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(71) Applicant *(for all designated States except US)*: **F. HOFFMANN-LA ROCHE AG** [CH/CH]; Grenzacherstrasse 124, CH-4070 Basel (CH).

(71) Applicant *(for US only)*: **GENENTECH, INC.** [US/US]; 1 DNA Way, South San Francisco, California 94080 (US).

(72) Inventors: **HOEFLICH, Klaus P.**; c/o Genentech, Inc., 1 DNA Way, South San Francisco, California 94080 (US). **JUBB, Adrian M.**; c/o Genentech, Inc., 1 DNA Way, South San Francisco, California 94080 (US). **KOEPPEN, Hartmut**; c/o Genentech, Inc., 1 DNA Way, South San Francisco, California 94080 (US). **ONG, Christy C.**; c/o Genentech, Inc., 1 DNA Way, South San Francisco, California 94080 (US).

(74) Agent: **SCHIRLIN, Julien**; Grenzacherstrasse 124, CH-4070 Basel (CH).

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(54) Title: METHODS OF TREATING MELANOMA WITH PAK1 INHIBITORS

(57) Abstract: The present invention provides methods and compositions for the treatment of melanoma using a PAK1 inhibitor. In some embodiments, PAK1 is overexpressed and/or amplified in the melanoma. In some embodiments, the melanoma is a wild-type BRAF melanoma.

METHODS OF TREATING MELANOMA WITH PAK1 INHIBITORS

Malignant melanoma accounts for approximately 80 percent of deaths from skin cancer. Although melanoma is surgically curable when discovered at early stages, regional and systemic spread of the disease considerably worsens the prognosis with only 14% of metastatic melanoma patients surviving for five years (American Cancer Society. Cancer facts & figures, 2011). The mitogen-activated protein kinase (MAPK) pathway has recently been elucidated as a critical growth pathway in several melanoma subtypes (Lopez-Bergami P. *Pigment Cell Melanoma Res.* 2011, 24(5):902-921). For instance, from a pooled analysis of data from 4493 patients the occurrence of BRAF (v-Raf murine sarcoma viral oncogene homolog B1) mutation is 41% in cutaneous melanomas (Lee JH, *et al.*, *Br J Dermatol.* 2011, 164(4):776-784). The most frequent BRAF somatic mutation in malignant melanoma is substitution of valine at residue 600 to confer constitutive catalytic activity and signaling (Davies H, *et al.*, *Nature.* 2002; 417(6892), 949-954.). Genetic studies have confirmed that BRAF is required for initiation and maintenance of melanoma in preclinical model systems (Davies H, *et al.*, 2002, *ibid*; Hoeflich KP, *et al.*, *Cancer Res.* 2006, 66(2):999-1006; Dankort D, *et al.*, *Genes Dev.* 2007, 21(4):379-3844-6). These discoveries prompted a flurry of drug discovery activity to develop small molecule inhibitors of BRAF, including GDC-0879, PLX-4720, PLX-4032/vemurafenib (Zelboraf™) and GSK2118436 (Hoeflich KP, *et al.*, *Cancer Res.* 2009, 69(7):3042-3051;Tsai J, *et al.*, *Proc Natl Acad Sci U.S.A.* 2008, 105(8):3041-3046; Bollag G, *et al.*, *Nature* 2011, 467(7315):596-599; Ribas A, & Flaherty KT., *Nature Rev.* 2011, 8(7):426-433). These inhibitors selectively decrease the growth of BRAF oncogene addicted tumor cells and provide hope for patients with the subset of melanoma that has activating mutations in the BRAF oncogene (Ribas A, & Flaherty KT., 2011, *ibid*). However, significantly less anti-tumor efficacy with current BRAF small molecule inhibitors is observed for wild-type BRAF melanoma cells (Hoeflich KP, *et al.*, 2009, *ibid*:3042-3051;Tsai J, *et al.*, *ibid*), raising the need to identify additional melanoma-associated driver genes to provide new insights into the biology, oncogenic signaling and possible therapeutic targets for disease management of melanoma patients of all classifications. The RAF kinase family is comprised of three members, ARAF, BRAF and CRAF, which play a pivotal role in transducing signals in the canonical MAPK signaling pathway from RAS to downstream kinases, MEK1/2 and ERK1/2. However, additional kinases have been reported to also play a role in ERK activation. In particular, several groups have reported that group-I p21-

activated kinases (PAKs) contribute to MAPK pathway activation via phosphorylation of both CRAF at Ser338, a critical residue for activation, and MEK1 at Ser298, a site that is proximal to the activation loop residues Ser217/Ser221 that are substrates for the RAF kinases (King AJ, *et al.*, *Nature*. 1998; 396(6707), 180-183; Tang Y, *et al.*, *Mol Cell Biol.* 1999, 19(3):1881-1891; Frost JA, *et al.*, *EMBO J.* 1997, 16(21):6426-6438). The pathway crosstalk between PAKs and the MAPK pathway signaling in epithelial cells can be induced by a variety of conditions, including growth factor stimulation and cell adhesion to the extracellular matrix (Slack-Davis JK, *et al.*, *J Cell Biol*. 2003, 162(2):281-291; Zang M, *et al.*, *J Biol Chem*. 2001, 276(27):25157-25165; Beeser A, *et al.*, *J Biol Chem.* 2005, 280(44):36609-36615). As a major downstream effector of the Rho family small GTPases Cdc42 and Rac1, PAK1 also plays a fundamental role in linking extracellular signals to changes in actin cytoskeleton organization, cell shape and adhesion dynamics (Arias-Romero LE, & Chernoff J., *Biology Cell*. 2008, 100(2):97-108; Kumar R, *et al.*, *Nat Rev Cancer* 2006, 6(6):459-471; Ong CC, *et al.*, *Oncotarget*. 2011, 2(6):491-496). PAK1 is widely expressed in a variety of normal tissues and expression is significantly increased in breast and lung cancers (Holm C, *et al.*, *J Natl Cancer Inst*. 2006, 98(10):671-680; Arias-Romero LE, *et al.*, *Oncogene* 2010, 29(43):5839-5849; Ong CC, *et al.*, *Proc Natl Acad Sci U.S.A.* 2011, 108(17):7177-7182). Functional studies have also implicated PAK1 in cell transformation (Vadlamudi RK, *et al.*, *J Biol Chem.* 2000, 275(46):36238-36244) and tumor growth (Ong CC, *et al.*, 2011, *ibid*; Yi C, *et al.*, *Cancer Res*. 2008, 68(19):7932-7937; Chow HY, *et al.*, *PloS One* 2010, 5(11):e13791). These findings indicate that PAK1 may contribute to tumorigenesis in some disease contexts.

The present invention relates to methods for treating a melanoma in an individual comprising contacting the melanoma with a therapeutically effective amount of a PAK1 inhibitor. In some embodiments, the melanoma is a wild-type BRAF melanoma. In some embodiments, PAK1 is overexpressed in the tumor compared to non-cancerous skin cells. In some embodiments, PAK1 is amplified in the tumor. In some embodiments, the melanoma is a wild-type BRAF melanoma wherein PAK1 is overexpressed in the melanoma. In some embodiments, the melanoma is a wild-type BRAF melanoma wherein PAK1 is amplified in the melanoma. In some embodiments, the melanoma is a wild-type BRAF melanoma wherein PAK1 is overexpressed in the melanoma and PAK1 is amplified in the melanoma. In some embodiments, PAK1 is overexpressed in the melanoma and PAK1 is amplified in the melanoma. In some embodiments, the melanoma is a mutant BRAF melanoma. In some embodiments, the individual is a human. In some embodiments, the invention provides methods for treating melanoma in an individual

comprising administering to the individual a therapeutically effective amount of a PAK1 inhibitor.

In some embodiments, the invention provides methods for treating a melanoma in an individual comprising contacting the melanoma with a therapeutically effective amount of a PAK1 inhibitor wherein the PAK1 inhibitor is a small molecule, a nucleic acid, or a polypeptide. In some embodiments, the invention provides methods for treating a melanoma in an individual comprising administering to the individual a therapeutically effective amount of a PAK1 inhibitor wherein the PAK1 inhibitor is a small molecule, a nucleic acid, or a polypeptide.

In some embodiments, the invention provides methods for treating a melanoma in an individual comprising contacting the melanoma with a therapeutically effective amount of a PAK1 inhibitor wherein the PAK1 inhibitor is used in combination with a therapeutic agent. In some embodiments, the invention provides methods for treating a melanoma in an individual comprising administering to the individual a therapeutically effective amount of a PAK1 inhibitor wherein the PAK1 inhibitor is used in combination with a therapeutic agent.

In some aspects, the invention provides uses of PAK1 inhibitors for the treatment of melanoma in an individual. The invention provides uses of PAK1 inhibitors in the manufacture of a medicament for the treatment of melanoma. In some embodiments, the melanoma is a wild-type BRAF melanoma. In some embodiments, PAK1 is overexpressed in the tumor compared to non-cancerous skin cells. In some embodiments, PAK1 is amplified in the tumor. In some embodiments, the melanoma is a wild-type BRAF melanoma wherein PAK1 is overexpressed in the melanoma. In some embodiments, the melanoma is a wild-type BRAF melanoma wherein PAK1 is amplified in the melanoma. In some embodiments, the melanoma is a wild-type BRAF melanoma wherein PAK1 is overexpressed in the melanoma and PAK1 is overexpressed in the melanoma. In some embodiments, PAK1 is overexpressed in the melanoma and PAK1 is amplified in the melanoma. In some embodiments, the melanoma is a mutant BRAF melanoma. In some embodiments, the individual is a human.

In some aspects, the invention provides compositions and kits comprising a PAK1 inhibitor for use in the treatment of melanoma. Various embodiments relating to these treatment methods are described herein and apply to compositions and kits. In some embodiments, the melanoma is a wild-type BRAF melanoma. In some embodiments, PAK1 is overexpressed in the tumor compared to non-cancerous skin cells. In some embodiments, PAK1 is amplified in the tumor. In some embodiments, the melanoma is a wild-type BRAF melanoma wherein PAK1 is overexpressed in the melanoma. In some embodiments, the melanoma is a wild-type BRAF melanoma wherein PAK1 is amplified in the melanoma. In some embodiments, the melanoma is a wild-type BRAF melanoma wherein PAK1 is overexpressed in the melanoma and PAK1 is

overexpressed in the melanoma. In some embodiments, PAK1 is overexpressed in the melanoma and PAK1 is amplified in the melanoma. In some embodiments, the melanoma is a mutant BRAF melanoma. In some embodiments, the individual is a human.

In some embodiments, the invention provides methods of inhibiting CRAF signaling and/or MEK signaling in a melanoma in an individual comprising contacting the melanoma with a therapeutically effective amount of a PAK1 inhibitor. In some embodiments, the melanoma is a wild-type BRAF melanoma. In some embodiments, PAK1 is overexpressed in the tumor compared to non-cancerous skin cells. In some embodiments, PAK1 is amplified in the tumor. In some embodiments, PAK1 is overexpressed in the tumor compared to non-cancerous skin cells. In some embodiments, PAK1 is amplified in the tumor. In some embodiments, the melanoma is a wild-type BRAF melanoma wherein PAK1 is overexpressed in the melanoma. In some embodiments, the melanoma is a wild-type BRAF melanoma wherein PAK1 is amplified in the melanoma. In some embodiments, the melanoma is a wild-type BRAF melanoma wherein PAK1 is overexpressed in the melanoma and PAK1 is overexpressed in the melanoma. In some embodiments, PAK1 is overexpressed in the melanoma and PAK1 is amplified in the melanoma. In some embodiments, the melanoma is a mutant BRAF melanoma. In some embodiments, the individual is a human. In some embodiments, the invention provides methods for treating melanoma in an individual comprising administering to the individual a therapeutically effective amount of a PAK1 inhibitor.

In some aspects, the invention provides, methods of identifying a human melanoma patient suitable for treatment with a PAK1 inhibitor comprising determining the BRAF genotype of the melanoma, wherein a melanoma comprising a wild type BRAF indicates that the patient is suitable for treatment with a PAK1 inhibitor. In some aspects, the invention provides methods of identifying a human melanoma patient suitable for treatment with a PAK1 inhibitor comprising determining the expression of PAK1 in the melanoma, wherein overexpression of PAK1 in the melanoma compared to non-cancerous skin cells indicates that the patient is suitable for treatment with a PAK1 inhibitor. In some aspects, the invention provides methods of identifying a human melanoma patient suitable for treatment with a PAK1 inhibitor comprising determining the copy number of PAK1 in the melanoma, wherein amplification of PAK1 in the melanoma indicates that the patient is suitable for treatment with a PAK1 inhibitor. In some aspects, the invention provides methods of identifying a human melanoma patient suitable for treatment with a PAK1 inhibitor comprising determining the BRAF genotype of the melanoma and determining the expression of PAK1 in the melanoma, wherein the presence of a wild-type BRAF and/or the overexpression of PAK1 in the melanoma compared to non-cancerous skin cells indicates that the patient is suitable for treatment with a PAK1 inhibitor. In some aspects, the invention

provides methods of identifying a human melanoma patient suitable for treatment with a PAK1 inhibitor comprising one or more of determining the BRAF genotype of the melanoma, determining the expression of PAK1 in the melanoma, and determining the copy number of PAK1 in the melanoma wherein one or more of the presence of a wild-type BRAF, the overexpression of PAK1 in the melanoma compared to non-cancerous skin cells, and amplification of PAK1 in the melanoma indicates that the patient is suitable for treatment with a PAK1 inhibitor.

In some aspects, the invention provides methods for treating a human melanoma patient with a PAK1 inhibitor comprising: (a) selecting a patient based on the BRAF genotype of the melanoma, wherein a melanoma comprising a wild type BRAF indicates that the patient is suitable for treatment with a PAK1 inhibitor; and (b) administering to the selected patient a therapeutically effective amount of a PAK1 inhibitor.

In some aspects, the invention provides methods for treating a human melanoma patient with a PAK1 inhibitor comprising: (a) selecting a patient based on the PAK1 expression level of the melanoma, wherein an overexpression of PAK1 in the melanoma compared to non-cancerous cells indicates that the patient is suitable for treatment with a PAK1 inhibitor; and (b) administering to the selected patient a therapeutically effective amount of a PAK1 inhibitor.

In some aspects, the invention provides methods for treating a human melanoma patient with a PAK1 inhibitor comprising: (a) selecting a patient based on the copy number of PAK1 in the melanoma, wherein amplification of PAK1 in the melanoma indicates that the patient is suitable for treatment with a PAK1 inhibitor; and (b) administering to the selected patient a therapeutically effective amount of a PAK1 inhibitor.

In some aspects, the invention provides methods for treating a human melanoma patient with a PAK1 inhibitor comprising: (a) selecting a patient based on the BRAF genotype of the melanoma and PAK1 expression level of the melanoma, wherein a melanoma comprising a wild type BRAF and/or overexpression of PAK1 in the melanoma compared to non-cancerous cells indicates that the patient is suitable for treatment with a PAK1 inhibitor; and (b) administering to the selected patient a therapeutically effective amount of a PAK1 inhibitor.

In some aspects, the invention provides methods for treating a human melanoma patient with a PAK1 inhibitor comprising: (a) selecting a patient based on one or more of the BRAF genotype of the melanoma, PAK1 expression level of the melanoma, and copy number of PAK1 in the melanoma, wherein a melanoma comprising one or more of a wild type BRAF, overexpression of PAK1 in the melanoma compared to non-cancerous cells, and amplification of PAK1 indicates that the patient is suitable for treatment with a PAK1 inhibitor; and (b) administering to the selected patient a therapeutically effective amount of a PAK1 inhibitor.

In some aspects, the invention provides methods of adjusting treatment of melanoma in a patient undergoing treatment with a PAK1 inhibitor, said method comprising assessing the PAK1 expression in the melanoma, wherein overexpression of PAK1 in the melanoma indicates that treatment of the individual is adjusted until PAK1 overexpression is no longer detected. In some embodiments the melanoma is a wild-type BRAF melanoma. In some embodiments, PAK1 is amplified in the melanoma. In some embodiments, the melanoma is a wild-type melanoma and PAK1 is amplified in the melanoma.

Figure 1 shows that PAK1 is highly expressed in human melanoma. (A) Analysis of 11q13 copy number gains in human melanoma tissues. Vertical red line represents chromosome location of the PAK1 gene. (B) PAK1 DNA copy and mRNA expression (226507_at Affymetrix MAS 5.0 signal) correlated for melanoma tumor samples. (C) Representative images of PAK1 immunohistochemistry in primary human malignant melanomas. Cytoplasmic expression score: 0 (I), 1 (II), 2 (III) and 3 (IV). Chromogen deposition indicates immunoreactivity against a hematoxylin counterstain. PAK1 expression was also seen in stromal cells (III) and cells intercalating within the epidermis that may represent Langerhan's cells (IV).

Figure 2 demonstrates PAK1 playing a critical role in proliferation of BRAF wild-type melanoma cells. (A) Proliferation of melanoma cells following transfection with siRNA oligonucleotides was measured by Cell TiterGlo ATP consumption assay. PAK1 was required for cell growth and the data were normalized to control and shown as the mean ± SD. (B) In a panel of melanoma cell lines, PAK1 inhibition selectively impaired growth of cells without BRAF(V600E) mutation (n = 5; 537MEL, Hs940T, MeWO, SK-MEL2, SK-MEL23, SK-MEL30) compared to those with BRAF(V600E) mutation (n = 9; p = 0.07; 624MEL, 888MEL, 928MEL, RPMI-7951, A375, Colo829, LOX-IMVI, Malme-3M, A375). (C) Inhibition of PAK1/2 decreased ERK1/2 and MEK1/2 phosphorylation and accumulation of cyclin D1. (D) PAK1/2 inhibition in SK-MEL23 BRAF wild-type melanoma cells decreases signaling to the cytoskeletal, MAPK, proliferation and NF-κB pathways as determined via reverse phase protein array (RPPA) analysis. Normalized RPPA results are presented as mean ± SD. siNTC = non-targeting control siRNA. siNRAS = NRAS-specific siRNA. siPAK1 = PAK1-specific siRNA. ΔPAK1 = chromosomal deletion of PAK1 gene.

Figure 3 depicts a series of immunoblots demonstrating that PAK1 is required for CRAF activation in BRAF wild-type melanoma cells. (A) PAK1- and PAK2-selective or non-targeting control (NTC) siRNA oligonucleotides were transfected into SK-MEL23 and 537MEL melanoma cells. After 48 h, endogenous MEK1 (A), MEK2 (B) or CRAF (C) proteins were immunoprecipitated and the complexes were immunoblotted to detect phosphorylation of residues critical for catalytic activation. Total protein levels in the immunocomplexes were also

determined as loading controls. (D) Cells were treated with DMSO or 5 μM PF-3758309 for 4 h and endogenous CRAF was immunoprecipitated and immunoblotted for Ser338 phosphorylation. Total CRAF levels in the immunocomplexes are also shown. (E) SK-MEL23 cells were treated with DMSO, 5 μM PF-3758309 or 20 μM IPA-3 for 4 h. CRAF immunocomplexes were incubated with inactive MEK1 protein in kinase buffer for 30 minutes. Levels of phospho-MEK1 (Ser217/Ser221) were determined and CRAF catalytic activity is reported as the levels of MEK1 phosphorylation normalized to total CRAF protein.

Figure 4 contains images demonstrating PAK is required for melanoma cell migration. Following non-targeting control (NTC) or PAK1/2 siRNA oligonucleotide transfection for 72 h, confluent WM-266-4 melanoma cell were wounded and images were recorded when wounds were made (dark shading) and after incubation for 28 h (bright field). Differences in relative wound density were statistically significant ($p < 0.001$; n=3).

Figure 5 depicts a series of immunoblots demonstrating in vitro differential sensitivity of MAPK signaling in BRAF wild-type and BRAF(V600E) melanoma cells treated with PAK inhibitors. (A) SK-MEL23 and A375 cells were treated with DMSO, 5 μM PF-3758309 or 0.2 μM PLX-4720 for 4 h and lysates were analyzed for phosphorylation of MAPK pathway components. Lighter and darker exposures of p-MEK1/2(S217/S221) immunoblots are shown. (B) Ectopic expression of Flag epitope-tagged PAK1 drove MAPK pathway activation in A375 cells. Specificity was demonstrated using PF-3758309 inhibitor treatment as a control.

Figure 6 depicts a series of graphs demonstrating decreased viability of BRAF wild-type melanoma cells due to treatment with in-house PAK inhibitors. Catalytic inhibition of PAK1 via I-007, I-054, I-087 and PF-3758309 treatment was tested in vitro using (A) SK-MEL23 and (B) 537MEL cells using a 4-day Cell TiterGlo (Promega) viability assay.

Figure 7 shows in vivo differential sensitivity of MAPK signaling due to PAK inhibition in xenograft tumor mouse models of BRAF wild-type and BRAF(V600E) melanoma. (A) Pharmacodynamic response of BRAF wild-type and mutant tumors measured by phosphorylation of CRAF(Ser338) following either vehicle or 35 mg/kg PF-3758309 administration. (B) Anti-tumor efficacy of 10, 15 and 25 mg/kg PF-3758309 i.p. daily dosing in the SK-MEL23 preclinical tumor model of BRAF wild-type melanoma.

Figure 8 depicts a series of graphs demonstrating individual tumor data for the SK-MEL23 preclinical tumor model of BRAF wild-type melanoma. (A) Tumor growth inhibition and (B) body weight loss are shown for individual animals treated with 10, 15 and 25 mg/kg PF-3758309. To analyze the repeated measurement of tumor volumes from the same animals over time, cubic regression splines were used to fit a non linear profile to the time courses of $\log_2$ tumor volume at each dose level. These non linear profiles were then related to dose within the

mixed model. Cubic regression splines were used to fit a non linear profile to the time courses of log_2 tumor volume at each dose level. These non linear profiles were then related to dose within the mixed model. Tumor growth inhibition as a percentage of Vehicle (%TGI) was calculated as the percentage of the area under the fitted curve (AUC) for the respective dose group per day in relation to the vehicle, using the formula: $\%TGI = 100 \times (1 - AUC_{dose}/AUC_{veh})$. Plotting was performed and generated using R version 2.8.1 and Excel, version 12.0.1 (Microsoft). Data were analyzed using R version 2.8.1 (R Foundation for Statistical Computing; Vienna, Austria), and the mixed models were fit within R using the nlme package, version 3.1-89.

Figure 9 shows immunoblots demonstrating differing pharmacodynamic responses of BRAF wild-type tumors treated with either G945 BRAF inhibitor or PF-3758309. Phosphorylation of CRAF(Ser338) was determined for SK-MEL23 xenograft tumors following administration of either 35 mg/kg PF-3758309 i.p. or 10 mg/kg G945 (BRAF inhibitor) p.o. compounds. Tumors were harvested 1 hour post dosing and flash frozen. Each lane represents tumor lysate from an individual xenograft mouse.

Figure 10 is a diagram depicting the mechanism of action for PAK1 in BRAF wild-type melanoma. (A) In the context of oncogenic mutation, BRAF strongly drives activation of the MAPK signaling pathway and these tumor cells are sensitive to inhibition of this kinase. (B) In melanomas in which BRAF is not mutated, elevated expression and genomic amplification of PAK1 is frequent and results in increased signaling to CRAF-MEK-ERK and potentially additional effector pathways. This subset of melanoma is relatively insensitive to BRAF inhibition and proliferative capacity is dependent on PAK1.

The present invention provides methods and compositions for the treatment of melanoma in an individual contacting the melanoma with a therapeutically effective amount of a PAK1 inhibitor. The invention also provides such methods of treatment comprising administering to the individual, a therapeutically effective amount of a PAK1 inhibitor. In some embodiments, the melanoma is a wild-type BRAF melanoma. In some embodiments, the melanoma overexpresses PAK1 compared to non-cancerous cells. In some embodiments, PAK1 is amplified in the melanoma. In some embodiments, the melanoma is a wild-type BRAF melanoma and overexpresses PAK1 compared to non-cancerous cells. In some embodiments, the melanoma is a wild-type BRAF melanoma, the melanoma overexpresses PAK1 compared to non-cancerous cells and PAK1 is amplified in the melanoma.

All references cited herein, including patent applications, patent publications, and Genbank Accession numbers are herein incorporated by reference, as if each individual reference were specifically and individually indicated to be incorporated by reference.

