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Synthesis and Biological Evaluation of 8-Oxoadenine Derivatives as Toll-like Receptor 7 Agonists Introducing the Antedrug Concept

Ayumu Kurimoto,*,† Kazuki Hashimoto,† Tomoaki Nakamura,† Kei Norimura,† Haruhisa Ogita,† Haruo Takaku,† Roger Bonnert,‡ Tom McInally,‡ Hiroki Wada,‡ and Yoshiaki Isobe†

†Chemistry Research II, Chemistry Research Laboratories, Dainippon Sumitomo Pharma Co., Ltd., 3-1-98 Kasagade-naka, Konahana-ku, Osaka 554-0022, Japan, and ‡AstraZeneca R&D Charnwood, Bakewell Road, Loughborough, Leicestershire LE11 5RH, U.K.

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Systemic administration of a Toll-like receptor 7 (TLR7) agonist is effective to in suppressing Th2 derived inflammation, however systemic induction of various cytokines such as IL-6, IL-12, and type I interferon (IFN) is observed. This cytokine induction would be expected to cause flu-like symptoms. We have previously reported adenine compounds (**3**, **4**) as interferon inducing agents acting as TLR7 agonists. To identify potent anti-inflammatory compounds without systemic side effects, a labile carboxylic ester as an antedrug functionality onto the *N*(9)-benzyl group of the adenine was introduced. We found that **9e** was a potent TLR7 agonist (EC_{50} 50 nM) and rapidly metabolized by human plasma ($T_{1/2}$ 2.6 min) to the pharmacologically much less active carboxylic acid **16**. Intratracheal administration of **9e** effectively inhibited allergen-induced airway inflammation without inducing cytokines systemically. Therefore, the TLR7 agonist with antedrug characteristics **9e** (SM-324405) is a novel candidate for immunotherapy of allergic diseases.

Introduction

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[a]) 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 TLRs have been reported which are fundamental in recognition of pathogen-associated molecular patterns (PAMPs).[1,2]

Imiquimod (**1**) and Resiquimod (**2**) belong to the imidazoquinolines, a class of low molecular weight IFN inducing agents. The compounds are ligands for TLR7 and/or TLR8[3−5] that have been considered as good drug candidates to suppress the Th2 cell dependent immune response due to enhancement of the Th1 response.[6] Resiquimod has been shown to inhibit allergen-induced airway inflammation and hyper-reactivity by modulating Th1/Th2 immune responses and therefore might be used as a powerful adjuvant for the specific immunotherapy of allergic disorders.[7,8]

We have reported the discovery of adenine derivatives as a novel class of IFN inducing agents.[9−12] Furthermore, 9-benzyl-2-(2-methoxyethoxy)-8-oxoadenine (**3**) has been identified as a TLR7 agonist in the literature.[13] Thus adenine derivatives could be considered as a novel class of agents for the treatment of allergic diseases by modulating Th1/Th2 immune responses. However, administration of a TLR7 agonist is associated with systemic side effect such as flu-like symptoms caused by induction of cytokines including IL-6, IL-12, and type I IFN.[14]

To reduce systemic side effects, we applied the antedrug concept to adenine derivatives. The term of "antedrug" is described as a drug designed to exert the desired topical effects at the site of administration after which it is quickly converted into inactive metabolite(s) upon entry into the systemic circulation.[15,16] Ester functionalities based on the antedrug concept have been described in the literature.[17−20]

Among the previously reported adenine derivatives, 9-benzyl-2-butoxy-8-oxoadenine (**4**) was identified as a potent IFN inducer (Figure 1).[9] Introduction of hydrophilic substituents into the *C*(2)-side chain of the adenine was not effective to increase activity,[11,12] therefore we initiated work to modify the substituent at the *N*(9)-position. The aim of this study was to combine the adenine derivative with a labile carboxylic ester to identify a novel candidate for immunotherapy of allergic diseases with antedrug characteristics.

Chemistry

The synthetic routes to the 2-butoxy-8-oxoadenine derivatives having an ester moiety are shown in Schemes 1−5. The reaction of **5** with sodium *n*-butoxide gave **6**. Compounds **7** were obtained by alkylation of **6** with an appropriate benzyl bromide **10** in the presence of potassium carbonate as base. Bromination at the *C*(8)-position of the adenine was carried

*To whom correspondence should be addressed. Phone: +81-6-6466-5203. Fax: +81-6-6466-5483. E-mail: ayumu-kurimoto@ds-pharma.co.jp.

[a]Abbreviations: BALF, bronchoalveolar lavage fluid; EC_{50}, half-maximal effective concentration; ELISA, enzyme-linked immunosorbent assay; Hu, human; IFN, interferon; IL-6, interleukin-6; IL-12, interleukin-12; i.t., intratracheal; MEC, minimum effective concentration; NF-kB, nuclear factor-kappa B; OVA, ovalbumin; PAMPs, pathogen-associated molecular patterns; PBMC, peripheral blood mononuclear cells; $T_{1/2}$, half-life; Th1, helper T-cells type 1; Th2, helper T-cells type 2; TLR7, toll-like receptor 7; TLR8, toll-like receptor 8; TLRs, toll-like receptors; VSV, vesicular stomatitis virus.

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out with bromine in the presence of sodium acetate. The desired compounds **9a**−**g** and **9i** were prepared by the treatment of corresponding 8-bromo compounds **8** with sodium methoxide, followed by heating with sulphuric acid in methanol.

In the case of **9j** and **9k**, the corresponding benzoate **15**[21] was reacted with **6**. Homologation of **7j** and **7k** was carried out by reduction of the ester moiety with lithium aluminum hydride to the alcohol, chlorination with thionyl chloride, and displacement of chlorine with sodium cyanide to give **13**. The reaction of **13** with bromine in the presence of sodium acetate afforded **14**. The desired compounds **9j** and **9k** were synthesized by the reaction of the corresponding precursors **14** with sodium methoxide, followed by reflux with sulphuric acid in methanol.

Compound **9h** was prepared from **7e** using a similar homologation procedure as shown in Scheme 3.

The hydrolysis of **9e** with sodium hydroxide gave the corresponding acid **16**, and the ethyl ester **9l** was prepared by heating **16** with sulphuric acid in ethanol (Scheme 4).

As shown in Scheme 5, compound **17** was prepared using a similar method to the synthesis of 2-butoxy compound **9e**.

