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Sesquiterpene lactone from *Wunderlichia crulsiana* inhibits the respiratory burst of leukocytes triggered by distinct biochemical pathways

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Abstract

The sesquiterpene lactone tubiferin was chemically purified from the brazilian native plant *Wunderlichia crulsiana* and identified by NMR and GC/MS data. Its ability to inhibit the respiratory burst of peritoneal inflammatory polymorphonuclear leukocytes (PMN) stimulated upon addition of phorbol miristate acetate (PMA), opsonized zymosan (OZ), and N-formyl-methionyl-leucyl-phenylalanine (fMLP) was evaluated. The tubiferin inhibition was more pronounced when PMN were stimulated through the protein kinase C pathway (PMA) compared to the alternative complement pathway (OZ). The inhibition when PMN were triggered by a chemoatractant stimulus (fMLP) was similar to that achieved with OZ-stimulated phagocytes. Tubiferin showed dose-dependent effects on the PMN respiratory burst triggered by the three different substances, and also decreased substancially the carrageenan-induced mice paw edema.

Keywords: Anti-inflammatory; Tubiferin ^{13}C NMR data; Sesquiterpene lactone; Superoxide anion; Polymorphonuclear leukocyte

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Introduction

Phagocytic cells, such as macrophages and polymorphonuclear leukocytes (PMN), release superoxide anion ($O_2^{\bullet -}$) as part of their cytocidal response against inflammatory stimuli through an enzymatic respiratory burst. This process, achieved by the membrane complex NADPH oxidase, can be triggered by soluble compounds, for instance, the co-carcinogen ester phorbol 12-myristate 13-acetate (PMA), through the protein kinase C pathway or by particulate agents, such as opsonised zymosan (OZ) that act via complement. Many chemotatic peptides, i.e., N-formyl-methionyl-leucyl-phenylalanine (fMLP) have a similar action on phagocytic cells, imitating parts of certain bacterial proteins (Babior, 1992).

Although the oxidative burst represents an essential physiological response to injury, acute or prolonged inflammatory processes may increase host tissue destruction. Therefore, an extensive search is focused on the development and characterization of pharmacological compounds that exhibit anti-inflammatory activities. Recently, some activities have been described for sesquiterpene lactones (SL) obtained from several plants on different inflammatory parameters such as synthesis and release of interleukin (Sobota et al., 2000), development of edema (Silván et al., 1996; Recio et al., 2000), increase of vascular permeability (Silván et al., 1996), and activation of NF-k*B* transcription factor (Rüngeler et al., 1999; Castro et al., 2000). However, there are only a few studies of SL effects on reactive nitrogen species (RNS) and/or reactive oxygen species (ROS) release by phagocytic cells during an inflammation process (Matsuda et al., 1996; Pérez-Garcia et al., 1996; Abdelghaffar et al., 1997; Ryu et al., 1998; Cho et al., 2000; Giannini et al., 2001).

Wunderlichia crulsiana belongs to Mutisieae tribe, Asteraceae family, which is well known to produce sesquiterpene lactones (Bohlmann et al., 1981, 1984; Catalan et al., 1996). In the present study we examined the effect of tubiferin, a SL from the brazilian native plant *W. crulsiana* on the respiratory burst of PMN elicited from inflammatory foci and triggered by different stimuli.

Materials and methods

Chemicals

Horse heart ferricytochrome c type VI (cyt c), phorbol-12-myristate-13-acetate (PMA), D-glucose, bovine blood superoxide dismutase (SOD), zymosan A (from *Saccharomyces ceresivae*, OZ), N-formyl-methionyl-leucyl-phenylalanine (fMLP), carrageenan, and the reference drug indomethacin was purchased from Sigma Chemicals Co. (St. Louis, MO). Sodium caseinate was obtained from ICN Biochemicals (Cleveland, OH). All other chemicals utilized were of analytical grade.

Plant material and sample preparation

The plant material was collected in Morro do Pai Inácio (S 12° 27′ 326 and W 41° 28′ 509), Chapada Diamantina, Bahia State, Brazil, on September 19th, 1998. Prof. Dr. Maria Lenise Guedes and Prof. Dr. J. Pirani made the plant material's identification. Voucher specimens are kept in the SPF Herbarium of the Instituto de Botânica da Universidade de São Paulo, SP, Brazil.

