

Identification of Inhibitors of NOD1-Induced Nuclear Factor- κ B Activation

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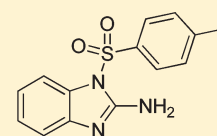
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S Supporting Information

ABSTRACT: NOD1 (nucleotide-binding oligomerization domain 1) protein is a member of the NLR (NACHT and leucine rich repeat domain containing proteins) protein family, which plays a key role in innate immunity as a sensor of specific microbial components derived from bacterial peptidoglycans and induction of inflammatory responses. Mutations in NOD proteins have been associated with various inflammatory diseases that affect NF- κ B (nuclear factor κ B) activity, a major signaling pathway involved in apoptosis, inflammation, and immune response. A luciferase-based reporter gene assay was utilized in a high-throughput screening program conducted under the NIH-sponsored Molecular Libraries Probe Production Center Network program to identify the active scaffolds. Herein, we report the chemical synthesis, structure–activity relationship studies, downstream counterscreens, secondary assay data, and pharmacological profiling of the 2-aminobenzimidazole lead (compound 1c, ML130) as a potent and selective inhibitor of NOD1-induced NF- κ B activation.

KEYWORDS: NOD1, NF- κ B activation, 2-aminobenzimidazole, hit-to-probe, ML130, MLPCN



ML130

NOD1 IC₅₀ = 0.56 ± 0.04 μ M
NOD2 IC₅₀ = >20 μ M
TNF- α IC₅₀ = >20 μ M
NOD1 to NOD2 fold Selectivity >36

The mammalian innate immune system serves as a first line of defense and relies on the detection of specific microbial ligands or “pathogen-associated molecular patterns” (PAMPs) by pattern recognition molecules (PRMs), acting as microbial sensors.^{1–7} In this context, among the major classes of PRMs is the NLR (NACHT and leucine rich repeat domain containing proteins) family, which has emerged as a key player in innate immunity responses. The NLR family comprises a large number of proteins from both vertebrate and invertebrate animal species, with >20 human proteins recognized.^{8–15} It has been shown that some NLR proteins detect bacterial cell wall components such as lipopolysaccharides and/or peptidoglycan (NOD1 or -2) as well as bacterial flagellin (IPAF, NAIP).^{16–19} Indeed, NOD2 is a general bacterial sensor that participates in the innate immunity against Gram-positive bacteria (*Streptococcus pneumoniae* and *Listeria monocytogenes*), Gram-negative bacteria (*Salmonella typhimurium*), and mycobacteria (*Mycobacterium tuberculosis*), while NOD1 recognizes primarily Gram-negative bacteria (*Escherichia coli*, *Chlamydia*, and *Helicobacter pylori*). NOD1 and 2 have also been involved in the induction of NF- κ B activation, caspase-1 activation, and apoptosis.^{20–22} Mutations in NOD genes are also associated with a number of human inflammatory diseases such as Crohn's disease (CD) and Blau syndrome.^{23–28} Hence, NLR proteins present an interesting avenue for the discovery and development of novel therapeutics for autoimmune and inflammatory diseases.^{29,30} Our efforts toward the discovery and optimization of a 2-aminobenzimidazole

scaffold-based inhibitor that specifically inhibits NOD1 induced NF- κ B activation are described.

A library of ~290,000 compounds from the NIH Molecular Libraries Small Molecule Repository compound collection (MLSMR)³¹ was evaluated using a cell-based NF- κ B driven luciferase reporter gene activity as a measure of NOD1 modulation. The goal was to identify compounds (chemical probes) that inhibited NOD1-mediated NF- κ B activation with an IC₅₀ of $\leq 1 \mu$ M and with dose–response Hill slopes between 0.5 and 1.4. The hit validation criteria also included 10-fold inhibition selectivity over NOD2 and a 5-fold target-based selectivity over TNF- α (tumor necrosis factor α)-mediated NF- κ B activation. The compounds satisfying the above-mentioned criteria were further subjected to a series of counterscreen assays including an Alamar blue cytotoxicity filter (AID1849).³² Because multiple cellular stimuli, acting through various pathways, can lead to NF- κ B activation, the TNF- α assay was designed to identify hits specific to TNF- α modulated pathways (hence, non-NOD modulated). Secondary assays were also performed to confirm that these compounds (a) inhibit interleukin-8 (IL-8) secretion, which is a biologically relevant downstream target of NOD1-stimulated signaling pathway (AID2250), and (b) selectively inhibit NOD1-dependent NF- κ B activation in other cell lines (AID2264).³² Ultimately, the high-throughput screening (HTS) campaign resulted in the

