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RESEARCH PAPER

Therapeutic action and underlying mechanisms of a combination of two pentacyclic triterpenes, α- and β-amyrin, in a mouse model of colitis

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Background and purpose: α- and β-amyrin are pentacyclic triterpenes found in plants and are known to exhibit pronounced anti-inflammatory effects. Here, we evaluated the effects of a 1:1 mixture of α- and β-amyrin (α,β-amyrin) on an experimental model of colitis in mice.
Experimental approach: Colitis was induced in *Swiss* male mice by trinitrobenzene sulphonic acid (TNBS) and followed up to 72 h; animals were treated systemically with α,β-amyrin, dexamethasone or vehicle. Macro- and microscopic damage, myeloperoxidase activity and cytokine levels were assessed in colons. Histological sections were immunostained for cyclooxygenase-2 (COX-2), vascular endothelial growth factor, phospho-p65 nuclear factor-κB (NF-κB) and phospho-cyclic AMP response element-binding protein (CREB)
Key results: TNBS-induced colitis was associated with tissue damage, neutrophil infiltration and time-dependent increase of inflammatory mediators. Treatment with α,β-amyrin (3 mg·kg^{-1}, i.p.) or dexamethasone (1 mg·kg^{-1}, s.c.) consistently improved tissue damage scores and abolished polymorphonuclear cell infiltration. α,β-Amyrin, like dexamethasone, significantly diminished interleukin (IL)-1β levels and partially restored IL-10 levels in colon tissues 72 h after colitis induction, but only α,β-amyrin reduced vascular endothelial growth factor expression by immunohistochemistry. The colonic expression of COX-2 at 24 h and that of phospho-NF-κB and phospho-CREB (peaking at 6 h) after colitis induction were consistently inhibited by both α,β-amyrin and dexamethasone.
Conclusions and implications: Systemic administration of α,β-amyrin exerted a marked and rapid inhibition of TNBS-induced colitis, related to the local suppression of inflammatory cytokines and COX-2 levels, possibly via inhibition of NF-κB and CREB-signalling pathways. Taken together, our data suggest a potential use of α,β-amyrin to control inflammatory responses in bowel disease.
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Keywords: α,β-amyrin; inflammation; interleukins; cyclooxygenase-2; vascular endothelial growth factor; nuclear factor-κB; cAMP response element-binding protein; mouse colitis; inflammatory bowel disease

Abbreviations: CREB, cAMP response element-binding protein; IBD, inflammatory bowel disease; IL, interleukin; MPO, myeloperoxidase; NF-κB, nuclear factor-κB; VEGF, vascular endothelial growth factor

Introduction

Inflammatory bowel disease (IBD) is a chronic condition of unknown aetiology involving multiple immune, genetic and environmental factors (McGuckin *et al.*, 2008). The inflammation of the intestinal mucosa is characterized by an influx of neutrophils and macrophages, accompanied by the activation of the transcription factors, nuclear factor-κB (NF-κB) and the cyclic AMP (cAMP) response element-binding protein (CREB), and consequent production of interleukin (IL)-1β, IL-6, tumour necrosis factor-α, interferon-γ and other pro-inflammatory agents that are secreted in the colonic mucosa of IBD patients. This process gives rise to an uncontrolled production of additional inflammatory mediators (McGuckin *et al.*, 2008), such as nitric oxide, reactive oxygen species, the eicosanoid prostaglandin E_2 (PGE_2) and other cytokines including the vascular endothelial growth factor (VEGF) (Danese *et al.*, 2006; Sandor *et al.*, 2006; Tolstanova *et al.*, 2008). The colonic inflammation induced by 2,4,6-trinitrobenzene sulphonic acid (TNBS) is a widely used animal

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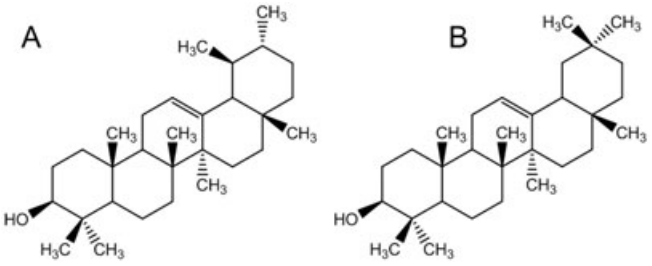

Figure 1 Chemical structure of α-amyrin (A) and β-amyrin (B) isolated from *Protium kleinii*.

model that produces an inflammatory process that is similar to that in human IBD (Morris *et al.*, 1989). In this model, TNBS penetrates into the colon and causes transmural lesions, ulcerations, necrosis, loss of crypt epithelium, thickening of the bowel wall, oedema, decreased colon length, and a massive infiltration of polymorphonuclear leucocyte (PMN) cells, characterizing macroscopic and histological damages (Selve and Wohrmann, 1992; Bento *et al.*, 2008).

In Brazilian folk medicine, the resins and leaves of *Protium kleinii* (Burseraceae) are used to alleviate inflammatory symptoms (Corrêa, 1984). In the last 10 years, studies have shown a systemic anti-inflammatory action of the essential oil from leaves and resins of some *Protium* species (Siani *et al.*, 1999; Rudiger *et al.*, 2007). The main bio-active components in those resins, α-amyrin and β-amyrin (Figure 1), are pentacyclic triterpenes isolated from *P. kleinii* as well other plant species (Oliveira *et al.*, 2005; Otuki *et al.*, 2005b; do Vale *et al.*, 2005). Some experimental evidence has pointed that α-amyrin exhibits systemic anti-nociceptive, anti-inflammatory, antipruritic, hepatoprotective and gastroprotective properties, when assessed *in vivo* (Kweifio-Okai *et al.*, 1994; Recio *et al.*, 1995; Oliveira *et al.*, 2004a,b). Our group has previously shown that a mixture of the triterpenes, α-amyrin and β-amyrin, produces consistent anti-nociception in rodents, especially when assessed in inflammatory models of pain (Otuki *et al.*, 2005a). We also reported that α-amyrin exhibits pronounced topical anti-inflammatory properties against 12-O-tetradecanoylphorbol-acetate-induced ear oedema, an experimental model of skin inflammation (Otuki *et al.*, 2005b). Such effects were mediated by inhibition of NF-κB, cyclooxygenase-2 (COX-2) and mitogen-activated protein kinases (Medeiros *et al.*, 2007). However, the mechanisms underlying the anti-inflammatory properties of the mixture of triterpenes, α,β-amyrin are, so far, only partially understood.

Therefore, we postulated that α,β-amyrin could have therapeutic benefits in IBD, and the present study was designed to evaluate the preventive and therapeutic effects of α,β-amyrin on TNBS-induced colonic inflammation in mice. Furthermore, we have also investigated some of the relevant mechanisms through which α,β-amyrin exerts its systemic anti-inflammatory action, which seem to be related to the modulation of local cytokines and reduction of COX-2 expression, possibly via inhibition of NF-κB and CREB-signalling pathways.

