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Structural requirements for TLR7-selective signaling by 9-(4-piperidinylalkyl)-8-oxoadenine derivatives

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ABSTRACT

We report the synthesis and biological evaluation of a new series of 8-oxoadenines substituted at the 9-position with a 4-piperidinylalkyl moiety. In vitro evaluation of the piperidinyl-substituted oxoadenines **3a**–**g** in human TLR7- or TLR8-transfected HEK293 cells and in human PBMCs indicated that TLR7/8 selectivity/potency and cytokine induction can be modulated by varying the length of the alkyl linker. Oxoadenine **3f** containing a 5-carbon linker was found to be the most potent TLR7 agonist and IFNα inducer in the series whereas **3b** possessing a 1-carbon linker was the most potent TLR8 agonist.

Toll-like receptors (TLR) are a family of structurally related receptors that detect highly conserved microbial components common to large classes of pathogens. These receptors are expressed on immune cells and upon activation mobilize defense mechanisms aimed at eliminating the invading pathogens. Of the more than 10 known TLRs that have been identified in humans, five are associated with the recognition of bacterial components (TLRs 1, 2, 4, 5, 6) and four others (TLRs 3, 7, 8, 9) appear to be restricted to cytoplasmic compartments and are involved in the detection of viral RNA (TLRs 3, 7, 8) and unmethylated DNA (TLR9).[1,2] Activation of TLRs regulates intracellular signaling pathways leading to the expression of inflammatory cytokines/chemokines and type I interferons (IFNα/β), which can lead to the preferential enhancement of innate anti-microbial responses and antigen-specific humoral and cell-mediated immune responses.

In the case of TLR7 and TLR8 activation, a few different classes of small molecule mimetics of the natural uridine- and/or guanosine-rich viral ssRNA ligands have been identified,[3–5] including 1*H*-imidazo[4,5-*c*]quinolines[6] and 8-hydroxyadenines.[7] Through screening of a library of purine derivatives, Hirota[7] discovered that 9-benzyl-8-hydroxyadenine **1** (Fig. 1; shown as favored keto/oxo tautomer) possessed IFN-inducing activity in vitro. Further evaluation of structure-activity relationships (SAR) in the 9-benzyl hydroxyadenine or 'oxoadenine' series revealed that a hydroxy group at the 8-position is essential for IFN-inducing activity and that an alkylated heteroatom at the 2-position dramatically increases IFN activity (e.g., compound **2**, Fig. 1).

While an extensive evaluation of SAR in the 9-arylmethyl and heteroarylmethyl oxoadenine series has been carried out over the past several years,[8–16] to our knowledge no systematic studies have been performed on the corresponding saturated derivatives (i.e., cycloalkyl or heterocycloalkyl-substituted oxoadenines). The few reported oxoadenines with cycloalkyl groups attached to the nitrogen at the 9-position (directly or via a methylene unit) have demonstrated weak or diminished IFN induction.[7,11] Since alkyl linker length on the corresponding N1 of 1-phenylalkylimidazoquinolines is known to profoundly effect IFN-inducing activity,[6] we were particularly interested in the synthesis and biological evaluation of a series of 4-piperidinylalkyl derivatives of **2** in which the length of the N9 alkyl linker was systematically varied (compounds **3a**–**g**, Fig. 1). In addition to the ability to form water-soluble salts, the 4-piperidinyl moiety of oxoadenines **3a**–**g** also provides a suitable handle for potential N-derivatization and conjugation as substitution of the aromatic amino group in both the oxoadenine and imidazoquinoline series abolishes IFN-inducing activity.[6,17] Conjugation of TLR7/8 agonists to lipids, proteins, and other molecules is known to enhance immune responses and decrease toxic effects.[6,18,19]

The piperidinylalkyl oxoadenines **3a**–**g** were synthesized from 2-*n*-butoxy-8-methoxyadenine (**6**)[20] and *N*-*t*-butoxycarbonyl (Boc)-4-piperidinyl derivatives **10** or **11** in a convergent manner as shown in Scheme 1. Adenine **6** was prepared in 6 steps and in

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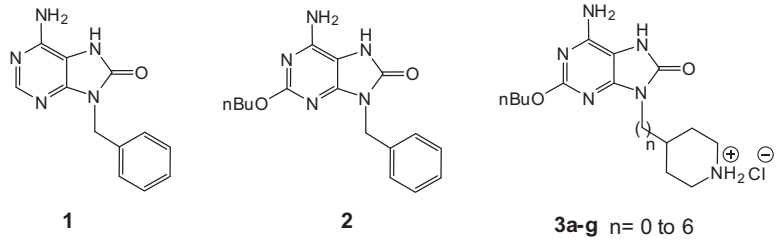
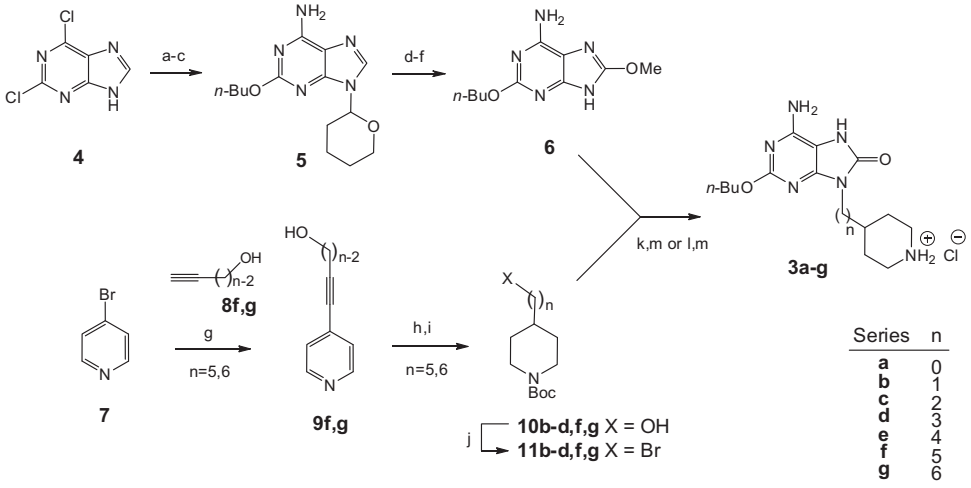


