Identification of 4-anilino-3-quinolinecarbonitrile inhibitors of mitogen-activated protein/extracellular signal-regulated kinase 1 kinase

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Abstract

A high-throughput screen for Ras–mitogen-activated protein kinase (MAPK) signaling inhibitors identified two series (class 1 and 2) of substituted 4-anilino-3-quinolinecarbonitriles as potent (IC_{50}s <10 nmol/L) mitogen-activated protein/extracellular signal-regulated kinase 1 (MEK1) kinase inhibitors. These compounds had cyanoquinoline cores, but differed in their respective aniline groups [1a, 1b: 4-phenoxyphenylaniline; 2a, 2b: 3-chloro-4-(1-methylimidazol-2-sulfanyl)aniline]. These compounds were competitive inhibitors of ATP binding by MEK1 kinase, and they had minimal or no effect on Raf, epidermal growth factor receptor (EGFR), Akt, cyclin-dependent kinase 4 (CDK4), or MK2 kinases at concentrations >100-fold higher than those that inhibited MEK1 kinase. Both class 1 and 2 compounds inhibited *in vitro* growth of human tumor cell lines. A class 2 compound (2b) was the most potent inhibitor of human tumor cell growth *in vitro*, and this effect was linked to distinct suppression of MAPK phosphorylation in cells. Compound 2b did not affect phosphorylation status of other kinases, such as EGFR, Akt, and stress-activated protein (SAP)/c-*jun*-NH kinase (Jnk); nor did it affect overall tyrosine phosphorylation level in cells. However, compound 2b did inhibit MEK1 phosphorylation in cells. Inhibition of MEK1 phosphorylation by 2b was not due to a major effect on Raf kinase activity, because enzyme assays showed minimal Raf kinase inhibition. We believe compound 2b inhibits kinase activity upstream of Raf, and thereby affects MEK1 phosphorylation in cells. Even with the dual effect of 2b on MEK and MAPK phosphorylation, this compound was well tolerated and significantly inhibited growth of the human colon tumor cell line LoVo (at 50 and 100 mg/kg BID, i.p.) in a nude mouse xenograft model. [Mol Cancer Ther 2004;3(6):755–62]

Introduction

Raf/mitogen-activated protein/extracellular signal-regulated kinase (MEK)/mitogen-activated protein kinase (MAPK) proteins play crucial roles in cellular signaling processes. The Ras-MAPK signaling cascade (MAPK module) is found in all eukaryotic organisms and is involved in transmitting signals from the extracellular compartment into the cytosol and nucleus (1, 2). This cascade is activated by GTP-loaded Ras that recruits Raf proteins (A, B, and C) to the inner cell membrane where Raf is activated by phosphorylation. Activated Raf phosphorylates and activates the dual specificity kinases MEK1 and MEK2 (MAP kinase kinase; refs. 3, 4). Activated MEK phosphorylates and activates the MAPKs extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2), which can translocate to the nucleus, and through the phosphorylation of a variety of substrates, modulate cytoplasmic events, such as cell proliferation and differentiation (4, 5).

Because oncogenic forms of Ras are associated with 30% of all cancers, Ras and the downstream kinase effectors of Ras represent attractive targets for pharmacologic intervention (6). Both *in vitro* and *in vivo* studies have shown that Raf and/or MEK are important pharmacologic targets (7-9). Raf/MEK/MAPK cascade assays, as well as individual Raf, MEK, or MAPK activity assays, have been described (10-12). We developed a sensitive, high-throughput Raf/MEK1/MAPK cascade ELISA (13), and extensively screened small molecule libraries to identify inhibitors of this signaling cascade. Using the Raf/MEK1/MAPK cascade ELISA, several potent and specific MEK1 inhibitors were found. For example, both 10*E*-hymenialdisine and 10*Z*-hymenialdisine were potent MEK1 inhibitors (IC_{50}s of 3 and 6 nmol/L, respectively; ref. 14). In cellular assays, 10*E*-hymenialdisine and 10*Z*-hymenialdisine inhibited growth of the human colon tumor line LoVo (14). Further screening also identified 4-anilino-3-quinolinecarbonitriles (CNQ) as potent and specific inhibitors of MEK1 kinase. Two series of CNQ compounds inhibited MEK1 kinase enzymatic activity with IC_{50}s <10 nmol/L. These were the 4-phenoxyphenylaniline (class **1**) CNQs (15, 16) and the 3-chloro-4-(1-methylimidazol-2-ylsulfanyl) aniline (class **2**) CNQs. In this study, we examined the specificity of these compounds for MEK1 kinase inhibition, and characterized the nature of CNQ compound inhibition of MEK1

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kinase (i.e., allosteric versus competitive). We examined the potency of these compounds concerning their ability to inhibit the growth of a diverse panel of human tumor cell lines *in vitro*. Additionally, in cellular assays, we evaluated the CNQ compound effect on phosphorylation status of RAS-MAPK module proteins, as well as other non-Ras-MAPK module signaling proteins. *In vivo* antitumor activity, and a potent and apparently Ras-MAPK module specific activity profile, for a class **2** CNQ compound suggests that this type of MEK1 inhibitor could become a candidate for clinical evaluation in treatment of tumors with an activated Ras-MAPK pathway.

Materials and Methods

Materials

The 4-phenoxyphenylaniline series (class **1** compounds) were prepared by previously described methods (15, 16). A similar method was used to prepare the 3-chloro-4-(1-methylimidazol-2-sulfanyl)aniline series (class **2** compounds; ref. 17).

