Inhibition of Protein Kinase C-Driven Nuclear Factor-κB Activation: Synthesis, Structure—Activity Relationship, and Pharmacological Profiling of Pathway Specific Benzimidazole Probe Molecules

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A unique series of biologically active chemical probes that selectively inhibit NF-κB activation induced by protein kinase C (PKC) pathway activators have been identified through a cell-based phenotypic reporter gene assay. These 2-aminobenzimidazoles represent initial chemical tools to be used in gaining further understanding on the cellular mechanisms driven by B and T cell antigen receptors. Starting from the founding member of this chemical series 1a (notated in PubChem as CID-2858522), we report the chemical synthesis, SAR studies, and pharmacological profiling of this pathway-selective inhibitor of NF-κB activation.

Introduction

Members of the nuclear factor-kappa B (NF-κB) family of transcription factors control many crucial physiological and pathological processes including host-defense, immune response, inflammation, and cancer. A number of intracellular signaling pathways leading to NF-κB activation have been elucidated and have been the subject of several excellent reviews. Most NF-κB activation pathways converge on IκB-α kinase (IKK) activation, resulting in phosphorylation of IκB-α, and thereby targeting this protein for ubiquitination with proteasome-dependent destruction. This cascade releases NF-κB heterodimers from the IκB-α complex in the cytosol and allows for translocation into nucleus where transcription of various pro-inflammatory genes is initiated. The various pathways upstream of IKK activation are diverse, with most of them linked to cell surface and intracellular receptors that sense cell damage and pathogens as well as activation in response to cytokines. Accordingly, small molecule pathway selective inhibitors can serve as powerful chemical tools to dissect these molecular networks which are crucial for normal cellular survival but are dysregulated in specific disease states.

The NF-κB pathway activated by antigen receptors is critical for adaptive immunity contributing to T and B lymphocyte activation, proliferation, cell survival, and effector functions. Dysregulated NF-κB activation in lymphocytes contributes to development of a variety of autoimmune-based disease states, chronic inflammation, and lymphoid malignancy. The NF-κB activation pathway linked to antigen receptors involves a cascade of adapter and signal transduction proteins that at minimum include a CARMA family protein, Bcl-10, MALT (paracaspase), TRAF6, Ubc13, caspase-8, and c-Flip. Formation of this complex is initiated by protein kinase C (PKC)-mediated phosphorylation of CARMA proteins. In T and B cells, this pathway is initiated by PKC-θ and PKC-β, respectively, leading ultimately to IKK activation through a mechanism potentially involving lysine 63-linked polyubiquitination of IKK-γ. Thus, the antigen receptor pathway for NF-κB activation is both initiated and concluded by activation of protein kinases, namely the PKCs and IKKs, respectively.

Although the IKK complex and its individual subunits represent logical targets for potential drug discovery, small-molecule inhibitors of IKKs suppress all known NF-κB activation pathways. This approach lacks the selectivity required to inhibit lymphocyte responses without simultaneously interfering with overall innate immune responses and leads to general immunosuppression attended by risk of pathogenic infection as well as other severe side effects.

