Biological Characterization of ARRY-142886 (AZD6244), a Potent, Highly Selective Mitogen-Activated Protein Kinase Kinase 1/2 Inhibitor

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Abstract

Purpose: The Ras-Raf-mitogen-activated protein kinase kinase (MEK) pathway is overactive in many human cancers and is thus a target for novel therapeutics. We have developed a highly potent and selective inhibitor of MEK1/2. The purpose of these studies has been to show the biological efficacy of ARRY-142886 (AZD6244) in enzymatic, cellular, and animal models.

Experimental Design: The ability of ARRY-142886 to inhibit purified MEK1 as well as other kinases was evaluated. Its effects on extracellular signal-regulated kinase (ERK) phosphorylation and proliferation in several cell lines were also determined. Finally, the inhibitor was tested in HT-29 (colorectal) and BxPC3 (pancreatic) xenograft tumor models.

Results: The IC_{50} of ARRY-142886 was determined to be 14 nmol/L against purified MEK1. This activity is not competitive with ATP, which is consistent with the high specificity of compound for MEK1/2. Basal and epidermal growth factor – induced ERK1/2 phosphorylation was inhibited in several cell lines as well as 12-*O*-tetradecanoylphorbol-13-acetate – induced ERK1/2 phosphorylation in isolated peripheral blood mononuclear cells. Treatment with ARRY-142886 resulted in the growth inhibition of several cell lines containing B-Raf and Ras mutations but had no effect on a normal fibroblast cell line. When dosed orally, ARRY-142886 was capable of inhibiting both ERK1/2 phosphorylation and growth of HT-29 xenograft tumors in nude mice. Tumor regressions were also seen in a BxPC3 xenograft model. In addition, tumors remained responsive to growth inhibition after a 7-day dosing holiday.

Conclusions: ARRY-142886 is a potent and selective MEK1/2 inhibitor that is highly active in both *in vitro* and *in vivo* tumor models. This compound is currently being investigated in clinical studies.

Excessive growth factor signaling leads to unregulated growth that can contribute to the pathogenesis of human cancer. The signaling cascade is initiated by the binding of peptide growth factors to their tyrosine kinase receptors at the plasma membrane. The receptor kinases are activated and through the recruitment of the growth factor receptor binding protein 2/son of sevenless complex to autophosphorylated sites on the receptors, the G protein Ras is induced to its active GTP-bound state. Ras recruits the serine/threonine kinase Raf to the plasma membrane, where it is then able to phosphorylate and activate mitogen-activated protein kinase kinases (MEK) 1 and 2, which are dual specificity protein kinases that phosphorylate serine/threonine and tyrosine residues. The MEK kinases in turn phosphorylate and activate their only currently known substrates, extracellular signal-regulated kinases (ERK) 1 and 2. ERK1/2 proteins translocate to the nucleus where they phosphorylate and activate effector proteins and transcription factors, resulting in diverse cellular responses, including proliferation.

The overexpression and/or mutation of epidermal growth factor (EGF) receptor (EGFR), erbB2, platelet-derived growth factor receptor, RET, and other growth factor receptors have been observed in many types of cancer and have been associated with elevated levels of phospho-ERK1/2 (1–4). Various members of the Ras gene family, which includes some of the first proto-oncogenes identified, are frequently mutated in many human cancers, including up to 90% of pancreatic cancers, 50% of colorectal cancers, 30% of lung cancers, and 15% to 30% of melanomas (5–7). These mutations, most often found in K-Ras, result in increased levels of active GTP-bound Ras (8). Raf is also a proto-oncogene. Although mutations in c-Raf and A-Raf are rare, point mutations in B-Raf were identified recently in two thirds of melanoma cases (9–11). Subsequently, B-Raf point mutations have also been identified in other cancers including 40% to 70% of papillary thyroid cancers, 60% of low-grade ovarian tumors, and 4% to

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16% of colorectal cancers (12). The most abundant B-Raf point mutation is V600E, which results in a hyperactive B-Raf, presumably by mimicking phosphorylation of the regulatory activation loop (13). MEK1/2 is not frequently mutated but the high incidence of elevated phospho-ERK1/2 in numerous human cancer tissues and cell lines reflects the multiple upstream events that can lead to the increased activity of MEK1/2 (14).

Because the Raf-MEK-ERK kinase signaling module is activated as a consequence of several gene amplifications and genetic mutations identified in human cancer, it is an attractive target for small-molecule intervention. The optimal way to evaluate the role of this signaling axis may be through the use of selective MEK1/2 inhibitors because there are currently no potent and selective Ras, Raf, or ERK inhibitors in clinical development. The MEK1/2 inhibitor CI-1040 reached phase II clinical evaluation but its development was stopped due to insufficient efficacy (15). Two second generation MEK1/2 inhibitors, ARRY-142886 (AZD6244) and PD0325901, which have shown better efficacy than CI-1040 in preclinical models, are currently in early clinical development (16, 17). The characterization of ARRY-142886 in enzymatic, cellular, and preclinical animal studies will be discussed here.

Materials and Methods

Compound synthesis

6-(4-Bromo-2-chloro-phenylamino)-7-fluoro-3-methyl-3H-benzoimidazole-5-carboxylic acid (2-hydroxy-ethoxy)-amide (ARRY-142886; Fig. 1A) was prepared according to the procedure described in patent application WO 03/77914 (example 10).

Cell lines

Cell lines were obtained from American Type Culture Collection (Manassas, VA). Medium was supplemented with 10% FCS and penicillin/streptomycin.

Enzymatic assays

MEK1. NH_2-terminal hexahistidine tagged, constitutively active MEK1 (S218D, S222D ΔR4F; ref. 18) was expressed in baculovirus-infected Hi5 insect cells and purified by immobilized metal affinity chromatography (Talon, Invitrogen, Carlsbad, CA), ion exchange (Resource Q, Amersham Biosciences, Piscataway, NJ), and gel filtration (Superdex 200, Amersham Biosciences). The activity of MEK1 was assessed by measuring the incorporation of [γ-^{33}P]phosphate from [γ-^{33}P]ATP onto ERK2. The assay was carried out in a 96-well polypropylene plate with an incubation mixture (100 μL) composed of 25 mmol/L HEPES (pH 7.4), 10 mmol/L $MgCl_2$, 5 mmol/L β-glycerolphosphate, 100 μmol/L sodium orthovanadate, 5 mmol/L DTT, 5 nmol/L MEK1, 1 μmol/L ERK2, and 0 to 80 nmol/L compound (final concentration of 1% DMSO). The reactions were initiated by the addition of 10 μmol/L ATP (with 0.5 μCi [γ-^{33}P]ATP/well) and incubated at room temperature for 45 min. An equal volume of 25% trichloracetic acid was added to stop the reaction and precipitate the proteins. Precipitated proteins were trapped onto glass fiber B filter plates, excess labeled ATP was washed off with 0.5% phosphoric acid, and radioactivity was counted in a liquid scintillation counter (Perkin-Elmer, Wellesley, MA). ATP dependence was determined by varying the amount of ATP in the reaction mixture. The data were globally fitted using SigmaPlot (SPSS, Inc., Chicago, IL). Values were calculated using the following equation for noncompetitive inhibition: $v = [V_{max} \times S / (1 + I / K_i)] / (K_m + S)$.

