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Effects of furocoumarins from *Cachrys trifida* on some macrophage functions

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Abstract

Phytochemical and biological studies aimed at the discovery and development of novel anti-inflammatory agents from natural sources have been conducted in our laboratory for a number of years. In this communication, three naturally occurring furocoumarins (imperatorin, isoimperatorin and prantschimgin) were evaluated as potential inhibitors of some macrophage functions involved in the inflammatory process. These furocoumarins have been tested in two experimental systems: ionophore-stimulated mouse peritoneal macrophages serve as a source of cyclooxygenase-1 and 5-lipoxygenase, and mouse peritoneal macrophages stimulated with *E. coli* lipopolysaccharide are the means of testing for anti-cyclooxygenase-2 and nitric-oxide-synthase activity. All above-mentioned furocoumarins showed significant effect on 5-lipoxygenase (leukotriene C_4) with IC50 values of $< 15\ \mu$M. Imperatorin and isoimperatorin exhibited strong-to-medium inhibition on cyclooxygenase-1- and cyclooxygenase-2-catalysed prostaglandin E_2 release, with inhibition percentages similar to those of the reference drugs, indometacin and nimesulide, respectively. Of the three furocoumarins, only imperatorin caused a significant reduction of nitric oxide generation. Imperatorin and isoimperatorin can be classified as dual inhibitors, since it was evident that both cyclooxygenase and lipoxygenase pathways of arachidonate metabolism were inhibited by these compounds. However, selective inhibition of the 5-lipoxygenase pathway is suggested to be the primary target of action of prantschimgin.

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Introduction

The coumarins (also known as benzopyrones) consist of fused benzene and α-pyrone rings and represent a large group of phenolic compounds occurring in green plants, as well as in fungi and bacteria (Murray et al 1982). They have shown antimicrobial, antitumoral and anti-aggregant activity and have been reported to be potent antioxidants endowed with anti-inflammatory properties (Hoult et al 1994; Payá et al 1994; Ng et al 1996; Hoult & Payá 1996; Cai et al 1997; Su et al 1998; Kang et al 1999). Furanocoumarins, alternatively known as furocoumarins, are a category of coumarins which occur as plant constituents predominantly in two families, the Umbelliferae and the Rutaceae. Species of these families have deep figurative roots in medicinal folklore and alchemy. Furocoumarins are present in these species as the major secondary metabolite, suggesting an important role for these compounds in the traditional preparations. Although biological activity of furocoumarins and the clinical use of plants containing these compounds for the

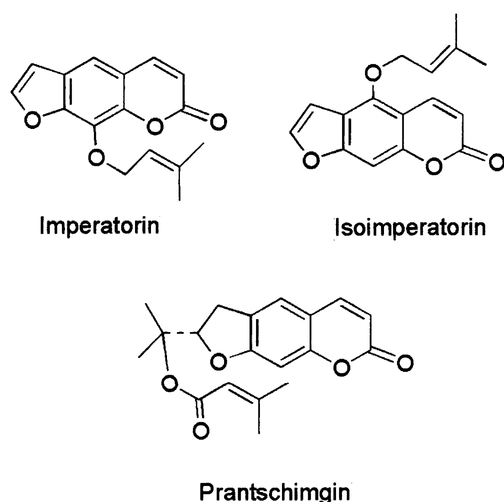

Figure 1 Structure of furocoumarins.

treatment of skin lesions have been previously investigated (Shin et al 1993; Chen et al 1995; Cheu et at 1996; Okugawa et al 1998; Fujioka et al 1999; Potapenko et al 1999), there are no pharmacological data concerning their anti-inflammatory properties.

