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Sepiapterin reductase inhibition selectively reduces inflammatory joint pain and increases urinary sepiapterin

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Abstract

Objective—To evaluate the anti-inflammatory and analgesic effect of sepiapterin reductase (SPR) inhibition in a mouse model of inflammatory joint disease and to evaluate sepiapterin as a non-invasive, translational biomarker of SPR inhibition/target engagement in mice and healthy human volunteers.

Methods—The collagen antibody-induced arthritis (CAIA) mouse model was used to induce joint inflammation. The effect of pharmacological inhibition of SPR on heat, cold and mechanical threshold sensitivity and signs of inflammation was tested in mice. Urine from mice and healthy volunteers treated with SPR inhibitors were analyzed by HPLC for changes in sepiapterin levels.

Results—CAIA presented joint inflammation with heat, mechanical and cold pain hypersensitivity (inflammatory acute phase), and heat, mechanical but not cold hypersensitivity without joint inflammation (post-inflammation; late phase). SPR inhibition significantly attenuated CAIA-induced heat hyperalgesia in both phases, and mechanical allodynia in the late phase. Signs

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MF, DdLS, BLT and NA performed behavior, DdLS, AL, BLT and NA performed HPLC, SJFC, MF, DdLS, AL and NA performed statistical analysis and biomarker calculations, SJFC, MC, AL, CW, AL and NA contributed to experimental design and writing.

of inflammation were unaffected by SPR inhibition. Urinary BH4 levels were increased (100%; $p<0.05$) during inflammation and reduced ($p<0.05$) by SPR inhibition. Increased urinary sepiapterin levels in the presence of SPR inhibition were associated with high sensitivity (70–85%) and specificity (82–88%) in both mice and healthy volunteers.

Conclusion—SPR inhibition reduces pain associated with joint inflammation, showing potential utility as an analgesic strategy for inflammatory joint pain. SPR inhibition also increases urinary sepiapterin, indicating the potential of this measurement as a non-invasive biomarker of target engagement of SPR inhibitors, such as sulfasalazine (SSZ), a disease-modifying anti-rheumatic drug used as a first line treatment for rheumatoid arthritis (RA).

Introduction

Tetrahydrobiopterin (BH4) has been traditionally described as a mandatory co-factor for aromatic amino acid hydroxylases, all nitric oxide synthase isoforms and alkylglycerol monooxygenase [for a review see (1,2)]. The enzyme sepiapterin reductase (SPR) plays a dual role in the regulation of BH4 intracellular levels: SPR catalyzes the last step of the *de novo* BH4 synthetic pathway, which initiates from GTP, and also participates in the BH4 biosynthetic salvage pathway, using sepiapterin and 7,8-dihydrobiopterin (BH2) as metabolic intermediates (2,3).

Increased BH4 levels in injured sensory neurons and inflamed tissues correlate with pain scores in humans and mice (4) and reducing BH4 production by treatment of mice with a small molecule SPR inhibitor (SPRi3) reduces pain and inflammation in models of granulomatous skin (complete Freund's adjuvant; CFA) or joint inflammation as well as reducing release of NO from macrophages *in vitro* (5). Recently, it was also discovered that SPR inhibition reduces T cell proliferation and decreases both autoimmune and type 2 allergic inflammation (6).

In the present study we investigated whether inhibition of SPR by treatment with two chemically distinct SPR inhibitors, SPRi3 and QM385, would reduce pain in the CAIA model of inflammatory joint pain. The model differs from the granulomatous CFA model because it is induced by injection of a cocktail of five mouse monoclonal antibodies that recognize conserved individual epitopes on LyC1 and LyC2 of the CB11 fragment of various species of type II collagen (7). After a trigger injection of lipopolysaccharide (LPS), there is rapid onset of clinical signs of arthritis (swelling, redness with a peak at 8–12 days and lasting for up to 28 days post-induction) accompanied by persistent mechanical and thermal hypersensitivity lasting at least 55 days post-induction (7).

Biomarkers have many essential uses including confirmation of target engagement, aiding dose selection for efficacy, and minimizing adverse effects (8). We have shown that sepiapterin levels in plasma and sensory neurons reflect the degree of SPR inhibition *in vitro* and *in vivo* (5,6), suggesting that sepiapterin could be used as a biomarker for SPR inhibition. In this study we assessed the reliability of sepiapterin in urine as a biomarker for SPR inhibition both in mice and humans. Additionally, in a human volunteer study we used sulfasalazine (SSZ), a small molecule, disease-modifying anti-rheumatic drug (DMARD) commonly recommended as a first line treatment for RA patients(9). SSZ inhibits SPR *in*

vitro at high concentrations (10) and we have now extended these findings to determine if it inhibits SPR *in vivo*.

