Inhibition of Delayed-Type Hypersensitivity by Cucurbitacin R Through the Curbing of Lymphocyte Proliferation and Cytokine Expression by Means of NFAT Translocation to the Nucleus

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Running Title

a) Cucurbitacin R inhibits NFAT translocation

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d) Abbreviations

DAPI, 6,4’-Diamidino-2-phenylindole dihydrochloride; DNFB, dinitrofluorobenzene; DTH, delayed-type hypersensitivity; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbertent assay; EMSA electrophoretic mobility shift assay; JAK2, Janus kinase-2; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NFAT, nuclear factor-AT; NF-κB, nuclear factor-κB; RT-PCR, reverse transcription-polymerase chain reaction; SRBC, sheep red blood cells; STAT3, signal transducer and activator of transcription-3; TCR, T cell receptors; TNF-α, tumour necrosis factor-α; TPA, 12-O-tetradecanoylphorbol 13-acetate.
ABSTRACT

Cucurbitacin R is known to exhibit an anti-inflammatory effect in different experimental models of inflammation. In this paper, we outline the effect of cucurbitacin R on T lymphocyte proliferation, cytokine production, and nuclear factor activation as well as its influence on various experimental models of delayed-type hypersensitivity (DTH) in mice. Cucurbitacin R reduced the proliferation of phytohemagglutinin A-stimulated human T lymphocytes (IC₅₀ 18 μM), modifying the cell cycle as well as the production of cytokines (interleukin-2, interleukin-4, interleukin-10, and especially interferon-γ) and the induction of the principal cyclins implicated in the cell cycle (A₁, B₁, D₂, and E). These effects are brought on by a novel, selective inhibition of NFAT by cucurbitacin R, with no concomitant effect on other transcription factors such as AP-1. In addition, we tested the in vivo effects of cucurbitacin R in three experimental models of DTH as well as its effects on T lymphocyte proliferation, the cell cycle, cytokines, and cyclins. While cucurbitacin R was found to reduce the inflammatory response brought on by both oxazolone and dinitrofluorobenzene (DNFB), its activity was even more pronounced against sheep red blood cells (SRBC)-induced oedema in mouse paws, with a clear reduction in the production of interleukin-1β, interleukin-4, and tumour necrosis factor (TNF-α) in the inflamed paw. In conclusion, cucurbitacin R has the potential to be a new immunosuppressive agent with antiproliferative effects through the inhibition of the NFAT with anti-inflammatory activity in DTH reactions.
Introduction

Delayed-type hypersensitivity (DTH), a peripheral expression of cell-mediated immunity, is triggered by antigen-specific T cells and can be induced by different allergens such as oxazolone, dinitrofluorobenzene (DNFB), and sheep red blood cells (SRBC). The first two compounds lead to contact hypersensitivity, which occurs in two phases. In the induction phase, the hapten reacts with protein to form a complex that is recognized by Langerhans cells, which then migrate to the lymph nodes, where CD4+ lymphocytes recognize the antigen. The elicitation phase occurs after further contact with the hapten, giving rise to the recruitment of antigen-specific T cells and resulting in severe dermal inflammation (Scott et al., 2002). This, in turn, leads to the release of different mediators such as interferon-γ, tumour necrosis factor (TNF)-α, interleukin-2, interleukin-4, and interleukin-10 by T lymphocytes; interleukin-12 and interleukin-18 by Langerhans cells (Garaczi et al., 2004); nitric-oxide by keratinocytes and Langerhans cells (Ross and Reske-Kunz, 2001); and interleukin-1β (Zunic et al., 1998), leukotrienes, and prostaglandins by macrophages (Meurer et al., 1988). In contrast, the DTH induced by SRBC is an immunological reaction mediated by specific, committed T cells. The reaction peaks consistently at 18 h, eliciting on the fourth day after immunization to give the maximal response, and then decreases progressively until the fourth month (Hurtrel et al., 1984).

In previous studies, we have demonstrated the anti-inflammatory and anti-arthritic properties of cucurbitacin R (Recio et al., 2004; Escandell et al., 2007a). The results from these studies suggest that cucurbitacin R may be a potential anti-inflammatory agent that acts by means of a mechanism in which the inhibition of TNF-α in lymphocytes is implicated (Amin et al., 1999). Likewise, the activation of both Janus kinase-2 (JAK2) and signal transducer and activator of transformation-3 (STAT3) is involved, as previously demonstrated for other closely related
Indeed, Escandell et al. (2007a) reported that the anti-arthritic effect of cucurbitacin R was due to the inhibition of STAT3 activation, the reduction of TNF-\(\alpha\), and the decrease of both nitric-oxide and prostaglandin E\(_2\) production, as well as the inhibition of the induction of nitric-oxide synthase, but not of cyclooxygenase-2. However, this same study did not corroborate previous descriptions of cucurbitacin R as an inhibitor of the transcriptional activation of nitric-oxide synthase induction through the blocking of nuclear factor-\(\kappa\)B (NF-\(\kappa\)B) (Park et al., 2001; 2004).

Nuclear factor-AT (NFAT) is a family of transcription factors that regulates the inducible expression of different cytokines and cell surface receptors critical for the immune response. Four of the five known NFAT members are regulated by calcineurin, which is a key intermediate of the calcium signalling pathway (Lee and Park, 2006). The activation of cell surface receptors coupled to calcium-signalling pathways sets off a mechanism in which the activated calcineurin dephosphorylates NFAT proteins and induces the movement of these proteins into the nucleus, where they cooperate with other proteins to form complexes on the DNA of the cell. This type of nuclear importation is opposed by kinases such as glycogen synthase kinase 3, thereby rendering transcription that is continuously responsive to receptor occupancy. It has been reported that in both activated and resting T cells, the phosphorylation levels of NFAT are determined by a dynamic equilibrium between trough calcineurin activation and kinase inhibition; thus, any stimulus results in dephosphorylation of NFAT and nuclear localization of the transcription factor (Crabtree and Olson, 2002).

