Inhibitory Effects of a New 1H-Pyrrolo[3,2-c]pyridine Derivative, KIST101029, on Activator Protein-1 Activity and Neoplastic Cell Transformation Induced by Insulin-Like Growth Factor-1

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Diarylureas and diarylamides derivatives are reported to have antitumor activity. Encouraged by the interesting antiproliferative activity of diarylurea and diarylamine derivatives, we synthesized a new series of diarylureas and diarylamides containing pyrrolo[3,2-c]pyridine scaffold. In this study, we demonstrate that a N-(4-benzamido-1H-pyrrolo[3,2-c]pyridin-1-yl)morpholin-4-yl)[3,2-c]pyridin-1-yl)phenyl)-4-morpholino-3-((trifluoromethyl)benzamide, KIST101029, inhibits neoplastic cell transformation induced by insulin-like growth factor 1 (IGF-1) in mouse epidermal JB6 Cl41 cells. The KIST101029 compound inhibited mitogen-activated protein kinases/extracellular signal-regulated kinase kinases (MEKs), c-jun N-terminal kinases (JNKs), and mammalian target of rapamycin (mTOR) signaling pathways induced by IGF-1 in JB6 Cl41 cells, resulting in the inhibition of c-fos and c-jun transcriptional activity. In addition, the KIST101029 inhibited the associated activator protein-1 (AP-1) transactivation activity and cell transformation induced by IGF-1 in JB6 Cl41 cells. Consistent with these observations, in vivo chorioallantoic membrane assay also showed that the KIST101029 inhibited IGF-1-induced tumorigenicity of JB6 Cl41 cells. Importantly, KIST101029 suppressed the colony formation of A375 cells in soft agar. Taken together, these results indicate that a KIST101029 might exert chemopreventive effects through the inhibition of phosphorylation of MAPK and mTOR signaling pathway.

Key words KIST101029; insulin-like growth factor 1; activator protein-1; cell transformation; c-Fos; c-Jun

The authors declare no conflict of interest.

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MATERIALS AND METHODS

Reagents and Antibodies  Eagle’s minimal essential medium (MEM), Dulbecco’s modified Eagle’s medium (DMEM), l-glutamine, gentamicin, insulin-like growth factor 1 (IGF-1), and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA, U.S.A.). 3-[4,5-Dimethylthiazol-2-thiazoyl]-2,5-diphenyltetrazolium bromide (MTT) was from Sigma-Aldrich (St. Louis, MO, U.S.A.). Cell Proliferation ELISA, BrdU (colorimetric) kit was from Roche Applied Science (Indianapolis, IN, U.S.A.). Polyvinylidene difluoride (PVDF) membrane was from Millipore (Bedford, MA, U.S.A.). Antibodies against phospho-MEK1/2, -ERK1/2, -p90RSK, -JNK, -c-Jun (Ser63), MEK1/2, ERK1/2, and JNK1/2 were purchased from Cell Signaling Tech. Inc. (Beverly, MA, U.S.A.); antibodies against p90RSK, c-Jun, c-Fos, goat anti-mouse immunoglobulin G (IgG) horse-radish peroxidase (HRP), and goat anti-rabbit IgG HRP were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The jetPEI cationic polymer transfection reagent was from Polyplus-transfection (New York, NY, U.S.A.). The Dual-luciferase reporter assay kit was purchased from Promega (Madison, WI, U.S.A.).

Cell Culture and Transfection  JB6 Cl41 mouse epidermal cells were cultured in supplemented with 5% FBS, at 37°C in humidified air containing 5% CO₂, A375 human melanoma cells were cultured in DMEM supplemented with 10% FBS, at 37°C. The DNA transfection of the cells was performed using a jetPEI cationic polymer transfection reagent (Polyplus-transfection). Dimethyl-sulfoxide (DMSO) was used as a vehicle to dissolve the compound and a final concentration of 0.1% DMSO (v/v) was used for each treatment.

