

GSK2646264, a spleen tyrosine kinase inhibitor attenuates the release of histamine in *ex vivo* human skin.

Running (short) title: SYKi GSK2646264 attenuates histamine release in human skin

BACKGROUND AND PURPOSE

Chronic Spontaneous Urticaria presents as a heterogeneous syndrome characterised by wheals, angioedema or both for greater than six weeks. Spleen tyrosine kinase (SYK) mediates allergen-induced mast cell degranulation via the IgE signalling pathway, a central component of wheal formation and inflammation. In this study, we demonstrate that perfused or topically administered GSK2646264 dose dependently attenuates IgE-mediated histamine release from mast cells in an *ex vivo* human skin model.

EXPERIMENTAL APPROACH

Using a novel SKIP device *ex vivo* human skin from mastectomy surgeries was challenged with anti-IgE, C5a and buffer to induce histamine release from skin mast cells. Histamine was collected via microdialysis fibers and measured fluorometrically. GSK2646264, was delivered via perfusion either using the microdialysis fibers or topically in a cream. Drug concentrations in the skin were measured by LC-MS and a PK/PD relationship was developed.

KEY RESULTS

Perfused GSK2646264 significantly inhibited anti-IgE (but not C5a) induced histamine release in a concentration dependent manner ($IC_{50} = 0.7 \mu M$, $IC_{90} = 6.8 \mu M$). The 0.5, 1 and 3% cream delivered GSK2646264 to the dermis above the IC_{90} (donor range 9.4 – 550 μM) and dose dependently attenuated the anti-IgE induced histamine release.

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CONCLUSIONS AND IMPLICATIONS

GSK2646264 administered topically or direct to the dermis blocked histamine release from *in situ* skin mast cells. A PK/PD relationship curve suggests that dermal concentrations above 6.8 μM should lead to approximately 90% inhibition of histamine release from skin mast cells following Fc ϵ RI activation implicating a potential use for the compound in skin mast cell diseases such as urticaria.

ABBREVIATIONS

Chronic Spontaneous Urticaria (CsU), Complement 5a (C5a), Fc-epsilon receptor 1 (Fc ϵ RI), Hydrochloric acid (HCL), Intra-probe drug delivery (IPDD), Laser Capture Mass spectrometry (LC-MS), Lower limit of quantification (LLQ), Multiple reaction monitoring (MRM), Pharmacokinetic/Pharmacodynamic (PK/PD), Revolutions per minute (RPM), Spleen tyrosine kinase (SYK), Ultra performance liquid chromatography (UPLC),

INTRODUCTION

Urticaria is a common skin disorder, affecting between 15-30% of the population and it is characterised by itchy wheals and sometimes angioedema. It is divided into acute and chronic forms and in the latter, which affects around 0.5-1% of the population, Chronic Spontaneous Urticaria (CsU) persists almost daily for at least 6 weeks. The pathophysiology of CsU is complex and in over half of the patients there is no known cause for the condition. However, in approximately 30-50% of patients, IgG autoantibodies against either IgE or its high affinity receptor Fc ϵ RI can be detected and there is literature to suggest that these auto-antibodies can cross link and dimerise Fc ϵ RI (Hide et al 1993) leading to activation of cutaneous mast cells and blood basophils and subsequent release of many pro-inflammatory mediators. (Niimi et al 1996). Inhibition of the Fc ϵ RI signalling pathway and the activation of SYK on mast cells and basophils will reduce three main mast cell functions: the release of preformed mediators such as histamine, the secretion of cytokines including TNF alpha and the production of leukotrienes and prostaglandins (Turner and Kinet 1999).

The involvement of the Fc ϵ RI pathway in CsU has been further validated by the clinical efficacy observed following omalizumab treatment, a recombinant humanized monoclonal

antibody that binds to the Fc portion of free IgE (Kaplan et al 2008, Saini et al 2015). Omalizumab rapidly reduces free IgE levels which consequently leads to the downregulation of FcεRI levels in mast cells (Beck et al 2004, Metz et al 2017) and basophils (MacGlashan DW 1997) and can inhibit the increase in FcεRI expression and function associated with elevated IgE expression in human skin mast cells (Gomez et al 2007). Furthermore, Serrano-Candelas et al (2016) reported that SYK phosphorylation along with downstream signalling pathways were also reduced when IgE sensitised LAD2, a human mast cell line, were incubated with omalizumab.

Inhibition of SYK kinase with a small molecule inhibitor should lead to a reduction in the signalling cascade post activation of the IgE- FcεRI complex causing a reduction in the aforementioned mediators normally released from a degranulating mast cell.

GSK2646264 is a small molecule inhibitor of spleen tyrosine kinase (SYK) recently discovered at GlaxoSmithKline (GSK) (Barker et al, 2018) as part of an extension to previous efforts to identify oral SYK inhibitors (Liddle et al. 2011, Garton et al 2016). *In vitro*, GSK2646264 demonstrated consistent potency across assays relevant to the mechanism of SYK inhibition including an IgE stimulated LAD2 assay and good selectivity over a range of closely related kinases with at least 30-fold selectivity over 10 other unrelated kinases (Barker et al, 2018).

This manuscript discloses the development of GSK2646264 as a topical agent for treatment of IgE mediated skin diseases such as chronic spontaneous and inducible urticaria and demonstrates its dose-dependent inhibition via topical and intra dermal administration in an anti-IgE-stimulated histamine release *ex-vivo* human skin model. Integrated data from the different models has allowed the development of a PK/PD model in human *ex-vivo* skin.

