Imperatorin Inhibits HIV-1 Replication through an Sp1-dependent Pathway*

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Rocío Sancho‡, Nieves Márquez‡, Marta Gómez-Gonzalo§, Marco A. Calzado‡, Giorgio Bettoni‡,
Maria Teresa Coiras‡, José Alcamí§, Manuel López-Cabreras§, Giovanni Appendino‡, and Eduardo Muñoz‡**

From the 3Departamento de Biología Celular, Fisiología e Inmunología, Universidad de Córdoba, Facultad de Medicina,
Avda. de Menéndez Pidal s/n, 14004 Córdoba, Spain, §Universidad de Biología Molecular, Hospital Universitario de la
Princesa, 28006 Madrid, Spain, *Università degli Studi del Piemonte Orientale, Dipartimento di Scienze Chimiche,
Alimentari, Farmaceutiche e Farmacologiche, Viale Ferrucci 33, 28100 Novara, Italy, and ‡Centro de Biología
Fundamental, Instituto Carlos III, C/td. Majadahonda a Pozuelo, 28220, Majadahonda, Madrid, Spain

Coumarins and structurally related compounds have been recently shown to present anti-human immunodeficiency virus, type 1 (HIV-1) activity. Among them, the dietary furanocoumarin imperatorin is present in citrus fruits, in culinary herbs, and in some medicinal plants. In this study we report that imperatorin inhibits either vesicular stomatitis virus-pseudotyped or gp160-encapsidated recombinant HIV-1 infection in several T cell lines and in HeLa cells. These recombinant viruses express luciferase as a marker of viral replication. Imperatorin did not inhibit the reverse transcription nor the integration steps in the viral cell cycle. Using several S’ long terminal repeat-HIV-1 constructs where critical response elements were either deleted or mutated, we found that the transcription factor Sp1 is critical for the inhibitory activity of imperatorin induced by both phorbol 12-myristate 13-acetate and HIV-1 Tat. Moreover in transient transfections imperatorin specifically inhibited phorbol 12-myristate 13-acetate-induced transcriptional activity of the Gal4-Sp1 fusion protein. Since Sp1 is also implicated in cell cycle progression we further studied the effect of imperatorin on cyclin D1 gene transcription and protein expression and in HeLa cell cycle progression. We found that imperatorin strongly inhibited cyclin D1 expression and arrested the cells at the G1 phase of the cell cycle. These results highlight the potential of Sp1 transcription factor as a target for natural anti-HIV-1 compounds such as furanocoumarins that might have a potential therapeutic role in the management of AIDS.

Furanocoumarins are abundant in citrus fruits, umbelliferous vegetables, and certain herbal medicines (1). Interest in these compounds has long been limited to psoralen, the mainstay of photodynamic therapy, but over the past few years there has been a growing interest in their penetrated derivatives spurred by the potent activity on drug metabolism of dietary compounds such as bergamottin and its dimeric analogs. While bergamottin is mainly contained in citrus plants, its isomer imperatorin is more widespread, occurring not only in lemon and lime oils, but also in the medicinal plant Angelica dahurica (2) and in popular culinary herbs such as parsley, fennel (1, 3). Despite its occurrence in edible plants, imperatorin shows potent pharmacological activity and has been studied for its anti-inflammatory and antitumoral activities (4–6). Moreover imperatorin and its close analog hernaculine have been reported to show anti-HIV activity with EC50 (effective concentration 50) in the micromolar range (7). However, nothing is known about the molecular mechanism underlying this light-independent activity. Since the coumarin calanolide A shows unique anti-HIV activity and is currently undergoing clinical trials in AIDS patients (8), we became interested in the molecular mechanisms underlying the activity of imperatorin, the archetypal antiviral dietary coumarin.

The human immunodeficiency virus, type 1 (HIV-1) is the etiologic agent of AIDS and is a member of the lentivirus family of retroviruses. The HIV-1 genome contains three structural and six regulatory genes, which encode structural viral proteins and six unique regulatory/accessory proteins that play a critical role in HIV-1 gene expression, transmission, and pathogenesis (9). The HIV-1 enters permissive cells by fusion of its envelope with the plasma membrane subsequent to gp120 binding to the CD4 receptor molecule. This interaction induces a conformational change that promotes secondary gp120 binding to the coreceptors CXCR4 or CCR5, and subsequently gp41 undergoes conformational changes that allow the interaction of the NH2-terminal fusion peptide of gp41 with the cell membrane (10, 11). Early after primary infection the viral genome is retrotranscribed and integrated into the host genome (12). The postintegration phase of the viral cycle preferentially occurs in activated cells and is regulated by the collaborative action of viral regulatory proteins and cellular factors on the long terminal repeat (LTR) promoter, which determines the extent of HIV-1 gene transcription and the level of viral replication in the infected cells (13, 14).

The HIV-1-LTR promoter is ~640 nucleotides long and has

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** To whom correspondence should be addressed. Tel.: 34-957-218267; Fax: 34-957-218229; E-mail: f1imuibe@uco.es.

