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Denbinobin, a naturally occurring 1,4-phenanthrenequinone, inhibits HIV-1 replication through an NF-κB-dependent pathway

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ABSTRACT

Anthraquinones and structurally related compounds have been recently shown to exert antiviral activities and thus exhibit a therapeutic potential. In this study we report the isolation of the 1,4-phenanthrenequinone, denbinobin, from a variety of *Cannabis sativa*. Denbinobin does not affect the reverse transcription and integration steps of the viral cycle but prevents HIV-1 reactivation in Jurkat T cells activated by TNFα, mAbs anti-CD3/CD28 or PMA. In addition, denbinobin inhibits HIV-1-LTR activity at the level of transcription elongation and also TNFα-induced HIV-1-LTR transcriptional activity. We found that denbinobin prevents the binding of NF-κB to DNA and the phosphorylation and degradation of NF-κB inhibitory protein, IκBα, and inhibits the phosphorylation 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 natural anti-HIV-1 compounds such as 1,4-phenanthrenequinones, which could serve as lead compounds for the development of an alternative therapeutic approach against AIDS.

1. Introduction

Human immunodeficiency virus (HIV) is the etiological agent of acquired immunodeficiency syndrome (AIDS) and this immunological disease remains the number one cause of mortality produced by infectious agents [1–3]. 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 to the release and maturation of progeny virions [4,5]. The detailed characterization of the HIV-1 genome and precise knowledge about the role of viral proteins and cellular factors involved in viral replication have opened new avenues to design and develop new antiviral drugs for the treatment of AIDS.

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 [6]. This current antiviral therapy presents important limitations [7–9] and, therefore, the development of new anti-HIV-1 agents is focusing on novel structures and/or new action

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mechanisms. In this sense, plant-derived natural products are emerging as potent anti-HIV-1 lead compounds [10,11]. For instance, Calanolide A and the betulinic acid derivative PA-457 are currently undergoing clinical trials in AIDS patients [11,12].

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) which represents the main binding site for the HIV-1 Tat protein [13,14]. Through interaction with TAR, Tat recruits the positive transcriptional elongation factor (p-TEFb), which phosphorylates the C-terminal domain of the RNA polymerase II [15–17]. 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 [18–20]. 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) [14,21]. The core promoter region of the HIV-1-LTR contains three tandem Sp1-binding sites and the core enhancer has two κB elements that are critical in the regulation of HIV-1 transcription [22,23].

NF-κB is a family of transcription factors involved in the control of a variety of cellular processes, such as immune and inflammatory responses, development, cellular growth, apoptosis and HIV-1 gene expression [24–26]. NF-κB is an inducible transcription factor made up of homo- and heterodimers of p50, p65, p52, relB and c-rel subunits that interact with a family of inhibitory IκB proteins, of which IκBα is the best characterised. In most cell types, these proteins sequester NF-κB in the cytoplasm by masking its nuclear localization sequence. Stimulation of cells with a variety of physiological or pathogenic stimuli leads to phosphorylation, ubiquitination, and the subsequent degradation of IκBα proteins [27,28]. Phosphorylation of IκBα at serines 32 and 36 is a key step involved in the activation of NF-κB complexes. This event is mediated by IκB kinases (IKKs), which are formed by a high-molecular weight complex (IKC) containing at least two kinase subunits (IKKα and IKKβ), and the associated modulatory protein NEMO/IKKγ [24,28,29]. 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 [30,31].

Although phenanthrenequinones of non-terpenoid origin occur relatively rarely in the plant kingdom, it has been shown that synthetic 1,4-phenanthrenequinones exhibit significant inhibitory activity against retrovirus [32]. The natural 1,4-phenanthrenequinone denbinobin (5-hydroxy-3,7-dimethoxy-1,4-phenanthrenequinone) has been extracted and purified from several *Dendrobium* or *Ephemerantha* (Orchidaceae) species, such as *D. nobile* [33], *D. moniliforme* [34], and *E. lonchophylla* [35] and was recently demonstrated to induce cell death in several cancer cell lines [36,37]. Denbinobin has also been reported to have other biological effects such as antioxidation [35], and anti-inflammation [34]. Also, the total synthesis of denbinobin has been reported [38].

In the present study we have isolated for the first time denbinobin from a variety of the plant *Cannabis sativa* and explored its anti-HIV-1 activity. We demonstrated that both synthetic and natural denbinobin inhibit the transcriptional activity of the HIV-1-LTR promoter through a signaling pathway involving the activation of the NF-κB transcription factor.

2. Materials and methods

2.1. Cell lines and reagents

The Jurkat T leukemia cell line was grown at 37 °C and 5% CO_2 in supplemented RPMI 1640 medium (Cambrex Co., Barcelona, Spain), containing 10% heat-inactivated fetal bovine serum, 2 mM glutamine, penicillin (50 U/ml) and streptomycin (50 μg/ml). The Jurkat-LAT-GFP cell line is a Jurkat derived clone latently infected with a recombinant virus containing the GFP gene driven by the HIV-LTR promoter and its full characterization was previously described [39]. After stimulation the percentage of GFP^+ cells was analyzed by flow cytometry in an EPIC XL flow cytometer (Beckman-Coulter Inc., CA, USA). The 5.1 clone line is a Jurkat derived clone stably transfected with a plasmid containing the luciferase gene driven by the HIV-LTR promoter and was maintained in complete RPMI medium supplemented with G418 (200 μg/ml). This cell line is highly responsive to TNFα but does not respond to PMA because it is defective in PKC signaling. The Hela-Tat-Luc is a stably transfected cell line with the plasmids pLTR-Luc and pcDNA3-Tat and was described previously [40]. The anti-IκBα mAb 10B was a gift from R.T. Hay (St. Andrews, Scotland), the anti-phospho-p65 (3031S) and anti-phospho-IκBα mAbs were from New England Biolabs (Hitchin, UK). The mAb anti-tubulin was purchased from Sigma Co. (St. Louis, MO, USA) and the mAb anti-p65 (sc-8008) was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-CD3 and anti-CD28 mAbs were purified from ATCC hybridomas (clone OKT3 and clone 15E8). [γ-^{32}P]ATP (3000 Ci/mmol) was from MP Biomedicals (Irvine, CA, USA). All other reagents were from Sigma.

