GSK1120212 (JTP-74057) Is an Inhibitor of MEK Activity and Activation with Favorable Pharmacokinetic Properties for Sustained In Vivo Pathway Inhibition


Abstract

**Purpose:** Despite their preclinical promise, previous MEK inhibitors have shown little benefit for patients. This likely reflects the narrow therapeutic window for MEK inhibitors due to the essential role of the P42/44 MAPK pathway in many nontumor tissues. GSK1120212 is a potent and selective allosteric inhibitor of the MEK1 and MEK2 (MEK1/2) enzymes with promising antitumor activity in a phase I clinical trial (ASCO 2010). Our studies characterize GSK1120212's enzymatic, cellular, and in vivo activities, describing its unusually long circulating half-life.

**Experimental Design:** Enzymatic studies were conducted to determine GSK1120212 inhibition of recombinant MEK, following or preceding RAF kinase activation. Cellular studies examined GSK1120212 inhibition of ERK1 and 2 phosphorylation (p-ERK1/2) as well as MEK1/2 phosphorylation and activation. Further studies explored the sensitivity of cancer cell lines, and drug pharmacokinetics and efficacy in multiple tumor xenograft models.

**Results:** In enzymatic and cellular studies, GSK1120212 inhibits MEK1/2 kinase activity and prevents Raf-dependent MEK phosphorylation (S217 for MEK1), producing prolonged p-ERK1/2 inhibition. Potent cell growth inhibition was evident in most tumor lines with mutant BRAF or Ras. In xenografted tumor models, GSK1120212 orally dosed once daily had a long circulating half-life and sustained suppression of p-ERK1/2 for more than 24 hours; GSK1120212 also reduced tumor Ki67, increased p27Kip1/CDKN1B, and caused tumor growth inhibition in multiple tumor models. The largest antitumor effect was among tumors harboring mutant BRAF or Ras.

**Conclusions:** GSK1120212 combines high potency, selectivity, and long circulating half-life, offering promise for successfully targeting the narrow therapeutic window anticipated for clinical MEK inhibitors.

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Introduction

Activating mutations in the mitogen-activated protein kinase (p42/p44 MAPK) pathway represent an important and unsolved therapeutic target in multiple solid tumors (1–5). The combined activating mutations in KRAS, BRAF, NRAS, and HRAS occur frequently in malignant melanomas (64%), carcinomas of the pancreas (73%), colon (51%), and lung (19%–estimates from COSMIC, 2010).

Inhibitors of MEK1 and MEK2 (MEK1/2) have long shown promise as targeted therapies for tumors dependent on these activating mutations in the MAPK pathway, but so far they have disappointed with their lack of clinical activity. The failure is presumed to be due to a narrow therapeutic window, constrained by the essential nature of the p42/p44 MAPK pathway in nontumor cells. The elusive goal has been a clinically active MEK inhibitor that combines sustained high level of MEK inhibition in tumors with limited systemic toxicity due to MEK inhibition in nontargeted organs. We hypothesize that the preferred pharmacokinetic (PK) profile for a MEK inhibitor would combine prolonged exposure and a low $C_{\text{max}}$ (peak concentration) to $C_{\text{trough}}$ (trough concentration) ratio, delivering sustained levels of sufficient dose and potentially avoiding toxicities that might result from high transient peak exposure. Previous MEK inhibitors and several new MEK inhibitors currently being evaluated in early clinical trials, PK profiles have generally been reported to have relatively high $C_{\text{max}}$ and short half-life in blood, requiring twice-daily dosing for activity (6–10). A repeated high $C_{\text{max}}$ may be a concern for systemic toxicity, whereas a short-circulating half-life may be insufficient to achieve the necessary sustained pathway inhibition in tumors.

Authors’ Affiliation: GlaxoSmithKline, Collegeville, Pennsylvania

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Corresponding Author: Aidan G. Gilmartin, GlaxoSmithKline, 1250 S. Collegeville Rd, Collegeville, PA 19426. Phone: 610-917-4078; Fax: 610-917-4181. E-mail: Aidan.G.Gilmartin@GSK.com
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Translational Relevance

Activating mutations in the p42/p44 MAPK pathway, particularly in Ras and BRAF, represent some of the most important unsolved therapeutic targets in multiple solid tumors. MEK inhibitors have long shown promise as targeted therapies for tumors driven by mutant Ras and Raf, but have so far disappointed with their lack of clinical activity. Here we describe GSK1120212 (JTP-74057), an allosteric inhibitor of MEK1 and MEK2 (MEK1/2) activity that has shown promising early clinical activity in tumors with activating mutations in BRAF. We characterize the biochemical, cellular, and in vivo activities of GSK1120212 in preclinical models. We also suggest several properties of GSK1120212 that may translate to superior clinical activity including (i) a long circulating half-life with low peak concentration to trough concentration ratio and (ii) the ability to inhibit MEK1/2 activation by preventing Raf phosphorylation of MEK on S217 (for MEK1).