Methods

Animals

All animal care and experimental protocols used in this study were approved by the local Ethics Committee. The experiments were conducted using male Swiss mice (35–40 g) bred in our own animal house. Animals were kept in a 12 h light/dark cycle, with controlled humidity (60–80%) and temperature (22 ± 1°C). Food and water were freely available. Experiments were performed during the light phase of the cycle. The animals were acclimatized to the experimental laboratory for at least 1 h before testing.

Induction and evaluation of experimental colitis

Colitis was induced by intracolonic administration of TNBS as previously described (Fiorucci *et al.*, 2007; Hara *et al.*, 2008). Briefly, one-day fasted mice received 100 μL of 2.5 mg TNBS (Sigma, St. Louis, MO, USA) in 50% ethanol via a polyethylene PE50 catheter, which was carefully inserted intrarectally 4 cm from the anus into the lumen. The animals were then kept in a head-down vertical position for 2 min. Control mice received 100 μL of 0.9% NaCl solution. Four hours later, the animals were given free access to food and water. After killing, rectum and colon were excised and opened longitudinally and carefully washed in saline to observe macroscopic alterations due to the induced colitis. The severity of the macroscopic damage was evaluated 72 h after TNBS administration, as previously described (Wallace *et al.*, 1989; Hara *et al.*, 2007). Briefly, inflammation level was scored from 0 to 10 as follows: 0, no inflammation; 1, hyperaemia without ulcers; 2, hyperaemia and wall thickening without ulcers; 3, one ulceration site without wall thickening; 4, two or more ulceration sites; 5, 0.5 cm extension of inflammation or severe damage; 6–10, 1 cm extension of inflammation or severe damage and the score was increased by one for each 0.5 cm of damage up to a maximal score of 10; 0 or 1, absence or presence of diarrhoea; 0 or 1, absence or presence of stricture; 0–2, absence or presence of medium or severe adhesion. In order to determine the peak of protein expression of VEGF, COX-2, phospho-p65 NF-κB and phospho-CREB for subsequent immunohistochemistry studies, another group of animals were killed 0, 2, 6, 12, 24, 48 and 72 h after TNBS instillation and colon tissues processed as normal.

In vivo *treatment protocols*

The 1:1 mixture of α,β-amyrin was diluted in 5% Tween 80 plus 5% ethanol in phosphate-buffered saline (PBS), just before use (Otuki *et al.*, 2005a). To evaluate the potential therapeutic effect of α,β-amyrin in experimental colitis, animals received different doses of α,β-amyrin twice daily (0.3, 1 and 3 $mg \cdot kg^{-1}$, i.p.), from 24 h after TNBS administration. Both control and another group of colitic mice were treated with 10 $mL \cdot kg^{-1}$ vehicle i.p. only (5% Tween 80 plus 5% ethanol in PBS), while dexamethasone (1 $mg \cdot kg^{-1}$, s.c., twice daily) was used as the positive control of treatment in all protocols. In a previous study (Otuki *et al.*, 2005b), we found

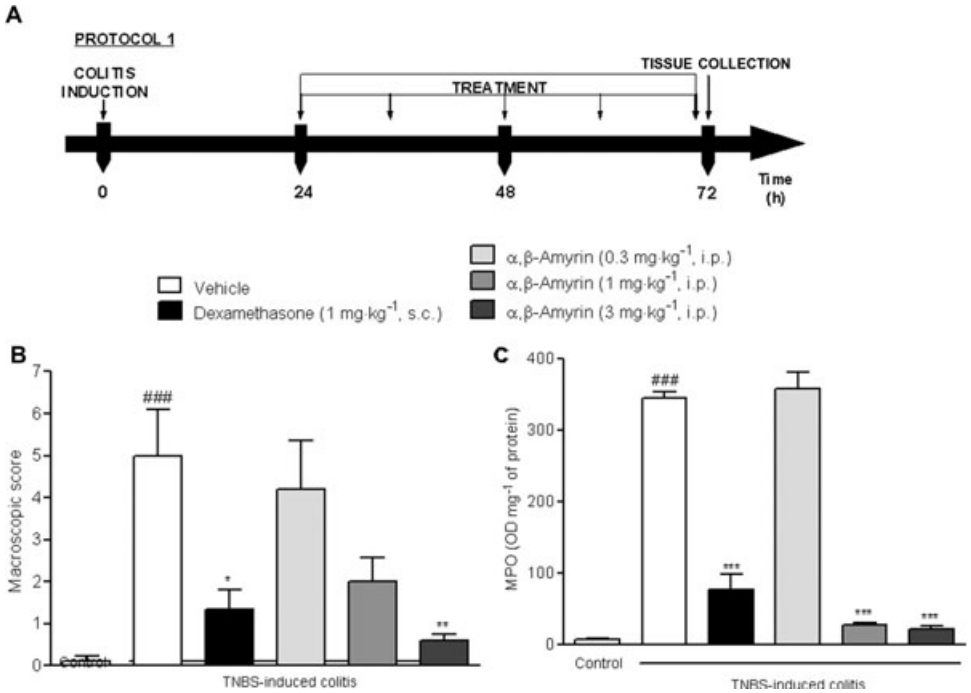

Figure 2 Therapeutic effect of different doses of α,β-amyrin on trinitrobenzene sulphonic acid (TNBS)-induced colitis in mice. (A) Protocol design for colitis treatment. Twenty-four hours after TNBS administration, separate groups of animals received a 2 day treatment with α,β-amyrin (0.3, 1 or 3 mg·kg^{-1}, i.p., twice daily), dexamethasone (1 mg·kg^{-1}, s.c.) or vehicle, and then were killed 72 h after colitis induction. A group of control mice received vehicle only, intracolonically at 0 h. (B) Both α,β-amyrin (3 mg·kg^{-1}) and dexamethasone (1 mg·kg^{-1}) significantly reduced the macroscopic damage. Data are expressed as mean ± SEM ($n = 5$). ###$P < 0.001$, compared with the control group; *$P < 0.05$, **$P < 0.01$, compared with vehicle-treated colitic mice (Kruskal-Wallis followed by Dunn's test). (C) Both α,β-amyrin (1 and 3 mg·kg^{-1}) and dexamethasone (1 mg·kg^{-1}) significantly reduced myeloperoxidase (MPO) activity. Data are expressed as mean ± SEM ($n = 5$) ###$P < 0.001$, compared with the control group; ***$P < 0.001$, compared with vehicle-treated colitic mice; one-way ANOVA followed by Student Newman-Keuls test.

that the mixture of α,β-amyrin was active orally, but in the present study we chose to give it by i.p. injection, due to the limited availability of the compound. Seventy-two hours following TNBS administration, the animals were killed and the colon was excised for macroscopic scoring and then processed for histological analysis or subsequent studies using tissue homogenate, according to protocol 1 (Figure 2A). Thus, in order to identify the possible mechanisms of α,β-amyrin involved in prevention of TNBS colitis, further sets of experiments were performed based on those time–course curves (Figure 3). In accordance with the immunohistochemistry results obtained in the time–course study, protocol 2 was designed to evaluate the expression of COX-2 in α,β-amyrin-pretreated mice (3 mg·kg^{-1}, i.p., –0,5, +12 and +20 h from TNBS instillation) and its effects on colitis development at 24 h. Furthermore, to evaluate NF-κB and CREB activation, we performed protocol 3, which consisted of pretreatment with α,β-amyrin (3 mg·kg^{-1}, i.p.) at 24, 12 and 0.5 h, prior to TNBS instillation and sample collection at 6 h after TNBS. Colonic tissues were then removed for immunohistochemical analysis.

