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Design and Synthesis of Tetrahydropyridopyrimidine Based Toll-Like Receptor (TLR) 7/8 Dual Agonists

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Abstract. In a continuing effort to discover novel TLR agonists, herein we report on the discovery and structure-activity relationship of novel tetrahydropyridopyrimidine TLR 7/8 agonists. Optimization of this series towards dual agonist activity and a high clearance profile resulted in the identification of compound **52a1**. Evaluation *in vivo* revealed an interferon stimulated response (ISG) in mice with limited systemic exposure and demonstrated the potential in antiviral treatment or as a vaccine adjuvant.

Key words: Immunomodulator, Toll-Like Receptor, TLR7/8, tetrahydropyridopyrimidine.

Toll-like receptors (TLRs) have the important role of recognizing molecular patterns that are present in foreign pathogens and to subsequently activate an immune response.[1] Single-stranded viral RNA is recognized by TLR7 and 8, the agonism of which induces a robust T_H1-type immune response. TLR 7 is expressed in plasmacytoid dendritic cells (pDCs) in humans, inducing the production of endogenous IFNα, and on B cells, to a lesser extent, inducing the proliferation and secretion of antibodies. TLR 8 however, is principally expressed in monocytes and myeloid dendritic cells (mDCs), and activation also results in a

strong T_H1 response primarily due to secretion of IL-12.[2,3,4] Therefore, TLR7/8 agonists show potential in the treatment of viral infections and other indications, such as potential adjuvants for therapeutic vaccines.[5,6,7]

The objective of this study was to identify an agonist with equal activity on both TLRs 7 and 8, based on the data generated from HEK293 cells transfected with hTLR7 or 8, and be structurally different to previously reported dual agonists. As a verification of cytokine production, a second *in vitro* assay was employed on human peripheral blood mononuclear cells (hPBMCs) where activation of human TLR7 on plasmacytoid dendritic cells (pDCs) effected the production of IFN and other cytokines, after which the conditioned media was added to the HCV replicon system measuring antiviral activity. Data for both assays are reported as least effect concentrations (LEC) defined as the concentration that induces an effect at least two-fold above the standard deviation of the assay (see supplementary data). Finally, a high first pass effect was targeted *in vivo*, to display a pharmacodynamic effect and limit risk factors associated with systemic exposure of TLR agonists.[8,9]

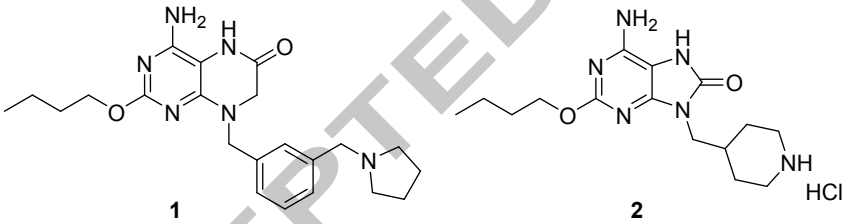


Figure 1
Structure of selected TLR agonists containing an aliphatic amine.

GS-9620 (**1,** Figure 1) is a selective TLR 7 agonist that has been studied in clinical trials for the treatment of chronic HBV.[10] It is a potent bicyclic agonist, with a pendent benzyl group further substituted by a methylenepyrrolidine, and displays a high first pass effect. The hydroxypurine series are described as TLR7 selective agonists.[11,12] An exception to this selective series was noted when the purine scaffold and pendant piperidine were separated by one methylene unit (**2**), that afforded potency on both TLR7 and 8 (Figure 1).[13] It was hypothesized that the position of the amine may be a factor influencing the selectivity for TLR7

vs 8. In the conceptual design of a new scaffold, targeting both TLR7 and 8, the pyrimidine core structure from previously described TLR7/8 agonist series (**3, 4**)[14,15] was combined with a piperidine ring fused across the 5,6-bond of the pyrimidine scaffold to afford novel agonist **5** (Figure 2).[15b] The piperidine is thus included into the bicyclic scaffold to provide a more rigid structure with greater control over the conformation to empirically learn about TLR 7 vs. 8 selectivity following varied substitution.