Here we describe GSK1120212 (JTP-74057), an allosteric inhibitor of MEK1/2 activity that has shown promising early clinical activity in tumors with activating mutations in BRAF (ASCO 2010). GSK1120212 is orally bioavailable, potent, and specific, and has a long circulating half-life with low Cmax to C trough ratio. We observe potent inhibition of ERK phosphorylation in all cell lines tested and cell growth inhibition among most cancer cell lines with activating mutations in the MAPK pathway. In addition to inhibiting MEK-dependent ERK phosphorylation, GSK1120212 inhibits MEK1/2 activation by preventing Raf phosphorylation of MEK on S217 (for MEK1). Finally, we observed significant growth inhibition in multiple tumor xenografts, and particularly in those harboring activating mutations in BRAF or KRAS.

Materials and Methods

MEK1 enzyme assays

Inhibitors were dissolved in dimethyl sulfoxide (DMSO) and were present at final concentrations of 0.015 to 5,000 nmol/L with a final DMSO concentration 2.5%. Activated diphosphorylated MEK (pp-MEK) assays contained 40 μmol/L 32P-γATP (AppKm8.5 ± 1.8 μmol/L), 0.5 nmol/L human-activated MEK1 or MEK2 (Millipore), 1 μmol/L kinase-dead ERK2 (K52R; Millipore; AppKm 0.73 ± 0.07 μmol/L). All assays were in buffer containing 20 mmol/L HEPES (pH 7.2), 0.15 mg/mL BSA, 10 mmol/L MgCl2, 0.8 mmol/L CHAPS (Sigma), and 1 mmol/L TCEP (Thermo), with kinase-dead ERK2 as substrate with 32P-γATP in reaction buffer. The kinase reactions were stopped after 60 minutes by transferring 30 μL of reaction mixture to a Durapore filter plate, and spun 5× with 10× volume dilutions for buffer exchange to effectively remove GSK1120212 and ATP (reduced to 50 pmol/L and 5 nmol/L, respectively). SB590885 was maintained at 1 μmol/L throughout the buffer exchange. MEK1 kinase assay was carried out in a buffer containing 20 mmol/L HEPES (pH 7.2), 0.15 mg/mL BSA, 10 mmol/L MgCl2, 0.8 mmol/L CHAPS (Sigma), and 1 mmol/L TCEP (Thermo), using kinase-dead ERK2 as substrate with 32P-γATP in TCA precipitation filter binding assay. MEK kinase reactions were stopped after 40 minutes by transferring 30 μL of reaction mixture to a Durapore 0.45-μm filters plates (Millipore) containing 12.5% TCA. Filters were dried and read with liquid scintillant on a TopCount (Perkin Elmer). Concentration response data were analyzed by nonlinear regression using a 3-parameter equation with the bottom set to zero for IC50 estimation using GraphPad Software.

For IC50 determination of initially unphosphorylated MEK (u-MEK), 0.2 nmol/L recombinant human MEK1 or MEK2 (GlaxoSmithKline) was preincubated with vehicle or with GSK1120212 for 40 minutes in reaction buffer. Phosphorylation/activation was then initiated by the addition of 20 nmol/L final B-Raf (University of Dundee) and 30 μmol/L final ATP for 10 minutes. B-Raf activity was then quenched by addition of the B-Raf inhibitor SB590885 (100 nmol/L final, 500 × ki). MEK kinase activity was assayed by the addition of 1 μmol/L KD-ERK2 (K52R; Upstate) and 0.02 μCi/μL 32P-γATP in reaction buffer. The kinase reactions were stopped after 90 minutes by transferring 30 μL of reaction mixture to a Durapore filter plate, and read as above.

Activity comparison of mono- and diphosphorylated MEK1

u-MEK1 was incubated with vehicle or 5 μmol/L final GSK1120212 for 30 minutes. Activation was then initiated by the addition of 50 nmol/L final B-Raf wild type (WT; University of Dundee) and 0.5 mmol/L final ATP. Activation reactions were quenched after 60 minutes with addition of 1 B-Raf inhibitor SB590885. At this time, 5 μmol/L GSK1120212 was added to the vehicle-treated samples to control for washout completion. Each reaction was loaded in centrifugal spin columns (10 kD MWCO; Millipore Cat) and spun 5× with 10× volume dilutions for buffer exchange to effectively remove GSK1120212 and ATP (reduced to 50 pmol/L and 5 nmol/L, respectively). SB590885 was maintained at 1 μmol/L throughout the buffer exchange. MEK1 kinase assay was carried out in a buffer containing 20 mmol/L HEPES (pH 7.2), 0.15 mg/mL BSA, 10 mmol/L MgCl2, 0.8 mmol/L CHAPS (Sigma), and 1 mmol/L TCEP (Thermo), using kinase-dead ERK2 as substrate with 32P-γATP in TCA precipitation filter binding assay. MEK kinase reactions were stopped after 40 minutes by transferring 30 μL of reaction mixture to a Durapore 0.45-μm filters plates (Millipore) containing 12.5% TCA. Filters were dried and read with liquid scintillant on a TopCount (Perkin Elmer). Concentration response data were analyzed by nonlinear regression using a 3-parameter equation with the bottom set to zero for IC50 estimation using GraphPad Software. Rates of ERK2 phosphorylation were determined as measures of relative activity.

