATCCGTGGGCGATAGGGTGACCATCA
 CATGCAGGGCATCTCAGAGCGTGTCTAGCGCAGTGGCATGGTACCAGCAGAAGCCAGGCAAGGCCCCCTAA
 GCTGCTGATCTACAGCGCCTCCTCTCTGTATTCCGGAGTGCCTTCTCGGTTCTCCGGCAGCCGGAGCGGA
 ACCGACTTTACCCGTGACAATCAGCTCCCTGCAGCCAGAGGATTTGCGCCACATACTATTGCCAGCAGCACT
 CCTACCCCATCACCTTTGGCCAGGGCACAAAGGTGGAGATCAAGGGAGGAGGCAGCGGAGGAGGCTCCGG
 AGGCGGCTCTGAGGTGCAGCTGGTGGAGTCCGGAGGAGGACTGGTGCAGCCTGGAGGCAGCCTGAGGCTG
 TCCTGTGCAGCATCTGGCTTCAACATCTCTTACTCTAGCATCCACTGGGTGCGCCAGGCACCAGGCAAGG
 GCCTGGAGTGGGTGGCTCTATCTACTCCTATTCTGGCAGCACCTACTATGCCGACAGCGTGAAGGGCCG
 GTTTACAATCAGCGCCGATACCTCCAAGAACACAGCCTATCTGCAGATGAATTCCCTGAGGGCAGAGGAC
 ACCGCAGTGTACTATTGTGCCAGATACGGCGCATGGATTATTGGGGCCAGGGCACCCCTGGTGACAGTGT
 CCTCTTAATCTAGA

2B1, SEQ ID NO:89

GGATCCGACATCCAGATGACCCAGTCCCCAAGCTCCCTGAGCGCATCCGTGGGCGATAGGGTGACCATCA
 CATGCAGGGCATCTCAGAGCGTGTCTAGCGCAGTGGCATGGTACCAGCAGAAGCCAGGCAAGGCCCCCTAA
 GCTGCTGATCTACAGCGCCTCCTCTCTGTATAGCGGCGTGCCATCCCGGTTCTCCGGCAGCCGGAGCGGA
 ACCGACTTTACCCGTGACAATCAGCTCCCTGCAGCCGAGGATTTGCGCCACATACTATTGCCAGCAGTACT
 CCGGCTCTGGCCACTATCTGATCACCTTTGGCCAGGGCACAAAGGTGGAGATCAAGGGAGGAGGCTCTGG
 AGGAGGCAGCGGAGGCGGCTCCGAGGTGCAGCTGGTGGAGTCCGGCGGCGGCTGGTGCAGCCTGGAGGC
 TCTCTGAGGCTGAGCTGTGCAGCATCCGGCTTCAACATCTACTCTAGCTCCATGCACTGGGTGCGCCAGG
 CACCAAGCAAGGGCCTGGAGTGGGTGGCCAGCATCTAGCTCCTCTAGCTACACCTCTTATGCCGACAG
 CGTGAAGGGCCGGTTTACAATCTCCGCCGATACCTCTAAGAACACAGCCTATCTGCAGATGAATTCCCTG
 AGGGCAGAGGACACCGCAGTGTACTATTGTGCCAGAGGCTACTGGTACTATTGGGCTGGTGGGCCAGCG
 CCATGGATTATTGGGGCCAGGGCACCCCTGGTGACAGTGTCTCTTAATCTAGA

2D9, SEQ ID NO:90

GGATCCGACATCCAGATGACCCAGAGCCCAAGCTCCCTGAGCGCATCCGTGGGCGATAGGGTGACCATCA
 CATGCAGGGCATCTCAGAGCGTGTCTAGCGCAGTGGCATGGTACCAGCAGAAGCCAGGCAAGGCCCCCTAA
 GCTGCTGATCTACAGCGCCTCCTCTCTGTATTCCGGAGTGCCTTCTCGGTTCTCCGGCAGCCGGAGCGGA
 ACCGACTTTACCCGTGACAATCAGCTCCCTGCAGCCAGAGGATTTGCGCCACATACTATTGCCAGCAGTGGT
 CCCACTATCTGATCACCTTTGGCCAGGGCACAAAGGTGGAGATCAAGGGAGGAGGCAGCGGAGGAGGCTC
 CGGAGGCGGCTCTGAGGTGCAGCTGGTGGAGTCCGGAGGAGGACTGGTGCAGCCTGGAGGCAGCCTGAGG
 CTGTCTGTGCAGCATCTGGCTTCAACCTGTACTATTACTCTATGCACTGGGTGCGCCAGGCACCAGGCA
 AGGGCCTGGAGTGGGTGGCCTCCATCTCTCCAGCTACGGCTATACCAGCTACGCCGACTCCGTGAAGGG
 CCGGTTTACAATCTCTGCCGATACCAGCAAGAACACAGCCTATCTGCAGATGAATTCCCTGCGGGCCGAG
 GACACCGCCGTGTATTACTGTGCCAGATCTAGCTTTTATTACTATGCCCTGGATTACTGGGGCCAGGGAA
 CCCTGGTGACAGTGTCTCTTAATCTAGA.

[0035] *Cloning and procedures for in cell expression* - The characterized mAbs were cloned to mammalian expression vectors (pCDNA3.1 or pMSCV PIG: Puro IRES GFP) in a reduced Fab form constructed by linear two copies of single chain variable Fragments (scFv) to ensure proper folding and expression. pCDNA3.1 is available from Addgene (Cambridge, MA) and GenScript (Piscataway, NJ) used it to clone the Fab sequences. Memorial Sloan Kettering Cancer Center (New York, NY) subcloned the Fab sequences from pCDNA3.1 to pMSCV. To easily detect the expression of Fabs and use them in pull-down experiments, 6 Myc tag sequences were included as an N-terminal tag to each scFv sequence during the cloning process. Transient expression of five Fabs in Mouse Embryo Fibroblasts (MEF) demonstrated that all Fabs are well expressed at similar levels. For more efficient transfection, Fab plasmids were transduced with retroviral transfection and stable Fab-

expressing clones were selected using GFP as a selection marker. Co-immunoprecipitation experiments using anti-myc agarose beads pulled down the Fabs and demonstrated their capacity to bind BAX in cells using anti-BAX Western analysis.

[0036] *Direct binding ELISAs* - BAX (1 μ g per well) was first immobilized on 96-well EIA/RIA plate (Corning Incorporated, Corning, NY) at room temperature for 1 h or at 4°C for overnight. PBS containing 3% BSA was used to block the wells after BAX immobilization (incubation for 1.5 h at room temperature). The Fabs were diluted into PBS buffer (pH 7.0), applied to the wells, and incubated for 1 h at room temperature. The plates were then washed with PBS and incubated for 1 h with horseradish peroxidase/anti-FLAG M2 antibody conjugate (binding to the FLAG tag at the C-terminus of Fab light chain). The wells were washed with PBS, developed with 3,3',5,5'-tetramethylbenzidine (TMB) substrate, and quenched with 0.5 M H₂SO₄. The absorbance at 450 nm was determined. The data were fit to a standard four parameter logistic equation by using GraphPad Prism (GraphPad Software, La Jolla, CA). The half-maximal binding (EC₅₀) values were obtained from the inflection point of the curve.

[0037] *Competitive Binding ELISAs* - Six Fab clones (3H1, 3G11, 3E8, 2A5, 2D9 and 2B1) were chosen for the competition ELISAs. Two different approaches were used. In the first approach, non-biotinylated Fabs were used to compete with the binding of biotinylated 3G11 (b3G11). To immobilized BAX on the 96-well EIA/RIA plate (Corning Incorporated, Corning, NY), a mixture of 50 nM b3G11 and increasing amount of each of the six selected Fabs were added. After washes, only the binding of b3G11 was monitored using horseradish peroxidase/streptavidin conjugate and TMB substrate. In the second approach, purified Fab proteins were used to compete with Fabs displayed at the phage surface. Similarly, to immobilized BAX on the 96-well EIA/RIA plate (Corning Incorporated, Corning, NY), a mixture of Fab-displayed phage (with a titer of $\sim 10^{12}$ pfu/mL) and 100 nM of each of the six selected Fab proteins were added. Only the binding of Fab-phage complex binding was monitored using horseradish peroxidase/anti-M13 antibody conjugate and TMB substrate.

[0038] *Biolayer Interferometry* - The forteBio BLItz system (Pall Corporation, Menlo Park, CA) was used to determine the binding kinetics and affinity between BAX and Fab proteins. Ni-NTA biosensors (Pall Corporation, Menlo Park, CA) were used for initial Fab protein immobilization, which was followed by BAX association and dissociation interaction analysis. 10 μ g/mL Fab was used for immobilizing at pH 7. For each Fab protein, at least three different BAX concentrations, varying from 30 nM to 1.2 μ M, were used, and

subsequently global fitting was used to generate the k_a (association rate constant), k_d (dissociation rate constant) and K_D (equilibrium dissociation constant) values.

[0039] *Size-exclusion Chromatography* - Superdex 75 10/300 GL and 200 10/300 GL (GE Healthcare) columns were used for size exclusion chromatography of recombinant BAX and Fab-BAX complex. Proteins were injected in columns equilibrated with a buffer containing 20 mM HEPES pH 7.2, and 150 mM KCl.

[0040] *Liposome Permeabilization Assay* - Liposomes were composed of the following molar percentages of lipids (Avanti Polar Lipids): phosphatidylcholine, 48%; phosphatidylinositol, 10%; phosphatidylethanolamine, 28%; dioleoyl phosphatidylserine, 10%; and tetraoleoyl cardiolipin, 4% and were loaded with ANTS/DPX (Molecular Probe) upon extrusion. Fabs at the indicated concentrations in 96-well format (Corning), with and without BAX (400 nM) or BAX (400nM) and tBID (30 nM) and then liposomes were added (10 μ L from 1 mL lipid stock) in assay buffer (10 mM HEPES, pH 7, 200 mM KCl, and 1 mM $MgCl_2$) to a final volume of 100 μ L. ANTS/DPX release was quantified based on the increase in fluorescence intensity that occurs when the ANTS fluorophore is separated from the DPX quencher upon release from the liposomes into solution. Fluorescence (λ_{ex} = 355 nm and λ_{em} = 520 nm) was measured over time at 30°C using a Tecan Infinite M1000 plate reader. The percentage release of ANTS/DPX at 90 min was calculated as percentage release = $((F - F_0)/(F_{100} - F_0)) \times 100$, where F_0 and F_{100} are baseline and maximal fluorescence, respectively. 1% Triton treatment is used to determine the maximum amount of liposomal release per assay, and this value sets the 100% value for the kinetic curve figures whereas the calculated percent release value for the bar graphs was calculated by using the percent release value of tBid-induced BAX mediated ANT/DPX release as 100%.

[0041] *BAX translocation assay* - Mitochondria from liver of *Bak^{-/-}* mice (0.75mg/ml) were resuspended in experimental buffer (125 mM KCl, 10 mM Tris-MOPS [pH 7.4], 5 mM glutamate, 2.5 mM malate, 1 mM KPO_4 , 0.1mM EGTA-Tris [pH 7.4]) and treated with the indicated concentrations of 3G11 recombinant BAX (200 nM) and tBid (100nM), singly and in combination, and incubated at room temperature for 20 min. The supernatant fractions were isolated by centrifugation at 5500xg for 10 min and the mitochondrial pellets resuspended and washed with 0.1M sodium carbonate (pH 11.5) for 20 min, centrifuged at 13,000xg for 10 min at 4°C, and then solubilized in 1% Triton X-100/PBS for 1 h at 4°C. Mitochondrial supernatant and pellet fractions were separated by 4-12% NuPage (Invitrogen) gels and analyzed by immunoblotting with anti-BAX antibody (Cell Signaling Cat. 2772).