In this paper, we offer a description and an analysis of the effects of cucurbitacin R on the lymphocyte cell cycle and proliferation to outline a possible mechanism of action and to determine the influence of the NFAT activity. In addition we tested the effect of cucurbitacin R
on DTH reactions induced by oxazolone, DNFB, and SRBC in order to gain insight into the compound’s anti-inflammatory activity in different types of hypersensitive reactions. We have also studied its effects on the pro-inflammatory mediators implicated in DTH.

**Methods**

**Reagents.** Cucurbitacin R was previously isolated from *Cayaponia tayuya* roots by Recio et al. (2004). Biochemicals, chemicals, reagents, and materials were purchased from Anaspec (San Jose, CA), eBioscience (San Diego, CA), GE Healthcare (Buckinghamshire, UK), Invitrogen (Langley, OK), LSAB2 (Glostrup, Denmark), Nunc (Raskilde, Denmark), Panreac (Barcelona, Spain), PharMingen (San Diego, CA), Roche (Basel, Switzerland), Santa Cruz Biotechnologies (Santa Cruz, CA), and Sigma-Aldrich (St Louis, MO). Enzyme linked immunoabsorbent assay (ELISA) kits for cytokine detection were purchased from eBioscience (San Diego, CA) and biotin-conjugated goat anti-rabbit immunoglobulin G and avidin-biotin peroxidase complex LSAB 2 System-HRP were obtained from Dako (Glostrup, Denmark).

**Animals.** Groups of six female Swiss mice weighing 25-30 g (8 weeks old) from Harlan Interfauna Iberica (Barcelona, Spain) were used for all the experiments. All animals were fed a standard diet *ad libitum*. Housing conditions and all in vivo experiments were approved by the Institutional Ethics Committee of the Faculty of Pharmacy (University of Valencia, Spain) in accordance with the guidelines established by the European Union on Animal Care (CEE Council 86/609).

**DTH induced by oxazolone, DNFB, and SRBC.** Female mice were sensitized through topical application onto their shaved abdomens of 150 μl of a 3% solution of oxazolone (Sigma-Aldrich) in acetone on day 1. 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 ears. Cucurbitacin R was tested at doses of 0.1, 0.3, and 0.5 mg per ear while dexamethasone (Sigma-Aldrich) was administered at a dose of 0.025 mg per ear. Both test compounds dissolved in acetone were applied topically (20 µl) to the ears 2, 24, and 48 h after challenge (single application). Ear thickness measurements of both the treatment and control groups were taken with a micrometer (Mitutoyo Series 293, Kawasaki, Japan); 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. The mice were challenged on day 6 through application of 20 µl of 0.2% DNFB in acetone to the inner and outer ear surfaces. Cucurbitacin R was tested at doses of 0.1, 0.3, and 0.5 mg per ear while dexamethasone (Sigma-Aldrich) was administered at a dose of 0.025 mg per ear. Both test compounds dissolved in acetone were applied topically (20 µl) to the ears 2, 24, and 48 h after challenge (single application). 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.

SRBC (2 × 10⁷, Sigma-Aldrich) in phosphate buffered-saline was injected s.c. into the shaved backs of the mice. Challenge was performed 5 days later through injection of 1 × 10⁸ red blood cells into the right hind paws of the mice; their left paws were injected with the same volume of vehicle. Cucurbitacin R (10 mg/kg) and dexamethasone (10 mg/kg) dissolved in EtOH-Tween 80-water (1:1:10) were administered i.p. immediately before (0 h) 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 (inflamed) and left (non-inflamed) paws. The control group was challenged with SRBC and treated with saline only.

Inhibitions are expressed as the % of the degree of the inflammation response with respect to the inflammation induced by each agent (oxazolone, DNFB, SRBC), set as 100%. The mice were sacrificed by means of cervical dislocation just after the last measurements and samples from ear punches or paws were taken from each animal. The samples were placed in 4% formalin (Panreac) and histological sections were prepared.

**Histology and immunohistochemistry.** Ear samples fixed in 4% neutral-buffered formalin were cut longitudinally into equal halves. One half was then embedded in paraffin (Sigma-Aldrich), cut into 3 to 4 μm sections, and stained with trichromic Masson prepared with products from Sigma-Aldrich. Experimental protocols were carried out as previously described in Bas et al. (2007). The inflammatory cells – lymphocytes, macrophages, and neutrophils – were counted in the papillary and in the reticular dermis/subcutis layers within an area of 1000 μm². For all immunohistochemistry assays (Escandell et al., 2007b), antibodies (Santa Cruz biotechnologies) of CD4⁺ (SC1140) and CD8⁺ (SC7188) were used.

**Cytokine measurement from ex vivo samples.** To determine the presence of cytokines in ears and paws from the control, cucurbitacin R-treated, and dexamethasone groups, samples were treated following the protocol described in Escandell et al. (2007a). TNF-α, interleukin-1β, and interleukin-4 were measured with the aid of an enzyme immunoassay (eBioscience) employed according to the manufacturer’s instructions.