Due to the sparse and rare availability of fresh human skin during the time allocated for this project, some of the results, which incorporate a dose response, presented in this manuscript are less than n=5. When this is the case, no statistical analysis was performed on the data. No randomisation or blinding of the experiments was possible. In accordance with the journal guidelines, these results are considered exploratory but sufficient to support the conclusions presented.

METHODS

Human skin for microdialysis ex vivo experiments

Skin specimens were obtained from women undergoing mastectomy surgery after the diagnosis of breast cancer. Before each experiment, the skin specimens were visually assessed and excluded if skin surface was affected or the tissue showed any signs of tumor infiltration. Ethical approval for use of the skin was obtained in accordance with Danish law and all women gave their informed oral and written consent to the use of the skin tissue (Ethics # H-3-2010-095).

Human skin for ex vivo skin penetration experiments

Human skin was obtained from cosmetic reduction surgery. Ethical approval for use of the skin was obtained in accordance with UK law and all donors gave their informed oral and written consent to the use of the skin tissue. The frozen skin was thawed at room temperature, subcutaneous fat was removed mechanically and the skin was sectioned with a dermatome to a thickness of $400 \pm 100 \mu\text{m}$ using a Nouvag TCM 3000 cutter.

Microdialysis experimental setup

Nine 23-gauge cannulas (Terumo, Europe N.V., Leuven, Belgium) were introduced in the upper dermis of an intact skin slice (sized 3 x 10 cm) and were placed 1 cm apart and at a depth of approximately 0.7 mm and a length of 10 mm. A microdialysis probe (EP Low Flux Probe 2 kDa, EP Medical) was introduced through each cannula and the cannula removed to leave the dialysis probe in the skin. The inlet of each dialysis probe was connected to individual syringes mounted in micro-injection pumps (CMA/100; CMA/Microdialysis, Stockholm, Sweden). The probes were perfused in parallel at a rate of $3.0 \mu\text{L}/\text{min}$ with PIPES buffer (Reflab, Denmark), and a recovery phase of 10 min was allowed before initiation of the experiments to wash out any histamine released due to insertion trauma from the cannula.

Intra-probe drug delivery (IPDD) and inhibition of histamine production in skin challenged with anti-IgE and C5a.

Intra-probe drug delivery (compound perfusion) was performed using the dialysis probes at a rate of 3.0 $\mu\text{L}/\text{min}$ with GSK2646264 at concentrations ranging from 0.2 to 25 $\mu\text{g}/\text{mL}$. During the perfusion with the compound, eluates from the probes were collected for each 60 minute perfusion to assess background histamine levels and any histamine release caused by the compound. After a pre-defined drug-delivery time of 3 h, the skin sites above the probes received intradermal injections of 50 μL PIPES buffer, Anti-IgE (5 ng/mL) or C5a (8.3 ng/mL). Concentrations used were found to give optimal histamine release in skin which is in accordance with previous findings (Petersen et al, 1994, Petersen et al, 1997). Following challenge, dialysates were collected for 30 min and analyzed for histamine concentration.

Due to methodological failure (congestion of dialysis fibers, high histamine basal levels or no response to anti-IgE or C5a), three experiments were excluded from calculations. Results reported are from five anti-IgE ($n=5$) and six C5a experiments ($n=6$). % of histamine release was calculated in relation to the mean histamine levels released by each donor after challenge in the absence of GSK2646264, normalised to 100%. Data was evaluated for normality with Shapiro-Wilk's test and for difference with a t-test for matched pairs. A significant difference was defined as $p<0.05$.

Determination of compound concentration in skin from anti-IgE microdialysis experiments

As the measurement of compound concentration in the skin was introduced while the experiments were already on going (two experiments were performed where the skin was not collected for compound concentration determination), only three out of the five donors were used for the anti-IgE and C5a experiments where whole depth skin biopsies were taken from the area surrounding the microdialysis tubing and transferred to a vial containing 1 mL extraction fluid (800 μL acetonitrile and 200 μL water). Vials were vortexed briefly and left for extraction overnight (for a minimum of 12 h) at room temperature on a roller mixer. 500 μL of extract was removed for compound analysis and the rest, including the biopsy, was discarded. The biopsy extracts were stored at -20°C until analysis. GSK2646264

concentration in the biopsy extracts was determined by reverse phase liquid chromatography coupled to atmospheric pressure ionization tandem mass spectrometry (LC-MS/MS) using a heat assisted electrospray interface operating in positive ion mode. A 25 μ L aliquot of each sample was removed and assayed against matrix matched calibration standards prepared in 80:20 acetonitrile:water (v/v) over the range 0.5 to 1000 ng/mL. To the samples and calibration standards 200 μ L of 5% acetonitrile (aq) containing Labetalol (62.5 ng/mL) was added for use as an extraction solution and internal standard. All prepared samples and standards were run on a LC gradient with chromatographic separation achieved by a Phenomenex Kinetex C18 100 Å, 2.6 μ m, 50 x 2.1 mm column. An API4000 (Applied Biosystems) mass spectrometer was used as detector and the nominal MRM transitions for GSK2646264 and internal standard (Labetalol) were 375 to 107 and 329 to 162 respectively. GSK2646264 concentration data was generated and processed using Analyst 1.4.2 software. Intermediate steps for data transfer/manipulation were performed using Microsoft Excel 2007. RRID: SCR_014271

Determination of compound concentration in the dermis from the in vitro permeation experiments

