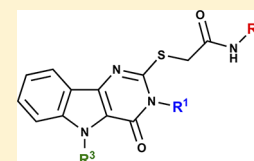


Identification of Substituted Pyrimido[5,4-*b*]indoles as Selective Toll-Like Receptor 4 Ligands

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ABSTRACT: A cell-based high-throughput screen to identify small molecular weight stimulators of the innate immune system revealed substituted pyrimido[5,4-*b*]indoles as potent NFκB activators. The most potent hit compound selectively stimulated Toll-like receptor 4 (TLR4) in human and mouse cells. Synthetic modifications of the pyrimido[5,4-*b*]indole scaffold at the carboxamide, N-3, and N-5 positions revealed differential TLR4 dependent production of NFκB and type I interferon associated cytokines, IL-6 and interferon γ-induced protein 10 (IP-10) respectively. Specifically, a subset of compounds bearing phenyl and substituted phenyl carboxamides induced lower IL-6 release while maintaining higher IP-10 production, skewing toward the type I interferon pathway. Substitution at N-5 with short alkyl substituents reduced the cytotoxicity of the leading hit compound. Computational studies supported that active compounds appeared to bind primarily to MD-2 in the TLR4/MD-2 complex. These small molecules, which stimulate innate immune cells with minimal toxicity, could potentially be used as adjuvants or immune modulators.



INTRODUCTION

The innate immune response is the first line of defense against microbial pathogens such as viruses, bacteria, fungi, and protozoa. A critical component of the innate immune response is the NFκB family of transcription factors.^{1,2} The Toll-like receptors (TLRs) are critical components of the innate immune system that regulate NFκB activation. In general, the TLRs recognize macromolecules that are associated with pathogens and with cell stress. These pathogen-associated molecular patterns (PAMPs) and their corresponding TLRs include: lipopeptides (TLR2), double-stranded RNA (TLR3), lipopolysaccharide (LPS, TLR4),³ bacterial flagellin (TLR5), guanine and uridine-rich single-stranded RNA (TLR7, 8), and hypo-methylated CpG rich DNA (TLR9).⁴ Innate immune cells use pattern recognition receptors (PRRs) such as TLRs to promote a rapid response to perceived threats before pathogen-specific adaptive immunity can be established. Indeed, this rapid response, involving multiple components of the innate immune system, has been recognized to guide the type of adaptive immune response that is most effective for the specific pathogenic threat.

The discovery that certain small molecules could serve as specific innate immune receptor ligands has expanded the possibility of designing drugs that may modulate the immune response at its earliest stages. Notable examples of such small molecules are the TLR7 agonists imiquimod,⁵ isatorabine,⁶ and the more recent 8-oxo-9-benzyladenines,⁷ as well as the TLR7/8 agonist resiquimod.⁸ A useful application of this concept is the incorporation of adjuvants into vaccines. Adjuvants are added to antigens in a vaccine setting to provide enhanced response to poorly immunogenic antigens, to increase seroconversion rates in populations with reduced responsive-

ness due to age (infants and the elderly) or disease (diabetes, renal failure), to facilitate the use of smaller doses of antigen, and to permit immunization with fewer doses of vaccine.⁹ Macromolecular activators of the innate immune system, such as monophosphoryl lipid A (MPLA), a TLR4 ligand, are utilized as adjuvants,¹⁰ but development of small molecular weight nonlipid ligands might have several advantages to address different immunological requirements for vaccines or immune therapeutics.

As part of our studies on small molecules that can activate TLRs, we conducted a high through-put screening (HTS) campaign in which a commercially available library of compounds was screened in a human cell-based NFκB activation assay. Many innate signaling pathways converge on the transcription factor NFκB. Hence it was used in the primary screen as a broad indicator of small molecule agonism of innate immunity. The specific innate receptor for the lead compounds was determined using genetically modified cells and primary mouse and human blood or bone marrow mononuclear cells. Here we report our discovery and initial structure–activity relationship (SAR) studies of the pyrimido[5,4-*b*]indole class of ligands and their characterization as TLR4/MD-2 agonists.

RESULTS AND DISCUSSION

High-Throughput Screen Study Design. A library of compounds was acquired from the University of California, San Francisco, Small Molecule Discovery Center consisting of about 170000 compounds from eight suppliers (<https://smdc.ucsf.edu>). The library was screened in three phases at a

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commercial HTS screening facility using the THP-1 human monocytic leukemia cell line, which contained a β -lactamase reporter gene under the control of the NF κ B response element that had been stably integrated into the cells. All screens were performed in activator mode using LPS as a positive control, achieving typical Z' values above 0.75. The three-phase screening process consisted of (1) a pilot screen of about 10000 compounds selected as representative of the entire primary library, (2) the primary screen of the entire library, and (3) a confirmation screen of about 2000 hits found in the primary screen. Compounds identified as active in two screens were considered to be confirmed hits. An analysis of the cluster enrichment methods for hit selection has been recently reported.¹¹

Discovery of Pyrimido[5,4-*b*]indoles as Activators of NF κ B. Following the cluster enrichment analysis, 225 compounds were selected for further in vitro biological evaluation involving cytokine induction assays in primary cells, including human peripheral blood mononuclear cells (hPBMC), mouse splenocytes, mouse bone marrow derived dendritic cells (mBMDC), and mouse bone marrow derived macrophages (mBMDM). These cells were incubated in triplicate, with each of the 225 compounds at a single concentration (1 μ M for splenocytes and 5 μ M for all other mouse cells and human cells), and the supernatants were tested for the presence of NF κ B dependent cytokines, IL-8 or IL-6, released from the human or mouse cells, respectively. Thirty-nine of the 225 compounds stimulated the human and mouse cells to secrete IL-8 or IL-6 above the detectable limit. To further confirm activity, these compounds were repurchased and retested by stimulating hPBMC and mBMDC with titrated doses and assaying for IL-8 and IL-6.

A few structurally diverse library scaffolds were identified in these cytokine assays as having reproducible responses in mouse and human stimulation assays. Among these scaffolds, the pyrimido[5,4-*b*]indoles emerged as the most potent and diverse class of compounds in the mouse cell assays with clear evidence of SAR. Within this scaffold cluster, the leading hit from the initial primary and secondary screens was a substituted acetamide attached to the pyrimidoindole ring system through a thioether linkage (Figure 1). Thus, compound 1 provided a starting point for structure–activity studies.

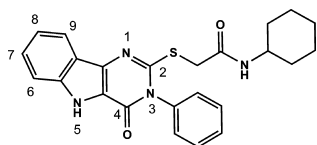


Figure 1. Structure of hit compound 1.

Target Receptor Identification. To identify the target receptor, we used HEK293 cells stably transfected with individual human (h) TLRs: TLR2, TLR3, TLR4/MD-2/CD14, TLR5, TLR7, TLR8, or TLR9 expressing HEK293 cells, along with an NF κ B activation reporter producing secreted embryonic alkaline phosphatase (SEAP). Among the tested TLR transfected cells, only those expressing TLR4/MD-2/CD14 responded to pyrimidoindoles, as shown in Figure 2A for compound 1. Because TLR4/MD-2/CD14 was the receptor complex for the active compounds in this series, it was important to rule out the possibility that activity might have been caused by LPS contamination. Therefore, compound 1

(and all active derivatives) was assayed for LPS (endotoxin) levels using a commercially available detection system and found to contain less than 10 endotoxin units (EU)/ μ mol compound. To further exclude contamination, compound 1 was resynthesized according to Scheme 1. Both samples of compound 1 displayed indistinguishable physicochemical and biological properties, indicating that the positive biological activity was not due to LPS or another contaminant. Full titration curves of the resynthesized compound were performed using the SEAP assay with mouse and human TLR4 transfected HEK293 cell lines, confirming dose-dependent activation (Figure 2B, C).

To confirm that TLR4 was indeed the receptor, compound 1 was assayed for IL-6 production in mBMDCs from wild-type and TLR4 deficient mice (Figure 2D). Genetic disruption of TLR4 completely abrogated IL-6 secretion induced by compound 1. The binding of compound 1 to the TLR4/MD-2/CD14 complex was further confirmed using a competitive antagonist for the TLR4 binding complex, LPS-RS (LPS from *Rhodococcus sphaeroides*).¹² LPS-RS inhibited the activation by compound 1 in a dose-dependent manner, indicating that compound 1 bound to the TLR4 complex (Figure 2E).

TLR4 signals through two distinct pathways, leading respectively to NF κ B dependent cytokines and type I interferon (IFN) production. Several naturally occurring TLR4 ligands and MPLA have been reported to require CD14 to activate the type I IFN regulatory pathway.¹³

The hTLR4 transfected HEK293 cell line (Figure 2A) also overexpresses MD-2 and CD14, which are TLR4 accessory proteins.^{14,15} Compound 1, however, was not dependent on CD14 for either IL-6 or type I IFN production, as demonstrated using CD14 deficient cells (Figure 2F, G). The supernatants from mBMDCs stimulated with graded doses of compound 1 were also tested for IP-10 as a surrogate marker of type I IFN release. Results showed a dose-dependent response for type I IFN (Figure 3A) production, which paralleled that of IP-10 (Figure 3B).

The above assays were utilized to compare the derivatives of the lead pyrimidoindole for their ability to activate mouse and human TLR4. Mouse TLR4 activation was assessed using primary mBMDC and IL-6 release (Figure 4) and confirmed using mTLR4 transfected HEK293 cells (Figure 5A, C). The values shown in the Tables 1–3 for IL-6 release, and mTLR4 activation are area under the curve (AUC) values for titrated doses of compounds from 312 nM to 10 μ M. Each cytokine induction curve was first converted to a percent activity curve, and then the AUC of the percent activity curve was calculated. The process of converting to a percent activity curve allowed subtracting background and adjusting for plate-to-plate variation. Finally, the AUC values were normalized to the activity of compound 1 within each experiment, set at 100. Human TLR4 activation is shown for stimulation of PBMC (IL-8) and hTLR4 HEK293 transfectomas (Figure 5B,D) at 10 μ M, as these assays were not sufficiently sensitive at lower concentrations to make AUC comparisons. The levels of hTLR4 activation by SAR derivatives are expressed relative to compound 1, set at 100.

SAR Studies. The primary HTS library contained a family of 452 compounds in the pyrimido[5,4-*b*]indole class and therefore represented a valuable initial indication of structural features important for activation of NF κ B. Upon inspection of the NF κ B activation values in the initial HTS relative to the

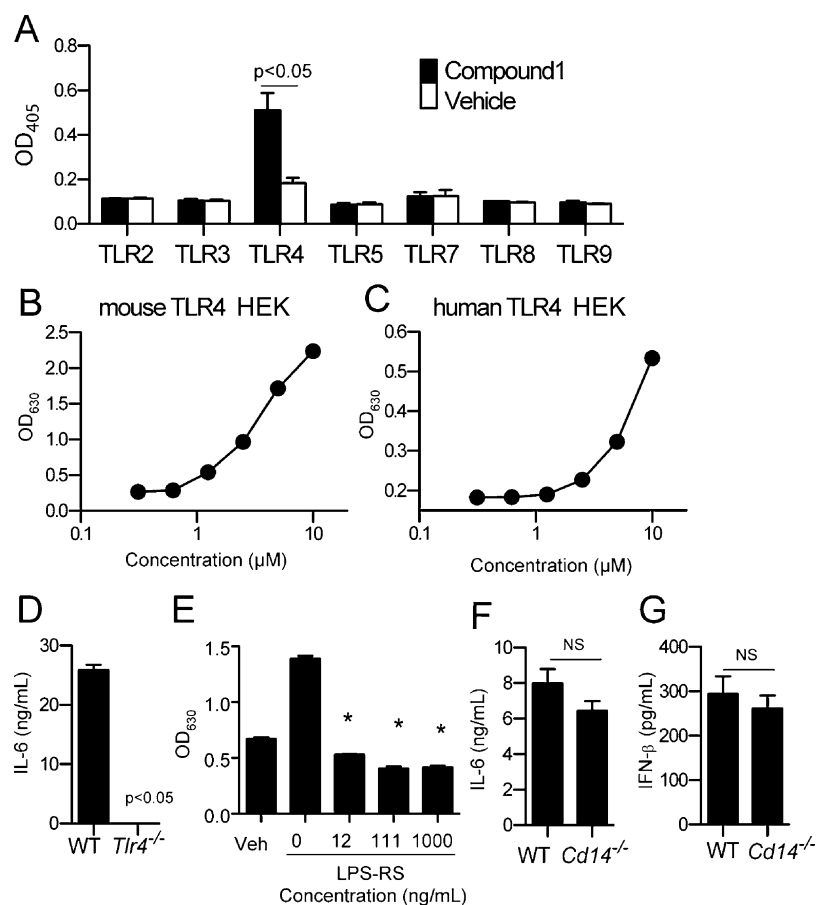
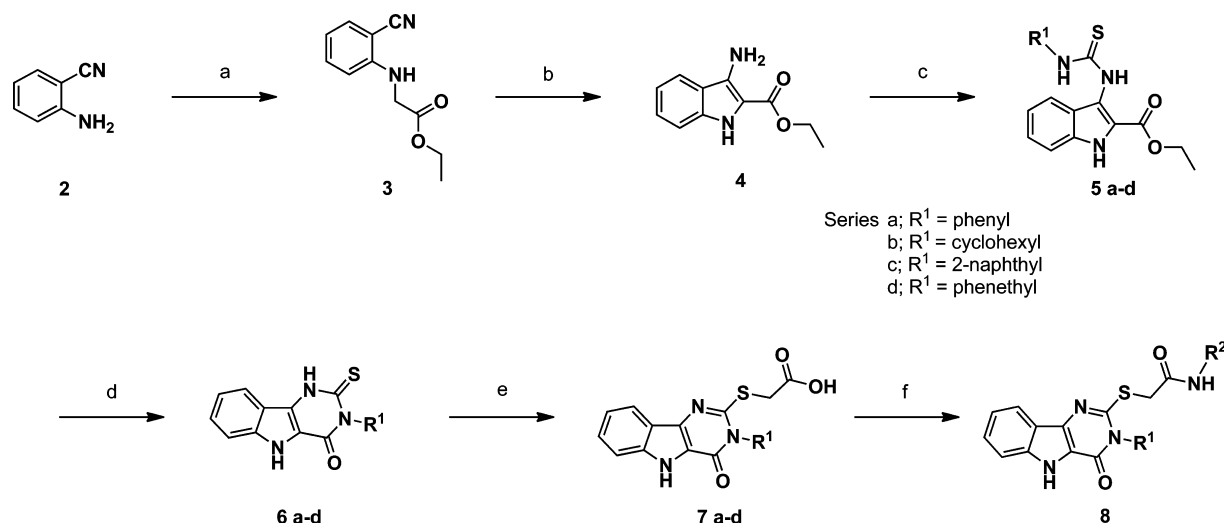


Figure 2. Target identification of compound **1** using human TLR HEK293 reporter cell lines and genetically deficient cells. (A) Human TLR2, TLR3, TLR4/MD-2/CD14, TLR5, TLR7, TLR8, and TLR9 HEK 293 Blue cells or NF- κ B/SEAPorter cells were incubated with compound **1** (10 μ M) for 20–24 h, and activation was evaluated by SEAP secretion in the culture supernatants by colorimetric assay at OD₄₀₅. Data shown are mean \pm SEM of triplicates and representative of two to three independent experiments showing similar results. $p < 0.05$ was considered significant compared to the vehicle control using Student's t test. (B,C) Mouse (B) or human (C) TLR4 HEK transfectomas were incubated with graded concentrations of compound **1**. TLR4 mediated NF κ B activation was measured by SEAP secretion in the culture supernatant. (D) WT and *Tlr4*^{-/-} mBMDC were incubated with compound **1** (10 μ M) for 18 h. IL-6 in the culture supernatants was measured by ELISA. (E) Mouse TLR4 transfectoma cells were incubated with 2.5 μ M compound **1** in the presence or absence of TLR4 antagonist LPS-RS (12, 111, 1000 ng/mL). Activation of the TLR4/NF κ B pathway was evaluated by SEAP secretion in the culture supernatants. * denotes $p < 0.05$ considered as significant compared to vehicle using one way ANOVA with Dunnett's post hoc testing. (F,G) WT and *Cd14*^{-/-} mBMDC were incubated with compound **1** (3.7 μ M) overnight. IL-6 in the culture supernatants was measured by ELISA (F) and type I IFN (IFN- β) measured by luciferase release in from an ISRE reporter cell line (G). $p < 0.05$ was considered as significant compared to vehicle using Student's t test. NS denotes "not significant". Data shown are mean \pm SEM of triplicates and representative of two independent experiments showing similar results.

various structural features, several trends were apparent. There was a requirement for a hydrophobic moiety in the region of the cyclohexyl group of compound **1**. In this same region, the carboxamide function was essential. Furthermore, a hydrophobic group at N-3, preferably a phenyl group, was also required for activity. Substitutions on the N-3 phenyl, other than fluorine atoms, resulted in loss of activity. Removal of the benzo ring of the indole portion of the scaffold also resulted in loss of activity. When the C-4 oxo was replaced by NH to form a triazine ring, loss of activity was observed. Finally, exchange of the entire acetamide moiety on the C-2 thiol with the N-3 phenyl group, such that the acetamide was attached at N-3 and the phenyl was attached at the C-2 thiol, resulted in loss of activity. With these SAR features as an initial guide, we elected to investigate modifications of hit compound **1** at three regions while maintaining the core pyrimido[5,4-*b*]indole ring system: the N-substitution of the S-acetamide, the N-3 substituent, and the N-5 substituent (Figure 6).

N-Substitutions at the carboxamide moiety were first undertaken to probe the limitations of the hydrophobic group requirement at this position with respect to optimization of cytokine induction (Table 1). While keeping all other structural features of hit compound **1** constant, we prepared a series of carboxamides substituted with various alkyl, cycloalkyl, aromatic, and heteroaromatic groups. The synthesis began with construction of the appropriately substituted pyrimido[5,4-*b*]indole ring system as shown in Scheme 1. 2-Amino-benzonitrile (**2**) was reacted with ethyl bromoacetate to yield ethyl 2-((2-cyanophenyl)amino)acetate (**3**) followed by base catalyzed ring closure to the aminoindole^{16,17} (**4**). At this point in the synthesis, a variety of substituents, represented by R¹ in the scheme, may be introduced that will determine the respective N-3 substituent following annulation of the pyrimidine ring. Thus, reaction of compound **4** with an isothiocyanate, such as phenylisothiocyanate, provided the substituted thioureidoindole^{16,18} (**5**), where R¹ was phenyl in this example. Ring closure of **5** using polyphosphoric acid

Scheme 1. General Synthetic Route^a

^aReagents and conditions: (a) BrCH₂COOEt, NaHCO₃, EtOH, reflux; (b) *tert*-BuOK, THF, <30 °C; (c) R¹-NCS, EtOH, reflux; (d) PPA, 110 °C; (e) ClCH₂COOH, KOH/EtOH, reflux; (f) R²-NH₂ (for R² substitutions refer to Table 1), HATU, DMF, room temp.

