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# A chemical switch for transforming a purine agonist for Toll-like receptor 7 to a clinically relevant antagonist

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Supporting Information Placeholder

**ABSTRACT:** Toll-like receptor 7 (TLR7) is an established therapeutic target in myriad autoimmune disorders, but no TLR7 antagonist is available for clinical use to date. Herein we report a purine scaffold TLR7 antagonist, first-of-its-kind to our knowledge, which was developed by rationally dissecting the structural requirements for TLR7-targeted activity for a purine scaffold. Specifically, we identified a singular chemical switch at C-2 that could make a potent purine scaffold TLR7 agonist to loose agonism and acquire antagonist activity, which could further be potentiated by the introduction of an additional basic center at C-6. We ended up developing a clinically relevant TLR7 antagonist with favorable pharmacokinetics and 70.8% oral bioavailability in mice. Moreover, the TLR7 antagonists depicted excellent selectivity against TLR8. To further validate the *in vivo* applicability of this novel TLR7

antagonist we demonstrated its excellent efficacy in preventing TLR7-induced pathology in a preclinical murine model of psoriasis.

#### **INTRODUCTION**

Toll-like receptors (TLRs) are the critical first line of immune defense that are mostly expressed on antigen presenting cells, e.g. dendritic cells and recognize molecular signature patterns associated with pathogens.<sup>1-3</sup> The members of the TLR family of receptors that can recognize nucleic acid molecules, both of self and non-self origins, viz. TLR7, TLR8 and TLR9, are located inside the acidic endosomal compartments of the immune cells.<sup>2,4-6</sup> Among them, TLR7, which recognizes singlestranded RNA, is expressed in humans selectively in plasmacytoid dendritic cells (pDCs) and B lymphocytes, and when activated in pDCs drives type I interferon production from pDCs.<sup>4</sup> pDC-derived type I IFNs are critical innate immune events in a number of autoimmune diseases, viz. systemic lupus erythematosus, psoriasis, Sjögren's syndrome, systemic sclerosis etc.<sup>7-11</sup> Thus the endosomal TLRs in pDCs, e.g. TLR7, are important therapeutic targets for these clinical contexts.<sup>12-14</sup> Application of the TLR7 agonist imiquimod on the skin leads to cutaneous autoimmunity in mice, which mimics the human skin autoimmunity in psoriasis, and consequently is an established preclinical model for psoriasis.<sup>15</sup> But a suitable TLR7 antagonist is yet to be available for clinical use.<sup>16</sup>

Both receptor agonists and antagonists are important therapeutic molecules in different clinical contexts and in many cases agonists and antagonists share overlapping binding region in their target molecules. Thus, agonistic chemical scaffolds are often used as a template for designing antagonists with shared region-specificities for the receptors.<sup>17-19</sup> For example, development of  $\beta$ -adrenergic receptor antagonists from the endogenous agonist adrenalin,<sup>20</sup> histamine receptor 2 antagonists from histamine<sup>21</sup> and opioid receptor antagonists from opioids.<sup>22</sup> TLR7 mediated immune activation has implication in autoimmune diseases like systemic lupus erythematosus (SLE), psoriasis. In SLE autoreactivity against RNA and RNA binding proteins are very prevalent and these immune complexes

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activate TLR7 in both pDCs and B cells, thereby perpetuating the inflammatory cascade.<sup>10</sup> In psoriasis self-RNA molecules released by dying keratinocytes form complexes with cationic antimicrobial peptides. These complexes have been shown to activate both plasmacytoid and conventional dendritic cells thus fuelling the cutaneous inflammation.<sup>8,10</sup> Hence, blocking TLR7 activation with suitable antagonist is sought for, which may intercept one of the key pathogenic nodes in these diseases. Purine scaffold is extensively studied for its potent TLR7 agonist activity.<sup>23-26</sup> Interestingly, guanosine, an endogenous purine class molecule, is also an agonist for TLR7.<sup>27</sup> The mechanism of TLR7 activation by guanosine has been shown through crystallographic study which is of great value in design and development of TLR7 ligands.<sup>28</sup> Previous efforts directed towards the purine scaffold have led to potent agonists' thorough understanding about the region-specificities of critical substituents. From crystallographic evidence and structural development of TLR7 agonist, it is understandable that -NH<sub>2</sub> group at C-6 position is crucial for scaffold recognition by TLR7.<sup>28</sup> Thus, logically we did not change -NH<sub>2</sub> group at C-6 position in our initial SAR development. Development of 2-substituted-8hydroxyadenine as IFNα inducer via TLR7 pathway revealed the profound effect of four carbon long alkoxy chain at C-2 position on the agonistic activity.<sup>25,29</sup> Among various substituents, O-alkyl groups at C-2 position have been found to have superior ability to modulate the agonistic activity in combination with N-benzyl or N-alkylamine at N-9 position.<sup>30-32</sup> Previous study on pteridinone core based TLR7 agonist showed that C-2 position butoxy linker bind to the conserved hydrophobic pocket of TLR7.<sup>33</sup> The N-9 substitutions modulate both activity and pharmacokinetic profile of purine,<sup>23,24</sup> perhaps because the weak base gets protonated and trapped in the acidic endosomal compartment,<sup>34,35</sup> harbouring TLR7. Throughout the literature the C-2 position was substituted and subsequently modification was done in N-9 position but not the other way round. Keeping this in mind, we intended to evaluate the fate of the agonist where C-2 substituent is absent keeping the known relevant N-9 substituents and -NH<sub>2</sub> group at C-6 position. Although extensive focus has been devoted towards development of TLR7 agonist, there is no reported TLR7 antagonist of purine scaffold, to our knowledge. Imiquimod belongs to

imidazoquinoline class of molecules, which also includes other well-known TLR7 agonists like gardiquimod and resiquimod.<sup>36</sup> TLR7 agonist gardiquimod was successfully transformed into TLR7 antagonist by converting to its 3H regioisomer.<sup>18</sup>

We have initiated our study from a representative purine agonist **7** containing essential TLR7 agonistic features at C-2, C-6, C-8 and N-9. We've explored the minimal structural features that impart antagonism in compound **7**, for mapping the synthetic path to a novel purine scaffold antagonist. We have depicted that a small structural modification in the ligand can lead to major changes in their functional activity. For assessing the TLR7-targeted activity (both agonism and antagonism) of the molecules, we used engineered HEK293 cells that expressed exogenous human TLR7 and reported TLR7-induced NF-kB activation in a secreted alkaline phosphatase-driven colorimetric assay. We have used a non-selective TLR7 antagonist hydroxychloroquine as a positive control. Through SAR development, we identified clinically relevant TLR7 antagonist **23** that has favourable pharmacokinetic properties with 70.8% oral bioavailability in mice. The nature of antagonism was determined by subjecting these antagonists to agonism assay. The TLR7 antagonists reported herewith showed excellent selectivity against TLR8. *In vivo* efficacy of **23** was demonstrated by its ability to prevent TLR7-induced pathology in a preclinical murine model of psoriasis.

#### CHEMISTRY

Chemical synthesis of TLR7 agonist 7 and 9. C-6 position of compound  $1^{30}$  was substituted with *n*-butoxy linkage via sodium *t*-butoxide treatment to obtain compound 2. The methoxy group at C-8 was introduced by bromination of 2 using *N*-bromosuccinimide followed by refluxing with a 5 M solution of sodium methoxide in methanol to provide compound 3 (Scheme 1). Deprotection of pyran ring from compound 3 using 10% TFA followed by 3-chloropropyl substitution in compound 4 using 1-bromo-3-chloropropane yielded compound 5. Chlorine group in compound 5 was then substituted with

either piperidine or ethylpiperazine to give compound **6** and **8**. Finally, C-8 position methoxy group was deprotected using 4 M HCl in 1,4-dioxane to get compound **7** and **9**.

# Scheme 1<sup>*a*</sup>



<sup>*a*</sup>Reagents and conditions: (i) Sodium *t*-butoxide (4.0 equiv.), *n*-BuOH, 100 °C, 12 h, 77%; (ii) a. *N*-bromosuccinimide (1.05 equiv.), CHCl<sub>3</sub>, 3 h, rt, b. sodium methoxide (2.7 equiv.), CH<sub>3</sub>OH, reflux, 4 h, 80%; (iii) 10% TFA in methanol, rt, 72 h, 73%; (iv) 1-bromo-3-chloropropane (2.0 equiv.), potassium carbonate (2.6 equiv.), rt, 16 h, 67%; (v) piperidine for compound **11** or ethylpiperazine for compound **12** (3.0 equiv.), potassium carbonate (1.5 equiv.), rt, 6 h, 67% - 73%; (vi) 4 M HCl in 1,4-dioxane, 3 h, 87% - 91%.

Chemical synthesis of weak TLR7 antagonist 14. In the present scheme 2, we successfully standardized a protocol for the synthesis of compound 14 by avoiding protection-deprotection chemistry at N-9 position in purine scaffold. Bromo group of 8-bromo adenine 10<sup>37</sup> was substituted with methoxy group by refluxing with a 5 M solution of sodium methoxide in methanol to obtain compound 11, which on treatment with 1-bromo-3-chloropropane provided compound 12. Chlorine group was substituted by ethylpiperazine to yield compound 13 followed by deprotection of methoxy group gave compound 14.

# Scheme 2<sup>*a*</sup>



<sup>*a*</sup>Reagents and conditions: (i) 5 M solution of sodium methoxide in methanol (10 equiv.), reflux, 12 h, 72%; (ii) 1-bromo-3-chloropropane (2.0 equiv.), potassium carbonate (2.6 equiv.), rt, 16 h, 78%; (iii) ethylpiperazine (3.0 equiv.), potassium carbonate (1.5 equiv.), rt, 6 h, 63%; (iv) 4 M HCl in 1,4-dioxane 3 h, 86%.

**Chemical synthesis of potent TLR7 antagonist.** Substitution of 3-carbon linker with ethylpiperazine group at N-9 position of 6-chloropurine (**15**) resulted in the intermediate compound **16** (Scheme 3). In the formation of compound **16** there is a mechanistic possibility of getting two structural isomers namely N-9 isomer and N-7 isomer. Here we obtained exclusively N-9 isomer and structure was confirmed by the X-ray crystallographic structure of hydrochloride salt of intermediate compound **16** (SI, Fig S6). Thereafter, the chloride group at C-6 position was replaced by free –NH<sub>2</sub> group by treating with ammonia in ethanol at 60 °C to derive compound **17**. Reduction of C-6 chloro group using palladium-carbon in **16** gave compound **18**. The chloro group at C-6 of intermediate **16** was substituted with different organic amine such as dimethylamine, piperidine, morpholine, piperazine, ethylpiperazine, and cyclopentylpiperazine yielded compound **19**, **20**, **21**, **22**, **23** and **24** respectively. The N-9 position of **25** was treated with 1-bromo-3-chloropropane to get chloro intermediate **26**, which was subsequently reacted with various azacyclic bases such as piperazine, morpholine, piperidine and pyrroline to yield compound **27**, **28**, **29** and **30** respectively. Compound **31** with propyl substitution at N-9 position was made from **25** using the same protocol used for the preparation of **26**. Structural

conformation of compound **23** was made by the analysis of NOESY, HMBC spectra, the detail is in supplementary information.

