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Selective benzimidazole inhibitors of the antigen receptor-mediated NF-κB activation pathway

Karl J. Okolotowicz [a,*,†], Ranxin Shi [b,†], Xueying Zheng [a], Mary MacDonald [a], John C. Reed [b], John R. Cashman [a]

[a] Human BioMolecular Research Institute, 5310 Eastgate Mall, San Diego, CA 92121, USA
[b] Burnham Institute for Medical Research, 10901 N. Torrey Pines Rd., La Jolla, CA 92037, USA

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ABSTRACT

Dysregulated antigen receptor-mediated NF-κB activation can contribute to development of autoimmunity, chronic inflammation, and malignancy. A chemical biology screening strategy has identified a substituted benzimidazole that selectively inhibits antigen receptor-mediated NF-κB activation without blocking other NF-κB activation pathways. A library of analogs was synthesized and the structure–activity relationship and metabolic stability for the series is presented.

1. Introduction

Nuclear factor-kappa B (NF-κB) is a member of a family of transcription factors that play a critical role in many pathways including host-defense, immune responses, inflammation, and cancer. Currently, nine pathways are known for the activation of NF-κB in mammals that include: (1) a canonical (classical) tumor necrosis factor (TNF) pathway;[1] (2) a non-canonical (alternative) pathway activated by select TNF-family receptors;[2] (3) the Toll-like receptor (TLR) pathway that involves the TLR domain-containing adapters and interleukin-1 receptor associated kinase (IRAK) family of protein kinases;[3] (4) an exogenous RNA activated pathway that is of importance for host-defenses against viruses;[4] (5) a DNA damage pathway involving the p53-induced protein with a death domain (PIDD);[5] (6) a pathway that involves the nucleotide-binding oligomerization domain (NOD)-family of proteins, cytosolic proteins that oligomerize in response to microbial-derived molecules;[6] (7) an antigen receptor-activated pathway in B-cells and T-cells;[7] (8) inhibition of apoptosis (IAP2)-mucosa associated lymphoid tissue lymphoma translocation gene 1 through tumor necrosis factor (TNF)-receptor-associated factor 6 (TRAF6); and (9) X-linked inhibition of Apoptosis Protein (XIAP) working via a transforming growth factor β activated kinase 1 (TAB1) pathway.[8] The core event that these NF-κB activation pathways converge upon is the activation of Inhibitor of κB Kinases (IKKs) that are typically comprised of a complex of IKK-alpha, IKK-beta, and the scaffold protein, IKK-gamma. In all but the non-canonical NF-kB pathway, IKK activation results in phosphorylation of IκB-alpha, targeting this protein for ubiquitination and proteasome-dependent destruction, thus releasing p65/p50 NF-κB heterodimers from IκB-alpha in the cytosol, and allowing translocation into the nucleus where they initiate transcription of various target genes.

The NF-κB pathway activated by antigen receptors is critical for acquired (as opposed to innate) immunity and contributes to T- and B-lymphocyte activation, proliferation, survival, and effector functions. Dysregulated NF-κB activation in lymphocytes can contribute to development of autoimmunity, chronic inflammation, and lymphoid malignancy.[7,9] The NF-κB activation pathway linked to antigen receptors involves a cascade of adapter and signal transducing proteins that minimally includes Carma1, Bcl-10, Paracaspase (MALT1), TRAF6, and Ubc13. In T- and B-cells, the NF-κB pathway is initiated by protein kinase C (PKC)-theta and PKC-beta, respectively, that induces phosphorylation of components of this signaling pathway, leading ultimately to IKK activation through a mechanism involving lysine 63-linked polyubiquitination of IKK-gamma.[10] Thus, the antigen receptor pathway for NF-κB activation is initiated and concluded by activation of protein kinases (i.e., PKCs and IKKs, respectively). With respect to drug discovery, although IKKs represent logical drug targets, chemical inhibitors of IKKs suppress all reported NF-κB activation pathways, and thus lack the selectivity required to inhibit lymphocyte responses without simultaneously interfering with innate immunity and creating broad immunosuppression with considerable risk of infection.[11]

* Corresponding author. Tel.: +1 858 458 9305; fax: +1 858 458 9311.
E-mail address: kokolotowicz@hbri.org (K.J. Okolotowicz).
† Contributed equally to the manuscript.

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A chemical biology strategy for identification of chemical compounds that selectively inhibit antigen receptor-mediated NF-κB activation was devised.[12] Two libraries comprising a total of 110,000 compounds[13] were screened for inhibitors of antigen receptor-mediated NF-κB activation. Compounds with an IC_{50} <3 μM were further characterized by twelve additional counter-screens that helped determine inhibition selectivity. From the initial screens, three hits with an IC_{50} <2 μM were identified and confirmed as selective for the antigen receptor activation pathway by the counter-screen assays. Of the three hits, benzimidazole (**1**) (Fig. 1) was found to be a highly potent and selective inhibitor of antigen receptor-mediated NF-κB activation. The successful synthesis of a diverse library of compounds based on benzimidazole **1** and their biological evaluation as inhibitors of NF-κB was accomplished and is reported herein.

