Journal Pre-proofs

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PII: S0960-894X(20)30044-5
DOI: https://doi.org/10.1016/j.bmcl.2020.126984
Reference: BMCL 126984

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 29 October 2019
Revised Date: 17 January 2020
Accepted Date: 19 January 2020

Please cite this article as: Bazin, H.G., Bess, L.S., Livesay, M.T., Li, Y., Cybulski, V., Miller, S.M., Johnson, D.A., T. Evans., J., Optimization of 8-oxoadenines with toll-like-receptor 7 and 8 activity, *Bioorganic & Medicinal Chemistry Letters* (2020), doi: https://doi.org/10.1016/j.bmcl.2020.126984

Optimization of 8-oxoadenines with toll-like-receptor 7 and 8 activity.

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KEYWORDS. Toll-Like-Receptor, TLR7, TLR8, oxoadenine.

ABSTRACT: Toll-like receptors 7 and 8 (TLR7/8) agonists are potent immunostimulants that are attracting considerable interest as vaccine adjuvants. We recently reported the synthesis of a new series of 2-*O*-butyl-8-oxoadenines substituted at the 9-position with various linkers and *N*-heterocycles, and showed that TLR7/8 selectivity, potency and cytokine induction could be modulated by varying the alkyl linker length and the *N*-heterocyclic ring. In the present study, we further optimized the oxoadenine scaffold by investigating the effect of different substituents at the 2-position of the oxoadenine on TLR7/8 potency/selectivity, cytokine induction and DC maturation in human PBMCs. The results show that introducing a 1-(*S*)-methylbutoxy group at the 2-position of the oxoadenine significantly increased potency for TLR7/8 activity, cytokine induction and DC maturation.

Despite the success of vaccines in eradicating or reducing the incidence of some infectious diseases such as smallpox, polio and measles, there is still an urgent need for the development of effective vaccines against endemic pathogenic viruses (HIV, influenza), newly emerging diseases (Zika, Chikungunya viruses) and global killers (tuberculosis and cancer). The development of new

adjuvants that are able to shape, enhance and prolong specific immune responses are critical to the success of new vaccines. Adjuvants currently approved as part of licensed vaccines[1] are safe and effective at increasing humoral immunity, but have limited capacity to generate potent and durable cell-mediated immune response, which is critical for the protection against many viral diseases and cancers.[2]

Toll-like receptors (TLR) are expressed on immune cells and recognize many different exogenous pathogen-associated molecular patterns (PAMPs). Because TLRs play a crucial role in innate and adaptive immunity, their agonists are being extensively investigated as vaccine adjuvants.[3,4] Upon recognition of PAMPs, TLRs trigger the induction of cytokines and co-stimulatory molecules, leading to the recruitment of cellular mediators critical for initiating innate and adaptive immune responses. There are ten known human TLRs including five TLRs (1, 2, 4, 5, 6) that sense bacterial components and four TLRs (3, 7, 8, 9) that are located in cytoplasmic compartments and recognize viral RNA (TLRs 3, 7, 8) and unmethylated DNA (TLR9).[5] Human TLR7 is mostly found on plasmacytoid DC and B cells and upon activation triggers the IRF7 and NFκB pathways, leading to the induction of IFNα and proinflammatory cytokines, respectively. Expression of human TLR8 mainly occurs on monocytes, macrophages, neutrophils and conventional DCs and its activation leads to proinflammatory cytokines. 1*H*-imidazo[4,5-*c*]quinolines[6] (Imiquimod and Resiquimod, Figure 1) and 8-hydroxyadenines[7] (SM360320 and GSK2245035, Figure 1) are small molecule mimetics of ssRNA that activate TLR7/8. Imiquimod is the only approved TLR7/8 agonist and is used for the topical treatment of certain skin conditions.[8] The use of imiquimod and resiquimod as vaccine adjuvants has led to mixed results.[4] Some clinical trials have also been suspended over safety concerns[9-13] following serious side-effects observed upon administration of TLR7/8 agonists due to rapid systemic cytokine

distribution. Thus there is a need to develop safer and more effective TLR7/8 agonists as vaccine adjuvants.

