British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2011.01790.x
www.brjpharmacol.org

RESEARCH PAPER

Biological characterization of a novel class of toll-like receptor 7 agonists designed to have reduced systemic activity

M Biffen[1], H Matsui[4], S Edwards[1], AJ Leishman[1], K Eiho[4], E Holness[1], G Satterthwaite[1], I Doyle[1], H Wada[2], NJ Fraser[1]*, SL Hawkins[1], M Aoki[4], H Tomizawa[4], AD Benjamin[1], H Takaku[4], T McInally[2] and CM Murray[3]†

[1]*Department of Bioscience, AstraZeneca R&D Charnwood, Loughborough, Leicestershire, UK,* [2]*Department of Medicinal Chemistry, AstraZeneca R&D Charnwood, Loughborough, Leicestershire, UK,* [3]*AstraZeneca plc, Alderley Park, Cheshire, UK, and* [4]*Pharmacology Research Lab, Dainippon Sumitomo Pharma Co., Ltd, Osaka, Japan*

Correspondence
Clare Murray, AstraZeneca plc, Alderley Park, Cheshire SK10 4TF, UK. E-mail: clare.murray@astrazeneca.com

*Current address: Vertex Pharmaceuticals (Europe) Ltd, 88 Milton Park, Abingdon, Oxfordshire, OX14 4RY, UK.
†AstraZeneca plc, Alderley Park, Cheshire SK10 4TF, UK.

Keywords
allergy; cytokines; lung; reporter cell; mononuclear cells; splenocytes; TLR7

Received
18 April 2011
Revised
30 September 2011
Accepted
12 October 2011

BACKGROUND AND PURPOSE

Toll-like receptor 7 (TLR7) agonists have potential in the treatment of allergic diseases. However, the therapeutic utility of current low molecular weight TLR7 agonists is limited by their systemic activity, resulting in unwanted side effects. We have developed a series of TLR7-selective 'antedrugs', including SM-324405 and AZ12441970, which contain an ester group rapidly cleaved in plasma to reduce systemic exposure.

EXPERIMENTAL APPROACH

Agonist activity at TLR7 of the parent ester and acid metabolite was assessed *in vitro* in reporter cells and primary cells from a number of species. Pharmacokinetics following a dose to the lungs was assessed in mice and efficacy evaluated *in vivo* with a mouse allergic airway model.

KEY RESULTS

Compounds were selective agonists for TLR7 with no crossover to TLR8 and were metabolically unstable in plasma with the acid metabolite showing substantially reduced activity in a number of assays. The compounds inhibited IL-5 production and induced IFN-α, which mediated the inhibition of IL-5. When dosed into the lung the compounds were rapidly metabolized and short-term exposure of the 'antedrug' was sufficient to activate the IFN pathway. AZ12441970 showed efficacy in a mouse allergic airway model with minimal induction of systemic IFN-α, consistent with the low plasma levels of compound.

CONCLUSIONS AND IMPLICATIONS

The biological and metabolic profiles of these TLR7-selective agonist 'antedrug' compounds are consistent with a new class of compound that could be administered locally for the treatment of allergic diseases, while reducing the risk of systemic side effects.

LINKED ARTICLE

This article is commented on by Kaufman and Jacoby, pp. 569–572 of this issue. To view this commentary visit http://dx.doi.org/10.1111/j.1476-5381.2011.01758.x

Abbreviations

BChE, butyrylcholinesterase; FCS, fetal calf serum; IL-1RA, IL-1 receptor antagonist; OVA, ovalbumin; PBMC, peripheral blood mononuclear cell; PHA, phytohaemagglutinin; SEAP, secretory alkaline phosphatase; Th1, type-1 T-helper; Th2, type-2 T-helper; TLR, toll-like receptor

Introduction

The global burden of allergic diseases such as asthma has continued to rise over the past decade (Pearce *et al.*, 2007). Epidemiological studies have suggested a link with reduced microbial exposure of children leading to the 'hygiene hypothesis' (Braun-Fahrlander *et al.*, 2002; Romagnani, 2004). The rationale behind this theory is that reduced exposure to microbes results in an immune deviation leading to an enhanced type-2 T-helper (Th2) cell-mediated immune response. This is characterized by the cytokines IL-4, IL-5, IL-13, which drive the allergic response (Holgate, 2008). Exposure to microbes modulates the adaptive immune response via interaction of pathogen-specific components with toll-like receptors (TLRs; nomenclature follows Alexander *et al.*, 2011) expressed on cells of the innate immune system. These cells include dendritic cells, monocytes, macrophages and B-lymphocytes. Activation of TLRs expressed on these cells results in production of cytokines and expression of co-stimulatory molecules that modulate the adaptive immune response (Iwasaki and Medzhitov, 2010).

Ten distinct TLRs have been identified (Takeda *et al.*, 2003) including TLR7, a receptor that recognizes single-stranded RNA (Diebold *et al.*, 2004; Lund *et al.*, 2004). Stimulation of TLR7 is also mediated by low molecular weight compounds including the imidazoquinolines imiquimod, resiquimod (R848) (Hemmi *et al.*, 2002), and 2-substituted-8-hydroxyadenine derivatives (Isobe *et al.*, 2003). Apart from TLR7 activity, R848 also has TLR8 activity in man (Jurk *et al.*, 2002). TLR7 is expressed on plasmacytoid dendritic cells, B-lymphocytes and monocytes and elicits cell-specific responses including production of IFN-α from plasmacytoid dendritic cells, proliferation of B-lymphocytes and production of cytokines including IL-12 and TNF-α from monocytes (Gorden *et al.*, 2005; Hanten *et al.*, 2008). *In vitro*, TLR7 agonists have been shown to change the phenotype of allergen-specific Th2 cells towards type-1 T-helper (Th1)/type-0 T-helper (Th0) effector cells (Fili *et al.*, 2006). Furthermore, in the Brown Norway rat allergic model the TLR7 agonist, R848, suppressed goblet cell hyperplasia and this was preceded by a reduction of both Th1 and Th2 cytokine production in the lungs (Camateros *et al.*, 2007). In a murine model the TLR7 agonist SA-2 reduced lung inflammation, lung eosinophilia and reduced ovalbumin (OVA)-specific IgE (Vultaggio *et al.*, 2009).

Although there is precedent for TLR7 agonists to modulate allergic reactions, an issue with currently available TLR7 agonists is that, although they are safe and tolerated, they induce systemic cytokines resulting in undesirable side effects. In clinical studies, the TLR7 agonists resiquimod (Pockros *et al.*, 2007), isatoribine (Horsmans *et al.*, 2005) and PF-4878691 (852A) (Fidock *et al.*, 2011) have been tested for the treatment of hepatitis C and 852A (Dudek *et al.*, 2007) has been tested in cancer patients. While demonstrating efficacy, levels of compound in the plasma were linked to cytokine induction and side effects included fever, fatigue, headache, shivering and influenza-like symptoms.

A strategy to minimize side effects linked to systemic exposure would be to limit the compound's effects to the location where it is applied. This has been achieved by tethering a TLR7 agonist to mouse serum albumin. When administered to the lung, this produced a local cytokine production in the bronchoalveolar lavage fluid with no systemic production of cytokines (Wu *et al.*, 2007). Another approach is to develop an 'antedrug'. This is defined as a locally active compound that is designed to be rapidly metabolized to an inactive form upon entry into the circulation (Lee and Soliman, 1982). The synthesis of a TLR7 agonist, SM-324405, with these characteristics has been reported (Kurimoto *et al.*, 2010). Within this series of 8-oxoadenine derivatives the compound AZ12441970 has also been synthesized and, here, we have characterized the biological activity of both SM-324405 and AZ12441970 as representatives of this series. These compounds are esters with TLR7 agonist activity and cleavage of the ester link by plasma esterases results in an acid with substantially reduced activity. When tested in a mouse allergic airway model *in vivo*, AZ12441970 was efficacious with minimal systemic cytokine production. Therefore, these compounds provide a basis for potential development as therapeutic agents for the treatment of allergic diseases by acting locally, while minimizing the induction of unwanted systemic side effects.

