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(54) **Title**: A METHOD OF TREATMENT

(57) **Abstract**: The present specification relates generally to a method of treatment. In particular, but not exclusively, the present invention provides a method for treating or preventing drug resistance in a subject with melanoma.

A METHOD OF TREATMENT

FIELD OF THE INVENTION

The present specification relates generally to a method of treatment. In particular, but not exclusively, the present invention provides a method for treating or preventing drug resistance in a subject with melanoma.

DESCRIPTION OF RELATED ART

Mutation of codon 600 (V600E) of the serine/threonine kinase BRAF is the most common genetic aberration that occurs in melanoma, with mutations identified in approximately 50% of patients with advanced melanoma (Davies et al. (2002) Nature 417: 949). This mutation results in the constitutive activation of the BRAF protein, which drives cellular proliferation and survival through the MAPK pathway via the activation of downstream kinases MEK and ERK. Other mutations of the MAPK pathway in melanoma include mutation of NRAS, occurring in about 10-30% of melanomas and the genetic aberration of HRAS by mutation and gene amplification during melanoma tumourigenesis.

The targeted inhibition of MAPK pathway members, such as BRAF and MEK is highly effective in achieving clinical responses in patients with BRAFV600-mutant metastatic melanoma, with clinical benefit observed in 80-90% of patients (Flaherty et al. (2010) *New England Journal of Medicine* 363: 809). However, of the patients that respond to treatment with MAPK inhibitors, the majority only display partial responses. These patients eventually develop adaptive or acquired resistance and progress with drug resistant disease. Furthermore, an additional 10-20% of patients exhibit inherent resistance to MAPK inhibition and do not respond to targeted therapy.

The mechanisms of drug resistance are multi-factorial. Several reports have described that acquired resistance to BRAF and/or MEK inhibitors occurs via the re-activation of the ERK pathway, by activating NRAS mutations, MEK mutations, BRAF amplification, alternative splicing of BRAF or activation of receptor tyrosine kinases (RTKs). Alternative MAP3K proteins are also able to reengage the MAPK pathway in the presence of BRAF

inhibitors. Early adaptive drug resistance, thought to be the main reason for residual disease and incomplete responses in the majority of patients, can also be mediated by an increase in P-ERK activity or metabolic changes. Although it has been suggested that the restoration of ERK signalling is sufficient to maintain cell survival during drug treatment during both the early drug adaptive stage and during disease progression, the precise mechanisms associated with inherent or adaptive drug resistance in cancer cells remain unclear.

The present inventors have shown that increased c-JUN expression and activity mediates cell survival associated with inherent and adaptive drug resistance to MAPK pathway inhibition in BRAFV600-mutant melanoma. The identification of factors that contribute to the survival of cells during drug treatment, either in the context of inherent resistance to treatment with targeted therapeutics directed against the MAPK pathway, or as part of the process of the development of acquired resistance in cells that survive treatment can be used to improve therapeutic regimens to achieve more complete and durable responses to targeted therapeutics.

SUMMARY OF THE INVENTION

In one aspect, the present specification provides a method for treating or preventing resistance to an inhibitor of the MAPK pathway in a subject, the method comprising administering to the subject an effective amount of an inhibitor of c-JUN.

Another aspect of the invention is the use of an inhibitor of c-JUN for the preparation of a medicament for the treatment or prevention of resistance to an inhibitor of the MAPK pathway.

In a further aspect, the present specification provides a method for treating BRAFV600mutant melanoma in a subject comprising administering to the subject an effective amount of an inhibitor of the MAPK pathway in combination with an inhibitor of c-JUN.

The present invention also relates to the use of an inhibitor of the MAPK pathway in

combination with an inhibitor of c-JUN for the preparation of a medicament for the treatment of BRAFV600-mutant melanoma.

In yet another aspect of the present invention there is provided a method of treating recurrent BRAFV600-mutant melanoma comprising administering to a subject who has previously presented with BRAFV600-mutant melanoma an effective amount of an inhibitor of c-JUN.

The invention also relates to the use of an inhibitor of c-JUN for the preparation of a medicament for the treatment of recurrent BRAFV600-mutant melanoma.

The present invention also provides a pharmaceutical composition comprising an inhibitor of c-JUN for the treatment or prevention of resistance to an inhibitor of the MAPK pathway in a subject and a pharmaceutically-acceptable carrier.

The present invention also provides a kit when used for the treatment or prevention of resistance to an inhibitor of the MAPK pathway in a subject, comprising an inhibitor of c-JUN and a pharmaceutically-acceptable carrier, together with instructions for use.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a photographic representation of western blots that show elevated c-JUN and P-c-JUN expression is strongly associated with resistance to vemurafenib. (A) Vemurafenib resistant cell lines (IC50 > 10^6 M; LOXIMVI, CO57-M1, HS294T and RPMI7951) have significantly elevated protein levels of key modulators of the JNK signalling pathway. The representative Tubulin blot shows comparable protein loaded for each cell line across the panel. The sizes (kDa) of protein markers are shown on the left of the blots. (B) Vemurafenib resistant cell have significantly high gene expression levels of c-*JUN* and CD274 (the gene that encodes for the PD-L1 protein). The heatmap shows relative gene expression with respect to samples. Red indicates high relative gene expression and blue represents low gene expression levels.

Figure 2 shows that inhibition of c-JUN or JNK treatment in combination with vemurafenib enhances response in drug resistant cell lines. (A) A graphical representation of concentration of vemurafenib (log10M; x-axis) against cell number (% of control; y-axis) showing that combination treatment with JNK-IN-8 and vemurafenib results in synergistic activity to re-sensitise resistant cell lines to vemurafenib treatment. (B-C) Photographic representations of western blot analyses of key modulators of resistance which demonstrates that drug treatment with vemurafenib results in increased JNK pathway and JUN activity, and combination treatment with JNK inhibitors SP600125 or JNK-IN-8 or the targeted knock down of JUN using siRNA prior to vemurafenib treatment prevents JUN activation (P-C-JUN). (D) A graphical representation of concentration of vemurafenib (log10M; x-axis) against cell number (% of control; y-axis) that show the targeted knock down of JUN using siRNA prior to vemurafenib treatment also enhances the response to vemurafenib treatment in resistant cell lines.

Figure 3 shows that increased c-JUN expression and activity is associated with development of acquired resistance to vemurafenib. (A) A graphical representation of time (h; x-axis) against fold change relative to GAPDH (y-axis) showing that c-JUN gene expression increases following a time course of treatment with vemurafenib in sensitive cell lines. (B) A photographic representation of a western blot analysis of c-JUN expression (C-JUN) and activity (P-C-JUN) across the same time course demonstrating

that the increase in gene expression was correlated with an increase in the protein expression and activity of c-JUN. The representative Tubulin blot shows comparable protein loaded for each cell line across the panel. (C) A graphical representation of c-JUN gene expression relative to GAPDH (y-axis) from A375 xenografts from animals treated with control chow (green) or chow complexed with PLX4720 (a precursor drug compound to vemurafenib used for in vivo studies) (red). Each symbol represents the average of triplicate data for each xenograft tumour and the horizontal bar of the same colour indicates the average +/-the SEM for the three mice on each plot. (D) A graphical representation of relative gene expression to GAPDH (y-axis) of c-JUN in melanoma samples from matched patient samples taken at pre-treatment (PRE), early-on-treatment (EARLY) and at progression of disease (PROG). The average increase in c-JUN levels between PRE and EARLY samples was significantly different ($P = 0.038$). (E) A graphical representation of gene expression of c-JUN relative to GAPDH (y-axis) as a fold change to DMSO treatment in A375 cells treated with either 500 nM vemurafenib (V), 500 nM MEK inhibitor selumetinib (M), 100 nM ERK inhibitor SCH772984 (E) or combinations of V, M and E as indicated (x-axis) shown directly above the corresponding photographic representation of a western analysis of the same conditions following 48 h of drug treatment. The representative Tubulin blot shows comparable protein loaded for each cell line across the panel.

Figure 4 shows that the drug induced increase in c-JUN mediates cell survival during the development of early adaptive resistance. (A) A photographic representation of a western blot analysis demonstrating the efficient knock-down of c-JUN expression and activity following pre-treatment with siRNA targeting c-JUN. The representative Tubulin blot shows comparable protein loaded for each cell line across the panel. (B) A graphical representation of vemurafenib-associated cell death comparing the proportion of dead cells (% cells PI positive; y-axis) with pre-treatment with siRNA targeting c-JUN.

Figure 5 shows that JNK inhibition reverses the changes associated with adaptive resistance to vemurafenib. (A) A graphical representation of dose responsiveness for various combinations of vemurafenib and JNK-IN-8 in A375 cells comparing vemurafenib concentration (log10M; x-axis) against cell number (% of control; y-axis). (B) A graphical representation of cell death following treatment with the various combinations comparing

the proportion of dead cells (% cells PI positive; y-axis) with combination treatment in A375 cells following 48 h treatment. (C) A graphical representation of cell migration comparing cell index (x-axis) against time (h; y-axis) as quantified using the Xcelligence system and cells cultures in CIM plates. (D) A photographic representation of a western blot analysis comparing the activity of c-JUN in A375 cells treated for 48 h with various combinations. The representative Tubulin blot shows comparable protein loaded for each cell line across the panel.

Figure 6 shows Bliss analysis of cell death. (A). Percentage of cells PI+ (dead) following treatment with various combinations of vemurafenib and JNK-In-8 in A375 cells following 2 days treatment. The A375 cells were pre-treated with either DMSO or 500nM vemurafenib for 2-4 days prior to combination treatment. The data shown is the Average +/- SEM for triplicate experiments. (B) Bliss analysis of cell death data shown in Figure 6A. Each drug dose combination was assessed by the Bliss excess, which is the difference between the measured relative activity and the relative activity predicted from the assumption of Bliss independence, fBliss = f1 + f2 - f1×f2. The highest single activity (HSA) excess was calculated as the difference between the observed fractional activity and the highest of the fractional activities from single doses of each drug. The percentage Bliss excess showing the combined drug activity for each of the various drug dose combinations. The red colour indicates synergistic activity. Average cell death taken from Figure 5 from triplicate experiments in A375 cells after 2 days treatment with various drug combinations of vemurafenib and JNKIn8 following pre-treatment for 2 days with DMSO (DMSO pre) or 500nM vemurafenib (VEM pre).

DETAILED DESCRIPTION OF THE INVENTION

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as acknowledgment or

admission or any form of suggestion that that prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of endeavour to which this specification relates.

It is to be understood that unless otherwise indicated, the subject invention is not limited to specific manufacturing methods, formulation components, dosage regimens, or the like, as such may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

It must be noted that, as used in the subject specification, the singular forms "a", "an" and "the" include plural aspects unless the context clearly dictates otherwise. Thus, for example, reference to "a mutation" includes a single mutation, as well as two or more mutations; reference to "an inhibitor" includes a single inhibitor, as well as two or more inhibitors; reference to "the disclosure" includes single and multiple aspects taught by the disclosure; and so forth.

Aspects taught and enabled herein are encompassed by the term "invention". All aspects are enabled within the width of the present invention.

Method of treating or preventing drug resistance

The present invention is predicated on the inventors' surprising findings that both inherent and early adaptive resistance to MAPK inhibitor therapy is mediated through increased c-JUN expression and activity in BRAFV600-mutant melanoma.

Accordingly, the present specification teaches a method of treating or preventing resistance to an inhibitor of the MAPK pathway in a subject, the method comprising administering to the subject an effective amount of an inhibitor of c-JUN. Use of an inhibitor of c-JUN may also be used in the preparation of a medicament for the treatment or prevention of resistance to an inhibitor of the MAPK pathway.

Melanoma

Melanoma is a cancer arising from the malignant transformation of melanocytes, pigment producing cells of the skin, eye, mucosal epithelia and meninges. It is among the most

aggressive and treatment-resistant cancers. The development of novel targeted therapies and immunotherapies has significantly improved patient outcomes, particularly for those with advanced disease. However, the incidence of melanoma continues to rise.

Melanoma is generally detected by physical examination of skin lesions after symptoms have developed. The presence of melanoma is typically confirmed by microscopic analysis of skin tissue obtained by biopsy.

Reference to the terms "symptoms", "clinical symptoms" or "signs" refers to any and all biological or physiological effects that are specific to melanoma. Symptoms of melanoma include a change in an existing mole, the development of a new pigmented or unusual-looking growth on the skin, irregular shaped moles, moles with irregular, notched or scalloped borders, growths with many colours or an uneven distribution of colour, new growth in a mole, itching in a mole and blood or other discharges from a mole.

Histologically, melanomas are classified into five distinct stages: (i) common acquired and congenital nevi without dysplastic changes; (ii) dysplastic nevi with structural and architectural atypia; (iii) radial-growth phase (RGP) melanoma; (iv) vertical-growth phase (VGP) melanoma; and (v) metastatic melanoma. Benign and dysplastic nevi are characterised by the disruption of the epidermal melanin unit, leading to an increased number of melanocytes in relation to keratinocytes. RGP melanoma, or in situ melanoma, grow laterally and remain confined to the epidermis. VGP melanoma invades the upper layer of the epidermis and beyond, and penetrates into the underlying dermis and subcutaneous tissue through the basement membrane, forming expansive nodules of malignant cells. Metastatic melanoma represents the final stage of disease progression whereby the cancer has spread from the primary site to nearby tissues and more distant lymph nodes, or has metastasised to other organs such as the lungs and brain.

In an embodiment, the melanoma contemplated by the methods of the present specification is metastatic melanoma. In another embodiment, the metastatic melanoma is BRAFV600mutant melanoma.

