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Structure–activity relationships of benzimidazole-based selective inhibitors of the mitogen activated kinase-5 signaling pathway

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ABSTRACT

In a prior communication we identified a novel class of benzimidazole-based inhibitors of EGF-induced phosphorylation of ERK5. In this paper we examine the biological activity of several 1-isopropyl-4-amino-6-ether linked benzimidazole-based compounds for their ability to selectively inhibit EGF-mediated ERK5 phosphorylation; potential utility of variation at the 6-position was indicated by the initial structural feature survey. Modification of EGF-induced formation of pERK1/2 and pERK5 in HEK293 cells were analyzed by Western blot analysis. Subsequent analysis of selected compounds in a high-throughput multiple kinase scan and the NCI 60-cell-line screen is presented.

1. Background

The mitogen activated protein kinase (MAPK) signaling pathway is a complex intersecting series of phosphorylation events that couple an extracellular signaling event such as growth factor binding, chemical mitogen presence, or stress with an appropriate cellular response such as MefC, Mef2A, or CREB upregulation.[1–4] These cellular responses can be either cytosolic responses or nuclear responses. The localization of the sub-cellular response is dependent on several factors including the degree of phosphorylation of the final signaling kinase known as extracellular signal-regulated kinase (ERK), binding of signaling kinases and/or ERK to scaffold proteins, and additional proteins associated with the phosphorylated ERK (pERK) scaffold complex.[5] Typically the sequence of signaling events proceeds as follows: (1) growth factor binding, (2) receptor dimerization and autophosphorylation, (3) mitogen activated kinase kinase kinase (MEKK) phosphorylation, (4) mitogen activated kinase kinase (MEK) phosphorylation, and (5) resultant ERK phosphorylation. There are considerable cross-interactions between the receptors, MEKK phosphorylation, MEK phosphorylation, and pERK effects. The most specific interaction in these cascades is the MEK mediated phosphorylation of ERK; for example, MEK5 is the only kinase that can phosphorylate ERK5 and ERK5 is the only known substrate for MEK5.[6]

The complexity of the signaling pathway and the relevance of the MAPK signaling pathway to human disease states including cancers,[5,7] specifically squamous cell carcinoma,[8] prostate,[9] and breast cancer[10]—in the case of MEK5 mediated phosphorylation of ERK5—suggest this would be a opportune area for the development of new therapeutic agents. There have been two reports of MEK5 inhibitors recently.[11,12] One group[11] presented a series of compounds of ATP-competitive oxindole inhibitors, represented by BIX02188 **1**, the second was our early communication[12] where compound **2** was identified as a novel inhibitor of EGF-mediated ERK phosphorylation. This communication examined compounds originally designed in the context benzimidazole-based ATP-site competitive inhibitors for the enzyme cyclin-dependent kinase-5 (CDK5). The rationale for cross-assaying was that early MEK inhibitors were derived from existing CDK2 inhibitors and that both MEK5 and CDK5 are ATP utilizing serine/threonine kinases. Analysis of how structural variation over the entire benzimidazole core inhibited the EGF-induced formation of pERK1/2 and pERK5, indicated that a 1-isopropyl group was better than a larger substituent, 4-mono-benzyl amine compounds were better than 4-dibenzyl amine compounds, and that a simple 6-methoxy compound was more potent than more complicated alkoxy or benzyl derivatives; see Figure 1. This last point stands in stark contrast to the emerging SAR on 1-isopropyl-4-benzylamino-6-substitutedbenzimidazoles at the enzyme CDK5.[13–16] For this core to have activity at CDK5, a hydrogen bond donor has been found essential for activity; this same structural feature results in inactivity in EGF-mediated

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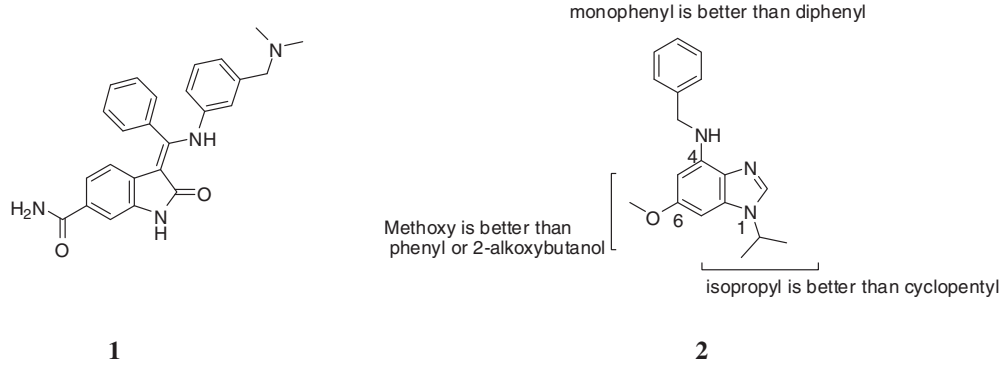


Figure 1. Known inhibitors of EGF-induced ERK5 phosphorylation.

phosphorylation of ERK. In a parallel observation, 6-methoxy compound **2** was inactive as a CDK5 inhibitor.

The marked divergence of the SAR between CDK5 and EGF-induced pERK5 formation derived from the presence of the 6-methoxy moiety of **2** encouraged further exploration of substitution at this position; see Figure 2. Our approach took a two-prong strategy. First, selected 6-alkoxy compounds **3–6** prepared[13] as a part of a structure-based-molecular modeling assisted program directed at identifying new CDK5 inhibitors were counter-screed against MEK5. As the SAR at MEK5 began to emerge, we recognized that the alkoxy derivatives followed a pattern that had been observed for known receptor tyrosine kinase (RTK) inhibitors such as gefitinib and erlotonib.[17–21] Examination of the side-chains previously utilized in RTK inhibitors suggested that compounds **7–10** should be prepared as these side-chains had displayed activity in several RTK SAR studies.