Immunoblotting

Cell lysates were prepared using RIPA buffer (TEKnova) supplemented with protease (ROCHE) and phosphatase inhibitor cocktails (SIGMA), immunoblotted according to standard procedures, and membranes were imaged with an Odyssey imager. Primary antibodies included diphospho ERK1/2 (T202/Y204), total ERK1/2 (137F5), phospho MEK1/2, total MEK1/2, p27kip1 and phospho C-Raf.
Cell culture and proliferation assays

Cell lines were obtained from ATCC and grown in the recommended media at 37°C, 5% CO₂ in a humidified incubator. Cells were seeded in triplicate 96-well microtiter plates at 1,000 cells per well. GSK1120212 dissolved in DMSO or negative control (0.1% DMSO) were added the following day and one plate was harvested with 50 μL of CellTiter-Glo (Promega) for a time 0 (T = 0) measurement. Remaining duplicate cell plates were typically incubated for 72 hours. Cells were then lysed with 50 μL CellTiter-Glo, and chemiluminescent signal was read on the Wallac EnVision 2100 plate reader. All data were normalized to signal at the time of compound addition (T = 0). Curves were analyzed using the XLfit (IDBS Ltd.) tool, fitting to a 4-parameter curve to determine the gI₅₀ (concentration of 50% growth inhibition relative to T = 0 and Yₘₐₓ values), the gl-Maximum (concentration giving maximum growth inhibition), and the Yₘᵢₙ (bottom of the 4-parameter curve at gl-Maximum). On the basis of Yₘᵢₙ value we defined the maximum biological response for each cell line as "cytotoxic" (Yₘᵢₙ < 90% reflecting a net-cell decrease), "cytostatic" (Yₘᵢₙ = 90%–200%, where cells undergo 1 or less complete divisions), or "resistant" (Yₘᵢₙ > 200%).

Site-specific analysis of MEK phosphorylation by mass spectrometry

For cellular studies, endogenous MEK1 was immunoprecipitated from 1 mg of whole cell lysate with a MEK1 (61B12) antibody and protein G beads. For in vitro kinase reactions, 7 μmol/L recombinant MEK1 was preincubated with vehicle or GSK1120212 (10 μmol/L) for 30 minutes, and treated with an equal volume containing constitutively active C-Raf (50 nmol/L; Millipore) for 120 minutes in kinase buffer (20 mmol/L MOPS, 0.05 mg/ml BSA, 0.01% Tween-20, 10 mmol/L MgCl₂, 5 mmol/L EGTA, 1 mmol/L DTT). Reactions were quenched by adding LDS loading buffer and boiling for 10 minutes. The samples were loaded onto a Bis-Tris Gel and visualized by Coomassie Blue staining.

Gel bands corresponding to endogenous MEK1 (immunoprecipitated) or recombinant MEK1 (kinase reaction) were excised, reduced, alkylated, and digested overnight with trypsin (Promega). After organic extraction, samples were injected on an Agilent 1100 nanoLC system. The nanoLC was interfaced to a LTQ-Orbitrap-XL mass spectrometer (ThermoFisher). Tryptic peptides were separated on a Dionex 100-μm RP-PSDVB monolithic column using a gradient of 0% to 30% acetonitrile/0.2% formic acid over 40 minutes. Mass spectrometry (MS)-based peptide sequencing was accomplished by tandem mass spectrometry either using data-dependent LC/MS/MS or by targeted MS/MS sequencing of selected precursors. Uninterpreted tandem MS spectra were searched for protein matches against an in-house protein sequence database using Mascot (Matrix Science). Alternatively, selected MS/MS spectra were manually interpreted to determine the site of phosphorylation within a specific peptide. Apparent phosphorylation stoichiometry was determined from the area under the LC/MS peaks corresponding to the phosphorylated form(s) of the peptide and the unphosphorylated counterpart. Stoichiometry was calculated using the formula:

\[
\text{Stoichiometry For } P_n = \frac{A(P_n)}{A(P_1) + A(P_2) + A(P_3) + A(NP)}
\]

where \(A(P_n)\) is the abundance of either \(P_1\) or \(P_2\), \(A(P_1)\) is the abundance of phosphopeptide 206–227 containing pSer217, \(A(P_2)\) is the abundance of peptide 206–227 containing pSer221, \(A(P_3)\) is the abundance of the doubly phosphorylated peptide, and \(A(NP)\) is the abundance of nonphosphorylated peptide 206–227.

