

The Anti-HIV Potency of Cyclotriazadisulfonamide Analogs Is Directly Correlated with Their Ability to Down-Modulate the CD4 Receptor

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ABSTRACT

9-Benzyl-3-methylene-1,5-di-*p*-toluenesulfonyl-1,5,9-triazacyclododecane (CADA) has been identified as a novel antiviral lead compound with significant anti-human immunodeficiency virus and anti-human herpesvirus 7 activity. Surprisingly, this compound selectively decreased the expression of the CD4 glycoprotein, the primary receptor needed for the entry of both viruses. Herein, we describe the CD4 down-modulating and antiviral potencies of more than 25 CADA derivatives. Flow

cytometric evaluation of cellular CD4 receptor expression in T cells demonstrated the specific CD4 down-modulating capacity of the CADA derivatives, with IC_{50} values similar to those obtained in the antiviral assays. The close correlation observed between the CD4 down-regulating and anti-HIV potencies of the CADA derivatives further points to CD4 receptor down-modulation as the primary mode of antiviral action for this group of compounds.

It is well known that infection of target cells by human immunodeficiency virus (HIV) is dependent on the presence of the CD4 surface molecule, which serves as the main virus receptor (Dalglish et al., 1984; Klatzmann et al., 1984). Also, human herpesvirus 7 (HHV-7) uses the CD4 receptor for viral entry (Furukawa et al., 1994; Lusso et al., 1994). Although CD4 is the primary receptor for HIV entry, several CD4-independent HIV-1 strains have been reported (Dumonceaux et al., 1998; Hoffman et al., 1999; Kolchinsky et al., 1999; LaBranche et al., 1999). These viruses, derived by passage on CD4-negative, CCR5-positive, or CXCR4-positive cells, can infect their target cells in the absence of the CD4 receptor by using a chemokine receptor. Interestingly, CD4-independent HIV isolates can be obtained from HIV-infected persons but these viruses show an enhanced sensitivity to antibody mediated neutralization (Hoffman et al., 1999; Edwards et al., 2001; Kolchinsky et al., 2001).

CD4 is a type I integral membrane glycoprotein that is expressed mainly on the surface of thymocytes, T helper lymphocytes, and cells of the macrophage/monocyte lineage

(Maddon et al., 1986). It participates in the maturation of T lymphocytes and, as an intercellular adhesion molecule, plays an important role in the stabilization of the interaction between T cell receptors on T cells and MHC II complexes on antigen-presenting cells. After the antigenic stimulation of T lymphocytes, CD4 also provides a physical noncovalent link to p56^{lck} protein tyrosine kinase, resulting in cell proliferation and interleukin-2 production (reviewed by Weiss and Littman, 1994).

The expression of the CD4 receptor is tightly regulated in various physiological processes. During the development of T cells in the thymus, the CD4[−]CD8[−] double-negative cells become CD4⁺CD8⁺ double-positive before they differentiate into single positive T cells (Zuniga-Pflucker et al., 1989). Also, after antigen-induced T cell activation, CD4 is quickly internalized via clathrin-coated pits, leading to a temporary desensitization of the cell (Pelchen-Matthews et al., 1992). In nonlymphoid cells, CD4 is slowly but constitutively endocytosed and recycled to the cell membrane (Pelchen-Matthews et al., 1991). In addition, viral encoded proteins may exert a profound effect on CD4 expression. Establishment of HIV infection is accompanied by the down-modulation of the CD4 molecule from the cell surface (Dalglish et al., 1984; Maddon et al., 1986), and this effect is mainly regulated by three

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ABBREVIATIONS: HIV, human immunodeficiency virus; HHV-7, human herpesvirus-7; CADA, 9-benzyl-3-methylene-1,5-di-*p*-toluenesulfonyl-1,5,9-triazacyclododecane (cyclotriazadisulfonamide); tosyl, toluenesulfonyl; mAb, monoclonal antibody; CPE, cytopathic effect; ELISA, enzyme-linked immunosorbent assay; MFI, mean fluorescence intensity.

HIV-1 proteins, Env, Nef, and Vpu (reviewed by Piguet et al., 1999).

In a previous report, we described the activity of 9-benzyl-3-methylene-1,5-di-*p*-toluenesulfonyl-1,5,9-triazacyclododecane (CADA) against HIV and HHV-7 and tentatively attributed this antiviral activity to a specific CD4 down-modulating potency (Vermeire et al., 2002). Binding studies with HIV-1 revealed that CADA did not directly interact with the CD4 receptor and/or viral envelope glycoproteins. Also, from time course experiments, it became clear that CADA differs in its mode of action from aurointricarboxylic acid and phorbol myristate acetate, two compounds that have been reported to interact with CD4 (Acres et al., 1986; Schols et al., 1989). Further analysis of CD4 mRNA levels suggested that CADA is not involved in the regulation of CD4 expression at the transcriptional level but most probably interacts at a (post)translational level (Vermeire et al., 2002).

In this study, we investigated a series of CADA analogs for their antiviral potency as well as their CD4 down-regulating capacity. From a comparative analysis of the structure-function relationship of the CADA derivatives with regard to both their anti-HIV and their CD4 down-modulating activities, we can conclude that the antiviral potency of the CADA analogs depended primarily on the down-regulation of CD4 receptor expression.