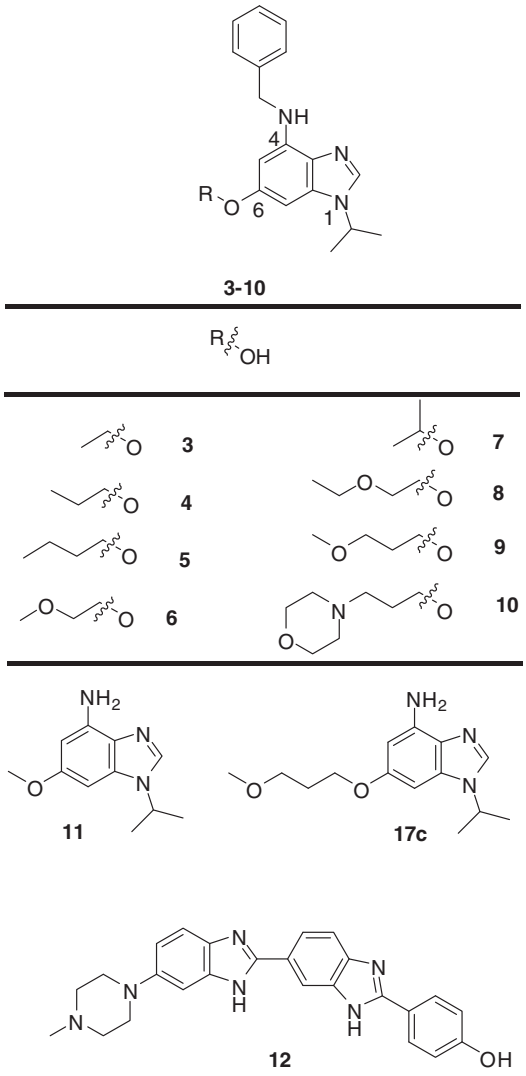
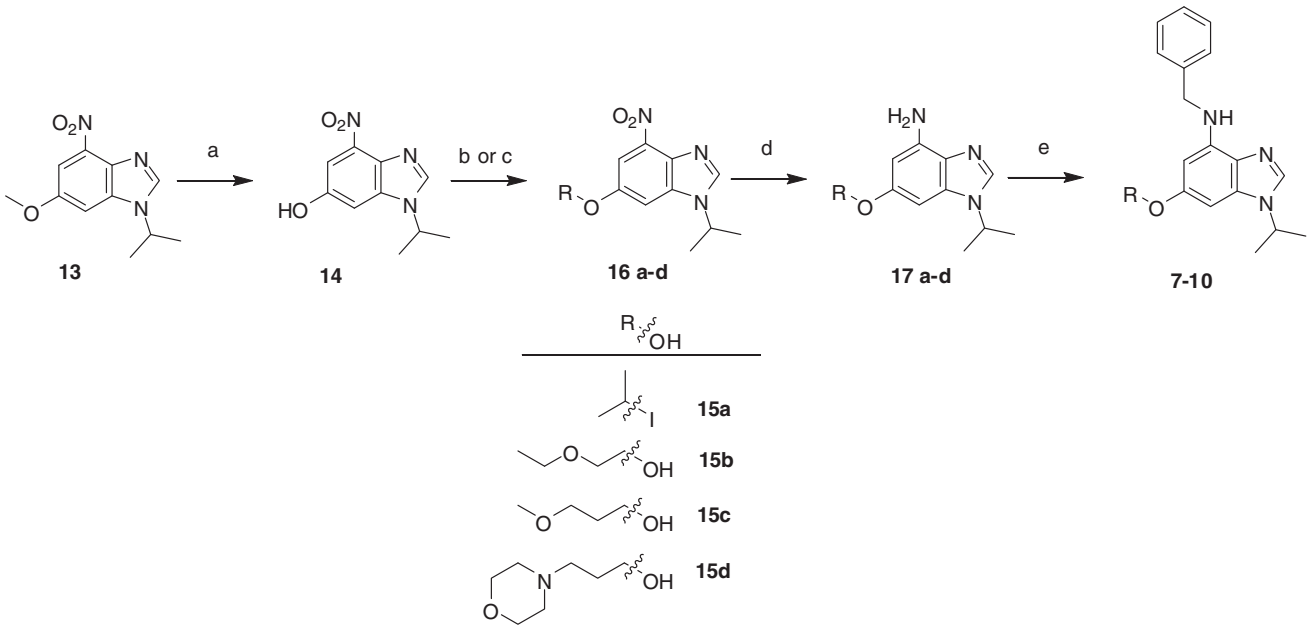


Figure 2. Compounds prepared and assayed for inhibition of EGF-induced ERK phosphorylation.