Definitions

The techniques and procedures described or referenced herein are generally well understood and commonly employed using conventional methodology by those skilled in the art, such as, for example, the widely utilized methodologies described in Sambrook *et al.*, Molecular Cloning: A Laboratory Manual 3rd. edition (2001) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (F. M. Ausubel, *et al.* eds., (2003)); the series METHODS IN ENZYMOLOGY (Academic Press, Inc.): PCR 2: A PRACTICAL APPROACH (M. J. MacPherson, B. D. Hames and G. R. Taylor eds. (1995)), Harlow and Lane, eds. (1988) ANTIBODIES, A LABORATORY MANUAL, and ANIMAL CELL CULTURE (R. I. Freshney, ed. (1987)); Oligonucleotide Synthesis (M. J. Gait, ed., 1984); Methods in Molecular Biology, Humana Press; Cell Biology: A Laboratory Notebook (J. E. Cellis, ed., 1998) Academic Press; Animal Cell Culture (R. I. Freshney), ed., 1987); Introduction to Cell and Tissue Culture (J. P. Mather and P. E. Roberts, 1998) Plenum Press; Cell and Tissue Culture Laboratory Procedures (A. Doyle, J. B. Griffiths, and D. G. Newell, eds., 1993-8) J. Wiley and Sons; Handbook of Experimental Immunology (D. M. Weir and C. C. Blackwell, eds.); Gene Transfer Vectors for Mammalian Cells (J. M. Miller and M. P. Calos, eds., 1987); PCR: The Polymerase Chain Reaction, (Mullis *et al.*, eds., 1994); Current Protocols in Immunology (J. E. Coligan *et al.*, eds., 1991); Short Protocols in Molecular Biology (Wiley and Sons, 1999); Immunobiology (C. A. Janeway and P. Travers, 1997); Antibodies (P. Finch, 1997); Antibodies: A Practical Approach (D. Catty., ed., IRL Press, 1988-1989); Monoclonal Antibodies: A Practical Approach (P. Shepherd and C. Dean, *eds.*, Oxford University Press, 2000); Using Antibodies: A Laboratory Manual (E. Harlow and D. Lane (Cold Spring Harbor Laboratory Press, 1999); The Antibodies (M. Zanetti and J. D. Capra, eds., Harwood Academic Publishers, 1995); and Cancer: Principles and Practice of Oncology (V. T. DeVita *et al.*, *eds.*, J.B. Lippincott Company, 1993).

Unless defined otherwise, technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Singleton *et al.*, Dictionary of Microbiology and Molecular Biology 2nd ed., J. Wiley & Sons (New York, N.Y. 1994), and March, Advanced Organic Chemistry Reactions, Mechanisms and Structure 4th ed., John Wiley & Sons (New York, N.Y. 1992), provide one skilled in the art with a general guide to many of the terms used in the present application.

"PAK," as used herein, refers to a family of non-receptor serine/threonine protein kinases (STKs). The p21-activated protein kinase (PAK) family of serine/threonine protein kinases plays important roles in cytoskeletal organization, cellular morphogenesis, cellular processes and

cell survival (Daniels *et al.*, *Trends Biochem. Sci.* 1999 24: 350-355; Sells *et al.*, *Trends Cell. Biol.* 1997 7: 162-167). The PAK family consists of six members subdivided into two groups: PAK 1-3 (group I) and PAK 4-6 (group II) which are distinguished based upon sequence homologies and the presence of an autoinhibitory region in group I PAKs. p21-Activated kinases (PAKs) serve as important mediators of Rac and Cdc42 GTPase function as well as pathways required for Ras-driven tumorigenesis. (Manser *et al.*, *Nature* 1994 367:40-46; B Dummler *et al.*, *Cancer Metathesis Rev.* 2009 28:51-63; R. Kumar *et al.*, *Nature Rev. Cancer* 2006 6:459-473).

"PAK1" or "p21-activated protein (Cdc42/Rac)-activated kinase 1" as used herein refers to a native PAK1 from any vertebrate source, including mammals such as primates (*e.g.*, humans) and rodents (*e.g.*, mice and rats), unless otherwise indicated. The terms encompass the genomic location (*e.g.* 11q13-q14 cytogenetic band, chromosome 11:77033060-77185108, and/or GC11M077033), "full-length," unprocessed PAK1 as well as any form of PAK1 that result from processing in the cell. The term also encompasses naturally occurring variants of PAK1, *e.g.*, splice variants or allelic variants. The sequence of an exemplary human PAK1 nucleic acid is NC_000011.9. An exemplary human PAK1 amino acid sequence is NP_0011220921 or NP_002567.3. The sequence of an exemplary mouse PAK1 nucleic acid is NC_000073.6 or an exemplary mouse PAK1 amino acid sequence NP_035165.2. The sequence of an exemplary rat PAK1 nucleic acid is NC_005100.2 or an exemplary rat PAK1 amino acid sequence NP_058894.1. The sequence of an exemplary dog PAK1 nucleic acid is NC_006603.3 or an exemplary dog PAK1 amino acid sequence XP_849651.1. The sequence of an exemplary cow PAK1 nucleic acid is AC_000186.1 or NC_007330.5. An exemplary cow PAK1 amino acid sequence is NP_001070366.1. The sequence of an exemplary rhesus monkey PAK1 nucleic acid is NC_007871.1. An exemplary rhesus monkey PAK1 amino acid sequence is XP_001090310.1 or NP_001090423.2. The sequence of an exemplary chicken PAK1 nucleic acid is NC_006088.3 or an exemplary chicken PAK1 amino acid sequence NP_001155844.1

"BRAF" or "Serine/threonine-protein kinase B-Raf," as used herein, refers to as used herein refers to a native BRAF from any vertebrate source, including mammals such as primates (*e.g.*, humans) and rodents (*e.g.*, mice and rats), unless otherwise indicated. The terms encompass the genomic location (*e.g.*, 7q34 cytogenetic band, chromosome 7:140433812-140624564, and/or GC07M140424), "full-length," unprocessed BRAF as well as any form of BRAF that result from processing in the cell. The term also encompasses naturally occurring variants of BRAF, *e.g.*, splice variants or allelic variants. The sequence of an exemplary human BRAF nucleic acid is NC_000007.13 or an exemplary human BRAF amino acid sequence NP_004324.2. The sequence of an exemplary mouse BRAF nucleic acid is NC_000072.6 or an exemplary mouse

BRAF amino acid sequence NP_647455.3. The sequence of an exemplary rat BRAF nucleic acid is NC_005103.2 or an exemplary rat BRAF amino acid sequence XP_231692.4. The sequence of an exemplary dog BRAF nucleic acid is NC_006598.3 or an exemplary dog BRAF amino acid sequence XP_532749.3. The sequence of an exemplary chicken BRAF nucleic acid is NC_006088.3 or an exemplary chicken BRAF amino acid sequence NP_990633.1. The sequence of an exemplary cow BRAF nucleic acid is AC_000161.1 or an exemplary cow BRAF amino acid sequence XP_002687048.1. The sequence of an exemplary horse BRAF nucleic acid is NC_009147.2 or an exemplary horse BRAF amino acid sequence XP_001496314.2.

"Wild-type BRAF" refers herein to a naturally occurring BRAF (including naturally occurring variants) not associated with melanoma. An example of wild-type human BRAF is provided by GenBank Accession No. NP_004324.2. As is known in the art, with respect to BRAF melanomas can be categorized and classified by BRAF type: wild-type BRAF and mutant BRAF.

"Mutant BRAF" as used herein refers to a BRAF protein with one or more mutations which is associated with melanoma. An example of a mutant BRAF is one where a valine at position 600 is replaced with a glutamate (V600E). As is known in the art, melanomas can be categorized by BRAF type: wild-type BRAF and mutant BRAF.

"CRAF" or "v-raf leukemia viral oncogene 1" as used herein, as used herein refers to a native CRAF from any vertebrate source, including mammals such as primates (*e.g.*, humans) and rodents (*e.g.*, mice and rats), unless otherwise indicated. The terms encompass the genomic location (*e.g.*, 3p25 cytogenetic band, chromosome 3:12625100-12705700, and/or GC03M012625), "full-length," unprocessed CRAF as well as any form of CRAF that result from processing in the cell. The term also encompasses naturally occurring variants of CRAF, *e.g.*, splice variants or allelic variants. The sequence of an exemplary human CRAF nucleic acid is NC_000003.11 or an exemplary human CRAF amino acid sequence NP_002871.1.

"MEK" or "mitogen-activated protein kinase kinase," as used herein, refers to a family of kinase enzymes which phosphorylate mitogen-activated protein kinase (MAPK). There are seven genes: MAP2K1 (MEK1), MAP2K2 (MEK2), MAP2K3 (MKK3), MAP2K4 (MKK4), MAP2K5 (MKK5), MAP2K6 (MKK6), and MAP2K7 (MKK7). The activators of p38 (MKK3 and MKK6), JNK (MKK4 and MKK7), and ERK (MEK1 and MEK2) define independent MAP kinase signal transduction pathways. The sequence of an exemplary human MEK1 nucleic acid is NC_000015.9 or an exemplary human MEK1 amino acid sequence NP_002746.1. The sequence of an exemplary human MEK2 nucleic acid is NC_000019.9 or an exemplary human MEK2 amino acid sequence NP_109587.1. The sequence of an exemplary human MEK3 nucleic acid is NC_000017.10. An exemplary human MEK3 amino acid sequence is

NP_002747.2 or NP_659731.1. The sequence of an exemplary human MEK4 nucleic acid is NC_000017.10 or an exemplary human MEK4 amino acid sequence NP_003001.1. The sequence of an exemplary human MEK5 nucleic acid is NC_000015.9. An exemplary human MEK5 amino acid sequence is NP_001193733.1, NP_002748.1, or NP_660143.1. The sequence of an exemplary human MEK6 nucleic acid is NC_000017.10 or an exemplary human MEK6 amino acid sequence NP_002749.2. The sequence of an exemplary human MEK7 nucleic acid is NC_000019.9 or an exemplary human MEK7 amino acid sequence NP_660186.1.

"Polynucleotide," or "nucleic acid," as used interchangeably herein, refer to polymers of nucleotides of any length, and include DNA and RNA. The nucleotides can be deoxyribonucleotides, ribonucleotides, modified nucleotides or bases, and/or their analogs, or any substrate that can be incorporated into a polymer by DNA or RNA polymerase, or by a synthetic reaction. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and their analogs. If present, modification to the nucleotide structure may be imparted before or after assembly of the polymer. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after synthesis, such as by conjugation with a label. Other types of modifications include, for example, "caps", substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages (*e.g.*, methyl phosphonates, phosphotriesters, phosphoamidates, carbamates, etc.) and with charged linkages (*e.g.*, phosphorothioates, phosphorodithioates, etc.), those containing pendant moieties, such as, for example, proteins (*e.g.*, nucleases, toxins, antibodies, signal peptides, ply-L-lysine, etc.), those with intercalators (*e.g.*, acridine, psoralen, etc.), those containing chelators (*e.g.*, metals, radioactive metals, boron, oxidative metals, etc.), those containing alkylators, those with modified linkages (*e.g.*, alpha anomeric nucleic acids, etc.), as well as unmodified forms of the polynucleotide(s). Further, any of the hydroxyl groups ordinarily present in the sugars may be replaced, for example, by phosphonate groups, phosphate groups, protected by standard protecting groups, or activated to prepare additional linkages to additional nucleotides, or may be conjugated to solid or semi-solid supports. The 5' and 3' terminal OH can be phosphorylated or substituted with amines or organic capping group moieties of from 1 to 20 carbon atoms. Other hydroxyls may also be derivatized to standard protecting groups. Polynucleotides can also contain analogous forms of ribose or deoxyribose sugars that are generally known in the art, including, for example, 2'-O-methyl-, 2'-O-allyl, 2'-fluoro- or 2'-azido-ribose, carbocyclic sugar analogs, ?-anomeric sugars, epimeric sugars such as arabinose, xyloses or lyxoses, pyranose sugars, furanose sugars, sedoheptuloses, acyclic analogs and abasic nucleoside analogs such as methyl riboside. One or more phosphodiester linkages may be replaced by alternative linking

groups. These alternative linking groups include, but are not limited to, embodiments wherein phosphate is replaced by P(O)S("thioate"), P(S)S ("dithioate"), (O)NR2 ("amidate"), P(O)R, P(O)OR', CO or CH2 ("formacetal"), in which each R or R' is independently H or substituted or unsubstituted alkyl (1-20 C) optionally containing an ether (-O-) linkage, aryl, alkenyl, cycloalkyl, cycloalkenyl or araldyl. Not all linkages in a polynucleotide need be identical. The preceding description applies to all polynucleotides referred to herein, including RNA and DNA.

"Oligonucleotide," as used herein, generally refers to short, single stranded, polynucleotides that are, but not necessarily, less than about 250 nucleotides in length. Oligonucleotides may be synthetic. The terms "oligonucleotide" and "polynucleotide" are not mutually exclusive. The description above for polynucleotides is equally and fully applicable to oligonucleotides.

The term "primer" refers to a single stranded polynucleotide that is capable of hybridizing to a nucleic acid and following polymerization of a complementary nucleic acid, generally by providing a free 3'-OH group.

The term "small molecule" refers to any molecule with a molecular weight of about 2000 daltons or less, preferably of about 500 daltons or less.

The term "antibody" herein is used in the broadest sense and encompasses various antibody structures, including but not limited to monoclonal antibodies, polyclonal antibodies, multispecific antibodies (*e.g.*, bispecific antibodies), and antibody fragments so long as they exhibit the desired antigen-binding activity.

The term "detection" includes any means of detecting, including direct and indirect detection.

The term "biomarker" as used herein refers to an indicator, *e.g.*, predictive, diagnostic, and/or prognostic, which can be detected in a sample. The biomarker may serve as an indicator of a particular subtype of a disease or disorder (*e.g.*, cancer) characterized by certain, molecular, pathological, histological, and/or clinical features. For example, biomarkers for melanoma include, but are not limited to, the presence of wild-type BRAF, overexpression of PAK1 and amplification of PAK1.

The "amount" or "level" of a biomarker associated with an increased clinical benefit to an individual is a detectable level in a biological sample. These can be measured by methods known to one skilled in the art and also disclosed herein. The expression level or amount of biomarker assessed can be used to determine the response to the treatment.

The terms "level of expression" or "expression level" in general are used interchangeably and generally refer to the amount of a biomarker in a biological sample. "Expression" generally refers to the process by which information (*e.g.*, gene-encoded and/or epigenetic) is converted into the structures present and operating in the cell. Therefore, as used herein, "expression" may refer to transcription into a polynucleotide, translation into a polypeptide, or even polynucleotide

and/or polypeptide modifications (*e.g.*, posttranslational modification of a polypeptide). Fragments of the transcribed polynucleotide, the translated polypeptide, or polynucleotide and/or polypeptide modifications (*e.g.*, posttranslational modification of a polypeptide) shall also be regarded as expressed whether they originate from a transcript generated by alternative splicing or a degraded transcript, or from a post-translational processing of the polypeptide, *e.g.*, by proteolysis. "Expressed genes" include those that are transcribed into a polynucleotide as mRNA and then translated into a polypeptide, and also those that are transcribed into RNA but not translated into a polypeptide (for example, transfer and ribosomal RNAs).

"Elevated expression," "elevated expression levels," "elevated levels" and "overexpressed" refers to an increased expression or increased levels of a biomarker in an individual relative to a control, such as an individual or individuals who are not suffering from the disease or disorder (*e.g.*, cancer) or an internal control (*e.g.*, housekeeping biomarker). In some examples, elevated expression or overexpression is the result of gene amplification.

"Reduced expression," "reduced expression levels," or "reduced levels" refers to a decrease expression or decreased levels of a biomarker in an individual relative to a control, such as an individual or individuals who are not suffering from the disease or disorder (*e.g.*, cancer) or an internal control (*e.g.*, housekeeping biomarker).

The term "housekeeping biomarker" refers to a biomarker or group of biomarkers (*e.g.*, polynucleotides and/or polypeptides) which are typically similarly present in all cell types. In some embodiments, the housekeeping biomarker is a "housekeeping gene." A "housekeeping gene" refers herein to a gene or group of genes which encode proteins whose activities are essential for the maintenance of cell function and which are typically similarly present in all cell types.

"Amplification," as used herein generally refers to the process of producing multiple copies of a desired sequence. "Multiple copies" mean at least two copies. A "copy" does not necessarily mean perfect sequence complementarity or identity to the template sequence. For example, copies can include nucleotide analogs such as deoxyinosine, intentional sequence alterations (such as sequence alterations introduced through a primer comprising a sequence that is hybridizable, but not complementary, to the template), and/or sequence errors that occur during amplification. Diploid cells typically contain two copies of a given gene, one on each chromosome. In some aspects of the invention, "amplification" or a chromosomal gene in a cell refers to a process where more than two copies of the gene are present in the cell.

The term "multiplex-PCR" refers to a single PCR reaction carried out on nucleic acid obtained from a single source (*e.g.*, an individual) using more than one primer set for the purpose of amplifying two or more DNA sequences in a single reaction.

The term "diagnosis" is used herein to refer to the identification or classification of a molecular or pathological state, disease or condition (*e.g.*, cancer). For example, "diagnosis" may refer to identification of a particular type of cancer. "Diagnosis" may also refer to the classification of a particular subtype of cancer, *e.g.*, by histopathological criteria, or by molecular features (*e.g.*, a subtype characterized by expression of one or a combination of biomarkers (*e.g.*, particular genes or proteins encoded by said genes)).

The term "aiding diagnosis" is used herein to refer to methods that assist in making a clinical determination regarding the presence, or nature, of a particular type of symptom or condition of a disease or disorder (*e.g.*, cancer). For example, a method of aiding diagnosis of a disease or condition (*e.g.*, cancer) can comprise measuring certain biomarkers in a biological sample from an individual.

By "correlate" or "correlating" is meant comparing, in any way, the performance and/or results of a first analysis or protocol with the performance and/or results of a second analysis or protocol. For example, one may use the results of a first analysis or protocol in carrying out a second protocols and/or one may use the results of a first analysis or protocol to determine whether a second analysis or protocol should be performed. With respect to the embodiment of polynucleotide analysis or protocol, one may use the results of the polynucleotide expression analysis or protocol to determine whether a specific therapeutic regimen should be performed.

"Individual response" or "response" can be assessed using any endpoint indicating a benefit to the individual, including, without limitation, (1) inhibition, to some extent, of disease progression (*e.g.*, cancer progression), including slowing down and complete arrest; (2) a reduction in tumor size; (3) inhibition (*i.e.*, reduction, slowing down or complete stopping) of cancer cell infiltration into adjacent peripheral organs and/or tissues; (4) inhibition (*i.e.* reduction, slowing down or complete stopping) of metastasis; (5) relief, to some extent, of one or more symptoms associated with the disease or disorder (*e.g.*, cancer); (6) increase in the length of progression free survival; and/or (9) decreased mortality at a given point of time following treatment.

The term "prediction" or "predicting" is used herein to refer to the likelihood that a patient will respond either favorably or unfavorably to a particular anti-cancer therapy. In one embodiment, prediction or predicting relates to the extent of those responses. In one embodiment, the prediction or predicting relates to whether and/or the probability that a patient will survive or improve following treatment, for example treatment with a particular therapeutic agent, and for a certain period of time without disease recurrence. The predictive methods of the invention can be used clinically to make treatment decisions by choosing the most appropriate treatment modalities for any particular patient. The predictive methods of the present invention are

valuable tools in predicting if a patient is likely to respond favorably to a treatment regimen, such as a given therapeutic regimen, including for example, administration of a given therapeutic agent or combination, surgical intervention, steroid treatment, *etc.*, or whether long-term survival of the patient, following a therapeutic regimen is likely.

The term "substantially the same," as used herein, denotes a sufficiently high degree of similarity between two numeric values, such that one of skill in the art would consider the difference between the two values to be of little or no biological and/or statistical significance within the context of the biological characteristic measured by said values (*e.g.*, K_d values or expression). The difference between said two values is, for example, less than about 50%, less than about 40%, less than about 30%, less than about 20%, and/or less than about 10% as a function of the reference/comparator value.

The phrase "substantially different," as used herein, denotes a sufficiently high degree of difference between two numeric values such that one of skill in the art would consider the difference between the two values to be of statistical significance within the context of the biological characteristic measured by said values (*e.g.*, K_d values). The difference between said two values is, for example, greater than about 10%, greater than about 20%, greater than about 30%, greater than about 40%, and/or greater than about 50% as a function of the value for the reference/comparator molecule.

The word "label" when used herein refers to a detectable compound or composition. The label is typically conjugated or fused directly or indirectly to a reagent, such as a polynucleotide probe or an antibody, and facilitates detection of the reagent to which it is conjugated or fused. The label may itself be detectable (*e.g.*, radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition which results in a detectable product.

An "effective amount" of an agent refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic or prophylactic result.

A "therapeutically effective amount" of a substance/molecule of the invention, agonist or antagonist may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the substance/molecule, agonist or antagonist to elicit a desired response in the individual. A therapeutically effective amount is also one in which any toxic or detrimental effects of the substance/molecule, agonist or antagonist are outweighed by the therapeutically beneficial effects

A "prophylactically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result. Typically but not necessarily, since

a prophylactic dose is used in subjects prior to or at an earlier stage of disease, the prophylactically effective amount will be less than the therapeutically effective amount.

In the case of melanoma, the therapeutically effective amount of the PAK1 inhibitor may reduce the number of cancer cells; reduce the primary tumor size; inhibit (*i.e.*, slow to some extent and preferably stop) cancer cell infiltration into peripheral organs; inhibit (*i.e.*, slow to some extent and preferably stop) tumor metastasis; inhibit or delay, to some extent, tumor growth or tumor progression; and/or relieve to some extent one or more of the symptoms associated with the disorder. To the extent the drug may prevent growth and/or kill existing cancer cells, it may be cytostatic and/or cytotoxic. For cancer therapy, efficacy in vivo can, for example, be measured by assessing the duration of survival, time to disease progression (TTP), the response rates (RR), duration of response, and/or quality of life.

To "reduce" or "inhibit" is to decrease or reduce an activity, function, and/or amount as compared to a reference. In certain embodiments, by "reduce" or "inhibit" is meant the ability to cause an overall decrease of 20% or greater. In another embodiment, by "reduce" or "inhibit" is meant the ability to cause an overall decrease of 50% or greater. In yet another embodiment, by "reduce" or "inhibit" is meant the ability to cause an overall decrease of 75%, 85%, 90%, 95%, or greater. Reduce or inhibit can refer to the symptoms of the disorder being treated, the presence or size of metastases, the size of the primary tumor, or the size or number of the blood vessels in angiogenic disorders.

The term "pharmaceutical formulation" refers to a preparation which is in such form as to permit the biological activity of the active ingredient to be effective, and which contains no additional components which are unacceptably toxic to a subject to which the formulation would be administered. Such formulations may be sterile.

A "sterile" formulation is aseptic or free from all living microorganisms and their spores.

A "pharmaceutically acceptable carrier" refers to an ingredient in a pharmaceutical formulation, other than an active ingredient, which is nontoxic to a subject. A pharmaceutically acceptable carrier includes, but is not limited to, a buffer, excipient, stabilizer, or preservative.

As used herein, "treatment" is an approach for obtaining beneficial or desired clinical results. For purposes of this invention, beneficial or desired clinical results include, but are not limited to, any one or more of: alleviation of one or more symptoms, diminishment of extent of disease, preventing or delaying spread (*e.g.*, metastasis, for example metastasis to the lung or to the lymph node) of disease, preventing or delaying recurrence of disease, delay or slowing of disease progression, amelioration of the disease state, and remission (whether partial or total). Also encompassed by "treatment" is a reduction of pathological consequence of a proliferative

disease. The methods of the invention contemplate any one or more of these aspects of treatment.

The term "melanoma" refers to a tumor of high malignancy that starts in melanocytes of normal skin or moles and metastasizes rapidly and widely. The term "melanoma" can be used interchangeably with the terms "malignant melanoma", "melanocarcinoma", "melanoepithelioma", and "melanosarcoma".

"Tumor," as used herein, refers to all neoplastic cell growth and proliferation, whether malignant or benign, and all pre-cancerous and cancerous cells and tissues. The terms "cancer", "cancerous", "cell proliferative disorder", "proliferative disorder" and "tumor" are not mutually exclusive as referred to herein.

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia or lymphoid malignancies. More particular examples of such cancers include, but not limited to, squamous cell cancer (*e.g.*, epithelial squamous cell cancer), lung cancer including small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung and squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastric or stomach cancer including gastrointestinal cancer and gastrointestinal stromal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, cancer of the urinary tract, hepatoma, breast cancer, colon cancer, rectal cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney or renal cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma, anal carcinoma, penile carcinoma, melanoma, superficial spreading melanoma, lentigo maligna melanoma, acral lentiginous melanomas, nodular melanomas, multiple myeloma and B-cell lymphoma (including low grade/follicular non-Hodgkin's lymphoma (NHL); small lymphocytic (SL) NHL; intermediate grade/follicular NHL; intermediate grade diffuse NHL; high grade immunoblastic NHL; high grade lymphoblastic NHL; high grade small non-cleaved cell NHL; bulky disease NHL; mantle cell lymphoma; AIDS-related lymphoma; and Waldenstrom's Macroglobulinemia); chronic lymphocytic leukemia (CLL); acute lymphoblastic leukemia (ALL); hairy cell leukemia; chronic myeloblastic leukemia; and post-transplant lymphoproliferative disorder (PTLD), as well as abnormal vascular proliferation associated with phakomatoses, edema (such as that associated with brain tumors), Meigs' syndrome, brain, as well as head and neck cancer, and associated metastases. In certain embodiments, cancers that are amenable to treatment by the antibodies of the invention include breast cancer, colorectal cancer, rectal cancer, non-small cell lung cancer, glioblastoma, non-Hodgkins lymphoma (NHL), renal cell cancer, prostate cancer, liver cancer, pancreatic cancer, soft-tissue sarcoma, kaposi's

sarcoma, carcinoid carcinoma, head and neck cancer, ovarian cancer, mesothelioma, and multiple myeloma. In some embodiments, the cancer is selected from: small cell lung cancer, glioblastoma, neuroblastomas, melanoma, breast carcinoma, gastric cancer, colorectal cancer (CRC), and hepatocellular carcinoma. Yet, in some embodiments, the cancer is selected from: non-small cell lung cancer, colorectal cancer, glioblastoma and breast carcinoma, including metastatic forms of those cancers.