**T Lymphocyte proliferation assay, cell cycle analysis, cell viability, and cytotoxicity.**

Peripheral lymphocytes were obtained from healthy human blood buffy coat residues. An
analysis of the cell cycle as well as various assays to determine cell proliferation, viability, and cytotoxicity were performed following the protocols previously described by Bas et al. (2007). Briefly, T lymphocytes were isolated with the aid of the Ficoll–Paque gradient density method under sterile conditions. Monocytes were removed by culturing the cells for 4 hours and then discarding the adherent cells. The lymphocyte yield was at least 95% before starting the experiment. For the proliferation assay, cucurbitacin R was added to the cells at concentrations ranging from 10 to 30 μM. For the cell cycle analysis, cucurbitacin R was used at 30 μM and either dexamethasone (5 μM) or aphidicolin (Sigma-Aldrich, 0.75 μM) was used as a positive control.

**Determination of cytokine production in T lymphocytes.** T lymphocytes (2 × 10⁵ cells per well) were cultured with phytohemagglutinin A (12.5 μg/ml, Sigma-Aldrich) alone or in combination with various concentrations of cucurbitacin R for 4 days. The cell supernatants were then collected and assayed for interleukin-2, interleukin-4, interleukin-10, and interferon-γ concentrations with the aid of enzyme-linked immunosorbent assay (ELISA, eBioscience).

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR).** Lymphocytes (1×10⁶/ml) were treated with cucurbitacin R (30 μM) for 18 h, after which the RNA was extracted on RNeasy mini spin (50) columns (Qiagen, Hilden, Germany). 1 μg of RNA was transformed to cDNA and the aliquots were amplified with the aid PCR, which was performed in an air thermocycler in accordance with the manufacturer’s instructions. Briefly, 2.5 μl of the first-strand complementary deoxyribonucleic acid (cDNA) was mixed with 0.75 μM primers (Invitrogen) of interferon-γ (sense: 5’-GCATCGTTTTGGGTCTTGTTACTGC-3’; antisense: 5’-CTCCTTTTTCGCTTCTGCTGCTGG-3’), IL-2 (sense: 5’-AACAGTGACCTACTTCAAG-3’; antisense: 5’-GTTGAGATGATGCTTTGACA-3’), IL-4
(sense: 5'-ATGGGTCTCACCTCCCAACTGCT-3'; antisense: 5'-GAACACTTTGAATATTTC TCTCTCAT-3'), IL-10 (sense: 5'-ATCCCCCAAGCTGAGAACCAAGACCCA-3'; antisense: 5'-TCTCAAGGGGCTGGGTCAGCTATCCCA-3'), and β-actin (sense: 5'-GCAGAGCAAGAGAGGCCATCC-3'; antisense: 5'-CTGTGGGTGGAAGCTGTAG-3'). The thermocycler conditions were 94 °C for 1 min, with an annealing temperature of 60 °C for 1 min and an elongation temperature of 72 °C for 1 min for the first 30 cycles, followed by an elongation temperature of 72 °C for 10 min more. After the reaction was completed, the amplified product was removed from the tubes and run on 2% agarose gel (Sigma-Aldrich).

**Western blot analysis.** Whole cell lysates from lymphocytes incubated for 24 h with phytohemagglutinin A (12.5 μg/ml) and cucurbitacin R (30 μM) were obtained as previously described by Escandell et al. (2007a). Antibodies for cyclins A (Santa Cruz, H-230), B (Santa Cruz, H-433), D2 (Santa Cruz, H-289), and E (Santa Cruz, C-19) were used along with those for β-actin. In addition, Jurkat cells and human lymphocytes were treated with cucurbitacin R for 1 h at 50 and 30 μM, respectively, and then stimulated with phytohemagglutinin A for 1 h. Whole cell extracts or nuclear and cytosolic cell extracts of lymphocytes and Jurkat cells were obtained as described by Ishiguro et al. (2007) and tested for NFAT antibody (Millipore 07-136, Billerica, MA). Western blot quantification was carried out with the aid of Scion image software, version 1.0.0.1 (Frederick, MA).

**Immunofluorescence.** T lymphocytes and Jurkat cells were grown separately on chamber slides and were either left untreated or treated with cucurbitacin R for 1 h and then stimulated with phytohemagglutinin A for one additional h. The cells were fixed in methanol-acetic acid (95:5) for 20 min at –20 °C and then incubated with NFAT antibody for 2 h at 37 °C. The slides were then washed with phosphate-buffered saline and incubated with a secondary anti-rabbit
antibody conjugated to Alexa 485 (Invitrogen) for 45 min at 37 °C. 6,4’-Diamidino-2-phenylindole dihydrochloride (DAPI, Sigma-Aldrich) was used to stain DNA in order to determine whether the NFAT was localized in the cytoplasm or the nucleus. Samples were then examined and analyzed with a fluorescent microscope (Nikon, Tokyo, Japan).

**NFAT reporter gene assay.** Stably transfected Jurkat cells with an NFAT/luc reporter gene were kindly provided by Prof. Baldari (University of Siena, Italy). The cells were treated with various concentrations of cucurbitacin R for 1 h and then treated with phytohemagglutinin A for 24 h. Luciferase activity in the cell extracts was quantified with the aid of a kit from Promega (Madison, WI). Direct quantification of luciferase activity in transfected cells was carried out at room temperature as follows: first, the cells were pelleted in microcentrifuge tubes, washed once in phosphate-buffered saline, and resuspended in 100 μl Promega luciferase reagent buffer (20 mM Tricine, 1.07 mM (MgCO₃)Mg(OH)₂×5·H₂O, 2.67 mM MgSO₄, 0.1 mM EDTA, 33.3 mM dithiothreitol, 270 μm coenzyme A, 470 μm luciferin, 530 μm ATP, 0.2% Triton X-100, pH 7.8). Light emission was then measured as relative light units in a BioOrbit 1253 luminometer (Turku, Finland).

