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Mesuol, a natural occurring 4-phenylcoumarin, inhibits HIV-1 replication by targeting the NF-κB pathway

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Abstract

Coumarins and structurally related compounds have been recently shown to inhibit replication of human immunodeficiency virus (HIV) and thus, exhibit a therapeutic potential. In this study we report that mesuol and isomesuol, two 4-phenyl coumarins, isolated from the tree *Marila pluricostata*, suppress HIV-1 replication in Jurkat T cells. These coumarins do not affect the reverse transcription and intregration steps of the viral cycle and their antiviral effect is additive with that of azidothymidine (AZT). In addition, mesuol inhibits TNFα-induced HIV-1-LTR transcriptional activity by targeting the nuclear factor-κB (NF-κB) pathway. While mesuol does not prevent either the binding of NF-κB to DNA or the phosphorylation and degradation of NF-κB inhibitory protein, IκBα, it inhibits the phosphorylation and the transcriptional activity of the NF-κB p65 subunit in TNFα-stimulated cells. These results highlight the potential of the NF-κB transcription factor as a target for anti-HIV-1 compounds such as 4-phenyl coumarins, which could serve as lead compounds for the development of additional therapeutic approaches against AIDS.

Keywords: 4-Phenyl-coumarins; Mesuol; HIV-1; LTR; NF-κB

1. Introduction

Retrovirus life cycle is commonly divided into two phases; the early phase refers to the steps of infection from cell binding to the viral integration into the cell genome, whereas the late phase begins with the expression of viral genes and continues through the release and maturation of progeny virions. The human immunodeficiency virus type 1 (HIV-1), the etiologic agent of AIDS, is a retrovirus that enters permissive cells through cell surface receptors and following viral entry, the HIV-1 RNA genome is reverse transcribed to a double-stranded DNA molecule that enters the nucleus and integrates into the host chromatin (Frankel and Young, 1998; Nisole and Saib, 2004). The post-integration phase of the viral cycle preferentially occurs in activated cells and is regulated by the collaborative action of the viral regulatory protein Tat (Transactivator of transcription) and cellular factors interacting with the long terminal repeat promoter (LTR), which determines the extent of HIV-1 gene transcription and the level of viral replication in the infected cells (Gaynor, 1995; Pereira et al., 2000). The HIV-1-LTR promoter is approximately 640 nucleotides long and has binding sites for many cellular transcription factors and a *cis*-activating stem-loop RNA structure called transactivating response element (TAR) that represents the main binding site for the HIV-1 Tat protein (Hauber and Cullen, 1988; Pereira et al., 2000). Through interaction with TAR, Tat recruits the positive transcriptional elongation fac-

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tor (p-TEFb), which phosphorylates the C-terminal domain of the RNA polymerase II (Chun and Jeang, 1996; Wei et al., 1998; Zhou et al., 2001). Recruitment of p-TEFb to TAR has been proposed to be both necessary and sufficient for activation of transcription elongation from the HIV-1-LTR promoter (Zhu et al., 1997; Mancebo et al., 1997; Zhou and Rana, 2002).

In addition to the TAR element, the HIV-1-LTR promoter contains three additional functional regions related to the regulation of HIV-1 transcription: the basal or core promoter (nt −78 to −1), the core enhancer (nt −105 to −79) and a modulatory region (nt −454 to −104) (Gaynor, 1992; Pereira et al., 2000). The core promoter region of the HIV-1-LTR contains three tandem Sp1-binding sites and the core enhancer has two κB elements that have been shown to be involved in the regulation of HIV-1 transcription (Berkhout and Jeang, 1992; Hiscott et al., 2001). Evidently, early events in the HIV-1 transcription are firstly regulated by a direct interaction between cellular transcription factors and the *cis*-acting elements located in the viral promoter that allows the accumulation of sufficient amounts of Tat protein, that, in turn, drives a strong HIV-1 replication (Nisole and Saib, 2004). Thus, new biomolecules impairing the function of cellular proteins required for efficient HIV-1 replication should be considered in the search of new anti-HIV-1 agents.

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 (Yeni et al., 2002). This current antiviral therapy presents important limitations (Ptak, 2002; De Clercq, 2002; Mansky, 2003) and, therefore, the development of new anti-HIV-1 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-1 lead compounds (Yu et al., 2003). We have now studied the anti-HIV-1 effects of two 4-phenyl coumarins, mesuol and isomesuol, isolated from leaves and stems of *Marila pluricostata* and we have shown that both compounds are potent inhibitors of HIV-1 replication in the human Jurkat T cell line. We also present evidence that mesuol inhibits the transcriptional activity of the HIV-1-LTR promoter through a signalling pathway that involves the phosphorylation of the p65 subunit of the NF-κB transcription factor.