The term "anti-cancer therapy" refers to a therapy useful in treating cancer. Examples of anti-cancer therapeutic agents include, but are limited to, *e.g.*, chemotherapeutic agents, growth inhibitory agents, cytotoxic agents, agents used in radiation therapy, anti-angiogenesis agents, apoptotic agents, anti-tubulin agents, and other agents to treat cancer , anti-CD20 antibodies, platelet derived growth factor inhibitors (*e.g.*, Gleevec™ (Imatinib Mesylate)), a COX-2 inhibitor (*e.g.*, celecoxib), interferons, cytokines, antagonists (*e.g.*, neutralizing antibodies) that bind to one or more of the following targets PDGFR-beta, BlyS, APRIL, BCMA receptor(s), TRAIL/Apo2, and other bioactive and organic chemical agents, etc. Combinations thereof are also included in the invention.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (*e.g.*, At^{211}, I^{131}, I^{125}, Y^{90}, Re^{186}, Re^{188}, Sm^{153}, Bi^{212}, P^{32} and radioactive isotopes of Lu), chemotherapeutic agents *e.g.*, methotrexate, adriamicin, vinca alkaloids (vincristine, vinblastine, etoposide), doxorubicin, melphalan, mitomycin C, chlorambucil, daunorubicin or other intercalating agents, enzymes and fragments thereof such as nucleolytic enzymes, antibiotics, and toxins such as small molecule toxins or enzymatically active toxins of bacterial, fungal, plant or animal origin, including fragments and/or variants thereof, and the various antitumor or anticancer agents disclosed below. Other cytotoxic agents are described below. A tumoricidal agent causes destruction of tumor cells.

A "toxin" is any substance capable of having a detrimental effect on the growth or proliferation of a cell.

A "chemotherapeutic agent" refers to a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and cyclosphosphamide (CYTOXAN®); alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, triethylenephosphoramide, triethylenethiophosphoramide and trimethylomelamine; acetogenins (especially bullatacin and bullatacinone); delta-9-tetrahydrocannabinol (dronabinol, MARINOL®); beta-lapachone; lapachol; colchicines; betulinic acid; a camptothecin (including the synthetic analogue topotecan

(HYCAMTIN®), CPT-11 (irinotecan, CAMPTOSAR®), acetylcamptothecin, scopolectin, and 9-aminocamptothecin); bryostatin; callystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); podophyllotoxin; podophyllinic acid; teniposide; cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CB1-TM1); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, chlorophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosoureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimnustine; antibiotics such as the enediyne antibiotics (e. g., calicheamicin, especially calicheamicin gamma1I and calicheamicin omegaI1 (see, *e.g.*, Nicolaou *et al.*, Angew. Chem Intl. Ed. Engl., 33: 183-186 (1994)); CDP323, an oral alpha-4 integrin inhibitor; dynemicin, including dynemicin A; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antibiotic chromophores), aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabicin, carminomycin, carzinophilin, chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin (including ADRIAMYCIN®, morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin, doxorubicin HCl liposome injection (DOXIL®), liposomal doxorubicin TLC D-99 (MYOCET®), peglylated liposomal doxorubicin (CAELYX®), and deoxydoxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peplomycin, porfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate, gemcitabine (GEMZAR®), tegafur (UFTORAL®), capecitabine (XELODA®), an epothilone, and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; eniluracil; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elfornithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; 2-ethylhydrazide; procarbazine; PSK® polysaccharide complex (JHS Natural Products, Eugene, OR); razoxane;

rhizoxin; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2'-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine (ELDISINE®, FILDESIN®); dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); thiotepa; taxoid, *e.g.*, paclitaxel (TAXOL®), albumin-engineered nanoparticle formulation of paclitaxel (ABRAXANETM), and docetaxel (TAXOTERE®); chloranbucil; 6-thioguanine; mercaptopurine; methotrexate; platinum agents such as cisplatin, oxaliplatin (*e.g.*, ELOXATIN®), and carboplatin; vincas, which prevent tubulin polymerization from forming microtubules, including vinblastine (VELBAN®), vincristine (ONCOVIN®), vindesine (ELDISINE®, FILDESIN®), and vinorelbine (NAVELBINE®); etoposide (VP-16); ifosfamide; mitoxantrone; leucovorin; novantrone; edatrexate; daunomycin; aminopterin; ibandronate; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoids such as retinoic acid, including bexarotene (TARGRETIN®); bisphosphonates such as clodronate (for example, BONEFOS® or OSTAC®), etidronate (DIDROCAL®), NE-58095, zoledronic acid/zoledronate (ZOMETA®), alendronate (FOSAMAX®), pamidronate (AREDIA®), tiludronate (SKELID®), or risedronate (ACTONEL®); troxacitabine (a 1,3-dioxolane nucleoside cytosine analog); antisense oligonucleotides, particularly those that inhibit expression of genes in signaling pathways implicated in aberrant cell proliferation, such as, for example, PKC-alpha, Raf, H-Ras, and epidermal growth factor receptor (EGF-R); vaccines such as THERATOPE® vaccine and gene therapy vaccines, for example, ALLOVECTIN® vaccine, LEUVECTIN® vaccine, and VAXID® vaccine; topoisomerase 1 inhibitor (*e.g.*, LURTOTECAN®); rmRH (*e.g.*, ABARELIX®); BAY439006 (sorafenib; Bayer); SU-11248 (sunitinib, SUTENT®, Pfizer); perifosine, COX-2 inhibitor (*e.g.*, celecoxib or etoricoxib), proteosome inhibitor (*e.g.*, PS341); bortezomib (VELCADE®); CCI-779; tipifarnib (R11577); orafenib, ABT510; Bcl-2 inhibitor such as oblimersen sodium (GENASENSE®); pixantrone; EGFR inhibitors (see definition below); tyrosine kinase inhibitors (see definition below); serine-threonine kinase inhibitors such as rapamycin (sirolimus, RAPAMUNE®); farnesyltransferase inhibitors such as lonafarnib (SCH 6636, SARASARTM); and pharmaceutically acceptable salts, acids or derivatives of any of the above; as well as combinations of two or more of the above such as CHOP, an abbreviation for a combined therapy of cyclophosphamide, doxorubicin, vincristine, and prednisolone; and FOLFOX, an abbreviation for a treatment regimen with oxaliplatin (ELOXATINTM) combined with 5-FU and leucovorin.

Chemotherapeutic agents as defined herein include "anti-hormonal agents" or "endocrine therapeutics" which act to regulate, reduce, block, or inhibit the effects of hormones that can promote the growth of cancer. They may be hormones themselves, including, but not limited to:

anti-estrogens with mixed agonist/antagonist profile, including, tamoxifen (NOLVADEX®), 4-hydroxytamoxifen, toremifene (FARESTON®), idoxifene, droloxifene, raloxifene (EVISTA®), trioxifene, keoxifene, and selective estrogen receptor modulators (SERMs) such as SERM3; pure anti-estrogens without agonist properties, such as fulvestrant (FASLODEX®), and EM800 (such agents may block estrogen receptor (ER) dimerization, inhibit DNA binding, increase ER turnover, and/or suppress ER levels); aromatase inhibitors, including steroidal aromatase inhibitors such as formestane and exemestane (AROMASIN®), and nonsteroidal aromatase inhibitors such as anastrazole (ARIMIDEX®), letrozole (FEMARA®) and aminoglutethimide, and other aromatase inhibitors include vorozole (RIVISOR®), megestrol acetate (MEGASE®), fadrozole, and 4(5)-imidazoles; lutenizing hormone-releaseing hormone agonists, including leuprolide (LUPRON® and ELIGARD®), goserelin, buserelin, and tripterelin; sex steroids, including progestines such as megestrol acetate and medroxyprogesterone acetate, estrogens such as diethylstilbestrol and premarin, and androgens/retinoids such as fluoxymesterone, all transretionic acid and fenretinide; onapristone; anti-progesterones; estrogen receptor down-regulators (ERDs); anti-androgens such as flutamide, nilutamide and bicalutamide; and pharmaceutically acceptable salts, acids or derivatives of any of the above; as well as combinations of two or more of the above.

The term "prodrug" as used in this application refers to a precursor or derivative form of a pharmaceutically active substance that is less cytotoxic to tumor cells compared to the parent drug and is capable of being enzymatically activated or converted into the more active parent form. See, *e.g.*, Wilman, "Prodrugs in Cancer Chemotherapy" Biochemical Society Transactions, 14, pp. 375-382, 615th Meeting Belfast (1986) and Stella *et al.*, "Prodrugs: A Chemical Approach to Targeted Drug Delivery," Directed Drug Delivery, Borchardt *et al.*, (ed.), pp. 247-267, Humana Press (1985). The prodrugs of this invention include, but are not limited to, phosphate-containing prodrugs, thiophosphate-containing prodrugs, sulfate-containing prodrugs, peptide-containing prodrugs, D-amino acid-modified prodrugs, glycosylated prodrugs, β-lactam-containing prodrugs, optionally substituted phenoxyacetamide-containing prodrugs or optionally substituted phenylacetamide-containing prodrugs, 5-fluorocytosine and other 5-fluorouridine prodrugs which can be converted into the more active cytotoxic free drug. Examples of cytotoxic drugs that can be derivatized into a prodrug form for use in this invention include, but are not limited to, those chemotherapeutic agents described above.

A "growth inhibitory agent" when used herein refers to a compound or composition which inhibits growth of a cell (*e.g.*, a melanoma call). Examples of growth inhibitory agents include agents that block cell cycle progression (at a place other than S phase), such as agents that induce G1 arrest and M-phase arrest. Classical M-phase blockers include the vincas (vincristine and

vinblastine), taxanes, and topoisomerase II inhibitors such as doxorubicin, epirubicin, daunorubicin, etoposide, and bleomycin. Those agents that arrest G1 also spill over into S-phase arrest, for example, DNA alkylating agents such as tamoxifen, prednisone, dacarbazine, mechlorethamine, cisplatin, methotrexate, 5-fluorouracil, and ara-C. Further information can be found in The Molecular Basis of Cancer, Mendelsohn and Israel, eds., Chapter 1, entitled "Cell cycle regulation, oncogenes, and antineoplastic drugs" by Murakami *et al.* (WB Saunders: Philadelphia, 1995), especially p. 13. The taxanes (paclitaxel and docetaxel) are anticancer drugs both derived from the yew tree. Docetaxel (TAXOTERE®, Rhone-Poulenc Rorer), derived from the European yew, is a semisynthetic analogue of paclitaxel (TAXOL®, Bristol-Myers Squibb). Paclitaxel and docetaxel promote the assembly of microtubules from tubulin dimers and stabilize microtubules by preventing depolymerization, which results in the inhibition of mitosis in cells.

By "radiation therapy" is meant the use of directed gamma rays or beta rays to induce sufficient damage to a cell so as to limit its ability to function normally or to destroy the cell altogether. It will be appreciated that there will be many ways known in the art to determine the dosage and duration of treatment. Typical treatments are given as a one time administration and typical dosages range from 10 to 200 units (Grays) per day.

An "individual" or "subject" is a mammal. Mammals include, but are not limited to, domesticated animals (*e.g.*, cows, sheep, cats, dogs, and horses), primates (*e.g.*, humans and non-human primates such as monkeys), rabbits, and rodents (*e.g.*, mice and rats). In certain embodiments, the individual or subject is a human.

Administration "in combination with" one or more further therapeutic agents includes simultaneous (concurrent) and consecutive or sequential administration in any order.

The term "concurrently" is used herein to refer to administration of two or more therapeutic agents, where at least part of the administration overlaps in time. Accordingly, concurrent administration includes a dosing regimen when the administration of one or more agent(s) continues after discontinuing the administration of one or more other agent(s).

By "reduce or inhibit" is meant the ability to cause an overall decrease of 20%, 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, or greater. Reduce or inhibit can refer to the symptoms of the disorder being treated, the presence or size of metastases, or the size of the primary tumor.

The term "package insert" is used to refer to instructions customarily included in commercial packages of therapeutic products, that contain information about the indications, usage, dosage, administration, combination therapy, contraindications and/or warnings concerning the use of such therapeutic products. In some embodiments, the invention package insert comprises instructions to treat melanoma with a PAK1 inhibitor.

An "article of manufacture" is any manufacture (*e.g.*, a package or container) or kit comprising at least one reagent, *e.g.*, a medicament for treatment of a disease or disorder (*e.g.*, cancer), or a probe for specifically detecting a biomarker described herein. In certain embodiments, the manufacture or kit is promoted, distributed, or sold as a unit for performing the methods described herein.

A "target audience" is a group of people or an institution to whom or to which a particular medicament is being promoted or intended to be promoted, as by marketing or advertising, especially for particular uses, treatments, or indications, such as individuals, populations, readers of newspapers, medical literature, and magazines, television or internet viewers, radio or internet listeners, physicians, drug companies, etc.

As is understood by one skilled in the art, reference to "about" a value or parameter herein includes (and describes) embodiments that are directed to that value or parameter per se. For example, description referring to "about X" includes description of "X".

It is understood that aspect and embodiments of the invention described herein include "consisting" and/or "consisting essentially of" aspects and embodiments. As used herein, the singular form "a", "an", and "the" includes plural references unless indicated otherwise.

An "individual," "subject," or "patient" is a vertebrate. In certain embodiments, the vertebrate is a mammal. Mammals include, but are not limited to, farm animals (such as cows), sport animals, pets (such as cats, dogs, and horses), primates, mice and rats. In certain embodiments, a mammal is a human.

The term "sample," or "test sample" as used herein, refers to a composition that is obtained or derived from a subject of interest that contains a cellular and/or other molecular entity that is to be characterized and/or identified, for example based on physical, biochemical, chemical and/or physiological characteristics. For example, the phrase "disease sample" and variations thereof refers to any sample obtained from a subject of interest that would be expected or is known to contain the cellular and/or molecular entity that is to be characterized. In one embodiment, the definition encompasses blood and other liquid samples of biological origin and tissue samples such as a biopsy specimen or tissue cultures or cells derived therefrom. The source of the tissue sample may be solid tissue as from a fresh, frozen and/or preserved organ or tissue sample or biopsy or aspirate; blood or any blood constituents; bodily fluids; and cells from any time in gestation or development of the subject or plasma. Samples include, but are not limited to, primary or cultured cells or cell lines, cell supernatants, cell lysates, platelets, serum, plasma, vitreous fluid, lymph fluid, synovial fluid, follicular fluid, seminal fluid, amniotic fluid, milk, whole blood, blood-derived cells, urine, cerebro-spinal fluid, saliva, sputum, tears, perspiration,

mucus, tumor lysates, and tissue culture medium, tissue extracts such as homogenized tissue, tumor tissue, cellular extracts, and combinations thereof.

The term "sample," or "test sample" includes biological samples that have been manipulated in any way after their procurement, such as by treatment with reagents, solubilization, or enrichment for certain components, such as proteins or polynucleotides, or embedding in a semi-solid or solid matrix for sectioning purposes. For the purposes herein a "section" of a tissue sample is meant a single part or piece of a tissue sample, *e.g.* a thin slice of tissue or cells cut from a tissue sample. In one embodiment, the sample is a clinical sample. In another embodiment, the sample is used in a diagnostic assay. In some embodiments, the sample is obtained from a primary or metastatic tumor. Tissue biopsy is often used to obtain a representative piece of tumor tissue. Alternatively, tumor cells can be obtained indirectly in the form of tissues or fluids that are known or thought to contain the tumor cells of interest; for instance, skin samples.

By "tissue sample" or "cell sample" is meant a collection of similar cells obtained from a tissue of a subject or individual. The source of the tissue or cell sample may be solid tissue as from a fresh, frozen and/or preserved organ, tissue sample, biopsy, and/or aspirate; blood or any blood constituents such as plasma; bodily fluids such as cerebral spinal fluid, amniotic fluid, peritoneal fluid, or interstitial fluid; cells from any time in gestation or development of the subject. The tissue sample may also be primary or cultured cells or cell lines. Optionally, the tissue or cell sample is obtained from a disease tissue/organ. The tissue sample may contain compounds which are not naturally intermixed with the tissue in nature such as preservatives, anticoagulants, buffers, fixatives, nutrients, antibiotics, or the like.

A "reference sample", "reference cell", "reference tissue", "control sample", "control cell", or "control tissue", as used herein, refers to a sample, cell, tissue, standard, or level that is used for comparison purposes. In one embodiment, a reference sample, reference cell, reference tissue, control sample, control cell, or control tissue is obtained from a healthy and/or non-diseased part of the body (*e.g.*, tissue or cells) of the same subject or individual. For example, healthy and/or non-diseased cells or tissue adjacent to the diseased cells or tissue (*e.g.*, cells or tissue adjacent to a tumor). In some embodiments, the reference sample is non-cancerous skin cells. In another embodiment, a reference sample is obtained from an untreated tissue and/or cell of the body of the same subject or individual. In some embodiments, the reference sample is non-cancerous skin cells of the body of the same subject or individual. In yet another embodiment, a reference sample, reference cell, reference tissue, control sample, control cell, or control tissue is obtained from a healthy and/or non-diseased part of the body (*e.g.*, tissues or cells) of an individual who is not the subject or individual. In some embodiments, the reference sample is non-cancerous skin

cells of an individual who is not the subject or individual. In even another embodiment, a reference sample, reference cell, reference tissue, control sample, control cell, or control tissue is obtained from an untreated tissue and/or cell of the body of an individual who is not the subject or individual.

In certain embodiments, a reference sample is a single sample or combined multiple samples from the same subject or patient that are obtained at one or more different time points than when the test sample is obtained. For example, a reference sample is obtained at an earlier time point from the same subject or patient than when the test sample is obtained. Such reference sample may be useful if the reference sample is obtained during initial diagnosis of cancer and the test sample is later obtained when the cancer becomes metastatic.

In certain embodiments, a reference sample includes all types of biological samples as defined above under the term "sample" that is obtained from one or more individuals who is not the subject or patient. In certain embodiments, a reference sample is obtained from one or more individuals with an angiogenic disorder (*e.g.*, cancer) who is not the subject or patient.

In certain embodiments, a reference sample is a combined multiple samples from one or more healthy individuals who are not the subject or patient. In certain embodiments, a reference sample is a combined multiple samples from one or more individuals with a disease or disorder (*e.g.*, an angiogenic disorder such as, for example, cancer) who are not the subject or patient. In certain embodiments, a reference sample is pooled RNA samples from normal tissues or pooled plasma or serum samples from one or more individuals who are not the subject or patient. In certain embodiments, a reference sample is pooled RNA samples from tumor tissues or pooled plasma or serum samples from one or more individuals with a disease or disorder (*e.g.*, an angiogenic disorder such as, for example, cancer) who are not the subject or patient.

For the purposes herein a "section" of a tissue sample is meant a single part or piece of a tissue sample, *e.g.* a thin slice of tissue or cells cut from a tissue sample. It is understood that multiple sections of tissue samples may be taken and subjected to analysis, provided that it is understood that the same section of tissue sample may be analyzed at both morphological and molecular levels, or analyzed with respect to both polypeptides and polynucleotides.

Expression levels/amount of a gene or biomarker can be determined qualitatively and/or quantitatively based on any suitable criterion known in the art, including but not limited to mRNA, cDNA, proteins, protein fragments and/or gene copy number. In certain embodiments, expression/amount of a gene or biomarker in a first sample is increased as compared to expression/amount in a second sample. In certain embodiments, expression/amount of a gene or biomarker in a first sample is decreased as compared to expression/amount in a second sample. In certain embodiments, the second sample is reference sample.

In certain embodiments, the terms "increase" or "overexpress" refer to an overall increase of about any of 5%, 10%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or greater, in the level of protein or nucleic acid, detected by standard art known methods such as those described herein, as compared to a reference sample. In certain embodiments, the terms "increase" or "overexpress" refer to the increase in expression level/amount of a gene or biomarker in the sample wherein the increase is at least about any of 1.5×, 1.75×, 2×, 3×, 4×, 5×, 6×, 7×, 8×, 9×, 10×, 25×, 50×, 75×, or 100× the expression level/amount of the respective gene or biomarker in the reference sample.

In certain embodiments, the term "decrease" herein refers to an overall reduction of about any of 5%, 10%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or greater, in the level of protein or nucleic acid, detected by standard art known methods such as those described herein, as compared to a reference sample. In certain embodiments, the term decrease refers to the decrease in expression level/amount of a gene or biomarker in the sample wherein the decrease is at least about any of 0.9×, 0.8×, 0.7×, 0.6×, 0.5×, 0.4×, 0.3×, 0.2×, 0.1×, 0.05×, or 0.01× the expression level/amount of the respective gene or biomarker in the reference sample.

"Detection" includes any means of detecting, including direct and indirect detection.

In certain embodiments, by "correlate" or "correlating" is meant comparing, in any way, the performance and/or results of a first analysis or protocol with the performance and/or results of a second analysis or protocol. For example, one may use the results of a first analysis or protocol in carrying out a second protocols and/or one may use the results of a first analysis or protocol to determine whether a second analysis or protocol should be performed. With respect to the embodiment of gene expression analysis or protocol, one may use the results of the gene expression analysis or protocol to determine whether a specific therapeutic regimen should be performed.

The word "label" when used herein refers to a compound or composition which is conjugated or fused directly or indirectly to a reagent such as a nucleic acid probe or an antibody and facilitates detection of the reagent to which it is conjugated or fused. The label may itself be detectable (*e.g.*, radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition which is detectable.

The term "polypeptide" refers to polymers of amino acids of any length. The polymer may be linear or branched, it may comprise modified amino acids, and it may be interrupted by non-amino acids. The terms also encompass an amino acid polymer that has been modified naturally or by intervention; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification, such as conjugation with a labeling

component. Also included within the definition are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids, *etc.*), as well as other modifications known in the art. The term "polypeptide" as used herein specifically encompasses a "protein". The terms "polypeptide" and "protein" as used herein specifically encompass antibodies.

An "isolated" nucleic acid molecule is a nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the polypeptide nucleic acid. An isolated nucleic acid molecule is other than in the form or setting in which it is found in nature. Isolated nucleic acid molecules therefore are distinguished from the nucleic acid molecule as it exists in natural cells. However, an isolated nucleic acid molecule includes a nucleic acid molecule contained in cells that ordinarily express the polypeptide where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

A "gene," "target gene," "target biomarker," "target sequence," "target nucleic acid" or "target protein," as used herein, is a polynucleotide or protein of interest, the detection of which is desired. Generally, a "template," as used herein, is a polynucleotide that contains the target nucleotide sequence. In some instances, the terms "target sequence," "template DNA," "template polynucleotide," "target nucleic acid," "target polynucleotide," and variations thereof, are used interchangeably.

A "native sequence" polypeptide comprises a polypeptide having the same amino acid sequence as a polypeptide derived from nature. Thus, a native sequence polypeptide can have the amino acid sequence of naturally occurring polypeptide from any mammal. Such native sequence polypeptide can be isolated from nature or can be produced by recombinant or synthetic means. The term "native sequence" polypeptide specifically encompasses naturally occurring truncated or secreted forms of the polypeptide (*e.g.*, an extracellular domain sequence), naturally occurring variant forms (*e.g.*, alternatively spliced forms) and naturally occurring allelic variants of the polypeptide.

An "isolated" polypeptide or "isolated" antibody is one that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would interfere with diagnostic or therapeutic uses for the polypeptide, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In certain embodiments, the polypeptide will be purified (1) to greater than 95% by weight of polypeptide as determined by the Lowry method, or more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or

nonreducing conditions using Coomassie blue, or silver stain. Isolated polypeptide includes the polypeptide in situ within recombinant cells since at least one component of the polypeptide's natural environment will not be present. Ordinarily, however, isolated polypeptide will be prepared by at least one purification step.

A polypeptide "variant" means a biologically active polypeptide having at least about 80% amino acid sequence identity with the native sequence polypeptide. Such variants include, for instance, polypeptides wherein one or more amino acid residues are added, or deleted, at the N- or C-terminus of the polypeptide. Ordinarily, a variant will have at least about 80% amino acid sequence identity, more preferably at least about 90% amino acid sequence identity, and even more preferably at least about 95% amino acid sequence identity with the native sequence polypeptide.

The term "benefit" is used in the broadest sense and refers to any desirable effect and specifically includes clinical benefit as defined herein.

Clinical benefit can be measured by assessing various endpoints, *e.g.*, inhibition, to some extent, of disease progression, including slowing down and complete arrest; reduction in the number of disease episodes and/or symptoms; reduction in lesion size; inhibition (*i.e.*, reduction, slowing down or complete stopping) of disease cell infiltration into adjacent peripheral organs and/or tissues; inhibition (*i.e.* reduction, slowing down or complete stopping) of disease spread; decrease of auto-immune response, which may, but does not have to, result in the regression or ablation of the disease lesion; relief, to some extent, of one or more symptoms associated with the disorder; increase in the length of disease-free presentation following treatment, *e.g.*, progression-free survival; increased overall survival; higher response rate; and/or decreased mortality at a given point of time following treatment.

Methods of the invention

The present invention provides methods for treating melanoma in an individual comprising contacting the melanoma with a therapeutically effective amount of a PAK1 inhibitor. In some embodiments, the method comprises administering to the individual a therapeutically effective about of a PAK1 inhibitor.

