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Further Exploration of the Structure-Activity Relationship of Imidazoquinolines; Identification of Potent C7 Substituted Imidazoquinolines

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Abstract

Small molecule agonists of TLR7/8, such as imidazoquinolines, are validated agonists for the treatment of cancer and for use in vaccine adjuvants. Imidazoquinolines have been extensively modified to understand the structure-activity relationship (SAR) at the N1- and C2-positions resulting in the clinical drug imiquimod, resiquimod, and several other highly potent analogues. However, the SAR of the aryl ring has not been fully elucidated in the literature. This initial study examines the SAR of C7-substituted imidazoquinolines. These compounds not only demonstrated that TLR7/8 tolerate changes at the C7 position but can increase potency and change their cytokine profiles. The most notable TLR7/8 agonists developed from this study **5**, **8**, and **14** which are up to 4-fold and 2-fold more active than resiquimod for TLR8 and/or TLR7, respectively, and up to 100-fold more active than the FDA approved imiquimod for TLR7.

Graphical Abstract

	R ₁	R ₂	R ₃	TLR7	EC ₅₀	TLR8
Res	H	OH	CH ₂ OC ₂ H ₅	0.23 ± 0.02		2.2 ± 0.1
5	MeO	OH	<i>n</i> -Bu	0.058 ± 0.003		0.96 ± 0.05
8	Cl	OH	<i>n</i> -Bu	0.081 ± 0.01		0.87 ± 0.03
13	CN	OH	CH ₂ OC ₂ H ₅	4.4 ± 0.2		3.6 ± 0.3
14	OH	H	<i>n</i> -Bu	0.068 ± 0.005		Not Active

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Declaration of Competing Interest

The authors declare no competing financial interests.

Appendix. A Supplementary data
Supplementary data file included.

Keywords

Imidazoquinolines; Toll-like Receptor 7; Toll-like Receptor 8; Adjuvants

Toll-like Receptors (TLRs) are a validated target for developing new immunostimulatory drugs.^{1,2} TLRs are a part of the innate immune system, called pattern recognition receptors (PRRs), that help prime the adaptive immune system.³ PRRs detect pathogen-associated molecular patterns (PAMPs), which are conserved features common to many pathogenic microorganisms and viruses, but are not expressed by the host organism (e.g. flagellin, lipopolysaccharides, bacterial DNA, and viral RNA).⁴ In humans, there are 10 TLRs (1–10), which can broadly be separated into endosomal (TLR3, 7, 8 and 9) and cell surface receptors (TLR1, 2, 4, 5, 6 and 10).⁴ The endosomal TLRs are natively activated by nucleic acid constructs not normally found or tightly controlled in the host, including double stranded RNA (TLR3), single stranded RNA (TLR7/8) and unmethylated CpG oligodeoxynucleotides (TLR9).⁵ TLR7/8 are expressed in a subset of immune system leukocytes, including monocytes, macrophages, natural killer (NK) cells, and B- and T-lymphocytes.⁶ TLR7/8 are canonical activators of the MyD88 pathway, which induces expression of pro-inflammatory type I interferons.⁷ Activation of Toll-like Receptor 7 and 8 is associated with a Th-1 biased (cell-mediated) immune response, as opposed to a Th-2 humoral (antibody) response.⁸ The cell-mediated immune response primarily helps eliminate intracellular pathogens, such as viruses, that may have limited exposure to humoral defenses.⁹ The cell-mediated immune response is also crucial in the host regulation of improper cell replication and development of cancer.¹⁰ For these reasons, small molecule agonists of TLR7/8 are highly sought after for the development of new vaccine adjuvants or antitumor agents.