Raf/MEK1/MAPK ELISA

Production of proteins and ELISA protocol was as described previously (13). Activated Raf was used to phosphorylate and activate glutathione *S*-transferase (GST)-MEK1 that in turn phosphorylated GST-MAPK. The phosphorylation of GST-MAPK by GST-MEK1 was detectable in ELISA format by a phospho-specific MAPK antibody (Sigma-Aldrich Corp., St. Louis, MO). The Sigma phospho-specific MAPK antibody recognized phosphorylation of the TEY sequence at amino acids 202 to 204 on MAPK. The ELISA protocol has been described (13). Various kinase inhibitors or random small molecules were prepared in 100% DMSO and were diluted from stock solutions into assay reactions so that DMSO concentration never exceeded 1% of total reaction volume. Reactions were incubated (0 to 300 min) at 25°C, and reactions were stopped by addition of EDTA (final concentration = 70 mmol/L). Data were collected from a Wallac Victor model Plate Reader and analyzed in Excel for single point percentage inhibition and IC_{50} determinations. The ELISA was DELFIA (dissociation-enhanced lanthanide fluorescence immunoassay) in format.

Assays for Raf and MEK1 Enzyme Selectivity

Raf. Activated Raf phosphorylation of MEK1 (30 nmol/L) was carried out in assay buffer with 100 μmol/L ATP. Kinase inhibitors, when present, were at 1 to 10,000 nmol/L, with final DMSO (compound solvent) concentration of 1% in all reactions. Raf kinase reactions were run for 0 to 300 minutes at 25°C, and reactions were stopped by addition of EDTA (final concentration = 70 mmol/L). MEK1 phosphorylation was determined by Western analysis using a-phospho-MEK1 antibody (Cell Signaling Technology, Beverly, MA). MEK1 phosphorylation was quantified by densitometry of X-ray film from MEK1 Western blots (13).

MEK1. MEK1 phosphorylation of MAPK was directly measured by use of activated MEK1 (Upstate Biotech, Lake Placid, NY) in an ELISA protocol that omitted addition of activated Raf. All other aspects of the MEK1/MAPK ELISA were identical to the Raf/MEK1/MAPK ELISA. When kinase inhibitor compounds were tested in the MEK1/MAPK ELISA, they were at 1 to 1,000 nmol/L, with final DMSO (compound solvent) concentration of 1% in all reactions.

Cellular Assays

Human tumor cell lines from American Type Culture Collection used were: LoVo (colon), CaCo-2 (colon), HCT116 (colon), LNCap (prostate), T24 (bladder), HT119 (bladder), MiaPaca (pancreas), and BXPC3 (pancreas). These cell lines were exposed to varying concentrations of CNQ compounds. Cells were grown in RPMI 1640 or DMEM with 10% fetal bovine serum supplemented with L-glutamine and penicillin/streptomycin. Cyanoquinoline compounds were prepared as a 10 mg/ml stock solution in 100% DMSO, and then diluted with complete growth media to desired concentrations and applied to cells; final DMSO concentration never exceeded 0.25%. Growth inhibition was measured by staining cells with sulforhodamine B (14).

Cell exposure to compounds for Western blot analysis of phosphoproteins was for either short (4 hours) or long (96 hours) duration. Exposure of cells to compounds was in complete media with serum. Antibodies used were from Sigma or Cell Signaling Technology.

In vivo Tumor Xenografts

Athymic *nu/nu* female mice were implanted s.c. with LoVo (colon) human tumor cells. When tumors reached an average mass between 80 and 200 mg (day 0), animals were randomized into treatment groups each containing either 5 or 10 animals. Mice were treated i.p. with the following: compound **1b** or **2b** prepared in a vehicle containing 2% Tween 80/D5W or vehicle alone (2% Tween 80/D5W). Compound was given to mice at 50 and 100 mg/kg doses twice daily (BID). Tumor mass [(length × width^2)/2] was determined approximately once a week for up to 28 days. Animal body weight and behavior was monitored for the duration of *in vivo* experiments.

Results

Inhibition of the Raf/MEK1/MAPK Cascade ELISA by 1a, 1b, 2a, and 2b

The Raf/MEK1/MAPK cascade ELISA found CNQ class **1** (**1a**, **1b**) and class **2** (**2a**, **2b**) compounds (Fig. 1) to be potent inhibitors of assay signal. The ELISA end point was the measurement of MAPK phosphorylation on Threonine (T) 202 and Tyrosine (Y) 204 by a phospho-specific monoclonal antibody. The sensitivity of the Raf/MEK1/MAPK ELISA was confirmed by testing specific and generic kinase inhibitors, such as staurosporine, tyrphostin, olomoucine, genistein, lavendustin A, and apigenin (Table 1). The Raf/MEK1/MAPK ELISA sensitivity to staurosporine (IC_{50} = 3 nmol/L), and lack of response to tyrphostin, olomoucine, genistein, lavendustin A, and apigenin confirmed assay performance. Table 1 lists ELISA data for **1a**, **1b**, **2a**, and **2b**. All of these compounds had

IC_{50} values below 10 nmol/L. In comparison, the commercially available MEK1 inhibitors PD98059 and U0126 had IC_{50}s of 2800 and 800 nmol/L in the Raf/Mek1/MAPK ELISA.

