Potent anti-inflammatory effects of an H_2S-releasing naproxen (ATB-346) in a human model of inflammation

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NON-STANDARD ABBREVIATIONS

ANOVA – Analysis of variance

BD – Becton Dickinson

CCR – C-C chemokine receptor

CD – Cluster of differentiation

COX - cyclooxygenase

CRP – C-reactive protein

EDTA - Ethylenediaminetetraacetic acid

ELISA – Enzyme-linked immunosorbent assay

FBC – Full blood count

FCS – Fetal calf serum

GI – Gastrointestinal

H_2S – Hydrogen sulfide

IL – Interleukin

inHg – Inches of mercury

LFT – Liver function tests

LPS – Lipopolysaccharide

MFI – Mean fluorescence intensity

MP – Mononuclear phagocyte

NADPH – Nicotinamide adenine dinucleotide phosphate

NHS – National Health Service

NSAID – Non-steroidal anti-inflammatory

OD – Optical density

PBS – Phosphate-buffered saline

PGE_2 – Prostaglandin E_2

SEM – Standard error of the mean

TNF-α – Tumour necrosis factor alpha

U+E – Urea and electrolytes

UV-KEc – Ultraviolet-killed *Escherichia coli*

ABSTRACT

ATB-346 is a hydrogen sulfide-releasing non-steroidal anti-inflammatory drug (H_2S-NSAID) derived from naproxen, which in preclinical studies has been shown to have markedly reduced gastrointestinal adverse effects. However, its anti-inflammatory properties in humans compared to naproxen are yet to be confirmed. To test this, we used a dermal model of acute inflammation in healthy, human volunteers, triggered by ultraviolet-killed *Escherichia coli*. This robust model allows quantification of the cardinal signs of inflammation along with cellular and humoral factors accumulating within the inflamed skin. ATB-346 was non-inferior to naproxen in terms of its inhibition of cyclooxygenase activity as well as pain and tenderness. ATB-346 significantly inhibited neutrophil infiltration at the site of inflammation at 4h, compared to untreated controls. Subjects treated with ATB-346 also experienced significantly reduced pain and tenderness compared to health controls. Furthermore, both classical and intermediate monocyte subsets infiltrating the site of inflammation at 48h expressed significantly lower levels of CD14 compared to untreated controls, demonstrating a shift towards an anti-inflammatory phenotype. Collectively, we have shown for the first time in humans that ATB-346 is potently anti-inflammatory and propose that ATB-346 represents the next generation of H_2S-NSAIDs, as a viable alternative to conventional NSAIDs, with reduced adverse effects profile.

INTRODUCTION

Inflammation is a protective response against infection or injury. However, when it becomes dysregulated as a consequence of environmental factors, genetic abnormalities or the ageing process, our immune system has the capacity to cause extensive damage (1). Inflammatory arthritis (2), asthma (3), chronic obstructive pulmonary disease (4), Alzheimer's disease and even cancer, while aetiologically disparate, are diseases unified by a dysregulated immune component (5). A key strategy for treating inflammation is based, largely, upon inhibiting the factors that drive this process and reducing pain. Such treatments include non-steroidal anti-inflammatory drugs (NSAIDs) such as naproxen, glucocorticoid steroids (prednisolone) and 'biologic' drugs such as infliximab (anti-tumour necrosis factor-alpha; TNF-α), anakinra (anti-interleukin-1) and tocilizumab (anti-interleukin-6) (6).

Although these treatments ameliorate disease symptoms, they do not bring a 'cure' and are ineffective in a significant subset of patients. This can lead to patients turning to opioids, which whilst often effective at reducing pain, can lead to increasing tolerance and dependence (7, 8). Furthermore, adverse effects can hamper endogenous homeostatic systems, predisposing to serious infection (9–11). Of note, NSAIDs increase cardiovascular risk (12–15) as well as causing unacceptably high levels of gastrointestinal (GI) upset (13, 15), with mucosal irritation and even ulcer formation, often leading to cessation of drug use. Such GI effects may, to an extent, be ameliorated by the co-administration of gastroprotective drugs, such as proton pump inhibitors (16), but given the growing problem of polypharmacy and the propensity for untoward drug interactions, clearly single agents are preferable where at all possible. Thus, there is a need to develop more efficient and effective therapeutics; with one approach being to harness the body's own healing process for therapeutic gain. Further, given the ongoing opioid crisis (17), non-addictive, efficacious alternatives are becoming increasingly relevant.