3-[4,5-Dimethylthiazol-2-thiazoyl]-2,5-diphenyltetrazolium Bromide (MTT) Assay  The MTT assay was performed to check cell viability. In brief, cells (1×10⁴ cells/mL) were seeded in 96-well plates with 100 μL of cell suspension in each well. After culturing for 24 h, cells were treated with KIST101029. The cells were then treated with 5 μg/mL MTT solution (10 μL/well) and incubated for 3 h, after which the purple formazan formed by the live cells was dissolved in 0.1% DMSO in isopropanol (100 μL/well). The absorbance was measured at 570 nm on a TriStar LB 941 (Berthold Techn. GmbHand Co., KG, Germany). Polyvinylidene difluoride (PVDF) membrane was from Millipore (Bedford, MA, U.S.A.).

Cell Proliferation Assay (Bromodeoxyuridine (BrdU) Incorporation)  JB6 Cl41 cells were seeded (1×10⁵ cells/well) in 96-well plates in 100 μL of 5% FBS-MEM. After 24 h, the cells were either treated or not treated with 50 ng/mL IGF-1 together with KIST101029 for 48 h, labeled with 10 μL/well BrdU labeling solution, and then reincubated for additional 4 h at 37°C in a 5% CO₂ atmosphere. After the cells migrated to the bottom of the well, the media was removed, and the cells were fixed with 100 μL of 4% paraformaldehyde for 30 min, followed by washing with 100 μL of 1× PBS. The cells were then incubated with BrdU detection buffer (100 μL) for 30 min. Anti-BrdU antibody was added, and the cells were incubated at RT for 30 min. Anti-BrdU antibody was followed by incubation with horseradish peroxidase-conjugated secondary antibody for 30 min, followed by incubation with 100 μL of 3,3-diaminobenzidine (DAB) for 5 min. The slides were stained with hematoxylin, washed with distilled water, and dried by flaming.

Immunoblot Analysis  The cells were disrupted in lysis buffer [50 mM Tris (pH 7.5), 5 mM ethylenediaminetetraacetic acid (EDTA), 150 mM NaCl, 1 mM dithiothreitol, 0.01% Nonidet P-40, 0.2 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail]. The proteins were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. The membranes were blocked and hybridized with the appropriate primary antibody overnight at 4°C. The protein bands were visualized using chemiluminescence detection kit (Amersham HRP Chemiluminescent Substrates, Amersham Biosciences, Piscataway, NJ, U.S.A.) after hybridization with the HRP-conjugated secondary antibody from rabbits or mice. The LAS4000-mini (FUJIFILM, Tokyo, Japan) was used for chemiluminescence detection.

Reporter Gene Assays  The reporter gene assay for firefly luciferase activity was performed using lysates from AP-1-, c-jun-, or c-fos-luc-transfected JB6 Cl41 cells. In addition, the reporter gene vector pRL-TK-luciferase plasmid (Promega) was co-transfected into each cell line and the renilla luciferase activity generated by this vector was used to normalize the results for transfection efficiency. Cell lysates were prepared by first washing the transfected JB6 Cl41 cells once with phosphate-buffered saline (PBS) at RT. After removing the PBS completely, passive lysis buffer (PLB, Promega) was added, and then cells were incubated at RT for 1 h with gentle shaking. The supernatant fraction was used to measure firefly and renilla luciferase activities. Cell lysates (50 μL each) were mixed with 50 μL of luciferase assay II reagent (Promega), and firefly luciferase light emission was measured by TriStar LB 941 (Berthold Tech. GmbHand Co., KG, Germany). Subsequently, 50 μL of renilla luciferase substrate was added in order to normalize the firefly luciferase data. The c-fos-luc promoter (pFos-WT GL3) and c-jun-luc promoter (JC6GL3) were kindly provided by Dr. Ron Prywes (Columbia University, New York, NY, U.S.A.). The AP-1 luciferase reporter plasmid (−73/+63 collagenase-luciferase) was kindly provided by Dr. Dong Zigang (Hormel Institute, University of Minnesota, Austin, MN, U.S.A.).

Architectural-Independent Cell Transformation Assay (Soft Agar Assay)  The effect of KIST101029 in the IGF-1 induced cell transformation was investigated in JB6 Cl41 cells. In brief, 8×10⁴ cells were exposed to IGF-1 (50 ng/mL) with or without KIST101029 in 1 mL of 0.3% basal medium Eagle (BME) agar containing 10% FBS, 2 mM l-glutamine, and 25 μg/mL gentamicin. The cultures were maintained at 37°C, in a 5% CO₂ incubator for 2 weeks, and the cell colonies were scored using an Axiovert 200 fluorescence microscope and Axio Vision software (Carl Zeiss Inc., Thornwood, NY, U.S.A.).