2. Results and discussion

2.1. Chemistry

Identification of regions in the 'hit' (i.e., compound **1**) to diversify by chemical synthesis was guided by biological evaluation of a number of closely related compounds obtained from a commercial supplier (ChemBridge Corporation, San Diego, CA). The results of inhibition studies of antigen receptor-mediated NF-κB activation from a small collection of commercial compounds suggested all three regions (i.e., **A**, **B**, **C**) of the benzimidazole core were important for NF-κB inhibitory potency (Fig. 2).

Two synthetic approaches (Schemes 1 and 2), were designed and utilized to vary either **B** or **C** portions of **1** during the last step of the synthesis.

The appropriate phenylenediamine (**2** or **3**) was treated with potassium ethyl xanthate in EtOH/H_2O to give 2-mercaptobenzimidazole **4** or **5**.[14] 2-Mercaptobenzimidazole, **4** or **5**, was further treated with HBr/Br_2 in acetic acid to afford the 2-bromobenzimidazole **6** or **7**.[15] Diversification of the **C** region was accomplished by addition of bromoacetophones **8a–d** in the presence of potassium carbonate in DMF to provide compounds **9a–d** and **10a–d**. To further diversify region **B**, compounds **9a–d** or **10a–d** were heated at 125 °C with the desired cyclic amine to give compounds **11a–d** and **12a–d** as a library of tertiary amines. It was found that an undesirable cyclic byproduct (i.e., **13**) was isolated as the major product when acyclic primary amines (e.g., 3-aminopropanol) were used in the synthesis of **1** (Fig. 3).[16]

To help minimize the undesired cyclic byproduct, the synthesis shown in Scheme 1 was further optimized as shown in Scheme 2. Heating 3-aminopropanol with **7** at 125 °C gave **14**. Compounds **14** and **15** were heated in *n*-butanol at 115 °C to give **1** as the major product, with only ~5% of **13** isolated. This optimized method gave increased yields of the desired product over the method shown in Scheme 1. For example, the method of Scheme 2 gave a ratio of 95:5 of **1**/**13**, whereas the method of Scheme 1 only gave a ratio of 2:98 of **1**/**13**. The optimized method of Scheme 2 allowed for synthesis of diversity of the **C** region using primary amine side-chains on the benzimidazole ring system.

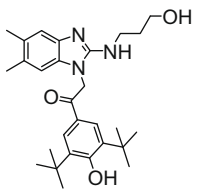

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Figure 1. An amino benzimidazole 'hit' from a screen of inhibition of antigen receptor-mediated NF-κB activation.

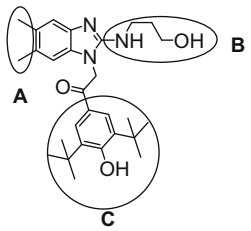
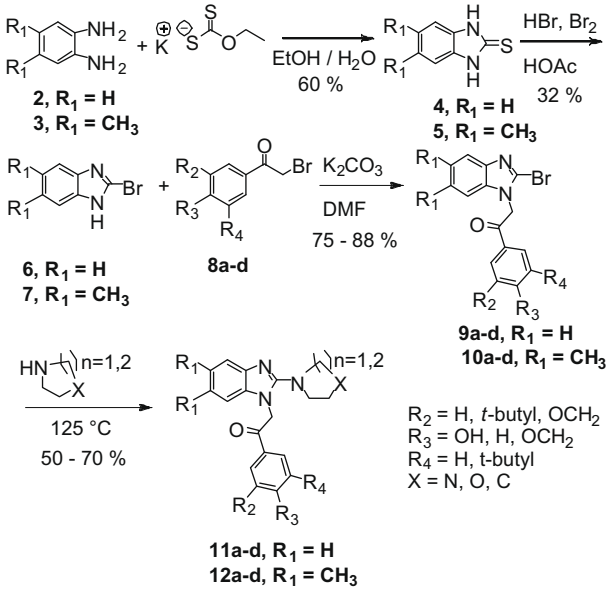
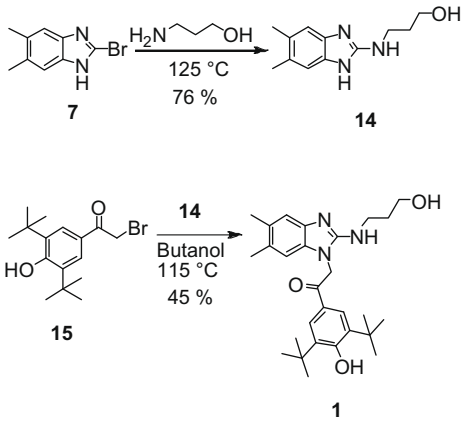


Figure 2. Three regions (i.e., **A**, **B** and **C**) of library diversification in 'hit' compound **1**.



