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(54) Title: INHIBITING B-CELL LYMPHOMA 2 (BCL-2) AND RELATED PROTEINS

(57) Abstract: Novel compounds inhibiting anti-apoptosis proteins B-cell lymphoma 2 (Bcl-2) and Bcl-XL include compounds of formula (I) and formula (II) disclosed herein, as well as liposome compositions comprising Bcl-2 inhibitor compounds. These compositions are useful for the treatment of cancer.

Inhibiting B-cell Lymphoma 2 (Bcl-2) and Related Proteins

Related Applications

[0001] This application claims priority to U.S. Provisional Patent Application No. 62/277,248, filed January 11, 2016, and U.S. Provisional Patent Application No. 62/444,168, filed January 9, 2017, both of which are incorporated by reference into the present application in their entirety and for all purposes.

Field

[0002] This disclosure relates to compounds and related methods of inhibiting B-cell lymphoma 2 (Bcl-2) and related proteins, including methods and compounds useful for the treatment of cancer.

Background

[0003] Apoptosis is recognized as an essential biological process for tissue homeostasis of all living species. In mammals in particular, it has been shown to regulate early embryonic development. Later in life, cell death is a default mechanism by which potentially dangerous cells (e.g., cells carrying cancerous defects) are removed. Several apoptotic pathways have been uncovered, and one of the most important involves the B-cell lymphoma 2 (Bcl-2) family of proteins, which are key regulators of the mitochondrial (also called "intrinsic") pathway of apoptosis. See, Danial, N. N. and Korsmeyer, S. J. Cell (2004) 116, 205-219. Targeted cancer therapy research has been reported against members of the Bcl-2 protein family, which are central regulators of programmed cell death. For example, the interaction of pro-apoptosis proteins (Bax, Bad, Bid, Bim, Bik, Puma, Noxa, etc.) with anti-apoptosis proteins (Bcl-2 family: Bcl-2, Bcl-X_L, Mcl-1, etc.) is believed to play a role in cell survival and death.

[0004] It has been believed that Bcl-2 is an excellent target for hematological cancers while Bcl-X_L is a target for solid tumors. It was also speculated that observed thrombocytopenia was caused by inhibiting Bcl-X_L (Cell, 2007, 128, 1173-1186). The Bcl-2 family members that inhibit apoptosis are over-expressed in cancers and contribute to tumorigenesis. Bcl-2 expression has been strongly correlated with resistance to cancer therapy and decreased survival. The Bcl-2 family of proteins can be further classified into three subfamilies depending on how many of the homology domains each protein contains and on its biological activity (i.e., whether it has pro- or anti-apoptotic function). The first subgroup contains

proteins having all 4 homology domains, i.e., BH1, BH2, BH3 and BH4. Their general effect is anti-apoptotic, that is to preserve a cell from starting a cell death process. Proteins such as, for example, Bcl-2, Bcl-X_L, and Mcl-1 are members of this first subgroup.

[0005] There remains a need for the development of therapies to deliver Bcl-2 family (e.g., inhibitors of both Bcl-2 and Bcl-X_L) inhibitor compounds for the treatment of cancer, either as single agents or as part of combination therapies (e.g., in combination with chemotherapy and/or radiation therapy). In particular, there remains a need for therapeutically effective Bcl-2 family inhibitor compositions without dose-limiting thrombocytopenia (e.g., acceptably high platelet counts after administration to a subject), without inducing tumor lysis syndrome or other unacceptable side effects.

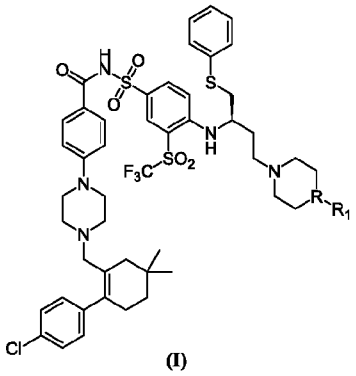
Summary

[0006] Applicants have discovered novel chemical compounds useful for inhibiting the B-cell lymphoma 2 (Bcl-2) family of proteins and the treatment of cancer, and liposome formulations of certain inhibitors of Bcl-2 having desirable properties (e.g., extended half-life in blood circulation and efficacy in treating tumors), including novel compounds that inhibit both Bcl-2 and Bcl-X_L. The inventions are based in part on the discovery of certain novel compounds for inhibiting the B-cell lymphoma 2 (Bcl-2) family of proteins, as well as extended plasma half-lives and enhanced antitumor efficacy of certain liposomal formulations of Bcl-2 inhibitor compounds. The compounds can inhibit both Bcl-2 and Bcl-X_L with an activity measured by an IC_{50} value of less than about 100 nM at Bcl-2 and less then about 100 nM at Bcl-X_L, as measured by the target activity assay of Example 1 ("Bcl-2/Bcl-X_L inhibitors"). Unless otherwise indicated herein, the activity of the Bcl-2/Bcl-X_L inhibitor compounds have a ratio of Bcl-2 to Bcl-XL activity of between about 0.1-1.0, as measured by the assay of Example 1.

[0007] In a first embodiment, novel Bcl-2/Bcl-X_L inhibitor compounds are provided. The Bcl-2/Bcl-X_L inhibitor compounds can have a chemical structure of formula (I) or formula (II) as disclosed herein and an IC_{50} value of less than about 100 nM against both Bcl-2 and Bcl-X_L as measured by the assay of Example 1. Representative examples of the Bcl-2/Bcl-X_L inhibitor compounds include compounds disclosed in Example 8.

[0008] In some examples, the novel Bcl-2/Bcl-X_L inhibitor compounds have the chemical structure of formula (I), or pharmaceutically acceptable salts thereof, wherein R is a moiety comprising an amine moiety with a pK_a of greater than 7.0 (preferably greater than 8.0, and

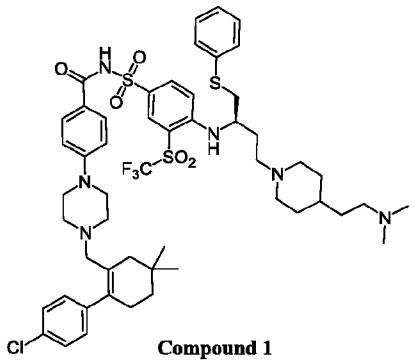
most preferably at least about 9.5) selected to provide the compound of formula (I) with an IC_{50} value of less than about 100 nM for Bcl-2 and an IC_{50} value of less than about 100 nM for Bcl-X_L, as measured by the assay of Example 1:



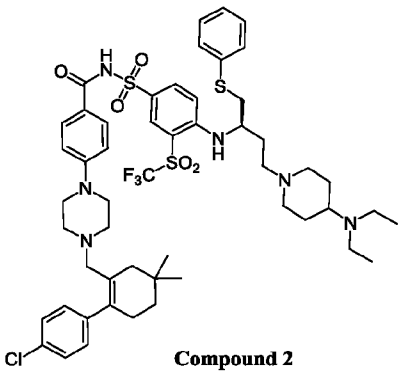
(I)

The compound of formula (I) can include R as N or CH, and R_1 as a moiety comprising an alkyl-substituted amine, preferably a tertiary alkyl-substituted amine. In some examples, R can be N or CH and R_1 can be $-N(R^a)(R^b)$ or $-(A)-N(R^a)(R^b)$ where -(A)- is a (C_1-C_4) aliphatic moiety (e.g., methylene or ethylene), and R^a and R^b are independently lower alkyl (e.g., C_1-C_4 alkyl, preferably methyl or ethyl), one of R^a or R^b is hydrogen and the other is C_1-C_4 alkyl, or R^a and R^b together form a cyclic ring (e.g., a nitrogen-containing heterocyclic ring). In other examples, R^a and R^b together form a heterocyclic ring comprising one or more heteroatoms. In yet other examples, R_1 can be a heterocycloalkyl moiety comprising an alkyl-substituted (e.g., methyl-substituted) tertiary nitrogen.

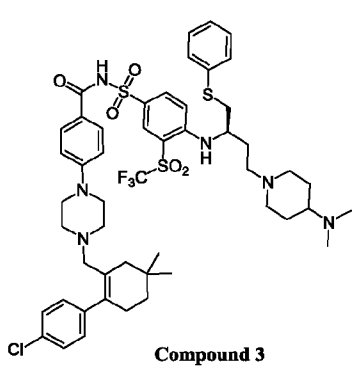
[0009] In particular, the compounds of formula (I) include Compounds 1-4, and pharmaceutically acceptable salts thereof:



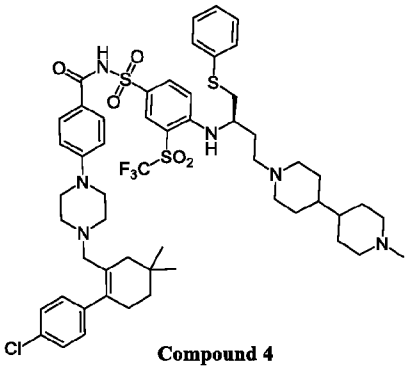
Compound 1



Compound 2

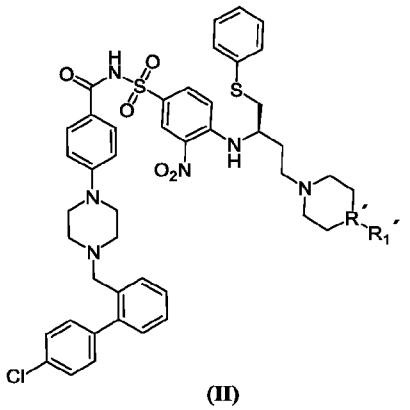


Compound 3



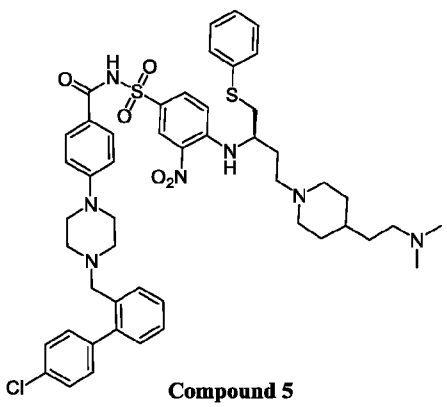
Compound 4

[0010] Additional novel Bcl-2 inhibitor compounds have the chemical structure of formula (II) or pharmaceutically acceptable salts thereof, wherein R′ and R_1′ together form a moiety comprising an amine with a pK_a of greater than 7.0 (preferably greater than 8.0, and most preferably at least about 9.5), selected to provide the compound of formula (I) with an IC_{50} value of less than about 100 nM for Bcl-2 and an IC_{50} value of less than about 100 nM for Bcl-X_L, as measured by the assay of Example 1:

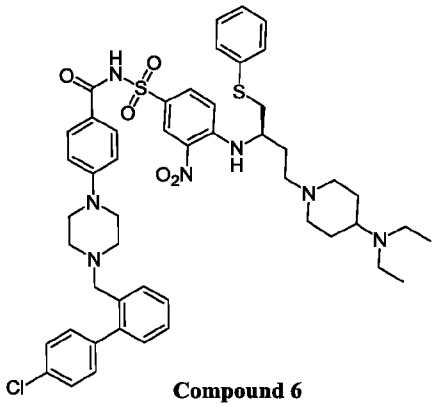


(II)

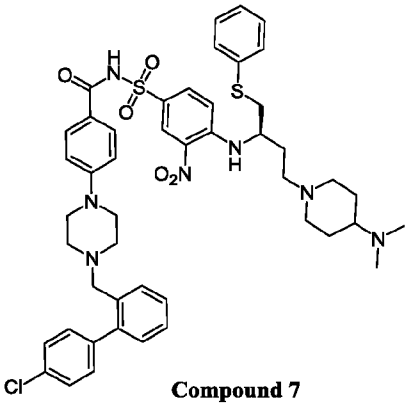
The compound of formula (II) can include R′ as N or CH, and R_1′ as a moiety comprising a tertiary alkyl-substituted amine. In some examples, R_1′ can be –N($R^{a'}$)($R^{b'}$) or –(A′)-N($R^{a'}$)($R^{b'}$) where –(A′)- is a (C_1-C_4) aliphatic moiety (e.g., methylene or ethylene), and Ra′ and Rb′ are independently lower alkyl (e.g., C_1-C_4 alkyl, preferably methyl or ethyl), one of Ra′ or Rb′ is hydrogen and the other is C_1-C_4 alkyl, or Ra′ and Rb′ together form a cyclic ring (e.g., a nitrogen-containing heterocyclic ring). In yet other examples, R_1′ can be a heterocycloalkyl moiety comprising an alkyl-substituted (e.g., methyl-substituted) tertiary nitrogen. In other examples, R_1′ can be heteroaryl such as pyrrolidine or piperidine when R′ is N. In particular, the compounds of formula (II) include Compounds 5-8, and pharmaceutically acceptable salts thereof:



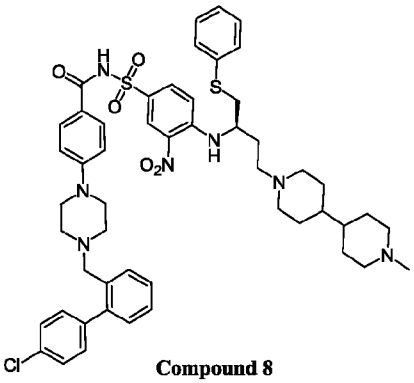
Compound 5



Compound 6

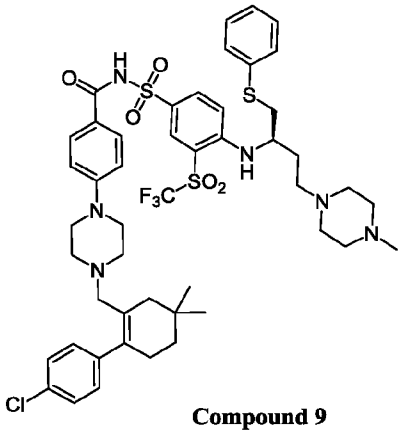


Compound 7

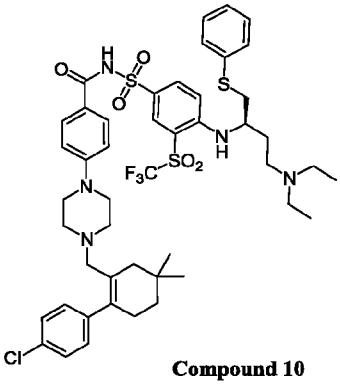


Compound 8

[0011] Additional compounds provided herein include Compound 9 and Compound 10:



Compound 9



Compound 10

Compound 9 and Compound 10 can be in the free base form, or in the form of a pharmaceutically acceptable salt.

[0012] In a second embodiment, liposome formulations of Bcl-2/Bcl-X_L inhibitor compounds are provided. The liposome formulation can encapsulate the Bcl-2/Bcl-X_L inhibitor compounds, including the compounds of the first embodiment, in liposome vesicles. Representative examples of liposome formulations of certain Bcl-2/Bcl-X_L inhibitor compounds are provided in Example 4. The liposome formulation can comprise liposomes that encapsulate at least 95%, preferably at least 99%, of the Bcl-2/Bcl-X_L inhibitor

compound in the liposome formulation. Non-encapsulated Bcl-2/Bcl-X_L inhibitor compound can be removed from the liposome formulation after the Bcl-2/Bcl-X_L inhibitor compound is encapsulated within the liposome. Preferably, the liposome is formulated to extend the circulation time of the Bcl-2/Bcl-X_L inhibitor and/or reduce the release of the Bcl-2/Bcl-X_L inhibitor compounds from the liposome in plasma. In one aspect, the liposome is contacted with albumin (e.g., BSA) under conditions effective to increase the concentration of the Bcl-2/Bcl-X_L inhibitor compound in mouse plasma over at least 4 hours, and preferably over at least 48 hours, as described in FIG. 3 and Example 5. In another aspect, the Bcl-2/Bcl-X_L inhibitor is formulated in a liposome in order to decrease undesirable thrombocytopenic effect of the inhibitor.

[0013] Novel liposome formulations comprising one or more compounds of formula (I) and/or formula (II) encapsulated in a liposome are useful for inhibiting Bcl-2 and Bcl-X_L, and the treatment of cancer. Particularly preferred examples include liposomes comprising compounds selected from the group consisting of compounds 1, 2, 3, 4, 5, 6, 7, or 8. The liposome formulation can be treated with serum albumin (e.g., BSA) after encapsulation of the novel compounds of formula (I) and/or formula (II). Preferably, the liposome formulation comprises a compound of formula (II) encapsulated in a liposome that is treated with serum albumin after encapsulation of the compound of formula (II) and prior to administration to a patient. In one example, a novel liposome formulation comprises Compound 6 in an albumin-treated liposome. The liposome can comprise one or more serum albumin protein on the outside of the liposome surface.

[0014] The liposome formulation can include a compound of formula (I) and/or formula (II) encapsulated in a unilamellar vesicle formed from one or more liposome-forming lipids (e.g., distearoyl phosphatidylcholine (DSPC)), cholesterol and a hydrophilic polymer-conjugated lipid (e.g., methoxy-poly(ethylene glycol)-1,2-distearyl-sn-glyceryl (PEG2000-DSG)). The liposome-forming lipid preferably comprises one or more phospholipids and, optionally, a sterol, such as cholesterol, with the ratio of the phospholipid(s) and the cholesterol selected to provide a desired amount of liposome membrane rigidity while maintaining a sufficiently reduced amount of leakage of the compound of formula (I) from the liposome. The type and amount of polymer-conjugated lipid can be selected to provide desirable levels of protein binding, liposome stability and circulation time in the blood stream. In some examples, the liposome vesicle comprises DSPC and cholesterol in a 3:2 molar ratio. In particular, the liposome can comprise a vesicle consisting of DSPC, cholesterol and PEG2000-DSG in a 3:2:0.3 molar ratio. The Bcl-2 inhibitor compound(s) (e.g., a compound of formula (I) or

formula (II)) can be entrapped within the liposome. One preferred example is a composition comprising a liposome having a vesicle formed from DSPC, cholesterol and PEG2000-DSG in a 3:2:0.3 molar ratio, encapsulating Compound 6.

[0015] The liposome formulation can include a compound of formula (I) and/or formula (II) encapsulated in a unilamellar vesicle formed from one or more liposome-forming lipids, a hydrophilic polymer-conjugated lipid (e.g., methoxy-poly(ethylene glycol)-oxycarbonyl-1,2-distearoyl-sn-phosphatidylethanolamine (PEG2000DSPE), and containing essentially no cholesterol. The liposome-forming lipid preferably comprises a neutral phospholipid (e.g., distearoyl phosphatidylcholine (DSPC)), an anionic lipid (e.g., distearoylphosphatidylglycerol (DSPG)), with the ratio of the phospholipid(s) selected to provide small (70-120 nm) and stable liposome size and increase resistance of the liposomes against aggregation and fusion in the presence of an encapsulated compound of formula (II). The type and amount of polymer-conjugated lipid can be selected to provide desirable levels of protein binding, liposome stability and circulation time in the blood stream. In some examples, the liposome vesicle comprises DSPC and DSPG in a 2:1 molar ratio. In particular, the liposome can comprise a vesicle consisting of DSPC, DSPG, and PEG2000-DSPE or PEG2000-DSG in a 2:1:0.2 molar ratio. The liposome can optionally comprise a lipid fluorescent label, such as DiIC18(3)-DS (Life Technologies, USA), in the amount of 0..05-0.3 mol.% relative to phospholipid. The Bcl-2 inhibitor compound(s) (e.g., a compound of formula (I) or formula (II)) can be entrapped within the liposome by a triethylammonium sucrose octasulfate gradient or an ammonium sulfate gradient at the drug/phospholipid ratio of less than 300 g/mol, preferably less than 250 g/mol, and more preferably at 100-200 g/mol. One preferred example is a composition comprising a liposome having a vesicle formed from DSPC, DSPG, PEG2000-DSPE, and DiIC18(3)-DS in a 2:1:0.2:0.002 molar ratio, encapsulating Compound 2 in the form of a sulfate or sucrose octasulfate salt at the drug/phospholipid ratio of 150 g/mol.

Description of the Drawings

[0016] **FIG. 1A** is a graph showing the cellular activity of certain Bcl-2 inhibitor compounds, including compounds of formula (I), against certain cancer cell lines, obtained using the assay of Example 1.

[0017] **FIG. 1B** is a graph showing the cellular activity of certain Bcl-2 inhibitor compounds, including compounds of formula (II), against certain cancer cell lines, obtained using the assay of Example 1.

[0018] FIG. 1C is a table showing the cellular activity of certain Bcl-2 inhibitor compounds, including compounds of formula (I) and formula (II), against certain cancer cell lines, obtained using the assay of Example 1.

[0019] FIGs. 2A, **2B**, **2C,** and **2D** are each graphs showing the dose-response of docetaxel in a MDA-MB231 breast carcinoma cells when combined with certain Bcl-2 inhibitor compounds tested at three different concentrations.

[0020] FIG. 3 is a graph showing the drug pharmacokinetics of free Compound 6 (not in a liposome), a first liposome formulation of compound Compound 6 (not pre-treated with albumin) and a second liposome formulation of compound Compound 6 (pre-treated with albumin).

[0021] FIG. 4 is a bar graph showing the platelet (PLT) count (in 1000's of PLT per microliter) in mice after administration of the first liposome formulation of compound Compound 6 (not pre-treated with albumin) and a second liposome formulation of compound Compound 6 (pre-treated with albumin), compared to normal PLT levels (dashed line).

[0022] FIGs. 5A, 5B, and **5C** are graphs showing the effect of dose escalation of the first liposome formulation of Compound 6 (not pre-treated with albumin) and the second liposome formulation of Compound 6 (pre-treated with albumin) on body weight measured in mice.

[0023] FIGs. 6A, 6B, and **6C** are graphs showing the effect of dose escalation of the first liposome formulation of Compound 6 (not pre-treated with albumin) and the second liposome formulation of Compound 6 (pre-treated with albumin) on platelet count in mice.

[0024] FIGs. 7A, 7B, and **7C** are graphs showing the effect of dose escalation of the formula (I) compound, Compound 2, vs the formula (II) compound, Compound 6, at 1 mg/kg (FIG. 7A), 2 mg/kg (FIG. 7B) and 5 mg/kg (FIG. 7C) on platelet count in mice.

[0025] FIGs. 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, and **18** each show a chemical reaction scheme useful in the synthesis of the disclosed Bcl-2 inhibitors, as described in the Examples herein.

[0026] FIG. 18A, 18B, 18C, and **18D** are graphs showing the fluorescent peptide titrations at multiple protein concentrations, used in developing the assay of Example 1.

[0027] FIGs. 19A, 19B, 19C, and **19D** are graphs showing protein titrations at two f-peptide concentrations, used in developing the assay of Example 1.

[0028] FIGs. 20A, 20B, 20C, and **20D** are graphs showing extended range protein titrations at single f-peptide concentration, used in developing the assay of Example 1.

[0029] FIGs. 21A, 21B, 21C, and **21D** are graphs showing the signal stability over time, used in developing the assay of Example 1.

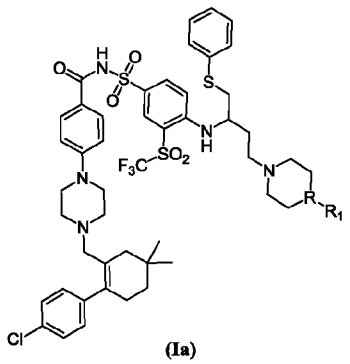
[0030] FIGs. 22A, 22B, 22C, 22D, 22E, and **22F** are graphs showing data from ~60 min incubation was used for EC_{50} determinations, used in developing the assay of Example 1.

[0031] FIG. 23 is a bar graph showing the platelet count (in 1000's of platelets (PTL) per microliter) in mice after intravenous administration of saline vehicle (Naïve Control), free Compound 6, or liposomally formulated Compound 6 at 10 mg/kg, compared to normal platelet levels (dashed line).

Detailed Description

[0032] Novel Bcl-2/Bcl-X_L inhibitor compounds can be obtained by methods described in the Examples. As used herein, and unless otherwise indicated, the term "Bcl-2/Bcl-X_L inhibitor compound" refers to a compound having an IC_{50} value of less than about 100 nM with both Bcl-2 and Bcl-X_L in the assay of Example 1. Preferably, the Bcl-2/Bcl-X_L inhibitor compounds have a ratio of IC_{50} values with Bcl-2 and Bcl-XL of between about 0.1 and 10.0 (including, for example, ratios between about 0.2 and 5.0, and ratios therebetween) measured by the assay of Example 1.

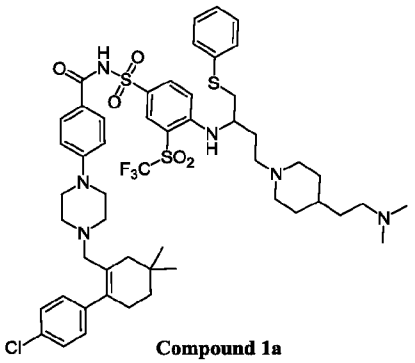
[0033] Provided herein are novel compounds having the chemical structure of formula (Ia), or pharmaceutically acceptable salts thereof, wherein R is a moiety comprising an amine with a pK_a of greater than 7.0 (preferably greater than 8.0, and most preferably at least about 9.5):



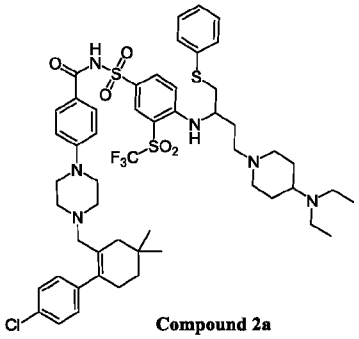
(Ia)

The compound of formula (Ia) can include R as N or CH, and R_1 as a moiety comprising a tertiary alkyl-substituted amine. In some examples, R can be N or CH and R_1 can be –$N(R^a)(R^b)$ or –(A)-$N(R^a)(R^b)$ where –(A)- is a (C_1-C_4) aliphatic moiety (e.g., methylene or ethylene), and R^a and R^b are independently lower alkyl (e.g., C_1-C_4 alkyl, preferably methyl or ethyl), or one of R^a or R^b is hydrogen and the other is C_1-C_4 alkyl, or R^a and R^b together form a cyclic ring (e.g., a nitrogen-containing heterocyclic ring). In other examples, R^a and

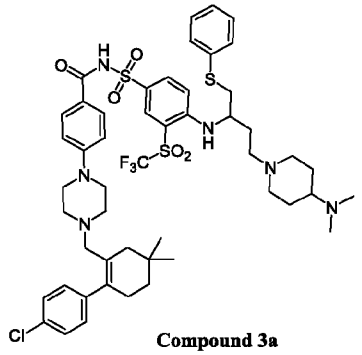
R^b together form a heterocyclic ring comprising one or more heteroatoms. In yet other examples, R_1 can be a heterocycloalkyl moiety comprising an alkyl-substituted (e.g., methyl-substituted) tertiary nitrogen. In particular, the compounds of formula (Ia) include Compounds 1a-4a, and pharmaceutically acceptable salts thereof:



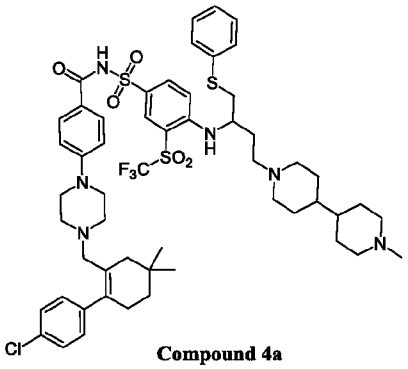
Compound 1a



Compound 2a

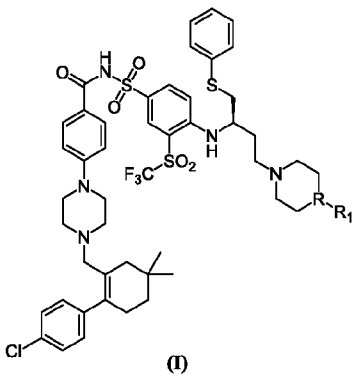


Compound 3a



Compound 4a

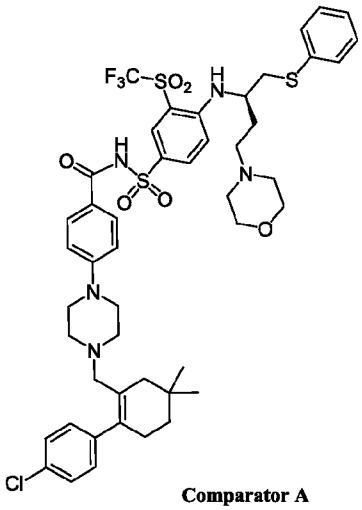
[0034] In one embodiment, the compounds have the chemical structure of formula (I), where R is a moiety comprising an amine with a pK_a of greater than 7.0 (preferably greater than 8.0, and most preferably at least about 9.5) selected to provide the compound of formula (I) with an IC_{50} value of less than about 100 nM for Bcl-2 and an IC_{50} value of less than about 100 nM for Bcl-X_L, as measured by the assay of Example 1:



(I)

[0035] The activity of representative examples of compounds of formula (I) at Bcl-2 and Bcl-X_L shown in Table 1 were measured using the assay in Example 1 (Activity Assay). Notably,

the activity of the compounds of formula (I) is sensitive to substitution of the morpholine moiety at the R-R_1 position in the Comparator A compound:



Comparator A

For example, the activity of compounds of formula (I) at Bcl-2 ranged from over 5-times more potent than Comparator A (e.g., Compound 2 and Compound 3) to more than 10-times less potent than Comparator A (e.g., Compound 4). Similarly, the activity of compounds of formula (I) at Bcl-X_L ranged from more than 5-times more potent than Comparator A (e.g., Compound 2 and Compound 3) to more than 30-times less potent than Comparator A (e.g., Compound 4). Compounds of formula (I) include compounds having a ratio of activity at Bcl-2/Bcl-X_L from about 70-105% of the comparable Bcl-2/Bcl-X_L activity ratio for Comparator A (e.g., Compound 1, Compound 2, and Compound 3).

[0036] In some examples, the Bcl-2/Bcl-X_L inhibitor compounds are compounds of formula (I) having IC_{50} values with Bcl-2 measured by Example 1 of less than that measured for Comparator A (i.e., less than 2.46, including about 0.1-2.46 and about 0.4-2.46 and about 0.1-0.5). In some examples, the Bcl-2/Bcl-X_L inhibitor compounds are compounds of formula (I) having IC_{50} values with Bcl-X_L measured by Example 1 of less than that measured for Comparator A (i.e., less than 47.88, including about 0.1-47.88 and about 0.25-47.88 and about 0.1-47.88).

[0037] The Bcl-2 and Bcl-X_L activities in Table 1 are selected examples of novel Bcl-2/ Bcl-X_L active compounds. In addition, other novel compounds can be identified based on this information. In particular, various novel Bcl-2/ Bcl-X_L inhibitor compounds with alternative structures at the R-R_1 binding site with similar chemical structures providing comparable size and shape to the examples shown in Table 1. The identification of such compounds is based in part on the discovery that the R-R_1 binding site can be a critical component of Bcl-2/Bcl-X_L activity, which is unexpected given its position in the molecule distal to the large

hydrophobic contact surface of the protein-protein interaction at the biological target site. Reviewing the data in Table 1, it is apparent that compounds with more bulky and more rigid substituents, e.g., Compound 1 and Compound 4 are much less potent compared to compounds with smaller substituents, e.g., Compound 2 and Compound 3. This information permits one of skill in the art to envisage a collection of additional compounds providing desired activity at the Bcl-2 and/or Bcl-X_L site, as identified with the assays disclosed herein. In some embodiments, the compounds of formula (I) include a substituted amine at the R-R_1 position, including a primary or secondary substituted amine (e.g., a amino moiety substituted with one or more alkyl moieties), wherein the R-R_1 moiety is selected to provide desired liposome loading characteristics (e.g., a substituted amino moiety having a pKa of greater than 7, 8 or 9, including a pKa value of 7-10, 8-10, or 9-10). In some embodiments, the compounds can have the structure at R-R_1 in formula (I) having one or two tertiary alkyl substituted amine moieties, including R-R_1 moieties selected from the group consisting of:

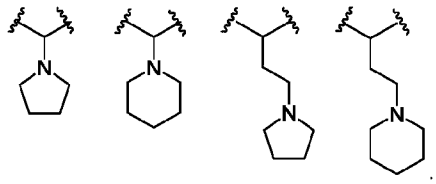
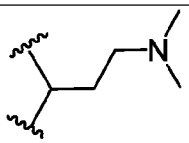
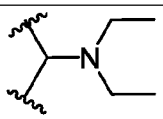
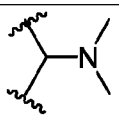
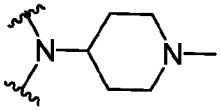
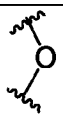
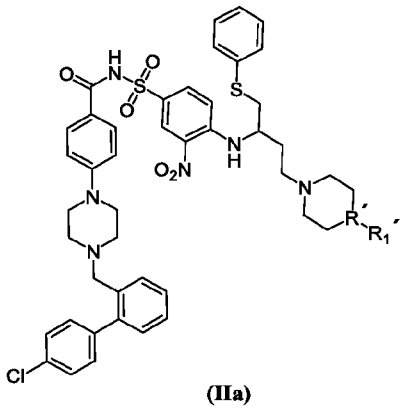


Table 1

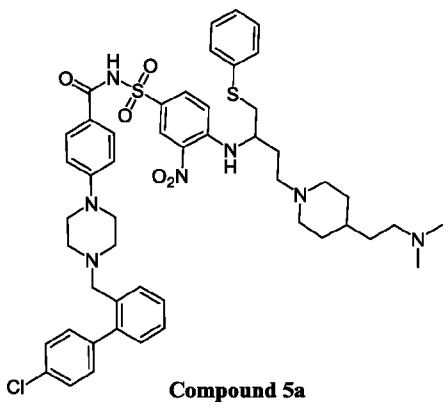
Compound	R-R_1 structure	Bcl-2/fbak (nM)	Bcl-X_L/fBad (nM)	Bcl-2/Bcl-X_L ratio
Compound 1		7.06	5.84	1.21
Compound 2		0.46	0.26	1.77
Compound 3		0.44	0.29	1.52
Compound 4		27.75	47.88	0.58
Comparator A		2.46	1.46	1.68

[0038] Also provided herein are novel compounds having the chemical structure of formula (IIa) or pharmaceutically acceptable salts thereof, whereing R′ and R_1′ together form a moiety comprising an amine with a pK_a of greater than 7.0 (preferably greater than 8.0, and most preferably at least about 9.5):

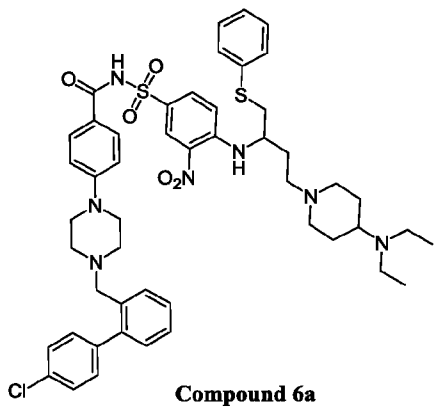


(IIa)

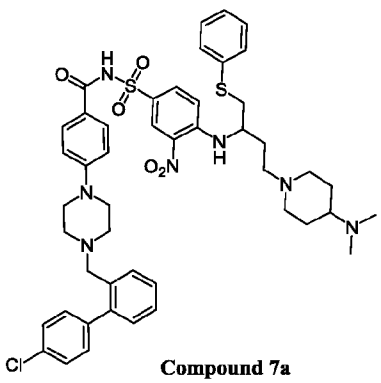
The compound of formula (IIa) can include R′ as N or CH, and R_1′ as a moiety comprising a tertiary alkyl-substituted amine. In some examples, R_1′ can be $-N(R^{a'})(R^{b'})$ or $-(A')-N(R^{a'})(R^{b'})$ where –(A′)- is a (C_1-C_4) aliphatic moiety (e.g., methylene or ethylene), and Ra′ and Rb′ are independently lower alkyl (e.g., C_1-C_4 alkyl, preferably methyl or ethyl), one of Ra′ or Rb′ is hydrogen and the other is C_1-C_4 alkyl, or Ra′ and Rb′ together form a cyclic ring (e.g., a nitrogen-containing heterocyclic ring). In yet other examples, R_1′ can be a heterocycloalkyl moiety comprising an alkyl-substituted (e.g., methyl-substituted) tertiary nitrogen. In other examples, R_1′ can be heteroaryl such as pyrrolidine or piperidine when R′ is N. In particular, the compounds of formula (IIa) include Compounds 5a-8a, and pharmaceutically acceptable salts thereof:



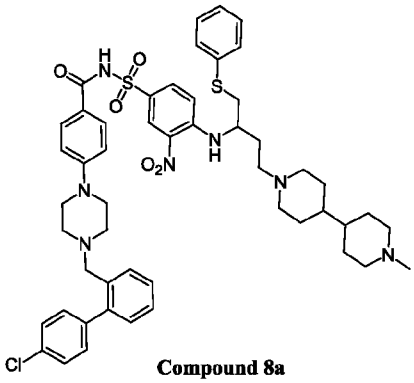
Compound 5a



Compound 6a

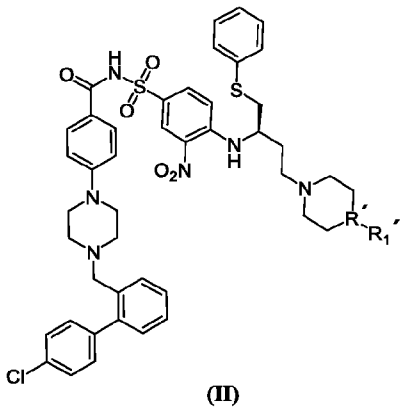


Compound 7a



Compound 8a

[0039] Additional novel Bcl-2 inhibitor compounds have the chemical structure of formula (II), where R′ is a moiety comprising an amine with a pK_a of greater than 7.0 (preferably greater than 8.0, and most preferably at least about 9.5) selected to provide the compound of formula (I) with an IC_{50} value of less than about 100 nM for Bcl-2 and an IC_{50} value of less than about 100 nM for Bcl-X_L, as measured by the assay of Example 1:



(II)

where R′ is N or CH, and R_1′ as a moiety comprising an alkyl-substituted amine, preferably a tertiary alkyl-substituted amine. In some examples, R_1′ can be –N($R^{a'}$)($R^{b'}$) or –(A′)-N($R^{a'}$)($R^{b'}$) where –(A′)- is a (C_1-C_4) aliphatic moiety (e.g., methylene or ethylene), and $R^{a'}$ and $R^{b'}$ are independently lower alkyl (e.g., C_1-C_4 alkyl, preferably methyl or ethyl), one of Ra′ or Rb′ is hydrogen and the other is C_1-C_4 alkyl, or $R^{a'}$ and $R^{b'}$ together form a cyclic ring (e.g., a nitrogen-containing heterocyclic ring), wherein the R′-R_1′ moiety in formula (II) is selected to provide inhibitor activity measured as a value of less than about 100 nM at both Bcl-2 and Bcl-X_L as measured in Example 1. In some examples, the compound of formula (II) can include R′ as CH, and R_1′ as a moiety comprising a tertiary alkyl-substituted amine. In some examples, R′ is CH and R_1′ can be –N($R^{a'}$)($R^{b'}$) or –(A′)-N($R^{a'}$)($R^{b'}$) where –(A′)- is a (C_1-C_4) aliphatic moiety (e.g., methylene or ethylene), and $R^{a'}$ and $R^{b'}$ are independently lower alkyl (e.g., C_1-C_4 alkyl, preferably methyl or ethyl). In other examples, R′ is N or CH

and R_1' can be a heterocycloalkyl moiety comprising an alkyl-substituted (e.g., methyl-substituted) tertiary nitrogen.

[0040] In some examples, the Bcl-2/Bcl-X_L inhibitor compounds are compounds of formula (II) having IC_{50} values with Bcl-2 measured by Example 1 of less than that measured for Comparator B (i.e., less than 0.32, including about 0.1-0.32 and about 0.2-0.32. In some examples, the Bcl-2/Bcl-X_L inhibitor compounds are compounds of formula (I) having IC_{50} values with Bcl-X_L measured by Example 1 of less than that measured for Comparator B (i.e., less than 0.23, including about 0.1-0.23 and about 0.18-0.23).

[0041] The activity of representative examples of compounds of formula (II) at Bcl-2 and Bcl-X_L shown in Table 2 were measured using the assay in Example 1 (Activity Assay) (e.g., Compounds 5, 6, 7, and 8). Notably, the activity of the compounds of formula (II) is sensitive to substitution of the dimethylamino moiety at the position occupied by the ring containing the R′-R_1' position in the formula (II) compounds. The Bcl-2 and Bcl-X_L activities in Table 2 are selected examples of novel Bcl-2/ Bcl-X_L active compounds. In addition, other novel compounds can be identified based on this information. In particular, various novel Bcl-2/ Bcl-X_L inhibitor compounds with alternative structures at the R′-R_1' binding site with similar chemical structures providing comparable size and shape to the examples shown in Table 2. The identification of such compounds is based in part on the discovery that the R′-R_1' binding site can be a critical component of Bcl-2/ Bcl-X_L activity, which is unexpected given its position in the molecule distal to the large hydrophobic contact surface of the protein-protein interaction at the biological target site. Reviewing the data in Table 2, it is apparent that compounds with more bulky and more rigid substituents, e.g., Compound 5 and Compound 8 are much less potent compared to compounds with smaller substituents, e.g., Compound 6 and Compound 7. This information permits one of skill in the art to envisage a collection of additional compounds providing desired activity at the Bcl-2 and/or Bcl-X_L site, as identified with the assays disclosed herein.

[0042] In some embodiments, the compounds of formula (II) include a substituted amine at the R′-R_1' position, including a primary or secondary substituted amine (e.g., a amino moiety substituted with one or more alkyl moieties), wherein the R′-R_1' moiety is selected to provide desired liposome loading characteristics (e.g., a substituted amino moiety having a pK_a of greater than 7, 8 or 9, including a pK_a value of 7-10, 8-10, or 9-10) and inhibitor activity of less than about 100 nM at both Bcl-2 and Bcl-X_L as measured in Example 1. In some embodiments, the compounds can have the structure at R′-R_1' in formula (II) having one or

two alkyl substituted amine moieties (e.g., one or more tertiary alkyl substituted amine moieties), including R′-R_1′ moieties selected from the group consisting of:

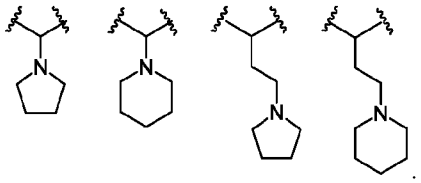
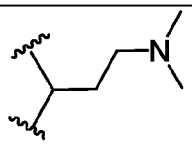
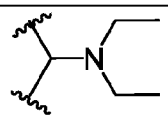
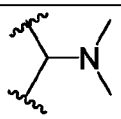
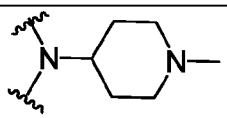
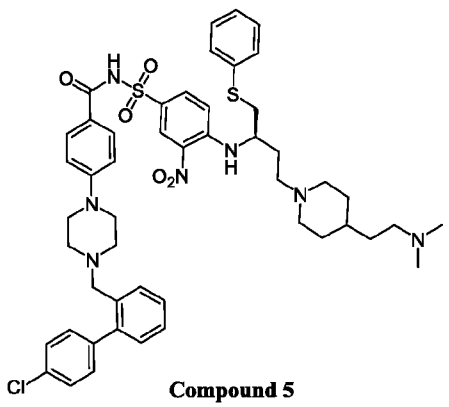


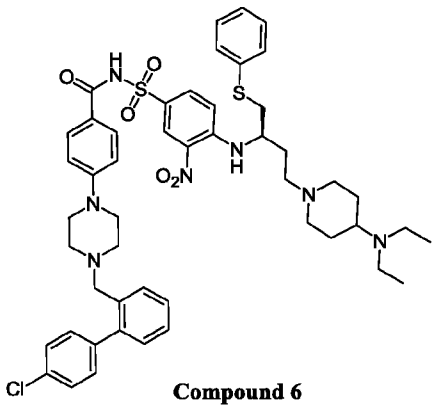
Table 2

Compound	R′-R_1′ **structure**	**Bcl-2/fbak (nM)**	**Bcl-X_L/fBad (nM)**	**Bcl-2/Bcl-X_L ratio**
Compound 5		8.87	5.55	1.60
Compound 6		0.20	0.18	1.11
Compound 7		0.39	0.33	1.18
Compound 8		26.9	33.3	0.81
Comparator B	n/a	0.32	0.23	1.39

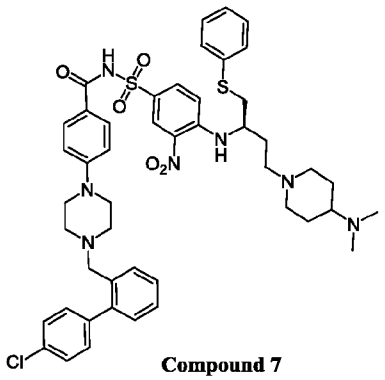
[0043] In particular, the compounds of formula (II) include compounds 5-8:



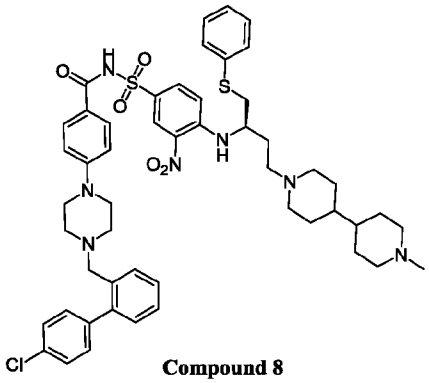
Compound 5



Compound 6

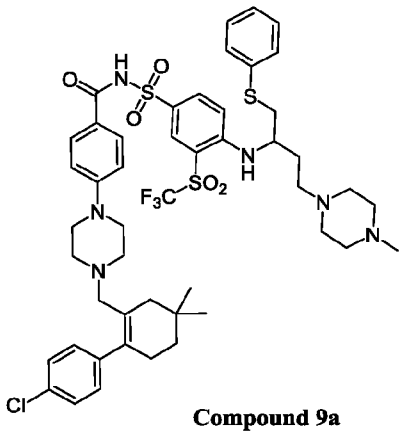


Compound 7

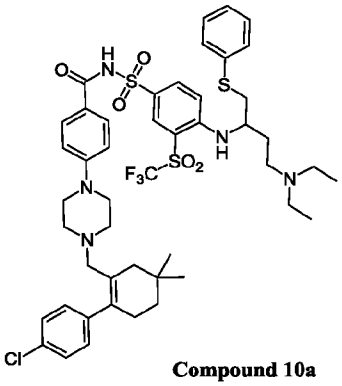


Compound 8

[0044] Additional compounds provided herein include Compound 9a and Compound 10a:



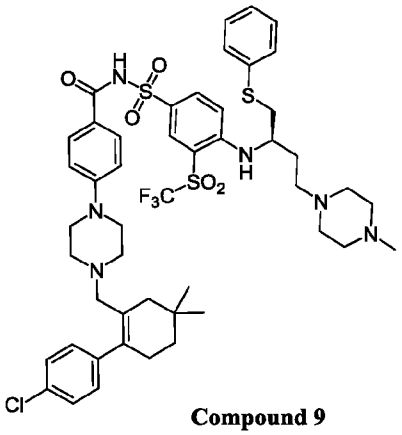
Compound 9a



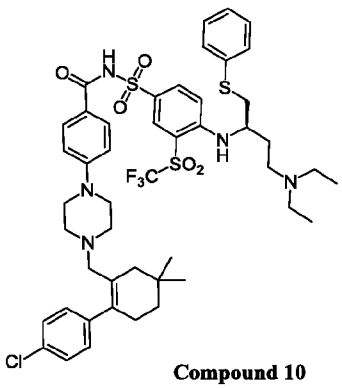
Compound 10a

Compound 9a and Compound 10a can be in the free base form, or in the form of a pharmaceutically acceptable salt.

