BIOCHEMICAL PHARMACOLOGY 73 (2007) 1738–1748

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HIV-1 protease inhibitors do not interfere with provirus transcription and host cell apoptosis induced by combined treatment TNF-α + TSA

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ARTICLE INFO

Article history:
Received 21 December 2006
Accepted 21 February 2007

Keywords:
HIV-1 protease inhibitor
NF-κB
Apoptosis

ABSTRACT

HIV-1 latency represents a major hurdle to the complete eradication of the virus from patients under highly active anti-retroviral therapy (HAART) regimens. One solution to this problem would be to eliminate the latently infected cellular reservoirs by forcing gene expression in presence of HAART to prevent spreading of the infection by the newly synthesized viruses. Many studies have reported that a combination of a histone deacetylase inhibitor (HDACi) (i.e. TSA, NaBut, Valproic acid, . . .) with a pro-inflammatory cytokine (i.e. TNFα, IL-1, . . .) reactivates in a synergistic manner HIV-1 transcription in latently infected cells. The aim of the present study was to determine whether HIV-1 protease inhibitors (PIs) used in HAART (such as Saquinavir, Indinavir, Nelfinavir, Lopinavir, Ritonavir and Amprenavir) could interfere with the potential purge of the cellular reservoirs induced by a combined treatment involving TSA and TNFα. We showed, in two HIV-1 latently infected cell lines (ACH-2 and U1) that all PIs efficiently inhibited release of mature viral particles but did neither affect cell apoptosis nor NF-κB induction and HIV-1 transcription activation following combined treatment with TNFα + TSA. This study is encouraging in the fight against HIV-1 and shows that PIs should be compatible with an inductive adjuvent therapy for latent reservoir reduction/elimination in association with efficient HAART regimens.

1. Introduction

HIV-1 infected individuals are currently treated by highly active anti-retroviral therapy (HAART). This therapy most often based on a combination of one inhibitor of the viral protease with two inhibitors of HIV-1 reverse transcriptase has permitted to reduce plasma virus to undetectable levels in many infected patients [1]. Unfortunately, interruption of HAART even after

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Abbreviations: HIV-1, human immunodeficiency virus type 1; LTR, long terminal repeat; HDACi, histone deacetylase inhibitor; TNFα, tumor necrosis factor alpha; IL-1, interleukin-1; HAART, highly active anti-retroviral therapy; PI, protease inhibitor; TSA, trichostatin A; CPT, camptothecin; EMSA, electrophoretic mobility shift assay; SQV, saquinavir; IND, indinavir; NFV, nelfinavir; LPV, lopinavir; RTV, ritonavir; APV, amprenavir

0006-2952/$ – see front matter
doi:10.1016/j.bcp.2007.02.011

prolonged periods of full viral suppression invariably results in rebounds of viral replication indicating that antiretroviral therapy is unable to completely eliminate HIV-1 [2–4]. This failure has been principally attributed to a long-lived, stable population of latently infected resting memory CD4+ T cells that carry an integrated viral genome, or provirus, that is transcriptionally silent [5,6]. Macrophages and monocytes also constitute reservoirs for HIV-1 [7]. These latent reservoirs have such a slow rate of decay during HAART that their eradication during a lifespan is unlikely [8]. It has been proposed that one possible solution to the problem of HIV-1 latency is to purge these reservoirs by deliberately forcing HIV-1 gene expression in these latently infected cells in presence of HAART to prevent spreading of the infection by the newly synthesized viruses [9]. Such an inductive treatment could reduce the number of latently infected cells by the cytopathic action of the virus or by cytotoxic T cells directed against viral antigens.

One element responsible for HIV-1 latency in resting $CD4^+$ T cells is the absence of transcription factors that are required for gene expression activation from the 5′ LTR [10,11]. Indeed, the enhancer located in the LTR U3 region contains two binding sites for the transcription factor NF-κB which plays a central role in the activation pathway of the HIV-1 provirus. In unstimulated cells, NF-κB whose the most abundant form is the heterodimer p50/RelA, is sequestered in the cytoplasm in an inactive form through interaction with inhibitory proteins of the IκB family [12]. Upon activation of NF-κB by various stimuli including pro-inflammatory cytokines (TNFα, IL-1) and bacterial lipopolysaccharides (LPS), IκBα is rapidly phosphorylated by a macromolecular IκB kinase complex (IKK), ubiquitinated and degraded by the 26*S* proteasome [12]. NF-κB then translocates to the nucleus and activates transcription from a wide array of promoters.

Chromatin structure also plays a crucial role in HIV-1 transcriptional repression during latency. Indeed, a nucleosome called nuc-1 located immediately downstream of the transcription start site is specifically and rapidly disrupted during transcriptional activation [13,14]. Several studies have demonstrated the transcriptional activation of HIV-1 promoter in response to histone deacetylase inhibitors (HDACi) such as trichostatin A (TSA), trapoxin (TPX), valproic acid (VPA) and sodium butyrate (NaBut) [15]. HIV-1 transcriptional activation following treatment with HDACi is associated with nuc-1 remodeling [13,16–18].

An important optimization of anti-AIDS therapy would consist in eliminating the pool of latently infected cells by activation of HIV-1 gene expression in these cells, while maintaining an efficient HAART regimen. We have previously demonstrated a strong synergistic activation of HIV-1 transcription by TNFα + TSA(NaBut) both in a cell line model of post-integration latency and in the context of a de novo viral infection [19]. The molecular mechanism underlying the TNFα + TSA synergism was partially elucidated and involves the prolongation of IKK complex activity which leads to the extended presence of NF-κB in the nucleus [20].

Based on these previous results, we have proposed the administration of HDACi together with a continuous HAART as a new potential therapeutic perspective to decrease the pool of latent reservoirs [18,19]. HDACi could synergize with TNFα whose levels are increased in the serum of HIV infected patients [21]. However, some HIV-1 protease inhibitors (PIs) used in HAART could interfere with eradication of the latent reservoirs. Indeed, some PIs, such as Saquinavir and Ritonavir have been reported to inhibit the proteasome [22–24]. By preventing IκBα proteolysis, these PIs could decrease NF-κB activation and provirus transcription, which could interfere with the purge of latent reservoirs induced by HDACi. Secondly, some PIs have been reported to affect both spontaneous and stimuli-induced apoptosis which could also lead to a less efficient purge of HIV-1 reservoirs [25–30]. These data are consistent with the *in vivo* observation that apoptosis both in peripheral blood and lymphatic tissues is rapidly decreased following initiation of PI based therapy independently of their antiviral effect [28].