Compounds **2–6** have been prepared and characterized previously.[13,22] Synthesis for compounds **7–10** followed this sequence. Selective reductive alkylation of the known precursor diamine, then Philips cyclization in a mixed-acid system of formic acid and concentrated hydrochloric acid gave the previously described **13**.[22] See Scheme 1. 6-Methoxy deprotection proceeded in concentrated HBr with microwave irradiation.[22] Alkylation of the resultant phenol **14** proceeded either via Mitsunobu alkylation utilizing the precursor alcohol **15b–d** or by sodium phenolate attack on the alkyl iodide **15a**. The choice of method employed was determined by commercial availability of the alkyl precursor. However the sodium phenolate alkylation was far cleaner permitting direct isolation and characterization of the ether product **16a**. When the Mitsunobu coupling protocol was employed, the resultant ethers **16b–d** co-eluted with attempted silica column chromatography with coupling side products notably triphenylphosphine oxide. These compounds were carried on as the crude as indicated in Section 3 and characterized via combustion analysis at subsequent synthetic steps. Catalytic reduction of the 4-nitro group to the corresponding 4-amino moiety was smoothly affected utilizing palladium on carbon as the catalyst under 50 PSI in a shaker apparatus to give compounds **17a–d**. Final reductive alkylation gave the 4-amine to the monobenzyl amines **7–10**. All compounds were prepared as the 4-amino mono benzyl derivatives based on prior observation[12] of this feature as a requirement for inhibition of EFG-mediated ERK5 phosphorylation. Mono alkylation was achieved routinely by the careful use a single stoichiometric equivalent of benzaldehyde, the use of sodium triacetoxborohydride as the hydride source, and the absence of an acid catalyst normally employed to activate the intermediate imine to the more reactive iminium species. For the new compounds prepared, no additional protective group strategy was necessary. Non-benzylated 4-amino derivatives were prepared during the synthesis as intermediates, were tested, and are presented when relevant. Compounds were prepared and tested as their free base or as their hydrochloride salt on the basis of which form a generated compound of purity adequate for combustion analysis.

To determine the capacity for compounds **2–12** and **17c** to inhibit EGF-mediated ERK phosphorylation, HEK293 cells were treated with 50 ng/mL of EGF to activate all MEK/ERK pathways. EGF was added to begin the phosphorylation cascade. After 30 min an



Scheme 1. Preparation of additional 6-alkoxy analogs of **1**. Reagents and conditions: (a) 48% HBr, microwave irradiation, 120 °C, 2.5 h, 97%[22]; (b) NaH, DMF, 23 °C, 2 h, 53%; (c) ROH (2.5 equiv), PPh_3 (2.5 equiv), DIAD (2.5 equiv) 30–46%; (d) 50 psi H_2, EtOH, 5% Pd/C, 23 °C, 12 h, 90–98%; (e) benzaldehyde (1.1 equiv), $NaHB(OAc)_3$ (2.1 equiv), DCE, 23 °C, 63–65%.

aliquot of test compound, initially prepared as a 10 mmol solution in DMSO, was added to generate a final concentration of 10 μM in the cellular incubate. Phosphorylation of ERK1/2 by MEK1/2 and phosphorylation of ERK5 by MEK5 were examined by Western blot analysis for phosphorylated (i.e., activated) pERK. Differentiation between pERK1/2 and pERK5 was cleanly provided by prior SDS–PAGE separation on the basis of molecular weight; pERK1/2 is approximately 44 kDa while pERK5 is much larger with a molecular weight of approximately 115 kDa. See Table 1.

Given that these compounds utilized a structural feature derived from the SAR of RTKs[17–19,21] a concern was that these compounds could be exerting their effects via inhibition of these kinases. The capacity for compound **9** to inhibit the EGF-mediated phosphorylation of MEK5 in an isoform selective manner and the ability of the different compounds to differentially affect phosphorylation of different ERK isoforms is inconsistent with this postulate. However, to further examine this possibility, compound **9** was submitted to a high-throughput screen at Ambit biosciences[23–25] (Kinomescreen). These assays examine the capacity for test compounds to competitively displace a solid-support ATP mimic from each specific enzyme's active site.[24,25] Two specific branches of the kinase family tree, or kinome, were selected for screening: RTKs and STEs. RTKs were selected to survey possible interactions at the level of receptor activation. STEs were selected as they represent the family of related kinases that include the various MEK isoforms.[24] At a 10 μm concentration no reasonable affinity was identified for the RTKs examined. See Supplementary data. Curiously the capacity for compound **9** to compete for the MEK5 ATP binding site was not seen. This raises questions regarding the specific site(s) of activity for compounds **2**, **9**, and **12** either on MEK5 or perhaps on another biological target mediating the EGF-induced phosphorylation of MEK5. One possibility is that these compounds could be allosteric inhibitors of MEK5. Other possibilities include the possibility of interaction with other yet unidentified biological targets.

Table 1
Cellular assay of inhibition of EGF-mediated formation of pERK isoforms

Compounds	pERK1/2 (%)	Inhibition (%)	pERK5 (%)	Inhibition (%)
2	46	54	15	85
3	246	Activation	37	63
4	313	Activation	76	24
5	153	Activation	95	5
6	170	Activation	115	Activation
7	100	0	78	22
8	0	100	25	75
9	487	Activation	50	51
10	9	92	100	0
11	82	18	58	42
17c	0	100	32	68
12	78	22	70	30
Staurosporine	18	82	78	22
U0126	0	100	73	27
NT	0	100	0	100
+	100	0	100	0

Average of three experiments, error ±5%.
NT = no treatment with EGF.
+ = treatment with EGF.

Compounds **2** and **12** have previously[12] been submitted to the NCI's 60-cell-line screen[26] (single dose inhibition study). Compare profile analysis identified compound **12** as being the most similar compound in the NCI's open access small molecule database to compound **2**. Interestingly both compounds contain a benzimidazole core. Compound **12** is the known DNA minor groove binding dye Hoechst 33258.[27] This commercially available compound was examined in the assay for EGF-mediated ERK phosphorylation and was shown to inhibit both pERK1/2 and pERK5 formation by about 30%. Curiously when compound **9**, a selective inhibitor of pERK5 formation, was tested in the NCI 60-cell-line screen and analyzed using the compare profile, it exhibited a much lower similarity to either the methoxy compound **2** or Hoechst 33258 than they displayed amongst themselves; see Supplementary data.