[0045] Further compounds provided herein include Compound 9 and Compound 10:



Compound 9



Compound 10

Compound 9 and Compound 10 can be in the free base form, or in the form of a pharmaceutically acceptable salt.

[0046] In a second embodiment, liposome formulations of Bcl-2/Bcl-X_L inhibitor compounds are provided. The liposome formulation can encapsulate the Bcl-2/Bcl-X_L inhibitor compounds of the first embodiment in liposome vesicles. Representative examples of

liposome formulations of certain Bcl-2/Bcl-X_L inhibitor compounds are provided in Example 4. The liposome formulation can comprise liposomes that encapsulate at least 95%, preferably at least 99%, of the Bcl-2/Bcl-X_L inhibitor compound in the liposome formulation. Non-encapsulated Bcl-2/Bcl-X_L inhibitor compound can be removed from the liposome formulation after the Bcl-2/Bcl-X_L inhibitor compound is encapsulated within the liposome. Preferably, the liposome is formulated to reduce the release of the Bcl-2/Bcl-X_L inhibitor compounds from the liposome in plasma. In one aspect, the liposome is contacted with albumin (e.g., BSA) under conditions effective to increase the concentration of the Bcl-2/Bcl-X_L inhibitor compound in mouse plasma over at least 4 hours, and preferably over at least 48 hours, as described in FIG. 3 and Example 5.

[0047] The liposome formulations can be selected to reduce incidence of thrombocytopenia (e.g., compositions tested in the data presented in FIGs. 4, 6A, 6B, 6C, 7A, 7B, 7C, and 23). In addition, the liposome formulations can be selected to provide extended blood circulation times, for example as measured in a mouse model in the data shown in FIG. 3. The liposome average size can be selected to lie below 0.2 micron (200 nm) to provide for convenient sterilization of the liposome composition by microfiltration. In other embodiments, the liposome size can be less than 170 nm, less than 150 nm, preferably less than 130 nm, or more preferably in the range of 70-120 nm, to provide for good blood circulation properties and uptake by tumors. Preferably, the liposome formulations provide a level of PLT count that is normal or within a medically acceptable range within a therapeutically relevant time period (e.g., any time post injection, within the duration of a chemotherapy treatment cycle, within 14-28 days after injection, or the like). Preferably, the liposome formulations provide a clearance rate of the Bcl-2/Bcl-X_L inhibitor compounds characterized by the plasma half-life of greater than about 5 hours (e.g., 10-100 hours) after administration (e.g., intravenous administration). The blood circulation half-life of the Bcl-2/Bcl-X_L inhibitor compounds in the liposome formulation is preferably at least about 5 hours, including blood circulation half-lives of about up to about 10 hours and 11-15 hours. FIG. 3 provides a representative example of measurement of the Bcl-2/Bcl-X_L inhibitor compound concentration in plasma of a mouse for two liposome formulations. Preferably, the liposome formulation is contacted with albumin prior to administration under conditions effective to enhance the amount of the Bcl-2/Bcl-X_L inhibitor compound present in the plasma after administration of the liposome (e.g., to reduce the reduction in the Bcl-2/Bcl-X_L inhibitor compound concentration in the plasma within about 4 hours after administration of the liposome).

[0048] Liposomes typically comprise vesicles containing one or more lipid bilayers enclosing an aqueous interior. Liposome compositions usually include liposomes in a medium, such as an aqueous fluid exterior to the liposome. Liposome lipids can include amphiphilic lipid components that, upon contact with aqueous medium, spontaneously form bilayer membranes, such as phospholipids, for example, phosphatidylcholines. Liposomes also can include membrane-rigidifying components, such as sterols, for example, cholesterol. In some cases, liposomes also include lipids conjugated to hydrophilic polymers, such as, polyethyleneglycol (PEG) lipid derivatives that may reduce the tendency of liposomes to aggregate and also have other beneficial effects.

[0049] A variety of liposomal properties or formulation methods play important roles in determining the degree of stability, and hence the rate of drug release from the liposomal carrier. The incorporation of highly saturated phospholipids, such as distearoylphosphatidylcholine or hydrogenated soy phosphatidylcholine in liposomal formulations of amphipathic drugs improves stability considerably when compared to liposomes containing unsaturated phospholipids. The inclusion of cholesterol reduces destabilizing interactions with plasma proteins, and also participates in regulating the permeability of liposomal membranes to small molecules. Spingomyelin-based liposomes have also demonstrated superior drug retention and activity when compared to phosphatidylcholine-based formulations, likely resulting in part from intermolecular hydrogen bonding with neighboring cholesterol molecules and the reduced hydrolysis of sphingomyelin when compared to phospholipids.

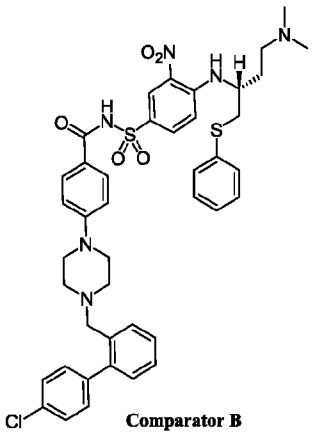
[0050] The liposome membrane composition of the present invention can be made by any suitable method known to or later discovered by one skilled in the art. In general, a variety of lipid components can be used to make the liposomes of the present invention. Lipid components usually include, but are not limited to (1) uncharged lipid components, e.g., cholesterol, ceramide, diacylglycerol, acyl(poly ethers) or alkylpoly(ethers); (2) neutral phospholipids, e.g., diacylphosphatidylcholines, sphingomyelins, and diacylphosphatidylethanolamines, (3) anionic lipids, e.g., diacylphosphatidylserine, diacylphosphatidylglycerol, diacylphosphatidate, cardiolipin, diacylphosphatidylinositol, diacylglycerolhemisuccinate, diaclyglycerolhemigluratate, cholesteryl hemisuccinate, cholesterylhemiglutarate, and the like; (4) polymer-conjugated lipids, e.g., N-[methoxy-(poly(ethylene glycol)diacylphosphatidylethanolamine, poly(ethylene glycol)-diacylglycerol, poly(ethylene glycol)-ceramide; and (5) cationic lipids, e.g., 1,2,-diacyl-3-trimethylammonium-propane (DOTAP), dimethyldioctadecylammonium bromide (DDAB),

and 1,2-diacyl-sn-glycero-3-ethylphosphocholine. Monoacyl-substituted derivatives of these lipids, as well as di- and monoalkyl-analogs can be also employed.

[0051] Various lipid components can be selected to fulfill, modify or impart one or more desired functions. For example, phospholipid can be used as principal vesicle-forming lipid. Inclusion of cholesterol is useful for maintaining membrane rigidity and decreasing drug leakage. Polymer-conjugated lipids can be used in the liposomal formulation to increase the lifetime of circulation via reducing liposome clearance by liver and spleen, or to improve the stability of liposomes against aggregation during storage, in the absence of circulation extending effect..

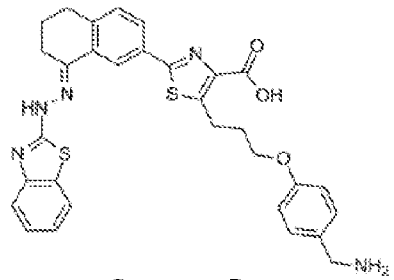
[0052] The liposome formulation can include a Bcl-2/Bcl-XL inhibitor compound (e.g., a compound of formula (II) or formula (I)) encapsulated in a unilamellar vesicle formed from one or more liposome-forming lipids (e.g., hydrogenated soy phosphatidylcholine (HSPC)), cholesterol and a hydrophilic polymer-conjugated lipid. In one embodiment, the hydrophilic polymer conjugated to the lipid is poly-ethylene glycol (PEG) (e.g., methoxy-poly(ethylene glycol)-1,2-distearyl-sn-glyceryl (PEG2000-DSG). Hydrophilic polymers, e.g., PEG with molecular weights from about 200 to about 30,000 can be employed. In one embodiment, the molecular weight of PEG moiety of a PEG-lipid is 2000. The PEG-lipid can be neutral, such as PEG2000-DSG, or ionically charged, such as N-methoxy-poly(ethylene glycol)oxycarbonyl-1,2-distearoyl phosphatidylethanolamine (PEG2000-DSPE). The liposome-forming lipid preferably comprises one or more phospholipids, with the ratio of the phospholipid(s) and the cholesterol selected to provide a desired amount of liposome membrane rigidity while maintaining a sufficiently reduced amount of leakage of the compound of formula (I) from the liposome. For example, R_1 can be a moiety of the formula –(A)-N(R^a)(R^b) where A is a linear or branched alkyl moiety, and R^a and R^b are independently C_1-C_4 alkyl, one of Ra′ or Rb′ is hydrogen and the other is C_1-C_4 alkyl, or R^a and R^b together form a heterocyclic ring.

[0053] Bcl-2 protein inhibitors include the following compounds: N-(4-(4-((4′ -chloro(1,1′ -biphenyl)-2-yl)methyl)piperazin-1-yl)benzoyl)-4-(((1R)-3-(dimethylamino)-1-((phenylsulfanyl)methyl)propyl)amino)-3-nitrobenzenesulfonamide) (referred to herein as "Comparator B"):



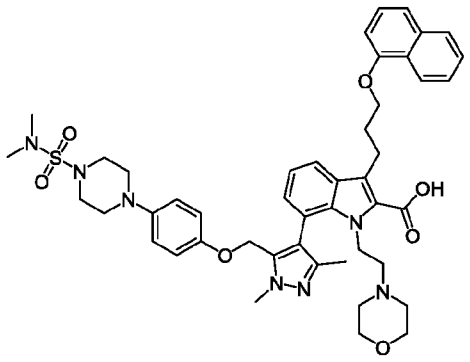
Comparator B

and N-(4-(4-((2-(4-chlorophenyl)-5,5-dimethyl-1-cyclohex-1-en-1-yl)methyl)piperazin-1-yl)benzoyl)-4-(((1R)-3-(morpholin-4-yl)-1-((phenylsulfanyl)methyl)propyl)amino)-3-((trifluoromethyl)sulfonyl)benzenesulfonamide (referred to herein as "Comparator A"). (Tse et al. (2008), Cancer Research 68: 3421-3428). Comparator A has shown dose-limiting thrombocytopenia (platelets counts) in clinical trials. This class of compound suffers from toxicity (thrombocytopenia) due to inhibition of Bcl-X_L (Bajwa et al. (2012), Expert Opin. Ther. Patents 22: 37-55) and the emergence of resistance. Bcl-X_L specific inhibitor with the following structure (referred to herein as "Comparator D"):



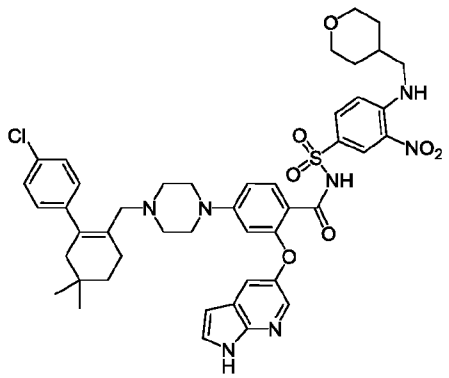
Comparator D

was explored for solid tumor. Mcl-1 upregulation has been implicated in the resistance of cancer cells to therapeutics targeting Bcl-2 and Bcl-X_L. Several companies are also pursuing Mcl-1 inhibitors, for example, a compound of the following structure (referred to herein as "Comparator E"):



Comparator E

[0054] Bcl-2 specific inhibitor of the following structure (referred to herein as "Comparator C"):



Comparator C

has less Bcl-X_L activity and has shown dose-dependent Tumor Lysis Syndrome in clinical trials, a serious side effect.

[0055] AZD4320 is a dual Bcl-2/ Bcl-X_L inhibitor (for IV dosing), that has shown transient platelet counts reduction observed in mouse models, returning to baseline at 72 hrs.

[0056] Liposomes typically have the size in a micron or submicron range and are well recognized for their capacity to carry pharmaceutical substances, including anticancer drugs, such as irinotecan, and to change their pharmaceutical properties in various beneficial ways. Methods of preparing and characterizing pharmaceutical liposome compositions are known in the field (see, e.g., Lasic D. Liposomes: From physics to applications, Elsevier, Amsterdam 1993; G. Grerориadis (ed.), Liposome Technology, 3rd edition, vol. 1-3, CRC Press, Boca Raton, 2006; Hong et al., US Pat. 8,147,867, incorporated by reference herein in their entirety for all purposes).

[0057] Bcl-2 inhibitor compound liposomes can be prepared by a process that includes the steps of (a) preparing a liposome containing a gradient-generating salt such as ammonium or a substituted ammonium salt (e.g., ammonium sulfate), and (b) subsequently contacting the

ammonium-sulfate containing liposome with a Bcl-2 inhibitor compound (e.g., a compound of formula (II) or formula (I)) under conditions effective for the irinotecan to enter the liposome and to permit a corresponding amount of ammonia to leave the liposome (thereby exhausting or reducing the concentration gradient of ammonium sulfate across the resulting liposome). Alternatively, the Bcl-2 inhibitor-loaded liposomes of the present invention can be prepared without the use of a transmembrane ion gradient, i.e., by direct entrapment, such as, by forming the liposome in the presence of concentrated aqueous solution of the inhibitor, whereby the drug solution is sequestered in the inner aqueous space of the liposome; by forming the liposome from the mixture of lipids with added drug, whereby the drug is entrapped in the liposome lipid membrane, or by incubation of the liposome with the drug, whereby a portion of the drug is distributed from the micellar phase into the liposome membrane.

[0058] Liposomes can be prepared in multiple steps comprising the formation of an ammonium sulfate containing liposome, followed by loading of a Bcl-2 inhibitor compound into the liposome as the ammonium sulfate leaves the liposome. The first step can include forming the ammonium sulfate containing liposome by hydrating and dispersing the liposome lipids in the solution of ammonium sulfate. This can be performed, for example, by dissolving the lipids, including DSPC and cholesterol, in heated ethanol, and dispersing the dissolved and heated lipid solution in the ammonium sulfate aqueous solution at the temperature above the transition temperature (T_m) of the liposome lipid, e.g., 60 °C or greater. The lipid dispersion can be formed into liposomes having the average size of 75-125 nm (such as 80-120 nm, or in some embodiments, 90-115 nm), by extrusion through track-etched polycarbonate membranes with the defined pose size, e.g., 100 nm. The ammonium sulfate solution can have a concentration of about 0.25M, and a pH (e.g., about 5.2-5.3) that is selected to prevent unacceptable degradation of the liposome phospholipid during the dispersion and extrusion steps (e.g., a pH selected to minimize the degradation of the liposome phospholipid during these steps). Then, the non-entrapped ammonium sulfate can be removed from the liposome dispersion, e.g., by dialysis, gel chromatography, ion exchange or ultrafiltration prior to irinotecan encapsulation. The resulting Bcl-2 inhibitor compound liposomes can contain a sulfate salt of the Bcl-2 inhibitor compound(s) of formula (I) or formula (II). These Bcl-2 inhibitor liposomes can be stabilized by loading enough of the Bcl-2 inhibitor compound into the liposomes to reduce the amount of ammonium in the resulting liposome composition to a level that results in less than a given maximum level of lyso-PC formation after 180 days at 4 °C, or less than a given maximum level of lyso-PC

accumulation rate in the liposome composition during storage in a refrigerator at about 4 °C, or, more commonly, at 5 ± 3 °C, measured, e.g., in mg/mL/month, or % PC conversion into a lyso-PC over a unit time, such as, mol% lyso-PC/ month. Next, the ammonium exchanged from the liposomes into the external medium during the loading process, along with any unentrapped Bcl-2 inhibitor compound, is typically removed from the liposomes by any suitable known process(es) (e.g., by gel chromatography, dialysis, diafiltration, ion exchange or ultrafiltration). The liposome external medium can be exchanged for an injectable isotonic fluid (e.g. isotonic solution of sodium chloride), buffered at a desired pH.

[0059] The liposomes encapsulating the Bcl-2 inhibitor compounds disclosed herein preferably interrupt protein-protein interaction (PPI) of pro-apoptosis proteins (Bid, Bim, Bad, Bak, Bax, Noxa, etc.) with anti-apoptosis proteins (Bcl-2 family: Bcl-2, Bcl-X_L, Mcl-1, etc.) leading to cell apoptosis (intrinsic apoptosis pathway), bind at a large hydrophobic, flexible contact surface (protein/protein), and/or have higher molecular weights about 1000. The liposomes can be used as mono or combination therapies (with chemo agents).

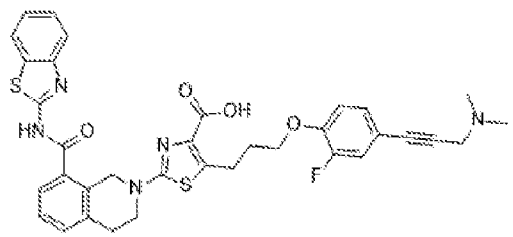
[0060] In particular, preferred liposomes are PEGylated liposomal formulations with reduced major distribution into platelets (to reduce the possibility of thrombocytopenia) compared to the free (non-encapsulated) form of the encapsulated Bcl-2 inhibitor compound.

[0061] Novel Bcl-2 inhibitor compounds were identified using the assay in Example 1, including compounds of formula (I) listed in Table 3. The cellular activity of certain Bcl-2 inhibitor compounds were measured against cell lines

Examples

[0062] The following examples illustrate some embodiments of the invention. The examples and preparations which follow are provided to enable those skilled in the art to more clearly understand and to practice these and other embodiments present invention. They should not be considered as limiting the scope of the invention, but merely as being illustrative and representative thereof.

[0063] A Bcl-X_L specific inhibitor of the following structure:



(referred to herein as "Comparator F") was used as an excellent tool compound for inducing thrombocytopenia. Platelet count in in vivo could be used as an indication of target engagement.

[0064] The examples and specification include illustrative examples of certain embodiments of the inventions disclosed herein.

[0065] In some embodiments, novel Bcl-2/Bcl-X_L inhibitor compounds are disclosed, including compounds of formula (I) and forumula (II) as described herein. The examples of the compounds of formula (I) and (II) are provided herein permit one to identify numerous additional Bcl-2/Bcl-X_L inhibitor compounds within the scope of formula (I) or formula (II) in addition to those described in Example 8 and elsewhere herein, including: (a) additional compounds of formula (I) where R is C or a heteroatom (such as N) and R_1 is a moiety comprising an amine (e.g., an alkyl substituted amine having a pK_a of at least about 7.0, preferably about 8.0-10.5 and most preferably about 9.0-10.0) selected to provide an IC_{50} value of less than 100 nM against each of Bcl-2 and Bcl-X_L (as measured by the assay of Example 1 below); and (b) additional compounds of formula (II) where R′ is C or a heteroatom (such as N) and R_1′ is a moiety comprising an amine (e.g., an alkyl substituted amine having a pK_a of at least about 7.0, preferably about 8.0-10.5 and most preferably about 9.0-10.0) selected to provide an IC_{50} value of less than 100 nM against each of Bcl-2 and Bcl-X_L (as measured by the assay of Example 1 below).

[0066] In other embodiments, liposomes encapsulating Bcl-2/Bcl-X_L inhibitor compounds are provided, where the liposome-encapsulated Bcl-2/Bcl-X_L inhibitor compounds have an activity against each of Bcl-2 and Bcl-X_L measured by an IC_{50} value of up to about <1 - 100nM using the assay of Example 1. In some examples, the liposomes encapsulate the novel Bcl-2/Bcl-X_L inhibitor compounds disclosed herein (e.g., Bcl-2/Bcl-X_L inhibitor compounds of formula (I) and formula (II)). In other examples, the liposomes encapsulate a Bcl-2/Bcl-X_L inhibitor Comparator compound disclosed herein.

[0067] The Bcl-2/Bcl-X_L inhibitor liposomes can provide an extended release of the encapsulated Bcl-2/Bcl-X_L inhibitor compound, as described in Example 5. In addition, the Bcl-2/Bcl-X_L inhibitor liposomes disclosed herein can be prepared to reduce incidence of thrombocytopenia in the mouse models disclosed herein to levels that are acceptable to advance the Bcl-2/Bcl-X_L inhibitor compounds as clinical candidates for human testing for the treatment of cancer. For example, the administration of Bcl-2/Bcl-X_L inhibitor encapsulated in liposomes as disclosed herein to a mouse model can result in PLT reduction over 3 days post administration that is less than the resulting PLT reduction of the non-

encapsulated Bcl-2/Bcl-X_L inhibitor. Preferably, the Bcl-2/Bcl-X_L inhibitor encapsulated in a liposome does not result in the reduction of PLT levels below about 400 PLT/microliter (preferably not below about 500 PLT/microliter and most preferably not below about 600 PLT/microliter) for at least about 21 days after injection in a mouse in any one of the test described in Examples 6 and 7 below.

Example 1: Determining Target Activity at Bcl-2, Bcl-X_L and Mcl-1

[0068] Compounds having inhibitory activity at Bcl-2, Bcl-X_L and/or Mcl-1 can be indentified using the assay of Example 1. The activity of compounds at targets Bcl-2, Bcl-X_L and Mcl-1 was determined using the assay of example 1. Unless otherwise indicated, the activity of compounds at Bcl-2, Bcl-X_L and Mcl-1 were determined using the following TR-FRET based assay.

[0069] Synthetic peptides specific for Bcl-2, Bcl-X_L and MCL-1 can be purchased or otherwise obtained by custom peptide synthesis. Synthetic genes can be obtained for GST-tagged Bcl-2, Bcl-X_L and Mcl-1 fragments. Proteins can be purified and used for assay development.

[0070] Materials:

- Bcl-2 (aa 1-207) E. coli expressed, RBC custom production
- Bcl-X_L (aa 1-209) E. coli expressed, RBC custom production
- Mcl-1 (aa 171-327) E. coli expressed, RBC custom production
- Custom peptides: All produced by Tufts University Core facility
- fBak Ac-GQVGRQLAIIGDK(5,6-FAM)-INR-NH2
- fBad Ac-NLWAAQRYGRELRRMSDK(5,6-FAM)-FVD-NH2
- fNoxa (6-FAM)-GELEVEFATQLRRFGDKLNF-NH2
- HTRF reagent: MAb Anti GST-Tb cryptate 61GSTTLA (CisBio)

A. Protein Production Summary

[0071] Bcl-2, Bcl-X_L and MCL-1 protein fragments were designed with a C-terminal GST tag. Summaries for protein production and purification are shown below. In case detailed protocol is required for any of the steps, it can be provided separately.

PROTEIN ID: Bcl-2 (C-GST)
MW (Da): 49245.20 **Expression:** BL21(DE3) cells were induced with 0.5 mM IPTG and grown in TB at 18 °C overnight **Purification:** Transformed cells were lysed in buffer containing 50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 10% glycerol, 1 mM TCEP, 1 mM PMSF, and benzamidine protease inhibitor cocktail. GST-tagged Bcl-2 was purified using a Glutathione Superflow Agarose (Pierce) column and then a Superdex HiLoad 16/600 SEC200 column. Protein was concentrated up to 2.52 mg/mL in the final formulation buffer. **Final formulation:** 50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 10% glycerol, 1 mM TCEP

PROTEIN ID: Bcl-X_L **(C-GST)**
MW (Da): 49777.2 **Expression:** BL21(DE3) cells were induced with 0.5 mM IPTG and grown in TB at 18 °C overnight **Purification:** Transformed cells were lysed in buffer containing 50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 10% glycerol, 1 mM TCEP and benzamidine protease inhibitor cockatil. GST-tagged Bcl-X_L was purified using a Glutathione Superflow Agarose (Pierce) column employing a step gradient from 0-25 mM Gluthatione in buffer containing 50 mM Tris-HCL (pH 8.0), 550 mM NaCl, 10% glycerol and 1mM TCEP. Protein was buffer exchanged and concentrated up to 3.88mg/mL in the final formulation buffer. **Final formulation:** 50 mM Tris-HCl pH 8.0, 500 mM NaCl, 10% glycerol, 1 mM TCEP

PROTEIN ID: Lot 2159b (Mcl-1s-GST)
MW (Da): 44436.00 **Expression:** BL21(DE3) cells were induced with 0.5 mM IPTG and grown in 2XTY at 37 °C for 4 h. **Purification: (change text)** Transformed cells were lysed in buffer containing 50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 10% glycerol, 1 mM TCEP and benzamidine protease inhibitor cockatil. GST-tagged Mcl-1s was purified using a Glutathione Superflow Agarose (Pierce) column followed by HiLoad 26-600 Superdex 200 column. Solution concentrated up to 3.93 mg/mL in the final formulation buffer. **Final formulation:** 50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 10% glycerol, 1 mM TCEP

B. Assay Buffer

[0072] The main objective of this assay development was to closely mimic assay set up described in Tao, Z.-F. et al. *Apoptosis-inducing agents for the treatment of cancer and immune and autoimmune diseases* 2011, Abbott Laboratories. Due to limited amount of experimental information in the patent, publications related to that work were used as references for buffer selection. Buffer 1 was selected as main buffer choice and buffer 2 was used as a close alternative.

Buffer 1: 100 mM Potassium Phosphate pH 7.5; 50 mM NaCl; 1 mM EDTA; 0.01% NP40 (see, Zhang, H., et al. Anal Biochem, 2002. **307**(1): p. 70-5 and Leverson, J.D., et al., Cell Death Dis, 2015. **6**: p. e1590).

Buffer 2: 20 mM Tris, pH 7.5; 50 mM NaCl; 0.01% NP40 (see Du, Y., et al. Assay Drug Dev. Technol, 2011. **9**(4): p. 382-93.)

C. Peptide selection

Protein target	Peptide
Bcl-2	fBak
Bcl-X_L	fBad
Mcl-1	fNoxa
Mcl-1	fBak

D. f-peptide titrations at fixed protein concentrations

[0073] f-peptide titrations were performed in order to evaluate signal window, select optimum peptide concentration and confirm if published peptide concentrations are within the desirable concentration range for use with in-house produced proteins and custom synthesis peptides. Mcl-1/Noxa was also tested in buffer 2 and showed identical behavior (data not shown). FIGs. 18A, 18B, 18C, and 18D are graphs showing the fluorescent peptide titrations at multiple protein concentrations.

E. Protein titration at fixed concentration of peptide

[0074] Based on the results of peptide titrations, proteins were titrated at peptide concentrations: C1= ~ K_d and C2= >K_d (100 nM, published assay peptide concentrations). Higher concentrations of peptides were selected for further experiments in order to maximize signal window. In all instances, selected peptide concentrations were sub-saturating. FIGs. 19A, 19B, 19C and 19D are graphs showing protein titrations at two f-peptide concentrations.

[0075] Protein titrations were repeated with an extended peptide range in order to get an accurate affinity constant estimation. FIGs. 20A, 20B, 20C, and 20D are graphs showing extended range protein titrations at single f-peptide concentration (see table below for protein/peptide pair concentrations).

F. Signal stability with time

[0076] HTRF technology enables multiple plate readings without negative effect on signal. In order to establish optimum incubation time, plates were scanned every 30 min for 2.5 hours. Signal stability as a function of time is shown below. At least 30 min incubation is required prior to reading in order to achieve maximum signal in assay. Bcl-2/fbak shows highest decrease in signal with prolonged incubation, while other pairs are less affected. A 60 minute incubation would be acceptable for all protein/peptide pairs. FIGs. 21A, 21B, 21C and 21D are graphs showing the signal stability over time.

G. Control compound displacement of probe peptide in final assay condition

[0077] Fluorescent peptide displacement by control compounds was tested in buffer 1. Protein and peptide concentrations were as shown in Table 2. Compounds were serially diluted in 100% DMSO and 15 nL of each compound concentration was delivered into protein solution using ECHO (Labcyte). Following 15 min pre-incubation, peptide was added to assay wells and mixture was further incubated for 15 min prior to addition of anti-GST-Tb

mAb. Plates were read every 30 min for 2.5 h. Data from ~60 min incubation was used for EC50 determinations. EC50 plots are shown below (FIGs. 22A, 22B, 22C, 22D, 22E and 22F). Mcl-1/fbak pair showed similar behavior to Mcl-1/fNoxa. EC50 plots for Mcl-1/fNoxa are shown. Data is summarized below (Table 2B).

Table 2B

	Compound IC_{50}* (M):			
	BC12/fbak	**BclXL/fBad**	**Mcl1/fNoxa**	**Mcl1/fbak**
Comparator A	2.75E-09	6.90E-10		
Comparator B	6.72E-11	2.46E-10		
Comparator C	5.20E-11	1.56E-07		
Comparator E			3.70E-08	4.8E-08
Comparator D		2.634E-10		

*Empty cells indicate no inhibition

H. Final Assay Conditions

[0078] The following final assay conditions were used (described in Tables 2C and 2D).

Table 2C

Buffer	100 mM potassium phosphate, pH 7.5; 50 mM NaCl 1 mM EDTA 0.01% NP40
Assay volume	15 uL
peptide	Protein dependent, see Table 2
Protein concentration	See Table 2
Incubation time	1 h
Reader	Envision (Perkin Elmer)

Table 2D. Selected protein concentrations for displacement IC_{50} determinations.

	Protein concentration, nM	Peptide used	Peptide concentration nM
Bcl-2	2.5	fBak	100
Bcl-XI	2	fBad	40
Mcl-1 Mcl-1	10 2.5	fNoxa fBak	100 100

Using the assay of Example 1 above, the following target activity was measured for the compounds listed in Table 3:

Table 3.

Compound ID	Bcl-2/fbak (nM)	Bcl-X_L /fBad (nM)
Compound 1	7.06	5.84
Compound 2	0.46	0.26
Compound 3	0.44	0.29
Compound 4	27.75	47.88
Compound 5	8.87	5.55
Compound 6	0.20	0.18
Compound 7	0.39	0.33
Compound 8	26.9	33.3
Compound 9	0.96	0.95
Compound 10	1.18	1.06
Reference Controls		
Comparator A	2.46	1.46
Comparator B	0.32	0.23
Comparator C (Bcl-2 selective)	0.15	
Comparator D (Bcl-X_L selective)	no inhibition	0.32

Example 2: Cellular activity of compounds of Formula (I) and Formula (II) against selected cell lines

[0079] FIG. 1C is a table (Table 3A) of the IC_{50} values measured for compounds on cell viability in multiple myeloma cells incubated with drug for 24 h, followed by washing and additional 72 h incubation. The IC_{50} values for compounds of formula (I) are plotted in the graph of FIG. 1A. The IC_{50} values for compounds of formula (II) are plotted in the graph of FIG. 1B.

Culture/treatment condition:

[0080] In vitro efficacy study was done using CellTiter-Glo® Luminescent Cell Viability Assay (Promega) with Corning Cat #3707 384 well White Clear bottom plates. Cells were plated (1000 cells/well) in 384 well format and allowed to incubate at 37 °C for 24 hours. Monotherapy drugs were added at the 24 h time point and then allowed to incubate at 37 °C for 24 hours. At the 48 hour time point or the appropriate time point the drugs in media were removed, washed with PBS, and fresh media was added. Cells were then allowed to incubate at 37 °C for 72 hours. For combination studies, cells were exposed to docetaxel for 24 hours, then docetaxel was removed and cells were exposed to the secondary compound (Bcl-2 inhibitor) for 24 hours. Cells were cultured in fresh media for an additional 48 hours. At the 120 hour time point media was removed and CellTiter-Glo (CTG) reagent was added (1:1 ratio with PBS). Plates were read using a luminometer (Envision Multilabel reader).

Data analysis:

[0081] Data was analyzed using an in-house algorithm developed using Matlab (Mathworks, Natick MA). In summary, average CTG mean luminescent values were computed for four replicate wells. Outlier detection was performed by computing the coefficient of variation (CV>20%) and outliers were removed from the average. CTG values were normalized based on a control non-treated well. Drug concentration in micromolar (uM) was log transformed prior to fitting to a four parameter logistic curve.

$$y = b + \frac{(a-b)}{\left(1+10^{(IC50-C)*slope}\right)}$$

Where C: concentration of drug, y: normalized CTG value, a: top asymptote (represents maximum cell kill), b: bottom asymptote (constrained 0.8-1.2), IC50, slope: logistic curve slope.

Data quality control was performed to ensure that the concentration range is optimal according to these rules: (1) if the lowest concentration kills more than 70% of the cells the concentration range is deemed too potent (2) if the highest concentration kills less than 30% of the cells, the concentration range is deemed low or the cell line is too resistant. Additionally, goodness of the fit was evaluated using R^2 and $R^2<0.9$ is flagged as a bad fit. Statistical analysis was performed using JMP (SAS Institute Inc., NC) and $p<0.05$ was considered significant.

Example 3: Dose-Response in combination with docetaxel

[0082] Figures 2A-2D are graphs showing the dose response of docetaxel (DTX) in MDA-MB231 breast carcinoma cells when combined with Comparator C, Compound 6, Compound 2, or Comparator E at three different concentrations.

[0083] This shows that the combination of Compounds 2 or 6 are able to synergize with docetaxel to significantly improve the antiproliferative activity with the respective combinations, and that this potentiation was greater for both compounds that for Compartors C or E. Finally it demonstrates that Compound 2 has a greater potentiating effect on docetaxel than Compound 6 in MDA-MB231 cells.

Culture/treatment condition:

[0084] In vitro efficacy study was done using CellTiter-Glo® Luminescent Cell Viability Assay (Promega) with Corning Cat #3707 384 well White Clear bottom plates. Cells were plated (1000 cells/well) in 384 well format and allowed to incubate at 37 °C for 24 hours. Monotherapy drugs were added at the 24 hour time point and then allowed to incubate at 37 °C for 24 hours. At the 48 hour time point or the appropriate time point the drugs in media were removed, washed with PBS, and fresh media was added. Cells were then allowed to incubate at 37 °C for 72 hours. For combination studies, cells were exposed to docetaxel for 24 hours, then docetaxel was removed and the cells were exposed to the secondary compound (Bcl-2 inhibitor) for 24 hours. Cells were cultured in fresh media for an additional 48 hours. At the 120 hour time point media was removed and CellTiter-Glo (CTG) reagent was added (1:1 ratio with PBS). Plates were read using a luminometer (Envision Multilabel reader).

Data analysis:

[0085] Data was analyzed using an in-house algorithm developed using Matlab (Mathworks, Natick MA). In summary, average CTG mean luminescent values were computed for four replicate wells. Outlier detection was performed by computing the coefficient of variation (CV>20%) and outliers were removed from the average. CTG values were normalized based on a control non-treated well. Drug concentration in microMolar (uM) was log transformed prior to fitting to a four parameter logistic curve.

$$y = b + \frac{(a-b)}{\left(1 + 10^{(IC50-C)*slope}\right)}$$

Where C: concentration of drug, y: normalized CTG value, a: top asymptote (represents maximum cell kill), b: bottom asymptote (constrained 0.8-1.2) , IC_{50}, slope: logistic curve slope.

[0086] Data quality control was performed to ensure that the concentration range is optimal according to these rules: (1) if the lowest concentration kills more than 70% of the cells the concentration range is deemed too potent (2) if the highest concentration kills less than 30% of the cells, the concentration range is deemed low or the cell line is too resistant.

Additionally, goodness of the fit was evaluated using R^2 and $R^2<0.9$ is flagged as a bad fit. Statistical analysis was performed using JMP (SAS Institute Inc., NC) and $p<0.05$ was considered significant.

Example 4: Liposome Preparations

Example 4-L1. Aqueous stock solution of Compound 6 dimesylate.

[0087] Free base Compound 6 (68.7 mg, 0.0743 mmol) in a powder form was thoroughly mixed with 0.1485 ml (0.1485 mmol) of 1 N aqueous methanesulfonic acid. To the resulting orange cake, 2 ml of distilled water was added, stirred with intermittent 1-2 min heating on a 65 °C water bath and subjected to 2 cycles of bath-sonication (5-10 sec.) until the clear solution having about 32 mg/ml of Compound 6 was obtained. The solution was adjusted to 20 mg/ml Compound 6 (as free base) by adding 1.29 ml of distilled water, and passed through a 0.45-micron polyvinylidenefluoride (PVDF) syringe filter. The solution had pH 2.80.

[0088] Stock solutions (20 mg/ml) of Compound 5, Compound 7, Compound 8, as well as Compound 1, Compound 2, Compound 3, and Compound 4, were obtained in the form of dimesylates in a similar manner. Prior to volume adjustment (to 20 mg/ml of the drug), formation of clear aqueous solutions of dimesylate salts of Compound 1 at about 89 mg/ml, and of Compound 5 at about 75 mg/ml was observed.

Example 4-L2. Injection solution of the "free" Compound 6.

[0089] In a 15-ml test tube, 6.92 ml of distilled water, 1.1 ml of 50% Dextrose USP, 0.33 ml of 1 M 4-hydroxyethylpiperazinoethanesulfonate (HEPES)-Na buffer, pH 7.0, and 1.65 ml of 20 mg/ml Compound 6 were mixed by vortexing and aseptically passed through 0.2-micron polyethersulfone (PES) syringe filter. The drug was assayed by spectrophotometry at 308 nm in 70% aqueous isopropanol-0.1 N HCl (1 OD unit – 0.0334 mg/ml Compound 6 free base). Found 3.307 ± 0.056 mg/ml (100.2 % of theory), pH 5.83.

Example 4-L3. Formulation of Compound 6 into PEGylated liposomes using ammonium sulfate gradient – 52 mg scale.

[0090] Distearoyl phosphatidylcholine (DSPC; Lipoid GMBH, Germany; 1002.4 mg), cholesterol "high purity" (Chol; Dishman, China; 327.3 mg), and PEG(2000)-

distearoylglycerol (PEGDSG; Sunbright GS-020, NOF, Japan; 334 mg) were combined in a closed glass vial with 2.5 ml of 100% ethanol (Sigma, USA, molecular biology grade) and heated on a 70 °C water bath until the lipids were completely dissolved. The target molar ratio of lipid components was DSPC:Chol:PEGDSG 3:2:0.3. The ethanolic solution of the lipids was quickly added to 22.8 g of 0.25 M ammonium sulfate, pH 5.27, pre-heated to 67.8 °C, with intensive stirring, and the stirring continued on a 70 °C water bath to form a suspension of multilamellar liposomes (MLV) as about 50 mM phospholipid. The MLV suspension was extruded through the stack of 4x100-nm and 1x200 nm track-etched polycarbonate filters (Whatman Nuclepore, USA) three times using Lipex thermobarrel extruder (Northern Lipids, Canada) with circulating water at 70 °C. The extruded liposomes were allowed to reach the room temperature and passed through 0.2-micron PES sterile filter. Particle size was determined by DLS using Malvern Zeta-sizer Nano; average liposome size (Xz, by the cumulants method) was 100.2 nm, polydispersity index (PdI) 0.036.

[0091] The extruded liposomes were chromatographed on a Sephadex G-25 (GE Healthcare, USA) column in distilled water as eluent. The void volume fraction containing liposomes was collected, and the phospholipid concentration was determined by acid digestion-blue phosphomolybdate spectrophotometric method to be 25.3 mM. In a 50-ml glass flask with a stirring bar, 15.7 ml of the post-Sephadex liposomes were combined with 5.06 ml of 50% Dextrose USP (to the final 88.4 mg/ml dextrose), 2.38 ml of distilled water (for volume adjustment), 0.26 ml of 1 M morpholinoethanesulfonic acid (MES)-Na OH buffer pH 5.2 (to the final 10 mM MES), and 2.6 ml of 20 mg/ml Compound 6 bis-mesylate solution (prepared according to Example 4-L1) to the final 2 mg/ml of the drug and drug/phospholipid ratio of 130.9 g/mol). The drug-liposome mixture was heated with stirring on a 65 °C water bath for 22 min. and chilled on ice. Ionic strength (IS) was adjusted to 0.1 M NaCl by adding 0.9 ml of 3 M NaCl stock solution. The IS-adjusted loading mixture (pH 4.86) was titrated with 1 M HEPES-Na buffer, pH 7.0, total 0.25 ml, to the final pH 5.62, and passed through a sterile 0.2-micron PES syringe filter. The filtered, pH- and ionic strength-adjusted loading mixture was purified from an non-encapsulated drug by column chromatography on a 10x volume of Sepharose CL-4B (GE Healthcare, USA), using 5 mM HEPES-Na, 144 mM NaCl, pH 6.5 buffer (HBS-6.5) for elution. The void volume fractions containing drug-loaded liposomes were combined and manually concentrated about 3-fold by diafiltration through a hollow fiber cartridge (C02-E500-10-N, Spectrum, USA). Liposome size Xz = 108.7 nm, PdI 0.028, the drug

concentration (determined as in Example 4-L2) 3.34 mg/ml; drug/phospholipid ratio 139.5 ± 2.6 g/mol (quantitative loading).

Example 4-L4. Treatment of Compound 6 liposomes with serum albumin.

[0092] The concentrated Compound 6 liposomes were treated with serum albumin as follows. Bovine serum albumin (BSA; A7906, Aldrich, USA) was dissolved in 0.85% aqueous NaCl to give 5% (w/w) solution, and the solution was passed through 0.2-micron syringe sterile filter. One volume part of the BSA solution was added to four volume parts of the Compound 6 liposomes to achieve the final 10 mg/ml of BSA, and the mixture was stirred at 37 °C for 50 min. The liposomes were purified from BSA using Sepharose CL-4B column chromatography, concentrated on a hollow fiber cartridge essentially as described above for the removal of non-encapsulated drug, and aseptically passed through 0.2-micron PES sterile filter. The albumin-treated, concentrated and filter-sterilized liposomes had 3.87 mg/ml of encapsulated Compound 6, drug/phospholipid ratio of 136.7 ± 0.8 g/mol, average liposome size Xz = 109.6 nm, PdI 0.027. The diafiltrate from the last the concentrating step was spectrophotometrically assayed for the free drug (at 308 nm) and residual free albumin (at 280 nm). Found free Compound 6 0.0065 mg/ml (0.17%), residual albumin 0.056 mg/ml. Treatment with albumin caused only a very minor loss of the liposome-associated drug (2.0%), which is within the error range of the drug/phospholipid ratio assay.

Example 4-L5. Formulation of Compound 6 into PEGylated liposomes using ammonium sulfate gradient – 2 mg scale.

