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CADA Inhibits Human Immunodeficiency Virus and Human Herpesvirus 7 Replication by Down-modulation of the Cellular CD4 Receptor

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The novel antiviral agent cyclotriazadisulfonamide (CADA) inhibited human immunodeficiency virus (HIV) (IC_{50}, 0.3–3.2 μM) and human herpesvirus 7 (HHV-7) infection (IC_{50}, 0.3–1.5 μM) in T-cell lines and PBMCs. When T-cells were pretreated with CADA for 24 h, they became markedly protected from viral infection. Flow cytometric analysis revealed a significant decrease in the expression of the CD4 glycoprotein, the primary receptor needed for entry of both viruses. Moreover, the antiviral activity of CADA correlated with its ability to down-modulate the CD4 receptor. CADA did not alter the expression of any other cellular receptor (or HIV coreceptor) examined. Time course experiments showed that CD4 down-modulation by CADA differs in mechanism from the effects of aurintricarboxylic acid, which binds directly to CD4, and phorbol myristate acetate, which activates protein kinase C. Further analysis of CD4 mRNA levels suggested that CADA was not involved in the regulation of CD4 expression at a transcriptional level, but very likely at (post) translational levels. This unique mechanism of action makes CADA an important lead in developing new drugs for treatment of AIDS, autoimmune diseases, and inflammatory disorders.

Key Words: HIV; HHV-7; CD4 receptor; down-modulation; antiviral.

INTRODUCTION

CD4 is a 55-kDa glycoprotein that is expressed on the membrane of helper T-cells, monocytes, as well as some nonlymphocytic leukemia cell lines (Maddon *et al.,* 1985, 1986; Neudorf *et al.,* 1989). It comprises four extracellular immunoglobulin-like domains, a single spanning transmembrane region, and a short cytoplasmic tail. CD4 has been identified as the main receptor for human immunodeficiency virus (HIV) (Dalgleish *et al.,* 1984; Klatzmann *et al.,* 1984) and human herpesvirus 7 (HHV-7) (Furukawa *et al.,* 1994; Lusso *et al.,* 1994). During HIV infection, three HIV-1 proteins, Nef, Env, and Vpu, contribute to the down-regulation of CD4 (Piguet *et al.,* 1999). Also HHV-7 can down-regulate the CD4 expression in SupT1 cells and $CD4^+$ T-lymphocytes (Furukawa *et al.,* 1994; Secchiero *et al.,* 1997). Aurintricarboxylic acid (ATA), which has been shown to bind directly to CD4, also has activity against HIV-1 and HHV-7 (Schols *et al.,* 1989a; Zhang *et al.,* 1999). In addition, phorbol myristate acetate (PMA), which can rapidly down-modulate CD4 expression in T-lymphocytes, at both the transcription and translation levels by activating protein kinase C (PKC), can inhibit HIV-1 and HHV-7 infection *in vitro* (Acres *et al.,* 1986; Chowdhury *et al.,* 1990; Golding *et al.,* 1994; Hoxie *et al.,* 1986; Neudorf *et al.,* 1991; Touraine *et al.,* 1992; Yasukawa *et al.,* 1995).

It is of particular interest to develop agents that block binding of the HIV virion to the host cell or entry of the virus by membrane fusion, because such drugs may be used to prevent viral infection upon exposure to the virus. The bicyclam AMD3100 and TAK-779 are compounds that were found to block HIV entry by binding to the HIV coreceptors CXCR4 and CCR5, respectively (Baba *et al.,* 1999; Schols *et al.,* 1997). Several peptides have been designed to inhibit conformational changes in gp41 (Chan *et al.,* 1998; Jiang *et al.,* 1993; Wild *et al.,* 1994). Viral resistance may still become a problem with these approaches and it may be difficult to develop drugs for these targets that can be taken orally.

Cyclotriazadisulfonamide (CADA) is a synthetic macrocycle (Fig. 1) (Choi, 1989; Samala, 1999; Sodoma, 1996) that was submitted to the U.S. National Cancer Institute (NCI) anti-HIV screening program. Based on its broad range of activity against several strains of HIV in various cell lines and its novel molecular structure, CADA was selected by NCI for initial, preclinical anti-HIV drug development studies. It was found to be soluble in human,

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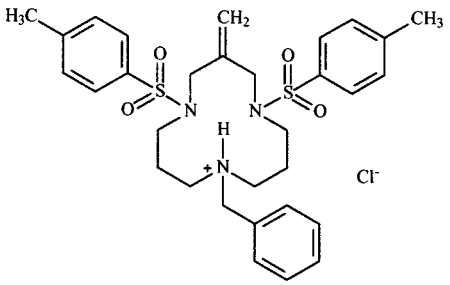

FIG. 1. Chemical structure of CADA (9-benzyl-3-methylene-1,5-di-*p*-toluenesulfonyl-1,5,9-triazacyclododecane).

rat, and mouse plasma at 1.9–3.1 μM, to be stable in plasma (>200 h), and to be detectable in the bloodstream up to 2 h after intravenous injection in mice.

Here, we report that CADA specifically down-modulates cell surface and intracellular CD4 expression. For a period of up to 4 days, CADA completely blocked CD4 expression in the MT-4 T-cell line, the SupT1 T-cell line, and peripheral blood mononuclear cells (PBMCs). This CD4 down-regulating effect resulted in a marked inhibition of HIV and HHV-7 infection.

RESULTS

Anti-HIV and anti-HHV-7 activity of CADA

In order to confirm the antiviral data of CADA obtained from the NCI anti-HIV screening program, we evaluated CADA in our antiviral assays. First, the anti-HIV-1 activity of CADA was tested. Cells were given fivefold dilutions of the compound and infected with an optimized dose of the virus. HIV replication was monitored and when strong CPE was observed in untreated infected cells, all samples were then scored for CPE and supernatant was collected for HIV-1 core antigen (p24 Ag) ELISA. As summarized in Table 1, CADA inhibits HIV-1 replication (strains IIIB, RF, and NL4.3) at an IC_{50} ranging from 0.3 to 3.2 μM when evaluated in different $CD4^+$ T-cell lines (such as MT-4 and SupT1) and in PBMCs. Furthermore, for two HIV-2 strains (ROD and EHO), an IC_{50} of 0.2 μM was obtained in MT-4 cells.

Comparable data were obtained when CADA was evaluated for its anti-HHV-7 activity. The IC_{50}s of CADA for HHV-7 were 0.3 and 1.5 μM when evaluated in SupT1 cells and PBMCs, respectively. For $CD8^+$ cell-depleted PBMCs an IC_{50} of 1.0 μM was measured. The CC_{50} values of CADA in MT-4 cells, SupT1 cells, and PBMC were 39, 142, and 73 μM, respectively. Thus, CADA equally inhibited HIV and HHV-7 infection.

Specific down-modulation of cell surface CD4 by CADA

Since the cellular CD4 receptor is the primary receptor needed for entry of HIV and HHV-7, the effect of CADA on CD4 expression was evaluated. A selective down-modulation of CD4 receptor expression in MT-4 T-cells was observed (Fig. 2A). MT-4 cells were treated with 3.2 μM CADA for 20 h and CD4 expression was then measured by flow cytometry using a FITC-labeled anti-CD4 monoclonal antibody (mAb). As depicted in Fig. 2A, the expression of the cellular CD4 receptor on CADA-treated cells was significantly down-modulated compared with the untreated cells [mean fluorescence intensities (MFI) were 10.6 and 28.8, respectively]. To measure the background staining, an isotype control was included (MFI, 3.5). In addition, quantification of the CD4 receptor on MT-4 cells was performed. Untreated MT-4 cells showed an antibody binding capacity (ABC) (i.e., the number of primary mouse antihuman CD4 monoclonal antibodies per cell) of 43,434, whereas the ABC of CADA-treated cells dramatically dropped to 5158. Thus, treatment of MT-4 cells with CADA (3.2 μM) results in an 88% reduction in CD4 expression.