If cancer cells are found in a tissue sample, an assessment is usually undertaken to

determine the stage, or extent, of the disease, with respect to the size and spread of the melanoma. The TNM is often employed for this purpose, where (T) denotes the thickness and ulceration of the melanoma, (N) denotes the spread of the melanoma to the lymph nodes and (M) denotes the spread of the melanoma to different parts of the body.

Once the TNM is determined, a stage of 0, I, II, III or IV is assigned to the melanoma. Stage 0 is the earliest stage of melanoma and is limited to in situ tumours with no detectable cancer cells in the regional lymph nodes or metastases at distant sites. Stage I tumours are localised melanomas that are classified into two distinct sub-stages, stage IA tumours are less than 1 mm thick without ulceration; and stage IB tumours may range from less than 1 mm to 2 mm thick both with and without ulceration. Stage II tumours are also localised melanomas that are classified into three distinct sub-stages, stage IIA may range from 1.01 to 4.00 mm thick both with and without ulceration; stage IIB tumours may range from 2.01 to > 4.00 mm thick both with and without ulceration; and stage IIC tumours are > 4.00 mm thick with ulceration. Stage III tumours are of any thickness or ulceration with macro-or micro-metastatic burden in the lymph nodes. Finally, stage IV tumours represent the most advanced stage of disease and are characterised by a primary tumour of any thickness or ulceration with macro-or micro-metastatic burden in the lymph nodes and metastases in the skin, subcutaneous tissue, nodal region, lung, other visceral metastases and distant metastases.

Treatment of melanoma

The therapeutic regimen for the treatment of melanoma can be determined by a person skilled in the art and will typically depend on factors including, but not limited to, the age, weight, family history and general health of the subject in addition to the type, size, stage and molecular characteristics of the melanoma, for example, the mutation status of BRAF.

Reference to the terms "treat", "treatment", "treating", "prevent", "preventing" and "prevention" refer to any and all uses which remedy a condition or symptom, prevent the establishment of a condition of a disease, or otherwise prevent, hinder, retard, abrogate or reverse the onset or progression of a condition or disease or other undesirable symptoms in any way whatsoever. Thus, the terms "treating" and "preventing" and the like are to be considered in their broadest possible context. For example, treatment does not necessarily

imply that a subject is treated until total recovery or cure. In conditions that display or are characterised by multiple symptoms, the treatment or prevention need not necessarily remedy, prevent, hinder, retard, abrogate or reverse all of said symptoms, but may remedy, prevent, hinder, retard, abrogate or reverse one or more of said symptoms. In the context of inherent or acquired resistance to treatment, the agents, uses ,methods and protocols of the present disclosure that involve treatment or prevention may prevent, reduce, ameliorate or otherwise delay the resistance to treatment, or of a highly undesirable event associated with resistance to treatment or an irreversible outcome of resistance to treatment, but may not itself prevent resistance to treatment in melanoma or an outcome associated therewith (e.g. a symptom associated with melanoma). Accordingly, treatment and/or prevention include amelioration of the symptoms of resistance to treatment in melanoma or preventing or otherwise reducing the risk of resistance to treatment.

The term "inhibiting" and variations thereof, such as "inhibition" and "inhibits", as used herein, do not necessarily imply the complete inhibition of the specified event, activity or function. Rather, the inhibition may be to an extent, and/or for a time, sufficient to produce the desired effect. Inhibition may be prevention, retardation, reduction, abrogation downregulation or otherwise hindrance of an event, activity or function. Such inhibition may be in magnitude and/or be temporal in nature. In particular contexts, the terms "inhibit" and "prevent", and variations thereof may be used interchangeably.

It would be appreciated by the person of skill in the art that the inhibition of a specified event, activity or function (e.g. c-JUN expression, activity or function) can be a result of inhibition, either by reducing absolute levels of the specified event, activity or function or by antagonising the specified event, activity or function such that effectiveness is decreased. Even the partial antagonism of the specified event, activity or function may act to reduce, although not necessarily eliminate, effectiveness.

In terms of achieving inhibition, means for achieving this objective would be well known to the person of skill in the art and include, but are not limited to:

(i) Introducing into a cell a proteinaceous or non-proteinaceous molecule which inhibits or downregulates the transcriptional and/or translational regulation of a gene, wherein this gene may be gene encoding c-JUN or functional portion thereof or some

other gene or gene region (e.g. promoter region) which directly or indirectly modulates the expression of c-JUN; or

(ii) Introducing a proteinaceous or non-proteinaceous molecule which functions as an antagonist to c-JUN.

The proteinaceous molecules described above may be derived from any suitable source such as natural, recombinant or synthetic sources and includes fusion proteins or molecules which have been identified following, for example, screening. The reference to non-proteinaceous molecules may be, for example, a reference to a nucleic acid molecule or it may be a molecule derived from natural sources, such as for example, by screening, or may be a chemically synthesised molecule. The present invention contemplates small molecules capable of acting as antagonists.

Antagonists may be any compound capable of blocking, inhibiting or otherwise preventing c-JUN from carrying out its normal biological function. Antagonists include monoclonal antibodies and antisense nucleic acids which prevent transcription or translation of activin genes or mRNA in mammalian cells. Modulation of expression may also be achieved utilising antigens, RNA, ribosomes, DNAzymes, aptamers, antibodies or molecules suitable for use in cosuppression. Suitable antisense oligonucleotide sequences (single stranded DNA fragments) of activin may be created or identified by their ability to suppress the expression of activin. The production of antisense oligonucleotides for a given protein is described in, for example, Stein and Cohen, 1988 (*Cancer Res* 48:2659-2668) and van der Krol *et al.*, 1988 (*Biotechniques* 6:958-976). Antagonists also include any molecule that prevents c-JUN activity/function.

The term "expression" refers to the transcription and translation of a nucleic acid molecule. Reference to "expression product" is a reference to the product produced from the transcription and translation of a nucleic acid molecule.

"Derivatives" of proteinaceous or non-proteinaceous agents include fragments, parts, portions or variants from either natural or non-natural sources. Non-natural sources include, for example, recombinant or synthetic sources. By "recombinant sources" is meant that the cellular source from which the subject molecule is harvested has been

genetically altered. This may occur, for example, in order to increase or otherwise enhance the rate and volume of production by that particular cellular source. Parts or fragments include, for example, active regions of the molecule. Derivatives may be derived from insertion, deletion or substitution of amino acids. Amino acid insertional derivatives include amino and/or carboxylic terminal fusions as well as intrasequence insertions of single or multiple amino acids. Insertional amino acid sequence variants are those in which one or more amino acid residues are introduced into a predetermined site in the protein although random insertion is also possible with suitable screening of the resulting product. Deletional variants are characterised by the removal of one or more amino acids from the sequence. Substitutional amino acid variants are those in which at least one residue in a sequence has been removed and a different residue inserted in its place. Additions to amino acid sequences include fusions with other peptides, polypeptides or proteins, as detailed above.

Derivatives also include fragments having particular epitopes or parts of the entire protein fused to peptides, polypeptides or other proteinaceous or non-proteinaceous molecules. Analogues of the molecules contemplated herein include, but are not limited to, modification to side chains, incorporating of unnatural amino acids and/or their derivatives during peptide, polypeptide or protein synthesis and the use of crosslinkers and other methods which impose conformational constraints on the proteinaceous molecules or their analogues.

Derivatives of nucleic acid sequences which may be utilised in accordance with the method of the present invention may similarly be derived from single or multiple nucleotide substitutions, deletions and/or additions including fusion with other nucleic acid molecules. The derivatives of the nucleic acid molecules utilised in the present invention include oligonucleotides, PCR primers, antisense molecules, molecules suitable for use in cosuppression and fusion of nucleic acid molecules. Derivatives of nucleic acid sequences also include degenerate variants.

A “variant” or “mutant” should be understood to mean molecules which exhibit at least some of the functional activity of the form of molecule (e.g. follistatin) of which it is a

variant or mutant. A variation or mutation may take any form and may be naturally or non-naturally occurring.

Chemical and functional equivalents should be understood as molecules exhibiting any one or more of the functional activities of the subject molecule, which functional equivalents may be derived from any source such as being chemically synthesised or identified via screening processes such as natural product screening. For example chemical or functional equivalents can be designed and/or identified utilising well known methods such as combinatorial chemistry or high throughput screening of recombinant libraries or following natural product screening (see further below). Antagonistic agents can also be screened for utilising such methods.

Therapeutic regimens

Suitable therapeutic regimens would be known to persons skilled in the art. In the context of melanoma, most subjects will have surgery to remove the cancerous tissue. For early stage melanomas, surgery will generally be sufficient to cure the disease. However, for more advanced disease, other treatments such as chemotherapy, radiotherapy, immunotherapy and targeted molecular therapy may also be required.

Reference to "surgery" includes wide local excision, Moh's micrographic surgery, lymph node dissection and other surgical interventions to remove metastatic tumour tissue from distant sites.

Reference to "radiotherapy" or "radiation therapy" includes both external radiation therapy and internal radiation therapy used to damage cancer cells and inhibit their proliferation. For example, external radiation is commonly delivered to patients in the form of x-rays directed to the tumour site by a machine. By contrast, internal radiation is generally delivered to patients using multiple small tubes and/or catheters inserted at the tumour site. Radiotherapy is generally used in combination with other treatment modalities, commonly surgery and chemotherapy.

Reference to "chemotherapy" means any agent that is administered to inhibit the growth of cancer cells or induce cancer cell death. Chemotherapeutic treatments for melanoma

include darcarbazine, temozolomide, nab-paclitaxel, paclitaxel, carmustine, cisplatin, carboplatin and vinblastine. Chemotherapeutic agents may be administered as single agents or in combination with one or more agents.

Reference to "targeted therapy" includes any targeted therapeutic agent that is specifically designed to interfere with molecular alterations that are specific to cancer cells. Targeted therapeutic agents may include, but are not limited to, monoclonal antibodies and small molecule inhibitors. For example, vemurafenib, and dabrafenib are small molecule inhibitors that inhibit the function of BRAF; and trametinib is a small molecule inhibitor that inhibits the function of MEK.

Modulatory agents

The proteinaceous and non-proteinaceous molecules referred to, above, are herein collectively referred to as “modulatory agents”. The term “modulatory agent” may be used interchangeably with the e terms "inhibitor", "drug" "composition", "agent", “therapeutic agent”, "medicament" and "active". A “modulatory agent” encompasses a chemical compound or biological molecule or cellular composition that induces a desired pharmacological and/or physiological effect by completely or partially inhibiting the expression or activity of a target molecule, for example, c-JUN. Any of the aforesaid terms encompass pharmaceutically acceptable and pharmacologically active ingredients including, but not limited to, salts, esters, amides, pro-drugs, active metabolites, analogues and the like. The term includes genetic, protein or lipid molecules or analogues thereof, in addition to the cellular compositions previously mentioned.

Modulatory agents may be targeted therapeutic agents that may be administered as single agents or in combination with one or more agents. For example, dabrafenib and trametinib; vemurafenib and trametinib; vemurafenib and cobimetinib and encorafenib and binimetinib are combinations of targeted therapeutic agents that may be used in the treatment of melanoma.

The agents which are utilised in accordance with the method of the present invention may take any suitable form. For example, proteinaceous agents may be glycosylated or unglycosylated, phosphorylated or dephosphorylated to various degrees and/or may contain a range of other molecules used, linked, bound or otherwise associated with the proteins such as amino acids, lipid, carbohydrates or other peptides, polypeptides or proteins. Similarly, the subject non-proteinaceous molecules may also take any suitable form. Both the proteinaceous and non-proteinaceous agents herein described may be linked, bound otherwise associated with any other proteinaceous or non-proteinaceous molecules. For example, in one embodiment of the present invention said agent is associated with a molecule which permits its targeting to a localised region.

The subject proteinaceous or non-proteinaceous molecule may act either directly or indirectly to downregulate/inhibit gene expression or activity of the gene product. Said molecule acts directly if it associates with the protein-encoding nucleic acid molecule or its expression product to modulate expression or activity, respectively. Said molecule acts indirectly if it associates with a molecule other than the protein-encoding nucleic acid molecule or expression product which other molecule either directly or indirectly downregulates/inhibits the expression or activity of the protein-encoding nucleic acid molecule or expression product, respectively.

Screening for modulatory agents

Screening for the modulatory agents can be achieved by any one of several suitable methods including, but in no way limited to, contacting a cell comprising the a gene of interest (e.g. the gene encoding c-JUN) or functional equivalent or derivative thereof with an agent and screening for the downregulation/inhibition of protein production or functional activity, downregulation/inhibition of the expression of a nucleic acid molecule encoding the protein of interest, or downregulation/inhibition of the activity or expression of a downstream activin cellular target. Detecting such downregulation can be achieved utilising techniques such as Western blotting, electrophoretic mobility shift assays and/or the readout of reporters of activin activity such as luciferases, CAT and the like.

It should be understood that the gene of interest or functional equivalent or derivative thereof may be naturally occurring in the cell which is the subject of testing or it may have

been transfected into a host cell for the purpose of testing. Further, the naturally occurring or transfected gene may be constitutively expressed - thereby providing a model useful for, inter alia, screening for agents which down regulate activin activity, at either the nucleic acid or expression product levels, or the gene may require activation - thereby providing a model useful for, inter alia, screening for agents which up-regulate activin expression. Further, to the extent that an activin nucleic acid molecule is transfected into a cell, that molecule may comprise the entire activin gene or it may merely comprise a portion of the gene such as the portion which regulates expression of the activin product. For example, the promoter region of the gene of interest (e.g. the gene encoding c-JUN) may be transfected into the cell which is the subject of testing. In this regard, where only the promoter is utilised, detecting modulation of the activity of the promoter can be achieved, for example, by ligating the promoter to a reporter gene. For example, the promoter may be ligated to luciferase or a CAT reporter, the downregulation of expression of which gene can be detected via modulation of fluorescence intensity or CAT reporter activity, respectively. In another example, the subject of detection could be a downstream regulatory target of the gene of interest, rather than the gene itself.

