Novel inhibitors of histone deacetylase (hdac), and methods, compositions and uses related thereto

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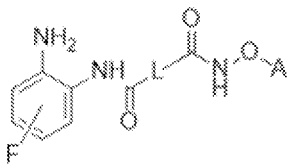

Formula A

(57) Abstract: The invention relates to the field of medicinal chemistry and pharmacology, specifically to novel inhibitors of histone deacetylase (HDAC), and to compositions, methods of making, and using them, for instance in the treatment of chronic obstructive pulmonary disease (COPD). Provided is a compound of the formula A or a pharmaceutically acceptable salt, hydrate, or solvate thereof, wherein A is H or an in vivo hydrolysable group, and L is a linker group.

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Title: Novel inhibitors of histone deacetylase (HDAC), and methods, compositions and uses related thereto.

The invention relates to the field of medicinal chemistry and pharmacology. More specifically, it relates to novel inhibitors of histone deacetylase (HDAC), to methods of making and using them, in particular in the treatment of chronic obstructive pulmonary disease (COPD).

COPD is a chronic inflammatory disease that is characterized by an influx of inflammatory cells in the lungs and an associated abundance of secreted cytokines. It is mainly caused by exposure to noxious airborne particles, for instance through smoking. The main symptoms include shortness of breath and cough with mucus production. Shortness of breath is the result of poor airflow due to a breakdown of lung tissue and concomitant airway remodeling (a process also known as emphysema). Inflammation in COPD mostly involves neutrophils and killer T cells (Buist et al. Eur. Respir. J. 21 (2003) 30S-35s).

Approximately 65 million people worldwide suffer from moderate to severe COPD and it leads to approximately 3 million deaths per year (Lõpez-Campos et al., Respirology. 21 (2016) 14–23). The World Health Organization predicts COPD to be the fourth main cause of death by 2030 in high-income countries, behind ischaemic heart disease, Alzheimer disease and stroke.

At present, no pharmaceutical agents are available which can effectively stop disease progression of COPD. Together with the high disease prevalence, this means that there is a huge medical need to treat COPD.

One of the most important steps in the management of COPD is to stop smoking. Other than that, and for non-smoking COPD patients, the treatment consists of dry-powder or metered-dose inhalation of short or long acting bronchodilators. Initially, short acting beta-2 agonists, like

salbutamol or terbutaline, together with short acting muscarinic antagonists, like ipratropium are prescribed. When these fail, treatment with long acting equivalents such as formoterol or salmeterol (in the case of beta-2 agonists) or tiotropium (in the case of muscarinic antagonists) is often indicated. Patients who have at least two disease exacerbations per year receive corticosteroids either orally or pulmonary (like prednisolon or fluticasone, respectively), to reduce the rate of exacerbations. In the end stage of the disease, patients often require additional oxygen and thus carry around a pressurized oxygen tank.

In vitro and in vivo models of inflammatory pulmonary diseases, including COPD and asthma, have shown beneficial effects of inhibitors of histone deacetylase (HDAC). See Zwinderman et al. (Epigenomes, 2019, 3, 19) and references cited therein. Classically, HDACs are known to deacetylate histones and therefore epigenetically impact gene expression. However, principally any protein with a lysine can undergo a dynamic process of acetylation and deacetylation at some point during its lifetime (Drazic et al., Biochim. Biophys. Acta - Proteins Proteomics. 1864 (2016) 1372–1401).

There are 18 known human histone deacetylases, grouped into four classes based on the structure of their accessory domains. Class I includes HDAC1, HDAC2, HDAC3, and HDAC8 and have homology to yeast RPD3. HDAC4, HDAC5, HDAC7, and HDAC9 belong to class IIa and have homology to yeast. HDAC6 and HDAC10 contain two catalytic sites and are classified as class IIb. Class III (the sirtuins) includes SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6, and SIRT7. HDAC11 is another recently identified member of the HDAC family and has conserved residues in its catalytic center that are shared by both class I and class II deacetylases and is sometimes placed in class IV.

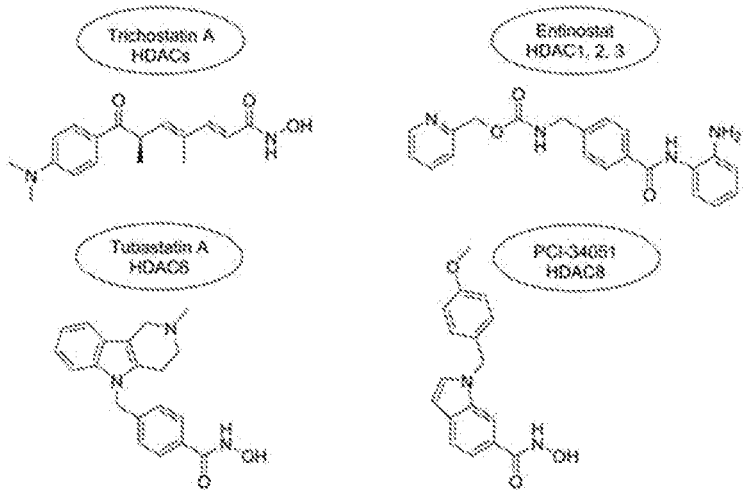
WO2017/118137 discloses benzamide derivatives as inhibitors of histone deacetylase. Methot, J.L., et al, Bioorganic & Medicinal Chemistry

Letters, vol. 18, pages 973-878 discloses exploration of the internal cavity of histone deacetylase (HCAC) with selective HDAC1/HDAC2 inhibitors. WO2005/030705 discloses further compounds and methods for inhibiting histone deacetylase enzymatic activity. EP1402888 discloses the use of substituted carbocyclic compounds as rotamases inhibitors. Katsutra, Y., et al, Chem. Pharm. Bull., 1992, vol. 40, p. 371-380 discloses synthesis and antiulcer activities of imidazo[1,21]pyridinil-2-alkylaminobenzoxazoles and 5,6,7,8-tetrahydroimidazo[1,2-a]pyridinyl derivatives. EP0196005 discloses pyridazinones, their preparation and pharmaceutical compositions containing them.

Currently, over 100 inhibitors of HDAC (HDACi) are in clinical trials for cancer therapy (Mottamal et al. Molecules. 20 (2015) 3898–3941) and various reports suggest that HDACi could also be effectively used to modulate inflammatory diseases, in part because the underlying disease mechanics in cancer overlap with inflammation (Kumar et al., Encycl. Immunobiol., 2016: pp. 406–415; Dekker et al., Drug Discov. Today. 19 (2014) 654–660). Moreover, the anti-inflammatory effects of HDACi are observed at concentrations that are 10-100 fold lower than their cell killing properties observed in cancer (Dinarello et al. Mol. Med. 17 (2011) 333–352).

Given that HDAC3 is a positive regulator of NF-κB mediated inflammation, inhibitors of HDAC3 have been proposed as novel therapeutics to combat inflammation in COPD and asthma (Leus et al., , Curr. Opin. Chem. Biol. 33 (2016) 160–168). In support of this, selective inhibition of HDAC3 with the inhibitor RGFP966 in LPS/IFN-γ stimulated macrophages attenuated the NF-kB transcriptional activity and demonstrated anti-inflammatory effects (Leus et al., Biochem. Pharmacol. 108 (2016) 58–74). Joint inhibition of HDAC1, 2 and 3 by entinostat (see molecular structure of entinostat in Scheme 1) in LPS/IFN-γ induced macrophages in a COPD mouse model led to increased acetylation of NF-κB, increased translocation towards the anti-inflammatory IL-10 promoter and

subsequent increased expression of IL-10. Additionally, it was shown in activated mouse macrophages that joint inhibition of HDAC1, 2 and 3 enables the fine-tuning of the NF-κB pathway towards an anti-inflammatory output, mediated by upregulation of IL-10 (Leus et al., Sci. Rep. 7 (2017) 1–18). Thus, joint inhibition of HDAC1/2/3 reduces cigarette smoke-induced airway inflammation in mice and therefore shows potential for the treatment of COPD.



Scheme 1. Histone deacetylase inhibitors used in in vivo models of asthma or chronic obstructive pulmonary disease.

Besides HDAC1, 2 and 3, also HDAC6 and HDAC8 appear to be implicated in the regulation of cellular processes in inflammation. For example, HDAC8 is known to deacetylate cortactin. Selective inhibition of HDAC8 with inhibitor PCI-34051 (Scheme 1) has been shown to attenuate airway hyperresponsiveness and inflammation, and to counteract airway remodeling (Ren et al., Inflamm. Res. 65 (2016) 995–1008). Another HDAC that is important in airway remodeling is HDAC6, whose primary function is the deacetylation of α-tubulin. The importance of HDAC6 is exemplified by knock-out mouse models that display impeded macrophage migration

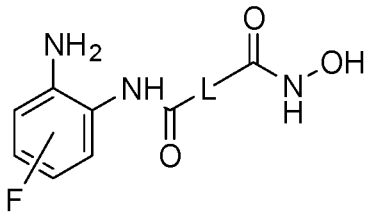
and motility (Yan et al., Theranostics. 8 (2018) 2927–2938. Treatment of asthmatic mice with the HDAC6 selective inhibitor tubastatin A (Scheme 1) mitigated airway hyperresponsiveness and inflammation along with a decrease in airway remodeling markers (Ren et al. 2016). These findings suggest that targeting of HDAC6 and HDAC8 with selective HDACi can be used to combat airway remodeling and inflammation in both asthma and COPD.

Current investigations are mostly aimed at defining the importance of specific HDAC isoforms in terms of their structural role or their catalytic role. Selective inhibition of the catalytic activity of HDAC6 could potentially alleviate airway remodeling and decrease inflammatory cell motility. Additionally, joint inhibition of HDAC1/2/3 might be required to attenuate airway inflammation. Altogether, a combination of HDAC1/2/3 inhibition and HDAC6 inhibition could attenuate both inflammation as well as airway remodeling in COPD and thereby offer a unique treatment option.

The present inventors aimed at providing a novel approach, wherein a single compound attenuates both inflammation as well as airway remodelling in COPD and related diseases.

Surprisingly, this resulted in the design and manufacture of a novel "dual warhead" HDACi showing a prolonged inhibition of one or more Class I HDACs (HDAC1/2/3), preferably at least HDAC3, in the low micromolar range, combined with a potent ability to inhibit HDAC6 (IC50 < 50 nM). Unexpectedly, the dual warhead HDACi inhibits HDAC1/2/3 with slow-on/slow-off kinetics, and HDAC6 with fast-on/fast-off kinetics.

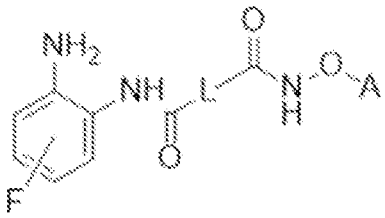
Accordingly, in one embodiment the invention provides a compound of the formula A'



Formula A'

or a pharmaceutically acceptable salt, hydrate, or solvate thereof, wherein L is a linker moiety comprising or consisting of an aliphatic or a 5- or 6-membered cyclic (hetero)aliphatic or (hetero)aryl moiety, and which compound has the ability to inhibit HDAC3 and HDAC6, preferably HDAC1, HDAC2, HDAC3 and HDAC6.

Also provided is a compound of the formula A



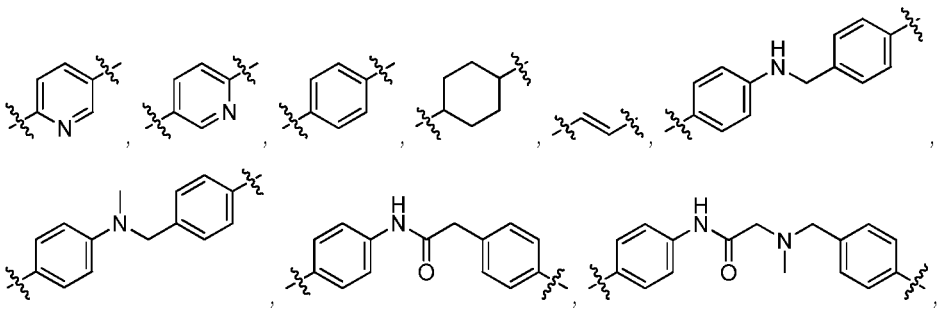
Formula A

or a pharmaceutically acceptable salt, hydrate, or solvate thereof,

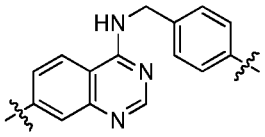
wherein

A is H or an in vivo hydrolysable group,

L is a linker group selected from the group consisting of



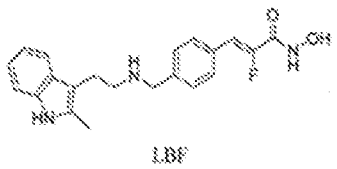
and



Preferably, the fluor-substituent is at the para-position relative to the amine-substituent on the aminobenzamide ring.

A dual warhead HDACi of the invention is not taught or suggested in the art.

WO2011/084991 relates to fluorinated deacetylase inhibitors of general Formula (I), (II) or (III) and to methods of preparing and using the compounds. WO2011/084991 only exemplifies the synthesis of a single Formula I compound referred to as "LBF" having the following structure:



LBF

Also disclosed are various hypothetical compounds, among which two N-hydroxy-benzamide (Formula III) compounds differing from those of the present invention through the fact that the F-atom is present on the phenyl

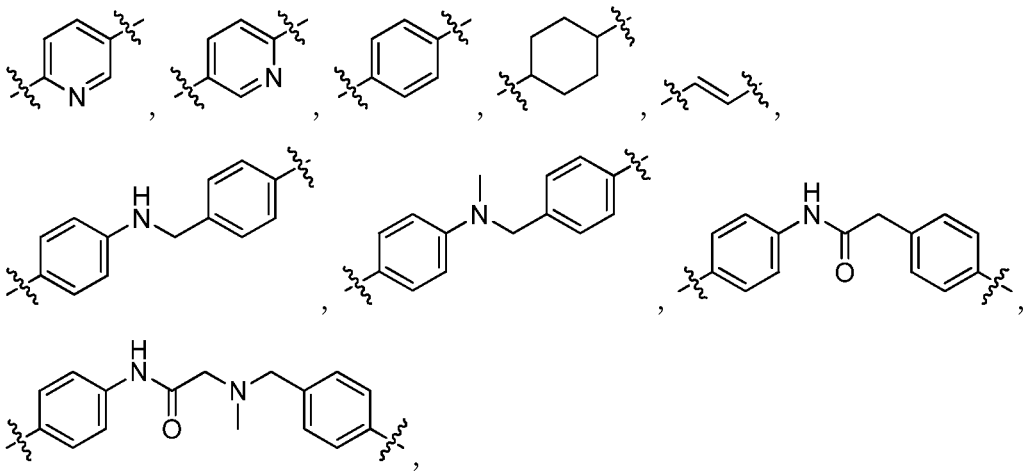
(pyridine) ring proximal to the hydroxylamide function instead of being present on the diaminophenyl moiety. Notably however, there is no enabling disclosure of how any of the proposed fluorinated N-hydroxy-benzamide compounds can be chemically synthesized. Clearly, the precursors and synthetic strategy depicted in Figure 3 of WO2011/084991 is not suitable. What is more, despite serious attempts, the present inventors did not succeed in obtaining the fluorinated compounds drawn in [00147] and [00148] of WO2011/084991. These compounds have thus not been made available to the public, and are therefore not comprised in the state of the art.