[0093] For preliminary in vivo studies, the protocol of Example 4-L3 was performed on the scale of 2 mg of the drug (1 ml of the liposome loading mixture) at the drug/phospholipid ratio of about 125 g/mol, and with omission of the concentrating step. Post-load mixture (pH 4.82) was adjusted to pH 5.61 before the Sepharose CL-4B size exclusion chromatography purification step. Following the loading step, 1-ml portions of IS- and pH adjusted loading mixture were applied onto PD-10 columns (GE Healthcare, USA) filled with 10 ml of Sepharose CL-4B. The liposome fraction was collected between 3.0 and 4.5 ml, while the free drug fraction, being micellar in nature (volume-average micelle diameter about 8-15 nm), appears at the elution volume of 5.5 ml. The combined liposome fractions were sterilized by passage through 0.2-micron PES syringe filter, and analyzed for particle size, drug, and phospholipid concentrations. Found cumulants-

average liposome size Xz = 106.8 nm, PdI 0.034, volume-average liposome size Dv = 101.6 nm, drug concentration 1.15 mg/ml, phospholipid concentration 8.38 mM, drug/phospholipid ratio 137.2 g/mol (quantitative loading). A portion of this liposome formulation was treated with BSA essentially as described in Example 4-L4, except that the hollow fiber concentration step was omitted.

Example 4-L6. Formulation of Compound 1 into PEGylated liposomes using ammonium sulfate gradient.

[0094] Liposomes composed of DSPC, Cholesterol, and PEGDSG in the molar ratio 3:2:0.3, containing entrapped 0.25 M ammonium sulfate were prepared essentially as described in Example 4-L3; Xz = 100.3 nm, PdI = 0.067; Dv = 93.2 nm. Phospholipid (PhL) 45.0 mM. The extruded liposomes were chromatographed on a Sephadex G-25 (GE Healthcare, USA) column in distilled water as eluent. The void volume fraction containing liposomes was collected, and the phospholipid concentration was determined to be 27.1 mM. In a 12x75 glass tube with a stirring bar, 0.295 ml of the post-Sephadex liposomes were combined with 0.097 ml of 50% Dextrose USP (to the final 88.4 mg/ml dextrose), 0.052 ml of distilled water (for volume adjustment), 0.005 ml of 1 M morpholinoethanesulfonic acid (MES)-NaOH buffer pH 5.2 (to the final 10 mM MES), and 0.05 ml of 20 mg/ml Compound 1 bis-mesylate solution (prepared according to Example 4-L1) to the final 2 mg/ml of the drug and drug/phospholipid ratio of 125 g/mol). The drug-liposome mixture was heated with stirring on a 65 °C water bath for 20 min. and chilled on ice. Ionic strength (IS) was adjusted to 0.1 M NaCl by adding 0.0175 ml of 3 M NaCl stock solution. The IS-adjusted loading mixture (pH 4.30) was purified from an non-encapsulated drug by column chromatography on a 10x volume of Sepharose CL-4B (GE Healthcare, USA), using 5 mM HEPES-Na, 144 mM NaCl, pH 6.5 buffer (HBS-6.5) for elution. Liposome size Xz = 121.9 nm, PdI = 0.086, Dv = 118.0 nm; drug concentration 0.57 mg/ml; drug/phospholipid ratio 96.1 ± 0.4 g/mol (loading efficiency 76.9%). Loading efficiency (LE) is defined as the percent of the added drug that becomes encapsulated, and is calculated as follows: $LE = DL/DL_0*100\%$, where DL_0 is drug/phospholipid ratio before removal of non-encapsulated drug, and DL is drug/phospholipid ratio in the liposomes purified from non-encapsulated drug.

Example 4-L7. Formulation of Compound 5 into PEGylated liposomes using ammonium sulfate gradient.

[0095] The protocol of Example 4-L6 was followed to encapsulate Compound 5, except that the amount of 1 M morpholinoethanesulfonic acid (MES)-NaOH buffer pH 5.2 was increased to 0.01 ml (to the final 20 mM MES). The IS-adjusted loading mixture (pH 3.90) was purified from an non-encapsulated drug by column chromatography on a 10x volume of Sepharose CL-4B (GE Healthcare, USA), using 5 mM HEPES-Na, 144 mM NaCl, pH 6.5 buffer (HBS-6.5) for elution. Liposome size Xz = 102.2 nm, PdI = 0.033, Dv = 95.9 nm; drug concentration 0.57 mg/ml; drug/phospholipid ratio 109.4 ± 1.2 g/mol (loading efficiency 87.5%).

Example 4-L8. Formulation of Compound 4 into PEGylated liposomes using ammonium sulfate gradient.

[0096] The protocol of Example 4-L6 was followed to encapsulate Compound 4, except that the amount of 1 M morpholinoethanesulfonic acid (MES)-NaOH buffer pH 5.2 was increased to 0.01 ml (to the final 20 mM MES). The IS-adjusted loading mixture (pH 4.70) was purified from an non-encapsulated drug by column chromatography on a 10x volume of Sepharose CL-4B (GE Healthcare, USA), using 5 mM HEPES-Na, 144 mM NaCl, pH 6.5 buffer (HBS-6.5) for elution. Liposome size Xz = 183.4 nm, PdI = 0.208, Dv = 234.2 nm; drug concentration 0.51 mg/ml; drug/phospholipid ratio 111.5± 0.7 g/mol (loading efficiency 89.2%).

Example 4-L9. Formulation of Compound 7 into PEGylated liposomes using ammonium sulfate gradient.

[0097] The protocol of Example 4-L6 was followed to encapsulate Compound 7, except that the IS-adjusted loading mixture (pH 4.75) prior to Sepharose CL-4B chromatography was further adjusted to pH 5.50 with 0.005 ml of 1 M HEPES-Na buffer pH 7. Liposome size Xz = 106.7 nm, PdI = 0.021, Dv = 101.3 nm; drug concentration 0.82 mg/ml; drug/phospholipid ratio 131.5± 1.1 g/mol (quantitative loading).

Example 4-L10. Formulation of Compound 8 into PEGylated liposomes using ammonium sulfate gradient.

[0098] Liposomes composed of DSPC , Cholesterol, PEGDSG, and a fluorescent lipid label DiIC18(3)-DS (Molecular probes, USA) in the molar ratio of 3:2:0.3:0.0015, respectively, containing entrapped 0.25 M ammonium sulfate were prepared essentially as

described in Example 4-L3, except that the calculated amount of DiIC18(3)-DS was added to the ethanolic solution of other lipids as 25 mg/ml stock solution in dimethylacetamide. After removal of unentrapped ammonium sulfate on a Sephadex G-25 column, the liposome PhL concentration was 23.6 mM. In a 12x75 glass tube with a stirring bar, 0.678 ml of the post-Sephadex liposomes were combined with 0.195 ml of 50% Dextrose USP (to the final 88.4 mg/ml dextrose), 0.017 ml of distilled water (for volume adjustment), 0.01 ml of 1 M MES-NaOH buffer pH 5.2 (to the final 10 mM MES), and 0.1 ml of 20 mg/ml Compound 8 bis-mesylate solution (prepared according to Example 4-L1) to the final 2 mg/ml of the drug and drug/phospholipid ratio of 125 g/mol). The drug-liposome mixture was heated with stirring on a 65 deg. C water bath for 23 min. and chilled on ice. Ionic strength (IS) was adjusted to 0.1 M NaCl by adding 0.035 ml of 3 M NaCl. The IS-adjusted loading mixture (pH 4.86) was purified from an non-encapsulated drug by column chromatography on a 10x volume of Sepharose CL-4B (GE Healthcare, USA), using HBS-6.5 buffer for elution. Liposome size Xz = 104.4 nm, PdI = 0.022, Dv = 98.6 nm; drug concentration 1.13 mg/ml; drug/phospholipid ratio 134.5 ± 1.8 g/mol (quantitative loading).

Example 4-L11. Formulation of Compound 2 into PEGylated liposomes using ammonium sulfate gradient.

[0099] Liposomes composed of DSPC , Cholesterol, PEGDSG, and a fluorescent lipid label DiIC18(3)-DS (Molecular probes, USA) in the molar ratio of 3:2:0.3:0.0015, respectively, containing entrapped 0.25 M ammonium sulfate were prepared as in Example 4-L10. After removal of unentrapped ammonium sulfate on a Sephadex G-25 column, the liposome PhL concentration was 25.3 mM. In a 12x75 glass tube with a stirring bar, 0.316 ml of the post-Sephadex liposomes were combined with 0.097 ml of 50% Dextrose USP (to the final 88.4 mg/ml dextrose), 0.031 ml of distilled water (for volume adjustment), 0.005 ml of 1 M MES-NaOH buffer pH 5.2 (to the final 10 mM MES), and 0.05 ml of 20 mg/ml Compound 2 bis-mesylate solution (prepared according to Example 4-L1) to the final 2 mg/ml of the drug and drug/phospholipid ratio of 125 g/mol). The drug-liposome mixture was heated with stirring on a 65 deg. C water bath for 20 min. and chilled on ice. Ionic strength (IS) was adjusted to 0.1 M NaCl by adding 0.0172 ml of 3 M NaCl. The IS-adjusted loading mixture was stirred with approx. 300 mg of dry ion-exchange resin Dowex 1x8-200 in Cl- form, the liposomes were purified from non-encapsulated drug by

column chromatography on a 10x volume of Sepharose CL-4B (GE Healthcare, USA), eluted with 5 mM MES-NaOH, 140 mM NaCl, pH 5.26 (MBS-5.2 buffer), and passed through 0.2-micro-m polyethersulfone syringe filter. Liposome size Xz = 152.7 nm, PdI = 0.125, Dv = 164.4 nm; drug concentration 0.35 mg/ml; drug/phospholipid ratio 70.0 ± 0.3 g/mol (loading efficiency 56.0%).

Example 4-L12. Formulation of Compound 1 into PEGylated liposomes using triethylammonium sucrose octasulfate gradient

[00100] Potassium sucrose octasulfate was dissolved in water and passed through an ion-exchange column with Dowex 50Wx8 100-200 mesh in H+-form to obtain a solution of sucrose octasulfuric acid at the concentration of about 1 N. The solution was titrated with neat triethylamine to pH 6.5 and diluted to 0.43 N (0.05375 M) sucrose octasulfate to make triethylammonium sucrose octasulfate (TEA-SOS) solution. Hydrogenated soy phosphatidylcholine (HSPC, Lipoid, Germany), cholesterol, PEGDSG, and DiIC18(3)-DS at the molar ratio of HSPC:Chol:PEGDSG: DiIC18(3)-DS of 3:2:0.15:0.003 were combined with 100% ethanol (1.43 ml for each 1 g of the total lipid) and heated in a closed vial on a 70 deg.C water bath until complete dissolution. DiIC18(3)-DS was added as a 25 mg/ml stock solution in dimethylacetamide. The lipid solution was added to the stirred TEA-SOS solution preheated to more than 65 deg. C on a 68 deg. C water bath to achieve the final phospholipid concentration of about 60 mM. The obtained suspension of multilamellar vesicles was twice extruded through the stack of four 100-nm and two 200-nm track-etched polycarbonate filters (Whatman Nuclepore) in a Lipex thermobarrel extruder (Northern Lipids, Canada), heated at 68 deg. C, at the pressure of 450 psi. The extruded liposomes were cooled to ambient temperature and filtered through 0.2-micro-m Acrodisc HT syringe filter (Pall); liposome size Xz = 104.4 nm, PdI = 0.024, phospholipid concentration 47.2 mM. The liposomes were purified from unentrapped TEA-SOS by gel-chromatography on Sepharose CL-4B in water; the elimination of non-encapsulated TEA-SOS was verified by conductivity measurements in the liposome-containing void volume fraction (1 micro-S/cm or less). After purification, TEA-SOS liposomes had phospholipid concentration of 26.4 mM. A volume of 0.316 ml of the purified TEA-SOS liposomes was mixed with 0.11 ml of 50% dextrose USP (to final 50 mg/ml dextrose), 0.324 ml of distilled water (for volume adjustment), 0.25 ml of 20 mg/ml Compound 1 (Example 4-L1) to final 5 mg/ml of the drug and drug/phospholipid ratio 600 g/mol, and 0.007 ml of 1 M

MES-NaOH buffer pH 5.2 to the final pH of 3.78. The mixture was incubated with stirring on a 65 deg.C water bath for 30 min. and chilled on ice. One-half ml of the post-incubation loading mixture was adjusted to 0.14 M NaCl by adding 0.0245 ml 3 M NaCl and chromatographed on Sepharose CL-4B, eluent HBS-6.5, to remove non-encapsulated drug. The drug-loaded liposomes obtained in the void volume fractions were passed through 0.45 micro-m and 0.2 micro-m polyethersulfone syringe filters. Liposome size Xz = 194 nm, PdI = 0.052, drug concentration 1.84 mg/ml; drug/phospholipid ratio 586.7 ± 8.3 g/mol (loading efficiency 97.8%).

Example 4-L13. Formulation of Compound 8 into PEGylated liposomes using 0.25 M triethylammonium sulfate gradients at various drug-lipid ratios.

[00101] Solution of triethylammonium sulfate was prepared by neutralizing calculated amount of 2 N sulfuric acid (volumetric standard) with neat triethylamine to pH 6.0-6.5, and diluting the neutralized solution with distilled water to the final triethylammonium sulfate concentration of 0.25 M. Liposomes with entrapped 0.25 M triethylammonium sulfate were prepared essentially as described in Example 4-L3, except that DSPC:Chol:PEGDSG molar ratio was 3:2:0.18, 0.25 M 0.25 M triethylammonium sulfate solution was used instead on 0.25 M ammonium sulfate, and extrusion step included 4 passages through the polycarbonate filter stack. The extruded liposomes were purified from non-encapsulated triethylammonium sulfate by chromatography on Sephadex G-25 in water and had 24.1 mM phospholipid. Various amounts of triethylammonium sulfate liposomes were combined with Compound 8 dimesylate solution (Example 4-L1) at the final drug concentration of 2 mg/ml in the presence of 88.4 mg/ml dextrose and 10 mM MES-NaOH buffer (pH 5.2 at 1 M MES) to obtain drug/phospholipid ratios of 200, 300, 400, and 600 g/mol. The pH of drug-liposome mixtures were carefully adjusted with 1 M NaOH to pH 5.3-5.4, the mixtures were incubated with stirring on a 65 deg. C water bath for 20 min, chilled on ice, and 3 M NaCl was added to the final NaCl concentration of 0.1 M. The liposomes were purified from non-encapsulated drug by the column chromatography on Sepharose CL-4B, eluent HBS-6.5. Characteristics of the obtained Compound 8 liposomes are summarized in Table 4:

Table 4.

Drug/phospholipid ratio, g/mol		Drug, mg/ml	Encapsulation efficiency, %	Liposome size	
Pre-purification	Post-purification			Xz, nm	PdI
200	147.1	0.61	73.5	225.8	0.177
300	211.4	0.65	70.5	194.8	0.169
400	234.8	0.51	58.7	171.5	0.102
600	369.7	0.54	61.6	150.5	0.121

Example 4-L14. Formulation of Compound 8 into PEGylated liposomes using 0.35 M triethylammonium sulfate gradients at various drug-lipid ratios.

[00102] Solution of triethylammonium sulfate was prepared by neutralizing calculated amount of 2 N sulfuric acid (volumetric standard) with neat triethylamine to pH 6.0-6.5, and diluting the neutralized solution with distilled water to the final triethylammonium sulfate concentration of 0.35 M. Liposomes at various drug/phospholipid ratios were prepared as in Example 4-L13, except that the concentration of triethylammonium sulfate solution was 0.35 M. Characteristics of the purified drug-loaded liposomes are summarized in Table 4A:

Table 4A.

Drug/phospholipid ratio, g/mol		Drug, mg/ml	Encapsulation efficiency, %	Liposome size	
Pre-purification	Post-purification			Xz, nm	PdI
200	155.0	0.72	77.5	208.8	0.111
300	209.6	0.66	69.9	192.5	0.198
400	244.0	0.54	61.0	197.8	0.172
600	381.2	0.56	63.5	165.2	0.135

Example 4-L15. Formulation of Compound 8 into PEGylated liposomes in the absence of ammonium gradient.

[00103] Liposomes of DSPC, Cholesterol and PEGDSG were prepared according to Example 4-L13, except that instead of 0.25 M triethylammonium sulfate, 0.85% solution

of sodium chloride was used. The extruded liposomes were incubated with Compound 8 in the same way as described in Example 4-L13 at the drug/phospholipid ratio of 600 g/mol. After removal of extraliposomal drug on the Sepharose CL-4B column, the liposomes had the following characteristics: Xz = 263.7 nm, PdI = 0.230, drug concentration 0.12 mg/ml, drug/phospholipid ratio 85.7 g/mol (encapsulation efficiency 14.3%).

Example 4-L16. Formulation of Compound 8 into PEGylated liposomes containing ionic PEG-lipid derivative and 0.25 M ammonium sulfate

[00104] A 100-nm extruded liposomes of HSPC, cholesterol, and N-methoxy-PEG(2000)-oxycarbonyl)-distearoylphosphatidylethanolamine (PEGDSPE) at the molar ratio of 3:2:0.3, containing 0.25 M ammonium sulfate, were purified from unentrapped ammonium sulfate on a Sephadex G-25 column (eluent water), and incubated 24 min. with stirring on a 68 deg. C water bath in the presence of 2 mg/ml Compound 8 in 88.4 mg/ml dextrose, 10 mM MES-Na (pH 5.2 at 1 M MES)and drug/phospholipid ratio of 100 g/mol, at pH 4.40. The resulting liposomes were chilled on ice, adjusted to 0.1 M NaCl by addition of 3M NaCl stock solution, and purified from non-encapsulated drug by chromatography on a Sepharose CL-4B column, eluent HBS-6.5. The purification step was repeated once again, and the void volume fraction containing drug-loaded liposomes was analyzed for the drug and phospholipid content. Drug/phospholipid ratio in the loaded, purified liposomes was 68.5 g/mol, or 68.5% of its pre-purification value (encapsulation efficiency 68.5%).

Example 4-L17. Formulation of compound Comparator B into PEGylated liposomes using ammonium sulfate gradient.

[00105] FIG. 23 shows data obtained from a study comparing the effects of the free and liposomal Compound 6 (formulation 1) at 10 mg/mg, showing that liposomal drug has less thrombocytopenic effect. Commercially obtained compound Comparator B (Selleck Chemicals) was mixed with two equivalents of 1 M methanesulfonic acid, distilled water was added to achieve 10 mg/ml of Comparator B, and the suspension was agitated, with intermittent warming up on a 65 °C water bath and brief sonication in an ultrasound bath until most of the drug was dissolved. The solution was passed through 0.2-micron polyethersulfone filter, and the drug concentration in the filtrate (8.96 mg/ml) was determined by spectrophotometry (see Example 4-L2), using dilutions of 10 mg/ml Comparator B

solution in DMSO as standards; 1 OD at 308 nm was found to correspond to 0.0308 mg/ml of Comparator B. The drug was loaded into the liposomes of DSPC, Cholesterol, and PEG(2000)DSG essentially as described in Example 4-L5, except that the aliquots of the components were calculated to achieve, prior to the 65°C incubation step, the final concentration of 1.9-2.0 mg/ml of the drug, 88.2 mg/ml of dextrose, 10 mM MES-NaOH buffer (pH 5.2 at 1 M MES), and the liposome phospholipid at the drug/phospholipid ratios of 125, 200, or 300 g/mol. Post-incubation mixtures were adjusted to 0.1 M NaCl, and the pH was adjusted to pH 5.4-5.5 with 1 M HEPES_Na buffer. The liposome were purified from the non-encapsulated drug by Sepharose gel chromatography as in Example 4-L5, and the purified liposomes were passed through 0.45- micron PVDF filter. Only the 125 g/mol formulation was partially passable through 0.2-micron PES filter, while two other were not. The liposomes had the following properties (Table 4B):

Table 4B.

Drug/phospholipid ratio, g/mol		Drug, mg/ml	Encapsulation efficiency, %	Liposome size	
Pre-purification	Post-purification			Xz, nm	PdI
123.8	103.0	0.70	83.2	199.9	0.223
201.7	165.9	0.67	82.3	269.7	0.239
292	227.3	0.77	77.8	368	0.245

Example 4-L18. Formulation of Compound 2 into cholesterol-free PEGylated liposomes with an acidic phospholipid using ammonium sulfate gradient.

[00106] DSPC, Distearoylphosphatidylglycerol (DSPG Na-salt, Avanti Polar Lipids, AL, USA), PEGDSPE, and a fluorescent lipid label DiIC18(3)-DS (Molecular probes, USA) in the molar ratio of 2:1:0.2:0.002 were co-dissolved in a chloroform-methanol mixture, and the solution was evaporated to dryness in vacuum at 60°C. The lipid residue was dissolved in 100% ethanol, mixed with 0.25 M ammonium sulfate and processed to form liposomes as described in Example 4-L3, except that the number of passes through the polycarbonate membrane stack was five. After removal of unentrapped ammonium sulfate on a Sephadex G-25 column, eluted with deionized water, the liposomes (12.8 mM phospholipid, Xz=96.1 nm, PdI=0.066) were combined with 50% Dextrose USP to reach the final 50 mg/ml dextrose, and mixed with 34.8 mg/ml Compound 2 bis-mesylate stock solution (prepared as described

in Example 4-L1) to reach the drug/phospholipid ratios of 150, 225, or 300 g/mol (calculated for the drug free base content). To the drug-liposome mixtures 1 M Morpholinoethanesulfonic acid (MES)-NaOH buffer (pH 5.2) was added to the final MES concentration of 10 mM, the mixtures were heated with stirring on a 65 deg. C water bath for 30 min. and chilled on ice. The loaded liposomes were purified from unencapsupated drug by chromatography on a 10x volume of Sepharose CL-4B (GE Healthcare, USA) columns eluted with 5% aqueous dextrose. Ionic strength (IS) of the liposomes (eluted at the void volume of the columns) was adjusted to 0.1 M NaCl with 3 M NaCl, and the pH was adjusted to 7.0 by adding 1 M HEPES-NaOH buffer, pH 7.3, to 10 mM HEPES. The pH- and IS-adjusted liposomes were passed through 0.2-micro-m polyethersulfone syringe filter. The liposomes had the following characteristic:

Table 4C.

Drug/phospholipid ratio, g/mol		Drug,	Encapsulation	Liposome size	
Pre-purification	Post-purification	mg/ml	efficiency, %	Xz, nm	PdI
150	143.30	0.92	95.5	97.4	0.053
225	206.9	1.31	91.9	100.1	0.039
300	239.6	1.44	79.9	97.3	0.052

[00107] Notably and unexpectedly, the liposomes prepared without cholesterol and with an acidic phospholipid (DSPG), showed very effective loading of Compound 2 via ammonium sulfate gradient method (>90% loading), while keeping the average liposome size small (z-average particle size 97-100 nm) and narrow size distribution (PdI <0.1) up to the drug/phospholipid ratio of 225 g/mol, in spite of the fact that an ionic PEG-lipid derivative (PEGDSGE)was used instead of PEGDSG for liposome PEGylation. This is in contrast with the findings of Example 4-L11, where the cholesterol-containing liposomes without an acidic phospholipid showed only a moderate loading efficiency for Compound 2 (56%), a larger particle size of 152.7 nm, and a wider size distribution (PdI=0.125) at the drug/phospholipid ratio of 125 g/mol.

Example 4-L19. Formulation of Compound 2 into cholesterol-free PEGylated liposomes with an acidic phospholipid using triethylammonium sucrose octasulfate gradient.

[00108] Solution of TEA-SOS was prepared as described in Example 4-L12, except that the concentration of TEA-SOS was 0.46 N (0.0575 M). DSPC, DSPG, PEGDSPE, and DiIC18(3)-DS in the molar ratio of 2:1:0.2:0.002 were co-dissolved in a chloroform-methanol mixture, and the solution was evaporated to dryness in vacuum at 60°C. The lipid residue was dissolved in 100% ethanol, mixed with 0.46 N TEA-SOS, and processed to form liposomes as described in Example 4-L17. Unentrapped TEA-SOS war removed by repeated chromatography on a Sepharose CL-4B column eluted with 5% aqueous dextrose until the conductivity of the liposome eluate was less than 12 micro-S/cm. The eluted liposomes (13.7 mM phospholipid, Xz=88.7 nm, PdI=0.003) were mixed with 34.8 mg/ml Compound 2 bis-mesylate stock solution (prepared as described in Example 4-L1) to reach the drug/phospholipid ratios of 150, 200, 250, or 300 g/mol (calculated for the drug free base content). The drug-liposome mixtures (pH 5.15.-5.23, no buffer added) were heated with stirring on a 68 deg. C water bath for 30 min. and chilled on ice. The loaded liposomes were purified from unencapsupated drug by chromatography on a 10x volume of Sepharose CL-4B (GE Healthcare, USA) columns eluted with 5% aqueous dextrose. Ionic strength (IS) of the liposomes (eluted at the void volume of the columns) was adjusted to 0.1 M NaCl with 3 M NaCl, and the pH was adjusted to 7.0 by adding 1 M HEPES-NaOH buffer, pH 7.3, to 10 mM HEPES. The pH- and IS-adjusted liposomes prepared at drug/phospholipid ratios of 150 and 200 g/mol were passed through 0.2-micro-m polyethersulfone syringe filter. The liposomes prepared at drug/phospholipid ratios of 250 and 300 g/mol did not pass through 0.2-micro-m polyethersulfone syringe filter and were characterized as is.The liposomes had the following characteristics:

Table 4D.

Drug/phospholipid ratio, g/mol		Drug,	Encapsulation	Liposome size	
Pre-purification	Post-purification	mg/ml	efficiency, %	Xz, nm	PdI
150	149.0	1.09	101.5	95.9	0.051
200	188.7	1.37	96.4	117.2	0.084
250	246.9	1.79	100.9	143.5	0.146
300	292.3	2.02	99.5	184.6	0.172

Example 4-L20. Formulation of Compound 2 into cholesterol-free PEGylated liposomes with an acidic phospholipid using triethylammonium sucrose octasulfate gradient at various concentrations of TEA-SOS.

[00109] Solution of TEA-SOS having the concentration of 0.43 N, 0.65, N, and 1.083 N were prepared as described in Example 4-L12. DSPC, DSPG, PEGDSPE, and DiIC18(3)-DS in the molar ratio of 2:1:0.2:0.003 were co-dissolved in a chloroform-methanol mixture, the solution was divided into three portions and evaporated to dryness in vacuum at 60°C. The lipid residues were dissolved in 100% ethanol, mixed with 0.43 N, 0.65 N, or 1.083 N TEA-SOS, and processed to form liposomes as described in Example 4-L17 (four passes through the polycarbonate membrane stack). Unentrapped TEA-SOS was removed by repeated chromatography on a Sepharose CL-4B column eluted with 5.1%, 7.8%, or 14.0% aqueous dextrose, respectively, until the conductivity of the liposome eluate was less than 13 micro-S/cm. The eluted liposomes (11.7-14.3 mM phospholipid, Xz 87.6-92.4 nm, PdI 0.049-0.076) were mixed with 37.4 mg/ml Compound 2 bis-mesylate stock solution (prepared as described in Example 4-L1) to reach the drug/phospholipid ratios of 150 or 300 g/mol (calculated for the drug free base content). The drug-liposome mixtures were heated with stirring on a 65 deg. C water bath for 30 min. and chilled on ice. The loaded liposomes were purified from unencapsupated drug by chromatography on a 10x volume of Sepharose CL-4B (GE Healthcare, USA) columns eluted with 5.1%, 7.8%, or 14.0% aqueous dextrose, respectively. The liposomes prepared at drug/phospholipid ratio of 150 g/mol were passed through 0.2-micro-m polyethersulfone syringe filter. The liposomes prepared at drug/phospholipid ratios of 300 g/mol did not pass through 0.2-micro-m polyethersulfone (PES) syringe filter and were characterized as is.The liposomes had the following characteristics:

Table 4E.

Entrapped TEA-SOS, N	Post-purification Drug/phopspholipid ratio, g.mol	Drug, mg/ml	Encapsulation efficiency, %	Liposome size	
Pre-purification drug/phospholipid ratio 150 g/mol				Xz, nm	PdI
0.43	143.7	1.09	95.8	94.4	0.048
0.65	148.6	1.37	99.1	97.4	0.031
1.083	147.1	1.79	98.0	99.9	0.069

Pre-purification drug/phospholipid ratio 300g/mol					
0.43	284.7	1.42	94.9	205.8	0.164
0.65	286.3	1.55	95.4	168.6	0.153
1.083	284.2	1.58	94.7	170.0	0.157

Example 4-L21. Formulation of Compound 2 into cholesterol-free PEGylated liposomes with various amounts of acidic phospholipid using triethylammonium sucrose octasulfate gradient.

[00110] Solution of TEA-SOS (0.43 N) was prepared as described in Example 4-L12. DSPC, DSPG, PEGDSPE, and DiIC18(3)-DS were co-dissolved in a chloroform-methanol mixture at the molar ratios of 2.4:0.6:0.2:0.003 ("20mol.% DSPG"), 2.7:0.3:0.2:0.003 ("10mol.% DSPG"), and 2.85:0.15:0.2:0.003 ("5 mol.% DSPG"). Also, a lipid composition at 10 mol.% DSPG containing PEGDSG instead of PEGDSPE in the same molar amount was prepared. The solutions were evaporated to dryness in vacuum at 60°C. The lipid residues were dissolved in 100% ethanol, mixed with 0.43 N TEA-SOS, and processed to form liposomes as described in Example 4-L17. Unentrapped TEA-SOS war removed by repeated chromatography on a Sepharose CL-4B column eluted with 5% aqueous dextrose until the conductivity of the liposome eluate was less than 25 micro-S/cm. The eluted liposomes (23.5-25.0 mM phospholipid) were mixed with 40.3 mg/ml Compound 2 bis-mesylate stock solution (prepared as described in Example 4-L1) to reach the drug/phospholipid ratio of 150 g/mol (calculated for the drug free base content). The drug-liposome mixtures (pH 4.5.-5.4, no buffer added) were heated with stirring on a 65 deg. C water bath for 30 min. and chilled on ice. The loaded liposomes were purified from unencapsupated drug by chromatography on a 10x volume of Sepharose CL-4B (GE Healthcare, USA) columns eluted with 5% aqueous dextrose. The eluted liposomes were passed through 0.2-micro-m PES syringe filter. The liposomes had the following characteristics:

Table 4F.

DSPG, mol. %	Post-purification Drug/phopspholipid ratio, g.mol	Drug, mg/ml	Encapsulation efficiency, %	Liposome size	
PEGDSPE compositions				Xz, nm	PdI
20	147.1	1.77	98.1		

10	145.8	1.67	97.2		
5	148.1	1.29	98.8		
PEGDSG composition					
10	147.4	1.745	98.2		

Example 4-L22. Formulation of Compound 2 into the liposomes containing the drug in a membrane-entrapped form.

[00111] Due to their relatively hydrophobic nature, compounds of the invention can be formulated to be contained within the bilayer membrane of the liposomes, that is, in the membrane-entrapped form, by the following general protocol. The drug (e.g., Compound 2) is dissolved in chloroform-methanol mixture (1:1 by volume) at the concentration of 10-50 mg/ml, and brought into dihydrichloride salt form by addition of the calculated amount of 1.25 M HCl solution in isopropanol (Sigma-Aldrich, USA). The solution is added to the liposome lipids dissolved in chloroform, optionally with addition of methanol, and the drug-lipid solution is evaporated in vacuum at 40-60 deg. C to dryness. The dry lipid cake is dissolved in 100% ethanol to form approximately 50% (w/w) solution at 70-75 deg. C, and mixed, at this temperature, with 10 volumes of pre-heated 5 mM HEPES-NaOH buffer, 144 mM NaCl, pH 6.5 (HBS-6.5 buffer). The drug-lipid suspension is extruded through polycarbonate membranes as described in Example 4-L3. The extruded liposome suspension is passed through a Sepharose CL-4B column, eluted with 10 mM HEPES-NaOH, 140 mM NaCl buffer pH 7.25 (HBS-7.25 buffer) to purify the liposomes from extraliposomal (e.g., micellar) drug. The purified liposomes are sterilized by passage through 0.2 micro-m (e.g., PES) filter. The following liposomes containing Compound 2 in the membrane-entrapped form were prepared:

Table 4G.

Drug, mol. parts	Lipid, mol parts:			Drug, mg/ml	DL ratio, g/mol	Encapsulation efficiency, %	Particle size	
	DSPC	Chol	DSPG				Xz, nm	PdI
1	2	1	0	2.32	468.9	98.6	1600	0.952

1	2	0	1	2.32	323.5	99.0	101.3	0.059
0.6	2	0	1	2.19	199.1	101.5	97.2	0.045
0.3	2	0	1	1.50	103.2	105.2	99.6	0.014
1	1.5	0	1.5	2.48	327.5	100.2	93.8	0.029
1	1	0	2	2.23	348.8	106.7	94.7	0.082
1	0.5	0	2.5	2.07	316.2	96.7	93.0	0.051

[00112] All lipid compositions also included 0.2 mol. parts of PEGDSPE and 0.002 mol. parts of DiIC18(3)-DS. Chol: cholesterol. DL ratio – drug/phospholipid ratio.

Example 4-L23. Formulation of Compound 2 into the liposomes containing the drug in a membrane-entrapped form in the presence of various anionic lipids.

[00113] Liposomes containing Compound 2 (1 mol part), DSPC (2 mol.parts), PEG2000DSPE (0.2 mol. parts), DiIC18(3)-DS (0.002 mol parts) , and 1 mol part an anionic lipid cholesteryl hemisuccinate (CHEMS, Sigma-Aldrich, USA), dimyristoyl phosphatidic acid (DMPA Na salt, Avanti Polar Lipids, USA), or cholesteryl sulfate ($CHSO_4$, Na salt, Avanti Polar Lipids, USA) were prepared according to Example 4-L22. The Sepharose-purified and 0.2 micro-m filtered liposomes had the following characteristics:

Table 4H.

Anionic lipid	Post-purification Drug/phospholipid ratio, g/mol	Drug, mg/ml	Encapsulation efficiency, %	Liposome size	
				Xz, nm	PdI
CHEMS	518.9	1.32	96.6	98.0	0.035
DMPA	320.2	1.11	90.2	73.8	0.045
$CHSO_4$	Not extrudable through polycarbonate membrane				

Example 5: Blood pharmacokinetics of novel liposomal Bcl-2/Bcl-X_L inhibitors

[00114] Liposomal formulations of Bcl-2 inhibitor Compound 6 (Formulation #1 - without BSA-treatment, and Formulation #2 - with BSA treatment) were prepared as

described in Example 4-L5. Free drug injection solution was prepared according to Example 4-L2. The liposomes and the free drug were administered intravenously at a dose of 15 mg drug/kg or 10 mg/kg, respectively, to three 6 week-old female CD-1 mice (Charles River) (body weight about 25 g). Blood samples were collected into lithium heparin tubes by bleeding from saphenous vein at 0.08, 0.25, 1.5, 4, 8 24 and 48h (post injection, p.i.) time points. Plasma was separated from the cell fraction by centrifugation at 10000 rpm for 5 min. Drugs were extracted by incubation of plasma samples with 200 µl of 1% acidic acid in methanol (1%Ac/MeOH) at least 2 hours at -80oC. Plasma proteins were spin down by centrifugation at 15000 rpm for 20 min. Then 75 µl of supernatant was transferred to HPLC vials (Thermo Scientific, Cat# C4011-LV1) and additional 75 µl of 1% Ac/MeOH were added. Drug content was analyzed by HPLC with each sample measured in duplicate. The data were expressed as the drug concentration in plasma plotted against post injection time in hours (Figure 3). PK parameters were calculated for each individual mouse using Pharmacokinetics data analysis package "PK Solutions" (Summit Research Services, CO, USA) and average values for each treatment group are presented in Table 5.

[00115] Pharmacokinetic profiles of two liposomal formulations of Compound 6 were compared with that of the free drug. As shown in FIG. 3 liposomal formulations of Bcl-2 inhibitor demonstrate superior in vivo circulation times relative to free (nonencapsulated) drug. Plasma half-live was improved from 0.35 hours for free drug to 11.1 hours for the drug encapsulated into liposomes (Table 5).

Table 5. Pharmacokinetics parameters of liposomal Bcl-2 inhibitor

Drug ID:	**Ls-Compound 6 (Formulation 1)**	**Ls-Compound 6 (Formulation 2)**	**free Compound 6**
	Average ± SD	**Average ± SD**	**Average ± SD**
Mice per group	3	3	3
Dosage, µg/kg	15000	15000	10000
C_{max}, µg/ml	379 ± 46.5	482.1 ± 59.5	68.3 ± 7.5
AUC, µg-hr/ml	2299 ± 72	8990 ± 1273	12.2 ± 1.5
Vd, ml	3.8 ± 0.6	1.5 ± 0.3	10.0 ± 2.9
CL, ml/hr	0.2 ± 0.01	0.1 ± 0.01	19.5 ± 2.0
Half-life, hr	11.09 ± 1.7	15.4 ± 4.2	0.35 ± 0.06

[00116] Preferably, the liposome is formulated to reduce the blood clearance rate of the Bcl-2/Bcl-X_L inhibitor compounds from the blood and/or to reduce the release of the Bcl-2/Bcl-X_L inhibitor compounds from the liposome in plasma. In one aspect, the liposome is

contacted with albumin (e.g., BSA) under conditions effective to increase the concentration of the Bcl-2/Bcl-X_L inhibitor compound in mouse plasma over at least 4 hours, and preferably over at least 48 hours, as described in FIG. 3 and Example 5.

[00117] The further improvement in PK properties of the liposomal drug was achieved by pretreatment of drug-loaded liposomes with BSA (Formulation #2). As shown in Figure 3, the drug concentration in plasma after injection of the Compound 6-loaded liposomes treated with BSA (Formulation #2) was significantly higher over time compared to liposomes without BSA treatment (Formulation # 2). This observation was related to the fast initial clearance phase that lasted until about between 1.5 and 4 hours post injection. Formulation #1 lost about 55% of the injected dose in the first 15 minutes after injection comparing to 0.1% for Formulation #2. Therefore, BSA-pretreatment stabilized the liposomal formulation of Bcl-2 inhibitor against fast initial clearance from the blood and/or fast initial drug release from the liposomes leading to better PK properties in vivo.

Without being bound by a theory, it is suggested that treatment with albumin may help to remove a portion of the inhibitor that remains bound to the outer leaflet of the liposome bilayer membrane and potentially creates either a membrane defect that, upon contact with blood, facilitates drug leakage from the liposome interior, or promotes opsonization of the liposomes resulting in faster liposome clearance. Sequestration of Bcl-2 inhibitors by serum albumin is documented (Vogler M, et al., Blood, vol. 117, p. 7145-7154, 2011).

Example 6: Effect of liposomal encapsulation on the thrombocytopenic activity of Bcl-2/Bcl-X_L inhibitors

[00118] It is known that thrombocytopenia, the decrease in blood platelet counts, is a dangerous side effect of the treatment of patients with Bcl-2 inhibitors associated with their toxicity against the platelets. the effect of liposome encapsulation on the thrombocytopenic activity of the Bcl-2 inhibitor Compound 6 in mice was evaluated. In the first study, 5-6 week-old female CD-1 mice (Charles River) were intravenously injected with the free or liposomal Compound 6 (Formulation #1) at the dose of 10 mg/kg. at a single dose of 10 mg/kg. Blood samples (50-100 μL) were drawn into micro-collection tubes with EDTA dipotassium salt (SARSTEDT lot# 4072801) by bleeding from saphenous vein at 0 (pre-injection), 6, 24, 48 and 72 hour post injection. In the control group, the mice received injection of the buffered physiological saline, and the blood was collected and processed similarly. Platelets were counted immediately after blood collection using a Veterinary

Hematology System "Hemavet 950" (The Americas Drew Scientific Inc, Oxford, CT). Quality control was performed using MULTI-TROL Controls Dog blood samples (Drew Scientific). As shown on FIG. 23, in the free drug group, the average platelet counts dropped from the pre-treatment value of 900,00-1,100,000 cells/micro-L to about 250,000 cells/micro-L at 6 hours post injection and stayed at approximately the same value at 24 hours post injection, while in the liposome group, the initial drop was significantly less pronounced: to about 550,000 cells/micro-L at 6 hours (free vs. liposomal drug, p=0.015), and at 24 hours the platelet count was about 900,000 cells/micro-L (free vs. liposomal drug, p=0.005), i.e., within the range of untreated controls. At 48 and 72 hours post injection the platelet counts were in the pre-treatment range (about 1,000,000 cells/micro-L) in both groups (FIG. 23). Thus, liposomal encapsulation of the Bcl-2 inhibitor compound 6 resulted in the significant decrease of its thrombocytopenic activity. This result was contrary to the expectations from previously reported data on the high endocytosis of liposomes by platelets (Male R., Vannier W.E, Baldeschwieler J.D., Proc. Natl Acad. Sci. USA, vol. 89, p. 9191-9195, 1992), suggesting that liposome encapsulation of a thrombocytotoxic drug would increase, not decrease, its adverse effect on the platelets; however, to our surprise, with regard to the liposomes of the present invention, it proved not to be the case.

[00119] In the second study, the effect of albumin treatment on the thrombocytopenic activity of the liposomal formulations of the present invention were compared. Compound 6 liposomes without BSA-treatment (Formulation #1) or with BSA-treatment (Formulation #2) were injected intravenously to mice at the dose of 15 mg/kg, the blood was drawn and analyzed essentially in the same way as in the first study (above). The results, shown on Figure 4, demonstrated that by treatment with albumin the thrombocytopenic effect of the liposomal Bcl-2 inhibitor was further decreased. Given that albumin treatment also eliminated the fast initial blood clearance phase in the pharmacokinetics of the liposomal Bcl-2 inhibitor, it is suggested, without being bound by a theory, that treatment with albumin prevents quick release of a portion of the encapsulated drug shortly after the injection, further reducing the exposure of platelets to the free (released) inhibitor.

[00120] In the third study, thrombocytopenic effect of free and liposomal Bcl-2 inhibitor of the present invention was compared in a multiple injection setting at various doses of the drug. Female CD-1 mice, in groups of three, received four tail vein injections, at the intervals of 7 days, of liposomal Bcl-2 inhibitor Compound 6 (Formulation #2) or free (nonencapsulated) Compound 6 at the doses of 10, 18 and 32.5 mg drug/kg. Blood samples were collected and processed as described in the first study (above). Additionally, to assess

treatment-related toxicity, the animals were weighted daily. The results are shown on Figures 5A, 5B and 5C, and Figures 6A, 6B and 6C.. At all dose levels, the initial post-injection drop in platelet counts was significantly more pronounced in the free drug groups (typically to 200,000-400,000 cells/micro-L), while in the liposome groups the platelet counts stayed above 400,000 cells/micro-L, typically at 500,000 cells/micro-L or more, with pre-treatment values being 700,000-800,000 cells/micro-L. There was no apparent cumulative effect on the platelet toxicity in either free or liposomal inhibitor treatment group. Thus, formulation of the Bcl-2 inhibitor in liposomes strongly improved the negative effect of the drug on platelets' survival comparing to free drug in the full range of the applied doses (Figures 6A, 6B and 6C). The animals in 10 mg/kg and 32.5 mg/kg liposomal drug dose groups showed retardation of growth compared to the animals in the free drug groups, suggesting that liposomal encapsulation may have increased some non-platelet related physiological effects of the drug; however, except for one single data point in the group receiving the highest dose of the liposomal drug, no body weight loss was observed in any of the groups (Figures 5A, 5B, and 5C), indicating that all treatments were well tolerated.

Example 7: Comparison of the thrombocytopenic activity of free (nonencapsulated) compounds Compound 2 and Compound 6 in mice.

[00121] Compound 2 or Compound 6 were dissolved in water as dimesylate salts (see Example 4-L1), further diluted in 5% Dextrose USP, and the pH was adjusted to about pH = 6 with 1M HEPES buffer.. Drug solutions were sterilized with 0.2 micron Nalgene™ 13mm Syringe Filters. Drug concentration in the solutions was determined by HPLC. 5-6 week-old female CD-1 mice (Charles River) were injected intravenously via the tail vein with a single bolus of free (non-encapsulated) Compound 2 or Compound 6 at 1, 2 or 5 mg/kg. Blood samples (50-100 µL) were collected into tubes pre-filled with EDTA dipotassium salt (SARSTEDT, lot# 4072801) by bleeding from saphenous vein at 0, 6, 24, 48 and 72 hour time points. Platelets were counted immediately after blood collection using a Veterinary Hematology System "Hemavet 950" (The Americas Drew Scientific Inc, Oxford, CT). Quality control was performed using MULTI-TROL Controls Dog blood samples (Drew Scientific Inc, lot # 600066).