Methods

Preparation of test compounds

The test compounds, SM-324405: methyl 2-(3-{[6-amino-2-butoxy-8-oxo-7H-purin-9(8H)-yl]methyl}phenyl)acetate, SM-324406: 2-(3-{[6-amino-2-butoxy-8-oxo-7H-purin-9(8H)-yl]methyl}phenyl)acetic acid, AZ12441970: methyl 2-(3-(((3-(6-amino-2-butoxy-8-oxo-7H-purin-9(8H)-yl)propyl)(3-(dimethylamino)propyl)amino)phenyl)acetate and AZ12443988: 2-(3-(((3-(6-amino-2-butoxy-8-oxo-7H-purin-9(8H)-yl)propyl)(3-(dimethylamino)propyl)amino)phenyl) acetic acid were synthesized in the Medicinal Chemistry Facilities, AstraZeneca R&D, Charnwood, UK and Dainippon Sumitomo Pharma Co., Ltd, Osaka, Japan.

For all assays, test compounds were made up in DMSO and serially diluted in DMSO to concentrations 1000-fold greater than the final incubation concentration. Stocks were diluted 100-fold into medium to generate 10× stock concentrations. DMSO vehicle controls were similarly prepared.

Animals

Animal studies were conducted according to the 'Animal Care and Use Committee' of Dainippon Sumitomo Pharma Co., Ltd. or in accordance with UK Home Office legislation under licence PPL40/2238; Procedure 5 and PPL40/2891 Procedure 2. Mice and rats at AstraZeneca were group housed in a controlled environment (temperature 19 ± 2°C; relative humidity 55% ± 10%; 15–20 air changes per hour and a 12:12 h light : dark cycle) with access to pelleted diet (RM1; Special Diet Services) and water *ad libitum*. Experimental procedures were conducted in accordance with the Animal (Scientific Procedures) Act 1986. For intranasal dosing, mice were anaesthetized with isofluorane (National Veterinary Supplies Ltd., Stoke on Trent, UK) and anaesthesia assessed by lack of reflex response. Terminal anaesthesia was achieved by intraperitoneal injection of sodium pentobarbital (20 mg) (National Veterinary Supplies Ltd.) followed by exsanguination once anaesthesia had been achieved.

Mice at Dainippon Sumitomo Pharma Co., Japan were housed in a controlled environment (temperature 23 ± 2°C; relative humidity 55% ± 10%; 10–20 air changes per hour and a 12:12 h light : dark cycle) with access to pelleted diet (CE-2; Clea Japan Inc., Tokyo, Japan) and water *ad libitum*. For intratracheal dosing of C57B/6 mice, the mice were anaesthetized with diethylether and anaesthesia assessed by lack of reflex response. Mice were killed under anaesthesia, by exsanguination.

TLR reporter assays

HEK293 cells, stably transfected with human TLR7 (pUNO expression vector) and pNiFty2-SEAP reporter plasmid were maintained in Dulbecco's modified Eagle's medium, FCS 10% (v/v), 2 mM *l*-glutamine, non-essential amino acids, 10 μg·mL^{-1} blasticidin S and 10 μg·mL^{-1} zeocin (Invitrogen, Paisley, Scotland). The sequence used was represented by the European Molecular Biology Laboratory Nucleotide Sequence Database sequence AF240467. Cells were seeded in tissue culture treated clear flat bottom polystyrene 96 well plates (Thermo Fisher Scientific, Loughborough, UK) (Costar, 3598) at 10 000 cells per well. Dose–response curves were generated by addition of test compounds and incubation for 20 h at 37°C in an atmosphere of 5% CO_2. The secretory alkaline phosphate (SEAP) released was quantified using *p*-nitrophenyl phosphate (Sigma, Dorset, UK) as a substrate, and the absorbance at 405 nm was determined by a microplate reader.

HEK293-human TLR8 cells were similarly constructed using the human TLR8 sequence identical to GenBank sequence AAZ95441.1.

Rat TLR7 reporter cells were generated with the same expression and reporter plasmids using the coding sequence of rat TLR7 identical to the sequence represented by GenBank Accession No. EF032637.

All methodologies were the same as for human TLR7 reporter cells, except that human TLR8 reporter cells were used at 20 000 cells per well, and rat TLR7 reporter cells were used at 25 000 cells per well.

Plasma stability determinations

The test compounds (initial concentration of 1 μM) were added to human or rat plasma (prepared by centrifuging blood collected in EDTA tubes at 1800× *g*) at 37°C in a total volume of 0.5 mL. Incubations were for 10 min at 37°C with samples taken at 0, 20 s, 40 s and 1, 2, 3, 5 and 10 min into acetonitrile. Supernatants were analysed by LC/MS/MS for the remaining parent compound, and $t_{1/2}$ of the parent compound was determined.

Splenocyte preparations

Spleens were removed from CO_2 asphyxiated male Brown Norway rats (Harlan, Bicester, UK) or from naïve female Balb/c mice (Harlan), following cervical dislocation, and placed in a Petri dish containing RPMI 1640. The spleen was gently pushed through a 70 μm BD Falcon Cell Strainer to obtain a single cell suspension. Cells were centrifuged at 400× *g* for 5 min to obtain a cell pellet, the supernatant removed and cells resuspended in fresh RPMI 1640. The cells were centrifuged again and the cells resuspended in complete medium (RPMI-1640, fetal calf serum (FCS) 5% (v/v), 2 mM *l*-glutamine, 10 U·mL^{-1} penicillin, 10 μg·mL^{-1} streptomycin and 50 μM 2-mercaptoethanol).

Peripheral blood mononuclear cell (PBMC) preparations

Blood from healthy, consenting volunteers was collected into heparin and layered onto Lymphocyte Separation Medium 1077 (PAA, Pasching, Austria) and centrifuged at 700× *g* for 25 min. The PBMC layer was removed, diluted to 50 mL with PBS and centrifuged at 400× *g* for 10 min. The supernatant was removed, the pellet resuspended in 50 mL PBS and centrifuged at 300× *g* for 5 min. Finally the cells were washed in 50 mL PBS and the cells recovered by centrifuging at 200× *g* for 5 min. PBMCs were finally resuspended in assay medium (RPMI 1640 with 25 mM HEPES, FCS 10% (v/v), 2 mM *l*-glutamine, 10 U·mL^{-1} penicillin and 10 μg·mL^{-1} streptomycin).

Dog PBMC were prepared from dog blood (Animal Facilities, AstraZeneca R&D) collected into heparin and the PBMC prepared using the same protocol as for human PBMC.

Splenocyte incubations

Twenty microlitres of test compound or complete RPMI 1640 with DMSO 1% (v/v), vehicle control, were added to each well followed by 180 μL of splenocyte cell suspension (2×10^5 cells) in complete RPMI prepared as described earlier. Splenocytes and compound were incubated at 37°C in an atmosphere of air/CO_2 (95/5 v/v) for the defined period of time.

Splenocyte proliferation was determined by addition of 0.0185MBq [^{3}H]-thymidine (Amersham, Buckinghamshire, UK) to cellular assays at 44 h. After a further 6 h incubation, the cells were harvested onto glass fibre filter mats using a Tomtec filtration apparatus. The mats were dried, Betaplate Scint (Perkin Elmer, Pangbourne, UK) added, and filter-bound radioactivity was quantified with a MicroBeta 1450 Trilux (Perkin Elmer).

Mouse splenocyte IL-5 and IFN-γ

Naïve female Balb/c mice were immunized by injection of 10 μg OVA + 1 mg $Al(OH)_3$ in 100 μL by intraperitoneal injection on day 0. Eight days after immunization, spleens from OVA/$Al(OH)_3$ sensitized mice were collected into RPMI 1640 medium and splenocytes prepared and incubated as described earlier. OVA was added to give a final concentration of 1 mg OVA mL^{-1} and incubations were for 5 d. The supernatant was removed for determination of the amount of IL-5 and IFN-γ produced.

PBMC incubations

Twenty microlitres of test compound or assay medium with dimethyl sulfoxide (DMSO) 1% (v/v), vehicle control, were added to each well followed by 180 μL of PBMC cell suspension (prepared as mentioned earlier) in assay medium (200 000 cells). PBMC and compound were incubated at 37°C in an atmosphere of air/CO_2 (95/5 v/v) for the defined period of time.

For induction of IL-5, human PBMC were prepared and plated out with compounds as described earlier. Phytohaemagglutinin (PHA) (Sigma) was added at a final concentration of 5 μg·mL^{-1} and incubated for 44 h when the supernatant was removed for determination of the amount of IL-5 produced.