Tumor xenograft studies

Cells were implanted in Nude mice and grown as tumor xenografts. Dosing began when tumors achieved approximately 150 to 200 mm³. GSK1120212 or vehicle was administered by oral gavage at a dose volume of 0.2 mL/20 g body weight in 0.5% hydroxypropylmethylcellulose (Sigma) and 0.2% Tween-80 in distilled water (pH 8.0). Dosing was daily for 14 consecutive days (qdx14). Results are reported as mean tumor volume for 7 to 8 mice/group. Tumors were measured twice weekly with Vernier calipers, and tumor volume was estimated from 2-dimensional measurements using a prolate ellipsoid equation [Tumor volume (mm³) = (length × width²) × 0.5]. The maximum tolerated dose (MTD) was defined as the highest dose that produced less than 20% mortality and less than 20% weight loss (~4 g). Antitumor activity was defined as tumor growth inhibition (TGI), partial regression (PR), or complete regression (CR). TGI represents the percent volume differential between the treated and control tumors at the time vehicle tumors exceeded a volume of 1,000 mm³. PR was defined as a 50% decrease in an individual tumor from the initial starting volume for at least 1 week (3 consecutive measurements). CR was defined as a greater than 93% decrease in an individual tumor volume for at least 1 week. All studies were conducted after review by the GSK Institutional Animal Care and Use Committee and in accordance with the GSK Policy on the Care, Welfare and Treatment of Laboratory Animals.

Pharmacokinetic analysis

For mouse PK studies, tumor-bearing mice were euthanized at specified timepoints postdose and blood was drawn and combined immediately with an equal volume of water to hemolyze the sample. Brains were harvested, flash frozen on dry ice, and weighed (with minimal handling to prevent tissues from thawing). Required water volume was then calculated on the basis of the brain weight and added to the frozen tissues (4 volumes of water per volume tissue), and samples were homogenized using a Polytron homogenizer. Aliquots of the homogenized brain and
blood were flash frozen and subsequently evaluated for GSK1120212 concentration by HPLC/MS/MS analysis. In both mouse and rat studies, compound half-life in blood is evaluated after multiple oral doses, and the "effective half-life" is calculated as previously defined (11, 12).

For rat PK studies, Sprague–Dawley rats (3 females) were orally dosed for 3 weeks (21 days) at a constant dose volume of 40 mL/m²/day to a dose of 1.0 mg/m², where body surface area (m²) was estimated as (0.105) × (body weight in kg). On day 21, serial blood samples were collected into tubes containing EDTA as an anticoagulant at times 0 (predose day 21), 0.5, 1, 2, 4, 8, and 24 hours postdose. Plasma was isolated by centrifugation, and all resulting samples were snap frozen on dry ice and then stored at −20 °C until analyzed for GSK1120212 by HPLC/MS/MS analysis.

**Pharmacodynamic (PD) measurement of p-ERK levels in blood and brain tissues**

Tissues were harvested and homogenized using Medi-machine (BD Bioscience) with 1 mL of lysis buffer [25 mmol/L Tris-HCl (pH 7.5), 2 mmol/L EDTA (pH 8.0), 2 mmol/L EGTA (pH 8.0), 1% Triton X-100, 0.1% SDS, 50 mmol/L Disodium β-glycerol phosphate, 2 mmol/L Na₃VO₄, 4 mmol/L Na-pyrophosphate, 2× phosphatase inhibitor cocktail], and kept on ice. Following homogenization of all samples, homogenates were centrifuged at 14,000 rpm for 15 minutes at 4 °C. Clarified lysates were flash frozen for later analysis. All samples were analyzed with a duplex ELISA (MescoScale Discovery) measuring total ERK1/2 and phospho ERK1/2 according to the manufacturer’s instructions. Plates were read on an MSD. SI6000.

**Immunohistochemistry**

Xenografted A-375PF11 tumor tissue was harvested 4 hours postdose with GSK1120212 or vehicle after dose 1 or dose 4. Tumor tissue cut to approximately 100 mm³ samples was fixed in 10% formalin (neutral-buffered; VWR) for 24 hours, and then transferred to 70% ethanol for fixation and retained in the BRAF-mutant SK-MEL-28 cells, in the A-375P cell lines (Fig. 1C). Although decreased phospho MEK is sustained in the A-375P cells from 6 to 48 hours posttreatment, indicative of cells exiting the cell cycle. Notably, inhibition of p-ERK1/2 is observed in cells irrespective of their response to GSK1120212 in a 3-day proliferation assay (Supplementary Fig. 2); this indicates that differential antiproliferative activity is due to differences in the cellular dependence on MEK1/2 signaling rather than on cellular differences in the inhibition of the MEK1/2 by GSK1120212.