Next, the effect of CADA was evaluated in different cell types. Figure 2B shows the CD4 receptor down-modulation in the MT-4 T-cell line, the SupT1 T-cell line, and PBMCs after incubation with CADA for 1 to 4 days. When MT-4 cells were incubated with CADA (3.2 μM) and stained with anti-CD4 mAb (Leu3a-FITC), a 6-fold decrease in surface CD4 antigen expression was observed after treatment for 1 day. After 4 days of incubation, the MFI was reduced 9-fold, meaning a significant down-modulation of the CD4 receptor expression. For the SupT1 cells (Fig. 2B), a 24-h treatment with CADA (3.2 μM) resulted in a 3-fold decrease in the surface CD4 expression. A longer incubation with CADA, i.e., 4 days, markedly down-modulated the cellular CD4 expression (an 11-fold reduction in MFI). When CADA was tested in other cell lines (lymphocytic MOLT-4 and monocytic THP-1), a down-modulation in CD4 expression was also observed (6- and 7-fold, respectively). In PBMCs, a 7-fold decrease in the CD4 expression was measured after

TABLE 1

Antiviral Activity of CADA Against Different HIV Strains[a]

	IC_{50} (μM)		
HIV strains	MT-4	SupT1	PBMC
HIV-1 IIIB	0.3	1.5	0.6
HIV-1 RF	1.5	1.8	1.9
HIV-1 NL4.3	0.5	3.2	1.6
HIV-2 ROD	0.2	ND[b]	ND
HIV-2 EHO	0.2	ND	ND

[a] Cells were given fivefold dilutions of the compound and infected with an optimized dose of the virus. HIV replication was monitored and when strong CPE was observed in the positive control (i.e., after 3–5 days for MT4, and 5–7 days for SupT1, 7 days for PBMC), all samples were scored for CPE and supernatant was collected for determination of HIV-1 core antigen (p24 Ag) by ELISA.

[b] ND, not determined.

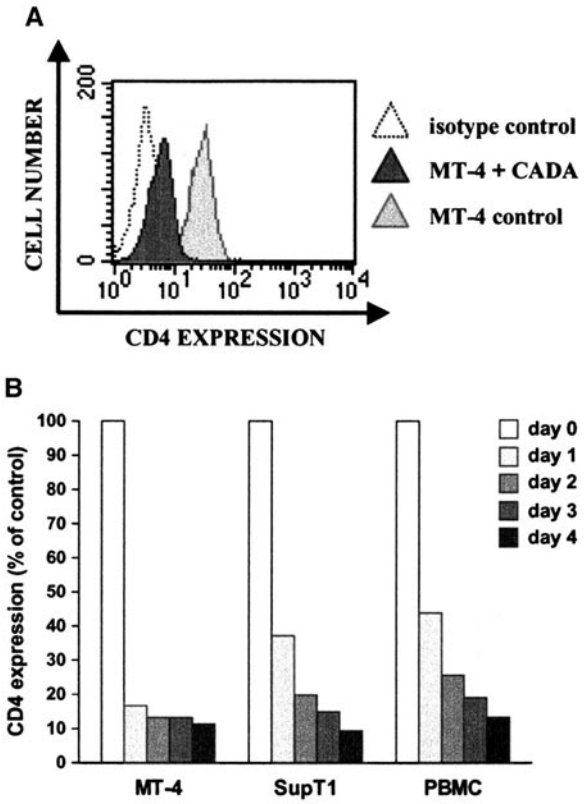

FIG. 2. (A) CD4 down-modulation in MT-4 cells after incubation with 3.2 μM CADA for 20 h. The figure shows the cell surface CD4 expression of untreated and CADA-treated cells after staining with Leu3a-FITC. Similar results were obtained in several repeat experiments. (B) Cell surface CD4 down-modulation in MT-4 and SupT1 cells and PBMCs after incubation with CADA for 1 to 4 days. On day 0, CADA (3.2 μM for MT-4 and SupT1; 16 μM for PBMC) and control medium were administered to the cell cultures. At the indicated time points, cells were stained with Leu3a-FITC (MT-4 and SupT1) or Leu3a-PE mAb (PBMCs). The figure shows the cell surface CD4 expression of CADA-treated cells after 1, 2, 3, and 4 days of incubation. Each bar represents the MFI of the CADA-treated cells as a percentage of the MFI of the untreated cells. One representative experiment of two is shown.

incubation with CADA (16 μM) for 4 days (Fig. 2B). Further flow cytometric analysis of PBMCs revealed a CD4 down-regulating effect of CADA not only in lymphocytes but also in monocytes (7- and 8-fold reductions of MFI, respectively). Thus, CADA significantly down-modulated CD4 expression when evaluated in different human $CD4^+$ cell lines and in PBMCs. Comparable results were obtained with two other anti-CD4 mAbs, OKT4a and OKT4; the latter also binds to a different region of CD4 (data not shown).

CADA down-regulates intracellular CD4

The effect of CADA on the intracellular presence of the CD4 receptor was also investigated. In these experiments, MT-4 cells were cultured in medium alone, in medium with CADA (3.2 μM), or in medium with PMA (8 nM). After 20 h of incubation, cells were washed and CD4 receptors were stained either intracellularly or extracellularly (Fig. 3). For the measurement of intracellular CD4, cells were first incubated with unlabeled anti-CD4 mAb (Leu3a) in order to block the CD4 molecules on the extracellular surface. After fixation and permeabilization of the cells, FITC-labeled anti-CD4 mAb (Leu3a-FITC) was administered and CD4 expression was analyzed flow cytometrically. For comparison, extracellular CD4 was also assessed. Briefly, cells were directly stained with Leu3a-FITC, fixed, permeabilized (without administration of an intracellular mAb), and analyzed. As shown in Fig. 3, CADA clearly affected the intracellular detection of the CD4 molecule (a sevenfold reduction in the MFI). In fact, the down-modulating effect of CADA on cell surface and intracellular CD4 expression was very similar. In contrast, the effect of PMA, a phorbol ester with CD4 down-modulating capability, on the expression of intracellular CD4 was almost nil (the MFI was 81.5% of that of the untreated cells), whereas PMA clearly down-regulated cell surface CD4 receptor expression.