Specificity of Class 1 and Class 2 Compounds

The specific target of compound inhibition in the Raf/MEK1/MAPK ELISA was identified by kinase assays that directly measured Raf phosphorylation of MEK1, or MEK1 phosphorylation of MAPK, using either MEK1 or MAPK phospho-specific antibodies. Additionally, this group of cyanoquinoline compounds was tested in epidermal growth factor receptor (EGFR), MK2, Akt, and cyclin-dependent kinase 4 (CDK4) kinase assays. Table 2 lists data from this battery of kinase assays. It was found that **1a**, **1b**, **2a**, and **2b** all inhibited MEK1 at IC_{50} values of <10 nmol/L, in good agreement with cascade ELISA data (Table 1). Detectable, but less potent, inhibition of both Raf kinase and EGFR kinase (IC_{50}s = 700 to 850 nmol/L) was observed for these compounds. Only one compound, **1a**, had MEK1 and EGFR IC_{50} values that were within 100-fold of each other (Table 2).

Kinetic Studies: Site of Action of Class 1 (1b) and Class 2 (2b) Compounds

To determine what type of MEK1 inhibitors class **1** and class **2** compounds were, we carried out double reciprocal analysis varying protein substrate, ATP, and inhibitor concentrations in kinase enzyme experiments. Data were analyzed by using the Enzyme Kinetics module of Sigmaplot 8.0. Figure 2 shows a representative graph indicating that compound **2b** behaved as a competitive inhibitor of ATP binding by MEK1 when ATP concentration was varied and MAPK held constant. A K_i value of 3.9 nmol/L was derived by plotting the slopes of the double reciprocal analysis against the concentration of **2b** (not shown). When

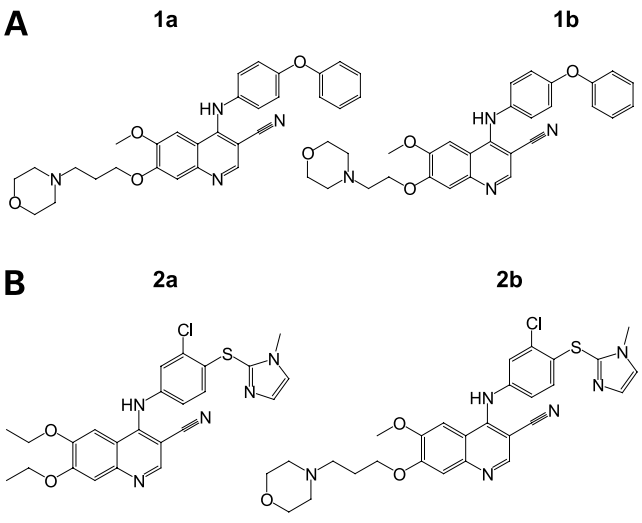

Figure 1. High-throughput screening efforts identified potent Ras-MAPK pathway inhibitors. MEK1 specific CNQ leads found by high-throughput screening include class **1** compounds **1a** and **1b** (4-phenoxyphenylaniline compounds) (**A**) and class **2** compounds **2a** and **2b** [3-chloro-4-(1-methylimidazol-2-ylsulfanyl)aniline compounds] (**B**).

Table 1. Raf/MEK1/MAPK ELISA signal inhibition by various kinase inhibitors and CNQ compounds

Compound	IC_{50} (nmol/L)	Comment
SKB-203580	160	Raf/p38 inhibitor
PD 98059	2,800	MEK1 inhibitor
PP3	300	Lck inhibitor
EKI-569	2,000	EGFR inhibitor
Staurosporine	3	Generic kinase inhibitor
Olomoucine	>10,000	Serine/Threonine kinase inhibitor
Tyrphostin	>10,000	Tyrosine kinase inhibitor
Genistein	>10,000	Tyrosine kinase inhibitor
Lavendustin A	>10,000	Tyrosine kinase inhibitor
Apigenin	>10,000	Serine/Threonine kinase inhibitor
Wyeth Compounds	IC_{50} (nmol/L)	Comment
1a	5	MEK1 inhibitor
1b	6	MEK1 inhibitor
2a	5	MEK1 inhibitor
2b	1	MEK1 inhibitor

MEK1 activity was assayed at various MAPK concentrations (ATP constant) and increasing amounts of **2b**, this class **2** compound was not competitive for MAPK binding by MEK1 (not shown). Similar analysis of **1a**, **1b**, and **2a** showed that they also were competitive inhibitors of ATP binding by MEK1, and they were not competitive for MAPK substrate binding (data not shown).

Cell Growth Inhibition by Class 1 and Class 2 Compounds

Cell growth inhibition by these MEK1 inhibitors was tested in a group of human tumor cell lines that were both wild-type (wt) mutant at the *ras* locus. Previously, we found that the human colon tumor line LoVo was sensitive to low nanomolar concentrations of FTI-276, a protein farnesyl transferase inhibitor that impairs Ras function (14, 18). In contrast CaCo-2 (wt at the *ras* locus) were >20-fold less sensitive to FTI-276 (14). We, therefore, assessed whether the MEK1 inhibitors described here had the same effect on LoVo and CaCo-2, and we also evaluated compound effect on the several additional tumor cell lines (wt and mutant at the *ras* locus) listed in Table 3.