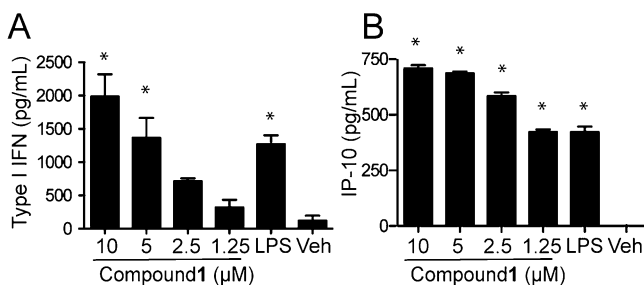


Figure 3. Type I IFN induction by compound 1. Wild-type mBMDCs were incubated with graded concentrations of compound 1 overnight. DMSO 0.5% served as the vehicle control. The levels of type I IFN were determined using an L929-ISRE luciferase reporter cell line and a mIFN β standard (A). IP-10 levels were determined by ELISA (B). Data shown are mean \pm SEM of triplicates and representative of two independent experiments showing similar results. * denotes $p < 0.05$ compared to vehicle using one way ANOVA with Dunnett's post hoc testing.

yielded the pyrimido[5,4-*b*]indole (6) bearing the N-3 substituent R¹. Alkylation of the 2-thioxo function with chloroacetic acid provided the versatile intermediate 7, which was then used to prepare the final test compounds (8) bearing a variety of N-substitutions at the acetamide moiety, designated

as R² in Scheme 1. Compounds 1, 13, 14, 15, 16, 17, as well as the N-3 derivatives 37 and 38, discussed later, were registered with Chemical Abstracts Service, but no literature references were found for any of these compounds. The hit compound 1 was resynthesized according to Scheme 1, as discussed above. Thus, by this synthetic method, compounds shown in Table 1, with R¹ held constant as a phenyl group, were prepared and evaluated in a cytokine induction assay for IL-6 in mBMDC and compared to compound 1. Examples of the data obtained from typical cytokine induction assays and NF κ B activation assays are shown in Figures 4 and 5, wherein compounds 1, 11, 12, 36, and 39 are compared for IL-6 production by mBMDC and hPBMC.

Inspection of the IL-6 AUC values relative to the hydrophobic group R² revealed that in general, compounds bearing the larger cycloalkyl groups, such as cyclooctyl (9), cycloheptyl (10), and cyclohexyl (1), were the most active, followed by branched alkyls and then straight chain alkyls and aromatic and heteroaromatic groups. A notable exception would be the *p*-fluorophenyl (14) and *o*-fluorophenyl (15) compounds. For the R² group, there appeared to be a strict "hydrophobic volume" requirement for activity. Hence, the 3,3-dimethylbutyl compound (28) was among the most active of the alkyls, with isopentyl (27), butyl (23), and isobutyl (26)

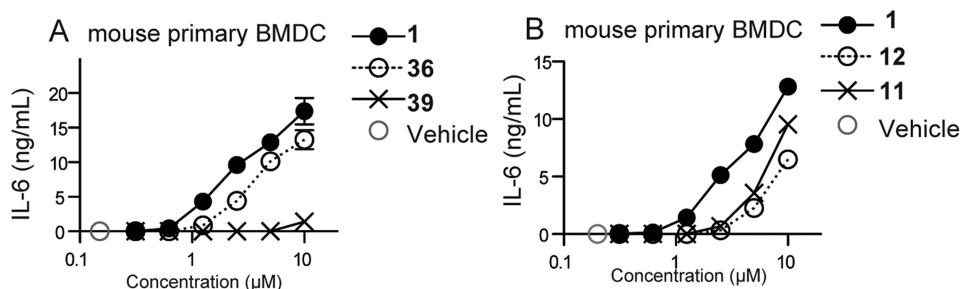


Figure 4. Representative data of SAR compound screening using mouse primary dendritic cells. Biological screening of SAR compounds was conducted using primary mBMDC. The cells were incubated with graded concentrations of the indicated compounds 1, 36, and 39 (A) or 11 and 12 (B) for 18 h. DMSO 0.5% served as the vehicle control. IL-6 levels in culture supernatants were measured by ELISA. Data shown are mean \pm SEM of triplicate.

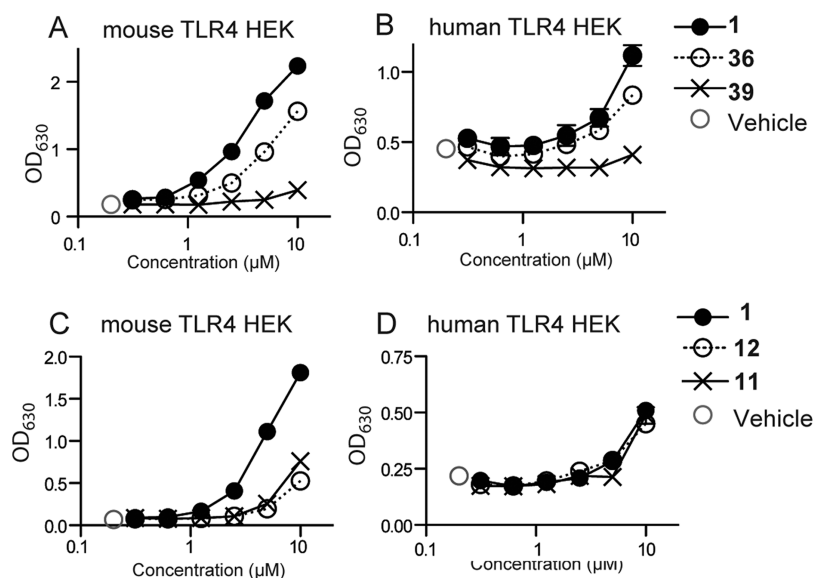


Figure 5. Representative data of SAR compound screening using TLR4 transfectomas. Mouse TLR4 (A,C), and hTLR4 (B,D) HEK transfectomas were incubated with graded concentrations of the indicated compounds **1**, **36**, and **39** (A) or **11** and **12** (B) for 18 h. DMSO 0.5% served as the vehicle control. The specific activation of the reporter cell lines was measured by SEAP activity in the supernatant by absorption at 630 nm. Data shown are mean \pm SEM of triplicate data.

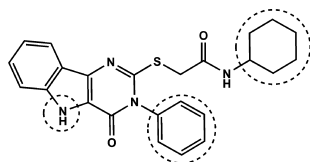


Figure 6. SAR regions of modification of hit compound **1**.

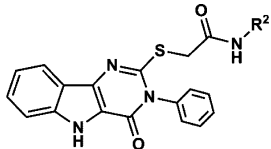
being somewhat less active. Interestingly, when the alkyl chain length of R^2 was extended to more than five carbons or reduced to three or fewer carbons, whether branched or not, significant loss of activity was observed (Table 1).

Encouraged by the SAR trends at the carboxamide moiety, we then addressed the N-3 position (Table 2). As mentioned above, data from the NF κ B primary screen indicated that a hydrophobic group at N-3, preferably an unsubstituted phenyl, was required for activity. However, to draw that conclusion with confidence, more examples of compounds bearing similar hydrophobic groups than were represented in the primary compound collection were needed. We therefore elected to prepare and evaluate a few additional derivatives of hit compound **1** with variation only at N-3. Thus, the R^1 group in compound **5** was varied by reaction of **4** with the appropriate isothiocyanate, and the remaining three steps for each derivative were completed as outlined in Scheme 1 while maintaining the R^2 group as cyclohexyl in each case. Table 2 compares the IL-6 inducing activity of compound **1** with the N-3 derivatives. As the IL-6 data indicate, replacing the N-3 phenyl in compound **1** with a cyclohexyl group (**36**) caused some loss of activity, while substitution on the N-3 phenyl (**37** and **38**) resulted in greater loss of activity. Replacing N-3 phenyl with a larger aromatic, such as naphthyl (**39**), or extending the phenyl with an alkyl chain, as in phenethyl (**40**), yielded total loss of activity. The combined substitution of R^1 with cyclohexyl and R^2 with cyclopentyl (**41**) resulted in partial loss of activity.

The third area of interest for modification was the N-5 position. None of the compounds in the original HTS library

had modifications at this position to guide the SAR. We elected to prepare a few simple N-5 alkyl derivatives of compound **1**. The N-5 indole-like nitrogen can be alkylated if the proton is first removed by a strong base, such as sodium hydride. Starting from compound **1**, Scheme 2 shows the preparation of the N-5 methyl derivative (**42**) that also produced a dimethyl side product (**43**) wherein the second methylation occurred at the carboxamide function, yielding the N,N -disubstituted derivative. The N-5 methyl butyrate ester (**44**) of compound **1** was also prepared by this method. Table 3 compares the IL-6 inducing activity of the N-5 derivatives relative to compound **1**. Interestingly, simple methylation at N-5 did not abrogate the immune stimulatory activity of compound **1**. Moreover, methylation at N-5 decreased the toxicity of compound **1** (Figure 7) as measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability assay. Dimethylation, on the other hand, resulted in total loss of IL-6 activity (compound **43**). To further probe the N-5 position, we elected to study a few N-5 alkylated derivatives of compound **1** while avoiding dialkylation in the process. Accordingly, we prepared the *t*-butyl ester of **7a** (compound **45**) and then alkylated the N-5 position with several primary alkyl halides, as depicted in Scheme 3. The resulting *t*-butyl esters (**46a–d**) were easily hydrolyzed to the corresponding free carboxylic acids (**47a–d**) and then converted to the *N*-cyclohexylcarboxamides (**48–51**) by the same method as described for compound **1**. Thus, using this strategy, the N-5 *n*-propyl, *n*-pentyl, *n*-dodecyl, and cyanomethyl derivatives were prepared. Finally, the cyanomethyl derivative (**51**) was converted to the N-5 acetamide derivative (**52**). All N-5 alkyl derivatives were found to be less active than the N-5 methyl (**42**), with the *n*-propyl (**48**) being the next most active of the series. The trend in the toxicity profile for these N-5 alkyl derivatives was confirmed, at least for the shorter chain alkyls, in that the N-5 methyl (**42**), cyanomethyl (**51**), and *n*-propyl (**48**) derivatives did not reduce cell viability at concentrations up to 10 μ M (Figure 7, shown for **42**, data not shown for **48** and **51**).

Table 1. N-Substituted Carboxamide Derivatives






























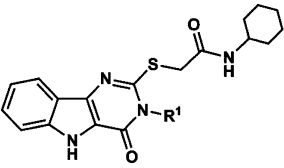
Compound	R ²	mouse			human	
		IL-6 ^a	IP-10 ^b	TLR4 ^c	IL-8 ^d	TLR4 ^e
1		100	100	100	100	100
9		126	107	99	118	100
10		114	98	95	73	64
11		53	52	28	5	23
12		35	66	18	19	32
13		11	44	21	15	31
14		103	70	81	107	108
15		36	54	43	61	71
16		14	45	11	13	39
17		19	55	13	54	116
18		<1	<1	2	<1	21
19		18	26	5	15	27
20		3	<1	<1	7	<1
21		1	<1	2	5	12
22		<1	<1	<1	4	8
23		49	61	31	29	32
24		40	40	42	25	23
25		<1	<1	4	7	8
26		44	53	24	11	21
27		61	62	56	53	38
28		99	85	126	95	72
29		30	45	29	23	17
30		12	55	17	6	13








Table 1. continued

Compound	R ²	mouse			human	
		IL-6 ^a	IP-10 ^b	TLR4 ^c	IL-8 ^d	TLR4 ^e
31		10	26	8	<1	11
32		<1	<1	<1	5	6
33		23	54	21	8	13
34		<1	<1	<1	17	<1
35	DOPE	<1	<1	<1	<1	<1

^aAUC values normalized to compound 1. Stimulation with LPS (10 ng/mL), and 5 μ M of 1 released an average of 20.6 ± 4.8 and 10.5 ± 1.3 ng/mL of IL-6, respectively. ^bAll compounds tested at 5 μ M, values normalized to 1. Stimulation with LPS 10 ng/mL and 5 μ M of 1 released averages of 234.8 ± 8.6 and 590 ± 11 pg/mL of IP-10, respectively. ^cAUC values normalized to compound 1. TLR4 cell activation stimulated with 10 ng/mL LPS and 5 μ M compound 1 resulted in OD₆₃₀ of 1.69 ± 0.08 and 1.52 ± 0.03 , respectively. ^dAll compounds tested at 10 μ M, values normalized to 1. Stimulation with LPS 10 ng/mL and 10 μ M of 1 released averages of 14.5 ± 0.6 and 7.8 ± 0.8 ng/mL of IL-8, respectively. ^eAll compounds tested at 10 μ M, values normalized to 1. TLR4 cell activation stimulated with 10 ng/mL LPS and 10 μ M compound 1 resulted in OD₆₃₀ of 1.90 ± 0.04 and 0.88 ± 0.02 , respectively.

Table 2. N-3 Derivatives

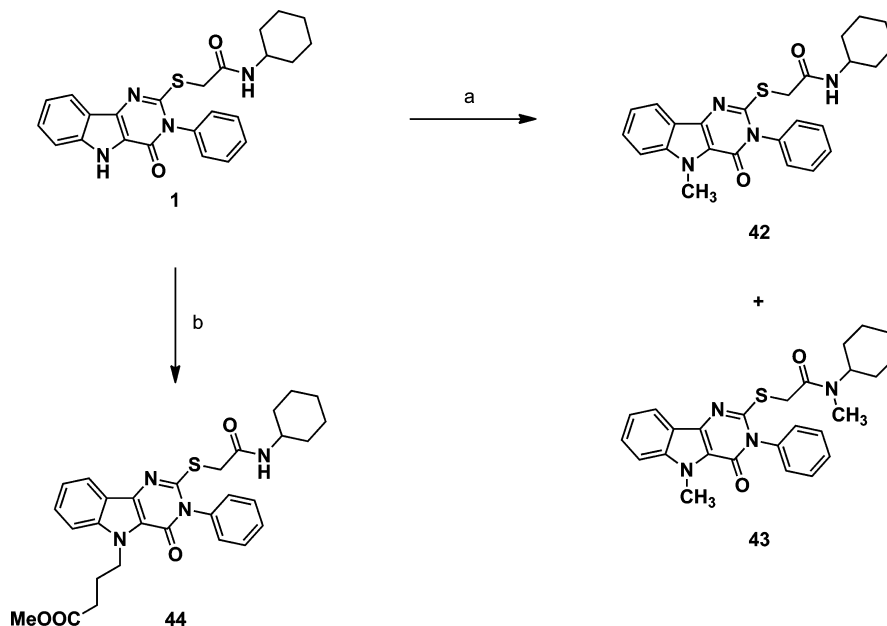


Compound	R ¹	mouse			human	
		IL-6 ^a	IP-10 ^b	TLR4 ^c	IL-8 ^d	TLR4 ^e
1		100	100	100	100	100
36		71	61	56	27	40
37		48	72	41	5	51
38		<1	<1	2	6	2
39		3	<1	6	<1	3
40		<1	<1	1	5	1
41	 , N-cyclopentyl	47	47	19	14	33

^aAUC values normalized to compound 1. Stimulation with LPS (10 ng/mL) and 5 μ M of 1 released an average of 20.6 ± 4.8 and 10.5 ± 1.3 ng/mL of IL-6, respectively. ^bAll compounds tested at 5 μ M, values normalized to 1. Stimulation with LPS 10 ng/mL and 5 μ M of 1 released averages of 234.8 ± 8.6 and 590 ± 11 pg/mL of IP-10, respectively. ^cAUC values normalized to compound 1. TLR4 cell activation stimulated with 10 ng/mL LPS and 5 μ M compound 1 resulted in OD₆₃₀ of 1.69 ± 0.08 and 1.52 ± 0.03 , respectively. ^dAll compounds tested at 10 μ M, values normalized to 1. Stimulation with LPS 10 ng/mL and 10 μ M of 1 released averages of 14.5 ± 0.6 and 7.8 ± 0.8 ng/mL of IL-8, respectively. ^eAll compounds tested at 10 μ M, values normalized to 1. TLR4 cell activation stimulated with 10 ng/mL LPS and 10 μ M compound 1 resulted in OD₆₃₀ of 1.90 ± 0.04 and 0.88 ± 0.02 , respectively.

The TLR4 transfectoma lines transmit their reporter signal after activation of the proinflammatory transcription factor, NF κ B. As one might expect, the IL-6 secretion induced by the compounds in primary mBMDs correlated well with their respective activities in mouse TLR4 transfectoma cell lines. With few exceptions, the most active compounds in the IL-6 assays were also those that showed the highest activity in the mTLR4 transfectoma line (Tables 1–3). In addition to

stimulating the production of inflammatory cytokines (e.g., IL-6) by NF κ B activation, TLR4 signaling can induce the production of type I IFN via the TRIF pathway.^{19,20} This pathway results in the activation of interferon regulatory factors (IRF), which are nuclear transcription factors that promote the transcription of IFNs. Type I IFN is important for the activation of antigen presenting dendritic cells, leading to good adjuvant activity, and also promotes cellular defenses against a

Scheme 2. N-5 Derivatives^a

^a(a) NaH, DMF, room temp, then CH₃I; (b) NaH, DMF, then Br(CH₂)₃COOCH₃

Table 3. N-5 Derivatives

Compound	R ³	mouse			human	
		IL-6 ^a	IP-10 ^b	TLR4 ^c	IL-8 ^d	TLR4 ^e
1	H	100	100	100	100	100
42	CH ₃	101	99	100	79	92
43	CH ₃ , N-methyl	<1	<1	1	5	<1
44	MeO-C(=O)-(CH ₂) ₄ -*	4	19	9	124	19
48	-(CH ₂) ₃ -*	49	45	43	83	117
49	-(CH ₂) ₄ -*	5	<1	6	1	11
50	-(CH ₂) ₈ -*	<1	<1	4	<1	10
51	N≡C-*	98	64	55	30	19
52	H ₂ N-C(=O)-*	1	1	8	4	11

^aAUC values normalized to compound 1. Stimulation with LPS (10 ng/mL) and 5 μM of 1 released an average of 20.6 ± 4.8 and 10.5 ± 1.3 ng/mL of IL-6, respectively. ^bAll compounds tested at 5 μM, values normalized to 1. Stimulation with LPS 10 ng/mL and 5 μM of 1 released averages of 234.8 ± 8.6 and 590 ± 11 pg/mL of IP-10, respectively. ^cAUC values normalized to compound 1. TLR4 cell activation stimulated with 10 ng/mL LPS and 5 μM compound 1 resulted in OD₆₃₀ of 1.69 ± 0.08 and 1.52 ± 0.03, respectively. ^dAll compounds tested at 10 μM, values normalized to 1. Stimulation with LPS 10 ng/mL and 10 μM of 1 released averages of 14.5 ± 0.6 and 7.8 ± 0.8 ng/mL of IL-8, respectively. ^eAll compounds tested at 10 μM, values normalized to 1. TLR4 cell activation stimulated with 10 ng/mL LPS and 10 μM compound 1 resulted in OD₆₃₀ of 1.90 ± 0.04 and 0.88 ± 0.02, respectively.

