Biliverdin and bilirubin sulfonate inhibit monosodium urate induced sterile inflammation in the rat

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Abstract

Background: Biliverdin, a by-product of haem catabolism, possesses potent endogenous antioxidant and anti-inflammatory properties. Bilirubin-C10-sulfonate (BRS), an active metabolite formed after enteral administration of BV in the rat, also possess antioxidant properties. Therefore, we investigated the anti-inflammatory and antioxidant activity of BV and BRS in an *in vivo* model of monosodium urate induced sterile inflammation.

Methods: Subcutaneous air pouches were created on the dorsal flanks of Wistar rats (10-12 weeks of age). Prior to stimulation of the 6-day old pouch with monosodium urate (25 mg), groups were pre-treated with intraperitoneal BRS (27 mg/kg) and BV (27 mg/kg). Total and differential leukocyte counts were determined in pouch fluid aspirate at 1, 6, 12, 24 and 48 h after monosodium urate stimulation. Biliverdin (BV), BRS and unconjugated bilirubin (UCB) concentrations in the serum and pouch fluid were quantified using liquid chromatography-mass spectrometry. Pouch fluid cytokine concentrations (IL-1 β , IL-1 α , TNF- α , IL-17A, IL-12, GM-CSF, IL-33, IFN- γ , IL-18, IL-10, MCP-1, CXCL-1 and IL-6) were assessed after 6 h. In addition, 24 h protein carbonyl and chloramine concentrations were assessed in pouch fluid using ELISA and spectrophotometry, respectively.

Results: BRS and BV significantly ($p < 0.05$) inhibited leukocyte (total, neutrophil and macrophage) infiltration into the pouch fluid from 6 to 48 h. For example, after 6 h neutrophil counts decreased following BRS ($0.32 \pm 0.11 \times 10^6$ cells mL⁻¹) and BV ($0.17 \pm 0.03 \times 10^6$ cells mL⁻¹) compared to MSU only ($3.51 \pm 1.07 \times 10^6$ cells mL⁻¹). Both BV and BRS significantly ($p < 0.05$) reduced pouch GM-CSF (BV: 5.8 ± 1.2 pg mL⁻¹, BRS: 6.9 ± 1.5 pg mL⁻¹ vs MSU only: 13.0 ± 1.9 pg mL⁻¹) and MCP-1 concentrations at 6 h (BV: 1804 ± 269 pg mL⁻¹, BRS: 7927 ± 2668 pg mL⁻¹ vs MSU only: 17290 ± 4503 pg mL⁻¹), whilst BV additionally inhibited IL-6 (4354 ± 977 pg mL⁻¹ vs MSU only: 25070 ± 5178 pg mL⁻¹) and IL-18 (17.6 ± 2.0 pg mL⁻¹ vs MSU only: 81.5 ± 19.9 pg mL⁻¹) concentrations at 6 h ($p < 0.05$). Despite these differences, no change in pouch chloramine or protein carbonyl concentrations occurred at 24 h ($p > 0.05$). Serum BV concentrations rapidly diminished over 6 h, however, BRS was readily detected in the serum over 48 h, and in pouch fluid over 12 h.

Conclusions: This study is the first to elucidate anti-inflammatory activity of BRS and the efficacy of BV administration in a model of gouty inflammation. Reduced leukocyte infiltration and cytokine production in response to sterile inflammation further support the

importance of these molecules in physiology and their therapeutic potential in sterile inflammation.

Introduction

Tetrapyrroles are involved in cellular metabolic processes including respiration and photosynthesis, and are evolutionarily conserved across all kingdoms of life.⁽¹⁾ From a precursor for phycobilin synthesis in cyanobacteria and algae⁽²⁾, to a part of the haem catabolic pathway/anti-inflammatory mechanism in mammals^(3, 4), these tetrapyrroles appear to be vital for the survival of all organisms that synthesise them.

Tetrapyrrolic bile pigments such as biliverdin (BV) and unconjugated bilirubin (UCB) are formed during haem catabolism following red blood cell breakdown, an event associated with acute tissue injury.⁽⁵⁾ The innate inflammatory responses to injury occurs in the early phases of tissue injury, however tissue injury is accompanied by the production of BV and UCB, which attenuates the subsequent inflammatory response and improves wound healing.⁽⁶⁾ Additionally, Gilbert's Syndrome (GS) is a condition presenting with a mildly elevated circulating UCB concentration caused by a reduced uridine glucuronosyltransferase 1A1 (UGT1A1) deficiency due to a polymorphism (~%60 reduction in function), and is associated with protection against oxidative processes underpinned by a greater antioxidant capacity in plasma which contributes to the 10.1% lower incidence of cardiovascular disease within the GS population.⁽⁷⁻¹¹⁾

