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Vitex agnus-castus dry extract BNO 1095 (Agnucaston®) inhibits uterine hyper-contractions and inflammation in experimental models for primary dysmenorrhea

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Abstract

Background: For many women, the monthly suffering induced by menstrual "cramps" is severe enough to profoundly disrupt their quality of life. In the case of primary dysmenorrhea, a condition related to premenstrual syndrome (PMS), intense uterine contractions are thought to trigger moderate to intense pain despite the absence of an underlying infection or other medically-identifiable disease states. The associated uterine hyper-contractility is reminiscent of labor, and associated pain is likely to be mediated by the release of prostaglandins, leukotrienes and the infiltration of leukocytes that normally accompany the breakdown of the endometrial lining. Standardized extracts of *Vitex agnus-castus* berries (VAC extracts of chaste tree, or chaste berries) are clinically effective in treating the symptoms of PMS, yet the mechanisms of how the chemically complex mixture acts are largely unknown.

Methods: Using an in vivo dysmenorrhea model rats were treated with 10 mg/kg estradiol-benzoate i.p. once daily for 12 days and with 2.1, 10.3 or 20.7 mg/kg VAC dry extract p.o. once daily for 7 days prior to induction of convulsions. Uterine contractions where induced with 2 IU/kg oxytocin i.p., followed by monitoring of abdominal convulsions and signs of pain on the last day of the experiment. Moreover, in vitro methods were applied that are described in the methods section.

Results: Here, we show that the VAC herbal dry extract BNO 1095 (commercially available as Agnucaston®) targets the uterine myometrial tissue and inflammatory signaling molecules of associated migratory/inflammatory cells. Specifically, BNO 1095 dose-dependently inhibited oxytocin-induced uterine contractions in a rat dysmenorrhea model in vivo and drug-induced contractions in isolated human and rat uterine tissue in vitro. Furthermore, BNO 1095 showed a promising anti-inflammatory capacity by potently inhibiting 5-lipoxygenase activity and leukotriene production and by reducing the production of reactive oxygen species and inflammatory cytokines in vitro.

Conclusion: These results provide evidence that BNO 1095 effectively treats menstruation-related complaints including primary dysmenorrhea.

Keywords: Agnucaston, Cytokines, Dysmenorrhea, Leukotriene, Lipoxygenase, Menstrual complaints, Premenstrual syndrome, Spasmolytic, Vitex agnus-castus

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Background

Primary dysmenorrhea refers to menstrual cramping in the absence of pelvic pathology [1] and is closely linked to a large group of related menstrual conditions that are collectively termed premenstrual syndrome (PMS) [2]. Primary dysmenorrhea represents a common gynecological complaint especially in young women, with a prevalence estimated to be as high as 40–50 % in women from this age group [1]. The intensity of pain associated with uterine cramps in affected women may range from moderate to severe, and cramps are frequently accompanied by back pain, nausea, vomiting and diarrhea. In some cases, the suffering in affected women prevents them from working, going to school, or normal daily activities.

Physiologically, the pain is most intense in the days just prior to menstrual flow, and has been proposed to arise from at least three temporally-related biological processes. First, the spasmodic uterine contractions in affected women are often more intense, more frequent, and of longer duration compared to non-dysmenorrheic women, and in some cases are described as "labor-like" [1]. Second, these uterine contractions may constrict the oxygenated blood supply by vasoconstriction within the endometrium, leading to enhanced cell death, ischemia [3] and the production of reactive oxygen species (ROS). Finally, even in the absence of an infection, there is a concomitant "immune-like" influx of inflammatory/migratory cells including mast cells, eosinophils, neutrophils, and macrophages immediately prior to menses that likely assists in the breakdown of endometrial tissue through the release of proteases and pro-inflammatory prostaglandins (PGs), leukotrienes, cytokines, and chemokines [3–6]. While these three processes plausibly act in concert to intensify the pain experienced during menstrual cramping, molecular details of how this process occurs are complex and poorly understood [1].

Amongst various pro-inflammatory mediators, $PGF_{2\alpha}$ and PGE_2 are known to play a central role in modulating uterine contraction and vasoconstriction in non-pregnant and pregnant females, respectively. Intrauterine administration of $PGF_{2\alpha}$ (but not PGE_2) during the secretory phase of the menstrual cycle has been shown to increase uterine contractility, and implicates this PG as a key player in primary dysmenorrhea and pain [3, 7]. There are also highly complex interactions between hormones and mediators, basal body temperature, sleep patterns, and the central nervous system which are not completely understood [8, 9].

In one possible molecular scheme for primary dysmenorrhea, the withdrawal of estrogen and progesterone leads to pro-inflammatory and pro-contractile cascades, as well as a reduction in superoxide dismutase (SOD) enzyme levels [10] that normally keep ROS levels low and protect cells against ROS-induced damage [4]. The menstrual cycle-dependent increase in ROS production activates transcription factor NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells), resulting in increased prostaglandin (especially $PGF_{2\alpha}$), chemokine and pro-inflammatory cytokine production [4, 11]. Finally, $PGF_{2\alpha}$-induced uterine contraction and vasoconstriction leads to hypoxia and pain in the respective myometrial tissue. This, together with the expression of pro-inflammatory mediators, used for (and by) the recruitment and activation of leukocytes results in inflammation, tissue breakdown and ultimately a characteristic menstrual bleeding [4, 6, 12], after which the pain tends to subside.

Nonsteroidal anti-inflammatory drugs (NSAIDs, such as naproxen sodium or ibuprofen) are widely used to treat PMS and primary dysmenorrhea by reducing PG production via the inhibition of cyclooxygenases (COX-1 and COX-2). However, most prescription-free NSAIDs can be associated with gastrointestinal side effects such as nausea and gastric ulceration as well as renal dysfunction [2]. Also 10–30 % of dysmenorrheic women are not responsive to COX inhibitors [13], suggesting the role of other mediators in this condition. For example, an elevation of leukotrienes observed in women with primary dysmenorrhea has been suggested to contribute to uterine hypercontraction in humans [14] and as shown in a guinea pig experimental model [15]. However, no medicinal products acting on the 5-lipoxygenase pathway have been approved for treatment of PMS so far.

