Dihydrocucurbitacin B Inhibits Delayed Type Hypersensitivity Reactions by Suppressing Lymphocyte Proliferation

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ABSTRACT

We have studied the effects of dihydrocucurbitacin B, a triterpene isolated from Cayaponia tayuya roots, on different models of delayed type hypersensitivity (DTH) in mice, as well as on T-lymphocyte proliferation and the mediators involved. In experiments with mice, dihydrocucurbitacin B inhibited the inflammatory reactions induced by oxazolone, dinitrofluorobenzene, and sheep red blood cells, reducing both the edema and cell infiltration. Moreover, the analysis of inflamed tissues showed that dihydrocucurbitacin B reduced the presence of the most relevant cytokines implicated in these processes, including interleukin-1β, interleukin-4, and tumor necrosis factor-α. Dihydrocucurbitacin B was also found to inhibit the proliferation of phytohemagglutinin-stimulated human T lymphocytes (IC50 = 1.48 μM), halting the cell cycle in the G0 phase. In addition, the triterpene reduced the production of interleukin-2, interleukin-4, interleukin-10, and interferon-γ in human T lymphocytes, and it hampered the induction of the principal cyclins involved in the cell cycle, including A1, B1, D2, and E2. Finally, dihydrocucurbitacin B was found to exert a selective inhibition on the nuclear factor of activated T cells (NFAT) in human lymphocytes without affecting the calcium influx. Taken together, these results suggest that dihydrocucurbitacin B curbs DTH reactions by inhibiting NFAT, which in turn suppresses the proliferation of the most relevant cells involved in DTH reactions, namely the T cells.

Cucurbitacins, a group of phytochemicals with anti-inflammatory activities, have been shown to exert specific effects against various inflammatory processes, including contact dermatitis (Rios et al., 2000, 2005). 23,24-Dihydrocucurbitacin B, a triterpenoid isolated from tayuya (Cayaponia tayuya) roots, has been shown to exert anti-inflammatory activity on different experimental models of inflammation (Recio et al., 2004). For example, it has a significant effect on adjuvant-induced arthritis in rats, reducing both inflammation and tissue damage and inhibiting the expression of several enzymes and mediators implicated in the inflammatory process, including elastase, cyclooxygenase, nitric-oxide synthase-2, and tumor necrosis factor (TNF)-α (Escandell et al., 2006). Moreover, dihydrocucurbitacin B has been found to possess potential anticancer properties, and it could have applications as an immunodepressant, acting by means of a mechanism in which the inhibition of both TNF-α and interleukin-1β in lymphocytes is implicated. It is also possible that dihydrocucurbitacin B operates by activating both the Janus kinase-2 pathway along with that of the signal transducer and activator of transcription-3. This mechanism seems likely, since a closely related compound, cucurbitacin B, has been shown to activate these pathways in diverse tumor cell lines (Sun et al., 2005).

Delayed type hypersensitivity (DTH) is a reaction triggered by antigen-specific T cells that can be induced by different allergens. The most common of these allergens are oxazolone and dinitrofluorobenzene, which both induce contact dermatitis, and sheep red blood cells (SRBC), which lead to tuberculinic reactions. Contact hypersensitivity, a type of DTH, occurs in two phases: induction and elicitation. In the first 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. Further contact with the hapten gives rise to the recruitment of antigen-specific T cells, resulting in erythema, edema, and, consequently, severe dermal inflammation.

ABBREVIATIONS: TNF, tumor necrosis factor; DTH, delayed type hypersensitivity; SRBC, sheep red blood cell(s); Th, T helper; NFAT, nuclear factor of activated T cells; DHC, dihydrocucurbitacin B; IL, interleukin; PHA, phytohemagglutinin; Tc, T cytotoxic.
The pharmacological response to different agents depends on the mechanism implicated in the anti-inflammatory process after a DTH reaction. Thus, contact hypersensitivity reactions may be reduced either by specific inhibitors of cytokine expression, including interleukin-1β and TNF-α (Zunic et al., 1998), or by proinflammatory enzymes, such as inhibitors of inducible nitric-oxide synthase (Ross and Reske-Kunz, 2001) and corticosteroids (Belsito, 2000). Interestingly, although specific inhibitors of cyclooxygenase or 5-lipoxigenase were found to reduce the presence of individual mediators, they had no effect on the swelling in oxazolone-induced DTH in mice. In fact, only corticosteroids reduced both the edema and the presence of inflammatory mediators in this case (Meurer et al., 1988).

