5α-TETRAHYDROCORTICOSTERONE: A TOPICAL ANTI-INFLAMMATORY GLUCOCORTICOID WITH AN IMPROVED THERAPEUTIC INDEX IN A MURINE MODEL OF DERMATITIS

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RESEARCH ARTICLE

5α-Tetrahydrocorticosterone: A topical anti-inflammatory glucocorticoid with an improved therapeutic index in a murine model of dermatitis

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Abstract

Background and Purpose: Glucocorticoids are powerful anti-inflammatory drugs, but are associated with many side-effects. Topical application in atopic dermatitis leads to skin thinning, metabolic changes, and adrenal suppression. 5α-Tetrahydrocorticosterone (5αTHB) is a potential selective anti-inflammatory with reduced metabolic effects. Here, the efficacy and side-effect profile of 5αTHB were compared with hydrocortisone in preclinical models of irritant dermatitis.

Experimental Approach: Acute irritant dermatitis was invoked in ear skin of male C57BL/6 mice with a single topical application of croton oil. Inflammation was assessed as oedema via ear weight following treatment with 5αTHB and hydrocortisone. Side-effects of 5αTHB and hydrocortisone were assessed following chronic topical steroid treatment (28 days) to non-irritated skin. Skin thinning was quantified longitudinally by caliper measurements and summarily by qPCR for transcripts for genes involved in extracellular matrix homeostasis; systemic effects of topical steroid administration also were assessed. Clearance of 5αTHB and hydrocortisone were measured following intravenous and oral administration.

Key Results: 5αTHB suppressed ear swelling in mice, with ED_{50} similar to hydrocortisone (23 μg vs. 13 μg). Chronic application of 5αTHB did not cause skin thinning, adrenal atrophy, weight loss, thymic involution, or raised insulin levels, all of which were observed with topical hydrocortisone. Transcripts for genes involved in collagen synthesis and stability were adversely affected by all doses of hydrocortisone, but only by the highest dose of 5αTHB (8× ED_{50}). 5αTHB was rapidly cleared from the systemic circulation.

Conclusions and Implications: Topical 5αTHB has potential to treat inflammatory skin conditions, particularly in areas of delicate skin.

Abbreviations: 5αTHB, 5alpha-tetrahydrocorticosterone; ACTH, adrenocorticotrophic hormone; GR, glucocorticoid receptor; GRE, glucocorticoid response element; HC, hydrocortisone; HPA, hypothalamic pituitary adrenal axis.

KEYWORDS
5α-tetrahydrocorticosterone, anti-inflammatory, dissociated steroid, glucocorticoid, selective glucocorticoid receptor modulator

What is already known

- Glucocorticoids effectively treat skin inflammation but cause many adverse effects, including skin thinning.
- There is great interest in developing glucocorticoids with more selective actions.

What does this study add

- Topical 5α-tetrahydrocorticosterone can alleviate dermatitis in murine models.
- Topical 5α-tetrahydrocorticosterone causes less skin thinning and systemic side-effects than hydrocortisone.

What is the clinical significance

- 5α-Tetrahydrocorticosterone may be a prototype for developing safer topical therapies of skin inflammation.

1 | INTRODUCTION

Glucocorticoids have been used extensively to treat inflammatory conditions since the 1960s. They are highly effective and have broad clinical application, including atopic dermatitis, asthma and rheumatoid arthritis, but their use is limited by deleterious side-effects. Most of their actions are mediated through the **glucocorticoid receptor** (GR; systematic nomenclature NR3C1, Cain et al., 2023), which is ubiquitously expressed. There has been great interest in designing selective GR modulators, to act as safer anti-inflammatory agents (De Bosscher et al., 2010; McMaster & Ray, 2008).

Ligand-driven changes in GR action offer exciting opportunities in drug design and, indeed, previous studies manipulating the A-ring of glucocorticoids have demonstrated interactions with alternative ligand binding pockets within GR's active site (Biggadike et al., 2009). Efforts from both academia and industry through in vitro screening have identified steroidal and non-steroidal compounds with selective properties (Bäumer et al., 2017; Chirumamilla et al., 2017; Coghlan et al., 2011; Dack et al., 2020; Schacke et al., 2004, 2009; Vollmer et al., 2012; Zhang et al., 2009).

Understanding the molecular mechanisms underpinning the diverse actions of GR has aided in this search (Nixon et al., 2011; Ratman et al., 2013). Transactivation of metabolic genes is mainly mediated through GR-dimers, whereas inflammation may be suppressed by glucocorticoids acting via GR monomers, attenuating the actions of other pro-inflammatory transcription factors, such as NFκB and AP-1, by tethering (Louw, 2019; Souffriau et al., 2018; Yang et al., 2021). Therefore, distinction of transactivation and transrepression has been the cornerstone of ligand screening (Schacke et al., 2006). Although ligands with a selective transrepression profile in vitro have been developed, unfortunately translation into systemic therapy in vivo has failed to demonstrate an improved therapeutic index, and none of these potential candidates have progressed to clinic, albeit several having been tested in clinical trials (Schacke et al., 2009; Vollmer et al., 2012). A potential explanation for this may be that some of the beneficial anti-inflammatory properties of glucocorticoids require activation of genes, for example, induction of dual specificity phosphatase 1 (*DUSP1*) (Abraham et al., 2006; Joanny et al., 2012), which would not be driven by agents selective for effects on transrepression.

Selective anti-inflammatory actions are particularly challenging to achieve with systemic administration, but a better safety profile may be a more realistic aim in the context of topical therapy. Atopic dermatitis affects 2%–10% of adults and 15%–20% of children, being particularly prevalent in young children (Abuabara et al., 2018), where it mainly affects the delicate facial skin. Steroid therapy is not recommended for infants under 2 years and even then is limited to **hydrocortisone**, the mildest treatment and for short time periods of up to 1 week. There is anxiety among patients' families and clinicians over skin thinning and systemic side-effects with steroid use in children, due to significant absorption of steroid across the delicate skin in infants (Charman et al., 2000).