2. Discussion

Modification of the 6-alkoxy substituent on the 1-isopropyl-4-benzylaminobenzimidazole core did modify the observed inhibition of EGF-mediated phosphorylation of ERK. This SAR is divergent from that observed for CDK5. Of the compounds exam-

ined compounds **3**, **4**, **7**, and **9** displayed the most selectivity for inhibition of EGF-induced ERK5 phosphorylation versus inhibiting pERK1/2 formation. Significantly these compounds did not inhibit MEK1/2 as did the previously identified compound **2**. The morpholino-derivative, **10**, was noticeably inactive consistent with a cationic group ablating modification of pERK levels. It is unclear if this is a direct effect or an artifact of cellular transport. Of these compounds **3** and **9** were equipotent within the limitations of the assay (63% and 50% inhibition of EGF-induced formation of pERK5, respectively). Given that the 4-amino debenzylated compounds **11** and **17c** also displayed activity it was a concern if this could be a complicating factor as these compounds were examined in further studies with in vivo systems. Compound **11**, the 4-amiodebenzylated derivative of **2**, displays inhibition of both pERK1/2 and pERK5 as did its 4-aminobenzyl congener **2**. Compound **17** is devoid of the ability to block EGF induction of pERK1/2 and is the 4-amino debenzylation derivative of compound **9**. Of note is that a considerable number of compounds could actually increase pERK1/2 levels. It is know that inhibition of MEK1/2 can result in increased MEK5 levels, but modification of MEK1/2 levels by inhibiting MEK5 levels has not been shown. This upregulation of pERK1/2 appears to be independent of the decrease in pERK5 levels and suggests a secondary interaction for these compounds mediating this effect rather than a direct compensatory mechanism. Additional work is required to better understand this upregulation of pERK1/2 by these compounds. In an attempt to better understand the role of selective inhibition of MEK5 versus MEK1/2, compound **9** was selected for additional testing; this compound would have the greatest decrease in MEK5 relative to MEK1/2. The determination of inhibition of pERK1/2 and pERK5 utilized is a cellular assay so it is likely that the differential inhibition of pERK isoforms represents a direct effect on the biological target rather than an artifact arising from differential membrane permeability or biological partitioning.

The relatively low ATP-site affinity of these compounds in the high-throughput ATP-site assay raises some interesting possibilities. The previously reported series of oxindole compounds,[11] of which **1** is an example, did display an ATP-competitive nature in a similar high-throughput assay and did attenuate pERK5 in vivo. It is possible that the benzimidazole-derived compounds presented may be exerting their attenuation of EGF-mediated pERK5 formation by binding to a site other than the ATP site on MEK5. This possibility would be consistent with the inability of the isolated enzyme and an ATP-competitive assay to detect a previously indentified in vivo inhibitor of EGF-mediated pERK5. Recently a kinetic analysis of MEK1/2 has been published,[28] and it has long been known that allosteric inhibitors of MEK1/2 exist.[1,3,4,7,29] An X-ray crystal structure of such a complex has been deposited in the PDB: 1S9J. This confirms the necessity to conduct both isolated enzyme and in vivo kinase assays to adequately characterize the origin of EGF-mediated MEK5 inhibition.

The differential activity in the NCI's 60-cell screen and the activity of Hoescht 33258 in attenuating the EGF-mediated formation of pERK5 again suggests some interesting possibilities. Hoescht 33258 is a known DNA minor groove binder and finds routine utility as an assay dye for DNA. The marked difference of compound **9** from compound **1** in the 60-cell-line screen could arise from either the selective inhibition of MEK5 or perhaps from the observed upregulation of pERK1/2. Regardless the conversion of the 6-alkoxy substituent does modify the selectivity for the inhibition of pERK5 formation relative to pERK1/2 formation and may underlie the observed difference in these cancer cell types.

The capacity of these compounds to uniquely raise or lower the level of ERK5 phosphorylation might not be mediated directly at the level of MEK5 phosphorylation of ERK5, but may arise from a yet unidentified biological target. Current studies are underway to more closely examine this series of compounds in isolated enzymes including MEK5 assays which will permit a kinetic analysis and a better determination of the nature of these compounds' observed in vivo inhibition of EGF-mediated phosphorylation of MEK5. A pull-down analysis of the potential biological site(s) of interaction should assist in determining the basis for attenuation of EGF-mediated pERK5 formation and is planned. Additional analysis of this series using in vitro models more representative of native human tumors will also be examined.

3. Experimental

All solvents and reagents were used as received unless noted otherwise. Tetrahydrofuran (THF) was distilled from Na–benzophenone ketyl radical under a blanket of argon prior to use. All reactions were conducted in dry glassware and under an atmosphere of argon unless otherwise noted. Microwave reactions were conducted in sealed tube and utilized a multimode Milestone Start apparatus for irradiation with power and control parameters as noted. Melting points were determined on a MelTemp apparatus and are uncorrected. All proton NMR spectra were obtained with an 500 MHz Oxford spectrospin cryostat, controlled by a Bruker Avance system, and were acquired using Bruker Topsin 2.0 acquisition software. Acquired FIDs were analyzed using MestReC 3.2. Elemental analyzes were conducted by Atlantic Microlabs and are ±0.4 of theoretical. All HRMS mass spectral analyzes were conducted at Duquesne University with a nano ESI chip cube TOF HRMS and are ±0.004 of theoretical. All ^{1}H NMR spectra were taken $CDCl_3$ unless otherwise noted and are reported as ppm relative to TMS as an internal standard. Coupling values are reported in Hertz.

