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(71) Applicants: **INSERM (INSTITUT NATIONAL DE LA SANTE ET DE LA RECHERCHE MEDICALE)** [FR/FR]; 101, rue de Tolbiac, 75013 Paris (FR). **UNIVERSITÉ PARIS DIDEROT– PARIS 7** [FR/FR]; 5 rue Thomas Mann, 75013 Paris (FR). **ASSISTANCE PUBLIQUE-HÔPITAUX DE PARIS** [FR/FR]; 3 Avenue Victoria, 75004 Paris (FR).

(72) Inventors: **DUMAZ, Nicolas**; HOPITAL SAINT LOUIS - INSERM U976, 1 avenue Claude Vellefaux, Pavillon Bazin, 75475 Paris 10 (FR). **BENSUSSAN, Armand**; HOPITAL SAINT LOUIS - INSERM U976, 1 avenue Claude Vellefaux, Pavillon Bazin, 75475 Paris 10 (FR). **DELYON, Julie**; HOPITAL SAINT LOUIS - INSERM U976, 1 avenue Claude Vellefaux, Pavillon Bazin, 75475 Paris 10 (FR). **MOURAH, Samia**; HOPITAL SAINT LOUIS - INSERM U976, 1 avenue Claude Vellefaux, Pavillon Bazin, 75475 Paris 10 (FR). **LEBBE, Celeste**; HOPITAL SAINT LOUIS - INSERM U976, 1 avenue Claude Vellefaux, Pavillon Bazin, 75475 Paris 10 (FR).

(74) Agent: **COLLIN, Mathieu**; INSERM TRANSFERT, 7 rue Watt, 75013 Paris (FR).

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(54) Title: METHODS AND PHARMACEUTICAL COMPOSITIONS FOR THE TREATMENT OF BRAF INHIBITOR RESISTANT MELANOMAS

(57) Abstract: The present invention relates to methods and pharmaceutical compositions for the treatment of BRAF inhibitor resistant melanomas. In particular, the present invention relates to a method of treating a melanoma resistant to BRAF inhibitors in a subject in need thereof comprising administering to the subject a therapeutically effective amount of a PDE4 inhibitor.

METHODS AND PHARMACEUTICAL COMPOSITIONS FOR THE TREATMENT OF BRAF INHIBITOR RESISTANT MELANOMAS

FIELD OF THE INVENTION:

The present invention relates to methods and pharmaceutical compositions for the treatment of BRAF inhibitor resistant melanomas.

BACKGROUND OF THE INVENTION:

Melanoma is the most aggressive skin cancer. It often induces metastasis and its incidence is rapidly growing and continues to rise alarmingly. Surgery is curative in nearly all cases before metastatic stage, but when metastasis appears, surgery, radiotherapy and conventional chemotherapy have little curative effects and patient survival is usually short. Molecular alterations are frequent in melanomas. Activating mutations in the serine/threonine kinase BRAF and in particular the BRAFV600E mutation occurs in about 50% of melanomas. This BRAF mutation induces activation of the MAPK pathway, which is involved in the tumoral development. Therefore several promising new therapies have been developed essentially based on targeted chemotherapy using inhibitors of MAPK pathway and in particular specific anti-BRAFV600E inhibitors: Vemurafenib (PLX4032) and Dabrafenib. Preclinical studies indicate that Vemurafenib and Dabrafenib block the mutated BRAF protein, inducing cell growth arrest and cell death in tumors carrying this mutation. Clinical trials of Vemurafenib and Dabrafenib have shown therapeutic effect in more than 50% of patients with BRAFV600E positive metastatic melanomas. However, unfortunately, in most patients melanoma cells outbreak and progress again once resistance to anti-BRAFV600E inhibitors is acquired. Therefore, identification and characterization of unknown pathways mediating resistance to BRAF inhibitors are essential for the rational design of targeted strategies to prevent and overcome said resistance across this entire population of patients.

SUMMARY OF THE INVENTION:

The present invention relates to methods and pharmaceutical compositions for the treatment of BRAF inhibitor resistant melanomas. In particular, the present invention is defined by the claims.

DETAILED DESCRIPTION OF THE INVENTION:

The inventors focused on the role of phosphodiesterases (PDE) as a potent therapeutic target in melanoma. Phosphodiesterases are enzymes, which modulate the cAMP pathway that plays a major role in melanocyte differentiation. The inventors have previously shown that in RAS-mutated melanoma (20% of melanoma), the overexpression of PDE4 enzymes was critical for the MAPK activation by oncogenic RAS, and that PDE4 inhibition induced cell death in melanoma cells (Marquette et al. Nat Struct Mol Biol 2011). The inventors have shown that PDE4D is overexpressed in melanoma tumours and that inhibiting PDE4D inhibited melanoma proliferation in clonogenic assays and in melanoma grown in 3D as spheroids. Moreover, the inventors demonstrated that PDE4 inhibitors inhibited the proliferation of melanoma cell lines resistant to BRAF inhibitors used to treat BRAF-mutated melanoma. The results demonstrate that PDE4 inhibitors could be used to treat melanoma resistant to BRAF inhibitors.

Accordingly, an object of the present invention relates to a method of treating a melanoma resistant to BRAF inhibitors in a subject in need thereof comprising administering to the subject a therapeutically effective amount of a PDE4 inhibitor.

As used herein, "melanoma" refers to a condition characterized by the growth of a tumor arising from the melanocytic system of the skin and other organs. Most melanocytes occur in the skin, but are also found in the meninges, digestive tract, lymph nodes and eyes. When melanoma occurs in the skin, it is referred to as cutaneous melanoma. Melanoma can also occur in the eyes and is called ocular or intraocular melanoma. Melanoma occurs rarely in the meninges, the digestive tract, lymph nodes or other areas where melanocytes are found. 40-60 % of melanomas carry an activating mutation in the gene encoding the serine-threonine protein kinase B-RAF (BRAF). Among the BRAF mutations observed in melanoma, over 90 % are at codon 600, and among these, over 90 % are a single nucleotide mutation resulting in substitution of glutamic acid for valine (BRAFV600E).

As used herein, the term "resistant" refers to the repeated outbreak of melanoma, or a progression of the melanoma independently of whether the disease was cured before said outbreak or progression.

As used herein, the term "BRAF inhibitor" refers to an agent that is capable of inhibiting BRAF kinase or mutated BRAF kinase activity (one or more mutated forms of

serine-threonine protein kinase B-RAF (BRAF)) (e.g. BRAFV600E). Accordingly, the term "BRAF inhibitors" encompasses within its scope a compound that is capable of inhibiting BRAF or its mutated form; or a compound that is capable of inhibiting V600 mutated form of BRAF. Examples of BRAF inhibitors include but are not limited to BAY43-9006 (sorafenib, Bayer), vemurafenib (PLX4032, Plexxikon; RG7204, RO5185426, Hofmann-LaRoche), GDC-0879 (GlaxoSmithKline), dabrafenib (GSK21 18436, GlaxoSmithKline), PLX4720 (Hofmann-LaRoche), BMS-908662 (XL281 , Bristol-Myers Squibb), LGX818 (Novartis), PLX3603 (RO5212054, Hofmann-LaRoche), ARQ-736 (ArQule), DP-4978 (Deciphera) or RAF265 (Novartis).