1 The abbreviations used are: HIV-1, human immunodeficiency virus, type 1; DRB, 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole; LTR, long terminal repeat; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/ERK kinase; PMA, phorbol 12-myristate 13-acetate; pTEFb, positive transcription elongation factor b; RLU, relative light unit(s); TAR, trans-activating response element; VSV, vesicular stomatitis virus; Luc, luciferase; PBS, phosphate-buffered saline; EMSA, electrophoretic mobility shift assay; GST, glutathione S-transferase; wt, wild type; DBD, DNA binding domain; CBP, CREB-binding protein (CREB)-binding protein.
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binding sites for many cellular transcription factors and a cis-activating stem-loop RNA structure called trans-activating response element (TAR), which is located from positions +1 to +59 of the HIV-1-LTR (15, 16). The TAR element represents the main binding site for the HIV-1 regulatory protein Tat (17–19). Through interaction with TAR, Tat recruits a host cell protein kinase complex, pTEFb, comprised of cyclin-dependent kinase 9 and cyclin T1, which binds to the loop region of TAR (20–22). As a consequence of the pTEFb recruitment to the HIV-1 promoter complex, the COOH-terminal domain of the RNA polymerase II is phosphorylated (21, 23), thereby increasing the efficiency of transcription elongation (24–26).

In addition to the TAR binding activity, Tat also interacts with other cellular transcription factors that regulate the transcriptional activity of the HIV-1-LTR promoter (16, 27, 28). The core promoter region of the HIV-1-LTR contains three tandem Sp1 binding sites and two xB elements located upstream of the TATA box. Although the xB enhancer has been considered the main inducible cis-acting element (29), several reports suggest that the interaction between Sp1 and Tat is required for Tat-mediated HIV-1-LTR transactivation (30, 31). In this sense, mutations of these Sp1 sites affect Tat-induced LTR transcriptional activity (32). The molecular mechanisms by which Tat interacts with Sp1 are controversial, and although some authors have reported a physical association between Tat and Sp1 (33), others failed to detect such an interaction, suggesting that bridge proteins are required for Tat–Sp1 complex formation (34). For instance, modulation of Sp1 phosphorylation by Tat-mediated recruitment of this factor to DNA-dependent protein kinase complex results in up-regulated expression of the HIV-1-LTR (35). Moreover it has been recently shown that pTEFb may be recruited to the preinitiation complexes through physical association of cyclin T1 with DNA-bound Sp1. This interaction allows robust HIV-1-LTR activation irrespective of pTEFb–Tat–TAR ternary complex formation (36), highlighting the importance of Sp1 in HIV-1 replication by both Tat-dependent and -independent pathways and its potential as a target for the development of new anti-HIV compounds.

Efforts to find an effective anti-HIV chemotherapy have been mainly focused on the development of chemicals targeting viral proteins, which are essential for HIV-1 replication (37). This antiviral therapy presents important limitations (26), and therefore the development of new anti-HIV agents is focusing on novel structures and/or new action mechanisms. In this sense, plant-derived natural products such as the coumarin derivatives are emerging as potent anti-HIV agents (8). In this study we investigated the anti-HIV effects of several furanocoumarins, and we showed that imperatorin is a potent inhibitor of HIV-1 replication in both primary T lymphocytes and transformed cell lines. We present evidence that imperatorin inhibits the transcriptional activity of the HIV-1-LTR promoter through a signaling pathway involving the activation of the Sp1 transcription factor.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents—MT-2 and Jurkat cells (American Type Culture Collection, Manassas, VA) were cultured in RPMI 1640 medium (Invitrogen) containing 10% heat-inactivated fetal bovine serum, 2 mm L-glutamine, penicillin (50 units/ml), and streptomycin (50 μg/ml) maintained at 37 °C in a 5% CO2 humidified atmosphere, and splinted twice a week. HeLa and 293T cells (ATCC) were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with fetal bovine serum and antibiotics at 37 °C in a 5% CO2 humidified atmosphere and splinted when confluent. Imp and 293T cells were inoculated with Opuntia chihurensis (28). P450 and all other reagents not cited above or later were purchased from Sigma.

Plasmids—The AP-1-luciferase (AP-1-Luc) plasmid was constructed by inserting three copies of an SV40 AP-1 binding site into the Xhol site of pGL-2 promoter vector (Promega, Madison, WI). The KBF-Luc construct contains three copies of the major histocompatibility complex enhancer xB site upstream of the ovalbumin promoter followed by the luciferase gene (39). The vector pNL4-3.Luc.R′ E′ (AIDS Research and Reference Reagent Program, NIAID, National Institutes of Health) from N. Landau, as described in Ref. 40, contains the firefly luciferase gene inserted into the pNL4-3 nef gene. Two frameshifts (5′ Env and Vpr amino acid 26) render this clone Env′ and Vpr′. The pcDNA-PSV contains the vesicular stomatitis virus G protein and was obtained from Dr. Arenzana-Seisdedos (Institute Pasteur, Paris, France), and the pcDNA-Tat expression vector has been described previously (41). The plasmid pEPNL3 was constructed by EcoRIV/IXhol digestion of pNL4-3-Luc.E′ together with either the pcDNA-PSV or the plasmid pNL3 plasmid encoding the vesicular stomatitis virus G protein of HIV-1 envelope, using the calcium phosphate transfection system as described before (46). Supernatants, containing virus stocks, were harvested 48 h post-transfection, centrifuged 5 min at 500 × g to remove cell debris, and stored at −80 °C until use. Cell-free viral stock was tested using an enzyme-linked immunosassay for antigen HIV-p24 detection (INNOTEST™ HIV-Ag, INNOGENETICS, Barcelona, Spain). Cultures were infected at a dose of 200 ng of HIV-1 gag p24 protein.

