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Synthesis and immunostimulatory activity of 8-substituted amino 9-benzyladenines as potent Toll-like receptor 7 agonists

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Abstract—Several 9-benzyl adenine derivatives bearing various substituted amines at the 8-position have been prepared and evaluated for interferon induction in peripheral blood mononuclear cells (PBMC) from healthy human donors. The 8-bromoadenine derivative **5** was used as a versatile intermediate for all substitutions. The most active 8-substituted amino compound was found to be the 8-morpholinoethylamino derivative **19** which had an EC_{50} in the submicromolar range.

In the mid 1980s, our laboratory and others began studying a group of synthetic nucleosides that had the unique ability to activate the innate immune response. These compounds were mainly derivatives and analogs of guanosine. Structure–activity studies within this class showed that certain compounds in the thiazolo[4,5-*d*]pyrimidine,[1] pyrazolo[3,4-*d*]pyrimidine,[2] purine,[3] 7-deazapurine,[4] and 9-deazapurine[5] ring systems were all active. Examples in each group were found to be potent antiviral agents in mouse models due to their ability to rapidly induce the production of interferon.[4,6,7] Among these guanosine analogs, 7-thia-8-oxoguanosine (**1**, TOG, Fig. 1) was studied in greatest detail and shown to exhibit broad spectrum antiviral activity and to activate natural killer cells, macrophages, and B-lymphocytes.[8] Other guanosine analogs extensively studied were 7-deazaguanosine[9] and 7-allyl-8-oxoguanosine (loxoribine).[10] The exact mechanism of innate immune potentiation by these guanosine-like compounds had not been elucidated and we were prompted to investigate the possibility that these small molecules could be acting via signaling through one or more of the known Toll-like receptors (TLRs), of which there are more than ten now identified, which recognize molecular patterns commonly associated with many microbial agents. As a result of those studies, we recently showed that TOG and certain other guanosine analogs activate immune cells via TLR7.[11] Interestingly, our earlier studies also showed that a sugar moiety was not required for immune system potentiation, as certain alkylated purines were also effective.[3] Since that time, alkylated adenine derivatives have been discovered[12–14] which are even more potent interferon inducers than the guanines and guanosines. Compounds of both classes, guanines and adenines, might be clinically useful in immune-based therapy or prophylaxis against viral diseases and other infectious diseases and cancer. Indeed, TOG, also known as isatoribine, has recently been reported to be effective at reducing the plasma virus concentration in patients with chronic hepatitis C virus (HCV) infections[15] with minimal side effects. Our own recent studies provide evidence that TLR7-mediated immunity against HCV involves at least two mechanisms: one depends on type 1 interferon production by leukocytes, and the other is mediated by TLR7 expressed by virally infected hepatocytes.[16] Currently, one TLR7/8 agonist (Imiquimod, **2**) is approved for use in human subjects for certain viral infections and skin cancers, and other agonists are in advanced stages of clinical development.[17] TLR agonists might be exploited for use against bioterror attacks on the basis of their mechanism of action. However, excessive activation of the innate immune system can result in severe side effects, autoimmune disease, and septic shock.[18] Indeed, the related molecule R848 (Resiquimod, **3**), a more potent analog of **2**, was reported to lack antiviral effects in HCV-infected patients, perhaps due to the poor tolerability that limited the dose level and frequency.[15] There-

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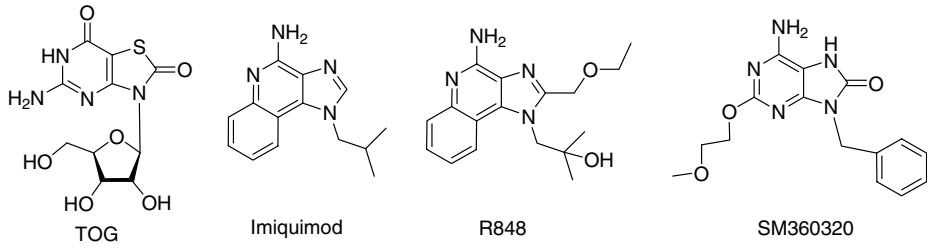