Scheme 1. Synthetic pathway for the variation of the **B** and **C** regions of **1** (Fig. 2).



Scheme 2. Optimized synthesis of benzimidazole analog **1**.

2.2. SAR discussion

5,6-Dimethyl-2-bromobenzimidazole (**7**) and 2-bromobenzimidazole (**6**) were chosen as the core moieties for library construction because of the commercial availability of their phenylenediamine

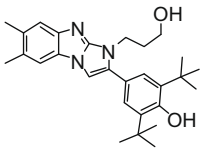


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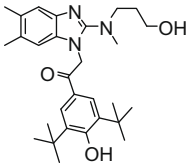
Figure 3. Cyclic benzimidazole byproduct from the synthesis of compound **1** using Scheme 1.



36

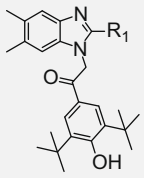
Figure 4. *N*-Methyl analog of benzimidazole **1**.

precursors (i.e., compounds **2** and **3**). The benzimidazole cores were alkylated with synthetically derived and/or commercially available bromoacetophenones (**6a–d**) to further diversify the library. Substituted and unsubstituted cyclic amines (e.g., piperidines, morpholines, and piperazines) were added in the last step to give the final compounds for two reasons: (a) to diversify the library and (b) to preclude ring cyclization to **13**. Preparation of this focused library afforded 144 compounds each with a purity of >90%. All 144 compounds were prepared and tested as inhibitors against antigen receptor-mediated NF-κB activation. However, this focused library contained only weakly potent compounds (IC_{50} >5 μM). Table 1 shows a representative sample from the 144 compounds synthesized for the initial SAR study. We hypothesized that the NH at the 2-position of the benzimidazole was a key interaction for the receptor and tertiary amines at this position decreased potency.

To test the hypothesis that a secondary amine was required for optimal potency, the *N*-methyl analog of benzimidazole **1** was synthesized (compound **36**, Fig. 4). However, it was also found to be weakly potent (i.e., IC_{50} >5 μM) and similar to weakly potent members of the cyclic tertiary amine focus library. Consequently, secondary amines at the 2-position of benzimidazoles were utilized exclusively in further studies.

A second focused library of secondary amine analogs of **1** provided considerable SAR information for the benzimidazole core. Eleven additional analogs were synthesized to further probe the SAR at the 2-amino and the acetophenone ring positions affording only secondary alkylamines. Table 2 summarizes the biological results for these analogs.

Table 1
NF-κB inhibition by selected tertiary amine compounds

Structure	Compound	R_1	IC_{50} (μM)
	**16**	Piperidine	>5
	17	Morpholine	>5
	18	Pyrrollidine	>5
	19	(*S*)-2(Methoxymethyl)-pyrrollidine	>5
	20	(*S*)-1-(2-Pyrrolidinylmethyl)-pyrrollidine	>5
	21	2,4-Dimethyl-3-ethylpyrrollidine	>5
	22	3-Hydroxypyrrollidine	>5
	23	2-(Hydroxymethyl)piperidine	>5
	24	4-Benzylpiperidine	>5
	25	3-Methylpiperidine	>5
	26	4-Hydroxypiperidine	>5
	27	2-Methylpiperidine	>5
	28	3,5-Dimethylpiperidine	>5
	29	4-Methylpiperidine	>5
	30	*N,N*-Dimethyl-1-piperidin-2-ylmethanamine	>5
	31	*N*-Methylpiperazine	>5
	32	*N*-Ethylpiperazine	>5
	33	*N*-Benzylpiperazine	>5
	34	*N*-Hydroxyethylpiperazine	>5
	35	*N*-(2-Methoxyphenyl)-piperazine	>5

The data of Table 2 show that compound **1** possessed a very strict SAR. Placement of a methyl group at the phenolic oxygen atom provided compound **37** that was a very potent NF-κB inhibitor, (IC_{50} = 0.07 μM). Incorporation of a methyl group into the aliphatic hydroxyl side chain oxygen atom afforded compound **38** that was somewhat less potent (i.e., the potency was decreased approximately fivefold). Based on these results, analogs were synthesized that were focused around modifying the aliphatic amino side chain. It was hypothesized that the terminal end of the aliphatic substituent required an atom that possessed properties of both a hydrogen donor and acceptor. Replacement of the terminal nitrogen for a methyl group was predicted to decrease the inhibitory nature of the molecule. However, the butyl analog (compound **44**) possessed an IC_{50} = 0.1 μM. This result showed that the terminal position was slightly more sensitive to the number of atoms than the requirement for terminal atoms to act in hydrogen bond donation or acceptor interactions.

