Pergamon

SCIENCE DIRECT®

Bioorganic & Medicinal Chemistry 11 (2003) 3165–3170

BIOORGANIC & MEDICINAL CHEMISTRY

Synthesis and MEK1 Inhibitory Activities of Imido-Substituted 2-Chloro-1,4-naphthoquinones

Oladapo Bakare,[a,*] Curtis L. Ashendel,[b] Hairuo Peng,[c] Leon H. Zalkow[c] and Edward M. Burgess[c]

[a]*Department of Chemistry, Howard University, 525 College Street, Washington DC 20059, USA*
[b]*Department of Medicinal Chemistry and Molecular Pharmacology, School of Pharmacy, Purdue University, West Lafayette, IN 47907, USA*
[c]*School of Chemistry and Biochemistry, Georgia Institute of Technology, Atlanta, GA 30332, USA*

Received 13 July 2001; accepted 3 April 2003

Abstract—Mitogen activated protein kinases are of interest as research tools and as therapeutic target for certain physiological disorders. In this study, we found 2-chloro-3-(*N*-succinimidyl)-1,4-naphthoquinone **6** to be a selective inhibitor of MEK1 with an IC_{50} of 0.38 μM. An open-chain homologue, **10**, showed selective cytotoxicity against renal cancer in the NCI in vitro tumor screening. Structure–activity relationship study of eight compounds showed the cyclic imido-substituted chloro-1,4-naphthoquinone as more potent and selective MEK1 inhibitors than the open chain homologues. The imido-substituted chloro-1,4-naphthoquinones were synthesized in a straightforward fashion by refluxing 2-amino-3-chloro-1,4-naphthoquinone with the appropriate acid chloride or diacyl dichloride.

Introduction

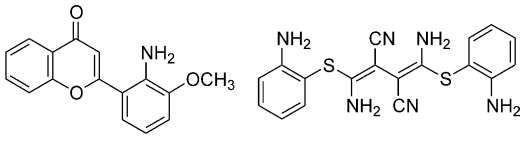
Over the past few years, the mitogen activated protein kinases (MAPKs) have been at the forefront of a rapid advance in understanding of cellular events in growth factors and cytokine receptor signaling. The MAPKs are a group of protein serine/threonine kinases that are activated in response to a variety of extracellular stimuli and mediate signal transduction from cell surface to nucleus.[1] In combination with several other signaling pathways, they can differentially alter the phosphorylation status of transcription factors. A controlled regulation of MAP kinase cascades is thus involved in cell proliferation, differentiation and apoptosis, whereas an unregulated activation can result in oncogenesis.[1–4]

Several lines of evidence support the critical role for the activation of the Raf-1/MEK/MAPK pathway in oncogenic transformation. For example, constitutive activation of the MAPK cascade was found to be associated with the carcinogenesis of human renal cell carcinomas.[5] Significantly increased expression and functional activities of MEK1, MEK2, and ERK1, ERK2 was found in human hepatocellular carcinoma.[6] In addition, mammary tumors initiated by neu, v-Ha-ras and c-Myc showed high levels of active MAPK, whose activation may be correlated with MEK activation. The anchorage independent growth of these tumor cells are strongly inhibited by MEK inhibitor **1** (PD098059).[7,8] In addition to the Park Davis inhibitor PD098059,[8] Favata and his co-workers[9] have identified the selective inhibition of MEK1 by UO126, **2**, while Williams et al.[10] reported the inhibitor RO 09-2210 (**3**). Compound **3** was able to block anti-CD3 induced peripheral blood T-cell activation and antigen induced IL-2 secretion by the inhibition of MEK1. This suggests that in addition to being useful in the control of tumor growth, MEK1 inhibitors could be important in controlling the progression of other diseases such as rheumatoid arthritis and multiple sclerosis which are linked to T-cell proliferation. Recently, Taher et al.[11] reported that cell signaling in response to γ-radiation is transduced through the MEK1/2/p42/44 MAP kinase pathway to increase HIV gene expression in a HeLa cell clone. Hence, apart from being useful in the understanding of cellular activities, all these compounds serve as leads to the development

*Corresponding author. Tel.: +1-202-806-6882; fax: +1-202-806-5442; e-mail: obakare@howard.edu