Macrophages play a major role in host defence against infection and tumour development and this activity is regulated through the production of several mediators. Activation of macrophages causes the release of various cytokines and chemicals such as nitric oxide (NO), prostaglandins and leukotrienes, which are important effector molecules that initiate inflammation. The oxidative metabolism of arachidonic acid leads to the synthesis of leukotrienes and prostaglandins through the cyclooxygenase and lipoxygenase pathways. Leukotrienes are a family of highly potent biological mediators generated by the enzyme 5-lipoxygenase (5-LOX). The ability of leukotrienes to mimic certain aspects of human disease and the recovery of these products from sites undergoing pathological reactions strongly suggest their important role in mediating a spectrum of inflammatory and immune diseases (Harris et al 1995). Prostaglandins are inflammatory mediators generated by the enzyme cyclooxygenase. In the last few years, it has been recognized that mammalian cells express two forms of cyclooxygenase: a constitutive enzyme (COX-1), responsible for the normal production of prostaglandins in the digestive tract, kidney and elsewhere and an inducible isoform (COX-2), induced in response to pro-inflammatory cytokines and bacterial cell components, found in elevated levels in inflammatory exudates (Payá et al 1997). These observations have led to the hypothesis that COX-1 is mainly associated with homeostasis and COX-2 with oedematous, nociceptive and pyretic effects of inflammation.

NO, synthesized by the enzyme nitric oxide synthase (NOS), has been reported as a mediator of inflammation and seems to be involved in both acute and chronic inflammation (Dugas et al 1995). After stimulation with bacterial lipopolysaccharide (LPS) many cells, including endothelial cells and macrophages, express the inducible isoforms of either COX-2 or NOS (iNOS), which are responsible for the production of large amounts of prostaglandins and NO, respectively. These inducible enzymes are essential components of the inflammatory response and are implicated in the pathogenesis of several inflammatory diseases (Vane et al 1994).

In this paper, we have examined the effect of three naturally occurring furocoumarins on some macrophage functions to study their mechanism of action as potential anti-inflammatory molecules.

Materials and Methods

Materials

Furocoumarins were isolated from *Cachrys trifida* Miller (Umbelliferae) and identified as imperatorin, isoimperatorin and prantschimgin by their physical (melting point, specific rotation) and spectroscopic data (UV, ^{1}H NMR and MS). Their structures are shown in Figure 1 (Murray 1978; Hernández & Rodríguez 1981). Cell-culture reagents were purchased from Life Technologies (Barcelona, Spain). Enzyme-linked immunosorbent assays (ELISA) kits for the determination of prostaglandin E_2 (PGE_2) and leukotriene C_4 (LTC_4) were provided by Cayman Chemical Co. (USA). Other reagents were purchased from Sigma Chemical Co. (USA).

Measurement of cell viability

Cytotoxicity studies were assessed by the mitochondrial-dependent reduction of the tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan (Woerdenbag et al 1994). Macrophages (5×10^5 cells) diluted in Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated fetal calf serum (FCS) were pipetted into 96-well microtiter plates and incubated overnight at 37°C and 5% CO_2–95% air. Cells were exposed to various concentrations of test compounds (0–100 μM) for 20 h under the same incubation conditions. MTT (0.5 mg mL^{-1} in

phosphate-buffered saline, PBS) was added and further incubated for 4 h at 37°C. After carefully aspirating the medium, cells were solubilized in dimethyl sulfoxide (DMSO; 100 μL). The extent of reduction of MTT to formazan within cells was quantitated by measurement of OD (optical density) at 550 nm using a microplate reader.

PGE_2 and LTC_4 measurement in A23187-stimulated macrophages

Macrophages from Swiss male mice (20 ± 5 g) were collected after peritoneal lavage with PBS. Cells were suspended in DMEM supplemented with 10% FCS and seeded into 24-well plates at a concentration of 10^6 cells mL^{-1}. After adhering to plates (24 h at 37°C in an atmosphere of 5% CO_2–95% air), non-adherent cells were washed off and then cultured in DMEM lacking FCS. Cells were pre-treated for 1 h at 37°C with test compounds or vehicle and then stimulated for a further 2 h by adding calcium ionophore A23187 (final concentration 1 μM) (Bickel et al 1994). The medium was withdrawn from each well and the level of PGE_2 and LTC_4 was quantified using ELISA kits according to the manufacturer's instructions.

