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Iodinated *N*-Acylvanillamines: Potential "Multiple-Target" Anti-Inflammatory Agents Acting via the Inhibition of T-Cell Activation and Antagonism at Vanilloid TRPV1 Channels

Nieves Márquez, Luciano De Petrocellis, Francisco J. Caballero, Antonio Macho, Aniello Schiano-Moriello, Alberto Minassi, Giovanni Appendino, Eduardo Muñoz, and Vincenzo Di Marzo

Departamento de Biología Celular, Fisiología e Inmunología, Facultad de Medicina, Universidad de Córdoba, Córdoba, Spain (N.M., F.J.C., A.M., E.M.); Endocannabinoid Research Group, Istituto di Cibernetica and Istituto di Chimica Biomolecolare, Consiglio Nazionale delle Ricerche, Pozzuoli, Italy (L.D.P., A.S.-M., V.D.M.); and Università degli Studi del Piemonte Orientale, DiSCAFF, Novara, Italy (A.M., G.A.)

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ABSTRACT

Synthetic *N*-acylvanillamines were designed and developed as metabolically stable compounds with pharmacological potential in analgesia and inflammation because of their interaction with cannabinoid receptors and the vanilloid receptor (TRPV1). Here, we show that arvanil inhibits early events in T-cell receptor (TCR)-mediated T-cell activation, such as calcium mobilization and nuclear factor of activated T-cell activation, and in late events in TCR-mediated activation, such as interleukin (IL)-2 gene transcription, IL-2R expression, and cell-cycle progression. Arvanil also prevents tumor necrosis factor-α-induced nuclear factor-κB (NF-κB) activation by direct inhibition of IκBα degradation, NF-κB binding to DNA, and NF-κB-dependent transcription. Aromatic iodination meta to the phenolic hydroxyl (on the 6′-carbon atom) converts arvanil and olvanil from TRPV1 agonists into antagonists. However, this structural modification did not affect the immunosuppressive and proapoptotic activity of these compounds. In summary, we described here novel activities of arvanil on T-cell functions and the development of two novel inhibitors of neurogenic inflammation (6′-I-olvanil and 6′-I-arvanil) endowed with a unique combination of TRPV1 antagonistic-, immunosuppressive-, and NF-κB-inhibitory properties. Our findings provide new mechanistic insights into the biological activities of *N*-alkylvanillamines and should foster the synthesis of improved analogs amenable to pharmaceutical development as analgesic and anti-inflammatory agents.

The pathological and physiological bases for initiation and maintenance of neurogenic inflammation can be traced to bidirectional cross-talks between the nervous and immune systems. Sensory nerves are specialized for the transmission of the pain signal to the central nervous system and to release pronociceptive and inflammatory neuropeptides such as substance P, neurokinin A, and calcitonin gene-related peptide (Holzer, 1988). Over the past few years, the endocannabinoid system has raised great interest because of the pleiotropic neurological and immunological effects associated with the manipulation of its complex network. This system is composed of cannabinoid and vanilloid receptors, their endogenous ligands (named endocannabinoids), and the enzymes responsible for the biosynthesis and metabolism of these compounds (De Petrocellis et al., 2000b). Anandamide (*N*-arachidonoyl ethanolamine) (AEA), the first endocannabinoid to be discovered, acts mainly by signaling through the CB_1/CB_2 receptors and type 1 vanilloid receptors (VR1 or TRPV1) (Zygmunt et al., 1999; De Petrocellis et al., 2004). TRPV1 is a nonselective cation channel gated by noxious heat, extracellular protons, and both exogenous and endoge-

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ABBREVIATIONS: AEA, *N*-arachidonoyl ethanolamine; HIV-1-LTR, human immunodeficiency virus-1 long terminal repeats; TNFα, tumor necrosis factor α; NADA, *N*-arachidonoyl-dopamine; NFAT, nuclear factor of activated T cells; TRPV1, vanilloid receptor type 1; RLU, relative light units; CB, cannabinoid receptor; NF-κB, nuclear factor κB; TCR, T-cell receptor; IL, interleukin; NAVA, *N*-acylvanillamine; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cell; SEB, staphylococcal enterotoxin B; PBS, phosphate-buffered saline; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; CP-55,940, (1*R*,3*R*,4*R*)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol; TdR, thymidine.

nous ligands (Caterina et al., 1997). The thermal and ligand sensitivity of TRPV1 is drastically increased under inflammatory conditions, and the activation of TRPV1 by endovanilloid/endocannabinoid molecules such as AEA and *N*-arachidonoyl-dopamine (NADA) is seemingly involved in the hyperalgesia subsequent to inflammation (Szallasi, 2001; Amaya et al., 2003; Dinis et al., 2004; Van Der Stelt and Di Marzo, 2004). On the other hand, pharmacological desensitization of TRPV1 with agonists, including AEA and NADA, also causes analgesic and anti-inflammatory effects (Toth et al., 2005). The manipulation of the endocannabinoid system has therapeutic potential for several conditions, including neuropathic pain and neurological inflammation (Iversen and Chapman, 2002; Rice et al., 2002), and research activity has been mainly focused on the development of CB agonists (Iversen and Chapman, 2002), TRPV1 agonists/antagonists (Wahl et al., 2001; Appendino et al., 2002, 2003), inhibitors of endocannabinoid inactivation (Hillard and Jarrahian, 2000; Bisogno et al., 2002), and compounds endowed with dual CB_1 and TRPV1 activity.

Arvanil (*N*-[3-methoxy-4-hydroxy-benzyl]-arachidonoyl-amide) was designed as a metabolically stable TRPV1/CB_1 hybrid ligand with a pharmacological profile suitable for the development of ultrapotent analgesics (Di Marzo et al., 2002). Indeed, arvanil is a relatively potent inhibitor of endocannabinoid cellular reuptake, whereas there is controversy regarding its capability to also inhibit fatty acid amide hydrolase, the enzyme responsible for the hydrolysis of AEA and other endocannabinoids (Melck et al., 1999; Glaser et al., 2003). Furthermore, both in vitro and in vivo experiments have demonstrated that arvanil also exerts CB_1- and TRPV1-independent biological activities such as NF-κB inhibition (Sancho et al., 2003a), induction of apoptosis (Sancho et al., 2003b), and analgesia (Brooks et al., 2002), Taken together, these data qualify arvanil as a pleiotropic agent with a complex pattern of bioactivity that transcends CBs and TRPV1 interaction.

The transcription factor NF-κB is one of the key regulators of genes involved in the immune/inflammatory response and in survival from apoptosis (Karin and Ben-Neriah, 2000). NF-κB is an inducible transcription factor made up of homo- and heterodimers of p50, p65 (RelA), p52, RelB, and c-rel subunits that interact with a family of inhibitory IκB proteins, of which IκBα is the best characterized (Karin and Ben-Neriah, 2000). In most cell types, these proteins sequester NF-κB in the cytoplasm by masking its nuclear localization sequence. There are two major pathways for NF-κB activation, known as the canonical and noncanonical activation pathways. In the canonical pathway, which is activated in T cells by TNFα or by TCR/CD28 signaling, phosphorylation by IκB kinases and degradation of IκB triggers the translocation of NF-κB from the cytoplasm to the nucleus (Karin and Ben-Neriah, 2000; Li et al., 2005). In addition to the control of NF-κB activity exerted at the nuclear translocation level, there is increasing evidence for another complex level of regulation that is mediated by direct phosphorylation of the p65 subunit transactivation domain. This phosphorylation not only regulates the DNA binding and transactivation properties of p65 but also the interactions between the transcription factor and the regulatory proteins (Schmitz et al., 2004).