Materials & Methods

Animals:

BALB/c male mice (9–10 weeks old, Jackson Laboratory) were housed in groups of five with food and water available *ad libitum* in a temperature-controlled room maintained on 12 h light/dark cycle (lights on 07:00 to 19:00h, 22±1°C, 50–60% humidity) and used after at least one week of acclimatization. All animal procedures were approved by the Boston Children's Hospital Institutional Animal Care and Use Committee (IACUC).

Healthy volunteers:

A group of 10 pain-free subjects (male n=4, female n=6, age range 31.9 ± 6.7) from Florianópolis, Brazil, were recruited. None were taking any medication for pain complaints and none reported any symptoms of pain prior to or during the study. Each volunteer collected one sample of urine before starting the SSZ administration ("pre"), and then on the following 3 days took a 500mg tablet, approximately every 6 h (total 2g/day) and collected the first voided urine each following morning. After the third day of SSZ treatment, volunteers did not take any further doses of SSZ but continued to collect the first voided urine of the day for 4 more days. A total of 8 urine samples per volunteer were collected for analysis. All treatment and urine collections were completed between June 2017 and July 2017. The healthy human volunteers study was performed in agreement with the Declaration of Helsinki principles. Written informed consent was received from participants prior to inclusion (Ethical Committee for Research in Humans, Universidade Federal de Santa Catarina, Brazil, Protocol # CAAE: 54297916.7.0000.0121).

CAIA model induction

Mice were randomly assigned to either CAIA or control (injected with non-specific IgG and LPS and tested together with experimental mice in a blinded manner) groups with both treatments represented in every cage. On Day 0, control mice were injected with non-specific IgG and model mice received an injection (1.5mg) of a cocktail of 5 monoclonal antibodies (clone A2–10 (IgG2a), F10–21 (IgG2a), D8–6 (IgG2a), D1–2G (IgG2b), and D2–112 (IgG2b) recognizing the conserved epitopes on various species of type II collagen (Chondrex Inc.; Redmond, WA, USA). On Day 3 all mice were injected i.p. with LPS (Chondrex Inc; 50 µg/mouse) to enhance arthritis induction (7).

Behavioral Testing for responses to thermal, mechanical and cold stimuli

To ensure equal baseline data prior to dosing of test compound, treatments were allocated a letter (A or B) and mice ranked from low to high, in terms of heat latencies or mechanical sensitivity. Treatments were then allocated in order, resulting in random allocation across cages. All behavioral testing was performed by a person blinded to model status (CAIA or control) and compound treatment (someone different performed the treatments from the person measuring behavioral responses).

Overall, two separate CAIA experiments were performed. Experiment 1 tested the effect of SPRi3 on pain hypersensitivity in both early and late phases of the CAIA model. Experiment 2 focused on the early phase of the model where joint inflammation is the key characteristic, and we tested the effect of a chemically distinct SPR inhibitor, QM385, that is a more potent inhibitor of SPR than SPRi3. Effect of pharmacological treatment on urinary or plasma sepiapterin levels was measured in both experiments.

Radiant heat:

Mice were assessed for responses to radiant heat (IITC Life Science Inc., USA) (Experiment 1: Day 0 to Day 60; Experiment 2: Day 0 and Day 10)(5). Testing was performed at the same time of day as when baseline responses were measured. After habituation on a glass surface (30°C; 30–60 min) a radiant heat source was applied to one hind paw and the latency to withdraw the paw from the heat stimulus was recorded.

von Frey:

Mice were assessed for responses to mechanical stimuli (Experiment 1: Day 0 to Day 60; Experiment 2: Day 0 to Day 9), as previously reported (5). Testing was performed at the same time of day as when baseline responses were measured. After habituation to test cages (7.5 × 7.5 × 15 cm; 30–60 min), baseline mechanical sensitivity was determined with eight von Frey filaments (bending force of 0.02, 0.04, 0.07, 0.16, 0.4, 0.6, 1 and 2 g) applied to the central part of the hind paw. Following a response, filaments were applied 3 s after the mouse returned the paw to the floor. For mechanical threshold the minimal force filament to which animals responded (at least 5 of the 10 stimulations) determined the response threshold.

Acetone test:

Mice were assessed for nocifensive responses to cooling by acetone evaporation (Experiment 1: Day 0 to Day 60; Experiment 2: Day 0 to Day 14)(5). Testing was performed at the same time of day as baselines. After habituation to test cages (7.5 × 7.5 × 15 cm; 30–60 min) a drop of acetone was applied to the hind paw and the duration of flinching, lifting and licking was recorded for one minute.