In one aspect, the dual warhead compound as provided herein inhibits HDAC1, HDAC2 and HDAC3 with a residence time (τ) > 60 minutes, preferably > 120 minutes and which exhibits the ability to inhibit HDAC6 with a residence time < 60 minutes, preferably < 45 minutes. As used herein, the HDAC isoforms refer to the human HDAC isoforms. Residence time (τ) is the time that a drug remains bound to its target before dissociating. Residence time is the reciprocal of dissociation rate (k_{off}).

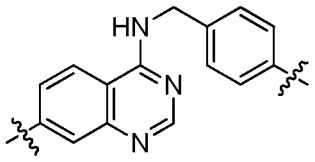
Preferably, the dual warhead compound exhibits the ability to inhibit any one of HDAC1, HDAC2, HDAC3 with an IC50 value $\leq$ 5 μM and /or HDAC6 with an IC50 value $\leq$ 1 μM, preferably $\leq$ 100 nM, more preferably < 60nM.

The L moiety is based on a structure having one or two 5- or 6- membered cyclic (hetero)aliphatic or (hetero)aryl moieties, preferably one or two 6-membered cyclic (hetero)aliphatic or (hetero)aryl moieties.

More in particular, compounds of the present invention encompass those wherein L is selected from the group consisting of



and



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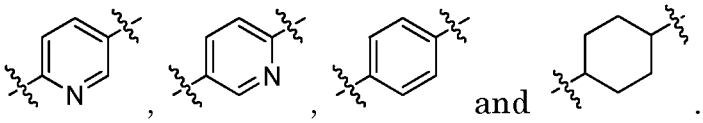
Preferred L-moieties include



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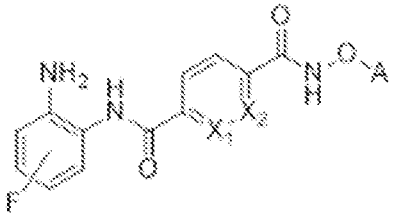
The invention also relates to a "partial pro-drug form" (hereinafter also "pro-drug") wherein the HDAC6 warhead is "masked" by a protecting group A, which masking/protecting group is hydrolysed (metabolized) in vivo, thereby becoming gradually available to inhibit HDAC6. This improves the half-life and membrane permeability of the compound and compensates for the difference in binding kinetics of both warheads. Importantly, in the pro-drug form the inhibitory activity of the HDAC1/2/3 warhead is unaltered. In the active dual warhead form both the HDAC1/2/3 and HDAC6 warheads

are available. The dual HDACi and the partial prodrug form are thus two different compounds with different pharmacokinetic and pharmacodynamic profiles, contributing to a successful therapy for e.g. COPD.

The invention also provides a compound of formula I



Formula I

wherein

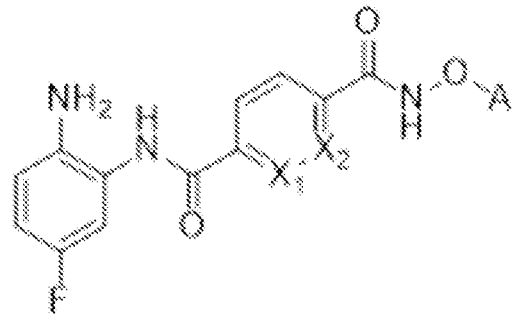
X_1 and X_2 are independently selected from C and N, provided that at least one of X_1 and X_2 is C;

A is H or an in vivo hydrolysable group,

or a pharmaceutically acceptable salt thereof.

The term "in vivo hydrolysable group" is meant to refer to a masking group that can be gradually released from the remainder of the molecule to yield the active dual warhead compound wherein A is H.

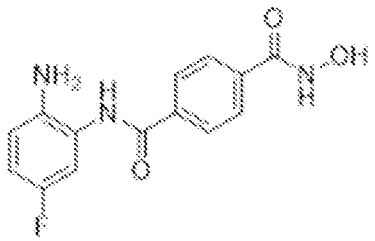
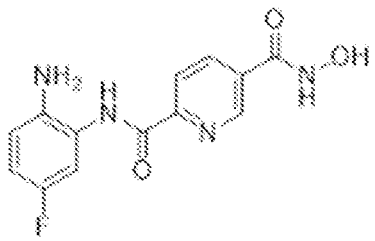
In a specific aspect, the pro-drug warhead compound is of the formula II



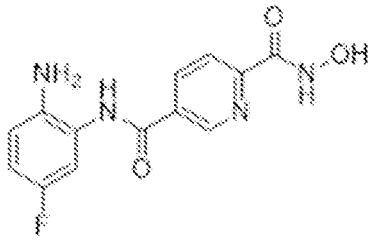
Formula II

Both X_1 and X_2 can be C. Alternatively, one of X_1 and X_2 is C, the other being N. For example, X_1 is N and X_2 is C, or X_1 is C and X_2 is N.

In one aspect, the invention provides an active dual warhead HDACi of the Formula II wherein A is H. The dual warhead HDACi is preferably selected from the group consisting of

and



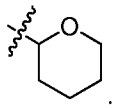
or a pharmaceutically acceptable salt thereof.

In a further aspect, the invention provides a pro-drug dual warhead HDACi of the Formula II, wherein the hydrolysable masking group A is -C(O)Y,

wherein Y is NR_1R_2, $OCR_1R_2R_3$ or $CR_1R_2R_3$,

and wherein R_1, R_2 and R_3 are independently selected from H; a cyclic or acyclic, substituted or unsubstituted aliphatic group; a cyclic or acyclic, substituted or unsubstituted heteroaliphatic group; a substituted or unsubstituted aryl, or a substituted or unsubstituted heteroaryl group, or if Y is NR_1R_2, wherein R_1 and R_2 together form a substituted or unsubstituted heterocycloalkyl group.

Alternatively, the hydrolysable group A is a tetrahydropyranyl



The term "aliphatic", as used herein, includes both saturated and unsaturated, straight chain *(i.e.,* unbranched) or branched aliphatic hydrocarbons, which are optionally substituted with one or more functional groups. As will be appreciated by one of ordinary skill in the art, "aliphatic" is intended herein to include, but is not limited to, alkyl, alkenyl, and alkynyl moieties. Thus, as used herein, the term "alkyl" includes straight and branched alkyl groups. An analogous convention applies to other generic terms such as "alkenyl", "alkynyl", and the like.

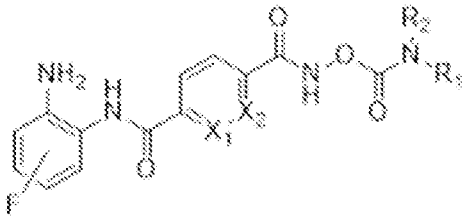
The term "heteroaliphatic," as used herein, refers to aliphatic moieties in which one or more carbon atoms in the main chain have been substituted with a heteroatom. Thus, a heteroaliphatic group refers to an aliphatic chain which contains one or more oxygen, sulfur, nitrogen, phosphorus or silicon atoms, *e.g.,* in place of carbon atoms. Heteroaliphatic moieties may be linear or branched, and saturated or unsaturated.

The term "aryl", as used herein, does not differ significantly from the common meaning of the term in the art, and refers to an unsaturated cyclic moiety comprising at least one aromatic ring. In certain embodiments, "aryl" refers to a mono- or bicyclic carbocyclic ring system having one or two aromatic rings including phenyl, naphthyl, tetrahydronaphthyl, indanyl and indenyl.

The term "heteroaryl", as used herein, does not differ significantly from the common meaning of the term in the art, and refers to a cyclic aromatic radical having from five to ten ring atoms of which one ring atom is selected

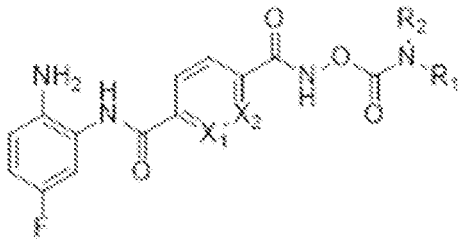
from S, O, and N; zero, one, or two ring atoms are additional heteroatoms independently selected from S, O, and N; and the remaining ring atoms are carbon, the radical being joined to the rest of the molecule via any of the ring atoms, such as, for example, pyridyl, pyrazinyl, pyrimidinyl, pyrrolyl, pyrazolyl, imidazolyl, thiazolyl, oxazolyl, isooxazolyl, thiadiazolyl, oxadiazolyl, thiophenyl, furanyl, quinolinyl, isoquinolinyl.

Good results are obtained with pro-drug compounds of the formula IV wherein masking group A is $C(O)NR_1R_2$, giving rise to a hydrolysable carbamate moiety , and wherein R_1 and R_2 are as defined above.



Formula IV

Specific exemplary compounds have the formula V



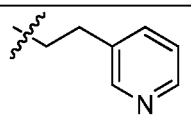
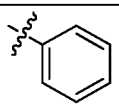
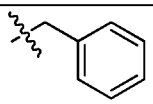
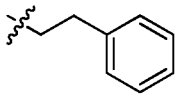
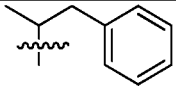
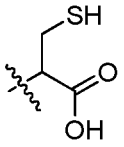
Formula V.

In one aspect, both X_1 and X_2 in a Formula IV or V compound are C-atoms.

For example, provided is a pro-drug HDACi compound of Formula IV or V comprising a R_1/R_2 combination selected from entries 1-17 according to Table 1:

Table 1

entry	R_1	R_2
1	H	
2	H	
3	H	
4	H	
5	H	
6	H	
7	H	

8	H	
9	H	
10	H	
11	H	
12	H	
13	H	
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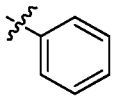
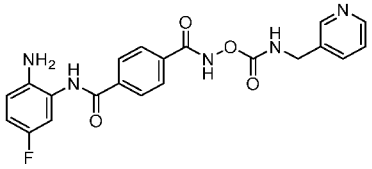
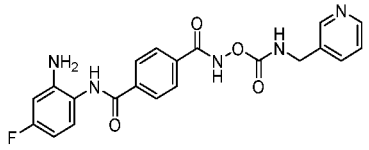
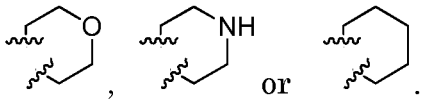
15	CH_3	CH_2CH_3
16	CH_2CH_3	CH_2CH_3
17	CH_3	

For example, the pro-drug HDACi is of the formula



or





In one embodiment, the invention provides a compound of Formula IV, preferably Formula V, wherein R_1 is H and R_2 is $-(CH_2)n$-Z, wherein n is 1-4 and Z is a 5- or 6-membered substituted or unsubstituted (hetero)aryl, preferably an unsubstituted pyridine. For example, see entries 5 to 8 in the table herein above. Also provided is the use of a compound according to the invention as an inhibitor of HDAC, preferably targeting HDAC1, HDAC2, HDAC3 and HDAC6.

Compounds of the invention plus various compounds for reference only can be evaluated using a variety of methods known in the art. For example, the following methods can be used to evaluate compounds of the invention: the inhibition of HDAC activity can be determined using HDAC activity assays known in the art. For example, Doodipala Samba Reddy et al. (Curr Protoc Pharmacol. 2018 Jun; 81(1): e41) describe a robust method for identifying HDAC inhibitors. In this simple and sensitive assay, HDAC activity in tissue lysates can be assessed fluorometrically using a Boc-Lys(Ac) HDAC activity kit. HDACs catalyze the deacetylation of the substrate, Boc-Lys(Ac)-AMC. Addition of a trypsin-containing developer or Lys-C protease converts the deacetylated product to a quantifiable fluorophore that can be used both as a screening method to identify putative HDAC inhibitors. As is exemplified herein below, the dual warhead HDACi or pro-drug form thereof is preferably evaluated in vitro using recombinant (human) HDAC enzymes. The ability of the compounds to modulate tubulin acetylation levels can be tested in vitro using human RPE-1 cells (Ran et al., *Sci. Rep.* 5 (2015) 12917).

Effects of the dual HDAC inhibitors on lung fibrosis can be assessed by marker gene expression in murine precision cut lung slices (PCLS). Pro-fibrotic markers of particulate interest include extracellular matrix protein fibronectine (FN), collagen 1a1 (Col1a1) and α-Smooth muscle actin (aSMA). Control markers may encompass growth factor CTGF and the protease inhibitor PAI-1.

As will be appreciated by a person skilled in the art, the unique dual warhead compounds, e.g, in their pro-drug form, as herein disclosed are advantageously used in preventive and therapeutic methods and compositions.

Pharmaceutically acceptable acid addition salts of the invention can be formed by reacting a compound of the invention with an equimolar or excess amount of acid. Alternatively, hemi-salts can be formed by reacting a compound of the invention with the desired acid in a 2:1 ratio, compound to acid. The reactants are generally combined in a mutual solvent such as diethylether, tetrahydrofuran, methanol, ethanol, isopropanol, benzene, or the like. The salts normally precipitate out of solution within about one hour to about ten days and can be isolated by filtration or other conventional methods.

Inorganic acids commonly employed to form such salts include hydrochloric acid, hydrobromic acid, hydroiodic acid, sulfuric acid, phosphoric acid, and the like. Organic acids commonly employed to form such salts include p-toluenesulfonic acid, methanesulfonic acid, oxalic acid, p-bromophenylsulfonic acid, carbonic acid, succinic acid, citric acid, benzoic acid, acetic acid and the like. Examples of such pharmaceutically acceptable salts thus are the sulfate, pyrosulfate, bisulfate, sulfite, bisulfite, phosphate, monohydrogenphosphate, dihydrogenphosphate, metaphosphate, pyrophosphate, chloride, bromide, iodide, acetate, propionate, decanoate, caprylate, acrylate, formate, isobutyrate, caproate, heptanoate, propiolate,

oxalate, malonate, succinate, hemisuccinate, suberate, sebacate, fumarate, maleate, butyne-1,4-dioate, hexyne-1,6-dioate, benzoate, chlorobenzoate, methylbenzoate, dinitrobenzoate, hydroxybenzoate, methoxybenzoate, phthalate, sulfonate, xylenesulfonate, phenylacetate, phenylpropionate, phenylbutyrate, citrate, lactate, ß-hydroxybutyrate, glycollate, tartrate, methanesulfonate, propanesulfonate, naphthalene-1-sulfonate, naphthalene-2-sulfonate, mandelate and the like.

Some of the compounds of the present invention may exist in unsolvated as well as solvated forms such as, for example, hydrates.

"Solvate" means a solvent addition form that contains either a stoichiometric or non-stoichiometric amounts of solvent. Some compounds have a tendency to trap a fixed molar ratio of solvent molecules in the crystalline solid state, thus forming a solvate. If the solvent is water the solvate formed is a hydrate, when the solvent is alcohol, the solvate formed is an alcoholate. Hydrates are formed by the combination of one or more molecules of water with one of the substances in which the water retains its molecular state as H_2O, such combination being able to form one or more hydrate.