Materials and Methods

Compounds and Monoclonal Antibodies. The compounds were synthesized as described in the following references and used in the free base or salt forms: CADA.HCl (Choi, 1989; Sodoma, 1996); HJC321.HCl (Choi, 1989); ASN6P6.2HCl, 95-211.HCl, AS112, AS117.HCl, ASPB127.HCl, and 95-213 (Sodoma, 1996); QJ023.HCl, QJ027, QJ028.HCl, QJ030.2HCl, QJ040.HCl, QJ041.HCl, QJ029.HCl, QJ035.HCl, QJ036, QJ037.HCl, QJ038.HCl, QJ033, 97-269, 98-035, and 98-037.2HCl (Jin, 1997); MFS010.HCl, MFS117.HCl, MFS105.HCl, MFS-PB001, and 95-210.HCl (Samala, 1999). The structures of these compounds are shown in Fig. 1. Stock solutions of each compound were prepared by dissolving 10 mg in 10 ml of dimethylsulfoxide (VWR International, Leuven, Belgium).

The monoclonal antibody (mAb) CD4 (SK3) was purchased from BD Biosciences (Erembodegem, Belgium). The HIV-1 p24 antigen ELISA kit was purchased from PerkinElmer Life Sciences (Zaventem, Belgium).

Viruses and Cell Cultures. The HIV-1 T-tropic (X4) molecular clone NL4.3 was obtained from the National Institute of Allergy and Infectious Disease AIDS reagent program (Bethesda, MD). The CD4⁺ T-cell lines MT-4 and SupT1 were obtained from the American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 medium (Invitrogen, Gaithersburg, MD) with 10% heat-inactivated fetal calf serum (Biowhittaker, Verviers, Belgium) and 2 mM L-glutamine (Invitrogen, Carlsbad, CA). The cell cultures were maintained at 37°C in a humidified, CO₂-controlled atmosphere and subcultivations were done every 2 to 3 days. The HIV-1 stock was obtained from the culture supernatant of HIV infected MT-4 cells.

Antiviral Assays. MT-4 cells were infected with the HIV-1 strain NL4.3. Briefly, 5-fold dilutions of the compounds (in 100 μ l) were added to 96-well flat-bottomed plates (International Medical, Brussels, Belgium). Then, to each well, 7.5×10^4 MT-4 cells were added in 50 μ l of medium, followed by 50 μ l (500 pg/ml p24 Ag) of diluted HIV-1 stock (strain NL4.3). Cytopathic effect (CPE) induced by the virus was checked microscopically at regular times. When strong CPE was observed (after 3 to 5 days of incubation) in untreated HIV-1-infected cells, the supernatant of all samples was collected (at the same time), stored at -20°C, and analyzed for HIV-1 core anti-

gen by p24 Ag ELISA (PerkinElmer Life Sciences). Finally, the IC₅₀ value of the compounds (i.e., the concentration of the compound required for 50% reduction of HIV replication as measured by the p24 antigen ELISA) was calculated.

Flow Cytometric Analyses. To study the effect of the CADA derivatives on surface CD4 antigen expression, MT-4 and SupT1 cells were incubated with a serial 5-fold dilution of the compounds (75, 15, 3, 0.6, and 0.12 μ M) or medium at 37°C. Cell surface CD4 antigen expression was analyzed at day 3 (MT-4) or day 4 (SupT1). Briefly, after washing with phosphate-buffered saline containing 2% fetal calf serum, cells were incubated with FITC-conjugated anti-CD4 (SK3) mAb for 30 min at 4°C. As a negative control for aspecific background staining, cells were stained in parallel with Simultest Control γ_1/γ_{2a} (BD Biosciences). Then the cells were washed, fixed with 1% aqueous formaldehyde solution and analyzed by flow cytometry with a FACScalibur (BD Biosciences, San Jose, CA). Data were acquired and analyzed with CellQuest software (BD Biosciences). For the calculation of the CD4 receptor expression, the mean fluorescence intensity (MFI) of each sample was expressed as percentage of the MFI of control cells (after subtracting the MFI of the isotype control). Finally, the IC₅₀ values of the compounds (i.e., the concentrations of the compounds required for 50% inhibition of cell surface CD4 expression) were calculated.

Cytotoxicity Assay. Cellular toxicity of the compounds was measured after 3 or 4 days of incubation by trypan blue exclusion and also by propidium iodide by flow cytometry, in parallel with the measurement of CD4 antigen expression. The CC₅₀ values of the CADA analogs correspond to the concentrations (micromolar) required to reduce the viability of the cells by 50%.

Statistical Analysis. For correlations between two parameters, the predicted lines were determined by simple linear regression analysis. The *P* values and Pearson's linear correlation coefficient (*r*) were calculated by Fig.P statistical package (Biosoft, Cambridge, UK).

Results

CD4 Down-Modulating Activity of CADA Analogs.

The CD4 down-modulating activity of CADA and 27 derivatives thereof are shown in Table 1. The prototype compound CADA markedly down-modulates CD4 receptor expression in MT-4 and SupT1 cells with IC₅₀ values of 0.80 and 1.03 μ M, respectively. Removal of two double bonds in the benzyl group of CADA, as in compound QJ023, slightly enhanced the CD4 down-modulating activity. If a cyclohexylmethylene group replaced the benzyl group of CADA, as in compound QJ028, the CD4 down-regulating potency was somewhat more pronounced (2-fold). In fact, QJ028 was the most potent of the CADA derivatives tested so far. The cytotoxic concentration (CC₅₀) of QJ028 was 29.3 μ M in MT-4 cells, which is about a 86-fold higher than its IC₅₀ value (Table 1) [selectivity index (CC₅₀/IC₅₀) = 86].