Clinical signs of inflammation

Body weight and clinical arthritis scores were assessed by an observer, blinded to the treatments, over 60 days in Experiment 1. For experiment 2 the measures were performed from Day 0 to Day 13. A 16 point-scoring system was used (0: normal paw; 1: one toe inflamed and swollen; 2: mild swelling of entire paw; 3: entire paw inflamed and swollen; 4: very inflamed, swollen and ankylose paw) (7). All pharmacological treatments were randomized within the home cage.

Paw edema

Right and left hind paw edema was measured from Day 0 to Day 13 in Experiment 2 as paw volume (in mL) using a Digital Water Plethysmometer (Bioseb, Pinellas County, FL, USA).

The mouse was gently restrained, and each hind paw placed separately into a water bath. The increase in paw volume (mean of 2 measures) was calculated daily by the difference from the baseline volume.

Joint Histology

Mice were euthanized on Day 13 and 60 min after QM385 administration (see compound section for regimen), hind paws removed and placed in 4% paraformaldehyde. Paws were decalcified, sectioned and stained with hematoxylin and eosin. The hematoxylin/eosin-stained specimens were analyzed by the presence of inflammatory cells, proliferation of new bone and cartilage around and within the joint. All fields of the sections were examined and evaluated carefully by a pathologist blind to the treatments. The degree of histologic changes were graded semi-quantitatively (0: no visible inflammation; 1: some inflammation; 2: more inflammation; 3: moderate inflammation; 4: extensive inflammation) (7).

Sample collections and HPLC measurements

Blood collection:

Mouse blood samples were collected 1 h after SPR inhibitor administration (on Day 13 in Experiment 2) by cardiac puncture in tubes containing anticoagulant (Ethylenediaminetetraacetic acid) and centrifuged ($5,000 \times g$ for 15 min at room temperature) to isolate plasma. Plasma samples were precipitated by the addition of 1 volume (v/v) of 5% trichloroacetic acid plus 6.5mM dithioerythritol. Afterwards, samples were centrifuged ($10,000 \times g$ for 10 min at 4°C) and 20uL analyzed for sepiapterin levels.

Urine Collection:

Animals: Urine was collected from unrestrained mice by placing mice in the same device used for von Frey, (a plastic cage on a wire grid floor). Urine was collected by pipette on a plastic sheet under the rack on Days 0 and 7, 13, 14, 21, 28, and 35, 1 h after SPRi3 administration, and on Days 1 and 2 after SSZ administration and stored at -80°C until analysis.

Healthy volunteers: Each volunteer collected a sample of their first urination of the day, which was stored at -20°C until all 8 samples of the volunteer were collected. Then all samples were submitted to the laboratory and stored at -80°C until analysis. Samples were treated as described above before sepiapterin measurements.

BH4 measurements: Urine samples were precipitated by the addition of one volume of 5% trichloroacetic acid (TCA), containing 6.5mM dithioerythritol (DTE). Samples were centrifuged at $16,000 \times g$ for 10 minutes at 4°C. Ten microliters of supernatant were transferred to a HPLC vial for analysis. The HPLC analysis of BH4 was carried out by using a Waters Atlantis dC-18 5 μm reverse phase column ($4.6 \times 250 \text{ mm}$), with a flow rate set at 0.60 mL/min and an isocratic elution of 6.5 mM sodium phosphate buffer, 6 mM citric acid, 1 mM sodium octyl sulfate (OSA), 2.5 mM diethylenetriaminepentaacetic acid (DTPA), 160 μM DTE and 12% acetonitrile, pH 3.0. The OSA was used in the mobile phase as an ion-pairing reagent which acts as an anionic counter ion for the separation and resolution of

positively charged analytes. The DTPA was added to chelate transition metals to prevent oxidation of the analytes, and the DTE was used as a reductant to further stabilize the reduced form of BH4. The Beckman Coulter Inc. HPLC system including a Model 125 Solvent Delivery Module and a Model 508 Autosampler controlled via 32 Karat Software 8.0. The electrochemical detector (Thermo Scientific - Dionex Coulochem III) with two-sensor electrochemical cells was routinely operated at +50 and +450 mV. The +450 mV channel provided the most sensitive response for measuring the BH4. The results were expressed as μmol of BH4 per mmol creatinine ($\mu\text{mol}/\text{mmol}$ creatinine).