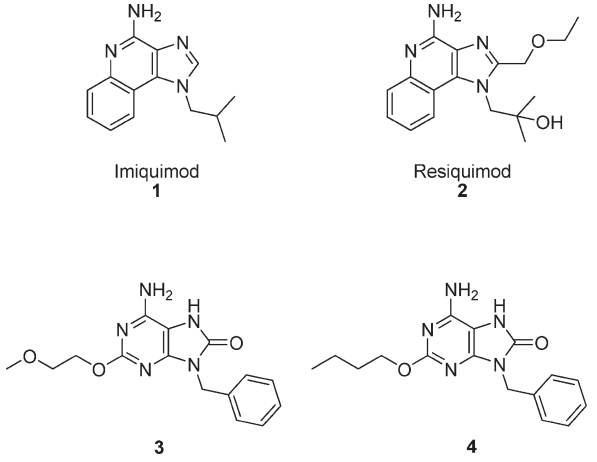


Figure 1

Results and Discussions

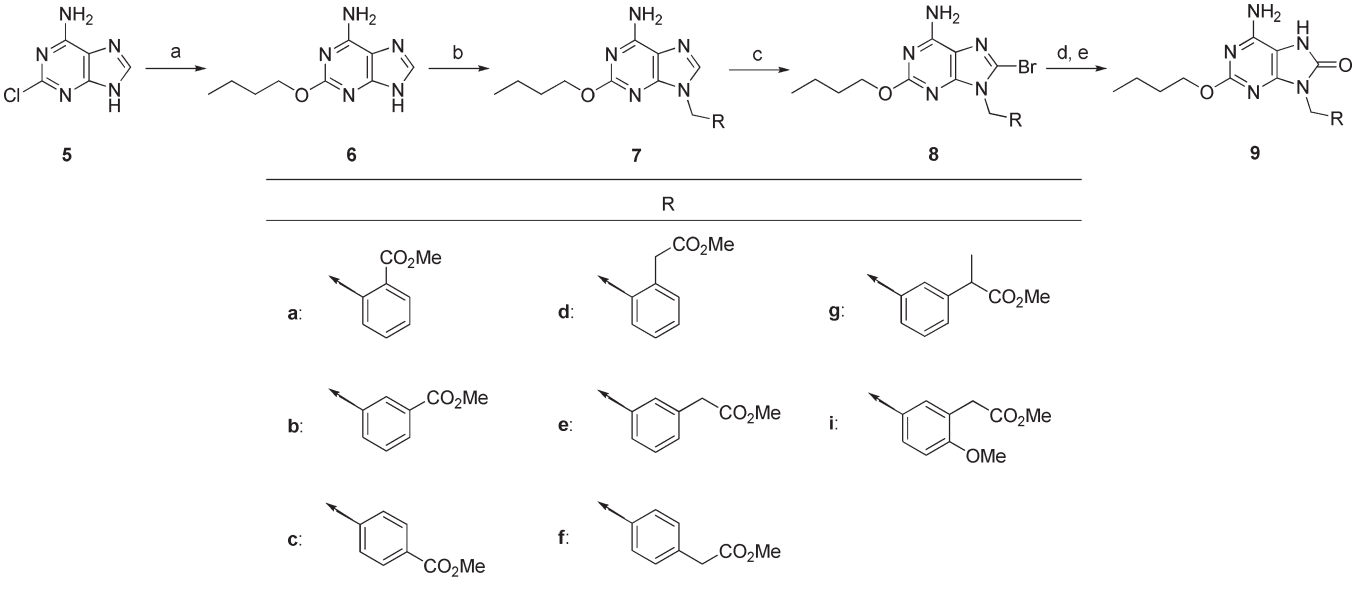
The in vitro Hu TLR7 agonist activities of the prepared compounds were evaluated by a reporter gene assay using HEK293 cells, which were stably transfected with human TLR7 along with the NF-*k*B SEAP reporter.[22] Metabolic stability was evaluated by incubating with human plasma and are expressed as the half-life ($T_{1/2}$) calculated by the least-squares method.

Biological results are summarized in Tables 1 and 2.

Using the potent and metabolically stable TLR7 agonist compound **4** as a starting point, we replaced the benzyl group at the *N*(9)-position with various benzoates. Both *meta*- and *para*-substituted compounds (**9b**, **9c**) were equipotent with unsubstituted **4**, whereas the *ortho*-substituted compound **9a** gave a reduction in activity. However, all of these benzoate compounds were stable in human plasma. In an attempt to find carboxylic ester functionalities which were labile in human plasma, we examined the introduction of linkers such as methylene, 1,2-ethylene, and 1,1-ethylene between the ester moiety and phenyl group (**9d**−**h**). The same activity trend as benzoates (**9a**−**c**) was noted, with *meta*- and *para*-substitution better than *ortho*-substitution. The highest activity was observed when the methyl acetate group was introduced at the *para*-position of the phenyl group (**9f**), Hu TLR7 EC_{50} = 10.3 nM. These acetate ester analogues did show decreased stability in human plasma, and it was found that compound **9e** was metabolized to the corresponding acid in human plasma with $T_{1/2}$ = 2.6 min. It was observed that compound **9g**, having a methyl group in the α position of the phenylacetate, was stable in human plasma. This suggested that the presence of a steric group blocked the access of the esterase to the carbonyl moiety. Compound **9h**, which has an ethylene linker at the *meta*-position, was also stable in human plasma. These results indicated that a *meta*-phenylacetate moiety as the *C*(9)-substituent was the most favorable to introduce an antedrug function with good Hu TLR7 agonist activity.

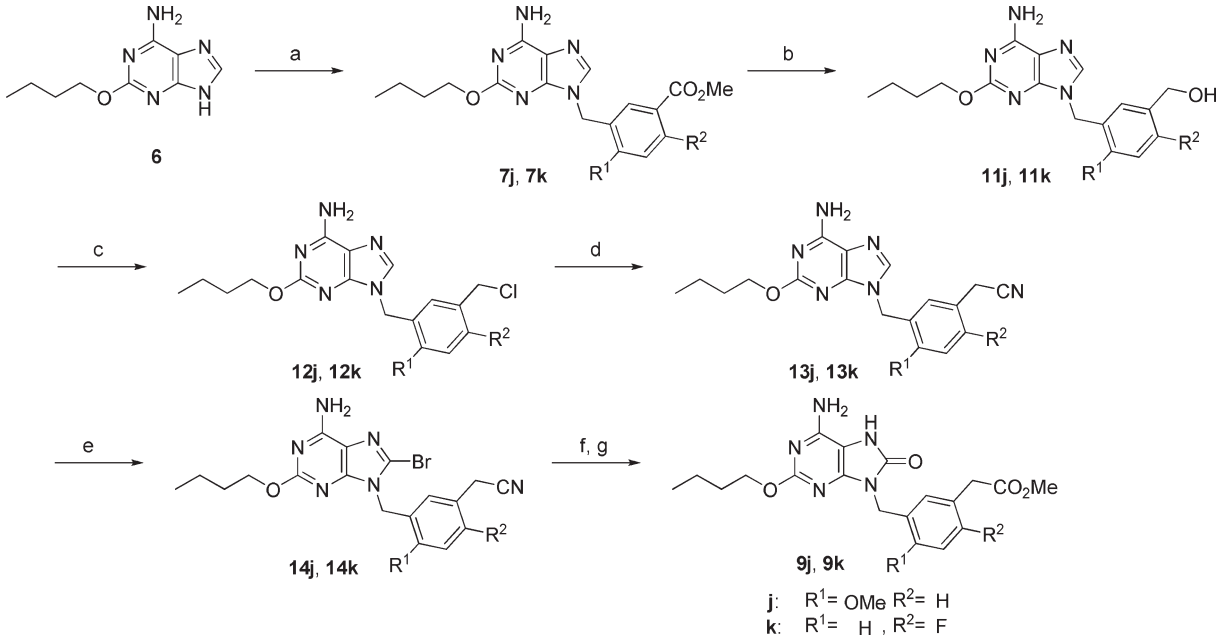
To identify more potent compounds, we investigated the introduction of substituents into the phenyl ring. We prepared