Aromatic iodination at C-6′ reverts the vanilloid activity of *N*-acylvanillamines (NAVAs), turning them into TRPV1 antagonists (Appendino et al., 2003, 2005). Because vanilloid antagonists have great potential for the treatment of pain, it was interesting to assess the effect of aromatic iodination of arvanil and olvanil, the archetypical NAVAs, on the activation of CB_1, the induction of apoptosis, and the immunosuppressive activity.

Materials and Methods

Cell Lines, Reagents, and Plasmids

The 5.1 cell line is a Jurkat-derived clone stably transfected with a plasmid containing the luciferase gene driven by the HIV-1-LTR promoter and was maintained in exponential growth in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 1 mM HEPES, and antibiotics (Sancho et al., 2003). Jurkat cells (American Type Culture Collection, Manassas, VA) were maintained in RPMI 1640 medium. The anti-IκBα mAb 10B was a gift from R. T. Hay (St. Andrews, Scotland, UK), the mAb anti-tubulin was purchased from Sigma Co. (St. Louis, MO), and the anti-phospho-p65 (3031S) was from New England Biolabs (Hitchin, UK). Fluorescein isothiocyanate-12-deoxy-2-uridine triphosphate and terminal deoxynucleotidyltransferase were from Roche (Indianapolis, IN). Arvanil and olvanil were obtained from Alexis (Carlsbad, CA), whereas 6′-iodo-arvanil and 6′-iodo-olvanil were prepared according to literature (Appendino et al., 2005) (Fig. 1). [γ-^{32}P]ATP (3000 Ci/mmol) and [^{3}H]TdR were from MP Biomedicals (Irvine, CA).

Plasmids

The NFAT-Luc plasmid contains three copies of the NFAT binding site of the IL-2 promoter fused to the luciferase gene. The KBF-Luc contains three copies of the major histocompatibility complex enhancer κB site upstream of the conalbumin promoter followed by the luciferase gene, and the IL-2-Luc (−326 to +45 of the IL-2 promoter) plasmid was described previously (Sancho et al., 2004). All other reagents were from Sigma.

Isolation of Human Peripheral Mononuclear Cells and T-Cell Proliferation Assays

Human peripheral blood mononuclear cells (PBMC) from healthy adult volunteer donors were isolated by centrifugation of venous blood on Ficoll-Paque Plus density gradients (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). Cells (10^5) were cultured in triplicate in 96-well round-bottomed microtiter plates (Nunc, Roskilde, Denmark) in 200 μl of complete medium and stimulated with staphylococcal enterotoxin B (SEB) (1 μg/ml) in the presence or absence of increasing concentrations of the selected compounds. SEB-activation model was used because this superantigen is presented by B cells and macrophages and activates T cells through TCR and costimulators. The cultures were carried out for 3 days and were pulsed with 0.5 μCi [^{3}H]TdR/well for the last 12 h of culture. Radioactivity incorporated into DNA was measured by liquid scintillation counting. Statistical analysis was performed using analysis of variance followed by the Student-Newman-Keuls method with values of $P < 0.05$ considered to be significant.

Cytofluorimetric Analyses of Cell-Surface Activation Antigens and Cell Cycle

For cell-cycle analyses and measurement of CD25 (IL-2Rα chain) expression, peripheral mononuclear cells (10^6/ml) were stimulated with SEB (1 μg/ml) in 24-well plates in a total volume of 2 ml of complete medium for 48 h in the presence or absence of different concentrations of either arvanil or olvanil. Antigen cell-surface fluorescence was measured by using a fluorochrome-labeled anti-CD25 mAb and analyzed by flow cytometry in an EPIC XL flow cytometer

(Coulter, Hialeah, FL). For DNA profile analyses, cells were washed in PBS, fixed in ethanol (70% for 24 h at 4°C) followed by RNA digestion (Rnase-A, 50 U/ml) and propidium iodide (20 μg/ml) staining, and analyzed by cytofluorometry. Ten thousand gated events were collected per sample, and the percentage of cells in every phase of the cell cycle was determined. The frequency of cells having undergone chromatinolysis was calculated by determining the sub-G_0/G_1 fraction.

Ca^{2+} Mobilization Assay in Jurkat Cells

Cells (10^7 cells/ml) were incubated for 1 h at 37°C in Tyrode's salt solution (137.0 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 12.0 mM $NaHCO_3$, and 5.6 mM D-glucose) containing 5 μM Indo-1-AM (Invitrogen) and immediately analyzed in a spectrofluorimeter (Hitachi F-2500; Hitachi Ltd., Yokohama, Japan) under continuous stirring and at a constant temperature of 37°C using a water-jacketed device. After 5-min accommodation to equilibrate temperatures, samples were excited at 338 nm, and emission was collected at 405 and 485 nm, corresponding to the fluorescence emitted by Ca^{2+}-bound and free Indo-1, respectively. $[Ca^{2+}]_i$ was calculated using the ratio values between bound and free Indo-1 fluorescence and assuming an Indo-1 K_d for Ca^{2+} of 0.23 μM. Maximum and minimum ratio values for calculations were determined by the addition at the end of the measurements of 10 μM ionomycin or 4 mM EGTA, respectively.

Transient Transfections and Luciferase Assays

Jurkat cells (10^7/ml) were transfected with the indicated plasmids using Lipofectamine (Invitrogen Life Technologies) according to the manufacturer's recommendations. Twenty-four hours after transfection, the cells were pretreated with arvanil for 30 min and stimulated for 6 h with a mix of anti-CD3 (clone OKT-3) (2.5 μg/ml) and anti-CD28 (clone 15E8) (1 μg/ml) mAbs cross-linked with protein A (10 μg/ml). Then, the cells were lysed in 25 mM Tris-phosphate, pH 7.8, 8 mM $MgCl_2$, 1 mM DTT, 1% Triton X-100, and 7% glycerol. Luciferase activity was measured using an Autolumat LB 9510 (EG&G Berthold, Bad Wildbad, Germany) following the instructions of the luciferase assay kit (Promega, Madison, WI). The experiments were repeated three times, and the results were expressed as a fold induction over nonstimulated cells. To determine NF-κB-dependent transcription of the HIV-1-LTR-luc, 5.1 cells were preincubated for 30 min with the compounds tested as indicated, followed by stimulation with TNFα (2 ng/ml) for 6 h. Then, the cells were lysed in luciferase buffer, and protein concentration was measured by the Bradford method. The background obtained with the lysis buffer was subtracted from each experimental value, the relative light units per microgram of protein was calculated, and the specific transactivation was expressed as the percentage of transcriptional activity compared with TNFα alone (100%). All of the experiments were repeated at least six times.

Western Blots

5.1 cells (1×10^6 cells/ml) were stimulated with TNFα (2 ng/ml) in the presence or absence of arvanil for the indicated period of time. Cells were then washed with PBS, and proteins were extracted in 50 μl of lysis buffer (20 mM HEPES, pH 8.0, 10 mM KCl, 0.15 mM EGTA, 0.15 mM EDTA, 0.5 mM Na_3VO_4, 5 mM NaFl, 1 mM DTT, leupeptin 1 μg/ml, pepstatin 0.5 μg/ml, aprotinin 0.5 μg/ml, and 1 mM phenylmethylsulfonyl fluoride) containing 0.5% Nonidet P-40. Protein concentration was determined by the Bradford assay (Bio-Rad, Richmond, CA), and 30 μg of proteins was boiled in Laemmli buffer and electrophoresed in 10% SDS/polyacrylamide gels. Separated proteins were transferred to nitrocellulose membranes (0.5 A at 100 V, 4°C) for 1 h. Blots were blocked in Tris-buffered saline solution containing 0.1% Tween 20 and 5% nonfat dry milk overnight at 4°C, and immunodetection of specific proteins was carried out with primary antibodies using an ECL system (Amersham).