Melanoma is a malignant tumor of melanocytes, *e.g.*, cells that produce melanin, a dark pigment which is responsible for the color of skin. Melanomas predominantly occur in skin, but are also found in other parts of the body, including the bowel and the eye *e.g.* uveal melanoma). Melanoma can originate in any part of the body that contains melanocytes. Examples of melanoma includes, but are not limited to superficial spreading melanoma, nodular melanoma, Lentigo maligna melanoma, and Acral lentiginous melanoma. Melanoma may be staged

depending on a number of criteria including size, ulceration, spread to lymph nodes, and/or spread to other tissues or organs. In some embodiments, the invention provides methods of treating a Stage I melanoma in an individual by contacting the melanoma with an inhibitor of PAK1. In some embodiments, the invention provides methods of treating a Stage II melanoma in an individual by contacting the melanoma with an inhibitor of PAK1. In some embodiments, the invention provides methods of treating Stage III melanoma in an individual by contacting the melanoma with an inhibitor of PAK1. In some embodiments, the invention provides methods of treating Stage IV melanoma in an individual by contacting the melanoma with an inhibitor of PAK1. In some embodiments, the invention provides methods of treating metastatic melanoma in an individual by contacting the melanoma with an inhibitor of PAK1. In some embodiments, the invention provides methods of treating recurrent melanoma in an individual by contacting the melanoma with an inhibitor of PAK1. In some embodiments, the method comprises administering to the individual a therapeutically effective amount of the PAK1 inhibitor. In some embodiments, the PAK1 inhibitor is a small molecule inhibitor of PAK1. In some embodiments, the individual is a mammal. In some embodiments the individual is a human. In some aspects, the invention provides methods of treating melanoma in an individual wherein the melanoma is a wild-type BRAF melanoma. In some embodiments, the invention provides methods of treating wild-type BRAF melanoma comprising contacting the melanoma with a therapeutically effective amount of a PAK1 inhibitor. In some embodiments, the invention provides methods of treating wild-type BRAF melanoma comprising administering to the individual a therapeutically effective amount of a PAK1 inhibitor. BRAF is a member of the Raf kinase family of serine/threonine-specific protein kinases. BRAF plays a role in regulating the MAP kinase/ERKs signaling pathway (the RAF-MEK-ERK pathway), which affects cell division, differentiation, and secretion. RAF-MEK-ERK signaling is frequently dysregulated in cancer. More than 30 mutations of the BRAF gene associated with human cancers have been identified. The frequency of BRAF mutations varies widely in human cancers from more than 80% in melanomas, to as little as 0-18% in other tumors, such as 1-3% in lung cancers and 5% in colorectal cancer. A common mutation found in cancers, particularly melanoma is a substitution of valine at codon 600 with glutamate (*i.e.*, V600E). For example, a thymine is substituted with adenine at nucleotide 1799 which leads to the V600E mutation. V600 mutations of BRAF lead to constitutive BRAF kinase activity. Methods to determine the genotype of BRAF in a melanoma are known to those in the art; for example, the nucleotide sequence of the BRAF gene from the melanoma may be determined using standard sequencing methods or by using the KASP SNP genotyping system (KBioscience). In some embodiments the invention provides methods of treating melanoma in an individual wherein the melanoma is a wild-type BRAF

melanoma. In some embodiments the invention provides methods of treating melanoma in an individual wherein the melanoma is a wild-type BRAF melanoma and the melanoma overexpresses PAK1 compared to non-cancerous cells. In some embodiments the invention provides methods of treating melanoma in an individual wherein the melanoma comprises a wild-type BRAF and PAK1 is amplified in the melanoma. In some embodiments, the melanoma is a wild-type BRAF melanoma wherein PAK1 is overexpressed in the melanoma compared to non-cancerous cells and PAK1 is amplified in the melanoma. In some embodiments the invention provides methods of treating melanoma in an individual wherein the melanoma is a mutant BRAF melanoma. In some embodiments the invention provides methods of treating melanoma in an individual wherein the melanoma is a mutant BRAF melanoma and the melanoma overexpresses PAK1 compared to non-cancerous cells. In some embodiments the invention provides methods of treating melanoma in an individual wherein the melanoma comprises a mutant BRAF and PAK1 is amplified in the melanoma. In some embodiments the invention provides methods of treating melanoma in an individual wherein the melanoma comprises a mutant BRAF wherein the mutant BRAF is not a V600E mutant BRAF. In some embodiments, the individual in a mammal. In some embodiments, the individual is a human. In some aspects, the invention provides methods of treatment of melanoma in an individual by contacting the melanoma with a therapeutically effective amount of PAK1 inhibitor. In some aspects, the invention provides methods of treatment of melanoma in an individual by administering to the individual a therapeutically effective amount of PAK1 inhibitor. PAKs participate in a number of pathways that are commonly deregulated in human cancer cells. PAK1 is a component of the mitogen-activated protein kinase (MAPK), JUN N-terminal kinase (JNK), steroid hormone receptor, and nuclear factor (NF) signaling pathways, which all have been associated with oncogenesis. PAKs activate MEK and RAF1 by phosphorylating them on serine 298 and serine 338, respectively. The increase of Ras-induced transformation by PAK1 correlated with its effects on signaling through the extracellular signal-regulated kinase (ERK)-MAPK pathway, and was dissociable from effects on the JNK or p38-MAPK pathways. (R. Kumar *et al. Nature Rev. Cancer* 2006 6:459). Constitutive activation of the ERK/MEK pathway is implicated in the formation, progression and survival of tumors and furthermore is associated with an aggressive phenotype, characterized by uncontrolled proliferation, loss of control of apoptosis and poor prognosis (J.A. Spicer, *Expert Opin. Drug Discov.* 2008 3:7). Tumor formation and progression require the inactivation of pro-apoptotic signals in cancer cells. PAK activity has been shown to downregulate several important pro-apoptotic pathways. PAK1 phosphorylation of RAF1 induces RAF1 translocation to mitochondria, where it phosphorylates the pro-apoptotic protein BCL2-antagonist of cell death (BAD). PAK1, PAK2,

PAK4 and PAK5 have also been reported to directly phosphorylate and inactivate BAD in selected cell types, such as CV-1 (simian) in origin and carrying the SV40 (COS) kidney, Chinese hamster ovarian (CHO) and human embryonic kidney (HEK) 293T cells (R. Kumar *et al.*, *ibid*). However, the relevant pathways downstream of PAK1 in human tumor cells remain only partially understood.

PAK1 is widely expressed in a variety of normal tissues; however, expression is significantly increased in ovarian, breast and bladder cancer. (S. Balasenthil *et al.*, *J. Biol. Chem.* 2004 279:4743; M. Ito *et al.*, *J. Urol.* 2007 178:1073; P. Schraml *et al.*, *Am. J. Pathol.* 2003 163:985). In luminal breast cancer, genomic amplification of PAK1 is associated with resistance to tamoxifen therapy, possibly occurring as a result of direct phosphorylation and ligand-independent transactivation of estrogen receptor by PAK1 (S. K. Rayala *et al.*, *Cancer Res.* 2006. 66:1694-1701).

In some aspects, the invention provides methods of treating melanoma in an individual by contacting the melanoma with a therapeutically effective amount of a PAK1 inhibitor. In some aspects, the invention provides methods of treating melanoma in an individual by administering to the individual a therapeutically effective amount of a PAK1 inhibitor. In some embodiments, the PAK1 gene is amplified in the melanoma. In some embodiments, the copy number of the PAK1 in the melanoma is about any of 2.5, 3.0, 3.5, 4.0, 4.5, 5.0 or greater than 5.0. Methods of determining the copy number of the PAK1 gene in a melanoma are known in the art. For example, the copy number of the PAK1 gene may be determined by using SNP arrays such as the Affymetrix 500K SNP array analysis. In some embodiments, the invention provides methods of treating melanoma in an individual wherein the copy number of PAK1 in the melanoma is greater than about 2.5. In some embodiments, the invention provides methods of determining the copy number of PAK1 in a melanoma subsequent to treatment with a PAK1 inhibitor. In some embodiments, the copy number of PAK1 in a melanoma is compared to the copy number of PAK1 in non-cancerous cells; for example, non-cancerous skin cells. In some embodiments, PAK1 is amplified in the melanoma and the melanoma overexpresses PAK1. In some embodiments, PAK1 is amplified in the melanoma and the melanoma is a wild-type BRAF melanoma. In some embodiments the individual is a mammal. In some embodiments, the individual is a human.

In some aspects, the invention provides methods of treating melanoma in an individual by contacting the melanoma with a therapeutically effective amount of a PAK1 inhibitor wherein PAK1 is overexpressed in the melanoma. In some aspects, the invention provides methods of treating melanoma in an individual by administering to the individual a therapeutically effective amount of a PAK1 inhibitor wherein PAK1 is overexpressed in the melanoma. Methods to

determine expression of PAK1 are known in the art. Examples of methods to determine expression levels of PAK1 in a melanoma include, but are not limited to immunohistochemistry, reverse-phase protein array (RPPA), quantitative PCR, immunoassays, and the like. Levels of PAK1 expression can be compared to other tumors and cells by using the Gene Expression Omnibus (GEO) database.

In some embodiments, the invention provides methods for treating melanoma in an individual by contacting the melanoma with a PAK1 inhibitor wherein PAK1 is overexpressed in the melanoma compared to non-cancerous cells. In some aspects, the invention provides methods of treating melanoma in an individual by administering to the individual a therapeutically effective amount of a PAK1 inhibitor wherein PAK1 is overexpressed in the melanoma. In some embodiments, expression of PAK1 in the melanoma is about any of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% or greater than 100% expression in non-cancerous cells. In some embodiments, expression of PAK1 in the melanoma is about any of 1.5-fold, 2.0-fold, 2.5-fold, 3, 3.5-fold, 4.0-fold, 4.5-fold, 5.0-fold, 6.0-fold, 7.0-fold, 8.0-fold, 9.0-fold, 10-fold or greater than 10-fold compared to expression of PAK1 in non-cancerous cells. In some embodiments, the melanoma overexpresses PAK1 compared to non-cancerous cells and the melanoma is a wild-type BRAF melanoma. In some embodiments, the melanoma overexpresses PAK1 compared to non-cancerous cells and PAK1 is amplified in the melanoma. In some embodiments, the melanoma overexpresses PAK1 compared to non-cancerous cells and the melanoma is a wild-type BRAF melanoma amd PAK1 is amplified in the melanoma. In some embodiments the individual is a mammal. In some embodiments, the individual is a human.

In some aspects, the invention provides methods of inhibiting CRAF signaling in a melanoma in an individual comprising contacting the melanoma with a therapeutically effective amount of a PAK1 inhibitor. In some aspects, the invention provides methods of inhibiting CRAF signaling in a melanoma in an individual comprising administering to the individual a therapeutically effective amount of a PAK1 inhibitor. Methods of measuring CRAF signaling are known in the art. For example, CRAF activation can be determined by immunoblot of CRAF isolated from a melanoma from an individual before and/or after treatment with a PAK1 inhibitor. Activation of CRAF may be measured using phospho-CRAF(Ser338) antibodies. In some embodiments, the melanoma is a wild-type BRAF melanoma. In some embodiments, PAK1 is over expressed in the melanoma. In some embodiments, the melanoma is a wild-type BRAF melanoma wherein PAK1 is overexpressed in the melanoma. In some embodiments, the melanoma is a wild-type BRAF melanoma wherein PAK1 is amplified in the melanoma. In some embodiments, the melanoma is a wild-type BRAF melanoma wherein PAK1 is overexpressed in the melanoma and PAK1 is amplified in the melanoma. In some embodiments, PAK1 is overexpressed in the

melanoma and PAK1 is amplified in the melanoma. In some embodiments, the individual is a mammal. In some embodiments, the individual is a human.

In some aspects, the invention provides methods of inhibiting MEK signaling in a melanoma in an individual comprising contacting the melanoma with a therapeutically effective amount of a PAK1 inhibitor. In some aspects, the invention provides methods of inhibiting MEK signaling in a melanoma in an individual comprising administering to the individual a therapeutically effective amount of a PAK1 inhibitor. Methods of measuring MEK signaling are known in the art. For example, MEK activation can be determined by immunoblot of MEK isolated from a melanoma from an individual before and/or after treatment with a PAK1 inhibitor. Activation of MEK may be measured using phospho-MEK1/1(Ser217/Ser221) antibodies. In some embodiments, the melanoma is a wild-type BRAF melanoma. In some embodiments, PAK1 is over expressed in the melanoma. In some embodiments, the melanoma is a wild-type BRAF melanoma wherein PAK1 is overexpressed in the melanoma. In some embodiments, the melanoma is a wild-type BRAF melanoma wherein PAK1 is amplified in the melanoma. In some embodiments, the melanoma is a wild-type BRAF melanoma wherein PAK1 is overexpressed in the melanoma and PAK1 is amplified in the melanoma. In some embodiments, PAK1 is overexpressed in the melanoma and PAK1 is amplified in the melanoma. In some embodiments, the individual is a mammal. In some embodiments, the individual is a human.

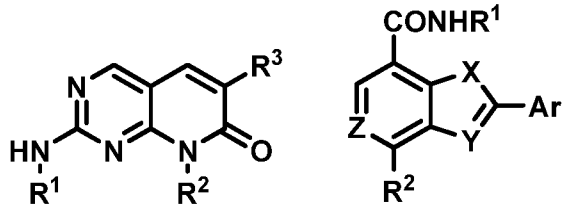
Inhibitors of PAK1

Provided herein are inhibitors of PAK1 (*e.g.* PAK1 antagonists) useful in the methods described herein. In some embodiments, the PAK1 inhibitor is a small molecule, a nucleic acid, a polypeptide or an antibody. Examples of PAK inhibitors are provided in WO 2007/072153, and WO 2010/07184 both of which are incorporated herein by reference.

Small molecules

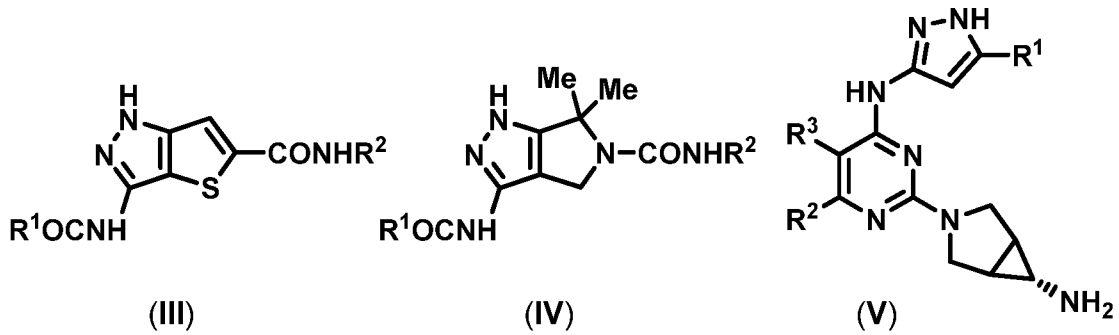
Provided herein are small molecules for use as PAK1 inhibitors for the treatment of melanoma. Small molecules are preferably organic molecules other than binding polypeptides or antibodies as defined herein that bind to PAK1 or interfere with PAK1 signaling as described herein. Binding organic small molecules may be identified and chemically synthesized using known methodology (see, *e.g.*, PCT Publication Nos. WO 00/00823 and WO 00/39585). Binding organic small molecules are usually less than about 2000 daltons in size, alternatively less than about 1500, 750, 500, 250 or 200 daltons in size, wherein such organic small molecules that are capable of binding, preferably specifically, to a polypeptide as described herein may be identified without undue experimentation using well known techniques. In this regard, it is noted

that techniques for screening organic small molecule libraries for molecules that are capable of binding to a polypeptide target are well known in the art (see, *e.g.*, PCT Publication Nos. WO 00/00823 and WO 00/39585). Binding organic small molecules may be, for example, aldehydes, ketones, oximes, hydrazones, semicarbazones, carbazides, primary amines, secondary amines, tertiary amines, N-substituted hydrazines, hydrazides, alcohols, ethers, thiols, thioethers, disulfides, carboxylic acids, esters, amides, ureas, carbamates, carbonates, ketals, thioketals, acetals, thioacetals, aryl halides, aryl sulfonates, alkyl halides, alkyl sulfonates, aromatic compounds, heterocyclic compounds, anilines, alkenes, alkynes, diols, amino alcohols, oxazolidines, oxazolines, thiazolidines, thiazolines, enamines, sulfonamides, epoxides, aziridines, isocyanates, sulfonyl chlorides, diazo compounds, acid chlorides, or the like. Small molecule inhibitors of PAK kinases have been described (see WO2006072831, WO2007023382, WO2007072153, WO2010/071846, US20090275570).



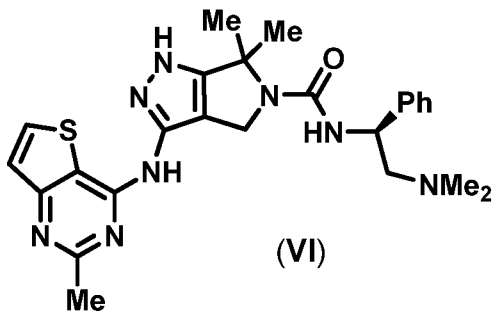
(I) (II)

A series of PAK1 selective inhibitors elaborated on the 2-aminopyrido[2,3-d]pyrimidin-7(8H)-one (I) scaffold have been disclosed by Afraxis, Inc. in a series of patent applications (WO2009086204, WO2010071846, WO2011044535, WO2011156646, WO2011156786,WO2011156640, WO2011156780,WO2011156775, WO2011044264) AstraZeneca has disclosed bicyclic heterocyclic PAK1 inhibitors of formula II (see WO2006106326).



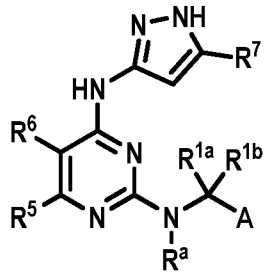
(III) (IV) (V)

Pfizer has disclosed PAK inhibitors elaborated on 1H-thieno[3,2-c]pyrazole (III), 3-amino-tetrahydropyrrolo[3,4-c]pyrazole (IV) and N4-(1H-pyrazol-3-yl)pyrimidine-2,4-diamine (V) (see WO 2004007504, WO 2007023382, WO2007072153, and WO2006072831).



(VI)

PF-3758309 (VI) is a potent ATP-competitive inhibitor of PAK1, 4, 5 and 6 that has been in clinical testing. (B. W. Murray *et al.*, Proc. Natl. Acad. Sci USA 2010 107(20):9446; Rosen L *et al.* Phase 1, dose escalation, safety, pharmacokinetic and pharmacodynamic study of single agent PF-03758309, an oral PAK inhibitor, in patients with advanced solid tumors [abstract]. In: Proceedings of the AACR-NCI-EORTC International Conference on Molecular Targets and Cancer Therapeutics; 2011 Nov 12-16; San Francisco, CA. Philadelphia (PA): AACR; Mol Cancer Ther 2011;10(11 Suppl):Abstract nr A177.



(VII)

A series of N2-bicyclic indolyl, indazolyl and benzimidazolyl derivatives of N4-(1H-pyrazol-3-yl)pyrimidine-2,4-diamines (VII) (U.S. Ser. No.: 61/527,453 filed 08/25/2011) and aza-indolyl, indazolyl and benzimidazolyl derivatives thereof (U.S. Ser. No. 61/579,227, filed 12/22/2011) have been disclosed and those references are incorporated by reference in their entirety. (A = indolyl, indazolyl and benzimidazolyl or aza derivatives thereof).

Nucleic acids

The invention provides herein polynucleotide antagonists of PAK1 for the treatment of melanoma in an individual. The polynucleotide can be an RNAi such as siRNA or miRNA, an antisense oligonucleotides, an RNAzymes, a DNAzymes, an oligonucleotides, a nucleotides, or any fragments of these, including DNA or RNA (*e.g.*, mRNA, rRNA, tRNA) of genomic or synthetic origin, which may be single-stranded or double-stranded and may represent a sense or antisense strand, to peptide nucleic acid (PNA), or to any DNA-like or RNA-like material, natural or synthetic in origin, including, *e.g.*, iRNA, ribonucleoproteins (*e.g.*, iRNPs). In some embodiments, the polynucleotide targets PAK1 expression (*e.g.* targets PAK1 mRNA).

The polynucleotide may be an antisense nucleic acid and/or a ribozyme. The antisense nucleic acids comprise a sequence complementary to at least a portion of an RNA transcript of PAK1. However, absolute complementarity, although preferred, is not required.

A sequence "complementary to at least a portion of an RNA," referred to herein, means a sequence having sufficient complementarity to be able to hybridize with the RNA, forming a stable duplex; in the case of double stranded PAK1 antisense nucleic acids, a single strand of the duplex DNA may thus be tested, or triplex formation may be assayed. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the larger the hybridizing nucleic acid, the more base mismatches with an PAK1 RNA it may contain and still form a stable duplex (or triplex as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

Polynucleotides that are complementary to the 5' end of the message, *e.g.*, the 5' untranslated sequence up to and including the AUG initiation codon, should work most efficiently at inhibiting translation. However, sequences complementary to the 3' untranslated sequences of mRNAs have been shown to be effective at inhibiting translation of mRNAs as well. See generally, Wagner, R., 1994, *Nature* 372:333-335. Thus, oligonucleotides complementary to either the 5'- or 3'-non-translated, non-coding regions of the PAK1 gene, could be used in an antisense approach to inhibit translation of endogenous PAK1 mRNA. Polynucleotides complementary to the 5' untranslated region of the mRNA should include the complement of the AUG start codon. Antisense polynucleotides complementary to mRNA coding regions are less efficient inhibitors of translation but could be used in accordance with the invention. Whether designed to hybridize to the 5'-, 3'- or coding region of PAK1 mRNA, antisense nucleic acids should be at least six nucleotides in length, and are preferably oligonucleotides ranging from 6 to about 50 nucleotides in length. In specific aspects the oligonucleotide is at least 10 nucleotides, at least 17 nucleotides, at least 25 nucleotides or at least 50 nucleotides.

In one embodiment, the PAK1 antisense nucleic acid of the invention is produced intracellularly by transcription from an exogenous sequence. For example, a vector or a portion thereof, is transcribed, producing an antisense nucleic acid (RNA) of the PAK1 gene. Such a vector would contain a sequence encoding the PAK1 antisense nucleic acid. Such a vector can remain episomal or become chromosomally integrated, as long as it can be transcribed to produce the desired antisense RNA. Such vectors can be constructed by recombinant DNA technology methods standard in the art. Vectors can be plasmid, viral, or others know in the art, used for replication and expression in vertebrate cells. Expression of the sequence encoding PAK1, or fragments thereof, can be by any promoter known in the art to act in vertebrate, preferably

human cells. Such promoters can be inducible or constitutive. Such promoters include, but are not limited to, the SV40 early promoter region (Bernoist and Chambon, *Nature* 29:304-310 (1981), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto *et al.*, *Cell* 22:787-797 (1980), the herpes thymidine promoter (Wagner *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 78:1441-1445 (1981), the regulatory sequences of the metallothionein gene (Brinster, *et al.*, *Nature* 296:39-42 (1982)), etc.

Small inhibitory RNAs (siRNAs) can also function as PAK1 inhibitors for use in the treatment of melanoma. PAK1 expression can be by contacting the melanoma with a small double stranded RNA (dsRNA) or a vector or construct that causes the production of small double-stranded RNA, such that expression of PAK1 is specifically inhibited. Methods for selecting an appropriate dsRNA or dsRNA-encoding vector are well known in the art (Tuschi, T et al (1999) *Genes Dev.* 13(24):3191-3197; Elbashir, SM *et al.*, (2001) *Nature* 411:494-498; Hannon, GF (2002) *Nature* 418:244-251; McManus MT and Sharp, PA (2002) *Nature Reviews Genetics* 3:737-747; Bremmelkamp, TR *et al.* (2002) *Science* 296:550-553,US Patents Nos. 6,573,099 and 6,506,559 and International Patent Publications WO01/36646, WO 99/32619 and WO 01/68836. Examples of PAK1 siRNA oligonucleotide sequences include, but are not limited to 1) GAAGAGAGGTTCAGCTAAA, 2) GGAGAAATTACGAAGCATA, 3) ACCCAAACATTGTGAATTA, 4) GGTTTATGATTAAGGGTTT, all obtained from Dharmacon, Inc.

Polypeptides

The invention provides polypeptide inhibitors of PAK1 activity for the treatment of melanoma in an individual. For example, binding polypeptides are polypeptides that bind, preferably and specifically to PAK1 as described herein. In some embodiments, the binding polypeptides are PAK1 antagonists. Binding polypeptides may be chemically synthesized using known polypeptide synthesis methodology or may be prepared and purified using recombinant technology. Binding polypeptides are usually at least about 5 amino acids in length, alternatively at least about 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 100 amino acids in length or more, wherein such binding polypeptides that are capable of binding, preferably specifically, to a PAK1, as described herein. Binding polypeptides may be identified without undue experimentation using well known techniques. In this regard, it is noted that techniques for screening polypeptide libraries for binding polypeptides that are capable of specifically

binding to a polypeptide target are well known in the art (see, *e.g.*, U.S. Patent Nos. 5,556,762, 5,750,373, 4,708,871, 4,833,092, 5,223,409, 5,403,484, 5,571,689, 5,663,143; PCT Publication Nos. WO 84/03506 and W O84/03564; Geysen *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 81:3998-4002 (1984); Geysen *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 82:178-182 (1985); Geysen *et al.*, in *Synthetic Peptides as Antigens*, 130-149 (1986); Geysen *et al.*, J. Immunol. Meth., 102:259-274 (1987); Schoofs *et al.*, *J. Immunol.*, 140:611-616 (1988), Cwirla, S. E. *et al.* (1990) *Proc. Natl. Acad. Sci. USA*, 87:6378; Lowman, H.B. *et al.* (1991) *Biochemistry*, 30:10832; Clackson, T. *et al.* (1991) *Nature*, 352: 624; Marks, J. D. *et al.* (1991), *J. Mol. Biol.*, 222:581; Kang, A.S. *et al.* (1991) *Proc. Natl. Acad. Sci. USA*, 88:8363, and Smith, G. P. (1991) *Current Opin. Biotechnol.*, 2:668).