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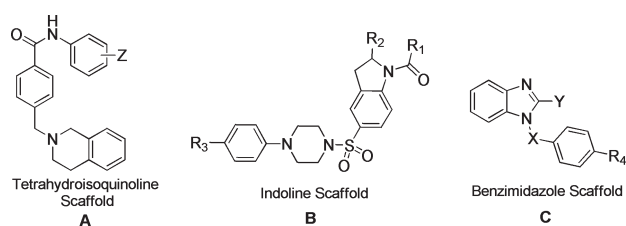


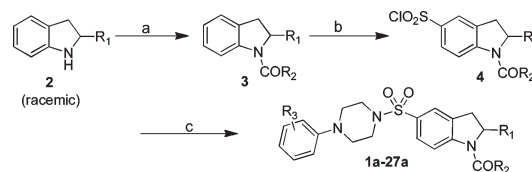
Figure 1. Three scaffolds ($IC_{50} \leq 10 \mu M$) identified in the HTS campaign.

identification of three different scaffolds, namely, tetrahydroisoquinoline (A), indoline (B), and benzimidazole (C) scaffolds that met the initial confirmatory screening criteria of inhibition of NOD1-mediated NF- κ B activation with IC_{50} of $\leq 10 \mu M$ (Figure 1). Of these three compound classes, only compounds represented by scaffold C fulfilled the final probe criteria of activity ($<1 \mu M$) and selective inhibition of NOD1-induced NF- κ B activation. Details of the comprehensive screening platform and pathway specific selectivity analysis are described in a separate publication.³³

After confirmation of the initial results, structure–activity relationship (SAR) development was initiated for the indoline scaffold using both an “analogue-by-catalog” approach and synthetic efforts. This class of compounds was prepared via acylation of the starting indoline (**2**) with select acid chlorides in the presence of pyridine (Table 1). Generation of the sulfonyl chloride (**4**) was achieved by a sequential treatment of the corresponding indoline derivative with chlorosulfuric acid in the presence of phosphorus pentachloride.^{34,35} The resulting indoline sulfonyl chloride (**4**) was reacted with various substituted *N*-phenylpiperazines, under basic conditions, to furnish the final products (**1a–27a**, Table 1) in high yields (85–95%).

The indoline scaffold was modified at three key positions annotated as R₁–R₃ (Table 1). In the first set of compounds (R₁ = Me, **1a–7a**, **9a–16a**, Table 1), analogues were synthesized to test the effect of the size of the aliphatic group at R₂ (Me vs Et vs cyclopropyl) and the stereoelectronic influence of the different groups at R₃—electron-deficient [Cl, NO₂, C(O)Me, and CN] vs electron-rich or neutral groups (OMe and Me). Subsequently, similar analogues with R₁ being H were prepared to study the effect of the indoline substitution. It was observed that the bioactivity of these compounds (**1a–27a**) is dependent on the nature of the substituent R₁. Compound **4a** with R₁ = Me exhibited an $IC_{50} = 1.87 \pm 0.41 \mu M$, but replacing the methyl group in compound **4a** with hydrogen (**23a**, Table 1) resulted in complete loss of activity. A similar trend was observed with another set of compounds (**5a** vs **18a**, **7a** vs **20a**, and **12a** vs **21a**, Table 1). The effect of substituent R₂ on the activity of these compounds depends on the electronic nature of R₃. For example, if R₃ = OMe (electron-donating group), the cyclopropyl analogue (**2a**, Table 1) shows superior activity as compared to the corresponding methyl analogue (**15a**, Table 1). On the other hand, if R₃ = C(O)Me (electron-withdrawing group), the cyclopropyl analogue (**7a**, Table 1) is less active than the corresponding methyl analogue (**6a**, Table 1). The nature of substituent R₃ also influenced the activity of these compounds. Having R₃ as an electron-withdrawing group such as NO₂ (**9a**, Table 1) and C(O)Me (**6a**, Table 1) improved the activity of these compounds. Replacing R₃ with neutral or electron-donating substituents decreases the NOD1 inhibitory activity (**9a** vs **14a**