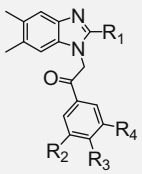
Compounds **40–43** were synthesized to probe the effects of substituents on the phenyl ring. Compounds **40–43** were found to be weak inhibitors of antigen receptor-mediated NF-κB activation. This result suggested that bulky aliphatic groups may be necessary for potent inhibition. Compound **46**, was synthesized to determine the influence of the presence of a ketone group on the potency of **1** (Fig. 5). The alcohol compound **46** was found to be weakly potent with an IC_{50} >5 μM and the conclusion was that the ketone group was necessary for greater potency.

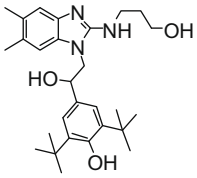
To further explore the SAR of the terminal portion of the aliphatic side chain, an aminopropargyl group was appended by synthesis. However, compound **45** was found to be weakly potent, (IC_{50} >5 μM) suggesting the amino side chain does not tolerate groups that can form strong pi interactions. While several of the analogs were found to have similar potency as benzimidazole **1** at inhibiting the antigen receptor-mediated NF-κB activation pathway, none showed a greater inhibitory effect. Modification of certain regions of **1** (e.g., *t*-butyl, keto, NH), were found to have significant impact on the inhibition of antigen receptor-mediated NF-κB activation while other groups, (e.g., 5,6-dimethyl substituent of the benzimidazole ring) did not have much effect. These results suggested that benzimidazole **1** possessed a strict SAR and small modifications of the core significantly affected its inhibitory potency.

2.3. Metabolic stability study

Inspection of the molecule suggested that several sites in compound **1** could be prone to metabolic attack and possibly decrease the potency of the parent molecule during planned in vivo evaluation. Metabolic stability studies were thus undertaken for the most potent compounds to determine the utility of **1** and close analogs for studies in vivo. In vitro metabolic stability studies in the presence of both human liver microsomes (HLM) and S9 and a reduced nicotinamide adenine dinucleotide phosphate (NADPH) generating system were done with the oxalate salt of compounds **1**, **37**, and **38**. Table 3 summarizes the results from these studies.

Table 2
Inhibition of antigen receptor-mediated NF-κB by compounds **1** and **37**–**45**

Structure	Compound	R_1	R_2	R_4	R_3	IC_{50} (μM)
	**1**	3-Hydroxypropylamino	*t*-Butyl	*t*-Butyl	OH	0.07
	37	3-Hydroxypropylamino	*t*-Butyl	*t*-Butyl	OMe	0.07
	38	3-Methoxypropylamino	*t*-Butyl	*t*-Butyl	OH	0.25
	39	3-Methoxypropylamino	*t*-Butyl	*t*-Butyl	OMe	0.25
	40	3-Hydroxypropylamino	Methyl	Methyl	OH	>5
	41	3-Methoxypropylamino	Methyl	Methyl	OH	>5
	42	3-Hydroxypropylamino	H	H	OH	>5
	43	3-Methoxypropylamino	H	H	OH	>5
	44	*n*-Butylamino	*t*-Butyl	*t*-Butyl	OH	0.1
	45	Propargylamino	*t*-Butyl	*t*-Butyl	OH	>8



46

Figure 5. Chemical structure of the ketone reduction product of **1**.

Metabolic stability studies showed compounds **1**, **37**, and **38** were quite stable to hepatic metabolism compared with testosterone that was run as a positive control. One possible reason for this result was that **1**, **37**, or **38** inhibited its own metabolism. To explore this point, inhibition of cytochrome 3A4-mediated microsomal hydroxylation of testosterone (known to be hydroxylated by cytochrome P-450, the major cytochrome P-450 in adult human liver microsomes) by **1**, **37**, or **38** was studied. Table 4 summarizes the results from the study of the inhibition of testosterone hydroxylase by **1**, **37**, and **38**.

Results of Table 4 show that **1**, **37**, and **38** possess only modest inhibitory activity of testosterone hydroxylase functional activity. From these results and the knowledge that imidazoles are potent inhibitors of cytochrome P-450'[17,18] any significant inhibition of cytochrome P-450 3A4 by compounds **1**, **37**, and **38** could account for the metabolic stability observed. That potent inhibition of cytochrome P-450 3A4 was not observed suggested that inhibition of its own metabolism was not occurring for **1**, **37**, and **38**. However, it is possible that some of the metabolic stability of compounds **1**, **37**, and **38** was due at least in part to inhibition of other cytochrome P-450s that are more prominent in the metabolism of **1**, **37**, and **38**. Based on the results presented in Table 4, sufficient metabolic stability is present for **1**, **37**, and **38** for these compounds to be taken forward in animal studies for efficacy testing.