In this regard, attention has turned to an emerging class of compounds with significant anti-inflammatory effects, the hydrogen sulfide (H_2S)-releasing NSAIDs (H_2S-NSAIDs). These consist of a conventional NSAID to which an H_2S-reasing moiety is covalently attached (18). One such H_2S-NSAID, ATB-346, has shown a markedly reduced gastrointestinal adverse effect profile (in terms of ulceration and bleeding) in animal studies (19). Furthermore, it was shown that after oral administration of ATB-346, plasma levels of naproxen derived from the

compound were much lower than equimolar doses of naproxen but exhibited cyclooxygenase (COX) inhibition and analgesic effects (18). Indeed, ATB-346 is in the order of 6-times more potent than naproxen in humans and is as effective at suppressing COX activity over a 24-hour period with a single dose (versus twice-daily naproxen) (20). In a recent Phase 2 trial in healthy volunteers (21), the incidence of upper GI ulceration in subjects taking standard dose twice daily naproxen was over 42%; significantly greater than the rate of ulceration of only 2.5% ($p < 0.001$) in subjects taking an equi-effective (in terms of COX inhibition) dose of ATB-346 daily.

Using a novel model of ultraviolet (UV)-killed *Escherichia coli* (UV-KEc)-triggered resolving dermal acute inflammation (22), we set out to investigate the anti-inflammatory role of ATB-346 in humans. Comparing this to its native counterpart, naproxen, and untreated controls we aimed to evaluate whether next generation H_2S-NSAIDs, whilst demonstrating lower GI adverse effect, are still able to retain their anti-inflammatory effect in humans.

MATERIALS AND METHODS

Ethics statement

The study was approved by University College London (UCL) Institutional Ethics Committee (Project Number: 10527/001). Written, informed consent was taken from all volunteers prior to voluntary participation in the study. All data were used and stored in line with the General Data Protection Regulation (2018). All procedures were carried out in line with the Helsinki Declaration, adopted by the 18th World Medical Assembly, as amended in 1983.

Volunteers

Twenty-three healthy, male volunteers aged 18-50 were recruited. Exclusion criteria included chronic inflammatory illness, allergy to NSAIDs, recent illness (within the last seven days), use of regularly prescribed medication or over-the-counter medication within the last seven days, vaccination within the last three months, smokers, use of recreational drugs, and those enrolled in another study. During the study, participants were asked to refrain from caffeinated products, alcohol and heavy exercise.

Volunteers were randomly allocated to one of three treatment arms in this single-blind study, with the experimental subjects unaware of which arm they had been allocated to. Seven volunteers were assigned to the naproxen arm and took 500 mg twice daily for three days prior to UV-KEc injection. Seven volunteers were assigned to the ATB-346 arm and took 250 mg once daily (the equi-effective dose to naproxen) for three days prior to injection. Seven volunteers took no drug during the study and served as the untreated control group. One volunteer from each of the naproxen and untreated control groups dropped out before completion of the study; one giving no reason and the other because they were already enrolled in another study.

UV-KEc preparation and injection

E. coli (Strain: The National Collection of Type Cultures 10418, Source: Public Health England, UK) were prepared as previously described (22, 23). In brief, bacteria were incubated overnight in Luria Broth (Sigma-Aldrich, Missouri, USA) at 37 °C. The following day they

were washed twice in sterile phosphate-buffered saline (PBS) (2500 *g* for 20 minutes at 4 °C). Counts were determined by optical density (OD) measurement where OD_{600} = 0.365 equated to 10^8 *E. coli*/mL. Bacteria were re-suspended in a sterile Petri dish and exposed to UV light for 60 minutes (302 nm, ChemiDoc, Bio-Rad laboratories, California, USA). The UV-KEc were re-suspended in 0.9% sodium chloride and aliquotted into sterile Eppendorf tubes obtaining a final count of 1.5 x 10^7/100 µL. Samples were confirmed be to non-viable by the Environmental Research Laboratory, University College London Hospitals NHS Foundation Trust and stored at -80 °C. Aliquots were thawed immediately prior to intradermal injection.

