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Discovery of potent, orally bioavailable *in vivo* efficacious antagonists of the TLR7/8 pathway

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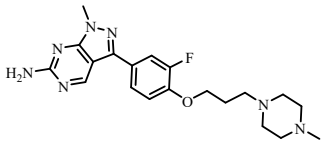
ABSTRACT

Antagonism of the Toll-like receptors (TLRs) 7 and TLR8 has been hypothesized to be beneficial to patients suffering from autoimmune conditions. A phenotypic screen for small molecule antagonists of TLR7/8 was carried out in a murine P4H1 cell line. Compound **1** was identified as a hit that showed antagonistic activity on TLR7 and TLR8 but not TLR9, as shown on human peripheral blood mononuclear cells (hPBMCs). It was functionally cross reactive with mouse TLR7 but lacked oral exposure and had only modest potency. Chemical optimization resulted in **2,** which showed *in vivo* efficacy following intraperitoneal administration. Further optimization resulted in **8** which had excellent *in vitro* activity, exposure and *in vivo* activity. Additional work to improve physical properties resulted in **15**, an advanced lead that had favorable *in vitro* and exposure properties. It was further demonstrated that activity of the series tracked with binding to the extracellular domain of TLR7 implicating that the target of this series are endosomal TLRs rather than downstream signaling pathways.

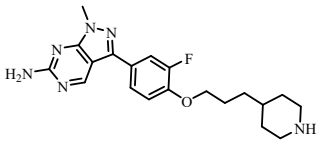
Toll-like Receptors (TLRs) are critical components of the innate immune response to Danger Associated Molecular Patterns (DAMPs)[1], which are molecular signatures of infection. For example, endosomal hypomethylated CpG rich DNA and single-stranded RNA (ssRNA) can indicate the presence of infection by viruses or bacteria. As such, TLR7 and TLR8 are capable of detecting endosomal ssRNA, and TLR9 is capable of detecting endosomal CpG-rich DNAs, to initiate innate responses against pathogens. In recent years, several lines of evidence suggested that in some autoimmune diseases, the body's own RNA or DNA may gain access to endosomes, aberrantly activate TLR7/8/9 and support the development of chronic inflammation.[2] Therefore, inhibiting endosomal TLRs has been proposed as a novel therapeutic approach for autoimmune diseases.[2] There have since been significant efforts in this space.[3-8] Here we describe the identification and characterization of a novel series of low molecular weight inhibitors of the TLR7/8 pathway.

In search of small molecule TLR7/8 inhibitors, two million compounds were screened on the murine dendritic cell (mDC) line P4H1[9], for the ability to inhibit IL-6 production induced by the low molecular weight TLR7 agonist, imiquimod.[10] Compounds showing activity of <10 μM and not interfering with cell viability were further studied on P4H1 cells for inhibition of IL-6 production induced by the TLR4 agonist, Lipopolysaccharide (LPS).[11] Only compounds without activity in this assay were further pursued.



1
(Hit compound)

mDC (TLR7): 0.17 μM
mDC (Tox): 0.90 μM
mSplenocyte (TLR7): 1.0 μM
mSplenocyte (TLR9): 2.0 μM
hPBMC (TLR7): 0.31 μM
hPBMC Tox: 30 μM



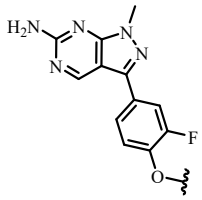
2

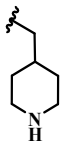
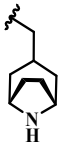
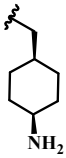
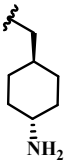
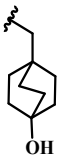
hPBMC (TLR7): 0.053 μM
THP-1 (TLR8): 0.026 μM
hPBMC (TLR9): 2.3 μM
mSplenocyte (TLR7): 0.097 μM
mSplenocyte (TLR9): 1.3 μM

Figu For derivative used in an *in vivo* experiment. Values are IC_{50}.