[0042] *Mitochondrial release assay* - Mitochondria from liver of *Bak*^{-/-} mice (1.5mg/ml) were resuspended in experimental buffer (125 mM KCl, 10 mM Tris-MOPS [pH 7.4], 5 mM glutamate, 2.5 mM malate, 1 mM KPO₄, 0.1mM EGTA-Tris [pH 7.4]) and treated with the indicated concentrations of 3G11 recombinant BAX (400 nM) and tBid (30nM), singly and in combination, and incubated at room temperature for 60 min. The supernatants were isolated by centrifugation at 5500xg for 10 min and the mitochondrial pellets solubilized in 1% Triton X-100/PBS. Mitochondrial supernatant and pellet fractions were separated by 4-12% NuPage (Invitrogen) gels and analyzed by immunoblotting with anti-cytochrome c antibody (BD Biosciences Cat. 556433).

[0043] *Western Blotting* - Samples from the mitochondria and translocation assay were electrophoretically separated on 4-12% NuPage (Invitrogen) gels, transferred to mobilon-FL PVDF membranes (Millipore) and subjected to immunoblotting. For visualization of proteins with Odyssey Infrared Imaging System (LI-COR Biosciences) membranes were blocked in PBS containing 3% milk powder. Primary BAX antibody (Cell Signaling 2772S) was incubated overnight at 4°C in a 1:1,000 dilution. After washing, membranes were incubated with an IRdye800-conjugated goat anti-rabbit IgG secondary antibody (LI-COR Biosciences) in a 1:10,000 dilution. Proteins were detected with Odyssey Infrared Imaging System.

[0044] *NMR samples and spectroscopy* - Uniformly ¹⁵N-labeled full-length human BAX was generated as previously described . Protein samples of BAX and BAX-3G11 mixtures at indicated concentrations were prepared in 25 mM sodium phosphate, 50 mM NaCl solution at pH 6.0 in 5% D₂O. Correlation 1H-¹⁵N HSQC and 1H-¹⁵N TROSY spectra were acquired at 25°C on a Bruker 600 MHz NMR spectrometer equipped with a cryogenic probe, processed using Topsin, and analyzed with CCPNMR. BAX wild type cross-peak assignments were applied as previously reported (9).

[0045] *Hydrogen/Deuterium exchange mass spectrometry* - Prior to hydrogen-deuterium exchange experiments, the quench condition for best sequence coverage of BAX was optimized as previously described (28). Briefly, 3 µl of stock solution of BAX at 1.0 mg/ml was mixed with 9 µl of H₂O buffer (8.3 mM Tris, 150 mM NaCl, in H₂O, pH7.2) at 0 °C and then quenched with 18 µl of ice cold quench solutions of 0.8% formic acid, 16% Glycerol, and GdnHCl at final concentrations of 0.05 M, 0.5 M, 1.0 M and 2.0 M. The quenched samples were frozen on dry ice and then subjected to an immobilized pepsin column (1x20mm, 30 mg/ ml porcine pepsin (Sigma)) for online digestion for 40 sec. The resulting peptides were collected on a C18 trap (Michrom MAGIC C18AQ 0.2x2) and separated using a reversed phase C18 column (Michrom MAGIC C18AQ 0.2x50 3µm 200Å) with a 30 min

linear gradient of 0.046% (v/v) trifluoroacetic acid, 6.4% (v/v) acetonitrile to 0.03% (v/v) trifluoroacetic acid, 38.4% (v/v) acetonitrile. The effluent was directed into an Orbitrap Elite mass spectrometer (Thermo-Fisher Sci. Inc) for MS analysis. The instrument was operated in positive ESI mode and the resolution of the instrument was set at 60,000. Proteome Discoverer software (Thermo Fisher Sci. Inc.) was used to identify the sequence of the resulting peptides. The optimal quench condition with the best coverage map of BAX (0.08M GuHCl in 0.8% formic acid) was used for subsequent functionally deuterated studies. Hydrogen-deuterium exchange reactions were initiated by diluting 3 μ l of pre-chilled protein stock solution (free BAX, 1 mg/ml, or antibody-bound BAX, 2mg/ml) into 9 μ l D₂O buffer (8.3 mM Tris, 150 mM NaCl, in D₂O, pDREAD 7.2). The samples were incubated at 0 °C for 10 sec, 100 sec and 1000 sec. The exchange reaction was terminated by the addition of 18 μ l of optimized quench solution at 0 °C and samples were immediately frozen on dry ice and stored at -80°C. In addition, un-deuterated samples and equilibrium-deuterated control samples were also prepared as previously described (29). The deuterated samples were then loaded onto above instrument for DXMS analysis. The centroids of the isotopic envelopes of un-deuterated, functionally deuterated, and equilibrium deuterated peptides were measured using HDExaminer, and then converted to corresponding deuteration levels with corrections for back-exchange (30).

[0046] *Structure calculations* - Structure calculations were performed with Crystallography and NMR system solve (CNS) within the HADDOCK web server using the prediction interface (31). HADDOCK calculations generated models of the complex that are in agreement with experimental distance restraints and have optimal electrostatic and van der Waals interactions based on a combination of molecular dynamics and energy minimization. HADDOCK docking was performed using the BAX NMR structural ensemble imported directly from the PDB (PDB ID: 1F16). The 3G11 structural model was generated using PIGS software based on structural homology modeling of known Fab structures of the same family that have different amino acid composition of CDR regions (32). Calculations were performed with ambiguous interaction restraints (AIR) derived from the hydrogen deuterium exchange mass spectrometry data and residues of the CDR regions of 3G11. For BAX AIR calculations, only residues that exhibited significant protection of hydrogen deuterium exchange upon titration and solvent accessibility over 50% as determined by the program NACCESS (Hubbard and Thornton, 1993) were defined as active residues; passive residues were automatically assigned by the HADDOCK web interface as those residues surrounding the active residues. For 3G11, AIR restraints were assigned based on residues involved in the

CDRH1, CDRH2, CDRH3, and CDRL3. Specifically, active residues for BAX included residues 19-25 and 123-142; active residues for 3G11 were defined as Heavy Chain: 26-32 (Loop 1), 52-54 (Loop 2), 96-101 (loop 3) and Light Chain; 141-148, 165-168, and 206-211. In each HADDOCK structure calculation, 1000 orientations/structures of the complex were generated by rigid-body docking energy minimization of the individual structures. The 188 lowest energy structures were semi-flexibly refined in torsion angle space and then refined in explicit solvent. 132 structures formed the most populous cluster (HADDOCK score = -140.5 ± 12.1 and Z-score = -1.7) with RMSD from the overall lowest energy structure (1.9 ± 1.1 Å) calculated on the backbone (CA, C, N, O, P) atoms of all residues involved in intermolecular contact using a 10 Å cut-off. In all cases 3G11 was localized to N-terminal trigger site. Ribbon diagrams and molecular models were depicted using PYMOL (Schrodinger).

Results

[0047] *Discovery of 14 Novel Synthetic Antibodies Targeting Monomeric BAX* - A restricted diversity phage antigen-binding fragment (Fab) library ("Library F") was used to select a panel of Fabs that bind to monomeric BAX (9). Library F was designed to contain mostly binomial Tyr/Ser diversity in CDR-H1 and H2, and expanded diversity, encoding the nine amino acids Tyr/Ser/Gly/Ala/Phe/Trp/His/Pro/Val in a 5/4/4/2/1/1/1/1/1 ratio, at CDR-L3 and H3 (33). In addition, the CDR-L3 and H3 loops vary in size. The expanded diversity in CDR-L3 and H3 in Library F was designed to mimic the distribution of amino acids in functional CDR-H3 segments of natural antibodies. Library F was screened against the monomeric BAX, which that was purified after utilizing chitin-column affinity purification and size exclusion chromatography (10). After 2-3 rounds of stringent selection, a panel of 14 BAX-specific Fabs with diverse CDR sequences was obtained (Fig. 1A). These clones were isolated from two distinct panning regimes, one in which biotinylated BAX was immobilized onto streptavidin-coated wells, and another in which BAX was coated directly on the wells. The properties of the selected clones were similar from either screening regime and thus further analyzed as a single conglomerated panel. Sequence comparison between the 14 Fabs revealed high diversity of residues and loop sizes in many positions with no apparent sequence homology in CDR segments from any two clones, demonstrating the diverse modes of interaction with BAX.

[0048] Fabs were expressed and purified and the half-maximal binding titers (EC_{50}) for BAX using ELISA were determined. The EC_{50} values ranged from high affinity 2.3 nM (2B1) to moderate affinity 250 nM (2A5) with most Fabs having single or double digit nM

nanomolar EC_{50} (Fig. 1A). Select Fabs 3G11, 3E8, 2A5 and 2B1 were evaluated for binding kinetics to monomeric BAX using Biolayer Interferometry (Fig. 1B). The binding affinities (K_D values) and ranking of the Fab proteins based on binding correlate with the EC_{50} from ELISA. In both assays, Fabs 3G11, 3E8 and 2B1 were the highest-affinity binders to BAX, while Fab 2A5 was the lowest-affinity binder (Fig. 1A, 1B). Hence, an array of 14 high-affinity antibodies against monomeric BAX was identified.

[0049] To explore if the Fabs engaged overlapping or distinct epitopes on BAX, competitive ELISA was performed using six of the clones (3H1, 3G11, 3E8, 2A5, 2D9 and 2B1) whose EC_{50} values cover the entire affinity range. A competitive ELISA was performed in which Fab 3G11 was first biotinylated (b3G11) and binding of b3G11 to BAX in the presence of other non-biotinylated Fab proteins was analyzed. All Fabs except Fab 2A5 demonstrated competitive inhibition of b3G11 binding with IC_{50} s ranging from nM to μ M (Fig. 1C). Fab 2A5 does not compete b3G11 binding likely because it is a much weaker binder than 3G11. Furthermore, a study was also conducted of how binding of phage-expressed Fabs (Φ) can be competitively inhibited by free Fab proteins. The data showed that phage-expressed Fab binding was inhibited to varying degrees in the presence of 100 nM Fab protein for many of the clones and that, in each case, the phage-expressed Fab for a particular clone could be inhibited by its own Fab protein (Fig. 1D). Again, Fab 2A5, the lowest affinity clone ($EC_{50} = 250$ nM) had little effect on Fab phage binding for all the other clones, but all the other Fabs proteins could significantly inhibit Fab 2A5 phage binding. On the contrary, Fabs 3E8 and 2B1, the highest affinity clones (EC_{50} s = 2.6 nM and 2.3 nM, respectively) showed the strongest inhibition on all Fab phage binding. Therefore, the competitive ELISA data matches predictions based on ELISA half-maximal binding titers and K_D values. These results indicate that, to a large extent, these Fabs have overlapping epitopes on the BAX surface with a range of affinities, but many in the low nanomolar range.

