British Journal of Pharmacology (2007) 152, 1272–1282
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www.brjpharmacol.org

RESEARCH PAPER

Demethylnobiletin inhibits delayed-type hypersensitivity reactions, human lymphocyte proliferation and cytokine production

E Bas[1], MC Recio[1], RM Giner[1], S Máñez[1], C López-Ginés[2], R Gil-Benso[2] and JL Ríos[1]

[1]*Departament de Farmacologia, Facultat de Farmàcia, Universitat de València, Burjassot, Spain and* [2]*Departament de Patologia, Facultat de Medicina, Universitat de València, Burjassot, Spain*

Background and purpose: Our aim was to examine the effect of demethylnobiletin on various experimental models of delayed-type hypersensitivity (DTH) reactions and to determine its influence on the mediators and enzymes involved in these reactions.
Experimental approach: DTH was induced in mice by oxazolone, dinitrofluorobenzene (DNFB) and sheep red blood cells (SRBC). The effect of demethylnobiletin on the ensuing DTH was studied, especially in relation to oedema formation, cell infiltration and tissue damage. Its activity on different mediators implicated in DTH reactions was also determined and its effect on nitric oxide synthase (NOS)-2 analysed. Finally, its influence on T lymphocyte proliferation, apoptosis and caspase 3 activity was tested.
Key results: DTH reactions were all reduced by demethylnobiletin. The experimental results suggest that the compound may act by reducing cell infiltration and by suppressing mediators such as interleukin-2 ($IC_{50} = 1.63$ μM), interleukin-4 ($IC_{50} = 2.76$ μM), tumour necrosis factor-α ($IC_{50} = 0.66$ μM), interferon-γ ($IC_{50} = 1.35$ μM), and interleukin-1β (46% at 2.5 μM) and by concomitantly increasing the production of the anti-inflammatory cytokine, interleukin-10. In addition, while demethylnobiletin affected nitric oxide production, it did not modify NOS-2 expression. Finally, demethylnobiletin inhibited proliferation of T cells and induced their apoptosis.
Conclusions and implications: Demethylnobiletin decreased DTH reactions induced by various agents. This finding, along with the fact that the compound has a low toxicity and exhibits several other interesting properties, could pave the way for other structurally related citroflavonoids to be used as pharmacological agents in complementary therapies.
British Journal of Pharmacology (2007) **152,** 1272–1282; doi:10.1038/sj.bjp.0707500; published online 15 October 2007

Keywords: delayed-type hypersensitivity; lymphocyte proliferation; interleukins; tumour necrosis factor; interferon; demethylnobiletin

Abbreviations: DNFB, 2,4-dinitrofluorobenzene; DTH, delayed-type hypersensitivity; NOS, nitric oxide synthase; PBS, phosphate-buffered saline; PHA, phytohaemagglutinin; SRBCs, sheep red blood cells; TNF-α, tumour necrosis factor-α

Introduction

Delayed-type hypersensitivity (DTH) reactions can be induced by various allergens, including oxazolone, 2,4-dinitrofluorobenzene (DNFB) and sheep red blood cells (SRBCs). In DTH reactions, T cells are first recruited into tissues and then activated by antigen-presenting cells to produce cytokines that mediate local inflammation (Kalish and Askenase, 1999). The biochemical processes are similar in both oxazolone- and DNFB-induced DTH reactions, albeit with several variations, mostly in relation to the time frame and the increased expression of mRNA transcription for interleukins detected in the epidermis (Zunic *et al*., 1998). DTH reactions induced by SRBCs, on the other hand, are tuberculinic-type reactions mediated by specific, committed T cells. In this type of DTH, the immunological reaction appears 18 h after challenge, reaches a maximum 96 h after challenge and then progressively decreases (Hurtrel *et al*., 1992). In general, although, after challenge with any of these topical allergens, mice develop contact hypersensitivity, which is characterized by swelling and by increased tissue levels of eicosanoids and cytokines (Thomson *et al*., 1993; Xu *et al*., 1997; Bruch-Gerharz *et al*., 1998; Ross *et al*., 1998).

Correspondence: Professor JL Ríos, Departament de Farmacologia, Facultat de Farmàcia, Universitat de València, Av. Vicent Andrés Estellés s/n, Burjassot, Valencia 46100, Spain.
E-mail: riosjl@uv.es
Received 11 June 2007; revised 24 July 2007; accepted 2 August 2007; published online 15 October 2007

These mediators are relevant for the development of contact hypersensitivity in mice as can be seen by the fact that stimulation with DNFB, for example, increases the levels of both interleukin-1β release and interleukin-1β receptor immunoreactivity without changing the mRNA levels of this protein (Matos *et al*., 2005). In addition, interleukin-4 plays an important role in directing Th2 cell development and preventing the differentiation of Th1 cells. In contrast, interferon-γ and interleukin-12 promote the differentiation of Th1 cells. The differential production of cytokines is thus a critical determinant for the nature of the immune response, which is why an unbalanced Th1/Th2 ratio is seen in many immunological diseases, including infectious diseases and both autoimmune and allergic responses (Wang *et al*., 2000).

Flavonoids have been reported to be anti-inflammatory and antiallergic agents, with their mechanism of action usually being correlated to their capacity to inhibit arachidonic acid metabolism (Middleton *et al*., 2000; Chi *et al*., 2001; Havsteen, 2002), their antioxidant activity (Harborne and Williams, 2000; Heim *et al*., 2002), their ability to modulate the complement system (Lasure *et al*., 1994) and their inhibitory effect on histamine release from mast cells (Di Carlo *et al*., 1999; Havsteen, 2002). However, no reports on their effect on DTH reactions have been published to date. Demethylnobiletin (Figure 1) is a common flavonoid present in many species of the genus *Citrus*, but for our purposes it was isolated from *Sideritis tragoriganum* ssp. *mugronensis*. There are few reports on the pharmacological activity of this flavonoid, but those that exist include studies of its capacity to suppress the production and gene expression of matrix metalloproteinase 9 (Ishiwa *et al*., 2000), its inhibition of non-enzymatic lipid peroxidation (Mora *et al*., 1990) and its capacity to scavenge superoxide radicals in a non-enzymatic system (Huguet *et al*., 1990). It has also been shown to exhibit anti-inflammatory activity against dermatitis induced by repeated application of 12-*O*-tetradecanoylphorbol 13-acetate to mouse ears, and the oedema in mouse paws induced by phospholipase A_2 and carrageenan (Bas *et al*., 2006).

In the present study, we report the effects of demethylnobiletin on the DTH reactions induced by oxazolone, DNFB and SRBCs, specifically on oedema formation, cell infiltration and tissue damage. We have also examined the effects of this compound on the different mediators and enzymes involved in these hypersensitivity reactions, including nitric oxide, nitric oxide synthase-2 (NOS-2), tumour necrosis factor-α (TNF-α), interferon-γ, interleukin-1β, interleukin-2, interleukin-4 and interleukin-10. In addition, we have determined the influence of demethylnobiletin on both T lymphocyte proliferation and the cell cycle, as well as its proapoptotic activity and its effect on caspase 3 activity.

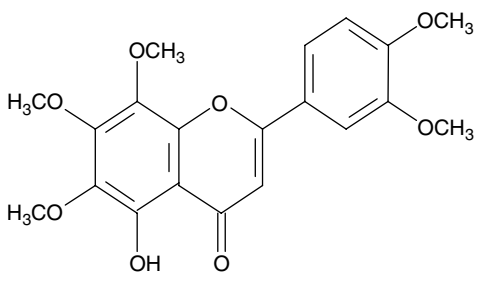

Figure 1 Structure of demethylnobiletin.

