



Discovery of 6-Amino-2-[(1*S*)-1-methylbutyl]oxy}-9-[5-(1-piperidinyl)pentyl]-7,9-dihydro-8*H*-purin-8-one (GSK2245035), a Highly Potent and Selective Intranasal Toll-Like Receptor 7 Agonist for the Treatment of Asthma

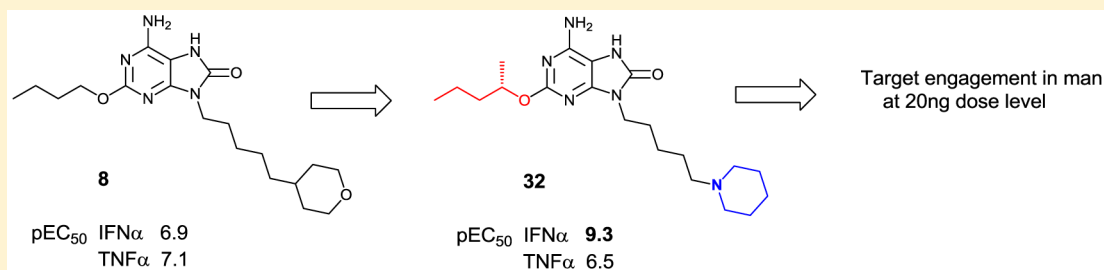
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S Supporting Information



ABSTRACT: Induction of IFNα in the upper airways via activation of TLR7 represents a novel immunomodulatory approach to the treatment of allergic asthma. Exploration of 8-oxoadenine derivatives bearing saturated oxygen or nitrogen heterocycles in the *N*-9 substituent has revealed a remarkable selective enhancement in IFNα inducing potency in the nitrogen series. Further potency enhancement was achieved with the novel (*S*)-pentylxy substitution at C-2 leading to the selection of GSK2245035 (32) as an intranasal development candidate. In human cell cultures, compound 32 resulted in suppression of Th2 cytokine responses to allergens, while *in vivo* intranasal administration at very low doses led to local upregulation of TLR7-mediated cytokines (IP-10). Target engagement was confirmed in humans following single intranasal doses of 32 of ≥20 ng, and reproducible pharmacological response was demonstrated following repeat intranasal dosing at weekly intervals.

■ INTRODUCTION

Allergic rhinitis (AR) and asthma are two closely related and very common chronic diseases of the airways. Their incidence continues to increase with 10–30% of the world's population suffering from one or both conditions. Asthma is by far the more serious of the two diseases and is responsible for approximately 250,000 deaths annually worldwide.¹ The current standard of care for allergic asthma consists of inhaled corticosteroids, to tackle the underlying inflammation, along with short and long acting inhaled β-2 agonist bronchodilators to relieve constriction of the airways. While such treatment delivers reliable control of asthma symptoms and exacerbations for the majority of patients, it requires continuous use of medication while the underlying immune pathology remains unaltered.

Allergic asthma is a complex disease which is characterized by an aberrant type-2 T-helper cell (Th2) immune response to

otherwise innocuous environmental proteins (allergens). The resulting overproduction of Th2 cytokines (e.g., IL-4, IL-5, IL-9, and IL-13) drives the differentiation, survival, and functions of the major effector cells, mast cells, basophils, and eosinophils that are responsible for the manifestation of symptoms.² Furthermore, Th2 cells play an essential role in the generation of allergen-specific IgE by B cells, which mediates the type 1 hypersensitivity reactions, and in the orchestration of local chronic allergic inflammation. Restoration of a more balanced Th2/Th1 and regulatory T cell response toward respiratory allergens could prevent the development of allergic symptoms and potentially lead to long-term modification of disease pathogenesis. Currently, allergen-specific immunotherapy (SIT) is the only treatment that has been shown to modify

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this aberrant immune response to allergens, resulting in allergen “desensitization” but usually requires long-term administration (3–5 years) of the allergen(s) to which the patient is sensitive and is accompanied by significant safety drawbacks.³ Consequently, alternative approaches aiming to modulate the immune milieu of the airways have begun to be explored. To this end, scientific interest has recently been directed toward the manipulation of Toll-like receptor (TLR) function.⁴

Human TLRs comprise a family of 10 transmembrane glycoproteins that are widely expressed on immune and epithelial cells and act as “danger sensors” by recognizing conserved microbial components known as pathogen-associated molecular patterns (PAMPs). Activation of TLRs results in mobilization of both innate and adaptive immune mechanisms that eliminate “danger” and preserve immune homeostasis.⁵ Because of their critical role in immune regulation, TLRs have been considered attractive targets for therapeutic intervention in a wide variety of disease settings.⁶ In the context of allergic respiratory diseases, local stimulation of TLRs in the airways resulting in the production of Th1 directing cytokines, in particular type 1 interferons (IFNs), has the potential to modulate the aberrant immune response to allergens. A deficiency in this regulatory mechanism due to insufficient microbial exposure in childhood is thought to be a key driver in the increasing incidence of allergy, the so-called “Hygiene Hypothesis”.⁷ The value of TLR activation in the treatment of allergic diseases is now well recognized and is clearly illustrated by the enhanced efficacy seen when TLR4 or TLR9 agonists are included as adjuvants in SIT.^{8,9}

Stand-alone application of TLR agonists to the upper airways, the main site of exposure to allergens, is a novel concept for the treatment of respiratory allergies that has only recently begun to be evaluated in clinical trials.^{10,11} The “United Airways Disease” hypothesis leads us to believe that triggering an immunomodulatory response in the nose will translate to benefit in both allergic rhinitis and asthma.¹² Compared to SIT, this approach has the advantage of theoretically providing protection from all aeroallergens to which a patient is naturally exposed during treatment, thereby eliminating the need for adjustment of treatment to each patient’s sensitization. Administration of TLR agonists to the airways has the potential to induce immune changes that will restrict the existing allergen-specific Th2 responsiveness and rebalance it toward a prophylactic Th1/Treg phenotype.¹³ Consequently, in addition to providing short-term symptom control, this approach has the potential to deliver long-term disease remission via allergen dependent T-cell and B-cell memory responses. Evidence for short-term reduction in allergic reactivity using this approach has been obtained through early clinical evaluation of the intranasal TLR8 agonist, 2-amino-*N,N*-dipropyl-8-[4-(1-pyrrolidinylcarbonyl)phenyl]-3*H*-1-benzazepine-4-carboxamide (VTX-1463) in allergic rhinitis¹¹ and with the intranasal plasma labile TLR7 agonist “antidrug” methyl [3-({[3-(6-amino-2-butoxy-8-oxo-7,8-dihydro-9*H*-purin-9-yl)propyl][3-(4-morpholinyl)propyl]amino}-methyl)phenyl]acetate (AZD8848)^{10a} in both allergic rhinitis^{10b,c} and asthma.^{10d} However, duration of effect of longer than 1 week has yet to be demonstrated following a course of intranasal TLR treatment (up to 8 weeks).

Among the TLRs, TLR7 is of particular importance in respiratory allergic diseases. TLR7 polymorphism has been strongly linked to atopy and asthma,¹⁴ while reduced function of TLR7 has been demonstrated in asthmatic adolescents.¹⁵

Furthermore, studies in rodent models of allergic airway disease have shown that local delivery of small molecule TLR7 agonists results in inhibition of both acute inflammation and chronic remodelling and in direct relaxation of airway smooth muscle and reduction of airway hyperreactivity.¹⁶ More importantly, comparison of the action of multiple classes of TLR ligands in an experimental asthma model concluded that TLR7 agonists have, in addition to the strongest antiallergic effect, the lowest adverse pro-inflammatory potential.¹⁷ The powerful antiviral potential of TLR7 agonists may also offer extra benefit in asthma patients through the better control of virus-driven asthma exacerbations.¹⁸ Finally, from a drug discovery perspective, TLR7 has the distinct advantage of being amenable to activation by small molecule ligands.¹⁹

TLR7 recognizes naturally single stranded viral RNA and, along with the other TLRs which recognize microbial nucleic acids (TLR3, 8 and 9), is activated within the endosomal compartment of the cell. Upon ligand binding, TLR7 dimerizes resulting in a signaling cascade via the adapter protein MyD88 culminating in the production of IFN α via an IRF-7 mediated pathway and pro-inflammatory cytokines including TNF α via an NF κ -B mediated pathway (Figure 1). TLR7 is expressed

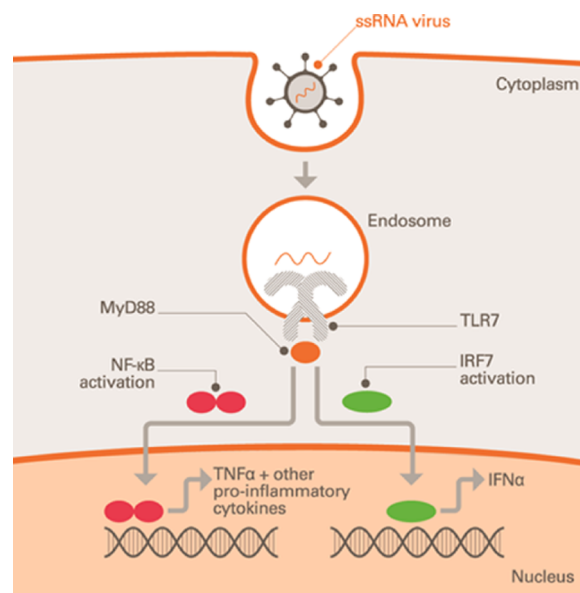


Figure 1. TLR7 signaling pathways.

primarily on plasmacytoid dendritic cells (pDC) which on activation produce large amounts of type-1 interferons, and it is engagement of this cell type in the nasal mucosa that is believed will drive the desired immunomodulatory response. Our objective therefore was to identify a small molecule agonist of TLR7, suitable for intranasal delivery, to explore this hypothesis preclinically and, if successful, in human subjects.

■ SMALL MOLECULE TLR7 AGONISTS

Since the discovery in 1957 of type-1 interferon and its potential antiviral and antitumor benefits,²⁰ a number of small molecule inducers of this key cytokine have been discovered. Many of these have subsequently been shown to be agonists of TLR7 and/or TLR8. Unsurprisingly, given that the natural ligand for TLR7/8 is single stranded RNA, many of these small molecule agonists resemble nucleosides or nucleoside bases (Figure 2). Simple pyrimidine based IFN inducers, typified by

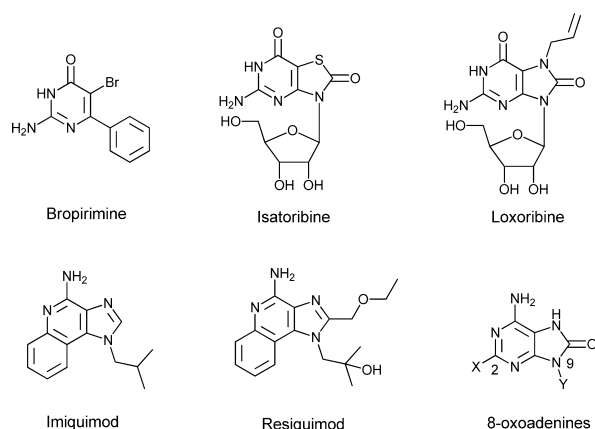


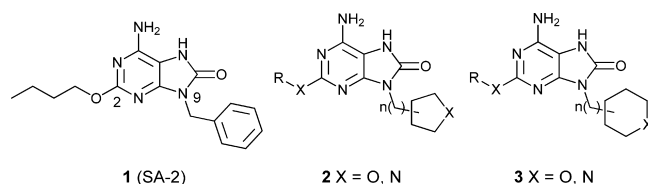
Figure 2. Representative small molecule TLR7 agonists.

bropirimine, were discovered by Upjohn in the 1970s,²¹ while guanine nucleoside analogues, including isatoribine and loxoribine, were discovered in the 1980s and shown to exert their effects via TLR7 in 2003.²² The imidazoquinoline analogues, also discovered in the 1980s, provided the first small molecule IFN inducer to be introduced into clinical practice with imiquimod launched in 1997 as a topical treatment (Aldara) for a variety of skin diseases including genital warts, actinic keratosis, and basal cell carcinoma.²³ Resiquimod is a more potent imidazoquinoline with dual TLR7/8 agonist activity which has progressed to clinical evaluation for a variety of indications but which has yet to deliver an approved medicine.²⁴ In 2002, Sumitomo discovered the 8-oxoadenine class of interferon inducers,²⁵ and this series was confirmed to act via TLR7 in 2006.²⁶ We became interested in TLR7 as an immunomodulatory mechanism in 2007 and conducted our own high throughput screen for IFN inducers but failed to identify any novel leads. Faced with the known scaffolds, we chose to investigate 8-oxoadenines as this series offers versatility by allowing activity and physicochemical properties to be manipulated by modification of both the C-2 and N-9 substituents. Subsequently, a variety of novel TLR7 agonist scaffolds retaining the key pharmacophore of 8-oxoadenines have been discovered and evaluated in the search for oral treatments for hepatitis B and C.²⁷ These include deazaadenine derivatives from Pfizer²⁸ and 6,6-pteridinone analogues from Gilead, an example of which (36, GS-9620) is currently in the clinic for HBV.²⁹

MEDICINAL CHEMISTRY STRATEGY

Published SAR on the 8-oxoadenine series had demonstrated considerable scope for modification of the C-2 substituent on the 8-oxoadenine template with *n*-butyloxy identified as a preferred substituent.³⁰ There appeared to be less scope for modification of the N-9 substituent with benzyl being established as a particularly favored potency enhancing group. Compound 1 (SA-2), combining these two activity enhancing features, shows activity following intraperitoneal dosing in a mouse model of Th2 mediated airway inflammation³¹ and, as such, provided a useful benchmark for our novel analogues. Exploration of the N-9 substituent had centered primarily on substituted benzyl and pyridylmethyl analogues,³² and there were no examples of saturated heterocycles having been incorporated at this position.³³ We therefore embarked on a program of work to explore both 5- and 6-membered saturated oxygen and nitrogen heterocycles at N-9 linked via methylene

chains of varying length as represented by the generic structures 2 and 3.



Whereas the reported modifications of the 8-oxoadenine series had been directed toward the discovery of orally active TLR7 agonists, we were looking to identify compounds that would selectively engage TLR7 locally in the nose after intranasal delivery with minimal systemic exposure. Consequently, our target compound profile was very different. We were aiming to identify a potent TLR7 agonist with high clearance and low oral bioavailability to minimize the risk of unwanted systemic cytokine induction from drug entering the circulation via nasal absorption or via gut absorption of any swallowed portion of the intranasal dose. This approach would also serve to minimize any risk of autoimmunity that might arise from systemic TLR7 activation.³⁴ In addition, to minimize the risk of a local inflammatory response, we ideally wanted the compound to be selective for induction of IFN α over TNF α , a pro-inflammatory cytokine implicated in many aspects of airway pathology in asthma,³⁵ although it was not clear at the outset how this selectivity might be achieved. Finally, the compound, or a suitable salt, needed to be sufficiently soluble and stable in water to allow delivery as an intranasal solution formulation.