**Electrophoretic mobility shift assay (EMSA) and determination of calcium cell influx into the cells.** Human lymphocytes or Jurkat cells were incubated with various concentrations of cucurbitacin R for 1 h. The cells were then stimulated for 1 h with phytohemagglutinin A or 12-O-tetradecanoylphorbol 13-acetate (TPA)/ionomycin, after which the nuclear protein was extracted. The protein content of the supernatant was determined with the aid of Bradford reagent, after which 10 μg of nuclear protein were loaded onto a 6% non-denatured polyacrylamide gel. EMSA were carried out with a DIG-gel shift kit (Roche Diagnostics, Mannheim, Germany) in accordance with the manufacturer’s protocol.
Lymphocytes \((0.5 \times 10^6)\) were placed into supplemented RPMI with 1.5 mM Ca\(^{2+}\) and then 4 μM of FLUO-3 (Invitrogen) were added for each incubation. The cells were treated with cucurbitacin R at different concentrations and the resulting suspensions were mixed and incubated for 1 h at 37 °C in 5% CO\(_2\) in air. Loaded cells were then measured for 30 s before the administration of lectins, after which changes in the intensity of relative Fluo-3 fluorescence were recorded at every 10-s interval for 300 s with a Beckman Coulter Epics flow cytometer (Fullerton, CA). All flow cytometric experiments were repeated at least three times.

**Statistics.** Statistical analysis was performed by means of a one-way analysis of variance (ANOVA) followed by Dunnett’s \(t\)-test for multiple comparisons. Values of \(P < 0.05\) were considered significant. Inhibition percentages (\%I) were calculated from the differences between drug treated groups and control animals treated only with the inflammatory agent. IC\(_{50}\) values were calculated from the dose/response linear regression plots.

**Results**

**Effects of cucurbitacin R on lymphocyte proliferation and the cell cycle.** Previous studies revealed cucurbitacin R (Fig.1A) as a new anti-inflammatory and immunosuppressive agent. To test its effect on lymphocyte proliferation, a phytohemagglutinin A-stimulated T lymphocyte assay was performed. Addition of cucurbitacin R to activated T lymphocytes inhibited cell proliferation in a concentration-dependent manner. Thus, at 30 μM, cucurbitacin R inhibited T cell proliferation by 100%, giving an IC\(_{50}\) value of 18 μM \((r^2 = 0.9998,\) range assayed 30 – 10 μM, 5 dilutions, \(n = 3\)). A detailed cell cycle analysis revealed that while the inhibitor aphidicolin stopped the cell cycle in the S phase of lymphocyte proliferation, cucurbitacin R and
Dexamethasone arrested the cycle of phytohemagglutinin A-stimulated cells in the G0 phase (Fig. 1B).

Time course experiments were performed to determine at what point in the activation process cucurbitacin R inhibited T cell proliferation. After stimulation with phytohemagglutinin A, T lymphocytes generally enter into the G0/G1 phase within 2-4 h, then into the S phase at 18-24 h, finally reaching the G2/M phase after 36-48 h (data not shown). Cucurbitacin R was thus added to cultures at 0, 3, 6, 10, 18, 24, 48, and 72 h and the cell cycle and proliferation assays were performed at 96 h. The results indicated that after stimulation, addition of cucurbitacin R between 0 and 6 h appeared to exert the highest suppressor effect on T cell proliferation (Fig. 1C and 1D). In contrast, addition of cucurbitacin R to the cultures 10 or 18 h after activation only slightly attenuated cell proliferation and cell cycle progression. The fact that cucurbitacin R had the higher suppressor effect when added between 0 – 10 h suggests that the inhibitory action of cucurbitacin R may be related either to the blocking of biochemical events or to the inhibition of the gene expression necessary for T cell proliferation which is otherwise activated with phytohemagglutinin A during this time.

**Effect of cucurbitacin R on the cytokine transcription and protein synthesis implicated in the human T lymphocyte cycle.** To evaluate the relevance of cytokine production for the inhibitory effects of cucurbitacin R on T cell proliferation after stimulation with phytohemagglutinin A, T cells were incubated with or without various concentrations of cucurbitacin R for 4 days. Supernatants were then collected and the production of interleukin-2, interleukin-4, interleukin-10, and interferon-γ was assayed with the aid of ELISA. Cytokine production (pg/ml) after treatment with cucurbitacin R was then compared with that of a blank group (non-treated cells), a control group (phytohemagglutinin A-stimulated cells), and a
positive control group with dexamethasone (10 μM). Cucurbitacin R clearly inhibited the production of interleukin-2 (Fig. 2A), interleukin-4 (Fig. 2B), interleukin-10 (Fig. 2C), and interferon-γ (Fig. 2D) by stimulated T lymphocytes. In light of this finding, we decided to test whether cucurbitacin R affected the expression of cytokine mRNA in activated T cells. We thus examined the effect of cucurbitacin R on the expression of mRNA for the four cytokines assayed and compared the results with the control group (phytohemagglutinin A-stimulated cells, Fig. 3A, lane 2) and the blank group (no stimulation, Fig. 3A, lane 1). In all cases, the cytokine mRNA expression was clearly reduced with respect to the control group (Fig. 3A, lane 4). Moreover, cucurbitacin R had no effect on this expression in the absence of a stimulus (Fig. 3A, lane 3).

To determine whether this observed effect on cell cycle progression was related to the expression of cyclins implicated in the different phases of the cell cycle in stimulated T lymphocytes, the cells were first incubated with or without cucurbitacin R and then the total cell proteins were extracted. The results demonstrated that while cucurbitacin R did not affect the expression of cyclins in non-stimulated cells (Fig. 3B, lane 3), it clearly reduced the expression of cyclins A₁, B₁, D₂, and E in stimulated cells with respect to the control group, as shown in Fig. 3B, lane 4.