None of the MEK1 inhibitors described here were potent inhibitors of CaCo-2 growth (IC_{50}s > 1,000 nmol/L). In contrast, the class **1** and class **2** compounds did inhibit growth of the other tumor cell lines listed in Table 3. Growth inhibition varied between compound classes, with compound **2b** showing the most potent and broadest range of tumor cell growth inhibition. Tumor cell lines affected by **2b** were from a diverse group of tissues that varied in *ras* status. LoVo (colon) was the most sensitive cell line (IC_{50} = 5 nmol/L), and HCT116, another human colon

Table 2. Specificity of class 1 (1a, 1b) and class 2 (2a, 2b) CNQs in kinase assays

Kinase	Class	Compound	IC_{50} (nmol/L)
Raf	**1**	**1a**	800
	1	**1b**	850
	2	**2a**	850
	2	**2b**	850
MEK1	**1**	**1a**	7
	1	**1b**	6
	2	**2a**	5
	2	**2b**	1
EGFR	**1**	**1a**	700
	1	**1b**	700
	2	**2a**	700
	2	**2b**	700
MK2	**1**	**1a**	>1,000
	1	**1b**	>1,000
	2	**2a**	>1,000
	2	**2b**	>1,000
Akt	**1**	**1a**	>1,000
	1	**1b**	>1,000
	2	**2a**	>1,000
	2	**2b**	>1,000
CDK4	**1**	**1a**	>1,000
	1	**1b**	>1,000
	2	**2a**	>1,000
	2	**2b**	>1,000

tumor line, was the least sensitive (IC_{50} = 450 nmol/L) to the growth inhibitory effects of **2b**. Compound **2b** also potently inhibited growth of LNCap (prostate) and BXPC3 (pancreas) cell lines (IC_{50}s ≤ 50 nmol/L).

Additional class **1** and class **2** compound analogues[3] tested in cellular assays confirmed that class **2** compounds were indeed more potent inhibitors of tumor cell line cell growth than class **1** compounds. The class **2** compound exception was **2a**, which did show good activity versus LoVo (IC_{50} = 15 nmol/L), but due to poor solubility in aqueous solutions (i.e., cell growth media) had limited *in vitro* activity versus most tumor cell lines (Table 3).

Class 1 and Class 2 Compound Specificity in Cells

Compounds **1b** and **2b** were examined for their ability to suppress MAPK phosphorylation in LoVo cells. Cell exposure to these compounds was for either short (4 hours) or long (96 hours) duration. With either protocol, both of these compounds effectively suppressed MAPK phosphorylation (data shown in Figs. 3 and 4 are from 96 hours exposure to compounds). Figure 3 shows that the class **1** CNQ, **1b**, suppressed MAPK phosphorylation (p-MAPK) in LoVo cells, without diminishing total MAPK protein. The IC_{50} for this effect on p-MAPK was 300 nmol/L (Table 4), in good agreement with data showing **1b** inhibitory effect on LoVo cell growth listed in Table 3. Compound **1b** had no effect on MEK1 phosphorylation (p-MEK1) levels, nor did it affect overall MEK1 protein levels (Fig. 3). These data indicate no gross toxicity by **1b**.

In contrast, in LoVo cells exposed to the compound **2b**, not only was p-MAPK inhibited at a considerably lower concentration (IC_{50} = 0.01 nmol/L; Table 4), but **2b** also unexpectedly suppressed p-MEK1 levels (Fig. 4). Compound **2b** effect on p-MEK1 (IC_{50} = 8 nmol/L, Table 4) correlated with the IC_{50} observed for LoVo cell growth inhibition (Table 3). There was no effect on MAPK or MEK1 total protein levels by **2b** (Fig. 4), indicating that compound effect on p-MAPK and p-MEK1 levels was not due to compound toxicity.

Because **2b** suppressed p-MEK1 and p-MAPK in LoVo cells, yet had only minimal activity versus Raf kinase in enzyme assays, we tested specificity of both class **2** and class **1** compounds by examining the phosphorylation status of signaling proteins outside the Ras-MAPK pathway in LoVo cells. Table 4 summarizes phospho-blot data for LoVo cells exposed (96 hours) to these compounds. Compound **2b** had no effect on phosphorylation status of stress-activated protein (SAP), Akt, or EGFR kinases; nor did they affect overall cell phospho-tyrosine levels at concentrations from 1 to 1,000 nmol/L. The class **1** CNQs also had no effect on phosphorylation status of stress-activated protein (SAP), Akt, or EGFR kinases; nor did

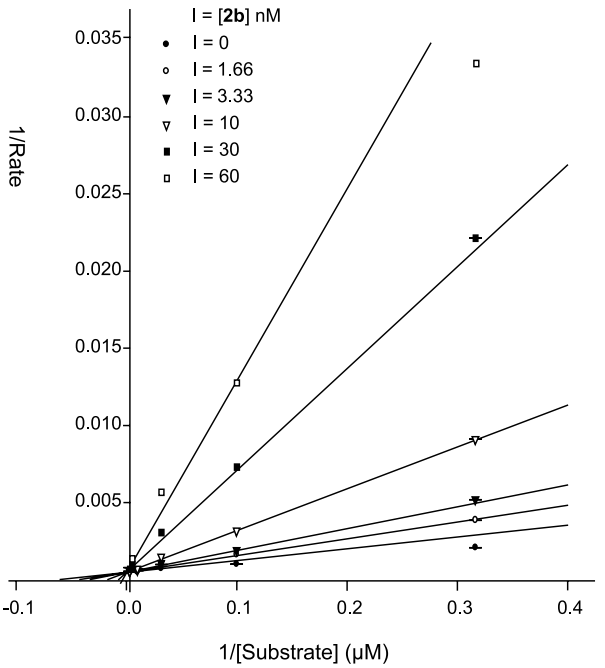

Figure 2. Compound **2b** (class **2**) was a competitive inhibitor of ATP binding by MEK1. Data were analyzed by using the Enzyme Kinetics module of Sigmaplot 8.0. Double reciprocal plots from experiments in which MEK1 and MAPK concentrations were held constant, whereas ATP concentration was varied, yielded lines that intersected the *Y* axis in unison, indicating competitive inhibition.