In this study, we describe the pharmacological effects of dihydrocucurbitacin B on three models of DTH induced by different agents to gain insight into the anti-inflammatory activity of this compound on different DTH processes. In addition, we outline the effects of dihydrocucurbitacin B on different proinflammatory mediators and on the lymphocyte cell cycle to better understand its possible mechanism of action.

**Materials and Methods**

**Reagents and Chemicals.** Dihydrocucurbitacin B (Fig. 1) was previously obtained from tayuya roots (Recio et al., 2004). Chemical and biochemical reagents were purchased from Merck (Darmstadt, Germany), Panrec (Barcelona, Spain), and Sigma-Aldrich (St. Louis, MO). Antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Enzyme-linked immunosorbent assay 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-horseradish peroxidase were obtained from Dako Denmark A/S (Glostrup, Denmark).

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

**DTH Induced by Oxazolone, Dinitrofluorobenzene, and SRBC.** Female mice were sensitized through topical application of 20 μl of 0.2% (v/v) dinitrofluorobenzene (Sigma-Aldrich) on days 1 and 2. Challenge was then performed on day 6 by applying dinitrofluorobenzene (20 μl; 0.2%, v/v) on the inner and outer ear surfaces. Dihydrocucurbitacin B (0.1, 0.3, and 0.5 mg/ear) in acetone was topically applied (20 μl) to the ear 2, 24, 48, and 72 h after challenge. Ear thickness measurements of the treated and control groups were taken with a micrometer (Series 293; Mitutoyo, Kawasaki, Japan), and the edema was calculated for each ear as the difference in thickness before treatment and 24, 48, and 72 h after challenge.

In the oxazolone-induced delayed hypersensitivity assay (Bas et al., 2007), mice were treated with dihydrocucurbitacin B at 0.1, 0.3, and 0.5 mg/ear, whereas in the SRBC assay (Bas et al., 2007), dihydrocucurbitacin B was administered i.p. at 10 mg/kg. Inhibition percentages are expressed with respect to the control group, which was treated only with the inducing agent (oxazolone, dinitrofluorobenzene, or SRBC). Doses were established according to previous data obtained for this compound by Recio et al. (2004) and Escandell et al. (2006).

**Histology and Immunohistochemistry.** Ear samples fixed in 4% neutral-buffered formalin were cut longitudinally as described previously by Escandell 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., 2007), antibodies (Santa Cruz Biotechnology, Inc.) of CD4⁺ (SC1140) and CD8⁺ (SC7188) were used.

**Cytokine Measurement from ex Vivo Samples.** Tests to determine the presence of cytokines in ears and paws from the control and dihydrocucurbitacin B-treated groups were performed. The skin was removed from the ears and paws, which were then homogenized with a Polytron (Kinematica AG, Luzern, Switzerland) in 2.5 ml of 10 mM HEPES (Sigma-Aldrich), 2 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich), 100 mM EDTA (Sigma-Aldrich), 0.32 M sucrose (Sigma-Aldrich), 1 mM diithiothreitol (Sigma-Aldrich), 2 mg/ml aprotinin (Sigma-Aldrich), and a complete mini EDTA-free protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The mixture was sonicated (3 cycles in 10 s) in a Branson 150 sonifier (Branson, Danbury, CT) and centrifuged (centrifuge 5810R; Eppendorf, Hamburg, Germany) at 10,000g for 20 min at 4°C. TNF-α, interleukin-1β, and interleukin-4 were measured with the aid of an enzyme immunoassay (eBioscience) used according to the manufacturer's instructions.