[0050] *Synthetic Antibodies Inhibit BH3-triggered BAX activation and MOMP* – To examine how binding to BAX by Fabs proteins modulates BAX function, liposomal assays were performed that explicitly evaluate how BH3-triggered BAX forms a pore in a membrane environment made from similar lipid composition of mitochondria without the contribution of other mitochondrial factors (34). Selected Fabs have the potential to activate BAX by engaging one of the activation sites of BAX either at its N-terminal or C-terminal surface (10,15,35,36) or inhibit BAX activation by inhibiting the BAX binding surface of the activator protein tBID or conformational changes associated with BAX activation (12,15,16,20,37). Therefore, the capacity of the Fabs to either activate BAX or inhibit BAX

activation induced by tBID was examined. None of the 14 Fabs had an effect on the liposomal integrity alone; neither does an unrelated vascular-endothelial growth factor (VEGF)-specific Fab (YADS1, negative control). Furthermore, none of the Fab proteins activated BAX and induced liposomal release (Fig. 2A). However, when tBID and BAX are combined with liposomes, liposomal release is robust as expected (Fig. 2A, 2B). In contrast, high affinity Fabs when combined with tBID and BAX inhibited tBID-triggered BAX activation significantly, or completely, at 2 μ M, in all cases, except with the exception of the lower affinity clone Fab 2A5 and the negative control YADS1 (Fig. 2B). Three of the highest binding Fabs 3G11, 3E8 and 2B1 yielded dose-responsive and time-dependent inhibition of liposomal release whereas the weaker binder 2A5 had no effect even at 2 μ M (Fig. 2C-2F).

[0051] To examine whether Fabs can inhibit BAX activation in the presence of mitochondrial membranes loaded with anti-apoptotic Bcl-2 or other mitochondrial proteins, mouse liver mitochondria were isolated from *Bak*^{-/-} mice to perform a mitochondrial release assay. Combination of tBID and BAX was exposed to several doses of Fab 3G11, which demonstrated dose-responsive inhibition of mitochondrial cytochrome c release induced by activated BAX, as assessed by separation of the supernatant and mitochondrial fractions and western analysis (Fig. 3A). Moreover, using the mitochondria assay, BAX localization was determined to examine whether inhibition by Fab 3G11 was due to prevention of BAX translocation to the membrane or blocking of the membrane integration and oligomerization on the outer mitochondrial membrane. Fab 3G11 dose-responsively inhibited the capacity of BAX for mitochondrial translocation suggesting that 3G11's high affinity binding to the monomeric BAX either competes binding of tBID or prevents conformational changes of BAX required for its mitochondrial translocation (Fig. 3B). Taken together, these data indicate that these Fab proteins inhibit BH3-triggered BAX activation and MOMP by restraining mitochondrial membrane translocation of BAX.

[0052] *Fab 3G11 Protein Forms a Stable and Stoichiometric Complex with BAX* - To investigate the effect of synthetic Fab proteins on the structure of BAX, the stability and stoichiometry of several Fab-BAX complexes were studied upon mixing the protein at various concentrations and analyzing by size-exclusion chromatography (SEC) (data not shown). Based on gel SEC and SDS-PAGE analysis, binding of the Fab 3G11 to BAX results in a stable 1:1 stoichiometric complex. 3G11 was selected for further structural analysis because of its high affinity and low dissociation constant (Fig. 1B). Next, structural effects of the 3G11 were analyzed based on 1H-15N HSQC spectra of full length BAX monomer at several doses of 3G11. The HSQC spectra are in agreement with the 3G11 and BAX forming

a stable complex, with a 1:1 stoichiometry and in slow exchange on the NMR time scale as evidenced by the dose-dependent loss of intensity of the cross peaks of the monomeric BAX and lack of new chemical shifts upon 3G11 titration. Despite the 3G11-BAX complex formation evidenced by the NMR titration, it is not possible to observe the ^1H - ^{15}N cross peaks of the complex in the ^1H - ^{15}N HSQC spectra due to the size of the complex (70 KDa).

[0053] *Fab 3G11 Protein Binds the N-terminal Surface of BAX to Prevent BAX Activation* - To further analyze the Fab-BAX interaction, the changes on the BAX structure were analyzed by measuring the solvent accessibility and hydrogen-deuterium exchange of the backbone amide hydrogens using hydrogen-deuterium exchange mass spectrometry (HXMS). First, the deuterium exchange of unbound BAX was analyzed in solution, which underlined the different deuterium exchange rates for exposed or unfolded regions (N-terminus, $\alpha 1$ - $\alpha 2$ loop) and more solvent-protected or structured regions including $\alpha 2$, $\alpha 3$ - $\alpha 4$, $\alpha 5$, $\alpha 6$ - $\alpha 8$. Upon 3G11-BAX complex formation at stoichiometric levels, deuterium exchange, sample digestion, preparation and analysis was performed in the same conditions as with free BAX. Interestingly, HXMS analysis of the Fab-bound BAX in solution highlighted significant solvent protection in helix $\alpha 1$ and $\alpha 6$ and the $\alpha 1$ - $\alpha 2$ loop while other regions of the BAX structure had little to no change in the deuterium incorporation upon interaction with the 3G11. Furthermore, HXMS showed a modest increase in solvent accessibility for residues in helices $\alpha 7$, $\alpha 8$ and partially $\alpha 9$ and $\alpha 2$ that are found at the C-terminal surface of BAX. Binding of the 3G11 Fab to full-length BAX (BAX WT) and to the C-terminal helix $\alpha 9$ -truncated BAX (BAX ΔC) was determined with similar dissociation constant K_D (Table 1), suggesting that major contacts of 3G11 occur elsewhere from the $\alpha 9$ or the canonical hydrophobic groove of BAX.

[0054] The HXMS analysis suggests that the binding region of the 3G11 is localized on the N-terminal surface of the BAX structure and overlaps with the 1) N-terminal trigger site of BAX ($\alpha 1/\alpha 6$) that controls a series of conformational changes upon BH3 domain activation (11-12) and 2) the binding epitope (residues 12-24) that is recognized by the 6A7 antibody only on the conformational active BAX (Fig. 4). Interestingly, the $\alpha 1$ - $\alpha 2$ loop, whose displacement was determined to be essential for the initiation of conformational changes upon BH3-triggered BAX activation (12), is protected by the Fab binding interaction. Thus, the binding interaction of 3G11 to BAX is localized to the N-terminal surface of BAX, overlapping with the N-terminal trigger site and preventing conformational changes that lead to activation of the monomeric BAX.

[0055] Although recent structural studies based on peptides from the cytomegalovirus protein vMIA (38) and BCL-2 (20) proteins suggested potential sites that intervene with BAX activation, interestingly, none of these peptides directly block the N-terminal activation site of the soluble BAX or the C-terminal activation site of the mitochondrial associated BAX. A protein-protein structure calculation approach (39) was used to further confirm this novel interaction of 3G11 bound to the N-terminal surface of BAX. HADDOCK structure calculations were performed using ambiguous interaction restraints between BAX residues determined by HXMS to be most protected from solvent upon 3G11 binding (residues of the $\alpha 1$, $\alpha 6$ and the $\alpha 1$ - $\alpha 2$ loop) and residues of the CDR regions present in the 3G11 protein sequence. Furthermore, calculations included an NMR structural ensemble of BAX with different loop conformations. Based on these calculations, the 3G11 binds more favorably in a direct interaction with N-terminal trigger site of BAX and the closed conformation of $\alpha 1$ - $\alpha 2$ loop (10,12). 3G11 protein makes interactions of hydrophobic and hydrophilic nature with the solvent-exposed hydrophobic residues and polar/charged residues of the BAX trigger site covering a large interface surface of (1640Å).

[0056] To validate the 3G11 direct interaction with the N-terminal trigger site, it was tested whether mutations on BAX can disrupt binding to 3G11 and affect its inhibitory activity of 3G11 in liposomal ANTS/DPX release assays. The 3G11-BAX structural model was used. Residues on BAX were selected that form contacts with residues of 3G11 using a 3Å cutoff. Residues K21 in helix $\alpha 1$ and R134 in helix $\alpha 6$ have previously been shown to interact with the stapled BIM BH3 peptide that activates BAX through the N-terminal trigger site (10). Further analysis of the structural model showed that R134 residue forms hydrogen bonds with four residues (S30, Y31, Y32, S33) in 3G11 CDR-H1 loop while the K21 residue is predicted to form one hydrogen bond interaction with residue S143 in 3G11. Consistently with the predicted contributions of each residue to the interaction with 3G11 Fab, liposomal ANTS/DPX release experiments showed that the inhibition effect of 3G11 on BAX WT activation by tBID is weakened with the presence of the K21E mutation, but it is was completely abolished with the R134E mutation (Fig. 5A-5E). Likewise, the double mutation R134E/K21E also abolished the capacity of 3G11 to inhibit BAX activation (Fig. 5D, 5E). The inability of 3G11 to inhibit activation of these BAX mutants is consistent with the decreased affinity of 3G11 to these BAX mutants as determined by ELISA binding experiments (Fig. 5F). Thus, the data suggest that 3G11 binds and blocks access to the N-terminal trigger site of BAX and therefore directly prevents BAX activation and its associated conformational changes. In accordance, mapping the predicted BAX interacting

residues based on the 3G11-BAX structural model and residues of the trigger site that interact with the stapled BIM BH3 peptide as determined by NMR studies demonstrates an extensive overlap (10). Taken together, these results suggest a novel mechanism of BAX inhibition and demonstrate the capacity of the reported Fabs as structural and functional probes of BAX.

Discussion

[0057] Decision of cellular life or death through the mitochondrial apoptotic pathway, in physiological or disease conditions, is mainly controlled by the interactions among the BCLL-2 family proteins. Activation of pro-apoptotic BAX is essential for apoptosis to proceed through mitochondrial dysfunction and caspases activation. Here, synthetic antibody technology was harnessed to generate high affinity BAX binding antibody fragments (Fabs). The screen identified at least 14 different Fabs with sequence diversity in CDR regions that bind with nM affinity to BAX. Interestingly, the competitive ELISAs confirmed that the identified Fab proteins bind to overlapping regions of the BAX surface. Selected Fabs bind full length BAX, which represents cytosolic BAX (9). It was demonstrated that 3G11 binds C-terminal truncated BAX, which mimics the mitochondrial-associated BAX (15). Mitochondrial inserted BAX oligomerizes and undergoes dramatic conformational changes of the monomeric BAX to such extent that the N-terminal surface of BAX is not available for binding to Fabs (13,15,16,40). Therefore, selected Fabs are expected to be conformational specific for cytosolic and mitochondrial-associated BAX and inhibit both conformations from proceeding along the BAX activation process.

[0058] All of the identified BAX-binding Fabs inhibit BAX activation triggered by pro-apoptotic tBID in liposomal and mitochondrial assays. Further investigation of the mechanism, using Fab 3G11 protein, indicated that the Fabs bind to the N-terminal surface of BAX without causing significant conformational changes on BAX. HADDOCK calculations and mutagenesis show that 3G11 binds to an extended surface on BAX that includes the N-terminal trigger site (helices $\alpha 1/\alpha 6$), which BIM, BID, and PUMA pro-apoptotic BH3 helices bind to trigger BAX activation (10,11,36,38,41). The data indicate that 3G11 competitively inhibits tBID-mediated BAX activation by blocking access to the N-terminal trigger site and preventing N-terminal conformational changes associated with BAX activation (11,36). Indeed, 3G11-binding prevents mitochondrial translocation of BAX, which requires significant conformational changes and integration into the membrane (13,15,16,40).