We also observe that GSK1120212 induces a decrease in MEK1/2 phosphorylation (phospho MEK) in several tumor lines (Fig. 1C). Although decreased phospho MEK is sustained in the BRAF-mutant SK-MEL-28 cells, in the A-375P (F11) and the KRAS-mutant HCT-116 cell lines, in the A-375P cells, the decrease in phospho MEK is transient, with levels rebounding by 48 hours. Similar inhibition of MEK phosphorylation has been reported for some other MEK inhibitors (16;17). In contrast, phospho MEK levels are not decreased in A549 cells, but rather show a time-dependent increase that corresponds with an increase in an activating phosphorylation on C-Raf (S338) kinase upstream of MEK1/2 (Supplementary Fig. 3). The gradual increase in MEK1/2 and Raf phosphorylation, evident at later timepoints, and detected by immunoblotting is consistent with reports for other allosteric MEK1/2 inhibitors and is attributed to inhibition of a negative feedback mechanism involving Sprouty and Dual Specificity Phosphatases (18). Taken together, these results suggest that besides inhibiting MEK phosphorylating ERK1/2, in some cells, GSK1120212 is partially inhibiting the activating phosphorylation of MEK by Raf.
GSK1120212 inhibits activating phosphorylation of MEK on S217

We further investigated the effect of GSK1120212 on the activation of MEK in an in vitro assay, examining MEK1 phosphorylation using mass spectrometry (MS). Previous studies indicated that MEK1 is maximally activated on dual phosphorylation of S217 and S221, with the monophosphorylated proteins being only fractionally activated (19). Phospho-specific antibodies to MEK1/2 generally are not suitable to resolve the monophosphorylated MEK (p-MEK) from pp-MEK(S217/S221), and are therefore inadequate for the current detailed analysis. In contrast, mass spectrometry, when combined with high resolution liquid chromatography, allowed development of an assay permitting discrimination between all 3 forms of a tryptic phosphopeptide containing phosphoserines 217 and/or 221. The MS-based analysis of in vitro kinase reactions showed that GSK1120212 completely prevents phosphorylation of MEK1(S217) by either C-Raf (Fig. 2A) or B-Raf (data not shown) but had no effect on S221 phosphorylation. We then attempted to determine the relative activity of p-MEK1(S221) compared with the pp-MEK1. MEK1 was activated with B-Raf in the presence or absence of GSK1120212 for 2 hours, compound was removed by repeated washes (in the presence of a B-Raf inhibitor SB590885A to prevent further activation), a fraction of protein was set aside for MS-analysis confirmation of phosphorylation, and finally the relative MEK1 kinase activities were compared for the rate of ERK2 phosphorylation. These results confirmed that p-MEK1(S221) was 32-fold more active than u-MEK, but only fractionally as active (1/83-fold) as pp-MEK1 (Supplementary Fig. 4A). Significantly, we also observed that the IC₅₀ for GSK1120212 was at least 6-fold lower for purified p-MEK1(S221) than for
of S217 on the MEK1 activation loop reduces the binding affinity of MEK1 for GSK1120212 presumably by altering the compound binding site.

To investigate whether GSK1120212 inhibited MEK1 phosphorylation at S217 in cells, A549 and A-375P(F11) cells were treated with 250 nmol/L compound for 0, 6, 24, 48, and 72 hours. MEK1 was immunoprecipitated and phosphorylation on S217 and S221 was monitored by mass spectrometry. As we observed by immunoblot analysis, phospho S221 levels gradually increased in both A549 and A-375P(F11). In contrast, S217 phosphorylation decreased on treatment with GSK1120212 and showed no increase over time. (Fig. 2C and D). These results are consistent with both our observation of a feedback response increase in MEK phosphorylation and the in vitro biochemical assay findings that GSK1120212 decreased phosphorylation of MEK1 at S217. We conclude that GSK1120212 fully inhibits MEK activity and partially inhibits MEK activation by Raf. Because GSK1120212 does not directly inhibit either C-Raf or B-Raf activity in enzymatic assays, we believe that GSK1120212 must bind to MEK in a way that specifically blocks the accessibility of S217 to Raf kinases.

Profiling cells sensitive to GSK1120212

To evaluate the therapeutic utility of GSK1120212 in cancers with various mutational profiles, we conducted 72-hour proliferation assays on a panel of cell lines. For our analysis we considered the curve parameters GI50 (concentration resulting in 50% growth inhibition) indicating the effective biological drug concentration and the Ymin (% cell number at the maximum effective dose relative to that at time 0) indicating the achievable biological effect. The growth and apoptotic response of 2 cell lines, SK-MEL-28 (BRAFV600E) and A549 (KRASG13D), were compared in more detail with daily cell growth curves (CellTiter-Glo). In SK-MEL-28 cells (Fig. 3A), GSK1120212 caused minimal effect on growth within 24 hours, but caused a clear net cell decrease (Ymin = -70% at 72 hours) at 48 and 72 hours. By comparison, in A549 cells (Fig. 3B), GSK1120212 caused primarily growth inhibition (Ymin = 182% at 72 hours). These results indicate that MEK inhibition can produce a time-dependent delay in growth and that the effect on net cell survival can vary across cell lines according to their intrinsic sensitivity.