ERK2. To measure inhibition of ERK2, the kinase activity of ERK2 was first activated by MEK1. Wild-type (WT) ERK2 containing an NH_2-terminal hexahistidine tag was overexpressed in *Escherichia coli* and purified by immobilized metal affinity chromatography (Talon), ion exchange (Resource Q), and gel filtration (Superdex 200). To activate WT ERK2, 2 mg WT ERK2 was mixed with 17 μg of constitutively active MEK1 in 4 mL of 25 mmol/L HEPES (pH 7.5) containing 1 mmol/L ATP. The reaction mixture was incubated at room temperature for 40 min, and the addition of two phosphates was confirmed by mass spectrometry. Activated WT ERK2 was further purified by ion exchange (Resource Q). ERK2 activity was assayed as described for constitutively active MEK, using 10 nmol/L activated ERK2. The substrate used was myelin basic protein (Sigma, St. Louis, MO) at a concentration of 1 μmol/L.

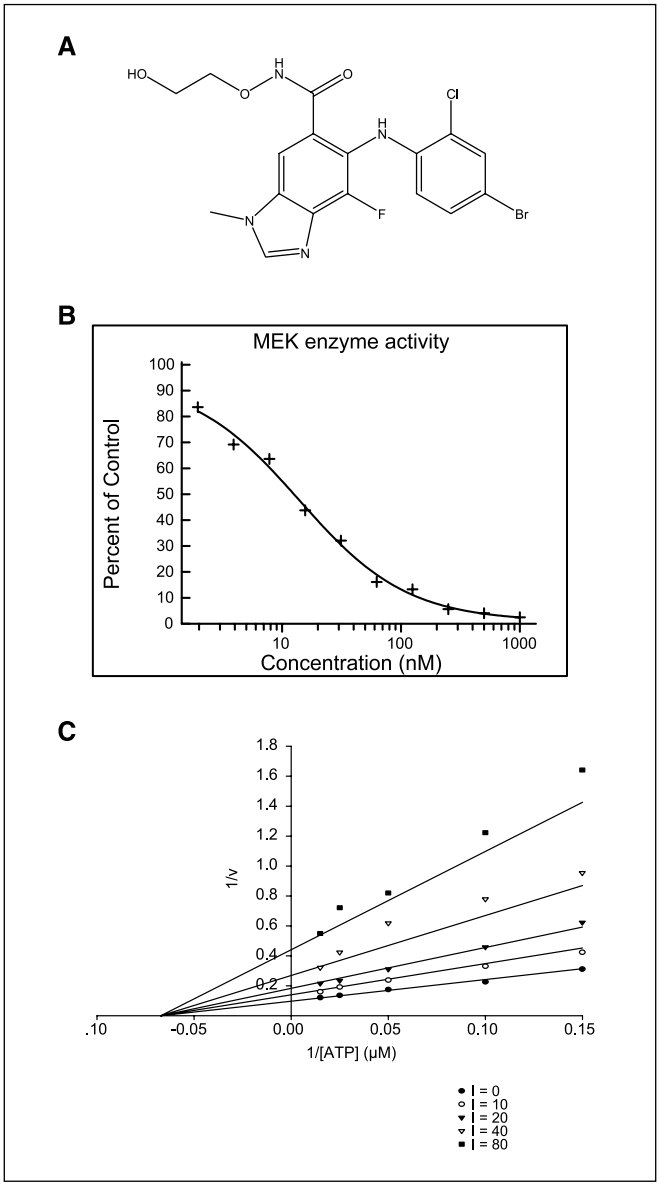

Fig. 1. Structure of ARRY-142886 and its ability to inhibit enzymatic MEK1 activity. *A,* chemical structure of 6-(4-Bromo-2-chloro-phenylamino)-7-fluoro-3-methyl-3H-benzoimidazole-5-carboxylic acid (2-hydroxy-ethoxy)-amide. *B,* MEK1 activity, in the presence of increasing concentrations of ARRY-142886, was assessed by measuring the incorporation of [γ-^{33}P]phosphate from [γ-^{33}P]ATP into ERK2. The IC_{50} was determined to be 14.1 nmol/L ± 0.79 (n = 9). Representative graph. *C,* Lineweaver-Burk plot for the inhibition of MEK1 activity by ARRY-142886 at varying concentrations of ATP. Concentrations of ARRY-142886 used were 0 nmol/L (●), 10 nmol/L (○), 20 nmol/L (▲), 40 nmol/L (Δ), and 80 nmol/L (■).

p38α. p38α activity was assayed at room temperature in a 100 μL reaction mixture containing 5 nmol/L p38α enzyme [previously activated with constitutively active MEK6 (MKK6)], 1 μmol/L activating transcription factor 2 fusion protein, 25 mmol/L HEPES (pH 7.4), 100 μmol/L vanadate, 1 mmol/L DTT, 10 mmol/L $MgCl_2$, and 10 μmol/L [γ-^{33}P]ATP (~0.1 μCi ^{33}P/reaction). The reaction was terminated after 40 min by adding trichloracetic acid to a final concentration of 125 mmol/L, incubated for 5 min, and then transferred directly to a glass fiber B membrane filter plate. The filter was washed with 0.5% phosphoric acid and dried under vacuum, and radioactivity was determined in a liquid scintillation counter.

MKK6. The kinase activity of MKK6 activity was determined using a time-resolved fluorescence resonance energy transfer–based assay. A glutathione *S*-transferase–tagged fusion protein of full-length p38α was used as the substrate. The assay was done using a 50 mmol/L HEPES and 20 mmol/L $MgCl_2$ buffer (pH 7.5) at room temperature. MKK6 (2 nmol/L) and glutathione *S*-transferase–p38α (750 nmol/L) were incubated with 2 μmol/L ATP and ARRY-142886 for 10 min. The reaction was quenched with 40 mmol/L EDTA/8.5 mmol/L Tris-HCl containing an anti–glutathione *S*-transferase antibody conjugated to europium (AD0065, Perkin-Elmer, Wellesley, MA) and an anti-phosphotyrosine antibody (PY20) conjugated to allophycocyanin (PJ254, Prozyme, San Leandro, CA). The reaction mixture was incubated for 60 min. Phosphorylation of glutathione *S*-transferase–p38α by MKK6 was quantitated by measuring the emissions at 615 and 665 nm on excitation at 340 nm.