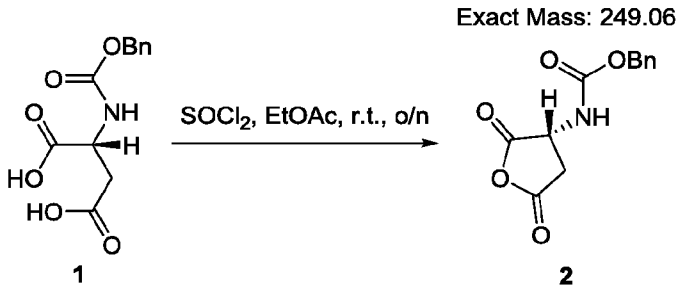
[00122] As shown in In Figures 7A, 7B, and 7C, Compound 2 is more active towards platelets compared to Compound 6 and causes severe thrombocytopenia even at doses as low as 1-2 mg of drug/kg. Referring to FIGs. 7A, 7B, and 7C, the first data point obtained at the "-1" time was the background measurement taken from blood drawn one day prior to the

injection of each specified test compound. The second data point for each curve was obtained 6 hours after injection of the test compound into the mouse in each experiment. (This is different from the data obtained in FIG. 4, where the background blood reading was taken on the same day as the first post injection blood reading 6 hours after administration of the test formulations 1 and 2). Referring to FIG. 4, the measurements at time 0 represent a background blood test performed prior to injection of the test liposome Formulations 1 and 2. The measurements at 6 hours were taken 6 hours after injection of test liposome Formulations 1 and 2 on the same day as the background blood test reading taken at time 0. (This is different from the method of obtaining data shown in FIGs. 7A, 7B and 7C, where the background reading indicated as time "-1" in each graph was obtained the day before administration of the test compounds, and the next data point in each curve was obtained 6 hours after administration of the test compound on the day after the background reading was obtained.)

Example 8: Synthesis of Intermediates and Compounds

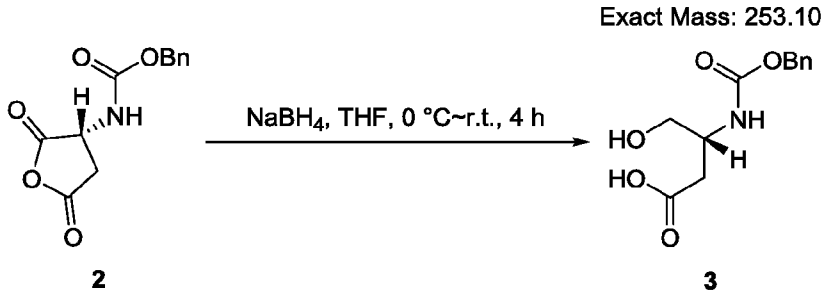
[00123] The synthesis of intermediates 4, 13, 18, 20 was accomplished according to the literature (Cheol-Min Park, et al. J. Med. Chem. 2008, vol. 51, 6902-6915 and reference therein)

1. Synthesis of intermediate 2



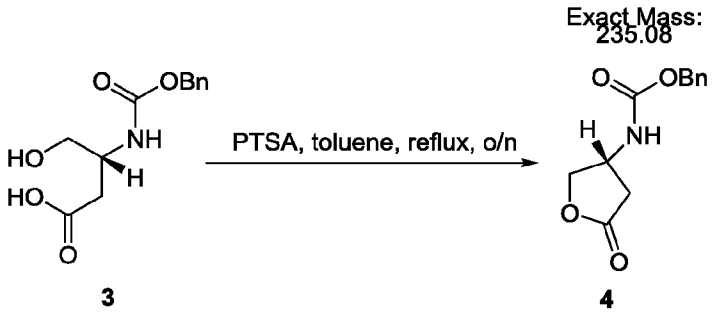
[00124] To the suspension of 1 (30.0 g, 112.3 mmol) in ethyl acetate (200 mL) was drop-added $SOCl_2$ (80 mL) at room temperature. After the addition was completed, the mixture was stirred at room temperature overnight. The solvent was removed in vacuo and the residue was suspended in ether/hexane (100 mL/100 mL) and stirred for two hours. The suspension solution was filtered and intermediate 2 (26.1 g, 93%) was afforded as a white solid.

2. Synthesis of intermediate 3



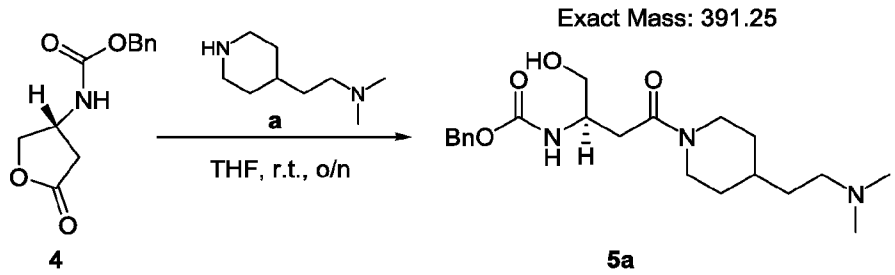
[00125] To the mixture of $NaBH_4$ (4.76 g, 125 mmol) in THF (150 mL) at 0 °C was added a suspension of intermediate 2 (26.0 g, 104 mmol) in THF (150 mL) in two hours. Then the mixture was stirred at 0°C for 1 hour and warmed to room temperature, stirred at room temperature for 3 hours. The mixture was acidified to pH = 2~3 with HCl (conc.) and concentrated to remove the organic solvent. Water (300 mL) was added and the result solvent was extracted with Et_2O (150 mL x 4). The organic layer was combined and concentrated to dryness, which was directly used for the next step without any further purification.

3. Synthesis of intermediate 4



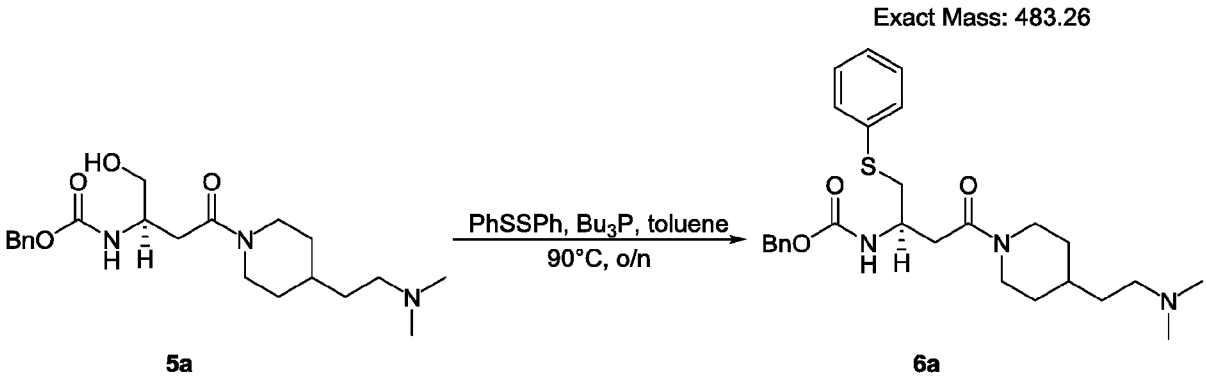
[00126] To the crude residue of 3 was added toluene (150 mL) and p-TsOH (150 mg). The mixture was refluxed for 6 hours equipped with a Dean-Stark apparatus. The solvent was removed in vacuo and Et_2O (200 mL) was added. The mixture was stirred at room temperature for 2 hours and then filtered to afford 4 (18.9 g, 77% for 2 steps) as a white solid.

4. Synthesis of intermediate 5a



[00127] To the solution of intermediate 4 (4.8 g, 20.4 mmol) in THF (80 mL) was added a (3.2 g, 20.4 mmol), and the solution was stirred at room temperature overnight. After completion of the reaction, the solvent was removed in vacuo to afford intermediate 5a (7.2 g, 100%) as yellow oil, which was used in the next reaction without any further purification.

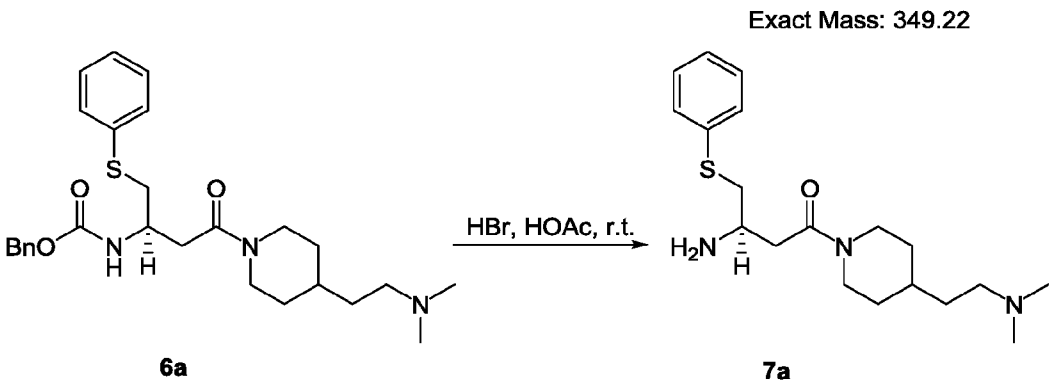
5. Synthesis of intermediate 6a



5a 6a

[00128] The crude 5a (6.7 g, 17.1 mmol) was dissolved in the toluene, and Bu_3P (7.0 g, 34.2 mmol), PhSSPh (5.6 g, 25.7 mmol) were added. The mixture solution was stirred at 90°C overnight. The solvent was removed in vacuo and the residue was purified by chromatography column (eluted with PE/EA = 5/1) to afford intermediate 6a (6.8 g, 82%) as a yellow oil.

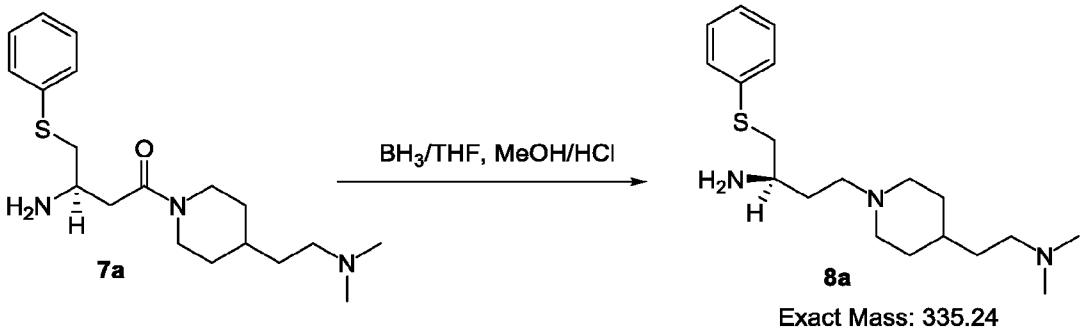
6. Synthesis of intermediate 7a



6a 7a

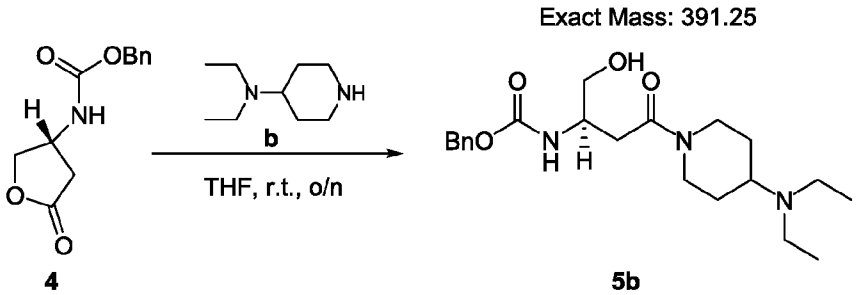
[00129] To the solution of intermediate 6a (6.8 g, 14.1 mmol) in HOAc (21 mL) was added HBr (7 mL), and the mixture solution was stirred at room temperature overnight. Then removed the solvent at reduced pressure, the residue 7a (4.7 g, 96%) was directly used for the next step.

7. Synthesis of intermediate 8a



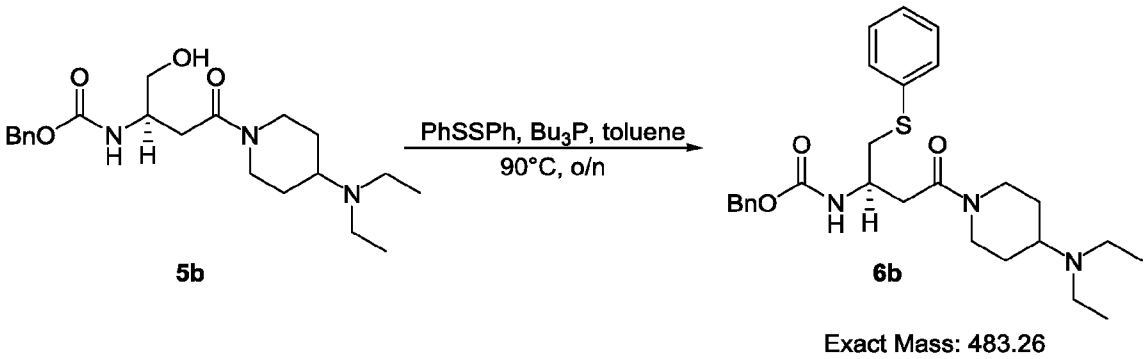
[00130] To the solution of 7a (3.0 g, 8.6 mmol) in THF (25 mL) was added BH_3/THF (1.0 M, 17.2 mL), the mixture was stirred at room temperature overnight. After completion of addition, MeOH (5 mL) was added slowly to quench the reaction, and then removed the solvent at reduced pressure, redissolved the residue in MeOH (25 mL) and HCl (3 mL). The mixture was reflux for 4 hours. Concentrated the solvent to afford 8a (3.9 g, 100%) as yellow solid, which was directly used for the next step.

8. Synthesis of intermediate 5b



[00131] To the solution of intermediate (7.0 g, 30.0 mmol) in THF (80 mL) was added b (4.7 g, 30.0 mmol), and the solution was stirred at room temperature overnight. After completion of the reaction, the solvent was removed in vacuo, the residue was purified by chromatography column (PE/EA = 2/1) to afford intermediate 5b (7.3 g, 63%) as yellow oil.

9. Synthesis of intermediate 6b



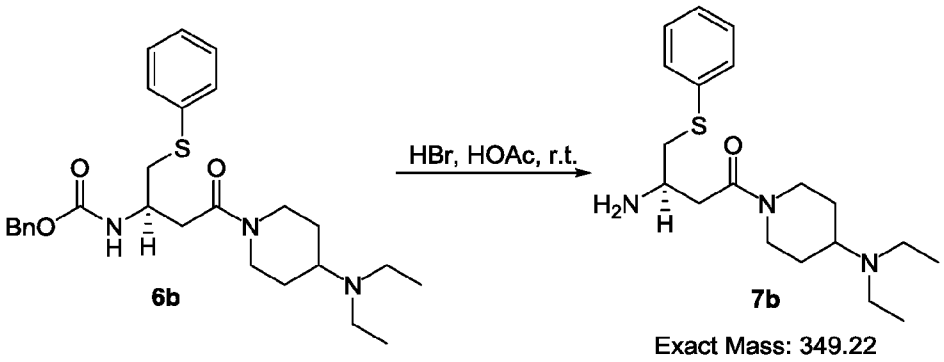
5b

6b

Exact Mass: 483.26

[00132] The crude intermediate 5b (7.0 g, 17.9 mmol) was dissolved in the toluene, and Bu_3P (7.2 g, 35.8 mmol), PhSSPh (5.9 g, 26.9 mmol) were added. The mixture solution was stirred at 90 °C overnight. The solvent was removed in vacuo and the residue was purified by chromatography column (eluted with PE/EA = 4/1) to afford intermediate 6b (6.5 g, 75%) as a yellow oil.

10. Synthesis of intermediate 7b



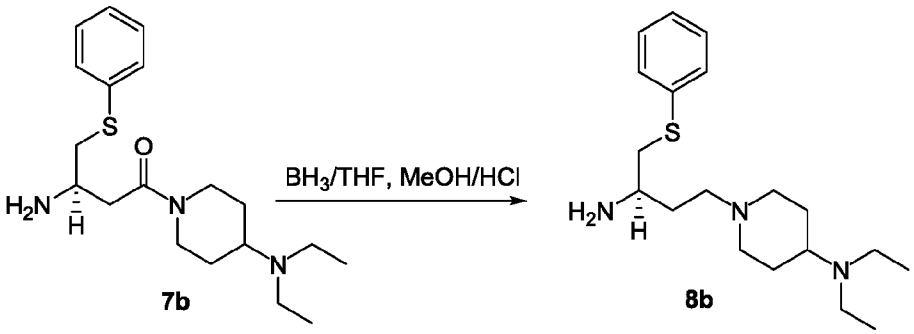
6b

7b

Exact Mass: 349.22

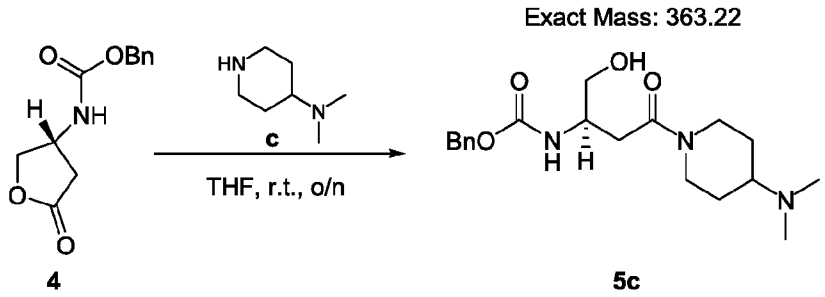
[00133] To the solution of intermediate 6b (6.5 g, 13.5 mmol) in HOAc (21 mL) was added HBr (7 mL), and the mixture solution was stirred at room temperature overnight. Then removed the solvent at reduced pressure, the residue 7b (4.5 g, 96%) was directly used for the next step.

11. Synthesis of intermediate 8b



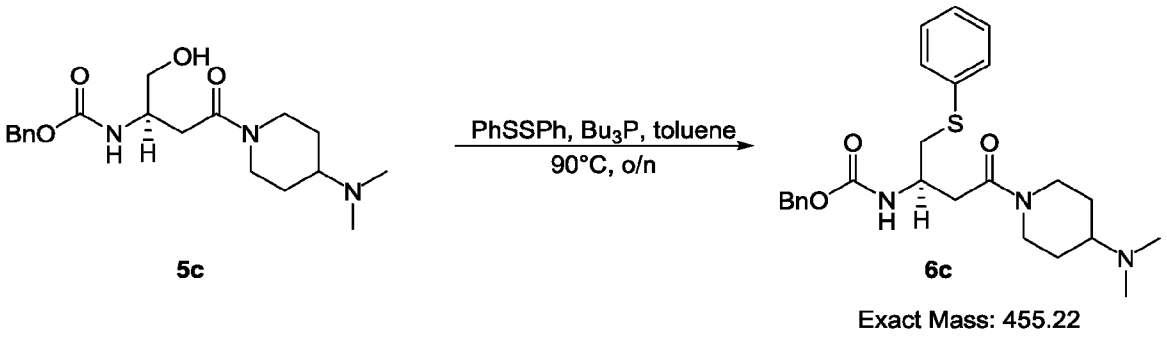
[00134] To the solution of 7b (3.0 g, 8.6 mmol) in THF (25 mL) was added BH_3/THF (1.0 M, 17.2 mL), the mixture was stirred at room temperature overnight. After completion of addition, MeOH (5 mL) was added slowly to quench the reaction, and then removed the solvent at reduced pressure, re-dissolved the residue in MeOH (25 mL) and HCl (3 mL). The mixture was reflux for 4 hours. Concentrated the solvent to afford intermediate 8b (4.0 g, 100%) as white solid, which was directly used for the next step. MS (M+H)+ 336.4

12. Synthesis of intermediate 5c



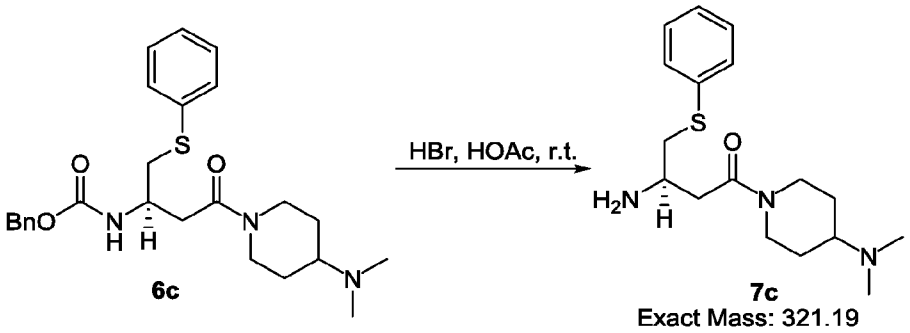
[00135] To the solution of intermediate 4 (6.5 g, 27.7 mmol) in THF (100 mL) was added intermediate c (3.6 g, 27.7 mmol), and the solution was stirred at room temperature overnight. After completion of the reaction, the solvent was removed in vacuo, the residue was purified by chromatography column (PE/EA = 3/1) to afford intermediate 5c (7.2 g, 72%) as yellow oil.

13. Synthesis of intermediate 6c



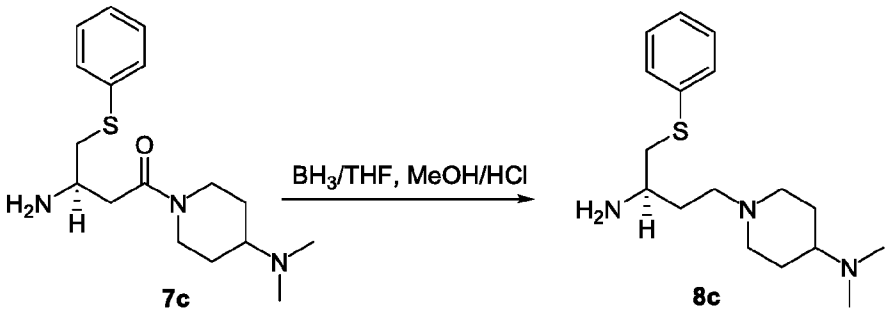
[00136] The crude intermediate 5c (7.2 g, 19.8 mmol) was dissolved in the toluene, and Bu_3P (8.0 g, 39.6 mmol), PhSSPh (6.5 g, 29.7 mmol) were added. The mixture solution was stirred at 90 °C overnight. The solvent was removed in vacuo and the residue was purified by chromatography column (eluted with PE/EA = 4/1) to afford intermediate 6c (6.7 g, 74%) as a yellow oil.

14. Synthesis of intermediate 7c



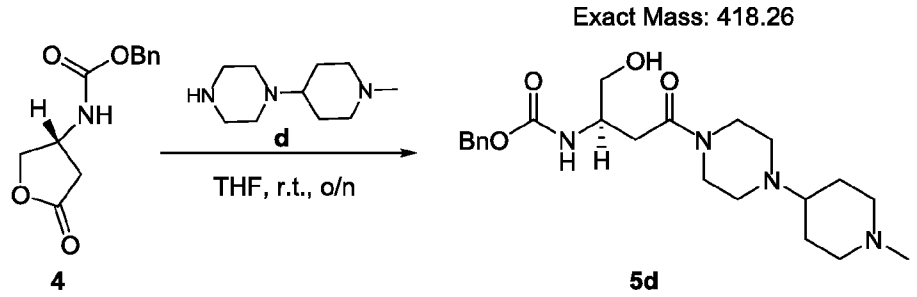
[00137] To the solution of intermediate 6c (6.7 g, 14.7 mmol) in HOAc (21 mL) was added HBr (7 mL), and the mixture solution was stirred at room temperature overnight. Then removed the solvent at reduced pressure, the residue 7c (2.9 g, 62%) was directly used for the next step.

15. Synthesis of intermediate 8c



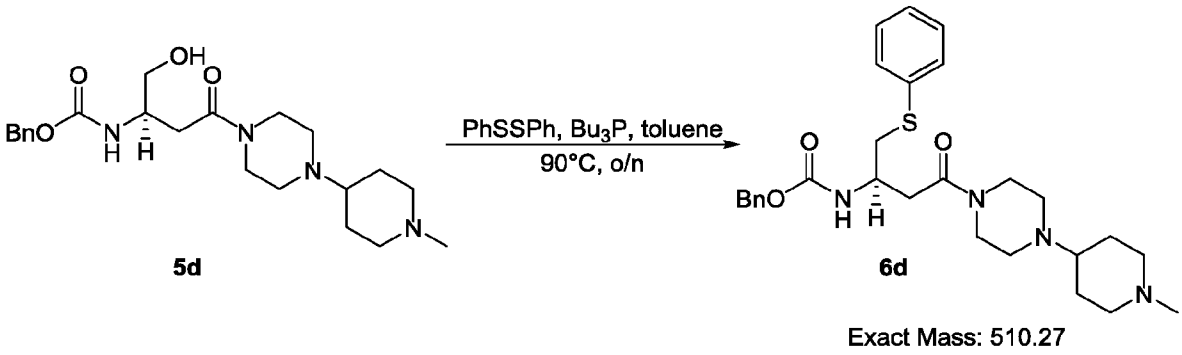
[00138] To the solution of intermediate 7c (0.9 g, 2.8 mmol) in THF (25 mL) was added BH_3/THF (1.0 M, 5.6 mL), the mixture was stirred at room temperature overnight. After completion of addition, MeOH (5 mL) was added slowly to quench the reaction, and then removed the solvent at reduced pressure, re-dissolved the residue in MeOH (25 mL) and HCl (3 mL). The mixture was reflux for 4 hours. Concentrated the solvent to afford 8c (1.0 g, 100%) as white solid, which was directly used for the next step.

16. Synthesis of intermediate 5d



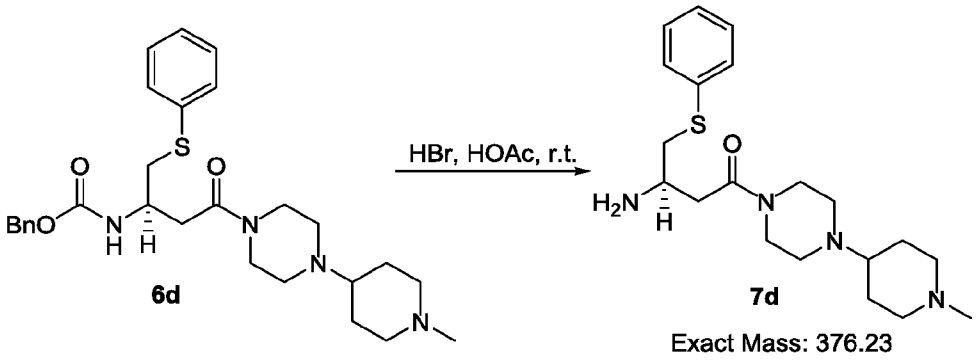
[00139] To the solution of intermediate 4 (6.5 g, 27.7 mmol) in THF (60 mL) was added d (5.0 g, 27.7 mmol), and the solution was stirred at room temperature overnight. After completion of the reaction, the solvent was removed in vacuo, the residue was purified by chromatography column (PE/EA = 1/1) to afford intermediate 5d (7.6 g, 66%) as yellow oil. MS (M+H)+ 419.4

17. Synthesis of intermediate 6d



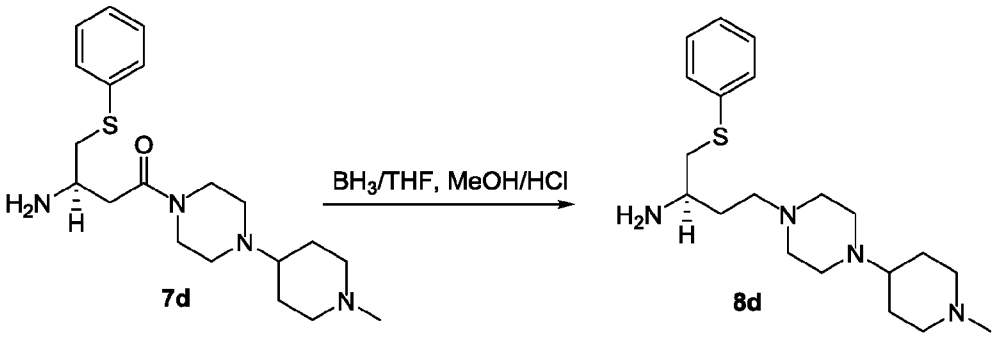
[00140] The crude intermediate 5d (7.6 g, 18.2 mmol) was dissolved in the toluene, and Bu_3P (7.4 g, 36.4 mmol), PhSSPh (6.0 g, 27.3 mmol) were added. The mixture solution was stirred at 90 °C overnight. The solvent was removed in vacuo and the residue was purified by chromatography column (eluted with PE/EA = 2/1) to afford intermediate 6d (7.4 g, 80%) as a yellow oil.

18. Synthesis of intermediate 7d



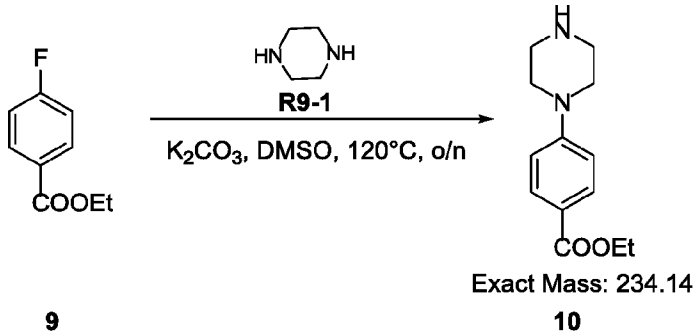
[00141] To the solution of intermediate 6d (7.4 g, 14.5 mmol) in HOAc (21 mL) was added HBr (7 mL), and the mixture solution was stirred at room temperature overnight. Then removed the solvent at reduced pressure, and the residue of intermediate 7d (4.5 g, 82%) was directly used for the next step.

19. Synthesis of intermediate 8d



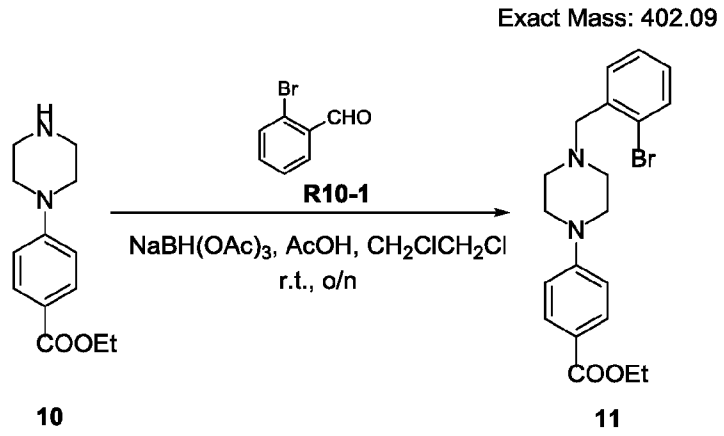
[00142] To the solution of 7d (4.5 g, 12.0 mmol) in THF (25 mL) was added BH_3/THF (1.0 M, 24 mL), the mixture was stirred at room temperature overnight. After completion of addition, MeOH (5 mL) was added slowly to quench the reaction, and then removed the solvent at reduced pressure, re-dissolved the residue in MeOH (25 mL) and HCl (3 mL). The mixture was reflux for 4 hours. Concentrated the solvent to afford intermediate 8d (2.6 g, 60%) as white solid, which was directly used for the next step.

20. Synthesis of intermediate 10



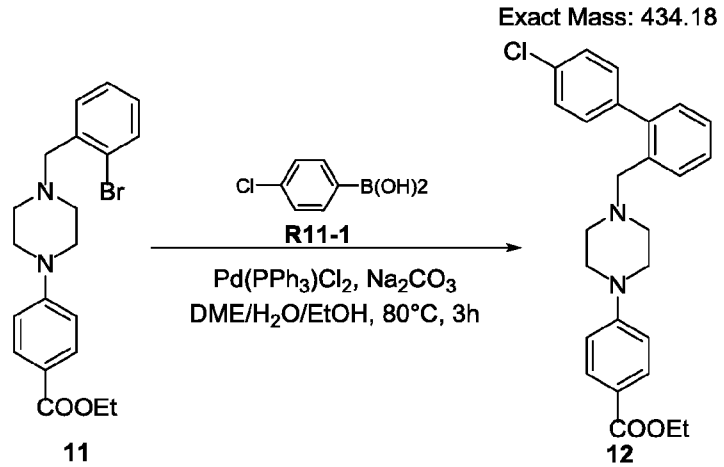
[00143] To a mixture of intermediate 4 (40.0 g, 238.1 mmol) in DMSO (400 mL) was added piperazine (82.0 g, 953.5 mmol). The mixture was stirred at 120 °C overnight, after cooling to room temperature; the mixture was poured into water (50 mL). The solid was collected by filtration to afford intermediate 10 (35.0 g, 90%) as a white solid.

21. Synthesis of intermediate 11



[00144] To a solution of intermediate 10 (10.0 g, 54.3 mmol) in dichloroethane (100 mL) was added R10-1 (12.6 g, 54.1 mmol) and acetic acid (5 mL). $NaBH(OAc)_3$ (45.8 g, 216.2 mmol) was added and the reaction was stirred at room temperature overnight. The reaction was quenched with Sat. NH_4Cl, EA was added and the solution was washed with Sat.$NaHCO_3$, brine, dried over Na_2SO_4 and concentrated to dryness. The residue was flushed through a silica gel with PE/EA (15/1) to give a white solid, intermediate 11 (17.0 g, 77%).

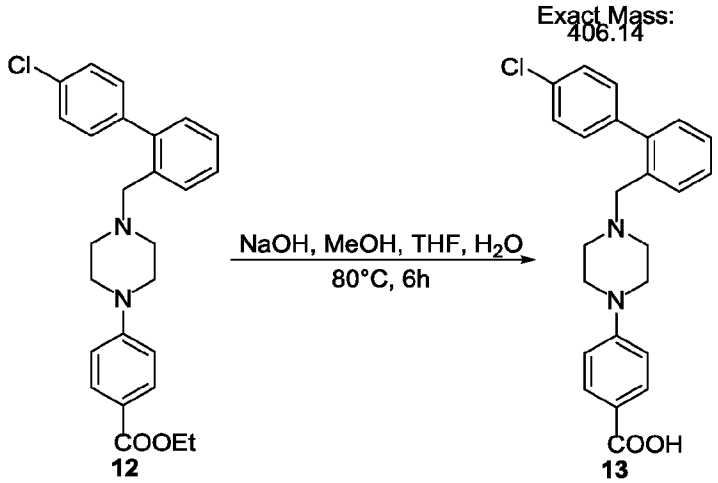
22. Synthesis of intermediate 12



[00145] To the solution of intermediate 11 (12.0 g, 29.9 mmol) and R11-1 (7.0 g, 44.9 mmol) in DME/EtOH/H_2O (30 mL/30 mL/30 mL) was added Na_2CO_3 (4.0 g, 38.1 mmol) and $Pd(PPh_3)_2Cl_2$. The mixture was flushed with argon three times and stirred at 80 °C for three hours. The mixture was concentrated to dryness and the residue was purified by silica gel

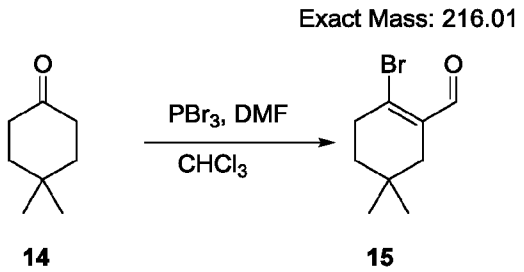
(eluted with PE/EA = 8/1) to afford intermediate 12 (8.7 g, 67%) as white solid. MS (M+H)+ 435.2

23. Synthesis of intermediate 13



[00146] To the solution of intermediate 12 (8.0 g, 18.4 mmol) in MeOH/THF/H_2O (30 mL/30 mL/ 30 mL) was added NaOH (2.2 g, 55 mmol). Then the mixture was reflux for 6 hours and the solvent was removed in vacuo, water (60 mL) was added and the pH value was adjusted to 2~3 with HCl (aq. 2M). The solid was collected by filtration, and then suspended in PE (50 mL) stirred for 1 hour, filtered to afford intermediate 13 (6.7 g, 89%) as a white solid. MS (M+H)+ 407.3

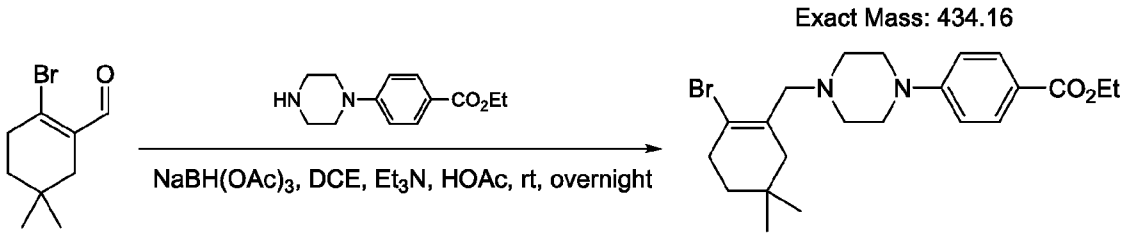
24. Synthesis of intermediate 15



[00147] To the solution of DMF (25 ml) in $CHCl_3$ (150 mL) at 0 oC was added PBr_3 dropwise. After the addition, the mixture was warmed to 10 °C slowly and then heated to 70 °C. The reaction was stirred for 30 minutes and then cooled to r.t.. The solution of intermediate 14 in $CHCl_3$ (30 mL) wad added dropwise and the mixture was heated to 70 °C again. After stirring for 90 minutes, the mixture was cooled to r.t. and added into aqueous

KOAc (4M, 150 mL). The pH value was adjusted to 7 with NaOH (aq, 5M). Then the mixture was extracted with heptane (400 mL x 5). The organic layer was washed with brine (400 mL), dried over Na_2SO_4, filtered and concentrated to afford 14 g of intermediate 15 as a red oil. ^1H NMR (400MHz, DMSO-d6): δ 9.85 (s, 1H), 2.7 (t, 2H), 2.01 (s, 2H), 1.46(t, 2H), 0.81 (s, 6H).

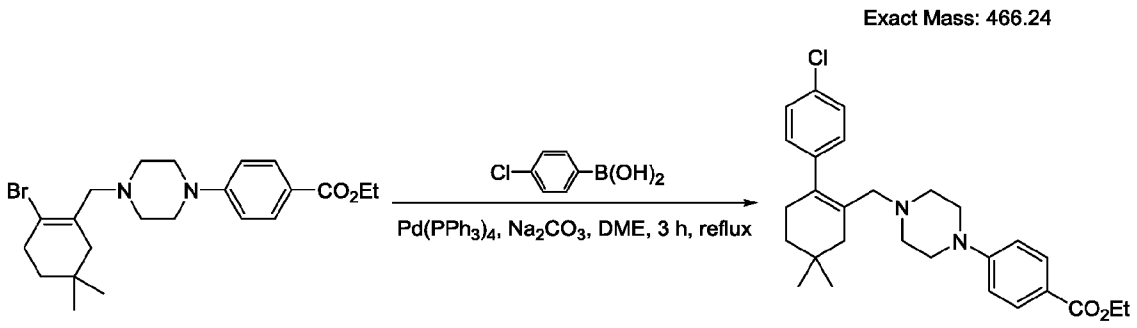
25. Synthesis of intermediate 16



15 16

[00148] To the solution of ethyl 4-(piperazin-1-yl)benzoate (15.2 g, 64.8 mmol) and intermediate 15 (14.0 g, 64.8 mmol) in EtOH (100 mL) was added $NaCNBH_3$ (4.9 g, 77.8 mmol). The mixture was adjusted to pH = 5 with HOAc. Then the resultant was stirred at r.t. overnight. The solvent was removed in vacuo. The residue was purified by silica-gel column to afford 10.9 g of intermediate 16 as a colorless oil. MS (M+H)+ 437.1

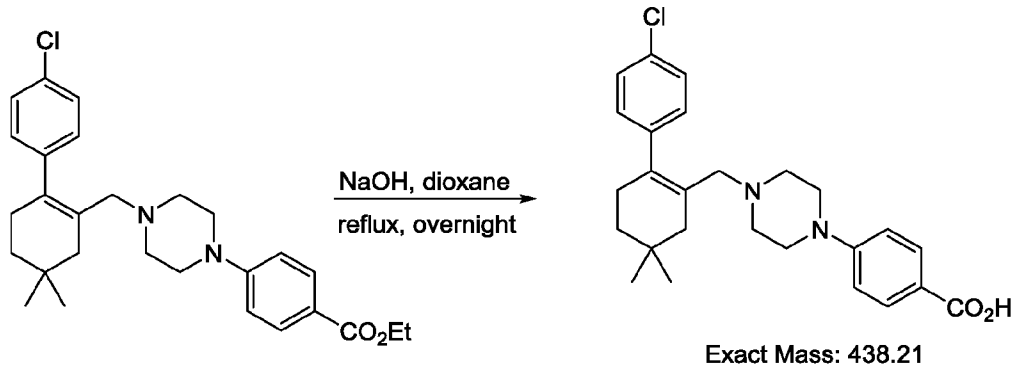
26. Synthesis of intermediate 17



16 17

[00149] To the solution of intermediate 16 (10.9 g, 25 mmol) and 4-chlorophenylboronic acid (4.7 g, 30 mmol) in DME/MeOH/H_2O (70 mL/30 mL/20 mL) was added Na_2CO_3 (3.2 g, 30 mmol) and $Pd(PPh_3)_4$. The mixture was flushed with N_2 three times and stirred at 80 °C for three hours. The mixture was concentrated to dryness. The residue was purified by silica-gel column to afford 9.0 g of intermediate 17 as a light yellow oil. MS (M+H)+ 467.2

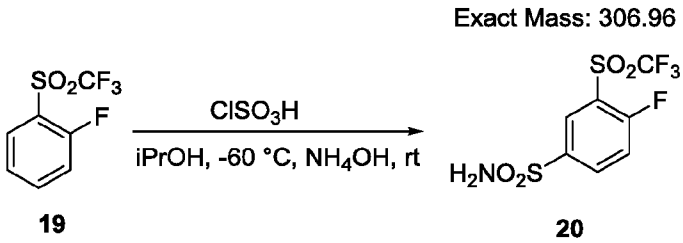
27. Synthesis of intermediate 18



17 18

[00150] To the solution of intermediate 17 (8.0 g, 17.2 mmol) in dioxane/water (3:1, 80 mL) was added NaOH (2.1 g, 51.5 mmol). Then the mixture was refluxed for 18 hours. The solvent was removed in vacuo. Water (60 mL) was added and the pH value was adjusted to 2~3 with HCl (aq, 2 M). The solid was collected by filtration, suspended in PE (50 mL) and filtered again to afford 5.0 g of intermediate 18 as a white solid.

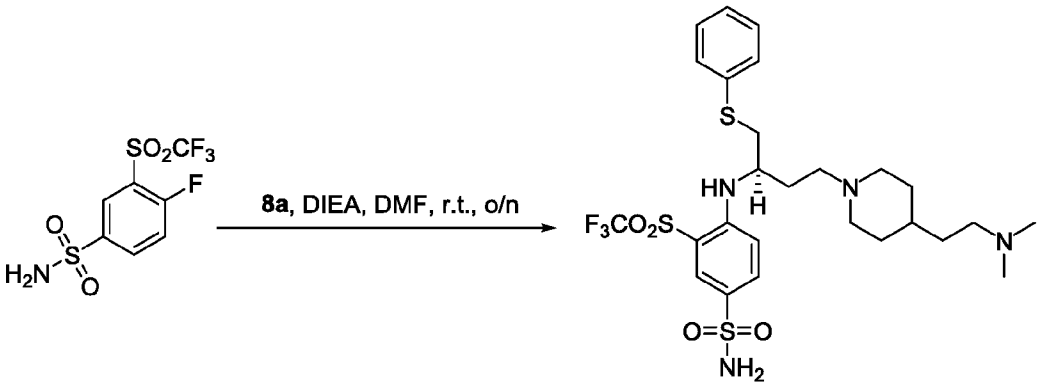
28. Synthesis of intermediate 20



19 20

[00151] The solution of 1-fluoro-2-(trifluoromethylsulfonyl)benzene 19 (2.0 g) in $ClSO_3H$ (13 mL) was stirred at 120 °C overnight. The reaction mixture was cooled to r.t. and poured into ice/water. The resultant was extracted with EA (50 mL x 3). The organic layer was washed with water (40 mL x 2) and brine (40 mL), dried over Na_2SO_4, filtered and concentrated to afford 2.5 g of white solid, which was redissolved in isopropanol (70 mL). The solution was cooled to -60 °C. Ammonium hydroxide (11 mL) was added dropwise. After stirring at -60 °C for 1 hour, HCl (6 M, 8 mL) was added to quench the reaction. The reaction mixture was warmed to r.t. and concentrated to dryness. Water (50 mL) was added and the mixture was extracted with EA (50 mL X 4). The organic layer was washed with water, dried over $MgSO_4$, filtered and concentrated to afford 2.3 g of intermediate 20 as a white solid.