Histological analysis and evaluation of microscopic damage

Each excised portion of distal colon was immediately fixed in 10% formaldehyde solution. All tissues were processed by using conventional histochemical techniques, embedded in paraffin wax and then sectioned at 5 μm thicknesses, mounted on glass slides and deparaffinized. For general histology and morphometric analysis, slices were stained by using haematoxylin–eosin by standard techniques. Samples were analysed by light microscopy. Distal portions of colon were histologically examined in cross sections at ×200 magnification. In each specimen, six random fields of view were examined and assessed by two observers, without knowledge of the treatments. Histological changes in each sample were graded from 0 to 4 as described by Neurath *et al*. (1995): 0, no inflammation; 1, very low level; and 2, low level of leucocytic infiltration; 3, high level of leucocytic infiltration, high vascular density, thickening of the colon wall; 4, transmural infiltrations, loss of goblet cells, high vascular density, thickening of the colon wall.

Myeloperoxidase activity assay

Neutrophil infiltration into the colon was assessed indirectly by measuring the myeloperoxidase (MPO) activity (De Young *et al*., 1989). Full colon segments were homogenized in EDTA/NaCl buffer (pH 4.7) and centrifuged at 10 000× *g* for 15 min at 4°C. The pellet was resuspended in 0.5% hexadecyltrimethyl ammonium bromide buffer (pH 5.4), and the samples were frozen in liquid nitrogen and thawed three times. Upon thawing, the samples were recentrifuged (10 000× *g*, 15 min, 4°C), and 25 μL of the supernatant was used for the MPO

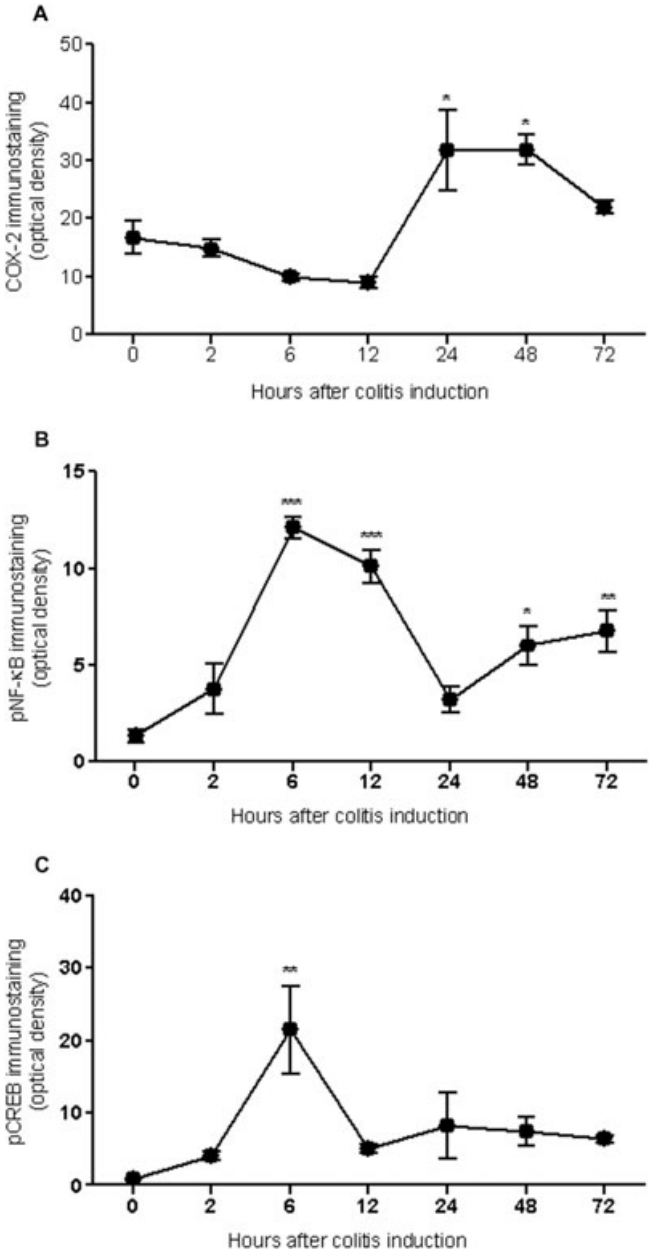

Figure 3 Time–course of expression of inflammatory factors elicited by intracolonic TNBS administration in mice. One-day fasted mice were killed at the indicated hours following TNBS instillation for colitis induction. Colonic tissues were processed for immunohistochemical analysis by using primary antibodies against the COX-2 isoenzyme (A) and the phosphorylated transcription factors pNF-κB (p65) (B) and pCREB (C). Both staining intensity and stained area of each immunoreaction are expressed as the percentage of immunostaining. Data represent mean ± SEM (n = 3–4); *P < 0.05, **P < 0.01, ***P < 0.001 versus hour 0; one-way ANOVA followed by Student Newman-Keuls test. COX-2, cyclooxygenase-2; CREB, cAMP response element-binding protein; NF-κB, nuclear factor-κB; TNBS, trinitrobenzene sulphonic acid.

assay. The enzymatic reaction was assessed by addition of 1.6 mM tetramethylbenzidine, 80 mM $NaPO_4$ and 0.3 mM hydrogen peroxide. The MPO activity was measured spectrophotometrically at 650 nm, and results were expressed as optical density (OD) values mg^{-1} tissue protein.

Determination of cytokine levels

Briefly, full-thickness colon samples were homogenized in phosphate buffer containing 0.05% Tween 20, 0.1 mM phenylmethylsulphonyl fluoride, 0.1 mM benzethonium chloride, 10 mM EDTA and 20 KIU aprotinin A. The homogenate was centrifuged at 3000× g for 10 min, and supernatants were stored at –80°C until further analysis. IL-1β and IL-10 levels were evaluated by using ELISA kits according to the manufacturer's recommendations. The results were expressed as $pg \cdot mg^{-1}$ tissue protein.