These methods provide a mechanism for performing high throughput screening of putative modulatory agents such as the proteinaceous or non-proteinaceous agents comprising synthetic, combinatorial, chemical and natural libraries. These methods will also facilitate the detection of agents which bind either the activin nucleic acid molecule or expression product itself or which modulate the expression of an upstream molecule, which upstream molecule subsequently downregulates/inhibits expression or expression product activity. Accordingly, these methods provide a mechanism of detecting agents which either directly or indirectly modulate gene expression and/or activity.

Libraries containing small organic molecules may be screened, wherein organic molecules having a large number of specific parent group substitutions are used. A general synthetic scheme may follow published methods (e.g., Bunin *et al.* 1994, *Proc Natl Acad Sci USA* 91:4708-4712; DeWitt *et al.* 1993, *Proc Natl Acad Sci USA* 90:6909-6913). Briefly, at each successive synthetic step, one of a plurality of different selected substituents is added to each of a selected subset of tubes in an array, with the selection of tube subsets being such as to generate all possible permutation of the different substituents employed in

producing the library. One suitable permutation strategy is outlined in US. Patent No. 5,763,263.

There is currently widespread interest in using combinational libraries of random organic molecules to search for biologically active compounds (see for example U.S. Patent No. 5,763,263). Ligands discovered by screening libraries of this type may be useful in mimicking or blocking natural ligands or interfering with the naturally occurring ligands of a biological target. By use of techniques, such as that disclosed in U.S. Patent No. 5,753,187, millions of new chemical and/or biological compounds may be routinely screened in less than a few weeks. Of the large number of compounds identified, only those exhibiting appropriate biological activity are further analysed.

With respect to high throughput library screening methods, oligomeric or small-molecule library compounds capable of interacting specifically with a selected biological agent, such as a biomolecule, a macromolecule complex, or cell, are screened utilising a combinational library device which is easily chosen by the person of skill in the art from the range of well-known methods, such as those described above. In such a method, each member of the library is screened for its ability to interact specifically with the selected agent. In practising the method, a biological agent is drawn into compound-containing tubes and allowed to interact with the individual library compound in each tube. The interaction is designed to produce a detectable signal that can be used to monitor the presence of the desired interaction. Preferably, the biological agent is present in an aqueous solution and further conditions are adapted depending on the desired interaction. Detection may be performed for example by any well-known functional or non-functional based method for the detection of substances.

Antibodies

In the context of antibodies, the present invention envisages the use of any suitable form of antibody including catalytic antibodies or derivatives, homologues, analogues or mimetics of said antibodies. Such antibodies may be monoclonal or polyclonal and may be selected from naturally occurring antibodies or subunits or may be specifically raised to target antigen. In the case of the latter, the antigen may first need to be associated with a carrier

molecule. Alternatively, fragments of antibodies may be used such as Fab fragments or Fab'$_2$ fragments.

Furthermore, the present invention extends to recombinant and synthetic antibodies and to antibody hybrids. A "synthetic antibody" is considered herein to include fragments and hybrids of antibodies. The antigen can also be used to screen for naturally occurring antibodies.

Both polyclonal and monoclonal antibodies are obtainable by immunization with the antigen or derivative, homologue, analogue, mutant, or mimetic thereof, and either type is utilizable therapeutically. The methods of obtaining both types of sera are well known in the art. Polyclonal sera are less preferred but are relatively easily prepared by injection of a suitable laboratory animal with an effective amount of the antigen, or antigenic parts thereof, collecting serum from the animal, and isolating specific sera by any of the known immunoadsorbent techniques. Although antibodies produced by this method are utilizable, they are generally less favoured because of the potential heterogeneity of the product.

The use of monoclonal antibodies is particularly preferred because of the ability to produce them in large quantities and the homogeneity of the product. The preparation of hybridoma cell lines for monoclonal antibody production derived by fusing an immortal cell line and lymphocytes sensitized against the immunogenic preparation can be done by techniques which are well known to those who are skilled in the art. (See, for example Douillard and Hoffman 1981, Basic Facts about Hybridomas, in *Compendium of Immunology* Vol II, ed. by Schwartz; Kohler and Milstein 1975, *Nature* 256:495-499; Kohler and Milstein 1976, *Eur J Immun* 6:511-519).

Preferably, an antibody within the scope of the present invention specifically binds its target antigen. By "specifically binds" is meant high avidity and/or high affinity binding of an antibody to a specific antigen. Antibody binding to its epitope on this specific antigen is stronger than binding of the same antibody to any other epitope, particularly those that may be present in molecules in association with, or in the same sample, as the specific antigen of interest. Antibodies that bind specifically to a polypeptide of interest may be capable of binding other polypeptides at a weak, yet detectable, level (e.g. 10% or

less of the binding shown to the polypeptide of interest). Such weak binding, or background binding, is readily discernible from the specific antibody binding to the polypeptide of interest, e.g. by use of appropriate controls.

Aptamers

The present invention is also directed to useful aptamers. In one embodiment, an aptamer is a compound that is selected *in vitro* to bind preferentially to another compound (in this case the identified proteins), in one aspect, aptamers are nucleic acids or peptides. Random sequences can be readily generated from nucleotides or amino acids (naturally occurring and/or synthetically made) in large numbers but of course they need not be limited to these. In another aspect, the nucleic acid aptamers are short strands of DNA that bind protein targets, such as oligonucleotide aptamers. Oligonucleotide aptamers are oligonucleotides which can bind to a specific protein sequence of interest. A general method of identifying aptamers is to start with partially degenerate oligonucleotides, and then simultaneously screen the many thousands of oligonucleotides for the ability to bind to a desired protein. The bound oligonucleotide can be eluted from the protein and sequenced to identify the specific recognition sequence. Transfer of large amounts of a chemically stabilized aptamer into cells can result in specific binding to a polypeptide of interest, thereby blocking its function. [For example, see the following publications describing *in vitro* selection of aptamers: Klug *et al.* 1994, *Mol Biol Rep* 20:97-107; Wallis *et al.* 1995, *Chem Biol* 2:543-552; Ellington 1994, *Curr Biol* 4:427-429; Lato *et al.* 1995, *Chem Biol* 2:291-303; Conrad *et al.* 1995, *Mol Divers* 1:69-78; and Uphoff *et al.* 1996, *Curr Opin Struct Biol* 6:281-287].

RNA inhibiting agents

Certain RNA inhibiting agents may be utilized to inhibit the expression or translation of messenger RNA ("mRNA") that is associated with a phenotype of interest. In an embodiment, an inhibitor may be a molecule that functions as a mediator of RNA interference. "RNA interference" or "RNAi" describes a mechanism of gene silencing that is based on degrading or otherwise preventing the translation of mRNA in a sequence specific manner that is dependent on small, non-coding RNA approximately 18 to 30-nucleotide (nt) in length. Three classes of small RNA can regulate genes by targeting transcripts in the cytoplasm: microRNAs (miRNAs), which are hairpin-derived RNAs with

imperfect complementarity to targets and that cause translational repression; small interfering RNAs (siRNAs), which have perfect complementarity to targets and cause transcript degradation; and PIWI-interacting RNAs (piRNAs), which target transposon transcripts in animal germ lines. All three classes of small RNA share a common mode of action, the minimal effector is a ribonucleoprotein complex comprising an Argonaute family protein bound to a singlestranded ~18 to 30 nt RNA that exhibits specificity by base-pairing interactions with the gene target. In miRNA and siRNA pathways, this is known as the RNA-induced silencing complex (RISC) and it drives the silencing of a target mRNA by degradation and/or transcriptional repression.

Despite the similarities in processing of siRNA and miRNA, miRNAs are endogenously expressed from the genome, whereas siRNAs may be endogenous or arise from viral infection or other exogenous sources. Furthermore, siRNA duplexes feature perfect base-pairing, while miRNA helices contain mismatches and more extended terminal loops. In the cytoplasm, the processing pathways converge for endogenous miRNAs and for typically exogenous siRNAs. Both types of RNAi precursors are cleaved down by a Dicer enzyme to a dsRNA duplex of the appropriate size for loading onto an Argonaute protein. The resulting dsRNA is a duplex of 21-to 25-nt strands, with a 2-nt overhang at each 3' terminus and a phosphate group at each recessed 5' terminus. The bound duplex and Argonaute protein are subsequently loaded into the RISC complex in a strand dependent manner. One strand, the guide strand, of the duplex is bound to Argonaute to direct silencing and the other strand, the passenger strand, is discarded. The RISC performs cellular surveillance, binding single-stranded RNA (ssRNA) such as mRNA with complementarity to the guide strand. Guide strand nucleotides 2-6 constitute the seed sequence and initialize binding to the target.

piRNAs are produced and processed by a completely distinct pathway, known as the 'ping pong cycle'. Briefly, piRNA genomic clusters are transcribed to produce the piRNA precursors. In the cytoplasm, these precursors are cleaved into short 23-29-nt antisense piRNAs. These short, single stranded RNAs (ssRNAs) are loaded into PIWI family Argonaute proteins AUB and PIWI. The loaded AUB or PIWI proteins then target the mRNA of active transposons for cleavage to produce sense piRNAs. The sense piRNAs are loaded into the PIWI-specific Argonaut protein AGO3, which then directs cleavage of

primary piRNA precursors and the subsequent production of more antisense piRNAs, completing the 'ping pong cycle'.

The RNAi molecules contemplated by the present invention should be understood to encompass all RNAi gene silencing mechanisms. The induction of RNAi to inhibit a target gene could be achieved by administering, in accordance with the method or use of the present invention, exogenous RNA oligonucleotides that can induce an RNAi mechanism. Reference to a "RNAi molecule" should therefore be understood as a reference to an RNA nucleic acid molecule that is double stranded or single stranded and is capable of effecting the induction of an RNAi mechanism to knock down the expression of a gene targeted or down regulating or preventing the onset of such a mechanism. In this regard, the subject RNAi molecule may be capable of directly mediating an RNAi mechanism, or it may require further processing. The subject RNAi molecule may be double stranded or single stranded. Examples of RNAi molecules that are suitable for use in the present application include, but are not limited to, long double stranded RNA (dsRNA), hairpin double stranded RNA (hairpin dsRNA), short interfering RNA (siRNA), short hairpin RNA (shRNA); microRNA (miRNA); and small temporal RNA (stRNA).

It will be appreciated that a person skilled in the art can determine the most suitable RNAi molecule for use in any given situation. For example, although it is preferable that the subject RNAi molecule exhibits 100% complementarity to its target nucleic acid molecule, the RNAi molecule may nevertheless exhibit some degree of mismatch to the extent that hybridization sufficient to induce an RNAi response in a sequence specific manner can be effected. Accordingly, it is preferred that the RNAi molecule of the present invention comprises at least 70%-100% sequence complementarity. Reference to "at least 70%-100%" means 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100%.

Methods for designing double stranded RNA to inhibit gene expression in a target cell are known (see, e.g., U.S. Pat. No. 6,506,559; Elbashir *et al.* 2002, *Methods* 26:199-213; Chalk *et al.* 2004, *Biochem Biophys Res Commun* 319:264-274; Cui *et al.* 2004, *Comput Methods Programs Biomed* 75:67-73; Wang *et al.* 2004, *Bioinformatics* 20:1818-1820).

For example, design of siRNAs (including hairpins) typically follow known thermodynamic rules (see, e.g., Schwarz, *et al.* 2003, *Cell* 115:199-208; Reynolds *et al.* 2004, *Nat Biotechnol.* 22:326-330; Khvorova *et al.* 2003, *Cell* 115:209-216). Many computer programs are available for selecting regions of a sequence that are suitable target sites. These include programs available through commercial sources such as Ambion, Dharmacon, Promega, Invitrogen, Ziagen, and GenScript as well as non-commercial sources such as EMBOSS, The Wistar Institute, Whitehead Institute, and others.

For example, design can be based on the following considerations. Typically, shorter sequences, less than about 30 nucleotides are selected. The coding region of the mRNA is usually targeted. The search for an appropriate target sequence optionally begins 50-100 nucleotides downstream of the start codon, as untranslated region binding proteins and/or translation initiation complexes may interfere with the binding of the siRNA endonuclease complex. Some algorithms, e.g., based on the work of Elbashir *et al.* 2000 (*Methods* 26:199-213) search for a selected sequence motif and select hits, with approximately 50% G/C-content (30% to 70% has also worked). If no suitable sequences are found, the search is extended.

Other nucleic acids, e.g., ribozymes, antisense, can also be designed based on known principles. For example, Sfold (see, e.g., Ding, *et al.*, *Nucl Acids Res* 32 Web Server issue, W135-W141; Ding & Lawrence 2003, *Nucl Acids Res* 31:7280-7301; and *Ding & Lawrence* 2001, *Nucl Acids Res* 20:1034-1046) provides programs relating to designing ribozymes and antisense, as well as siRNAs.

Immunotherapy

Reference to "immunotherapy" includes any agent that is specifically designed to induce, enhance or suppress an immune response in a subject to destroy cancer cells. "Activation immunotherapies" are immunotherapies designed to elicit or amplify an immune response. "Suppression immunotherapies" reduce or suppress an immune response.

Immunotherapeutic agents may include, but are not limited to, recombinant, synthetic and natural preparations of interleukins, cytokines, chemokines, cytosine phosphate-guanosine, oligodeoxynucleotides and glucans, cell-based immune therapies, autologous immune

enhancement therapy, engineered T-cells, adoptive T-cells, vaccines and monoclonal antibodies. For example, high-dose interleukin 2 (IL-2), adoptive cell transfer with autologous T cells and immune checkpoint inhibitors such as ipilimumab, a humanised CTLA-4 blocking monoclonal antibody and monoclonal antibodies that antagonize PD-1 or PD-L1 (e.g. pembrolizumab, nivolumab) have been successfully used to treat melanoma.