In this report, we studied the effect of six different PIs used in HAART, Saquinavir (SQV), Indinavir (IND), Nelfinavir (NFV), Lopinavir (LPV), Ritonavir (RTV) and Amprenavir (APV) on mature viral particles production, synthesis of viral mRNA and NF-κB activation as well as on apoptotic cell death in HIV-1 latently infected promonocytic (U1) and lymphocytic (ACH-2) cell lines after the combined treatment TNFα + TSA.

2. Materials and methods

2.1. Reagents

TNFα was purchased from Roche. TSA and CPT were obtained from Sigma–Aldrich. Anti-IκBα monoclonal antibody was a kind gift from R. Hay (Scotland). Anti-β-actin monoclonal antibody was obtained from Sigma–Aldrich. Horseradish peroxidase-conjugated antibodies were obtained from Dako (Danemark).

2.2. HIV-1 protease inhibitors

Saquinavir was obtained from Roche (Basel, Switzerland), Indinavir from MSD (New Jersey, USA), Lopinavir and Ritonavir from Abbott (Chicago, USA), Nelfinavir from Pfizer (Groton, UK) and Amprenavir from Glaxosmithkline (Harlow, UK). Protease inhibitors were solubilized in DMSO at a stock concentration of 40 mM.

2.3. Cell lines

The T-cell line ACH-2 and the promonocytic cell line U1, both HIV-1 latently infected, as well as non-infected T-cell lines, CEM and Jurkat, and non-infected monocytic cell line U937 were cultivated in RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum, 1% penicillin and 1% streptomycin at 37 °C in a 5% CO_2 incubator.

2.4. p24 determination

Viral p24 antigen levels in culture supernatants were determined by ELISA (Innotest HIV Antigen mAb kit, Innogenetics) according to the manufacturer's instructions.

2.5. Cell viability determination

Cell viability was measured by using the cell proliferation reagent WST-1 (Boehringer Mannheim).

2.6. Apoptosis detection

Apoptosis was monitored by flow cytometry detecting active intracellular caspase-3 (BD Pharmigen) or by Annexin V and Propidium Iodide staining (Roche, Germany) according to the manufacturer's instructions.

2.7. Nuclear and cytoplasmic protein extraction

Cells were washed with cold phosphate-buffered saline and resuspended in lysis buffer containing 10 mM HEPES–KOH pH 7.9, 2 mM $MgCl_2$, 0.1 mM EDTA, 10 mM KCl, 0.5% IGEPAL, 1 mM PMSF, 1 mM DTT and protease inhibitors (Complete, Roche). After incubation on ice for 10 min and centrifugation at 14,000 rpm for 30 s, the supernatant containing cytoplasmic proteins was harvested and stored at −80 °C. The pellet was resuspended in saline buffer (50 mM HEPES–KOH, pH 7.9, 2 mM $MgCl_2$, 0.1 mM EDTA, 50 mM KCl, 400 mM NaCl, glycérol 10% (v/v), 1 mM PMSF, 1 mM DTT and protease inhibitors) and incubated for 30 min on ice. After centrifugation at 14,000 rpm for 15 min, the supernatant containing the nuclear proteins was harvested and stored at −80 °C. The protein content was measured by the Bio-Rad protein assay kit (Biorad laboratories GmbH, Munchen).

2.8. Electrophoretic Mobility Shift Assay (EMSA)

Five micrograms of nuclear proteins were incubated for 20 min at room temperature in binding buffer (20 mM HEPES-KOH, 75 mM NaCl, 1 mM EDTA, 5% (v/v) glycerol, 0.5 mM $MgCl_2$, 1 mM DTT) with BSA (2 μg), poly(dIdC)-poly(dIdC) (1.25 μg) and 0.2 ng of ^{32}P-labeled double strand oligonucleotide carrying the LTR κB site (sense, 5′-GGTTACAAGGGACTTTCCGCTG-3′; antisense, 3′-TGTTCCCTGAAAGGCGACGGTT-5′). DNA-protein complexes were then resolved by electrophoresis on native 6% polyacrylamide gel. Dried gels were revealed by autoradiography.

2.9. Western blotting

Cytoplasmic proteins were resolved by SDS-PAGE, transferred onto a polyvinylidene fluoride membrane and blotted with anti-IκBα monoclonal antibody. Detection of bands was visualized by chemiluminescence using the ECL kit (Amersham, UK).

2.10. RNA extraction and RT-PCR

Total RNA was extracted from 10^6 cells by using the RNeasy Mini Kit (Qiagen) and quantified with a Nanodrop. RNA (1 μg) was then reverse transcribed for 1 h at 37 °C in the presence of M-MLV reverse transcriptase. cDNA were amplified by real-time PCR using the SyberGreen Mix method (Applied Biosystems, Warrington, UK). The following primers were used: HIV-1 LTR-Forward (5′-GCCTCAATAAAGCTTGCCTTGA-3′) and HIV-1 LTR-Reverse (5′-GGCGCCACTGCTAGAGATTTT-3′) [31]. These primers amplified a conserved concensus region of 161 nt (nt68 to nt189). The primers encompassed partly the R region (nt68 to nt97) and completely the U5 region (nt97 to nt180) of the LTR. p-Values were calculated with a *t* test (www.graphpad.com).