# Scheme 3<sup>*a*</sup>



<sup>*a*</sup>Reagents and conditions: (i) 1-(3-chloropropyl)-4-ethylpiperazine (2.0 equiv.), potassium carbonate (2.0 equiv.), DMF, 60 °C, 12 h, 84%; (ii) ammonia in ethanol (7.0 equiv.), 60 °C, 12 h, 67%; (iii) H<sub>2</sub>, Pd/C, THF, rt, 2 h, 91%; (iv) respective amine, potassium carbonate (2.0 equiv.), CH<sub>3</sub>CN, reflux, 12 h, 73% - 95%; (v) 1-bromo-3-chloropropane for (**26**) and 3-bromopropane for (**31**) potassium carbonate (2.0 equiv.), DMF, rt, 12 h, 71% - 78%; (vi) respective amine, potassium carbonate (2.0 equiv.), DMF, rt, 12 h, 71% - 78%; (vi) respective amine, potassium carbonate (2.0 equiv.), DMF, 60 °C, 12 h, 78% - 93%.

# **RESULTS AND DISCUSSION**

The importance of butoxy at C-2,  $-NH_2$  at C-6 and hydroxyl at C-8 in TLR7-agonism by purine chemotypes is established.<sup>38,39</sup> We selected compound **7** as a starting point as it contains all the essential

features of a TLR7 agonist. The synthetic strategy (Scheme 1) for compound **7** allowed us to readily introduce an additional basic center at N-9. Replacing piperidine with ethylpiperazine, led to the enhancement in agonism in **9** (Scheme 1), which had an EC<sub>50</sub> of 0.9  $\mu$ M as opposed to 2.6  $\mu$ M for **7** (Fig 1a, 1b. Table 1). Based on the previous SAR studies it has been established that substituents such as butoxy at C-2 position are a key feature for TLR7 agonism.<sup>23,25</sup> As discussed before, we hypothesized that C-2 butoxy may be targeted to transform the potent TLR7 agonist **9** into a TLR7 antagonist. Indeed, removal of C-2 butoxy in **14** (Scheme 2), led to the disappearance of TLR7 agonism and acquisition of considerable antagonism (Fig 1c, 1d. Table 1). Thus, we were able to identify a 'chemical switch' at C-2 that could make a potent purine scaffold TLR7 agonist to loose agonistic properties and acquire antagonistic properties towards TLR7.



Figure 1. Discovery of TLR7 antagonist from known TLR7 agonist. (a,c,e) Conversion of TLR7 agonist 7 (EC<sub>50</sub> = 2.6  $\mu$ M) to a more potent TLR7 agonist 9 (EC<sub>50</sub> = 0.9  $\mu$ M) by the addition of ethylpiperazine group (a) compound 9 to weak TLR7 antagonist 14 (IC<sub>50</sub> = 43.1  $\mu$ M) by the elimination of butoxyl group (c) 3 to potent TLR7 antagonist 23 (IC<sub>50</sub> = 4.7  $\mu$ M) by systematic SAR study (e). (b,d,f) HEK-Blue<sup>TM</sup> TLR7 reporter cells were incubated overnight in the presence of indicated concentrations of given compounds and on the following day the culture supernatants were subjected to a colorimetric assay to measure the degree of TLR activation/inhibition. Data is represented as Mean ± SEM.

This novel purine scaffold molecule 14, despite showing significant antagonism to human TLR7, had an IC<sub>50</sub> (43.1 µM) too high for a therapeutically relevant antagonist. So, we explored if omission of other essential agonist-specific molecular features from 14 can help to develop more potent antagonists with translational potential. We found that removal of the hydroxyl group at C-8 led to rather a loss of all activity against TLR7 in compound 17 at 50  $\mu$ M. The importance of the  $-NH_2$  group was evident from the crystal structure of guanosine as well as the chemical ligands from imidazoquinoline class too.<sup>27</sup> Also, the SAR study at C-6 position of purines has depicted the significance of -NH<sub>2</sub> group.<sup>40</sup> Accordingly, we focused on the modification at C-6 position. Neither complete deletion of -NH<sub>2</sub> group from C-6 in compound 18 nor converting the -NH<sub>2</sub> at C-6 to dimethylamino in compound 19 brought antagonistic activity towards TLR7 (Table 1) at 50 µM. Next, we extended the dimethylamino group of compound **19** with different azacylic moieties like piperidine, morpholine and piperazine. Interestingly, piperidine substitution at C-6 position in compound 20 regained the TLR7 antagonistic effect and the compound shows moderate TLR7 antagonism with an  $IC_{50}$  value of 23.9  $\mu$ M. Replacement of piperidine with morpholine in compound 21 showed comparable TLR7 antagonism with an  $IC_{50}$  of 25 µM. Since morpholine cannot be further extended at 4' position we introduced piperazine in compound 22 with an aim for further derivatization, but the TLR7 antagonistic potency decreased to 39.3  $\mu$ M. However, ethyl substitution on piperazine NH group in compound 23 regained the TLR7 antagonistic effect in single digit micromolar level with an IC<sub>50</sub> value of 4.7  $\mu$ M. Of note here, an ethylpiperazine group at N-9 led to enhancement of TLR7 agonism, while introducing the same group at C-6 potentiated TLR7 antagonism. Further logical extension of ethyl group in compound 23 with more hydrophobic cyclopentane group in compound 24 led to slight decrease in the TLR7 antagonist activity (IC<sub>50</sub> value of 6.2 µM, Table 1). Figure 1 is a snapshot of our SAR development depicting minimal structural requirements and a singular chemical switch that could transform a TLR7 agonist into a TLR7

antagonist in the same purine scaffold, which is represented in a TLR7 reporter assay done in parallel using an established TLR7 agonist (CL264), the purine agonists **7** and **9** as well as the TLR7 antagonist **23** which has been shown to inhibit CL264-induced TLR7 activation (Fig 1g).

# Table 1. SAR depicting the conversion of agonist to antagonist



Comp					TLR7	TLR7
No	$\mathbb{R}^1$	$R^2$	$\mathbb{R}^3$	$\mathbf{R}^4$	Agonism	Antagonism
NO.					(µM) EC <sub>50</sub>	(µM) IC <sub>50</sub>
7	-OBu	$-NH_2$	-OH	- <u></u> ¥N	$2.6 \pm 0.05$	а
9	-OBu	$-NH_2$	-OH	-N_N_	$0.9 \pm 0.01$	а
14	Н	$-NH_2$	-OH	-N_N_	b	43.1±4.1
17	Н	$-NH_2$	Η		b	а
18	Н	Н	Н	-N_N_	b	а
19	Н	NMe <sub>2</sub>	Η	-N_N_	b	а
20	Н	-N	Н	-N_N_	b	23.9±3.1
21	Н	-N_O	Н	-N_N_	b	25.0±3.5
22	Н		Н		b	39.3±3.7
23	Н	-N_N-	Н	-N_N-	b	4.7±0.4
24	Н	-N_N-	Н	-N_N_	b	6.2±1.5
27	Н	-N_N_	Н		b	37% <sup>c</sup>
28	Н	-N_N_	Η	-N_O	b	40.0±4.2
29	Н	-N_N-	Η	—N	b	$15.8 \pm 1.8$
30	Н	-N_N-	Н	-N	b	48.3±4.9
31	Н	-N_N-	Н	Н	b	а
Hydrox	Hydroxychloroquine				NT	8.6±1.2

<sup>a</sup>No significant inhibition at 50  $\mu$ M. <sup>b</sup>No significant activation at 50  $\mu$ M. <sup>c</sup> % Inhibition at 50  $\mu$ M. NTnot tested. Hydroxychloroquine is a positive control

Initially, in compound **9** it was observed that the introduction of weak base at N-9 can modulate TLR7 activity. Thus in our next development, we further explored the role of weak base at N-9 position

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with different groups such as morpholine, pyrrolidine and piperidine. Replacing ethylpiperazine group in compound **23** with piperazine in **27** led to drastic fall in TLR7 antagonist activity (37% inhibition at 50  $\mu$ M). The result suggests that hydrophobic ethyl group is essential for TLR7 antagonistic activity. The result correlates well with our first set of SAR results where unsubstituted piperazine at C-6 in compound **22** showed 8 fold less potency than ethyl substituted piperazine group in **23**. Further substitution with morpholine, piperidine and pyrrolidine yielded less potent TLR7 antagonist compound **28**, **29** and **30** with IC<sub>50</sub> 40.0, 15.8, 48.27  $\mu$ M respectively. Expectedly, elimination of weak base at N-9 in compound **31** led to complete loss of TLR7 antagonism at 50  $\mu$ M (Table 1). To ensure the validity of TLR7 antagonism assay, the ability of different doses of hydroxychloroquine to inhibit TLR7 activation was studied as a positive control. Comparison between compound **17** with **23** suggests that introduction of ethylpiperazine group with an additional basic center and an extended hydrophobic attachment at C-6 had profound potentiating effect on TLR7 antagonism in purine scaffold, whereas additional basic center at N-9 can modulate the TLR7-targeted activity (either agonism as in **7** and **9**, or antagonism as in **23** and **29**).

None of the TLR7 antagonists showed any TLR7 agonism upto 50  $\mu$ M dose (SI, Fig S2) which ruled out the possibility of partial agonism. As TLR7 and TLR8 show high degree of structural similarity,<sup>19</sup> next we monitored the efficacy of our TLR7 antagonists in TLR8 antagonistic assay and we found that none of the TLR7 antagonists showed significant TLR8 inhibition (SI, Fig S3) up to 50  $\mu$ M. The result strongly suggests that the TLR7 antagonists reported in the manuscript are selective for TLR7 when compared with TLR8 antagonism.<sup>41</sup> TLR7 antagonistic effect of compound **23** was further assessed by evaluating the ability of our compound to inhibit the production of TLR7 triggered proinflammatory cytokines such as IL-6 and TNF $\alpha$ .<sup>41,42</sup> Compound **23** showed 42.9 % inhibition (Fig 2a) of IL-6 gene transcription at IC<sub>50</sub> dose (5  $\mu$ M) in HEK-blue TLR7 cell line. Inhibition of proinflammatory cytokines was measured by real time PCR using selective TLR7 agonist CL264 as

positive control. For further validation employing ex vivo human blood-derived model, we observed the inhibition of TLR7 driven TNF $\alpha$  induction in primary human peripheral blood mononuclear cells (hPBMCs) by ELISA (Fig 2b). These results led us to conclude that our compound of interest is a potent TLR7 antagonist capable of inhibiting TLR7 mediated immune responses as depicted by the inhibition of IL-6 and TNF $\alpha$  production in response to TLR7 activation. Thus, we have successfully identified hitherto unknown site-specific substitutions that can switch a well-established purine scaffold TLR7 agonist into a TLR7 antagonist, as demonstrated through the TLR7 reporter assay.