We have previously reported that a novel endogenous steroid, **5α-tetrahydrocorticosterone** (5αTHB), demonstrates dissociated properties (Gastaldello et al., 2017; Yang et al., 2011). 5αTHB is a metabolite of **corticosterone** (compound B), the principal endogenous glucocorticoid in rodents and also formed in low levels in humans. When administered systemically to mice, it suppresses inflammation in thioglycolate-induced peritonitis and causes generalised immune suppression, but without adverse metabolic side-effects, at least in the short term (Yang et al., 2011). When administered topically to mice, 5αTHB effectively suppresses irritant dermatitis, reducing inflammatory cell infiltrate and **myeloperoxidase** activity, indicative of a particular decrease in neutrophils, and increasing expression of the anti-inflammatory mediators lipocortin and DUSP1 (Gastaldello et al., 2017). In addition, in both in vitro and in vivo models of angiogenesis, 5αTHB was less angiostatic than corticosterone (Abernethie et al., 2022). Here, we use pre-clinical models to compare 5αTHB with the commonly used treatment hydrocortisone and assess

pharmacokinetics, efficacy to treat topical inflammation caused by irritant dermatitis, and also the associated side-effect profile.

2 | METHODS

2.1 | Test systems

2.1.1 | Mouse models

C57BL/6 male mice (male, aged 8–10 weeks, approx. 25 g) were obtained from Harlan UK and housed in groups of two to three per cage at 18–22°C, in Techniplast 1284 mouse cages with 2HK bedding and DFS shavings, with environmental enrichment of rodent rolls and fun tunnels. Animals were kept in standard 12-h light–dark cycles and given free access to drinking water and standard chow (Special Diet Services, Witham, UK). Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the *British Journal of Pharmacology* (Lilley et al., 2020). Experiments were performed under Home Office License following review by the University of Edinburgh Animal Welfare and Ethical Review Body. At cull (9 AM, by asphyxiation with CO_2), thymus and adrenal glands were excised, fixed in formalin and weighed; plasma was prepared from trunk blood collected in EDTA. Both ears were excised along the proximal margin of the pinnae, weighed and snap frozen on dry ice within 1 min. Frozen samples were stored at −80°C.

2.2 | Experimental protocols and design

All protocols adhere to *BJP* guidelines (Curtis et al., 2018). Mice were randomly assigned to groups of equal size. For studies of irritant dermatitis, groups sizes of six gave a power of >95% to detect a 50% reduction in inflammation with hydrocortisone. For studies of chronic steroid exposure, groups sizes of six gave a power of >95% to detect a 10% reduction in skin thickness. The C57BL/6 strain was selected as a mouse strain sensitive to croton oil-induced dermatitis and responsive to steroids (Gastaldello et al., 2017). All in vivo measurements and post-mortem analysis (but not in vivo applications) were conducted by scientists blinded to the nature of the treatment.

2.2.1 | Efficacy to treat acute irritant dermatitis

Mild inflammation in mice (n = 6 per group) was induced with the application of croton oil (300 µg in 10 µl of 95% ethanol:5% isopropyl myristate) to the pinna of the right ear. A steroid (either 5αTHB or hydrocortisone; dose range 1–100 µg) was incorporated into the croton oil solution and co-applied. After 24 h, mice were culled, as described above. Ear inflammation is calculated as the difference in weight between the treated (right) and untreated (left) ear, thus reflecting local oedema, and is presented as the percentage of the maximum swelling seen in the group treated with croton oil alone for each batch of mice on which the experiment was conducted. This approach was approved by our veterinary ethical review panel, in order to allow for day-to-day variance in absolute measures of swelling and so increase the statistical power of our experiment, and thus reduce the number of animals required. Direct comparisons of potency were repeated using 15 and 25 µg (approximate ED_{50}) of either 5αTHB or hydrocortisone. Negative controls were conducted in pilot studies and demonstrated no significant effect of vehicle treatment on apparent ear swelling compared with untreated ears (ear 'swelling' in vehicle group 1.53 ± 0.78 mg vs. untreated group 1.10 ± 1.36 mg, $P = 0.52$; n = 16 and 7, respectively). In inflammation experiments, the mean magnitude of ear swelling in response to croton oil was a weight difference between inflamed and non-inflamed ears of individual mice of 24.57 ± 2.53 mg (n = 20). The untreated, non-inflamed (left) ears weighed 34.37 ± 1.57 mg compared with the treated, inflamed (right) ears, which weighed 58.97 ± 3.74 mg.

2.2.2 | Side-effects of chronic treatment on healthy skin

Responses to steroid were assessed following chronic application of either 5αTHB or hydrocortisone (25–200 µg, in 10 µl 95% ethanol:5% isopropyl myristate) to the right ear of mice daily for 28 days (n = 6 per group). The left ear was untreated. Thickness of both ears (using calipers; Kafer digital thickness gauge, Mapra Technik, UK) and body weight were assessed at 2- to 3-day intervals. Tissues were collected as described above.

2.2.3 | Pharmacokinetic profiles of 5αTHB and hydrocortisone

Pharmacokinetics of 5αTHB and hydrocortisone were assessed following intravenous (penile vein) or oral (gavage) administration (0.1 mg of steroid in 100 µL of 10% ethanol in 0.9% w/v saline). Mice were culled at timed intervals following administration, to 120 min, trunk blood collected and plasma prepared (n = 8 per time point for each steroid and route of administration). Mean data for concentration versus time were fitted to a one compartment model, and half-life and area under the curve were calculated using Kinetica® software (Thermo Scientific, Hemel Hempstead, UK). The ratios of the area under the curve for the oral versus intravenous bolus were expressed as a percentage, representing oral bioavailability.

2.2.4 | Laboratory methods

Insulin, corticosterone and **adrenocorticotrophic hormone** (ACTH) were quantified in plasma by ELISA (Crystalchem, Illinois, USA, and Enzo Life Sciences, Exeter, UK, respectively). RNA was extracted from quarter ears following tissue disruption with a MagNA Lyser (Roche Diagnostics, Burgess Hill, UK) and 250 ng of RNA reverse transcribed and subjected to quantitative real-time PCR, in triplicate, according to MIQE guidelines, and as previously published (Gastaldello et al., 2017; Livingstone et al., 2015). Data are presented relative to the mean of the reference genes *Tbp*, ***Ppia*** and *Rn18s*, which did not differ between treatment groups, and comparisons are made to the vehicle treated group.