Methods

Animals

All animal procedures were conducted in accordance with the guidelines established by the European Union on Animal Care (CEE Council 86/609). Housing conditions and all *in vivo* experiments were approved and monitored by the Institutional Ethics Committee of the Faculty of Pharmacy (University of Valencia, Spain). Female Swiss mice weighing 25–30 g (Harlan Interfauna Iberica, Barcelona, Spain) were acclimatized for 7 days in individual Plexiglas cages before the experiments, during which time they were fed Purina standard rat chow (Purina Co., St Louis, MO, USA) and given free access to water. Animals were fasted overnight before the experiments.

DTH induced by oxazolone, DNFB and SRBCs

Female mice were sensitized on day 1 through topical application of 150 μl of a 3% solution of oxazolone (Sigma-Aldrich, St Louis, MO, USA) in acetone to the shaved abdomen. On day 2, ear thicknesses were measured (data for ears without inflammation). Challenge was performed on day 6 through application of 20 μl of 1% oxazolone in acetone to the inner and outer surfaces of both the ears. Demethylnobiletin (0.5 mg per ear) and dexamethasone (0.025 mg per ear; Sigma-Aldrich) dissolved in acetone were applied topically (20 μl) to the ears 6, 24 and 48 h after challenge (single application). Ear thickness measurements of the treated and control groups were taken with a micrometer (Mitutoyo Series 293; Kawasaki, Japan) and the oedema was calculated for each ear as the difference in thickness before treatment (0 h) and 24, 48 and 72 h after challenge. The control group was treated only with oxazolone.

In the DNFB test, the sensitization phase was induced by topical application of 20 μl of 0.2% DNFB (Sigma-Aldrich) in acetone onto the shaved abdomen on days 1 and 2. Mice were challenged on day 6 through application of 20 μl of 0.2% DNFB in acetone to the inner and outer ear surfaces. Demethylnobiletin (0.5 mg per ear) and dexamethasone (0.025 mg per ear) dissolved in acetone were applied topically (20 μl) to the ears 2, 24 and 48 h after challenge. The oedema was calculated for each ear as the difference in thickness before treatment (0 h) and 24, 48 and 72 h after challenge. The control group was treated only with DNFB.

Sheep red blood cells (2×10^7; Sigma-Aldrich) in phosphate buffer solution were injected subcutaneously into the shaved backs of the mice. Challenge was performed 5 days later through injection of 1×10^8 red blood cells into the right hind paws of the mice. Demethylnobiletin (15 mg kg^{-1}) and dexamethasone (10 mg kg^{-1}) dissolved in ethanol–Tween 80–water (1:1:10) were administered intraperitoneally immediately before and 16 h after challenge. The paw volumes were measured with a plethysmometer (Ugo Basile,

Comerio, Italy) 18, 24 and 48 h after challenge. The oedema was calculated as the difference between the volume of the right and left paws. The control group was treated only with SRBCs.

In all three experiments, the mice were killed by means of cervical dislocation, after which paws or ear punches from each animal were used for histological analysis. In each experiment, activity is expressed as an inhibition percentage with reference to the control group.

Histology

Ear samples were fixed in 4% neutral-buffered formalin (Panreac, Barcelona, Spain). Each sample was cut longitudinally into half. One half was embedded in paraffin (Panreac), cut into 3 to 4 μm sections and stained with hematoxylin–eosin, trichrome stain, periodic acid Schiff and toluidine blue (all from Sigma-Aldrich). Paws and knees were removed and fixed in 10% formalin. The paws were then trimmed, placed in decalcifying solution for 24 h, embedded in paraffin, sectioned at 5 μm and stained with trichromic Masson (prepared with products from Sigma-Aldrich). Epithelium thickness was evaluated under $\times 100$ magnification and expressed as the mean ± s.e.mean of the number of epidermal layers from the basal to the granulous stratum, both inclusive. A representative area of the inflammatory cellular response was then selected for semi-quantitative cell counting in 20 fields at $\times 40$ magnification. The inflammatory cells, lymphocytes, macrophages and neutrophils were counted in the papillary as well as in the reticular dermis/subcutis layers, and conventionally expressed as 1–2–3 units according to their relative abundance. Samples were studied with the aid of light microscopy (Dialux 22 Leitz, Wetzlar, Germany).

Immunohistochemistry

The organs obtained were trimmed, placed in decalcifying solution for 24 h and then 8 μm sections were prepared from paraffin-embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% H_2O_2 in 60% methanol for 30 min. The sections were permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) in phosphate-buffered saline (PBS; Sigma-Aldrich) for 20 min. Nonspecific adsorption was minimized by incubating the section in 2% normal goat serum (Sigma-Aldrich) in PBS for 20 min. Endogenous biotin- or avidin-binding sites were blocked by means of sequential incubation for 15 min with avidin and biotin (LSAB2, Glostrup, Denmark). The sections were then incubated overnight with primary anti-cyclooxygenase-2 (1:500) (SC-1747; Santa Cruz, CA, USA) with control solutions. Control included buffer alone or non-specific purified rabbit IgG (Sigma-Aldrich). Specific labelling was detected with a biotin-conjugated goat anti-rabbit IgG (LSAB2) and avidin–biotin peroxidase complex (LSAB2).

Determination of cell toxicity

The cytotoxicity of demethylnobiletin on cells (murine RAW 264.5 macrophages and human neutrophils and lymphocytes) was tested with the aid of the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay. Cells (1×10^{-6} cells per ml) were exposed to the product at concentrations of 25, 10, 5 and 2.5 μM in a microplate for 30 min (neutrophils), 24 h (macrophages) and 4 days (lymphocytes). The medium was then removed and 100 μl per well of a 0.5-mg ml^{-1} solution of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide were added and incubated at 37 °C until blue deposits were visible. The coloured metabolite was then dissolved in dimethyl sulphoxide. Absorbance was measured at 490 nm using a Labsystems Multiskan EX plate reader (Helsinki, Finland). Results were expressed in absolute absorbance readings; a decrease indicated a reduction in cell viability.

T lymphocyte isolation and proliferation

Lymphocytes from human blood buffy coat residues were isolated by means of the Ficoll–Paque gradient density method. Under sterile conditions, cells were resuspended to a concentration of 10^6 cells ml^{-1} in RPMI-1640 medium (Invitrogen, Langley, OK, USA), supplemented with 2% heat-inactivated foetal calf serum (Invitrogen), 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (Invitrogen). A volume of 200 μl of cell suspension was applied onto each well of a 96-well flat-bottomed plate (Nunc, Raskilde, Denmark) with or without 0.5 μg ml^{-1} phytohaemagglutinin (PHA). Demethylnobiletin was added to the cells at concentrations ranging from 5, 2.5 and 1.25 μM along with dexamethasone (positive control at 1 μM). The plates were incubated in 5% CO_2-air-humidified atmosphere at 37 °C for 4 days, after which time T-cell proliferation was determined with the aid of a modified colorimetric 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay. The formazan product thus formed was dissolved in dimethyl sulphoxide by shaking on a microtitre plate shaker. The optical density was measured with an enzyme-linked immunosorbant assay reader (Labsystems Multiskan EX) at 490 nm. Controls consisted of lymphocytes with either PHA (100% activity) or medium (0% activity), or of samples with non-stimulated lymphocytes.

Cell cycle analysis

To analyse the cell cycle, T lymphocytes were first adjusted to a final volume of 10^6 cells per ml before use, and then 1 ml of cell suspension was placed into each well of a 24-well flat-bottomed plate (Nunc) with or without PHA (0.5 μg ml^{-1}) after treatment with the products. Then two different cell cycle experiments were conducted. First, either demethylnobiletin (25, 10 and 5 μM) or cycloheximide (30 μM; Sigma-Aldrich) was added to the cells and the plates were incubated in 5% CO_2-air-humidified atmosphere at 37 °C for 24 or 72 h. In this experiment, we were looking for the sub G_0 peak as an index of apoptosis, and then for the G_1, G_2 and S peaks. The cells were harvested by means of centrifugation, washed in PBS (pH 7.2) and then fixed in 70% ethanol for 30 min at −20 °C. After washing the cells once with PBS, DNA was stained with propidium iodide (4 μg ml^{-1}; Sigma-Aldrich) containing 100 μg ml^{-1} of ribonuclease A (Sigma-Aldrich). For the second experiment, demethylnobiletin (10 μM) was

added at different times (0, 3, 6, 18, 24, 48 and 72 h) after the cells had been stimulated with PHA. The plates were then incubated in 5% CO_2-air-humidified atmosphere at 37 °C for 72 h, and the protocol described above was carried out. In this second test, we wanted to observe at which times and in which phases the cell cycle was blocked. Flow cytometry analysis was conducted with the aid of a Beckman Coulter EPICS XCL (Fullerton, CA, USA); 10^4 cells were counted for each test sample.