In one embodiment, the invention provides a pharmaceutical composition comprising at least one (pro-drug) compound according to the invention, and a pharmaceutically acceptable carrier, vehicle or diluent. The composition may be used on their own or in the form of appropriate medicinal preparations for administering by a route selected from oral, parenteral, intramuscular, intranasal, sublingual, intratracheal, inhalation, ocular, vaginal, rectal, and intracerebroventricular. In one aspect, the pharmaceutical composition is formulated for administration to the upper and lower airways, preferably by inhalation.

For administration by inhalation, the compounds for use according to the present invention may be conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of e.g. gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch. Also contemplated herein is pulmonary delivery of the compounds of the invention. The compound is delivered to the lungs of a mammal while inhaling and traverses across the lung epithelial lining to the blood stream.

One preferred route of administration is by inhalation, which is possible through nebulizing an aqueous solution of the active HDACi (pro-dug) compound or by inhaling a dry-powder or aerosolized form of the compound.

Contemplated for use in the practice of this invention are a wide range of mechanical devices that are known in the art for pulmonary delivery of therapeutic products. These include nebulizers, metered dose inhalers, and powder inhalers.

A dry-powder formulation or an aerosolized form is preferred since it is easier to use for patients. This is readily achieved by using a pharmaceutically acceptable salt of a HDACi and including it in any type of suitable generic dry-powder inhaler. In a specific aspect, the Cyclops™ disposable dry-powder inhaler is used.

All such devices require the use of formulations suitable for the dispensing of compound. Typically, each formulation is specific to the type of device employed and may involve the use of an appropriate propellant

material, in addition to the usual diluents, and/or carriers useful in therapy. Also, the use of liposomes, microcapsules or microspheres, inclusion complexes, or other types of carriers is contemplated. Chemically modified compound may also be prepared in different formulations depending on the type of chemical modification or the type of device employed. Formulations suitable for use with a nebulizer, either jet or ultrasonic, will typically comprise compound dissolved in water at a concentration of about 0.1 to 25 mg of biologically active compound per mL of solution. The formulation may also include a buffer and a simple sugar (e.g., for stabilization and regulation of osmotic pressure). The nebulizer formulation may also contain a surfactant, to reduce or prevent surface induced aggregation of the compound caused by atomization of the solution in forming the aerosol.

In a further embodiment, the invention provides a metered-dose inhaler or dry-powder inhaler (DPI) comprising a warhead HDAC inhibitor or a pro-drug form thereof as herein disclosed. A DPI is a device that delivers medication to the lungs in the form of a dry powder. DPIs are commonly used to treat respiratory diseases such as asthma, bronchitis, emphysema and COPD.

Formulations for use with a metered-dose inhaler device typically generally comprise a finely divided powder containing the compound suspended in a propellant with the aid of a surfactant. The propellant may be any conventional material employed for this purpose, such as a chlorofluorocarbon, a hydrochlorofluorocarbon, a hydrofluorocarbon, or a hydrocarbon. Examples include trichlorofluoromethane, dichlorodifiuoromethane, dichlorotetrafluoroethanol, and 1,1,1,2-tetrafluoroethane, or combinations thereof.

In a further embodiment, the invention provides a compound as herein disclosed for use as medicament, in particular for use in a method of treating, alleviating, and/or preventing a pulmonary disease, preferably an airway inflammatory disease or lung cancer, such as COPD, asthma, idiopathic pulmonary fibrosis, non-small-cell lung cancer or small-cell lung cancer.

Thus, also provided is a method of treatment or prevention of a pulmonary disease, preferably an airway inflammatory disease or lung cancer, such as COPD, asthma, idiopathic pulmonary fibrosis, non-small-cell lung cancer or small-cell lung cancer, comprising administering to a subject in need thereof a pharmaceutically effective dose of a pharmaceutical composition of the invention.

As used herein, the term "treat," "treating," "alleviate," or "alleviating" herein, is meant decreasing the symptoms, markers, and/or any negative effects of a condition in any appreciable degree in a patient who currently has the condition. In some embodiments, treatment may be administered to a subject who exhibits only early signs of the condition for the purpose of decreasing the risk of developing the disease, disorder, and/or condition.

As used herein, the term "prevent," "prevention," or "preventing" refers to any method to partially or completely prevent or delay the onset of one or more symptoms or features of a disease, disorder, and/or condition. Prevention may be administered to a subject who does not exhibit signs of a disease, disorder, and/or condition.

As used herein, "subject" means a human or animal (in the case of an animal, more typically a mammal). In one aspect, the subject is a human. Such subject can be considered to be in need of treatment with a dual warhead HDAC inhibitor or a pro-drug form thereof.

For the above-mentioned uses the dosage administered will, of course, vary with the composition employed, the mode of administration and the treatment desired. However, in general, satisfactory results will be obtained when the active components are administered at a daily dosage of from 0.1 mg to 20 mg per kg of mammalian body weight, preferably given in divided doses 1 to 4 times a day. For man, the total daily dose is in the range of from 5 mg to 1,400 mg, more preferably from 10 mg to 100 mg, and unit dosage forms suitable for oral administration comprise from 2 mg to 1,400 mg of the active components admixed with a solid or liquid pharmaceutical carrier or diluent.

In certain embodiments, compounds of the invention are administered at dosage levels greater than about 0.001mg/kg, such as greater than about 0.01 mg/kg or greater than about 0.1 mg/kg. For example, the dosage level may be from about 0.001 mg/kg to about 50 mg/kg such as from about 0.01 mg/kg to about 25 mg/kg, from about 0.1 mg/kg to about 10 mg/kg, or from about 1 mg/kg to about 5 mg/kg of subject body weight per day, one or more times a day, to obtain the desired therapeutic effect. It will also be appreciated that dosages smaller than 0.001 mg/kg or greater than 50 mg/kg (for example 50-100 mg/kg) can also be administered to a subject.

In one embodiment, the compound of the invention is administered once-daily, twice-daily, or three-times daily. In one embodiment, the compound of the invention is administered continuously (i.e., every day) or intermittently (e.g., 3-5 days a week). In another embodiment, administration could be on an intermittent schedule.

Further, administration less frequently than daily, such as, for example, every other day may be chosen. In additional embodiments, administration with at least 2 days between doses may be chosen. By way of example only, dosing may be every third day, bi-weekly or weekly. As another example, a

single, acute dose may be administered. Alternatively, compounds of the invention can be administered on a non-regular basis e.g., whenever symptoms begin. For any compound described herein the effective amount can be initially determined from animal models.

The invention includes combination therapies including the compounds and compositions for use in methods of treating, alleviating, and/or preventing conditions described herein. Combination therapy includes administering one or more compounds of the invention in combination with one or more pharmaceutically active ingredients for treating, alleviating, or preventing a pulmonary disease. The combination therapies comprise the administration of an effective amount of one or more (e.g. one) compounds of the invention and the administration of an effective amount of one or more (e.g., one) other pharmaceutically active ingredients (e.g., drugs). The compounds of the invention and the other pharmaceutically active ingredients can be administered separately (i.e., each is in its own separate dosage form), or the compounds of the invention can be combined with the other pharmaceutically active ingredients in the same dosage form. Pharmaceutically active ingredients that are useful in combination therapies of the invention include e.g., short acting beta-2 agonists, like salbutamol or terbutaline, short acting muscarinic antagonists, like ipratropium, or long acting equivalents such as formoterol or salmeterol (in the case of beta-2 agonists) or tiotropium (in the case of muscarinic antagonists).

The compounds of the invention are also useful in combination with known pharmaceutically active ingredients such as anti-(lung)cancer agents, or anti-airway inflammatory disease.

Combinations of the presently disclosed compounds with other anti-cancer or chemotherapeutic agents are within the scope of the invention. A person of ordinary skill in the art would be able to discern which combinations of agents would be useful based on the particular characteristics of the drugs and the cancer involved. Such anti-cancer agents include drugs and combination therapies approved for Non-Small Cell Lung Cancer: Abraxane (Paclitaxel Albumin-stabilized Nanoparticle Formulation) Afatinib Dimaleate, Afinitor (Everolimus),Afinitor Disperz (Everolimus) Alecensa (Alectinib),Alectinib, Alimta (Pemetrexed Disodium), Alunbrig (Brigatinib), Atezolizumab, Avastin (Bevacizumab), Bevacizumab, Brigatinib,Capmatinib Hydrochloride, Carboplatin, Cemiplimab-rwlc Ceritinib, Crizotinib, Cyramza (Ramucirumab), Dabrafenib Mesylate Dacomitinib, Docetaxel, Doxorubicin Hydrochloride, Durvalumab, Entrectinib, Erlotinib Hydrochloride, Everolimus, Gavreto (Pralsetinib) Gefitinib, Gilotrif (Afatinib Dimaleate), Gemcitabine Hydrochloride Gemzar (Gemcitabine Hydrochloride), Imfinzi (Durvalumab), Infugem (Gemcitabine Hydrochloride), Ipilimumab, Iressa (Gefitinib), Keytruda (Pembrolizumab), Libtayo (Cemiplimab-rwlc), Lorbrena (Lorlatinib) Lorlatinib, Mekinist (Trametinib Dimethyl Sulfoxide), Methotrexate Sodium Mvasi (Bevacizumab), Necitumumab, Nivolumab, Opdivo (Nivolumab) Osimertinib Mesylate, Paclitaxel, Paclitaxel Albumin-stabilized Nanoparticle Formulation, Paraplat (Carboplatin), Paraplatin (Carboplatin) Pembrolizumab, Pemetrexed Disodium, Portrazza (Necitumumab), Pralsetinib, Ramucirumab, Retevmo (Selpercatinib), Rozlytrek (Entrectinib) Selpercatinib, Tabrecta (Capmatinib Hydrochloride), Tafinlar (Dabrafenib Mesylate), Tagrisso (Osimertinib Mesylate), Tarceva (Erlotinib Hydrochloride), Taxotere (Docetaxel), Tecentriq (Atezolizumab), Tepmetko (Tepotinib Hydrochloride), Tepotinib Hydrochloride, Trametinib Dimethyl Sulfoxide, Trexall (Methotrexate Sodium), Vizimpro (Dacomitinib)

Vinorelbine Tartrate, Xalkori (Crizotinib), Yervoy (Ipilimumab), Zirabev (Bevacizumab), Zykadia (Ceritinib), CARBOPLATIN-TAXOL, GEMCITABINE-CISPLATIN.

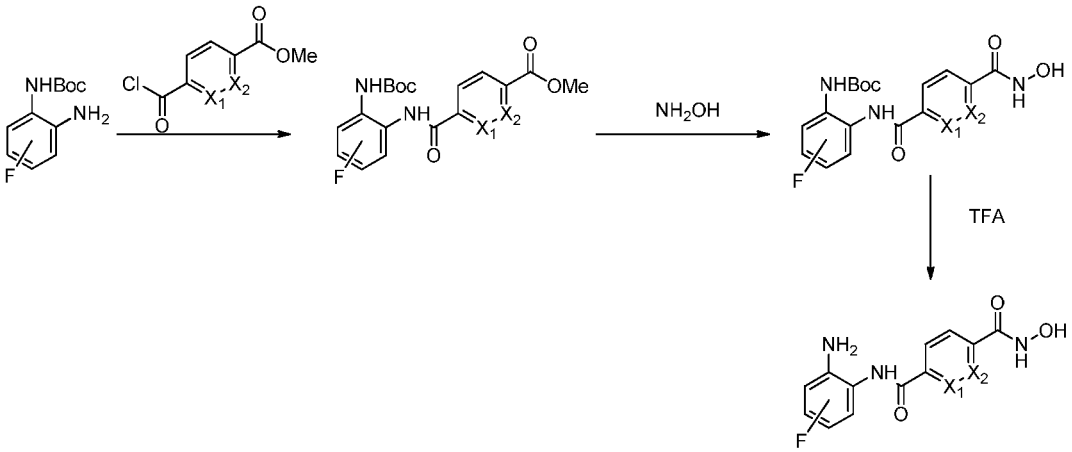
Drugs Approved for Small Cell Lung Cancer: Afinitor (Everolimus), Atezolizumab, Doxorubicin Hydrochloride, Durvalumab, Etopophos (Etoposide Phosphate), Etoposide, Etoposide Phosphate, Everolimus Hycamtin (Topotecan Hydrochloride), Imfinzi (Durvalumab), Lurbinectedin Methotrexate Sodium, Nivolumab, Opdivo (Nivolumab), Tecentriq, (Atezolizumab), Topotecan Hydrochloride, Trexall (Methotrexate Sodium) Zepzelca (Lurbinectedin).

The compounds of the invention are also useful when co-administered with radiation therapy.

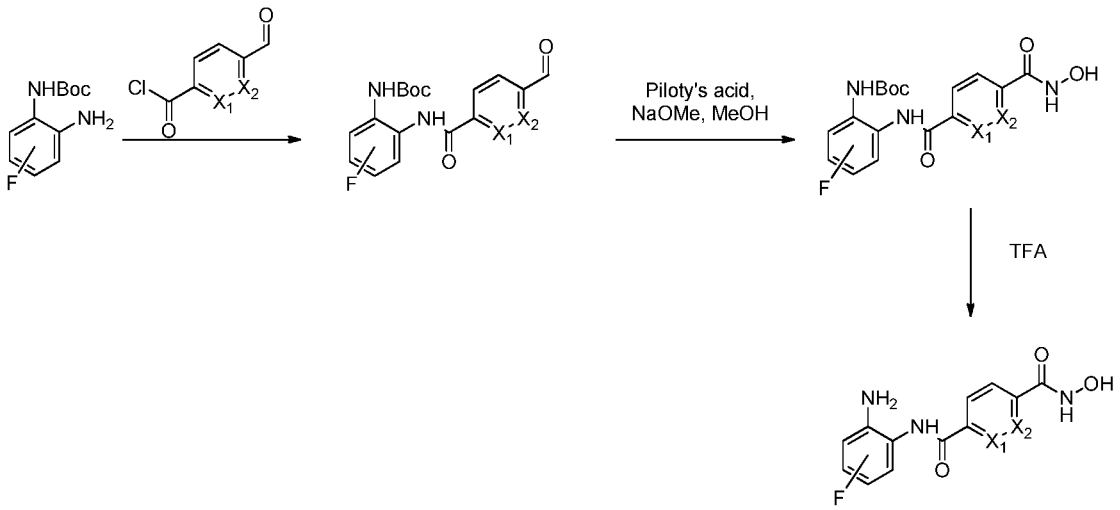
In one aspect, the invention provides a kit containing one or more compounds of the invention or a pharmaceutically acceptable salt, hydrate, or solvate, thereof. In one aspect, the kit further contains a pharmaceutically active ingredient. There is also provided a process for the preparation of such a pharmaceutical composition which comprises mixing the ingredients simultaneously or sequentially.

A further aspect of the invention relates to a method of synthesizing a compound of the invention or a pharmaceutically acceptable salt, hydrate, or solvate, thereof.