Skin penetration studies to support the development of drugs for topical application is a common practice as exemplified by Karpanen et al., 2008 and Morais Ruela et al., 2016. Two full scale permeation and penetration experiments (with different time points, up to 4 and 24 h, respectively) using skin from three donors were performed using Franz diffusion cells (average surface area of approximately 0.6 cm² and a volume of approximately 2.0 mL were employed). The skin was mounted between the donor and receiver compartments and the cells were sealed together using Parafilm[®] and clips, with the integrity of the donor and receiver checked. The Franz cells were filled with receiver fluid (purified water) and each cell was subsequently equilibrated to ensure a surface temperature of 32°C (external skin surface temperature) for at least 30 min prior to dosing (water bath temperature of 37°C). A displacement pipette was used to apply the cream (~8 mg) to the plunger of a 1 mL syringe. The cream was applied to the skin surface and spread over the diffusion area using the plunger. Prior to and after application the weight of the plunger was recorded and the dose per cell was calculated. Receiver fluid (200 μ L) was removed at the following time points: 0, 2 and 4 h (experiment 1) or 0, 2, 4, 6, 8, 10, 22 and 24 h (experiment 2) and stored frozen (-20°C) before analysis by LC-MS/MS. Following the final time points (4 h and 24 h), the

cells were dismantled and three cotton swabs were used to remove the cream from the surface of the skin. The top layers of the skin (stratum corneum) was removed with Six tape strips (D-Squame®) and the remaining epidermis and dermis were placed into an incubator at 60 °C for 2 min before being manually separated by peeling the two layers apart. The dermis samples were placed into Precellys24 homogeniser vials and 1 mL of extraction diluent (80:20, acetonitrile:water with 0.1% formic acid) was added and the contents homogenised at 5,800 revolutions per minute (RPM) for 2 x 20s at a temperature between 2-8 °C. The contents were emptied into a glass vial and a further 1 mL of extraction diluent was added, vortex mixed for ca 30 seconds and emptied into the same glass vial. All vials were shaken on an orbital shaker at ambient temperature for 16 to 20 h. Following the extraction procedure, the extraction solvent system was removed from the vials and centrifuged at 16,060 g for 10 min to remove all undissolved materials and particulates. The supernatant was recovered and an aliquot (100 µL) was transferred into a vial for analysis and spiked with 100 µL of the internal standard solution [50 ng/mL of R(+)-SCH-23390, HCl (Sigma) in a solution of 80:20, 1 % v/v formic acid (aq):acetonitrile in a 1:1 ratio] and mixed using a vortex mixer.

Samples and calibration standards were run on a UPLC gradient with chromatographic separation achieved by an Acquity BEH C18, 1.7 µm, 50 x 2.1 mm column. A Waters Xevo TQ mass spectrometer was used as detector and the nominal MRM transitions for GSK2646264 and internal standard were 375 to 106 and 288 to 91 respectively. GSK2646264 concentration data was generated and processed using Masslynx version 4.1 software. RRID: SCR_014271

Topical drug delivery and inhibition of histamine release following anti-IgE skin challenge

As *ex vivo* skin does not readily absorb fluids or compounds, a device for facilitating penetration of a topically administered compound into the skin called SkiP (Skin Penetration) (see supplemental figure 1) was developed. The SkiP device was inspired by the conventional Franz penetration/diffusion cell, but with 9 apertures for topical application. Immediately after removal, human breast tissue skin slices (3 x 10 cm) were excised and placed in RPMI 1640 medium (room temperature), subcutaneous fat was removed from the skin specimens and the skin placed over the reservoirs of SkiP device. The outline of the 9

apertures of the device was marked on the skin with a permanent marker. A 23 gauge cannula was placed through the marked apertures of the skin without perforating the area of the skin in contact with the fluid in the reservoirs, and 25 μL of serum from a healthy control was injected intradermally around the cannula in order to minimize any potential stress due to dehydration during the SkiP-procedure. The cannula was slowly removed from the skin to allow serum to disperse in the skin and the skin locked in place by a plate. The SkiP device was flipped over in order to fill the reservoirs with culture medium (RPMI 1640 supplemented with 1 mg/mL glucose). The device was flipped back to the original position with skin side up and 10 mg of the 0.1, 0.5, 1 and 3% (w/w) of GSK2646264 cream formulations were topically applied using a syringe to each area of the aperture (1 cm^2). Each formulation was applied onto two skin sites (to obtain a spontaneous and anti-IgE challenge) and evenly distributed throughout the dosing area using a plastic rod. The SkiP was incubated at 37°C in a humidified atmosphere for a total of 4 h, before the skin was removed and microdialysis fibers (216 μm diameter, 2 kDa molecular weight cut off and a length of 10 mm) were inserted guided by 23 gauge cannulas. After insertion, each fibre was perfused at 3 $\mu\text{L}/\text{min}$ with Pipes buffer using CMA/100 pumps (CMA Microdialysis, Stockholm, Sweden). The skin was perfused for 10 minutes to reduce any trauma induced by placing the microdialysis fibre and eluates sampled to obtain baseline histamine values. Following a further 30 min perfusion, the skin was then injected intradermally with 50 μL Pipes buffer (spontaneous histamine release) or 50 μL anti-IgE (5 ng/mL; anti-IgE induced histamine release). The perfusion was continued for 30 min and eluates were sampled to determine histamine release caused by the Pipes buffer and the anti-IgE respectively.