As used herein, the term "treatment" or "treat" refer to both prophylactic or preventive treatment as well as curative or disease modifying treatment, including treatment of patient at risk of contracting the disease or suspected to have contracted the disease as well as patients who are ill or have been diagnosed as suffering from a disease or medical condition, and includes suppression of clinical relapse. The treatment may be administered to a subject having a medical disorder or who ultimately may acquire the disorder, in order to prevent, cure, delay the onset of, reduce the severity of, or ameliorate one or more symptoms of a disorder or recurring disorder, or in order to prolong the survival of a subject beyond that expected in the absence of such treatment. By "therapeutic regimen" is meant the pattern of treatment of an illness, e.g., the pattern of dosing used during therapy. A therapeutic regimen may include an induction regimen and a maintenance regimen. The phrase "induction regimen" or "induction period" refers to a therapeutic regimen (or the portion of a therapeutic regimen) that is used for the initial treatment of a disease. The general goal of an induction regimen is to provide a high level of drug to a patient during the initial period of a treatment regimen. An induction regimen may employ (in part or in whole) a "loading regimen", which may include administering a greater dose of the drug than a physician would employ during a maintenance regimen, administering a drug more frequently than a physician would administer the drug during a maintenance regimen, or both. The phrase "maintenance regimen" or "maintenance period" refers to a therapeutic regimen (or the portion of a therapeutic regimen) that is used for the maintenance of a patient during treatment of an illness, e.g., to keep the patient in remission for long periods of time (months or years). A maintenance regimen may employ continuous therapy (e.g., administering a drug at a regular intervals, e.g., weekly, monthly, yearly, etc.) or intermittent therapy (e.g., interrupted

treatment, intermittent treatment, treatment at relapse, or treatment upon achievement of a particular predetermined criteria [e.g., disease manifestation, etc.]).

As used herein, the term "PDE4 inhibitor" has its general meaning in the art and refers to any compound which inhibits selectively the type 4 phosphodiesterase. In some embodiments, the PDE4 inhibitor of the present invention when compared to other known types of phosphodiesterases, e.g. type 1, 2, 5 etc., whereby the compound has a lower IC50 for the type 4 phosphodiesterase by a factor of 10 compared to the IC50 for the inhibition of other known phosphodiesterases, e.g. type 1, 2, 5, etc. PDE4 inhibitors are well known in the art (see e.g. Peter Norman PDE4 inhibitors 1998 Journal: Expert Opinion on Therapeutic PatentsVolume 8, Issue 7, July 1998, pages 771-784; Peter Norman PDE4 inhibitors 1999 Journal: Expert Opinion on Therapeutic PatentsVolume 9, Issue 8, August 1999, pages 1101-1118; Novel 4-aminopyrazolo[3,4-b]pyridine PDE4 inhibitors Journal: Expert Opinion on Therapeutic PatentsVolume 15, Issue 1, January 2005, pages 111-114; Joshua O Odingo Inhibitors of PDE4: a review of recent patent literature Journal: Expert Opinion on Therapeutic PatentsVolume 15, Issue 7, July 2005, pages 773-787; Lluís Pagès, Amadeu Gavaldà & Martin D Lehner PDE4 inhibitors: a review of current developments (2005 – 2009) Journal: Expert Opinion on Therapeutic Patents Volume 19, Issue 11, November 2009, pages 1501-1519; Peter Norman PDE4 inhibitors 2001. Patent and literature activity 2000 - September 2001 Journal: Expert Opinion on Therapeutic PatentsVolume 12, Issue 1, January 2002, pages 93-112; Amadeu Gavaldà & Richard S Roberts Phosphodiesterase-4 inhibitors: a review of current developments (2010 – 2012) Journal: Expert Opinion on Therapeutic PatentsVolume 23, Issue 8, August 2013, pages 997-1016)

Examples of PDE4 inhibitors are disclosed in the following patent application publications: EP2070913; EP2196465; EP2226323; EP2380890; EP2394998; US20090036518; US20090130076; US20090131530; US20090197871; US20090258905; US20100081646; US20100159034; US20100227853; US20110065691; US20110275622; US20110275623; US20120178708; US20140187555; US20140275553; US20140301999; US8324394; US8791267; US8865723; WO2006025920; WO2009003669; WO2009037302; WO2009067600; WO2009077068; WO2009089027; WO2009094528; WO2009095773; WO2009098320; WO2009100166; WO2009100167; WO2009100169; WO2009100170; WO2009106419; WO2009111676; WO2009115874; WO2009144494; WO2009147476; WO2010003084;WO2010004319; WO2010027975; WO2010028005; WO2010029299;

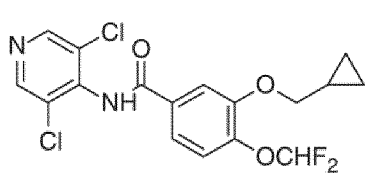
WO2010035745; WO2010046791; WO2010055083; WO2010059836; WO2010059838; WO2010069322; WO2010069504; WO2010076564; WO2010084402; WO2010089107; WO2010097172; WO2010106494; WO2010106495; WO2010130224; WO2010144416; WO2010147922; WO2011018510; WO2011073231; WO2011092547; WO2011114103; WO2011134468; WO2011136192; WO2011160632; WO2012016845; WO2012016889; WO2012040258; WO2012083153; WO2012097116; WO2012133492; and WO2012168226.

In some embodiments, the PDE4 inhibitor of the present invention is selected from the group consisting of:

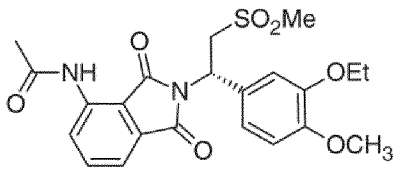
- N-(3,5-dichloropyrid-4-yl)-3-cyclopentyloxy-4-methoxybenzamide [INN: PICLAMILAST] and its salts; the preparation of this compound and its pharmaceutically acceptable salts as well as their use as PDE4 inhibitors is disclosed in the international patent application WO92/12961
- 3-[3-(cyclopentyloxy)-4-methoxybenzyl]-6-(ethylamino)-8-isopropyl-3H-purine [Research Code: V-11294A]; the preparation of this compound and its pharmaceutically acceptable salts as well as their use as PDE4 inhibitors is disclosed in the international patent application WO95/00516
- N-[9-methyl-4-oxo-1-phenyl-3,4,6,7-tetrahydropyrrolo[3,2,1-jk][1,4]benzo-diazepin-3(R)-yl]pyridine-4-carboxamide [Research Code: CI-1018]; the preparation of this compound and its pharmaceutically acceptable salts as well as their use as PDE4 inhibitors is disclosed in the international patent application WO96/11690.
- 3,7-dihydro-3-(4-chlorophenyl)-1-propyl-1H-purine-2,6-dione [INN AROFYLLINE]; the preparation of this compound and its pharmaceutically acceptable salts as well as their use as PDE4 inhibitors is disclosed in the European patent application EPO435811.
- N-(3,5-dichloro-4-pyridinyl)-2-[1-(4-fluorobenzyl)-5-hydroxy-1H-indol-3-yl]-2-oxoacetamide [Research Code: AWD-12-281]; the preparation of this compound and its pharmaceutically acceptable salts as well as their use as PDE4 inhibitors is disclosed in the international patent application WO98/09946
- N-(3,5-dichloropyridin-4-yl)-2-[5-fluoro-1-(4-fluorobenzyl)-1H-indol-3-yl]-2-oxoacetamide [Research Code: AWD-12-343]; the preparation of this compound and its pharmaceutically acceptable salts as well as their use as PDE4 inhibitors is disclosed in the international patent application WO98/09946.