IN-VITRO PROFILING

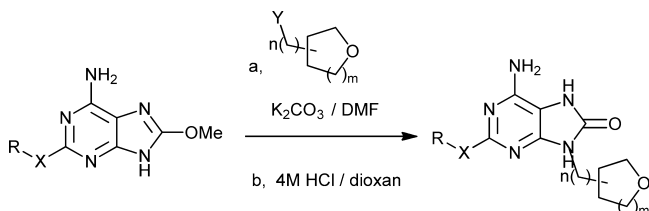
A variety of screening strategies have been employed in the search for novel IFN α inducing small molecules. Historically, these involved directly monitoring IFN α induction or the resulting antiviral activity after dosing compounds *in vivo*, usually in mice.²¹ Subsequently, measuring IFN α induction *in vitro* following incubation of compounds with mouse splenocytes has been employed, for example, in the early optimization of the 8-oxoadenine series.²⁵ More recently, and specifically for TLR7/8 agonists, reporter assays have been employed giving readouts of activation of the NF κ -B arm of TLR7/8 signaling, most commonly in a HEK293 cell line.³⁶ Although reproducible and amenable to high throughput, we chose not to employ such reporter assays as they provide a readout only of activation of the undesired pathway leading to TNF α induction. We chose instead to evaluate compounds using a human peripheral blood mononuclear cell (PBMC) assay to provide a direct readout of IFN α induction in a more relevant cell population including the primary target plasmacytoid dendritic cells. Incubation of test compounds with cryopreserved human PBMC (method A) for 24 h followed by MSD analysis for IFN α gave dose–response curves from which pEC₅₀ values were derived. This assay format was used to guide SAR development during the early phase of the program, and subsequently, to gain insight into selectivity of TLR7 activation, the assay was adapted using freshly isolated PBMC (method B) to give readouts of both IFN α and TNF α induction.

OXYGEN HETEROCYCLES

Analogues first investigated contained saturated oxygen heterocycles in the N-9 substituent and explored the impact of chain

length and position of attachment to the oxygen heterocycle in the 2-*n*-butyloxy series. Compounds were prepared using the methodology shown in Scheme 1 and were profiled using cryopreserved human PBMC to give pEC₅₀ values for IFN α induction following 24 h of incubation.

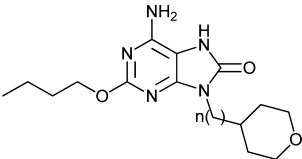
Scheme 1. Synthetic Route to Oxygen Heterocycles^a



^aX = O, NH; Y = Br or methanesulfonate ester leaving group.

This initial data set revealed remarkable tolerance of a variety of saturated oxygen heterocyclic substituents at the N-9 position (Tables 1 and 2). Encouraging activity was seen with

Table 1. 4'-THP Chain Length SAR^a



Compound	<i>n</i>	IFN α pEC ₅₀
4	1	6.7 ^b
5	2	6.7
6	3	7.2
7	4	7.0
8	5	7.3
9	6	6.1

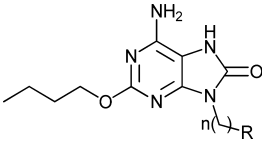
^aPBMC assay method A; pEC₅₀ values (negative log of the molar EC₅₀) are geometric means of at least two independent measurements unless otherwise indicated. ^b*n* = 1.

4'-linked tetrahydropyran (THP) derivatives linked through chains of 1 to 6 carbon atoms with activity superior to the benchmark 9-benzyl derivative **1** (pEC₅₀ 6.9) seen with the *n*-propyl, *n*-butyl, and *n*-pentyl-linked derivatives (**6**, **7**, and **8**) (Table 1).

Examination of alternatively linked THP and tetrahydrofuran (THF) analogues indicated that the ring size and position of attachment also had little impact on activity (Table 2). Thus, the 3-linked THP **10** showed comparable activity to the 4-linked counterpart **7**, whereas the 2-linked isomer **11** was slightly less active. Comparable activity was seen with THF analogues with the 3-linked isomer **12** again appearing more active than the 2-linked analogue **13**. Also, where investigated, chirality at the point of attachment to the heterocycle appeared to have little effect on activity as illustrated by the THF enantiomers **14** and **15**.

Encouraged by these initial results we explored the SAR of THF and THP analogues more extensively using an array approach which additionally incorporated investigation of the impact of the C-2 substituent (Figure 3). Five different C-2 groups were investigated; R² = *n*-butyloxy and *n*-butylamino as standards and three novel C-2 substituents; R² = cyclopropylethoxy, (*R*)-2-pentyloxy and (*S*)-2-pentyloxy (Figure 3).

Table 2. THP/THF Isomer SAR^a



Compound	R	<i>n</i>	IFN α pEC ₅₀ ^a
7		4	7.0
10		4	7.4
11		4	6.5
12		4	7.2
13		4	6.8
14		2	6.9
15		2	7.0

^aPBMC assay method A; pEC₅₀ values are geometric means of at least two independent measurements unless otherwise indicated.

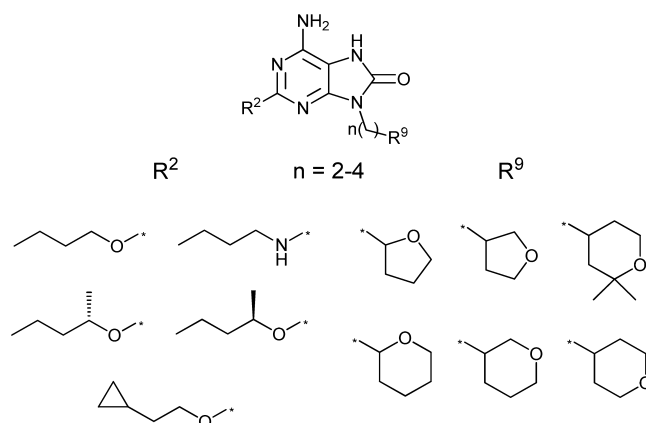


Figure 3. Composition of the N-9 oxygen heterocycle array.

The latter pair of isomers was chosen based on earlier encouraging data with this C-2 substituent in racemic form.

Data from this array of compounds (Figure 4) reinforced the earlier observations that good IFN α potency could be achieved with a wide variety of THF or THP containing N-9 substituents. Furthermore, some interesting trends were observed regarding SAR of the 2-substituent. Consistent with data in the literature, *n*-butyloxy was found to be superior to *n*-butylamino, while the cyclopropylethoxy substituent provided potency similar to that of *n*-butyloxy. More interesting was the clear distinction between the novel (*R*) and (*S*)-pentyloxy analogues with the latter isomer being clearly the more potent and also more potent than the standard *n*-butyloxy substitution at this center. This learning was taken forward into the N-9

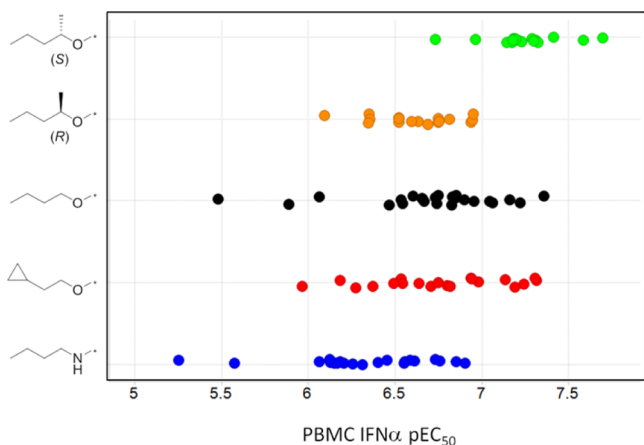
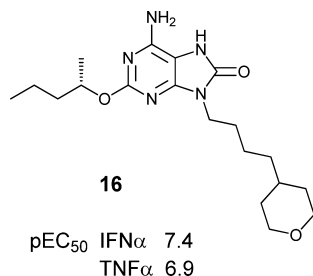


Figure 4. Spotfire plot of oxygen heterocycle array. PBMC assay method A; pEC₅₀ values are the geometric means of at least two independent measurements unless otherwise indicated.

amino heterocyclic series where further exploration of the C-2 substituent was undertaken.

Examination of the cytokine induction profiles of the more potent examples from the saturated oxygen heterocyclic series revealed only modest selectivity for induction of IFN α over the unwanted pro-inflammatory cytokine TNF α . For example, the 2-(S)-pentyloxy 4'-linked THP analogue **16** showed pEC₅₀ values for IFN α and TNF α induction in human PBMC of 7.4 and 6.9, respectively.



NITROGEN HETEROCYCLES

Concerns that incorporation of a basic amine into the N-9 substituent might restrict permeability and prevent access to TLR7 located in the endosome were quickly allayed when a variety of nitrogen heterocycles were found to show enhanced IFN α potency compared to that of their oxygen counterparts in cellular assays. Surprisingly, this enhancement in IFN α potency was not mirrored by an enhanced TNF α response, and consequently, the amine derivatives displayed much improved IFN α /TNF α selectivity (Table 3). As with the oxygen heterocycles, there was found to be a wide tolerance for a variety of nitrogen heterocycles at this position including N- and C-linked piperidines (**17**–**20**) and N-linked piperazines (**21**). Pyrrolidines were also active and selective, although the N-linked derivative **22** was somewhat less potent than its 6-membered counterpart **17**, while expansion to the 7-membered azepane **23** resulted in a slight enhancement in activity. However, the morpholino derivative **24** was markedly less potent and provided the first indication that the basic amine was key to the enhanced IFN α potency in this series.

The N-linked piperidine series was selected for more detailed examination beginning with a study of the effect of the length of the linking chain. In contrast to the oxygen heterocyclic series,

Table 3. Nitrogen Heterocycle SAR^a

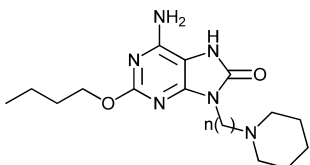
Compound	R	IFN α pEC ₅₀		TNF α pEC ₅₀	
17		8.2		5.2	
18		8.6		6.0	
19		9.2		6.2	
20		9.1		5.2	
21		8.6		5.2	
22		7.7		4.8	
23		8.8		5.2	
24		6.7		5.4	

^aPBMC assay method B; pEC₅₀ values are geometric means of at least two independent measurements unless otherwise indicated.

there was a significant reduction in IFN α potency with linking chains of less than 4 carbon atoms, but high levels of potency and selectivity were seen with chains of 4–6 carbon atoms (Table 4).

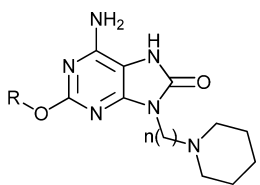
Final optimization of the C-2 substituent was undertaken in the N-linked piperidine series linked by either the *n*-butyl or *n*-pentyl chain (Table 5). The enhancement in potency seen earlier in the oxygen series with the 2-(S)-pentyloxy substituent was again observed with examples **29** and **32** being approximately 10-fold more potent than their *n*-butyloxy counterparts **17** and **27**, respectively. The longer 2-(S)-hexyloxy derivative **30** offered no advantage while shortening to the 2-(S)-butyloxy derivative **33** resulted in a slight drop in potency. Further truncation to give the isopropoxy derivative **34** was accompanied by a more marked reduction in IFN α potency

Table 4. N-Linked Piperidine Chain Length SAR



Compound	<i>n</i>	IFN α pEC ₅₀	TNF α pEC ₅₀ ^b
25	2	5.6 ^a	NT
26	3	6.5 ^a	NT
17	4	8.2 ^b	5.2
27	5	8.1 ^b	5.3
28	6	8.8 ^b	5.7

^aPBMC assay method A. ^bPBMC assay method B; pEC₅₀ values are geometric means of at least two independent measurements unless otherwise indicated. NT = not tested.

Table 5. C-2 Substituent SAR in N-Linked Piperidine Series^a


Compound	R	<i>n</i>	IFN α pEC ₅₀	TNF α pEC ₅₀
17		4	8.2	5.2
29		4	9.1	5.6
30		4	9.2	5.6
31		4	6.0	4.9
27		5	8.1	5.3
32 (GSK2245035)		5	9.3	6.5
33		5	9.0	5.0
34		5	7.6	4.9

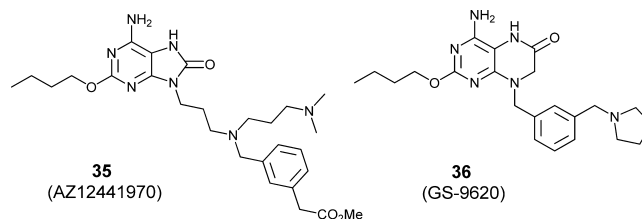
^aPBMC assay method B; pEC₅₀ values are geometric means of at least two independent measurements unless otherwise indicated.

while cyclization to the cyclopentyloxy analogue **31** resulted in a dramatic loss in activity of ca. 1000-fold.

Compound **32** (GSK2245035)³⁷ was selected from this series as a development candidate as it combines excellent potency and selectivity with the desired pharmacokinetic and pharmaceutical developability properties (vide infra).

N-9 MODIFICATION: STRUCTURAL CONSIDERATIONS

The remarkable tolerance of a wide variety of substitutions at the *N*-9 position of the 8-oxoadenine template has also been observed by others, most notably by the Sumitomo/AstraZeneca group who have used this position to incorporate elaborate plasma labile ester “ante-drug” functionality. For example, compound **35** (AZ12441970) is characterized by a very bulky *N*-9 substituent incorporating not only a plasma labile ester but also basic amine functionality.³⁸ This tolerance is further illustrated by the retention of TLR7 agonist activity with 8-oxoadenines containing a fluorophore linked at *N*-9.³⁹ Similar tolerance has been demonstrated at the corresponding position on the imidazoquinoline scaffold.³⁹ Activation of TLR7 (and 8) by a variety of small molecule ligands is remarkable given that the natural ligand for these key components of our immune defense is the complex macromolecule ssRNA. A crystal structure of TLR7 has yet to be reported, but the first TLR8 ectodomain crystal structure revealed a site in which imidazoquinoline agonists, including resiquimod, bind and cause a conformational reorganization of the dimeric structure bringing the C-termini into closer proximity.⁴⁰ This crystal structure has been used to derive TLR7 homology models⁴¹ which have been used to dock other small molecule ligands. For example, the Gilead group have shown that the pteridinone derivative **36** is able to bind into the same ligand binding site and that this model could accommodate the bulky amine containing substituent of this compound.²⁹



Very recently, a second TLR8 crystal structure from the Tokyo University group has revealed a surprising aspect to activation of TLR8 by ssRNA.⁴² This new X-ray structure suggests that activation of TLR8 by the natural ligand does not involve the binding of intact ssRNA but instead involves the binding of two degradation products thereof. Uridine is shown to bind at the same site at the imidazoquinoline ligands while a short oligonucleotide binds to a second site on the receptor. Occupation of both sites is required for activation of TLR8 by ssRNA. It remains to be seen whether TLR7 activation also relies on the binding of fragments of the natural ligand at more than one binding site, and therefore, TLR7 homology modeling based on the TLR8 crystal structure must be questionable.

N-9 MODIFICATION: ENHANCED IFN α POTENCY/SELECTIVITY

Enhancement of TLR7 agonist activity, and aqueous solubility, of 8-oxoadenines by incorporation of a basic amine into the *N*-9 substituent has been reported by the Sumitomo/AZ group⁴³ and also by the Pfizer group on a closely related 3-deaza oxoadenine template.⁴⁴ However, both of these groups assessed TLR7 activity using an NF κ -B reporter assay and do not report on the IFN α /TNF α selectivity of their analogues. In contrast, Gilead have employed a screening strategy similar to our own

one, measuring both IFN α and TNF α induction in human PBMC in discovering the *N*-9 amino containing oral HBV asset 36, on the related 6,6-pteridinone template.²⁹ In this series, IFN α /TNF α selectivity up to 100-fold was reported (based on minimum effective concentration (MEC) values), but selectivity was much less consistent than in our 8-oxoadenine series. They ascribe TNF α induction to TLR8 activity and show, with compound 36, a reasonable correlation of IFN α and TNF α MEC values with TLR7 and TLR8 reporter assay EC values, respectively.