EGFR and ErbB2. The assays for the determination of EGFR and ErbB2 kinase activities are based on an ELISA. ARRY-142886 (final DMSO concentration of 1%), 0.3 units/mL EGFR from A431 cells (SE-116, Biomol, Plymouth Meeting, PA) or 250 ng/mL recombinant protein of the cytoplasmic domain of ErbB2, and 15 μmol/L ATP in assay buffer [50 mmol/L HEPES (pH 7.3), 125 mmol/L NaCl, 24 mmol/L $MgCl_2$, 0.1 mmol/L Na_3VO_4] were incubated on a 0.25 mg/mL poly(Glu/Tyr) 4:1 (Sigma)–coated plate for 30 min at room temperature. After washing, the phosphorylated polymer substrate was detected with 0.2 μg/mL horseradish peroxidase–conjugated monoclonal antibody to phosphotyrosine (Invitrogen). After the addition of 1 mol/L phosphoric acid to stop the development, the chromogenic substrate, 3,3′,5,5′-tetramethylbenzidine, was quantitated by spectrophotometry at 450 nm.

B-Raf. B-Raf kinase activity was assessed by quantitating the incorporation of ^{33}P into inactive fluorosulfonylbenzoyladenosine-modified MEK from [γ-^{33}P]ATP. The assay was done using a 25 mmol/L HEPES buffer (pH 7.5) at room temperature. B-Raf (V600E; 20 nmol/L) and 1 μmol/L fluorosulfonylbenzoyladenosine-MEK were preincubated with ARRY-142886 for 15 min. The reaction was initiated with 4 μmol/L [γ-^{33}P]ATP (~0.1 μCi ^{33}P/reaction). The reaction was terminated after 40 min by adding trichloracetic acid to a final concentration of 200 mmol/L, incubated for 5 min, and then transferred directly to a glass fiber B membrane filter plate. The filter was washed twice for 30 s with 0.5% phosphoric acid and dried under vacuum. Approximately 30 μL scintillant was added per well to the filter plate, and radioactivity was quantified.

Other kinases. Assays were done at the Upstate Kinase Profiler Specificity Testing Service according to their established protocols (Upstate Cell Signaling Solutions, Waltham, MA).

Cellular ERK1/2 phosphorylation

Cells were grown in 24-well plates, incubated with ARRY-142886 for 1 h, and lysed in radioimmunoprecipitation assay buffer [50 mmol/L Tris (pH 8), 150 mmol/L NaCl, 1% NP40, 0.5% deoxycholic acid, 0.05% SDS, 2 mmol/L EDTA]. If appropriate, cells were also stimulated with 100 ng/mL EGF for 5 min before lysis. Cell lysates were evaluated by Western analysis using an antibody to phospho-ERK1/2 (Cell Signaling Technology, Danvers, MA). The blot was stripped and reprobed with pooled antibodies to ERK1 and ERK2 (Santa Cruz Biotechnology, Santa Cruz, CA). Signals were visualized by enhanced chemiluminescence. For the 96-well assay, Malme-3M cells were plated and, on the next day, treated with ARRY-142886 for 1 h. The cells were fixed in 3.7% formaldehyde, permeabilized with 100% methanol, and incubated with an antibody to phospho-ERK1/2 and an antibody to total ERK1/2 (Santa Cruz Biotechnology) followed by fluorescently tagged secondary antibodies (A21058, Invitrogen, Carlsbad, CA and 611-132-122, Rockland Immunochemicals, Boyertown, PA). Fluorescent signals were visualized and quantified using the LI-COR imager (LI-COR Biosciences, Lincoln, NE).

Cellular viability assays

Cells plated in 96-well plates were incubated with multiple concentrations of compound for 3 days. The number of viable cells was determined using the CellTiter 96 Aqueous Nonradioactive Cell Proliferation Assay (Promega, Madison, WI).

ERK phosphorylation in 12-*O*-tetradecanoylphorbol-13-acetate–stimulated peripheral blood mononuclear cells from whole blood samples

Whole blood (stored at 4°C overnight before analysis) was treated with ARRY-142886 for 1 h at 37°C followed by treatment with 400 nmol/L 12-*O*-tetradecanoylphorbol-13-acetate (TPA) for 10 min and fixation in 2% formaldehyde for 10 min. Peripheral blood mononuclear cells (PBMC) were isolated using a Ficoll/Hypaque density gradient, washed twice with PBS, permeabilized, and stored in 90% methanol at −20°C. For antibody staining, cells were collected by centrifugation, washed in PBS/4% FCS, and incubated with an antibody to phospho-ERK (Cell Signaling Technology) followed by a FITC-conjugated antibody fragment to rabbit IgG (Caltag Laboratories, Burlingame, CA). Using flow cytometry, the lymphocyte population was selected using forward and side scatter and analyzed for fluorescence content.

HT-29 and BxPC3 xenograft studies

HT-29 human colon carcinoma or BxPC3 human pancreatic tumor fragments were implanted s.c. in the flank of nude mice and allowed to grow to 100 to 150 mg. Mice (n = 10 per group) were randomized to treatment groups to receive vehicle (10 mL/kg and 10% ethanol/10% cremophor EL/80% D5W) or ARRY-142886 (10, 25, 50, or 100 mg/kg, oral, BID) on days 1 to 21. Tumors $[(W^2 \times L) / L]$ were measured twice weekly. Tumor growth inhibition was calculated as 1 − (tumor $\text{size}_{\text{treated}}$ / tumor $\text{size}_{\text{vehicle}}$) on each measurement day. Four hours after the last dose on day 21, three mice per group were euthanized to evaluate pharmacokinetic/pharmacodynamic responses. Tumors were excised and flash frozen. Homogenates were analyzed for phospho-ERK1/2 and ERK1/2 expression by Western blotting as described above. For the HT-29 study, monitoring of tumor regrowth was continued for the remaining seven mice per group until tumors reached 1,000 mm^3, when mice would be sacrificed. There were two BxPC3 tumor xenograft studies. For the first study, one group of mice was treated with the clinical standard of care, gemcitabine (Gemzar), at 160 mg/kg, i.p., every 3rd day for a total of four doses. This dose was determined to be the maximum tolerated dose for gemcitabine in the BxPC3 model on this dosing schedule. To evaluate whether previously treated tumors would be refractory to a second cycle of treatment, a second BxPC3 xenograft study was carried out. Mice were treated with vehicle or ARRY-142886 at 25 or 50 mg/kg, BID, for 21 days. Treatment was stopped and tumors were allowed to grow for an additional 7 days before treatment resumed for another 21-day cycle.