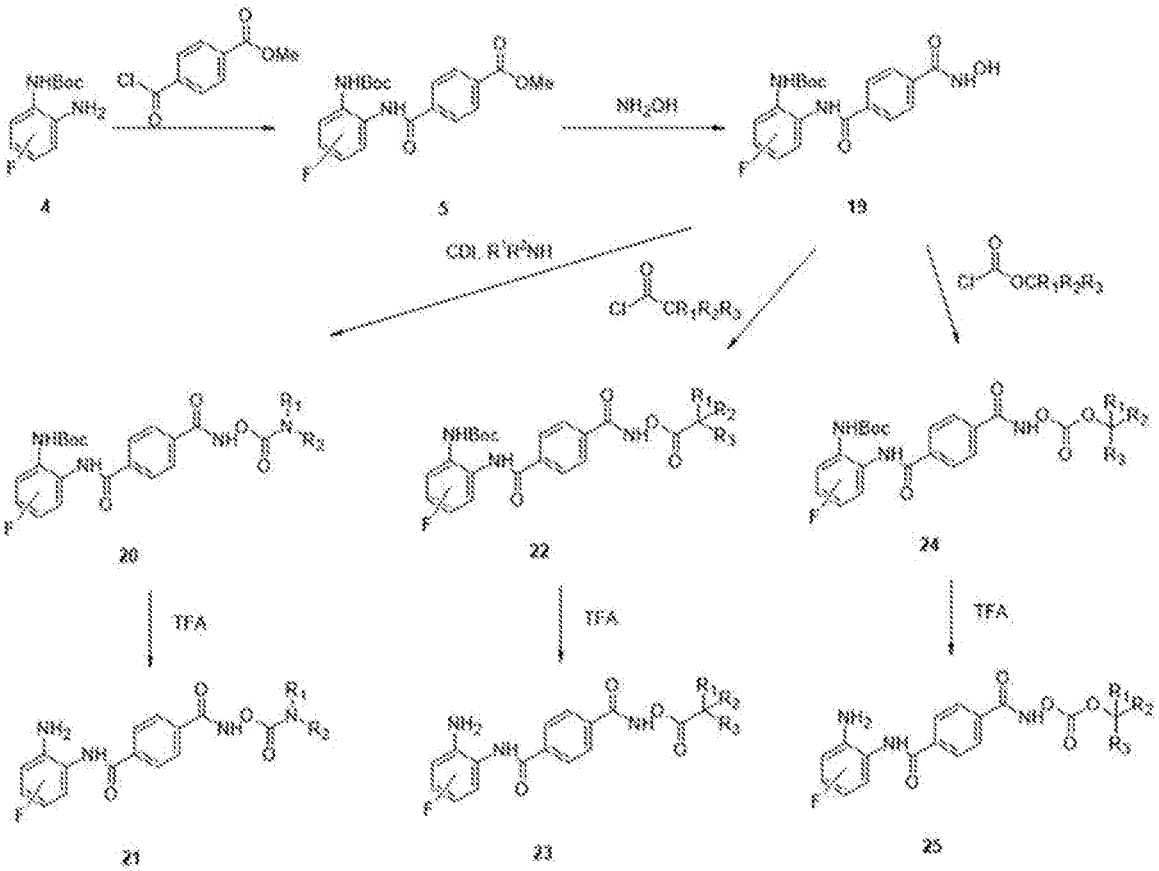
In one embodiment, the invention provides a method for providing an active dual HDACi compound according to Formula I, comprising the steps depicted in the following scheme:



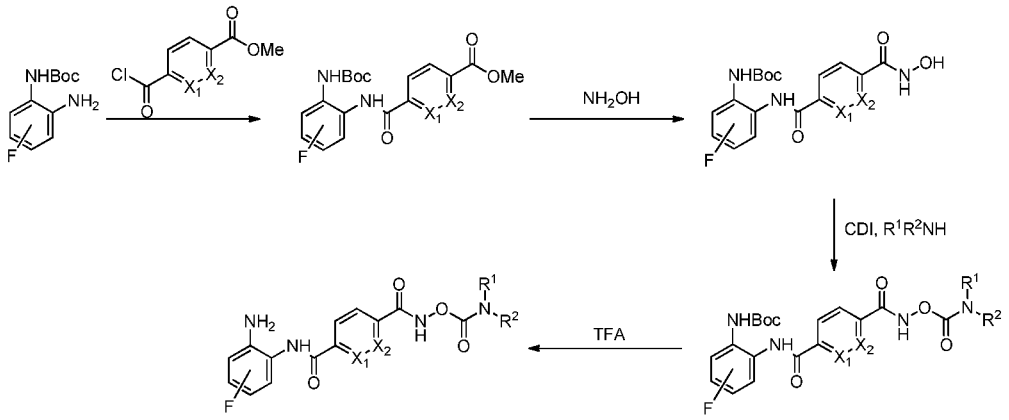
or comprising the steps of



In another embodiment, the invention provides a method for providing a pro-drug dual HDACi compound according to Formula II, according to a synthetic route as depicted in the following scheme :



For example, the invention provides a method for providing a pro-drug dual HDACi compound according to Formula IV or V, comprising the steps depicted in the following scheme:



LEGEND TO THE FIGURES

Figure 1: Exemplary dual HDACi compounds

Figure 2: HDAC inhibition curve of dual HDACi with IC50 of inhibition of HDAC1, 2 and 3.

Figure 3: HDAC inhibition curve of prodrug HDACi with IC50 of inhibition of HDAC1, 2 and 3.

Figure 4: HDAC inhibition curve of dual HDACi with IC50 of inhibition of HDAC6.

Figure 5: Dose-response of dual HDACi or its prodrug form on acetylation levels of tubulin in human RPE-1 cells. Human RPE-1 cells were treated with the dual HDACi or the prodrug form of the dual HDACi at various concentrations for 18 h. Tubulin acetylation levels were evaluated with Western blot analysis.

Figure 6: Effects of the dual HDACi on the expression of fibrotic markers in murine precision cut lung slices (PCLS). Murine PCLS were exposed to a

cocktail of cytokines (TGF-ß1 5 ng/ml, 10 ng/ml TNF-α, 30 ng/ml PDGF-AB, 5 µM LPA), to induce a fibrotic response. The inhibitor (10 µM) significantly reduced the expression of the pro-fibrotic markers extracellular matrix protein fibronectin (FN; panel A), collagen 1a1 (Col1a1; panel B) and α-Smooth muscle actin (aSMA; panel C). Expression of the growth factor CTGF (panel D) and the protease inhibitor PAI-1 (panel E) were unaffected. Overall, this indicates that the HDAC inhibitor reduces fibrosis.

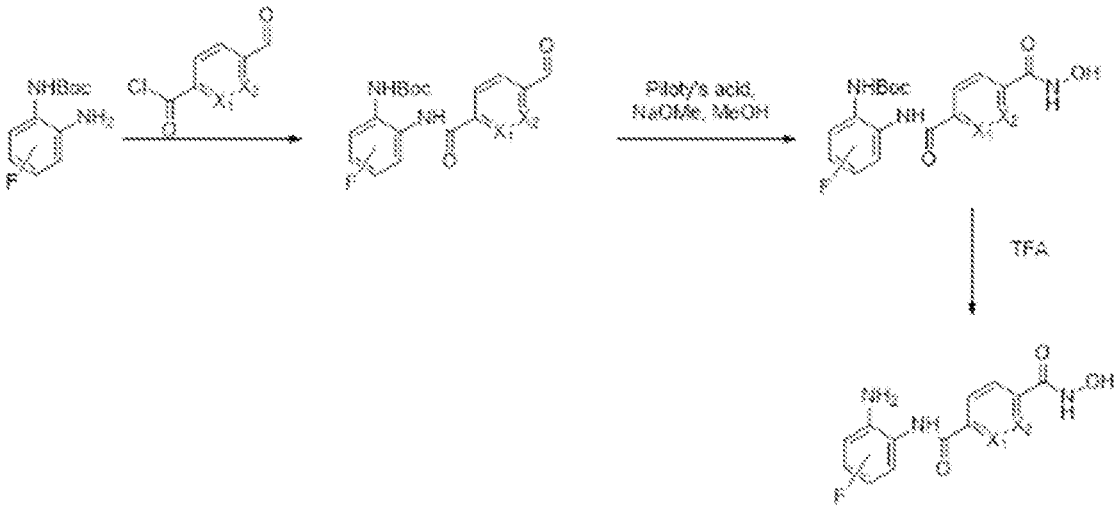
Figure 7: Compound stability in human, mouse or rat microsomes (panel A) or plasma (panel B).

Figure 8: Gene expression of collagen I α1 (panel A), fibronectin (panel B) and α-smooth actin (panel C) after treatment of lung slices with fibrosis cocktail, or fibrosis cocktail in combination with investigational compounds (10 µM) for 48h. Data represent normalized mean with SEM relative to vehicle, n=6 animals.

EXPERIMENTAL SECTION

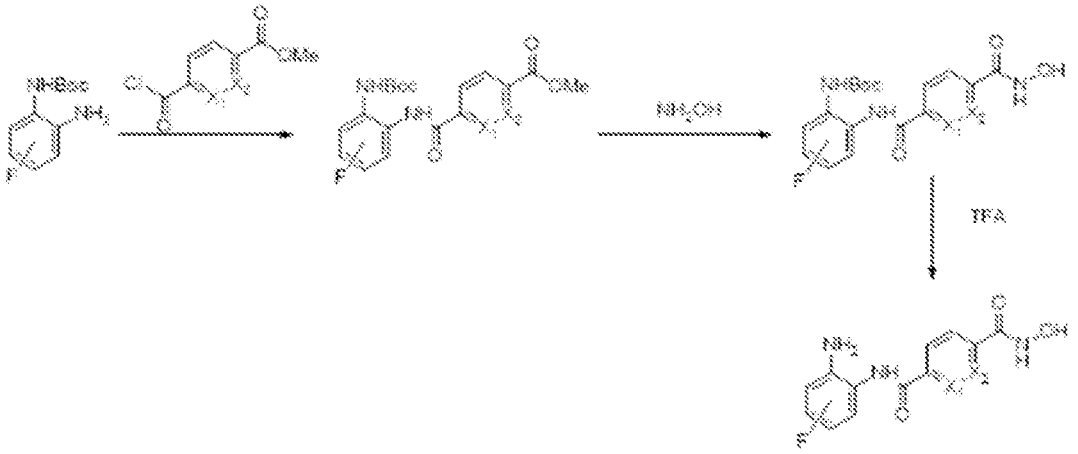
EXAMPLE 1: Synthesis of Dual HDACi

Dual warhead HDACi compounds can be synthesized according to the general Schemes 2 and 3 herein below.



Scheme 2: Angeli-Rimini reaction route towards the hydroxamic acid series.

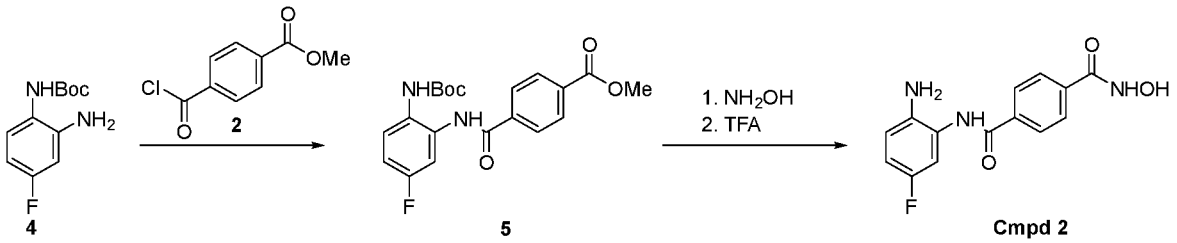
In scheme 2, a mono-Boc protected dianilino species is coupled with an appropriate acid chloride. Subsequent reaction of the resultant aldehyde with Piloty's acid and an acid mediated Boc cleavage affords the desired hydroxamic acid.



Scheme 3: Condensation reaction approach towards the hydroxamic acid series.

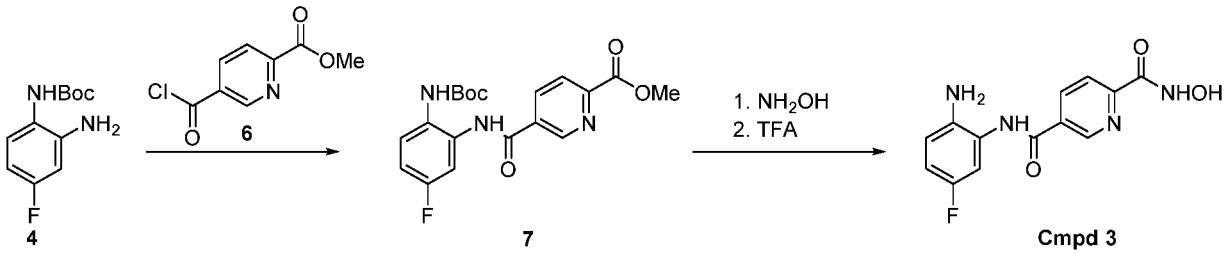
In scheme 3, analogously to scheme 2, a mono-Boc protected dianilino species is coupled with an appropriate acid chloride. The resultant ester is subsequently reacted with hydroxylamine to afford the intermediary hydroxamic acid. Acidic cleavage of the Boc group realizes the final compound.

For example, **Cmpd 2** can be accessed in a three step sequence from commercially available aniline **4** (CAS 579474-47-8). Coupling of amine **4** with acid chloride **2** will give ester **5**. Reaction of **5** with hydroxylamine and subsequent treatment with TFA will afford **Cmpd 2**.



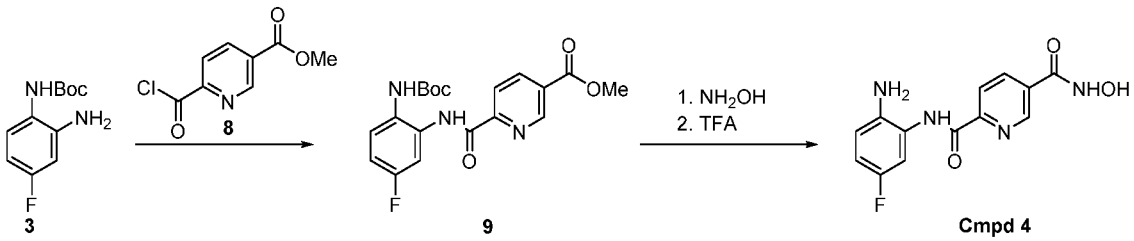
*Scheme 4: Three step synthesis towards **Cmpd 2**.*

In an analogous method; aniline **3** is reacted with acid chloride **6** to afford pyridyl **7**. Acid chloride **6** is accessible in one step from the commercially available carboxylic acid (CAS 17874-76-9). Reaction of **7** with hydroxylamine, followed acid mediated deprotection with TFA will afford the desired product **Cmpd 3**.