3. Results

3.1. PIs efficiently inhibit the release of mature viral particles from TNFα + TSA-treated ACH-2 and U1 cells

Several studies have previously reported a synergistic activation of HIV-1 transcription following the combined treatment TNFα + TSA [13,19]. In this work, we used both the promonocytic (U1) [32] and lymphocytic (ACH-2) [33] cell lines as post-integration latency models. We tested six PIs which are routinely used in the clinic: SQV, IND, NFV, LPV, RTV and APV [34]. PIs block the cleavage of *gag* and *gag-pol* protein precursors which leads to the release of immature and non-infectious viral particles [35]. For each PI, we first determined the concentration range resulting in an efficient inhibition of mature virus release from TNFα + TSA-treated ACH-2 and U1 cells. The production of mature viral particles was monitored by ELISA determination of the capside protein p24 level in culture supernatants. Both cell lines were left untreated or were treated with TNFα (100 U/mL) alone, with TSA (450 nM) alone or with TNFα + TSA in the presence or not of each PI for 16 h. As previously described [19], treatment of both cell lines by TNFα or TSA alone stimulated virus production which was further increased after the combined treatment TNFα + TSA (Fig. 1). While TNFα and TSA had an additive effect in ACH-2 cells (Fig. 1A), they strongly synergized in U1 cells (Fig. 1B). Fig. 1 shows the dose-dependent inhibition of the viral p24 production by IND between 0.1 and 10 μM. The EC_{50} were determined to be 0.32 μM for U1 cells and 0.48 μM for ACH-2 cells. The inhibition profiles for the other PIs (SQV, NFV, LPV, RTV and APV) were relatively similar (data not shown). In each case, the inhibition was complete at 10 μM. Since PIs have been described to bind a wide range of plasma and serum proteins [36], the unbound concentration reached in our experiment is less than 10 μM and close to the clinically relevant concentration. Accordingly, we used this pharmacological protein free concentration in the following experiments.

On the other hand, a concentration of 0.1 μM led to only a weak inhibition. Table 1 shows the EC_{50} for each PI on U1 or ACH-2 cells. SQV and APV were the most efficient PIs in both cell lines, followed by NFV, RTV, IND and LPV in U1 cells. However, IND appeared to be more effective against HIV-1 than NFV, RTV and LPV in ACH-2 cells. For each PI, the EC_{50} were lower for U1 than for ACH-2 cells. This more pronounced effect of PIs on HIV-1 production in U1 cells compared with ACH-2 cells may be due to the lower level of virus production by the former (data not shown).

3.2. PIs do not interfere with cell death after HIV-1 reactivation induced by the combined treatment TNFα + TSA

To ensure an efficient purge of viral reservoirs, it is mandatory that the antiviral combination does not prevent infected cells to be directly killed by the cytopathic action of the virus or to be destroyed by the immune system.

To determine the cytopathic effect of HIV-1, we measured cell viability after the combined treatment with TNFα + TSA for 24 h both in latently infected cells (ACH-2, U1) and in parental non-infected cell lines, CEM and U937, respectively.

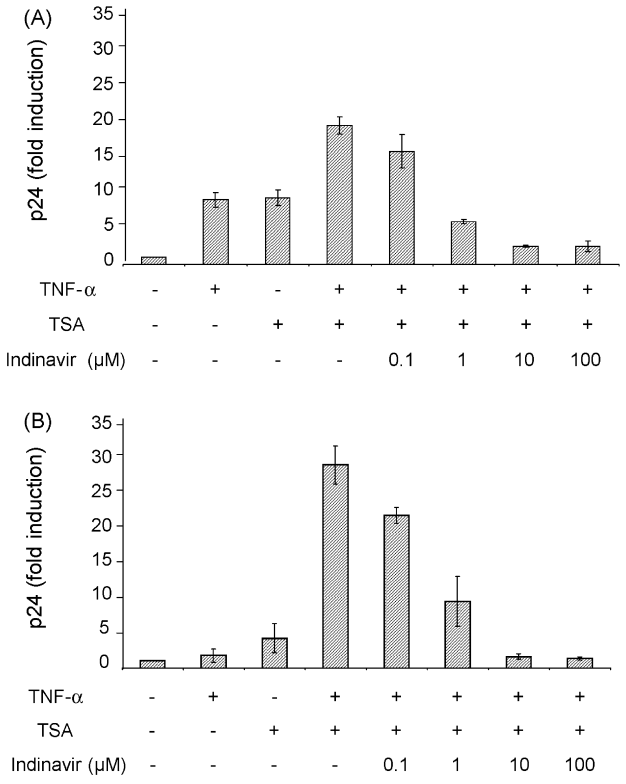

Fig. 1 – Effect of Indinavir on the release of mature viral particles following combined treatment with TNFα + TSA. ACH-2 (A) and U1 (B) cell lines were untreated or treated with TNFα (100 U/mL) or TSA (450 nM) or TNFα + TSA in the presence or not of IND (0.1, 1, 10 and 100 μM). p24 levels were determined by ELISA in the supernatants at 16 h post-induction. Values represent means of three independent experiments. The error bars show the standard errors of the mean.

Cell viability was determined by the WST-1 assay [37]. The treatment of non-infected T lymphocytes (CEM) by TNFα (100 U/mL) or TSA alone induced a low mortality which was not further increased by the combination of both agents (Fig. 2A). However, in the latently infected T lymphocytes ACH-2, these same treatments resulted in a loss of viability which was proportional to the virus production (Figs. 1 and 2A). Approximately, 70% of ACH-2 cells compared to only 20% of CEM cells died after the combined treatment TNFα + TSA indicating that viral reactivation was cytopathic for ACH-2 cells. However, whatever the treatment, viabilities of U1 and U937 cells were similar and were not significantly affected meaning that promonocytes U1 were not susceptible to HIV-1 cytopathic effects (Fig. 2B).

Table 1 – PI concentration that inhibits 50% of HIV-1 particles release (EC50) from U1 and ACH-2 cell lines stimulated by the combined treatment TNFα/TSA

	EC_{50} (μM)	
	U1	ACH-2
Saquinavir	0.17	0.34
Amprenavir	0.2	0.46
Nelfinavir	0.27	0.55
Ritonavir	0.28	0.63
Indinavir	0.32	0.48
Lopinavir	0.41	1.3

We showed that the PIs efficiently inhibited the release of mature viral particles from TNFα + TSA-treated ACH-2 and U1 cells (Fig. 1). In addition to their protective role in the strategy based on the use of HDACi, PIs could interfere with the purging effect of this therapeutic approach. Consequently, we studied the effect of each PI on the viability of ACH-2 cells after HIV-1 reactivation by the combined treatment TNFα + TSA. Fig. 2C shows that IND tested over a broad range of concentrations (0.1–20 μM) neither antagonized nor synergized with the cytopathic effect of HIV-1. The other PIs used at concentrations which are relevant *in vivo* (0.1–10 μM) did not interfere with the viability of ACH-2 cells after TNFα + TSA treatment (data not shown). SQV and NFV were cytotoxic by themselves above 10 μM (data not shown).

