European Journal of Pharmacology 587 (2008) 296–301

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Anti-inflammatory effects of a bioavailable compound, Artepillin C, in Brazilian propolis

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ARTICLE INFO

Article history:
Received 2 August 2006
Received in revised form 15 February 2008
Accepted 20 February 2008
Available online 29 February 2008

Keywords:
Artepillin C
Propolis
Inflammation
NF-κB
Prostaglandin E_2
Nitric oxide

ABSTRACT

Artepillin C is the major compound in the Brazilian green propolis from *Baccharis dracunculifolia*. Our aim in this study was to investigate the anti-inflammatory effects, absorption, and bioavailability of Artepillin C in mice. The animals used were male Swiss mice subjected to: paw oedema by carrageenan (300 μg/paw), carrageenan-induced peritonitis, and prostaglandin E_2 determination. We also measured *in vitro* nitric oxide production by RAW 264.7 cells and NF-κB activity in HEK 293 cells. Finally, we measured the absorption and bioavailability of Artepillin C in plasma from mice by means of GC-MS after a single oral dose (10 mg/kg). *In vivo*, Artepillin C produced a maximal inhibition of 38% after 360 min on paw oedema. Artepillin C also decreased the number of neutrophils during peritonitis (IC_{50}: 0.9 (0.5–1.4) mg/kg). Treatment with Artepillin C decreased prostaglandin E_2 by 29±3% and 58±5% at 1 and 10 mg/kg, respectively, with a mean ID_{50} of 8.5 (8.0–8.7)mg/kg). Similarly, in *in vitro* models, Artepillin C (3, 10, or 100 μM) decreased nitric oxide production by RAW 264.7 cells with a mean IC_{50} of 8.5 (7.8–9.2) μM. In HEK 293 cells, Artepillin C reduced NF-κB activity with a mean IC_{50} of 26 (22–30) μg/ml), suggesting anti-inflammatory activity, particularly during acute inflammation. Lastly, Artepillin C was absorbed after an oral dose (10 mg/kg) with maximal peaks found at 1 h (22 μg/ml). Collectively, Artepillin C showed anti-inflammatory effects mediated, at least in part, by prostaglandin E_2 and nitric oxide inhibition through NF-κB modulation, and exhibited bioavailability by oral administration.

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1. Introduction

Artepillin C [3-{4-hydroxy-3,5-di(3-methyl-2-butenyl)phenyl}-2(*E*)-propenoic acid] is a low-molecular weight phenolic compound isolated from Brazilian propolis. Bees collect exudates from *Baccharis dracunculifolia* in order to produce green propolis, which contains a large concentration of this compound (Marcucci et al., 2001; Park et al., 2004). We have previously shown that Brazilian propolis contains high levels of the phenolic compounds Artepillin C (Fig. 1) and the derivatives 2,2-dimethyl-6-carboxyethenyl-8-prenyl-2*H*-1-benzopyran, 3-prenyl-4-hydroxycinnamic, *p*-coumaric, caffeic acid, and caffeoylquinic acids in addition to cinnamic acids and the flavonoids pinobanksin and kaempferol (Marcucci et al., 2000). The flavonoid content corresponds to 22.37 mg/g of dried extract. Artepillin C possesses anti-microbial (Salomão et al., 2004), anti-tumor (Kimoto et al., 1996, 2000, 2001a,b; Shimizu et al., 2005; Orsolic et al., 2006), apoptosis-inductor (Matsuno et al., 1997), immunomodulator (Gekker et al., 2005; Kimoto et al., 1998), and anti-oxidant properties (Hayashi et al., 1999; Nakanishi et al., 2003; Simões et al., 2004).

Uto et al. (2002) reported the first total synthesis of Artepillin C through *o,o′*-diprenylation of *p*-halophenols in water. This process includes the synthesis of other important compounds with significant pharmacological effects (Uto et al., 2002; Shimizu et al., 2004; Uto et al., in press). A synthetic intermediate, 2,6-diprenyl-4-halophenol, with potent anti-oxidant effects can be produced in this manner (Pan and Hori, 1994; Uto et al., 2002).