Figure 1. Structures of prototypical oxoadenines **1** and **2** and piperidinyl-substituted oxoadenines **3a**–**g**.

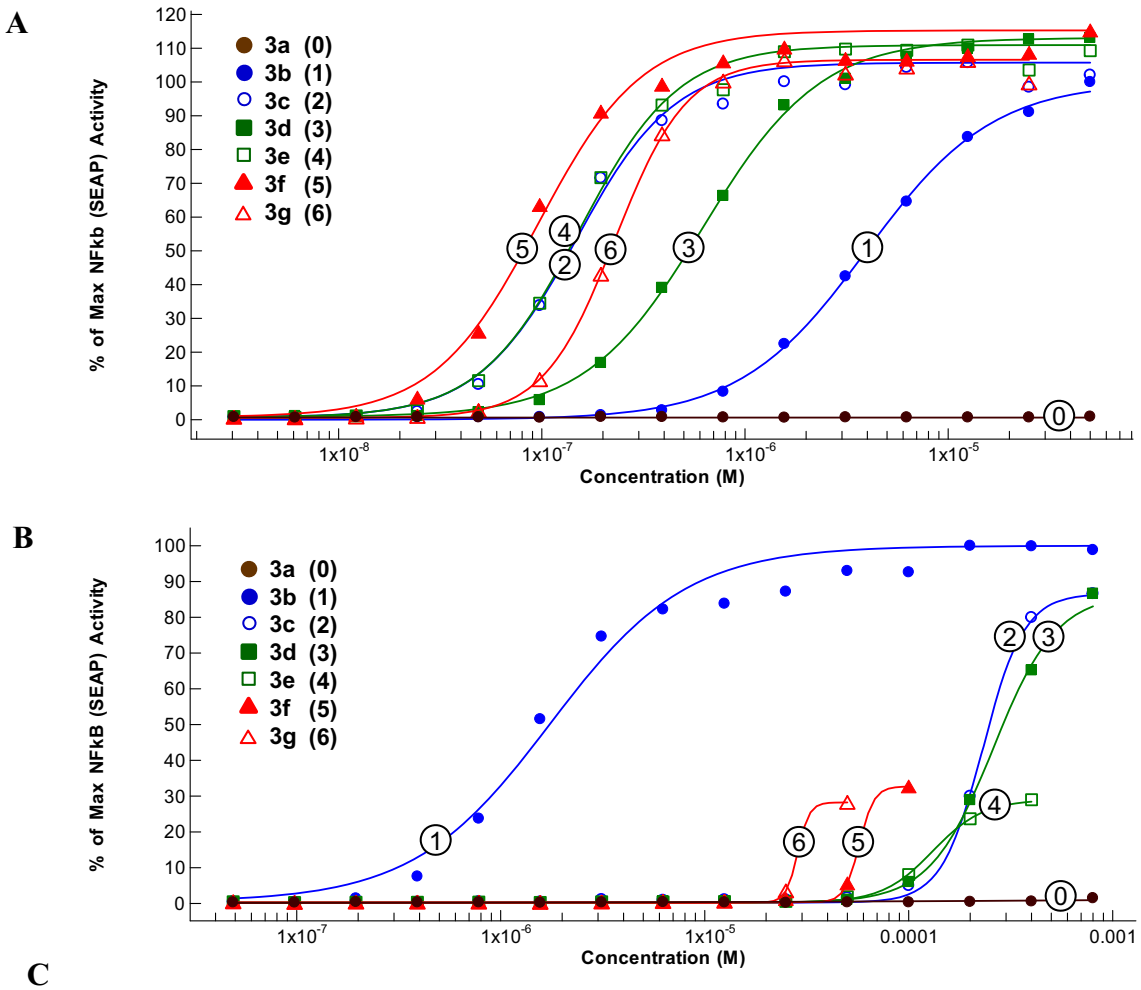
56% overall yield from commercially available 2,6-dichloropurine (**4**).[21] N-protection of **4** as the tetrahydropyranyl (THP) aminal, followed by sequential displacement of the 6- and 2-chloro groups with ammonia and *n*-butoxide gave the THP-protected adenine **5**[20] in 73% overall yield from **4**. Adenine **5** was then converted to **6** in 3 steps and in 77% overall yield by 8-bromination, bromide displacement with methoxide, and THP-deprotection with trifluoroacetic acid (TFA). The requisite Boc-protected 4-piperidinylalkyl bromides were either purchased commercially (**11a**,**e**) or prepared from the corresponding alcohols using Appel conditions (Ph_3P/CBr_4). The two piperidinyl alcohols not commercially available (**10f**,**g**) were prepared in 3 steps from 4-bromopyridine (**7**) by Sonogashira coupling of **7** with acetylenic alcohols **8f**,**g**, followed by hydrogenation of the alkynyl pyridines **9f**,**g** and *N*-Boc protection of the resulting piperidinyl alkanols.[22] Subsequent N-alkylation of adenine **6** with the Boc-protected piperidinylalkyl bromides **11b**–**g**[23] in the presence of potassium carbonate in dimethylformamide (DMF) followed by cleavage of the Boc and methoxy groups with 4 N HCl in dioxane afforded the desired oxoadenine hydrochloride salts **3b**–**g**[24] in 41–84% overall yield from **6**. Since N-alkylation of **6** with 1-Boc-4-bromopiperidine (**11a**) failed to give any product under these conditions (K_2CO_3, DMF), oxoadenine **3a**[25] was conveniently prepared according to a modification of a literature method[26] by Mitsunobu reaction of **6** with 1-Boc-4-hydroxypiperidine (**10a**) in presence of diisopropyl azodicarboxylate (DIAD) and PPh_3 followed by acidic deprotection.

