CADA, a novel CD4-targeted HIV inhibitor, is synergistic with various anti-HIV drugs *in vitro*

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Objective: To evaluate the anti-HIV-1 activity of the cyclotriazadisulfonamide CADA against primary isolates *in vitro* and the combination of CADA with approved anti-HIV drugs for potential synergy.

Methods: Peripheral blood mononuclear cells (PBMC) were treated with CADA and infected with 16 different clinical isolates. After 8 days of infection, the median inhibitory concentration (IC_{50}) was calculated from the p24 viral antigen content in the supernatant. MT-4 cells were infected with HIV-$1_{NL4.3}$ and then cultured with CADA or other antiretroviral drugs (i.e., several reverse transcriptase, protease and entry inhibitors), alone and in combination. After 4 days, IC_{50} was determined for the various drugs in replicate assays. Analysis of combined effects was performed using the median effect principle (CalcuSyn; Biosoft).

Results: The entry inhibitor CADA exerted a potent and consistent anti-HIV-1 activity against a wide range of R5, R5/X4 and X4 primary isolates in PBMC. From the two-drug studies, combination indices showed synergy between CADA and reverse transcriptase inhibitors (zidovudine, stavudine, lamivudine, zalcitabine, didanosine, abacavir, tenofovir, nevirapine, delavirdine and efavirenz), and protease inhibitors (lopinavir, saquinavir, indinavir, nelfinavir, amprenavir and ritonavir). In addition, the combination of CADA with the gp41 fusion inhibitor T-20 (enfuvirtide), the CXCR4 antagonist AMD3100 and the gp120-specific interacting plant lectins from *Galanthus nivalis* (GNA) and *Hippeastrum* hybrid (HHA) also resulted in a synergistic inhibition.

Conclusions: Compounds that can specifically downmodulate the CD4 receptor in PBMC have broad-spectrum anti-HIV activity against primary isolates and act synergistically when used in conjunction with currently available antiretroviral drugs. They deserve further study as potential candidate anti-HIV drugs.

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Introduction

The use of highly active antiretroviral therapy (HAART) with double or triple drug combinations chosen from the reverse transcriptase (RT) and protease inhibitors has significantly improved the survival of patients with AIDS. However, the emergence of drug resistance in the virus and both short- and long-term drug-related side effects are among the main reasons for continuing the development of new classes of effective anti-HIV drug that target the replicative cycle at different sites. One of the most promising targets is the viral entry–fusion process, in which the attachment of viral gp120 to the cellular CD4 receptor is the initial step of this complex multistep process [1,2]. Binding of HIV to the CD4 receptor induces a conformational change that brings gp120 into proximity with a cellular coreceptor. The chemokine receptors CCR5 and

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CXCR4 were identified as the principal coreceptors for viral entry into T lymphocytes and macrophages [3,4]. Following the interaction of the viral gp120 with the chemokine receptor, a more dramatic conformational change in the gp120–gp41 complex leads to the formation of the trimer-of-hairpins structure in gp41, enabling the viral envelope to fuse with the cell membrane and, subsequently, releasing the viral capsid into the cytoplasm of the target cell [5].

The synthetic macrocycle cyclotriazadisulfonamide (CADA) has consistent CD4-downmodulating activity in T cell lines (i.e., MT-4, SupT1, MOLT-4 and Jurkat), CD4-transfected U87 cells and in peripheral blood mononuclear cells (PBMC) [6,7]. Interestingly, CADA does not alter the expression of any other cellular receptor (or HIV coreceptor) examined [6]. It was shown earlier that CADA has potent activity against a broad range of HIV strains (i.e., laboratory-adapted HIV-1 and HIV-2 strains) [6]. Pretreatment of the cells with the drug for 24 h further enhanced the anti-HIV activity of CADA. As reported previously, the antiviral activity of CADA has been tentatively attributed to the specific CD4-downregulating potency of this compound and, importantly, correlated with its ability to downmodulate the CD4 receptor [6]. In fact, a close correlation was observed between the CD4-downmodulating and anti-HIV potencies of 17 different CADA derivatives, further pointing to CD4 receptor downregulation as the primary and unique mode of antiviral action of this novel group of HIV inhibitors [8].