5αTHB and corticosterone in plasma were quantified following extraction (10:1 chloroform:aqueous) of plasma (200 µl), following addition of internal standards (epi-cortisol and epi-

tetrahydrocorticosterone; 1 μg) and derivatised to form methoxyamine-trimethylsilyl derivatives (Homer et al., 2017). Analysis was performed using a Quantum Ultra (Thermo Scientific, Hemel Hempstead, UK) gas chromatograph triple quadrupole mass spectrometer, equipped with DB17MS column (30 m, 0.25 mm, 0.25 μm, Agilent, Wokingham, UK). Samples were injected using a programmed temperature vapourisation injector at 120°C and increased to 270°C at 14.5°C·min^{-1}. The oven temperature was initially 120°C and then increased (30°C·min^{-1}) to achieve 200°C and then at 5°C·min^{-1} to achieve 300°C, which was maintained for 5 min. The interface and source temperatures were 280°C and electron voltage 70 eV. Ions were detected in selective reaction monitoring mode, using the following transitions (collision energies): derivative of hydrocortisone (m/z 605 → 515; 20 V), 5αTHB (m/z 474 → 384; 15 V), d4F (m/z 609 → 519; 20 V), d8B (m/z 556 → 525; 15 V). Amounts were calculated versus calibration curves constructed from peak area ratios of standards/internal standards versus amount, processed similarly.

2.3 | Statistical analysis

The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2018). Statistical tests (Student's *t* tests, one- and two-way and repeated-measure ANOVA, with Fisher's LSD post hoc analysis only if ANOVA $P < 0.05$) were performed using Statistica v12 (Statsoft, Bedford, UK). Differences from ANOVA are reported for all doses of each steroid and are referred to as combined steroid groups. Statistical significance was defined as $P < 0.05$. Non-linear regression was performed using GraphPad Prism v6 (La Jolla, USA) to assign ED_{50} values using a robust fit log dose response. Pharmacokinetic modelling was performed using Kinetica v5.1 (Thermo Scientific, Hemel Hempstead, UK).

2.4 | Materials

Chemicals were obtained from Sigma Aldrich (Poole, UK) or VWR Chemical (Lutterworth, UK). Steroids were from Steraloids (Newport, Rhode Island, USA).

2.5 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in https://www.guidetopharmacology.org, and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/2022 (Alexander et al., 2021).

3 | RESULTS

Comparison was made between the efficacy and side-effect profiles of 5αTHB and hydrocortisone following topical application. Pharmacokinetics were compared following oral or intra-venous administration of steroids.

3.1 | 5αTHB suppressed topical acute inflammation associated with croton-oil induced dermatitis

5αTHB prevented swelling induced by croton oil, in a dose responsive manner (Figure 1a), with an ED_{50} value equivalent to that of hydrocortisone (fitted as 23.3 vs. 12.7 μg, respectively), with similar maximal efficacy (THB 65.1% vs. HC 76.4% suppression at the highest concentration tested). When doses representing the fitted ED_{50} values were retested, the extent of suppression of inflammation achieved was comparable for both steroids (Figure 1b).

3.2 | 5αTHB did not cause skin thinning upon topical application

3.2.1 | Treatment of healthy skin

Daily administration of hydrocortisone at the ED_{50} (25 μg) caused substantial thinning of the ear by day 3 of treatment, whereas the same dose of 5αTHB did not cause significant skin thinning at any point during the 28 day period (Figure 2a). Higher doses of hydrocortisone (100 μg and 200 μg) disrupted the integrity of the skin of the ear to such an extent that reliable measurement of thickness by calipers became impossible by day 17 (Figure 2b–d) and three animals in the highest dose group were culled early, under the advice of University of Edinburgh Veterinary Scientific Services, due to the severity of skin atrophy. Although higher doses of 5αTHB cause slight skin thinning at days 6 and 13, this effect was transient and had fully recovered by day 17 (Figure 2a). A similar pattern was observed in the ear weights at cull, with hydrocortisone causing a significant reduction in ear weight at all doses, whereas 5αTHB did not cause any reduction (Figure 2e). Hydrocortisone suppressed abundance of transcripts encoding collagen (***Col1a1***, *Col1a2* and ***Col3a1***) in doses of 25 μg and above (Table 1), whereas the effects of 5αTHB were only seen at the highest dose tested (200 μg) and, even then, were of lower magnitude. Transcript abundance for the collagen chaperone protein, *Serpinh1*, also was suppressed by hydrocortisone, but not by 5αTHB. Transcript abundances for **lysyl oxidases** (*Loxl1*, *2*, *3* and *4*), which catalyse cross-linking of collagen, were suppressed by hydrocortisone but not by 5αTHB. mRNA for *Leprel2*, which is involved in collagen chain assembly, was suppressed by hydrocortisone at all doses, but only at the highest dose tested (8× ED_{50}) of 5αTHB.

3.2.2 | 5αTHB did not cause systemic side-effects upon topical administration

Hydrocortisone caused substantial skin thinning in the untreated contralateral ear following 28 days administration (Figure 3a), whereas this effect was significantly less apparent with 5αTHB, and there was no significant effect at any time point for any individual dose. Furthermore, mice receiving hydrocortisone, but not 5αTHB, lost weight (Figure 3b) and had elevated circulating insulin (Figure 3c).