From the women's perspective, effective alternatives, particularly herbal medicines with fewer side effects are preferred [16] to treat PMS-related symptoms. Fruits of *Vitex agnus-castus* tree, also known as chasteberry, have traditionally been used [17] to treat minor symptoms related to the monthly pain, swelling, and mood disorders that are collectively termed PMS [2]. Standardized herbal extracts from *Vitex agnus-castus* fruits (VAC dry extracts) are recognized and sold in Europe as "well-established use" herbal medicinal products that are effective for relief of PMS with fewer side effects than NSAIDs. The efficacy and safety of VAC dry extracts in treating PMS symptoms such as irregular menstruation and breast pain (mastodynia) have been confirmed by several controlled clinical studies [18–21].

The mechanism of action of VAC herbal extracts in treating menstrual symptoms has not been completely elucidated, perhaps due to the large number of potentially active extract constituents, including iridoids, flavonoids, and diterpenes [22–26]. For example, a large body of evidence suggests that VAC extracts acts in the brain at dopamine D_2 receptors of the hypothalamus/pituitary gland axis to inhibit the systemic release of prolactin from the pituitary gland resulting in the relief of PMS symptoms [27–31]. However, the potential for localized anti-contractive and anti-inflammatory actions

of VAC extracts in primary dysmenorrhea has not been investigated up to now.

Therefore, in the present studies, we investigated the ability of the standardized VAC extract BNO 1095 (commercially-available as Agnucaston®) to modulate a number of potential mechanisms responsible for dysmenorrhea using in vivo, in vitro, and enzymatic assays, with a special emphasis on uterine contraction, pain, and the production and release of the pro-inflammatory molecules PGs, leukotrienes, cytokines and ROS.

Methods

Materials and reagents

The VAC dry extract BNO 1095 (DER 7–11:1) is commercially available as Agnucaston® and was provided by Bionorica SE, Neumarkt, Germany. For in vitro studies, solubilization of the test item was obtained by resuspension in 50 % ethanol (v/v) at a concentration of 40 mg/ml and homogenized by 5 min of vortexing, followed by a 30 min incubation at room temperature (RT) in an ultrasonic bath. The suspension was centrifuged at 3000 g for 10 min at RT and the supernatant was used immediately for the experiments.

Unless otherwise specified, all reagents were obtained from Sigma-Aldrich (Taufkirchen, Germany).

In vivo measurements of uterine contraction, pain, and locomotor sedation in a rat dysmenorrhea model

Nulliparous, non-pregnant female Sprague Dawley rats were purchased at the age of 12 to 14 weeks from Charles River (Sulzfeld, Germany) and housed under specific pathogen-free conditions, with sterilized water and food given ad libitum. 12 days prior to the start of the experiments, the animals were randomly allocated to approximately-equal control (saline), treatment (BNO 1095), or comparator (carprofen) groups ($n = 12–14$ per group) and acclimatized in groups of 4 animals to their new cages. On Day 1 to Day 12, all animals were i.p. injected once-daily with 10 mg/kg body weight estradiol-benzoate. On Day 6 to Day 12, animals assigned to the treatment groups were dosed once-daily with an oral suspension (in sterile water) of BNO 1095 via oral gavage at one of three doses: 2.1 mg/kg body weight (comparable to five times the recommended human equivalent dose, 5× HED), 10.3 mg/kg body weight (25× HED) or 20.7 mg/kg body weight (50× HED) in a volume of 10 ml/kg, while animals of the control group received an equal amount of water. On Day 12, animals from the control, treatment and comparator groups received the following (all volumes equal between groups, respectively): water p.o. and a subcutaneous (s.c) injection of saline; BNO 1095 extract p.o. 3 hours after the last application of estradiol-benzoate and an s.c. injection of saline, or p.o. water and a s.c. injection of 5 mg/kg body weight of the NSAID carprofen (Pfizer, Berlin, Germany).

On Day 12, 1 hour after the vehicle or carprofen s.c. injection, oxytocin (10 I.U./ml, Hexal AG, Holzkirchen, Germany) was applied i.p. at a dose of 2,000 mI.U./kg (3.33 μg/kg) body weight to the animals of all groups to induce uterine contractions. Latency time and number of abdominal convulsions, indicated by writhings, after administration of oxytocin were monitored to estimate the degree of myometrial stimulation. In addition, the pain reaction was quantified according to the grimace scale as described by Sotocinal et al. [32], a method chosen due to its ease of automation and its ability to differentiate between spontaneous and evoked (e.g., in response to abdominal prodding) pain. Briefly, changes in orbital tightening, nose/cheek flattening, ear change and whisker change were quantified in a scale of 0 (normal), 1 (moderate) or 2 (obvious). In addition to measurements of uterine contraction and pain, a beam walking test was performed, as described by Goldstein et al. [33] with modifications according to Flierl et al., [34] to analyze gait and latency while crossing a wooden bar to measure any potential influence of BNO 1095 or carprofen administration on motor coordination (a subjective scale from 0 to 16, where a score of 7 = normal and 3 = motor disorder) or sedation (measured by the amount of time between placing the animal on the beam and moving forward to cross the bar).

Rat experiments were conducted in accordance with the regulations for the care and use of laboratory animals and approved by the institutional animal ethics committee: a) EU: Directive 2010/63/EU of the European Parliament and of the Council of September 22, 2010 on the Protection of Animals Used for Scientific Purposes; b) Directive CETS No. 123 (ETS 123): European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes Council of Europe (1986), Appendix A (2006).

In vitro organ bath contraction experiments using excised rat uterine tissue

For the analysis of anti-convulsive activity using isolated rat uterine strips, female nulliparous Sprague–Dawley rats with body weights in the range of 250–275 g at delivery were obtained from Charles River Laboratories (Saint Germain sur l'Arbresle, France). Rats were treated with diethylstilbestrol (0.25 mg/kg, i.p.) 18 h prior to experiment (to synchronize hormonal conditions). Diethylstilbestrol was dissolved at the concentration of 0.5 mg/ml in corn oil (batch MKBH4894V, Sigma-Aldrich) and a volume of 0.5 ml/kg was injected. On the day of the experiment, rats were sacrificed by CO_2 asphyxiation. The uterus was excised, cleaned from connecting tissues and longitudinal strips of uterus were rapidly dissected

from each horn (2 strips per horn) and placed in 5 ml organ baths containing oxygenated Krebs solution of the following composition (in mM): NaCl 114, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11.7 (pH 7.4, aerated with 95 % O_2 and 5 % CO_2 at 37 °C). Uterine tissues were allowed to equilibrate at 1.0 g resting tension for at least 60 min, during which time the Krebs solution was replaced every 15 min and the tension readjusted if necessary. Following this equilibration period, myometrial contractions were induced by adding 0.5 nM (0.5 ng/ml) oxytocin, 1 μM (354.5 ng/ml) $PGF_{2\alpha}$ or 50 nM (54.2 ng/ml) vasopressin to the baths. After contractions stabilized in amplitude and frequency, cumulative concentration-response curves to 10–1000 μg/ml BNO 1095 (dry extract solubilized as described above), positive control ritodrine (Sigma-Aldrich, Saint-Quentin Fallavier, France) and the vehicle control (aqueous ethanol solution) were recorded. Ethanol concentration in organ baths were at most 0.5 % for the maximal drug (or extract) concentrations tested. Data are presented as percent of control (relaxation before addition of test substance or vehicle).