Workers at Novartis used fluorescently tagged 8-oxoadenine and imidazoquinolines to investigate intracellular localization in human pDC and showed these compounds to localize in endosomes.³⁹ The Pfizer group postulate that the enhancement in IFN α potency with their basic analogues may be the result of concentration in the acidic endosomal compartment where TLR7 activation occurs, and we also believe this to be the case, but this alone would not explain the selective enhancement of IFN α induction over TNF α induction if the latter is also driven by endosomal TLR7 activation. However, recent studies have shown that in murine bone marrow derived pDC incubated with vaccinia or flu virus the TLR7/IRF7 pathway driving IFN α induction and the TLR7/NF- κ B pathway leading to TNF α induction occur in *different* endosomal compartments. Importantly, activation to give IFN α occurs in the lysosome related organelle (LRO), a process reported to be dependent on the trafficking of TLR7 by adaptor protein 3 (AP-3) (Figure 5).⁴⁵ The highly acidic nature of the LRO (pH 4.5–5.5)⁴⁶

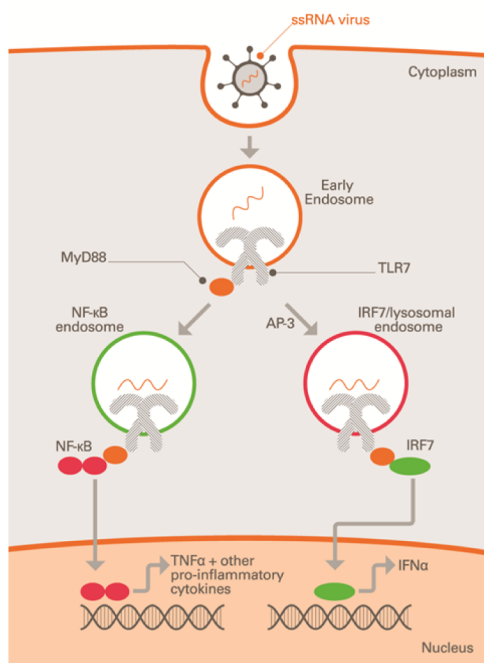


Figure 5. Compartmentalization of TLR7 signaling.

could result in selective concentration of the basic amine derivatives in this compartment and therefore account for their enhanced IFN α /TNF α selectivity. Supporting this hypothesis, we have shown selective loss of TLR7 activated IFN α but not NF- κ B driven IL-12 production by splenic pDC from Pearl mice which have an AP-3b1 mutation resulting in loss of function of AP-3 complex⁴⁷ (data not shown).

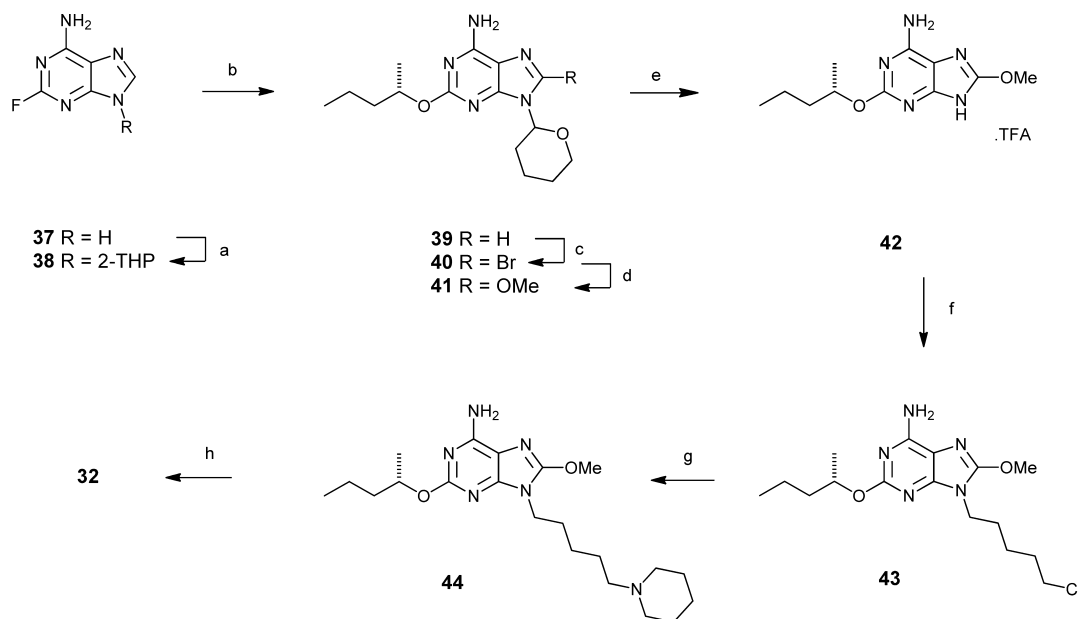
This important endosomal compartmentalization of the receptor is absent from TLR7 reporter assays which also usually provide a readout only of signaling via the NF- κ B pathway. The extraordinary potency and selectivity of the amino derivatives would therefore have been missed if the TLR7 reporter had been used as a primary assay, as illustrated by the weak activity seen with compound 32 in a TLR7 reporter assay in U20S cells (pEC_{50} = 5.7) compared to the very high IFN α potency seen in human PBMC (pEC_{50} = 9.3). Flow cytometry was used to identify the key cell types activated by 32 in human whole blood cultures. These studies showed IFN α to be produced exclusively by pDC while TNF α originated in pDC, myeloid DC (mDC), and in particular monocytes. As monocytes and mDC predominantly express TLR8 and not TLR7,⁴⁸ production of TNF α at higher drug concentrations is likely to be due to weak TLR8 agonist activity as demonstrated with compound 32 (*vide infra*).

■ SYNTHESIS

The synthetic route to compound 32 starting from 2-fluoroadenine 37 is outlined in Scheme 2. Initial protection of the *N*-9 position of 37 as the THP derivative 38 allowed the 2-(*S*)-pentyloxy side chain to be introduced by fluoro displacement with 2-(*S*)-pentanol in the presence of sodium *tert*-butoxide to give 39 in 95% yield. Bromination of 39 with *N*-bromosuccinimide afforded the 8-bromo intermediate 40 in 99% yield. The masked 8-oxo functionality was then introduced by treatment of 40 with sodium methoxide in methanol to give, after removal of the THP protection with TFA, the 8-methoxy core 42 in 79% yield for the two stages. Alkylation of 42 with 1-bromo-5-chloropentane occurred predominantly at *N*-9, and a small amount of *N*-7 alkylated material was observed, which was removed by chromatography, to give the chloropentyl intermediate 43 in 70% yield. Displacement of the chloro functionality in 43 with piperidine yielded the 8-methoxy precursor 44 (52%), which was finally deprotected under acidic conditions to give the clinical candidate 32 as the crystalline free base (73%). For safety assessment studies, and for clinical use, compound 32 was converted *in situ* into the more soluble and more stable maleate salt. Given the extraordinary potency of this molecule (1 g $\equiv$ 500 million 20 ng doses), unusually small amounts of drug substances were required for safety assessment studies and only tiny amounts for the provision of clinical supplies. This route was successfully scaled up to provide hundreds of grams of 32 with very strict containment controls being applied to ensure safe handling of this highly potent immunostimulatory agent.

■ PRECLINICAL PROFILING: IMMUNOLOGY

Cytokine Responses in Human PBMC and Whole Blood. More detailed cytokine profiling of compound 32 was conducted in human whole blood. Nanomolar IFN α potency was retained in human whole blood, and IFN α /TNF α selectivity was found to be even greater than that in PBMC (Table 6). Similar selectivity was seen over IL-1 β induction giving a profile expected to minimize the potential for pyrogenicity and inflammatory events associated with these pro-inflammatory cytokines. In addition compound 32 was shown to induce IL-12p70, IL-10, and IFN γ , cytokines known to play a role in the reduction of Th2 responses, enhancement of Th1 responses, and, in the case of IL-10, increase T regulatory responses.

Scheme 2. Synthesis of Compound 32^a

^aReagents and conditions: (a) *N,O*-bis(trimethylsilyl)acetamide, CH₃CN, reflux then tetrahydropyran-2-yl acetate, TMS-triflate, CH₃CN 0–10 °C, 49%; (b) 2(*S*)-pentanol, NaOBu^t 50 °C, 95%; (c) NBS, CHCl₃, <5 °C, 99%; (d) NaOMe, MeOH, reflux, 97%; (e) TFA, MeOH, 20 °C, 81%; (f) K₂CO₃, DMF, 50 °C then 1-bromo-5-chloropentane, 20 °C, 70%; (g) piperidine, NEt₃, DMF, 70 °C, 52%; (h) HCl, dioxan, MeOH, 20 °C then NaHCO₃, 73%.

Table 6. Human *in Vitro* Cytokine Profile (20 h of Incubation) for Compound 32

media	cytokine	pEC ₅₀	n
PBMC	IFN α	9.35	24
PBMC	TNF α	6.5	19
whole blood	IFN α	9.05	14
whole blood	TNF α	5.0	12
whole blood	IL-1 β	5.3	9
whole blood	IFN γ	5.9	13
whole blood	IL-10	6.6	8
whole blood	IL-12p70	7.2	8

Cross-Species Pharmacology. The pattern of TLR expression in hematopoietic cells and the cytokines produced by DC subsets are different in rodents and humans.⁴⁹ However, murine PDC do produce IFN α in response to TLR7 activation, and studies with compound 32 were undertaken to confirm bioactivity in rodents prior to testing *in vivo*.

Mouse: potency of compound 32 for murine IFN α induction was determined by flow cytometric analysis of intracellular IFN α in pDC from murine splenocyte cultures (*n* = 11) derived from naïve BALB/c mice. The murine potency was ca. 10-fold lower than that determined in an equivalent assay in human blood.

Rat: *in vitro* cultures of rat PBMC or whole blood with compound 32 resulted in variable and minimal cytokine responses (IL-1 β , TNF α , IL-6, and IP-10) indicating the rat to be a much poorer responder than the mouse. However, *in vivo* dose-related increases in IFN bioactivity were demonstrated in rats treated with 32 at 0.4 mg/kg and above, with the maximal response seen 2 h after dosing.

Monkey: data in the literature indicates that TLR patterns of expression in cynomolgus monkeys and their responses to TLR activators are similar to those in humans.⁵⁰ Comparison of

IFN α and TNF α responses in human and cynomolgus monkey whole blood incubated with compound 32 showed the potencies for IFN α to be very similar (pEC₅₀ values 7.5 vs. 7.1 for human and monkey, respectively). The absolute human IFN α potency in this assay was lower than that reported in Table 6, possibly due to the use of an assay with different antibody reagents from those used in the earlier studies. A direct comparison of the human and monkey TNF α responses showed comparable potencies in the two species (pEC₅₀ values 5.3 vs 4.8 for humans and monkeys, respectively), showing that the IFN α / TNF α selectivity seen in humans was reflected in cynomolgus monkey and giving confidence that *in vivo* studies in the monkey are likely to be most predictive of responses in humans.

TLR and Wider Selectivity Profiling. Selectivity for TLR7 over the other nucleic acid detecting TLRs (TLRs 3, 8, and 9) was assessed in a human osteosarcoma (U2OS) cell line transfected with cDNA expression vectors for TLR7 or 8 and in a human embryonic kidney (HEK) cell line transfected with cDNA expression vectors for TLR3 or 9 using NF- κ B-driven luciferase reporter systems. Compound 32 showed only modest activity (pEC₅₀ 5.9) in the TLR7 reporter system mirroring the lower potency of 32 for the induction of TNF α via the NF- κ B pathway. Compound 32 showed very weak agonist activity (pEC₅₀ 4.7) in the TLR8 reporter assay and was inactive when tested up to 50 μ M in HEK cells expressing TLR3 and TLR9. The wider selectivity profile of 32 was evaluated against a panel of 51 seven-transmembrane (7TM), enzyme, ion-channel, kinase, transporter, and nuclear receptor targets. Weak activity was detected against 12 targets, and these were evaluated in secondary assays where pK_i (affinity) values were determined, and all were shown to be <6.

***In Vitro* Immunomodulation.** To gain evidence that the immunomodulatory cytokines induced by compound 32 might deliver benefit in the treatment of allergic diseases, the impact

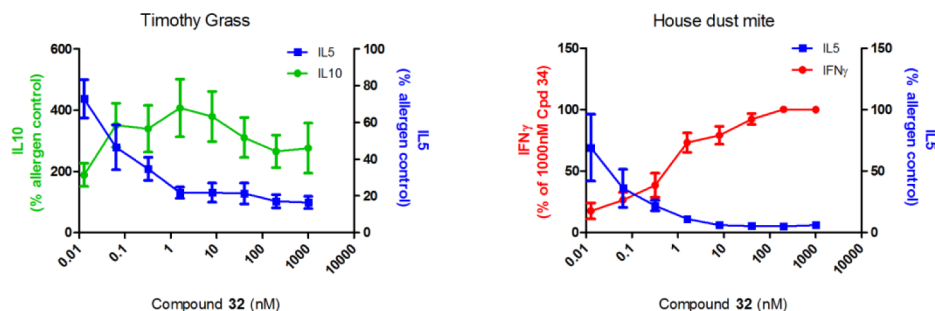


Figure 6. Impact of compound 32 on allergen driven cytokine responses in human PBMC.

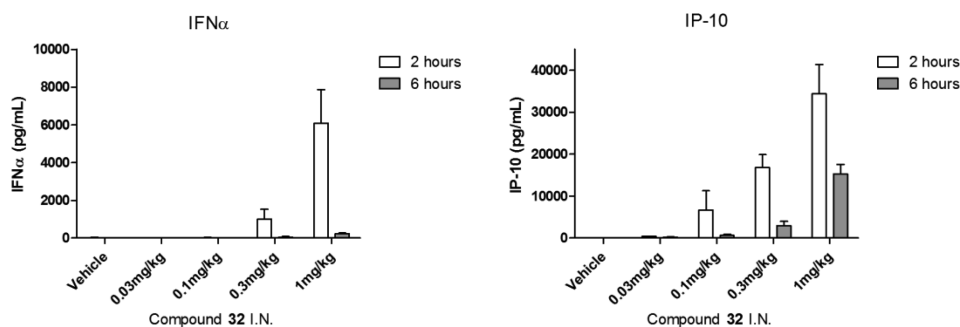


Figure 7. Serum IFN α and IP-10 responses following i.n. dosing of compound 32 in BALB/c mice.

of the compound on the cytokine response to allergen using PBMC from allergic individuals was investigated.⁵¹ Following incubation for 6 days, 32 reduced levels of the Th2 cytokines IL-5 and IL-13 released in response to Timothy grass ($n = 9$) or house dust mite ($n = 7$) in PBMC cultures derived from individuals allergic to these allergens, in a dose-dependent manner (Figure 6). Furthermore, 32 gave rise to enhanced production of IL-10 and IFN γ , indicating that, in the presence of allergen, this candidate has the potential to modify the nature of the T-cell responses.

In Vivo PD Studies. *In vivo* evaluation of the pharmacodynamic (PD) response to compound 32 was first investigated in the mouse. Female BALB/c mice were dosed intranasally (i.n.) with 32 in 0.2% Tween 80/saline and IFN α and IP-10 (IFN γ -inducible protein-10) levels monitored in serum and nasal lavage, 2 and 6 h after dosing. IP-10 is a chemokine commonly used as a downstream biomarker of IFN induction. Dose-related increases in IFN α levels in serum were detected at doses of 0.3 mg/kg and above at the 2 h time point which had subsided at 6 h (Figure 7). IP-10 provided a more sensitive biomarker confirming a clear serum response at 2 h at the 0.1 mg/kg dose level. IP-10, but not IFN α , could also be detected in nasal lavage samples, with maximum levels being present at 6 h post-treatment, but these levels were ~200-fold lower than those seen in serum (data not shown) and were considered insufficient to confirm target engagement locally in the nose.