Immunohistochemical analysis

Immunohistochemistry was performed in colon slices (5 μm), using the following primary antibodies and respective dilutions: anti-VEGF (1:200), anti-COX-2 (1:50), anti-phospho-p65 NF-κB (1:50) and anti-phospho-CREB (1:100). High temperature antigen retrieval was applied by immersion of the slides in a water bath at 95–98°C in 10 mM trisodium citrate buffer pH 6.0, for 45 min. The non-specific binding was blocked by incubating sections for 1 h with goat normal serum diluted in PBS. After overnight incubation at 4°C with primary antibodies, the slides were washed with PBS and incubated with the secondary antibody Envision plus, ready-to-use, for 1 h at room temperature. The sections were washed in PBS, and the visualization was completed by use of 3,3′-diaminobenzidine (DAB) or permanent red in chromogen solution and counterstained lightly with Harris's haematoxylin solution. Control and experimental tissues were placed on the same glass slide and processed under the same conditions. Images of colon sections stained with antibodies to VEGF, COX-2, phospho-p65 NF-κB or phospho-CREB were acquired using a Sight DS-5M-L1 digital camera (Nikon, Melville, NY, USA) connected to an Eclipse 50i light microscope (Nikon). Settings for image acquisition were identical for control and experimental tissues. We captured three images of colon sections per mouse, and a threshold optical density that best discriminated staining from the background was obtained using the NIH ImageJ 1.36b imaging software (National Institutes of Health, Bethesda, MD, USA). The total pixels intensity was determined, and data were expressed as optical density (OD).

Preliminary oral acute toxicity

Healthy male mice fasted overnight with water *ad libitum* were randomly divided into five groups (n = 3 per group). The α,β-amyrin was diluted in 5% Tween 80 plus 5% ethanol in PBS and administered orally by gavage to those groups of mice at doses of 5, 50, 300 or 2000 $mg \cdot kg^{-1}$ body weight, while the control group received vehicle only. The general behaviour of the mice and signs of toxicity were observed continuously for 30 min after the oral treatment, and then at hourly interval for 4 h and thereafter over a period of 24 h (OECD, 2001). Animals were further observed once a day up to 14 days following treatment for behavioural changes and signs of toxicity and/or death. The latency of death and the body weight were measured daily.

Statistical analysis

All data are expressed as means ± SEM. For non-parametric data, Kruskal-Wallis followed by Dunn's test was performed.

For parametric data, statistical significance of differences between the groups was determined by one-way ANOVA followed by Student Newman-Keuls test. Statistical analyses were performed by using GraphPad Prism 4 software (GraphPad Software Inc., San Diego, CA, USA). A *P*-value of less than 0.05 was considered to be statistically significant.

Materials

α,β-Amyrin (1:1 mixture) was isolated from resins of the plant *P. kleinii* as previously described with purity degree of higher then 95% (Otuki *et al*., 2005b). Control mice received 10 mg·kg^{-1} of vehicle solution containing 5% Tween 80 plus 5% ethanol in PBS, and this treatment had no significant effect. Dexamethasone, hexadecylmethylammonium bromide, tetramethylbenzidine, hydrogen peroxide, Tween 20, Tween 80, phenylmethylsulphonyl fluoride, benzethonium chloride, EDTA, aprotinin, PBS, eosin, haematoxylin and 2,4,6-TNBS were purchased from Sigma (St. Louis, MO, USA). Formaldehyde was obtained from Merck (Darmstadt, Germany). Mouse IL-10 and IL-1β/IL-1F2 DuoSet ELISA Development kits were obtained from R&D Systems (Minneapolis, MN, USA). The following primary antibodies were used: monoclonal mouse anti-VEGF (C-1, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), rabbit polyclonal anti-COX-2, rabbit polyclonal anti-phospho-p65 NF-κB (Ser276) and rabbit monoclonal anti-phospho-CREB (Ser133) (all from Cell Signaling Technology, Beverly, MA, USA). Secondary antibody Envision Plus (HRP or AP) and DAB or permanent red chromogens were both purchased from Dako Cytomation (Carpinteria, CA, USA). The drug/molecular target nomenclature conforms to the *BJP* Guide to Receptors and Channels (Alexander *et al*., 2008).

Results

Acute oral toxicity

Oral administration of α,β-amyrin (5–300 mg·kg^{-1}) to mice did not produce any relevant sign of acute toxicity when observed up to 14 days. In addition, the weight gain, water and food consumption also did not change compared with a control group (results not shown). However, at a much higher dose (2000 mg·kg^{-1}), one of three animals died within 14 days.

Effect of treatment with α,β-amyrin on morphological damage and MPO activity in colon tissue of mice with TNBS-induced colitis

In initial experiments, 2.5 mg TNBS given to the distal colon, was found to induce a severe acute colitis. TNBS produced high scores of macroscopic (5.6 ± 0.5 vs. 0.2 ± 0.2 in the control group; $P = 0.0001$; $n = 5$ per group) and microscopic damage (3.8 ± 0.2 vs. 0.4 ± 0.3 in the control group; $P = 0.0028$; $n = 3$ *per* group), when assessed 72 h following colitis induction. Morphologic damage was accompanied by a marked increase in MPO activity (4.7-fold elevation in colitis when compared with the control group; $P = 0.0001$; $n = 5$ in each group). These measures of damage induced by TNBS alone (protocol 1, Figure 2A) were not affected by treatment with vehicle, after 72 h (Figures 2B,C and 4).

