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(57) Abstract: The present invention relates to methods and pharmaceutical compositions for the treatment of melanoma.



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METHODS AND PHARMACEUTICAL COMPOSITIONS FOR THE TREATMENT OF MELANOMA

FIELD OF THE INVENTION:

The present invention relates to methods and pharmaceutical compositions for the treatment of melanoma.

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 (Genotyping of cutaneous melanoma. I C Glitza and M A Davies. Chin Clin Oncol. 2014, vol 3 p27- doi:10.3978/j.issn.2304-3865.2014.03.01). This BRAF mutation induces activation of the MAPK pathway, which is involved in essential cellular processes such as proliferation, differentiation and especially invasion, suggesting a relationship between BRAF mutation and metastatic potential (Driver mutations in melanoma: lessons learned from bench-to-bedside studies. Mehnert JM, Kluger HM. Curr Oncol Rep. 2012, vol 14: p 449-457). 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, only patients with this mutation benefit from these treatments. In the clinical context, the majority of patients first respond to these inhibitors and most of their metastases regress (Overcoming resistance to BRAF inhibition in BRAF-mutated metastatic melanoma. Francesco Spagnolo, Paola Ghiorzo and Paola Queirolo. Oncotarget, 2014, Vol. 5, No. 21 p102-21). Unfortunately, in most patients melanoma cells outbreak and progress again once resistance to anti-BRAFV600E inhibitors is acquired. (Targeting RAF kinases for cancer

therapy: BRAF-mutated melanoma and beyond. M. Holderfield et al. Nature Reviews Cancer 14, 455–467 (2014) doi:10.1038/nrc3760).

Novel immunotherapies using antibodies targeting inhibitor components of immune responses such as: anti-CTLA-4 (Ipilimumab) or anti-PD-1 (Nivolumab (BMS-936558) and Pembrolizumab) are also promising but occasionally cause dangerous side effects resulting from autoimmune reactions. Reliable treatments of metastatic melanoma are not yet available and the exploration of new chemotherapies or immunotherapies is still needed.

Therefore, the inventors searched for molecules with differential toxicity on normal cells and metastatic melanoma cells and in particular for molecules that could be able to reduce the development of metastases and potentiate the anti-melanoma effect of Vemurafenib and Dabrafenib.

SUMMARY OF THE INVENTION:

The present invention relates to methods and pharmaceutical compositions for the treatment of melanoma.

DETAILED DESCRIPTION OF THE INVENTION:

The role of macrocyclic lactones in melanoma was investigated by the inventors using human melanoma cell lines, murine melanoma cell lines, melanoma treatments with Vemurafenib and Dabrafenib, NMRI-nu/nu mice and C57BL/6-IFN- γ KO mice developing melanoma metastasis.

The inventors found that Ivermectin decreases metastasis implantation of murine metastatic melanoma B16F10 in C57BL/6 IFN γ -KO mice. The inventors also demonstrated that Ivermectin almost prevents completely metastasis implantation of human melanoma cells WM266-4 in NMRI nu/nu mice. The inventors also found that Ivermectin and several other macrocyclic lactones have the capacity to kill all tested melanoma cells (10 cell lines), including the BRAFV600E negative cells, demonstrating that Ivermectin is extremely promising against metastatic melanoma independently of the presence of the BRAF V600E mutation.

Moreover, the inventors surprisingly found that Ivermectin induce sensitization of melanoma cells to treatments with inhibitors of BRAFV600E such as Vemurafenib and Dabrafenib. The inventors demonstrated that low doses of Ivermectin (corresponding to 1/5 or 1/10 of its IC₅₀) potentiate the action of Vemurafenib on melanoma cells that do not carry the BRAFV600E mutation, and which are normally insensitive to Vemurafenib cytotoxicity. Therefore, a treatment protocol combining Vemurafenib with Ivermectin is expected to be

efficient for all metastatic melanoma patients. Furthermore this low dose of Ivermectin also potentiates the action of Dabrafenib on melanoma cells. The inventors also demonstrated that PAK1 is the Ivermectin target involved in melanoma treatment sensitivity.

Moreover, concentrations used by the inventors in the *in vivo* experiments are low (3,25mg/kg), corresponding to the doses used in anti parasitic treatments in humans, contrary to what had been previously shown with colorectal cancers (10, 50mg and even 100mg/kg/day) (WO 2012150543 A1).

Therapeutic methods and uses

Accordingly, the present invention relates to a compound selected from the group consisting of PAK-1 antagonists or PAK-1 expression inhibitors in combination with one or more melanoma treatment for use in the prevention or treatment of melanoma in a subject in need thereof.

As used herein, the term “subject” denotes a mammal. Typically, a subject according to the invention refers to any subject (preferably human) afflicted with or susceptible to be afflicted with a cancer. Typically a subject according to the invention is a subject afflicted or susceptible to be afflicted with melanoma or melanoma metastasis.

As used herein, the term "melanoma" has its general meaning in the art and refers to melanoma such as revised in the World Health Organisation Classification C43-C44. The term "melanoma" further encompasses both primary and metastatic melanoma. The term “melanoma” also relates to melanoma and metastatic melanoma with or without BRAF mutations including but not limiting to mutations such as BRAF V600E mutation.

As used herein, the term “melanoma treatment” has its general meaning in the art and refers to chemotherapy, immunotherapy, radiotherapy or targeted chemotherapy with therapeutic active agents used in the treatment of melanoma or metastatic melanoma. In one embodiment, the melanoma treatment is selected from the group consisting of but not limited to BRAF inhibitors such as Vemurafenib (PLX4032), Dabrafenib (GSK2118436), Raf-1 inhibitor such as Sorafenib, MEK inhibitors such as Trametinib, Selumetinib, and MAPK pathway inhibitors such as described in (Dossett LA, Kudchadkar RR, Zager JS. BRAF and MEK inhibition in melanoma. Expert Opin Drug Saf. 2015 Feb 4:1-12; Long GV. et al.,

Combined BRAF and MEK inhibition versus BRAF inhibition alone in melanoma. *N Engl J Med.* 2014 Nov 13;371(20):1877-88); Roulstone V. et al., BRAF- and MEK-targeted Small Molecule Inhibitors Exert Enhanced Anti-Melanoma Effects in Combination with Oncolytic Reovirus through ER Stress. *Mol Ther.* 2015 Jan 26; Ilieva KM. et al., Effects of BRAF mutations and BRAF inhibition on immune responses to melanoma. *Mol Cancer Ther.* 2014 Dec;13(12):2769-83).

As used herein, the term “PAK-1” has its general meaning in the art and refers to P21-Activated Kinase 1, also known as Serine/threonine-protein kinase PAK 1, or P21 protein (Cdc42/Rac)-activated kinase 1. PAK-1 is a member of p21-activated kinases family (PAKs) involved in the ERK activation, MAPK pathway activation and that are critical effectors that link the Rho GTPases to cytoskeleton reorganization and nuclear signaling and have been implicated in a wide range of biological activities. Several functions have been attributed to PAK-1 proteins, including roles in cell transformation, cell motility and morphology, tumor growth, and tumorigenesis (Hisashi Hashimoto et al. *Drug Discov Ther.* 2009; 3(6):243-246; Ong et al., 2013).

The term “expression” when used in the context of expression of a gene or nucleic acid refers to the conversion of the information, contained in a gene, into a gene product. A gene product can be the direct transcriptional product of a gene (e.g., mRNA, tRNA, rRNA, antisense RNA, ribozyme, structural RNA or any other type of RNA) or a protein produced by translation of a mRNA. Gene products also include messenger RNAs, which are modified, by processes such as capping, polyadenylation, methylation, and editing, and proteins (e.g., PAK-1) modified by, for example, methylation, acetylation, phosphorylation, ubiquitination, SUMOylation, ADP-ribosylation, myristilation, and glycosylation.

An “inhibitor of expression” refers to a natural or synthetic compound that has a biological effect to inhibit the expression of a gene such as pharmaceutical compounds or targeted siRNA).

The term “PAK-1 antagonist” refers to a compound that selectively blocks or inactivates PAK-1. As used herein, the term “selectively blocks or inactivates” refers to a compound that preferentially binds to and blocks or inactivates PAK-1 with a greater affinity and potency, respectively, than its interaction with the other sub-types or isoforms of the PAKs family.

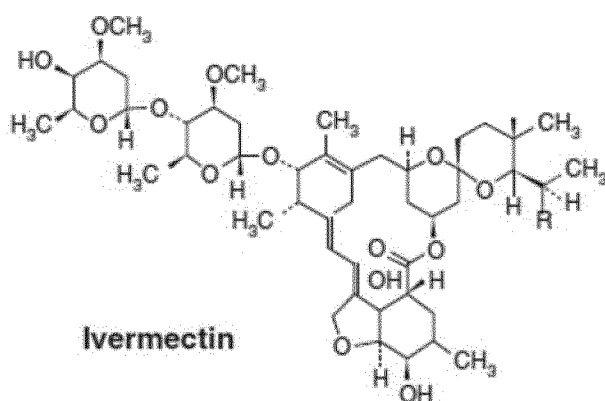
Compounds that prefer PAK-1, but that may also block or inactivate other PAKs, as partial or full antagonists, are contemplated. The “PAK-1 antagonist” refers to compounds that blocks PAK-1 interaction with proteins involved in ERK pathway and MAPK pathway such as RAF-1 (CRAF), inhibits its phosphorylation, and blocks MAPK cascade. Typically, a PAK-1 antagonist is a small organic molecule, a peptide, a polypeptide, an aptamer or an intra-antibody.

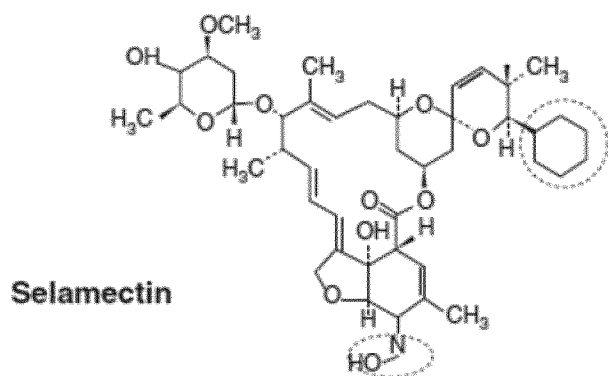
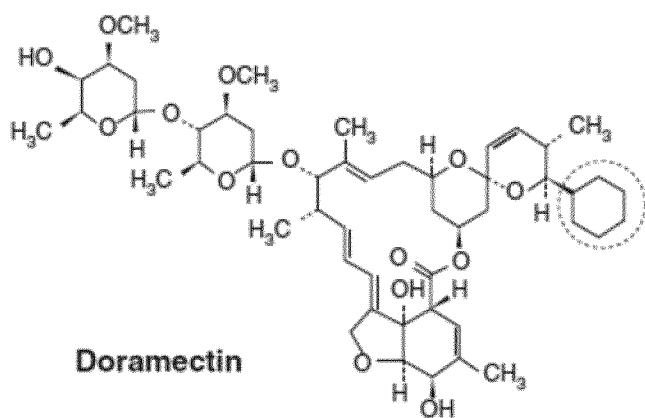
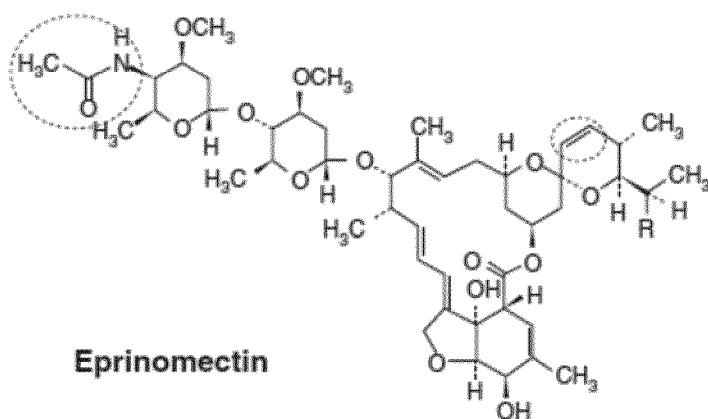
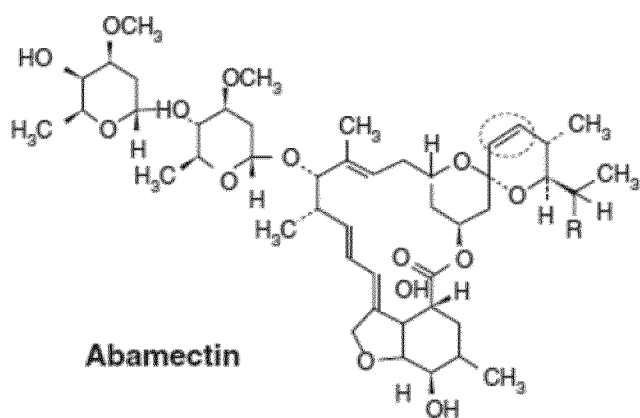
In one embodiment of the invention, PAK-1 antagonist is a macrocyclic lactone.

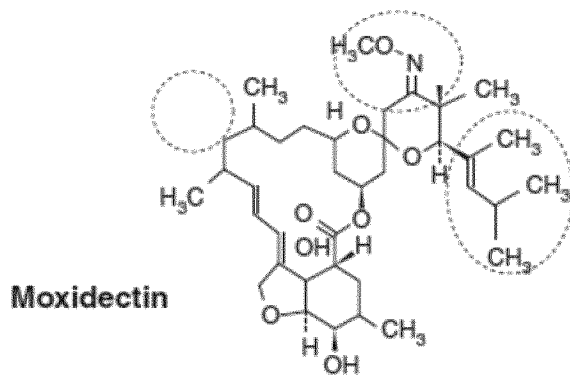
As used herein, the term “macrocyclic lactones” has its general meaning in the art and refers to macrocyclic lactones and macrocyclic lactones derivatives described in (Lespine A. Lipid-like properties and pharmacology of the anthelmintic macrocyclic lactones. Expert Opin Drug Metab Toxicol. 2013 Dec; 9(12):1581-95).

The term “macrocyclic lactones” also refers to macrocyclic lactones and macrocyclic lactones derivatives described in WO 2012/078605, WO 2012/150543, WO 2011/075592, WO 93/16189, WO 2012/028556 and US 3,503,994.

As used herein, the term “macrocyclic lactones” has its general meaning in the art and refers to compounds described in Lespine A. 2013 such as compounds having the formula:







In one embodiment of the invention, macrocyclic lactones include but are not limited to Ivermectin (Stromectol), Doramectin, Selamectin, Moxidectin, Milbemycin, Abamectin, Nemadectin and Eprinomectin.

Accordingly, the present invention also relates to a macrocyclic lactone in combination with one or more melanoma treatment for use in the prevention or treatment of melanoma in a subject in need thereof.

In one embodiment, the present invention relates to a macrocyclic lactone such as Ivermectin, Doramectin, Selamectin, Moxycyctin or Abamectin in combination with one or more melanoma treatment such as Vemurafenib or Dabrafenib for use in the prevention or treatment of melanoma in a subject in need thereof.

In a particular embodiment, the present invention relates to Ivermectin in combination with Vemurafenib and/or Dabrafenib for use in the prevention or treatment of melanoma in a subject in need thereof.

In another embodiment, the PAK-1 antagonist of the invention is an aptamer. Aptamers are a class of molecule that represents an alternative to antibodies in term of molecular recognition. Aptamers are oligonucleotide sequences with the capacity to recognize virtually any class of target molecules with high affinity and specificity. Such ligands may be isolated through Systematic Evolution of Ligands by EXponential enrichment (SELEX) of a random sequence library, as described in Tuerk C. and Gold L., 1990. The random sequence library is obtainable by combinatorial chemical synthesis of DNA. In this library, each member is a linear oligomer, eventually chemically modified, of a unique sequence. Possible modifications, uses

and advantages of this class of molecules have been reviewed in Jayasena S.D., 1999. Peptide aptamers consists of a conformationally constrained antibody variable region displayed by a platform protein, such as E. coli Thioredoxin A that are selected from combinatorial libraries by two hybrid methods (Colas et al., 1996). Then after raising aptamers directed against PAK-1 of the invention as above described, the skilled man in the art can easily select those inhibiting PAK-1.

In one embodiment, the compound of the invention is an inhibitor of PAK-1 expression.

Inhibitors of PAK-1 expression for use in the present invention may be based on antisense oligonucleotide constructs. Anti-sense oligonucleotides, including anti-sense RNA molecules and anti-sense DNA molecules, would act to directly block the translation of PAK-1 mRNA by binding thereto and thus preventing protein translation or increasing mRNA degradation, thus decreasing the level of PAK-1 proteins, 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 PAK-1 can be synthesized, e.g., by conventional phosphodiester techniques and administered by e.g., intravenous injection or infusion. Methods for using antisense techniques for specifically alleviating 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 PAK-1 expression for use in the present invention. PAK-1 gene expression can be reduced by contacting the 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 PAK-1 expression is specifically inhibited (i.e. RNA interference or RNAi). Methods for selecting an appropriate dsRNA or dsRNA-encoding vector are well known in the art for genes whose sequence is known (e.g. see Tuschl, T. et al. (1999); Elbashir, S. M. et al. (2001); Hannon, GJ. (2002); McManus, MT. et al. (2002); Brummelkamp, TR. et al. (2002); U.S. Pat. Nos. 6,573,099 and 6,506,559; and International Patent Publication Nos. WO 01/36646, WO 99/32619, and WO 01/68836).

Ribozymes can also function as inhibitors of PAK-1 expression for use in the present invention. Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by endonucleolytic cleavage. Engineered hairpin or hammerhead motif ribozyme molecules that specifically and efficiently catalyze endonucleolytic cleavage of PAK-1 mRNA sequences are thereby useful

within the scope of the present invention. Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage sites, which typically include the following sequences, GUA, GUU, and GUC. Once identified, short RNA sequences of between about 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site can be evaluated for predicted structural features, such as secondary structure, that can render the oligonucleotide sequence unsuitable. The suitability of candidate targets can also be evaluated by testing their accessibility to hybridization with complementary oligonucleotides, using, e.g., ribonuclease protection assays.

Both antisense oligonucleotides and ribozymes useful as inhibitors of PAK-1 expression can be prepared by known methods. These include techniques for chemical synthesis such as, e.g., by solid phase phosphoramidite chemical synthesis. Alternatively, anti-sense RNA molecules can be generated by in vitro or in vivo transcription of DNA sequences encoding the RNA molecule. Such DNA sequences can be incorporated into a wide variety of vectors that incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Various modifications to the oligonucleotides of the invention can be introduced as a means of increasing intracellular stability and half-life. Possible modifications include but are not limited to the addition of flanking sequences of ribonucleotides or deoxyribonucleotides to the 5' and/or 3' ends of the molecule, or the use of phosphorothioate or 2'-O-methyl rather than phosphodiesterase linkages within the oligonucleotide backbone.

Antisense oligonucleotides siRNAs 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 or ribozyme nucleic acid to the cells and preferably cells expressing PAK-1. Preferably, 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 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 rouse 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.

Preferred viral vectors are based on non-cytopathic eukaryotic viruses in which non-essential genes have been replaced with the gene of interest. Non-cytopathic viruses include retroviruses (e.g., lentivirus), the life cycle of which involves reverse transcription of genomic viral RNA into DNA with subsequent proviral integration into host cellular DNA. Retroviruses have been approved for human gene therapy trials. Most useful are those retroviruses that are replication-deficient (i.e., capable of directing synthesis of the desired proteins, but incapable of manufacturing an infectious particle). Such genetically altered retroviral expression vectors have general utility for the high-efficiency transduction of genes in vivo. Standard protocols for producing replication-deficient retroviruses (including the steps of incorporation of exogenous genetic material into a plasmid, transfection of a packaging cell lined with plasmid, production of recombinant retroviruses by the packaging cell line, collection of viral particles from tissue culture media, and infection of the target cells with viral particles) are provided in KRIEGLER (A Laboratory Manual," W.H. Freeman C.O., New York, 1990) and in MURRY ("Methods in Molecular Biology," vol.7, Humana Press, Inc., Clifton, N.J., 1991).