Sepiapterin measurements: Blood samples were precipitated by the addition of one volume of 5% TCA, containing 6.5mM DTE. Samples were centrifuged at $16,000 \times g$ for 10 minutes at 4°C. Ten microliters of supernatant were transferred to a HPLC vial for analysis. Urine samples were centrifuged ($16,000 \times g$ for 10 min at 4°C), the supernatant diluted in 10 volumes (v/v) of 15 mM phosphate buffer and 20 μL of the sample were analyzed. HPLC analysis of sepiapterin was done using an Alliance e2695, Waters, Milford, USA, a Waters Atlantis dC-18, 5 μm RP column (4.6×250 mm; temperature 35°C), a flow rate (0.7 mL/min) and isocratic elution of mobile phase (85% phosphate buffer (15mM); 15% acetonitrile, pH 6.4). Identification and quantification of sepiapterin was done using a multi-wavelength fluorescence detector (ex. 425 nm, em. 530 nm, module 2475, Waters, Milford, USA) and calculated as nmol of sepiapterin per liter of plasma (nmol/L), or μmol of sepiapterin per mmol of creatinine ($\mu\text{mol}/\text{mmol}$ creatinine).

Biomarker Calculations

Assessment of sepiapterin as a pharmacodynamic biomarker of SPR inhibition done by counting the number of true positives, true negatives, false positives and false negatives after SPRi3 or SSZ treatment. The cut-off value was defined as mean + 1 $\times$ standard deviation of all of the non-drug treated urine samples (Table 1).

Compounds

Compounds for mouse studies were prepared fresh on the day of testing and administered by someone other than the person performing the behavior testing. Collagen cocktail, IgG and LPS (supplied frozen) were defrosted immediately prior to injection. Compounds for systemic injection were administered in 10mL/kg.

SPRi3 (IC_{50} of 5.2 μM in a cell-based assay) (5) was dissolved (30mg/mL; i.p.) in 2-hydroxypropyl- β -cyclodextrin (50% w/v prepared in 0.9% sterile saline). SPRi3 is rapidly absorbed into the plasma (T_{max} 0.11 h, $T_{1/2}$ 3.95 h) after intra-peritoneal injection (6) and induces a maximum reduction of nociceptive hypersensitivity and plasma BH4 levels 1 h post-administration (5). We therefore administered SPRi3 1 h before behavioral and biomarker measurements. QM385 (IC_{50} of 35nM in a cell-based assay) was suspended (0.3mg/mL; p.o.) in 0.5% TWEEN 80/5% carboxymethyl cellulose in 0.9% saline. QM385 is also rapidly adsorbed into the plasma (T_{max} 1 h, $T_{1/2}$ 4 h) after oral administration (6). QM385 was therefore administered twice per day for 3 days and testing performed 1 h after the final dose. Sulfasalazine (10mg/mL; p.o.) for mice (Sigma, USA) prepared in 5%

dimethyl sulfoxide in 0.9% sterile saline. Sulfasalazine (Azulfine®) for human volunteers (Apsen Farmaceutica S/A, São Paulo, Brazil) was administered orally as a tablet. SPRI3, QM385 and SSZ are three structurally different SPR inhibitors (5,6).

Statistics

Mouse behavior sample sizes were determined *a priori* based on our experience with models of pain in mice (5) and biomarkers from the literature (11). Dose-dependent experiments were analyzed by One-way ANOVA with Dunnett post hoc and linear regression. Time courses analyzed by mixed ANOVA with post-hoc tukey tests for differences between groups. Paired and unpaired *t*-tests were used where only two, dependent or independent groups were tested. $P < 0.05$ as set as the level of significance.

Results

Injection of a cocktail of collagen antibodies (see Figure 1A) to mice induced, as reported before (7), a pain-related hypersensitivity syndrome consisting of two distinct phases; one with, and one without clinical signs of active joint inflammation.

Both controls and CAIA treated animals initially lost weight following injection of LPS, with CAIA mice losing significantly [Mixed ANOVA, Time; $F_{(7,224)} = 31.40$, $P < 0.01$] more weight between days 8 and 12; however, the rate of weight gain from day 12 was equivalent to the control animals (Figure 1B). The CAIA model resulted in marked disease activity [Mixed ANOVA, Time; $F_{(7, 224)} = 94.30$, $p < 0.01$; group; $F_{(2, 224)} = 416.5$, $p < 0.01$; interaction; $F_{(14, 224)} = 20.13$, $p < 0.01$] during the early phase (days 3–28; Figure 1C) as shown by swollen, red toe joints on both front and rear paws. The mean total score peaked 3 days after LPS injection (mean 13.1 ± 0.5), persisted until day 15 and then gradually declined to that of control mice by day 28. While cold allodynia followed the same time course as the arthritis score (early phase time course; Figure 1F), heat and mechanical hypersensitivity started 6 days after collagen antibody cocktail injection, and was maintained even when visible signs of inflammation had resolved (CAIA late phase; > 28 days; Figure 1D–F). Control mice did not show significant changes in arthritis scores, body weight or evoked hypersensitivity to heat, cold or mechanical stimuli compared with pre-treatment ($p > 0.05$ for each measure).