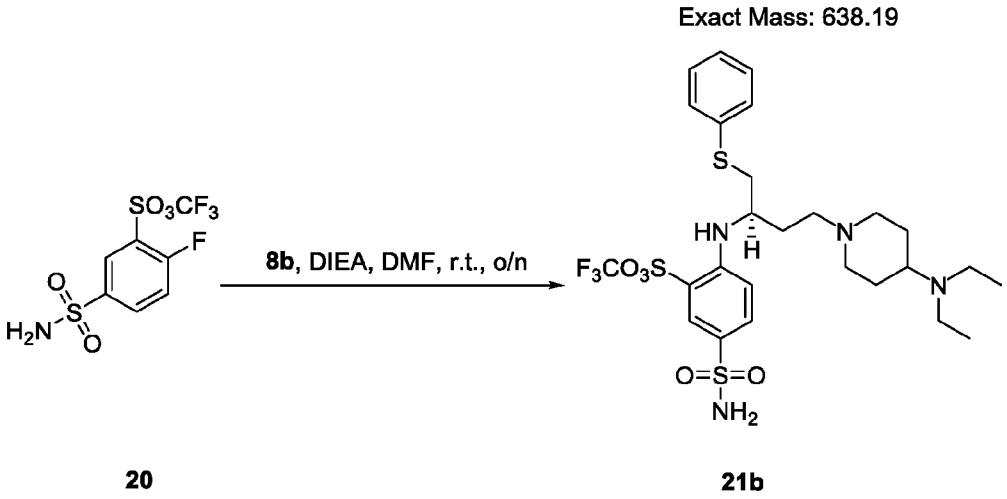
29. Synthesis of intermediate 21a



20 21a

[00152] To the solution of intermediate 20 (2.0 g) in DMF (100 mL) was added DIPEA (16 mL) and intermediate 8a (4.0 g). The solution was stirred at r.t. for 6 hours. The solvent was removed in vacuo. The residue was purified by reversed-phase column to afford 900 mg of intermediate 21a as a yellow oil.

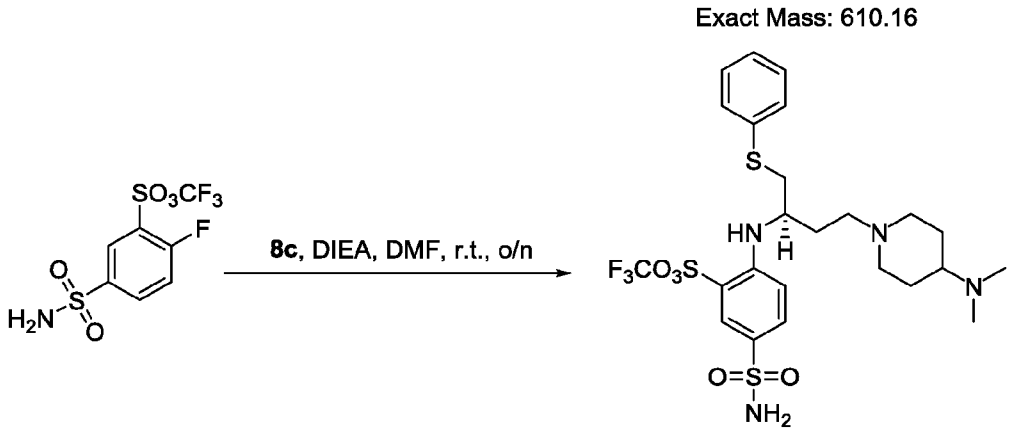
30. Synthesis of intermediate 21b



20 21b

[00153] To the solution of intermediate 20 (2.0 g) in DMF (100 mL) was added 8b (1.8 g, 5.4 mmol) and DIEA (1.7 g, 13.5 mmol). The mixture was stirred at room temperature overnight. The crude product was purified by by reversed-phase column to afford 850 mg of intermediate 21b as yellow solid.

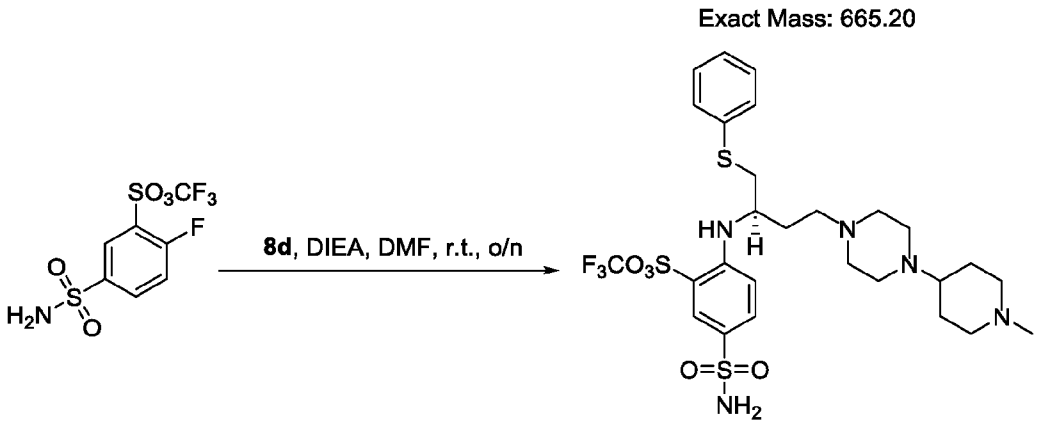
31. Synthesis of intermediate 21c



20 21c

[00154] To the solution of intermediate 20 (2.0 g) in DMF (100 mL) was added 8c (1.7 g, 5.4 mmol) and DIEA (1.7 g, 13.5 mmol). The mixture was stirred at room temperature overnight. The crude product was purified by by reversed-phase column to afford 930 mg of intermediate 21c as yellow solid. Exact Mass: 610.16

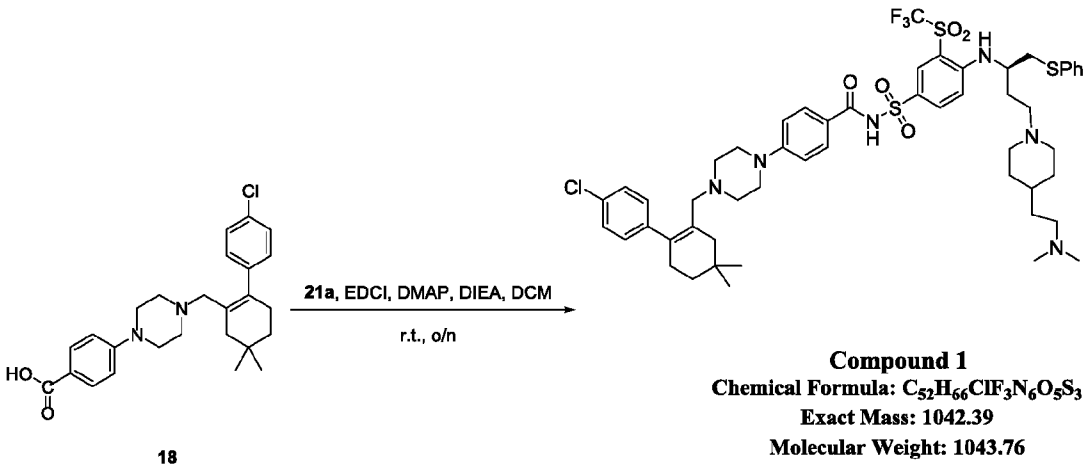
32. Synthesis of intermediate 21d



20 21d

[00155] To the solution of intermediate 20 (2.0 g) in DMF (100 mL) was added 8d (1.9 g, 5.4 mmol) and DIEA (1.7 g, 13.5 mmol). The mixture was stirred at room temperature overnight. The crude product was purified by by reversed-phase column to afford 700 mg of intermediate 21d as yellow solid. Exact Mass: 665.20

33. Synthesis of Compound 1

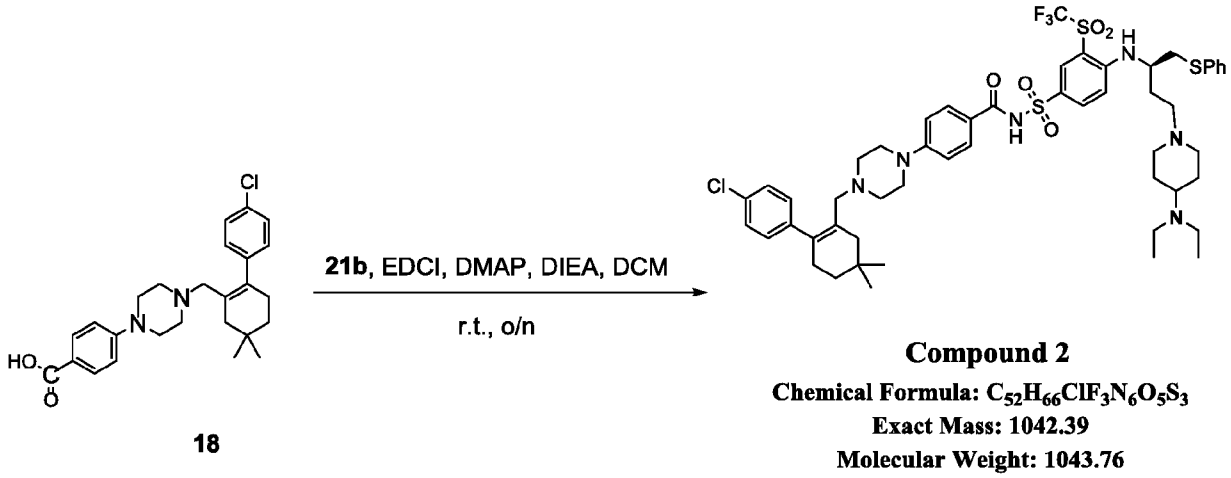


18

Compound 1
Chemical Formula: $C_{52}H_{66}ClF_3N_6O_5S_3$
Exact Mass: 1042.39
Molecular Weight: 1043.76

[00156] To the solution of intermediate 18 (0.6 g, 1.1 mmol) in DCM (30 mL) was added intermediate 21a (550 mg, 1.4 mmol), DMAP (0.4 g, 3.3 mmol), EDCI (450 mg, 2.2 mmol) and DIEA (700 mg, 4.4 mmol). The mixture was stirred at r.t. overnight. DCM (150 mL) was added and the organic layer was washed with water (30 mL X 3) and brine (30 mL), and then concentrated to dryness. The residue was purified by prep-HPLC to afford 250 mg of Compound 1 as a white solid. MS (M+H)+ 1043; ^{1}H NMR (400MHz, DMSO-d6): δ 8.05 (s, 1H), 7.90(d, 1H, J=8.8 Hz), 7.70 (d, 2H, J=8.8Hz), 7.15-7.5 (m, 7H),7.12 (d, 2H, J=8.0Hz), 6.76-6.85 (dd, 4H), 3.98 (m, 1H), 2.8-3.5 (m, 9H), 2.10-2.80 (m, 18H), 1.0-2.1(m, 16H), 0.98 (s, 6H).

34. Synthesis of Compound 2



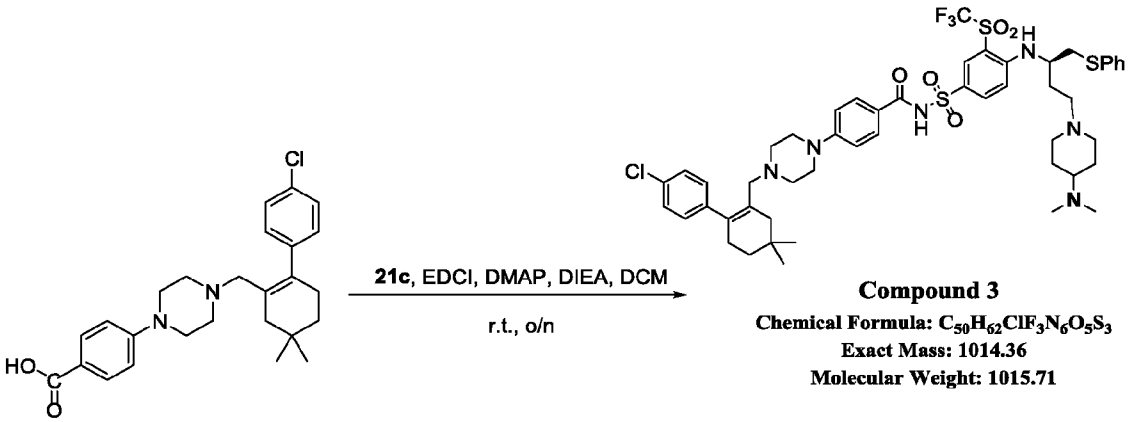
18

Compound 2
Chemical Formula: $C_{52}H_{66}ClF_3N_6O_5S_3$
Exact Mass: 1042.39
Molecular Weight: 1043.76

[00157] To the solution of intermediate 18 (0.6 g, 1.1 mmol) in DCM (30 mL) was added intermediate 21b (550 mg, 1.4 mmol), DMAP (0.4 g, 3.3 mmol), EDCI (450 mg, 2.2

mmol) and DIEA (700 mg, 4.4 mmol). The mixture was stirred at r.t. overnight. DCM (150 mL) was added and the organic layer was washed with water (30 mL x 3) and brine (30 mL), and then concentrated to dryness. The residue was purified by prep-HPLC to afford 350 mg of Compound 2 as a white solid. MS (M+H)+ 1043; ^{1}H NMR (400MHz, DMSO-d6): δ 8.02 (s, 1H), 7.91(d, 1H), 7.69 (d, 2H), 7.10-7.5 (m, 9H),6.6-6.9 (m, 4H), 3.98 (m, 1H), 2.8-3.4 (m, 12H), 2.6-2.8(m, 3H), 2.18-2.38 (m, 8H), 1.6-2.1(m, 8H), 1.35-1.60 (m, 4H), 1.05-1.20 (t, 6H), 0.98 (s, 6H). High resolution mass (Thermo ScientificTM Q ExactiveTM hybrid quadrupole-Orbitrap mass spectrometer): Calculated for C52H66ClF3N6O5S3 + 2Proton (2*1.00728) = 1044.4041, theoretical m/z of doubly charged ion: 522.2021; Found: 522.2020.

35. Synthesis of Compound 3



18

Compound 3

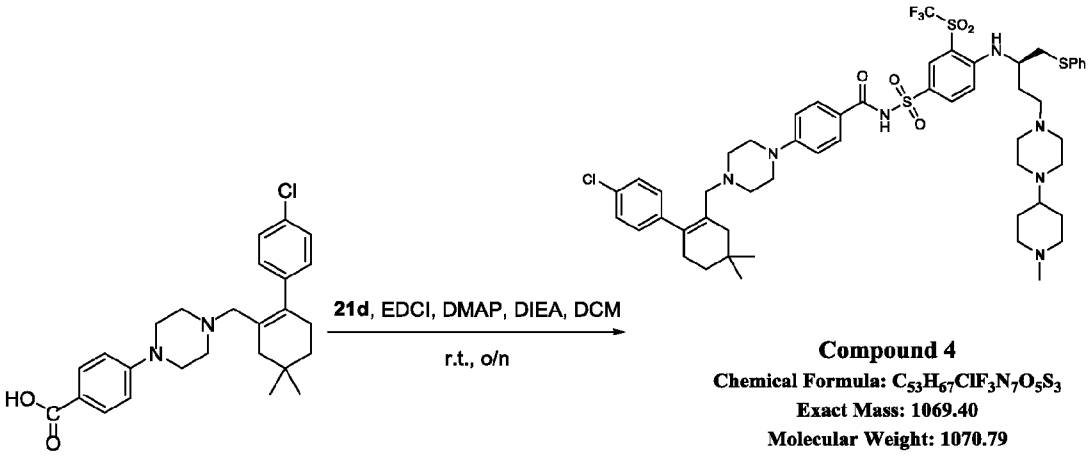
Chemical Formula: $C_{50}H_{62}ClF_3N_6O_5S_3$

Exact Mass: 1014.36

Molecular Weight: 1015.71

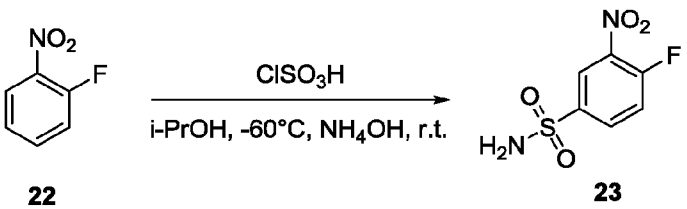
[00158] To the solution of intermediate 18 (0.6 g, 1.1 mmol) in DCM (30 mL) was added intermediate 21c (520 mg, 1.4 mmol), DMAP (0.4 g, 3.3 mmol), EDCI (450 mg, 2.2 mmol) and DIEA (700 mg, 4.4 mmol). The mixture was stirred at r.t. overnight. DCM (150 mL) was added and the organic layer was washed with water (30 mL X 3) and brine (30 mL), and then concentrated to dryness. The residue was purified by prep-HPLC to afford 280 mg of Compound 3 as a white solid. MS (M+H)+ 1015; ^{1}H NMR (400MHz, DMSO-d6): δ 8.04 (s, 1H), 7.93(d, 1H, J=8 .8), 7.71 (d, 2H, J=8.8Hz), 7.20-7.4 (m, 9H),6.6-6.8 (m, 4H), 3.98 (m, 1H), 2.6-3.7 (m, 17H), 2.1-2.4(m, 8H), 1.5-2.0 (m, 8H), 1.2-1.5(m, 4H), 0.98 (s, 6H).

36. Synthesis of Compound 4



[00159] To the solution of intermediate 18 (0.6 g, 1.1 mmol) in DCM (30 mL) was added intermediate 21d (560 mg, 1.4 mmol), DMAP (0.4 g, 3.3 mmol), EDCI (450 mg, 2.2 mmol) and DIEA (700 mg, 4.4 mmol). The mixture was stirred at r.t. overnight. DCM (150 mL) was added and the organic layer was washed with water (30 mL x 3) and brine (30 mL), and then concentrated to dryness. The residue was purified by prep-HPLC to afford 320 mg of Compound 4 as a white solid. MS (M+H)+ 1070; ^{1}H NMR (400MHz, DMSO-d6): δ 8.03 (s, 1H), 7.95(d, 1H, J=8.8 Hz), 7.68 (d, 2H, J=8.8Hz), 7.16-7.4 (m, 9H),6.7-6.9 (m, 4H), 3.98 (m, 1H), 3.0-3.4 (m, 10H), 2.1-2.9(m, 22H), 2.18-2.38 (m, 8H), 1.3-2.0(m, 10H), 0.98 (s, 6H).

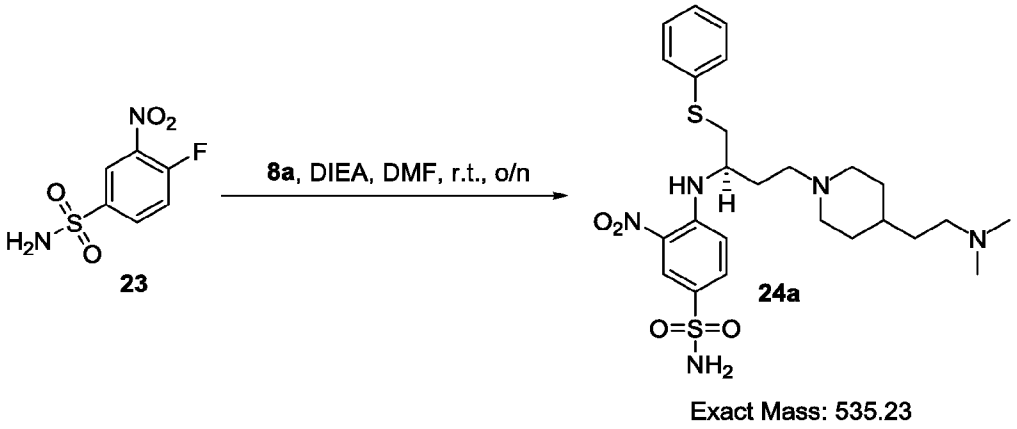
37. Synthesis of intermediate 23



[00160] The solution of 22 (4.0 g, 28.4 mmol) in $ClSO_3H$ (25 mL) was stirred at 120 °C overnight, and the reaction was cooled to room temperature and poured into ice water. Then the resultant was extracted with EA (50 mL x 3), combined the organic layers, removed the solvent at reduced pressure, and the crude residue was re-dissolved in i-PrOH. The solution was cooled to -60°C. Ammonium hydroxide was added drop-wise, and stirred at this temperature for 1 hour, HCl (6M, 8 mL) was added to quenched the reaction, the reaction

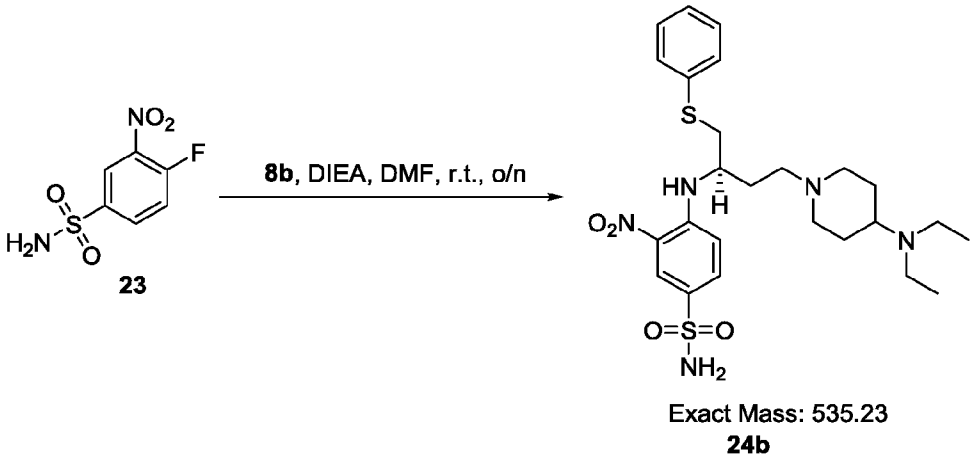
was warmed to room temperature and concentrated to dryness. The intermediate 23 (5.1 g, 82 %) was obtained as white solid. ^{1}H NMR (400MHz, DMSO-d6): δ8.52 (dd, 1H), 8.20(dq, 1H), 7.84 (dt, 1H), 7.73(s, 2H).

38. Synthesis of intermediate 24a



[00161] To the DMF (10 mL) solution was added intermediate 23 (1.0 g, 4.5 mmol), intermediate 8a (1.8 g, 5.4 mmol) and DIEA (1.7 g, 13.5 mmol), and then the mixture solution was stirred at room temperature overnight. The crude product was purified by Prep-HPLC, and intermediate 24a (600 mg, 25%) was obtained as yellow solid.

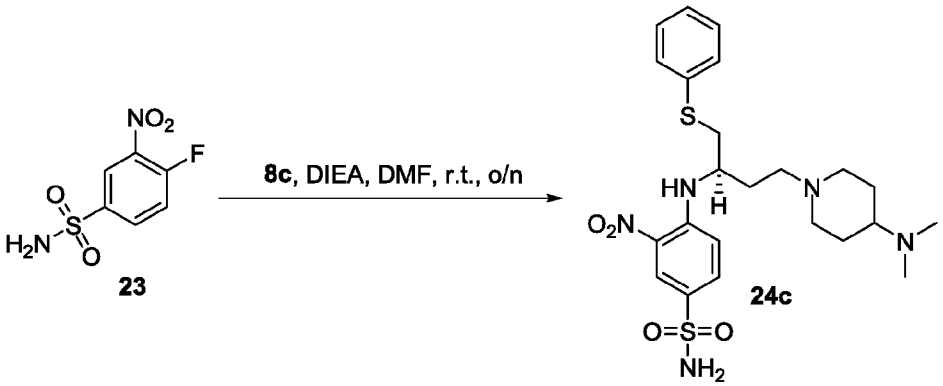
39. Synthesis of intermediate 24b



[00162] To the DMF (10 mL) solution was added intermediate 23 (1.0 g, 4.5 mmol), intermediate 8b (1.8 g, 5.4 mmol) and DIEA (1.7 g, 13.5 mmol), and then the mixture solution was stirred at room temperature overnight. The crude product was purified by Prep-

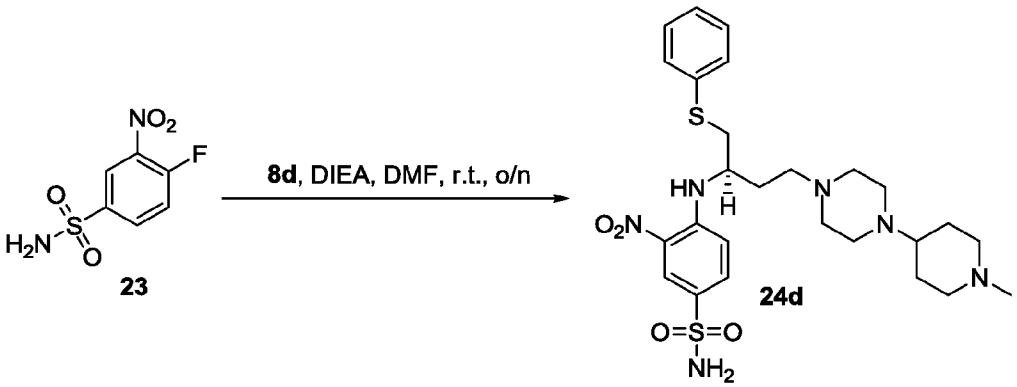
HPLC, and intermediate 24b (810 mg, 33%) was obtained as yellow solid. MS (M+H)+ 536.3.

40. Synthesis of intermediate 24c



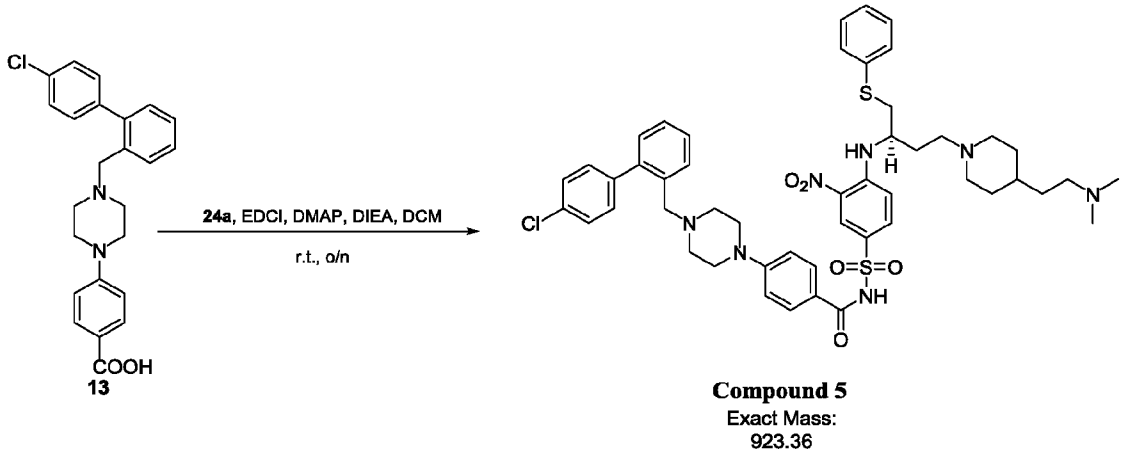
[00163] To the DMF (10 mL) solution was added intermediate 23 (1.0 g, 4.5 mmol), intermediate 8c (1.7 g, 5.4 mmol) and DIEA (1.7 g, 13.5 mmol), and then the mixture solution was stirred at room temperature overnight. The crude product was purified by Prep-HPLC and intermediate 24c (930 mg, 40%) was obtained as yellow solid.

41. Synthesis of intermediate 24d



[00164] To the DMF (10 mL) solution was added intermediate 23 (1.0 g, 4.5 mmol), intermediate 8d (1.9 g, 5.4 mmol) and DIEA (1.7 g, 13.5 mmol), and then the mixture solution was stirred at room temperature overnight. The crude product was purified by Prep-HPLC, and intermediate 24d (690 mg, 27%) was obtained as yellow solid.

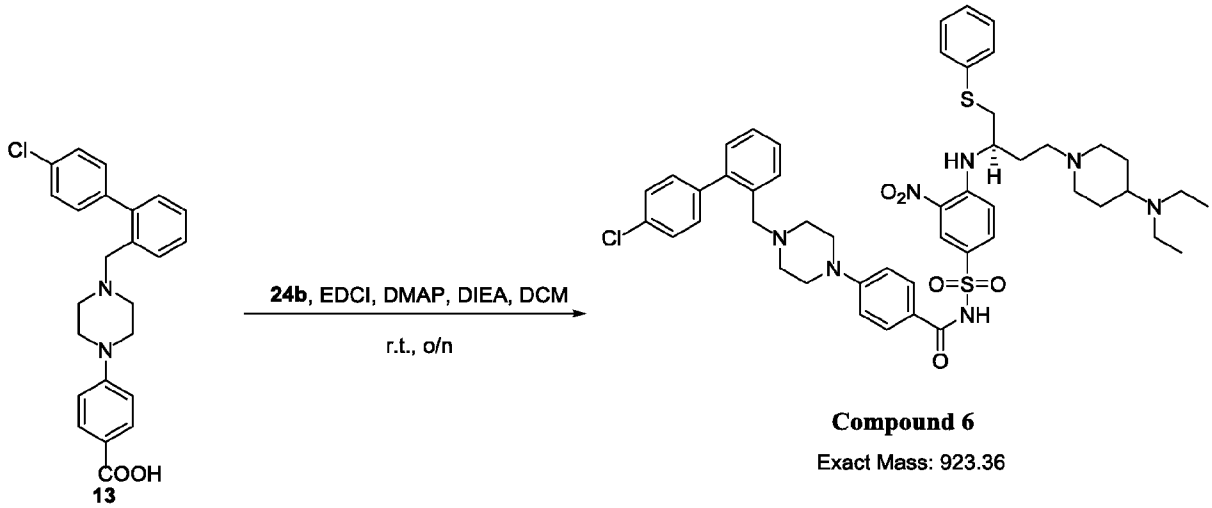
42. Synthesis of Compound 5



Compound 5
Exact Mass:
923.36

[00165] The intermediate 13 (550 mg, 1.4 mmol) and intermediate 24a (722 mg, 1.4 mmol) were dissolved in DCM (30 mL), DMAP (484 mg, 4.0 mmol), DIEA (700 mg, 5.4 mmol) and EDCI (516 mg, 2.7 mmol) were added, and then the mixture solution was stirred at room temperature overnight, the solvent was removed at reduced pressure and the residue was purified by Prep-HPLC, Compound 5 (70 mg, 6%) was obtained as yellow solid. MS (M+H)+ 924; ^{1}H NMR (400MHz, DMSO-d6): δ 8.42 (s, 1H), 7.70-7.76(m, 3H), 7.1-7.6 (m, 14H), 6.92 (d, 1H),6.8 (d, 2H), 4.05 (m, 1H), 3.3-3.8 (m, 5H), 3.3 (m, 4H), 2.8-3.0 (m, 4H), 2.4(m, 4H), 1.0-2.1 (t, 8H).

43. Synthesis of Compound 6

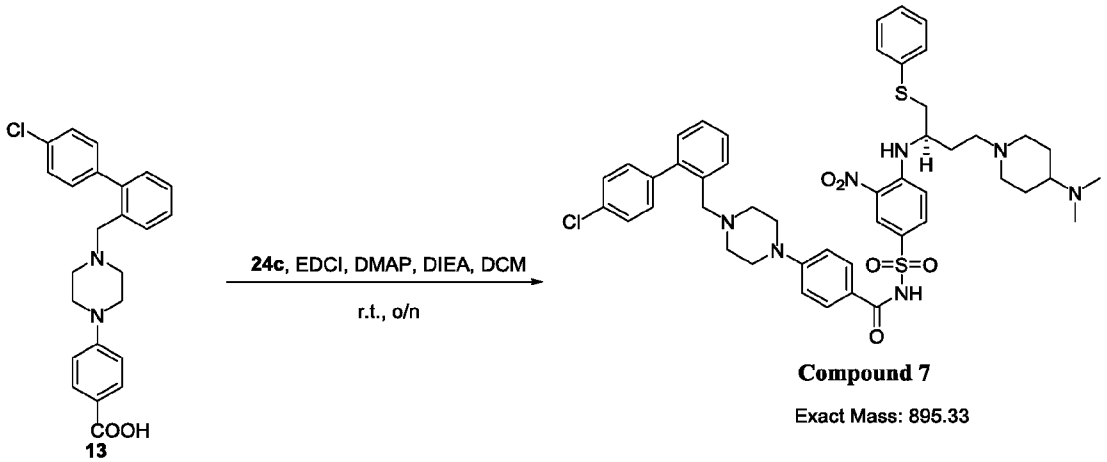


Compound 6
Exact Mass: 923.36

[00166] The intermediate 13 (609 mg, 1.5 mmol)and intermediate 24b (500 mg, 1.5 mmol) were dissolved in DCM (25 mL), DMAP (545 mg, 4.5 mmol), DIEA (774 mg, 6.0 mmol) and EDCI (573 mg, 3.0 mmol) were added, and then the mixture solution was stirred

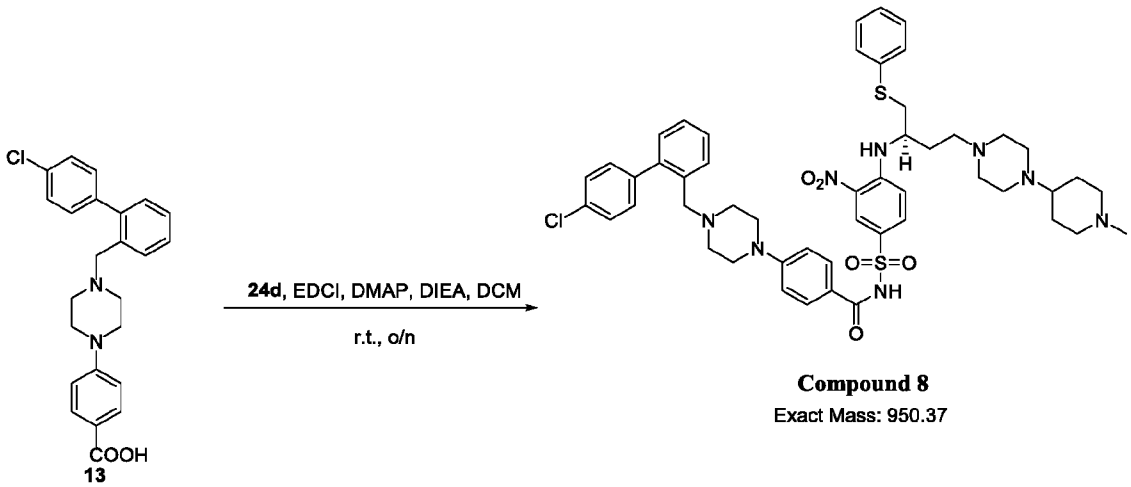
at room temperature overnight, the solvent was removed at reduced pressure and the residue was purified by Prep-HPLC, Compound 6 (350 mg, 26%) was obtained as yellow solid. MS (M+H)+ 924; ^{1}H NMR (400MHz, DMSO-d6): δ 8.48(s, 1H), 8.40 (d, 1H), 7.70-(dd , 3H), 7.1-7.6 (m, 14H), 6.92 (d, 1H),6.8 (d, 2H), 4.05 (m, 1H), 2.5-3.5 (m, 12H), 1.6-2.4 (m, 14H), 1.0-1.6 (m, 10H). High resolution mass (Thermo ScientificTM Q ExactiveTM hybrid quadrupole-Orbitrap mass spectrometer): Calculated for C49H58ClN7O5S2 + 2Proton (2*1.00728) = 925.3775, theoretical m/z of doubly charged ion: 462.6887; Found: 462.6883. Chiral HPLC purity: 100% (Chiralcel column, OD-3; Column Size 4.6*100mm, 3um; 0.1% DEA in Methanol/CAN (1:1 ratio).

44. Synthesis of Compound 7



[00167] The intermediate 13 (390 mg, 1.0 mmol) and intermediate 24c (514 mg, 1.0 mmol) were dissolved in DCM (25 mL), DMAP (351 mg, 2.9 mmol), DIEA (490 mg, 3.8 mmol) and EDCI (218 mg, 1.8 mmol) were added, and then the mixture solution was stirred at room temperature overnight, the solvent was removed at reduced pressure and the residue was purified by Prep-HPLC, Compound 7 (310 mg, 36%) was obtained as yellow solid. MS (M+H)+ 896; ^{1}H NMR (400MHz, DMSO-d6): δ 8.43(s, 1H), 8.36 (d, 1H), 7.70-(dd , 3H), 7.1-7.6 (m, 14H), 6.88 (d, 1H),6.80 (d, 2H), 4.05 (m, 1H), 2.6-3.5 (m, 12H), 2.62 (s, 6H), 2.2-2.5 (m, 7H), 1.0-2.2 (m, 9H)

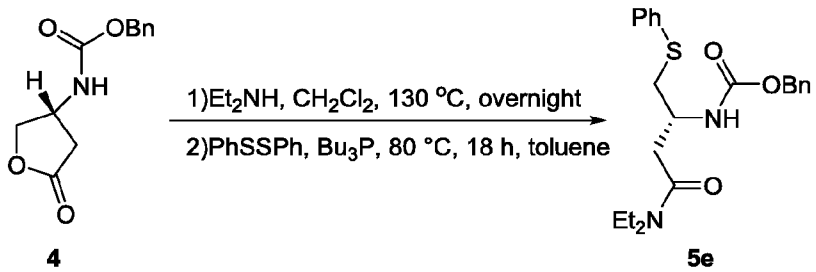
45. Synthesis of Compound 8



[00168] The intermediate 13 (406 mg, 1.0 mmol) and intermediate 24d (562 mg, 1.0 mmol) were dissolved in DCM (25 mL), DMAP (363 mg, 3.0 mmol), DIEA (516 mg, 4.0 mmol) and EDCI (382 mg, 2.0 mmol) were added, and then the mixture solution was stirred at room temperature overnight, the solvent was removed at reduced pressure and the residue was purified by Prep-HPLC, Compound 8 (405 mg, 43%) was obtained as yellow solid. MS (M+H)+ 951; ^{1}H NMR (400MHz, DMSO-d6): δ 8.43(s, 1H), 8.36 (d, 1H), 7.70-(dd , 3H), 7.1-7.6 (m, 14H), 6.88 (d, 1H), 6.80 (d, 2H), 4.05 (m, 1H), 3.0-3.6 (m, 10H), 2.2-2.8 (m, 10H), 1.0-2.1 (m, 7H).

[00169] Compound 9 and Compound 10 can be synthesized by steps similar to the other compounds disclosed herein.

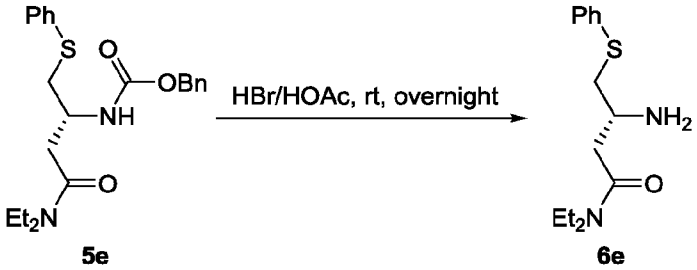
46. Synthesis of intermediate 5e



[00170] To the solution of intermediate 4 (12.0 g, 51 mmol) in THF (100 mL) was added diethylamine (20 mL). The solution was stirred at 130 °C in a sealed tube overnight. The solvent was removed in vacuo to afford 13.0 g of colorless oil. To the solution of above

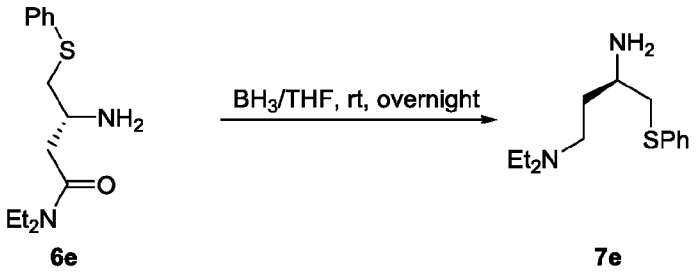
oil in toluene (120 mL) was added Bu_3P (18 g, 89 mmol) and PhSSPh (14 g, 64 mmol) successively. The mixture was stirred at 90 ℃ overnight. The solvent was removed in vacuo and the residue was purified by silica-gel column to afford intermediate 10 g of 5e as a brown oil.

47. Synthesis of intermediate 6e



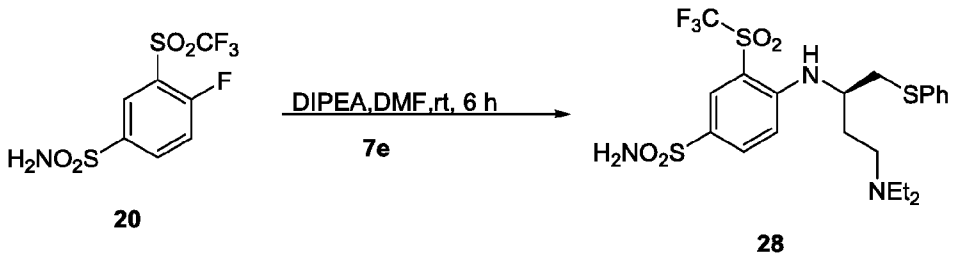
[00171] To the solution of intermediate 5e (8.0 g) in HOAc (50 mL) was added a solution of HBr in HOAc (33%, 20 mL). The mixture was stirred at r.t. overnight. The solvent was removed in vacuo. The residue was purified by reversed-phase column to afford 4.5 g brown oil.

48. Synthesis of intermediate 7e



[00172] To the solution of intermediate 6e (3.0 g, 11.3 mmol) in THF (50 mL) was added a solution of BH_3 in THF (1M, 45 mL).The mixture was stirred at r.t. overnight. MeOH (5 mL) was added to quench the reaction. The mixture was concentrated to dryness. MeOH (40 mL) and HCl (conc. 40 mL) was then added. The mixture was refluxed for 4 hours and then concentrated to dryness to afford 3.2 g of intermediate 7e as a yellow oil, which was used for next step directly.

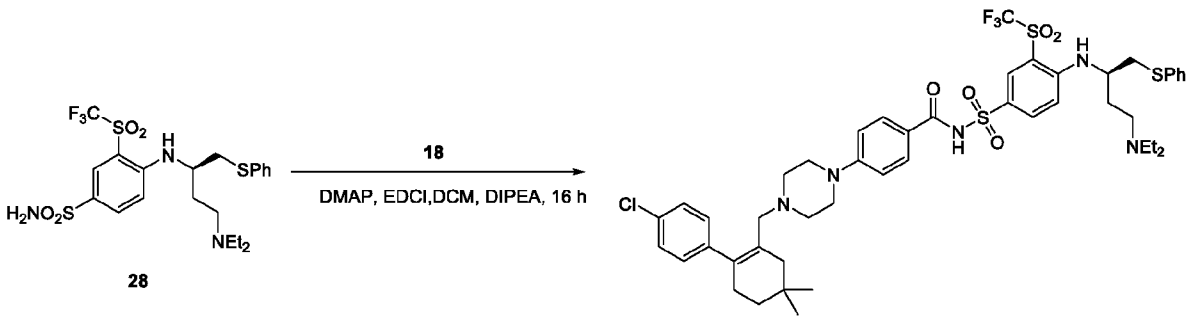
49. Synthesis of intermediate 28



20 28

[00173] To the solution of intermediate 20 (2.0 g) in DMF (100 mL) was added DIPEA (16 mL) and intermediate 6b (4.0 g). The solution was stirred at r.t. for 6 hours. The solvent was removed in vacuo. The residue was purified by reversed-phase column to afford 900 mg of 28 as a yellow oil.

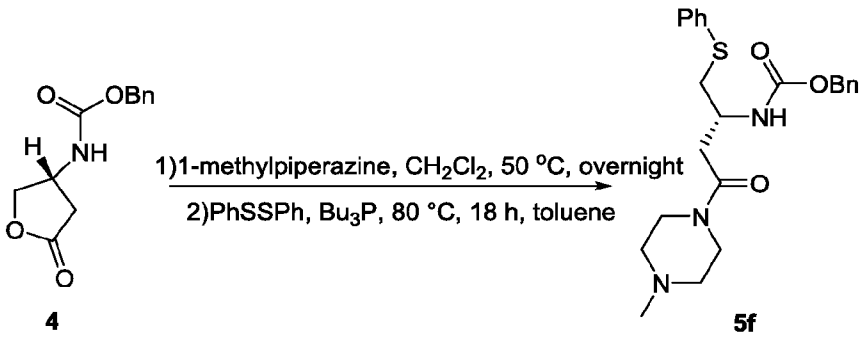
50. Synthesis of Compound 10



28 **Compound 10**
Exact Mass: 959.32

[00174] To the solution of intermediate 28 (1.2 g, 2.2 mmol) in DCM (30 mL) was added intermediate 18 (1.1 g, 2.7 mmol), DMAP (0.8 g, 6.6 mmol) and EDCI (0.9 g, 4.4 mmol). The mixture was stirred at r.t. overnight. DCM (150 mL) was added and the organic layer was washed with water (30 mL x 3) and brine (30 mL), and then concentrated to dryness. The residue was purified by prep-HPLC to afford 1.1 g of Compound 10 as a white solid. MS (M+2H)+ 961; ^{1}H NMR (400MHz, DMSO-d6): δ 9.05 (br s, 1H), 8.10 (s, 1H), 8.00 (d, 1H), 7.72 (d, 2H), 7.29-7.41 (m, 5H), 7.22 (t, 1H), 7.14 (d, 2H), 6.93 (d, 1H), 6.80 (d, 2H), 6.70 (d, 1H), 7.07 (d, 2H), 4.03-4.07 (m, 1H), 2.80-3.15 (m, 9H), 2.78 (s, 2H), 2.23-2.30 (m, 6H), 2.00-2.10 (m, 4H), 1.45 (t, 2H), 1.26 (s, 6H), 1.00-1.24 (m, 5H), 0.99 (s, 6H).

