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Synthesis and Structure–Activity Relationships of 3-Cyano-4-(phenoxyanilino)quinolines as MEK (MAPKK) Inhibitors

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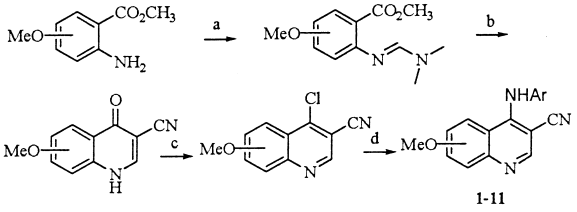
Abstract—A series of 3-cyano-4-(phenoxyanilino)cyanoquinolines has been prepared as MEK (MAP kinase kinase) inhibitors. The best activity is seen with alkoxy groups at both the 6- and 7-positions. The lead compounds show low nanomolar IC_{50}'s against MAP kinase kinase, and have potent inhibitory activity in tumor cells.

Introduction

The mitogen-activated protein kinase (MAPK) pathway is a major player in the kinase signaling cascade from growth factors to the cell nucleus.[1] The MAPK pathway involves a heirarchy of kinases at multiple levels: MAP kinases (or ERKs, extracellular signal-regulated kinases) and MAP kinase kinases (or MEKs, MAPK/ERK kinases). MEK is activated by phosphorylation on serine 218 and 222 residues by upstream kinases such as those in the Raf family. Once activated, MEK catalyzes phosphorylation on a TEY site on ERK. The activated ERK then phosphorylates and activates transcription factors in the nucleus such as c-Myc and Elk-1/TCF. Constitutively active MEK mutants are known to induce cell transformation and produce tumors in nude mice.[2] Over-expression and/or over-activation of MEK or ERK protein has been found to be associated with various human cancers, including kidney, breast, colon, and oral carcinomas, leukemias, and glial neoplasms.[3] Inhibition of the MAPK pathway through inhibition of MEK or ERK presents a unique opportunity to block uncontrolled cell growth and, thus, has potential therapeutic utility in cancer treatment.[4–6] This strategy has been pursued by other pharmaceutical companies, such as Parke-Davis[5] and DuPont.[6] We have been pursuing a series of 4-anilino-3-cyanoquinolines[7] as inhibitors of MAP kinase kinase. The initial leads came from selective evaluation of compounds prepared as putative inhibitors of other kinases.[7b] We now report the synthesis and structure–activity relationships of this series of compounds as MEK (MAP kinase kinase) inhibitors.

Chemistry

The synthesis of compounds **1**–**11** is shown in Scheme 1. Starting from methyl anthranilates, condensation in refluxing dimethylformamide dimethylacetal provided amidine methyl esters. Treatment with lithium acetonitrile gave cyclized quinolones, which underwent chlorination and aniline replacement, yielding the desired 4-anilino-3-cyanoquinolines.



Scheme 1. (a) DMF-DMA, reflux; (b) $LiCH_2CN$, THF, −78 °C, then HOAc, rt; (c) $POCl_3$, reflux; (d) $ArNH_2$, $EtOCH_2CH_2OH$, reflux.

It was reported by AstraZeneca[8] that replacing a 7-methoxy with 7-morpholinoalkoxy substituents on a 4-anilinoquinazoline greatly increased potency against

