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Syntheses of fluorescent imidazoquinoline conjugates as probes of Toll-like receptor 7

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ABSTRACT

Toll-like receptor (TLR)-7 agonists show prominent immunostimulatory activities. The synthesis of a TLR7-active N^1-(4-aminomethyl)benzyl substituted imidazoquinoline **5d** served as a convenient precursor for the covalent attachment of fluorophores without significant loss of activity. Fluorescence microscopy experiments show that the fluorescent analogues are internalized and distributed in the endosomal compartment. Flow cytometry experiments using whole human blood show differential partitioning into B, T, and natural killer (NK) lymphocytic subsets, which correlate with the degree of activation in these subsets. These fluorescently-labeled imidazoquinolines will likely be useful in examining the trafficking of TLR7 in immunological synapses.

Toll-like receptors (TLRs) are pattern recognition receptors that recognize specific molecular patterns present in molecules that are broadly shared by pathogens, but are structurally distinct from host molecules.[1] There are 10 TLRs in the human genome.[2] The ligands for these receptors are highly conserved microbial molecules such as lipopolysaccharides (LPS) (recognized by TLR4), lipopeptides (TLR2 in combination with TLR1 or TLR6), flagellin (TLR5), single stranded RNA (TLR7 and TLR8), double stranded RNA (TLR3), CpG motif-containing DNA (recognized by TLR9), and profilin present on uropathogenic bacteria (TLR 11).[3] The activation of TLRs by their cognate ligands leads to activation of innate immune effector mechanisms, including the production of pro-inflammatory cytokines, up-regulation of MHC molecules and co-stimulatory signals in antigen-presenting cells, resulting in amplification of specific adaptive immune responses involving both T- and B-cell effector functions.[4–6] Thus, TLR stimuli serve to link innate and adaptive immunity[4] and can therefore be exploited as powerful adjuvants in eliciting both primary and anamnestic immune responses.

Our point of departure in the systematic evaluation of TLR agonists as vaccine adjuvants[7–9] focuses on identifying chemotypes which are strongly immunostimulatory, and yet devoid of dominant pro-inflammatory cytokine-inducing activities;[7] the TLR7-agonistic imidazoquinolines have thus far seemed ideal in meeting these requirements.[7] Continuing work on characterizing the immunostimulatory activities of TLR7 agonists show, as expected, clear involvement of plasmacytoid dendritic cells,[10] but we have also observed a set of accessory cell-independent direct responses in $CD4^+$ and $CD8^+$ T and $CD3^-CD56^+$ natural killer (NK) lymphocytes (to be published elsewhere). We are specifically desirous of examining the uptake, intracellular distribution, and trafficking of the imidazoquinoline in immunological synapses,[11,12] and it became necessary to develop probes of TLR7 that are fluorescently-labeled.

Our earlier SAR study on the TLR7-agonistic activities had exhaustively explored C2 and C4 substituents on the imidazoquinoline scaffold (Fig. 1),[13] but proved unsuccessful in identifying potential positions that would tolerate the introduction of bulky aryl groups without compromising activity.

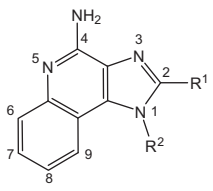

Figure 1. General structure of 1*H*-imidazo[4,5-*c*]quinolin-4-amine. The C2 and N^1 substituents that were found to correspond to optimal TLR7-agonistic activity were *n*-butyl and benzyl, respectively (Ref. 13).