It has been suggested that Artepillin C is easily incorporated albeit difficult to conjugate within cells (Kasai, 2002). Shimizu et al. (2004) described that prenyl groups introduced into a molecule increase the affinity for cell membranes *in vitro*. Furthermore, they showed that Artepillin C modified with prenyl groups had high affinity for lipid

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doi:10.1016/j.ejphar.2008.02.067

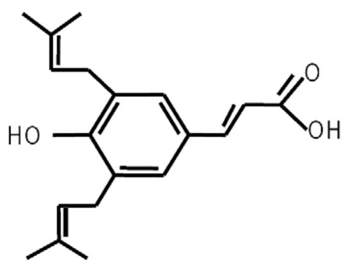

Fig. 1. Chemical structure of Artepillin C (3,5-diprenyl-4-hydroxycinnamic acid).

membranes and was able to insert partially into cells. This raised the prospect of using Artepillin C for the treatment of pain and inflammatory diseases.

The purpose of this study was to evaluate the anti-inflammatory effects of Artepillin C in animal models. In addition, the absorption and bioavailability of Artepillin C was determined.

2. Materials and methods

2.1. Preparation and chemical characterization of Artepillin C

Synthetic Artepillin C was supplied by the Department of Biological Sciences & Technology, Faculty of Engineering, The University of Tokushima, Tokushima, Japan. Uto et al. (2002) were the first to report total synthesis of Artepillin C by *o*,*o′*-diprenylation of *p*-halophenols in water.

2.2. Pharmacological assays

2.2.1. Animals

Adult male Swiss mice (18–30 g) were utilized in the experiments. The animals were maintained in an environment under controlled temperature (21±2 °C) under daylight supplemented with electric light from 6:00 a.m. to 6:00 p.m., with access to food and water *ad libidum*. Throughout the experiments, animals were handled using the principles and guidelines for the care of laboratory animals according to Zimmermann (1983) and following the guidelines of an Institutional Review Board.

2.2.2. Measurement of paw oedema

Measurement of paw oedema was performed according to Calixto et al. (1991) with slight modifications. Under slight anesthesia with 2.2.2 tribromoethanol (0.12 g/kg), male Swiss mice received a 20 µl i.d. injection in one hindpaw of phosphate buffered saline (composition mmol/l: sodium chloride 137, potassium chloride 2.7, and phosphate buffer 10) containing carrageenan (300 µg/paw). The contralateral paw received 20 µl of saline and was used as the control. Oedema was measured by the use of a plethysmometer (Ugo Basile) at several times after injection of the irritant. Oedema is expressed in ml as the difference between the test and control paws. Animals were treated with Artepillin C (1 or 10 mg/kg, i.p.) 30 min before the injection of irritant. Control groups received the same volume of the vehicle (sterile saline solution).

2.2.3. Acute carrageenan-induced inflammatory reaction in the peritoneal cavity of mice

Animals were treated with Artepillin C (0.1, 1 or 10 mg/kg, i.p) or indomethacin (1 mg/kg, i.p.) 30 min before induction of inflammation by means of carrageenan (100 µg/ml, i.p.) (Sigma-Aldrich, São Paulo, Brazil).

After 4 h, the peritoneal fluid was collected in sterile and heparinized PBS (2 ml) for quantification of the cell numbers using a Neubauer chamber and the differential counting of leukocytes after concentration of the exudate and staining with May-Grünwald-Giemsa. Myeloperoxidase (MPO) activity was quantified by the H_2O_2-dependent oxidation of TMB by addition of 25 µl peritoneal exudate, 25 µl 1.6 mM TMB, and 100 µl 0.3 mM H_2O_2 in 96-well plates. The optical density was measured at 450 nm and the enzyme activity calculated by comparison with a standard curve. Cell migration was quantified as previously described by Carvalho et al. (1999).

2.2.4. Prostaglandin E_2 determination in peritoneal inflammation in mice

Animals were treated with Artepillin C (0.1, 1, or 10 mg/kg, i.p) or indomethacin (1 mg/kg, i.p.) 30 min before induction of inflammation by means of carrageenan (100 µg/ml, i.p.) (Sigma-Aldrich, São Paulo, Brazil).

After 4 h, the peritoneal fluid was collected in sterile and heparinized phosphate buffered saline (2 ml), then the amount of PGE_2 in the supernatant was measured by radioimmunoassay.