Intradermal injection of UV-KEc

All procedures were carried out under aseptic, non-touch technique under the supervision of the study clinician. The volar aspect of both forearms of each volunteer was cleaned using 70% isopropyl alcohol and 1.5 x 10^7/100 µL UV-KEc in 100 µL of 0.9% sodium chloride were injected via the intradermal route into the marked site 7 cm from the antecubital fossa. This dose is known to elicit a consistent, safe and localised reaction, leading to redness (measuring 5-10 cm at maximum in diameter), mild tenderness and warmth at the site of the UV-KEc injection. Inflammation was allowed to ensue and resolve for the duration of the study with clinical measurements, laser Doppler imaging and peripheral venous blood taken at pre-defined time-points representing baseline (0 hr), onset of inflammation (4h) and resolution phases (24h and 48h) (Figure 4C).

Peripheral blood analysis

A full blood count (FBC), urea and electrolytes (U+E), liver function tests (LFT) and C-reactive protein (CRP) were taken for each volunteer during the screening process. At each of the time-points, an FBC and CRP were collected using ethylenediaminetetraacetic acid (EDTA)-anti-coagulated and spray-coated silica and polymer gel-containing Vacutainers® (Becton Dickinson, New Jersey, USA), respectively. All peripheral blood samples were processed at The Doctor's Laboratory Ltd. (The Halo Building, London, UK). At each time-point, heparin-anticoagulated blood was centrifuged at 2000 *g* for 10 minutes at room temperature to separate the plasma, which was split into 500 µL aliquots using Protein LoBind Eppendorfs and stored at -80 °C for subsequent soluble mediator analysis.

Laser Doppler imaging

A laser Doppler imager (moor LDI-HIR, Moor Instruments Ltd., Devon, UK) was used to acquire data quantifying microvascular hyper-reactivity. At the pre-defined time-points described above, the forearm was placed under the scanner at a set distance of 58 cm. The scanner emits a laser which is scattered by erythrocytes. The resulting Doppler shift is dependent on the velocity and concentration of cells at the site and therefore represents blood flow and thus leucocyte trafficking. The obtained data are displayed as colour-coded images, which can then be analysed using the moorLDI software (Version 6.1) representing arbitrary perfusion units (blood flow), a hitherto unappreciated marker of resolution at the site of inflammation. Total flux (blood flow) was calculated by multiplying the number of 'valid' pixels (those demonstrating blood flow above the background level of 300 perfusion units) and the mean blood flow of these valid pixels (Figure 4C).

Clinical measurements

As a further parameter of inflammation (and thus resolution), a visual analogue score (0-10) was used to quantify the pain experienced at the site of inflammation (where 0 = no pain; 10 = worst pain imaginable). This was repeated for elicited tenderness with the application of a 100 g weight. Temperature was measured using an electronic thermometer (Thermofocus® 01500A3, Tecnimed Srl, Varese, Italy) both centrally (forehead) and at the site of the injection. The clinical procedures room where all measurements were taken, was kept at a constant, ambient temperature of 21 °C using an air conditioning unit.

Skin blister formation and inflammatory exudate collection

Inflammatory exudate was obtained from the site of the UV-KEc injection by formation of a blister at two time-points, 4h and 48h, as previously described (22). This process involves placing a suction blister chamber centred over the site of UV-KEc deposition, connected to a negative pressure instrument (NP-4, Electronic Diversities Ltd., Maryland, USA). The suction chamber at the base has an orifice plate with a 10 mm aperture, through which the blister is formed by a standardised, gradually incrementing negative pressure, starting at 2 inches of mercury (inHg) until a uniloculated blister begins to form. The maximum pressure used was 9 inHg and after the blister had fully formed, the pressure was gradually reduced back to baseline following a standardised protocol, with the whole process taking approximately 1.5 to 2h. The

chamber was then removed, and the blister pierced using a sterile 20 gauge hypodermic needle, along the lateral border. The exudate was rolled out using a sterile 1 mL syringe and the contents aspirated using a 200 µL pipette tip. The fluid was immediately transferred into a V-bottom 96-well plate containing 50 µL of 3% sodium citrate (Sigma-Aldrich, Missouri, USA) in PBS (Gibco). Extra holes were created in the overlying skin to prevent re-accumulation of blister fluid and it was cleaned once more and covered with a sterile dressing (9 x 10 cm dressing, Mepore). The plate was then centrifuged at 1000 *g* for 5 minutes at 4 °C. An empty sterile Protein LoBind tube (Eppendorf, Hamburg, Germany) was weighed, and the supernatant added to this tube which was re-weighed. The difference in weight gave the blister volume. The fluid was split into 30 µL aliquots and stored at -80 °C for soluble mediator analysis. The pellet was re-suspended immediately in 100 µL polychromatic flow cytometric staining buffer (5% fetal calf serum [FCS] [Gibco] in PBS [Gibco] with 2 mM EDTA [Sigma-Aldrich, Missouri, USA]).