Table 3
Metabolic stability of oxalate salts for compounds **1**, **37** and **38**

Cofactor system	Enzyme	Half life (h)			
		1	**33**	**34**	Testosterone
+NADPH	HLM[a]	5.5	2.9	3.1	0.8
	S9[a]	15.2	4.4	4.6	
+UDPG	HLM	Stable[b]	Stable	Stable	Not determined
	S9				
+PAPS	HLM				
	S9				

[a] HLM, human liver microsomes, S-9 is the supernatant from a 10,000*g* centrifugation of a human liver homogenate.
[b] Stable indicates no detectable loss of compound in 1 h as judged by HPLC.

3. Conclusions

In summary, a library of >160 compounds was synthesized as inhibitors of the antigen receptor-mediated NF-κB activation pathway. The results showed that several compounds exhibited the same range of potency as compound **1**. A concise SAR was determined from the library of compounds that was synthesized. The SAR was found to be very steep and small changes made in **1** afforded significant changes in the potency of inhibition. Finally, metabolic stability studies were done on select compounds. The results showed compounds **1**, **37**, and **38** were metabolically stable, and only very modestly inhibited their own metabolism. It is likely that compounds such as **1**, **37**, and **38** possess sufficient pharmacological and pharmaceutical properties (i.e., potency, solubility, and metabolic stability) to test as inhibitors of inflammation in appropriate animal models.

4. Experimental

4.1. Chemistry

^{1}H NMR (300 MHz) spectra were determined on a Varian Mercury 300 instrument in the indicated solvent. ^{13}C NMR (125 MHz) spectra were determined on a Bruker AMX-500 II in the indicated solvent. Mass spectra were recorded with a Hitachi M8000. The active compounds were checked for purity using LC/MS at two wavelengths (220 and 254 nm), (Platform: Agilent: equipped with an autosampler, detector Diode Array Detector (monitored at 220 and 254 nm), a MS detector (Perkin Elmer API 150EX); HPLC column: Sunfire C18, 3.5 μm, 2.1 × 50 mm; HPLC gradient: 800 μL/min, from 10% acetonitrile in water to 90% acetonitrile in water in for 5.5 min. Both acetonitrile and water have 0.025% TFA). t_R is retention time (TIC% purity). All compounds were checked by TLC on silica gel G plates with fluorescent indicator at 254 nm. Developed plates were visualized by UV light. Solvents were of reagent grade and, when necessary, were purified and dried by standard methods. Concentration of the reaction solutions involved the use of rotary evaporator under reduced pressure. Compound **1** was purchased from Chembridge (San Diego).

4.1.1. 2-Mercapto-5,6-benzimidazole (5)

A mixture of 10 g (73 mmol) of 4,5-dimethylphenylenediamine, 16 g (111 mmol) of potassium ethyl xanthate, 100 mL ethanol, and 14 mL of water were added to a 500 mL Erlenmeyer flask and heated to reflux. After 3 h, 3.4 g of charcoal (Norit A) was added and refluxed for an additional 10 min. The Norit was filtered and the filtrate was heated to 60–70 °C. To the warm solution was added 100 mL of warm water and 8 mL of acetic acid in 16 mL of water with efficient stirring. Upon the addition of the acetic acid solution, the mixture became a foamy solid. The mixture was placed in a 4 °C refrigerator for 3 h. The solid was filtered and dried

Table 4
Inhibition of testosterone hydroxylase in the presence of HLM[a] and an NADPH generating system

Compound	Calculated IC_{50} (μM)
1	85
37	67
38	97

[a] HLM, human liver microsomes.

over P_2O_5 to give 8.1 g (62%) of a tan solid. The compound was used without further purification. ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.21 (s, 6H), 6.91 (s, 2H), 12.31 (br s, 2H). MS: calculated $C_9H_{10}N_2S$ m/z = 178.06 found m/z = 179.25 [M+H].

4.1.2. 2-Bromo-5,6-benzimidazole (7)

To a cooled solution of 40 mL of acetic acid and 4.2 mL (37 mmol) of 48% aqueous HBr was added 5 g (28 mmol) of compound **5**. To this slurry was slowly added 5.2 mL (101 mmol) of bromine dropwise over 10 min. The reaction turned orange and became unstirrable and after half of the bromine was added, manual or mechanical agitation was needed to break up solids. After the addition of the bromine, 80 mL of acetic acid was added and the mixture was stirred at room temperature. After 4.5 h, the mixture was diluted with 90 mL of water and cooled to 0 °C. The pH of the mixture was adjusted to pH 4 by the addition of solid NaOH. Upon basification a solid precipitated out of solution. The solid was filtered and dried overnight to give 2 g (32%) of product as a light orange solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.25 (s, 6H), 7.20 (s, 2H), 13.0 (br s, 1H). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 138.0, 130.8, 125.2, 114.5, 19.8. MS: calculated $C_9H_9BrN_2$ m/z = 223.99, 225.99 found m/z = 225.24, 227.25 [M+H].