[3] Mallon R, Wojciechowicz D, Collins K, Berger D, Dutia M, and Powell D. *In vitro* and *in vivo* evaluation of 4-anilino-3-quinolinecarbonitrile inhibitors of MEKI kinase, manuscript in preparation.

Table 3. Humlan tumor cell line growth inhibition by class 1 (1a, 1b) and class 2 (2a, 2b) CNQs

IC_{50} (nmol/L)							
Cell Line	Origin	*Ras* Status	FTI-276	Class **1** **1a**	Class **1** **1b**	Class **2** **2a**	Class **2** **2b**
LoVo	Colon	K-*ras* 13D	100 ± 22	400 ± 27	380 ± 22	15 ± 7	7 ± 3
HCT116	Colon	K-*ras* 13D	50 ± 14	>1,000	900 ± 133	900 ± 230	450 ± 61
LNCap	Prostate	wt (wild-type)	>1,000	1,000 ± 167	>1,000	600 ± 121	50 ± 15
T24	Bladder	H-*ras* 12V	100 ± 13	950 ± 141	900 ± 168	450 ± 70	100 ± 26
MiaPaca	Pancreas	K-*ras* 12C	>1,000	500 ± 49	>1,000	350 ± 75	200 ± 90
BXPC3	Pancreas	wt	>1,000	500 ± 58	410 ± 29	300 ± 90	40 ± 10
CaCo-2	Colon	wt	>1,000	>1,000	>1,000	>1,000	>1,000

they affect overall cell phospho-tyrosine levels at concentrations from 1 to 1,000 nmol/L. Poor solubility of **2a** in aqueous solutions (cell culture media) prevented accurate evaluation of this compound's *in vitro* cellular effects. Finally, compound **2b** effect on both MEK1 and MAPK phosphorylation was not confined to LoVo cells because this same effect was observed when BXPC3 (pancreas) and SW620 (colon) tumor cells were exposed to **2b** (data not shown).

In vivo

Figure 5 shows that compound **2b** inhibited LoVo tumor growth when given to animals by i.p. route at both 50 and 100 mg/kg BID. Compound administration was stopped at day 21, at which time there was 78% tumor growth inhibition at the 100 mg/kg dose level, and 54% tumor growth inhibition at the 50 mg/kg dose level. Tumors were measured 1 week after compound dosing stopped and tumor growth inhibition of 81% and 56% was still observed for animals treated at 100 and 50 mg/kg dose levels, respectively. All animals treated with compound **2b** showed no gross toxicities, no weight loss, nor any abnormal behavior. All tumor inhibition results were found to be statistically significant compared with control group (vehicle only) as determined by Student's two-tailed *t* test analysis of data.

Compound **1b** was also tested *in vivo* against LoVo tumors at both 50 and 100 mg/kg BID by i.p. route. This compound did not show any *in vivo* efficacy (data not shown). Compound **2b** had poor solubility at the 100 mg/kg

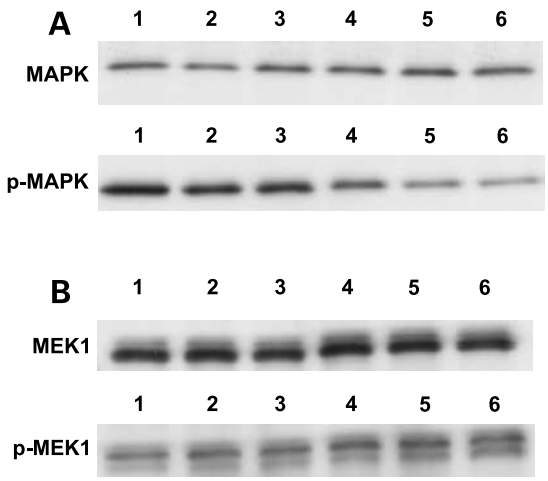

Figure 3. Compound **1b** (class **1**) inhibited MAPK phosphorylation in LoVo (human colon tumor) cells. **A,** LoVo cells were exposed to **1b** for 96 hours at concentrations of (*1*) 0 nmol/L, (*2*) 10 nmol/L, (*3*) 30 nmol/L, (*4*) 100 nmol/L, (*5*) 300 nmol/L, and (*6*) 1,000 nmol/L. The IC_{50} for compound **1b** inhibition of phosphorylated MAPK (*p-MAPK*) was 300 nmol/L (by densitometry). **B,** compound **1b** had no effect on MEK1 or phosphorylated-MEK1 (*p-MEK1*) levels at any compound concentration tested [(*1*) 0 nmol/L, (*2*) 10 nmol/L, (*3*) 30 nmol/L, (*4*) 100 nmol/L, (*5*) 300 nmol/L, and (*6*) 1000 nmol/L].