2. Materials and methods

2.1. Cell lines and reagents

Jurkat cells (American Type Culture Collection, Manassas, VA) were cultured in RPMI 1640 medium (Invitrogen, Barcelona, Spain), containing 10% heat-inactivated fetal bovine serum, 2 mM glutamine, penicillin (50 U/ml) and streptomycin (50 μg/ml) and were maintained at 37 °C in a 5% CO_2 humidified atmosphere and split twice a week. The 5.1 cell line (obtained from Dr. N. Israël, Pasteur Institute, Paris, France) 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 complete medium supplemented with G418 (200 μg/ml). The assayed phenylcoumarins were isolated from the dichloromethane extract of leaves and stems of *M. pluricostata* (Clusiaceae), a tree growing in Colombia, Panama and Costa Rica (D'Arcy, 1980) with restricted distribution. Isomesuol and mesuol (Chakraborty and Das, 1966; Reutrakul et al., 2003) were obtained in pure form through several chromatographic separations and crystallizations. Their characterization and structural assignment will be reported elsewhere, along with those of other 13 closely related compounds identified in the extract. The anti-IκBα mAb 10B was a gift from R.T. Hay (St. Andrews, Scotland), and the polyclonal anti-phospho-p65 (3031S) was from New England Biolabs (Hitchin, UK). All other reagents not cited above or later were purchased from Sigma–Aldrich (Barcelona, Spain).

2.2. Isolation and activation of peripheral mononuclear cells

Human peripheral blood mononuclear cells (PBMCs), from healthy adult volunteer donors, were isolated by centrifugation of venous blood on Ficoll-Hypaque® density gradients (Pharmacia LKB Biotechnology, Piscataway, NJ). Cells (2.5×10^6/ml) were treated with SEB for 72 h and then collected and used for recombinant virus infection assays as previously described (Sancho et al., 2004).

2.3. Plasmids

The vector pNL4-3.Luc.R^-E^- (AIDS Research and Reference Reagent program, NIAID, National Institutes of Health) from N. Landau was previously described (Connor et al., 1995). This vector contains the firefly luciferase gene inserted into the pNL4-3 nef gene and two frameshifts (5′ Env and Vpr amino acid 26) render this clone Env^- and Vpr^-. The $pcDNA_3$-VSV plasmid contains the cDNA encoding the vesicular stomatitis virus (VSV) G protein and was obtained from Dr. Arenzana-Seisdedos (Pasteur Institute, Paris, France). The Gal4-Luc reporter plasmid includes five Gal4 DNA binding sites fused to the luciferase gene (Schmitz et al., 1995). The Gal4-p65 contains the C-terminal region of the human p65 (amino acids 286–551) fused to the Gal4 binding domain and was obtained from M.L. Schmitz (University of Bern, Switzerland) and the Gal4-VP16 plasmid has been previously described (Minden et al., 1995).

2.4. Production of VSV-pseudotyped recombinant viruses and infection assays

High titer VSV-pseudotyped recombinant virus stocks were produced in 293T cells as previously described (Sancho

et al., 2004). Briefly, the cells were co-transfected with the pNL4-3.Luc.R^- E^- plasmid along with the pcDNA$_3$-VSV plasmid by the calcium phosphate transfection method. Supernatants, containing virus stocks, were harvested 48 h post-transfection and were 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 immunoassay for antigen HIV-1 p24 and cultures were infected with 200 ng of HIV-1 gag p24 protein as follows. Jurkat cells or isolated PBMCs (10^6/ml in 24-well plates) were pre-treated with the compounds for 30 min and then inoculated with the virus stocks. Twenty-four hours later the cells were washed twice in PBS and lysed in 25 mM Tris–phosphate pH 7.8, 8 mM $MgCl_2$, 1 mM DTT, 1% Triton X-100 and 7% glycerol during 15 m at RT. Then the lysates were spun down and the supernatants were used to measure luciferase activity using an Autolumat LB 9510 (Berthold, Bad Wildbad, Germany) following the instructions of the luciferase assay kit (Promega, Madison, WI). The results are represented as the percentage of activation (considering the infected and untreated cells 100% activation). Results represent mean ± standard deviation (S.D.) of four independent experiments.

2.5. Transient transfections and HIV-1-LTR Luciferase assays

Jurkat cells were transfected with the indicated plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. At 24 h post-transfection, cells were pretreated with mesuol for 30 min, and treated or not with PMA for 6 h. To determine NF-κB dependent transcription of the HIV-1-LTR-luc, 5.1 cells were preincubated for 30 min with the compounds as indicated, followed by stimulation with TNFα for 6 h. 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 detected as above described and protein concentration was measured by the Bradford method (Bio-Rad, Hercules, CA). The background obtained with the lysis buffer was subtracted from each experimental value, the RLU/μg of protein was calculated and the specific transactivation expressed as fold induction over untreated cells. All the experiments were repeated at least four times.