4.1.3. General procedure for the alkylation of bromobenzimidazole (8a–d)

To a flask was added the appropriate bromobenzimidazole (0.6 mmol), brominated acetophenone (0.66 mmol), and K_2CO_3 (1.3 mmol) in 1.2 mL DMF. The solution was stirred at room temperature overnight. The reaction mixture was poured into 10% citric acid and extracted 3 times with EtOAc. The organic layers were combined, dried over Na_2SO_4 and concentrated in vacuo. The crude material was purified via column chromatography with the appropriate solvent system, generally 4:1 hexane/EtOAc.

4.1.4. General procedure for the synthesis of the benzimidazole library (9a–d, 10a–d)

To a 1-dram vial was added the appropriate alkylated bromobenzimidazole **8a–d** (0.01 mmol) and the cyclic amines (0.1 mmol) and heated to 125 °C. After 4 h, the reactions were cooled to room temperature and the products were isolated. The reaction mixture was dissolved in 0.5 mL EtOAc and washed with 2 M HCl (0.5 mL). The acid was neutralized with saturated sodium bicarbonate extracted twice with EtOAc (0.5 mL). The organic layers were combined, dried over sodium sulfate and concentrated to dryness. The isolated material was used without further purification.

4.1.5. 3-(5,6-Dimethyl-1*H*-benzo[*d*]imidazol-2-ylamino)propan-1-ol (14)

50 mg (0.22 mmol) of **7** and 100 μL (1.3 mmol) 3-aminopropanol were added to a 1-dram vial and heated to 125 °C until the reaction was complete based on TLC analysis. The reaction was cooled to room temperature, extracted with saturated sodium bicarbonate/EtOAc. The organic layer was dried over sodium sulfate and concentrated to give a brown residue. The crude product was purified via preparative TLC using 10% MeOH/CH_2Cl_2. A band isolated with an R_f = 0.25 gave 37.3 mg (76%) of a light brown residue. ^{1}H NMR (DMSO-d_6, 300 MHz) δ 1.62–1.70 (m, 2H), 2.18 (s, 6H), 3.25–3.33 (m, 2H), 3.43–3.47 (m, 2H), 4.86 (br s, 1H), 6.33 (br s, 1H), 6.87 (s, 2H), 10.46 (br s, 1H). MS: calculated $C_{12}H_{17}N_3O$ m/z = 219.14 found m/z = 220.56 [M+H].

4.1.6. General procedure for the synthesis of the target benzimidazoles

To a 1-dram vial, 1 equiv of the appropriate aklylaminobenzimidazole and 1.2 equiv of the appropriate bromoacetophenone **5** were added to 0.5 mL butanol. The reaction was heated to 115 °C until the reaction was complete based on TLC analysis. Butanol was removed and the crude mixture was extracted with saturated sodium bicarbonate/EtOAc. The organic layer was dried over sodium sulfate and concentrated to dryness. The crude mixture was purified via preparative TLC using 5% MeOH/CH_2Cl_2. Yields range from 30% to 50%.

4.1.6.1. 2-(2-(3-Hydroxypropylamino)-5,6-dimethyl-1*H*-benzo[*d*]-imidazol-1-yl)-1-(3,5-di-*tert*-butyl-4-hydroxyphenyl)ethanone (1). R_f = 0.3. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.87–1.00 (m, 2H), 1.49 (s, 18H), 2.22 (s, 3H), 2.26 (s, 3H), 3.52 (t, J = 5.6, 2H), 3.61 (t, J = 5.5, 2H), 5.60 (s, 2H), 6.87 (s, 1H), 7.05 (s, 1H), 7.97 (s, 2H). LC/MS t_R = 2.39 (99%). MS: calculated $C_{28}H_{39}N_3O_3$ m/z = 465.30, found m/z = 466.82 [M+H].