Chorioallantoic Membrane (CAM) Assay  Briefly, fertilized chicken eggs were transferred to an egg incubator and allowed to grow for 10d. After this, JB6 Cl41 cells (2×10⁵) together with IGF-1 or KIST101029 were placed on the exposed CAM, the eggs were incubated in a humidified incubator at 37°C for 7 d. After 7 d, images were digitally recorded at ×15 magnifications with an SZ-61 zoom stereomicroscope (Olympus, Center Valley, PA, U.S.A.). Tumor areas were analyzed with Image J digital imaging software (download from the NIH website).

Statistical Analysis  Statistical calculations were carried out with Prism 4 for Macintosh (GraphPad Software Inc., La Jolla, CA, U.S.A.). Results are expressed as the mean± standard error of triplicate measurements of three independent experiments. Student’s t-test was used for statistical analyses;
RESULTS

**KIST101029 Inhibits IGF-1-Induced Cell Proliferation of JB6 Cl41 Cells via Its Direct Interaction with Raf-1**

Sorafenib (BAY 43–9006) is a synthetic molecule that can be broadly defined as a bi-aryl urea, which was originally identified through inhibition of Raf-1 kinase biochemical and cellular mechanistic assays. Therefore, we designed and synthesized a new sorafenib analog, KIST101029, possessing 1H-pyrrolo[3,2-c]pyridine scaffold (Fig. 1A), and evaluated the effects of its derivative, KIST101029, on the cell viability and cell proliferation of JB6 Cl41 mouse epidermal cells by MTT assay and BrdU incorporation assay, respectively. KIST101029 at lower concentrations from 0.01 µM to 0.5 µM did not affect cell viability at 96h in JB6 Cl41 cells, whereas significantly decreased the cell viability of JB6 Cl41 cells at higher concentrations from 1 µM to 10 µM, dose-dependently (Fig. 1B).

To also examine the effect of KIST101029 on cell proliferation induced by insulin like growth factor-1 (IGF-1), JB6 Cl41 cells were treated with IGF-1 in an absence or presence of KIST101029. The result showed that KIST101029 significantly reduced the IGF-1-induced cell proliferation after treatment of KIST101029, at concentrations from 0.1 µM to 0.5 µM, dose-dependently (Fig. 1C). To determine whether the inhibition of IGF-1-induced cell proliferation induced by KIST101029 was caused by a directed interaction with Raf-1, we next performed pull-down assay. We transfected Myc-tagged Raf-1 in HEK 293 cells. The cells lysates were incubated with KIST101029-biotin and then blotted with the anti-Myc antibody. The results showed that the exogenously expressed Raf-1 was found in the KIST101029-biotin-immunoprecipitated-sepharose beads, but not in the sepharose beads (Fig. 1D), suggesting that KIST101029 directly bind with Raf-1 in vitro.

**KIST101029 Suppresses Signaling Cascade of MEKs/ERKs, JNKs/c-Jun, and mTOR/p70S6K Induced by IGF-1 in JB6 Cl41 Cells**

IGF-1 exerts both proliferative and anti-apoptotic effects via activating two distinct signaling pathways, mitogen activated protein kinase (MAPK) and phosphoinositol 3-kinase (PI3K). Therefore, we hypothesized that KIST101029 might inhibit these signaling pathway leading to anti-proliferative effects on JB6 Cl41 cells. To examine the effect of KIST101029 on phosphorylation of MAPKs induced by IGF-1, JB6 Cl41 cells were treated with IGF-1 in the absence or presence of various concentrations of KIST101029 and immunoblotted with anti-phospho antibodies against MEK1/2, ERK1/2, and p90RSK. The results showed that KIST101029 suppressed IGF-1-induced phosphorylation of MEK1/2, ERK1/2, and p90RSK in a dose dependent manner (Fig. 2A). Constitutively active ERK signaling pathway upregulates JNK and activates c-Jun oncogene and its downstream targets including RACK1 and cyclin D1. We next
examined whether KIST101029 downregulates JNKs pathways induced by IGF-1. The results showed that KIST101029 dose dependently inhibited IGF-1-induced phosphorylation of JNK1/2 as well as c-Jun (Fig. 2B). We next investigated whether KIST101029 inhibits AKT/mTOR/p70S6K signaling. As shown in Fig. 3C, KIST101029 did not inhibit the phosphorylation of AKT induced by IGF-1 (Fig. 2C). However, KIST101029 inhibited IGF-1-induced phosphorylation of p70S6K via activation of Raptor (Fig. 2D). These overall data suggest that KIST101029 has inhibitory effects on the signaling pathway of MAPK as well as mTOR.

