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Synthesis and Evaluation of 4-Anilino-6,7-dialkoxy-3-quinolinecarbonitriles as Inhibitors of Kinases of the Ras-MAPK Signaling Cascade

Dan Berger,[a,*] Minu Dutia,[a] Dennis Powell,[a] Biqi Wu,[a] Allan Wissner,[a] Diane H. Boschelli,[a] M. Brawner Floyd,[a] Nan Zhang,[a] Nancy Torres,[a] Jeremy Levin,[a] Xuemei Du,[a] Donald Wojciechowicz,[b] Carolyn Discafani,[b] Constance Kohler,[b] Steven C. Kim,[b] Larry R. Feldberg,[b] Karen Collins[b] and Robert Mallon[b]

[a]*Chemical Sciences, Wyeth Research, Pearl River, NY 10965, USA*
[b]*Discovery Oncology, Wyeth Research, Pearl River, NY 10965, USA*

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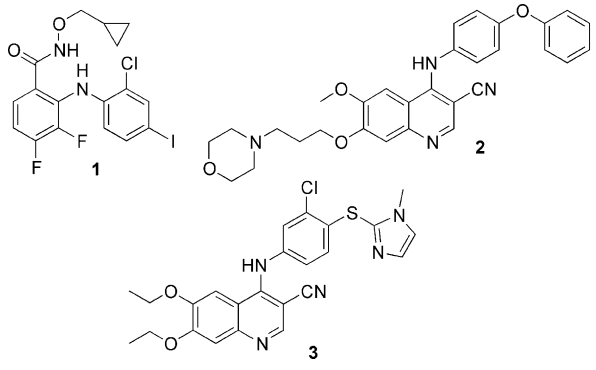
Abstract—4-[3-Chloro-4-(1-methyl-1H-imidazol-2-ylsulfanyl)]anilino-6,7-diethoxy-3-quinolinecarbonitrile (**3**) was identified as a MEK1 kinase inhibitor with exceptional activity against LoVo cells. The structure–activity relationships of the C-4 aniline substituents were explored, and water-solubilizing groups were added at the C-7 position to improve physical properties. Secondary cellular assays revealed that a compound possessing the appropriate aniline substituents inhibited MEK1 as well as MAPK phosphorylation, thereby acting as a dual inhibitor of the Ras-MAPK signaling cascade.

The Ras-MAPK signaling cascade transmits mitogenic stimuli from growth factor receptors and activated Ras to the cell nucleus. Research on the Ras-MAPK signaling pathway identified Raf as the downstream effector of Ras.[1] Activated Raf phosphorylates two serine residues on MAP kinase kinase (MEK1), which in turn activates Mitogen Activated Protein Kinases (MAPK) by phosphorylation of threonine and tyrosine residues.[2,3]



Activated MAPK translocates to the nucleus, mediating cell growth and proliferation through the phosphorylation of various substrates.[4] Inappropriate Ras activation is associated with ~30% of all human cancers, making the kinase components of the Ras-MAPK signaling cascade attractive targets for pharmaceutical intervention.[5]

Researchers at Pfizer (formerly Parke-Davis) identified **1** as a potent MEK1 kinase inhibitor with oral activity in vivo against selected tumor lines.[6] This compound, currently known as CI-1040, has entered clinical trials.[7] Recently, researchers at Wyeth have reported 4-anilino-3-quinolinecarbonitrile analogues with potent activity against MEK1 kinase, such as **2**.[8,9] The activity of these inhibitors was determined by a direct MEK1-MAPK fluorescent ELISA assay.[8]

We have developed a high throughput, non-radioactive Raf/MEK1/MAPK cascade ELISA assay to detect Raf and/or MEK1 kinase inhibitors.[10] For this assay, the end point was the measurement of MAPK phosphorylation on Threonine (T) 202 and Tyrosine (Y) 204 by a phospho-specific monoclonal antibody. The specific

*Corresponding author. Tel.: +1-845-602-3435; fax: +1-845-602-5561; e-mail: bergerd@wyeth.com

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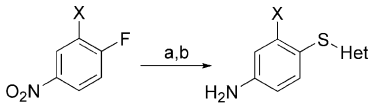
target of Raf/MEK1/MAPK ELISA inhibition was identified by enzymatic assays which directly measured Raf phosphorylation of MEK1, or MEK1 phosphorylation of MAPK.[10] Using this system, **3** was identified as a potent MEK1 inhibitor (IC_{50}: 10 nM), with exceptional activity (IC_{50}: 7 nM) against LoVo cells (a K-*ras* mutant human colon tumor line).