Introduction of a nitrogen atom at position 3 or 4 of the benzene ring of the benzyl group (3-pyridylmethylene and 4-pyridylmethylene group in compounds ASN6P6 and QJ030, respectively) had a detrimental effect on the CD4 down-modulating activity (7-fold decrease). Furthermore, the presence of a nitrogen atom at position 2 of the aromatic group (2-pyridylmethylene in compound 98-037) gave a complete loss of activity. In contrast, compound QJ027, which has a nitrogen atom at the same position (position 2) but in a smaller aromatic ring (2-pyrrolylmethylene group), still had CD4 down-modulating potency, although 10-fold less than CADA. Smaller cyclic structures (e.g., cyclopropylmethylene

in compound QJ041) substituted for the cyclohexylmethylene group of QJ028 reduced the activity by 7-fold.

The CD4 down-modulating potency of CADA was affected in different ways when the benzyl group was replaced by an

aliphatic chain. Substitution by a longer open chain, such as an isopentyl group, as in compound QJ038, maintained the CD4 down-regulating activity. However, compounds with a short-chain (isopropyl in compound QJ035) were clearly less

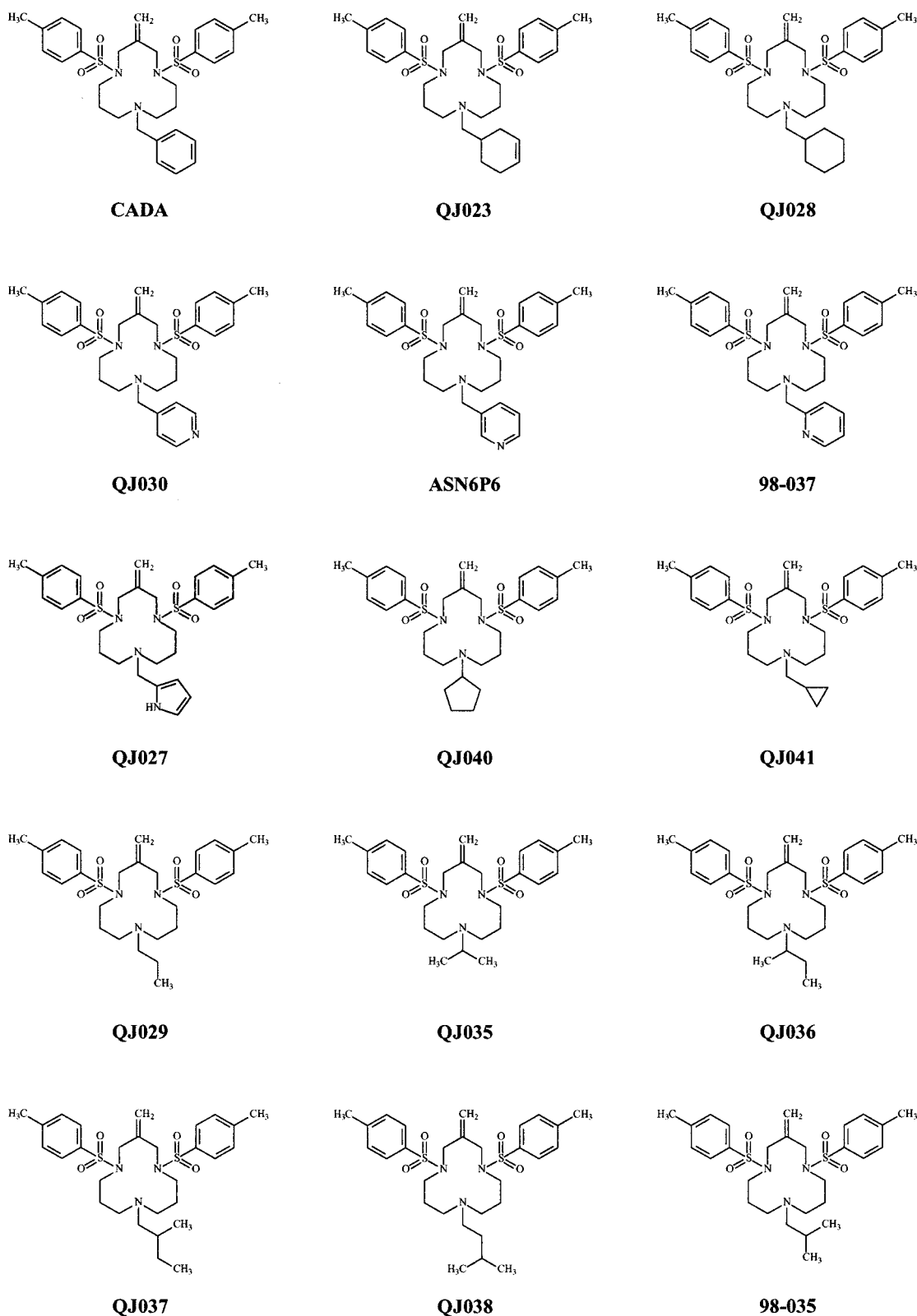


Fig. 1. Chemical structures of CADA analogs.