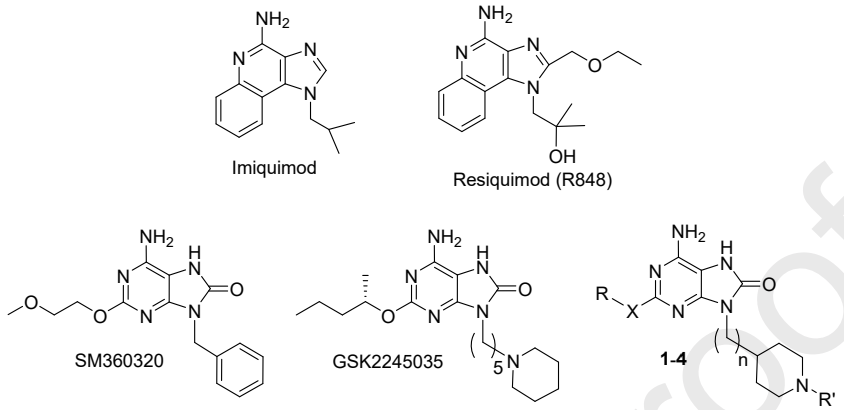


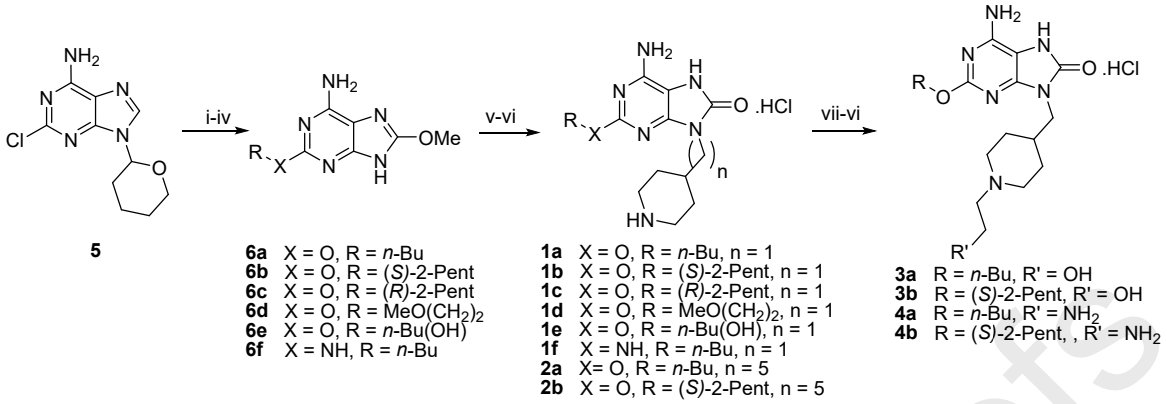
Figure 1. Structures of known imidazoquinolines and oxoadenines, and oxoadenines **1-4**.

In the course of our own program aimed at developing safe and effective TLR7/8 agonists as vaccine adjuvants, we synthesized and evaluated a series of twenty-seven new 2-*O*-butyl 8-oxoadenines substituted at the *N*-9 position with different *N*-heterocycles linked to the oxoadenine via alkyl linkers.[14,15] We demonstrated that TLR7/8 selectivity/potency and cytokine induction could be modulated by varying the length of the alkyl linker and correlated to the *N*-heterocycle ring size while the ring chirality had little effect on biological activity. We also reported that the *N*-heterocycle ring could be further substituted with amino- or hydroxyl-alkyl groups (Scheme 1, **3a** and **4a**) without negatively impacting biological activity.[15] We are interested in developing such *N*-heterocyclic oxoadenines for several reasons. In addition to their water-soluble salt forming ability, the NH and OH groups in oxoadenines **1-4** (Figure 1) can be further derivatized. Derivatization that can increase the cellular uptake of TLR7/8 agonists into endosomal/lysosomal compartments of DCs where TLR7 and TLR8 reside[16] are of considerable interest. To this end, we are investigating lipid conjugation of imidazoquinolines[17,18] and oxoadenines since lipid conjugation of nucleoside drugs, including TLR7/8 agonists,[19,20] is known to facilitate endocytosis

and decrease toxic side effects. Additionally, formulation of such nucleolipids into liposomes and other nanoparticle systems is expected to help protect the drug from degradation, further reduce toxicity and improve immunogenicity via a depot effect.[21]

In this letter, we report further optimization of the 8-oxoadenine scaffold by investigating the effect of different substituents at the C-2 position of the 8-oxoadnine scaffold on TLR7/8 potency and selectivity, and on cytokine (IFNα and TNFα) induction in human peripheral blood mononuclear cells (hPBMCs). The ability of these oxoadenines to induce DC maturation was also evaluated by measuring expression levels of major histocompatibility complex (MHC) molecules[22] (MHC-I and HLA-DR) and co-stimulatory ligands[23] (CD80 and CD86) in hPBMC.