**T Lymphocyte Proliferation Assay.** Peripheral lymphocytes were obtained from human blood buffy-coats. Cells were isolated as described by Bus et al. (2007). A volume of 200 μl of cell suspension was applied onto each well of a 96-well flat-bottomed plate (NUNC A/S, Roskilde, Denmark) with or without 25 μg/ml phytohemagglu-
tinin (Sigma-Aldrich). Either dihydrocucurbitacin B at concentrations ranging from 1.0 to 2.5 μM or 5 μM dexamethasone were added to the cells. The plates were incubated in 5% CO₂, air-humidified atmosphere at 37°C for 4 days, after which T cell proliferation was determined with the aid of a modified colorimetric 3-(4,5-di-methythiazol-2-yl)-2,5-diphenyltetrazolium assay. Controls consisted of lymphocytes with phytohemagglutinin (100% activity) or medium (0% activity). There were also control samples with non-stimulated lymphocytes. The kinetic studies were performed after the addition of dihydrocucurbitacin B (final concentration, 3 μM) at 0, 3, 6, 10, 18, 24, 48, 72, and 96 h after stimulation. Lymphocyte proliferation measurements were performed as described above.

**Cell Cycle Analysis.** T lymphocytes were adjusted to a final relation of (1 × 10⁶) cells/ml before use, and then 1 ml of cell suspension was placed into each well of a 24-well flat-bottomed plate (NUNC A/S), with or without 25 μg/ml phytohemagglutinin. Then, either 2.5 μM dihydrocucurbitacin B, 5 μM dexamethasone, or 0.75 μM aphidicolin (Sigma-Aldrich) was added to the cells. The plates were incubated under a 5% CO₂, air-humidified atmosphere at 37°C for different time periods, after which the cells were harvested through centrifugation, washed in phosphate-buffered saline, pH 7.2, and fixed in 70% ethanol (Sigma-Aldrich) for 30 min at −20°C. The cells were then washed and stained once with phosphate-buffered saline. DNA was stained with 4 μg/ml propidium iodide (Sigma-Aldrich) containing 100 μg/ml ribonuclease A (Sigma-Aldrich). Flow cytometry analysis was carried out on an Epics XL-MCL device (Beckman Coulter, Fullerton, CA).

**Determination of Cell Viability and Cytotoxicity.** T lymphocytes were cultured to a final relation of 1 × 10⁶ cells/ml in a supplemented medium, and then they were stimulated with 25 μg/ml phytohemagglutinin. After 4 days, 100 μl of a solution of 50 μg/ml propidium iodide was added, and cell viability was measured 30 min later with a flow cytometry Epics XL-MCL device (Beckman Coulter).

**Determination of Cytokine Production in T Lymphocytes.** T lymphocytes (2 × 10⁵ cells/well) were cultured with phytohemagglutinin alone or in combination with various concentrations of dihydrocucurbitacin B for 4 days. The cell supernatants were then collected and assayed for the presence of interleukin-2, interleukin-4, interleukin-10, and interferon-γ by means of an enzyme-linked immunoabsorbent assay (detection limit of immunoassay, 4 pg/ml) (eBioscience).

**Extraction of Total Cellular RNA.** Cells (5 × 10⁶) were treated with or without phytohemagglutinin and cultured with dihydrocucurbitacin B for 18 h. T cells were collected, and total RNA was extracted with the aid of RNeasy mini spin (50) columns (QIAGEN GmbH, Hilden, Germany) according to the manufacturer’s instructions, and then RNA was redissolved in diethyl pyrocarbonate (Sigma-Aldrich)-treated H₂O. The concentration of the extracted RNA was calculated by measuring the optical density at 260 nm. The ratio of the optical density at 260 nm to that at 280 nm was always higher than 1.8.