Scheme 1. Synthesis of Compounds **9a**−**g** and **9i**



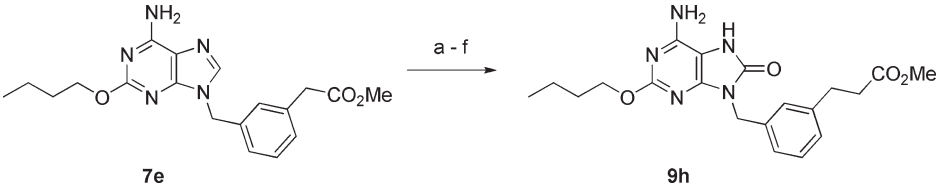
Reagents and conditions: (a) NaOnBu, nBuOH; (b) RCH_2Br (**10**), K_2CO_3, DMF, rt; (c) Br_2, AcONa, $CHCl_3$, rt; (d) 5N NaOH, MeOH, reflux; (e) H_2SO_4, MeOH, reflux.

Scheme 2. Synthesis of Compounds **9j** and **9k**



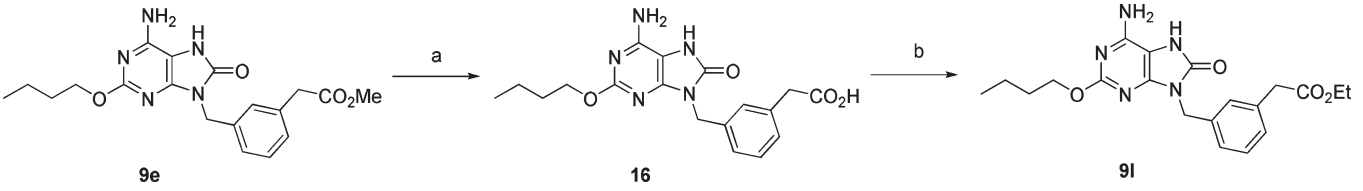
Reagents and conditions: (a) $ArCH_2Br$ (**15**), K_2CO_3, DMF, rt; (b) $LiAlH_4$, THF, 0 °C; (c) $SOCl_2$, $CHCl_3$, reflux; (d) NaCN, DMF, rt; (e) Br_2, AcONa, $CHCl_3$, rt; (f) 5N NaOH, MeOH, reflux; (g) H_2SO_4, MeOH, reflux.

Scheme 3. Synthesis of Compound **9h**



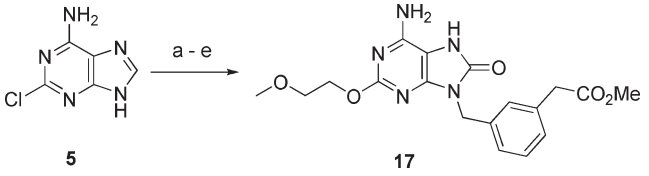
Reagents and conditions: (a) $LiAlH_4$, THF, 0 °C; (b) $SOCl_2$, $CHCl_3$, reflux; (c) NaCN, DMF, rt; (d) Br_2, AcONa, $CHCl_3$, rt; (e) 5N NaOH, MeOH, reflux; (f) H_2SO_4, MeOH, reflux.

Scheme 4. Synthesis of Compounds **16** and **9l**



Reagents and conditions: (a) NaOH, MeOH, rt; (b) EtOH, H_2SO_4, reflux.

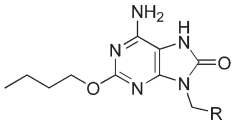
Scheme 5. Synthesis of Compound **17**

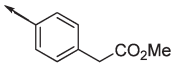
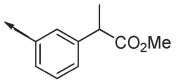
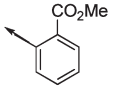
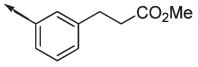
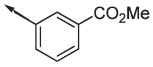
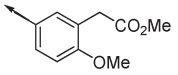
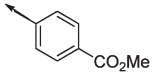
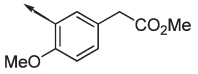
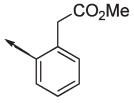
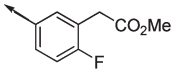
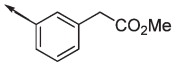
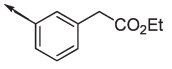


Reagents and conditions: (a) $NaO(CH_2)_2OMe$, $MeO(CH_2)_2OH$; (b) **10e**, K_2CO_3, DMF, rt; (c) Br_2, AcONa, $CHCl_3$, rt; (d) 5N NaOH, MeOH, reflux; (e) H_2SO_4, MeOH, reflux.

compounds with methoxy as an electron-donating group and fluorine as an electron-withdrawing group. The introduction of methoxy at the *para*-position (**9i**) significantly increased the activity to EC_{50} 8.1 nM, whereas the *ortho*-isomer (**9j**) showed much reduced activity (EC_{50} 108 nM), however a significant increase in metabolic stability was observed $T_{1/2}$ = 98 min. Introduction of the fluorine atom, as shown in (**9k**), increased the activity by 2-fold (EC_{50} 25 nM) with an increase in $T_{1/2}$ to 13.1 min. Comparing the plasma stability of **9e**:H, **9k**:F, and **9i**:OMe, it was observed that stability increased with the increase in size of the substituent at the *para*-position, OMe > *F* > H. We therefore reasoned that steric hindrance around the ester had a major effect on the metabolic rate as previously shown for **9g**. We changed **9e** to the larger ethyl ester **9l**, and again the potency and lability in human plasma were not improved compared to **9e**. Thus, we conclude that the methyl ester gives the optimal balance of activity and metabolic instability.

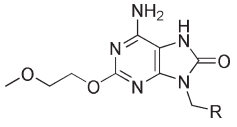
Table 1. Human TLR 7 Activities and Metabolic Stabilities in Human Plasma[a]

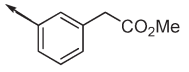


compd	R	EC_{50} (nM)	$T_{1/2}$ (min)	compd	R	EC_{50} (nM)	$T_{1/2}$ (min)
2		260.1	NT	**9f**		10.3	21.9
4		63.7	NT	**9g**		75.0	> 98
9a		609.1	> 98	**9h**		87.5	> 98
9b		51.5	> 98	**9i**		8.1	> 98
9c		72.9	> 98	**9j**		108.0	14.4
9d		466.8	> 98	**9k**		25.7	13.1
9e		50.1	2.6	**9l**		187.9	9.0

[a] NT: not tested.

Table 2. Human TLR 7 Activities and Metabolic Stabilities in Human Plasma[a]



compd	R	EC_{50} (nM)	$T_{1/2}$ (min)	compd	R	EC_{50} (nM)	$T_{1/2}$ (min)
3		399.4	NT	**17**		312.3	1.4

[a] NT: not tested.