Since several proteins encoded by HIV-1 (Tat, Vpr, ...) can trigger apoptosis [27,38], we determined the percentages of apoptotic ACH-2 cells after HIV-1 reactivation induced by TNFα or TSA or the combined treatment TNFα + TSA. Apoptosis was monitored after 24 h by flow cytometry detecting active intracellular caspase-3. The treatments with TNFα alone, with TSA alone or a combination of both led to significant percentages of apoptotic cells of about 51, 27 and 71, respectively (Fig. 3A).

The effect of each PI on the percentages of apoptotic cells was evaluated in ACH-2 cells treated by the combination TNFα + TSA. IND, from 0.1 to 20 μM did not disrupt the apoptotic process triggered by HIV-1 reactivation after TNFα + TSA treatment (Fig. 3A). The percentages of apoptotic cells were similarly not affected by the other PIs at a concentration of 10 μM (Fig. 3B). A small increase in apoptotic cell numbers was even observed for SQV, NFV and LPV above 10 μM (data not shown). The failure of each PI to inhibit apoptosis of ACH-2 cells after HIV-1 reactivation by the combined treatment TNFα + TSA was confirmed by determining the percentages of caspase 3 positive cells earlier than 16 h (Fig. 3C). The effect of a pretreatment for 6 h with each PI was also tested. Fig. 3C shows the preincubation for 6 h with NFV (10 μM) did not decrease but instead slightly increased the percentages of apoptotic cells after 6, 9 and 12 h.

Since several PIs are known to inhibit various apoptotic pathways [25–30,39–41], we wanted to check that our experimental conditions allowed PIs to exert their protective effect. We tested the effect of NFV known to inhibit camptothecin (CPT)-mediated apoptosis [41]. Jurkat T cells were preincubated for 6 h with NFV 10 μM before addition of CPT. The percentages of apoptotic cells were measured after 16 h either by annexin V and propidium iodide (PI) or active caspase 3 staining using flow cytometry. As illustrated in Fig. 3D and as previously described [42], CPT induced apoptosis in Jurkat T cells. In our experiment, the percentages of apoptotic cells were different according to the staining method. CPT induced about 30% of Annexin V^+/PI^- cells, 45% of Annexin V^+/PI^+ cells and approximately 65% of caspase-3^+ cells. As previously described, NFV induced a significant decrease (from about 30

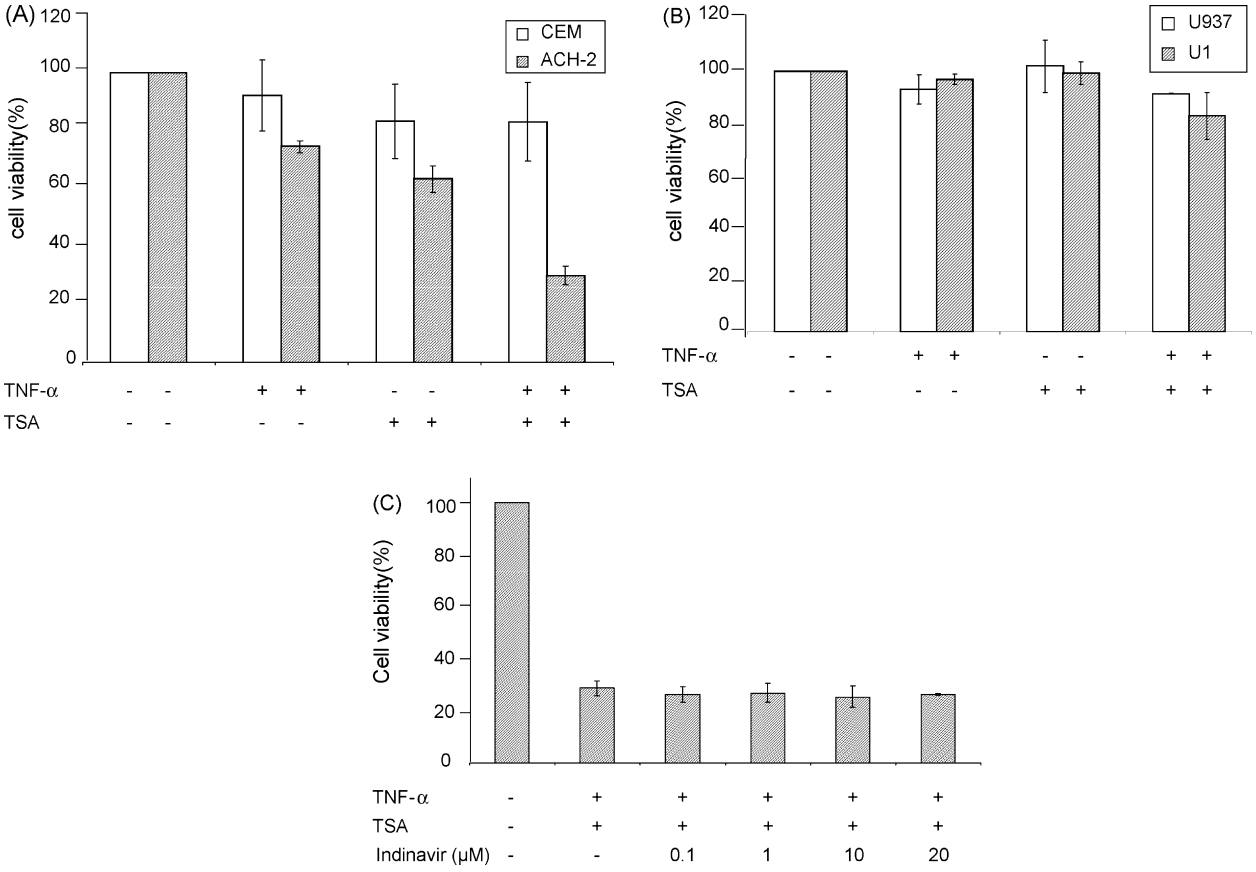

Fig. 2 – Cell viability after the combined treatment TNF-α + TSA in the presence or not of Indinavir. Infected (ACH-2) or not infected (CEM) T lymphocytes (A) as well as infected (U1) or not infected (U937) monocytes (B) were treated or not with TNFα (100 U/mL) or TSA (450 nM) or with the combination TNFα + TSA. The effect of IND was studied on the viability of ACH-2 cells after TNFα + TSA treatment (C). The percentages of cell viability were determined by WST-1 test after 24 h. Assays were performed in triplicate. The error bars show the standard errors of the mean.