Intranasal dosing of compound 32 in the cynomolgus monkey provided similar evidence for TLR7 activation but at very much lower doses than in the mouse. Plasma IP-10 measured 6 h after dosing provided the most sensitive biomarker of target engagement with raised levels of this chemokine detected at doses of 30 ng/kg and above (Figure 8). No TNF α was detected, even at the 3000 ng/kg dose level, reflecting the IFN α /TNF α selectivity seen *in vitro*.

In addition, target engagement in nasal tissue following intranasal dosing in the monkey was investigated by tran-

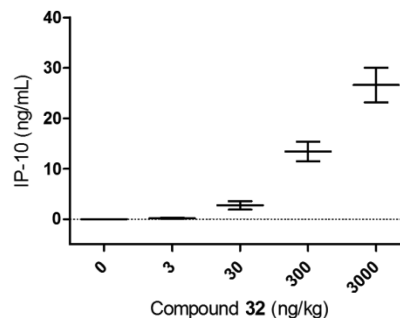


Figure 8. Plasma IP-10 responses 6 h after i.n. dosing of 32 in cynomolgus monkeys.

scriptomic analysis. Thus, nasal scrapes were collected 6 and 24 h after intranasal dosing with 32 for TaqMan gene expression analysis using a panel of 18 interferon stimulated genes (ISG). Dose- and time-dependent increases in mRNA mean fold expression were observed for ISG; Table 7 shows representative data from genes showing the largest fold increases, relative to the vehicle mean. This data provided clear evidence for engagement of TLR7 locally in the nose at a dose level of 3 ng/kg. With the exception of minimal MCP-1 induction in a single monkey, compound 32 did not induce any increase in plasma cytokines at this dose level indicating that at low doses it is possible to activate TLR7 in the nasal tissue without inducing a systemic cytokine response.

Data collected from the cardiovascular and respiratory safety study in the cynomolgus monkey showed that doses ≥ 30 ng/kg were associated with mild, dose-dependent increases in body temperature which returned to baseline within 24 h.⁵² This transient fever response is entirely consistent with the TLR7 induced cytokine response and was subsequently also observed during the clinical evaluation of 32 (*vide infra*). On the basis of this *in vitro* and *in vivo* data, the cynomolgus monkey was selected as the most relevant species from which to determine

Table 7. TaqMan Gene Expression Analysis of IFN Responsive Genes in Nasal Scrapes of Monkeys 24 h after Intranasal Dosing with Vehicle or Compound 32^a

IFN responsive gene	treatment group		
	vehicle	3 ng/kg	30 ng/kg
CCL8	1.0	3,972	19,315
GBP1	2.4	68.5	97.6
IFIT2	2.1	108.5	123.8
CXCL10	1.6	230	638
OAS1	1.2	38.9	53.7
OAS2	1.0	128.2	179.9
OAS3	0.9	86.3	101.2

^aValues represent mean fold change of gene expression at 24 h ($n = 3/\text{group}$) vs average vehicle treated expression. CCL, chemokine (C–C motif) ligand; GBP, interferon-induced guanylate-binding protein; IFIT, interferon-induced protein with tetratricopeptide repeats; CXCL, chemokine (C-X-C motif) ligand; OAS, 2'-5'-oligoadenylate synthetase.

the minimal anticipated biological effect level (MABEL). This was set at 3 ng/kg based on the detection of minimal systemic levels of the most sensitive and reproducible PD biomarker IP-10, and this figure was in turn used to calculate the starting intranasal dose of 2 ng for the first time in human (FTIH) study.

■ PRECLINICAL PROFILING: DMPK

Compound **32** shows desirable DMPK characteristics for a topically active intranasal drug with high systemic clearance (mouse 107, rat 96 mL/min/kg) and negligible oral bioavailability (rat, 1%). Very low levels of **32** were detected in the hepatic portal vein following oral administration to rats, indicating negligible passage of intact parent across the gastrointestinal tract and probably resulting largely from complete ionization of the basic piperidine amine (pK_a 9.7) in the gut. In contrast, bioavailability following intranasal administration of a solution of compound **32** is estimated to be 42% in the mouse, 12% in the rat, and predicted to be 100% in the cynomolgus monkey. Furthermore, an early T_{max} of ≤ 5 min (first sampling occasion) after intranasal dosing in rats and cynomolgus monkeys indicates rapid absorption across nasal tissue where the target plasmacytoid dendritic cells are located. Compound **32** was highly turned over by mouse, rat, monkey, and human liver microsomes with a good *in vitro/in vivo* correlation for mouse and rat, indicating that *in vivo* clearance is likely to be due mainly to metabolism. Following incubation *in vitro* with hepatocytes from rats, humans, and monkeys, no major qualitative differences were observed in the routes of metabolism of **32** between the species. The metabolism was complex with routes identified including hydroxylation, dehydrogenation, and a combination of hydroxylation and/or dehydrogenation of the pentane side chain and piperidine moiety, *N,N*-depentylation, *N*-depiperinidation with subsequent oxidation of the hexyl chain, glucuronidation, oxidations, and *N*-acetylation.

■ PRECLINICAL PROFILING: SAFETY ASSESSMENT

Compound **32** (maleate salt) has been evaluated in nonclinical studies for up to 8 weeks of weekly intranasal dosing in Sprague–Dawley rats and cynomolgus monkeys and up to 14 days of daily oral dosing in CD-1 mice, Sprague–Dawley rats, and New Zealand White rabbits. Standard genotoxicity assays

and safety pharmacology studies have also been conducted. Additional dermal and ocular irritancy studies were also performed in CD-1 mice and cynomolgus monkeys, respectively, using the intranasal formulation.

Species selection was based on comparative *in vitro* pharmacology studies looking at cytokine profiles on incubation of **32** with PBMC from different species. As reported in the literature, and shown above in the [Preclinical Profiling: Immunology](#) section, the cynomolgus monkey was found to most closely mirror the IFN α and TNF α responses seen in human PBMC. The lack of suitable cytokine assays prevented evaluation of the dog as a nonrodent species, and the mini-pig was ruled out due to poor IFN α /TNF α selectivity (data not shown). The rabbit is known not to respond to TLR7 agonists.²³ The rat and monkey were therefore selected as the most appropriate preclinical species for toxicity testing; the rat was selected because as a poor responder, it would allow higher systemic exposures to be achieved without initiating potentially dose limiting pharmacologically mediated effects.

The main challenges faced during the toxicological assessment of compound **32** were ensuring sufficient containment of the extraordinarily potent immunostimulatory agonist and the known tolerization of the response when repeat doses were administered too frequently. High containment practices were implemented to protect operators dispensing and delivering the intranasal spray formulation. Tolerization was investigated in the early dose ranging studies and was determined by comparison of once, twice, or three-times weekly dosing with daily dosing in the mouse and comparison of once daily and weekly dosing in the monkey. The optimal dosing regimen required to maintain pharmacological activity (cytokine response) was weekly dosing because tolerization was rapid with daily dosing resulting in a loss of cytokine response after only 3 days of administration.

Genotoxicity assessments indicated that **32** does not present a hazard to humans, and there were no safety pharmacology findings of concern. This toxicological assessment is predominantly based on the results of the definitive 8 week intranasal toxicity studies in the rat and monkey.

The principal local effect noted in rats and monkeys after repeat intranasal dosing was nasal irritancy, believed to be mechanism related and driven by local cytokine induction; this was only apparent at very high dose levels/concentrations in the rat and was the primary finding in the determination of the no observed adverse effect level (NOAEL) in both species. These findings comprised slight to marked inflammation and minimal to moderate exudate, erosion/ulceration of squamous and respiratory epithelium, squamous epithelium regeneration/hyperplasia with and without keratinization, squamous metaplasia of respiratory epithelium, and regeneration/hyperplasia of respiratory epithelium in the monkey. The NOAEL in the rat was 453/772 $\mu\text{g/kg/day}$ for 14 days in male and female, respectively, and 406/698 $\mu\text{g/kg/week}$ for 8 weeks in male and female, respectively. The NOAEL in the monkey was very much lower at 35.9 ng/kg/week for 8 weeks reflecting the much greater sensitivity of the monkey compared to that in the rat. These NOAELs provided adequate cover with regard to nasal exposure over the whole range of proposed doses in the clinic.

The principal systemic effect was increased levels of specific cytokines (IFN α , IP-10, IL-6, IL-1R antagonist, and MCP-1, G-CSF, and CRP); all other effects were considered to be secondary to this primary response (i.e., increased body

temperature, increased heart rate, alterations in splenic and lymph node cellularity, and minor changes in some clinical chemistry parameters). With the exception of the splenic and lymph node effects, all of the observed changes would be readily monitored in humans. The effects that were seen in the spleen and lymph node were of low severity and were shown to be fully reversible on withdrawal from treatment in the monkey. The observed cytokine response is consistent with the known TLR7 pharmacology of compound **32**.

Similar pharmacologically mediated effects were seen after oral administration in rats and mice; these mainly comprised altered cellularity in selected lymphoid tissues (spleen, mesenteric and mandibular lymph nodes, and thymus). TLR7 agonism leads to changes in lymphoid cell populations, either directly or due to downstream effects of cytokine production.

There was no evidence of dermal irritancy in the mouse after a single 4 h exposure to the clinical intranasal formulation at concentrations up to 1000 $\mu\text{g/mL}$; the clinical intranasal formulation is therefore classified as nonirritating according to the Draize scheme. The mouse has previously been shown to be predictive for adverse skin reactions resulting from exposure to other TLR7 agonists.⁵³ The risk of adverse skin reactions in the clinic from accidental skin exposure to relevant concentrations of the clinical intranasal formulation was therefore considered to be minimal.

There were a wide variety of adverse ocular effects in the monkey after a single exposure to the clinical intranasal formulation at a concentration of 1000 $\mu\text{g/mL}$. These included swollen and red skin (peri-orbital region), corneal opacity/edema/staining, pupil dilation/pupil miotic, conjunctival congestion/swelling/discharge, and hyperaemic retina. The onset of the majority of these effects were delayed until at least 48 h after dosing, although some changes could be detected by ophthalmic examination at 6 h after dosing (aqueous cells and aqueous flare). A lower dose of 10 $\mu\text{g/mL}$ was well tolerated. There is, therefore, a theoretical possibility of concentration-dependent ocular effects after accidental exposure in humans; however, any ocular symptoms can be easily reported and monitored in the clinic.

There were therefore no effects to preclude the intranasal administration of **32** to humans in proposed clinical trials with appropriate monitoring of nasal inflammation and/or discomfort.

■ PRECLINICAL PROFILING: PHARMACEUTICAL FORMULATION

While suspension formulations can be successfully delivered using conventional nasal spray devices, an aqueous solution formulation is very much preferred. This requires the compound to be sufficiently soluble and stable in water (with potential excipients to aid solubility or stability) in order to provide a pharmaceutical product with a suitable shelf life. In addition, consistent delivery of the drug from the device on repeated actuation and over time is required, which can be compromised by any adsorption of the drug to components of the device; this becomes a more significant issue for a very low dose product. The early clinical evaluation of **32** required an extraordinarily low strength formulation of 0.01 $\mu\text{g/mL}$ (100 μL per nostril) to deliver the starting dose of 2 ng, while the highest dose studied in the monkey intranasal safety study (320 $\mu\text{g/kg}$) required a formulation of 10 mg/mL. The free base, compound **32**, is an off-white crystalline solid (melting point 207 °C) with a relatively low aqueous solubility (50 $\mu\text{g/mL}$),

which can be enhanced to give sufficient solubility (200 $\mu\text{g/mL}$ at pH 7) to cover clinical doses by inclusion of the surfactant Tween 80. However, solutions of the free base were found to show unacceptable instability, believed to be due to *N*-oxide formation. This problem was overcome by *in situ* formation of the maleate salt for early studies. Subsequently, preparation of **32** as the maleate salt revealed this version to be a highly soluble compound with sufficient solubility (11 mg/mL at pH7) to cover the highest doses in preclinical safety assessment. Furthermore, formulations prepared with the maleate salt have delivered a pharmaceutical product displaying good stability (≥ 18 months at temperatures up to 30 °C) and which gives very reproducible delivery across the dose range from a device fitted with a Valois VP7 Pump. The very low strength formulations presented significant analytical challenges, but these were overcome by the development of a suitably sensitive assay (LLQ ca.1 ng/mL) for **32**, which has allowed the stability of even the lowest strength formulations to be successfully monitored.

■ CLINICAL STUDIES

The FTIH study was a randomized, double-blind, placebo-controlled study exploring the safety, tolerability, and pharmacokinetics of single escalating intranasal doses of **32** in healthy volunteers (HVT) and patients with active pollen-driven AR (NCT01480271).⁵² In addition, target engagement was explored by monitoring the induction of TLR7-mediated biomarkers both locally in the nose (nasal scrapes and/or nasal lavage) and also in peripheral blood. The starting dose for this study was set at 2 ng using a MABEL approach based on extrapolation from safety and PD findings in the monkey and applying a safety factor of 10.⁵² Doses of 2, 20, 40, 60, and 100 ng were explored in HVT and doses of 2, 6, 20, and 40 ng in AR patients (further doses were not evaluated due to the end of the pollen season). No serious adverse events (AEs) occurred in either group. Mild to moderate and transient “flu-like” symptoms potentially related to TLR7-mediated cytokine release syndrome (CRS) were seen in HVT at doses of ≥ 40 ng with a dose-dependent increase incidence. Doses higher than 100 ng were not tested as the CRS-related AEs induced at this level were not considered acceptable for the target population and met the predefined stopping criteria.⁵² In AR patients, only few sporadic mild CRS-related AEs were observed without a clear dose relationship. Activation of the TLR7 pathway was confirmed in both populations at doses ≥ 20 ng, by a dose-dependent increase of serum and nasal lavage IP-10 levels and upregulation of IFN α -stimulated genes in nasal scrapes. IP-10 monitoring in nasal lavage provides a very convenient and sensitive method of confirming TLR7 engagement locally in the nose and has been successfully employed to assess target engagement in subsequent studies.