- Tetrahydro-5-[4-methoxy-3-[(1S,2S,4R)-2-norbornyloxy]phenyl]-2(1H)-pyrimidone [INN: ATIZO-RAM]; the preparation of this compound and its pharmaceutically acceptable salts as well as their use as PDE4 inhibitors is disclosed in the European patent application EP0389282.
- β-[3-(cyclopentyloxy)-4-methoxyphenyl]-1,3-dihydro-1,3-dioxo-2H-isoindole-2-propanamide [Research Code: CDC-801]; the preparation of this compound and its pharmaceutically acceptable salts as well as their use as PDE4 inhibitors is disclosed in the international patent application WO97/23457.
- Methanesulfonic acid 2-(2,4-dichlorophenylcarbonyl)-3-ureidobenzo-furan-6-yl ester [INN: LIRIMILAST]; the preparation of this compound and its pharmaceutically acceptable salts as well as their use as PDE4 inhibitors is disclosed in the European patent application EP0731099.
- 3,5-dichloro-4-[8-methoxy-2-(trifluoromethyl)quinolin-5-ylcarbox-amido]pyridine-1-oxide [Research Code: SCH-351591]; the preparation of this compound and its pharmaceutically acceptable salts as well as their use as PDE4 inhibitors is disclosed in the international patent application WO00/26208;
- cis-4-cyano-4-[3-cyclopentyloxy-4-methoxyphenyl]cyclohexane-1-carboxylic acid [INN: Cilomilast], the preparation of this compound and its pharmaceutically acceptable salts as well as their use as PDE4 inhibitors is disclosed in the international patent application WO93/19749
- 3-Cyclopropylmethoxy-4-difluoromethoxy-N-(3,5-dichloropyrid-4-yl)-benzamide [INN: ROFLUMILAST] as well as its N-oxide [3-cyclopropylmethoxy-4-difluoromethoxy-N-(3,5-dichloro-1-oxypyrid-4-yl)benzamide, the preparation of these compounds and their pharmaceutically acceptable salts as well as their use as PDE4 inhibitors is disclosed in the international patent application WO95/01338
- and the pharmaceutically acceptable salts of the above listed compounds.

In some embodiments, the PDE4 inhibitor of the present invention is selected from the group consisting of:



1 Roflumilast



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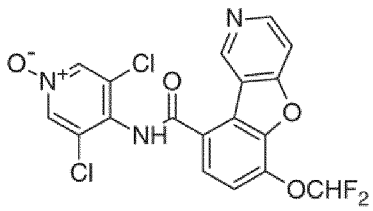
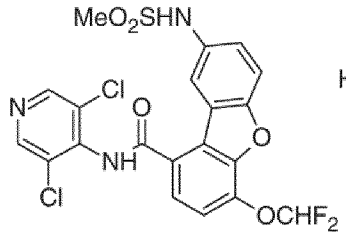
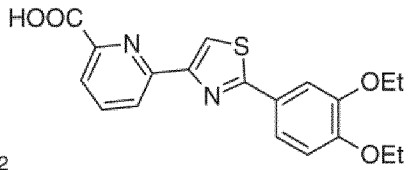


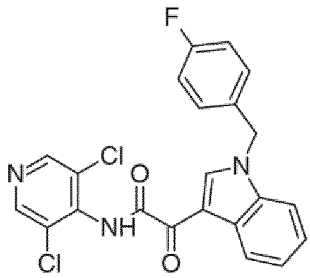
3 Revamilast



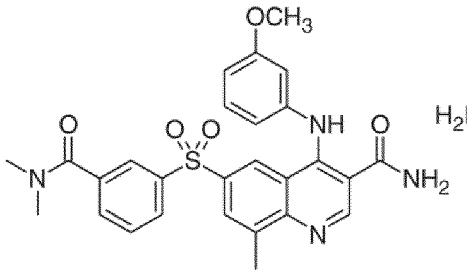
4 Oglemilast



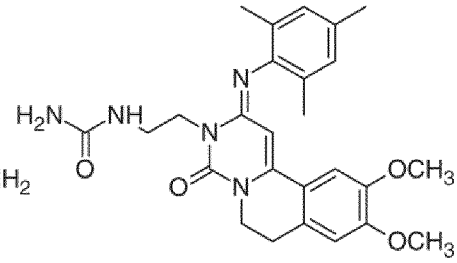
5 Tetomilast



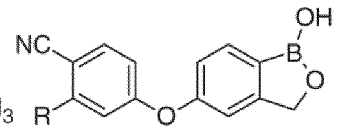
6 Ronomilast



7 GSK256066



8 RPL554



9 R = H, AN2728
10 R = CN, AN2898

In some embodiments, the PDE4 inhibitor is an inhibitor of PDE4 expression. An "inhibitor of expression" refers to a natural or synthetic compound that has a biological effect to inhibit the expression of a gene. In a preferred embodiment of the invention, said inhibitor of gene expression is a siRNA, an antisense oligonucleotide or a ribozyme. For example, anti-sense oligonucleotides, including anti-sense RNA molecules and anti-sense DNA molecules, would act to directly block the translation of PDE4 mRNA by binding thereto and thus preventing protein translation or increasing mRNA degradation, thus decreasing the level of PDE4, and thus activity, in a cell. For example, antisense oligonucleotides of at least about 15 bases and complementary to unique regions of the mRNA transcript sequence encoding PDE4 can be synthesized, e.g., by conventional phosphodiester techniques. Methods for using antisense techniques for specifically inhibiting gene expression of genes whose sequence is known are well known in the art (e.g. see U.S. Pat. Nos. 6,566,135; 6,566,131; 6,365,354; 6,410,323; 6,107,091; 6,046,321; and 5,981,732). Small inhibitory RNAs (siRNAs) can also

function as inhibitors of expression for use in the present invention. PDE4 gene expression can be reduced by contacting a subject or cell with a small double stranded RNA (dsRNA), or a vector or construct causing the production of a small double stranded RNA, such that PDE4 gene expression is specifically inhibited (i.e. RNA interference or RNAi). Antisense oligonucleotides, siRNAs, shRNAs and ribozymes of the invention may be delivered in vivo alone or in association with a vector. In its broadest sense, a "vector" is any vehicle capable of facilitating the transfer of the antisense oligonucleotide, siRNA, shRNA or ribozyme nucleic acid to the cells and typically cells expressing PDE4. Typically, the vector transports the nucleic acid to cells with reduced degradation relative to the extent of degradation that would result in the absence of the vector. In general, the vectors useful in the invention include, but are not limited to, plasmids, phagemids, viruses, other vehicles derived from viral or bacterial sources that have been manipulated by the insertion or incorporation of the antisense oligonucleotide, siRNA, shRNA or ribozyme nucleic acid sequences. Viral vectors are a preferred type of vector and include, but are not limited to nucleic acid sequences from the following viruses: retrovirus, such as moloney murine leukemia virus, harvey murine sarcoma virus, murine mammary tumor virus, and rous sarcoma virus; adenovirus, adeno-associated virus; SV40-type viruses; polyoma viruses; Epstein-Barr viruses; papilloma viruses; herpes virus; vaccinia virus; polio virus; and RNA virus such as a retrovirus. One can readily employ other vectors not named but known to the art.

As used herein, the expression “therapeutically effective amount" meant a sufficient amount of the active ingredient (i.e. the PDE4 inhibitor) for treating or reducing the symptoms at reasonable benefit/risk ratio applicable to any medical treatment. It will be understood that the total daily usage of the compounds and compositions 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 with the active ingredients; 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, typically 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.

Typically the active ingredient of the present invention (e.g. PDE4 inhibitor) is combined with pharmaceutically acceptable excipients, and optionally sustained-release matrices, such as biodegradable polymers, to form pharmaceutical compositions. 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. 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 vegetables 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. In the pharmaceutical compositions of the present invention, the active ingredients of the invention can be administered in a unit administration form, as a mixture with conventional pharmaceutical supports. 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.

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. PDE4D is overexpressed in human melanoma tumors. PDE4D mRNA expression assessed by qRT-PCR in 43 melanoma tumor samples (related to housekeeping mRNA PPIA).