Results

Enzymatic kinase activity and selectivity assays. The effect of ARRY-142886 (AZD6244; Fig. 1A) on the enzymatic activity of purified constitutively active MEK1 was measured using a ^{33}P-based radioactive assay (Fig. 1B). The IC_{50} was determined

Table 1. Activity of ARRY-142886 (10 μmol/L) against other kinases

Kinase	Percentage of control
p38α	88
MKK6	100
EGFR	90
ErbB2	100
ERK2	100
B-Raf	100
CDK2/cyclinA	93
c-Raf	98
c-Src	95
Insulin receptor kinase	103
JNK2α2	100
MAPKAP-K2	101
PDGFRα	99
PKBα	97
PKCα	93

Abbreviations: PDGFR, platelet-derived growth factor receptor; CDK, cyclin-dependent kinase; JNK, c-Jun NH_2-terminal kinase; PKBα, protein kinase Bα; PKCα, protein kinase Cα.

to be 14.1 ± 0.79 nmol/L. Experiments conducted to determine the competitive nature of the inhibition exhibited by ARRY-142886 indicate that the compound is not competitive with respect to ATP (Fig. 1C). The data were globally fitted; the best fit was for noncompetitive inhibition (K_m^{ATP} = 14.9 ± 1.1 μmol/L and K_i = 22.6 ± 1.1 nmol/L). To explore the selectivity of this compound, its activity against other serine/threonine and tyrosine kinases was evaluated. No inhibition was observed at 10 μmol/L against more than 40 kinases. A subset of these results is shown in Table 1.

Inhibition of cellular phosphorylation. The ability of ARRY-142886 to inhibit MEK1/2 in their cellular environment was assayed by measuring the phosphorylation state of ERK1/2 (phospho-ERK), the direct substrates of MEK1/2, in treated cells. ARRY-142886 was active in inhibiting ERK1/2 phosphorylation in all cell lines examined, with IC_{50} concentrations <40 nmol/L (Fig. 2A). Inhibition of the high basal ERK1/2 phosphorylation in Malme-3M cells was further quantified using a 96-well format immunofluorescence-based assay. After a 1-h incubation with compound, the cells were fixed and stained for phospho-ERK and total ERK. The IC_{50} was determined to be 10.3 ± 2.0 nmol/L (Fig. 2B).

Next, the ability of ARRY-142886 to inhibit growth factor–induced phospho-ERK was examined in EGFR-overexpressing A431 cells. As shown in Fig. 2C, EGF-induced ERK1/2 phosphorylation was inhibited by ARRY-142886. ERK5 phosphorylation was also induced. In contrast to ERK1/2 phosphorylation, no inhibition of ERK5 phosphorylation was seen up to 10 μmol/L ARRY-142886 (Fig. 2C).

TPA induces ERK1/2 phosphorylation by activating Raf through a Ras-independent mechanism. To investigate the ability of ARRY-142886 to inhibit TPA-induced phospho-ERK, whole blood samples were treated *ex vivo* with compound, stimulated with TPA, and immediately fixed in formaldehyde to preserve ERK phosphorylation. Isolated PBMCs were analyzed for their phospho-ERK content by flow cytometry. TPA stimulates phospho-ERK in 74% of PBMCs under these conditions (Fig. 2D). The presence of 100 nmol/L ARRY-142886 reduces the percentage of cells with elevated

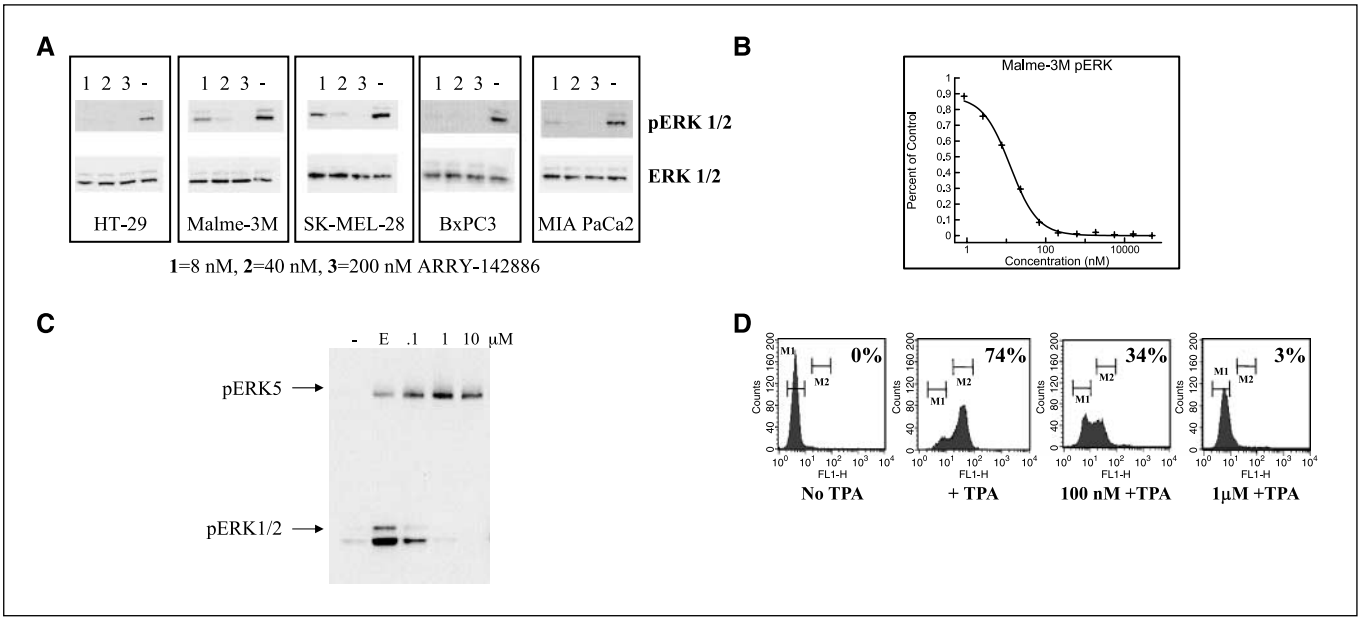

Fig. 2. Inhibition of basal and induced ERK1/2 phosphorylation in human cancer cell lines and PBMCs. *A,* cells plated in 24 wells were incubated with the indicated concentrations of ARRY-142886 for 1 h at 37°C. Phospho-ERK (*pERK*) levels were determined by Western blotting of the cell lysates. Blots were then stripped and reprobed for total ERK. *B,* Malme-3M cells were treated as above but in 96-well format. Fixed cells were stained for phospho-ERK and quantified by a fluorescence imager. Phospho-ERK is normalized to total ERK. The IC_{50} was determined to be 10.3 nmol/L ± 2.0 (n = 5). Representative graph. *C,* EGFR-overexpressing A431 cells were incubated with ARRY-142886 at the indicated concentrations for 1 h before EGF stimulation. Cell lysates were analyzed for phospho-ERK by Western blotting. This antibody also cross-reacts with phospho-ERK5. *D,* human whole blood was incubated with ARRY-142886 at 100 nmol/L and 1 μmol/L for 1 h and stimulated with TPA. PBMCs were isolated, stained for phospho-ERK, and analyzed by flow cytometry. Upper right hand percentages indicate the percentage of cells in the M2 gate containing induced phospho-ERK.