Figure 4. Compound **2b** (class **2**) inhibited MAPK (**A**) and MEK1 (**B**) phosphorylation in LoVo (human colon tumor) cells. **A,** LoVo cells were exposed to **2b** for 96 hours at concentrations of (*1*) 0 nmol/L, (*2*) 0.01 nmol/L, (*3*) 0.1 nmol/L, (*4*) 1.0 nmol/L, and (*5*) 10.0 nmol/L. Compound **2b** had an IC_{50} value of 0.01 nmol/L (densitometry) for inhibition of phosphorylated-MAPK (*p-MAPK*). **B,** compound **2b** also reduced phosphorylated-MEK1 (*p-MEK1*) when LoVo cells were exposed to (*1*) 0 nmol/L, (*2*) 0.01 nmol/L, (*3*) 0.1 nmol/L, (*4*) 1.0 nmol/L, and (*5*) 10.0 nmol/L compound concentrations. The IC_{50} = 8 nmol/L (by densitometry) corresponds well with concentrations that caused LoVo cell growth inhibition.

Table 4. Summary IC_{50} (nmol/L) phospho-blot data from LoVo (Human Colon Tumor) cells exposed to CNQs

	MEK	MAPK	SAP	AKT	EGF-R	P-TYR
Class **1** Compound						
1a	>1,000	380	>1,000	>1,000	>1,000	>1,000
1b	>1,000	300	>1,000	>1,000	>1,000	>1,000
Class **2** Compound						
2b	8	0.01	>1,000	>1,000	>1,000	>1,000
2a	nd*	nd	nd	nd	nd	nd

NOTE: All IC_{50} data derived from densitometry done on Western blots (see Materials and Methods).
*nd, not done due to poor compound solubility.

dose level, because dosing solutions had turbid appearance. We believe that the antitumor activity of compound **2b** was directly related to its more potent profile in *in vitro* cellular assays.

Discussion

In this report, we have shown that two types of CNQs, the 4-phenoxyphenylaniline (class **1**) and 3-chloro-4-(1-methylimidazol-2-sulfanyl)aniline (class **2**) compounds were potent inhibitors of the MEK1 kinase *in vitro* (Tables 1 and 2). The class **1** and class **2** compounds inhibited MEK1 kinase at IC_{50} concentrations below 10 nmol/L (Table 1). The potency of these compounds compares favorably with the Pfizer MEK1 inhibitor, CI-1040, which has a reported IC_{50} of 17 nmol/L (8). CI-1040 was advanced to clinical trials (19). Unlike the MEK1 inhibitors CI-1040, PD98059, or U0126 that all apparently act by an allosteric mechanism (20), the CNQs were competitive inhibitors of ATP binding by MEK1 (Fig. 2). In direct enzyme assays, class **1** and **2** compounds inhibited MEK1 phosphorylation of MAPK, but had a comparatively modest effect on Raf kinase phosphorylation of MEK1 (Table 2).

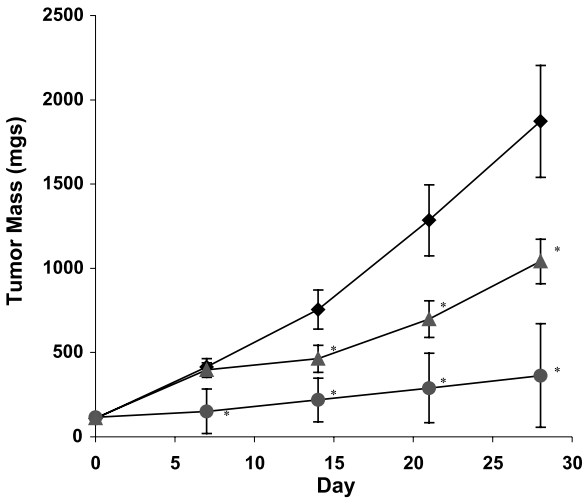

Figure 5. *In vivo* efficacy of compound **2b**. Female *nu/nu* mice were injected with 1.5×10^6 LoVo colon tumor cells. Mice bearing LoVo tumors of ~100 mg were treated BID by i.p. route with compound **2b** in 2% Tween 80/D5W at 50 mg/kg (▲) and 100 mg/kg (●) for 21 days. Control group (♦) received vehicle (2% Tween 80/D5W) only. Statistical significance was determined by Student's two-tailed *t* test for all doses compared with vehicle control. *, $P \leq 0.02$.

The CNQs had different potency profiles when tested for growth inhibition of human tumor cell lines. The class **2** compound, **2b**, had the best potency against the broadest range of human tumor cell lines (IC_{50}s from 5 to 450 nmol/L; Table 3). The most sensitive lines were LoVo (colon), LNCap (prostate), BXPC3 (pancreas), and HT1197 (bladder) tumor cells (IC_{50}s <60 nmol/L). In comparison, the farnesyl transferase inhibitor FTI-276 (18) only showed a potency advantage over **2b** against the human colon tumor cell line HCT116 (Table 3). The MEK1 inhibitors U0126 and PD98059 both had IC_{50} values >1,000 nmol/L when tested against the cell lines listed in Table 3 (data not shown).

Compound **2b** caused cell growth inhibition in cells with both mutant and wt *ras*, indicating that MEK1 inhibition is an important target in tumors with Ras-MAPK pathway activation whether or not such activation originates from mutant *ras*. It is likely that tumors that have Ras-MAPK pathway activation resulting from mutant or inappropriately activated forms of receptor tyrosine kinases (21), or mutant forms of Raf [e.g., B-Raf (22)] will be susceptible to MEK1 and/or Raf inhibitors. Pre-clinical data on the *in vitro* and *in vivo* profiles of the Bayer Raf inhibitor (BAY43-9006), and the Pfizer MEK1 inhibitor (CI-1040) on human tumor cells (both *ras* mutant and wt) support this hypothesis (8, 9, 23).