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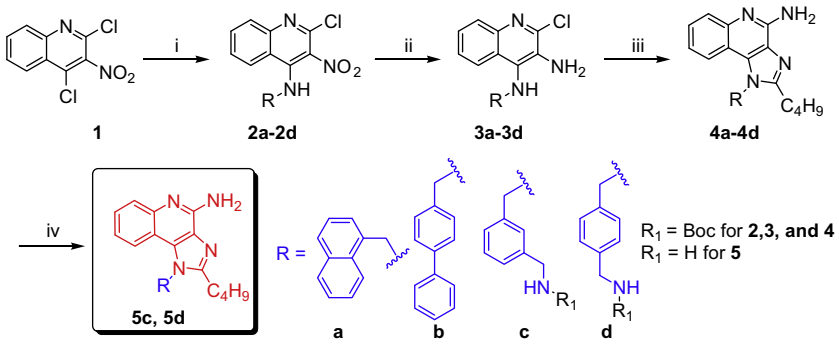
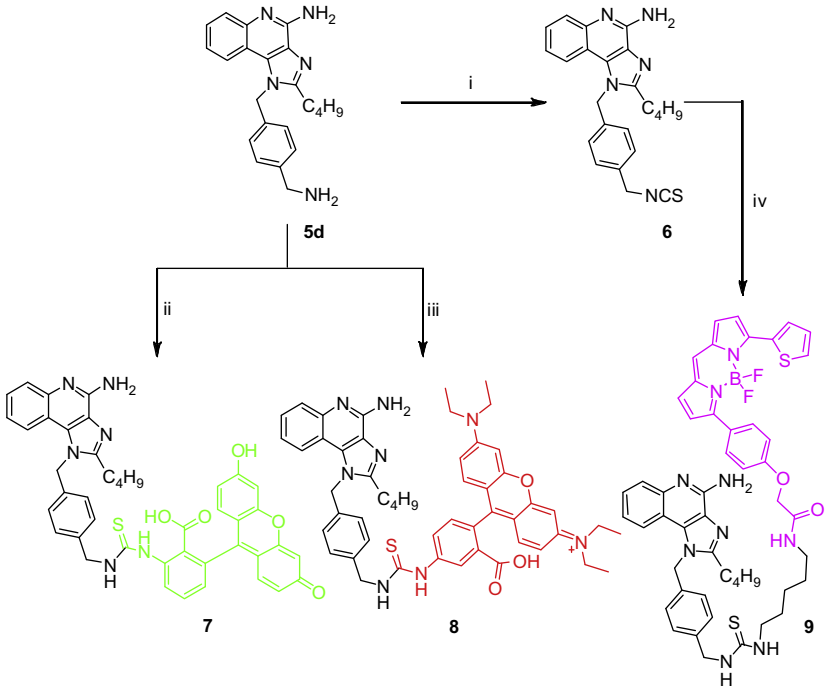


Scheme 1. Syntheses of N^1-substituted imidazoquinolines. Reagents: (i) RNH_2, Et_3N, CH_2Cl_2; (ii) Pt/C, H_2, Na_2SO_4, EtOAc; (iii) (a) C_4H_9COCl, Et_3N, THF; (b) NH_3/MeOH, 150 °C; (iv) HCl/dioxane.



Scheme 2. Syntheses of fluorescent analogues of **5d**. Reagents: (i) CS_2, Et_3N, DMAP, $(Boc)_2O$, CH_2Cl_2; (ii) fluorescein isothiocyanate, Et_3N, MeOH; (iii) rhodamine B isothiocyanate, Et_3N, CH_2Cl_2; (iii) BODIPY® TR cadaverine, pyridine.

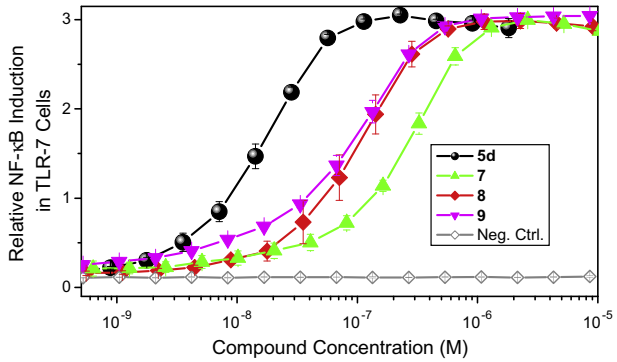


Figure 2. Activities of **5d**, **7**, **8**, and **9** in reporter gene assays using human TLR7.

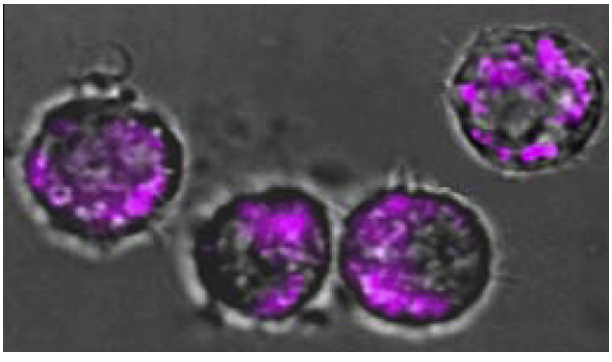


Figure 3. Murine J774 cells treated with 100 nM of **9**. An overlay of phase-contrast and epifluorescence images is depicted. An excitation filter at 562 nm and a long-pass emission filter (601–800 nm) were used.