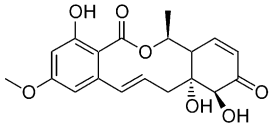
0968-0896/03/$ - see front matter © 2003 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0968-0896(03)00267-0

of therapeutically useful candidates. In our in-house screening of a number of compounds, we identified the chloro-1,4-naphthoquinone derivative **4**, as a MEK1 inhibitor. This led to the design and synthesis of analogues of **4** as potential inhibitors of the MAP kinase cascade. Some compounds with the chloro-1,4-naphthoquinone skeleton were recently reported to possess antineoplastic,[12] antihypoxic,[13] antiischemic,[13] anti-platelet, anti-inflammatory and anti-allergic activities;[14,15] while others have been shown to inhibit the human cytomegalovirus (HCMV) protease.[16] We here report a novel MEK1 selective inhibitor, 2-chloro-3-(*N*-succinimidyl)-1,4-naphthoquinone (**6**), and various homologues with a chloro-1,4-naphthoquinone skeleton. These compounds provide new leads for the development of potent and pharmacologically useful MEK inhibitors.

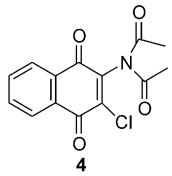


PD098059 (**1**)

UO126 (**2**)



Ro 09-2210 (**3**)



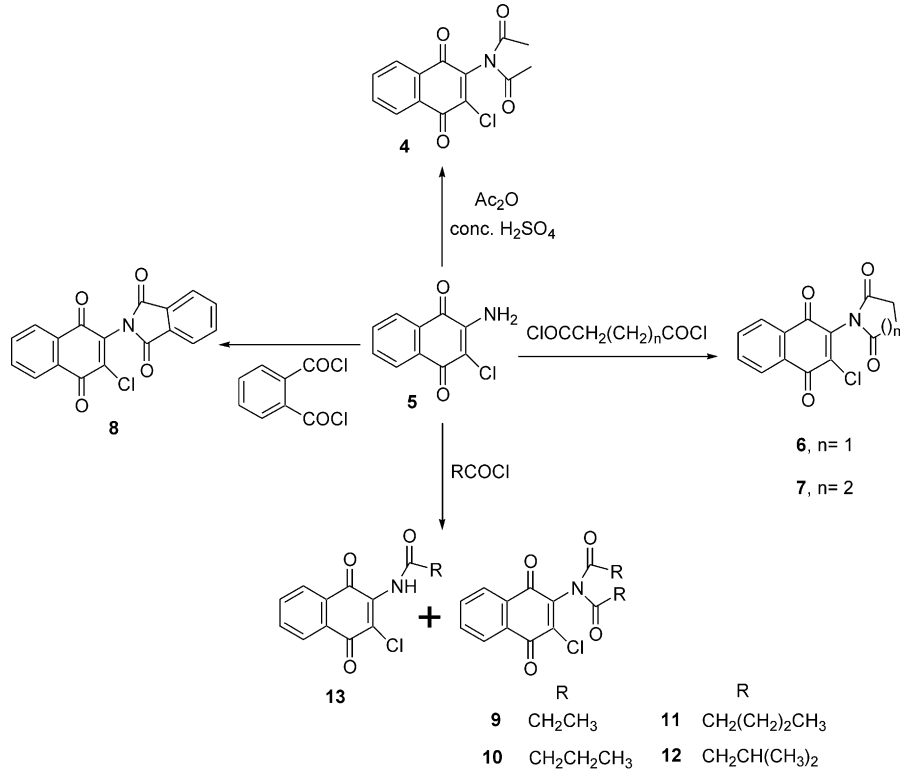
4

Chemistry

The synthesis of compounds **4** and **6–12** was accomplished in a straightforward way as shown in Scheme 1. Compound **4** was obtained by refluxing acetic anhydride and 2-amino-3-chloro-1,4-naphthoquinone with a catalytic amount of concd H_2SO_4. Compounds **6–12** were made by refluxing 2-amino-3-chloro-1,4-naphthoquinone in the appropriate acid chloride or diacyl dichloride for 30–60 min. In the case of **9–12**, the mono-substituted amide- derivatives were also isolated and separation was achieved by silica gel chromatography. 2-Amino-3-chloro-1,4-naphthoquinone **5** is commercially available, but could easily be obtained from 2,3-dichloro-1,4-naphthoquinone and ammonia in a mixture of concd ammonium hydroxide and ethanol.