TLR7 and TLR8 share almost similar ligand binding domain. Both the TLRs have one small molecule ligand binding site and another ssRNA binding site.<sup>27</sup> Zhang et al.<sup>19</sup> reported a third binding site for the inhibition of TLR8 in the rest state. The binding model was extrapolated for finding new antagonist binding zone in TLR7. Karroum et al. validated this new antagonist binding site in TLR7 through systematic computational analysis of Imidazo[1,2-a]pyrazines, Imidazo[1,5-a]quinoxalines, and Pyrazolo[1,5-a]quinoxalines class TLR7 antagonist.<sup>41</sup> Due to unavailability of human TLR7 crystal structure we have built homology model structure of TLR7 (SI, Fig S7). Molecular docking study and validation was performed using Discovery Studio suite 4.1 (SI, Fig S8a), which shows that our TLR7 antagonists also bind in the same region attaining similar pattern of interactions along with newer interactions (Fig 2c, 2d). The molecular docking indicates that the purine ring of the lead molecule 23 is sandwiched between two hydrophobic residues Phe329 and Tyr1374 forming  $\pi$ - $\pi$  stacking interactions (Fig 2c). Hydrogen bonding between hydroxyl group of Ser452 with nitrogen of C-6 position of ethylpiperazine was observed in compound 23 as one of the key important interaction signifying the importance of terminal nitrogen in piperazine group as observed in the structure-activity relationships between compounds 19, 20 and 21 with dimethylamino, piperidine and morpholine group respectively. N-9 Position of the alkyl linker with pendent hydrophobic amine was stabilised by the hydrophobic interaction of Leu331 and Phe329. The significance of ethyl group can be observed by comparing the

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inhibitory potency of compound **23** and **27**. Compound **23** showed IC<sub>50</sub> value of 4.7  $\mu$ M whereas compound **27** without the ethyl group showed 37% inhibition at 50  $\mu$ M. Similar binding interactions were observed for other potent TLR7 antagonist compound **24** (Fig 2d). From the molecular docking analysis of our other TLR7 antagonists we can conclude that along with hydrogen bonding with Ser452, hydrophobic interactions play a vital role in determining TLR7 antagonism (SI, Fig S8).

b L-6 mRNA expression а 15· 200relative to 18S  $TNF\alpha(pg/ml)$ 100-Gardiquimod(5µg/ml) CL264(5µg/ml) compound 23(5µM) compound 23(5µM) С **SER452** d SER452 TYR1374\* **TYR13 PHE327** PHE3 THR384 PHE32 **PHE329** LEN3

Figure 2. Inhibition of proinflammatory cytokines and docking study of compound 23 with homology modelled hTLR7 (a) HEK-Blue<sup>TM</sup> TLR7 reporter cells were treated with 5  $\mu$ M compound 23, one hour prior to stimulation with CL264, 6 hours after which, the cells were harvested and subjected to gene expression studies to monitor the mRNA levels of the IL6 gene relative to that of 18S

(housekeeping gene) in the presence and absence of compound treatment. Data is represented as Mean  $\pm$  SEM, \*p<0.05, n=6. Unpaired Student's t-test was done to measure statistical significance. (b) Primary human PBMCs were treated with 5  $\mu$ M compound 23, one hour prior to stimulation with Gardiquimod and incubated overnight. On the following day the culture supernatants were subjected to ELISA to measure the degree of TLR activation/inhibition in terms of TNF $\alpha$  production. Data is represented as Mean  $\pm$  SEM, \*p<0.05, n=4. Paired Student's t-test was done to measure statistical significance. (c) Interaction between TLR7 ectodomain (homology model template PDB ID: 5WYZ) and compound 23. (d) Interaction between TLR7 ectodomain and compound 24. Hydrogen bonds and hydrophobic interactions are shown by green dash line and grey dash line respectively. \*marks residues are in chain B.

Selectivity of TLR7 antagonist **23** over TLR8 can be anticipated through docking analysis. TLR8 and TLR7 share similar antagonistic binding site except for one single residue change.<sup>41</sup> Polar residue Thr384 for TLR7 is replaced by nonpolar bulky Ile403 in TLR8. The molecular modeling of compound **23** in TLR7 indicates that the N-9 flexible linker is 5 Å away from Thr384 (Fig 2c). It can be anticipated that due to the steric bulk of Ile403 compound **23** is unable to bind to TLR8, which could be the probable reason for selectivity of our antagonists towards TLR7 over TLR8.

Table 2. In vitro pharmacokinetics of compound 23.

Aq. sol μg/ml clogF		gP Å <sup>2</sup>	NRB <sup>a</sup>	Caco-2 Permeability $P_{app} (10^{-6} \text{ cm/s})$		Plasma stability		Microsomal stability				
	clogP			$A \rightarrow B^b  B \rightarrow A$		efflux ratio	human <sup>d</sup>	mice <sup>d</sup>	human		mice	
					B→A <sup>c</sup>				T <sub>1/2</sub> (mi n)	QH%	T <sub>1/2</sub> (min )	QH%
200.3	1.38	52.6	7	3.57	9.28	2.6	95%	99.2%	90.3	26.5	111.8	14.4
<sup><i>a</i></sup> Number of rotatable bolds. <sup><i>b</i></sup> Apical to basal. <sup><i>c</i></sup> Basal to apical. <sup><i>d</i></sup> remaining after 2h.												



**Figure 3. Pharmacokinetics profiling of potent TLR7 antagonist 23.** (a) Doses of 15 mg/kg and 10 mg/kg body weight of compound **23** was orally administered to 3 sets of male C57BL/6 mice. (b) The dose of 15 mg/kg body weight of compound **23** was intravenously administered to 3 male C57BL/6 mice. (c) Calculated pharmacokinetics parameter in tabulated formed. 2% Ethanol in PBS (0.01M, pH = 7.4) buffer used as vehicle. The concentration of compound present in serum at indicated time points was quantified by LC-MS/MS (Figure S4).

We selected compound 23 for further investigation. Initially we aimed at gathering insights about *in vitro* pharmacokinetics of 23 through a series of established assays. The aqueous solubility of 23 was found to be considerable (200  $\mu$ g/ml) at pH 7.4 and calculated clogP value was 1.38 (Table 2). The polar surface area (52.6 Å<sup>2</sup>) and seven rotatable bonds were also good indicators that compound 23 would have favourable oral bioavailability (Table 2).<sup>43</sup> On Caco-2 permeability assay the efflux ratio of 23 was found to be 2.6, suggesting that this molecule would have moderate membrane permeability and is not likely to be a substrate for efflux transporters, viz. P-glycoprotein. In a nut shell these results

reflect that compound **23** may have good adsorption in the gastrointestinal tract. Compound **23** also showed acceptable plasma stability as well as metabolic stability (liver microsomal assay), assessed in case of both mice and human (Table 2). Moreover, compound **23** showed no cytotoxicity on HEK293 cells *in vitro* up to a concentration of 100  $\mu$ M (Fig S4).

Finally, to assess *in vivo* applicability of this novel TLR7 antagonist **23** we checked the *in vivo* pharmacokinetics of the molecule on oral and intravenous administration to C57BL/6 mice in two doses of 10 mg/kg and 15 mg/kg. The maximum plasma concentration ( $C_{max}$ ) surpassed the in vitro IC<sub>50</sub> against TLR7 in 15 mg/kg oral dose whereas in 10 mg/kg dose  $C_{max}$  remained below the IC<sub>50</sub>. It was found that compound **23** was rapidly absorbed into body (Fig 3a) with a half-life of 4.6 hours upon 15 mg/kg oral dose (Fig 3c). As compound **23** is basic nature it shows good Vss (4.2 L/kg, Fig 2c) which reflect the high duration of drug in target site.<sup>44</sup> Finally all the favourable range of in vitro and in vivo pharmacokinetics (Fig 3) of compound **23** adds up to give excellent oral bioavailability (F = 70.8%, Fig 3a, 3b).



Figure 4. Efficacy of TLR7 antagonist in rodent psoriasis model. (a) Schematic representation of experimental protocol followed- Compound 23 or vehicle was orally administered for 7 consecutive days starting one day before (Day 0) topical application of imiquimod on the dorsal skin surface, which was done for 6 consecutive days. The following day mice were sacrificed, normal and psoriatic skin harvested and subjected to histopathological evaluation. (b) (left) Shaved normal dorsal skin before imiquimod application, (middle) shaved imiquimod treated dorsal skin of vehicle treated mouse on day 7 showing significant scale formation on skin, (right) shaved imiquimod treated dorsal skin of compound 23 treated mouse on day 7 showing markedly lesser scale formation compared to previous group. (c) Thickness of skin fold of vehicle treated mice (n=9) and compound treated mice (n=9) were recorded and plotted every day (3 readings each) to monitor the gradual changes taking place upon daily imiquimod application. Data is represented as Mean  $\pm$  SEM. 2% Ethanol in PBS (0.01 M, pH = 7.4) buffer was used as vehicle.

To validate *in vivo* efficacy of 23 for TLR7 antagonism in a clinically relevant context, we utilized an established preclinical model of the cutaneous autoimmune disease psoriasis. Daily

application of the TLR7 agonist imiquimod to the skin of C57BL/6 mice leads to a psoriasiform inflammation in 6 days, which closely mimics the human disease in terms of morphology, histopathology and the underlying immunocellular involvements.<sup>15</sup> This preclinical model has not only been the key support for the pathogenetic role of TLR7 in this clinical context, but also has been instrumental for discovery of other downstream pathogenetic mechanisms for the disease as well as validation of most of the clinically used targeted biologic therapies used clinically.<sup>45</sup> We administered 15 mg/kg body weight compound **23** by oral gavage to the mice daily from the first day of application of imiquimod cream and on the 6th day assessed disease development, in comparison with vehicle-administered control mice having developed imiquimod-driven psoriasis (Fig 4a). We found that oral administration of our TLR7 antagonist **23** could totally prevent the cutaneous pathology in terms of macroscopic morphology (Fig 4b). There was also significant retardation in skin thickening in the antagonist-treated mice (Fig 4c).



Figure 5. Histopathologic scoring of the H&E-stained skin sections of mice with psoriatic disease. (a) (upper) H&E stained, imiquimod exposed, dorsal skin section of vehicle treated mouse showing marked thickening of epidermis and presence of hyperkeratosis, papillomatosis and acanthosis, (lower) H&E stained, imiquimod exposed, dorsal skin section of compound 23 treated mouse showing appreciably lesser thickening of epidermis. (b) Representative images showing the key pathognomonic histopathologic features used for scoring. (c) All histological features were scored based on their severity and preponderance, 1 being least severe and 3 being most severe. (d) Imiquimod exposed dorsal skin sections from compound treated or vehicle treated mice were graded on the basis of different clinically relevant histopathological parameters such as hyperkeratosis, acanthosis, papillomatosis, suprapapillary thinning, Monros microabscess, spongiosis, pustule of Kogoj, vascular dilatation of control group (n=9) and compound treated group (n=9), as a measure of disease severity. (e) Data on scores for individual pathognomonic features compared between control and treatment groups. (\*\*p<0.01). Histopathologic scoring of the lesional skin in the treated mice revealed nearly complete absence of the pathognomonic features like hyperkeratosis, parakeratosis, acanthosis, spongiosis, papillomatosis, suprapapillary thinning, vascular dilatation, microabscesses and pustule formation (Fig 5a, 5b). On scoring the histopathology based on the listed features (Fig 5c), there was total amelioration of disease on oral administration **23** (Fig 5d, 5e).



Figure 6. Inhibition of pro-inflammatory TLR7 mediated gene expression in rodent model of psoriasis. (a-e) Dorsal skin sections obtained from C57BL/6 mice treated with vehicle (n=6) or treated with 15 mg/kg compound 23 (n=6) for 7 consecutive days were processed and subjected to gene expression studies to measure expression of indicated genes- TNF $\alpha$  (a), MX1 (b), MX2 (c), ISG15 (d) and IFIT1 (e). (\*p<0.05, \*\*p<0.01, ns = non significant).