A library of approximately 110,000 compounds from the NIH Molecular Libraries Small Molecule Repository (MLSMR) compound collection was screened using a HEK293 cell-based NF-κB luciferase reporter gene strategy. Primary hit compounds with an IC₅₀ ≤ 10 μM were further characterized by 14 additional cell-based counter-screens that helped determine pathway selectivity in the HEK293 cell line and other lymphocytes. Ultimately, three hit series emerged and were confirmed through an extensive counter-screen assay platform to be selective for the antigen receptor activation pathway (Figure 1A). Of the three primary hit classes identified in the HTS campaign, only the 2-aminobenzimidazole series represented by 1-(3,5-di-tert-butyl-4-hydroxyphenyl)-2-(2-(3-hydroxypropylamino)-5,6-dimethyl-1H-benzo[d]imidazol-1-yl)ethanone, 1a (CID-2858522), selectively inhibited the NF-κB pathway induced by PKC activators and...
antigen receptors. Compound 1a did not inhibit activation of NF-κB through TNF-α stimulation (Figure 1B). Details of the comprehensive screening platform and pathway specific cell-based selectivity assessment, as outlined above, are described in our accompanying paper. On the basis of the above information and selectivity profile, the 2-aminobenzimidazole series was selected for chemistry follow-up as it met our initial potency criteria (< 1 μM) and selective inhibition of antigen receptor-mediated NF-κB activation. We discuss the development of an efficient synthetic route to the 2-aminobenzimidazole scaffold, the design and synthesis of SAR analogues, as well as in vitro and in vivo pharmacological profiling of 1a. These structure—activity relationship studies provide molecular insight for further optimization and synthesis of proteomic probes, which include analogues to be used for the identification of the precise cellular target protein and subsequent mode of action studies.

Results and Discussion

The initial HTS hit compounds of interest (Figure 1) were resynthesized so that identity and cellular activity could be confirmed. Additional commercially available dry powder samples for closely related analogues were ordered (ChemBridge Corporation, San Diego, CA), and the quality, purity, and identity for each compound was confirmed. In the case where the commercial analogues were not homogeneous, they were purified by preparative HPLC to ≥98%. Each compound was assessed for bioactivity using a cell-based assay employing IC50 values in the micromolar to nanomolar range as shown in Figure 1S (see Supporting Information). The “analogue by catalogue” approach ultimately provided an initial set of 2-aminobenzimidazoles having NF-κB antigen receptor pathway induced by PMA (a PKC activator) vs TNF-α activation.

![Figure 1](image-url)

Figure 1. (A) Three scaffold classes (with IC50 < 10 μM, most active members shown) identified in the high throughput screening (HTS) campaign. (B) Selectivity of 1a for inhibition of the NF-κB antigen receptor pathway induced by PMA (a PKC activator) vs TNF-α activation.

We discuss the development of an efficient synthetic route to the 2-aminobenzimidazole scaffold, the design and synthesis of SAR analogues, as well as in vitro and in vivo pharmacological profiling of 1a. These structure—activity relationship studies provide molecular insight for further optimization and synthesis of proteomic probes, which include analogues to be used for the identification of the precise cellular target protein and subsequent mode of action studies.

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starting materials and then treated with intermediate 14a to access analogues II–In (Scheme 2B; see Supporting Information for details). To replace the 2-aminobenzimidazole core of 1a with related heterocycles, commercially available 5,6-dimethyl-1H benzimidazole and 5,6-dimethyl-1H-benzimidazol-2-one were alkylated with 15a in the presence of sodium hydride to furnish 18 and 19 in 63 and 55% yield, respectively (Scheme 3).

Through a systematic substitution process, we probed the individual contribution of the substituents at the 5- and 6-positions of the benzimidazole aryl moiety. It was found that substituents at the 5-position were better tolerated than at 6-position and activity of analogues was sensitive to both the electronic and steric nature of the substituents (1b–f and 17b–f, Table 1A). The results indicate that the 6-position is more sensitive to the steric bulk of the substituent than the 5-position because replacement of either methyl group with a bromine led to a 40–80-fold drop in activity (1a, 1b, and 17b, Table 1A).