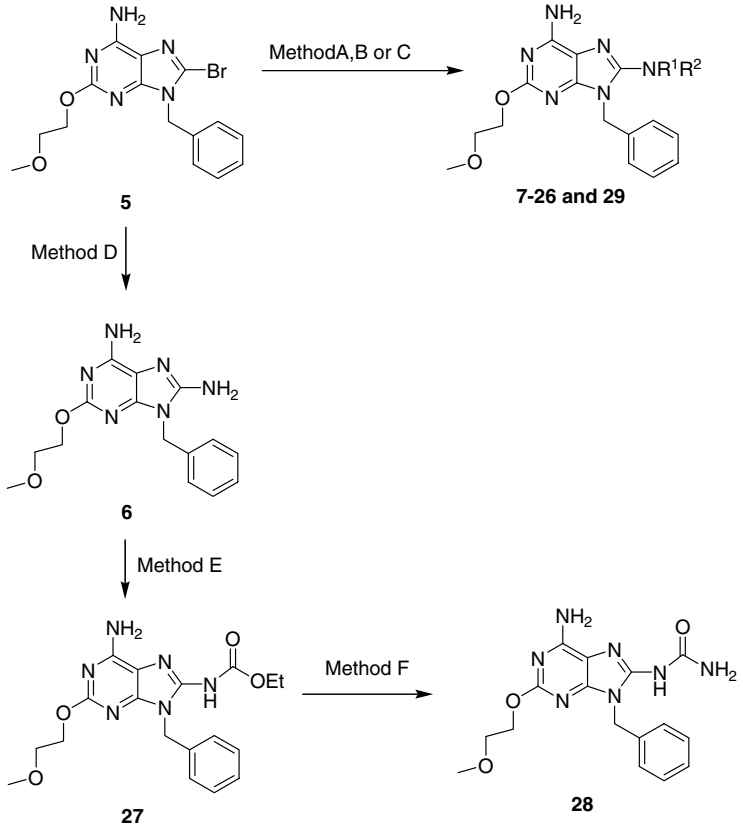
Figure 1.

fore, the ideal candidates as TLR agonists should have the right balance of activity and tolerability.

As part of our ongoing studies in the design and preparation of agonists of TLR signaling, we prepared a series of 8-substituted amino adenine compounds and evaluated them in mouse and human cell-based assays. The starting point for the selection of the compounds was based on recent reports describing the potent activity of 9-benzyl-8-oxo-2-alkoxyadenines as interferon inducers.[13,14] We first prepared 9-benzyl-2-methoxyethoxy-8-oxoadenine (**4**) according to the published procedure[14] and then confirmed that it was acting exclusively through TLR7 signaling.[16] A review of the literature revealed that apart from the 8-oxo group, no other modifications at the 8-position had been reported for this purine class of interferon inducers, with the exception of a few prodrugs that would eventually provide the 8-oxo function.[13] Thus, the reported modifications included those at the 2 and 9 positions only.[12] We elected to prepare and investigate the structure–activity function of a series of 8-substituted amino adenines while maintaining all other structural features constant.

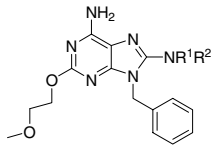
The versatile 8-bromoadenine derivative (**5**)[14] was used as an intermediate for all substitutions. The general procedures for the amine substitutions are depicted in Scheme 1 and the final products are listed in Table 1, each with the corresponding method of preparation indicated.

The synthesis of compounds **7**–**10**, **12**, **14**–**16**, **18**–**24**, **26**, and **29** by method A was carried out in an autoclave using water as solvent. The hydrazino compound **29** pre-



Scheme 1. Reagents and conditions: Method (A) NH(R^1R^2), H_2O, 110–125 °C, 12 h. Method (B) NH(R^1R^2), 150–160 °C, 6 h. Method (C) $Pd_2(dba)_3$, BINAP, secondary amine, K_2CO_3, *t*-butanol, 130 °C, 12 h. Method (D) 1—NaN_3, DMF, 100 °C, 7 h; 2—H_2/Raney-Ni, rt, 12 h. Method (E) ClCOOEt, pyridine, rt, 12 h. Method (F) NH_3/MeOH, 70 °C, 10 h.

Table 1.