In assays where butyrylcholinesterase (BChE) (Serotec, Kidlington, UK) was added to shorten the exposure time to antedrug, PBMC were plated out with BChE at a concentration of 1 U·mL^{-1} and incubations were initiated by addition of compound. After 24 h, 150 µL supernatant was removed for cytokine determinations and replaced with 150 µL fresh medium. At 44 h, [^{3}H]-thymidine was added and proliferation determined as described earlier.

Gene chip analysis

Balb/c mouse splenocytes, Brown Norway rat splenocytes or human PBMC were incubated with compound, and after 4 h stimulation RNA was extracted using TRIzol® Reagent (Invitrogen). Microarray analysis was performed on human (HG-U133 plus 2), mouse (MOE430), and rat (RAE230) Affymetrix chip sets according to standard protocols (Affymetrix, Santa Clara, CA, USA). Raw microarray data was normalized using the MAS5 algorithm within GeneChip Operating Software (Affymetrix).

Cytokine determinations

Cytokines were determined from splenocyte or PBMC supernatants using the following commercial kits and samples were processed by following the manufacturer's instructions.

Human IL-5 (555202), human TNF-α (555212), human IFN-γ (555142) and mouse IL-5 (555236) were from BD Biosciences, Oxford, UK; human IL-1RA (DY280), human IFN-α (41100) and mouse IL-1RA (MRA00) from R&D Systems, Abingdon, UK and RPN2789 from GE Healthcare Life Sciences, Little Chalfont, UK.

Human IL-13 (LHC0131), mouse IFN-γ (CMC4033), mouse IL-5 (CMC0053), mouse IL-6 and mouse TNF-α multiplex (LCM0001) were from Invitrogen.

Mouse IFN-α levels in supernatants were quantified by a bioassay using mouse L929 cells transfected with cDNA encoding the IFN-α inducible p2′5′-oligoadenylate synthase promoter and luciferase gene (L929/OAS cells). Supernatants were incubated with these cells for 20 h and the luciferase produced was quantified following addition of Bright-Glo Luciferase Assay System (E2610, Promega, Southampton, UK) and determining the luminescence compared with a standard curve of mouse IFN-α (HC1040a; Hycult Biotech, Uden, the Netherlands).

Determination of pharmacokinetics In vivo

AZ12441970 was formulated in 0.1% Tween80/0.6% NaCl/ 50 mM phosphate buffer pH 6.0 at a concentration of 0.5 mg·mL^{-1}. Six female BALB/c mice were briefly anaesthetized with isoflurane then dosed intranasally with 50 µL of the formulation, giving a dose of 1 mg·kg^{-1} per mouse. This volume is sufficient to be inhaled into the lung rather than remain in the nasal cavity. At each time point, two animals were killed by an overdose of pentobarbital and blood taken from the vena cava into sodium fluoride (0.2 M final concentration) to prevent hydrolysis by esterase enzymes before mixing with the anticoagulant EDTA. Samples were quenched in methanol and frozen at –20°C. Lungs were excised and placed in vials containing 1 mL sodium fluoride (1.2 M) and immediately frozen at –20°C. The lungs were homogenized with eight parts water, and aliquots of the homogenate quenched with methanol. Standard curves were prepared from a known weight of the test compound AZ12441970 and the acid metabolite AZ12443988, added to lung homogenate or blood containing sodium fluoride and treated as earlier samples.

All samples were centrifuged and the supernatant analysed by LC/MSMS and the concentrations of AZ12441970 and AZ12443988 quantified.

Mouse OVA-induced allergic airways model

Female C57BL/6 mice (Charles River Japan Inc., Hino, Japan) were sensitized by subcutaneous injection of 10 µg of OVA adsorbed with 4 mg aluminium hydroxide adjuvant in 100 µL on Day 0 and 14. Animals were challenged by intratracheal (20 µL) administration of OVA (0.5 mg·mL^{-1}) on Day 22. AZ12441970 (40 µL, dissolved in 0.1% Tween80/ 0.6% NaCl/50 mM phosphate buffer pH 6.0) was administered via the intratracheal route 24 h and 2 h prior to OVA challenge. Animals were killed under anaesthesia 48 h after the OVA challenge, and the number of eosinophils in bronchoalveolar lavage fluid was measured by FACS analysis as described previously (van Rijt *et al.*, 2004). Briefly, bronchoalveolar lavage fluid cells were pre-incubated with anti-mouse CD16/CD32 monoclonal antibody 2.4G2 (BD Bioscience, San Diego, CA, USA) at 4°C for 15 min, then incubated with anti-mouse FITC-CD4(L3T4), FITC-CD8, FITC-B220 and PE-CCR3 (BD Bioscience). Number of CD4$^-$ CD8$^-$ B220$^-$ CCR3$^+$ eosinophils was determined using a Becton Dickinson FACScan (Becton Dickinson). IL-5 in bronchoalveolar lavage fluid was measured by ELISA (BD Bioscience).

Systemic cytokine induction in mice

AZ12441970 and R848 (dissolved in 0.1% Tween80/0.6% NaCl/50 mM phosphate buffer pH 6.0) were administered to naïve female C57BL/6 mice via the intratracheal route in a volume of 20 µL per mouse. Blood was collected 90 min later into heparinized syringes and plasma was prepared by centrifugation. Plasma was stored frozen until analysis. IFN-α in plasma was measured by the reporter assay system using L929/OAS cells and cytokines were determined by ELISA or Luminex technologies.

Data analysis

Results are shown as means ± SEM, unless otherwise stated. Differences between means were assessed by two-tailed, paired Students t-test, using $P < 0.05$ as showing significance.

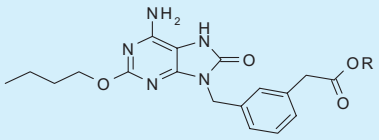
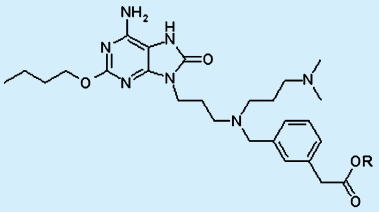
Results

Characterization of the TLR agonist activity of SM-324405, AZ12441970 and their metabolites

A synthetic chemistry program was undertaken that led to TLR7 agonist antedrugs that were rapidly metabolized in plasma (Kurimoto *et al.*, 2010). A subsequent research programme led to the identification of an alternative series of compounds exemplified by AZ12441970. This paper profiles the biological activity and mechanism of action of this series

Table 1

Structure, potency and plasma stability of antedrug compounds and R848

Compound	Structure	R
SM-324405		Me
SM-324406		H
AZ12441970		Me
AZ12443988		H

Compound	Human TLR7 pEC_{50}	Rat TLR7 pEC_{50}	Human TLR8 pEC_{50}	Human plasma $t_{1/2}$ (min)	Rat plasma $t_{1/2}$ (min)
R848	6.8	6.4	5.2	N/A	N/A
SM-324405	7.3	6.6	Inactive	2.6	0.19
SM-324406	7.2	5.6	Inactive	N/A	N/A
AZ12441970	6.8	6.6	Inactive	1.2	0.07
AZ12443988	<5.0	<5.0	Inactive	N/A	N/A

Dose–response curves of compounds (20 nM to 10 μM) were assayed for activity in HEK293 cells stably expressing human TLR7, rat TLR7 or human TLR8. Activity was calculated from the release of secretory alkaline phosphatase whose expression was linked to an NF-κB promoter. Plasma $t_{1/2}$ was determined by incubating the compounds in human or rat plasma and determining disappearance of parent compound by LC/MS/MS. Determinations are the mean of ≥3 individual experiments.
N/A, not applicable.

as demonstrated by the TLR7 agonists, SM-324405 and AZ12441970, along with their metabolites (Table 1). These compounds were rapidly metabolized in human plasma with a $t_{1/2}$ of 1–3 min and in rat plasma with a $t_{1/2}$ of less than 1 min (Table 1).

AZ12441970 and SM-324405 showed activity that was equivalent to or greater than the well characterized TLR7/8 ligand, R848, in human and rat TLR7 reporter assays (Table 1). Whereas R848 was active at human TLR8, neither of the other two compounds had human TLR8 activity, as assessed by ability to activate NF-κB in a reporter cell line. The product generated following metabolism in plasma is an acid that, in the case of SM-324405, still showed significant activity in the human and rat TLR7 reporter assays. However, the design of AZ12441970 was such that its acid metabolite, AZ12443988, showed a greater than 60-fold reduction in potency in the human TLR7 reporter assay. Neither of the acids showed any detectable TLR8 activity.