Triplicate samples of 15 μL were transferred to test plates for fluorometric detection of histamine as previously described (*Petersen et al. 1996*). Assay sensitivity for histamine was 5 ng/mL and CV% < 10%. The initial intention was to generate data for six donors, however two donors showed low responsiveness to anti-IgE challenge, one donor in particular had large amount of scar tissue that would have affected the distribution of mast cells in the skin. The reported results were generated from four donors.

Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS

Guide to PHARMACOLOGY (Harding *et al.*, 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.*, 2017).

RESULTS

GSK2646264 inhibits anti-IgE (but not C5a) induced histamine release from mast cells

To address whether GSK2646264 inhibition of histamine release from the mast cells was selectively due to SYK inhibition, GSK2646264 was perfused via the microdialysis tubing into the *ex vivo* human skin and subsequently challenged with either vehicle (PIPES buffer), anti-IgE or C5a. The results are displayed in figures 1A and 1B. After perfusion with compound at different concentrations the baseline histamine release mean values were below the lower limit of quantification (LLQ) <5 ng/ml (1.7 ± 2.3 for the control used in the anti-IgE challenged experiment and 4.8 ± 3.1 ng/mL for the control used in the C5a experiment).

As expected, in the absence of compound, the anti-IgE and C5a challenges induced histamine release within a similar order of magnitude and variability, 130 ± 90 ng/mL and 133 ± 37 ng/mL respectively. The maximum histamine production values from each challenge on individual skin donors were used to normalise the data and calculate the % of inhibition achieved by the SYK inhibitor GSK2646264 represented in figures 1A and 1B.

As shown in figure 1A, GSK2646264 perfused at different concentrations (200, 1000, 5000, 25000 ng/mL equivalent to 0.53, 2.67, 13.4 and 66.8 μ M respectively) was found to significantly ($p < 0.05$) inhibit anti-IgE induced histamine release in all 5 donors in a concentration dependent manner after 3 hour perfusion. As displayed in figure 1B, GSK2646264 did not inhibit C5a-induced histamine release in the 6 tested donors with any concentration of GSK2646264 tested.

GSK2646264 increased skin concentration correlates with inhibition of anti-IgE stimulated histamine release from skin mast cells

GSK2646264 concentrations in the skin biopsy samples were measured by LC-MS/MS as described in the materials and methods sections.

The relationship between the compound concentration in the biopsy and the percentage inhibition of histamine release was investigated in three out of the five donors used in the anti-IgE challenged experiments. The percentage inhibition of histamine release and biopsy concentration was processed using Phoenix WNL v6.1 and the resulting relationship is presented in Figure 2. This analysis produced an IC₅₀ value of 0.7 μ M which is within the range of the expected efficacy of GSK2646264 as previously reported potencies for GSK2646264 in cellular assays (pIC₅₀=7.1 in SYK lysate assay and pIC₅₀ = 6.7 in B cell pERK, Barker et al, 2018). The skin compound concentration for three of the C5a experiments was also determined confirming that the compound reached the dermis with concentration range between 2-20 fold higher than the IC₅₀s determined in the pERK cellular assay but with no effect on the histamine levels.

Topical formulation (cream) delivers GSK2646264 to the dermis at potentially efficacious concentrations

Following the previously reported skin penetration results (Barker et al, 2018) where GSK2646264 was shown to penetrate the skin when using a simple Ethanol/PBS formulation, an *in vitro* study to investigate the percutaneous properties of a clinical ready formulation containing GSK2646264 was performed. The creams were prepared at four different concentrations (0.1, 0.5, 1.0 and 3.0% (w/w)). Composition of the creams are provided in Supplementary Table 1. The four cream formulations were applied to the ex vivo human skin setup in Franz cells as described in materials and methods. The concentration measured in the dermis at 4 and 24 hours are presented in Table 1. All cream strengths tested showed a good linear relationship with the concentration of drug in the dermis and all achieved mean concentrations of GSK2646264 at or above 10 μ M. This data was integrated with the estimated skin IC₅₀ and IC₉₀ from figure 2 to visually depict that all the cream strengths applied at 10 mg/cm² achieve concentrations above IC₉₀ 24 hours after application (Supplemental Figure 2).

These predicted human skin exposures were used to set the strength of creams investigated in the clinical study (NCT02424799).

GSK2646264 cream achieves inhibition of anti-IgE histamine release in the *ex vivo* human skin

Following topical application of GSK2646264 in the cream formulations at four different strengths (0.1, 0.5, 1 and 3% (w/w)) the efficacy of GSK2646264 was further explored in the *ex vivo* human skin histamine release model using the SkiP device as described in the materials and methods section. Data displayed in figure 3 shows that histamine release from mast cells in the skin could be significantly inhibited in a dose-dependent manner with top three strengths exhibiting greater than 50% inhibition.

DISCUSSION AND CONCLUSIONS

The inhibition of mast cell degranulation with SYK inhibitors has been widely documented (Kovarova et al 2004, Masuda and Schmitz, 2008; Rossi et al., 2006) and numerous oral and inhaled SYK inhibitors have shown benefit pre-clinically in animal models of airway inflammation and autoimmune diseases (Wong et al., 2004; Matsubara et al., 2006; Yamamoto et al., 2003). However, the promise of a selective SYK inhibitor as a new oral medicine has not been possible so far, potentially due to dose limiting pre-clinical toxicological findings (Long et al 2016).

This paper describes the SYK inhibitor GSK2646264, formulated in a topically applied cream, which is currently in clinical development in chronic spontaneous urticaria, cold urticaria (NCT02424799) and cutaneous lupus erythematosus (NCT02927457).