No effect of CADA on other cell surface antigens

In addition, the expression of various other surface antigens was examined in MT-4 cells, SupT1 cells, and PBMCs after incubation with CADA (16 μM) for up to 4 days. As shown in Table 2, among all the surface antigens examined, only CD4 was markedly reduced after treatment of the cells with CADA for 4 days. In contrast, the surface antigen expression of the markers CD2, CD3, CD5, CD8, CD11b, CD25, CD26, CD28, CD38, CD45,

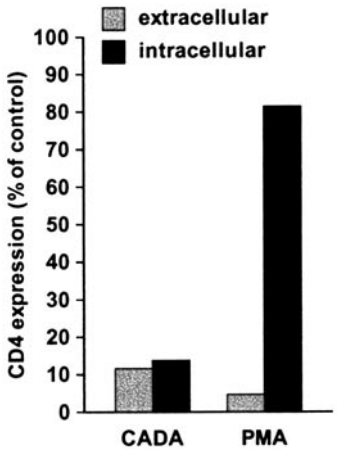

FIG. 3. Cell surface and intracellular CD4 expression in MT-4 cells after incubation with CADA or PMA. MT-4 cells were cultured for 20 h in medium alone, in medium with 8 nM PMA, or in medium with 3.2 μM CADA. Gray histograms (extracellular CD4 expression): cells were washed, stained with anti-CD4 mAb (Leu3a-FITC), and fixed. Black histograms (intracellular CD4 expression): cells were washed, incubated with anti-CD4 mAb (unlabeled Leu3a) in order to block surface antigen, fixed, permeabilized, and stained with anti-CD4 mAb (Leu3a-FITC). Each bar represents the MFI of the CADA- or PMA-treated cells as a percentage of the MFI of the untreated cells. Similar results were obtained in a repeat experiment and also in SupT1 cells.

TABLE 2

Cell Surface Antigen Expression in MT-4, and SupT1 Cells and PBMCs Cultured for 4 Days in the Absence or the Presence of CADA (16 μM)

	MT-4		SupT1		PBMC	
Surface molecule	Control	CADA	Control	CADA	Control	CADA
CD2	1.5[a]	2.6	2.4	0.5	96.2	90.7
CD3	2.3	5.9	1.4	2.7	89.7	89.4
CD4	95.6	6.3	96.4	1.2	60.3	0.2
CD5	99.6	99.1	96.1	93.9	82.0	80.6
CD8	2.8	3.5	98	92.3	25.3	25.1
CD11b	7.0	9.7	0.5	0.6	19.9	12.3
CD25	96.7	93.3	2.3	1	37.6	35.5
CD26	1.4	3.2	0.2	0.3	1.4	3.2
CD28	6.6	6.6	98.7	90.8	92.0	89.6
CD38	95	84.5	99.5	99.1	78.9	73.2
CD45	2.7	3.6	99.5	99.6	98.9	98.3
CD45RA	2.7	2.0	2.4	0.5	69	64.3
CD57	0.7	1.5	73	82.4	4.9	2.3
CD71	85.2	98.8	39.8	33.9	29.3	27.6
HLA-DR	98.7	98.7	1.9	1.7	28	27
TCRα/β	0.8	1.1	2.5	1.6	80.4	82.3

[a] Percentage of cells positive for cell surface antigen staining.

CD45RA, CD57, CD71, HLA-DR, and TCR-$\alpha\beta$ remained unaffected after treatment with CADA (Table 2). As depicted in Fig. 4, cell surface expression of the HIV coreceptor/chemokine receptor CXCR4 also remained unchanged after incubation of MT-4 cells with 3.2 μM CADA for 20 h (MFI were 38.4 and 37.0 for CADA-treated and control cells, respectively), whereas CD4 expression was clearly down-regulated in these cells. In contrast, when MT-4 cells were cultured in the presence of 8 nM phorbol ester PMA for 20 h, a significantly down-modulating effect of PMA on CD4 and on CXCR4 expression was observed (ninefold and fourfold decreases, respectively) (Fig. 4). Similar results were obtained in the SupT1 T-cell line (data not shown). Even a short exposure (i.e., 2 h) of MT-4 or SupT1 cells to PMA (160 nM) remarkably down-regulated the surface expression of CD4 as well as CXCR4, an observation which was described by Signoret *et al.* (1997). However, CADA only affected CD4 and not CXCR4 expression after 20 h of incubation. Additionally, in PBMCs CADA had no down-modulating effect on the cellular expression of either of the major HIV coreceptors CXCR4 and CCR5 (MFI of CXCR4 staining, 76 and 99 for control and CADA-treated cells, respectively). Furthermore, CADA (at 16 μM) did not interact with the stromal cell-derived factor 1α (SDF-1α) or regulate on activation normal T-cell-expressed and secreted (RANTES)-induced intracellular Ca^{2+} signaling in CXCR4- or CCR5-transfected cells (data not shown).

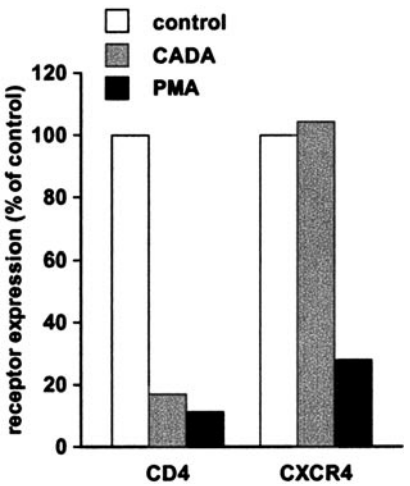

FIG. 4. Effect of CADA and PMA on the cell surface expression of CXCR4 and CD4. MT-4 cells were cultured for 20 h in medium alone (open histogram), in medium with 3.2 μM CADA (gray histogram), or in medium with 8 nM PMA (black histogram). Cells were washed and stained with anti-CD4 mAb (Leu3a-FITC) or anti-CXCR4 mAb (12G5-PE). Each bar represents the MFI as a percentage of the MFI of the untreated cells. The results shown are from one representative experiment. Comparable results were obtained in SupT1 cells.

CADA does not affect CD4 mRNA levels

Further attempts were made to elucidate the mechanism of CADA's action on CD4 expression. First, steady-state levels of total CD4 were compared in untreated and CADA-treated cells by Western blot analysis. In accordance with the results from flow cytometry (i.e., cell surface and intracellular CD4 measurement), the total CD4 protein levels were significantly lower in SupT1 and MT-4 cells treated with 0.64 μM CADA (Fig. 5, lanes 3 and 7), compared with untreated cells (lanes 1 and 5). A higher dose of the compound (3.2 μM) resulted in an almost complete loss of CD4 (lanes 4 and 8). Even a

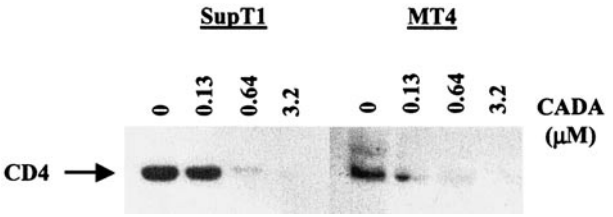

FIG. 5. Decreased protein level of CD4 in SupT1 and MT-4 cells after treatment with CADA. Cells were treated with different concentrations of CADA for 3 days and subjected to Western blotting analysis as described under Materials and Methods.

treatment with 0.13 μM CADA slightly reduced the CD4 level in these cells (Fig. 5, lanes 2 and 6).