Production of VSV-pseudotyped and HIV-1 Recombinant Viruses—High titer VSV-pseudotyped or HIV-1 recombinant virus stocks were produced in 293T cells by cotransfection of pNL4-3.Luc.R′ E′ together with either the pcDNA-PSV or the plasmid pNL3 plasmid encoding the vesicular stomatitis virus G protein of HIV-1 envelope, using the calcium phosphate transfection system as described before (46). Supernatants, containing virus stocks, were harvested 48 h post-transfection, centrifuged 5 min at 500 × g to remove cell debris, and stored at −80 °C until use. Cell-free viral stock was tested using an enzyme-linked immunosassay for antigen HIV-p24 detection (INNOTEST™ HIV-Ag, INNOGENETICS, Barcelona, Spain). Cultures were infected at a dose of 200 ng of HIV-1 gag p24 protein.

VSV-pseudotyped HIV-1 Infection Assay—Cells (106/ml) were plated on a 24-well plate and were pretreated with the compounds for 30 min. After pretreatment, cells were inoculated with virus stocks (200 ng of p24), and 24 h later cells were washed twice in PBS and lysed in 25 mM Tris-phosphate, pH 7.8, 8 mM MgCl2, 1 mM dithiothreitol, 1% Triton X-100, and 7% glycerol during 15 min at room temperature. Then the lysates were centrifuged, and the supernatant was used to measure luciferase activity using an Autolumat LB 9510 (Berthold, Bad Wildbad, Germany) following the instructions of the luciferase assay kit (Promega). The results are represented as the percentage of activation (considering the infected and untreated cells as 100% activation) or RLU. Results represent mean ± S.D. of four different experiments.

Isolation of Nuclear Extracts and Mobility Shift Assays—Human peripheral blood mononuclear cells from healthy adult volunteer donors were isolated by centrifugation of venous blood on Ficoll-Hypaque® density gradients (Amersham Biosciences). Cells (2 × 106/ml) were washed twice in PBS and lysed in 25 mM Trisophosphate, pH 7.8, 8 mM MgCl2, 1 mM dithiothreitol, 1% Triton X-100, and 7% glycerol for 15 min at room temperature. Then the lysates were centrifuged, and the supernatant was used to measure luciferase activity using an Autolumab 9510 (Berthold, Bad Wildbad, Germany) following the instructions of the luciferase assay kit (Promega). The results are represented as the percentage of activation (considering the infected and untreated cells as 100% activation) or RLU. Results represent mean ± S.D. of four different experiments.

Isolation of Nuclear Extracts and Mobility Shift Assays—HeLa cells were transfected with the indicated plasmids using LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s recommendations. 24 h post-transfection, cells were pretreated with imperatorin for 30 min and treated or not with PMB for 12 h. The cells were washed twice in PBS and lysed in 25 mM Trisophosphate, pH 7.8, 8 mM MgCl2, 1 mM dithiothreitol, 1% Triton X-100, and 7% glycerol for 15 min at room temperature in a horizontal shaker. Then the lysates were centrifuged, and the supernatant was used to measure luciferase activity using an Autolumab 9510 (Berthold, Bad Wildbad, Germany) following the instructions of the luciferase assay kit (Promega). The results are represented as the percentage of activation (considering the infected and untreated cells as 100% activation) or RLU. Results represent mean ± S.D. of four different experiments.

Isolation of Nuclear Extracts and Mobility Shift Assays—HeLa cells (106/ml) were pretreated with imperatorin at the indicated doses for 1 h (fatty acid free) in the presence or absence of PMB (50 ng/ml), and then washed twice with cold PBS, and proteins from nuclear extracts were isolated as described previously (47). Protein concentration was determined by the Bradford method (Bio-Rad). For the electrophoretic mobility shift assay (EMSA), double-stranded oligonucleotide containing the consensus site for Sp1, 5′-ATT CGA TCG GCG CGG GAC GAG-3′ (Promega), was end-labeled with [γ-32P]ATP. The reaction mixture contained 15 μg of total extracts, 0.5 μg of poly(dI-dC) (Amer sham Biosciences), 20 mM Hepes, pH 7, 70 mM NaCl, 2 mM dithiothreitol, 0.01% Nonidet P-40, 100 μg/ml bovine serum albumin, 4% Ficoll, and 100,000 cpm end-labeled DNA fragments in a total volume of 20 μl.
When indicated, 0.5 μl of rabbit anti-Spy (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or preimmune serum was added to the standard reaction before the addition of the radiolabeled probe. For cold competition, a 100-fold excess of the double-stranded oligonucleotide competitor was added to the binding reaction. After a 30-min incubation at 4 °C, the mixture was electrophoresed through a native 6% polyacrylamide gel containing 89 mM Tris base, 89 mM boric acid, and 2 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. For the Tat-TAR binding assay, RNA probe containing the 5′ bulge of TAR (48) was end-labeled with γ-<sup>32</sup>PATP and incubated with 20 μM recombinant GST-Tat protein in EMSA buffer for 30 min at 4 °C, and RNA-protein complexes were separated by a 6% denaturing polyacrylamide gels, dried, and exposed to x-ray film at ~80 °C.