We have previously shown that alternative *C*(2)-substituents can give potent compounds, therefore we investigated the introduction of the *meta*-phenylacetate on the methoxyethoxy adenine analogue **3**. Compound **17** was prepared and showed equivalent lability ($T_{1/2}$ 1.4 min) with the butoxy analogue **9e** while maintaining potency. These results suggest that a *meta*-phenylacetate moiety at the *N*(9)-position may be a useful substituent in various analogues to introduce an antedrug while maintaining TLR7 activity.

Furthermore, the in vitro activity against TLR8 was measured. We found that EC_{50} of compound **9e** was over 10 μM. In addition to specific potency and metabolic instability in human plasma, we confirmed that **9e** was quickly metabolized in rat plasma ($T_{1/2}$ < 0.04 min). These data led us to select compound **9e** for further evaluation.

The in vitro IFN inducing activity of compound **9e** and the corresponding acid **16** were measured by the following method. Human peripheral blood mononuclear cells (PBMC) were incubated with the compounds, and the amount of IFN in supernatants was measured by a human ELISA system. The IFN inducing activity in human PBMC is shown in Table 3.

Compounds **9e** and **16** induced IFN via TLR7 activation, and the minimum effective concentration (MEC) of compound **9e** and the corresponding acid **16** in human PBMC

Table 3. IFN Inducing Activity in Human PBMC (pg/mL)

		nM							
compd	volunteer	3	10	30	100	300	1000	3000	10000
9e	**A**	< 1.25	3.77	10.37	12.86	24.19	12.73		
	B	< 1.25	1.61	4.71	8.17	8.37	6.20		
	C	< 1.25	5.76	10.43	14.34	10.24	7.54		
16	**A**			< 1.25	< 1.25	< 1.25	< 1.25	< 1.25	1.98
	B			< 1.25	< 1.25	< 1.25	< 1.25	< 1.25	< 1.25
	C			< 1.25	< 1.25	< 1.25	< 1.25	2.25	12.35
2	**A**		< 1.25	1.42	11.72	18.38	24.46		
	B		< 1.25	< 1.25	5.61	7.26	7.75		
	C		< 1.25	1.32	16.44	22.47	32.07		

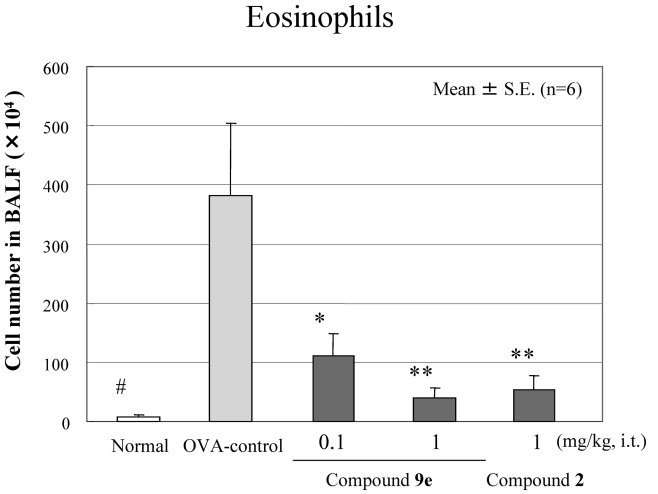


Figure 2. Anti-inflammatory activity of compound **9e** and **2** in a BN rat model. Results are expressed means ± SEM numbers of eosinophils in BALF. #: $P < 0.05$; significant difference from OVA-control (Welch test). *: $P < 0.05$; **: $P < 0.01$; significant difference from OVA-control (Dunnett's test).

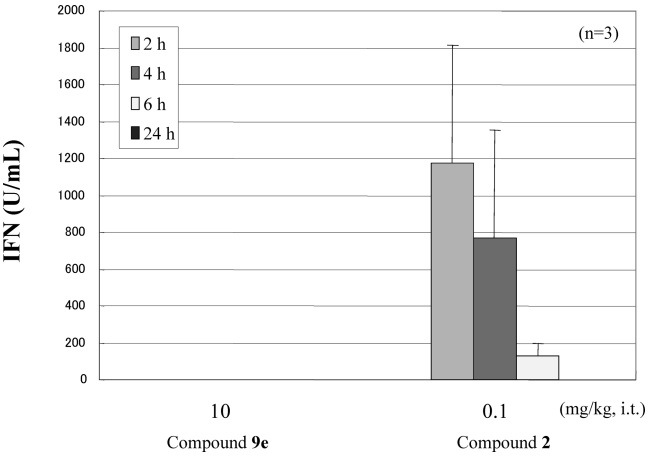


Figure 3. Systemic IFN induction in plasma after i.t. dosing.

were 10 and 3000 nM, respectively. There is a 300-fold difference in the activity of ester and acid, therefore we considered **9e** to show the desired antedrug properties.

To confirm efficacy in vivo, we assessed the efficacy of compound **9e** on allergic airway inflammation in a Brown Norway rat model by intratracheal (i.t.) administration. It has been reported that plasma stable TLR7 agonists inhibit inflammation in several animal models.[8,23] Our aim was to confirm the efficacy of a TLR7 agonist with the antedrug concept.

Brown Norway rats were sensitized by ovalbumin (OVA) together with aluminum hydroxide adjuvant. The sensitized animals were challenged by OVA aerosol exposure. In allergic asthma, an influx of eosinophils in the airway occurs following inhalation of allergen. Two hours before antigen challenge, **9e** and **2** were intratracheally administered to sensitized rats at 0.1 or 1 mg/kg. The number of eosinophils in the bronchoalveolar lavage fluid (BALF) were counted in a hemocytometer at 24 h after challenge. As shown in Figure 2, compound **2** at 1 mg/kg inhibited eosinophil influx into BALF, whereas **9e** also exhibited efficacy against eosinophilia in a dose dependent manner from 0.1 mg/kg compared with the control group. This result suggested that a compound with high rat plasma instability, which would therefore have a short exposure due to hydrolysis to the much less active acid, was sufficient to show efficacy.

Finally, **9e** and **2** were administered intratracheally to unsensitized rats to evaluate systemic IFN induction. The concentrations of IFN in plasma at 2, 4, 6, and 24 h after dosing were determined by a bioassay system.[24,25]

As shown in Figure 3, compound **2** induced a significant quantity of IFN in plasma at 0.1 mg/kg, whereas no IFN induction was observed when **9e** was administered to groups up to 10 mg/kg (detection limit is 6 U/mL). In viewing the results of Figure 2 and 3, no selectivity was seen between reduction in eosinophilia and induction of IFN in compound **2**, but **9e** showed greater than a 100-fold margin. In addition, **9e** was not detected in plasma samples, however the acid metabolite **16** was observed.

These results suggest that the antedrug concept is useful to achieve efficacy without observation of systemic side effects due to cytokine induction by the TLR7 agonist. Thus topical administration of compound **9e** (SM-324405) is considered to be effective for immunotherapy of allergic disease.

Conclusion

In this paper, the research for a novel class of agent for immunotherapy of allergic disease was described. A series of 2-butoxy-8-oxoadenines containing an ester moiety were prepared and evaluated for TLR7 agonist potency and stability in human plasma. We found that compound **9e** exhibited good potency and was rapidly metabolized to the much less active carboxylic acid **16**. Intratracheal administration of **9e** in a BN rat model significantly reduced the number of eosinophils in BALF and did not induce IFN systemically.