A possible mechanism of CD4 down-regulation by CADA could be the interaction of the compound on the transcriptional level. Therefore, CD4 mRNA levels in SupT1 cells relative to control mRNA were examined in the presence and the absence of CADA (3.2 μM). Total RNA was isolated from untreated and treated cells, reverse transcribed, and subjected to a CD4-specific PCR. cDNA preparations were serially diluted (3-fold) and amplified by PCR for CD4 and β-actin. As depicted in Fig. 6, similar mRNA levels for CD4 were obtained in CADA-treated cells and untreated cells, meaning that CADA did not affect the transcription of CD4 although the cell surface CD4 expression was significantly decreased as measured by flow cytometry (a 9-fold reduction in MFI). In addition, when the effect of CADA was tested on the U87.CD4$^+$ cell line, a transformed human glioma cell line constitutively expressing CD4 via a nonendogenous enhancer/promoter, a marked down-modulation of surface CD4 expression was observed (a 10-fold reduction in MFI), indicating that CADA does not act on the endogenous CD4 enhancer/promoter.

Kinetics of CD4 down-modulation by CADA

Next, the kinetics of the CD4 down-regulating activity of CADA was compared to those of several other compounds. The anionic polymer ATA can directly inhibit the binding of the OKT4a/Leu3a mAb to the CD4 receptor (Schols *et al.,* 1989a). When SupT1 cells were treated with ATA (118 μM) for 20 min, a 9-fold decrease in cell surface CD4 staining was observed. Similar results were obtained in PBMCs, where again ATA directly blocked the binding of Leu3a mAb to the CD4 receptor within 20 min of incubation (a 15-fold decrease). In contrast, when SupT1 cells were incubated with PMA (8 nM) for 30 min, only a slight CD4 decrease in these cells could be detected (the MFI was 87.3% of that of untreated cells). However, CD4 expression was dramatically decreased after incubation with PMA for 4 h (a 6-fold reduction in MFI) (Fig. 7). Compared to PMA, CADA had a different time-dependent effect on the down-modulation of CD4. The CD4 expression in SupT1 cells treated with CADA (3.2 μM) for 1 h was still as high as that in untreated cells (Fig. 7), whereas PMA treatment resulted in a CD4 down-modulation of more than 50%. Remarkably, in CADA-treated SupT1 cells a more than 50% decrease in CD4 expression became only clearly visible after an incubation of 12 h (Fig. 7). The CD4 expression dropped beneath 10% of the control after a 4-day treatment with CADA (see Fig. 2). Thus, the kinetics of the CD4 receptor down-regulation elicited by CADA treatment differed from that of PMA and ATA treatment. Also, the PKC blocker staurosporine, which can block the PMA effect on down-modulation of CD4, did not show any effect on the down-regulation of CD4 with CADA (data not shown).

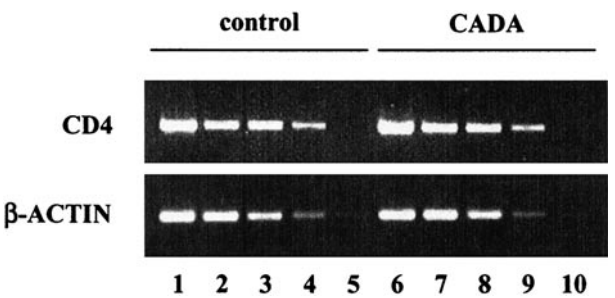

FIG. 6. RT–PCR analysis of CD4 mRNA levels in untreated (lanes 1–5) or CADA-treated (lanes 6–10) SupT1 cells. Cells were given medium or 3.2 μM CADA for 4 days and then subjected to total RNA isolation. cDNA preparations were serially diluted (threefold) and amplified by PCR for CD4 and β-actin. Lanes 1 and 6, dilution 1/1; lanes 2 and 7, 1/3; lanes 3 and 8, 1/9; lanes 4 and 9, 1/27; and lanes 5 and 10, 1/81. Comparable results were obtained in MT-4 cells.

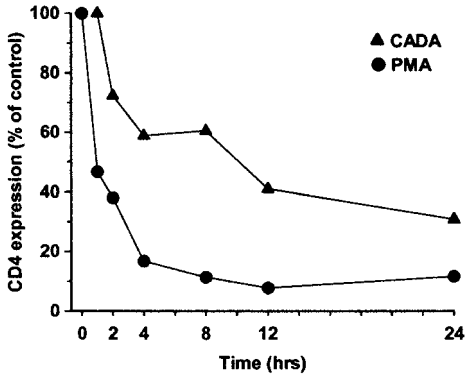

FIG. 7. Kinetics of CADA- and PMA-induced CD4 modulation in SupT1 cells. The MFI of the Leu3a-FITC (anti-CD4 mAb) staining are depicted as a percentage of the MFI of untreated cells. Cells were exposed to CADA (3.2 μM) or PMA (8 nM), and at the indicated time points after administration of the compounds the CD4 receptor expression was assessed by flow cytometry. One representative experiment of three is shown.

The antiviral potency of CADA correlates with its ability to down-modulate CD4

The question arises whether the antiviral potency of CADA was primarily due to its ability to down-regulate the CD4 receptor. A dose–response effect of CADA on

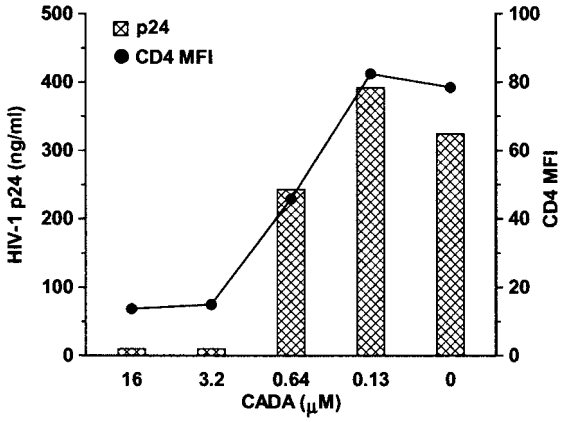

FIG. 8. Correlation between anti-HIV potency and CD4 down-modulating capability of CADA. MT-4 cells were infected with NL4.3 in the presence of different doses of CADA. After 4 days, supernatant was collected and analyzed for its p24 content (vertical bars). In parallel, uninfected MT-4 cells were treated with the same doses of CADA, and CD4 expression was analyzed flow cytometrically after 4 days of incubation. The MFI of the Leu3a-FITC staining is depicted for the different doses of CADA (line). One representative experiment of two is shown.

HIV infection and on CD4 down-modulation is combined in Fig. 8. MT-4 cells were infected with the HIV-1 strain NL4.3 in the presence of different doses of CADA (16, 3.2, 0.64, and 0.13 μM). After 4 days of incubation, when CPE was clearly visible, supernatant was collected and viral replication was measured by p24 Ag ELISA. As shown in Fig. 8, CADA at concentrations of 16 and 3.2 μM completely blocked viral replication, whereas at 0.64 μM 25% inhibition of virus production was detected. A lower dose of the compound (i.e., 0.13 μM) had no inhibitory effect on HIV-1 replication. Thus, the IC_{50} of CADA in this experiment was 1.1 μM. When the CD4 receptor expression was measured on CADA-treated MT-4 cells, a similar dose-dependent effect of CADA on the MFI was observed. High concentrations of CADA (i.e., 16 and 3.2 μM) resulted in a significant down-modulation in CD4 receptor expression, as evident from the low MFI values. At 0.64 μM the MFI was 54% of that of untreated cells, resulting in an IC_{50} of 0.76 μM. These results demonstrate that the CD4 down-regulating activity of CADA directly correlates with its anti-HIV potency.