Next, the antagonist activity of the TLR4-inactive hits derived from the above TLR7 screen was confirmed on mouse splenocytes using ssRNA40 as TLR7 agonist and IL-6 as a readout. Selectivity was ascertained on mouse splenocytes using the TLR9 agonist CpG1585 (TLR9) and IL-6 as a readout. The next step was to this purpose, human peripheral blood mononuclear cells (hPBMCs) were stimulated with the TLR7/8 ligand ssRNA40, with the TLR7-dependent cytokine IFN-α as a readout It was found that most of the hits from the mDC screen were not cross reactive to hPBMCs.

Table 1. Structure activity relationship of the basic amine bearing tail group[a]



Assay[b,c]	**3** 	**4** 	**5** 	**6** 	**7** 	**8** 	**9** 
hPBMC (TLR7)	0.17	0.095	0.50	0.071	0.025	0.006	0.20
THP-1 (TLR8)	0.06	0.088	0.01	0.02	0.005	0.003	0.075
hPBMC (TLR9)	3.4	1.2	6.5	2.2	1.4	2.7	5.3
mSplenocyte (TLR7)	0.22	0.12		0.15	0.11	0.038	1.2
mSplenocyte (TLR9)	2.0	1.2	1.2	1.2	1.9	0.84	2.8

[a]Values are IC_{50} in μM.

[b]Details of assay conditions are in Supplemental Information.

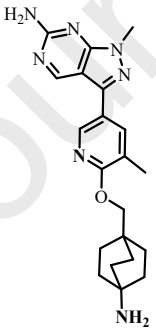
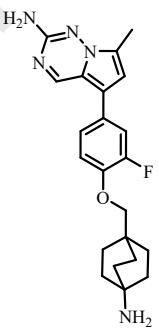
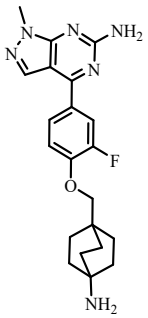
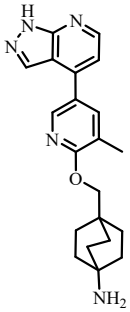
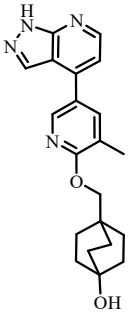
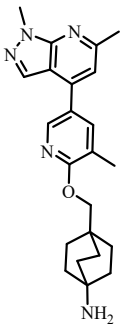
[c]Cellular toxicity was run as a negative control, and was not observed up to the highest level of compound tested (10 μM)

Compound **1**[12] shown in Figure 1 was found to be an exception. This hit not only confirmed in the screening assay using the synthetic TLR7 agonist imiquimod but also demonstrated moderate antagonist activity in mouse splenocytes using ssRNA as a TLR7 agonist. Importantly, TLR7 antagonist activity was maintained in hPBMC's, and this without an indication of cellular toxicity. Therefore **1** was selected for further optimization.

With the substitution of the methyl piperazine for piperidine, **2** was obtained. At this point, two additional assays were used to enhance the characterization suite. Mice do not express a functional TLR8[13], so to investigate TLR8 activity in human cells THP-1 cells[14] , were stimulated with the synthetic TLR7/8 agonist resiquimod (R848)[10] and the TLR8-dependent cytokine TNFα was read out. To investigate TLR9 activity in human cells, hPBMCs were stimulated with the TLR9 agonist ODN2216 reading out IFNα.

It was found that **2** demonstrated 97 nM activity on TLR7 in mouse splenocytes with substantial selectivity over TLR9. In human cells, the potency on TLR7 and TLR8 was even slightly better with a high degree of selectivity over TLR9. Although **2** did not have adequate oral exposure (Table 3), exposure following i.p. dosing was considered sufficient to demonstrate *in vivo* efficacy.