Polychromatic flow cytometric analysis

Blister cells suspended in staining buffer as above, were incubated with a cocktail of appropriate antibodies specific to cell surface markers for 30 minutes (4 °C in the dark), along with appropriate fluorescence minus one (FMO) controls. Stained cells were then washed using staining buffer at 1000 *g* for 3 minutes at 4 °C. Cell profiles were then acquired immediately using a flow cytometer (LSR Fortessa™, Becton Dickinson [BD], New Jersey, USA). Polychromatic flow cytometric data were analysed using FlowJo software (BD, New Jersey, USA).

Blister fluid soluble mediator analysis

The supernatant from the inflammatory blister exudate was diluted in the appropriate assay diluent and all assays were performed as per manufacturer's instructions. For prostaglandin E_2 (PGE_2) quantification, an enzyme-linked immunosorbent assay (ELISA) was used (Cayman Chemical, Michigan, USA). For interleukin-10 (IL-10) and TNF-α quantification, a customised two-plex Magnetic Luminex® assay was used (R&D Systems Inc., Bio-techne, Minnesota, USA).

Statistical analyses

Prism's GraphPad software (Version 8.2.1, GraphPad Software Inc., California, USA) was used for generating figures and for statistical analysis. Data are expressed as individual values with the mean and standard error of the mean (SEM) on a linear scale. The unpaired data were tested for normal distribution using normality tests recommended by the software. For normally distributed data, differences between groups were probed using ordinary one-way analysis of variance (ANOVA) and Tukey's multiple comparisons test. For non-normally distributed data, differences were probed for using Kruskal-Wallis test and Dunn's multiple comparisons test. p value of < 0.05 was taken as the threshold of significance with graphical representation of $p < 0.05$ = *, $p < 0.01$ = ** and $p < 0.001$ = ***.

RESULTS

Baseline characteristics

The mean age of all volunteers was 25 years and there were no significant differences in age between treatment groups as shown in Table 1. The majority of volunteers were white British (67%). The medications were well tolerated, with no adverse effects reported for either of the drug treatment groups. Approximately 20% of total volunteers complained of a heaviness in their axillae between 4h and 24h following injection with UV-KEc. These cases were associated with linear, erythematous markings tracking from the site of injection towards the axillae, presumably representing lymphatic tracking, although this was pain-free. The mean blister volume across for all participants at 4h was 129.73 µL and 135.43 µL at 48h, with no significant differences in the formed blister volumes between volunteers across all three groups. Baseline volunteer characteristics and blister volumes are shown in Table 1. No differences were seen in renal function, liver function, full blood count or CRP between groups at any time-point.

Gating strategy for identification of infiltrating cells at site of UV-KEc triggered acute inflammation

To identify leucocyte subsets during the different phases of inflammation, blister exudate was obtained following suction using a negative pressure cup. Cells were separated by centrifugation and characterised using polychromatic flow cytometry and a gating strategy focussing on cells of the myeloid lineage, as shown in representative dot plots in Figure 1. After exclusion of debris (Figure 1i) and doublets (Figure 1ii), cells of haematopoietic origin (cluster of differentiation 45 [$CD45^+$]) were identified (Figure 1iii). Neutrophils (Figure 1vii) were identified as $Lineage^-$ (Lin^-: CD3, CD19, CD20 and CD56), $HLA\text{-}DR^-$, $CD16^+$, and confirmed as $Siglec8^-$. Total mononuclear phagocytes (MPs) were identified as Lin^- and $HLA\text{-}DR^+$. Further classification into MP subtypes was obtained based on the expression of CD14 and CD16: classical monocytes ($CD14^+$, $CD16^-$), intermediate monocytes ($CD14^+$, $CD16^+$), non-classical monocytes ($CD14^-$, $CD16^+$) and dendritic cells ($CD14^-$, $CD16^-$) (Figure 1vi). Lin^+ cells were further divided into B cells ($HLA\text{-}DR^+$) and T/NK cells ($HLA\text{-}DR^-$) based on expression of HLA-DR (Figure 1viii).