Results and Discussion

The inhibitory activities of the compounds studied are listed in Table 1. Structure–activity relationship study showed the derivatives with cyclic substituents **6**, **7**, and **8** to have higher potency than the open chain substituted derivatives. These compounds also show better MEK1 activity than PD098059, **1**, which displayed an IC_{50} of 3.8 μM in our assay. The succinimydyl derivative **6** with IC_{50} of 0.38 μM was found to be the most potent inhibitor of MEK1 in this study. This shows a considerable selectivity over the inhibition of ERK1 with IC_{50} of 82.9 μM and raf1 at 34.5 μM. Inhibition of bovine heart PKA and PKC by this compound was



Scheme 1.

Table 1. IC_{50} (μM) of the 2-chloro-1,4-naphthoquinone derivatives (**4** and **6–12**) on PKA, PKC, ERK1, MEK1 and raf1

Compd	PKA (bovine heart)	PKA (mouse rec.)	PKC (rat brain)	ERK1	MEK1	Raf1
4	8.1	7.6	60.3	99.4	7.5	54.9
6	16.3%*	—	—	82.9	0.38	34.5
7	9.2	8.9	>50	112	1.1	62.6
8	14.3%*	—	—	>296	3.6	41.5
9	—	—	—	106.3	10.3	71.9
10	6.2	10.6	>60	126.5	6.6	31.63
11	7.7	26.0%*	4.7%*	130.4	15.7	71.8
12	7.9	44.7%*	3.3%*	98.5	10.9	61.2

Positive controls were used in all the studies: For MEK this was PD098059 with IC_{50} of 3.8 μM. For raf1, it was staurosporine, which was tested at 20 μg/mL but failed to inhibit at this concentration. Staurosporine also served as control for ERK1, PKA and PKC with IC_{50} of 6.2, 0.37 and 0.01 μM, respectively.
*% Inhibition obtained at 10 μM.

negligible. The glutarimydyl 6-membered ring substituted derivative **7**, inhibited MEK1 with an IC_{50} of 1.1 μM and also showed slightly less selectivity than **6**. Likewise, the phthalimydyl substituted derivative **8** showed lower potency and selectivity than **6** as shown in Table 1. The open chain imides generally showed lower inhibitory activity for MEK1 and higher inhibitory activity for bovine heart PKA making them less selective than those with cyclic substituent. The most potent open chain substituted compound in this series was the dibutylamino derivative **10** with an IC_{50} of 6.6 μM for MEK1. This compound is also the best inhibitor of PKA in the whole series displaying an activity with IC_{50} of 6.2 μM but was an order of magnitude less potent for PKC (IC_{50} greater than 60 μM). While the imido-substituted chloronaphthoquinones in this study showed reasonable activities on bovine heart PKA, they displayed little or no activities on PKC.

Evaluation of **10** against 50 human cancer cell lines in the NCI in vitro screening revealed a selective cytotoxicity against the renal cancer cell lines. ACHN and CAXI-1 cell lines were the most sensitive to **10** with total growth inhibitory potency (log TGI) of −5.59 and −5.46, respectively (Table 2). The effect of **10** in this group of renal cancer cell lines was least on SN12C with log TGI of −4.01 where the mean value for all the 50 cancer cell lines screened was −4.28. Most of the remaining 42 cell lines screened in this study displayed less sensitivity to **10**. The selectivity of **10** for renal carcinoma cell lines is consistent with findings that MAPKs are constitutively activated in some renal cell carcinomas,[5] suggesting that **10** has potential for significant clinical impact on this disease.