Dried powdered stems (850 g) were extracted with dichloromethane (CH_2Cl_2). The CH_2Cl_2 extract (24 g) was submitted to chromatography on silica gel-60 using a gradient of CH_2Cl_2 in n-hexane,

CH_2Cl_2, and a gradient of methanol (MeOH) in CH_2Cl_2. The fraction eluted with pure CH_2Cl_2 showed a mixture of SL when analyzed by ^{1}H-NMR. This fraction was partitioned with hexane/(MeOH/H_2O 95:5) 1:1. The hydroalcoholic phase was submitted to chromatography on Sephadex LH-20 eluted with MeOH, which contains a mixture of three SLs. To purify tubiferin from that fractions, a preparative TLC, eluted 6 times with hexane/acetone 8:2 was made. The purity of tubiferin was checked by GC analysis and identified by NMR and compared with literature data (Bermejo Barrera et al., 1967; Bohlmann and Van, 1977; Marco and Carda, 1987; Ahmad et al., 1990).

Chemical identification

The GC measurements were performed using a Hewlett Packard 5890 series II, automatic injector HP 7673 and integrator HP 3396A, 30 m capillary column (5% phenyl in 95% of methyl-silicon), Helium as carrier gas and FID detector. The oven temperature was 200 °C (0 min)-10 °C/min-220 °C (10 min)-10 °C/min-280 °C, injector temperature: 250 °C, detector temperature: 310 °C. The ^{1}H- and ^{13}C-NMR spectra were recorder in a Bruker instrument (500 and 125 MHz, respectively) using $CDCl_3$ as solvent and TMS as internal reference. To our knowledge, only ^{1}H-NMR data of tubiferin were reported in literature (Bermejo Barrera et al., 1967). We determined the ^{13}C-NMR data ($CDCl_3$, 125 MHz): 157.76 (C-1), 126.78 (C-2), 200.32 (C-3), 42.08 (C-4), 52.34 (C-5), 81.99 (C-6), 50.22 (C-7), 21.22 (C-8), 37.26 (C-9), 38.50 (C-10), 138.41 (C-11), 170.00 (C-12), 117.32 (C-13), 19.29 (C-14), and 14.60 (C-15).

Animals and PMN preparation

Swiss male mice (25–28 g, 6–8 weeks old) were obtained from the local breeding colonies at the Instituto de Química, SP, and the Escola de Farmácia e Odontologia de Alfenas, MG, Brazil. The animals were housed in plastic cages, and water and food were available ad libitum. PMN were obtained and purified as previously described (DePierre and Karnovsky, 1974). Briefly, the animals were injected intraperitoneally with 1.0 mL of 12% (w/v) sodium caseinate in isotonic saline solution, always at 13:00 hrs to avoid circadian oscilation (Brigagão and Colepicolo, 1996). After 4 h, the animals were sacrificed by cervical dislocation and peritoneal cells harvested with 5.0 mL of ice-cold phosphate buffered saline (PBS). The cell suspensions were pelleted by centrifugation (250 × *g* for 10 min), resuspended in 1.0 mL D-PBS-G (1.35 mg/mL D-glucose), and the number of cells was determined by counting in an AO Spencer hematocytometer chamber. The cell population obtained was 95% PMN and the viability after each experiment was greater than 90% (stained by May-Grünwald-Giemsa and Trypan Blue), as judged by optical microscopy. Cytotoxicity assays were carried out with tubiferin in yeast cultures (Gunatilaka et al., 1992).

$O_2^{\bullet-}$ release measurement

Cell suspensions (1×10^7 PMN/mL) were incubated with 20 μL of the SL tubiferin (140 μg dissolved in *iso*-propanol) and 0.75 mM of cyt c at 37 °C for 10 min. Controls were incubated with the same concentration of *iso*-propanol and cyt c. PMA (20 ng/mL dissolved in DMSO), OZ (100 μg/mL) or fMLP (1 μM) were added to trigger the respiratory burst. The reduction of cyt c was followed spectrophotometrically for 1 min at 550 nm against preparations containing 5U of SOD (Cohen and Chovaniec, 1987). The dose-dependence of inhibition of $O_2^{\bullet-}$ release by PMN was determined with

different amounts of SL (20–140 μg) and the ED_{50} (defined as the dose effective to inhibit 50% of $O_2^{\bullet-}$ release by $1x10^7$ PMN) was calculated by non-linear regression.