ATB-346 is potently anti-inflammatory in a UV-KEc-triggered model

Consistent with previous work in our laboratory, polychromatic flow cytometric characterisation of inflammatory cell infiltrate following injection of UV-KEc into the skin revealed a robust inflammatory response with infiltration of neutrophils peaking at 4h in the untreated control group (24) and clearing by 48h (Figure 2A). ATB-346 significantly reduced neutrophil numbers at the peak of the onset of inflammation (4h), however, no significant differences were noted at 48h. In the naproxen group, there was also a significant reduction in neutrophil infiltration at 4h compared to the untreated controls, albeit to a lesser extent than that seen with ATB-346. Again, at 48h there were no significant differences in neutrophil numbers. Using the above gating strategy, cells of the lymphoid lineage were split into T/NK cells and B cells, based on their expression of HLA-DR (Figure 2B and 2G, respectively). The HLA-DR$^-$ T/NK cell numbers were significantly lower in those subjects treated with ATB-346 compared to untreated controls at 48h, although no such difference was seen at 4h. The numbers of classical and intermediate monocytes were increased up to 48h following inflammatory stimulus (Figure 2C and 2D, respectively). ATB-346 and naproxen caused a trend to a reduction in numbers of classical monocytes and intermediate monocytes at 48h, although this was not significant (Figure 2E). Dendritic cell numbers were also lower in the ATB-346 group compared to both untreated controls and those treated with naproxen, although this was not significant (Figure 2F). The remaining lymphoid cells, HLA-DR+ B cells, showed no significant differences in numbers at either time-point (Figure 2G).

ATB-346 significantly alters monocyte subset phenotype but not neutrophils

Having noted a trend towards a reduction in all monocyte subtypes at 48h following UV-KEc injection in both ATB-346 and naproxen groups compared to untreated controls, we further investigated surface expression levels of cluster of differentiation 14 (CD14), a co-receptor for Toll-like receptor 4 needed for lipopolysaccharide (LPS) detection by cells of the immune system (25). At 48h, both classical and intermediate monocytes exhibited a significant reduction ($p < 0.05$) in their expression of CD14 based on MFI, in both naproxen and ATB-346 treated groups, compared to untreated controls (Figures 3B and 3C). Despite the significant reduction in blister neutrophil numbers at 4h in both the ATB-346 and naproxen-treated volunteers, there was no difference in CD62L expression between groups (Figure 3D).

ATB-346 significantly alters perception of pain and tenderness

A volunteer-reported visual analogue score was used to assess pain and elicited tenderness at the site of inflammation. Volunteers treated with ATB-346 reported significantly lower pain scores at the time-point of maximal neutrophil infiltration (4h) compared to untreated controls; effects also observed with naproxen (Figure 5A). Subjects exposed to ATB-346 also reported significantly lower tenderness scores compared to untreated controls, following the application of a 100 g weight at the site of inflammation (Figure 5B). Temperature at the site of inflammation measured using a digital thermometer followed the same course in both ATB-346 and naproxen groups, peaking at 24h. In the untreated control group, the peak was at 4h, however neither of these differences were significant (Figure 5C).

ATB-346 does not significantly alter vascular hyper-reactivity

Blood flow at the site of UV-KEc-triggered inflammation was assessed using laser Doppler imaging (Figure 4C). As previously shown, vascular hyper-reactivity as demonstrated by perfusion units, peaked at 24h in all groups, but declined back to similar levels seen at 4h by 48h. This indicates efficient resolution of acute inflammation and suggests that neither treatment significantly altered the trajectory of resolution, despite higher numbers of neutrophils in untreated controls at 4h (Figure 4A). At all time-points, most notably at 24h (Figure 4B), there was a trend towards increased hyper-reactivity in the ATB-346 group compared to naproxen and untreated control group, although this was not significant.