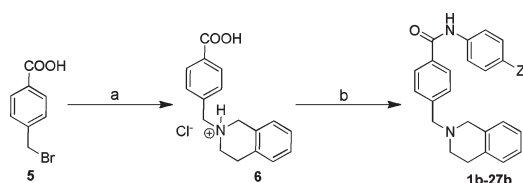
Table 1. SAR Analysis of the Indoline Scaffold^a



entry ^b	R ₁	R ₂	R ₃	n	IC ₅₀ (μM)		
					NOD1	NOD2	TNF-α
1a	Me	Me	Cl	3	2.57 ± 0.40	1.75 ± 0.07	5.76 ± 1.50
2a	Me	cyclopropyl	OMe	2	0.51 ± 0.15	0.39 ± 0.01	3.85 ± 1.50
3a	Me	cyclopropyl	H	2	>20	17.4 ± 1.83	15.3 ± 0.46
4a	Me	Et	NO ₂	3	1.87 ± 0.41	1.55 ± 0.28	4.92 ± 1.52
5a	Me	cyclopropyl	Cl	3	5.6 ± 1.12	3.63 ± 0.58	7.26 ± 1.6
6a	Me	Me	COMe	3	4.42 ± 0.48	3.72 ± 0.21	6.0 ± 0.91
7a	Me	cyclopropyl	COMe	5	10.26 ± 3.51	7.90 ± 1.6	>20
8a	H	Et	Cl	2	>20	>20	>20
9a	Me	Me	NO ₂	3	1.7 ± 0.28	1.41 ± 0.16	3.27 ± 0.58
10a	Me	Et	Cl	2	1.01 ± 0.23	0.6 ± 0.12	0.58 ± 0.03
11a	Me	Et	COMe	2	2.0 ± 0.04	1.58 ± 0.11	1.63 ± 0.23
12a	Me	cyclopropyl	NO ₂	2	0.26 ± 0.05	0.20 ± 0.02	0.24 ± 0.01
13a	Me	Me	H	2	11.15 ± 0.39	8.27 ± 0.06	14.1 ± 1.41
14a	Me	Me	Me	2	5.76 ± 0.57	3.45 ± 0.06	4.32 ± 0.05
15a	Me	Me	OMe	2	3.63 ± 0.11	3.29 ± 0.25	4.3 ± 0.27
16a	Me	cyclopropyl	Me	2	>20	>20	>20
17a	H	Et	CN	2	>20	>20	>20
18a	H	cyclopropyl	Cl	2	>20	>20	>20
19a	H	cyclopropyl	CN	2	>20	>20	>20
20a	H	cyclopropyl	COMe	2	>20	>20	>20
21a	H	cyclopropyl	NO ₂	2	6.5 ± 0.27	8.45 ± 0.14	>20
22a	H	Et	COMe	2	>20	>20	>20
23a	H	Et	NO ₂	2	>20	>20	>20
24a	H	Me	CN	2	5.87 ± 0.14	9.24 ± 0.23	8.79 ± 0.55
25a	H	Me	COMe	2	17.4 ± 1.69	>20	>20
26a	H	Me	NO ₂	2	>20	>20	>20
27a	H	Me	Cl	2	>20	>20	>20

^aAll structures were inactive in an Alamar blue cytotoxicity assay (0% activity at 20 μM). (a) R₂COCl, pyridine, room temperature, 12 h, ~80–90%. (b) ClSO₃H, PCl₅, 0 °C, 75–95%. (c) *N*-phenylpiperidine, Et₃N, room temperature, 6 h, 85–95%. ^bThe racemic mixture of compounds (not the individual enantiomers) was subjected to the biological assays.

and **15a**, Table 1). The data shown in Table 1 also indicate that the compounds belonging to indoline scaffold are generally

Scheme 1. Synthesis of the Tetrahydroisoquinoline Scaffold and Its Analogues 1b–26b^a

^a (a) (i) Two equivalents of tetrahydroisoquinoline, THF, reflux, 12 h, 80–85%; (ii) aqueous NaOH; (iii) 10% HCl. (b) Anilines, HBTU, DIPEA, DMF, room temperature, 12 h, 55–75%.

nonselective toward NOD1 and NOD2 and also have an inhibitory activity in the TNF- α assay, which suggests that these analogues act via a nonselective mechanism. These observations, along with the poor pH 7.4 buffer solubility of the series ($\sim 0.1 \mu\text{g/mL}$), guided our decision to cease pursuit of this scaffold for probe generation.