Similarly, the 5-nitro analogue was 20-fold less active than 1a while the 6-nitro analogue was found to be inactive (1e and 17e, Table 1A). Installation of a planar phenyl group resulted in analogues with activity approaching that of 1a, suggesting a positive hydrophobic interaction at the 5-position (1c and 17c, Table 1A). An unexpected result was that the 5- and 6-amino analogues were the most active analogues in this substitution series, suggesting access to H-bonding interactions (1f and 17f, Table 1A). It was found that while O-methylation of the 2-aminopropanol side chain retained most of the activity (1g, Table 1B), removal of the hydroxyl substituent in the 2-butyloximino analogue led to a moderate loss of activity (1h, Table 1B), suggesting that the electronegative oxygen atom is not essential for activity. Truncation of the butyl group in the 2-methylamino analogue led to improved activity and was found to not be essential for activity, further confirming that NF-κB inhibition is not due to antioxidant activity. The SAR for aryl substitution at this position is steep. Elimination or replacement of one of the tert-butyl groups with a tert-butoxy group resulted in completely inactive analogues, suggesting that the di-tert-butyl phenyl group might represent a conformational lock and is extremely important for binding/activity (1i and 1n, Table 1). Replacement of the 2-alkylamino moiety with hydrogen led to a significant loss of activity, however, the tert-butoxy group did not result in complete loss of activity (1j, Table 1B). The presence of a tertiary amine at the benzimidazole 2-position was found to abolish activity (1k, Table 1B). These results, taken together, suggest that the entire 2-aminopropanol substituent is likely solvent exposed and may not be completely required (except the NH moiety) for binding to the cellular target protein.

Replacement of the N1-3,5-dimethyl-4-hydroxyacetophenone with either simple acetophenones or 3,5-dimethyl-4-hydroxyacetophenone resulted in complete loss of activity (6–10, Figure 1, see Supporting Information). It was found that the des-4-phenoxy analogue retained complete activity (11, Table 1C), and hence the 4-phenoxy substituent is not essential for activity, further confirming that NF-κB inhibition is not due to antioxidant activity. The SAR for aryl substitution at this position is steep. Elimination or replacement of one of the tert-butyl groups with a tert-butoxy group resulted in completely inactive analogues, suggesting that the di-tert-butyl phenyl group might represent a conformational lock and is extremely important for binding/activity (1m and 1n, Table 1). Replacement of the 2-alkylamino moiety with hydrogen led to a significant loss of activity, however, the 2-benzimidazolone analogue was essentially equipotent to 1a (18 and 19, Figure 2). These results indicate that a polar heteroatom is needed at the 2-position for activity, yet the 2-amino group is completely replaceable. This observation has important bearing on future probe SAR because it allows further derivatization at the N1 position allowing exploration of additional chemical space via alkylation or acylation.
The SAR indicates the entire 2-aminopropyl chain does not contribute significantly to bioactivity. Because the precise cellular protein target is unknown, we capitalized on this information to synthesize two analogues that have an azide and a terminal alkyne incorporated in the 2-aminoealkyl side chain (1o, 1p, Figure 2). The objective was to utilize these as building blocks for the synthesis of pull down probes for protein target identification. We were gratified that both analogues retain sufficient bioactivity (within 6-fold) and are currently being utilized in ongoing studies involving biocoujugation via “click chemistry” to furnish biotinylated derivatives for affinity studies.

**In Vitro and In Vivo Pharmacological Profiling.** Compound 1a was subjected to in vitro ADME/T assays to profile its general pharmacological properties (Table 2). Compound 1a exhibits good to moderate affinity toward plasma protein binding, possibly limiting cellular protein target is unknown, we capitalized on this information to synthesize two analogues that have an azide and a terminal alkyne incorporated in the 2-aminopropyl side chain (1o, 1p, Figure 2). The objective was to utilize these as building blocks for the synthesis of pull down probes for protein target identification. We were gratified that both analogues retain sufficient bioactivity (within 6-fold) and are currently being utilized in ongoing studies involving biocoujugation via “click chemistry” to furnish biotinylated derivatives for affinity studies.

