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(54) Title: COMPOUNDS TARGETING THE BFL-1 ANTI-APOPTOTIC PROTEIN AND USES THEREOF FOR THE TREATMENT OF CANCER

(57) Abstract: The present invention relates to compounds targeting the Bfl-1 anti-apoptotic protein and uses thereof for the treatment of cancer. In particular, the present invention relates to at least one compound selected from the group consisting of BDM-49234, BDM 53787 and pharmaceutical acceptable salts thereof for use in a method of treatment of cancer in a subject in need thereof.



COMPOUNDS TARGETING THE BFL-1 ANTI-APOPTOTIC PROTEIN AND USES
THEREOF FOR THE TREATMENT OF CANCER

5 **FIELD OF THE INVENTION:**

The present invention relates to compounds targeting the Bfl-1 anti-apoptotic protein and uses thereof for the treatment of cancer.

BACKGROUND OF THE INVENTION:

10 Many of the cellular events that initiate malignant transformation of a normal cell (e.g., activation of oncogenes) also activate oncogenic stress pathways that usually cause the cell to enter apoptosis. In order for these cells to survive and cause cancer, they typically must have acquired changes in other cellular pathways to prevent apoptosis. Through their capacity to interact within each other, pro- and anti-apoptotic proteins of the Bcl-2 family
15 are important regulators and executioners of the intrinsic apoptotic pathway. Expression of the Bcl-2 proteins is often deregulated in cancer and thus dictates whether or not many current cancer therapies are effective, because most of these treatments result in stress signals that ultimately must activate the apoptosis machinery of cancer cells. Increased expression of pro-survival members is recognized as a hallmark of many cancers, and drugs that can
20 prevent their action would be very valuable. One approach currently being developed in anti-cancer drug discovery is to search for BH3 mimetics capable of occupying and blocking the hydrophobic pocket of anti- apoptotic Bcl-2 family members necessary for interacting with pro-apoptotic proteins. Illustration of such strategy is the identification of ABT-737, a small molecule with high affinity for Bcl-2, Bcl-xl and Bcl-w ¹ and more recently the
25 development of ABT-199 a selective inhibitor of Bcl-2 ². Navitoclax an orally bioavailable derivative of ABT-737 is currently evaluated in phase 2 clinical trials ³⁻⁵. Besides a dose-limiting toxicity towards platelets for navitoclax, a limitation for the use of ABT-199 and navitoclax is the expression by tumor cells of Mcl-1 or Bfl-1 (BCL2A1) anti-apoptotic proteins that was shown to confer resistance to ABT-737 ⁶⁻⁸, thus emphasizing the need for
30 BH3 mimetics specific for Mcl-1 or Bfl-1.

Since its discovery in 1995 as a gene overexpressed in stomach cancer ⁹, high expression of Bfl-1 has been documented in various types of cancers (for review see ⁸) and particularly in lymphoid malignancies, as a sub-group of diffused large B-cell

lymphoma (DLBCL) ¹⁰, mediastinal B cell lymphoma (MLBCL) ¹¹ and mantle cell lymphoma ¹². Bfl-1 was also implicated in the emergence of resistance of B-CLL subjects to fludarabine treatment ^{13, 14}. Further studies using RNA interference strategies demonstrated that inhibition of Bfl-1 sensitize fresh B-CLL or malignant B-cell lymphoma cell lines to
5 chemotherapeutic agents such as Cisplatin and Fludarabine, and therefore validated Bfl-1 as a therapeutic target in B cell malignancies ¹³⁻¹⁵. Peptide aptamers that specifically interact with the hydrophobic groove of Bfl-1 were recently identified. Said aptamers disrupt the interaction of Bfl-1 with its pro- apoptotic partners such as the pro-survival protein Bax. As for RNA interference strategy it was demonstrated that anti-Bfl-1 aptamers sensitize
10 malignant B-cell lymphoma cell lines to chemotherapeutic agents ¹⁶. Thus, disrupting interactions of Bfl-1 with pro-apoptotic partners appears to be an efficient strategy to overcome its pro-survival activity in malignant cells.

SUMMARY OF THE INVENTION:

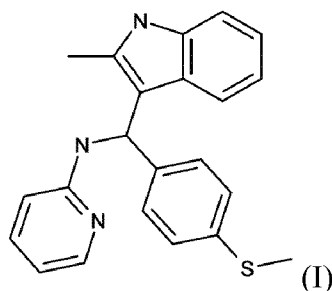
15 The present invention relates to compounds targeting the Bfl-1 anti-apoptotic protein and uses thereof for the treatment of cancer. In particular, the present invention is defined by the claims.

DETAILED DESCRIPTION OF THE INVENTION:

20 Here the inventors describe the discovery of small molecules targeting Bfl-1 anti-apoptotic protein using high-throughput screening (HTS) approach of a chemical library composed of molecules with relaxed drug-like properties as it is known that modulators of protein-protein interactions usually fall slightly outside well-known rule of thumb such as the Lipinski rule of 5. The inventors found two compounds (BDM_49234, BDM_53787)
25 that display electrophilic functions, specifically interact with Bfl-1, inhibit Bfl-1 protective activity and promote cell death of malignant B cells. Of particular interest, the inventors observed a synergistic effect of those compounds with ABT-737 in Bfl-1 expressing lymphoma cell lines.

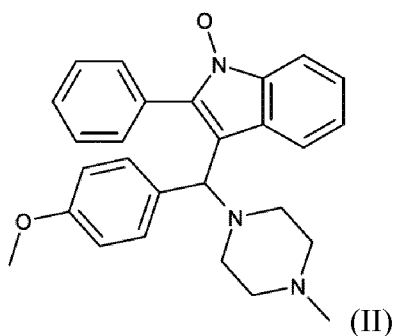
30 Accordingly, the present invention relates to a method of the treatment of cancer in a subject in need thereof comprising administering the subject with a therapeutically effective amount of at least one compound selected from the group consisting of BDM_49234, BDM_53787 and pharmaceutical acceptable salts thereof.

As used herein the term “BDM_49234” refers to the compound having the general formula (I):



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As used herein the term “BDM_53787” refers to the compound having the general formula (II):



This compound was previously described in Christensen QH, Grove TL, Booker SJ, Greenberg EP. A high-throughput screen for quorum-sensing inhibitors that target acyl-homoserine lactone synthases. Proc Natl Acad Sci U S A. 2013 Aug 20;110(34):13815-20. doi: 10.1073/pnas.1313098110. Epub 2013 Aug 7.

Preparing the compounds of the invention is within the skill of the organic chemistry art. The compounds described herein can be conveniently prepared from commercially available starting materials, compounds known in the literature, or readily prepared intermediates, by employing standard synthetic methods and procedures known to those skilled in the art. Standard synthetic methods and procedures for the preparation of organic molecules and functional group transformations and manipulations can be readily obtained from the relevant scientific literature or from standard textbooks in the field. It will be appreciated that where typical or preferred process conditions (i.e., reaction temperatures, times, mole ratios of reactants, solvents, pressures, etc.) are given, other process conditions can also be used unless otherwise stated. Optimum reaction conditions may vary with the particular reactants or solvents used, but such conditions can be determined by one skilled in

the art by routine optimization procedures. Those skilled in the art of organic synthesis will recognize that the nature and order of the synthetic steps presented may be varied for the purpose of optimizing the formation of the compounds described herein. The preparation methods can be monitored according to any suitable method known in the art. For example, product formation can be monitored by spectroscopic means, such as nuclear magnetic resonance spectroscopy (e.g., ^1H or ^{13}C), infrared spectroscopy (FT -IR), spectrophotometry (e.g., UV -visible), or mass spectrometry (MS), or by chromatography such as high performance liquid chromatography (HPLC) or thin layer chromatography (TLC). Preparation of compounds can involve the protection and deprotection of various chemical groups. The need for protection and deprotection, and the selection of appropriate protecting groups can be readily determined by one skilled in the art. The reactions of the processes can be carried out in suitable solvents which can be readily selected by one of skill in the art of organic synthesis. Suitable solvents can be substantially nonreactive with the starting materials (reactants), the intermediates, or products at the temperatures at which the reactions are carried out, i.e., temperatures which can range from the solvent's freezing temperature to the solvent's boiling temperature. A given reaction can be carried out in one solvent or a mixture of solvents. Depending on the particular reaction step, suitable solvents for a particular reaction step can be selected.

Pharmaceutically acceptable salts of the compounds of formula (I) or (II) include the acid addition and base salts thereof. Suitable acid addition salts are formed from acids, which form non-toxic salts. Examples include the acetate, aspartate, benzoate, besylate, bicarbonate/carbonate, bisulphate/sulphate, borate, camsylate, citrate, edisylate, esylate, formate, fumarate, gluceptate, gluconate, glucuronate, hexafluorophosphate, hibenzate, hydrochloride/chloride, hydrobromide/bromide, hydroiodide/iodide, isethionate, lactate, malate, maleate, malonate, mesylate, methylsulphate, naphthylate, 2-napsylate, nicotinate, nitrate, orotate, oxalate, palmitate, pamoate, phosphate/hydrogen phosphate/dihydrogen phosphate, saccharate, stearate, succinate, tartrate, tosylate and trifluoroacetate and xinafoate salts. For a review on suitable salts, see "Handbook of Pharmaceutical Salts: Properties, Selection, and Use" by Stahl and Wermuth (Wiley-VCH, Weinheim, Germany, 2002). Typically the pharmaceutically acceptable salts of compounds of formula (I) or (II) may be prepared by one or more of three methods by reacting the compound of formula (I) or (II) with the desired acid or base. The resulting salt may precipitate out and be collected by

filtration or may be recovered by evaporation of the solvent. The degree of ionization in the resulting salt may vary from completely ionized to almost non-ionized.

The compounds of the invention may also exist in both unsolvated and solvated forms.

5 The term "solvate" is used herein to describe a molecular complex comprising the compound of the invention and a stoichiometric amount of one or more pharmaceutically acceptable solvent molecules, for example, ethanol. The term "hydrate" is employed when said solvent is water.

10 Hereinafter all references to compounds of formula (I) or (II) include references to salts, solvates and complexes thereof and to solvates and complexes of salts thereof.

The compounds of the invention include compounds of formula (I) or (II) as hereinbefore defined, including all polymorphs and crystal habits thereof, prodrugs and
15 isomers thereof whenever relevant. So-called "pro-drugs" of the compounds of formula (I) or (II) are also within the scope of the invention. Thus certain derivatives of compounds of formula (I) or (II) which may have little or no pharmacological activity themselves can, when administered into or onto the body, be converted into compounds of formula (I) or (II) having the desired activity, for example, by hydrolytic cleavage. Such derivatives are referred to as
20 "prodrugs". Prodrugs in accordance with the invention can, for example, be produced by replacing appropriate functionalities present in the compounds of formula (I) or (II) with certain moieties known to those skilled in the art as "pro-moieties" as described, for example, in "Design of Prodrugs" by H. Bundgaard (Elsevier, 1985).

25 In some embodiments, the subject suffers from a cancer selected from the group consisting of breast cancer, prostate cancer, lymphoma, skin cancer, pancreatic cancer, colon cancer, melanoma, malignant melanoma, ovarian cancer, brain cancer, primary brain carcinoma, head-neck cancer, glioma, glioblastoma, liver cancer, bladder cancer, non-small cell lung cancer, head or neck carcinoma, breast carcinoma, ovarian carcinoma, lung
30 carcinoma, small-cell lung carcinoma, Wilms' tumor, cervical carcinoma, testicular carcinoma, bladder carcinoma, pancreatic carcinoma, stomach carcinoma, colon carcinoma, prostatic carcinoma, genitourinary carcinoma, thyroid carcinoma, esophageal carcinoma, myeloma, multiple myeloma, adrenal carcinoma, renal cell carcinoma, endometrial carcinoma, adrenal cortex carcinoma, malignant pancreatic insulinoma, malignant carcinoid

carcinoma, choriocarcinoma, mycosis fungoides, malignant hypercalcemia, cervical hyperplasia, leukemia, acute lymphocytic leukemia, chronic lymphocytic leukemia, chronic granulocytic leukemia, acute granulocytic leukemia, acute myelogenous leukemia, chronic myelogenous leukemia, hairy cell leukemia, neuroblastoma, rhabdomyosarcoma, Kaposi's sarcoma, polycythemia vera, essential thrombocytosis, Hodgkin's disease, non-Hodgkin's lymphoma, soft-tissue sarcoma, osteogenic sarcoma, primary macroglobulinemia, and retinoblastoma.

In some embodiments, the subject suffers from a haematological malignancy selected from the group consisting of leukemia, lymphoma or myeloma.

In some embodiments, the lymphoma is a mature (peripheral) B-cell neoplasm. In specific embodiments, the mature B-cell neoplasm is selected from the group consisting of B-cell chronic lymphocytic leukemia/small lymphocytic lymphoma; B-cell prolymphocytic leukemia; Lymphoplasmacytic lymphoma; Marginal zone lymphoma, such as Splenic marginal zone B-cell lymphoma (+/-villous lymphocytes), Nodal marginal zone lymphoma (+/-monocytoid B-cells), and Extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT) type; Hairy cell leukemia; Plasma cell myeloma/plasmacytoma; Follicular lymphoma, follicle center; Mantle cell lymphoma; Diffuse large cell B-cell lymphoma (including Mediastinal large B-cell lymphoma, Intravascular large B-cell lymphoma, and Primary effusion lymphoma); and Burkitt's lymphoma/Burkitt's cell leukemia.