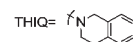
The tetrahydroisoquinoline scaffold can be obtained via alkylation of the 4-bromomethyl benzoic acid **5** using various secondary amines.³⁶ The corresponding amine was isolated as a hydrochloride salt **6**, which was coupled with various anilines using standard coupling conditions (HBTU/DIPEA) to yield the final products **1b–27b** (Scheme 1). It was observed that the bioactivity of compounds belonging to tetrahydroisoquinoline series is highly dependent on the nature of the substituent Y. Having Y as a tetrahydroisoquinoline moiety (**1b–5b**, Table 2) is pertinent for the activity. Replacement of Y with other *N*-heterocycles such as piperidine (**6b**, **7b**, **10b**, **12b**, and **15b**, Table 2) or indoline (**8b**, **9b**, **11b**, **14b**, and **17b**, Table 2) resulted in complete loss of activity. Also, increasing the carbon chain length resulted in loss of activity (**4b** vs **19b** and **3b** vs **20b**, Table 2). Substituent “X” as the $-\text{C}(\text{O})\text{NH}-$ group is imperative as reversal of the amide group [replacement by $-\text{NHC}(\text{O})-$] results in loss of the NOD1/2 and TNF- α inhibitory activity (**2b** vs **26b** and **4b** vs **22b**). Varying the type of substituent Z shows that the electron-donating substituents provided the more active compounds from the series (**1b**, **4b**, **13b**, and **16b** vs **5b**, **21b**, Table 2). The data shown in Table 2 also indicate the nonselective nature of the tetrahydroisoquinoline scaffold toward inhibition of NOD1- and NOD2-mediated NF- κ B activation. This series also exhibit inhibitory activity in the TNF- α assay, which indicates that these analogues may act via a nonselective mechanism, similar to the indoline scaffold (Table 1).

The 2-aminobenzimidazole scaffold (C) was also explored for optimization and SAR development (Table 3). Commercially available 2-aminobenzimidazole **7** was treated with various sulfonyl chlorides, in the presence of pyridine, to obtain the corresponding sulfonamides.³⁷ The nature of substituent Y and linker X also influenced the activity of the 2-aminobenzimidazole analogues (Table 3). The presence of substituent Y as the NH_2 group on the benzimidazole ring is imperative as replacement renders the compound inactive (**6–8c**, Table 3). For example, the absence of the NH_2 group in compound **6c** decreases NOD1 inhibitory activity by 10-fold. Replacement of NH_2 group with a methyl (**7c**) or SH group (**8c**) results in loss of activity ($\text{IC}_{50} > 20 \mu\text{M}$). Replacing the substitutions R_1 and R_2 with methyl groups instead of hydrogen also jeopardizes the bioactivity (**8–9c**, Table 3). Varying the type of substituent X shows that the sulfonyl group provides the most active compound from the series (**1c** vs **10–15c**, Table 3). Compound **10c** with a methylene linker or compound **13c** with an ethoxy linker exhibited a