Table 2. Inhibition of cell growth in human cell lines

Name	Type	Ras/Raf mutation (reported)	IC_{50} (nmol/L) ± SE
HT-29	Colon	B-Raf	175 ± 42
Malme-3M	Melanoma	B-Raf	59 ± 4
MIA PaCa-2	Pancreatic	K-Ras	473 ± 169
SK-MEL-2	Melanoma	N-Ras	200 ± 35
SK-MEL-28	Melanoma	B-Raf	93 ± 17
BxPC3	Pancreatic	None reported	>8,000
BT474	Breast	WT	>50,000
Zr-75-1	Breast	WT	>50,000
Malme-3	Normal fibroblast	None reported	>50,000

phospho-ERK to 34%, whereas 1 μmol/L compound blocks nearly all TPA-induced phospho-ERK.

Inhibition of cellular growth. The functional consequences of blocking MEK1/2 on cellular viability were evaluated in several cell lines (Table 2). ARRY-142886 was more potent in cell lines containing activating B-Raf and Ras mutations, with IC_{50} values ranging from 59 to 473 nmol/L. In contrast, ARRY-142886, at concentrations of up to 50 μmol/L, had minimal effect on other cell lines, including Malme-3, which is the normal counterpart to the melanoma cell line Malme-3M. These results show that inhibition of cell growth by this compound is not due to general cytotoxicity.

Activity of ARRY-142886 in* in vivo *mouse xenograft models. After showing enzymatic and cellular potency, ARRY-142886 was evaluated in mice for antitumor activity against a HT-29 xenograft, a colorectal tumor model carrying a B-Raf mutation. Figure 3A shows that ARRY-142886 is effective in inhibiting tumor growth at all doses tested. The time to the tumor growth end point (i.e., 1,000 mm^3) was 36 days for the two highest dose groups compared with 18 days for the vehicle control group ($P < 0.01$, t test). Tumor growth after 11 days (last day where all control tumors were <1,000 mm^3) of dosing was inhibited by 55% at the low dose of 10 mg/kg and by 70% at the high dose of 100 mg/kg. Recovery of tumor growth was observed after cessation of ARRY-142886 administration (Fig. 3A). Tumor regrowth was significantly delayed in the 100 mg/kg dose group.

The ERK1/2 phosphorylation levels of the tumors were also evaluated. Four hours after the last dose on day 21, tumors from three mice per group were excised and analyzed for phospho-ERK and total ERK. Inhibition of phospho-ERK was seen in tumors of mice that were treated with compound when compared with tumors of vehicle-treated mice (Fig. 3B). Total ERK levels were comparable in all groups.

We next evaluated the activity of ARRY-142886 against the BxPC3 pancreatic tumor xenograft model. The study design was similar to the HT-29 study reported above with the addition of a standard-of-care treatment group (gemcitabine/Gemzar). Figure 4A shows that ARRY-142886 was not only more effective than gemcitabine but also able to induce tumor regressions at the concentrations tested. Tumor growth inhibition with ARRY-142886 at all doses and on all days was significantly better than that seen with gemcitabine ($P < 0.01$). At the 50 mg/kg dose, 94% tumor growth inhibition was observed, with 6 of 10 animals showing tumor regressions. As with the HT-29 tumors, inhibition of phospho-ERK1/2 was seen (Fig. 4B), showing that in both animal models, the efficacy of ARRY-142886 as an antitumor agent correlates with MEK inhibition in the tumors.

The second BxPC3 xenograft study was carried out to evaluate tumor sensitivity to ARRY-142886 after a treatment-free interval. ARRY-142886 at the 25 or 50 mg/kg dose showed significant inhibition of tumor growth at both doses for the first 21-day treatment cycle. After treatment cessation, tumor regrowth was apparent within 4 days, with a noticeable lag in tumor growth in the 50 mg/kg dose group. On resumption of ARRY-142886 treatment, tumor growth was again inhibited, with regressions seen in both dosing groups (Fig. 4C).

Discussion

The identification of activating Ras and B-Raf mutations, as well as elevated levels of ERK1/2 phosphorylation, in both primary cancer tissues and cancer cell lines has provided strong evidence for the role of the Raf-MEK-ERK pathway in human cancers (5, 12, 14). Recently, several MEK inhibitors have entered human clinical testing, including CI-1040, PD0325901, and the subject of this article, ARRY-142886 (15–17, 19, 20).

ARRY-142886 is a potent and selective inhibitor of both MEK1 and MEK2. Its IC_{50} against purified constitutively active MEK1 is 14 nmol/L. The IC_{50} values against Raf-activated MEK1 and MEK2 are similar, showing that ARRY-142886 does not distinguish between the two closely related kinases (data not