**Effect of cucurbitacin R on NFAT in human lymphocytes.** NFAT plays a crucial role in T lymphocyte regulation. It is activated in response to protein kinase C activation and calcium mobilization, both of which are triggered by T cell receptor (TCR) stimulation. In vitro, a combination of a phorbol ester such as TPA and a calcium ionophore or stimulation with lectins can mimic TCR-generated signals. In addition, phytohemagglutinin A-stimulated T lymphocytes have been shown to activate proliferation through calcium mobilization and NFAT
dephosphorylation. We thus decided to examine the effect of cucurbitacin R on NFAT with the aid of EMSA and Western blot techniques. We found that while cucurbitacin R did not affect calcium mobilization (data not shown), it inhibited activation of NFAT (Fig. 4A). Surprisingly, cucurbitacin R did not inhibit NFAT dephosphorylation (Fig. 4B).

To confirm the cellular localization of NFAT in cucurbitacin R-treated cells, we performed NFAT immunofluorescence assays in human T lymphocytes. We found that in non-stimulated cells (blank), NFAT was located in the cytoplasm while in phytohemagglutinin A-stimulated cells it was clearly translocated to the nucleus. In phytohemagglutinin A-stimulated cells treated with cucurbitacin R, however, NFAT remained in the cytoplasm. Cells that are treated with cucurbitacin R but not stimulated with phytohemagglutinin A thus have the same characteristics as cells from the blank group (Fig. 5).

In order to confirm the effect of cucurbitacin R on NFAT, we carried out various tests, including an NFAT reporter gene assay, immunofluorescence and EMSA assays, and Western blot analysis in Jurkat cells. To test whether cucurbitacin R inhibited T cell proliferation and to determine the role of NFAT in this process, we used stably transfected Jurkat cells with the gene that encodes firefly luciferase under the control of a trimer of the NFAT binding site on the interleukin-2 gene promoter. We found that cucurbitacin R inhibited NFAT activity at a concentration of 50 µM in a dose-dependent manner (Fig. 6A). Moreover, cucurbitacin R did not inhibit the dephosphorylation of NFAT. However, the NFAT remained in the cytoplasm and was not translocated to the nucleus (Fig. 6B). To corroborate these findings, we performed an immunofluorescence assay of NFAT on Jurkat cells. As was the case in human T lymphocytes, the NFAT remained in the cytoplasm (Fig. 7).
To test the specificity of cucurbitacin R on NFAT inhibition, we performed EMSA for both NFAT and AP-1 on Jurkat cells activated with various stimuli. We found that cucurbitacin R inhibited the activation of NFAT in phytohemagglutinin A-stimulated Jurkat cells (Fig. 8A) and also in Jurkat cells stimulated with ionomycin/TPA (Fig. 8B). However, in no case did cucurbitacin R affect the activation of AP-1 (data not shown). These results demonstrate the specificity of the mechanism used by cucurbitacin R to inhibit T cell proliferation.

**Effects of cucurbitacin R on DTH induced by DNFB, oxazolone, and SRBC.** DTH is a cell-mediated immunity in which T cells are implicated. To test the antiproliferative effect of cucurbitacin R in lymphocytes and its NFAT inhibitory effect we performed three different DTH models to test cucurbitacin R effect in vivo. At doses of 0.3 and 0.5 mg per ear, cucurbitacin R inhibited the DTH reactions induced by oxazolone and DNFB in mouse ears, but had no effect at 0.1 mg per ear. In the oxazolone test, the effect manifested itself 24 h after challenge at doses of 0.3 mg and 0.5 mg per ear, but in the resolution phase, only the higher dose was effective. In contrast, the effect in the DNFB test was slight, but significant at 0.3 mg and 0.5 mg per ear in both the early and late phases of the reaction. In the DTH reaction induced by SRBC in mouse paws, cucurbitacin R at 12.5 mg/kg significantly reduced oedema formation at 18 h, 24 h, and 48 h after challenge; these values are similar to those obtained with dexamethasone at 10 mg/kg (Table 1). The effect of cucurbitacin R on cytokine reduction for TNF-α was higher in the SRBC test, for IL-1β in the oxazolone experiment, and for IL-4 in the DNFB test (Table 2).

**Histology and histochemistry.** The histological examination of the paws of animals treated only with SRBC (control group) showed no inflammatory lesion in the articular tissue. There was, however, an inflammatory lesion in the conjunctive tissue, fascia, and muscles, as well as
intense, acute inflammation with an abundance of polymorphonuclear leukocytes and macrophages (data not shown) as well as CD4+ and CD8+ lymphocytes (Fig. 9A). The paws treated with cucurbitacin R presented a mild inflammation with a reduced infiltrate of a mixture of polymorphonuclear leukocytes and lymphocytes (data not shown), with no clear predominance of either CD4+ or CD8+ cells (Fig. 9B). The paws of mice treated with dexamethasone (Fig. 9C) showed characteristics similar to those from the cucurbitacin R-treated group.

**Discussion**

Cucurbitacins are an interesting group of natural triterpenes with anti-inflammatory and cytotoxic properties. Depending on their double bonds and substituents, their degree of potency and/or toxicity can vary. In the case of cucurbitacin R, the absence of both a double bond at C23-C24 and an acetyl in C25, clearly reduce the range of cytotoxicity with respect to other cucurbitacins. However, no specific studies on its toxicity in animals have been reported. In one previous study (Ríos et al., 1990), the acute toxicity of a mixture of cucurbitacins was described in mice, with the authors obtaining LD50 values of 375 mg/kg per os and 67 mg/kg intraperitoneally. This data, along with the cytotoxicity values observed in the in vitro experiments reported here and previous in vivo data concerning the anti-arthritic potential of cucurbitacin R all indicate that this compound is a potential anti-inflammatory agent.