2.6. Electrophoretic mobility shift assays

Jurkat (10^6/ml) were pre-treated with mesuol at the indicated doses for 30 min and then were stimulated or not with TNFα (5 ng/ml) for 30 min. Cells were then washed twice with cold PBS, and proteins from total cell extracts were isolated as previously described (Sancho et al., 2003). Protein concentration was determined by the Bradford method. For gel retardation a double-stranded oligonucleotide containing the consensus site for NF-κB (Promega) was end-labelled with [γ-^{32}P]ATP. The binding reaction mixture contained 15 μg of total extracts, 0.5 μg poly (dI-dC) (Amersham Biosciences Inc.), 20 mM Hepes pH 7, 70 mM NaCl, 2 mM DTT, 0.01% NP-40, 100 μg/ml BSA, 4% Ficoll and 100,000 cpm of end-labelled DNA fragments in a total volume of 20 μl. After 30 min incubation at room temperature, 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.

2.7. Western blot

Jurkat cells (10^6/ml) were treated as indicated. Cells were then washed with PBS and proteins extracted from cells 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 NaF, 1 mM DTT, leupeptin 1 μg/ml, pepstatin 0.5 μg/ml, aprotinin 0.5 μg/ml and 1 mM PMSF) containing 0.5% NP-40. Protein concentration was determined by the Bradford method 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 °C) for 1 h. The blots were blocked in TBS solution containing 0.1% Tween 20 and 5% nonfat dry milk overnight at 4 °C and immunodetection of IκBα and phospho-p65 was carried out with specific mAbs and HRP-labelled secondary antibody using an ECL system (Amersham Biosciences Inc.).

2.8. Semiquantitative PCR analysis

Reverse transcriptase products were detected as previously described (François and Klotman, 2003), with minor modifications. Briefly, Jurkat cells were infected with VSV-pseudotyped recombinant virus for 24 h and the DNA extracted. PCR amplification of R/U5 (short) and LTR/gag (long) reverse transcriptase product were done as previously described (Sancho et al., 2004) and using the following primers; to amplify short retrotranscribed product (amplicon size: 140 bp): R/U5 (forward), 5′-GGCTAACTAGGGAACCCACTG-3′; R/U5 (reverse), 5′-CTGCTAGAG ATTTTCCACACTGAC-3′; and to amplify long retrotranscription product (amplicon size: 200 bp): R/U5 (forward), LTR/gag (reverse), 5′-CCTGCCTCGAGAGAGCTGCTC TGG-3′. As a control, genomic DNA was subjected to β-actin amplification and PCR products were electrophoresed on a 2% (w/v) agarose gel.

2.9. Analysis of HIV integrated DNA by nested Alu-PCR

Genomic DNA from Jurkat VSV-pseudotyped HIV-1-infected cells and Jurkat control cells was extracted and quantified as above described. The detection of HIV-1-LTR integrated into the cell genome was performed as previously described (Van Maele et al., 2003) with slight modifications. The first PCR was carried out by using primers Alu-LTR

5′ (5′-TCCCAGCTACTCGGGAGGCTGAGG-3′) from conserved human Alu sequences and Alu-LTR 3′ from conserved HIV-1-LTR sequences (5′-TTTCAGGTCCCTGTTCGGGC GCCA-3′). Each PCR amplification was performed in a 50 μl PCR reaction mixture containing DNA (50 ng), 1× PCR buffer, 1.2 mM $Mg(OAc)_2$, 200 μM dNTPs, 25 pmol of each primer and 1.6 units of rTth DNA polymerase XL (Roche Diagnostics, Barcelona, Spain). The mixtures were amplified in a MultiGene cycler IR system (Labnet, Woodbridge, NJ). Samples containing all PCR reaction components except rTth DNA polymerase were submitted to an initial 2-min cycle at 94 °C. rTth was then added and the samples were subjected to denaturation at 94 °C for 3 min, 22 cycles of 30 s denaturation at 94 °C, 30 s annealing at 66 °C and 5 min extention at 70 °C with 10 min at 72 °C for the final extention step. Following the initial PCR, a second nested PCR amplification was carried out by using an aliquot equivalent to 1/25 of the 22-cycle product. The second PCR was performed by semiquantitative PCR analysis with primers R/U5 (forward) and R/U5 (reverse) (described above). Each PCR amplification was performed in a 50 μl PCR reaction mixture containing 2 μl from the first round Alu-PCR product, 1× PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.2 μM of each primer and 2.5 units of recombinant TaqDNA 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° and final extension step of 7 min. As a control, genomic DNA was subjected to β-actin amplification and used to normalize the nested PCR products, which were submitted to electrophoresis on a 2% (w/v) agarose gel.