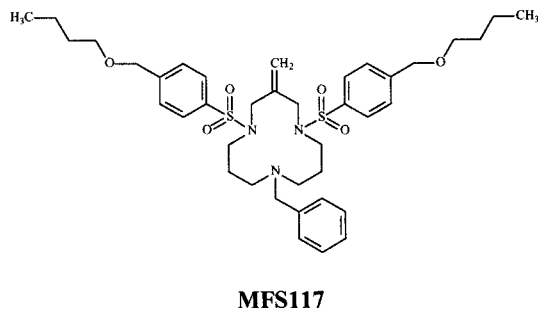
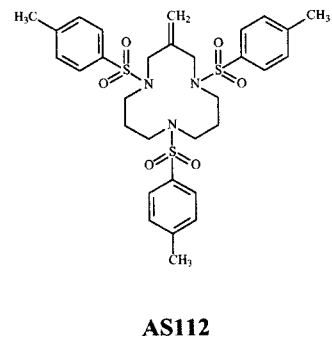
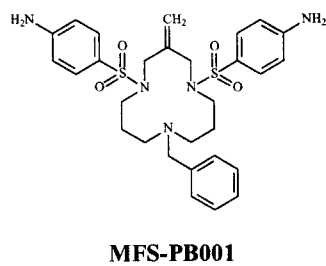
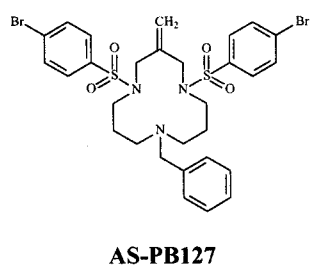
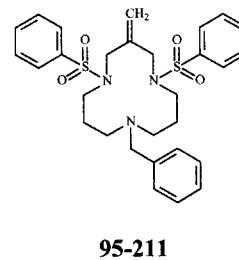
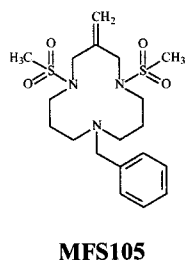
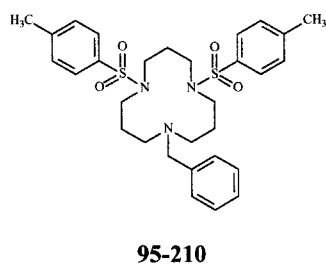
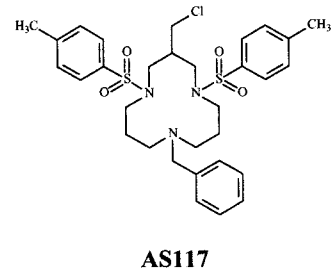
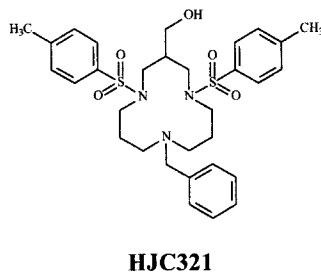
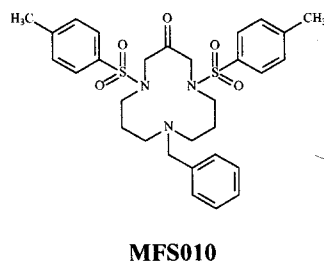
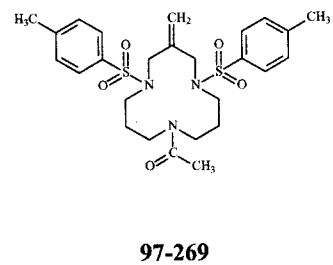
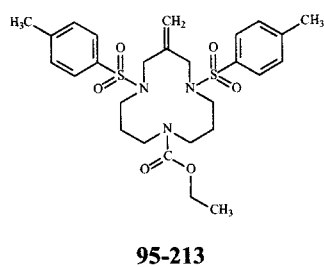
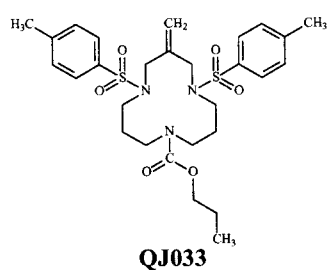


Fig. 1. Continued.

TABLE 1

CD4 down-modulating capability and anti-HIV-1 activity of the different CADA analogs

The compounds were used in the free base or salt forms as listed under *Materials and Methods*. IC₅₀ for CD4 down-modulation was the concentration of the compound required for 50% inhibition of cell surface CD4 expression in MT-4 or SupT1 cells, as measured by flow cytometry. IC₅₀ for HIV-1 (NL4.3) infection in MT-4 cells was concentration of the compound required to reduce viral HIV-1 replication by 50% as measured by the p24 Ag ELISA. CC₅₀ was the 50% cytotoxic concentration (i.e., the concentration of the compound required to reduce the viability of the MT-4 or SupT1 cells by 50%). Data represent mean values \pm S.D. for three or four independent experiments.