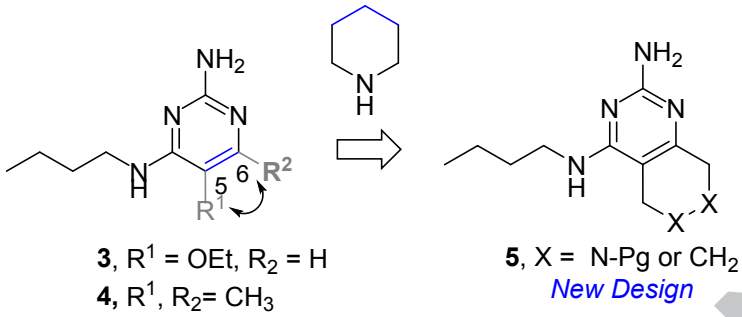


Figure 2.
Novel series of tetrahydropyridopyrimidine TLR 7/8 agonists derived from described pyrimidine series.[14,15]

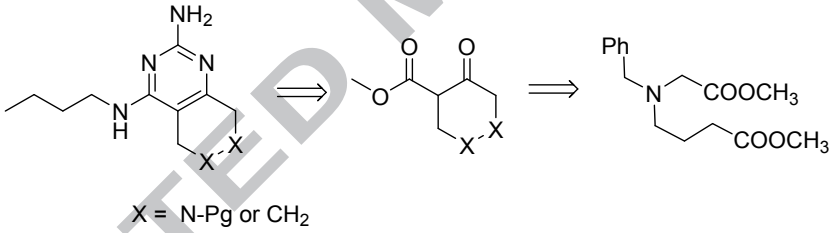
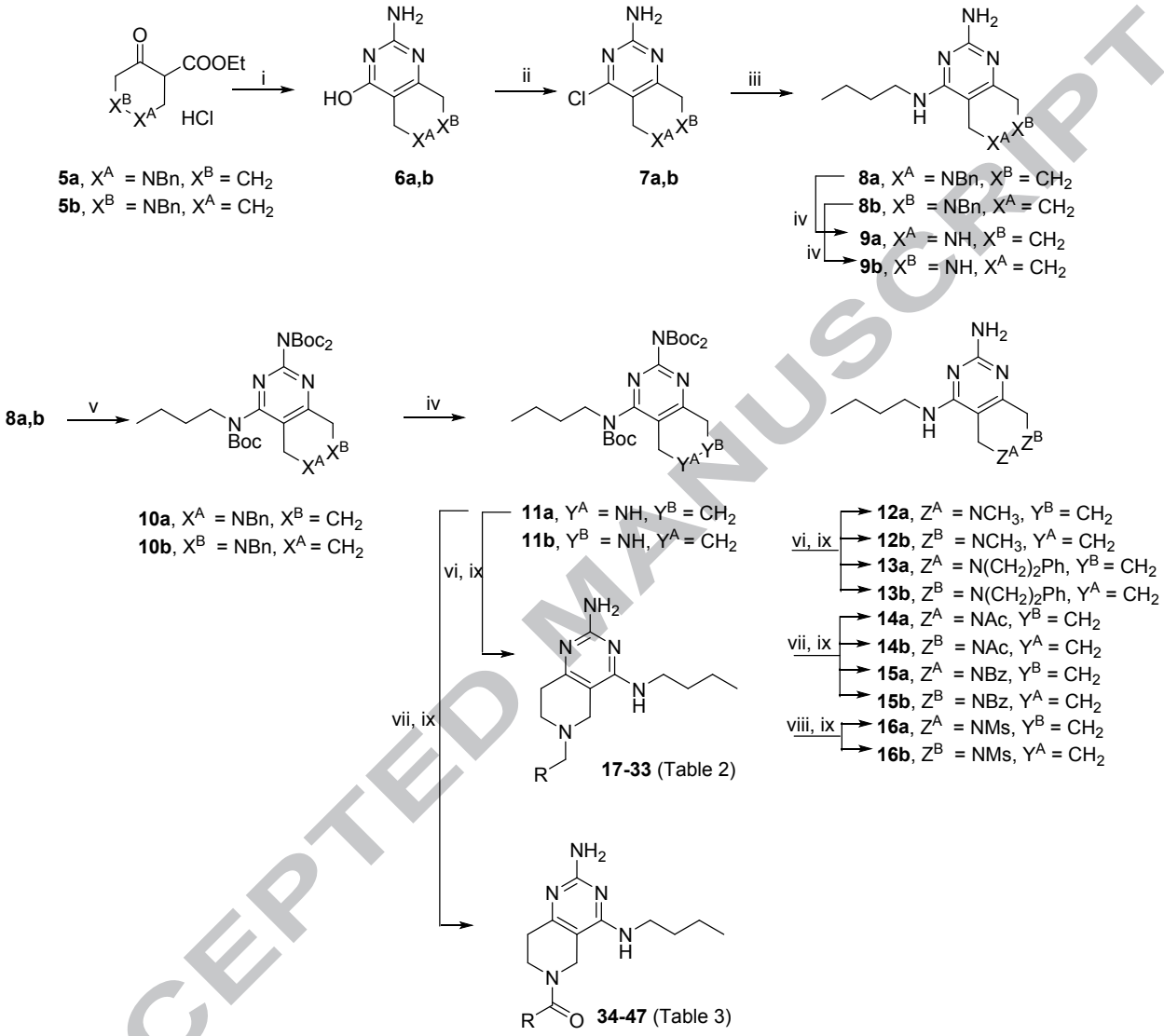