Preferred viruses for certain applications are the adeno-viruses and adeno-associated viruses, which are double-stranded DNA viruses that have already been approved for human use in gene therapy. The adeno-associated virus can be engineered to be replication deficient and is capable of infecting a wide range of cell types and species. It further has advantages such as, heat and lipid solvent stability; high transduction frequencies in cells of diverse lineages, including hemopoietic cells; and lack of superinfection inhibition thus allowing multiple series of transductions. Reportedly, the adeno-associated virus can integrate into human cellular DNA in a site-specific manner, thereby minimizing the possibility of insertional mutagenesis and variability of inserted gene expression characteristic of retroviral infection. In addition, wild-type adeno-associated virus infections have been followed in tissue culture for greater than 100 passages in the absence of selective pressure, implying that the adeno-associated virus genomic integration is a relatively stable event. The adeno-associated virus can also function in an extrachromosomal fashion.

Other vectors include plasmid vectors. Plasmid vectors have been extensively described in the art and are well known to those of skill in the art. See e.g., SANBROOK et al., "Molecular Cloning: A Laboratory Manual," Second Edition, Cold Spring Harbor Laboratory Press, 1989. In the last few years, plasmid vectors have been used as DNA vaccines for delivering antigen-encoding genes to cells in vivo. They are particularly advantageous for this because they do not have the same safety concerns as with many of the viral vectors. These plasmids, however, having a promoter compatible with the host cell, can express a peptide from a gene operatively

encoded within the plasmid. Some commonly used plasmids include pBR322, pUC18, pUC19, pRC/CMV, SV40, and pBlueScript. Other plasmids are well known to those of ordinary skill in the art. Additionally, plasmids may be custom designed using restriction enzymes and ligation reactions to remove and add specific fragments of DNA. Plasmids may be delivered by a variety of parenteral, mucosal and topical routes. For example, the DNA plasmid can be injected by intramuscular, intradermal, subcutaneous, or other routes. It may also be administered by intranasal sprays or drops, rectal suppository and orally. It may also be administered into the epidermis or a mucosal surface using a gene-gun. The plasmids may be given in an aqueous solution, dried onto gold particles or in association with another DNA delivery system including but not limited to liposomes, dendrimers, cochleate and microencapsulation.

A further aspect of the present invention relates to the compound selected from the group consisting of PAK-1 antagonists or PAK-1 expression inhibitors in combination with one or more melanoma treatment for use in the prevention of melanoma metastasis in a subject in need thereof.

In one embodiment, the present invention relates to the macrocyclic lactone in combination with one or more melanoma treatment for use in the prevention of melanoma metastasis in a subject in need thereof.

In one embodiment, the present invention relates to the macrocyclic lactone such as Ivermectin, Doramectin, Selamectin, Moxydectin or Abamectin in combination with one or more melanoma treatment such as Vemurafenib or Dabrafenib for use in the prevention of melanoma metastasis in a subject in need thereof.

A further aspect, the compound selected from the group consisting of PAK-1 antagonists or PAK-1 expression inhibitors of the invention sensitizes melanoma cells to melanoma treatment.

Accordingly, the present invention relates to the compound selected from the group consisting of PAK-1 antagonists or PAK-1 expression inhibitors for use in a method for enhancing therapeutic efficacy of melanoma treatment in a subject in need thereof.

In one embodiment, the present invention relates to the macrocyclic lactone according to the invention for use in a method for enhancing therapeutic efficacy of melanoma treatment in a subject in need thereof.

5 In one embodiment, the present invention relates to the macrocyclic lactone such as Ivermectin, Doramectin, Selamectin, Moxydectin or Abamectin for use in a method for enhancing therapeutic efficacy of melanoma treatment such as Vemurafenib or Dabrafenib in a subject in need thereof.

10 In a particular embodiment, the present invention relates to Ivermectin for use in a method for enhancing therapeutic efficacy of Vemurafenib and/or Dabrafenib in a subject in need thereof.

15 Typically the compound selected from the group consisting of PAK-1 antagonists or PAK-1 expression inhibitors of the present invention and the melanoma treatment as described above are administered to the subject in a therapeutically effective amount.

20 By a "therapeutically effective amount" of the compound of the present invention as above described is meant a sufficient amount of the compound. It will be understood, however, 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, 25 general health, sex and diet of the subject; the time of administration, route of administration, and rate of excretion of the specific compound employed; the duration of the treatment; drugs used in combination or coincidental with the specific compound employed; and like factors well known in the medical arts. For example, it is well within the skill of the art to start doses of the compound at levels lower than those required to achieve the desired therapeutic effect and to gradually increase the dosage until the desired effect is achieved. However, the daily 30 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 compound of the present invention 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 compound of the present invention, preferably from 1 mg to about 100 mg of the compound of the present invention. 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.

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In a particular embodiment, the compound selected from the group consisting of PAK-1 antagonists or PAK-1 expression inhibitors according to the invention may be used in low doses. Typically, the term "low doses" refers to macrocyclic lactones concentration (such as Ivermectin) between 0.01 μM and 20 μM . Particularly, the term "low doses" refers to
10 macrocyclic lactones concentration of 0.01, 0.05, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 20.0 μM . In a particular embodiment, the term "low doses" refers to macrocyclic lactones concentrations from 1/10 to 1/3 of their IC₅₀, particularly, from 1/10 to 1/5 of their IC₅₀.

The therapeutically effective amount of the melanoma treatment of the invention is well
15 known in the art. Typically, the therapeutically effective amount of the melanoma treatment relates to melanoma treatment concentration (such as Vemurafenib or Dabrafenib) between 0.01 μM to 6 μM . Particularly, the therapeutically effective amount of the melanoma treatment relates to melanoma treatment concentration of 0.01, 0.05, 0.1, 0.5, 1.0, 1.5, 2, 3, 4, 5, 6 μM , IC₅₀ from 0,5 to 4,5 μM .

20

According to the present invention, the compound selected from the group consisting of PAK-1 antagonists or PAK-1 expression inhibitors of the invention is administered sequentially or concomitantly with one or more melanoma treatment.

25 In a particular embodiment, the compound selected from the group consisting of PAK-1 antagonists or PAK-1 expression inhibitors of the invention is administered sequentially or concomitantly with one or more melanoma treatment, and one or more therapeutic active agent such as chemotherapeutic, immunotherapeutic or radiotherapeutic agents.

30 In some embodiments, the therapeutic active agent is a chemotherapeutic agent. The term "chemotherapeutic agent" refers to chemical compounds that are effective in inhibiting tumor growth. Examples of chemotherapeutic agents include alkylating agents such as Dacarbazine, thiotepa and cyclophosphamide; alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa;

ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramidate, triethylenethiophosphoramide and trimethylolmelamine; acetogenins (especially bullatacin and bullatacinone); a carnotecicin (including the synthetic analogue topotecan); bryostatin; calystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); cryptophycins (particularly cryptophycin 1 and cryptophycin 8);
5 dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CBI-TMI); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine,
10 trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine; antibiotics such as the enediyne antibiotics (e.g. calicheamicin, especially calicheamicin (11 and calicheamicin 211, see, e.g., Agnew Chem Intl. Ed. Engl. 33:183-186 (1994); dynemicin, including dynemicin A; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antibiotic
15 chromomorphores), aclacinomysins, actinomycin, anthramycin, azaserine, bleomycins, cactinomycin, carubicin, canninomycin, carzinophilin, chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxydoxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins, mycophenolic acid,
20 nogalarnycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine,
25 carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine, 5-FU; androgens such as calusterone, dromostanolone propionate, epitestosterone, mepitostane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; amsacrine; bestabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elfornithine; elliptinium acetate;
30 an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidamine; maytansinoids such as maytansine and ansamitocins; mitoguanine; mitoxantrone; mopidamol; nitracrine; pento statin; phenamet; pirarubicin; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK®; razoxane; rhizoxin; sizofiran; spirogennanin; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verrucarin A, rosidin A and

anguidine); urethan; vindesine; dacarbazine; mannomustine; mitobromtol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids, e.g. paclitaxel (TAXOL®, Bristol-Myers Squibb Oncology, Princeton, N.J.) and doxorubicin (TAXOTERE®, Rhone-Poulenc Rorer, Antony, France); chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitomycin C; mitoxantrone; vincristine; vinorelbine; navelbine; novantrone; teniposide; daunomycin; aminopterin; xeloda; ibandronate; CPT-11; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoic acid; capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

Also included in this definition are antihormonal agents that act to regulate or inhibit hormone action on tumors such as anti-estrogens including for example tamoxifen, raloxifene, aromatase inhibiting 4(5)-imidazoles, 4-hydroxytamoxifen, toremifene, keoxifene, LY117018, onapristone, and toremifene (Fareston); and anti-androgens such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

In some embodiments, the therapeutic active agent is a targeted cancer therapy. Targeted cancer therapies are drugs or other substances that block the growth and spread of cancer by interfering with specific molecules ("molecular targets") that are involved in the growth, progression, and spread of cancer. Targeted cancer therapies are sometimes called "molecularly targeted drugs", "molecularly targeted therapies", "precision medicines", or similar names. In some embodiments, the targeted therapy consists of administering the subject with a tyrosine kinase inhibitor as defined above.

In some embodiments, therapeutic active agent is an immunotherapeutic agent. The term "immunotherapeutic agent," as used herein, refers to a compound, composition or treatment that indirectly or directly enhances, stimulates or increases the body's immune response against cancer cells and/or that decreases the side effects of other anticancer therapies. Immunotherapy is thus a therapy that directly or indirectly stimulates or enhances the immune system's responses to cancer cells and/or lessens the side effects that may have been caused by other anti-cancer agents. Immunotherapy is also referred to in the art as immunologic therapy, biological therapy biological response modifier therapy and biotherapy. Examples of common immunotherapeutic agents known in the art include, but are not limited to, cytokines, cancer vaccines, monoclonal antibodies and non-cytokine adjuvants. Alternatively the

immunotherapeutic treatment may consist of administering the subject with an amount of immune cells (T cells, NK, cells, dendritic cells, B cells...). Immunotherapeutic agents can be non-specific, i.e. boost the immune system generally so that the human body becomes more effective in fighting the growth and/or spread of cancer cells, or they can be specific, i.e. targeted to the cancer cells themselves immunotherapy regimens may combine the use of non-specific and specific immunotherapeutic agents. Non-specific immunotherapeutic agents are substances that stimulate or indirectly improve the immune system. Non-specific immunotherapeutic agents have been used alone as a main therapy for the treatment of cancer, as well as in addition to a main therapy, in which case the non-specific immunotherapeutic agent functions as an adjuvant to enhance the effectiveness of other therapies (e.g. cancer vaccines). Non-specific immunotherapeutic agents can also function in this latter context to reduce the side effects of other therapies, for example, bone marrow suppression induced by certain chemotherapeutic agents. Non-specific immunotherapeutic agents can act on key immune system cells and cause secondary responses, such as increased production of cytokines and immunoglobulins. Alternatively, the agents can themselves comprise cytokines. Non-specific immunotherapeutic agents are generally classified as cytokines or non-cytokine adjuvants. A number of cytokines have found application in the treatment of cancer either as general non-specific immunotherapies designed to boost the immune system, or as adjuvants provided with other therapies. Suitable cytokines include, but are not limited to, interferons, interleukins and colony-stimulating factors. Interferons (IFNs) contemplated by the present invention include the common types of IFNs, IFN-alpha (IFN- α), IFN-beta (IFN- β) and IFN-gamma (IFN- γ). IFNs can act directly on cancer cells, for example, by slowing their growth, promoting their development into cells with more normal behaviour and/or increasing their production of antigens thus making the cancer cells easier for the immune system to recognise and destroy. IFNs can also act indirectly on cancer cells, for example, by slowing down angiogenesis, boosting the immune system and/or stimulating natural killer (NK) cells, T cells and macrophages. Recombinant IFN-alpha is available commercially as Roferon (Roche Pharmaceuticals) and Intron A (Schering Corporation). Interleukins contemplated by the present invention include IL-2, IL-4, IL-11 and IL-12. Examples of commercially available recombinant interleukins include Proleukin® (IL-2; Chiron Corporation) and Neumega® (IL-12; Wyeth Pharmaceuticals). Zymogenetics, Inc. (Seattle, Wash.) is currently testing a recombinant form of IL-21, which is also contemplated for use in the combinations of the present invention. Colony-stimulating factors (CSFs) contemplated by the present invention include granulocyte colony stimulating factor (G-CSF or filgrastim), granulocyte-macrophage

colony stimulating factor (GM-CSF or sargramostim) and erythropoietin (epoetin alfa, darbepoietin). Treatment with one or more growth factors can help to stimulate the generation of new blood cells in subjects undergoing traditional chemotherapy. Accordingly, treatment with CSFs can be helpful in decreasing the side effects associated with chemotherapy and can allow for higher doses of chemotherapeutic agents to be used. Various-recombinant colony stimulating factors are available commercially, for example, Neupogen® (G-CSF; Amgen), Neulasta (pelfilgrastim; Amgen), Leukine (GM-CSF; Berlex), Procrit (erythropoietin; Ortho Biotech), Epogen (erythropoietin; Amgen), Arnesp (erythropoietin). In addition to having specific or non-specific targets, immunotherapeutic agents can be active, i.e. stimulate the body's own immune response, or they can be passive, i.e. comprise immune system components that were generated external to the body. Passive specific immunotherapy typically involves the use of one or more monoclonal antibodies that are specific for a particular antigen found on the surface of a cancer cell or that are specific for a particular cell growth factor. Monoclonal antibodies may be used in the treatment of cancer in a number of ways, for example, to enhance a subject's immune response to a specific type of cancer, to interfere with the growth of cancer cells by targeting specific cell growth factors, such as those involved in angiogenesis, or by enhancing the delivery of other anticancer agents to cancer cells when linked or conjugated to agents such as chemotherapeutic agents, radioactive particles or toxins. Monoclonal antibodies currently used as cancer immunotherapeutic agents that are suitable for inclusion in the combinations of the present invention include, but are not limited to, rituximab (Rituxan®), trastuzumab (Herceptin®), ibritumomab tiuxetan (Zevalin®), tositumomab (Bexxar®), cetuximab (C-225, Erbitux®), bevacizumab (Avastin®), gemtuzumab ozogamicin (Mylotarg®), alemtuzumab (Campath®), and BL22. Other examples include anti-CTLA4 antibodies (e.g. Ipilimumab), anti-PD1 antibodies, anti-PDL1 antibodies, anti-TIMP3 antibodies, anti-LAG3 antibodies, anti-B7H3 antibodies, anti-B7H4 antibodies or anti-B7H6 antibodies. In some embodiments, antibodies include B cell depleting antibodies. Typical B cell depleting antibodies include but are not limited to anti-CD20 monoclonal antibodies [e.g. Rituximab (Roche), Ibritumomab tiuxetan (Bayer Schering), Tositumomab (GlaxoSmithKline), AME-133v (Applied Molecular Evolution), Ocrelizumab (Roche), Ofatumumab (HuMax-CD20, Gemnab), TRU-015 (Trubion) and IMMU-106 (Immunomedics)], an anti-CD22 antibody [e.g. Epratuzumab, Leonard et al., Clinical Cancer Research (Z004) 10: 53Z7-5334], anti-CD79a antibodies, anti-CD27 antibodies, or anti-CD19 antibodies (e.g. U.S. Pat. No. 7,109,304), anti-BAFF-R antibodies (e.g. Belimumab, GlaxoSmithKline), anti-APRIL antibodies (e.g. anti-human APRIL antibody, ProSci inc.), and

anti-IL-6 antibodies [e.g. previously described by De Benedetti et al., J Immunol (2001) 166: 4334-4340 and by Suzuki et al., Europ J of Immunol (1992) 22 (8) 1989-1993, fully incorporated herein by reference]. The immunotherapeutic treatment may consist of allografting, in particular, allograft with hematopoietic stem cell HSC. The immunotherapeutic treatment may also consist in an adoptive immunotherapy as described by Nicholas P. Restifo, Mark E. Dudley and Steven A. Rosenberg "Adoptive immunotherapy for cancer: harnessing the T cell response, Nature Reviews Immunology, Volume 12, April 2012). In adoptive immunotherapy, the subject's circulating lymphocytes, NK cells, are isolated amplified *in vitro* and readministered to the subject. The activated lymphocytes or NK cells are most preferably be the subject's own cells that were earlier isolated from a blood or tumor sample and activated (or "expanded") *in vitro*.

In some embodiments, the therapeutic active agent is a radiotherapeutic agent. The term "radiotherapeutic agent" as used herein, is intended to refer to any radiotherapeutic agent known to one of skill in the art to be effective to treat or ameliorate cancer, without limitation. For instance, the radiotherapeutic agent can be an agent such as those administered in brachytherapy or radionuclide therapy. Such methods can optionally further comprise the administration of one or more additional cancer therapies, such as, but not limited to, chemotherapies, and/or another radiotherapy.

The present invention also relates to a method for preventing or treating melanoma in a subject in need thereof, comprising the step of administering to said subject the compound selected from the group consisting of PAK-1 antagonists or PAK-1 expression inhibitors in combination with one or more melanoma treatment.

The present invention also relates to a method for preventing melanoma metastasis in a subject in need thereof, comprising the step of administering to said subject the compound selected from the group consisting of PAK-1 antagonists or PAK-1 expression inhibitors in combination with one or more melanoma treatment.

The present invention also relates to a method for enhancing therapeutic efficacy of melanoma treatment in a subject in need thereof, comprising the step of administering to said subject the compound selected from the group consisting of PAK-1 antagonists or PAK-1 expression inhibitors.

Pharmaceutical composition and kits of the invention

The compound selected from the group consisting of PAK-1 antagonists or PAK-1 expression inhibitors in combination with one or more melanoma treatment of the invention may be used or prepared in a pharmaceutical composition.

In one embodiment, the invention relates to a pharmaceutical composition comprising the compound selected from the group consisting of PAK-1 antagonists or PAK-1 expression inhibitors in combination with one or more melanoma treatment of the invention and a pharmaceutical acceptable carrier for use in the prevention or treatment of melanoma in a subject in need thereof.

In a further aspect, the invention relates to a pharmaceutical composition comprising the compound selected from the group consisting of PAK-1 antagonists or PAK-1 expression inhibitors in combination with one or more melanoma treatment of the invention and a pharmaceutical acceptable carrier for use in the prevention of melanoma metastasis in a subject in need thereof.

In a further aspect, the invention relates to a pharmaceutical composition comprising the compound selected from the group consisting of PAK-1 antagonists or PAK-1 expression inhibitors of the invention and a pharmaceutical acceptable carrier for use in a method for enhancing therapeutic efficacy of melanoma treatment in a subject in need thereof.

Typically, the compounds of the invention may be combined with pharmaceutically acceptable excipients, and optionally sustained-release matrices, such as biodegradable polymers, to form therapeutic compositions.

"Pharmaceutically" or "pharmaceutically acceptable" refer 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.

In the pharmaceutical compositions of the present invention for oral, sublingual, subcutaneous, intramuscular, intravenous, transdermal, local or rectal administration, the active

principle, alone or in combination with another active principle, can be administered in a unit administration form, as a mixture with conventional pharmaceutical supports, to human beings. Suitable unit administration forms comprise oral-route forms such as tablets, gel capsules, powders, granules and oral suspensions or solutions, sublingual and buccal administration forms, aerosols, implants, subcutaneous, transdermal, topical, intraperitoneal, intramuscular, intravenous, subdermal, transdermal, intrathecal and intranasal administration forms and rectal administration forms.

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

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

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

The compounds of the invention can be formulated into a composition in a neutral or salt form. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

The carrier can also be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and

the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminium monostearate and gelatin.

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

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

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

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

According to the present invention, said compound selected from the group consisting of PAK-1 antagonists or PAK-1 expression inhibitors of the invention and melanoma treatments are contained in the same composition or administrated separately for simultaneous, separate or sequential use in the prevention or treatment of melanoma.