Compound	R^1	R^2	Method	IFN-α[a]	SEM
4				418.00	53.94
6	H	H	D	35.25	23.19
7	H	Me	A	[b]	
8	H	Et	A	80.30	34.22
9	H	*n*-Pr	A	8.80	4.44
10	H	*n*-Bu	A	0.73	0.73
11	Et	Et	C	[b]	
12	H	2-Hydroxylethyl	A	99.21	36.11
13	2-Hydroxylethyl	2-Hydroxylethyl	B	0.82	0.00
14	H	3-Hydroxy-*n*-propyl	A	113.18	39.73
15	H	4-Hydroxy-*n*-butyl	A	[b]	
16	H	Tetrahydrofurfuryl	A	[b]	
17	H	Benzyl	B	[b]	
18	H	Phenylethyl	A	1.33	0.00
19	H	2-(Morpholino)ethyl	A	110.48	30.16
20	H	2-(Piperidin-1-yl)ethyl	A	[b]	
21	H	2-Methoxyethyl	A	[b]	
22	H	Diethanolaminoethyl	A	[b]	
23	H	Diethanolaminopropyl	A	[b]	
24	H	Cyclohexylmethyl	A	[b]	
25		Morpholino	C	0.41	0.00
26	H	2-(1*H*-Indol-3-yl)ethyl	A	[b]	
27	H	Ethoxycarbonyl	E	1.03	0.00
28		Carbamoyl (8-ureido)	F	[b]	
29	H	NH_2 (8-Hydrazino)	A	0.21	0.21
DMSO				0.26	0.26

[a] Interferon concentration in pg/mL. All compounds compared at 1 μM; see Ref. 24.
[b] Below lower limit of detection.

cipitated from a reaction using 20% aqueous hydrazine, while compound **6** was the major product if a concentration of hydrazine lower than 10% was used. Products **13** and **17** were afforded by reaction of **5** with a large excess of diethanolamine or benzylamine, respectively, as reagent and solvent at elevated temperatures. Compounds **11** and **25** were obtained in good yield by the palladium-catalyzed reaction of the corresponding secondary amine with **5** under anhydrous conditions. To our knowledge, this is the first example of palladium-catalyzed amination of an adenine system by a hindered amine. There are existing methods for 8-aminoadenine preparation.[19–23] Reaction of 8-bromo compound **5** with NaN_3 followed by Raney-Ni-catalyzed hydrogenation furnished **6** in good yield. Acylation of **6** with ethylchloroformate led to the ethylcarbamate **27** which was converted to the ureido compound **28** by reaction with methanolic ammonia.

All compounds were tested for their ability to induce the production of interferon α in human peripheral blood mononuclear cells (PBMC) compared to compound **4** as a positive control.[24] As indicated in Table 1, each compound was tested at 1 μM and the most active compounds were found to be **8**, **12**, **14**, and **19**. Detailed analytical data for these compounds are provided in the references section below.[25–28] In addition, the EC_{50} (the concentration of compound at which 50% of the maximal IFN concentration was achieved) was determined for these four most active compounds and these are shown relative to compound **4** in Table 2[29] along with the average maximal IFN produced by each compound. These values are a composite of data from several healthy donors.

The simple replacement of the 8-oxo function in compound **4** with an amino group (compound **6**) completely removed all interferon inducing activity.

Table 2.

Compound	EC_{50}[a] (μM)	Maximal IFN[b]
4	0.14	220
6	>10	132
8	4.42	141
12	5.33	168
14	3.20	206
19	0.79	176

[a] See Ref. 29.
[b] Average value in pg/mL of interferon from PBMC of three healthy donors. All compounds compared at 10 μM.

However, further substitution of this amino function yielded several active compounds with structure–activity trends that are apparent even within this relatively small group. First, the homologous series of *N*-methyl, *N*-ethyl, *N*-*n*-propyl, and *N*-*n*-butyl derivatives showed that, by comparison, the 2-carbon chain (compound **8**) was superior to the others. However, when one adds a second ethyl group (compound **11**), the activity is lost. When a terminal hydrophilic group, such as OH, is added to the ethyl group (compound **12**), the activity is enhanced somewhat. In this series, the hydroxypropyl is about equally active but the hydroxybutyl is devoid of activity. Addition of a second hydroxyalkyl group (compound **13**) again abrogates activity. The ring-containing secondary amine, morpholino compound **25**, was also not active. Interestingly, addition of a 2-carbon alkyl chain between the 8-amino and the morpholino ring (compound **19**) resulted in the highest activity of the entire group of 8-substituted amino compounds studied to date, with an EC_{50} of 0.79 μM. Other compounds with ring-chain combinations besides morpholinoethyl, such as phenylethyl (**18**), piperidin-1-ylethyl (**20**), and 2-(1*H*-indol-3-yl)ethyl (**26**), were not active. All active compounds are thought to be working exclusively through TLR7 signaling, as was shown earlier for compound **4**,[16] since experiments in which bone marrow derived macrophages from TLR7 knockout mice were exposed to the active compounds showed no enhancement of cytokine (TNFα, IL-6, IL-12, etc.) production (data not shown). Studies to optimize the lead compound **19** are presently underway.