In some embodiments, the lymphoma is selected from the group consisting of multiple myeloma (MM) and non Hodgkin's lymphoma (NHL), mantle cell lymphoma (MCL), follicular lymphoma, Waldenstrom's macroglobulinemia (WM) or B-cell lymphoma and diffuse large B-cell lymphoma (DLBCL). In some embodiments, Non-Hodgkin's Lymphoma (NHL) falls into one of two categories, aggressive NHL or indolent NHL. Aggressive NHL is fast growing and may lead to a subject's death relatively quickly. Untreated survival may be measured in months or even weeks. Examples of aggressive NHL includes B-cell neoplasms, diffuse large B-cell lymphoma, T/NK cell neoplasms, anaplastic large cell lymphoma, peripheral T-cell lymphomas, precursor B-lymphoblastic leukemia/lymphoma, precursor T-lymphoblastic leukemia/lymphoma, Burkitt's lymphoma, Adult T-cell lymphoma/leukemia (HTLV1+), primary CNS lymphoma, mantle cell lymphoma, polymorphic post-

transplantation lymphoproliferative disorder (PTLD), AIDS-related lymphoma, true histiocytic lymphoma, and blastic NK-cell lymphoma. The most common type of aggressive NHL is diffuse large cell lymphoma. Indolent NHL is slow growing and does not display obvious symptoms for most subjects until the disease has progressed to an advanced stage.

5 Untreated survival of subjects with indolent NHL may be measured in years. Nonlimiting examples include follicular lymphoma, small lymphocytic lymphoma, marginal zone lymphoma (such as extranodal marginal zone lymphoma (also called mucosa associated lymphoid tissue—MALT lymphoma), nodal marginal zone B-cell lymphoma (monocytoid B-cell lymphoma), splenic marginal zone lymphoma), and lymphoplasmacytic lymphoma
10 (Waldenstrom's macroglobulinemia). In some cases, histologic transformation may occur, e.g., indolent NHL in subjects may convert to aggressive NHL.

In some embodiments, the leukemia is selected from the group consisting of acute lymphocytic leukemia (ALL), acute myeloid leukemia (AML), chronic lymphocytic leukemia
15 (CLL), and small lymphocytic lymphoma (SLL). Acute lymphocytic leukemia is also known as acute lymphoblastic leukemia and may be used interchangeably herein. Both terms describe a type of cancer that starts from the white blood cells, lymphocytes, in the bone marrow.

20 In some embodiments, the compounds of the invention are used in combination with a chemotherapeutic agent. Chemotherapeutic agents include, but are not limited to alkylating agents such as thiotepa and cyclophosphamide; alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine,
25 triethylenephosphoramide, triethylenethiophosphoramide and trimethylolomelamine; acetogenins (especially bullatacin and bullatacinone); a camptothecin (including the synthetic analogue topotecan); bryostatin; callystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CB1-TM1);
30 eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimustine; antibiotics such as the

enediynes antibiotics (e.g., calicheamicin, especially calicheamicin gammall and calicheamicin omegall; dynemicin, including dynemicin A; bisphosphonates, such as clodronate; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antibiotic chromophores, aclacinomysins, actinomycin, 5 anthrarnycin, azaserine, bleomycins, cactinomycin, carabycin, caminomycin, carzinophilin, chromomycinis, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxy doxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalamycin, olivomycins, 10 peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, 15 dideoxyuridine, doxifluridine, enocitabine, floxuridine; androgens such as calusterone, dromostanolone propionate, epitio stanol, mepitio stanol, testolactone; anti-adrenals such as aminogluthethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; eniluracil; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elformithine; 20 elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK polysaccharide complex); razoxane; rhizoxin; sizofuran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2''-trichlorotriethylamine; trichothecenes 25 (especially T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids, e.g., paclitaxel and doxetaxel; chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum coordination complexes such as cisplatin, oxaliplatin and carboplatin; vinblastine; platinum; etoposide (VP- 16); 30 ifosfamide; mitoxantrone; vincristine; vinorelbine; novantrone; teniposide; edatrexate; daunomycin; aminopterin; xeloda; ibandronate; irinotecan (e.g., CPT-1 1); topoisomerase inhibitor RFS 2000; difluoromethylomithine (DMFO); retinoids such as retinoic acid; capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

In some embodiments, the compounds of the invention are administered to the subject in combination with a compound which targets a member of Bcl-2 family. Typically, said compound is selected from the group consisting of ABT-199, ABT-263 and ABT-737.

5 As used herein the term "ABT-737" has its general meaning in the art and refers to 4-{4-[(4'-Chlorobiphenyl-2-yl)methyl]piperazin-1-yl}-N-{[4-({(1R)-3-(dimethylamino)-1-[(phenylsulfanyl)methyl]propyl}amino)-3-nitrophenyl]sulfonyl}benzamide.

10 As used herein the term "ABT-199" has its general meaning in the art and refers to 4-[4-[[2-(4-Chlorophenyl)-4,4-dimethylcyclohex-1-en-1-yl]methyl]piperazin-1-yl]-N-[[3-nitro-4-[[tetrahydro-2H-pyran-4-yl)methyl]amino]phenyl]sulfonyl]-2-[(1H-pyrrolo[2,3-b]pyridin-5-yl)oxy]benzamide.

15 As used herein, the term "ABT-263" or "Navitoclax" has its general meaning in the art and refers to R)-4-(4-((4'-chloro-4,4-dimethyl-3,4,5,6-tetrahydro-[1,1'-biphenyl]-2-yl)methyl)piperazin-1-yl)-N-((4-((4-morpholino-1-(phenylthio)butan-2-yl)amino)-3-((trifluoromethyl)sulfonyl)phenyl)sulfonyl)benzamide.

20 In some embodiments, compounds of the invention are administered to a subject having a refractory cancer, i.e. a cancer that is resistant to chemotherapeutic agents. In particular the subject suffers from a cancer which is resistant to a fludarabine or cisplatin treatment. In some embodiments, the subject suffers from a cancer that is resistant to a BCL-2 inhibitor (e.g. ABT-737, ABT-199 or ABT-263).

25 By a "therapeutically effective amount" is meant a sufficient amount of the compound to treat cancer at a reasonable benefit/risk ratio applicable to any medical treatment.

30 It will be understood that the total daily usage of the compounds of the present invention will be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effective dose level for any particular subject will depend upon a variety of factors including the disorder being treated and the severity of the disorder; activity of the specific compound employed; the specific composition employed, the age, body weight, general health, sex and diet of the subject; the time of administration, route of administration, and rate of excretion of the specific compound employed; the duration of

the treatment; drugs used in combination or coincidental with the specific compound employed; and like factors well known in the medical arts. For example, it is well within the skill of the art to start doses of the compound at levels lower than those required to achieve the desired therapeutic effect and to gradually increase the dosage until the desired effect is achieved. However, the daily dosage of the products may be varied over a wide range from 0.01 to 1,000 mg per adult per day. Typically, the compositions contain 0.01, 0.05, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 25.0, 50.0, 100, 250 and 500 mg of the active ingredient for the symptomatic adjustment of the dosage to the subject to be treated. A medicament typically contains from about 0.01 mg to about 500 mg of the active ingredient, preferably from 1 mg to about 100 mg of the active ingredient. An effective amount of the drug is ordinarily supplied at a dosage level from 0.0002 mg/kg to about 20 mg/kg of body weight per day, especially from about 0.001 mg/kg to 7 mg/kg of body weight per day.

The compounds of the invention are administered as a formulation in association with one or more pharmaceutically acceptable excipients to form pharmaceutical composition.

As used herein, the term "Pharmaceutically" or "pharmaceutically acceptable" refers to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to a mammal, especially a human, as appropriate. A pharmaceutically acceptable carrier or excipient refers to a non-toxic solid, semi-solid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type.

Pharmaceutical compositions suitable for the delivery of compounds of the present invention and methods for their preparation will be readily apparent to those skilled in the art.

In the pharmaceutical compositions of the present invention for oral, sublingual, subcutaneous, intramuscular, intravenous, transdermal, local or rectal administration, the active principle (i.e. a compound of the invention), alone or in combination with another active principle, can be administered in a unit administration form, as a mixture with conventional pharmaceutical supports, to animals and human beings. Suitable unit administration forms comprise oral-route forms such as tablets, gel capsules, powders, granules and oral suspensions or solutions, sublingual and buccal administration forms, aerosols, implants, subcutaneous, transdermal, topical, intraperitoneal, intramuscular,

intravenous, subdermal, transdermal, intrathecal and intranasal administration forms and rectal administration forms.

In particular, the pharmaceutical compositions contain vehicles which are pharmaceutically acceptable for a formulation capable of being injected. These may be in particular isotonic, sterile, saline solutions (monosodium or disodium phosphate, sodium, potassium, calcium or magnesium chloride and the like or mixtures of such salts), or dry, especially freeze-dried compositions which upon addition, depending on the case, of sterilized water or physiological saline, permit the constitution of injectable solutions.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; formulations including sesame oil, peanut oil or aqueous propylene glycol; and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi.

Solutions comprising compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

Compounds of the invention can be formulated into a composition in a neutral or salt form as above described.

The carrier can also be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many

cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminium monostearate and gelatin.

5

Sterile injectable solutions are prepared by incorporating the compounds of the invention in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

15

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above, but drug release capsules and the like can also be employed.

20

For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion. Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject.

30

Compounds of the invention may be formulated within a therapeutic mixture to comprise about 0.0001 to 1.0 milligrams, or about 0.001 to 0.1 milligrams, or about 0.1 to 1.0 or even about 10 milligrams per dose or so. Multiple doses can also be administered.

In addition to the compounds formulated for parenteral administration, such as intravenous or intramuscular injection, other pharmaceutically acceptable forms include, e.g. tablets or other solids for oral administration; liposomal formulations; time release capsules ;
5 and any other form currently used.

The invention will be further illustrated by the following figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

FIGURES:

Figure 1. Chemical library selection.

Figure 2. Screening to identify molecules targeting Bfl-1 and displaying cytotoxic properties. 25 000 compounds were screened at 30 μ M to find inhibitors of Bfl-1 (30 nM) binding to Bim BH3 (15 nM).

(A) Screening flow chart. On the basis of the results of the screening algorithm, BDM_49234 and BDM_53787 were selected and tested in apoptotic cellular assays and for mechanism of action studies. * Structural analogs for additional Dose-Response Curve (DRC) experiments were chosen in the group of screening positives with percentage inhibition values included between 40% and 60%. QC: quality control of the library samples.

(B) Screening primary data analysis. The number of positive compounds was reported in function of Bfl-1/Bim inhibition percentage. Wells with fluorescence value higher than 3800 UF were excluded from analysis.

(C) DRC of the 4 primary selected compounds in TR-FRET assay. Experiment was made with resupplied compounds. Data are mean of replicates.

(D) Cytotoxic activity of the 4 primary selected compounds. BP3 and IM9 cells were incubated 24 h with compounds at 25 μ M. Cell viability was evaluated using a metabolic assay (ATP Lite) and data are presented as percent of viable cell compared to untreated control. Data are presented as mean $\pm$ SEM of 3 independent experiments.

Figure 3. Inhibitory effect of BDM_49234 and BDM_53787 in FP assays against other apoptotic and anti-apoptotic proteins of the Bcl-2 family. BDM_49234 (A) and

BDM_53787 (B) effect on Bfl-1 binding to Bim BH3 or to other pro-apoptotic peptides (Bax and Bak). BDM_49234 (C) and BDM_53787 (D) effect on Bfl-1 or other anti-apoptotic proteins (Bcl-2, Bcl-xl and Mcl-1) binding to Bim BH3. Increasing concentrations of compounds were pre-incubated for 30 minutes with anti-apoptotic proteins before BH3 peptide addition in PBS buffer, pH 7.4, 1% DMSO, 0.01% triton X100. The concentrations of anti-apoptotic proteins used in the different protein/protein interactions are the followings: Bfl-1 (30 nM) / Bim (15 nM), Bfl-1 (300 nM) / Bax (10 nM), Bfl-1 (400 nM) / Bak (10 nM), Bcl-2 (15 nM) / Bim (15 nM), Bcl-xl (10 nM) / Bim (15 nM), Mcl-1 (10 nM) / Bim (15 nM). FP was measured after 15 minutes. Data are mean of 2 incubates.

Figure 4. Pro-apoptotic effect of BDM_49234 and BDM_53787 compounds towards lymphoma cell lines. BP3 and IM9 cells were treated 24h with increasing concentrations of BDM_49234 (A) and BDM_53787 (B) molecules. Percent of viable cells was evaluated by propidium iodide / Annexin V double staining and flow cytometry analysis. IM9 (C, D) and BP3 (E, F) cells were incubated in the presence of ABT-737 and BDM_49234 or BDM_53787 at the indicated concentrations. Percent of viable cells was evaluated as in A. Data are presented as mean +/-SEM of 3 independent experiments.

EXAMPLE:

Materials and Methods:

Chemical library selection and preparation:

The compounds used in the experimental screening (fluorescence polarization assay) were selected from the Enamine HTS collection (Sept 2009) and processed with the program Pipeline Pilot v7.5. The selection is based on a hierarchical procedure combining the compliance with physicochemical thresholds (molecular weight, logP, etc.) and the absence of chemical moieties commonly associated with toxicity and an increase in false positive rates. The physicochemical thresholds used for the compound selection are the following: number of Hydrogen bonds acceptors < 13; number of Hydrogen bonds donors < 8; 150 < molecular weight < 700; logP < 6; TPSA < 160; and number of rotatable bonds < 13. Later in the process a fingerprint-based criterion was employed to ensure the chemical diversity of the resulting collection followed by a random selection to end up with 25,000 compounds for purchasing.

The chemical library was formatted in barcoded racks of 96 tubes and stored at ambient temperature or -20°C for dried or 10 mM DMSO-dissolved copies, respectively. The workstation to dilute and aliquot the library was a CyBI-Well® (CyBio, Savigny Le Temple, France) liquid handler. The sample management system avoids repeated freeze thaw cycles and ensures the longest possible lifetime for all the samples. Structures, compound ID and library manipulations were recorded in Isis and access bases.