KIST101029 Inhibits IGF-1-Induced c-fos, c-jun, and AP-1 Activation c-Jun and c-Fos are nuclear proto-oncoproteins whose expression is stimulated by a variety of growth-promoting agents and activated oncogenes. AP-1 is mainly composed with Jun (c-Jun, JunB, and JunD) and Fos (c-Fos, FosB, Fra-1, and Fra-2) and induced by several external stimuli, such as EGF, which increase MAPK activity. To investigate whether KIST101029 suppresses the IGF-1-induced c-fos or c-jun transcriptional activity, we took advantage of the availability of the reporter plasmid carrying the luc gene under the control of the murine c-fos or c-jun promoter. Twenty-four hours after transfection with these reporters in JB6 Cl41 cells, cells were starved for another 12h by incubating in serum-deprived MEM at 37°C in a 5% CO2 atmosphere. At 12h of starvation, cells were pretreated or not treated with KIST101029 for 12h, and then treated or not treated with IGF-1 (50 ng/mL) for additional 12h. The results showed that IGF-1-induced c-fos or c-jun transcriptional activity was significantly suppressed by KIST101029 (Figs. 3A, 3B). To determine whether the antitumorigenic effect of KIST101029 is responsible for the inhibition of AP-1 activation response to IGF-1, we next cotransfected the AP-1 luciferase reporter plasmid and pRL-TK-luciferase plasmid into cells. The IGF-1-induced AP-1 activity was significantly inhibited by KIST101029 similar to that observed for c-jun or c-fos activity (Fig. 3C). These data indicated that the suppression of the c-jun, c-fos, and AP-1 promoters by IGF-1 is one of the mechanisms explaining the anti-proliferative effect of KIST101029.

KIST101029 Suppressed Cell Transformation of JB6 Cl41 Cells Induced by IGF-1 AP-1 is major transcription factor involved in neoplastic cell transformation of JB6 Cl41 cells induced by various tumor promoters. AP-1 is major transcription factor involved in neoplastic cell transformation of JB6 Cl41 cells induced by various tumor promoters. We next examined the effect of KIST101029 on IGF-1-induced cell transformation. JB6 Cl41 cells were treated separately with 50 ng/mL IGF-1 in the absence or presence of various concentrations of KIST101029 in a soft agar matrix and incubated at 37°C in a 5% CO2 incubator for 14d. Our results showed that KIST101029 significantly inhibited the IGF-1-induced cell transformation of JB6 cells in a dose-dependent manner (Figs. 4A, 4B). These data strongly indicated that KIST101029 plays an inhibitory role of neoplastic cell transformation in epidermal mouse skin cells stimulated with IGF-1.

KIST101029 Inhibited the IGF-1-Induced Tumor Progression of JB6 Cl41 Cells in Vivo The in vivo CAM assay with JB6 Cl41 cells was subsequently performed in the absence or presence of IGF-1 with/without KIST101029. The representative images showed that IGF-1 induced the tumor formation in CAM as evident from tumor area. Interestingly, we observed that treatment of KIST101029 significantly inhibited the tumor formation induced by IGF-1 (Fig. 5A). For statistical evaluation, images of sections were digitally recorded and tumor areas were analyzed (Fig. 5B). Similar to ours in vitro soft agar assay, these results indicated that the KIST101029 treatment significantly inhibited IGF-1-induced tumor progression of JB6 Cl41 cells in CAM of chicken embryos compared with control group.