Comparison of SM-324405 and R848 activity in primary cells from different species

We carried out a gene expression study to determine whether the new 8-oxoadenine-compounds, as exemplified by SM-324405, showed activity in primary cells across a number of species. R848 has been studied extensively (see Introduction for references) as a TLR7/8 agonist and was included as a positive control. All compounds were tested at concentrations considered to give a maximal response. SM-324405 induced a gene induction profile in human PBMC, as well as rat and mouse splenocytes, similar to that induced by R848 (Figure 1). TLR7 stimulation of plasmacytoid dendritic cells results in production of type-I IFN and both R848 and SM-324405 induced >10-fold increases in mRNA for *Ifnα* and *Ifnβ* in human PBMCs. Responses for induction of *Ifnα* and *Ifnβ* in mouse and rat splenocytes were not as robust, though there was clear induction of the IFN-regulated genes *Cxcl10*, *Ifit2* and *Oas* in all species with both agonists. A range of cytokine and chemokine genes including *Ccl3*, *Il10*, *Il12*, *Ifnγ* and *TNF-α* were also induced by SM-324405 and R848 across all three species. *Tlr7* and its downstream signalling molecules *Myd88* and *Irf7* also showed equivalent levels of induction by both agonists in all three species. These data confirmed that, from the 8-oxoadenine series of compounds, SM-324405 had a similar biological activity profile to that of R848 in human, rat and mouse cells.

The mouse mRNA data did not show changes in *Ifnα* with either agonist. This may have been the result of poor detection by the probe, so human PBMC and mouse splenocytes were stimulated with R848 and SM-324405 and IFN-α deter-

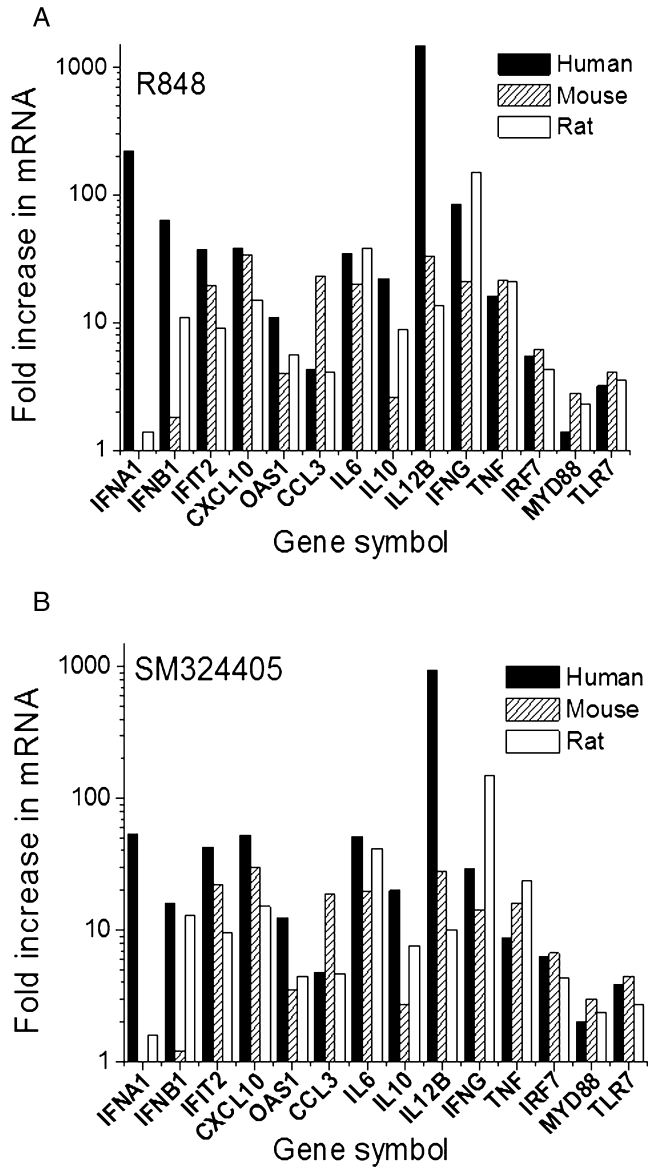


Figure 1

Induction of mRNA by R848 (A) or SM-324405 (B) in human, mouse and rat cells. Human PBMC were incubated with 1 μM SM-324405 or 10 μM R848 for 4 h and RNA extracted and analysed. Balb/c mouse splenocytes or Brown Norway rat splenocytes were incubated with 100 nM SM-324405 or R848 and after 4 h stimulation RNA was extracted and analysed by microarray analysis. Levels of gene expression induced by the compounds were expressed as a fold increase over the control incubation. Results are from a single experiment in each species and are representative of 3 such determinations.

mined by ELISA or bioassay (Figure 2A and B). The data confirmed that both agonists were inducers of IFN-α. In addition IFN-γ protein was determined and showed that apart from changes at the mRNA level, there were also equivalent effects at the protein level (Figure 2C and D). The activity of the acid metabolite was at least 10- to 30-fold less than that of the parent compound in inducing IFN-α and IFN-γ from human and mouse cells (Figure 2).

The induction of IFN-α was consistent with activation of TLR7 on plasmacytoid dendritic cells. TLR7 agonists have also been shown to stimulate proliferation of B-cells and the compound potencies were assessed for induction of proliferation in splenocytes from mice and rats (Figure 3A and B). R848 and SM-324405 showed similar activity with SM-324405 having pEC_{50} values of 8.4 and 8.2 in mouse and rat respectively. In mouse and rat splenocytes the acid potency was 6.8 and <6.0, respectively, giving an ester/acid potency ratio of 40 and >200. The determination of a proliferative response is a useful indicator of activity in species where reagents for determining products such as cytokines are limited. We therefore determined activity in dog PBMC (Figure 3C) and found that SM-324405 had a pEC_{50} of 7.9 showing that the compounds are active in the dog. The activity of the acid metabolite was not determined in dog PBMC as dog plasma lacks an esterase capable of cleaving SM-324405, so the compounds do not behave as antedrugs in this species (data not shown).

AZ12441970 has improved antedrug characteristics

The antedrug concept used in these compounds relies on the metabolite (acid) having reduced activity, compared with its parent (ester), and it was clear from the reporter assay for human and rat TLR7 (Table 1) that the acid of SM-324405 was, at best, only 10-fold less active than its parent ester. Having established that this adenine series of compounds showed biological activity comparable with R848, we sought compounds where the activity of the metabolite was further reduced and this led to a series of compounds exemplified by AZ12441970 (Table 1). This compound had a 0.5 log reduction in human TLR7 potency compared with SM-324405, though the rat TLR7 potency, pEC_{50} = 6.6, was maintained (Table 1). The acid metabolite, AZ12443988, was less active than the acid of SM-324405 and had an ester/acid potency ratio of >60-fold for both human and rat TLR7. The true extent of the potency ratio value could not be determined as the activity of AZ12443988 was so low that no pEC_{50} could be determined over the range of concentrations up to 10 μM. As AZ12441970 also has a shorter plasma $t_{1/2}$ than SM-324405, overall, AZ12441970 exhibited improved antedrug properties *in vitro*.