We first evaluated five 2-substituted analogs of the 2-*O*-butyl-9-methylpiperidinyl-8-oxoadenine **1a**.[14] The substituents investigated are 1-(*S*)-methylbutoxy (**1b**), 1-(*R*)-methylbutoxy (**1c**), methoxyethoxy (**1d**), 4-hydroxybutoxy (**1e**) and *n*-aminobutyl (**1f**). We choose to limit the linear length of the 2-substituent to 4 and 5 atoms since optimal activity of similar oxoadenines has been observed with chain length ranging from 4 to 6 atoms.[7,24] Oxoadenines **1a-f** were prepared in two steps by alkylation of the corresponding intermediate 2-substituted-8-methoxyadenines **6a-f** with the requisite *N*-*t*-butoxycarbonyl (Boc)-protected 1-bromomethylpiperidine and acidic deprotection (Scheme 1). Intermediates **6a-f** were prepared from 2-chloro-6-amino-9-tetrahydropyran adenine **5**[25] in 4 steps following published procedures[26-28] (Scheme 1).



5

6a X = O, R = *n*-Bu
6b X = O, R = (*S*)-2-Pent
6c X = O, R = (*R*)-2-Pent
6d X = O, R = $MeO(CH_2)_2$
6e X = O, R = *n*-Bu(OH)
6f X = NH, R = *n*-Bu

1a X = O, R = *n*-Bu, n = 1
1b X = O, R = (*S*)-2-Pent, n = 1
1c X = O, R = (*R*)-2-Pent, n = 1
1d X = O, R = $MeO(CH_2)_2$, n = 1
1e X = O, R = *n*-Bu(OH), n = 1
1f X = NH, R = *n*-Bu, n = 1
2a X= O, R = *n*-Bu, n = 5
2b X = O, R = (*S*)-2-Pent, n = 5

3a R = *n*-Bu, R' = OH
3b R = (*S*)-2-Pent, R' = OH
4a R = *n*-Bu, R' = NH_2
4b R = (*S*)-2-Pent, , R' = NH_2

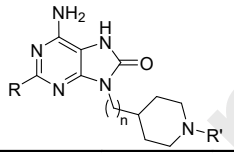
Scheme 1. Reagents and conditions: (i) *t*-BuONa, R-OH, 100 °C (**6a,d**) or 70 °C **(6b-c)**, 1-4 days, 58-85%; or $TBSO(CH_2)_4OH$, *t*-BuONa, 55 °C, 2.5 days, 63% **(6e)**; or *n*-$BuNH_2$, *n*-PrOH, 130 °C, 8 h, 96% (**6f**); (ii) NBS, $CHCl_3$, 0 °C to rt, 4 h; (iii) NaOMe, CH_3OH, Δ, 4 h, 75-78% (2 steps); (iv) TFA, CH_3OH, rt, 3 days, 83-90%; (v) K_2CO_3, 4-bromoalkyl-*N*-Boc-piperidine, DMF, 50 °C, 16 h; (vi) 4 N HCl/dioxane, CH_3OH, rt, 1 h, 61-90% (2 steps); (vii) K_2CO_3, $Br(CH_2)_2OTBS$ or $Br(CH_2)_2NHBoc$, DMF, 50 °C, 16 h, 53-70%.