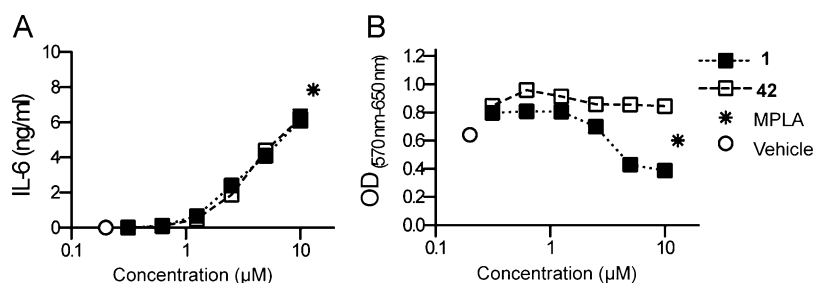
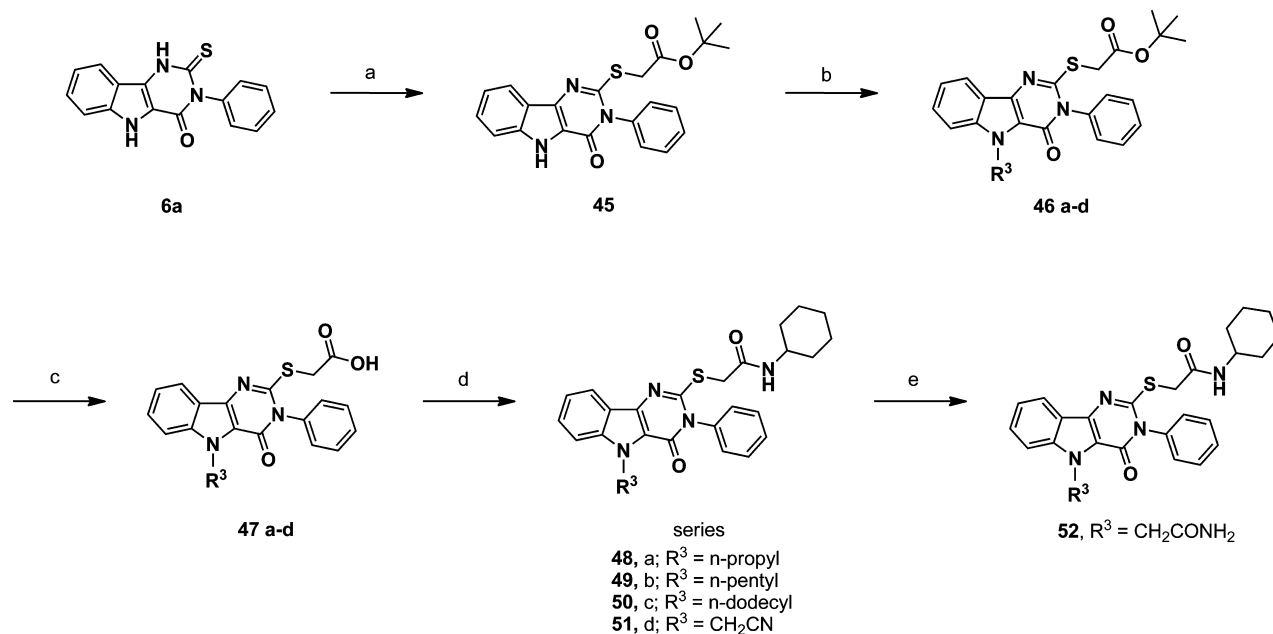


Figure 7. Assessment of cytotoxicity of compounds 1 and 42. Cytotoxicity of the compounds was evaluated by MTT assay as a measure of viability. mBMDC (10^5 /well) were incubated with graded concentrations of the compounds for 18 h and compared with vehicle (0.5% DMSO) and MPLA ($1 \mu\text{g/mL}$). (A) IL-6 levels in the culture supernatants were determined by ELISA. (B) The cells were lysed after the overnight incubation with MTT reagents, and absorbance at 570 nm was measured, subtracting the reference absorbance at 650 nm. Data shown are mean \pm SEM of triplicates and are representative of two independent experiments showing similar results.

Scheme 3. N-5 Alkyl Derivatives^a



^a(a) $\text{ClCH}_2\text{COO } t\text{-Bu}$, KOH, DMA, H_2O ; (b) NaH, DMF, then $R^3\text{X}$ (iodopropane or iodopentane or bromododecane or bromoacetonitrile); (c) TFA, DCM or CH_3CN ; (d) cyclohexyl- NH_2 , HATU, DMF, room temp; (e) compound 51, H_2SO_4 , H_2O .

variety of pathogens, particularly RNA viruses. This host defense is aided by circulating interferon inducible factors such as IP-10. The TLR4 transfectoma reporter lines in this study did not utilize an interferon reporter system; hence, the IP-10 data does not correlate as closely as the IL-6 data.

Interestingly, several of the compounds, namely 12, 13, 15, 16, 17, 29, 30, and 33, induced diminished IL-6 release compared to compound 1 but had nearly equivalent induction of IP-10 (Tables 1 and 2). These results suggest that there may be a possibility of separating the two activities on the basis of structural features. The correlation of IL-6 and IP-10 production of the selected compounds is shown graphically in Figure 8. Two distinct groups of compounds are observed, shown as circled regions of the plot. Thus, the group at the upper right area (dotted circle) comprises compounds that induce high IL-6 and high IP-10 production, including compounds 1, 9, 10, 28, and 42. The other group is composed of compounds that induce low IL-6 but relatively higher IP-10 production. These two clusters were selected based on mean \pm SD of the normalized IL-6 and IP-10 values of all SAR compounds. For IL-6, the mean was 32 ± 37 . For IP-10, the

mean was 37 ± 33 . The cluster in the dotted circle includes those compounds whose values were at least one SD above the mean for both cytokines. The other cluster includes those compounds whose values were below the mean for IL-6 and were above the mean for IP-10. It was notable that when the carboxamide substituent was phenyl or substituted phenyl (13, 15, 16, 17), with the exception of *p*-fluorophenyl (14), IL-6 release was reduced while IP-10 production was maintained at a relatively higher level. A few compounds bearing branched aliphatic carboxamide substituents showed a similar trend, as noted above for 29, 30, and 33.

In general, the active compounds exhibited greater activity in the murine cell systems than in human cells. Although the innate immune system is highly conserved among species, there are differences between human and mouse TLR4.^{21–23} Similar to the active compounds reported here nonsynthetic ligands have been noted to have species specific behavior. Notably, tetraacylated lipid IVa, a synthetic lipid A precursor, has been reported to act as a weak agonist to mouse TLR4/MD-2 but as an antagonist to human TLR4/MD-2.^{24,25} Recently mouse and human TLR4/MD2 crystal structures with this ligand have

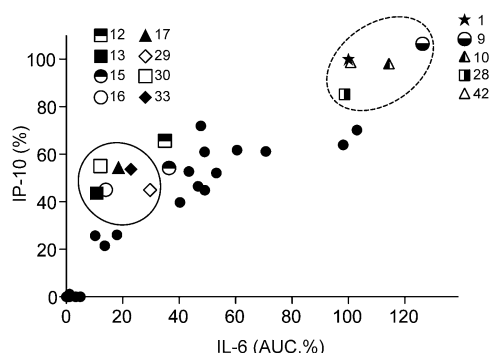


Figure 8. Correlation plot of IL-6 versus IP-10 induced by SAR derivatives. IL-6 (AUC) and IP-10 values in Tables 1–3 were plotted. Two distinct groups, compounds in the dotted circled (upper right) and in solid (left) circled areas, were selected based on the induction of IL-6 and IP-10. Numbers in the legends indicate compound ID. These clusters were selected based on mean \pm SD of the normalized IL-6 and IP-10 values of all SAR compounds. For IL-6, the mean \pm SD was 32 ± 37 . For IP-10, the mean \pm SD was 37 ± 33 . The group at the upper right area comprises compounds that induce high IL-6 and high IP-10 production, including compounds **1**, **9**, **10**, **28**, and **42**, whose values were at least one SD above the mean for both cytokines. The other cluster is composed of compounds (**13**, **16**, **17**, **29**, **30**, and **33**) that induce low IL-6 but relatively higher IP-10 production, whose values were below the mean for IL-6 and were above the mean for IP-10.

suggested that the different charge distributions of mouse and human TLR4/MD-2 affect the positions of the phosphate groups of lipid IVa, orienting them in a manner that would limit receptor dimerization by human TLR4.^{22,26}

Computational Studies. TLR4, in association with MD-2, is responsible for the physiological recognition of LPS.^{27,28} The structural basis of receptor specificity and of the mechanism of activation by LPS have recently been elucidated by determining the crystal structure of the TLR4/MD-2-LPS complex at 3.1 Å resolution.²⁹ Binding of agonistic ligands such as LPS causes dimerization of the extracellular domains to form a TLR4/MD-2-LPS macromolecular complex. Like the extracellular domains of other TLRs, TLR4 contains leucine-rich repeats and adopts a characteristic horseshoe-like shape. MD-2 is noncovalently bound to the side of the horseshoe ring and also directly interfaces with the ligand. MD-2 has a β -cup fold structure composed of two antiparallel β -sheets forming a large hydrophobic pocket for ligand binding. LPS binds to this pocket and directly mediates dimerization of the two TLR4/MD-2 complexes. TLR4 can be activated by structurally diverse LPS molecules, which have been predicted to occupy this pocket in MD-2.

Small molecule hit compounds discovered in the present study were also thought to bind to the TLR4/MD-2 complex in such a way as to facilitate dimerization. Accordingly, we examined the predicted binding mode(s) of one of the most active compounds to the murine TLR4/MD-2 complex by conducting molecular docking of compound **28** to the crystal structure of the mouse complex (PDB 2Z64) using the programs HEX³⁰ and AMPAC.³¹ We selected the best configurations of **28** bound to this complex based on molecular surface shape complementarity and the most favorable intermolecular energy of interactions. It is noteworthy that the best docking position for **28** was within the LPS pocket.²⁹ Figure 9 shows the predicted binding mode of compound **28** in the TLR4/MD-2 model, while the set of binding interactions

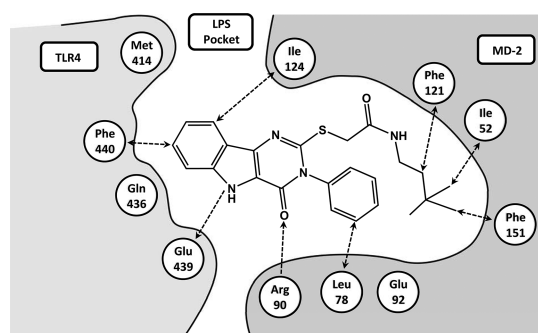


Figure 9. Predicted binding mode of compound **28** to mouse TLR4/MD-2 complex.

that may keep the compound in the MD-2 pocket bound to both TLR4 and MD-2 is depicted in Figure 10. There is a set of

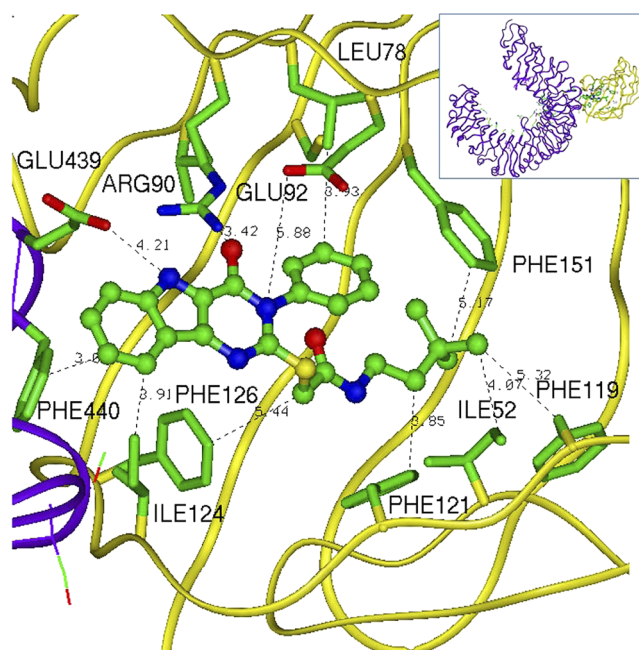


Figure 10. Predicted binding interactions of compound **28** with mouse TLR4/MD-2 complex (PDB 2Z64).

favorable electrostatic interactions resulting in possible hydrogen bonds formed by the residues Glu439 of TLR4 and Arg90 of MD-2 with compound **28**, although the former would approach a hydrogen bond interaction due to the flexibility of the Glu439 side chain. There are also multiple potential hydrophobic interactions with MD-2 and TLR4. Such interactions of the compound with two proteins can improve the free energy of complex formation by approximately 8–10 kcal/mol.

Interestingly, the predicted binding model for the pyrimidoindoles is similar to that proposed for tricyclic antidepressant, amitriptyline.³² This molecule also has some TLR4/MD-2 binding and has a similar three-ring scaffold. Other small molecules such as paclitaxel,³³ opioids,³⁴ and a peptide³⁵ have also been reported to bind to the MD-2 binding pocket.

CONCLUSION

In the course of an HTS designed to identify activators of innate immunity, a series of substituted pyrimido[5,4-*b*]indoles

were discovered as selective TLR4 ligands. Small molecules of this class are unique among TLR4 activators in that they are “non-lipid-like”. Structure–activity evaluation in both mouse and human cells revealed that, to maintain activity, the carboxamide region of this scaffold must contain a hydrophobic moiety of significant “volume”. Interestingly, a subset of the compounds bearing phenyl and substituted phenyl carboxamides induced lower NF κ B-dependent inflammatory cytokine release while maintaining interferon-dependent IP-10 production. Varying the substituents at N-3 indicated an even greater restriction for a hydrophobic moiety at this position, with a phenyl group being preferred for activity. Finally, N-5 substitution revealed that short alkyl substituents at this position attenuate cell toxicity relative to the corresponding nonsubstituted derivative while maintaining TLR4 activity. Both the inflammatory cytokine and type I IFN inducing activities of these compounds were CD14-independent. Computational studies with one of the active compounds predicted binding primarily to MD-2 in the murine TLR4/MD-2 complex. Lead optimization studies to improve the activity of the compounds using computational methods are currently underway in our laboratories. To date, no single adjuvant can elicit the optimal immune response to all pathogens in vaccines. Here we have described a panel of small molecules that stimulate immune cells to produce distinct profiles of NF κ B and interferon associated cytokines. This panel of molecules may allow for differential analysis of the relative pathway induction needed for adequate immunoprotection and immunotherapy for diverse pathogens.

■ EXPERIMENTAL SECTION

Chemistry. Materials. Reagents were purchased as at least reagent grade from Sigma-Aldrich (St. Louis, MO) unless otherwise specified and used without further purification. Solvents were purchased from Fischer Scientific (Pittsburgh, PA) and were either used as purchased or redistilled with an appropriate drying agent. HTS compound library was obtained from UCSF Small Molecule Discovery Center (San Francisco, CA). Commercial HTS service was provided by Invitrogen (Grand Island, NY). Compounds 13, 14, 15, 16, 17, 37, and 38 were purchased from Life Chemicals (Burlington, ON, Canada). All synthesized compounds, intermediates, and purchased compounds from Life Chemicals were determined to be >95% pure by HPLC utilizing an Agilent 1100 LC/MSD. Endotoxin levels of active compounds were measured with Endosafe-PTS (Charles River, Wilmington, MA) and found to have less than 10 EU/ μ mol. Reaction room temperature was maintained between 22 and 24 °C.

Instrumentation. Analytical TLC was performed using precoated TLC silica gel 60 F₂₅₄ aluminum sheets purchased from EMD (Gibbstown, NJ) and visualized using UV light. Flash chromatography was carried out on EMD silica gel 60 (40–63 μ m) system or with a Biotage Isolera One (Charlotte, NC) using the specified solvent. Reactions were monitored using an Agilent 1100 LC/MSD (Santa Clara, CA) with either a Supelco Discovery HS C18 column (Sigma-Aldrich) or an Onyx Monolithic C18 (Phenomenex, Torrance, CA) with purity above 98% by percent area. All synthesized compounds and intermediates were analyzed by high-resolution MS using an Agilent 6230 ESI-TOFMS (Santa Clara, CA). Selected compounds were analyzed by IR (KBr window method) to confirm presence of functional groups having useful diagnostic frequencies using a Perkin-Elmer 1600 series. ¹H NMR spectra were obtained on a Varian Mercury 300 or 400 (Varian, Inc., Palo Alto, CA). ¹³C spectra were obtained on Varian 500 with Xsens probe. The chemical shifts are expressed in parts per million (ppm) using suitable deuterated NMR solvents in reference to TMS at 0 ppm. The 3D structures were prepared and optimized using the AMPAC semiempirical quantum chemistry program (Accelrys, San Diego, CA).

General Procedure A for the Synthesis of Compound 5. To a solution of compound 4 (1 equiv) in warm EtOH was added the appropriate isothiocyanate (1.1 equiv) dropwise with stirring. The reaction was refluxed for 6 h and cooled overnight. Solids were filtered, washed with EtOH, and dried overnight in vacuo to give compound 5.