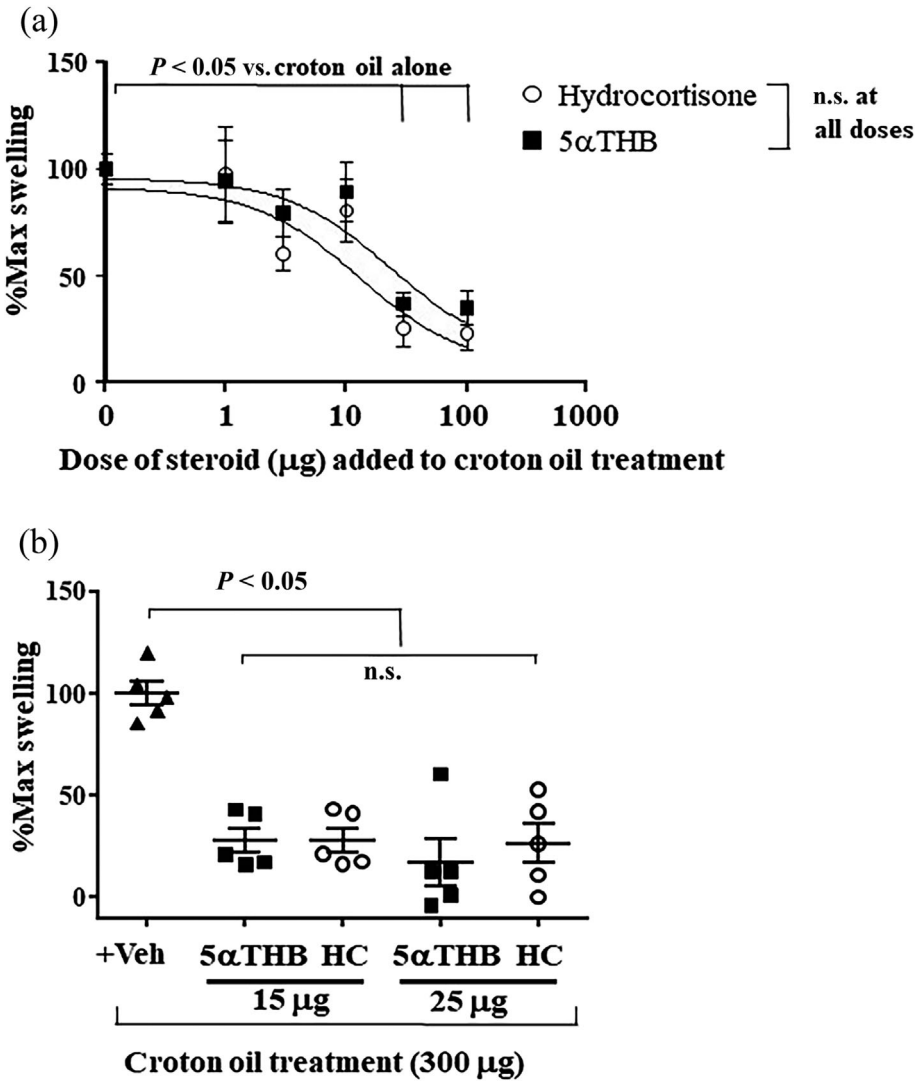

FIGURE 1 5αTHB is effective at suppressing croton-oil induced inflammation. (a) Hydrocortisone and 5αTHB both prevented swelling induced by croton oil, in a dose-dependent manner, and were similarly potent and effective. The ED_{50} value for each steroid was fitted to the mean data as 23.3 μg for 5αTHB and 12.7 μg for hydrocortisone. Data are mean ± SEM, n = 8 per group, and comparison of steroid treatment to croton oil alone was conducted by *t* test at each dose, with multiple comparison correction. $P < 0.05$ for 30 μg and 100 μg doses compared with control. (b) Both 15 and 25 μg doses (as approximate ED_{50}), hydrocortisone and 5αTHB were equally efficacious as preventing swelling in response to croton oil treatment. Data are individual data points, and mean ± SEM, n = 5 per group compared by one way ANOVA with Fisher's post hoc test. Overall ANOVA $P < 0.05$, all steroid treatments $P < 0.05$ compared with croton oil alone (+Veh), comparison of 5αTHB with hydrocortisone was n.s. +Veh, croton oil alone (300 μg croton oil in 10 μl of 95% ethanol: 5% isopropyl myristate); 5αTHB, croton oil plus 5α-tetrahydrocorticosterone (at dose indicated); HC, croton oil plus hydrocortisone (at dose indicated); n.s., not significant.

Hypothalamic–pituitary–adrenal (HPA) axis suppression occurred with hydrocortisone, reflected by a smaller thymus and smaller adrenal glands (Figure 3d,e) and, again, these effects were significantly less apparent with 5αTHB. There also was a trend towards decreased ACTH concentrations in hydrocortisone treated mice compared with 5αTHB groups (0.80 ± 0.18 $pg{\cdot}ml^{-1}$ in combined hydrocortisone groups vs. 2.54 ± 0.87 $pg{\cdot}ml^{-1}$ in combined 5αTHB groups; $P = 0.09$ vs. 2.34 ± 1.25 $pg{\cdot}ml^{-1}$ in vehicle group; $P = 0.3$) although corticosterone concentrations were maintained (101.3 ± 22.5 nM in vehicle group; 129.2 ± 13.9 nM in combined 5αTHB groups and 106.4 ± 11.2 combined hydrocortisone groups).

3.2.3 | 5αTHB was rapidly cleared systemically and poorly orally bioavailable

Following an intravenous bolus, concentrations of 5αTHB declined more rapidly than hydrocortisone, with a much shorter half-life (2.4 vs. 31 min; Figure 4a). Following an oral bolus, 5αTHB was detected in very few mice and only at time points in the range 5–15 min; hydrocortisone was detected reliably with an oral bioavailability of 46% (Figure 4b).

4 | DISCUSSION AND CONCLUSIONS

These in vivo data demonstrate that 5αTHB is an effective topical anti-inflammatory agent with fewer local and systemic side-effects than hydrocortisone. Its rapid clearance is beneficial for a topical agent because any drug that is absorbed will be rapidly inactivated and, thus, 5αTHB may be suitable for development for topical treatment of conditions where standard steroid therapy is contraindicated, for example, facial skin and paediatric applications.