The present invention further contemplates a combination of treatments, such as the administration of inhibitory/downregulating agent together with other proteinaceous or non-proteinaceous molecules which may facilitate the desired therapeutic or preventative outcome.

The inhibitory/downregulating agent may be administered as a single dose or may be administered as multiple sequential doses or it may be continuously infused. Where more than one molecule is administered, there may be simultaneous administration in the same formulation or in different formulations via the same or different routes or sequential administration via the same or different routes. By "sequential" administration is meant a time difference of from seconds, minutes, hours or days.

In an aspect of the present specification there is provided a method of treating BRAFV600mutant melanoma comprising administering to a subject an effective amount of an inhibitor of the MAPK pathway in combination with an inhibitor of c-JUN.

In another aspect of the present specification there is provided the use of an inhibitor of the MAPK pathway and an inhibitor of c-JUN for the preparation of a medicament for the treatment of BRAFV600-mutant melanoma.

In a further aspect of the present specification there is provided a method of treating recurrent BRAFV600-mutant melanoma comprising administering to a subject that has previously presented with BRAFV600-mutant melanoma an effective amount of an inhibitor of c-JUN.

In yet a further aspect of the present specification there is provided the combined use of an inhibitor of c-JUN with one or more other immunotherapeutic agents, such as monoclonal antibody directed against PD-L1, for the preparation of a medicament for the treatment of BRAFV600-mutant melanoma.

In yet a further aspect of the present specification there is provided the a method of treating recurrent BRAFV600-mutant melanoma comprising administering to a subject that has previously presented with BRAFV600-mutant melanoma the combination of an inhibitor of c-JUN with one or more other immunotherapeutic agents, such as monoclonal antibody directed against PD-L1.

Reference to the term "recurrent" or "persistent" includes any relapse in the disease of a melanoma subject that occurs following treatment with a targeted therapy. This is inclusive of melanoma subjects that have inherent or acquired resistance to the targeted therapy.

In an embodiment, the inhibitor of the MAPK pathway is selected from the group consisting of: BRAF, MEK, ERK, RTK and/or RAS inhibitors. In another embodiment, the inhibitor of the MAPK pathway is a BRAF inhibitor. In a further embodiment, the BRAF inhibitor is vemurafenib.

In an embodiment, the inhibitor of c-JUN is an inhibitor of the JNK pathway.

The MAPK pathway

Melanoma is one of the most treatment-resistant tumours and generally, traditional treatment modalities such as chemotherapy and radiotherapy have not been particularly effective in treating melanoma. Therefore, the development of targeted therapeutics and immunotherapies has revolutionised treatment for melanoma patients. The most successful targeted therapies used for the treatment of melanoma are inhibitors of the MAPK signalling pathway.

The MAPK pathway is a signal-transduction pathway that transmits mitogenic signals from activated cell surface growth factor receptors and is recognised to be a key regulator of cellular growth and survival. Briefly, in normal cells, growth factors bind to surface

receptor tyrosine kinases (RTKs) that transduce their growth promoting signals through the activation of the small G protein RAS, which leads to the activation of the serine/threonine kinase RAF, and then to the activation of MEK. MEK then phosphorylates and activates MAPK, also known as ERK.

Inhibitors of the MAPK pathway have been shown to be highly effective in achieving clinical responses in patients with BRAF mutant melanoma. For example, inhibitors of the MAPK pathway include, but are not limited to, RAF inhibitors such as sorafenib, SB590885, PLK4720, XL281, RAF265, encorafenib, dabrafenib and vemurafenib; MEK inhibitors such as XL518, CI-1040, PD035901, MEK162, selumetinib andtrametinib (GSK1120212); ERK inhibitor SCH772984; RTK inhibitors such as afatinib, axitinib, bosutinib, crizotinib, dasatinib, erlotinib, gefitinib, imatinib, lapatinib, nilotinib, pazopanib, sorafenib and sunitinib; and RAS inhibitors such as lonofarnib, tipifarnib, salirasib, deltarasin and PDEd. However, it will be appreciated that any inhibitor of the MAPK pathway is within the scope of the present specification.

Despite the initial response to treatment with inhibitors of the MAPK pathway, the majority of patients display only partial responses and eventually progress due to the development of acquired resistance to targeted therapy. Furthermore, 10-20% of patients with melanoma do not respond to treatment with inhibitors of the MAPK pathway as a result of inherent resistance to the treatment.

Reference to "resistance" or "resistant" includes inherent, acquired and adaptive drug resistance. Cancer cells can exhibit inherent resistance to a drug which is present before treatment with a given drug begins as a result of inherited hereditary genetic characteristics; develop acquired resistance whereby cancer cells that were originally sensitive to a given drug become resistant to treatment as a result of genetic mutation; or develop adaptive resistance, which involves a temporary (i.e. reversible) increase in the ability of a cancer cell to survive treatment with a given drug as a result of altered gene or protein expression due to exposure to an environmental trigger, for example, stress, nutrient conditions, growth state and suboptimal levels of the drug itself. Adaptive drug resistance differs from inherent or acquired drug resistance as it is a transient state that usually reverts where the environmental trigger is removed.

Inherent and acquired resistance to inhibitors of the MAPK pathway has been reported to occur through mechanisms the re-activate the ERK pathway, such as activating NRAS mutations, MEK mutations, BRAF amplification, alternative splicing of BRAF or activation of RTKs. However, the mechanisms of adaptive drug resistance remain unclear.

Resistance to MAPK inhibitors

The present inventors have made the surprising discovery that up-regulation of c-JUN expression and activity is responsible for mediating resistance to inhibitors of the MAPK pathway. Gene expression, in vitro, in vivo and patient analyses resulted in the identification of c-JUN as a key molecule in resistance to inhibitors of the MAPK pathway. Therefore, c-JUN, or up-stream activators of c-JUN, such as the JNK pathway, which potentiates the transcriptional activity of c-JUN by phosphorylation of serine's 63 and 73, may be targeted for the treatment or prevention of resistance to treatment with inhibitors of the MAPK pathway in melanoma.

c-JUN

The term "jun proto-oncogene", "JUN" or "c-JUN" refers to the protein or polypeptide that is encoded by the JUN gene. The term "c-JUN" may be used herein to refer to either or both c-JUN polypeptide or a gene (polynucleotide) encoding a c-JUN polypeptide, interchangeably. Those skilled in the art will recognise from the context of the disclosure whether the polypeptide or polynucleotide (gene) is the subject of the disclosure.

c-JUN is a component of the AP1 transcription factor complex, comprising 331 amino acids with a molecular weight of approximately 35 kDa. c-JUN consists of a basic leucine zipper domain that is essential for DNA binding. Importantly, c-JUN is the mammalian counterpart of v-JUN retroviral oncogene, and is recognised to act as a proto-oncogene in various mammalian cancers. Aberrant activity and expression of c-JUN is responsible for the induction of a number of target genes that are involved in regulating cell cycle progression, migration and survival during tumourigenesis.

Without wanting to be bound by any particular theory or mode of action, it is proposed that inhibition of c-JUN in malignant melanoma can treat or prevent resistance to vemurafenib

by overcoming the re-activation of ERK signalling.

The term "c-JUN" includes vertebrate and non-vertebrate c-JUN. Suitable vertebrates that fall within the scope of the invention include, but are not limited to, any member of the subphylum Chordata including primates, rodents (e.g. mice, rates, guinea pigs), lagomorphs (e.g. rabbits, hares), bovines (e.g. cattle), ovines (e.g. sheep), caprines (e.g. goats), porcines (e.g. pigs), equines (e.g. horses), canines (e.g. dogs), felines (e.g. cats), avians (e.g. chickens, turkeys, duck, geese, companion birds such as canaries, budgerigars, etc.), marine mammals (e.g. dolphins, whales), reptiles (e.g. snakes, frogs, lizards, etc.), and fish. In an embodiment, c-JUN is human c-JUN.

In an embodiment, the human c-JUN is a protein encoded by the mRNA sequence represented by GenBank Accession numbers NM_002228.3.

Reference to the term c-JUN also includes homologs thereof. The term "homolog" typically refers to peptides with similar biological activity, although differing in amino acid sequence at one or more amino acid positions when the sequences are aligned. For example, the amino acid sequences of two homologous c-JUN peptides may differ only by one amino acid residue with in the aligned amino acid sequences of five to ten amino acids. Alternatively, two homologous c-JUN peptides of fifteen to twenty or more amino acids can differ by up to three amino acid residues when aligned. Homologous c-JUN peptides may also differ by up to approximately 5%, 10%, 20% or 25% of the amino acid residues when the amino acid sequences of the two peptide homologs are aligned.

Homologs of c-JUN may be found in the same species (i.e. between two or more individuals of the same species), in related species and/or sub-species, or in different species. For example, for a human c-JUN, homologs include those found in non-human vertebrates and non-vertebrates. Suitable vertebrates that fall within the scope of the invention include, but are not limited to, any member of the subphylum Chordate including primates, rodents (e.g. mice, rates, guinea pigs), lagomorphs (e.g. rabbits, hares), bovines

(e.g. cattle), ovines (e.g. sheep), caprines (e.g. goats), porcines (e.g. pigs), equines (e.g. horses), canines (e.g. dogs), felines (e.g. cats), avians (e.g. chickens, turkeys, duck, geese,

companion birds such as canaries, budgerigars, etc.), marine mammals (e.g. dolphins, whales), reptiles (e.g. snakes, frogs, lizards, etc.), and fish. A preferred homolog is one found in a primate (e.g. a human, ape, monkey, chimpanzee). Alternatively, a c-JUN homolog may be from the same species (e.g. human).

Generally, homologs will have at least about 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to a particular amino acid or nucleotide sequence, as determined, for example, by sequence alignment programs known in the art using default parameters.

The JNK pathway

The term "Jun N-terminal kinase", "JNK" or "stress-activated protein kinase" pathway refers to a MAPK signalling pathway that is related to the MAPK pathway as described above.

The JNK pathway is a signalling pathway that is activated by environmental and genotoxic stresses to control cell proliferation, differentiation, survival and the migration of different cell types. The JNK proteins are encoded by three genes, MAPK8 (which encodes JNK1), MAPK9 (which encodes JNK2) and MAPK10 (which encodes JNK3), which are alternatively spliced to produce at least ten different isoforms. JNK1 and JNK2 are ubiquitously expressed throughout the body, whereas JNK3 is generally expressed in the brain. Following the activation of RTKs on the surface of cells in response to growth factors, environmental stresses or inflammatory cytokines, JNKs are activated by upstream MKK4 and MKK7 kinases. The major target of activated JNK is the transcription factor AP1, which comprises c-FOS and c-JUN. JNKs phosphorylate c-JUN, which then combine with FOS to form the AP1 transcription factor.

Without wanting to be bound by any particular theory or mode of action, it is proposed that inhibition of the JNK signalling pathway can prevent the phosphorylation of c-JUN, which is known to stabilise c-JUN levels, thereby providing an alternative therapeutic strategy for preventing or treating resistance mediated through c-JUN.

Subject

The terms "subject", "individual" and "patient" are used interchangeably herein to refer to any subject to which the present disclosure may be applicable, particularly a vertebrate subject, and even more particularly a mammalian subject. Suitable vertebrate animals that fall within the scope of the invention include, but are not restricted to, any member of the subphylum Chordate including primates, rodents (e.g. mice, rates, guinea pigs), lagomorphs (e.g. rabbits, hares), bovines (e.g. cattle), ovines (e.g. sheep), caprines (e.g. goats), porcines (e.g. pigs), equines (e.g. horses), canines (e.g. dogs), felines (e.g. cats), avians (e.g. chickens, turkeys, duck, geese, companion birds such as canaries, budgerigars, etc.), marine mammals (e.g. dolphins, whales), reptiles (e.g. snakes, frogs, lizards, etc.), and fish. In some embodiments, the subject is a primate (e.g. a human, ape, monkey, chimpanzee). In a preferred embodiment, the subject is a human.

Methods for treating or preventing resistance to MAPK inhibitors

In an aspect, the present specification teaches a method for treating or preventing resistance to an inhibitor of the MAPK pathway in a subject, said method comprising administering to the subject an effective amount of an inhibitor of c-JUN.

In another aspect, the present specification teaches the use of an inhibitor of c-JUN for the preparation of a medicament for the treatment or prevention of resistance to an inhibitor of the MAPK pathway.

Reference to an "effective amount" means the amount of an inhibitor of c-JUN when administered to a subject, in particular a human subject, in need of such treatment, is sufficient to effect treatment or prevent resistance to an inhibitor of the MAPK pathway.

In an embodiment, the resistance to an inhibitor of the MAPK pathway is inherent resistance. In another embodiment, the resistance to an inhibitor of the MAPK pathway is acquired resistance. In yet another embodiment, the resistance to an inhibitor of the MAPK pathway is adaptive resistance.

In an embodiment, the subject has been administered an inhibitor of the MAPK pathway for the treatment of cancer.

In another embodiment, the subject has been administered an inhibitor of the MAPK pathway for the treatment of BRAFV600-mutant melanoma.

Reference to "BRAFV600-mutant melanoma" means melanoma with a V600E or V600K mutation in BRAF. Genomic analyses have identified activating BRAF mutations in as many as 60% of human melanomas. Importantly, the majority of these mutations (80%) cluster in the kinase-activation domain (V600E) of BRAF and result in a single phosphomimetic substitution of that is known to confer constitutive activation of BRAF.

Activated BRAF phosphorylates and activates MEK proteins (MEK1 and MEK2), which then activates downstream MAP kinases.