[0059] BAX is shown to have two different activation sites depending on its cytosolic or mitochondrial associated conformations; the N-terminal BH3 pocket (trigger site) and the C-

terminal BH3 pocket. Although several proteins have been reported to directly bind BAX and inhibit its activation, only two other studies reported structural evidence of the binding interaction on the surface of BAX. A stapled helical peptide of the BH4 domain of BCL-2 protein binds to a cleft formed by select residues of the $\alpha 1$ - $\alpha 2$ loop, $\alpha 2$, $\alpha 3$, $\alpha 5$ and $\alpha 6$ (20). A helical peptide of the cytomegalovirus protein vMIA binds to a distinct site at the same side of the BAX structure that includes the loops of $\alpha 3$ - $\alpha 4$ and $\alpha 5$ - $\alpha 6$ (38). In both cases, the peptides bind to a geographically distinct site that have no overlap with either of the N-terminal or C-terminal activation site. Therefore, these mechanisms of BAX inhibition reflect allosteric mechanisms that suppress conformational changes upon BAX activation. The present data indicate that the direct inhibition mechanism of BAX by 3G11 Fab through interaction with the N-terminal activation site is a feasible and effective mechanism of BAX inhibition.

[0060] Pharmacological targeting of BAX whether to either promote or inhibit its activation has been proposed to be a promising therapeutic strategy. However, discovery of pharmacological modulators of BAX has been challenging due to limited insights or lack of appropriate probes to use for small molecule discovery. Recently, small molecule BAX activators that bind to the BAX trigger site have been identified using a competitive binding assay of the stapled BIM BH3 peptide activator that binds to the same site (42). Likewise, the application of synthetic antibodies to BAX provides a novel opportunity to use Fabs as probes for screening small molecule libraries using a competitive binding assay between the identified inhibitory Fabs and BAX. Such small molecule inhibitors that bind directly to the BAX trigger site will be effective for development of therapeutics.

Table 1. Binding kinetics and thermodynamics of Fab 3G11 with BAX WT and BAX ΔC .

BAX	k_{on} ($M^{-1} s^{-1}$)	k_{off} (s^{-1})	K_D (nM)
BAX WT	$(3.0 \pm 1.5) \times 10^5$	$(1.1 \pm 0.1) \times 10^{-2}$	38 ± 12
BAX ΔC	$(2.9 \pm 0.5) \times 10^5$	$(1.8 \pm 0.1) \times 10^{-2}$	65 ± 13

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What is claimed is:

1. A synthetic fragment antigen-binding (Fab) antibody that binds to an N-terminal activation site of BCL-2-associated X-protein (BAX) and inhibits BAX activation.
2. The synthetic Fab antibody of claim 1, wherein the antibody maintains BAX in its inactive, monomeric form.
3. The synthetic Fab antibody of claim 1 or 2, wherein the antibody binds to residues of helices $\alpha 1$ and $\alpha 6$, and to residues of $\alpha 1$ - $\alpha 2$ loop of BAX.
4. The synthetic Fab antibody of any of claims 1-3, wherein the antibody binds to BAX with a half-maximal binding (EC_{50}) affinity of 2nM-70nM.
5. The synthetic Fab antibody of any of claims 1-4, wherein the antibody blocks the interaction of BAX with a BAX activating partner.
6. The synthetic Fab antibody of claim 5, wherein the BAX activating partner is selected from the group consisting of tBID, PUMA, BIM and NOXA.
7. The synthetic Fab antibody of any of claims 1-6, wherein the antibody is selected from the group consisting of
2B1 Fab, having a CDRL3 region comprising the amino acid sequence QYSGSGHYLI (SEQ ID NO:1), a CDRH1 region comprising the amino sequence IYSSSM (SEQ ID NO:2), a CDRH2 region comprising the amino acid sequence SISSSSSYTS (SEQ ID NO:3) and a CDRH3 region comprising the amino acid sequence RGYWYYWAWWASAMD (SEQ ID NO:4);
3E8 Fab, having a CDRL3 region comprising the amino acid sequence QSSYSLI (SEQ ID NO:5), a CDRH1 region comprising the amino sequence LSYYSM (SEQ ID NO:6), a CDRH2 region comprising the amino acid sequence SISPYYGITY (SEQ ID NO:7) and a CDRH3 region comprising the amino acid sequence RGGAYYFGYYGSGSYAMD (SEQ ID NO:8);
3G11 Fab, having a CDRL3 region comprising the amino acid sequence QWSFGPI (SEQ ID NO:9), a CDRH1 region comprising the amino sequence ISYYSM (SEQ ID NO:10).

NO:10), a CDRH2 region comprising the amino acid sequence SIYPYSSSTY (SEQ ID NO:11) and a CDRH3 region comprising the amino acid sequence RSSAMD (SEQ ID NO:12);

2D9 Fab, having a CDRL3 region comprising the amino acid sequence QWSHYLI (SEQ ID NO:13), a CDRH1 region comprising the amino sequence LYYYSM (SEQ ID NO:14), a CDRH2 region comprising the amino acid sequence SISPSYGYTS (SEQ ID NO:15) and a CDRH3 region comprising the amino acid sequence RSSFYYYALD (SEQ ID NO:16);

3G9 Fab, having a CDRL3 region comprising the amino acid sequence QHYYYSPWPI (SEQ ID NO:17), a CDRH1 region comprising the amino sequence LYSYYI (SEQ ID NO:18), a CDRH2 region comprising the amino acid sequence SISPYYSSTY (SEQ ID NO:19) and a CDRH3 region comprising the amino acid sequence RSSYSYAGMD (SEQ ID NO:20);

2C11 Fab, having a CDRL3 region comprising the amino acid sequence QSYVSPI (SEQ ID NO:21), a CDRH1 region comprising the amino sequence ISSYYI (SEQ ID NO:22), a CDRH2 region comprising the amino acid sequence SISSYYSSTY (SEQ ID NO:23) and a CDRH3 region comprising the amino acid sequence RVSYGHAYVGYSSGMD (SEQ ID NO:24);

3H4 Fab, having a CDRL3 region comprising the amino acid sequence QSWYYSYPI (SEQ ID NO:25), a CDRH1 region comprising the amino sequence LSYSSM (SEQ ID NO:26), a CDRH2 region comprising the amino acid sequence SISSYYSYTS (SEQ ID NO:27) and a CDRH3 region comprising the amino acid sequence RYYGYGGGID (SEQ ID NO:28);

2A6 Fab, having a CDRL3 region comprising the amino acid sequence QSAGGYPLI (SEQ ID NO:29), a CDRH1 region comprising the amino sequence IYYSSM (SEQ ID NO:30), a CDRH2 region comprising the amino acid sequence SISPYSSYTS (SEQ ID NO:31) and a CDRH3 region comprising the amino acid sequence RSFGYGWAFD (SEQ ID NO:32);

3H1 Fab, having a CDRL3 region comprising the amino acid sequence QHSYPI (SEQ ID NO:33), a CDRH1 region comprising the amino sequence ISYSSI (SEQ ID NO:34), a CDRH2 region comprising the amino acid sequence SIYSYSGSTY (SEQ ID NO:35) and a CDRH3 region comprising the amino acid sequence RYGAMD (SEQ ID NO:36);

2A2 Fab, having a CDRL3 region comprising the amino acid sequence QYYYYPI (SEQ ID NO:37), a CDRH1 region comprising the amino sequence ISSSSI (SEQ ID NO:38), a CDRH2 region comprising the amino acid sequence SIYSYGYTY (SEQ ID NO:39) and a CDRH3 region comprising the amino acid sequence RYSAMD (SEQ ID NO:40);

3G3 Fab, having a CDRL3 region comprising the amino acid sequence QGAWSGGHLI (SEQ ID NO:41), a CDRH1 region comprising the amino sequence LSYSSM (SEQ ID NO:42), a CDRH2 region comprising the amino acid sequence YISPYYGYTY (SEQ ID NO:43) and a CDRH3 region comprising the amino acid sequence RGWAYYYGYWGPSGLD (SEQ ID NO:44);

2D5 Fab, having a CDRL3 region comprising the amino acid sequence QWGYSHSLI (SEQ ID NO:45), a CDRH1 region comprising the amino sequence ISYSS (SEQ ID NO:46), a CDRH2 region comprising the amino acid sequence SISPYYGSTY (SEQ ID NO:47) and a CDRH3 region comprising the amino acid sequence RSHFGALD (SEQ ID NO:48);

2D2 Fab, having a CDRL3 region comprising the amino acid sequence QSYWVSPF (SEQ ID NO:49), a CDRH1 region comprising the amino sequence LYYSSI (SEQ ID NO:50), a CDRH2 region comprising the amino acid sequence SIYPYSGSTY (SEQ ID NO:51) and a CDRH3 region comprising the amino acid sequence RSYGYAMD (SEQ ID NO:52); and

2A5 Fab, having a CDRL3 region comprising the amino acid sequence QYHYWYYPI (SEQ ID NO:53), a CDRH1 region comprising the amino sequence ISYSSM (SEQ ID NO:54), a CDRH2 region comprising the amino acid sequence SISPYYGSTY (SEQ ID NO:55) and a CDRH3 region comprising the amino acid sequence RAGAMD (SEQ ID NO:56).

8. A method of inhibiting apoptotic cell death by contacting BCL-2-associated X-protein (BAX) with the synthetic Fab antibody of any of claims 1-7 in an amount effective to inhibit activation of BAX and apoptotic cell death.

9. The method of claim 8, wherein the synthetic Fab antibody is conjugated to a therapeutic agent.

10. The method of claim 8 or 9, wherein the synthetic Fab antibody is conjugated to an agent that facilitates transport across a cell membrane.

11. The method of any of claims 8-10, wherein the synthetic Fab antibody is administered to a subject using lentiviral or adenoviral expression, a plasmid, nanoparticles or liposomes.
12. The method of any of claims 8-10, wherein the synthetic Fab antibody is administered to a subject having a disease or disorder selected from the group consisting of a cardiovascular disease or disorder, a neurodegenerative or neurological disease or disorder, a liver disease or disorder, a kidney disease or disorder, a metabolic disease or disorder, an immunological disorder, ischemia, infertility, a blood disorder, renal hypoxia, diabetes, hepatitis, asthma and AIDS.
13. A method for measuring inactive monomeric BAX levels in a tissue sample from a subject comprising contacting the sample with the synthetic Fab antibody of any of claims 1-7 and measuring the amount of antibody bound to the sample, wherein the amount of bound antibody is indicative of the level of inactive monomeric BAX in the tissue sample.
14. A method for screening for small molecules that bind to an N-terminal activation site of BAX comprising contacting BAX with the synthetic Fab antibody of any of claims 1-7 in the presence and in the absence of a small molecule and measuring the amount of antibody that binds to BAX, wherein a decrease in the amount of antibody binding to BAX in the presence of the small molecule indicates that the small molecule is a candidate for binding to an N-terminal activation site of BAX.
15. The method of claim 14, wherein the method is a competitive ELISA screening assay.
16. A method of predicting the ability of a cancer therapy to promote apoptotic cell death comprising contacting a cancerous tissue sample with the synthetic Fab antibody of any of claims 1-7, with and without the cancer therapy, and measuring the amount of antibody that binds to the tissue sample, wherein a decrease in the amount of bound antibody with the cancer therapy indicates that the cancer therapy promotes activation of BAX and apoptotic cell death, and the amount of bound antibody without the cancer therapy can predict the capacity of the cancer therapy to promote activation of BAX and apoptotic cell death in the cancerous tissue.