Protein Preparation:

GST fusion proteins containing Δ -Cter-Bfl-1, Δ -Cter-Bcl-2, Δ -Cter-Bcl-XL or Δ -Cter-Mcl-1 were expressed from pGEX4T-1 plasmid in XL1-Blue cells (Stratagene). The cells were grown in 1 liter of TY media (1.6% (w/v) tryptone, 1% (w/v) yeast extract, and 85 mM NaCl) with 50 μ g/mL ampicillin at 37 °C to an A600 nm of 0.8 followed by the addition of isopropyl- β -D-thiogalactopyranoside (0.4 mM) and incubated at 25 °C for 7 h. The cells were recovered in 10 ml lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Tween 20, 0.1% 2- β -mercaptoethanol supplemented with protease inhibitor mixture (RocheDiagnostics), 0.5 mg/mL lysozyme and benzonase (250 units/ μ L)) 30 min at 30 °C, followed by three freezing cycles. The cellular debris were removed by centrifugation at 6,000 x g for 20 min, and the resulting supernatants were incubated with 2mL of glutathione-Sepharose (GE Healthcare) at 4 °C for 3 h. The resin was washed three times with buffer (20mM Tris-HCl, pH 8.0, 150mM NaCl, 0.1% Tween 20, and 0.1% 2- β -mercaptoethanol) followed by elution of GST fusion proteins in 10 mM of reduced glutathione dissolved in 50 mM Tris-HCl, pH 8.0.

Recombinant proteins in solutions were thereafter dialyzed in PBS buffer containing 1mM DTT using Amicon Ultra-4 10K columns (Millipore) and glycerol was added to a final concentration of 10% to stabilize proteins.

Fluorescence Polarization (FP) Assays

FP assays were performed in 10 mM PBS pH 7.4, 100 mM NaCl, 2.7 mM KCl, 0.01% Triton X100 and 1% DMSO in black 96-well microplates (Corning, Amsterdam, The Netherlands). Twenty five μ L Bcl-2 family proteins were incubated with 25 μ L test compound at room temperature for 30 minutes. Then, 25 μ L FITC-BH3 peptide were added. The BH3 peptides, FITC-Bim (FITC-DMRPEIWIAQELRRIGDEFNAYYAR), FITC-Bax (FITC-VPQDASTKKLSECLKRIGDELDSNMELQR) and FITC-Bak (FITC-KGGGQVGRQLAIIGDDINRRYDS) were synthesized by GeneCust (Luxembourg). After

an incubation of 15 to 45 min at room temperature, direct fluorescence and FP were measured using a Victor3™ V1420 Perkin Elmer plate reader (excitation 485 nm and emission filter 535 nm). The polarization signal was defined in equation (1) where S_c and P_c were the signals corrected in plan S and P by subtracting the buffer (without labeled-ligand in the well) signal. The « g factor » was a correction factor depending on plate reader and fluorophore used.

$$FP = 1000 \times \left(\frac{S_c - g.P_c}{S_c + g.P_c} \right) \quad (1)$$

Minimal and maximal polarization values (n=6) were obtained in each test plates by incubating FITC-BH3 alone (positive control) or with the Bcl-2 family protein (negative control), respectively. Data were normalized intra-plate with these controls and percentage inhibitions were calculated by the following equation (2):

$$\%Inhibition = 100 - \left(\frac{(x - Mean_{positivecontrol}) \times 100}{Mean_{negativecontrol} - Mean_{positivecontrol}} \right) \quad (2)$$

Gambogic acid (Tocris Biosciences, Bristol, United Kingdom) was used as a positive reference compound (2 μ M and 100 μ M). Z' factors were calculated according to the Zhang et al. method¹⁷.

Plates were validated if their respective Z' factors were ≥ 0.5 and if PPI inhibition with 100 μ M or 2 μ M gambogic acid were $\geq 80\%$ and $\geq 40\%$, respectively.

IC₅₀ values were calculated from concentration-response curves by a nonlinear regression analysis at four parameters (3) using XL fit™ 5.2.0.0. from IDBS (Guilford, United Kingdom) or GraphPad Prism 5.02 (San Diego, USA).

$$y = A + \frac{B - A}{1 + \left(\frac{10^C}{x} \right)^D} \quad (3)$$

A, minimum y value; B, maximum y value; C, LogIC₅₀ value; D, slope factor.

Data were recorded in Access bases and analysed with Pipeline Pilot™ 8.5 (Accelrys, San Diego, USA).

Time-Resolved Fluorescence Resonance Energy Transfer Assay (TR-FRET):

The TR-FRET assay was conducted in black 384-well microplates (Corning, Amsterdam, The Netherlands). Five μ L recombinant human Bfl-1 protein (120 nM) were incubated with 5 μ L compounds at room temperature for 30 minutes. Then, 5 μ L LanthaScreen™ Tb-anti-GST_Tag Antibody (Invitrogen) and 5 μ L FITC-Bim peptide were added at 20 nM and 40 nM, respectively. Buffer was PBS pH 7.4 with 0.01% Triton X100 and 1% DMSO. After 60 min incubation at room temperature, the plates were read using a Mithras LB 940 Multimode Microplate Reader (Berthold Technologies) with a TR-FRET program (excitation at 340 nm, emission for FITC signal at 485 nm and emission for terbium signal at 520 nm). Minimal TR-FRET value (negative control) was obtained by incubating FITC-Bim and Tb-anti-GST_Tag Antibody in the absence of Bfl-1. Maximal TR-FRET value (positive control) was obtained by incubating Bfl-1, Tb-anti-GST_Tag Antibody and FITC-Bim. Gambogic acid (IC₅₀ 2 μ M) was used as a reference inhibitor at 5 μ M and 100 μ M in each plate. The TR-FRET signal was expressed as Delta F according to equation (4).

$$\Delta F = \left(\frac{R_{\text{sample}} - \text{Mean}(R_{\text{NegCtrl}})}{\text{Mean}(R_{\text{NegCtrl}})} \right) \times 100 \quad (4)$$

$$\text{With } R = \frac{\text{em520}}{\text{em485}}$$

With Percentage inhibition and IC₅₀ values were calculated as described for the FP Assay.

Glutathione Stability Experiments:

Compound (10 mM DMSO) and L-glutathione (5 mM) were incubated 1h at 37°C under mixing, with final concentrations of 330 μ M and 2.5 mM for the compound and the L-glutathione respectively. After incubation for 1 h, analysis was performed thanks to a LC-MS system (Waters Alliance-ZQ2000) under full scan detection (the substrate and the expected glutathione adduct masses were extracted from the full scan spectrum). HPLC analysis was performed using a XBridge C18 (50*4,6 mm, 5 μ m) column. The mobile phase solvents used were: (A) H₂O 5mM ammonium formate buffer pH 3,8; (B) CH₃CN 5mM ammonium formate buffer pH 3,8. The following mobile phase gradient was applied: 2% B during 30s, 2-

98% (B) in 6'; hold at 98% (B) for 2'; 98%-2% B in 10s; 2% B hold for 1'20. The injection volume was 20 μ L and the flow rate of 1 mL/min.

Cell Lines and Products:

5 All media and cell culture reagents were purchased from Life technologies. BP3, IM9, and peripheral blood mononuclear cells (PBMCs) were cultured in RPMI supplemented with 10% fetal bovine serum, 2mM glutamine, 10mM Hepes and 40 μ g/mL gentamycin. PBMCs were collected from healthy donors (Etablissement Français du Sang). Separation of PBMCs was obtained by standard Ficoll density gradient centrifugation. After 2 washes in phosphate-
10 buffered saline, PBMCs were cultured in RPMI with molecules as indicated. Mouse embryonic fibroblasts (MEF) either wild-type (WT) or bak^{-/-} bax^{-/-} double knock-out (DKO) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 10 mM Hepes and 40 μ g/mL gentamycin. All cells were cultured at 37°C in humidified atmosphere with 5% CO₂.
15 ABT-737 and Obatoclax were purchased from Euromedex and Gambogic acid from TEBU.

Cytotoxic Assay:

Cells were cultured at a density of 5.10⁵ cells/mL with indicated molecule
20 concentrations. ATP monitoring was performed 24h after treatment using ATPlite assay (Perkin Elmer) according to manufacturer instructions. This assay is based on ATP measurement that is a marker for cell viability. Briefly, cells are lysed using buffer provided by the kit and incubated 5 minutes with shaking. Then substrate solution containing Luciferase and D-luciferin (reacting with ATP to produce light) is added to the wells and
25 plate is incubated 10 min in the dark. Luminescence is thereafter measured on a Tecan Infinite M200 plate reader. Luminescent signal is proportional to viable cell number in each well.

Cell Death Assay

Cells were treated with molecules for 24 h. Cell death was evaluated by propidium
30 iodide / Annexin V double staining (BD Pharmingen) and analyzed by flow cytometry with FlowJo software (TreeStar). Viable cells are gated as propidium iodide/ Annexin V double negative cells.

Docking Experiments

The two enantiomers of BDM53787 were docked into the binding pocket of Bfl-1 using the programs MOE-DOCK 2012.10 (Chemical Computing Group Inc., Montreal, Canada) and Autdock 4.2¹⁸. The structure of Bfl-1 (pdb code 2VM6) was prepared using MOE 2012.12 and the structure preparation protocol with defaults settings. MGL-Tools 1.5.6 was also used to prepare the protein structure prior to its use with Autodock 4.2. Figures were generated with MOE 2012.10.

The selected complex proposed by Autodock was also investigated within MOE 2012.10 and a full minimization of the complex was carried out. Then, the molecular surface of the protein in the vicinity of the binding site and the electrostatic map were calculated to picture the pocket of Bfl-1 and the polarity of the favored interacting regions with BDM53787.

Results:

Chemical library selection and characteristics:

The hierarchical protocol used for compound selection allowed us to prioritize the purchase of 25,000 compounds among the 1,100,307 compounds of the Enamine HTS collection (Sept 2009). The number of compounds along the selection process and the distribution of some major physicochemical properties for the resulting database are shown in

Fig. 1.

Development of Bfl-1 FP Assay

A FP assay was set up to follow the interaction of the recombinant human GST-Bfl-1- Δ Cter protein with FITC-Bim BH3. Preliminary experiments with various concentrations of Bim BH3 incubated in PBS buffer showed that reproducible and similar polarization values could be obtained with concentrations going from 15 nM to 50 nM (106 ± 8 mP at 15 nM to 99 ± 2 mP at 50nM). Consequently we chose to use Bim BH3 peptide at 15 nM which is the lowest concentration giving a low and stable polarization value. As expected we observed that addition of the Bfl-1 protein to Bim BH3 peptide increased the polarization value. We observed that DMSO, the vehicle of tested compounds, has no significant effect on the FP assay when added at 1%, the percentage planned to be used for the chemical library dilution, whereas polarization signal was highly increased in Bim BH3 incubates when Triton X100, that is used to reduce colloidal aggregation of small molecules and non-specific effects, was introduced at concentration higher than 0.02%. Finally, when used at 0.01%, triton did not

significantly affect the EC₅₀ value calculated from a Bfl-1 concentration response curve (EC₅₀ value of 30 nM vs 24 nM with or without 0.01% triton, respectively). We thus decided to carry on the assay development using PBS pH7.4, 1% DMSO, 0.01% triton X100 as a buffer. Using these experimental conditions we observed that FP values of Bim BH3 with Bfl-1 titration were similar when measured 15 to 60 min after Bfl-1 addition. To go further in the investigation of the robustness of the FPA in screening conditions, we distributed Bim BH3 alone or together with Bfl-1 in 96-well plates according to a chess board map. We observed that no plate effect occurred. Then, we used the Z' factor to evaluate the sensibility and the reproducibility in the assay. This factor should be higher than 0.5 and the closest to 1, its maximal theoretical value, for a screening campaign. We found that using Bim at 15nM and Bfl-1 at 30 nM, a Bfl-1 concentration very close to its EC₅₀, a good Z' factor value of 0.7 could be achieved. Consequently screening of the chemical library was done by run of 10 plates, with 30 min pre-incubation with Bfl-1 and FP measurement done in a period of time ranging from 15 to 45 min after Bim addition. The selected screening conditions are summarized in **Suppl. Table 1**. In these conditions the IC₅₀ value of gambogic acid used as a positive reference compound was around 1µM.

Suppl. Table 1

Category	Parameter	Description
Assay	Type of assay	Fluorescence polarization assay (FPA)
	Target	Recombinant Human Bfl-1
	Primary measurement	Fluorescence polarization
	Key reagents	Recombinant human GST-Bfl-1-dCterm protein was produced in house. FITC-Bim BH3 was synthesized by GeneCust (Luxembourg) (FITC-DMRPEIWIAQELRRIGDEFNAYYAR).
	Assay protocol	25 µL Bfl-1 protein at 90 nM were pre-incubated with 25 µL compounds at 90 µM at room temperature in 10 mM PBS pH 7.4, 100 mM NaCl, 2.7 mM KCl, 0.01% Triton X100. After 30 minutes 25 µL FITC-Bim peptide at 45 nM were added. Fluorescence polarization was measured after an incubation of 15 to 45 min at room temperature.
Library	Additional comments	Vehicle: DMSO 1%
	Library size	25 000
	Library composition	Molecules with relaxed drug like properties
	Source	Enamine Ltd, Kiev, Ukraine
	Additional comments	10 mM DMSO stock solutions stored at -20°C in barcoded plates (single used copy)
Screen	Format	96-well microtiter plates (dark, non-binding surface)
	Concentration(s) tested	30 µM
	Plate controls	Minimal and maximal polarization values were obtained by incubating FITC-Bim BH3 alone (positive control) or with Bfl-1 (negative control), respectively. Gambogic acid was used as a positive reference compound (2 µM and 100 µM).

Post-HTS analysis	system	Reagent/ compound dispensing	CyBI™ -Well (CyBio).
	software	Detection instrument and	Victor™3V (Perkin Elmer) ☐ ex 485 nm, and ☐ em 535 nm, counting time 1 sec
		Assay validation/QC	Z' factor > 0.5; PPI inhibition with 100μM gambogic acid > 80%; PPI inhibition with 2 μM gambogic acid > 40%
		Correction factors	None
		Normalization	Data were normalized using positive and negative controls from the same plate
		Additional comments	Final results expressed as percentage PPI inhibition. Screening data were stored in access data bases and analyzed using pipeline pilot™ (Accelrys)
		Hit criteria	> 60% inhibition and < 3800UF
		Hit rate	0.16%
		Additional assay(s)	Retest in triplicate and DRC on crude library hits
	structure	Confirmation of hit purity and	LCMS
		Additional comments	IC ₅₀ values were calculated from concentration-response curves by a nonlinear regression analysis using XL fit™ from IDBS or GraphPad Prim 5.