Following the perfusion of GSK2646264 by IPDD a whole depth biopsy of the skin was taken and the concentration of the drug quantified using LC-MS/MS technology. Although there are some unknowns and caveats around the determination of the total concentration of compound in the skin biopsies (e.g. unknown extraction recovery and compound distribution across the skin layers), a relationship between biopsy concentration and the percentage inhibition of histamine release was performed for three out of the five donors used in the anti-IgE challenged experiments. This analysis indicated an IC_{50} value of 0.7 μM and an IC_{90} value of 6.8 μM which is within the range of the expected efficacy of GSK2646264 as previously reported in other cellular assays (Barker et al, 2018).

One of the key failures of topically applied drugs is the inability to penetrate through to the dermis of the skin. In order to determine if the cream formulation being prepared for administration to human subjects was able to penetrate through the different layers of skin, it was applied topically to ex vivo human skin at four strengths using Franz diffusion cells. As most of the mast cells reside in the dermis, the skin layers were separated into dermis and epidermis 4 and 24 hours after application of GSK2646264 to the top of the skin. Although compound concentrations in *in-vitro* penetration experiments can be extremely variable all dermal values exceed the IC₅₀ value of 0.7 µM and the integrated data from the human *in vitro* dermal penetration and histamine release at 4 and 24h after topical administration are predicted to lead to >90% inhibition of histamine release from the skin mast cells (Supplementary figure 2). As far as we are aware this is the first time such an approach to measure the relationship between drug concentration and percentage inhibition of histamine following skin mast cell activation has been undertaken.

To further validate this approach, we examined the effect of the same 4 strengths of GSK2646264 on skin stimulated with anti-IgE and confirmed that a strength dependent inhibition of histamine in the novel SkiP ex-vivo skin model was also achievable (figure 3). Due to the variability of human skin to respond to IgE, only donor skin which released histamine were included in the final analysis. However, in this experiment, following one application of a 3% strength cream, a mean inhibition of 70% was observed after 4 hours suggesting that the PK/PD relationship was relatively accurate. A higher inhibition may have been achievable if the compound could have been left on the skin for longer but the integrity of the skin over longer periods of time in the SkiP device did not allow this.

Furthermore, this paper highlights that an integrated analysis of data from IPDD microdialysis in combination with traditional skin microdialysis and topical application are suitable techniques for investigating the effects of compounds delivered directly to primary *in situ* skin cells. These data helped to model the predicted efficacious dose and support the use of GSK2646264 for the potential treatment of IgE-mediated mast cell driven disorders such as chronic urticaria.

SUMMARY:

What is already known? SYK mediates allergen-induced mast cell degranulation via the IgE signalling pathway.

What this study adds? Model of the predicted efficacious dose in human skin.

Clinical Significance: Supports use of GSK2646264 for the potential treatment of IgE-mediated mast cell driven disorders.

Author contributions

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Conflicts of interest

Marion Dickson, Cesar Ramirez Molina, Edward Hooper-Greenhill and Mike Barker are GSK employees.

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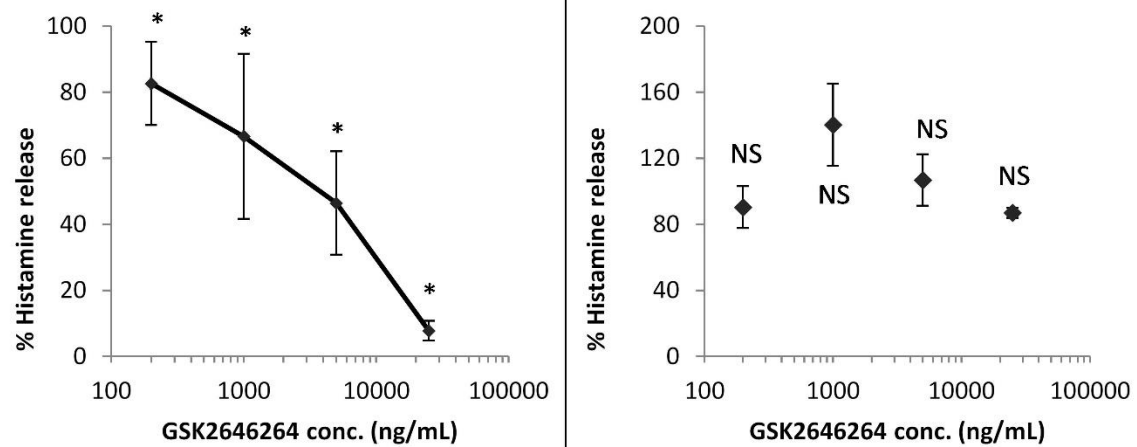


Figure 1. GSK2646264 concentration dependent inhibition of challenge induced histamine release from mast cells in human skin (A, n=5; B, n=6). % of histamine release was calculated in relation to the mean histamine levels released by each donor after challenge in the absence of GSK2646264. Graph displays mean $\pm$ SD. * $P < 0.05$. NS: not significant.