Figure 3.
Retrosynthetic analysis of novel TLR7/8 agonists. Pg; protecting group.

Tetrahydropiperidinopyrimidines are described as drug-like scaffolds concerning a variety of drug targets, such as calcium-calmodulin protein kinase inhibitors,[16] as phosphodiesterase 10A inhibitors for the treatment of schizophrenia,[17] and towards the potential treatment of cystic fibrosis.[18] Retrosynthetically, the tetrahydropyridopyrimidine scaffold can be derived from the corresponding oxo-piperidinecarboxylates, which in turn could be formed by a Dieckman condensation from a protected aminodiester (Figure 3). The synthesis pathway was analogous to methods described in the literature.[18,19] Initial exploration in potency and selectivity originated from a library of electrophiles on the piperidine nitrogen. Further modifications

to the carbons of the fused piperidine ring, if needed, could arise from alteration of the precursors or be introduced using other methods.[19b]

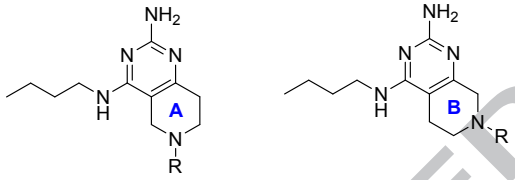


Scheme 1. Synthesis of TLR agonists. Reagents and conditions: (i) guanidine carbonate, EtOH, reflux, 18h (ii) $POCl_3$, 100ºC, 4h (iii) Al_2O_3, 1,4-dioxane, *n*-butylamine 120ºC, 18h (iv) H_2, 10% Pd/C, CH_3OH, rt, 2h (v) Boc_2O, DMAP, THF, 80 ºC, 4h (vi) RCHO, $NaHB(OAc)_3$, DCE, rt, 18h (vii) RCOCl, DMAP, Et_3N, rt, 16h (viii) MsCl, DMAP, Et_3N, 0ºC to rt, 18h (ix) 4M HCl in dioxane, rt, 18h.