KIST101029 Suppressed Tumorigenicity of A375 Human...
Increased signaling through the RAF/MEK/ERK pathway, as a result of autocrine stimulation by basic fibroblast growth factor and hepatocyte growth factor, is implicated in melanocytic tumorigenesis. To confirm that inhibition of the Raf-1/MEK/ERK signaling pathway by KIST101029 leads to the suppression of tumorigenesis, we first examined whether KIST101029 as well as sorafenib, a RAF inhibitor, could inhibit cell viability of A375 human melanoma cells. Results indicate that KIST101029 and sorafenib inhibited cell growth with an IC\(_{50}\) of 0.96 µM and 5.12 µM in A375 cells, respectively (Fig. 6A). These findings further supported the evidence showing that KIST101029 and sorafenib inhibited the phosphorylation of ERK1/2 as well as MEK1/2 (Fig. 6B). Next, we further examined the inhibitory effect of KIST101029 and sorafenib on the colony formation of A375 cells using soft agar assay. Representative images demonstrate a profound reduction of the colony formation mediated by the treatment of KIST101029 and sorafenib compared with untreated control group (Fig. 6C). For statistical evaluation, the numbers of colonies were digitally recorded and counted. The results showed that KIST101029 and, to a lesser extent, sorafenib significantly inhibited colony formation of A375 cells (Fig. 6D).

### DISCUSSION

There is a significant unmet medical need for the development of effective therapies that can stabilize or slow the progression of solid tumors. The bi-aryl urea sorafenib is an oral multikinase inhibitor that inhibits cell surface tyrosine kinase receptors, such as vascular endothelial growth factor receptors (VEGFR) and platelet-derived growth factor receptor (PDGF), and downstream intracellular serine/threonine kinases, such as Raf-1, wild-type B-Raf and mutant B-Raf, which are involved in tumor cell proliferation and tumor angiogenesis. Currently, sorafenib is the only effective drug for advanced hepatocellular carcinoma (HCC). However, accumulating clinical evidence has suggested that this therapeutic agent rarely causes tumor shrinkage. The reason for the reduced effect of sorafenib in reducing the size of the tumors is unknown. Basic research studies have suggested that the blockade of Raf signaling might result in unexpected molecular events, such as the reciprocal activation of the Akt oncoprotein. In the present study, we identified KIST101029, a new diarylurea and diarylamide derivative, as novel RAF kinase inhibitor that targets tumor cell proliferation and tumorigenesis. KIST101029 exhibited a strong inhibitory effect on the signaling pathway of MAPK as well as mTOR induced...
by IGF-1. Also, KIST101029 blocked IGF-1-induced c-fos, c-jun, and AP-1 activation, and thereby effectively suppressed neoplastic transformation.

Ras activation is the first step in activation of the MAPK cascade. Following oncogenic Ras activation, Raf kinase family composed of three members, A-RAF, B-RAF, and Raf-1 (also termed c-Raf), is recruited to the cell membrane through binding to Ras to cause tumorigenic transformation.30) Raf proteins directly activate MEK1/2 via phosphorylation of multiple serine residues. MEK1 and MEK2 are themselves tyrosine and threonine/serine dual-specificity kinases that subsequently phosphorylate threonine and tyrosine residues in ERK1/2 resulting in activation.31) A significant role for the MAPK in cancer biology has been well established. The Ras proteins were initially identified as the transforming component of oncogenic viruses for K-Ras and H-Ras, whereas N-Ras was identical as the transforming component of a neuroblastoma.32) Additional support for the importance of the MAPK pathway in oncogenesis comes from the prevalence of activating mutations among family members in multiple cancer types. Ras mutations are found in up to 30% of all cancers and are particularly common in pancreatic cancers and colon

Fig. 5. *In Vivo* Effects of KIST101029 on the IGF-1-Induced Tumorigenesis

(A and B) Fertilized chicken eggs were implanted with JB6 Cl41 cells with/without IGF-1 and KIST101029 for 15d. Representative pictures of the CAM (A) and measured tumor area (B) are shown. Error bars indicate the means±standard deviation of six samples per group from two independent experiments. * p<0.05, compared with control groups or only IGF-1-treated group, respectively.