Compound	IC ₅₀		Anti-HIV-1	CC ₅₀	
	CD4 Down-Modulation			Toxicity	
	MT-4	SupT1		MT-4	SupT1
	μM			μM	
CADA	0.80 ± 0.35	1.03 ± 0.21	1.21 ± 0.07	40.7 ± 5.4	>75
QJ023	0.57 ± 0.27	0.68 ± 0.16	0.60 ± 0.19	32.7 ± 6.4	40.4 ± 10.7
QJ027	7.86 ± 1.55	10.49 ± 0.13	8.02 ± 0.43	35.3 ± 3.3	37.0 ± 3.0
QJ028	0.34 ± 0.06	0.43 ± 0.07	0.29 ± 0.05	29.3 ± 7.8	36.4 ± 23.9
QJ029	4.08 ± 0.82	5.80 ± 1.04	2.39 ± 1.59	36.4 ± 4.3	37.9 ± 3.1
QJ030	5.46 ± 2.06	11.12 ± 3.64	6.13 ± 2.20	32.4 ± 1.4	>75
QJ033	2.12 ± 0.26	4.18 ± 1.13	2.60 ± 0.69	44.1 ± 1.2	>75
QJ035	11.00 ± 1.50	>15	10.59 ± 1.22	38.9 ± 1.9	40.4 ± 0.9
QJ036	3.04 ± 1.57	3.48 ± 0.55	3.52 ± 1.91	42.6 ± 6.3	>75
QJ037	0.98 ± 0.16	1.34 ± 0.34	0.98 ± 0.25	34.5 ± 7.8	43.1 ± 10.9
QJ038	0.67 ± 0.09	0.96 ± 0.33	0.77 ± 0.35	32.5 ± 1.6	13.1 ± 5.1
QJ040	8.29 ± 1.21	13.06 ± 1.51	6.39 ± 1.00	30.3 ± 4.4	34.1 ± 4.1
QJ041	6.57 ± 2.87	7.36 ± 0.91	6.62 ± 0.47	36.1 ± 8.5	36.6 ± 2.8
AS-N6P6	5.44 ± 1.43	5.76 ± 1.42	5.60 ± 2.79	32.6 ± 11.9	>75
95–213	11.09 ± 0.69	16.15 ± 2.14	27.15 ± 18.20	>75	>75
98–035	10.33 ± 3.33	10.93 ± 1.88	19.30 ± 14.86	>75	>75
HJC321	3.65 ± 1.73	4.95 ± 0.32	7.31 ± 1.72	41.0 ± 1.0	>75
AS117	1.90 ± 0.48	2.26 ± 0.25	2.20 ± 0.63	46.9 ± 9.7	>75
AS-PB127	1.76 ± 0.26	3.78 ± 2.60	1.84 ± 0.04	47.7 ± 13.2	>75
95–210	10.94 ± 2.93	>75	>15	38.0 ± 2.5	>75
98–037	>75	>75	>75	>75	>75
AS112	>15	>75	>15	43.0 ± 1.4	>75
97–269	>75	>75	>75	>75	>75
MFS010	>15	>75	>15	47.2 ± 6.7	>75
MFS117	>15	>75	>15	30.5 ± 11.6	>75
MFS105	>75	>75	>75	>75	>75
95–211	>15	>75	>15	40.2 ± 1.9	>75
MFS-PB001	>15	>15	>15	34.3 ± 0.8	49.2 ± 12.7

active than CADA (14-fold less active for the CD4 down-regulation in MT-4 cells and not active in SupT1 cells). CADA analogs with an *n*-propyl or 1-methylpropyl side chain (e.g., compounds QJ029 and QJ036) had CD4 down-modulating potency lying in between. Shift of the methyl branch from position 1 (QJ036) to position 2 (98-035) of the side chain at *N*⁹ of triazacyclododecane decreased potency, but increasing the size of the alkyl group to five carbons (QJ037 and QJ038) restored activity to almost the level of CADA.

It thus seems that the length of the aliphatic chain of the CADA derivatives is crucial for their CD4 down-regulating potency. This is further emphasized by comparison of the compounds QJ033 and 95-213. Again, compound QJ033, with its longer propyloxycarbonyl group, had 5-fold higher CD4 receptor down-modulating activity than compound 95-213, with its shorter ethyloxycarbonyl group. Substitution by an acetyl group, as in compound 97-269, led to a complete loss of CD4 down-regulating activity.

Modifications of the three-carbon bridge between the two toluenesulfonyl (tosyl) groups of CADA, had differential effects on the CD4 down-modulating potency. Removal of the exocyclic 3-methene bound (compound 95-210) decreased by 14-fold the down-regulating potency of CADA in MT-4 cells. Furthermore, substitution of the 3-methene by 3-oxo (MFS010) abrogated CD4 down-regulating activity. If the 3-methene unit was replaced by a hydroxymethylene or chloromethylene group (compounds HJC321 and AS117, respec-