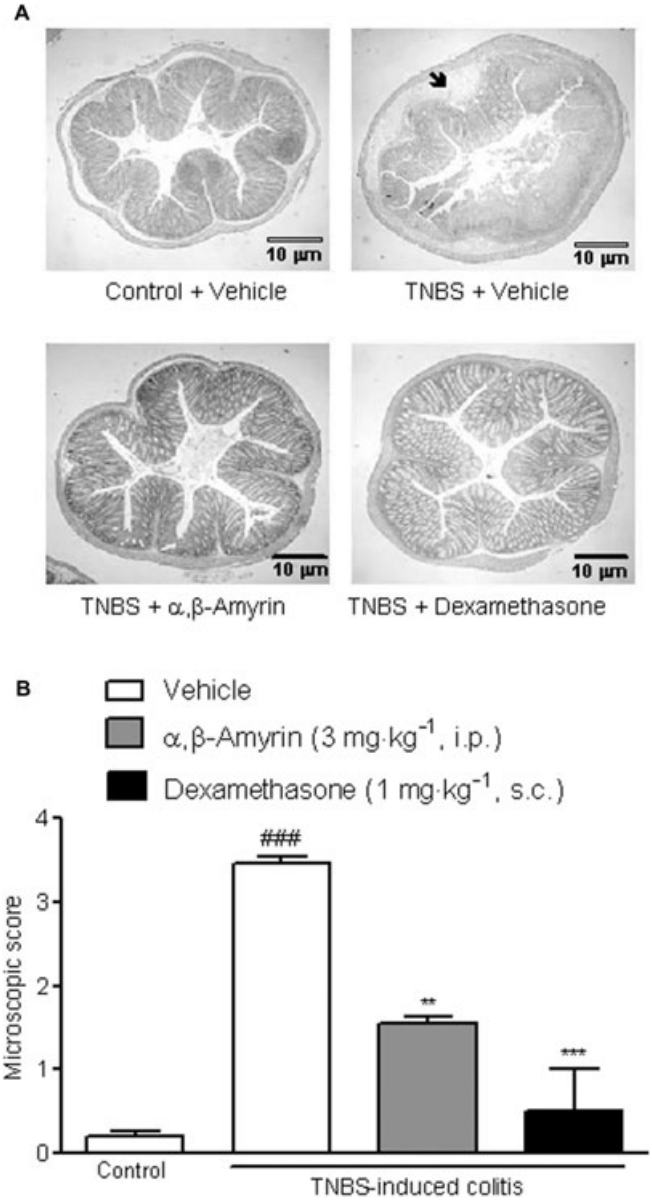

Figure 4 Effect of α,β-amyrin treatment on microscopic damage to colon, 72 h after colitis induction in mice. (A) Representative histological images of haematoxylin–eosin staining of colon tissue sections after treatment as indicated, which were interpreted by means of the inflammatory microscopic score in (B). Arrow indicates submucosal infiltration of inflammatory cells. Original magnification, ×400. (B) Both α,β-amyrin (3 mg·kg^{-1}) and dexamethasone (1 mg·kg^{-1}) treatment ameliorated the histological damage induced by trinitrobenzene sulphonic acid (TNBS) on colonic tissues. Data are expressed as mean ± SEM ($n = 3$); ###$P < 0.001$, compared with the control group; **$P < 0.01$, ***$P < 0.001$, compared with vehicle-treated colitic mice (Kruskal-Wallis followed by Dunn's test).

The systemic treatment of TNBS colitis with α,β-amyrin (0.3, 1 and 3 mg·kg^{-1}) caused a dose-related improvement of the macroscopic damage, compared with vehicle-treated mice, reaching a statistically significant effect at the dose of 3 mg·kg^{-1} (Figure 2B), equivalent to 90% inhibition of the TNBS effect. As expected, dexamethasone (1 mg·kg^{-1})

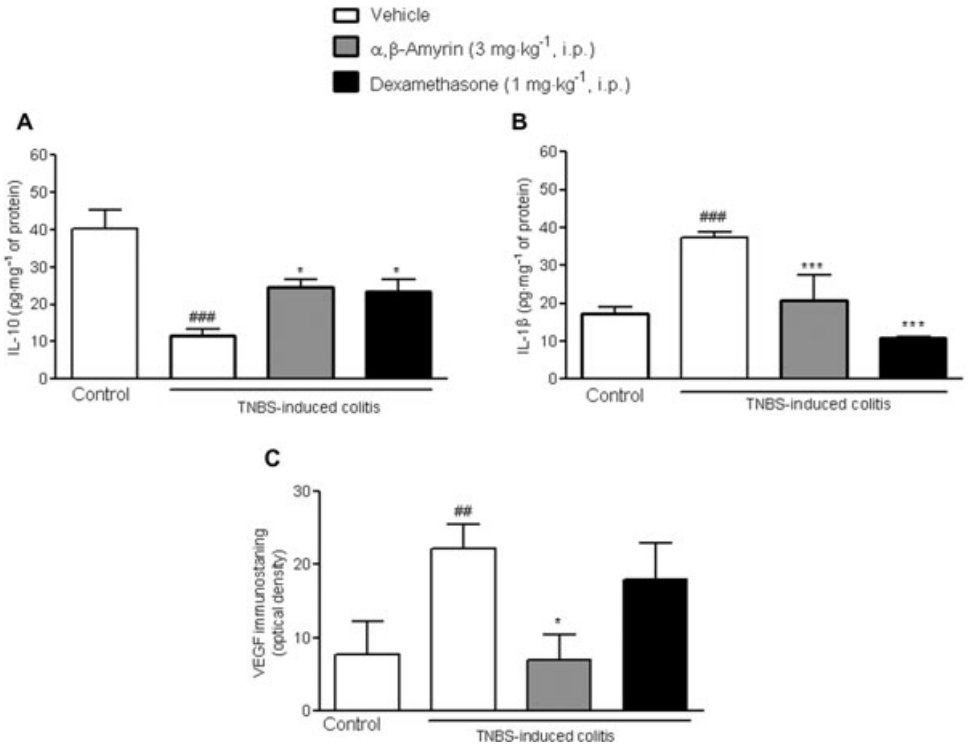

Figure 5 Effect of α,β-amyrin treatment on local synthesis of cytokines 72 h after colitis induction in mice. Both α,β-amyrin (3 mg·kg^{-1}) and dexamethasone (1 mg·kg^{-1}) treatment restored IL-10 levels (A) and reduced IL-1β levels (B) assessed in colon tissue homogenates by enzyme-linked immunosorbent assay. The VEGF expression (C) in colon sections was reduced by α,β-amyrin (3 mg·kg^{-1}) treatment, but not by dexamethasone (1 mg·kg^{-1}). Staining intensity and stained area of VEGF immunoreaction (Figure S1) are expressed as the percentage of immunostaining. Data represent mean ± SEM (n = 3–4); ##P < 0.01, ###P < 0.001, compared with the control group; *P < 0.05, ***P < 0.001 compared with vehicle-treated colitic mice. Error bar in the dexamethasone-treated group (B) is hidden (SEM = 0.2842, n = 3); one-way ANOVA followed by Student Newman-Keuls test. IL, interleukin; TNBS, trinitrobenzene sulphonic acid; VEGF, vascular endothelial growth factor.

treatment also significantly reduced the macroscopic score, by about 75%. In addition, the colonic accumulation of neutrophils, indirectly indicated by the MPO activity, was not affected by treatment with 0.3 mg·kg^{-1} α,β-amyrin treatment, but was markedly inhibited by the doses of 1 and 3 mg·kg^{-1} (Figure 2C). Treatment with dexamethasone also significantly inhibited MPO activity. Based on these results, α,β-amyrin was used at 3 mg·kg^{-1} in subsequent experiments to investigate some of the mechanisms underlying its anti-inflammatory and immunomodulatory effects.