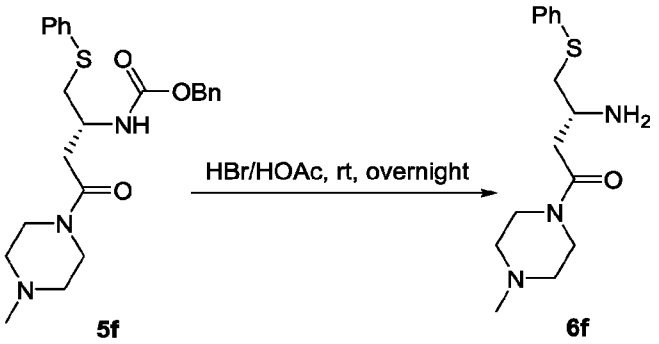
51. Synthesis of intermediate 5f



4 5f

[00175] To the solution of intermediate 4 (8.5 g, 36 mmol) in THF (100 mL) was added 1-methylpiperazine (14.5 g, 145 mmol). The solution was stirred at 50 °C in a sealed tube overnight. The solvent was removed in vacuo to afford 17.0 g of colorless oil. To the solution of above oil in toluene (200 mL) was added Bu_3P (24 g, 119 mmol) and PhSSPh (18 g, 82 mmol) successively. The mixture was stirred at 90 °C overnight. The solvent was removed in vacuo. The residue was purified by silica-gel column to afford 11 g of intermediate 5f as a brown oil.

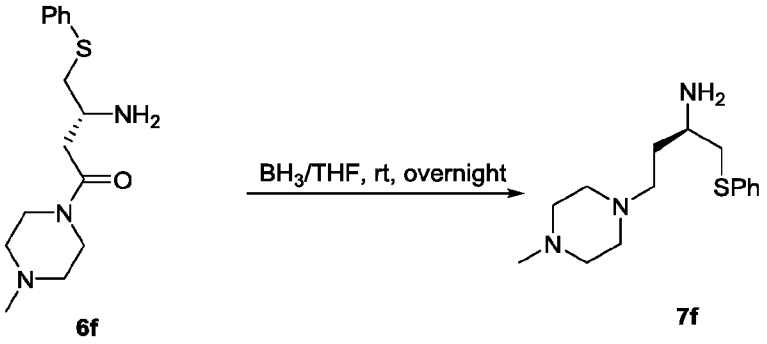
52. Synthesis of intermediate 6f



5f 6f

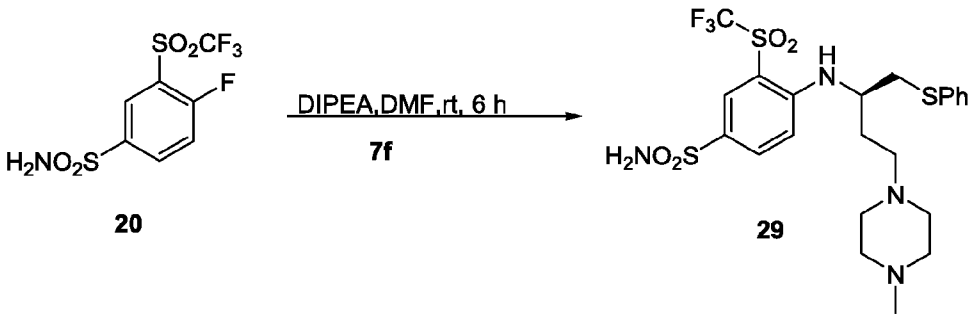
[00176] To the solution of intermediate 5f (11.0 g) in HOAc (60 mL) was added a solution of HBr in HOAc (33%, 20 mL). The mixture was stirred at r.t. overnight. The solvent was removed in vacuo. The residue was purified by reversed-phase column to afford 7.1 g of intermediate 6f as a brown oil.

53. Synthesis of intermediate 7f



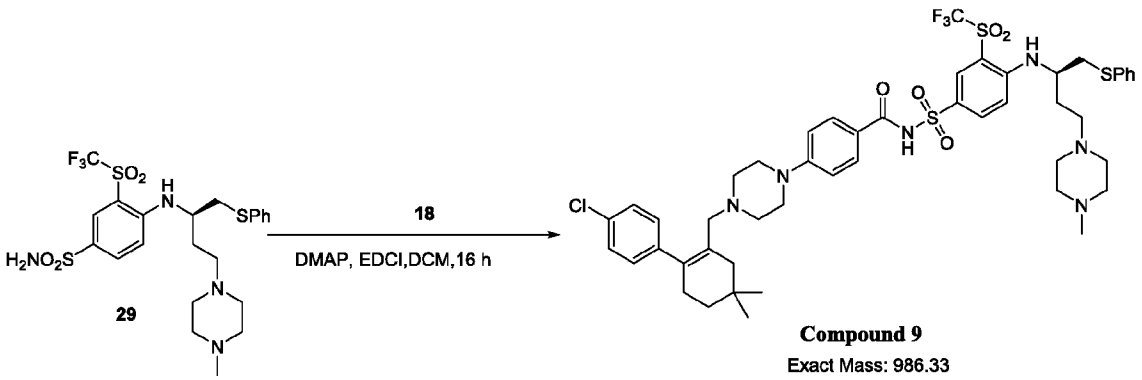
[00177] To the solution of intermediate 6f (3.0 g) in THF (50 mL) was added a solution of BH_3 in THF (1M, 45 mL). The mixture was stirred at r.t. overnight. MeOH (5 mL) was added to quench the reaction. The mixture was concentrated to dryness. MeOH (40 mL) and HCl (conc. 40 mL) was then added. The mixture was refluxed for 4 hours and then concentrated to dryness to afford 3.1 g of intermediate 7f as a yellow oil, which was used for next step directly.

54. Synthesis of intermediate 29



[00178] To the solution of intermediate 20 (1.5 g) in DMF (40 mL) was added DIPEA (6 mL) and intermediate 7f (3.0 g). The solution was stirred at r.t. for 6 hours. The solvent was removed in vacuo. The residue was purified by reversed-phase column to afford 1.9 g of intermediate 29 as a yellow oil.

55. Synthesis of Compound 9



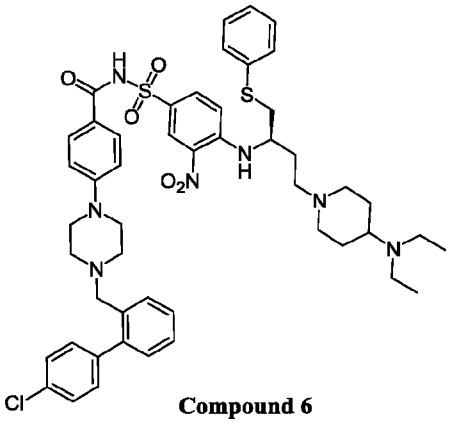
[00179] To the solution of intermediate 29 (0.90 g, 1.6 mmol) in DCM (20 mL) was added intermediate 18 (0.84 g, 1.9 mmol), DMAP (0.58 g, 4.77 mmol) and EDCI (0.61 g, 3.2 mmol). The mixture was stirred at r.t. overnight. DCM (150 mL) was added and the organic solvent was washed with water (30 mL x 3) and brine (30 mL), and then concentrated to dryness. The residue was purified by prep-HPLC to afford 400 mg of Compound 9 as a white solid. MS (M+H)+ 987; ^{1}H NMR (400MHz, CDCl3): δ 8.32 (d, 1H), 7.95 (d, 1H), 7.86 (d, 2H), 7.24-7.38 (m, 5H), 6.98 (d, 2H), 6.88 (d, 1H), 6.76 (d, 2H), 6.40 (d, 1H), 3.80-3.69 (m, 1H), 3.20-3.28 (m, 4H), 2.87-3.01 (m, 2H), 1.64-2.99 (m, 25H), 1.45 (q, 1H), 1.26 (t, 2H), 0.94 (d, 6H).

[00180] While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure that come within known or customary practice within the art to which the invention pertains and may be applied to the essential features set forth herein.

Claims

We claim:

1. A liposome composition comprising Compound 6



Compound 6

or a pharmaceutically acceptable salt thereof, wherein Compound 6 or the pharmaceutically acceptable salt thereof is encapsulated in a liposome.

2. The composition of claim 1, wherein the liposome comprises distearoyl phosphatidylcholine (DSPC) and cholesterol.

3. The composition of claim 2, wherein the liposome further comprises PEG(2000)-distearoylglycerol (PEG-DSG).

4. The composition of claim 3, wherein the liposome comprises DSPC, cholesterol and PEG-DSG in a molar ratio of about 3:2:0.3.

5. The composition of claim 1, obtained by a process comprising contacting the liposome composition with albumin to obtain albumin-treated liposomes, and subsequently separating the albumin-treated liposomes from the albumin unattached to the liposomes.

6. The composition of claim 5, wherein the albumin is bovine serum albumin and the process further comprises removing Compound 6 that is not encapsulated in the liposome.

7. The composition of claim 6, wherein the albumin is a 5% w/w solution of the BSA.

8. The composition of claim 7, wherein the albumin has a concentration of about 10 mg/mL BSA, and the albumin-treated liposomes are obtained by contacting the liposome composition with the albumin for about 50 minutes at about 37 °C.

9. The composition of claim 5, comprising less than about 0.1 mg/mL residual free albumin.

10. The composition of claim 9, wherein at least about 95% of Compound 6 in the liposome composition is encapsulated in a liposome.

11. The composition of any one of claims 1-10, comprising a total of about 125 g of Compound 6 per mol of total phospholipid in the composition.

12. The composition of claim 11, having a pH of about 5-6.

13. The composition of any one of claims 11-12, wherein the liposomes have a particle size of about 100-110 nm.

14. The composition of any one of claims 1-13, obtained by a process comprising the steps of

a. forming a liposome encapsulating ammonium sulfate encapsulated in a vesicle comprising a phospholipid and cholesterol; and
b. contacting the liposome from step (a) with Compound 6 under conditions effective to load Compound 6 into the lipsosome.

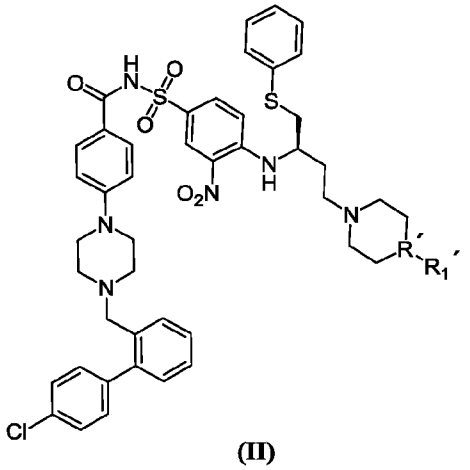
15. The composition of claim 14, wherein ammonium sulfate has a concentration of about 0.25 M prior to contacting the liposome with Compound 6.

16. The composition of claim 15, wherein the bis-mesylate salt of Compound 6 is contacted with the liposome to load the Compound 6 into the liposome.

17. A composition comprising Compound 6, or a pharmaceutically acceptable salt thereof

18. A bis-mesylate salt of Compound 6.

19. A compound of formula (II)



(II)

or a pharmaceutically acceptable salt thereof,

wherein R′ is N or CH, and R_1′ is a moiety comprising a tertiary alkyl-substituted amine having a pK_a of greater than 7.0 and IC_{50} activity at Bcl-2 of less than 0.32 (measured according to Example 1).

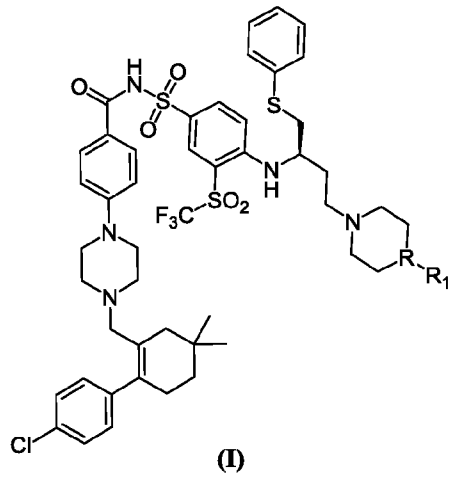
20. The compound of claim 19, wherein R_1′ is –N($R^{a'}$)($R^{b'}$) or –(A′)-N($R^{a'}$)($R^{b'}$) where –(A′)- is a (C_1-C_4) aliphatic moiety, and $R^{a'}$ and $R^{b'}$ are independently lower alkyl.

21. The compound of claim 19, wherein R′ is CH and R_1′ is –N($R^{a'}$)($R^{b'}$).

22. The compound of claim 21, wherein $R^{a'}$ and $R^{b'}$ are independently selected from ethyl and methyl.

23. The compound of claim 22, wherein $R^{a'}$ and $R^{b'}$ are each ethyl.

24. A compound of formula (I)



(I)

or a pharmaceutically acceptable salt thereof,

wherein R is N or CH, and R_1 is a moiety comprising a tertiary alkyl-substituted amine having a pK_a of greater than 7.0 and IC_{50} activity at Bcl-2 of less than 2.46 nM (measured according to Example 1).

25. The compound of claim 24, wherein R_1 is $-N(R^a)(R^b)$ or $-(A)-N(R^a)(R^b)$ where $-(A)-$ is a (C_1-C_4) aliphatic moiety, and R^a and R^b are independently lower alkyl.

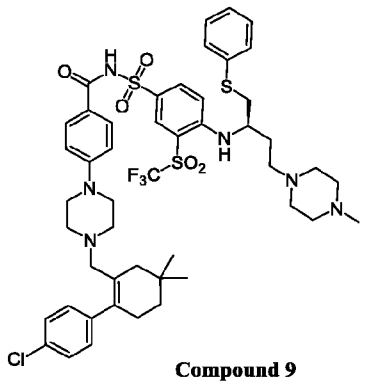
26. The compound of claim 25, wherein R is CH and R^1 is $-N(R^a)(R^b)$.

27. The compound of claim 26, wherein R^a and R^b are independently selected from ethyl and methyl.

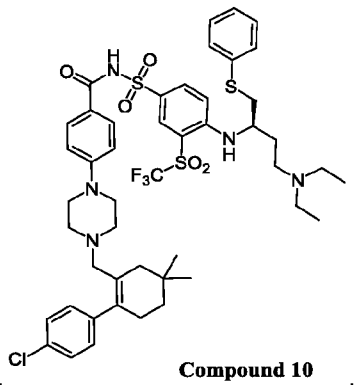
28. The compound of claim 27, wherein R^a and R^b are each ethyl.

29. The compound of claim 27, wherein R^a and R^b are each methyl.

30. A compound selected from



Compound 9



Compound 10

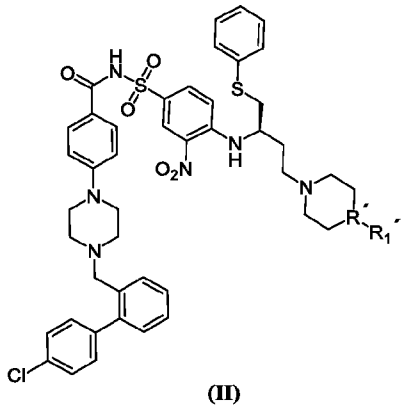
and pharmaceutically acceptable salts thereof.

31. A composition comprising a liposome having a Bcl-2/Bcl-X_L inhibitor compound encapsulated in a unilamellar vesicle formed from one or more liposome-forming lipids, cholesterol and a hydrophilic polymer-conjugated lipid, wherein the injection of a dose of 10 mg/kg of the liposome encapsulated Bcl-2/Bcl-X_L inhibitor compound in a mouse according to Example 6 results in a blood platelet drop of less than about 20% measured 24 hours after injection of the liposome encapsulated Bcl-2/Bcl-X_L inhibitor compound.

32. A composition comprising a liposome having a Bcl-2/Bcl-X_L inhibitor compound encapsulated in a unilamellar vesicle formed from one or more liposome-forming lipids, cholesterol and a hydrophilic polymer-conjugated lipid, wherein the injection of a dose of 10 mg/kg of the liposome encapsulated Bcl-2/Bcl-X_L inhibitor compound in a mouse according to Example 6 results in a blood platelet counts measured above about 400,000 PLT/microliter blood measured 72 hours after injection of the liposome encapsulated Bcl-2/Bcl-X_L inhibitor compound.

33. The composition of claim 32, wherein the Bcl-2/Bcl-X_L inhibitor compound is a compound of formula (I) or formula (II).

34. A composition comprising a liposome having a Bcl-2/Bcl-X_L inhibitor compound of formula (II)



(II)

or a pharmaceutically acceptable salt thereof, wherein R´ is N or CH, and R_1´ is a moiety comprising a tertiary alkyl-substituted amine, and the compound of formula (II) is encapsulated in a unilamellar vesicle formed from one or more liposome-forming lipids, cholesterol and a hydrophilic polymer-conjugated lipid, wherein the injection of a dose of 10 mg/kg of the liposome encapsulated Bcl-2/Bcl-X_L inhibitor compound in a mouse according to Example 6 results in a blood platelet counts measured above about 400,000 PLT/microliter blood measured 72 hours after injection of the liposome encapsulated Bcl-2/Bcl-X_L inhibitor compound.

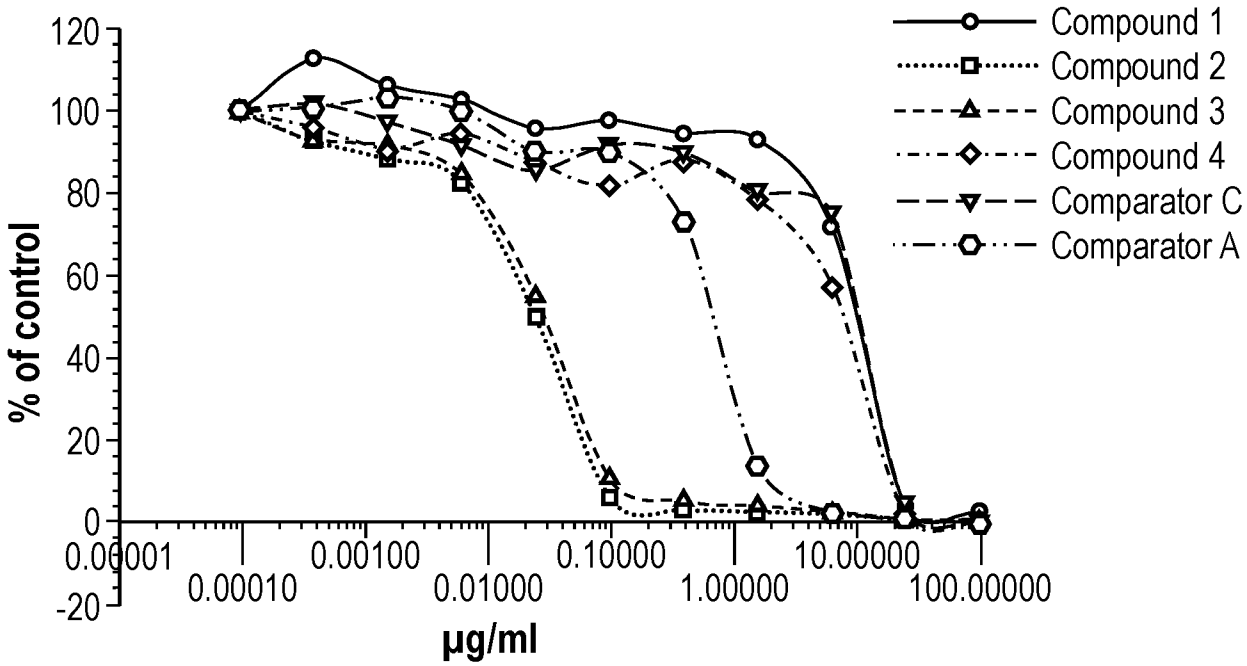

Compound 1
Compound 2
Compound 3
Compound 4
Comparator C
Comparator A
% of control
120
100
80
60
40
20
0
-20
0.00001
0.00010
0.00100
0.01000
0.10000
1.00000
10.00000
100.00000
µg/ml

Fig. 1A

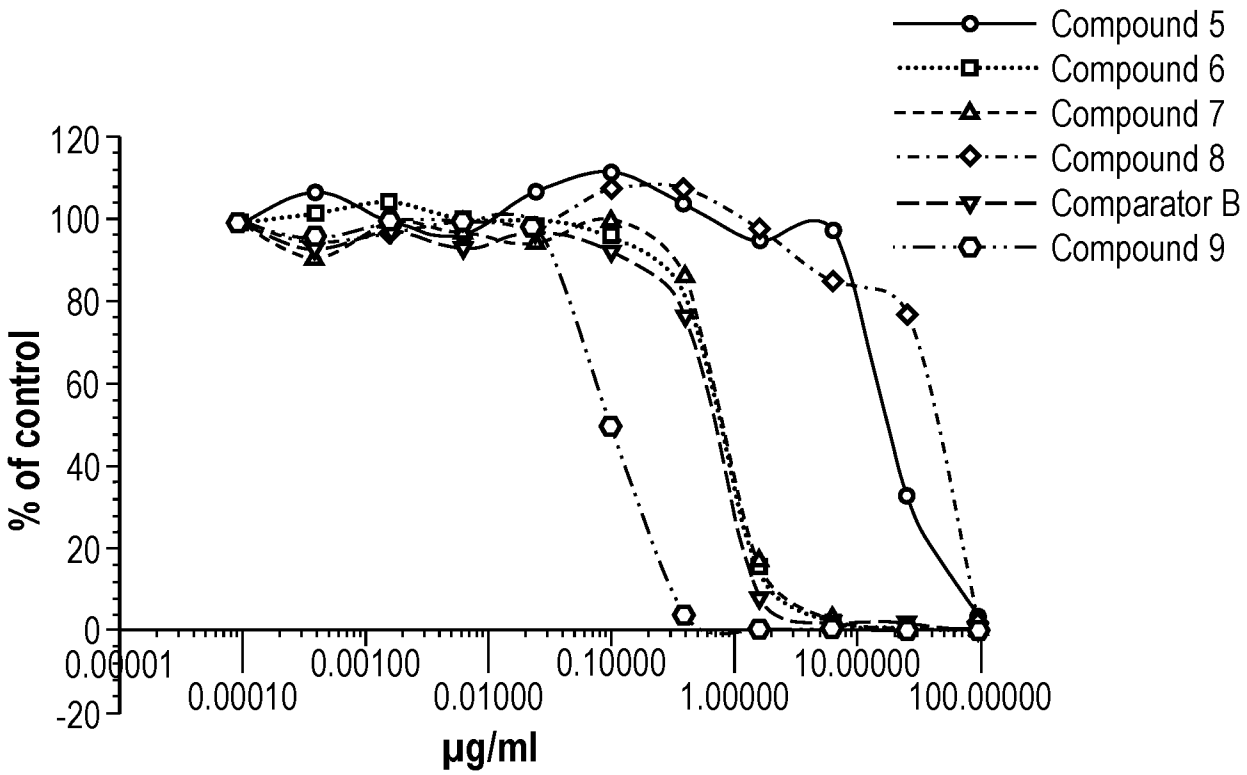

Compound 5
Compound 6
Compound 7
Compound 8
Comparator B
Compound 9
% of control
120
100
80
60
40
20
0
-20
0.00001
0.00010
0.00100
0.01000
0.10000
1.00000
10.00000
100.00000
µg/ml

Fig. 1B

Table 3A

IC50 (log10 uM)	Scale	-3 => nM	0 => µM	3 => mM							
Cell Line	Compar. A	Comp. 1	Comp.2	Comp. 3	Comp. 4	Compar. B	Comp. 5	Comp. 6	Comp. 7	Comp. 8	Compar. C
AMO-1	1.07	-0.10	1.03	1.03		1.38		1.45	1.81		1.06
Colo677	0.31	0.97	-0.31	-0.24	0.91	0.59	1.60	0.88	0.87	1.61	1.04
EJM	0.60		0.01	0.24		0.71	1.81	0.90	1.00	1.71	
JJN3	0.47	0.96	0.04	0.10	0.90	0.70	1.62	0.96	1.01	1.59	0.75
KMS-12BM	-0.27	0.95	-1.15	-1.02	0.93	-0.57	1.74	0.00	0.04		-1.08
KMS-12PE	-1.15	0.72	-2.38	-2.23	0.50	-1.68	1.61	-0.74	-0.61		-3.37
L363	0.94		0.36	0.86		1.17	-0.52		1.43	1.70	1.21
LP-1	0.66		0.95	0.96		1.00	1.44			1.70	1.06
MM-1R	-0.40	0.95	-1.34	-1.24	0.87	-0.11	1.52	0.30	0.15	1.64	0.99
MM-1S	-0.52	0.90	-1.62	-1.48	0.89	-0.24	1.37	-0.10	0.04	1.58	1.00
MOLP2	0.46		-0.29	-0.14	0.92	0.46	1.63	0.49	0.59	1.72	0.94
MOLP8	0.95	1.06	0.56	0.86	1.03	1.32					1.14
NCI-H929	1.06	1.04	0.99	1.00		1.31	1.64	1.20	1.23	1.73	
OPM2	0.74	0.99	0.57	0.52		0.95	1.45	1.16	1.30	1.64	1.04
RPMI-8226	-0.41	0.97	-1.23	-1.05	1.07	0.07	1.76	0.32	0.42	1.78	0.96
SKMM2	-1.88	0.05	-2.47	-2.63	0.35	-2.64	0.85	-0.99	-1.28	1.12	-3.42
U266B1	1.11	-0.12	0.57	0.67	0.99	1.36	1.58	1.13	1.43	1.69	1.72
mean	**0.22**	**0.72**	**-0.34**	**-0.22**	**0.85**	**0.34**	**1.41**	**0.50**	**0.63**	**1.63**	**0.34**
sem	**0.21**	**0.12**	**0.28**	**0.29**	**0.07**	**0.27**	**0.15**	**0.20**	**0.22**	**0.05**	**0.42**
t-test		vs. Compar. A	vs. Compar. A	vs. Compar. A	vs. Compar. A		vs. Compar. B	vs. Compar. B	vs. Compar. B	vs. Compar. B	
		0.04603	***0.00003***	***0.00019***	0.00130		0.00256	***0.02062***	***0.00097***	0.00041	
t-test	vs. Compar. C	vs. Compar. C	vs. Compar. C	vs. Compar. C	vs. Compar. C	vs. Compar. C	vs. Compar. C	vs. Compar. C	vs. Compar. C	vs. Compar. C	
	0.47069	0.31214	***0.01372***	***0.02558***	0.14282	0.66435	0.04386	0.45505	0.29853	0.02617	

statistically significantly more potent

statistically significantly less potent

Fig. 1C

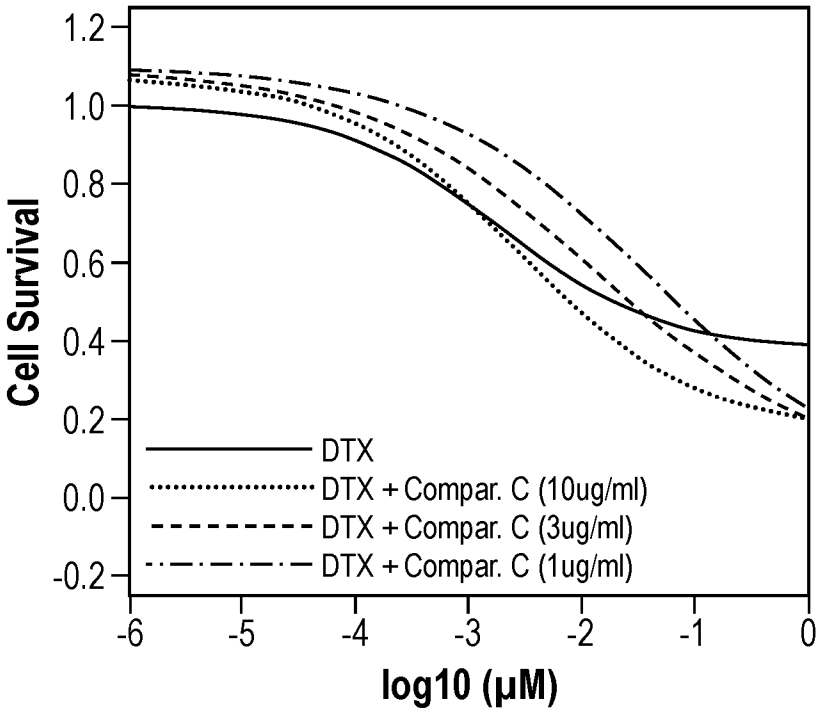

1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
Cell Survival
DTX
DTX + Compar. C (10ug/ml)
DTX + Compar. C (3ug/ml)
DTX + Compar. C (1ug/ml)
-6
-5
-4
-3
-2
-1
0
log10 (μM)

Fig. 2A

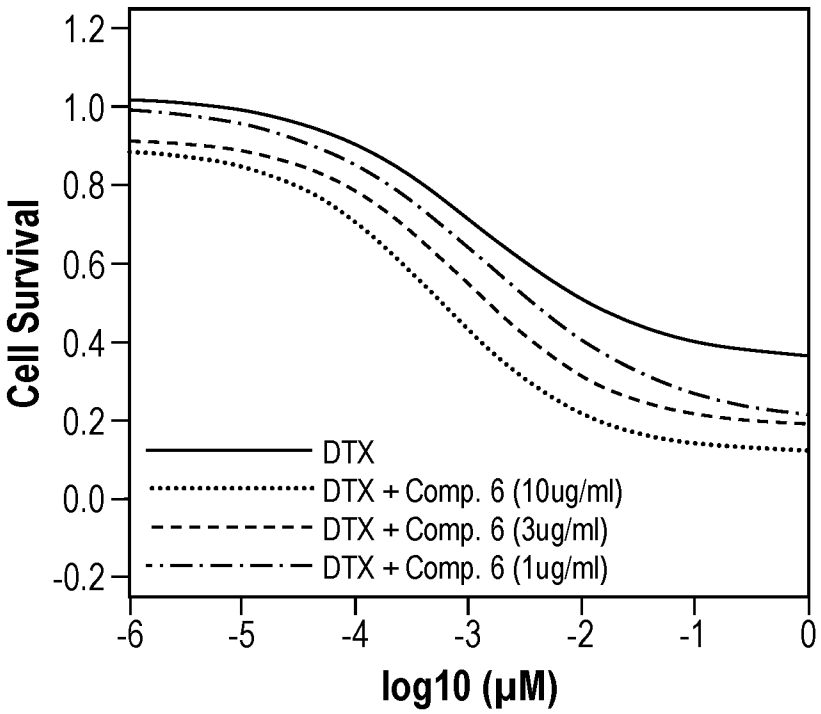

1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
Cell Survival
DTX
DTX + Comp. 6 (10ug/ml)
DTX + Comp. 6 (3ug/ml)
DTX + Comp. 6 (1ug/ml)
-6
-5
-4
-3
-2
-1
0
log10 (μM)

Fig. 2B

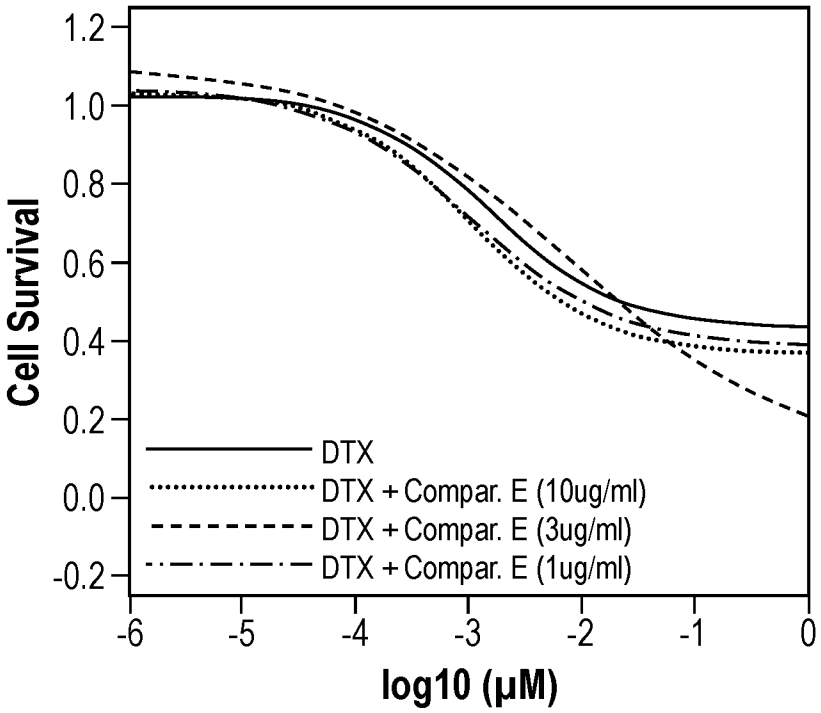

1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
Cell Survival
DTX
DTX + Compar. E (10ug/ml)
DTX + Compar. E (3ug/ml)
DTX + Compar. E (1ug/ml)
-6
-5
-4
-3
-2
-1
0
log10 (μM)

Fig. 2C

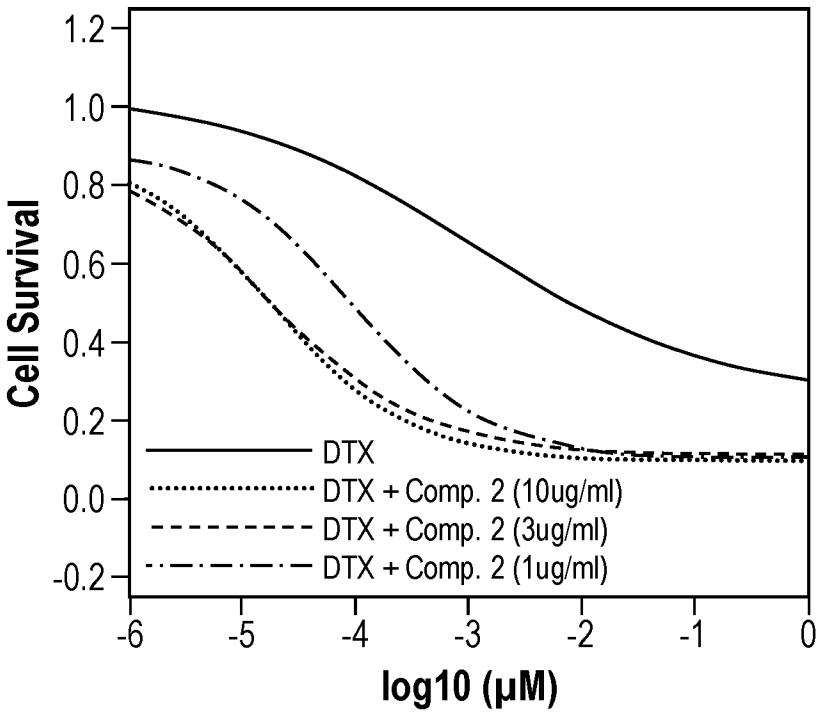

1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
Cell Survival
DTX
DTX + Comp. 2 (10ug/ml)
DTX + Comp. 2 (3ug/ml)
DTX + Comp. 2 (1ug/ml)
-6
-5
-4
-3
-2
-1
0
log10 (μM)

Fig. 2D

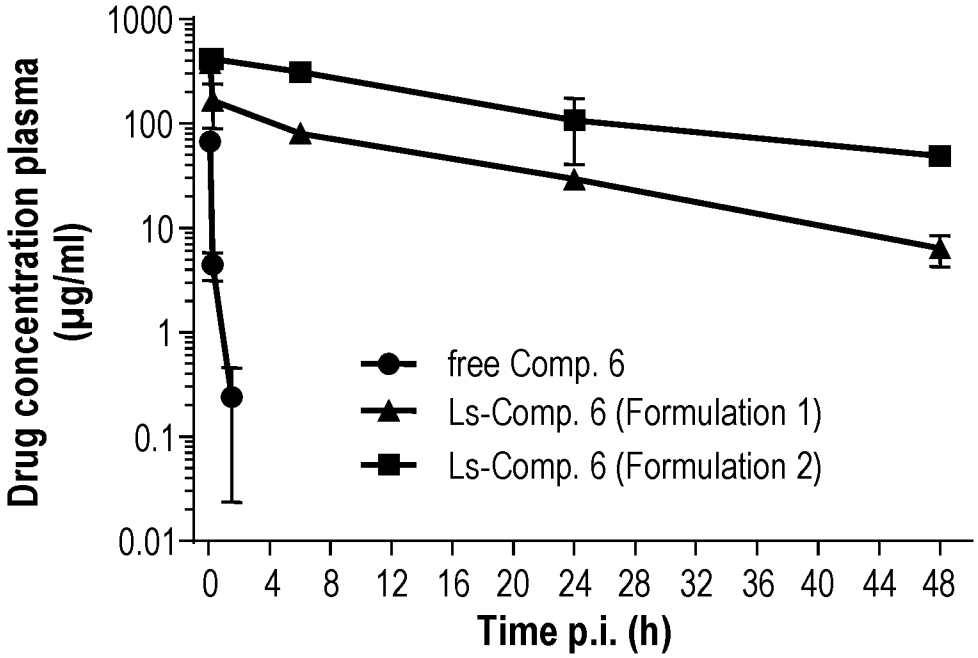

Drug concentration plasma (µg/ml)
1000
100
10
1
0.1
0.01
0 4 8 12 16 20 24 28 32 36 40 44 48
Time p.i. (h)
free Comp. 6
Ls-Comp. 6 (Formulation 1)
Ls-Comp. 6 (Formulation 2)

Fig. 3

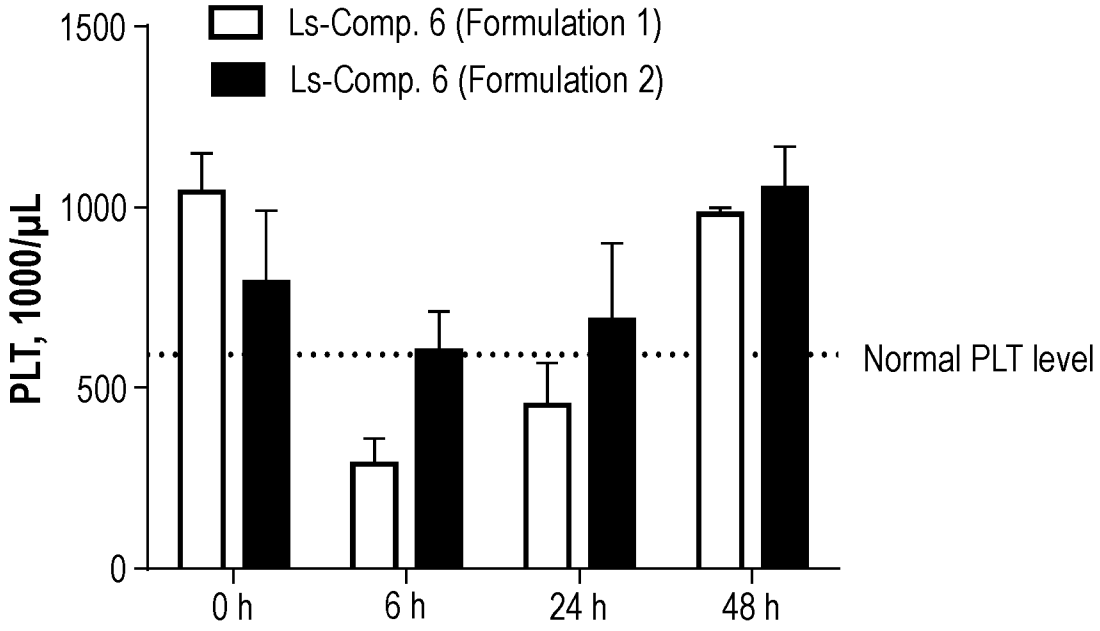

Ls-Comp. 6 (Formulation 1)
Ls-Comp. 6 (Formulation 2)
PLT, 1000/µL
1500
1000
500
0
0 h
6 h
24 h
48 h
Normal PLT level