Inhibition of the Th2 cytokine, IL-5, by AZ12441970

TLR7 agonists have the potential to treat allergic diseases, which are characterized by a Th2 phenotype, by rebalancing the immune response. We used PHA to polyclonally stimulate human PBMC and assessed the ability of the compounds to inhibit IL-5 production as a marker of Th2 cytokine modulation (Figure 4A and B). R848 dose-dependently inhibited the production of IL-5 with a pIC_{50} of 7.7. AZ12441970 and SM-324405 were potent inhibitors of IL-5 with a pIC_{50} of 8.7 and 7.9 respectively. The acid metabolite of AZ12441970 (AZ12443988) was much less active (pIC_{50} = 5.4) compared with SM-324406 (pIC_{50} = 6.8) as an inhibitor of IL-5 production, giving an ester/acid ratio of 1900 for AZ12441970. This

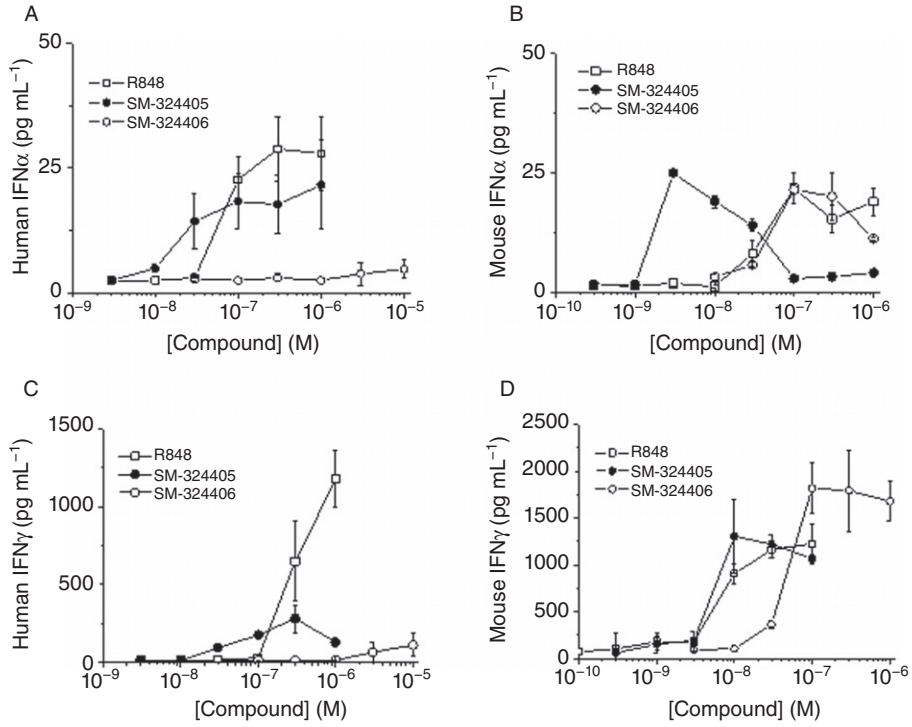


Figure 2

Induction of IFN-α and IFN-γ in human and mouse cells by TLR7 agonists. Human PBMC or mouse splenocytes were incubated with R848, SM-324405 or SM-324406, over a range of concentrations. Cell culture supernatants were removed after 24 h to assay human IFN-α (A), human IFN-γ (C) and mouse IFN-α (B) or after 5 d, to assay mouse IFN-γ (D). Cytokines were determined by ELISA. Incubations were in triplicate and are representative of three separate experiments. Data shown are means ± SD.

was an improvement on the ratio of 13 for SM-324405/SM-324406. PHA also induced IL-13 in this assay and in an assessment of 10 TLR7 agonists we observed equivalent inhibition of IL-5 and IL-13 (Supporting Information Figure S1). IL-4, however, was not induced. In a murine assay where IL-5 was induced by addition of the antigen (OVA), R848 and AZ12441970 potently inhibited IL-5 production with pIC_{50} of 8.7 and 7.5 respectively (Figure 4C). AZ12443988 inhibited IL-5 production with a pIC_{50} of 6.0. Therefore, this class of TLR7 agonist suppresses Th2 cytokine production in both human and mouse T-cells.

Identifying potential biomarkers of TLR7 agonist activity

As TLR7 stimulation of PBMC results in production of IFN-α, we investigated whether this might be a mediator in the inhibition of IL-5. In both human PBMC and mouse splenocyte assays, addition of IFN-α resulted in inhibition of IL-5 (Figure 5). Therefore, IFN-α is a potential biomarker for TLR7 stimulation of plasmacytoid dendritic cells as well as a marker of suppression of Th2 cytokines. IFN-α has a short $t_{1/2}$ *in vivo* making it difficult to measure as a potential biomarker. To overcome this, we investigated gene products that are secondary markers of IFN-α activity. Addition of AZ12441970 to human PBMC induced IL-1RA (Figure 6A), and this induction was blocked with a neutralizing anti-IFN-α/β receptor antibody (Figure 6A). We confirmed that IFN-α induced IL-1RA (Figure 6B), and demonstrated that inclusion of the neutralizing antibody blocked the production of IL-1RA by IFN-α. This confirmed IL-1RA as a downstream marker of IFN-α production.

Short-term compound exposure favours induction of IFN-α

To investigate the pharmacokinetic profile of AZ12441970, it was given intranasally to mice in a volume that ensured delivery to the lung. AZ12441970 is unstable in mouse plasma with a $t_{1/2}$ of 0.22 min. 10 min after dosing, 90% of the compound had been metabolized in the lung and the acid metabolite generated (Table 2). Both the ester and acid levels declined further up to 150 min. Plasma levels of AZ12441970 were of the order of 400-fold lower than lung levels at the three time points studied. Although levels of plasma AZ12443988 were higher than those of the parent compound, the acid was also cleared from the blood.

From the pharmacokinetic data it was evident that not only was AZ12441970 rapidly metabolized in the plasma, but that it was hydrolysed when deposited in the lung. BChE had been confirmed as the plasma esterase responsible for cleavage of the antedrug ester as the BChE selective inhibitor, ethephon, blocked conversion of AZ12441970 to its acid metabolite in plasma (Supporting Information Figure S2). The IC_{50} of 24 μM was consistent with a value of 11 μM determined by Haux *et al.* (2000). To determine whether

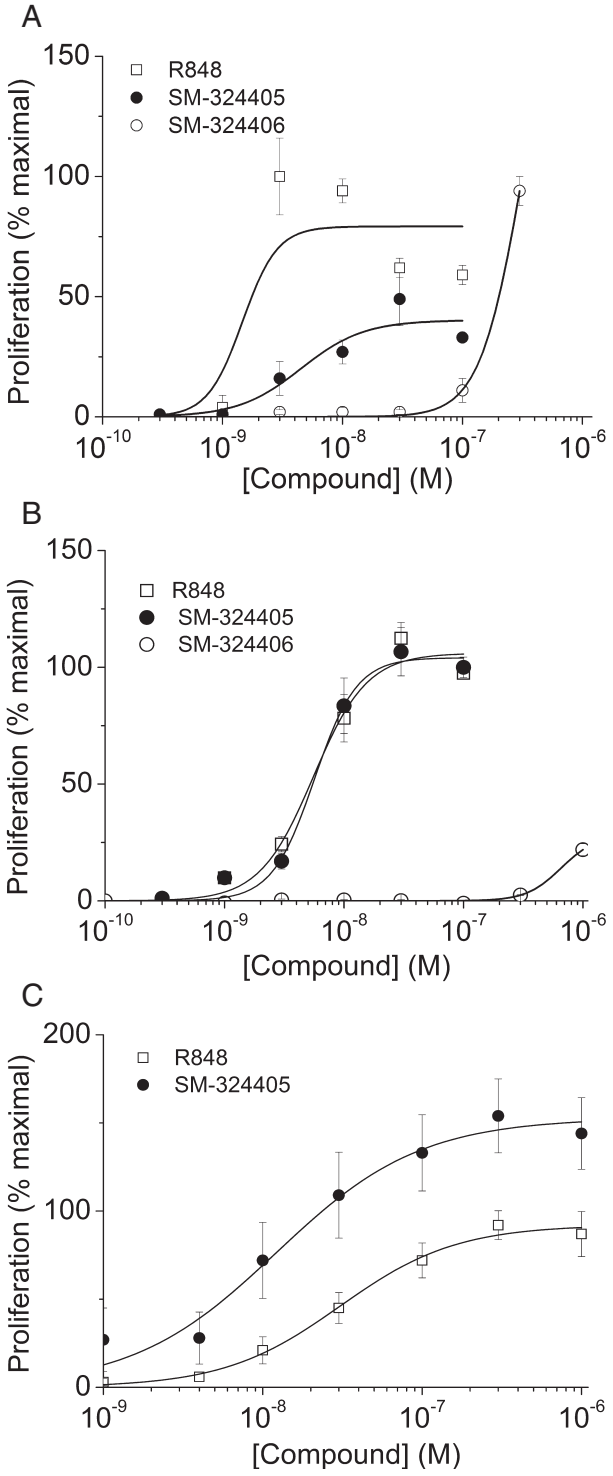


Figure 3

Potency of TLR7 agonist antedrug and its metabolite, as inducer of proliferation in mouse, rat and dog cells. Mouse splenocytes (A), rat splenocytes (B) or dog PBMC (C) were incubated in triplicate with the compounds for 2 d. At this time, cell proliferation was determined by incorporation of [^{3}H]-thymidine. In each individual experiment the maximal induction of proliferation induced by R848 was set at 100% and all values normalized to this value. Data shows mean values ± SEM from three separate determinations for mouse and rat, and four experiments for dog.