Administration of BV protects from inflammatory and oxidant-mediated damage *in vivo*.⁽¹²⁻¹⁵⁾ Specifically, BV administration protects via reducing the expression or release of inflammatory cytokines IL-6, MCP-1 and TNF- α in models of ischemia-reperfusion injury.^(13, 16, 17) Additionally, BV protects from Forssmann reagent induced anaphylaxis⁽¹⁸⁾, tissue transplantation⁽¹⁵⁾, and endotoxin-induced acute lung injury⁽¹⁴⁾ with a recent study demonstrating that BV and related bile pigments neutralise the harmful effects of superoxide.⁽¹⁹⁾ Interestingly, intraduodenal (i.d.) and intraperitoneal (i.p.) administration of BV in rodents also leads to the formation of bilirubin-10-sulfonate (BRS)^(20, 21), a novel antioxidant with the potential to confer protection against oxidative and, potentially, inflammatory processes.⁽²⁰⁾ Testing the anti-inflammatory therapeutic potential of these bile pigments clearly requires further *in vivo* investigation in models of sterile inflammation with clinical relevance to human inflammatory conditions in order to determine their potential physiological importance and their suitability as novel therapeutics. Gouty arthritis

is a common clinically relevant sterile inflammatory condition, with many patient's disease being refractory to treatment. In the United States alone, the financial burden of gouty arthritis in 2013 was ~\$1 billion, with acute gouty arthritis attacks accounting for 32% of this cost.⁽²²⁾ New treatments could potentially aid in the treatment of this condition, however, it remains unknown whether bile pigments can influence the course of this condition or its pathophysiology.

MSU crystals can activate the NLRP3 inflammasome via lysosomal leakage of cathepsins into the cytosol.⁽²³⁾ Furthermore, it is well documented that MSU crystals induce inflammatory cytokine and chemokine production^(24, 25) and initiate an acute inflammatory response mimicking the condition of gout in humans.^(25, 26) This response is likely induced by multiple mechanisms, including direct activation of leukocytes, membrane lysis and complement activation.⁽²⁷⁾ During the acute phase of the inflammatory response, inflammatory cytokines monocyte chemoattractant protein-1 (MCP-1), interferon-gamma (IFN- γ), interleukin-6 (IL-6), interleukin-1 β (IL-1 β), myeloperoxidase (MPO), macrophage inflammatory protein-1 α (MIP-1 α), tumour necrosis factor- α (TNF- α), chemokine ligand 1 and 2 (CXCL-1 and CCL-2 respectively), interleukin-8 (IL-8), and macrophage inflammatory protein-2 (MIP-2) have been reported to increase in MSU crystal air pouch inflammation models.⁽²⁴⁾ Bile pigments attenuate MCP-1, IL-6 and MPO responses, therefore, it was hypothesised that BRS and BV administration to the MSU subcutaneous air pouch model of inflammation would significantly reduce both leukocyte infiltration, inflammatory cytokines and biomarkers of oxidative stress/modifications within the inflammatory milieu of the pouch.

Materials and Methods

Materials

Biliverdin hydrochloride and bilirubin sulfonate were obtained from Frontier Scientific (Utah, USA). All other reagents were obtained from Sigma-Aldrich (Australia), unless otherwise stated.

Sodium biliverdinate synthesis

50.07 mg of NaOH (1.252 mmol) was added to 400 mL of ethanol and sonicated for 10 min at 32°C. This solution was then added to a flask containing 250 mg of BV-HCl (0.404 mmol). This solution was again sonicated for 10 min at 32°C, then 20 mL aliquots of the 0.625 mg mL⁻¹ BV solution were transferred to 50 mL Falcon tubes, with each containing 12.5 mg of BV. These solutions were then evaporated to dryness (Speedyvac centrifuge vacuum concentrator; LabGear, Australia) at 400 × g, 32°C for 12 h at a pressure of ~2 millibar. The dry aliquots were sealed, transferred to a -80°C freezer and reconstituted in PBS immediately prior to use. All solutions were protected from light during preparation using aluminium foil and a darkened laboratory.

Monosodium Urate Crystal synthesis

Monosodium urate crystals were synthesised using previously published methods.⁽²⁸⁾ Uric acid (1.680 g; Abcam, United Kingdom) was added to 400 mL of double distilled H₂O and stirred at 15 RPM for 5 s. Then, 2.25 mL of 5 M NaOH was added and the solution was stirred at 15 RPM for 5 s. The solution was then heated to 60°C, and then left at room temperature for 24 h. Following this, the solution was filtered under light vacuum (grade 5 Whatman paper, Thomas Scientific, USA) and the resulting crystalline solids were washed with 200 mL of 100% ethanol before being dried under vacuum. The crystals were observed via Scanning Electron Microscopy (SEM) at 15 kV under high vacuum with images taken at 10 random fields of view (15 kV × 1000). In each field, 10 crystals were counted, and their respective length and width quantified (Neoscope JCM-5000, Jeol, Japan) and were observed to have the characteristic needle-like appearance and dimensions observed in clinical gout (length: 11.4 ± 5.52 µm, n=100, width: 2.09 ± 0.73 µm, n=100)^(28, 29), (Figure 1). MSU crystals were gently pressed and distributed over electrical tape and loaded onto a

sample stub, which was then coated with gold for 2 min using a NeoCoater (MP-19020NCTR, Jeol, Japan). Administered MSU crystals were tested via limulus amebocyte lysate kit and were endotoxin free (<0.03 EU mg^{-1} ; Genscript, USA).