An unexpected result from this study was the effect of compound **2b** on MEK1 phosphorylation in LoVo cells. Compound **2b** inhibited MEK1 phosphorylation in LoVo cells at an IC_{50} of 8 nmol/L (Table 4), and this corresponded with the IC_{50} for cell growth inhibition by this compound on LoVo (IC_{50} = 5 nmol/L, Table 3). This occurred despite enzyme assay data showing minimal compound effect on Raf kinase. Additionally, inhibition of MAPK phosphorylation occurred at a >100-fold lower level than that observed for **2b** effect on MEK1 phosphorylation (Table 4). Compound **2b** effects on a target (or targets) other than Raf are likely the basis of these effects.

The effects of 2b on target(s) upstream of Raf must cause a reduced pool of activated MEK1 available to phosphorylate MAPK. This effect, in conjunction with the direct effect of **2b** on MEK1 kinase activity, may account for the potent inhibition (IC_{50} = 0.01 nmol/L) of MAPK phosphorylation by **2b** in LoVo cells. This observation corresponds with the predicted sensitivity of the Raf/MEK1/MAPK signaling cascade to inhibitors (i.e., Raf > MEK1 > MAPK; refs. 24, 25). This sensitivity profile results from the distributive (non-processive) mechanism of both Raf and MEK1 in which the rate of MEK1 activation depends on the concentration of Raf squared; and similarly the rate of MAPK activation is dependent on the concentration of MEK1 squared (13, 24, 25).

We have not yet determined the mechanism by which **2b** suppresses MEK1 phosphorylation and also causes very potent suppression of p-MAPK in cells. Certainly a component of the effect on p-MAPK in cells must be the direct inhibition of MEK1 by **2b** (Tables 1 and 2). But we expect **2b**, like many kinase inhibitors, has additional activities beyond those for which it was originally targeted. Gleevec inhibits the activity of at least three kinases *in vitro*: Abl, c-Kit, and PDGFR (26). As an even more relevant example, a recent report showed that the MEK1 inhibitors U0126 and PD98059 inhibited KSR (kinase suppressor of Ras) associated phosphorylation of Raf in HL-60 human promyeloblastic leukemia cells (27). We are, therefore, testing **2b** for inhibitory effects on KSR or non-receptor kinases implicated in Raf activation (e.g., Pak1 or C-Tak1; refs. 28-34). Additionally, we are extending analysis of **2b** to examine any effects on receptor tyrosine kinases besides EGFR. These receptors are present on many tumor cell lines (35), and possible attenuation of their activity by **2b** could help account for the potent inhibitory effects on MEK1 and MAPK phosphorylation.

Suppression of p-MEK1 and p-MAPK levels by **2b** did not translate into general compound toxicity at the cellular or *in vivo* (below) levels. *In vitro*, two sets of experimental data support this conclusion. One, CaCo-2 cell growth was not affected by **2b** at concentrations >1,000 nmol/L (Table 3). And secondly, phosphorylation status of non-Ras-MAPK module signaling proteins, including overall tyrosine phosphorylation levels, were not affected by **2b** at concentrations >1,000 nmol/L (Table 4). Compound **2b** inhibition of MEK1 phosphorylation was not unique to LoVo cells, because the same effect was observed *in vitro* in BXPC3 and SW620 (colon) tumor cells exposed to **2b** (not shown).

Despite the unresolved mechanism of action in cells, significant *in vivo* antitumor activity of compound **2b** was observed at doses of both 50 and 100 mg/kg BID (i.p.) in the LoVo xenograft model. *In vivo* activity of **2b** correlated with both its potent inhibition of MAPK phosphorylation, and its growth inhibitory effects on the LoVo human colon tumor cell line *in vitro* (Tables 3 and 4). Compound **2b** was well tolerated by mice at both dose levels, suggesting that the relatively selective nature of this compound for the Ras-MAPK pathway could yield a clinical candidate with an acceptable safety profile. Compound **1b** had no *in vivo* activity, which correlated with its less potent *in vitro* profile (Tables 3 and 4). Compound **2b** did have poor solubility at 100 mg/kg dose level concentrations. We are addressing this problem both by altering the formulation for *in vivo* delivery of this compound, and by structural changes to the **2b** molecule.

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References

1. McCormick F, Wittinghofer A. Interactions between Ras proteins and their effectors. Curr Opin Biotechnol 1996;7:449-56.

2. Robinson MJ, Cobb MH. Mitogen-activated protein kinase pathways. Curr Opin Cell Biol 1997;9:180-6.

3. Lewis TS, Shapiro PS, Ahn NG. Signal transduction through MAP kinase cascades. Adv Cancer Res 1998;74:49-139.

4. Kolch W. Meaningful relationships: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions. Biochem J 2000;351:289-305.

5. Lavoie JN, L'Allemain G, Brunet A, Muller R, Pouyssegur J. Cyclin D1 expression is regulated positively by the p42/p44 MAPK and negatively by the p38/HOGMAPK pathway. J Biol Chem 1996;271:20608-16.

6. Barbacid M. RAS genes. Annu Rev Biochem 1987;56:779-822.

7. O'Dwyer PJ, Stevenson JP, Gallagher M, et al. c-Raf-1 depletion and tumor responses in patients treated with the c-Raf-1 antisense oligodeoxynucleotide ISIS 5132 (CGP 69846A). Clin Cancer Res 1999;5:3977-82.