In experiment 1 with the SPR inhibitor, SPRI3, the treatment significantly reduced heat hyperalgesia in both the early [paired *t*-test, $t_{(19)} = 5.5$; $p < 0.01$] and late [paired *t*-test, $t_{(13)} = 6.0$; $P < 0.01$] phases of the CAIA model (Figures 2A and 2E), while mechanical hypersensitivity was only reduced [paired *t*-test, $t_{(15)} = 2.9$; $p < 0.05$] in the late phase (Figures 2B and 2F). Cold hypersensitivity analyzed in the early phase, when physical signs of joint inflammation were present, was not modified ($p > 0.05$) by SPRI3 treatment (Figure 2C). As a marker of inflammatory pain, the levels of BH4 were assessed in the urine 1 h after SPR inhibition on Day 7. SPRI3 significantly reduced [unpaired *t*-test, $t_{(14)} = 2.3$, $p < 0.05$] BH4 levels (Figure 2D), correlating with the analgesic activity of the compound

In experiment 2, we also studied the effect of SPR inhibition on the signs of inflammation evident during the early phase of the CAIA model, by using a recently developed inhibitor

of the BH4 synthesis, QM385, which was recently shown to have immune suppressive properties in experimental autoimmune models (6). Initially, the inhibitory effect on SPR activity was confirmed by measuring the levels of plasma sepiapterin in response to QM385 administration in naïve mice. As shown in Figure 3A the effect of QM385 was dose-dependent, with a minimum effective dose of 0.3mg/kg [$F_{(5, 30)} = 42.70$, $p < 0.01$] and a maximum one of 3 mg/kg (Figure 3A; $\beta = 0.71$; $P < 0.001$). We then tested QM385 on the pain hypersensitivity and signs of inflammation during the early phase of the CAIA model. QM385 (3mg/kg p.o) inhibited SPR, as shown by increased [one way ANOVA, $F_{(2, 17)} = 53.22$, $p < 0.01$] levels of sepiapterin (Figure 3B). Similarly to SPRi3, QM385 administration reduced heat [paired t-test $df=5$, $t_{(5)}=2.9$; $p < 0.05$] but not mechanical [$p > 0.05$] pain hypersensitivity or cold allodynia [$p > 0.05$] in the CAIA early phase (Figures 3C, 3D and 3E, respectively). The analgesic effect of QM385 was not related to anti-inflammatory properties of the compound, since QM385 had no effect on the clinical arthritis score (Figure 3F), the external appearance of the joints (redness, swelling; Figures 3G and 3H) or the extent of inflammation scored from histological sections (Figure 3I); ($p > 0.05$) in each case. Altogether, the results of experiment 2, confirmed that SPR inhibition induces a selective reduction in the pain hypersensitivity present at the time of heightened inflammation in this model of joint inflammation, without reducing the inflammation *per se*. The data acquired using two chemically unrelated SPR inhibitors extend the analgesic profile of SPR inhibition from the previously published work on neuropathic and soft tissue inflammation (5,6), to include joint inflammation. Combining the biomarker and effects on evoked pain we conclude that SPR inhibition is sufficient to produce analgesia in an RA-like model of joint disease, differentiating this mechanism of action from currently known DMARDs.

Sepiapterin levels have been previously shown to be increased in the CSF, blood and urine of patients affected by mutations in the SPR gene, leading to compromised SPR content or activity (11). The increased sepiapterin levels indicate that SPR function can be assessed non-invasively by measuring urinary sepiapterin levels. To test if urinary sepiapterin levels could be used to reliably monitor pharmacological inhibition of SPR in mouse and human urine, we measured the metabolite following administration of three structurally unrelated SPR inhibitors (SPRi3 and SSZ in rodents and SSZ in humans).