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The invention also provides kits comprising at least one compound selected from the group consisting of PAK-1 antagonists or PAK-1 expression inhibitors in combination with one or more melanoma treatment of the invention. Kits containing the compounds of the invention find use in therapeutic methods.

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Screening method

In a further aspect, the present invention relates to a method of screening a candidate compound for use as a drug for the prevention or treatment of melanoma in a subject in need thereof, wherein the method comprises the steps of: i) providing candidate compounds and ii) selecting candidate compounds that blocks the action of PAK-1.

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The present invention also relates to the candidate compound according to the invention in combination with one or more melanoma treatment for use in the prevention or treatment of melanoma in a subject in need thereof.

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In a further aspect, the present invention relates to a method of screening a candidate compound for use as a drug for the prevention or treatment of melanoma in a subject in need thereof, wherein the method comprises the steps of:

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- providing a cell, tissue sample or organism expressing the PAK-1,
 - providing a candidate compound such as small organic molecule, intra-antibodies, peptide or polypeptide,
 - measuring the activity of the PAK-1,
 - and selecting positively candidate compounds that blocks the action of PAK-1,
- inhibits PAK-1 expression.

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Methods for measuring the activity of the PAK-1 are well known in the art. For example, measuring the PAK-1 activity involves determining ERK and MAPK pathway activation on the PAK-1 cloned and transfected in a stable manner into a CHO cell line or measuring RAF-1

phosphorylation in the presence or absence of the candidate compound (Hisashi Hashimoto et al. Drug Discov Ther. 2009; 3(6):243-246; Ong et al., 2013).

Tests and assays for screening and determining whether a candidate compound is a PAK-1 antagonist are well known in the art. In vitro and in vivo assays may be used to assess the potency and selectivity of the candidate compounds to reduce PAK-1 activity.

Activities of the candidate compounds, their ability to bind PAK-1 and their ability to inhibit PAK-1 activity may be tested using isolated melanoma cells expressing PAK-1, CHO cell line cloned and transfected in a stable manner by the human PAK-1.

Cells expressing another PAKs than PAK-1 may be used to assess selectivity of the candidate compounds.

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: Ivermectin is cytotoxic against LB39-MEL cells but not on normal human fibroblast.

The human melanoma cells LB39-MEL and human normal fibroblasts MRC5 (ATCC CCL171) were seeded at 1×10^4 cells/well and treated with vehicle alone (RPMI/DMSO 0,5%) or with Ivermectin at 5 μ M for 72h. Number of cells after treatment was quantified using a Cellomics Arrayscan microscope.

Figure 2: Pictures illustrating Ivermectin cytotoxicity on LB39-MEL cells

Multifield reconstruction pictures of the effect on LB39-MEL cells of 72h incubation with vehicle (RPMI/DMSO 5%) or Ivermectin (5 μ M) on cell survival. Pictures were taken on a Cellomics Arrayscan microscope (20X objective). 20 fields are displayed for vehicle treated cells and 49 for Ivermectin treated cells.

Figure 3: Ivermectin cytotoxicity on different melanoma cell lines

Ten human melanoma cell lines: LB1319-MEL (wild type NRAS and BRAF); WM266-4 (wild type NRAS and mutated BRAFV600E); BB74-MEL; LB2033-MEL; A375 (wild type

NRAS and mutated BRAFV600E); LB1829-MEL; MZ2-MEL.3 (BRAF-wt and NRAS Q61K); LB583-MEL; LB1757-MEL and LB33-MEL (wild type NRAS and BRAF) and a murine melanoma cell line B16F10 (wild type NRAS and BRAF) were used in this experiment. Freshly extracted mouse thymocytes were used as normal control cells. Cells were cultivated 72h with increasing concentrations of Ivermectin or with vehicle (RPMI/DMSO 0,25%). Surviving cells were counted using a Cell Counter (Coulter).

Figure 4: Intraperitoneal injections of Ivermectin are not toxic for C57Bl/6-IFN γ KO mice

Twenty two C57Bl/6 IFN γ -KO mice were weighed before and after intraperitoneal (IP) injections. Before injections, the average weight of mice was 18,2 grams (left), the group was then split in two groups that received one intraperitoneal injection/day for 7 days of 0,1 mL of vehicle alone (PBS/DMSO 0,25%) or vehicle with Ivermectin at 3,25 mg/kg. After vehicle injections the average weight of mice was 18,3 g (middle). After Ivermectin injections the average weight of mice was 16,7 g (right).

Figure 5: Intraperitoneal injections of Ivermectin are not toxic for NMRI-nude mice

Thirteen NMRI-nu/nu mice were weighed before and after intraperitoneal injections. Before injections the average weight of mice was 25,8 grams (left), the group was then split in two groups that received one intraperitoneal injection/day for 20 days of 0,1 mL of vehicle alone (PBS/DMSO 0,25%) or vehicle with Ivermectin at 3,25 mg/kg. After vehicle injection, the average weight of mice was 27,7 grams (middle). After Ivermectin injections, the average weight of mice was 26,7 grams (right).

Figure 6: Intraperitoneal Ivermectin injections decrease lung metastases implantation of B16F10 murine melanoma cells in C57BL/6 IFN γ -KO mice

B16F10 cells (1×10^5 cells/mouse) were injected intravenously in the tail vein of twenty C57BL/6 IFN γ -KO mice and left untreated for 48h. Then mice were subsequently treated by intraperitoneal injections 1x/day for 7 days with 0,1 mL of vehicle alone (PBS/DMSO 0,25%) or with 3.25mg/kg of Ivermectin. Mice were sacrificed at Day 10, their lungs were removed and the number of metastasis counted in double blind experiments. Number of

metastases/mouse is represented in vehicle treated condition (average at 79) on the left and Ivermectin treated conditions (average at 41) on the right.

Figure 7: Intraperitoneal Ivermectin injections decrease lung metastases implantation of WM266-4 human melanoma cells in Nude mice

WM266-4 cells (1×10^6 cells/mouse) were injected intravenously in the tail vein of thirteen NMRI nu/nu mice and left untreated for 48h. Then mice were subsequently treated by intraperitoneal injection 1x/day for 17 days with 0,1 mL of vehicle alone (PBS/DMSO 0,25%) or with 3.25mg/kg of Ivermectin. Mice were sacrificed at Day 20, their lungs were removed and the number of metastasis was detected using immunohistochemical staining with KBA.62 mAb and counted in double blind experiments. Number of metastases/mouse is represented in vehicle treated condition on the left and Ivermectin treated condition on the right.

Figure 8: Macrocyclic lactones cytotoxicity on BRAF-wt melanoma cells

LB1319-MEL cells were seeded at 5×10^3 cells/well and treated with increasing concentration of Ivermectin, Doramectin, Selamectin, Moxidectin, or Abamectin. After 72h of treatment, cell number was quantified using a Cellomics Arrayscan microscope. Number of cells in the vehicle treated condition was used to normalize results.

Figure 9: Macrocyclic lactones cytotoxicity on BRAFV600E mutated melanoma cells

WM266-4 cells were seeded at 5×10^3 cells/well and treated with increasing concentration of Ivermectin, Doramectin, Selamectin, Moxidectin, or Abamectin. After 72h of treatment, cell number was quantified using a Cellomics Arrayscan microscope. Number of cells in the vehicle treated condition was used to normalize results.

Figure 10: Combination of low doses of Ivermectin with growing concentrations of Vemurafenib do not increase its cytotoxicity to mutated melanoma cells

Effect of Ivermectin alone, Vemurafenib (PLX-4032) alone or Ivermectin plus Vemurafenib combination on BRAFV600E positive melanoma cells. WM266-4 cells were treated with increasing concentrations of Ivermectin alone (0,37; 1; 3 and 10 μ M), or with increasing concentrations of PLX-4032 alone (0,07; 0,22; 0,66 and 2) or with a fixed concentration of Ivermectin (0,37 μ M) plus increasing concentrations of PLX4032 (0,07; 0,22;

0,66 and 2). After 72h of treatment, cell number was quantified using a Cellomics Arrayscan microscope. Number of cells in the untreated condition was used to normalize results.

Figure 11: Combination of a low dose of Ivermectin with increasing doses of Vemurafenib do not enhance its cytotoxicity against WM266-4 mutated melanoma cells

Effect of Ivermectin alone, Vemurafenib (PLX-4032) alone or Ivermectin plus Vemurafenib combination on the survival of BRAFV600E positive melanoma cells. WM266-4 cells were treated with increasing concentrations of Ivermectin alone (0,37; 1; 3 and 10 μ M), or with increasing concentrations of PLX-4032 alone (0,07; 0,22; 0,66 and 2) or with a fixed concentration of Ivermectin (0,37 μ M) plus increasing concentrations of PLX4032 (0,07; 0,22; 0,66 and 2). Surviving cells were counted after 72h incubation using a Cell Counter.

Figure 12: Low doses of Ivermectin, Moxidectin or Abamectin do not potentiate Vemurafenib activity on BRAFV600E mutated melanoma cells

Effect of Ivermectin (I), Moxidectin (M) and Abamectin (A) plus Vemurafenib (V) combinations on the survival of BRAFV600E mutated melanoma cells. WM266-4 cells were treated with a fixed concentration of Vemurafenib (0,66 μ M) alone or with combinations of fixed concentrations (0,37 μ M) of Ivermectin (I0,3), Moxidetin (M0,3) or Abamectin (A0,3) plus a fixed concentration of Vemurafenib (0,66 μ M) (V0,6). Surviving cells were counted after 72h incubation using a Cell Counter. Ivermectin, Moxidectin and Abamectin did not potentiate Vemurafenib activity on BRAFV600E mutated cells.

Figure 13: Combination of a low dose of Ivermectin with increasing doses of Vemurafenib increases its cytotoxicity to BRAF-wt melanoma cells

Effect of Ivermectin alone, Vemurafenib (PLX-4032) alone or Ivermectin plus Vemurafenib combination on BRAF-wt melanoma cells. LB1319-MEL cells were treated with increasing concentrations of Ivermectin alone (0,37; 1; 3 and 10 μ M), or with increasing concentrations of PLX-4032 alone (0,07; 0,22; 0,66 and 2) or with a fixed concentration of Ivermectin (0,37 μ M) plus increasing concentrations of PLX4032 (0,07; 0,22; 0,66 and 2). After 72h of treatment, cell number was quantified using a Cellomics Arrayscan microscope. Number of cells in the untreated condition was used to normalize results.

Figure 14: Combination of a low dose of Ivermectin with increasing doses of Vemurafenib increases its cytotoxicity to BRAF-wt melanoma cells

Pictures were taken on a Cellomics Arrayscan microscope (20X objective) during the experiment presented in Figure 13.

Figure 15: Combination of two low doses of Ivermectin with increasing concentrations of Vemurafenib increases its cytotoxicity to BRAF-wt melanoma cells

Effect of Ivermectin alone, Vemurafenib (PLX4032) alone or Ivermectin plus Vemurafenib combination on the survival of BRAFV600E negative melanoma cells. LB1319-MEL cells were treated with increasing concentrations of Ivermectin alone (0,37; 1; 3 and 10 μ M), or with increasing concentration of Vemurafenib alone (0,07; 0,22; 0,66 and 2) or with fixed concentrations of Ivermectin 1 μ M (I1) or 0,37 μ M (I0,3) plus increasing concentration of Vemurafenib (V) (0,07; 0,22 and 0,66). Surviving cells were counted after 72h incubation using a Cell Counter. Ivermectin potentiates Vemurafenib activity on BRAFV600E negative cells.

Figure 16: Low doses of Ivermectin, Moxidectin or Abamectin potentiate Vemurafenib activity on BRAF-wt melanoma cells

Effect of Ivermectin (I), Moxidectin (M) and Abamectin (A) plus Vemurafenib (V) combinations on the survival of BRAFV600E negative melanoma cells. LB1319-MEL cells were treated with a fixed concentration of Vemurafenib (0,66 μ M) alone or with combinations of fixed concentrations (0,37 μ M) of Ivermectin (I0,3), Moxidectin (M0,3) or Abamectin (A0,3) plus a fixed concentration of Vemurafenib (0,66 μ M) (V0,6). Surviving cells were counted after 72h incubation using a Cell Counter. Ivermectin, Moxidectin and Abamectin potentiate Vemurafenib activity on BRAFV600E negative cells.

Figure 17: Combination of a low dose of Ivermectin with increasing doses of Dabrafenib increases its cytotoxicity to melanoma cells

Effect of Ivermectin alone, Dabrafenib alone (from 0,01 to 0,8 μ M) or Ivermectin (0,37 μ M) plus Dabrafenib (from 0,01 to 0,8 μ M) combinations on WM266-4 melanoma cells. 72h after treatment of melanoma cells, cell number was quantified using a Cellomics Arrayscan microscope. Number of cells in the untreated condition was used to normalize results.

Figure 18: Combination of a low dose of Ivermectin with increasing doses of Dabrafenib increases its cytotoxicity to melanoma cells

Effect of Ivermectin alone, Dabrafenib alone or Ivermectin plus Dabrafenib combination on the survival of BRAFV600E positive melanoma cells. WM266-4 cells were treated with increasing concentrations of Ivermectin alone (0,37; 1; 3 and 10 μ M), or with increasing concentrations of Dabrafenib alone (0,03; 0,09; 0,27 and 0,8) or with a fixed concentration of Ivermectin (0,37 μ M) plus increasing concentrations of Dabrafenib (0,03; 0,09; 0,27 and 0,8). Surviving cells were counted after 72h incubation using a Cellulomics Arrayscan microscope.

Figure 19: Ivermectin potentiates Dabrafenib cytotoxicity on melanoma cells

Ivermectin potentiates Dabrafenib activity on BRAFV600E mutated cells. WM266-4 cells were treated with Dabrafenib alone or in combination with Ivermectin. The cells were treated with Dabrafenib (0,27 or 0,8 μ M) (D0,27 or D0,8) alone or with 0,37 μ M of Ivermectin (I0,37). Surviving cells were counted after 72h incubation using a Cell Counter.

Figure 20: Anti-PAK1 cytotoxicity against melanoma cells

LB39-MEL were seeded at 1×10^3 /well and treated with vehicle alone (RPMI/DMSO 0,5%) or with IPA-3 at 5 μ M for 72h. Number of cells after treatment was quantified using a Cellomics Arrayscan microscope.

Figure 21: Cytotoxicity against melanoma cells of another anti-PAK1 (FRAX 597)

Effect of FRAX 597 (a small molecule pyridopyrimidinone, which is a potent inhibitor of the group I PAKs) on the survival of LB1319-MEL and WM266-4 melanoma cells. Cells were treated with increasing concentrations of FRAX 597 (0,3; 1; 3; 10 and 20 μ M). Surviving cells were counted after 72h incubation using a Cell Counter.

Figure 22 : PAK1 is the molecular target of Ivermectin involved in its cytotoxicity to melanoma cells.

(A) LB1319-MEL cells were untreated or treated for 72 h with PLX-4032 at 0,3 μ M or with Ivermectin at 0,3 μ M, then analyzed by Western Blot for ERK and phospho-ERK expressions. (B) Quantification of p-ERK expression compared to ERK expression is shown.

(C) LB1319-MEL cells were untreated or treated for 72 h with PLX-4032 at 0,3 μ M or with Ivermectin at 0,3 μ M, then analyzed by Western Blot for phospho-RAF1 expression. Actin was used as a loading control. (D) Quantification of p-RAF1 compared to actin is shown.

EXAMPLE:**Material & Methods*****Mice***

Six- to eight-wk-old female NMRI nu/nu (Nude) mice (JANVIER Labs) and C57BL/6 IFN- γ KO mice produced in our animal facility (agreement n°B.31.555.26) were used for *in vivo* experiments. These immunodepressed mice were used to limit the *in vivo* immune response against melanoma cells. All experiments involving mice were done using appropriate conditions of husbandry, experimentation and care, supervised by the Ethic Comity of the Institut Claudius Regaud, under the control of the Regional Comity of Midi-Pyrénées (France). Our protocol was validated and received the agreement numbers ICR-2009-0011 and ICR-2009-0020.

Cell lines

Nine human melanoma cell lines were provided by Pr. T. Boon (Ludwig Institut for Cancer Research, Brussels): LB1319-MEL (wild type NRAS and BRAF); LB39-MEL (wild type NRAS and mutated BRAFV600E); LB33-MEL (wild type NRAS and BRAF); BB74-MEL; LB2033-MEL; LB1829-MEL; MZ2-MEL.3 (BRAF wt and NRAS Q61K); LB583-MEL and LB1757-MEL. One murine melanoma cells B16F10 (wild type NRAS and BRAF) and two human melanoma cells: WM266-4 (wild type NRAS and mutated BRAFV600E) and A375 (wild type NRAS and mutated BRAFV600E) were purchased from the American Type Culture Collection (ATCC).

All tumor cells were maintained in culture by serial passages in culture medium composed of RPMI 1640 medium supplemented with 10% fetal bovine serum (FCS), 1mM glutamine, 1% penicillin-streptomycin-amphotericin B. They were monthly tested to be mycoplasma-free.

Thymuses were harvested from four-wk-old female C57BL/6 IFN- γ KO mice, minced and single-cell suspensions of thymocytes were prepared.

Screening of compounds library.

Library screening was performed on 1280 commercially available components (Sigma Lopack 1280) and 560 proprietary molecules freshly diluted from stock plates. Daughter plates at 1mM were used to screen for potential anti melanoma compounds. Products were distributed

on cells (1 μ L in 199 μ L of medium (RPMI 1640 medium supplemented with 10% fetal bovine serum), at 5 μ M final concentration) using an automatic pipetting robot (Beckman NxP). Cells were treated with compounds for increasing periods of time prior to fixation. Following primary screening, detected hits were re-ordered (all from Sigma) and tested again using freshly prepared compounds.

Evaluation of differential toxicity of Ivermectin on a human melanoma cell line and normal human fibroblasts

Human primary fibroblasts MRC5 (ATCC CCL171) and LB39-MEL melanoma cells were plated at t=0 in 96 well plates (glass bottom slides, Corning) at 1×10^4 per well and treated with vehicle: RPMI-1640 medium supplemented with 10% fetal bovine serum, 1mM glutamine, 1% penicillin-streptomycin-amphotericin B with 0,25% of DMSO (RPMI/DMSO 0,25%) alone or with 5 μ M Ivermectin, final concentration in RPMI/DMSO 0,25%. 24h or 72h post treatment, cells were washed with PBS and incubated with RPMI-1640 medium containing Hoechst 33342 to measure nuclei number, surface and intensity. Nuclei count thus represents cell count in our condition. Cells were scored on a Cellomics Arrayscan Vti automated fluorescence microscope using the cell cycle algorithm. Results are normalized according to the corresponding cell number in vehicle (RPMI/DMSO 0,25%). Results are presented as means $\pm$ standard deviation (SD).

Evaluation of the in vitro killing of melanoma cells or normal murine thymocytes by Ivermectin

Ten human melanoma cell lines, one murine melanoma cell line and normal murine thymocytes were treated *in vitro* with Ivermectin at different doses from 0,5 to 25 μ M for 72h. Thymocytes were plated at 1 or 2×10^6 /well in 24 well plates. Melanoma cells were trypsinized, washed once and plated in culture in 24 well plates. 1,5 mL/well of RPMI 1640 medium supplemented with 10% fetal bovine serum, 1mM glutamine, 1% penicillin-streptomycin-amphotericin B (RPMI) was added with vehicle only (RPMI/DMSO 0,25%) or with increasing doses of Ivermectin. Surviving cells were counted 72 hours later using a Cell Counter (Coulter). The number of cells surviving in vehicle alone was used to normalize results. All conditions were done in triplicate and the experiment was repeated twice.