17. The method of any of claims 13-16, wherein the synthetic Fab antibody is labeled with a fluorescent label or a radioactive label.

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Clone	EC ₅₀ (nM)	Sequences			
		CDRL3	CDRH1	CDRH2	CDRH3
<i>Kabat numbering</i>		90....	.30...	50..a.55..	94
2B1	2.3	QYSGSGHYLI	IYSSSM	SISSSSSYTS	RGYWYYWAWWASAMD
3E8	2.6	QSSYSLI	LSYYSM	SISPYYGTY	RGGAYYFGYYGSGSYAMD
3G11	5.5	QWSFGPI	ISYYSM	SIYPYSSSTY	RSSAMD
2D9	7.6	QWSHYLI	LYYYSM	SISPSYGYTS	RSSFYYYALD
3G9	9.5	QHYYSPWPI	LYSYYI	SISPYSSSTY	RSSYSYAGMD
2C11	10	QSYVSP	ISSYYI	SISYYSSSTY	RVSYGHAYVGYSSGMD
3H4	11	QSWYYSYPI	LSYSSM	SISYYSYTS	RYGYGGGID
2A6	13	QSAGGYPLI	IYYSSM	SISPYSSYTS	RSFGYGWAFD
3H1	19	QHSYPI	ISYSSI	SIYSYSGSTY	RYGAMD
2A2	24	QYYYPI	ISSSSI	SIYSYGYTY	RYSAMD
3G3	26	QGAWSGGHLI	LSYSSM	YISPYYGTY	RGWAYYYGYWGSPGLD
2D5	46	QWGYSHSHLI	ISYSSI	SISPYYGSTY	RSHFGALD
2D2	66	QSYWVSPF	LYYSSI	SIYPYSGSTY	RSYGYAMD
2A5	250	QYHYWYPI	ISYYSM	SISPYYGSTY	RAGAMD

FIG. 1A

Fab	BLITZ			ELISA
	k_a (M ⁻¹ s ⁻¹)	k_d (s ⁻¹)	K_D (nM)	EC ₅₀ (nM)
2B1	2.5×10^5	2.6×10^{-3}	10	2.3
3E8	2.9×10^5	7.5×10^{-3}	26	2.6
3G11	3.0×10^5	1.1×10^{-2}	38	5.5
2A5	5.4×10^4	9.1×10^{-3}	169	250

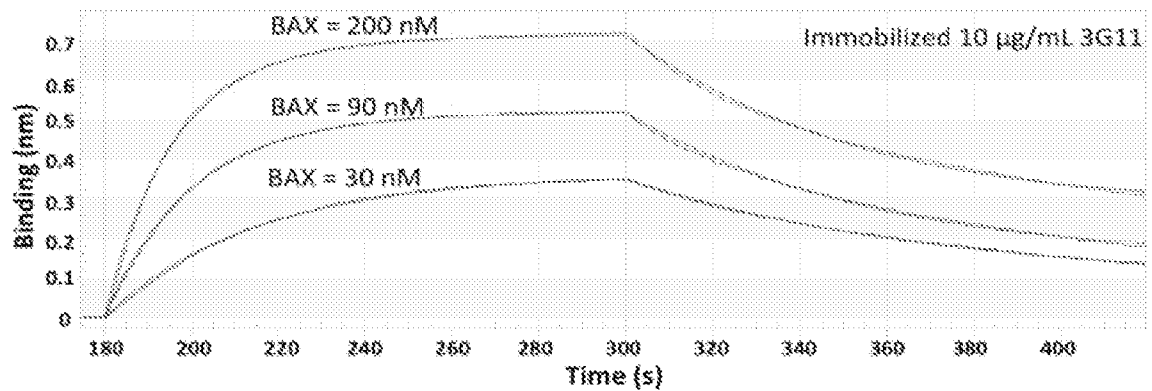


FIG. 1B

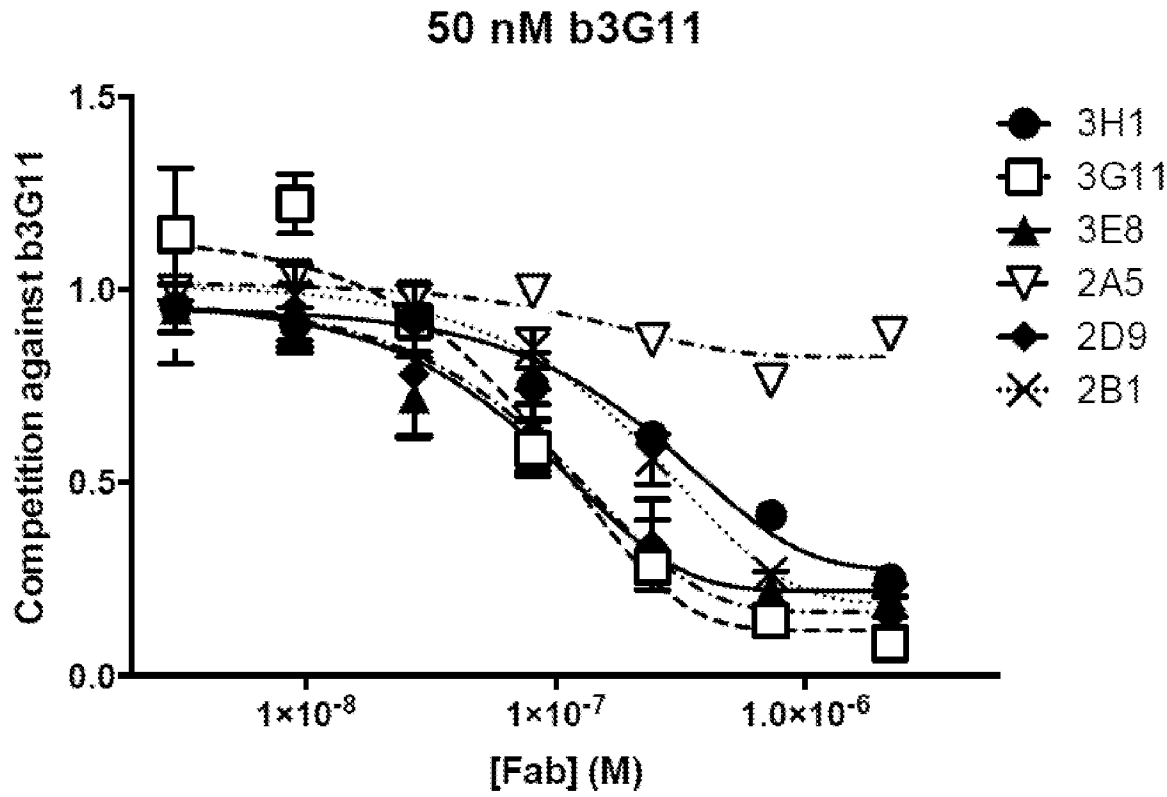


FIG. 1C

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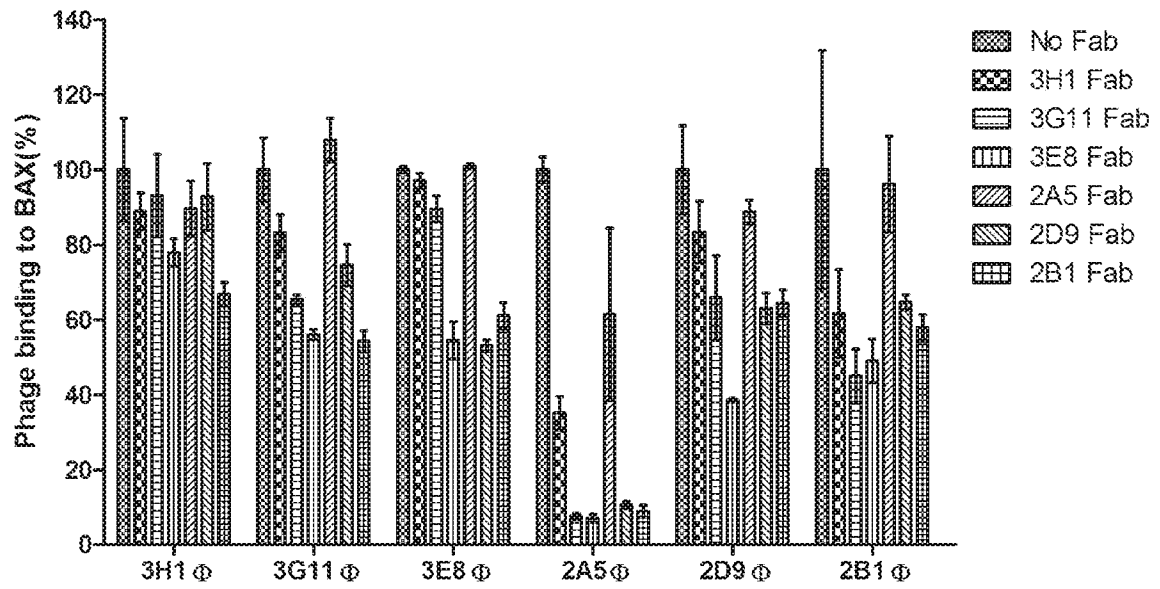