All experiments using rat uterine tissue were performed in accordance with French legislation concerning the protection of laboratory animals and in accordance with a currently valid license for experiments on vertebrate animals, issued by the French Ministry for Agriculture and Fisheries.

In vitro organ bath contraction experiments using excised human uterine tissue

Samples of human uterus, obtained with informed consent, were taken from non-pregnant pre-menopausal donors. All uterine strips (approximately 15 mm length), were suspended in 25 ml organ baths containing physiological salt solution (PSS, 119.0 mM NaCl, 4.7 mM KCl, 1.2 mM $MgS0_4$, 24.9 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$ and 11.1 mM glucose), aerated with 95 % O_2 and 5 % CO_2 and maintained at approximately 37 °C. One side of the tissue strip was attached to an isometric transducer that records changes in tension. The uterine strips were allowed to equilibrate for at least 30 min. Uterine strips were then normalized to a standard passive tension (20 mN, approximately 2 g) to reduce signal variability prior to pharmacological intervention. The strips were then allowed to equilibrate for at least 1 hour, with washes with PSS every 15 min, to allow the development of spontaneous contractions. Any uterine strips failing to produce spontaneous contractions after tension was applied in the organ bath were rejected.

0.2 μM (0.2 μg/ml) oxytocin was added to increase the size and frequency of the spontaneous contractions. After contractions stabilized in amplitude and frequency, cumulative concentration-response curves to 10–400 μg/ml BNO 1095, the dihydropyridine calcium-channel blocker isradipine (positive control) and the vehicle control (aqueous ethanol solution) were recorded. The concentration of ethanol in organ baths was within the range of 0.013 to 0.5 % for the maximal concentration tested. Data are presented as percent of control (relaxation before addition of test substance or vehicle).

Inhibition of purified 5-lipoxygenase (5-LO) and cellular leukotriene biosynthesis in isolated human monocytes

In the cell-free (purified enzyme) assay, human recombinant 5-lipoxygenase (5-LO) was expressed in *E. coli* Bl21 (DE3) cells that were transformed with pT3–5LO, and purified by ATP-agarose column [35]. For determination of the enzymatic activity, the purified enzyme was added to 1 ml of a 5-LO assay mix (PBS, pH 7.4, 1 mM EDTA, 1 mM ATP). After incubation for 10 min at 4 °C with vehicle (0.5 % ethanol) or BNO 1095, samples were pre-warmed for 30 s at 37 °C in the presence of 2 mM $CaCl_2$ followed by addition of 20 μM (6.1 μg/ml) arachidonic acid. The reaction was stopped after 10 min at 37 °C by addition of 1 ml ice-cold methanol, and 200 ng PGB_1 were added. Formed metabolites were extracted and analyzed by HPLC as previously described [36]. 5-LO products include LTB_4 all-trans isomers and 5-H(p)ETE. Results are reported as percentage of vehicle control.

Analysis of 5-LO inhibition in a cell-based assay was performed using human monocytes isolated from freshly withdrawn peripheral blood samples. Blood samples were obtained from consenting healthy donors (Institute of Transfusion Medicine, University Hospital Jena, Germany) who had declared that they had not taken any anti-inflammatory drugs within 10 days of providing the sample. The venous blood was centrifuged at 4,000 g for 20 min at 20 °C, PBMC were isolated by dextran sedimentation and centrifugation on Nycoprep cushions, and monocytes were collected by adherence as described above. Monocytes were finally resuspended in PGC buffer at the cell density of 2×10^6 cells/ml and incubated for 15 min at 37 °C with BNO 1095, vehicle or zileuton (3 μM or 0.71 μg/ml). Then, cells were stimulated at 37 °C with the Ca^{2+}-ionophore A23187 (5 μM or 2.6 μg/ml). After 10 min, the reaction was stopped on ice and samples were centrifuged at 500 g for 10 min at 4 °C. Supernatants were collected and formed LTC_4 was analyzed by ELISA (Enzo Life Sciences GmbH, Lörrach, Germany) according to manufacturer's instructions. For analysis of LTB_4, all trans LTB_4 and 5-H(p)ETE using HPLC, 750 μL supernatant were mixed with 750 μL methanol and 22.5 μL of 1 N HCl, 150 ng PGB_1, and 375 μL of PBS were added. Formed 5-LO products were extracted, analyzed by HPLC as described elsewhere [36] and presented as sum of all 5-LO products (i.e., LTB_4, its all-trans isomers, and 5-H(p)ETE). Results are reported as normalized percentages

vs. vehicle control (=100 %). Data represent three independent experiments, each collecting single data points.

Analysis of cytokine release from cultured human PBMCs

Cryopreserved PBMCs (Eurofins Panlabs Inc., Bothwell, WA, USA) were thawed and seeded into 96 well plates in culture media (RPMI 1640, 10 % FBS, 1 % penicillin/streptomycin, 2 mM L-alanyl-L-glutamine) at a cell density of 5×10^4 cells/well. After incubation for 1 h at 37 °C, 10–300 μg/ml BNO 1095, vehicle control (0.3 % ethanol final concentration) or the positive control 100 nM (39.25 ng/ml) dexamethasone was added and incubated again for 1 h at 37 °C. After addition of 50 ng/ml LPS, PBMCs were incubated for 24 h at 37 °C. Collected supernatants were analyzed for cytokines using ProcartaPlex multiplex bead arrays for human IL-1β, IL-6, IL-8, MIP-1α and TNFα (Affymetrix, Santa Clara, CA, USA). Data represent two independent experiments in duplicate measurements.