General Procedure B for the Synthesis of Compound 6b–d. Compound 5 was dissolved in polyphosphoric acid and stirred at 110 °C for 3.5 h. Solution was added to ice-cold water and extracted with EtOAc and dried over MgSO₄. The solid was then dried in vacuo overnight to give compound 6.

General Procedure C for the Synthesis of Compound 7. In a flame-dried flask, compound 6a (1 equiv) and KOH (2 equiv) were dissolved in anhydrous EtOH with heat. In a separate flame-dried flask, chloroacetic acid (1 equiv) was added to anhydrous EtOH. Chloroacetic acid solution was then added to the reaction mixture and refluxed for 6 h. The reaction was concentrated by half and acidified with 3 M HCl to pH 4. The solids were collected, washed with water, and dried in vacuo to give compound 7.

General Procedure D for the Synthesis of Compound 8. Compound 7 (1 equiv), triethylamine (2 equiv), and the appropriate amine (1.1 equiv) were dissolved in anhydrous DMF. To this solution, HATU (1.1 equiv) dissolved in DMF was added and stirred until complete and concentrated in vacuo. The crude material was then recrystallized in MeOH to give compound 8.

General Procedure E for the Synthesis of Compound 46. NaH (1.1 equiv) was added to a solution of compound 45 (1 equiv) in DMF. The reaction mixture was stirred for 5 min, and then the appropriate alkyl halide (1.1 equiv) was added and stirred until complete. The crude product was extracted with EtOAc and dried over MgSO₄. Crude material was finally recrystallized with MeOH to give compound 46.

General Procedure F for the Synthesis of Compound 47. Compound 46 was dissolved in 1:1 acetonitrile/trifluoroacetic acid and stirred at room temperature overnight. Depending on the N-alkyl substitution, crude material either precipitated as pure product or was purified by chromatography to give compound 47.

N-Cyclohexyl-2-((4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-b]indol-2-yl)thio)acetamide (1). Compound 7a (50 mg, 0.14 mmol), triethylamine (40 μ L, 0.28 mmol), and cyclohexylamine (18 μ L, 0.16 mmol) were dissolved in anhydrous DMF (1 mL). HATU (59.5 mg, 0.16 mmol) dissolved in 0.2 mL of DMF was added to the reaction mixture and stirred for 20 min and then concentrated in vacuo. The crude material was recrystallized with MeOH to give 61 mg in near quantitative yield. ¹H NMR (300 MHz, DMSO-*d*₆) δ ppm 0.97–1.33 (m, 4 H), 1.51 (d, *J* = 8.53 Hz, 1 H), 1.56–1.80 (m, 4 H), 3.48 (m, 1 H), 3.87 (s, 2 H), 7.24 (t, *J* = 7.29 Hz, 1 H), 7.38–7.67 (m, 6 H), 8.05 (d, *J* = 7.98 Hz, 1 H), 8.15 (d, *J* = 7.70 Hz, 1 H), 12.09 (s, 1 H). HRMS calcd for C₂₄H₂₄N₄O₂SNa (M + Na)⁺, 455.1512; found, 455.1511.

Ethyl 2-((2-Cyanophenyl)amino)acetate (3). Anthranilonitrile (30.29 g, 256 mmol), ethyl bromoacetate (29.46 mL, 267 mmol), and sodium bicarbonate (25.6 g, 300 mmol) were combined in anhydrous EtOH (90 mL) and refluxed for 42 h. After cooling slightly, solution was decanted from precipitate into a prewarmed flask. Further cooling of the decantate yielded crystals, which were then filtered and washed with cold water to give 23.6 g of white crystalline product. ¹H NMR (500 MHz, DMSO-*d*₆) δ ppm 1.13–1.27 (m, 3 H), 4.04 (d, *J* = 6.41 Hz, 2 H), 4.12 (q, *J* = 7.22 Hz, 2 H), 6.36 (t, *J* = 6.25 Hz, 1 H), 6.64 (d, *J* = 8.54 Hz, 1 H), 6.69 (t, *J* = 7.47 Hz, 1 H), 7.41 (t, *J* = 7.78 Hz, 1 H), 7.49 (dd, *J* = 7.93, 1.53 Hz, 1 H). HRMS calcd for C₁₁H₁₂N₂O₂Na (M + Na)⁺, 227.0791; found, 227.0794.

Ethyl 3-Amino-1H-indole-2-carboxylate (4). In a flame-dried flask, a suspension of potassium *t*-butoxide (7.124 g, 63.5 mmol) in anhydrous THF was stirred and maintained below 30 °C under argon. To this solution was added a solution of compound 3 (16 g, 63.5 mmol) in anhydrous THF over 45 min and stirred for an additional 2 h. The reaction was then poured into ice water, extracted with EtOAc, and dried over MgSO₄. The solid was then dissolved in minimal EtOH, and water was added dropwise until just cloudy and allowed to precipitate at room temperature. Precipitate was filtered and washed

with cold EtOH to give 6.6 g of compound **4** in 51% yield. ^1H NMR (500 MHz, DMSO- d_6) δ ppm 1.33 (t, J = 7.02 Hz, 3 H), 4.29 (q, J = 7.12 Hz, 2 H), 5.68 (s, 2 H), 6.88 (ddd, J = 8.08, 5.80, 1.98 Hz, 1 H), 7.15–7.25 (m, 2 H), 7.74 (d, J = 7.93 Hz, 1 H), 10.34 (s, 1 H). HRMS calcd for $\text{C}_{11}\text{H}_{12}\text{N}_2\text{O}_2$ ($\text{M} + \text{H}$) $^+$, 205.0972; found, 205.0973.

Ethyl 3-(3-Phenylthioureido)-1H-indole-2-carboxylate (5a). Compound **4** (5.21 g, 25.5 mmol) was reacted with phenyl isothiocyanate (3.36 mL, 28.1 mmol) in warm EtOH (75 mL) according to general procedure A to give 6.732 g compound **5a** in 75% yield. ^1H NMR (400 MHz, DMSO- d_6) δ ppm 1.32 (t, J = 7.07 Hz, 3 H), 4.32 (q, J = 7.31 Hz, 2 H), 7.09 (dt, J = 14.75, 7.50 Hz, 2 H), 7.22–7.38 (m, 3 H), 7.43 (d, J = 8.29 Hz, 1 H), 7.51 (d, J = 7.80 Hz, 2 H), 7.56 (d, J = 8.29 Hz, 1 H), 9.40 (s, 1 H), 9.69 (br s, 1 H), 11.79 (s, 1 H). HRMS calcd for $\text{C}_{18}\text{H}_{17}\text{N}_3\text{O}_2\text{SNa}$ ($\text{M} + \text{Na}$) $^+$, 362.0934; found, 362.0935.

Ethyl 3-(3-Cyclohexylthioureido)-1H-indole-2-carboxylate (5b). Compound **4** (100 mg, 0.49 mmol) was reacted with cyclohexyl isothiocyanate (169.15 mg, 0.54 mmol) in warm EtOH (750 μL) according to general procedure A to give 104 mg of compound **5b** in 61.8% yield. ^1H NMR (400 MHz, DMSO- d_6) δ ppm 1.01–1.42 (m, 9 H), 1.49–1.62 (m, 1 H), 1.62–1.74 (m, 2 H), 1.77–1.96 (m, 2 H), 4.13 (br s, 1 H), 4.29 (q, J = 7.09 Hz, 2 H), 7.05 (t, J = 7.33 Hz, 1 H), 7.25 (t, J = 8.07 Hz, 1 H), 7.41 (d, J = 8.07 Hz, 1 H), 7.49 (d, J = 8.07 Hz, 1 H), 8.91 (s, 1 H), 11.71 (br s, 1 H). HRMS calcd for $\text{C}_{18}\text{H}_{23}\text{N}_3\text{O}_2\text{S}$ ($\text{M} + \text{H}$) $^+$, 345.1511; found, 345.15142.

Ethyl 3-(3-Naphthalen-1-ylthioureido)-1H-indole-2-carboxylate (5c). Compound **4** (50 mg, 0.28 mmol) was reacted with 1-naphthyl isothiocyanate (49.9 mg, 0.27 mmol) in warm EtOH (750 μL) according to general procedure A to give 58.6 mg of compound **5c** in 61.5% yield. HRMS calcd for $\text{C}_{22}\text{H}_{19}\text{N}_3\text{O}_2\text{SNa}$ ($\text{M} + \text{Na}$) $^+$, 412.1090; found, 412.1091.

Ethyl 3-(3-Benzylthioureido)-1H-indole-2-carboxylate (5d). Compound **4** (100 mg, 0.49 mmol) was reacted with phenethyl isothiocyanate (80.4 μL , 0.54 mmol) in warm EtOH (1.5 mL) according to general procedure A to give 147 mg of compound **5d** in 81.8% yield. ^1H NMR (400 MHz, DMSO- d_6) δ ppm 1.31 (t, J = 6.97 Hz, 3 H), 4.27 (q, J = 7.21 Hz, 2 H), 7.06 (t, J = 7.70 Hz, 1 H), 7.09–7.33 (m, 7 H), 7.43 (dd, J = 8.07, 4.03 Hz, 2 H), 7.56 (br s, 1 H), 9.12 (br s, 1 H), 11.79 (br s, 1 H). HRMS calcd for $\text{C}_{20}\text{H}_{22}\text{N}_3\text{O}_2\text{S}$ ($\text{M} + \text{H}$) $^+$, 368.1427; found, 368.1431.

3-Phenyl-2-thioxo-2,3-dihydro-1H-pyrimido[5,4-b]indol-4(5H)-one (6a). To a flame-dried flask with cold anhydrous EtOH (75 mL) was added cold acetyl chloride (7 mL, 98.5 mmol) under argon with stirring. In a separate flame-dried flask charged with argon, compound **5a** (6.5 g, 19 mmol) was dissolved in anhydrous EtOH (25 mL) and added to the acetyl chloride solution. The reaction was refluxed for 12 h and cooled upon completion. Precipitate was filtered and recrystallized with EtOH to give 3.77 g of compound **6a** in 75% yield. ^1H NMR (400 MHz, DMSO- d_6) δ ppm 7.09–7.31 (m, 3 H), 7.31–7.61 (m, 6 H), 8.20 (d, J = 8.07 Hz, 1 H), 12.17 (br s, 1 H). HRMS calcd for $\text{C}_{16}\text{H}_{12}\text{N}_3\text{OS}$ ($\text{M} + \text{H}$) $^+$, 294.0696; found, 294.0698.

3-Cyclohexyl-2-thioxo-2,3-dihydro-1H-pyrimido[5,4-b]indol-4(5H)-one (6b). Compound **5b** (70 mg, 0.2 mmol) and 1 mL of polyphosphoric acid was reacted according to general procedure B to give 60.6 mg of compound **6b** in quantitative yield. ^1H NMR (400 MHz, DMSO- d_6) δ ppm 1.07–1.49 (m, 5 H), 1.53–1.70 (m, 1 H), 1.70–1.84 (m, 2 H), 2.01 (s, 2 H), 3.86–4.09 (m, 1 H), 7.11 (t, J = 8.07 Hz, 1 H), 7.29–7.54 (m, 2 H), 7.87 (d, J = 8.07 Hz, 1 H), 8.06 (d, J = 6.97 Hz, 1 H), 11.60 (s, 1 H). HRMS calcd for $\text{C}_{16}\text{H}_{18}\text{N}_3\text{OS}$ ($\text{M} + \text{H}$) $^+$, 300.1165; found, 300.1169.

3-Naphthalen-1-yl-2-thioxo-2,3-dihydro-1H-pyrimido[5,4-b]indol-4(5H)-one (6c). Compound **5c** (150 mg, 0.38 mmol) and 1 mL of polyphosphoric acid was reacted according to general procedure B to give 132 mg of compound **6c** in quantitative yield. ^1H NMR (400 MHz, DMSO- d_6) δ ppm 7.02–7.18 (m, 1 H), 7.33–7.48 (m, 2 H), 7.48–7.67 (m, 3 H), 7.74 (d, J = 8.07 Hz, 1 H), 7.87 (d, J = 8.07 Hz, 1 H), 7.84 (d, J = 7.70 Hz, 1 H), 7.94–8.03 (m, 1 H), 8.03–8.18 (m, 1 H), 11.83 (s, 1 H). HRMS calcd for $\text{C}_{20}\text{H}_{14}\text{N}_3\text{OS}$ ($\text{M} + \text{H}$) $^+$, 344.0852; found, 344.0855.

3-Phenethyl-2-thioxo-2,3-dihydro-1H-pyrimido[5,4-b]indol-4(5H)-one (6d). Compound **5d** (50.7 mg, 0.138 mmol) and 1 mL of

polyphosphoric acid was reacted according to general procedure B. Crude product was purified by automated flash chromatography (Biotage) (DCM/MeOH = 99:1) to give 27.1 mg of compound **6d** in 61.1% yield. ^1H NMR (400 MHz, DMSO- d_6) δ ppm 2.95 (t, J = 7.33 Hz, 2 H), 3.67 (t, J = 6.20 Hz, 2 H), 6.96–7.63 (m, 8 H), 7.91 (d, J = 7.70 Hz, 1 H), 8.31 (d, J = 4.77 Hz, 1 H), 11.50–11.89 (m, 1 H). HRMS calcd for $\text{C}_{18}\text{H}_{15}\text{N}_3\text{OS}$ ($\text{M} + \text{H}$) $^+$, 321.0936; found, 321.0939.

2-((4-Oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-b]indol-2-yl)thio)acetic Acid (7a). Compound **6a** (3.77 g, 12.9 mmol), KOH (1.44 g, 25.8 mmol), chloroacetic acid (1.25 g, 12.9 mmol, 6.5 mL of anhydrous EtOH), and 115 mL of anhydrous EtOH were reacted according to general procedure C to give 3.66 g of compound **7a** in 81% yield. IR: 3251 (OH), 1702 (CO carboxyl), 1660 (CO amide) cm^{-1} . ^1H NMR (400 MHz, DMSO- d_6) δ ppm 3.94 (s, 2 H), 7.14–7.36 (m, 1 H), 7.38–7.84 (m, 7 H), 7.93 (d, J = 7.70 Hz, 1 H), 12.09 (br s, 1 H). HRMS calcd for $\text{C}_{18}\text{H}_{13}\text{N}_3\text{O}_3\text{SNa}$ ($\text{M} + \text{Na}$) $^+$, 374.0570; found, 374.0572.

2-((3-Cyclohexyl-4-oxo-4,5-dihydro-3H-pyrimido[5,4-b]indol-2-yl)thio)acetic Acid (7b). Compound **6b** (165.8 mg, 0.55 mmol), KOH (62 mg, 1.1 mmol), chloroacetic acid (52.3 mg, 0.55 mmol, 3 mL of anhydrous EtOH), and 62 mL of anhydrous EtOH were reacted according to general procedure C to give 35.6 mg of compound **7b** in 17.9% yield. ^1H NMR (400 MHz, DMSO- d_6) δ ppm 1.05–1.52 (m, 4 H), 1.59–2.05 (m, 6 H), 3.94 (s, 2 H), 4.20–4.46 (m, 1 H), 7.15 (t, J = 7.33 Hz, 1 H), 7.30–7.59 (m, 2 H), 7.88 (d, J = 8.07 Hz, 1 H), 11.79 (br s, 1 H). HRMS calcd for $\text{C}_{18}\text{H}_{19}\text{N}_3\text{O}_3\text{SNa}$ ($\text{M} + \text{Na}$) $^+$, 380.1039; found, 380.1042.

2-((3-Naphthalen-2-yl)-4-oxo-4,5-dihydro-3H-pyrimido[5,4-b]indol-2-yl)thio)acetic Acid (7c). Compound **6c** (120 mg, 0.35 mmol), KOH (39.1 mg, 70 mmol), chloroacetic acid (33 mg, 0.35 mmol, 3 mL of anhydrous EtOH), and 62 mL of anhydrous EtOH were reacted according to general procedure C to give 67.1 mg of compound **7c** in 47.8% yield. ^1H NMR (400 MHz, DMSO- d_6) δ ppm 3.90 (s, 2 H), 7.26 (t, J = 7.40 Hz, 1 H), 7.33–7.66 (m, 5 H), 7.67–7.80 (m, 2 H), 7.98 (d, J = 7.70 Hz, 1 H), 8.09 (d, J = 8.43 Hz, 1 H), 8.17 (t, J = 4.77 Hz, 1 H), 12.14 (br s, 1 H). HRMS calcd for $\text{C}_{22}\text{H}_{15}\text{N}_3\text{O}_3\text{SNa}$ ($\text{M} + \text{Na}$) $^+$, 424.0726; found, 424.0730.

2-((4-Oxo-3-phenethyl-4,5-dihydro-3H-pyrimido[5,4-b]indol-2-yl)thio)acetic Acid (7d). Compound **6d** (271 mg, 0.84 mmol), KOH (94.5 mg, 1.69 mmol), chloroacetic acid (79.8 mg, 0.84 mmol, 3 mL of anhydrous EtOH), and 62 mL of anhydrous EtOH were reacted according to general procedure C to give 160 mg of compound **7d** in 50% yield. ^1H NMR (400 MHz, DMSO- d_6) δ ppm 3.02 (t, J = 7.80 Hz, 2 H), 4.02 (s, 2 H), 4.35 (t, J = 7.31 Hz, 2 H), 6.87–7.83 (m, 8 H), 7.95 (d, J = 7.31 Hz, 1 H), 11.92 (br s, 1 H). HRMS calcd for $\text{C}_{20}\text{H}_{17}\text{N}_3\text{O}_3\text{SNa}$ ($\text{M} + \text{Na}$) $^+$, 402.0883; found, 402.0885.

N-Cycloheptyl-2-((4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-b]indol-2-yl)thio)acetamide (9). Compound **7a** (50 mg, 0.14 mmol), cycloheptylamine (39.86 μL , 0.16 mmol), triethylamine (39.63 μL , 0.28 mmol), and HATU (59.52 mg, 0.16 mmol) were reacted according to general procedure D to give 12.46 mg in 18.5% yield. ^1H NMR (300 MHz, DMSO- d_6) δ ppm 1.18–1.65 (m, 10 H), 1.66–1.88 (m, 2 H), 3.59–3.77 (m, 1 H), 3.87 (s, 2 H), 7.25 (t, J = 7.01 Hz, 1 H), 7.40–7.55 (m, 4 H), 7.56–7.68 (m, 3 H), 8.06 (d, J = 7.98 Hz, 1 H), 8.20 (d, J = 7.70 Hz, 1 H), 12.10 (s, 1 H). ^{13}C NMR (126 MHz, DMSO- d_6) δ ppm 24.12, 28.28, 34.69, 37.17, 50.51, 113.31, 119.7, 120.48, 120.78, 120.81, 127.77, 130.00, 130.01, 130.32, 136.51, 137.68, 139.35, 153.01, 155.39, 165.92. HRMS calcd for $\text{C}_{25}\text{H}_{26}\text{N}_4\text{O}_2\text{SNa}$ ($\text{M} + \text{Na}$) $^+$, 469.1669; found, 469.1670.