Sepiapterin levels (Figure 4A) were significantly increased in the urine of CAIA mice, 1 h after receiving SPRi3 (300mg/kg) on day 7 (Controls: 3.7 $\mu\text{mol}/\text{mmol}$ creatinine, 95% CI [2.7 – 4.8]; SPRi3 treated mice: 6.7 $\mu\text{mol}/\text{mmol}$ creatinine, 95% CI [4.8 – 8.6]; $P < 0.05$) and on day 45 (Controls: 3.9 $\mu\text{mol}/\text{mmol}$ creatinine, 95% CI [2.6 – 5.1]; SPRi3 treated mice: 8.9 $\mu\text{mol}/\text{mmol}$ creatinine 95% CI [4.6 – 13.1]; $P < 0.05$). Sepiapterin levels returned to baseline levels in the absence of SPRi3 (measured on days 14, 21, 28, 35) indicating the specificity of sepiapterin for SPR inhibition. Biomarker parameters were calculated (Table 2) using a cut-off value of 3.3 $\mu\text{mol}/\text{mmol}$ creatinine (equivalent to the mean + 1 $\times$ SD of the level of sepiapterin measured in urine of mice not treated with SPRi3; $n = 78$ samples). An AUC-ROC plot depicts the clear effectiveness of sepiapterin as a biomarker for SPRi3 inhibition (Figure 4B). When samples from day 7 and day 45 are combined, urine levels of sepiapterin were associated with an overall sensitivity of 80%, specificity of 86.7% and accuracy of 85.7% to predict SPRi3 treatment *i.e.* SPR inhibition and that these measures were similar for the day 7 or day 45 data examined separately (Table 2).

SSZ has been used for more than six decades as a first line treatment for RA because of its disease modifying properties (9) and more recently has been found to be an inhibitor of SPR (10,11). We have previously demonstrated that SSZ treatment elicited tissue and plasma accumulation of sepiapterin in mice (5). Here we administered SSZ to mice as well as to healthy human volunteers. The dose of SSZ used for the mouse biomarker measurements was selected based on our previous experiments showing analgesic effects in the intra-plantar CFA model of inflammation (5). Figure 4C shows that 1–3 h after SSZ (100 mg/kg p.o.) on each of two separate days, sepiapterin levels increased [unpaired t-tests, Day 1; $t_{(12)}=2.05$; $p=0.065$; Day 2; $t_{(16)}=3.0$; $p<0.05$] in urine relative to vehicle treated mice. Biomarker parameters (Figure 4D and Table 2) show that Specificity, False Negative Rates and False Positive Rates were in close agreement with the calculations from the SPRi3 experiment (see Table 2 for magnitude of effects); however, accuracy and sensitivity were lower, which may reflect the lower potency of SSZ relative to SPRi3. The standard initial dose of SSZ in the clinic is 2g/day (given as $4 \times 500\text{mg}$, every 6 h), which was the dose selected for the study reported here in healthy volunteers, takes several days to reach steady state levels. After administration of SSZ to healthy volunteers (2g/day; 3 consecutive days) sepiapterin levels increased in the urine each day, reaching significance [unpaired t-test, $t_{(18)}=1.8$; $p<0.05$] on Day 3 of sampling following administration of SSZ (Figure 3E). Sepiapterin levels immediately returned to pre-treatment levels on cessation of treatment with SSZ. The timing of the change in sepiapterin levels in the urine is in line with the clinical observation that SSZ is required to be administered for at least 3–5 days to achieve therapeutically relevant levels of SSZ in the patient. BH4 levels in the human urine samples were also measured and found not to change in response to SSZ treatment (data not shown) underlining the value of urinary sepiapterin specifically as a biomarker of SPR inhibition.

Discussion

SPR inhibition reduced heat and mechanical pain hypersensitivity resulting from systemic inflammation in a mouse model of RA-like joint inflammation, extending earlier work on the analgesic effects of SPR inhibition in granulomatous inflammation (5). SPR inhibitors also increased sepiapterin levels in the urine and plasma of mice and in the urine of healthy human volunteers, revealing for the first time that sepiapterin is a specific and sensitive pharmacodynamic biomarker of SPR inhibition in humans. The data also confirm that SSZ inhibits SPR activity at a dose used therapeutically in the clinic as a DMARD. The biomarker findings were translated from mouse to healthy humans implying the potential utility of this measure of target engagement for clinical monitoring and future studies of novel SPR inhibitors.

Our data are in agreement with previous findings from other groups showing that injection of a cocktail of collagen antibodies induces a pain syndrome consisting of two distinct phases. The acute phase shows marked inflammation and pain and the late phase shows no joint inflammation but pain is present (12–14), supporting the notion that active inflammation *per se* is not necessarily the direct cause for pain hypersensitivity in this model (15). Consequently, this may not be an accurate model of the pathophysiology of RA-like diseases. Furthermore, conventional and biological DMARDs, e.g. SSZ and anti-IL-1 β antibody, do not relieve pain unlike shown here by SPR inhibitors (16).