**Reverse Transcriptase-Polymerase Chain Reaction.** Aliquots of 1 μg of RNA were transformed to first-strand cDNA with Moloney murine leukemia virus reverse transcriptase (Promega Biosciences, San Luis Obispo, CA). Then, 2.5 μl of the resulting cDNA was mixed with 0.75 μM primers (Invitrogen, Langley, OK) of interferon-γ (sense: 5′-GGATGCTTGGGCTCCTGCTGGTACT CG-3′; antisense: 5′-CTCCTTCTGGTGGGTTTTAGCTGTCG-3′), interleukin-2 (sense: 5′-AAAGTGCACTACTCAAG-3′; antisense: 5′-GTTAGATGATCTGGTGACCA-3′), interleukin-4 (sense: 5′-ATGTCCTGCTCCTCAACCTGCT-3′; antisense: 5′-GGACCTTTGAAATTCTCCTCTCAT-3′), interleukin-10 (sense: 5′-ATCCCCAAGCTGGAGAACAGCCA-3′; antisense: 5′-TCTCAAGGGGGTGCTG TTCAGCTATCCCA-3′), and β-actin (sense: 5′-GACAAGCAAGAAGAGCATCC-3′; antisense: 5′-CTGTGTTGGTGAACCTGTAG-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. After the reaction, the amplified product was removed from the tubes and run on 2% agarose gel (Sigma-Aldrich).

**Western Blot Analysis.** Cellular lysates from lymphocytes incubated for 24 h with phytohemagglutinin were obtained as described previously by Escandell et al. (2007). For cyclins A (H-230; Santa Cruz Biotechnology, Inc.), B (H-453; Santa Cruz Biotechnology, Inc.), D₁ (H-289; Santa Cruz Biotechnology, Inc.), and E (C-19; Santa Cruz Biotechnology, Inc.), membranes were incubated with their respective polyclonal antibodies (1:200 dilution). For β-actin (Sigma-Aldrich), membranes were incubated with monoclonal antibody (1:10,000 dilution). Western blot quantification was carried out with the aid of Scion Image software, version 1.0.0.1 (Scion Corporation, Frederick, MD).

**Electrophoretic Mobility Shift Assays.** Human lymphocytes were incubated with 2.5 μM dihydrocucurbitacin B for 1 h. The cells were then stimulated with phytohemagglutinin for an additional 1 h, after which the nuclear protein was extracted. The protein content of the supernatant was determined with the aid of Bradford reagent, and then 10 μg of nuclear protein was loaded into a 6% nondenatured polyacrylamide gel. Electrophoretic mobility shift Assays were carried out with a DIG-gel shift kit (Roche Diagnostics).

**Determination of Calcium Cell Influx.** Lymphocytes (0.5 × 10⁶) were plated into supplemented RPMI 1640 medium with 1.5 mM Ca²⁺, and then 4 μM FLUO-3 (Invitrogen) was added for each incubation. The cells were then treated with 2.5 μM dihydrocucurbitacin B, and the suspension was mixed and incubated for 1 h at 37°C in 5% CO₂ in air. The FLUO-3-loaded cells were then measured for 30 s before administering lectins. Changes in relative fluorescence intensity were recorded at 10-s intervals for 300 s with the aid of a Flows flow cytometer (Beckman Coulter). All flow cytometric experiments were repeated at least three times.

**Statistics.** Data were expressed as mean ± S.E.M. First, a one-way analysis of variance was performed, followed by Dunnett’s t test for multiple comparisons. When making comparisons with the control group, values of P less than 0.05 were considered significant. Inhibition percentages were calculated from the differences between the drug-treated group and control animals treated only with the inflammatory agent. Inhibitory concentration-50 (IC₅₀) and effective dose-50 (ED₅₀) values were calculated from dose-response linear regression plots.