The human (h) TLR7/8 activity of new oxoadenines **3a**–**g** was assessed by a reporter gene assay using HEK293 cells stably transfected with either hTLR7 or hTLR8 and the NFκB SEAP (secreted embryonic alkaline phosphatase) reporter (Fig. 2).[27] This assay measures NFκB mediated SEAP production following TLR7- or TLR8-specific activation. It should be noted that the HEK reporter assay only measures the NFκB pathway so additional assay systems are necessary to evaluate IRF7 pathway activation. The hTLR7 and hTLR8 specificity and potency (EC_{50}) of oxoadenines **3a**–**g** are shown in Figure 2. Oxoadenine **3a** was not active on hTLR7 or hTLR8 but the other oxoadenines **3b**–**g** were all active. While increasing the linker length beyond one carbon dramatically increased hTLR7 potency, no linear correlation between linker length and hTLR7 potency was observed in this assay. The 5-carbon linker oxoadenine **3f** was the most potent hTLR7 agonist of the series while the 1-carbon linker oxoadenine **3b** was the most potent hTLR8 agonist of the series, with hTLR8 potency significantly decreasing with longer linkers.

The loss of hTLR8 activity observed after stimulation with higher doses of oxoadenines **3e**–**g** suggested possible toxicity or activation induced cell death in HEK293-hTLR8 cells. Live/dead® fixable Aqua staining was used to evaluate potential cell death following HEK293-hTLR8 stimulation with oxoadenines **3e**–**g**. Aqua staining (cell death) at 24 h following stimulation correlated with both oxoadenine linker length and dose (Fig. 3). Cell toxicity was not observed after 24 h stimulation with the shorter linker oxoadenines **3b**–**d** (data not shown). The oxoadenine dose triggering HEK293-hTLR8 cell toxicity was the lowest with the 6-carbon linker (**3g**) and increased with decreased linker length (**3e**,**f**). However, the longer linker length oxoadenines (**3e**–**g**) induced modest NFκB activation in the HEK293-hTLR8 cells suggesting that the toxicity observed in HEK293-hTLR8 cells is dissociated from NFκB activity and possibly associated with activation of another intracellular signaling pathway via TLR8.



Scheme 1. Reagents and conditions: (a) 3,4-dihydropyran, *p*-TsOH, AcOEt, 50 °C; (b) 2 M NH_3 in *i*-PrOH, 60 °C, 86% (2 steps); (c) *t*-BuONa, *n*-BuOH, 100 °C, 85%; (d) *N*-bromosucccinimide, $CHCl_3$, rt, 88%; (e) NaOMe, MeOH, reflux; (f) TFA, MeOH, rt, 87% (2 steps); (g) $(PPh_3)PdCl_2$, CuI, Et_3N, Δ, 82% (**9f**), 21% (**9g**); (h) 10% $Pd(OH)_2$/C, H_2, AcOH, 90 °C; (i) Boc_2O, Et_3N, CH_2Cl_2, rt, 80% (2 steps); (j) CBr_4, PPh_3, CH_2Cl_2, rt, 92–99%; (k) Et_3N, PPh_3, DIAD, DMF, 70 °C, 63%; (l) K_2CO_3, DMF, 50 °C; (m) 4 N HCl/dioxane, MeOH, rt, 59% (**3a**), 75% (**3b**), 83% (**3c**), 41% (**3d**), 63% (**3e**), 64% (**3f**), 84% (**3g**).



