Arch Virol (2008) 153:561–565
DOI 10.1007/s00705-007-0023-4

BRIEF REPORT

Two immunomodulators, curcumin and sulfasalazine, enhance IDV antiretroviral activity in HIV-1 persistently infected cells

D. A. Riva · P. N. Fernández-Larrosa · G. L. Dolcini · L. A. Martínez-Peralta · F. C. Coulombié · S. E. Mersich

Received: 19 February 2007 / Accepted: 17 December 2007 / Published online: 4 January 2008

Abstract Since the appearance of resistance to antiretroviral treatment is unavoidable, the host cell's transcription factor NF-kappaB is a novel HIV target. The goal of this study was to characterize the effect of two immunomodulators, curcumin (Cur) and sulfasalazine (Sul), with a protease inhibitor, indinavir (IDV), on HIV-1 persistently infected CD4+ T-cells. Viral p24 antigen production, viral infectivity (tested on MAGI cells) and viral relative infectivity (viral infectivity/p24) were analysed. When used alone, both immunomodulators were able to reduce viral infectivity. When in combination, both 10 μM IDV plus 10 μM Cur and 10 μM IDV plus 250 μM Sul showed a significant reduction in viral infectivity and viral relative infectivity when compared to the reduction produced by IDV alone. Thus, the use of immunomodulators with IDV could help to reduce HIV-1 production in persistently infected cells.

Two major obstacles to the elimination of HIV-1 infection are latently infected cells and cells harbouring replicating HIV-1 that can persist following antiviral therapy [1]. Also, even if highly active antiretroviral therapy (HAART) greatly improves clinical outcome for HIV-1-infected patients, failures due to viral mutation call for new drugs that attack novel HIV targets. One such target is the host cell's transcription factor nuclear factor-kappa B (NF-kappaB), which plays an important role in the regulation of HIV-1 gene expression [18].

HIV protease inhibitors (PIs) have been considered one of the most significant advances in controlling viral infection. Indinavir (IDV) is a potent PI that, when used in combination with reverse transcriptase (RT) inhibitors, has been shown to effectively suppress viral replication, reduce morbidity and prolong survival in HIV-infected patients. However, concern is growing about complications caused by prolonged use of PIs. The effects of five PIs on vasomotor function, nitric oxide synthase expression and oxidative stress in porcine coronary arteries have already been studied [9].

Curcumin (Cur), 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione; diferuloylmethane, is a major active component of food-flavouring turmeric (*Curcuma longa*). It has been shown to be non-toxic, to have antioxidant activity and to inhibit such mediators of inflammation as NF-kappaB, cyclooxygenase-2, lipooxygenase and inducible nitric oxide synthase [2].

Another potent and specific inhibitor of the transcription factor NF-kappaB with many immunomodulatory actions is sulfasalazine (Sul), 5-[4-(2-Pyridylsulfamoyl)phenylazo] salicylic acid, a sulfone analogue [25]. Also, sulfone derivatives have been shown to inhibit HIV-1 RT [7] and to down-modulate CD4 receptor [24].

In the present study, we characterized the action of two immunomodulators (Cur and Sul) on HIV-1 persistently infected CD4+ T-cells as a model for HIV cell reservoirs, in the presence of a representative PI such as IDV.

D. A. Riva (✉) · F. C. Coulombié · S. E. Mersich
Laboratory of Virology, Department of Biochemistry, School of Science, University of Buenos Aires, Int. Güiraldes 2160, Pab2 P4, Ciudad Universitaria, C1428EGA, Buenos Aires, Argentina
e-mail: diegor@qb.fcen.uba.ar

P. N. Fernández-Larrosa · G. L. Dolcini · L. A. Martínez-Peralta
National Reference Center for AIDS, Department of Microbiology, School of Medicine, University of Buenos Aires, Buenos Aires, Argentina

Non-infected H9 cells and HIV-infected H9/HTLVIIIB cells (persistently infected with HIVHXB2 strain: H9+) were cultured in complete RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin, at 37°C in a humidified atmosphere (5% CO_2 in air). Cells were collected during the log phase of growth, when cell viability was over 95%, and immediately used for the experiments described below. Multinuclear Activation of Galactosidase Indicator (MAGI) cells were kept in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum, G418 and hygromycin. H9, H9+ and MAGI cell lines were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH.