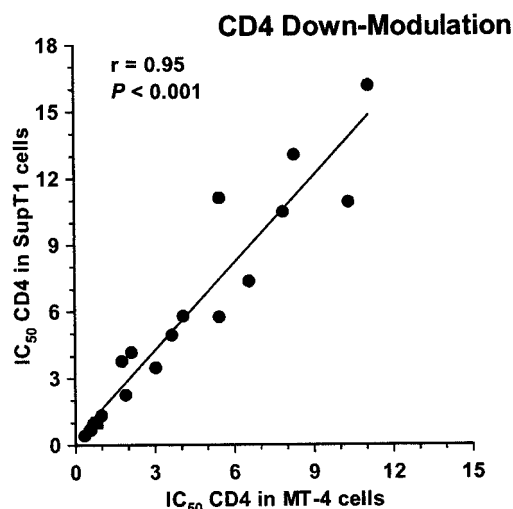


Fig. 2. Correlation of the CD4 expression down-modulation of the different CADA analogs (i.e., CADA, QJ023, QJ027, QJ028, QJ029, QJ030, QJ033, QJ036, QJ037, QJ038, QJ040, QJ041, AS-N6P6, 95-213, 98-035, HJC321, AS117, and AS-PB127) in MT-4 cells versus SupT1 cells as assessed by linear regression analysis. For each analog, the CD4 down-modulating activity in MT-4 cells (IC₅₀ value in micromolar as calculated from the mean fluorescence intensity of MT-4 cells labeled with the FITC-conjugated anti-CD4 mAb) was plotted against the CD4 down-modulating capability in SupT1 cells (micromolar IC₅₀ value).

tively), only a slight diminution of the CD4 down-regulating potency was observed (5- and 2-fold, respectively).

Finally, the importance of the toluenesulfonyl groups was investigated. The 1,5-di-methanesulfonyl analog MFS105 was completely devoid of any CD4 down-regulating activity. Deletion of the *p*-methyl from the tosyl entity (compound 95-211) or replacement of the *p*-methyl by a more bulky group (*n*-butyloxymethylenephylsulfonyl in compound MFS117) led to a complete loss of the CD4 down-regulating activity. The presence of a third tosyl group instead of the 9-benzyl substituent (compound AS112) also had a negative impact on the activity, possibly because of decreased basicity of the substituted nitrogen. Replacement of the tosyl methyls by bromines (as in compound AS-PB127) decreased the CD4 down-regulating activity by 2- to 4-fold, whereas substitution of the *p*-methyl by amino (as in compound MFS-PB001) made the compound inactive in CD4 down-regulation.

As listed in Table 1, the IC_{50} values for CD4 receptor down-modulation in the MT-4 T cell line are comparable with those obtained in SupT1 cells. When the IC_{50} values of the CADA analogs in MT-4 cells were plotted as a function of their IC_{50} values for CD4 down-regulation in SupT1 cells (on a linear-linear scale) (Fig. 2), regression analysis showed a strong linear correlation ($r = 0.95$) between the CD4 down-modulating activities in both cell lines, demonstrating that the effect of the CADA analogs on the CD4 receptor expression is not restricted to a specific T cell line.

Antiviral Activity of CADA Analogs against HIV-1.

Table 1 also presents the anti-HIV-1 activity of the different CADA derivatives. The prototype compound CADA inhibited HIV-1 NL4.3 replication in MT-4 cells at an IC_{50} of 1.21 μ M.

As was the case for the CD4 down-regulating activity, compound QJ028 also seemed to be the most active analog when evaluated for its antiviral potency. Also, the anti-HIV-1 activity of compound QJ023 correlated with its CD4 down-regulating activity. The dose-response effects of CADA, QJ023, QJ028, and QJ033 on HIV inhibition and CD4 down-modulation are shown in Fig. 3. MT-4 cells were treated with different concentrations of each compound (16, 3.2, 0.64, and 0.13 μ M). After 4 days of incubation, CD4 receptor expression was measured by flow cytometry. As shown in Fig. 3, CADA at a concentration of 16 and 3.2 μ M significantly down-modulated CD4 receptor expression, whereas at 0.64 μ M, 58% down-regulation was measured. A lower dose of the compound (i.e., 0.13 μ M) had no inhibitory effect on CD4 receptor expression. When the anti-HIV-1 activity of CADA was measured in MT-4 cells, a similar dose-dependent effect of CADA on the NL4.3 infection was observed. Thus, MT-4 cells were infected with the HIV-1 strain NL4.3 in the presence of decreasing concentrations of each compound (16, 3.2, 0.64, and 0.13 μ M). After 4 days of incubation, when CPE was clearly visible, the supernatant was collected and viral replication was measured by p24 Ag ELISA. High concentrations of CADA (i.e., 16 and 3.2 μ M) resulted in a significant inhibition of viral replication (Fig. 3). CADA at a dose of 0.64 μ M resulted in a 25% inhibition of virus production, whereas a lower dose of the compound (i.e., 0.13 μ M) had no anti-HIV-1 activity, as seen from the p24 core antigen values (392 ng/ml compared with 325 ng/ml for the infected control). These results demonstrate that the CD4 down-regulating activity of CADA directly correlated with its anti-HIV potency. A similar correlation was observed for other com-

