Identification of Human Toll-like Receptor 2-Agonistic Activity in Dihydropyridine–Quinolone Carboxamides

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S Supporting Information

ABSTRACT: Using a multiplexed, reporter gene-based, high-throughput screen, we identified 9-fluoro-7-hydroxy-3-methyl-5-oxo-*N*-(pyridin-3-ylmethyl)-2,3-dihydro-1*H*,5*H*-pyrido-[3,2,1-*ij*]quinoline-6-carboxamide as a TLR2 agonist. Preliminary structure–activity relationship studies on the carboxamide moiety led to the identification of analogues that induce chemokines and cytokines in a TLR2-dependent manner. These results represent new leads for the development of vaccine adjuvants.

KEYWORDS: Vaccine adjuvants, Toll-like receptor 2, chemokines, cytokines, dihydropyridine–quinolones

The sustained decrease in mortality caused by infectious diseases is largely attributable to immunization, and vaccines continue to play an indispensable role in decreasing the burden of infectious diseases worldwide.¹ The major categories of vaccines are those that contain live attenuated microbes, those that contain killed microbes, and those that contain one or more of an antigenic subunit (such as protein or polysaccharide) derived from the microbe.² Modern vaccines increasingly rely on well-defined, highly purified subunit and recombinant antigens, and often require the incorporation of appropriate immune potentiators (also termed adjuvants), along with the antigen. Adjuvants initiate early innate immune responses and thereby enhance immunogenicity, leading to more robust and long-lasting adaptive immune responses.³

Toll-like receptors (TLRs) are a family of pattern recognition receptors that serve as key sentinels of innate immune system. There are 10 TLRs in the human genome; these trans-membrane proteins recognize pathogen-associated molecular patterns that are shared by pathogens but are sufficiently different so as to be distinguishable from host molecules.^{4–6} The engagement of innate immune receptors plays a role in the action of vaccine adjuvants such as monophosphoryl lipid A (TLR4 agonist)⁷ and Adjuvant 1018 (TLR9 agonist),^{8,9} highlighting the importance of innate immune stimulatory molecules in the design and development of novel vaccines.

Using a recently described multiplexed, reporter gene-based, high-throughput screen (HTS) capable of detecting agonists of TLR2, TLR3, TLR4, TLR5, TLR7, TLR8, TLR9, NOD1, and NOD2,¹⁰ we identified a novel immunostimulatory chemotype, typified by the hit compound **2** (9-fluoro-7-hydroxy-3-methyl-5-oxo-*N*-(pyridin-3-ylmethyl)-2,3-dihydro-1*H*,5*H*-pyrido[3,2,1-*ij*]quinoline-6-carboxamide, Scheme 1). Subsequent deconvolution of activity in individual TLR/NLR screens indicated TLR2 activity with no detectable activity for other

TLR/NLR targets in counter-screens. Other than numerous canonical TLR2 agonists of the thioacylglycerol lipopeptide chemotypes that we had previously explored,^{11–14} heterocyclic small molecules capable of engaging TLR2 have not been identified as of yet. An examination of the HTS results for the nearest neighbors of **2** suggested that the amide-linked aryl group could be a productive venue for exploration. We report here a preliminary structure–activity relationship (SAR) study on varying the 6-carboxamide moiety.

The tricyclic dihydropyridine–quinolone core was synthesized using an elegant one-pot method developed by Ukrainets et al.^{15,16} A concerted nucleophilic addition–cyclization reaction of 6-fluoro-2-methyl-1,2,3,4-tetrahydroquinoline with triethylmethanetricarboxylate afforded the ester **1**, which was subsequently displaced with a variety of amines to yield analogues **2** (hit compound) through **57** (Scheme 1). We began our SAR exploration by evaluating substituted pyridines (analogues **3–21**), an examination of which initially appeared to suggest that substituents at C2 and C6 of the pyridine ring were poorly tolerated, whereas those at C4 and C5 were well tolerated. However, **14**, with a 6-aminopyridylmethyl substituent was found to be equipotent to **2** in primary human TLR2-specific reporter gene assays, and compound **11** bearing a 6-chloropyridylmethyl substituent was also found to be weakly active (Figure 1). Surprisingly, **16**, with a 2-dimethylaminopyridylmethyl group, but not **15** with a 6-dimethylaminopyridylmethyl functional group retained TLR2-agonistic activity (Figure 1A).

Taken together, these results appeared to point to potential H-bonding interactions with the ring nitrogen of the pyridine in a particular rotameric configuration, a surmise that was consistent

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Scheme 1^a

^aActive compounds are depicted in red. Their corresponding EC₅₀ values in human TLR2 screens are listed in Figure 1. Reagents and conditions: (i) triethylmethanetricarboxylate, diphenyl ether, microwave, 180 °C, 1 h; (ii) alkyl/aryl methanamines, DMF, microwave, 100 °C, 30 min (for 2–8, 10–12, 14–26, 28–33, 35, 37–45, 47–52, and 55); alkyl/aryl methanamine (hydrochloride salts), DIPEA, DMF, microwave, 100 °C, 30 min (for 9, 13, 27, 34, 36, and 46); N-Boc protected heterocycloalkyl methanamine, DMF, microwave, 100 °C, 30 min, 4 M HCl in dioxane, 2 h (for 53, 54, 56, and 57). Compounds 27 and 51–57 were obtained as the racemates.