The BRAF genotype of melanoma patients may be determined by routine methods well known to a person skilled in the art, for example, polymerase chain reaction (PCR) using appropriate primers or gene sequencing methods.
In an embodiment, the inhibitor of the MAPK pathway is selected from the group consisting of: RAF, MEK, ERK, RTK and RAS inhibitors.

RAF kinases are a family of serine/threonine-specific protein kinases that are related to retroviral oncogenes. The three RAF kinase family members are ARAF, BRAF and CRAF.

MEK is a dual specificity protein kinase that is encoded by the MAP2K7 genes. This kinase specifically activates the JNK pathway to mediate cellular responses to proinflammatory cytokines and environmental stresses.

ERK is an extracellular signal related kinase that is encoded by MAPK1 gene. This kinase acts as an integration point for the transduction of biochemical signals associated with the cellular processes of proliferation, differentiation, transcription regulation and development.

RTKs are high affinity surface receptors that transduce extracellular signals for many polypeptide growth factors, cytokines and hormones. They are regulators of normal

cellular processes and also play a role in tumour development and progression. For example, KIT, ERBB2, the EPH and FGFR are frequently mutated in melanoma.

The RAS genes are the most frequently mutated oncogenes in human cancer. There are three RAS genes in humans, HRAS, KRAS and NRAS. Each encodes small GTPases that are involved in signal transduction.

In another embodiment, the inhibitor of the MAPK pathway is a BRAF inhibitor.

Reference to "BRAF" means "B-Raf proto-oncogene, serine/threonine kinase", which is a RAF serine/threonine protein kinase that regulates the MAPK pathway. As described above, mutations in the BRAF gene are associated with oncogene-driven tumourigenesis in melanoma.

In a further embodiment, the BRAF inhibitor is vemurafenib.

The terms "vemurafenib", "Zelboraf", "PLX4032", "RG7204" or "RO5185426" refer to a reversible, ATP-competitive small molecule inhibitor of the kinase domain of mutant BRAF. For example, in cancer cells with the V600E mutation, vemurafenib inhibits the phosphorylation of MEK by BRAF and induces growth-inhibition or apoptotic cell death.

In an embodiment, the inhibitor of the MAPK pathway is administered prior to the inhibitor of c-JUN. For example, the MAPK pathway may be administered 2-10 days prior to the inhibitor of c-JUN. Reference to "2-10 days prior" means 2, 3, 4, 5, 6, 7, 8, 9 or 10 days prior.

Inhibitors of c-JUN include, but are not limited to, c-JUN peptide inhibitors. Inhibitors of the JNK pathway include, but are not limited to SP6000125, JNK-IN-8, CC-401, SU3327, AS601245, PGL5001 and BI78D3. However, it will be appreciated that any inhibitor of the JNK pathway and c-JUN is within the scope of the present specification.

In an embodiment, an inhibitor of c-JUN may be administered in combination with

chemotherapy, radiotherapy, targeted therapy and/or immunotherapy. In another embodiment, an inhibitor of c-JUN may be administered in combination with two or more treatment modalities (i.e. chemotherapy, radiotherapy, targeted therapy and/or immunotherapy). Treatment modalities will typically be selected with a view to treating melanoma and/or melanoma recurrence and/or the development of resistance to MAPK inhibitors. For example, an inhibitor of c-JUN may be administered in combination with an immunotherapy, such as a monoclonal antibody directed against PD-L1.

Pharmaceutical compositions

The pharmaceutical composition contemplated by the present specification comprises an inhibitor of c-JUN, which may be prepared in a manner known in the art and are those suitable for enteral, such as oral or rectal, and parental administration to a subject, particularly a human subject, comprising an effective amount of an inhibitor of c-JUN alone, or in combination with one or more pharmaceutically acceptable carriers or diluents, especially suitable for enteral or parental application.

The pharmaceutical compositions of the invention can be administered in a variety of unit dosage forms depending upon the method of administration. Dosages for typical modulatory pharmaceutical compositions are well known to those of skill in the art. Such dosages are typically advisory in nature and are adjusted depending on the particular therapeutic context, patient or organ tolerance, etc. The amount of agent adequate to accomplish this is defined as a “therapeutically effective dose”. The dosage schedule and amounts effective for this use, i.e., the “dosing regimen,” will depend upon a variety of factors, including the pharmaceutical formulation and concentration of active agent, and the like. In calculating the dosage regimen, the mode of administration also is taken into consideration. The dosage regimen must also take into consideration the pharmacokinetics, i.e., the pharmaceutical composition’s rate of absorption, bioavailability, metabolism, clearance, and the like. (See, e.g., the latest version of Remington: The Science and Practice of Pharmacy, edited by Allen, Loyd V., Jr; Egleton and Davis; *Peptides* 18:1431-1439; Langer 1990 *Science* 249:1527-1533).

Suitable pharmaceutical compositions contain from about 0.1% to about 99.9% of the active ingredient. Reference to "about 0.1% to about 99.9%" means 0.1, 0.2, 0.3, 0.4, 0.5,

0.6, 0.7, 0.8, 0.9, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 99.9%.

The composition may contain any suitable carriers, diluents or excipients. These include all conventional solvents, dispersion media, fillers, solid carriers, coatings, antifungal and antibacterial agents, dermal penetration agents, surfactants, isotonic and absorption agents and the like. It will be understood that compositions of the invention may also include other supplementary physiologically active agents.

The carrier must be pharmaceutically "acceptable" in the sense of being compatible with the other ingredients of the composition and not injurious to the subject. Compositions include that suitable for oral, rectal, nasal, topical (including buccal and sublingual), vaginal or parental (including subcutaneous, intramuscular, intravenous and intradermal) administration. The compositions may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. Such methods include the step of bringing into association the active ingredient with the carrier. In general, the compositions are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and then if necessary shaping the product.

In an embodiment, the composition is suitable for parental administration. In another embodiment, the composition is suitable for intravenous administration. In a further embodiment, the composition is suitable for subcutaneous administration.

Compositions suitable for parental administration include aqueous and non-aqueous isotonic sterile injection solutions which may contain anti-oxidants, buffers, bactericides and solutes which render the composition isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The compositions may be presented in unit-dose or multi-dose sealed containers, for example, ampoules and vials, and may be stored in freeze-dried

(lyophilised) condition requiring only the addition of the sterile liquid carrier, for example, water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets.

Preferred unit dosage compositions are those containing a daily dose or unit, daily sub-dose, or an appropriate fraction thereof, of the active ingredient.

In an embodiment, the pharmaceutical composition further comprises an inhibitor of the MAPK pathway. In another embodiment, the inhibitor of the MAPK pathway is selected from the group consisting of RAF, MEK, ERK, RTK and/or RAS inhibitors. In a further embodiment, the inhibitor of the MAPK pathway is a BRAF inhibitor. In an embodiment, the BRAF inhibitor is vemurafenib.

In an embodiment, the pharmaceutical composition comprises an inhibitor of c-JUN that is an inhibitor of the JNK pathway.

Kits

The present invention contemplates a kit when used for the treatment or prevention of resistance to an inhibitor of the MAPK pathway in a subject comprising an inhibitor of c-JUN and a pharmaceutically-acceptable carrier, together with instructions for use.

In an embodiment, the inhibitor of c-JUN is an inhibitor of the JNK pathway.

All essential materials and reagents required for treating or preventing resistance to an inhibitor of the MAPK pathway may be assembled together in a kit. The kits may optionally include appropriate therapeutic agents to be administered in combination with an inhibitor of c-JUN, including, but not limited to an inhibitor of the MAPK pathway.

In an embodiment, the inhibitor of the MAPK pathway is selected from the group consisting of RAF, MEK, ERK, RTK and/or RAS inhibitors. In another embodiment, the inhibitor of the MAPK pathway is a BRAF inhibitor. In a further embodiment, the BRAF inhibitor is vemurafenib.

Those skilled in the art will appreciate that the invention described is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications which fall within the scope. The invention also includes all of the features, steps, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

EXAMPLES

Aspects of certain embodiments of the present invention are further described by reference to the following non-limiting Examples.

Materials and Methods

Cell lines

Melanoma cell lines (Table 1) were obtained from American Type Culture Collection (ATCC) or the Australasian Biospecimen Network-Oncology Cell Line Bank at the Queensland Institute of Medical Research Berghofer Medical Research Institute (QIMR Berghofer).

Short tandem repeat (STR) genotyping using 6 STR loci was performed to confirm the identity of each cell line.

IC_{50} values shown in Table 1 are representative of average IC50 values from multiple experiments. An average IC_{50} value of 1000 nM (1 μM) was used to separate the sensitive and resistant cell lines.

Table 1. Melanoma cell lines

Cell line	Response to PLX4032	IC_{50} (nM PLX4032)
RPMI7951	Resistant	2772
HS294T	Resistant	2274
CO57-M1	Resistant	2188
LOXIMVI	Resistant	1801
D17	Sensitive	724
A02	Sensitive	367
C055	Sensitive	303
D28	Sensitive	282
WM2664	Sensitive	265

CO91	Sensitive	251
SKMEL1	Sensitive	212
D41	Sensitive	179
COLO829	Sensitive	166
HT144	Sensitive	157
CO74	Sensitive	110
SKMEL24	Sensitive	98
CO88	Sensitive	95
SKMEL28	Sensitive	87
CO89	Sensitive	68
A375	Sensitive	65
A15	Sensitive	64
MALME	Sensitive	56

Detection of BRAF mutations

BRAF mutations in exon 15 were detected by Sanger sequencing. The sequencing products were run on a 3700 Genetic Analyser (Applied Biosystems) and the sequencing data analysed using Sequencer 4.6 (Gene Codes Corporation).

siRNA transfection

Cell lines were transfected with 50 nM of c-JUN siRNA (Dharmacon siGENOME Smart Pool Cat. No. M-003268-03-0005) complexed with 0.1% DharmaFECT 1 lipid transfection reagent. Lipid and siRNA were each diluted separately in non-supplemented media for 5 mins and then complexed for 15 mins prior to addition to cells.

Sulphorhodamine B (SRB) drug dose response assay

Cell lines were seeded into 96-well microtitre plates for 48 hrs prior to the addition of drug. Cells were treated for 72 hrs with drug ranging in concentration from 0.01 nM to 30 μM and analysed for total cell number according to SRB absorbance. Briefly, cells were fixed in situ by the addition of cold trichloroacetic acid (TCA) and stained using 0.4% (w/v) SRB/ 1% (v/v) acetic acid. SRB absorbance was measured at 515 nM in 10 mM tris(hydroxymethyl)aminomethane (TRIS) to generate dose response curves. IC50 values

for each cell line were calculated as the drug dose resulting in 50% reduction in SRB absorbance relative to solvent treated control cells.

Western blot analysis

Whole cell lysates were prepared in lysis buffer containing 50 mM 4-(2-hydroxyethyl)-1piperazineethanesulphonic acid (HEPES), 150 mL NaCl, 1 mM ethylene glycol tetraacetic acid (EGTA), 1.5 mM MgCl2, 1% Triton-X-100 (v/v), 10% Glycerol (v/v), 50 mM NaF, 10 mM Na3VO4 and complete mini protease inhibitor cocktail. Protein concentrations were quantified using the DC Protein Assay (BioRad). 20 µg protein aliquots were separated on 10-12% sodium dodecyl sulphate-polyacrylamide (SDS) gels by electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were then blocked with 0.1% TRIS buffered saline-Tween-20 v/v; TBST) containing 5% (w/v) skim milk powder and probed with antibodies. Bound antibodies were detected using horseradish peroxidase conjugated secondary antibodies incubated with enhanced chemoluminescence (ECL) or ECL-Plus Western Blotting Detection Reagents (GE Healthcare), which were exposed to X-Ray film (Fugifilm) and developed by autoradiography.

Gene Expression

Gene expression data for the panel of melanoma cell lines accessible through NCBI Gene Expression Omnibus GEO Series accession number GSE45558, were obtained using Affymetrix 1.0ST expression arrays, RMA normalized and background corrected.

Patient samples

Fresh frozen tumour samples were obtained prior, during and on progression, from patients participating in clinical trials involving vemurafenib, dabrafenib, trametinib or a combination of both dabrafenib and trametinib undertaken at the Melanoma Institute Australia and Westmead Hospital (NSW, Australia). Studies had local institutional review board approval and all patients provided written informed consent.

All samples were pathologically reviewed and scored for tumour content. Only tumours with > 80% tumour content were selected for this study.

Gene expression analysis by RT-QPCR

Gene expression analysis of cell lines and xenograft tumours was performed using RTQPCR. Briefly, total RNA was extracted using the PureLink RNA Mini Kit (Life Technologies). Total mRNA was used for reverse transcription using the High Capacity cDNA Reverse Transcription kit (Life Technologies). 30 ng of reverse transcribed cDNA was used for quantitative PCR using Fast SYBR Green PCR Master Mix and gene specific primers. All reactions were performed in triplicate using the StepOnePlue Real-Time PCR System (Applied Biosystems). Relative expression was determined according to the comparative CT method followed by normalisation to GAPDH.

Xenograft tumour growth and treatment of mice

4 x 10^6 A375 cells were mixed in a 1:1 ratio with high concentration Matrigel and injected subcutaneously into the right flank of female NOD-SCID-ILγ mice. Tumours were monitored every 2-3 days and tumour volume measurements taken according to the length and width of tumours.

Treatment commended 10 days post-injection of A375 cells and the average volume of tumours observed was 183.4 mm3 (range: 39.4 – 401 mm3). Test animals were fed a diet of vemurafenib (PLX4720; n = 3) and were matched to non-treated control mice (n = 3). Mice were culled 1 week after the commencement of treatment and tumours were surgically resected. Tumours were prepared for analysis using the Mellwain tissue chopper, snap frozen in liquid nitrogen and stored at -80°C. RNA was extracted from tumour pellets and analysed for gene expression as described above.