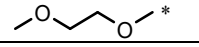
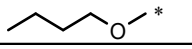
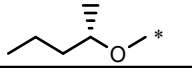
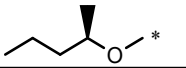
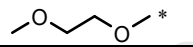
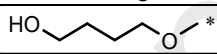
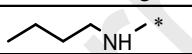
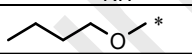
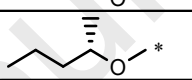
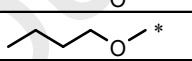
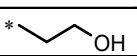
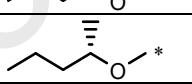
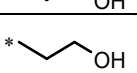
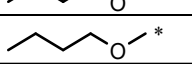
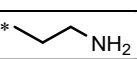
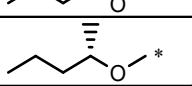
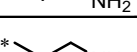
Oxoadenines **1a-f** were first assessed for human (h) TLR7 and TLR8 activity, and for cytokine induction (IFNα and TNFα) in human PBMCs, and compared to the known benchmarks imidazoquinoline resiquinod (R848, a dual TLR7/8 agonist) and Sumitomo oxoadenine SM360320 (TLR7 agonist). The hTLR7 and hTLR8 activity of the compounds 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. This assay measures NFκB mediated SEAP production following TLR7- or TLR8-specific activation. Since the HEK reporter assay only measures the NFκB pathway, additional assay systems would be necessary to evaluate the IRF7 pathway activation by TLR7 agonists. Of note, the TLR7/8 potency values we previously published[14] for **1a** and **2a** were determined using an in-house derived stably transfected HEK293 cell line. The current study uses commercially obtained HEK293-hTLR7 and -hTLR8 NFkB-SEAP reporter cells from Novus Biologicals (Littleton, CO) and Invivogen (San Diego, CA), respectively, resulting in some changes in EC_{50} values and in TLR7/8 specificity from the previously published findings.

Replacing the O atom at the 2-position (**1a**) with a N atom (**1f**) led to a two-fold reduction in hTLR7 EC_{50} (Table 1 and Figure S1). Substituting the third C atom in the 2-*O*-butoxy group (**1a**)

with an oxygen (**1d**) or adding a terminal hydroxyl group to the 2-*O*-butoxy group (**1e**) led to a significant decrease in hTLR7 activity, and the corresponding EC_{50} for **1e** and **1f** could not be calculated within the μM dose range evaluated. Introducing a (*S*)-methyl group α to the 2-*O* atom (**1b**) increased hTLR7 potency seven-fold while the corresponding *R*-isomer **1c** was about two times less potent than **1a**. Oxoadenines **1a-f** were less hTLR7/NFκB potent than the benchmark TLR7/8 ligands R848 and SM360320. Similar results were observed for hTLR8 activity.

Table 1. Chemical Structures, TLR7 and TLR8 Activity and Cytokines induction of 8-Oxoadenines



Compound	R	n	R'	TLR7 EC_{50} (μM)	TLR8 EC_{50} (μM)	IFNα MEC (μM)	IFNα PC (μM)	IFNα PL (pg/mL)	TNFα MEC (μM)	TNFα PL[a] (pg/mL)
R848	--	--	--	0.75±0.08	5.87±0.40	0.08	10	1408	2.0	19750
SM360320		--	--	1.17±0.30	--	0.08	0.08	1444	2.0	1663
1a		1	H	19.8±2.6	326±626	0.08	0.4	5899	2.0	2861
1b		1	H	2.68±0.57	2.53±1.31	0.0032	0.016	5473	0.4	12456
1c		1	H	35.8±4.6	619±115	0.08	0.4	6210	10	185
1d		1	H	>500	>500	10	10	2240	--	--
1e		1	H	>500	--	--	--	--	--	--
1f		1	H	40.0±15.2	>500	0.08	0.4	5983	--	--
2a		5	H	0.45±0.10	--	0.00064	0.016	4678	0.4	2874
2b		5	H	0.24±0.08	--	<0.00001	0.0032	2642	0.08	3015
3a		1		23.1±2.2	12.5±3.9	0.4	0.4	4731	10	2738
3b		1		4.06±0.56	2.14±0.51	0.016	0.08	4746	2.0	10257
4a		1		30.0±4.8	13.0±5.4	0.4	0.4	3045	2.0	6133
4b		1		6.71±1.13	2.13±0.61	0.016	0.08	4415	2.0	19307

EC_{50}s are mean values and SD of three (TLR7) or four independent experiments (TLR8) conducted with HEK293-hTLR7 or HEK293-hTLR8 cells treated for 24 hours with the indicated compounds. [a] PL (peak cytokine level) at 10 μM; MEC (minimum effective concentration; lowest dose tested that induced cytokine), PC (peak concentration, dose inducing the highest PL). PL, MEC and PC are representative data from one out of three independent donors.