Table 2. Inhibitory potencies [Log GI_{50} (M)[a], Log TGI (M)[b] and Log LC_{50} (M)[c]] of 2-chloro-3-dibutyrylamino-1,4-naphthoquinone (**10**) on Renal Cancer Cell Lines from the NCI in vitro screening

Cell line	Log GI_{50}	Log TGI	Log LC_{50}
786-0	−5.04	−4.40	>−4.00
A498	−4.97	−4.41	−4.06
ACHN	−5.97	−5.59	−5.21
CAXI-1	−5.76	−5.46	−5.26
RXF393	−5.64	−5.33	−5.02
SN12C	−4.78	−4.01	>−4.00
TK-10	−4.39	−4.82	−4.39
UO-11	−5.79	−5.22	−4.61
Mean[d]	−5.37	−4.28	−4.15
Range[e]	2.02	1.59	1.22

[a]GI_{50}, drug molar concentration causing 50% cell growth inhibition.
[b]TGI, drug molar concentration causing total growth inhibition.
[c]LC_{50}, drug molar concentration causing 50% cell death.
[d]Mean values over all cell lines tested.
[e]The difference in value of the least sensitive cell and the most sensitive cell of all the cells tested.

Conclusion

We have synthesized and tested eight imido-substituted 2-chloro-1,4-naphthoquinones as potent selective MEK1 inhibitors. The cyclic substituted derivatives were generally more potent and selective that the open chain homologues. All the three cyclic imido- substituted derivatives **6**, **7**, and **8** in this study were selective and more potent MEK1 inhibitor than PD098059, with **6** being the most potent and selective MEK1 inhibitor in the series. An open-chain homologue **10** was found to exhibit selective cytotoxicity on a panel of renal cancer lines. These compounds provide new leads for the development of potent and pharmacologically useful selective inhibitor of the MAP kinase cascade.

Experimental

Melting points were determined on a Koffler hot-stage equipped with a digital thermometer and are uncorrected. IR spectra were recorded on a Nicolet 520 FT-IR spectrometer using KBr disc for solids and NaCl plate for liquids. ^{1}H NMR spectra were recorded in $CDCl_3$ or DMSO-d_6 with a Varian Gemini-300 (300 MHz) spectrometer and chemical shifts are reported (δ) relative to TMS (0 ppm) as internal standard. Mass spectra were recorded on a VG analytical 70-SE spectrometer equipped with 11-250J data system, and all exact mass determinations were recorded at 5000 resolution. Microanalysis were performed by Atlantic Microlab, Norcross, GA, USA.

2-Chloro-3-diacetylamino-1,4-naphthoquinone (4). Concentrated sulfuric acid (0.5 mL) was added dropwise (over 10 min) to a mixture of 2-amino-3-chloro-1,4-naphthoquinone **5** (1.5 g, 7.23 mmol) and acetic anhydride (200 mL) at −5 °C. The resulting mixture was stirred at room temperature for 10 min and heated at 100–110 °C for 50 min after which it was concentrated in vacuo and the residue added to ice water. The resulting mixture was filtered, washed with small amount of acetic acid followed by water to obtain a yellow solid. The crude product was re-crystallized from ethanol to give yellow crystalline material (1.29 g, 83.7%). Mp 155–158 °C; IR (cm^{-1}) 3445, 3332, 3102, 3078, 2936, 1739, 1708, 1676, 1609, 1589, 1420, 1368, 1320, 1231, 1122, 1067, 1037, 974, 865, 714, 627, 598. ^{1}H NMR ($CDCl_3$) δ 2.39 (s, 6H), 7.27–8.20 (m, 2H), 8.23–8.28 (m, 2H); MS *m/e* 293.043991 (293.0454858 M + 2 expected for $C_{14}H_{10}NO_4Cl$). Anal. calcd for $C_{14}H_{10}NO_4Cl$: C, 57.65; H, 3.46; N, 4.80; found C, 57.73; H, 3.47; N, 4.73.