N-Cyclooctyl-2-((4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-b]indol-2-yl)thio)acetamide (10). Compound **7a** (25 mg, 0.07 mmol), cyclooctylamine (24.15 μL , 0.08 mmol), triethylamine (19.82 μL , 0.14 mmol), and HATU (29.76 mg, 0.08 mmol) were reacted according to general procedure D to give 30.77 mg in quantitative yield. ^1H NMR (400 MHz, DMSO- d_6) δ ppm 1.23–1.89 (m, 14 H), 3.68–3.82 (m, 1 H), 3.87 (s, 2 H), 7.24 (t, J = 6.97 Hz, 1 H), 7.37–7.75 (m, 7 H), 8.06 (d, J = 8.07 Hz, 1 H), 8.18 (d, J = 7.70 Hz, 1 H), 12.10 (br s, 1 H). ^{13}C NMR (126 MHz, DMSO- d_6) δ ppm 23.75, 25.47, 27.25, 31.87, 37.16, 49.49, 113.32, 119.7, 120.48, 120.78, 120.81, 127.78, 130.00, 130.01,

130.32, 136.51, 137.68, 139.36, 153.02, 155.39, 165.90. HRMS calcd for $C_{26}H_{29}N_4O_2S$ ($M + H$)⁺, 461.2006; found, 461.2010.

N-Cyclopentyl-2-((4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-*b*]indol-2-yl)thio)acetamide (**11**). Compound **7a** (50 mg, 0.14 mmol), cyclopentylamine (31 μ L, 0.16 mmol), triethylamine (39.63 μ L, 0.28 mmol), and HATU (59.52 mg, 0.16 mmol) were reacted according to general procedure D to give 48.9 mg in 76% yield. ¹H NMR (300 MHz, DMSO-*d*₆) δ ppm 1.32–1.55 (m, 4 H), 1.55–1.70 (m, 2 H), 1.71–1.86 (m, 2 H), 3.87 (s, 2 H), 3.97 (dd, *J* = 13.07, 6.46 Hz, 1 H), 7.25 (t, *J* = 7.29 Hz, 1 H), 7.35–7.72 (m, 7 H), 8.05 (d, *J* = 7.98 Hz, 1 H), 8.26 (d, *J* = 6.88 Hz, 1 H), 12.10 (s, 1 H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ ppm 23.95, 32.74, 37.13, 51.12, 113.32, 119.69, 120.5, 120.76, 120.79, 127.77, 129.99, 130.01, 130.32, 136.50, 137.67, 139.35, 153.02, 155.39, 166.64. HRMS calcd for $C_{25}H_{26}N_4O_2SNa$ ($M + Na$)⁺, 441.1356; found, 441.1367.

N-Cyclobutyl-2-((4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-*b*]indol-2-yl)thio)acetamide (**12**). Compound **7a** (50 mg, 0.14 mmol), cyclobutylamine (26.73 μ L, 0.16 mmol), triethylamine (39.63 μ L, 0.28 mmol), and HATU (59.52 mg, 0.16 mmol) were reacted according to general procedure D to give 41.6 mg in 73.5% yield. ¹H NMR (300 MHz, DMSO-*d*₆) δ ppm 1.48–1.72 (m, 2 H), 1.93 (q, *J* = 9.70 Hz, 2 H), 2.05–2.26 (m, 2 H), 3.85 (s, 2 H), 4.08–4.26 (m, 1 H), 7.26 (t, *J* = 7.29 Hz, 1 H), 7.37–7.70 (m, 6 H), 8.07 (d, *J* = 7.98 Hz, 1 H), 8.56 (d, *J* = 7.70 Hz, 1 H), 12.10 (s, 1 H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ ppm 15.11, 30.72, 37.03, 44.72, 113.32, 119.70, 120.52, 120.81, 127.78, 130.00, 130.01, 130.33, 136.49, 137.69, 139.35, 152.95, 155.4, 166.21. HRMS calcd for $C_{22}H_{20}N_4O_2SNa$ ($M + Na$)⁺, 427.1199; found, 427.1200.

N-(Naphthalen-2-yl)-2-((4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-*b*]indol-2-yl)thio)acetamide (**18**). Compound **7a** (50 mg, 0.14 mmol), 2-aminonaphthalene (44.82 mg, 0.16 mmol), triethylamine (39.63 μ L, 0.28 mmol), and HATU (59.52 mg, 0.16 mmol) were reacted according to general procedure D to give 40.8 mg in 61% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 4.16 (br s, 2 H), 7.11 (t, *J* = 7.70 Hz, 1 H), 7.26–8.07 (m, 14 H), 8.31 (br s, 1 H), 10.65 (br s, 1 H), 12.10 (br s, 1 H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ ppm 37.99, 113.27, 115.48, 119.71, 120.21, 120.49, 120.68, 120.72, 125.07, 126.92, 127.71, 127.74, 127.90, 128.89, 130.01, 130.06, 130.18, 130.41, 133.84, 136.49, 137.16, 137.63, 139.30, 152.89, 155.37, 166.96. HRMS calcd for $C_{28}H_{20}N_4O_2SNa$ ($M + Na$)⁺, 499.1199; found, 499.1201.

N-(Furan-2-ylmethyl)-2-((4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-*b*]indol-2-yl)thio)acetamide (**19**). Compound **7a** (50 mg, 0.14 mmol), furfurylamine (27.66 μ L, 0.16 mmol), triethylamine (39.63 μ L, 0.28 mmol), and HATU (59.52 mg, 0.16 mmol) were reacted according to general procedure D to give 50.37 mg in 83.6% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 3.95 (s, 2 H), 4.29 (d, *J* = 5.50 Hz, 2 H), 6.25 (d, *J* = 3.30 Hz, 1 H), 6.32 (t, *J* = 2.57 Hz, 1 H), 7.24 (t, *J* = 6.97 Hz, 1 H), 7.35–7.72 (m, 7 H), 7.97 (d, *J* = 7.70 Hz, 1 H), 8.73 (t, *J* = 5.32 Hz, 1 H), 12.10 (br s, 1 H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ ppm 36.46, 36.76, 107.46, 107.48, 107.49, 110.86, 110.87, 110.89, 113.23, 119.71, 120.60, 120.80, 120.88, 127.76, 130.01, 130.02, 130.34, 136.50, 137.69, 139.34, 142.64, 152.32, 152.81, 155.41, 167.44. HRMS calcd for $C_{23}H_{18}N_4O_3SNa$ ($M + Na$)⁺, 453.0992; found, 453.0995.

N-(1*H*-Indazol-6-yl)-2-((4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-*b*]indol-2-yl)thio)acetamide (**20**). Compound **7a** (50 mg, 0.14 mmol), 2-aminoindazole (41.68 mg, 0.16 mmol), triethylamine (39.63 μ L, 0.28 mmol), and HATU (59.52 mg, 0.16 mmol) were reacted according to general procedure D to give 32.7 mg in 50% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 4.11 (s, 2 H), 7.09 (t, *J* = 7.33 Hz, 1 H), 7.17 (d, *J* = 8.43 Hz, 1 H), 7.32–7.77 (m, 9 H), 7.87–8.01 (m, 2 H), 8.11 (s, 1 H), 10.55 (br s, 1 H), 12.07 (br s, 1 H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ ppm 38.00, 99.22, 113.29, 114.43, 119.60, 119.65, 120.55, 120.69, 121.03, 121.24, 127.81, 129.95, 130.10, 130.45, 133.80, 136.42, 137.66, 137.73, 139.31, 140.74, 148.09, 152.88, 155.37, 162.89, 167.00. HRMS calcd for $C_{25}H_{18}N_6O_2SNa$ ($M + Na$)⁺, 489.1104; found, 489.1106.

2-((4-Oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-*b*]indol-2-yl)thio)-*N*-(thiazol-2-yl)acetamide (**21**). Compound **7a** (50 mg, 0.14

mmol), 2-aminothiazole (31.35 mg, 0.16 mmol), triethylamine (39.63 μ L, 0.28 mmol), and HATU (59.52 mg, 0.16 mmol) were reacted according to general procedure D to give 12.82 mg in 21.1% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 4.17 (s, 2 H), 6.90–7.27 (m, 2 H), 7.30–7.78 (m, 8 H), 7.83 (d, *J* = 7.70 Hz, 1 H), 12.03–12.16 (m, 1 H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ ppm 20.55, 28.58, 36.97, 46.95, 113.30, 119.71, 120.54, 120.77, 120.80, 127.76, 130.01, 130.32, 136.49, 137.68, 139.35, 152.91, 155.40, 167.27. HRMS calcd for $C_{21}H_{15}N_5O_2S_2Na$ ($M + Na$)⁺, 456.0559; found, 456.0562.

N-Ethyl-2-((4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-*b*]indol-2-yl)thio)acetamide (**22**). Compound **7a** (50 mg, 0.14 mmol), ethylamine (17.73 μ L, 0.16 mmol), triethylamine (39.63 μ L, 0.28 mmol), and HATU (59.52 mg, 0.16 mmol) were reacted according to general procedure D to give 22.9 mg in 43.3% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 1.02 (t, *J* = 7.15 Hz, 3 H), 3.04–3.16 (m, 2 H), 3.88 (s, 2 H), 7.26 (t, *J* = 7.33 Hz, 1 H), 7.39–7.54 (m, 3 H), 7.56–7.69 (m, 3 H), 8.05 (d, *J* = 8.07 Hz, 1 H), 8.26 (m, 1 H), 12.11 (s, 1 H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ ppm 15.12, 34.34, 37.01, 113.30, 119.70, 120.59, 120.74, 120.81, 127.76, 130.01, 130.32, 136.49, 137.68, 139.35, 152.88, 155.41, 167.02. HRMS calcd for $C_{20}H_{18}N_4O_2SNa$ ($M + Na$)⁺, 401.1043; found, 401.1046.

N-Butyl-2-((4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-*b*]indol-2-yl)thio)acetamide (**23**). Compound **7a** (50 mg, 0.14 mmol), butylamine (30.94 μ L, 0.16 mmol), triethylamine (39.63 μ L, 0.28 mmol), and HATU (59.52 mg, 0.16 mmol) were reacted according to general procedure D to give 18.6 mg in 44.7% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 0.78 (t, *J* = 7.31 Hz, 3 H), 1.17–1.30 (m, 2 H), 1.32–1.46 (m, 2 H), 2.98–3.13 (m, 2 H), 3.89 (s, 2 H), 7.25 (t, *J* = 6.83 Hz, 1 H), 7.39–7.55 (m, 4 H), 7.60 (m, *J* = 5.40, 5.40 Hz, 3 H), 8.04 (d, *J* = 7.80 Hz, 1 H), 8.17–8.25 (m, 1 H), 12.10 (s, 1 H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ ppm 13.61, 19.53, 31.2, 36.52, 38.7, 112.84, 119.26, 120.08, 120.32, 120.38, 127.3, 129.54, 129.55, 129.86, 136.05, 137.25, 138.93, 152.43, 154.96, 166.7. HRMS calcd for $C_{22}H_{22}N_4O_2SNa$ ($M + Na$)⁺, 429.1356; found, 429.1359.

2-((4-Oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-*b*]indol-2-yl)thio)-*N*-pentylacetamide (**24**). Compound **7a** (25 mg, 0.07 mmol), *n*-amylamine (20 μ L, 0.16 mmol), triethylamine (19.82 μ L, 0.14 mmol), and HATU (29.76 mg, 0.08 mmol) were reacted according to general procedure D to give 19.5 mg in 66% yield. ¹H NMR (300 MHz, DMSO-*d*₆) δ ppm 0.74 (t, *J* = 7.15 Hz, 3 H), 1.07–1.25 (m, 4 H), 1.31–1.45 (m, 2 H), 2.98–3.15 (m, 2 H), 3.88 (s, 2 H), 7.24 (t, *J* = 6.88 Hz, 1 H), 7.40–7.54 (m, 3 H), 7.56–7.68 (m, 3 H), 8.04 (d, *J* = 7.98 Hz, 1 H), 8.20 (t, *J* = 5.36 Hz, 1 H), 12.10 (s, 1 H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ ppm 14.26, 22.29, 29.03, 29.25, 36.92, 113.27, 119.69, 120.52, 120.78, 120.81, 127.76, 130.00, 130.32, 136.48, 137.69, 139.35, 152.85, 155.40, 167.16. HRMS calcd for $C_{23}H_{25}N_4O_2S$ ($M + H$)⁺, 421.1693; found, 421.1689.

N-Hexyl-2-((4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-*b*]indol-2-yl)thio)acetamide (**25**). Compound **7a** (25 mg, 0.07 mmol), hexylamine (21 μ L, 0.16 mmol), triethylamine (19.82 μ L, 0.14 mmol), and HATU (29.76 mg, 0.08 mmol) were reacted according to general procedure D to give 20 mg in 65% yield. ¹H NMR (300 MHz, DMSO-*d*₆) δ ppm 0.76 (t, *J* = 6.46 Hz, 3 H), 1.01–1.49 (m, 8 H), 3.06 (q, *J* = 6.60 Hz, 2 H), 3.87 (s, 2 H), 7.24 (t, *J* = 6.80 Hz, 1 H), 7.41–7.55 (m, 3 H), 7.55–7.66 (m, 3 H), 8.05 (d, *J* = 7.70 Hz, 1 H), 8.19 (t, *J* = 5.23 Hz, 1 H), 12.10 (s, 1 H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ ppm 14.29, 22.40, 26.49, 29.51, 31.40, 36.82, 113.28, 119.63, 120.55, 120.79, 120.81, 127.82, 129.94, 130.04, 130.36, 136.41, 137.72, 139.36, 152.80, 155.41, 167.33. HRMS calcd for $C_{24}H_{27}N_4O_2S$ ($M + H$)⁺, 435.1849; found, 435.1846.

N-Isobutyl-2-((4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-*b*]indol-2-yl)thio)acetamide (**26**). Compound **7a** (50 mg, 0.14 mmol), isobutylamine (31.11 μ L, 0.16 mmol), triethylamine (39.63 μ L, 0.28 mmol), and HATU (59.52 mg, 0.16 mmol) were reacted according to general procedure D to give 17.71 mg in 31% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 0.72–0.92 (m, 6 H), 1.68 (dt, *J* = 13.47, 6.64 Hz, 1 H), 2.91 (t, *J* = 6.60 Hz, 2 H), 3.93 (s, 2 H), 7.25 (t, *J* = 6.97 Hz, 1 H), 7.40–7.56 (m, 3 H), 7.61 (d, *J* = 6.23 Hz, 3 H), 8.04 (d, *J* = 7.70 Hz, 1 H), 8.23 (t, *J* = 6.23 Hz, 1 H), 12.11 (br s, 1 H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ ppm 20.55, 28.58, 36.97, 46.95, 113.30, 119.71,

120.54, 120.77, 120.80, 127.76, 130.01, 130.32, 136.49, 137.68, 139.35, 152.91, 155.40, 167.27. HRMS calcd for $C_{22}H_{22}N_4O_2SNa$ ($M + Na$)⁺, 429.1356; found, 429.1359.

N-Isopentyl-2-((4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-*b*]indol-2-yl)thio)acetamide (**27**). Compound **7a** (25 mg, 0.07 mmol), isopentylamine (20 μ L, 0.16 mmol), triethylamine (19.82 μ L, 0.14 mmol), and HATU (29.76 mg, 0.08 mmol) were reacted according to general procedure D to give 20 mg in 67% yield. ¹H NMR (300 MHz, DMSO-*d*₆) δ ppm 0.78 (d, *J* = 6.60 Hz, 6 H), 1.29 (q, *J* = 6.88 Hz, 2 H), 1.54 (tt, *J* = 13.38, 6.84 Hz, 1 H), 3.08 (s, 2 H), 3.88 (s, 2 H), 7.24 (t, *J* = 7.01 Hz, 1 H), 7.36–7.68 (m, 6 H), 8.04 (d, *J* = 7.98 Hz, 1 H), 8.17 (t, *J* = 5.50 Hz, 1 H), 12.10 (s, 1 H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ ppm 22.71, 25.45, 36.86, 37.68, 38.43, 113.29, 119.64, 120.55, 120.77, 120.80, 127.83, 129.95, 130.03, 130.35, 136.42, 137.71, 139.34, 152.82, 155.40, 167.23. HRMS calcd for $C_{23}H_{23}N_4O_2S$ ($M + H$)⁺, 421.1693; found, 421.1689.

N-(3,3-Dimethylbutyl)-2-((4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-*b*]indol-2-yl)thio)acetamide (**28**). Compound **7a** (25 mg, 0.07 mmol), 3,3-dimethylbutylamine (20 μ L, 0.16 mmol), triethylamine (19.82 μ L, 0.14 mmol), and HATU (29.76 mg, 0.08 mmol) were reacted according to general procedure D and purified by reverse-phase chromatography (C18, 10:90 to 90:10 water:methanol gradient) to give 18 mg in 60% yield. ¹H NMR (300 MHz, DMSO-*d*₆) δ ppm 0.83 (s, 9 H), 1.26–1.47 (m, 2 H), 3.00–3.16 (m, 2 H), 3.86 (s, 2 H), 7.24 (t, *J* = 7.29 Hz, 1 H), 7.39–7.55 (m, 3 H), 7.56–7.70 (m, 3 H), 8.04 (d, *J* = 7.98 Hz, 1 H), 8.17 (t, *J* = 5.50 Hz, 1 H), 12.10 (s, 1 H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ ppm 24.12, 28.28, 34.68, 37.17, 50.51, 113.31, 119.70, 120.48, 120.78, 120.81, 127.77, 130.00, 130.01, 130.32, 136.51, 137.68, 139.35, 153.01, 155.39, 165.92. HRMS calcd for $C_{24}H_{27}N_4O_2S$ ($M + H$)⁺, 435.1849; found, 435.1846.