We have identified potent TLR7 agonists antedrugs and shown data which indicate that the TLR7 agonist **9e** with antedrug properties is a novel candidate for the immunotherapy of allergic diseases.

Experimental Section

1. Chemistry. Melting points were measured on a Thomas−Hoover capillary melting point apparatus and are uncorrected. Nuclear magnetic resonance (NMR) spectra were recorded at ambient temperature on a Bruker AVANCE 400 FT NMR spectrometer. Chemical shifts are expressed in δ values (ppm) relative to tetramethylsilane as an internal standard and signals are expressed as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), or br (broad). Elemental analyses were performed by the Analytical Science Group, Dainippon Sumitomo Pharma, Osaka, Japan. The purities of tested compounds were assessed as being >95% using analytical LC, which was performed using a Waters 2790 separations module equipped with XTerra MS C18 2.5 μm (2.1 mm × 20 mm) column at 254 nm and eluted with a gradient of 2−96% solvent B (acetonitrile) in solvent A (water) and 3% solvent C (2% formic acid in acetonitrile) over 3.5 min at 1 mL/min. Commercial reagents and solvents were of reagent grade and used without further purification. Thin-layer chromatography (TLC) was performed on Merk Kieselgel 60 F_{254} precoated plates, and components were visualized using UV light. Flash chromatography was conducted using Merk Kieselgel 60 F_{254} or Cica-Reagent Silica Gel 60.

2-Butoxy-9*H*-purin-6-amine (6). Sodium (67.79 g, 2.948 mol) was dissolved in *n*-BuOH (1000 mL) and 2-chloropurin-6-amine (50.00 g, 0.295 mol) added. The mixture was stirred at 140 °C for 10 h and water added. The organic layer was separated, concentrated in vacuo, and water added to the residue. HCl was added, and the precipitate formed was collected by filtration and washed with EtOH to give compound **6** (45.85 g, 75%) as a colorless solid. ^{1}H NMR (DMSO-d_6) δ 7.88 (1H, brs), 7.08 (2H, brs), 4.17 (2H, t, J = 6.6 Hz), 1.65 (2H, m), 1.40 (2H, m), 0.92 (3H, t, J = 7.4 Hz).

Methyl [3-(Bromomethyl)phenyl]acetate (10e). An ice-cooled solution of (3-tolyl)acetic acid (50.00 g, 0.333 mol) in MeOH (500 mL) was treated with $SOCl_2$ (5 mL) and stirred at room temperature for 2 h. The reaction mixture was concentrated in vacuo, saturated aq $NaHCO_3$ was added, and the product was extracted with $CHCl_3$. The organic layer was dried over Na_2SO_4 and evaporated to give methyl (3-tolyl)acetate (54.19 g, 99%) as a colorless oil, which was dissolved in CCl_4 (330 mL), *N*-bromosuccinimide (58.74 g, 0.330 mmol), and a catalytic amount of benzoyl peroxide (1.00 g) were added. The mixture was refluxed for 6 h, cooled to room temperature, and filtered. The filtrate was concentrated in vacuo and purified by silica gel column chromatography using hexane−Et_2O (10:1) as eluent to give compound **10e** (48.38 g, 60%) as a colorless oil. ^{1}H NMR ($CDCl_3$) δ 7.23 (4H, m), 4.48 (2H, s), 3.70 (3H, s), 3.63 (2H, s).

Methyl {3-[(6-Amino-2-butoxy-9*H*-purin-9-yl)methyl]phenyl}acetate (7e). To a suspension of compound **6** (20.72 g, 0.100 mol) and K_2CO_3 (69.11 g, 0.500 mol) in DMF (400 mL) was added compound **10e** (29.17 g, 0.120 mol) and stirred at room temperature for 8 h. The reaction mixture was concentrated in vacuo, water and $CHCl_3$ were added, and the organic layer was separated, dried over Na_2SO_4, and evaporated. The residue was recrystallized from MeOH−H_2O to give compound **7e** (33.58 g, 91%) as a colorless solid. ^{1}H NMR (DMSO-d_6) δ 8.03 (1H, s), 7.20 (6H, m), 5.24 (2H, s), 4.21 (2H, t, J = 6.6 Hz), 3.65 (2H, s), 3.58 (3H, s), 1.65 (2H, m), 1.40 (2H, m), 0.92 (3H, t, J = 7.5 Hz).

Methyl {3-[(6-Amino-8-bromo-2-butoxy-9*H*-purin-9-yl)methyl]phenyl}acetate (8e). To a suspension of compound **7e** (10.00 g, 27.07 mol) and NaOAc (11.10 g, 135.35 mmol) in $CHCl_3$ (300 mL) was added bromine (5 mL, 97.58 mmol) and stirred at room temperature for 1 h. To the reaction mixture was added 10% aq $Na_2S_2O_3$ and saturated aq $NaHCO_3$. The organic layer was separated, dried over Na_2SO_4, and evaporated. The residue was purified by silica gel column chromatography using MeOH−$CHCl_3$ as eluent to give compound **8e** (10.40 g, 86%) as a colorless solid. ^{1}H NMR (DMSO-d_6) δ 7.46 (2H, brs), 7.30(1H, m), 7.19 (2H, m), 7.12 (1H, m), 5.24 (2H, s), 4.21 (2H, t, J = 6.6 Hz), 3.65 (2H, s), 3.58 (3H, s), 1.65 (2H, m), 1.38 (2H, m), 0.91 (3H, t, J = 7.3 Hz).

Methyl {3-[(6-Amino-2-butoxy-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl]phenyl}acetate (9e). To a solution of compound **8e** (10.40 g, 23.20 mol) in MeOH (400 mL) was added 5N NaOH (200 mL) and refluxed for 5 h. The reaction mixture was neutralized with HCl and concentrated in vacuo. To the residue was added MeOH (300 mL) and H_2SO_4 (20 mL) and then refluxed for 1 h. The reaction mixture was neutralized with aq NH_3 and water added. The precipitate was collected by filtration to give compound **9e** (6.92 g, 77%) as a colorless solid; mp 246−251 °C. ^{1}H NMR (DMSO-d_6) δ 10.01 (1H, brs), 7.19 (4H, m), 6.47 (2H, brs), 4.83 (2H, s), 4.14 (2H, t, J = 6.8 Hz), 3.64 (2H, s), 3.59 (3H, s), 1.62 (2H, m), 1.36 (2H, m), 0.90 (3H, t, J = 7.3 Hz). Anal. ($C_{19}H_{23}N_5O_4\cdot 0.5H_2O$) C, H, N.

Compounds **9a**−**d**, **9f**−**g**, and **9i** were prepared from compound **6** using a similar procedure as for compound **9e**.

Methyl 2-[(6-Amino-2-butoxy-8-oxo-7,8-dihydro-9*H*-purin-9-yl)methyl]benzoate (9a). Yield 33%; mp 228−240 °C. ^{1}H NMR (DMSO-d_6) δ 10.03 (1H, brs), 7.93 (1H, d, J = 7.8 Hz), 7.53 (1H, t, J = 7.8 Hz), 7.41 (1H, t, J = 7.8 Hz), 6.95 (1H, d, J = 7.8 Hz), 6.50 (2H, brs), 5.23 (2H, s), 4.07 (2H, t, J = 6.6 Hz), 3.90 (3H, s), 1.57 (2H, m), 1.33 (2H, m), 0.87 (3H, t, J = 7.3 Hz). Anal. ($C_{18}H_{21}N_5O_4\cdot 0.25H_2O$) C, H, N.