2.2. Isolation of denbinobin

The *C. sativa* chemotype (CARMA) was cultivated at the I.S.I.C. (Rovigo, Italy). Young plants were selected for health and perfect form and the growing period was from May to September 2005. Whole plants were cut and air-dried for two weeks until the humidity was reduced to 10–12%. Flowers and leaves were divided from the stalk and branches by hand and seeds were eliminated. To complete the drying step, the plant material was stored in the dark in tissue bags, in a cold room (10 °C) with low humidity (30% RH). The plant material (200 g) was heated in an oven at 120 °C for two hours. After cooling, it was exhaustively extracted with acetone to afford a dark-black residue (16.4 g) that was dissolved in methanol (70 mL) and filtered over 40 g of RP18 silica gel. The filtration bed was washed with further 50 mL of methanol, and the pooled filtrates were evaporated, to afford 11.8 g of residue. This was fractionated by gravity column chromatography on silica gel (petroleum ether–ethyl acetate gradient), to afford four subfractions (A–D). Subfraction B was crystallized from ether to afford 10 mg of denbinobin as a dark black powder. From the mother liquors, further 12 mg of denbinobin were

obtained after preparative HPLC (hexane–ethyl acetate, 7:3) purification. Denbinobin was identified according to its physical and spectroscopic properties [34]. Synthetic denbinobin was prepared as previously described [38].

2.3. Plasmids

The KBF-Luc contains three copies of the MHC enhancer κB site upstream of the conalbumin promoter, followed by the luciferase gene. The vector pNL4-3.Luc.R$^-$E$^-$ (AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Disease, National Institutes of Health) from N. Landau, as described in reference [41], contains the firefly luciferase gene inserted into the pNL4-3 nef gene. Two frameshifts (5′ Env and Vpr aa 26) render this clone Env$^-$ and Vpr$^-$. The pcDNA$_3$-VSV encoded the vesicular stomatitis virus G protein and the plasmids pXP1LTRwt (nucleotides −554 to +77), pXP1LTRΔκB-Luc (nucleotides −554 to +77 with κB enhancer element deleted), and pXP1κB-Sp I + II + III-Luc (nucleotides −105 to + 77 with the three Sp1-binding sites mutated) have been previously described [42].

2.4. Production of VSV-pseudotyped and HIV-1 recombinant viruses

High titre VSV-pseudotyped recombinant virus stocks were produced in 293T cells by cotransfection of pNL4-3.Luc.R$^-$E$^-$ together with either the pcDNA$_3$-VSV or the epNL3 plasmid encoding the vesicular stomatitis virus G-protein using the calcium phosphate transfection system. 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-p24 detection (INNOTEST ™ hiv-Ag, INNOGENETICS, Barcelona, Spain). Cultures were infected at a dose of 200 ng of HIV-1 gag p24 protein.

2.5. VSV-pseudotyped HIV-1 infection assay

Cells (10^6/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 and 24 h later cells were washed twice in PBS and lysed in luciferase buffer (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 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 Co., MA, USA). The results are represented as the % of activation (considering the infected and stimulated cells in the absence of denbinobin as the 100% activation). Results represent mean ± SD of four different experiments.

2.6. Transient transfections and luciferase assays

Jurkat cells were transfected with the indicated plasmids using Rottifect reactives (ROTH, Karlsruhe, Germany) according to the manufacturer's recommendations. 48 h post-transfection, cells were pretreated with denbinobin for 30 min, and stimulated with anti-CD3/anti-CD28/Protein-A mixture (4 μg/2 μg/10 μg), TNFα (20 ng/ml) or PMA (50 ng/ml). The cells were washed twice in PBS and lysed in luciferase buffer during 15 m at RT in a horizontal shaker. Then the lysates were spun down and the supernatants were used to measure luciferase activity. Results are represented as RLU or fold induction over untreated control. Results represent mean ± SD of at least four different experiments.

2.7. Isolation of nuclear extracts and mobility shift assays

Jurkat cells (10^6/ml) were pretreated with denbinobin at the indicated doses for 30 min and then were stimulated or not with TNFα (20 ng/ml) for the indicated times. Cells were then washed twice with cold PBS and proteins from nuclear extracts isolated as previously described [43]. For the electrophoretic mobility shift assay (EMSA), double stranded oligonucleotide containing the consensus oligonucleotide probes NF-κB, 5′-AGTTGAGGGGACTTTCCCAGG-3′ and the commercial SP1 site (Promega) were end-labelled with [γ-^{32}P]ATP. The binding reaction mixture contained 5 μg of nuclear extracts, 0.5 μg poly(dI-dC), 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 of 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.

2.8. Western blot

Jurkat cells (10^6/ml) were treated with denbinobin at the indicated doses for 12 h. 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 NaFl, 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 Bradford assay 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 proteins was carried out with specific mAbs and HRP-labelled secondary antibody using an ECL system (Amersham Biosciences Inc.).