The 1-(*S*)-methyl-butoxy oxoadenine **1b** was again the most potent oxoadenine of the series with over a 100-fold increase in hTLR8 potency compared to the *n*-butyloxy derivative **1a** (Table 1 and Figure S1). Oxoadenine **1c** was about two-fold less potent than **1a** while oxoadenines **1d-f** had negligible activity and their EC_{50}s could not be calculated in the μM range evaluated. The much larger increase of potency for TLR8 (>100-fold) over TLR7 (7-fold) observed when adding the (*S*)-methyl substituent to **1a** indicates that changes at the 2-position of the oxoadenine scaffold have a much greater effect on the hTLR8 receptor than on the hTLR7 receptor. Oxoadenine **1b** was about two-fold more hTLR8 potent than R848. As expected, these compounds did not activate hTLR3 or hTLR9, as shown by the lack of SEAP induction observed after stimulation of HEK293-hTLR3 or HEK293-hTLR9 cell lines with compounds **1a-e** (Figure S2), confirming the TLR7/8 selectivity of the compounds evaluated.

The 1-(*S*)-methylbutoxy oxoadenine **1b** was the most potent IFNα inducer of the series with a minimum effective concentration (MEC) 25-fold lower than observed for the butyloxy oxoadenine **1a** (Table 1 and Figure S2). Butyloxy (**1a**), 1-(*R*)-methylbutoxy (**1c**) and butylamine- (**1f**) analogs displayed similar IFNα induction profile (similar MEC, peak concentration (PC) and peak level (PL)) while the methoxyethoxy oxoadenine **1d** induced IFNα only at the highest dose tested. These results are consistent with the literature data.[7,24,27] The hydroxybutyloxy derivative (**1e**) was inactive across the dose range evaluated. Oxoadenines **1a-c**,**f** were more potent IFNα inducers than R848 and SM360320. The IFNα production from hPBMCs, which occurs via activation of the TLR7/IRF7 pathway, cannot be correlated to the hTLR7 activity observed in the HEK293-hTLR7 assay because the HEK assay only provides a read-out for the TLR7/NFκB pathway. In terms of TNFα induction from primary human PBMCs, the 1-(*S*)-methylbutoxy oxoadenine **1b** was also the most potent oxoadenine of the series (Table 1 and Figure S2) followed by the butyloxy

oxoadenine **1a**. The 1-(*R*)-methylpentoxy oxoadenine **1c** induced a very small amount of TNFα at the highest dose tested while the remaining oxoadenines were inactive. These results mirror the hTLR8 (NF-κB) responses from the HEK293 cells. Comparing cytokine induction observed for **1a** and **1f** indicates that when changing the heteroatom at the 2-position from oxygen to nitrogen, IFNα induction is maintained while TNFα induction is abrogated. Oxoadenines **1a-f** were less inflammatory than the benchmark imidazoquinoline compound R848.

In order to investigate if the enhancement of TLR7/8 potency and cytokine induction observed upon introducing a (*S*) methyl group to the 2-butyloxy chain is maintained across different 9-*N* substituted oxoadenines, we also synthesized the 1-(*S*)-methylbutoxy analogs (**2b-4b**) of oxoadenines **2a**,[14] **3a**[15,29] and **4a**.[15,29] Oxoadenine **2b**[30] was prepared by *N*-alkylation of intermediate **6b** with the requisite 1,1-dimethylethyl 4-(5-bromopentyl)-1-piperidine carboxylate[14] followed by acidic deprotection (Scheme 1). Oxoadenines **3b** and **4b** were prepared in two steps from **1b** by alkylation of the piperidinyl N atom with (2-bromoethyl)-*t*-butyldimethylsilane or 2-[(*t*-butoxycarbonyl) amino] ethyl bromide and acidic deprotection (Scheme 1). Introducing a (*S*)-methyl group to the 2-butyloxy chain of oxoadenines **2a-4a** increased hTLR7 potency as shown by the 4- to 6-fold reduction in EC_{50}s of oxoadenines **2b**-**4b** (Table 1 and Figure S1). This potency increase was similar to the one (7-fold) observed between **1a** and **1b**. hTLR8 potency of oxoadenines **3b** and **4b** was also enhanced 6-fold. hTLR8 activity of **2a** and **2b** was very low (Figure S1) and the corresponding hTLR8 EC_{50}s could not be calculated in the μM dose range evaluated. Oxoadenines **3a**, **3b**, **4a** and **4b** were slightly TLR8-selective. Both 1-(*S*)-methylbutoxy oxoadenines **3b** and **4b** had an IFNα MEC 25-fold lower than the corresponding butyloxy oxoadenines **3a** and **4a**, similar to the MEC decrease (25-fold) observed between **1a** and **1b**. While IFNα peak levels for 1-(*S*)-methylpentoxy oxoadenines **1b**, **3b** and **4b** were similar or higher than