Isolation of Nuclear Extracts and Mobility Shift Assays

5.1 cells (10^6/ml) were stimulated with the agonists in complete medium as indicated. Cells were then washed twice with ice-cold PBS, and proteins from nuclear extracts were isolated as described previously (Sancho et al., 2003). Protein concentration was determined by the Bradford method (Bio-Rad). For the electrophoretic mobility shift assay (EMSA), the consensus oligonucleotide probes NF-κB, 5′-AGTTGAGGG GACTTTCCCAGG-3′, and the commercial SP1 site (Promega) were end-labeled with [γ-^{32}P]ATP. The binding reaction mixture contained 3 μg of nuclear extract, 0.5 μg of poly(dI-dC) (Pharmacia Fine Chemical, Piscataway, NJ), 20 mM HEPES, pH 7, 70 mM NaCl, 2 mM DTT, 0.01% Nonidet P-40, 100 μg/ml bovine serum albumin, 4% Ficoll, and 100,000 cpm of end-labeled DNA fragments in a total volume of 20 μl. After 30 min of incubation at 4°C, the mixture was electrophoresed through a native 6% polyacrylamide gel containing 89 mM Tris-borate, 89 mM boric acid, and 1 mM EDTA. Gels were pre-electrophoresed for 30 min at 225 V and then for 2 h after loading the samples. These gels were dried and exposed to X-Ray film at −80°C.

Detection of DNA Strand Breaks by the TUNEL Method

Treated cells (1×10^6) were fixed in 4% paraformaldehyde in PBS for 24 h at 4°C, washed twice in PBS, and permeabilized in 0.1% sodium citrate containing 0.1% Triton X-100 for 20 min. Fixed cells were washed three times in PBS and resuspended in a final volume of 50 μl of TUNEL buffer (0.3 nmol fluorescein isothiocyanate-12-deoxy-2-uridine triphosphate, 3 nmol dATP, 50 nmol $CoCl_2$, 5 U terminal deoxynucleotidyltransferase, 200 mM potassium cacodylate, 250 μg/ml bovine serum albumin, and 25 mM Tris-HCl, pH 6.6). The cells were incubated for 1 h at 37°C and then washed twice in PBS and analyzed by flow cytometry. Ten thousand gated events were collected per sample, and the percentage of apoptotic cells was determined.

Assay of Agonist and Antagonist Activity at TRPV1

Human embryonic kidney 293 cells overexpressing the human TRPV1 were kindly donated by GlaxoSmithKline (Uxbridge, Middlesex, UK). Cells were grown as monolayers in minimum essential medium supplemented with nonessential amino acids, 10% fetal calf serum, and 0.2 mM glutamine, and maintained under 95% O_2/5% CO_2 at 37°C. The effect of the substances on $[Ca^{2+}]_i$ was determined by using Fluo-3, a selective intracellular fluorescent probe for Ca^{2+} (De Petrocellis et al., 2000a). One day before experiments, cells were transferred into six-well dishes coated with poly-(L-lysine) (Sigma) and grown in the culture medium mentioned above. On the day of the experiment, the cells (50–60,000 per well) were loaded for 2 h at 25°C with 4 μM Fluo-3 methylester (Molecular Probes, Carlsbad, CA) in dimethyl sulfoxide containing 0.04% Pluoronic. After loading, cells were washed with Tyrode's solution, pH 7.4, trypsinized, resuspended in Tyrode's solution and transferred to the cuvette of the fluorescence detector (PerkinElmer LS50B; PerkinElmer Life and Analytical Sciences, Boston, MA) under continuous stirring. Experiments were carried out by measuring cell fluorescence at 25°C (λ_{EX} = 488 nm, λ_{EM} = 540 nm) before and after the addition of the test compounds at various concentrations. The efficacy of the agonists was determined by comparing it to the analogous effect observed with 4 μM ionomycin. In antagonism experiments, varying concentrations of 6′-I-olvanil or 6′-I-arvanil were added 10 min before capsaicin (100 nM). Concentration-response curves for capsaicin in the presence of increasing concentrations of 6′-I-olvanil or 6′-I-arvanil were also constructed to calculate Schild's plots. Data were expressed, for the agonists, as EC_{50} values and, for the antagonists, as IC_{50} values or as K_b constants, both calculated by GraphPad Prism (GraphPad Software Inc., San Diego, CA).

Assay of Direct or Indirect Agonist Activity at CB_1 and CB_2 Receptors

Binding Assays. For CB_1 and CB_2 receptor binding, [^{3}H]CP-55,940 (K_d = 690 nM) was incubated with P_2 membranes from whole rat brains as described elsewhere (Di Marzo et al., 2002). Displacement curves were generated by incubating drugs with 0.5 nM [^{3}H]CP-55,940. In all cases, K_i values were calculated by applying the Cheng-Prusoff equation to the IC_{50} values (obtained with the use of GraphPad Prism) for the displacement of the bound radioligand by increasing concentrations of the test compounds.

Fatty Acid Amide Hydrolase Assays. The effect of compounds on the enzymatic hydrolysis of [^{14}C]AEA (6 mM) was studied by using membranes prepared from rat brain incubated with increasing concentrations of compounds in 50 mM Tris-HCl, pH 9, for 30 min at 37°C. [^{14}C]Ethanolamine produced from [^{14}C]AEA hydrolysis was measured by scintillation counting of the aqueous phase after extraction of the incubation mixture with 2 volumes of $CHCl_3/CH_3OH$ (2:1 by volume) (Di Marzo et al., 2002).

AEA Cellular Uptake Assays. The effect of compounds on the uptake of AEA by rat C6 glioma cells was studied as described previously (Di Marzo et al., 2002). Cells were incubated with [^{14}C]AEA (4 μM) for 5 min at 37°C, in the presence or absence of varying concentrations of the compounds. Residual [^{14}C]AEA in the incubation media after extraction with $CHCl_3/CH_3OH$ (2:1 by volume) was used as a measure of the AEA that was taken up by cells. Data are expressed as the IC_{50} value of AEA uptake calculated with the use of GraphPad Prism.

Results

Arvanil Inhibits Antigen-Specific T-Cell Proliferation and Cell-Cycle Progression. We studied the effects of arvanil and olvanil on several T-cell activation events induced by stimulation with SEB. This enterotoxin is a T-cell superantigen that mimics T-cell activation induced by TCR and costimulators (Marrack and Kappler, 1990). DNA synthesis measured by [^{3}H]TdR uptake in SEB-stimulated T cells was markedly inhibited by arvanil in a concentration-dependent manner. In contrast, olvanil did not inhibit SEB-induced T-cell proliferation at the concentrations tested (Fig. 2A). As a consequence of cell activation, primary T cells progress to the S-phase and G_2/M phases of the cell cycle. Three days after activation with SEB, T cells were fully cycling and progressed through the S, G_2, and M phases of the cell cycle (32.5%). Moreover, TCR activation also leads to the apoptosis of a significant percentage of activated cells (16.2% subdiploid cells). As expected, pretreatment with arvanil, but not with olvanil, significantly prevented the entry of the SEB-activated cells into the S and G_2/M phases of the cell cycle. We also found that arvanil did not increase the percentage of subdiploid cells compared with control SEB-treated cells (Fig. 2B), thus confirming our previous report showing that arvanil induces apoptosis in cancer but not in primary T cells (Sancho et al., 2003). To investigate the effects of arvanil on TCR-induced Ca^{2+} mobilization, Jurkat T cells were loaded with the indicator dye Indo-1 and treated with arvanil for 15 s at 37°C before CD3 stimulation. CD3 stimulation by anti-CD3 mAb (OKT-3), followed by cross-linking with protein A (10 μg/ml), transiently increased Ca^{2+} levels, and the incubation of Jurkat T cells with arvanil (10 μM) gave much lower Ca^{2+} fluxes than untreated cells (Fig. 3A). T-cell activation involves the induction of several surface molecules such as CD69, CD25, or intercellular adhesion molecule-1, whose gene transcriptional regulation is highly dependent on NF-κB. Thus, the effect of the NAVAs on the cell-surface expression of the CD25 activation marker was

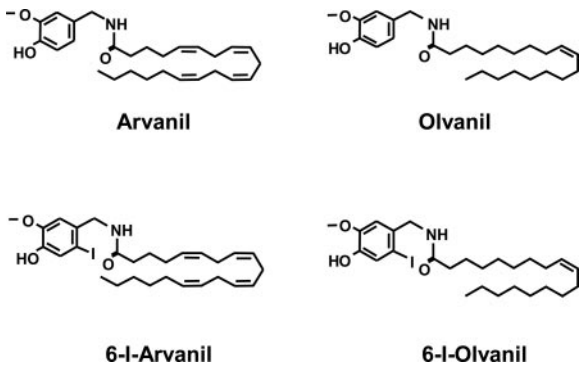

Fig. 1. Chemical structures of the NAVAs used in this study.