Development of FP assays for compound selectivity characterization

To characterize compound selectivity for Bfl-1/Bim interaction we developed a panel of FP assays dedicated to Bcl-2 proteins. We kept all the Bfl-1/Bim assay conditions previously established, optimizing only the concentrations of FITC-peptides and proteins to be used. The lowest peptide concentrations giving a low and stable polarization value were chosen. Protein concentrations were selected after analysis of protein concentration curves obtained by incubating increasing protein concentrations with FITC peptide. We chose protein concentrations closed to the K_D, but for which a robust Z' value (above 0.5) could be calculated. Consequently, the concentrations of anti-apoptotic and pro-apoptotic partners used in each complex were as followed: Bfl-1 (300 nM) / Bax (10 nM), Bfl-1 (400 nM) / Bak (10 nM), Bcl-2 (15 nM) / Bim (15 nM), Bcl-xl (10 nM) / Bim (15 nM), Mcl-1 (10 nM) / Bim (15 nM). Finally we validate all FP assays using gambogic acid as a positive reference compound.

HTS to identify Bfl-1-targeting compounds displaying cytotoxic properties

The 25,000 compounds of the library were screened at the concentration of 30 μM for their capacity to disrupt Bfl-1/Bim interaction (**Fig. 2**). The FP assay-based HTS demonstrated robust performance with an average Z' factor of 0.81± 0.08 and an average percentage inhibition value of 68 ± 9 for gambogic acid at 2μM used as positive reference compound. As the fluorescence of compounds could interfere with the FP assay, we take into account only data associated with a fluorescence value below 3800 UF, a limit that

corresponded to the mean fluorescence of all negative controls ± 3 SD. Fourty one compounds were found to inhibit more than 60% of the Bfl-1/Bim interaction (**Fig. 2B**). They were defined as primary positives and advanced in confirmatory sequential steps including 1) repeat single-dose testing in triplicate, 2) dose-responsive competitive binding and 3) confirmation of identity and purity of the library samples (**Fig. 2A**). This process yielded 15 positives with $IC_{50} \leq 30 \mu M$. To further confirm these positives we cherry picked 10 structural analogs in the group of compounds giving 40% to 60% inhibition in HTS, and found 6 additional compounds. After activity analysis (sigmoidal aspect of the dose response curve (DRC), maximal inhibition effect, slope at inflection point) and taking account of chemical information we selected 3 chemical series of 12 compounds (indoles, phenol and benzimidazoles) that were also active in a secondary TR-FRET assay (**Suppl. Table 2**). The TR-FRET secondary assay investigated the same molecular interaction between GST-Bfl-1- ΔC ter protein and FITC-Bim BH3, but in this assay FITC fluorescence is excited by the long-life time terbium-conjugated anti-GST when brought into proximity. This assay is less prone to interference with fluorescent tested compounds.

Suppl. Table 2.

ID_Structure	series	FPA pIC ₅₀ 95% CI	TR-FRET % inhibition at 30 μM (n=3)
BDM_53787	indole	5.9-6.2	49%
BDM_49234	indole	5.0-5.1	73%
BDM_53322	indole	4.6-4.7	54%
BDM_53351	indole	4.4-4.6	60%

BDM_53323	indole	4.6-4.7	60%
BDM_53325	indole	4.6-4.8	57%
BDM_53321	indole	4.7-5.0	42%
BDM_56776	phenol	5.1-5.2	81%
BDM_47391	phenol	5.3-5.4	79%
BDM_46699	phenol	4.8-4.9	76%
BDM_56919	phenol	4.6-4.9	40%
BDM_57859	benzimidazole	5.2-5.5	65%

Two prototypes of the indole series, BDM_53787 and BDM_49234 with respective IC_{50} of 1.4 μM and 2.5 μM in TR-FRET (**Fig. 2C**), one compound from the phenol series, BDM_56776 (IC_{50} of 4.8 μM), and the benzimidazole compound, BDM_57859 (IC_{50} of 5.8

μM) were resupplied and evaluated for their cytotoxic activity towards B cell lines by using ATPlite metabolic assay. We used the diffused large B cell lymphoma (DLBCL) cell line BP3 and the B lymphoblastoid cell line IM9 that highly express Bfl-1 mRNA and protein and that are sensitive to Bfl-1 down regulation¹⁵). These cell lines also express other anti apoptotic Bcl-2 protein family members (Bcl-xL and Mcl-1 for BP3 and Bcl2 and Bcl-xL for IM9)¹⁵). As shown in **Fig. 2D**, BDM_49234 and BDM_53787 demonstrated strong activity against BP3 and IM9 cell lines as less than 10% treated cells are still viable following 24h treatment with 25 μM compound. BDM_57859 and BDM_56776, less active in this assay, were not progressed.

Compound inhibition of Bfl-1 interaction with other pro-apoptotic partners

It is known that Bfl-1 interacts with multiple pro-apoptotic proteins, potentially through the same BH3 domain binding site. As shown in **Fig. 3A** and **Fig. 3B** we observed that BDM_49234 and BDM_53787 displaced Bfl-1 from its interaction with FITC-Bax or FITC-Bak BH3 peptides. Whatever the pro-apoptotic protein, IC₅₀ values are in the same range for BDM_49234 (6 μM, 16 μM and 8 μM, for Bfl-1/Bim, Bfl-1/Bax and Bfl-1/Bak, respectively). Interestingly, BDM_53787 is ten fold more potent to displace Bim BH3 (IC₅₀ of 0.8 μM) than Bax (IC₅₀ of 10 μM) or Bak BH3 peptides (IC₅₀ of 8 μM) from their complex with Bfl-1.

Characterization of compound selectivity for Bfl-1 anti-apoptotic protein

We next evaluated BDM_49234 and BDM_53787 for their capacity to inhibit FITC-Bim BH3 binding to Bcl-2, Bcl-xl and Mcl-1 proteins (**Fig. 3C** and **Fig. 3D**). Both compounds showed lower inhibition of Bcl-2/Bim interaction compared to Bfl-1/Bim interaction. Only 65 % of the binding is inhibited by 100 μM BDM_49234 with an estimated IC₅₀ ≥ 40 μM (vs an IC₅₀ of 8 μM for Bfl-1/Bim), while 83% of the binding is inhibited by 100 μM BDM_53787 with an IC₅₀ value of 13 μM (vs 0.8 μM for Bfl-1 binding). Inhibition of Bim binding to Bcl-xl and Mcl-1 protein by BDM_49234 and BDM_53787 were not significant with estimated IC₅₀ ≥ 100 μM.

Biological activity of selected compounds towards B cell lymphoma.

In order to further characterize their biological activity we tested the capacity of compounds BDM_49234 and BDM_53787 to induce apoptosis of B lymphoma cell lines. For this purpose, BP3 and IM9 cells were cultured for 24 hours in the presence of increasing

concentrations of compounds BDM_49234 or BDM_53787 and apoptosis was assessed by propidium iodide/Annexin V double staining and flow cytometry analysis (**Fig. 4A and 4B**). We observed that both compounds induced an efficient apoptosis of BP3 and IM9 cells with IC50 values below 10 and 1 μ M for molecule BDM_49234 or BDM_53787 respectively.

As competitive FP assays showed that BDM_49234 and BDM_53787 compounds preferentially interacted with Bfl-1, we next tested whether these two compounds could be combined with the selective Bcl-2/Bcl-xl ABT-737 molecule to exert an additive or a synergistic pro-apoptotic effect. BP3 and IM9 cells were treated with sub-optimal concentrations of ABT-737 and compound BDM_49234 or BDM_53787, and percentage of viable cells determined after 24 hours. We observed that both compounds synergized with ABT-737 to induce IM9 cell death (**Fig. 4C and 4D**), while only BDM_53787 synergized with ABT-737 to induce cell death of BP3 lymphoma cell line (**Fig. 4E and 4F**).

Anti-apoptotic Bcl-2 members suppress apoptosis by inhibiting Bax and Bak pro-apoptotic Bcl-2 members, and cells lacking both Bax and Bak proteins are resistant to apoptotic stimuli that act through Bax/Bak-dependent disruption of mitochondrial function¹⁹. To assess the specificity of our anti-apoptotic Bcl-2 family inhibitors for this pathway we used murine embryonic fibroblasts (MEF) either WT or Bax/Bak DKO. Similarly to ABT-737, compound BDM_49234 did not induce death of WT or DKO MEFs for concentrations up to 6.25 μ M, a dose that efficiently induced apoptosis of IM9 B lymphoma. Interestingly we observed that at those concentrations compound BDM_49234 was also not toxic for PBMC. In contrast, compound BDM_53787 induced apoptosis of both types of MEFs, suggesting that its pro-apoptotic effect may also depend on other pathways than blockade of Bcl-2 anti apoptotic proteins. This behavior has already been reported for gambogic acid and obatoclax.

Reactivity of compounds with glutathione

As the screening provided Bfl-1/Bim inhibitors displaying potentially electrophilic Mannich base functions, we investigated the reactivity of BDM_53787 and BDM_49234 with the thiol group of cysteine (Cys). Indeed 5 Cys are present in the human full length Bfl-1 protein, and the crystal structure of Bfl-1 in complex with Bim revealed that Trp147 of the amphipathic helix of BH3-Bim stacks onto a surface patch formed by Leu52 and Cys55 in the Bfl-1 groove²⁰. We thus incubated the compounds at 330 μ M in PBS buffer, pH7.4 with 2.5 mM excess of glutathione, which is an ideal substrate to follow compound addition reaction. After 1 hour at 37 °C an HPLC-MS analysis was performed. HPLC-MS indicated that BDM_49234 reacts rapidly with glutathione, the glutathione adduct being identified at 4'21 at

m/z 573 $[M - N\text{-pyridine} + \text{GSH}]^+$, while few remaining parent compound is detectable. At the difference from what observed with BDM_49234, BDM_53787 shows almost no glutathione adduct after incubation for 1h and almost all the initial amount of substrate is remaining. These results suggested that BDM_49234, but not BDM_53787, display reactivity with the thiol group of Cys that may result in a less reversible chemical interaction with Bfl-1.

To go further in the mechanism of inhibition of Bfl-1/Bim interaction by BDM_49234 or BDM_53787, we designed two types of experiments to assess the potential reversibility of compounds binding to Bfl-1 and consequently disruption of Bfl-1/Bim interaction. We first followed time dependence inhibition of Bfl-1/Bim interaction. Incubations were performed with Bfl-1, Bim and BDM_53787 or BDM_49234 and FP measurements were made each 90 seconds during 2 hours. We observed that the inhibitory effect of BDM_49234 is time-dependent consistent with the requirement for a slow but tight binding to the protein. Such an effect was not observed with BDM_53787. A second assay was designed to assess the reversibility of compound binding to Bfl-1. This assay measures the reversibility of compound binding upon dilution. We observed that both compounds incubated at 10 IC_{50} (in black) completely inhibit Bim binding to Bfl-1, whereas only 20% of Bim binding inhibition is observed at 0.1 IC_{50} (in pale grey). When the same weakly inhibitory concentrations are obtained from a 100-fold dilution of compound pre-incubated at 10 IC_{50} , BDM_49234 shows a significantly higher residual binding than BDM_53787 to Bfl-1. This is consistent with the chemical reactivity of BDM_49234, and its slower binding to Bfl-1. Also the IC_{50} measured for strongly BDM_49234 depend upon pre-incubation time.

Docking of BDM_53787 compound into Bfl-1 binding pocket.

In the docking procedure we decided to only dock compound BDM_53787 and not BDM_49234. The main reason is that because of its putative transient link to the Cys-55 of Bfl-1, only the reaction product of BDM_49234 binds to Bfl-1 and not the parent compound such that it would be highly speculative to attempt to propose a binding mode for this molecule. On the contrary, the two enantiomers of BDM_53787 were docked into the binding pocket of Bfl-1 using two different programs MOE-DOCK and Autodock 4.2¹⁸ to propose a variety of binding modes for visual inspection and pose selection. Conversely, to assist the selection of the binding pose of BDM_53787, the crystal structure of Bfl-1 in complex with the interacting alpha-helix of Bim (pdb code 2VM6) was used to predict the hot spot residues of Bim interacting with the binding pocket of Bfl-1 using the virtual alanine scanning DrugscorePPI server²¹. Several hot spot residues were predicted including Bim-Ile148, Bim-

Leu152, and Bim-Arg153 with predicted $\Delta\Delta G$ superior to 1.5 kcal.mol⁻¹. The selection of the BDM_53787 docking pose was therefore made with the attempt to mimic the binding mode of BIM with regards to these three hot spot residues.

The proposed binding mode of BDM_53787 was selected among the Autodock poses of the docked ligand and corresponds to the enantiomer R of BDM_53787. In this binding mode, the phenyl moiety of BDM_53787 occupies the hydrophobic sub-pocket of BIM-Leu152, the 4-methoxyphenyl moiety occupies the hydrophobic sub-pocket of BIM-Ile148 and the piperazine moiety occupies a region of the Bfl-1 binding pocket compatible with the type of interaction that Bim-Arg153 makes with Bfl-1 including a salt-bridge with residue Glu80 of Bfl-1.