the corresponding butyloxy oxoadenines **1a**, **3a** and **4a**, the most potent 1-(*S*)-methylpentoxy oxoadenines **2b** induced lower IFNα peak levels than the corresponding butyloxy oxoadenine **2a**. Oxoadenines **2-4** were all more potent IFNα inducer than R848 and oxoadenines **2a** and **2-4b** were more potent IFNα inducer than SM360320. Although oxoadenines **3b** and **4b** displayed higher MEC than SM360320, these oxoadenines induced higher IFNα peak levels. As observed for **1a**/**1b**, introduction of a (*S*)-methyl group to oxoadenines **2a** and **3a** led to a 5-fold decrease in TNFα MEC (Table 1 and Figure S2). While introducing the (S) methyl group to **4a** did not decrease the TNFα MEC concentration of **4b**, this small substitution drastically increased TNFα PL induced by **4b**, and **4b** was equivalent to R848 for TNFα induction in the dose range tested. Oxoadenines **2a-b** had the lowest TNFα MEC but also induced the lowest TNFα peak levels.

Oxoadenines **1-4** were also evaluated for their ability to induce DC maturation at a single 0.1 μM dose in hPBMCs, by measuring the levels of major histocompatibility complex (MHC) molecules[22] (MHC-I and HLA-DR) and co-stimulatory ligands[23] (CD80 and CD86) by flow cytometry. As expected, the least TLR7/8 active oxoadenines **1d,e** did not stimulate the production of any of the four DC maturation markers investigated in either mDCs or pDCs (Figures 2 and 3). In mDCs, increase of MHC-I stimulation was only observed for oxoadenines **2a**,**b**, albeit at a modest level (1.3 to 1.4-fold, Figure 2A). Oxoadenines **1b**, **2a,b** and **3b** also led to a 1.6- 2.7- and 1.5-fold increase of CD80 stimulation in mDCs, respectively (Figure 2B), although the large variance observed between the three donors tested would require testing in additional donors to draw a firm conclusion. Oxoadenines **1-4** induced no HLA-DR or CD86 in mDCs (Figures 3A-B). While R848 was also ineffective at inducing HLA-DR (Figure 3A) and CD80 in mDCs (Figure 2B), it induced a 1.4- and 1.9-fold increase in MHC-I (Figure 2A) and CD86 (Figure 3B) levels in mDCs, respectively.