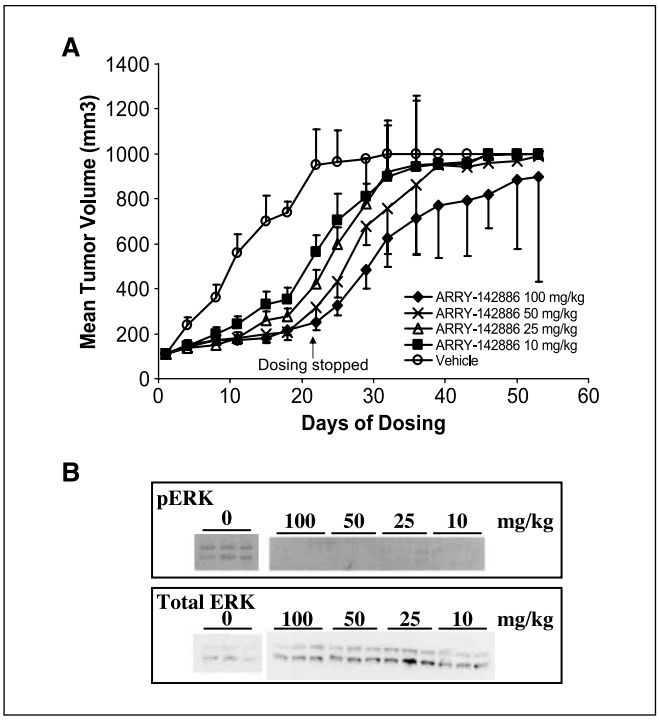

Fig. 3. Inhibition of tumor growth and decreased tumor phospho-ERK1/2 levels in a mouse HT-29 xenograft model. *A,* mice bearing HT-29 tumors were dosed with vehicle or ARRY-142886 (10, 25, 50, or 100 mg/kg, oral, BID) for 21 d (n = 10 per group). Animals were sacrificed when tumors reached 1,000 mm^3 in size. The larger error bars at end of study are due to the reduced number of animals left on study. *B,* 4 h after the last dose on day 21, three mice per group were euthanized. Homogenates from excised tumors were analyzed for phospho-ERK1/2 and ERK1/2 expression by Western blotting.

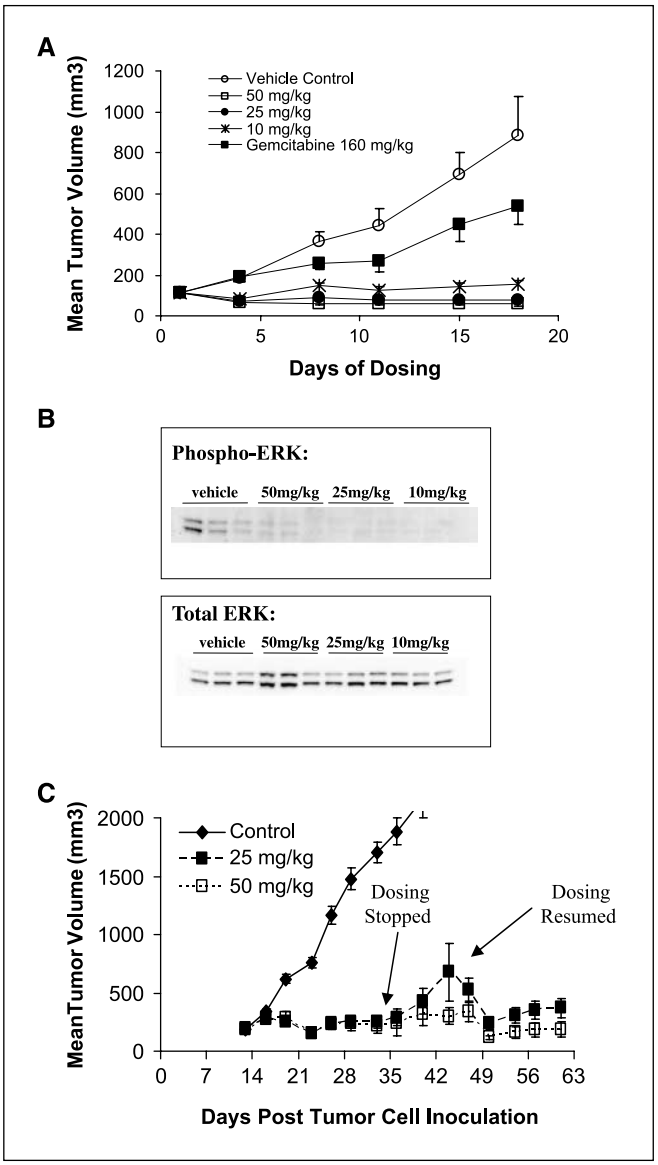

Fig. 4. Tumor growth inhibition in the mouse BxPC3 xenograft model. *A,* mice bearing BxPC3 tumors were orally dosed with vehicle or ARRY-142886 (10, 25, or 50 mg/kg, oral, BID) for 18 d (n = 10 per group). Note that dosing was terminated before day 21 due to excessive tumor growth in the control animals. A positive control group was dosed with gemcitabine at 160 mg/kg, i.p., every 3rd day for a total of four doses. *B,* 4 h after the last dose on day 18 in the study above, three mice per group were euthanized. Homogenates from excised tumors were analyzed for phospho-ERK1/2 and ERK1/2 expression by Western blotting. *C,* dosing of mice bearing BxPC3 tumors occurred for 21 d (25 or 50 mg/kg, oral, BID) followed by a 7-d dosing holiday to allow for tumor regrowth. Dosing was then resumed for an additional 21 d.

shown). Mechanistic enzymology studies showed that MEK inhibition by ARRY-142886 is not competitive with respect to ATP. Molecular modeling data are consistent with the binding of ARRY-142886 to the allosteric inhibitor binding site in MEK1/2 that has been described previously (21). The presence of inhibitor in this unique site is proposed to lock MEK1/2 into an inactive conformation that enables binding of ATP and substrate but disrupts both the molecular interactions required for catalysis and the proper access to the ERK activation loop.

This novel kinase inhibitor binding site has not been identified in other kinase structures and translates to exquisite selectivity for ARRY-142886, as shown by the lack of inhibition at 10 μmol/L against more than 40 protein kinases (Table 1; data not shown). In addition, ARRY-142886 does not seem to inhibit MEK5 because EGF-induced phosphorylation of ERK5 was not inhibited at concentrations up to 10 μmol/L. Similar results were seen in BT474 cells, which express elevated basal levels of phospho-ERK5 (data not shown). The phosphorylation of MEK1/2, whether basal or EGF induced, was not inhibited by treatment with ARRY-142886 (data not shown), supporting the notion that this compound inhibits MEK by directly inhibiting enzymatic activity and not by blocking its phosphorylation and activation by Raf (22).

ARRY-142886 was able to potently inhibit basal ERK1/2 phosphorylation in all tested cell lines. In addition, this compound inhibited EGF-induced (Ras mediated) and TPA-induced (non-Ras mediated) ERK1/2 phosphorylation. The effect of ARRY-142886, however, on cellular proliferation varied from cell line to cell line. We observed that melanoma and/or cell lines with the B-Raf V600E point mutation were the most sensitive to MEK inhibition, whereas the growth of BT474 and Zr-75-1, cell lines which have WT Ras and B-Raf, was unaffected. These findings are consistent with other reports. For example, melanoma cell lines, where B-Raf mutations are highly present (~66%), from the NCI 60 cell line panel were found to be the most growth sensitive to PD98059, an early MEK inhibitor compound (23). The correlation of mutant B-Raf with growth inhibition of cell lines by the MEK inhibitor, CI-1040, was also shown recently (24, 25). The correlation of response to Ras mutations is less apparent, which is probably due to the fact that activated Ras can induce other Raf-independent pathways involving phosphatidylinositol 3-kinase, RalGEF proteins, and others (26). Thus, the identification of MEK-driven proliferation may be critical in optimizing the efficacy of a MEK inhibitor (27).