N-(3-Methylpentyl)-2-((4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-*b*]indol-2-yl)thio)acetamide (**29**). Compound **7a** (25 mg, 0.07 mmol), 3-methylpentylamine (20 μ L, 0.16 mmol), triethylamine (19.82 μ L, 0.14 mmol) and HATU (29.76 mg, 0.08 mmol) according to general procedure D and purified by reverse-phase chromatography (C18, 10:90 to 90:10 water:methanol gradient) to give 17 mg in 55% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 0.67–0.85 (m, 6 H), 0.96–1.11 (m, 1 H), 1.13–1.26 (m, 2 H), 1.31 (dd, *J* = 12.65, 6.42 Hz, 1 H), 1.37–1.51 (m, 1 H), 2.99–3.17 (m, 2 H), 3.87 (s, 2 H), 7.24 (t, *J* = 7.52 Hz, 1 H), 7.41–7.55 (m, 4 H), 7.56–7.69 (m, 3 H), 8.04 (d, *J* = 8.07 Hz, 1 H), 8.17 (t, *J* = 5.50 Hz, 1 H), 12.10 (s, 1 H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ ppm 11.42, 19.22, 29.22, 31.70, 36.16, 37.49, 113.27, 119.69, 120.50, 120.81, 127.77, 130.00, 130.01, 130.33, 136.48, 137.69, 139.36, 152.84, 155.40, 167.11. HRMS calcd for $C_{24}H_{26}N_4O_2SNa$ ($M + Na$)⁺, 457.1669; found, 457.1670.

2-((4-Oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-*b*]indol-2-yl)thio)-*N*-(pentan-2-yl)acetamide (**30**). Compound **7a** (25 mg, 0.07 mmol), 2-aminopentane (18.54 μ L, 0.16 mmol), triethylamine (19.82 μ L, 0.14 mmol), and HATU (29.76 mg, 0.08 mmol) according to general procedure D to give 19.1 mg in 65% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 0.74 (t, *J* = 6.97 Hz, 3 H), 1.01 (d, *J* = 6.60 Hz, 3 H), 1.13–1.62 (m, 4 H), 3.64–3.81 (m, 1 H), 3.89 (s, 2 H), 7.24 (t, *J* = 7.33 Hz, 1 H), 7.41–7.72 (m, 6 H), 8.05 (t, *J* = 6.60 Hz, 2 H), 12.09 (br s, 1 H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ ppm 14.25, 19.32, 21.23, 37.11, 38.84, 44.94, 113.28, 119.68, 120.47, 120.77, 120.84, 127.75, 130.01, 130.32, 136.51, 137.68, 139.35, 152.97, 155.39, 166.36. HRMS calcd for $C_{23}H_{24}N_4O_2SNa$ ($M + Na$)⁺, 443.1512; found, 443.1514.

2-((4-Oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-*b*]indol-2-yl)thio)-*N*-(pentan-3-yl)acetamide (**31**). Compound **7a** (25 mg, 0.07 mmol), 3-aminopentane (18.24 μ L, 0.16 mmol), triethylamine (19.82 μ L, 0.14 mmol), and HATU (29.76 mg, 0.08 mmol) were reacted according to general procedure D to give 19.44 mg in 65% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 0.77 (d, *J* = 6.83 Hz, 6 H), 1.21–1.62 (m, 4 H), 3.47–3.58 (m, 1 H), 3.93 (s, 1 H), 7.24 (t, *J* = 6.83 Hz, 1 H), 7.37–7.76 (m, 6 H), 7.93 (d, *J* = 7.31 Hz, 1 H), 8.05 (d, *J* = 7.80 Hz, 1 H), 12.10 (br s, 1 H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ ppm 10.87, 27.40, 37.00, 52.36, 113.29, 119.64, 120.50, 120.73, 120.86, 127.82, 129.95, 130.05, 130.36, 136.43, 137.71, 139.34, 152.98, 155.40,

167.06. HRMS calcd for $C_{23}H_{25}N_4O_2S$ ($M + H$)⁺, 421.1693; found, 421.1695.

N-(*tert*-Butyl)-2-((4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-*b*]indol-2-yl)thio)acetamide (**32**). Compound **7a** (50 mg, 0.14 mmol), *t*-butylamine (32.9 μ L, 0.16 mmol), triethylamine (39.63 μ L, 0.28 mmol), and HATU (59.52 mg, 0.16 mmol) were reacted according to general procedure D to give 22.6 mg in 39.7% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 1.15–1.37 (m, 9 H), 3.85 (s, 2 H), 7.26 (t, *J* = 6.83 Hz, 1 H), 7.41–7.56 (m, 4 H), 7.57–7.71 (m, 3 H), 7.93 (s, 1 H), 8.07 (d, *J* = 7.80 Hz, 1 H), 12.11 (s, 1 H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ ppm 28.86, 37.86, 50.89, 113.34, 119.68, 120.54, 120.74, 120.78, 127.76, 129.99, 130.00, 130.31, 136.54, 137.68, 139.37, 153.13, 155.40, 166.60. HRMS calcd for $C_{22}H_{22}N_4O_2SNa$ ($M + Na$)⁺, 429.1356; found, 429.1359.

2-((4-Oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-*b*]indol-2-yl)thio)-*N*-(tetrahydrofuran-2-yl)methylacetamide (**33**). Compound **7a** (50 mg, 0.14 mmol), tetrahydrofurfurylamine (32.31 μ L, 0.16 mmol), triethylamine (39.63 μ L, 0.28 mmol) and HATU (59.52 mg, 0.16 mmol) were reacted according to general procedure D to give 35 mg in 57.5% yield. IR: 3372, 3193 (NH), 1651 (CO) cm^{-1} . ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 1.32–1.61 (m, 1 H), 1.63–2.06 (m, 3 H), 2.96–3.26 (m, 2 H), 3.52–3.64 (m, 1 H), 3.64–3.75 (m, 1 H), 3.76–3.86 (m, 1 H), 3.92 (s, 2 H), 7.25 (t, *J* = 6.97 Hz, 1 H), 7.35–7.71 (m, 6 H), 8.07 (d, *J* = 8.06 Hz, 1 H), 8.30 (t, *J* = 5.50 Hz, 1 H), 12.10 (br s, 1 H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ ppm 25.12, 28.4, 36.38, 43.03, 67.15, 77.11, 112.81, 119.25, 120.11, 120.39, 127.32, 129.55, 129.57, 129.88, 136.05, 137.25, 138.92, 152.4, 154.95, 167.14. HRMS calcd for $C_{23}H_{22}N_4O_3SNa$ ($M + Na$)⁺, 457.1305; found, 457.1308.

2-((4-Oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-*b*]indol-2-yl)thio)-*N*-(piperidin-4-yl)acetamide (**34**). Compound **7a** (25 mg, 0.07 mmol), 4-aminopiperidine (16.6 μ L, 0.16 mmol), triethylamine (19.82 μ L, 0.14 mmol), and HATU (29.76 mg, 0.08 mmol) were reacted according to general procedure D to give 17 mg in 65% yield. IR: 3372, 3193 (NH), 1651 (CO) cm^{-1} . ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 1.27 (d, *J* = 11.36 Hz, 1 H), 1.46 (d, *J* = 9.90 Hz, 1 H), 1.77–2.05 (m, 2 H), 2.64–2.85 (m, 1 H), 4.00–4.43 (m, 3 H), 7.14–7.38 (m, 2 H), 7.41–7.80 (m, 6 H), 8.04 (d, *J* = 7.70 Hz, 1 H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ ppm 33.41, 35.16, 36.00, 36.46, 40.91, 44.69, 45.37, 48.25, 113.30, 119.70, 120.67, 120.75, 120.79, 127.76, 130.00, 130.03, 130.33, 136.51, 137.63, 137.68, 139.36, 152.93, 152.97, 155.40, 165.44, 166.23. HRMS calcd for $C_{23}H_{24}N_4O_2S$ ($M + H$)⁺, 434.1645; found, 434.1648.

(*Z*)-3-((Hydroxy(2-((4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-*b*]indol-2-yl)thio)acetamido)ethoxyphosphoryl)oxy)propane-1,2-diyl Dioleate (**35**). Compound **7a** (50 mg, 0.14 mmol), DOPE (32.9 μ L, 0.16 mmol), triethylamine (39.63 μ L, 0.28 mmol), and HATU (59.52 mg, 0.16 mmol) were reacted according to general procedure D to give 16.4 mg in 10.9% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 0.84 (t, *J* = 6.83 Hz, 6 H), 1.05–1.75 (m, 50 H), 1.96 (br s, 8 H), 2.23 (t, *J* = 7.31 Hz, 4 H), 3.02 (s, 3 H), 3.14–3.26 (m, 2 H), 3.59–3.83 (m, 3 H), 3.90 (s, 2 H), 4.02–4.17 (m, 1 H), 4.26 (d, *J* = 9.26 Hz, 1 H), 5.06 (br s, 1 H), 5.30 (br s, 3 H), 7.24 (t, *J* = 7.31 Hz, 1 H), 7.38–7.75 (m, 6 H), 8.09 (d, *J* = 7.80 Hz, 1 H), 8.87 (br s, 1 H), 10.12 (br s, 1 H), 12.08 (br s, 1 H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ ppm 8.84, 14.38, 14.39, 22.58, 24.85, 24.91, 27.02, 27.05, 28.88, 28.91, 28.98, 29.00, 29.07, 29.12, 29.19, 29.33, 29.43, 29.45, 29.57, 31.77, 33.82, 34.00, 37.11, 45.62, 62.78, 63.05, 70.84, 70.89, 113.19, 119.66, 120.61, 120.86, 120.89, 127.62, 129.96, 130.00, 130.27, 136.50, 137.72, 139.36, 152.72, 155.40, 167.22, 172.67, 172.91. HRMS calcd for $C_{59}H_{89}N_4O_{10}PSNa$ ($M + Na$)⁺, 1099.5929; found, 1099.5930.

N-Cyclohexyl-2-((3-cyclohexyl-4-oxo-4,5-dihydro-3H-pyrimido[5,4-*b*]indol-2-yl)thio)acetamide (**36**). Compound **7b** (17.7 mg, 0.05 mmol), triethylamine (13.8 μ L, 0.1 mmol), and cyclohexylamine (6.2 μ L, 0.06 mmol) were dissolved in anhydrous DMF (0.5 mL). HATU (20.74 mg, 0.06 mmol) dissolved in 0.2 mL of DMF was added to the reaction mixture and stirred for 20 min and concentrated in vacuo. The crude material was recrystallized in MeOH to give 9.1 mg in 41% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 0.99–2.17 (m, 16 H),

2.60–2.88 (m, 4 H), 3.99 (br s, 2 H), 4.17–4.45 (m, 1 H), 7.20 (t, $J = 7.33$ Hz, 1 H), 7.34–7.65 (m, 2 H), 8.01 (d, $J = 8.07$ Hz, 1 H), 8.22 (d, $J = 6.97$ Hz, 1 H), 11.86 (br s, 1 H). ^{13}C NMR (126 MHz, DMSO- d_6) δ ppm 19.02, 24.92, 25.28, 25.63, 26.36, 28.79, 32.87, 37.21, 48.49, 56.47, 61.95, 113.22, 120.27, 120.56, 120.72, 120.82, 127.59, 136.47, 139.26, 151.90, 155.96, 166.22. HRMS calcd for $\text{C}_{24}\text{H}_{30}\text{N}_4\text{O}_2\text{SNa}$ ($\text{M} + \text{Na}$) $^+$, 461.1982; found, 461.1983.

N-Cyclohexyl-2-((3-(naphthalen-2-yl)-4-oxo-4,5-dihydro-3H-pyrimido[5,4-*b*]indol-2-yl)thio)acetamide (**39**). Compound **7c** (26.1 mg, 0.065 mmol), triethylamine (18.1 μL , 0.13 mmol), and cyclohexylamine (8.2 μL , 0.071 mmol) were dissolved in anhydrous DMF (0.1 mL). HATU (27.2 mg, 0.071 mmol) dissolved in 0.2 mL of DMF was added to the reaction mixture and stirred for 20 min and concentrated in vacuo. The crude material was recrystallized in MeOH to give 27 mg in 86% yield. ^1H NMR (400 MHz, DMSO- d_6) δ ppm 0.76–1.38 (m, 5 H), 1.42–1.95 (m, 5 H), 3.43–3.55 (m, 1 H), 3.74–4.00 (m, 2 H), 7.29 (t, $J = 6.97$ Hz, 1 H), 7.41 (d, $J = 8.43$ Hz, 1 H), 7.47–7.66 (m, 4 H), 7.68–7.81 (m, 2 H), 8.01–8.36 (m, 4 H), 12.17 (s, 1 H). ^{13}C NMR (126 MHz, DMSO- d_6) δ ppm 24.82, 25.57, 31.23, 32.72, 32.75, 36.27, 36.97, 48.36, 113.39, 119.55, 120.69, 120.81, 120.84, 122.13, 126.31, 127.24, 127.99, 128.25, 128.88, 129.03, 130.07, 130.92, 132.83, 134.46, 138.05, 139.48, 153.40, 155.47, 162.87, 166.19. HRMS calcd for $\text{C}_{28}\text{H}_{26}\text{N}_4\text{O}_2\text{SNa}$ ($\text{M} + \text{Na}$) $^+$, 505.1669; found, 505.1671.

N-Cyclohexyl-2-((4-oxo-3-phenethyl-4,5-dihydro-3H-pyrimido[5,4-*b*]indol-2-yl)thio)acetamide (**40**). Compound **7d** (25 mg, 0.066 mmol), triethylamine (19.5 μL , 0.13 mmol), and cyclohexylamine (8.2 μL , 0.073 mmol) were dissolved in anhydrous DMF (0.1 mL). HATU (27.2 mg, 0.073 mmol) dissolved in 0.2 mL of DMF was added to the reaction mixture and stirred for 20 min and concentrated in vacuo. The crude material was recrystallized in MeOH to give 18.28 mg in 60% yield. ^1H NMR (400 MHz, DMSO- d_6) δ ppm 0.83–1.42 (m, 5 H), 1.44–2.08 (m, 5 H), 2.87–3.17 (m, 2 H), 3.95–4.24 (m, 2 H), 4.25–4.62 (m, 2 H), 7.33 (br s, 6 H), 7.87–8.48 (m, 3 H), 11.99 (s, 2 H). ^{13}C NMR (126 MHz, DMSO- d_6) δ ppm 24.91, 25.63, 32.83, 34.09, 36.63, 45.66, 48.51, 113.26, 119.40, 120.37, 120.67, 120.84, 127.16, 127.76, 129.10, 137.30, 138.26, 139.36, 151.68, 154.92, 166.19. HRMS calcd for $\text{C}_{26}\text{H}_{28}\text{N}_4\text{O}_2\text{SNa}$ ($\text{M} + \text{Na}$) $^+$, 483.1825; found, 483.1827.

2-((3-Cyclohexyl-4-oxo-4,5-dihydro-3H-pyrimido[5,4-*b*]indol-2-yl)thio)-*N*-cyclopentylacetamide (**41**). Compound **7b** (25 mg, 0.07 mmol), triethylamine (19.5 μL , 0.14 mmol), and cyclopentylamine (8.82 μL , 0.08 mmol) were dissolved in anhydrous DMF (0.5 mL). HATU (29.3 mg, 0.08 mmol) dissolved in 0.2 mL of DMF was added to the reaction mixture and stirred for 20 min and concentrated in vacuo. The crude material was recrystallized in MeOH to give 9.3 mg in 30.3% yield. ^1H NMR (400 MHz, DMSO- d_6) δ ppm 0.97–2.09 (m, 18 H), 2.61–2.83 (m, 4 H), 4.02 (br s, 1 H), 4.17–4.37 (m, 1 H), 7.20 (t, $J = 7.30$ Hz, 1 H), 7.36–7.56 (m, 2 H), 8.00 (d, $J = 8.06$ Hz, 1 H), 8.32 (d, $J = 7.33$ Hz, 1 H), 11.86 (br s, 1 H). ^{13}C NMR (126 MHz, DMSO- d_6) δ ppm 23.97, 25.25, 26.34, 28.78, 32.73, 37.02, 51.21, 61.99, 113.23, 120.31, 120.54, 120.67, 120.79, 127.66, 136.48, 139.24, 151.93, 155.94, 166.85. HRMS calcd for $\text{C}_{23}\text{H}_{28}\text{N}_4\text{O}_2\text{SNa}$ ($\text{M} + \text{Na}$) $^+$, 447.1825; found, 447.1827.

N-Cyclohexyl-2-((5-methyl-4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-*b*]indol-2-yl)thio)acetamide (**42**) and *N*-Cyclohexyl-*N*-methyl-2-((5-methyl-4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-*b*]indol-2-yl)thio)acetamide (**43**). To a solution of compound **1** (50 mg, 0.12 mmol) in DMF (1 mL) in a flame-dried flask was added NaH 60% dispersion in mineral oil (9.24 mg, 0.24 mmol) and stirred at room temperature for 10 min. Methyl iodide (14.3 μL , 0.24 mmol) was then added to the solution and stirred overnight. Reaction mixture was concentrated and purified by preparative thin-layer chromatography (40:60 ethyl acetate:hexane) to give 18.18 mg in 33.9% yield of compound **42** and 5.3 mg in 9.6% yield of compound **43**. ^1H NMR (400 MHz, DMSO- d_6) δ ppm 0.93–1.37 (m, 5 H), 1.40–1.90 (m, 5 H), 3.44–3.58 (m, 1 H), 3.88 (s, 2 H), 4.15 (s, 3 H), 7.31 (t, $J = 7.33$ Hz, 1 H), 7.39–7.53 (m, 2 H), 7.53–7.78 (m, 5 H), 8.05–8.27 (m, 2 H). ^{13}C NMR (126 MHz, DMSO- d_6) δ ppm 24.90, 25.62, 31.47, 32.82, 37.14, 48.41, 111.31, 119.12, 120.09, 120.74, 120.92, 128.03,

129.99, 130.34, 136.35, 137.47, 140.40, 153.27, 155.75, 166.14. HRMS calcd for $\text{C}_{25}\text{H}_{27}\text{N}_4\text{O}_2\text{S}$ ($\text{M} + \text{H}$) $^+$, 447.1849; found, 447.1852. **43**: ^1H NMR (400 MHz, DMSO- d_6) δ ppm 0.76–1.98 (m, 10 H), 2.10 (s, 3 H), 3.97 (s, 2 H), 4.23 (s, 3 H), 7.16 (t, $J = 7.52$ Hz, 1 H), 7.26–7.85 (m, 8 H), 8.07 (d, $J = 7.70$ Hz, 1 H). ^{13}C NMR (126 MHz, DMSO- d_6) δ ppm 24.85, 25.60, 31.43, 32.28, 38.02, 50.24, 62.03, 111.44, 119.63, 120.34, 120.85, 121.08, 128.20, 129.74, 130.25, 135.43, 136.73, 139.34, 155.99, 156.50, 169.99. HRMS calcd for $\text{C}_{26}\text{H}_{29}\text{N}_4\text{O}_2\text{S}$ ($\text{M} + \text{H}$) $^+$, 461.2006; found, 461.2007.