General synthesis of imido-substituted 2-chloro-1,4-naphthoquinones 6-12 as exemplified by the synthesis of 2-chloro-3-(*N*-succinimidyl)-1,4-naphthoquinone (6). A mixture of 2-amino-3-chloro-1,4-naphthoquinone **5** (280 mg, 1.35 mmol) and succinyl chloride (10 mL) was refluxed gently for 35 min. The resulting solution was frozen at −15 °C and treated with diethyl ether (100 mL) to precipitate a yellow solid. The mixture was filtered under suction and the precipitate washed with diethyl ether. The product was re-crystallized from methanol to obtain **6** as a yellow solid (155 mg, 59.6%). Mp 199–202 °C. IR (cm^{-1}) 3493, 3012, 2947, 1760, 1722, 1689, 1617, 1387, 1275, 1163, 870, 730, 623, 426. ^1H NMR (DMSO-d_6) δ 3.03 (s, 4H), 7.92–7.99 (m, 2H), 8.05–8.10 (m, 1H), 8.12–8.16 (m, 1H). MS *m*/*e* 289.0118 (289.0142 expected M^+ for $C_{14}H_8NO_4Cl$). Anal. calcd for $C_{14}H_8NO_4Cl$: C, 58.05; H, 2.78; N, 4.84; found C, 58.05; H, 2.85; N, 4.83.

2-Chloro-3-(*N*-glutarimidyl)-1,4-naphthoquinone (7). Melting point dec. >160 °C. IR (cm^{-1}) 3434, 2960, 2624, 1738, 1689, 1673, 1640, 1620, 1594, 1404, 1365, 1280, 1247, 1150, 1120, 1005, 832, 711. ^1H NMR ($CDCl_3$) δ 2.15–2.24 (m, 2H), 2.85 (t, 4H, $J=7.0$ Hz), 7.78–7.85 (m, 2H), 8.12–8.17 (m, 1H), 8.21–8.27 (m, 1H). MS *m*/*e* 304.0335 (304.0377 expected M+1 for $C_{15}H_{10}NO_4Cl$). Anal. calcd for $C_{15}H_{10}NO_4Cl$: C, 59.32; H, 3.32; N, 4.61; found C, 59.27; H, 3.30; N, 4.63.

2-Chloro-3-(*N*-phthalimidyl)-1,4-naphthoquinone (8). Melting point 243–245 °C. IR (cm^{-1}) 3435, 1792, 1730, 1681, 1617, 1374, 1275, 1102, 1084, 888, 862, 718, 710, 628. ^1H NMR ($CDCl_3$) δ 7.82–7.89 (m, 4H), 7.97–8.02 (m, 2H), 8.17–8.22 (m, 1H), 8.25–8.30 (m, 1H). MS *m*/*e* 337.0123 (337.0142 expected M^+ for $C_{18}H_8NO_4Cl$); Anal calcd for $C_{18}H_8NO_4Cl$: C, 64.01; H, 2.39; N, 4.15; found C, 63.95; H, 2.42; N, 4.18.

2-Chloro-3-dipropionylamino-1,4-naphthoquinone (9). Melting point 139–143 °C. IR (cm^{-1}) 3457, 3341, 2983, 2944, 2881, 1742, 1708, 1677, 1610, 1591, 1462, 1354, 1313, 1284, 1258, 1180, 1152, 1111, 1075, 1033, 1013, 914, 859, 797, 720, 632. ^1H NMR ($CDCl_3$) δ 1.16 (t, 6H, $J=7.2$ Hz), 2.69 (m, 4H), 7.85 (m, 2H), 8.19 (m, 1H), 8.25 (m, 1H). MS *m*/*e* 319.0595 (319.0611 expected M^+ for $C_{16}H_{14}NO_4Cl$)

2-Chloro-3-dibutyrylamino-1,4-naphthoquinone (10). Melting point 111–113 °C. IR (cm^{-1}) 3466, 3335, 2966, 2933, 2874. 1742, 1709, 1683, 1611, 1591, 1255, 1183, 1156, 1123, 1097, 860, 722, 630. ^1H NMR ($CDCl_3$) δ 0.95 (t, 6H, $J=7.4$ Hz), 1.64–1.76 (m, 4H). MS *m*/*e* 348.0974 (348.1003 expected M+1 for $C_{18}H_{18}NO_4Cl$). Anal. calcd for $C_{18}H_{18}NO_4Cl$: C, 62.16; H, 5.22; N, 4.03; found C, 61.98; H, 5.16; N, 3.95.