CADA inhibits HIV-1 entry

In addition, an HIV-1 entry PCR was set up in order to further prove that CADA can efficiently block HIV infection at a very early step. A semiquantitative HIV-1 LTR R/U5-specific PCR was performed on total DNA isolated from SupT1 or MT-4 cells at 2 h after infection with the HIV-1 strain NL4.3. Synthesis of the LTR R/U5 DNA transcript, also known as "strong-stop DNA," is the first step in the reverse transcription process and is accomplished at a very early stage of the infection, immediately after viral entry and uncoating. Thus, MT-4 and SupT1 cells were treated with CADA (3.2 μM) for 2 and 4 days, respectively, resulting in significantly reduced CD4 expression (an eightfold reduced MFI compared to the nontreated cells). As shown in Fig. 9, CADA-pretreated cells were markedly less susceptible to viral entry, demonstrating that CADA acts as a viral entry inhibitor by reducing the expression of the main HIV receptor. As a control for the viral entry assay, treatment of the cells with AMD3100 (12 μM), a strong and specific antagonist of the HIV coreceptor CXCR4, also blocked HIV-1 entry (Fig. 9).

Enhanced anti-HIV activity of CADA after pretreatment of the cells

As could be expected from the time course of the CD4 down-modulating effect of CADA, preincubation of MT-4 cells with CADA for 24 h markedly enhanced its antiviral activity. Table 3 presents the results for MT-4 cells pretreated with CADA (8 μM) for 1 day and then infected with the HIV-1 strain NL4.3 or RF in the absence or the presence of CADA. HIV-1 infection in CADA-pretreated MT-4 cells was substantially decreased, compared to that in the untreated MT-4 cells (a 4.5-fold reduction in p24 core antigen levels). Also, when the cells were pretreated with CADA, administration of CADA at 0.64 μM then completely blocked viral replication. Comparable results were obtained with SupT1 cells and PBMCs (data not shown).

For HHV-7 infection, pretreatment of SupT1 cells with CADA (8 μM) for 1 day resulted in a decrease in HHV-7 infection of more than threefold compared to that in

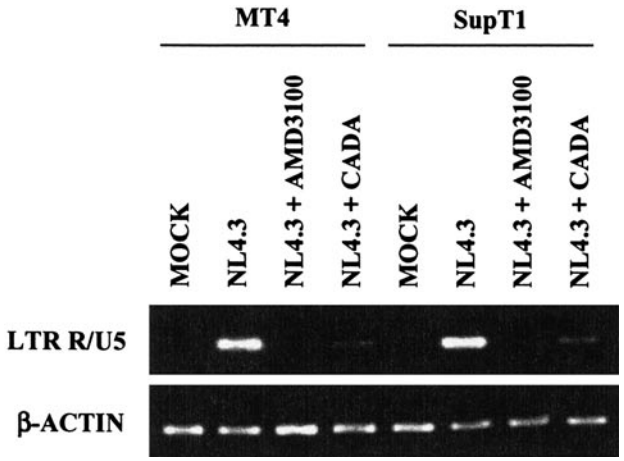

FIG. 9. PCR-based detection of virus entry into MT-4 and SupT1 cells. Cells were treated with 3.2 μM CADA for 2 (MT-4) or 4 (SupT1) days, resulting in significantly reduced CD4 expression. For comparison, cells were also treated with 12 μM AMD3100 for 30 min. Then cells were infected with HIV-1 NL4.3, and 2 h after infection total DNA was extracted from the cells and analyzed by a semiquantitative PCR using HIV-1-specific primers from the LTR R/U5 region. DNA samples from mock-infected cells produced no signal (lanes 1 and 5). DNA recovery was controlled by PCR with β-actin-specific primers.

TABLE 3

Levels of p24 Antigen (pg/ml) in the Supernatant of MT-4 Cells Infected with HIV-1 Strains NL4.3 and RF

	NL4.3		RF	
	Pretreated[a]	Untreated	Pretreated[a]	Untreated
Medium				
CADA	34,839	158,627	42,853	100,002
(3.2 μM)	<5	<5	<5	<5
(0.64 μM)	<5	63,098	<5	56,139
(0.13 μM)	33,785	127,838	35,894	75,118

[a] Cells were pretreated with CADA (8 μM) for 24 h, and the compound was added again after HIV infection at the indicated concentrations.

untreated SupT1 cells. Thus, as expected from the data presented in Fig. 2, pretreatment of the cells with CADA for 24 h clearly enhanced its antiviral potency.

DISCUSSION

Incubation of $CD4^+$ T cells with CADA resulted in a down-modulation of the cell surface CD4 expression. In addition, intracellular CD4 expression was also strongly blocked after incubation with CADA. Further Western blotting analysis of the total CD4 protein levels confirmed the CD4 down-regulating activity of the compound. Except for the CD4 receptor, CADA did not have effect on any of the other cellular receptors evaluated so far. As in SupT1 cells, a significant down-modulating effect on CD4 expression was also detected in PBMCs. Thus, in all cell lines tested, CADA acted as a very specific down-modulator of CD4. Interestingly, even in the U87.$CD4^+$ cell line, a transformed human glioma cell line constitutively expressing CD4 via a nonendogenous enhancer/promoter (Clapham *et al.,* 1991), a marked down-modulated of surface CD4 expression was observed. This suggested that interaction of CADA on CD4 mRNA regulation seemed very unlikely. Indeed, when the effect of CADA was evaluated on the transcriptional level of CD4, no differences in CD4 mRNA production were measured in CADA-treated T-cells compared to untreated cells (Fig. 6). The data with the kinetics of CADA indicate that the timing of the CD4 down-regulating effect of CADA varied somewhat from one cell line to another (in MT-4 cells the CD4 down-modulation began to occur after 12 h, whereas in SupT1 cells and PBMCs the effect of CADA was more pronounced after 4 days of incubation). This relatively long incubation time before modulation of CD4 becomes clearly visible suggests that CADA may affect CD4 expression at the translation level.

As three CD4 binding events are needed to efficiently activate HIV-1 Env trimers (Layne *et al.,* 1990), multimeric CD4 binding is required for HIV infection, further implying that receptor density plays a crucial role in the efficiency of viral infectivity (Platt *et al.,* 1997). Davis *et al.* reported that $CD4^+$ T-cells bind approximately 49,000 CD4 (Leu3a) antibody molecules and that this binding is bivalent, suggesting 98,000 CD4 antigen molecules on the surface of these cells (Davis *et al.,* 1998). Our data concerning the quantification of the CD4 receptor showed a similar result (approximately 43,000 CD4 antibody (Ab) molecules/cell). Interestingly, after treatment with CADA the amount of CD4 Ab bound to the cells dropped to approximately 5000/cell. Thus, CADA is able to reduce the CD4 receptor density by almost 90%. It has been reported that CD4 receptor density will significantly impact the efficiency of viral entry and when the CD4 level becomes limiting (less than 10,000 CD4 molecules/cell) it strongly inhibits viral infection (Kabat *et al.,* 1994; Platt *et al.,* 1997); primary HIV isolates especially seem to be much more dependent on the level of CD4 expression (Kabat *et al.,* 1994; Platt *et al.,* 2000). When evaluated against six different primary HIV-1 isolates in PBMCs, CADA showed a potent (but variable) activity ranging from 0.002 to 2.3 μM. Although CD4-independent viruses have been described (Hoffman *et al.,* 1999), these are much more sensitive to neutralization by antibodies, which could explain the rarity of CD4-independent wild-type HIV variants (Edwards *et al.,* 2001; Kolchinsky *et al.,* 2001). A CD4-lowering drug, such as CADA, not only inhibits viral infection by blocking viral entry (Fig. 9), but also can make the virus more prone to elimination by the neutralizing action of antibodies. Furthermore, as several domains of CD4 play an important role in regulating HIV entry of cells (Poulin *et al.,* 1991), a specific down-modulator of the complete CD4 molecule may be considered an effective antiviral agent.