Detection of apoptosis in human lymphocytes

T lymphocytes (1 ml of 2×10^6 cells per ml) were placed into each well of a 24-well flat-bottomed plate (Nunc) with or without 0.5 μg ml^{-1} PHA. Demethylnobiletin (10 μM) was added to the cells and the plates were incubated in 5% CO_2-air-humidified atmosphere at 37 °C for 72 h. The cells were collected by means of centrifugation and the pellet was resuspended in lysis buffer (100 mM EDTA, 10 mM Tris-HCl); then 10% sodium dodecyl sulphate and 100 μg ml^{-1} proteinase K (Sigma-Aldrich) were added to the homogenate, which was subsequently incubated for 1 h at 50 °C. Proteins were precipitated by the addition of 5 M NaCl and the samples were centrifuged at 20 000 *g* for 15 min. The supernatant was mixed with an equal volume of phenol–chloroform–isoamyl alcohol (25:24:1) and centrifuged at 20 000 *g* for 15 min. The uppermost layer was collected in a new vial, mixed with an equal volume of chloroform–isoamyl alcohol (24:1) and centrifuged at 20 000 *g* for 5 min. Finally, the newly formed uppermost layer was mixed with 2-propanol, 10 mM $MgCl_2$ and 10% glycogen (1 μl) and placed on ice for 15 min. DNA fragments were collected from this layer. To these fragments, 70% ethanol was added and the mixture was centrifuged at 20 000 *g* for 10 min. The desiccated pellet of DNA fragments was then dissolved in 25 μl of Tris–EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4). Contaminating RNA was removed by incubating the samples with RNAse A (1 mg ml^{-1}) at 37 °C for 20 min. Purified DNA (1 μg) was subjected to electrophoresis on a 2% agarose gel. The gel was stained with 0.5 μg ml^{-1} ethidium bromide solution, viewed on a UV transilluminator and photographed.

Annexin V apoptosis assay

The detection of phosphatidylserine on the cell surface of human lymphocytes was performed with the Annexin V–fluorescein isothiocyanate apoptosis detection kit (BD Biosciences Pharmingen, San Diego, CA, USA), in accordance with the manufacturer's instructions. Briefly, non-stimulated and PHA-stimulated cells (10^6 ml^{-1}) were cultured in 24-well flat-bottomed plates and subsequently exposed (or not) to demethylnobiletin (25, 10 and 5 μM) and 30 μM cycloheximide for 24 h. The cells were collected, washed with cold PBS and resuspended in binding buffer. The cells were then stained with Annexin V–fluorescein isothiocyanate and propidium iodide. After a 30 min incubation period, the cells were analysed with the aid of flow cytometry.

Analysis of caspase 3 activity

The activity of caspase 3 was determined with the cell-permeable fluorogenic substrate Ac-Asp–Glu–Val–Asp-AMC (Ac-DEVD-AMC; EnzoLyte AMC Capase-3 Assay kit; Anaspec, San Jose, CA, USA), which was used according to the manufacturer's instructions. The activity was quantified by measuring fluorescence intensity, after cleavage by caspase 3, at Ex/Em = 354/442 nm. Purified lymphocytes (10^6 cells per ml) were isolated and cultured in a 96-well black tissue culture microplate, and treated with demethylnobiletin (25, 10 and 5 μM) and either cycloheximide (30 μM; Sigma-Aldrich) or Ac-DEVD-CHO (10 μM). They were then stimulated with PHA for 24 h. The caspase 3 substrate (Ac-DEVD-AMC) was added to the cells and the fluorescence was measured with a Victor Fluorescence microplate reader (Perkin Elmer, Wellesley, MA, USA).

Determination of cytokine production in T lymphocytes

T lymphocytes (10^6 cells per ml) were cultured with PHA alone or with demethylnobiletin at various concentrations (10.0–0.3 μM) for 1 or 3 days. The cell supernatants were then collected and assayed at 24 h for interleukin-1β, interleukin-2, interleukin-4 and interleukin-10, and again at 72 h for TNF-α and interferon-γ. To this end, an enzyme immunoassay kit from eBioscience (San Diego, CA, USA) was used in accordance with the manufacturer's instructions.

Prostaglandin E_2 production in RAW 264.7 macrophages

Macrophages (RAW 264.7) at 1×10^6 cells ml^{-1} were co-incubated in a 96-well culture plate (200 μl) with either lipopolysaccharide (1 μg ml^{-1}) alone or with lipopolysaccharide (1 μg ml^{-1}) and demethylnobiletin at concentrations ranging from 10 to 2.5 μM for 24 h. The cell supernatants were then collected and assayed for prostaglandin E_2 with a specific enzyme immunoassay kit (ECL; GE Healthcare Buckinghamshire, UK) used according to the manufacturer's instructions. In this experiment, prostaglandin E_2 production was assessed as an index of cyclooxygenase-2 activity.

Nitrite production and nitric oxide activity induction in intact RAW 264.7 macrophages

RAW 264.7 murine macrophages were cultured in Dulbecco's Modified Eagle's medium containing 2 mM L-glutamine, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 10% foetal bovine serum (all from Invitrogen). Cells were removed from the tissue culture flask with a cell scraper and resuspended until a final volume of 1×10^6 cells ml^{-1} in free serum medium was reached. Nitrite production was assessed as the index of nitric oxide generation in the induction phase. Macrophages (RAW 264.7) were co-incubated in 96-well culture plates (200 μl) with 1 μg ml^{-1} of lipopolysaccharide (Sigma-Aldrich) at 37 °C for 24 h in the presence of demethylnobiletin (10, 5 and 2.5 μM), dexamethasone (5 μM) or vehicle (PBS). The concentration of nitrites was determined in the culture supernatant with the Griess reagent (Sigma-Aldrich).

Macrophages (RAW 264.7) at 1×10^6 cells ml^{-1} were stimulated with lipopolysaccharide in the absence of test compounds. After 24 h, the cells were washed with fresh medium and incubated for 2 h in the presence of the test

compounds. Supernatants were collected in order to measure the nitrite accumulation during the last 2 h. The concentration of nitrite was determined in the culture supernatant with the Griess reagent. In this experiment, nitrite production was assessed as the index of NOS activity.

Western blot analysis

To study the inhibition of NOS induction, RAW 264.7 macrophages (10^6 cells per well) were co-incubated in a 24-well culture plate (200 μl) with 1 μg ml^{-1} lipopolysaccharide at 37 °C for 24 h in the presence of demethylnobiletin (10 μM), dexamethasone (5 μM) or vehicle (PBS). Cellular lysates were obtained with lysis buffer (Complete mini EDTA-free protease inhibitor cocktail; Roche, Basel, Switzerland). Following centrifugation (10 000 *g*, 10 min), the proteins present in the supernatants were quantified. Equal amounts of protein (30 μg) were then loaded on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis gel, transferred onto polyvinylidene difluoride membranes and kept for 90 min at 125 mA. The membranes were then blocked in PBS–Tween 20 containing 3% defatted milk and incubated with anti-NOS-2 polyclonal antibody (1:1000 dilution). Blots were washed and incubated with peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:20 000 dilution). The immunoreactive bands were visualized with an enhanced chemiluminiscence system (ECL; GE Healthcare). Western blot quantification was carried out with Scion image software version 1.0.0.1 (Frederick, MA, USA).