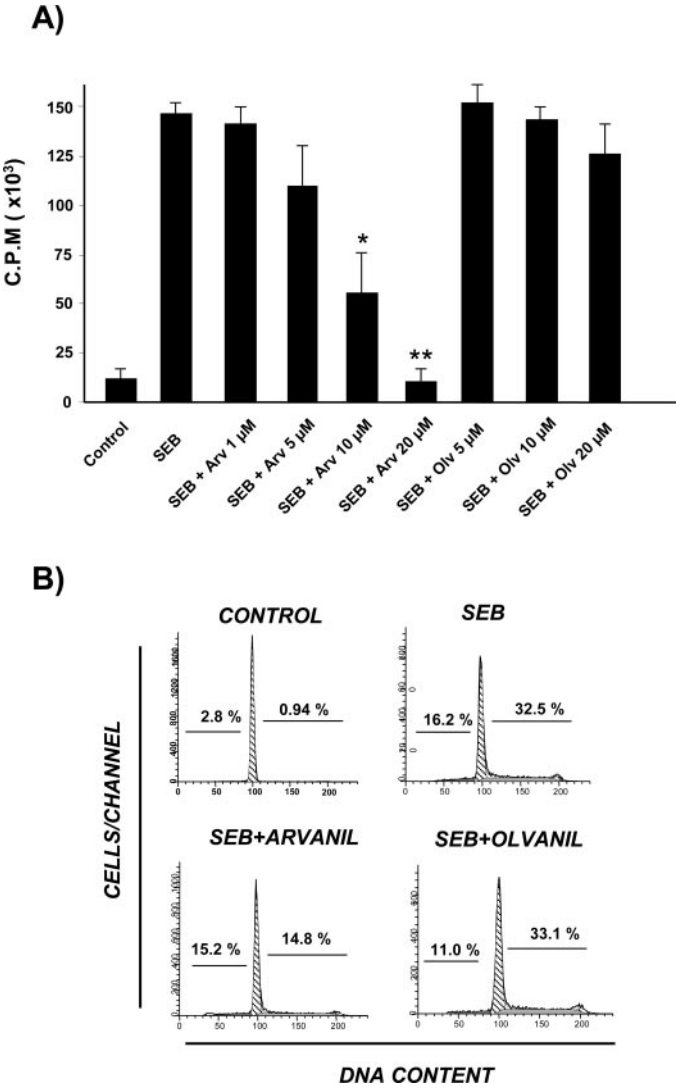

Fig. 2. Effect of arvanil and olvanil on T-cell proliferation and cell cycle progression. A, human PBMCs were stimulated with SEB (1 μg/ml) in the presence or absence of increasing concentrations of arvanil and olvanil for 72 h. [^{3}H]Thymidine incorporation was measured by liquid scintillation counting and represented as the mean of cpm ± S.E. of three different experiments. *, $P \leq 0.05$, and **, $P \leq 0.01$ to SEB. B, PBMCs were pretreated with either arvanil (10 μM) or olvanil (10 μM) and stimulated with SEB (1 μg/ml) for 72 h. The percentage of subdiploid cells and cells entering the S and G_2/M phases of the cell cycle is indicated. The results are representative of four independent experiments.

studied in SEB-stimulated primary T cells. We show in Fig. 3B that stimulation with SEB causes an increase of CD25-positive cells (58 ± 7%) that was markedly inhibited by arvanil (16 ± 8%). Arvanil was also effective at inhibiting the expression of CD69 and intercellular adhesion molecule-1 in SEB-activated T cells (data not shown).

Effect of Arvanil on IL-2 Promoter Activity. IL-2 gene expression is regulated mainly at the transcriptional level. To evaluate whether the inhibitory effect of Arvanil on T cell activation and proliferation was mediated by the inhibition of cytokines at the transcriptional level, we investigated the regulation of IL-2 promoter in Jurkat cells transiently transfected with the promoter reporter plasmid IL-2-Luc. After transfection, cells were preincubated with arvanil for 30 min, activated with a mix of CD3/CD28 mAbs for 6 h, and tested for luciferase activity. Arvanil efficiently inhibited CD3/CD28-induced luciferase expression driven by the IL-2 promoter in a dose-dependent manner (Fig. 4). Transcriptional activity of IL-2 gene depends on the coordinated activation of several transcription factors, including NFAT and NF-κB, and especially the NFAT activation pathway is highly dependent on calcium signaling. Thus, we evaluated the effect of arvanil on the transcriptional activity of those factors by using luciferase reporter constructs under the control of minimal promoters containing binding sites for each of them.

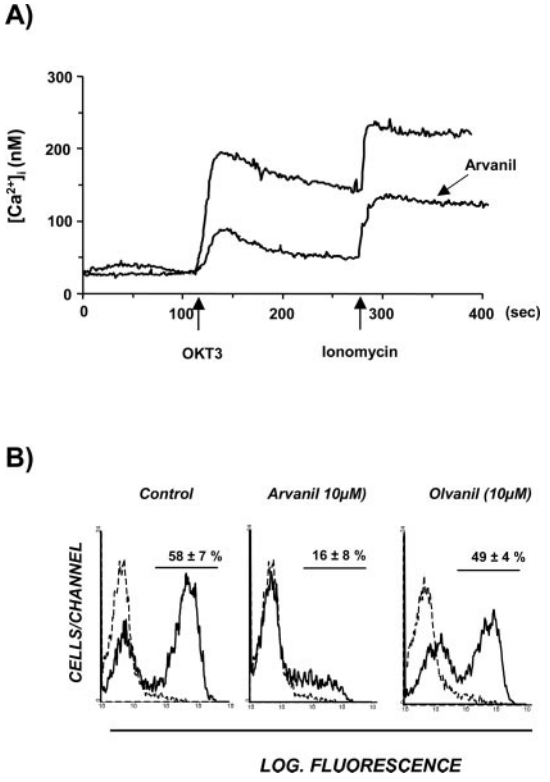

Fig. 3. Effect of arvanil on Ca^{2+} mobilization in Jurkat cells and CD25 expression in primary T cells. A, Jurkat cells were loaded with Indo-1-AM, treated with OKT-3 in the presence or absence of arvanil (10 μM), and the calcium mobilization was measured by ratiometric fluorescence as indicated under *Materials and Methods*. Vertical arrow indicates the time of OKT-3 addition, and arvanil was added 10 s before. After approximately a 5-min recording, ionomycin (1 μg/ml) was added to standardize results. Data are representative of at least four independent experiments. B, PMBCs were treated as indicated for 48 h and the expression of CD25 detected by flow cytometry. The numbers represent the percentage ± S.D. of $CD25^+$ cells measured in three different experiments.