Fig. 6. Effects of KIST101029 on Tumorigenicity of A375 Cells

(A) Cells were seeded and cultured for 96h in 10% FBS/DMEM at 37°C in 5% CO₂ atmosphere. Then, the cells were treated with sorafenib and KIST101029 dose-dependently as indicated. Cell viability was measured by MTT assay, as described in Materials and Methods. Data are represented as the means±S.D. as determined from triplicate experiments. (B) Cells were starved for 24h, pretreated with sorafenib and KIST101029 at the indicated concentrations for 24h, and harvested. The levels of phosphorylated and total proteins related with MEK1/2 and ERK1/2 in whole cell lysates were determined by immunoblotting analysis using specific antibodies against the corresponding proteins, respectively. (C and D) A375 cells were treated or not treated with sorafenib and KIST101029, as indicated concentration, in soft agar. The representative colonies from three separate experiments were photographed (C). The colonies were counted under a microscope with the aid of the Image-Pro Plus software program (D). Columns, mean of triplicate samples; bars, S.D. * p<0.05, compared with only DMSO treated cells.
cancers. B-RAF mutations have a more narrow distribution, but are prevalent in a few specific malignancies including melanoma, papillary thyroid cancers, and low-grade ovarian cancers. Based on the well-defined role for the Ras-Raf-MEK-ERK pathway in human cancers, therapeutic targeting has been an area of intense investigation.

AP-1 is dimeric transcription factors composed of Jun, Fos or activating transcription factor (ATF) subunits that bind to a common DNA site, the AP-1 binding site. AP-1 is regulated mainly by MAPKs. Previous studies have demonstrated that importance of the involvement of ERK1/2 in neoplastic cell transformation of JB6 P+ (promotion-sensitive) cells, whereas JB6 P- (promotion-resistance) cells do not respond to EGF as well as TPA due to low level of phosphorylated ERK. Constitutively active mitogen-activated protein (MAP) kinases, including Raf, MEK and ERK, are sufficient to promote cell transformation through activated AP-1-regulated transcription.

In this study, KIST101029 inhibited IGF-1-induced phosphorylation of MEK and ERK in JB6 Cl41 cells, and this inhibition of the MEK-ERK pathway led to the suppression of neoplastic transformation through the inhibition of AP-1. In addition, JNK involved AP-1 action and neoplastic transformation in JB6 P+ cells. Other studies have found that JNK2-deficient mice failed to induce skin tumorigenesis in response to TPA, which also supports the important role of JNK in skin tumorigenesis. This prompted us to examine the effect of KIST101029 on IGF-1-induced JNK phosphorylation in JB6 Cl41 cells. KIST101029 inhibited IGF-1-induced JNK as well as e-Jun phosphorylation. These results indicated that the inhibition of ERK as well as JNK by KIST101029 might be responsible for KIST101029’s strong inhibition of neoplastic transformation in soft agar and CAM. Further, in vitro pull-down assays revealed that KIST101029 bound with Raf-1, which may be contribute to the observed reduced kinase activities of MAPKs pathway.

In summary, KIST101029 inhibited IGF-1-induced cell proliferation and neoplastic transformation of JB6 Cl41 cells via its binding with Raf-1. This inhibition was mediated mainly through the blocking of the MEK-ERK, JNK-e-Jun, and mTOR-p70S6K pathway, and subsequent suppression of AP-1 activity. KIST101029 binds with Raf-1. In addition, KIST101029 significantly inhibited the tumor formation induced by IGF-1 in CAM. Together these results suggested that Raf-1 is the most potent molecular target of KIST101029 for suppressing neoplastic transformation.