Carrageenan-induced mice paw edema

Paw edema was induced by a subplantar injection of 50 μL of 3% (w/v) carrageenan into the mice right paws, with or without tubiferin (560 μg/100 g body weight) dissolved in *iso*-propanol/Tween 80/water (2:2:20, v/v) or the standard drug indomethacin that is an inhibitor of cyclooxygenase (COX) catalyzed prostaglandin biosynthesis (560 μg/100 g body weight) (Sugishita et al., 1981). Paw volumes were measured on a plethysmometer (Ugo Basile) 4 h after injections. The results were expressed as an increase in paw volume due to carrageenan injection and the action of tubiferin or indomethacin.

Statistical analysis

All values of biological data are expressed as mean ± S.E.M. of three separate experiments performed in triplicate. The Kruskal-Wallis test for unpaired observation between control and experimental samples was carried out for statistical evaluation of a difference; p values of 0.05 or less were considered as statistically significant.

Results

Fig. 1 shows the chemical structure of the SL tubiferin identified by NMR and GC/MS data, whose purity degree was 90%.

When stimulated with PMA, PMN release $O_2^{\bullet-}$ at a rate of 3.5 ± 0.5 nmol/min. Addition of tubiferin (140 μg) decreased the rate by 61%. OZ stimulated-PMN release 0.98 ± 0.1 nmol $O_2^{\bullet-}$/min. This rate was significantly decreased upon the addition of the SL by 36%. In a similar manner, the finding of a decrease was also observed when PMN were stimulated with fMLP in presence of SL (35%) relative to the control PMN (5.8 ± 0.1 nmol $O_2^{\bullet-}$/min), as shown in Fig. 2. Either DMSO or *iso*-propanol had no effect on the $O_2^{\bullet-}$ release by PMN. Also, at the experimental concentrations used, tubiferin was not able to reduce cyt c,

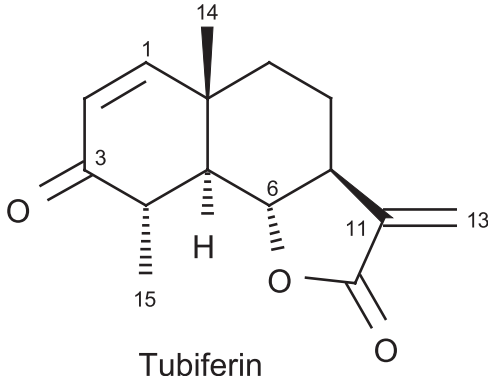

Fig. 1. Chemical structure of SL tubiferin purified from *W. crulsiana* stems.

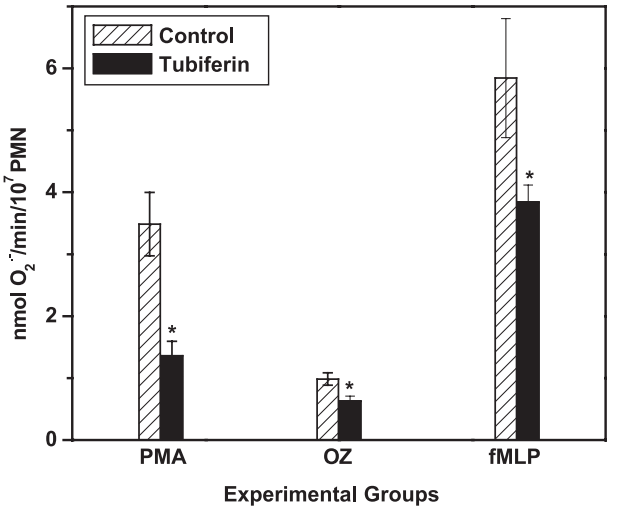

Fig. 2. Inhibitory action achieved by the SL tubiferin on the release of $O_2^{\bullet-}$. The inflammatory PMN (10^7 cells) were triggered by PMA, OZ, and fMLP without (control) or with previous tubiferin (140 μg) incubation. The results are expressed as the mean ± S.E.M. of three experiments, n = 3. *$p < 0.001$.

to cause direct dismutation of $O_2^{\bullet-}$ or to inhibit yeast cell proliferation. It was verified that PMN viability was not imparied with SL and/or *iso*-propanol treatment in the concentrations used in this study as determined by Trypan Blue exclusion (data not shown).