As a reaction triggered by antigen-specific T cells, DTH is an important in vivo manifestation of a cell-mediated immune response. The DTH reaction is characterized by the expansion of antigen-specific Th1 type CD4+ T cells during the initial phase and an inflammation response by Th1 cytokines released from CD4+ T cells during the effector phase (Baumer et al., 2003; Kermani et al., 2000; Grabbe and Schwarz, 1998). In the present study, we have demonstrated
that cucurbitacin R reduces the inflammatory response in DTH, especially in the tuberculinic-type reaction induced by SRBC, in a range similar to that of dexamethasone. This effect may be related to the reduction of the Th1 cytokine TNF-\(\alpha\) in inflamed tissue; it may also be enhanced by the inhibition of other Th1 cytokines such as interleukin-2 and especially interferon-\(\gamma\) in stimulated human T lymphocytes. Once stimulated, T lymphocytes secrete a range of cytokines, including interferon-\(\gamma\) and interferon-2, which promote local recruitment of cells from the blood (Rosen et al., 1989). These, in turn, release new mediators, notably interferon-\(\gamma\), interleukin-2, interleukin-4, interleukin-10, and TNF-\(\alpha\), which induce the symptoms of the reactions. Of these mediators, interferon-\(\gamma\) is a key cytokine for immunomodulation and, along with interleukin-2, it is a relevant effector of the immune response (Grassegger and Högfl, 2004). However, as in clinically delayed hypersensitivity reactions, cytokine pathways are often overlapped, with one preferential reaction dominating at the final reaction. Interleukin-1\(\beta\) is required for hapten-specific T cell priming in the sensitization phase after challenge and for the induction and production of TNF-\(\alpha\), which is necessary for the induction of local inflammation during the elicitation phase (Nakae et al., 2003). Furthermore, the production of TNF-\(\alpha\) and interleukin-1\(\beta\) regulates the expression of the pro-inflammatory enzymes nitric-oxide synthase and cyclooxygenase-2 (Bruch-Gerharz et al., 1998). For its part, interleukin-4 is a key cytokine in the development of T cells, especially Th2 cells (Nagai et al., 2000). Because of its effects on these three mediators, cucurbitacin R could be an interesting new agent for the treatment of DTH induced by various agents.

To determine exactly how cucurbitacin R exerts these effects, we have to examine the principal event in the generation of the immune response, namely the activation and clonal expansion of T lymphocytes. These are spontaneously arrested in the \(G_0/G_1\) phase and remain quiescent for long
periods until they are exposed to specific agents (Chen et al., 2007). The activation of T cells with agents such as antigens or phytohemagglutinin A triggers the biochemical events that induce resting T cells to enter into the cell cycle after proliferation and differentiation (Kuo et al., 2003), during which the transcription factor NFAT is involved. Thus, before stimulation, lymphocytes are in the G0/G1 phase while after stimulation, they pass to the S phase and finally to the M phase. This cycle is accompanied by the expression of a series of genes such as those for interleukin-2, interferon-\(\gamma\), and the cyclins, which are expressed in a carefully controlled order as the cells pass through the G0, G1, and S phases (Ajchenbaum et al., 1993; Liu et al., 2004). Indeed, cell cycle activation is in part coordinated by the D-type cyclins, which are rate limiting and essential for the progression through the G1 phase of the cycle (Chen et al., 2007). We found that addition of cucurbitacin R affected the cell cycle of stimulated T lymphocytes without exerting any effect on cell viability. Indeed, the cell cycle analysis showed that cucurbitacin R inhibited the entry of stimulated cells into the S phase of the cycle, as could be seen by the T cell suppression. As discussed above, cell stimulation leads to T cell proliferation, which is controlled at specific stages of the cell cycle by different cyclin-dependent kinases. These enzymes are activated by cyclins D and E during the G1 phase, cyclin A in the S phase, and cyclin B in the transition from the G2 to the M phase (Chen et al., 2007). When lymphocytes were treated with cucurbitacin R, the expression of cyclins A1, B1, D2, and E was suppressed, together with that of the cytokines interleukin-2 and interferon-\(\gamma\), thus arresting the cell cycle in the G0/G1 phase. The inhibition of the transcription factor NFAT by cucurbitacin R is involved in all these effects.

The NFAT family of transcription factors plays a pivotal role in the development and function of the immune system as a whole. The activation process of these transcription factors plays is
tightly regulated by calcium-dependent phosphatase calcineurin and has been a target of the immunosuppressive drugs cyclosporin A and tacrolimus (FK-506). In spite of their toxicity, both of these drugs exhibit a potent immunosuppressive activity and have revolutionized the field of organ transplants. Tacrolimus has also recently been approved for use in the treatment of atopic dermatitis (Nghiem et al., 2002). However, while the clinical use of these drugs has dramatically increased the success of organ transplants, their therapeutic use is limited by their severe side effects (Crabtree and Olson, 2002). For this reason, NFAT continues to be a worthy research target for developing new immunosuppressant drugs. The most important factor in the toxicity of the existing drugs is their ability to inhibit calcineurin in cells outside of the immune system (Proksch et al., 2005). Unlike cyclosporine A and tacrolimus, however, cucurbitacin R neither modifies calcineurin activity nor does it produce dephosphorylation of NFAT, thereby avoiding the translocation of NFAT from the cytoplasm to the nucleus. This mechanism is similar to that reported for the acetyl-derivative in Jurkat cells, which inhibits NFAT activation through importin β1 interference (Ishiguro et al., 2007). As a result, cucurbitacin R is able to inhibit the production of cytokines such as interleukin-2, interleukin-4, TNF-α, and interferon-γ both in vivo and in vitro. These effects, which we have described above in the case of DTH induced by various agents, have previously been observed in experimental arthritis (Escandell et al., 2007a). Thus, interleukin-2 production increases after NFAT translocation, inducing clonal expansion and promoting the production of other cytokines such as TNF-α and interferon-γ, which are associated with various autoimmune and inflammatory diseases. For its part, interleukin-4 plays an important role in the promotion of other pro-inflammatory cytokines (Proksch et al., 2005). However, because of the conditions under which our experiments were performed, cucurbitacin R did not modify NF-κB activity in Jurkat cells (data not shown). In a previous paper (Escandell
et al., 2007a) we reported that cucurbitacin R at 29 μM reduced cytokine production through the inhibition of STAT-3 activation without affecting NF-κB or AP-1 activation. Park et al. (2001) have shown that a related compound, dihydrocucurbitacin D, inhibited NF-κB at 80 μM. However, since cucurbitacin R is toxic at this concentration, we did not study its effect at higher doses.