Further studies with HT-29 and Malme-3M cells showed that a 24-h incubation with the compound induced a G_1-S cell cycle arrest (Fig. 1; Supplementary Data). In addition, after a 2-day incubation period, ARRY-142886 was able to induce apoptosis in several cell lines. Interestingly, this effect is independent of B-Raf mutational status. Treatment with the MEK inhibitor activated caspase-3 and caspase-7 in the melanoma cell lines, Malme-3M (V600E) and SK-MEL-2 (mutant N-Ras), whereas no activation was seen in either HT-29 (V600E) or SK-MEL-28 (V600E; Fig. 2; Supplementary Data; data not shown). Induction of apoptosis by MEK inhibition has also been reported for M14-MEL, Ocl-AML3, HL-60, NB40, HT1080, RPMI-SE, HepG2, and other cell lines (23, 28–31). Other factors, such as mutations in the phosphatidylinositol 3-kinase/Akt survival pathway, may determine how cells will respond to MEK inhibition.

It was shown previously that MEK inhibition resulted in reduced growth of human HT-29 xenograft tumors in nude mice (32). ARRY-142886 was tested in this model in a similar manner. Tumor growth after 11 days (last day where all control tumors were <1,000 mm^3) of dosing was inhibited by 55% at doses as low as 10 mg/kg (oral, BID), suggesting better *in vivo* efficacy than CI-1040, which was reported to inhibit HT-29 tumor growth by 59% to 69% when administered for 14 days (49-200 mg/kg oral, BID; ref. 32). Inhibition of tumor ERK1/2 phosphorylation by ARRY-142886 correlated with decreased tumor growth. Continuous dosing of

ARRY-142886 was necessary to maintain tumor growth inhibition in this model as tumor growth resumed once dosing stopped. These observations are consistent with cellular studies, in which the removal of compound results in the rapid reestablishment of phospho-ERK in both HT-29 and Malme-3M cells (data not shown).

Because an overactive Ras-Raf-MEK pathway is present in many pancreatic cancers, ARRY-142886 was also tested in a pancreatic BxPC3 xenograft model. Tumor growth was inhibited by 84% to 94% at doses of 10, 25, and 50 mg/kg. Tumor stasis was also seen at 3 mg/kg in another study (33). In addition, in the BxPC3 study that was carried out to evaluate a 3-week on, 1-week off treatment regimen, tumors were shown to remain responsive to ARRY-142886 after a 7-day holiday from treatment.

In the BxPC3 model, tumor regressions were seen at the 25 and 50 mg/kg doses by day 4 of treatment initiation. This observation was especially interesting because in cell culture studies, the compound had minimal effect on cell viability. This discrepancy between minimal *in vitro* cell viability and potent *in vivo* tumor efficacy has also been observed for PANC-1 (34) and Zr-75-1 cell lines. Fairly low doses of ARRY-142886 (10 or 25 mg/kg, BID) was shown to inhibit the growth of Zr-75-1 tumors *in vivo* (Fig. 3; Supplementary Data) despite little effect on cell viability (Table 2). Apparently, the efficacy seen in the tumor models may be due, in part, to *in vivo* inhibition of angiogenesis. In support of this hypothesis, we have observed antiangiogenic activity for a structurally related compound in an *in vivo* basic fibroblast growth factor–induced Matrigel angiogenesis model (35). It is also possible that *in vivo* tumor growth is more sensitive to MEK inhibition due to the interplay between tumor and host factors and the role of MEK1/2 in this process.

Efficacy has been shown with ARRY-142886 in other human xenograft models, including MIA PaCa-2, HCT-116, LOX, Calu6, and A549 (34, 36). Notably, tumor regressions were seen in models for pancreatic cancer and melanoma, cancer types where activating mutations of the MEK pathway have been identified.

In this report, we have described the biological characterization of this compound, including its activity against both purified and cellular MEK1/2, its ability to inhibit cellular growth and induce apoptosis, and, finally, its efficacy against tumor growth in *in vivo* xenograft models. The pharmacologic activity of ARRY-142886 in humans has been shown by the observation of concentration-dependent inhibition of TPA-induced ERK1/2 phosphorylation in PBMCs isolated from cancer patients (19). A MEK inhibitor would presumably be most effective in MEK-driven cancers, which may be identified by the presence of activating mutations in B-Raf, Ras, or growth factor receptors and/or elevated levels of ERK1/2 phosphorylation. These cancers include pancreatic, colon, lung, melanoma, and papillary thyroid cancers, showing the potential widespread therapeutic potential of a MEK inhibitor.

In conclusion, we have shown that ARRY-142886 (AZD6244) is a potent and highly selective MEK1/2 inhibitor with good efficacy in *in vitro* and *in vivo* models. This compound is currently being evaluated in human clinical studies.