Activation through CD3/CD28 increased the luciferase gene expression driven by these promoters in Jurkat cells, and we found that arvanil effectively inhibited each of these promoters in a dose-dependent manner, with NFAT being the most sensitive transcription factor to the inhibitory activity of arvanil (Fig. 4).

Effect of Arvanil and 6′-I-Arvanil on NF-κB Activation. We have reported recently that NADA, an endogenous lipid mediator that is a structural isomer of arvanil, is a potent inhibitor of the NF-κB pathway (Sancho et al., 2004, 2005). This NF-κB pathway is activated in T cells both by costimulation with mAbs anti-CD3 and anti-CD28 or TNFα (Li et al., 2005). Therefore, to study the effects of arvanil and its halogenated analog on the activation of the NF-κB pathway, we used the cloned 5.1 cell line that is highly sensitive to TNFα. First, The NF-κB DNA binding activity was studied by EMSA assays in nuclear proteins extracted from 5.1 cells that were preincubated with increasing concentrations of either arvanil or 6′-I-arvanil and further stimulated for 30 min with TNFα. As depicted in Fig. 5A, arvanil was more efficient at inhibiting the TNFα-induced NF-κB binding activity. We have shown previously that the heterodimer p50/p65 is the main complex induced by TNFα in this cell line (Sancho et al., 2003a). Next, to identify the molecular target of these compounds on the NF-κB activation pathway, 5.1 cells were stimulated with TNFα for 10 min in the absence or presence of increasing concentrations of either arvanil or 6′-I-arvanil, and the total extracts were analyzed for IκBα degradation and Ser536 p65 phosphorylation induced by TNFα. We observed that in this case, both IκBα degradation and p65 phosphorylation were inhibited by arvanil in a concentration-dependent manner. In contrast, 6′-I-arvanil did not prevent TNFα-induced IκBα degradation, but it was a potent inhibitor of Ser536 p65 phosphorylation. The steady-state levels of α-tubulin were not affected (Fig. 5B). Because Ser536 p65 phosphorylation is required for the NF-κB transcriptional activity, we analyzed the effects of arvanil and 6′-I-arvanil on NF-κB-mediated HIV-1 promoter activation, because this promoter contains two NF-κB binding sites that are absolutely required for TNFα-induced transactivation. Thus, 5.1 cells were preincubated with increasing concentrations of the compounds (see structures in Fig. 1) and next were stimulated with TNFα for 6 h. The cells were then lysed, and the luciferase activity was measured. Pretreatment with arvanil or 6′-I-arvanil resulted in a clear inhibition of the TNFα-mediated HIV-1-LTR gene transcription in a concentration-dependent manner (Fig. 5C). This inhibition was much less evident in the case of olvanil and 6′-I-olvanil, indicating that the structure of the fatty acid chain could play a critical role in this effect, which is very likely to be independent of CB_1 and TRPV1 receptors, because these are not expressed in 5.1 cells (Sancho et al., 2003a; data not shown).

6-I′-Arvanil Inhibits T-Cell Proliferation in Primary Lymphocytes and Induces Apoptosis in Tumoral Cells. We investigated the antiproliferative activity of iodinated *N*-acylvanillamines in the model of SEB-induced T-cell proliferation. We observed that both arvanil and 6′-I-arvanil (10 μM) inhibited T-cell proliferation measured by $[^3H]$TdR uptake, with 6-I-arvanil being more potent than its parent compound. In contrast, neither olvanil nor 6-I-olvanil affected the antigen-induced proliferation in human primary T cells (Fig. 6A). In a previous report, we demonstrated that arvanil induces apopto-

sis in Jurkat cells but not in primary T cells. Arvanil proapoptotic activity was dependent on reactive oxygen production and was mediated through the Fas-associated death domain/caspase 8 pathway (Sancho et al., 2003). Because these biological effects are CB_1- and TRPV-1-independent, it was interesting to assess whether, and to what extent, aromatic iodination could affect them. To this purpose, cells were treated for 18 h with arvanil, olvanil, and their iodo-derivatives at 10 μM concentrations. Specific DNA fragmentation, the hallmark of apoptosis, was measured by the TUNEL method and flow cytometry. As expected, arvanil induced a strong increase in the percentage of apoptotic cells (60%), and this proapoptotic activity was further enhanced by 6′-iodination. Remarkably, 6′-iodination enhanced also the proapoptotic activity of olvanil, a weaker inducer of apoptosis compared with arvanil (Fig. 6B). Taken together, these results strongly suggest that the vanilloid moiety is critical for the induction of apoptosis and that vanillyl modification can modulate this activity. The observa-

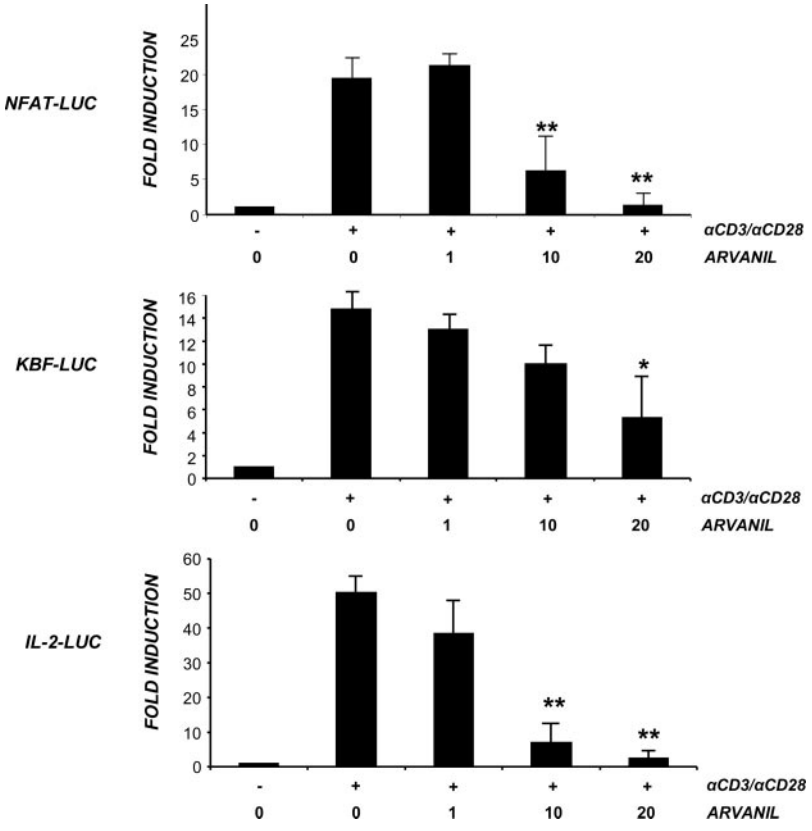

Fig. 4. Effects of arvanil on IL-2 gene regulation, NFAT and NF-κB transactivation. Jurkat T cells transfected with either the IL-2 promoter luciferase reporter plasmid or with the luciferase reporter plasmid NFAT-luc or KBF-Luc as described under *Materials and Methods*. Forty-eight hours after transfection, the cells were treated for 30 min with increasing concentrations of arvanil and then stimulated with a mix of mAbs anti-CD3/anti-CD28 for 6 h, and luciferase activity was measured in the cell lysates. Results are the means ± S.E. of three determinations expressed as fold induction (experimental RLU-background RLU/basal RLU-background RLU. **, $P \leq 0.01$ to fold induction values in the absence of arvanil.