2.2.5. Nitric oxide and cell viability quantification

Raw 264.7 cells obtained from the American Type Culture Collection (ATCC, Maryland, USA) were cultured in Dulbecco's Modified Essential Medium with 4 mM L-glutamine and 4.5 g/l glucose (DMEM, endotoxin level < 0.005 EU/ml, Bio Whittaker, Bioproducts, Heidelberg, Germany) supplemented with 10% heat-inactivated fetal calf serum (Gibco/BRL Life Technologies, Eggenstein, Germany). Cells were maintained at 37 °C, 5% CO_2 and used for experiments between passage 4 and 12. Confluent cells were stimulated with 1 µg/ml lipopolysaccharide (LPS) (*E. coli*, Serotype 055:B5 Sigma, Deisenhofen, Germany). Isoprenomics were dissolved in DMEM at the time the experiments were carried out.

RAW 264.7 cells were seeded in 96-well plates (4×10^4 cells/well), and after 2 days were stimulated or not with 1 µg/ml LPS in the presence or absence of Artepillin C (1, 3, 10, or 100 µg/ml) for 20 h. The generation of nitric oxide was assessed in the supernatant of cell cultures by quantification of nitrite using the Griess reaction (Green et al., 1984). Briefly, 100 µl of each supernatant was added to wells of 96-well plates, each containing 90 µl 1% sulfanilamide in 5% phosphoric acid plus 90 µl 0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride in water. The optical density was measured at 550 nm. Cell viability was determined by mitochondrial-dependent reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to formazan quantified at 550 nm. After removal of supernatant for nitrite determination, the cells were incubated with 0.5 mg/ml MTT at 37 °C for 45 min, the medium was aspirated, and 250 µl of dimethyl sulfoxide was added to the reaction mixture for 3 h in the absence of light for solubilization of formazan.

2.2.6. NF-κB transactivation activity

HEK 293 cells (human embryonic kidney lineage, ACC 305, DSMZ-German Collection of Microorganisms and Cell Cultures) were maintained in DMEM supplemented with 10% fetal bovine serum (Biochrom KG, Germany), 2 mM L-glutamine (Merck, Germany), 100 U/ml penicillin, and 100 µg/ml streptomycin (PAN, Biotech, Germany). Cells were split 1:10 at 85–90% confluence using 0.05% trypsin plus 0.02% EDTA in PBS. The cells (5×10^5 cells) were seeded in Petri dishes (diameter = 6 cm) and after 24 h they were transfected with pNF-κBluc and pRSV-β-gal plasmid using the Ca^{2+}-phosphate method (Dirsch et al., 2004). The cells were plated in 24-well plates (1×10^5 cells/well) and after incubation for 16 h, submitted to treatment for 2 h with 3, 10, or 30 µg/ml of Artepillin C or MG132 (10 µM), a specific inhibitor of nuclear factor κB (NF-κB). Subsequently, the cells were stimulated for 6 h with 1 ng/ml TNF-α, washed with PBS, and lysed with passive lysis buffer. The NF-κB activity was measured by the luciferase assay system according to the manufacturer's instructions (Promega, Heidelberg, Germany) using an AutoLumat Plus luminometer (Berthold, Bad Wildbad, Germany).

2.2.7. Mass spectrometry

The plasma of animals treated with Artepillin C (10 mg/kg) was analysed using a chromatograph column DB-5ms (30 m×0.25 mm ID×0.25 FT) from Agilent. Gas chromatography employed a Focus GC model from Thermo-Finnigan. Mass spectrometry was performed by means of a Focus DSQ model from Thermo-Finnigan. The entrance initial capillary temperature was 50 °C without isotherm and with a discharge of 10 °C/min to 320 °C. The source temperature of the injector was 290 °C and the temperature of mass spectrometry was 230 °C.

2.3. Drugs and reagents

Formic acid, methanol, indomethacin, formalin, 2,2,2 tribromoethanol, prostaglandin E_2, lambda carrageenan grade IV, phosphate buffer solution (pH 7.6, composition mM: NaCl 137.0, KCl 2.0, and phosphate buffered salts 10.0), saline, TMB solution, L-glutamine, glucose, LPS, FBS, penicillin, streptomycin, NEAA, insulin, sodium pyruvate, HEPES, sulfanilamide, H_3PO_4, *N*-(1-naphthyl) ethylenediamine dihydrochloride, MTT, DMSO, TRAIL, and lactacystin were acquired from Sigma Chemical Co., St Louis, MO, USA.