Acknowledgments

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24. Human blood samples were obtained from the San Diego Blood Bank. PBMC were isolated by density-gradient centrifugation over Ficoll-Hypaque (Amersham Pharmacia). Cells were resuspended in RPMI 1640 medium, supplemented with 10% fetal bovine serum (FBS), L-glutamine, and penicillin/streptomycin (RP10; Invitrogen, Carlsbad, CA), plated at 10^6 cells/well in 96-well plates, and stimulated with compounds at 1 μM final concentration for 24 h at 37 °C, 5% CO_2. The IFN-α level in the supernatants was measured by Luminex (Austin, TX) using the Beadlyte Human MultiCytokine kit (Upstate, Charlottesville, VA), according to the manufacturer's instructions. Results presented in the table are averages of data composited from four different donors.
25. Selected data for compound **12**: mp: 145–146 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.17–7.33 (m, 5 H), 5.18 (s, 2H), 5.11 (s, 2H), 4.44 (t, J = 5 Hz, 2H), 4.36 (br, 1H), 3.75 (q, J = 5 and 5 Hz, 4H), 3.48 (t, J = 5 Hz, 2H), 3.41 (s, 3H). MS (ESI) *m/z*: 359.3 (MH^+). Anal. Calcd for $C_{17}H_{22}N_6O_3$: C, 56.97; H, 6.19; N, 23.45%. Found: C, 56.59; H, 6.29; N, 23.06%.

26. Compound **8**: mp: 156–158 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.19–7.35 (m, 5H), 5.15 (s, 2 H), 5.11 (s, 2H), 4.45 (t, J = 5 Hz, 2H), 3.74 (t, J = 5 Hz, 2H), 3.69 (br, 1H), 3.41 (s, 3H), 3.37 (m, 2H), 1.13 (t, J = 5 Hz, 3H). MS (ESI) m/z: 343.4 (MH^+). Anal. Calcd for $C_{17}H_{22}N_6O_2 \cdot 0.5\ H_2O$: C, 58.05; H, 6.54; N, 23.90%. Found: C, 58.45; H, 6.44; N, 23.64%.
27. Compound **14**: mp: 128–130 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.18–7.33 (m, 5H), 5.11 (br, 4 H), 4.44 (t, J = 5 Hz, 2H), 3.73 (t, J = 5 Hz, 2H), 3.52 (m, 4H), 3.40 (s, 3H), 1.61 (m, 2H). MS (ESI) m/z: 373.8 (MH^+). Anal. Calcd for $C_{18}H_{24}N_6O_3 \cdot 0.5H_2O$: C, 56.63; H, 6.55; N, 22.02%. Found: C, 57.01; H, 6.42; N, 22.22%.
28. Compound **19**: mp: 148–151 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.23–7.34 (m, 5H), 5.19 (br, 2H), 5.12 (s, 2H), 4.75 (br, 1H), 4.46 (t, J = 5 Hz, 2H), 3.75 (t, J = 5 Hz, 2H), 3.47 (m, 4H), 3.41 (s, 3H), 3.38 (t, J = 5 Hz, 2H), 2.51 (t, J = 5 Hz, 3H), 2.30 (m, 4H). MS (ESI) m/z: 450.6 (MNa^+). Anal. Calcd for $C_{21}H_{29}N_7O_3 \cdot 0.2H_2O$: C, 58.45; H, 6.81; N, 22.73%. Found: C, 58.51; H, 6.85; N, 22.51%.
29. For dose response study, cells were treated with compounds at concentrations ranging from 10 μM to 10 nM for 24 h. Results are a composite from three different donors and non-linear regression curve fit analysis was performed using GraphPad Prism software version 4.0b (San Diego, CA) to determine the EC_{50}.