to 11%) of the percentages of Annexin V^+/PI^- cells. Nevertheless, NFV increased to the same extent the percentages of Annexin V^+/PI^+ cells and the percentages of caspase-3^+ cells. Whereas Annexin V staining represents cells in early apoptosis, double positive Annexin V/PI and active caspase-3 determination evaluates the percentage of cells in late apoptosis. NFV decreased early apoptosis induced by CPT but had a synergistic effect on late apoptosis induced by CPT and by TNFα + TSA treatment. Altogether, these results indicate that none of the six PIs prevented late apoptosis of ACH-2 cells undergoing HIV-1 reactivation after TNFα + TSA treatment. It is also important that the protease inhibitors do not cause the apoptosis of the bystanders non infected cells to allow a good recovery of the immune system in treated patients.

3.3. *Effect of PIs on NF-κB activation induced by combined treatment TNFα + TSA*

The aim of the therapeutic approach using HDACi in the presence of HAART is to stimulate viral transcription in latently infected cells in order to purge these latent reservoirs by a direct cytopathic effect caused by the virus or by the immune system. The transcriptional activation by a combined treatment TNFα + TSA in latent provirus-containing cells requires the LTR 5′ transactivation by the transcription factor NF-κB. In unstimulated cells, NF-κB is sequestered in the cytoplasm in an inactive form through interaction with IκBα. Upon activation of NF-κB by TNFα, IκBα is rapidly phosphorylated by the IκB kinase (IKK) complex, ubiquitinated and degraded by the 26S proteasome. The released NF-κB then translocates to the nucleus, where it can activate transcription from the HIV-1 LTR.

Several studies have demonstrated an inhibition of the human proteasome by PI [22–24] suggesting that some PIs could affect the NF-κB activation and HIV-1 transcription following combined treatment with TNFα + TSA and could not fit within the scope of the HDACi concept.

Each PI was evaluated for its ability to interfere with the NF-κB activation in ACH-2 and U1 cells in response to the combined treatment TNFα + TSA. NF-κB activation was estimated by two ways: first by determining the DNA-binding activity of nuclear extracts on a probe carrying the LTR HIV-1 κB site (electromobility shift assay:EMSA) and secondly by following the proteolysis of cytoplasmic IκBα (immunoblot anti-IκBα). Fig. 4A shows the effect of IND (0.1–20 μM) on NF-κB nuclear translocation and IκBα proteolysis following the combined treatment TNFα + TSA of ACH-2 cells. As expected, TNFα alone unlike TSA or IND, induced a significant NF-κB