Regardless of the electrophile substitution on the nitrogen of regioisomers A and B, agonists **8-16** showed a varied degree of TLR8 selectivity (Table 1) and less potency on TLR7 in comparison with in-house data on Resiquimod (TLR7 LEC = 0.1 μM, TLR8 LEC = 0.3 μM). The most potent analogs were indeed the

more basic benzyl, methyl and phenethyl analogs (**8a, 12a, 13a, 13b** respectively). Although these agonists provided intriguing potency, the desired 1:1 ratio of TLR7:8 agonism was not observed. Furthermore, concern was raised about agonists containing a basic piperidine moiety, a potential cationic center that may have an impact on physicochemical properties and pharmacokinetics, in particular, an increase in the volume of distribution, and the possible risk of phospholipidosis.[20,21] This could result in a longer terminal half-life, potentially risking toxicity associated with systemic cytokine activation.[8] The greatest differences in TLR8 activity between regioisomers A and B were the unsubstituted scaffold (**9a vs 9b**) and the methanesulfonamide (**16a vs 16b**). Finally, the benzamide substituent **15a** of the regioisomer A subseries gave the desired equal ratio of TLR7 to 8 activities, while its congener **15b**, in the regioisomer B subseries, was five-fold more selective for TLR8. Thus, exploration continued solely on the regioisomer A subseries.

Table 1
Electrophile scan on regioisomers A and B



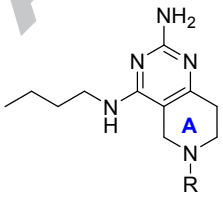
Entry*	R	Regio-isomer	LEC[a] hTLR7 (μM)	LEC[a] hTLR8 (μM)
8a	Bn	A	1.6	0.06
8b	Bn	B	11	0.4
9a	H	A	>25	7.2
9b	H	B	8.9	0.6
12a	CH_3	A	9.6	0.03
12b	CH_3	B	10	0.2
13a	$Ph(CH_2)_2$-	A	1.8	0.2
13b	$Ph(CH_2)_2$-	B	6.5	0.2
14a	Ac	A	4.9	0.5
14b	Ac	B	5.8	0.4
15a	Bz	A	1.5	1.4
15b	Bz	B	3.9	0.8
16a	Ms	A	>25	9.9
16b	Ms	B	20	0.8

*All compounds had CC_{50} > 24 μM
[a]LEC; least effective concentration (see supplementary data)

Before initiating further exploration into benzamide analogs of **15a** in the regioisomer A subseries, benzyl substituents were briefly examined because of the numerous possibilities for variation from readily available reagents. Indeed, with benzyl substituents, one can influence the conformation of the ring in space by groups on the 2-phenyl position, and influence the electronics by changing the ring substituents. The library approach provided facile identification of possible protein interactions, via substituent effects, or π-stacking, that could affect TLR potency and selectivity, or could reveal differences in physicochemical properties.

The synthesis of a library of benzylamine products on regioisomer A, started from Boc-protected intermediate **10a** (Scheme 1). The variable benzyl group was installed via reductive alkylation employing the corresponding aldehyde in DCE, using sodium triacetoxyborohydride as a reducing agent, to afford agonists **17-23** (Scheme 1). Overall, the activity of the benzylamine regioisomer A subseries had greater selectivity for TLR8 (Table 2). Compounds containing a substituent at the 2-position of the benzene ring (**17-20**) showed the best potency on this subseries but were not able to influence the selectivity vs. that of the unsubstituted benzene ring (**8a**). Substitution at the benzylic-4-position (e.g. **22**) was deleterious to activity on TLR7, especially with larger groups (**23-26**), but left little effect on TLR 8 agonist potential. The crowded 1-napthyl analog (**27**) shared the same selectivity as **8a**. Heterocyclic ring analogs (**28-33**) also led to decreased activity versus **8a**, and showed no compounds having equal ratio of TLR7 vs 8 agonism. Attention thus returned to forming a library of amides on regioisomer A to identify additional agonists with equal potency on TLR7 vs 8.

Table 2
Activity of the benzyl substituents on regioisomer A



Entry*	R	LEC[a] hTLR7	LEC[a]	LEC[a]