Figure 2. PDE4 inhibition significantly reduces growth of BRAF-mutated melanoma cell line. (A) Clonogenic assay in BRAF-mutated SkMel28 treated with BRAF inhibitor vemurafenib 3μM, PDE4 inhibitor rolipram 10μM + forskolin 1μM, or DMSO as a control. Bars represent mean +-SD. (B) Size of SkMel28 and M74 melanospheres treated with PDE4 inhibitor rolipram 10μM + forskolin 1μM, or DMSO in spheroid culture medium for 2 weeks. Bars represent mean +-SD. (C) Proliferation assay of melanospheres dissociated after 2 weeks of treatment with PDE4 inhibitor rolipram 10 μM + forskolin 1 μM, or DMSO in spheroid culture medium. Luminescence was measured after 96 hours without any additional treatment. Bars represents mean +-SD.

Figure 3. PDE4D inhibition by RNA interference significantly BRAF-mutated melanoma cell lines. (A) Clonogenic assay in WM266.4 silenced for PDE4D (mi1 or mi2PDE4D) or not (miCTL). Bars represent mean +-SD. (B) Proliferation assay of melanospheres dissociated after 2 weeks of culture in spheroid culture medium. Luminescence was measured after 96. Bars represents mean +-SD.

Figure 4. PDE4 inhibition significantly reduces growth of BRAF-mutated melanoma cell lines resistant to BRAF inhibitors. (A) Clonogenic assay in SkMel28 resistant to vemurafenib (BRAF Res. and BRAF RAS) treated with BRAF inhibitor vemurafenib 3μM, vemurafenib 10μM, PDE4 inhibitor rolipram 10μM + forskolin 1μM, or DMSO as a control. Bars represent mean +-SD. (B) Clonogenic assay in SkMel28 resistant to vemurafenib (BRAF Res. and BRAF RAS) treated with BRAF inhibitor vemurafenib 10μM,

PDE4 inhibitor rolipram 10μM + forskolin 1μM, PDE4 inhibitors (rolipram, apremilast, roflumilast 10μM) or DMSO as a control. Bars represent mean +-SD.

EXAMPLE:

Methods

Human tumour samples:

From 2000 to 2005, paraffin-embedded (FFPE) tissue specimens were collected from dermatology department of Saint-Louis hospital, Paris, France for 43 patients with primary melanoma (n=33) and lymph node metastases (n=10). All patients gave informed written consent.

Quantitative RT-PCR:

RNA from paraffin-embedded (FFPE) tissue sections was extracted from five 10μm sections using RNeasy FFPE extraction kit (Qiagen) after xylene treatment according to the manufacturer's protocol. RNA quantity and quality was assessed using the Nanodrop-ND-1000 (Nanodrop Technologies, Wilmington). First-strand cDNA was synthesized using a High-Capacity cDNA Archive Kit (Applied-Biosystems) according to the manufacturer's protocol. Transcript levels were measured by qRT-PCR using Perfect Master Mix-Probe (AnyGenes, France) on LightCycler-480 (Roche). Transcript levels were normalized to the housekeeping PPIA (peptidylprolyl isomerase A) and β-ACTIN transcripts.

Immunohistochemistry:

Six primary melanoma samples (Breslow index<1mm, n=3 and Brelow index>4mm, n=3) were stained for PDE4D using immunohistochemistry to illustrate results obtained from qPCR. Five micrometer-thick sections cut from the formalin-fixed, paraffin-embedded block were dewaxed in xylene and rehydrated through decreasing concentrations of alcohol. Antigen retrieval was carried out in 10mM citrate sodium buffer (pH 6) by heating in a water bath for 15 min at 95°C. Slides were blocked with 2.5% normal horse serum, incubated with anti-PDE4D antibody (1:200, Abcam) overnight at 4°C, then incubated with biotinylated secondary antibody followed by incubation with peroxidase-streptavidin complex (Universal Quick Kit RTU, Vector Labs). Color development was performed with DAB (Vector Labs) and counterstaining with hematoxylin.

Cell culture and reagents:

BRAF-mutated melanoma cell lines human melanoma cell lines SkMel28 and WM266.4 were cultured in respectively RPMI or DMEM supplemented with 10% fetal calf serum (FCS).

Forskolin, PDEs inhibitors (rolipram, apremilast, roflumilast) and BRAF inhibitor (vemurafenib) were obtained from Selleckchem and dissolved in dimethylsulphoxide (DMSO).

We generated 2 melanoma cell lines derived from SkMel28 with acquired resistance to vemurafenib. SkMel28 were transfected with plasmid containing the cDNA of oncogenic RAS (G12V) (SkMel28RAS) or the empty vector (SkMel28Res) and selected by exposure to increased doses of vemurafenib concentration over 2 months.

Transfection/miRNA miPDE4D:

miR-PDE4D containing vectors were obtained by cloning two complementary oligonucleotides specifics of PDE4D into a BLOCK-iT™ Pol II miR RNAi Expression Vector with EmGFP, according to manufacturer instructions (Life technologies, Carlsbad, CA, USA). WM266.4 melanoma cell lines stably expressing a miRNA control (mi-CTL) or targeting PDE4D (mi1-PDE4D and mi2-PDE4D) were obtained by transfection with JetPEI (Polyplus-transfection, Illkirch, France) according to the manufacturer's instructions and selection by blasticidin (10 μg ml−1; GE Healthcare Europe GmbH, Velizy-Villacoublay, France). Resistant cells were then sorted by fluorescence with an ARIA III cell sorter from Becton Dickinson (BD Biosciences, Franklin Lakes, NJ, USA), the most fluorescent cells (about 1%) were amplified. mi-CTL and mi-PDE4D cells were cultured in complete medium and expression of PDE4D and actin were analysed by Western Blotting.

Clonogenic assays:

Cells were plated at low density (2000 cells per well in 6-well tissue culture plates) in fresh medium. Cells were treated every 48 hours with DMSO or inhibitors at the indicated concentrations, in duplicate. After 10 days, cells were stained with 0.5% crystal violet, and the number of colonies was counted.

Melanoma sphere culture:

SkMel28, M74 and WM266.4 were seeded at a concentration of 2000 cells/ml in DMEM/F12 serum free medium supplemented with B27, 5 µg/ml insulin, 20 ng/ml epidermal growth factor, 20 ng/ml fibroblast growth factor and 1% penicillin-streptomycine on ultra low-attachment 6-well plates. Cells were treated every 48 hours with DMSO or inhibitors. Medium was replaced weekly. To analyse sphere-forming capacity, the largest sphere diameters were measured after 1 week and 2 weeks of treatment on light microscope images of each well at 10x magnification. After 2 weeks, primary melanoma spheres were dissociated using PBS-EDTA and seeded in 1% agarose-coated 96-well plates for proliferation experiment. Cells were allowed to proliferate for 96 hours. Cell viability was measured using a CellTiter-Glo Luminescent assay following the manufacturer's instructions.

Results

PDE4D is expressed in human melanoma tumours

We first analysed the expression of PDE4D in human melanoma tumours by quantitative RT-PCR and immunohistochemistry (Figure 1). PDE4D mRNA was significantly expressed in primary melanomas at early stage (Breslow index<1mm), advanced stage (Breslow index>4mm) and in lymph node metastases. PDE4D mRNA expression level was significantly associated with tumour stage (P<0.0001) (Figure 1). Using immunohistochemistry, PDE4D was also detected in melanoma cells at the protein level. PDE4D was expressed more abundantly in advanced melanomas (data not shown).

PDE4 inhibitor rolipram significantly reduces clone formation in BRAF-mutated melanoma cell lines

BRAF-mutated cell line SkMel28 was cultured at low density in the presence of inhibitors for 10 days to assess cell clone formation in comparison with DMSO as a control (Figure 2A). As expected the BRAF inhibitor, vemurafenib at 3µM, significantly inhibited SkMel28 clone formation. Re-activation of the cAMP pathway with rolipram 10µM, a PDE4 inhibitor, and a sub-optimal dose forskolin, an adenylyl cyclase activator, (1µM) reduced the ability of SkMel28 to form clones by 82%.