2.4. Statistical analysis

For the *in vivo* studies, the results are presented as mean±standard error, except for the ID_{50} values (i.e. the doses of extract necessary to reduce the response by 50% relative to the control value), which are expressed as mean±standard deviation. The differences between the experimental groups were evaluated using analysis of variance followed by Dunnett's multiple comparison test or by Student's *t*-test. When appropriate, the ID_{50} values were estimated from individual experiments by the use of the least squares method. For *in vitro* studies, the responses are expressed as the statistical difference between control and treatment groups. *P* values less than 0.05 were considered significant.

3. Results

3.1. Effect of Artepillin C on inflammatory models in vivo

In order to investigate the anti-inflammatory effects of Artepillin C *in vivo*, we employed animal models. Animals were initially treated with Artepillin C followed by carrageenan-induced paw oedema measured at several time points (30 min to 360 min). In this model, 1 or 10 mg/kg Artepillin C (i.p.) significantly inhibited oedema induced by carrageenan, with a maximal average inhibition of 38% after 360 min (Fig. 2).

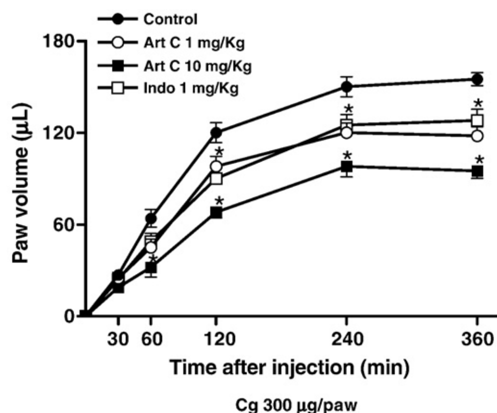

Fig. 2. Effects of Artepillin C (1 or 10 mg/kg) or indomethacin (1 mg/kg) on paw oedema models induced by carrageenan. Each value represents the mean±S.E.M. of 6 animals, and asterisks indicate significant inhibition of the paw weight in relation to the corresponding untreated groups, $P<0.05$.

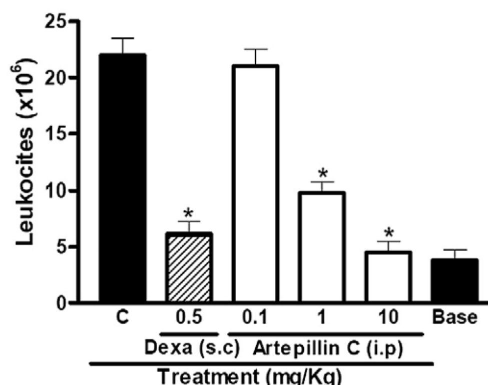

Fig. 3. Effects of the administration of Artepillin C (0.1, 1, or 10 mg/kg, i.p.) or dexamethasone (0.5 mg/kg, s.c.) on acute carrageenan-induced inflammatory reactions, measured by the concentration of cells in the peritoneal fluid (peritonitis). Each value represents the mean±S.E.M. of 6 animals, and asterisks indicate significant inhibition of the total number of cells in the peritoneal cavity in relation to the untreated groups, $P<0.05$.

To further evaluate anti-inflammatory effects suggested by the paw oedema experiment, we tested Artepillin C in a carrageenan-induced model of peritonitis. The basal concentration of cells in the peritoneal cavity was 0.6×10^6 cells/ml but 4 h after carrageenan administration, this concentration increased to 13.9×10^6 cells/ml, which we defined as 100% cell migration. The percentage of neutrophils (84±2%) in the peritoneal exudate was higher than that of mononuclear cells (16±3%), an expected pattern in carrageenan-induced peritonitis (data not shown). When the animals were treated with Artepillin C (0.1, 1, or 10 mg/kg i.p) or dexamethasone (0.5 mg/kg, s.c.), a statistically significant decrease in the total number of cells in the peritoneal cavity was observed, demonstrating inhibition of the inflammatory process (Fig. 3). The decrease in the number of neutrophils was 55±4% and 80±5% after 1 and 10 mg/kg Artepillin C, respectively, with an estimated IC_{50}=0.9 (0.5–1.4) mg/kg. Dexamethasone at 0.5 mg/kg caused a decrease in neutrophil migration by 72±6%. By both routes of administration, Artepillin C and dexamethasone led to statistically significant decreases in the number of neutrophils, which was proportional to decreases in myeloperoxidase activity (data not shown).