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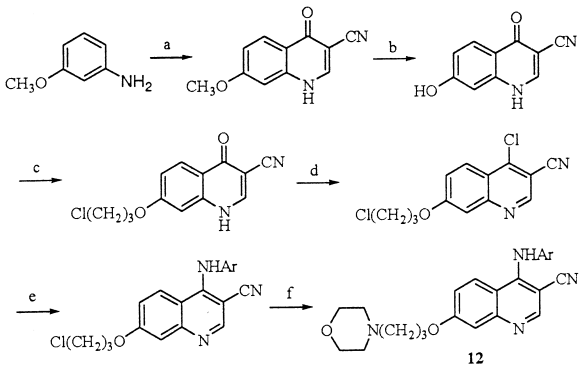
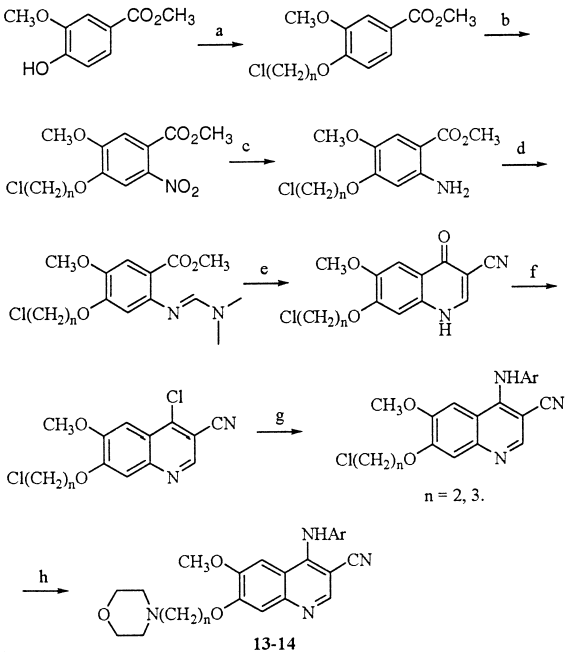
VEGF receptor tyrosine kinases. We adopted the same strategy and incorporated morpholinoalkoxy substituents at the 7-position. The synthesis of compounds **12**–**14** was accomplished in a similar manner, as shown in Schemes 2 and 3. 2-Chloroethoxy or 3-chloropropoxy groups were introduced to the 7-position in cyanoquinolines, with the chloro group as the precursor for the morpholino group. After aniline replacement, the chloro group was replaced by morpholine in the presence of a catalytic amount of sodium iodide.



Scheme 2. (a) Ethyl (ethoxymethylene)cyanoacetate, Dowtherm, reflux; (b) pyridine hydrochloride, 220 °C; (c) $Cl(CH_2)_3OTs$, NaH, DMF rt; (d) $POCl_3$, reflux; (e) $ArNH_2$, $EtOCH_2CH_2OH$, reflux; (f) orpholine, NaI, DME, reflux.

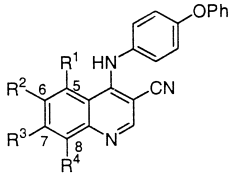


Scheme 3. (a) $Cl(CH_2)_nOTs$, K_2CO_3, $(C_8H_{17})_3N^+CH_3Cl^-$, acetone, reflux; (b) HNO_3, HOAc, 50 °C; (c) Fe, NH_4Cl, CH_3OH, reflux; (d) DMF–DMA, reflux; (e) $LiCH_2CN$, THF, −78 °C, then HOAc, rt; (f) $POCl_3$, reflux; (g) $ArNH_2$, $EtOCH_2CH_2OH$, reflux; (h) morpholine, NaI, DME, reflux.

Results and Discussion

The compounds were tested in two related enzyme assays: a coupled Raf-MEK-ERK fluorescent ELISA assay[9] (in which an activated Raf was used to phosphorylate MEK) and a direct MEK-ERK fluorescent ELISA assay[10] (in which a previously activated MEK was used to activate ERK). The IC_{50} values for compounds **1**–**9** and **12**–**14** in these two assays are shown in Table 1.

Table 1. Inhibitory activity of compounds **1**–**9** and **12**–**14**



Compd	R^1	R^2	R^3	R^4	IC_{50} (nM) Coupled MEK[9]	IC_{50} (nM) Direct MEK[10]
1	H	H	H	H	125	680
2	OMe	H	H	H	98	450
3	H	OMe	H	H	18	35
4	H	H	OMe	H	90	125
5	H	H	H	OMe	>10,000	>10,000
6	H	OMe	OMe	H	9	30
7	OMe	H	OMe	H	60	185
8	H	OMe	OMe	OMe	>10,000	>10,000
9	OMe	H	H	OMe	>10,000	>10,000
12	H	H	$O(CH_2)_3$morph	H	28	68
13	H	OMe	$O(CH_2)_2$morph	H	8	25
14	H	OMe	$O(CH_2)_3$morph	H	2.4	7

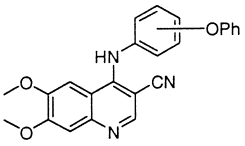
Values are means of two experiments.