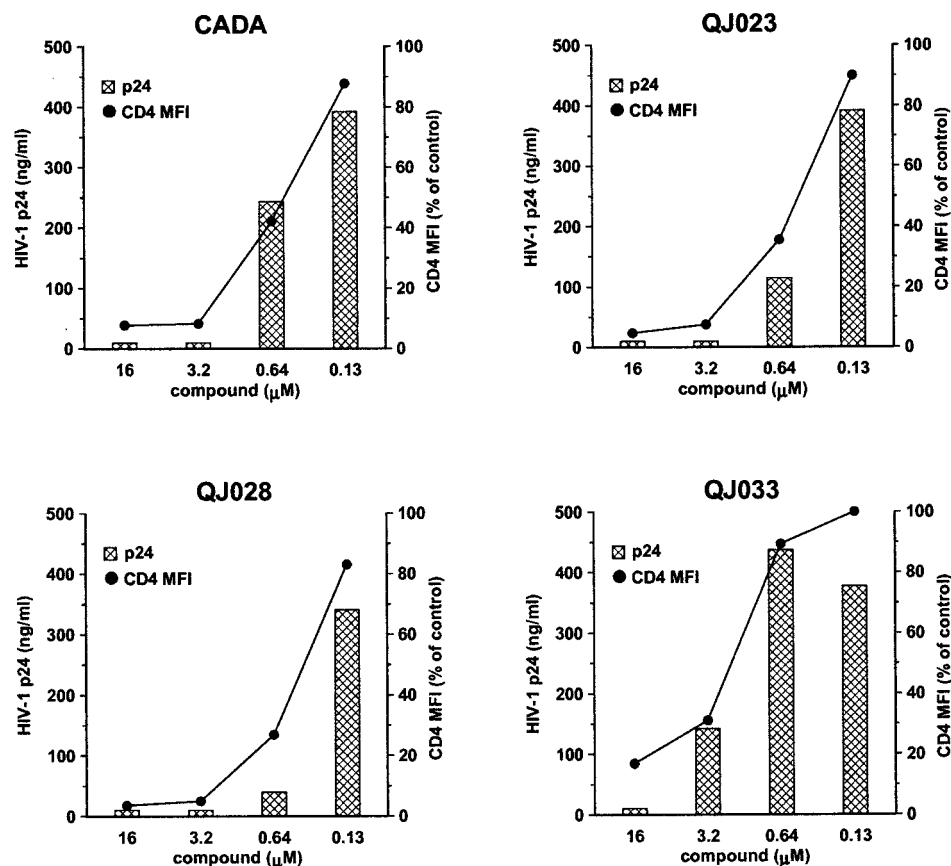


Fig. 3. Correlation between anti-HIV potency and CD4 down-modulating capability of CADA, QJ023, QJ028, and QJ033. MT-4 cells were infected with NL4.3 in the presence of different concentrations of the compounds. After 4 days, the supernatant was collected and analyzed for its p24 viral Ag content (vertical bars). In parallel, uninfected MT-4 cells were treated with the same concentrations of the analogs, and CD4 expression was analyzed flow cytometrically after 4 days of incubation (line). The MFI of the Leu3a-FITC staining was calculated for the different compounds, and is expressed as percentage of the MFI of control MT-4 cells. One representative experiment of three is shown.

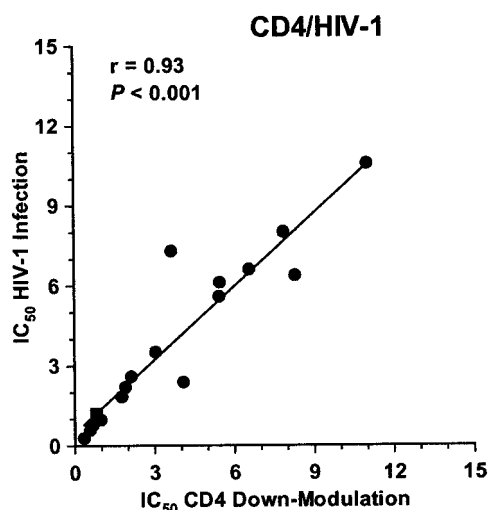


Fig. 4. Correlation of the anti-HIV-1 (NL4.3) activity and CD4 expression down-modulation by the different CADA analogs (i.e., CADA, QJ023, QJ027, QJ028, QJ029, QJ030, QJ033, QJ035, QJ036, QJ037, QJ038, QJ040, QJ041, AS-N6P6, HJC321, AS117, and AS-PB127) in MT-4 cells, as assessed by linear regression analysis. For each analog, the anti-HIV-1 activity (micromolar IC_{50} value) was plotted against the CD4 down-modulating capability (micromolar IC_{50} value calculated from the mean fluorescence intensity of MT-4 cells labeled with the FITC-conjugated anti-CD4 mAb).

pounds (Fig. 3). Administration of $0.64 \mu\text{M}$ QJ028 to the cell cultures resulted in 88% inhibition of NL4.3 infection and 73% down-regulation of the CD4 receptor. When compound QJ033 was tested for its antiviral activity and CD4 down-modulating potency, inhibition levels of 56 and 69%, respectively, could be measured at a concentration of $3.2 \mu\text{M}$, whereas at $0.64 \mu\text{M}$, neither antiviral activity nor CD4 down-regulation were observed (Fig. 3). Also, for the other CADA analogs, comparable IC_{50} values were obtained for CD4 down-regulation and inhibition of virus replication (Table 1).

Correlation between HIV-1 and CD4 Down-Modulation. The IC_{50} values of the CADA derivatives for CD4 receptor down-modulation were compared with their IC_{50} values for inhibition of HIV-1 replication. There was a close correlation among the inhibitory effects of the compounds on HIV-1 NL4.3 infection and CD4 receptor expression. When IC_{50} values of the CADA analogs for HIV-1 replication were plotted as a function of their IC_{50} values for CD4 down-regulation (on a linear-linear scale) (Fig. 4), regression analysis showed a strong linear correlation ($r = 0.93$) between the inhibitory effects on HIV-1 infection and on CD4 receptor expression.