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	3a	3b	3c	3d	3e	3f	3g
hTLR7 EC_{50} (μM)	--	3.95 ± 0.17	0.15 ± 0.02	0.62 ± 0.03	0.15 ± 0.01	0.10 ± 0.02	0.23 ± 0.02
hTLR8 EC_{50} (μM)	--	1.8 ± 0.37	231 ± 5.6	260 ± 8.8	131 ± 3.4	57 ± 19.9	29 ± 23.5

Figure 2. NFκB response of (A) HEK293-hTLR7 and (B) HEK293-hTLR8 cells treated for 24 h with oxoadenines **3a**–**g**; (C) hTLR7 and hTLR8 EC_{50} values for oxoadenines **3a**–**g** are shown with 95% confidence interval. Each data point was done in triplicate and averaged. EC_{50} were calculated after generating dose-response curves in XL*fit* (IBDS). Data is representative of four independent experiments. The maximum activity of **3b** (used as a reference standard) was set to 100% NFκB and all other compound values are relative to **3b**.

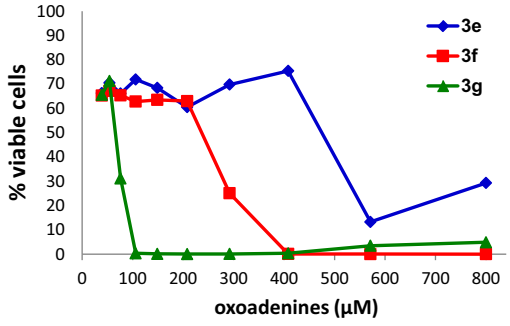

Figure 3. After 24 h stimulation with oxoadenines **3e**–**g**, HEK293-hTLR8 cells were incubated with Aqua dye for 30 min. After extensive wash, the stained cells were analyzed for viability using BD LSRII.

The induction of cytokines in human peripheral blood mononuclear cells (hPBMCs)[28] after 24 h stimulation with oxoadenines **3e**–**g** was evaluated using cytokine ELISA and intracellular cytokine staining (ICS).[29] Induction of TNFα is shown in Figure 4A. A clear increase in TNFα secretion with increasing linker length was observed, with maximal TNFα secretion observed for the 5-carbon linker (Fig. 4A). ICS was also used to examine the activation status and cytokine contributions of distinct cell subsets. A similar pattern was observed in myeloid dendritic cells (mDCs) (HLA-DR^+ $CD11c^+$ $CD123^-$) when comparing IL-6 (Fig. 4B), TNFα and IFNγ induction (not shown). Taken together, these data demonstrate an increase in proinflammatory cytokines as the length of the alkyl linker is increased to 5 carbons. The 6-carbon linker oxoadenine **3g** induced less TNFα than **3f** possessing 5 carbons but more than the 4-carbon linker oxoadenine **3e**.

Given the nonlinear relationship observed between the linker length and the hTLR7 EC_{50} values obtained with oxoadenines **3a**–**g** in the HEK293 system (which is only indicative of the NFκB side of TLR7 signaling), IFNα induction from hPBMCs was expected to be more representative of the hTLR7 activity of these compounds.

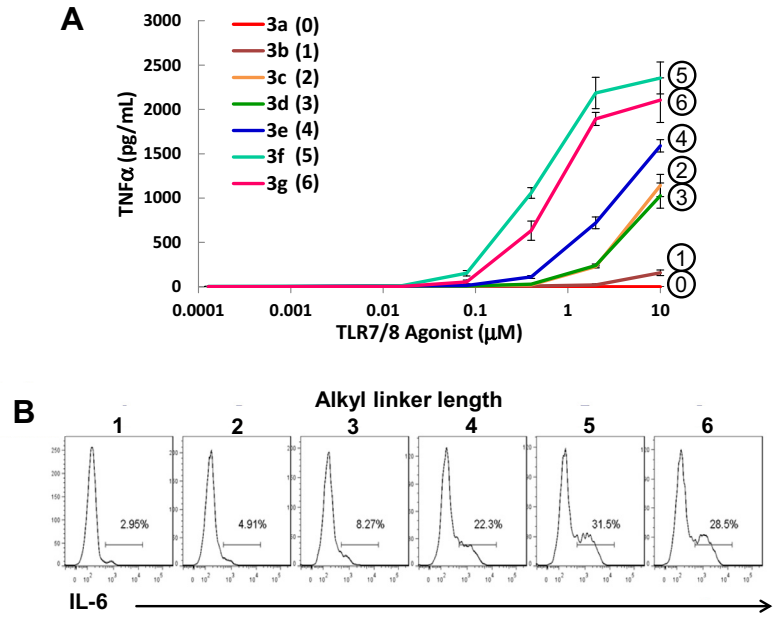

Figure 4. (A) TNFα induction in hPBMCs, and (B) IL-6 expression in mDCs after stimulation with oxoadenines **3a**–**g**. Data shown is representative of hPBMCs from three different healthy donors evaluated in triplicate. Circled numbers indicate the linker length. Error bars indicate SEM. TNFα was assayed using FluoroKine multiplex kits and IL-6 levels were measured by ICS in mDCs (HLA-DR$^+$ CD11c$^+$ CD123$^-$).