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In a repeat experiment too, the amelioration of cutaneous pathology with compound 23 was evident in terms of reduction in skin fold thickness (Figure S5) as well as in terms of reduction in the tissue expression of the proinflammatory cytokine TNF $\alpha$  (widely implicated in the pathogenesis of psoriasis) and four major type I IFN signature genes (ISGs) MX1, MX2, ISG15, IFIT1 (surrogate markers for type I IFN induction in situ) (Fig 6a-e).



**Figure 7. Short-term visceral toxicity assessment**. (a-f) Representative images of H&E stained sections of liver specimens (a,b), lung specimens (c,d) and kidney specimens (e,f) obtained from C57BL/6 mice treated with vehicle (a,c,e) or 15 mg/kg compound **23** (b,d,f) for 7 consecutive days.

Moreover, histomorphology of lung, liver, kidney did not show any remarkable pathologic changes following oral administration of compound **23** for 7 consecutive days at a dose rate of 15 mg/kg as compared to vehicle administration (Fig 7). No notable toxicity-induced pathology was discernible, viz. changes in parenchymal architecture of the liver, hepatocyte survival, fatty infiltration or inflammation in compound treated mice as compared to vehicle treated mice (Fig 7a, 7b). Similarly, no discernable changes were observed in lung microanatomy as well. No evidence of immune infiltration or alveolar architecture was observed in the lungs (Fig 7c, 7d). In kidneys no glomerular hypertrophy, mesangial proliferation, capsular space obliteration or inflammation was observed in the compound treated mice as compared to control mice (Fig 7e, 7f). We also did not observe any behavioral or physiological abnormalities in experimental animals within the study period, thus indicating that oral administration of compound **23** does not cause any apparent short-term visceral cytotoxity in C57BL/6 mice. Thus this novel TLR7 antagonist **23** was very efficient in preventing a TLR7-driven TNF $\alpha$  and type I IFN induction and the autoimmune pathology *in vivo*.

#### CONCLUSION

In summary, we have been able to define minimal structural requirements in a purine scaffold TLR7 agonist **1** and identify a singular 'chemical switch' in the scaffold that could transform the molecule to a TLR7 antagonist. We established that a small structural modification in TLR7 ligand can lead to reversal in their functional activity. We further developed on that and could design a potent TLR7 antagonist **23**, which had favourable *in vitro* and *in vivo* pharmacokinetics with excellent oral bioavailability (70.8%) in mice. Interestingly, ethylpiperazine group at N-9 led to enhancement of TLR7 agonism, while introducing the same at C-6 led to potentiation of TLR7 antagonism. Introduction of an additional basic center at C-6 has profound potentiating effect on TLR7 antagonism in purine scaffold. Our SAR studies successfully identified site-specific substitutions that can switch a well-established

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purine scaffold TLR7 agonist into TLR7 antagonist. None of the TLR7 antagonist showed any TLR7 agonism, which signifies that they are pure antagonists. It is worthwhile to mention that all the TLR7 antagonists reported herein showed excellent selectivity against TLR8. The docking analysis of our TLR7 antagonists showed that the purine ring is sandwiched between two hydrophobic residues Phe329 and Tyr1374 forming  $\pi$ - $\pi$  stacking interactions along with hydrogen bonding with Ser452, which plays vital role in determining TLR7 antagonism. Finally, we could validate the *in vivo* efficacy of this TLR7 antagonist in a clinically relevant murine model of psoriasis. The histomorphology of lung, liver, kidney did not show any remarkable pathologic changes following oral administration. The TLR7 antagonist **23** thus appears to be a very promising lead molecule for further development into clinically translatable TLR7 antagonists that can be *first-in-class* therapeutic agents in the relevant clinical contexts, which include a number of autoimmune diseases accounting for significant morbidity worldwide.

## EXPERIMENTAL

Aqueous solubility assay. 6  $\mu$ l of 50 mM DMSO stock was added to the deep well plate containing 594  $\mu$ l of pH 7.4 0.25 M phosphate buffer (including DMSO control), mixed and incubated for 18 hours at room temperature with constant mixing at 300 rpm. The plate was sealed well during the incubation process. After incubation, the samples were centrifuged for 20 min at 4000 rpm. 400  $\mu$ L of supernatant was analyzed by HPLC-UV. The DMSO content in the sample was 1.0% and the final concentration of the compound in deep-well plate was 500  $\mu$ M. the spectrum was read using Shimadzu UV spectrophotometer at 240 nm. (Calculation is in Table S1).

**Caco-2 permeability assay**. 10 mM stock of compound in DMSO was diluted with HBSS buffer pH 7.4 to a final concentration of 10  $\mu$ M. For the proliferation of cells, 250  $\mu$ l of DMEM was added to the basal compartment of 96 well multi-screen Caco-2 plate and seeded 12000 cells/well (0.16  $\times$  106 cells/ml) in all the apical wells placed in CO<sub>2</sub> incubator at 37 °C. On the day of assay, the medium

was removed and washed twice with HBSS Buffer. 250  $\mu$ l of HBSS buffer with 2% BSA was added to basal wells and 75  $\mu$ l of the test compound was added to apical wells. 25  $\mu$ l of basal samples were collected at 120 min and processed as mentioned below. 250  $\mu$ l of the test compound and 75  $\mu$ l of HBSS buffer with 2% BSA was added to basal wells and apical wells respectively. At 120 min 25  $\mu$ l of apical samples were collected and processed as stated below. Single point calibration curve in HBSS buffer with 2% BSA was used. Donor samples and receiver samples were diluted 1:1 with HBSS separately while former contain 2% BSA. It was precipitated with 200  $\mu$ l of acetonitrile containing internal standard and vortexed for 5 min @ 1000 rpm, centrifuged at 4000 rpm for 10 min. Finally, 100  $\mu$ l of supernatant was diluted with 200  $\mu$ l of water and submitted for LC-MS/MS analysis.(Calculation is in Table S2)

**Plasma stability assay**. 25  $\mu$ M stock of test compound was prepared in acetonitrile from the stock solution of 10 mM in DMSO. By diluting acetonitrile:water (50:50) from the previously prepared 10 mM stock, 125  $\mu$ M sample of 200  $\mu$ l was prepared. For 0 min samples, plasma was heat inactivated at 56 °C for 45 min and to it 3  $\mu$ l of 25  $\mu$ M test compound was added to make the final volume 75  $\mu$ l. In 200  $\mu$ l acetonitrile, a 25  $\mu$ l aliquot of the mixture was taken and crashed. At 0, 15, 30, 60 and 120 min, a 25  $\mu$ l aliquot of the sample was precipitated immediately with 200  $\mu$ l of acetonitrile, centrifuged at 4000 X RCF, 4 °C for 20 minutes and analyzed on LC-MS/MS. (Calculation for human plasma stability in Table S3 and mice plasma stability Table S4).

**Microsomal stability**. 10 mM stock of test compound in DMSO was diluted with 1:1 water acetonitrile to get 200  $\mu$ l 1 mM stock solution. 100  $\mu$ M working concentration of test compound was prepared by diluting 1 mM stock solution of test compound in DMSO with acetonitrile: water (1:1). 2.5 mM NADPH cofactor solution was prepared from 16 mM stock solution and 3.33 mg/ml working stock of liver microsomes was prepared from 20 mg/ml liver microsome master stock solution. 85  $\mu$ l of phosphate buffer of pH 7.4 was mixed with 2.5  $\mu$ l of test compound (100  $\mu$ M) and 75  $\mu$ l 3.33 mg/ml

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HLM in a deep well plate for pre-incubation of 10 min at 37 °C. 32.5 μl mixture was added to 17.5 μl buffer incubate for 60 min without NADPH cofactor. For zero minute sample, 16.5 μl preincubated mixture was crashed with 200 μl acetonitrile and 8.75 μl cofactor was added to it. 62 μl NADPH-cofactor was added to remaining incubation mixture and kept for 60 min at 37 °C. For other time point samples, 25 μl of incubation mixture at 5, 10, 30, 60 and 60 min without cofactor was precipitated immediately with 200 μl of acetonitrile containing internal standard and vortex for 5 min at 1200 RPM. The samples were centrifuged at 4000 x RCF, 4 °C for 10 min. Supernatant (150 μl) was diluted with 150 μl of water and analyzed on LC-MS/MS (Calculation for human microsomal stability in Table S5 and mice microsomal stability Table S6).

TLR7 agonism and antagonism assay using HEK-Blue<sup>TM</sup> hTLR7 reporter cell line. The TLR7 agonistic or antagonist activity of synthesized compounds was assayed using a HEK293 cell line engineered to express human TLR7 as well as a NF- $\kappa$ B induced secreted alkaline phosphatase (SEAP). The assay was based on the principle that TLR activation with TLR agonist leads to downstream NF- $\kappa$ B activation which in turn induces SEAP production and secretion into culture supernatant, causing colour change upon interaction with the detection media.  $7 \times 10^4$  cells/well were seeded in 100 mg/ml Normocin supplemented complete DMEM medium and allowed to adhere to the substrate for 5 hours at 37 °C and 5% CO<sub>2</sub>. For antagonism assays, CL264 (TLR7 agonist, 5 µg/ml) was added to the cells pre-incubated for 1 hour with indicated doses of compounds, whereas for agonism assays, cells were only treated with indicated doses of compounds. Following overnight incubation, 50 µl of culture supernatant from each well, was added to 200 µl of Quanti-Blue detection media and constantly monitored for colour change. After 1-2 hours of incubation, spectrophotometric reading at 655 nm wavelength was recorded. To ensure the validity of TLR7 antagonism assay, the ability of different doses of hydroxychloroquine to inhibit TLR7 activation was studied as a positive control. Two-tailed Paired Student's t-test was

performed using GraphPad Prism software version 5.0. HEK-Blue<sup>™</sup> hTLR7 reporter cell line, Normocin and CL264 were purchased from Invivogen, USA.

TLR8 antagonism assay using HEK-Blue<sup>TM</sup> hTLR8 reporter cell line. To ensure the specificity of the synthesized antagonists for human TLR7 and exclude the possibility of nonspecific antagonism toward human TLR8 which is structurally very similar to human TLR7, we performed reporter assays using HEK-Blue<sup>TM</sup> hTLR8 reporter cell line. The protocol used to perform this experiment was exactly same as that for the antagonism assay in HEK-TLR7 cell line, described above. The only difference was the usage of a TLR8 specific agonist CL075 (3 µg/ml) as the stimulant.

Cytotoxicity assay in HEK293 cell line. 1  $\mu$ M, 5  $\mu$ M, 10  $\mu$ M, 20  $\mu$ M, 40  $\mu$ M, 60  $\mu$ M, 80  $\mu$ M and 100  $\mu$ M of indicated compounds were added to  $6 \times 10^4$  HEK293 cells cultured incomplete DMEM at 37 °C and 5% CO<sub>2</sub> overnight, following which cells were harvested in PBS (0.01 M, pH = 7.4), stained with 2.5 mg/ml PI for 1 min and percentage of cells showing PI uptakein a flow cytometer (BD LSR Fortessa) was measured.