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References

1. Blume-Jensen P, Hunter T. Oncogenic kinase signalling. Nature 2001;411:355–65.
2. Choong NW, Ma PC, Salgia R. Therapeutic targeting of receptor tyrosine kinases in lung cancer. Expert Opin Ther Targets 2005;9:533–59.
3. Porter AC, Vaillancourt RR. Tyrosine kinase receptor-activated signal transduction pathways which lead to oncogenesis. Oncogene 1998;17:1343–52.
4. Robertson SC, Tynan J, Donoghue DJ. RTK mutations and human syndromes: when good receptors turn bad. Trends Genet 2000;16:368.
5. Bos JL. ras oncogenes in human cancer: a review. Cancer Res 1989;49:4682–9.
6. Gray-Schopfer VC, da Rocha Dias S, Marais R. The role of B-RAF in melanoma. Cancer Metastasis Rev 2005;24:165–83.
7. Fransen K, Klintenas M, Osterstrom A, Dimberg J, Monstein HJ, Soderkvist P. Mutation analysis of the BRAF, ARAF, and RAF-1 genes in human colorectal adenocarcinomas. Carcinogenesis 2004;25:527–33.
8. Friday BB, Adjei AA. K-ras as a target for cancer therapy. Biochim Biophys Acta 2005;1756:127–44.
9. Davies H, Bignell GR, Cox C, et al. Mutations of the BRAF gene in human cancer. Nature 2002;417:949–54.
10. Emuss V, Garnett M, Mason C, Marais R. Mutations of C-RAF are rare in human cancer because C-RAF has a low basal kinase activity compared with B-RAF. Cancer Res 2005;65:9719–26.
11. Lee JW, Soung YH, Kim SY, et al. Mutational analysis of the ARAF gene in human cancers. APMIS 2005;113:54–7.
12. Beeram M, Patnaik A, Rowinsky EK. Raf: a strategic target for therapeutic development against cancer. J Clin Oncol 2005;23:6771–90.
13. Wan PT, Garnett MJ, Roe SM, et al. Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. Cell 2004;116:855–67.
14. Hoshino R, Chatani Y, Yamori T, et al. Constitutive activation of the 41-/43-kDa mitogen-activated protein kinase signaling pathway in human tumors. Oncogene 1999;18:813–22.
15. Rinehart J, Adjei AA, Lorusso PM, et al. Multicenter phase II study of the oral MEK inhibitor, CI-1040, in patients with advanced non-small-cell lung, breast, colon, and pancreatic cancer. J Clin Oncol 2004;22:4456–62.
16. Lorusso P, Krishnamurthi S, Rinehart J, et al. A Phase 1-2 clinical study of a second generation oral MEK inhibitor, PD 0325901 in patients with advanced cancer [abstract]. Proceedings of the American Society of Clinical Oncology Annual Meeting; 2005; Orlando, FL.
17. Chow L, Eckhardt S, Reid J, et al. A first in human dose-ranging study to assess the pharmacokinetics, pharmacodynamics, and toxicities of the Mek inhibitor, ARRY-142886 (AZD6244), in patients with advanced solid malignancies [abstract]. Proceedings of the AACR-NCI-EORTC International Conference on Molecular Targets and Cancer Therapeutics; 2005; Philadelphia, PA.
18. Mansour SJ, Candia JM, Matsuura JE, Manning MC, Ahn NG. Interdependent domains controlling the enzymatic activity of mitogen-activated protein kinase kinase 1. Biochemistry 1996;35:15529–36.
19. Doyle M, Yeh T, Brown S, et al. Validation and use of a biomarker for clinical development of the MEK1/2 inhibitor ARRY-142886 (AZD6244) [abstract]. Proceedings of the American Society of Clinical Oncology Annual Meeting; 2005; Orlando, FL.
20. Lorusso PM, Adjei AA, Varterasian M, et al. Phase I and pharmacodynamic study of the oral MEK inhibitor CI-1040 in patients with advanced malignancies. J Clin Oncol 2005;23:5281–93.
21. Ohren JF, Chen H, Pavlovsky A, et al. Structures of human MAP kinase kinase 1 (MEK1) and MEK2 describe novel noncompetitive kinase inhibition. Nat Struct Mol Biol 2004;11:1192–7.
22. Ahn NG, Nahreini TS, Tolwinski NS, Resing KA. Pharmacologic inhibitors of MKK1 and MKK2. Methods Enzymol 2001;332:417–31.
23. Koo HM, VanBrocklin M, McWilliams MJ, Leppla SH, Duesbery NS, Woude GF. Apoptosis and melanogenesis in human melanoma cells induced by anthrax lethal factor inactivation of mitogen-activated protein kinase kinase. Proc Natl Acad Sci U S A 2002;99:3052–7.
24. Pohl G, Ho CL, Kurman RJ, Bristow R, Wang TL, Shih IM. Inactivation of the mitogen-activated protein kinase pathway as a potential target-based therapy in ovarian serous tumors with KRAS or BRAF mutations. Cancer Res 2005;65:1994–2000.
25. Solit DB, Garraway LA, Pratilas CA, et al. BRAF mutation predicts sensitivity to MEK inhibition. Nature 2005;439:358–62.
26. Repasky GA, Chenette EJ, Der CJ. Renewing the conspiracy theory debate: does Raf function alone to mediate Ras oncogenesis? Trends Cell Biol 2004;14:639–47.

27. Ballif BA, Blenis J. Molecular mechanisms mediating mammalian mitogen-activated protein kinase (MAPK) kinase (MEK)-MAPK cell survival signals. Cell Growth Differ 2001;12:397–408.

28. Brassard DL, English JM, Malkowski M, Kirschmeier P, Nagabhushan TL, Bishop WR. Inhibitors of farnesyl protein transferase and MEK1,2 induce apoptosis in fibroblasts transformed with farnesylated but not geranylgeranylated H-Ras. Exp Cell Res 2002;273:138–46.

29. Hoshino R, Tanimura S, Watanabe K, Kataoka T, Kohno M. Blockade of the extracellular signal-regulated kinase pathway induces marked G_1 cell cycle arrest and apoptosis in tumor cells in which the pathway is constitutively activated: up-regulation of p27(Kip1). J Biol Chem 2001;276:2686–92.

30. Milella M, Kornblau SM, Estrov Z, et al. Therapeutic targeting of the MEK/MAPK signal transduction module in acute myeloid leukemia. J Clin Invest 2001;108:851–9.

31. Mitsui H, Takuwa N, Maruyama T, et al. The MEK1-ERK map kinase pathway and the PI 3-kinase-Akt pathway independently mediate anti-apoptotic signals in HepG2 liver cancer cells. Int J Cancer 2001;92:55–62.

32. Sebolt-Leopold JS, Dudley DT, Herrera R, et al. Blockade of the MAP kinase pathway suppresses growth of colon tumors *in vivo*. Nat Med 1999;5:810–6.

33. Winkler J, Lee P, Wallace E, et al. Anti-tumor activity, pharmacokinetic and pharmacodynamic effects of the MEK inhibitor ARRY-142886 (AZD6244) in a BxPC3 pancreatic tumor xenograft model [abstract]. Proceedings of the AACR-NCI-EORTC International Conference on Molecular Targets and Cancer Therapeutics; 2004; Geneva, Switzerland.

34. Lee P, Wallace E, Yeh T, et al. ARRY-142886, a potent and selective MEK Inhibitor: III) Efficacy Against Human Xenograft Models Correlates with Decreased ERK phosphorylation [abstract]. Proceedings of the 95th Annual AACR Meeting; 2004; Orlando, FL.

35. Wallace E, Lyssikatos J, Blake J, et al. 4-(4-Bromo-2-fluorophenylamino)-1-methyl-pyridin-2(1H)ones: potent and selective MEK 1,2 inhibitors [abstract]. Proceedings of the AACR-NCI-EORTC International Conference on Molecular Targets and Cancer Therapeutics; 2005; Philadelphia, PA.

36. Lee P, Wallace E, Yeh T, et al. Demonstration of broad *in vivo* anti-tumor activity of ARRY-142886 (AZD6244), a potent and selective MEK inhibitor [abstract]. Proceedings of the AACR-NCI-EORTC International Conference on Molecular Targets and Cancer Therapeutics; 2004; Geneva, Switzerland.

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