2.10. Cytotoxicity assays

Jurkat cells or PBMCs (10^5/ml) were seeded in 96-well plates in complete medium and treated with increasing doses of the compounds for 24 h. Samples were then diluted with 300 μl of PBS and incubated for 1 min at room temperature in the presence of propidium iodide (10 μg/ml). After incubation, cells were immediately analyzed by flow cytometry.

3. Results

3.1. Effects of mesuol and isomesuol in HIV-1 replication in lymphoid cells

Others and we have shown that some plant-derived coumarins exert anti-HIV activity (Yu et al., 2003; Sancho et al., 2004). To study the anti-HIV activity of isomesuol and mesuol, two 4-phenylcoumarins isolated from the tree *M. pluricostata* (Fig. 1), we infected Jurkat 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

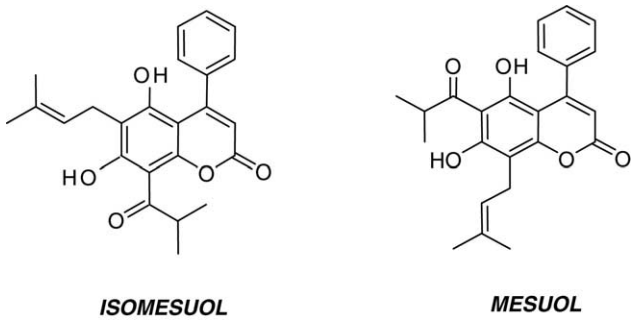

Fig. 1. Chemical structures of the 4-phenylcoumarins isomesuol and mesuol.

support robust HIV-1 replication (Canki et al., 2001; Sancho et al., 2004). 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 pre-treatment of Jurkat cells 30 min prior infection with increasing doses of either mesuol or isomesuol resulted in a dose-dependent inhibition of luciferase activity with an IC50 ranging from 2 to 2.5 μM and reaching a complete inhibition at 15 μM (Fig. 2A). Mesuol also showed a potent anti-HIV activity in peripheral blood mononuclear cells acutely infected with this HIV-1 clone (Fig. 2A). The anti-HIV-1 effect of these 4-phenylcoumarins was not due to cytotoxicity, since cell viability assays did not reveal a significant decrease in the percentage of cell viability in infected cells treated for 24 h with increasing concentrations of both compounds (Fig. 2B).

Although the VSV-pseudotyped HIV-1 clone bypasses the natural mode of HIV-1 entry into mammalian cells, it needs the reverse transcription and integration steps. Therefore, we studied whether these steps were affected by either mesuol or isomesuol. First, semiquantitive 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 products, respectively. As depicted in Fig. 3A, neither mesuol nor isomesuol at 15 μM decreased the amount of both R/U5 and LTR/gag products obtained following Jurkat cells infection with VSV-pseudotyped HIV-1. Azidothymidine (AZT) inhibited the amplification of the full-length (LTR-Gag) product (data not shown and Sancho et al., 2004). Next, we investigated the effect of these compounds in HIV-1 integration; the DNA of infected Jurkat cells was extracted and subjected to a first round of Alu-PCR followed by nested PCR using internal LTR primers as described in Section 2. β-Actin was also amplified and used to normalize the amount of integrated HIV-1. Jurkat infection with VSV-pseudotyped HIV-1 resulted in viral integration, which was not prevented by either mesuol or isomesuol (Fig. 3A). To confirm the lack of effects on the viral reverse transcription and integration steps we performed functional luciferase-based analyses. Jurkat cells were pretreated with AZT