2.9. Semiquantitative PCR analysis

Reverse transcriptase products were detected as previously described [44], with minor modifications. Briefly, Jurkat cells were infected with VSV-pseudotyped recombinant virus (200 ng p24 inoculum) for 24 h as indicated and total DNA was extracted with 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 reaction mixture containing DNA (50 ng), 1× PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.2 μM 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 final extension step of 7 min. The following primers were used to amplify short retrotranscription product (amplicon size: 140 bp): R/U5 (forward), 5′-GGCTAACTAGGGAACCCACTG-3′; R/U5 (reverse), 5′-CTGCTAGAGATTTTCCACACTGAC-3′, and long retrotranscription product (amplicon size: 200 bp): R/U5 (forward), LTR/gag (reverse), 5′-CCT GCC TCG AGA GAG CTG CTC 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.

2.10. Analysis of HIV-1 integrated DNA by nested Alu-PCR assay

Genomic DNA from Jurkat VSV-pseudotyped HIV-1-infected cells was extracted by using the QIAamp DNA minikit (QIAGEN GmbH, Hilden, Germany). The detection of HIV-1-LTR integrated into the cell genome was performed as previously described [43]. The first PCR was carried out by using primers LA1, from conserved sequences of HIV-1-LTR and LA2, from conserved human Alu sequences. Sequences of the primers are as follows: LA1 5′-TGTGTGCCCGTCTGTTGTGT-3′ (forward) and LA2 5′-TGCTGGGATTACAGGCGTGAG-3′ (reverse). Each PCR amplification was performed in a 50 μl PCR reaction mixture containing 0.5 μg of total DNA, 2 mM $MgSO_4$, 300 μM each of dATP, dGTP, dCTP, and dTTP, 20 pmol of primers LA1 and LA2, 5 μl of 10× reaction buffer and 1.25 U of AmpliTaq DNA Polymerase. 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′-GTGCCCGTCTGTTGTGTGACT-3′ (forward) and NL2 5′-CCGAGTCCTGCGTCGAGAGA-3′ (reverse) (amplicon size: 142 bp). A 3 μl aliquot from the first PCR amplification was added to a final volume of 50 μl of the nested PCR mixture. It contained 2 mM $MgCl_2$, 200 μM each of dATP, dGTP, dCTP, and dTTP, 20 pmol of primers NL1 and NL2, and 1.0 U AmpliTaq DNA Polymerase. Before PCR, samples were heated to 95 °C for 10 min. Cycling conditions were 40 cycles: 94 °C for 45 s; 50 °C for 1.5 min; 68 °C for 4 min, and a final incubation of 68 °C for 10 min. As a control, genomic DNA was subjected to β-actin amplification and used to normalize the nested PCR products, which were electrophoresed on a 3% (w/v) agarose gel.

2.11. RNA extraction and analysis of HIV transcripts

Jurkat Lat GFP cells (6×10^6 cells) were stimulated with PMA (50 ng/ml) for 2 h in the presence or the absence of denbinobin. Total RNA extraction was performed with RNAeasy mini kit (QIAGEN) according to the manufacturer's instructions. 2 μg of total RNA was submitted to DNAseI digestion for 15 min at room temperature and retrotranscriptase reaction was performed with Superscript II (SSII, Invitrogen) and random primers and the cDNA was employed in Real Time RT-PCR reaction using iQ SYBR Green Supermix (Bio-Rad, CA, USA) in an iCycler Thermal Cycler instrument (Bio-Rad). The PCR Amplification protocol was a denaturation step (95 °C, 3 min), followed by 40 cycles (95 °C for 20 s; 55 °C for 20 s; 72 °C for 20 s) and a final incubation of 72 °C for 30 s. The primers used were HIV-TAR-Up (5′-GTTAGACCAGATCTGAGCCT-3′) and HIV-TAR-Low (5′-GTGGGTTCCCTAGTTAGCCA-3′) for initiation of transcription, HIV-TAT-Up (5′-GGAAACCAGAGGAGCTCTCT-3′) and HIV-TAT-Low (5′-GAGTCTGACTGCCTTGAGGA-3′) for elongation; β-actin-Up (5′-GCTCCTCCTGAGCGCAAG-3′) and β-actin-Low (5′-CATCTGCTGGAAGGTGGACA-3′), HPRT1-Up (5′-ATGGGAGGCCATCACATTGT-3′) and HPRT1-Low (5′-ATGTAATCCAGCAGGTCAGCAA-3′). Relative quantification ($2^{-\Delta C_t}$) was calculated according to the formula: $\Delta C_t = C_t$ (elongation) − avg. avg. [C_t (housekeeping)].

2.12. Cytotoxicity assays

Jurkat cells were seeded in 96-well plates in complete medium and treated with increasing doses of denbinobin for six hours. Samples were then diluted with 300 μl of PBS and incubated 1 min at RT in the presence of PI (10 μg/ml). After incubation, cells were immediately analyzed by flow cytometry in an EPICS XL flow cytometer (Coulter, Hialeah, FL).

2.13. Statistical analysis

At least three independent experiments were used for data analysis. Original data were converted into %-values of LPS control and mean ± S.E.M. were calculated. Values were compared using *t*-test (two groups) or one-way ANOVA with *post hoc* Student–Newman–Keuls test (multiple comparisons). The level of statistical significance was set at $p < 0.05$.

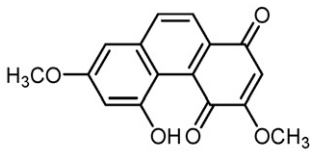
3. Results

3.1. Isolation of denbinobin from a variety of C. sativa

Denbinobin was obtained as black crystals (22 mg from 100 g of powdered plant material) from a cannabinoid-free polar fraction of hemp (cannabigerol-rich strain). Purification was greatly facilitated by the crystalline nature of denbinobin, a relatively minor constituent of the crude plant extract (Fig. 1).

