Discovery of Imidazoquinolines with Toll-Like Receptor 7/8 Independent Cytokine Induction

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S *Supporting Information*

ABSTRACT: Toll-like receptors (TLRs) are key targets in the design of immunomodulating agents for use as vaccine adjuvants and anticancer treatments. The imidazoquinolines, imiquimod and resiquimod, have been shown to activate TLR-7 and -8, which in turn induce cytokine production as part of the innate immune response. Herein, we report the synthesis and discovery of a C7-methoxycarbonyl derivative of imiquimod that stimulates cytokine production but is devoid of TLR-7/8 activity. Data are presented that shows that this analogue not only induces IL-12p40 and TNF production, similar to that of imiquimod and resiquimod, but greatly enhances the production of IL-1β, a key cytokine involved in the activation of CD4 T cells. It is further demonstrated that TLR-7/8 activation can be recovered by the addition of a C2-alkyl substituent to this newly discovered analogue. The results support the existence of an alternative mechanism of action by which imidazoquinolines can stimulate cytokine production.

KEYWORDS: *imidazoquinolines, toll-like receptor 7/8, cytokine induction*
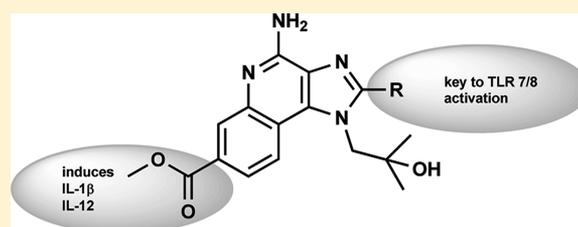
Toll-like receptors 7 and 8 (TLR 7/8) are key targets for the design of small molecule immunomodulators for use as vaccine adjuvants and anticancer/antiviral agents.[1−3] These receptors trigger the nuclear factor κ light-chain enhancer of activated B cells (NF-κB) mediated production of proflammatory cytokines and chemokines in response to the presence of viral single-stranded ribonucleic acid (ssRNA) within the endosome of B cells and dendritic cells.[4] The imidazoquinolines imiquimod (**1**) and resiquimod (**2**) (Figure 1) are potent agonists of this pathway. These compounds first appeared in the patent literature in the 1980s.[5,6] Topical imiquimod cream was FDA approved in 1997 for the treatment of basal cell carcinoma, and there remains extensive interest in optimizing this class of compounds for multiple indications.[7,8] Although their ability to induce interferon-α (IFNα) was known at that time, the mechanism of action was not discovered until 2002 when Hemmi et al. definitively linked the imidazoquinolines to TLR 7 function.[9] They reported that both **1** and **2** failed to induce cytokine production in TLR 7-deficient mice. The results were further supported and extended by Gorden et al. and Jurk et al. who disclosed that resiquimod also activates the TLR 8 signaling pathway.[10,11]

Following the discovery that imidazoquinolines activate TLR-7 and -8, Gerster et al. reported the synthesis and evaluation of a large series of analogues that included many compounds from the original patent literature.[12] Although the compounds were evaluated for IFNα production, TLR 7/8 functional data were not reported. The most potent analogues were found to contain a short alkyl chain at the C-2 position (with *n*-butyl showing the highest potency) and short, hydroxyl alkyl chains at N-1. In terms of aryl substitutions, it was

Figure 1. Imidazoquinolines.

determined that the amino group at C-4 was required for activity. Although the chemistry was limited, simple substitutions to the remaining aryl positions were found to abolish activity, except in the case of C-7. The C-7 methoxy, hydroxyl, and methyl derivatives retained modest IFNα production. In more recent work, Shukla et al. verified the TLR 7 activity of a series of C-2 and N-1 substituted imdazoquinolines, including imiquimod.[13] Their results indicated that the most potent and TLR 7 selective compounds contained an *n*-butyl group at C-2 (as suggested by Gerster et al.) and either a 2-hydroxy-2-methylpropyl or a benzyl group at N-1. Although it was reported that all of the C-2 alkyl compounds were TLR 7-selective, a systematic evaluation of TLR 8 binding was not presented.

One of the limiting factors in the design and synthesis of imidazoquinolines for screening is the cumbersome nature of the synthetic route. Prior reports have used traditional heterocyclic chemistry that is borrowed from purine base synthesis.[14] The approach applies a modified Traube reaction to annulate diaminoquinolines by condensation with ortho esters or acid chlorides (yielding the desired imidazoquinoline tricycle). While this approach allows substitutions to be efficiently introduced to the imidazole ring system, modifications to the quinoline ring are more complicated, requiring additional synthetic steps to be added to obtain the substituted precursors. Functional group modifications must also be compatible with the reaction conditions used in subsequent steps, further limiting the synthetic pathway. To address this problem, we have developed an alternative route that exploits the Suzuki–Miyaura reaction to efficiently synthesize highly substituted imidazoquinolines in four steps. In this report, we describe the application of this chemistry to produce a set of C-7 methoxy carbonyl derivatives (**5** and **6**) in addition to unsubstituted analogues (**3** and **4**). We also present TLR 7/8 and cytokine screening data that indicates that this series functions through a unique pathway in activating cytokine production.