Evaluation of differential cytotoxicity of Ivermectin and four other macrocyclic lactones (mectins) on human melanoma cells BRAF-wt or BRAFV600E mutated

LB1319-MEL (wild type NRAS and BRAF) and WM266-4 (wild type NRAS and mutated BRAFV600E) were treated *in vitro* for 72h with Ivermectin, Doramectin, Selamectin, Moxidectin or Abamectin at different doses from 0,315 to 20 μ M. Surviving cells were counted after 72 hours of culture with Cellomics Arrayscan microscope and IC50 was calculated for each product.

Pulmonary metastases implantation

To evaluate the impact of repeated intraperitoneal (IP) Ivermectin injections on metastases development:

Twenty C57BL/6-IFN- γ KO mice were injected intravenously (iv) in the tail vein with 1×10^5 B16F10 cells. Two days later and every following day mice were IP injected with 0,1mL of vehicle (PBS/DMSO 0,25%) or 0,1 mL of vehicle containing 3,25 mg/kg of Ivermectin. Mice were sacrificed 10 days later; all mice survived the experiment and did not significantly loose weight. Macroscopic black metastases were detected visually and quantified. These quantifications were done by double blind scoring. The experiment included 10 mice/group.

Thirteen NMRI-nu/nu mice were injected iv in the tail vein with 1×10^6 WM266-4 cells. Two days later and every following day mice were IP injected with 0,1mL of vehicle (PBS/DMSO 0,25%) or 0,1 ml of vehicle containing 3,25 mg/kg of Ivermectin. Mice were sacrificed 20 days later; all mice survived the experiment and did not loose weight. The lungs were removed, fixed in formalin and paraffin embedded to visualize microscopic metastases. Lung metastases were quantified after immunohistochemical (IHC) staining by a pathologist and a biologist. These analyses were done in double blind. The IHC staining was done with KBA.62 mAb ready to use from DAKO revealed by FLEX/HRP (20 min incubation) then by FLEX DAB+ Sub-Chromo (10 min incubation) and FLEX Hematoxylin (5 min incubation). The experiment included 6 or 7 mice/group.

Determination of the therapeutic synergy of Ivermectin with Vemurafenib (PLX4032) and with Dabrafenib

WM266-4 and LB1319-MEL cells were plated at 5×10^3 per well in 96 well plates. Treatments were realized using a two-entry matrix with concentrations of Vemurafenib ranging from 2 μ M to 0,0625 μ M by two-fold serial dilutions (lines contained Vemurafenib dose response). Ivermectin starting concentration was fixed to 10 μ M and decreased to 0,315 μ M by two fold dilutions (columns contained Ivermectin dose response). 72h post treatment, cells were washed with PBS and incubated with RPMI medium containing Hoechst 33342, to measure

nucleus number, surface and intensity. Cells were scored on a Cellomics Arrayscan Vti automated fluorescence microscope using the cell cycle algorithm.

LB1319-MEL and WM266-4 cells were treated *in vitro* for 72h with Ivermectin alone (at 0,37; 1; 3 and 10 μ M) or with Vemurafenib (PLX4032) alone (at 0,07; 0,22; 0,66 and 2 μ M) or with combinations of fixed concentrations of Ivermectin (0,37 or 1 μ M) with increasing concentrations of Vemurafenib. Surviving cells were counted 72 hours later using a Cell Counter (Coulter).

WM266-4 cells were treated *in vitro* for 72h with Ivermectin alone (at 0,37; 1; 3 and 10 μ M) or with Dabrafenib alone (at 0,01; 0,03; 0,09; 0,27 and 0,8 μ M) or with combinations of a fixed concentration of Ivermectin (0,37 μ M) with increasing concentrations of Dabrafenib. Surviving cells were counted 72 h later using a Cell Counter (Coulter) or a Cellomics Arrayscan Vti automated fluorescence microscope.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software. Significance of analyses was assessed by t-test or Tukey one-way ANOVA test. All statistic tests were two-sides. The values are expressed as means $\pm$ standard deviation (SD) in the figures. P-values less than .05 were considered statistically significant.

Results

Screening compounds having a differential toxicity on melanoma cells and normal human fibroblasts

We tested 1280 commercial products and 560 proprietary compounds for their ability to preferentially kill human metastatic melanoma cells (LB39-MEL) versus normal human fibroblasts (MRC5). LB39-MEL and MRC5 cells were incubated with these molecules at 5 μ M for 24h. Cell survival was scored on a Cellomics Arrayscan.

We performed a secondary screening of the identified compounds to eliminate false positive that could be related to technical problems during the acquisition or the experiment itself. The compounds were tested at different times (24h and 72h) and at different concentrations (5 μ M and 0,5 μ M). Cells were also cultivated for 72h with vehicle alone (RPMI/DMSO at 0,5%).

We have highlighted the Ivermectin molecule, which has an IC₅₀ of less than 5 μ M and which has a differential toxicity on LB39-MEL versus MRC5 cells (Figure 1 and illustrated in

Figure 2). Ivermectin (trade name: Stromectol), a FDA approved molecule that is already used for its anti helminthic capacities.

Ivermectin is specifically toxic to melanoma cells

5 To verify that the cytotoxic effect of Ivermectin is not restricted to the screening cell line LB39-MEL, we tested the toxicity of this molecule on ten different human melanoma cell lines and on one murine melanoma cell line B16F10. We used freshly extracted mouse thymocytes as control normal cells. Thymocytes were used as control because T lymphocytes are very sensitive to anticancer therapy and white blood cells often collapse in patients receiving
10 chemotherapy. All cells were cultivated for 72h with increasing concentrations of Ivermectin or with vehicle (RPMI/DMSO 0,25%). Surviving cells were counted using a Cell Counter (Coulter). Thymocytes are resistant to Ivermectin up to the dose of 8 μ M. Conversely all melanoma cells were sensitive to Ivermectin and 5 μ M is a discriminator dose (Figure 3).

Ivermectin is cytotoxic to melanoma cells independently of BRAF status, as cells that
15 do not carry BRAF V600E mutation and mutated cells are killed by Ivermectin.

Ivermectin is not toxic in vivo

To determine whether Ivermectin could be used in preclinical phases for the treatment of metastatic melanoma, we investigated the toxicity of this molecule in mice. The molecule
20 was daily administered by intraperitoneal (IP) injections in C57Bl/6-IFN γ -KO and NMRI-nu/nu mice.

Twenty two C57Bl/6 IFN γ -KO mice were weighed before and after the daily and repeated IP injections (7 days) with vehicle (PBS/DMSO 0,25%) or with Ivermectin at 3,25 mg/kg. Before injections, the average weight of mice was 18,2 grams, after the vehicle
25 (PBS/DMSO 0,25%) injections the average weight was 18,3 g and after Ivermectin injections at 3,25 mg/kg the average weight was 16,7 g. These results show that weight loss (8.8%) induced by Ivermectin injections in C57Bl/6-IFN γ -KO mice is less than 10% and therefore acceptable (Figure 4).

30 Thirteen NMRI-nu/nu mice were weighed before and after the daily and repeated IP injections (20 days) with vehicle (PBS/DMSO 0,25%) or with Ivermectin at 3,25 mg/kg. Before injections the average weight of mice was 25,8 grams. After vehicle injections the average weight was 27,7 grams and after Ivermectin injections the average weight was 26,7 grams.

These results show that mice have taken a bit of weight during the course of experience and that weight loss associated with Ivermectin injections compared to vehicle injections is less than 4% and not significant (Figure 5).

Furthermore, the quantification of white blood cells in peripheral blood of C57Bl/6-IFN γ KO and NMRI-nu/nu mice after these IP injections showed that there was no decrease in circulating white blood cells after Ivermectin injections. Altogether these results confirm that Ivermectin, an FDA approved molecule, is not toxic *in vivo* at the concentration used in our experiments.

Ivermectin decreases metastases implantation in mice

In order to test the efficacy of Ivermectin against metastatic melanoma development *in vivo*, we used two different melanoma models. We initially used the murine metastatic melanoma B16F10 that we injected intravenously (iv) in immunodepressed syngeneic mice C57BL/6 IFN γ -KO at a dose of 1×10^5 cells/mouse. The absence of interferon- γ leads to a very significant decrease of the immune response in the mutant mice. These IFN γ -KO mice were used to ensure that Ivermectin is efficient by itself, without the need of the immune response against the tumor. Following injections into the tail vein, B16F10 cells will produce lung metastases. After these iv injections of B16F10 cells, mice were left untreated for 48 hours, in order to allow the B16F10 melanoma cells to perform the early stages of metastasis (extravasation and implantation) into the lungs. Then the mice were injected intraperitoneally (IP) 1x/day for 7 days with 3.25mg/kg of Ivermectin. Control mice similarly injected with B16F10 cells were only treated with vehicle solution (PBS/DMSO 0.25%). The mice were sacrificed ten days after the injections of B16F10 cells, their lungs were removed and the number of metastasis counted in double blind experiments. The results presented in Figure 6 show that Ivermectin decreases the occurrence of lung metastases *in vivo*. Indeed, mice injected with vehicle alone developed an average of 79 lung metastases. While this number is reduced to 41 in mice treated with Ivermectin at 3,25 mg/kg (Figure 6).

Ivermectin prevents metastasis implantation of human melanoma cells in NMRI nu/nu mice

To test the efficiency of Ivermectin to inhibit the metastases from human melanoma cells *in vivo*, we used NMRI nu/nu mice in which we injected iv WM266-4 cells (1×10^6 cells/mouse). These mice are immunodepressed and human WM266-4 melanoma cells injected

into their tail vein produce lung metastases. After WM266-4 iv injections, mice were left untreated for 48 hours in order to allow these tumor cells to perform the early stages of metastasis into the lungs. The mice were then injected intraperitoneally (IP) 1x/day for 17 days with 3,25mg/kg of Ivermectin or with vehicle (PBS/DMSO 0,25%) alone. The experiment is longer because human melanomas have a longer time of implantation and multiplication in nude mice than B16F10 cells injected in syngeneic C57BL/6 mice. Mice were sacrificed twenty days after the injections of WM266-4, their lungs were removed and the number of metastasis was quantified after immunohistochemical staining with KBA.62 mAb. This anti-melanoma antibody is used in diagnosis of primary and metastatic melanomas but also to detect micro-metastases in sentinel lymph node. The results show that Ivermectin is very efficient to decrease the occurrence of lung metastases. Indeed, the seven mice IP injected with vehicle developed a total of 31 metastases, whereas a single metastasis was observed in one mouse among the six IP injected with Ivermectin at 3,25 mg/kg and five mice did not display any metastasis (Figure 7).

Anti melanoma activity of Ivermectin and several other macrocyclic lactones (mectins)

The anti-melanoma specific activity of Ivermectin and four other mectins: Doramectin, Selamectin, Moxidectin and Abamectin were compared *in vitro* on LB1319-MEL (BRAF-wt) and WM266-4 (BRAFFV600E mutated) melanoma cells.

LB1319-MEL cells were treated with increasing concentrations (from 0,315 to 20 μ M) of Ivermectin or the four other molecules. After 72h incubation, surviving cells were quantified using a Cellomics ArrayScan microscope. All mectins are efficient to kill *in vitro* LB1319-MEL melanoma cells. This allowed us to determine their IC₅₀ on BRAF-wt melanoma cells. Ivermectin and Moxidectin are slightly more efficient at killing these melanoma cells (IC₅₀ = 3 μ M), than the other mectins (IC₅₀ = 4 μ M) (Figure 8).

WM266-4 cells were also treated with Ivermectin and the four other mectins and analyzed as previously. The results obtained with this BRAFFV600E mutated melanoma cell line show that Ivermectin and all the other mectins are effective to kill melanoma cells bearing the BRAF V600E mutation. In this tumor model also, the Moxidectin is the most efficient molecule with an IC₅₀ of 2,5 μ M versus 4 or 5 μ M for the other molecules (Figure 9). Moxidectin has the highest activity and the simplest chemical structure. Therefore it potentially represents the minimal structure required for anti melanoma activity of these macrocyclic

lactones, but deep structure relationship determination will be required to confirm this hypothesis.

Cytotoxic sensitization of Vemurafenib action by Ivermectin

5 Vemurafenib (PLX-4032) is a pharmacological molecule designed to inhibit the constitutive activation of the BRAFV600E mutated protein involved in melanoma metastasis. It has a therapeutic effect only in patients with metastatic melanomas bearing this mutation, and its effect is often temporary. We have tested the capacity of Ivermectin to potentiate the anti melanoma activity of Vemurafenib on human melanoma cells. Melanoma cells were treated *in*
10 *vitro* for 72 hours with increasing doses of Vemurafenib (PLX4032) and Ivermectin, alone or in combination. These experiments were performed on WM266-4 melanoma cells carrying the BRAF-V600E mutation and on LB1319-MEL cells, which do not carry this mutation.

WM266-4 cells were treated with increasing concentrations of Vemurafenib alone (from 0,0625 to 2 μ M), or with increasing concentrations of Ivermectin alone (from 0,315 to 10 μ M)
15 or with increasing combinations of both molecules. An illustration is presented in Figure 10 showing no increase in cytotoxicity induced by a 72h treatment with a combination of a fixed concentration at 0,315 μ M of Ivermectin, which does not induce tumor cells death, associated with increasing concentrations of Vemurafenib (from 0,0625 to 2 μ M). The number of WM266-4 cells was quantified using a Cellomics ArrayScan microscope.

20 WM266-4 cells were also treated with increasing concentrations of Vemurafenib alone (0,07; 0,22; 0,66 and 2 μ M), or with increasing concentrations of Ivermectin alone (from 0,37; 1; 3 and 10 μ M) or with a fixed concentration at 0,37 μ M of Ivermectin, which does not induce tumor cells death, associated with increasing concentrations of Vemurafenib (0,07; 0,22; 0,66 and 2 μ M) after 72h incubation, the number of WM266-4 cells was quantified using a Cell
25 Counter (Figure 11).

The results of these treatments show that both Ivermectin (as also previously shown in Figure 3) and Vemurafenib are cytotoxic to melanoma cells carrying the mutation BRAFV600E, and that the combination Ivermectin plus Vemurafenib is not more cytotoxic to mutated melanoma cells than Vemurafenib alone.

30 The capacity of low doses of Moxidectin and Abamectin (0,37 μ M) to increase the cytotoxicity of Vemurafenib on BRAFV600E mutated WM266-4 melanoma cells was also tested. As Ivermectin, these two other macrocyclic lactone did not potentiate the Vemurafenib-induced killing of the mutated melanoma cells (Figure 12).

LB1319-MEL cells were similarly treated and analyzed but the obtained results were different from those observed with the mutated BRAFV600E melanoma cell line. Indeed as previously described, Vemurafenib is almost not cytotoxic to the BRAF-wt melanoma cells. Conversely, Ivermectin alone is capable of killing these melanoma cells in a dose dependent manner (as also previously shown in Figure 3). However, the most interesting result is that Ivermectin potentiates the action of Vemurafenib on melanoma cells that do not carry the BRAFV600E mutation (Figures 13, 14 and 15). Indeed, as illustrated in Figure 13 for a combination of Ivermectin and Vemurafenib, the association of a fixed and very low dose of Ivermectin ineffective by itself ($0,315 \mu\text{M}$, $= 1/10 \text{ IC}_{50}$ alone), with Vemurafenib triggers a significant cytotoxicity against un-mutated melanoma cells. In Figure 14 are presented pictures showing sensitization to lysis by Vemurafenib induced by a low dose of Ivermectin ($0,3 \mu\text{M}$) on LB1319-MEL. This sensitization to Vemurafenib cytotoxicity is also observed with surviving LB1319-MEL cells counted after 72h incubation using a Cell Counter (Figure 15). These melanoma cells were treated with Ivermectin alone ($0,3$, 1 , 3 or $10 \mu\text{M}$) or with Vemurafenib alone ($0,07$, $0,22$, $0,66$, $1 \mu\text{M}$) or with two combinations of low doses of Ivermectin (1 and $0,3 \mu\text{M}$) with the increasing concentrations of Vemurafenib.

Then the inventors tested the capacity of two other macrocyclic lactones: Moxidectin and Abamectin to increase the cytotoxicity of Vemurafenib on LB1319-MEL melanoma cells, which are not mutated on BRAFV600E and consequently insensitive to Vemurafenib killing. LB1319-MEL cells were treated with Vemurafenib alone ($0,66 \mu\text{M}$) or with combinations of a low dose of the macrocyclic lactone ($0,3 \mu\text{M}$) with $0,66 \mu\text{M}$ of Vemurafenib. As Ivermectin these two other mectins potentiated the Vemurafenib-induced killing of these BRAF-wt melanoma cells (Figure 16).

Therefore, combinations of low concentration of Ivermectin or other macrocyclic lactones with normal concentration of Vemurafenib lead to cytotoxic potentialization that convert a Vemurafenib resistant cell line into a sensitive one. This result opens the field for combinatory therapies being independent of the BRAF mutation status.

Cytotoxic sensitization of Dabrafenib action by Ivermectin.

Dabrafenib, like Vemurafenib, is a promising pharmacological molecule inhibiting the constitutive activation of the BRAFV600E mutated protein and having a therapeutic effect in patients with metastatic melanomas bearing this mutation. We have tested the capacity of Ivermectin to potentiate the anti melanoma activity of Dabrafenib on human melanoma cells.

5 WM266-4 melanoma cells carrying the BRAF-V600E mutation were treated *in vitro* for 72 hours with increasing doses of Dabrafenib and Ivermectin, alone or in combination.

WM266-4 cells were treated with concentrations of Dabrafenib alone (from 0,01 to 0,8 μ M) or with concentrations of Ivermectin alone (from 0,37 to 10 μ M) or with combinations of both molecules: a fixed concentration at 0,37 μ M of Ivermectin, which does not induce tumor cells death, associated with increasing concentrations of Dabrafenib (from 0,01 to 0,8 μ M). After 72h incubation, the number of WM266-4 cells was quantified using a Cellomics ArrayScan microscope. The results show that the combination Ivermectin plus Dabrafenib is more cytotoxic to mutated melanoma cells than Dabrafenib alone. This effect is most visible for the low concentrations of Dabrafenib. Thus, if Dabrafenib alone achieves maximal effect at 0,8 μ M and is linked to side effects in patients, our results show that combining Ivermectin with a nine fold reduction in Dabrafenib concentration (0,09 μ M) triggers the maximal cytotoxic effect (Figures 17 and 18).

WM266-4 cells were also treated with two concentrations of Dabrafenib alone (0,27 and 0,8 μ M) or with two combinations of a fixed concentration at 0,37 μ M of Ivermectin, which does not induce tumor cells death, associated with Dabrafenib at 0,27 and 0,8 μ M. After 72h incubation, the number of WM266-4 cells was quantified using a Cell Counter. The results confirmed that the combination Ivermectin plus Dabrafenib is more cytotoxic to melanoma cells than Dabrafenib alone (Figures 19).

Therefore, combinations of low concentrations of Ivermectin with Dabrafenib should lead to an increased therapeutic effect. Therefore, lower doses of Dabrafenib can be used, limiting adverse effects.

Molecular target of Ivermectin

A previous publication has shown that the Serine/threonine p21-activated protein kinase 1 (PAK1) is a new target of Ivermectin and that Ivermectin blocks the PAK1- dependent growth of human ovarian cancer and NF2 tumor cell lines (*Hisashi Hashimoto et al. Drug Discov Ther. 2009; 3(6):243-246*). Ivermectin concentrations needed to inactivate PAK-1 in ovarian and

NF2 tumors (IC50 between 5-20 μ M) are similar to the concentrations used in our tests to induce melanoma killing, suggesting that Ivermectin target could be the same in these tumors. This hypothesis was strengthened by the fact that two other PAK1 inhibitors: IPA3 and FRAX597 are similarly able to kill melanoma cells as we observed with LB39-MEL and WM266-4 melanoma cells (Figure 20 and 21).

PAK proteins, a family of serine/threonine p21-activated kinases, include PAK1, PAK2, PAK3 and PAK4. They are critical effectors that link the Rho-Rac-Cdc42 GTPases to cytoskeleton reorganization and nuclear signaling and have been implicated in a wide range of biological activities. PAK1 is a key player in several pathways, whose perturbation is known to be oncogenic. Among others functions PAK1 regulates cell motility and morphology.