Table 2. SAR Analysis of the Tetrahydroisoquinoline Scaffold^a

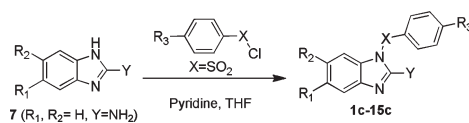
Entry	Z	X	Y	n	NOD1 IC ₅₀ (μM)	NOD 2 IC ₅₀ (μM)	TNF- α IC ₅₀ (μM)
1b	Me	CONH	THIQ	2	3.44±1.10	>20	>20
2b	Cl	CONH	THIQ	2	0.83±0.09	3.54±0.41	0.49±0.21
3b	COOEt	CONH	THIQ	2	0.15±0.01	0.40±0.02	0.14±0.01
4b	OEt	CONH	THIQ	3	1.13±0.26	0.57±0.17	2.05±0.39
5b	F	CONH	THIQ	3	3.11±0.65	1.85±0.32	2.88±0.75
6b	F	CONH	Piperidinyl	3	>20	>20	>20
7b	OEt	CONH	Piperidinyl	3	>20	>20	>20
8b	OEt	CONH	Indolyl	3	>20	>20	>20
9b	F	CONH	Indolyl	3	>20	>20	>20
10b	OMe	CONH	Piperidinyl	2	>20	>20	>20
11b	OMe	CONH	Indolyl	2	>20	>20	>20
12b	SMe	CONH	Piperidinyl	3	>20	>20	>20
13b	SMe	CONH	THIQ	3	0.24±0.08	0.26±0.02	0.37±0.16
14b	SMe	CONH	Indolyl	2	>20	>20	>20
15b	NMe ₂	CONH	Piperidinyl	2	>20	>20	>20
16b	NMe ₂	CONH	THIQ	2	1.61±0.26	3.58±0.13	1.83±0.66
17b	NMe ₂	CONH	Indolyl	3	>20	>20	>20
18b	OCF ₃	CONH	THIQ	4	0.08±0.01	0.03±0.01	0.03±0.01
19b	OEt	CONH	THIQ	2	>20	>20	>20
20b	COOEt	CONH		2	10.6±0.97	>20	10.77±1.15
21b	SO ₃ H	CONH	THIQ	2	>20	>20	>20
22b	OEt	NHCO	THIQ	2	>20	>20	>20
23b	OMe	CONH	THIQ	2	0.51±0.01	0.78±0.02	0.65±0.15
24b	Br	CONH	THIQ	2	1.03±0.14	1.62	1.50±0.17
25b	Cl	CONH	THIQ	2	>20	>20	>20
26b	Cl	NHCO	THIQ	2	>20	>20	>20

^a All structures were inactive in an Alamar blue cytotoxicity assay (0% activity at 20 μM).



reduction in activity (15-fold) or loss of activity. Compound **11c** with a carbonyl linker results in loss of selectivity, showing similar activity in NOD1 (2.8 μM) and NOD2 (3.8 μM) assays. Also, changing the linker to $-\text{CH}_2\text{CO}$ (**14c**) or $-\text{COCH}_2$ (**15c**) resulted in loss of activity. It was observed that the bioactivity of **1c** and its analogues is highly dependent on the nature of the substituent R_3 (Table 3). The absence of the 4-substituent (**4c**) or presence of other substituents such as 4-OMe (**3c**) or 4- NO_2 (**5c**) reduces the compound potency by 4–20-fold.

In summary, compound **1c** selectively (>36-fold) inhibits NOD1-dependent activation of NF- κ B as ascertained through γ -tri-DAP-stimulated luciferase signaling in a NF- κ B-linked reporter assay (AID2333) in HEK293T cells containing endogenous NOD1 levels with submicromolar potency ($\text{IC}_{50} = 0.56 \pm 0.04 \mu\text{M}$), while not inhibiting either MDP-stimulated (NOD2-dependent, AID2334) signaling in reporter cell lines containing both endogenous and overexpressed NOD2 proteins or TNF- α -induced NF- κ B activation (AID2337) (Table 3). Furthermore, compound **1c** also shows selective inhibition of NOD1-(γ -tri-DAP)-dependent IL-8 secretion but neither NOD2-dependent nor TNF- α -dependent IL-8 secretion in biologically relevant MCF-7 cells (Figure 1 and Table 1 in the Supporting Information). Compound **1c** also showed selective NOD1-dependent inhibition of NF- κ B activation via NOD1 (DAP, AID2264) modulation but did not inhibit NF- κ B activation via PMA/ionomycin (AID2261) and doxorubicin

Table 3. SAR Analysis of the 2-Amino Benzimidazole Scaffold^a

entry	R ₁	R ₂	R ₃	X	Y	n	IC ₅₀ (μM)		
							NOD1	NOD2	TNF-α (NOD1-dependent)
1c	H	H	Me	SO ₂	NH ₂	6	0.56 ± 0.04	>20	>20
2c	H	H	Cl	SO ₂	NH ₂	2	0.09 ± 0.01	19.9 ± 0.15	>20
3c	H	H	OMe	SO ₂	NH ₂	4	2.7 ± 0.69	>20	>20
4c	H	H	H	SO ₂	NH ₂	4	2.2 ± 0.21	>20	>20
5c	H	H	NO ₂	SO ₂	NH ₂	6	14.0 ± 1.8	>20	>20
6c	H	H	Me	SO ₂	H	2	6.3 ± 0.81	>20	>20
7c	H	H	Me	SO ₂	Me	2	>20	>20	>20
8c	Me	H	Me	SO ₂	SH	2	>20	>20	>20
9c	Me	Me	Me	SO ₂	NH ₂	4	>20	>20	>20
10c	H	H	Cl	CH ₂	NH ₂	2	7.7 ± 0.82	11.9 ± 0.5	>20
11c	H	H	2,4-diCl	CO	NH ₂	2	2.8 + 0.57	3.8 ± 1.5	3.2
12c	H	H	F	CO	NH ₂	2	18.0 ± 2.0	>20	>20
13c	H	H	Cl	(CH ₂) ₂ CO	NH ₂	2	16.3 ± 3.7	>20	>20
14c	H	H	OMe	CH ₂ CO	NH ₂	2	>20	>20	>20
15c	H	H	H	COCH ₂	NH ₂	2	>20	>20	>20