Native ligands of TLR7/8 are guanosine and uridine-rich sequences common to viral single stranded RNA (ssRNA).¹¹ In the case of TLR7, guanosine binds a first docking site on the receptor, which primes a second site to bind guanosine with subsequent dimerization and activation.¹² Activation of TLR8, which is always in the dimerized state, occurs when uridine and ssRNA bind and cause a conformational change.¹³ Binding of either guanosine or uridine alone is insufficient for activation. However, several small molecules have been described with sufficient affinity to activate TLR7/8 after binding of the first (guanosine) site alone, including imidazoquinolines, thiazoquinolines, benzoazepines, benzonaphthyridine and guanine analogs.^{14–17} The most recognizable imidazoquinolines are imiquimod (**1**) and resiquimod (**2**) shown in Figure 1. Imiquimod is the only FDA-approved TLR7 agonist and is the active ingredient in the topical ointment Aldara®, used to treat skin conditions such as superficial basal cell carcinoma and actinic keratosis.¹⁸ Resiquimod is a dual TLR7/8 agonist that is in clinical trials for treatment of cutaneous T-cell lymphoma and melanoma.^{19–21}

Since imidazoquinolines have been established as useful immunostimulants, significant effort has been put forth to develop the structure-activity relationship (SAR) and to help identify potent and effective imidazoquinolines suitable for vaccine adjuvants and cancer treatments.^{22–26} To date, the SAR investigations of imidazoquinolines have mostly focused on the N1 and C2 positions. The N1 position has been substituted with several different

groups with differing results of selective TLR7, TLR8 or mixed TLR7/8 agonists.^{23,25–27} TLR7 tolerates many changes at N1 and C2 and are not required for activity, however, substitutions at these positions typically increase potency.^{27,28} TLR8 typically prefers N1 alkyl hydroxy substituents and either C2 *n*-butyl or *n*-pentyl with *n*-pentyl being more potent to produce mixed TLR7/8 agonists.²⁵ The C4 amine is required for activity as changes to this position result in no activity.²⁷ One position that has been relatively under-explored is the C7 position. Gerster et al. found that imidazoquinolines with a C7 methoxy, hydroxyl, and/or methyl group retained similar IFN α production when compared to **1**.²⁷ Recently, Larson et al. showed that imidazoquinolines **3a-c** (Figure 1) are TLR8 selective.²⁶ In addition, the disclosure of the crystal structure of **2** bound to the TLR8 receptor (Figure 2) provides further evidence that the C7 position might tolerate more functional groups than currently observed.²⁹ As seen in Figure 2, phenylalanine 405 (F405) and tyrosine 353 (Y353) are conserved in the binding site and may be useful residues to help increase the agonist activity of C7 substituted imidazoquinolines. To investigate this hypothesis, we synthesized various C7 substituted derivatives and evaluated their activities in TLR7/8 reporter cell lines and their cytokine induction in donor canine leukocytes. In this initial study, we explored the influence of electronic effects at the C7 position through the addition of electron withdrawing and electron donating groups (EWGs and EDGs) as well as hydrogen bond donor and acceptors.

Our initial synthetic targets for this investigation were aimed at C7 methoxy (**4** and **5**), chloro (**6–9**), nitrile (**10–13**), and hydroxyl (**14**) analogs as shown in Figure 3. The C7 methoxy group was selected due to its strong electron donating properties as well as its hydrogen bond accepting ability. The C7 chloro was picked for its lipophilic properties as well as its resonance into the aryl ring system. The C7 nitrile was selected for its strong electron withdrawing properties and polarizing nature. The C7 hydroxy was chosen for its strong electron donating properties and its hydrogen bond accepting and donating abilities. Also, in this study, the N1 substituent was changed from an isobutyl to the 2-hydroxy-2-methylpropyl while the C2 was changed from the *n*-butyl and ethoxymethyl. These N1 and C2 substitutions were mixed to observe if changes at C7 would change the known SAR of the N1 and C2 positions.

To obtain our targets, synthesis was started with the commercially available 7-methoxyquinolin-4-ol (**15a**) and 7-chloroquinolin-4-ol (**15b**) outlined in Scheme 1. First, **15a** and **15b** were subjected to 70% HNO₃ in propionic acid to obtain the 3-nitroquinolinols (**16a, b**). Next, **16a** and **16b** were chlorinated using POCl₃ and alkylated with the appropriate aminoalkyl chain to obtain **17a-d**. Reduction of the nitro group in **17a-d** using iron dust and ammonia chloride gave the diamines which were immediately used in the subsequent step to form the imidazoquinolines **18a-f** by condensation with the appropriate acid under refluxing conditions. *N*-oxides **19a-f** were made by oxidizing **18a-f** with *m*-CPBA. Subsequent treatment of **19a-f** with *p*-TsCl and ammonium hydroxide afforded compounds **4–9**. The overall yield for the 7-step sequence of reaction was approximately 30%. The C7 chloro analogues (**6–9**) were converted into the corresponding C7 nitriles (**10–13**) in yields of at least 76% using a mixture of Pd(TFA)₂, rac-2-(Di-tert-butylphosphino)-1,1'-binaphthyl (TrixiePhos), Zn dust and Zn(CN)₂.³⁰ Finally, the

