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Synthesis and evaluation of 8-oxoadenine derivatives as potent Toll-like receptor 7 agonists with high water solubility

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ABSTRACT

We report the discovery of novel series of highly potent TLR7 agonists based on 8-oxoadenines, **1** and **2** by introducing and optimizing various tertiary amines onto the N(9)-position of the adenine moiety. The introduction of the amino group resulted in not only improved water solubility but also enhanced TLR7 agonistic activity. In particular compound **20** (DSR-6434) indicated an optimal balance between the agonistic potency and high water solubility. It also demonstrated a strong antitumor effect in vivo by intravenous administration in a tumor bearing mice model.

The immune system is comprised of innate and acquired immunity, both of which work cooperatively to protect the host from microbial infections. Toll-like receptors (TLRs) are a family of type I transmembrane receptors and are the central component of the innate immune system. TLRs stimulate immune cells via the MyD88-dependent interleukin-1 receptor signaling pathway. So far, 13 subtypes of TLRs have been reported and each is known to be fundamental in the recognition of pathogen-associated molecular patterns.[1,2] Ligation of TLRs on antigen-presenting cells, such as dendritic cells (DCs), leads to production of proinflammatory cytokines, DC maturation and priming of the adaptive immune system.[3] TLR7 is expressed by plasmacytoid dendritic cells (pDCs) and ligand recognition leads to the secretion of type I interferon (IFN).[4]

Imiquimod has been reported as the first synthetic TLR7 agonist and preclinical studies indicate that it is likely to function through the induction of type I IFN and IFN-inducible genes, which in turn can have direct effects on tumor cell growth and/or harness components of the adaptive immune system. TLR7 activation can also enhance antitumor effects through inhibition of angiogenesis, NK cell-mediated cytotoxicity and direct apoptosis of tumor cells.[5–7] Although Imiquimod has been used to treat several dermatological cancers, such as superficial basal cell carcinoma by topical application, it is not applicable for other types of cancer by systemic administration.[8] 852A, an Imiquimod analogue, has also been evaluated in a phase II clinical trial for the treatment of several types of cancer, such as ovarian cancer, lung cancer and metastatic melanoma.[9,10] Despite the positive clinical responses to 852A observed in some patients, further development has been suspended. We hypothesized that a narrow therapeutic window due to its weak TLR7 agonistic activity (EC_{50} 2657 nM in our screen) may have resulted in its discontinuation of the clinical trial.

We have previously reported several 8-oxoadenines **1** and **2** as novel structural class IFN inducers, a mechanism of action of them was TLR7 agonism and EC_{50} toward TLR7 of 166 and 32 nM, respectively (Fig. 1).[11,12] However, these compounds were poorly soluble in water (<1 μg/mL at pH 7.4 buffer), which would require the co-injection of a solubilising agent for intravenous administration. In this manuscript we describe our studies is to identify potent TLR7 agonists with improved water solubility by the molecular modification of the 8-oxoadenine.

In general, the aqueous solubility of small molecules depends on their hydrophobicity. We have prepared and evaluated several adenines which have hydrophilic group at N(9)-position, that is, hydroxyl **3**,[11] carboxylic acid **4**,[13] dimethylamino **5**,[11] and methoxy **6**,[11] however, improved solubility was not seen. Thus, we investigated the use of basic amines as a solubilising functional agents. We prepared 8-oxobenzyladenine **7** with a dimethylamino group on a C(2)-thio linked side chain. But the IFN inducing activity of it was decreased significantly.[14] Since an unsubstituted amino group at the C(6)-position and an oxo group at C(8)-position of the adenine contributed to their activity, we initiated a program of work to introduce an amino group to the adenine N(9)-position.

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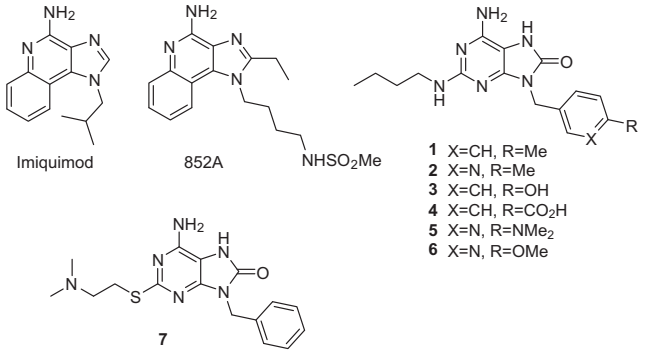


Figure 1. Structures of Imiquimod 852A, and compounds **1**–**7**.