Discussion:

Novel small compounds targeting Bcl-2 and/or Bcl-xl anti-apoptotic protein of the Bcl-2 family such as Navitoclax, an orally bioavailable derivative of ABT-737, or ABT-199 are now in clinical trials³⁻⁵. Interestingly, it has been demonstrated that long-term exposure to ABT-737 result in increased levels of Bfl-1 and Mcl-1 and development of resistance in lymphoma cell lines that were initially sensitive⁶. Furthermore the use of a highly sensitive and quantitative RT-PCR assay to examine Bcl-2, Bcl-xl, Bcl-w, Bcl-b, Mcl-1 and Bfl-1 defined that the (Bfl-1 + Mcl-1)/Bcl-2 ratio as the most predictive marker for the response of primary B-CLL and leukemic cell lines to ABT-737 treatment⁷. Thus the efficacy of anti-Bcl-2 and Bcl-xl compounds is limited by the expression of Mcl-1 or Bfl-1, underlying the need for Mcl-1 and/or Bfl-1 chemical inhibitors. Based on the strong characterization of Bfl-1 in chemoresistance associated with its over-expression in DBCL and B-CLL where it stands as an innovative target¹³⁻¹⁶, we focused on the identification of Bfl-1 inhibitors. We described here 2 compounds that specifically target the Bfl-1 hydrophobic BH3-binding groove, and thereby efficiently disrupt its interaction with pro-apoptotic partners, such as Bim but also Bax and Bak. Both compounds inhibit Bfl-1 protective activity and promote cell death of malignant B cells, either alone or in combination with ABT-737. The synergistic effect of those compounds with ABT-737 is of particular interest since Bfl-1 overexpression was clearly identified as a limit to its efficacy.

The 25,000 compound chemical collection was designed to favor the chemical diversity and adequacy of compounds physicochemical profiles for this target type. To this end, the choice of “soft” physicochemical thresholds like those described in the method

section are inspired by several studies showing that indeed inhibitors of protein-protein interactions and more precisely those of the Bcl-2 family are characterized by significantly higher molecular weight and hydrophobicity^{22, 23}. Thus, taking out such compounds from the library prior to screening would have diminished our chances of success. But as observed in **Fig.1**, the number of compounds removed by the physicochemical thresholds is relatively low (22,636 out of 1,110,307). This can be attributed to the fact that most commercial compound catalogs have been designed to comply with some general chemistry rules such as Lipinski's RO5²⁴ and or Veber rules²⁵. The other important step in the selection process deals with the detection of some problematic chemical moieties usually associated to toxicity or the observation of false positives or frequent hitters such as Michael acceptors or epoxides. This selection criterion is responsible for removing a more important number of compounds (~240,000 compounds).

Screening of such a library with "relaxed" drug like properties provides 3 structurally different series of Bfl-1/Bim inhibitors, amongst which 2 contain Mannich base function. (Ar-CHR-NR₁R₂), where the amine fragment can be substituted by a sulfur nucleophile. Bfl-1 contains at position 55 a Cys residue close to the binding groove, that could explain why we and others described electrophilic Bfl-1 inhibitors²⁶⁻²⁸. The indol series was progressed and tested in different cell lines. Positioning of the ligand in the groove is a prerequisite for activity: in the Mannich base family described here, one of the compounds is not thiol reactive and a reversible inhibitor, while its close analogue is thiol reactive and a partial reversible binder. The reversible compound (BDM_53787) was more potent in the cell-free and cell based assays, and molecular modeling clearly supports the experimental work and proposes a likely binding mode of Bfl-1. The partially reversible compound (BDM_49234) was less potent, but it was also less toxic, suggesting that chemical reactivity and irreversibility does not imply a non-specific cell toxicity.

The thiol reactivity should not be by itself a reason to discard a hit before medicinal chemistry efforts to improve potency and explore chemical reactivity are done. In this context, a great care to incubation times should be given when measuring activities on target and comparing compounds with very different kinetic properties. Later in development, at equivalent affinity to the target, binding kinetics and pharmacokinetics should be jointly analyzed: slow tight binders may be of interest if the C_{max} and elimination rates are high. Therefore having in hand compounds with various binding kinetics early in the drug discovery is favorable.

Most of the inhibitors that have been identified on the Bcl-2 family are characterized by a physicochemical profile that places them outside of the usual drug-like chemical space i.e with a higher molecular weight (> 600 g/mol) or a higher lipophilicity (logP > 5-6). This can impede their future as an actual drug especially if they are meant to be orally administrated. The present study demonstrates that it is indeed possible to identify active compounds on Bfl-1 that possess reasonable physichemical properties. As matter of fact, compounds BDM_49234 and BDM_53787 are both characterized by acceptable size (~400 g/mol) and lipophilicity (logP ~4).

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Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.

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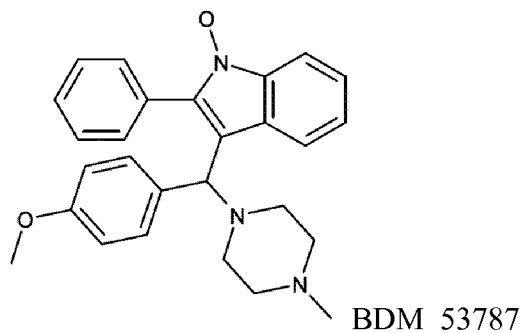
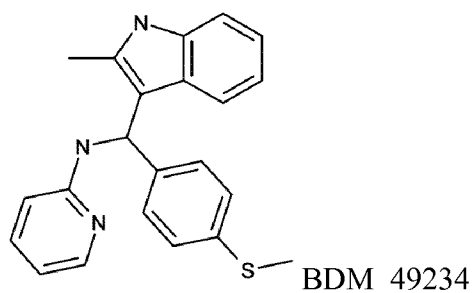
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CLAIMS:

1. A method of the treatment of cancer in a subject in need thereof comprising administering the subject with a therapeutically effective amount of at least one compound selected from the group consisting of BDM_49234, BDM_53787 and pharmaceutical acceptable salts thereof wherein BDM_49234 and BDM_53787 have the general formulae:



2. The method of claim 1 wherein the subject suffers from a cancer selected from the group consisting of breast cancer, prostate cancer, lymphoma, skin cancer, pancreatic cancer, colon cancer, melanoma, malignant melanoma, ovarian cancer, brain cancer, primary brain carcinoma, head-neck cancer, glioma, glioblastoma, liver cancer, bladder cancer, non-small cell lung cancer, head or neck carcinoma, breast carcinoma, ovarian carcinoma, lung carcinoma, small-cell lung carcinoma, Wilms' tumor, cervical carcinoma, testicular carcinoma, bladder carcinoma, pancreatic carcinoma, stomach carcinoma, colon carcinoma, prostatic carcinoma, genitourinary carcinoma, thyroid carcinoma, esophageal carcinoma, myeloma, multiple myeloma, adrenal carcinoma, renal cell carcinoma, endometrial carcinoma, adrenal cortex carcinoma, malignant pancreatic insulinoma, malignant carcinoid carcinoma, choriocarcinoma, mycosis fungoides, malignant hypercalcemia, cervical hyperplasia, leukemia, acute

lymphocytic leukemia, chronic lymphocytic leukemia, chronic granulocytic leukemia, acute granulocytic leukemia, acute myelogenous leukemia, chronic myelogenous leukemia, hairy cell leukemia, neuroblastoma, rhabdomyosarcoma, Kaposi's sarcoma, polycythemia vera, essential thrombocytosis, Hodgkin's disease, non- Hodgkin's lymphoma, soft-tissue sarcoma, osteogenic sarcoma, primary macroglobulinemia, and retinoblastoma.

3. The method of claim 1 wherein the subject suffers from a haematological malignancy selected from the group consisting of leukemia, lymphoma or myeloma.
4. The method of claim 1 wherein the compound is used in combination with chemotherapeutic agent selecting from the group consisting of alkylating agents such as thiotepa and cyclophosphamide; alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethylenethiophosphoramide and trimethylolomelamine; acetogenins (especially bullatacin and bullatacinone); a camptothecin (including the synthetic analogue topotecan); bryostatin; callystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CB1-TM1); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimustine; antibiotics such as the enediyne antibiotics (e.g. , calicheamicin, especially calicheamicin gammall and calicheamicin omegall ; dynemicin, including dynemicin A; bisphosphonates, such as clodronate; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antiobiotic chromophores, aclacinomysins, actinomycin, authrarnycin, azaserine, bleomycins, cactinomycin, carabycin, caminomycin, carzinophilin, chromomycinis, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxy doxorubicin), epirubicin, esorubicin,

idarubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues
 5 such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine; androgens such as calusterone, dromostanolone propionate, epitio stanol, mepitio stanane, testolactone; anti-adrenals
 10 such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; eniluracil; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elformithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids such as maytansine and
 15 ansamitocins; mitoguazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK polysaccharide complex); razoxane; rhizoxin; sizofuran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and anguidine); urethan;
 20 vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids, e.g., paclitaxel and doxetaxel; chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum coordination complexes such as cisplatin, oxaliplatin and carboplatin; vinblastine; platinum; etoposide (VP- 16); ifosfamide; mitoxantrone;
 25 vincristine; vinorelbine; novantrone; teniposide; edatrexate; daunomycin; aminopterin; xeloda; ibandronate; irinotecan (e.g., CPT-1 1); topoisomerase inhibitor RFS 2000; difluoromethylomithine (DMFO); retinoids such as retinoic acid; capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

5. The method of claim 1 wherein the compound of the invention is administered to the
 30 subject in combination with a compound which targets a member of Bcl-2 family.
6. The method of claim 5 wherein the compound which targets a member of Bcl-2 family is selected from the group consisting of ABT-199, ABT-263 and ABT-737.

7. The method of claim 1 wherein the compound is administered to a subject having a refractory cancer.
8. The method of claim 1 wherein the subject suffers from a cancer which is resistant to a fludarabine or cisplatin treatment.
- 5 9. The method of claim 1 wherein the subject suffers from a cancer that is resistant to a BCL-2 inhibitor such as ABT-737, ABT-199 or ABT-263.