**Results**

**Effects on DTH Induced by Oxazolone, Dinitrofluorobenzene, and SRBC.** The administration of dihydrocucurbitacin B at different doses (0.1, 0.3, and 0.5 mg/ear) reduced the oxazolone-induced DTH reaction, but it had no clear dose-response effect. Thus, at 24 h, edema inhibition was 39% (0.3 mg/ear), with no increase in the effect observed at higher doses. At 72 h, the effect was actually higher at a dose of 0.3 mg/ear than at a dose of 0.5 mg/ear (Fig. 2A). For dinitrofluorobenzene-induced DTH, the compound inhibited the reaction at doses of 0.3 mg/ear (29, 30, and 47% at 24, 48, and 72 h, respectively) and 0.5 mg/ear (26, 54, and 60% at 24, 48, and 72 h, respectively), but it had no significant effect at 0.1 mg/ear (Fig. 2B). Interestingly, it reduced the DTH reaction by 56% when administered together with the allergenic product (data not shown). In the DTH reaction induced by SRBC in mouse paws, dihydrocucurbitacin B at a dose of 10 mg/kg i.p. clearly and significantly reduced the reaction by 54% at 18 h and by 57% at 24 h, but the effect disappeared at 48 h (Fig. 2C). Finally, the levels of TNF-α, interleukin-1β, and interleukin-4 were all reduced by dihydrocucurbitacin B in animals treated with oxazolone (Fig. 2D) or dinitrofluorobenzene (Fig. 2E). In both cases, there was a correlation...
between the reduction of cytokines and the antiedema effect, although the effect of dihydrocucurbitacin B was higher in the dinitrofluorobenzene model (Fig. 2E).

The histological study of the ears from the control group treated only with oxazolone showed a characteristic inflammatory lesion that affected the dermis with both edema and cell infiltration in which the ratio of neutrophils/lymphocytes/macrophages was 3:2:1 (Fig. 3A1). The ear treated with dihydrocucurbitacin B (Fig. 3B1) presented histological changes with respect to the control group. The differences included the infiltration of fewer neutrophils, a milder inflammatory lesion, a reduction of edema and inflammatory cell infiltration, and a reduction of epithelium thickness. Other parameters of the inflammatory process, such as papillomatosis and spongiosis, were also attenuated in both dihydrocucurbitacin B-treated groups. There was no clear difference in the CD4^+ and CD8^+ populations between the treated and control groups (data not shown).

The ears from mice treated only with dinitrofluorobenzene (control group) showed a characteristic inflammatory lesion similar to that described above (Fig. 3A2). In contrast, the ears treated with dihydrocucurbitacin B (Fig. 3B2) presented a milder inflammatory lesion along with reduced edema and inflammatory cell infiltration. Papillomatosis and spongiosis were also attenuated in the dihydrocucurbitacin B-treated group. Although dihydrocucurbitacin B reduced T-cell infiltration in inflamed tissue, once again there was no clear effect on the CD4^+ and CD8^+ populations in the treated group (data not shown).

![Fig. 2.](image1.png) A to C, effect of dihydrocucurbitacin B (DHCB) on DTH induced in mice by oxazolone, expressed as percentage of inhibition of ear swelling (A); DNFB, expressed as percentage of inhibition of ear swelling (B); and SRBC, expressed as the increase in paw swelling (C). D and E, effect of DHCB on interleukin (IL)-1β, IL-4, and TNF-α production in the ears of mice treated with oxazolone (D) and dinitrofluorobenzene (E). The vertical lines indicate the S.E.M. Statistically significant difference with respect to the control is expressed as **, \( P < 0.01 \) (Dunnett's t test). \( n = 5 \) or 6 animals.

![Fig. 3.](image2.png) Oxazolone-induced DTH reaction in mice. Hematoxylin- and eosin-stained section of ears. A, control group treated with oxazolone only. B, dihydrocucurbitacin B-treated group. DNFB-induced DTH reaction in mice. Hematoxylin- and eosin-stained section of ears. A, control group treated with DNFB only. B, dihydrocucurbitacin B-treated group.
The histological examination of the paws of animals treated only with SRBC (control group) showed a characteristic inflammatory lesion in the connective periarticular tissues, as well as intense, acute inflammation with an abundance of polymorphonuclear leukocytes (data not shown). The paws treated with dihydrocucurbitacin B presented a mild inflammation with reduced infiltrate of a mixture of polymorphonuclear leukocytes and lymphocytes (data not shown).