Statistics

The data are expressed as mean ± s.e.mean for *in vivo* experiments and mean ± s.d. for *in vitro* experiments. Statistical analysis was performed with a two-way analysis of variance followed by the Bonferroni test or a one-way analysis of variance and Dunnett's *t*-test for multiple comparisons, depending on the experiment. When making comparisons with the control group, values of $P<0.05$ were considered significant. Inhibition percentages (%I) were calculated from the differences between the drug-treated group and the control animals treated only with the inflammatory agent. The IC_{50} value was calculated from the dose-effect linear regression plot made with at least four concentrations. Statistical analysis was performed with the aid of a GraphPad Prism 4 statistics package (GraphPad Software, San Diego, CA, USA).

Reagents

Demethylnobiletin (5-*O*-demethylnobiletin; Figure 1) was obtained from *S. tragoriganum* (see Bas *et al*., 2006). Biochemicals, chemicals, reagents and materials were purchased from Anaspec, eBioscience, GE Healthcare, Invitrogen, LSAB2, Nunc, Panreac, Roche, Santa Cruz and Sigma-Aldrich.

Results

Effects on DTH reactions induced by oxazolone, DNFB and SRBCs

Demethylnobiletin (Figure 1) was applied to the ears of mice at a dose of 0.5 mg per ear and it reduced the DTH reaction induced in the ear by oxazolone by 29% (24 h), 23% (48 h) and 28% (72 h), whereas the reference drug dexamethasone (0.025 mg per ear) inhibited the reaction by 90, 98 and 90% at the same times (Figure 2a). The DTH reaction induced by DNFB in mice decreased by 44% (24 h), 41% (48 h) and 36% (72 h) after the application of demethylnobiletin at 0.5 mg per ear, whereas dexamethasone (0.025 mg per ear) inhibited the reaction by 84, 96 and 91% (Figure 2b). In the DTH reaction induced by SRBCs in mouse paws, demethylnobiletin at 15 mg kg^{-1} (intraperitoneal) significantly reduced the oedema by 57% 48 h after challenge, but had no effect at either 18 or 24 h. In contrast, dexamethasone (10 mg kg^{-1}) significantly inhibited the oedema formation at 18, 24 and 48 h by 64, 67 and 48%, respectively (Figure 2c).

Seventy-two hours after challenge, the ears treated only with oxazolone (Figure 3a; Table 1) showed a characteristic lesion with oedema, along with diffuse cell infiltration in a 1:1 ratio of neutrophils to lymphocytes with no macrophages in the dermis. The epidermis exhibited both papillomatosis and acanthosis without spongiosis and exocytosis; there was also a mild degree of hypertrophy and hyperplasia of fibroblasts and subpapillar fibrosis (Figure 3a1). The ear treated with demethylnobiletin (Figure 3a2) presented histological changes with respect to the control group, including a reduction of oedema, focal infiltration with a reduction of lymphocytes and neutrophils, and the suppression of both papillomatosis and acanthosis. The dexamethasone-treated group (Figure 3a3) presented neither oedema nor focal or diffuse infiltration. Papillomatosis and acanthosis were both observed, but to a lesser degree than in the control group.

The analysis of ears 72 h after the application of DNFB to the animal (Figure 3b; Table 1) showed an inflammatory lesion that affected the dermis with oedema, produced cell infiltration in which the ratio of neutrophils–lymphocytes–macrophages was 2:2:1 and led to papillar fibrosis. The epidermis itself presented minimal to mild papillomatosis, acanthosis and exocytosis (Figure 3b1). The ear treated with demethylnobiletin (Figure 3b2) presented histological changes with respect to the control group. These changes were similar to those found in the dexamethasone-treated group (Figure 3b3), which had isolated neutrophils, a minimal inflammatory lesion and reductions of both the oedema and epithelium thickness (2.4 ± 0.1 cells). Other parameters of the inflammatory process, including papillomatosis and acanthosis, were also attenuated in both the flavone- and dexamethasone-treated groups.

The histological study of paws (Figure 3c; Table 1) obtained from the control group (48 h after challenge with SRBCs) showed characteristic signs of inflammation, with mixed focal infiltration (neutrophils, macrophages and lymphocytes) and marked oedema. The lesion principally affected connective tissue in the dermis, muscle tissue, fascia and tendons (Figure 3c1). There was also minimal articular inflammation with pannus bridging <20% along with isolated cysts with fibrin. The effect on cartilage and bone was minimal, with less than 5% of the bone surfaces (osteoclasts) affected. Neither osteoid nor periosteal regeneration was present (Figure 3c1). Mice treated with demethylnobiletin exhibited similar symptoms, but with

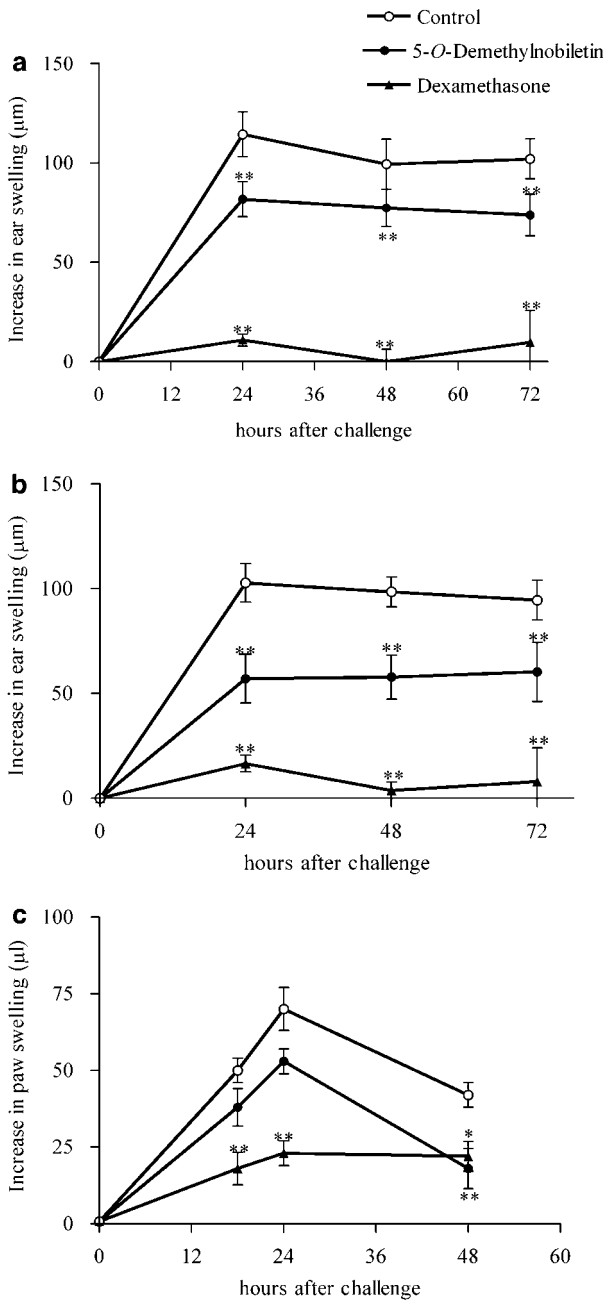

Figure 2 Effects of demethylnobiletin (0.5 mg per ear) and dexamethasone (0.025 mg per ear) on DTH reactions induced by oxazolone (**a**), DNFB (**b**) and SRBC (**c**) in mice; $n=6$ animals; ear swelling increase in μm (**a, b**); paw volume increase in μl (**c**). Each measure represents the mean ± s.e.mean of the increase in tissue swelling calculated with respect to the same group on day 0, before application of the irritant. Statistical significance of difference from the control $^{**}P<0.01$, $^{*}P<0.05$ by Dunnett's multiple comparison test.

neither cysts nor osteoclasts. The inflammatory lesion was attenuated and focalized in the connective tissue. The inflammatory infiltrate was formed by lymphocytes, macrophages and neutrophils (Figure 3c2). Animals treated with dexamethasone showed an inflammatory profile similar to that of the group treated with the flavonoid. In the articulations, there was a discrete oedema and sparse inflammatory infiltrate, which was predominantly made up of lymphocytes (Figure 3c3).