		(μM)	hTLR8 (μM)	hPBMC (μM)
17	2-chlorobenzyl	1.5	0.05	0.15
18	2-chloro-3-methoxybenzyl	1.0	0.04	0.05
19	2,3-dichlorobenzyl	1.5	0.18	0.25
20	2,6-dichlorobenzyl	2.4	0.14	0.19
21	3,5-difluorobenzyl	3.5	0.23	0.53
22	4-methoxybenzyl	2.1	0.08	0.17
23	4-(trifluoromethoxy)benzyl	4.7	0.24	0.52
24	4-trifluoromethylbenzyl	2.8	0.23	0.54
25	4-*t*-butylbenzyl	19	0.66	2.79
26	4-phenyl-benzyl	14	0.50	4.49
27	1-napthyl-CH_2-	1.3	0.43	0.43
28	2-pyridyl-CH_2-	3.3	0.07	0.14
29	3-pyridyl-CH_2-	3.0	0.13	0.15
30	4-pyridyl-CH_2-	5.2	0.29	0.50
31	2-thiophen-CH_2-	2.5	0.14	0.16
32	2-furyl-CH_2-	3.7	0.08	0.07
33	3-indole-CH_2-	>20	0.22	0.18

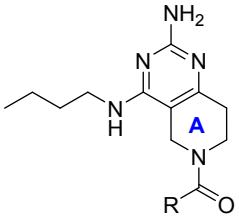
*All compounds had CC_{50} >24 μM

[a]LEC; least effective concentration (see supplementary data).

The library of amide analogs on regioisomer A (**34-47**) were prepared by reacting the variable acid chloride with **10a** under basic conditions in dichloromethane with DMAP at ambient temperature (Scheme 1, Table 3). Alkyl amides (**34-36**) proved active but TLR8 selective. The 2 or 4-pyridyl analogs (**37, 38**) led to less active agonists, and the same for the pyrazine congeners **39**, and **40**. The thiophene amide **41** proved to be more potent than the isosteric benzamide (**15a**), had a 1:1 ratio of agonist activity, and was the most potent of the series. The 4-thiazole derivative **42**, showed desirable activity. However, the addition of a 2-amino group to the heterocyclic amide, as in **43**, led to a significant loss in potency on both TLR7 and 8. Activity was regained in the 2-methyl-4-thiazole analog **44**. The opposite observation in relative activity was made in the 5-thiazole amides (**45** vs **46**) where the amino-substituent proved more active than the methylthiazole analog. Heterocyclic amides **47-49** displayed activity above 1 μM, and were found to be nearly equipotent on the PBMC assay. In summary, **41** and **44** had the desired 1:1 ratio of agonist activity but showed insufficient selectivity over the hERG antitarget (hERG IC_{50} = 0.9 and 1.3 μM respectively).[22] While low plasma concentrations of **41** and **44** (99 and 88 percent metabolized in mouse liver microsomes after 15 minutes at 1μM concentration, respectively) were expected after oral

administration, however, an effort was made to remove this off-target activity. Further derivatives were made to address the potential cardiovascular liability of **41** and **44**.

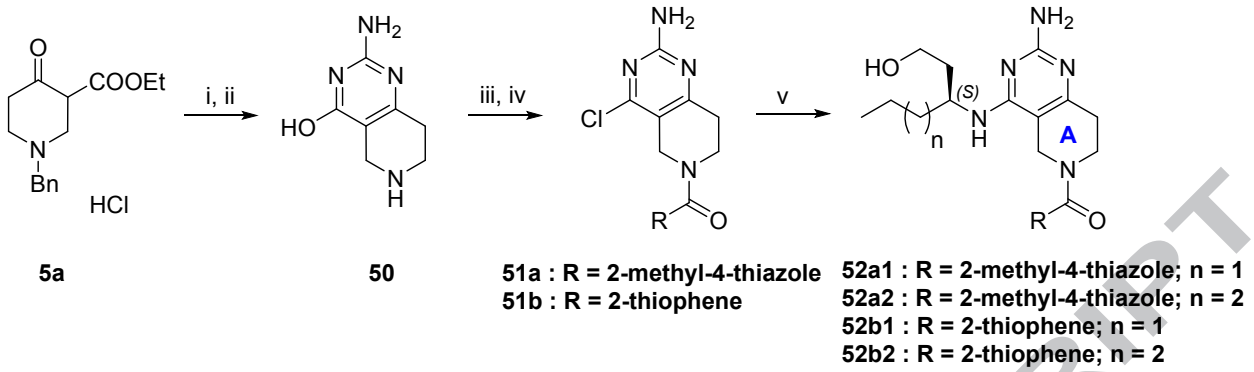
Table 3
Activity of the aromatic amide series on regioisomer A.