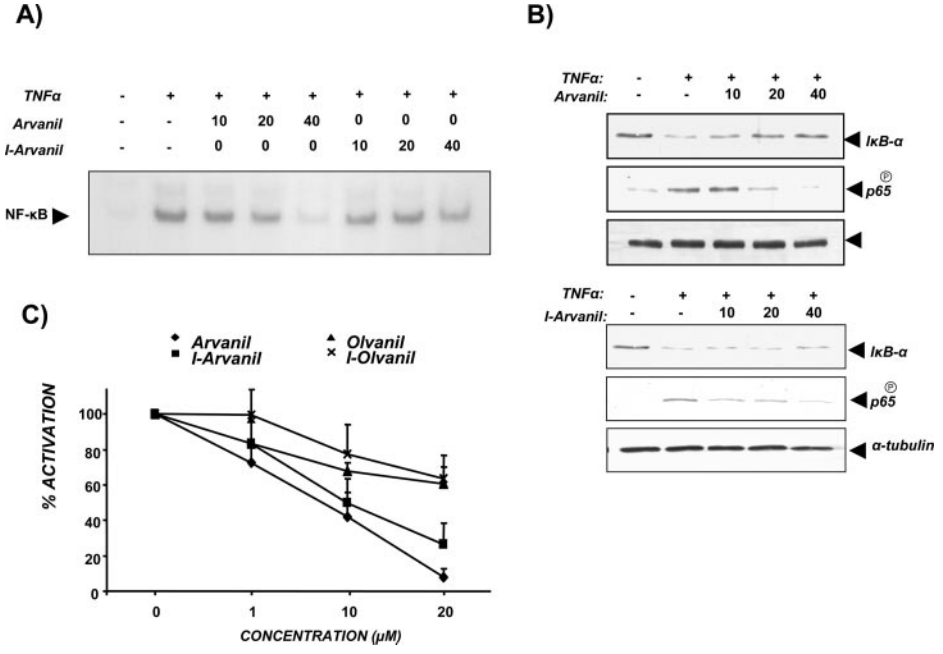

Fig. 5. Effects of arvanil and I-arvanil on NF-κB activation. A, 5.1 cells, either untreated or pretreated with arvanil and I-arvanil at the concentrations indicated, were incubated with TNFα for 30 min. The nuclear extracts from these cells were then assayed by EMSA using γ-^{32}P-labeled NF-κB. B, 5.1 cells were incubated for 30 min with arvanil and then stimulated with TNFα for 10 min, and protein from whole cells was extracted and analyzed for p65 phosphorylation (Ser536) and for IκBα phosphorylation and degradation by Western blot analysis. C, 5.1 cells were preincubated with arvanil, olvanil, 6′-I-arvanil, and 6′-I-olvanil at the indicated concentrations and stimulated with TNFα (2 ng/ml) for 6 h. The luciferase activity was measured, and results are the mean ± S.E. of at least six determinations and expressed as the percentage of activation compared with the values obtained with TNFα alone.

tion that neither AEA nor arachidonic acid could induce apoptosis in 5.1 cells at a 10 μM concentration supports these conclusions, although AEA was found to induce apoptosis when the cells were incubated for a period of time longer than 30 h (data not shown).

6′-Iodination Reverses the TRPV1 Agonist Activity of NAVAs into Antagonist Activity. As reported previously (De Petrocellis et al., 2000a), both olvanil and arvanil are very potent agonists at the recombinant human TRPV1, as assayed by measuring TRPV1-mediated increases in $[Ca^{2+}]_i$ in human embryonic kidney 293 cells transfected with the cDNA encoding for this receptor. However, iodination on the 6′-position of the vanillyl group totally reversed the agonistic activity into a relatively potent antagonistic activity (Table 1 and Fig. 7, A–C). In fact, both 6′-I-olvanil and 6′-I-arvanil were more potent than capsazepine as TRPV1 antagonist against capsaicin under these conditions. The two compounds also efficaciously antagonized the agonist action of the two endovanilloids AEA and NADA (Fig. 7, D–I). With the former agonist, used at a 1 μM concentration, 6′-I-olvanil and 6′-I-arvanil exhibited IC_{50} values of 6.7 ± 0.9 and 30.1 ± 4.9, respectively, whereas with NADA used at a 0.3 μM concentration, the estimated IC_{50} values were 67.0 ± 12.5 and 39.4 ± 7.6, respectively. Both compounds behaved as competitive agonists with AEA, but not with capsaicin or NADA (Fig. 7, B, C, E, F, H, and I). With 6′-I-olvanil, slopes of the Schild's plots were 0.46 ± 0.04 and 0.50 ± 0.09 with capsaicin and NADA, respectively, and 0.93 ± 0.10 with AEA. With 6′-I-arvanil, slopes of the Schild's plots were 0.53 ± 0.09 and 0.77 ± 0.09 with capsaicin and NADA, respectively, and 1.16 ± 0.16 with AEA. The K_b values for 6′-I-olvanil and 6′-I-arvanil against AEA were 1.6 and 6.0 nM, respectively.

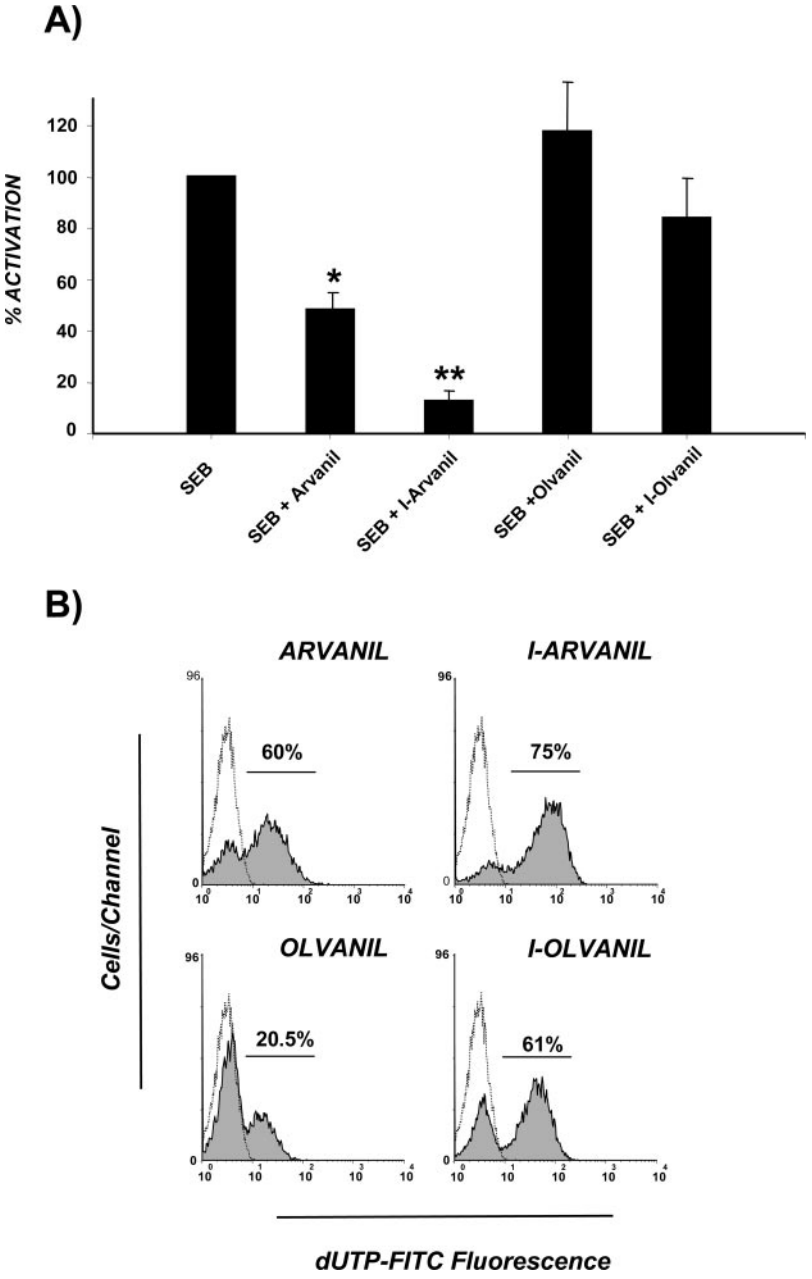

Fig. 6. Effect of NAVAs on T-cell proliferation and apoptosis. A, human PBMCs were stimulated with SEB (1 μg/ml) in the presence or absence of the compounds (10 μM) for 72 h. [^{3}H]Thymidine incorporation was measured by liquid scintillation counting and is represented as the mean of percentage activation compared with SEB ± S.E. of three different experiments. *, $P \leq 0.05$, and **, $P \leq 0.01$ to SEB. B, 5.1 cells were treated with 10 μM of each compound for 18 h, and the percentage of apoptotic cells (DNA fragmentation) was detected by TUNEL staining and flow cytometry. The numbers represent the percentage of apoptotic cells and are representative of three different experiments.