Discussion

In an earlier study, we demonstrated that the lead compound, CADA, has anti-HIV and anti-HHV-7 activity (Vermeire et al., 2002). Interestingly, CADA was shown to specifically down-modulate the CD4 receptor but had no effect on 20 other cellular surface antigens examined, including the HIV coreceptors CXCR4 and CCR5. Analysis of CD4 mRNA levels suggested that CADA was not involved in the regulation of CD4 expression at a transcriptional level (Vermeire et al., 2002). Presumably, the drug interferes at the (post)translational level of CD4 expression, but the exact mechanism of this interaction is still subject of further investigation.

From previous work (Vermeire et al., 2002), it was suggested that the antiviral activity of CADA must be attributable solely to its down-regulating effect on the CD4 receptor expression, because the CD4 molecule is the main receptor for entry of HIV (Dalglish et al., 1984; Klatzmann et al., 1984). Therefore, a number of CADA derivatives have been tested for their effect on CD4 receptor down-modulation and HIV-1 infection. The CD4 down-regulating activity of 18 CADA analogs could be detected not only in the MT-4 T cell line but also in SupT1 cells. Furthermore, almost similar IC_{50} values were measured in both cell lines (Fig. 2), indicating that the effect of the different CADA derivatives on CD4 receptor expression is not restricted to one specific cell type. These data are in accordance with earlier findings that the CD4 down-modulating activity of the lead compound CADA could be detected in all cell types examined (T cells, monocytes/macrophages, PBMCs, and CD4-transfected U87 cells) (Vermeire et al., 2002).

The activity of the CADA compounds seemed to depend on the substituent at N^9 of triazacyclododecane (ideally the cyclohexylmethylene group in compound QJ028), which is probably needed for the interaction with its target. Small modifications of this cyclohexylmethylene group (e.g., insertions of one or two double bonds into the benzyl group or substitution by smaller cyclic structures) had slight detrimental effects on the CD4 down-modulating and antiviral activity. Also, introduction of a nitrogen atom at different positions of the aromatic ring (e.g., compounds QJ030 and 98-037) affected the CD4 down-regulating potency in different ways, possibly because of altered distribution of the positive and negative charges in the ring structure. Replacement of the benzyl group by an aliphatic chain (as in compounds QJ035–QJ038) usually diminished the activity of the compounds. For these analogs, the length of the open chain seemed to be crucial for their CD4 down-regulating potency; i.e., the size of the alkyl group had to be 5 carbons to reach a level of activity similar to that of CADA. This led us to suggest that the length of the substituent, and thus its bulkiness, may influence the efficiency of interference with the CD4 receptor expression. In addition, the presence of the two toluenesulfonyl groups also seemed to be of major importance for the activity of the compounds. This might explain that modifications of the tosyl groups as well as alterations of the three-carbon bridge between the tosyl groups (resulting in disturbance of the distance between the 2 tosyl groups) usually led to a complete loss of activity.

Here, we suggest that the CADA analogs, which possess activity against HIV-1, most probably act through down-regulation of CD4 receptor expression. We demonstrated that CADA derivatives that are able to down-modulate CD4 inhibit HIV-1 infection. The most active agents that emerged were QJ028 and QJ023. Similar IC_{50} values were obtained for HIV-1 inhibition and for down-modulation of CD4 expression, which means that the CADA analogs interfere with HIV entry through a CD4 down-regulating mechanism. An almost perfect correlation could be observed between their anti-HIV and CD4 down-modulating activity for a wide range of CADA derivatives (Fig. 4).

HIV infection requires the binding of the envelope glycoprotein gp120 to the primary receptor CD4 (Dalglish et al., 1984; Klatzmann et al., 1984) and is, consequently, more efficient when the CD4 receptor is abundantly expressed on

the surface of its target cells. It has been described that primary T cell-tropic viruses infect a panel of HeLa-CD4 cell clones that differ in CD4 quantities over a broad range with efficiencies that are linearly dependent on cell surface densities of CD4 (Kabat et al., 1994; Kozak et al., 1997). Because multimeric CD4 binding is required for efficient HIV infection (Layne et al., 1990), CD4 receptor density must play a crucial role in the efficiency of viral infectivity (Platt et al., 1997). Thus, drugs with CD4 down-modulating activity, such as CADA, can strongly inhibit virus entry by reducing the CD4 receptor density below a level that is required for infection. Although several CD4-independent HIV-1 strains have been described previously (Dumonceaux et al., 1998; Hoffman et al., 1999; Kolchinsky et al., 1999; LaBranche et al., 1999), these viruses show higher infectivity and replicative ability when CD4 is present. In addition, CD4 independence of HIV has been correlated with enhanced sensitivity to antibody mediated neutralization (Hoffman et al., 1999; Edwards et al., 2001; Kolchinsky et al., 2001).

In conclusion, our data demonstrate that the antiviral activity of the CADA analogs is mediated by their specific CD4 down-regulating potency. Because these compounds are not available for oral administration, they might not be immediate candidates for clinical HIV trials. However, the CADA derivatives will be helpful in improving our understanding of the involvement of the CD4 receptor in a number of immune responses and human diseases (e.g., rheumatoid arthritis). Thus, down-modulation of the CD4 receptor might be an interesting approach to diminish the immune reactivity of CD4⁺ T cells, which opens new perspectives for future drug design based on interference with CD4 receptor-mediated processes.

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