*Scheme 5: Four step synthesis towards **Cmpd 3**.*

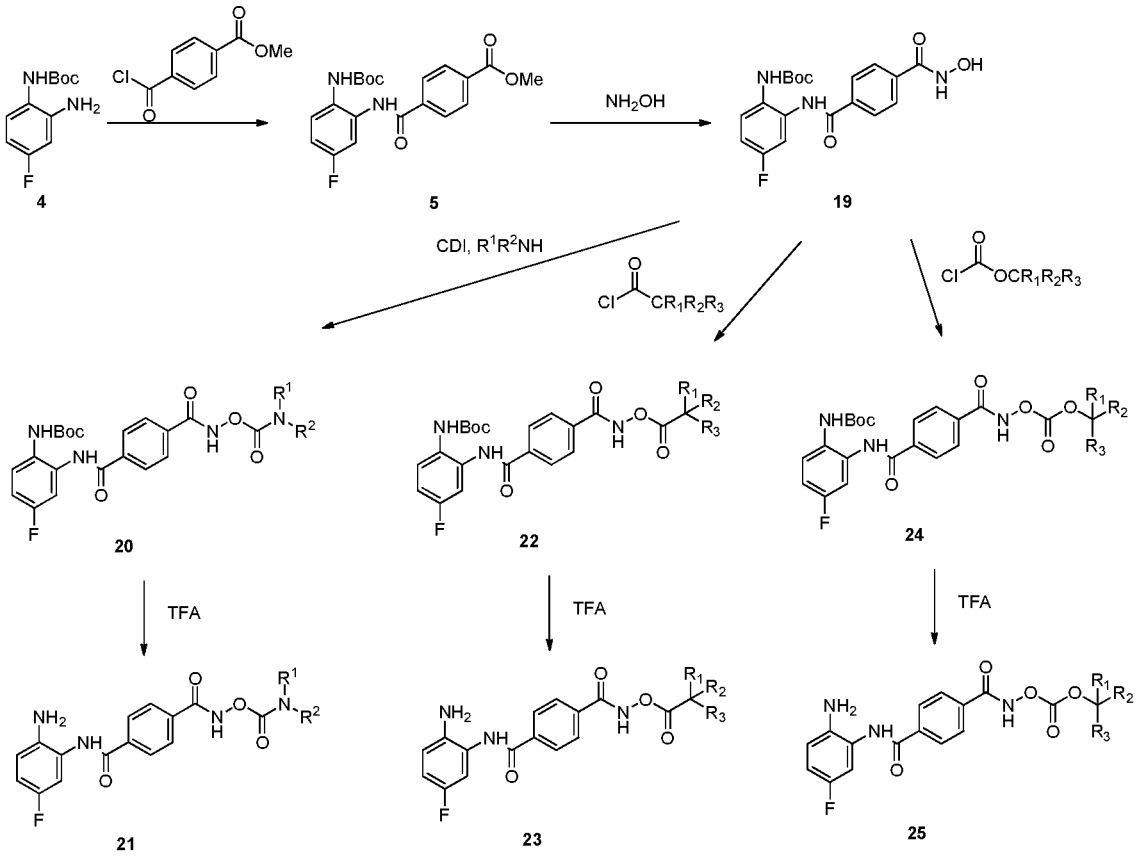
Intermediate **9** is accessible by reaction of aniline **3** with acid chloride 8. Reactant 8 is available in one step from the corresponding, commercially available, carboxylic acid (CAS 17874-79-2). Exposure of intermediate **9** to hydroxylamine followed by treatment with TFA will yield **Cmpd 4**.



*Scheme 6: Four step synthesis towards **Cmpd 4**.*

EXAMPLE 2: Synthesis of Pro-drug Dual HDACi

A suitable synthetic route towards exemplary pro-drug forms of a dual warhead HDACi is outlined in general scheme 7 below. It utilizes a precursor of **Cmpd 2** (see above) for the synthesis of common intermediate **19**, which is then provided with masking group A of the formula -C(O)Y, wherein Y is NR1R2 (compound series **21**), CR1R2R2 (compound series **23**) or OCR1R2R3 (compound series **25**)



*Scheme 7: 4 step synthesis towards Cmpd series **21, 23 and 25**.*

Common intermediate **19** is accessible in two-steps from commercially available aniline **4** (CAS 579474-47-8). Coupling of hydroxamic acid **19** with a CDI activated amine will yield intermediate **20**. Subsequent Boc

deprotection with TFA will afford the desired compound series **21**. Alternatively, coupling of intermediate **19** with an acyl chloride yields intermediate **23**, whereas coupling of compound **19** with a chloroformate yields intermediate **24**. Subsequent Boc deprotection with TFA affords the desired compound series **23** and **25**, respectively.

EXAMPLE 3: Properties of Dual HDACi

Materials and methods

HDAC inhibition assays

Black 96-well flat bottom microplates (Corning® Costar®, Corning Incorporated, NY) were used. Human recombinant C-terminal FLAG-tag, C-terminal His-tag HDAC 1 (BPS Bioscience, Catalog #: 50051), human recombinant C-terminal FLAG-tag HDAC 2 (BPS Bioscience, Catalog #: 50052), human recombinant C-terminal His-tag HDAC 3/NcoR 2 (BPS Bioscience, Catalog #: 50003) or human recombinant C-terminal FLAG-tag HDAC 6 (BPS Bioscience, Catalog #: 50056) were diluted in incubation buffer (25 mM Tris-HCl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1.0 mM MgCl, 0.01% Triton-X and 1 mg/mL BSA). A total of 40 µL of this dilution was incubated with 10 µL of a two-fold serial dilution of inhibitors in 5% DMSO/incubation buffer and 50 µL of the fluorogenic Boc-Lys(ε-Ac)-AMC (20 µM, Bachem, Germany) at 37 °C. After 90 min incubation 50 µL of the stop solution (25 mM Tris-HCl (pH 8), 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl2, 0.01% Triton-X, 6.0 mg/mL trypsin from porcine pancreas Type IX-S, lyophilized powder, 13,000-20,000 BAEE units/mg protein (Sigma Aldrich) or 2 mM Lys-C peptidase developer and 200 µM vorinostat was added. After incubation at 37 °C for 30 min, the fluorescence intensity was measured on a Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek,

USA) with a gain of 70 and an excitation wavelength of 370 nm and an emission wavelength of 460 nm. GraphPad Prism 5.0 (GraphPad Software, Inc.) was used for the determination of the IC50 of each compound. Nonlinear regression was used for data fitting.

Assessment of acetylated tubulin levels in RPE-1 cells

Human RPE-1 cells were obtained from the American Type Culture Collection (ATCC; Wesel, Germany) and cultured in 96-well plates or T75 flasks (Costar Europe, Badhoevedorp, The Netherlands) at 37 °C under 5% CO_2/95% air in Dulbecco's Modification of Eagle's Medium (DMEM) containing GlutaMAX™ (Gibco® by life Technologies, Bleiswijk, The Netherlands) supplemented with 10% (v/v) heated fetal bovine serum (FBS; Invitrogen, Breda, The Netherlands), 2 mM additional GlutaMAX™ (Gibco® by life Technology, Bleiswijk, The Netherlands), 100 U/ml penicillin (Gibco® by life Technologies, Bleiswijk, The Netherlands) and 100 mg/ml streptomycin (Gibco® by life Technologies, Bleiswijk, The Netherlands). RPE-1 cells were used between passage 5 and 16. RPE-1 cells were treated with different concentrations of the dual warhead HDACi or a partial prodrug form for 18 h, after which the cells were harvested. Tubulin acetylation levels were evaluated with Western blot analysis using an acetyl-tubulin antibody.

Precision-cut lung slices and treatment

Precision-cut lung slices were prepared as described previously (Oenema et al., 2013). Animals were euthanized by subcutaneous injection with ketamine (40 mg/kg, Alfasan, Woerden, The Netherlands) and dexdomitor (0.5 mg/kg, Orion Pharma, Mechelen, Belgium). Following euthanization, the trachea was cannulated, and the animal was ex-sanguinated via the aorta abdominalis. Subsequently, the lungs were inflated through the cannula with a low melting-point agarose solution (1.5% final concentration

(Gerbu Biotechnik GmbH, Wieblingen, Germany) in CaCl2 (0.9 mM), $MgSO_4$ (0.4 mM), KCl (2.7 mM), NaCl (58.2 mM), NaH_2PO_4 (0.6 mM), glucose (8.4 mM), $NaHCO_3$ (13 mM), HEPES (12.6 mM), sodium pyruvate (0.5 mM), glutamine (1 mM), MEM-amino acids mixture (1:50), and MEM-vitamins mixture (1:100), pH = 7.2).

Following inflation, lungs were placed on ice for 15 min, so that the agarose could solidify for slicing. Next, the lungs were separated into individual lobes. These lobes were used to prepare tissue cores, after which the lobes were sliced at a thickness of 250 µm, which was the same for all further experimental procedures. Slicing was performed in medium composed of $CaCl_2$ (1.8 mM), $MgSO_4$ (0.8 mM), KCl (5.4 mM), NaCl (116.4 mM), NaH_2PO_4 (1.2 mM), glucose (16.7 mM), $NaHCO_3$ (26.1 mM), HEPES (25.2 mM), pH = 7.2, using a tissue slicer (CompresstomeTM VF- 300 microtome, Precisionary Instruments, San Jose CA, USA).

Lung slices were incubated in a humid atmosphere under 5% CO_2/95% air at 37°C. Every 30 min slices were washed (four times in total). PCLS were incubated in DMEM supplemented with sodium pyruvate (1 mM), MEM non-essential amino acid mixture (1:100; Gibco® by Life Technologies), gentamycin (45 µg/ml; Gibco® by Life Technologies), penicillin (100 U/ml), streptomycin (100 µg/ml), and amphotericin B (1.5 µg/ml; Gibco® by Life Technologies). Slices were cultured at 37°C in a humidified atmosphere under 5% CO_2/95% air in 12-well tissue culture plates, using three to four slices per well. Matched slices from the same mouse were treated with TGF-ß (2 ng/ml) for 48h. The slices were then treated with a compound at an appropriate concentration for an appropriate amount of incubation time. Finally, the mRNA expression of α-smooth muscle actin, collagen 1α1, fibronectin, surfactant protein C and HopX was determined following known protocols and compared to an untreated control. Previous work (unpublished) demonstrated that mouse lung slice viability is preserved after 72 h of culturing, as mitochondrial activity did not change.

This indicates that the lung slice is viable for at least 3 days. Our experiments were all performed within 56 h after sacrifice.

Results

The dual HDACi core compound (Cmpd 1) inhibits human recombinant HDAC1, 2 and 3 with a low micromolar potency (Figure 2) as expected from an aminobenzamide type HDACi (entinostat IC50 = 0.4 μM). The partial prodrug form (Cmpd 1 partial prodrug, Figure 1) also inhibits HDAC1, 2 and 3 in the low micromolar range (Figure 3), indicating that masking the HDAC6 warhead has minimal effect on the inhibition of HDAC1/2/3 (as expected from the pharmacophore).

Furthermore, the dual HDACi potently inhibits (human) HDAC6 with an IC50 of 39 nM (Figure 4). Additionally, it increases the acetylation level of tubulin (the direct downstream target of HDAC6) in human RPE-1 cells (Figure 5) in a concentration-dependent manner, showing that the dual HDACi also potently inhibits HDAC6 in human cells.

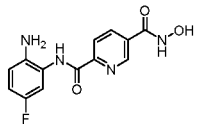
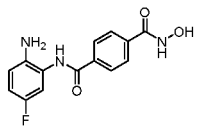
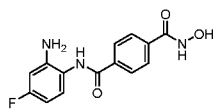
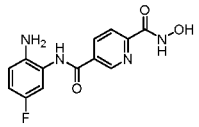
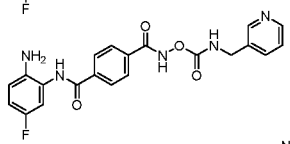
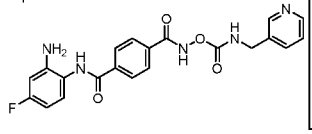
Interestingly, the prodrug form increases tubulin acetylation to a higher degree than the non-masked form (Figure 5), which indicates a slow-release of the HDAC6 warhead over time and a resulting prolonged inhibition of HDAC6.

Finally, a significant decrease in the expression of several fibrotic markers was observed in murine precision cut lung slices exposed to a cocktail of cytokines upon treatment with the dual HDACi (Figure 6).

EXAMPLE 4: Compound solubility, membrane permeability and stability.

This example illustrates three pharmacologically relevant properties (solubility, membrane permeability and stability) of exemplary dual HDACi compounds and their prodrug forms.

Table 1

Structure	*ID*	*Solubility*[1]	*Permeability*[2]	*Stability*[3]
	GR-21-0004	>80	2	M: 240, 27, 70 P: 108, 540, 540
	GR-21-0002	>80*	2	M: 240, 116, 240 P: 155, 540, 540
	GR-22-0002	>80	2	M: 240, 240, 240 P: 540, 540, 540
	GR-21-0003	-	-	-
	GR-22-0006	>80	2	M: 153, 224, 157 P: 299, 540, 285
	GR-22-0008	68	1	M: 220, 155, 224 P: 255, 172, 208

[1] Solubility in an aqueous phosphate buffered solution at pH 7.4 incubated at room temperature for 4 hours (μM).

[2] Parallel artificial membrane permeability values, P_{app} (10^{-6} cm/s) at pH 7.4.

[3] Half-life (min) in microsomes (M) and plasma (P). Values are given in the order: human, mouse and rat.

Solubility and membrane permeability

To determine the kinetic solubility, a pre-dissolved solution in an organic solvent (DMSO) of each of the compounds was prepared. The pre-solution was subsequently diluted into an aqueous phosphate buffered solution at pH 7.4 and incubated at room temperature for 4 h. Samples were centrifuged and supernatants analyzed using LC-UV.

The membrane permeability was assessed with a parallel artificial membrane permeability assay (PAMPA). This assay was first introduced by Kansy et al., (J. Med. Chem. 1998, 41, 7, 1007–1010) has been widely used in the pharmaceutical industry as a high throughput permeability assay to predict oral absorption. It is a relatively fast and easy method to determine the permeability of substances from a donor compartment, through a lipid-infused artificial membrane, into an acceptor compartment. A multi-well microtiter plate is used for the donor, and a membrane/acceptor compartment is placed on top to form a sandwich assembly. At the beginning of the test, the test compound is added to the donor compartment, and the acceptor compartment is compound-free. After an incubation period, the sandwich is separated and the amount of test compound is measured in each compartment. Ketoprofen and verapamil, which are both highly absorbed in the gut (>90%), were included as reference compounds.

Stability in plasma and microsomes

Exemplary test compounds were incubated in duplicate with human, mouse and rat liver microsomes or plasma at 37°C at a final concentration of 1 μM.

Control incubations with reference compounds were included for each experiment to assure the expected enzyme activity levels. Aliquots were taken at different time points (0, 5, 15, 30 and 45 min) and subjected to LC-MS analysis.

The percentage of remaining compound was defined as the ratio of compound peak area at a specific time point to the peak area at time zero, multiplied by 100%. The metabolic stability was evaluated by plotting the natural logarithm of the percentage of compound remaining versus time and performing linear regression. Using this graph, the elimination constant, half-life ($t_{1/2}$) and *in vitro* intrinsic clearance were calculated.

Results

Table 1 herein above summarizes the results obtained.

The majority of compounds had a solubility >80 µM. The solubility of the most soluble reference compound ketoprofen was also >80 µM. For compounds GR-21-0002 and GR-22-0008 additional peaks were observed which may be the result of the compounds' instability under the assay conditions used. A solubility of >80 µM roughly corresponds to a >25 mg/L solubility of the parent compounds and a solubility of >30 mg/L of the prodrugs.