3.2. Effect of denbinobin on HIV-1 viral cycle

The anti-viral activity of several phenanthrenequinones structurally related to denbinobin has been recently reported [32]. To study the anti-HIV activity of denbinobin, we infected Jurkat cells with the pNL4-3 HIV-1 clone pseudotyped with the VSV envelope, which bypasses the natural mode of HIV-1



Denbinobin

Fig. 1 – Chemical structure of denbinobin.

entry into these cells that support robust HIV-1 replication [45]. 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 12 h after cellular infection with the VSV-pseudotyped HIV-1 clone, and pretreatment of Jurkat cells 60 min prior infection with increasing doses of denbinobin did not inhibit significantly the luciferase activity (Fig. 2A). Also, preincubation of the viral stock with denbinobin for 3 h did not prevent HIV-1 infection and LTR viral transcription (data not shown). Interestingly, integrated HIV-1 reactivation induced by the phorbol ester PMA, a general protein kinase C activator, was clearly inhibited by denbinobin in a concentration dependent manner (Fig. 2B). Denbinobin was not cytotoxic at the time and concentrations tested. However, at higher concentrations denbinobin induces apoptosis and interferes with the luciferase assays. To further confirm that denbinobin did not affect HIV-1 reverse transcription or integration steps 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. Denbinobin did not decrease the amount of both R/U5 and LTR/gag products obtained following Jurkat cells infection with VSV-pseudotyped HIV-1. However, AZT at 10 μM clearly inhibited the amplification of the full-length (LTR-gag) product (Fig. 2C) [43,44]. Next, we studied the effects of denbinobin on HIV-1 integration in Jurkat cells infected with the VSV-pseudotyped HIV-1 proviral clon. The cells were pretreated with AZT or denbinobin 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 in Section 2. β-Actin was also amplified and used to normalize the amount of integrated HIV-1. We show in Fig. 2D that Jurkat infection with VSV-pseudotyped HIV-1 resulted in viral integration that was clearly prevented by AZT pretreatment, while denbinobin up to 5 μM did not inhibit such viral cycle step. Taken together, these results indicate that denbinobin interfere with the signaling pathway that activates integrated HIV-1, whereas it does not affect basal HIV-1 transcription.

3.3. *Denbinobin inhibits HIV-1 reactivation*

To analyze whether the inhibitory effects of denbinobin on HIV-1 reactivation were mediated at the HIV-1-LTR transcriptional

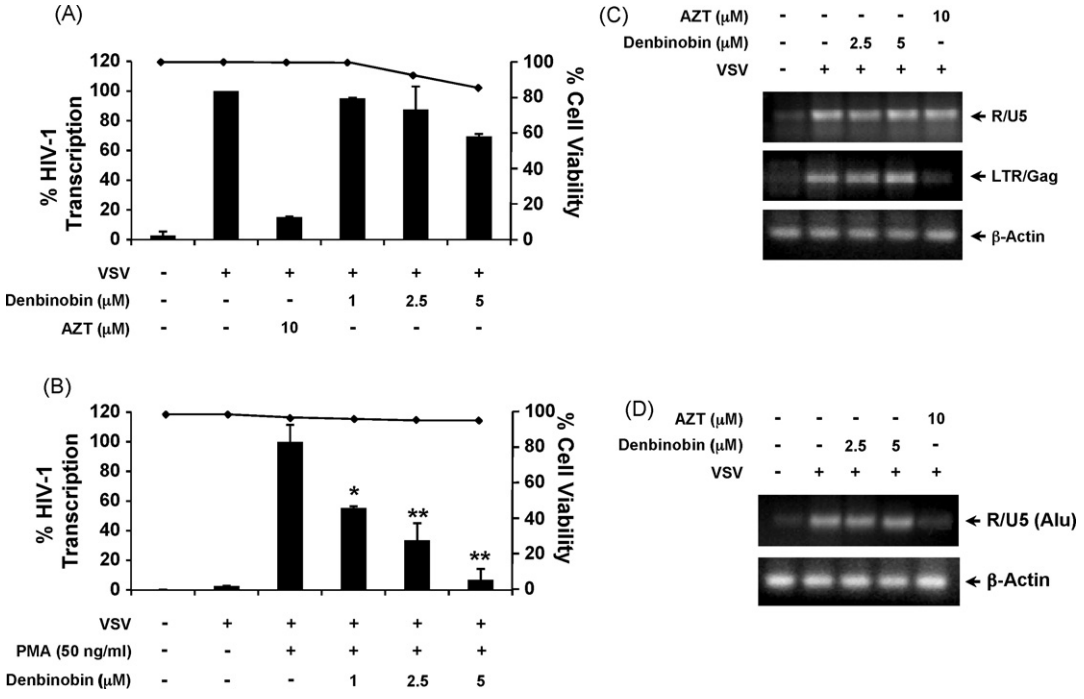


Fig. 2 – Effects of denbinobin on HIV-1 replication, retrotranscription and integration. (A) Jurkat cells (10^6/ml) were pretreated with AZT or denbinobin for 30 min and then infected with VSV-pseudotyped-pNL4-3.Luc.R^-E^- (200 ng p24) for 12 h. Luciferase activity in cell extracts was determined and results represented as percentage of activation ± SD compared to non-treated infected cells (100% activation). (B) Jurkat cells were infected as in panel A and 12 h later stimulated with PMA for 6 h in the presence or absence of denbinobin. The luciferase activity was measured and expressed as the % of inhibition compared to PMA alone (100% activation). (C) Semiquantitative PCR was performed on DNA extracted from Jurkat cells 12 h post-virus infection following treatment with denbinobin or 10 μM AZT. Primers were used to amplify the HIV-1 reverse transcriptase short products R/U5 (top panel) and long products LTR/gag (middle panel) as well as the β-actin (bottom panel) as a control. (D) Detection of integrated DNA by using Alu-LTR PCR. Genomic DNA from Jurkat cells treated as in C was extracted and was subjected to PCR by using the primers LA1 and LA2. An aliquot of the first PCR product was subjected to second round of PCR by using nested HIV-1-LTR-specific primers (NL1 and NL2) and the products visualized by agarose gel electrophoresis (upper panel). $^*p < 0.05$, $^{**}p < 0.01$ with respect to PMA treatment.