**Table 1. Analogue SAR** (A) 5 and 6-Monosubstitution of the Benzimidazole Core, (B) Substitution at the 2-Position, and (C) Substitution on the Aryl N1-Acetophenone Moiety

| Entry | X | R1 | R2 | IC50 (μM) |
|-------|----|----|----|-----------|
| 1a    | Me | Me | 0.07 |
| 1b    | Br | H  | 3   |
| 1c    | Ph | H  | 0.6 |
| 1d    | COOMe| H | 1.8 |
| 1e    | NO2| H  | 2   |
| 1f    | NH2| H  | 0.8 |
| 1g    | H  | Br  | 6   |
| 1h    | H  | Ph  | 0.9 |
| 1i    | H  | COOMe| N-A |
| 1j    | H  | NO2| > 8 |

**Table 2. In Vitro ADME Data for 1a**

| solubility (μg/mL) | permeability* Pd (× 10^-6 cm/s) | plasma protein binding (% bound) | plasma stability* (% remaining) | microsome stability* (% remaining) |
|---------------------|---------------------------------|---------------------------------|---------------------------------|----------------------------------|
| pH 5.0/6.2/7.4      | pH 5.0/6.2/7.4                  | human 10 μM/1 μM mouse 10 μM/1 μM | human/mouse                     | human 10 μM/1 μM mouse 10 μM/1 μM |
| 42/0.5/0.7          | 299/710/441                    | 99.8/99.9/99.9                   | 100/100                         | 55/26                            |

*Compound at 50 μM. **PAMPA** Pd: low 5 × 10^-5, moderate 250 × 10^-5, high 1000 × 10^-5. Plasma/PBS; compound at 40 μM, 3 h. °% remaining at 1 h.

**Figure 2. Bioactivity of 2H-benzimidazole 18, 2-benzimidazolone 19, azide 1o, and alkynyl ester 1p.**

**Table 3. In Vivo Exposure Data for 1a**

| dose (mg/kg) | concentration (μM) 0.5 h | concentration (μM) 3 h |
|-------------|--------------------------|------------------------|
| 10          | 1.81 ± 0.41              | 0.50 ± 0.04            |
| 30          | 8.28 ± 0.20              | 3.64 ± 0.76            |
| 50          | 5.93 ± 1.30              | 6.96 ± 0.76            |

performed to determine the overall blood levels of compound (Table 3). Probe compound 1a exhibited nonlinear pharmacokinetics, showing higher serum levels at the 0.5 h measurement time for the 30 mg/kg dose compared to 50 mg/kg but displaying typical dose-dependent behavior when measured at t = 3 h. The increasing accumulation seen at a dose of 50 mg/kg may be due to a depot effect created by CYP3A4 inhibition. The cohort exhibited clear signs of morbidity at t = 3 h at the 50 mg/kg dose. On the basis of the above data, blood levels can be expected to reach maximum concentrations approximately 10-fold higher than IC50 at a dose of 30 mg/kg, thus defining a promising dose for future applications in short-term, acute biomarker studies such as in vivo measurements of cytokine production.

**General Target Inhibition Profile.** Protein kinases play critical roles in NF-κB activation. PKCs are proximal kinases in the NF-κB pathways activated by PMA/ionomycin and by T-cell and B-cell antigen receptors, while the IKKs are distal kinases operating in the terminal segments of these and other NF-κB activation pathways. At concentrations up to 8 μM, 1a failed to suppress PKC-θ (the PKC family members implicated in TCR/BCR signaling), and IKK-θ (a component of the IKK complex), while known PKC and IKK inhibitors and the broad-spectrum kinase inhibitor staurosorine afforded potent inhibition. In addition, 1a did not inhibit endogenous PKC activity or phosphorylation of downstream substrates. Probe 1a did not directly inhibit PKC-β, PKC-θ, or IKK-β. A broad kinome screen was then conducted using KINOMEscan (Ambit Biosciences) platform. Of 353 protein kinases surveyed, 1a inhibited only three by more than 50% at 10 μM; Raf (57% inhibition), TLK1 (70% inhibition), and JAK2 (53% inhibition), none of which are clearly implicated in NF-κB regulation.