Because of its potent cellular activity, compound **3** served as a particularly promising new lead structure. We present here our exploration of the SAR of a series of analogues of **3**, as well as our efforts to improve the aqueous solubility of this compound. Specifically, we varied the substitution on the aniline 'headpiece' to determine how this would affect both enzyme and cellular activity. Based on the SAR of our previously described MEK1 kinase inhibitors, it was anticipated that the C-7 position of the 4-anilino-3-quinolinecarbonitriles would be optimal for the addition of water-solubilizing substituents. It was anticipated that such substituents could improve the physical properties of **3**, which was highly insoluble in water (<0.1 μg/mL) at pH 7.4, as well as a variety of organic solvents. Also of interest was to determine why compound **3** was significantly more active against LoVo cells than **2**, while possessing equivalent MEK1 kinase activity (Table 1).

Scheme 1 outlines the methods by which the majority of the substituted anilines were constructed. The fluoride substituents of **4a–d** were readily displaced by a variety of heterocyclic thiols to provide intermediates that were subsequently reduced by iron/ammonium chloride to provide anilines **5a–h**. Compounds **5a**,**b** and **5h** have been previously described.[11]

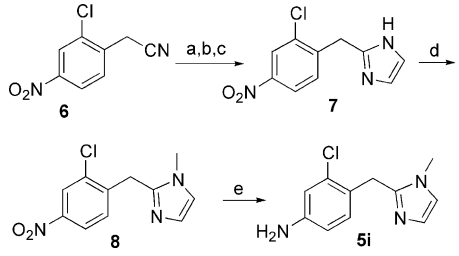


4a X = H
4b X = Cl
4c X = Me
4d X = Br

5a X = Cl, Het = 2-(1-methylimidazole)
5b X = H, Het = 2-(1-methylimidazole)
5c X = Me, Het = 2-(1-methylimidazole)
5d X = Br, Het = 2-(1-methylimidazole)
5e X = Cl, Het = 2-(1,5-dimethylimidazole)
5f X = Cl, Het = 2-(1,4,5-trimethylimidazole)
5g X = Cl, Het = 2-thiazole
5h X = Cl, Het = 2-pyridine

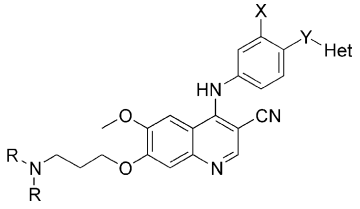
Scheme 1. (a) HS-Het, NaH, DMF or NMP; (b) Fe/NH_4Cl, MeOH, H_2O.

The synthesis of aniline **5i** is outlined in Scheme 2. Compound **6**[12] was converted to intermediate **7** by reaction with ethanolic HCl, followed by treatment with 2-aminoacetaldehyde dimethyl acetal and the subsequent addition of acetic acid and HCl gas.[13] Heating **7** in toluene with 1.5 equiv of dimethyl sulfate provided **8**, which was reduced with iron/ammonium chloride to afford **5i**.



Scheme 2. (a) EtOH/HCl; (b) $NH_2CH_2CH(OCH_3)_2$; (c) HCl gas, AcOH; (d) dimethylsulfate, toluene; (e) Fe/NH_4Cl, MeOH, H_2O.

Table 1. Inhibition in the Raf/MEK1 coupled assay and cellular activity for compounds **1–3**, **10a–l**



Compd	NRR	Het	X	Y	Raf/MEK1 IC_{50} (nM)[a]	LoVo IC_{50} (nM)[a]	CaCo IC_{50} (nM)[a]
10a	*N*-Morpholine	2-(1-Methylimidazole)	Cl	S	2	5	>1000
10b	*N*-Morpholine	2-(1-Methylimidazole)	H	S	5	43	4700
10c	*N*-Morpholine	2-(1-Methylimidazole)	Me	S	28	36	3200
10d	*N*-Morpholine	2-(1-Methylimidazole)	Br	S	2	5	5200
10e	*N*-Morpholine	2-(1,5-Dimethylimidazole)	Cl	S	1	325	>1000
10f	*N*-Morpholine	2-(1,4,5-Trimethylimidazole)	Cl	S	2	342	900
10g	*N*-Morpholine	2-Thiazole	Cl	S	9	56	2700
10h	*N*-Morpholine	2-Pyridine	Cl	S	8	200	>1000
10i	*N*-Morpholine	2-(1-Methylimidazole)	Cl	CH_2	212	380	5500
10j	1-(4-Ethylpiperazine)	2-(1-Methylimidazole)	Cl	S	3	7	>1000
10k	1-(Thiomorpholine-4-oxide)	2-(1-Methylimidazole)	Cl	S	2	36	>1000
10l	*N*-Pyrrolidine	2-(1-Methylimidazole)	Cl	S	4	10	>1000
1	—	—	—	—	2	131	>1000
2	*N*-Morpholine	Phenyl	H	O	4	400[16]	5500
3	—	—	—	—	10	7	>1000

[a]The IC_{50} values reported represent the means of at least two separate determinations with typical variations of less than 40% between replicate values.