The systemic cytokine response is believed to result from spillover of locally produced cytokines since, with the exception of one sample, no quantifiable systemic exposure to **32** was detected despite a very sensitive assay (LLQ 2 pg/mL). The observed “flu-like” symptoms are therefore also considered to be driven by the local TLR7 engagement, which is consistent with the observation by AstraZeneca of similar AEs with their intranasal TLR7 agonist “ante-drug” designed to be rapidly inactivated in plasma.^{10b}

Having identified a well-tolerated single dose of compound **32** that activates the TLR7 cascade in the nose, the safety and pharmacodynamics of repeat intranasal administration was next

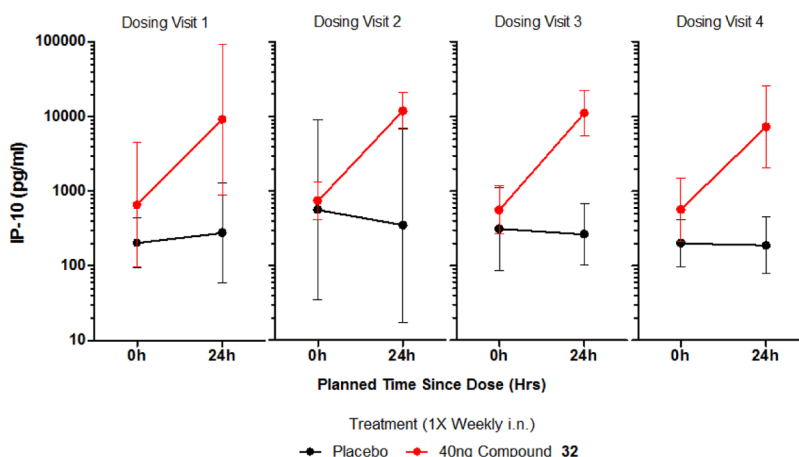


Figure 9. 4-Week repeat dose study: IP-10 levels in nasal lavage pre (0 h)- and 24 h post-intranasal administration of 40 ng of compound **32** on dosing visits 1–4.

investigated. Repeated stimulation of TLRs is known to be associated with tachyphylaxis, and our preclinical evaluation had shown that dosing with **32** more frequently than once-weekly resulted in a marked reduction of the pharmacodynamic response. In contrast, the opposite phenomenon was observed in a clinical study with the oral TLR7 agonist *N*-(4-(4-amino-2-ethyl-1*H*-imidazo[4,5-*c*]quinolin-1-yl)butyl)-methanesulphonamide (PF-4878691), which was terminated due to the dangerous amplification of cytokine production following twice weekly repeat dosing.⁵⁴ To evaluate the effects of repeat dosing with compound **32**, a randomized, double-blind, placebo-controlled study was conducted in patients with symptomatic AR and mild asthma during the pollen season (NCT01607372).⁵² Compound **32** was administered intranasally at weekly intervals for 4 weeks. Doses of 40 and 80 ng were studied, and both dose levels were shown to be well tolerated with only mild “flu-like” symptoms seen in some subjects (67%, 14%, and 20% of subjects in 40 ng, 80 ng, and placebo groups, respectively).⁵² There was no evidence of nasal irritancy in this study in any of the treatment groups. The serum and nasal lavage IP-10 levels very clearly increased in a consistent manner after each weekly dosing with no evidence for amplification or tolerization of the response. No dose-dependent effect on IP-10 induction was observed; nasal lavage data for the placebo and 40 ng cohorts is shown in Figure 9.

Compound **32** has now progressed into an 8 week clinical study treating subjects with respiratory allergies and investigating the safety, pharmacodynamics, and effect on allergic reactivity in response to allergen challenge 1 and 3 weeks, plus 1 year after treatment (NCT01788813, NCT02446613).

CONCLUSIONS

Optimization of both the C-2 and N-9 substituents on the 8-oxoadenine scaffold had provided the intranasal clinical candidate **32**, which displays extraordinary IFN α inducing potency and IFN α /TNF α selectivity. 2-(*S*)-Pentyloxy was identified as a novel potency enhancing 2-substituent, but the major breakthrough came with the introduction of basic amine functionality in the N-9 substituent. Thus, switching from saturated oxygen heterocycles to saturated nitrogen heterocycles in the N-9 side chain was accompanied by a dramatic and selective enhancement of IFN α potency. SAR of the N-9 substituent strongly suggests that the origin of this potency enhancement is not the result of specific interactions with

TLR7, and our favored hypothesis is that this IFN α potency is driven by the concentration of these basic derivatives in the highly acidic endosomal compartment where TLR7 induced IRF7 activation to generate IFN α takes place. Incorporation of PD biomarkers into the preclinical safety studies with compound **32** proved invaluable in setting the starting dose and dosing regimen for the clinic as well as establishing methodologies to assess target engagement following intranasal dosing in humans. The extreme potency of **32** presented additional challenges, but the compound shows excellent pharmaceutical properties that allow reliable delivery of doses as low as 1 ng per actuation of an intranasal spray formulation. Early clinical evaluation of compound **32** in healthy volunteers and patients with allergic rhinitis by the intranasal route has confirmed local activation of TLR7 at very low, well tolerated, doses, and a reproducible pharmacological response has been confirmed following repeat dosing at weekly intervals. An 8 week clinical study with compound **32** is nearing completion, which will provide the first indications of the impact of repeated TLR7 activation in the nose on allergic reactivity and duration of effect. The results of this study will be presented in due course.

EXPERIMENTAL SECTION

TLR7 Agonists. The highly potent immunostimulatory TLR7 agonists described herein should be handled with appropriate containment controls particularly when manipulating powder samples which may become easily airborne.

Biology. The PBMC assay A measured IFN α induction following incubation of test compounds for 24 h with cryopreserved human PBMC. The PBMC assay B measured both IFN α and TNF α induction following incubation of test compounds for 24 h with freshly prepared human PBMC. Full details of these primary screening assays along with methods used for additional *in vitro* and *in vivo* immunology profiling of compound **32** can be found in the [Supporting Information](#). All *in vivo* studies were conducted in accordance with the GSK Policy on the Care, Welfare and Treatment of Laboratory Animals and were reviewed by the Institutional Animal Care and Use Committee at GSK, and/or by the ethical review process at the institution where the work was performed.

Chemistry. TLC was performed on Merck 0.25 mm Kieselgel 60 F254 plates using UV light and/or staining with aqueous KMnO₄ solution for visualization. LCMS analysis of reactions and products was conducted using one of three methods (A–C) which are described in detail in the [Supporting Information](#) along with conditions used for mass-directed autoperative HPLC (MDAP). Chromatographic

purification was typically performed using prepacked silica gel cartridges on a flash chromatography system such as the Flashmaster II system (Argonaut Technologies Ltd.). Solvent removal using a stream of nitrogen was performed at 30–40 °C on a GreenHouse Blowdown system available from Radleys Discovery Technologies. Unless otherwise stated, ¹H NMR spectra were recorded at 400 MHz in either chloroform-*d* or DMSO-*d*₆ on either a Bruker DPX 400 or Bruker Avance DRX or Varian Unity 400 spectrometer. Spectra at 600 MHz were recorded using a Bruker AVII+ spectrometer. The internal standard used was either tetramethylsilane or the residual protonated solvent at 7.25 ppm for chloroform-*d* or 2.50 ppm for DMSO-*d*₆. The purity of all compounds screened in biological assays was assessed by LCMS and NMR analysis and was ≥95% as unless otherwise specified.

Synthesis of Compound 32. 2-Fluoro-9-(tetrahydro-2H-pyran-2-yl)-9H-purin-6-amine (**38**). *N*,*O*-Bis(trimethylsilyl)acetamide (975 mL, 3.988 mol) was added to a stirred suspension of 2-fluoro-9H-purin-6-amine (**37**) (200 g, 1.306 mol) in anhydrous acetonitrile (4 L) in a 10 L controlled laboratory reactor and the resulting mixture heated to reflux and maintained at that temperature for 2 h. The circulator was then reprogrammed and the reaction mixture cooled to 0 °C. A solution of tetrahydropyran-2-yl acetate (preparation described in *Tetrahedron Lett.* **2006**, 47, 4741–4741) (282 g, 1.959 mol) in anhydrous acetonitrile (500 mL) was then added slowly via a dropping funnel followed by trimethylsilyl trifluoromethanesulfonate (283 mL, 1.567 mol) dropwise via a dropping funnel. No significant exotherm was observed. The circulator temperature was readjusted to 10 °C and stirring maintained for a further 1 h. The mixture was then quenched by the addition of 1 M sodium carbonate (4 L). A solid precipitate was observed and the pH checked to be basic. Additional water was added to the suspension (1 L), and on standing, the layers separated with the aqueous layer containing significant solid inorganics. The majority of the aqueous and inorganic solid was separated. The organic layer still contained significant solid and was cooled to 0 °C with stirring to encourage further precipitation. This solid was then collected by filtration, and the pad was washed very well with water then dried *in vacuo* at 40 °C overnight to give the title compound as a cream colored solid (152.8 g, 49.4%). LCMS (System C): *t*_{RET} = 1.71 min; *MH*⁺ = 238. ¹H NMR δ (chloroform-*d*) 7.98 (s, 1H), 5.90 (br s, 2H), 5.59–5.66 (m, 1H), 4.13–4.21 (m, 1H), 3.71–3.81 (m, 1H), 1.91–2.17 (m, 3H), 1.60–1.84 (m, 3H+) (integral obscured by solvent).

2-[[[(1*S*)-1-Methylbutyl]oxy]-9-(tetrahydro-2H-pyran-2-yl)-9H-purin-6-amine (**39**). Sodium *t*-butoxide (206 g, 2.144 mol) was added to (*S*)-2-pentanol (720 mL, 6.58 mol) in a 2 L round bottomed flask. The mixture was stirred at 50 °C until all of the sodium *t*-butoxide had dissolved. Compound **38** (130 g, 548 mmol) was then added in portions over 5 min. After 3 h, LCMS analysis indicated complete consumption of the starting material, and the mixture was poured into ice/water (3 L) and then extracted with methyl *t*-butyl ether. This resulted in emulsion formation, the mixture was filtered through Celite, and the organic phase was separated. The aqueous layer was then treated with solid NaCl and then re-extracted with methyl *t*-butyl ether. The organic extracts were combined and washed with brine, dried over anhydrous magnesium sulfate, filtered, and then evaporated to yield the title compound as a pale brown gum (158.59 g, 95%). LCMS (System C): *t*_{RET} = 2.65 min; *MH*⁺ = 306. ¹H NMR δ (chloroform-*d*) 7.84 (s, 1H), 5.61–5.66 (m, 1H), 5.55 (br s, 2H), 5.18 (sxt, *J* = 6.2 Hz, 1H), 4.10–4.17 (m, 1H), 3.69–3.78 (m, 1H), 1.37–2.13 (m, 10H+) (integral obscured by impurities), 1.31–1.36 (m, 3H), 0.94 (t, *J* = 7.3 Hz, 3H) [mixture of diastereoisomers; signals grouped together and treated as a single component for integration].

8-Bromo-2-[[[(1*S*)-1-methylbutyl]oxy]-9-(tetrahydro-2H-pyran-2-yl)-9H-purin-6-amine (**40**). *N*-Bromosuccinimide (12.16 g, 68.3 mmol) was added portionwise over 5 min to a stirred solution of compound **39** (14.9 g, 48.8 mmol) in chloroform (80 mL) at <5 °C under an atmosphere of nitrogen. The reaction mixture was stirred at <5 °C for 5 h then washed with saturated sodium hydrogen carbonate solution (80 mL) then water (80 mL) and evaporated. The resulting foam was dissolved in DCM (50 mL) and washed with water (50 mL) and then brine (50 mL). The combined aqueous phases were washed

with DCM (50 mL). The combined organic layers were dried through a hydrophobic frit, and the solvent removed *in vacuo* to yield the title compound as an orange foam (18.5 g, 99%). LCMS (System C): *t*_{RET} = 3.06 min; *MH*⁺ = 384/386. ¹H NMR δ (chloroform-*d*) 5.61 (dd, *J* = 11.0, 2.5 Hz, 1H), 5.36 (br s, 2H), 5.07–5.17 (m, 1H), 4.12–4.20 (m, 1H), 3.65–3.74 (m, 1H), 2.95–3.10 (m, 1H), 2.05–2.14 (m, 1H), 1.58 (s, 8H+) (integral obscured by water), 1.32–1.37 (m, 3H), 0.91–0.97 (m, 3H) [mixture of diastereoisomers; signals are grouped together and treated as a single component for integration].

2-[[[(1*S*)-1-Methylbutyl]oxy]-8-(methyloxy)-9-(tetrahydro-2H-pyran-2-yl)-9H-purin-6-amine (**41**). Compound **40** (7.1 g, 18.48 mmol) was dissolved in anhydrous methanol (70 mL), and a solution of sodium methoxide (25%) in methanol (8 mL) was added dropwise under an atmosphere of nitrogen. The solution was heated to reflux at 90 °C for 4 h under an atmosphere of nitrogen. Additional sodium methoxide in methanol (25% solution, 3 mL) was added, and the reaction was stirred at 60 °C for a further 16 h. An additional portion of sodium methoxide in methanol (25% solution, 5 mL) was added, and the reaction was stirred at 90 °C for a further 7 h. The solvent was removed on a rotary evaporator, and the crude product was partitioned between ethyl acetate (75 mL) and saturated ammonium chloride solution (75 mL). The organic layer was washed with brine (75 mL). The solvent was removed on a rotary evaporator to yield the title compound as a pale orange foam (6 g, 97%). LCMS (System B): *t*_{RET} = 1.14 min; *MH*⁺ = 336, 337. ¹H NMR δ (chloroform-*d*) 5.50 (dd, *J* = 11.3, 2.3 Hz, 1H), 5.06–5.18 (m, 3H), 4.07–4.15 (m, 4H), 3.64–3.73 (m, 1H), 2.68–2.83 (m, 1H), 1.98–2.08 (m, 1H), 1.64 (br s, 8H+) (integral obscured by water), 1.30–1.36 (m, 3H), 0.89–0.97 (m, 3H) [mixture of diastereoisomers; signals are grouped together and treated as a single component for integration].

2-[[[(1*S*)-1-Methylbutyl]oxy]-8-(methyloxy)-9H-purin-6-amine Tri-fluoroacetate Salt (**42**). Compound **41** (6 g, 17.89 mmol) was dissolved in methanol (50 mL). Trifluoroacetic acid (20.67 mL, 268 mmol) was added dropwise and the mixture stirred at 20 °C for 72 h under an atmosphere of nitrogen. The solvent was removed *in vacuo*, and the resulting solid was washed with ethyl acetate and filtered. The filtrate was stripped and the residue washed with ethyl acetate. The combined solid residues were dried in a vacuum oven for 2 h to give the title compound as an off white solid (5.3 g, 81%). LCMS (System B): *t*_{RET} = 0.76 min; *MH*⁺ = 252, 253. ¹H NMR δ (DMSO-*d*₆) 7.63 (br s, 2H), 5.07–5.15 (m, 1H), 4.05 (s, 3H), 1.51–1.75 (m, 2H), 1.32–1.47 (m, 2H), 1.30 (d, *J* = 6.0 Hz, 3H), 0.90 (t, *J* = 7.5 Hz, 3H).

9-(5-Chloropentyl)-2-[[[(1*S*)-1-methylbutyl]oxy]-8-(methyloxy)-9H-purin-6-amine (**43**). Compound **42** (4.430 g, 12.13 mmol) was dissolved in DMF (70 mL). Potassium carbonate (4.30 g, 31.1 mmol) was added, and the reaction mixture was heated and stirred at 50 °C for 2 h and then cooled to room temperature. 1-Bromo-5-chloropentane (1.598 mL, 12.13 mmol) was added and the mixture stirred at room temperature for 48 h. The solvent was then removed, and the residue was partitioned between DCM (100 mL) and 6% NaHCO₃ solution (100 mL), and the layers were separated. The aqueous layer was washed with additional DCM (2 × 50 mL), and the organic extracts were combined, dried by passage through a hydrophobic frit, and the solvent removed. The residue (ca. 6 g) was loaded in dichloromethane and purified by aminopropyl SPE (70 g, three cartridges) using a 0–100% ethyl acetate/cyclohexane gradient. The appropriate fractions were combined and evaporated *in vacuo* to give the title compound as a yellow oil (3.037 g, 70.4%). LCMS (System A): *t*_{RET} = 2.58 min; *MH*⁺ = 356, 358. ¹H NMR δ (chloroform-*d*) 5.06–5.21 (m, 3H), 4.11 (s, 3H), 3.93 (t, *J* = 7.0 Hz, 2H), 3.51 (t, *J* = 7.0 Hz, 2H), 1.73–1.86 (m, 5H), 1.37–1.61 (m, 5H), 1.32 (d, *J* = 6.0 Hz, 3H), 0.93 (t, *J* = 7.5 Hz, 3H).