IDV (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH), Sul and Cur (Sigma Chemical Co. St. Louis, MO) were dissolved in DMSO, so that the final concentration of DMSO in the cell culture medium never exceeded 0.1%. H9 and H9+ cells were collected as described above, resuspended at 10^5 cells per ml in RPMI 1640, 5% fetal calf serum and 2 mM L-glutamine containing the test compound at the appropriate concentration. After 24 h of incubation, H9 and H9+ cells were centrifuged and cytotoxicity assays were performed. H9+ cell supernatants (SN) from the untreated and drug-treated cultures were used for virus production assays.

To evaluate the effect of each individual compound on cell viability, the MTT assay was performed as described elsewhere [10]. The absorption at 640 nm was measured and compaired with control replicates (no agent added) (Table 1). Interestingly, the CC50 of IDV in H9 cells was slightly higher than its published activity of 198 ± 18 μM [11]. The CC50 and CC20 of Cur were similar for HIV-infected (H9+) and non-infected (H9) cell lines. In contrast, the CC50 and CC20 of both IDV and Sul were significantly different ($P < 0.05$) for HIV-infected and non-infected cells. In this report, tests were done in the presence of non-cytotoxic doses (concentrations lower than the CC20) of each compound.

To determine the effect of the different treatments on virus production, H9+ cells were removed by centrifugation and the SN was analysed for both p24 antigen and infectivity. Viral p24 antigen was measured by enzyme-linked immunosorbent assay (ELISA) (Vironostika, Biomerieux). Results were expressed as percentage of p24 in the presence of the test compound with respect to the p24 level in untreated cells. Control untreated cells were set as 100% and represented 57.0 ± 1.6 ng p24/10^6 H9+ cells.

Infectious-virus titrations of the SN were performed with MAGI cells as already described [16]. The CD4-LTR/B-gal indicator cells were infected with the SN of H9+ treated with the different agents or a combination of them.

Table 1 Observed cell toxicity of the agents. H9 and H9+ cells were treated in the presence of different concentrations of the agents

	CC50 (μM)		CC20 (μM)	
Agent	H9	H9+	H9	H9+
IDV	573 ± 169[a]	1,289 ± 180[a]	229 ± 69[a]	516 ± 129[a]
Cur	76 ± 9[b]	67 ± 8[b]	30 ± 6[b]	27 ± 5[b]
Sul	742 ± 75[a]	1,101 ± 109[a]	373 ± 49[a]	692 ± 4[a]

After 24 h, cell viability was determined by MTT as indicated in Materials and methods. Results are expressed as mean ± SD of three independent experiments. CC50 and CC20 represent the concentration of the agent (μM) which affected cell viability by 50% or 20%, respectively

[a] Significant differences between H9 and H9+ ($P < 0.05$)

[b] No significant differences between H9 and H9+ ($P > 0.1$)

Blue cells were counted under a microscope at a magnification of 100× and results were expressed as percentage of blue cells in the presence of the test compound with respect to blue cells in untreated cells. Control untreated cells were set as 100% and represented 2,600 ± 120 blue cells/10^6 H9+ cells. The MAGI assay monitors a subset of steps in the viral replication cycle from initial fusion to gene expression. Infection of MAGI cells with HIV-1 provides a source of newly synthesized Tat, which activates a promoter that allows the β-galactosidase protein to accumulate [22]. Agents used in this paper did not interfere with a control infectivity assay. When 50 μM IDV, 250 μM Sul or 10 μM Cur were added to the SN of H9+ untreated cells just before MAGI infection, the number of blue cells was not affected. In contrast, other components of HAART treatment, such as any RT inhibitor, interfere with the MAGI assay and were not included in the present report.

Also, a relative infectivity ratio was calculated as the percentage blue cells/percentage p24 antigen. This ratio has been proposed as an indication of the relative HIV infectivity of the viral progeny in different cell lines [4].

Data represent means ± SD of three independent experiments for each treatment. Data from the different treatments were compared using paired Student's t-test (two-tailed) and significance was considered at $P < 0.05$.