Target compounds **10a–l** were synthesized by heating **9**[8] with anilines **5a–i** in the presence of pyridine hydrochloride, followed by amine displacement of the alkyl chloride moiety (Scheme 3).[14]

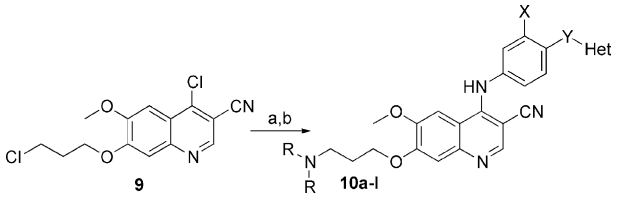
All compounds were tested in the high-throughput Raf/MEK1/MAPK ELISA assay. Cellular proliferation assays (sulforhodamine B staining) were carried out using LoVo (K-*ras* mutant) and CaCo (K-*ras* wild-type) human colon tumor cell lines.[15] It was anticipated that selective MEK1 inhibitors would be significantly more active against the K-*ras* mutant LoVo cell line, and that active, but non-selective or cytotoxic compounds, would have similar activity against both cell lines. These results are summarized in Table 1.

Compound **10a**, with the same aniline substitution as **3**, but possessing a water-solubilizing group at C-7, was particularly potent in the enzyme and cellular assays (Raf/MEK1 IC_{50}: 2 nM, LoVo: 5 nM). Replacement of the 3-chloro substituent on the aniline ring with hydrogen or methyl resulted in a modest decrease in activity (compounds **10b** and **10c**, respectively), while bromo was equipotent (**10d**). Given the size difference between the chloro and bromo substituents, it appears that these groups provided a beneficial electronic effect, rather than providing additional binding interactions to the enzyme.

Compounds **10e** and **10f**, which possess additional methyl groups on the imidazole ring, were very active in the enzyme assay (Raf/MEK1 IC_{50}s: 1 and 2 nM, respectively), but only modestly active against LoVo cells (IC_{50}s: 325 and 342 nM, respectively). Two heterocyclic groups other than *N*-methylimidazole that provided moderate cellular activity were the 2-thiazole substituted **10g** (LoVo IC_{50}: 56 nM) and the 2-pyridyl substituted **10h** (LoVo IC_{50}: 200 nM). When the thioether linker to the *N*-methylimidazole moiety was replaced by a methylene group (**10i**), activity was substantially reduced (Raf/MEK1 IC_{50}: 212 nM, LoVo: 380 nM).

Water-solubilizing groups other than morpholine provided analogues of **10a** with similar potency in vitro (**10j–l**). These compounds were more soluble in aqueous media than **3** (for example, aqueous solubility of **10j** = 2 μg/mL, **10l** = 55 μg/mL, at pH 7.4). The Pfizer compound **1** was comparable to our most potent analogues in the Raf/MEK1 assay with an IC_{50} of 2 nM, while being less active against LoVo cells (IC_{50} 131 nM).

To determine why compounds with similar MEK1 activity have significantly different inhibitory effects on



Scheme 3. (a) **5a–i**, pyridine-HCl, 2-ethoxyethanol; (b) RRNH, KI, DMF.

LoVo cell proliferation, further mechanistic studies were carried out with **10a** and **2**.[17] LoVo cells were treated with compounds **10a** and **2** to determine their ability to inhibit the phosphorylation of MEK1, MAPK and other selected kinases. The results are summarized in Table 2. Compound **10a** inhibited the phosphorylation of both MEK1 and MAPK with IC_{50}s of 8 and 0.01 nM, respectively. Presumably, the potent action of **10a** on phospho-MAPK levels was due to the dual effect of decreasing phospho-MEK1 levels, and inhibition of MAPK phosphorylation by MEK1. In contrast, **2** inhibited the phosphorylation of MAPK with an IC_{50} of 380 nM, but did not affect phospho-MEK1. Neither compound had an effect on the phosphorylation status of Akt or EGFR kinases; nor did they affect overall cell phospho-tyrosine levels at concentrations from 1 to 1000 nM. Furthermore, it was demonstrated that the decrease in phospho-MEK1 levels by **10a** was not due to Raf kinase inhibition (Raf IC_{50}: 850 nM). While the source of this inhibition of phospho-Mek1 levels is still under investigation, it suggests that this dual activity of **10a** is responsible for its potent antiproliferative effects on LoVo cells.