**Analysis of Cell Toxicity and Effect of Dihydrocucurbitacin B on T Lymphocyte Proliferation.** Dihydrocucurbitacin B exhibited no cell toxicity at doses of 10 μM or lower. In the cell proliferation assay, T lymphocytes were first incubated with phytohemagglutinin for 4 days, which stimulated cell proliferation by 300%. Addition of dihydrocucurbitacin B to these activated T lymphocytes totally inhibited cell proliferation. Thus, the compound was found to suppress phytohemagglutinin-treated cell proliferation by 100% at 2.5 μM, with an IC₅₀ value of 1.49 μM (r² = 0.9998; range assayed, 2.5–1.0 μM; five dilutions; n = 3).

**Effect of Dihydrocucurbitacin B on the Cell Cycle.** T lymphocytes stimulated with phytohemagglutinin typically enter into the G₁ phase within 2 to 4 h, the S phase at 18 to 24 h, and finally reach the G₂/M phase after 36 to 48 h. Because dihydrocucurbitacin B was found to inhibit cell proliferation totally (see above), we set out to determine in which phase the T cell cycle was arrested. We found that resting T lymphocytes stayed mainly in the G₀/G₁ phase 96 h after incubation with or without dihydrocucurbitacin B. Cells incubated without the compound and stimulated with phytohemagglutinin changed from the G₀/G₁ phase to the S and G₂/M phases (Fig. 4A), whereas stimulated cells that had been previously treated with 2.5 μM dihydrocucurbitacin B generally remained in the G₀/G₁ phase, with only a very reduced passage to the S phase. Dexamethasone (5 μM) also modified the cycle of phytohemagglutinin-stimulated cells, halting it in the G₀ phase of lymphocyte proliferation. In a parallel assay, 0.75 μM aphidicolin stopped the cycle in the S phase. In addition, a blank group was assayed to determine the normal evolution of the cell cycle (Fig. 4A). After running all the assays, we calculated the percentage of T cells in the G₀/G₁, S, and G₂/M phases in each case. The results are shown in Fig. 4B. In an additional study, the effect of dihydrocucurbitacin B on the cell cycle and proliferation of T lymphocytes after phytohemagglutinin stimulation was examined. The results indicate that addition of 2.5 μM dihydrocucurbitacin B 0 and 3 h after phytohemagglutinin stimulation inhibited the passage of the cells from G₀/G₁ to S phase by 96 and 97%, respectively. In contrast, addition of 2.5 μM dihydrocucurbitacin B between 6 and 72 h produced a gradual increase in the percentage of cells in the S and G₂/M phases (Fig. 5A). Again, the percentage of T cells in the various phases was calculated in each case (Fig. 5B).

**Effects of Dihydrocucurbitacin B on Cytokine Production in Human T Lymphocytes.** We then went on to study the production of interferon-γ, interleukin-2, interleukin-4, and interleukin-10 by T lymphocytes treated with dihydrocucurbitacin B at different concentrations (2.5–1.0 μM) to gain insight into the relationship among these cytokines, cell proliferation, and cell cycle progression as well as to determine the implications of their inhibition on the inflammatory process. The effects of dihydrocucurbitacin B on cytokine production (picograms per milliliter) were compared with those from the blank group (nontreated cells), the control group (phytohemagglutinin-stimulated cells), and the positive control group (phytohemagglutinin-stimulated cells treated with 5 μM dexamethasone). The compound was found to inhibit significantly the production of interferon-γ, interleukin-2, and interleukin-10 in stimulated cells (Fig. 6, Fig. 4. Effect of 2.5 μM DHCB, 0.75 μM aphidicolin (aphi), and 5 μM dexamethasone (dex) on T lymphocyte proliferation and cell cycle. A, cells were stained with propidium iodide, and the DNA content was analyzed by means of flow cytometry. B, results were analyzed with the aid of the Chylcred program to determine cell phase percentage. Each bar is the mean of three independent experiments. Values represent the percentage of cells in each phase of the cell cycle ± S.E.
Effects of Dihydrocucurbitacin B on Cyclin Expression and Transcription Factor in Human Lymphocytes and on Calcium Cell Influx.

Avera, B–D). There was also a slight reduction of interleukin-4 production, but the effect was not significant (Fig. 6A).