Entry*	R	LEC[a] hTLR7 (μM)	LEC[a] hTLR8 (μM)	LEC[a] hPBMC (μM)
34	Et	2.3	0.4	0.53
35	Ipr	8.1	1.6	1.80
36	cyclobutyl	0.8	0.4	0.23
37	2-pyridyl	9.5	2.9	2.21
38	4-pyridyl	>25	>25	3.56
39	2-pyrazinyl	3.7	2.2	2.38
40	2-pyrazinyl-5-methyl	11	6.8	1.07
41	2-thiophene	0.2	0.4	0.07
42	4-thiazole	1.2	0.5	0.17
43	2-amino-4-thiazole	8.0	6.9	1.05
44	2-methyl-4-thiazole	2.0	1.8	0.53
45	2-amino-5-thiazole	2.1	4.4	0.38
46	4-methyl-5-thiazole	13	5.6	2.23
47	1,2,3-thiadiazole	0.9	0.1	0.15
48	4-oxazole	0.7	0.2	0.17
49	3-methylisoxazole	0.8	0.4	0.22

[a]LEC; least effective concentration (see supplementary data). All compounds had CC_{50} >24 μM

It was previously described in a related series of TLR agonists that the presence of a branched (*S*)-configured aminoalcohol aided in the reduction of off-target activity.[14] Thus, the same strategy was applied to this scaffold. The favored 2-thiophene (**41**), and 2-methyl-4-thiazole (**44**) amides were held constant in the next phase of exploration where aminoalcohol derivatives were formed to explore their potential to reduce hERG binding.

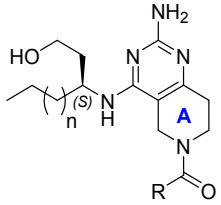


Scheme 2. Synthesis of TLR agonists with an aminoalcohol variation. Reagents and conditions: (i) guanidine carbonate, EtOH, reflux, 18h, (ii) H_2, 10%Pd/C, methanol, rt, 2h (iii) R-COCl, Et_3N, DMAP, CH_2Cl_2, rt, 16h (iv) $POCl_3$, 100ºC, 4h (v) 3-aminohexanol or 3-aminoheptanol, IPA, 120ºC, 18h.

Akin to the chemistry described in scheme 1, **5a** was converted to **50** in two steps (Scheme 2). The amines were acylated with the preferred acid chlorides, and the 4-chloro species **51a** and **51b** were afforded after treatment with phosphorousoxychloride at reflux. **51a** was then reacted with 3-aminohexanol or 3-aminoheptanol to afford **52a1** and **52a2**, respectively. Likewise, **51b** was also reacted with the two γ-aminoalcohols to afford **52b1** and **52b2**. The resulting products had acceptable agonist potential and showed a clear reduction in hERG potential. The aminoalcohol modification in amide products **52a1**, and **52a2**, resulted in over 20-fold reduction in hERG binding compared to the *n*-butylamine congener **44**. Analogs containing the larger (*S*)-3-aminoheptanol (**52a2**, **52b2**) trended toward TLR8 selectivity when compared to the (*S*)-3-aminohexanol congeners (**52a1**, **52b1**). Noteworthy, all four compounds shared the same potency in the PBMC assay (Table 4). In summary, **52a1** represented nearly equal agonism on both TLR7 and 8, with a potentially reduced cardiovascular risk. To validate these *in vitro* results, compound **52a1** was examined *in vivo* for its pharmacokinetic and pharmacodynamic properties. **52a1** was administered orally to healthy C57Bl/6 mice at a dose of 5mg/kg. **52a1** was found to be readily absorbed (T_{max} = 0.5h) and rapidly cleared, in line with our target product profile, resulting in low plasma concentrations but considerably higher compound concentration in the liver. Despite the low C_{max} seen in mice, pharmacodynamic results showed a response in interferon γ-induced protein (IP-10), in plasma and in the liver, where the T_{max} was measured at 4h. This data suggests that compound **52a1** induced an antiviral innate immune response. This is reassuring regarding the safety

of **52a1** in that at low dose antiviral cytokines could be observed without the negative aspects of systemic IFNα.[23]