Compound 1a was screened for general functional activity to determine effects on second messenger systems using a subset of cloned human or rodent receptors, ion channels, and transporters (NIMH-PDSP, University of North Carolina Chapel Hill). Of the 92 targets screened, 1a showed greater than 50%
inhibition at 10 μM for only eight targets (see Supporting Information for details). For all active targets assessed, the subsequent IC_{50} values were greater than 5 μM, highlighting that 1a does not exhibit significant off-target activity.20

Conclusions
A series of substituted 2-amino-benzimidazoles has been identified that serve as promising research tools as pathway-selective chemical inhibitors of NF-κB activation induced by activators of PKC signaling through B and T cell antigen receptors. Interestingly, the MLPCN probe candidate (1a) was identified directly from the screening hit set and only lesser or equipotent analogues have been prepared, indicating a limited SAR for the series. Compound 1a was found to be nontoxic and inhibited IL-8 production induced by PKC activators in HEK293 cells (IC_{50} < 0.1 μM), while failing to inhibit NF-κB reporter gene activation by agonists of the other NF-κB activation pathways. Compound 1a attenuated CD3/CD28 and PMA/ionomycin-induced production of IL-2 by Jurkat T-cells (IC_{50} < 5 μM) and anti-lgM-stimulated proliferation of murine B-lymphocytes (IC_{50} ~ 2 μM). We have used the SAR data to generate the azide and alkyln containing derivatives 1o and 1p. These have been utilized in the synthesis of biotinylated conjugates directed toward affinity-based target identification and future mode of action studies that will be reported separately. In addition, the equipotent 2-benzimidazole analogues (19) provide a modified scaffold with an additional N2-site for future exploration. Compound 1a and its analogues represent new chemical tools that may be useful toward further development of therapeutically useful pathway selective modulators of NF-κB activity that may find application in disease models of inflammation, cancer and other autoimmune diseases.

Experimental Section

General Procedure (Method B, Scheme 1) of N2-Alkylation of 2-Aminobenzimidazoles for the Synthesis 1a, 1b, 1d, 1e, 1g, 1h, 17a, 17b, 17d, and 17e as Illustrated by Synthesis of 1-(3,5-Di-tert-butyl-4-hydroxyphenyl)-2-(2-(3-hydroxypropylamino)-5,6-dimethyl-1H-benzimidazol-1-yl)ethanone, 1a. To a 20 dram vial equipped with a stir bar was added 2-amino-benzimidazole 14a (0.37 g, 1.6 mmol) followed by corresponding 2-bromoacetophenone 15a (0.62 g, 1.9 mmol). To the mixture was added 10 mL of methanol and solid NaHCO3 (3 equiv). The mixture was stirred at 23 °C for 3 days, after which the solvent was evaporated to a suitable volume and subsequent purification by silica gel flash column chromatography (hexanes to 1:4 hexanes/ethyl acetate) resulted in a yellow film, which on lyophilization from a 2:8 acetone/water mixture afforded 1a as white solid (0.61 g, 78%).1H NMR (400 MHz, acetone-d6) δ 7.87 (s, 2H), 6.95 (s, 1H), 6.77 (s, 1H), 5.94 (s, 1H), 5.52 (s, 2H), 3.55–3.47 (m, 2H), 3.42 (t, J = 5.6 Hz, 2H), 2.14 (s, 2H), 2.12 (s, 3H), 1.56 (p, J = 6.0 Hz, 2H), 1.40 (s, 18H).13C NMR (100 MHz, acetone-d6) δ 191.58, 159.66, 156.73, 141.17, 137.58, 134.43, 128.85, 127.29, 127.19, 126.16, 116.74, 108.72, 57.65, 48.47, 39.19, 34.90, 34.67, 19.77, 19.74. LRMS (ESI): calcd for C18H16N2O3 [M + H] = 466.3, obsd [M + H] = 466.1. HRMS (ESI): calcd for C18H16N2O3 [M + H] = 466.3070, obsd [M + H] = 466.3091.

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Supporting Information Available: Detailed experimental procedures for the biology, pharmacology, and chemistry sections and spectroscopic characterization of compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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