Several conclusions can be drawn from the testing results. A methoxy substituent at the 5- and 7-position increases activity moderately (**2**, **4**, or **7** versus **1**), while a methoxy at the 8-position totally diminishes activity (**5**, **8**, and **9**). A methoxy group at the 6-position increases activity by about one order of magnitude (**3** versus **1**; **14** versus **12**). The best activity is achieved when both the 6- and 7-positions are substituted with methoxy (**6**) or alkoxy groups (**13** and **14**). An important finding is that enzyme inhibition is retained upon replacing R^3 OMe by $O(CH_2)_{2\text{-}3}$morph (**6** versus **13** and **14**). For the alkyl chain length between the morpholine and the oxygen at the 7-position, a three methylene unit provides better activity compared with a two methylene unit (**13** versus **14**). The same trend was observed at the 7-position of a 4-anilinoquinazoline prepared as VEGF receptor tyrosine kinase inhibitors by AstraZeneca.[8]

The role of the *para*-phenoxyaniline substitution pattern at the 4-position was also studied by comparison with the corresponding *meta*- and *ortho*- analogues (**6** versus **10** and **11**, Table 2). Moving the phenoxy group from a *para*- to a *meta*-position decreases the activity (**6** versus **10**), while the *ortho*-substitution essentially abolishes activity (**11**).

These 3-cyano-4-(phenoxyanilino)quinoline compounds appear to be selective inhibitors of the MEK enzyme. The activity in the direct MEK assay is about 2–4-fold less potent than in the coupled MEK assay. Greater potency in the coupled assay may reflect either some

Table 2. Inhibitory activity of compounds **6**, **10**, and **11**

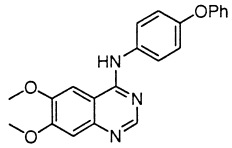


Compd	OPh	IC_{50} (nM) Coupled MEK[9]	IC_{50} (nM) Direct MEK[10]
6	*para*	9	30
10	*meta*	95	100
11	*ortho*	>10,000	>10,000

Values are means of two experiments.

Raf inhibition or greater sensitivity of response in coupled systems.[11] However, more direct evidence for selective MEK inhibition by some of these compounds comes from data showing more potent inhibition of MEK phosphorylation of ERK than Raf phosphorylation of MEK as measured by phosphoimaging of [^{33}P]ATP bands on SDS-PAGE gels (data not shown). In addition, in vitro selectivity for other enzymes was studied. The most active compounds, **6** and **13**, were tested in an ERK2 kinase assay,[12] and found to have greatly reduced activity (IC_{50} >10 μM). They were also found to have little effect on certain tyrosine kinases {EGFR (epidermal growth factor receptor), ECK (epithelial cell kinase), and KDR (VEGF related kinase insert domain-containing receptor)} or serine/threonine kinases such as cyclin dependent kinases (cdk2, cdk4) and AKT (target for PI3K) (data not shown). Studies on inhibition of other related MAPK kinase family members is ongoing.

The cyanoquinoline core is necessary for inhibition of MEK activity. Compound **15**, the quinazoline analogue of compound **6**, was found to be weakly active (IC_{50} >100 μM).



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Compounds **6**, **13**, and **14** were tested for their ability to inhibit cell growth in Colo205, Lovo, and SW620 human colon tumor lines in culture.[13] The activity of these compounds is shown in Table 3. Compounds **13** and **14**, with a morpholinoalkoxy group at the 7-position, showed better cell inhibitory activity than the corresponding dimethoxy compound, **6**. Cells are checked microscopically for morphological and cytotoxic changes at each concentration. Viability is checked using trypan blue dye staining and counting on a hemacytometer. Compounds are not cytotoxic at concentrations near the IC_{50} value; there is no evidence of cytotoxicity at concentrations below 5 μM. Compounds **13** and **14** showed evidence of cytotoxicity only at concentrations greater than 5 μM. This represents a window of 10–15 fold between the 50% efficacious concentration and the toxic concentration. The data do indicate a difference in IC_{50} values for enzyme activity and effects on cell proliferation on the order of 50–100-fold. Higher IC_{50} values in cells relative to enzyme may suggest a problem with availability and uptake in cells, or alternatively, reflect that net inhibition of the MEK/ERK pathway is in the context of cell specific biochemistry (e.g. compensatory alternative growth signals). Separate studies to be reported elsewhere, show that these compounds do inhibit ERK phosphorylation in tumor cells at concentrations less than 1 μM.