Figure 1. (A) Human TLR2-specific agonistic potencies of active analogues in primary screens (reporter gene assay). (B) Human MCP-1 induction (EC₅₀ values) in human TLR2-expressing cells by select analogues. PAM₂CSK₄ (100 ng/mL) and DMSO (diluent) were used as positive and negative controls, respectively, in both assays. Data shown are means of quadruplicates.

with the finding that the homologated analogue **26**, as well as conformationally constrained derivative **27** also retained activity. Activity was lost in all of the regioisomeric pyridinylmethyl analogues (**19–21**, **25**), as well as pyridazinylmethyl (**22**, **24**) and the pyrimidinylmethyl compound **23** (Figure 1A).

Congeners with indolylmethyl substituents (**28–31**) as well as the benzimidazolylmethyl derivative **33** were inactive. The *N*-methylpyrazolylmethyl analogue **42** and the morpholinyl

derivative **57**, a variety of heterocyclic (**32**, **32–41**, **43–50**) and saturated heterocyclic compounds (**51–56**) were also inactive (Scheme 1).

In ongoing studies aimed at delineating “signatures” that are diagnostic of innate immune activation regardless of the innate immune sensor involved, we have found using next-generation RNA sequencing of global transcriptomal programs (and subsequently confirmed by analyte-specific immunoassays)

Figure 2. Dose–response profiles of cytokine and chemokine induction in THP-1 cells. Data shown are means of triplicates.

Figure 3. TLR2-agonistic activity of enantiopure compounds. Data shown are means of triplicates.

prominent upregulation of CC and CXC chemokines by virtually the entire repertoire of innate immune stimuli.¹⁷ We therefore examined MCP-1 induction in human TLR2-expressing reporter cells to confirm the observations of our primary screens.

We were gratified to find that MCP-1 induction was largely concordant with TLR2-agonistic potencies derived from

Scheme 2^a

^aReagents and conditions: (i) (a) *N*-tosyl-L-proline, oxalyl chloride, DMF, toluene, RT, overnight; (b) DIPEA, DCM, reflux, 30 min; (ii) NaOEt, EtOH, reflux, overnight; (iii) triethylmethanetricarboxylate, diphenyl ether, microwave, 180 °C, 1 h; (iv) 3-picolylamine (for **61** and **63**), or *S*-(aminomethyl)pyridine-2-amine (for **62** and **64**), or *S*-(5-fluoropyridin-3-yl)methanamine (for **65**), DMF, microwave, 100 °C, 30 min.

Figure 4. (A) NF-κB induction by PAM₂CSK₄ (canonical TLR2 agonist) and the enantiopure compounds **63**–**65** in THP-1 Blue reporter cells showing relative potencies of the two compounds. (B) Additive effect of PAM₂CSK₄ and **63**. Dose–response profiles to **63** in THP-1 Blue reporter cells are enhanced in the presence of increasing concentrations (0.015 to 9.83 pM) of PAM₂CSK₄. Data shown are means of quadruplicates. Similar enhancements were also observed for **64** and **65**.

reporter gene assays (Figure 1A,B). However, we observed differences in rank-order potencies between the assay platforms for a few compounds, in particular. Compounds **14** and **10** were considerably weaker in eliciting MCP-1 (Figure 1B) than would be expected from their corresponding hTLR2 EC₅₀ values (Figure 1A); conversely, analogue **26** was considerably more potent in eliciting MCP-1 (Figure 2).

In order to identify the eutomer and distomer (and eudysmic ratios) of the racemic TLR2-active compounds, we initially undertook the syntheses of *R*- and *S*-enantiomers of **2** and **14** (Scheme 2). *N*-Acylation of 6-fluoro-2-methyl-1,2,3,4-tetrahydroquinoline with tosyl-*S*-prolinoyl chloride resulted in easily separable diastereoisomers **58a** and **58b** (Scheme 2), whose characterization exactly matched the findings of Sonawane and co-workers.¹⁸ Solvolysis with NaOEt furnished enantiomeric intermediates **59a** and **59b**, which furnished the enantiopure targets **61**–**65** (Scheme 2). Examination of these compounds in TLR2-specific reporter gene assays revealed that the *S*-enantiomers were eutomeric, which was confirmed again in **65**, the *S*-enantiomer of **10** (Figure 3).

Given that cells of myeloid lineage are the primary responder cells for TLR2 stimuli,^{19–21} we sought to re-examine select active enantiopure compounds in THP-1 cells of monocytic lineage using multiplexed cytokine/chemokine assays (Figure 2). Consistent with results depicted in Figure 1B, the *S*-enantiomeric compounds **63**–**65** were found to be prominent inducers of chemokines MCP-1, MIP-1α, and MIP-1β (Figure S1).

Given that the dihydropyridine–quinolone carboxamides represent a novel class of TLR2 agonists, it was of particular interest to explore if these compounds bind to TLR2 at a site distinct from that of the canonical lipopeptides. While definitive conclusions must await crystallographic studies, costimulation experiments (Figure 4) clearly indicate additive effects of **63** in the presence of PAM₂CSK₄. Similar additive results with PAM₂CSK₄ were also observed for **64** and **65** (Figure S2). These results suggest that the binding site of the dihydropyridine–quinolone carboxamides are likely to be distinct.

These results represent exciting new leads of noncanonical small-molecule agonists of TLR2 and charts a course forward for a detailed exploration of SAR of both the dihydropyridine and quinolone rings of the tricyclic core.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmmedchemlett.8b00540.

Syntheses and experimental procedures, and characterization of compounds (PDF)

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

HTS, high-throughput screen; IL-1 α, Interleukin-1 α; MCP-1, monocyte chemoattractant protein 1 (CCL2); MIP-1 α/β, macrophage inflammatory protein 1-α/β; NOD-1/2, nucleotide-binding oligomerization domain-containing protein 1/2; NLR, NOD-like receptor; TLR, Toll-like receptor; TNF-α, tumor necrosis factor α.

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