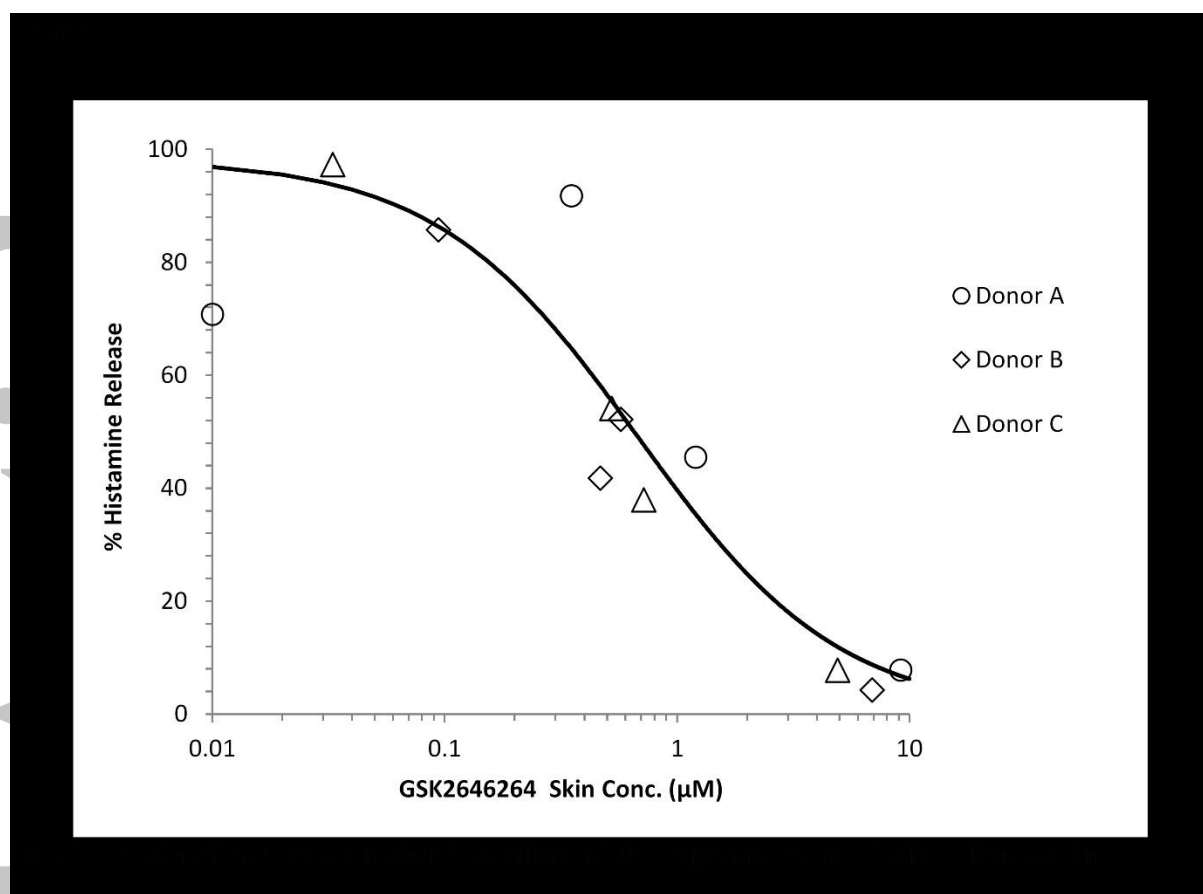


Figure 2. GSK2646264 concentration from three of the experiments (n=3) where human skin was challenged with anti-IgE and its relationship to the % of histamine release inhibition as described by the Inhibitory Effect E0 at C0 model in Phoenix WinNonLin (WNL) $E=E0*(1-(C/(C+IC50)))$. The estimated IC50 of 0.7 μM has a standard error and %CV of 0.2 and 21.5 respectively. The model calculated the IC90 = 6.8 μM.