Western Blot—HeLa cells (10/5 ml) were treated with imperatorin at the indicated doses for 12 h. Cells were then washed with PBS, and proteins were extracted from cells in 50 μl of lysis buffer (20 mM Hepes, pH 8.0, 10 mM KC1, 0.15 mM EGTA, 0.15 mM EDTA, 0.5 mM NaVO<sub>4</sub>, 5 mM NaF, 1 mM dithiothreitol, 1 μg/ml leupeptin, 0.5 μg/ml pepstatin, 0.5 μg/ml aprotinin, and 1 mM phenylmethylsulfon fluoride) containing 0.5% Nonidet P-40. Protein concentration was determined by a Bradford assay (Bio-Rad), and 30 μg of proteins were 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 h for first) and blocked in TBS solution containing 0.1% Tween 20 and 5% nonfat dry milk overnight at 4 °C, and immunodetection of cyclin D1 was carried out with monoclonal antibody α-CyclD1 (Sigma) and horseradish peroxidase-labeled secondary antibody using the ECL system (Amersham Biosciences).

Semiquantitative PCR Analysis—Reverse transcriptase products were detected as described previously (49) with minor modifications. Briefly HeLa cells were infected with VSV-pseudotyped recombinant virus (200 ng of p24 inoculum) for 24 h as indicated, and total DNA was extracted with the QIAamp DNA minikit (Qiagen GmbH, Hilden, Germany) and quantified by UV spectrophotometry at 260 nm. Each PCR amplification was performed in a 50-μl PCR mixture containing DNA (50 ng), 1.5 mM MgCl2, 200 μM dNTPs, 0.5 μM concentration of each primer, and 2.5 units of recombinant Taq DNA polymerase (Invitrogen). The mixtures were amplified in a MultiGene cycler IR system (Labnet, Woodbridge, NJ) for an initial 2 min denaturation step at 91 °C and then 35 cycles consisting of 1 min at 91 °C, 2 min at 65 °C, and 1 min at 72 °C and a final extension step of 7 min. The following primers were used to amplify short retrotranscription product (amplonc size, 140 bp): R/U (forward), 5′-GCC TAA CTA GGA AAC CCA CTG-3′; R/U (reverse), 5′-CTG CTA GAG ATT TTC CAC ACT GAC-3′. The following primer was used to amplify long retrotranscription product (amplonc size, 200 bp): R/U (forward), LTRgag (reverse), 5′-GAG CCC TGC AAG AGA GCT CTC CTG-3′. As a control, genomic DNA was extracted from HeLa cells, subjected to β-actin amplification, and PCR products were electrophoresed on a 2% (w/v) agarose gel.

**Analysis of HIV-1 Integrated DNA by Nested Alu-PCR Assay—** Genomic DNA from HeLa VSV-pseudotyped HIV-1-infected cells and HeLa control cells was extracted by using the QIAamp DNA minikit (Qiagen GmbH) and quantified by UV spectrophotometry at 260 nm. The detection of HIV-1-LTR integrated into the cell genome was performed as described previously (50) with slight modifications. The first PCR was carried out by using primers LA1, from conserved sequences of HIV-1-LTR, and LA2, from conserved human Alu sequences. The sequence of the primers are as follows: LA1, 5′-TGTGGCCTGCCTTTGTGGT-3′ (forward); and LA2, 5′-TGGTGGATCACGCCTTGT-5′ (reverse). Each PCR amplification was performed in a 50-μl PCR mixture containing 0.5 μg of total DNA, 2 mM MgSO4 (Applied Biosystems, Foster City, CA); a 300 μM concentration of each of dATP, dGTP, dCTP, and dTTP (Amersham Biosciences); 20 pmol of primers LA1 and LA2, 5 μl of 10× reaction buffer (Applied Biosystems); and 1.25 units of AmpliTaq DNA polymerase (Applied Biosystems). Amplifications were carried out in an MJ Research PTC-100 thermocycler (Saratoga, CA) in a GeneAmp PCR System 2700 (Applied Biosystems). Samples were subjected to an initial cycle of 94 °C for 10 min. Cycling conditions of the PCR were 50 cycles: 94 °C for 45 s, 53 °C for 1.5 min, 72 °C for 30 s, and a final incubation of 72 °C for 10 min. The second PCR (LTR-nested) was performed by using LTR internal primers: NL1, 5′-CTGGCTGCTCGAGGAAA-3′ (reverse) (amplonc size, 142 bp), A-3′-CCAGTGCTGCTCGAGGAA-3′ (reverse) (amplonc size, 200 bp), with a 2 μl aliquot of the first PCR amplification, and PCR products were electrophoresed on a 2% (w/v) agarose gel.