Table 2. Stucture Activity Relationships for the rest of the scaffold[a]

Assay[b]	**10** 	**11** 	**12** 	**13** 	**14** 	**15** 
hPBMC (TLR7)	<0.001	0.046	0.045	<0.001	0.003	<0.001
THP-1 (TLR8)	0.001	0.040	0.018	<0.001	<0.001	0.002
hPBMC (TLR9)	3.3	3.4	3.7	1	>5	ND
mSplenocyte (TLR7)	ND	0.093	0.30	0.004	0.055	ND
mSplenocyte (TLR9)	ND	0.46	0.80	1.2	>10	ND

[a]Values are IC_{50} in μM., ND: No Data.

[b]Details of assay conditions are in Supplemental Information.

female 129/Sv mice, blocked both IFN-α and IL6 secretion in serum after a challenge with ssRNA006. The secretion of the same cytokines in blood in response to CpG1585 was only modestly affected. The exposure of **2** at 2 hours was 1.5 µM, consistent with the *in vitro* IC50 (Figure 2).

Based on these results, the SAR for the amine containing portion of the molecule was further explored (Table 1).

Shortening the linker to the piperidine to 1 carbon afforded **3** which was slightly less active but did exhibit very modest but measurable p. o. exposure in mouse. Metabolite ID studies pointed to robust metabolism of the piperidine ring so modifications were pursued. Introduction of a bicyclic bridge afforded **4** which maintained activity. The spirocyclic derivative **5** lost some activity while moving the amine function out of the ring in **6** and **7** maintained or slightly improved activity. None of these changes were effective in improving p. o. exposure in mouse (Supplementary Material).

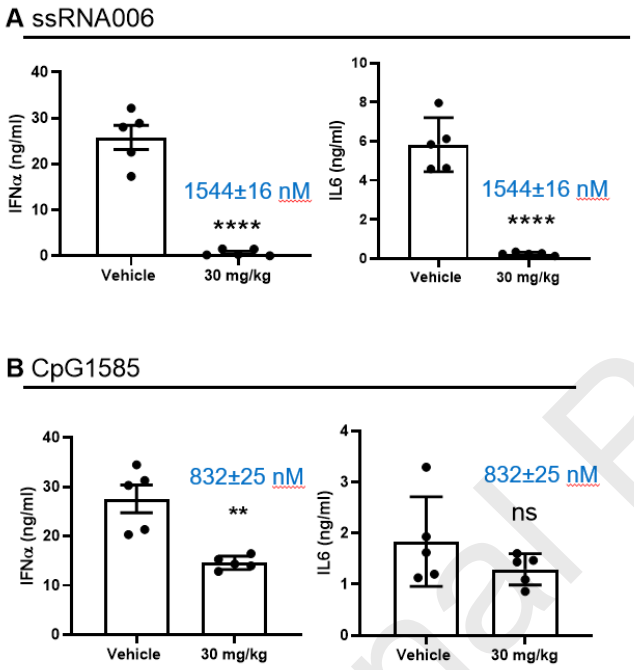


Figure 2: Compound 2 inhibits (A) TLR7/ssRNA006- and (B) TLR9/CpG1585-mediated cytokines in mice

Bars represent means of n=5 ± SD; corresponding blood exposures ± SD are shown in blue above the respective bars. **, p<0.01, ****, p<0.0001, unpaired t test.

The [2,2,2] bicyclic derivative **8** was made to introduce steric bulk next to the amine functionality with the hypothesis that this would mitigate metabolism and fortuitously resulted in a significant boost in activity both in human and mouse cellular assays. Gratifyingly, **8** also had very good oral exposure when dosed in suspension to Balb/c male mice (Table 3). Up to this point, it had not been possible to identify any derivatives in this series without an amine. As an exception, **9** was found to still have an appreciable level of antagonism for TLR7 and TLR8, though not in mouse cells.

When **8** was dosed as the HCl salt p. o. at 5 mg/kg to female 129/Sv mice (Figure 3), both IFN-α and IL6 secretion were completely blocked in blood 2 h after challenge with ssRNA006. As mentioned above, TLR8 is not functional in mice, which would indicate that TLR7 is the major driver of both IFN-α and IL6 expect that ssRNA would drive IFN-α and IL6 via TLR7 and TLR8, respectively. The secretion of the same cytokines in response to CpG1585 was only modestly affected. The exposure **2** at 2 hours was 300 nM, making the *in vivo* data consistent with the *in vitro* derived activity data.