The immunohistochemical assay showed the expression of cyclooxygenase-2 in the cell cytoplasm of mononuclear cells in the control group, with a reduction of cyclooxygenase-2 expression in ears treated with demethylnobiletin (Figure 4b). The group treated with dexamethasone also presented a reduction in the number of cells expressing this enzyme (Figure 4c). The immunohistochemical characterization of lymphocytes indicated a mixed cell infiltrate with a predominance of lymphocyte $CD4^+$ over $CD8^+$ (data not shown).

Determination of cell toxicity, T lymphocyte proliferation assay and cell cycle analysis

In the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay, demethylnobiletin at 10 μM showed no toxicity against human neutrophils, human lymphocytes or murine macrophages (RAW 264.7).

Moreover, demethylnobiletin significantly inhibited T cell proliferation 72 h after PHA stimulation, with an IC_{50} value of 2.76 μM (10–1 μM; $r^2=0.9999$, $P=0.0074$, $n=3$), whereas dexamethasone inhibited T cell proliferation by 78% at 1 μM (control 170 ± 31; blank 57 ± 5**; dexamethasone 82 ± 10**; $n=3$). After stimulation with PHA and with no further treatment, T cells generally enter the G_1 phase within 2–4 h, the S phase after approximately 18–24 h and finally reach the G_2/M phase between 36–48 h. After 24 and 72 h of incubation either with or without demethylnobiletin, the cell cycle was analysed with the aid of a propidium iodide reagent before undergoing flow cytometry analysis. Our results indicated that the resting T cells stayed mainly in the G_0/G_1 phase, whereas cells stimulated with phytohemagglutinin first entered the S phase and then the G_2/M phase. In addition, some cells actually became apoptotic after stimulation (sub G_0 phase). Demethylnobiletin did not modify the cell cycle at 24 h, as can be seen from the fact that at that point the cells behaved similar to those of the control group, but at 72 h, treated cells at all concentrations were still in the G_0/G_1 phase, a result similar to that obtained when cycloheximide was used as a reference drug (Table 2).

To establish the exact point at which demethylnobiletin inhibits cell proliferation, different time-course experiments were performed. Thus, 10 μM of test compound was added to cell cultures at times ranging from 0 to 72 h. After 72 h of incubation, the cell cycle of all groups was analysed with the aid of a propidium iodide reagent and subsequent flow cytometry analysis. The flavonoid was found to halt the cell cycle between the G_0/G_1 and S phases, usually before the 24-h mark (Table 3). Moreover, at 25 and 10 μM, demethylnobiletin induced apoptosis of T lymphocytes (Figure 5). This effect was confirmed by means of the Annexin V assay, in which the flavonoid was found to increase the number of apoptotic cells with respect to the blank and the control groups (Table 4).

Furthermore, demethylnobiletin had a notable effect on caspase 3 activity, inhibiting it by 52% at 25 μM (control 21.6 ± 2.6 r.f.u.; blank 5.5 ± 0.1** r.f.u.; demethylnobiletin 12.7 ± 1.0** r.f.u.; $n=3$). The response was dose dependent and decreased as the final concentration was reduced. In the

Table 1 Quantification of inflammatory lesions induced by oxazolone or DNFB in mouse ear or by SRBCs in mouse paws, according to a semiquantitative scale

	Oxazolone		*DNFB*		*SRBC*	
Control	Epidermis	2	Epidermis	2/3	Cartilage	0
	Papillary dermis	3	Papillary dermis	3	Connective tissue	3
	Reticular dermis	3	Reticular dermis	3	Synovial tissue	1/2
Demethylnobiletin	Epidermis	0/1	Epidermis	0/1	Cartilage	0
	Papillary dermis	1/2	Papillary dermis	1	Connective tissue	2
	Reticular dermis	1	Reticular dermis	1	Synovial tissue	1
Dexamethasone	Epidermis	0	Epidermis	0	Cartilage	0
	Papillary dermis	0/1	Papillary dermis	0/1	Connective tissue	1/2
	Reticular dermis	0/1	Reticular dermis	0/1	Synovial tissue	1

Abbreviations: DNFB, dinitrofluorobenzene; SRBC, sheep red blood cell.
These values represent the mean of 20 fields at $\times$ 40. Grades of inflammation are as follows: 0, no inflammation; 1, weak; 2, mild 3, severe. Inflammatory effects of oxazolone or DNFB were assessed in ear tissue 72 h after challenge; demethylnobiletin or dexamethasone was applied topically to the ear at 6, 24 and 48 h after challenge. Inflammation after SRBC treatment was assessed in paws 48 h after challenge; demethylnobiletin or dexamethasone was given intraperitoneally immediately before, and 16 h after, challenge.

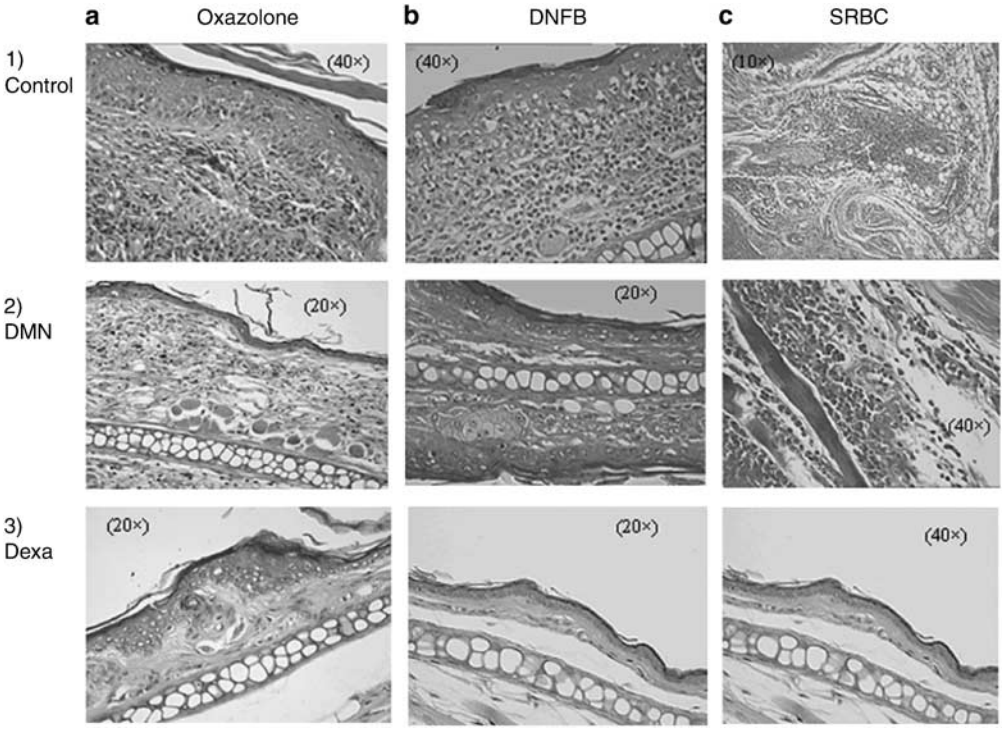

Figure 3 Haematoxylin–eosin-stained section of ears after application of oxazolone (72 h after challenge) (**a**), DNFB (72 h after challenge) (**b**) and paws after sensitization with SRBCs (48 h after challenge) (**c**). (**1**) Control: ears and paws treated only with oxazolone (**a1**) or DNFB (**b1**), and paws treated only with SRBC (**c1**). (**2**) Ears treated with demethylnobiletin (DMN) in the DTH induced by oxazolone (**a2**) or DNFB (**b2**), and paws treated with demethylnobiletin in the DTH induced by SRBC (**c2**). (**3**) Ear treated with dexamethasone (Dexa) in the DTH induced by oxazolone (**a3**) or DNFB (**b3**), and paws treated with dexamethasone in the DTH induced by SRBC (**c3**).

case of the reference drugs, the reduction of caspase 3 activity was 27% for cycloheximide at 30 μM (17.1 ± 0.9** r.f.u., $n = 3$) and 98% for the specific caspase 3 inhibitor, Ac-DEVD-CHO, at 10 μM (0.42 ± 0.02** r.f.u.; $n = 3$).