**IL-6 mRNA expression measurement through RT-PCR Analysis.** HEK-Blue<sup>TM</sup> TLR7 cells were seeded in a 96 well plate at a density of  $1 \times 10^5$  cells/well and allowed to attach overnight. Next morning, cells were pre-incubated with 5 µM of compound **23** for one hour before being stimulated with 5 µg/ml of TLR7 agonist-CL264 for 6 hours, following which cells were harvested in TRIzol reagent (Invitrogen). Total RNA was isolated using the manufacturer's protocol followed by cDNA preparation using the Applied Biosystems kit and real time PCR was done using BioRad SYBR Green Master Mix to measure the expression levels of IL6 gene relative to that of 18S (housekeeping gene). Primer sequences used, Hu-18S: Forward: 5'-GTTGGTTTTCGGAACTGAGG-3'Reverse:5'-TCGTTTATGGTCGGAACTACG-3' and Hu-IL6: Forward: 5'-CACAGACAGCCACTCACCTCTTC-3' Reverse: 5'-TTTGCTGCTTTCACACATGTTACTC-3'.

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TLR7 antagonism assay in primary human PBMCs. Primary human peripheral blood mononuclear cells (PBMCs) were isolated from blood taken from healthy individuals after obtaining their informed consent as well as ethical approval from the Institutional Ethics Committee, in accordance with the guidelines mentioned in the Declaration of Helsinki. PBMCs were obtained by performing density gradient centrifugation and seeded at a density of  $2 \times 10^5$  cells/well in a 96 well plate. PBMCs were pre-incubated with 5 µM of compound 23 for one hour before being stimulated with 5 µg/ml of TLR7 agonist-Gardiquimod overnight. Next day, culture supernatant was collected and subjected to TNF $\alpha$  ELISA according to the protocol outlined by the Manufacturer (Mabtech).

**Homology modeling and docking study.** Homology structure of human TLR7 ectodomain was build using sequence Q9NYK1 retrieve from UNIPROT. Homology model structure of hTLR7 was built using TLR8 (PDB 5WYZ) as a template in discovery studio modeler suite, according to the reported procedure.<sup>41</sup> Docking experiment was performed using Discover Studio 4.1 C-DOCKER module. Docking method was optimized by analyzing reported TLR7 antagonist binding pose. Random conformers of compounds were searched using dynamics of 1000 steps. Docking simulation was performed using default parameter of C-DOCKER module in Discover Studio 4.1. The docked results were fully minimized using CHARM force field with conjugate gradient 1.0. Pictorial view of docking pose was made using PyMOL.

*In-vivo* pharmacokinetics and bioavailability study. Nine 8-10 weeks old male C57BL/6 mice were recruited for the experiment. They were divided into 3 groups each having three mice. The compound was reconstituted in 2% ethanol in PBS (0.01 M, pH = 7.4). The mice belonging to the first group were given oral gavage with 15 mg/kg compound, the mice belonging to the second group were given oral gavage with 10 mg/kg compound and the mice belonging to the third group were given intravenous injections of 15 mg/kg compound. Blood samples were drawn at 0 h, 0.5 h, 1 h, 2 h, 4 h, 6 h, 12 h and 24 h after compound administration and serum was isolated for the mice administered orally

with the compound. On the other hand, blood samples were drawn at 0 h, 0.25 h, 0.5 h, 1 h, 2 h, 4 h, 6 h, 12 h and 24 h after compound administration and serum was isolated from the mice injected intravenously with the compound.10  $\mu$ l of serum from each mouse was extracted with 30  $\mu$ l of LC/MSgrade acetonitrile + 0.1% formic acid (J.T. Baker) by alternate vortexing and chilling. Next, the supernatant was collected by centrifugation at 14,000 rpm for 10 min and analyzed by LC-MS/MS to measure the concentration of compound **23** in serum. Standards of compound **23** ranging from 74.688 pg to 5975 pg was prepared in serum and extracted in acetonitrile + 0.1% formic acid. Mass spectrometry was carried out in LTQ ORBITRAP XL using Hypersil Gold C18 column having a diameter of 100 X 2.1 mm with particle size being 1.9  $\mu$ . The column formed the stationary phase and a mixture of solution A (acetonitrile + 0.1% formic acid) and solution B (HPLC grade H<sub>2</sub>0 + 0.1% formic acid) formed the mobile phase. Liquid chromatography (injection volume 2  $\mu$ l) was done using an isocratic solution containing 40% acetonitrile and 0.1% formic acid. The intact compound had a retention time of 0.76 mins and (m/z) of 387.2988, while (m/z) of fragmented compound after MS/MS was 273.1852. All data was analyzed using the Thermo Xcalibur software.

*In-vivo* efficacy study. 18 wild type C57BL/6 male mice aged 8-10 weeks, provided by CSIR-IICB animal house facility were divided into two groups; 1. Control group-containing 9 mice with induced psoriasis, treated with 2% ethanol in sterile PBS (0.01 M, pH = 7.4) (vehicle) and 2. Treatment group- containing 9 mice with induced psoriasis treated with compound 23. Psoriasis was induced by daily topical application of 62.5 mg 5% w/w imiquimod cream (Imiquad; Glenmark, India) on a patch of shaved dorsal skin of the mice for 6 consecutive days. Vehicle and compound (15 mg/kg) were administered orally daily for 7 days, beginning one day prior to the start of imiquimod application. Skin fold measurement of the affected area was performed by a person blind to the experimental details and outcomes, with a standard Vernier caliper on a daily basis starting prior to imiquimod application and continuing till the end of the experiment. The experimental animals were sacrificed on the 7<sup>th</sup> day of

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Imiquimod application and portions of affected skin as well as normal skin were collected from each animal (in 4% paraformaldehyde) for histopathological evaluation by H&E staining. All animal handling and mouse experiments were conducted in accordance with national and international guidelines and upon approval from the institutional ethics committee. All recommendations enlisted in the ACS Ethical Guidelines were followed while performing the mouse experiments.

**RNA isolation, cDNA preparation and Quantitative PCR**. Lesional skin tissue derived from 6 control C57BL/6 mice and 6 compound **23** (15 mg/kg) treated mice, following 7 consecutive days of oral administration (according to a protocol identical to that described in *in-vivo* efficacy study), was minced into tiny pieces, frozen in liquid nitrogen, crushed using a mortar & pestle in TRIzol Reagent (Invitrogen) and RNA was isolated according to the manufacturer's protocol. cDNA was produced using the High- Capacity cDNA Reverse Transcription Kit (Applied Bisosystems). qPCR was performed to study the expression of the specified genes using SyBR Green Master Mix (BioRad), (normalization was done with respect to the expression of  $\beta$ -actin- housekeeping gene).

## **Primer sequences:**

Mu-β actin	F: 5'-GAGGTATCCTGACCCTGAAGTA-3'
	R: 5'-GCTCGAAGTCTAGAGCAACATAG-3'
Mu-TNFα	F: 5'-AATGGCCTCCCTCTCATCAGTT-3'
	R: 5'-CCACTTGGTGGTTTGCTACGA-3'
Mu-MX1	F: 5'-TTCAAGGATCACTCATACTTCAGC-3'
	R: 5'-GGGAGGTGAGCTCCTCAGT-3'
Mu-MX2	F: 5'-TTCCAGCATCTGAATGCCTAC-3'

	R: 5'-ACTGGATGATCAAGGGAACG-3'
Mu-ISG15	F: 5'-ACGGTCTTACCCTTTCCAGTC-3'
	R: 5'-CCCCTTTCGTTCCTCACCAG-3'
Mu-IFIT	F: 5'-AGGCTGGAGTGTGCTGAGAT-3'
	R: 5'-TCTGGATTTAACCGGACAGC-3'

**Short-term** *in vivo* **toxicity studies:** Lung, liver, kidney specimens of mice, following oral administration of compound **23** and vehicle for 7 consecutive days at a dose rate of 15 mg/kg, were collected and preserved in 10% formalin. Hematoxylin and Eosin staining of tissue sections was performed for histomorphological evaluation. Images were acquired in an Olympus CKX41 brightfield inverted microscope at an objective magnification of 10X and monitored to study histomorphological parameters to deduce the visceral toxicity associated with compound **23** administration.

**Chemical synthesis and methods.** General Methods. All starting materials, reagents, and solvents were purchased from commercial suppliers and used without further purification. Air-sensitive reactions were carried out under dry nitrogen or argon atmosphere. For TLC on silica gel plates (Merck silica gel 60, F254) was used. RediSepRf silica gel columns was used for column chromatographic purification on the Teledyne ISCO CombiFlashRf system using 230-400 mesh silica gel. <sup>1</sup>H NMR was recorded at 300 MHz (Bruker-DPX), 400 MHz (Jeol) and 600 MHz (Bruker-Avance) frequency and <sup>13</sup>C NMR spectra were recorded at 75 MHz (Bruker-DPX), 100 MHz (Jeol) and 150 MHz (Bruker-Avance) using TMS as the internal standard. High-resolution mass spectra, HRMS (m/z) were measured using EI (Jeol-JMS 700 mass spectrometer), ESI (Q-Tof Micro mass spectrometer) techniques and ESI (LTQ Orbitrap XL mass spectrometer). The purity of all the compounds was determined to be > 95% analyzed

 by Hitachi HPLC using column Xtimate C18 (4.6 mm  $\times$  150 mm, 5.0  $\mu$ m) using gradient elution of acetonitrile in water 0-90% for 12 min and flow rate 1 ml/min with detection at 254 nm wavelength.

Synthesis of 2-butoxy-9-(tetrahydro-2*H*-pyran-2-yl)-9*H*-purin-6-amine (2).<sup>31</sup> Sodium *t*butoxide (0.8 g, 7.8 mmol) was added portion wise to the solution of 6 ml *n*-BuOH and compound **1** (0.5 g, 2.0 mmol) in rt. The reaction mixture was then stirred for 12 h at 100 °C and concentrated under vacuum. Reaction mixture was purified by column chromatography to obtain pure compound **2** as yellowish liquid (77%). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>)  $\delta$  7.78 (s, 1H), 6.51(s, 2H), 5.54 (dd, *J* = 2.4, 9.0 Hz, 1H), 4.23 (t, *J* = 6.6 Hz, 2H), 4.04 (d, *J* = 12.6 Hz, 1H), 3.69-3.61 (m, 1H), 1.99-1.88 (m, 3H), 1.72-1.59 (m, 4H), 1.44-1.36 (m, 2H), 1.14-1.09 (m, 1H), 0.87 (t, *J* = 7.2 Hz, 3H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>)  $\delta$  162.3, 156.6, 151.0, 136.6, 115.5, 81.4, 68.6, 67.0, 31.7, 31.1, 24.9, 22.9, 19.3, 13.9. HRMS (ESI) *m*/*z* (M+H) calculated for C<sub>14</sub>H<sub>22</sub>N<sub>5</sub>O<sub>2</sub> 292.1773 found 292.1766.