We applied this analysis of proliferation assay curves across 94 cancer cell lines, evaluating sensitivity to GSK1120212 on the basis of GI50 and Ymin. The latter parameter was used to define cell response as cytotoxic (net cell loss), cytostatic (<1 net cell doubling), or less than cytostatic (>1 net cell doubling). Among the cell lines tested, those with the BRAFV600E mutation represented the most sensitive group of cells. Of the BRAFV600E-mutant cells, 10 of 10 had GI50 less than 50 nmol/L GSK1120212 (Fig. 3C), and 7 of 10 had cytotoxic response (Fig. 3D). Among the other cell lines that showed the greatest sensitivity to GSK1120212, the predominant determinants were activating mutations in KRAS or NRAS. Among cell lines

Figure 2. GSK1120212 partially inhibits Raf phosphorylation activation of MEK. A, 120 minutes in vitro incubation of C-Raf with MEK1 causes a majority of u-MEK (gray) to be phosphorylated on S221, S217 (black), or both (white); inclusion of GSK1120212 (10 μmol/L) prevents phosphorylation of MEK1 on S217 but not on S221 (MS analysis, B and C, MS analysis of cellular MEK1 phosphorylation in (B) A-375P(F11) and (C) A549 cells treated with 250 nmol/L GSK1120212 shows phospho S221 (black) levels increased in both cell lines at later timepoints; in contrast, phospho S217 (gray) levels decreased to limit of detection.

pp-MEK1 (Supplementary Fig. 4B and C); an accurate IC50 could not be determined for u-MEK due to its low kinase activity. This suggests that the additional phosphorylation
with activating KRAS mutation (specifically alterations in G12, G13, or Q61), 16 of 25 (64%) had gI50 less than 50 nmol/L. SKMEL22012 and 19 of 25 (76%) cell lines had cytoytic or cytostatic responses. Of the 4 cell lines with activating NRAS mutations (Q61L/R), 2 showed sensitivity to GSK1120212 (gI50 < 10 nmol/L; cytotoxic to cytostatic response). Taken together, these data suggest that activating BRAF and Ras mutations are determinant of sensitivity to GSK1120212.

The pharmacokinetic profile and pharmacodynamic activity of GSK1120212 following oral administration

The capability of GSK1120212 to inhibit ERK1/2 phosphorylation (p-ERK) in vivo was assessed in A-375P(F11) (melanoma encoding BRAF V600E mutation) xenograft model. GSK1120212 (3 mg/kg), the 14-day MTD in nude mice, was administrated daily by oral gavage. Blood, brain, and tumor were harvested at 2, 4, 8, and 24 hours after day 1 and day 7 administration. Concentrations were determined from blood and tissue homogenates by LC/ MS/MS analysis, whereas inhibition of p-ERK was measured in tumor and brain lysates by ELISA (Fig. 4A; Supplementary Fig. 5). The data show that on day 1 a single dose of GSK1120212 significantly reduces ERK phosphorylation for more than 8 hours. We further observe that blood levels of GSK1120212 accumulate following multiple drug administrations (with steady state being reached on day 4; data not shown). As a consequence of the higher steady state levels of GSK1120212, inhibition of p-ERK is greater following the day 7 dose with reduced p-ERK levels sustained over 24 hours. The sustained inhibition of ERK phosphorylation is consistent with the PK profile in blood, which was characterized by a gradual increase in GSK1120212 blood concentration to the Cmax (Cmax average = 1,410 nmol/L at 4.0 hours day 7), followed by a gradual decline in concentrations, resulting in a long effective half-life (day 7 estimated mean T1/2 = 33 hours). Brain samples showed no inhibition of ERK phosphorylation, with low levels of GSK1120212 detected at day 7 in the brain (Supplementary Fig. 5).

By immunohistochemistry (IHC) of the tumors, we confirmed both the immediate inhibition of ERK phosphorylation as well as the longer-term antiproliferative activity. Consistent with the p-ERK ELISA results, IHC staining for ERK phosphorylation (diphospho ERK1/2) confirmed significant inhibition 4 hours after doses 1 and 4 with 3 mg/kg GSK1120212 (Fig. 5). Additionally, IHC staining for levels of Ki67 and p27Kip1/CDKN1B (markers of cell proliferation and cell cycle arrest, respectively) confirmed inhibition of cell proliferation (reduced Ki67) and G1 cell cycle arrest (elevated p27Kip1/CDKN1B) following 4 days GSK1120212 treatment.