The synthesis of **3**−**6** (Scheme 1) begins with the multicomponent condensation of the aminomalononitrile (**7**), orthoester (**8a/b**), and β-amino alcohol (**9**) to afford the 5-aminoimidazole-4-carbonitriles (**10a/b**).[15,16] The key iodoimidazole intermediates (**11a/b**) are obtained in good yield using a modified Sandmeyer reaction employing isoamyl nitrite and diiodomethane.[17] Suzuki coupling of **11a/b** with 2-aminophenylboronic acid (**12a**) or 2-amino-4-methoxycarbonylphenylboronic acid (**12b**) furnishes the atropisomeric intermediates (**13**−**16**). Intramolecular cyclization is achieved by treatment of the heterobiaryl intermediates with either anhydrous HCl or NaOMe to afford the desired imidazoquinolines (**3**−**6**). Acid catalysis is preferred for substrates **15** and **16** due to partial saponification of the methyl esters under the basic conditions. Although this approach provides an efficient method to install novel functionality on the benzene ring of the quinoline, we found that the efficiency of the cross-coupling step was influenced by the C-2 alkyl substituent. It was noted that **11b** reacted sluggishly with a significant portion undergoing competitive protio-dehalogenation while **11a** furnished the desired cross-coupled products (**13** and **14**) in 3 h with high yields. To avoid steric hindrance with the bulky C-2 substituent, the N-1 group in **11b** likely is directed toward the 5-iodo substituent, thereby hindering the palladium-catalyzed cross-coupling and explaining its lower intrinsic reactivity.

Scheme 1. Synthesis of Imidazoquinoline Analogues[a]
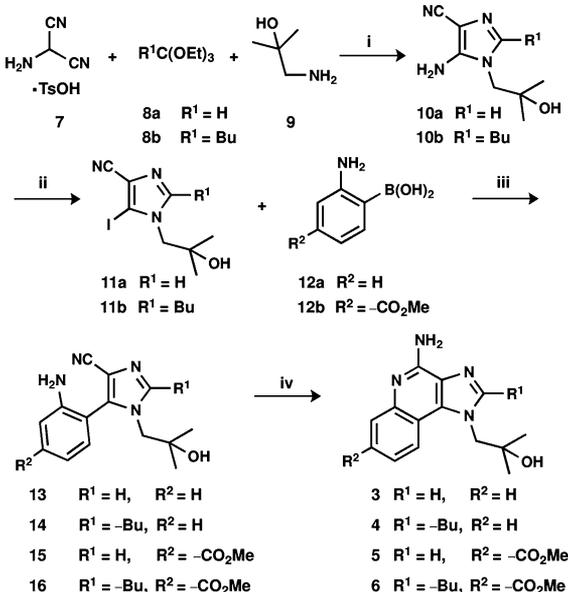
[a]Reagents and conditions: (i) NEt₃, THF, reflux to rt, 15 h, 65−88%. (ii) CH₂I₂, isoamylnitrite, CHCl₃, 80 °C, 30 min, 65−75%. (iii) Pd(OAc)₂, PPh₃, Na₂CO₃, DME−H₂O, 100 °C, 3−15 h, 34−95%. (iv) 4 N HCl in dioxane (excess), 80 °C, 85−93%.

Compounds **1**−**6** were initially screened using TLR 7 and TLR 8 reporter cell lines for their ability to induce the NF-κB pathway. Consistent with prior studies, imiquimod (**1**) was determined to be a selective TLR 7 agonist, whereas resiquimod (**2**) triggered both TLR7 and TLR 8 (as reported in Table 1). Resiquimod also showed an increased potency at TLR 7 as compared to that of imiquimod. This increased potency may be linked to the C-2 substituent as suggested by the activity of compound **3**. Deletion of the C-2 ethoxymethyl moiety of resiquimod resulted in a 67-fold drop in TLR 7 agonist activity and abolished TLR 8 activation entirely. Further evidence to support the vital role C-2 substitution plays in both potency and selectivity can be found in the data reported for **4**. Both TLR 7 and 8 function was restored and enhanced by addition of a C-2 butyl group to **3**. The C-2 butyl substitution in **4** increased TLR 7 and 8 potency by approximately 10- and 60-fold, respectively, when compared to resiquimod.