In conclusion, our results suggest that cucurbitacin R is a new type of anti-inflammatory agent that could be useful in treating pathologies in which the immune system is implicated, including DTH and arthritis. Its molecular mechanism involves the inhibition of NFAT and the consequent inhibition of cytokines and cyclins regulated by this transcription factor. Although the in vitro IC₅₀ value of this compound is high, the in vivo tests demonstrated that its effect is similar to that of dexamethasone in the SRBC test. Future research will be focused on testing related compounds such as cucurbitacins I and E, which have been shown to be effective in a nanomolar to fentomolar range in several in vitro assays of inflammation and cytotoxicity.

Acknowledgements

We are indebted to Prof. Baldari (University of Siena, Italy) for his kind donation of stably transfected Jurkat cells with an NFAT/luc reporter gene and to the Centre de Transfusions de la Comunitat Valenciana (Valencia, Spain) for its generous supply of human blood. We thank Luis Orta for his useful comments on the manuscript.
References


Footnotes

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Legends for Figures

Figure 1. Effects of cucurbitacin R in lymphocytes proliferation and cell cycle. (A) Chemical structure of cucurbitacin R. (B) Percentage of cell cycle phase in which the cycle was arrested in the case of non-stimulated cells (blank) and those treated with cucurbitacin R (CCR, 30 μM), aphidicolin (aphi, 0.75 μM), and dexamethasone (dex, 5 μM) on T lymphocyte after 96 hours phytohemagglutinin A (PHA) stimulation; cells were stained with propidium iodide and the DNA content was analyzed with the aid of flow cytometry. Results were analyzed with the aid of the Chylcred software to determine cell phase percentages. (C) Effect of cucurbitacin R (CCR, 30 μM) on T lymphocyte proliferation after cucurbitacin R addition following phytohemagglutinin A stimulation. Control cells are phytohemagglutinin A-stimulated lymphocytes after 96 hours. Time is expressed in hours after stimulation. The proliferation index was measured as absorbance with the aid of the MTT assay. (D) Cell cycle progression after cucurbitacin R addition following phytohemagglutinin A stimulation. Each bar is the mean of three independent experiments. Values represent absorbance ± SEM. Statistically significant difference with respect to the control is expressed as * P < 0.05 and ** P < 0.01 (Dunnett’s t-test).

Figure 2. Cytokine production in T lymphocytes treated with cucurbitacin R at different concentrations. Effects on cytokine production: (A) Interleukin (IL)-2; (B) IL-4; (C) IL-10; and (D) interferon (IFN)-γ. Bars represent cytokine production in pg/ml in the presence or absence of test compounds with standard error and statistical significance. Blank (B, untreated cells), control (C, phytohemagglutinin A-stimulated cells), 10 μM dexamethasone (D)-treated
phytohemagglutinin A-stimulated cells, and cucurbitacin R-treated phytohemagglutinin A-stimulated cells (range of cucurbitacin R concentrations: 10 – 30 μM for IL-2, IL-10 and IFN-γ, and 2.75 – 40 μM for IL-4).

**Figure 3.** Effect of cucurbitacin R on interleukin (IL)-2, IL-4, IL-10 and interferon (IFN)-γ expression and cyclins induction. (A) Effects of cucurbitacin R on cytokine mRNA expression in T lymphocytes as detected with the aid of RT-PCR analysis. T cells were stimulated with phytohemagglutinin A (PHA, lanes 2 and 4) in the presence (lane 4) or absence (lane 2) of cucurbitacin R (CCR, 30 μM). Lane 1 shows mRNA expression in cells without treatment and lane 3 represents the effect of cucurbitacin R (30 μM) on non-stimulated T cells. (B) Effects of cucurbitacin R on cyclin expression in human T lymphocytes as detected with the aid of Western blot techniques. T cells were stimulated with phytohemagglutinin A (PHA, lanes 2 and 4) in the presence (lane 4) or absence (lane 2) of cucurbitacin R (CCR, 30 μM). Lane 1 shows protein expression in cells without treatment. RT-PCR and Western blot were quantified with the aid of the Scion image software. Values represent absorbance ± SEM. Statistically significant difference with respect to the control (phytohemagglutinin A) is expressed as * P < 0.05 and ** P < 0.01 (Dunnett’s t-test). Figures are representative of three experiments performed with similar results.