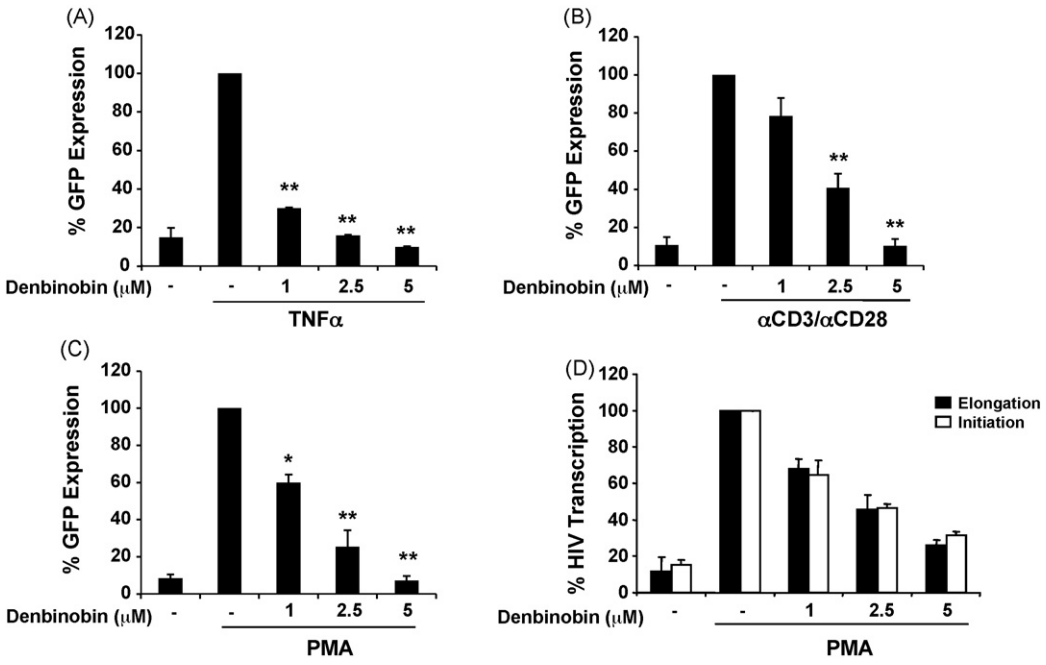


Fig. 3 – Effects of denbinobin on HIV-1 reactivation. Jurkat-LAT-GFP cells were preincubated with increasing concentrations of denbinobin, followed by stimulation with TNFα (A), phorbol esters (B) or α-CD3/α-CD28 mAbs (C) for 6 h. Then GFP expression was analyzed by flow cytometry. (D) Jurkat-LAT-GFP cells were stimulated with PMA for 2 h in the presence or absence of denbinobin. Total RNA was extracted and Real Time RT-PCR assay was performed with primers targeting the first HIV-TAT exon. Results are presented as the percentage of elongation compared to only PMA treated cells (100%) ±SD of three different experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ with respect to the respective agonist treatment.

activity level, we used Jurkat-LAT-GFP cell line which represents a lymphocyte-based model of HIV post-integration latency. This cell line contains a full-length latent HIV provirus containing GFP in place of Nef; thus, transcriptional activation of the latent provirus can be readily detected in individual cells by flow cytometry. Following treatment with TNFα, PMA or the agonistic α-CD3/α-CD28 mAbs GFP expression was induced to various and characteristic degrees in this cell line [39]. In Fig. 3 (A–C) it is shown that denbinobin inhibited in a concentration dependent manner the expression of GFP in Jurkat-LAT-GFP cells stimulated with TNFα, PMA or the α-CD3/α-CD28 mAbs. Interestingly, denbinobin was more effective to inhibit TNFα-induced HIV-1 activation (IC50 < 1 μM) compared to PMA or α-CD3/α-CD28 mAbs stimulation (1.5 and 2.5 μM respectively). To assess the effect of denbinobin on HIV transcription from the latent promoter, RNA was extracted from Jurkat-LAT-GFP under repressed (−PMA) and activated (+PMA) conditions in the presence or absence of increasing concentrations of denbinobin, and initiated and elongated transcripts were quantified. PMA increased elongated transcripts were clearly inhibited by denbinobin (Fig. 3D).

3.4. *Natural and synthetic denbinobin inhibits TNF-induced but not Tat-induced promoter HIV-1-LTR activity*

One of the major tasks in natural product research is the total synthesis of chemical metabolites. In this sense, the synthesis of denbinobin has already been described [38] and therefore we have studied the anti-HIV-1 activity of both natural and synthetic denbinobin in two stably HIV-LTR transfected cell lines, namely 5.1, a lymphoid T cell line, in which the luciferase gene under control of the HIV-1-LTR that is activated by TNFα, and HeLa-Tat-Luc cells, in which the HIV-1-LTR is directly activated by the HIV-1 Tat protein [46]. 5.1 cells were preincubated with increasing concentrations of denbinobin and then stimulated with TNFα (5 ng/ml) for 6 h and luciferase activity was measured. As depicted in Fig. 4A, pretreatment with denbinobin resulted in a dose-dependent inhibition of TNFα-induced LTR activation. Both natural and synthetic denbinobin showed identical biological activities (IC50 < 1 μM). The inhibitory effects of denbinobin were not due to interference with the transcriptional machinery or with the *in vitro* activity of the luciferase enzyme, since the inducible expression of luciferase mediated by Tat in Hela-Tat-Luc cells was not affected by this compound at any of the concentrations tested. As expected the high levels of luciferase activity induced by Tat were inhibited by the pTEFb inhibitor 5,6-dichloro-1-β-D-ribofuranosyl benzimidazole (DRB) (Fig. 4B).