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REFERENCES

1) Brunelleschi S, Penengo L, Santoro MM, Gaudino G. Receptor tyrosine kinases as target for anti-cancer therapy. Curr. Pharm. Des., 8, 1959–1972 (2002).
2) Magné N, Fischel JL, Dubreuil A, Formento P, Poupon MF, Laurent-Puig P, Milano G. Influence of epidermal growth factor receptor (EGFR), p53 and intrinsic MAP kinase pathway status of tumour cells on the antiproliferative effect of ZD1839 (“Iressa”). Br. J. Cancer, 86, 1518–1523 (2002).
3) Kahler S, Neldling S, van Eickels M, Vetter H, Meyer R, Grohe C. Estrogen receptor alpha rapidly activates the IGF-1 receptor pathway. J. Biol. Chem., 275, 18447–18453 (2000).
4) Lokker NA, Sullivan CM, Hollenbach SJ, Israel MA, Giese NA. Platelet-derived growth factor (PDGF) autocrine signaling regulates survival and mitogenic pathways in glioblastoma cells: evidence that the novel PDGF-C and PDGF-D ligands may play a role in the development of brain tumors. Cancer Res., 62, 3729–3735 (2002).
5) Meadows KN, Bryant P, Pumiglia K. Vascular endothelial growth factor induction of the angiogenic phenotype requires Ras activation. J. Biol. Chem., 276, 49289–49298 (2001).
6) Schlessinger J. Cell signaling by receptor tyrosine kinases. Cell., 103, 211–225 (2000).
7) Maurer G, Tarkowski B, Baccarini M. Raf kinases in cancer-roles and therapeutic opportunities. Oncogene, 30, 3477–3488 (2011).
8) Malumbres M, Pellicer A. RAS pathways to cell cycle control and cell transformation. Front. Biosci., 3, d887–d912 (1998).
9) Dong Z, Birrer MJ, Watts RG, Matrisian LM, Colburn NH. Blocking of tumor promoter-induced AP-1 activity induces inhibition in JB6 mouse epidermal cells. Proc. Natl. Acad. Sci. U.S.A., 91, 609–613 (1994).
10) Huang C, Ma WY, Young MR, Colburn N, Dong Z. Shortage of mitogen-activated protein kinase is responsible for resistance to AP-1 transactivation and transformation in mouse JB6 cells. Proc. Natl. Acad. Sci. U.S.A., 95, 156–161 (1998).
11) Rendahub F, Pierie K, Mamat M, Minard G, Khan KD, Luttell LM, Transactivation of the EGFr receptor mediates IGF-1-stimulated phosphorylation and ERK1/2 activation in COS-7 cells. J. Biol. Chem., 275, 22583–22589 (2000).
12) Marshall CJ. MAP kinase kinase, MAP kinase kinase and MAP kinase. Curr. Opin. Genet. Dev., 4, 82–89 (1994).
13) Sette C, Barchi M, Bianchini A, Conti M, Rossi P, Geremia R. Activation of the mitogen-activated protein kinase ERK1 during metastatic progression of mouse pachytyene spermatocytes. J. Biol. Chem., 274, 33571–33579 (1999).
14) Wilhelm S, Chen DS. BAY 43-9006: preclinical data. Curr. Pharm. Des., 8, 2255–2257 (2002).
15) Egberts F, Kahler KC, Livingstone E, Hauschild A. Metastatic melanoma: scientific rationale for sorafenib treatment and clinical results. Onkologie, 31, 398–403 (2008).
16) Wilhelm SM, Carter C, Tang L, Wilkie D, McNabola A, Rong H, Chen C, Zhang X, Vincent P, McHugh M, Cao Y, Shujat J, Gawlak S, Eveleigh D, Rowley B, Liu L, Adnan L, Lynch M, Alaiadr C, Taylor I, Gedrich R, Voznesensky A, Riedl B, Post LE, Bollag G, Trail PA. BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. Cancer Res., 64, 7099–7109 (2004).
17) Wilhelm SM, Adnan L, Newell P, Villanueva A, Llovet JM, Lynch M. Preclinical overview of sorafenib, a multikinase inhibitor that targets both Raf and VEGF and PDGF receptor tyrosine kinase signaling. Mol. Cancer Ther., 7, 3129–3140 (2008).
18) Wilhelms BJ, Blackwell SJ, Miller JH, Mancoff JS, Dardano T, Tran A, Phillips LG. Do not use epinephrine in digital blocks: myth or truth? Plast. Reconstr. Surg., 107, 393–397 (2001).
19) El-Gamal MI, Jung MH, Oh CH. Discovery of a new potent bisamide FMS kinase inhibitor. Bioorg. Med. Chem. Lett., 20, 3216–3218 (2010).
20) Menu E, Kooijman R, Valkenhorst EV, Asosingh K, Bakkus M, Camp BV, Vanderkerken K. Specific roles for the PI3K and therapeutic opportunities, and pathway cross-talk. Mol. Cancer Ther., 7, 3129–3140 (2008).
21) Jiang MH, El-Gamal MI, Abdel-Maksoud MS, Sim T, Yoo KH, Oh CH. Design, synthesis, and antiproliferative activity of new 1H-pyrido[3,2-c]pyridine derivatives against melanoma cell lines. Part
23) Lopez-Bergami P, Huang C, Goydos JS, Yip D, Bar-Eli M, Herlyn M, Smalley KS, Mahale A, Eroshkin A, Aaronson S, Ronai Z. Rewired ERK-JNK signaling pathways in melanoma. Cancer Cell, 11, 447–460 (2007).