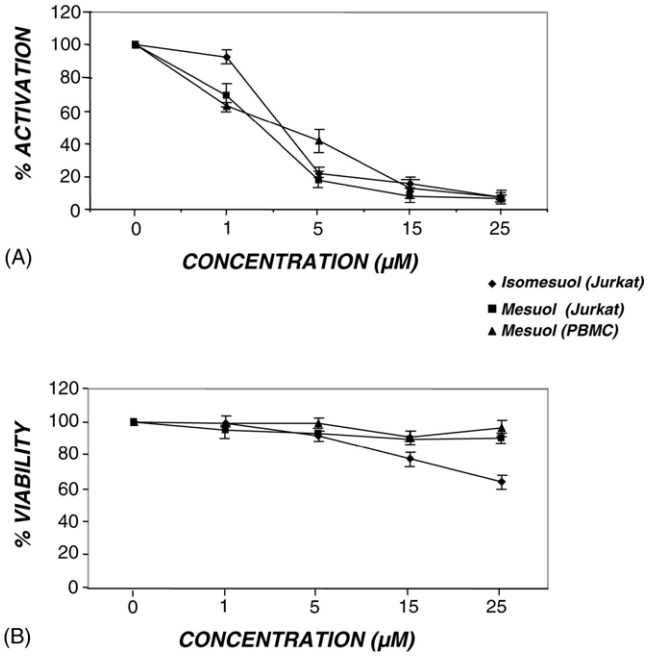

Fig. 2. Effects of mesuol and isomesuol on recombinant virus replication and cell viability. (A) Jurkat cells or mitogen-activated PBMCs (10^6/ml) were pretreated with mesuol and isomesuol for 30 min and then infected with the VSV-pseudotyped-HIV recombinant virus for 24 h. Luciferase activity in cell extracts was determined and results represented as percentage of activation ± S.D. compared to non-treated infected cells (100% activation). (B) Jurkat cells or mitogen-activated PBMC (10^6/ml) were pretreated as in A, harvested and the percentage of cell death was determined by propidium iodide staining followed by flow cytometry analysis. Results are represented as the percentage of cell viability and are the mean ± S.D. of three different experiments.

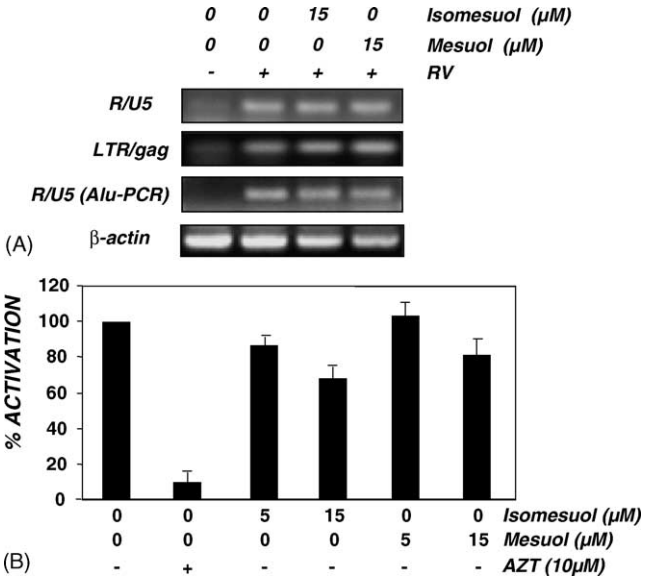

Fig. 3. Effects of mesuol and isomesuol on viral reverse transcription and integration. (A) Jurkat cells were pretreated with mesuol and isomesuol (15 μM) and infected with 200 ng/p24 of the recombinant VSV-pseudotyped HIV virus (RV) for 24 h. DNA was extracted and semiquantitative PCR was performed. Primers were used to amplify the HIV-1 reverse transcriptase short products (R/U5) and long products (LTR/gag) as well as the β-actin as a control. HIV-1 DNA integration was detected using primers Alu-LTR 5′and Alu-LTR 3′. An aliquot of the first PCR product was subjected to the second round of PCR by using nested HIV-1-LTR-specific primers (R/U5 (forward) and R/U5 (reverse)) and the products visualized by agarose gel electrophoresis. (B) Jurkat cells (10^6/ml) were pretreated with mesuol, isomesuol or AZT for 30 min and then infected with RV for 12 h. Cells were then washed, cultured again and luciferase activity measured after 48 h. Results are represented as percentage of activation ± S.D. compared to non-treated infected cells (100% activation).

(10 μM), mesuol and isomesuol and then infected with the VSV-pseudotyped HIV-1 clone. Twelve hours post-infection the compounds were removed from the culture medium by washing the cells three times with PBS, then the cultures were continued for another 48 h and finally the luciferase activity was measured as described above. As shown in Fig. 3B the inhibitory effect of AZT was maintained even after extensive washing, indicating that viral reverse transcription and integration steps fundamentally occur during the first 12 h after infection. On the contrary, neither mesuol nor isomesuol were effective in inhibiting HIV-1 replication under these culture conditions. Taken together these data strongly suggest that these 4-phenylcoumarins inhibit HIV-1 replication by suppressing HIV-1 gene expression at the transcriptional level. Further experiments were done using mesuol.

Since AZT and mesuol seem to inhibit HIV-1 replication by targeting different steps in the viral life cycle, either a synergy or an additive effect between both compounds could be expected. To investigate this possibility we tested the effects of sub-optimal concentrations of AZT and mesuol either separately or in combination. In Fig. 4 it is shown that AZT at 1 nM and mesuol at 1.25 μM partially inhibited the luciferase activity when tested separately (between 30 and 40% inhibition), while a clear additive anti-HIV effect (∼80% inhibition) was found with a combination of both compounds.