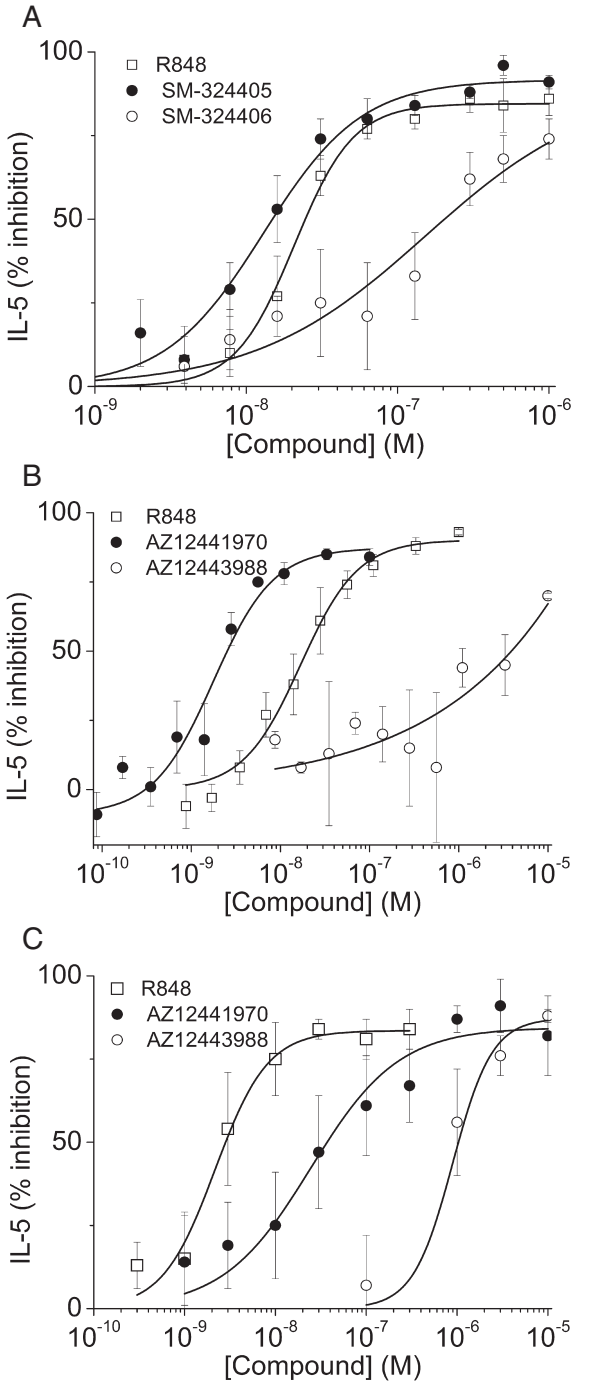


Figure 4

Suppression of Th2 cytokines in human PBMC and mouse splenocytes by TLR7 agonists. Human PBMC (A,B) were incubated with the compounds indicated and stimulated with PHA for 2 d, at which point cell culture supernatants were removed and the IL-5 produced determined by ELISA. In (C), OVA-sensitized mouse splenocytes were incubated with the compounds indicated and OVA (1 mg·mL^{-1}) for 5 d before determination of IL-5 in the cell culture supernatant by ELISA. Levels of IL-5 produced in the presence of compound were calculated as % inhibition based on the level of IL-5 produced with vehicle only in the presence of stimulus (PHA or OVA). Data are presented as mean ± SEM from four individual experiments (human) and three individual experiments (mouse).

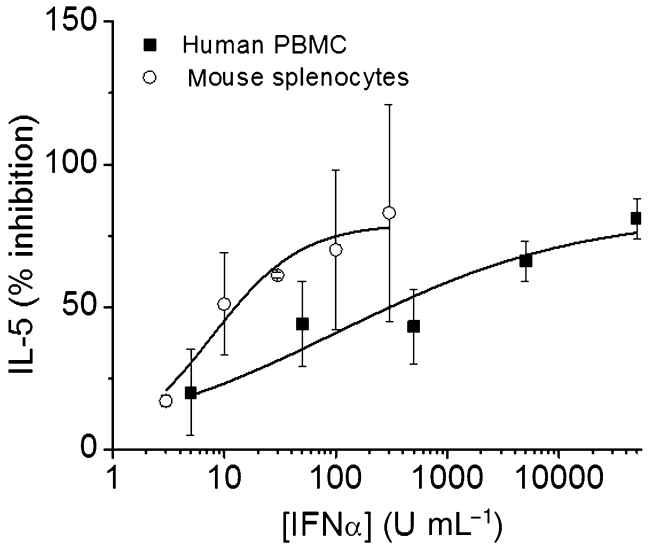


Figure 5

Suppression of IL-5 in human and mouse cells by IFN-α. Human PBMC or mouse splenocytes were incubated with different concentrations of IFN-α in the presence of PHA or OVA respectively. Supernatants were removed after 5 d (mouse) or 2 d (human) and IL-5 determined by ELISA. Data are presented as mean ± SEM from three separate experiments.

short-term exposure of a TLR7 agonist would mediate a biological effect, we incubated PBMCs in the presence of BChE to enable rapid metabolism of the ester and thereby reduce the time of exposure. Incubations with equivalent concentrations of the metabolite AZ12443988 demonstrated the extent to which any acid produced from the cleavage of ester would have biological activity. In the presence of BChE, higher concentrations of AZ12441970 were required for the production of both IFN-α and its downstream marker IL-1RA (Figure 7A and B). The levels of these mediators produced in the presence of BChE at 1 μM AZ12441970 were equivalent to the levels induced in the absence of BChE. None of this activity could be attributed to the acid, which was inactive at concentrations up to 1 μM (Figure 7B). When other endpoints of TLR7 stimulation, such as TNF-α production and proliferation of PBMC, were determined the inclusion of BChE resulted in almost complete suppression of any TLR7-mediated effect, even at the highest dose of AZ12441970 tested. This data shows that short-term exposure results preferentially in induction of IFN-α production compared with TNF-α production and proliferation.

AZ12441970 shows efficacy in vivo *with minimal systemic activity*

AZ12441970 activity *in vivo* was determined by giving it intratracheally to OVA-sensitized C57BL/6 mice that were then challenged with intratracheal antigen (Table 3). Pretreatment with 1 mg·kg^{-1} AZ12441970 by intratracheal administration significantly reduced eosinophilia in the

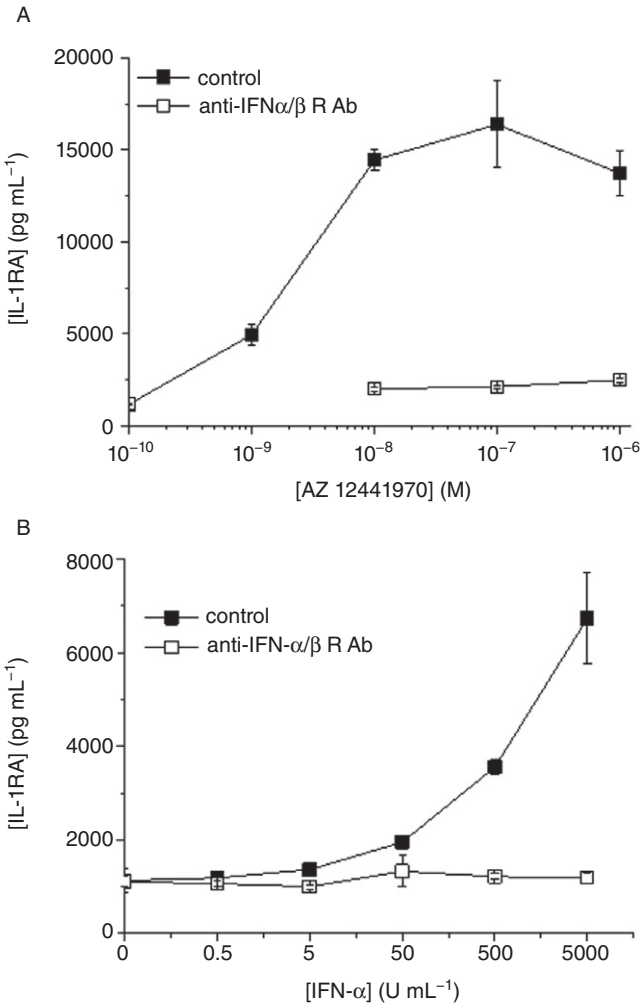


Figure 6

Induction of IL-1RA by AZ12441970 is mediated by IFN-α. Human PBMC were incubated (in triplicate) with AZ12441970 (A) or IFN-α (B) in the presence or absence of 1 μg·mL^{-1} neutralizing IFN-α/β receptor antibody. After 24 h, incubation supernatants were removed and IL-1RA determined by ELISA. Results (means ± SD) are representative of three similar experiments.