A prerequisite in the development of new anti-HIV agents is that they possess potent antiviral activity against clinical isolates. Also, they should retain their activity in the presence of other antiretroviral drugs without exerting antagonistic interactions. In this report, we demonstrate the consistent anti-HIV activity of CADA against a set of R5, R5/X4, and X4 clinical isolates in PBMC. The combination of CADA with a variety of approved anti-HIV drugs acting at different steps in the HIV replicative cycle was evaluated. Two-drug combination assays were performed with multiple RT, protease, as well as entry–fusion inhibitors to examine *in vitro* synergistic anti-HIV activity between CADA and all other antiretroviral agents.

Materials and methods

Viruses

The T-tropic (X4) HIV-1 molecular clone NL4.3 was obtained from the NIAID AIDS Reagent Program (National Institutes of Health Bethesda, Maryland, USA). Virus stocks of the clinical isolates (6–72) were generated by coculture of PBMC from healthy donors with lymphocytes from an HIV-1-infected person. Coreceptor usage of the viruses was determined by viral replication in CXCR4- and CCR5-transfected U87.CD4 cells.

Cells

The CD4 cell line MT-4 was obtained from the American Type Culture Collection (Rockville, Maryland, USA) and cultured in RPMI 1640 medium (Gibco BRL, Gaithersburg, Maryland, USA) with 10% heat-inactivated fetal calf serum (Biowhittaker Europe, Verviers, Belgium) and 2 mmol/l L-glutamine (Gibco BRL). Buffy coat preparations from healthy donors were obtained from the Blood Bank in Leuven. PBMC were first isolated by density gradient centrifugation over Lymphoprep ($d = 1.077$ g/ml) (Nycomed, Oslo, Norway) and then stimulated with 2 μg/ml phytohemagglutinin (Sigma Chemical, Bornem, Belgium) for 3 days at 37°C.

Flow cytometric analysis

To study the effect of CADA on surface CD4 receptor expression, uninfected PBMC were incubated with a serial fivefold dilution of the compound at 37°C. After 3 days, cells were washed with phosphate-buffered saline (PBS) containing 2% fetal calf serum and incubated with fluoroscein isothiocyanate- or phycoerythrin-labeled monoclonal antibody (BD, San Jose, California, USA) for 30 min at 4°C. As a negative control for unspecific background staining, cells were stained in parallel with Simultest Control γ_1/γ_{2a} (BD). Then the cells were washed, fixed with 1% formaldehyde, and analyzed by flow cytometry with a FACScalibur (BD). Data were acquired and analyzed with CellQuest software (BD) to obtain the percentage of CD4 fluorescent cells and the mean fluorescence intensity of each sample.

Compounds and drugs

CADA was synthesized as described elsewhere [8,9] and dissolved at 16 mmol/l in dimethylsulfoxide (DMSO). For the RT and protease inhibitors, all drugs were provided in their prescription form. Zidovudine (Retrovir; GlaxoSmithKline, Research Triangle Park, North Carolina, USA) was dissolved at 10 mmol/l in PBS, stavudine (Zerit; Bristol-Myers Squibb, Princeton, New Jersey, USA) at 50 mmol/l in DMSO, lamivudine (Epivir-HBV; GlaxoSmithKline/Shire Pharmaceuticals, Basingstoke, UK) at 87.22 mmol/l in DMSO, zalcitabine (Hivid; Roche Laboratories, Nutley, New Jersey, USA) at 50 mmol/l in PBS, didanosine (Videx EC; Bristol-Myers Squibb) at 10 mmol/l in PBS, abacavir sulfate (Ziagen; GlaxoSmithKline) at 50 mmol/l in DMSO, tenofovir disoproxil fumarate (Viread; Gilead Sciences, Foster City, California, USA) at 10 mmol/l in PBS, nevirapine (Viramune; Boehringer Ingelheim Pharmaceuticals, Ridgefield, Connecticut, USA) at 67.56 mmol/l in DMSO, delavirdine