Effects of ATB-346 on soluble mediators and PGE_2 levels

Following UV-KEc-triggered inflammation, the supernatant was probed for differences in relevant cytokines and lipid mediators. While there was a trend towards an increase in TNF-α (Figure 6A) and a reduction in IL-10 (Figure 6B) in both treatment groups, these were not significant. There was, however, a significantly reduced concentration of PGE_2 in both ATB-346- and naproxen-treated volunteers compared to untreated controls (Figure 6C).

DISCUSSION

In this study, we found that ATB-346, exerts a potent anti-inflammatory effect as shown by a significant reduction in neutrophil numbers at 4h compared to untreated controls. Whilst we know that NSAIDs inhibit neutrophil infiltration due to the downstream effects of inhibited prostanoid synthesis and reduced neutrophil trafficking (26–28), the exact mechanism for a reduction in neutrophils at this time point remains unknown. Neutrophils, being the most abundant of human circulating phagocytes, are known to be the first cells to infiltrate into sites of acute inflammation. The purpose of this is to phagocytose invading microorganisms and debris (29). In order to 'switch off' inflammation, these effete cells then undergo programmed cell death by apoptosis and are cleared by MPs, beginning the resolution phase (30). However, more recently it has been shown that certain populations of neutrophils may re-enter the circulation following initial extravasation, migrating to lymph nodes to facilitate antigen presentation (31, 32), and thereby bridging the innate and adaptive immune systems.

Given that we have shown, under the influence of both ATB-346 and naproxen (both NSAIDs), a significant reduction in neutrophil numbers at 4h (the onset of resolution) compared to untreated controls, it is possible that both drugs act directly on neutrophils to reduce their infiltration into the site of inflammation through the mechanism described above. A significant reduction in neutrophil numbers at this time-point could also be due to reduced efflux from the bone marrow or such reverse transmigration, however, the data presented here are not substantiated enough to draw any firm conclusions. Further studies are needed to further understand this process.

CD14-expressing classical and intermediate monocyte numbers were higher at 48h, with numbers trending downwards between untreated controls and those treated with naproxen and further still for ATB-346. Whilst this trend was not significant, we noted a significant reduction in CD14 expression for both cell subtypes, in both drug treatment groups compared to untreated controls at this time point. It is known that CD14 is involved in LPS-induced monocyte activation and plays a crucial role in fighting infection and inflammation, as well as regulating numerous cellular responses (25, 36). A significant reduction in expression of CD14 on both classical and intermediate monocytes at this time could represent a switch to a more anti-

inflammatory phenotype. The data presented here suggests that this observation is due to exposure to an NSAID rather than a drug-specific effect of ATB-346.

From a clinical point of view, both ATB-346 and naproxen significantly reduced pain at the site of inflammation at 4h compared to untreated controls, the time-point of maximal neutrophil infiltration. In addition, ATB-346 also significantly reduced elicited tenderness at this site, again at 4h. This suggests that a reduction in the number of inflammatory cells and inflammatory mediators during inflammation may contribute to the analgesic effect of both drugs. Temperature, one of the other cardinal signs of acute inflammation (37), was not significantly different between groups at any time-point; however, it was of interest that both ATB-346 and naproxen were able to delay the peak temperature in local tissue from 4h in untreated volunteers compared to those treated with an NSAID. This would be consistent with an NSAID-mediated reduction in prostanoids normally involved in vasodilatation and thus local elevation in temperature, including prostacyclin and PGE_2 (26, 27).

It was observed at both 4h and 24h that there was increased blood flow, as measured by Doppler perfusion units, in volunteers treated with ATB-346 compared to both untreated controls and those treated with naproxen. Counterintuitively, this seemingly represented increased blood flow at the site of UV-KEc injection in those treated with an anti-inflammatory drug. Paradoxically, this increased blood flow correlated with significantly reduced pain and tenderness scores in these volunteers. H_2S is one of the three classical gaseous mediators (33, 34), along with nitric oxide and carbon monoxide, playing important roles in both physiological and pathophysiological processes (34, 35). It is feasible that the H_2S moiety released from ATB-346 induced vasodilatation at the site of UV-KEc, hence accounting for the difference between the two drug treatment groups. The clinical significance of this remains unknown and further investigation is needed to better understand this process.