SkMel28, M74 and WM266.4 were cultured in a three-dimensional melanospheres that in vitro partially recapitulate tumorigenic melanoma growth and that are known to contain an increased proportion of melanoma-initiating cells (Sette 2013, Perego 2010). PDE4 inhibition with rolipram 10µM + forskolin 1µM significantly reduced the growth of SkMel28

and M74 melanosphere (Figure 2B). Cell viability was assessed in SkMel28 and WM266.4 melanospheres using a proliferation assay after 2 weeks of treatment with rolipram 10μM + forskolin 1μM in spheroid culture medium. After 96 hours of culture in a 96-well plate without any additional treatment, cell viability remained inhibited in melanospheres previously treated with rolipram+forskolin (Figure 2C). The PDE4 inhibitor rolipram durably impaired the ability to grow as melanospheres. These data suggest that inhibiting PDE4 could be effective to reduce viability of melanoma cells with stem cell features.

PDE4 inhibition with miRNA significantly reduces tumour cell growth in BRAF-mutated melanoma

To confirm the effect of PDE4 inhibitor on BRAF-mutated melanoma cell lines was due to inhibition of PDE4 and not a side effect, we silenced PDE4D in WM266.4 using 2 different specific miRNA (mi1-PDE4D and mi2-PDE4D). We performed clonogenic assays and sphere proliferation assays on WM266.4 cell lines depleted of PDE4D (mi1-PDE4D and mi2-PDE4D) and control cells (mi-CTL). We showed that the silencing of PDE4D expression significantly reduced the ability of melanoma cells to form clones (Figure 3A) and to grow as spheroids (Figure 3B). These results were consistent with those we obtained with the PDE4 inhibitor.

PDE4 inhibition significantly reduces tumour growth in BRAF mutated-melanoma cell lines resistant to BRAF inhibitor, vemurafenib

In order to investigate the effects of PDE4 inhibition in melanoma resistant to BRAF inhibitors, we generated 2 independent cell lines derived from SkMel28 with acquired resistance to vemurafenib (SkMel28 Res and SkMel28 RAS). We showed that rolipram 10μM + forskolin 1μM reduced by 89% (SkMel28 Res) and 86% (SkMel28 RAS) the ability of resistant melanoma cell lines to form clones whereas vemurafenib 3μM had no inhibitory effect (Figure 4A). Finally, we compared the effect of rolipram to other more potent inhibitors of PDE4 inhibitors (apremilast and roflumilast). We showed that all PDE4 inhibitors were able to inhibit clone formation in sensitive and resistant melanoma cell lines, with Roflumilast being the most potent (Figure 4B).

REFERENCES:

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.

CLAIMS:

1. A method of treating a melanoma resistant to BRAF inhibitors in a subject in need thereof comprising administering to the subject a therapeutically effective amount of a PDE4 inhibitor.

2. The method of claim 1 wherein the melanoma is resistant to vemurafenib.

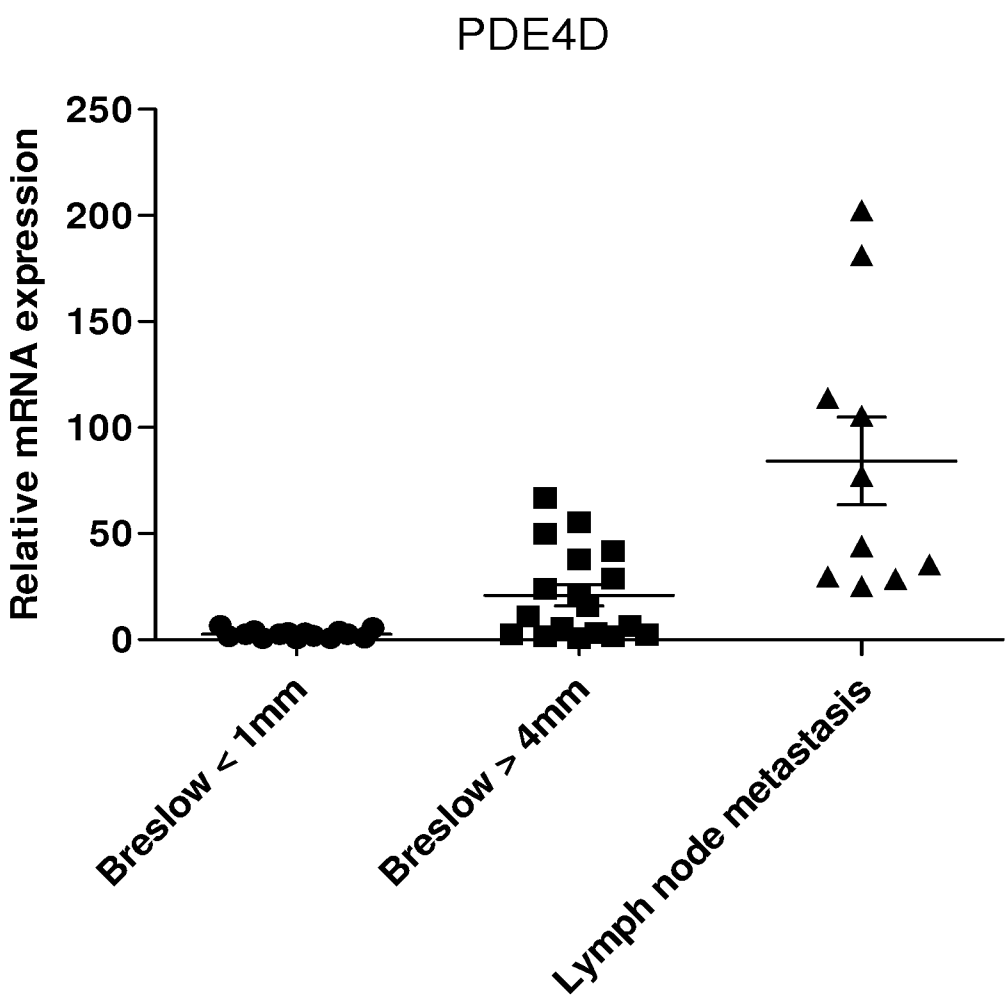

PDE4D
Relative mRNA expression
250
200
150
100
50
0
Breslow < 1mm
Breslow > 4mm
Lymph node metastasis

Figure 1

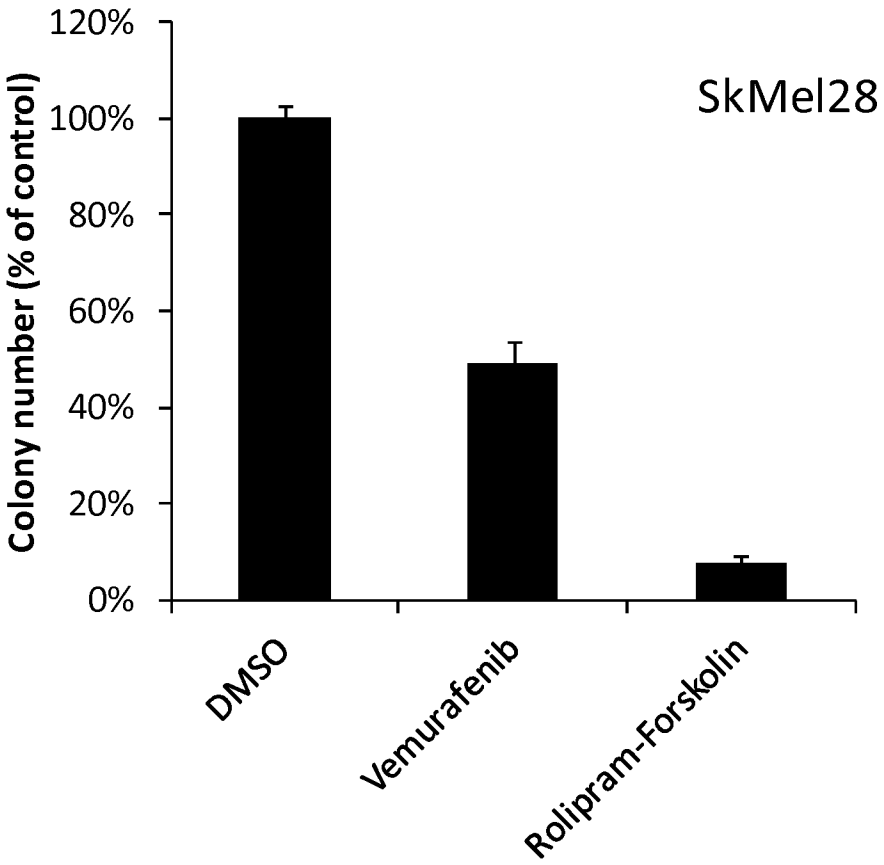

120%
100%
80%
60%
40%
20%
0%
Colony number (% of control)
SkMel28
DMSO
Vemurafenib
Rolipram-Forskolin