Synthesis of 2-butoxy-8-methoxy-9-(tetrahydro-2*H*-pyran-2-yl)-9*H*-purin-6-amine (3). *N*-Bromosuccinimide (0.06 g, 0.36 mmol) was added to the solution of compound 2 (0.1 g, 0.3 mmol) in 0.6 ml CHCl<sub>3</sub> at 0 °C. Reaction was allowed to warm room temperature and stirred for 4 h at rt. Reaction mixture was diluted with CHCl<sub>3</sub> and washed with sodium thiosulfate. Evaporation of CHCl<sub>3</sub> layer gave TLC wise pure bromo substituted compound and the next step was forwarded without further purification. This bromo derivative was refluxed with a 5 M solution of sodium methoxide in methanol (2.7 equiv.) for 4 h and reaction mixture was concentrated under vacuum. The reaction mixture was then purified by column chromatography to give pure compound **3** as brownish semi solid (80%).<sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>)  $\delta$  5.54 (dd, *J* = 1.8, 11.1 Hz, 1H), 5.28 (s, 2H), 4.28 (t, *J* = 6.6 Hz, 2H), 4.17-4.12 (m, 4H), 3.73-3.65 (m, 1H), 2.84-2.72 (m,1H), 2.06-2.02 (m, 1H), 1.79-1.63 (m, 6H), 1.58-1.46 (m, 3H), 0.97 (t, *J* = 7.5 Hz, 3H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>)  $\delta$  160.7, 154.3, 153.9, 151.5, 110.9, 81.3,

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68.9, 66.9, 56.9, 31.2, 28.6, 24.9, 23.5, 19.3, 13.9 HRMS (ESI) m/z (M+H) calculated for  $C_{15}H_{24}N_5O_3322.1879$  found 322.1884.

Synthesis of 2-butoxy-8-methoxy-9*H*-purin-6-amine (4). Compound 3 (0.2 g, 0.5 mmol) was added to 1 ml solution of 10% TFA in methanol and stirred for 72 h. Completion of reaction was monitored by TLC and white precipitation was obtained after dilution of reaction mixture with ethyl acetate. Precipitation was filtered to obtain pure compound 4 as TFA salt (73%). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub> + 1 drop CD<sub>3</sub>OD)  $\delta$  4.34 (t, *J* = 6.6 Hz, 2H), 4.05 (s, 3H), 1.74-1.64 (m, 2H), 1.41-1.33 (m, 2H), 0.88 (t, *J* = 7.5 Hz, 3H). HRMS (ESI) *m/z* (M+H) calculated for C<sub>10</sub>H<sub>26</sub>N<sub>5</sub>O<sub>2</sub> 238.1304 found 238.1311.

#### General procedure A for synthesis of 2-butoxy-9-(3-chloropropyl)-8-methoxy-9H-purin-6-

**amine (5)**. Compound **4** (0.1 g, 0.5 mmol) and potassium carbonate (0.2 g, 1.5 mmol) were dissolved in 1 ml DMF and the reaction mixture was heated at 50 °C for 2 h. 1-Bromo-3-chloropropane was added to the reaction mixture at room temperature and stirred for 16 h. The solvent was then removed and reaction was partitioned between CHCl<sub>3</sub> and water. Organic layer was then dried and column chromatography was performed to get pure compound **5** as white solid (67%, mp. 115 °C). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>)  $\delta$  5.32 (s, 2H), 4.26 (t, *J* = 6.6 Hz, 2H), 4.11-4.06 (m, 5H), 3.52 (t, *J* = 6.6 Hz, 2H), 2.28-2.20 (m, 2H), 1.77-1.70 (m, 2H), 1.51-1.44 (m, 2H), 0.95 (t, *J* = 7.2 Hz, 3H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>)  $\delta$  160.8, 154.6, 153.8, 151.9, 110.9, 67.0, 56.8, 41.8, 38.7, 32.0, 31.2, 19.3, 13.9 HRMS (ESI) m/z (M+H) calculated for C<sub>13</sub>H<sub>21</sub>N<sub>5</sub>O<sub>2</sub>Cl 314.1384 found 314.1387.

General procedure B for synthesis of 2-butoxy-8-methoxy-9-(3-(piperidin-1-yl)propyl)-9*H*purin-6-amine (6). Compound 5 (0.1 g, 0.3 mmol), potassium carbonate (0.1 g, 0.7 mmol) and piperidine (0.1 ml, 1.0 mmol) were dissolved in 0.5 ml DMF. The reaction mixture was then stirred for 6 h at rt. Completion of reaction was confirmed by TLC and column chromatography was performed to obtain pure compound 6 as semi solid (73%).<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  5.22 (s, 2H), 4.24 (t, *J* = 6.8

 Hz, 2H), 4.07 (s, 3H), 3.94 (t, *J* = 7.2 Hz, 2H), 2.37-2.34 (m, 6H), 2.00-1.92 (m, 2H), 1.77-1.70 (m, 2H), 1.59-1.53 (m, 4H), 1.49-1.43 (m, 2H) 1.41-1.35 (m, 2H), 0.93 (t, *J* = 7.2 Hz, 3H). <sup>13</sup>C NMR (100 MHz, CDC13) δ 160.8, 154.8, 153.7, 152.0, 110.9, 66.9, 56.7, 56.2, 54.5, 39.6, 31.2, 26.1, 25.7, 24.2, 19.3, 14.0. HRMS (ESI) m/z (M+H) calculated for C<sub>18</sub>H<sub>31</sub>N<sub>6</sub>O<sub>2</sub> 363.2508 found 363.2512.

General procedure C for synthesis of 6-amino-2-butoxy-9-(3-(piperidin-1-yl)propyl)-9*H*purin-8-ol (7). Compound 6 (0.1 g, 0.03 mmol) was dissolved in 0.5 ml 4 M HCl in dioxane at rt for 3 h. Reaction mixture was then neutralized with ammonia and concentrated in vacuum. Column chromatography was performed using 5% ammonia in methanol and CHCl<sub>3</sub> as a mobile phase to obtain pure compound 7 as white solid (87%, mp. > 250 °C). <sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>)  $\delta$  5.84 (s, 1H), 4.22 (t, *J* = 6.6Hz, 2H), 3.87 (t, *J* = 6.6 Hz, 2H), 2.61-2.59 (m, 6H), 2.07-2.04 (m, 2H), 1.74-1.70 (m, 2H), 1.67-1.65 (m, 4H), 1.46-1.43 (m, 4H), 0.94 (t, *J* = 7.2 Hz, 3H). <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub> + 1 drop CD<sub>3</sub>OD)  $\delta$  160.7, 153.7, 149.7, 147.9, 99.0, 67.0, 55.8, 54.1, 38.0, 31.0, 25.0, 24.8, 23.5, 19.2, 13.8. HRMS (ESI) *m/z* (M+H) calculated for C<sub>17</sub>H<sub>29</sub>N<sub>6</sub>O<sub>2</sub> 349.2352 found 349.2351. HPLC purity 97.9%.

#### Synthesis of 2-butoxy-9-(3-(4-ethylpiperazin-1-yl)propyl)-8-methoxy-9H-purin-6-amine (8).

Compound **6** (0.1 g, 0.3 mmol), potassium carbonate (0.1 g, 0.7 mmol) and ethylpiperazine (0.13 ml, 1.0 mmol) were dissolved in 0.5 ml DMF. Reaction was then performed according to general procedure B to obtain pure compound **8** as semi solid (67%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  5.31 (s, 2H), 4.22 (t, *J* = 6.4 Hz, 2H), 4.05 (s, 3H), 3.93 (t, *J* = 6.8 Hz, 2H), 2.48-2.32 (m, 12H), 1.93-1.88 (m, 2H), 1.74-1.67 (m, 2H), 1.49-1.41 (m, 2H), 1.03 (t, *J* = 7.2 Hz, 3H), 0.91 (t, *J* = 7.2 Hz, 3H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>)  $\delta$  160.8, 154.8, 153.7, 151.9, 110.8, 66.9, 56.7, 55.5, 53.0, 52.7, 52.3, 39.6, 31.2, 26.2, 19.3, 13.9, 11.9. HRMS (ESI) *m*/<sub>z</sub> (M+H) calculated for C<sub>19</sub>H<sub>34</sub>N<sub>7</sub>O<sub>2</sub> 392.2774 found 349.2773.

# Synthesisof6-amino-2-butoxy-9-(3-(4-ethylpiperazin-1-yl)propyl)-9H-purin-8-ol(9).Compound 8 (0.1 g, 0.3 mmol) was dissolved in 0.5 ml 4 M HCl in dioxane at rt for 3 h. Reaction was

then performed according to general procedure C to obtain pure compound **9** as white solid (91%, mp. > 250 °C). <sup>1</sup>H NMR (400 MHz, CD<sub>3</sub>OD)  $\delta$  4.25 (t, *J* = 6.8 Hz, 2H), 3.85 (t, *J* = 6.8 Hz, 2H), 2.76-2.04 (m, 12H), 1.95-1.88 (m, 2H), 1.74-1.66 (m, 2H), 1.51-1.43 (m, 2H), 1.04 (t, *J* = 7.2 Hz, 3H), 0.94 (t, *J* = 7.2 Hz, 3H). <sup>13</sup>C NMR (100 MHz, CD<sub>3</sub>OD)  $\delta$  160.8, 153.8, 149.9, 148.4, 98.5, 66.6, 55.5, 52.2, 52.0, 51.9, 38.2, 30.9, 24.8, 18.9, 12.8, 10.3. HRMS (ESI) *m/z* (M+H) calculated for C<sub>18</sub>H<sub>32</sub>N<sub>7</sub>O<sub>2</sub> 378.2617 found 378.2617. HPLC purity 99.2%.

Synthesis of 8-methoxy-9*H*-purin-6-amine (11). Compound 10 (0.2 g, 6.3 mmol) was refluxed with a 5 M solution of sodium methoxide in methanol (63 mmol, 10 ml) for 12 h. Reaction mixture was then concentrated in vacuum and diluted with 2 ml water. Reaction mixture was neutralized and the precipitation was filtered to obtain pure compound 11 as brown solid (72%, mp. > 250 °C). <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ )  $\delta$  8.06 (s, 1H), 7.38 (s, 2H), 6.81 (s,1H), 3.98 (s, 3H). <sup>13</sup>C NMR (100 MHz, CD<sub>3</sub>OD)  $\delta$  154.5, 151.4, 150.5, 148.5, 106.3, 57.2. HRMS (ESI) m/z (M+H) calculated for C<sub>6</sub>H<sub>8</sub>N<sub>5</sub>O 166.0729 found 166.0732.

Synthesis of 9-(3-chloropropyl)-8-methoxy-9*H*-purin-6-amine (12). The reaction was performed according to general procedure A using compound 11 (0.2 g, 1.2 mmol), potassium carbonate (0.5 g, 3.6 mmol) and 1-bromo-3-chloropropane in 3 ml DMF to obtain compound 15 as white semi solid (78%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  8.22 (s, 1H), 5.38 (s, 2H), 4.17-4.14 (m, 5H), 3.54-3.49 (m, 2H), 2.30-2.23 (m, 2H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>)  $\delta$  155.6, 152.9, 151.3, 150.2, 115.7, 57.2, 41.7, 39.0, 31.9. HRMS (ESI) m/z (M+H) calculated for C<sub>6</sub>H<sub>13</sub>N<sub>5</sub>OCl 242.0809 found 242.0809.