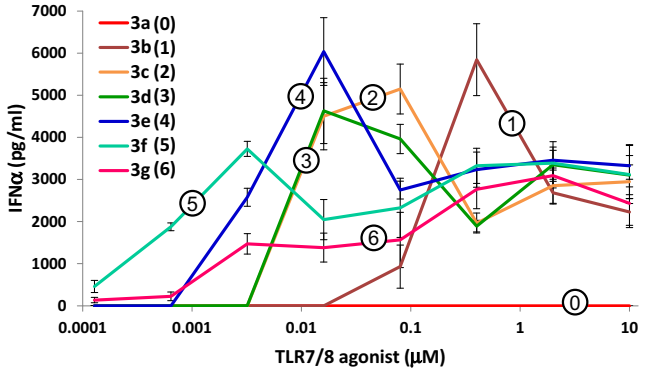

Figure 5. IFNα induction in hPBMCs after stimulation with oxoadenines **3a**–**g**. IFNα levels were measured using human IFNα VeriKine ELISA kit. Data shown is representative of hPBMCs from three different healthy donors evaluated in triplicate. Circled numbers indicate the linker length. Error bars indicate SEM.

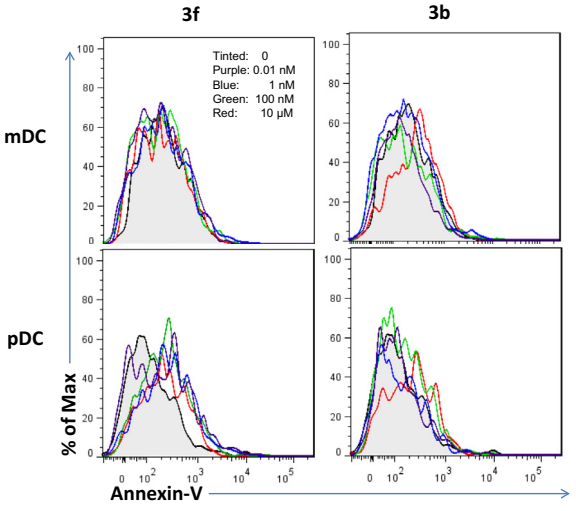

Figure 6. Annexin-V staining of hPBMCs after stimulation with **3b** or **3f** and cell phenotyping (mDC: HLA-DR$^+$ CD11c$^+$ CD123$^-$ and pDC: HLA-DR$^+$ CD11c$^-$ CD123$^+$).

A bimodal pattern for IFNα expression was observed for all but one of these compounds (Fig. 5). Each compound exhibited a bell-shaped dose–response curve from peak to base within a 100 μM dose range, except oxoadenine **3a** which was inactive. The dose required for peak IFNα induction decreased with increasing linker length, but higher concentrations led to a dose-responsive decrease in IFNα. Concurrently, TNFα levels in the same cell culture supernatants increased in a dose-dependent fashion (Fig. 4A). The dichotomy observed between TNFα and IFNα induction in the same hPBMC supernatants could be due to the differential expression of TLR8 on human mDCs and TLR7 on human pDCs. The pDC specific suppression of the TLR7-IRF7 signaling pathway via a regulatory feedback loop or cell-type specific activation induced cell death (AICD) could be responsible for the unique cytokine pattern observed in this study. To evaluate these hypotheses, hPBMCs were stimulated with various doses of **3b** (1-carbon linker) or **3f** (5-carbon linker) and the cells evaluated for activation induced apoptosis by Annexin-V staining. Oxoadenine **3f** was associated with a dose-dependent increase in Annexin-V staining in pDC starting at 1 nM but not in mDC (Fig. 6). In contrast, only the highest dose of **3b** (10 μM) was associated with Annexin-V positive cells in both pDC and mDC subsets. This observed cell type specific apoptosis correlated with the dose-dependent IFNα and TNFα induction curves and the intracellular cytokine staining of IFNα and TNFα in pDCs and mDCs, respectively (data not shown). Overall, these results demonstrated that increasing the carbon linker from 1 to 5 carbons increases the potency for IFNα induction from pDC but also reduces the dose threshold for apoptosis while leaving TNFα induction from mDC largely unaltered.

In summary, we have described the synthesis and the structure-activity relationship of a series of oxoadenines substituted at the 9-position with a 4-piperidinylalkyl moiety. A minimum of 1-carbon linker was required for hTLR7 and hTLR8 activity.

The 5-carbon linker oxoadenine was the most potent hTLR7 agonist while the 1-carbon linker was the most potent hTLR8 agonist of the series. Proinflammatory cytokines and IFNα induction in hPBMCs increased with increasing linker length, with the 5-carbon linker oxoadenine being the most active cytokine inducer. These results suggest that it is possible to modulate hTLR7/8 specificity and cytokine induction in the oxoadenine series with non-aromatic groups at N9 using minor structural modification. Insights from these types of studies will help to identify safer, more selective TLR7/8 agonists with tailored biological properties. More extensive structure–activity relationship studies in the 8-oxoadenine series and molecular modeling studies using the recently described TLR8 crystal structure[30] are currently underway.

Author contributions: All authors participated in the design or implementation or analysis, and interpretation of the study results. All authors were involved in drafting the manuscript or revising it critically for important intellectual content. All authors had full access to the data and approved the manuscript before it was submitted by the corresponding author.