As the opioid crisis continues in the developed world (7, 8), it is vitally important that we are able to find new therapeutics that are both efficacious, but also agreeable to patients in terms of adverse effects. This is especially important, particularly long-term, given major issues with tolerance and dependence (8). Conventional NSAIDs are effective at reducing the signs of inflammation as shown here, but have been limited in their use, predominantly due to gastrointestinal, cardiovascular and renal complications (12–15). This issue could potentially be negated through the addition of the H_2S moiety in drugs such as ATB-346.

Whilst ATB-346 significantly reduced numbers of infiltrating neutrophils at the peak of onset of acute inflammation, it was reassuring that the immune cells were not suppressed as a consequence of this treatment. We have previously shown that inhibiting COX activity with NSAIDs primes innate immune responses, increasing phagocytosis and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-mediated bacterial clearing. This was associated with elevated TNF-α (38). Measurement of TNF-α revealed comparably increased levels of this pro-inflammatory cytokine in the blister fluid of volunteers treated with both ATB-346 and naproxen, further highlighting that ATB-346 does not compromise host immunity, unlike glucocorticoid steroids (11). Suppression of PGE_2 seen at 4h at the site of inflammation in both ATB-346 and naproxen-treated volunteers suggests that both drugs are capable of suppressing cyclooxygenase enzymes.

The strength of this study lies in the reproducible model and study design to allow a robust interrogation of cellular and humoral responses in healthy human volunteers exposed to the experimental pharmacological agent ATB-346. One limitation is that, whilst the experimental subjects were blind to their treatment arm, some inferences could have been made by the volunteer owing to the differing dosing regimens of the two randomly allocated drug groups. This study is further limited that we were only able to perform two blisters per volunteer representing only two time-points during inflammation. It is possible we may have observed further differences in cellular infiltrates between groups at the 24h time-point, particularly given that this was the maximal time-point for vascular hyper-reactivity in all groups.

In summary, we used a UV-KEc-triggered model of acute dermal inflammation to demonstrate ATB-346 is potently anti-inflammatory. Specifically, ATB-346 was able to significantly inhibit neutrophil infiltration into the site of injected bacteria compared to untreated controls, with greater significance than naproxen when comparing to untreated controls. Whilst more work is needed to further explore the mechanism underpinning the reduction of neutrophils at 4h in those treated with ATB-346, we propose that ATB-346 represents the next generation of H_2S-NSAIDs, as a viable alternative to conventional NSAIDs, with reduced gastrointestinal adverse effects, exerting its effect through multiple potential facets of the inflammatory pathway and a potential novel mode of action mediated through the H_2S moiety.

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AUTHOR CONTRIBUTIONS

J. R.W. Glanville and D. W. Gilroy designed the study. J. R.W. Glanville performed the majority of the experiments, analysed the data, prepared the figures and wrote the manuscript. P. Jalali, A. A. Hosin, A. A. Patel and A. A. Maini helped with volunteer recruitment and performing the experiments. J. D. Flint contributed reagents, analytic tools and training of the techniques used. J. L. Wallace provided the ATB-346, regulatory documentation and expert advice on its use.

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TABLE AND FIGURE LEGENDS

Table 1. Baseline characteristics of study participants. Age, ethnicity and blister volume at both 4h and 48h for all 21 healthy, male volunteers recruited are shown below. No significant differences were seen between participants within in each or between groups for either age or blister volume at either time-point; $n = 21$.

Figure 1. Characterisation of inflammatory cell infiltrate into skin blisters. Inflammatory exudate was obtained using a negative pressure suction cup at the site of UVKEc-triggered resolving acute inflammation, with cells separated by centrifugation. Cells were incubated with a fluorophore-tagged antibody cocktail. Stained cells were acquired using BD LSR Fortessa™ and analysed using FlowJo software (BD, New Jersey, USA). Representative dot plots for polychromatic flow cytometric gating strategy are shown. Following exclusion of debris (i), the gating strategy employed allowed identification of single cells (ii), C45+ haematopoietic immune cells (iii), total lymphocytes (iv, top left box), neutrophils (vii), total mononuclear phagocytes (v, top box), mononuclear phagocyte subsets (classical, intermediate, non-classical monocytes and dendritic cells [vi]), B cells (viii, left box) and T/NK cells (viii, right box). Arrows indicate gating strategy.