The anionic polymer ATA inhibits HIV-1 replication by a direct interaction with the CD4 receptor (Schols *et al.,* 1989a). It can also inhibit HHV-7 infection, probably due to its interference with the CD4 receptor (Zhang *et al.,* 1999). Inhibition of CD4 expression by ATA can be detected immediately after incubating the cells with ATA. In contrast, our data show that incubation of the cells with CADA for 1 h did not affect CD4 receptor expression, suggesting that the down-modulation of CD4 expression by CADA is not due to direct binding to the surface CD4 receptor. Previous studies have shown that the phorbol ester PMA can down-regulate CD4 expression at the transcriptional and translational levels (Neudorf *et al.,* 1991). Phorbol esters can also down-modulate the CD4 receptor by activating PKC (Chowdhury *et al.,* 1990). In addition, it has been shown that PMA can inhibit HHV-7 infection (Yasukawa *et al.,* 1995) and HIV-1 infection (Touraine *et al.,* 1992). Furthermore, phorbol esters inhibit HIV-induced syncytia formation through down-modulation of the surface CD4 receptor (Chowdhury *et al.,* 1990) or by modulating an accessory component in the $CD4^+$ cells (Golding *et al.,* 1994). From our data we can conclude that the down-modulation of CD4 by CADA has a

different time-dependent course than that of PMA. First, PMA affects the cell surface CD4 expression in a much faster way than CADA does. Second, CADA also down-modulated intracellular CD4 expression, an effect that was not seen when cells were treated with PMA. PMA also down-regulated CXCR4 quickly, whereas CADA is not capable of doing so. Finally, the PKC inhibitor staurosporine does not influence the down-modulation of CD4 expression by CADA. This suggests that CD4 receptor down-regulation by CADA does not occur through activation of PKC.

Gangliosides, which are acidic glycolipids, can also induce a selective loss of CD4 without affecting other surface molecules and can block CD4-mediated HIV-1 infection (Chieco-Bianchi *et al.,* 1989; Kawaguchi *et al.,* 1989; Offner *et al.,* 1987). Yet the effects of gangliosides on CD4 are neutralized in the presence of serum albumin (Chieco-Bianchi *et al.,* 1989). Therefore, the effect of human serum on the anti-HIV activity of CADA was studied in our antiviral assays, and we found that 10% human serum did not significantly alter the antiviral potency of CADA.

Persistently HIV-infected cells express HIV glycoproteins on their cell surfaces and thus bind to the CD4 molecule of uninfected $CD4^+$ cells. To elicit syncytium formation, the viral envelope protein must bind to the CD4 receptor on the surface of the $CD4^+$ T cell, and compounds shown to interact with CD4–gp120 binding have activity in these cell assays (Schols *et al.,* 1989b). When administered directly into the syncytia formation assay, CADA was not able to prevent the formation of giant cells (data not shown), leading to the conclusion that CADA does not interfere directly with the CD4–gp120 interaction. Furthermore, this conclusion got more support when CADA was tested in our virus binding assay, where again no inhibitory effect of the compound could be observed on the virus–cell interaction, whereas administration of pentosan polysulfate (PPS) resulted in a marked blocking of viral binding, as expected (Schols *et al.,* 1989a) (p24 values were 5.1, 5.4, and 1.3 ng/ml for control and CADA- and PPS-treated cells, respectively). However, when SupT1 cells were pretreated with CADA in order to down-regulate the cellular CD4 expression before being added to (untreated) HIV-infected HUT78 cells, a clearly inhibitory effect of CADA on HIV-induced giant cell formation was observed, as evident from a significantly reduced CPE.

That the antiviral activity of CADA can be related to the specific target CD4 has been further stressed by the evaluation of CADA against several resistant HIV-1 strains. When CADA was tested in an antiviral assay using dextran sulfate-resistant NL4.3 virus (Esté *et al.,* 1997), AMD3100-resistant NL4.3 virus (De Vreese *et al.,* 1996), or SDF-1α-resistant NL4.3 virus (Schols *et al.,* 1998), no loss of activity was detected compared to that in the wild-type NL4.3 virus (data not shown). Thus, CADA seems to retain its anti-HIV activity even if HIV-1 strains used were resistant to polyanionic or CXCR4-antagonistic compounds. Also, when NL4.3 wild-type virus stock was pretreated with CADA prior to infection, a similar CPE was observed to that with untreated virus, indicating again that CADA does not directly interact with the virion. Furthermore, a strong correlation between CD4 down-modulation and the antiviral activity of several CADA analogs has been observed, supporting the conclusion that the antiviral activity is solely due to their CD4 down-regulating potency (K. Vermeire, manuscript in preparation). The selection of an HIV-1 strain resistant to CADA, which has currently been in progress for 4 months without success, lets us suspect that resistance of HIV to CADA will develop slowly, as could be expected from the crucial role of the CD4 receptor in viral entry.

In conclusion, CD4 structure-based drug design may lead to new roads for antiviral research (Li *et al.,* 1998). Our study has demonstrated that CADA down-modulates CD4 by a unique and very specific mechanism and, consequently, inhibits HIV-1 and HHV-7 infection. This discovery may open the door to a new approach to the chemotherapy of AIDS, autoimmune diseases, and inflammatory disorders, such as rheumatoid arthritis, psoriasis, and multiple sclerosis.

MATERIALS AND METHODS

Viruses and cell cultures

The IIIB, RF, and NL4.3 strains of HIV-1 and the EHO and ROD strains of HIV-2 were obtained from the NIAID AIDS Reagent Program (Bethesda, MD). The KHR strain of HHV-7 was kindly provided by Dr. K. Yamanishi (Department of Microbiology, Osaka University School of Medicine, Osaka, Japan). The $CD4^+$ cell lines MT-4, SupT1, HUT-78, MOLT-4, and THP-1 were obtained from the American Type Culture Collection (Rockville, MD) and cultured in RPMI 1640 medium (Gibco BRL, Gaithersburg, MD) with 10% heat-inactivated fetal calf serum (FCS) (Biowhittaker Europe, Verviers, Belgium) and 2 mM L-glutamine (Gibco BRL). Human astroglioma U87 cells expressing human CD4 (U87.CD4) (Clapham *et al.,* 1991) were kindly provided by Dr. Dan R. Littman (Skirball Institute of Biomolecular Medicine, New York University Medical Center, New York, NY) and were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Paisley, UK) containing 10% FCS, 0.01 M HEPES buffer (Invitrogen), and 0.2 mg/ml geneticin (G-418 sulfate) (Invitrogen). The HHV-7 stock was made in the SupT1 cell line, whereas the HIV-1 stocks were made in MT-4 cells. Buffy coat preparations from healthy donors were obtained from the Blood Bank in Leuven. Peripheral blood mononuclear cells (PBMCs) were first isolated by density gradient centrifugation over Lymphoprep (d = 1.077 g/ml) (Nycomed, Oslo, Norway). CD8 magnetic beads (Dynal AS, Oslo, Norway) were added to remove the

$CD8^+$ T lymphocytes. The PBMCs and $CD8^+$ T-cell-depleted PBMCs were stimulated with 2 μg/ml of phytohemagglutinin (PHA) (Sigma Chemical Co., Bornem, Belgium) for 3 days at 37°C.