In this regard, bacteriophage (phage) display is one well known technique which allows one to screen large polypeptide libraries to identify member(s) of those libraries which are capable of specifically binding to a target PAK1. Phage display is a technique by which variant polypeptides are displayed as fusion proteins to the coat protein on the surface of bacteriophage particles (Scott, J.K. and Smith, G. P. (1990) *Science*, 249: 386). The utility of phage display lies in the fact that large libraries of selectively randomized protein variants (or randomly cloned cDNAs) can be rapidly and efficiently sorted for those sequences that bind to a target molecule with high affinity. Display of peptide (Cwirla, S. E. *et al.* (1990) *Proc. Natl. Acad. Sci. USA*, 87:6378) or protein (Lowman, H.B. *et al.* (1991) *Biochemistry*, 30:10832; Clackson, T. *et al.* (1991) *Nature*, 352: 624; Marks, J. D. *et al.* (1991), *J. Mol. Biol.*, 222:581; Kang, A.S. *et al.* (1991) *Proc. Natl. Acad. Sci. USA*, 88:8363) libraries on phage have been used for screening millions of polypeptides or oligopeptides for ones with specific binding properties (Smith, G. P. (1991) *Current Opin. Biotechnol.*, 2:668). Sorting phage libraries of random mutants requires a strategy for constructing and propagating a large number of variants, a procedure for affinity purification using the target receptor, and a means of evaluating the results of binding enrichments. U.S. Patent Nos. 5,223,409, 5,403,484, 5,571,689, and 5,663,143.

Although most phage display methods have used filamentous phage, lambdoid phage display systems (WO 95/34683; U.S. 5,627,024), T4 phage display systems (Ren *et al.*, *Gene*, 215: 439 (1998); Zhu *et al.*, *Cancer Research*, 58(15): 3209-3214 (1998); Jiang *et al.*, Infection & *Immunity*, 65(11): 4770-4777 (1997); Ren *et al.*, *Gene*, 195(2):303-311 (1997); Ren, *Protein Sci.*, 5: 1833 (1996); Efimov *et al.*, *Virus Genes*, 10: 173 (1995)) and T7 phage display systems (Smith and Scott, Methods in Enzymology, 217: 228-257 (1993); U.S. 5,766,905) are also known.

Additional improvements enhance the ability of display systems to screen peptide libraries for binding to selected target molecules and to display functional proteins with the potential of

screening these proteins for desired properties. Combinatorial reaction devices for phage display reactions have been developed (WO 98/14277) and phage display libraries have been used to analyze and control bimolecular interactions (WO 98/20169; WO 98/20159) and properties of constrained helical peptides (WO 98/20036). WO 97/35196 describes a method of isolating an affinity ligand in which a phage display library is contacted with one solution in which the ligand will bind to a target molecule and a second solution in which the affinity ligand will not bind to the target molecule, to selectively isolate binding ligands. WO 97/46251 describes a method of biopanning a random phage display library with an affinity purified antibody and then isolating binding phage, followed by a micropanning process using microplate wells to isolate high affinity binding phage. The use of Staphlylococcus aureus protein A as an affinity tag has also been reported (Li *et al.* (1998) *Mol Biotech.*, 9:187). WO 97/47314 describes the use of substrate subtraction libraries to distinguish enzyme specificities using a combinatorial library which may be a phage display library. A method for selecting enzymes suitable for use in detergents using phage display is described in WO 97/09446. Additional methods of selecting specific binding proteins are described in U.S. Patent Nos. 5,498,538, 5,432,018, and WO 98/15833.
Methods of generating peptide libraries and screening these libraries are also disclosed in U.S. Patent Nos. 5,723,286, 5,432,018, 5,580,717, 5,427,908, 5,498,530, 5,770,434, 5,734,018, 5,698,426, 5,763,192, and 5,723,323.

Antibodies

In some embodiments of the invention, the PAK1 inhibitor for the treatment of melanoma in an individual is an isolated antibodies that bind to PAK1. In some embodiments, the antibody is humanized. In a further aspect of the invention, an anti-PAK1 antibody or an antibody that inhibits PAK1 function. In some embodiments the antibody is a monoclonal antibody, including a chimeric, humanized or human antibody. In some embodiments, the antibody is an antibody fragment, *e.g.*, a Fv, Fab, Fab', scFv, diabody, or F(ab')2 fragment. In another embodiment, the antibody is a full length antibody, *e.g.*, an intact IgG1" antibody or other antibody class or isotype as defined herein.
In certain embodiments, amino acid sequence variants of the antibodies and/or the binding polypeptides provided herein are contemplated. For example, it may be desirable to improve the binding affinity and/or other biological properties of the antibody and/or binding polypeptide. Amino acid sequence variants of an antibody and/or binding polypeptides may be prepared by introducing appropriate modifications into the nucleotide sequence encoding the antibody and/or binding polypeptide, or by peptide synthesis. Such modifications include, for example, deletions from, and/or insertions into and/or substitutions of residues within the amino acid sequences of

the antibody and/or binding polypeptide. Any combination of deletion, insertion, and substitution can be made to arrive at the final construct, provided that the final construct possesses the desired characteristics, *e.g.*, target-binding.

In certain embodiments, antibody variants and/or binding polypeptide variants having one or more amino acid substitutions are provided. Sites of interest for substitutional mutagenesis include the HVRs and FRs. Amino acid substitutions may be introduced into an antibody and/or binding polypeptide of interest and the products screened for a desired activity, *e.g.*, retained/improved antigen binding, decreased immunogenicity, or improved ADCC or CDC.

One type of substitutional variant involves substituting one or more hypervariable region residues of a parent antibody (*e.g.*, a humanized or human antibody). Generally, the resulting variant(s) selected for further study will have modifications (*e.g.*, improvements) in certain biological properties (*e.g.*, increased affinity, reduced immunogenicity) relative to the parent antibody and/or will have substantially retained certain biological properties of the parent antibody. An exemplary substitutional variant is an affinity matured antibody, which may be conveniently generated, *e.g.*, using phage display-based affinity maturation techniques such as those described herein. Briefly, one or more HVR residues are mutated and the variant antibodies displayed on phage and screened for a particular biological activity (*e.g.*, binding affinity).

Alterations (*e.g.*, substitutions) may be made in HVRs, *e.g.*, to improve antibody affinity. Such alterations may be made in HVR "hotspots," *i.e.*, residues encoded by codons that undergo mutation at high frequency during the somatic maturation process (see, *e.g.*, Chowdhury, *Methods Mol. Biol.* 207:179-196 (2008)), and/or SDRs (a-CDRs), with the resulting variant VH or VL being tested for binding affinity. Affinity maturation by constructing and reselecting from secondary libraries has been described, *e.g.*, in Hoogenboom *et al.* in *Methods in Molecular Biology* 178:1-37 (O'Brien *et al.*, ed., Human Press, Totowa, NJ, (2001).) In some embodiments of affinity maturation, diversity is introduced into the variable genes chosen for maturation by any of a variety of methods (*e.g.*, error-prone PCR, chain shuffling, or oligonucleotide-directed mutagenesis). A secondary library is then created. The library is then screened to identify any antibody variants with the desired affinity. Another method to introduce diversity involves HVR-directed approaches, in which several HVR residues (*e.g.*, 4-6 residues at a time) are randomized. HVR residues involved in antigen binding may be specifically identified, *e.g.*, using alanine scanning mutagenesis or modeling. CDR-H3 and CDR-L3 in particular are often targeted.

In certain embodiments, substitutions, insertions, or deletions may occur within one or more HVRs so long as such alterations do not substantially reduce the ability of the antibody to bind

antigen. For example, conservative alterations (*e.g.*, conservative substitutions as provided herein) that do not substantially reduce binding affinity may be made in HVRs. Such alterations may be outside of HVR "hotspots" or SDRs. In certain embodiments of the variant VH and VL sequences provided above, each HVR either is unaltered, or contains no more than one, two or three amino acid substitutions.

Combination therapy

The PAK1 inhibitors of the methods described herein can be used either alone or in combination with other agents in a therapy for the treatment of melanoma. For instance, a PAK1 inhibitor described herein may be co-administered with at least one additional therapeutic agent including another PAK1 inhibitor. In certain embodiments, an additional therapeutic agent is a chemotherapeutic agent. In some embodiments, the additional therapeutic agent may be Aldesleukin, Dacarbazine, DTIC-Dome (Dacarbazine), Ipilimumab, Proleukin (Aldesleukin), Vemurafenib, Yervoy (Ipilimumab), and/or Zelboraf (Vemurafenib). The example of the use of PAK1 inhibitors in combination therapies is provided by PCT/EP2011/070008 filed November 14, 2011.

Such combination therapies noted above encompass combined administration (where two or more therapeutic agents are included in the same or separate formulations), and separate administration, in which case, administration of the PAK1 inhibitor can occur prior to, simultaneously, and/or following, administration of the additional therapeutic agent and/or adjuvant. In some embodiments, PAK1 inhibitors are used for the treatment of melanoma in an individual in combination with radiation therapy. In some embodiments, PAK1 inhibitors are used for the treatment of melanoma in an individual in combination with surgical removal of all or a portion of the melanoma from the individual.

In some embodiments of the invention, the individual has been previously treated for melanoma, for example, using an anti-cancer therapy. In one example, the anti-cancer therapy is surgery. In another embodiment, the subject can be further treated with an additional anti-cancer therapy before, during (*e.g.*, simultaneously), or after administration of the PAK1 inhibitor. Examples of anti-cancer therapies include, without limitation, surgery, radiation therapy (radiotherapy), biotherapy, immunotherapy, chemotherapy, or a combination of these therapies.

Route of administration

The route of administration is in accordance with known and accepted methods, such as by single or multiple bolus or infusion over a long period of time in a suitable manner, *e.g.*,

injection or infusion by subcutaneous, intravenous, intraperitoneal, intramuscular, intraarterial, intralesional or intraarticular routes, topical administration, inhalation or by sustained release or extended-release means. In some embodiments, the invention provides for methods for the treatment of melanoma in an individual with a PAK1 inhibitor wherein the PAK1inhibitor is administered intravenously to the individual. In other embodiments, the invention provides for methods for the treatment of melanoma in an individual with a PAK1 inhibitor wherein the PAK1inhibitor is administered topically to the individual.

Pharmaceutical compositions

For the methods of the invention, therapeutic formulations of the invention are prepared for storage by mixing the PAK1 inhibitor having the desired degree of purity with optional physiologically acceptable carriers, excipients or stabilizers (Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980)), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptide; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (*e.g.*, Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG).

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. For example, it may be desirable to further provide an immunosuppressive agent. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsule prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsule and poly-(methylmethacylate) microcapsule, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-

particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences 16th edition, Osol, A. *Ed.* (1980).

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, *e.g.*, films, or microcapsule. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37°C, resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S--S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

In some aspects, the invention provides a composition comprising a PAK1 inhibitor for use in the treatment of melanoma. In some embodiments, the melanoma is a wild-type BRAF melanoma. In some embodiments, PAK1 is over expressed in the melanoma. In some embodiments, the melanoma is a wild-type BRAF melanoma wherein PAK1 is overexpressed in the melanoma compared to non-cancerous cells; for example, non-cancerous skin cells. In some embodiments, the melanoma is a wild-type BRAF melanoma wherein PAK1 is amplified in the melanoma. In some embodiments, the melanoma is a wild-type BRAF melanoma wherein PAK1 is overexpressed in the melanoma and PAK1 is amplified in the melanoma. In some embodiments, PAK1 is overexpressed in the melanoma and PAK1 is amplified in the melanoma. In some embodiments, the melanoma is a mutant BRAF melanoma. In some embodiments, the melanoma is a mutant BRAF melanoma and the melanoma overexpresses PAK1 compared to non-cancerous cells and/or PAK1 is amplified in the melanoma. In some embodiments, the invention provides a composition comprising PAK1 inhibitor for use in the treatment of melanoma in a mammal. In some embodiments, the invention provides a composition comprising PAK1 inhibitor for use in the treatment of melanoma in a human.

In some aspects, the invention provides a use for a PAK1 inhibitor in the manufacture of a medicament for the treatment of melanoma. In some embodiments, the melanoma is a wild-type BRAF melanoma. In some embodiments, PAK1 is over expressed in the melanoma. In some embodiments, the melanoma is a wild-type BRAF melanoma wherein PAK1 is overexpressed in the melanoma compared to non-cancerous cells; for example, non-cancerous skin cells. In some embodiments, the melanoma is a wild-type BRAF melanoma wherein PAK1 is amplified in the melanoma. In some embodiments, the melanoma is a wild-type BRAF melanoma wherein PAK1 is overexpressed in the melanoma and PAK1 is amplified in the melanoma. In some embodiments, PAK1 is overexpressed in the melanoma and PAK1 is amplified in the melanoma. In some embodiments, the melanoma is a mutant BRAF melanoma. In some embodiments, the melanoma is a mutant BRAF melanoma and the melanoma overexpresses PAK1 compared to non-cancerous cells and/or PAK1 is amplified in the melanoma. In some embodiments, the invention provides a use for a PAK1 inhibitor in the manufacture of a medicament for the treatment of melanoma in a mammal. In some embodiments, the invention provides a use for a PAK1 inhibitor in the manufacture of a medicament for the treatment of melanoma in a human.

Kits

The invention also provides kits, medicines, compositions, and unit dosage forms for use in any of the methods described herein.

Kits of the invention include one or more containers comprising a PAK1 inhibitor (or unit dosage forms and/or articles of manufacture) and in some embodiments, further comprise instructions for use in the treatment of melanoma in accordance with any of the methods described herein. The kit may further comprise a description of selection an individual suitable or treatment (*e.g.* selection based on BRAF genotype). Instructions supplied in the kits of the invention are typically written instructions on a label or package insert (*e.g.*, a paper sheet included in the kit), but machine-readable instructions (*e.g.*, instructions carried on a magnetic or optical storage disk) are also acceptable. In some embodiments, the kit further comprises another therapeutic agent.

The kits of the invention are in suitable packaging. Suitable packaging include, but is not limited to, vials, bottles, jars, flexible packaging (*e.g.*, sealed Mylar or plastic bags), and the like. Kits may optionally provide additional components such as buffers and interpretative information. The present application thus also provides articles of manufacture, which include vials (such as sealed vials), bottles, jars, flexible packaging, and the like.

Melanoma biomarkers and treatment

The invention provides methods to identify human melanoma patients suitable for treatment with a PAK1 inhibitor by determining the presence of one or more melanoma biomarkers. In some embodiments, the melanoma biomarker is overexpression of PAK1 in the melanoma, amplification of PAK1 in the melanoma, and/or the presence of wild-type BRAF in the melanoma. In some embodiments the overexpression of PAK1 is determined by comparison to non-cancerous tissue; for example non-cancerous skin tissue. In some embodiments, the biomarkers are detected in a test sample obtained from the individual. In some embodiments, the presence of the biomarker is determines by comparison of a test sample with a reference sample.

In one embodiment, the invention provides methods to identify human melanoma patients suitable for treatment with a PAK1 inhibitor by determining the expression of PAK1 in the melanoma wherein overexpression of PAK1 in the melanoma compared to non-cancerous cells indicated that the patient is suitable for treatment with a PAK1 inhibitor. In some embodiments, overexpression of PAK1 by about any of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% or greater than 100% in the melanoma compared to non-cancerous cells indicates that the patient is suitable for treatment with a PAK1 inhibitor. In some embodiments, overexpression of PAK1 by about any of 1.5-fold, 2.0-fold, 2.5-fold, 3, 3.5-fold, 4.0-fold, 4.5-fold, 5.0-fold, 6.0-fold, 7.0-fold, 8.0-fold, 9.0-fold, or 10-fold compared to expression of PAK1 in non-cancerous cells indicates that the patient is suitable for treatment with a PAK1 inhibitor. Methods to determine expression of PAK1 are known in the art. Examples of methods to determine expression levels of PAK1 in a melanoma include, but are not limited to immunohistochemistry, reverse-phase protein array (RPPA), quantitative PCR, immunoassays, and the like. Levels of PAK1 expression can be compared to other tumors and cells by using the Gene Expression Omnibus (GEO) database.

In another embodiment, the invention provides methods to identify human melanoma patients suitable for treatment with a PAK1 inhibitor by detecting the amplification of PAK1 in the melanoma wherein amplification of the PAK1 gene in the melanoma indicates that the patient is suitable for treatment with a PAK1 inhibitor. In some embodiments, a copy number of PAK1 of about any of 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10, or greater than 10 in the melanoma indicates that the patient is suitable for treatment with a PAK1 inhibitor. Methods to determine amplification of a gene are known in the art. For example, the copy number of the PAK1 gene may be determined by using SNP arrays such as the Affymetrix 500K SNP array analysis.

In another embodiment, the invention provides methods to identify human melanoma patients suitable for treatment with a PAK1 inhibitor by detecting the genotype of BRAF in the melanoma wherein wild-type BRAF in the melanoma indicates that the patient is suitable for

treatment with a PAK1 inhibitor. Methods to determine the genotype of the BRAF gene in the melanoma are known in the art; for example, the nucleotide sequence of the BRAF gene from the melanoma may be determined using standard sequencing methods or by using the KASP SNP genotyping system (KBioscience).

The invention provides methods of treating melanoma in a patient provided that the patient has been found to have a biomarker for melanoma selected from overexpression of PAK1 in the melanoma, amplification of PAK1 in the melanoma and/or the presence of wild-type BRAF in the melanoma; the method comprising administering to the patient a therapeutically effective amount of a PAK1 inhibitor. In some embodiments, the patient is a human patient. In some embodiments of the above embodiment, at least one of the biomarkers is overexpression of PAK1 wherein PAK1 is overexpressed by about any of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% or greater in the melanoma compared to non-cancerous cells. In some embodiments, expression of PAK1 in the melanoma is greater than about any of 1.5-fold, 2.0-fold, 2.5-fold, 3, 3.5-fold, 4.0-fold, 4.5-fold, 5.0-fold, 6.0-fold, 7.0-fold, 8.0-fold, 9.0-fold, or 10-fold compared to expression of PAK1 in non-cancerous cells. Methods to determine expression of PAK1 are known in the art. Examples of methods to determine expression levels of PAK1 in a melanoma include, but are not limited to immunohistochemistry, reverse-phase protein array (RPPA), quantitative PCR, immunoassays, and the like. Levels of PAK1 expression can be compared to other tumors and cells by using the Gene Expression Omnibus (GEO) database.

In some embodiments of the above embodiment, at least one of the biomarkers is amplification of PAK1 in the melanoma wherein a copy number of PAK1 of about any of 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10, or greater than 10 in the melanoma. Methods to determine amplification of a gene are known in the art. For example, the copy number of the PAK1 gene may be determined by using SNP arrays such as the Affymetrix 500K SNP array analysis.

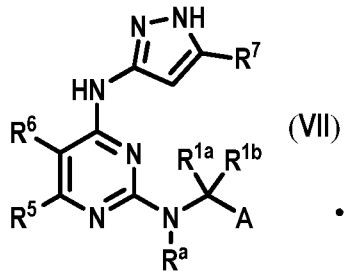
In some embodiments of the above embodiment, at least one of the biomarkers is the genotype of BRAF in the melanoma wherein the patient has a melanoma containing a wild-type melanoma. In some embodiments of the above embodiment, the presence of melanoma biomarkers in the patient had been previously determined prior to treatment with the PAK1 inhibitor.

The invention provides methods of adjusting treatment of melanoma in a patient undergoing treatment with a PAK1 inhibitor wherein the expression of PAK1 in the melanoma is determined. In some embodiments, the melanoma is a wild-type BRAF melanoma. In some embodiments, the overexpression of PAK1 in the melanoma indicates that treatment with the PAK1 inhibitor may continue. In some embodiments, the expression of PAK1 in a melanoma in a patient undergoing treatment with PAK1 is monitored over time. In some embodiments, the

expression of PAK1 in the melanoma is monitored at least daily, at least weekly, at least monthly. In some embodiments, the expression of PAK1 in a melanoma in a patient undergoing treatment with a PAK1 inhibitor is monitored over time. If PAK1 expression increases over the course of treatment with the PAK1 inhibitor, the amount of PAK1 inhibitor administered to the patient is increased or remains the same. In some embodiments, the amount of PAK1 inhibitor administered to the patient is increased until the level of PAK1 expression decreases or is no longer detected. If PAK1 expression decreases over the course of treatment with the PAK1 inhibitor, the amount of PAK1 inhibitor administered to the patient is decreased or remains the same. In some embodiments, the expression of PAK1 expression in a melanoma of a patient undergoing treatment with a PAK1 inhibitor is monitored over time where treatment with the PAK1 inhibitor is continued until PAK1 expression in the melanoma is no longer detected.

Exemplary embodiments

In some embodiments the invention provides methods for treating a melanoma in an individual comprising contacting the melanoma with a therapeutically effective amount of a PAK1 inhibitor. In further embodiments, the melanoma is a wild-type BRAF melanoma. In yet further embodiments, PAK1 is overexpressed in the tumor compared to non-cancerous skin cells. In further embodiments of any of the above embodiments, PAK1 is amplified in the tumor. In further embodiments, the copy number of the PAK1 in the tumor is greater than about 2.5. In further embodiments of any of the above embodiments, the inhibitor is a small molecule, a nucleic acid, or a polypeptide. In some embodiments, the small molecule is PF-3758309. In some embodiments, the small molecule is a compound of formula I.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In further embodiments, the small molecule is a compound of formula I and A is 4-indolyl, 5-indolyl, 4-indazolyl, 5-indazolyl, 4-benzimidazolyl or 5-benzimidiazolyl; R^a, R^{1a} and R^{1b} are independently hydrogen or C_{1-3} alkyl; R^5 is hydrogen or C_{1-6} alkyl; R^6 is hydrogen, halogen or C_{1-6} alkyl; and, R^7 is cycloalkyl optionally substituted by fluorine.

In further embodiments of any of the above embodiments, the individual is a human.

In further embodiments of any of the above embodiments, the PAK1 inhibitor is used in combination with a therapeutic agent.

The invention provides the use of a PAK1 inhibitor for the treatment of melanoma in an individual. In some embodiments of the use, the melanoma is a wild-type BRAF melanoma.

The invention provides compositions comprising a PAK1 inhibitor for use in the treatment of melanoma. In some embodiments of the composition, the melanoma is a wild-type BRAF melanoma. In some embodiments, the composition further comprises a pharmaceutically acceptable excipient.

The invention provides the use of a PAK1 inhibitor in the manufacture of a medicament for the treatment of melanoma. In some embodiments of the use, the melanoma is a wild-type BRAF melanoma.

The invention provides kits comprising a PAK1 inhibitor for use in treating melanoma comprising PAK1 inhibitor and directions for use in the treatment of melanoma. In some embodiments of the kit, the melanoma is a wild-type BRAF melanoma.

The invention provides methods of inhibiting CRAF signaling in a melanoma in an individual comprising contacting the melanoma with a therapeutically effective amount of a PAK1 inhibitor.

The invention provides methods of inhibiting MEK signaling in a melanoma tumor comprising contacting the melanoma with a therapeutically effective amount of a PAK1 inhibitor.

The invention provides methods of identifying a human melanoma patient suitable for treatment with a PAK1 inhibitor comprising determining the BRAF genotype of the melanoma, wherein a melanoma comprising a wild type BRAF indicates that the patient is suitable for treatment with a PAK1 inhibitor.

The invention provides methods of identifying a human melanoma patient suitable for treatment with a PAK1 inhibitor comprising determining the expression of PAK1 in the melanoma, wherein overexpression of PAK1 in the melanoma compared to non-cancerous skin cells indicates that the patient is suitable for treatment with a PAK1 inhibitor. In some embodiments of the method, the overexpression of PAK1 in the melanoma is 2.5-fold greater than the expression of PAK1 in the non-cancerous skin cellsThe invention provides methods for treating a human melanoma patient with a PAK1 inhibitor comprising: (a) selecting a patient based on the BRAF genotype of the melanoma, wherein a melanoma comprising a wild type BRAF indicates that the patient is suitable for treatment with a PAK1 inhibitor; and (b) administering to the selected patient a therapeutically effective amount of a PAK1 inhibitor.

The invention provides methods for treating a human melanoma patient with a PAK1 inhibitor comprising: (a) selecting a patient based on the PAK1 expression level of the melanoma,

wherein a overexpression of PAK1 in the melanoma indicates that the patient is suitable for treatment with a PAK1 inhibitor; and (b) administering to the selected patient a therapeutically effective amount of a PAK1 inhibitor. In some embodiments of the methods, the overexpression of PAK1 in the melanoma is 2.5-fold greater than the expression of PAK1 in the non-cancerous skin cells.