Figure 3. Profiling cellular response to GSK1120212. A, cells were treated with titrations of GSK1120212 to conduct 72 hours cell growth assays using CellTiter-Glo reagent. In highly sensitive SKMEL-28 cells (BRAFV600E), GSK1120212 initially causes a slight growth delay within 24 hours, with increased net cell death evident by 48 and 72 hours. B, in the intermediate-sensitive A549 cells (KRASG13D), GSK1120212 causes little effect in 24 hours, with growth inhibition and a slight decrease in net cells occurring at 48 and 72 hours. All data are normalized to the cell signal at time 0 (dashed gray line). Characterization of the cell response for 94 cell lines are based on the gI50 O and D, characterization of the cell response for 94 cell lines based on (C) the gI50 and (D) the maximum drug effect (Ymax of the 4-parameter growth inhibition curve) confirms that activating mutations in BRAF and KRAS predispose cells to sensitivity to GSK1120212.
To evaluate the PK profile of GSK1120212 in more detail, repeat dose PK studies were also conducted in rats. Female rats were orally dosed with 1.0 mg/kg GSK1120212 for 3 weeks (n = 3 rats). Following the day 1 and day 21 doses, blood was drawn serially at timepoints extending to 24 hours (Fig. 4B). Consistent with the mouse data, on each day we observed a gradual increase of GSK1120212 concentrations in plasma to the $C_{\text{max}}$ (average: 42.4 nmol/L) at $T_{\text{max}}$ followed by a gradual decline in concentrations. As observed in mouse studies, there was drug accumulation following repeated administration, and subsequent studies confirmed that steady state is achieved by day 4 (data not shown). In mice, the mean apparent half-life in blood was approximately 33 hours. B, more detailed PK analysis of plasma levels GSK1120212 in female SD rats dosed daily at 1.0 mg/m² (n = 3 per day). Serial draws on day 1 and day 21 reveals a gradual increase in plasma concentration, shallow $C_{\text{max}}$, and gradual decrease, resulting in drug accumulation. The mean apparent half-life in rats was approximately 36 hours.

GSK1120212 shows broad antitumor activity in multiple tumor xenograft models

In all tumor xenograft studies, nude mice harboring xenografted tumors (approximate tumor volume 200 mm³) were administered GSK1120212 by oral gavage daily for 14 days. Dosing in the BRAF-mutant A-375P(F11) xenograft model caused a dose-dependent TGI from 0.1 to 3.0 mg/kg GSK1120212 (MTD; Fig. 6A). Although treatment with GSK1120212 caused significant tumor
growth delay and some regressions, A-375P(F11) tumors generally resumed growth when drug treatment was interrupted (data not shown). Similarly in the KRAS-mutant HCT 116 xenograft model, GSK1120212 caused significant TGI at 1.0 or 3.0 mg/kg doses (Fig. 6B).

GSK1120212 was tested for activity in multiple additional tumor xenograft models with activating mutations in BRAF, KRAS, or neither (Supplementary Fig. 6) and showed antitumor activity in all models. In all experiments, 3.0 mg/kg GSK1120212 was orally administered daily for 14 days, and TGI was determined in comparison to the vehicle treated tumors at the time when mean tumor size exceeded 1,000 mm³. Among the xenograft lines tested, the BRAF-mutant Colo205, A-375P(F11), and HT29 models showed the most significant mean TGI (85%, 80%, and 87% TGI) with multiple complete and partial tumor regressions. Two KRAS-mutant xenograft models, HCT-116 and A549, also showed significant TGI (83% and 75% TGI) but without significant tumor regressions. As predicted by cell proliferation assays, tumor xenograft lines with WT RAF/RAS (PC3, BxPC3, and BT474) were relatively much less sensitive, showing only modest TGI with no tumor regressions (46%, 45%, and 44% TGI).

Discussion

Past studies of other allosteric MEK inhibitors have expressed conflicting results as to their ability to inhibit Raf phosphorylation of MEK (7,16,17,20). Although this may reflect real differences in the mechanism of action for different compounds, it also likely reflects the technique used for measuring MEK activation. Most commercial phospho MEK1/2 antibodies do not differentiate between p-MEK and pp-MEK, but these represent significantly different activation states (19). GSK1120212 inhibition of S217 phosphorylation contributes to the overall inhibition of MEK. At high concentration (5 μmol/L), both AZD6244 and PD0325901A also inhibited S217 phosphorylation in vitro (data not shown), but we have not investigated their cellular inhibition of MEK activation nor how phosphorylation affects the binding affinity of MEK for compounds other than GSK1120212.

In enzymatic assays we observed that GSK1120212 inhibits the initially u-MEK1/2 with IC₅₀s approximately 1/20th or 1/12th that of pp-MEK1 and pp-MEK2, respectively. Furthermore, we observed that GSK1120212 is at least a 6-fold more potent inhibitor of kinase activity for p-MEK1(S221) compared with pp-MEK1. This suggests that phosphorylation of S217 (and likely S221 as well) reduces the affinity of the compound for MEK1, likely by altering the adjacent activation loop spanning (for MEK1) L²⁰⁶CDFGVSQQLSDMANSFVGR²²⁷ that partially defines the compound binding pocket. This explanation is consistent with structural observations that an earlier MEK inhibitor, PD318088, stabilized the activation loop in an inactive conformation while simultaneously shifting a catalytically critical helix domain (15). Notably, multiple studies have now identified single mutations that confer resistance to allosteric MEK inhibitors by both reducing the binding affinity and simultaneously increasing the basal activity of MEK (20, 21; our unpublished results). Our data suggest that by partially inhibiting MEK activating
phosphorylation, GSK1120212 sustains its binding to MEK and inhibits the accumulation of fully activated pp-MEK that is triggered in some tumors by pathway feedback mechanisms.