*Methyl 4-(2-((2-(Cyclohexylamino)-2-oxoethyl)thio)-4-oxo-3-phenyl-3H-pyrimido[5,4-*b*]indol-5(4H)-yl)butanoate* (**44**). To a flame-dried flask, bromobutyric acid (50 μL , 0.43 mmol) was dissolved in anhydrous MeOH (3 mL) followed by the addition of TMSCl (272.45 μL , 2.15 mmol). The solution was stirred overnight at room temperature and concentrated in vacuo to remove all trace of MeOH and HCl. In a separate flame-dried flask, compound **1** (25 mg, 0.06 mmol) was dissolved in DMF (1 mL). NaH 60% dispersion (2.31 mg, 0.06 mmol) was added to the solution. After stirring at room temperature for 10 min, methyl iodide (3.6 μL , 0.06 mmol) was added and stirred for an additional 12 h. The reaction mixture was concentrated in vacuo and purified by preparative thin-layer chromatography to give 1.3 mg in 4% yield. IR: 3293 (NH), 1732 (CO ester), 1683 (CO amide) cm^{-1} . ^1H NMR (400 MHz, DMSO- d_6) δ ppm 0.99–1.32 (m, 5 H), 1.52 (d, $J = 12.19$ Hz, 1 H), 1.57–1.80 (m, 4 H), 1.91–2.10 (m, 2 H), 2.29 (t, $J = 7.31$ Hz, 2 H), 3.47 (s, 3 H), 3.47 (br s, 1 H), 3.89 (s, 2 H), 4.62 (t, $J = 6.83$ Hz, 2 H), 7.30 (t, $J = 7.56$ Hz, 1 H), 7.39–7.51 (m, 2 H), 7.52–7.68 (m, 4 H), 7.74 (s, 1 H), 8.11 (d, $J = 7.80$ Hz, 1 H), 8.17 (d, $J = 7.80$ Hz, 1 H). ^{13}C NMR (126 MHz, DMSO- d_6) δ ppm 24.88, 25.59, 26.00, 30.71, 32.79, 37.02, 51.71, 111.34, 118.74, 120.19, 120.80, 121.09, 128.18, 129.93, 130.02, 130.37, 136.29, 137.81, 139.77, 153.45, 155.47, 166.28, 173.19. HRMS calcd for $\text{C}_{29}\text{H}_{32}\text{N}_4\text{O}_4\text{SNa}$ ($\text{M} + \text{Na}$) $^+$, 555.2036; found, 555.2036.

*tert-Butyl 2-((4-Oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-*b*]indol-2-yl)thio)acetate* (**45**). Compound **6a** (100 mg, 0.34 mmol) and KOH (38.2 g, 0.68 mmol) were suspended in 2 mL of dimethylacetamide in a flame-dried flask with stirring. H_2O was added dropwise until KOH was completely dissolved. *t*-Butyl chloroacetate (49 μL , 0.34 mmol) was immediately added to the reaction mixture and stirred at room temperature monitoring with thin-layer chromatography (1:99 MeOH:DCM). Upon completion, reaction mixture was extracted with ethyl acetate (20 mL) and water (40 mL), dried over MgSO_4 , and concentrated in vacuo. EtOH (5 mL) was added to the resulting viscous liquid, and pure product was filtered to give 55 mg in 40% yield. IR: 3192 (NH), 1732 (CO ester), 1674 (CO amide) cm^{-1} . ^1H NMR (400 MHz, DMSO- d_6) δ ppm 1.32–1.48 (m, 9 H), 3.91 (s, 2 H), 7.26 (t, $J = 7.31$ Hz, 1 H), 7.42–7.55 (m, 4 H), 7.56–7.69 (m, 3 H), 7.96 (d, $J = 8.29$ Hz, 1 H), 12.13 (s, 1 H). HRMS calcd for $\text{C}_{22}\text{H}_{21}\text{N}_3\text{O}_3\text{SNa}$ ($\text{M} + \text{Na}$) $^+$, 430.1196; found, 430.1197.

*tert-Butyl 2-((4-Oxo-3-phenyl-5-propyl-4,5-dihydro-3H-pyrimido[5,4-*b*]indol-2-yl)thio)acetate* (**46a**). Compound **45** (50 mg, 0.12 mmol), NaH 60% dispersion in mineral oil (3.3 mg, 0.14 mmol), iodopropane (13.1 μL , 0.14 mmol), and DMF (1 mL) were reacted according to general procedure E and purified by silica gel chromatography (10:90 ethyl acetate:hexane) to give 32.8 mg in 61% yield. ^1H NMR (400 MHz, DMSO- d_6) δ ppm 0.82 (t, $J = 7.31$ Hz, 3 H), 1.40 (s, 9 H), 1.66–1.95 (m, 2 H), 3.91 (s, 2 H), 4.56 (t, $J = 6.58$ Hz, 2 H), 7.30 (br s, 1 H), 7.39–7.68 (m, 6 H), 7.75 (d, $J = 8.29$ Hz, 1 H), 7.99 (d, $J = 7.80$ Hz, 1 H). HRMS calcd for $\text{C}_{25}\text{H}_{27}\text{N}_3\text{O}_3\text{SNa}$ ($\text{M} + \text{Na}$) $^+$, 472.1665; found, 472.1668.

*tert-Butyl 2-((4-Oxo-5-pentyl-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-*b*]indol-2-yl)thio)acetate* (**46b**). Compound **45** (50 mg, 0.12 mmol), NaH 60% dispersion in mineral oil (3.3 mg, 0.14 mmol), iodopentane (17.64 μL , 0.14 mmol), and DMF (1 mL) were reacted according to general procedure E and purified by silica gel chromatography (10:90 ethyl acetate:hexane) to give 33.8 mg in 59% yield. ^1H NMR (400 MHz, DMSO- d_6) δ ppm 0.80 (t, $J = 6.83$ Hz, 3 H), 1.25 (d, $J = 6.83$ Hz, 4 H), 1.32–1.55 (m, 9 H), 1.64–1.83 (m, 2 H), 3.91 (s, 2 H), 4.59 (t, $J = 7.31$ Hz, 2 H), 7.30 (t, $J = 7.56$ Hz, 1 H), 7.46 (dd, $J = 7.56$, 1.71 Hz, 2 H), 7.54 (t, $J = 7.31$ Hz, 1 H), 7.57–7.67 (m, 3 H), 7.72 (d, $J = 8.78$ Hz, 1 H), 7.99 (d, $J = 7.80$ Hz, 1

H). HRMS calcd for $C_{27}H_{31}N_3O_3SNa$ ($M + Na$)⁺, 500.1978; found, 500.1975.

tert-Butyl 2-((5-Dodecyl-4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-b]indol-2-yl)thio)acetate (46c). Compound 45 (50 mg, 0.12 mmol), NaH 60% dispersion in mineral oil (3.3 mg, 0.14 mmol), bromododecane (32.4 μ L, 0.14 mmol), and DMF (1 mL) were reacted according to general procedure E and purified by silica gel chromatography (10:90 ethyl acetate:hexane) to give 37.4 mg in 54% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 0.84 (t, *J* = 6.83 Hz, 3 H), 1.21 (br s, 18 H), 1.40 (s, 9 H), 1.64–1.86 (m, 3 H), 3.91 (s, 2 H), 4.59 (t, *J* = 7.07 Hz, 2 H), 7.29 (t, *J* = 7.80 Hz, 1 H), 7.45 (dd, *J* = 7.31, 1.95 Hz, 2 H), 7.54 (t, *J* = 7.80 Hz, 1 H), 7.58–7.67 (m, 3 H), 7.72 (d, *J* = 8.29 Hz, 1 H), 7.98 (d, *J* = 7.80 Hz, 1 H). HRMS calcd for $C_{34}H_{45}N_3O_3SNa$ ($M + Na$)⁺, 598.3074; found, 598.3070.

tert-Butyl 2-((5-(Cyanomethyl)-4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-b]indol-2-yl)thio)acetate (46d). Compound 45 (318 mg, 0.78 mmol), NaH 60% dispersion (38.16 mg, 0.86 mmol), bromoacetonitrile (65.5 μ L, 0.86 mmol), and DMF (20 mL) were reacted according to general procedure E to give 247.8 mg in 71% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 1.41 (s, 9 H), 3.94 (s, 2 H), 5.87 (s, 2 H), 7.43 (t, *J* = 7.31 Hz, 1 H), 7.47–7.55 (m, 2 H), 7.58–7.72 (m, 4 H), 7.88 (d, *J* = 8.78 Hz, 1 H), 8.03 (d, *J* = 8.29 Hz, 1 H). HRMS calcd for $C_{24}H_{22}N_4O_3SNa$ ($M + Na$)⁺, 469.1305; found, 469.1303.

2-((4-Oxo-3-phenyl-5-propyl-4,5-dihydro-3H-pyrimido[5,4-b]indol-2-yl)thio)acetic Acid (47a). Compound 46a (29.74 mg, 0.07 mmol), acetonitrile (1 mL), and trifluoroacetic acid (1 mL) were reacted according to general procedure F to give 26 mg in quantitative yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 0.81 (t, *J* = 6.58 Hz, 3 H), 1.75 (m, 2 H), 3.96 (s, 2 H), 4.56 (t, *J* = 7.07 Hz, 2 H), 7.29 (t, *J* = 7.31 Hz, 1 H), 7.38–7.88 (m, 7 H), 7.99 (d, *J* = 8.29 Hz, 1 H). HRMS calcd for $C_{21}H_{19}N_3O_3SNa$ ($M + Na$)⁺, 416.1039; found, 416.1036.

2-((4-Oxo-5-pentyl-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-b]indol-2-yl)thio)acetic Acid (47b). Compound 46b (30.98 mg, 0.065 mmol), acetonitrile (1 mL), and trifluoroacetic acid (1 mL) were reacted according to general procedure F to give 27 mg in quantitative yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 0.81 (t, *J* = 6.58 Hz, 3 H), 0.99–1.43 (m, 4 H), 1.62–1.84 (m, 2 H), 3.96 (s, 2 H), 4.59 (t, *J* = 7.07 Hz, 2 H), 7.29 (t, *J* = 7.31 Hz, 1 H), 7.45 (br s, 2 H), 7.51–7.68 (m, 4 H), 7.72 (d, *J* = 8.29 Hz, 1 H), 7.98 (d, *J* = 8.29 Hz, 1 H). HRMS calcd for $C_{23}H_{23}N_3O_3SNa$ ($M + Na$)⁺, 444.1352; found, 444.1351.

2-((5-Dodecyl-4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-b]indol-2-yl)thio)acetic Acid (47c). Compound 46c (34.81 mg, 0.06 mmol), acetonitrile (1 mL), and trifluoroacetic acid (1 mL) were reacted according to general procedure F to give 28.6 mg in 92% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 0.82 (t, *J* = 6.83 Hz, 3 H), 1.19 (d, *J* = 7.80 Hz, 18 H), 1.71 (d, *J* = 6.34 Hz, 2 H), 3.94 (s, 2 H), 4.56 (t, *J* = 7.07 Hz, 2 H), 7.27 (t, *J* = 7.80 Hz, 1 H), 7.36–7.47 (m, 2 H), 7.59 (s, 4 H), 7.70 (d, *J* = 8.29 Hz, 1 H), 7.96 (d, *J* = 7.80 Hz, 1 H), 12.74 (br s, 1 H). HRMS calcd for $C_{30}H_{37}N_3O_3SNa$ ($M + Na$)⁺, 542.2448; found, 542.2444.

2-((5-(Cyanomethyl)-4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-b]indol-2-yl)thio)acetic Acid (47d). Compound 46a (29.74 mg, 0.07 mmol), acetonitrile (1 mL), and trifluoroacetic acid (1 mL) were reacted according to general procedure F to give 26 mg in quantitative yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 3.84–4.15 (m, 2 H), 5.87 (s, 2 H), 7.42 (t, *J* = 7.56 Hz, 1 H), 7.47–7.56 (m, 2 H), 7.57–7.74 (m, 4 H), 7.88 (d, *J* = 8.29 Hz, 1 H), 8.03 (d, *J* = 7.80 Hz, 1 H), 12.89 (br s, 1 H). HRMS calcd for $C_{20}H_{14}N_4O_3SNa$ ($M + Na$)⁺, 413.0679; found, 413.0676.

N-Cyclohexyl-2-((4-oxo-3-phenyl-5-propyl-4,5-dihydro-3H-pyrimido[5,4-b]indol-2-yl)thio)acetamide (48). Compound 47a (29.4 mg, 0.075 mmol), HATU (31.3 mg, 0.082 mmol), triethylamine (20.8 μ L, 0.15 mmol), cyclohexylamine (9.43 μ L, 0.082 mmol), and DMF (1 mL) were reacted according to general procedure D to give 4 mg in 11% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 0.82 (t, *J* = 7.33 Hz, 3 H), 1.00–1.37 (m, 5 H), 1.42–1.99 (m, 7 H), 3.88 (s, 2 H), 4.56 (t, *J* = 6.97 Hz, 2 H), 7.29 (t, *J* = 7.70 Hz, 1 H), 7.41–7.50 (m, 1 H), 7.50–7.68 (m, 4 H), 7.74 (d, *J* = 8.43 Hz, 1 H), 8.10 (d, *J* = 8.07

Hz, 1 H), 8.17 (d, *J* = 8.07 Hz, 1 H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ ppm 11.35, 24.15, 24.89, 25.61, 32.83, 37.12, 45.80, 48.40, 111.49, 118.75, 120.09, 120.61, 120.99, 127.98, 129.96, 129.98, 130.29, 136.38, 137.68, 139.83, 153.35, 155.43, 166.14. HRMS calcd for $C_{27}H_{31}N_4O_2S$ ($M + H$)⁺, 475.2162; found, 475.2164.

N-Cyclohexyl-2-((4-oxo-5-pentyl-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-b]indol-2-yl)thio)acetamide (49). Compound 47b (34 mg, 0.08 mmol), HATU (34 mg, 0.088 mmol), triethylamine (22.5 μ L, 0.16 mmol), cyclohexylamine (10.2 μ L, 0.088 mmol), and DMF (1 mL) were reacted according to general procedure D to give 21 mg in 52% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 0.80 (t, *J* = 6.97 Hz, 3 H), 1.00–1.40 (m, 9 H), 1.43–1.92 (m, 7 H), 3.44–3.58 (m, 1 H), 3.88 (s, 2 H), 4.58 (t, *J* = 7.15 Hz, 2 H), 7.29 (t, *J* = 7.33 Hz, 1 H), 7.40–7.68 (m, 6 H), 7.72 (d, *J* = 8.43 Hz, 1 H), 8.03–8.32 (m, 2 H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ ppm 14.35, 22.29, 24.91, 25.62, 28.70, 30.56, 32.84, 37.11, 44.38, 48.42, 111.45, 118.70, 120.13, 120.64, 121.02, 128.02, 129.99, 130.31, 136.39, 137.70, 139.74, 153.36, 155.44, 166.16. HRMS calcd for $C_{29}H_{34}N_4O_2S$ ($M + H$)⁺, 502.24025; found, 502.24018.

N-Cyclohexyl-2-((5-dodecyl-4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-b]indol-2-yl)thio)acetamide (50). Compound 47c (28.6 mg, 0.055 mmol), HATU (23 mg, 0.061 mmol), triethylamine (15.33 μ L, 0.110 mmol), cyclohexylamine (7 μ L, 0.061 mmol), and DMF (1 mL) were reacted according to general procedure D and purified by silica gel chromatography (20:80 ethyl acetate:hexane) to give 22 mg in 67% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 0.73–0.92 (m, 4 H), 1.20 (d, *J* = 6.97 Hz, 22 H), 1.41–1.89 (m, 7 H), 3.45–3.61 (m, 1 H), 3.88 (s, 2 H), 4.58 (t, *J* = 6.60 Hz, 2 H), 7.28 (t, *J* = 7.52 Hz, 1 H), 7.36–7.80 (m, 7 H), 8.05–8.26 (m, 2 H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ ppm 11.85, 14.50, 22.63, 24.97, 25.70, 26.59, 29.19, 29.44, 30.88, 31.82, 32.90, 37.20, 44.48, 48.42, 111.54, 118.70, 120.22, 120.69, 121.09, 128.06, 130.05, 130.39, 136.46, 137.79, 139.83, 153.41, 155.50, 166.17. HRMS calcd for $C_{36}H_{49}N_4O_2S$ ($M + H$)⁺, 601.3571; found, 601.3570.

2-((5-(Cyanomethyl)-4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-b]indol-2-yl)thio)-N-cyclohexylacetamide (51). Compound 47d (150 mg, 0.39 mmol), HATU (161 mg, 0.42 mmol), triethylamine (59 μ L, 0.77 mmol), cyclohexylamine (49 μ L, 0.42 mmol), and DMF (5 mL) were reacted according to general procedure D to give 157.3 mg in 86.7% yield. IR: 3240 (NH), 1697 (CO) cm^{-1} . ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 0.97–1.33 (m, 5 H), 1.45–1.87 (m, 5 H), 3.45–3.58 (m, 1 H), 3.90 (s, 2 H), 5.87 (s, 2 H), 7.42 (t, *J* = 7.80 Hz, 1 H), 7.47–7.56 (m, 2 H), 7.57–7.74 (m, 4 H), 7.88 (d, *J* = 8.29 Hz, 1 H), 8.17 (dd, *J* = 13.16, 7.80 Hz, 2 H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ ppm 24.92, 25.62, 32.84, 33.04, 37.21, 48.45, 111.45, 116.75, 118.20, 121.01, 121.35, 122.12, 129.02, 129.90, 130.08, 130.56, 135.95, 138.98, 139.65, 155.44, 155.50, 166.02. HRMS calcd for $C_{26}H_{26}N_5O_2S$ ($M + H$)⁺, 472.1802; found, 472.1803.