The synthetic route for N(9)-benzyladenines is shown in Scheme 1. The reaction of **8** with 4-hydroxymethylbenzyl chloride in the presence of potassium carbonate gave **9**. Bromination at C(8)-position of **9** was carried out with bromine in the presence of sodium acetate and 8-oxoadenine **11** was obtained by heating the bromide **10** in concd HCl. Chlorination of the methylbenzylhydroxyl group at the N(9)-position of the **11** with thionyl chloride, followed by displacement of the chloro group with corresponding amines afforded the target compounds **13**–**16**. *meta*-Substituted compounds **17** and **18** were also prepared by using a similar method described above.

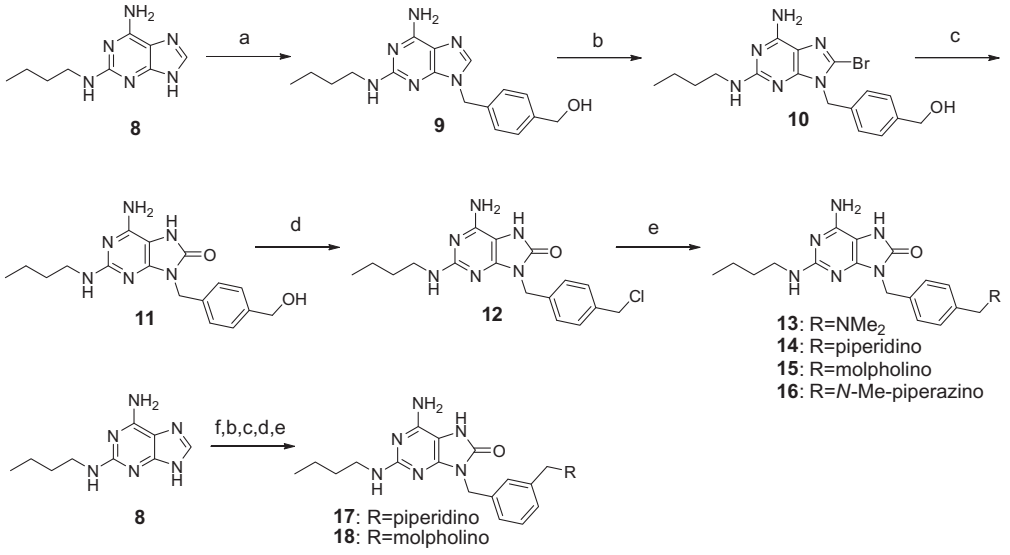
The synthetic route of pyridyl compounds is shown in Scheme 2. Compound **19** was prepared according to the reported method.[11] Target compounds **20**–**29** were prepared through the displacement of the chloro group in **19** with the corresponding amino alcohols in the presence of sodium hydride, or heating with the corresponding amines. All target compounds were recrystallized from alcoholic solvent and provided for the evaluation.

The in vitro human TLR7 agonistic activities of the prepared compounds were evaluated by a reporter gene assay using HEK293 cells, which were stably transfected with human TLR7 along with the NF-κB SEAP reporter.[15] The results of benzyl compounds are summarized in Table 1. The EC_{50} value of dimethylamine analogue **13** was evaluated as 9.3 nM, approximately 20-fold greater than that of **1**. In addition, the water solubility of **13** was determined to be 98 μg/mL, and **13** was found to be more soluble than **1** (<1 μg/mL). In contrast to the case of **7**, the introduction of a basic amino group to the adenine N(9)-position enhanced its TLR7 agonistic activity and this result encouraged us to optimize the amino group. The piperidine **14** showed comparable activity with **13**, whereas the morpholine **15** gave a decreased activity (EC_{50} = 58 nM), suggesting that stronger basicity is required for the higher potency. Compound **14** was less soluble than **13** probably due to its increased hydrophobicity. To increase hydrophilicity we prepared di-amino typed compound, *N*-methylpiperazine **16** which showed a similar level of activity with **13**, but the solubility was not improved.