Cell death assays

Following drug treatment, cells were harvested, pooled and stained with 1 µg/ml Propidium Iodide (PI) in PBS. Stained cells were analysed by flow cytometry using the Canto II (Becton Dickinson). The percentage of PI positive cells was assessed using FCS Express (De Novo Software).

Cell migration assays

Cell migration assay were performed using the RTCA DP Instrument (ACEA Bioscience, Inc.). Cells were passaged one day before the experiment to reach 60-80% confluence. The

lower chamber of migration CIM plates were filled with 160 µl of 10% FBS, RPMI + HEPES medium and the upper chamber was filled with 50 µl serum-free medium to cover the membrane. Both mediums contained the relevant drug at the appropriate concentration. A serum gradient was established prior to taking the baseline measurement. 15,000 cells diluted in drug were seeding into the upper chamber. The CIM-plates were then loaded into the RTCA DP Analyser in a tissue culture incubator. Measurements were taken at 15 min intervals for 300 repetitions (> 72 hr).

Statistical analysis

All statistical analysis was performed using the Excel (Microsoft), GraphPad Prism (GraphPad Software, Inc.) and R (R Core Team) software packages.

Results

Resistance to BRAF/MEK inhibitors is associated with JNK pathway activation

We assessed a panel of 22 BRAF V600-mutant melanoma cell lines that were either resistant or sensitive to vemurafenib (PLX4032) treatment. A comparison between the resistant and sensitive cell lines identified the JNK signalling pathway as a key modulator of resistance. Specifically, the vemurafenib resistant cell lines displayed activation of the JNK signalling pathway with higher levels of c-JUN (Figure 1). Regarding Figure 1B, gene expression of CD274 encoding PD-L1 is significantly higher in the resistant lines. Thus, high c-JUN expression correlates with high PD-L1 expression in the resistant lines.

Inhibition of JNK or reduction in c-JUN enhances drug response to vemurafenib in resistant cell lines

Combination treatment with vemurafenib and either the JNK inhibitor SP600125 or JNKIN-8 result in an enhanced response in resistant cell lines when compared to resistant cell lines treated with vemurafenib alone (Figure 2). Furthermore, JNK-IN-8 was also able to inhibit c-JUN phosphorylation in all of the resistant cell lines, which was associated with a reduction in vemurafenib-associated induction of p-ERK (Figure 2).

Transfection with siJUN prior to treatment with vemurafenib effectively reduced the level of c-JUN transcript and protein (Figure 2). The transient silencing of c-JUN results in an enhanced response to vemurafenib (Figure 2) and increased the proportion of apoptotic cell

death in resistant cell lines. These data suggest that JNK pathway signalling and elevated levels of c-JUN contribute to drug resistance in the vemurafenib resistant cell lines.

Increased c-JUN expression and activity is associated with the development of adaptive resistance to vemurafenib.

Cell lines that were sensitive to vemurafenib were exposed to drug. In residual cells remaining following treatment with vemurafenib, we observed an increase in c-JUN levels and c-JUN phosphorylation (Figure 3). This increase in c-JUN levels and activity was also detected at high doses of vemurafenib treatment *in vivo* (Figure 3).

Tumour samples collected from patients shortly after treatment with vemurafenib or dabrafenib (EARLY) have a statistically significant increase in JUN mRNA relative to that of the pre-treatment tumours from the same patients (Figure 3). These data are consistent with *in vitro* and *in vivo* data demonstrative that residual cells remaining following vemurafenib treatment express higher levels of c-JUN. Furthermore, combination treatment of A375 cells with BRAF, MEK and ERK inhibitors also result in an increase in c-JUN expression and activity (Figure 3), suggesting that the increase in c-JUN observed is not only an effect of BRAF inhibition, but rather a consequence of inhibition of the RAF-MEK-ERK signalling pathway.

Constitutive expression of c-JUN mediates resistance in vemurafenib sensitive cell lines

Enforced constitutive expression of c-JUN by retroviral transduction of A375 cells is sufficient to induce cellular changes associated with the development of resistance in vemurafenib sensitive cell lines. Increased c-JUN expression resulted in increased activity, as demonstrated by an increase in the levels of p-c-JUN (Figure 4). Interestingly, we also observed that the constitutive expression of c-JUN results in an increase in the levels of p-ERK (Figure 4), suggesting that c-JUN could contribute to the p-ERK rebound detected following drug treatment, which previous studies have reported to be a key component of the development of resistance (Lito et al. (2012) Cancer Cell, 22: 668).

Drug-induced increase in c-JUN mediates cell survival during the development of resistance

Reduction of drug-induced c-JUN levels and activity by pre-treatment with siRNA to JUN resulted in a significant increase in the level of vemurafenib-induced apoptotic cell death in A375, SKMEL28 and WM266-4 cells (Figure 5). These data indicate that c-JUN plays a critical role in cell survival during the development of adaptive resistance. The reduction in c-JUN levels by pre-treatment with siRNA also reduced the level of p-ERK, confirming the contribution of elevated c-JUN to the rebound in p-ERK, as described above.

JNK inhibition reverses the changes associated with adaptive resistance to vemurafenib

As a potential therapeutic strategy for overcoming adaptive resistance, we assessed the potential benefit of targeting the JNK pathway, which we previously observed to enhance the response to vemurafenib in resistant cell lines (Figure 2).

Combination treatment of vemurafenib and the JNK inhibitor, JNK-IN-8, enhanced the efficacy of vemurafenib treatment in sensitive A375 cells, displaying additive/synergistic effects (Figure 5B or Figure 6). Vemurafenib in combination with JNK-IN-8 increased cell death (Figure 5B)
and reduced the level of cell migration observed with vemurafenib treatment alone (Figure 5C). These data assessing JNK inhibitor combinatorial effects, together with the data demonstrating a reduction in vemurafenib induced c-JUN by siRNA, strongly support the notion for the critical role of c-JUN in mediating cell survival during the early drug adaptive process.

Combining vemurafenib with the JNK inhibitor, JNK-IN-8, enhanced the efficacy of vemurafenib treatment in A375 cells (Figure 5B and Figure 6). The assessment of cell death (Figure 6A) revealed that combination treatment could prevent early residual cell survival following single agent BRAF inhibitor treatment. The level of cell death with combination treatment with vemurafenib and JNK-IN-8 was higher and more synergistic after a short term "drug-adaptation" in vemurafenib (Figure 6B), suggesting that early drug adaptation resulting in high JUN and P-cJUN could increase the responsiveness to combination treatment of vemurafenib with JNK inhibitors.

Target sequences within the JNK/JUN pathway:

The list below provides nucleic acid sequences which may be targetable by inhibitory RNA and proteins targetable by small molecule inhibitors. Targeting such sequences/proteins is expected to inhibit or reduce c-JUN activity.

NM_ codes: NCBI RefSeq nucleotide sequence codes (mRNA sequences)
NP_ codes: NCBI RefSeq protein sequence codes

MAPK8 mitogen-activated protein kinase 8 [Homo sapiens (human)]
Gene ID: 5599
Also known as
JNK; JNK1; PRKM8; SAPK1; JNK-46; JNK1A2; SAPK1c; JNK21B1/2

NM_001278547.1 (GI:513788278) -> NP_001265476.1 mitogen-activated protein kinase 8 JNK1 beta2
NM_001278548.1 (GI:513788280) -> NP_001265477.1 mitogen-activated protein kinase 8 isoform 5
NM_002750.3 (GI:513788275) -> NP_002741.1 mitogen-activated protein kinase 8 isoform alpha1
NM_139046.2 (GI:513788276) -> NP_620634.1 mitogen-activated protein kinase 8 isoform beta1
NM_139049.2 (GI:513788277) -> NP_620637.1 mitogen-activated protein kinase 8 isoform alpha2

MAPK9 mitogen-activated protein kinase 9 [Homo sapiens (human)]
Gene ID: 5601
Also known as
JNK2; SAPK; p54a; JNK2A; JNK2B; PRKM9; JNK-55; SAPK1a; JNK2BETA; p54aSAPK; JNK2ALPHA

NM_001135044.1 (GI:205277411) -> NP_001128516.1 mitogen-activated protein kinase 9 isoform JNK2 gamma

NM_001308244.1 (GI:815891107) -> NP_001295173.1 mitogen-activated protein kinase 9 isoform gamma2

NM_002752.4 (GI:205277404) -> NP_002743.3 mitogen-activated protein kinase 9 isoform alpha2

NM_139068.2 (GI:205277406) -> NP_620707.1 mitogen-activated protein kinase 9 isoform alpha1

NM_139069.2 (GI:205277408) -> NP_620708.1 mitogen-activated protein kinase 9 isoform beta1

NM_139070.2 (GI:205277410) -> NP_620709.1 mitogen-activated protein kinase 9 isoform beta2

MAPK10 mitogen-activated protein kinase 10 [Homo sapiens (human)]

Gene ID: 5602

Also known as

JNK3; JNK3A; PRKM10; SAPK1b; p493F12; p54bSAPK

NM_001318067.1 (GI:969536251) -> NP_001304996.1 mitogen-activated protein kinase 10 isoform 5

NM_001318068.1 (GI:969536253) -> NP_001304997.1 mitogen-activated protein kinase 10 isoform 6

NM_001318069.1 (GI:969536247) -> NP_001304998.1 mitogen-activated protein kinase 10 isoform 1x

NM_002753.4 (GI:969536249) -> NP_002744.1 mitogen-activated protein kinase 10 isoform 2

NM_138980.3 (GI:969536250) -> NP_620446.1 mitogen-activated protein kinase 10 isoform 3

NM_138982.3 (GI:969536246) -> NP_620448.1 mitogen-activated protein kinase 10 isoform 1

MAP4K4 mitogen-activated protein kinase kinase kinase kinase 4 [Homo sapiens (human)]

Gene ID: 9448

Also known as
HGK; NIK; MEKKK4; FLH21957; HEL-S-31

NM_001242559.1 (GI:336020357) -> NP_001229488.1 mitogen-activated protein kinase kinase kinase kinase 4 isoform 4
NM_001242560.1 (GI:336020359) -> NP_001229489.1 mitogen-activated protein kinase kinase kinase kinase 4 isoform 5
NM_004834.4 (GI:336020352) -> NP_004825.3 mitogen-activated protein kinase kinase kinase kinase 4 isoform 1
NM_145686.3 (GI:336020354) -> NP_663719.2 mitogen-activated protein kinase kinase kinase kinase 4 isoform 2
NM_145687.3 (GI:336020356) -> NP_663720.1 mitogen-activated protein kinase kinase kinase kinase 4 isoform 3

MAP2K7 mitogen-activated protein kinase kinase 7 [Homo sapiens (human)]
Gene ID: 5609
Also known as
MEK; MKK7; JNKK2; MEK 7; MAPKK7; PRKMK7; SAPKK4; SAPKK-4
NM_001297555.1 (GI:662033892) -> NP_001284484.1 dual specificity mitogen-activated protein kinase kinase 7 isoform 1
NM_001297556.1 (GI:662033894) -> NP_001284485.1 dual specificity mitogen-activated protein kinase kinase 7 isoform 2
NM_145185.3 (GI:662033896) -> NP_660186.1 dual specificity mitogen-activated protein kinase kinase 7 isoform 3

JUN jun proto-oncogene [Homo sapiens (human)]
Gene ID: 3725
Also known as AP1; AP-1; c-Jun

NM_002228.3 (GI:44890066) -> NP_002219.1 transcription factor AP-1

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CLAIMS

1. A method for treating or preventing resistance to an inhibitor of the MAPK pathway in a subject, said method comprising administering to the subject an effective amount of an inhibitor of c-JUN.

2. Use of an inhibitor of c-JUN for the preparation of a medicament for the treatment or prevention of resistance to an inhibitor of the MAPK pathway.

3. The method according to claim 1 or use according to claim 2, wherein the resistance to an inhibitor of the MAPK pathway is inherent resistance.

4. The method according to claim 1 or use according to claim 2, wherein the resistance to an inhibitor of the MAPK pathway is acquired resistance.

5. The method according to claim 1 or use according to claim 2, wherein the resistance to an inhibitor of the MAPK pathway is adaptive resistance.

6. The method according to any one of claims 1 or 3 to 5 or use according to any one of claims 2 to 5, wherein the subject has been administered an inhibitor of the MAPK pathway for the treatment of cancer.

7. The method or use according to claim 6, wherein the subject has been administered an inhibitor of the MAPK pathway for the treatment of BRAFV600-mutant melanoma.

8. The method according to any one of claims 1 or 3 to 7 or use according to any one of claims 2 to 7, wherein the inhibitor of the MAPK pathway is selected from the group consisting of: RAF, MEK, ERK, RTK and/or RAS inhibitors.

9. The method of or use according to according to claim 8, wherein the inhibitor of the MAPK pathway is a BRAF inhibitor.

10. The method or use according to claim 9, wherein the BRAF inhibitor is vemurafenib.

11. The method according to any one of claims 1 or 3 to 10 or use according to any one of claims 2 to 10, wherein the inhibitor of c-JUN is an inhibitor of the JNK pathway.

12. A method of treating BRAFV600-mutant melanoma comprising administering to a subject an effective amount of an inhibitor of the MAPK pathway in combination with an inhibitor of c-JUN.

13. Use of an inhibitor of the MAPK pathway and an inhibitor of c-JUN for the preparation of a medicament for the treatment of BRAFV600-mutant melanoma.

14. The method according to claim 12 or use according to claim 13, wherein the inhibitor of the MAPK pathway is selected from the group consisting of: RAF, MEK, ERK, RTK and/or RAS inhibitors.

15. The method or use according to claim 14, wherein the inhibitor of the MAPK pathway is a BRAF inhibitor.

16. The method or use according to claim 15, wherein the BRAF inhibitor is vemurafenib.

17. The method according to any one of claims 12 or 14 to 16, or use according to any one of claims 13 to 16, wherein the inhibitor of c-JUN is an inhibitor the JNK pathway.