The average permeability values P_{app} (10^{-6}cm/s) at pH 7.4 were between 1 and 6 for all of test compounds. This was similar to ketoprofen (P_{app} = 1). The prodrugs have a primary aromatic amine with a predicted pKa of around 5, whereas the parent compounds have both a primary aromatic amine and an aromatic hydroxamic acid, the latter with a pKa of around 9.

This means that at pH 7.4 all of the test compounds will most likely not be ionized. Coupled to the relatively low P_{app} values, this indicates that there may be significant retention of the HDACi compounds in the artificial membrane. Conceivably, at lower pH values the ionization of the primary amine will result in a decrease in the membrane affinity and consequently a higher permeability. In agreement with this hypothesis, verapamil, the other reference compound with a high gut absorption, has a high Papp of 63 but is cationic at pH 7.4 due to the presence of a tertiary aliphatic amine with a pKa of around 10 (data not shown).

Figure 7 shows the half-life of the compounds in plasma and microsomes of human, mouse and rat. Marked differences in the stability of the compounds are observed, both when comparing compounds and when comparing species. In all, a relatively low human plasma and human microsome stability is preferred to reduce systemic exposure after pulmonary administration.

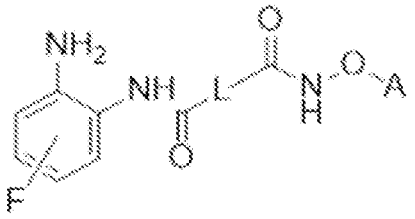
EXAMPLE 5: Fibrotic marker gene expression

The exemplary compounds of Example 4 were also evaluated with respect to their capacity to influence gene expression of several fibrotic markers. Mouse precision cut lung slices were prepared from naïve C57Bl/6 mice and exposed to a fibrosis cocktail (TGF-ß1 5 ng/ml, 10 ng/ml TNF-α, 30 ng/ml PDGF-AB, 5 µM LPA) for 48 h. Slices were treated with test compounds at 10 µM for 48 h. All compounds were assessed in one assay and 6 replicates were included, resulting in 6 animals/experiment. Read-out was gene expression using α-smooth muscle actin (α-SMA) as marker of myofibroblast differentiation and collagen 1α1 (COL1A1) and fibronectin (FN) as markers of extracellular matrix deposition.

Treatment with the fibrosis cocktail induced a significant increase in the gene expression of α-SMA, COL1A1 and FN, with a 2.4-fold, 3.7-fold and 4-fold increase, respectively (Figure 8). The test compounds did not significantly inhibit α-SMA expression nor FN expression at 10 μM in combination with the fibrosis cocktail (Figure 8). Yet, compounds GR-22-0006 and GR-21-0004 clearly reduced the expression of COL1A1 (Figure 8).

Claims

1. A compound of the formula A

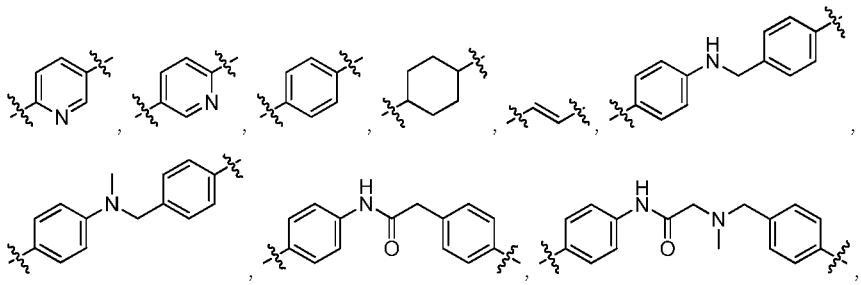


Formula A

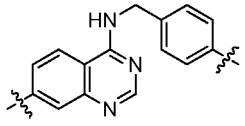
or a pharmaceutically acceptable salt, hydrate, or solvate thereof,

wherein

L is a linker group selected from the group consisting of



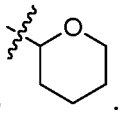
and



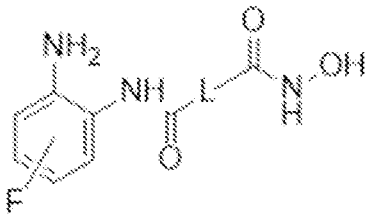
A is H or an *in vivo* hydrolysable group of the formula-C(O)Y, wherein Y is NR_1R_2, $OCR_1R_2R_3$ or $CR_1R_2R_3$,

wherein R_1, R_2 and R_3 are independently selected from H; a cyclic or acyclic, substituted or unsubstituted aliphatic group; a cyclic or acyclic, substituted or unsubstituted heteroaliphatic

group; a substituted or unsubstituted aryl, or a substituted or unsubstituted heteroaryl group; or wherein if Y is NR_1R_2, R_1 and R_2 together form a substituted or unsubstituted (hetero)cycloalkyl group,

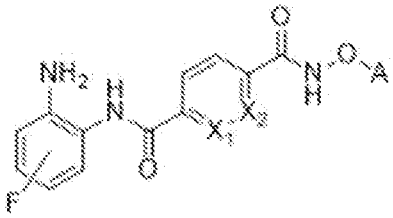
or wherein A is  .

2. Compound according to claim 1 of the formula B



Formula B

3. A compound according to claim 1 of the formula I



Formula I

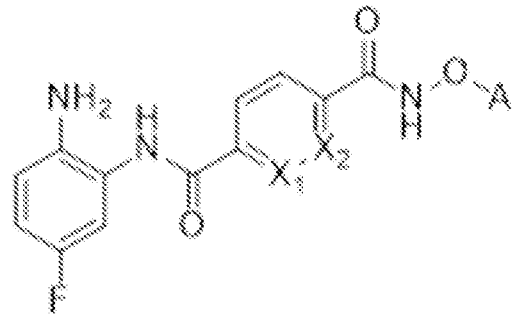
wherein

X_1 and X_2 are independently selected from C and N, provided that at least one of X_1 and X_2 is C; and

A is as defined in claim 1,

or a pharmaceutically acceptable salt, hydrate, or solvate thereof.

4. Compound according to claim 3, of the formula II

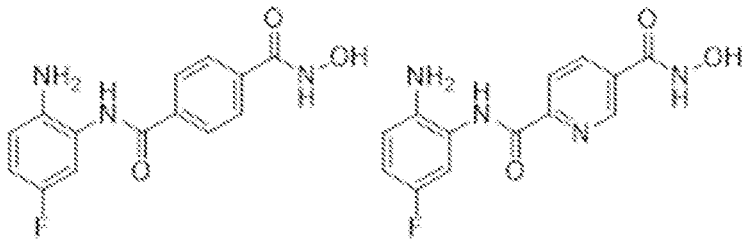


Formula II

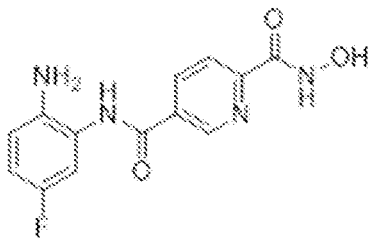
5. Compound according to claim 3 or 4, wherein X_1 and X_2 are C.

6. Compound according to any one of claims 3 to 5, wherein A is H.

7. Compound according to claim 6, selected from the group consisting of



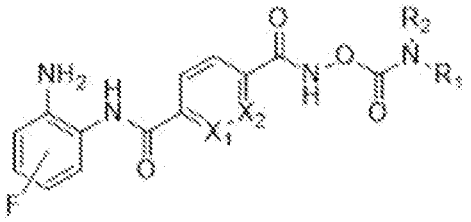
and



8. Compound according to any one of the preceding claims, wherein A is -C(O)Y, wherein Y is NR_1R_2, $OCR_1R_2R_3$ or $CR_1R_2R_3$, and wherein R_1, R_2 and R_3 are independently selected from H; a cyclic or acyclic, substituted or unsubstituted aliphatic group; a cyclic or acyclic, substituted or unsubstituted heteroaliphatic group; a substituted or unsubstituted aryl, or a substituted or unsubstituted

heteroaryl group; or wherein if Y is NR_1R_2, R_1 and R_2 together form a substituted or unsubstituted (hetero)cycloalkyl group.

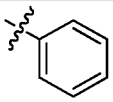
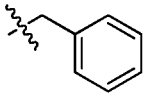
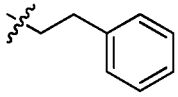
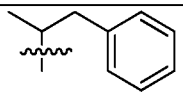
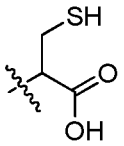
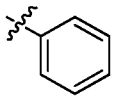
9. Compound according to claim 8, having the formula III



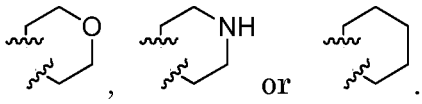
Formula III

10. Compound according to claim 9, wherein R_1 and R_2 are selected from entries 1 to 17 of the following table

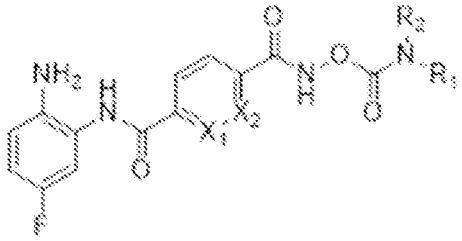
	R_1	R_2
1	H	
2	H	
3	H	
4	H	
5	H	
6	H	
7	H	
8	H	

9	H	
10	H	
11	H	
12	H	
13	H	
14	CH_3	CH_3
15	CH_3	CH_2CH_3
16	CH_2CH_3	CH_2CH_3
17	CH_3	

11. Compound according to claim 9, wherein R_1 and R_2 together form the structure


, or .

12. Compound of claim 10, having the formula IV



Formula IV.

13. Compound according to any one of claims 8-10, wherein R_1 is H and R_2 is $-(CH_2)n$-Y, wherein n is 1-4 and Y is a 5- or 6-membered substituted or unsubstituted (hetero)aryl, preferably an unsubstituted pyridine.

14. A pharmaceutical composition comprising a compound according to any one of the preceding claims, and a pharmaceutically acceptable carrier, vehicle or diluent.

15. Pharmaceutical composition according to claim 14, wherein the composition is formulated for administration to the upper and lower airways, preferably for administration by inhalation.

16. A dry-powder inhaler comprising a pharmaceutical composition according to claim 15.

17. The use of a compound according to any one of claims 1-13 as an inhibitor of HDAC, preferably targeting HDAC1, HDAC2, HDAC3 and HDAC6.

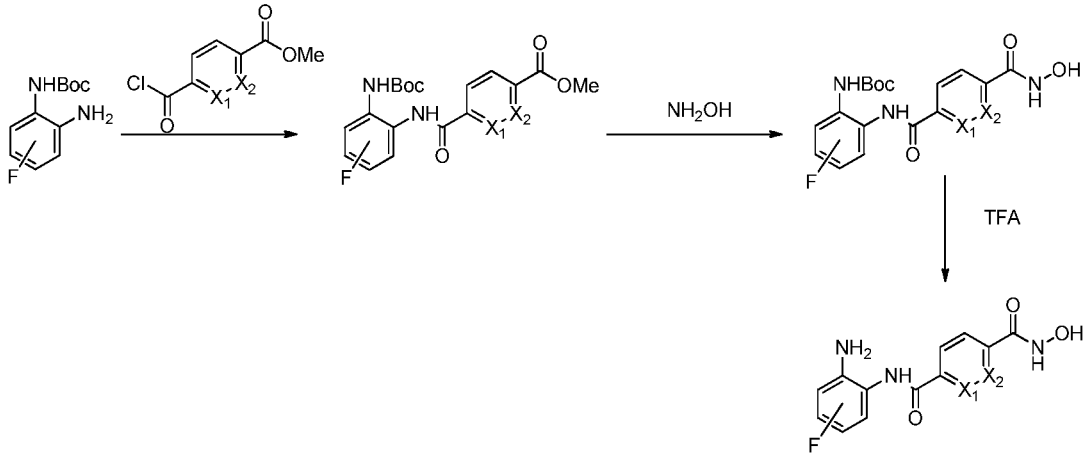
18. A compound according to any one of claims 1-13 for use in a method of treating, alleviating, and/or preventing a pulmonary disease, preferably an airway inflammatory disease or lung cancer, such as COPD,

asthma, idiopathic pulmonary fibrosis, non-small-cell lung cancer or small-cell lung cancer.

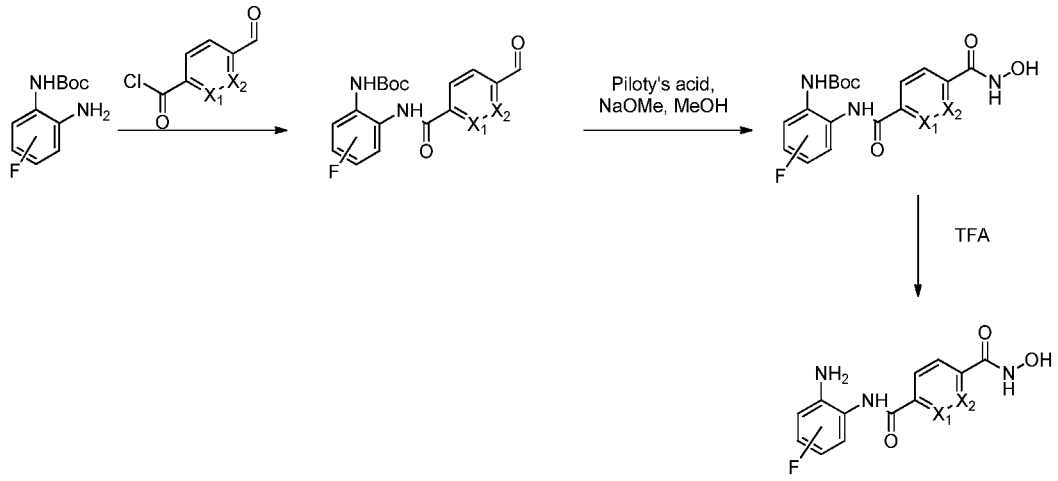
19. A method of treating, alleviating, and/or preventing a pulmonary disease, comprising administering to a subject in need thereof a pharmaceutically effective dose of a pharmaceutical composition according to claim 14 or 15.

20. Method according to claim 19, wherein said pulmonary disease is an airway inflammatory disease or lung cancer, preferably selected from the group consisting of COPD, asthma, idiopathic pulmonary fibrosis, non-small-cell lung cancer and small-cell lung cancer.