The most intriguing and novel findings were revealed when a methyl ester was added to the quinoline ring at C-7, generating compound **5**. This compound was entirely devoid of TLR 7 and TLR 8 agonist activity as measured by the reporter cells. This result was completely unexpected. On the basis of previous reports that indicated that C-7-substituted imidazoquinolines stimulated the release of IFNα, the compounds were further screened for cytokine production. The results given in Table 1 show that despite displaying no appreciable activity in triggering TLR 7 or 8, compound **5** induced the release of interleukin 1β (IL-1β) from mouse bone marrow-derived dendritic cells (BMDC) at levels 15 times greater than compounds **1**−**4**. In addition to IL-1β, compound **5** induced 2−3 times more tumor necrosis factor (TNF) α and IL-12p40 than imiquimod, demonstrating a broad induction of inflammatory cytokines (commonly linked with TLR activation). Once again, TLR 7 and 8 binding was restored by the addition of a C-2 butyl group to **5**. Although **6** was not the most potent TLR 7 and 8 agonist of the group, the ability to

Table 1. TLR 7/8 Agonist Activities of Analogurs 1−6 and Their Corresponding Cytokine Production Data[a]

compd	cytokine (pg/mL)				EC$_{50}$ (μM)	
	TNFα	IL-1β	IL-12p40	IL-10	TLR-7	TLR-8
1	593 ± 62	35 ± 1	889 ± 100	33 ± 2	12.1 ± 4.4	n/a[b]
2	2134 ± 266	38 ± 4	1491 ± 31	72 ± 16	1.4 ± 0.3	6.4 ± 2.6
3	2041 ± 31	61 ± 18	839 ± 35	60 ± 8	95 ± 20	n/a
4	1743 ± 165	73 ± 8.2	864 ± 187	181 ± 24	0.1 ± 0.02	0.100 ± 0.003
5	1451 ± 137	1144 ± 116	1773 ± 62	94 ± 14	n/a	n/a
6	1353 ± 552	893 ± 306	1286 ± 276	110 ± 45	1.2 ± 0.3	41.3 ± 3.6
control	91.3 ± 34.5	26.2 ± 3.7	260.6 ± 11.9	16.2 ± 4.7		

[a]Multiplexed cytokine production was measured in triplicate using analogues at 30 μM. [b]No activation (>270 μM).

significantly enhance IL-1β production was retained, suggesting the C-7 methyl ester derivatives trigger an alternative pathway to cytokine production.

To our knowledge, this is the first demonstration of an imidazoquinoline that triggers a profound induction of inflammatory cytokines in the absence of TLR 7 or 8 agonist activity. Although the mode of action of **5** is currently not known, some clues to the pathways involved may be found in high levels of IL-1β reported for **5** and **6**. The unique and important role of IL-1β has only recently been appreciated as "signal three" for activation of cluster of differentiation 4 (CD4) T cells. Specifically, IL-1 receptor-deficient CD4 T cells have inferior proliferative and functional capacities, demonstrating a direct effect of IL-1β on the T cell. Moreover, recombinant IL-1β can profoundly improve memory T cell formation and antibody responses against protein antigens in mice.[18] It is important to note that IL-1β release is dependent on activation of the inflammasome, an intracellular pathway that senses microbial infection including ssRNA.[19] Thus, it is reasonable to conclude that the alternative pathway accessed by the C-7 methyl ester derivatives may involve inflammasome activation. Considering that IL-1β has a unique capacity to increase antibody responses and modulate CD4 T cell differentiation, this discovery could be the first step in developing novel imidazoquinoline analogues that have superior activity as vaccine adjuvants or for single agent therapy. This hypothesis awaits validation in mouse models and human patients.

ASSOCIATED CONTENT

Ⓢ Supporting Information
Full experimental details and compound characterization data as well as biological methods and procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

ABBREVIATIONS

TLR, toll-like receptor; NF-κB, nuclear factor κ light-chain enhancer of activated B cells; ssRNA, single-stranded ribonucleic acid; IFN, interferon; BMDC, bone marrow-derived dendritic cell; CD4, cluster of differentiation 4; IL, interleukin; TNF, tumor necrosis factor

REFERENCES

(1) Parkinson, T. The future of toll-like receptor therapeutics. *Curr. Opin. Mol. Ther.* **2008**, *10*, 21−31.

(2) Czarniecki, M. Small molecule modulators of toll-like receptors. *J. Med. Chem.* **2008**, *51*, 6621−6626.

(3) Coffman, R. L.; Sher, A.; Seder, R. A. Vaccine adjvants: Putting innate immunity to work. *Immunity* **2010**, *33*, 492−503.