FIG. 1D

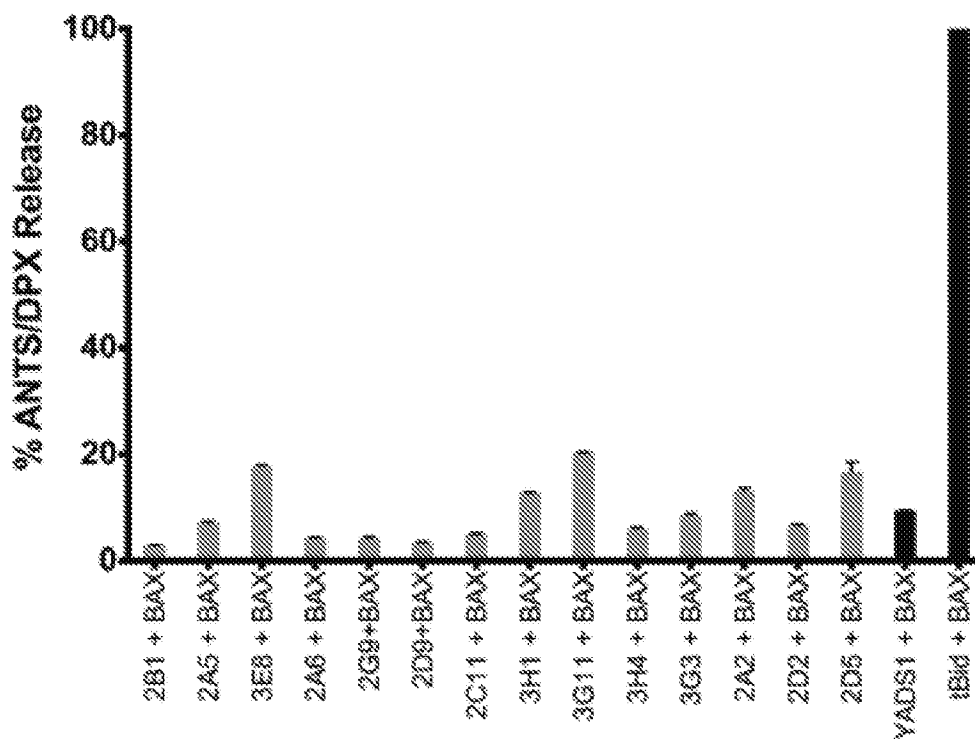


FIG. 2A

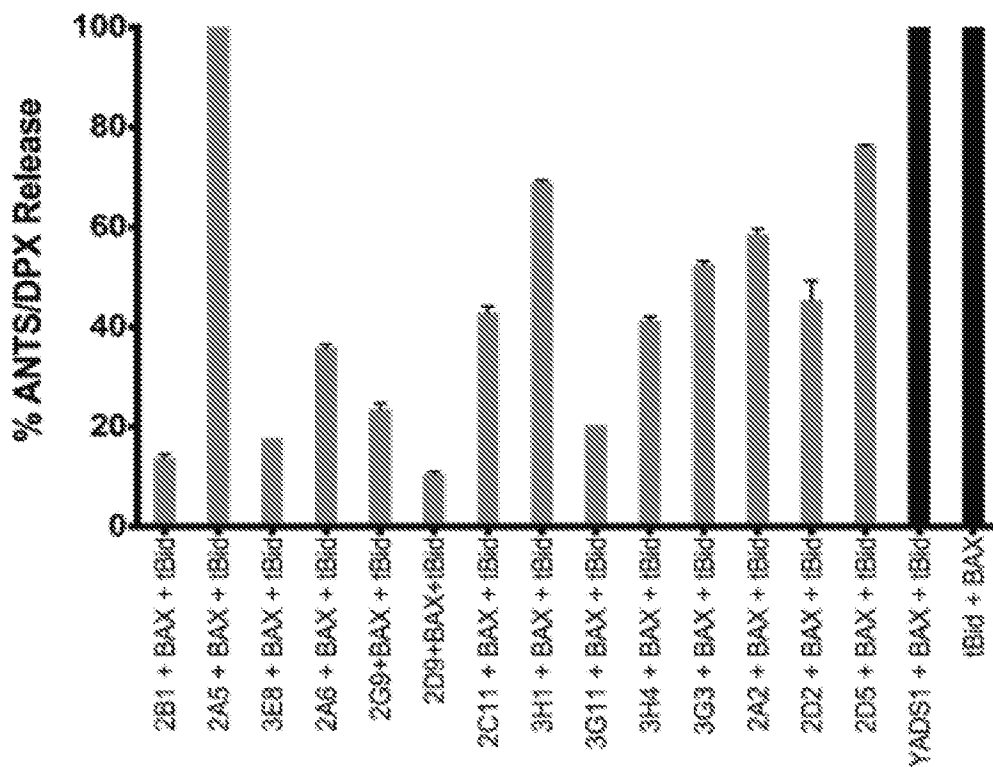


FIG. 2B

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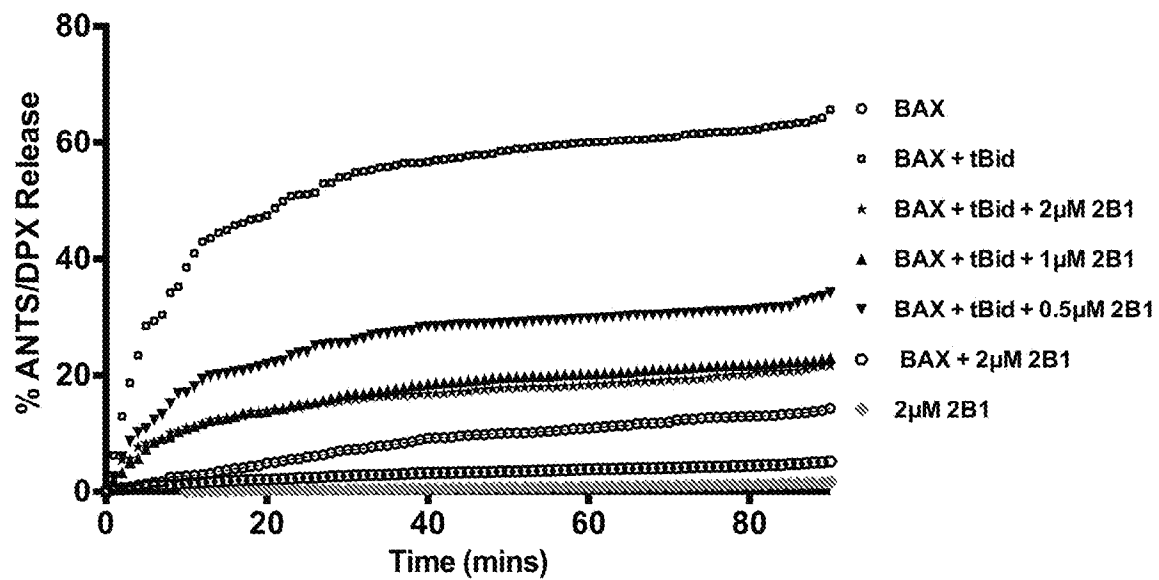


FIG. 2C

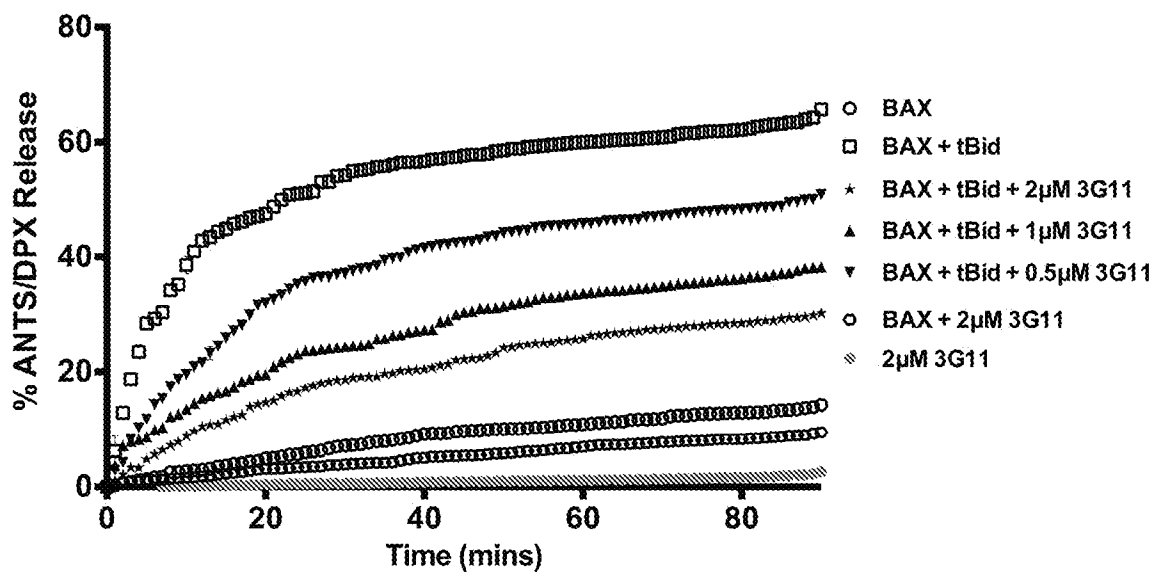


FIG. 2D

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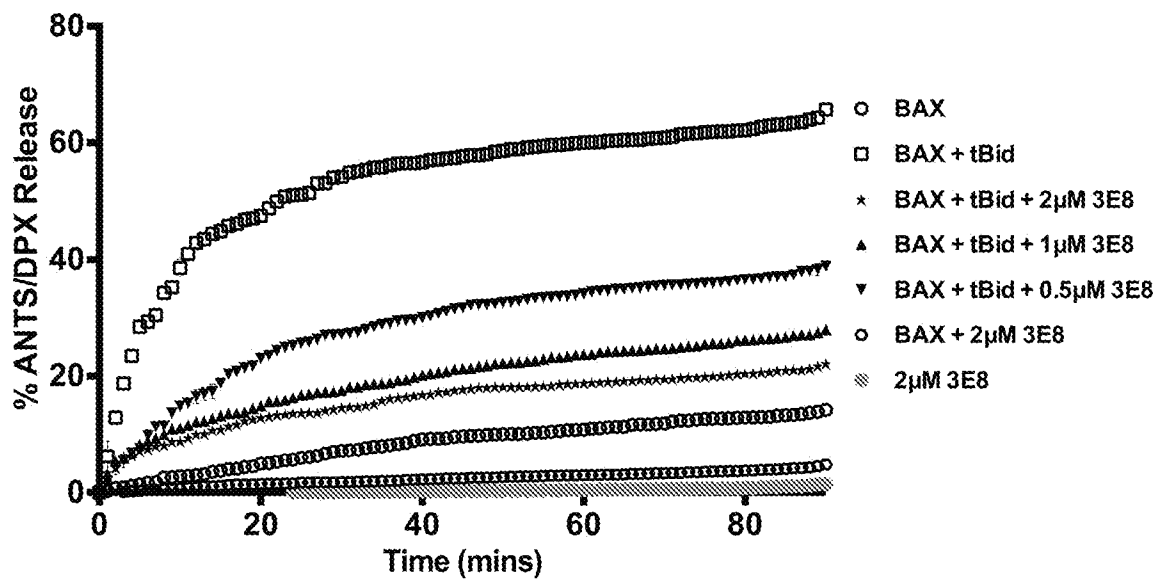