Because the production of cytokines in activated T lymphocytes decreased in the presence of dihydrocucurbitacin B, we also carried out a reverse transcription-polymerase chain reaction analysis to determine the effects of this compound on the cytokine mRNA expression in activated T cells. After extracting total cellular RNA from activated T lymphocytes in either the presence or absence of 2.5 μM dihydrocucurbitacin B, we found that mRNA levels of interferon-γ, interleukin-2, interleukin-4, and interleukin-10 from stimulated T lymphocytes were all attenuated by dihydrocucurbitacin B (Fig. 7A, lane 4) with respect to the control (Fig. 7A, lane 2), which measured β-actin mRNA expression (Fig. 7A). Moreover, a densitometry analysis demonstrated that the ratios of cytokine mRNA decreased with respect to β-actin mRNA in phytohemagglutinin-stimulated T lymphocytes (Fig. 7B).

Effects of Dihydrocucurbitacin B on Cyclin Expression and Transcription Factor in Human Lymphocytes and on Calcium Cell Influx. As indicated above, the phytohemagglutinin-stimulated cells remained in the G0/G1 phase, with only very reduced passage to the S phase when cocultured with dihydrocucurbitacin B. Because cyclins are involved in the progression of cells throughout the cell cycle, we tested the effects of dihydrocucurbitacin B on the most relevant cyclins in this process. At a concentration of 2.5 μM, dihydrocucurbitacin B was found to inhibit the expression of cyclins A1, B1, D2, and E1 (Fig. 8A) as was demonstrated by densitometry analysis (Fig. 8B).

We went on to test the effect of dihydrocucurbitacin B on the nuclear factor of activated T cells (NFAT) and the influx of calcium. NFAT is controlled by calcineurin, whereas a Ca2+-dependent phosphatase controls and activates the genes that encode cytokines and their receptors in response to antigenic stimulation of immune cells. Binding sites for NFAT proteins are present in the promoter/enhancer regions of several inducible genes, including the cytokines interleukin-2, interleukin-4, and interferon-γ, along with other genes related to the cell cycle. Although 2.5 μM dihydrocucurbitacin B inhibited the activation of NFAT in phytohemagglutinin-stimulated T lymphocytes (Fig. 9, lane 4) with respect to the control group (Fig. 9, lane 3), it did not modify the calcium influx into these same cells (data not shown).

Discussion

Application of a hapten such as oxazolone or dinitrofluorobenzene after a previous sensitization produces severe edema in the reticular dermis, mild edema in the papillary dermis, and severe mixed inflammatory cellular infiltration throughout the dermis and subcutis (Chapman et al., 1986). Different mediators are implicated in these processes, including nitric oxide, arachidonic metabolites, and proinflammatory cytokines such as interleukin-1β, interleukin-2, interleukin-4, TNF-α, and interferon-γ, which all play a relevant role in the development of contact hypersensitivity in mice after sensitization and production of TNF-α, which is necessary for the induction of local inflammation during the elicitation phase (Nakae et al., 2003). Furthermore, the production of TNF-α and interleukin-1β regulates the expression of nitric oxide synthase and cyclooxygenase-2 (Bruch-Gerharz et al., 1998; Ross et al., 1998).

Our results demonstrate that when administered topically, dihydrocucurbitacin B not only suppresses the inflammatory response, but also reduces both the edema and the cellular infiltrate while simultaneously reducing the expression of interleukin-1β, interleukin-4, and TNF-α in inflamed tissue. Interleukin-1β is required for hapten-specific T-cell priming in the sensitization phase after challenge as well as for the induction and production of TNF-α, which is necessary for the induction of local inflammation during the elicitation phase (Nakae et al., 2003). Furthermore, the production of TNF-α and interleukin-1β regulates the expression of nitric oxide synthase and cyclooxygenase-2 (Bruch-Gerharz et al., 1998; Amin et al., 1999). Interleukin-4, in contrast, is a key cytokine in the development of T cells, especially Th2 cells (Nagai et al., 2000). These data justify in part the in vivo effects of dihydrocucurbitacin B on oxazolone- and dinitrofluorobenzene-induced DTH. Moreover, dihydrocucurbitacin B has been found to reduce the production of interferon-γ,
interleukin-2, and interleukin-10 in human T lymphocytes in vivo. We had previously demonstrated that dihydrocucurbicitin B reduces TNF-α production in both lymphocytes and macrophages and also reduces nitric-oxide production in inflamed tissue as well as in RAW 264.7 macrophages (Escandell et al., 2006). However, in the latter case, dihydrocucurbicitin B exerted this activity only when added before the induction of nitric-oxide synthase, showing no activity when added after the enzyme had already been induced (Escandell et al., 2006). This is a clear indication that the compound does not act through direct inhibition of this particular synthase.