bronchoalveolar lavage fluid by 87% and, although not significant, there was also a trend towards a reduction in the production of IL-5. When given intratracheally to naïve C57BL/6 mice, 0.1 mg·kg^{-1} AZ12441970 did not induce any increase in systemic IFN-α (Table 4) and treatment with a higher dose (1 mg·kg^{-1}) of AZ12441970 resulted in only a modest increase in plasma IFN-α levels, when compared with the IFN-α induced by 1 mg·kg^{-1} R848. Plasma IL-1RA, IL-6 and TNF-α were similarly not induced by intratracheal dosing of AZ12441970, whereas all three cytokines were clearly induced by R848. This demonstrated the concept that local administration of AZ12441970 can mediate beneficial effects in a mouse allergy model with minimal systemic activation.

Table 2

Pharmacokinetics of AZ12441970 dosed intranasally in the mouse

Time (min)	% Dose in lung		Concentration in lung (nM)		Concentration in blood (nM)	
	AZ12441970	AZ12443988	AZ12441970	AZ12443988	AZ12441970	AZ12443988
10	10	9	20 900	19 600	56	546
30	5	7	9 800	12 800	27	167
150	1	1	1 500	1 200	3	40

AZ12441970 was given intranasally to yield a 1 mg·kg^{-1} dose to the lungs of mice. At the indicated times animals were killed and blood taken into sodium fluoride to prevent any further hydrolysis of ester. Lungs were excised and placed in vials containing sodium fluoride and immediately frozen at –20°C. AZ12441970 and its acid metabolite, AZ12443988, were quantified by LC/MS/MS.

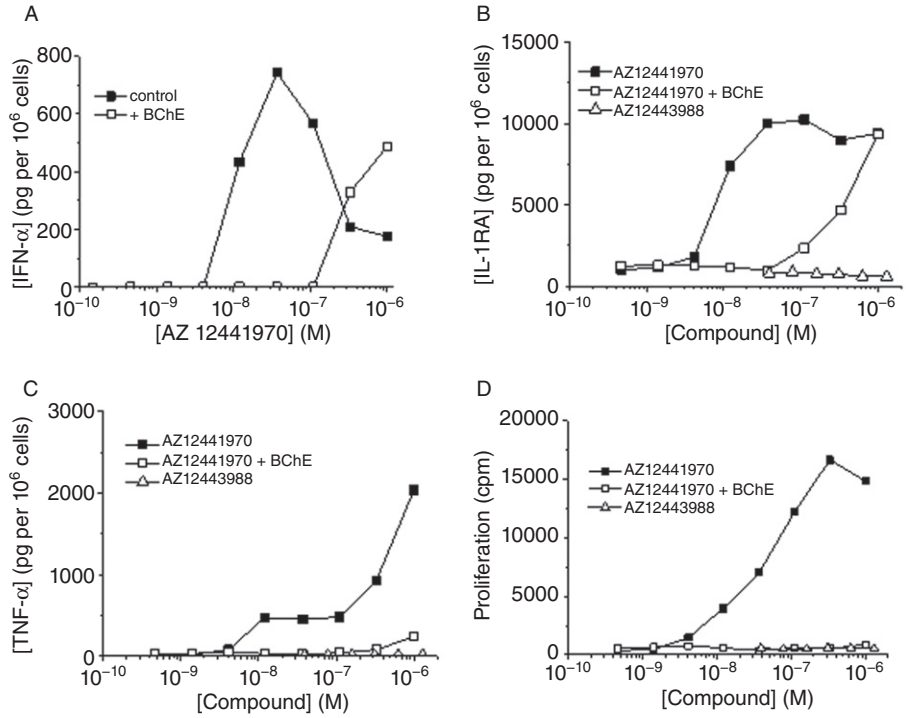


Figure 7

Cellular activation following short-term exposure to AZ12441970. Human PBMC were incubated with this TLR7 agonist antedrug in the presence or absence of 1 U·mL^{-1} BChE, or were incubated with the acid metabolite AZ12443988. After 24 h, incubation supernatants were removed and IFN-α (A), IL-1RA (B) and TNFα (C) determined by ELISA. To determine proliferation (D), PBMC were incubated for 2 d and proliferation determined following addition of [^{3}H]thymidine. Results (mean values) are representative of four similar experiments.

Discussion

TLR7 agonists have immunomodulating properties making them attractive as therapies for the treatment of a variety of indications including oncology, virology and allergic diseases (Camateros *et al.*, 2007; Dudek *et al.*, 2007; Pockros *et al.*, 2007). However, given concerns over the adverse events linked to systemic IFN responses observed for a TLR7 agonist in the treatment of hepatitis C (Fidock *et al.*, 2011), the use of current TLR7 ligands for allergic indications would be limited by side effects brought about by systemic activity of the compounds. We have produced TLR7 agonists with the potential to act locally at the site of administration and, through metabolism by plasma esterases to a less active form, to have reduced systemic activity. These compounds, exemplified by SM-324405 and AZ12441970, have activity comparable with the known TLR7 agonist, R848, and we have demonstrated their activity in a number of species by assays including induction of a range of IFN-regulated genes, cytokines, proliferation and suppression of IL-5. Unlike R848, these

Table 3

Inhibition of eosinophilia and IL-5 in bronchoalveolar lavage fluid by AZ12441970 in a mouse allergic airways OVA challenge model

Conditions		Eosinophils			IL-5		
Challenge	Compound	Mean	SEM	% inhibition	Mean (pg·mL^{-1})	SEM (pg·mL^{-1})	% inhibition
OVA	Vehicle	9135	2576	0	135	43	0
OVA	0.1 mg·kg^{-1} AZ12441970	1829	1132	80 (0.03)	63	15	53 (0.15)
OVA	1 mg·kg^{-1} AZ12441970	1228	588	87 (0.03)	27	16	80 (0.12)
Vehicle	Vehicle	254	71		0		

OVA systemically-sensitized C57BL/6 mice were dosed intratracheally with AZ12441970 24 and 2 h before intratracheal challenge with OVA. Forty-eight hours after antigen challenge eosinophil number and IL-5 levels in bronchoalveolar lavage fluid was determined. Animal group size for each treatment is five, apart from vehicle challenged mice where $n = 3$. Data is presented as mean ± SEM and P values (paired Student's T-test) are shown in brackets.

Table 4

Induction of systemic cytokines following intratracheal administration of R848 or AZ12441970

Treatment	IFN-α (pg·mL^{-1}) ($n = 4$)	IL-1RA (pg·mL^{-1}) ($n = 3$)	IL-6 (pg·mL^{-1}) ($n = 3$)	TNF-α (pg·mL^{-1}) ($n = 3$)
vehicle	7.3 ± 0.2	0 ± 0	0 ± 0	5.8 ± 2.2
0.1 mg·kg^{-1} AZ12441970	7.2 ± 0.2	Not determined	Not determined	Not determined
1 mg·kg^{-1} AZ12441970	77 ± 26	0 ± 0	0 ± 0	7.8 ± 2.1
1 mg·kg^{-1} R848	1625 ± 145	394 ± 197	868 ± 439	390 ± 197

R848 or AZ12441970 was given intratracheally to naïve C57BL/6 mice. Ninety minutes later blood was collected, plasma prepared and cytokines and IFN-α quantified. Animal group size is indicated for each group. Data are presented as mean ± SEM.

compounds were unable to activate TLR8 in a reporter assay coupled to NF-κB.