Figure 1: A representative scanning electron microscope image of monosodium urate crystals used to induce sterile inflammation within the air pouch model.

Sterile Inflammation Air Pouch Model

Adult male Wistar rats (10–12 weeks of age; 344–440 g) were housed at constant temperature, under a 12 h light-dark cycle and with free access to food and water. Prior to each procedure, animals were anaesthetised using 3% isoflurane (Pharmachem, Australia), at 1 L min^{-1} in 100% oxygen and maintained under anaesthesia via nose cone. Initially, the animals' dorsal flanks were shaved, and subcutaneous air pouches were formed immediately below the scapulae via subcutaneous delivery of 20 mL of sterile filtered air (0.22 μm syringe filter and a 27G needle). On the fourth day, a further 10 mL of filtered air was delivered to the pouch to maintain patency and assist the formation of a pseudosynovial joint capsule.⁽³⁰⁾

On the sixth day, under light anaesthesia, 5 mL of a 5 mg mL^{-1} sterile suspension of MSU crystals in 0.9% NaCl (Baxter, Australia) was injected into the air pouch of each rat via a 21G needle, except for the sham group ($n=3$), which received only 5 mL of saline. Thirty min prior to MSU delivery, rats received a 3.5 mL i.p. injection of BRS (27 mg kg^{-1} , $n=6$), BV (27 mg kg^{-1} , $n=5$, dissolved 1:1 in PBS:NaCl), ibuprofen (positive control; 8.1 mg kg^{-1} , $n=5$; Glentham Life Sciences, United Kingdom) or PBS (MSU only and sham groups) through a 5 mL syringe and

0.45 μ M syringe filter. The dose of BV and BRS were chosen following a recent pharmacokinetic study⁽²¹⁾ and the dose of ibuprofen was chosen as it represents molar equivalence with BV. All compounds were dissolved in PBS and injected with a 25G needle unless otherwise stated.

Samples of pouch exudate were taken under light anaesthesia periodically at 1, 6, 12, 24, and 48 h after MSU administration by injecting 5 mL of sterile saline into the pouch, gently massaging the pouch and withdrawing 4 mL of exudate which was collected into a 4 mL EDTA tube (5.3 mM EDTA; BD, USA) and stored on ice. Additionally, 200 μ L of blood was collected via a tail vein bleed at each timepoint. Leukocyte counts were estimated via hemocytometer, and the remaining fluid along with the blood sample were centrifuged for 8 min at 2000 $\times g$ at room temperature (RT). The supernatant and serum were aliquoted, placed on dry ice and stored at -80°C, and cell pellets were used for differential staining using a Rapid Diff stain (Australian Biostain, Australia) according to the manufacturer's instructions.

Inflammatory markers

Cytokines (IL-1 β , IL-1 α , TNF- α , IL-17A, IL-12, GM-CSF, IL-33, IFN- γ , IL-18, IL-10, MCP-1, CXCL-1 and IL-6) were quantified in the 6 h pouch exudate using a bead-based immunoassay sandwich ELISA method (LEGENDplex, Biolegend, USA). Samples were processed and quantification of soluble analytes performed utilising a BD SORP LSR II Fortessa flow cytometer, as per the manufacturer's instructions for use (IFU). A minimum of 500 events per analyte was acquired in the classification channel fluorophore APC (Ex 640nm Em: 670/30) with Median Fluorescence Intensity (MFI) of the reported fluorophore (PE; Ex 561 Em 588/12) interpolated against relevant standard curves using the proprietary Biolegend Software (LEGENDplex v8.0).

Biomarkers of oxidative stress/modification

Protein carbonyl concentration in pouch fluid was assessed at 24 h via ELISA (Biocell, New Zealand) and chloramine concentration was assessed using previously published methods.⁽³¹⁾ Protein concentration was assessed by Pierce BCA kit (Thermofisher, Australia). Myeloperoxidase (MPO) was assessed at 6, 12 and 24 h via ELISA (resolvingIMAGES,

Australia), however, the values were below the sensitivity of the assay and are not presented.

Bile pigment quantification

Serum samples were analysed for bile pigments via quantitative UHPLC-MS (LCMS-2020, Shimadzu, Japan). Separation of the targeted tetrapyrroles was achieved using a reverse phase C18 column (Vision HT C18HL, 2 mm × 100 mm, 1.5 µm, PhaseSep, Australia). The UHPLC column was preceded by a VisionHT C18HL guard column (2 mm × 5 mm, 1.5 µm, PhaseSep, Australia) and an UltraShield UHPLC precolumn filter (Restek, 0.2 µm, Shimadzu, Australia), respectively. The column oven and autosampler were set to 45°C and 4°C, respectively, and the flow rate was 0.35 mL min⁻¹.