Although the activity of **8** was encouraging, there were challenges with the physical properties of this molecule, especially the solubility (high throughput solubility of the HCl salt at pH 6.8: 0.023 µM). The team decided to pursue an exploration of derivatives that were meant to enhance the properties of the molecule. Table 2 shows some key compounds in this effort.

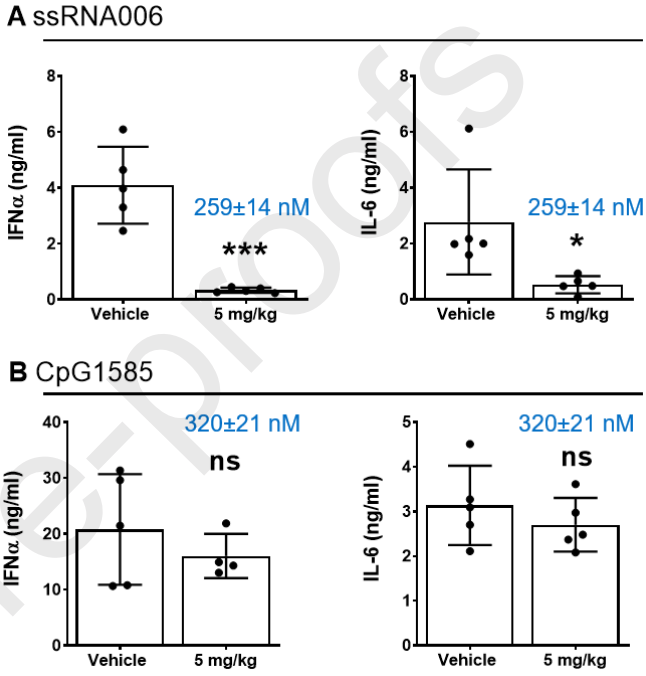


Figure 3: Compound 8 inhibits (A) TLR7/ssRNA006- but not (B) TLR9/CpG1585-mediated cytokines in mice

Bars represent means of n=5 ± SD; corresponding blood exposures ± SD are shown in blue above the respective bars. *, p<0.05, ****, p<0.0001, unpaired t test.

Replacement of the fluorophenyl group with a 3-methyl pyridine afforded **10** which gave a substantial boost to potency as well as allowing the formation of soluble salts. Head group exploration afforded **11** which retained activity. Moving the attachment point to the 4 position of the pyrazolo-pyrimidine ring system afforded **12** which similarly maintained activity. Additional exploration of the bicyclic head group combined with the 3-methyl pyridine result afforded **13** which had very potent activity in human and mouse cell lines. This optimized skeleton now allowed the substitution of an alcohol for an amine to retain activity (**14**). It was found that **13** was a substrate for aldehyde oxidase (Supplementary Material).[15] This would complicate development since this enzyme is only expressed in higher species and can result in highly compromised oral exposure once dosed in these species. For this reason, the putative site of metabolism was blocked with a methyl group on the 6-position of the pyrazolo-pyridine ring. Additionally, it was found that blocking of the NH was beneficial for p. o. exposure and the advanced lead **15** was synthesized which demonstrated a lack of aldehyde oxidase metabolism along with outstanding *in vitro* activity in both human and mouse cells.

compounds. It should be noted that in the case of **8**, a specific crystal polymorph needed to be used in order to get reproducible data. While both **8** and **10** exhibited roughly similar exposure parameters, switching to the pyrrolo pyridine (**13**) head group resulted in markedly improved exposure. The V_{ss} was also reduced from 15 to 5.1, a marked improvement. Substitution of the primary amine for and alcohol (**14**) maintained excellent exposure and further reduced the V_{ss} to 3.1. Methylation of the pyrazolo-pyridine head group (**15**) maintained good p. o. exposure and excellent human potency, but was not optimal in IV clearance and V_{ss}.