An important feature of the present compounds is the incorporation of a particular ester moiety into their structure to make them susceptible to cleavage by plasma esterase. The acid metabolite needs to have substantially reduced activity compared with the parent ester to give the required antedrug properties. In a number of assays, including induction of mouse IFN-α and IFN-γ and inhibition of human IL-5, the metabolite, SM-324406, was up to 30-fold less active than the parent SM-324405, thus meeting the criteria for an antedrug. However, in the human TLR7 reporter assay the ester and acid had equivalent activity. The different ratios of ester : acid potency in the various assays may well reflect the endosomal localization of the ligand binding domain of TLR7. In the case of TLR9, which is another endosomally located TLR, it has been demonstrated that TLR9 translocates between different endosomal vesicles (Latz *et al.*, 2004). So far it has not been possible to devise a binding assay for TLR7 agonists, so the cellular potency of the compounds reflects their ligand binding affinity, accessibility to the receptor, and as they are agonists, the receptor occupancy required to elicit a response. If TLR7, like TLR9, can translocate between different endocytic vesicles, which are likely to differ in their intracellular acidity, then none of these variables can be assumed to be the same from cell to cell. Therefore, the variation in ester : acid potency may well reflect differences in cell permeation and local binding conditions.

The human TLR7 reporter cell assay showed the least difference in ester : acid potency for SM-324405 and its acid, so this assay was used to identify compounds with improved ester : acid activity ratios. Following further research, AZ12441970 was identified as having an acid metabolite, AZ12443988, which was inactive in the human TLR7 reporter assay.

TLR7 agonists have been shown to suppress Th2 cytokine production (Wagner *et al.*, 1999; Fili *et al.*, 2006; Vultaggio *et al.*, 2009) and we confirmed that AZ12441970 inhibited IL-5 production in both mouse and human cellular systems. As allergic diseases, such as asthma, have been linked to an overproduction of Th2 cytokines (Holgate, 2008), TLR7 agonists are therefore candidates as potential therapeutic agents for such diseases, because these agonists reduce both IL-5 and IL-13. TLR7 activation of plasmacytoid dendritic cells produces IFN-α (Guiducci *et al.*, 2009) and IFN-α has been shown to block human Th2 development and inhibit cytokine secretion from committed Th2 cells (Huber *et al.*, 2010). Here, in both human and mouse *in vitro* assays, IFN-α was induced by the TLR7 agonist and IFN-α mediated the suppression of IL-5. Therefore, IFN-α is a potential biomarker that is associated with suppression of the Th2-phenotype that could be used to verify activity linked to TLR7 activation. IFN-α is transient in

its production *in vivo* (Reiter *et al.*, 1994) but there are a number of downstream IFN-regulated genes that could also be monitored. IL-1RA was induced by IFN-α in hepatocytes (Wan *et al.*, 2008), and we confirmed in human PBMC that IL-1RA was induced in an IFN-α-dependent manner, and that AZ12441970 induction of IL-1RA was totally inhibited by blocking IFN-α/β signalling. Whereas IL-1RA has been shown to be induced by a TLR7 agonist in human PBMC (Testerman *et al.*, 1995), our data demonstrates the association with IFN-α. Thus IL-1RA has utility as a biomarker of TLR7 activity linked to the inhibition of Th2 cytokines by IFN-α.

Although this new class of compound was designed to be metabolically unstable in plasma, we found that AZ12441970 was also metabolized in mouse lung with only 10% remaining after 10 min. As *in vitro* determinations were carried out in the presence of heat-inactivated FCS that does not metabolize the ester, efforts were made to simulate short-term compound exposure to determine how it affected TLR7 activation. To achieve this BChE was included in the *in vitro* incubations to increase the rate of metabolism of AZ12441970. This resulted in a rightward-shift in the dose–response curves. Reducing the time of exposure of the cells to the parent compound still resulted in a full IFN-α/ IL-1RA response at the higher levels of AZ12441970, though TNF-α production and proliferation was greatly diminished. Assays with the acid metabolite clearly demonstrated that converted acid could not account for any of the effects measured. Therefore, the differing effects on the endpoints measured all resulted from the transient exposure to the ester, when BChE was included. As all outcomes were determined from the same set of cells, these data suggested that signalling through TLR7 in plasmacytoid dendritic cells resulted in efficient coupling to the IFN-α pathway following short-term exposure to compound, compared with proliferation (potentially of B-cells) and TNF-α production (likely to also involve monocytes). Sasai *et al*. (2010) have shown that TLR9 located in a $LAMP2^+$ endosome activated IRF7 leading to the production of type-1 IFNs whereas TLR9 in $VAMP3^+$ endosomes leads to the production of NF-κB-regulated cytokines, such as TNF-α. Our data also point to distinct coupling mechanisms for the production of IFN-α and NF-κB-regulated processes, in our case identified by the length of time the cells are exposed to the TLR7 agonist. This could come about as the result of differential rates of permeation to the receptor in the different cell types, or it may indicate that longer receptor occupancy is required for the induction of NF-κB-regulated genes and proliferation of B-cells compared with activation of the IFN-α pathway. Further studies to elucidate the mechanisms in the different cell populations would be needed to clarify this point, though the lack of a suitable antibody for immunohistochemistry and technical difficulties in producing a fluorescently-tagged TLR7 currently hinder this approach.

In a mouse allergic airways model, AZ12441970 showed efficacy in the suppression of antigen-induced eosinophilia, with a trend to reduction of IL-5 levels in bronchoalveolar lavage. This was achieved at concentrations of AZ12441970 that induced either no, or negligible, levels of systemic cytokines when compared with the plasma-stable compound R848, which induced substantial systemic levels. This data reflects the pharmacokinetics of AZ12441970, which after administration to the lung via intranasal application, resulted in blood levels of AZ12441970 that were 400-fold lower than those in the lung. The level of acid metabolite in the blood was 10-fold higher than that of the ester, so to reduce any systemic activity attributed to the acid metabolite it is necessary to have an activity at least 10-fold less than that of the ester. The clearance of both ester and acid from the lung and blood also demonstrate that neither of these moieties is being accumulated in either of these tissues. Similar efficacy with the TLR7 antedrug SM-324405, dosed intratracheally, in a rat airways allergy model without induction of systemic cytokines has also been observed (Kurimoto *et al.*, 2010). Whereas efficacy by intratracheal dosing has been shown to prove the antedrug concept of these drugs, further studies to refine the method of administration, including nebulized inhalation, are ongoing to determine whether efficacy can be achieved using a more clinically relevant method of administration.

In conclusion, we have produced an 8-oxoadenine antedrug series that forms the basis of a class of compounds that could have utility for dosing locally, for example to the lung, for the treatment of allergic diseases while potentially minimizing systemic side effects. This is made possible by the compounds described having good TLR7 activity and the potential side effects are likely to be reduced by rapid metabolism of the ester in the plasma generating a metabolite with greatly reduced activity. Demonstration that short-term exposure favours the production of IFN-α rather than the induction of proinflammatory cytokines and proliferation would also act to limit unwanted side effects while still delivering efficacy through the generation of IFN-α.

Acknowledgements

Many thanks to Dr A. Berry for support in delivering the pharmacokinetic studies.

The work described in this paper was supported through a collaboration between AstraZeneca and Dainippon Sumitomo Pharma. The authors acknowledge Dr Ash Bahl for his vision in initiating the collaboration and support in carrying out this work.

Conflicts of interest

The authors state no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Comparative inhibition of IL-5 and IL-13 by TLR7 agonists. Dose response curves for 10 TLR7 agonists were determined in human PBMC stimulated with PHA. After 2 d

incubation, the cell culture supernatants were removed and the IL-5 produced determined by ELISA and IL-13 determined by Luminex. pIC50 values were determined for each compound and plotted below.

Figure S2 Inhibition of BChE by ethephon. AZ12441970, at an initial concentration of 1 μM, was added to human plasma at 37°C in a total volume of 0.5 mL with the indicated concentrations of ethephon. Incubations were for 15 s at 37°C at which point the reaction was stopped with acetonitrile. Supernatants were quantified by LC/MS/MS for acid (AZ12443988) production and the amount generated was normalized to that generated in the absence of ethephon (set to 100%).