The dose-response curves of the inhibitory effect of tubiferin on $O_2^{\bullet-}$ release by PMN are shown in Fig. 3. The SL was more effective at inhibiting the respiratory burst when the cells were stimulated by PMA (ED_{50}: 115 μg/10^7 cells) than by OZ (ED_{50}: 188 μg/10^7 cells) or fMLP (ED_{50}: 198 μg/10^7 cells).

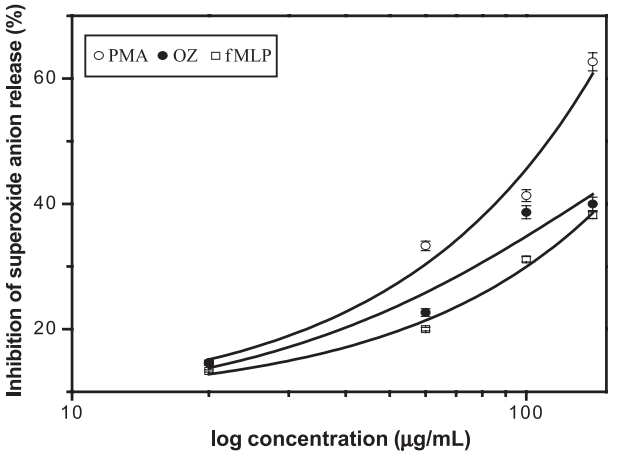

Fig. 3. Dose-response curves of the tubiferin inhibitory action on $O_2^{\bullet-}$ release by PMN. The phagocytic cells (1×10^7 cells/mL) were stimulated to release $O_2^{\bullet-}$ after previous incubation with different amounts (20–140 μg) of the SL. The results are expressed as the mean ± S.E.M. of three experiments, n = 3.

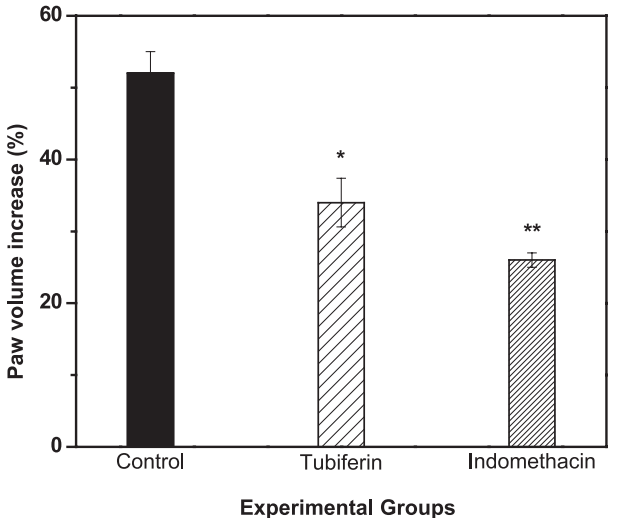

Fig. 4. Tubiferin in vivo anti-inflammatory effect on carrageenan-induced paw-edema. An inflammation process was induced (control) and indomethacin was used as an anti-inflammatory standard drug in order to compare its effects to tubiferin. Both substances were tested in the same dose (560 μg/100 g body weight). The results are expressed as the mean ± S.E.M. of three experiments, n = 3. *$p < 0.05$; **$p < 0.001$.

Fig. 4 shows the effect of the SL on carragenan-induced paw inflammation. Tubiferin inhibited paw swelling by 35%, near to that of indomethacin (50%), suggesting a topical anti-inflammatory activity in this co-injection carragenan and tubiferin or indomethacin model.

Discussion

ROS are generated at the phagocytic site as a major response of PMN during the inflammatory process (Karnovsky et al., 1994; Rabadji et al., 1996). Overproduction of ROS can damage the host tissues and lead to cell death. It is extremely important to find products that act efficiently as an anti-inflammatory compounds.