8. 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.

9. Lackey K, Cory M, Davis R, et al. The discovery of potent cRaf-1 kinase inhibitors. Bioorg & Med Chem Lett 2000;10:223-6.

10. Alessi DR, Cuenda A, Cohen P, Dudley DT, Saltiel AR. PD-098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase *in vitro* and *in vivo*. J Biol Chem 1995;270:27489-94.

11. Alessi DR, Cohen P, Ashworth A, Cowley S, Leevers SJ, Marshall CJ. Assay and expression of mitogen-activated protein kinase, MAP kinase kinase, and Raf. Methods Enzymol 1995;255:279-90.

12. McDonald OB, Chen WJ, Ellis B, et al. A scintillation proximity assay for the Raf/MEK/ERK kinase cascade: high-throughput screening and identification of selective enzyme inhibitors. Anal Biochem 1999;268:318-29.

13. Mallon R, Feldberg LR, Kim SC, et al. An enzyme-linked immunosorbent assay for the Raf/MEK1/MAPK signaling cascade. Anal Biochem 2001;294:48-54.

14. Tasdemir D, Mallon R, Greenstein M, et al. Aldisine alkaloids from the Philippine sponge *Stylissa massa* are potent inhibitors of mitogen-activated protein kinase kinase-1 (MEK1). J Med Chem 2002;45:529-32.

15. Zhang N, Wu B, Powell D, et al. Synthesis and structure-activity relationships of 3-cyano-4(phenoxyanilino)quinolines as MEK (MAPKK) inhibitors. Bioorg & Med Chem Lett 2000;10:2825-31.

16. Zhang N, Wu B, Eudy N, et al. MEK (MAPKK) inhibitors. Part 2. Structure-activity relationships of 4-anilino-3-cyano-6,7-dialkoxyquinolines. Bioorg & Med Chem Lett 2001;11:1407-13.

17. Berger D, Dutia M, Powell D, et al. Synthesis and evaluation of 4-anilino 6,7-dialkoxy-3-quinolinecarbonitriles as inhibitors of kinases of the Ras-MAPK signaling cascade. Bioorg & Med Chem Lett 2003;13:3031-4.

18. Lerner EC, Zhang TT, Knowles DB, Qian Y, Hamilton AD, Sebti SM. Inhibition of the prenylation of K-Ras, but not H- or N-Ras, is highly resistant to CAAX peptidomimetics and requires both a farnesyltransferase

and a geranylgeranyltransferase inhibitor in human tumor cell lines. Oncogene 1998;15:1283-8.

19. Sebolt-Leopold JS. Development of anticancer drugs targeting the MAP kinase pathway. Oncogene 2000;19:6594-9.

20. English JM, Cobb MH. Pharmacological inhibitors of MAPK pathways. Trends Pharmacol Sci 2002;23:40-5.

21. Herrera R, Sebolt-Leopold JS. Unraveling the complexities of the Raf/MAP kinase pathway for pharmacological intervention. Trends Mol Med 2002;8 Suppl:S27-31.

22. Davies H, Bignell GR, Cox C, et al. Mutations of the BRAF gene in human cancer. Nature 2002;417:949-54.

23. Lyons JF, Wilhelm S, Hibner B, Bollag G. Discovery of a novel Raf kinase inhibitor. Endocr Relat Cancer 2001;8:219-25.

24. Huang CY, Ferrell JE. Ultrasensitivity in the mitogen-activated protein kinase cascade. Proc Natl Acad Sci USA 1996;93:10078-83.

25. Burack WR, Sturgill TW. The activating dual phosphorylation of MAPK by MEK is nonprocessive. Biochemistry 1997;36:5929-33.

26. Atkins JH, Gershell LJ. From the analyst's couch: selective anticancer drugs. Nat Rev Cancer 2002;2:645-6.

27. Wang X, Studzinski GP. Phosphorylation of Raf-1 by kinase suppressor of ras is inhibited by ''MEK-specific'' inhibitors PD 098059 and U0126 in differentiating HL60 cells. Exp Cell Res 2001;268:294-300.

28. Roy F, Therrien M. MAP kinase module: the Ksr connection. Curr Biol 2002;12:R325-7.

29. Xing HR, Lozano J, Kolesnick R. Epidermal growth factor treatment enhances the kinase activity of kinase suppressor of Ras. J Biol Chem 2000;275:17276-80.

30. Anselmo AN, Bumeister R, Thomas JM, White MA. Critical contribution of linker proteins to Raf kinase activation. J Biol Chem 2002;277:5940-3.

31. Xing HR, Kolesnick R. Kinase suppressor of Ras signals through Thr269 of c-Raf-1. J Biol Chem 2001;276:9733-41.

32. Zang M, Waelde CA, Xiang X, Rana A, Wen R, Luo Z. Microtubule integrity regulates Pak leading to Ras-independent activation of Raf-1. Insights into mechanisms of Raf-1 activation. J Biol Chem 2001;276:25157-65.

33. Muller J, Ory S, Copeland T, Piwnica-Worms H, Morrison DK. C-TAK1 regulates Ras signaling by phosphorylating the MAPK scaffold, KSR1. Mol Cell 2001;8:983-93.

34. Dhillon AS, Kolch W. Untying the regulation of the Raf-1 kinase. Arch Biochem Biophys 2002;404:3-9.

35. Pawson T. Regulation and targets of receptor tyrosine kinases. Eur J Cancer 2002;38 Suppl 5:S3-10.

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