Figure 2. Cellular profile at the site of UVKEc-triggered resolving acute inflammation. Inflammatory exudate was obtained using a negative pressure suction cup at two time points (4h and 48h), one per forearm for each volunteer. Cells were separated from the soluble mediator-containing supernatant by centrifugation and incubated with a fluorophore-tagged antibody cocktail. Stained cells were acquired using BD LSR Fortessa™ and analysed using Flowjo software (BD, New Jersey, USA). Using the gating strategy shown in Figure 1, populations of neutrophils (A), T/NK cells (B), mononuclear phagocyte subsets (classical [C], intermediate [D], non-classical monocytes [E] and dendritic cells [F]) and B cells (G) were identified. Cell numbers are shown as cell count per millilitre. Cell phenotype is displayed above each cell type. Data are expressed as individual values with the mean and standard error of the mean at each time point; $n = 21$, $*p < 0.05$, $***p < 0.001$.

Figure 3. Mean intensity fluorescence (MFI) values for blister fluid cells. Healthy, male volunteers were randomised to receive either ATB-346 (250 mg daily) or naproxen (500 mg twice daily) or no drug for 48h prior to inflammation onset. Inflammatory exudate was obtained using a negative pressure suction cup at two time points (4h and 48h), one per forearm for each volunteer. Cells were separated from the soluble mediator-containing supernatant by centrifugation. MFI data are also shown for CD14 surface expression on classical monocytes (A) and intermediate monocytes (B) at the 48h time point. Representative, histograms showing CD14 surface expression on classical and intermediate monocytes at the 48h time-point are shown in Panel C. MFI data are shown for CD62L surface expression on blister neutrophils at 4h (D). Data are expressed as individual values with means and standard error of the mean; $n = 21$, $*p < 0.05$.

Figure 4. Assessment of vascular hyper-reactivity at the site of UV-KEc-triggered resolving acute resolution. Healthy, male volunteers were randomised to receive either ATB-346 (250 mg daily) or naproxen (500 mg twice daily) or no drug for 48 hours prior to inflammation. Acute inflammation was triggered by intradermal injection of 1.5×10^7 UV-KEc suspended in 100 µL 0.9% sodium chloride. Vascular response and total blood flow was assessed using a laser Doppler imager (moorLDI-HIR, Moor Instruments Ltd., Devon, UK) and are shown here as total mean perfusion units (A). Total mean flux at 24h are shown as individual data points (B). Representative flux images and accompanying photographs were acquired and analysed using moorLDI software (Moor Instruments Ltd., Devon, UK. Representative flux images and photographs are shown here from baseline (0h), 4, 24 and 48h (C). Data are expressed as mean values with standard error of the mean shown.

Figure 5. Clinical scores if pain, tenderness and temperature at the site of UV-KEc-triggered resolving acute resolution. Healthy, male volunteers were randomised to receive either ATB-346 (250 mg daily) or naproxen (500 mg twice daily) or no drug for 48 hours prior to inflammation onset. Volunteers were asked to score the level of pain experienced at the site of acute inflammation, triggered by intradermal injection of 1.5×10^7 UV-KEc suspended in 100 µL 0.9% sodium chloride. Scores were given from 0 – 10, with 10 being the worst pain imaginable and 0 being no pain at all. Tenderness scores were also obtained using the same

scale, following the application of a 100 g weight at the site, where † = untreated control 4h time-point and ‡ = ATB-346 time-point. Temperature was taken using a digital thermometer at the site of inflammation (Thermofocus® 01500A3, Tecnimed Srl, Varese, Italy). Results for all clinical scores are shown here from baseline (0h), 4, 24 and 4h. Data are expressed as individual values with means and standard error of the mean; $n = 21$, $*p < 0.05$, $***p < 0.001$.

Figure 6. Cytokine and PGE_2 concentrations at the site of UV-KEc-triggered resolving acute inflammation. Inflammatory exudate was obtained using a negative pressure suction cup at two time points (4h and 48h), one per forearm for each volunteer. Cells were separated from the soluble mediator-containing supernatant by centrifugation. Cytokines (TNF-α and IL-10) in the supernatant were probed using a two-plex ELISA (R&D Systems Inc., Bio-techne, Minnesota, USA). PGE_2 concentration was determined using an ELISA (Cayman Chemical, Michigan, USA). Data are expressed as individual values with means and standard error of the mean; $n = 17$, $*p < 0.05$.