Histological analysis of control mice revealed no sign, or only a very low level, of leucocyte infiltration into the colon, while the epithelial integrity was preserved. In contrast, 72 h after TNBS administration, the colonic wall became thick and the epithelial architecture was destroyed. Under microscopy, distortion of crypts, loss of goblet cells, submucosal oedema and infiltration of mono- and polymorphonuclear cells were observed (Figure 4A). Treatment with 3 mg·kg^{-1} of α,β-amyrin restored the histological appearance of colon sections to normal (Figure 4A) and significantly reduced the histological score of colitis by 62 ± 10%. Likewise, dexamethasone treatment was effective in inhibiting TNBS-induced colitis by 81 ± 10%.

Effect of α,β-amyrin on levels of inflammatory cytokines during colitis

To assess the effect of α,β-amyrin treatment on the balance of cellular mediators involved in the inflammatory response, colon samples were obtained at 72 h, according to protocol 1 (Figure 2A). First, we measured the tissue levels of the pro- and the anti-inflammatory cytokines, IL-1β and IL-10 respectively. Animals with TNBS-induced colitis exhibited twofold elevation of local IL-1β levels compared with saline-treated animals (Figure 5B). Those levels were markedly reduced by treatment with α,β-amyrin or dexamethasone. At the same time after TNBS, local production of IL-10 was clearly decreased, compared with the values in control tissues. Treatment of TNBS colitis with either α,β-amyrin or dexamethasone partially, but significantly (P = 0.04), raised the levels of IL-10 in colon towards the control values (Figure 5A).

We next evaluated the contribution of the cytokine VEGF, an inducer of vascular hyperpermeability, to the therapeutic responses in colitic mice. Expression of VEGF protein in the colon tissue of mice with TNBS-induced colitis was measured by immunohistochemical methods. The data in Figure 5C indicate that low VEGF levels were found in the colons from control animals and that they were increased by 3.1-fold at 72 h after TNBS. Of note, treatment with α,β-amyrin (3 mg·kg^{-1}, i.p.), but not with dexamethasone (1 mg·kg^{-1}, s.c.), significantly reduced the expression of VEGF to basal levels (Figure 5C). Cytokine levels including the VEGF expression pattern (Figure S1) were consistent with the morphological scores and MPO activity in control and colitic mice.

In order to define more clearly the cellular mechanism by which α,β-amyrin inhibited colon inflammation, we carried out immunohistochemical studies of some components of

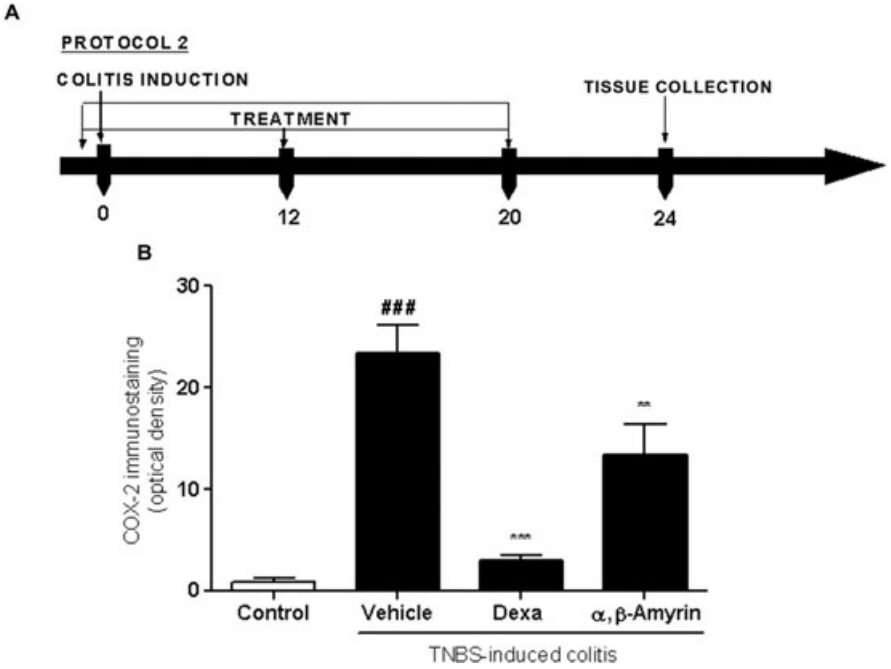

Figure 6 Effect of α,β-amyrin treatment on COX-2 expression 24 h after colitis induction in mice. (A) Animals were treated with α,β-amyrin, dexamethasone or vehicle, 0.5 h prior to and 12 and 20 h after TNBS instillation, as indicated. Tissue was collected 24 h after colitis induction. (B) COX-2 expression at 24 h, assessed by immunohistochemistry of colon sections (Figure S2), was reduced by dexamethasone (Dexa, 1 mg·kg^{-1}) and α,β-amyrin (3 mg·kg^{-1}) (A). Immunostaining intensity and area were quantified by image analysis and are expressed as arbitrary units (mean ± SEM, $n = 3$). ###$P < 0.001$, compared with the control group; **$P < 0.01$, ***$P < 0.001$, compared with vehicle-treated colitic mice; one-way ANOVA followed by Student Newman-Keuls test. COX-2, cyclooxygenase-2; CREB, cAMP response element-binding protein; TNBS, trinitrobenzene sulphonic acid.

the inflammatory response. A time–course of the expression of protein was generated for COX-2, pNF-κB and pCREB, by using immunoreactivity analysis. These analyses disclosed that COX-2 expression reached its peak 24 h after colitis induction (Figure 3A), whereas the transcription factors NF-κB and CREB presented a peak of phosphorylation at 6 h after TNBS instillation (Figure 3B,C).

Effect of preventive treatment with α,β-amyrin on COX-2 protein expression in the colon tissue of TNBS-induced colitis

High levels of pro-inflammatory PGs are produced by COX enzymes during the inflammatory response, and PGE_2 formation correlates with the level of COX-2 expression. Given that we had found a peak of local COX-2 expression at 24 h after TNBS (Figure 3A), a separate set of experiments was performed by using the protocol 2 (Figure 6A). At 24 h after TNBS, there was a marked increase of COX-2 expression (about 50-fold by immunohistochemistry), compared with control animals (Figure 6B). Systemic pretreatment with α,β-amyrin significantly inhibited the increased COX-2 expression by 41% ($P = 0.01$; $n = 5$), while treatment with dexamethasone completely inhibited this increase. The immunohistochemical localization of COX-2 in colon sections was in the cytoplasm and was predominantly found in epithelial cells (Figure S2).