demethylation of **4** to phenol **14** was accomplished in 85% yield using pyridinium hydrochloride.

The analogs (**4** – **14**) were screened for TLR7 and TLR8 activity *in vitro* using commercially available human embryonic kidney cells (HEK293) stably co-transfected to express either hTLR7 or hTLR8, along with a secreted embryonic alkaline phosphatase (SEAP) reporter gene induced by NF- κ B (HEK-Blue™, InvivoGen). TLR activation induces NF- κ B production and subsequent SEAP secretion, which is measured using HEK-Detection media (InvivoGen) containing a colorimetric substrate for SEAP (UV-Vis, 637 nm). Transfected null cell lines lacking the hTLR7 or hTLR8 receptors but possessing the NF- κ B induced SEAP reporter gene were used as a control to screen for non-specific activation of NF- κ B (Table 1). Our target compounds were not evaluated at concentrations higher than 100 μ M due to solubility issues.

As expected, imiquimod (**1**) and resiquimod (**2**) were found to be agonists for TLR7 and TLR8 receptors with values consistent with previous reports.^{24–27} Generally, we found that (1) TLR7 is more tolerant to changes than TLR8; (2) tertiary alcohols at the N1 position tend to increase the TLR7 activity when compared to those without the tertiary alcohol; and (3) TLR8 activity requires the tertiary alcohol at N1.²⁴ This is all consistent with previous investigations.^{23–27} We also observed that when C2 contains the ether substitution, TLR7/8 activity decreases when compared to the C2-butyl. This suggests that the changes made at C7 do not cause a change in the trend for N1 and C2 sites.

As others have found, TLR7/8 continues to tolerate C7 substitutions when the N1 and C2 are substituted appropriately.²⁵ The most important finding is that there is a general trend for C7 substitutions. It has been observed in this study that EDGs are stronger activators of TLR7/8 than EWGs. For TLR7 the range was from no statistically significant difference to a 13-fold decrease. For TLR8 the range was from no statistically significant difference to a 9-fold decrease. The observed decrease in activity could be due to the electron deficiency of the imidazoquinoline ring system.

Some notable compounds are the C7-methoxy compounds, **4** and **5**, and the C7-hydroxyl, **14**, as these are substantially more active agonists, which suggests that the increase of electron density of the imidazoquinoline ring system may be increasing the hydrogen bonding interactions of the C4-amine and N5 pyridine nitrogen, or the increase could be contributed to a potential hydrogen bond from the neighboring tyrosine in the binding pocket.²⁹

The imidazoquinolines were further screened for cytokine production in canine peripheral blood mononuclear cells (cPBMCs). Dog PBMCs were chosen for these evaluations based on the superiority of the dog to mouse models of human immunological responses; in addition, we are conducting on-going canine cancer immunotherapy trials with similar compounds.^{31,32} The agonists at 20 μ M concentrations were evaluated for TNF α , IL-12, IL-6, IL-10, IL-2, and IFN γ production by ELISA following incubation with 2×10^6 cPBMC/mL for 29 hours (Table 1). TNF α and IL-12 were chosen as they are typically seen in a Th-1 cytokine response. None of the compounds studied activated IL-6, IL-10 or IL-2