The rationale for PAK1 being Ivermectin target in melanoma cytotoxicity is due to the fact that Ivermectin inactivates PAK1, which inhibits the phosphorylation of the kinase RAF-1 (CRAF) at Ser338 (p-Raf1) and consequently MAPK cascade. MAPK pathway is involved in cancer induction, maintenance and progression. RAF proteins interact, including BRAF activates CRAF, and both proteins can activate MEK (*J. Downward et al. Nature Med 2011, vol 17, p 286-288*). So, in melanomas, the inhibition of RAF1 phosphorylation by Ivermectin, via PAK1, could inhibit MEK activation and consequently tumor development.

The inventors also demonstrated that PAK1 is the Ivermectin target involved in melanoma treatment sensitivity.

Conclusion

The low efficiency of conventional treatments against metastatic melanomas, such as Dacarbazine or IFN α , led clinicians to promote new targeted chemotherapy using inhibitors of MAPK pathway and particularly inhibitors of the mutated BRAFV600E protein (Vemurafenib or Dabrafenib). These inhibitors are active only in patients bearing melanoma expressing BRAF V600E mutated protein (about 50% of patients), where they induce a dramatic reduction in the number and size of metastases, which unfortunately in the majority of patients, is transient. Therefore, these treatments often do not significantly prolong survival of patients. In this case, patients are in therapeutic failure. Our results show that Ivermectin and other PAK1 inhibitors appear extremely promising against metastatic melanoma since their effects are independent of the presence of the BRAF V600E mutation. Indeed, among the melanoma lines we tested, LB33-MEL, LB1319-MEL, MZ2-MEL.3 and B16F10, among others, do not carry the BRAF mutation. Ivermectin and several macrocyclic lactones variants have the capacity to kill all the melanoma cells, including the BRAFV600E negative cells. Moreover Ivermectin and these

macrocyclic lactones have a second particularly interesting property. Our study of potentialization showed that addition of very low doses of Ivermectin, or the other mectins, at doses corresponding to 1/10 of their IC50, potentiate the action of Vemurafenib on melanoma cells that do not carry the BRAFV600E mutation, and which are normally insensitive to Vemurafenib induced cytotoxicity. Therefore, treatment protocols combining Vemurafenib with Ivermectin or the other mectins are expected to be efficient for all metastatic melanoma patients. Moreover this low dose of Ivermectin also potentiates the action of Dabrafenib on melanoma cells and should lead to an increased therapeutic effect or a drastic reduction of side effects in patients. Therefore treatments protocols combining Vemurafenib or Dabrafenib with Ivermectin or the other mectins could be done with reduced doses of BRAFV600E inhibitors, which is expected to reduce the toxicity of these treatments.

With a global market of melanoma expected to reach 3 billion dollars annually in 2021, the opportunity to include all patients in the same clinical protocol provides exceptional medical and financial perspectives. We therefore claim the use of Ivermectin, or other mectins, for the treatment of metastatic melanoma in combination with inhibitors of MAPK pathway. As Ivermectin concentrations used in our tests are similar to those used for its normal FDA approved anti helminthic use, this molecule should directly enter into a Phase I/II Fast Track Clinical Trial. If its combination with anti MAPK pathway products is proven to be efficient in patients, Ivermectin would provide an asset to current treatments and would potentiate their action. Ivermectin could become the reference molecule for treatment of metastatic melanoma not carrying BRAF V600E mutation.

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.

Dossett LA, Kudchadkar RR, Zager JS. BRAF and MEK inhibition in melanoma. Expert Opin Drug Saf. 2015 Feb 4;1-12

Francesco Spagnolo, Paola Ghiorzo and Paola Queirolo. Overcoming resistance to BRAF inhibition in BRAF-mutated metastatic melanoma. Oncotarget, 2014, Vol. 5, No. 21 p102-21

Glitza I C and Davies M A. Genotyping of cutaneous melanoma. Chin Clin Oncol. 2014, vol 3 p27- doi:10.3978/j.issn.2304-3865.2014.03.01

Hisashi Hashimoto et al. Drug Discov Ther. 2009; 3(6):243-246; Ong et al., 2013

Holderfield M. et al. Targeting RAF kinases for cancer therapy: BRAF-mutated
5 melanoma and beyond. Nature Reviews Cancer 14, 455–467 (2014) doi:10.1038/nrc3760

Ilieva KM. et al., Effects of BRAF mutations and BRAF inhibition on immune
responses to melanoma. Mol Cancer Ther. 2014 Dec;13(12):2769-83

Lespine A. Lipid-like properties and pharmacology of the anthelmintic macrocyclic
lactones. Expert Opin Drug Metab Toxicol. 2013 Dec; 9(12):1581-95

10 Long GV. et al., Combined BRAF and MEK inhibition versus BRAF inhibition alone
in melanoma. N Engl J Med. 2014 Nov 13;371(20):1877-88)

Mehnert JM, Kluger HM. Driver mutations in melanoma: lessons learned from bench-
to-bedside studies. Curr Oncol Rep. 2012, vol 14: p 449-457

Roulstone V. et al., BRAF- and MEK-targeted Small Molecule Inhibitors Exert
15 Enhanced Anti-Melanoma Effects in Combination with Oncolytic Reovirus through ER Stress.
Mol Ther. 2015 Jan 26

CLAIMS:

1. A method for preventing or treating melanoma in a subject in need thereof, comprising the step of administering to said subject a compound selected from the group consisting of
5 PAK-1 antagonists or PAK-1 expression inhibitors in combination with one or more melanoma treatment.
2. A method for preventing melanoma metastasis in a subject in need thereof, comprising the step of administering to said subject a compound selected from the group consisting of
10 PAK-1 antagonists or PAK-1 expression inhibitors in combination with one or more melanoma treatment.
3. A method for enhancing therapeutic efficacy of melanoma treatment in a subject in need thereof, comprising the step of administering to said subject the compound selected from the group consisting of PAK-1 antagonists or PAK-1 expression inhibitors.
4. The method according to any one of claims 1 to 3 wherein said PAK-1 antagonist is a
15 macrocyclic lactone.
5. The method according to claim 4 wherein said macrocyclic lactone is selected from Ivermectin, Doramectin, Selamectin, Moxidectin, Milbemycin, Abamectin, Nemadectin and Eprinomectin.
6. The method according to any one of claims 1 to 5 wherein said melanoma treatment is
20 Vemurafenib and/or Dabrafenib.
7. A pharmaceutical composition comprising a compound selected from the group consisting of PAK-1 antagonists or PAK-1 expression inhibitors in combination with one or more melanoma treatment and a pharmaceutical acceptable carrier for use in the prevention or treatment of melanoma in a subject in need thereof.
- 25 8. A pharmaceutical composition comprising a compound selected from the group consisting of PAK-1 antagonists or PAK-1 expression inhibitors and a pharmaceutical acceptable carrier for use in a method for enhancing therapeutic efficacy of melanoma treatment in a subject in need thereof.

9. A method of screening a candidate compound for use as a drug for the prevention or treatment of melanoma in a subject in need thereof, wherein the method comprises the steps of:

- providing a cell, tissue sample or organism expressing the PAK-1,
- providing a candidate compound such as small organic molecule, intra-antibodies,
5 peptide or polypeptide,
- measuring the activity of the PAK-1,
- and selecting positively candidate compounds that blocks the action of PAK-1,
inhibits PAK-1 expression.

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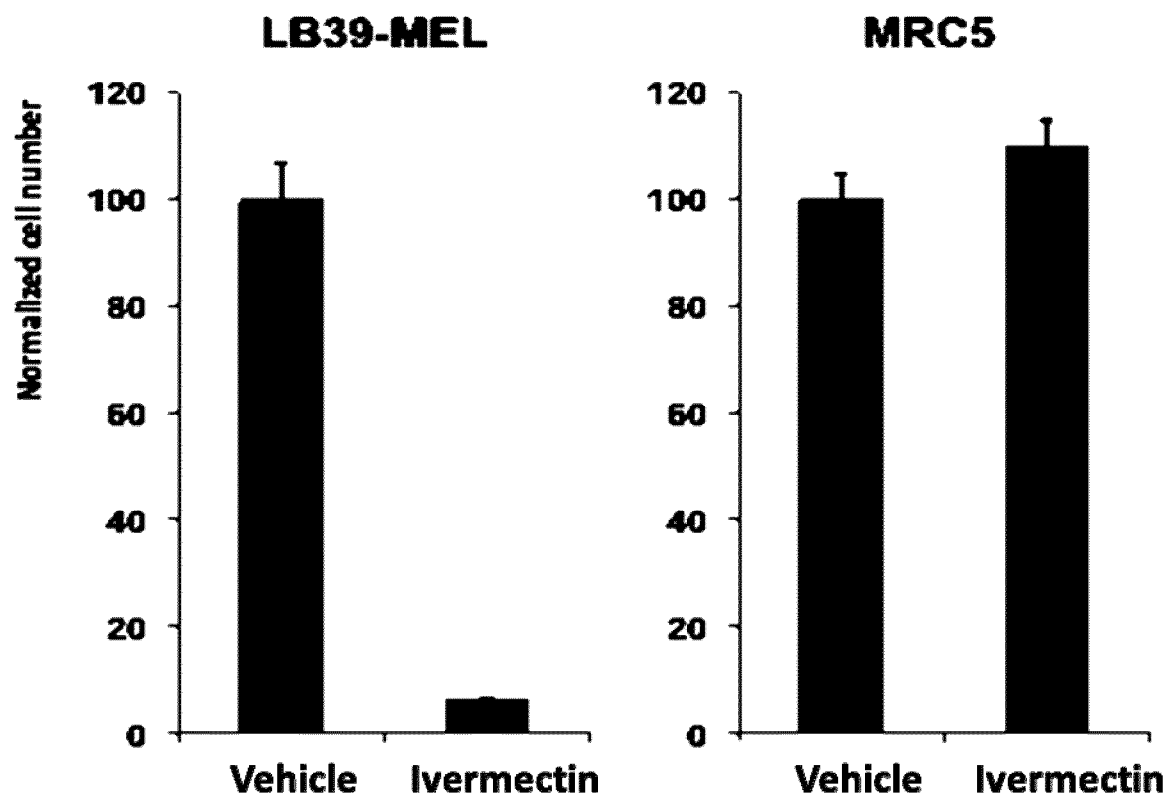
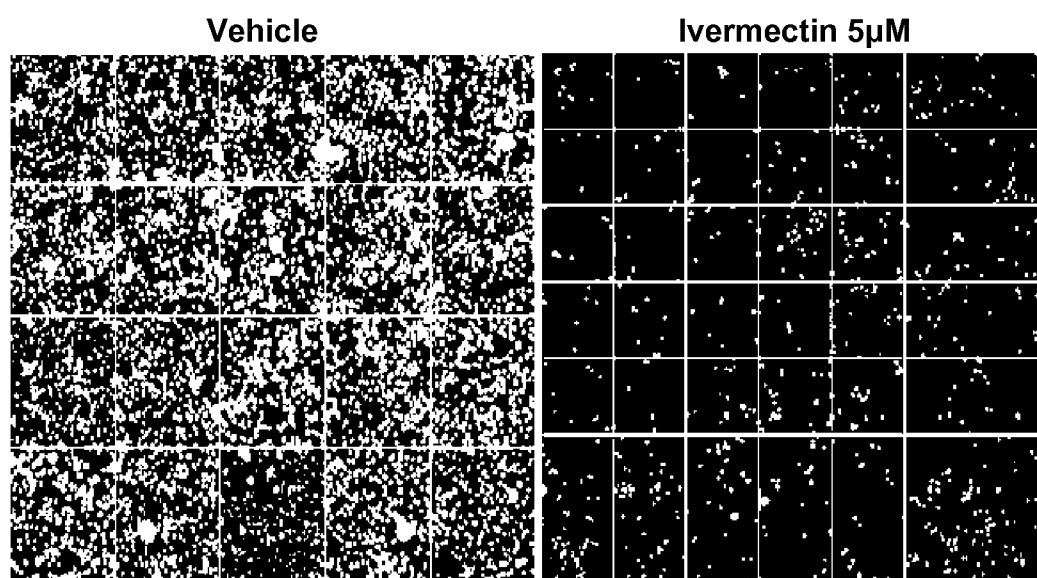


Figure 1

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**Figure 2**

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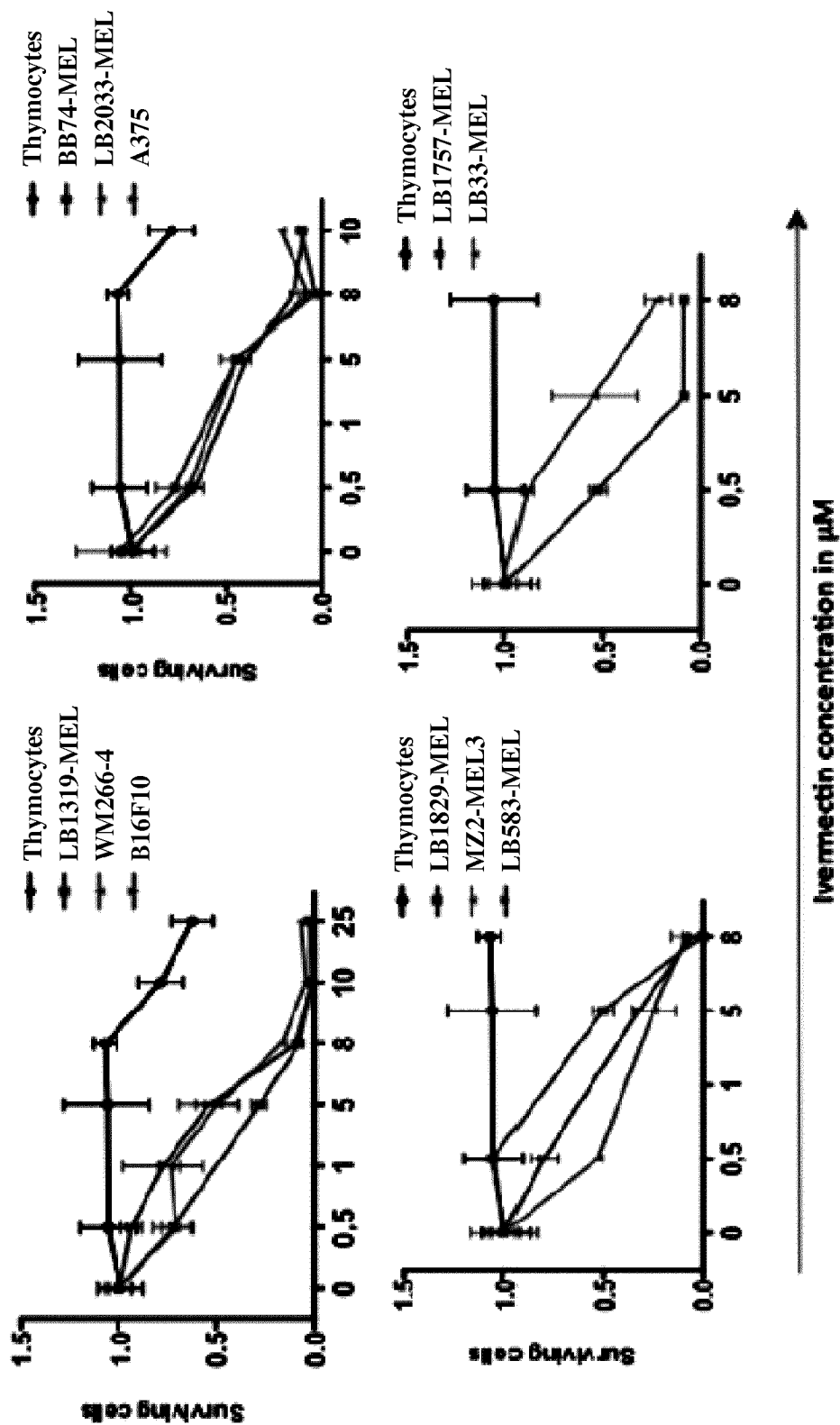


Figure 3

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C57BL6- $\text{INF}\delta\text{KO}$ mice weight

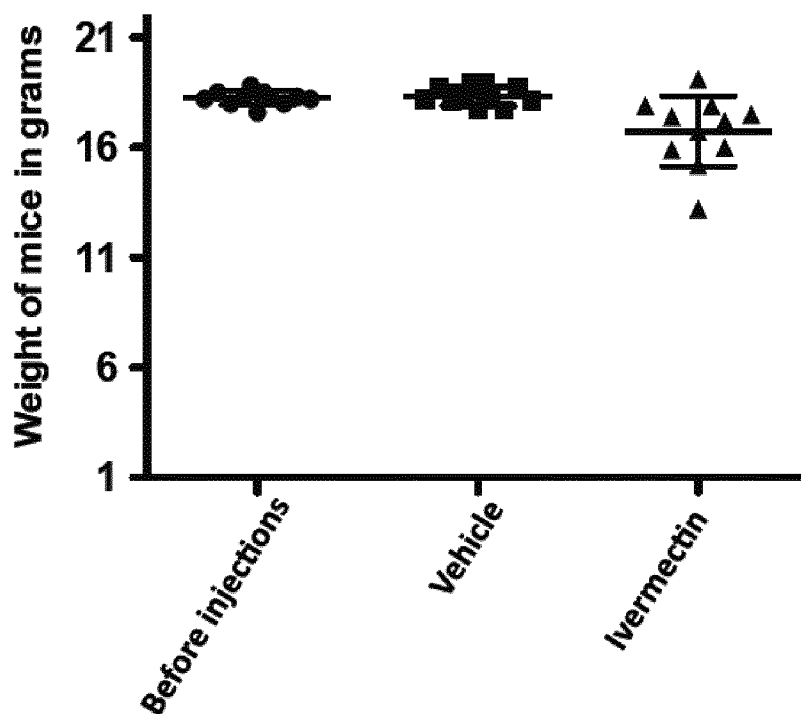


Figure 4

NMRI-nu mice weight

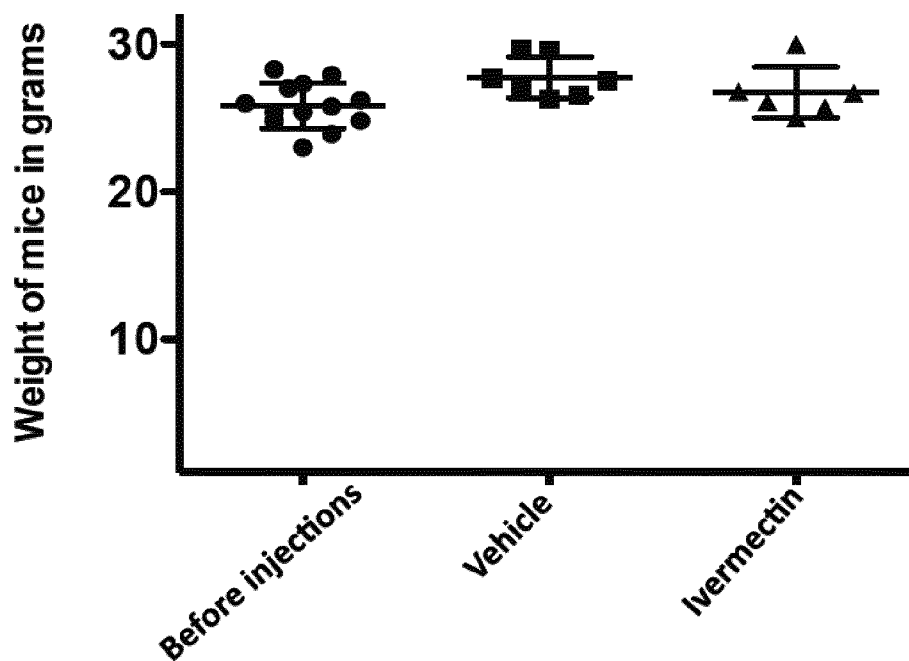


Figure 5

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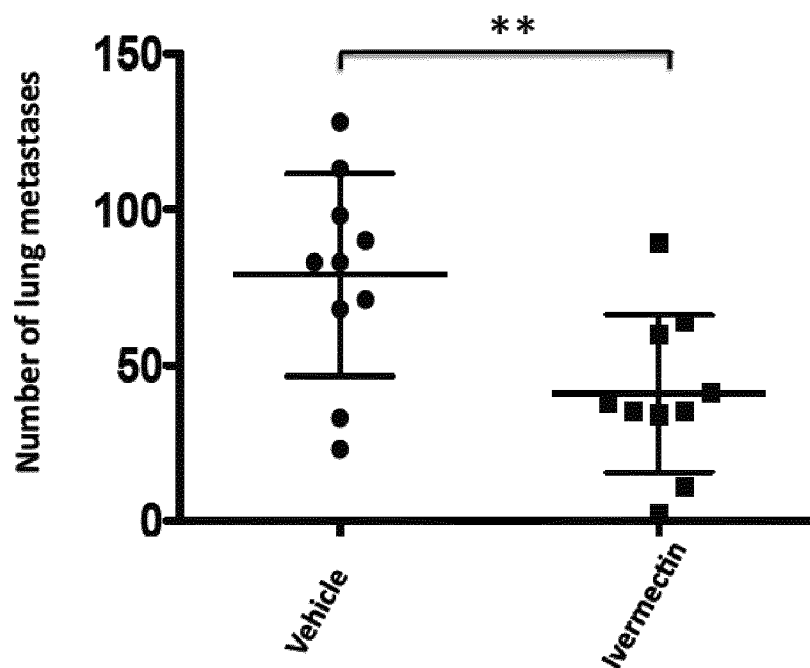
B16F10 metastases in C57BL/6-IFN γ KO mice