SPR inhibition, by administration of SPRI3, reduced heat hyperalgesia in both the early and late phases, had no effect on cold hypersensitivity and reduced mechanical hypersensitivity in the late phase of this model of arthritis. In a separate experiment, where we focused on the effect of SPR inhibition on pain and signs of inflammation in the early phase, we found that QM385, an SPR inhibitor chemically unrelated to SPRI3, also inhibited the heat hypersensitivity during the period of heightened inflammation. Interestingly, QM385 did not reduce the signs of inflammation (joint appearance, histological inflammation score and immune infiltrate) showing that SPR inhibition produces a selective effect on pain induced by inflammation. Since we have recently found that BH4 promotes T cell proliferation and that SPR inhibition decreases inflammation in mouse models of T cell transfer colitis, the experimental autoimmune encephalomyelitis model of multiple sclerosis and type 2 allergic inflammation in the lung and skin (6), we conclude that the CAIA model very likely does not include a T cell proliferative component and it is therefore unlikely to be a model of autoimmune, inflammatory polyarthritic disorders which are T cell dependent (17). Moreover, the direct administration of anti-collagen antibodies may only replicate the terminal steps of autoimmune joint inflammation. Nevertheless, the CAIA model generated pain, which was sensitive to SPR inhibition and this may reflect changes in BH4 activity in sensory neurons innervating inflamed joints.

A biomarker is a quantifiable trait of normal or pathogenic biological processes or of responses to an exposure or intervention, including therapeutic interventions. An ideal biomarker would be one that can be measured in a biological fluid that can be obtained from the patient non-invasively, *i.e.* urine, saliva, or skin wipe, and would be stable at room temperature (8). Our results indicate that measurement of sepiapterin in urine fits these criteria for a biomarker for monitoring target engagement (patient compliance) and/or safety if high exposure increases risk of on-target adverse effects.

Work by others has shown that DMARDs such as SSZ are able to bind to SPR and inhibit its activity *in vitro*, and this interaction was suggested to be responsible for some of the side-effects as a consequence of reduced BH4 levels in the central nervous system (19). Extrapolating from the mouse model results, our data suggest that SPR inhibition is also a likely mechanism for the analgesic action of SSZ. Further work in clinical populations evaluating the dose range of SSZ and other SPR inhibitors, and variance of therapeutic efficacy and biomarker response could establish the relationship between sepiapterin levels in urine and analgesic efficacy and side-effects. Clinical use of this biomarker could assist in precision medicine by detecting ranges associated with efficacy for RA, ulcerative colitis, inflammatory bowel disease and Crohn's disease, and perhaps also improve the management of the risk of adverse effects due to excessive inhibition of BH4. N-acetyltransferase 2 polymorphisms play a role in the differential response to SSZ (20), and a combination of biomarkers and genetics to maximize efficacy and reduce side effects is, therefore, now in reach.

In conclusion, we have shown that SPR inhibition reduces pain hypersensitivity in a mouse model of joint inflammation. Furthermore, we have demonstrated using three chemically distinct SPR inhibitors that urinary sepiapterin is a sensitive and specific biomarker of

pharmacological inhibition of SPR and could be used to improve the effectiveness of the use of SSZ in the clinic as a target engagement biomarker.

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Figure 1. Body weight, arthritis score and pain-related responses following collagen antibody cocktail administration

(A) Experimental timeline: (B) Time-course of body weight measurements (n=13), (C) arthritis clinical scores (n=13), (D) heat (controls, n=5; CAIA, n=19), (E) mechanical (n=11) and (F) acetone cooling thresholds (n=11). Measures were performed every 3 days, starting on Day 0 to Day 56. Control and CAIA indicate IgG-treated and antibody cocktail-treated mice, respectively. All graphs show mean $\pm$ SEM. Post-hoc Tukey * $P < 0.05$ vs. same control time point.

Figure 2. Effect of SPRI3 on pain-related responses and urinary BH4 levels in CAIA model of arthritis

Effects of SPRI3 on (A) heat (n=20), (B) mechanical (controls, n=24; CAIA vehicle; n=16; CAIA SPRI3; n=18), and (C) acetone cooling thresholds (controls, n=15; CAIA vehicle; n=7; CAIA SPRI3; n=8) in the inflammatory early phase of CAIA (controls, n=15; CAIA vehicle; n=7; CAIA SPRI3; n=8). (D) Urinary BH4 levels were measured on Day 7 (controls, n=6; CAIA vehicle; n=5; CAIA SPRI3; n=6–10). Most of these mice were also tested for (E) heat (n=14) and (F) mechanical thresholds (n=16) in the late non-inflammatory phase of CAIA. All graphs show mean $\pm$ SEM. Student's unpaired *t*-test, # *P* < 0.05, CAIA vehicle vs. CAIA SPRI3; Student's paired *t*-test 0 vs. 1h **P* < 0.05.