Our attention subsequently turned to exploring the effect of varying substituents at N^1, while holding the C2-*n*-butyl and C4-NH_2 groups constant since these have been shown to correspond to maximal activity.[13] The N^1-naphthylenemethyl-substituted compound **4a** was inactive, and the N^1-biphenyl-4-methyl compound **4b** was weakly active (EC_{50}: 396 nM); the N^1-(4-amino-

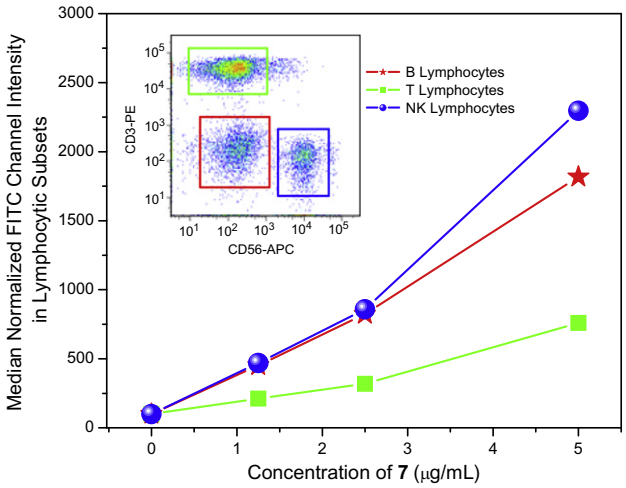


Figure 4. Uptake of **7** in lymphocytic subsets as examined by flow cytometry. Whole human blood was incubated with graded concentrations of **7** for 30 min, lymphocytes stained with cell surface markers (anti-CD3-phycoerythrin [PE], and anti-CD56-PE-allophycocyanin). Erythrocytes were lysed, and 10^5 total events were acquired per sample.

methyl)benzyl substituted analogue **5d** was considerably more active (EC_{50}: 20 nM; Scheme 1) than its N^1-(3-aminomethyl)benzyl regioisomer **5c** (EC_{50}: 110 nM). The free primary amine on the N^1 substituent of **5d** was covalently coupled directly to commercially-available fluorescein isothiocyanate and rhodamine B isothiocyanate (Scheme 2). Conversely, the amine on **5d** was converted first to the isothiocyanate **6**, allowing the subsequent coupling of amine-bearing fluorophores, such as the bora-diazaindacene dye, BODIPY-TR-cadaverine (Scheme 2). All three fluorescent conjugates retain TLR7-agonistic activity, although their potencies are slightly attenuated relative to the parent compound, **5d** (Fig. 2); the EC_{50} values of **7**, **8**, and **9** are, respectively, 247 nM, 115 nM, and 108 nM.

Incubation of murine macrophage J774.A1 cells with **8** or **9**, followed by intravital epi- and confocal fluorescence microcopy showed prominent perinuclear localization, which is consistent with the expected endosomal distribution of TLR7.[14] Shown in Figure 3 is a representative epifluorescence micrograph of J774 cells treated with **9** at 100 nM concentration.

Our earlier immunoprofiling of the TLR7-agonistic imidazoquinolines had shown a very prominent activation of B- and NK-cells, but minimal activation of T cells,[7] and we asked if a possible reason could be differential uptake of the TLR7 agonist in lymphocytic subsets. Flow cytometric analysis of the FITC-labeled **7** in experiments employing whole human blood indeed show a prominent uptake of **7** in $CD3^-CD56^+$ NK and $CD3^-CD56^-$ B lymphocytes as compared to $CD3^+CD56^-$ T lymphocytes (Fig. 4).

The syntheses of fluorescent imidazoquinoline analogues that retain TLR7-agonistic activity are expected to be useful probes in examining their potential immunostimulatory and adjuvantic properties.

Acknowledgment

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Supplementary data

Supplementary data (experimental methods and characterization of compounds (^1H, ^{13}C, and mass spectra)) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.09.093.

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