Analysis of bile pigments in serum and pouch exudate

The initial mobile phase consisted of 34% mobile phase B (HPLC grade methanol, Scharlau, Spain) and 66% mobile phase A (10 mM ammonium acetate in 25% HPLC grade methanol and 75% Milli-Q H₂O), representing a 50.5% initial methanol concentration. The gradient program applied for the analytical portion of the analysis was as follows: 0.3 min - 50% B; 1.5 min - 74% B; 1.8 min - 92% B; 3.1 min - 92% B. The re-equilibration portion of the analysis ran from 3.1 min to 6.5 min at 34% B, with the flow rate increased to 0.42 mL min⁻¹ at 3.5 min, held until 4.0 min, reducing linearly to 0.35 mL min⁻¹ at 4.4 min and remaining so until 6.5 min. The total run time including re-equilibration was 6 min, with retention times of 2.00, 2.25 and 2.76 min for BRS, BV and UCB, respectively. A 2 µL injection volume was used for all analyses. ESI operating conditions were: desolvation line temperature 250°C, heat block temperature 200°C, nebulizing gas flow 1.5 L min⁻¹ (N₂), drying gas flow 15 L min⁻¹ (N₂) with all detection voltages set via autotune using the manufacturer's tuning solution (Shimadzu, Japan). Data were collected in negative ion mode via single ion monitoring (SIM) of the m/z corresponding with the deprotonated cation [M-H]⁻ of each compound.

External and Internal Quantification Standards

Stock solutions of BRS, BV and UCB were dissolved individually at 8 mM in DMSO, then combined with additional DMSO to form a mixed stock solution containing 2 mM of each

compound. This mixed stock solution was further diluted with DMSO in order to form 'working solutions' with concentrations from 390 nM to 200 μ M and were made fresh prior to each standard curve.

Internal standards (IS) included mesobilirubin-10-sulfonate, mesobiliverdin and mesobilirubin (mBRS, mBV and mUCB, respectively; Frontier Scientific, USA). A mixed IS solution was created by initially dissolving each IS at 8 mM in DMSO, which were then combined and further diluted with DMSO to form the final IS solution with mBRS/mBV/mUCB concentrations of 80/80/40 μ M, respectively. Aliquots of 500 μ L of the IS solutions were made, and frozen in liquid nitrogen prior to storage at -80°C. Prior to use, IS aliquots were defrosted in darkness at room temperature.

Calibration

Matrix matched calibration standards were prepared by adding 10 μ L of 'working solution' to 390 μ L of fresh rat serum to form spiked serum containing BRS/BV/UCB at concentrations from ~391 nM to 200 μ M plus one DMSO only blank. After 10 s of mixing via vortex and 10 min of incubation in the dark at room temperature, 40 μ L of spiked serum was added to 10 μ L of IS solution followed by mixing for 5 s via vortex. To this solution, 150 μ L of 1:4 DMSO:methanol was added and mixed via vortex again for 10 s before being centrifuged (21500 $\times g$; 10 min). Prior to injection, the supernatant was passed through a 0.22 μ m syringe filter (PhaseSep, Australia) and 2 μ L of the filtrate was injected for analysis.

Quality control

Quality control (QC) standards were prepared as per the calibration standards above at BRS/BV/UCB concentrations of 1.56/12.5/50 μ M representing a low/medium/high concentration, and 40 μ L of each were aliquoted into 1.5 mL microtubes. Prior to flash freezing in liquid N₂ and storage at -80°C, 10 μ L of IS was spiked into each aliquot of rat serum spiked with mixed standard and vortexed for 10 s. Prior to each batch of sample analysis, one aliquot of each concentration level was defrosted while shielded from light, prepared as above and injected for analysis to confirm accuracy within acceptance criteria range of 85 -115%. Following every 10th sample injection, one QC standard was reanalysed to ensure assay validity over time.

Sample analysis

Prior to serum analysis, 10 μL of IS solution was added to 40 μL of sample serum or pouch exudate, mixed via vortex for 10 s before adding 150 μL of 1:4 DMSO:methanol and mixing via vortex again for 10 s before being centrifuged ($21500 \times g$; 10 min). The supernatant was then passed through a 0.22 μm syringe filter (PhaseSep, Australia) and 2 μL of the resulting filtrate was injected for analysis.