Cytokine production by human T lymphocytes

When non-stimulated T cells were treated with10 μM of demethylnobiletin, the basal levels of cytokines were not affected, except for that of interleukin-1β, the production of which increased by 50% with respect to the PHA-stimulated group (control). In stimulated T lymphocytes, however, the results were quite different. For example, at a concentration of 2.5 μM, demethylnobiletin decreased the production of interleukin-1β by 46%, while at higher concentrations the effect was lower (control 1175 ± 383 pg ml^{-1}; blank 26 ± 10**; and demethylnobiletin 1000 ± 26 at 10 μM, 719 ± 53** at 5 μM, 636 ± 40** at 2.5 μM and 802 ± 29**at 1.25 μM; $n = 3$). The production of interleukin-2 decreased depending on the concentration of the flavonoid, with an IC_{50} value of 1.63

(10.0–1.25 μM; $r^2=0.9193$, $P=0.0412$, $n=3$), whereas for interleukin-4 the effect was not significant (2.76 μM, 10.0–1.25 μM; $r^2=0.9749$, $P=0.1012$, $n=3$), respectively. In the case of interleukin-10, the presence of demethylnobiletin at a concentration of 10 μM increased the production of this cytokine by 46%, while the effect was slightly mitigated at lower concentrations (control 4039 ± 376 pg ml^{-1}; blank 675 ± 17**; and demethylnobiletin 5602 ± 161** at 10 μM, 5511 ± 152** at 5 μM, 5020 ± 507** at 2.5 μM and 4704 ± 353** at 1.25 μM; $n=3$). The production of TNF-α was drastically reduced by demethylnobiletin, with an IC_{50} value of 0.66 μM (10.0–0.3 μM; $r^2=0.9836$, $P=0.0083$, $n=3$). Similarly, the production of interferon-γ was clearly reduced by the flavone, with an IC_{50} value of 1.35 μM (10.0–0.3 μM; $r^2=0.9951$, $P=0.0447$, $n=3$). Values of cytokine inhibition by dexamethasone at 1 μM against interleukin-1β, interleukin-2, interleukin-4, interleukin-10, TNF-α and interferon-γ were 80% (256 ± 73** pg ml^{-1}, $n=3$), 79% (136 ± 14** pg ml^{-1}, $n=3$), 93% (22 ± 10** pg ml^{-1}, $n=3$),

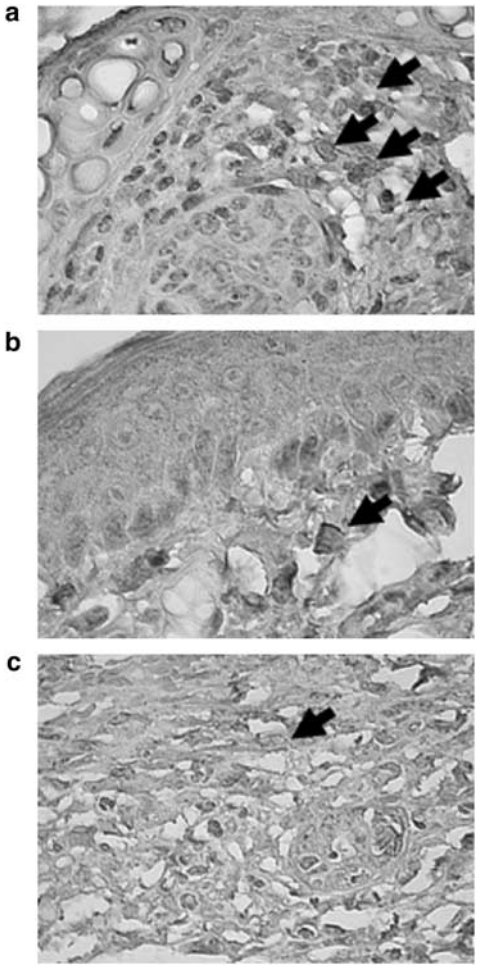

Figure 4 Immunohistochemical study of ears after sensitization with oxazolone (× 40). (**a**) Control: ear treated only with oxazolone, (**b**) ear treated with demethylnobiletin and (**c**) ear treated with dexamethasone. Cells with cytoplasmic staining for COX-2 are marked by an arrow.

Table 2 Modification of T cell proliferation by different concentrations of DMN at 24 and 72 h after stimulation with PHA

	Sub G_0	*G_0/G_1*	*S*	*G_2/M*
Cell cycle (24 h)				
Blank (vehicle)	3.2 ± 0.7***	92.7 ± 0.7***	0.3 ± 0.1	2.8 ± 0.1*
Control (PHA)	6.8 ± 1.0	82.4 ± 1.5	1.0 ± 0.9	4.6 ± 0.9
CHX (30 μM)	27.7 ± 1.9***	65.9 ± 0.8***	1.4 ± 0.2	2.4 ± 0.2**
DMN (25 μM)	6.7 ± 0.9	85.5 ± 1.0***	0.7 ± 0.2	3.7 ± 0.8
DMN (10 μM)	6.2 ± 0.9	85.2 ± 0.9***	0.9 ± 0.3	4.6 ± 0.6
DMN (5 μM)	4.9 ± 0.7**	86.2 ± 0.7***	0.8 ± 0.4	4.5 ± 0.8
Cell cycle (72 h)				
Blank (vehicle)	2.1 ± 0.3	97.2 ± 3.2***	0.9 ± 0.1***	0.3 ± 0.1***
Control (PHA)	5.0 ± 1.9	75.0 ± 2.8	12.5 ± 2. 7	9.5 ± 1.8
CHX (30 μM)	14.8 ± 2.7***	84.3 ± 1.9***	1.9 ± 0.4***	1.9 ± 0.4***
DMN (25 μM)	7.9 ± 0.6*	89.2 ± 1.7***	1.5 ± 0.4***	2.9 ± 1.0***
DMN (10 μM)	6.6 ± 1.0	89.1 ± 1.9***	2.5 ± 0.4***	3.0 ± 1.3***
DMN (5 μM)	5.8 ± 0.9	89.9 ± 1.0***	2.0 ± 0.3***	2. 9 ± 1.1***

Abbreviations: CHX, cycloheximide; DMN, demethylnobiletin; PHA, phytohaemagglutinin.
Blank represents the non-stimulated group, control represents the cells stimulated with PHA, CHX represents cells treated with PHA and cycloheximide, DMN represents cells treated with PHA and DMN at different concentrations. CHX was used as reference drug. Values shown in the table are the percentage of cells at the stage of cell cycle shown (mean ± s.d.; $n=3$); ***$P<0.001$, **$P<0.01$, *$P<0.05$ (Bonferroni test), compared with control values.