Measurement of PGE_2 and nitrite in LPS-activated macrophages

Swiss male mice (25 ± 5 g) were injected intraperitoneally with 1 mL of thioglycolate broth 4 days before use (López-Collazo et al 1998). Peritoneal macrophages were prepared as follows: light ether-anaesthetized mice (4–6) were killed by cervical dislocation and injected intraperitoneally with 5 mL of sterile RPMI 1640 (culture medium). The peritoneal fluid was carefully aspirated to avoid haemorrhage and kept at 4°C to prevent the adhesion of macrophages to the plastic. After centrifugation at 200 *g* for 10 min at 4°C, the cell pellet was washed twice with 45 mL of ice-cold PBS. Cells were seeded at 1×10^6 mL^{-1} in RPMI 1640 supplemented with 10% FCS. After incubation for 2 h at 37°C in 5% CO_2, non-adherent cells were removed by extensive washing with PBS. Cells were cultured in the same medium containing 0.5 μg mL^{-1} *E. coli* LPS with or without test sample for 17 h. Culture supernatants were used for PGE_2 and NO measurements. NO released was assessed spectrophotometrically as the stable end-product nitrite in the culture supernatant with the Griess reagent (Green et al 1982). Nitrite concentration was calculated by comparison with OD550 of standard solutions of $NaNO_2$ prepared in culture medium.

Data analysis

Results are expressed as mean $\pm$ s.e.m. One-way analysis of variance followed by Dunnett's *t*-test for multiple comparisons of unpaired data was used for statistical evaluation, $P < 0.05$ was considered significant.

Results

Effects of furocoumarins on PGE_2 (COX-1) and LTC_4 (5-LOX)

Addition of calcium ionophore A23187 to mouse peritoneal macrophages causes the generation of nanogram amounts of eicosanoids via both COX-1 and 5-LOX pathways, measured in terms of immunoassayable PGE_2 and LTC_4, respectively. Validation of this system for the identification of inhibitors of the two divergent pathways of arachidonate metabolism was obtained by using indometacin, a well-characterized cyclooxygenase inhibitor (IC50 (concentration giving 50% inhibition) = 2.35 μM) and nordihydroguaiaretic acid (NDGA), a known inhibitor of 5-LOX that potently reduces LTC_4 synthesis (IC50 = 0.49 μM). The compounds tested showed remarkable activity in both assays. In the PGE_2-release assay, both imperatorin and isoimperatorin exhibited a significant inhibitory effect with inhibition percentages slightly lower than the reference drug, indometacin (Table 1). Inhibition was more evident with imperatorin, which completely abolished PGE_2 production (95% inhibition at the highest non-cytotoxic dose 100 μM). Prantschimgin did not affect PGE_2 release. In the LTC_4 release assay, the three furocoumarins were potent inhibitors of this mediator, achieving inhibition percentages similar to those of the reference drug NDGA (Table 2). IC50 values were in increasing order of activity: prantschimgin 14.42 μM, isoimperatorin 1.62 μM and imperatorin 0.60 μM.

Effects of furocoumarins on NO production and PGE_2 (COX-2)

Treatment of cultured macrophages with LPS causes the release of large amounts of NO (as deduced by nitrite accumulation in the culture medium) and enhances COX-2 activity (measured as PGE_2 generation). Furocoumarins were tested with regard to their effects on these mediators. Test compounds assayed at 10 μM in the absence of LPS did not alter basal nitrite concentration. The production of nitrite by unstimulated macrophages was undetectable (< 2.5 μM). Activation of macrophages with LPS (0.5 μg mL^{-1}) for 24 h resulted

Table 1 Inhibition by furocoumarins of PGE_2 release from mouse peritoneal macrophages stimulated with calcium ionophore A23187 and *E. coli* LPS.