Synthesis of 9-(3-(4-ethylpiperazin-1-yl)propyl)-8-methoxy-9*H*-purin-6-amine (13). Compound 12 (0.1 g, 0.4 mmol), potassium carbonate (0.1 g, 0.7 mmol) and ethylpiperazine (0.2 ml, 1.1 mmol) were dissolved in 0.5 ml DMF. Reaction was then performed according to general procedure B to obtain pure compound 13 as semi solid (63%). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>)  $\delta$  8.21 (s, 1H), 5.49 (s,

 2H), 4.12 (s, 3H), 4.04 (t, J = 6.9 Hz, 2H), 2.45-2.36 (m, 12H), 1.97-1.92 (m, 2H), 1.07 (t, J = 7.2 Hz, 3H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>)  $\delta$  155.8, 152.8, 151.1, 150.3, 115.7, 56.9, 55.5, 52.9, 52.8, 52.3, 39.9, 26.2, 11.8. HRMS (ESI) m/z (M+H) calculated for C<sub>15</sub>H<sub>26</sub>N<sub>7</sub>O 320.2199 found 320.2195.

Synthesis of 6-amino-9-(3-(4-ethylpiperazin-1-yl)propyl)-9*H*-purin-8-ol (14). Compound 13 (0.1 g, 0.3 mmol) was dissolved in 0.5 ml 4 M HCl in dioxane at rt for 3 h. Reaction was then performed according to general procedure C to obtain pure compound 14 as white semi solid (86%). <sup>1</sup>H NMR (300 MHz, CD<sub>3</sub>OD)  $\delta$  8.09 (s, 1H), 3.95 (t, *J* = 6.6 Hz, 2H), 2.49-2.40 (m, 12H), 2.02-1.93 (m, 2H), 1.04 (t, *J* = 7.2 Hz, 3H). <sup>13</sup>C NMR (100 MHz, CD<sub>3</sub>OD)  $\delta$  153.5, 150.9, 147.9, 147.1, 100.0, 55.5, 52.2, 51.9, 51.8, 38.3, 24.7, 10.3. HRMS (ESI) *m*/*z* (M+H) calculated for C<sub>14</sub>H<sub>24</sub>N<sub>7</sub>O 306.2042 found 306.2038. HPLC purity 97.7%.

**6-Chloro-9-(3-(4-ethylpiperazin-1-yl)propyl)-9H-purine** (16). Compound 15 (0.1 g, 0.7 mmol) and 1-(3-chloropropyl)-4-ethylpiperazine (0.3 g, 1.2 mmol) were taken in dry 0.5 ml DMF and potassium carbonate (0.2 g, 1.2 mmol) was added to it. The reaction mixture was heated at 60 °C for 12 h. Water was added to the reaction mixture. The aqueous solution was extracted with chloroform and the organic layer was dried over Na<sub>2</sub>SO<sub>4</sub> and evaporated under vacuum. The residue was purified by silica gel column chromatography eluting with 4% methanol in chloroform to provide compound **16** as semi solid (84%). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>)  $\delta$  8.74 (s, 1H), 8.17 (s, 1H), 4.38 (t, *J* = 6.6 Hz, 2H), 2.51-2.37 (m, 8H), 2.30 (t, *J* = 6.6 Hz, 2H), 2.10-2.07 (m, 2H), 1.75-1.69 (m, 2H), 1.08 (t, *J* = 7.2 Hz, 3H). <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>)  $\delta$  151.9, 151.8, 150.9, 145.9, 131.7, 54.2, 52.4, 52.3, 52.2, 42.4, 29.7, 26.0, 11.5. HRMS (ESI) *m*/*z* (M+H) calculated for C<sub>14</sub>H<sub>22</sub>ClN<sub>6</sub> 309.1594 found 309.1588.

**9-(3-(4-Ethylpiperazin-1-yl)propyl)-9***H***-purin-6-amine (17)**. Compound **16** (0.1 g, 0.3 mmol) and 2 ml ammonia in ethanol (7 equiv.) were taken in pressure tube. The reaction mixture was heated at 60 °C for 12 h. Ethanol was evaporated and residue was purified by column chromatography to obtain

compound **17** as semi solid (67%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub> + CD<sub>3</sub>OD)  $\delta$  8.10 (s, 1H), 7.79 (s, 1H), 4.14 (t, *J* = 6.8 Hz, 2H), 3.01-2.81 (m, 6H), 2.70-2.53 (m, 4H), 2.36 (t, *J* = 6.8 Hz, 2H), 1.97-190 (m, 2H), 1.20 (t, *J* = 7.2 Hz, 3H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub> + CD<sub>3</sub>OD)  $\delta$  156.6, 153.5, 145.0, 123.0, 100.0, 57.0, 56.0, 55.2, 53.8, 53.7, 45.8, 30.3, 13.2, 4.8. HRMS (ESI) *m*/*z* (M+H) calculated for C<sub>14</sub>H<sub>24</sub>N<sub>7</sub> 290.2093 found 290.2084. HPLC purity 99.6%.

**9-(3-(4-Ethylpiperazin-1-yl)propyl)-9H-purin-6-amine (18)**. Compound **16** (0.1 g, 0.3 mmol) was dissolved in 10 ml THF and solution was degas using argon. 10% Palladium on carbon (10 mol%) was added to reaction mixture and then reaction mixture was stirred at room temperature in hydrogen atmosphere for 1 h. Completion of the reaction was confirmed by monitoring TLC and catalyst was separated using celite filtration. Filtrate was evaporated and residue was purified by column chromatography to obtain compound **18** as semi solid (91%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub> )  $\delta$  9.13 (s, 1H), 8.98 (s, 1H), 8.13 (s, 1H), 4.38 (t, *J* = 6.6 Hz, 2H), 2.58-2.38 (m, 10H), 2.32 (t, *J* = 6.6 Hz, 2H), 2.13-2.05 (m, 2H), 1.08 (t, *J* = 7.2 Hz, 3H). <sup>13</sup>C NMR (150 MHz, CDCl<sub>3</sub> )  $\delta$  152.5, 151.4, 148.5, 145.9, 134.1, 54.4, 52.6, 52.3, 41.7, 26.2, 11.7. HRMS (ESI) *m/z* (M+H) calculated for C<sub>14</sub>H<sub>23</sub>N<sub>6</sub> 275.1984 found 275.1973. HPLC purity 96.4%.

**9-(3-(4-Ethylpiperazin-1-yl)propyl)**-*N*,*N*-dimethyl-9*H*-purin-6-amine (19). Compound 16 (0.1 g, 0.3 mmol) and 2 ml dimethylamine in THF (2 M) were added to 3 ml acetonitrile in presence of base potassium carbonate (0.06 g, 0.62 mmol) The reaction mixture was refluxed for 12 h then solvent was evaporated and column chromatography was performed to obtain pure compound 19 as semi solid (89%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  8.31 (s, 1H), 7.71 (s, 1H), 4.22 (t, *J* = 6.8 Hz, 2H), 3.50 (s, 6H), 2.44-2.37 (m, 10H), 2.28 (t, *J* = 6.8 Hz, 2H), 2.04-1.99 (m, 2H), 1.07 (t, *J* = 7.2 Hz, 3H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>)  $\delta$  155.0, 152.4, 150.6, 138.8, 120.3, 54.5, 52.9, 52.8, 52.4, 41.6, 26.6, 11.9. HRMS (ESI) *m/z* (M+H) calculated for C<sub>16</sub>H<sub>26</sub>N<sub>7</sub> 318.2406 found 318.2404. HPLC purity 98.6%.

General procedure C 9-(3-(4-ethylpiperazin-1-yl)propyl)-6-(piperidin-1-yl)-9*H*-purine (20). Compound 16 (0.1 g, 0.2 mmol) was dissolved in acetonitrile (1 ml) and potassium carbonate (0.04 g, 0.28 mmol) and piperidine (0.02 g, 1.2 mmol) were added. The reaction was refluxed under the N<sub>2</sub> atmospheric condition for 12 h. Organic layer was extracted with CHCl<sub>3</sub> system and column chromatography was done by using CH<sub>3</sub>OH and CHCl<sub>3</sub> system to give compound 20 (73%) as a semi solid. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  8.29 (s, 1H), 7.72 (s, 1H), 4.24-4.20 (m, 6H), 2.51-2.41 (m, 8H), 2.31 (t, *J* = 6.8 Hz, 2H), 2.05-1.98 (m, 4H), 1.70-1.65 (m, 6H), 1.08 (t, *J* = 7.2 Hz, 3H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) $\delta$ 154.0, 152.4, 150.8, 138.6, 119.9, 54.5, 52.8, 52.7, 52.4, 41.6, 26.6, 26.2, 24.9, 11.8. HRMS (ESI) *m*/<sub>z</sub> (M+H) calculated for: C<sub>19</sub>H<sub>3</sub>oN<sub>7</sub> 358.2719 found 358.2721. HPLC purity 95.6%.

**4-(9-(3-(4-Ethylpiperazin-1-yl)propyl)-9H-purin-6-yl)morpholine (21)**. Compound **16** (0.1 g, 0.2 mmol) was dissolved in acetonitrile (1 ml) and potassium carbonate (0.04 g, 0.28 mmol) and morpholine (0.03 g, 1.2 mmol) were added. The reaction then proceeds according to general procedure C to get compound **21** (82%) as a semi solid. <sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>)  $\delta$  8.35 (s, 1H), 7.76 (s, 1H), 4.33-4.30 (m, 4H), 4.26 (t, *J* = 6.6 Hz, 2H), 3.83 (t, *J* = 4.8 Hz, 4H), 2.49-2.40 (m, 8H), 2.31 (t, *J* = 6.6 Hz, 2H), 2.06-2.02 (m, 2H), 1.97-1.91 (m, 2H), 1.08 (t, *J* = 7.2 Hz, 3H). <sup>13</sup>C NMR (150 MHz, CDCl<sub>3</sub>)  $\delta$ . 156.4, 154.7, 153.4, 141.6, 122.5, 69.5, 56.8, 55.3, 55.2, 54.7, 43.9, 28.8, 14.3. HRMS (ESI) *m/z* (M+H) calculated for: C<sub>18</sub>H<sub>30</sub>N<sub>7</sub>O 360.2512 found 360.2523. HPLC purity 95.1%.

**9-(3-(4-Ethylpiperazin-1-yl)propyl)-6-(piperazin-1-yl)-9H-purine (22)**. Compound **16** (0.1 g, 0.2 mmol) was dissolved in acetonitrile (1 ml) and potassium carbonate (0.04 g, 0.28 mmol) and piperazine (0.1 g, 1.2 mmol) were added. The reaction then proceeds according to general procedure C to get compound **22** (86%) as a semi solid. <sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>)  $\delta$  8.30 (s, 1H), 7.72 (s, 1H), 4.27-4.21 (m, 6H), 3.00-2.95 (m, 4H), 2.49-2.33 (m, 10H), 2.27 (t, *J* = 6.6 Hz, 2H), 2.04-1.98 (m, 2H), 1.05 (t, *J* = 7.2 Hz, 3H). <sup>13</sup>C NMR (150 MHz, CDCl<sub>3</sub>) $\delta$ . 156.4, 154.7, 153.3, 141.3, 122.4, 56.8, 55.4,

55.2, 54.7, 48.7, 43.7, 28.8, 14.4. HRMS (ESI) *m*/*z* (M+H) calculated for: C<sub>18</sub>H<sub>31</sub>N<sub>8</sub> 359.2672 found 35.92675. HPLC purity 99.4%.