The invention provides methods for treating a human melanoma patient comprising administering to the selected individual a therapeutically effective amount of a PAK1 inhibitor, wherein the genotype of the melanoma had been determined to be wild type for BRAF.

The invention provides methods for treating a human melanoma patient comprising administering to the patient a therapeutically effective amount of a PAK1 inhibitor, wherein the melanoma had been determined to overexpress PAK1 compared to non-cancerous skin cells. In some embodiments of the methods, the overexpression of PAK1 in the melanoma is 2.5-fold greater than the expression of PAK1 in the non-cancerous skin cells.

The invention provides methods of adjusting treatment of melanoma in a patient undergoing treatment with a PAK1 inhibitor, said method comprising assessing the PAK1 expression in the melanoma, wherein overexpression of PAK1 in the melanoma indicates that treatment of the individual is adjusted until PAK1 overexpression is no longer detected.

All of the features disclosed in this specification may be combined in any combination. Each feature disclosed in this specification may be replaced by an alternative feature serving the same, equivalent, or similar purpose. Thus, unless expressly stated otherwise, each feature disclosed is only an example of a generic series of equivalent or similar features.

Further details of the invention are illustrated by the following non-limiting Examples. The disclosures of all references in the specification are expressly incorporated herein by reference.

EXAMPLES

The examples below are intended to be purely exemplary of the invention and should therefore not be considered to limit the invention in any way. The following examples and detailed description are offered by way of illustration and not by way of limitation.

Example 1: Elevated PAK1 protein expression and genomic amplification in melanoma.

To determine the possible extent of PAK1 dysregulation in human melanoma, primary tumor tissue from 87 melanoma patients was assayed for DNA copy number changes using high-resolution single nucleotide polymorphism (SNP) arrays. Affymetrix 500K SNP array analysis, genomic DNA preparation, chip processing and data analysis were performed as published

previously (Harvey PM, *et al.*, (2008) *Genes Chromosomes Cancer*, 47(6):530-542) to measure copy gains of 11q13, the region of chromosome 11 that harbors the PAK1 gene, in the sampled melanoma tissue. To collect expression array data for matched tumor samples, RNA was extracted from frozen tumor tissue and applied to Affymetrix (Santa Clara, CA) HGU133 gene expression microarrays. The frequency of PAK1 amplification was 9% (8 of 87 specimens with copy number ≥ 2.5) in this tumor panel (Figure 1A). RNA was purified from 42 melanoma tumor and cell lines specimens and increased PAK1 copy number was correlated with mRNA expression (Pearson correlation = 0.75; Figure 1B). Dysregulated PAK1 expression was more frequent than would be predicted by genomic amplification alone, thereby suggesting that additional transcriptional or regulatory mechanisms increase PAK1 expression in this indication (Reddy SD, et al, (2008) *Cancer Res*, 68(20):8195-8200 and de la Torre-Ubieta L, *et al.*, (2010) *Genes Dev*, 24(8):799-813). Elevated expression of PAK1 in melanoma compared to normal skin tissues was also demonstrated using gene expression data deposited in the Gene Expression Omnibus database (GSE4587). Interestingly, PAK1 gene amplification was preferentially observed in tumors that lacked activating mutations in the BRAF oncogene at 22% versus 0% for BRAF wild-type or mutant, respectively ($p = 0.005$, two-sided t-test; Figure 1B). The levels of PAK1 mRNA expression differed between wild-type and BRAF(V600E) or BRAF(V600M) genotypes ($p = 0.006$ and $p=0.125$, respectively; Figure 1B). Taken together, this suggests that PAK1 could be a tumor-promoting "driver" gene in a subset of BRAF wild-type melanomas.

To further evaluate the extent of PAK1 dysregulation in human melanomas, PAK1 protein expression level and subcellular localization were ascertained via immunohischemistry (IHC) staining of a distinct set of tissue microarrays. Briefly, formalin-fixed paraffin-embedded tissue blocks and corresponding pathology reports were obtained for 92 primary melanomas resected between 1993 and 2009 (Oxford Radcliffe Hospitals, Oxford, UK). The melanoma series comprised 23 nodular, 3 lentigo maligna, 45 superficial spreading, 3 desmoplastic, 5 acral lentiginous and 13 unclassifiable melanoma specimens. Four cancers were stage pT1, 17 were stage pT2, 28 were stage pT3, 35 were stage pT4 and 8 cases could not be accurately staged. Tissue microarrays (TMAs) were assembled as described previously (Bubendorf L, *et al.*, (2001) *J Pathol*, 195(1):72-79). Approval was obtained for the use of all human tissue from the local research ethics committee (C02.216). Immunohistochemistry (IHC) was performed as described previously (Ong CC, *et al.*, (2011) *PNAS*, 108(17):7177-7182). Intensity of PAK1 expression was scored separately in the cytoplasm and nuclei of neoplastic cells on a scale of 0 to 3. The highest intensity score among replicate cores was used as the score for each patient. The same pathologist scored all cases, blind to the clinical data. The chi-squared test was used to evaluate associations between categorical variables. Robust and selective IHC reactivity of PAK1

antibody was previously demonstrated (Ong CC, *et al.*, (2011) *PNAS*, 108(17):7177-7182). In malignant melanoma, 46 of 92 (50%) primary tumor samples were positive for PAK1 expression and 26% of all cases showed staining of moderate (2+) or strong (3+) intensity in the malignant cells (Figure 1C, panels III and IV; Table 1). Nuclear localization of PAK1 was only evident in a very small proportion of melanomas. Identical results were seen with an alkaline phosphatase label and fast red chromogen in place of a horseradish peroxidase label and brown diaminobenzidine. PAK1 was weakly expressed in basal keratinocytes in normal skin, and lymphocytes and presumed Langerhans cells were positive for PAK1 expression (Figure 1C, panel IV). Together, these data show that PAK1 DNA copy number, mRNA and protein expression are broadly upregulated in human melanoma.

Example 2: Negative association between PAK1 over-expression and BRAF mutation in primary melanomas

Given the prevalence of oncogenic mutation of *BRAF* and *NRAS* in melanoma (Lee JH, *et al.*, (2011) *Br J Dermatol*, 164(4):776-784), melanoma tissues were genotyped for known hotspot mutations in BRAF (codon 600) and NRAS (codons 12, 13, 61 and 146) genes. Mutation status was determined for BRAF codon 600 and NRAS codons 12, 13 61 and 146 via KASPar (KBioscience, Herts, England) and conventional Sanger DNA sequencing methods. Genotype data for BRAF (39 Val600Glu, 1 Val600Lys and 46 wild-type) and NRAS (1 Gln61His, 7 Gln61Lys, 1 Gln61Lys + Gln61Arg + Leu59Ala, 1 Gln61Leu, 19 Gln61Arg, 2 Gln61Arg + Gln61Lys and 53 wild-type) were available for 86 and 84 tumors, respectively, and were consistent with the ranges of mutation frequencies that have been previously published for cutaneous melanoma (Lee JH, *et al.*, (2011) *Br J Dermatol*, 164(4):776-784). PAK1 IHC staining was scored blind to clinicopathological details and mutation status and results are summarized in Table 1. Notably, PAK1 protein expression was dysregulated selectively in BRAF wild-type tumors (19 of 46 were positive for strong IHC staining of PAK1) compared to melanomas expressing oncogenic V600E or V600K mutants (4 of 40 tumors with high IHC staining). This negative correlation between PAK1 expression and BRAF mutation was statistically significant ($p < 0.001$, Chi-squared 10.702). BRAF and NRAS mutations were not mutually exclusive, and presence of tumors with either mutation was also negatively associated with PAK1 protein expression ($p=0.004$, Chi-squared 8.128). A similar trend, albeit not statistically significant, was observed when dichotomizing samples into only NRAS mutant and non-mutant status ($p=0.45$, Chi-squared 0.569). There was no significant association between PAK1 protein expression and mitotic count ($p=0.61$ Student's T-test), pathological tumor (pT) stage ($p=0.14$ Chi-squared), Breslow thickness ($p=0.85$ Student's T-test) or ulceration ($p=0.91$

Chi-squared). Taken together, these results provide evidence that PAK1 dysregulation is strongly associated with cutaneous melanomas that lack oncogenic mutation of BRAF and define a subset of human melanoma for which there is no effective targeted therapy.

Table 1. PAK1 protein overexpression in BRAF wild-type melanoma

MAPK activator	Genotype	PAK1 IHC		P-value	Chi-squared
		0, 1	2, 3		
BRAF	WT	27	19	0.001	10.702
	Mutant	36	4		
NRAS	WT	37	16	0.45	0.569
	Mutant	24	7		

Example 3: PAK1 is required for proliferation of BRAF wild-type melanoma cells

Given the genomic and histologic data for elevated PAK1 expression in the subset of human melanoma that is wild-type for BRAF, PAK1 expression and the effect of RNAi-mediated knockdown of PAK1 was examined in a panel of melanoma cell lines in order to clarify the contribution of PAK1 towards tumor cell proliferation. Cell lines were acquired from the American Type Culture Collection (ATCC; Manassas, VA) and maintained at 37°C and 5% CO2 in Dulbecco's Modified Eagle Medium (DMEM) or Roswell Park Memorial Institute 1640 (RPMI 1640) media with 10% fetal bovine serum and 4 mM L-glutamine. Cell lines were transfected with commercially available short-interfering RNA (siRNA) oligonucleotide duplexes from Dharmacon RNAi Technologies (Chicago, IL) that were previously characterized for efficiency and selectivity of PAK1 and PAK2 knockdown (Ong CC, *et al.*, (2011) *PNAS*, 108(17):7177-7182). Cellular viability was assessed via ATP content using the CellTiter-Glo Luminescent Assay (Promega, Madison, WI) and results represent mean ± standard deviation from three experiments. Increased PAK1 protein expression in melanomas expressing wild-type versus mutant BRAF was also observed for immortalized cell lines in culture. Cell viability analysis demonstrated that 537MEL, MeWo, SK-MEL23 and SK-MEL30 melanoma cells expressed high levels of PAK1 protein and transient knockdown of PAK1 via a pool of multiple PAK1-selective siRNA oligonucleotides resulted in a 1.8- to 4.3-fold reduction in cell viability when compared with cells transfected with a non-targeting, negative control siRNA oligonucleotide ($p < 0.0001$; Figure 2A). Furthermore, inhibition of PAK1 generally reduced proliferation of BRAF wild-type melanoma cells relative to $BRAF^{V600E}$ cells ($p < 0.07$; n=14), further supporting a role for PAK1 as a driver of proliferation in this melanoma subtype (Figure

2B). To better assess the mechanism by which PAK1 contributes to proliferation, PAK-dependent cellular signaling was assessed in 537MEL and SK-MEL23 cells. Protein extracts from cell lysates were prepared at 4°C with Cell Extraction Buffer (Invitrogen, Carlsbad, CA), 1 mM phenylmethylsulphonyl fluoride (PMSF), Phosphatase Inhibitor Cocktail 1/2 (Sigma Aldrich, St. Louis, MO), and one tablet of Complete EDTA-free MiniTM protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). For Western blot analysis, proteins were resolved by 4-12% SDS-PAGE and transferred to nitrocellulose membranes (Millipore Corporation, Billerica, MA). Immunoblotting was performed using the indicated primary antibodies and analyzed using secondary antibodies for enhanced chemiluminescence (ECL). MAPK pathway activation, as determined by phosphorylation of ERK and MEK, was dramatically inhibited by PAK knockdown (Figure 2C). In agreement with this result, cyclin D1 levels (which are essential for regulating cyclin-dependent kinases and G1/S progression) were also diminished as a consequence of PAK1 ablation. PAK1 signaling in BRAF wild-type melanoma cells was further investigated using a reverse-phase protein array (RPPA) phosphoproteomics platform. Protein lysates were analyzed by RPPA (Theranostics Health, LLC) by first diluting all samples to a final concentration of 0.5 mg/mL. The sample dilutions were printed in duplicate on slides that were then subjected to immunostaining with a panel of antibodies primarily directed against specific phosphorylated or cleaved proteins. Each of these antibodies had previously undergone extensive validation for both phosphorylation and protein specificity using single band detection at the appropriate molecular weight by immunoblotting. The intensity value for each end point was determined by identifying spots for each duplicate dilution curve for each sample that were within the linear dynamic range of the staining after background subtraction with each spot (within slide local background and also against a slide stained with secondary antibody only). Each value was normalized relative to the total protein intensity value for that sample derived from a slide stained with Sypro Ruby (Invitrogen). RPPA data were processed by $\log_2$ transformation and linear scaling (z-score conversion) to ensure normality and linearity. RPPA analysis showed a decreased signaling to MAPK, nuclear factor-κB (NF-κB) and cytoskeletal pathways following PAK1 inhibition in BRAF wild-type (SK-MEL23), but not BRAF mutant (A375), melanoma cells (Figure 2D).

PAK1 has been shown to phosphorylate both CRAF (Ser338) and MEK1(Ser298) (17, 29-31). Hence, the molecular mechanism by which PAK1 triggers activation of the MAPK pathway in BRAF wild-type melanoma cells was investigated. Since phospho-specific antibodies that are raised to the Ser217/Ser221 activation loop sites on MEK proteins do not distinguish between MEK1 and MEK2, the MEK isoforms were immunoprecipitated from cells transfected with either control or PAK-selective siRNA oligonucleotides as previously described (Hatzivassiliou

G, *et al.*, (2010) *Nature*, 464(7287):431-435) and MEK activation was detected via immunoblotting with phospho-MEK1/2(Ser217/Ser221) antibodies. PAK knockdown diminished both MEK1 (Figure 3A) and MEK2 (Figure 3B) phosphorylation in 537MEL and SK-MEL23 cells. Since the Ser298 phosphorylation site on MEK1 is not conserved in MEK2, PAK-dependent activation of both MEK isoforms would suggest that upstream signaling to CRAF might be a driver of MAPK pathway regulation in BRAF wild-type melanoma cells. CRAF was immunoprecipitated from cells transfected with either control or PAK-selective siRNA oligonucleotides as previously described (Hatzivassiliou G, *et al.*, (2010) *Nature*, 464(7287):431-435) and CRAF activation was detected via immunoblotting with phospho-CRAF(Ser338) antibodies. Western analysis demonstrated that PAK ablation reduced phosphorylation of CRAF on Ser338, a residue critical for full activation of this kinase (Figure 3C). The dependence of CRAF(Ser338) phosphorylation (Figure 3D) and CRAF effector signaling (Figure 3E) on PAK catalytic activity was also confirmed using PF-3758309, an inhibitor of PAKs that is currently in clinical development (Murray BW, et al, (2010) *PNAS*, 107(20): 9446-9451), and IPA-3, an allosteric inhibitor that binds PAK1-3 and prevents activation by Rho family GTPases (Deacon SW, *et al.*, (2008) *Chemistry & Biology*, 15(4):322-331).

Additional loss-of-function studies to analyze the role of PAK1 in BRAF wild-type melanoma cells were conducted by investigating the contribution of PAK1 to melanoma cell migration. Briefly, WM-266-4 melanoma cells were transfected with non-targeting control (NTC) or PAK1/2 siRNA oligonucleotide for 72 h and confluent WM-266-4 melanoma cell were subsequently wounded. Images were recorded when wounds were made (dark shading) and 28 h after wounding (bright field). Differences in relative wound density were statistically significant ($p < 0.001$; n=3) revealing a requirement for PAK1 in melanoma cell migration (Figure 4). Taken together, the functional consequences of PAK1 blockade in BRAF wild-type melanoma cells encompasses pronounced cytostatic effects via reduced CRAF activation and subsequent MAPK pathway signaling.

Example 4: Differential sensitivity of BRAF wild-type melanoma cells to PAK and BRAF inhibition

To more closely investigate the activity and cellular mechanism of action of PAK signaling within sensitive and insensitive tumor types, small molecule inhibition of PAK and BRAF were compared using SK-MEL23 BRAF wild-type and A375 BRAF(V600E) cells. For analysis of pathway modulation, cells were treated with 5 μM PF-3758309 or with 0.2 μM PLX-4720, a BRAF inhibitor, for 4 h before cell lysates were analyzed for phosphorylation of MAPK pathway

components. Administration of PF-3758309 resulted in profound MAPK pathway modulation in SK-MEL23 cells (lane 2), but not A375 cells (lane 5), as determined by measurement of ERK1/2 and MEK1/2 phosphorylation on kinase loop residues that are critical for catalytic activity (Figure 5A). In comparison, analysis of PLX-4720-mediated signaling changes revealed only modest inhibition of ERK1/2 and MEK1/2 phosphorylation in SK-MEL23 cells (lane 3), whereas the same treatment conditions potently inhibited MAPK activation in BRAF(V600E) cells (lane 6). As a control, no differences were noted for total ERK1/2 or MEK1/2 protein levels in this experiment. Consistent with previous reports, MEK1-Ser298 was confirmed as a PAK-specific phosphorylation site but Ser298 phosphorylation was unlinked to MEK activation loop phosphorylation in BRAF(V600E) melanoma cells (lane 5). The biological consequence of PAK1 phosphorylation of MEK1-Ser298 is presently not well understood, however it has been shown that PAK1-MEK1 signaling can be mediated by cell-cell contact and adhesion (Slack-Davis JK, *et al.*, (2003) *J Cell Biol*, 162(2):281-291). PAK signaling was also induced via ectopic expression of Flag-PAK1 in BRAF(V600E) cells with only moderate endogenous expression of PAK1. Elevated PAK1 signaling in A375 cells resulted in a significant increase in CRAF and MEK phosphorylation that was reversible by addition of PF-3758309 (Figure 5B), suggesting that acquisition of PAK1 overexpression could be another mechanism to overcome dependence on oncogenic BRAF in melanoma (Johannessen CM, *et al.*, (2011) *Nature*, 468(7326):968-972).

To determine if PAK inhibitors decreased cell viability of wild-type BRAF melanoma cells, SK-MEL23 and 537MEL cells were assayed with the CellTiter-Glo Luminescent Assay (Promega, Madison, WI) after treatment with PF-3758309 or with (S)-N^2-(1-(1H-indol-5-yl)ethyl)-N^4-(5-cyclopropyl-1H-pyrazol-3-yl)-6-methylpyrimidine-2,4-diamine (I-007), N^2-((1H-indol-4-yl)methyl)-N^4-(5-cyclopropyl-1H-pyrazol-3-yl)-6-methylpyrimidine-2,4-diamine (I-054) and N^2-((4-chloro-1H-benzo[d]imidazol-5-yl)methyl)-N^4-(5-cyclopropyl-1H-pyrazol-3-yl)-N^2-methyl-pyrimidine-2,4-diamine (I-087). Inhibition of PAK1 by treatment with all tested PAK inhibitors significantly reduced cell proliferation indicating that inhibition of PAK signaling is a target for treatment of BRAF wild-type melanoma (Figure 6A and B).

To extend the in vitro observations, pharmacodynamic modulation by PAK small molecule inhibitors was evaluated using tumor xenograft models. Cultured SK-MEL-23, A2058.X1 and A375.X1 cells were removed from culture, suspended in Hank's buffered saline solution (HBSS), mixed 1:1 with Matrigel (BD Biosciences, USA), and implanted subcutaneously into the right flank of naïve female NCR nude (Taconic Farms, Hudson, NY) or Beige Nude XID (Harlan Laboratories, CA) mice. Animals with tumors of a mean volume of approximately 250 mm3 were grouped into treatment cohorts. Tumor volumes were calculated by the following

formula: Tumor Volume = $0.5 \times (a \times b^2)$, where 'a' is the largest tumor diameter and 'b' is the perpendicular tumor diameter. Tumor volume results are presented as mean tumor volumes ± the standard error of the mean (SEM). Percent growth inhibition (%INH) at the end of study (EOS) was calculated as %INH = 100 [(EOS Vehicle EOS Treatment)/(EOS Vehicle)]. Data analysis and generation of p values using the Dunnett t test was done using JMP software (SAS Institute, Cary, NC). All experimental procedures conformed to the guiding principles of the American Physiology Society and were approved by Genentech's Institutional Animal Care and Use Committee. Following tumor establishment, animals were either administered saline or PF-3758309 (25 mg/kg, i.p.) and tumors were harvested 1 h after dosing. Tumors were frozen and pulverized on dry ice using a small Bessman tissue pulverizer (Spectrum Laboratories, Rancho Dominguez, CA) and protein extracts were prepared at 4°C with Cell Extraction Buffer (Invitrogen, Carlsbad, CA), 1 mM phenylmethylsulphonyl fluoride (PMSF), phosphatase Inhibitor Cocktail 1/2 (Sigma Aldrich, St. Louis, MO), and one tablet of Complete EDTA-free MiniTM protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Proteins were subsequently resolved by 4-12% SDS-PAGE and transferred to nitrocellulose membranes (Millipore Corporation, Billerica, MA) for immunoblotting with the indicated antibodies. Treatment with PF-3758309 resulted in a substantial decrease in CRAF, MEK1/2 and ERK1/2 phosphorylation in SK-MEL23 tumors (Figure 7A). In A2058.X1 BRAF(V600E) tumors, decreased phosphorylation of CRAF(Ser338) was not observed following PF-3758309 dosing. The effect of PF-3758309 on growth and maintenance of BRAF wild-type tumors was also evaluated in an efficacy experiment for 21 days (Figure 7B and Figure 8A-B). Treatment with 10, 15 and 25 mg/kg PF-3758309 significantly impaired tumor growth (74%, 76% and 91% inhibition relative to the control cohort, respectively) relative to the vehicle cohort as measured on the final day of dosing (Dunnett's t-test, $p<0.0001$). In comparison, minimal anti-tumor efficacy and inhibition of CRAF phosphorylation were observed for SK-MEL23 tumors treated with a potent RAF inhibitor *in vivo* (Figure 9B)(Hoeflich KP, *et al.*, (2009) *Cancer Res*, 69(7):3042-3051). In addition, phosphoproteomic analysis of BRAF mutant (A375) and wild-type (SK-MEL23) cells treated with PLX-4720 BRAF inhibitor demonstrated that these cell subtypes of melanoma exhibit different signaling responses due to BRAF inhibition (Figure 9A). Together, the magnitude of MAPK pathway inactivation by PF-3758309 was correlated with anti-tumor efficacy in a BRAF wild-type melanoma xenograft model and these data support the conclusion that interfering with PAK signaling could have therapeutic efficacy in this subset of melanoma (model depicted in Figure 10).

CLAIMS

WHAT IS CLAIMED IS:

1. A method for treating a melanoma in an individual comprising contacting the melanoma with a therapeutically effective amount of a PAK1 inhibitor.

2. The method of claim 1, wherein the melanoma is a wild-type BRAF melanoma.

3. The method of claim 1 or 2, wherein PAK1 is overexpressed in the tumor compared to non-cancerous skin cells.

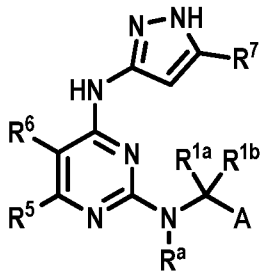
4. The method of any one of claims 1-3, wherein PAK1 is amplified in the tumor.

5. The method of claim 4, wherein the copy number of the PAK1 in the tumor is greater than about 2.5.

6. The method of any one of claims 1-5, wherein the inhibitor is a small molecule, a nucleic acid, or a polypeptide.

7. The method of claim 6, wherein the small molecule is PF-3758309.

8. The method of claim 6, wherein the small molecule is a compound of formula VII.


(VII)

9. The method of claim 8, wherein the small molecule is a compound of formula VII and A is 4-indolyl, 5-indolyl, 4-indazolyl, 5-indazolyl, 4-benzimidazolyl or 5-benzimidiazolyl; R^a, R^{1a} and R^{1b} are independently hydrogen or C_{1-3} alkyl; R^5 is hydrogen or C_{1-6} alkyl; R^6 is hydrogen, halogen or C_{1-6} alkyl; and, R^7 is cycloalkyl optionally substituted by fluorine.

10. The method of any one of claims 1-9, wherein the individual is a human.

11. The method of any one of claims 1-10, wherein the PAK1 inhibitor is used in combination with a therapeutic agent.

12. Use of a PAK1 inhibitor for the treatment of melanoma in an individual.

13. The use of claim 12, wherein the melanoma is a wild-type BRAF melanoma.

14. A composition comprising a PAK1 inhibitor for use in the treatment of melanoma.

15. The composition of claim 14, wherein the melanoma is a wild-type BRAF melanoma.

16. The composition of claim 14 or 15, further comprising a pharmaceutically acceptable excipient.

17. Use of a PAK1 inhibitor in the manufacture of a medicament for the treatment of melanoma.

18. The use of claim 17, wherein the melanoma is a wild-type BRAF melanoma.

19. A kit comprising a PAK1 inhibitor for use in treating melanoma comprising PAK1 inhibitor and directions for use in the treatment of melanoma.

20. The kit of claim 19, wherein the melanoma is a wild-type BRAF melanoma.

21. A method of inhibiting CRAF signaling in a melanoma in an individual comprising contacting the melanoma with a therapeutically effective amount of a PAK1 inhibitor.

22. A method of inhibiting MEK signaling in a melanoma tumor comprising contacting the melanoma with a therapeutically effective amount of a PAK1 inhibitor.