When both immunomodulators were analysed, a significant reduction in p24 (44%) and HIV infectivity (75%) was observed only at Sul concentrations as high as 250 μM (Fig. 1a). In contrast, no statistically significant differences in p24 antigen production or infectivity were observed between 0.1 and 1 μM Cur (Fig. 1b), while 10 μM Cur reduced p24 levels by 6% and viral infectivity by 36% (Fig. 1b). When used alone, IDV treatment on H9+ cells reduced both p24 production and viral infectivity in a dose-dependent manner: 50 μM IDV reduced p24 levels by 62% and blue cells by 92%, while 10 μM IDV reduced p24 levels by 44% and blue cells by 84% (Fig. 2a,b).

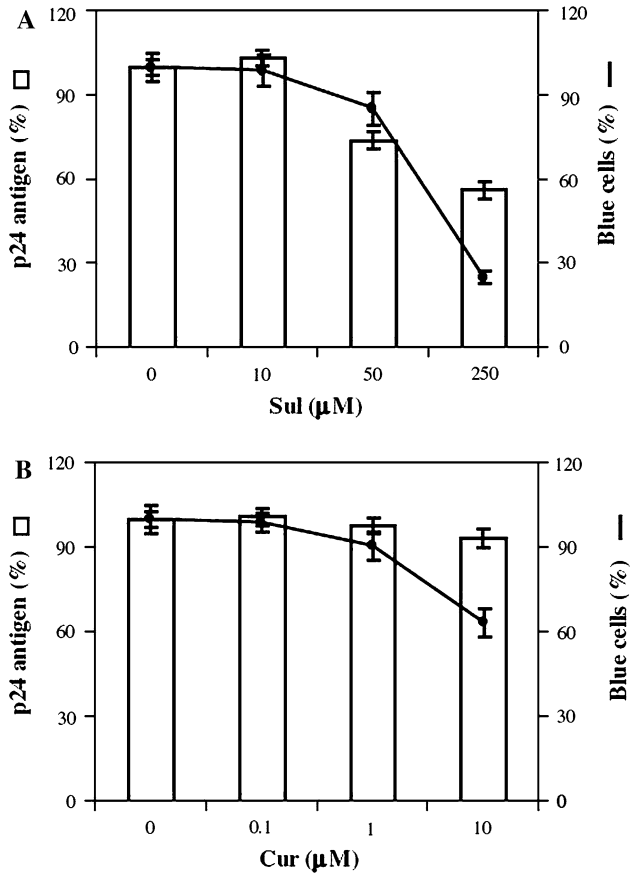

Fig. 1 Effect of the immunomodulators on viral infectivity and p24 levels. H9+ cells were incubated with various concentrations of (a) Sul or (b) Cur. After 24 h, aliquots of the SN were collected for p24 antigen determination and virus infectivity determination (*blue cells*). Data obtained from SN of control untreated H9+ cells were set as 100%