(4) Akira, S.; Takeda, K.; Kaisho, T. Toll-like receptors: Critical proteins linking innate and acquired immunity. *Nat. Immunol.* **2001**, *2*, 675−680.

(5) Gerster, J. F. 1*H*-Imidazol[4,5-*c*]quinolines and 1*H*-imidazo[4,5-*c*]quinolin-4-amines. EP 06/0145340, 1985.

(6) Gerster, J. F.; Crooks, S. L.; Lindstrom, K. J. Preparation of 1*H*-imidazo[4,5-*c*]quinoline-4-amines as virucides, neoplasm inhibitors, and interferon inducers. WO 92/15582, 1992.

(7) Miller, R. L.; Gerster, J. F.; Owens, M. L.; Slade, H. B.; Tomai, M. A. Imiquimod applied topically: a novel immune response modifier and new class of drug. *Int. J. Immunopharmacol.* **1999**, *21*, 1−14.

(8) Gaspari, A. A.; Tyring, S. K.; Rosen, T. Beyond a decade of 5% imiquimod topical therapy. *J. Drugs Dermatol.* **2009**, *8*, 467−474.

(9) Hemmi, H.; Kaisho, T.; Takeuchi, O.; Sato, S.; Sanjo, H.; Hoshino, K.; Horiuchi, T.; Tomizawa, H.; Takeda, K.; Akira, S. Small antiviral compounds activate immune cells via the TLR7MyD88-dependent signaling pathway. *Nat. Immunol.* **2002**, *3*, 196−200.

(10) Jurk, M.; Heil, F.; Vollmer, J.; Schetter, C.; Krieg, A. M.; Wagner, H.; Lipford, G.; Bauer, S. Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848. *Nat. Immunol.* **2002**, *3*, 499−499.

(11) Gorden, K. B.; Gorski, K. S.; Gibson, S. J.; Kedl, R. M.; Keiper, W. C.; Qiu, X.; Tomai, M. A.; Alkan, S. S.; Vasilakos, J. P. Synthetic TLR agonists reveal functional differences between human TLR7 and TLR8. *J. Immunol.* **2005**, *174*, 1259−1268.

(12) Gerster, J. F.; Lindstrom, K. J.; Miller, R. L.; Tomai, M. A.; Birmachu, W.; Bomersine, S. N.; Gibson, S. J.; Imbertson, L. M.; Jacobson, J. R.; Knafla, R. T.; Maye, P. V.; Nikolaides, N.; Oneyemi, F. Y.; Parkhurst, G. J.; Pecore, S. E.; Reiter, M. J.; Scribner, L. S.; Testerman, T. L.; Thompson, N, J.; Wagner, T. L.; Weeks, C. E.; Andre, J.-D.; Lagain, D.; Bastard, Y.; Lupu, M. Synthesis and structure-activity-relationships of 1*H*-imidazao[4,5-*c*]quinolines that induce interferon production. *J. Med. Chem.* **2005**, *48*, 3481−3491.

(13) Shukla, N. M.; Malladi, S. S.; Mutz, C. A.; Balakrishna, R.; David, S. A. Structure-activity relationships in human toll-like receptor 7-active imidazoquinoline analogues. *J. Med. Chem.* **2010**, *53*, 4450−4465.

(14) Robins, R. K.; Dille, K. J.; Willits, C. H.; Christensen, B. E. Purines. II. The synthesis of certain purines and the cyclization of

several substituted 4, 5-diaminopyrimidines. *J. Am. Chem. Soc.* **1952**, *75*, 263−266.

(15) Ruske, W.; Ruske, E. Nitromalonsäure-dinitril. *Chem. Ber.* **1958**, *91*, 2505−2512.

(16) Peinador, C.; Quintela, J. M.; Moreira, M. J. A short and facile synthesis of Heteromine A. *Tetrahedron* **1997**, *53*, 8269−8272.

(17) Minakawa, N.; Kojima, N.; Matsuda, A. Nucleosides and nucleotides. 184. Synthesis and conformational investigation of anti-fixed 3-deaza-3-halopurine ribonucleosides. *J. Org. Chem.* **1999**, *64*, 7158−7172.

(18) Ben-Sasson, S. Z.; Hu-Li, J.; Quiel, J.; Cauchetaux, S.; Ratner, M.; Shapira, I.; Dinarello, C. A.; Paul, W. E. IL-1 acts directly on CD4 T cells to enhance their antigen-driven expansion and differentiation. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 7119−7124.

(19) Burns, K.; Martinon, F.; Tschopp, J. New insights into the mechanism of IL-1β maturation. *Curr. Opin. Immunol.* **2003**, *15*, 26−30.