Methyl 3-[(6-Amino-2-butoxy-8-oxo-7,8-dihydro-9*H*-purin-9-yl)methyl]benzoate (9b). Yield 34%; mp 285−288 °C. ^{1}H NMR (DMSO-d_6) δ 10.02 (1H, brs), 7.93 (1H, s), 7.87 (1H, d, J = 7.6 Hz), 7.59 (1H, d, J = 7.6 Hz), 7.49 (1H, t, J = 7.6 Hz), 6.48 (2H, brs), 4.93 (2H, s), 4.14 (2H, t, J = 6.5 Hz), 3.84 (3H, s), 1.63 (2H, m), 1.36 (2H, m), 0.90 (3H, t, J = 7.3 Hz). Anal. ($C_{18}H_{21}N_5O_4$) C, H, N.

Methyl 4-[(6-Amino-2-butoxy-8-oxo-7,8-dihydro-9*H*-purin-9-yl)methyl]benzoate (9c). Yield 33%; mp 300−302 °C. ^{1}H NMR (DMSO-d_6) δ 10.21 (1H, brs), 7.92 (2H, d, J = 8.4 Hz), 7.39 (2H, d, J = 8.4 Hz), 6.54 (2H, brs), 4.93 (2H, s), 4.11 (2H, t, J = 6.8 Hz), 3.83 (3H, s), 1.62 (2H, m), 1.36 (2H, m), 0.90 (3H, t, J = 7.3 Hz). Anal. ($C_{18}H_{21}N_5O_4\cdot 0.25H_2O$) C, H, N.

Methyl {2-[(6-Amino-2-butoxy-8-oxo-7,8-dihydro-9*H*-purin-9-yl)methyl]phenyl}acetate (9d). Yield 13%; mp 227−229 °C. ^{1}H NMR (DMSO-d_6) δ 9.97 (1H, brs), 7.21 (4H, s), 6.43 (2H, brs), 4.85 (2H, s), 4.12 (2H, t, J = 6.6 Hz), 4.01 (2H, s), 3.58 (3H, s), 1.61 (2H, m), 1.36 (2H, m), 0.89 (3H, t, J = 7.3 Hz). Anal. ($C_{19}H_{23}N_5O_4$) C, H, N.

Methyl {4-[(6-Amino-2-butoxy-8-oxo-7,8-dihydro-9*H*-purin-9-yl)methyl]phenyl}acetate (9f). Yield 47%; mp 295−297 °C. ^{1}H NMR (DMSO-d_6) δ 10.11 (1H, brs), 7.22 (4H, m), 6.49 (2H, brs), 4.83 (2H, s), 4.14 (2H, t, J = 6.5 Hz), 3.63 (2H, s), 3.58 (3H, s), 1.62 (2H, m), 1.36 (2H, m), 0.90 (3H, t, J = 7.3 Hz). Anal. ($C_{19}H_{23}N_5O_4$) C, H, N.

Methyl 2-{3-[(6-Amino-2-butoxy-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl]phenyl}propanoate (9g). Yield 25%; mp 272−275 °C. ^{1}H NMR (DMSO-d_6) δ 9.96 (1H, brs), 7.29−7.12 (4H, m), 6.44 (2H, brs), 4.82 (2H, s), 4.13 (2H, t, J = 6.6 Hz), 3.75 (1H, q, J = 7.1 Hz), 3.54 (3H, s), 1.61 (2H, m), 1.36 (2H, m), 1.33 (3H, d, J = 7.1 Hz), 0.89 (3H, t, J = 7.3 Hz). Anal. ($C_{20}H_{25}N_5O_4$) C, H, N.

Methyl {5-[(6-Amino-2-butoxy-8-oxo-7,8-dihydro-9*H*-purin-9-yl)methyl]-2-methoxyphenyl}acetate (9i). Yield 39%; mp 231−235 °C. ^{1}H NMR (DMSO-d_6) δ 9.91 (1H, brs), 7.20 (1H, d, J = 8.4 Hz), 7.15 (1H, s), 6.91 (1H, d, J = 8.4 Hz), 6.42 (2H, brs), 4.75 (2H, s), 4.15 (2H, t, J = 6.4 Hz), 3.70 (3H, s), 3.55 (3H, s), 3.53 (2H, s), 1.62 (2H, m), 1.37 (2H, m), 0.90 (3H, t, J = 7.3 Hz). Anal. ($C_{20}H_{25}N_5O_5\cdot 0.75H_2O$) C, H, N.

Methyl 5-[(6-Amino-2-butoxy-9H-purin-9-yl)methyl]-2-fluorobenzoate (7k). To a suspension of compound **6** (600 mg, 2.90 mmol) and K_2CO_3 (802 mg, 5.80 mmol) in DMF (35 mL) was added methyl 5-(bromomethyl)-2-fluorobenzoate (1.27 g, 5.15 mmol) and stirred at room temperature for 14 h. The reaction mixture was concentrated in vacuo, water and $CHCl_3$ were

added, and the organic layer was separated, dried over Na_2SO_4, and evaporated. The residue was purified by silica gel column chromatography using MeOH−$CHCl_3$ as eluent to give compound **7k** (597 mg, 55%) as a colorless solid. ^{1}H NMR ($CDCl_3$) δ 7.95 (1H, dd, J = 6.7 Hz, 2.4 Hz), 7.61 (1H, s), 7.48 (1H, m), 7.12 (1H, dd, J = 10.3 Hz, 8.6 Hz), 5.55 (2H, brs), 5.27 (2H, s), 4.33 (2H, t, J = 6.6 Hz), 3.93 (3H, s), 1.78 (2H, tt, J = 7.6 Hz, 6.6 Hz), 1.51 (2H, tq, J = 7.6 Hz, 7.4 Hz), 0.97 (3H, t, J = 7.4 Hz).

{5-[(6-Amino-2-butoxy-9*H*-purin-9-yl)methyl]-2-fluorophenyl}-methanol (11k). To a suspension of lithium aluminum hydride (121 mg, 3.20 mmol) in THF (20 mL) was added dropwise a solution of compound **7k** (597 mg, 1.60 mmol) in THF (40 mL) at 0 °C and stirred for 1.5 h. The reaction mixture was quenched with H_2O (0.72 mL) and 1N NaOH (0.54 mL) and filtered through celite. The filtrate was concentrated in vacuo and the residue triturated with $CHCl_3$. The precipitate was collected by filtration to give compound **11k** (536 mg, 97%) as a colorless solid. ^{1}H NMR (DMSO-d_6) δ 8.03 (1H, s), 7.47 (1H, dd, J = 7.1 Hz, 2.2 Hz), 7.28 (1H, m), 7.11 (1H, dd, J = 10.2 Hz, 8.6 Hz), 5.23 (2H, s), 4.49 (2H, s), 4.21 (2H, t, J = 6.6 Hz), 1.65 (2H, tt, J = 7.5 Hz, 6.6 Hz), 1.38 (2H, tq, J = 7.5 Hz, 7.4 Hz), 0.91 (3H, t, J = 7.4 Hz).