Reactive oxygen species (ROS) release assay from isolated human macrophages

Human monocytes were isolated as described above. To obtain macrophages, the freshly isolated monocytes were incubated for 6 days with 20 ng/ml M-CSF at 37 °C in a 5 % CO_2 atmosphere. For analysis of ROS formation, cells (human macrophages or neutrophils) were preincubated with the peroxide-sensitive fluorescence dye 2′,7′-dichlorofluorescein-diacetate (1 μg/ml) for 10 min at 37 °C. Then, 5–75 μg/ml (macrophages) or 1 – 100 μg/ml (neurophils) of the test compound or vehicle (containing 0.5 % ethanol) was added and after 10 min, cells were stimulated by 100 nM (61.7 ng/ml) (macrophages) or 1.62 μM (1 μg/ml) (neutrophils) phorbol 12-myristate 13-acetate (PMA). The fluorescence emission at 530 nm was measured after excitation at 485 nm in a thermally controlled (37 °C) 96-or 24-well plate in a spectrofluorometer. Diphenylene iodonium (DPI, 5 μM or 1.4 μg/ml) was used as control inhibitor. Data represent three independent experiments, each collecting single data points.

DPPH colorimetric radical scavenging (ROS-reduction) assay

The reaction of DPPH (2,2-diphenyl-1-picrylhydrazyl) with an antioxidant or reducing compound produces the corresponding hydrazine $DPPH_2$, which is assessed by monitoring the photometric color change from purple to yellow. Briefly, 0.1–300 μg/ml BNO 1095 was added to 100 μl ethanol (blank controls) or to 100 μl of a solution of the stable free radical in ethanol buffered with acetate to pH 5.5 (50 μM, corresponding to 19.7 μg/ml), in a 96-well plate. Ascorbic acid (100 μL of 50 μM (8.8 μg/ml) solution ascorbic acid in ethanol, i.e. 5 nmol ascorbic acid) was used as reference compound. The absorbance was read at 520 nm after 30 min incubation under gentle shaking in the dark. Radical scavenging activity is expressed as % absorption of the subtracted blank control [$A_{520\ nm}$ (DPPH, 50 μM + extract)-$A_{520\ nm}$ (blank)] vs. [$A_{520\ nm}$ (DPPH, 50 μM + vehicle) - $A_{520\ nm}$ (blank)]. Data represent three independent experiments, each collecting single data points.

Statistical analysis

Unless otherwise stated, all values are presented as mean ± standard error of the mean (SEM) of at least two independent experiments analysed in triplicate measurements. Statistical significance was assessed using one-way ANOVA followed by Dunnett's multiple comparison *post-hoc* test and a p-value criteria of ≤ 0.05 was used to assess statistical significance. For in vitro experiments, IC_{50} values were calculated by non-linear regression using the equation (Y = 100/(1 + 10^((LogEC50-X)*HillSlope)), X = log of dose or concentration, Y = normalized response (0–100 %)). Curve fitting, IC_{50} value calculation, and statistical analyses were performed with GraphPad Prism version 5.04 (GraphPad Software Inc., San Diego, CA).

Results

BNO 1095 dose-dependently reduced the number of oxytocin-induced uterine contraction and resulting uterine pain without causing sedation or locomotor deficits

Primary dysmenorrhea is characterized by severe pain during intense uterus contractions. Nulliparous rats were treated for 1 week by daily oral gavage with 2.1–20.7 mg/kg BNO 1095 dry extract (corresponding to 5 to 50 times the recommended human equivalent dosage, or 5× to 50× HED) prior to induction of uterine cramps by i.p. injection of 2,000 mI.U./kg (3.33 μg/kg) body weight oxytocin. Immediately after oxytocin injection, signs of uterine contractions and pain were monitored and recorded. Specifically, the latency until onset, total number of contractions (abdominal convulsions, writhings), and perceived pain (quantified according to the "grimace scale", with a maximal score of 2 representing obvious pain) was compared among vehicle control, treatment (BNO 1095), and comparator (carprofen, an NSAID analgesic) groups.

Treatment with up to 20.7 mg/kg of BNO 1095 did not significantly alter the latency until the start of convulsions when compared to either the vehicle control or 5 mg/kg s.c. injection of the carprofen comparator groups (data not shown). However, progressively increasing doses of BNO 1095 (2.1 up to 20.7 mg/kg body weight) (Fig. 1a) led to a maximal reduction of convulsions by about 67 % versus the vehicle control group (mean number of convulsions ± SEM: vehicle, 9.72 ± 1.61; 20.7 mg/kg BNO 1095: 3.23 ± 1.16; carprofen: 1.77 ± 0.99) that was significantly different compared to the vehicle control ($p < 0.05$) and

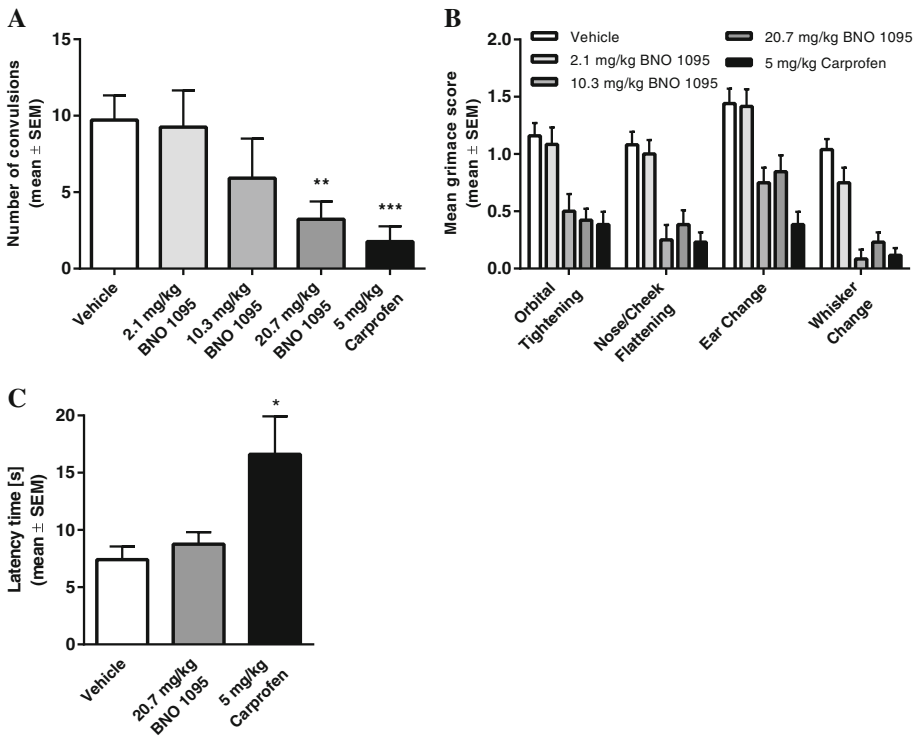

Fig. 1 Inhibition of contractions by BNO 1095 in vivo in a rat dysmenorrhea model. Effect of the *Vitex agnus castus* dry extract BNO 1095 on (**a**) number of abdominal convulsions and **b** the grimace scale after i.p. oxytocin injection. **c** Latency time until start of beam walking. Data are given as means ± SEM, $n = 12–14$. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$

roughly comparable to the inhibition seen with the carprofen group at the 20.7 mg/kg BNO 1095 dose.