2-Chloro-3-divalerylamino-1,4-naphthoquinone (11). Viscous liquid (gum). IR (cm^{-1}) 2959, 2933, 2881, 1730, 1683, 1610, 1600, 1472, 1318, 1286, 1259, 1180, 1146, 1089, 1040, 870, 797, 715, 636. ^1H NMR ($CDCl_3$) δ 0.88–0.93 (m, 6H), 1.28–1.40 (m, 4H), 1.57–1.70 (m, 4H), 2.50–2.72 (m, 4H), 7.80–7.88 (m, 2H), 8.15–8.20 (m, 1H), 8.22–8.28 (m, 1H). MS *m*/*e* 378.1472 (378.1472 expected M+3 for $C_{20}H_{22}N0_4Cl$).

2-Chloro-3-diisovalerylamino-1,4-naphthoquinone (12). Melting point 118–121 °C. IR (cm^{-1}) 3440, 2962, 2881, 1731, 1709, 1683, 1611, 1591, 1450, 1375, 1368, 1258, 1163, 1091, 873, 736, 636. ^1H NMR ($CDCl_3$) δ 0.95, 0.96 (d, 6H, $J=2.8$ Hz), 0.97, 0.98 (d, 6H, $J=2.8$ Hz), 2.14–2.30 (m, 2H), 2.44–2.62 (m, 4H), 7.83–7.88 (m, 2H), 8.17–8.28 (m, 2H). MS *m*/*e* 375.1217 (375.1237 expected M^+ for $C_{20}H_{22}NO_4Cl$). Anal. calcd for $C_{20}H_{22}NO_4Cl$: C, 63.91; H, 5.90; N, 3.73; found C, 63.79; H, 5.97; N, 3.75.

Materials and methods for bioassays

Reagents. Catalytic subunit of cAMP dependent protein kinase (PKA) isolated from bovine heart and protein kinase C isolated from rat brain were purchased from Pierce. Mouse recombinant cAMP dependent protein kinase catalytic subunit was purchased from Calbiochem. The dye-labeled substrates, Kemptide (Lissamine Rhodamine B-LRRASLG) and Myelin basic protein (MBP 4-14) (Lissamine Rhodamine B-EKRPSQRSKYL), were purchased from Pierce as a lyophilized powder with 1% bovine serum albumin in amber vials.

Mitogen activated protein kinase assay

Inhibitors were dissolved in DMSO to give 10 mg/mL solution and successive 10-fold dilutions were prepared. The test solution (1.0 μL) was placed in a plastic test tube and 30 μL enzyme solution (8 μg GST-ERK1, 0.32 mg/mL bovine serum albumin, 16 μg/mL leupeptin, 20 mM Tris pH 7.4 buffer) was added and the tube placed in ice. The reactions were started by adding to each tube 20 μL of substrate solution (ATP to give 50 μM final concentration of 1000–2000 cpm per pmol, porcine myelin basic protein to give 1.0 mg/mL final concentration, magnesium chloride to give 10 mM final concentration in the assay, and *p*-nitrophenylphosphate to give 2.5 mM in the assay) and the placement of the tubes at 30 °C for 30 min. Each reaction was terminated by removing a 5-μL portion and applying it to P81 phosphocellulose paper and immediately washing the paper with distilled water. The radioactivity on the washed and dried papers was determined by counting Cerenkov radiation in a liquid scintillation counter. Mouse ERK1 (as a recombinant glutathione S-transferase fusion protein) was prepared by glutathione-agarose affinity chromatography from extracts of JM109 *Escherichia coli* containing the plasmid pGEX-3X:ERK1. Porcine myelin basic protein was prepared from pig brain.

MEK1 and Raf1 assays

Inhibitors were dissolved and diluted in DMSO and 1 μL of dilution to give the indicated final concentration, was placed in a plastic test tube. 30 μL enzyme solution was added. To assay for raf-1, partially purified raf-1 (expressed in Sf9 insect cells infected with baculovirus directing expression of wt human c-raf-1) was supplied as the enzyme and a kinase-inactive mutant of GST-MEK1 was included as substrate. To assay MEK1, GST-MEK1 expressed in bacteria and purified