5αTHB binds to GR and displays properties suggestive of anti-inflammatory effects, both in vitro and in vivo, whilst not disrupting metabolism and in contrast to corticosterone (Gastaldello et al., 2017; Yang et al., 2011). In vivo, ACTH was suppressed by 5αTHB to a lesser extent than corticosterone in rats (McInnes et al., 2004), predicting fewer adverse effects on the HPA axis. Low

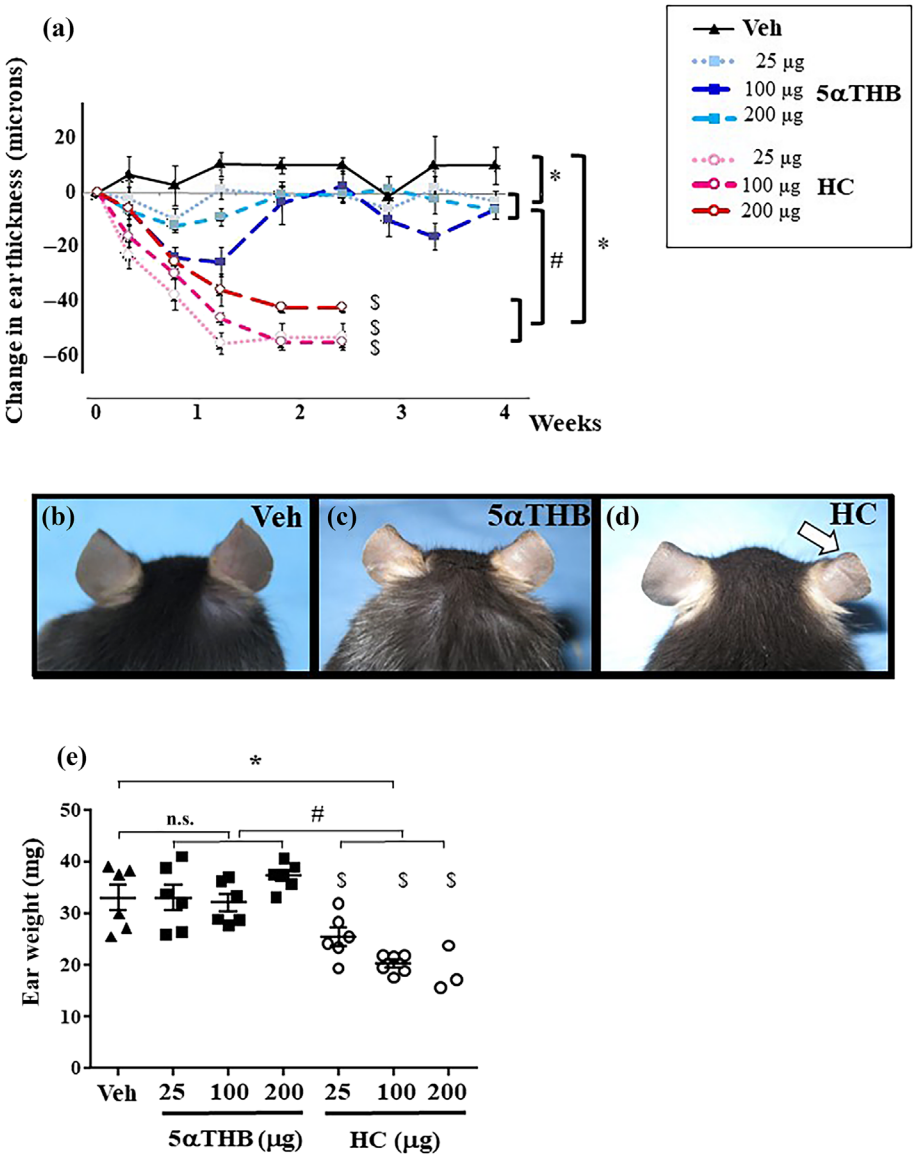

FIGURE 2 5αTHB does not cause skin thinning in effective pharmacological doses. Hydrocortisone caused significant skin thinning when applied daily to the ears of mice in doses of 25, 100 and 200 µg, reaching a maximal effect after 9–11 days (a). Measurements of skin thickness on the ears treated with hydrocortisone were no longer possible from 17 days due to atrophy: representative photographs of atrophy observed only in mice treated with hydrocortisone for 28 days (d) and not vehicle (b) or 5αTHB (c). 5αTHB overall caused only modest skin thinning over 28 days when tested in the dose range 25–200 µg, but no individual dose caused statistically significant thinning overall compared with vehicle and the thinning was significantly less in mice treated with 5αTHB than those treated with hydrocortisone. Ear weight at cull was significantly reduced by hydrocortisone treatment, but there was no effect of 5αTHB (e). Data are mean ± SEM, and for (e) are individual data points with mean ± SEM, compared by repeated measure or one way ANOVA with Fisher's post hoc test. n = 6 per treatment for all ear thickness measurements shown, and for ear weights except HC 200 µg where n = 3 (due to early termination of three of this group for health reasons as a result of effect of HC on skin) and thus not subject to statistical analysis. * indicates $P < 0.05$ on ANOVA when comparing all doses of steroid with the vehicle group, # indicates $P < 0.05$ on ANOVA when comparing all doses of hydrocortisone with all doses of 5αTHB and $ indicates $P < 0.05$ on Fisher's post hoc test for the specific steroid dose annotated compared with the vehicle group. Veh, vehicle (10 µl of 95% ethanol:5% isopropyl myristate); 5αTHB, 5α-tetrahydrocorticosterone; HC, hydrocortisone; n.s., not significant.

circulating levels of 5αTHB were detected following systemic infusion in studies by Yang et al. (2011) and, here, limited oral bioavailability of 5αTHB was demonstrated along with and rapid clearance, likely through conjugation upon first pass metabolism. Therefore, the anti-inflammatory actions of 5αTHB were investigated in a model of topical inflammation.

Steroid sensitive irritant dermatitis was induced by croton oil, a natural agent containing phorbol esters (Reichardt et al., 2001; Schacke et al., 2009). The efficacy and side-effect profile of 5αTHB were compared with hydrocortisone, the most commonly steroid used for paediatric skin inflammation. In previous studies, Gastaldello et al. (2017) demonstrated that 5αTHB suppressed inflammation in this model, but

TABLE 1 Transcriptional changes in treated mouse ears.