Figure 6

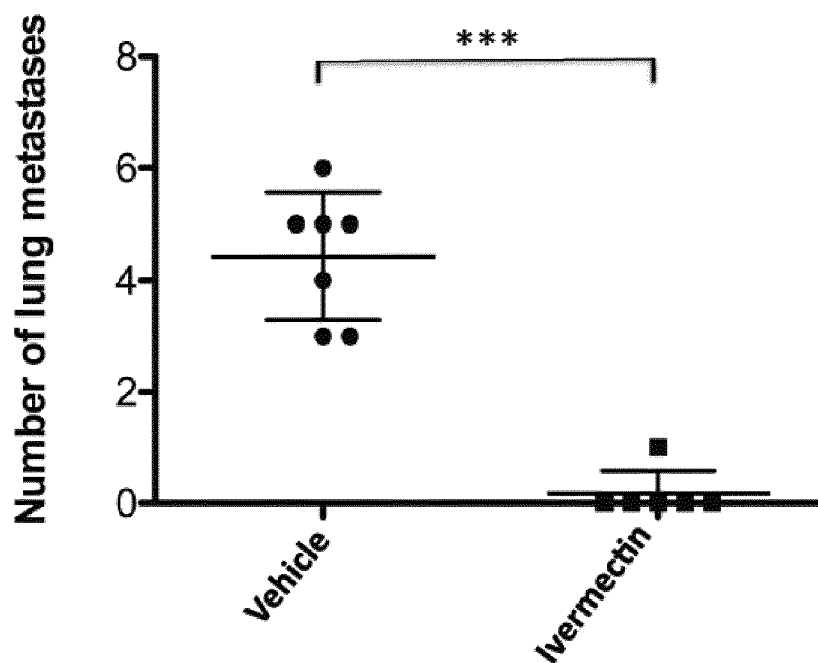
WM266-4 metastases in Nude mice

Figure 7

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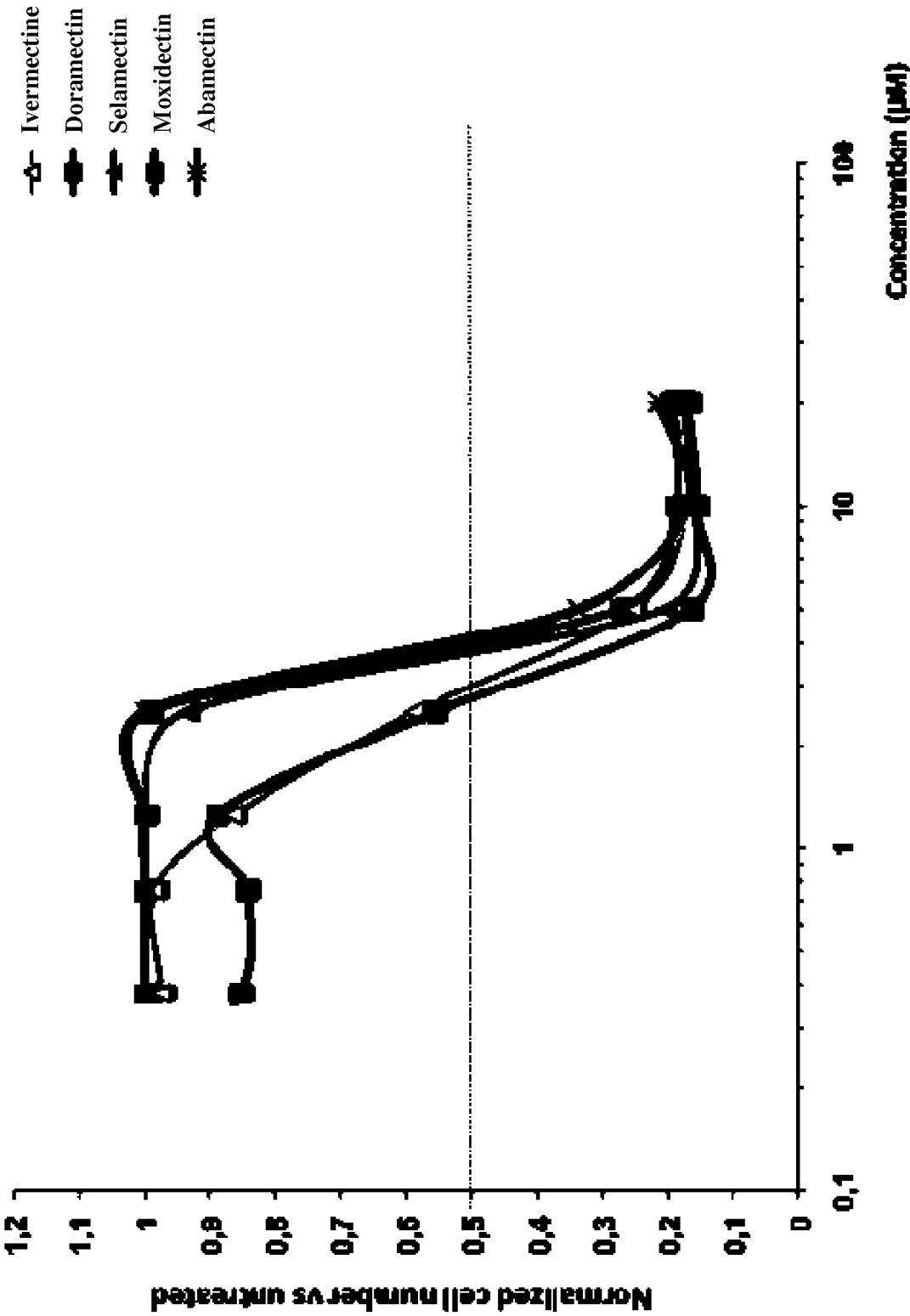


Figure 8

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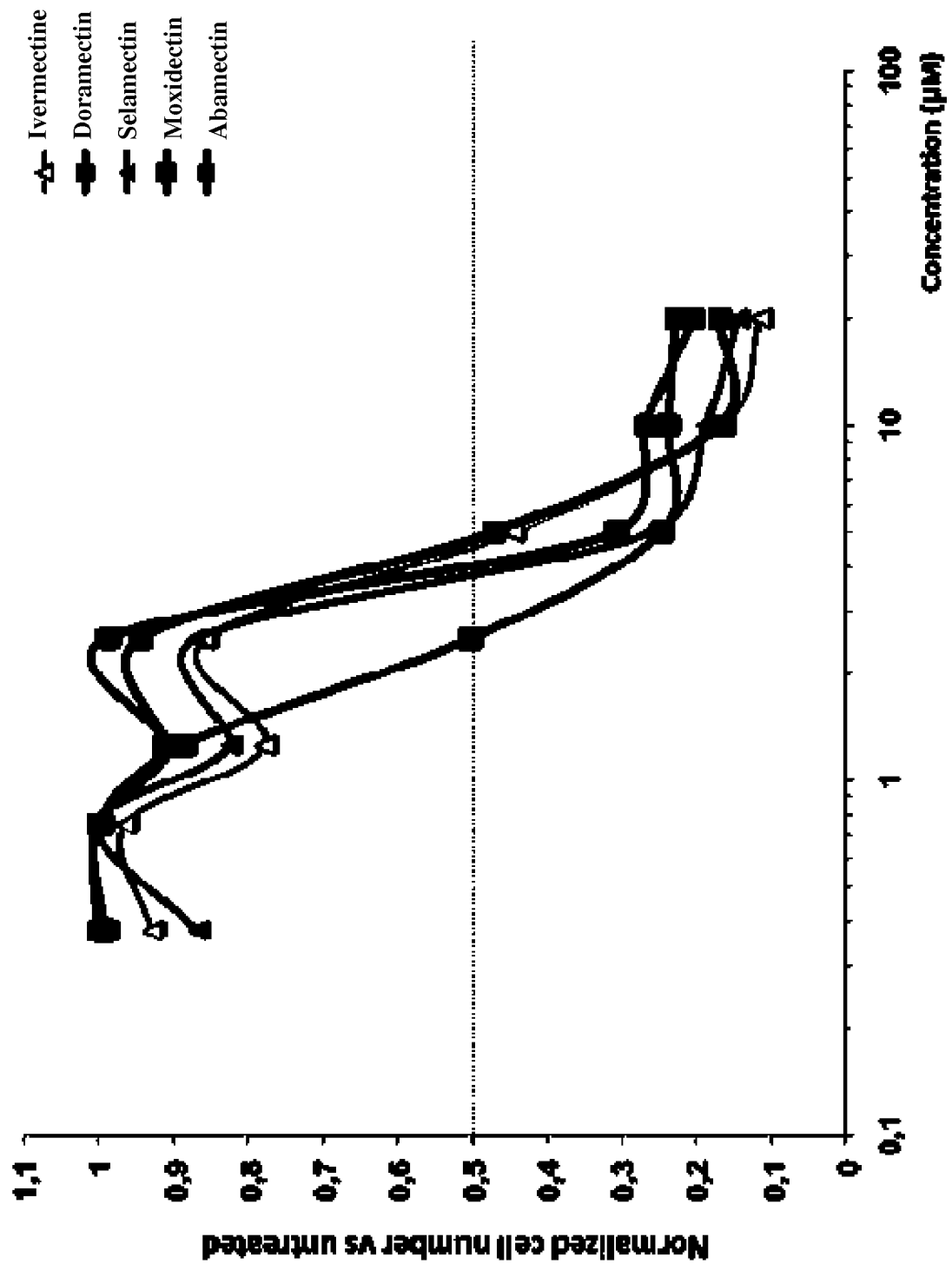


Figure 9

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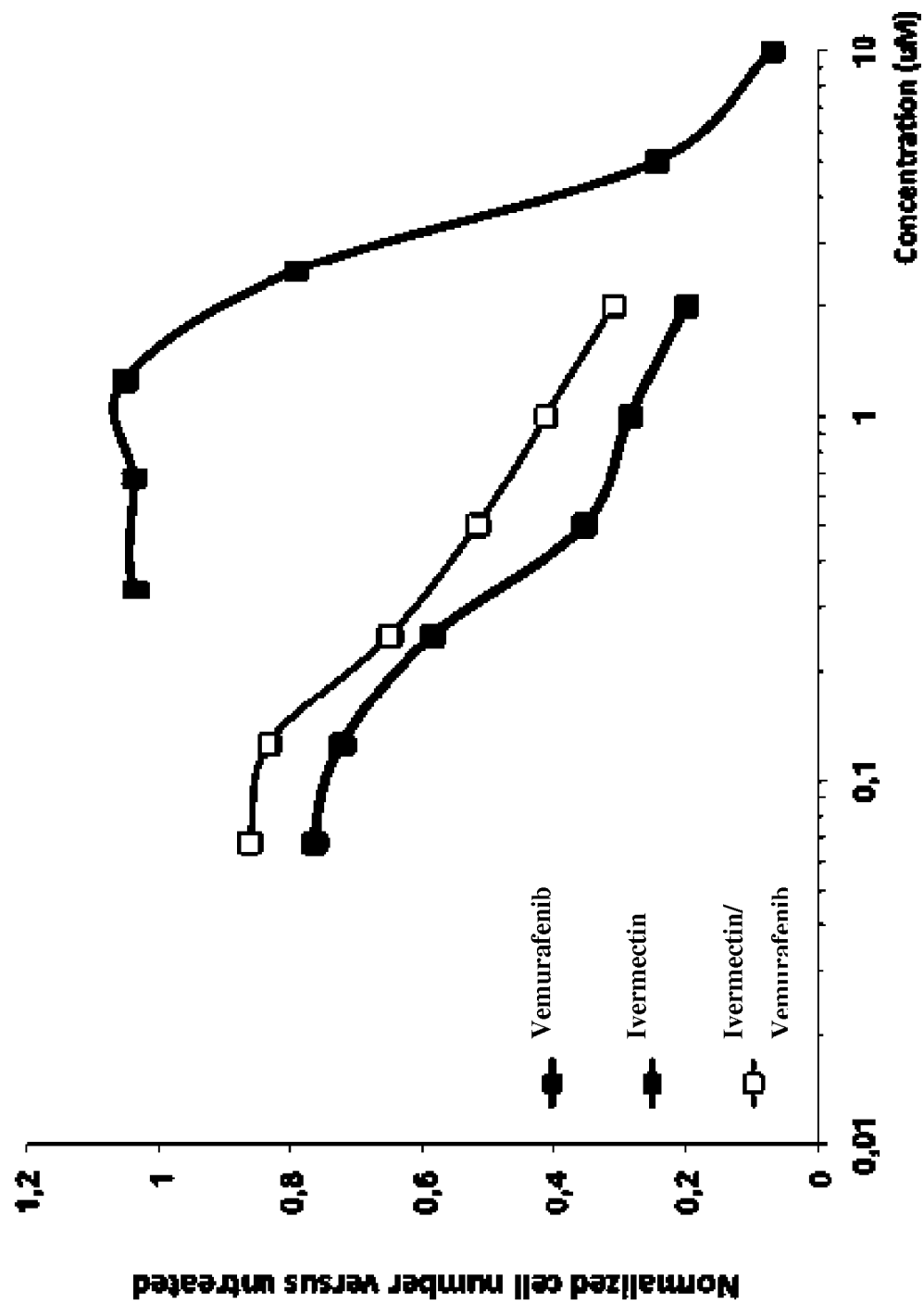


Figure 10

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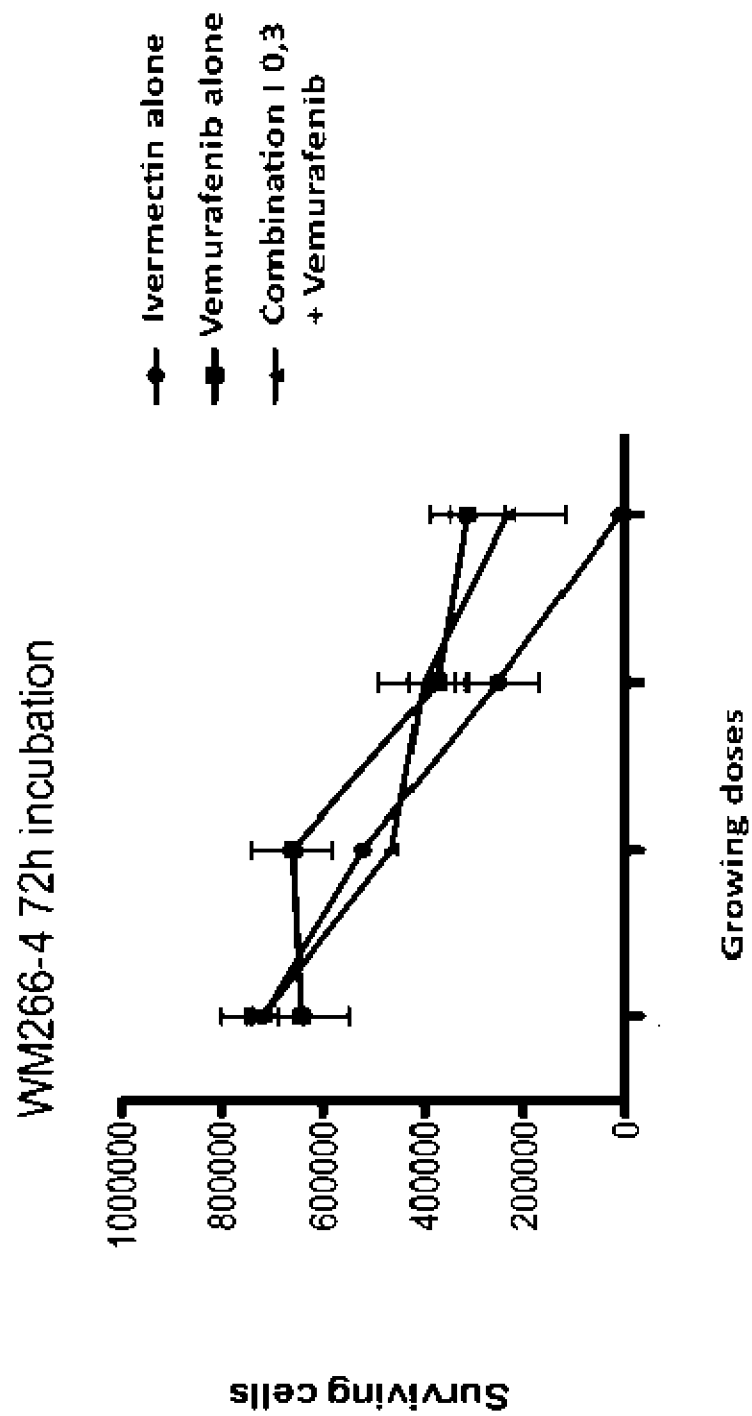