In the measurement of perceived pain (Fig. 1b), no values from the BNO 1095 or comparator groups reached statistical significance versus the control group, but 20.7 mg/kg BNO 1095 showed numerical decreases in the mean grimace scale values for orbital tightening, nose/cheek flattening, and whisker change reaching similar levels to those seen with carprofen. The mean value for ear change was not markedly reduced by BNO 1095 when compared to carprofen.

In the beam walking test (Fig. 1c), animals of all tested groups crossed the beam with a normal gait and with less than two foot-slips per beam crossing. In comparing the beam walk score for rats from the vehicle control, 20.7 mg/kg BNO 1095, and 5 mg/kg carprofen groups, rats from the BNO 1095 group (8.75 s ± 1.05) started to cross the beam 2 times faster than rats from the carprofen group (16.62 s ± 3.32) suggesting that rats from the latter group seem to be more sedated than the control ($p < 0.05$) and BNO 1095 groups. In contrast, the beam walk score for rats from the vehicle control, 20.7 mg/kg BNO 1095, and 5 mg/kg carprofen groups (not shown) indicated that the gait of all three groups were normal and almost identical (7.0 ± 0, 7.0 ± 0, 6.92 ± 0.08, respectively). Taken together, these results indicate that while rats treated with 20.7 mg/kg BNO 1095 showed a reduction of the number of contractions and a decrease in pain that was roughly comparable to that of 5 mg/kg carprofen, BNO 1095 at this dose did not induce noticeable sedation or other impairments in motor coordination.

BNO 1095 inhibited drug-induced contractions in excised rat and human uterine tissue

To study the direct effects of BNO 1095 on uterine contractions, isolated uterus strips from nulliparous rats were mounted in organ baths and smooth muscle contractions were induced by 0.5 nM (0.5 ng/ml) oxytocin (Fig. 2a), 1 μM (354.5 ng/ml) $PGF_{2\alpha}$ (Fig. 2b) or 50 nM (54.2 ng/ml) vasopressin (Fig. 2c). In each case, addition of 10–400 μg/ml BNO 1095 demonstrated a concentration-dependent inhibition of contractions of uterus strips which exhibited a maximal effect (ca. 100 % inhibition of control) that exceeded by far the maximal inhibition (ca. 20 % inhibition, Fig. 2b) observed with the vehicle control (max. 0.5 % ethanol). After fitting each respective concentration-response curve, IC_{50} values for the ability of BNO 1095 to inhibit contractions induced by oxytocin, $PGF_{2\alpha}$, and vasopressin were determined at 142 μg/ml, 131 μg/ml, and 100 μg/ml, respectively.

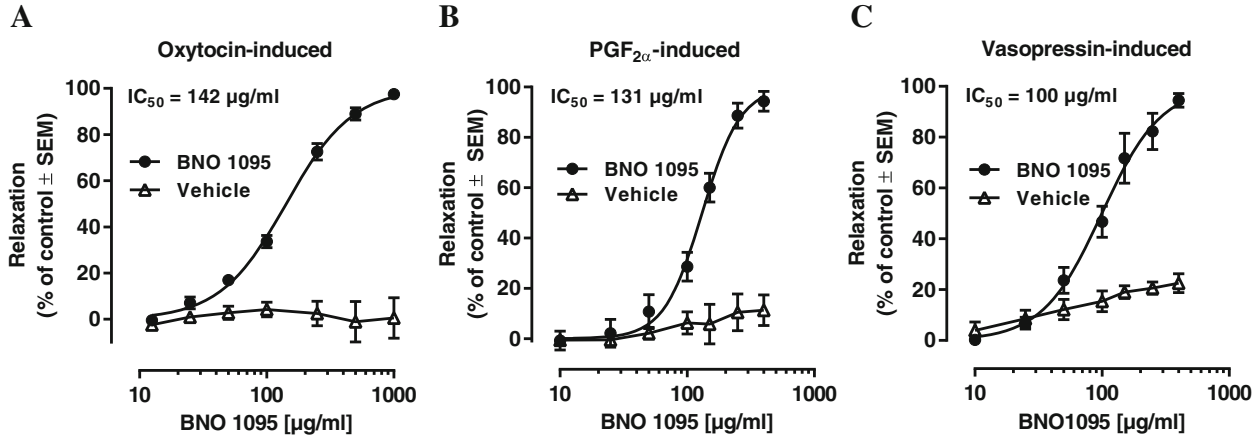

Fig. 2 Concentration-dependent inhibition of drug-induced contraction by BNO 1095 on rat uterine strips. Relaxation of **a** 0.5 nM (0.5 ng/ml) oxytocin-, **b** 1 μM (354.5 ng/ml) $PGF_{2\alpha}$-or **c** 50 nM (54.2 ng/ml) vasopressin-induced contractions of isolated rat uterus specimens. Up to 0.5 % ethanol was present in the vehicle control. Data are given as means ± SEM, $n = 3$

In addition, we analyzed the effect of BNO 1095 on isolated human myometrial strips obtained from women undergoing uterine resections. Analogous to the experiments described above, cumulative addition of 10–400 μg/ml BNO 1095 inhibited 200 nM (0.2 μg/ml) oxytocin-induced periodic smooth muscle contractions in a concentration-dependent manner with a curve-fitted IC_{50} value of 82 μg/ml (Fig. 3). Note that although myometrial strips were obtained from apparently healthy portions of the uterus, a longer preparation time and transport to the laboratory as for the rat tissue samples may contributed to the larger basal response elicited by the vehicle control (up to 40 %) compared to control responses observed in the rat tissue experiments. As in the rat tissue experiments, maximal ethanol concentrations in the vehicle control were 0.5 %.