23. A method of identifying a human melanoma patient suitable for treatment with a PAK1 inhibitor comprising determining the BRAF genotype of the melanoma, wherein a melanoma comprising a wild type BRAF indicates that the patient is suitable for treatment with a PAK1 inhibitor.

24. A method of identifying a human melanoma patient suitable for treatment with a PAK1 inhibitor comprising determining the expression of PAK1 in the melanoma, wherein overexpression of PAK1 in the melanoma compared to non-cancerous skin cells indicates that the patient is suitable for treatment with a PAK1 inhibitor.

25. The method of claim 24, wherein the overexpression of PAK1 in the melanoma is X% greater than the expression of PAK1 in the non-cancerous skin cells.

26. A method for treating a human melanoma patient with a PAK1 inhibitor comprising:

(a) selecting a patient based on the BRAF genotype of the melanoma, wherein a melanoma comprising a wild type BRAF indicates that the patient is suitable for treatment with a PAK1 inhibitor; and

(b) administering to the selected patient a therapeutically effective amount of a PAK1 inhibitor.

27. A method for treating a human melanoma patient with a PAK1 inhibitor comprising:

(a) selecting a patient based on the PAK1 expression level of the melanoma, wherein a overexpression of PAK1 in the melanoma indicates that the patient is suitable for treatment with a PAK1 inhibitor; and

(b) administering to the selected patient a therapeutically effective amount of a PAK1 inhibitor.

28. The method of claim 27, wherein the overexpression of PAK1 in the melanoma is 2.5-fold greater than the expression of PAK1 in the non-cancerous skin cells.

29. A method for treating a human melanoma patient comprising administering to the selected individual a therapeutically effective amount of a PAK1 inhibitor, wherein the genotype of the melanoma had been determined to be wild type for BRAF.

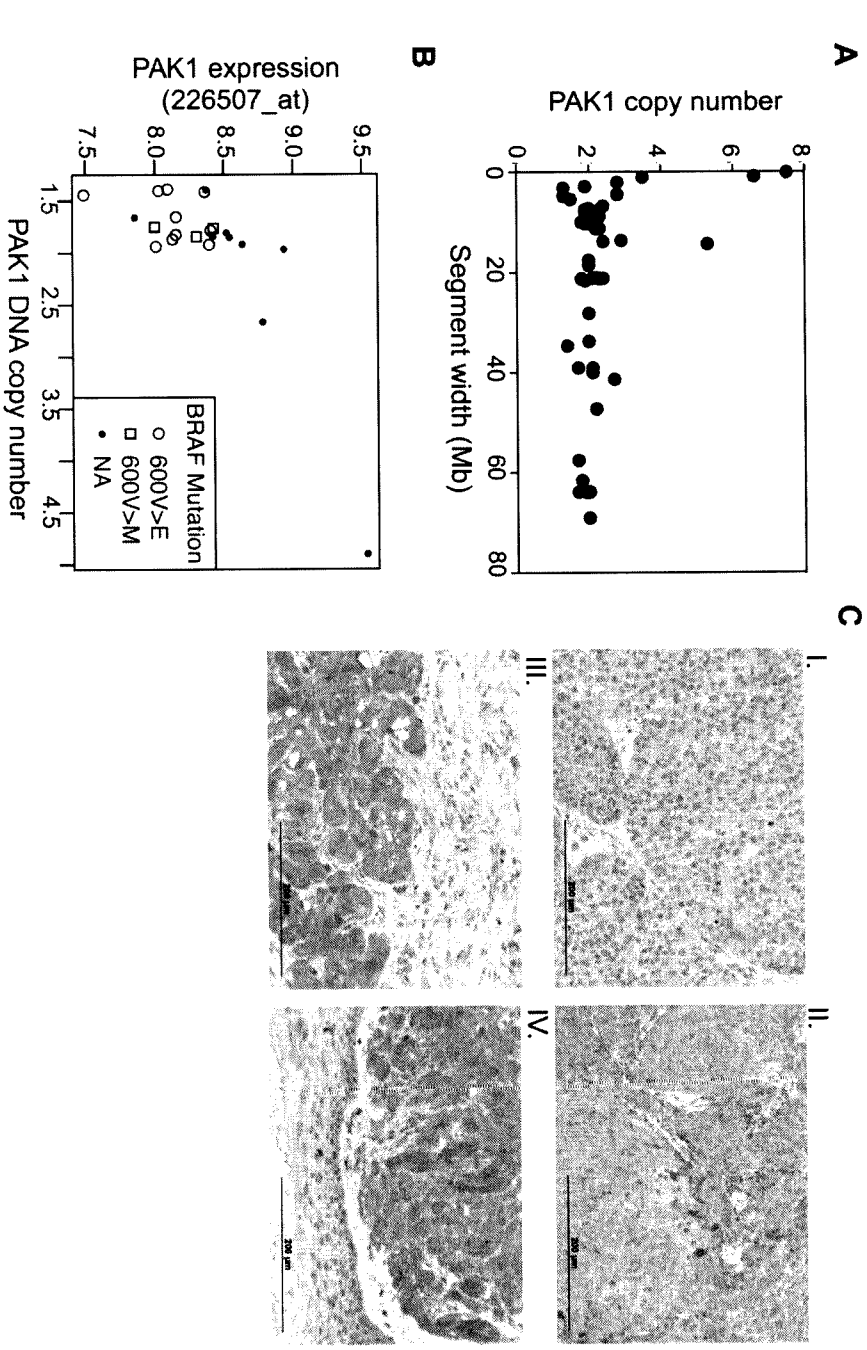
30. A method for treating a human melanoma patient comprising administering to the patient a therapeutically effective amount of a PAK1 inhibitor, wherein the melanoma had been determined to overexpress PAK1 compared to non-cancerous skin cells.

31. The method of claim 30, wherein the overexpression of PAK1 in the melanoma is 2.5-fold greater than the expression of PAK1 in the non-cancerous skin cells.

32. A method of adjusting treatment of melanoma in a patient undergoing treatment with a PAK1 inhibitor, said method comprising assessing the PAK1 expression in the melanoma, wherein overexpression of PAK1 in the melanoma indicates that treatment of the individual is adjusted until PAK1 overexpression is no longer detected.

33. The invention as hereinbefore described.

Figure 1


A
PAK1 copy number
Segment width (Mb)
B
PAK1 expression
(226507_at)
PAK1 DNA copy number
BRAF Mutation
600V>E
600V>M
NA
C
I.
II.
III.
IV.

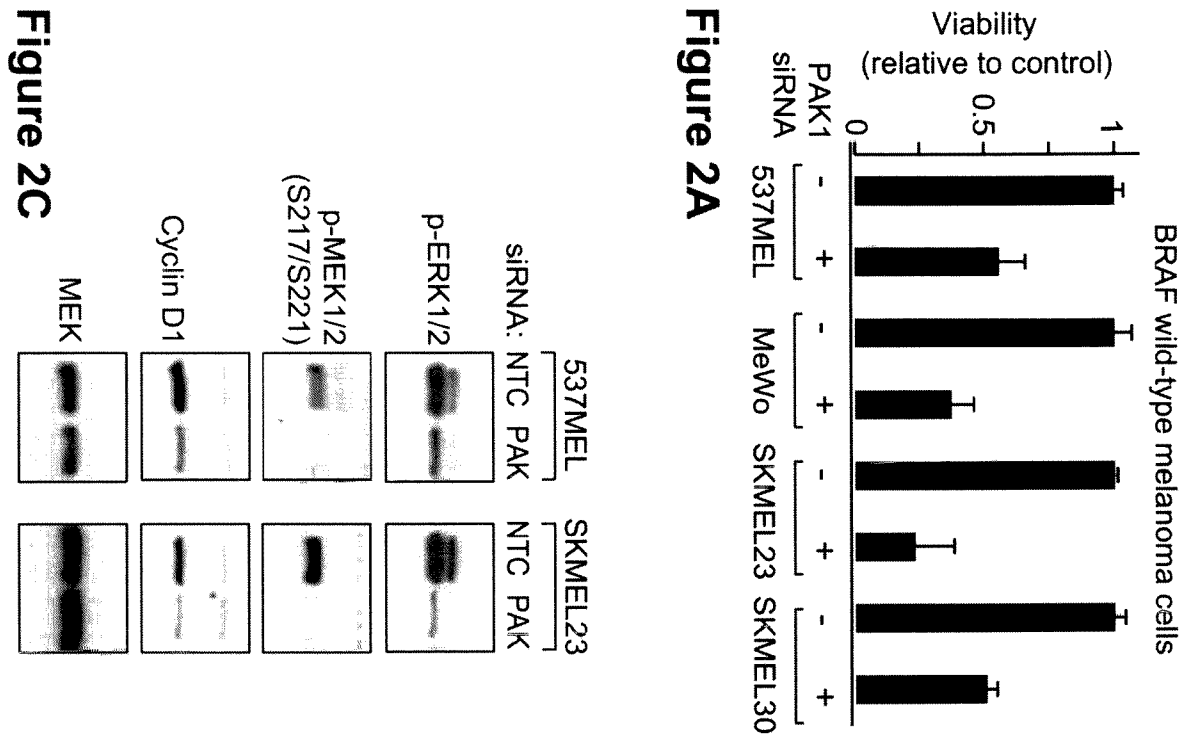


Figure 2A
Viability
(relative to control)
0
0.5
1
PAK1
siRNA
-
+
-
+
-
+
-
+
537MEL
MeWo
SKMEL23
SKMEL30
BRAF wild-type melanoma cells

Figure 2C
MEK
Cyclin D1
p-MEK1/2
(S217/S221)
p-ERK1/2
siRNA: NTC PAK NTC PAK
537MEL
SKMEL23

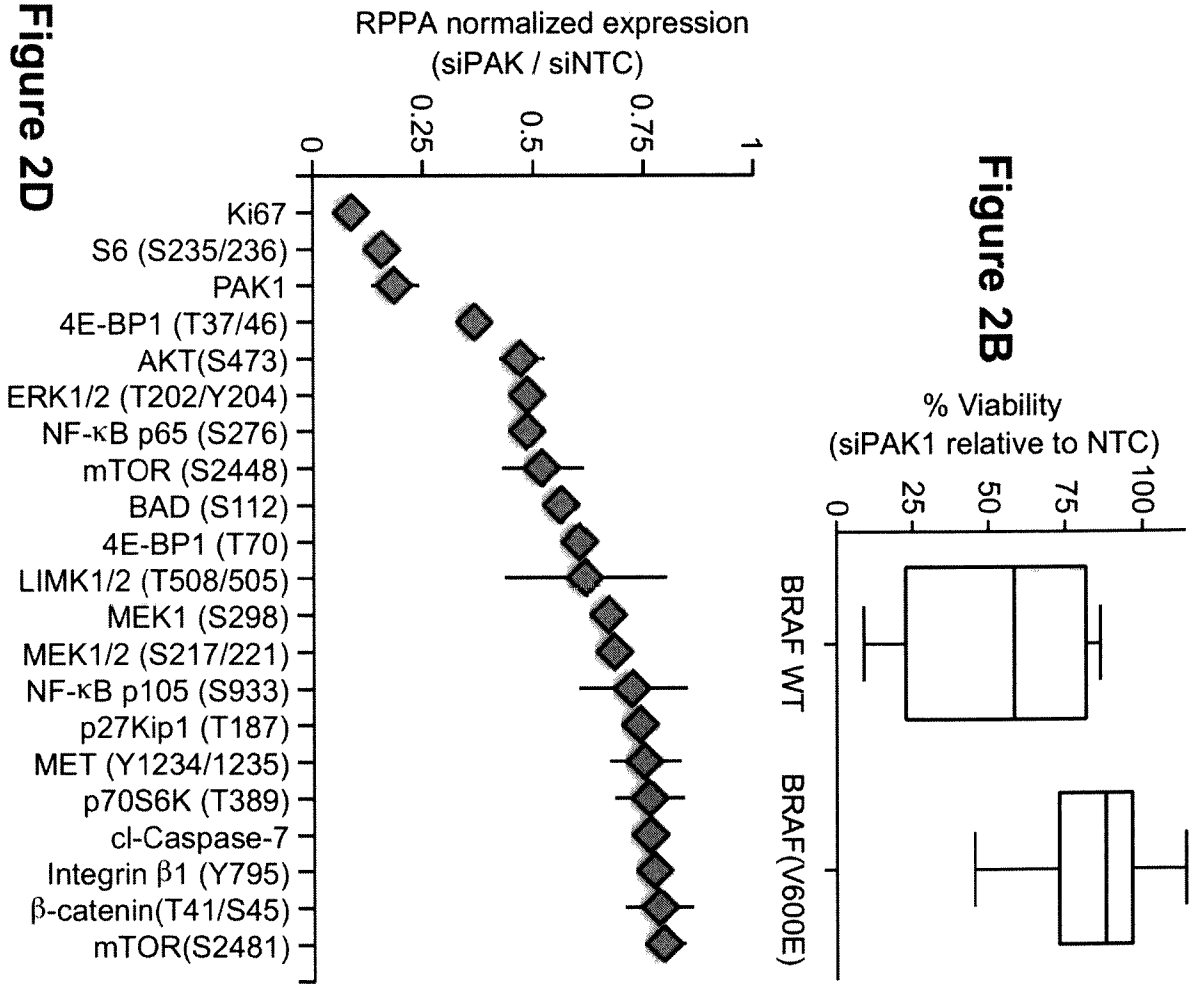


Figure 2D
RPPA normalized expression
(siPAK / siNTC)
0
0.25
0.5
0.75
1
Ki67
S6 (S235/236)
PAK1
4E-BP1 (T37/46)
AKT(S473)
ERK1/2 (T202/Y204)
NF-κB p65 (S276)
mTOR (S2448)
BAD (S112)
4E-BP1 (T70)
LIMK1/2 (T508/505)
MEK1 (S298)
MEK1/2 (S217/221)
NF-κB p105 (S933)
p27Kip1 (T187)
MET (Y1234/1235)
p70S6K (T389)
cl-Caspase-7
Integrin β1 (Y795)
β-catenin(T41/S45)
mTOR(S2481)

Figure 2B
% Viability
(siPAK1 relative to NTC)
0
25
50
75
100
BRAF WT
BRAF(V600E)

Figure 3

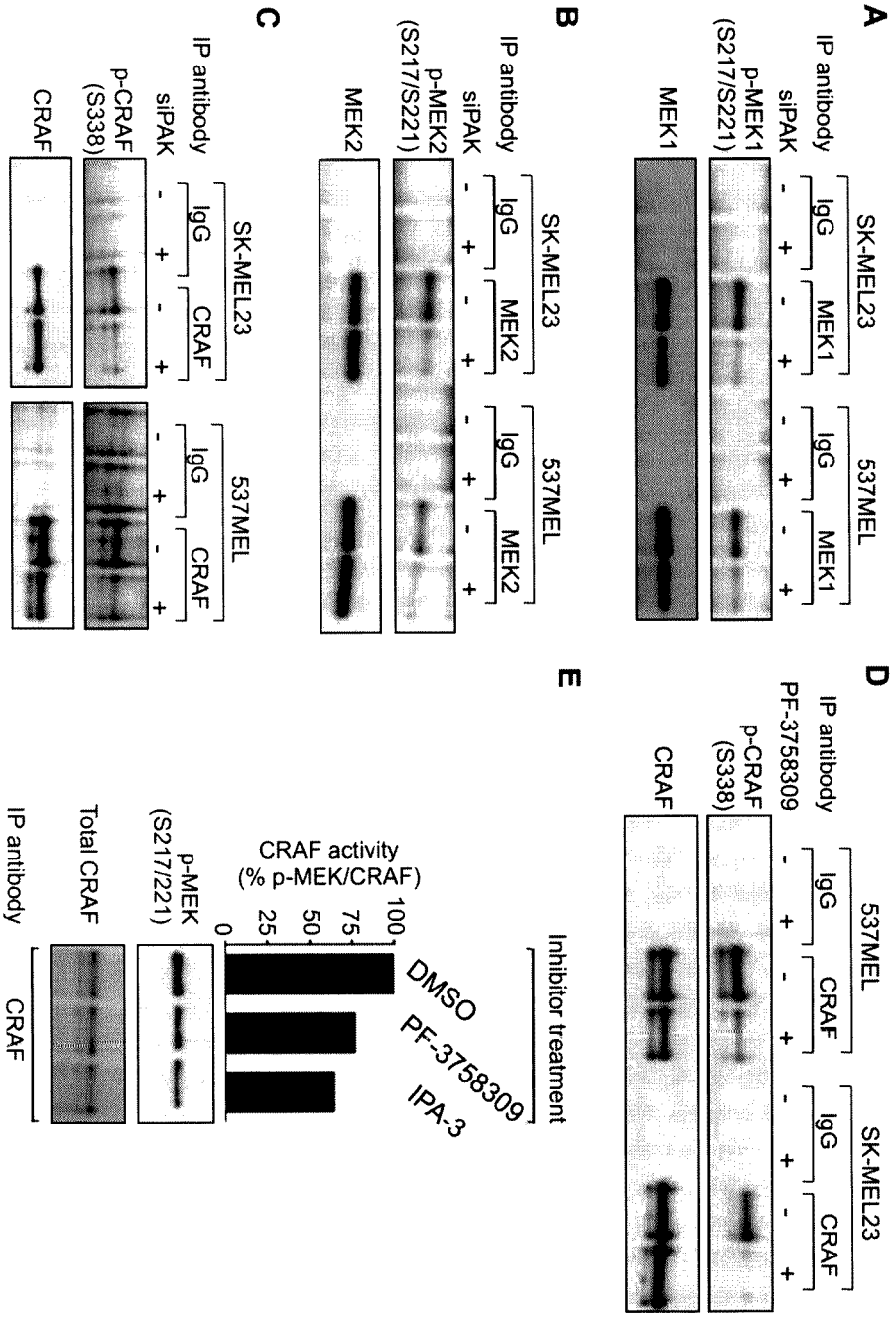

A
SK-MEL23
537MEL
IP antibody
IgG
MEK1
siPAK
p-MEK1 (S217/S221)
MEK1
B
SK-MEL23
537MEL
IP antibody
IgG
MEK2
siPAK
p-MEK2 (S217/S221)
MEK2
C
SK-MEL23
537MEL
IP antibody
IgG
CRAF
siPAK
p-CRAF (S338)
CRAF
D
537MEL
SK-MEL23
IP antibody
IgG
CRAF
PF-3758309
p-CRAF (S338)
CRAF
E
Inhibitor treatment
DMSO
PF-3758309
IPA-3
CRAF activity (% p-MEK/CRAF)
0
25
50
75
100
p-MEK (S217/221)
Total CRAF
IP antibody
CRAF

Figure 4

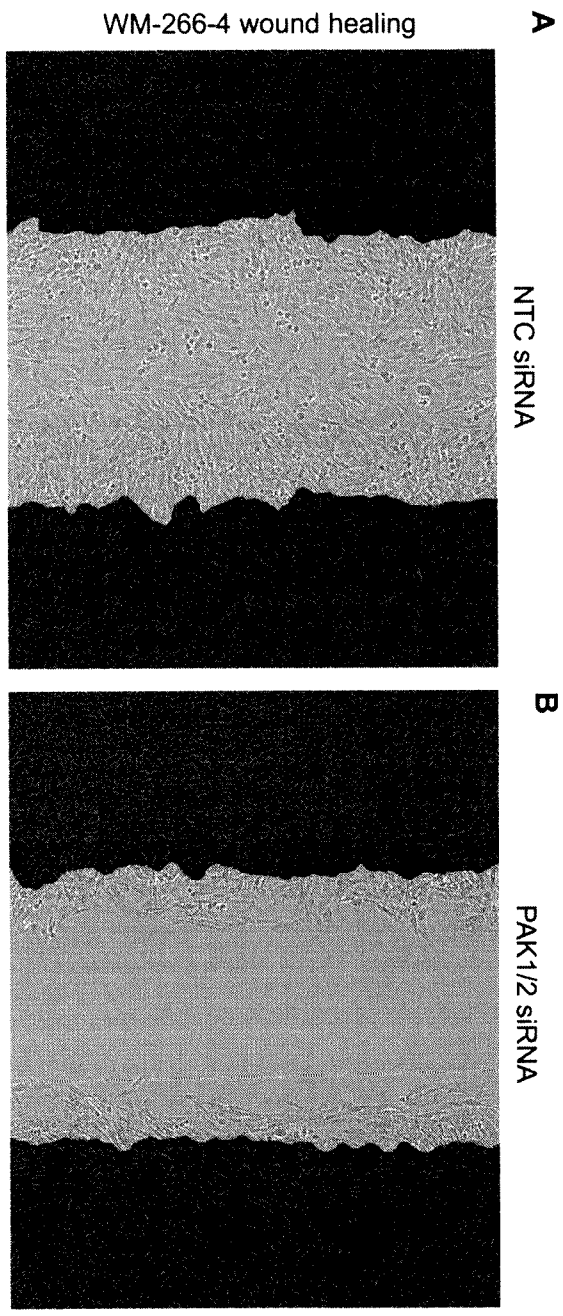


Figure 5

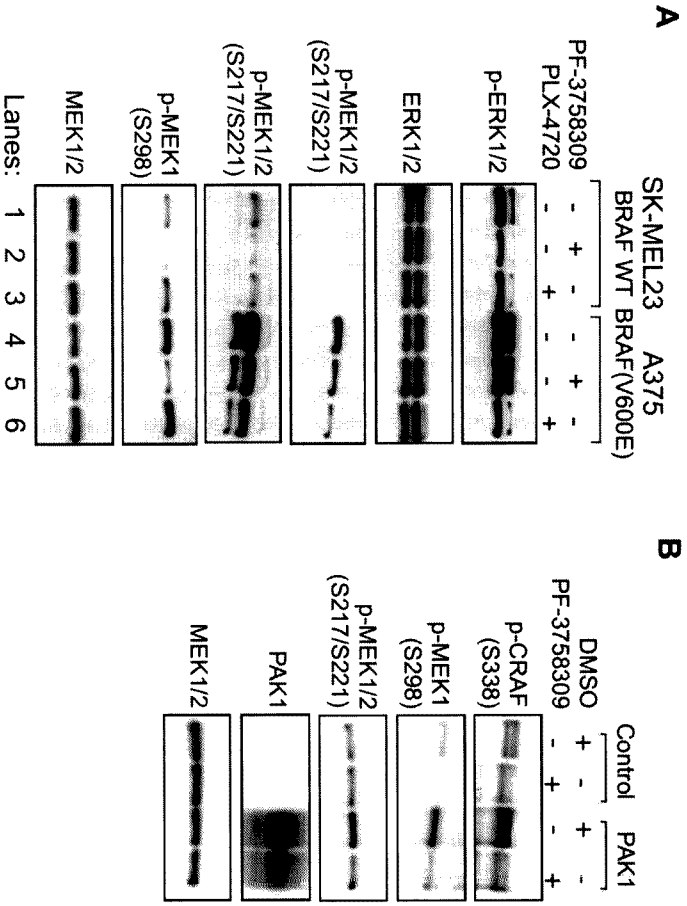
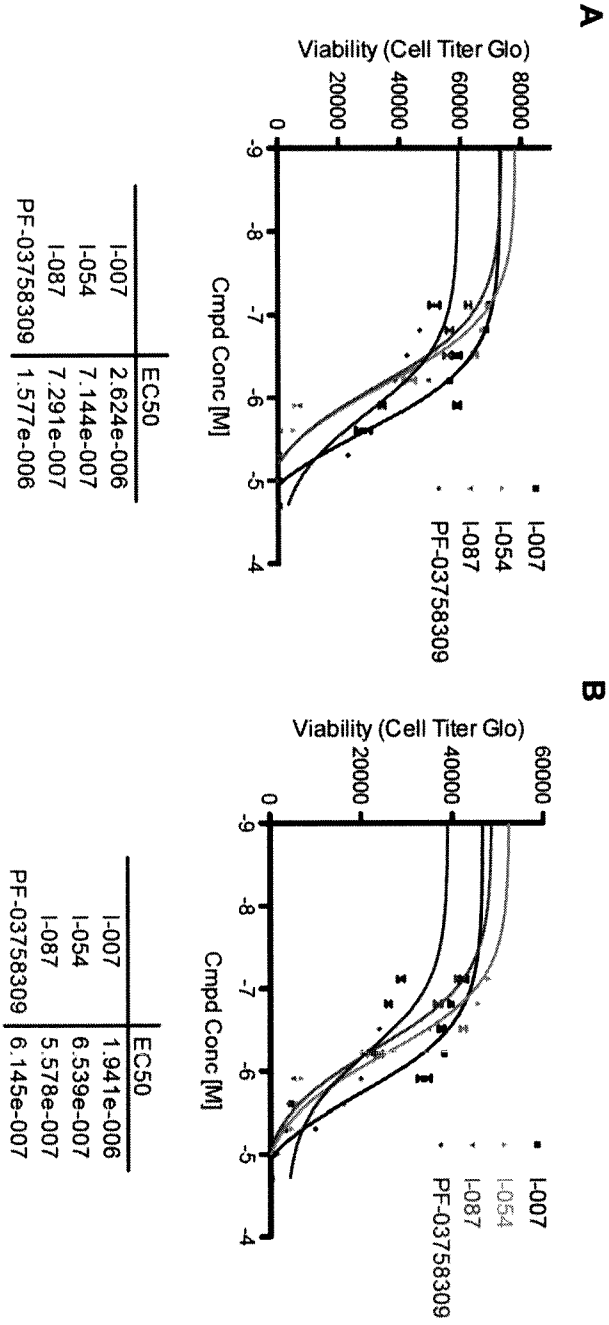