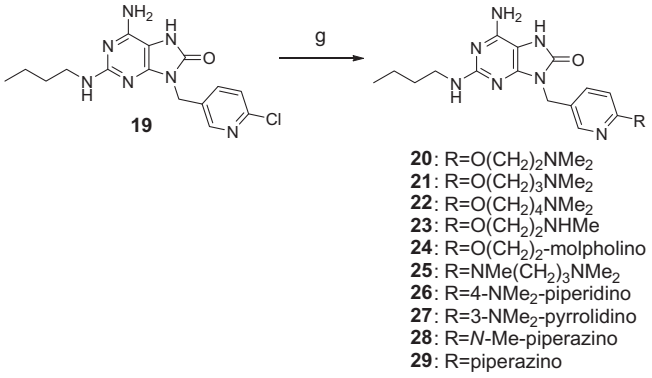
The increase of aqueous solubility might be caused by disruption of molecular symmetry.[16] As shown in Table 1, the amino group was placed onto the *meta*-position of the benzyl group. *meta*-Substituted compounds **17** and **18** showed weaker potency than the corresponding *para*-substituted compounds **14** and **16**, respectively. Addition of amino group to the N(9)-substituent of **1** enhanced both potency and solubility. We, therefore, investigated this further to optimize the balance between the aqueous solubility and the potency.

Previously we reported the structure–activity relationship of 9-pyridylmethyladenines. The order of potency was 3-pyridyl > 4-pyridyl > 2-pyridyl and especially, 6-substituted-3-pyridyl compound such as **2**, **5**, and **6** demonstrated highly potent IFN inducing activity in vitro.[11] Hence, we carried out the synthesis of 6-amino-substituted-3-pyridyl compounds in an attempt to attain further improvements in their profiles.

The results of pyridyl compound are summarized in Table 2. We were delighted to see that, as in the case of N(9)-benzyl compounds, the introduction of an amino group was useful, dimethylaminoethoxy analogue **20**[17] gave single figure nano-molar potency with improved solubility compared to methoxy compound **6**. We examined the methylene chain length and number of methyl groups based on **20**. Both C3 and C4 linked compounds, **21** and **22** showed approximately twofold weaker potencies with similar solubility as **20**, respectively, whereas the mono-methyl analogue **23** afforded a significantly decreased level of its activity. Potency of compound **24**, in which the dimethylamino group in **20** was replaced with morpholino, showed weaker potency than **20**. And the result was similar with that of compound **13** versus **15**, suggesting that basicity of the amino group have effects on the potency.

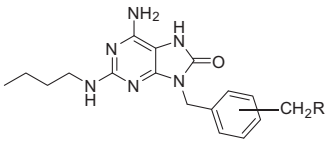


Scheme 1. Reagents and conditions: (a) 4-hydroxybenzylchloride, K_2CO_3, DMF (quant); (b) Br_2, NaOAc, $CHCl_3$ (77%); (c) c-HCl (68%); (d) $SOCl_2{\cdot}CH_2Cl_2$; (e) corresponding amines, DIEA (20–75%), $CHCl_3$; (f) 3-hydroxybenzylchloride, K_2CO_3, DMF (quant).



Scheme 2. Reagents and conditions: (g) corresponding alcohols, NaH, DMF, or corresponding amines (25–65%).

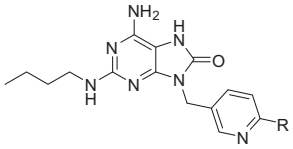
Table 1
Human TLR7 agonistic activity and solubility of N(9)-benzyl compounds

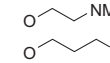
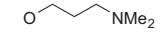
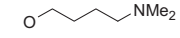
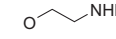
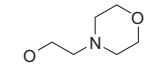
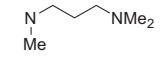
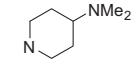
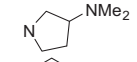
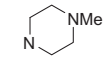


Compd	Position	R	hTLR7 EC_{50}[a] (nM)	Solubility[b] (mg/mL)
1	*para*	H	166	<1
13	*para*	NMe_2	9.3	98
14	*para*		7.6	19
15	*para*		58	8
16	*para*		7	108
17	*meta*		30.1	Not tested
18	*meta*		130	Not tested
852A			2657	Not tested

[a] Mean values of two independent experiments.
[b] Solubility at pH 7.4 buffer.

Table 2
Human TLR7 agonistic activity and solubility of N(9)-pyridylmethyl compounds



Compd	R	hTLR7 EC_{50}[a] (nM)	Solubility[b] (mg/mL)
2	Me	32	<1
6	OMe	28.1	<1
20		7.2	372
21		19.6	288
22		15.2	350
23		165.7	Not tested
24		50.2	34
25		72.8	725
26		9.4	71
27		7.4	53
28		5.4	7
29		48.5	63
852A		2657	Not tested

[a] Mean values of two independent experiments.
[b] Solubility at pH 7.4 buffer.

Table 3
Antitumor activity of **20** on HM-1 metastasis model

Compd	Dose (mg/kg)	% Inhibition of lung metastasis
20	0.1	78 ± 21*
	1	100 ± 0**
852A	1	6 ± 20
	10	55 ± 19

The data are expressed as the mean ± SD of 6 mice.
* P <0.05 versus vehicle (Wilcoxon test).
** P <0.01.