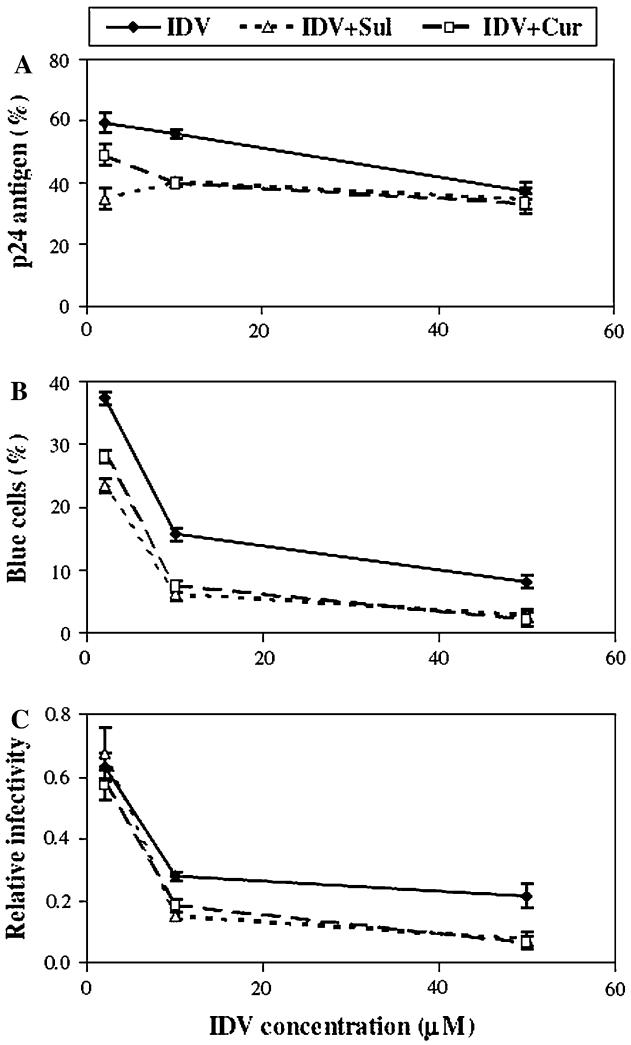

Fig. 2 Effect of Sul and Cur in combination with different concentrations of IDV. H9+ cells were treated with different concentrations of IDV plus 250 μM Sul or 10 μM Cur. After 24 h, aliquots of the supernatant were collected for **a** p24 antigen determination and **b** virus infectivity determination (*blue cells*). **c** Viral relative infectivity was calculated for each cell treatment (% blue cells/% p24 antigen). The ratio for untreated cell SN was set as 1

To exclude the possibility that the compounds enhance each other's cytotoxic effect, cellular viability was monitored as already described. No combination analysed had any effect on cell viability (data not shown). The combinations of IDV + Cur and IDV + Sul produced a reduction in p24 levels in H9+ cell SN that could be seen when the IDV concentration ranged from 2 to 10 μM (Fig. 2a).

When H9+ cells were incubated in the presence of the combination of 10 μM Cur plus various concentrations of IDV and examined for infectivity, the percentage of blue cells in cell SN incubated with 2, 10 and 50 μM IDV were 28, 7 and 2%, respectively (Fig. 2b). Similar results were observed when cells were incubated in the presence of 250 μM Sul plus various concentrations of IDV and examined for infectivity (Fig. 2b).

When H9+ cells were treated with IDV alone, the relative infectivity was reduced from 0.63 to 0.22 in the 2- to 50-μM IDV range (Fig. 2c). The reduction in viral relative infectivity of the combination of IDV+Cur could only be seen when the IDV concentration ranged from 10 to 50 μM (Fig. 2c). A high reduction in viral relative infectivity was observed when H9+ cells were incubated with 10 μM Cur in the presence of 50 μM IDV (0.07) with respect to IDV treatment alone (0.22) (Fig. 2c). Similarly, for the IDV + Sul combination, a reduction in HIV-1 relative infectivity was observed when IDV was included in a range of concentration from 10 to 50 μM (Fig. 2c). We observed a reduction in viral relative infectivity when 50 μM IDV plus 250 μM Sul was present in H9+ cell SN (0.08) when compared to 50 μM IDV treatment alone (0.22). We also showed that 10 μM IDV plus 250 μM Sul exhibited a reduction in relative infectivity (0.15) when compared to 10 μM IDV treatment alone (0.28) (Fig. 2c). On the other hand, the combination of Sul or Cur with a lower

concentration of IDV (2 μM) had no effect on viral relative infectivity (Fig. 2c).

Combined therapy, reverse transcriptase and protease inhibitors have presented the problem of resistance due to mutations in the virus. In turn, targeting NF-kappaB, which is a normal part of the human T-4 cell, is not subject to mutations [18]. Also, a possible association between HIV PIs and collateral effects including hyperlipidemia, lipodystrophy, and insulin resistance have been proposed [6].

Although sulfone therapy have been extensively applied [12,17], analogs of Sul with improved NF-kappaB-inhibiting activity have been recently synthesized [14]. On the other hand, Cur has been proposed as an agent for chemoprevention of cancer [20], but controversial data on its anti-HIV activity have been obtained [13,15].

In this study, we observed a differential cytotoxicity of IDV and Sul on HIV-1 persistently infected CD4+ T-cells in comparison with non-infected cells (Table 1). Similarly, different sensitivity to cell death after exposure to many stimuli has already been described in cells of lymphocytic origin chronically infected with HIV-1 [19,26]. In our hands, differences in IDV and Sul cytotoxicity observed in H9+ with respect to H9 could be related to an uncharacterised sequence of events that diminished cell death in persistently infected cells.