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21. *Synthesis of* ***6***: *p*-Toluenesulfonic acid (0.01 equiv) was added to a suspension of 2,6-dichloropurine in ethyl acetate (0.5 M). The mixture was heated to 50 °C and 3,4-dihydro-2*H*-pyran (1.5 equiv) was added. The reaction mixture was stirred at 50 °C for 2 h then concentrated and dried under vacuum. The resulting yellow solid was heated with 2 M ammonia in isopropanol (7.0 equiv) at 50 °C for 7 h and the reaction mixture poured into water. After standing at rt overnight, the yellow solid was filtered off, washed with isopropanol, dried under vacuum and purified by chromatography on silica gel (0–2% $CHCl_3$ in CH_3OH) to give 2-chloro-9-(tetrahydro-2*H*-pyran-2-yl)-9*H*-purin-6-amine[31] in 86% yield. Sodium *t*-butoxide (4.0 equiv) was added portionwise to *n*-butanol (20 equiv) (Note: exothermic reaction). The suspension was stirred until homogeneous (~20 min) and 2-chloro-9-(tetrahydro-2*H*-pyran-2-yl)-9*H*-purin-6-amine (1.0 equiv) was added. The reaction mixture was then stirred at 100 °C overnight and concentrated under vacuum. After aqueous work-up (CH_2Cl_2/H_2O) and purification by chromatography on silica gel (0–1.2% CH_3OH in $CHCl_3$), compound **5**[20] was isolated in 85% yield. ^1H NMR ($CDCl_3$): δ 7.86 (1H, s), 6.10 (broad s, 2H), 5.63 (d, J = 10.0 Hz, 1H), 4.36 (m, 2H), 4.14 (d, J = 9.6 Hz, 1H), 3.75 (t, J = 11.2 Hz, 1H), 2.06 (m, 3H), 1.37–1.81 (m, 7H), 0.92 (t, J = 7.2 Hz, 3H). *N*-Bromosuccinimide (1.05 equiv) was slowly added to a solution of **5** in $CHCl_3$ (0.65 M) at 0 °C. The reaction mixture was stirred at 0 °C for 20 min then at rt for 4 h. After aqueous work-up (CH_2Cl_2/H_2O) and purification by chromatography on silica gel (0–1.5% CH_3OH in $CHCl_3$) 8-bromo-2-butoxy-9-(tetrahydro-2*H*-pyran-2-yl)-9*H*-purin-6-amine[20] was obtained in 88% yield. A solution of 8-bromo-2-butoxy-9-(tetrahydro-2*H*-pyran-2-yl)-9*H*-purin-6-amine in CH_3OH (0.35 M) was heated to reflux with a solution of sodium methoxide in CH_3OH (2.7 equiv) for 4 h. The reaction mixture was concentrated and after aqueous work-up (ethyl acetate and saturated NH_4Cl solution) the crude was purified by chromatography on silica gel (0–1.5% CH_3OH in $CHCl_3$) to give 2-butoxy-8-methoxy-9-(tetrahydro-2*H*-pyran-2-yl)-9*H*-purin-6-amine.[20] TFA (10% v/v TFA/CH_3OH) was added to a solution of 2-butoxy-8-methoxy-9-(tetrahydro-2*H*-pyran-2-yl)-9*H*-purin-6-amine in CH_3OH (0.23 M) and stirred at rt for 3 days. The reaction mixture was concentrated, diluted with ethyl acetate and filtered. The yellow solid was washed with a small volume of ethyl acetate and dried under vacuum to give **6**[20] as an off-white solid in 87% yield (2 steps). ^1H NMR ($CD_3OD/CDCl_3$) δ 4.50 (t, J = 6.8 Hz, 2H), 4.15 (s, 3H), 1.80 (m, 2H), 1.48 (m, 2H), 0.99 (t, J = 7.2 Hz, 3H).
22. *Synthesis of* ***10f*** *and* ***10g***: 4-Bromopyridine hydrochloride (2.5 g) was partitioned between 1 N sodium hydroxide (20 mL) and ethyl acetate (3 × 20 mL). The organic layer was separated, dried over Na_2SO_4 and concentrated under vacuum. The resulting oil was dissolved in Et_3N (2.6 M) and degassed under nitrogen. 4-Pentyn-1-ol or 5-hexyn-1-ol (1.1 equiv) was added followed by bis(triphenylphosphine)palladium(II) chloride (0.01 equiv) and copper(I) iodide (0.02 equiv) and the reaction mixture stirred at reflux for 20 min. Aqueous work-up (ethyl acetate/H_2O) and purification by chromatography on silica gel (gradient 0–30% ethyl acetate in heptane) led to **9f** and **9g** in 82% and 21% yield, respectively. **9f**: ^1H NMR (400 MHz, $CDCl_3$) δ 8.52 (m, 2H), 7.25 (m, 2H), 3.82 (m, 2H), 2.58 (t, J = 7.2 Hz, 2H), 1.88 (m, 2H), 1.62 (m, 1H). **9g**: ^1H NMR (400 MHz, $CDCl_3$) δ 8.52 (m, 2H), 7.25 (m, 2H), 3.73 (m, 2H), 2.48 (t, J = 7.2 Hz, 2H), 1.74 (m, 4H), 1.51 (m, 1H). Compound **9f** or **9g** was dissolved in acetic acid (0.05 M) and the solution hydrogenated using a H-Cube®continuous-flow hydrogenation reactor (ThalesNano) (20% $Pd(OH)_2/C$ cartridge, 100 bars H_2, 90 °C, 1 mL/min). Once the hydrogenation was complete, the reaction mixture was concentrated and dried under vacuum. The resulting crude was dissolved in CH_2Cl_2 (0.4 M) and reacted with Et_3N (1.5 equiv) and di-*t*-butyl dicarbonate (1.2 equiv) at rt for 30 min. After aqueous work-up (CH_2Cl_2/H_2O) and purification by chromatography on silica gel (gradient 0–30% ethyl acetate in heptane), compounds **10f** and **10g** were isolated in 80% yield. **10f**: ^1H NMR (400 MHz, $CDCl_3$) δ 4.06 (s, 2H), 3.64 (t, J = 6.8 Hz, 2H), 2.66 (t, J = 11.4 Hz, 2H), 1.54–1.66 (m, 4H), 1.45 (s, 9H), 1.24–1.39 (m, 8H), 1.08 (m, 2H). **10g**: ^1H NMR (400 MHz, $CDCl_3$) δ 4.05 (s, 2H), 3.64 (dd, J = 6.2, 10.6 Hz, 2H), 2.63 (t, J = 12.0 Hz, 2H), 1.53–1.65 (m, 4H), 1.45 (s, 9H), 1.20–1.40 (m, 10H), 1.04 (m, 2H).
23. *Synthesis of* ***11f*** *and* ***11g***: PPh_3 (1.2 equiv) was slowly added to a cold solution (0 °C) of **10f** or **10g** and CBr_4 (1.6 equiv) in CH_2Cl_2 (0.45 M) and the resulting solution stirred at rt for 45 min. The concentrated reaction mixture was directly purified by chromatography on silica gel (gradient 0–30% ethyl acetate in heptane) and **11f** and **11g** were isolated in 92% and 99% yield, respectively. **11f**: ^1H NMR (400 MHz, $CDCl_3$) δ 4.06 (s, 2H), 3.41 (t, J = 6.7 Hz, 2H), 2.66 (t, J = 11.7 Hz, 2H), 1.86 (m, 2H), 1.65 (m, 2H), 1.45 (s, 9H), 1.29–1.42 (m, 5H), 1.25 (m, 2H), 1.07 (m, 2H). **11g**: ^1H NMR (400 MHz, $CDCl_3$) δ 4.06 (s, 2H), 3.41 (t, J = 7.0 Hz, 2H), 2.66 (t, J = 11.7 Hz, 2H), 1.85 (m, 2H), 1.63 (m, 2H), 1.45 (s, 9H), 1.20–1.43 (m, 7H), 1.23 (m, 2H), 1.05 (m, 2H).
24. *General procedure for N-alkylation and acidic deprotection for* ***3b–g***[32]: To a solution of **6** in DMF (0.25 M) was added K_2CO_3 (325 mesh, 3.0 equiv). The reaction mixture was sonicated several seconds to obtain a fine suspension and stirred at 60 °C for 1 h. After cooling to 50 °C, bromide **11b–g** (1.2 equiv) was added and the reaction mixture stirred overnight at 50 °C. After cooling to rt and aqueous work-up (ethyl acetate/H_2O) the resulting crude was purified by chromatography on silica gel (gradient 0–10% CH_3OH in $CHCl_3$). The purified product was dissolved in CH_3OH (0.1 M) and reacted with 4 N HCl in dioxane