Among the strategies to attenuate excessive inflammation, a great deal of interest has been paid to the possible modulation of oxidant production by phagocytes (Matsuda et al., 1996; Pérez-Garcia et al., 1996; Abdelghaffar et al., 1997; Cho et al., 2000; Giannini et al., 2001). In the current studies, the SL tubiferin (Fig. 1) was isolated, purified and chemically characterized from *W. crulsiana*. The inhibitory effect on $O_2^{\bullet -}$ released by PMN achieved by this eudesmanolide was significant (Fig. 2). We compared the biochemical pathway of stimulus for PMN. The greatest inhibitory activity of the SL was obtained when the phagocytic cells were stimulated with PMA. The activation by PMA of the Ca^{2+} and phospholipid-dependent PKC in several inflammatory mechanisms is well established, with resulting phospholipase A2 (PLA2)-dependent arachidonic acid release and eicosanoid production (Nishizuka, 1989). In recent reports, pseudoguaianolide SL showed selective activity against PMA analog induced ear edema, and other SL blocked PKC activation and the inflammatory process in mouse ear edema (Kuchera et al., 1993; Silván et al., 1996; Garcia Pastor et al., 1999). Our results suggest that among other inflammatory parameters inhibited by antagonist compounds of the PKC

pathway, such effective anti-inflammatory agents block oxygen metabolite formation in the respiratory burst of PMN.

In comparison with PMA-triggered cells, the inhibition of the respiratory burst triggered by the particulate agent OZ achieved by tubiferin was less, although significant than that obtained with PMA triggered cells. In another SL study, OZ was used in experiments to cause a rat air pouch inflammatory event that was inhibited in vitro and in vivo by cacospongionolide B, a metabolite isolated from marine sponge showing selectivity for inhibition of secretory PLA2 versus cytosolic PLA2 (Garcia Pastor et al., 1999).

We also tested the SL with PMN stimulated by fMLP, a peptide that activates G protein-coupled receptors and thereby imitates certain bacterial antigens to trigger $O_2^{\bullet-}$ release. Low-dose and long-term administrations of lactone-ring macrolides have been reported to be very effective in the treatment of chronic inflammatory sinusitis, suggesting that lactones are able to affect antigen-presenting cells (Miyazawa and Lino, 1997). However, fMLP evoked a biochemical pathway that was less affected by tubiferin from *W. crulsiana* than those in PMA-stimulated PMN.

A few previous studies have examined the effects of SL on generation of ROS by leukocytes. Cohen et al. (1997) reported that a butyrolactone down-regulated the response causing severe acute lung injury after reperfusion of ischemic tissue, a process involving neutrophil activation and oxygen radical generation. In addition to the anti-inflammatory in vivo activity in different inflammation models, the hydrogen peroxide production from blood leukocyte in vitro was significantly reduced by *Pluchea sagittalis* aqueous extract, and other lactones were able to inhibited the $O_2^{\bullet-}$ release by human PMN (Pérez-Garcia et al., 1996; Abdelghaffar et al., 1997; Giannini et al., 2001).

Tubiferin showed a dose dependent-action (Fig. 3) on NADPH oxidase activity. The shapes of the curve on the respiratory burst and the ED_{50} for PMA-stimulated PMN were different from those achieved by OZ- or fMLP-triggered phagocytes. These findings show that the inhibition caused by tubiferin might use a different mechanism when the PMN were stimulated by PMA compared with those stimuli that were membrane-dependent. Since tubiferin showed strong inhibitory effects on ex vivo PMN $O_2^{\bullet-}$ release, we further studied its actions over an in vivo inflammation model. Notably, tubiferin was able to cause a significant decrease in carrageenan-induced paw edema, near to that effect of the COX inhibitor indomethacin (Fig. 4).

Many data reinforces the relationship between the presence of the alpha-methylene-gamma-lactone group in SL structure and their anti-inflammatory action (Matsuda et al., 1996; Rüngeler et al., 1999; Castro et al., 2000; Cho et al., 2000). We showed here that tubiferin, an SL which has this alkylant chemical group, is able to inhibit the leukocyte NADPH oxidase system on ex vivo model, and its capability to reduce in vivo acute topic inflammation process.

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