**RESULTS**

**Imperatorin Inhibits HIV-1 Replication in Both Lymphoid and Non-lymphoid Cells—** Anti-HIV activity of several coumarins structurally unrelated to calanolides has been reported recently (8). Psoralen, imperatorin, heraclenin, and heracelenol are a set of structurally related bioactive compounds differing from each other in the prenylation and/or oxidation state of the prenyl group bound to the furanocoumarin core (8). To study the anti-HIV activity of these furanocoumarins we infected MT-2 cells with the pNL4-3 HIV-1 clone pseudotyped with the VSV envelope, which bypasses the natural mode of HIV-1 entry into these cells that support robust HIV-1 replication (51). Upon integration into host chromosomes, this recombinant virus expresses the firefly luciferase gene, and consequently luciferase activity in infected cells correlates with the rate of viral replication. Thus, high luciferase activity levels were detected 24 h after cellular infection with the VSV-pseudotyped HIV-1 clone, and pretreatment of MT-2 cells 30 min prior to infection with increasing doses of imperatorin, heraclenin, and heracelenol resulted in a dose-dependent inhibition of the luciferase activity, with imperatorin being the most effective inhibitor of HIV-1 replication, to an extent comparable to the cyclin-dependent kinase 9 kinase inhibitor DRB (5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole) (25). Interestingly, psoralen did not inhibit luciferase activity in MT-2 infected cells, suggesting that prenylation is critical for anti-HIV activity (Fig. 1A).

Next we assayed the anti-HIV activity of imperatorin in other T cell models, discovering that pretreatment of either Jurkat T cells (Fig. 1B) or staphylococcal enterotoxin B-stimulated human primary T cells (Fig. 1C) with imperatorin caused a dose-dependent inhibition of the luciferase activity associated to VSV-pseudotyped HIV-1 proviral clone replication. The VSV protein mediates cell entry via an endocytic pathway (52), and we were interested in studying whether and to what extent imperatorin could also inhibit the replication of a HIV-1 clone that infects T cells through a process of virus-cell membrane fusion. For this purpose, we generated an HIV-1-enveloped provirus by cotransfecting 293T cells with the pNL4-3.LucR-′ vector together with the epNL3 plasmid encoding the HIV-1 envelope gene. Then MT-2 cells were pretreated with imperatorin for 30 min and further infected with the HIV-1-enveloped packaged virus. Fig. 1D shows that imperatorin also inhibited the luciferase activity driven by a recombinant virus that uses the CD4 and CXCR4 cell surface HIV-1 recep-
tors for entry into the cells. The inhibitory activity of imperatorin on both provirus types (VSV-pseudotyped versus HIV-1 gp160-enveloped) indicates that this compound is not a fusion inhibitor and suppresses HIV-1 replication by affecting other viral cycle steps.

The effects of imperatorin on cellular toxicity were evaluated by propidium iodide staining and flow cytometry. Jurkat and HeLa cells were treated with increasing doses of imperatorin, and cell viability was tested after 24, 48, and 72 h. According to our previous results (38) this furanocoumarin induced cell death only at the highest tested concentration (100 μM) and after 48–72 h treatment (Fig. 2). This concentration is 4–5 times higher than the IC50 required to inhibit HIV-1 replication. Similar results were obtained using MT-2 or primary peripheral mononuclear cells (data not shown).

To identify the step inhibited by imperatorin in the viral cell cycle we compared the inhibitory activity of imperatorin in HeLa cells either infected with the VSV-pseudotyped HIV-1 provirus or transfected with the pNL4-3.Luc.R E' plasmid. While viral infection requires reverse transcription, integration, and transcription steps to induce luciferase activity, direct transfection with the pNL4-3.Luc.R E' plasmid only requires the transcription step to express luciferase. Treatment of HeLa cells with imperatorin or DRB before infection resulted in a strong luciferase activity inhibition (Fig. 3A), and a similar pattern was found in HeLa cells transfected for 24 h with the reporter plasmid and further incubated with imperatorin for 12 h (Fig. 3B). We further determined whether HIV-1 reverse transcription or integration steps were affected by imperatorin.

First, semiquantitative PCR was performed to amplify HIV-1 strong stop (R/U5) and full-length (LTR/gag) reverse transcriptase products, which represent early and late reverse transcriptase transcripts, respectively. Imperatorin at 50 μM did not decrease the amount of both R/U5 and LTR/gag products obtained following HeLa cell infection with VSV-pseudotyped HIV-1. However, azidothymidine at 10 μM clearly inhibited the amplification of the full-length (LTR-gag) product (Fig. 3C). In parallel the same HIV-1 DNA regions were amplified by PCR using the pNL4-3.Luc.R E' plasmid as template, and a standard curve was obtained. The amount of the RU/5 and LTR/gag product detected in HIV-1-infected cells was comparable to 10² copies of viral DNA. Thus the reverse transcriptase products detected represent de novo synthesis since less than 10 copies of the HIV-1 R/U5 and LTR/gag reverse transcribed DNA products were detected in the viral preparations (data not shown).

Next we studied the effects of imperatorin on HIV-1 integration in HeLa cells infected with the VSV-pseudotyped HIV-1 proviral clone. The cells were pretreated with azidothymidine or imperatorin before infection, and 24 h later the DNA was extracted and subjected to a first round of Alu-PCR followed by nested PCR using internal LTR primers as described under “Experimental Procedures.” β-Actin was also amplified and
used to normalize the amount of integrated HIV-1. We show in
Fig. 3 that HeLa infection with VSV-pseudotyped HIV-1 re-
sulted in viral integration comparable to the one observed in
DNA extracted from 8E5 cells, which contain a single inte-
grated copy of HIV-1 (53). HIV-1 integration was clearly pre-
vented by azidothymidine pretreatment (relative ratio, 0.24),
while imperatorin up to 50 \( \mu M \) did not inhibit such viral cycle
step. Taken together, these results demonstrated that impera-
torin inhibits HIV-1 replication by acting at the transcriptional
level and that HeLa cells can be a valid model to study the
molecular mechanisms targeted by this natural compound.