We also prepared and evaluated nitrogen atom linked compounds. *N*-Methyl analogue **25** showed a fourfold loss of potency compared to the oxygen atom linked compound **20**. Meanwhile, the cyclic amino compounds **26**–**28** demonstrated high potency with single figure nano-molar EC_{50} values but with reduced solubility compared to **25**, suggesting that appropriate structural flexibility may be necessary for improved aqueous solubility. As same as the cases of **20** versus **23**, compound **29** gave ninefold loss of the activity compared to **28**, indicating the secondary amino group was not preferable to show potent activity.

Among the compounds prepared, compound **20** exhibited the best balance of potency (EC_{50} = 7.2 nM) with high aqueous solubility (372 μg/mL). A small tertiary amino group with a short methylene linker is considered to be crucial to satisfy our criteria for compounds progression, that is, potent activity with high water solubility. It is of note that TLR7 agonistic potency of 852A, EC_{50} = 2672 nM is 370-fold weaker than that of the compound **20**.

Thus, we selected compound **20** for further evaluation. Before conducting an in vivo antitumor activity, we evaluated the activities of **20** and 852A towards mice TLR7 and established the pharmacokinetics/pharmacodynamics (PK/PD) profile in mice. Compound **20** showed potent agonistic activity toward mice TLR7 (EC_{50} = 4.6 nM), whereas 852A afforded weak activity (EC_{50} = 6224 nM) which is consistent with human TLR7. Compound **20** was dosed to Balb/c female mice intravenously at a dose of 1 mg/kg to confirm a PK/PD relationship. The quantity of IFN in plasma was measured by bio-assay using L929 cells with vesicular stomatitis virus.[18,19] It had notably high volumes of distribution (V_{dss} = 6.6 L/kg) and the $T_{1/2}$ was calculated as 1.3 h, however, a significant amount of IFN was induced into plasma (840 ± 32 IU/mL) at 6 h post dosing. In comparison, 852A induced IFN at a dose of 10 mg/kg, but to a lower extent (19 ± 3 IU/mL) than that of the compound **20**.

We evaluated the in vivo antitumor activity of **20** using a mice syngeneic melanoma lung metastasis tumor model. HM-1 ovarian cancer cells were injected into B6C3F1 mice subcutaneously on day 0, the primary tumor was surgically removed under anesthesia condition with isofluorane on day 10, test compounds were intravenously administered biweekly commencing on day 11 and the number of lung metastasis was counted on day 35. As shown in Table 3, the compound **20** suppressed the lung metastasis

significantly, 78% inhibition was seen at 0.1 mg/kg dosing (with no tumor metastasis at the 1 mg/kg group). However, the efficacy of 852A was limited, that is, 55% suppression at 10 mg/kg. These results correlate well with their in vitro activities and indicates that the in vivo efficacy of the compound **20** is >100-fold higher than that of 852A.

In summary, a study on the structure–activity relationship of 8-oxoadenines for TLR7 agonistic activity and solubility was conducted based upon the structures of **1** and **2**. The introduction of an alkylamino group on the N(9)-position of the adenine resulted in significantly improved aqueous solubility without loss of potency. In particular, the compound **20** (DSR-6434) was identified as a potent human TLR7 agonist, EC_{50} = 7.2 nM with high water solubility 372 μg/mL. In addition, **20** demonstrated a strong antitumor effect in an in vivo model by intravenous administration.

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References and notes

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17. *Spectral data for compound **20**:* mp = 248–249 °C; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 9.64 (1H, br s), 8.13 (1H, d, J = 2.2 Hz), 7.65 (1H, dd, J = 2.4, 8.4 Hz), 6.76 (1H, d, J = 8.4 Hz), 6.26 (1H, t, J = 5.6 Hz), 6.02 (2H, s), 4.74 (2H, s), 4.33 (2H, t, J = 5.8 Hz), 3.17 (2H, q, J = 6.8 Hz), 2.56 (2H, t, J = 5.8 Hz), 2.17 (6H, s), 1.45 (2H, m), 1.30 (2H, m), 0.88 (3H, t, J = 7.2 Hz); MS (ESI) m/z 401 (M+1); Anal. Calcd for $C_{19}H_{28}N_8O_2 \cdot 0.5H_2O$: C, 55.73; H, 7.14; N, 27.36. Found: C, 55.50; H, 7.12; N, 27.51 (recrystallized from MeOH).
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