significantly above vehicle. However, as expected nearly all compounds in this study activated IL-12 and TNF α . A trend is observed that IL-12 production increases when the tertiary alcohol of the N1 position is present, suggesting that TLR8 helps increase IL-12 production. Also, having a tertiary alcohol at the N1 position appears to activate INF γ production. It is also observed that EDGs are typically stronger activators of TNF α and IL-12 when compared to EWGs. One compound that is interesting is **14**. It did not follow the trends above. It has one of the highest TNF α responses but one of the lowest IL-12 responses observed despite that it is strongly electron donating and one of the strongest activators of TLR7. The meaning of this result is not clear. We chose ca. 29 hours to evaluate cytokine expression based on preliminary studies with similar compounds showing a rapid increase in inflammatory cytokines over the first 24 hours, peaking between 30 and 48 hours (data not shown). Studies of PBMC cytokine induction with *E. coli* showed cytokines IL-10, IL-6 and IL-2 plateauing near 30 hours, but IL-12 peaked then declined after 30 hours and TNF α rapidly declined after 12 hours³³. Due to the heterogeneous immune cell composition of PBMC preparations and the temporal complexity of cytokine responses, our assay may have missed the peak IL-12 response to **14**. The TLR7 assay indicated strong engagement, so further studies of this compound should include kinetic studies of PBMC stimulation. While the strong IL-12 response and limited activation of IL-10 is strongly associated with a Th-1 biased response, we do not have sufficient data to conclude none of the compounds induced a Th-2 differentiation. Currently, we are evaluating selected compounds *in vivo* for induction of tumor-infiltrating lymphocytes and natural killer cells in syngeneic mouse tumors, which is more indicative of pro-antitumor activity than the broader classification of compounds as Th-1 or Th-2 biased.

In this study, the SAR of the C7 position of imidazoquinolines was further evaluated by synthesizing compounds **4** – **14** with overall yields around 30% at the gram scale. We observed that changes to the N1 and C2 positions combined with the C7 position maintained activity trends consistent with findings from others. We also found that TLR7/8 tolerates all of the target C7 substitutions made in this study when the N1 and C2 are substituted appropriately. We also noted a trend that EDGs at C7 of imidazoquinolines are stronger activators of TLR7/8 when compared to EWGs. This study also discovered TLR7/8 activating substitutions generally increased TNF α and IL-12 cytokine responses and that TLR8 activation potentiated an IFN γ response. However, one compound, **14**, breaks this trend. Despite being one of the strongest TLR7 selective agonists **14** barely potentiates any cytokine induction. Other notable compounds produced from this study are **5**, **8**, and **14** which are up to 4-fold and 2-fold more active than resiquimod for TLR8 and/or TLR7, respectively, and up to 100x more active than the FDA approved imiquimod for TLR7. Only a few TLR7/8 agonists have been reported to date with < 100 nM activity, and this is the first report of high potency agonists based on modifications at the C7 position using the methoxy, chloro and nitrile functional groups. The results in cPBMCs show that different C7 functionalities alter the agonist-cytokine response profile. Additional investigations of the C7 position are currently underway and will be disclosed in due course.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS:

SAR	structure-activity relationship
TLR	toll-like receptor
PRR	pattern recognition receptor
PAMP	pathogen-associated molecular patterns
NK cells	natural killer cells
cPBMCs	canine peripheral blood mononuclear cells
EWG	electron withdrawing group
EDG	electron donating group
DMAC	dimethylacetamide
TrixiePhos	rac-2-(Di-tert-butylphosphino)-1,1'-binaphthyl

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Figure 1.
Representative imidazoquinolines.

Figure 2.

Resiquimod (**2**) bound to TLR8 crystal structure with proposed TLR7 binding site residues. The counterpart residues of human TLR7 are shown in parentheses. Nonconserved residues between TLR7 and TLR8 are written in red. The C atoms of **2** are yellow. Hydrogen bonds by dashed lines. The chemical structures of these ligands are shown at top right in each panel. From Tanji *et al*, PDB 3W3L.²⁹ Reprinted with permission from AAAS.

Figure 3.
Target imidazoquinolines.