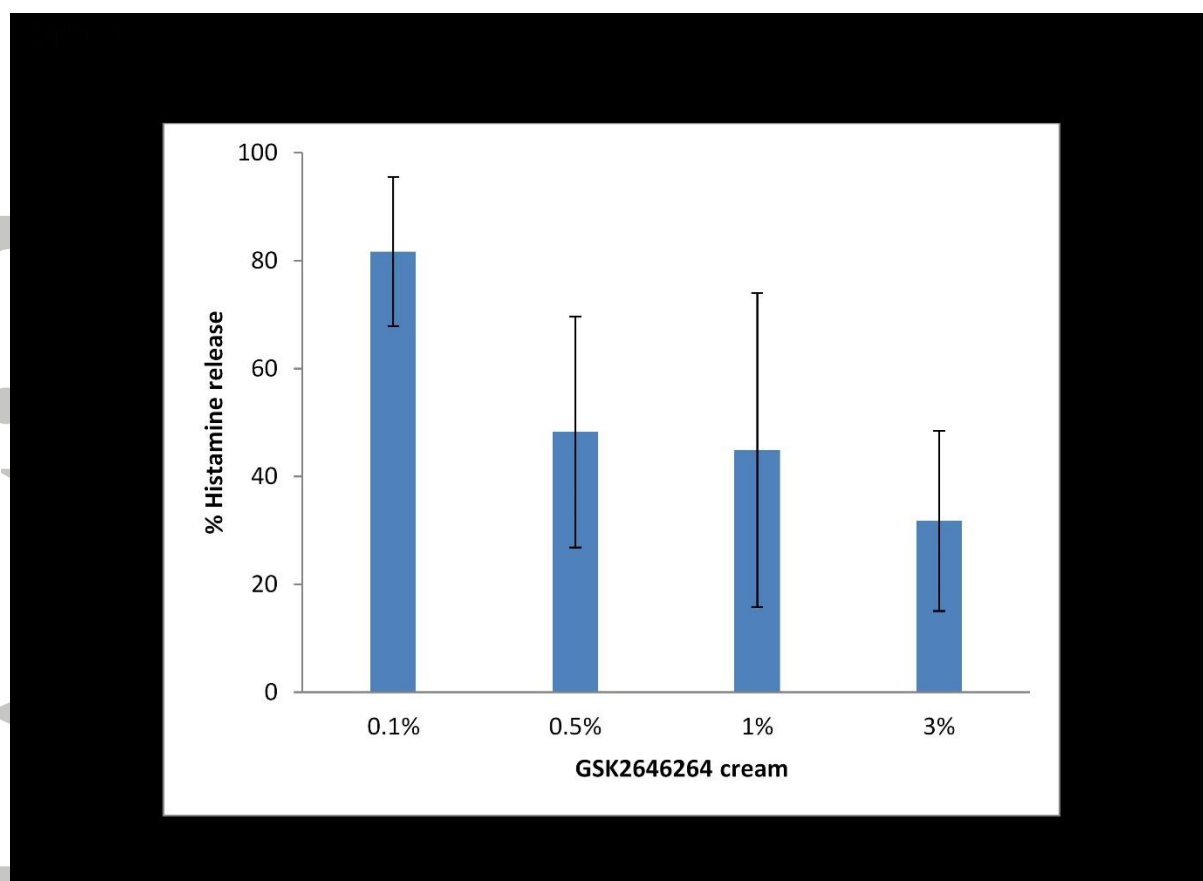


Figure 3. Inhibition of anti-IgE induced histamine release from mast cells in ex vivo human skin with four strengths of GSK2646264 cream. % of histamine release was calculated in relation to the histamine levels released by the skin donor after anti-IgE challenge in the presence of placebo (no drug cream). Experiments performed using the SkiP device, with data generated from four donors (n=4). Graph displays mean $\pm$ SD.

Table 1. Mean *in vitro* dermal GSK2646264 concentrations at 4 h and 24 h for Arlacel cream formulations in human skin dermis

Arlacel Cream Formulation Strength (% (w/w)) ^a	Dermal Concentration (μM) ^b					
	4 h			24 h		
	Mean	Range		Mean	Range	
		minimum	maximum		minimum	maximum
0.1	9.9	2.6	28.6	38.2	11.2	73.5
0.5	30.3	9.4	105.4	48.6	14.9	142.4
1.0	40.5	12.3	125.7	62.9	30.4	91.4
3.0	76.3	8.9	123.7	359.9	175.6	550.4

Data presented as mean values and range (minimum and maximum values determined) of three skin donors used for each cream strength (n=3), 1 to 3 replicates per donor. a: ArlacelTM cream strengths (% w/w) are given in terms of parent. b: Dermal concentration was calculated from dermal recovery assuming the following skin section dimensions; 0.6 cm² surface area, 0.03 cm depth, 0.018 cm³ volume (1 mL equivalent to 1 cm³). Amount recovered was then converted to μM.

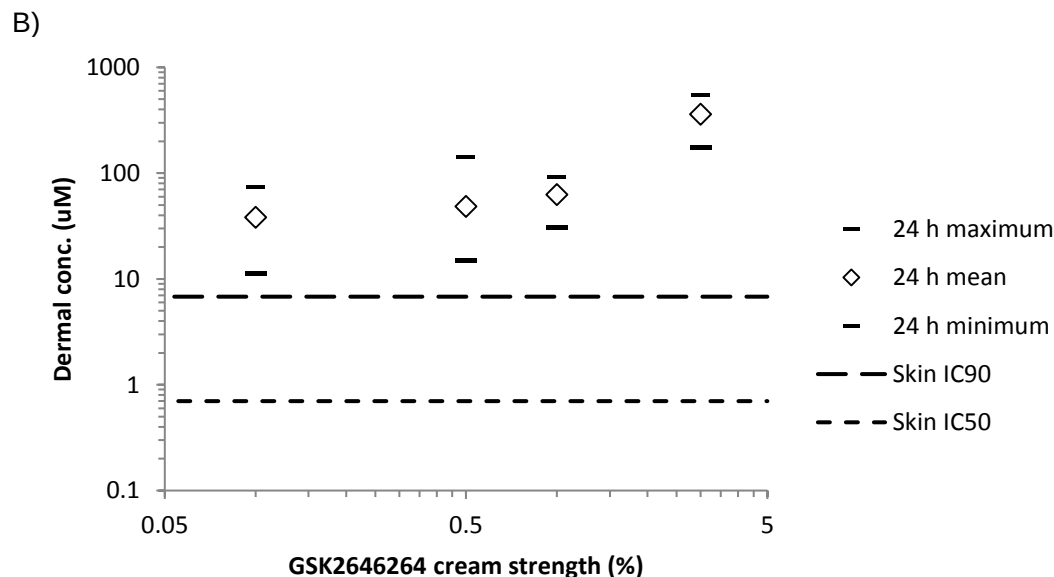
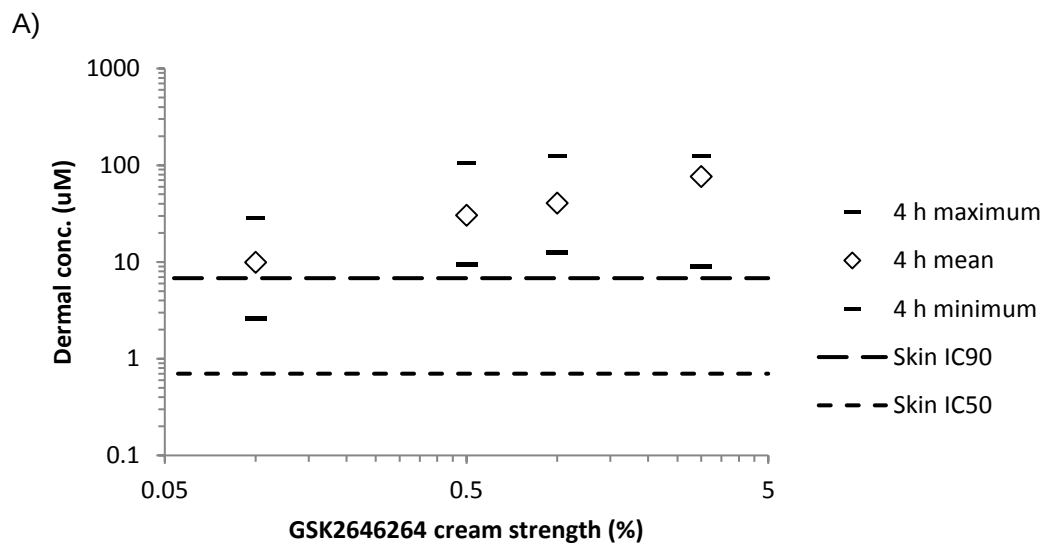
Supporting information