In profiling cell lines for response to GSK1120212, we clearly observe the elevated sensitivity of cell lines dependent on activating mutations in the MAPK pathway, particularly BRAF and KRAS. Although there were insufficient numbers for statistical significance, we also observed in several cell lines that activating mutations in PIK3CA correlate negatively with sensitivity in KRAS-mutant cell lines, consistent with recently published observations for MEK inhibitors (22). By far the most sensitive cellular subset, BRAF-mutant cell lines respond to low concentration GSK1120212 (low $gI_{50}$) with increased net cell death in a 72 hours proliferation assay; in contrast, most KRAS-mutant cell lines respond to low concentration GSK1120212 (low $gI_{50}$), but the net cell response is predominantly cytostasis or only growth delay. These results suggest that a MEK inhibitor may have the strongest single-agent therapeutic effect in BRAF-mutant tumors. Although our data show that there should be single-agent activity of GSK1120212 in some KRAS-mutant tumors, for other KRAS tumors MEK inhibition may be insufficient.

![Figure 6. GSK1120212 antitumor activity A. 14-day daily (QDx14) oral (po) dosing of GSK1120212 causes dose-dependent TGI in (A) A-375P(F11) (BRAF<sup>V600E</sup>) and (B) HCT 116 (KRAS<sup>G12D</sup>) tumor xenograft models.](image_url)
these tumors may require combination with inhibitors of the adjacent PI3K signal transduction pathway (e.g., inhibitors of PI3K, AKT, mTOR).

Recent clinical results with the B-Raf inhibitor PLX4032 have revealed promising activity in melanoma patients (23). In addition to showing therapeutic validity of targeting the MAPK pathway, these results suggest two potential benefits from a combination of B-Raf and MEK inhibitors. First, the combination approach has the potential to stave off drug resistance arising from individual mutations that represent an Achilles heel to most kinase inhibitors. Second, the combination may abrogate a reported toxicity of B-Raf inhibitors, increased frequency of squamous cell lesions, attributed to a mechanism in which one B-Raf inhibitor–bound member of a Raf dimer transactivates the other member resulting in downstream phosphorylation of MEK (24). Combination with a MEK inhibitor might be expected to block this effect in nontumor cells.

Multiple MEK inhibitors have now failed to show significant efficacy in clinical trials. CI-1040 produced a range of new recognizable on-target adverse events including skin rash, edema, and diarrhea (4), but unpromising activity. PD0325901 was discontinued due in part to toxicities that included retinal vein occlusions and neuropathy that apparently reflected intolerable drug levels passing the blood barriers of the retina and central nervous system; these toxicities were subsequently modeled in animal models where PD0325901 caused retinal vein occlusion in rabbits (25) and inhibition of p-ERK in the brain (10). We showed that GSK1120212 does not significantly penetrate intact brain nor inhibit brain p-ERK, suggesting that toxicity observed for PD0325901 might be avoided. Both PD0325901 and, more recently, AZD6244 (Arry-142886) showed promising inhibition of ERK phosphorylation in a subset of melanoma patients, but these failed to translate into improved patient outcome in single agent trials (26–29). One interpretation is that previous MEK inhibitor compounds have not achieved the drug properties required to hit a pharmacokinetic “sweet spot,” producing in vivo concentrations that remain within the narrow therapeutic window, raising the question of whether this goal is in fact achievable.

GSK1120212 is orally bioavailable, exceptionally potent and specific, and has a long half-life with a shallow C_{max} to C_{trough} PK profile. These characteristics suit the profile for an inhibitor of tumors dependent on activating mutations in the MAPK pathway, especially those harboring activating mutations in KRAS and BRAF. Because growth inhibition by a MEK inhibitor is generally fully reversible on drug removal, the presumed optimal profile is a sustained steady-state drug concentration in excess of the dose required for activity. In contrast to multiple other MEK inhibitors that required twice-daily dosing in mice to achieve sustained or near-sustained MEK inhibition, GSK1120212 dosed daily at the repeat-dose MTD achieves steady state blood levels that remain more than 250 nmol/L (5× our defined “sensitive” concentration in cells) for 24 hours, causing sustained inhibition of p-ERK. We showed that GSK1120212 does not significantly penetrate intact brain nor inhibit brain p-ERK, suggesting that brain penetration–related toxicities may be avoidable by a favorable drug distribution profile. Recent phase I clinical data for GSK1120212 indicates that it has shown favorable early activity in melanoma patients (30) and is currently being evaluated in phase II clinical trials.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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a rapidly cleared drug: as shown by once-daily divalproex-ER. Clin Drug Investig 2006;26:681–90.
Correction: GSK1120212 (JTP-74057) Is an Inhibitor of MEK Activity and Activation with Favorable Pharmacokinetic Properties for Sustained In Vivo Pathway Inhibition

In this article (Clin Cancer Res 2011;7:989–1000), which was published in the March 1, 2011, issue of Clinical Cancer Research (1), Fig. 4A was incorrectly labeled due to the following production error: The sequence for Day 1 and Day 7 was labeled “2h, 4h, 4h, 24h,” instead of “2h, 4h, 8h, 24h.” The corrected figure appears below. The online version has been corrected and no longer matches the print version.

![Corrected Figure 4A](image)

Reference


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