Table 3. Cell growth inhibitory activity of compounds **6**, **13**, and **14** in three human colon tumor lines[13]

Compd	IC_{50} (μM) Colo205	IC_{50} (μM) Lovo	IC_{50} (μM) SW620
6	1.43	0.63	1.50
13	0.63	0.25	0.70
14	0.36	0.19	0.38

In conclusion, we have developed a series of 3-cyano-4-(phenoxyanilino)cyanoquinolines as MEK (MAP kinase kinase) inhibitors. The most active compounds have alkoxy groups at both the 6- and 7- positions. Compounds with a methoxy group at the 6-position and a morpholinoalkoxy group at the 7- position maintain potent enzymatic activity compared with the methoxy analogues. These compounds represent novel structures with MEK inhibitory activity. The lead compounds show low nanomolar IC_{50}'s against MAP kinase kinase, and have potent activity on several tumor cell lines.

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9. Coupled MEK fluorescent immunoassay: The assay was a dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA) using Wallac reagents. Characterization of the assay will be described elsewhere. Compounds were diluted in DMSO. Six to eight concentrations were used to generate a response curve. Briefly, kinase reactions were conducted in 50 mM Tris buffer, pH 7.4 (Tris 50 mM, $MgCl_2$ 1 mM, DTT 1 mM and ATP 100 μM). Active human c-Raf (Upstate Biotech, Lake Placid, NY), inactive human recombinant GST-MEK1, and inactive human recombinant GST-ERK2 (both prepared in house and described elsewhere) were added and incubated with diluent or inhibitor in the presence of ATP for 60–90 min at 25 °C. The reaction was linear for up to 2 h. The supernatent was transferred to anti-GST coated Elisa plates for binding of phosphorylated product overnight at 4 °C. The contents were removed and the wells were blocked with Superblock (Pierce). Anti-phospho MAPK antibody (New England Biolabs or Sigma) was diluted in enzyme dilution buffer (Wallac) and incubated with samples overnight at 4 °C. The plates were washed in buffer (Wallac wash buffer) and the secondary antibody (europium tagged anti-rabbit or anti-mouse IgG, Wallac) was diluted in enzyme dilution buffer and incubated with samples for 1 h at 25 °C. After washing, enhancer solution (Wallac) was added, and readings were made on a Wallac Victor Model 1420 fluorescence plate reader. Readings were converted to percentage inhibition, and IC_{50} values were calculated from the concentration–response curve.
10. Direct MEK fluorescent immunoassay: The assay used a DELFIA format with Wallac reagents as described above and characterized elsewhere. Compound dilutions, reaction plates, Elisa plates and kinase buffer were prepared as described above. Activated human recombinant GST-MEK1 (UBI) and inactive human recombinant GST-ERK2 were added simultaneously with inhibitors. Incubation times, transfer to Elisa plates, processing with blocking buffer, primary and secondary antibodies, and plate readings and IC_{50} calculations were the same as described above.
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12. Radiolabeled ERK2 kinase assay: Inhibitors were incubated with p42 active ERK (UBI) in 50 mM Tris, pH 7.4 kinase buffer for 30 min on ice. The phosphorylation reaction was started by addition of Myc peptide substrate peptide (Anaspec) and [^{33}P]ATP (Amersham, Sp. Act. 1000–3000 Ci/mmol) and incubated for 30 min at 30 °C. The reaction was stopped and the samples were chilled for 15–30 min on ice. Supernatents were transferred to P81 phosphocellulose filter paper or an MTP plate with embedded P81 filter. Filters were washed three times with 1% acetic acid, 5 min each and then three times with water, 5 min each. After brief air drying, scintillation fluid was added to the filter discs or MTPs, and the samples were counted in a Packard Tri Carb 2100 TR liquid scintillation counter (vials) or Wallac Microbeta 1450 counter (MTPs) using a [^{33}P] program.
13. Human tumor cells were obtained from American Type Culture Collection (Rockville, MD) and plated in 6-well dishes at densities of approximately $1–2\times10^5$ cells/35 mm^2 well in RPMI 1640 media containing 10% fetal bovine serum and penicillin/streptomycin, glutamine and HEPES additives and allowed to attach overnight. The following day, inhibitor compounds were added at the appropriate concentration and cells exposed for 3 days in a 37 °C incubator. Cells were harvested by trypsinization and counted on an electronic particle counter (Coulter Model Z1). Prior to harvesting, cells were observed under a light microscope for morphological or cytotoxic changes. Cells were also checked for viability using trypan blue dye staining and observation under the microscope.