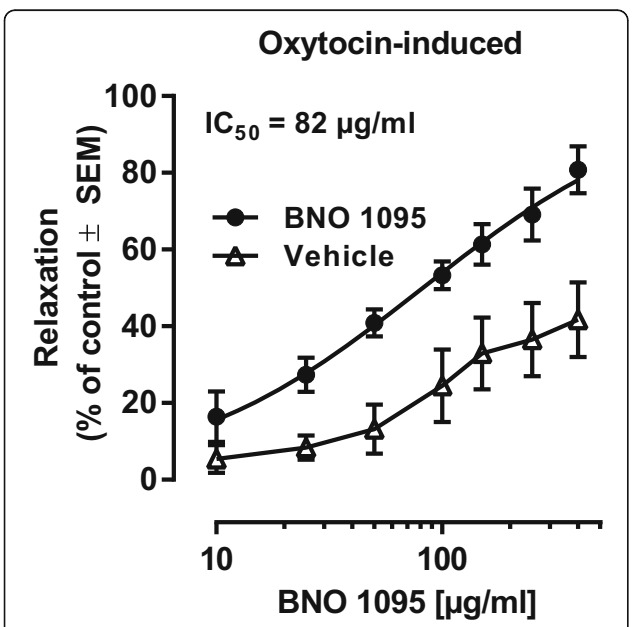

Fig. 3 Concentration-dependent anti-convulsive effect of BNO 1095 on human uterine strips. Relaxation of 200 nM (0.2 μg/ml) oxytocin-induced contractions of isolated human uterus specimens through BNO 1095. Up to 0.5 % ethanol was present in the vehicle control. Data are given as means ± SEM, $n = 5$

BNO 1095 did not substantially inhibit purified phosphodiesterase (PDE) enzyme activity

After confirming the ability of BNO 1095 in an in vivo rat model and in vitro excised uterine tissue experiments, we further investigated possible mechanisms of its action. A number of literature sources have suggested that PDE enzymes, responsible for the conversion of cyclic AMP (cAMP) into adenosine triphosphate (ATP), may enhance the properties of intrinsic relaxant molecules, especially in pregnant females. Specifically, human endometrial stromal cells express the PDE isoforms PDE4 and PDE8 [37], and PDE4-selective inhibitors such as rolipram have been shown to have spasmolytic effects in the myometrium [38–40].

Therefore, we analyzed the BNO 1095 extract regarding its inhibitory effect on the PDE subtypes PDE4B2, PDE4D1 and PDE8A1. However, for each of the tested enzymes subtypes, BNO 1095 inhibited PDE activity only at rather high concentrations, that is, 100 and 300 μg/ml (data not shown), compared to the pronounced ability of BNO 1095 to potently inhibit the production of leukotrienes, cytokines, and ROS (see below). Therefore, it appears unlikely that BNO 1095 inhibits uterine contractions via a reduction of PDE activity in the non-pregnant myometrium.

BNO 1095 potently suppresses leukotriene biosynthesis in human monocytes and inhibits the activity of purified 5-LO in a cell-free assay

Leukotrienes are synthesized from arachidonic acid involving 5-LO and represent important lipid mediators of the inflammatory immune response that are known to accompany menstruation [13, 15, 41]. We therefore investigated the effect of the extract on 5-LO product biosynthesis in a cell-free (purified enzyme) and cell-based (in human monocytes) model.

In a cell-free 5-LO inhibition assay, BNO 1095 concentration-dependently inhibited the activity of purified 5-LO with an IC_{50} value of 28.9 μg/ml (Fig. 4a). These results were confirmed and expanded using a cell-based assay, where 10–400 μg/ml BNO 1095 inhibited the formation of 5-LO products (LTB_4, trans LTB_4, epi-LTB_4 and 5-H(p)ETE) with an IC_{50} value of 22.2 μg/ml and blocked the formation of LTC_4 with an IC_{50} value of 63.5 μg/ml (Fig. 4b).

BNO 1095 did not substantially reduce COX-mediated prostaglandin synthesis in isolated human monocytes and macrophages

We investigated the effects of BNO 1095 on the biosynthesis of PGE_2 and TXB_2 in human monocyte-derived macrophages after stimulation with the pro-inflammatory agent LPS (1 μg/ml). Addition of up to 300 μg/ml BNO 1095 to macrophages after activation by LPS caused no observable inhibitory effect on the formation of COX-mediated biosynthesis of PGE_2 (LPS + BNO 1095: 70.0 % ± 30.3 vs. LPS alone set to 100 %) and TXB_2 (LPS + BNO 1095: 108.4 % ± 20.3 vs. LPS alone set to 100 %) (data not shown). The BNO 1095 extract (up to 300 μg/ml) also failed to significantly influence the release of $PGF_{2\alpha}$ from LPS-stimulated human monocytes (LPS + BNO 1095: 73.2 % ± 5.4 vs. LPS alone: 108.0 % ± 7.4) (data not shown). These results indicate that BNO does not target the COX pathway of the arachidonic acid cascade in intact cells.

Analysis of cytokine release from cultured human peripheral blood mononuclear cells (PBMCs)

Cytokines are potent regulators of inflammation. Analysis of human PBMCs revealed effective inhibition of cytokine release after pretreatment with 10–300 μg/ml BNO 1095 for 1 h prior to stimulation with 50 ng/ml LPS. As shown in Fig. 5, BNO 1095 concentration-dependently inhibited the release of IL-1β, IL-6, IL-8, TNFα and the chemokine MIP-1α (macrophage inflammatory protein-1α, or CCL3) with IC_{50} values of 20.1, 60.7, 98.4, 51.6 and 75.7 μg/ml, respectively. The release of the major inflammatory cytokine IL-1β was inhibited at lower concentrations when compared to the other four molecules, which is of interest, since IL-1β is known to stimulate PG and leukotriene biosynthesis [13, 42]. Higher BNO 1095 concentrations were required to inhibit IL-8 secretion, a chemokine known to recruit neutrophil migration and thought to be important for the endometrial remodeling during menstruation [43].

BNO 1095 reduced reactive oxygen species (ROS) release from isolated human macrophages and shows antioxidant properties in a cell-free assay

Certain phytomedicines have been reported to possess antioxidant capability, and are thus proposed to be protective against cell damage mediated by ROS. To test if BNO 1095 may interfere with ROS production, macrophages (Fig. 6a) or neutrophils (Fig. 6b) where analyzed for their capability to release ROS. A significant reduction of ROS formation in both cell types after pretreatment with BNO 1095 for 1 h prior to stimulation with PMA was observed. The cell-free DPPH radical scavenging assay demonstrated anti-oxidant activity of the BNO 1095 extract, with an IC_{50} value of 92.3 μg/ml (Fig. 6c).