Chronically infected cells are usually used to analyse mechanisms of pathogenesis and antiviral activities [5,26]. Persistently infected H9-derived cell lines produce abnormal particles with low infectivity [4]. Also, inhibitors of HIV-1 protease activity give rise to immature viral progeny, since protease activation is required for gag and pol processing leading to virus maturation [23]. In our hands, discrepancy between the percentage of p24 and blue cells could be related to these phenomena (Figs. 1,2). In this context, the study of nucleic-acid-based HIV-1 viral load would be unsuitable. Thus, determining virus infectivity seems to be more important than determining p24 to characterize HIV production in H9+ cells.

The drugs studied against NF-kappaB fall mainly into three categories: IkappaB phosphorylation and degradation inhibitors (such as Sul and Cur), antioxidants (such as Cur) and NF-kappaB DNA-binding inhibitors. Cur has a potential application in the cardiovascular system since oxidative stress is an important mechanism for lesion formation. It has been demonstrated that 10 μM Cur blocks ritonavir-induced overproduction of reactive oxygen species in porcine coronary arteries [8]. Although Cur modulates an antioxidant response in H9+ cells by affecting superoxide dismutase activity (data not shown), we cannot conclude that curcumiṅs antioxidant activity is the major factor involved. Previous studies have demonstrated that Cur also suppresses constitutive IkappaB-alpha phosphorylation and down-regulates NF-kappaB in different cell lines [3,21]. Thus, the reduction in viral infectivity and relative infectivity observed with Cur in the presence of IDV with respect to IDV treatment alone (Fig. 2b,c) could be related to both the modulation of NF-kappaB and the regulation of HIV-1 gene expression [18]. Similar effects were observed when Sul was present in H9+ cell SN. In conclusion, the use of Cur or Sul in combination with IDV could reduce infectivity in viral progeny.

Further investigations including different periods of incubation and other cell reservoir models are to be done. Also, it would be interesting to study new small molecule inhibitors of NF-kappaB [14].

The results presented in this study suggest that both immunomodulators, Sul and Cur, interfere with viral production. Since the appearance of resistance to antiretroviral treatment is unavoidable, the use of an immunomodulator with a different mechanism of action could help to reduce the persisting replication observed in the presence of antiviral therapy and the selection of resistant HIV-1 variants.

Acknowledgements H9, H9/HTLV-IIIB, MAGI cells and indinavir were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. This study was supported by grants X-227, UBA, Argentina and PICT 05-11734, ANPyCT, Argentina.

References

1. Belmonte L, Bare P, de Bracco MME, Ruibal-Ares BH (2003) Reservoirs of HIV replication after successful combined antiretroviral treatment. Curr Med Chem 10:303–312
2. Bengmark S (2006) Curcumin, an atoxic antioxidant and natural NFkappaB, cyclooxygenase-2, lipooxygenase, and inducible nitric oxide synthase inhibitor: a shield against acute and chronic diseases. J Parenter Enteral Nutr 30:45–51
3. Bharti AC, Donato N, Singh S, Aggarwal BB (2003) Curcumin (diferuloylmethane) down-regulates the constitutive activation of nuclear factor-kappa B and IkappaBalpha kinase in human multiple myeloma cells, leading to suppression of proliferation and induction of apoptosis. Blood 101:1053–1062
4. Bouyac M, Rey F, Nascimbeni M, Courcoul M, Sire J, Blanc D, Clavel F, Vigne R, Spire B (1997) Phenotypically Vif- human immunodeficiency virus type 1 is produced by chronically infected restrictive cells. J Virol 71:2473–2477
5. Briggs CJ, Ott DE, Coren LV, Oroszlan S, Tozser J (1999) Comparison of the effect of FK506 and cyclosporin A on virus production in H9 cells chronically and newly infected by HIV-1. Arch Virol 144:2151–2160
6. Calza L, Manfredi R, Chiodo F (2003) Hyperlipidaemia in patients with HIV-1 infection receiving highly active antiretroviral therapy: epidemiology, pathogenesis, clinical course and management. Int J Antimicrob Agents 22:89–99
7. Cancio R, Silvestri R, Ragno R, Artico M, De Martino G, La Regina G, Crespan E, Zanoli S, Hubscher U, Spadari S, Maga G (2005) High potency of indolyl aryl sulfone nonnucleoside inhibitors towards drug-resistant human immunodeficiency virus type 1 reverse transcriptase mutants is due to selective targeting