Figure 2A

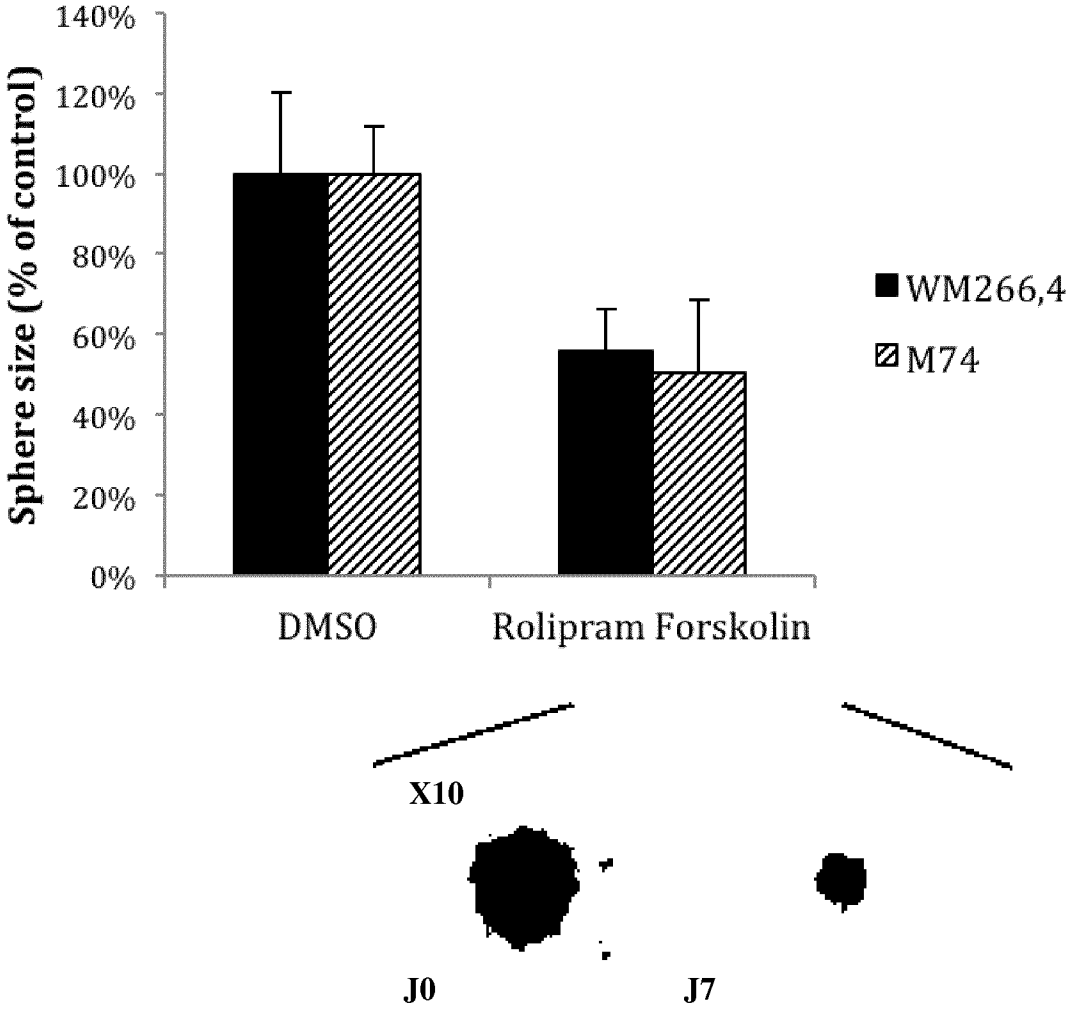

140%
120%
100%
80%
60%
40%
20%
0%
Sphere size (% of control)
WM266,4
M74
DMSO
Rolipram
Forskolin
X10
J0
J7

Figure 2B

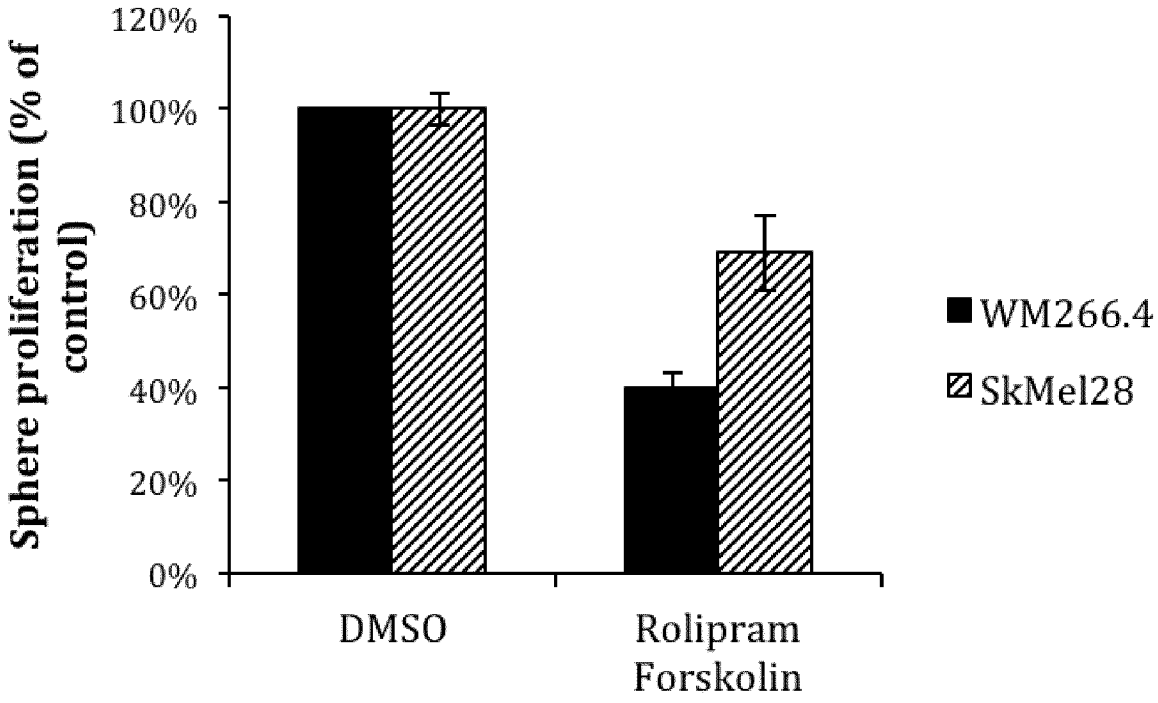

Sphere proliferation (% of control)
120%
100%
80%
60%
40%
20%
0%
DMSO
Rolipram
Forskolin
WM266.4
SkMel28