	R^1	R^2	R^3
4	MeO	H	<i>n</i> -Bu
5	MeO	OH	<i>n</i> -Bu
6	Cl	H	<i>n</i> -Bu
7	Cl	H	$CH_2OC_2H_6$
8	Cl	OH	<i>n</i> -Bu
9	Cl	OH	$CH_2OC_2H_6$
10	CN	H	<i>n</i> -Bu
11	CN	H	$CH_2OC_2H_6$
12	CN	OH	<i>n</i> -Bu
13	CN	OH	$CH_2OC_2H_6$
14	OH	H	<i>n</i> -Bu

Scheme 1. Synthesis of 7-substitued imidazoquinolines^a

^aReagents and conditions: (i) 70% HNO₃, propionic acid, reflux, 2 h, 55–75%. (ii) POCl₃, DMF, 50 °C, 30 min. (iii) NH₂CH₂(CH₃)₂R², Et₃N, EtOH, reflux 15 min, 70–90% over two steps. (iv) Fe (dust), NH₄Cl, H₂O, reflux 2 h, 70–99%. (v) R³COOH (neat), reflux 1–2 h, 60–80%. (vi) mCPBA, CHCl₃, 4 h, 75–85%. (vii) NH₄OH, *p*-TsCl, DCM, rt 30 min, 70–85%. (viii) Pd(TFA)₂, Zn (dust), TrixiePhos, Zn(CN)₂, dimethylacetamide (DMAC), 95 °C for 15 h, 76–90%. (ix) Pyr·HCl (neat), reflux 30 min, 85%.

Table 1.**TLR7/8 Agonist Activities of Analogs 4–13**

Compound	EC ₅₀ (M) ± SD ^a			Cytokine (pg/mL) ± SD ^b				
	TLR7	TLR8	TNFα	IL-12	IL-6	IL-10	IL-2	IFNγ
1	6.8 ± 0.8	-	25 ± 3	1200 ± 140	300 ± 48	16 ± 2	85 ± 37	18 ± 6
2	0.25 ± 0.03	3.0 ± 0.3	190 ± 30	2800 ± 520	380 ± 80	21 ± 2	120 ± 12	26 ± 7
4	0.11 ± 0.01	>100 ^c	49 ± 9	746 ± 130	500 ± 140	37 ± 13	30 ± 29	0
5	0.081 ± 0.009	1.7 ± 0.3	64 ± 12	2000 ± 160	290 ± 63	14 ± 2	82 ± 56	14.5 ± 6
6	0.3 ± 0.02	>100	91 ± 20	760 ± 50	240 ± 67	14 ± 4	63 ± 44	7 ± 1
7	0.91 ± 0.09	>100	114 ± 25	781 ± 40	280 ± 81	20 ± 1	50 ± 22	5 ± 5
8	0.084 ± 0.007	1.5 ± 0.2	22 ± 4	1700 ± 330	230 ± 44	7 ± 6	55 ± 30	13 ± 10
9	0.63 ± 0.03	4.9 ± 0.6	70 ± 8	1900 ± 130	320 ± 76	12 ± 3	76 ± 59	16 ± 2
10	0.99 ± 0.40	>100	10 ± 1	920 ± 170	350 ± 95	37 ± 10	60 ± 41	13 ± 5
11	6.8 ± 0.7	>100	7 ± 1	190 ± 62	71 ± 20	6 ± 2	57 ± 14	4 ± 3
12	0.79 ± 0.23	3.9 ± 0.2	44 ± 10	418 ± 72	290 ± 77	14 ± 3	45 ± 22	12 ± 4
13	3.5 ± 0.7	27 ± 6	80 ± 20	13 ± 3	214 ± 69	6 ± 3	46 ± 42	0.2 ± 0.4
14	0.073 ± 0.005	>100	270 ± 70	102 ± 65	330 ± 92	17 ± 3	79 ± 19	9 ± 8
Vehicle			8 ± 2	18 ± 8	310 ± 90	33 ± 16	47 ± 20	0.4 ± 0.7

^a Average EC₅₀ values were determined from three independent measurements at 10 or more concentrations, performed in quadruplicate using either hTLR7 or hTLR8 transfected HEK-Blue cells along with corresponding Null controls. Raw data and statistical methods available in SI.

^b Cytokine production was measured in triplicate at 20 μM using cPBMCs, full graphs and statistical analysis available in SI.

^c >100 = no activity observed at 100 μM. Vehicle is 1% DMSO in water.