3.5. *Inhibition of the HIV-1-LTR transactivation by denbinobin is mediated through the κB elements located in the LTR-enhancer*

The upstream LTR promoter contains binding sites for the transcription factor NF-κB, NFAT and Sp1 among others [14], and since PMA, TNFα and mAb anti-CD3 are well known activators of NF-κB, we addressed whether denbinobin could

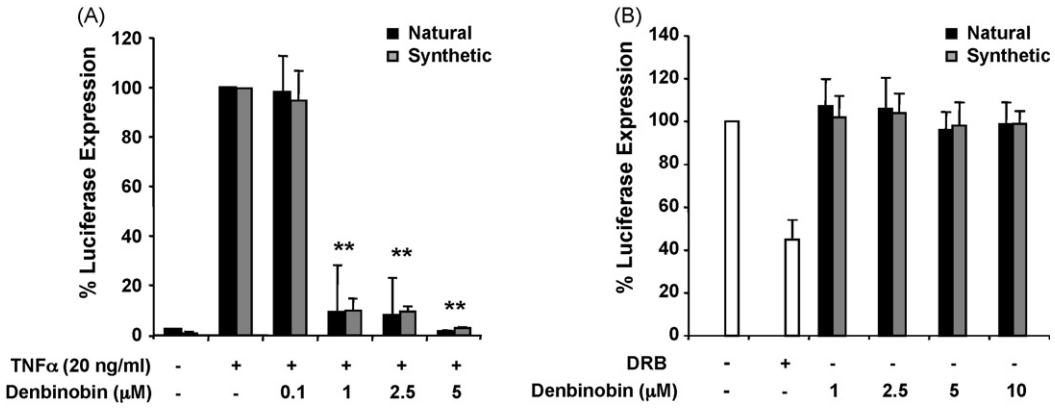


Fig. 4 – Effects of natural and synthetic denbinobin on TNFα and Tat-induced HIV-1-LTR activation. (A) 5.1 cells were preincubated with denbinobin at the indicated doses and stimulated with TNFα (2 ng/ml) for 6 h. The luciferase activity was measured and results are presented as the percentage of inhibition compared to TNFα in the absence of denbinobin. (B) Hela Tat-Luc cells were incubated with either DRB (50 μM) or denbinobin (natural and synthetic) at the indicated doses and after 12 h luciferase activity was measured. Results are presented as the percentage of inhibition relative to control (RLU activity in Hela-Tat-Luc cells). $^{*}p < 0.05$, $^{**}p < 0.01$ with respect to the TNFα treatment.

impair both NF-κB- and Sp1-dependent transcriptional activation by transfecting Jurkat cells with the plasmids wt-LTR-Luc, pXP1LTRΔκB-Luc and pXP1κB-Sp I + II + III-Luc (Fig. 5A). As shown in Fig. 5B denbinobin clearly inhibited the TNFα-induced activity of the wt-LTR-Luc and the pXP1κB-Sp I + II + III-Luc promoters in a dose-dependent manner. In contrast, and only at the higher concentration tested, denbinobin exhibited some inhibitory activity on the NF-κB mutated LTR promoter. These results indicate that denbinobin inhibits the HIV-LTR through an NF-κB-dependent pathway. To further confirm the anti-NF-κB activity of denbinobin Jurkat cells were transiently transfected with a luciferase reporter construct under the control of a minimal promoter containing three binding sites for NF-κB. Activation by PMA, TNFα, and mAbs anti-CD3/CD28 increased the luciferase gene expression driven by this artificial promoter in Jurkat cells and we found that denbinobin effectively inhibited this promoter activity in a dose-dependent manner (Fig. 6).

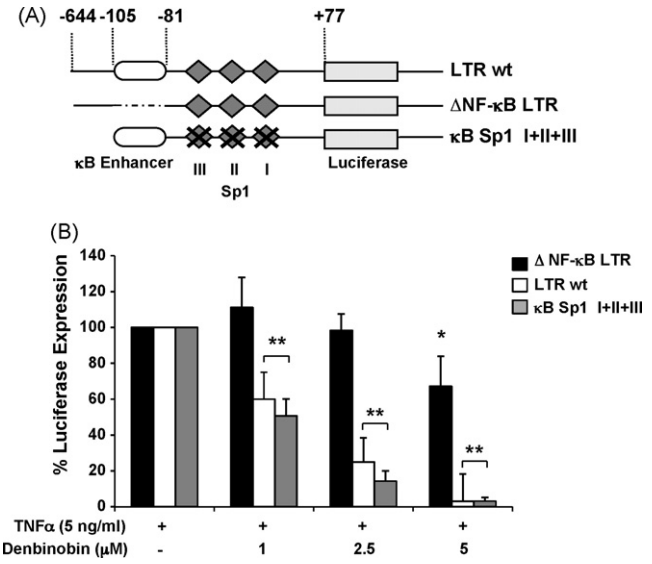


Fig. 5 – Denbinobin inhibition of HIV-1-LTR transactivation is dependent on the NF-κB elements (A) Schematic representation of plasmid constructions used. (B) Jurkat cells were transfected with the indicated reporter plasmids and 24 h after transfection the cells were preincubated with increasing concentrations of denbinobin and stimulated with TNFα for 6 h. Results are presented as the percentage of luciferase activity relative to control (100% for TNFα treatment). $^{*}p < 0.05$, $^{**}p < 0.01$ with respect to the TNFα treatment.