2-Butoxy-9-[3-(chloromethyl)-4-fluorobenzyl]-9*H*-purin-6-amine (12k). To a suspension of compound **11k** (535 mg, 1.55 mmol) in $CHCl_3$ (16 mL) was added $SOCl_2$ (0.57 mL, 7.80 mmol) and refluxed for 1 h. The reaction mixture was neutralized with saturated aq $NaHCO_3$ and separated. The organic layer was dried over Na_2SO_4 and evaporated to give compound **12k** (535 mg, 95%) as a pale-yellow solid. ^{1}H NMR ($CDCl_3$) δ 7.61 (1H, s), 7.40 (1H, dd, J = 6.9 Hz, 2.2 Hz), 7.25 (1H, m), 7.05 (1H, dd, J = 9.0 Hz, 8.8 Hz), 5.84 (2H, brs), 5.25 (2H, s), 4.59 (2H, s), 4.33 (2H, t, J = 6.6 Hz), 1.78 (2H, tt, J = 7.6 Hz, 6.6 Hz), 1.50 (2H, tq, J = 7.6 Hz, 7.4 Hz), 0.97 (3H, t, J = 7.4 Hz).

{5-[(6-Amino-2-butoxy-9*H*-purin-9-yl)methyl]-2-fluorophenyl}-acetonitrile (13k). To a solution of compound **12k** (535 mg, 1.47 mmol) in DMF (15 mL) was added NaCN (220 mg, 4.50 mmol) and stirred at room temperature for 15 h. The reaction mixture was neutralized with 1N HCl and concentrated in vacuo. Water and $CHCl_3$ were added, and the organic layer was separated, dried over Na_2SO_4, evaporated, and the residue triturated with $CHCl_3$−hexane. The precipitate was collected by filtration to give compound **13k** (457 mg, 88%) as a colorless solid. ^{1}H NMR ($CDCl_3$) δ 7.61 (1H, s), 7.44 (1H, dd, J = 7.0 Hz, 2.1 Hz), 7.29 (1H, m), 7.08 (1H, dd, J = 9.0 Hz, 8.8 Hz), 5.54 (2H, brs), 5.26 (2H, s), 4.34 (2H, t, J = 6.6 Hz), 3.75 (2H, s), 1.79 (2H, tt, J = 7.6 Hz, 6.6 Hz), 1.51 (2H, tq, J = 7.6 Hz, 7.4 Hz), 0.97 (3H, t, J = 7.4 Hz).

{5-[(6-Amino-8-bromo-2-butoxy-9H-purin-9-yl)methyl]-2-fluorophenyl}acetonitrile (14k). To a suspension of compound **13k** (455 mg, 1.28 mmol) and NaOAc (190 mg, 2.30 mmol) in $CHCl_3$ (37 mL) was added bromine (310 mg, 1.93 mmol) and stirred at room temperature for 3 h. To the reaction mixture was added 10% aq $Na_2S_2O_3$ and saturated aq $NaHCO_3$. The organic layer was separated, dried over Na_2SO_4, and evaporated. The residue was triturated with MeOH and the precipitate collected by filtration to give compound **14k** (426 mg, 77%) as a colorless solid. ^{1}H NMR ($CDCl_3$) δ 7.53 (1H, dd, J = 7.0 Hz, 2.1 Hz), 7.34 (1H, m), 7.06 (1H, dd, J = 9.0 Hz, 8.8 Hz), 6.01 (2H, brs), 5.28 (2H, s), 4.34 (2H, t, J = 6.6 Hz), 3.75 (2H, s), 1.77 (2H, tt, J = 7.6 Hz, 6.6 Hz), 1.51 (2H, tq, J = 7.6 Hz, 7.4 Hz), 0.97 (3H, t, J = 7.4 Hz).

Methyl{5-[(6-amino-2-butoxy-8-oxo-7,8-dihydro-9*H*-purin-9-yl)methyl]-2-fluorophenyl}acetate (9k). To a suspension of compound **14k** (372 mg, 0.86 mmol) in MeOH (18 mL) was added 5N NaOH (9 mL) and refluxed for 5 h. The reaction mixture was neutralized with 12N HCl and the precipitate collected by filtration. To the solid was added MeOH (15 mL) and H_2SO_4 (1 mL) and then refluxed for 3 h. The reaction mixture was neutralized with saturated aq $NaHCO_3$ and water added. The precipitate was collected by filtration to give compound **9k** (321 mg, 93%) as a colorless solid; mp 272−274 °C. ^{1}H NMR (DMSO-d_6) δ 9.96 (1H, brs), 7.29−7.23 (2H, m), 7.14 (1H, dd, J = 9.7, 8.4 Hz), 6.46 (2H, brs), 4.82 (2H, s), 4.14 (2H, t, J = 6.6 Hz), 3.70 (2H, s), 3.60 (3H, s), 1.62 (2H, m), 1.37 (2H, m), 0.90 (3H, t, J = 7.4 Hz). Anal. ($C_{19}H_{22}FN_5O_4$) C, H, N.

Compound **9j** was prepared from compound **6** using a similar procedure as for compound **9k**.

Methyl{3-[(6-amino-2-butoxy-8-oxo-7,8-dihydro-9*H*-purin-9-yl)methyl]-4-methoxyphenyl}acetate (9j). Yield 31%; mp 282−287 °C. ^{1}H NMR (DMSO-d_6) δ 10.00 (1H, brs), 7.13 (1H, d, J = 8.4 Hz), 6.97 (1H, d, J = 8.4 Hz), 6.67 (1H, s), 6.47 (2H, brs), 4.80 (2H, s), 4.08 (2H, t, J = 6.6 Hz), 3.83 (3H, s), 3.53 (3H, s), 3.50 (2H, s), 1.59 (2H, m), 1.33 (2H, m), 0.87 (3H, t, J = 7.4 Hz). Anal. ($C_{20}H_{25}N_5O_5$) C, H, N.

Compound **9h** was prepared from compound **7e** using a similar procedure as for compound **9k**.

Methyl 3-{3-[(6-Amino-2-butoxy-8-oxo-7,8-dihydro-9*H*-purin-9-yl)methyl]phenyl}propanoate (9h). Yield 44%; mp 269−271 °C. ^{1}H NMR (DMSO-d_6) δ 9.99 (1H, brs), 7.15 (4H, m), 6.46 (2H, brs), 4.81 (2H, s), 4.14 (2H, t, J = 6.6 Hz), 3.54 (3H, s), 2.80 (2H, t, J = 7.6 Hz), 2.58 (2H, t, J = 7.6 Hz), 1.62 (2H, m), 1.36 (2H, m), 0.90 (3H, t, J = 7.3 Hz). Anal. ($C_{20}H_{25}N_5O_4\cdot 0.25H_2O$) C, H, N.