3.1. 6-Isopropoxy-1-isopropyl-4-nitro-1*H*-benzo[*d*]imidazole (16d)

A dry one-necked flask was charged with 1-isopropyl-4-nitro-1*H*-benzo[*d*]imidazol-6-ol **14** (300.0 mg, 1.36 mmol), 2-iodopropane **15a** (0.27 mL, 2.72 mmol), and 2.5 mL of DMF. NaH (163.2 mg, 6.8 mmol) was added at 0 °C in two portions. This solution was stirred at 0 °C for 30 min and at 23 °C for an additional 2 h. The reaction mixture was poured into 10 mL EA and 10 mL K_2CO_3 (aq, satd). The mixture was extracted three times each with 10 mL portions of EA and the combined extracts were washed with NaCl (aq, satd) and dried over Na_2SO_4. The extract was decanted and the solvent was removed under reduced pressure. The crude material (177.1 mg) was isolated on SiO_2 and eluted with hexanes/EA. Recrystallization from EA and hexane gave 154.0 g (51.3%) of **16a** as a yellow solid. Mp: 78.9–80.1 °C. SiO_2 TLC R_f 0.55 (CH_2Cl_2/5% CH_3OH/0.1% NH_4OH). ^{1}H NMR ($CDCl_3$): δ 8.11 (s, 1H), 7.79 (d, J = 2.0 Hz, 1H), 7.21 (d, J = 2.0 Hz, 1H), 4.61 (m, 1H), 4.57 (m, 2H), 1.64 (d, J = 6.8 Hz, 6H), 1.40 (d, J = 6.8 Hz, 6H). Anal. Calcd for $C_{13}H_{17}N_3O_3 \cdot 1/6\ H_2O$: C, 58.63; H, 6.56; N, 15.78. Found: C, 58.85; H, 6.40; N, 15.94.

3.2. 6-Isopropoxy-1-isopropyl-1*H*-benzo[*d*]imidazol-4-amine (17a)

6-Isopropoxy-1-isopropyl-4-nitro-1*H*-benzo[*d*]imidazole **16a** (110 mg, 0.42 mmol) was dissolved into 75 mL EtOH and added to a Parr hydrogenation vessel previously charged with Pd/C (10% w/w, 6.2 mg). After three vacuum/purge cycles with H_2, the vessel was charged to 50 psi with H_2 and shaken for 5 h. The mixture was filtered through a pad of Celite and then washed with an additional 25 mL of EtOH. The solvent was removed under reduced pressure. 95.2 mg (97.7%) of **17a** as a pale brown, hygroscopic solid. Mp: 34.2–35.0 °C. SiO_2 TLC R_f 0.33 (CH_2Cl_2/5% CH_3OH/0.1% NH_4OH).

^{1}H NMR ($CDCl_3$): δ 7.75 (s, 1H), 6.27 (d, J = 2.0 Hz, 1H), 6.20 (d, J = 2.0 Hz, 1H), 4.47 (m, 1H), 4.05 (s, 2H, NH_2, 2H), 1.57 (d, J = 6.8 Hz, 6H), 1.35 (d, J = 6.8 Hz, 6H). Anal. Calcd for $C_{13}H_{19}N_3O{\cdot}1/4H_2O$: C, 65.66; H, 8.29; N, 17.67. Found: C, 65.73; H, 7.90; N, 17.62.

3.3. *N*-Benzyl-6-isopropoxy-1-isopropyl-1*H*-benzo[*d*]imidazol-4-amine (7)

6-Isopropoxy-1-isopropyl-1*H*-benzo[*d*]imidazol-4-amine **17a** (83.4 mg, 0.36 mmol) and solid $NaBH(OAc)_3$ (227.38 mg, 1.07 mmol) were added to 2.5 mL of DCE. Benzaldehyde (43.8 μL, 0.43 mmol) was added in one portion and the mixture was stirred for 12 h. The reaction mixture was quenched with the addition of 1 mL of $NaHCO_3$ (aq, satd) and stirred for an additional 10 min. The mixture was taken up into 25 mL of 5% K_2CO_3 (aq, satd) and then extracted three times with 15 mL portions of Et_2O. The combined extracts were washed three times with 5 mL portions of NaCl (aq, satd) and then dried over Na_2SO_4. The extract was decanted and the solvent was removed under reduced pressure. The crude material was isolated on SiO_2 and eluted with hexanes/EA. This material was recrystallized from EA and hexanes gave 75.7 mg (65.5%) of **7** as a white crystalline solid. Mp: 101.6–101.9 °C. SiO_2 TLC R_f 0.53 (CH_2Cl_2/5% CH_3OH/0.1% NH_4OH). ^{1}H NMR ($CDCl_3$): δ 7.70 (s, 1H), 7.41 (d, J = 2.0 Hz, 2H), 7.31 (t, J = 2.0 Hz, 2H), 6.20 (d, J = 2.0 Hz, 1H), 6.05 (d, J = 2.0 Hz, 1H), 5.23 (m, 1H), 4.48 (s, 2H), 3.95 (m, 1H), 1.58 (d, J = 6.8 Hz, 6H), 1.31 (d, J = 6.0 Hz, 6H). Anal. Calcd for $C_{20}H_{25}N_3O$: C, 74.27; H, 7.79; N, 12.99. Found: C, 74.13; H, 7.71; N, 12.99.