Effect of preventive treatment with α,β-amyrin on NF-κB and CREB in the colon tissue of mice with TNBS-induced colitis

To further define some signalling systems activated by inflammatory stimuli that could mediate the effect of α,β-amyrin, we studied the transcriptional factors NF-κB and CREB, which are able to modulate the expression of several inflammatory genes. As, in the colon tissue, the immunoreactivity for both phosphorylated factors peaked at 6 h in the development of TNBS-induced colitis (Figure 3B,C), another set of experiments was performed by using the protocol 3 (Figure 7A), to assess the phosphorylation state of NF-κB (p65) and CREB, using specific antibodies. TNBS-induced colitis, as expected, induced pronounced phosphorylation of NF-κB and CREB (Figure 7B,C) and their subsequent translocation to the nucleus of *lamina propria* and inflammatory cells (Figures S3 and 4). Interestingly, systemic pretreatment with α,β-amyrin (3 mg·kg^{-1}) significantly abolished both NF-κB and CREB activation (Figure 7B,C). Systemic treatment with dexamethasone (1 mg·kg^{-1}) had comparable effects, reducing the expression of the pNF-κB and that of pCREB to almost control levels.

Discussion

The use of medicinal plants or their active components is becoming an increasingly attractive approach for the treatment of various inflammatory disorders. A large number of plants and their isolated constituents have shown beneficial therapeutic properties including anti-inflammatory and immunomodulatory effects (Calixto *et al*., 2003; 2004). The results of this study have demonstrated, for the first time, the effectiveness of the plant triterpenes, α- and β-amyrin in ameliorating TNBS-induced colitis in mice, as shown by

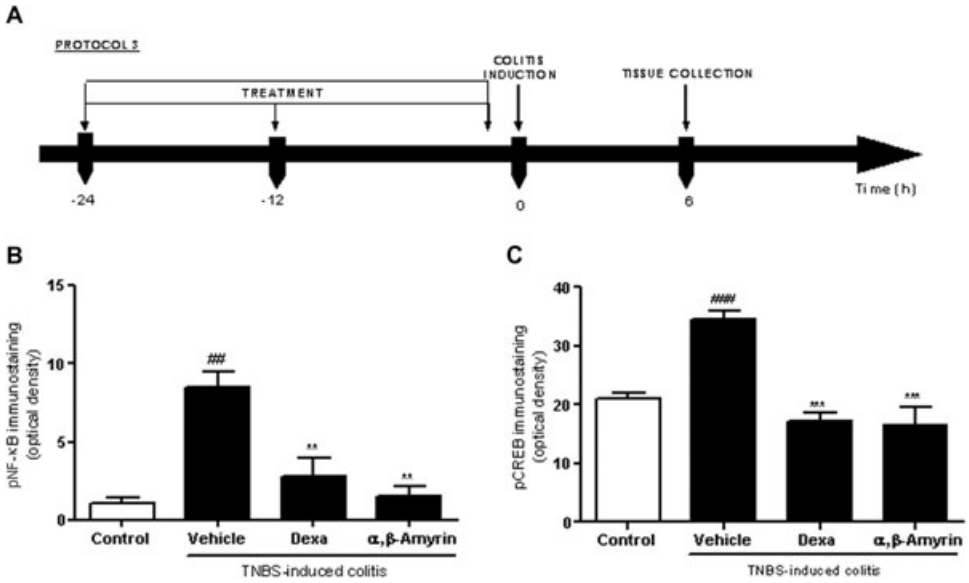

Figure 7 Effect of α,β-amyrin treatment on transcription factors 6 h after colitis induction in mice. (A) Animals were treated with α,β-amyrin, dexamethasone or vehicle (24, 12 and 0.5 h), prior to TNBS instillation. Then, colitis was induced and after 6 h, tissues were collected. Colonic expression levels of pNF-κB (p65) (B) and pCREB (C) were assessed after 1 day of treatment and at 6 h of colitis development. The immunoreactivity of pNF-κB (p65) and pCREB on colon sections (Figures S3 and 4 respectively) was reduced by both α,β-Amyrin (3 mg·kg^{-1}) and dexamethasone (Dexa, 1 mg·kg^{-1}). Immunostaining intensity and area were quantified by image analysis and are expressed as arbitrary units (mean ± SEM, $n = 3$). ##$P < 0.01$, ###$P < 0.001$, compared with the control group; **$P < 0.01$, ***$P < 0.001$, compared with vehicle-treated colitic mice; one-way ANOVA followed by Student Newman-Keuls test. CREB, cAMP response element-binding protein; TNBS, trinitrobenzene sulphonic acid; NF-κB, nuclear factor-κB.

macroscopic and histological examinations. The anti-inflammatory effects reported herein for the mixture of α,β-amyrin were accompanied by modulation of inflammatory mechanisms. These results confirm and largely extend the earlier reported anti-inflammatory effects of this natural product in other models of inflammatory diseases (Kweifio-Okai *et al.*, 1994; Recio *et al.*, 1995; Otuki *et al.*, 2005b; Medeiros *et al.*, 2007).

To gain further insights into the mechanisms through which α,β-amyrin exerted its beneficial effect in TNBS-induced colitis, we assessed (by means of histological, biochemical and immunohistochemical procedures) the colon levels of IL-1β and IL-10, as well as the expression of VEGF, COX-2 and the phosphorylated forms of NF-κB and CREB. Our data consistently show that, like dexamethasone, α,β-amyrin given intraperitoneally (3 mg·kg^{-1}), in the same range of doses previously shown to exert anti-inflammatory effects (Otuki *et al.*, 2005a), almost totally suppressed the influx of polymorphonuclear cells to the colon following TNBS injection, as assessed by the MPO activity. Accordingly, at the same dose and scheme of treatment, α,β-amyrin, similarly to dexamethasone, significantly inhibited the increase of the pro-inflammatory cytokine IL-1β in the colon after TNBS. Such data are relevant to potential use in human disease, as there are several pieces of experimental evidence indicating that IL-1β release is critically involved in IBD (Sartor, 2006). Also of interest were the data showing significant recovery of the anti-inflammatory cytokine IL-10 observed after treatment with either α,β-amyrin or with dexamethasone in mice with TNBS-induced colitis. A growing amount of evidence indicates that IL-10 plays a relevant role in the development of IBD, as knockout animals for IL-10 exhibited spontaneous colitis (Davidson *et al.*, 2000; McCafferty *et al.*, 2000).

Apart from the imbalance in the immune system, there are now indications that local microvasculature and inflammation-dependent angiogenesis exert a relevant role in both human and murine IBD (Danese *et al.*, 2006; Sandor *et al.*, 2006; Tsiolakidou *et al.*, 2008). To further evaluate the mechanisms of the anti-inflammatory action of α,β-amyrin on TNBS-induced colitis, we assessed through immunohistochemistry, the protein expression of the angiogenic cytokine VEGF. These experiments revealed that systemic treatment with α,β-amyrin, but not with dexamethasone, significantly and completely reversed the high tissue content of VEGF in colitis samples to control levels. Therefore, inhibition of VEGF synthesis or the prevention of injury-induced VEGF expression might constitute an additional mechanism, through which α-β-amyrin exerts its beneficial anti-inflammatory action against TNBS-induced colitis, in contrast to those involved in response to glucocorticoid treatment. In order to understand the significance of the difference between α,β-amyrin and dexamethasone on VEGF expression, further studies are required to elucidate which inducers of angiogenesis might be involved in the disruption of intestinal immune homeostasis and the VEGF-related pathogenic cascade.