Concentrations of tetrapyrroles in serum were calculated via the internal standard method using AUC integration of single ion monitoring (SIM) mass chromatograms at the relevant m/z to compare each analyte to its meso internal standard. Linear calibration curves ($r^2 > 0.9995$) were generated in Labsolutions software (version 5.87 sp1, Shimadzu, Japan) and the limit of quantification for bile pigments in serum and exudate approximated to be 250 nM for all analytes.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 8.1. Differences in leukocyte counts, cytokines and oxidative stress/damage markers were analysed via one-way ANOVA (Holm-Sidak *post hoc*) to test for differences between MSU only and treatment groups at each time point. Normality of data sets was assessed using the Shapiro-Wilk test. If data sets lacked normal distribution, equivalent non-parametric tests were conducted and outliers were removed using the ROUT test ($Q = 1\%$). All error bars are displayed as SEM throughout.

Results

An acute, sterile inflammatory response was observed in the air pouch of rats following the administration of MSU crystals, which was assessed by measuring leukocyte infiltration, cytokine concentrations and markers of oxidative stress. The population of the infiltrating leukocytes primarily consisted of neutrophils and monocytes, which peaked at 12 and 48 h respectively (Figures 2 and 3).

Leukocyte infiltration

Leukocyte infiltration was determined by measuring leukocyte counts in the pouch exudate at each timepoint via haemocytometer (Figures 2 and 3). A marked and significant ($p < 0.05$) increase in leukocyte infiltration was observed for the MSU only group when compared with the sham group from 6 to 48 h inclusive (Figure 2). All treatment groups (BRS, BV and ibuprofen) had significantly decreased total leukocyte and neutrophil counts compared with the MSU only group from 6 to 48 h inclusive (Figure 2).

Figure 2: Effects of 27 mg kg^{-1} i.p. pre-treatment of BRS (n=6, orange) and BV (n=5, blue) on leukocyte infiltration (A) and neutrophil infiltration (B) following MSU administration (25 mg in 5 mL sterile saline) to a 6 day old air pouch model of acute inflammation. Ibuprofen (8.11 mg kg^{-1} i.p. molar equivalent to BRS) was administered as a positive control (IBU n=5, grey), and PBS (3.5 mL, i.p.) was administered as a negative control (MSU only n=5, black). The sham group (SHAM, white) was pre-treated with 3.5 mL PBS i.p. and received 5 mL of saline into the pouch instead of MSU. * denotes significant difference ($p < 0.05$) compared to MSU only, † denotes significant difference ($p < 0.05$) compared to BRS at the same timepoint and ‡ denotes significant difference ($p < 0.05$) compared to BV for the same time point. From 6-48 h, the sham group was significantly lower than MSU only (not shown with symbol for neatness). Data are presented as mean $\pm$ SEM.

Figure 3: Effects of 27 mg kg⁻¹ i.p. pre-treatment of BRS (n=6, orange) and BV (n=5, blue) on monocyte infiltration (A) and lymphocyte infiltration (B) following MSU administration (25 mg in 5 mL sterile saline) to a 6 day old air pouch model of acute inflammation. Ibuprofen (8.11 mg kg⁻¹ i.p., molar equivalent to BRS) was administered as a positive control (IBU n=5, grey), and PBS (3.5 mL, i.p. n=3) was administered as a negative control (MSU only, n=5, black). * denotes significant difference ($p < 0.05$) compared with VEH, † denotes significant difference ($p < 0.05$) compared to BRS. From 12-48 h, the sham group was significantly lower than MSU only (not shown with symbol for neatness). Data are presented as mean $\pm$ SEM.

Inflammatory Cytokines

Concentrations of cytokines in pouch exudate at 6 h were determined using a multiplexed bead-based flow cytometric immunoassay. Only cytokines that were significantly ($p < 0.05$; unpaired t-test) elevated in MSU only groups versus sham and significantly decreased in treatment groups versus MSU only ($p < 0.05$; ANOVA) are reported here (Figure 4). All other cytokine responses can be found in Supplementary Figure 1.

Figure 4: Effects of 27 mg kg⁻¹ i.p. pre-treatment of BRS (n=6, orange) and BV (n=5, blue) on concentrations of IL-6 (A), MCP-1 (B), GM-CSF (C) and IL-18 (D) in pouch exudate following MSU administration (25 mg in 5 mL sterile saline) to a 6 day old air pouch model of acute inflammation at the 6 h time point. * denotes $p < 0.05$ (ANOVA) compared with MSU only (black). Data are presented as mean $\pm$ SEM.

Markers of Oxidative Stress

Chloramine and protein carbonyl concentrations in BRS and BV treated animals were not significantly different when compared to the MSU only (Figure 5 and 6).

Figure 5: Effects of 27 mg kg⁻¹ i.p. pre-treatment with MSU only (n=5, black), BRS (n=6, orange) and BV (n=5, blue) on concentrations of chloramine expressed in nmol chloramine per mg of protein in pouch exudate following MSU administration (25 mg in 5 mL sterile saline) into the 6 day old air pouch at the 24 h time point. No significant differences were detected between groups. Data are presented as mean $\pm$ SEM.