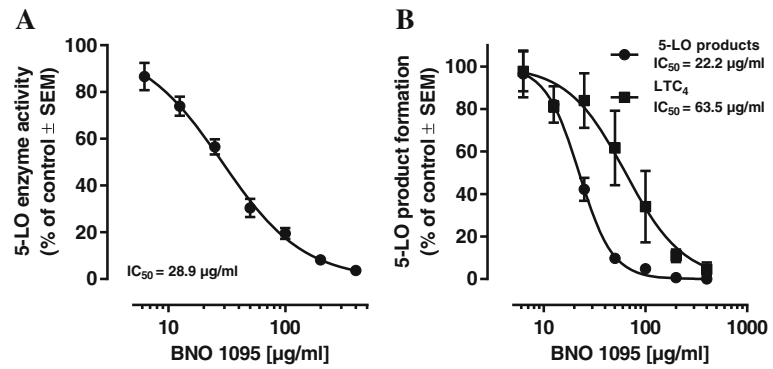

Fig. 4 Concentration-dependent inhibition of 5-LO (purified enzyme) activity and 5-LO product formation in human monocytes by BNO 1095. **a** Cell-free 5-LO enzyme inhibition by BNO 1095 using purified human recombinant 5-LO. **b** Inhibition of 5-LO product (sum of LTB_4, trans-LTB_4, epi-LTB_4 and 5-H(p)ETE) formation and LTC_4 production by BNO 1095 in a cell-based (human monocyte) in vitro assay. 0.5 % ethanol served as vehicle control. Data are given as means ± SEM, $n = 3$

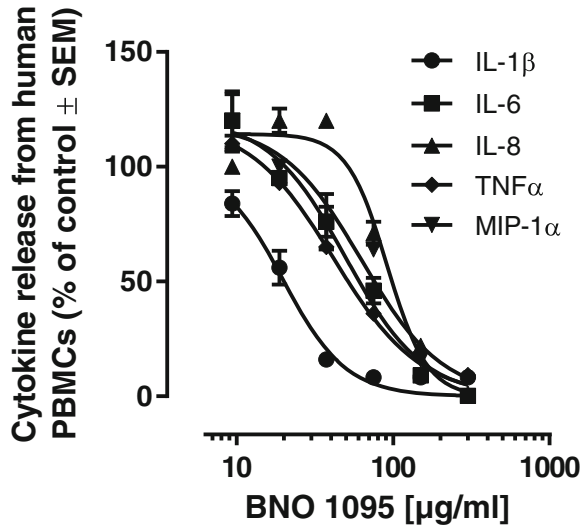

Fig. 5 Concentration-dependent inhibition of inflammatory cytokine release from human PBMCs by BNO 1095. LPS-induced inflammatory cytokine release from human PBMCs is inhibited by BNO 1095. Cells were pretreated with BNO 1095 for 1 h and subsequently stimulated with 50 ng/ml LPS for 24 h. 0.5 % ethanol served as vehicle control. Data are given as means ± SEM, $n = 2$

Discussion

Reduction of contractions and reduction of perceived pain by BNO 1095

In both humans and animal models, uterus contractions can be mediated by oxytocin, vasopressin or $PGF_{2\alpha}$ [44, 45], although the physiological relevance of each ligand is highly dependent on the implantation state (e.g., the female is pregnant or non-pregnant) and the temporal position in the pregnancy or menstrual/estrous cycle [42]. Other molecules are also important in the modulation and induction of menstruation, most notably progesterone and estrogen [4, 6] as well as PGE_2 [3]. While a consensus model underlying primary dysmenorrhea is still lacking, the clinical use of NSAIDs and oral contraceptives for the treatment of menstrual pain [46, 47] and PMS [2] suggest the involvement of these molecules in these conditions.

In our in vivo animal model system for dysmenorrhea rats were treated once-daily with 10 mg/kg body weight estradiol-benzoate to induce endometrial hyperplasia as a prerequisite for oxytocin-induced dysmenorrhea-like uterine contractions. It is worth mentioning that estradiol is known to induce prolactin secretion [48]. Bigazzi et al. [49] reported that human prolactin-increased frequency and amplitude of spontaneous contractions of rat uterus. This is in contrast to the report of Lessing et al. [50] demonstrating that rat prolactin has no significant effect on rat uterine muscle, indicating species specificity of prolactin. Thus, a potential effect of endogenous prolactin in this model may be negligible.

In our dysmenorrhea model, BNO 1095 decreased the number of oxytocin-induced uterine contractions and pain scores as measured by the rat grimace scale. Further evidence for a spasmolytic action of BNO 1095 was provided by in vitro experiments with human or rat uterine tissue strips, where BNO 1095 concentration-dependently inhibited contractions induced by oxytocin, vasopressin, or $PGF_{2\alpha}$. These data are supported by results from a study by Afifi et al. showing that isoorientin, a flavonoid found in the VAC whole fruit extract (BNO 1095) [26], concentration-dependently inhibits contractions of the rat and guinea pig uterus in vitro [22]. The anti-convulsive effect may contribute to the beneficial effects of BNO 1095 in relieving pain caused by menstrual cramps. Our results also complement data from several in vitro studies revealing a neuroendocrine mode of action of BNO 1095 extracts. Specifically, BNO 1095 extract preparations demonstrated specific binding to the dopamine D_2 receptor [27, 28], which would thereby inhibit prolactin release from the pituitary gland and down-regulate a systemic