2-[[[(1*S*)-1-Methylbutyl]oxy]-8-(methyloxy)-9-[5-(1-piperidinyl)-pentyl]-9H-purin-6-amine (**44**). Compound **43** (80 mg, 0.225 mmol), triethylamine (0.031 mL, 0.225 mmol), and piperidine (0.045 mL, 0.45 mmol) were suspended in DMF (3 mL) and the mixture heated to 70 °C for 18 h. The solvent was removed and the residue partitioned between DCM (4 mL) and saturated sodium bicarbonate (4 mL). The aqueous phase was re-extracted with further DCM, and the combined organic extracts were concentrated and the residue

dissolved in 1:1 MeOH/DMSO (1 mL) and purified by MDAP. The product containing fractions were combined and evaporated under a stream of nitrogen to give the title compound (47.2 mg, 51.9%). LCMS (System C): $t_{\text{RET}} = 3.11$ min; $\text{MH}^+ = 405$. ^1H NMR δ (chloroform- d) 5.08–5.17 (m, 3H), 4.10 (s, 3H), 3.91 (t, $J = 7.3$ Hz, 2H), 2.28–2.40 (m, 4H), 2.20–2.27 (m, 2H), 1.71–1.81 (m, 4H), 1.37–1.61 (m, 10H), 1.32 (d, $J = 6.0$ Hz, 3H), 1.24–1.31 (m, 2H), 0.93 (t, $J = 7.3$ Hz, 3H).

6-Amino-2-[[[(1S)-1-methylbutyl]oxy]-9-[5-(1-piperidinyl)pentyl]-7,9-dihydro-8H-purin-8-one (32). Compound 44 (2.5 g, 6.18 mmol) was dissolved in methanol (25 mL). 4 M Hydrogen chloride in 1,4-dioxane (38.6 mL, 154 mmol) was added, and the mixture was stirred at room temperature for 4 h. The solvent was evaporated *in vacuo* to leave an off-white solid (2.555 g). Then, 2.455 g of this material was partitioned between DCM (50 mL) and saturated NaHCO_3 solution (50 mL). The layers were separated, and the aqueous phase was washed with additional DCM (3×25 mL). The organic extracts were combined, dried (hydrophobic frit), and the solvent evaporated *in vacuo* to give the title compound as an off-white solid (1.756 g, 72.8%). LCMS (System A): $t_{\text{RET}} = 1.42$ min; $\text{MH}^+ = 391$. 1.7 g of this material was recrystallized from ethyl acetate (ca. 50 mL). The crystals were collected, washed with ice-cold ethyl acetate (15 mL), and dried *in vacuo* at 50 °C for 3 h to give the title compound as a cream crystalline solid (1.33 g). Melting point onset (DSC): 207.4 °C. LCMS (System A): $t_{\text{RET}} = 1.42$ min; $\text{MH}^+ = 391$. ^1H NMR δ (DMSO- d_6 , 600 MHz) 9.80 (s, 1H), 6.33 (br s, 2H), 4.99 (sxt, $J = 6.2$ Hz, 1H), 3.64 (t, $J = 7.0$ Hz, 2H), 2.23 (br s, 4H), 2.15 (t, $J = 7.3$ Hz, 2H), 1.57–1.68 (m, 3H), 1.27–1.52 (m, 11H), 1.19–1.25 (m, 5H), 0.88 (t, $J = 7.3$ Hz, 3H). ^{13}C NMR δ (DMSO- d_6 , 151 MHz) 159.7, 152.3, 149.4, 147.6, 97.9, 71.1, 58.4, 54.0, 38.9, 37.8, 27.6, 25.8, 25.5, 24.2, 23.9, 19.8, 18.2, 13.9.

Images of the DSC trace and also of the XRPD can be found in WO2010/018133.^{37a}

6-Amino-2-[[[(1S)-1-methylbutyl]oxy]-9-[5-(1-piperidinyl)pentyl]-7,9-dihydro-8H-purin-8-one, Maleate Salt (32 Maleate Salt). **Preparation 1.** Compound 32 (0.384 g, 0.98 mmol) was dissolved in isopropyl alcohol (4.6 mL) and heated to 40 °C. Maleic acid (0.114 g, 0.98 mmol) was added. A clear solution was obtained. During cooling to room temperature, precipitation occurred. The slurry was filtered, washing with isopropyl alcohol (5 mL), and dried under reduced pressure at 40 °C to constant weight to give the title compound as a white solid (0.305 g, 61%). ^1H NMR confirms a 1:1 ratio of maleic acid: 32 ^1H NMR δ (DMSO- d_6) 9.85 (s, 1H), 8.85 (br s, 1H), 6.39 (s, 2H), 6.02 (s, 2H), 5.00 (m, $J = 6.2$ Hz, 1H), 3.68 (t, $J = 6.8$ Hz, 2H), 3.40 (m, 2H), 2.98 (m, $J = 8.1$ Hz, 2H), 2.82 (br s, 2H), 1.85–1.24 (m, 16H), 1.21 (d, $J = 6.1$ Hz, 3H), 0.89 (t, $J = 7.3$ Hz, 3H).

Preparation 2. A solution of compound 32 (1.46 g, 3.74 mmol) in isopropyl alcohol (14.6 mL) was clarified (filtered at room temperature through a BondElut cartridge) and then heated to approximately 50 °C. A solution of maleic acid (0.434 g, 3.74 mmol) in isopropyl alcohol (2.9 mL) was added. The resulting solution was then seeded and cooled to 45 °C. Further seed was added. The resulting slurry was cooled to room temperature and held overnight (approximately 16 h), then cooled in an ice/water bath for 30 min. The slurry was filtered, washing with isopropyl alcohol (4.5 mL and then 3 mL). The product was dried under reduced pressure at 40 °C to constant weight to give the title compound (1.305 g, 69%). Analysis by XRPD indicated this sample to be crystalline. Images of the XRPD can be found in WO2011/098452.^{37b}

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.5b01647.

Synthesis and characterizing data for compounds 4–31, 33, and 34, LCMS and MDAP methods, details of human PBMC screening assays, and preclinical *in vitro* and *in vivo* profiling of compound 32 (PDF)

SMILES data (CSV)

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Notes

The authors declare the following competing financial interest(s): All authors were employees of GSK at the time the work was carried out. Keith Biggadike, Diane Coe, Bob Gibbon, Stephen Hermitage, Amanda Lucas, Charlotte Hardy, Stephen Smith, Naimisha Trivedi, and Xiao Lewell are co-inventors on patents covering compound 32 or its maleate salt.

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■ ABBREVIATIONS USED

AP-3, adaptor protein-3; AR, allergic rhinitis; CRS, cytokine release syndrome; FTIH, first time in human; HVT, healthy volunteer; IFN α , interferon- α ; IP-10, IFN γ -inducible protein-10; IRF-7, interferon regulatory factor-7; ISG, interferon stimulated genes; LLQ, lower limit of quantification; LRO, lysosome related organelle; MABEL, minimum anticipated biological effect level; MSD, meso scale discovery; MyD88, myeloid differentiation primary response protein 88; PAMP, pathogen-associated molecular pattern; PBMC, peripheral blood mononuclear cell; pDC, plasmacytoid dendritic cell; SIT, specific immunotherapy; Th1, type-1 T-helper cell; Th2, type-2 T-helper cell; ssRNA, single stranded RNA

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■ NOTE ADDED AFTER ASAP PUBLICATION

The version of this paper that was published ASAP February 10, 2016, contained an error in the caption of Figure 8. This was corrected, and the revised version was reposted February 18, 2016

SUPPORTING INFORMATION

Discovery of 6-Amino-2-[[[(1S)-1-methylbutyl]oxy]-9-[5-(1-piperidinyl)pentyl]-7,9-dihydro-8H-purin-8-one (GSK2245035), a Highly Potent and Selective Intranasal Toll-like Receptor 7 Agonist for the Treatment of Asthma

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Synthesis and characterising data for compounds 4-31, 33 and 34

PBMC screening assays

- Assay A - IFN α induction using cryopreserved human PBMC
- Assay B - IFN α /TNF α induction in freshly prepared human PBMC

Pre-clinical *in vitro* and *in vivo* profiling of compound 32.

- 20h Whole Blood and PBMC profiling of compound 32.
- Impact of compound 32 on allergen-driven cytokine response in PBMC from allergic donors.
- *In vivo* PD studies: Cytokine and transcriptomic responses following intranasal dosing of compound 32 in Cynomolgus monkeys.

LCMS and MDAP Methods

Synthesis and characterising data for compounds 4-31, 33 and 34

Compound number	Synthesis	Mass Spectrum MH ⁺	NMR
4	WO2008/101867 Example 1	322	¹ H NMR δ (400 MHz, DMSO- <i>d</i> ₆) 9.86 (s, 1H) 6.41 (br s, 2H) 4.14 (t, <i>J</i> =6.6 Hz, 2H) 3.76 - 3.86 (m, 2H) 3.55 (d, <i>J</i> =7.1 Hz, 2H) 3.17 - 3.27 (m, 2H) 1.94 - 2.10 (m, 1H) 1.58 - 1.69 (m, 2H) 1.32 - 1.50 (m, 4H) 1.14 - 1.29 (m, 2H) 0.92 (t, <i>J</i> =7.5 Hz, 3H)
5	WO2008/101867 Example 11	336	¹ H NMR δ (400 MHz, DMSO- <i>d</i> ₆) 9.84 (s, 1H) 6.40 (br s, 2H) 4.13 (t, <i>J</i> =6.7 Hz, 2H) 3.79 (dd, <i>J</i> =11.2, 2.9 Hz, 2H) 3.64 - 3.74 (m, 2H) 3.21 (t, <i>J</i> =10.9 Hz, 2H) 1.53 - 1.70 (m, 6 H) 1.32 - 1.46 (m, 3 H) 1.07 - 1.23 (m, 2 H) 0.91 (t, <i>J</i> =7.5 Hz, 3 H)
6	WO2008/101867 Example 84	350	¹ H NMR δ (400 MHz, DMSO- <i>d</i> ₆) 10.16 (br s, 1H) 6.44 (br s, 2H) 4.14 (t, <i>J</i> =6.6 Hz, 2H) 3.80 (dd, <i>J</i> =11.1, 3.5 Hz, 2H) 3.65 (t, <i>J</i> =7.1 Hz, 2H) 3.19 - 3.28 (m, 2H) 1.60 - 1.73 (m, 4H) 1.33 - 1.58 (m, 5H) 1.02 - 1.26 (m, 4H) 0.92 (t, <i>J</i> =7.3 Hz, 3H)
7	WO2008/101867 Example 85	364	¹ H NMR δ (400 MHz, DMSO- <i>d</i> ₆) 9.82 (br s, 1 H) 6.36 (s, 2H) 4.15 (t, <i>J</i> =6.7 Hz, 2H) 3.79 (dd, <i>J</i> =11.3, 2.8 Hz, 2H) 3.66 (t, <i>J</i> =7.0 Hz, 2H) 3.22 (td, <i>J</i> =11.7, 2.0 Hz, 2H) 1.59 - 1.69 (m, 4H) 1.45 - 1.53 (m, 2H) 1.33 - 1.44 (m, 3H) 1.17 - 1.30 (m, 4H) 1.01 - 1.15 (m, 2H) 0.92 (t, <i>J</i> =7.4 Hz, 3H)
8	Prepared similarly to Compound 7	378	¹ H NMR δ (400 MHz, DMSO- <i>d</i> ₆) 9.84 (br s, 1H) 6.38 (br s, 2H) 4.15 (t, <i>J</i> =6.8 Hz, 2H) 3.75 - 3.83 (m, 2H) 3.66 (t, <i>J</i> =7.0 Hz, 2H) 3.23 (td, <i>J</i> =12.0, 2.0 Hz, 2H) 1.59 - 1.70 (m, 4H) 1.47 - 1.55 (m, 2H) 1.01 - 1.45 (m, 12 H) 0.92 (t, <i>J</i> =7.3 Hz, 3H)
9	Prepared similarly to Compound 7	392	¹ H NMR δ (400 MHz, DMSO- <i>d</i> ₆) 9.90 (br s, 1H) 6.39 (br s, 2H) 4.13 (t, <i>J</i> =6.7 Hz, 2H) 3.78 (dd, <i>J</i> =11.3, 2.8 Hz, 2H) 3.64 (t, <i>J</i> =6.9 Hz, 2H) 3.22 (td, <i>J</i> =11.7, 2.0 Hz, 3H) 1.57 - 1.69 (m, 4H) 1.45 - 1.54 (m, 2H) 1.31 - 1.44 (m, 3H) 1.00 - 1.30 (m, 10H) 0.91 (t, <i>J</i> =7.4 Hz, 3H)

10	WO2008/101867 Example 83	364	¹ H NMR δ (400 MHz, DMSO- <i>d</i> ₆) 9.90 (br s, 1H) 6.40 (br s, 2H) 4.14 (t, <i>J</i> =6.5 Hz, 2H) 3.58 - 3.77 (m, 4H) 3.21 (td, <i>J</i> =11.0, 2.9 Hz, 1H) 2.90 (t, <i>J</i> =10.4 Hz, 1H) 1.69 - 1.79 (m, 1H) 1.55 - 1.68 (m, 4H) 1.33 - 1.54 (m, 5H) 0.97 - 1.31 (m, 5 H) 0.92 (t, <i>J</i> =7.3 Hz, 3H)
11	WO2008/101867 Example 81	364	¹ H NMR δ (400 MHz, DMSO- <i>d</i> ₆) 9.86 (br s., 1H) 6.37 (s, 2H) 4.15 (t, <i>J</i> =6.7 Hz, 2H) 3.76 - 3.85 (m, 1H) 3.64 (t, <i>J</i> =6.9 Hz, 2H) 3.08 - 3.19 (m, 1H) 1.56 - 1.79 (m, 4H) 1.17 - 1.54 (m, 8H) 1.03 - 1.16 (m, 1 H) 0.92 (t, <i>J</i> =7.4 Hz, 3H)
12	WO2008/101867 Example 79	350	¹ H NMR δ (400 MHz, DMSO- <i>d</i> ₆) 9.86 (br s, 1H) 6.40 (s, 2H) 4.14 (t, <i>J</i> =6.7 Hz, 2H) 3.52 - 3.78 (m, 5H) 3.16 (t, <i>J</i> =7.8 Hz, 1H) 1.98 - 2.13 (m, 1H) 1.85 - 1.97 (m, 1H) 1.56 - 1.71 (m, 4H) 1.15 - 1.46 (m, 7H) 0.91 (t, <i>J</i> =7.4 Hz, 3H)
13	WO2008/101867 Example 75	350	¹ H NMR δ (400 MHz, chloroform- <i>d</i>) 10.28 (br s, 1H) 5.79 (br s, 2H) 4.29 (t, <i>J</i> =6.8 Hz, 2H) 3.67 - 3.96 (m, 5H) 1.71 - 2.03 (m, 8H) 1.37 - 1.54 (m, 5H) 0.98 (t, <i>J</i> =7.4 Hz, 3H)
14	WO2008/101867 Example 77	322	¹ H NMR δ (400 MHz, DMSO- <i>d</i> ₆) 9.87 (br s, 1H) 6.38 (br s, 2H) 4.15 (t, <i>J</i> =6.7 Hz, 2H) 3.74 - 3.81 (m, 1H) 3.65 - 3.73 (m, 3H) 3.54 - 3.62 (m, 1H) 3.22 (dd, <i>J</i> =8.2, 6.9 Hz, 1H) 1.95 - 2.12 (m, 2H) 1.59 - 1.76 (m, 4H) 1.32 - 1.50 (m, 3H) 0.92 (t, <i>J</i> =7.4 Hz, 3H)
15	WO2008/101867 Example 76	322	¹ H NMR δ (400 MHz, DMSO- <i>d</i> ₆) 9.86 (br s, 1H) 6.38 (br s, 2 H) 4.15 (t, <i>J</i> =6.7 Hz, 2H) 3.75 - 3.81 (m, 1H) 3.65 - 3.73 (m, 3H) 3.54 - 3.62 (m, 1H) 3.22 (dd, <i>J</i> =8.3, 6.8 Hz, 1H) 1.95 - 2.11 (m, 2H) 1.60 - 1.76 (m, 4H) 1.33 - 1.49 (m, 3H) 0.92 (t, <i>J</i> =7.4 Hz, 3H)
16	WO2008/101867 Example 121	378	¹ H NMR δ (400 MHz, DMSO- <i>d</i> ₆) 9.91 (br s, 1H) 6.34 (s, 2H) 5.00 (sxt, <i>J</i> =6.0 Hz, 1H) 3.79 (dd, <i>J</i> =10.7, 3.4 Hz, 2H) 3.65 (t, <i>J</i> =7.0 Hz, 2H) 3.22 (td, <i>J</i> =11.7, 2.0 Hz, 2H) 1.55 - 1.68 (m, 3H) 1.18 - 1.54 (m, 13H) 1.02 - 1.15 (m, 2H) 0.89 (t, <i>J</i> =7.3 Hz, 3H)
17	WO2010/018133 Example 6	363	¹ H NMR δ (400 MHz, DMSO- <i>d</i> ₆) 10.01 (br s, 1H) 6.43 (br s, 2H) 4.15 (t, <i>J</i> =6.7 Hz, 2H) 3.67 (t, <i>J</i> =6.9 Hz, 2H) 2.16 - 2.30 (m, 6H) 1.58 - 1.71 (m, 4H) 1.27 - 1.51 (m, 10H) 0.92 (t, <i>J</i> =7.4 Hz, 3H)