{3-[(6-Amino-2-butoxy-8-oxo-7,8-dihydro-9*H*-purin-9-yl)methyl]-phenyl}acetic Acid (16). To a solution of compound **9e** (100 mg, 0.259 mmol) in MeOH (50 mL) was added 1N NaOH (20 mL) and stirred at room temperature for 2 h. The reaction mixture was neutralized with 1N HCl and concentrated in vacuo. Water was added to the residue and the precipitate collected by filtration to give compound **16** (92 mg, 96%) as a colorless solid; mp 259−262 °C. ^{1}H NMR (DMSO-d_6) δ 12.31 (1H, brs), 10.03 (1H, brs), 7.22 (4H, m), 6.47 (2H, brs), 4.83 (2H, s), 4.14 (2H, t, J = 6.8 Hz), 3.50 (2H, s), 1.60 (2H, m), 1.38 (2H, m), 0.90 (3H, t, J = 7.0 Hz). Anal. ($C_{18}H_{21}N_5O_4\cdot 0.25H_2O$) C, H, N.

Ethyl {3-[(6-Amino-2-butoxy-8-oxo-7,8-dihydro-9*H*-purin-9-yl)methyl]phenyl}acetate (9l). To a suspension of compound **16** (58 mg, 0.159 mmol) in EtOH (20 mL) was added H_2SO_4 (1 mL) under ice-cooling and refluxed for 3 h. After cooling to room temperature, the mixture was neutralized with aq NH_3 and extracted with $CHCl_3$. The organic layer was dried over Na_2SO_4 and evaporated. The residue was purified by silica gel column chromatography using MeOH−$CHCl_3$ as eluent to give compound **9l** (53 mg, 85%) as a colorless solid; mp 269−270 °C. ^{1}H NMR (DMSO-d_6) δ 9.98 (1H, brs), 7.27 (1H, t, J = 8.0 Hz), 7.16 (3H, m), 6.46 (2H, brs), 4.83 (2H, s), 4.13 (2H, t, J = 6.6 Hz), 4.03 (2H, q, J = 7.1 Hz), 3.58 (2H, s), 1.62 (2H, m), 1.36 (2H, m), 1.14 (3H, t, J = 7.1 Hz), 0.90 (3H, t, J = 7.3 Hz). Anal. ($C_{20}H_{25}N_5O_4\cdot 0.1H_2O$) C, H, N.

Compound **17** was prepared from compound **5** using a similar procedure as for compound **8e**.

Methyl {3-[(6-Amino-2-methoxyethoxy-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl]phenyl}acetate (17). Yield 13%; mp 238−241 °C. ^{1}H NMR (DMSO-d_6) δ 9.96 (1H, s), 7.27 (1H, dd, J = 7.6 Hz, 7.6 Hz), 7.20 (1H, s), 7.17 (1H, d, J = 7.6 Hz), 7.15 (1H, d, J = 7.6 Hz), 6.47 (2H, brs), 4.83 (2H, s), 4.25 (2H, t, J = 4.8 Hz), 3.65 (2H, s), 3.58 (3H, s), 3.58 (2H, t, J = 4.8 Hz), 3.26 (3H, s). Anal. ($C_{18}H_{21}N_5O_5\cdot 0.25H_2O$) C, H, N.

2. Biology. 2.1. TLR7 Reporter Gene Assay. HEK293-hTLR7 cells, stably transfected with human TLR7 (pUNO expression vector) and pNiFty2-SEAP reporter plasmid, were kindly gifted from AstraZeneca. The cells were seeded in 96-well plates at 2×10^4 cells/well in DMEM supplemented with 1% nonessential amino acid, 10 μg/mL blasticidin S (Invitrogen), 10 μg/mL zeocin (Invivogen), and 10% heat-inactivated FCS and then stimulated with various concentrations of test compounds and incubated for 20 h at 37 °C in 5% CO_2. Then *p*-nitrophenyl phosphate was added as a substrate into the plates and incubated at room temperature for 20 min. After the reaction was

stopped with 1N sodium hydroxide solution, the absorbance at 405 nm was measured by a microplate reader.

2.2. Plasma Stability Study. The test compounds were added to human or rat plasma preincubated for 5 min at 37 °C (final concentrations of compounds were 1 μM). After incubation for 5 or 15 min at 37 °C, reactions were stopped by adding 3 times volume of methanol. Then the sample were centrifuged and remaining parent compounds in supernatants were analyzed by LC-MS.

2.3. Human PBMC (IFN Inducing Activity). Blood anticoagulated with heparin was obtained from healthy volunteers in our laboratory that had provided informed consent prior to donation. PBMC were isolated by density gradient centrifugation using LymphoprepTM (Nycomed) as recommended by the manufacturer. The isolated PBMC were washed twice with PBS and resuspended with serum free RPMI1640 supplemented with 50 U/mL penicillin/50 μg/mL streptomycin (Invitrogen). Test compounds were dissolved in DMSO and added into culture medium of the PBMC (1×10^6 cells/mL) at various concentrations (final DMSO concentration was kept constant at 0.1%). After incubation for 18 h at 37 °C, 5% CO_2, supernatants were collected by centrifugation (1200 rpm for 5 min) and stored at −20 °C until analyzed for cytokines. IFN was assayed by an ELISA kit (Amersham, RPN2789).

2.4. Inhibition of Inflammatory Cells in BALF (Efficacy). Male 8−10 weeks old Brown Norway rats were sensitized by intraperitoneal injection of ovalbumin (1 mg) together with aluminum hydroxide adjuvant (100 mg) in saline (1 mL) on day 0 and 7. Control (unsensitized/unchallenged) animals received vehicle (saline) alone at the same time points. On any one-day between days 14 and 18, rats were challenged by exposure to ovalbumin aerosol for 15 min generated from a 10 mg/mL ovalbumin solution by a nebulizer (Ultrasonic nebulizer, NE-U17, Omron). Control animals were similarly exposed to saline aerosol for 15 min. Two hours before antigen challenge, rats were dosed with test compounds (suspended in saline) or vehicle by i.t. administration (dosing volume was 0.5 mL/kg). Twenty-four hours after antigen challenge, rats were sacrificed and the trachea was cannulated. The airway lumen was washed with 2 mL of saline, and this procedure was repeated six times (total volume of 12 mL). Infiltrated cells in BALF were stained with Turk solution and the number of nucleated cells was counted in a counting chamber. A differential count was made on a smear prepared with a cytocentrifuge (Cytospin II, Shandon Scientific Ltd.) and stained with Diff-Quick solution (May−Grunwald stain). At least 300 cells were counted in each BALF sample (magnification × 400).

2.5. Induction of Systemic IFN (Side Effect). Male 8−10 weeks old Brown Norway rats were dosed with test compounds (suspended in saline) by i.t. administration (dosing volume was 0.5 mL/kg). At 2, 4, 6, and 24 h after i.t. administration, rats were anaesthetized with ether, and heparinized blood samples (about 0.3 mL) were collected via the caudal vein. Then plasma samples were prepared by centrifugation (12000 rpm for 10 min), and stored at −20 °C until analyzed for IFN. IFN titers in the plasma samples were determined in a CPE reduction assay (bioassay) using L929 and vesicular stomatitis virus (VSV).

Supporting Information Available: Analytical data of tested compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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