Figure 6: Effects of 27 mg kg⁻¹ i.p. pre-treatment with MSU only (n=5, black), BRS (n=6, orange) and BV (n=5, blue) on concentrations of protein carbonyl (nmol carbonyl per mg of protein) in pouch exudate following MSU administration (25 mg in 5 mL sterile saline) into the a 6 day old air pouch. Data are presented as mean $\pm$ SEM.

Bile Pigment Concentrations

Concentrations of BRS, BV and UCB were determined in venous blood over the course of 48 h and are shown in Figure 7.

Figure 5: Effects of 27 mg kg⁻¹ i.p. pre-treatment of BRS (n=6, A, left) and BV (n=5, B, right) on serum concentrations of BRS, BV and UCB. Data is omitted where compound concentrations were not detectable. Data are presented as mean ± SEM.

Samples of pouch exudate were also analysed for bile pigment concentrations. No bile pigments were detected in the pouch exudate of MSU only or BV treated animals, however BRS was detected in pouch exudate of BRS treated animals between 1 and 24 h of MSU administration (Figure 8).

Figure 6: Effects of 27mg kg⁻¹ i.p. BRS pretreatment of (n=6) on exudate BRS concentrations. BV and MSU only treatment had no quantifiable bile pigment concentrations.

Discussion

The role of inflammation in chronic disease is well established, and bile pigments continue to show therapeutic potential across a range of models of non-sterile, sterile inflammation and oxidative damage.^(4, 11, 20, 21, 32, 33) This manuscript is the first to explore the anti-inflammatory potential of bile pigment administration against gouty inflammation, and the first to describe the anti-inflammatory activity of BRS. BV and BRS pre-treatment decreased leukocyte infiltration (Figure 2A), neutrophil and monocyte ingress (Figure 2B and 3A) from 6-48 h and cytokine concentrations in pouch exudate (IL-6, MCP-1, GM-CSF and IL-18; Figure 4A-D) at 6 h following treatment compared with the MSU only group. A large, yet select, number of cytokines were measured in this study and will assist in clarifying relevant mechanisms of action of biliverdin and bilirubin in future studies. Additionally, these data show that BRS is retained in the circulation, peaking at approximately 120 μM and remaining above 5 μM after 48 h, demonstrating a favourable pharmacokinetic profile and potential to confer protection over a greater time period compared to BV.

Leukocyte Infiltration

In addition to the significant decrease in leukocyte and neutrophil infiltration observed in the BV and BRS treatment groups, monocyte and lymphocyte infiltration into the pouch from 12-48 h were also significantly decreased (Figure 3). These data suggest initial suppression of the inflammatory cascade during the early phase of the inflammatory response may have reduced the severity of subsequent leukocyte infiltration promoting prompt resolution. At 12 h, the neutrophil (Figure 2A) and monocyte (Figure 3B) counts were significantly reduced in the BV group compared to BRS, suggesting more potent inhibition of leukocyte infiltration by BV. Interestingly, at 48 h BV and BRS both significantly reduced leukocyte infiltration compared with MSU only (Figures 2 and 3) and were similar with the effect observed with ibuprofen treatment, suggesting the capacity to acutely resolve inflammation is shared by both compounds and has a similar effect to that of ibuprofen. Furthermore, BRS significantly reduced neutrophil infiltration compared with BV at 48 h (Figure 2B), indicating that BRS administration may have been more effective in resolving and preventing neutrophil infiltration, or promoted neutrophil apoptosis/phagocytosis after 48 h compared to BV. Neutrophil infiltration into tissues during an inflammatory response occurs following rolling, adhesion and transmigration of neutrophils on endothelial cells.⁽³⁴⁾ Significant down-regulation of these events occurs following BV administration in adult Balb/c mice (i.p. 9 mg kg⁻¹).⁽³⁴⁾ BV pre-treatment in swine (single bolus i.v. 50 µmol kg⁻¹) also significantly inhibits neutrophil infiltration following hepatic ischaemic reperfusion injury.⁽¹⁵⁾ Furthermore, *in vitro* studies conclude that binding of BV to biliverdin reductase-A (BVR-A) on the surface of neutrophils and macrophages interferes with TLR4, PI3 kinase and Akt signalling pathways, thus reducing leukocyte infiltration via inhibition of chemotaxis.⁽³⁵⁻³⁹⁾ Further studies show that BVR deletion enhances macrophage chemotaxis, demonstrating an inhibitory role for BVR-A.⁽³⁸⁾ While there are no previous data regarding BRS, UCB is reported to inhibit leukocyte extravasation with no changes observed in ICAM-1 or VCAM-1 expression.⁽⁴⁰⁾ These data imply that UCB may have interfered with adhesion molecule signalling and contributed to the improved resolution seen here after 48 h. These data further corroborate BV's inhibitory effects on chemotaxis and demonstrate, for the first time, that BRS also exhibits similar effects which in part, may occur following the spontaneous decomposition of BRS to BV.⁽²⁰⁾

Inflammatory Cytokines

These data show that BV treatment significantly reduces IL-6, MCP-1, GM-CSF and IL-18 pro-inflammatory cytokine concentrations in pouch exudate 6 h post treatment (Figure 3) and BRS administration reduced MCP-1 and GM-CSF concentrations ($p < 0.05$; Figure 3).