Compounds and monoclonal antibodies

Aurintricarboxylic acid (ATA; M_r 422.3), phorbol myristate acetate (PMA; M_r 616.8), and pentosan polysulfate (PPS; M_r 3100) were purchased from Sigma Chemical Company. The bicyclam AMD3100 was synthesized as described previously (Bridger *et al.,* 1995). The compound CADA (M_r 618, Fig. 1) was synthesized by Dr. Thomas W. Bell (Department of Chemistry, University of Nevada, Reno, NV) by a modification of the methods developed in Stony Brook (Department of Chemistry, State University of New York, Stony Brook, NY). CADA was dissolved at 16 mM in DMSO.

The mAbs labeled with phycoerythrin (PE) or FITC used were the following: CD2, CD3, CD4 (Leu3a, RPA-T4), CD5, CD8, CD11b, CD25, CD26, CD26, CD28, CD38, CD45, CD45RA, CD57, CD71, HLA-DR, TCR-$\alpha\beta$, CXCR4, and CCR5. All mAbs were purchased from BD Biosciences (Erembodegem, Belgium). The anti-CD4 mAbs OKT4 and OKT4a were purchased from Ortho Diagnostic Systems (Beerse, Belgium). The HIV-1 p24 antigen ELISA kit was purchased from NEN (Brussels, Belgium). The specific mAb to HHV-7 (RK-4) (Advanced Biotechnologies, Columbia, MD) recognizing an early HHV-7 protein was used to detect HHV-7-infected cells.

Antiviral activity

For HIV-1, PBMCs were incubated with the HIV-1 strains IIIB, RF, and NL4.3 for 2 h at 37°C. Then the cells were washed with warm medium and seeded into 48-well flat bottom plates (Iwaki, Japan). Fivefold dilutions of the compound were added to each well, together with 1 ng/ml of IL-2 (R&D Systems Europe, Abingdon, Oxon, UK). The anti-HIV-1 assays were also performed with MT-4 and SupT1 cells. Fivefold dilutions of CADA in 100 μl were added to 96-well flat bottom plates (Iwaki). Then 6×10^4 MT-4 cells or SupT1 cells were added in 50 μl of medium, and finally 50 μl of diluted HIV-1 stocks (strains IIIB, RF, and NL4.3) was added to each well. Cytopathic effect induced by the virus was checked regularly microscopically. When strong CPE was observed in the positive control (i.e., untreated HIV-1-infected cells), all samples were scored for CPE at the same time regardless of the CPE present, and the supernatant of each sample was then collected (at the same time), stored at −20°C, and analyzed for HIV-1 core antigen by p24 Ag ELISA.

For HHV-7, fivefold dilutions of CADA were added in 500 μl of culture medium in 24-well flat bottom plates (Iwaki), whereupon 2×10^5 SupT1 cells were added in 400 μl of culture medium. After 30 min of incubation at room temperature, 100 μl of HHV-7 stock was added to each well. HHV-7-infected and mock-infected SupT1 cells were cultured in a final volume of 1 ml of medium in the absence of CADA. On day 4, half of the medium and the cells were replaced and fresh medium without new compound was added. This procedure was repeated every 2 or 3 days. In the anti-HHV-7 assay in PBMCs and $CD8^+$ T-cell-depleted PBMCs, fivefold dilutions of CADA were added in 250 μl of culture medium in 24-well plates, and then 2×10^6 cells were added in 400 μl of culture medium. After 30 min of incubation at room temperature, 100 μl of HHV-7 stock was added together with IL-2 (1 ng/ml). At day 4, 1 ml of fresh medium containing IL-2 (1 ng/ml) was added to each well. The CPE was checked regularly microscopically. When CPE was observed, the HHV-7 antigen expression was monitored by flow cytometry, as described previously (Zhang *et al.,* 1999).

Flow cytometric analyses

To study the effect of CADA on surface CD4 antigen expression, MT-4 cells, SupT1 cells, HHV-7-infected SupT1 cells, and PBMCs were incubated with a serial fivefold dilution of CADA (16, 3.2, 0.64, and 0.13 μM) or medium at 37°C. Cell surface CD4 antigen expression was analyzed at different time points (up to 4 days). After washing with phosphate-buffered saline (PBS) containing 2% fetal calf serum (FCS), cells were incubated with mAb for 20 min at 4°C. Then the cells were washed, fixed with 1% formaldehyde, and analyzed by a FACScalibur (BD, San Jose, CA).

For comparative assessments on CD4 receptor expression in T cells, experiments with ATA and PMA were also conducted. Briefly, CADA (16 or 3.2 μM), ATA (118 μM), and PMA (8 nM) were added to the cells for an incubation time between 20 min and 4 days, and cell surface CD4 antigen expression was analyzed as described above.

In order to investigate the effect of CADA on intracellular CD4 protein expression, cells were incubated with CADA (16 or 3.2 μM), PMA (8 nM), or medium at 37°C. The intracellular CD4 antigen expression was evaluated on day 1. Briefly, the cells were first washed with PBSA (PBS, 0.1% NaN_3, 2% NCS) and then incubated with unlabeled CD4 mAb (Leu3a) to block the surface CD4 expression. After 30 min of incubation at 4°C, the cells were washed and resuspended in 1% formaldehyde for 1 h at 4°C. Then the cell pellets were resuspended in 0.2% Tween 20 at 37°C. After 15 min of incubation, the cells were centrifuged and incubated with CD4 mAb (Leu3a-FITC) for 30 min at 4°C, washed again, and analyzed by flow cytometry.

Quantitative analysis of CD4 receptor

DAKO QIFIKIT (DAKO, Glostrup, Denmark) was used for the quantitative determination of cell surface CD4

receptor by flow cytometry. The antigen quantity is expressed in ABC units, i.e., the number of primary mouse monoclonal antibodies per cell or microbead. According to the manufacturer's user manual, 4×10^5 MT-4 cells were incubated (30 min, 4°C) with a saturating concentration (10 μl) of unconjugated primary mouse monoclonal antibody against human CD4 (Leu3a pure, BD). As a negative control, an irrelevant mouse monoclonal antibody of the same isotype (IgG1) was included (negative control, DAKO). Cells were washed and FITC conjugate (FITC-conjugated goat antimouse immunoglobulins, DAKO) was administered to the cells as well as to the Set-up and Calibration Beads. After an incubation of 45 min (4°C), cells and beads were washed, fixed with 1% formaldehyde, and analyzed by a FACScalibur (BD). For the calculation of the ABC of the samples, the MFI of each bead population of the Calibration Beads (bearing different but exact known numbers of monoclonal Ab molecules) was defined and used for the construction of the calibration curve.

Time-of-addition experiments

To further investigate the antiviral effect of CADA, time-of-addition assays were performed. Briefly, 5×10^6 SupT1 or MT-4 cells were pretreated with CADA at 8 μM. After 1 or 4 days of pretreatment with CADA, the cells were evaluated for CD4 expression level and infected with HHV-7 or HIV-1, as described above.