Fig. 4

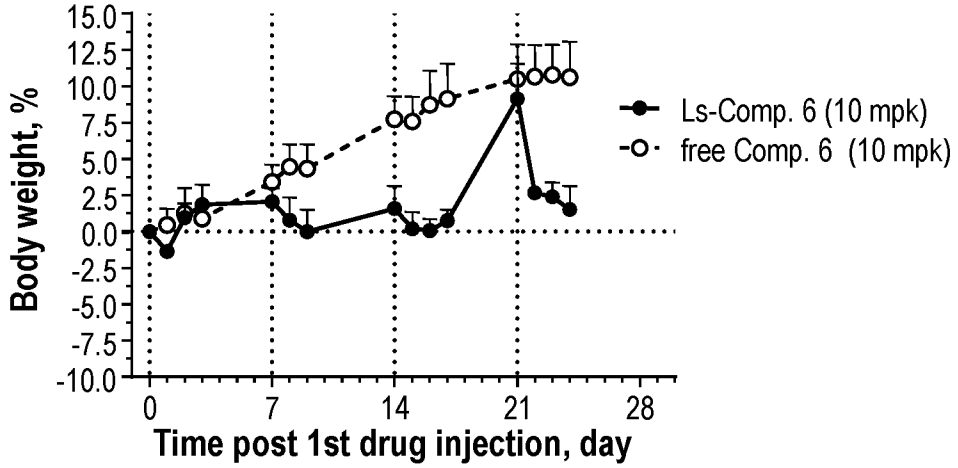


Fig. 5A

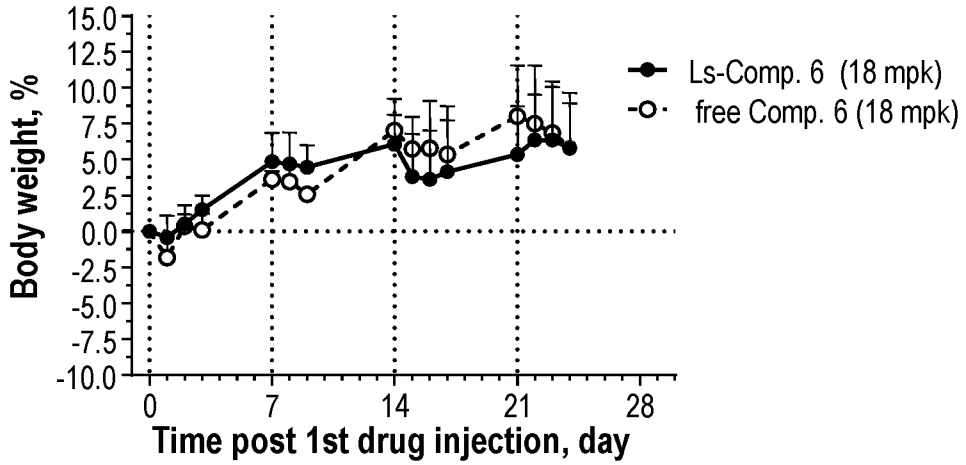


Fig. 5B

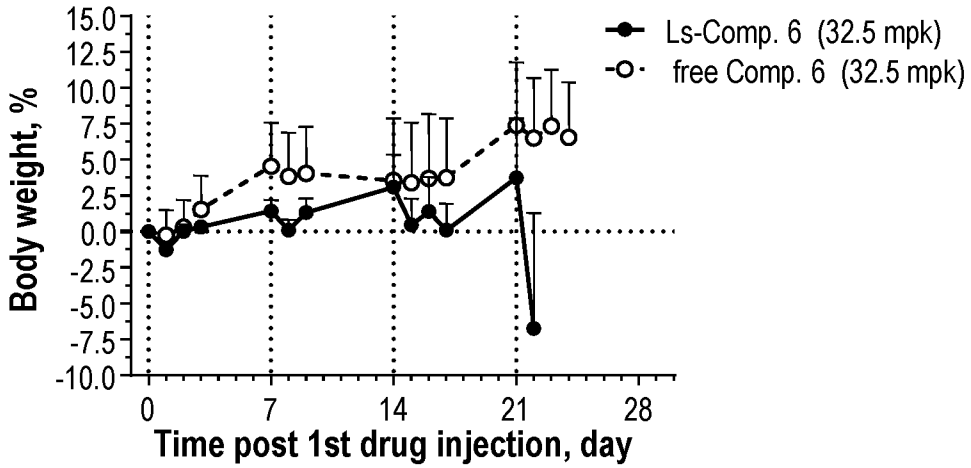


Fig. 5C

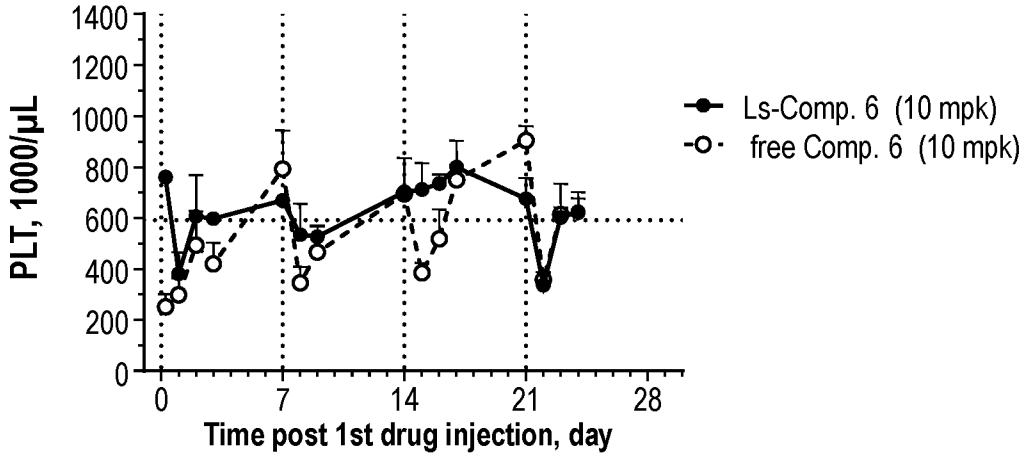


Fig. 6A

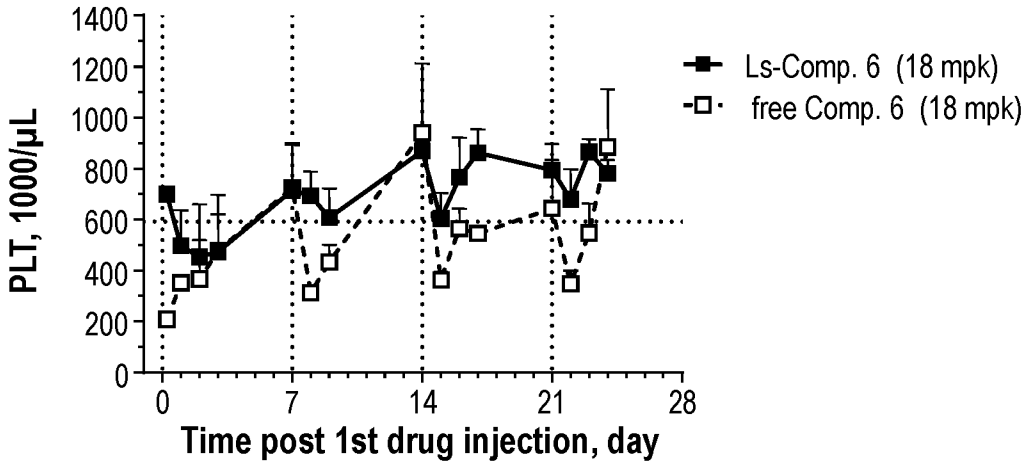


Fig. 6B

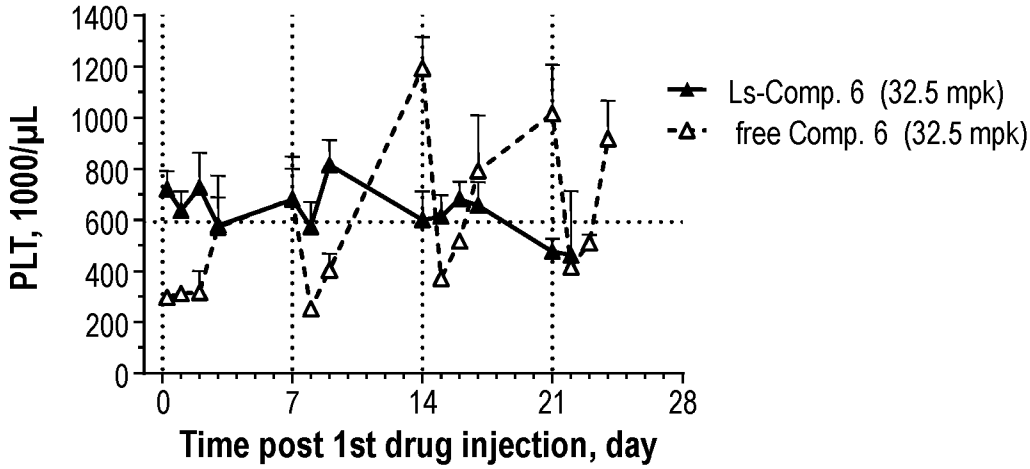


Fig. 6C

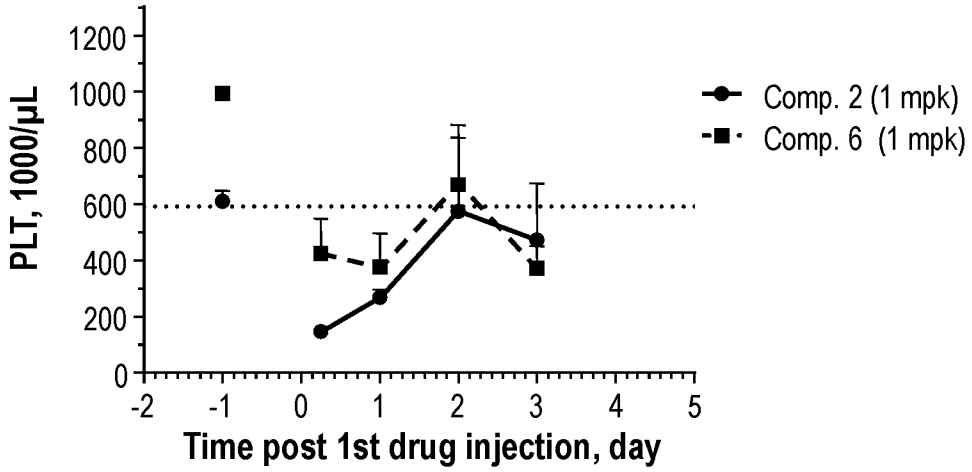


Fig. 7A

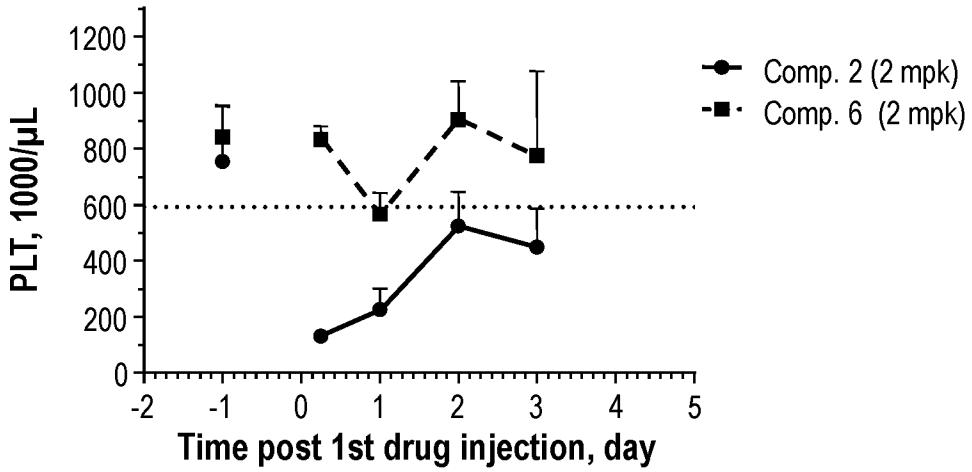


Fig. 7B

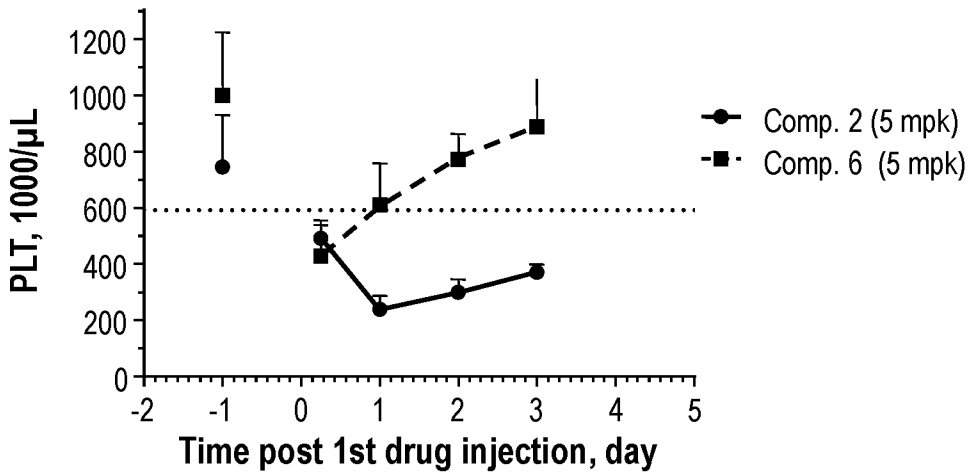


Fig. 7C

Scheme 1: synthesis of intermediate 4

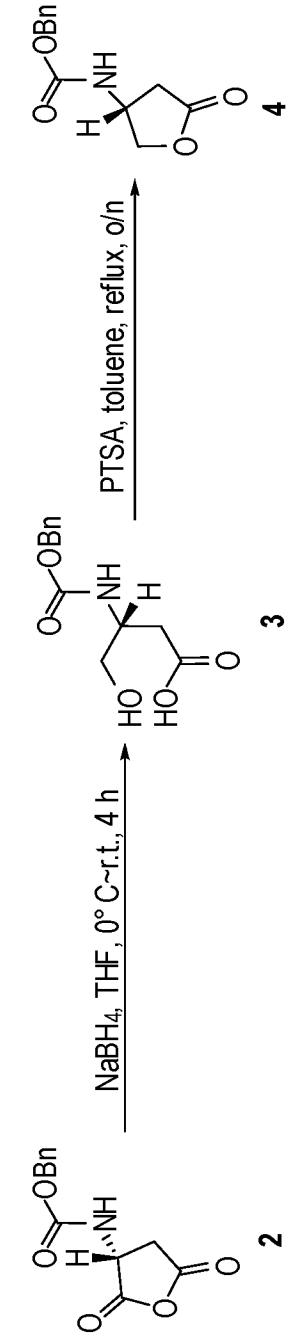


Fig. 8

Scheme 2: synthesis of intermediate 8a

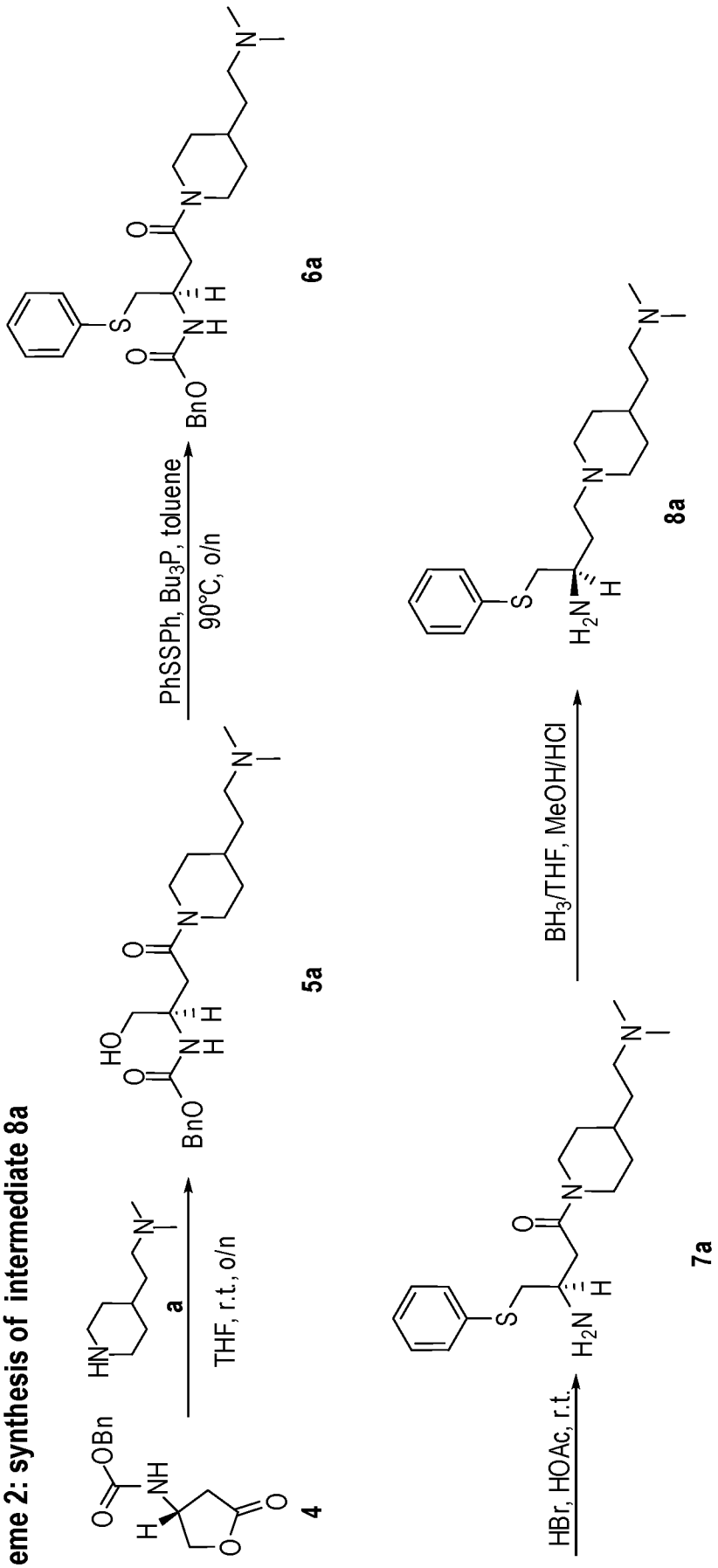


Fig. 9

Scheme 3: synthesis of intermediate 8b

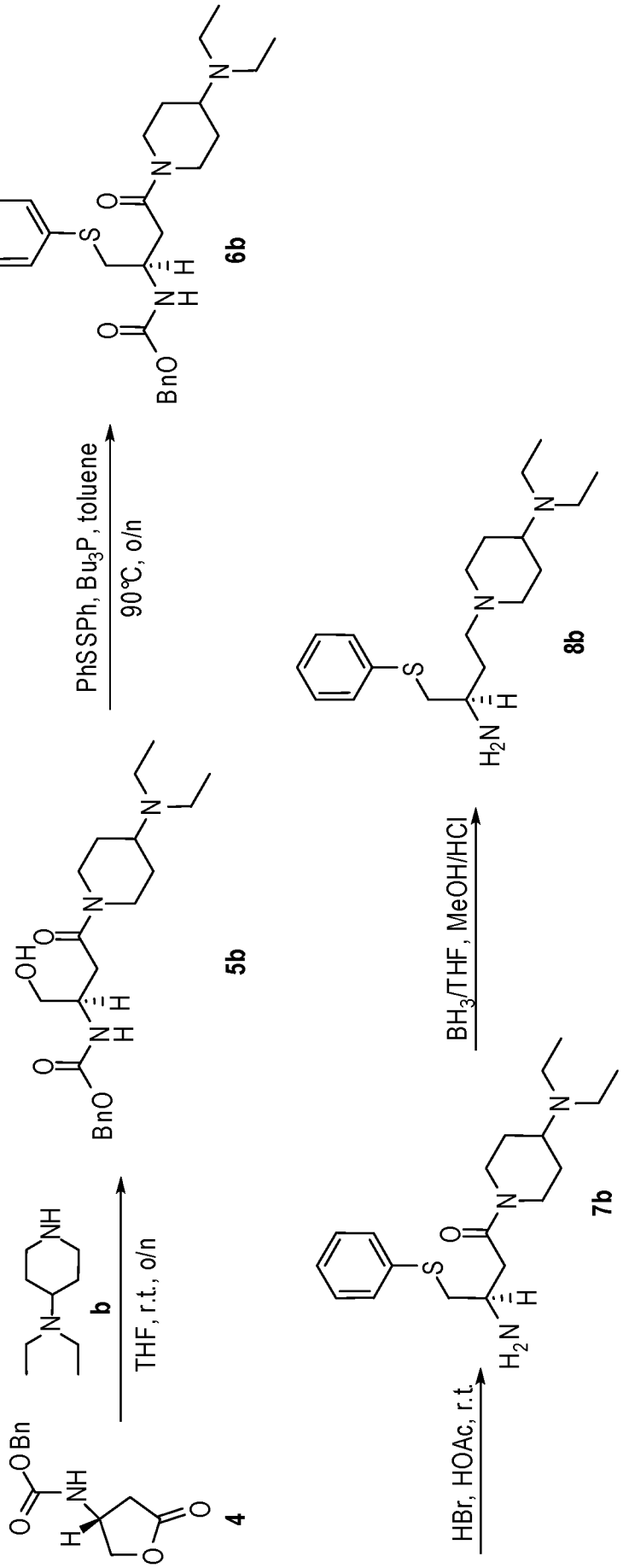


Fig. 10

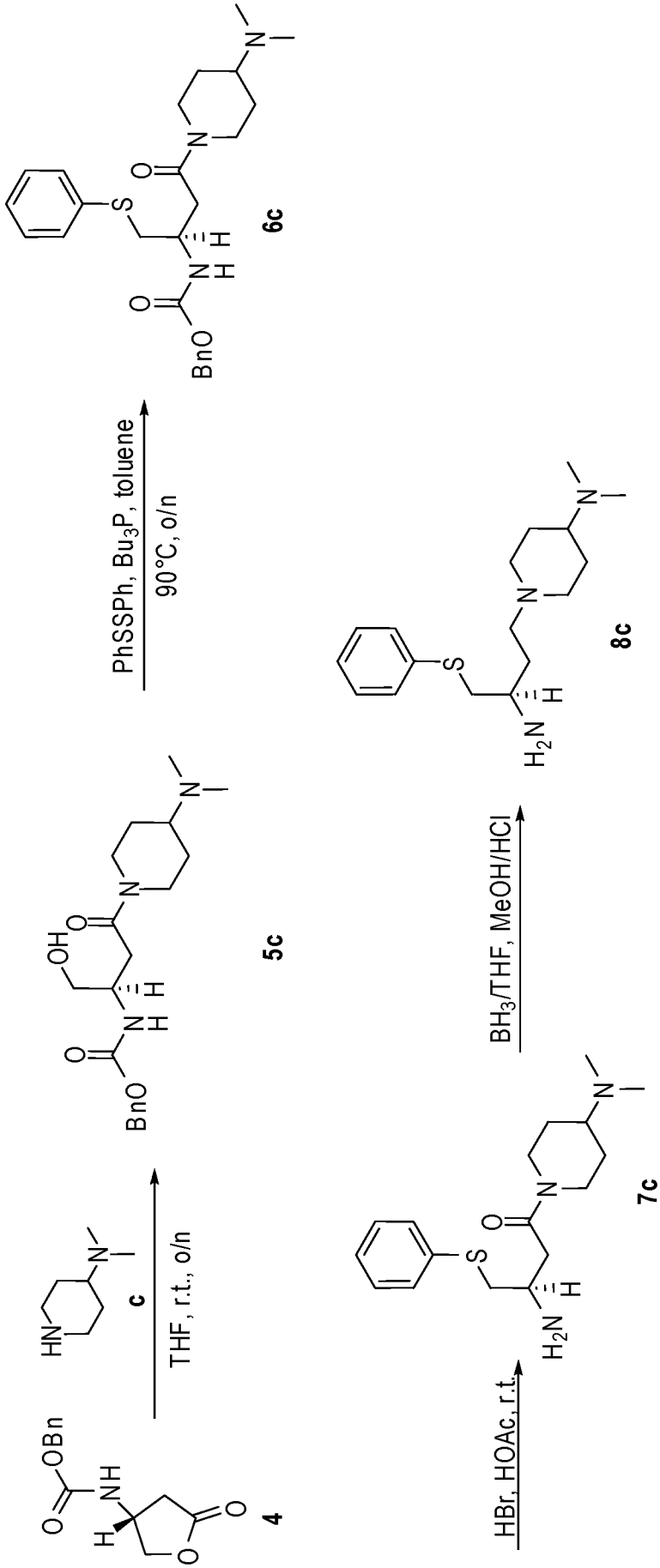

Scheme 4: synthesis of intermediate 8c
4
c
THF, r.t., o/n
5c
PhSSPh, Bu3P, toluene
90°C, o/n
6c
HBr, HOAc, r.t.
7c
BH3/THF, MeOH/HCl
8c

Fig. 11

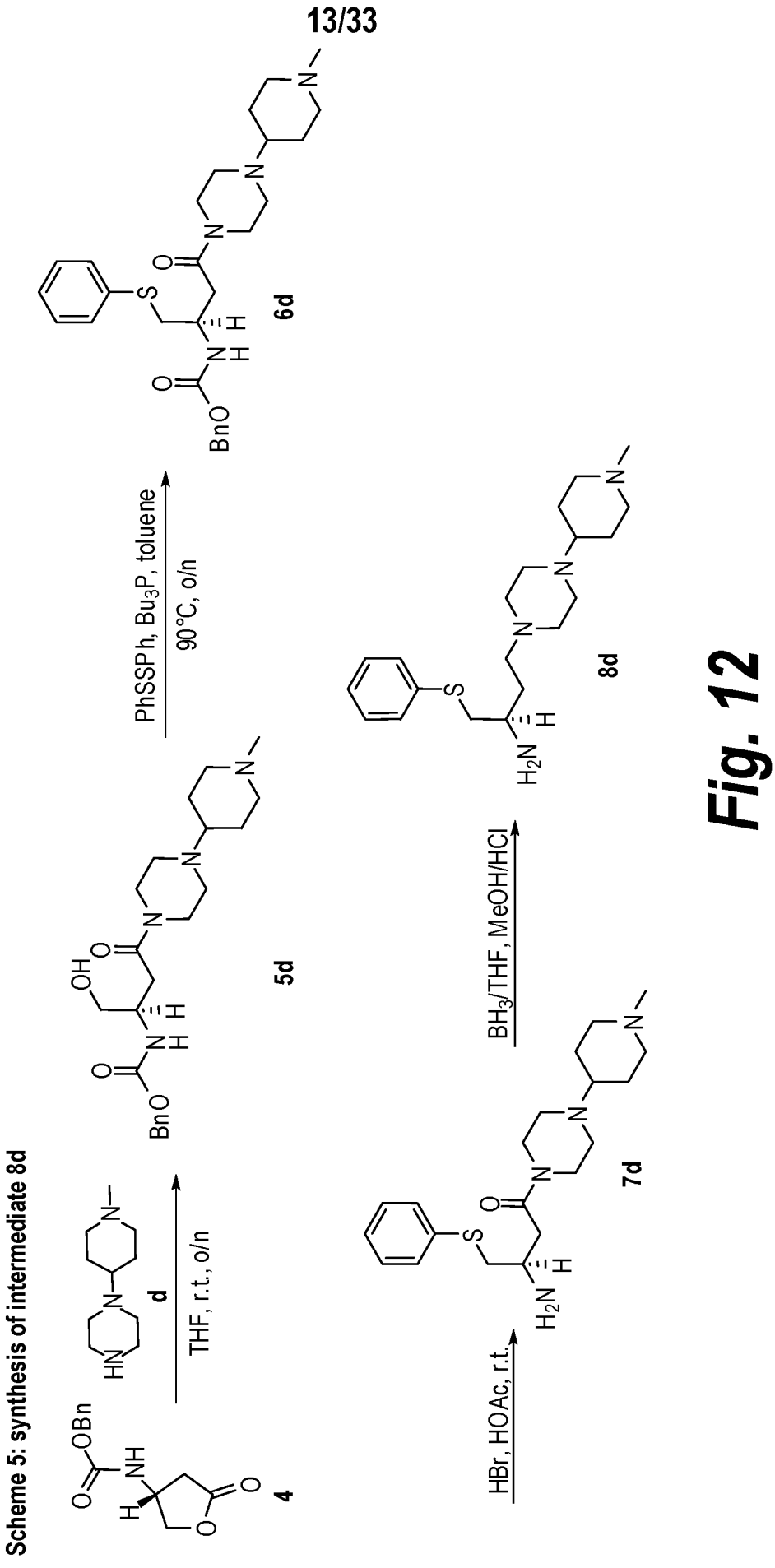

Scheme 5: synthesis of intermediate 8d
4
d
THF, r.t., o/n
5d
PhSSPh, Bu3P, toluene
90°C, o/n
6d
HBr, HOAc, r.t.
7d
BH3/THF, MeOH/HCl
8d

Fig. 12

Scheme 6: synthesis of intermediate 13

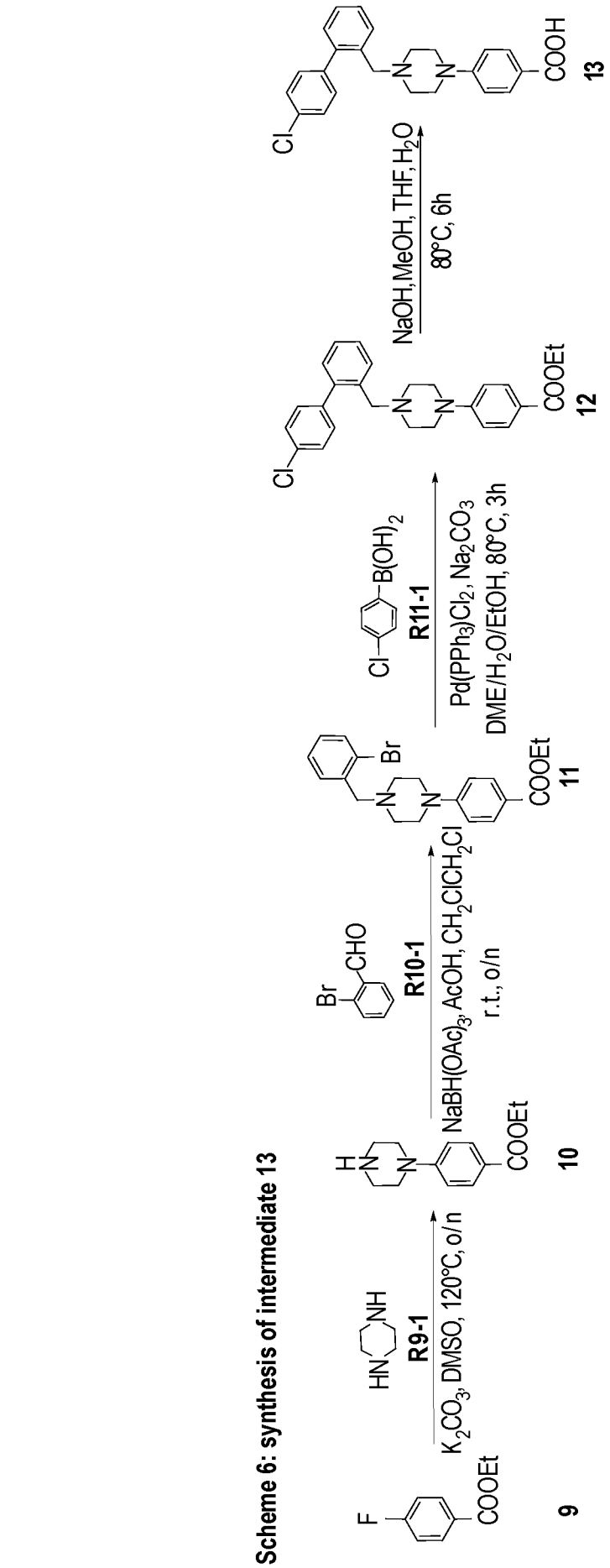
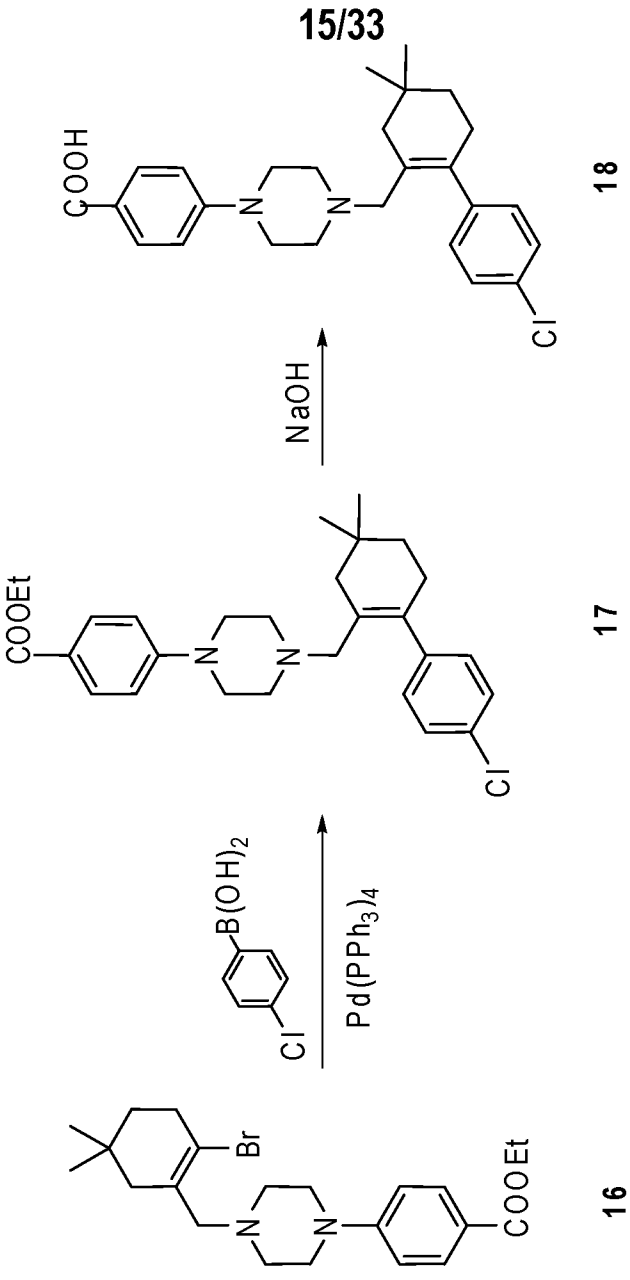
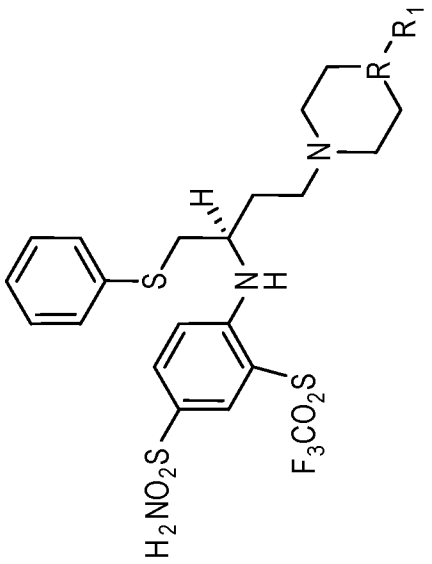


Fig. 13



Scheme 7: synthesis of intermediate 18

Fig. 14



21a, 21b, 21c, 21d

8a, 8b, 8c or 8d

DIPEA, DMF

20

1) $ClSO_3H$

2) NH_4OH

19

Scheme 8: synthesis of intermediates 21a, 21b, 21c, 21d

Fig. 15

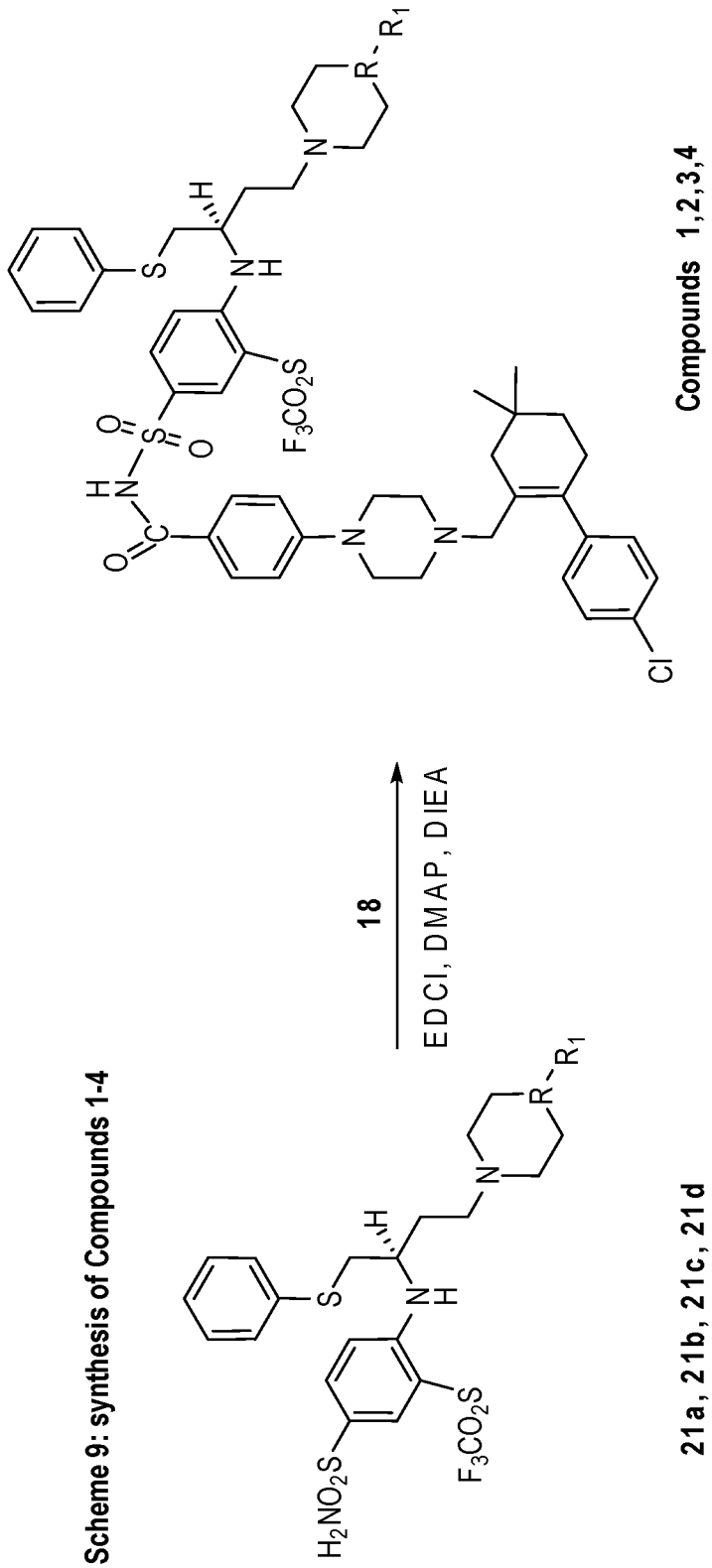
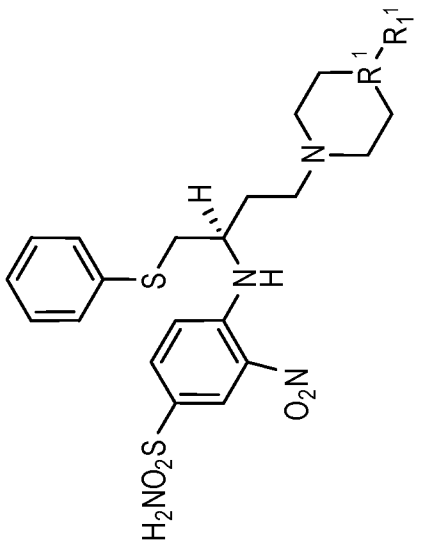

Scheme 9: synthesis of Compounds 1-4
21a, 21b, 21c, 21d
18
EDCI, DMAP, DIEA
Compounds 1,2,3,4

Fig. 16



Scheme 10: synthesis of intermediates 24a, 24b, 24c, 24d

Fig. 17

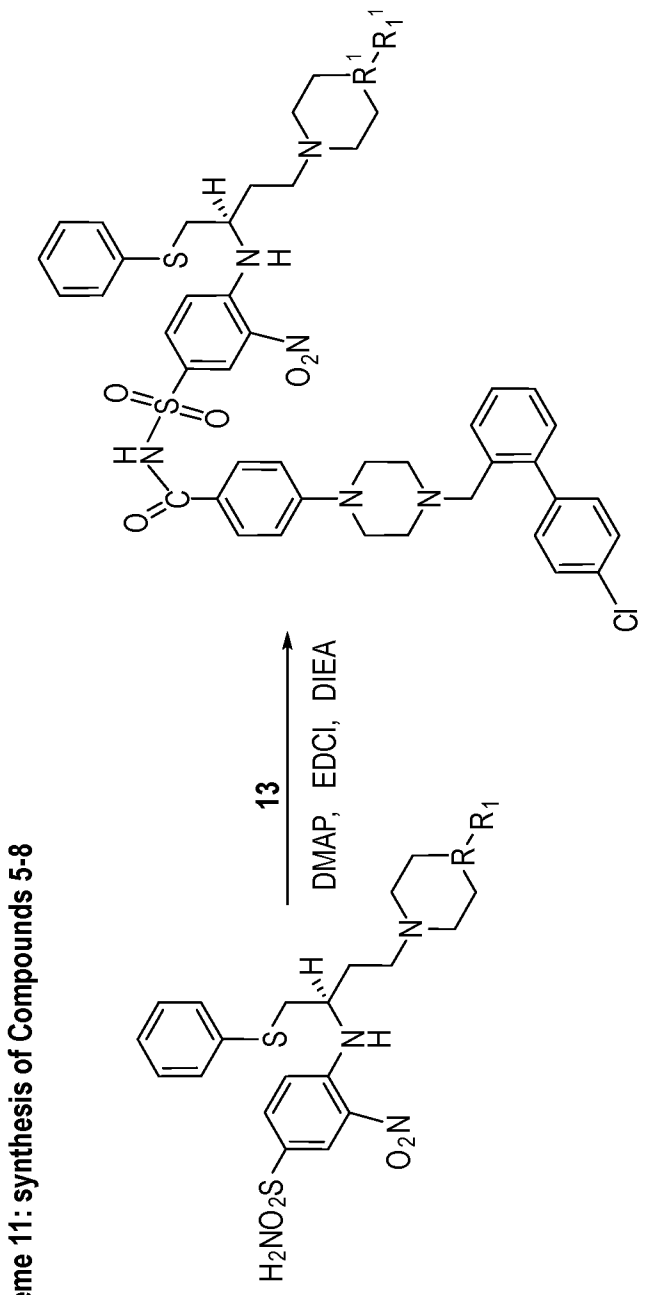
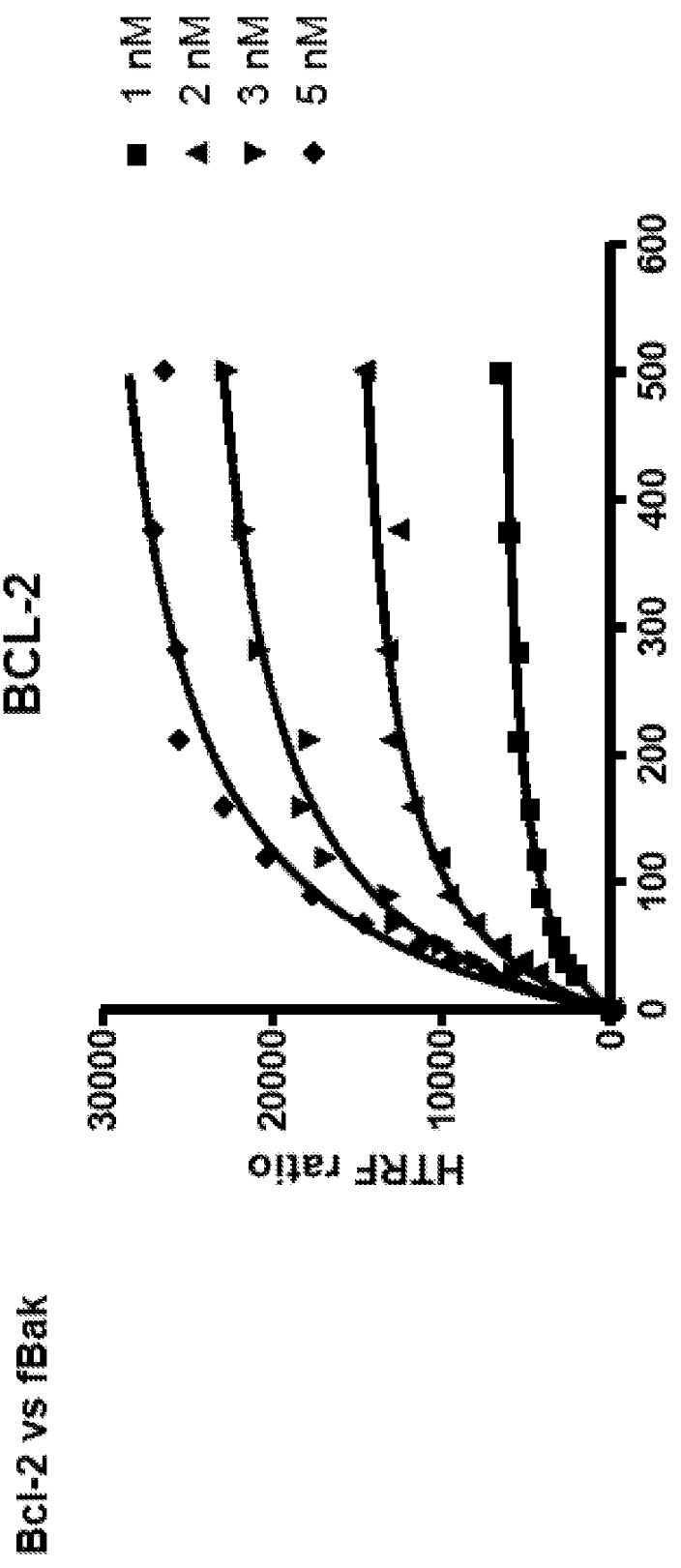

Scheme 11: synthesis of Compounds 5-8
24a, 24b, 24c, 24d
13
DMAP, EDCI, DIEA
Compounds 5, 6, 7, 8

Fig. 18



	1 nM	2 nM	3 nM	5 nM
One site binding (hyperbola)				
Best-fit values				
BMAX	7049	16429	26626	33187
KD	69.50	69.71	82.22	82.78

Fig. 18A

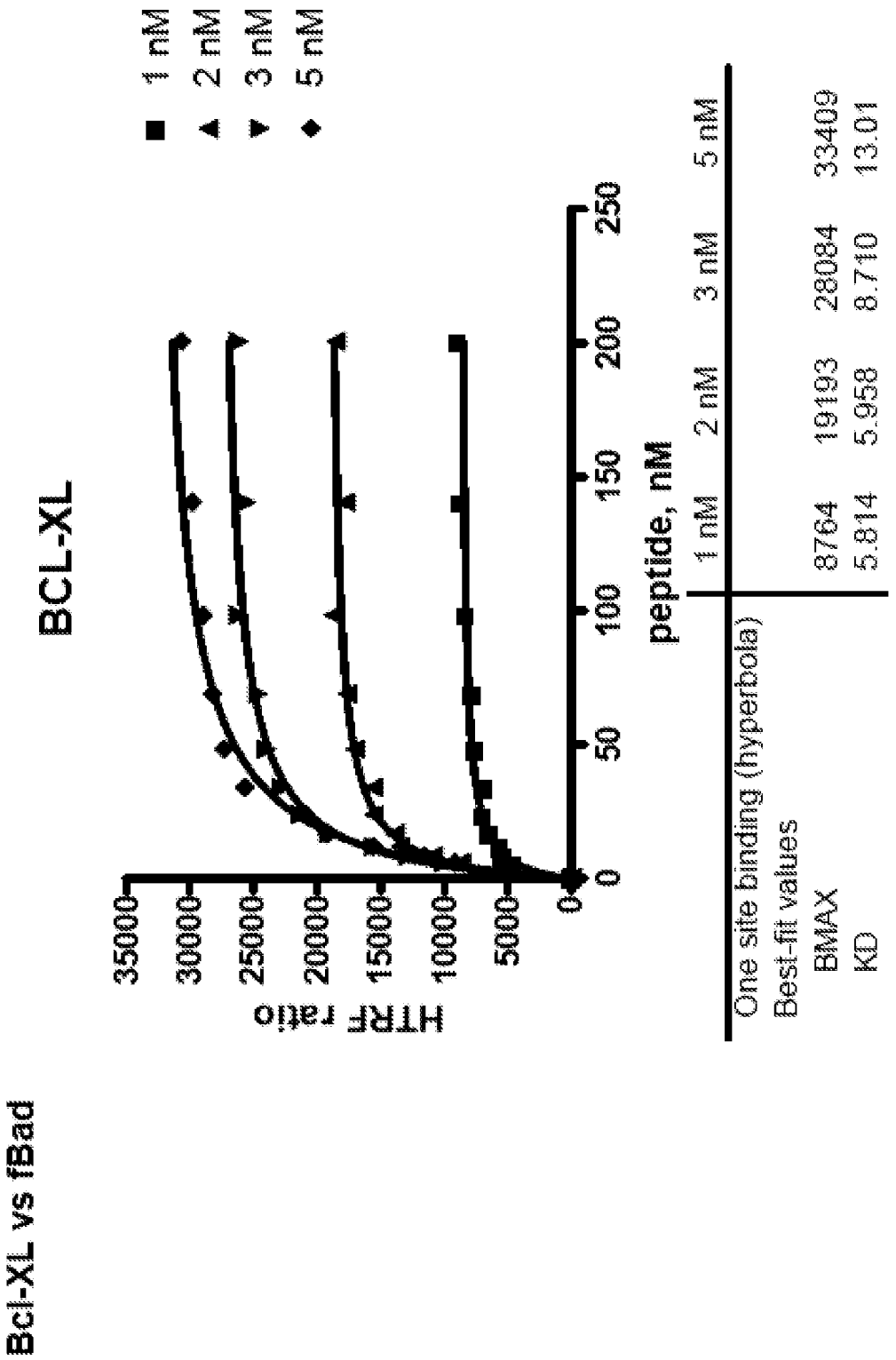

Bcl-XL vs fBad
BCL-XL
1 nM
2 nM
3 nM
5 nM
HTRF ratio
35000
30000
25000
20000
15000
10000
5000
0
0
50
100
150
200
250
peptide, nM
One site binding (hyperbola)
Best-fit values
BMAX
KD
1 nM
2 nM
3 nM
5 nM
8764
5.814
19193
5.958
28084
8.710
33409
13.01