FIG. 2E

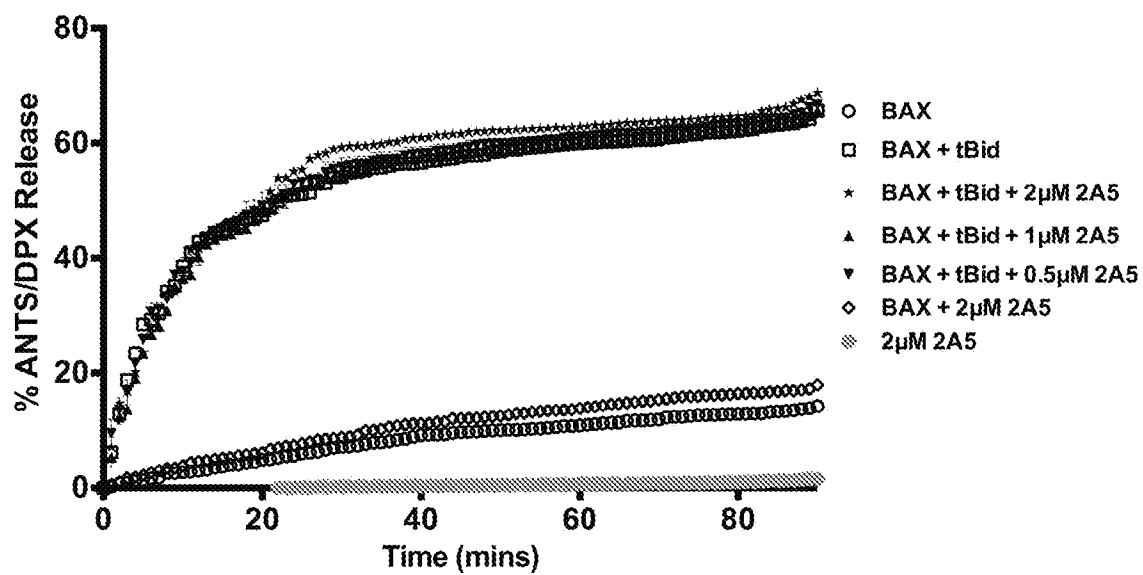


FIG. 2F

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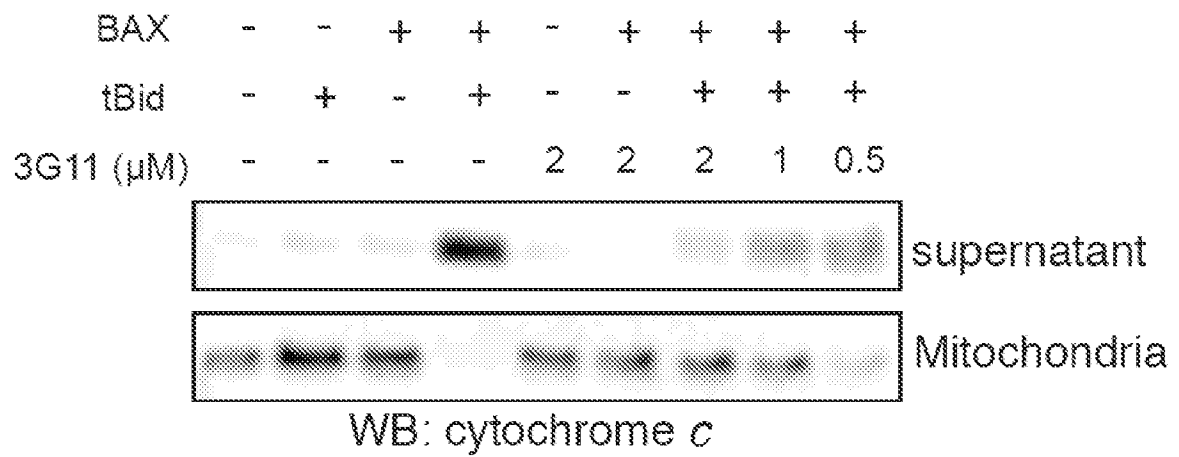
cytochrome *c* release

FIG. 3A

BAX translocation

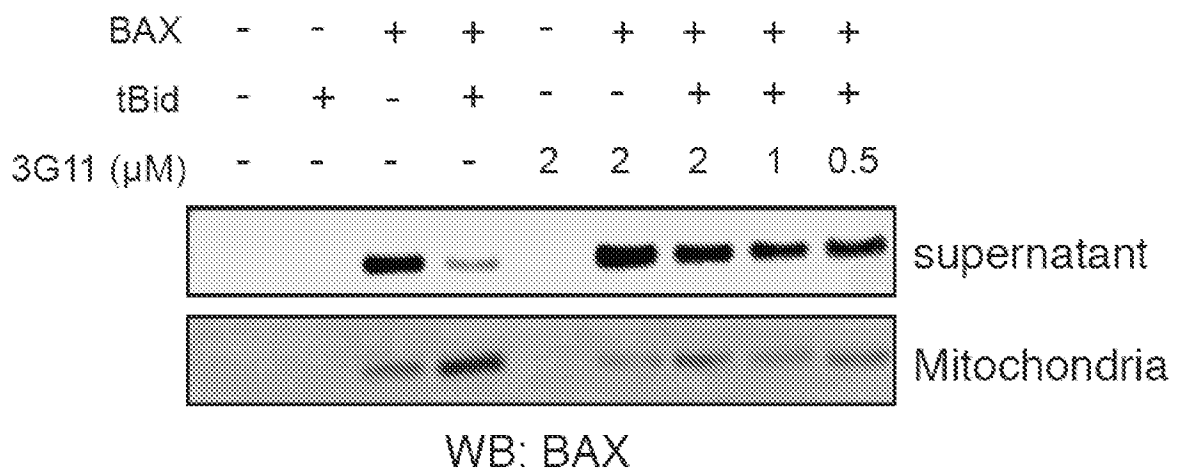


FIG. 3B

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1                               α1                α1-α2 loop
MDGSGEQPRG GGPTSSSEQIM KTGALLLQGF IQDRAGRMGG EAFELALDPV
51          α2                α3                α4
PQDASTKKLS ECLKRIGDEL DSNMELQRM IAAVITDSPRE VFFRVAADMF
101         α5                α6                α7
SDGNFNWGRV VALFYFASKL VLKALCTKVP ELIRTINGWT LDFLNERLLG
151         α8                α9
WIQLQGGWDG LLSYFGTPTW QTVTIFVAGV LTASLTIWKK MG

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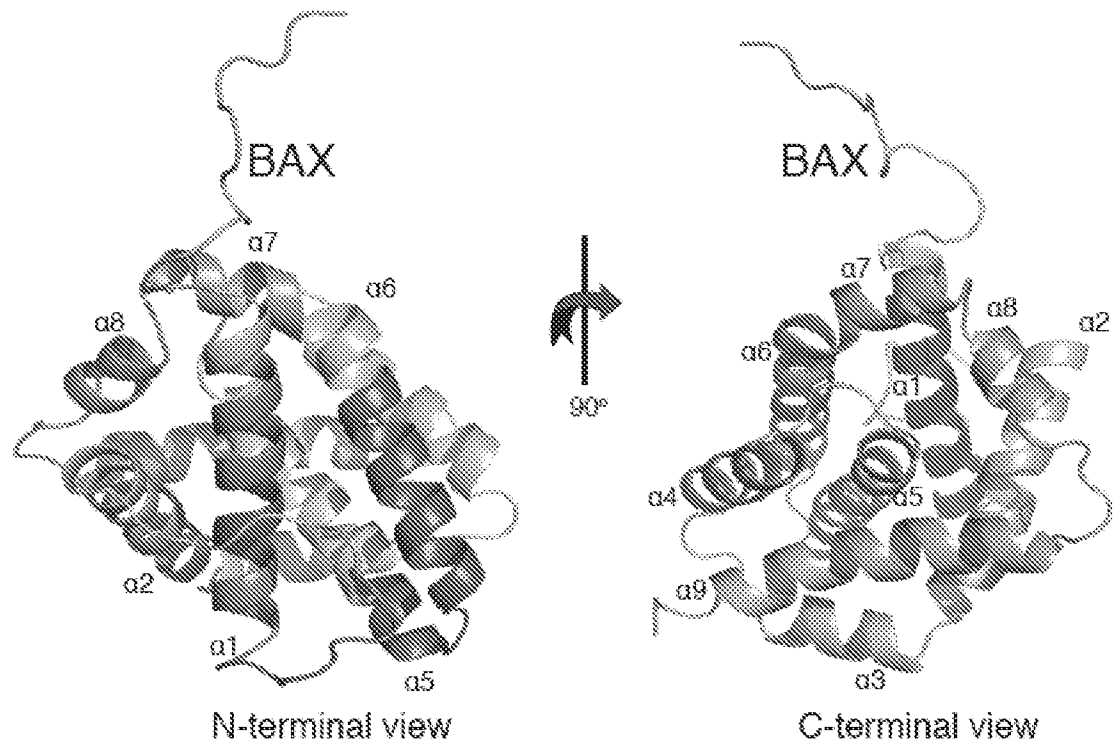