Table 3 Modification of the T cell cycle after stimulation with PHA by DMN added at different times after PHA

	Time	*Cell cycle*			
	(h)	*Sub G_0*	*G_0/G_1*	*S*	*G_2/M*
Blank	72	3.1 ± 0.6***	94.7 ± 1.2***	2.4 ± 0.5***	0.5 ± 0.1***
Control	72	5.4 ± 1.0	78.4 ± 1.2	7.9 ± 1.0	8.7 ± 1.1
Time of addition of DMN (10 μM) after stimulation with PHA	0	3.7 ± 0.8*	85.8 ± 0.8***	5.0 ± 0.8***	5.9 ± 0.3***
	3	4.2 ± 1.5	84.1 ± 1.2***	6.8 ± 1.7	5.2 ± 0.8***
	6	4.1 ± 1.5	82.9 ± 0.3***	7.5 ± 2.0	5.7 ± 0.2***
	18	5.3 ± 1.7	82.8 ± 1.1***	6.2 ± 0.9*	6.2 ± 0.9***
	24	4.1 ± 1.1	82.8 ± 0.8***	6.0 ± 1.0**	7.3 ± 1.3*
	48	5.8 ± 1.3	81.8 ± 1.8***	7.2 ± 0.5	5.5 ± 0.9***
	72	5.3 ± 1.1	77.6 ± 2.9***	9.5 ± 1.9*	7.6 ± 1.1

Abbreviations: DMN, demethylnobiletin; PHA, phytohaemagglutinin.
Blank represents the non-stimulated group and control represents the cells stimulated with PHA. DMN was added at different times (h) after PHA, as shown in the Table. Values shown in the table are the percentage of cells at the stage of cell cycle shown (mean ± s.d.; $n=3$); ***$P<0.001$, **$P<0.01$, *$P<0.05$ (Bonferroni test), compared with control values.

86% ($1146 \pm 94^{**}$ pg ml^{-1}, $n=3$), 79% ($356 \pm 20^{**}$ pg ml^{-1}, $n=3$) and 93% ($3.0 \pm 1.6^{**}$ ng ml^{-1}, $n=3$), respectively.

Effects of demethylnobiletin on arachidonic acid metabolism, nitrite production and NOS induction in intact RAW 264.7 macrophages

Demethylnobiletin was found to inhibit prostaglandin E_2 production in RAW 264.7 macrophages by 47% at 10 μM (control 258.3 ± 62.1; blank $68.3 \pm 11.4^{**}$; demethylnobiletin $168.7 \pm 41.4^{**}$, $n=3$). Interestingly, this inhibition did not diminish at lower concentrations: 50% at 5 μM and 46% at 2.5 μM (demethylnobiletin 5 μM $163.6 \pm 45.4^{**}$ and 2.5 μM $170.9 \pm 36.1^{**}$, $n=3$). Dexamethasone also inhibited prostaglandin E_2 production by 52% at 10 μM ($159.1 \pm 20.2^{**}$, $n=3$). In addition, demethylnobiletin reduced nitrite production in RAW 264.7 macrophages ($IC_{50}=7.53$ μM, range 10–2.5, $r^2=0.9958$, $P=0.0413$, $n=3$), but did not modify NOS-2 expression, as assessed by western blot analysis (data not shown).

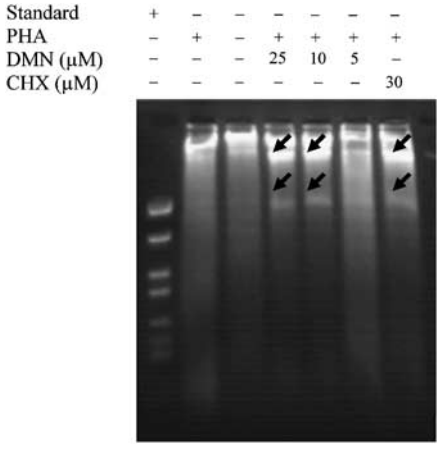

Figure 5 Analysis of genomic DNA fragmentation in human lymphocyte cells. Lane 1 corresponds to the standard. Lane 2 corresponds to the DNA ladder of cells treated with PHA (control) and lane 3 to the DNA ladder of non-treated cells (blank). Cells treated with demethylnobiletin (DMN) 25, 10 and 5 μM (lanes 4, 5 and 6) and cycloheximide 30 μM (CHX, lane 7).

Discussion

Our results show that demethylnobiletin not only mitigated the inflammatory reaction to the application of allergens such as oxazolone, DNFB and SRBC, but that it also reduced the activation and proliferation of human lymphocytes stimulated with PHA. Specifically, our findings demonstrate a reduction in the ear oedema induced by oxazolone and DNFB, with a decrease in both tissue damage and cell infiltration. Nevertheless, the best effect was against the inflammation induced by the application of SRBC, with effects in the last phase similar to those of dexamethasone. Moreover, these results demonstrate that demethylnobiletin exerts a proliferation-suppressive action, which is not related to cell viability. In fact, analysis of the cell cycle showed that demethylnobiletin actually inhibited the passage of PHA-stimulated lymphocytes into the S phase of the cell cycle 72 h after stimulation in much the same way as the reference drug cycloheximide. This effect may be directly related to the suppression of T cell proliferation. In addition, demethylnobiletin inhibited the production of proinflammatory cytokines such as interleukin-1β, interleukin-2, interleukin-4 and especially TNF-α and interferon-γ, while simultaneously increasing the production of the anti-inflammatory cytokine interleukin-10. In the case of interleukin-2, this cytokine, together with interleukin-1β, drives the proliferation of T cells and their differentiation to type-1 helper lymphocytes (Hou *et al*., 2006); these latter cells are crucial in the response to allergenic agents (Wang *et al*., 2000). In addition, interferon-γ induces both NOS-2 expression and nitric oxide synthesis by the T cells. However, during contact hypersensitivity reactions, nitric oxide is also produced by other inflammatory cells such as Langerhans cells and keratinocytes, and contributes to swelling and infiltration of effector cells (Ross and Reske-Kunz, 2001). Furthermore, interleukin-1β and TNF-α have a stimulatory effect on local endothelium, on the attraction of leukocytes and the upregulation of surface molecules on resident cells during the elicitation of contact hypersensitivity responses and, whereas interleukin-4 is the predominant cytokine in contact hypersensitivity, interferon-γ is strongly expressed in DTH reactions (Grabbe and Schwarz, 1998). Indeed, the

Table 4 Apoptosis in non-stimulated and PHA-stimulated T cells, and its modification by DMN and CHX

	Cells			
	Alive	*Apoptotic*	*Transition*	*Necrotic*
Blank (vehicle)	$85.9 \pm 0.3^{***}$	$10.8 \pm 0.6^{***}$	$1.9 \pm 0.2^{***}$	$1.5 \pm 0.3^{***}$
Control (PHA)	76.3 ± 0.5	16.6 ± 0.6	4.2 ± 0.5	2.9 ± 0.3
CHX (30 μM)	$78.1 \pm 0.4^{+++}$	$15.8 \pm 0.7^{+++}$	$3.4 \pm 0.3^{+++}$	$3.7 \pm 0.2^{+++}$
CHX (30 μM) + PHA	$62.8 \pm 0.7^{***}$	$27.6 \pm 0.5^{***}$	$5.7 \pm 0.5^{***}$	$3.9 \pm 0.4^{*}$
DMN (25 μM)	$76.1 \pm 0.5^{+++}$	$20.0 \pm 0.5^{+++}$	$3.3 \pm 0.3^{++}$	0.6 ± 0.2
DMN (25 μM) + PHA	$72.5 \pm 0.7^{***}$	$22.6 \pm 0.6^{***}$	3.5 ± 0.4	$1.3 \pm 0.2^{***}$
DMN (10 μM)	$84.8 \pm 0.7^{+}$	11.1 ± 0.7	1.8 ± 0.2	2.3 ± 0.4
DMN (10 μM) + PHA	$68.4 \pm 0.5^{***}$	$25.5 \pm 0.7^{***}$	4.9 ± 0.4	$1.3 \pm 0.2^{***}$
DMN (5 μM) + PHA	$71.2 \pm 0.6^{***}$	$22.2 \pm 0.5^{***}$	$5.3 \pm 0.6^{*}$	$1.2 \pm 0.2^{***}$

Abbreviations: CHX, cycloheximide; DMN, demethylnobiletin; PHA, phytohaemagglutinin.
Blank represents the non-stimulated group and control represents the cells stimulated with PHA only. Values shown in the table are the percentage of cells in each class (mean ± s.d.; $n=3$); $^{***}P<0.001$, $^{*}P<0.05$ as compared with the control group; $^{+++}P<0.001$, $^{++}P<0.01$, $^{+}P<0.05$ as compared with the blank group; Bonferroni test; $n=3$.

suppression of all these cytokines by demethylnobiletin may be directly related with the observed pharmacological effects of the compound.