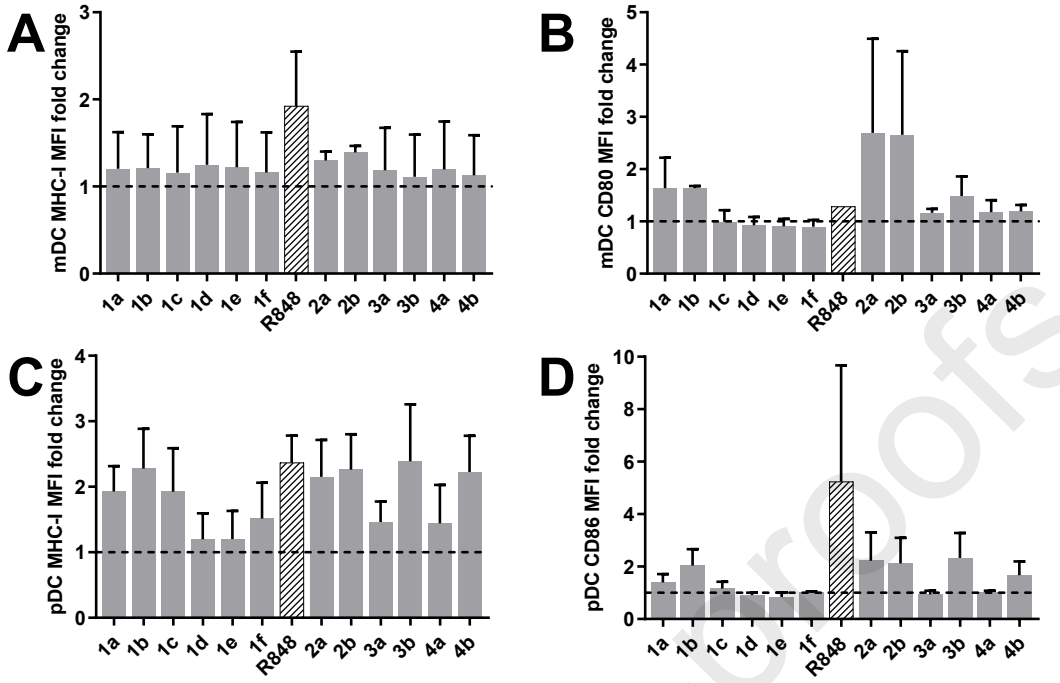


Figure 2. Fold-change of (A) MHC-I and (B) CD80 levels in mDCs; (C) MHC-I and (D) CD86 levels in pDCs, after 6 h stimulation with oxoadenines **1-4** compared to unstimulated cells. Data is mean values of three independent experiments in three different donors (2 donors for R848 except for CD80 with 1 donor). Error bars indicate SD.

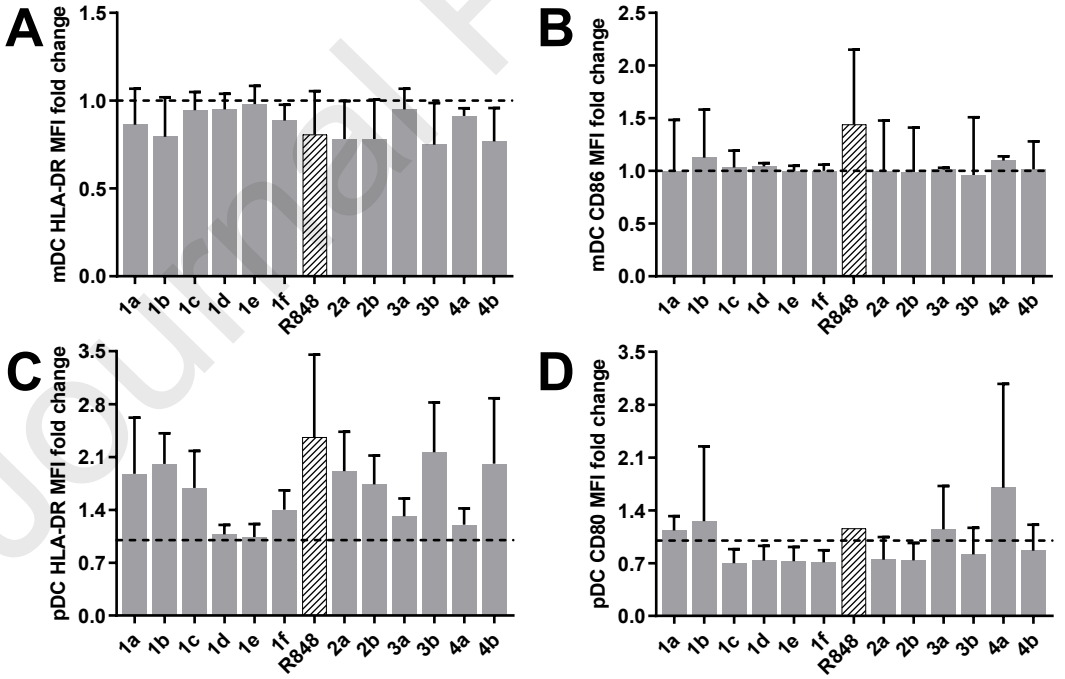


Figure 3. Fold-change of (A) HLA-DR and (B) CD86 levels in mDCs; (C) HLA-DR and (D) CD80 levels in pDCs, after 6 h stimulation with oxoadenines **1-4** compared to unstimulated cells. Data is mean values of three independent experiments in three different donors (2 donors for R848 except for CD80 with 1 donor). Error bars indicate SD.