3.4. 6-(2-Ethoxyethoxy)-1-isopropyl-1*H*-benzo[*d*]imidazol-4-amine (17b)

A dry one-necked flask was charged with 1-isopropyl-4-nitro-1*H*-benzo[*d*]imidazol-6-ol **14** (334.0 mg, 1.5 mmol), 2-ethoxyethanol **15b** (0.52 mL, 5.4 mmol), Ph_3P (985.0 g, 3.8 mmol), and 4 mL of anhydrous DMF. This solution was cooled to 0 °C. Neat DIAD (0.4 mL, 2.8 mmol) was added with drop-wise addition over 15 min. The solution was stirred for 30 min at 0 °C and then the ice-bath was removed. The reaction was stirred for an additional 2 h at 23 °C. The reaction was then cooled to 0 °C and an additional quantity of DIAD (0.4 mL, 2.8 mmol) was added at 0 °C in one portion. The reaction was stirred for 30 min at 0 °C, then at 23 °C for 12 h. The reaction mixture was poured into 10 mL EA and 10 mL K_2CO_3 (aq, satd), extracted three times with 10 mL portions of EA and then the combined organic extracts were washed with NaCl (aq, satd) and dried over Na_2SO_4. The extract was decanted and the solvent was removed under reduced pressure to give 4.9 g of crude **16b**. Crude **16b** was dissolved into 75 mL of EtOH and added to a Parr hydrogenation vessel previously charged with Pd/C (10% w/w, 120.2 mg). After three vacuum/purge cycles with H_2, the vessel was charged to 50 psi with H_2 and shaken for 5 h on a Parr hydrogenation apparatus. The mixture was filtered through a pad of Celite and then washed with an additional 25 mL of EtOH. The solvent was removed under reduced pressure. The crude oil was poured into 50 mL of 1 N HCl (aq) (pH 1) and washed three times with 10 mL portions of EA. The aqueous phase was neutralized and then basified with 6 N NaOH (aq) to a pH of 10 and then extracted three times with 15 mL portions of Et_2O. The combined Et_2O extracts were washed with NaCl (aq, satd) and dried over Na_2SO_4. The solvent was decanted and then removed under reduced pressure to give 119.0 mg (30%) of **17b** as a pale brown, hygroscopic solid was obtained. Mp: 37.4–38.0 °C. SiO_2 TLC R_f 0.31 (CH_2Cl_2/5% CH_3OH/0.1% NH_4OH). ^{1}H NMR ($CDCl_3$): δ 7.74 (s, 1H), 6.31 (d, J = 2.0 Hz, 1H), 6.23 (d, J = 2.0 Hz, 1H), 4.47 (m, 1H), 4.14 (t, J = 5.2 Hz, 2H), 3.80 (t, J = 5.2 Hz, 2H), 3.62 (q, J = 6.8 Hz, 2H), 1.57 (d, J = 6.8 Hz, 6H), 1.25 (t, J = 7.2 Hz, 3H). Anal. Calcd for $C_{14}H_{21}N_3O_2$: C, 62.29; H, 6.59; N, 9.47. Found: C, 62.13; H, 6.57; N, 9.36.

3.5. *N*-Benzyl-6-(2-ethoxyethoxy)-1-isopropyl-1*H*-benzo[*d*]imidazol-4-amine oxalate (8)

6-(2-Ethoxyethoxy)-1-isopropyl-1*H*-benzo[*d*]imidazol-4-amine **17b** (90.0 mg, 0.34 mmol) and solid $NaBH(OAc)_3$ (217.3 mg, 1.0 mmol) were added to 2.5 mL of DCE. Benzaldehyde (53.6 μL, 0.5 mmol) was added in one portion and the mixture was stirred for 12 h. The reaction mixture was quenched with the addition of 1 mL of $NaHCO_3$ (aq, satd) and then stirred for an additional 10 min. The mixture was taken up into 50 mL of 5% K_2CO_3 (aq, satd) and then extracted three times with 15 mL portions of Et_2O. The combined extracts were washed with NaCl (aq, satd) and then dried over Na_2SO_4. The extract was decanted and the solvent was removed under reduced pressure. The crude material was isolated on SiO_2 and eluted with hexanes/EA. The isolated product (50.2 mg, 0.14 mmol) was dissolved in 2.5 mL of EA, followed by addition of the solution of anhydrous oxalic acid (15.5 mg, 0.16 mmol) into 2.5 mL of EA. The solvent was removed under reduced pressure to afford 60.6 mg (40%) of **8** as a white crystalline solid. Mp: 158.7–158.8 °C. SiO_2 TLC R_f 0.57 (CH_2Cl_2/5% CH_3OH/0.1% NH_4OH). ^{1}H NMR (MeOD-d_4): δ 8.87 (s, 1H), 7.41 (d, J = 7.6 Hz, 2H), 7.33 (t, J = 7.2 Hz, 2H), 7.26 (t, J = 7.2 Hz, 1H), 6.56 (d, J = 2.0 Hz, 1H), 6.24 (d, J = 2.0 Hz, 1H), 4.80 (m, 1H), 4.46 (s, 2H), 4.11 (t, J = 4.4 Hz, 2H), 3.76 (t, J = 1.6 Hz, 2H), 3.56 (q, 2H), 1.63 (d, J = 6.8 Hz, 6H), 1.19 (t, J = 7.2 Hz, 3H). Anal. Calcd for $C_{29}H_{23}N_3O_6$: C, 62.29; H, 6.59; N, 9.47. Found: C, 62.13; H, 6.57; N, 9.36.