Figure 2C

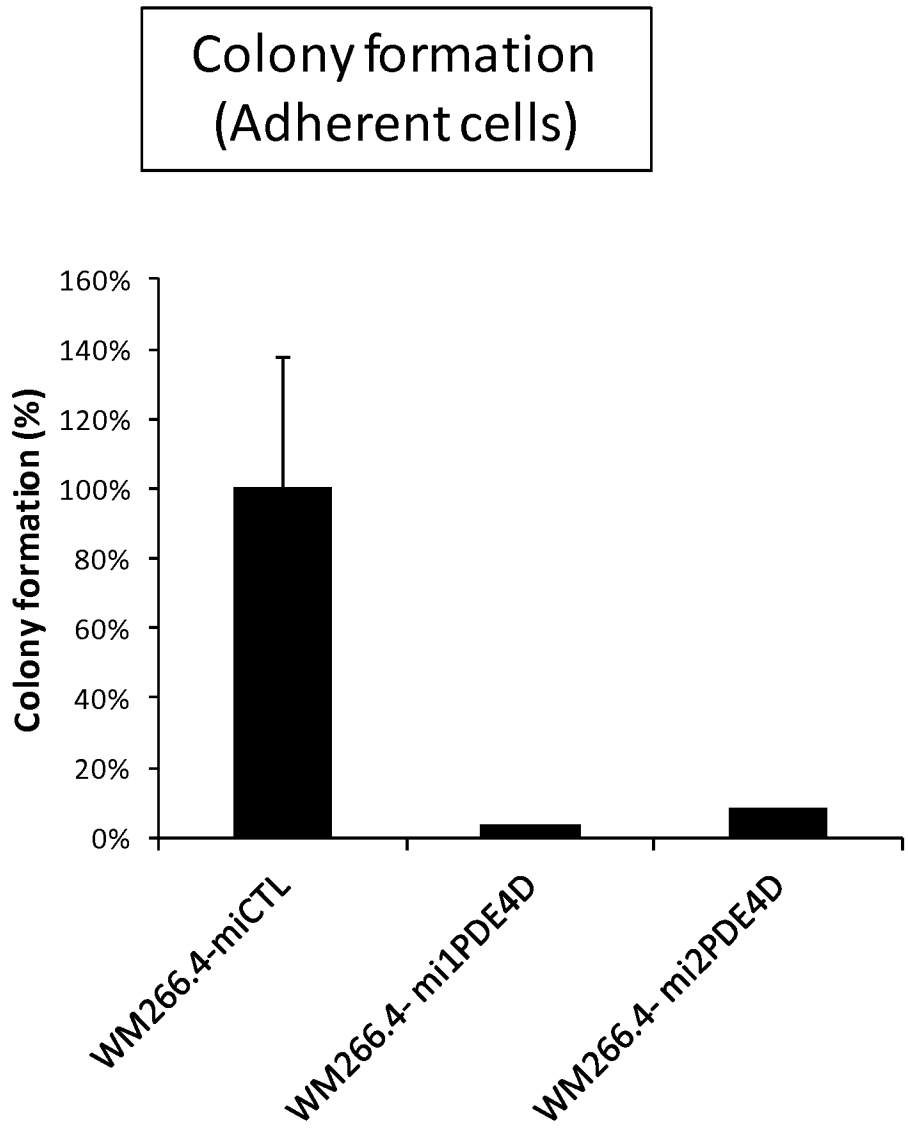

Colony formation
(Adherent cells)
160%
140%
120%
100%
80%
60%
40%
20%
0%
Colony formation (%)
WM266.4-miCTL
WM266.4- mi1PDE4D
WM266.4- mi2PDE4D

Figure 3A

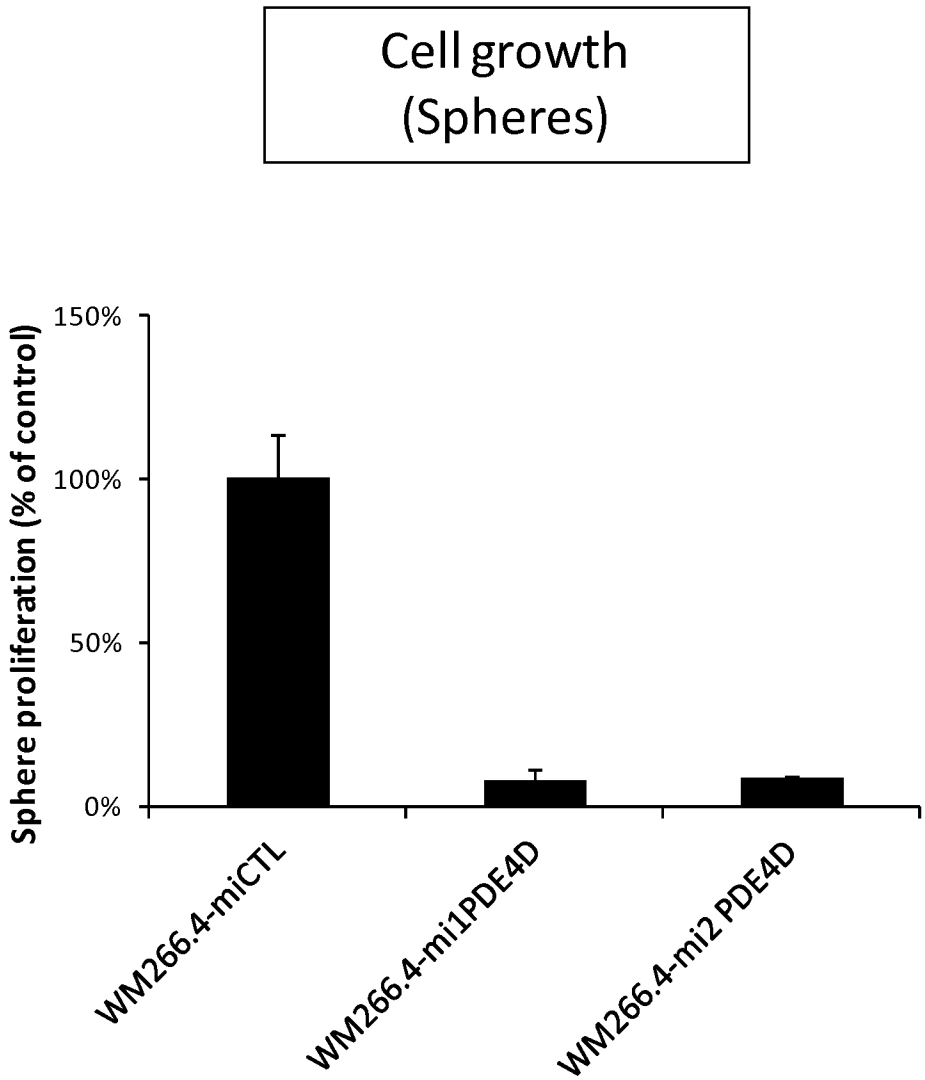

Cell growth
(Spheres)
Sphere proliferation (% of control)
150%
100%
50%
0%
WM266.4-miCTL
WM266.4-mi1PDE4D
WM266.4-mi2 PDE4D