6′-Iodination Does Not Greatly Modify the Capability of NAVAs to Directly or Indirectly Activate Cannabinoid Receptors. As reported previously (Di Marzo et al., 1998, 2000), neither olvanil nor arvanil is a very strong ligand of human recombinant cannabinoid receptors, and they are not efficacious inhibitors of AEA enzymatic hydrolysis through fatty acid amide hydrolase. However, the two compounds, and arvanil in particular, are good inhibitors of AEA cellular reuptake. In the case of arvanil, 6′-iodination of the vanillyl moiety did not strongly modify the capability of binding to CB_1 receptors, slightly improved the affinity for CB_2 receptors and abolished its inhibitory effect on AEA uptake (Table 1). In the case of olvanil, this chemical modification slightly improved both its binding activity at CB_1 receptors and its capability of inhibiting AEA uptake. However, in no case can the 6′-I-derivatives be defined as potent "direct" or "indirect" (i.e., via inhibition of AEA degradation) activators of cannabinoid receptors.

Discussion

In this study, we showed that arvanil, the archetypical NAVA endowed with CB_1 and TRPV1 agonistic activity, in-

TABLE 1

Summary of the effects of NAVAs and their 6′-iodo-derivatives as direct or indirect activators of cannabinoid receptors

Data are means ± S.E. of $n = 3$ experiments.

Compound	K_i		IC_{50}		EC_{50} for hTRPV1 Agonist Activity	IC_{50} for hTRPV1 Antagonist Activity[a]
	hCB_1	hCB_2	AEA Hydrolysis by Rat Brain	AEA Uptake from C6 Cells		
				μM		
Olvanil[b]	>5	>5	>50	19.9 ± 3.7	0.0005 ± 0.0003	N.A.
6′-I-Olvanil	2.5 ± 0.3	>5	>50	9.0 ± 1.1	>10	0.035 ± 0.006
Arvanil[b,c]	2.6 ± 0.5	>5	32.0 ± 4.1	11.2 ± 2.1	0.0005 ± 0.0002	N.A.
6′-I-Arvanil	1.9 ± 0.2	2.3 ± 0.3	>50	>25	>10	0.051 ± 0.007

N.A., not available; h, human.

[a] Calculated against 100 nM capsaicin. Under the same conditions, the IC_{50} value for capsazepine is 0.104 ± 0.014 nM.

[b] Data are from De Petrocellis et al. (2000b).

[c] Data are from Di Marzo et al. (2000).

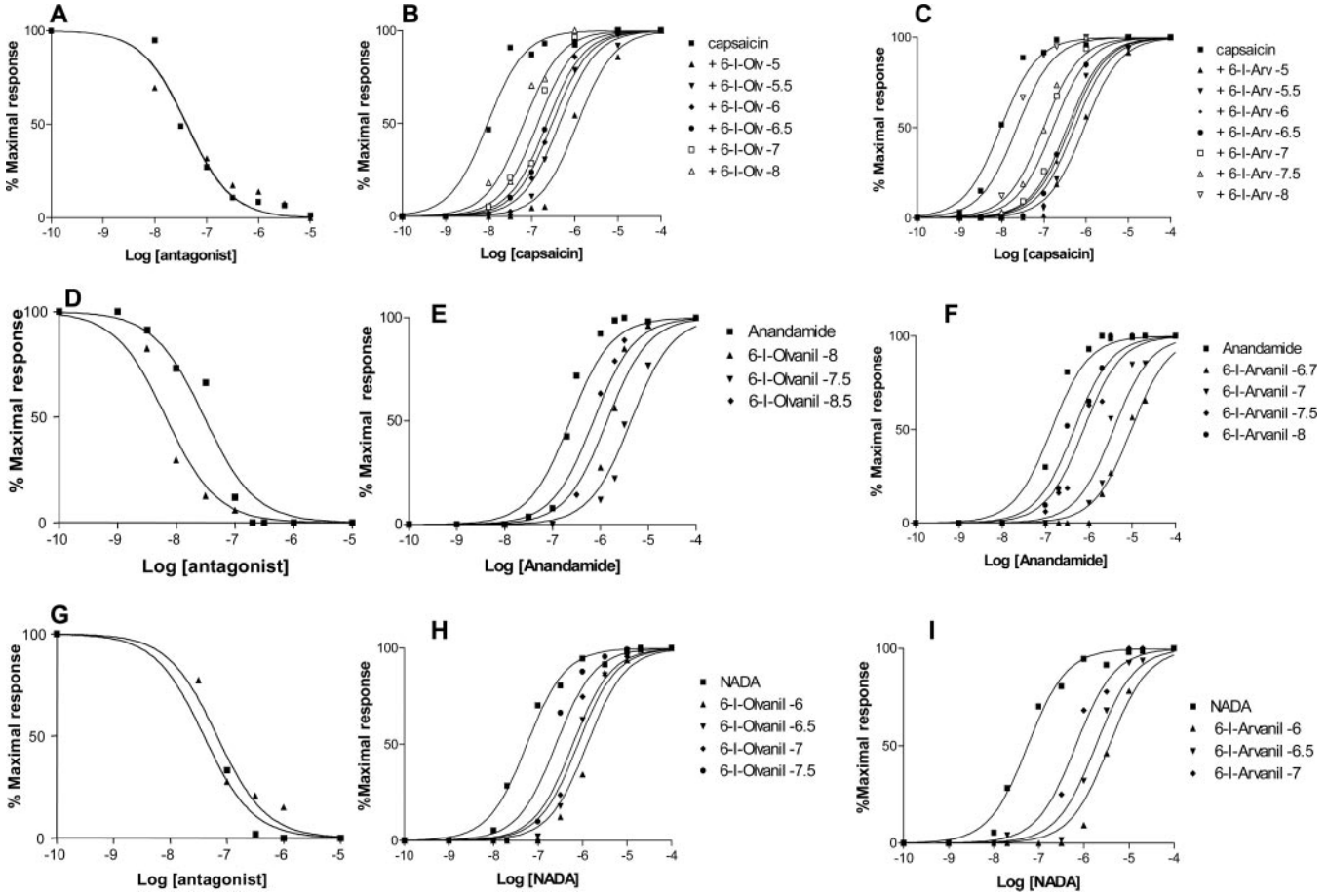

Fig. 7. TRPV1 antagonistic activities of 6′-I-olvanil and 6′-I-arvanil. A, D, and G, concentration-response curves for the inhibition by 6′-I-olvanil (▲) or 6′-I-arvanil (■) of capsaicin (100 nM, A), anandamide (1 μM, D), and NADA (0.3 μM, G). B, C, E, F, H, and I, inhibition by increasing concentrations (indicated as logarithms) of 6′-I-olvanil (B, E, and H) or 6′-I-arvanil (C, F, and I) on increasing concentrations of capsaicin (B and C), anandamide (E and F), or NADA (H and I). These curves were used to draw Schild's plots. Data are means of $n = 3$ experiments. Standard error bars are not shown for the sake of clarity and were never greater than 5%.

hibits antigen-specific-mediated T-cell proliferation by targeting TCR-mediated intracellular calcium mobilization, NFAT activation and IL-2 gene transcription. In addition, we show here that arvanil, olvanil, and their 6-I′ derivatives are pleiotropic agents, showing a series of CB_1- and TRPV1-dependent and independent activities. Aromatic iodination meta to the phenolic hydroxyl causes a dramatic switch in the vanilloid activity of arvanil and olvanil. Thus, whereas aromatic iodination meta to the phenolic hydroxyl caused a dramatic switch in the vanilloid activity of arvanil and olvanil, turning them from TRPV1 agonists into TRPV1 antagonists, the immunosuppressive, anti-NF-κB, and pro-apoptotic activities that are typical of olvanil and, particularly, arvanil were not affected by this structural modification and were still exerted at higher concentrations than those required to antagonize TRPV1.