of different mechanistic forms of the enzyme. Antimicrob Agents Chemother 49:4546–4554
8. Chai H, Yan S, Lin P, Lumsden AB, Yao Q, Chen C (2005) Curcumin blocks HIV protease inhibitor ritonavir-induced vascular dysfunction in porcine coronary arteries. J Am Coll Surg 200:820–830
9. Chai H, Yang H, Yan S, Li M, Lin PH, Lumsden AB, Yao Q, Chen C (2005) Effects of 5 HIV protease inhibitors on vasomotor function and superoxide anion production in porcine coronary arteries. J Acquir Immune Defic Syndr 40:12–19
10. Denizot F, Lang R (1986) Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J Inmunol Methods 89:271–277
11. Essey RJ, McDougall BR, Robinson WE Jr (2001) Mismatched double-stranded RNA (polyI-polyC(12)U) is synergistic with multiple anti-HIV drugs and is active against drug-sensitive and drug-resistant HIV-1 in vitro. Antiviral Res 51:189–202
12. Gaffo A, Saag KG, Curtis JR (2006) Treatment of rheumatoid arthritis. Am J Health Syst Pharm 63:2451–2465
13. Gilden D, Smart T (1996) Curcumin trial finds no activity. GMHC Treat Issues 10:9
14. Habens F, Srinivasan N, Oakley F, Mann DA, Ganesan A, Packham G (2005) Novel sulfasalazine analogues with enhanced NF-kB inhibitory and apoptosis promoting activity. Apoptosis 10:481–491
15. Jordan WC, Drew CR (1996) Curcumin—a natural herb with anti-HIV activity. J Natl Med Assoc 88:333
16. Kimpton J, Emerman M (1992) Detection of replication-competent and pseudotyped human immunodeficiency virus with a sensitive cell line on the basis of activation of an integrated beta-galactosidase gene. J Virol 66:2232–2239
17. Kovacs JA, Gill VJ, Meshnick S, Masur H (2001) New insights into transmission, diagnosis, and drug treatment of Pneumocystis carinii pneumonia. JAMA 286:2450–2460
18. Pande V, Ramos MJ (2003) Nuclear factor kappa B: a potential target for anti-HIV chemotherapy. Curr Med Chem 10:1603–1615
19. Pinti M, Biswas P, Troiano L, Nasi M, Ferraresi R, Mussini C, Vecchiet J, Esposito R, Paganelli R, Cossarizza A (2003) Different sensitivity to apoptosis in cells of monocytic or lymphocytic origin chronically infected with human immunodeficiency virus type-1. Exp Biol Med (Maywood) 228:1346–1354
20. Sharma RA, Gescher AJ, Steward WP (2005) Curcumin: the story so far. Eur J Cancer 41:1955–1968
21. Strasser EM, Wessner B, Manhart N, Roth E (2005) The relationship between the anti-inflammatory effects of curcumin and cellular glutathione content in myelomonocytic cells. Biochem Pharmacol 70:552–559
22. Venkateshwar Goud T, Srinivasa Reddy N, Raghavendra Swamy N, Siva Ram T, Venkateswarlu Y (2003) Anti-HIV active petrosins from the marine sponge Petrosia similis. Biol Pharm Bull 26:1498–1501
23. Ventoso I, Navarro J, Munoz MA, Carrasco L (2005) Involvement of HIV-1 protease in virus-induced cell killing. Antiviral Res 66:47–55
24. Vermeire K, Bell TW, Choi HJ, Jin Q, Samala MF, Sodoma A, De Clercq E, Schols D (2003) The anti-HIV potency of cyclotriazadisulfonamide analogs is directly correlated with their ability to down-modulate the CD4 receptor. Mol Pharmacol 63:203–210
25. Wahl C, Liptay S, Adler G, Schmid RM (1998) Sulfasalazine: a potent and specific inhibitor of nuclear factor kappa B. J Clin Invest 101:1163–1174
26. Wang YY, Ouyang DY, Huang H, Chan H, Tam SC, Zheng YT (2005) Enhanced apoptotic action of trichosanthin in HIV-1 infected cells. Biochem Biophys Res Commun 331:1075–1080