^a All compounds were inactive in an Alamar blue cytotoxicity assay (0% activity at 20 μM).

Table 4. In Vitro ADME Data for Compound 1c (ML130)

solubility (μg/mL)	permeability ^{a, b} Pe (×10 ⁻⁶ cm/s)	plasma protein binding (% bound)		plasma stability ^c (% remaining)	microsome stability ^d (% remaining)	
		human 10 μM/1 μM	mouse 10 μM/1 μM		human	mouse
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	mouse
2/2/2	491/562/382	97.7/97.5	95.5/95.0	100/100	41.8	0.8

^a Compound at 50 μM. ^b PAMPA Pe: low, 5 × 10⁻⁶; moderate, 250 × 10⁻⁶; high, 1000 × 10⁻⁶. ^c Plasma/PBS; compound at 1 μM, 3 h. ^d Percent remaining at 1 h.

(AID2255)-induced pathways (Figure 2 in the Supporting Information).

In vitro ADME data for probe compound 1c indicate low solubility and moderate to high “effective” cell permeability at the three pH levels tested (Table 4). Compound 1c also exhibits moderate plasma protein binding with excellent stability in both human and mouse plasma. It shows low stability in the presence of mouse microsomes but moderate stability in human microsomes and exhibits LD₅₀ >50 μM toward Fa2N-4 immortalized human hepatocytes. Preliminary in vivo dose–exposure data (using a rapid assessment of compound exposure method, “RACE”) were also obtained for compound 1c in conjunction with quantitative bioanalytical analysis to understand its pharmacokinetics in mice. Mice dosed with compound 1c (30 and 15 mg/kg, ip) exhibited significant compound exposure at *t* = 20 min, which rapidly decreased by *t* = 120 min. Mice dosed with compound 1c at 30 mg/kg, ip, exhibited a higher initial compound exposure than mice dosed at 15 mg/kg, ip (5.5 vs 1.2 μg/mL); however, plasma levels at *t* = 120 min were not improved (<0.5 μg/mL).

Compound 1c showed moderate activity (>50% inhibition) when tested at a single concentration (10 μM) across PDSP

panel (NIMH Psychoactive Drug Screening Program) that included 89% H1 (histamine), 92% 5HT6 (serotonin), and 69% 5HT2B receptors (see the Supporting Information, section D). Compound 1c also exhibited excellent selectivity in Ambit KinomeScreen showing little activity against 443 kinases at a 10 μM concentration. Inhibitory activity (reported as % control) was observed only for the following kinases: AURK (Aurora kinase) B and C (not A), 21 and 23%, respectively; CFLT3 (Fms-like tyrosine kinase), 27% C; and R1OK2 (right open reading frame), 6% C. Compound 1c was also found to be noncytotoxic in NCI 60 human tumor cell line anticancer drug screen.

In summary, a new class of 2-aminobenzimidazoles has been identified as potent and selective inhibitors of NOD1-induced NF-κB activation. Compound 1c has shown selective inhibition of NOD1-induced NF-κB activation in HEK293 cells with no cytotoxicity and was selected as a probe candidate molecule (notated in PubChem as ML130).³⁸ Compound 1c was also confirmed in secondary assays by selectively inhibiting NOD1-dependent IL-8 secretion and also selectively inhibiting the NOD1-dependent pathway to NF-κB activation. Thus, ML130 and related analogues provide new chemical tools for development

of pathway selective inhibitors of NF- κ B activation. Further work focused on improvement of potency and pharmacological profile of the probe molecule **1c** is underway.

■ ASSOCIATED CONTENT

S Supporting Information. Synthetic procedures, characterization of final products, biological assay protocols and data, and pharmacology profile. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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