Table 4
Activity of selected aromatic amides with an amino alcohol modification

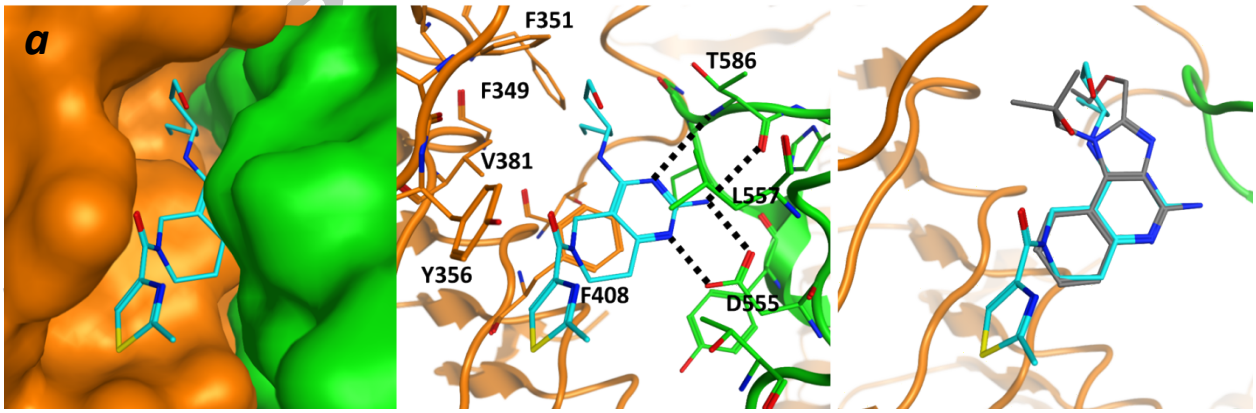


Entry	n	R	LEC[a] hTLR7 (μM)	LEC[a] hTLR8 (μM)	LEC[a] hPBMC (μM)	hERG IC_{50} (μM)	LM[b] (m,h)
52a1	1	2-methyl-4-thiazole	1.5	3.4	0.2	22	33, 21
52a2	2	2-methyl-4-thiazole	1.0	6.6	0.2	>50	71, 30
52b1	1	2-thiophene	0.2	1.1	0.2	7.6	73, 22
52b2	2	2-thiophene	0.2	1.7	0.2	6.9	90, 37

[a]LEC; least effective concentration (see supplementary data). All compounds had CC_{50} >24 μM
[b]LM; liver microsome stability (percent turnover at 1 μM, 15 min.). m; mouse. h; human.

Table 5
Mouse pharmacokinetic and pharmacodynamic parameters after oral administration of 5 mg/kg.

Entry	T_{max} (h)	C_{max}		AUC $_{0-last}$ (ng·h/mL)		mIFNα		IP-10		
	Plasma	Plasma (ng/mL)	Liver (ng/g)	Plasma (ng/mL)	Liver (ng/g)	Plasma Cmax (pg/mL)	Liver Cmax (pg/g)	Plasma Cmax (pg/mL)	Liver Cmax (pg/g)	T_{max} (h)
52a1	0.5	29 ± 9.0	711 ± 657	70	1410	0	0	20 ± 7	75 ± 21	4