It is now well recognized that TRPV1 functions as a molecular integrator of nociceptive stimuli, including heat, protons, endogenous ligands, and plant toxins. Potent, nonpungent synthetic TRPV1 agonists capable of immediately desensitizing this receptor can be used for the treatment of several pathologies, including inflammatory hyperalgesia (Szallasi, 2002). The mechanism underlying the analgesic activity of vanilloid agonists is that activation of TRPV1 is followed by rapid desensitization to further stimuli, thus leading to a clinical phenomenon known as "paradoxical analgesia" (Szallasi, 2002). However, TRPV1 is also expressed in the central nervous system and in non-nervous peripheral tissues and cells (De Petrocellis and Di Marzo, 2005), in which its persistent activation may lead to undesirable side effects. For instance, it has been shown that capsaicin (the major pungent compound of red hot chili pepper) induces angiogenesis in an animal model of acute neurogenic inflammation in a process partially mediated by the release of substance P and other neuropeptides (Seegers et al., 2003). Therefore, TRPV1 agonists can be of enormous interest for the development of new drugs for certain types of pathologies (e.g., bladder hyperreflexia), but these compounds may be not suitable for some types of long-term neurogenic inflammatory disorders in which their prolonged administration may exacerbate the disease. Moreover, the irritant sensation and airway irritations induced by TRPV1 agonists (i.e., capsaicin and resiniferatoxin) is not well tolerated by a significant percentage of patients (Szallasi, 2002). For these reasons, the development of TRPV1 antagonists rather than agonists is now in the pipeline for drug discovery against pain and inflammatory disorders (Appendino et al., 2003). We have now found that iodination of NAVAs, a general maneuver to revert TRPV1 antagonism, generates compounds endowed with cannabinoid and TRPV1 receptor-independent in vitro anti-inflammatory activities. Within these actions, 6′-iodination did not influence the capability, or lack thereof, of arvanil or olvanil to inhibit SEB-induced T-cell proliferation or to counteract NF-κB activity and NF-κB-dependent transcriptional activity and T-cell proliferation, although it improved the proapoptotic effect of both compounds on 5.1 leukemia cells.

Neurogenic inflammation is a very complex process in which the immune system plays a key role. For instance, invasion of lymphocytes into brain parenchyma is a common feature in different brain pathologies, including viral encephalitis, multiple sclerosis, stroke, and other post-traumatic processes (Szallasi, 2002). In addition, peripheral inflammation is regulated by the infiltration of activated immune cells into the inflammatory site. Thus, the role of the immune system in inflammatory diseases is a major area of research, and new therapeutic strategies based on immune modulation are under consideration, especially for peripheral neurogenic inflammation and autoimmune disorders such as multiple sclerosis (Behi et al., 2005). In this direction, we have found here that both arvanil and 6′-I-arvanil are potent inhibitors of antigen-induced T-cell proliferation, and the molecular basis for this immunosuppressive activity can be traced back to the inhibition of early events in TCR-mediated activation. Indeed, our results show that arvanil inhibits TCR-induced calcium mobilization, and, as a consequence, NFAT activation and IL-2 gene transcription are clearly prevented. Moreover, arvanil also affects the NF-κB activation pathway activated by both CD3/CD28 and TNFα-mediated signaling. Arvanil and 6′-I-arvanil differ also in the mechanism of their inhibition of the NF-κB pathway, because arvanil but not 6′-I-arvanil prevents TNFα-induced IκB degradation and NF-κB binding to DNA, although both compounds inhibit Ser536 phosphorylation of the p65 subunit. Whereas further research is required to identify the specific molecular targets for both compounds in the NF-κB pathway, we have demonstrated previously that endogenous endocannbinoids such are AEA and NADA inhibit this pathway by acting at a different level in NF-κB regulation (Sancho et al., 2003a, 2004). Notwithstanding, the NF-κB inhibitory activity of these two compounds is of utmost relevance because this transcription factor regulates not only T-cell activation but also many other events involved in the inflammatory process (Li et al., 2005). For instance, substance P modulates adhesion molecule gene transcription in microvascular endothelial cells through an NF-κB dependent pathway (Quinlan et al., 1999), and also activates NF-κB-dependent IL-8 gene expression (Lieb et al., 1997). Experiments are in progress in our laboratory to study the effects of arvanil, 6′-I-arvanil, and other iodo-derivatives of NAVAs in the signaling pathways activated by substance P in human macrophage and endothelial cells.

6′-Iodination did not quantitatively affect the in vitro anti-inflammatory properties of arvanil or substantially improve the much weaker similar effects exerted by olvanil. Unlike arvanil, this compound did not inhibit SEB-induced T-cell proliferation and did not affect NF-κB activity, NF-κB-dependent transcriptional activity, and T-cell proliferation. Nevertheless, this compound could still induce apoptosis of 5.1 leukemia cells, and this property was retained in its 6′-iododerivative.

Because arvanil and olvanil are capable of stimulating the activity of cannabinoid receptors either directly or indirectly (via inhibition of endocannabinoid inactivation mechanisms), we were interested in assessing whether 6′-iodination also would affect these properties of the two NAVAs. However, no major effect was found in this case, except for a strong decrease in the inhibitory action of arvanil on AEA cellular uptake. Thus, both olvanil and 6′-I-olvanil were very weak ligands of both cannabinoid receptor subtypes, although they shared the capability of inhibiting AEA cellular uptake, whereas 6′-I-arvanil exhibited slightly improved affinity for CB_2 receptors with respect to arvanil. Although these properties are unlikely to contribute to the in vitro anti-inflam-

matory and proapoptotic actions of the four compounds studied here, because these actions were observed in cell lines that do not express cannabinoid receptors or were found previously to not be sensitive to cannabinoid receptor antagonists, we cannot rule out that cannabinoid receptors might contribute and add further strength to the putative anti-inflammatory effects that might be observed in vivo with these compounds.

As a final note, it is interesting to remark that different structure-activity relationships underlie the pleiotropic properties of NAVAs that can be modulated independently and make it tempting to speculate that the apolar acyl moiety and the hydrophilic head group of these compounds interact with their receptors in a different way. Drug discovery in the realm of anti-inflammatory drugs has long been dominated by "magic bullet" strategies, as exemplified by cyclooxygenase-2 inhibitors. We have described here an alternative approach that led to the development of two compounds (6′-I-olvanil and 6′-I-arvanil) that, in in vitro systems, can inhibit neurogenic inflammation with a pleiotropic mechanism that includes TRPV1 antagonists and T-cell activation inhibition, and studies in vivo are now needed to show whether these compounds have indeed a potential as anti-inflammatory drugs.

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Address correspondence to: Dr. Eduardo Muñoz Blanco, Departamento Biologia Celular, Fisiologia e Inmunologia, Facultad de Medicina de Córdoba, Avda. Menéndez Pidal s/n, 14004 Córdoba, España. E-mail: fi1muble@uco.es