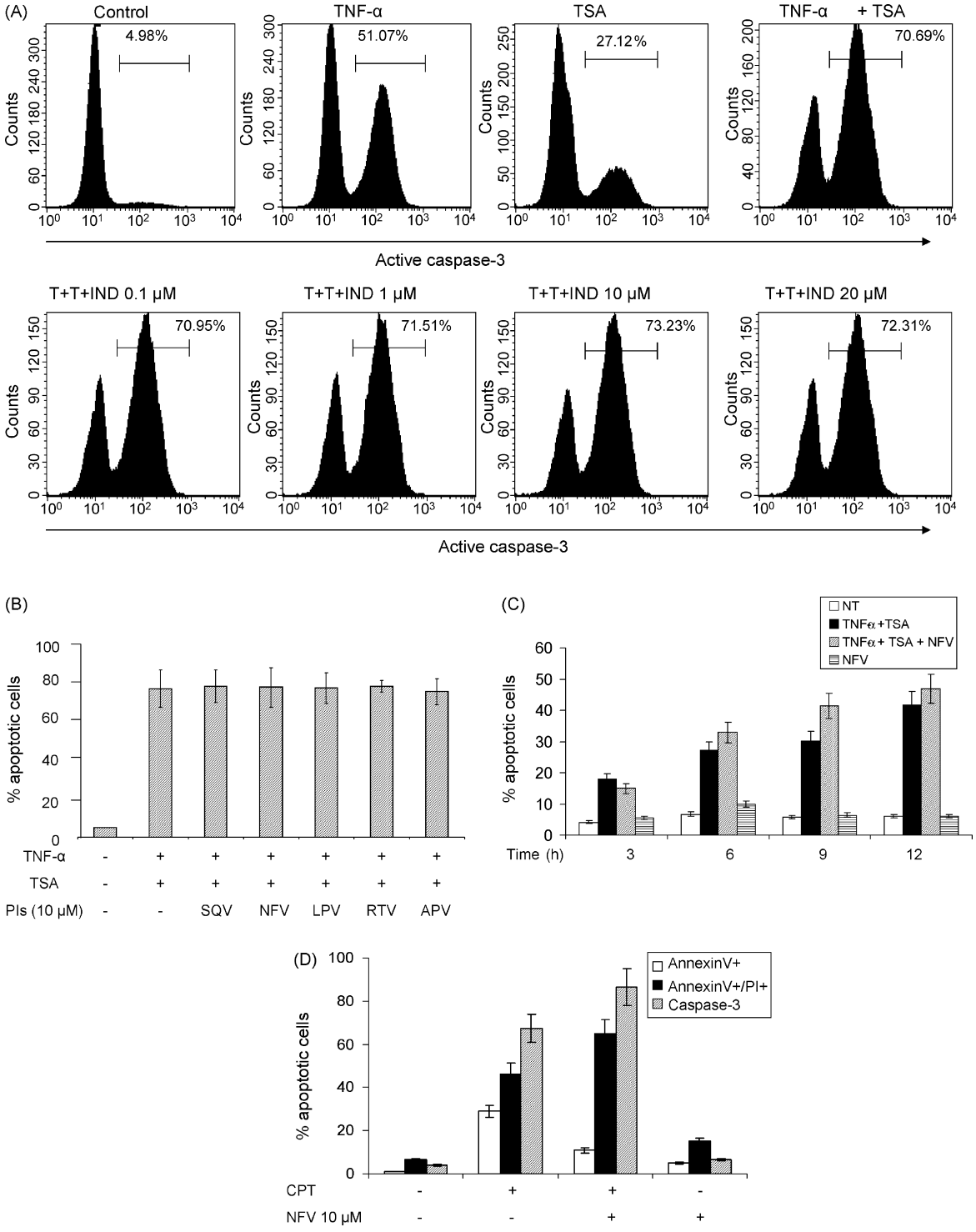

Fig. 3 – Effect of PIs on the apoptosis of ACH-2 cells induced by TNF-α + TSA treatment. (A) Effect of Indinavir at various concentrations. ACH-2 cells were treated or not for 24 h with TNFα (100 U/mL) or TSA (450 nM) or TNFα + TSA in the presence or not of IND (0.1, 1, 10 and 20 μM). Apoptotic cells were detected by active caspase-3 staining. (B) Effect of SQV, NFV, LPV, RTV and APV. ACH-2 cells were treated or not for 24 h with TNFα + TSA in the presence or not of SQV, NFV, LPV, RTV or APV. Apoptotic cells were detected by active caspase-3 staining. (C) Effect of NFV on apoptosis monitored at shorter times. ACH-2 cells, pretreated or not with NFV (10 μM) for 6 h, were stimulated or not with the combination TNF-α/TSA during 3, 6, 9 and 12 h. Apoptotic cells were detected by active caspase-3 staining using flow cytometry. (D) Effect of NFV on CPT-induced apoptosis monitored by annexin V/PI or caspase-3 staining. Jurkat T cells, pretreated or not with NFV (10 μM) for 6 h, were stimulated or not for 16 h with CPT (1 μM). Apoptotic cells were detected by AnnexinV/PI or caspase-3 staining. In all experiments, values represent the means of two independent experiments. The error bars show the standard errors of the mean.

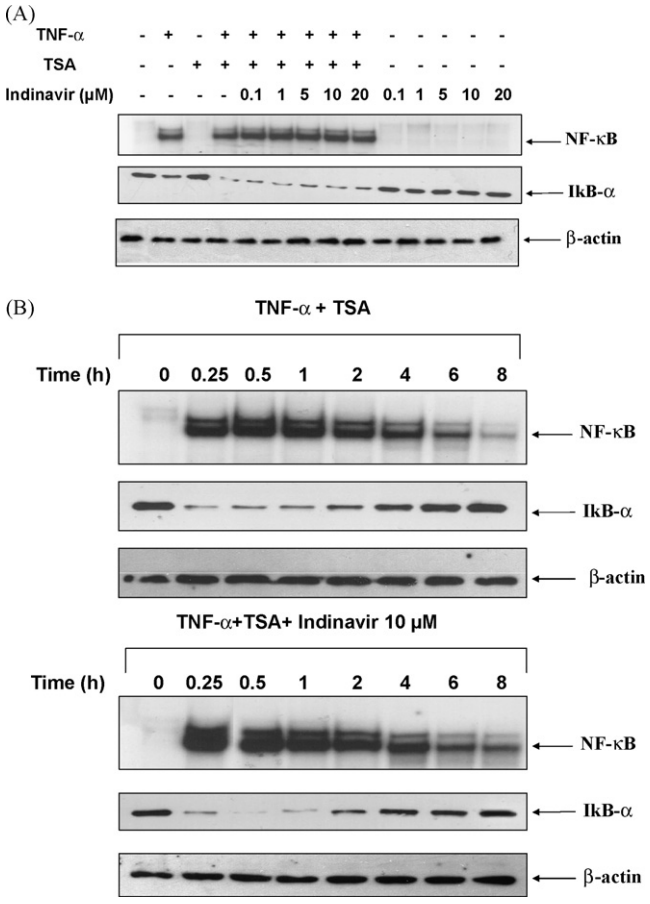

Fig. 4 – Effect of Indinavir on NF-κB nuclear translocation and on IκB-α degradation following treatment with TNFα + TSA. (A) ACH-2 cells were treated or not with TNFα (100 U/mL) or TSA (450 nM) or TNFα + TSA for 30 min in the presence or not of various concentrations of IND. (B) ACH-2 cells were treated or not with TNFα plus TSA in the presence or not of Indinavir (10 μM) for various times (0–8 h). NF-κB nuclear translocation was estimated by EMSA using a ^{32}P-labeled probe carrying the HIV-1 LTR κB site. IκBα proteolysis was determined by Western blot analysis of cytoplasmic extracts using an anti-IκBα antibody. A western blot analysis of the same cytoplasmic extracts with an anti-β-actin antibody was used as a loading control.

activation and IκBα degradation after 30 min. The addition of TSA did not further increase NF-κB activation in ACH-2 cells. No significant effect of IND, whatever its concentration, was observed on NF-κB translocation and IκBα degradation after TNFα + TSA stimulation for 30 min. NFV, LPV, and RTV tested in the same concentration range neither interfered with NF-κB activation determined in ACH-2 cells treated for 30 min with TNFα + TSA (data not shown). However, in the same conditions, a small decrease both in NF-κB translocation and IκBα proteolysis could be observed with SQV at 20 μM (data not shown). In U1 cells (data not shown), similar results were obtained.