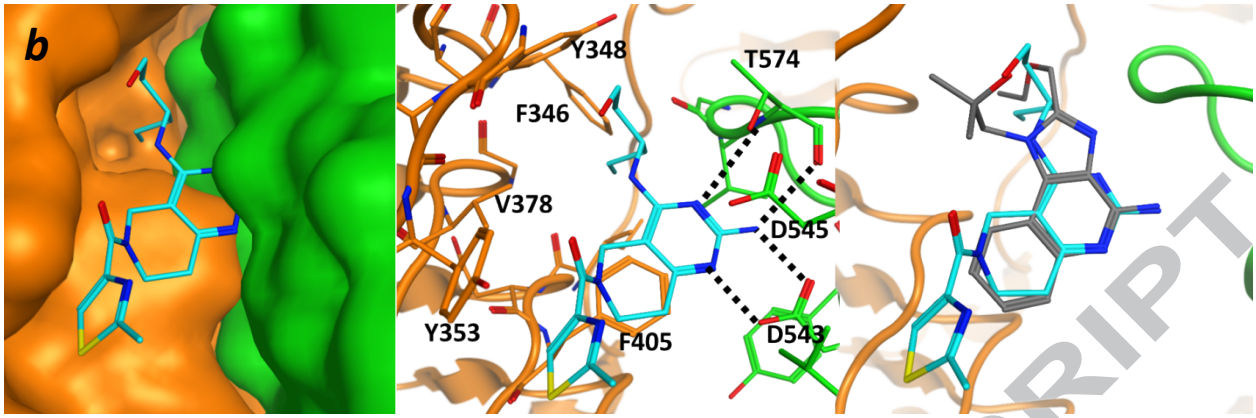


Figure 4. Compound **52a1** modeled in the Resiquimod binding pocket on (a) monkey TLR7 dimer interface (PDB ID:5GMH) & (b) human TLR8 dimer interface (PDB ID: 3W3N). The two monomers are colored orange and green, respectively. The surface representation is on the left, labelled key interactions in the middle, and an overlay with Resiquimod on the right. The carbon atoms of **52a1** are colored in cyan, while the carbon atoms of Resiquimod are in gray.

Figure 4 shows potential binding modes of compound **52a1** in the TLR7 and TLR8 dimer interfaces. Analogous to Resiquimod, **52a1** forms hydrogen-bonding interactions with aspartate residues (ASP555 in TLR7 and ASP543 in TLR8) and backbone atoms (THR586 in TLR7 and THR574 in TLR8), in both TLR7 and 8. Additionally, the pendant alkyl chain of **52a1**, makes a tight fit into the deep hydrophobic pocket, composed of residues including mainly valine, phenylalanine and tyrosine at the dimer interface in both TLR7 and TLR8. The alcohol chain, while having van der waals interactions, remains solvent exposed at the distal end, in both cases. Despite a good ligand based overlay with Resiquimod, there is an uncertainty in the orientation of the 2-methyl-4-thiazole amide substituent, as well as corresponding receptor residues around this moiety, due to observed clashes in the receptor crystal structures bound to directly overlaid conformations of **52a1**. Although in the current model, such clashes are relieved by selecting different lower-energy rotamers of tyrosine residues (Figure 4; TYR356 in TLR7 and TYR353 in TLR8) stacked with the thiazole moiety, it is possible that the conformations of the receptors are different, especially in the loops containing these tyrosine residues or some other form of conformational plasticity in this region, for instance, in the relative orientation of the TLR monomers. Overall, this model of **52a1** in complex with the receptor, provides a partial structural basis for the dual agonistic nature, as it shows how this compound engages conserved residues in both TLR7 and 8.

In conclusion, a series of dual TLR7/8 agonists were described, with many compounds showing inherent TLR8 selectivity. Among these, **52a1**, of the amide subseries on regioisomer A, was identified as a novel and potent dual agonist. The pharmacokinetic profile of **52a1** in mice confirmed the low systemic exposure, and target engagement was demonstrated by the induction of IP-10. In addition, **52a1** showed low plasma protein binding (30% bound in mice plasma) no inhibition of major CYP450 isozymes (CYP450 >10 μM: 3A4, 2C8, 2C9, 2D6, 1A2, 2C19) and lacked off-target activity across a multireceptor panel (>10 μM against histamine, dopamine, and serotonin subtypes). This series warrants further exploration as potential immunomodulating agents.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at ...

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Highlights

- A new series of TLR 7/8 dual agonists based on tetrahydropyridopyrimidines.
- A desired high first pass effect was observed to limit systemic exposure and cytokine activation.
- Target engagement was demonstrated by the induction of IP-10 after oral administration in mice.

24.