**Figure 4.** Cucurbitacin R inhibits NFAT activation after phytohemagglutinin A stimulation. (A) Effects of cucurbitacin R (CCR) on NFAT in human T lymphocytes after stimulation with phytohemagglutinin A as detected with the aid of EMSA. T cells were stimulated with phytohemagglutinin A (PHA) in the absence (lane 3) or presence (lane 4) of cucurbitacin R
(CCR, 30 μM). Lane 1 shows NFAT from cells without treatment (blank) and lane 2 shows the competition assay (CA); briefly, phytohemagglutinin A stimulated nuclear extracts were used in the reaction using 100 fold concentration of unlabeled probe respect to the DIG-labelled probe. (B) T lymphocytes were treated for 1 h with cucurbitacin R (CCR) at 30 μM and then stimulated with phytohemagglutinin A (PHA) for 1 h more. Samples were collected and tested with the aid of Western blot analysis. Phosphorylated and dephosphorylated NFAT were detected with β-actin as a control.

**Figure 5.** Localization of intracellular NFAT. Human lymphocytes were treated for 1 h with cucurbitacin R (CCR) at 30 μM and then stimulated with phytohemagglutinin A (PHA) for 1 h more. The cells were then fixed and stained with NFAT antibody and 4',6-diamidino-2-phenylindol dihydrochloride (DAPI).

**Figure 6.** Cucurbitacin R (CCR) inhibits NFAT translocation to the nucleus. (A) Reporter gene assay. Jurkat cells, stably transfected with NFAT/luc reporter gene were either not stimulated (Blank), phytohemagglutinin A-stimulated (control), or treated with cucurbitacin R at different concentrations for 1 h and stimulated with phytohemagglutinin A for 24 h. Luciferase activity was measured with standard protocols. (B) Jurkat cells were treated for 1 h with cucurbitacin R (CCR) at 50 μM and then stimulated with phytohemagglutinin A (PHA) for 1 h more. Whole cell extract or nuclear and cytoplasmic extract were collected and tested with the aid of Western blot analysis.
Figure 7. Localization of intracellular NFAT. Jurkat cells were treated for 1 h with cucurbitacin R (CCR) at 50 μM and then stimulated with phytohemagglutinin A (PHA) for 1 h more. The cells were then fixed and stained with NFAT antibody and 4’,6-diamidino-2-phenylindol dihydrochloride (DAPI).

Figure 8. Effects of cucurbitacin R (CCR) at different concentrations on activated Jurkat cells in NFAT of electrophoretic mobility shift assay (EMSA). (A) Jurkat cells were stimulated with phytohemagglutinin A (PHA) or (B) ionomycin/TPA in the absence (lane 1-2) or presence (lane 3-6) of cucurbitacin R (CCR) at different concentrations (50-100 μM). Lane 1 shows the activation of NFAT in cells without treatment (blank).

Figure 9. Immunohistochemical study of paws after sensitization with SRBC. Presence of CD4+ and CD8+ in paws treated with cucurbitacin R 48 h after challenge with SRBC. (A) control group treated with SRBC only; (B) cucurbitacin R-treated group; (C) dexamethasone-treated group.
Table 1. Effects of cucurbitacin R on DTH induced by oxazolone, DNFB, and SRBC. Edema as expressed as the increase in ear thickness (ΔT in μl ± S.E.M.) in the oxazolone and DNFB experiments, and increase in paw volume (ΔV in mg ± SEM) in the SRBC experiments. Percentages of inhibition (%I) for each measurement were calculated from values for the cucurbitacin R group with respect to those for the control. Statistically significant difference with respect to the control is expressed as * P < 0.05 and ** P < 0.01 (Dunnett’s t-test).

### DTH-induced by oxazolone

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<th>72 h</th>
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<td>%I</td>
<td>ΔT ± SEM</td>
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<td>209 ± 13</td>
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<td>186 ± 13</td>
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<td>94 ± 9**</td>
</tr>
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<td>Dexamethasone 0.025</td>
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### DTH-induced by DNFB

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<td>%I</td>
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<tr>
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<td>–</td>
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<td>23</td>
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### DTH-induced by SRBC

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<td>Cucurbitacin R 12.5</td>
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<td>63</td>
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Table 2. Effects of cucurbitacin R on TNF-α, interkeukin (IL)-1β and IL-4 in DTH-induced by oxazolone, DNFB and SRBC. Percentages of inhibition were calculated from values of test compounds minus blank (protein extract of animals treated with vehicle) versus control (protein extract of animals treated with DTH reagent) minus blank. Values are expressed as pg of each cytokine production per ml ± SEM; n.d. = no detected amounts. Statistically significant difference with respect to the control is expressed as * \( P < 0.05 \) and ** \( P < 0.01 \) (Dunnett’s t-test).

<table>
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<th>IL-4</th>
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<tr>
<td></td>
<td>pg/ml ± SEM</td>
<td>%I</td>
<td>pg/ml ± SEM</td>
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<tr>
<td><strong>DTH-induced by oxazolone</strong></td>
<td></td>
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<tr>
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<td>211 ± 36</td>
<td>–</td>
<td>32 ± 8</td>
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<tr>
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<td>44</td>
<td>114 ± 18*</td>
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<tr>
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<td>162 ± 19*</td>
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<td>30 ± 10*</td>
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<td><strong>DTH-induced by SRBC</strong></td>
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<td></td>
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<tr>
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<tr>
<td>Dexamethasone</td>
<td>293 ± 7*</td>
<td>101</td>
<td>219 ± 3*</td>
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</table>
Figure 2

A) IL-2 ± SEM (pg/ml)

B) IL-4 ± SEM (pg/ml)

C) IL-10 ± SEM (pg/ml)

D) IFN-γ ± SEM (pg/ml)
Figure 4

A)  

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B)  

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NFAT  

Dephosphorylated NFAT
Figure 6

B) Table and Western Blot Analysis

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<tr>
<td>CCR (50 μM)</td>
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- **NFAT**
  - Whole cell extract
  - Nuclear extract
  - Cytoplasmic Cell extract

- **β-actin**
Figure 8

A)  

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B)  

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