Next, we determined whether PI could affect the kinetics of NF-κB activation following TNFα + TSA treatment. As illustrated in Fig. 4B, translocation of NF-κB and IκBα proteolysis could be already observed after 15 min. IκBα neosynthesis associated with a decrease of the nuclear NF-κB levels began after 60 min. The addition of IND at a concentration of 10 μM did neither accelerate nor delay NF-κB translocation and IκBα degradation. SQV, NFV, LPV, RTV and APV tested at the same concentration neither interfered with the kinetics of NF-κB activation in ACH-2 cells treated by TNF-α + TSA (data not shown). Similar results were observed in monocytes (data not shown).

3.4. *Effect of PIs on HIV-1 provirus transcription induced by the combined treatment TNF-α + TSA*

Neither of the PIs, used at pharmacological concentrations, inhibited IκBα proteolysis by proteasome 26S and the following NF-κB translocation in TNFα + TSA-treated ACH-2 and U1 cells. Since PIs could affect other transcription factors and coactivators involved in viral transcription and because they have been shown able to exert side effects on cellular turnover and metabolism [43–45], we studied their effect on HIV-1 transcriptional activation by combined treatment TNFα + TSA in both latently infected cells. The viral mRNAs expression was followed by semi-quantitative RT-PCR using primers targeting HIV-1 LTR. The time course of HIV-1 mRNAs production in response to the combined treatment TNFα + TSA in the presence or not of IND is shown for ACH-2 cells in Fig. 5A. The combined treatment with TNFα + TSA led to a significant increase in viral mRNA levels up to 17- and 20-fold, at 4 and 6 h after stimulation, respectively. In the presence of IND, a further accumulation of viral mRNA was noticed at 4 and 6 h after stimulation (21- and 27-fold, respectively). The other PIs did not significantly affect the viral transcription except for the LPV which also had a synergistic effect on viral mRNA synthesis at 4 and 6 h after treatment (data not shown). Interestingly, both IND and LPV, unlike the other PIs, had the same synergistic effect on HIV-1 transcription in monocytes U1 (Fig. 5B). Therefore, none of the PIs tested in this work significantly abrogated viral transcription induced by TNFα + TSA.

4. Discussion

The persistance of latently HIV-infected cellular reservoirs harboring replication competent proviruses, despite prolonged HAART regimens, represents a major hurdle to virus eradication. Several approaches to purge cellular reservoirs have been investigated. One of them consists to deliberately force HIV-1 gene expression by HDACi administration in the presence of HAART to prevent the spreading of the infection by the newly synthesized viruses [9]. However, some PIs used in HAART have been reported to inhibit the proteasome [22–24] or to exert antiapoptotic effects [25–30], which could interfere with the purge of the cellular reservoirs. In this work, we examined the effect of six PIs currently used in HAART (SQV, IND, NFV, LPV, RTV, APV) on mature virus release, HIV-1 transcription and NF-κB activation as well as host cell apoptosis in two HIV-1 latency models after combined treatment TNFα + TSA. We have shown that all the tested

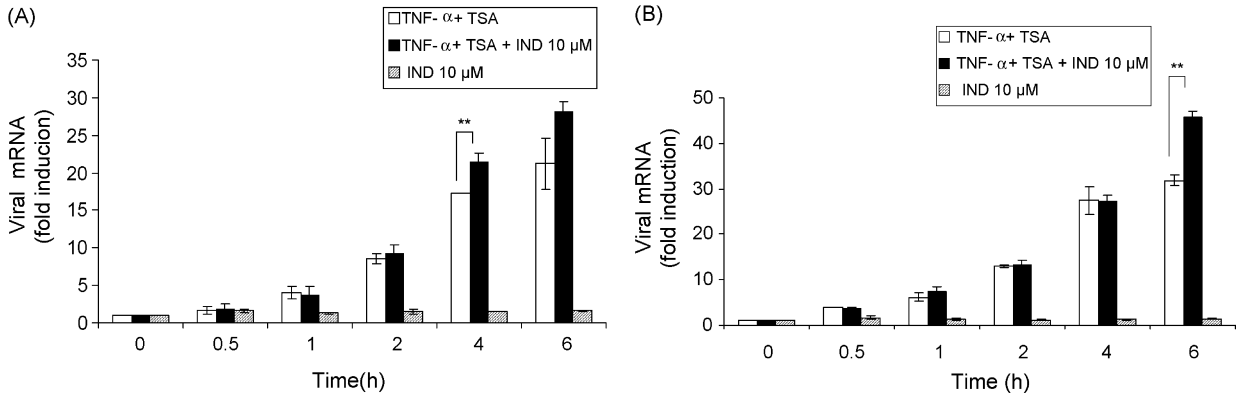

Fig. 5 – Effect of Indinavir on viral mRNAs expression following combined treatment TNF-α + TSA. Total RNA was extracted from ACH-2 (A) or U1 cells (B) treated or not with TNFα plus TSA in the presence or not of IND (10 μM) for various times (0–6 h). After reverse transcription, a real time PCR was performed with primers targeting HIV-1 LTR. Assay was performed in triplicate. The error bars show the standard errors of the mean. $^{**}p < 0.05$.

PIs inhibited mature virus release but they did not prevent IκBα degradation, NF-κB nuclear translocation, viral gene transcription and cell apoptosis caused by virus reactivation. Therefore, these data demonstrated that the purge of viral latent reservoirs by HDACis in the presence of PIs could be envisioned.

In the HIV-1 latently infected T lymphoid cell line ACH-2, the combined treatment TNFα + TSA induced a significant loss of viability resulting from apoptosis. The percentage of apoptotic cells correlated with the extent of virus reactivation estimated by the p24 levels in cell supernatants or by viral mRNA levels. Since the treatment of the uninfected parental cell line (CEM) by TNFα or TSA used separately did not significantly affect cellular viability and since the combination of both agents did not further decrease the viability percentages, our results suggest that ACH-2 apoptosis arises at least partially from virus reactivation. These observations are in agreement with several reports demonstrating that HIV-1 infection caused cell death by several mechanisms in both infected and non-infected cells [27] and that soluble HIV-1 products such as the accessory proteins Tat, Vpr, the protease or the gp120 participated to apoptosis [46].