Virus binding assay

MT-4 cells (5×10^5) were incubated with compound (16 μM CADA or 8 μM PPS) or medium for 30 min at 37°C. Next, supernatant containing 1×10^5 pg of p24 antigen of HIV-1 NL4.3 virus was added. One hour after infection, cells were washed three times with PBS (with 1% FCS) and lysed and p24 antigen bound to the cells was determined by a commercial p24 Ag ELISA test (NEN, Brussels, Belgium).

In order to investigate if CADA has a direct inhibitory effect on the virus, HIV-1 NL4.3 virus stock (650 ng/ml) was pretreated with 16 μM CADA for 2 h at 37°C prior to infection. Next, the virus stock was diluted to 0.5 ng/ml for the infection of MT-4 cells, leaving less than 16 nM residual CADA in the supernatant of the infected cells. When strong CPE was observed in the positive control (i.e., untreated HIV-1-infected cells), all samples were scored for CPE at the same time regardless of the CPE present, and the supernatant of each sample was then collected (at the same time), stored at −20°C, and analyzed for HIV-1 core antigen by p24 Ag ELISA.

Syncytium formation assay

Fivefold dilutions of CADA or anti-CD4 mAb (RPA-T4) were added in 500 μl of culture medium in 24-well flat bottom plates. Then 1×10^6 SupT1 cells were added in 250 μl of culture medium, followed by 1×10^6 HIV-1 (IIIB)-infected HUT-78 cells in 250 μl of culture medium. The HIV-1-induced cell fusion was checked microscopically 24 h after the start of the coculture. In some experiments, only the SupT1 cells were pretreated with CADA (3.2 μM) for 4 days and added to the (untreated) IIIB-infected HUT-78 cells.

HIV entry PCR

Cells (SupT1 or MT-4) were seeded in 24-well plates at 1×10^6 cells per well. Virus stocks (diluted to a p24 titer of 10,000 pg/ml) were treated with 500 U/ml of RNase-free DNase (Roche Molecular Biochemicals) for 1 h at room temperature. Then the cells in each well were infected with 1000 pg of p24. After incubation at 37°C for 2 h, the cells were washed twice with PBS and total DNA was extracted from the infected cells using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). The DNA was eluted from the QIAamp spin columns in a final volume of 200 μl of elution buffer. Then 10 μl of each DNA sample was subjected to 38 cycles of HIV-1 LTR R/U5-specific and 28 cycles of β-actin-specific PCR on a Biometra T3 Thermocycler. Each cycle comprised a 45-s denaturation step at 95°C, a 45-s annealing step at 61°C, and a 45-s extension step at 72°C. The primers used were LTR R/U5 sense primer 5′-GGCTAACTAGGGAACCCACTG-3′ (nucleotides 496 to 516, according to the HIV-1 HXB-2 DNA sequence, see Ratner *et al.,* 1985), LTR R/U5 antisense primer 5′-CTGCTAGAGATTTTCCACACTGAC-3′ (nucleotides 612 to 635), β-actin sense primer 5′-TCTGGCGGCACCACCATGTACC-3′ (nucleotides 2658 to 2679), β-actin antisense primer 5′-CGATGGAGGGGCCGGACTCG-3′ (nucleotides 2961 to 2980). The reaction mixtures contained PCR buffer (supplied with the enzyme), 200 μM concentrations of dATP, dGTP, dCTP, and dTTP (Life Technologies), 0.4 μM concentrations of the forward and reverse primers, and 0.5 U of SuperTaq DNA polymerase (HT Biotechnology, Cambridge, England) in a total volume of 25 μl. After gel electrophoresis through a 2% agarose gel, the amplified DNA fragments were visualized by ethidium bromide. In preliminary experiments, the exponential range of the PCR amplification curve was determined for both the HIV-1 LTR R/U5 and the β-actin PCRs by varying the amount of input DNA and the number of PCR cycles. Based on these experiments, appropriate conditions were chosen to perform the PCRs.

RT–PCR analysis of CD4 expression

Total RNA was isolated from approximately 5×10^6 cells using the RNeasy Mini Kit (QIAGEN). To eliminate any possible contamination by genomic DNA, DNase treatment (RNase-free DNase Set, QIAGEN) was included in the RNA purification protocol. The RNA concentration of the samples was determined spectropho-

tometrically at 260 nm. After a 1-min denaturation at 95°C, 2 μg of RNA was reversed transcribed by 4 U of Rous-associated virus (RAV-2) reverse transcriptase (Amersham-Pharmacia Biotech, Uppsala, Sweden) in a 50-μl mixture consisting of reaction buffer (supplied with the enzyme) with 250 μM concentrations of dATP, dGTP, dCTP, and dTTP (Invitrogen), 6 μg of random hexanucleotide primers (Invitrogen), and 130 U of human placental ribonuclease inhibitor (Amersham-Pharmacia Biotech). The reaction was allowed to proceed for 90 min at 45°C, whereafter the cDNA samples were stored frozen. Then, 2 μl of each DNA sample was subjected to 38 cycles of CD4-specific and 30 cycles of β-actin-specific PCR on a Biometra T3 Thermocycler (Biometra). For CD4, each cycle comprised a 45-s denaturation step at 95°C, a 45-s annealing step at 67°C, and a 45-s extension step at 72°C. For β-actin, each cycle comprised a 45-s denaturation step at 95°C, a 45-s annealing step at 63°C, and a 45-s extension step at 72°C. Oligonucleotide primer pairs for CD4 consisted of the following nucleotide sequences: 5′ primer (5′–3′) GTGAACCTGGTGGTGATGAGAGC and 3′ primer (5′–3′) GGGCTACATGTCTTCTGAAACCGGTG. β-Actin oligonucleotide primer pairs consisted of the 5′ primer (5′–3′) ATCCTCACCCTGAAGTACCCCA and the 3′ primer (5′–3′) GAAGGTCTCAAACATGATCTGGGT. The reaction mixtures contained PCR buffer (supplied with the enzyme), 100 μM concentrations of dATP, dGTP, dCTP, and dTTP (Life Technologies), 0.5 μM concentrations of the forward and reverse primers, and 0.5 U of SuperTaq DNA polymerase (HT Biotechnology) in a total volume of 25 μl. After gel electrophoresis through a 2% agarose gel, the amplified DNA fragments were visualized by ethidium bromide. In preliminary experiments, the exponential range of the PCR amplification curve was determined for both the CD4 and the β-actin PCRs by varying the amount of input DNA and the number of PCR cycles. Based on these experiments, appropriate conditions were chosen to perform the PCRs.

Western blotting analysis

Cells were lysed in 100 μl of PBS–0.5% Triton X-100 and sonnicated two times for 15 s at 0°C. Cell lysates were next centrifuged 15 min at 4°C (15,000 *g*), and the protein concentration in the supernatants was measured using the Bio-Rad Protein Assay (Bio-Rad Laboratories GmbH, München, Germany), with BSA as a standard. Aliquots of nonreduced protein were subjected to electrophoresis on 12.5% polyacrylamide gels containing SDS. The proteins were electroblotted onto PVDF membranes (PVDF-Plus, MSI Corp.) and blocked overnight in TBST + 5% nonfat dry milk. Blots were probed with 0.66 μg/ml of purified mouse antihuman CD4 mAb (BD Biosciences) for 1 h at RT. After incubation with a horseradish peroxidase-conjugated antimouse mAb (Jackson, PA, U.S.A.) blots were developed using chemiluminescent detection (ECL$^+$, Amersham).

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