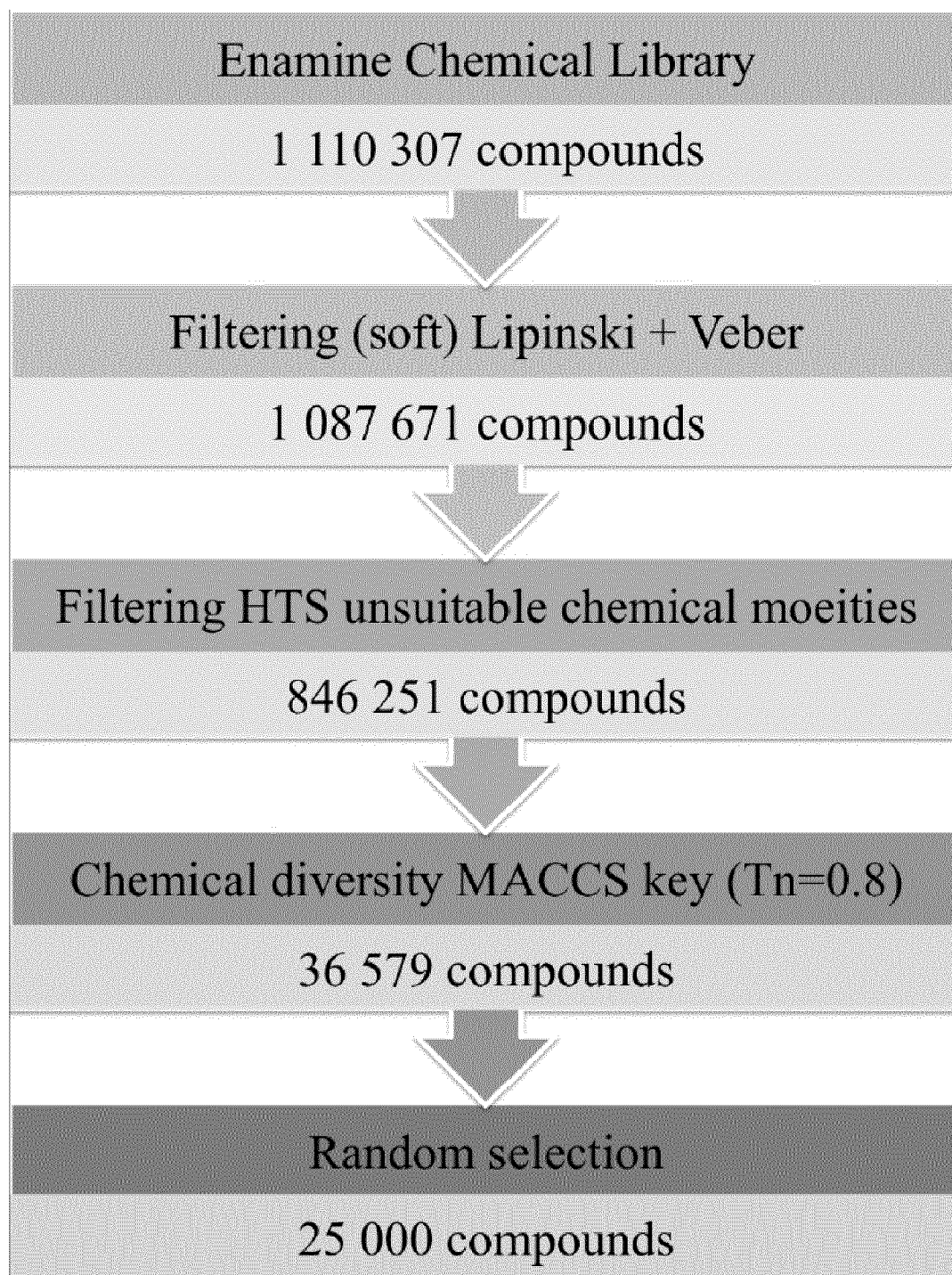


Figure 1

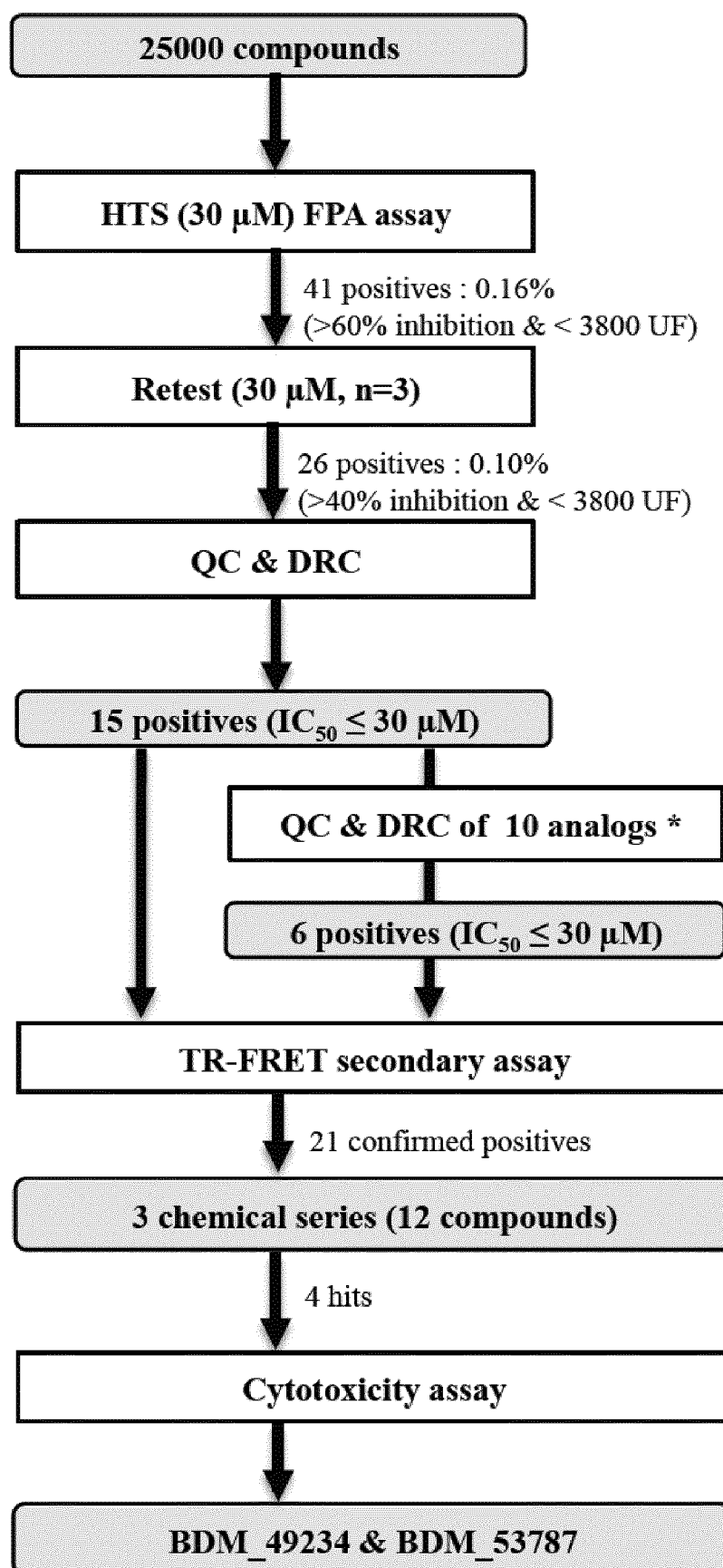


Figure 2A

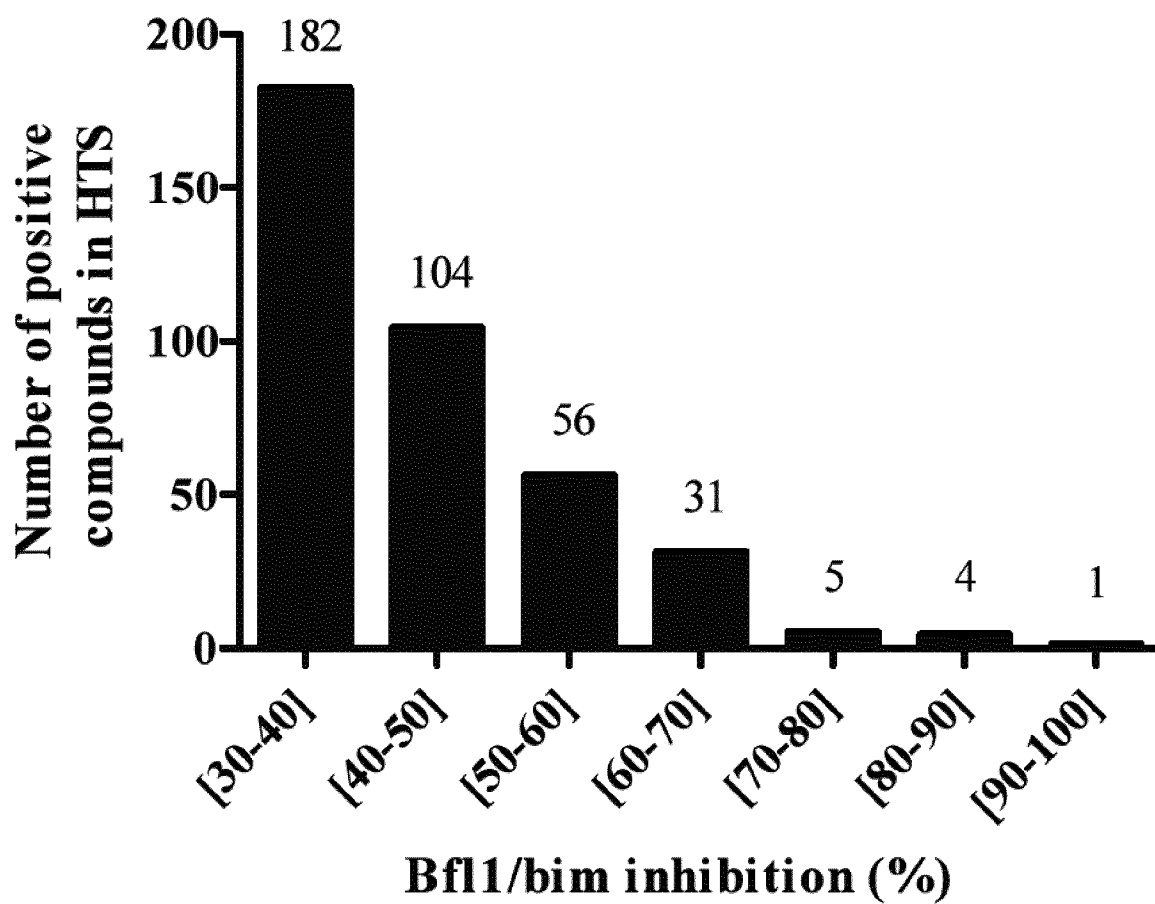


Figure 2B

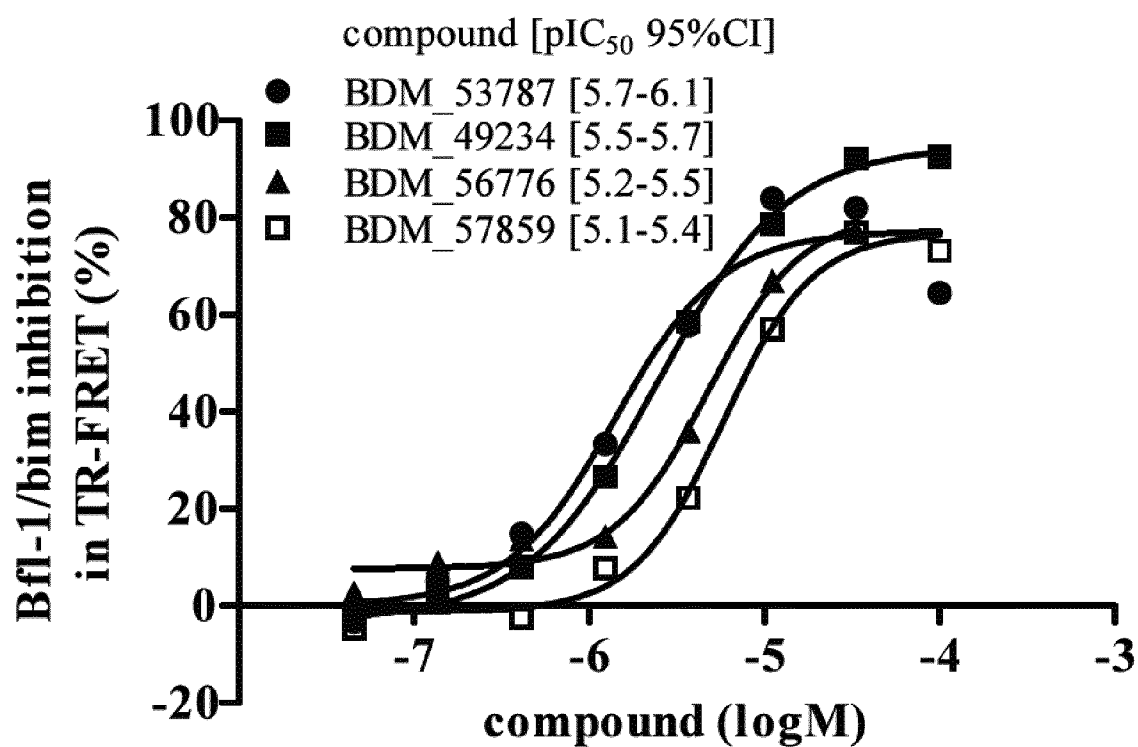


Figure 2C

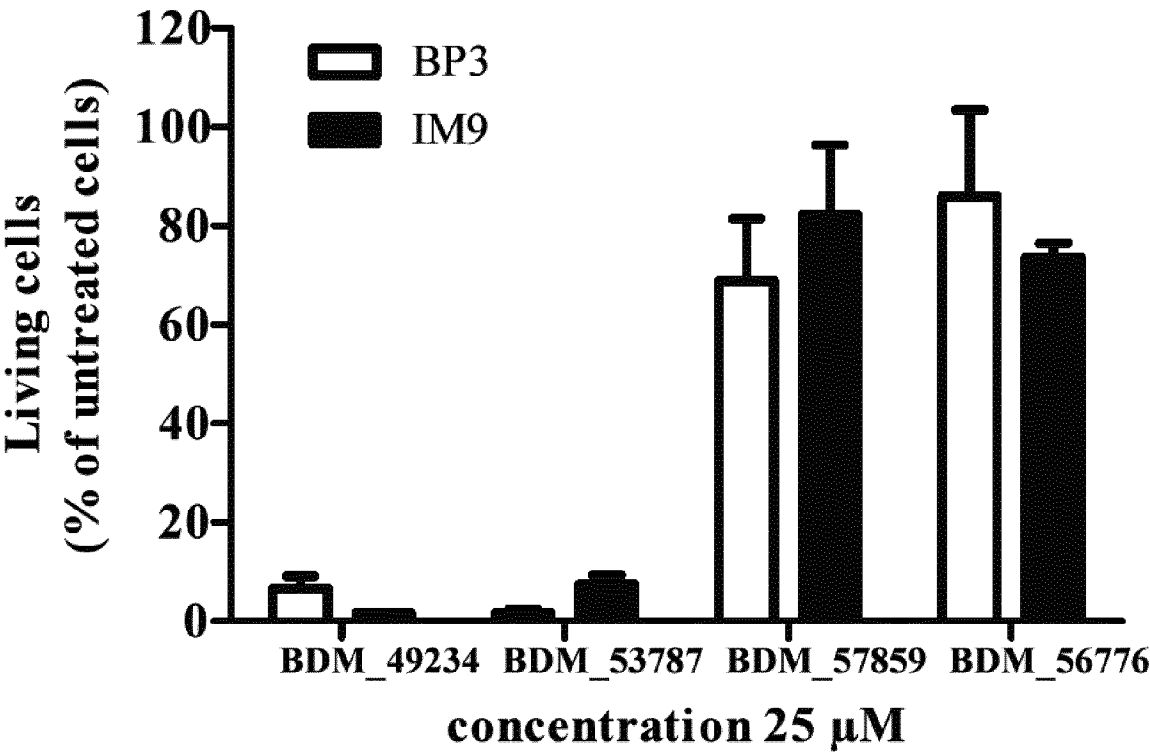


Figure 2D

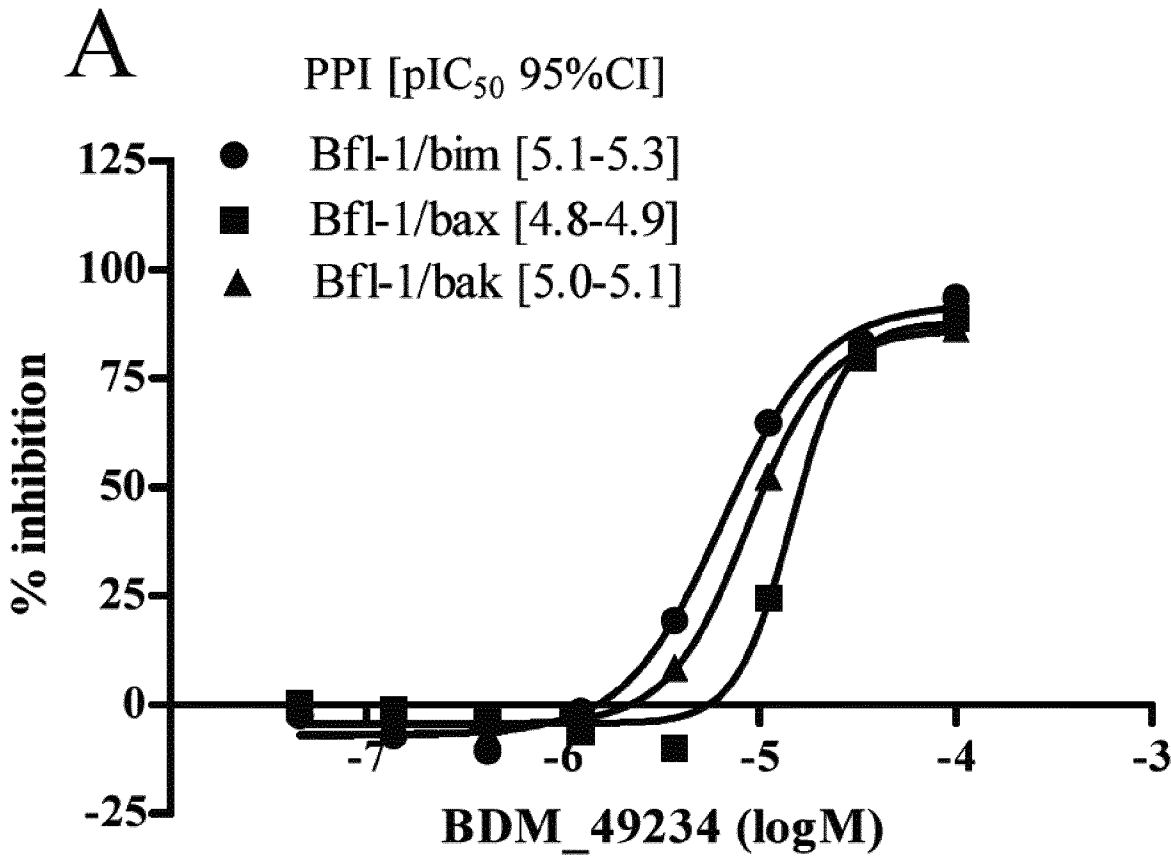


Figure 3A

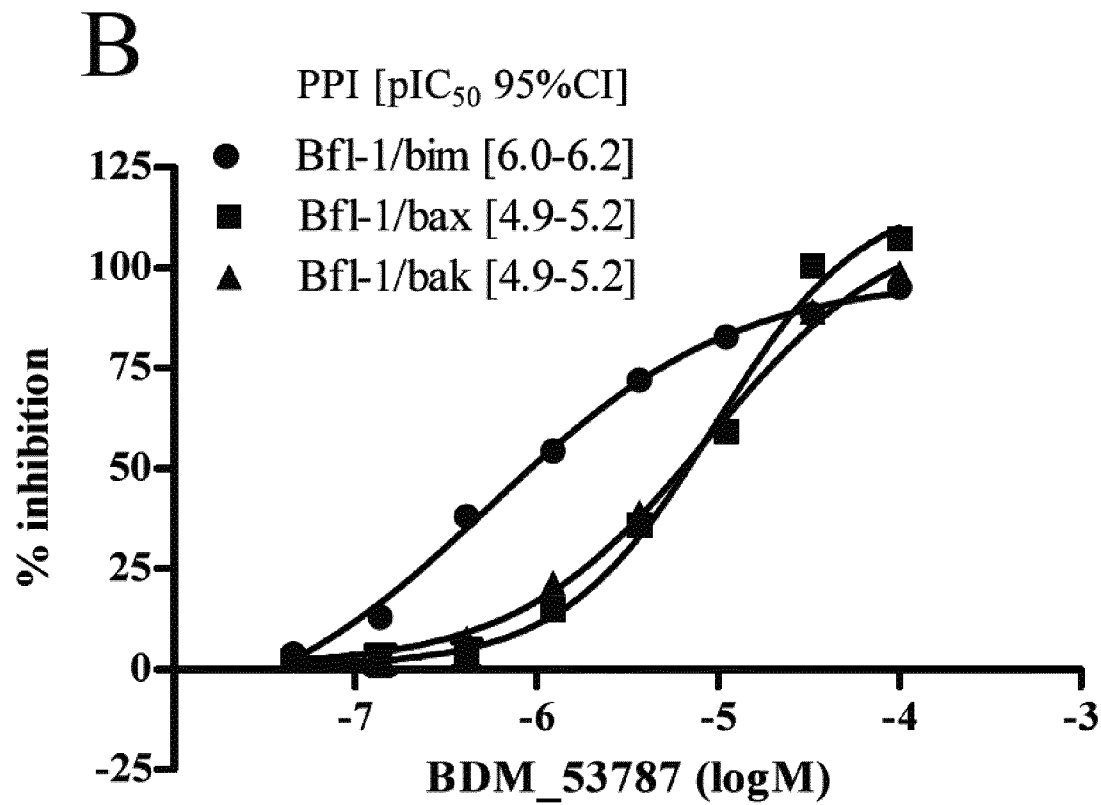


Figure 3B

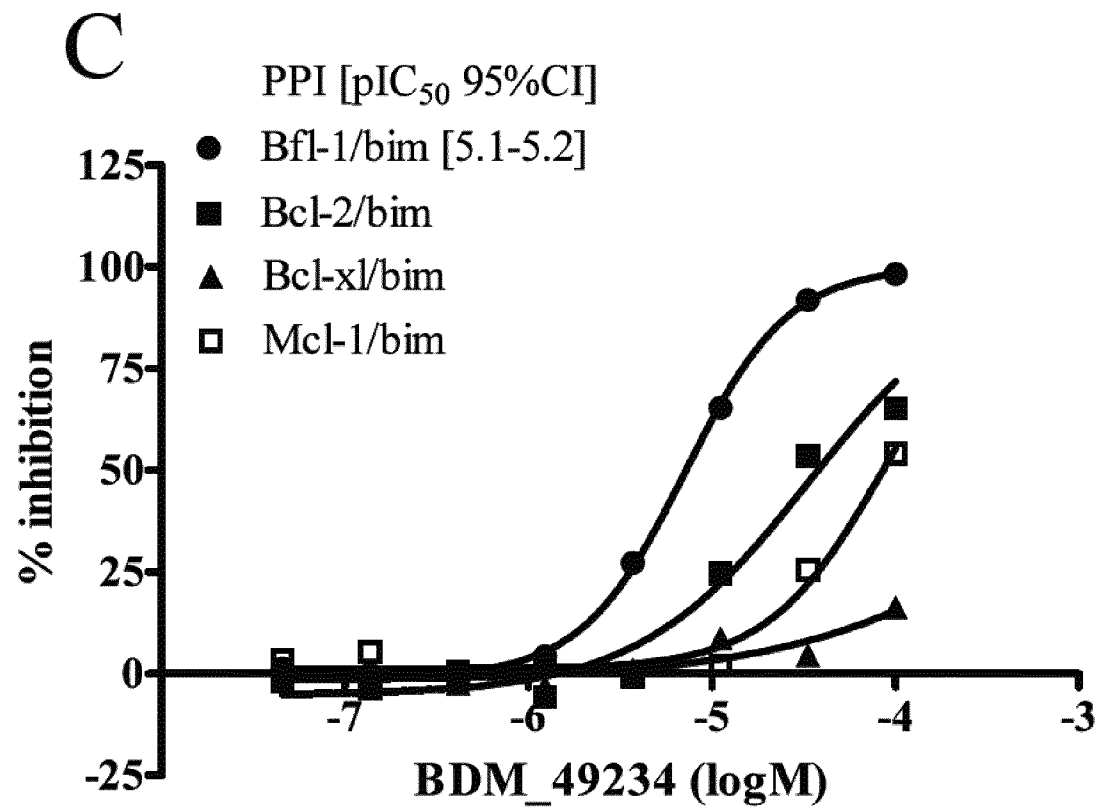


Figure 3C

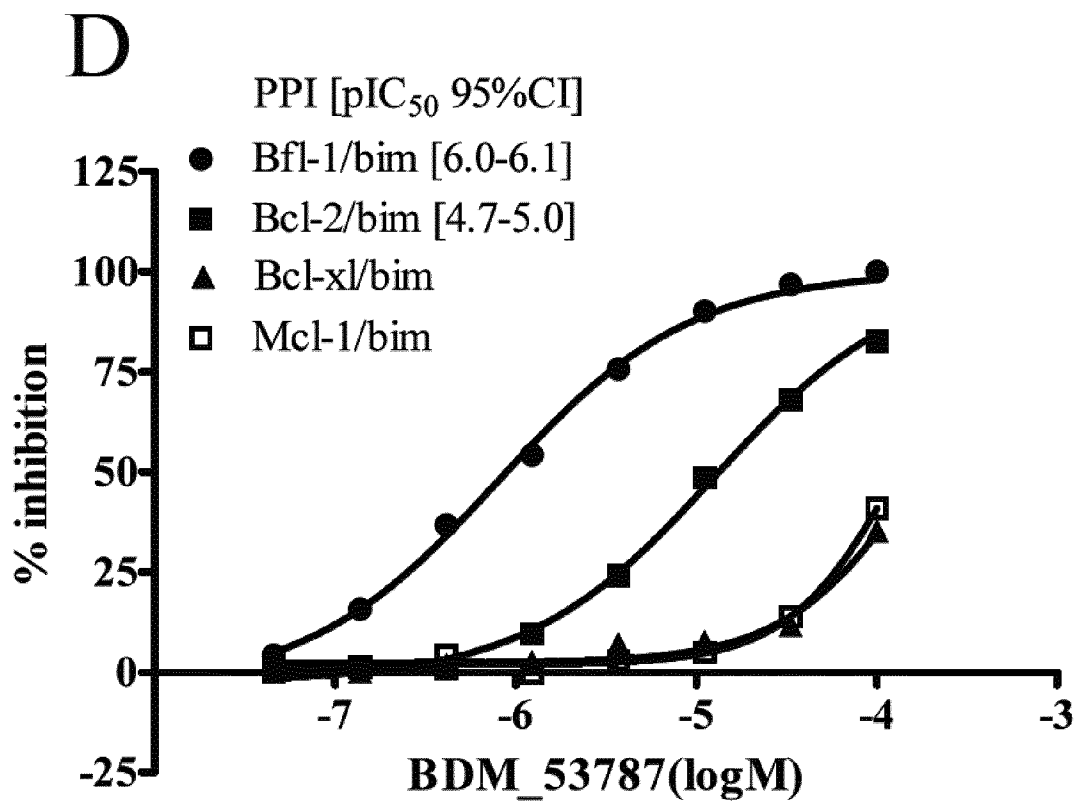


Figure 3D

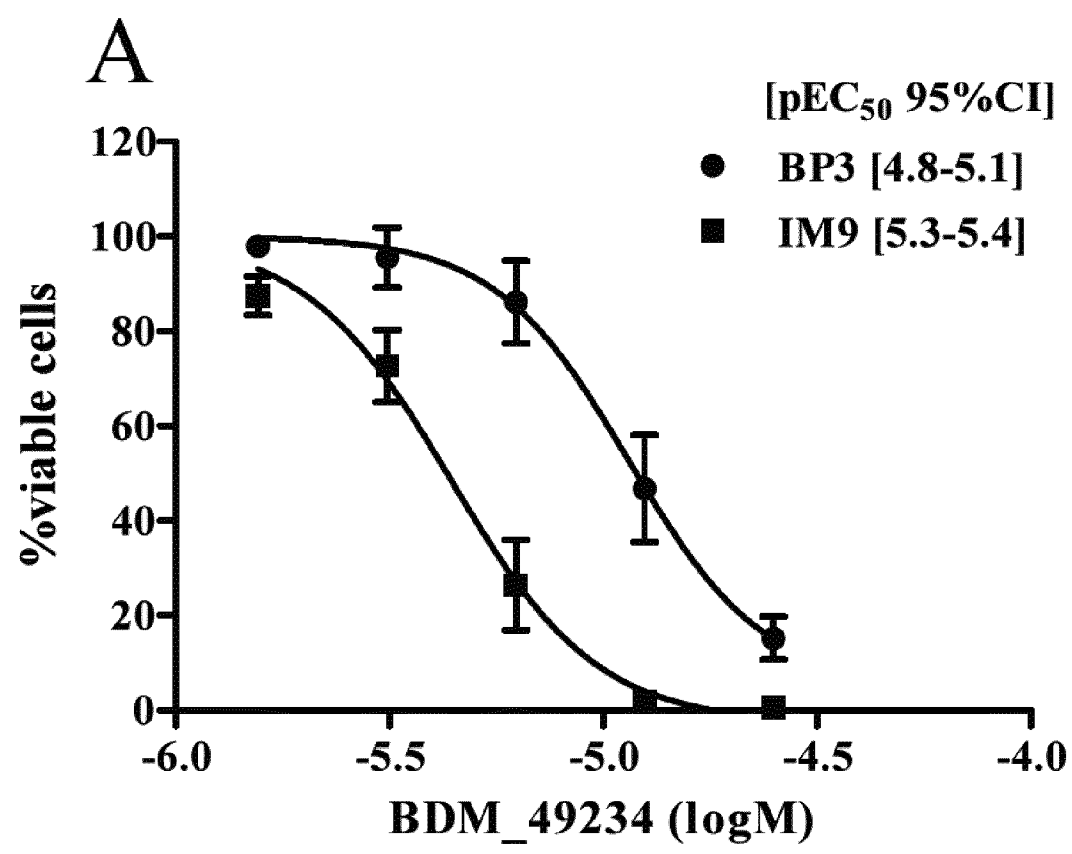


Figure 4A

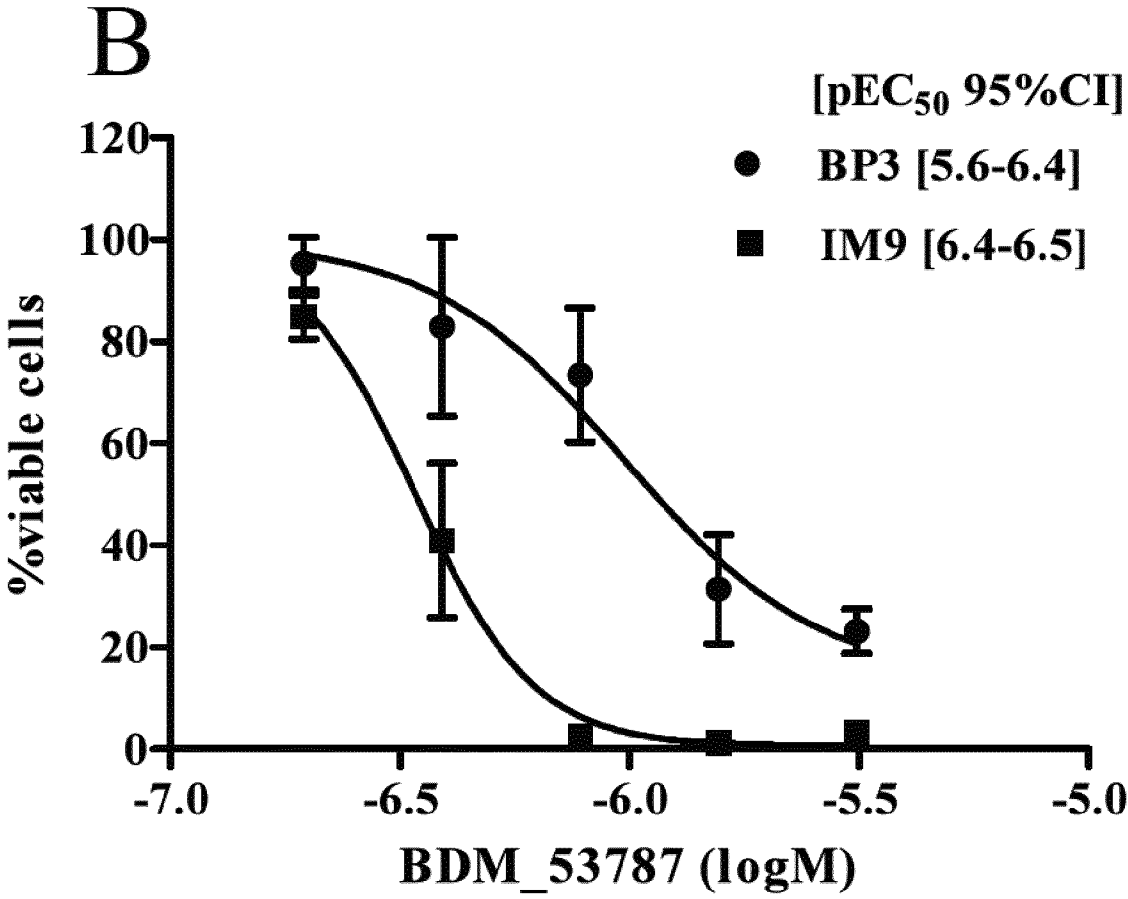


Figure 4B