18	WO2010/018134 Example 55	363	¹ H NMR δ (400 MHz, DMSO- <i>d</i> ₆) 6.40 (br s, 2H) 4.10 - 4.19 (m, 2H) 3.64 (t, <i>J</i> =6.8 Hz, 2H) 2.75 - 2.86 (m, 1H) 2.28 - 2.39 (m, 1H) 2.03 (t, <i>J</i> =10.9 Hz, 1H) 1.55 - 1.74 (m, 5H) 1.04 - 1.55 (m, 10H) 0.83 - 0.97 (m, 4H)
19	WO2010/018134 Example 107	363	¹ H NMR δ (400 MHz, DMSO- <i>d</i> ₆) 6.40 (br s, 2H) 4.14 (t, <i>J</i> =6.4 Hz, 2H) 3.65 (t, <i>J</i> =6.5 Hz, 2H) 2.88 (d, <i>J</i> =11.3 Hz, 2H) 2.40 (t, <i>J</i> =11.5 Hz, 2H) 1.57 - 1.69 (m, 4H) 1.51 (d, <i>J</i> =11.3 Hz, 2H) 1.33 - 1.45 (m, 2H) 1.20 (br m, 5H) 0.86 - 0.99 (m, 4H)
20	WO2010/018134 Example 109	405	¹ H NMR δ (600 MHz, DMSO- <i>d</i> ₆) 9.95 (br s, 1H) 6.42 (br s, 2H) 4.14 (t, <i>J</i> =6.6 Hz, 2H) 3.65 (t, <i>J</i> =7.0 Hz, 3H) 3.01 (d, <i>J</i> =11.0 Hz, 3H) 2.47 (br s, 1H) 1.56 - 1.73 (m, 6H) 1.34 - 1.43 (m, 2H) 1.12 - 1.33 (m, 7H) 1.07 (d, <i>J</i> =6.8 Hz, 6H) 0.91 (t, <i>J</i> =7.4 Hz, 3H)
21	WO2010/018131 Example 29	406	¹ H NMR δ (400 MHz, DMSO- <i>d</i> ₆) 9.84 (br s, 1H) 6.39 (br s, 2H) 4.15 (t, <i>J</i> =6.7 Hz, 2H) 3.67 (t, <i>J</i> =6.9 Hz, 2H) 2.15 - 2.44 (m, 10H) 1.58 - 1.72 (m, 4H) 1.31 - 1.46 (m, 4H) 0.86 - 1.00 (m, 9H)
22	WO2010/018133 Example 5	349	¹ H NMR δ (400 MHz, DMSO- <i>d</i> ₆) 9.91 (br s, 1H) 6.40 (br s, 2H) 4.15 (t, <i>J</i> =6.7 Hz, 2H) 3.66 (t, <i>J</i> =6.9 Hz, 2H) 2.31 - 2.40 (m, 6H) 1.58 - 1.72 (m, 8H) 1.33 - 1.44 (m, 4H) 0.92 (t, <i>J</i> =7.4 Hz, 3H)
23	WO2010/018133 Example 7	377	¹ H NMR δ (400 MHz, DMSO- <i>d</i> ₆) 10.01 (br s, 1H) 6.42 (br s, 2H) 4.14 (t, <i>J</i> =6.5 Hz, 2H) 3.66 (t, <i>J</i> =7.0 Hz, 2H) 2.40 (t, <i>J</i> =7.2 Hz, 2H) 1.58 - 1.71 (m, 4H) 1.45 - 1.56 (br m, 8H) 1.29 - 1.44 (m, 4H) 0.92 (t, <i>J</i> =7.4 Hz, 3H)
24	Prepared similarly to Compound 22	365	¹ H NMR δ (400 MHz, chloroform- <i>d</i>) 5.89 (br s, 2H) 4.28 (t, <i>J</i> =6.7 Hz, 2H) 3.88 (t, <i>J</i> =7.0 Hz, 2H) 3.67 - 3.74 (m, 4H) 2.31 - 2.45 (m, 6H) 1.71 - 1.87 (m, 4H) 1.43 - 1.59 (m, 4H) 0.97 (t, <i>J</i> =7.4 Hz, 3H)
25	Prepared similarly to Compound 22	335	¹ H NMR δ (400 MHz, methanol- <i>d</i> ₄) 4.53 (t, <i>J</i> =6.5 Hz, 2H) 4.32 (t, <i>J</i> =5.5 Hz, 2H) 3.80 (d, <i>J</i> =12.5 Hz, 2H) 3.54 (t, <i>J</i> =5.5 Hz, 2H) 2.96 - 3.08 (m, 2H) 1.77 - 2.01 (m, 7H) 1.46 - 1.61 (m, 3H) 1.01 (t, <i>J</i> =7.5 Hz, 3H)
26	WO2010/018133 Example 34	349	¹ H NMR δ (400 MHz, DMSO- <i>d</i> ₆) 6.40 (br s., 2H) 4.15 (t, <i>J</i> =6.7 Hz, 2H) 3.67 (t, <i>J</i> =7.0 Hz, 1H) 2.16 - 2.30 (m, 6H) 1.71 - 1.83 (m, 2H) 1.57 - 1.69 (m, 2H) 1.26 - 1.48 (m, 8H) 0.91 (t, <i>J</i> =7.4 Hz, 3H)

27	WO2010/018133 Example 10	377	^1H NMR δ (400 MHz, DMSO- d_6) 9.88 (br s, 1H) 6.39 (br s, 2H) 4.14 (t, J =6.5 Hz, 2H) 3.65 (t, J =6.9 Hz, 2H) 3.17 (d, J =5.0 Hz, 1H) 2.19 - 2.30 (m, 4H) 2.11 - 2.18 (m, 2H) 1.58 - 1.70 (m, 4H) 1.29 - 1.49 (m, 10H) 1.15 - 1.27 (m, 2H) 0.92 (t, J =7.3 Hz, 3H)
28	WO2010/018133 Example 14	391	^1H NMR δ (400 MHz, DMSO- d_6) 6.41 (br s, 2H) 4.14 (t, J =6.5 Hz, 2H) 3.64 (t, J =6.9 Hz, 2H) 2.18 - 2.28 (m, 4H) 2.14 (t, J =7.3 Hz, 2H) 1.58 - 1.68 (m, 4H) 1.17 - 1.50 (m, 15H) 0.91 (t, J =7.4 Hz, 3H)
29	WO2010/018133 Example 20	377	^1H NMR δ (400 MHz, DMSO- d_6) 9.82 (br s, 1H) 6.34 (br s, 2H) 5.00 (sxt, J =6.3 Hz, 1H) 3.66 (t, J =6.9 Hz, 2H) 2.16 - 2.29 (m, 6H) 1.55 - 1.69 (m, 3H) 1.26 - 1.54 (m, 11H) 1.21 (d, J =6.0 Hz, 3H) 0.89 (t, J =7.3 Hz, 3H)
30	WO2010/018133 Example 25	405	^1H NMR δ (400 MHz, DMSO- d_6) 9.87 (br s, 1H) 6.35 (br s, 2H) 4.98 (sxt, J =6.3 Hz, 1H) 3.65 (t, J =6.9 Hz, 2H) 2.16 - 2.29 (m, 6H) 1.56 - 1.70 (m, 3H) 1.25 - 1.48 (m, 13H) 1.21 (d, J =6.0 Hz, 3H) 0.82 - 0.89 (m, 3H)
31	WO2010/018133 Example 28	389	^1H NMR δ (400 MHz, DMSO- d_6) 9.86 (br s, 1H) 6.35 (s, 2H) 5.19 - 5.25 (m, 1H) 3.66 (t, J =6.9 Hz, 2H) 2.17 - 2.28 (m, 6H) 1.84 - 1.95 (m, 2H) 1.52 - 1.72 (m, 8H) 1.30 - 1.47 (m, 8H)
33	WO2010/018133 Example 33	377	^1H NMR δ (400 MHz, DMSO- d_6) 6.39 (br s, 2H) 4.90 (sxt, J =6.3 Hz, 1H) 3.64 (t, J =6.9 Hz, 2H) 2.19 - 2.28 (m, 4H) 2.12 - 2.18 (m, 2H) 1.50 - 1.70 (m, 4H) 1.18 - 1.48 (m, 13H) 0.89 (t, J =7.4 Hz, 3H)
34	WO2010/018133 Example 26	365	^1H NMR δ (400 MHz, DMSO- d_6) 9.85 (br s, 1H) 6.36 (s, 2H) 4.98 - 5.12 (m, 1H) 3.64 (t, J =7.1 Hz, 2H) 2.23 (br s, 4H) 2.15 (t, J =7.1 Hz, 2H) 1.58 - 1.70 (m, 2H) 1.29 - 1.49 (m, 8H) 1.16 - 1.28 (m, 8H)

PBMC screening assays

All blood samples used for experimental studies from human volunteers were collected in accordance with the Stevenage blood donation unit consent & ethics.

Assay A: Induction of IFN α using Cryopreserved Human PBMC

Preparation of PBMC. Blood samples of up to 200 mL were collected into sodium heparin (10 U/mL) from healthy human volunteers. Whole blood in 25 mL volumes was overlaid onto 15 mL Ficoll gradients in Leucosep tubes, and centrifuged at 1000g for 20 min. Cells in the band at the plasma/histopaque interface were carefully removed and washed twice with PBS (centrifuged at 400g for 5 min). The final pellet was resuspended in freezing medium (90% Heat-inactivated serum, 10% DMSO) to a cell concentration of 4×10^7 cells/mL. The re-suspended cells were then cryopreserved using a rate controlled freezer, and stored at -140 °C for up to 4 months.

Compound Preparation. Compounds were dissolved in DMSO. Serial 2-fold dilutions with DMSO were prepared and 0.25 μ L dispensed into 384-well clear Greiner polypropylene plates

Incubation and Assay for IFN α . Immediately prior to assay, vials of cryopreserved PBMC were thawed rapidly in a water bath at 37 °C. The PBMCs were then diluted in growth media [RPMI 1640 containing 10% fetal calf serum (Invitrogen), Penicillin+Streptavidin 100 U/mL, L-Glutamine 2 mM, and 1000 units/ml recombinant human IFN- γ (Preprotech catalogue #300-02)] to a density of 1×10^6 cells/ml, and 50 μ L/well dispensed to 384-well clear Greiner polypropylene plates containing 0.25 μ L DMSO or test compound in 0.25 μ L DMSO. Top final concentration of compound was typically 50 μ M or 5 μ M (to obtain curve fit for highly active compounds). Plates were incubated for 24 hours (37 °C, 95% air, 5% CO₂). A multi-isoform immunoassay was used to quantify IFN α in PBMC supernatants. Rabbit polyclonal antibody against human IFN α (catalogue number 31101, Stratech Scientific) was diluted 1:10000 in assay buffer (RPMI 1640 containing 10% fetal calf serum, Invitrogen) and 20 μ L was added to each well of an MSD (Meso-Scale Discovery) single small-spot 384-well GAR (goat anti-rabbit antibody coated) plate. The plate was incubated for 1 hour at room temperature with vigorous shaking. Following three washes with PBS, 20 μ L of cell supernatant were added to each well of the plate. The plate was then incubated for 1 hour at

room temperature with vigorous shaking. A pair of monoclonal antibodies to IFN α (catalogue numbers 21100 and 21112, Stratech Scientific) were labelled with sulfo-TAG (MSD), diluted 1:1000 in assay buffer and 20 μ L added to each well of the plate. The plate was further incubated for 1 hour at room temperature with vigorous shaking. Following three washes with PBS, 30 μ L of x2 T buffer (MSD) was added to each well and the plate was read on an MSD Sector 6000 plate reader. Data were normalised to internal plate controls of 1 μ M resiquimod (n=16) and DMSO (n=16). pEC₅₀ values were derived by 4-parameter curve fit with IRLS in ActivityBase, from 11-point, two-fold serial dilution of test compounds.

PBMC screening assay B: Induction of IFN α and TNF α using Fresh Human PBMC

Preparation of PBMC. Blood samples were collected from human volunteers into sodium heparin (10 U/mL). 25 mL volumes of whole blood were overlaid onto 15 mL Histopaque in Leucosep tubes which were centrifuged at 800g_{av} for 20 minutes and the band at the plasma/histopaque interface carefully removed. The collected cells were centrifuged at 800g_{av} for 10 minutes and the pellet resuspended in 10mL of media (RPMI 1640 (Low endotoxin) supplemented with 10% v/v foetal calf serum (FCS, low endotoxin) 100 U/mL penicillin G, 100 g/mL streptomycin, 10 mM L-glutamine and 1x non-essential amino acids. The PBMCs were diluted to give a final concentration of 2x10⁶/mL and 100 μ L of this cells suspension was added to assay wells.

Compound preparation. Compounds were dissolved and serially diluted in DMSO to give 100x the required concentration range using a Biomek 2000. 1 μ L of test compound was transferred into 96-well tissue culture plates using a Biomek FX. Each compound was assayed in duplicate for each donor. Each plate contained a dilution series of the TLR7/8 agonist Resiquimod as a standard and additional wells of 2 μ M final concentration Resiquimod, used to define the approximate maximal response.