3.2. Mesuol inhibits HIV-1-LTR transactivation through an NF-κB dependent pathway

To analyze whether the inhibitory effect of mesuol on HIV-1 replication is mediated at the HIV-1-LTR transcriptional ac-

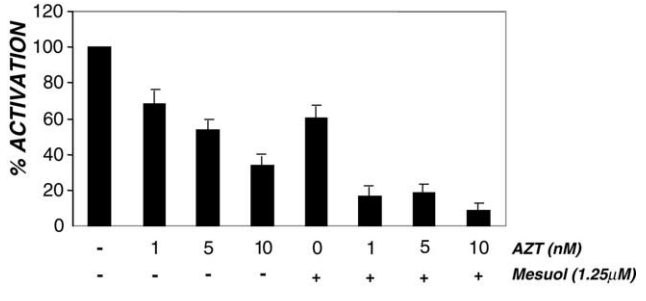

Fig. 4. Additive effect of mesuol and AZT. Jurkat cells (10^6/ml) were pretreated with AZT, mesuol or a combination of both for 30 min and then infected with RV for 24 h. Luciferase activity in cell extracts was determined and results represented as percentage of activation ± S.D. compared to non-treated infected cells (100% activation).

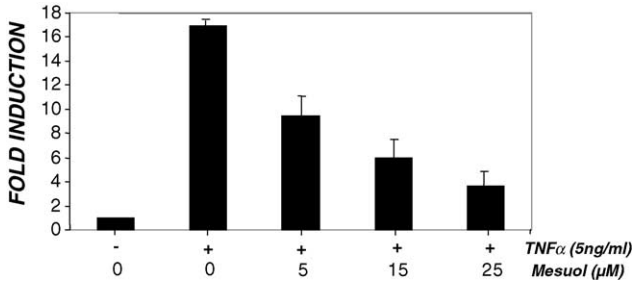

Fig. 5. Mesuol inhibits HIV-1-LTR transactivation in 5.1 cells. 5.1 cells were preincubated with mesuol at the indicated doses and stimulated with TNFα (5 ng/ml) for 6 h. The luciferase activity was measured and results are the mean ± S.E. of three determinations expressed as fold induction (experimental RLU-background RLU)/(basal RLU-background RLU).

tivity level, we used the cloned 5.1 cell line that contains the luciferase gene driven by the HIV-1-LTR promoter, which is responsive to TNFα through the NF-κB pathway. The HIV-1 promoter contains two NF-κB binding sites that are absolutely required for TNFα-induced transactivation (Alcami et al., 1995). The 5.1 cells were preincubated with increasing concentrations of mesuol and then stimulated with TNFα (5 ng/ml) for 6 h and luciferase activity was measured. As depicted in Fig. 5, pretreatment with mesuol resulted in a dose-dependent inhibition of TNFα-induced LTR activation suggesting that the NF-κB pathway is involved in the inhibitory mechanism of mesuol.

3.3. Mesuol inhibits the phosphorylation and the transcriptional activity of the NF-κB p65 subunit in stimulated Jurkat cells

The signaling pathways that activate NF-κB include a complex activation of regulatory kinases resulting in the phosphorylation and degradation of the IκB proteins and nuclear translocation of NF-κB (Karin and Ben-Neriah, 2000). Moreover, post-translational modifications of the p65 subunit in the nucleus are also required for NF-κB transcriptional activity (Vermeulen et al., 2002; Schmitz et al., 2004). To investigate the level at which mesuol exerts its inhibitory effect on NF-κB activation, we stimulated Jurkat cells with TNFα in the presence of increasing concentrations of this compound, and proteins from total cell extracts were analyzed for NF-κB DNA binding activity by gel retardation experiments. In Fig. 6A it is shown that TNFα-treatment led to a clear increase in NF-κB binding to DNA after 30 min of stimulation that was not affected by the presence of mesuol. The DNA-binding specificity was studied by supershift experiments with specific anti-p50 and anti-p65 (RelA) antibodies and by cold competition experiments with unlabelled competitors, and the heterodimer p50/p65 was identified as the main complex (data not shown, and Sancho et al., 2003). Next, we investigated in parallel the effects of mesuol on the steady state levels of IκBα and p65 protein phosphorylation by immunobloting. Jurkat cells were pretreated with mesuol (10 μM) for 15 min and then stimulated with TNFα at the indicated times. The ki-