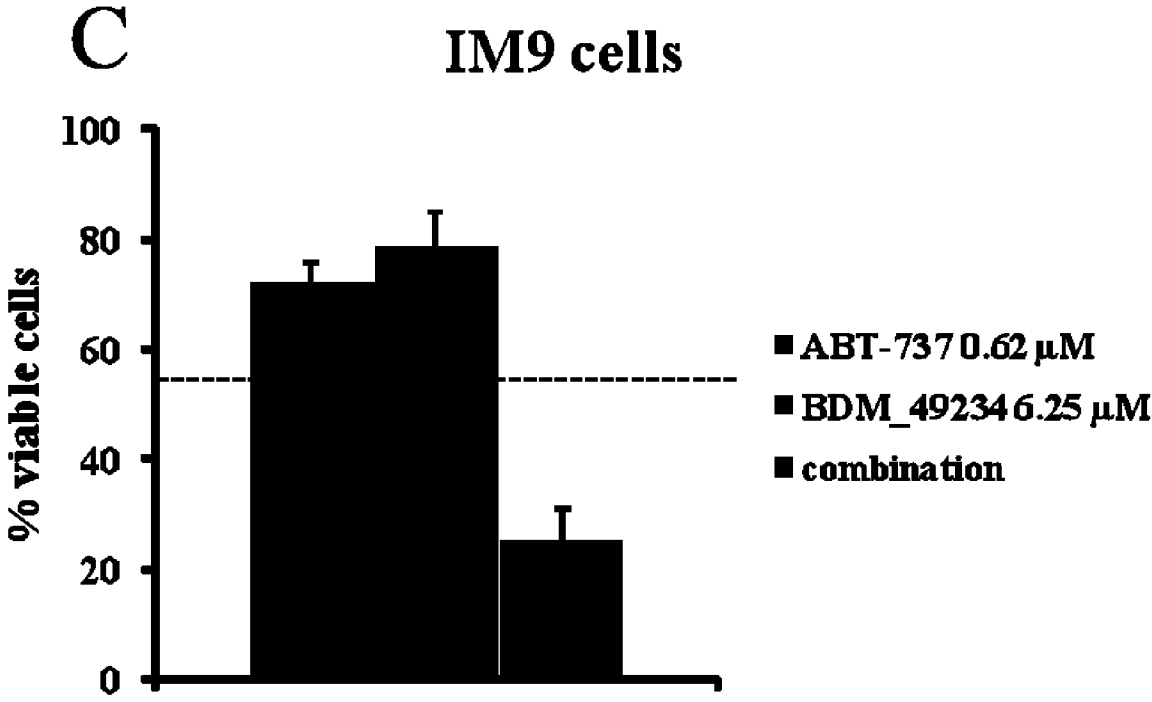


Figure 4C

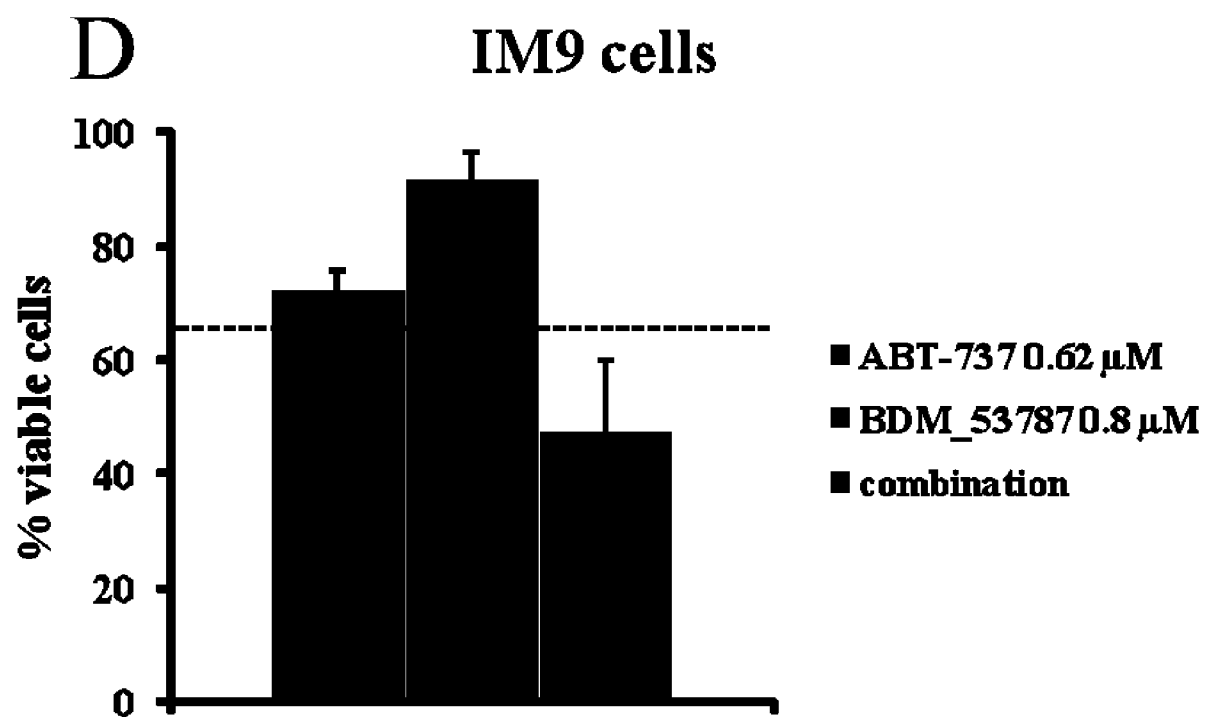


Figure 4D

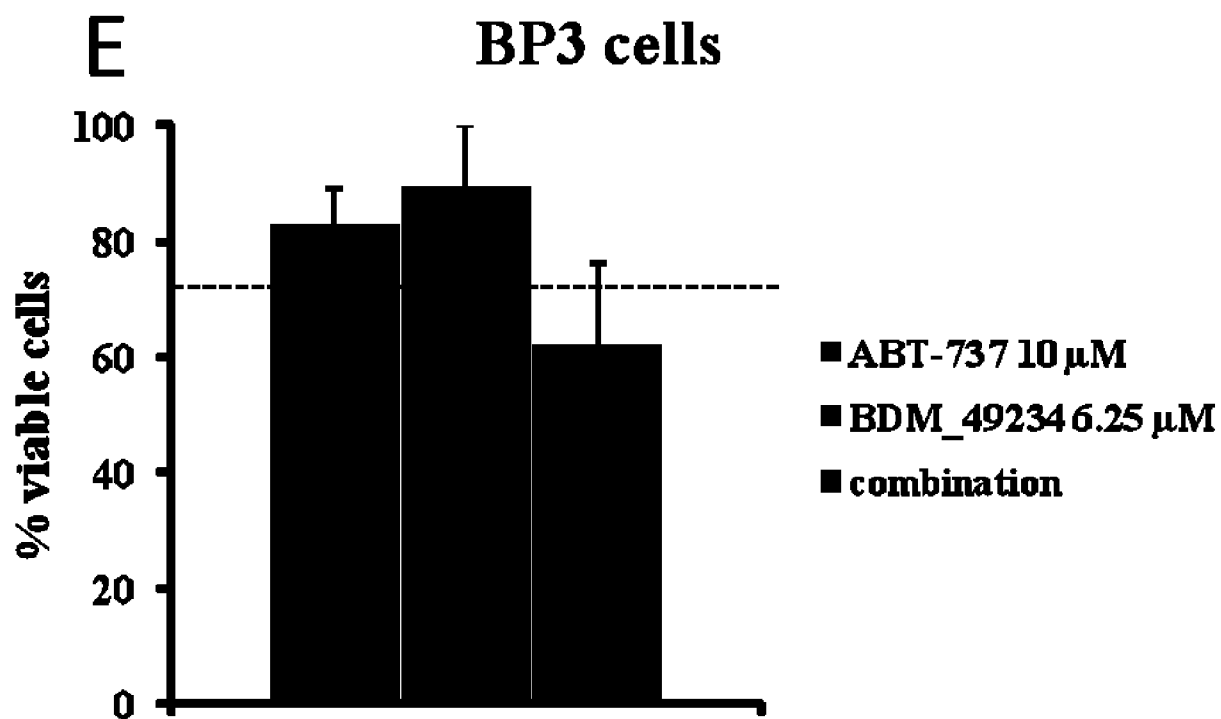


Figure 4E

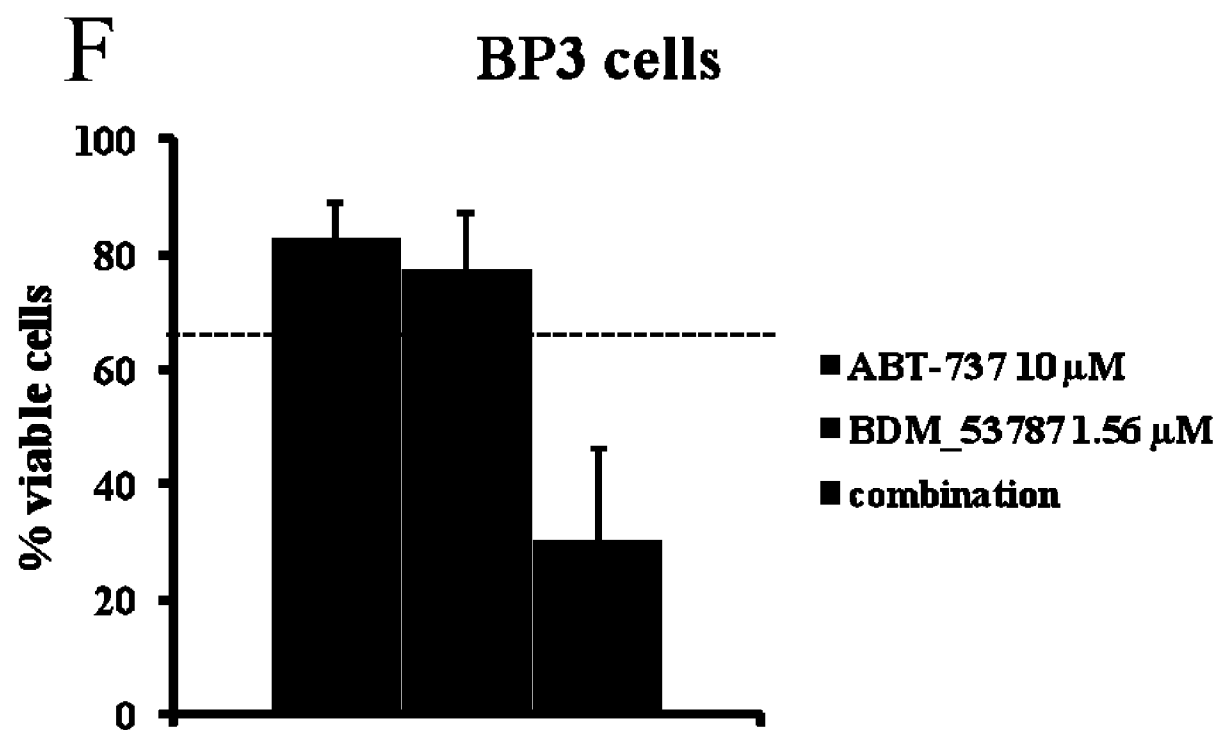


Figure 4F

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2014/077041

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K31/4439 A61K31/496 A61K45/06 A61K31/495 A61K31/5377
A61P35/00 A61P35/02

ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K

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

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

EPO-Internal, WPI Data, CHEM ABS Data, BIOSIS, EMBASE, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2013/032960 A2 (UWM RES FOUNDATION INC [US]; SIDHU PREETPAL SINGH [US]; NANDHIKONDA PR) 7 March 2013 (2013-03-07) page 9, paragraph [0026] page 33, paragraph [00129] page 35, paragraph [00139] - page 36, paragraph [00140] page 38, paragraph [00150] - page 42, paragraph [00151] page 49, paragraph [00176] - page 50, paragraph [00178] claims 63, 64, 72, 73, 79, 81, 82, 90, 91 ----- -/-	1-9

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2014/077041

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