Prostaglandins are involved in acute inflammation and can modulate oedema and cell migration. To investigate the role of prostaglandin E_2 on the anti-inflammatory effects of Artepillin C in mice, we measured the concentration of prostaglandin E_2 in the peritoneal exudate after the induction of inflammation. In these experiments, treatment with

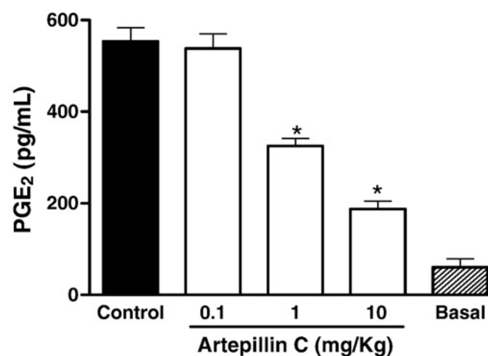

Fig. 4. Effects of treatment with Artepillin C (0.1, 1, or 10 mg/kg) on Prostaglandin E2 in peritoneal exudates of mice. Bars represent the mean±S.E.M. of three independent experiments performed in triplicate and asterisks indicate significant inhibition of enzyme activity in relation to the untreated group, $P<0.05$.

Artepillin C (1 and 10 mg/kg, i.p.) decreased prostaglandin E_2 production in the peritoneal exudate by 29±3% and 58±5%, respectively. The mean ID_{50} was 8.5 (8.0–8.7) mg/kg (Fig. 4).

3.2. Effect of Artepillin C on inflammatory models in vitro

To measure the influence of Artepillin C on the reactivity of inflammatory cells, we used the macrophage cell line RAW 264.7. In this *in vitro* model, treatment with Artepillin C (3, 10, or 100 μM) led to a decrease in nitrite concentration in the supernatant of RAW 264.7 macrophages with IC_{50} = 8.5 (7.8–9.2) μM, while in LPS-stimulated cells, the nitrite concentration was 57.5±5.9 μM (Fig. 5A). For concentrations up to 100 μM, Artepillin C did not interfere with the viability of RAW 264.7 cells, as shown by the MTT test for determining cell viability (Fig. 5B). Therefore, we conclude that the effect of Artepillin C on nitrite production was specific to responses by macrophage.

In another set of experiments, we tested the effect of Artepillin C on Nuclear Factor κ B (NF-κB) activity in TNF-α-stimulated HEK 293 cells transfected with a NF-κB-driven luciferase reporter gene. NF-κB is an important proteic target in the development of new strategies to treat inflammatory diseases. In our experiments, we observed that treatment with Artepillin C reduced luciferase activity with an IC_{50} = 26 (22–30) μg/ml (Fig. 6). Artepillin C did not affect the viability of HK 293 cells in culture at all concentrations tested (data not shown).

3.3. Evaluation of the plasma concentration of Artepillin C in mice

Finally, we measured how much Artepillin C was bioavailable in the blood of mice at several time points after administration. Mice received a single dose of Artepillin C, 10 mg/kg, and at several times we measured the chemical presence of Artepillin C. Our results indicated that absorption and bioavailability of Artepillin C was enough to produce biological effects. We observed that Artepillin C can be absorbed after oral administration and that the maximal peak occurred after 1 h with a mean plasma concentration of 22 μg/ml (Fig. 7).