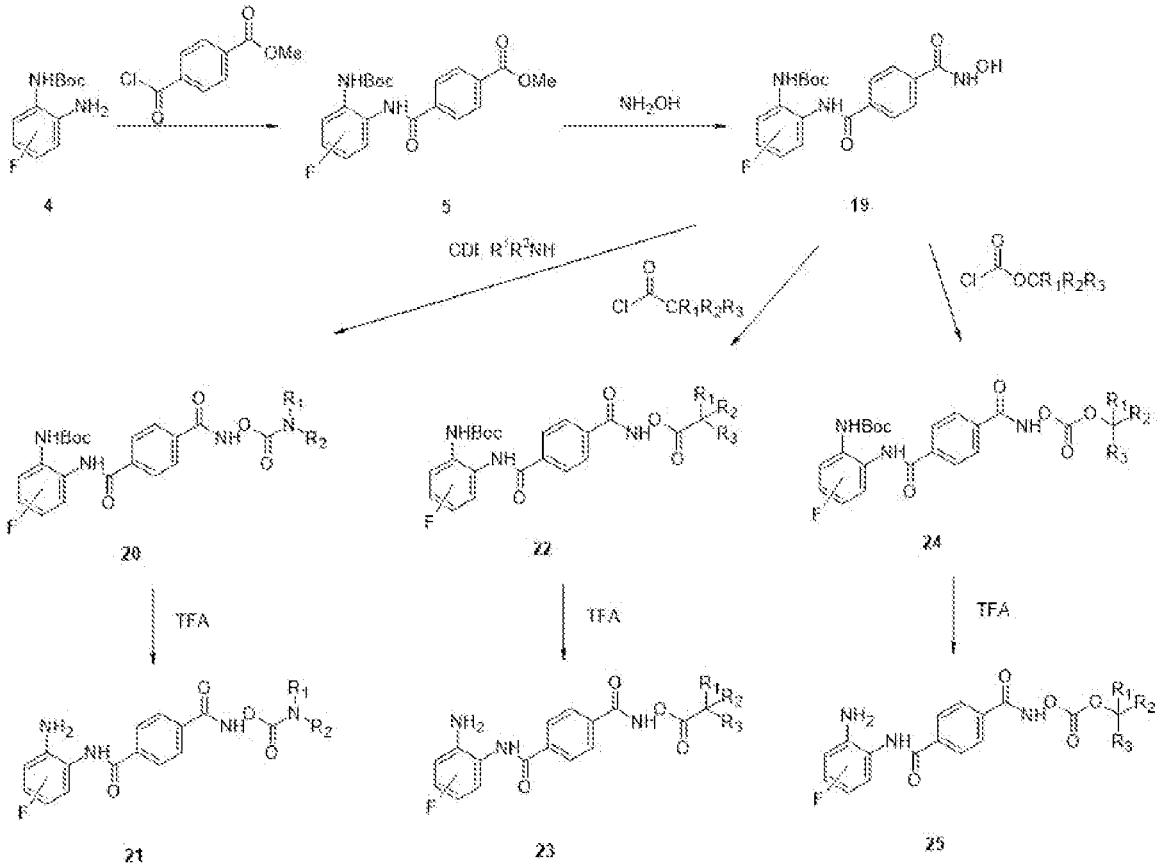
21. A method for providing a compound according to claim 6 or 7, comprising the steps of



or comprising the steps of



22. A method for providing a compound according to any one of claims 8-10, comprising one or more of the following reaction steps of:



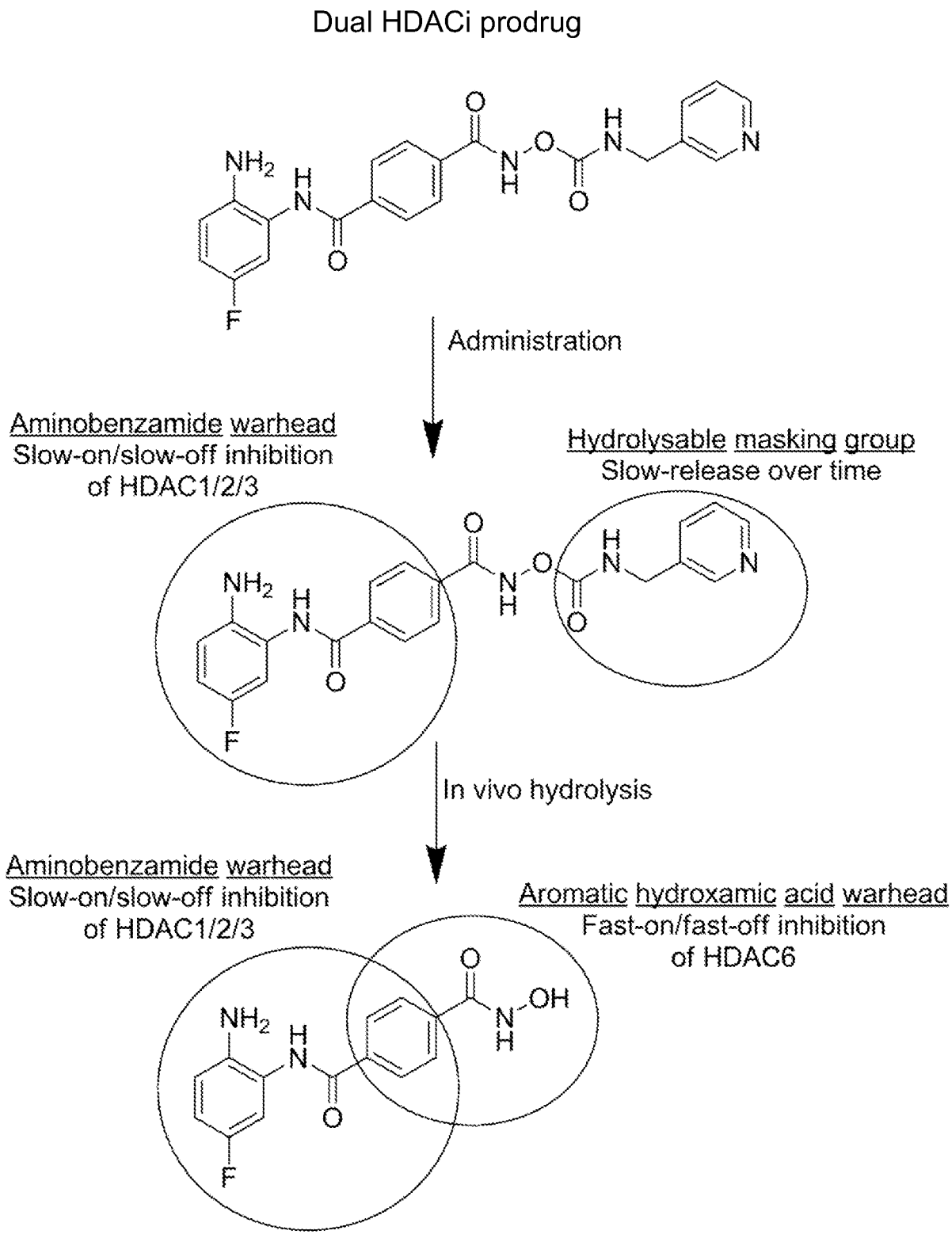

Dual HDACi prodrug
Administration
Aminobenzamide warhead
Slow-on/slow-off inhibition
of HDAC1/2/3
Hydrolysable masking group
Slow-release over time
In vivo hydrolysis
Aminobenzamide warhead
Slow-on/slow-off inhibition
of HDAC1/2/3
Aromatic hydroxamic acid warhead
Fast-on/fast-off inhibition
of HDAC6

Fig. 1

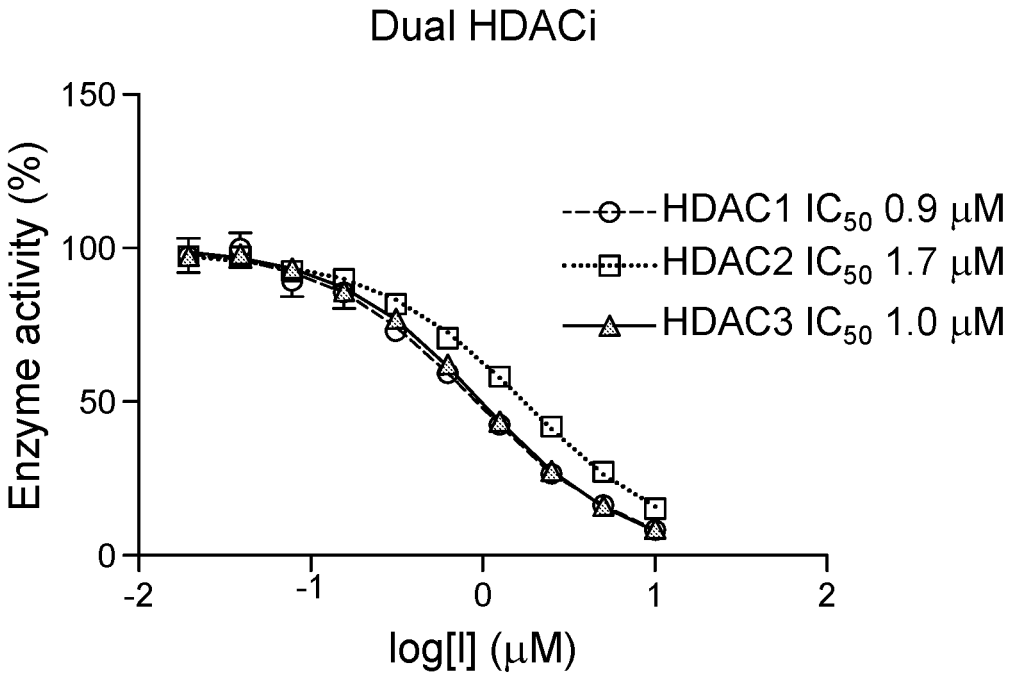

Dual HDACi
Enzyme activity (%)
150
100
50
0
-2
-1
0
1
2
log[I] (μM)
HDAC1 IC50 0.9 μM
HDAC2 IC50 1.7 μM
HDAC3 IC50 1.0 μM

Fig. 2

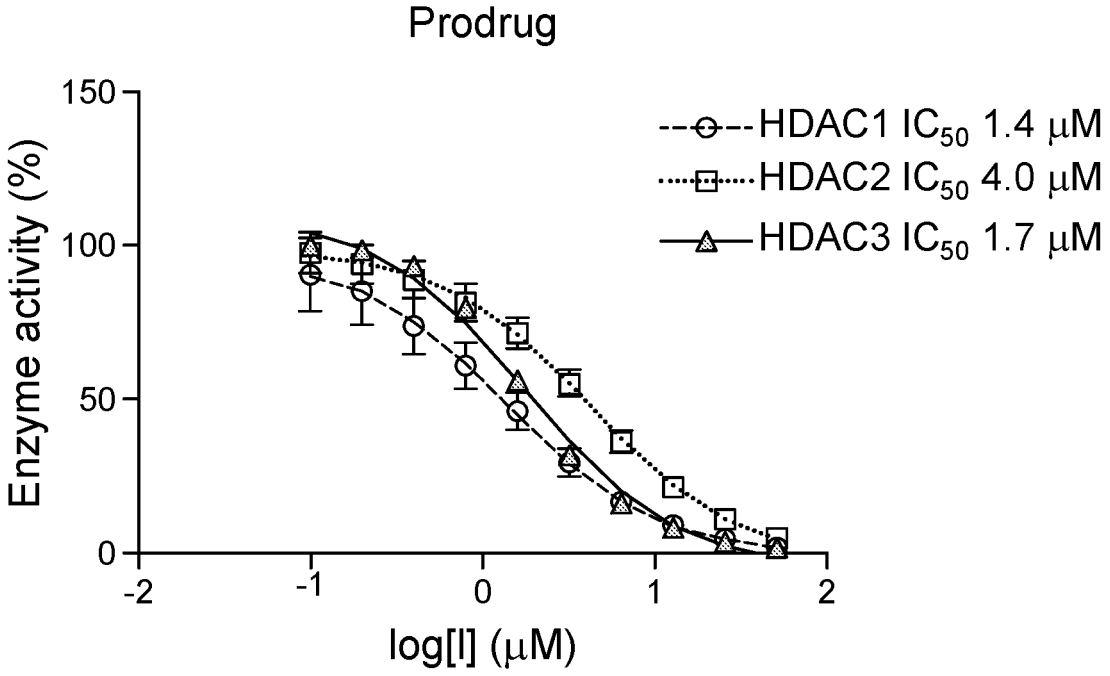

Prodrug
Enzyme activity (%)
150
100
50
0
-2
-1
0
1
2
log[I] (μM)
HDAC1 IC50 1.4 μM
HDAC2 IC50 4.0 μM
HDAC3 IC50 1.7 μM

Fig. 3

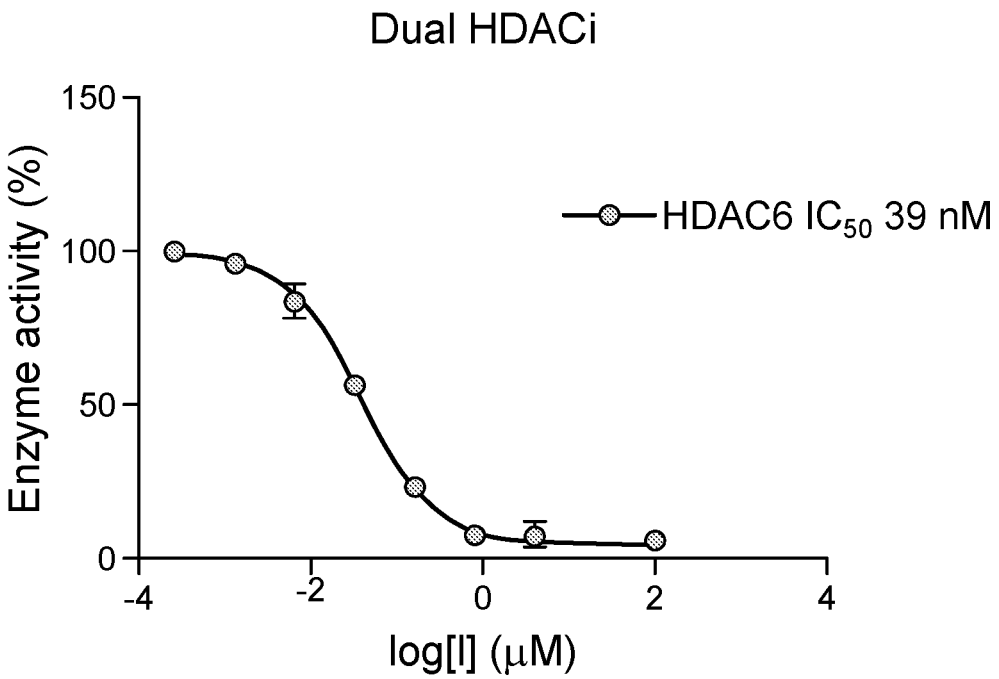

Dual HDACi
Enzyme activity (%)
150
100
50
0
-4
-2
0
2
4
log[I] (μM)
HDAC6 IC50 39 nM