4.1.6.2. 1-(3,5-Di-*tert*-butyl-4-methoxyphenyl)-2-(2-(3-hydroxypropylamino)-5,6-dimethyl-1*H*-benzo[*d*]imidazol-1-yl)ethanone (37). R_f = 0.4. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.43 (s, 18H), 1.70–1.77 (m, 2H), 2.27 (s, 3H), 2.28 (s, 3H), 3.56–3.60 (m, 2H), 3.61–3.65 (m, 2H), 3.72 (s, 3H), 5.23 (s, 2H), 6.78 (s, 1H), 7.20 (s, 1H), 7.94 (s, 2H). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 192.9, 165.7, 155.6, 145.3, 139.1, 132.7, 130.4, 129.3, 128.8, 127.5, 117.2, 108.1, 64.8, 58.7, 48.6, 39.8, 36.3, 33.8, 32.1, 20.4, 20.2. LC/MS t_R = 2.55 (96%). MS: calculated $C_{29}H_{41}N_3O_3$ m/z = 479.31, found m/z = 480.53 [M+H].

4.1.6.3. 2-(2-(3-Methoxypropylamino)-5,6-dimethyl-1*H*-benzo-[*d*]imidazol-1-yl)-1-(3,5-di-*tert*-butyl-4-hydroxyphenyl)ethanone (38). R_f = 0.4. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.49 (s, 18H), 1.89–1.97 (m, 2H), 2.28 (s, 3H), 2.29 (s, 3H), 3.25 (s, 3H), 3.52 (t, J = 5.6, 2H), 3.60 (t, J = 5.5, 2H), 5.16 (s, 2H), 6.79 (s, 1H), 7.27 (s, 1H), 7.91 (s, 2H). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 192.7, 165.5, 154.9, 145.16, 140.22, 132.94, 129.9, 129.4, 128.4, 127.4, 117.4, 108.0, 72.2, 64.8, 58.7, 48.7, 42.5, 36.3, 32.09, 29.2, 20.4, 20.3. LC/MS t_R = 2.65 (99%). MS: calculated $C_{29}H_{41}N_3O_3$ m/z = 479.31, found m/z = 480.23 [M+H].

4.1.6.4. 2-(2-(3-Methoxypropylamino)-5,6-dimethyl-1*H*-benzo[*d*]-imidazol-1-yl)-1-(3,5-di-*tert*-butyl-4-methoxyphenyl)ethanone (39). R_f = 0.6. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.44 (s, 18H), 1.89–1.97 (m, 2H), 2.28 (s, 3H), 2.29 (s, 3H), 3.25 (s, 3H), 3.52 (t, J = 5.6, 2H), 3.60 (t, J = 5.5, 2H), 3.72 (s, 3H), 5.18 (s, 2H), 6.79 (s, 1H), 7.27 (s, 1H), 7.95 (s, 2H). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 192.2, 159.7, 155.0, 140.4, 136.7, 133.0, 129.8, 128.2, 126.6, 126.3, 117.5, 107.9, 72.0, 58.9, 48.4, 42.3, 34.7, 30.3, 29.3, 20.4, 20.3. LC/MS t_R = 2.63 (99%). MS: calculated $C_{30}H_{43}N_3O_3$ m/z = 493.33, found m/z = 494.13 [M+H].

4.1.6.5. 1-(3,5-Di-*tert*-butyl-4-hydroxyphenyl)-2-(2-(butylamino)-5,6-dimethyl-1*H*-benzo[*d*]imidazol-1-yl)ethanone (44). R_f = 0.8. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.90–0.95 (m, 3H), 1.47 (s, 18H), 1.57–1.68 (m, 4H), 2.29 (s, 6H), 3.44–3.50 (m, 2H), 5.14 (s, 2H), 6.82 (s, 1H), 7.28 (s, 1H), 7.92 (s, 2H). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 192.9, 159.9, 155.0, 136.7, 132.8, 131.1, 130.4, 129.9, 128.3, 126.6, 117.5, 108.0, 48.4, 43.7, 34.7, 32.1, 30.3, 20.2, 20.4,

20.3, 14.1. LC/MS t_R = 2.57 (98%). MS: calculated $C_{29}H_{41}N_3O_2$ m/z = 463.32, found m/z = 464.35 [M+H].

4.2. Biology

Phorbol myristic acetate (PMA), ionomycin, the NADPH generating system, uridine diphosphoglucose (UDPG), alamethicin, and adenosine 3′-phosphate 5′-phosphosulfate lithium salt hydrate (PAPS) were from Sigma–Aldrich (St. Louis, MO). Lecithin was obtained from Calbiochem (La Jolla, CA). Human liver microsomes (HLM) and human liver S9 were purchased from B. D. Gentest (Woburn, MA). RP-HPLC analysis of synthetic compounds was done using a Hitachi HPLC system (D-7000 interface, L-7100 pump, L-7200 autosampler, and the L-7400 UV detector) and a HS Supelco column (4.6 mm × 250 mm, 5 μm). All of the chemicals or reagents were obtained in the highest purity available commercially.