Treatment	PGE_2 release (ng mL^{-1})		COX-2/COX-1 ratio
	COX-1	COX-2	
Cells alone	0.10 ± 0.20	0.85 ± 0.20	–
Control	3.80 ± 0.50	9.50 ± 0.50	–
Indometacin (100 μM)	0.15 ± 0.10*	–	
Nimesulide (100 μM)	–	0.12 ± 0.20*	
Imperatorin (100 μM)	0.13 ± 0.60*	0.35 ± 0.70*	0.90
Imperatorin (50 μM)	0.29 ± 0.10*	0.55 ± 0.10*	
Imperatorin (25 μM)	0.55 ± 0.10*	0.59 ± 0.30**	
Isoimperatorin (100 μM)	0.35 ± 0.50*	0.17 ± 0.10*	0.45
Isoimperatorin (50 μM)	0.40 ± 0.20*	0.65 ± 0.10**	
Isoimperatorin (25 μM)	1.13 ± 0.10**	0.72 ± 0.20**	
Prantschimgin (100 μM)	–	–	–

Mouse macrophages stimulated with calcium ionophore A23187 (1 μM) were used as a source of COX-1 and those stimulated with *E. coli* LPS (0.5 μg mL^{-1}) as a source of COX-2. All values are mean ± s.e.m. *$P \leqslant 0.01$, **$P \leqslant 0.05$, vs control.

Table 2 Inhibition by furocoumarins of LTC_4 release and NO release from mouse peritoneal macrophages stimulated with calcium ionophore A23187 and *E. coli* LPS.

Treatment	LTC_4 (ng mL^{-1})	NO (μM)
Cells alone	0.11 ± 0.20	0.50 ± 0.20
Control	35.00 ± 0.60	43.10 ± 3.10
NDGA (25 μM)	0.25 ± 0.70*	–
Dexamethasone (1 μM)	–	21.50 ± 2.50*
Imperatorin (100 μM)	0.23 ± 0.30*	0.21 ± 0.10*
Imperatorin (50 μM)	0.70 ± 0.10*	
Imperatorin (12.5 μM)	1.50 ± 0.1*	
Imperatorin (10 μM)		17.80 ± 0.50*
Imperatorin (1 μM)		39.80 ± 1.50
Isoimperatorin (100 μM)	0.25 ± 0.70*	–
Isoimperatorin (50 μM)	0.50 ± 0.30*	–
Isoimperatorin (25 μM)	2.40 ± 0.20*	–
Isoimperatorin (12.5 μM)	3.20 ± 0.30*	–
Prantschimgin (100 μM)	0.26 ± 0.20*	–
Prantschimgin (50 μM)	0.85 ± 0.10*	–
Prantschimgin (25 μM)	15.00 ± 0.20*	–
Prantschimgin (12.5 μM)	21.00 ± 0.40*	–

Mouse macrophages stimulated with calcium ionophore A23187 (1 μM) were used as a source of LTC_4 and those stimulated with *E. coli* LPS (0.5 μg mL^{-1}) as a source of NO. All values are mean ± s.e.m. *$P \leqslant 0.01$ vs control.

in an accumulation of nitrite (43.12 ± 3.1 μM). Pre-treatment of cells for 30 min with various concentrations of imperatorin caused a significant concentration-dependent reduction of NO (IC50 = 9.2 μM; Table 2). The ethanol concentration used as coumarin vehicle had no effect on nitrite production. Dexamethasone (1 μM) used as positive control strongly suppressed NO generation (50 % at 1 μM). Isoimperatorin and prantschimgin up to 100 μM did not show any inhibitory effect. Prantschimgin failed to modify PGE_2 production (Table 1). Imperatorin and isoimperatorin and the reference compound, nimesulide, significantly inhibited PGE_2 production in a dose-dependent manner. Inhibition was more evident with isoimperatorin (IC50 = 3.73 μM). Nimesulide, used as a positive control in the COX-2 assay, showed an IC50 value of 2.54 μM.

Among the furocoumarins, imperatorin was the most potent inhibitor for both PGE_2 and nitrite production in macrophages. Inhibition of these mediators was not due to toxicity, as determined by the MTT assay.

Discussion

Coumarins are naturally occurring plant metabolites whose biological activities have been extensively reviewed (Hoult & Payá 1996). However, the anti-inflammatory properties of furocoumarins are not well established. In this paper, three naturally occurring furocoumarins, imperatorin, isoimperatorin and prantschimgin, were examined for their effect on some macrophage functions of relevance to the inflammatory process.