In order to establish the utility of our most potent compounds, **10a** and **10j** were tested against other cell lines. Both **10a** and **10j** showed good activity against several cell lines, among them a pancreatic line: BXPC-3 (IC_{50}s: 29 and 7 nM, respectively), a prostate line: LnCAP (IC_{50}s: 40 and 45 nM, respectively) and a bladder line: HT1197 (IC_{50}s: 50 nM for both compounds).

Several compounds were tested against LoVo xenografts in nude mice. Compounds were dosed as suspensions in 0.5% Methocel/2% Tween 80 solutions and injected via an intraperitoneal route (ip) into groups of five mice.[18] One compound that produced a reasonably consistent response was **10j** (Fig. 1).

Table 2. Summary IC_{50} phospho-blot data from LoVo cells exposed to compounds **10a** and **2**

Compd	P-MEK1 (nM)	P-MAPK (nM)	P-Akt (nM)	P-EGFR (nM)	P-TYR (nM)
10a	8	0.01	>1000	>1000	>1000
2	>1000	380	>1000	>1000	>1000

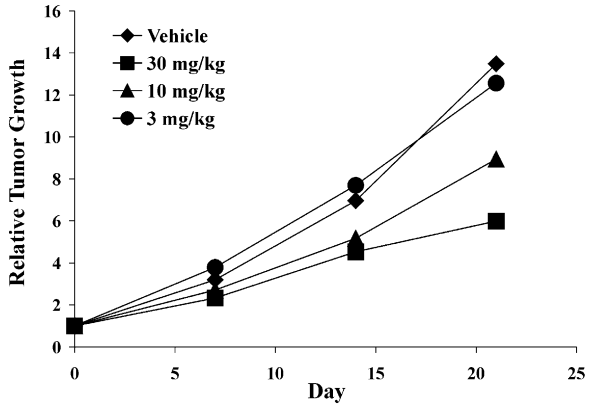


Figure 1. Effect of **10j** in a LoVo xenograft model.

When dosed at 30 mg/kg twice daily, a statistically significant ($P<0.01$) reduction in tumor growth was seen at day 21 (T/C 44%).

In summary, we have discovered a potent series of MEK1 inhibitors with exceptional cellular activity against several cell lines. These compounds possess additional activity that results in decreased phospho-MEK1 levels at low nanomolar concentrations. Compound **10j** showed activity in vivo against LoVo xenografts when dosed ip. We are currently further optimizing this series to improve the pharmacological properties and oral absorption.

Acknowledgements

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14. All final products were characterized by ^{1}H NMR, MS and elemental analysis. A representative example, compound **10k**: ^{1}H NMR (DMSO-d_6) δ 1.98 (m, 2H); 2.55 (t, $J=5.2$ Hz, 2H); 2.70 (m, 4H); 2.87 (broad s, 4H); 3.60 (s, 3H); 3.91 (s, 3H); 4.21 (t, $J=4.8$ Hz, 2H); 6.57 (d, $J=6.4$ Hz, 1H); 7.12 (d, $J=7.8$ Hz, 1H); 7.16 (s, 1H). 7.35 (s, 1H), 7.39 (s, 1H), 7.54 (s, 1H), 7.68 (s, 1H), 8.50 (s, 1H), 9.59 (s, 1H). MS (ES) m/z 597.2 $(M+H)^+$. Anal. for $C_{28}H_{29}ClN_6O_3S_2\cdot H_2O$, calcd: C 54.66; H 5.08; N 13.66, found: C 54.62H 4.93; N 13.60.
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18. Human Colon Carcinoma LoVo cells (American Type Culture Collection, Rockville, Maryland # CRL-155) were grown in tissue culture in RPMI (Gibco/BRL, Gaithersburg, MD) supplemented with 10% FBS (Gemini Bio-Products Inc., Calabasas, CA, USA). Athymic nu/nu female mice (Charles River, Wilmington, MA, USA) were injected SC in the flank area with 7×10^6 LoVo cells plus Matrigel (BD Biosciences, Billerica, MA, USA). When tumors attained a mass of between 130 and 180 mg, the mice were randomized into treatment groups (day zero), five animals per group. Animals were treated IP twice a day on days 1 through 20 post staging (day zero) with either 30, 10, or 3 mg/kg/dose of compound prepared in 0.5% Methocel/2% Tween 80 as the vehicle control. Tumor mass was determined every 7 days [(length$\times$width^2)/2] for 21 days post staging. Relative tumor growth (Mean tumor mass on days 7, 14 and 21 divided by the mean tumor mass on day zero) was determined for each treatment group. Statistical analysis (Student t-test) of log relative tumor growth was used to compare treated verses control group in each experiment. A p value ($p\leq0.05$) indicates a statistically significant reduction in relative tumor growth of treated group compared to the vehicle control.