Figure 11

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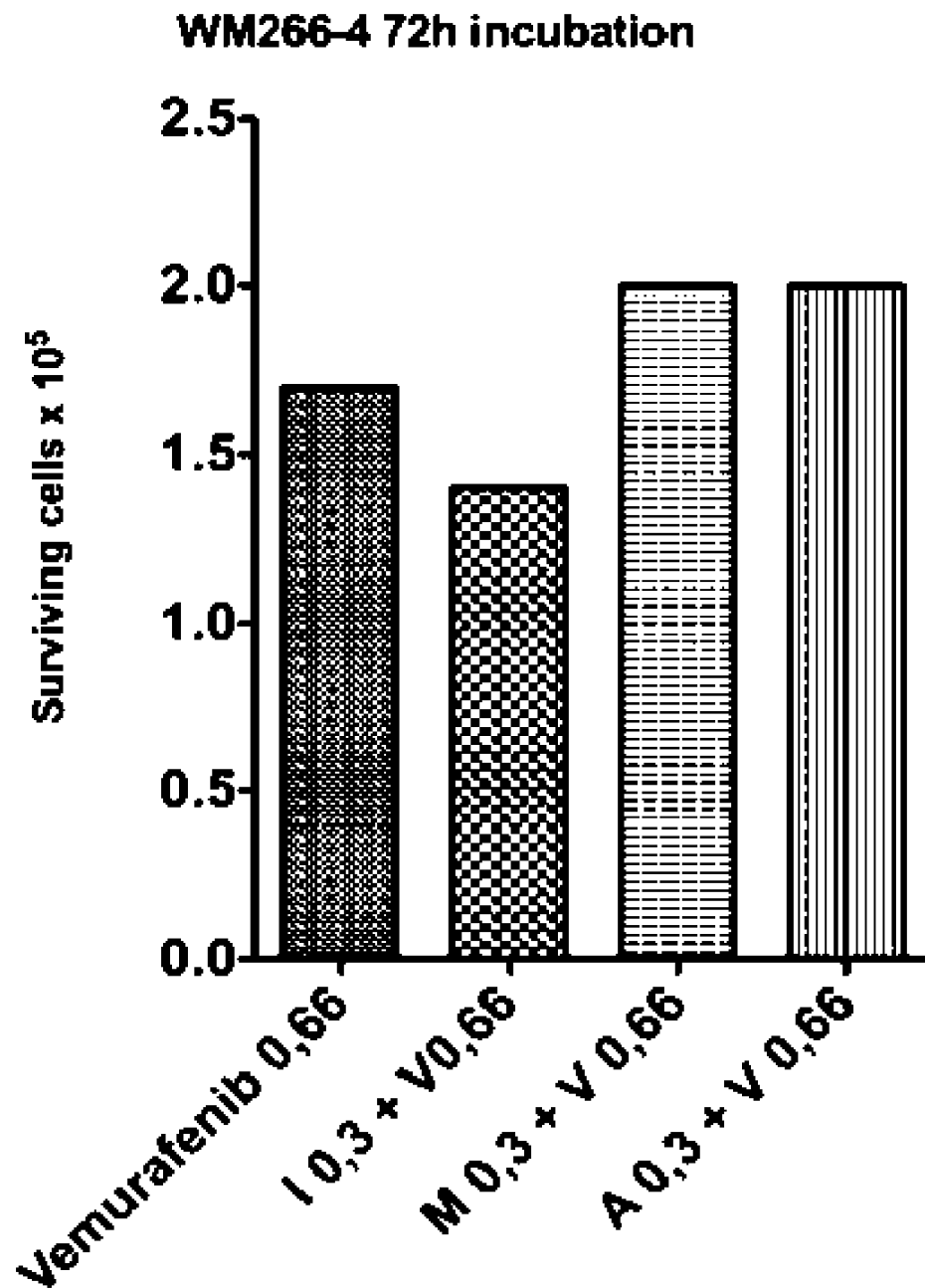


Figure 12

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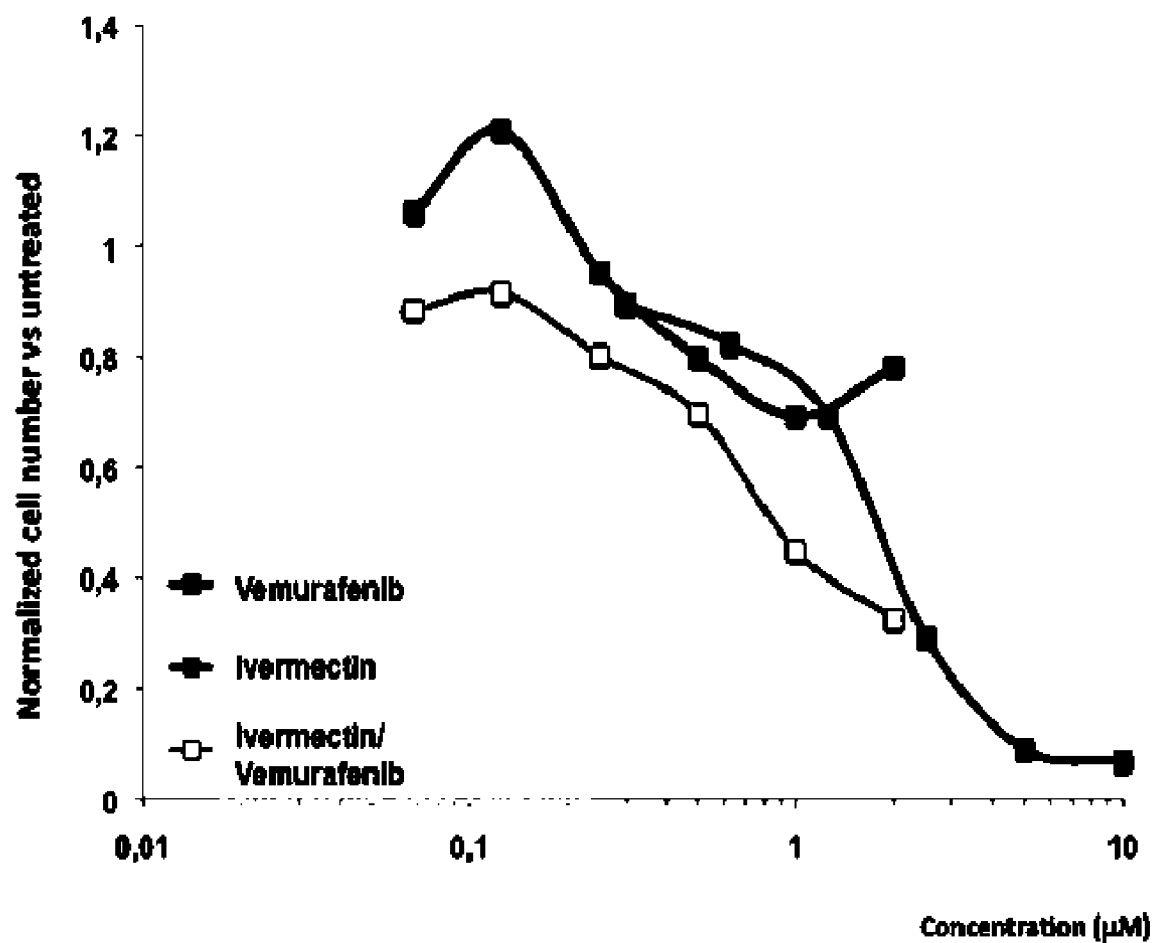


Figure 13

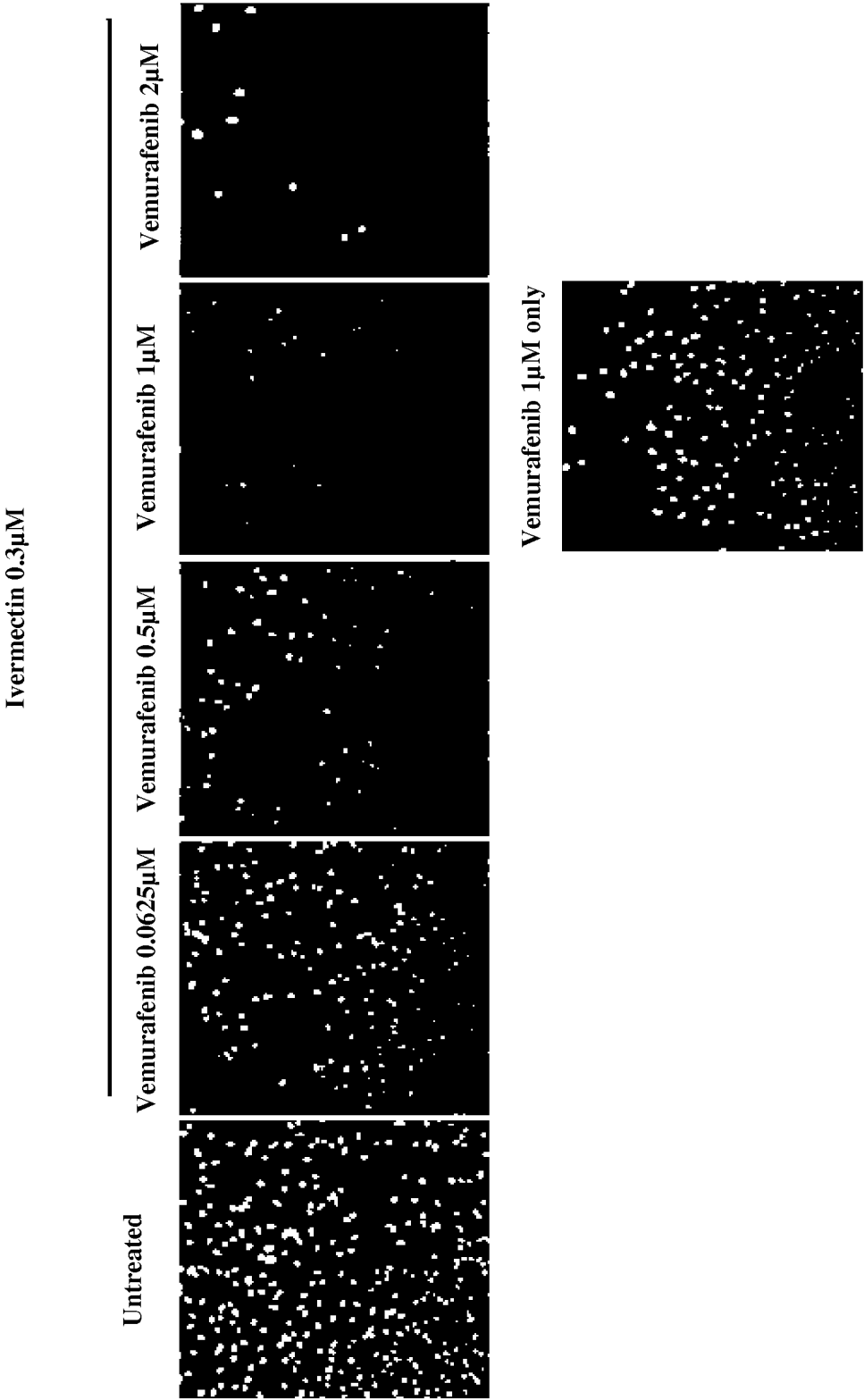


Figure 14

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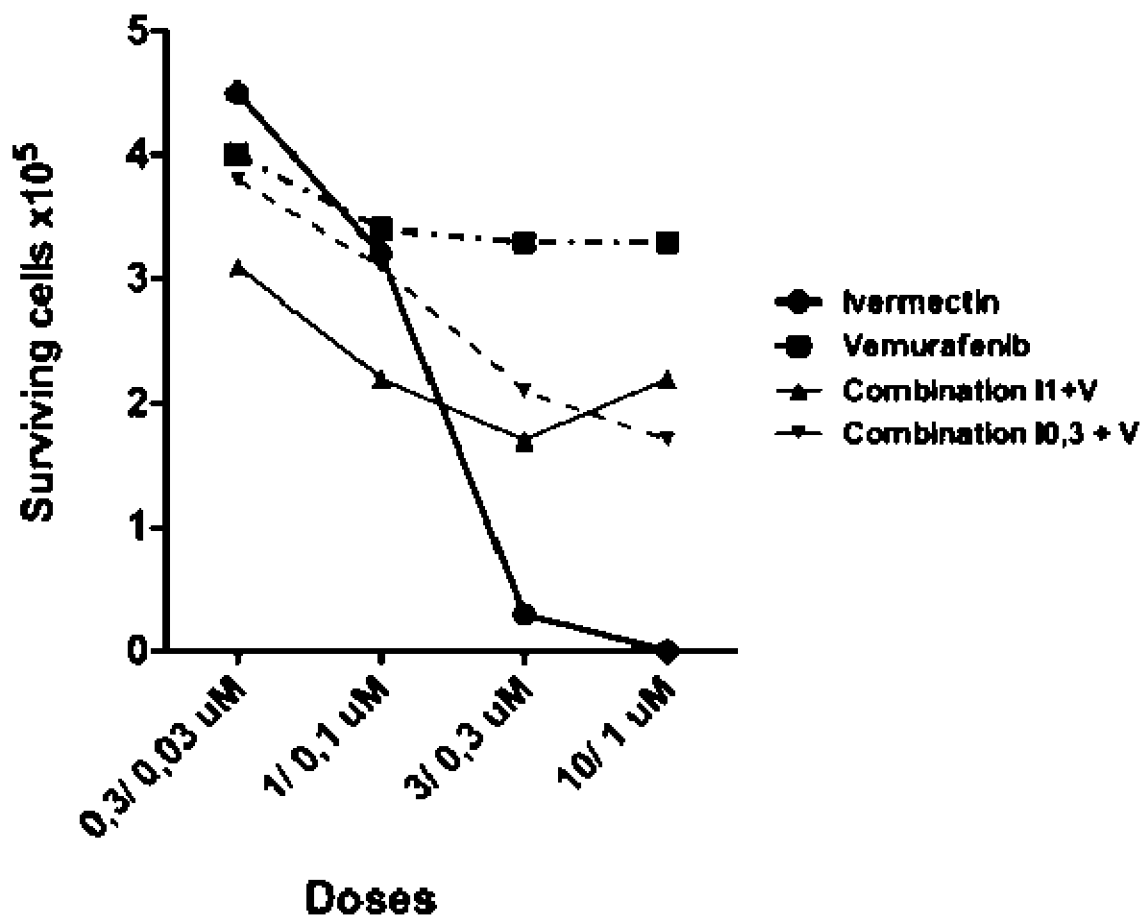
LB1319-MEL 72h incubation

Figure 15

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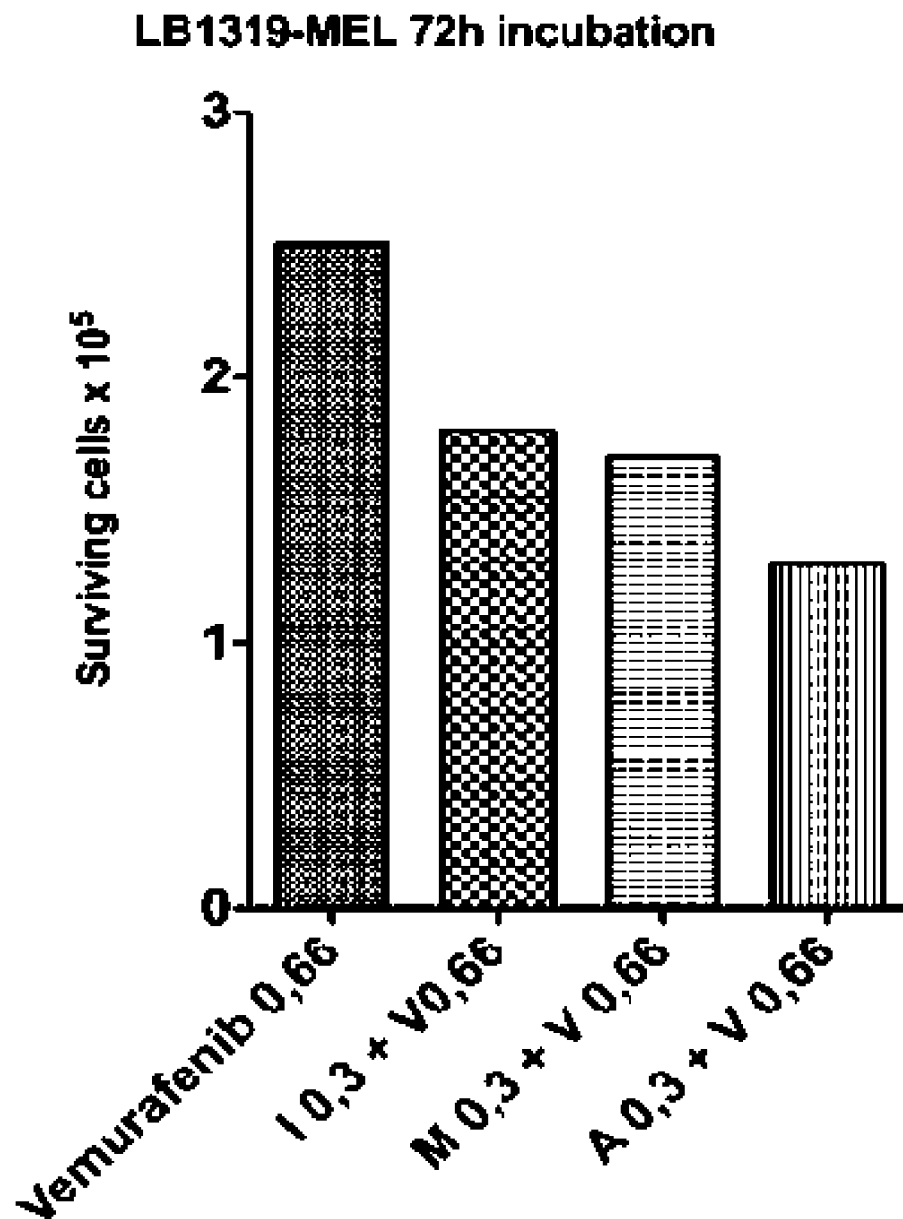


Figure 16

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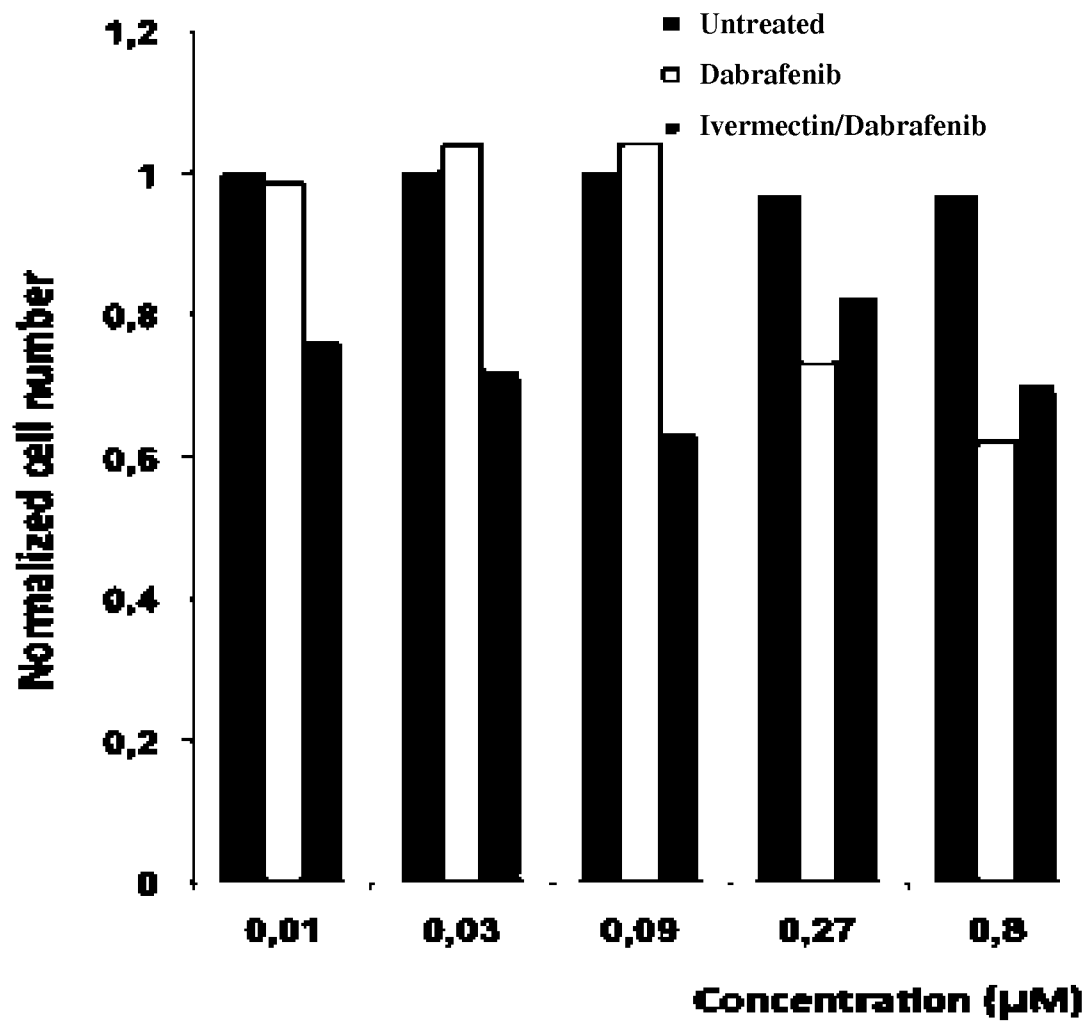