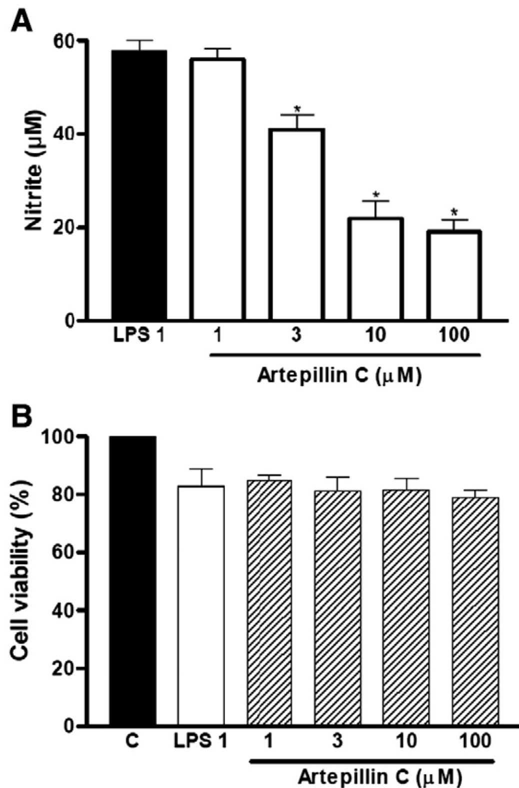

Fig. 5. Effects of treatment with Artepillin C (1, 3, 10, or 100 μM) on nitrite production by RAW 264.7 cells stimulated with 1 μg/ml LPS for 20 h (A) or on cell viability by MTT assay (B). Bars represent the mean±S.E.M. of three independent experiments performed in triplicate and asterisks indicate significant inhibition of enzyme activity in relation to the untreated group, $P<0.05$.

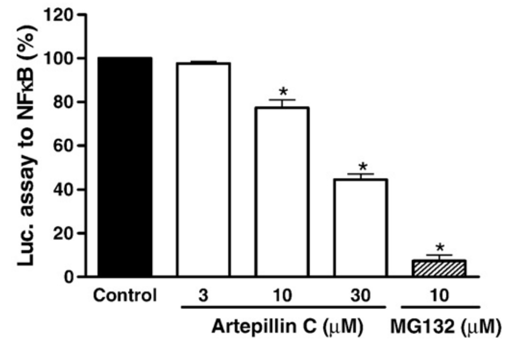

Fig. 6. Effects of treatment with Artepillin C (3, 10, or 30 μM) or MG 132 (10 μM) on luciferase activity in HEK 293 cells transiently transfected with a NF-κB-driven luciferase reporter gene. Bars represent the mean±S.E.M. of three independent experiments performed in triplicate and asterisks indicate significant inhibition of enzyme activity in relation to the untreated group, $P<0.05$.

4. Discussion

Brazilian propolis from southern Brazil has been previously evaluated by means of HPLC, where several chemical constituents were identified which possess biological activity. These include 3-prenyl-4-hydroxycinnamic acid, 2,2-dimethyl-6-carboxyethenyl-2*H*-1-benzopyrane, Artepillin C, and 2,2-dimethyl-6-carboxyethenyl-8-prenyl-2*H*-1-benzopyran (Marcucci et al., 2001; Ying et al., 2007). This profile is characteristic of propolis samples collected in southern Brazil, especially in Santa Catarina State. The botanical source of these propolis is a mixture of *B. dracunculifolia*, *Araucaria angustifolia*, and *Eucalyptus citriodora* (Bankova et al., 1999; Park et al., 2004).

Artepillin C has been identified as a major component of Brazilian propolis in the south and southeastern regions, and it exhibits several biological effects, including anti-oxidant (Shimizu et al., 2004, Nakanishi et al., 2003) and anti-tumor properties (Shimizu et al., 2005, Akao et al., 2003, Sugimoto et al., 2003, Ahn et al., 2007). Recently, Pisco et al. (2006) synthesized compounds with anti-proliferative activity as analogues of prenylated natural products present in Brazilian propolis, uncovering a new class of anti-tumor compounds.

During tissue inflammation, there is normally vasodilation and recruitment of capillaries and at least transient increases in capillary

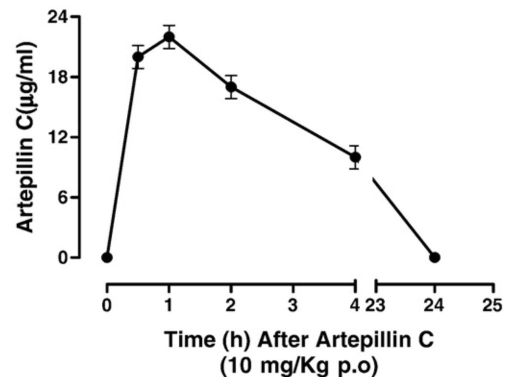

Fig. 7. Time course of oral absorption of Artepillin C after administration of 10 mg/kg. The results represent peaks of plasma concentrations at 30 min, 1, 2, 4, or 24 h after administration. Each point represents the average of triplicate experiments.

permeability. This leads to the extravasation of plasma proteins and to tissue oedema. The application of proinflammatory substances may also change the interstitial structure, contributing to this oedema (Carlsson and Rippe, 1999). We have shown in this study that treatment with Artepillin C can decrease paw oedema in mice, suggesting an important effect on plasma extravasation.