2-((5-(2-Amino-2-oxoethyl)-4-oxo-3-phenyl-4,5-dihydro-3H-pyrimido[5,4-b]indol-2-yl)thio)-N-cyclohexylacetamide (52). A KOH (6 mg, 0.11 mmol) solution in water (50 μ L) was added to a solution of compound 51 in dimethylacetamide (200 μ L) and stirred at rt overnight. Reaction mixture was acidified with 3 M HCl, extracted with ethyl acetate and water, dried over $MgSO_4$, and concentrated to dryness in vacuo. Further recrystallization in MeOH gives 20 mg in 37% yield. ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 0.94–1.36 (m, 5 H), 1.43–1.89 (m, 5 H), 3.43–3.62 (m, 1 H), 3.89 (s, 2 H), 5.24 (s, 2 H), 7.12–7.22 (m, 1 H), 7.30 (t, *J* = 7.33 Hz, 1 H), 7.42 (m, *J* = 7.30, 2.20 Hz, 2 H), 7.48–7.67 (m, 5 H), 8.10 (d, *J* = 7.70 Hz, 1 H), 8.18 (d, *J* = 7.70 Hz, 1 H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ ppm 24.90, 25.59, 32.82, 37.05, 46.85, 48.48, 111.36, 119.30, 120.36, 120.91, 120.95, 128.13, 129.87, 130.05, 130.41, 136.14, 137.82, 140.64, 153.47, 155.65, 166.26, 169.77. HRMS calcd for $C_{26}H_{27}N_5O_3SNa$ ($M + Na$)⁺, 512.1727; found, 512.1726.

Biological Studies. Animals. Seven–nine-week-old C57BL/6 (wild-type, WT) and *Cd14^{-/-}* (C57BL/6 background) were purchased from the Jackson Laboratories (Bar Harbor, MA). *Tlr4^{-/-}* mice were a gift from Dr. Shizuo Akira (Osaka University, Japan) and backcrossed for 10 generations onto the C57BL/6 background at University of California, San Diego (UCSD). All animal

experiments were approved by the UCSD Institutional Animal Care and Use Committee.

In Vitro Cytokine Induction in Bone Marrow Derived Dendritic Cells (BMDC). BMDC were prepared from C57BL/6 mice as described.³⁶ BMDC (10^5 cells per well) were plated in 96-well plates in 200 μ L of complete RPMI1640 supplemented with 10% fetal calf serum (FCS; Sigma Aldrich), 100 U/mL penicillin, and 100 μ g/mL streptomycin (Invitrogen). The cells were incubated with graded concentrations of the compounds for 18 h at 37 °C, 5% CO₂. After 18 h incubation, the cell culture supernatants were collected. LPS (purified LPS, Invivogen, San Diego, CA) or MPLA (1 μ g/mL synthetic MPLA, Invivogen, San Diego, CA) were used as positive controls. The levels of IL-6 in the culture supernatants were determined by ELISA (BD Biosciences, La Jolla, CA).³⁷ The AUC was calculated from BMDC dose–response curves using Prism 5 (GraphPad, San Diego). Each cytokine induction curve was first converted to a percent activity curve, and then the AUC of the percent activity curve was calculated. The process of converting to a percent activity curve allowed subtracting background and adjusting for plate-to-plate variation. Finally, the AUC values were normalized to the activity of compound 1 within each experiment, set at 100. The cultures stimulated with LPS 10 ng/mL, and 5 μ M compound 1 released an average of 20.6 ng/mL \pm 4.7 SD and 10.5 ng/mL \pm 1.3 SD of IL-6, respectively.

In Vitro Assays Using TLR Reporter Cell Lines. Murine or human TLR4 HEK Blue cells (Invivogen, 2.5×10^4 cells per well of a 96-well plate), or NF κ B/SEAPorter HEK 293 cells (Imgenex, San Diego, CA) for human TLR2, TLR3, TLR5, TLR7, TLR8, or TLR9 (5×10^4 cells per well of 96 well plate) were incubated with graded doses of compound 1. The culture supernatants were harvested after a 20–24 h incubation period. SEAP activity in the supernatants was determined by a colorimetric assay, using either the SEAPorter Assay Kit (Imgenex), with absorbance read at 405 nm, or QuantiBlue (Invivogen), with absorbance read at 630 nm. Stimulation of the human TLR4 cells with 10 ng/mL LPS resulted in OD₆₃₀ of 1.90 relative to 0.88 of cells stimulated with 10 μ M compound 1. The relative reporter activation for the murine TLR4 cells for cells stimulated with 10 ng/mL LPS and 5 μ M compound 1 was 1.70 ± 0.08 and 1.52 ± 0.03 , respectively.

In Vitro Activities in hPBMC. Human PBMC were isolated from buffy coats obtained from the San Diego Blood Bank (San Diego, CA) as described previously.^{37,38} PBMC (1×10^6 /mL) were incubated with various compounds in complete RPMI for 18 h at 37 °C, 5% CO₂, and culture supernatants were collected. The levels of IL-8 in the supernatants were determined by ELISA (BD Biosciences, La Jolla, CA). Human cell cultures were treated with 10 ng/mL LPS as the positive control. Cultures treated with LPS 10 ng/mL and compound 1 10 μ M released averages of 14.5 ± 0.6 ng/mL SD and 7.8 ± 0.8 ng/mL SD of IL-8, respectively.

Type I IFN Assay. L929 cells stably expressing an interferon sensitive response element (ISRE) luciferase reporter construct were kindly provided by Dr. B. Beutler (UT Southwestern, Texas).³⁹ The bioactivity of type I IFN in mBMDC supernatants was measured by luciferase assay using L929-ISRE cells as described previously.³⁹ L929-ISRE cells were plated at 5×10^4 cells per well in Dulbecco's Modified Eagle Medium (Invitrogen) supplemented with 10% FCS, 100 U/mL penicillin, and 100 μ g/mL streptomycin (DMEM-10) in a 96-well white-walled clear-bottom plate. Thus, 50 μ L of mBMDC supernatant was incubated with L929-ISRE cells in 50 μ L of DMEM for 6 h. Mu-IFN Beta Standard (PBL Interferon Source, Piscataway, NJ) was used as a standard. The luciferase activities were measured by Steady-Glo luciferase assay buffer (Promega, Madison, WI).

In addition, the levels of IP-10, a surrogate marker of type I IFN, in the supernatants were determined by ELISA (R&D Systems, Minneapolis, MN). The cultures stimulated with LPS 10 ng/mL, and 5 μ M compound 1 released an average of 234.8 ± 8.6 and 590.0 ± 10.5 pg/mL of IP-10 respectively.

Statistical Analysis. The data are represented as mean \pm standard error of the mean (SEM). Areas-under-curve (AUCs) were calculated using the trapezoid method. Prism 5 (GraphPad Software) statistical

software was used to obtain *p*-values for comparison between groups (*p* < 0.05 was considered significant). For the in vitro studies, two-tailed Student's *t* test was used to compare two groups, and one-way ANOVA Dunnett's test was used to compare multiple groups.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

AUC, area under the curve; HTS, high throughput screen; TLR, Toll-like receptor; IL, interleukin; TNF, tumor necrosis factor; LPS, lipopolysaccharide; MyD88, myeloid differentiation primary response gene 88; MD-2, myeloid differentiation protein-2; MPLA, monophosphoryl lipid A; PBMC, peripheral blood mononuclear cells; EtOH, ethanol; THF, tetrahydrofuran; TMSCl, trimethylsilyl chloride or chlorotrimethylsilane; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DMF, *N,N*-dimethylformamide; HATU, *O*-(7-azabenzotriazol-1-yl)-*N,N,N'*,*N'*-tetramethyluroniumhexafluorophosphate; DCM, dichloromethane; MeOH, methanol; TEA, triethylamine; BMDC, bone marrow derived dendritic cells; BMDM, bone marrow derived macrophages; NF κ B, nuclear factor κ B; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ISRE, interferon response element; IFN, interferon; SEAP, secreted embryonic alkaline phosphatase; ELISA, enzyme-linked immunosorbent assay; IP-10, interferon γ -induced protein 10; PRR, pattern-recognition receptors

REFERENCES

- (1) Dev, A.; Iyer, S.; Razani, B.; Cheng, G. NF- κ B and innate immunity. *Curr. Top. Microbiol. Immunol.* **2011**, *349*, 115–143.
- (2) Li, Q.; Verma, I. M. NF- κ B regulation in the immune system. *Nature Rev. Immunol.* **2002**, *2*, 725–734.
- (3) Fang, H.; Wu, Y.; Huang, X.; Wang, W.; Bing, A.; et al. Toll-like Receptor 4 (TLR4) Is Essential for Hsp70-like Protein 1 (HSP70L1) to Activate Dendritic Cells and Induce Th1 Response. *J. Biol. Chem.* **2011**, *286*, 30393–30400.
- (4) Hemmi, H.; Takeuchi, O.; Kawai, T.; Kaisho, T.; Sato, S.; et al. A Toll-like receptor recognizes bacterial DNA. *Nature* **2000**, *408*, 740–745.
- (5) Prins, R. M.; Craft, N.; Bruhn, K. W.; Khan-Farooqi, H.; Koya, R. C.; et al. The TLR-7 Agonist, Imiquimod, Enhances Dendritic Cell Survival and Promotes Tumor Antigen-Specific T Cell Priming: Relation to Central Nervous System Antitumor Immunity. *J. Immunol.* **2005**, *176*, 157–164.

- (6) Lee, J.; Chuang, T.-H.; Redecke, V.; She, L.; Pitha, P. M.; et al. Molecular basis for the immunostimulatory activity of guanine nucleoside analogs: activation of toll-like receptor 7. *Proc. Natl. Acad. Sci. U. S. A.* **2003**, *100*, 6646–6651.
- (7) Lee, J.; Wu, C. C. N.; Lee, K. J.; Chuang, T.-H.; Katakura, K.; et al. Activation of anti-hepatitis C virus responses via Toll-like receptor 7. *Proc. Natl. Acad. Sci. U. S. A.* **2006**, *103*, 1828–1833.
- (8) Smits, E. L. J. M.; Cools, N.; Lion, E.; Camp, K.; Ponsaerts, P.; et al. The Toll-like receptor 7/8 agonist resiquimod greatly increases the immunostimulatory capacity of human acute myeloid leukemia cells. *Cancer Immunol. Immunother.* **2010**, *59*, 35–46.
- (9) Coffman, R. L.; Sher, A.; Seder, R. A. Vaccine Adjuvants: Putting Innate Immunity to Work. *Immunity* **2010**, *33*, 492–503.
- (10) Mata-Haro, V.; Cekic, C.; Martin, M.; Chilton, P. M.; Casella, C. R.; et al. The Vaccine Adjuvant Monophosphoryl Lipid A as a TRIF-Biased Agonist of TLR4. *Science* **2007**, *316*, 1628–1632.
- (11) Pu, M.; Hayashi, T.; Cottam, H.; Mulvaney, J.; Arkin, M.; et al. Analysis of high-throughput screening assays using cluster enrichment. *Stat. Med.* **2012**, *31*, 4175–4189.
- (12) Coats, S. R.; Pham, T.-T. T.; Bainbridge, B. W.; Reife, R. A.; Darveau, R. P. MD-2 Mediates the Ability of Tetra-Acylated and Penta-Acylated Lipopolysaccharides to Antagonize *Escherichia coli* Lipopolysaccharide at the TLR4 Signaling Complex. *J. Immunol.* **2005**, *175*, 4490–4498.
- (13) Jiang, Z.; Georgel, P.; Du, X.; Shamel, L.; Sovath, S.; et al. CD14 is required for MyD88-independent LPS signaling. *Nature Immunol.* **2005**, *6*, 565–570.
- (14) Wright, S. D.; Ramos, R. A.; Tobias, P. S.; Ulevitch, R. J.; Mathison, J. C. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science* **1990**, *249*, 1431–1433.
- (15) Nagai, Y.; Akashi, S.; Nagafuku, M.; Ogata, M.; Iwakura, Y.; et al. Essential role of MD-2 in LPS responsiveness and TLR4 distribution. *Nature Immunol.* **2002**, *3*, 667–672.
- (16) Devani, M. B.; Shishoo, C. J.; Pathak, U. S.; Parikh, S. H.; Shah, G. F.; et al. Synthesis of 3-substituted thieno[2,3-*d*]pyrimidin-4(3*H*)-one-2-mercaptoacetic acids and their ethyl esters for pharmacological screening. *J. Pharm. Sci.* **1976**, *65*, 660–664.
- (17) Thurkauf, A.; Hutchison, A. Preparation of aryl heterocycl pyrimidines as GABA brain receptor ligands. (Neurogen Corp.) Cont. of U.S. Ser. No. 865,129, abandoned, 1994; 17 pp.
- (18) Monge, A.; Palop, J. A.; Recalde, I.; Martinez-Crespo, F.; Fernandez-Alvarez, E. Synthesis of 5*H*-pyrimido[5,4-*b*]indole derivatives and related compounds. *An. Quim., Ser. C: Quim. Org. Bioquim.* **1985**, *81*, 267–270.
- (19) Kawai, T.; Takeuchi, O.; Fujita, T.; Inoue, J.-I.; Muhlrad, P. F.; et al. Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. *J. Immunol.* **2001**, *167*, 5887–5894.
- (20) Yamamoto, M.; Sato, S.; Mori, K.; Hoshino, K.; Takeuchi, O.; et al. A novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN- I^2 promoter in the Toll-like receptor signaling. *J. Immunol.* **2002**, *169*, 6668–6672.
- (21) Ohto, U.; Fukase, K.; Miyake, K.; Shimizu, T. Structural basis of species-specific endotoxin sensing by innate immune receptor TLR4/MD-2. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109*, S7421–S7426.
- (22) Meng, J.; Drolet, J. R.; Monks, B. G.; Golenbock, D. T. MD-2 Residues Tyrosine 42, Arginine 69, Aspartic Acid 122, and Leucine 125 Provide Species Specificity for Lipid IVA. *J. Biol. Chem.* **2010**, *285*, 27935–27943.
- (23) Vasl, J.; Oblak, A.; Gioannini, T. L.; Weiss, J. P.; Jerala, R. Novel Roles of Lysines 122, 125, and 58 in Functional Differences between Human and Murine MD-2. *J. Immunol.* **2009**, *183*, 5138–5145.
- (24) Muroi, M.; Tanamoto, K.-i. Structural Regions of MD-2 That Determine the Agonist-Antagonist Activity of Lipid IVA. *J. Biol. Chem.* **2006**, *281*, 5484–5491.
- (25) Saitoh, S.-i.; Akashi, S.; Yamada, T.; Tanimura, N.; Kobayashi, M.; et al. Lipid A antagonist, lipid IVA, is distinct from lipid A in interaction with Toll-like receptor 4 (TLR4)-MD-2 and ligand-induced TLR4 oligomerization. *Int. Immunol.* **2004**, *16*, 961–969.
- (26) Walsh, C.; Gangloff, M.; Monie, T.; Smyth, T.; Wei, B.; et al. Elucidation of the MD-2/TLR4 interface required for signaling by lipid IVA. *J. Immunol.* **2008**, *181*, 1245–1254.
- (27) Shimazu, R.; Akashi, S.; Ogata, H.; Nagai, Y.; Fukudome, K.; et al. MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *J. Exp. Med.* **1999**, *189*, 1777–1782.
- (28) Medzhitov, R.; Preston-Hurlburt, P.; Janeway, C. A., Jr. A human homolog of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature* **1997**, *388*, 394–396.
- (29) Park, B. S.; Song, D. H.; Kim, H. M.; Choi, B.-S.; Lee, H.; et al. The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. *Nature* **2009**, *458*, 1191–1195.
- (30) Macindoe, G.; Mavridis, L.; Venkatraman, V.; Devignes, M.-D.; Ritchie, D. W. HexServer: an FFT-based protein docking server powered by graphics processors. *Nucleic Acids Res.* **2010**, *38*, W445–W449.
- (31) Dewar, M. J. S. AMPAC: a general program for chemical calculations using procedures developed by the Dewar group. *Mod. Tech. Comput. Chem.: MOTECC-91* **1991**, 455–467.
- (32) Hutchinson, M. R.; Loram, L. C.; Zhang, Y.; Shridhar, M.; Rezvani, N.; et al. Evidence that tricyclic small molecules may possess toll-like receptor and myeloid differentiation protein 2 activity. *Neuroscience* **2010**, *168*, 551–563.
- (33) Zimmer, S. M.; Liu, J.; Clayton, J. L.; Stephens, D. S.; Snyder, J. P. Paclitaxel Binding to Human and Murine MD-2. *J. Biol. Chem.* **2008**, *283*, 27916–27926.
- (34) Hutchinson, M. R.; Zhang, Y.; Shridhar, M.; Evans, J. H.; Buchanan, M. M.; et al. Evidence that opioids may have toll-like receptor 4 and MD-2 effects. *Brain, Behav., Immun.* **2009**, *24*, 83–95.
- (35) Liu, L.; Ghosh, N.; Slivka, P. F.; Fiorini, Z.; Hutchinson, M. R.; et al. An MD2 Hot-Spot-Mimicking Peptide that Suppresses TLR4-Mediated Inflammatory Response in Vitro and in Vivo. *ChemBioChem* **2011**, *12*, 1827–1831.
- (36) Wu, C. C.; Hayashi, T.; Takabayashi, K.; Sabet, M.; Smee, D. F.; et al. Immunotherapeutic activity of a conjugate of a Toll-like receptor 7 ligand. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104*, 3990–3995.
- (37) Chan, M.; Hayashi, T.; Kuy, C. S.; Gray, C. S.; Wu, C. C.; et al. Synthesis and immunological characterization of toll-like receptor 7 agonistic conjugates. *Bioconjugate Chem* **2009**, *20*, 1194–1200.
- (38) Hayashi, T.; Rao, S. P.; Takabayashi, K.; Van Uden, J. H.; Kornbluth, R. S.; et al. Enhancement of innate immunity against *Mycobacterium avium* infection by immunostimulatory DNA is mediated by indoleamine 2,3-dioxygenase. *Infect. Immun.* **2001**, *69*, 6156–6164.
- (39) Crozat, K.; Georgel, P.; Rutschmann, S.; Mann, N.; Du, X.; et al. Analysis of the MCMV resistome by ENU mutagenesis. *Mamm. Genome* **2006**, *17*, 398–406.