Figure 17

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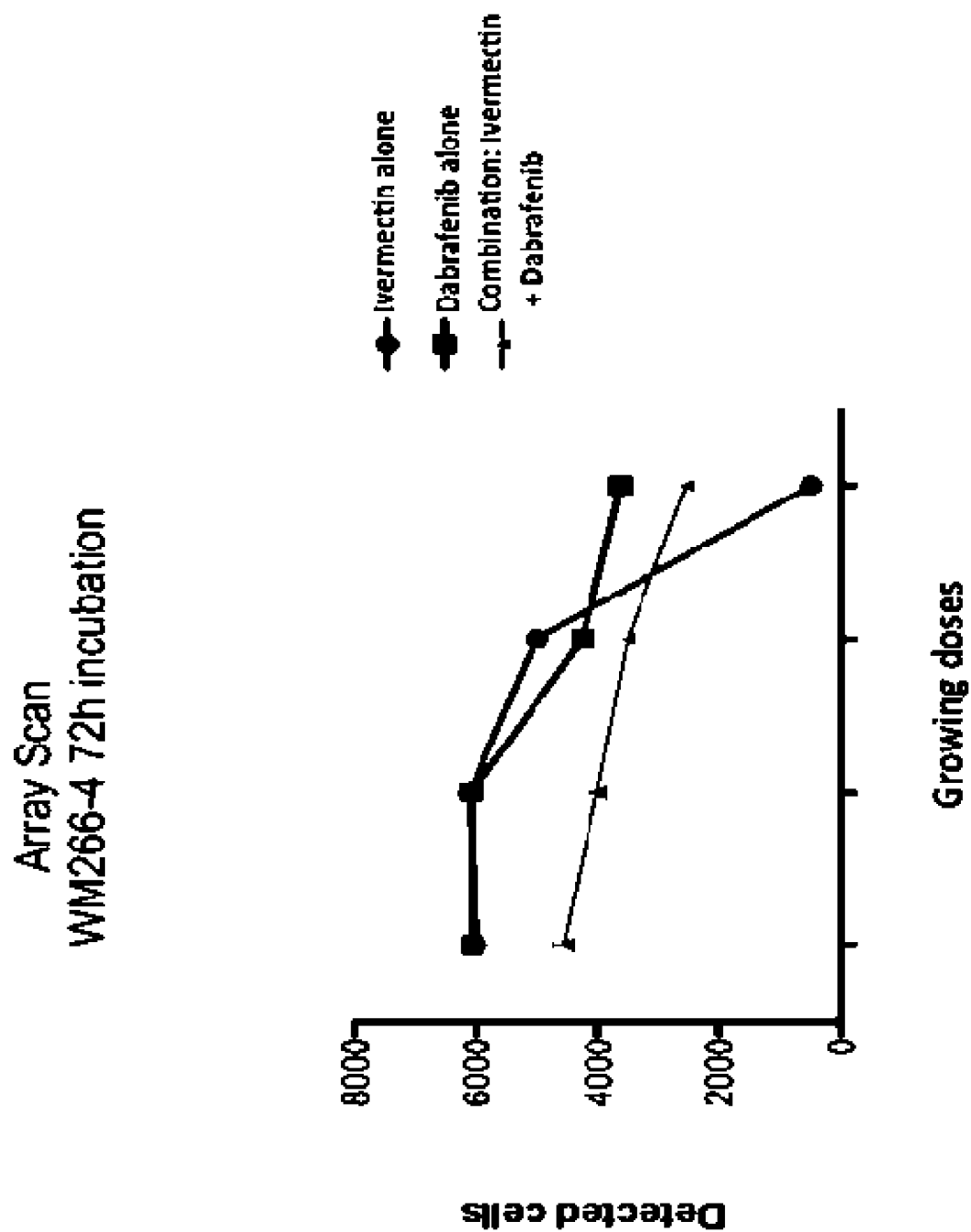


Figure 18

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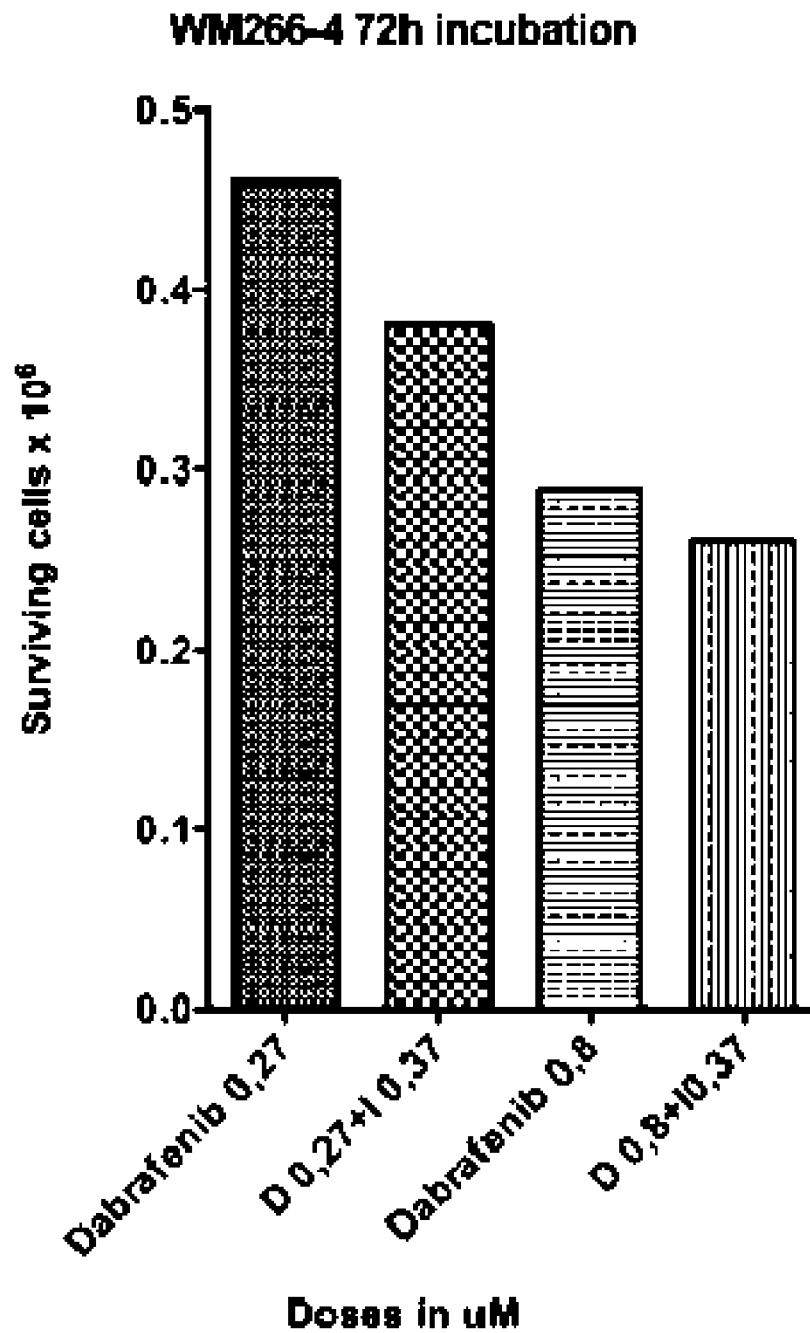


Figure 19

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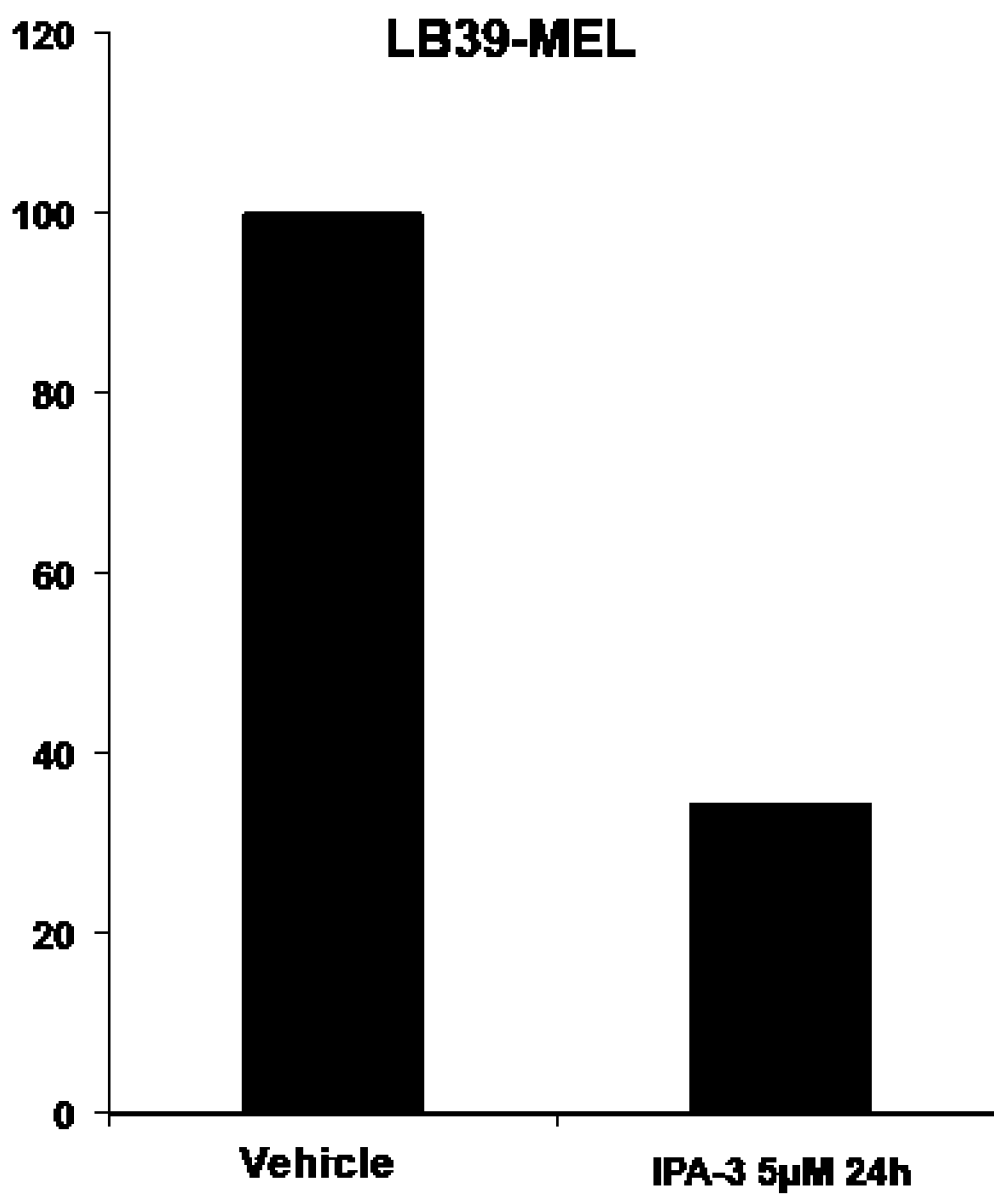


Figure 20

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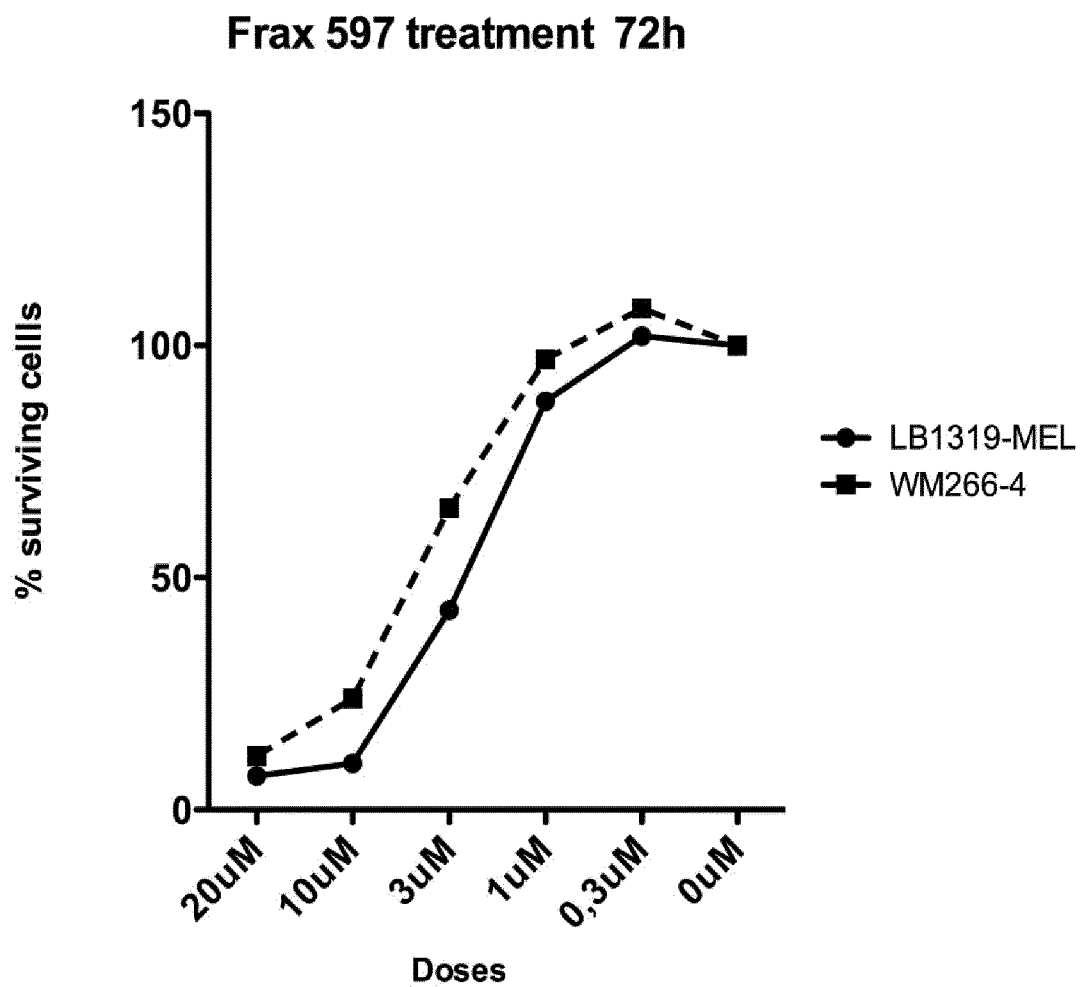


Figure 21

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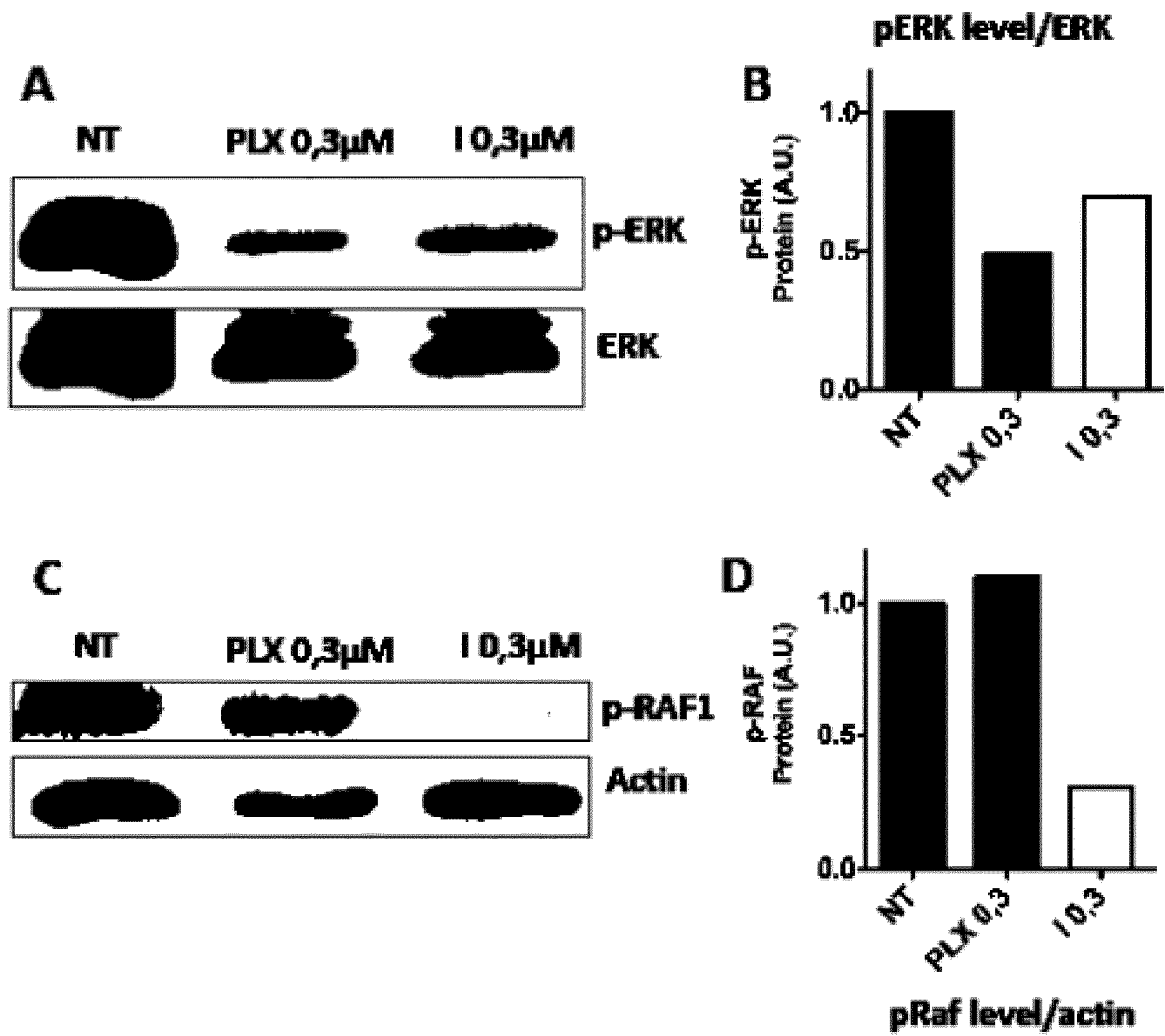


Figure 22

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/054615

A. CLASSIFICATION OF SUBJECT MATTER		
INV.	A61K45/06 C12Q1/48	A61K31/437 A61K31/506 A61K31/7048 A61P35/00
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 C12Q		
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, BIOSIS, EMBASE		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DRINYAEV V A ET AL: "Antitumor effect of avermectins", EUROPEAN JOURNAL OF PHARMACOLOGY, ELSEVIER SCIENCE, NL, vol. 501, no. 1-3, 6 October 2004 (2004-10-06), pages 19-23, XP004587589, ISSN: 0014-2999, DOI: 10.1016/J.EJPHAR.2004.08.009 abstract page 22, column 1, paragraph 2; figure 6 ----- -/--	1,3-5,7, 8
☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 31 March 2016		Date of mailing of the international search report 03/05/2016
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Tardi, Christine

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2016/054615

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HASHIMOTO H ET AL: "Ivermectin inactivates the kinase PAK1 and blocks the PAK1 dependent growth of human ovarian cancer and NF2 tumor cell lines", DRUG DISCOVERY TODAY: THERAPEUTIC STRATEGIES, ELSEVIER, AMSTERDAM, NL, vol. 3, no. 6, 1 December 2009 (2009-12-01), pages 243-246, XP002700324, ISSN: 1740-6773 abstract -----	5
X	WO 2013/135745 A1 (HOFFMANN LA ROCHE [CH]; GENENTECH INC [US]) 19 September 2013 (2013-09-19)	1-3,6-9
Y	page 42, paragraph 2-4; claim 11 page 30, lines 7-10 -----	5

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2016/054615

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2013135745 A1	19-09-2013	CA 2860994 A1	19-09-2013
		CN 104168898 A	26-11-2014
		EP 2844248 A1	11-03-2015
		HK 1200093 A1	31-07-2015
		JP 2015511598 A	20-04-2015
		KR 20140135198 A	25-11-2014
		WO 2013135745 A1	19-09-2013