6-(4-Ethylpiperazin-1-yl)-9-(3-(4-ethylpiperazin-1-yl)propyl)-9*H*-purine (23). Compound 16 (0.5 g, 0.7 mmol) was dissolved in acetonitrile (8 ml) and potassium carbonate (0.2 g, 1.5 mmol) and ethylpiperazine (0.13 ml, 1.2 mmol) were added. The reaction then proceeds according to general procedure C to get compound 23 (95%) as a semi solid. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 8.34 (s, 1H), 7.76 (s, 1H), 4.43-4.29 (m, 4H), 4.25 (t, J = 6.6 Hz, 2H), 2.60-2.57 (m, 4H), 2.51- 2.39 (m, 12H), 2.31 (t, J = 6.6 Hz, 2H), 2.08-2.01 (m, 2H), 1.16-1.06 (m, 6H). <sup>13</sup>C NMR (150 MHz, CDCl<sub>3</sub>) δ 153.9, 152.3, 150.9, 138.9, 120.0, 54.4, 53.0, 52.8, 52.7, 52.5, 52.3, 41.5, 26.5, 12.0. HRMS (ESI) m/z (M+H) calculated for: C<sub>20</sub>H<sub>35</sub>N<sub>8</sub> 387.2985 found 387.2979. HPLC purity 99.2%.

**6-(4-Cyclopentylpiperazin-1-yl)-9-(3-(4-ethylpiperazin-1-yl)propyl)-9H-purine** (24). Compound **16** (0.1 g, 0.4 mmol) was dissolved in acetonitrile (1 ml) and potassium carbonate (0.05 g, 0.4 mmol) and piperazine (0.03 g, 0.34 mmol) were added. The reaction then proceeds according to general procedure C to get compound **24** (86%) as a semi solid. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>)  $\delta$  8.33 (s, 1H), 7.74 (s, 1H), 4.46-4.30 (m, 4H), 4.25 (t, *J* = 6.6 Hz, 2H), 2.71-2.55 (m, 13H), 2.36 (t, *J* = 6.9 Hz, 2H), 2.09-2.00 (m, 2H), 1.91-1.87 (m, 4H), 1.78-1.68 (m, 2H), 1.62-1.49 (m, 4H), 1.19 (t, *J* = 7.2 Hz, 3H). <sup>13</sup>C NMR (150 MHz, CDCl<sub>3</sub>) $\delta$ .153.7, 152.3, 150.9, 138.8, 119.9, 67.6, 54.2, 52.3, 52.2, 51.6, 41.5, 30.1,26.4, 23.9, 24.0, 10.9. HRMS (ESI) *m/z* (M+H) calculated for: C<sub>23</sub>H<sub>39</sub>N<sub>8</sub>427.3298 found 427.3297. HPLC purity 97.3%.

**9-(3-Chloropropyl)-6-(4-ethylpiperazin-1-yl)-9H-purine (26)**. Compound **25** (1.6 g, 6.7 mmol) and potassium carbonate (1.9 g, 14 mmol) were dissolved in 1 ml DMF and the reaction mixture was heated at 50 °C for 2 h. 1-Bromo-3-chloropropane was added to the reaction mixture at room temperature and stirred for 12 h. The solvent was then removed and reaction was partitioned between

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CHCl<sub>3</sub> and water. Organic layer was then dried and column chromatography was performed to get pure compound **5** as semi solid (71%). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>)  $\delta$  8.17 (s, 1H), 7.63 (s, 1H), 4.25-4.12 (m, 6H), 3.35 (t, *J* = 6.0 Hz, 2H), 3.43 (t, *J* = 5.1 Hz, 4H), 2.35-2.28 (m, 2H), 2.25-2.16 (m, 2H), 0.98 (t, *J* =7.2 Hz, 3H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>)  $\delta$  153.8, 152.4, 150.7, 138.5, 120.1, 52.8, 52.4, 41.4, 40.8, 31.8, 11.9. HRMS (ESI) m/z (M+H) calculated for C<sub>14</sub>H<sub>22</sub>N<sub>6</sub>Cl 309.1594 found 309.1599.

**6-(4-Ethylpiperazin-1-yl)-9-(3-(piperazin-1-yl)propyl)-9H-purine (27)**. Compound **26** (0.1 g, 0.3 mmol), potassium carbonate (0.1 g, 0.7 mmol) and piperidine (0.11 ml, 1.0 mmol) were dissolved in 0.5 ml DMF. Reaction was then performed according to general procedure B to obtain pure compound **8** as semi solid (78%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  8.29 (s, 1H), 8.10 (s, 1H), 4.56-4.41 (m, 4H), 4.31 (t, *J* = 6.8 Hz, 2H), 3.12 (t, *J* = 5.2 Hz, 8H), 2.99-2.95 (m, 2H), 2.60-2.58 (m, 4H), 2.41 (t, *J* = 6.8 Hz, 2H), 2.08-2.01 (m, 2H), 1.19 (t, *J* = 7.2 Hz, 3H). <sup>13</sup>C NMR (150 MHz, CDCl<sub>3</sub>) $\delta$ 155.8, 154.0, 153.3, 143.2, 122.2, 56.7, 54.4, 53.9, 51.7, 45.9, 43.9, 28.4, 11.4. HRMS (ESI) *m/z* (M+H) calculated for: C<sub>18</sub>H<sub>31</sub>N<sub>8</sub> 359.2672 found 359.2675. HPLC purity 95.4%.

**4-(3-(6-(4-Ethylpiperazin-1-yl)-9***H***-purin-9-yl)propyl)morpholine (28)**. Compound **26** (0.1 g, 0.3 mmol), potassium carbonate (0.1 g, 0.7 mmol) and morpholine (0.09 ml, 1.0 mmol) were dissolved in 0.5 ml DMF. Reaction was then performed according to general procedure B to obtain pure compound **8** as semi solid (85%). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 8.31 (s, 1H), 7.72 (s, 1H), 4.34-4.31 (m, 4H), 4.24 (t, J = 6.8 Hz, 2H), 3.68-3.66 (m, 4H), 2.59-2.57 (m, 4H), 2.50-2.45 (m, 2H), 2.38-2.36 (m, 4H), 2.27 (t, J = 6.8 Hz, 2H), 2.05-1.98 (m, 2H), 1.12 (t, J = 7.2 Hz, 3H). <sup>13</sup>C NMR (150 MHz, CDCl<sub>3</sub>) δ 153.8, 152.3, 150.9, 138.8, 120.0, 66.9, 54.9, 53.5, 52.8, 52.4, 41.5, 29.6, 26.1, 11.8. HRMS (ESI) m/z (M+H) calculated for: C<sub>18</sub>H<sub>30</sub>N<sub>7</sub>O 360.2512 found 360.2523. HPLC purity 96.0%.

**6-(4-Ethylpiperazin-1-yl)-9-(3-(piperidin-1-yl)propyl)-9***H***-purine (29)**. Compound **26** (0.1 g, 0.3 mmol), potassium carbonate (0.1 g, 0.7 mmol) and piperidine (0.11 ml, 1.0 mmol) were dissolved in

olved in  0.5 ml DMF. Reaction was then performed according to general procedure B to obtain pure compound **8** as semi solid (93%). <sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>) δ 8.33 (s, 1H), 7.75 (s, 1H), 4.41-4.39 (m, 4H), 4.24 (t, *J* = 6.6 Hz, 2H), 2.59-2.57 (m, 4H), 2.49-2.45 (m, 2H), 2.42-2.35 (m, 4H), 2.31 (t, *J* = 6.6 Hz, 2H), 2.07-2.03 (m, 2H), 1.60-1.55 (m, 4H), 1.47-1.41 (m, 2H), 1.13 (t, *J* =7.2 Hz, 3H). <sup>13</sup>C NMR (150 MHz, CDCl<sub>3</sub>) δ 153.8, 152.2, 150.8, 138.9, 119.9, 55.0, 54.3, 52.9, 52.4, 41.5, 26.4, 25.6, 24.1, 11.8. HRMS (ESI) m/z (M+H) calculated for C<sub>19</sub>H<sub>32</sub>N<sub>7</sub> 358.2719 found 358.2713. HPLC purity 96.8%.

**6-(4-Ethylpiperazin-1-yl)-9-(3-(pyrrolidin-1-yl)propyl)-9H-purine (30)**. Compound **26** (0.1 g, 0.3 mmol), potassium carbonate (0.1 g, 0.7 mmol) and pyrrolidine (0.08 ml, 1.0 mmol) were dissolved in 0.5 ml DMF. Reaction was then performed according to general procedure B to obtain pure compound **8** as semi solid (93%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 8.30 (s, 1H), 7.74 (s, 1H), 4.38-4.28 (m, 4H), 4.25 (t, J = 6.8 Hz, 2H), 2.55 (t, J = 5.2 Hz, 4H), 2.48-2.41 (m, 8H), 2.08-2.00 (m, 2H), 1.77-1.72 (m, 4H), 1.10 (t, J = 7.2 Hz, 3H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ 153.9, 152.4, 150.9, 138.9, 120.0, 54.1, 53.0, 52.6, 52.5, 41.7, 28.7, 23.5, 12.0. HRMS (ESI) m/z (M+H) calculated for C<sub>18</sub>H<sub>30</sub>N<sub>7</sub> 344.2563 found 344.2569. HPLC purity 95.8%.

**6-(4-Ethylpiperazin-1-yl)-9-propyl-9H-purine (31)**. Compound **25** (1.0 g, 4.3 mmol) and potassium carbonate (1.2 g, 8.6 mmol) were dissolved in 1 ml DMF and the reaction mixture was heated at 50 °C for 2 h. 3-Bromopropane (0.7 ml, 6.5 mmol) was added to the reaction mixture at room temperature and stirred for 12 h. The solvent was then removed and reaction was partitioned between CHCl<sub>3</sub> and water. Organic layer was then dried and column chromatography was performed to get pure compound **5** as semi solid (78%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  8.35 (s, 1H), 7.73 (s, 1H), 4.44-4.26 (m, 4H), 4.14 (t, *J* = 7.2 Hz, 2H), 2.61-2.57 (m, 4H), 2.51-2.44 (m, 2H), 1.94-1.87 (m, 2H), 1.13 (t, *J* = 7.2 Hz, 3H), 0.96 (t, *J* = 7.2 Hz, 3H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>)  $\delta$  153.9, 152.3, 150.9, 138.4, 119.9,

52.9, 52.5, 45.4, 23.4, 11.9, 11.2. HRMS (ESI) m/z (M+H) calculated for  $C_{14}H_{23}N_6275.1984$  found 275.1977. HPLC purity 96.9%.

# ASSOCIATED CONTENTS

# Supporting Information

TLR7 Antagonistic assay, TLR7 Agonistic assay, TLR8 Antagonistic assay, cytotoxicity assay,X-ray crystallographic structure of HCl salt of compound **16**. LC-MS/MS spectrum analysis of compound **23**, aqueous solubility assay table, Caco-2 permeability assay table, plasma stability assay table, microsomal stability assay table, molecular docking, HPLC data,<sup>1</sup>H NMR and <sup>13</sup>C NMR spectra of compounds 1–31 and molecular formula strings.

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# **ABBREVIATIONS USED**

pDC, plasmacytoid dendritic cell; SLE, systemic lupus erythematosus; hPBMC, human peripheral blood mononuclear cell, LC-MS, liquid chromatography–mass spectrometry; ISG15, interferon-stimulated gene 15; DMEM, dulbecco's modified eagle medium; HBSS, hank's balanced salt solution; SEAP, secreted alkaline phosphatase, IFN, interferon.

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