Interestingly, the HIV-1 latently infected monocytic cell line U1 was not sensitive to apoptosis following combined treatment with TNFα + TSA, while the cells also produced significant amounts of virus in response to this treatment. This observation has been previously reported by others demonstrating that HIV-1 chronically infected cells belonging to the lymphocytic lineage unlike cells from monocytic origin showed an increased propensity to undergo apoptosis [47]. These authors also observed a very high expression level of the death receptor CD95 (APO-1/Fas) in ACH-2 cells while U1 monocytes exhibited a consistent lower CD95 expression. Others have recently shown that HIV-1 Nef protects human monocyte-derived macrophages from HIV-1 induced apoptosis [48]. Altogether, these results would explain why human macrophages, unlike lymphocytes, survive HIV infection and represent a cellular reservoir *in vivo* resisting HAART regimens. Accordingly, this resistance of monocytic cells represents a hurdle for an efficient elimination of the viral reservoirs by HDAC inhibitors-based therapy although these HIV-1 infected monocytes/macrophages could still be targeted by cytotoxic T cells directed against viral antigens. However, it would be worth finding a strategy to eliminate this potential HIV-1 reservoir.

In the majority of patients, plasma virus burdens decline substantially within weeks of PI therapy and CD4+ T-cell counts increase. Intriguingly, CD4+ T-cell increases can occur before viral titers drop, indicating that the antiviral and immune reconstitutive effects are not necessarily linked [29,30]. One hypothesis raised from these *in vivo* observations is the possibility for PIs to inhibit apoptosis in infected or non-infected cells independently of their effect on HIV-1 protease. Consequently, several PIs used at therapeutically relevant concentrations were shown to inhibit apoptosis induced by various stimuli such as TNFα, CD95, CPT, ...[26]. IND, for example, exerts its anti-apoptotic effects by directly inhibiting calpain [39]. RTV has an inhibitory effect on the expression of caspases and Fas-R [40]. NFV prevents mitochondrially driven apoptosis by inhibiting the opening of the permeability transition pore complex (PTPC) [41]. In the present study, however, neither of the six PIs used at pharmacological concentrations inhibited apoptosis in TNFα + TSA-treated ACH-2 cells. Some PIs such as NFV even showed pro-apoptotic effects. Such pro-apoptotic effects have already been described for SQV [49]. Our results obtained with NFV were unexpected since apoptosis in HIV-1 infection usually involves mitochondria and NFV is known to inhibit mitochondrially driven apoptosis [41]. The origin of this discordance between our results and those reported in the literature could be the use of different methods of monitoring apoptosis. In the majority of the works devoted to study the effects of PIs on apoptosis, authors evaluated the percentage of apoptotic cells by counting Annexin V^+/PI^- cells. This population represents cells in early apoptosis. In our work, we chose the staining of active caspase-3 which detects cells in late apoptosis. When we measured the percentages of apoptotic Jurkat cells following CPT treatment in the presence or absence of PIs, different results were obtained according to the method used. As reported in the literature, NFV induced a decrease in the

percentages of Annexin V^+/PI^- cells. However, when apoptosis was estimated by active caspase-3 staining, NFV showed by itself a pro-apoptotic effect. Another hypothesis explaining the failure of PIs to exert an anti-apoptotic effect would be the involvement of an apoptotic pathway bypassing the mitochondria in TNFα + TSA-treated ACH-2 cells. Such mitochondria-independent pathway leading to caspase-3 activation has been already described and involves caspase-8 [41].

Another side effect of the PIs that we discussed in our work is their potential interference with NF-κB activation. None of the six PIs at pharmacological concentrations significantly interfered with IκBα proteolysis by the proteasome 26S and with NF-κB translocation in the nucleus following TNFα + TSA treatment in the two post-integration latency model cell lines used. SQV has been previously reported to inhibit the 20S and 26S proteasomes [22]. Similarly, RTV could inhibit IκBα proteolysis by the 26S proteasome following induction with LPS in B lymphocytes [24]. However, these experiments with SQV and RTV have been carried out in the presence of very high PI concentrations (50–100 μM) which cannot be reached in the blood of patients in HAART regimens.

In order to confirm that PIs do not interfere with HIV-1 transcription, we examined their effect on the viral mRNA expression in response to the combined treatment TNFα + TSA in both latency models. Neither of the PIs used at pharmacological concentrations inhibited HIV-1 transcription in ACH-2 and U1 cells following combined treatment. Surprisingly, the addition of IND and LPV led to a further increase in the viral mRNA levels.

An explanation to these effects could be proposed by considering the enhancement of HIV-1 transcription by SerpinB2, a serine protease inhibitor [50]. SerpinB2 has recently been shown to have an intracellular role as a retinoblastoma protein (Rb)-binding protein that inhibits Rb degradation [51]. The SerpinB2-mediated elevation of Rb protein levels appeared to be responsible for enhancing HIV-1 transcription. The pRb is known to be degraded by the proteasomal pathway [52] and by other proteinases, such as calpaïn [53] and caspases [54]. Some PIs, such as IND used at therapeutically relevant concentrations, have been reported to inhibit calpain [39]. Increased HIV-1 transcription observed in the presence of IND could result from the inhibition of Rb cleavage by calpain leading to increased Rb levels. Since Rb is a ubiquitous regulator of transcription involved in many cellular activities including cell cycle control, apoptosis, differentiation and tumor suppression, the interference of some PIs with Rb-regulated transcription could explain some side effects of PIs observed in HAART-treated patients [43–45].

The PIs used in this work at pharmacological concentrations efficiently inhibited the release of mature viral particles but did not interfere with HIV-1 transcription and host cell apoptosis induced by combined treatment TNFα + TSA in ACH-2 and U1 cells. Altogether these results are encouraging and suggest that PIs would be compatible with a therapy designed to purge HIV-1 reservoirs by HDACi administration. Clinical studies on HDACi-treated patients in the presence of HAART will be required to demonstrate a consistent decrease of the pool of latently infected cells, preventing viral rebound following cessation of therapy.

Acknowledgements

This work was supported by a grant from the Walloon region (contract 215361). C.V. is a Ph.D. student supported by the Walloon region. S. L-P is Research Associate, J.P. and C.V.L. are Research Director of the FNRS (Brussels, Belgium).

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