Inflammatory cytokines that are clinically associated with gout-induced inflammation⁽⁴¹⁾ were decreased following BV treatment. These data supported the hypothesis and agree with published literature reporting administration of BV generally reduces pro-inflammatory cytokine expression. For example, 50 mg kg⁻¹ i.p. BV treatment prior to and immediately after small bowel transplant in Lewis rats significantly decreases serum IL-6.⁽⁴²⁾ Another study reports significant decreases in IL-6 and MCP-1 for i.p. BV (5 mg kg⁻¹) treatment in a caecal ligation model⁽⁴³⁾, with BV treatment delivered 8, 6, and 3 h before laparotomy, once immediately prior to laparotomy closure and 15 h postoperatively. This study repeatedly dosed BV, to ensure bioavailability of BV over a longer period, which was achieved in our study following single BRS treatment (Figure 7A). The decrease in MCP-1, a mediator of monocyte and lymphocyte recruitment associated with inflammation/insulin resistance⁽⁴⁴⁾, observed in both BV and BRS administered conditions here and in the caecal ligation study⁽⁴³⁾, support the decrease in monocyte and lymphocyte recruitment (Figure 3).

The significant decrease in IL-18 following BV treatment is a novel finding of this study. Canonically, IL-18 is an inducing factor for interferon gamma (IFN- γ)⁽⁴⁵⁾, suggesting that BV might have inhibit macrophage M1 polarisation as recently shown in BVR knockout macrophages.⁽³⁹⁾ The IL-18 receptor exerts similar effects to the IL-1 receptor and plays an important role in regulating the differentiation of cell types of the adaptive immune system⁽⁴⁶⁾ Interestingly, serum IL-18 concentrations are significantly reduced in aged UGT1A1-deficient Gunn rats (i.e. rats with naturally elevated serum UCB) compared to the wild type.⁽⁴⁷⁾ Biliverdin is rapidly reduced to UCB *in vivo* (Figure 7B), therefore, it is possible that physiological reduction of BV mediated the IL-18 response in this study.

Finally, GM-CSF, a mediator of neutrophil recruitment⁽⁴⁸⁾, was significantly reduced in pouch exudate at 6 h (Figure 4C) for BV and BRS, which supports the decreased leukocyte infiltration findings here. Additionally, a recent *in vitro* study reports that proinflammatory cytokine production, including GM-CSF, is downregulated by BV and phycocyanobilin (PCB;

an algal linear tetrapyrrole structurally similar to BV) pre-treatment of human peripheral blood mononuclear cells (PBMC) stimulated by a mixed lymphocyte reaction.⁽⁴⁹⁾

These data agree with recent reports that BV and enzymes involved in bile pigment metabolism (BVR/Haem Oxygenase-1) inhibit sterile inflammatory responses. For example, BV treatment protects rats from acetaminophen-induced toxicity⁽⁵⁰⁾, ameliorates cerebral ischemia reperfusion injury in rats⁽⁵¹⁾ and suppresses inflammatory cytokine release in ischemia reperfusion injury and insulin resistance models.⁽⁵²⁾ These studies, while they measure different outcomes, add to a growing body of evidence demonstrating that these bile pigments play a significant role in attenuating inflammation. Improved understanding of these compounds' roles within mammalian physiology could lead to their future development as safe and effective anti-inflammatory therapeutics.

Bile Pigments in Serum

Biliverdin administration induces potent anti-inflammatory effects^(12-14, 17); however, we recently demonstrated that i.p. and i.d. biliverdin administration results in appreciable bilirubin-10-sulfonate (~22%) conversion *in vitro* and *ex vivo*.⁽²⁰⁾ Following i.p. BV and BRS administration, bile pigments were systemically and readily available during the acute phase of inflammation. In addition, this manuscript brings very important information regarding longer term systemic concentrations of BRS retained in the circulation. In conscious animals, circulating concentrations ranged between 120 and 5 μ M over 48 h. While BV rapidly decreased over time (i.e., 24 h) with consequent formation of unconjugated bilirubin (presumably due to metabolism via BVR⁽²¹⁾) and a small amount of BRS (Figure 7B). After BRS administration however, serum BRS concentrations were elevated for 48 h, with only small amounts of BV present over 24 h (Figure 7A). These data demonstrate that BRS slowly decomposes, releasing BV, which could mediate longer term anti-inflammatory effects via BVR signalling.