Figure 3B

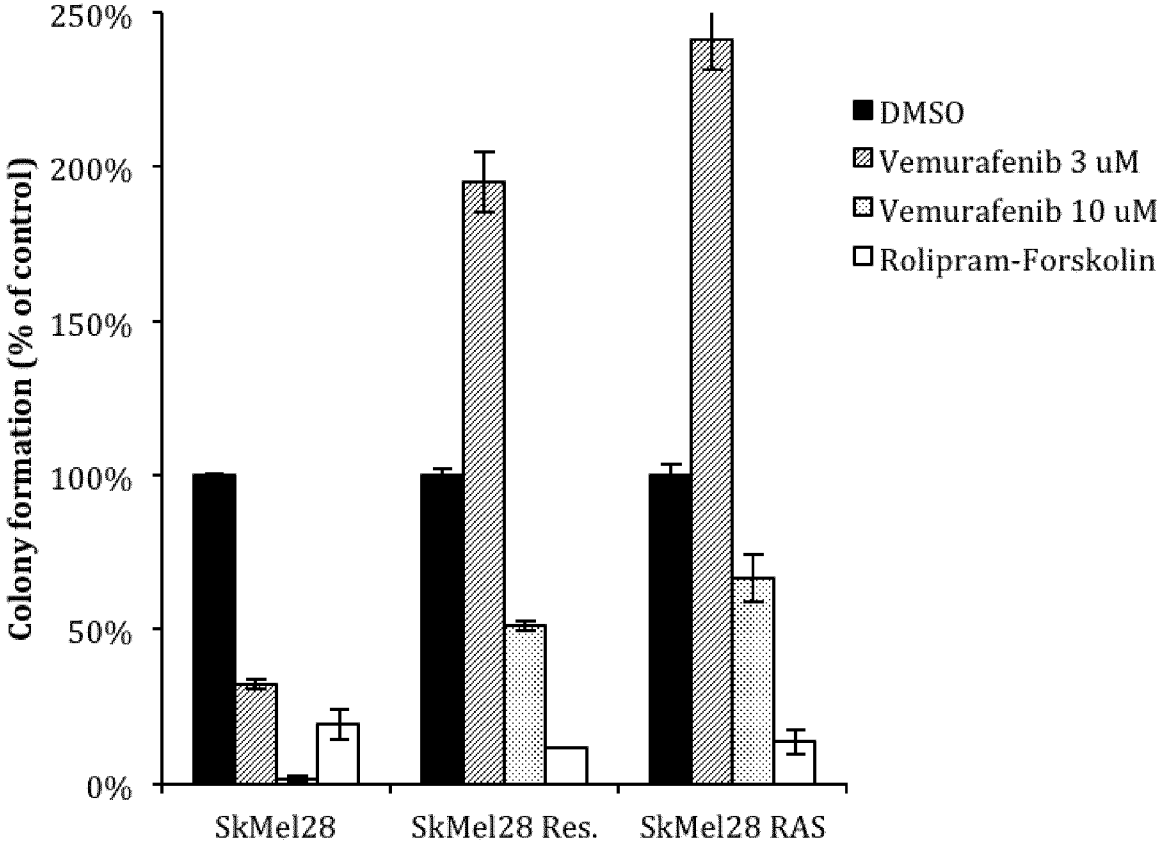

250%
200%
150%
100%
50%
0%
Colony formation (% of control)
DMSO
Vemurafenib 3 uM
Vemurafenib 10 uM
Rolipram-Forskolin
SkMel28
SkMel28 Res.
SkMel28 RAS

Figure 4A

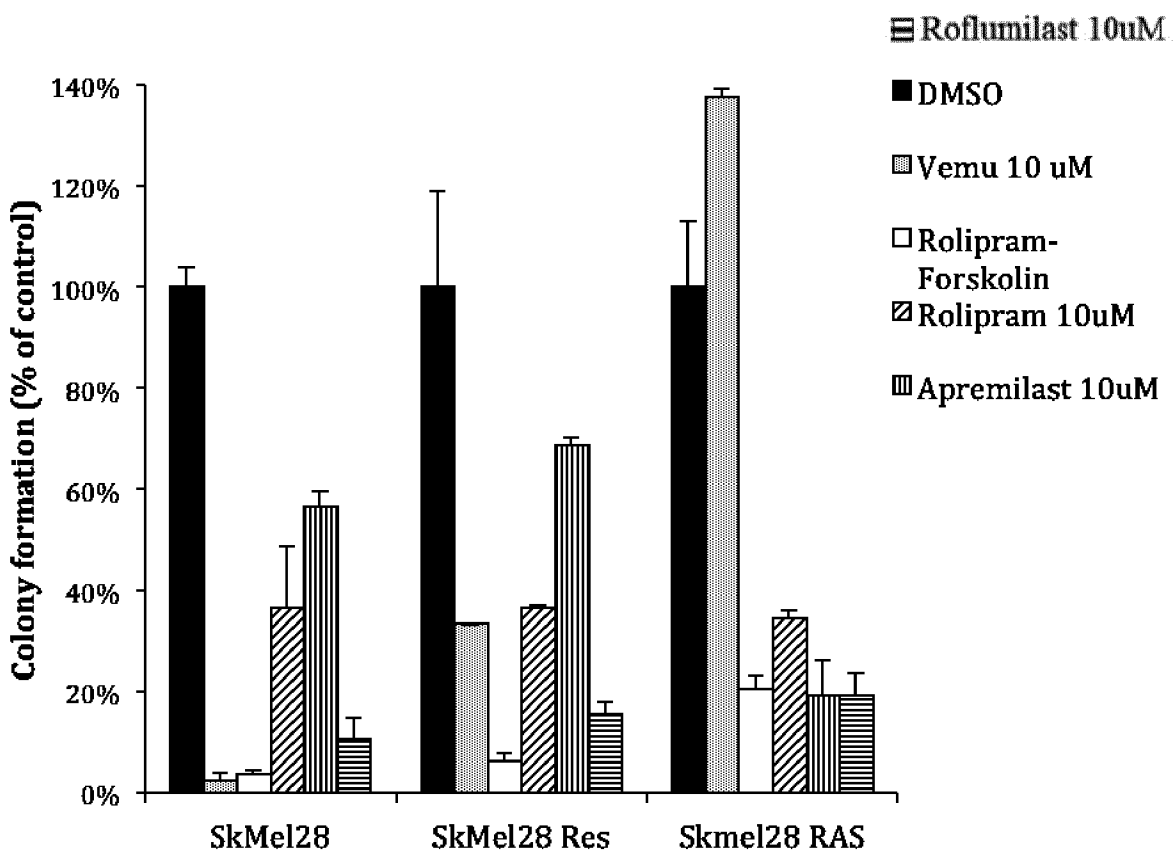

Roflumilast 10uM
DMSO
Vemu 10 uM
Rolipram-Forskolin
Rolipram 10uM
Apremilast 10uM
Colony formation (% of control)
140%
120%
100%
80%
60%
40%
20%
0%
SkMel28
SkMel28 Res
Skmel28 RAS

Figure 4B

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/078448

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K31/4015 A61K31/4035 A61K31/44 A61P35/00 A61K48/00
A61K31/7105
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 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, BIOSIS, CHEM ABS Data, EMBASE, WPI 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 2012/027716 A1 (COLLABRX INC [US]; LEHRER RAPHAEL [US]; COOPERSMITH ROBERT [US]) 1 March 2012 (2012-03-01) claims 1-7	1,2
Y	N Dumaz: "PDE4 interacts with FAK to control melanoma invasion", Journal of Investigative Dermatology, Vol. 133, Suppl. 1, 1 May 2013 (2013-05-01), page S241, XP055262646, Retrieved from the Internet: URL:http://www.jidonline.org/article/S0022-202X%2815%2936425-3/pdf [retrieved on 2016-04-04] the whole document	1,2

-/--

[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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 January 2017	18/01/2017

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	Scheithe, Rupert

1

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/078448

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	AMÉLIE MARQUETTE ET AL: "ERK and PDE4 cooperate to induce RAF isoform switching in melanoma", NATURE STRUCTURAL AND MOLECULAR BIOLOGY, vol. 18, no. 5, 1 May 2011 (2011-05-01), pages 584-591, XP055261971, US ISSN: 1545-9993, DOI: 10.1038/nsmb.2022 the whole document -----	1,2

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2016/078448

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2012027716 A1	01-03-2012	US 2012053185 A1	01-03-2012
		WO 2012027716 A1	01-03-2012