Imperatorin Inhibits HIV-1-LTR Transactivation through
NF-\( \kappa \)B, AP-1, and TAR-independent Pathways—To analyze
whether the inhibitory effects of imperatorin on HIV-1 replica-
tion were mediated at the HIV-1-LTR transcriptional activity level, HeLa cells were transiently transfected with the plasmid pXP1LTRwt, containing the complete HIV-1-LTR sequence (nucleotides 644 to 77) upstream of the luciferase reporter gene (Fig. 4A), or cotransfected with the same reporter plasmid along with the HIV-1 Tat expression vector pcDNA3-Tat. The pXP1LTRwt-transfected cells were treated with imperatorin for 30 min and then stimulated or not with PMA for 12 h, and the luciferase activity was measured in cell lysates. To study the effects of imperatorin in Tat-induced HIV-1-LTR activation, the cells were cotransfected with the indicated plasmids and after 24 h incubated with imperatorin for another 12 h, and then the luciferase activity was measured. As depicted in Fig. 4A, imperatorin clearly inhibits HIV-1-LTR transactivation induced by either PMA or HIV-1 Tat protein. In contrast, the HIV-1-LTR basal activity was weakly affected by this compound. The results also show that Tat-induced HIV-1-LTR activation was more efficiently inhibited by imperatorin than PMA-induced LTR transactivation.

The upstream LTR promoter contains binding sites for the transcription factors NF-κB, AP-1, nuclear factor of activated T-cells, and Sp1 among others (14), and since PMA activates signaling pathways leading to NF-κB and AP-1 activation among other transcription factors, we addressed whether imperatorin could impair both NF-κB- and AP-1-dependent transcriptional activation by transfecting HeLa cells with luciferase reporter constructs under the control of minimal promoters containing binding sites for each of them. PMA activation increased the luciferase gene expression driven by these promoters that was not significantly affected by the presence of imperatorin (Fig. 4, B and C). These results strongly suggest that inhibition of luciferase activity observed in either VSV-pseudotyped HIV-1 recombinant virus or LTR promoter is quite specific, and it is not the consequence of nonspecific imperatorin-mediated effects on the transcriptional machinery.

To further confirm the lack of participation of NF-κB and AP-1 transcription factors in the inhibitory mechanisms mediated by imperatorin, HeLa cells were transfected with the pXP1LTRΔκB plasmid (nucleotides -554 to +77 with κB enhancer element deleted, Fig. 5A), which strongly responds to either PMA or HIV-1 Tat protein. Again imperatorin was able to inhibit HIV-1-LTR transactivation in response to both stimuli (Fig. 5A). It is well known that Tat/TAR binding is a critical step for the potent Tat-induced HIV-1-LTR transactivation. Therefore we studied the effects of imperatorin on the “in vitro” binding activities of the protein complexes bound to TAR using HIV-1 Tat recombinant protein. We show that the presence of imperatorin in the binding reaction did not affect Tat-TAR binding complex formation indicating that the inhibitory activity of imperatorin on Tat-induced HIV-1-LTR transactivation was not mediated by a disruption of Tat-TAR complexes (Fig. 5C). To further confirm a TAR-independent pathway for the imperatorin inhibitory mechanism of HIV-1-LTR transactivation, HeLa cells were transiently transfected with the pXP1LTRΔTAR plasmid (deleted in the TAR region) with or without pcDNA3-Tat. As expected, Tat did not induce the tran-

![Fig. 4. Effects of imperatorin on Tat- and PMA-induced HIV-1-LTR activation.](image-url)