3.6. 1-Isopropyl-6-(3-methoxypropoxy)-1*H*-benzo[*d*]imidazol-4-amine (17c)

A dry one-necked flask was charged with 1-isopropyl-4-nitro-1*H*-benzo[*d*]imidazol-6-ol **14** (334.0 mg, 1.5 mmol), 3-methoxypropan-1-ol **15c** (0.52 mL, 5.4 mmol), Ph_3P (985.0 g, 3.8 mmol), and 4 mL of anhydrous DMF. This solution was cooled to 0 °C. Neat DIAD (0.4 mL, 2.8 mmol) was added with drop-wise addition over 15 min. The solution was stirred for 30 min at 0 °C and then the ice-bath was removed. The reaction was stirred for an additional 2 h at 23 °C. The reaction was then cooled to 0 °C and an additional quantity of DIAD (0.4 mL, 2.8 mmol) was added at 0 °C in one portion. The reaction was stirred for 30 min at 0 °C, then at 23 °C for 12 h. The reaction mixture was poured into 10 mL EA and 10 mL K_2CO_3 (aq, satd), extracted three times with 10 mL portions of EA and then the combined organic extracts were washed with NaCl (aq, satd) and dried over Na_2SO_4. The extract was decanted and the solvent was removed under reduced pressure to give 4.5 g of crude **16c**. Crude **16c** was dissolved into 75 mL of EtOH and added to a Parr hydrogenation vessel previously charged with Pd/C (10% w/w, 120.1 mg). After three vacuum/purge cycles with H_2, the vessel was charged to 50 psi with H_2 and shaken for 5 h on a Parr hydrogenation apparatus. The mixture was filtered through a pad of Celite and then the pad was washed with an additional 25 mL of EtOH. The solvent was removed under reduced pressure. The crude oil was poured into 50 mL of 1 N HCl (aq) (pH 1) and washed (3×10 mL) with EA. This was neutralized then basified with 6 N NaOH (aq) to a pH 10 and then extracted (3×15 mL) with Et_2O. The combined Et_2O extracts were washed with 5 mL of NaCl (aq, satd) and dried (Na_2SO_4). The solvent was decanted and then removed under reduced pressure to give 159.0 mg (40%) of **17c** as a pale brown, hygroscopic solid. Mp: 43.1–44.7 °C. SiO_2 TLC R_f 0.32 (CH_2Cl_2/5% CH_3OH/0.1% NH_4OH). ^{1}H NMR ($CDCl_3$): δ 7.74 (s, 1H), 6.28 (d, J = 2.0 Hz, 1H), 6.19 (d, J = 2.0 Hz, 1H), 4.47 (m, 1H), 4.06 (t, J = 6.4 Hz, 2H), 3.57 (t, J = 6.4 Hz, 2H), 3.36 (s, 3H), 2.06

(m, 2H), 1.57 (d, J = 6.8 Hz, 6H). Anal. Calcd for $C_{14}H_{21}N_3O_2 \cdot 1/3H_2O$: C, 62.43; H, 8.11; N, 15.60. Found: C, 62.64; H, 8.15; N, 15.38.

3.7. *N*-Benzyl-1-isopropyl-6-(3-methoxypropoxy)-1*H*-benzo[*d*]imidazol-4-amine (9)

1-Isopropyl-6-(3-methoxypropoxy)-1*H*-benzo[*d*]imidazol-4-amine **17c** (120.0 mg, 0.46 mmol) and solid $NaBH(OAc)_3$ (289.8 mg, 1.4 mmol) were added to 2.5 mL of DCE. Benzaldehyde (53.6 μL, 0.5 mmol) was added in one portion and the mixture was stirred for 12 h. The reaction mixture was quenched with the addition of 1 mL of $NaHCO_3$ (aq, satd) and then stirred for an additional 10 min. The mixture was taken up into 5% K_2CO_3 (aq, satd) and then extracted three times with 15 mL portions of Et_2O. The combined extracts were washed with NaCl (aq, satd) and then dried over Na_2SO_4. The extract was decanted and the solvent was removed under reduced pressure. The crude material was isolated on SiO_2 and eluted with hexanes/EA. Recrystallization from EA and hexane gave 101.4 mg (63%) of **9** as a white crystalline solid. Mp: 75.7–75.9 °C. SiO_2 TLC R_f 0.58 (CH_2Cl_2/5% CH_3OH/0.1% NH_4OH). ^1H NMR ($CDCl_3$): δ 7.70 (s, 1H), 7.40 (d, J = 7.6 Hz, 2H), 7.32 (t, J = 7.2 Hz, 2H), 7.29 (d, J = 7.2 Hz, 1H), 6.20 (d, J = 2.0 Hz, 1H), 6.04 (d, J = 2.0 Hz, 1H), 5.23 (m, 1H), 4.46 (m, 2H), 4.04 (t, J = 6.4 Hz, 2H), 3.55 (t, J = 6.0 Hz, 2H), 3.34 (s, 3H), 2.03 (m, 2H), 1.57 (d, J = 6.8 Hz, 6H). Anal. Calcd for $C_{21}H_{27}N_3O_2$: C, 71.36; H, 7.70; N, 11.89. Found: C, 71.22; H, 7.71; N, 11.89.