4.2.1. Cell engineering

HEK293 cells were co-transfected with pUC13-4xNF-κB-Luc and *p*-TK-puromycin-resistance plasmids. Stable clones were selected by culture in Dulbecco's Modified Eagle's Media (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Hyclone), 1% v/v penicillin–streptomycin mix (Invitrogen) containing 1 μg/mL puromycin. Individual clones were tested for responsiveness to PMA/ionomycin-induced NF-kB reporter gene activity, and a clone was selected.

PMA/Iono induced NF-κB luciferase assay was done in HEK293-NF-κB-Luc stable cells HEK293-NF-κB-Luc cells (10^4 cells in 90 μL medium) were seeded in 96-well white plates (Greiner Bio-one) overnight. Compounds were diluted in culture medium and 5 μL was added to the cells to reach a desired final concentration. 2 h later, 5 μL PMA/Iono (200 ng/mL) was added (final concentration = 10 ng/mL) and cells were treated for another 16 h. 50 μL BriteLite solution (Perkin–Elmer) was added to each well and luminescence was read using a luminometer (LJL Biosystems). The luminescence of cells treated by PMA/Ion was set to 100% and the data of each compound were processed and presented as the percentage of control. IC_{50} values were calculated using GraphPad Prism 5.0.

4.2.2. Metabolic stability studies

The in vitro oxidation, glucuronidation, and sulfation of **1** and analogs were studied with human liver microsomes or human liver S9 in the presence of 3 different cofactors: an NADPH generating system, UDPG and PAPS.[19–21] All incubations at different time points were measured in duplicate. All incubations were initiated by the addition of HLM or S9, and were conducted at 37 °C in a shaking incubator. Aliquots of the incubation were stopped at 5, 15, 30, 45, and 60 min by the addition of two volumes of cold acetonitrile. For the 0 min time point, samples were aliquoted into acetonitrile before adding the human liver microsomes or S9. Samples were analyzed using RP-HPLC with UV detection. Flow rate: 1.2 mL/min, UV wavelength was selected at 235 nm. Two solvent phases were used: Solvent A was 85% acetonitrile (ACN) with 15% H_2O and 0.1% trifluoroacetic acid (TFA), and Solvent B was 0.1% TFA in H_2O. The mobile phase was 90% Solvent A with 10% Solvent B for **37** with human liver S9 and NADPH incubation; retention time (t_R) was 11.2 min. The mobile phase was 100% Solvent A for all other incubation samples, and the *t*R for **1**, **37**, and **38** were 7.9, 10.5, and 10.3 min, respectively. The percent of **1** and analogs oxidized, glucuronidated, or sulfated were calculated based on the disappearance of the parent compound.

4.2.2.1. Microsomal oxidation. The final concentration of the incubation mixture contained 100 μM substrate, 1.6 mg/mL HLM (or S9), 400 μM $NADP^+$, 400 μM glucose-6-phosphate, 250 μM DETAPAC (used as an antioxidant), and 4 IU/mL glucose-6-phosphate dehydrogenase in potassium phosphate buffer (pH 7.4, 50 mM).

4.2.2.2. Glucuronidation with UDPG. Substrate (100 μM), 1.6 mg/mL HLM (or S9), 200 μM UDPG, 40 μg/mL alamethicin, and 72 μg/mL lecithin was incubated in potassium phosphate buffer (pH 7.4, 50 mM).

4.2.2.3. Sulfation with PAPS. Substrate (100 μM) was incubated with 1.6 mg/mL HLM (or S9) and 100 μM PAPS in Tris–HCl buffer (50 mM, pH 7.4).

4.2.2.4. Testosterone oxidation. Testosterone oxidation was studied to investigate the inhibitory effect of **1** and analogs. The incubation mixture contained 50 μM testosterone, 1.6 mg/mL human liver microsomes, 400 μM $NADP^+$, 400 μM glucose-6-phosphate, 250 μM DETAPAC, 4 IU/mL glucose-6-phosphate dehydrogenase, and 1 mM $MgCl_2$ in potassium phosphate buffer (pH 7.4, 50 mM). All incubations were initiated by the addition of substrate, and were run at 37 °C in a shaking incubator. The assay was terminated at 10 and 20 min by the addition of 1 mL of cold ethyl acetate. The terminated incubations were centrifuged for 5 min at 1800 rpm to separate the organic and aqueous layers. The organic layer was decanted into a new tube and concentrated under an argon stream until dry. The residue was reconstituted with 100 μL of methanol. When test compounds were added to the incubation to determine the IC_{50} of **1** or analogs, the buffer volume was reduced to maintain the over-all final volume at 100 μL. Samples were analyzed using RP-HPLC; mobile phase was 32% (acetonitrile + 0.1% TFA) and 68% (H_2O + 0.1% TFA), and the flow rate was 1.2 mL/min. Testosterone had a retention time of 13.4 min and the 6-OH testosterone eluted at 4.4 min. A gradient mobile phase was selected for the inhibition study to elute **1** and analogs.

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