For cyclooxygenase-2, a direct inhibition of the enzyme was hypothesized because the effect occurred both before and after the induction of the enzyme in RAW 264.7 macrophages (Escandell et al., 2006). These data are in agreement with a previous report by Jayaprakasam et al. (2003), who demonstrated that cucurbitacins with an acetyl group at C28 of the chain, as with dihydrocucurbitacin B, selectively inhibit cyclooxygenase-2. In a similar vein, we had previously demonstrated that leukotriene B4 production was not affected by rat neutrophils (Recio et al., 2004). In contact hypersensitivity induced by oxazolone or dinitrofluorobenzene in mice, levels of both prostaglandins and leukotrienes increase. Curiously, inhibitors of cyclooxygenase and lipoxygenase, which are responsible for the production of prostaglandins and leukotrienes, respectively, had no direct effect on the inflammation itself. Rather, these inhibitors acted through reduction of arachidonate metabolite levels. Indeed, only corticoids, which exert their influence at a higher level than the COX and LOX pathways, are capable of reducing swelling, cell infiltration, and levels of arachidonate metabolites altogether (Meurer et al., 1988; Zunic et al., 1998). However, in one of our previous studies (Recio et al., 2004), we observed no different effect for dihydrocucurbitacin B when the glucocorticoid receptor was blocked by mifepristone, a finding that indicates that the compound does not act by means of a corticoid-like mechanism. In fact, the most likely mechanism involved is protein synthesis because when applied together, cycloheximide reduces the activity of dihydrocucurbitacin B. We have now found additional support for invoking this mechanism, since a direct inhibition of NFAT was observed when we analyzed the proliferation of T lymphocytes. We thus have a clearer idea of the implications of NFAT on the immune response after treatment with dihydrocucurbitacin B and the consequent disruption of the cell cycle. This, in turn, is corroborated by several different lines of evidence,
which suggest that NFAT factors are major players in the control of the cell cycle of T cells (Serfling et al., 2000). In fact, the principal event in immune responses is the activation and clonal expansion of T lymphocytes, which in the process of differentiation, spontaneously arrest in the G0 phase and remain quiescent for long periods until exposed to specific antigens or mitogens, such as phytohemagglutinin, which stimulate the T lymphocyte receptors and initiate a cascade of events, including cell proliferation and differentiation (Kuo et al., 2000). After recognizing the stimulant agent, NFAT is dephosphorylated through calcium cell influx, producing the activation of the calmodulin-calcineurin system. Subsequently, NFAT migrates to the nucleus, and, together with activating protein-1, activates the synthesis of interleukin-2 and other mediators implicated in immune and inflammatory responses (Crabtree, 1999; Serfling et al., 2000). This stimulation leads to lymphocyte proliferation, which is controlled at specific stages of the cell cycle by different cyclin-dependent kinases. These are activated by specific cyclins during a particular phase of the cell cycle. The cyclins themselves are a family of proteins implicated in the cell cycle that are synthesized and destroyed during this process. When lymphocytes are stimulated, the proliferation machinery starts and the cyclin-dependent kinases are activated by cyclin D and E during the G1 phase, cyclin A in the S phase, and cyclin B for the transition from the G2 to the M phase. When lymphocytes are treated with dihydrocucurbitacin B, the expression of cyclins is clearly inhibited and the cell cycle starts and the cyclin-dependent kinases are activated by specific cyclins during a particular phase of the cell cycle. These are activated by specific cyclins.