The enzymes COX-1 and COX-2 are responsible for the transformation of arachidonic acid into PGs and have an important function in the inflammatory processes. In contrast to the constitutive form COX-1, COX-2 is generally, but not exclusively, induced in response to stimulators such as growth factors, cytokines and tissue injury (Sakamoto, 1998). Colitis induced by TNBS in rats leads to macroscopic damage

that was related to PGE_2 synthesis, COX-2 protein expression and leucocyte infiltration in the stroma of inflammatory colon, indicating that arachidonic acid metabolites derived from COX-2 exerted a critical role in this model of IBD (Martin *et al*., 2003). The results of the present study clearly show that the inhibition of the COX-2 protein expression in the mouse colon after administration of α,β-amyrin is indeed associated with its ability to prevent the inflammatory pattern of cytokines observed in colitic mice. Moreover, our data also show that rectal TNBS administration caused a time-dependent up-regulation of COX-2 protein expression and that systemic treatment with α,β-amyrin ameliorated TNBS-induced colitis at 24 h. These observations could explain, at least in part, the relatively strong and rapid onset of the systemically anti-inflammatory property reported for these plant triterpenes (Recio *et al*., 1995; Otuki *et al*., 2005a,b).

Activation of the NF-κB transcription factor process by inflammatory cytokines and intestinal microorganisms induces the phosphorylation and consequent degradation of IκB by its kinase that allows NF-κB translocation into the cell nucleus to activate gene expression for relevant inflammatory proteins (Ghosh and Karin, 2002). The activated form of this transcription factor has been detected in mononuclear and epithelial cells of inflamed colon (Rogler *et al*., 1998). In addition, selective down-regulation of the p65 subunit of NF-κB by specific antisense oligonucleotides prevented experimental colitis (Neurath and Pettersson, 1997). We have previously demonstrated that α,β-amyrin inhibits NF-κB activation and p65 translocation in a mouse model of skin inflammation (Medeiros *et al*., 2007). Our results using the phospho-p65 NF-κB antibody in the TNBS-induced colitis model confirm and extend the view that α,β-amyrin is able to inhibit the translocation of p65 into the nucleus, thus strongly suggesting that inhibition of NF-κB activation is a key mechanism through which these natural pentacyclic triterpenes modulate intestinal inflammation.

The transcription factor CREB is known to regulate a wide variety of genes by binding cAMP response elements to promoter regions, including that for the COX-2 gene (Schroer *et al*., 2002). In intestinal epithelial cells, the phosphorylation of CREB at Ser^{133} was induced by COX-2 expression, as well growth factors and other inflammatory mediators (Zhao, 2007; Pham *et al*., 2008). We found that TNBS administration markedly increased expression of nuclear phospho-CREB, while systemic treatment with α,β-amyrin, like dexamethasone, significantly modulated intracellular cAMP signalling and reversed such events. Conversely, the down-regulation of CREB activity, in later stages of colitis (Figure 3), could possibly induce a negative feedback of the inflammatory process caused by TNBS, which in turn results in an anti-inflammatory effect. However, additional studies are necessary to confirm these hypotheses. Considering the wide range of CREB effects in inflammatory processes, we postulate that rectal TNBS administration promotes a CREB-mediated up-regulation of inflammatory proteins, including COX-2, as previously discussed. Taken together, our results point to the anti-inflammatory effect of α,β-amyrin involving the inhibition of the activation of both CREB and NF-κB signalling at 6 h, then modulating the subsequent steps of protein synthesis that produce inflammatory mediators.

In this study we have shown the systemic preventive or therapeutic anti-inflammatory action of the pentacyclic triterpenes α- and β-amyrin in TNBS-induced colitis in mice. Our findings show that α,β-amyrin is as efficacious as dexamethasone in reversing the macroscopic and microscopic outcomes of TNBS-induced colitis, including the restoration of cytokine balance. Furthermore, the results also indicate that inhibition of NF-κB and CREB activation is certainly the main mechanism through which these triterpenes exert their anti-inflammatory action, along with a reduction of COX-2 expression. Finally, only systemic treatment with α,β-amyrin consistently reduced VEGF expression, an effect that could account for a decreased angiogenesis-induced inflammatory response on colon tissues. Moreover, our study suggested that oral administration of α,β-amyrin up to 300 $mg \cdot kg^{-1}$ did not cause any apparent acute toxicity, and collectively the present results suggest that α,β-amyrin might constitute a potential and relevant alternative for the treatment of IBD.

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Conflict of interest

None.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Representative immunohistochemical localization of vascular endothelial growth factor (VEGF) in distal colon tissue sections as indicated. Immunohistochemistry was performed by using the monoclonal antibody anti-VEGF (C-1) and peroxidase (HRP) polymer secondary antibody and diaminobenzidine as chromogen. Brown colour (arrow) indi-

cates area of positive cytoplasmic staining, mainly observed in colitic mice [trinitrobenzene sulphonic acid (TNBS) + vehicle]. There is a similar pattern in sections of normal colon (control + vehicle) and α,β-amyrin-treated colitic mice. Magnification, ×400.

Figure S2 Representative immunohistochemical localization of cyclooxygenase-2 (COX-2) in distal colon tissue sections of the four indicated groups. Immunohistochemistry was performed by using the monoclonal antibody anti-COX-2 and alkaline phosphatase (AP) polymer secondary antibody and permanent red chromogen. Red colour indicates area of COX-2-positive cytoplasmic staining, predominantly in epithelial cells, which has a similar pattern in sections of normal colon (control + vehicle), dexamethasone and α,β-amyrin-treated colitic mice. Magnification, ×800.

Figure S3 Panel shows representative sections of immunostained distal colon tissues for nuclear phospho-p65 nuclear factor-κB (NF-κB) (brown). Immunohistochemistry was performed using the monoclonal antibody anti-phospho-p65 NF-κB and peroxidase (HRP) polymer secondary antibody and diaminobenzidine chromogen. Systemic pretreatment with α,β-amyrin or dexamethasone abolished phospo-p65 NF-κB activation. Magnification, ×400.

Figure S4 Panel shows representative sections of distal colon tissues for nuclear phospho-cyclic AMP response element-binding protein (CREB) staining (brown). Immunohistochemistry was performed by using the monoclonal antibody anti-phospho-CREB and peroxidase (HRP) polymer secondary antibody and diaminobenzidine chromogen. Systemic pretreatment with α,β-amyrin or dexamethasone abolished CREB activation observed in epithelial cells and the submucosal infiltrate. Magnification, ×400.