Fig. 4

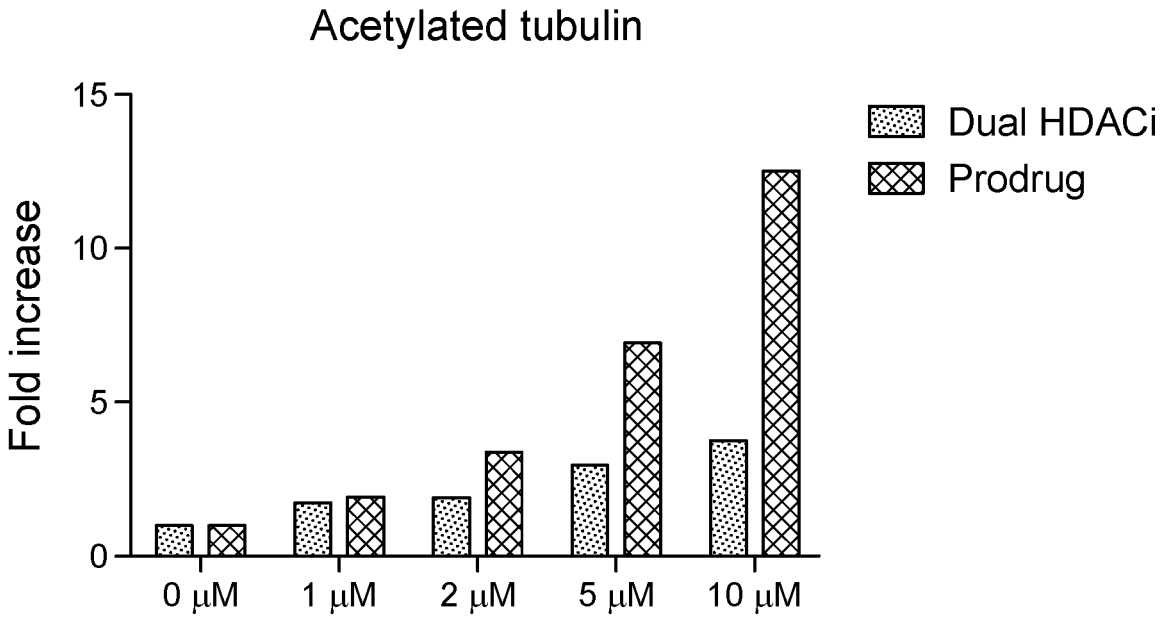

Acetylated tubulin
Fold increase
15
10
5
0
0 μM
1 μM
2 μM
5 μM
10 μM
Dual HDACi
Prodrug

Fig. 5

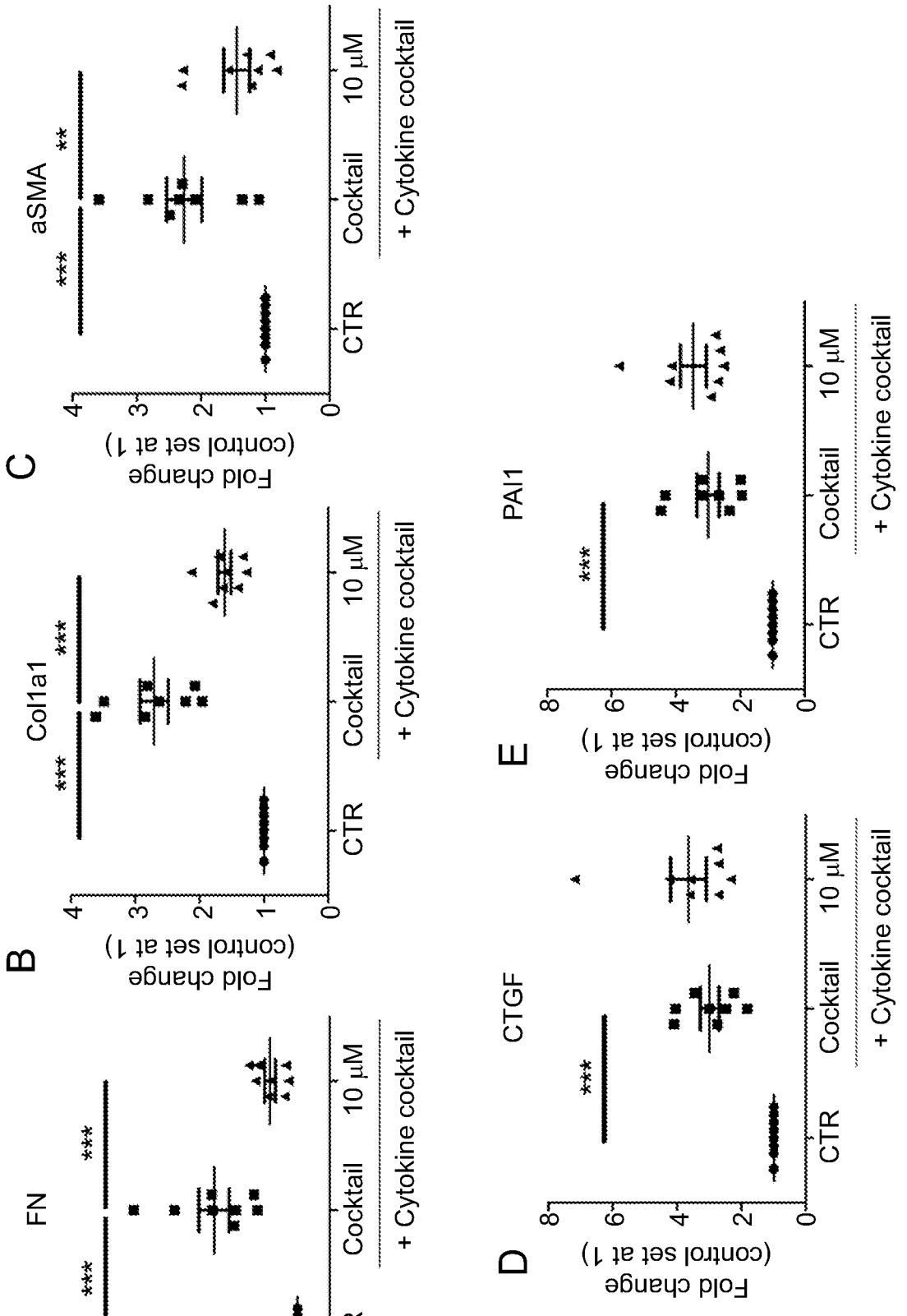

A
FN
B
Col1a1
C
aSMA
D
CTGF
E
PAI1
Fold change (control set at 1)
***
**
CTR
Cocktail 10 µM
+ Cytokine cocktail

Fig. 6

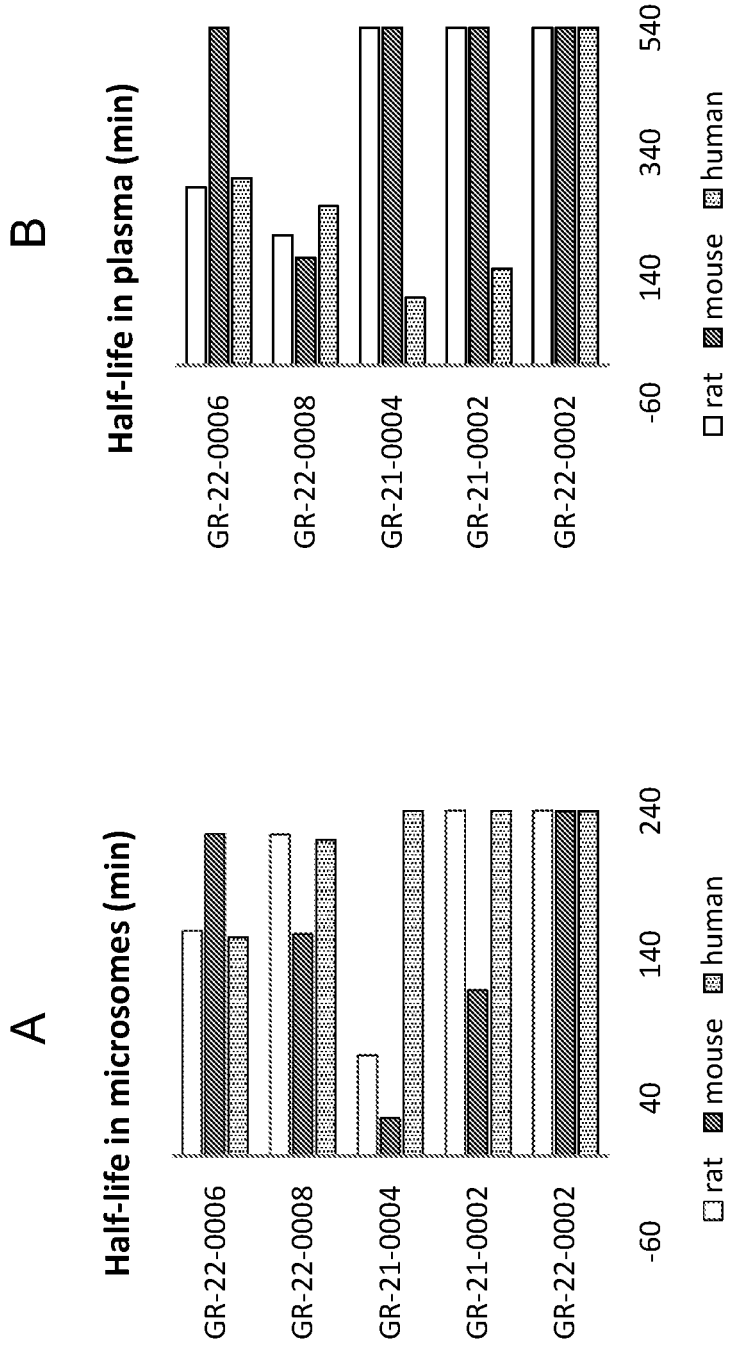

A
Half-life in microsomes (min)
GR-22-0006
GR-22-0008
GR-21-0004
GR-21-0002
GR-22-0002
-60
40
140
240
rat
mouse
human
B
Half-life in plasma (min)
GR-22-0006
GR-22-0008
GR-21-0004
GR-21-0002
GR-22-0002
-60
140
340
540
rat
mouse
human

Fig. 7

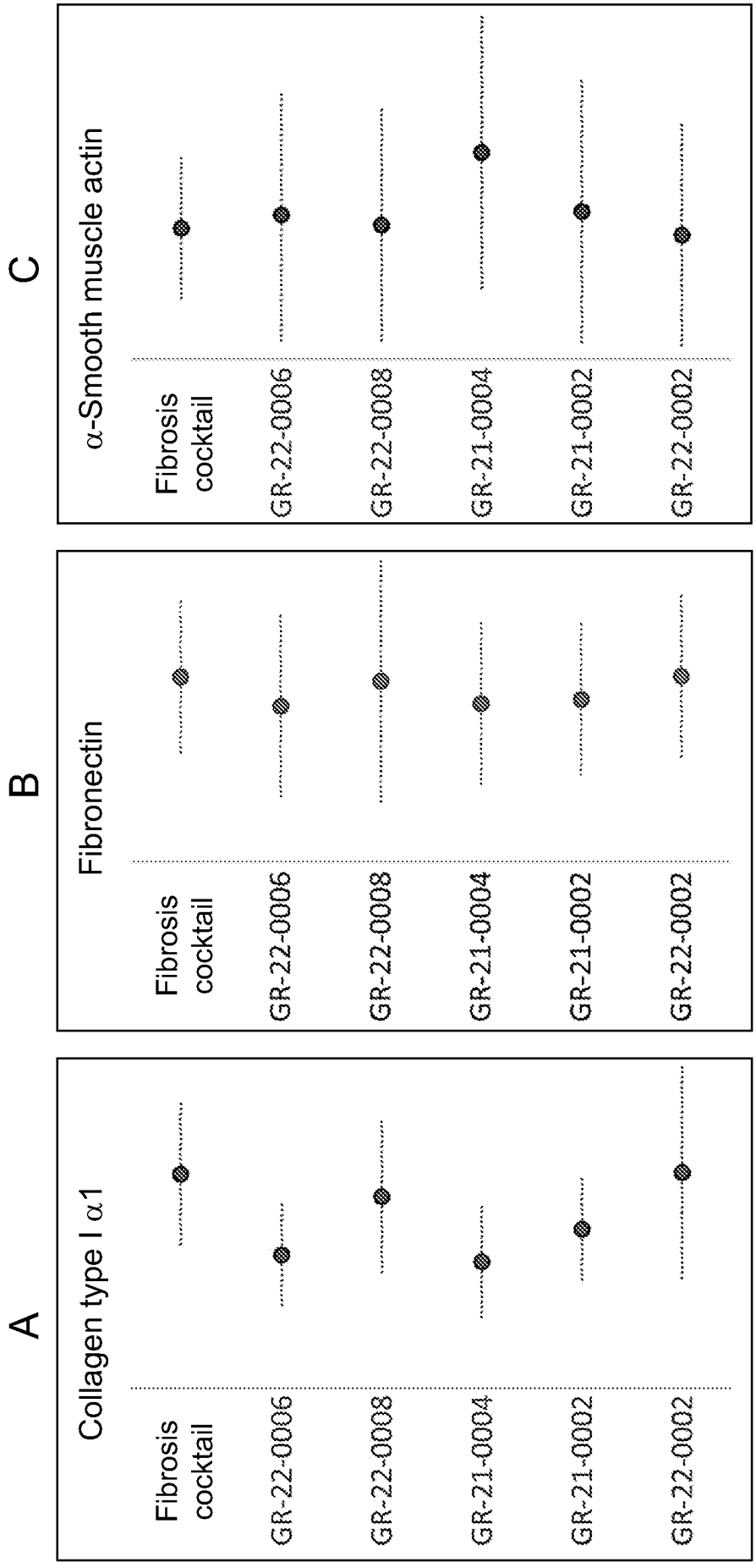

A
Collagen type I α1
Fibrosis cocktail
GR-22-0006
GR-22-0008
GR-21-0004
GR-21-0002
GR-22-0002
B
Fibronectin
Fibrosis cocktail
GR-22-0006
GR-22-0008
GR-21-0004
GR-21-0002
GR-22-0002
C
α-Smooth muscle actin
Fibrosis cocktail
GR-22-0006
GR-22-0008
GR-21-0004
GR-21-0002
GR-22-0002

Fig. 8

INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2022/050429

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07D213/75 C07C259/06 C07C259/10 C07D239/94 A61K31/16
A61K31/44 A61K31/517 A61P25/00 A61P29/00 A61P35/00
A61P9/00

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)
C07D C07C A61P

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2011/084991 A2 (HARVARD COLLEGE [US]; DANA FARBER CANCER INST INC [US] ET AL.) 14 July 2011 (2011-07-14) paragraphs: [0007]; [0147];[0148];[0217];[0218]; claims; examples	1-22
Y	WO 2004/069803 A2 (HOFFMANN LA ROCHE [CH]) 19 August 2004 (2004-08-19) page 24 - page 27; claims; examples	1-22
Y	WO 2006/097460 A1 (MENARINI INT OPERATIONS LU SA [LU]; ROSSI CRISTINA [IT] ET AL.) 21 September 2006 (2006-09-21) claims; examples	1-22

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[X] Further documents are listed in the continuation of Box C.

[X] 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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 October 2022	26/10/2022

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Gavriliu, Daniela

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INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2022/050429

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2005/097747 A1 (ATON PHARMA INC [US]; MILLER THOMAS A [US] ET AL.) 20 October 2005 (2005-10-20) claims; examples	1-22

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/NL2022/050429

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		JP 2008533091 A	21-08-2008
		KR 20070112240 A	22-11-2007
		MA 29389 B1	01-04-2008
		TW 200724529 A	01-07-2007
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		EP 1745022 A1	24-01-2007
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