(6.0 equiv) at rt for 1 h. The reaction mixture was concentrated and dried under vacuum and the residue purified by chromatography on silica gel (0–100% $CHCl_3/CH_3OH/H_2O$ 85/15/0.5 in $CHCl_3/CH_3OH/H_2O$ 75/25/1.0). **3b**: ^{1}H NMR (400 MHz, CD_3OD) δ 4.27 (t, J = 6.8 Hz, 2H), 3.77 (d, J = 7.2 Hz, 2H), 3.40 (d, J = 12.8 Hz, 2H), 2.96 (dt, J = 2.8, 13.0 Hz, 2H), 2.21 (bs, 1H), 1.93 (d, J = 12.0 Hz, 2H), 1.75 (q, J = 6.8 Hz, 2H), 1.55 (m, 2H), 1.50 (m, 2H), 0.98 (t, J = 7.2 Hz, 3H); positive ES TOF-MS calcd for $[M+H]^+$ 321.2039, found 321.2226; **3c**: ^{1}H NMR (400 MHz, CD_3OD) δ 4.26 (t, J = 6.4 Hz, 2H), 3.88 (t, J = 7.0 Hz, 2H), 3.34 (d, J = 12.8 Hz, 2H), 2.93 (dt, J = 3.2, 12.8 Hz, 2H), 2.08 (bd, J = 14.4 Hz, 2H), 1.71–1.77 (m, 4H), 1.60 (bs, 1H), 1.48 (m, 2H), 1.41 (m, 2H), 0.98 (t, J = 7.6 Hz, 3H); positive ES TOF-MS calcd for $[M+H]^+$ 335.2195, found 335.2182; **3d**: ^{1}H NMR (400 MHz, CD_3OD) δ 4.27 (t, J = 6.4 Hz, 2H), 3.81 (t, J = 6.8 Hz, 2H), 3.35 (d, J = 12.4 Hz, 2H), 2.95 (dt, J = 2.8, 13.0 Hz, 2H), 1.67–1.80 (m, 6H), 1.31-1.51 (m, 7H), 0.98 (t, J = 7.2 Hz, 3H); positive ES TOF-MS calcd for $[M+H]^+$ 349.2352, found 349.3217; **3e**: ^{1}H NMR (400 MHz, CD_3OD) δ 4.26 (t, J = 6.4 Hz, 2H), 3.81 (t, J = 6.8 Hz, 2H), 3.33 (t, J = 12.4 Hz, 2H), 2.93 (dt, J = 2.8, 13.0 Hz, 2H), 1.89 (d, J = 12.8 Hz, 2H), 1.36–1.74 (m, 13H), 0.97 (t, J = 7.2 Hz, 3H); positive ES TOF-MS calcd for $[M+H]^+$ 363.2508, found 363.2594; **3f**: ^{1}H NMR (400 MHz, CD_3OD) δ 4.28 (t, J = 6.4 Hz, 2H), 3.83 (t, J = 6.8 Hz, 2H), 3.35 (m, 2H), 2.93 (t, J = 13.2 Hz, 2H), 1.91 (d, J = 13.2 Hz, 2H), 1.75 (m, 4H), 1.31–1.60 (m. 11H), 0.98 (t, J = 7.4 Hz, 3H); positive ES TOF-MS calcd for $[M+H]^+$ 377.2665, found 377.3884; **3g**: ^{1}H NMR (400 MHz, CD_3OD) δ 4.28 (t, J = 6.4 Hz, 2H), 3.82 (t, J = 7.0 Hz, 2H), 3.38 (m, 2H), 2.94 (t, J = 12.4 Hz, 2H), 1.92 (d, J = 13.2 Hz, 2H), 1.75 (m, 4H), 1.35–1.58 (m. 13 H), 0.98 (t, J = 7.2 Hz, 3H); positive ES TOF-MS calcd for $[M+H]^+$ 391.2821, found 391.3733.