3.6. *Denbinobin inhibits the NF-κB pathway in 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 [24]. In addition to this pathway, a second level of NF-κB activation involves the phosphorylation of the p65 and the subsequent stimulation of NF-κB transactivation [25,28]. Thus, to investigate the level at which denbinobin exerted its inhibitory effect on NF-κB activation, we stimulated Jurkat cells with TNFα for different times in the presence or absence of denbinobin (2.5 μM), and proteins from nuclear cell extracts were analyzed for NF-κB DNA binding activity by EMSA, and for studying the steady state levels of IκBα and p65 proteins and their phosphorylated forms by Western blot. The EMSA experiments revealed a clear increase in NF-κB binding to DNA that was prevented by the presence of denbinobin. Conversely, no significant effect by denbinobin was observed on binding activity at the Sp1 site (Fig. 7A). In Jurkat cells we detected that TNFα induced a rapid phosphorylation of the cytoplasmic IκBα protein and the NF-κB subunit p65. Moreover, IκBα phosphorylation preceded the degradation of the protein that was more evident after 15–30 min of treatment with this cytokine. Interestingly, denbinobin inhibited both IκBα phosphorylation and degradation, and p65 phosphorylation indicating that this compound mediates

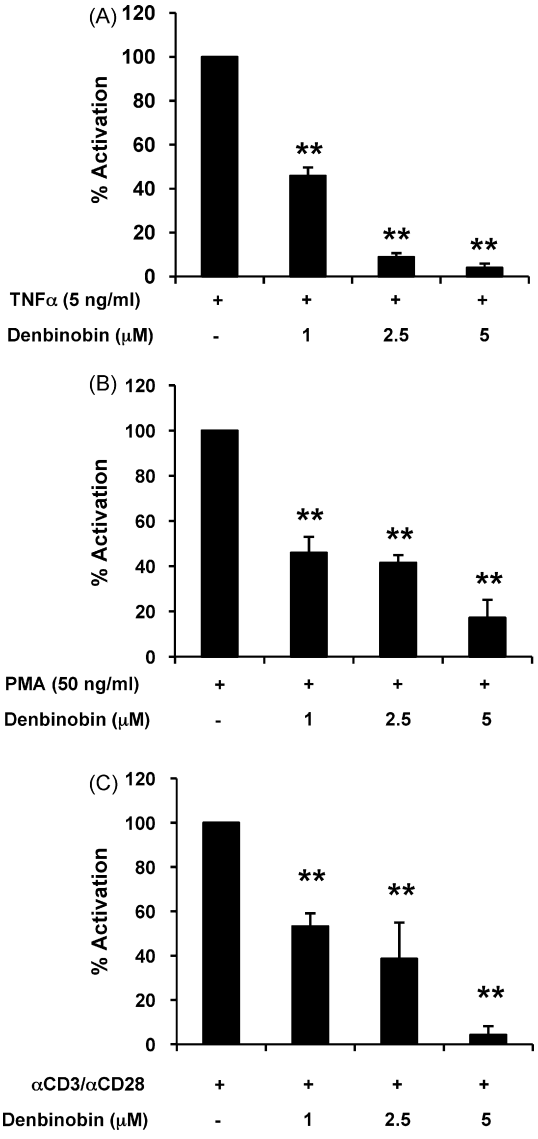


Fig. 6 – Regulation of NF-κB-mediated transactivation by denbinobin. Jurkat T cells were transiently transfected with the luciferase reporter plasmid KBF-Luc and 24 h after transfection the cells were preincubated for 30 min with denbinobin at the concentrations indicated, before stimulation with TNFα (A), PMA (B) or α-CD3/α-CD28 mAbs (C) for 6 h. Luciferase activity was measured and the results are presented as the percentage of luciferase activity relative to control (100% for stimuli treatment in the absence of denbinobin). $^{**}p < 0.01$ with respect to the respective agonist treatment.

its anti-NF-κB activity by targeting a step upstream of IκBα phosphorylation in the canonical pathway of NF-κB activation (Fig. 7B).

4. Discussion

The use of plant extracts to alleviate human pathologies is centuries old and continues nowadays. Thus, natural product research has paved the way to develop on a scientific basis single agent drugs that are 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 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 [7–9]. 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 [8,47]. 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 [48]. Here we show that denbinobin inhibits PMA-, TCR/CD28- and TNFα-induced HIV-1-LTR transcriptional activity in three different cellular models.

Phenanthrenes are uncommon aromatic metabolites which are probably generated by oxidative coupling of the aromatic rings of stilbene precursors or from diterpenoid precursors. Several plant-derived phenanthrenequinones have been described so far and some of them show interesting biological activities [49]. Denbinobin is one of the pure compounds isolated from the stems of *Dendrobium moniliforme*, known as Shi-Hu in Chinese medicine, which has been used for a long time to treat respiratory tract infection, as a tonic to nourish the stomach and to reduce fever. *Ephemerantha lonchophylla* is also as a source of Shi-Hu in Taiwan. In our screening program to study phytoextracts and natural products with anti-HIV-1 activities we have identified a variety of *C. sativa* L. (variety CARMA) with a very low content of psychoactive cannabinoid $^{9}\Delta$-Tetrahydrocannabinol. We found that an acetone extract from this plant is a potent inhibitor of TNFα-induced transcriptional activity of the HIV-1-LTR promoter. The phytochemical analysis of this extract revealed the presence of cannabinoids such as cannabidiol, cannabigerol and cannabichromene; stilbenoids such as canniprene, cannabispiranol and cannabispirane; prenylated flavones such as canflavin A and B; and the 1,4-phenanthrenequinone denbinobin. (The detailed phytochemical analysis of the extract will be described elsewhere.) The analysis of the biological activities of those compounds revealed that denbinobin is the principal anti-HIV-1 compound present in the phytoextract (data not shown) and therefore a detailed mechanistic study was undertaken.