**Fig. 4.** Effects of imperatorin on Tat- and PMA-induced HIV-1-LTR activation. A, schematic representation of pXP1LTRwt plasmid (upper panel). HeLa cells were transfected with pXP1LTRwt together with pcDNA3 (left) or pcDNA3-TAT (right). Twenty-four h post-transfection the cells were treated with imperatorin, and 30 min later PMA was added, where indicated, for 12 h. Cell extracts were used to measure luciferase activity, and results are represented as RLU ± S.D. of three different experiments. B, HeLa cells were transfected with the KBF-Luc plasmid, and 24 h later the cells were preincubated with imperatorin for 30 min and stimulated with PMA for 12 h. C, HeLa cells were transfected with the AP-1-Luc plasmid, and 24 h later the cells were treated as in Fig. 3B. Luciferase activity was measured and represented as RLU ± S.D. of three independent assays.
Inhibition of the HIV-1-LTR Transactivation by Imperatorin Is Mediated through the Sp1 Elements Located in the LTR-enhancer—Several reports indicate that the HIV-1-LTR Sp1 binding sites mediate the up-regulation of the transcriptional activity induced by HIV-1 Tat and other stimuli (35, 54). Thus, to analyze the relevance of the Sp1 binding sequences in the imperatorin HIV-1 inhibitory pathway we transiently transfected HeLa cells with two constructs containing the proximal region of HIV-1-LTR (nucleotides \(-81\) to \(+77\)) with either wt or mutated Sp1 binding sites. Imperatorin was found to be a potent inhibitor of the transcriptional activity of the Sp1wt construct in response to PMA, HIV-1 Tat, and a combination of both stimuli (Fig. 6A). Interestingly, in Fig. 6B, it is shown that the mutated Sp1 construct did not respond to either PMA or HIV-1 Tat stimuli separately, but it was activated by a combination of both stimuli, and this induction was not affected by imperatorin. However, this induction (4.5-fold) is minimal when compared with the induction observed using the construct containing all three Sp1 binding sites and the TAR element (Fig. 6A). These results strongly suggest that Sp1 is the main molecular target inhibited by imperatorin. Sp1-dependent transcription can be regulated by both DNA binding activity and post-translational modifications that enhance its transactivational properties (55). Thus, we were interested in studying the effects of imperatorin in both the DNA binding and the transcriptional activity of this factor. In Fig. 7A it is shown that Sp1 DNA binding was not modified by either imperatorin alone or in combination with PMA. The protein complexes bound to the Sp1 sites were identified by supershift and cold competition experiments. To further analyze whether imperatorin directly inhibits Sp1 transactivation properties, we performed cotransfection experiments using a set of constructs containing the full-length Sp1, Sp3, and Sp4 transcription factors fused to the yeast Gal4 transactivator DNA binding domain together with a reporter plasmid containing the luciferase gene under the control of a Gal4-responsive element (Gal4-Luc). The results presented in Fig. 7B revealed that Gal4-Sp1 transcriptional activity was increased (\(\approx 6\)-fold) upon PMA treatment, and this induction was inhibited by the presence of imperatorin. Moreover the levels of basal transcription induced by Gal4-Sp1 were not significantly affected by imperatorin. As expected, pretreatment with imperatorin did not affect the luciferase activity induced by the fusion protein Gal4-c-Jun in PMA-stimulated HeLa cells. Interestingly both Gal4-Sp3 and Gal4-Sp4 transcriptional activities were up-regulated by PMA although to a different extent, and only the Gal4-Sp4-induced activity was inhibited with the highest concentration of imperatorin (Fig. 7C). Altogether these results highlight the importance of Sp1 as the target for imperatorin.
Effects of Imperatorin in Cyclin D1 Expression and Cell Cycle Progression—Sp1 is a ubiquitous transcription factor showing different functional properties, and it fulfills specific roles in the regulation of biological processes by activating a number of genes (55), some of them implicated in cell cycle progression (56). Thus, we studied the effects of imperatorin in the transcriptional activity of the cyclin D1 gene promoter, which contains four functional Sp1 sites (56, 57). As depicted in Fig. 8A, imperatorin inhibited in a dose-dependent manner the transcriptional activity of a transiently transfected construct containing the cyclin D1 promoter (−1745 bp) followed by luciferase gene. Moreover the steady state levels of cyclin D1 protein in HeLa cells were greatly reduced in the presence of imperatorin, which did not affect housekeeping protein β-tubulin expression (Fig. 8B). Finally and since Sp1 has been identified as an important regulator of the cell cycle in G1 phase (58), we investigated the effects of imperatorin in the different phases of the cell cycle in HeLa cells. Therefore, the cells were treated with 25 μM imperatorin for 24 h and compared with untreated control cells, which were full cycling and progressed through the S, G2, and M phases of the cell cycle (45.8% of the cells). However, imperatorin-treated cells showed a clear decrease in the percentage of cells in the G2/M phase that paralleled an increase in the percentage of cells at the G0/G1 phase (Fig. 8C).

Although imperatorin inhibited the basal levels of cyclin D1 gene and protein expression, at present we cannot rule out that other transcription factors besides Sp1 could also be affected by this furanocoumarin.

**DISCUSSION**

Clinical treatment of AIDS patients with a combination of anti-HIV drugs has been successful in reducing the bloodstream viral load. Current antiretroviral drugs inhibit the HIV-1 replication by targeting viral enzymes (reverse transcriptase and protease), but this therapy has important limitations such as the severe side effects typical of long term treatments, the emergence of drug-resistant HIV-1 strains, and the lack of effects on the proviral burden (28). The use of natural or synthetic compounds targeting cellular proteins involved in HIV-1 replication has opened new research avenues in the management of AIDS (59). Within these agents, compounds interfering with both cell cycle checkpoint and HIV-1-LTR promoter regulatory proteins are of special interest since HIV-1 replication preferentially occurs in dividing cells (13, 60). Here we show that imperatorin inhibits the Sp1 transcriptional activity that plays an important role in both the cell cycle progression (58) and the HIV-1 replication (30).

Sp1 is a member of a multigene family that binds DNA GC boxes and related motifs through COOH-terminal zinc finger motifs (55). Two further members of this family, Sp3 and Sp4, bind with similar affinity to the same recognition sequence as Sp1 (61). Whereas Sp1 and Sp3 are practically present in all cell types, Sp4 expression is rather restricted to the central nervous system (62). In HeLa cells, two specific complexes were observed in the EMSA assay, namely complex I, composed by Sp1 and Sp3, and complex II, made exclusively by Sp3 (63). Moreover Sp3 has been shown to repress both basal and Tat-
induced expression of the HIV-1 promoter (63). Thus, it could be possible that imperatorin exerts its HIV-1-LTR inhibitory activity by inducing a switch in the ratio of Sp1/Sp3 bound to DNA toward an increase of repressor Sp3. Nevertheless high concentrations of imperatorin neither inhibit nor enhance the PMA-induced (Fig. 7A) or basal Sp3 transcriptional activity (not shown).