on glutathione agarose was used as the enzyme and a kinase-inactive mutant of His-6-ERK1 (expressed in bacteria and purified on Ni-NTA-agarose) was included as substrate. All enzyme solutions additionally included leupeptin at 10 μg/mL (final concentration in the assay). The reactions were started with the addition of 20 μL of substrate solution (ATP to give 50 μM final concentration of 1000–2000 cpm per pmol, magnesium chloride to give 10 mM final concentration in the assay, and *p*-nitrophenylphosphate to give 2.5 mM in the assay) and the placement of the tubes at 30 °C for 20 min. Each reaction was terminated by the addition of 50 μL of stop solution (10 mg/mL sodium dodecylsulfate, 10% 2-mercaptoethanol, 20 mM EDTA) and heating at 95 °C for 10 min. The phosphoproteins in the reactions were analyzed by separation on 10% polyacrylamide-SDS gels, electrophoretic transfer onto nitrocellulose membranes, and autoradiography with X-ray film. The radioactivity present in the substrate protein bands was determined by densitometry and the concentration of inhibitor needed for 50% inhibition of the enzyme was determined from the dose-response curve for each compound. Reactions containing solvent (DMSO) only were run as uninhibited controls. Each compound but **6** and **7** were tested for inhibition of MEK1 at 1 and 5 μg/mL, while **6** and **7** were tested at 0.2 and 1.0 μg/mL. All compounds were tested for inhibition of raf-1 at 10 and 30 μg/mL.

Protein kinase A and protein kinase C assays

The PKA assay and protein kinase C assay were performed using the colorimetric PKA assay kit and colorimetric PKC assay kit (SpinZyme™ Kit, Pierce), respectively. Standard curves were generated for both assays according to the instruction booklets that came with the kits. The best enzyme concentration was chosen from the standard curve to ensure that the amount of enzyme added to each assay tube phosphorylated the peptide substrate in a linear fashion over a 30-min incubation, and the UV absorbance of the phosphorylated substrate in the control is larger than 0.5 AU. For bovine heart PKA, 1.7 unit of PKA (a unit of activity is the amount of enzyme required to catalyze the transfer of 1 pmol of phosphate to casein in 1 min at pH 7.4) added to each tube. For mouse recombinant PKA, 0.17 unit of the enzyme (a unit is defined as the amount of enzyme that will transfer 1 pmol of phosphate to kemptide per min at 30 °C, pH 7.5) was chosen. For rat brain PKC, 0.05 unit of PKC (a unit is defined as the enzyme activity that catalyzes the transfer of 1 nmol of phosphate to histone H1 in one min at 30 °C) was chosen and added to each tube.

Cyclic AMP dependent protein kinase assay

The compounds were dissolved and diluted in DMSO to appropriate concentration and 1 μL of the solution was added to each tube followed by addition of buffers and substrate solutions. Finally timed additions (every 30 s) of enzyme solutions to each assay tube initiated the reaction. The 36 μL of final reaction mixture contained 2 mM ATP, 10 mM $MgCl_2$, 0.002% Triton X-100, 20 mM Tris, 100 μM cAMP, 310 μM Kemptide, and the enzyme (1.7 U for bovine heart PKA, 0.17 U for mouse recombinant PKA) with a pH of 7.4. Positive control contained 1 μL of DMSO instead of the inhibitor solution. Negative control contained the same volume of dilution buffer instead of the enzyme solution. The reaction mixtures were vortexed and incubated at 30 °C for 30 min. Then, timed aliquots (30 μL every 30 s) were removed from each assay tube and applied to the affinity membrane of an individual SpinZyme™ Affinity Separation Unit. Phosphopeptide binding buffer (2 × 250 μL, pH 5.0) that contained 0.1 M sodium acetate, 0.5 M sodium chloride, and 0.02% sodium azide, were added to each separation unit and incubated for 3 min. The buffer, which eluted non-phosphorylated peptide, was washed through the membrane by centrifugation at 5500–5800 rmp for 1 min. The membrane-containing bucket was moved to a new receptacle. Phosphopeptide elution buffer (2 × 250 μL, pH 8.0) that contained 0.1 mM ammonia bicarbonate and 0.02% sodium azide, was added to the bucket, and incubated for 3 min and centrifuged at 5500–5800 rmp for 1 min. The resulted 500 μL eluted solution of phosphorylated substrate was measured with a Shimadzu UV spectrometer at 570 nm against a blank containing 500 μL of elution buffer.