HIV viral cycle is divided into early and late events. Early events begin with the viral entry into the host cell and conclude with the integration of the HIV provirus into the cell genome. In a productive infection integrated provirus rapidly initiates HIV replication, a process that initiates the post-integration phase of the viral cycle that occurs in activated cells and is regulated by the collaborative action of viral regulatory proteins (i.e. HIV-1 Tat) and cellular factors (i.e. NF-κB and Sp1) on 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 [14,50]. Thus, viral regulatory proteins and cellular transcription factors could be possible targets for anti-HIV chemotherapy. Our results indicate that denbinobin does not interfere with the initial steps (retro-transcription and

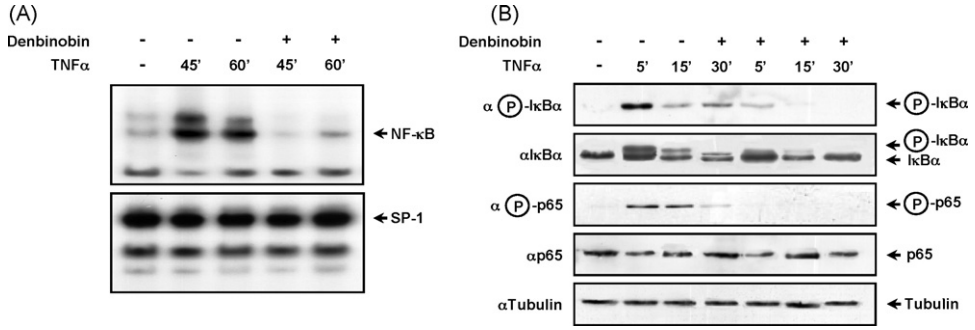


Fig. 7 – Effects of denbinobin on NF-κB activation pathway. (A) Jurkat cells were incubated with denbinobin at the indicated doses, treated with TNFα for 45 and 60 min and total protein extracted. Sp1 and NF-κB-DNA binding activities were determined by EMSA. (B) Western blot analysis of IκBα phosphorylation and degradation and p65 phosphorylation (Ser^{536}) performed in Jurkat cells preincubated with denbinobin (2.5 μM) and stimulated with TNFα for the indicated periods of time.

integration) of the pre-integration phase of the viral cycle, but strongly inhibits TNFα-, TCR/CD3- and PMA-induced HIV-1 reactivation (Fig. 8).

Since the activation of the NF-κB is a common pathway activated by TNFα, TCR or PMA we investigated the effects of denbinobin on the activation of this pathway. We clearly show that denbinobin inhibits the phosphorylation and degradation of IκBα and therefore prevents both the DNA binding and the transcriptional activities of the NF-κB subunit p65. Interestingly, denbinobin also inhibits the transcriptional activity of the LTR-Sp1 mutant construct and has no effect on HIV-1-Tat-induced LTR transactivation. Moreover, this compound did not affect the transcriptional activity of Gal4-Luc induced by the fusion protein Sp1-Gal4 (data not shown). We also found that denbinobin slightly inhibited the transcriptional activity driven by a promoter lacking the κB sites in the enhancer region. Interestingly, it has been shown that the p65 subunit of the NF-κB transcription factor is also involved in the LTR elongation and may be also targeted by denbinobin [51]. It has been suggested that Sp1 transcription factors in the presence of HIV-Tat protein are required for basal-level replication in HIV-1 infected cells [22,52,53] and therefore this could explain why denbinobin did not prevent basal-level replication in VSV-pseudotyped infected cells. In contrast, NF-κB is required for cytokine and TCR-induced HIV-1 replication [27,54,55] and our results strongly suggest that the activity of denbinobin is specific for the NF-κB pathway probably by targeting a kinase(s) upstream IκBα, and we are currently working on the identification of such kinase(s).

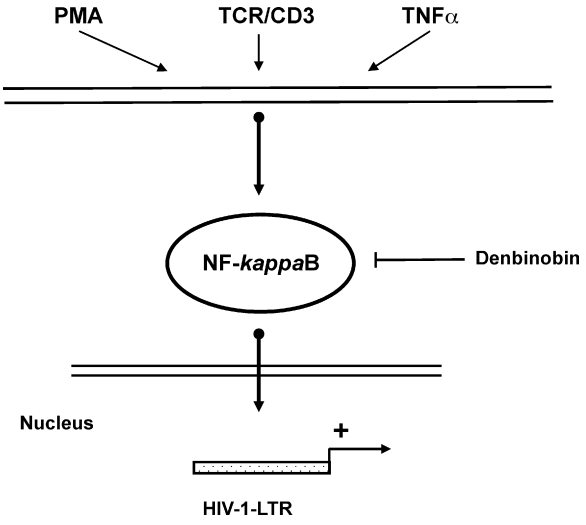


Fig. 8 – Schematic model summarizing the obtained data.

Numerous plant-derived compounds, including stilbenoid and terpenoid derivatives, have been shown to exert anti-HIV-1 activity by acting on both viral and cellular targets [30,31,56]. In addition, some natural compounds were reported to suppress HIV-1 gene expression and replication through the inhibition of NF-κB activation pathway, yet the precise mechanism of action is unknown for most of the compounds analyzed so far [57,58]. In addition to the NF-κB inhibitory activity denbinobin also targets other cellular processes [37,59], and therefore further SAR studies are required to identify the pharmacophore responsible for this activity. Preliminary results indicate that the benzoquinones structure is not required for NF-κB inhibition (Sánchez-Duffhues et al., manuscript in preparation).

Plant derived antiviral compounds interfering with the cellular machinery that regulates the HIV-LTR promoter are of special interest, since these drugs, if borne useful for patients, are unlikely to induce mutations in the host genome. Thus, natural compounds such as denbinobin and perhaps other 1,4-phenanthrenequinones might have a potential therapeutic role in the management of AIDS, most probably in combination with other anti-HIV drugs.

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