Sp1 inhibitors are potentially good candidates for mutation-insensitive antiviral drugs. For instance, the plant lignan 3'-O-methyl nordihydroguaiaretic acid, a natural suppressor of HIV-1 replication (64), was revealed by gel mobility shift studies not to affect NF-kB-DNA binding, but it rather inhibited Sp1. This Sp1 inhibitory mechanism seems to be different from the one mediated by imperatorin since the binding of Sp1 to DNA was not affected by this compound. However, our results with the Sp1 mutated HIV-1-LTR construct and the Gal4-Sp1 system clearly indicate that Sp1 represents one of the main targets for anti-HIV activity of imperatorin.

In addition to its DNA binding activity, Sp1 can be regulated at different levels. Thus, Sp1 is phosphorylated at threonines 453 and 739 by activation of the MEK/ERK pathways to either stimulate or repress gene transcription (65, 66). In this context, it is worth noting that HIV-1 Tat protein activates the ERK pathway (67). Moreover phosphorylation of serine 131 in Sp1 is crucial for Tat-induced Sp1-dependent HIV-1 promoter activation (35), and this phosphorylation may be induced by the DNA-dependent protein kinase in HIV-1 Tat-transfected HeLa cells (35). Therefore, Sp1 phosphorylation at different residues may represent one of the converging points for the different signaling pathways involved in HIV-1-LTR transactivation.

Since imperatorin inhibited both PMA (a known activator of the ERK and DNA-dependent protein kinase pathways) and Tat-induced Sp1-dependent HIV-1-LTR transcription, it is not unreasonable to assume that this coumarin could inhibit the activation of either ERK or DNA-dependent protein kinase or both. Sp1 is capable of interacting with several proteins including TATA-binding protein, dTAFII110/hTAFII130, YY1, Oct-1, E2F, and p107 (68), and a physical and functional interaction between Sp1 and cyclin T1 has been demonstrated to be sufficient to induce TAR-independent HIV-1-LTR activation (36). It is therefore possible that imperatorin prevents the physical and/or functional interaction between Sp1 and some of its co-activators, and experiments are in course to study in detail the mechanisms by which imperatorin inhibits Sp1 function.

The coactivator and acetyltransferase cAMP-response element-binding protein (CREB)-binding protein (CBP) and the paralog p300 are recruited to the HIV-1 promoter by Tat (69). Therefore, DNA-bound Sp1 protein can be acetylated by CBP/p300 in response to either PMA or HIV-1 Tat (70). In this context imperatorin could inhibit a common step in the signaling pathways activated by both PMA and Tat.

Finally another interesting finding is that imperatorin inhibits the expression of cyclin D1 and induces G1 cell cycle arrest in HeLa cells. Using the same cell type, it has been demonstrated that Sp1 is a transcription factor that regulates G1 cell cycle checkpoint (58). Since the block of endogenous Sp1 strongly inhibited the expression of CycD1 and the epidermal growth factor receptor, the induction of cell cycle arrest by natural or synthetic compounds has been proposed as a possible therapeutic alternative for HIV-1 infection (60). Thus, nat-

![Fig. 7. Effects of imperatorin on Sp1 binding to DNA and transcriptional activity. A, HeLa cells were preincubated with imperatorin at the indicated concentrations for 30 min followed by stimulation where indicated with PMA for 6 h. Sp1-DNA binding activity in nuclear cell extracts from stimulated cells was studied by EMSA (right panel). Supershift analysis by using α-Sp1 antibody or preimmune serum (PIS) and cold competition experiments by using unlabeled Sp1 and AP-1 probes were performed in nuclear extracts from HeLa control cells (right panel). B, HeLa cells were cotransfected with 0.7 μg of a Gal4-Luc reporter plasmid/ml together with 0.3 μg of the construct Gal4-Sp1 or Gal4-c-Jun. C, HeLa cells were cotransfected with 0.7 μg of a Gal4-Luc reporter plasmid/ml together with 0.3 μg of the construct Gal4-Sp3, Gal4-Sp4, or Gal4-DBD. After 24 h, cells were pretreated or not with increasing doses of imperatorin and further stimulated with PMA (20 ng/ml) for 6 h. Luciferase activity was measured, and the results are represented as means ± S.D. of three determinations expressed as -fold induction (observed experimental RLU/basal RLU in the absence of any stimuli). ns, nonspecific.](image-url)
Imperatorin Inhibits HIV-1 Replication

FIG. 8. Imperatorin inhibits cyclin D1 gene transcription and cyclin D1 protein expression and induces cell cycle arrest in G1 phase. A, HeLa cells were transiently transfected with the plasmid pGL2-cyclinD1. 24 h later the cells were treated with increasing concentrations of imperatorin for 12 h, and the luciferase activity was measured in the cell lysates as described. B, the steady state of cyclin D1 (CycD1) and α-tubulin protein expression was studied in imperatorin-treated HeLa cells by Western blot. C, HeLa cells were treated or not with imperatorin (25 μM) for 24 h, and the cell cycle distribution was examined by flow cytometry. The percentage of cells in each phase of the cell cycle was calculated using Cyclcheck Version 1.0.2 cell software.

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