Parameter	**2**	**8**	**10**	**13**	**14**	**15**
p. o. AUC (h*nM)	0	3046	2983	29600	36300[c]	28900
p. o. C_{max} (nM)	0	506	354	3230	4370[c]	1400
IV CL (mL/min/kg)	165	98	96	23	11	44
IV V_{ss} (L/kg)	29	15	15	5.1	3.1	13.8
IV T1/2 (h)	5.9	4.1	4.1	4.0	4.7	4.9
F (%)	0	36	34	75	43	150

Table 3. PK parameters in mouse[a,b,c]

[a]Balb\c male mice, 3 animals per cohort. 5mg/kg IV, 20 mg/kg p. o.. PEG300/D5W, 3:1 formulation.

[b]p.o. – orally dosed, AUC- area under the curve, C_{max} – maximum observed concentration, IV CL – observed clearance when dosed intravenously, IV V_{ss} – observed steady state volume when dosed intravenously, IV T1/2 – observed terminal half life when dosed intravenously, F – observed bioavailability.

[c]p. o. formulation was 2.5 mg/mL suspension in 0.5%MC/0.5% Tween

Although the overall parameters of **15** looked compelling, there were additional efforts ongoing to further increase the sp3 character of the scaffold[16,17] which ultimately delivered the preferred candidate. These efforts will be reported in a separate publication.

Concomitantly with the SAR efforts based on functional readouts, the team was conducting target identification activities for this series of TLR7/8 antagonists. It was hypothesized that selective inhibition of TLR7 and TLR8 over TLR9 would involve a signaling node unique to these TLRs. Alternatively, the inhibition could be mediated through binding to the TLRs themselves. To clarify this point, chimeric TLR domain swap mutants were expressed in HEK293[18] cells where the TLR7 extracellular domain (ECD) was fused to the TLR9 intracellular domain (ICD) and vice versa. The cells were then stimulated with either ssRNA40 for the TLR7 ECD or R848 for the TLR9 ECD and NFκB was read out. Table 4 shows the activity of 13 and 14 on these mutants.

Assay	**13**	**14**
TLR7 ECD, TLR9 ICD	< 0.001 μM	< 0.001 μM
TLR9 ECD, TLR7 ICD	>5 μM	1.5 μM

Table 4. TLR7/9 chimeric receptor activity

The fact that TLR7 potency tracked with the presence of the ligand binding TLR7 ECD and not the chimera with the TLR9 inhibition by interaction with TLR7 itself.

In summary, we have demonstrated how a pathway based screen served to find inhibitors of the human TLR7/8 pathway in mouse dendritic cells. The team started with the hit **1** which had modest activity and no oral exposure. After showing *in vivo* efficacy with the tool compound **2**, systematic SAR exploration led to both increased on target potency and vastly improved p. o. exposure culminating in the advanced lead **15**. The team also showed that the receptor itself was likely the target of the lead series in a series of cell-based domain swapping experiments.

Acknowledgements

The authors would like to acknowledge all of our team collaborators working at Novartis at the time of these efforts.

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Supplementary Material

Detailed synthetic procedures for all of the newly synthesized compounds as well as biochemical and *in vivo* and *ex vivo* assay procedures are provided in the Supplementary material.

Graphical Abstract

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Discovery of potent, bioavailable *in vivo* efficacious modulators of the TLR7/8 pathway

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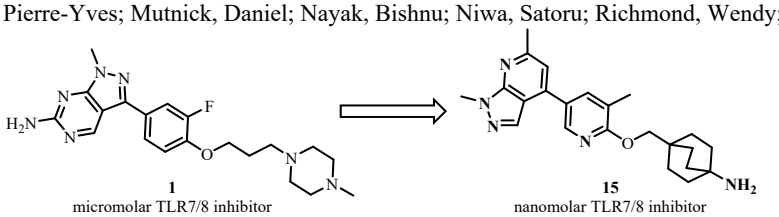

1
micromolar TLR7/8 inhibitor
with no PO exposure

15
nanomolar TLR7/8 inhibitor
with good PO exposure