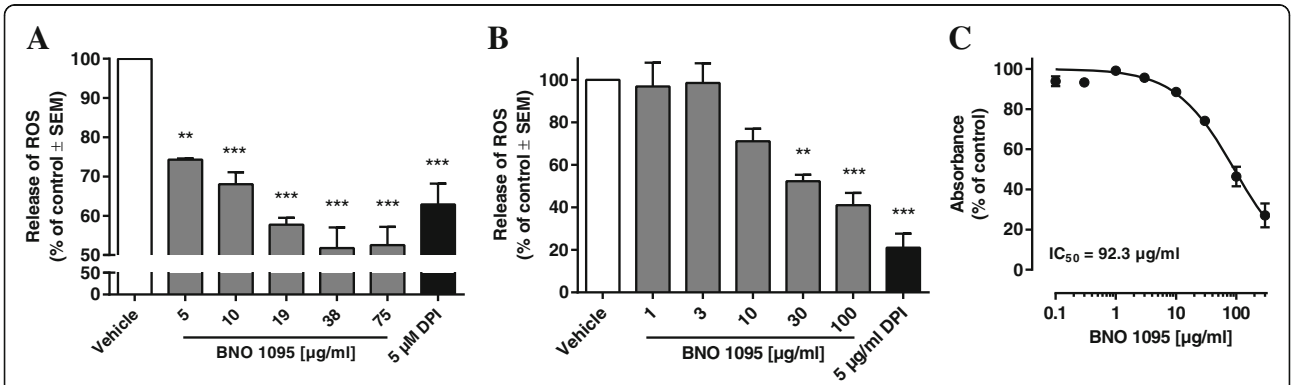

Fig. 6 Analysis of the effects of BNO 1095 on cellular ROS formation and ROS scavenging activity. Effects of BNO 1095 on cellular formation of ROS in 100 nM (1 µg/ml) PMA-activated human (**a**) macrophages and **b** neutrophils. **c** Anti-oxidative activity of BNO 1095 in a cell-free colorimetric assay. DPI (reference compound), 5 µM (1.4 µg/ml). 0.5 % ethanol was present in the vehicle control. Data are given as means ± SEM, $n = 3$. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$

hormonal cascade ultimately responsible for a wide range of menstrual symptoms in women suffering from PMS. Therefore, BNO 1095 shows not only a systemic, but also a local action in the uterus to control biological processes known to be active during menstruation.

Reduction of pro-inflammatory signaling molecules by BNO 1095

At present, it is difficult to determine which molecules and processes relate to primary dysmenorrhea versus the normal menstrual cycle, since dysmenorrhea is likely an "exaggeration" of healthy body processes. However, in addition to increased uterine contractions, it has also become increasingly clear that pro-inflammatory molecules and inflammatory cells are thought to play a role in endometrial breakdown and assist in the eventual removal of the endometrial lining in the absence of pregnancy. Although far from being a "disease state", such an inflammatory process along with leukocyte recruitment in the late secretory phase [5] may unfortunately increase the pain sensitivity and/or uterine contractions via intercoupled feedback mechanisms in patients with primary dysmenorrhea.

In our present study we used a combination of in vitro cell-based (primary composed of human monocytes, neutrophils and macrophages) and cell-free (enzymatic, colorimetric) assays in order to demonstrate inhibitory actions of BNO 1095 on the pro-inflammatory and pain-enhancing leukotrienes, cytokines/chemokines, and ROS. In light of the growing evidence for a role of inflammatory mediators to affect the immune response in facilitating endometrial breakdown during menstruation [3, 4, 6, 13, 43, 51], the ability of BNO 1095 to potently inhibit release of these inflammatory mediators may explain the beneficial effect of this herbal extract in the treatment of primary dysmenorrhea.

Leukotrienes have been postulated to increase the sensitivity to pain in the uterus [1, 4, 13, 14, 41] and to cause uterine contractions [15]. Indeed, higher circulating levels of LTC_4 and LTD_4 have been found in the menstrual blood of women with primary dysmenorrhea [13, 14] and thus, the inhibition of uterine contraction via a reduction of these cysteinyl-leukotrienes represents a plausible molecular mechanism for the BNO 1095 action, as observed in the rat in vivo experiments. Interestingly, our investigation of the COX-1/2-mediated branch of the arachidonic acid pathway supports the hypothesis that BNO 1095 does not significantly inhibit the release of PGE_2, $PGF_{2\alpha}$, or TXB_2 in cells associated with an increased migratory/inflammatory uterine response during menstruation [3–6].

Finally, our results suggest that antioxidant compounds within BNO 1095 may reduce pain by scavenging ROS, which are known to increase $PGF_{2\alpha}$, chemokine and pro-inflammatory cytokine production during menstruation. Specifically, ROS in conjunction with copper-zinc superoxide dismutase (Cu/Zn-SOD) [4, 10] and NF-kB have been proposed to serve as important signal transducers between the withdrawal of estrogen and progesterone and endometrial breakdown via a stimulation of COX-2 expression and promotion of $PGF_{2\alpha}$ release [11].

Anti-inflammatory and anti-oxidative effects have been previously reported for VAC extracts and several single VAC constituents [25, 52, 53]. For example, Choudary et al. demonstrated LO enzyme-inhibiting activity of casticin in vitro [54], and the flavonoid vitexin inhibited pro-inflammatory cytokine production and inflammation-induced pain [23] in an in vivo experimental inflammation model. This broad anti-inflammatory and anti-oxidative effects described for VAC extracts and its constituents could therefore contribute to the beneficial action of VAC extracts regarding PMS and painful menstrual complaints.

Conclusion

Our in vivo data from an experimental dysmenorrhea model and in vitro studies using isolated rat and human uterine specimens revealed significant spasmolytic properties of BNO 1095 via local actions that are complementary to the extract's previously-known neuroendocrine inhibition of prolactin release. Additionally, BNO 1095 showed potent anti-inflammatory activity in vitro as it suppressed leukotriene (but not prostaglandin) biosynthesis, cytokine release and ROS production from isolated human leukocytes. The ability of BNO 1095 to inhibit the release of these pro-inflammatory molecules from migratory/inflammatory cells complements the extract's spasmolytic action and supports the beneficial effects described for BNO 1095 for the treatment of menstrual disorders.

Abbreviations

COX: Cyclooxygenase; HED: Human equivalent dose; LO: Lipoxygenase; LT: Leukotriene; NF-kB: Nuclear factor kappa-light-chain-enhancer of activated B cells; PG: Prostaglandin; PMS: Premenstrual syndrome; ROS: Reactive oxygen species; SOD: Superoxide dismutase; VAC: Vitex agnus castus

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Authors' contributions

JR made substantial contributions to conception and interpretation of data and has been involved in drafting the manuscript. OW made substantial contributions to acquisition of data and has been involved in critical revision of the manuscript. AA has been involved in conception and critical revision of the manuscript. GK supervised the study and made substantial contributions to conception and interpretation of data and has been involved in critical revision of the manuscript. All authors read and approved the final manuscript.

Competing interests

J. Röhrl, A. Ammendola and G. Künstle are employees of Bionorica SE, Germany. O. Werz received funding from Bionorica SE, Germany. The authors declare that they have no further competing interests.

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