The low DC maturation activity induced by only a few of the oxoadenines tested on mDCs is not unexpected since mDCs express TLR8 and most of the oxoadenines tested are weak TLR8 agonists. In general, oxoadenines **1-4** were more active in pDCs, which was expected since these oxoadenines are TLR7 agonists and human pDCs express TLR7. In pDCs, oxoadenines **1a-c** and **2-4** induced a 1.2- to 2.4-fold increase in MHC-I (Figure 2C) and HLA-DR levels (Figure 3C). Introducing a 1-(*S*)-methyl group to the 2-butyloxy chain of oxoadenines **3a** and **4a** increased MHC-I and HLA-DR stimulation by 1.5-1.7-fold. Compounds **2a,b** induced a similar 1.7-2.3-fold increase in MHC-I and HLA-DR stimulation but introduction of the 2-α methyl group in **2b** did not increase stimulation levels as observed with **3**-**4**. Oxoadenines **1c-e**, **3a** and **4a** were mostly inactive for CD86 (Figure 2D) and CD80 (Figure 3D) activation, while **1a** and **1b** induced a 1.4- and 2.0-fold increase of CD86 levels, respectively. As previously observed for MHC-I and HLA-DR upregulation, adding the 2-α methyl group to **3a** and **4a** led to a 1.7- to 2.3-fold increase in CD86 levels, but did not further increase the activity of **2b**. The most potent oxoadenines tested were as potent as R848 with respect to MHCI and II upregulation in pDCs but less potent than R848 with respect to CD86 upregulation.

In conclusion, the data described herein show that the TLR7/8 activity of oxoadenines can be modulated by the substituent at the C-2-position. Replacing the oxygen atom at the 2-position with a nitrogen atom led to a less potent oxoadenine and abrogation of TNFα induction while introducing an oxygen or hydroxyl in the C-2 butyl side-chain drastically decreased TLR7/8 activity. Introducing an α-(*S*) methyl group on the 2-butyloxy side chain increased TLR7/8 potency, cytokine induction and upregulation of some DC maturation markers. The α-(*S*) methylated oxoadenines **3b** and **4b** were the most TLR8 active oxoadenines of the series with EC_{50}

< 5 μM. Oxoadenine **3b** has been selected for further phospholipidation and the resulting phospholipidated oxoadenine is being evaluated as a vaccine adjuvant.

Acknowledgements

The authors thank Lois Walsh and Rebekah Tee for formulation of the compounds described in this study, Clara Davison for performing the hTLR3 and hTLR9 assay, and GlaxoSmithKline where the compounds were first prepared and tested under NIAID contract HHSN272200900036C prior to Novation of the contract to the University of Montana for completion of the work described.

AppendixA. Supplementary data

Supplementary data to this article can be found online.

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Conflict of Interest Disclosure

HGB, LSB, MTL, YL, VC, DAJ and JTE were employees of the GSK group of companies at the time of the synthesis and initial in vitro characterization of the compounds (HEK293 and PBMC assays). HGB, LSB, MTL, VC and JTE became employees of the University of Montana where additional compound synthesis/characterization and in vitro testing (HEK293, PBMC and DC maturation markers) were completed. HGB, YL and DAJ are inventors on patents covering some of the oxoadenines described in this manuscript.

Funding Sources

This work was supported in part by the National Institute of Allergy and Infectious Diseases (NIAID) under contract HHSN272200900036C (to Corixa Corporation d/b/a GlaxoSmithKline Biologicals SA, novated to University of Montana) and by the Center for Translational Medicine at University of Montana. Any opinions, findings, conclusions or recommendations expressed in this article are those of the authors and do not necessarily reflect the views of the NIAID.

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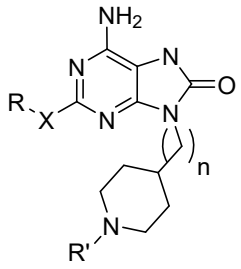
Graphical Abstract



	X	R	n	R'	EC_{50} (μM) hTLR7	hTLR8
1a	O	*n*-Bu	1	H	19.8	326
1b	O	(*S*)-2-Pent	1	H	2.68	2.53
1c	O	(*R*)-2-Pent	1	H	35.8	>500
1d	O	$CH_3O(CH_2)_2$	1	H	>500	>500
1e	O	$HO(CH_2)_4$	1	H	>500	--
1f	N	*n*-Bu	1	H	40.0	>500
2a	O	*n*-Bu	5	H	0.45	--
2b	O	(*S*)-2-Pent	5	H	0.24	--
3a	O	*n*-Bu	1	$(CH_2)_2OH$	23.1	12.5
3b	O	(*S*)-2-Pent	1	$(CH_2)_2OH$	4.06	2.14
4a	O	*n*-Bu	1	$(CH_2)_2NH_2$	30.0	13.0
4b	O	(*S*)-2-Pent	1	$(CH_2)_2NH_2$	6.71	2.13