The following three figures and one table are presented as supporting information because they are not essential to the results and discussion of the manuscript but provide details that are likely to be of interest to scientists who wish to know more about the way the experiments were performed, how the data was integrated, as well as the structure, IUPAC name and molecular weight of the compound.

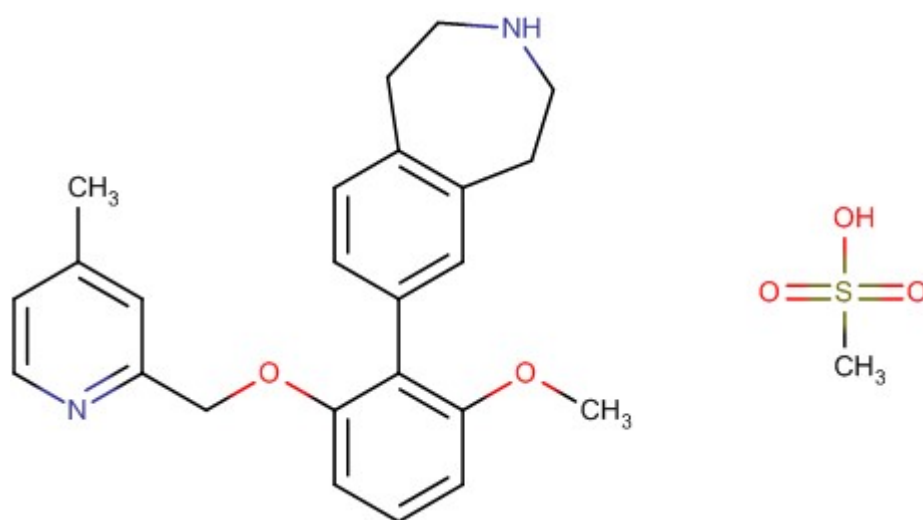
Supporting information Figure S1. SkiP device used to test the topical of application of GSK2646264 creams in the Topical drug delivery and inhibition of histamine release following anti-IgE skin challenge



Supporting information Figure S2. GSK2646264 mean and range of dermal concentrations from the *in vitro* human skin penetration study testing different strengths (0.1, 0.5, 1.0 & 3.0%) of the cream formulations following a 4 h (A) and 24 h (B) in Franz diffusion cells. The graphs integrate the estimated skin IC50 and IC90 as determined by the Inhibitory Effect E0 at C0 model described in Figure 2 for comparison.



Supporting information Figure 3. Compound structure (salt version) and IUPAC name for GSK2646264. 7-{2-methoxy-6-[(4-methylpyridin-2-yl)methoxy]phenyl}-2,3,4,5-tetrahydro-1H-3-benzazepine; methanesulfonic acid. Molecular Weight (salt): 470.581 Da. Molecular Weight of parent: 374.475 Da.



Supporting information Table S1. GSK2646264 creams composition (all creams prepared with GSK2646264D, the mesylate salt version of the free base GSK2646264).

	Creams composition (%)				
GSK2646264 free base	0.10	0.50	1.0	3.0	Placebo
GSK2646264 salt equivalent	0.13	0.63	1.26	3.77	0
Stearic acid	2.5	2.5	2.5	2.5	2.5
Crodamol GTCC	5.0	5.0	5.0	5.0	5.0
Cetostearyl alcohol	5.0	5.0	5.0	5.0	5.0
Isoproyl myristate	5.0	5.0	5.0	5.0	5.0
Dimethicone 350	1.0	1.0	1.0	1.0	1.0
Phosphate buffer pH 5.5	64.27	62.77	52.14	34.63	38.4
Arlacel 165	6.0	6.0	6.0	6.0	6.0
Propylene Glycol	5.0	5.0	5.0	20.0	20.0
Glycerol	5.0	5.0	5.0	5.0	5.0
Benzyl Alcohol	0	2.0	0	2.0	2.0
Ethanol	0	0	10.0	10.0	10.0
Phenoxyethanol	1.0	0	1.0	0	0
Butylated hydroxyanisole	0.1	0.1	0.1	0.1	0.1