FIG. 4

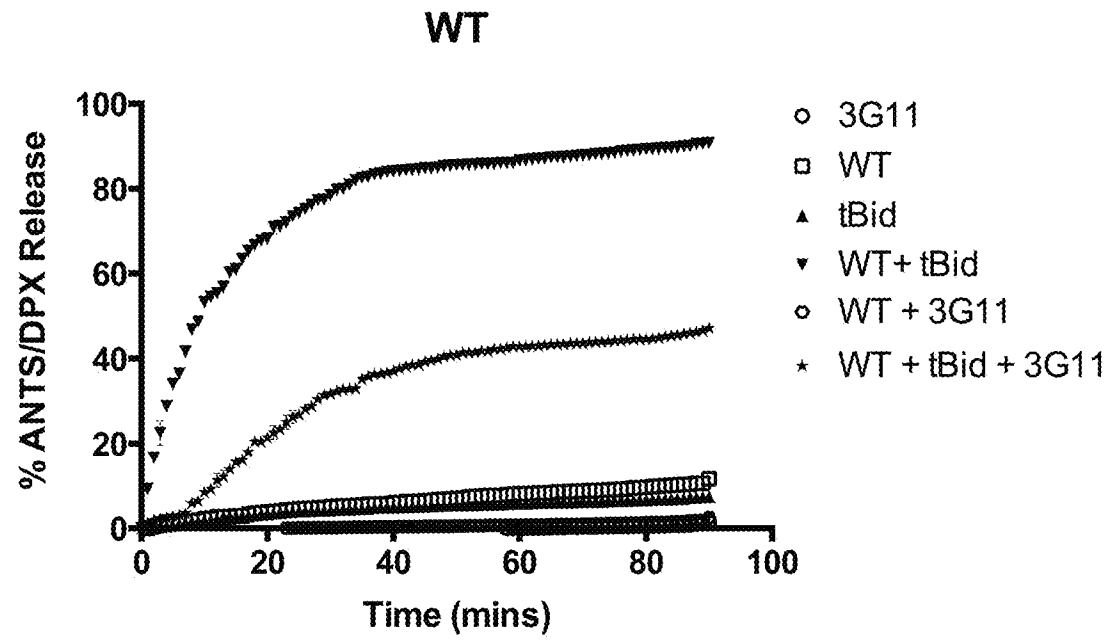


FIG. 5A

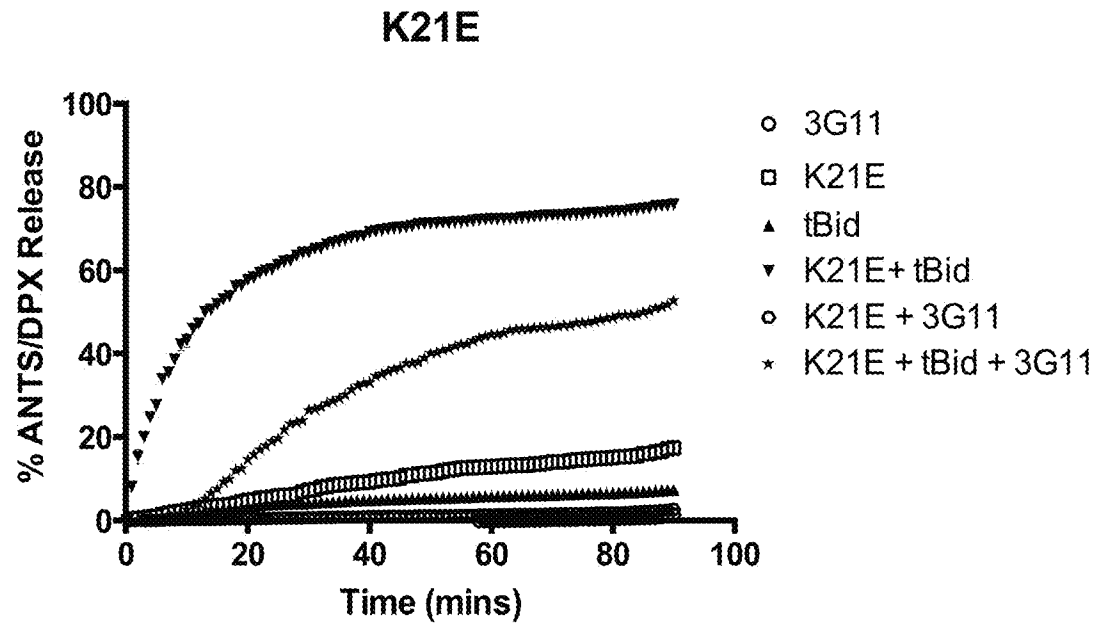


FIG. 5B

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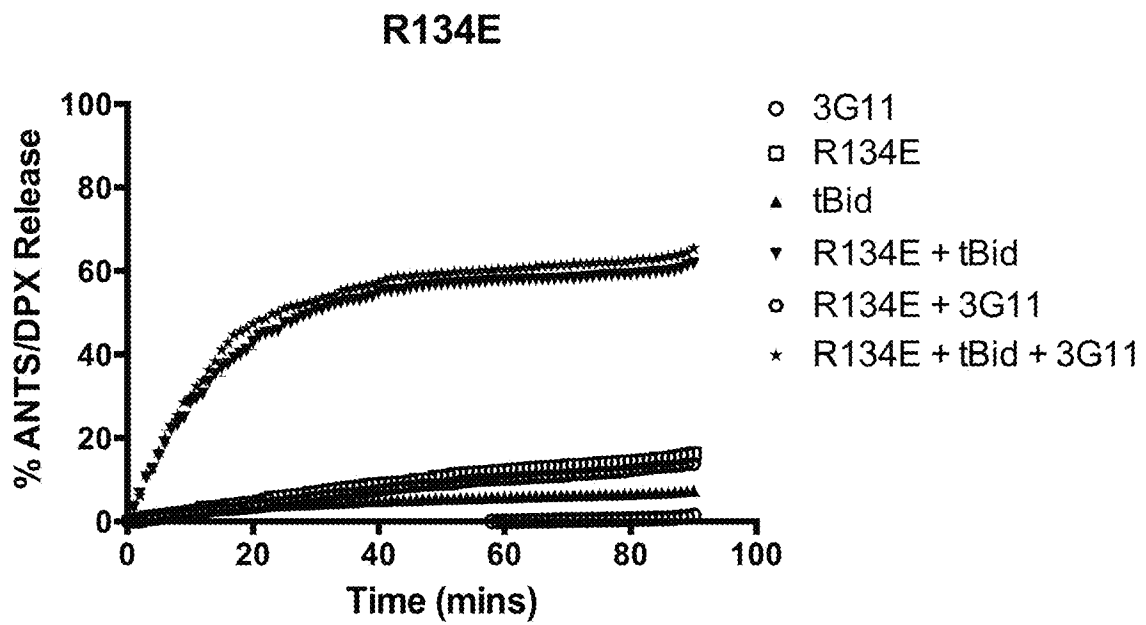


FIG. 5C

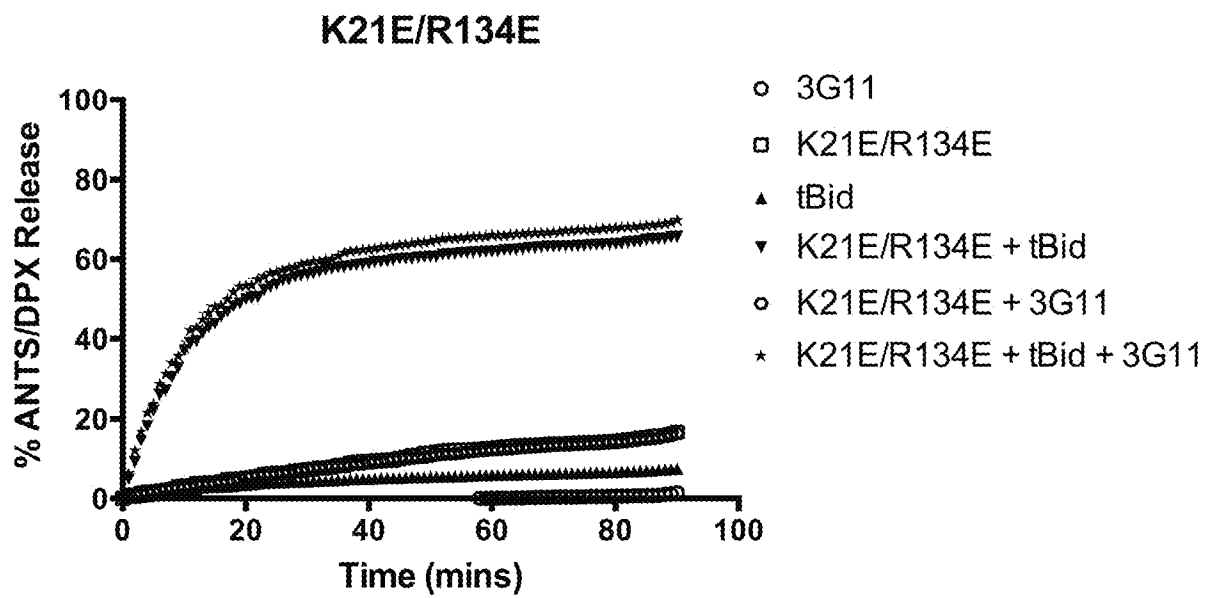


FIG. 5D

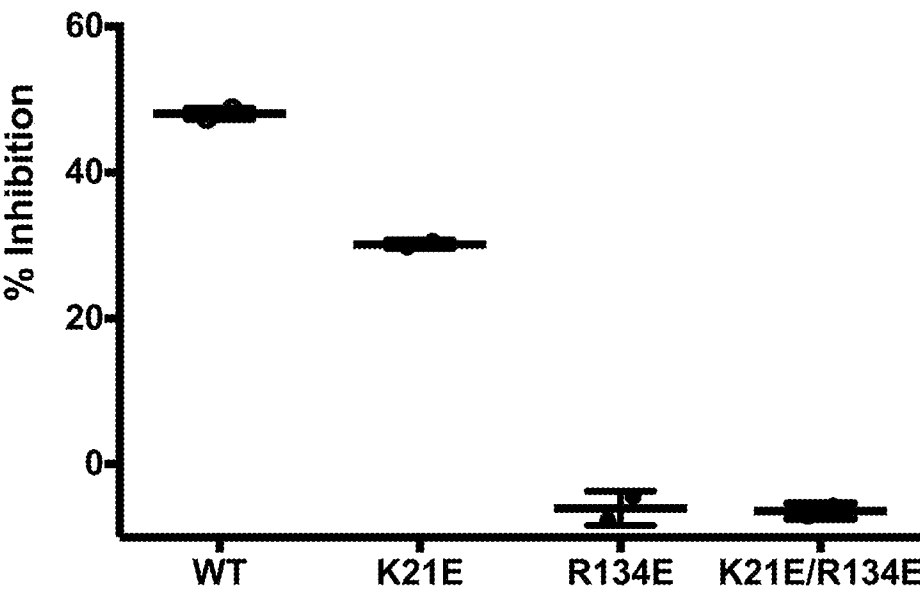


FIG. 5E

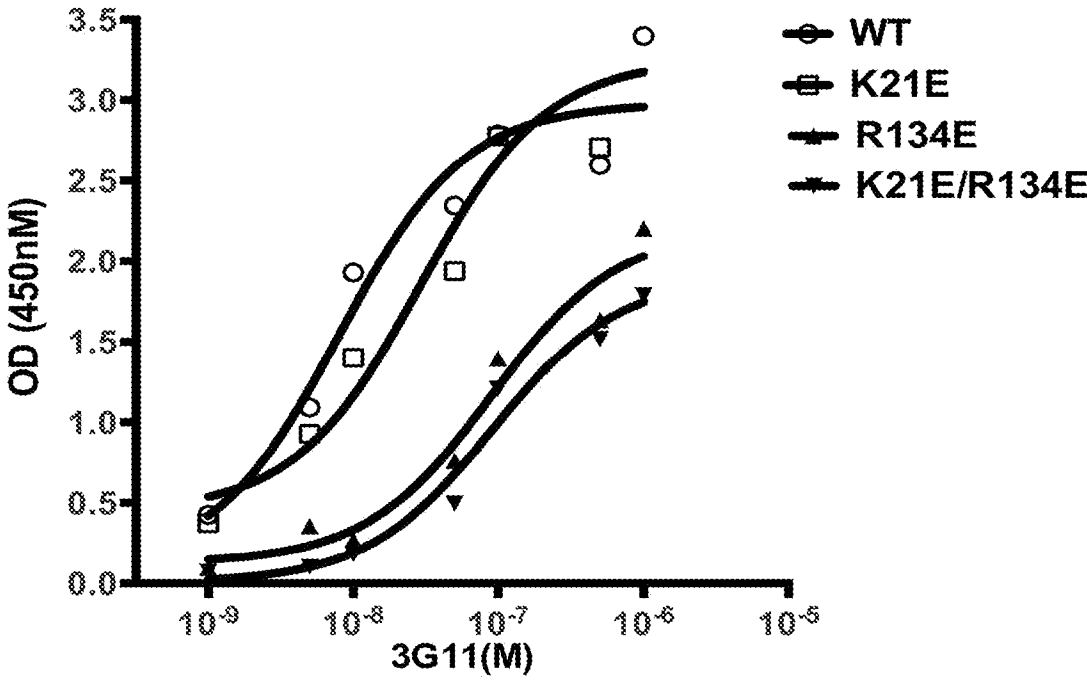


FIG. 5F

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US16/48508

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C07K 14/47, 16/00; A61K 38/17, 39/395; G01N 33/53 (2016.1)

CPC - C07K 14/47, 16/00; A61K 38/17, 39/395; G01N 33/53

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC: C07K 14/47, 16/00; A61K 38/17, 39/395; G01N 33/53 (2016.1)

CPC: C07K 14/47, 16/00; A61K 38/17, 39/395; G01N 33/53

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PatSeer (US, EP, WO, JP, DE, GB, CN, FR, KR, ES, AU, IN, CA, INPADOC Data); Google Scholar; EBSCO; PubMed

Terms: BAX, Fab, fragment, antibody, N-terminal, inhibit, synthetic, antigen, binding, domain, activation, monomer, loop, helices, protein

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X — Y	US 8,361,462 B2 (PANDEY, S. et al.) 29 January, 2013; column 5, lines 42-46; column 6, lines 3-7; column 7, lines 30-35; column 10, lines 37-38	1-2 — 3/1-2
Y	(GAVATHIOTIS, E et al.) BH3-Triggered Structural Reorganization Drives the Activation of Proapoptotic BAX. Molecular Cell. 12 November 2010, Vol. 40, No. 3; pages 481-492; summary; 482, 1st column, 2nd paragraph; page 482, 2nd column, 1st paragraph; page 486, 1st column, 2nd paragraph; page 490, 2nd column, 3rd paragraph; DOI: 10.1016/j.molcel.2010.10.019	3/1-2
P, X	(UCHIME, O et al.) Synthetic Antibodies Inhibit Bcl-2-associated X Protein (BAX) through Blockade of the N-terminal Activation Site. Journal of Biological Chemistry. 12 November 2015, Vol. 291, No. 1; pages 89-102; DOI: 10.1074/jbc.M115.680918	1-2, 3/1-2

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

19 October 2016 (19.10.2016)

Date of mailing of the international search report

15 NOV 2016

Name and mailing address of the ISA/US

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Facsimile No. 571-273-8300

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Shane Thomas

PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US16/48508

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 4-17
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.