		5αTHB			Hydrocortisone			ANOVA		
Gene	Vehicle	25 μg	100 μg	200 μg	25 μg	100 μg	200 μg	5αTHB vs. Veh	HC vs. Veh	5αTHB vs. HC
Loxl1	1.55 ± 0.44	2.89 ± 0.57	2.41 ± 0.52	0.92 ± 0.08	0.72 ± 0.14	0.78 ± 0.16	0.77 ± 0.06			#
Loxl2	1.29 ± 0.16	1.82 ± 0.12	2.18 ± 0.30	1.35 ± 0.16	0.57 ± 0.07	0.62 ± 0.05	0.81 ± 0.04	*	*	#
Loxl3	1.08 ± 0.28	1.68 ± 0.48	1.87 ± 0.37	1.09 ± 0.15	1.16 ± 0.29	0.67 ± 0.18	0.99 ± 0.21			#
Loxl4	1.21 ± 0.23	1.62 ± 0.32	2.11 ± 0.76	1.15 ± 0.21	0.71 ± 0.11	0.53 ± 0.06	0.54 ± 0.08			#
Leprel1	1.16 ± 0.12	1.80 ± 0.30	1.81 ± 0.50	1.17 ± 0.11	0.77 ± 0.02	0.94 ± 0.03	1.00 ± 0.05		*	#
Leprel2	2.57 ± 0.42	3.47 ± 0.73	4.27 ± 1.43	1.16 ± 0.19	0.84 ± 0.15	0.50 ± 0.07	0.42 ± 0.07		*	#
Serpinh1	1.11 ± 0.06	1.24 ± 0.12	1.54 ± 0.14	1.25 ± 0.13	0.63 ± 0.08	0.56 ± 0.04	0.67 ± 0.03	*		#
P4ha2	0.75 ± 0.05	1.12 ± 0.16	1.09 ± 0.17	1.14 ± 0.22	0.55 ± 0.05	0.78 ± 0.11	0.94 ± 0.05	*		#
Col1a1	1.06 ± 0.20	1.33 ± 0.31	1.79 ± 0.25	0.54 ± 0.07	0.25 ± 0.05	0.20 ± 0.03	0.47 ± 0.11		*	#
Col1a2	1.22 ± 0.16	1.37 ± 0.30	2.01 ± 0.28	0.70 ± 0.12	0.26 ± 0.06	0.21 ± 0.03	0.38 ± 0.07		*	#
Col3a1	1.43 ± 0.24	1.43 ± 0.37	2.02 ± 0.24	0.64 ± 0.12	0.22 ± 0.06	0.20 ± 0.03	0.41 ± 0.10		*	#

Note: Data are the ratio of the gene of interest to the mean of the reference genes *Tbp*, *Ppia* and *Rn18s*, which did not differ between treatment groups, and shown are mean ± SEM. n = 6 for each treatment group except hydrocortisone 200 μg (n = 3) . Groups were compared by one-way ANOVA, and if significant a further comparison of effect of steroid to vehicle was made with Fisher's post hoc test. Overall ANOVA $P < 0.05$.
Abbreviations: 5αTHB, 5α-tetrahydrocorticosterone; HC, hydrocortisone; Veh, vehicle.
*$P < 0.05$ for steroid groups (all doses of each steroid) compared with vehicle. #$P < 0.05$ for 5αTHB compared with hydrocortisone.

was less potent than the endogenous steroid corticosterone (which itself is not used therapeutically). Here, 5αTHB was as effective and potent as hydrocortisone in reducing swelling associated with irritant dermatitis, achieving up to ~85% suppression within 24 h.

Skin atrophy is one of the more debilitating side-effects of topical steroid use (Schoepe et al., 2006). At repeated high dose, this effect can become permanent, with the skin structure becoming irreversibly damaged. Thinning of the epidermis due to glucocorticoids is rapid due to high proliferation of keratinocytes, whereas the dermis thins more slowly due to the low proliferation rate of collagen types 1 and 3 (Cossmann & Welzel, 2006). Thinning due to glucocorticoids is characterised by decreased size of keratinocytes (Kolbe et al., 2001), reduced number of fibroblasts (Lehmann et al., 1983) and lipid depletion in the stratum corneum (Sheu et al., 1997). Depletion of extracellular matrix leaves vessels unsupported, which promotes bruising. A further insult to angiostasis brought about by steroids is impaired wound healing when skin is damaged, so a topical anti-inflammatory agent with less damaging effects on skin integrity is desirable. 5αTHB is already known to be less angiostatic (Abernethie et al., 2022; Gastaldello et al., 2017) and, therefore, skin thinning following chronic treatment was assessed in mouse ears over 4-week treatment, using doses that were therapeutic (ED_{50} to suppress inflammation) and also 4× and 8× higher, applied to healthy skin to simulate a toxic dose escalation. As anticipated, hydrocortisone showed substantial skin thinning at the therapeutic dose, with maximal thinning at 7–10 days. Kirby and Munro (1976) showed maximal thinning in mouse ears within 5 days, but this difference may relate to the solvent vehicle used here, which caused skin thinning on its own, whereas Kirby and Munro (1976) used an ointment.

This skin atrophy escalated in severity in a dose responsive manner, with ears becoming shrivelled and paper thin, to the extent that, at the higher doses, measurements were not deemed reliable. 5αTHB had a transient effect to thin the skin but, after 4 weeks of treatment, adverse effects were not seen at any dose tested. These findings were reflected with terminal ear weight, which was reduced substantially by hydrocortisone but not 5αTHB. Recovery also has been reported by Kirby and Munro (1976) who used other steroids, but the mechanism is unclear.

Profiling of gene transcripts underpinning pathways regulating extracellular matrix and inflammation revealed that hydrocortisone, but not 5αTHB, suppressed the abundance of mRNAs of genes encoding proteins responsible for maintenance of extracellular matrix. Tiganescu et al. (2013) demonstrated similar glucocorticoid-driven responses in mouse skin, and reduced transcripts of many similar genes are used as readouts in industrial screening of the therapeutic index of topical glucocorticoids in 3T3 fibroblasts and human keratinocytes (reviewed by Schoepe et al., 2006). Specifically, hydrocortisone down-regulated transcripts of *Col1a1* and *Col1a2*, responsible for synthesis of type 1 collagen (~80% of dermal collagen) and also *Col3a1*, encoding Type 3 collagen (10%–15% of skin collagen). Type 3 collagen is highly sensitive to down-regulation by glucocorticoids (Schoepe et al., 2006). In a previous study, these transcripts were not suppressed after 24 h of treatment with corticosterone (Gastaldello et al., 2017). However, following longer exposure to steroid in an implant model of angiogenesis, corticosterone suppressed collagen abundance (assessed by staining), as well as *Col1a1*, *1a2*, *3a1* and ***Col4a1*** transcripts, whereas 5αTHB had no effect on collagen abundance and more limited effects transcriptionally (Gastaldello et al., 2017). Similarly in in vitro models of angiogenesis, 5αTHB had far fewer transcriptional effects within blood vessels, which were found typical of corticosterone (Abernethie et al., 2022). Further evidence of protection from extracellular matrix depletion with 5αTHB treatment could be seen in this study, with minimal suppression of