Incubation and Assays for IFN α and TNF α . The cell preparations were incubated for 24 hours (37 °C, 95% air, 5% CO₂) after which a sample of the supernatant was removed using the Biomek FX and assayed for both IFN α and TNF α using the MSD (Mesoscale Discovery) electrochemiluminescence assay platform. The IFN α assay was carried out similarly to that described above. The TNF α assay was carried out as per kit instructions (Cat No K111BHB). Cytokine released was expressed as a percentage of the 2 μ M Resiquimod control (column 11). This percentage was plotted against compound concentration and the pEC₅₀ for the

response determined by non-linear least squares curve fitting. For the IFN α responses generally a 4 parameter logistic model was selected. For the TNF α responses where a clear maximum response was obtained (i.e. a well defined plateau in the response was observed) then a 4 parameter model was generally used. If the upper asymptote of the curve wasn't well defined then the curve fitting was generally constrained to a maximal response of 100% (i.e. to the response to 2 μ M Resiquimod) or to the response of the highest concentration tested if this was greater than the resiquimod response. Some curves were bell shaped for one or both cytokines and the cytokine data on the down slope of the bell shaped response (i.e. concentrations above those giving the maximal response) were generally excluded from the fit, usually with the exception of the concentration immediately above the peak response. Curve fitting thus concentrated on the up slope of the dose response curve. The control Resiquimod showed pEC₅₀ values for IFN α and TNF α induction of 7.0 and 6.1 respectively.

20h Whole Blood and PBMC profiling of compound 32 (Table 6)

Preparation of whole blood. Human whole blood was taken by venipuncture from healthy volunteers in accordance with the GSK Stevenage blood donation unit consent & ethics. Blood was heparinised with sodium heparin (10 IU/mL) (Leo Pharmaceuticals) and used undiluted in whole blood assays.

PBMC prepared as detailed in PBMC screening assay B.

Preparation of compound plates. Compound 32 and Resiquimod were supplied as 10mM stock solutions in dimethyl sulfoxide (DMSO). These were diluted to 100x final assay concentration (5 mM starting concentration) followed by serial dilutions (1 in 5) in DMSO across the plate using the Biomek 2000 robot. Diluted 32 was then stamped into 96 well daughter plates (1 μ L for PBMC assays and 1.5 μ L for whole blood assays) using the Biomek FX, giving a final concentration range between 50 μ M to 25.6 pM. Resiquimod (final concentration, 2 μ M) was present on each plate as a control to standardise cytokine responses between individuals.

Cell culture conditions

Whole blood plates. Heparinised whole blood (150 μ L) was added to the required test wells containing appropriately diluted compound 32 (1.5 μ L). The plate was incubated as above for

20 hours then mixed on an orbital shaker for 1 minute until the plasma and red blood cells recombined. Sterile PBS (70 μ L, containing 10 IU/mL heparin) was added to the wells containing blood and further mixed as above. The plate was centrifuged at $800g_{av}$ for 10 minutes and diluted plasma was collected using a Biomek FX liquid handling robot. The plasma cytokine levels were determined by MSD assays (IFN α , Th1/2 10-plex or TNF α or a custom 4 plex assay detecting TNF α , IFN γ , IL-10 and IL-1 β or IL-1 β , granulocyte-macrophage colony stimulating factor (GMCSF), IL-6 and TNF α).

PBMC plates. PBMC (100 μ L) at 2×10^6 cells/mL were added to a 96 well plate containing appropriately diluted compound **32** (1 μ L). The plate was incubated as above for 20 hours, then centrifuged at $800g_{av}$ for 10 minutes. Supernatants were collected using a Biomek FX liquid handling robot. The supernatant cytokine levels were determined by MSD assays (IFN α , Th1/2 10-plex or TNF α , or a custom 4 plex assay detecting TNF α , IFN γ , IL-10 and IL-1 β).

Cytokine analysis

IFN α MSD assay. Goat anti-rabbit IgG plates (MSD: cat L41RA-1) were blocked with 4% bovine serum albumin in PBS (150 μ L/well, BSA/PBS) for 1 hour at room temperature (RT) with orbital shaking. Capture rabbit polyclonal anti-human IFN α (Stratech Scientific Ltd: cat 31101-1, 1 in 4600 dilution; or in house, 1 in 10000 dilution, R012/008, animal Cassie821059) were diluted in MSD assay buffer and added onto the plate (25 μ L/well). The plate was left at 4 °C overnight. The plate was washed three times with PBS/Tween 20 (0.05% v/v) (PBS/Tw) and blotted dry. Supernatants from whole blood or PBMC assays were loaded onto the plate (25 μ L/well). In addition, each plate also contained an IFN α standard curve (0 to 10000pg/mL, 1 in 3 dilution) (R&D systems: cat 11100-1). Samples and standard were incubated for 2 hours at RT with orbital shaking followed by washing three times with PBS/Tw. Sulfo labelled mouse monoclonal anti-human IFN α (25 μ L/well; Stratech Scientific Ltd: MMHA-11 (cat 21112-9) and MMHA-2 (cat 21100-9)) antibody (1 in 600 or 1 in 1000 dilution, dependent on batch) were added and the plate was incubated for 1 hour at RT with orbital shaking. Following incubation, the plate was washed three times with PBS/Tw and 150 μ L/well of 2x read buffer T (MSD: cat R92TC-1) was added and quantified on the MSD Sector Imager 6000.

Th1/Th2 MSD 10-plex or TNF α MSD assays. Th1/Th2 cytokine plates (MSD: cat N05010B-1) or TNF α plates (MSD: cat L451BHB) were used according to manufacturer's instructions. Total secreted cytokine concentrations (pg/mL) from whole blood and PBMC were determined from cytokine standard curves prepared in each assay using MSD software. Assays were carried out in duplicate determinations in each individual and values were averaged before pEC₅₀ and EC₅₀ values were calculated (where applicable). In cases where samples from one individual were used more than once, pEC₅₀ values were determined separately and averaged. Values were fitted by non-linear least squares curves (4 parameter logistic equation) and pEC₅₀ values were determined using GraphPad Prism v5.01 (GraphPad software, La Jolla, CA, USA).

Impact of compound 32 on allergen-driven cytokine response in PBMC from allergic donors (Figure 6)

Preparation of PBMC. Defibrinated human blood was centrifuged at 800 g_{av} for 15 minutes. The upper layer of serum was collected and heat inactivated (HI) at 56 °C for 30 minutes. The cell pellet was resuspended in 50 mL PBS and 25 mL was layered onto 20 mL of Ficoll (GE Healthcare Biosciences) in 50 mL centrifuge tubes and centrifuged at 800 g_{av} for 25 minutes. PBMC were collected from the interface and washed with PBS by centrifugation (250 g_{av} , 10 minutes). The cell pellet was resuspended in assay medium (RPMI 1640 supplemented with 20% HI autologous serum, 2 mM glutamine, and 100 U/mL Penicillin/Streptomycin) at 4x10⁶ cells/mL.

Compound **32** was supplied as 10mM stock solution in DMSO. The compound was diluted in assay medium to give 4x final assay concentration (4 uM starting concentration) followed by serial dilutions (1 in 5) across the plate. The DMSO content (0.04%) was consistent in all samples. Triplicate cultures were set up at all concentrations.

Lyophilised allergen preparations (Aquagen SQ; HDM; *D.pteronyssinus* and Tg; *P.pratense*, both from Alk Abello) were added to cultures at a final concentration of 10 μ g/mL.

PBMC plates. PBMC (100 μ L, at 4x10⁶ cells/mL) were added to a 96 well plate containing medium or allergen (10 μ g/mL Tg or HDM [(Aquagen SQ; HDM; *D.pteronyssinus* and Tg; *P.pratense*, both from Alk Abello), with vehicle (0.04% DMSO)). These cultures represented

negative and positive controls respectively. Appropriately diluted compound **32** was added to allergen treated cultures at a final concentration range from 1uM to 64pM in a total volume of 200 μ L. The plate was incubated as above for 6 days. Supernatant was collected from each well and stored at -20 °C prior to analysis. Cytokine levels in supernatants were detected using Meso Scale Discovery (MSD) assays (Th1/Th2 10-plex) as described above..

Data Analysis. Cytokine concentrations (pg/mL) were determined from cytokine standard curves prepared in the assay using MSD software. A total of 14 experiments using 7 HDM allergic individuals, and 15 experiments using 9 Tg allergic individuals were analysed. In cases where an individual was used in more than one experiment, the data were averaged and counted as n=1. The lower three points of each curve was derived from n=3 HDM allergic and n=4 Tg allergic individuals.

IL-5, IL-13 and IL-10 data analysis. Individuals produce different levels of IL-5, IL-13 and IL-10 in response to allergen. To allow comparison of data from all individuals, responses for these cytokines were expressed as a percentage of the allergen driven, vehicle control, response to HDM or Tg.

IFN γ , IL-1 β , IL-12p70 and TNF α data analysis. IFN γ , IL-1 β , IL-12p70 and TNF α production in cultures was driven by compound **32**. Data for these cytokines were expressed as a percentage of the cytokine levels produced at 1uM compound **32**.

In vivo studies

All *in vivo* studies were conducted in accordance with the GSK Policy on the Care, Welfare and Treatment of Laboratory Animals and were reviewed by the Institutional Animal Care and Use Committee at GSK, and/or by the ethical review process at the institution where the work was performed.

Cytokine responses following intranasal dosing of compound 32 in BALB/c mice (Figure 7).

Mice (female BALB/c strain, 20 g body weight) were dosed intranasally (5 μ L administered between the nostrils) under light anaesthesia with either vehicle (0.2% Tween-80 in saline) or compound **32** (n=6/group). Compound **32** was prepared as an aqueous suspension (maximum 5 mg/mL) by adding 0.2% Tween-80 in saline. The animals were euthanized 2 or 6 hours after dosing by administration of an overdose of sodium pentobarbitone. The trachea was

exposed and cannulated towards the nose. Phosphate buffered saline (PBS, 0.5 mL) containing 3.72 g/L EDTA, 0.1% bovine serum albumin (BSA) and protease inhibitors was flushed through the nose and collected as it dripped from the nostrils (nasal lavage fluid). A blood sample was then taken by cutting the jugular veins to provide a serum sample. Nasal lavage fluid and serum samples were stored at -20 °C prior to assay of IFN α and IP-10 levels by ELISA.

IFN α ELISA assay. IFN α levels in serum and nasal lavage samples were determined by ELISA (PBL Mouse Interferon Alpha ELISA, R&D systems) according to the manufacturer's instructions.

IP-10 ELISA assay. IP-10 levels in serum and nasal lavage samples were determined by ELISA (Mouse IP-10 Quantikine, R&D systems) according to manufacturer's instructions.

***In vivo* PD studies: Cytokine and transcriptomic responses following intranasal dosing of compound 32 in Cynomolgus monkeys (Figure 8 and Table 7)**

Cytokine study. Data collected during an 8 week (Once weekly dosing) intranasal toxicity study. Compound 32 maleate salt was formulated as a solution at concentrations of 0.1, 1, 10 and 100 μ g/mL in 0.015% (w/w) Benzalkonium chloride (BKC) and 0.015% EDTA (w/w) in 0.9% (w/v) aqueous sodium chloride (pH 5 $\pm$ 0.5). Intranasal dosing of these solutions to Cynomolgus monkeys (3/sex/group) at a dose volume of 100 μ L/dose (50 μ L/nostril) gave nominal doses of 0 (vehicle), 3, 30, 300 or 3000 ng/kg (based on a 3 kg monkey). Blood samples (1.0 mL) were obtained by femoral venipuncture from all animals. Serial blood samples, nominally 0 (1 hour predose) 2, 6 and 24 hours postdose were collected on Day 1 of the study. The blood was collected into K2 EDTA coated tubes. Samples were mixed gently and placed on crushed wet-ice until centrifugation. The samples were centrifuged for approximately 10 minutes in a refrigerated centrifuge (approximately 4°C) at 2700 rpm. The resultant plasma was separated, transferred to uniquely labeled clear polypropylene tubes and frozen over solid carbon dioxide (dry ice) or in a freezer set to maintain -70°C or below. Cytokines IFN α , IFN γ , IP-10, MIP-1 β , TNF α , IL-6, MCP-1, IL-1R antagonist, sCD40L, G-CSF and CRP in plasma (25 to 100 μ L, neat or diluted as necessary) were measured by validated multiplex bead-based immunoassays or ELISA. CRP was measured using the Roche Diagnostics (Indianapolis, IN) CRP method on ADVIA 1650 Chemistry System (Siemens Corporation, Tarrytown, NY).

Transcriptomic study. Compound **32** maleate salt was formulated as a solution in 0.015% Benzalkonium chloride (BKC) and 0.015% EDTA in 0.9% aqueous sodium chloride with a final pH adjusted to approximately pH 5. Concentrations of 0.1 and 1 µg/mL administered once to each monkey at a dose volume of 50 µL/actuation/nostril resulted in nominal doses of 3 and 30 ng/kg, respectively (based on a 3 kg monkey). The device used for dosing was a clinical nasal spray applicator (Valois Type 7 pump) designed to deliver the correct dose (50 µL/shot). Compound **32** maleate salt was administered intranasally on Day 1 to male monkeys (3/group). Cytokines and related analytes (IFN α , IP-10, IL-6, IL-1R antagonist, MCP-1, TNF α and G-CSF) were measured in plasma from whole blood samples that were collected from monkeys on Day 1 (pre dose, 6 and 24 hours after dosing). A single nasal scrape from each monkey was collected on Day 1 (6 and 24 hours after dosing, from the left and right nostril, respectively) for TaqManTM gene expression analysis of interferon responsive genes including OAS1, OAS2, OAS3, IFIT2 (ISG-54), IFIT3, IFIT5, CCL2 (MCP-1), CCL8 (MCP-2), CXCL11, GBP-1, MIG (CXCL9), MX1, CXCL10, IRF1, IRF6, IRF7, IL1RN, ADAR, IFNA1, IFNA2, IFNA4, IFNB1, and IFNB2, and an inflammatory response gene TNF α .

LCMS and MDAP Methods

Three LCMS systems (A-C) were employed to monitor reactions and assess purity of products whilst preparative purification was conducted using Mass-directed autopreparative HPLC (MDAP). Mass spectra in each case were recorded on a Waters ZQ mass spectrometer using alternative-scan positive and negative mode electrospray ionisation, and the UV detection range was 210 to 350nm

LCMS System A

Sunfire C₁₈ column (30 mm x 4.6 mm ID, 3.5 µm), flow rate 3 mL/min, temperature 30 °C. UV detection range: Solvents, A: 0.1% v/v solution of formic acid in water and B: 0.1% v/v solution of formic acid in acetonitrile.

Gradient:

Time (min)	A%	B%
0	97	3
0.1	97	3
4.2	0	100
4.8	0	100
4.9	97	3
5.0	97	3

LCMS System B

Acquity UPLC BEH C₁₈Column (50 mm x 2.1 mm ID, 1.7 µm), flow rate 1 mL/min, temperature 40 °C. Solvents, A: 10 mM aqueous ammonium bicarbonate adjusted to pH10 with ammonia solution and B: acetonitrile

Gradient:

Time (min)	A%	B%
0	99	1
1.5	3	97
1.9	3	97
2.0	0	100

LCMS System C

XBridge C₁₈ column Column (50 mm x 4.6 mm ID, 3.5 µm), flow rate 3 mL/min, temperature 30 °C. Solvents, A: 10mM aqueous ammonium bicarbonate adjusted to pH10 with ammonia solution and B: acetonitrile

Gradient:

Time (min)	A%	B%
0	99	1
0.1	99	1
4.0	3	97
5.0	3	97

MDAP

XBridge C₁₈ column (typically 150 mm x 19 mm i.d. 5 µm packing diameter), flow rate 20 mL/min, ambient temperature. Solvents, A:10 mM aqueous ammonium bicarbonate adjusted to pH 10 with ammonia solution and B: acetonitrile run as a gradient over either 15 or 25 minutes.