During peritonitis, there is an increase in the transport of small and large solutes between plasma and the dialysate (Krediet et al., 1987; Morris, 1953) and production of a parallel cell migration. These changes may be explained by the vasodilation of capillaries in the peritoneal membrane and the acute opening of large pores in the microvessels caused by cells and inflammatory mediators, such as neutrophils and prostaglandin E_2, respectively (Woo et al., 1986).

The experiments with carrageenan clearly showed that Artepillin C inhibits neutrophil mobilization to the peritoneal cavity. The decrease in MPO activity corroborates this inhibition in Artepillin C-treated mice, since this enzyme is absent from other types of leukocytes (Dallegri and Ottonello, 1997). This observation is also supported by Tan-No et al. (2006) who showed that the anti-inflammatory activity of Chinese propolis is a result of inhibiting nitric oxide production in carrageenan-induced mouse paw oedema (Tan-No et al., 2006).

In addition, we have shown that treatment with Artepillin C can inhibit prostaglandin E_2 production during peritoneal inflammation. This effect can, at least in part, explain the anti-oedematogenic and anti-inflammatory effects of Artepillin C on paw oedema and peritonitis, respectively, induced by carrageenan in mice.

In vitro experiments demonstrated that Artepillin C decreased the nitric oxide level in the supernatant of RAW 264.7 macrophages and also inhibited NF-κB activity in TNF-α-stimulated HEK 293 transfected cells. We can hypothesize that Artepillin C in RAW 264.7 cells inhibits the inducible NOS (iNOS) expression, the enzyme activity and the binding activity of NF-κB, and that transfected cells inhibit iNOS promoter activity through its NF-κB sites, similar to what has been reported for Chinese propolis (Song et al., 2002). NF-κB is involved in the control of inflammatory responses by interfering with the production of iNOS and the inducible isoform of cyclooxygenase COX-2 (Paulino et al., 2003; Calixto et al., 2004). Phenolic compounds found at high concentrations in Brazilian propolis, including Artepillin C, exhibit a broad range of biological properties including the ability to act as an anti-oxidant by scavenging free radicals such as nitric oxide radicals, and also the capacity to interfere with inflammatory processes by inhibiting iNOS and COX-2 activities (Olszanecki et al., 2002; Rosenkranz and Thampatty, 2003; Banerjee et al., 2002).

Despite the existence of several pharmacological studies with Artepillin C, the present work demonstrates that this propolis component at low concentrations induces analgesic and anti-inflammatory effects in mouse models. These observations, together with the *in vitro* results, reinforce the hypothesis that the analgesic and anti-inflammatory action of Artepillin C may be due to inhibition of iNOS gene expression through interference with NF-κB sites in the iNOS promoter, and through the inhibition of prostaglandin E_2 production during the induction of pain and inflammation. Further experiments are now in progress to investigate the effects of other Artepillin C-derived compounds and their respective pharmacological properties and pharmacological mechanisms.

Recently, Krzek et al. (2006) showed that reverse-phase high-performance liquid chromatography is accurate enough to determine selected phenolic acids in propolis extracts in terms of standardization for drug manufacturing purposes. The absorption and bioavailability of Artepillin C in rats after oral administration was also shown *in vitro*, suggesting that Artepillin C can be absorbed through cell membranes (Konishi et al., 2005). Our results clearly show that Artepillin C is bioavailable and has an absorption with a maximal peak after 1 h when administered orally. These results suggest that Artepillin C can be used orally in a potential treatment for pain and inflammation.

Acknowledgements

This study was supported by grants from the Centro Universitário Barriga Verde (UNIBAVE), Santa Catarina, Brazil, and Deutscher Akademischer Austauschdienst (DAAD), Bonn, Germany. We would like to thank Mrs. Elfriede Eppinger (University of Munich, Germany) for her excellent technical assistance.

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