Figure 3. Effect of QM385 on pain-related responses on the acute inflammatory phase of the CAIA model of arthritis

Effects of SPR inhibition on (A) sepiapterin levels in the plasma of naïve mice treated with QM385 (crescent concentrations, p.o.; n=6), (B) sepiapterin levels in the QM385-treated mice in the early inflammatory phase of CAIA (controls, n=6; CAIA vehicle; n=6; CAIA QM385; n=8; performed on Day 13), (C) heat (controls, n=4; CAIA vehicle; n=6; CAIA QM385; n=6; performed on Day 10), (D) mechanical (controls, n=6; CAIA vehicle; n=8; CAIA QM385; n=6; performed on Day 9) and (E) acetone cooling thresholds (n=5; performed on Day 14), (F, G) paw edema (n=7; performed on Day 0 to Day 13), and (H) histopathological arthritis score (controls, n=7; CAIA vehicle; n=11; CAIA QM385; n=13; performed on Day 14). All graphs show mean $\pm$ SEM. A: One way ANOVA, post-hoc Dunnetts vs 0; C: Student's unpaired *t*-test, # *P* < 0.05, CAIA vehicle vs. CAIA SPRi3; Student's paired *t*-test 0 vs. 1h **P* < 0.05. B, D, and F: Mixed model repeated measures ANOVA with post hoc Dunnett's *P* < 0.05 vs control at same time point. G-I: One-way ANOVA followed by the post hoc Dunnett test; * *P* < 0.05.

Figure 4. Levels of urinary sepiapterin following treatment with SPR inhibitors

(A) Effect of SPRi3 administration (controls, n=4–7; CAIA vehicle, n=7–16; CAIA SPRi3; n=6–9; performed on Days 7, 14, 21, 28, 35, and 45), (C) of SSZ administered for 2 consecutive days in mice (Day 1, n=7; Day 2, n=9) and (E) of SSZ administration in humans during 3 days (n= 10, on Day “pre” to Day 7) on levels of urinary sepiapterin. A and C: Student’s unpaired *t*-test vs. control * $P < 0.05$. E: One-way ANOVA with Tukey post hoc test, * $P < 0.05$. B, D and F: AUC-ROC for sepiapterin as a biomarker of SPRi3 or SSZ. An ideal biomarker would have both sensitivity and specificity equal to 1 meaning all mice treated with SPRi3 showed sepiapterin levels in the urine higher than the cut-off value, and all samples taken from mice not treated with SPR inhibitor would have levels lower than the cut-off value. A biomarker with no predictive value (dotted line) would have equal sensitivity and false positive rate because a mouse with or without SPR inhibitor treatment would be equally likely to be test positive.

Table 1.

Parameters employed for the calculations of sepiapterin as a pharmacodynamic biomarker of SPR inhibition

True positive (TP)	Samples treated with SPRI3 or SSZ and sepiapterin levels above cut-off
False positive (FP)	Samples treated with vehicle and sepiapterin levels above cut-off
True negative (TN)	Samples treated with vehicle and sepiapterin levels below cut-off
False negative (FN)	Samples treated with SPRI3 or SSZ and sepiapterin levels below cut-off
Sensitivity TP/(TP+FN)	Probability of a positive test in animals dosed with SPR inhibitor
Specificity TN/(TN+FP)	Probability of a negative test in animals dosed with SPR inhibitor
False positive rate FP/(TN+FP)	Probability of a positive test among animals dosed with vehicle
False negative rate FN/(TP+FN)	Probability of a negative test among animals dosed with vehicle
Accuracy (TP+TN)/(TP+TN+FP+FN)	Probability of correct test results

Table 2.

Biomarker parameters for urinary sepiapterin as a biomarker of SPR inhibition

Experiment	Sensitivity	Specificity	False Negative Rate	False Positive Rate	Accuracy
SPRi3 in mice					
Day 7	77.7	86.7	13.3	22.2	85.8
Day 45	83.3	86.7	13.3	16.6	86.5
Overall	80	86.7	13.3	20	85.7
SSZ in mice					
Day 1	57.1	85.7	14.3	42.9	71.4
Day 2	85.7	88.8	11.1	33.3	77.7
Overall	62.5	87.5	16.7	12.5	75
SSZ in human volunteers					
Day 1	40	82.2	17.7	60	74.5
Day 2	30	82.2	17.7	70	72.7
Day 3	70	82.2	17.7	30	80
Overall	46.6	82.2	17.7	53.3	68