These compounds have been tested for their ability to inhibit the generation of several mediators in two ex-

perimental systems: calcium ionophore A23187-stimulated mouse peritoneal macrophages validated as a source of COX-1 and 5-LOX enzymes, and elicited mouse peritoneal macrophages stimulated with *E. coli* LPS for testing COX-2 and iNOS inhibitors. We chose mouse peritoneal macrophages as a cell model as it has the additional relevant advantages that cellular activation (for example by the calcium ionophore A23187) causes the generation of both 5-LOX (e.g. LTC_4) and COX-1 products (e.g. PGE_2) (Williams et al 1984). COX-2 is the main enzyme responsible for the production of PGE_2 in LPS-stimulated macrophages, as the selective compound nimesulide strongly reduced the levels of this eicosanoid (Tavares et al 1995). Glucocorticoids are known to inhibit NOS (Radomski et al 1990) without affecting constitutive enzymes. Dexamethasone showed inhibitory effects on NO synthesis in this model.

In the PGE_2-release assay (COX-1), only prantschimgin had no significant effect. Imperatorin and isoimperatorin exhibited strong-to-medium inhibition of PGE_2 release. Results of this study also showed that all the mentioned coumarins potently reduced LTC_4 generation and could be ranked according to their inhibitory potency as prantschimgin $<$ isoimperatorin $<$ imperatorin. Furocoumarins were further tested for COX-2- and iNOS-inhibiting properties. In this study, we demonstrated that two coumarins, imperatorin and isoimperatorin, suppressed LPS-induced PGE_2 release in a dose-dependent manner, an effect related to COX-2 activity. Of the three furocoumarins tested, only imperatorin was effective as an inhibitor of NO synthesis. This would give a relative selectivity ratio (COX-2/COX-1 ratio) for imperatorin of 0.90, expressing an equipotent effect. Isoimperatorin had a weaker activity towards COX-2, yielding a COX-2/COX-1 ratio of 0.45. These results appear to differ from those reported in the literature for isoimperatorin showing no inhibitory activity on microsomal COX-1 from ram seminal vesicles and 5-LOX from ionophore-stimulated intact porcine leucocytes (Liu et al 1998).

Our results identify some features concerning the inhibition of arachidonate metabolism by furocoumarins. If the magnitude of the anti-inflammatory effects is compared with the chemical structure of the furocoumarins tested, some relationships can be observed. The linear furocoumarin nucleus, with additional substituents in the body of the molecule, is one of the most positive characteristic for high 5-LOX activity. Substitution in the 5 or 8 positions of the coumarin nucleus did not reduce in-vitro activity, but a linearly annulated substituted compound with fused and coumarin rings resulted in decreased activity. In fact, the presence of a foreign radical at position 5′, together with the lack of unsaturation at C4′-C5′ decreases COX activity, as is apparent when the activity of imperatorin and isoimperatorin is compared with prantschimgin, which was inactive in the PGE_2-release assay. It is important to note that imperatorin and isoimperatorin are very close chemically, differing from each other by the substitution at position 5 and 10, respectively. The same features can be suggested for COX-2 inhibitory effects observed with furocoumarins. Previous studies have indicated that several coumarins modulate arachidonic acid metabolism in peritoneal leucocytes (Hoult & Payá 1996). Cellular mechanisms underlying these effects are still unclear but are thought to be linked to their antioxidant properties. In this study, we have demonstrated that furocoumarins have inhibitory activity on COX-1 and COX-2.

From these results, selective inhibition of the 5-LOX pathway is suggested to be the primary target of action of prantschimgin. It is unlikely that the activity of this compound is associated with an inhibitory effect on the COX pathway in our cellular systems. Moreover, imperatorin and isoimperatorin can be classified as dual inhibitors, since it was evident that both the COX and LOX pathways of arachidonate metabolism were inhibited by these compounds. In conclusion, furocoumarins, active constituents of *Cachrys trifida*, are potential anti-inflammatory agents. The possible relationship between the structural properties of furocoumarins and their anti-inflammatory activity deserves further investigation.

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