Fig. 18B

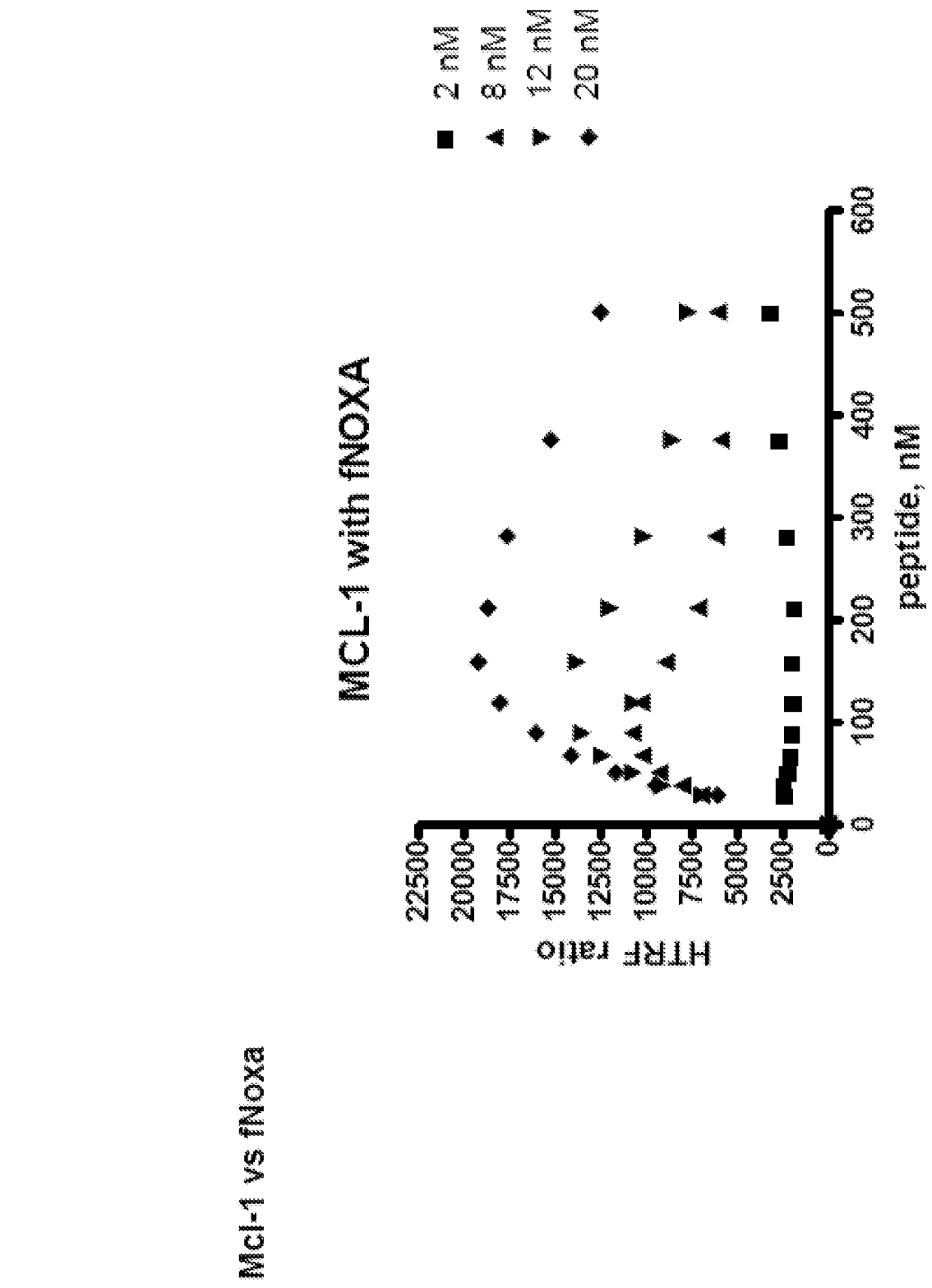
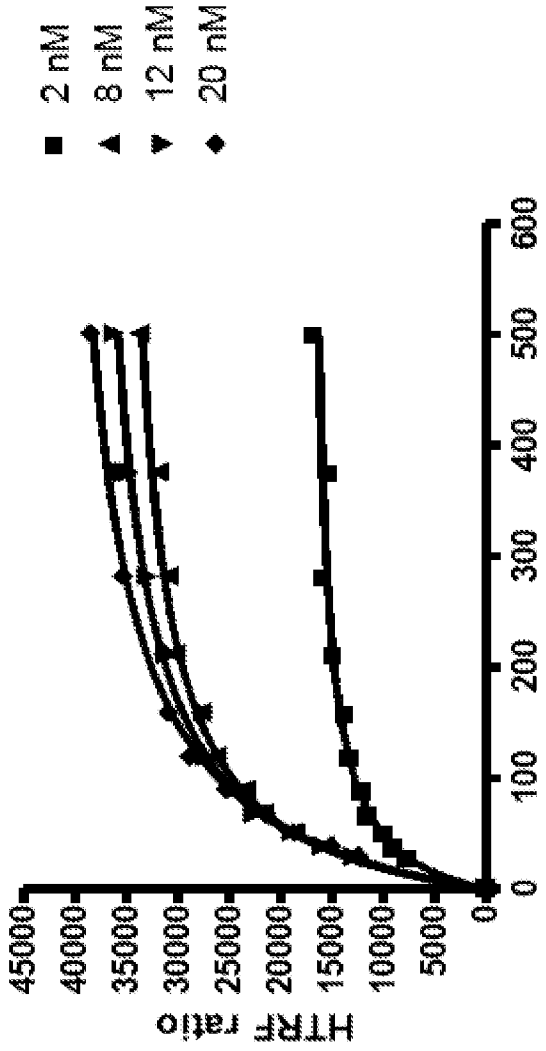

Mcl-1 vs fNoxa
MCL-1 with fNOXA
2 nM
8 nM
12 nM
20 nM
HTRF ratio
22500
20000
17500
15000
12500
10000
7500
5000
2500
0
0
100
200
300
400
500
600
peptide, nM

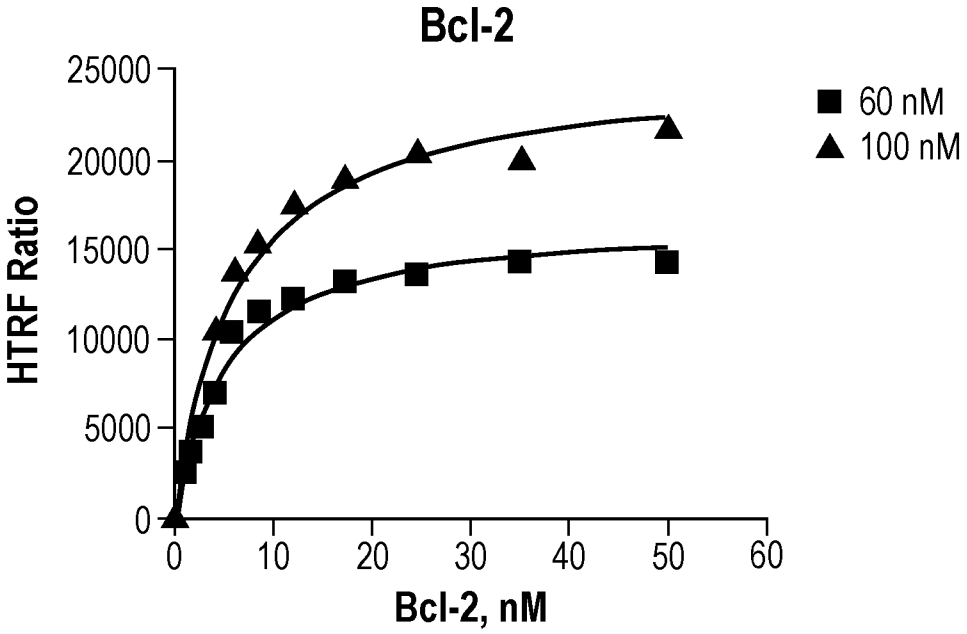
Fig. 18C

Mcl-1 vs fBak



One site binding (hyperbola)	2 nM	8 nM	12 nM	20 nM
Best-fit values				
BMAX	17475	36726	39788	43293
KD	36.65	48.44	55.27	65.72

Fig. 18D



	60 nM	100 nM
Bmax	16658	25184
Kd	5.126	6.238

Fig. 19A

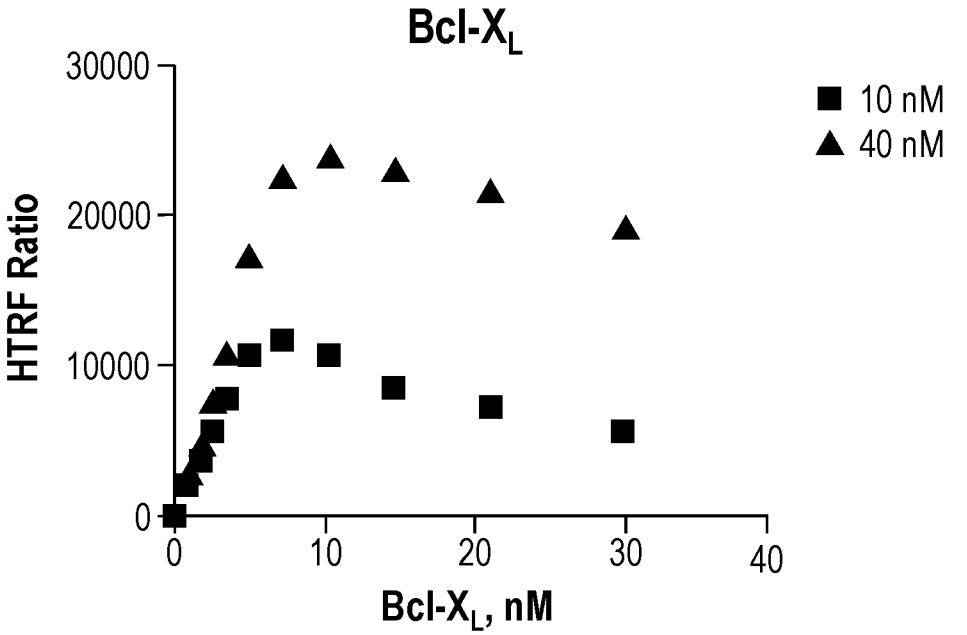


Fig. 19B

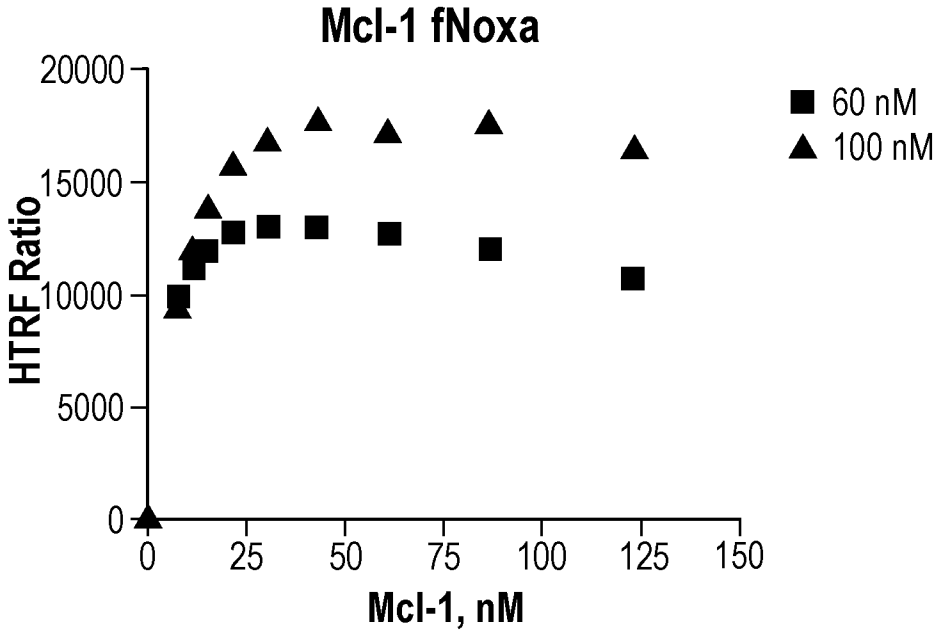
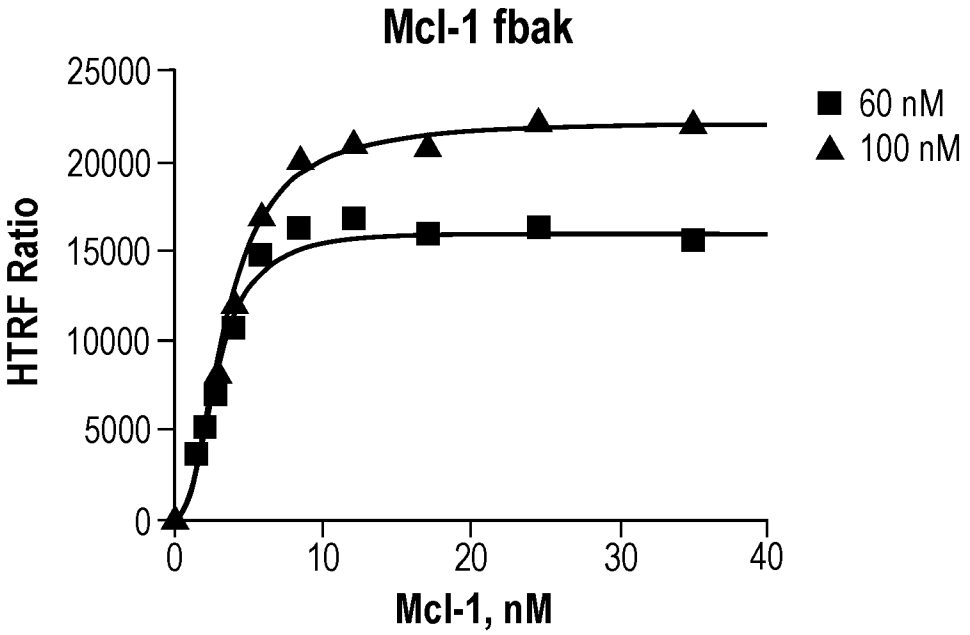
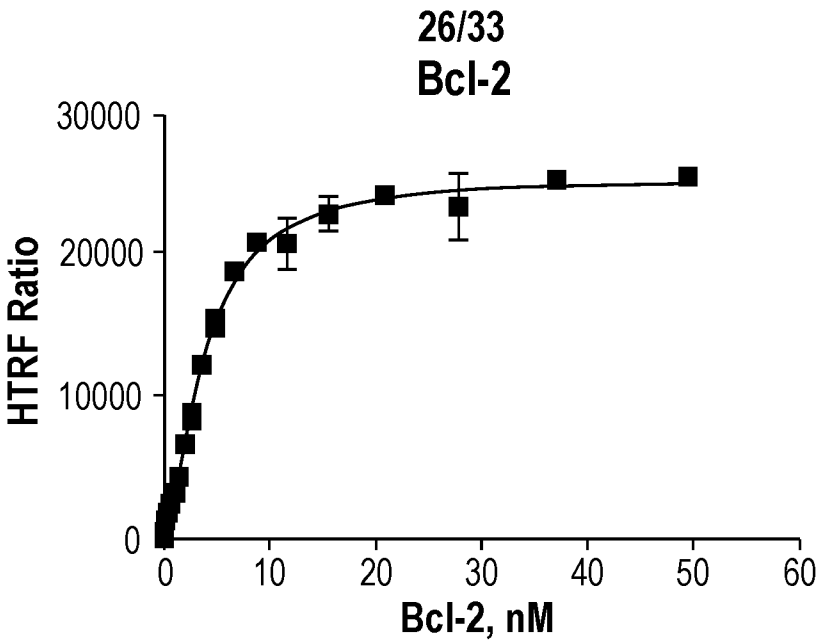


Fig. 19C



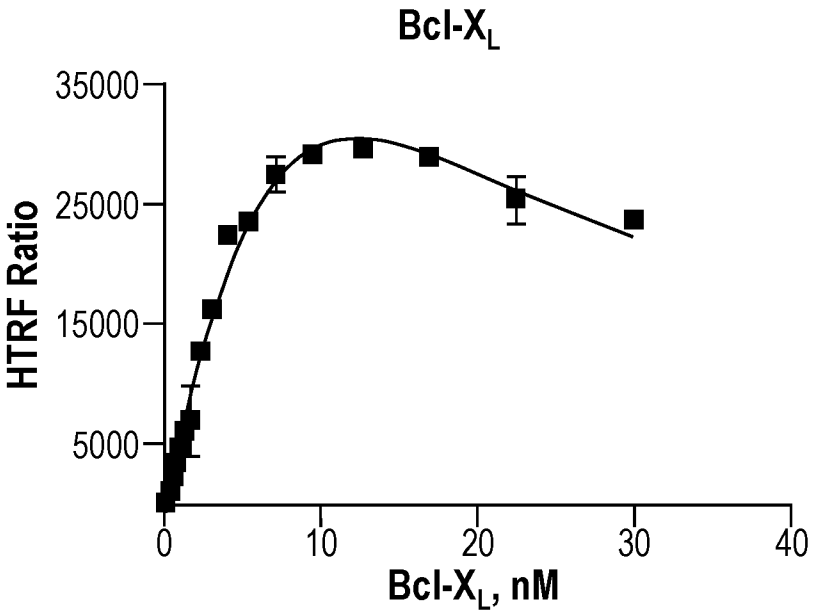
	60 nM	100 nM
One site – Specific binding with Hill slope		
Best-fit values		
Bmax	15000	22108
h	2.474	2.145
Kd	2.766	3.477

Fig. 19D



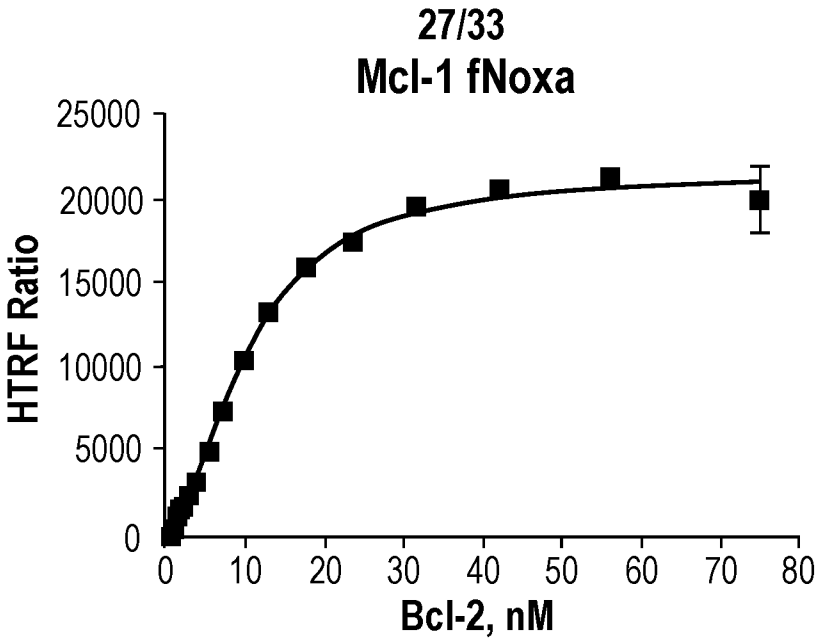
One site – Specific binding with Hill slope	
Best-fit values	
Bmax	25778
h	1.638
Kd	3.938

Fig. 20A



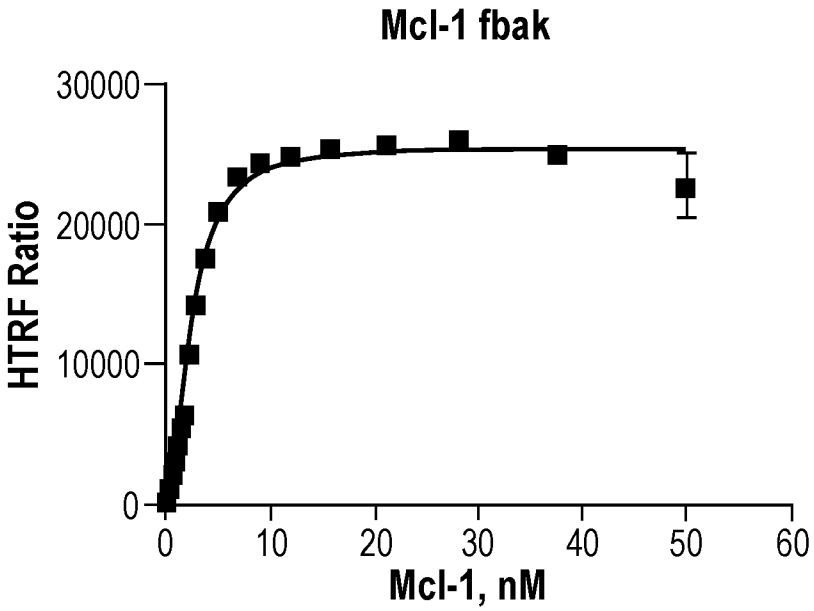
Substrate inhibition	
Best-fit values	
Vmax	209257
Km	35.93
Ki	4.167

Fig. 20B



One site – Specific binding with Hill slope	
Best-fit values	
Bmax	21708
h	1.868
Kd	10.38

Fig. 20C



One site – Specific binding with Hill slope	
Best-fit values	
Bmax	25547
h	1.918
Kd	2.416

Fig. 20D

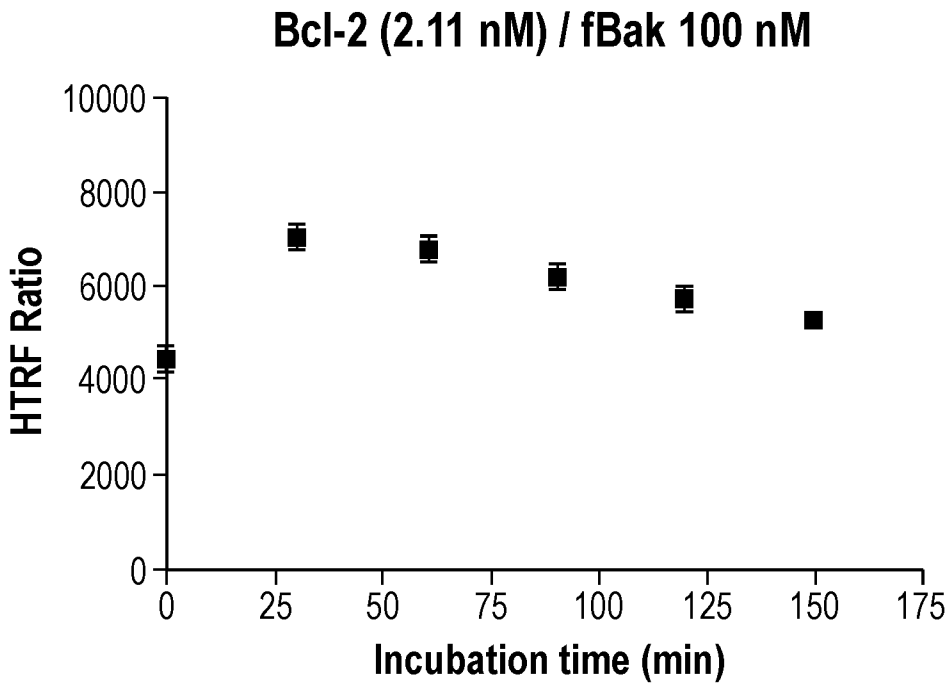


Fig. 21A

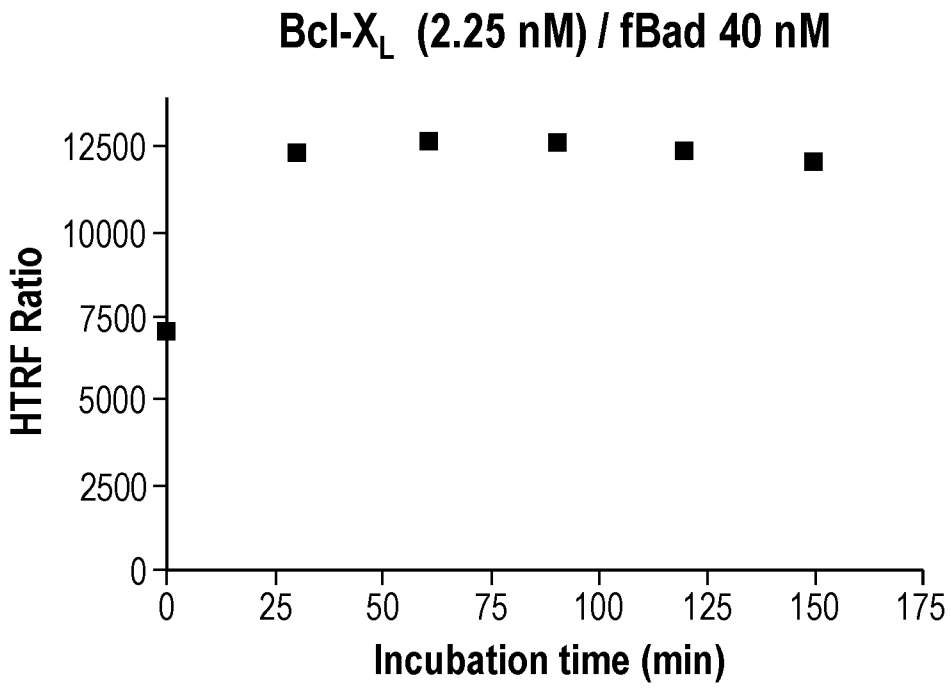


Fig. 21B

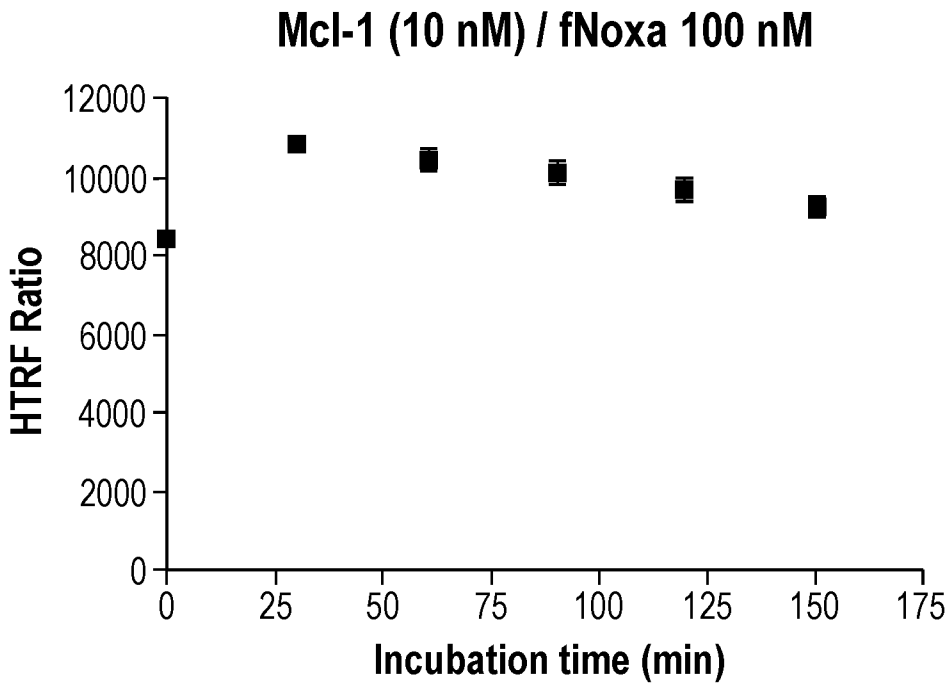


Fig. 21C

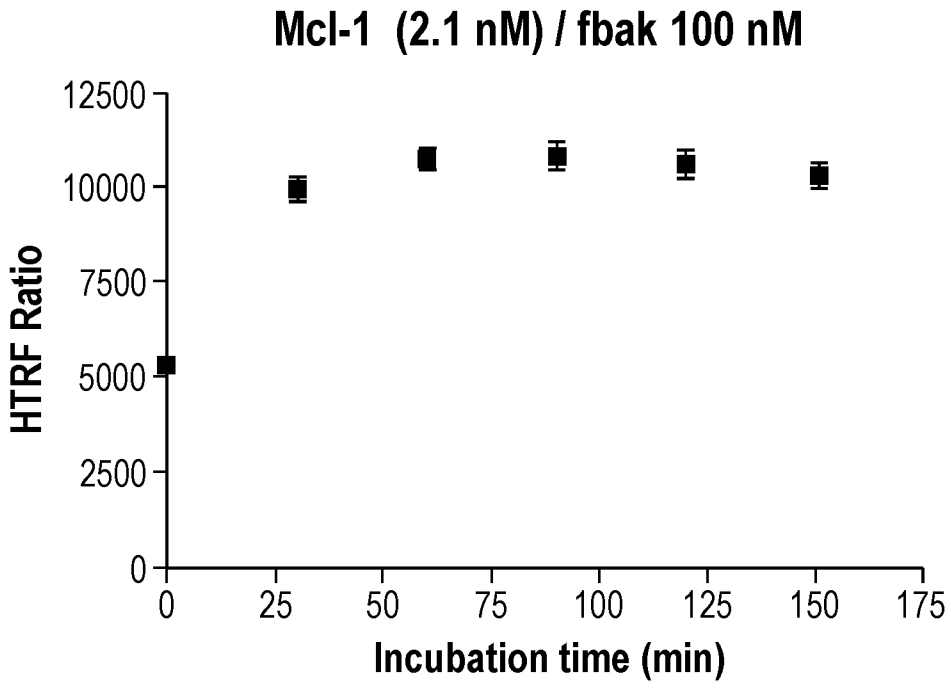
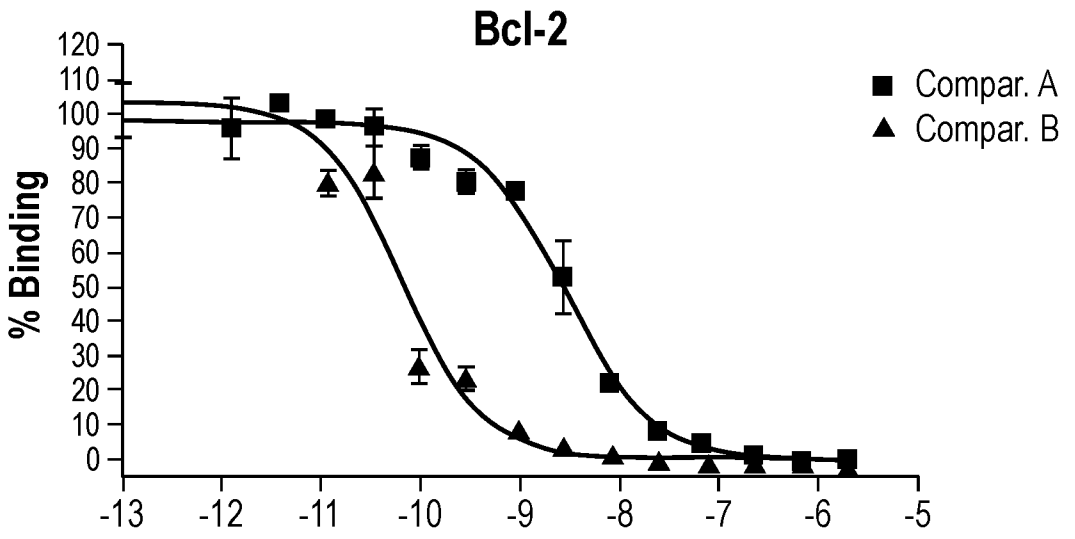
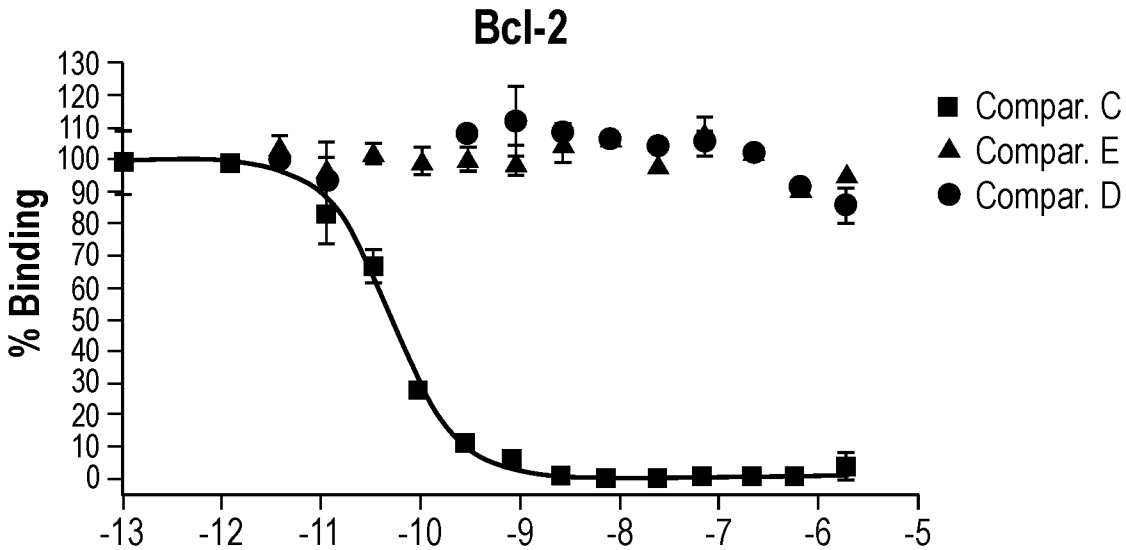


Fig. 21D



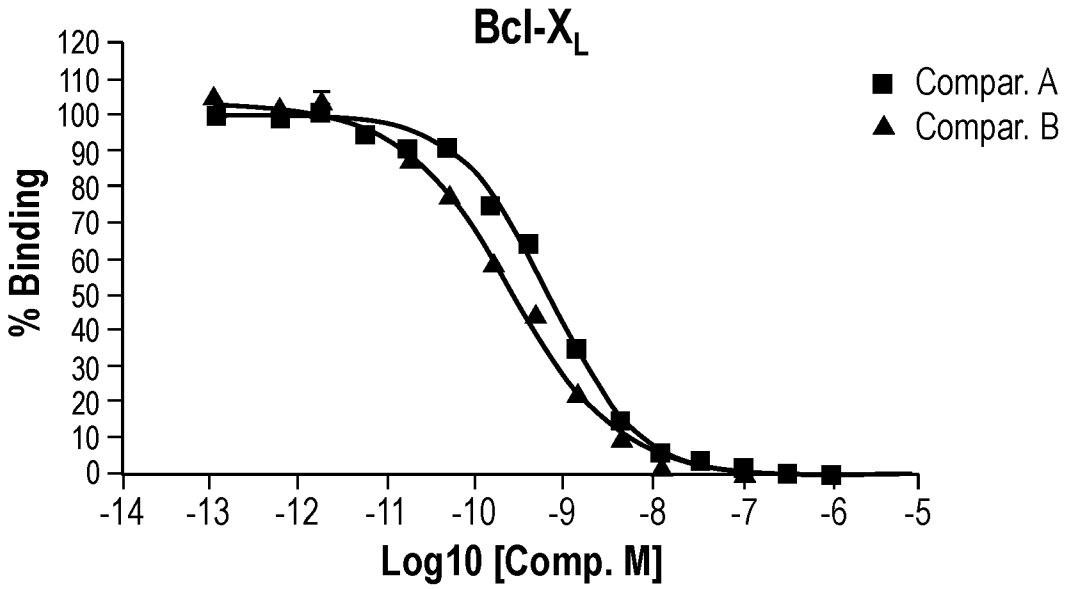
	Compar. A	Compar. B
HILLSLOPE	-1.003	-1.068
EC_{50}	2.752e-009	6.724e-011

Fig. 22A



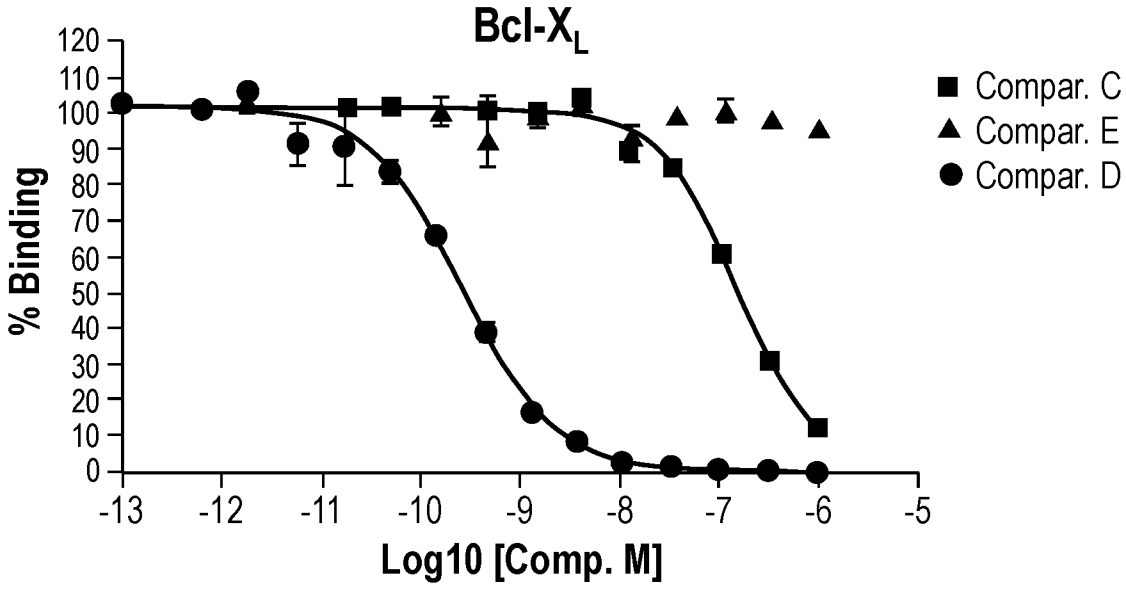
	Compar. C
HILLSLOPE	-1.263
EC_{50}	5.173e-011

Fig. 22B



	Compar. A	Compar. B
HILLSLOPE	-0.8558	-0.7049
EC_{50}	6.901e-010	2.461e-010

Fig. 22C



	Compar. C	Compar. D
HILLSLOPE	-1.090	-0.9335
EC_{50}	1.556e-007	2.634e-010

Fig. 22D

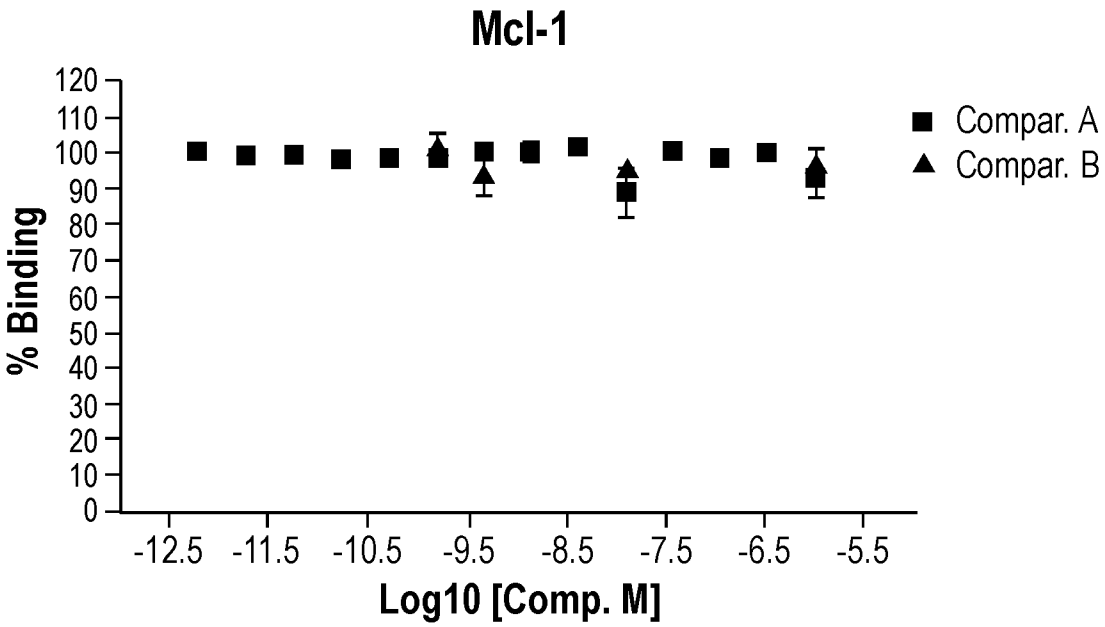
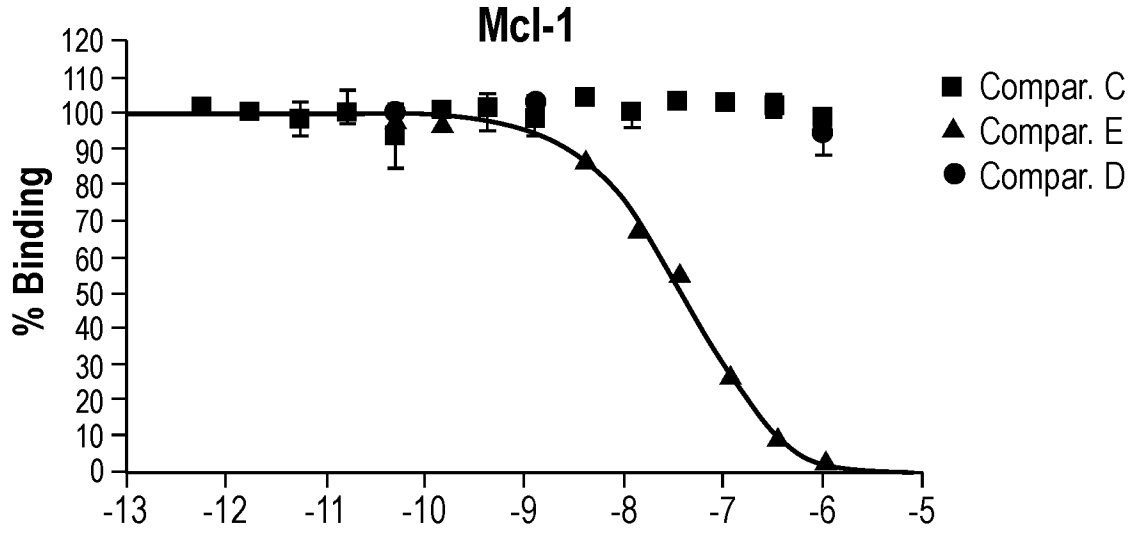
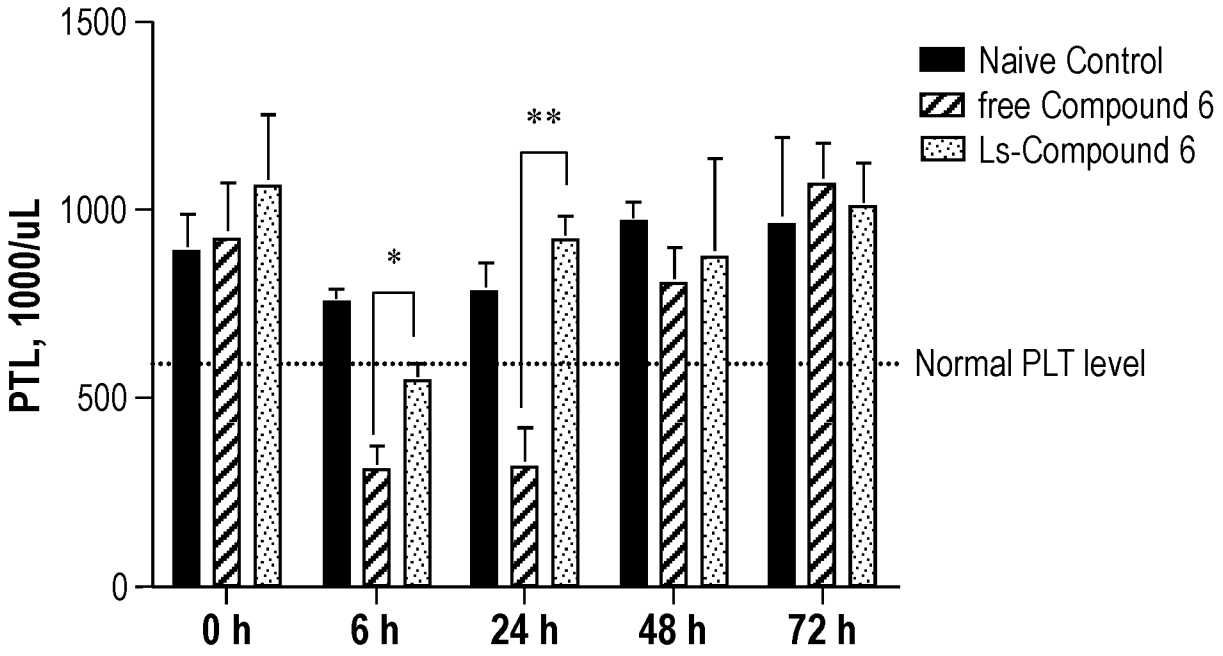


Fig. 22E



	Compar. E
HILLSLOPE	-0.8103
EC_{50}	4.346e-006

Fig. 22F



* p = 0.015
** p = 0.005

Fig. 23