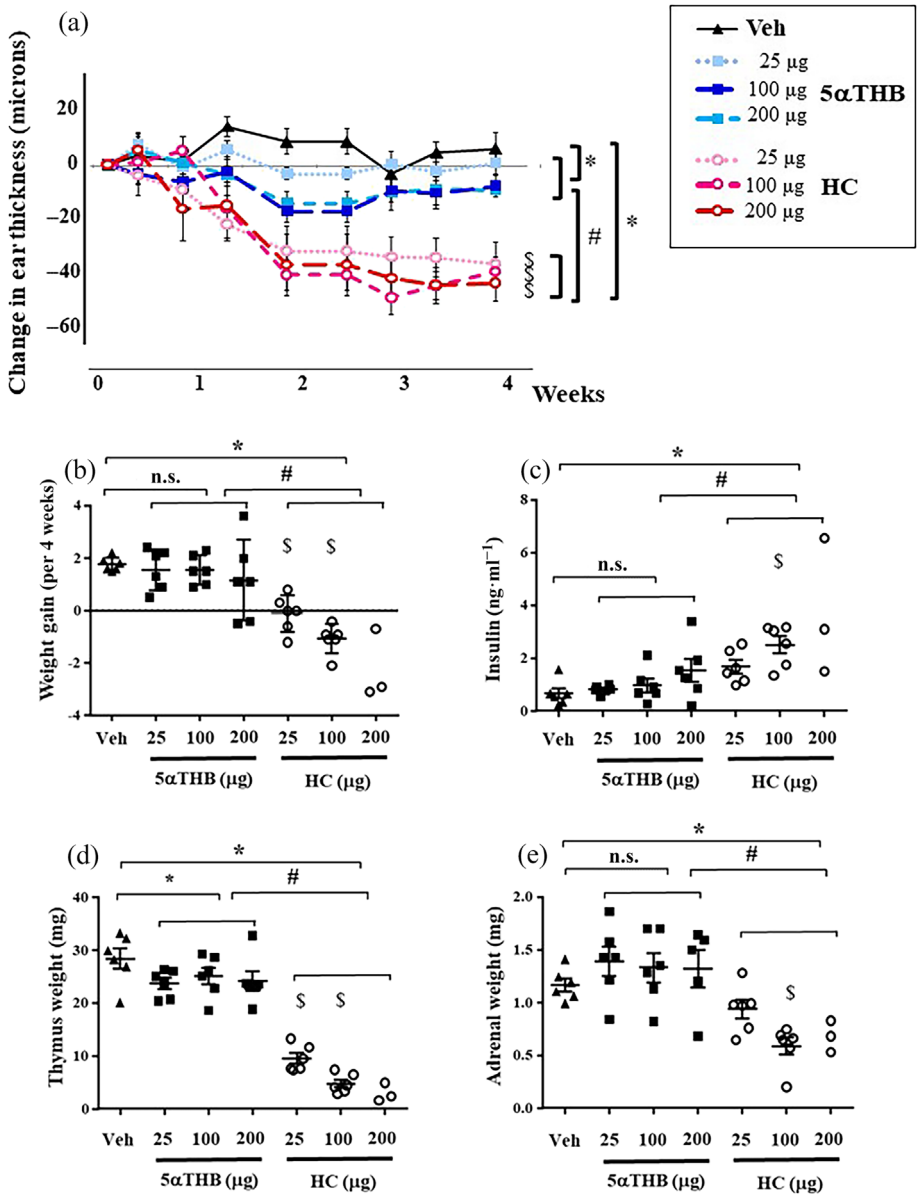

FIGURE 3 5αTHB does not cause system side-effects when administered to skin where the barrier has been disrupted. Following 28 days of administration hydrocortisone, but not 5αTHB, caused skin thinning in the untreated contralateral ear (a) and weight loss (b) and elevated circulating insulin (c). Hypothalamic–pituitary–adrenal axis suppression occurred with hydrocortisone but not 5αTHB, reflected in smaller thymus and adrenal glands (d, e). Data are mean ± SEM, and for (b)–(e) are individual data points with mean ± SEM, compared by repeated-measure or one-way ANOVA with Fisher's post hoc test. n = 6 per treatment, except HC 200 µg where n = 3 (due to early termination of three of this group for health reasons as a result of effect of HC on skin) and thus not subject to statistical analysis. * indicates $P < 0.05$ on ANOVA when comparing all doses of steroid with the vehicle group, # indicates $P < 0.05$ on ANOVA when comparing all doses of hydrocortisone with all doses of 5αTHB and $ indicates $P < 0.05$ on Fisher's post hoc test for the specific steroid dose annotated compared with the vehicle group. Veh, vehicle; 5αTHB, 5α-tetrahydrocorticosterone; HC, hydrocortisone.