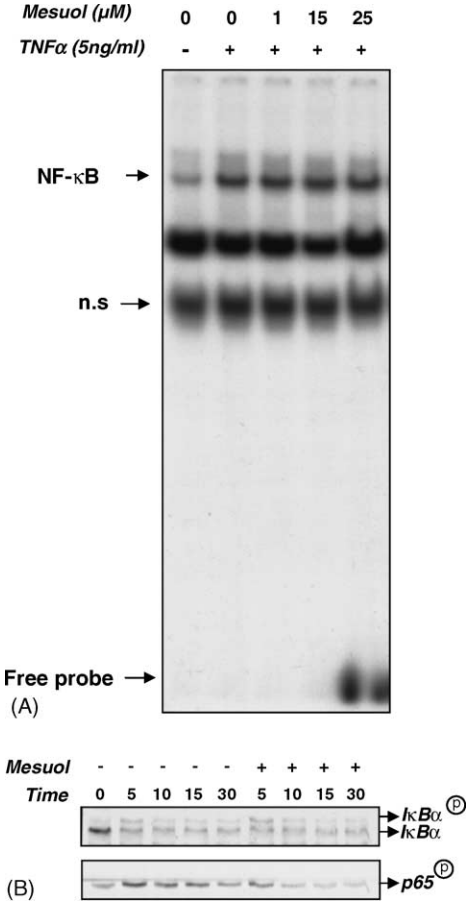

Fig. 6. Effects of mesuol on NF-κB activation. (A) Jurkat cells were pretreated with mesuol at the indicated doses, treated with TNFα for 30 min and protein extracts were tested for NF-κB binding activity by EMSA. (B) Western blot analysis of IκBα phosphorylation and degradation and p65 phosphorylation performed in Jurkat cells pretreated with mesuol (10 μM) and stimulated with TNFα for the indicated periods of time.

netic experiments revealed that TNFα induced a rapid phosphorylation and degradation of the IκBα protein that was not affected by the presence of mesuol. Interestingly, p65 (serine-536) was also rapidly phosphorylated in response to this cytokine and this phosphorylation was maintained even for 30 min after TNFα-stimulation. Pretreatment of the cells with mesuol did not affect early TNFα-induced p65 phosphorylation but clearly inhibited late phosphorylation of this protein (Fig. 6B). To further analyze whether mesuol inhibits directly p65-transcriptional activity, we performed cotransfection experiments using Gal4-p65, a fusion protein between the transactivation domain of p65 (amino acids 286–551) and the DNA binding domain of the yeast Gal4 transactivator, together with a reporter plasmid containing the luciferase gene under the control of a Gal4 –responsive element (Gal4-Luc). This system has the advantage that the Gal4 transactivator fusion protein is exclusively nuclear, and thus is regulated independently of IκBs (Schmitz et al., 1995). The results presented in Fig. 7A revealed that transcriptional activity of Gal4-p65 was increased (∼3-fold) upon treatment of the cells

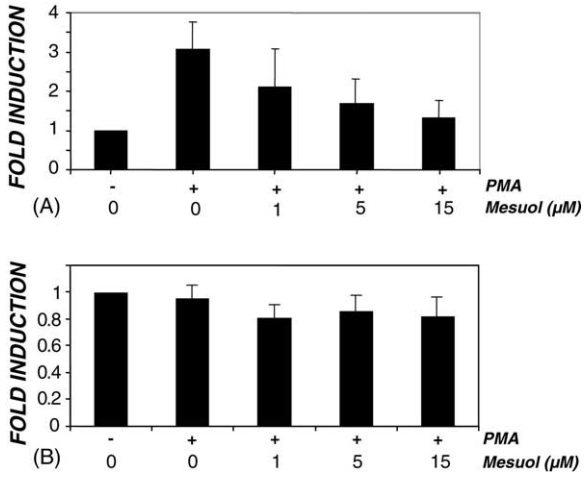

Fig. 7. Effect of mesuol on p65 transcriptional activity. Jurkat cells were transiently cotransfected with the plasmids Gal4-p65 and Gal4-Luc (A) or with the plasmids VP-16-Gal4 and Gal4-Luc (B). Twenty-four hours post-transfection, the cells were incubated for 30 min with increasing concentrations of mesuol, stimulated with PMA for 6 h, and the luciferase activity was measured. Results are the means ± S.D. of three different experiments and represented as fold induction over untreated control.

with PMA and this induction was significantly inhibited by the presence of mesuol in a concentration-dependent manner, while pretreatment with mesuol did not affect the luciferase activity induced by the fusion protein Gal4-VP16 (Fig. 7B).