18. The method according to any one of claims 12 or 14 to 17, or use according to any one of claims 13 to 17, wherein the inhibitor of the MAPK pathway is administered prior to the inhibitor of c-JUN.

19. A method of treating recurrent BRAFV600-mutant melanoma comprising administering to a subject that has previously presented with BRAFV600-mutant melanoma a therapeutically effective amount of an inhibitor of c-JUN.

20. Use of an inhibitor of c-JUN for the preparation of a medicament for the treatment of

recurrent BRAFV600-mutant melanoma.

21. The method according to claim 19 or use according to claim 20, wherein the recurrent BRAFV600-mutant melanoma is resistant to an inhibitor of the MAPK pathway.

22. The method according to claim 19 or 21 or use according to claim 20 or 21, wherein the recurrent BRAFV600-mutant melanoma is inherently resistant to an inhibitor of the MAPK pathway.

23. The method according to claim 19 or 21 or use according to claim 20 or 21, wherein the wherein the recurrent BRAFV600-mutant melanoma has acquired resistance an inhibitor of the MAPK pathway.

24. The method according to claim 19 or 21 or use according to claim 20 or 21, wherein the recurrent BRAFV600-mutant melanoma has adaptive resistance to an inhibitor of the MAPK pathway.

25. The method according to any one of claims 19 or 21 to 24 or use according to any one of claims 20 to 24, wherein the recurrent BRAFV600-mutant melanoma is resistant to an inhibitor of the MAPK pathway selected from the group consisting of: RAF, MEK, ERK, RTK and/or RAS inhibitors.

26. The method or use according to claim 25, wherein the inhibitor of the MAPK pathway is a BRAF inhibitor.

27. The method or use according to claim 25, wherein the BRAF inhibitor is vemurafenib.

28. The method according to any one of claims 19 or 21 to 27 or use according to any one of claims 20 to 27, wherein the inhibitor of c-JUN is an inhibitor of the JNK pathway.

29. The method according to any one of claims 1, 3 to 11, 19 or 21 to 28 or use according to any one of claims 2 to 11, or 20 to 28 wherein the inhibitor of c-JUN is administered in

combination with one or more treatment modality selected from the group comprising: chemotherapy, radiotherapy, targeted therapy and/or immunotherapy.

30. A pharmaceutical composition comprising an inhibitor of c-JUN for the treatment or prevention of resistance to an inhibitor of the MAPK pathway in a subject and a pharmaceutically-acceptable carrier.

31. The pharmaceutical composition according to claim 30, wherein the composition further comprises an inhibitor of the MAPK pathway.

32. The pharmaceutical composition according to claim 31, wherein the inhibitor of the MAPK pathway is selected from the group consisting of RAF, MEK, ERK, RTK and/or RAS inhibitors.

33. The pharmaceutical composition according to claim 32, wherein the inhibitor of the MAPK pathway is a BRAF inhibitor.

34. The pharmaceutical composition according to claim 33, wherein the BRAF inhibitor is vemurafenib.

35. The pharmaceutical composition according to any one of claims 30 to 34, wherein the inhibitor of c-JUN is an inhibitor of the JNK pathway.

36. The pharmaceutical composition according to any one of claims 30 to 35, further comprising one or more additional modulatory agents for the treatment of cancer.

37. A kit when used for the treatment or prevention of resistance to an inhibitor of the MAPK pathway in a subject comprising an inhibitor of c-JUN and a pharmaceutically-acceptable carrier, together with instructions for use.

38. The kit according to claim 37, wherein the kit further comprises an inhibitor of the MAPK pathway.

39. The kit according to claim 36, wherein the inhibitor of the MAPK pathway is selected from the group consisting of RAF, MEK, ERK, RTK and/or RAS inhibitors.

40. The kit according to claim 39, wherein the inhibitor of the MAPK pathway is a BRAF inhibitor.

41. The kit according to claim 39, wherein the BRAF inhibitor is vemurafenib.

42. The kit according to any one of claims 36 to 41, wherein the inhibitor of c-JUN is an inhibitor of the JNK pathway.

43. The kit according to any one of claims 36 to 42, further comprising one or more additional modulatory agents for the treatment of cancer.

44. The method according to any one of claims according to any one of claims 1, 3 to 12, 14 to 19 or 21 to 29, use according to any one of claims 2 to 11, 13 to 18 or 20 to 29, the composition according to any one of claims 30 to 36, or the kit according to any one of claims 37 to 43, wherein the inhibitor of c-JUN is a RNA inhibiting agent.

45. The method, use, composition or kit according to claim 44, wherein the RNA inhibiting agent is a miRNA, siRNA or piRNA, or combination thereof.

FIGURE 1

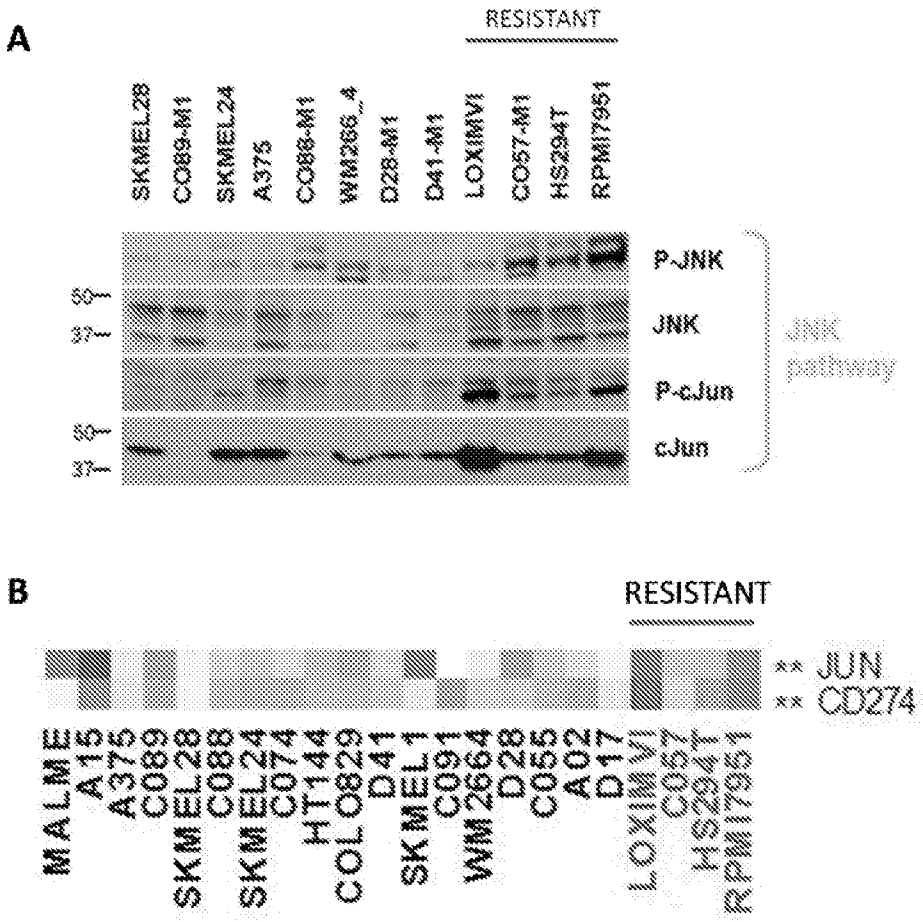

A
RESISTANT
SKMEL28
CO89-M1
SKMEL24
A375
CO88-M1
WM266_4
D28-M1
D41-M1
LOXIMVI
CO57-M1
HS294T
RPMI7951
50
37
50
37
P-JNK
JNK
P-cJun
cJun
JNK pathway
B
RESISTANT
** JUN
** CD274
MALME
A15
A375
CO89
SKMEL28
CO88
SKMEL24
CO74
HT144
COLO829
D41
SKMEL1
CO91
WM2664
D28
CO55
A02
D17
LOXIMVI
CO57
HS294T
RPMI7951

FIGURE 2

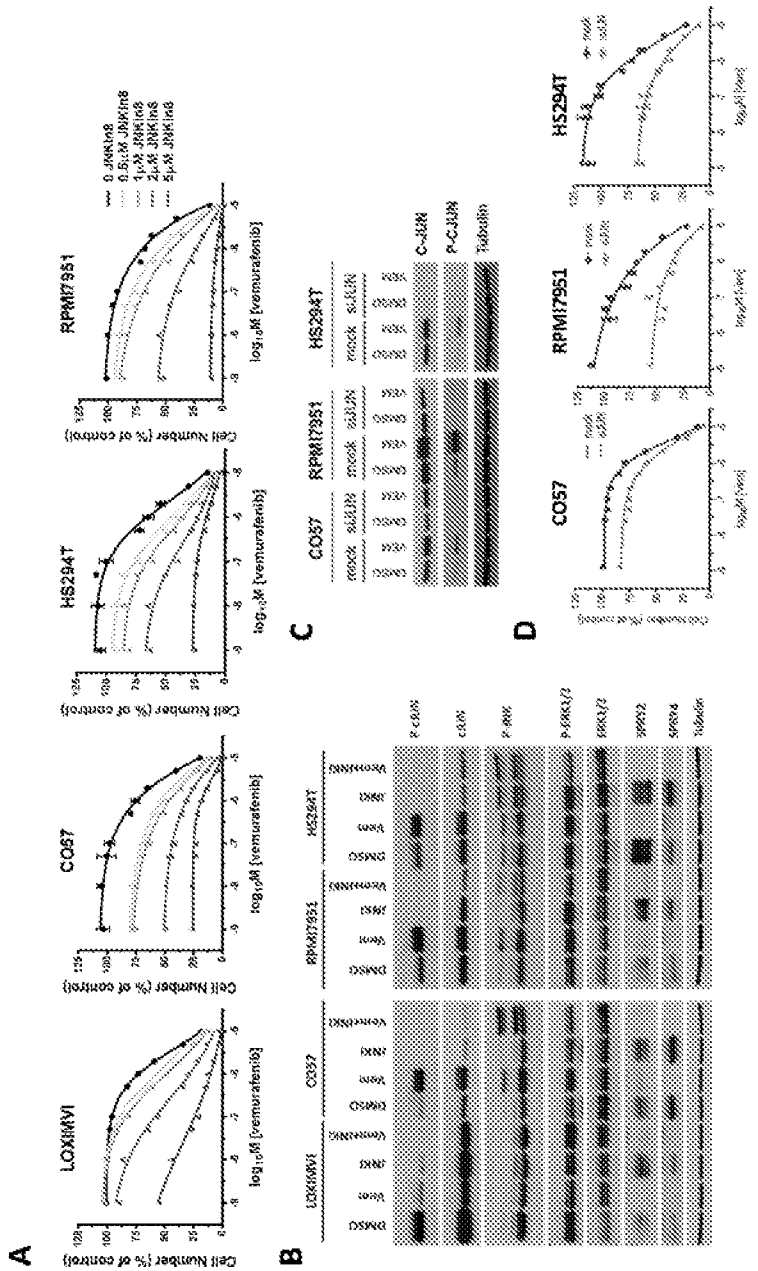

A
LOXIMVI
COS7
HS294T
RPMI7951
Cell Number (% of control)
log10M [vemurafenib]
0 JNKIn8
0.5μM JNKIn8
1μM JNKIn8
2μM JNKIn8
5μM JNKIn8
B
DMSO
JNKi
Vem
Comb
Vemurafenib
P-c-Jun
c-Jun
P-JNK
P-MKK7/7
MKK7/7
pMKK4
MKK4
Tubulin
C
COS7
RPMI7951
HS294T
mock
siJUN
C-JUN
P-C-JUN
Tubulin
D
COS7
RPMI7951
HS294T
+ mock
+ siJUN

FIGURE 3

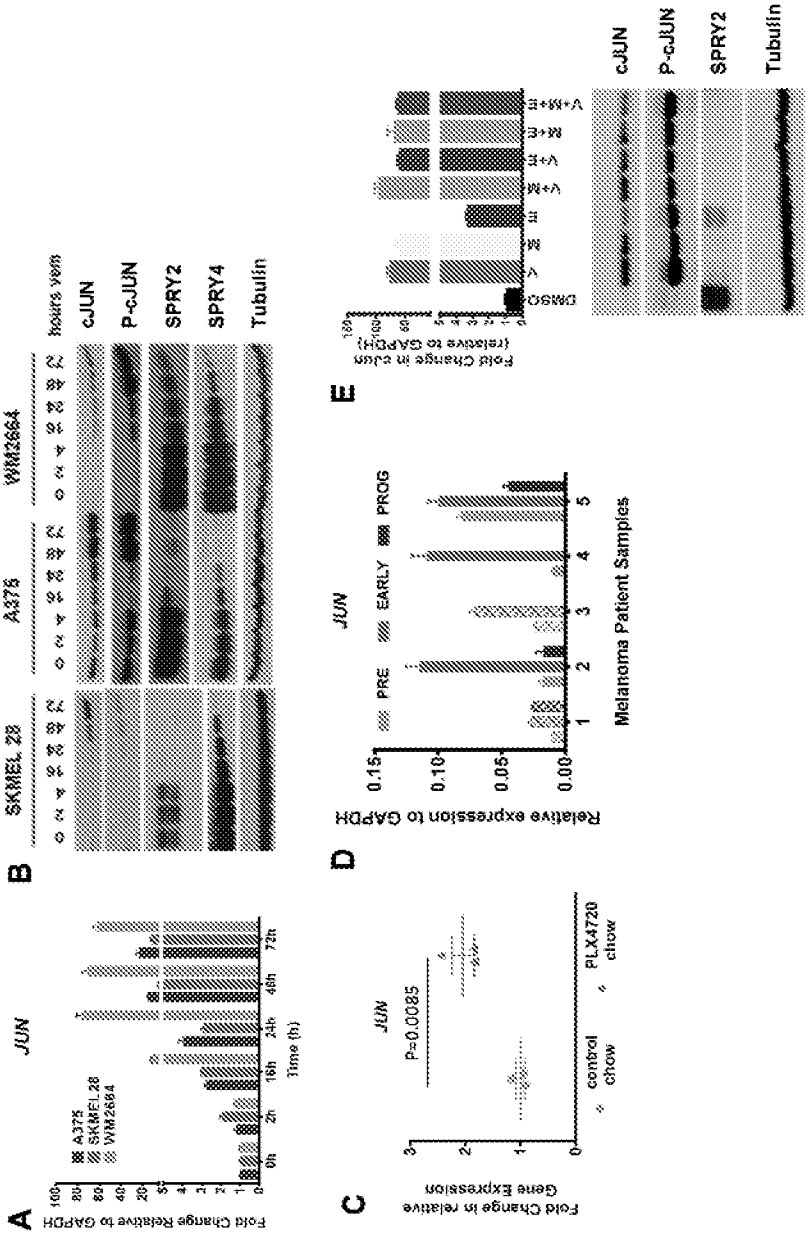

A
JUN
Fold Change Relative to GAPDH
A375
SKMEL28
WM2664
Time (h)
B
SKMEL 28
A375
WM2664
hours vem
cJUN
P-cJUN
SPRY2
SPRY4
Tubulin
C
JUN
P=0.0085
Fold Change in relative Gene Expression
control chow
PLX4720 chow
D
JUN
PRE
EARLY
PROG
Relative expression to GAPDH
Melanoma Patient Samples
E
Fold Change in cJun (relative to GAPDH)
DMSO
V
M
III
V+M
V+III
M+III
V+M+III
cJUN
P-cJUN
SPRY2
Tubulin