We also noted that demethylnobiletin modified lymphocyte recruitment in the tissue and that this event probably involved an apoptotic mechanism. This was supported by the observation of DNA fragmentation in the lymphocytes, as detected by ethidium bromide agarose gel electrophoresis. In addition, the flavonoid decreased the proliferation of activated T cells. While there are no studies to date on the effect of this flavonoid on cell proliferation, there is a previous report on the inhibition of cell proliferation by 3′-demethoxy nobiletin (tangeretin), which inhibited thymidine transport in stimulated lymphocytes and depressed the expression of class II histocompatibility antigens in human peripheral blood monocytes with a reversible effect (Middleton and Kandaswami, 1992). This effect may be the same as that of demethylnobiletin on the proliferation of lymphocytes in our assays.

To date, there have been no reports on the activity of demethylnobiletin on inflammations induced by DTH reactions. In a previous study, Delaney *et al*. (2001) reported the effect of a standardized mixture of polymethoxylated flavones, including demethylnobiletin, on SRBC-immunized mice. The mixture of flavonoids produced a mild suppression of natural killer cell activity, but did not suppress humoral immunity. These researchers demonstrated that the natural killer cell activity was actually reduced by an inhibition of TNF-α production. Similar results against DTH reactions induced by SRBCs and DNFB have been previously described for other related flavonoids, such as plantagoside, a pentahydroxyflavanone derivative that inhibited the *in vitro* immune response of mouse spleen cells to SRBC in a concentration-dependent manner (Middleton and Kandaswami, 1993). These data are in line with those obtained in our experiments, and emphasize the role of demethylnobiletin as an inhibitor of DTH reactions.

Mechanistic studies of the effects of polymethoxyflavones on DTH reactions are also very scarce. For example, Lin *et al*. (2003) demonstrated that nobiletin (the 5-methyl derivative of our compound) suppressed the interleukin-1-induced production of prostaglandin E_2 in human synovial fibroblasts, downregulated cyclooxygenase-2 (but not cyclooxygenase-1) mRNA expression and interfered with both lipopolysaccharide-induced production of prostaglandin E_2 and the gene expression of interleukin-1α, interleukin-1β, TNF-α and interleukin-6 in mouse J774A.1 macrophages. These researchers proposed a mechanism similar to that of dexamethasone for these anti-inflammatory and immunomodulatory properties. This mechanism could also be valid for demethylnobiletin, since both compounds have the same properties, albeit with different relative potencies (Ishiwa *et al*., 2000). In a similar vein, Manthey *et al*. (1999) reported the inhibition of TNF-α production by demethylnobiletin ($IC_{50} = 5\,\mu M$) in human lipopolysaccharide-stimulated monocytes. This compound, together with heptamethoxyflavone (same IC_{50} value), showed the highest potency of all the methoxyflavones tested, including nobiletin ($IC_{50} = 10\,\mu M$). This particular study thus demonstrated that the degree of methoxylation influences the inhibitory effect on TNF-α production; however, the authors only studied the effect of heptamethoxyflavone on the expression of interleukin-10 and the macrophage inflammatory protein-1α, since the compound did not inhibit the expression of interleukin-1β, interleukin-6 or interleukin-8. Nevertheless, heptamethoxyflavone did induce a substantial increase of cAMP levels in monocytes by directly inhibiting phosphodiesterase activity, which could, in part, provide a mechanism for the inhibition of cytokine production.

Different skin cells such as keratinocytes, Langerhans cells, melanocytes, fibroblasts and endothelial cells, are capable of synthesizing nitric oxide through several NOSs, including NOS-2 (Bruch-Gerharz *et al*., 1998). Nitric oxide plays a relevant role in contact dermatitis, causing an inflammatory response in the implicated tissues. After elicitation by means of allergen contact, there is a nonspecific phase characterized by low nitric oxide levels, in which both vasodilatation and attraction of neutrophils take place. Subsequently, the antigen-specific phase develops, marked by high nitric oxide levels. This in turn leads to the inhibition of mediator release by the mast cells, downregulation of adhesion molecules, disruption of neutrophil attraction and apoptosis of effector cells (Ross and Reske-Kunz, 2001). Since corticosteroids suppress NOS-2 synthesis, they serve as a therapy for contact dermatitis, but a more selective suppression might obtain similar results while avoiding the negative side effects of corticosteroids (Ahluwalia, 1998). Some flavonoids such as kaempferol (flavonol) and apigenin (flavone) are potent inhibitors of NOS-2 induction at the level of NOS-2 gene transcription, while concomitantly increasing the endothelial NOS-3 activity (Olszanecki *et al*., 2002). Nobiletin, as mentioned above, has been found to suppress the expression of NOS-2 by 52% and of cyclooxygenase-2 by 42% (Murakami *et al*., 2003). Interestingly, in our study, although demethylnobiletin at 5 μM decreased nitric oxide production ($IC_{50} = 7.53\,\mu M$), it did not modify the induction of the enzyme, which indicates that the reduction of nitric oxide is not due to an effect on NOS expression. Furthermore, it inhibited prostaglandin E_2 production in RAW 264.7 macrophages by 50% at 5 μM. In addition, we had previously found that demethylnobiletin had little effect on cyclooxygenase induction, but that it inhibited 5-lipoxygenase activity, decreasing leukotriene B_4 release ($IC_{50} = 0.35\,\mu M$) by directly inhibiting the enzyme (Bas *et al*., 2006). All these findings support a mechanism of action involving an inhibitory effect on arachidonic acid metabolism together with the inhibition of nitric oxide production. The compound did not affect the induction of the enzymes, but acted by directly inhibiting them. Moreover, while inhibitors of NOS-2 induction and nitric oxide production helped ameliorate the inflammatory process somewhat, specific cyclooxygenase and 5-lipoxygenase inhibitors had no effect on the inflammation, even when administered together, although the levels of prostaglandins and leukotrienes decreased. In the end, only corticoids reduced all the parameters of contact hypersensitivity, including swelling, cell infiltration and prostaglandin and leukotriene levels (Meurer *et al*., 1988; Zunic *et al*., 1998). For this reason, and because demethylnobiletin is a common non-toxic citroflavonoid, this polymethoxyflavone and its related compounds could be good alternatives for treating

inflammatory reactions induced by DTH, especially in order to avoid the side effects of corticosteroids.

The present results indicate that demethylnobiletin, which is present in the extracts of *S. tragoriganum* and species of *Citrus*, may reduce tissue inflammation in part by inhibiting T lymphocyte proliferation and the expression of different mediators. We hypothesize that the inhibitory effects of demethylnobiletin on PHA-activated lymphocyte proliferation are related to decreases in the production of cytokines, arachidonate metabolites and nitric oxide, and to the way in which the compound affects the T cell cycle by reducing entry into the S phase. Taken together, these findings explain the effect of demethylnobiletin as an anti-inflammatory agent in DTH reactions, as it reduces both cell infiltration and the production of different mediators.

Acknowledgements

This work was supported by the Spanish Government (SAF2006-06726). We are indebted to the Centre de Transfusions de la Comunitat Valenciana (Valencia, Spain) for its generous supply of human blood.

Conflict of interest

The authors state no conflict of interest.

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