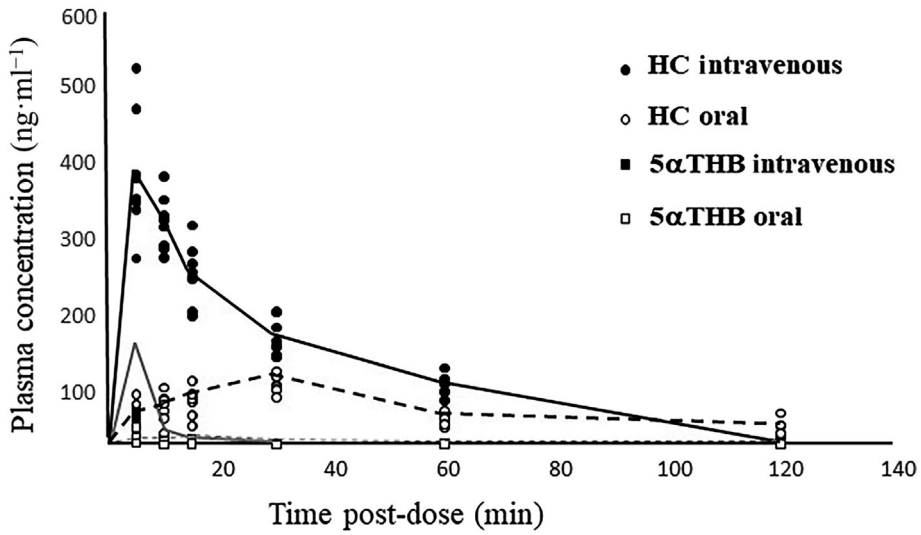

FIGURE 4 5αTHB is cleared more rapidly than hydrocortisone. Concentrations of 5αTHB and hydrocortisone in plasma following an intravenous bolus, showing more rapid clearance of 5αTHB than hydrocortisone. 5αTHB had highly limited oral bioavailability. Data shown are individual points in conjunction with lines representing the mean population data used to derive pharmacokinetic parameters by modelling as one compartment, n = 8 per group, with steroids not detected in all samples at every time point. 5αTHB, 5α-tetrahydrocorticosterone; HC, hydrocortisone.

transcripts for lysyl hydroxylases and **prolyl hydroxylases**, which form hydroxyproline to stabilise the collagen helix, and also *SerpinH1*, a collagen-specific chaperone protein. Hydrocortisone suppressed these transcripts whereas 5αTHB at the ED_{50} did not have any effects, although smaller changes were apparent at the maximum dose tested. These findings in conjunction with those of Gastaldello et al. (2017), demonstrating that 5αTHB is less angiostatic than corticosterone, suggest a portfolio of a safer anti-inflammatory drug with less adverse effects on skin integrity or wound repair.

Systemic absorption of steroids, even when given topically, can cause side-effects including altered metabolism and adrenal suppression, with children more susceptible due to greater absorption through their thinner skin. Metabolic systemic side-effects were seen in a dose-dependent manner with hydrocortisone, including loss of weight and increased circulating insulin. Hydrocortisone also suppressed the HPA axis, reflected in adrenal atrophy and thymic involution. Interestingly, skin thinning on the contralateral ear was observed with hydrocortisone, albeit developing more slowly than in the ears receiving steroid directly, presumably by delivery through the blood following absorption. This effect also had been previously noted by Kirby and Munro (1976). However, again, protection from systemic side-effects with 5αTHB was observed—akin to custom designed 'dual-soft' agonists (Dack et al., 2020). Even at the highest dose of 5αTHB, there were no significant effects on metabolic parameters or adrenal weight and only a modest decrease in thymus weight.

Several reasons may be proposed to explain the differential susceptibility to systemic side-effects. 5αTHB has a different profile of cellular responses to classical glucocorticoids, but also has different pharmacokinetic properties. The pharmacokinetic profile demonstrates that 5αTHB is rapidly cleared upon entering the systemic circulation, so blood levels would remain very low. Indeed, its elimination was 10 times faster than hydrocortisone, and 5αTHB was virtually undetectable in blood after oral administration: In contrast, hydrocortisone was ∼47% orally bioavailable. Hydrocortisone is absorbed into the blood following topical administration giving rise to severe systemic side-effects. While approaches to formulation may be employed to moderate this problem, the same adverse responses are not observed following treatment with 5αTHB. 5αTHB may either be cleared rapidly upon absorption or indeed not absorbed extensively through the skin. However, the physico-chemical properties of 5αTHB suggest it to be more lipophilic than hydrocortisone, with a logP of 2.76 versus 1.76 (Billich et al., 2004, 2005). The fact that absorption of topically administered compounds in rodents can exceed human by up to 10-fold bodes well for a lack of systemic side-effects in man treated with 5αTHB (Schoepe et al., 2006).

The mechanism of action of glucocorticoids to deplete collagen is poorly understood and often does not involve traditional transactivation. Col1a1, for example, does not have a GRE or negative GRE, and the effects of glucocorticoids may be mediated indirectly via tethering with Smad3 to negatively regulate gene transcription, whereas GR tethers with AP-1 to down-regulate transcription of *Col3a1* and **matrix metalloproteases**, as reviewed by Schoepe et al. (2006). GR tethering to AP-1 also is implicated in negative feedback effects of glucocorticoids on **CRH** (Malkoski & Dorin, 1999), as well as through GR monomer binding to negative GREs, and this may provide a common mechanism to explain the lack of effects of 5αTHB on collagen integrity and the HPA axis. It is also possible that alternative receptors may be involved; for example, MR antagonists can alleviate glucocorticoid-driven skin atrophy (Bigas et al., 2018; Lee et al., 2021; Maubec et al., 2015; Sevilla et al., 2020; Sevilla & Perez, 2018).

A key next step will be demonstrating a similar profile of efficacy with reduced side-effects in the context of established inflammation. These experiments are problematic using the mouse ear model of inflammation, due to conflation of the anti-inflammatory effect with the skin thinning effect of hydrocortisone, which is evident as early as days 3–6 in the experiments described above. This challenge is demonstrated in Figure S1, and reinforced by Yang et al. (2021), and we therefore suggest the next step should be undertaken in more translational models, such as pigs, as has been proposed by others in the field (e.g., Dack et al., 2020).

In conclusion, 5αTHB is associated with an in vivo profile of activity, which supports the glucocorticoid metabolite as a safe and effective anti-inflammatory steroid and a candidate for future drug development. Moreover, it may offer mechanistic insights into disorders where the generation of 5α-reduced glucocorticoids are enhanced, for example, in obesity (Andrew et al., 1998; Fraser et al., 1999), polycystic ovarian syndrome (Stewart et al., 1990) or suppressed, for example, fatty liver disease (Westerbacka et al., 2003).

AUTHOR CONTRIBUTIONS

D. E. W. Livingstone planned and performed all experiments, analysed data and wrote and revised the manuscript. R. Andrew and B. R. Walker planned experiments, aided in data interpretation and wrote and revised the manuscript. All other authors participated in planning, executing and interpreting subsections of the experimental work and commented on the manuscript.

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CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest for this manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. The data will be retained in full on secure servers for 6 years after the publication date.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design and Analysis, and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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