FIGURE 4

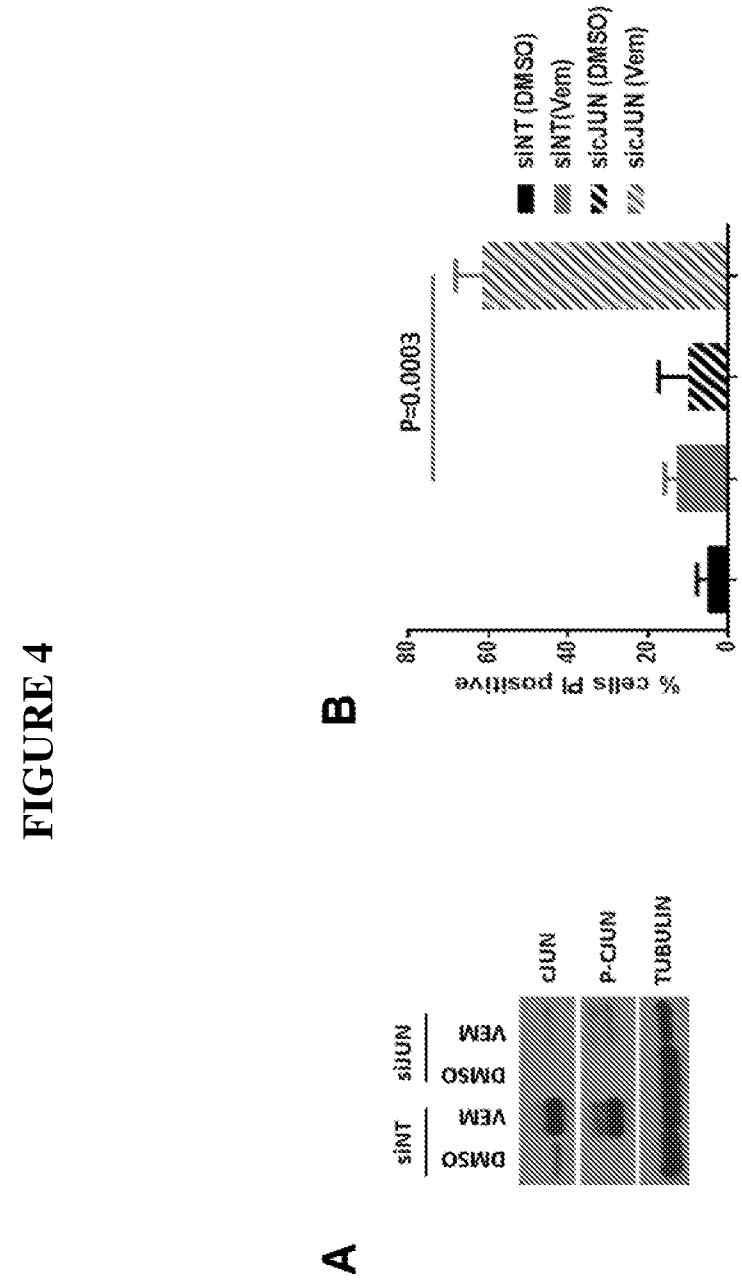

A
siNT
siJUN
DMSO
VEM
DMSO
VEM
cJUN
P-cJUN
TUBULIN
B
P=0.0003
siNT (DMSO)
siNT(Vem)
sicJUN (DMSO)
sicJUN (Vem)
% cells PI positive
0
20
40
60
80

FIGURE 5

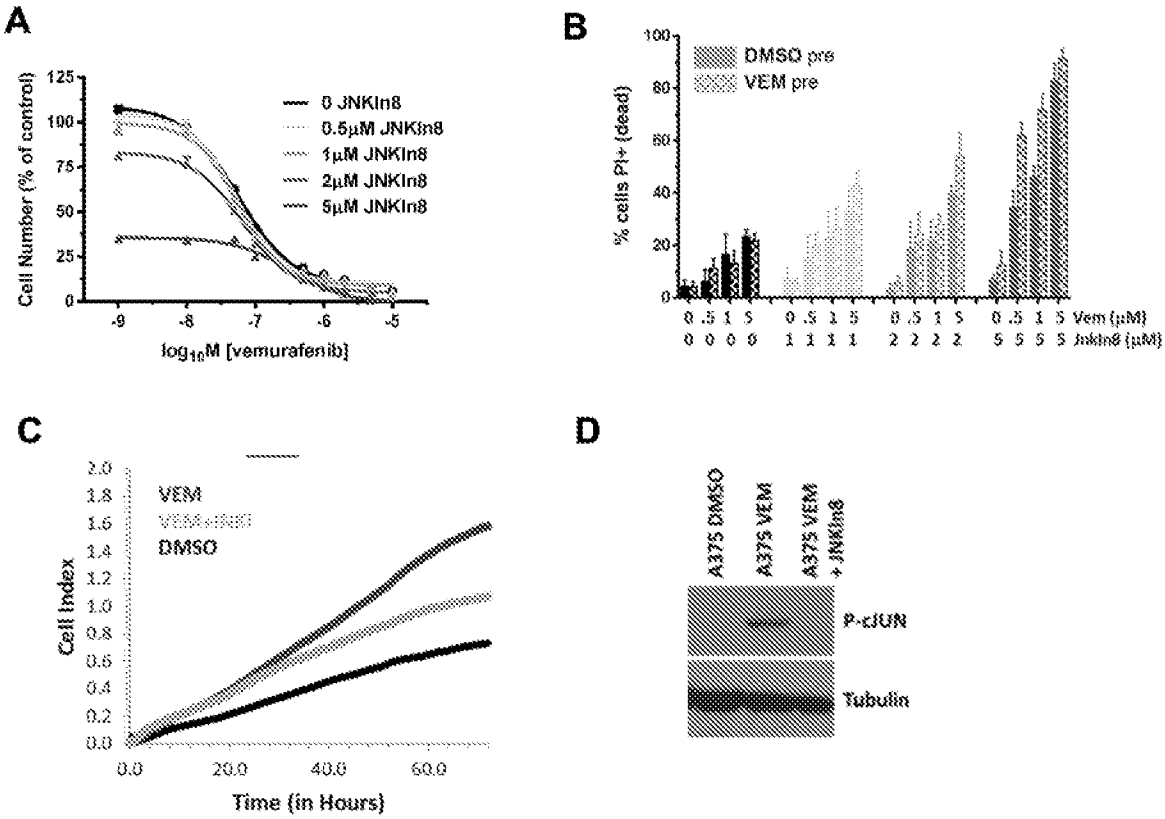
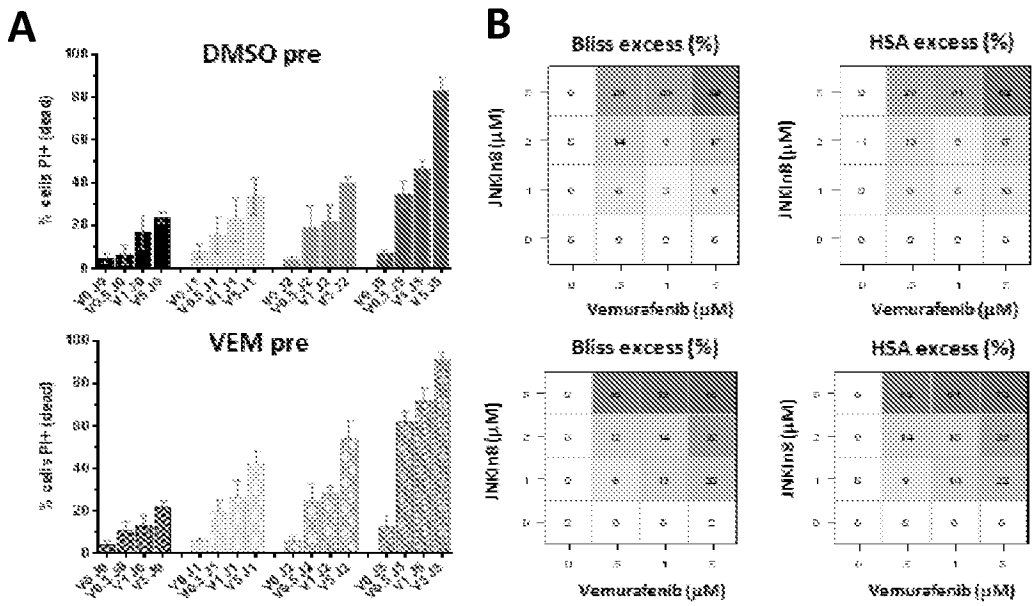

A
Cell Number (% of control)
125
100
75
50
25
0
-9
-8
-7
-6
-5
log10M [vemurafenib]
0 JNKIn8
0.5μM JNKIn8
1μM JNKIn8
2μM JNKIn8
5μM JNKIn8
B
% cells PI+ (dead)
100
80
60
40
20
0
DMSO pre
VEM pre
0 .5 1 5 0 .5 1 5 0 .5 1 5 0 .5 1 5 Vem (μM)
0 0 0 0 1 1 1 1 2 2 2 2 5 5 5 5 JNKIn8 (μM)
C
Cell Index
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
VEM
DMSO
0.0
20.0
40.0
60.0
Time (in Hours)
D
A375 DMSO
A375 VEM
A375 VEM + JNKIn8
P-cJUN
Tubulin

FIGURE 6


A
DMSO pre
VEM pre
B
Bliss excess (%)
HSA excess (%)
JNKIn8 (μM)
Vemurafenib (μM)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2016/050075

A. CLASSIFICATION OF SUBJECT MATTER

A61K 48/00 (2006.01) A61K 45/00 (2006.01) A61P 35/00 (2006.01)

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)

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)

Epoque - WPI, Medline, Epodoc

STN - CAPLUS, EMbase

Keywords, MAPK, C_JUN, JNK, jun terminal kinase, vemurafenib

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 May 2016	26 May 2016
Name and mailing address of the ISA/AU	**Authorised officer**
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustralia.gov.au	Johanna Lowery AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. 0262832968

INTERNATIONAL SEARCH REPORT C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		International application No. **PCT/AU2016/050075**
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2013152038 A1 (BUCK INSTITUTE FOR RESEARCH ON AGING.) 10 October 2013 Whole document, example 10 especially	1-45
X	NGUYEN, T. V. et al "Sorafenib resistance and JNK singnaling in carcinoma during extracellular matrix stiffening." Biomaterials, Vol. 35, page 5749-5759. (2014) Whole Document	1-6, 8, 11 and 29-45
A	US 2012/0301878 A1 (ROSEN et al) 29 November 2012	1-45
A	TAKASHIMA, A. et al "Protein Kinase Cδ is a Therapeutic Target in Malignant Melanoma with NRAS Mutation." ACS Chemical Biology, Vol. 9, page 1003-1014. 2014.	1-45
X	LOGRASSO, P. et al "Inhibitors of c-JUN-N-Terminal Kinase (JNK)." Mini-Reviews in Medicinal Chemistry, Vol. 8, No. 8, page 755-766. 2008. Whole Document	30-45
P,X	FALLAHI-SICHANI, M. et al "Systematic analysis of BRAFV600E melanomas reveals a role for JNK/C-Jun pathway in adaptive resistance to drug-induced apoptosis." Molecular Systems Biology, Vol. 11, Item 797. Published online March 26 2015 Whole document	1-45
P,X	RAMSDALE, R. et al "The transcription cofactor c-JUN mediates phenotype switching and BRAF inhibitor resistance in melanoma." Science Signalling, Vol. 8, Issue 390, Item ra82. 18 August 2015 Whole Document	1-45

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/AU2016/050075**

This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	**Publication Date**	**Publication Number**	**Publication Date**
WO 2013152038 A1	10 October 2013	WO 2013152038 A1	10 Oct 2013
		US 2013288981 A1	31 Oct 2013
US 2012/0301878 A1	29 November 2012	US 2012301878 A1	29 Nov 2012

End of Annex

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.