24) Eferl R, Wagner EF. AP-1: a double-edged sword in tumorigenesis. Nat. Rev. Cancer, 3, 859–868 (2003).

25) Li JJ, Westergaard C, Ghosh P, Colburn NH. Inhibitors of both nuclear factor-kappaB and activator protein-1 activation block the neoplastic transformation response. Cancer Res., 57, 3569–3576 (1997).

26) Satyamoorthy K, Li G, Gerrero MR, Borse MS, Volpe P, Weber BL, Van Belle P, Elder DE, Herlyn M. Constitutive mitogen-activated protein kinase activation in melanoma is mediated by both BRAF mutations and autocrine growth factor stimulation. Cancer Res., 63, 756–759 (2003).

27) Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, de Oliveira AC, Santoro A, Raoul JL, Forner A, Schwartz M, Porta C, Zeuzem S, Bolondi L, Galle PR, Seitz JF, van der Houwelingen ACH, Bruix J, Haussinger D, Galle PR, Seitz JF, Borbath I, Häussinger D, Giannaris T, Shan M, Moscovici M, Voliotis D, Bäsecke J, Cocco L, Evangelisti C, Marrelli G, Cervello M, McCubrey JA. Roles of the Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR pathways in controlling growth and sensitivity to therapy—implications for cancer and aging. Aging (Albany, NY, Online), 3, 192–222 (2011).

28) Steelman LS, Chappell WH, Abrams SL, Kempf RC, Long J, Laidler P, Mijatovic S, Maksimovic-Ivanic D, Stivala F, Mazzarino MC, Donia M, Fagone P, Malaponte G, Nicoletti F, Libra M, Miliana M, Tafuri A, Bonati A, Cocco L, Evangelisti C, Mantelli AM, Montalto G, Cevreli M, McCubrey JA. Roles of the Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR pathways in controlling growth and sensitivity to therapy—implications for cancer and aging. Aging (Albany, NY, Online), 3, 192–222 (2011).

29) Khorasani-Far R, White MA, Westwick JK, Solski PA, Chrzanowska-Wodnicka M, Van Aelst L, Wigler MH, Der CJ. Oncogenic Ras activation of Raf/mitogen-activated protein kinase-independent pathways is sufficient to cause tumorigenic transformation. Mol. Cell. Biol., 16, 3923–3933 (1996).

30) Roberts PJ, Der CJ. Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. Oncogene, 26, 3291–3310 (2007).

31) Friday BB, Adjemian AA. K-ras as a target for cancer therapy. Biochim. Biophys. Acta, 1756, 127–144 (2005).

32) Bos JL, Fearon ER, Hamilton SR, Vries MV-, van Boom JH, van der Eb AJ, Vogelstein B. Prevalence of ras gene mutations in human colorectal cancers. Nature, 327, 293–297 (1987).

33) Steelman LS, Chappell WH, Abrams SL, Kempf RC, Long J, Laidler P, Mijatovic S, Maksimovic-Ivanic D, Stivala F, Mazzarino MC, Donia M, Fagone P, Malaponte G, Nicoletti F, Libra M, Miliana M, Tafuri A, Bonati A, Cocco L, Evangelisti C, Mantelli AM, Montalto G, Cevreli M, McCubrey JA. Roles of the Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR pathways in controlling growth and sensitivity to therapy—implications for cancer and aging. Aging (Albany, NY, Online), 3, 192–222 (2011).

34) Mansour SJ, Matten WT, Hermann AS, Candia JM, Rong S, Fukuwaka K, Vande Woude GF, Ahn NG. Transformation of mammalian cells by constitutively active MAP kinase kinase. Science, 265, 966–970 (1994).

35) Dong Z, Ma W, Huang C, Yang CS. Inhibition of tumor promoter-induced activator protein 1 activation and cell transformation by tea polyphenols, (−)-epigallocatechin gallate, and theaflavins. Cancer Res., 57, 4414–4419 (1997).

36) Chen N, Nomura M, She QB, Ma WY, Bode AM, Wang L, Flavell RA, Dong Z. Suppression of skin tumorigenesis in c-Jun NH(2)-terminal kinase-2-deficient mice. Cancer Res., 61, 3908–3912 (2001).