25. *Synthesis of **3a***: DIAD (10 equiv) was added dropwise to a stirred solution of PPh_3 (10 equiv) in DMF (0.28 M) at 0 °C and the reaction mixture stirred at this temperature for 30 min. 1-Boc-4-hydroxypiperidine (**10a**, 10 equiv) was added and the reaction mixture stirred at 0 °C for 10 min then at rt for 30 min. A suspension of **6** (1.0 equiv) and Et_3N (2 equiv) in DMF (0.28 M) was added. The reaction mixture was stirred at 60 °C overnight, concentrated and purified by chromatography on silica gel (50–80% ethyl acetate in heptane followed by 0–2% CH_3OH in $CHCl_3$) to give the alkylated derivative in 63% yield. Subsequent acidic deprotection following the general procedure described above gave **3a** in 59% yield. **3a**: ^{1}H NMR (CD_3OD, 400 MHz) δ 4.53 (m, 1H), 4.29 (t, J = 6.4 Hz, 2H), 3.53 (d, J = 8.4 Hz, 2H), 3.14 (dt, J = 2.8, 10.4 Hz, 2H), 2.80 (dq, J = 3.8, 8.8 Hz, 2H), 2.01 (d, J = 10.8 Hz, 2H), 1.73 (m, 2H), 1.50 (m, 2H), 0.99 (t, J = 7.6 Hz, 3H).
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27. HEK293 cells expressing human TLR7 or TLR8 and NFκB responsive SEAP reporter gene were obtained from Invivogen (San Diego, CA). These cells were maintained in culture media of DMEM (Invitrogen, Grand Island, NY), 10% FBS (Sigma, St. Louis, Missouri) and selection antibiotics (Invitrogen, and Invivogen). HEK293 stably transfected with hTLR7 or hTLR8 were stimulated for 24 h with aqueous formulations of oxoadenines **3a**–**f** and culture supernatants were analyzed for NFκB activation using the colorimetric SEAP detection kit QuantBlue (Invivogen).
28. Primary human PBMCs were isolated from fresh blood from healthy donors via Ficoll gradient separation and plated at 0.5×10^6 cells/well in 96-well tissue culture plates (RPMI-1640 plus 10% FBS). hPBMCs were maintained with RPMI-1640 culture media (Invitrogen, Grand Island, NY), antibiotics (Invitrogen) and 10% FBS (Sigma). hPBMCs were stimulated for 24 h with aqueous formulations of oxoadenines **3a**–**f**. Culture supernatants were analyzed for cytokine/chemokine induction using multiplex kits (FluoroKine multiplex kits from R&D Systems, Minneapolis, MN) and human IFNα VeriKine ELISA kit (Pestka Biomedical Laboratories, Inc., Piscataway, NJ).
29. Human PBMCs were stimulated with aqueous formulation of oxoadenines **3a**–**g** for 24 h and GolgiPlug (10 ng/ml, BD Bioscience) was added for the final 6 h stimulation. After the 24 h stimulation cells were washed and stained for lineage-specific cell surface markers. BD Perm/Fix buffer (BD Bioscience) was used for permeabilization and fluorochrome conjugated anti-cytokine/chemokine antibodies were added. Data were acquired with a BD LSRII flow cytometer and analyzed with FlowJo (TreeStar, Boston, MA). Dead cells were excluded by FSC gating and Aqua exclusion.
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