In our previous study, we tested the pharmacokinetics of the investigated compounds over 3 h only in Wistar rats where the animal was fasted, under a surgical plane of anaesthesia and had the bile duct cannulated which effectively interrupted entero-hepatic circulation. Both studies dosed Wistar rats of a similar weight and age with the same amounts of both

compounds via the intraperitoneal route and the 1 h timepoints in both studies were comparable, with the peak serum concentration in both studies reaching in excess of 100 μM for BRS and $\sim 17 \mu\text{M}$ for BV. While both experiments demonstrated a rapid decline in circulating BV after administration, BRS maintained a serum concentration of $\sim 100 \mu\text{M}$ after 3 h (in anaesthetised rats), whereas in the present study concentrations fell to 50 μM after 6 h representing the gradual elimination of the compound from blood. As this present study administered BRS and BV to free-living and conscious animals, the pk data presented here are not influenced by the effects of anaesthetics, or interruption of the enterohepatic circulation, providing clearer data of each compound's longer-term bioavailability.

We also detected BRS in pouch exudate (Figure 8) over 24 h, indicating that it either leaked into the pouch with exudate formation, or diffused through tissues into this compartment. Surprisingly, markers of oxidative stress were not significantly impacted by BV or BRS (despite consistent reductions in protein carbonyl for BRS and BV) (Figures 5 and 6). These data are, however, most likely related to the relatively low local concentrations of bile pigments in the pouch environment. Future studies could investigate the effect of injecting the compounds directly into the pouch (akin to the joint) to determine whether local concentrations can similarly inhibit inflammatory and oxidative processes. Additionally, MPO concentrations in pouch exudate were below the limit of detection for our assay; however, it is possible that BRS, which possesses a reduced C10 bridge, behaves similar to UCB, which inhibits neutrophil burst and neutralises superoxide radicals.⁽¹⁹⁾ Furthermore, high urate concentrations in the pouch may also have competed for radical formation over the relatively low concentrations of BRS, impacting protection from pouch fluid oxidation. Therefore perhaps increased bile pigment concentrations in pouch fluid could inhibit MPO mediated chloramine and protein carbonyl formation⁽⁵³⁾, supporting the need for further studies to address this question.

Conclusion

Current human and clinical data indicates consistent benefits associated with mild elevation of UCB (as observed in Gilbert's Syndrome) as well as the potential of tetrapyrrole administration⁽³²⁾ to treat a myriad of inflammatory diseases including cardiovascular disease and type 2 diabetes mellitus.

This study describes the *in vivo* anti-inflammatory efficacy of BRS and BV in an MSU crystal induced model of sterile inflammation in the rat, as well as longer term serum pharmacokinetics in conscious animals for both compounds. The data presented demonstrates the importance of bile pigments as endogenous anti-inflammatory compounds that are actively produced during sterile inflammation and indicates that BV/BRS administration are potential therapeutic avenues for the treatment of sterile inflammation/gout. BRS administration confers similar benefits to BV administration while remaining in systemic circulation for up to 48 h following treatment. We propose that the administration of BRS, a hydrophilic compound, is a simple method to increase BV and UCB *in vivo*, which could help in realising the anti-inflammatory therapeutic potential of bile pigments.

Credit Statement

Ryan G. Shiels: Conceptualization, Methodology, Validation, Formal analysis, Writing - Original Draft, Writing - Review & Editing, Visualization, Project administration. **Wenu Hewage:** Investigation, Writing - Original Draft, Writing - Review & Editing. **Evan Pennell:** Investigation, Writing - Review & Editing. **Josif Vidimce:** Investigation, Writing - Review & Editing. **Andrew G. Pearson:** Writing - Review & Editing, Supervision. **Karl-Heinz Wagner:** Writing - Review & Editing, Supervision, Funding acquisition. **Michael Morgan:** Methodology, Writing - Review & Editing, Supervision. **Andrew C. Bulmer:** Conceptualization, Methodology, Investigation, Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

Author contribution statement

A.C.B., M.M. and R.G.S. conceived the design of this study. R.G.S. and W.H. performed animal experimentation and interpretation of data. R.G.S. and W.H. performed HPLC and LCMS analysis, pouch exudate analysis and statistical analysis of data. J.V. assisted with statistical analysis and performed quantification of protein carbonyl. E.N.P. assisted with the quantification of cytokines via flow cytometry. A.G.P., G.G. and K.H.W. assisted with concept, technical advice and input. A.C.B. and K.H.W. secured funding for the project. All authors contributed to revision of the MS and provided their final approval for manuscript submission.

Competing interests

The author(s) declare no competing interests.

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Animal Use

All animal care and experimental procedures complied with the Guidelines of the Australian National Health and Medical Research Council and were approved by the Griffith University Animal Ethics Committee (MSC/03/18/AEC).

Supplementary information

Supplementary Figure 7: Concentrations of cytokines in pouch exudate at 6 h were determined using a multiplexed bead-based flow cytometric immunoassay.

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