Averaged negative control absorbance values were subtracted from all inhibitor sample absorbance and the positive control absorbance before averaging their triplicate absorbance values. The% inhibition value for an inhibitor sample was calculated from the following equation:

$$\%\ \text{Inhibition} = (1 - \text{Inhibitor Absorbance Value}/\text{Positive Control Absorbance value}) \times 100\%$$

Protein kinase C assay

The procedure of protein kinase C assay is similar to that of PKA assay. The 36 μL of final reaction mixture contained 2 mM ATP, 10 mM $MgCl_2$, 0.002% Triton X-100, 20 mM Tris, 0.1 mM $CaCl_2$, 0.2 mg/mL phosphatidyl-L-serine, 350 μM Lissamine Rhodamine B labeled Myelin basic protein, and 0.05 unit of the enzyme PKC with a pH of 7.4. The phosphorylated peptide substrate was isolated from the unphosphorylated one using the SpinZyme™ Affinity Separation membrane. The% inhibition value for inhibitor samples were calculated with the same equation used for PKA assay.

Acknowledgements

We are grateful to the National Cancer Institute of the National Institute of Health for financial support.

References and Notes

1. Cobb, M. H.; Goldsmith, E. J. *J. Biol. Chem.* **1995**, *270*, 14843.

2. Sivaraman, V. S.; Wang, H. Y.; Nuovo, G. R.; Malbon, C. C. *J. Clin. Invest*. **1997**, *99*, 1478.
3. Das, R.; Vonderhaar, B. K. *Breast Cancer Res. Treat*. **1996**, *40*, 141.
4. Tong, L.; Pav, S.; White, D. M.; Rogers, S.; Crane, K. M.; Cywin, C. L.; Brown, M. L.; Pargellis, C. A. *Nat. Struct. Biol*. **1997**, *4*, 311.
5. Oka, H.; Chatani, Y.; Hoshino, R.; Ogawa, O.; Kakehi, Y.; Terachi, T.; Okada, Y.; Kawaichi, M.; Kohno, M.; Yoshida, O. *Cancer Res*. **1995**, *55*, 4182.
6. Schmidt, C. M.; McKillop, I. H.; Cahill, P. A.; Sitzmann, J. V. *Biochem. Biophys. Res. Commun*. **1997**, *236*, 54.
7. Amundadottir, L. T.; Leder, P. *Oncogene* **1998**, *16*, 737.
8. Alessi, D. R.; Cuenda, A.; Cohen, P.; Dudley, D. T.; Saltiel, A. R. *J. Biol. Chem*. **1995**, *270*, 27489.
9. Favata, M. F.; Horiuchi, K. Y.; Manos, E. J.; Daulerio, A. J.; Stradley, D. A.; Feeser, W. S.; Van Dyk, D. E.; Pitts, W. J.; Earl, R. A.; Hobbs, F.; Copeland, R. A.; Magolda, R. L.; Scherle, P. A.; Trzaskos, J. M. *J. Biol. Chem*. **1998**, *273*, 18623.
10. Williams, D. H.; Wilkinson, S. E.; Purton, T.; Lamont, A.; Flotow, H.; Murray, E. J. *Biochemistry* **1998**, *37*, 9579.
11. Taher, M. M.; Hershey, C. M.; Oakley, J. D.; Valerie, K. *Photochem. Photobiol*. **2000**, *71*, 455.
12. Chang, H.-X.; Chou, T.-C.; Savaraj, N.; Liu, L. F.; Yu, C.; Cheng, C. C. *J. Med. Chem*. **1999**, *42*, 405.
13. Kartoflitskaya, A. P.; Stepanyuk, G. I.; Yushkova, V. V.; Marintsova, N. G.; Novikov, V. P. *Pharm. Chem. J*. **1997**, *31*, 291.
14. Huang, L. J.; Chang, F. C.; Lee, K. H.; Wang, J. P.; Teng, C. M.; Kuo, S. C. *Bioorg. Med. Chem*. **1998**, *6*, 2261.
15. Lien, J. C.; Huang, L. J.; Wang, J. P.; Teng, C. M.; Lee, K. H.; Kuo, S. C. *Bioorg. Med. Chem*. **1997**, *5*, 2111.
16. Ertl, P.; Cooper, D.; Allen, G.; Slater, M. J. *Bioorg. Med. Chem. Lett*. **1999**, *9*, 2863.