4. Discussion

The use of plant extracts to alleviate human pathologies is centuries old and continue nowadays. Thus, natural product research has paved the way to develop a scientific basis for drugs used for the treatment of many diseases. Current antiretroviral drugs inhibit the HIV-1 replication by targeting viral enzymes (reverse transcriptase and protease), but this therapy has important limitations such as side effects typical of long-term treatments, the emergence of drug-resistant HIV-1 strains and the lack of effects on the proviral burden (Ptak, 2002; De Clercq, 2002; Mansky, 2003). 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 (De Clercq, 2002; Sadaie et al., 2004).

Numerous plant-derived compounds, including coumarins, have been evaluated for inhibitory effects on HIV-1 replication “in vitro” (Uchiumi et al., 2003; Cos et al., 2004). Anti-HIV-1 coumarins have been identified to inhibit different steps of the viral cell cycle (Yu et al., 2003), and one of them, calanolide A, is currently undergoing clinical trials in AIDS patients (Yu et al., 2003). Here we show that 4-phenylcoumarins, mesuol and isomesuol, inhibit HIV-1 replication in T cells by targeting the phosphorylation and transcriptional activity of the NF-κB p65 subunit that play an important role on HIV-1-LTR transcription (Hiscott et al., 2001).

The transcription factor NF-κB is one of the key regulators of genes involved in the immune/inflammatory response as well as in HIV-1 gene regulation. NF-κB is an inducible transcription factor made up of homo- and hetero-dimers 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 and upon stimulation trough the canonical pathway IκBα protein is phosphorylated by the IκBα kinase complex (IKC), ubiquitinated, and subsequently degraded by the proteasome, resulting in the translocation of NF-κB from the cytoplasm to the nucleus (Bonizzi and Karin, 2004). In addition to the control of NF-κB activity 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 (Schmitz et al., 2004). For instance, TNFα induces the phosphorylation of serine 536 (Sakurai et al., 2003) within the transactivation domain 1 of p65, and mutation of this serine impairs NF-κB-dependent transcription, highlighting the functional relevance of these phosphorylations (Yang et al., 2003). The IKKβ subunit of the IKC is involved not only in IκBα phosphorylation but also in p65 phosphorylation (serine 536) in TNFα-stimulated cells (Sakurai et al., 2003). In our experiments mesuol did not affect the activity of IKKβ, since TNFα-dependent IκBα phosphorylation and early p65 phosphorylation on serine 536 were not inhibited. However, sustained p65 phosphorylation and transactivation activity were clearly impaired suggesting that other kinase(s) responsible for serine 536 phosphorylation could be the potential target for the inhibitory activity of mesuol. However, we cannot discard the possibility that mesuol can inhibits the activity of other kinase(s) targeting additional and relevant residues such are serines 276, 311 and 529 within the p65 protein. Interestingly, mesuol and isomesuol also inhibit Tat-mediated HIV-1-LTR transactivation without interfering with the Tat–TAR binding interaction (unpublished results), and we have found that Tat can induce the transcriptional activity of a p65 form lacking serines 529 and 536 (Sancho et al., unpublished results). Therefore, mesuol could inhibit p65 regulation by acting at a common element involved in TNFα and HIV-1 Tat-signalling pathways, and this could explain the fact that mesuol is more effective inhibiting HIV-1 replication than TNFα-mediated LTR transactivation. This specific target could be transcriptional co-activators p300/CBP, since phosphorylation of serines 276 and 311 facilitates the interaction of p65 with the coactivator p300/CBP and enhances p65 function (Zhong et al., 2002; Duran et al., 2003), and a physical association between p300 and Tat has been also demonstrated (Benkirane et al., 1998). Experiments are in course to investigate this hypothesis.

Anti-HIV coumarins have been identified to inhibit different steps of the viral cycle (Vlietinck et al., 1998; Yu et al., 2003). However, in the majority of the cases the exact mechanism of actions needs to be confirmed. Recently, we

have shown that imperatorin is a potent inhibitor of HIV-1 replication by impairing the function of the Sp1 transcription factor (Sancho et al., 2004). Also, Uchiumi et al. (2003) have shown that glucocoumarin, a natural compound carrying a 3-phenylcoumarin scaffold, has a negative effect on HIV-1-LTR transcription, but the specific element in the viral promoter responsible for the negative effect of glycocoumarin was not identified. Taking into account the high structural similarity between glycocoumarin and mesuol it is possible that the anti-HIV-1 activity of both 3- and 4-phenylcoumarins is mediated by inhibition of the NF-κB pathway.

Plant-derived antiviral compounds interfering with HIV-1-LTR promoter regulatory proteins are of special interest, since these drugs, if proven useful for patients, are unlikely to generate drug-resistant HIV strains. Thus, natural compounds such as 4-phenyl-coumarins might have a potential therapeutic role in the management of AIDS most probably in combination with other anti-HIV drugs.

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