A
SK-MEL23 A375
BRAF WT BRAF(V600E)
PF-3758309 - + - - + -
PLX-4720 - - + - - +
p-ERK1/2
ERK1/2
p-MEK1/2 (S217/S221)
p-MEK1/2 (S217/S221)
p-MEK1 (S298)
MEK1/2
Lanes: 1 2 3 4 5 6
B
Control PAK1
DMSO + - + -
PF-3758309 - + - +
p-CRAF (S338)
p-MEK1 (S298)
p-MEK1/2 (S217/S221)
PAK1
MEK1/2

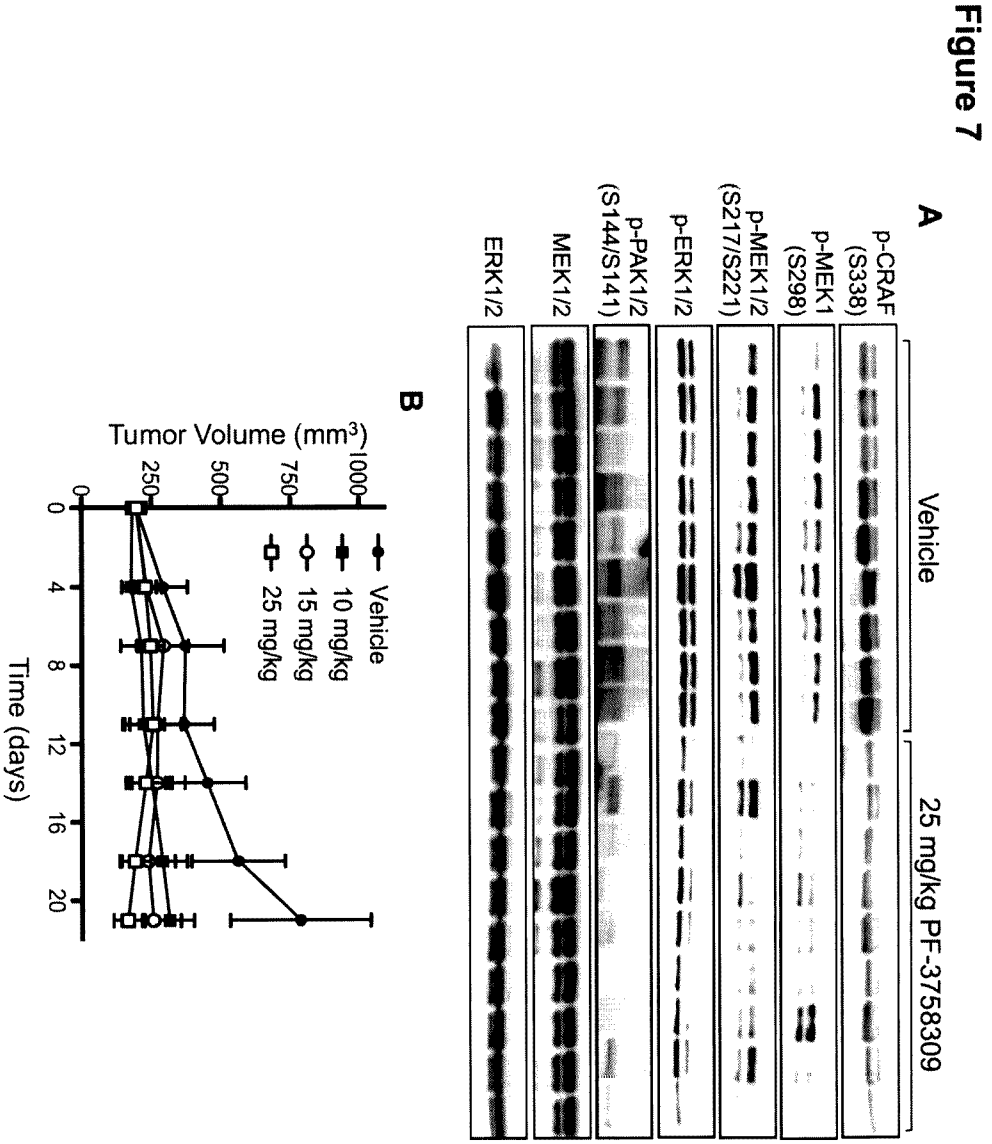
Figure 6



	EC50
I-007	2.624e-006
I-054	7.144e-007
I-087	7.291e-007
PF-03758309	1.577e-006

	EC50
I-007	1.941e-006
I-054	6.539e-007
I-087	5.578e-007
PF-03758309	6.145e-007

Figure 7


A
Vehicle
25 mg/kg PF-3758309
p-CRAF (S338)
p-MEK1 (S298)
p-MEK1/2 (S217/S221)
p-ERK1/2
p-PAK1/2 (S144/S141)
MEK1/2
ERK1/2
B
Tumor Volume (mm3)
0
250
500
750
1000
Time (days)
0
4
8
12
16
20
Vehicle
10 mg/kg
15 mg/kg
25 mg/kg

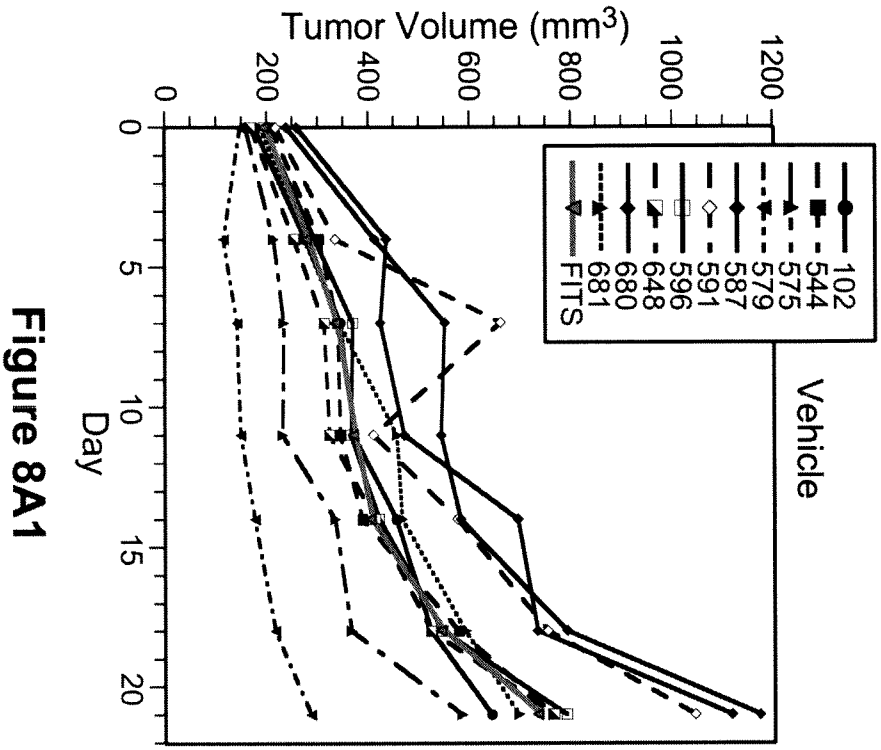


Figure 8A1

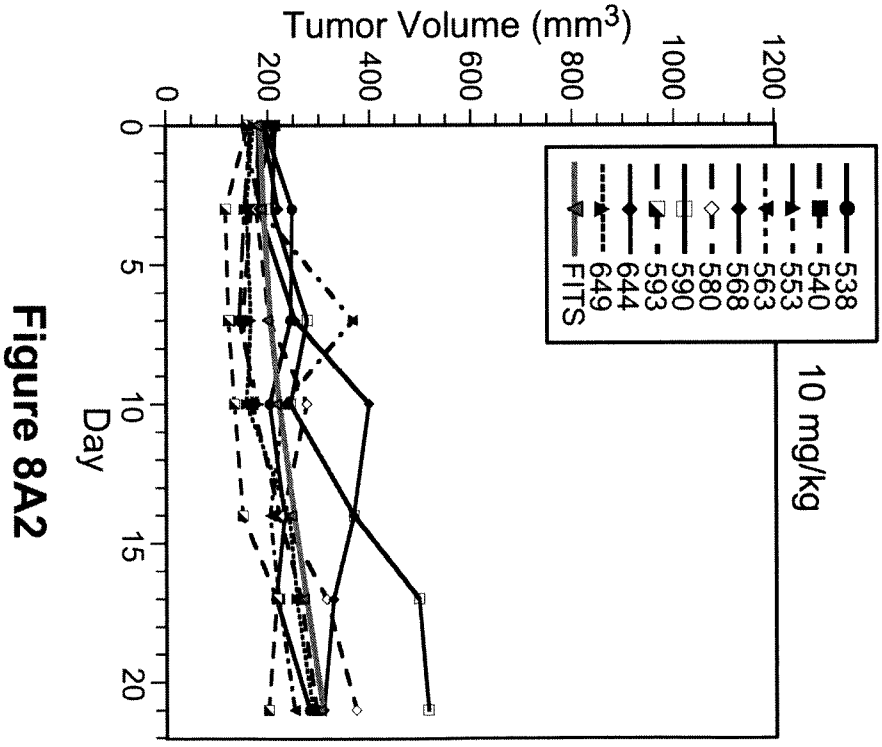


Figure 8A2

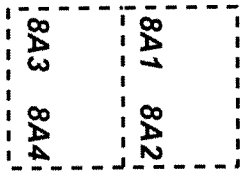


Figure 8A

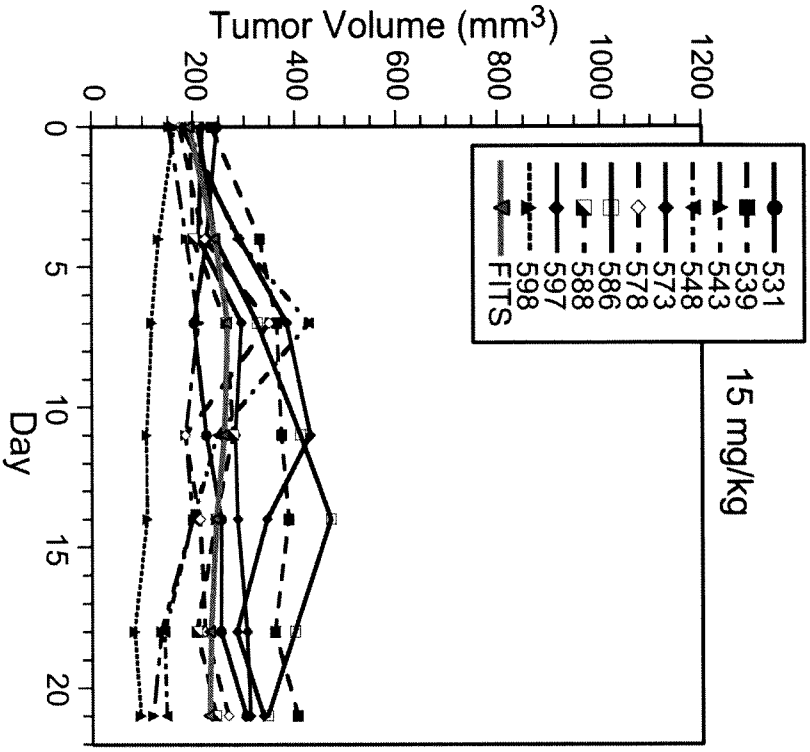


Figure 8A3

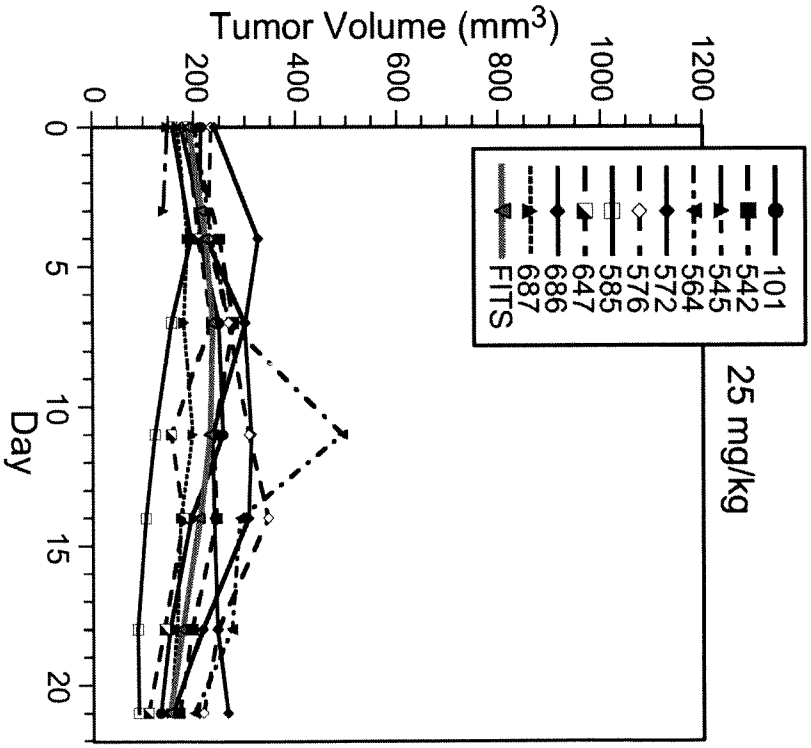


Figure 8A4

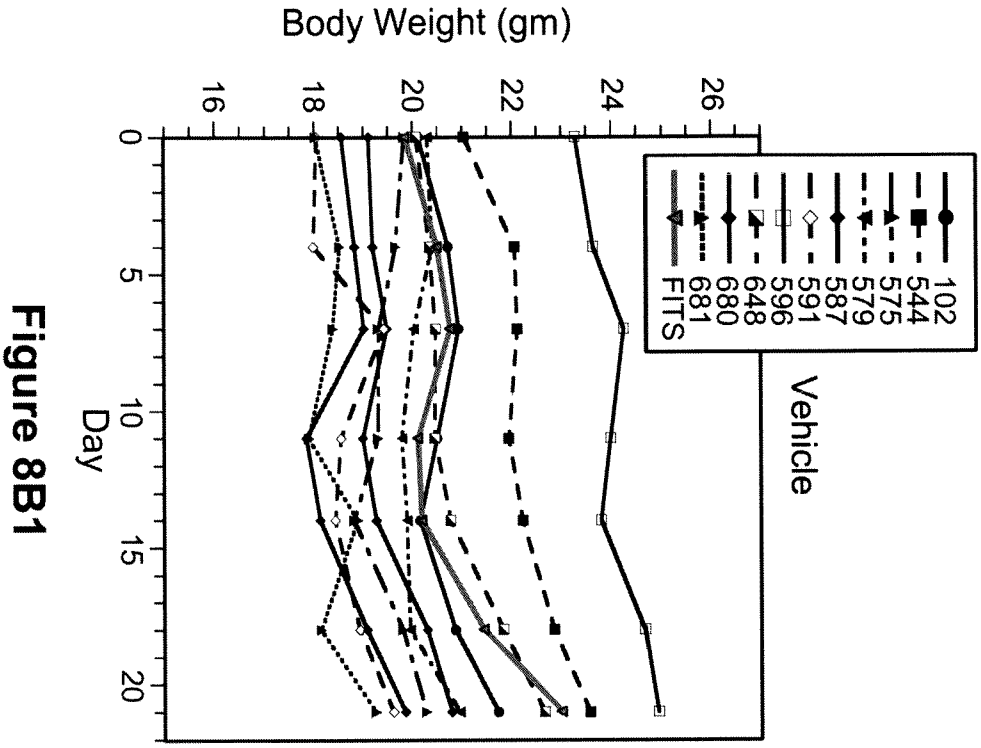


Figure 8B1

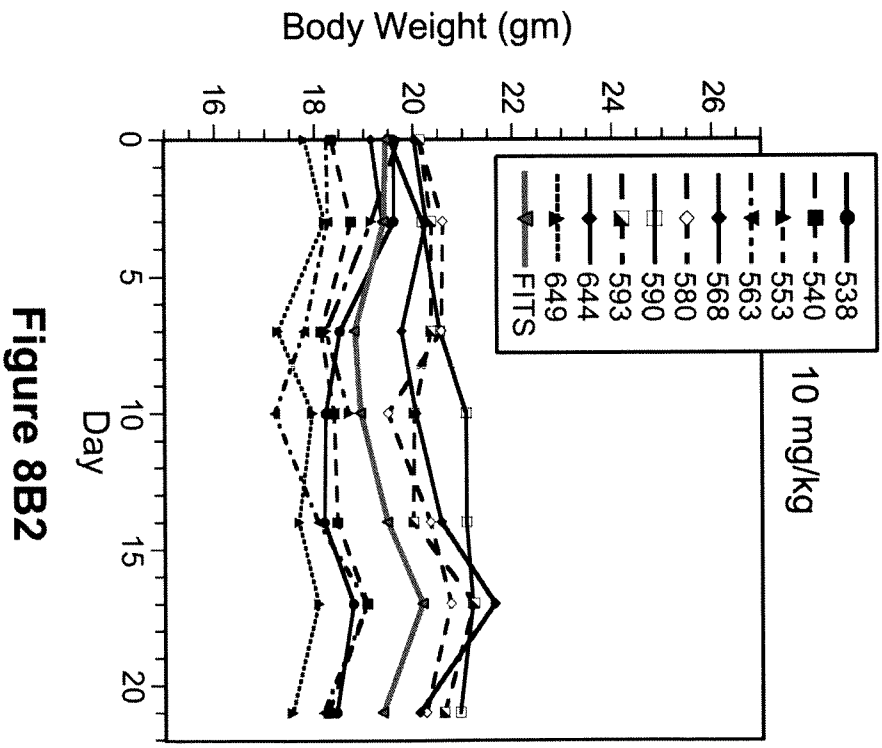


Figure 8B2

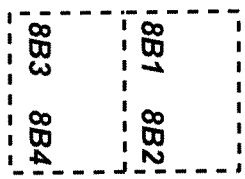


Figure 8B

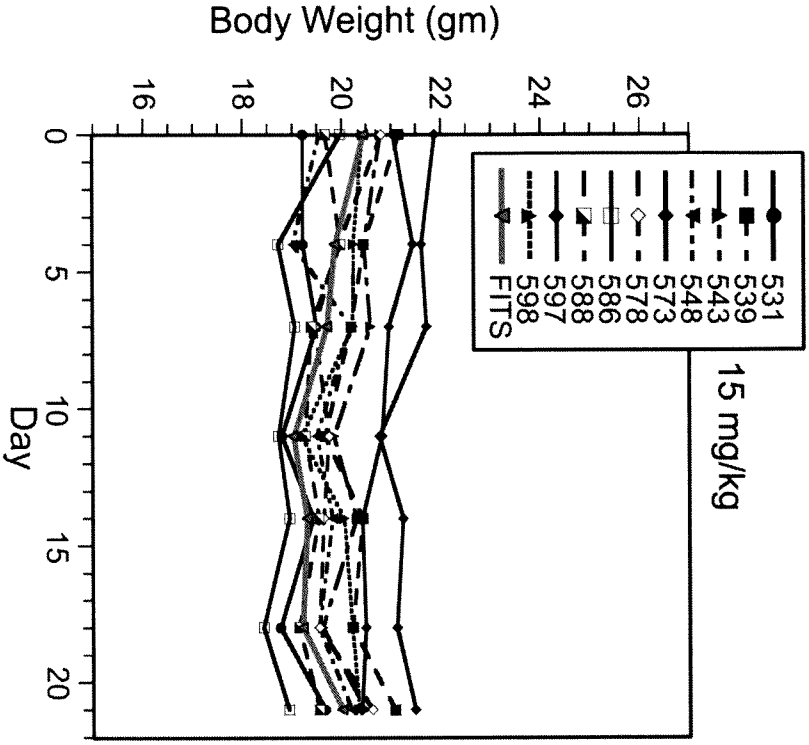


Figure 8B3

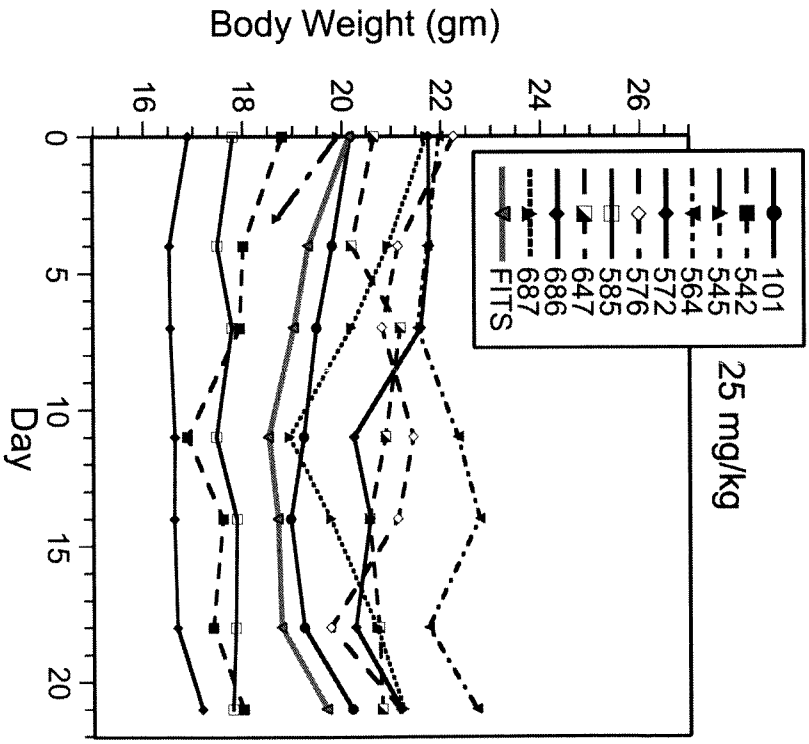


Figure 8B4

Figure 9

A

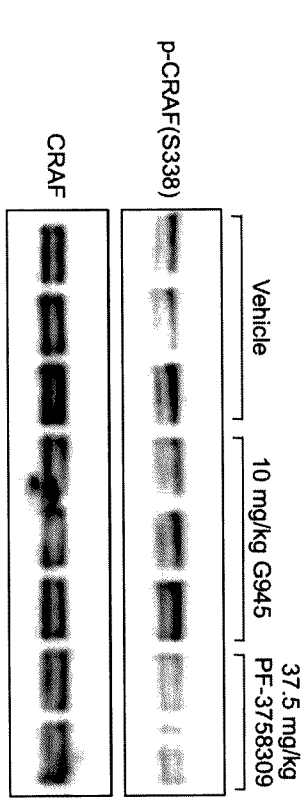
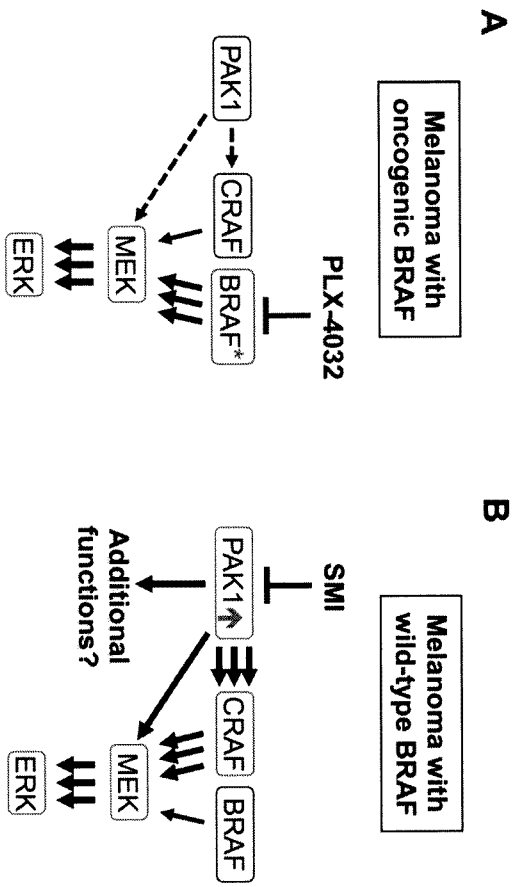


Figure 10


A
Melanoma with oncogenic BRAF
PLX-4032
PAK1
CRAF
BRAF*
MEK
ERK
B
Melanoma with wild-type BRAF
SMI
PAK1
CRAF
BRAF
MEK
ERK
Additional functions?

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2013/055085

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K31/41 A61P35/00
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2009/275570 A1 (DALY KEVIN [US] ET AL) 5 November 2009 (2009-11-05) paragraphs [0014], [0225]	1-33
X	WO 2006/072831 A1 (PFIZER [US]; BOUZIDA DJAMAL [US]; DONG LIMING [US]; GUO CHUANGXING [US) 13 July 2006 (2006-07-13) page 18, line 28	1-33
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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 July 2013	18/07/2013

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

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	MURRAY ET AL: "Small-molecule p21-activated kinase inhibitor PF-3758309 is a potent inhibitor of oncogenic signaling and tumor growth.", PNAS, vol. 107, no. 20, 18 May 2010 (2010-05-18) , - 18 May 2010 (2010-05-18), pages 9446-9451, XP002700325, abstract	1-33
X	WO 2011/156646 A2 (AFRAXIS INC [US]; CAMPBELL DAVID [US]; DURON SERGIO G [US]; VOLLRATH B) 15 December 2011 (2011-12-15) page 1, line 27 - paragraph 223	1-33

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Information on patent family members

International application No
PCT/EP2013/055085

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