3.8. *N*-Benzyl-1-isopropyl-6-(3-morpholinopropoxy)-1*H*-benzo[*d*]imidazol-4-amine hydrochloride (16)

A dry one-necked flask was charged with 1-isopropyl-4-nitro-1*H*-benzo[*d*]imidazol-6-ol **14** (249.8 mg, 1.1 mmol), 3-morpholinopropan-1-ol **15d** (0.45 mL, 3.3 mmol), Ph_3P (737.2 g, 2.75 mmol), and 2 mL of anhydrous DMF. This solution was cooled to 0 °C. Neat DIAD (0.32 mL, 1.65 mmol) was added with drop-wise addition over 15 min. The solution was stirred for 30 min at 0 °C and then the ice-bath was removed. The reaction was stirred for an additional 2 h at 23 °C. The reaction was then cooled to 0 °C and an additional quantity of DIAD (0.22 mL, 1.1 mmol) was added at 0 °C in one portion. The reaction was stirred for 30 min at 0 °C, then at 23 °C for 12 h. The reaction mixture was poured into 10 mL EA and 10 mL K_2CO_3 (aq, satd), extracted three times with 10 mL portions of EA and the combined organic extracts were washed with NaCl (aq, satd) and dried over Na_2SO_4. The extract was decanted and the solvent was removed under reduced pressure to give crude **16d** which was dissolved into 75 mL of EtOH and added to a Parr hydrogenation vessel previously charged with Pd/C (10% w/w). After three vacuum/purge cycles with H_2, the vessel was charged to 50 psi with H_2 and shaken for 5 h on a Parr hydrogenation apparatus. The mixture was filtered through a pad of Celite and washed with an additional 25 mL of EtOH. The solvent was removed under reduced pressure. The crude oil was poured into 50 mL of 1 N HCl (aq) (pH 1) and washed three times with 10 mL portions of EA. The aqueous phase was neutralized and then basified with 6 N NaOH (aq) to a pH of 10 and then extracted three times with 15 mL portions of Et_2O. The combined Et_2O extracts were washed with NaCl (aq, satd) and dried over Na_2SO_4. The solvent was decanted and then removed under reduced pressure to give 100 mg of **17d** as a crude product. ^1H NMR ($CDCl_3$): δ 7.29 (d, J = 7.2 Hz, 1H), 6.20 (d, J = 2.0 Hz, 1H), 6.04 (d, J = 2.0 Hz, 1H), 5.23 (m, 1H), 4.06 (m, 2H), 4.04 (t, J = 6.4 Hz, 2H), 3.67 (m, 4H), 3.34 (m, 2H), 2.46 (m, 2H), 2.03 (m, 4H), 1.57 (d, J = 6.8 Hz, 6H).

Crude **17d** (100.0 mg, 0.31 mmol) was dissolved into 2.5 mL of DCE. $NaBH(OAc)_3$ (199.5 mg, 0.93 mmol) and benzaldehyde (31.0 μL, 0.31 mmol) were added in one portion and the mixture was stirred for 12 h. The reaction mixture was quenched with the addition of 1 mL of $NaHCO_3$ (aq, satd) and then stirred for an additional 10 min. The mixture was taken up into 50 mL of 5% K_2CO_3 (aq, satd) and then extracted three times with 15 mL portions of Et_2O. The combined extracts were washed with NaCl (aq, satd) and then dried over Na_2SO_4. The extract was decanted and the solvent was removed under reduced pressure. The crude material was isolated on SiO_2 and eluted with hexanes/EA. Recrystallization from EA and hexane gave 60.0 mg (46.8%) of **16** as a white solid. Mp: 186.0–186.7 °C. SiO_2 TLC R_f 0.41 (CH_2Cl_2/5% CH_3OH/0.1% NH_4OH). ^1H NMR ($CDCl_3$): δ 7.70 (s, 1H), 7.40 (d, J = 7.6 Hz, 2H), 7.32 (t, J = 7.2 Hz, 2H), 7.29 (d, J = 7.2 Hz, 1H), 6.20 (d, J = 2.0 Hz, 1H), 6.04 (d, J = 2.0 Hz, 1H), 5.23 (m, 1H), 4.06 (m, 2H), 4.04 (t, J = 6.4 Hz, 2H), 3.67 (m, 4H), 3.34 (m, 2H), 2.46 (m, 2H), 2.03 (m, 4), 1.57 (d, J = 6.8 Hz, 6H). Anal. Calcd for $C_{14}H_{21}N_3O_2 \cdot 2H_2O$: C, 55.40; H, 7.40; N, 10.83. Found: C, 55.10; H, 7.13; N, 11.20.

3.9. EGF-induced phosphorylation of ERK1/2 and ERK5

Western blot analysis. Equal amounts of protein (60 μg) from each treatment were separated on 8% SDS gels and transferred to PVDF membrane [Millipore, Danvers, MA] for Western blot analysis. Blots were blocked in 5% non-fat milk in 1×TBS/0.1%Tween/0.02% NaN_3 for 1 h and then incubated overnight at 4 °C in primary antibody (1:1000 pERK1/2, 1:500 pERK5, Cell Signaling). Blots were washed with 1×TBS/0.1%Tween for 30 min and then incubated with secondary antibody (1:1000, horseradish peroxidase-conjugated goat anti-rabbit, Upstate). Proteins were visualized with enhanced chemiluminescence (Upstate). Films were scanned and quantified using MATLAB, v.7.1 (Mathworks, Natick, MA).

3.10. High-throughput assay of compound 9

This compound was submitted for analysis for solid-support bound ATP analog displacement according to previously described protocols. Full results are disclosed in Supplementary data.

NCI 60-cell-line screens were conducted according to their routine procedure. Results of single dose treatment are presented in Supplementary data.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.09.017.

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