mesylate (Rescriptor; Pfizer, New York, New Jersey and Agouron Pharmaceuticals, San Diego, California, USA) at 36.26 mmol/l in DMSO, and efavirenz (Sustiva; Bristol-Myers Squibb) at 52.66 mmol/l in DMSO. The following protease inhibitors were used: indinavir sulfate (Crixivan; Merck, Whitehouse Station, New Jersey, USA) dissolved at 15.87 mmol/l in DMSO, ritonavir (Norvir; Abbott Laboratories, North Chicago, Illinois, USA) at 12.33 mmol/l in DMSO, lopinavir (ABT-378; Abbott Laboratories) at 15.42 mmol/l in DMSO, saquinavir (Fortovase; Roche Laboratories) at 14.52 mmol/l in DMSO, nelfinavir mesylate (Viracept; Pfizer/Agouron Pharmaceuticals) at 17.02 mmol/l in DMSO, and amprenavir (Agenerase; GlaxoSmithKline/Vertex Pharmaceuticals, Cambridge, Massachusetts, USA) at 19.36 mmol/l in DMSO. The fusion inhibitor T-20 (Trimeris, Durham, North Carolina, USA) was dissolved at 2 mg/ml in PBS. The specific CXCR4 antagonist AMD3100 was synthesized as described previously [10] and was dissolved at 5 mg/ml in DMSO. The mannose-specific plant lectins *Galanthus nivalis* (GNA) and *Hippeastrum* hybrid (HHA) were derived and purified from the bulbs of these plants, as described elsewhere [11,12], and were dissolved at 1 mg/ml in distilled water.

Antiviral assays

For the anti-HIV activity of CADA against primary isolates, fivefold dilutions of the compound (in 250 μl medium) were added to each well of 48-well flat bottom plates (Iwaki Glass, Iwaki, Japan). Then PBMC were seeded in the tissue culture plates (5×10^5 cells in 200 μl medium) together with interleukin-2 (1 ng/ml) (R&D Systems Europe, Abingdon, UK) and 50 μl diluted virus stocks of the primary isolates at a final concentration of 1000 pg/ml. After 3 days, 100 μl fresh medium with interleukin-2 was added. The supernatant of each sample was collected after 8 days of incubation, stored at −20°C and analyzed for HIV-1 core antigen by p24 antigen enzyme-linked immunoassay (ELISA; Perkin Elmer, Boston, Massachusetts, USA). Cell viability of the PBMC was measured after 8 days of incubation by trypan blue exclusion. No cytotoxicity was observed in uninfected PBMC at the highest concentration of CADA (16 μmol/l).

The anti-HIV-1 activity of each drug in MT-4 cells was determined using a tetrazolium-based colorimetric assay [13]. Threefold dilutions of the drugs in 100 μl medium were added to duplicate wells of 96-well flat bottom plates (Iwaki Glass). Then MT-4 cells were seeded in the tissue culture plates (7.5×10^4 cells in 50 μl medium), and finally 50 μl diluted HIV-$1_{NL4.3}$ stock (20× the median tissue culture infective dose) was added to each well, resulting in a final volume of 200 μl. Cytopathic effect induced by the virus was checked regularly microscopically. After 4 days of infection, when a strong cytopathic effect was observed in the positive control (i.e., untreated) HIV-1-infected cells, the cell viability was assessed spectrophotometrically via the *in situ* reduction of the tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium inner salt, using the CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Madison, Wisconsin, USA). The absorbance was then recorded at 490 nm with a 96-well plate reader and compared with four cell control replicates (cells without virus and drugs) and four virus control wells (cells with virus but without drugs). Each assay was performed at least three times. The median inhibitory concentration (IC_{50}), or the concentration that inhibited HIV-induced cell death by 50%, was calculated from each dose–response curve.

Combination experiments and evaluation of synergy

After an IC_{50} was obtained for each drug, the anti-HIV-1 activity of each compound alone or in combination with CADA was tested. Assays were designed so that the IC_{50} value of the drug would occur in the middle of the dilution range (threefold dilutions). Therefore, dose–response curves from an ineffective concentration to a maximally effective concentration could be determined for each compound. The antiviral activity was assayed in a single 96-well plate with cell control, virus control, dilutions of CADA in duplicate, dilutions of the drug to be tested in duplicate, and three different fixed ratios of CADA and the drug. The ratios were based on the IC_{50} values for each antiviral drug alone. The assay was designed so that the first ratio of CADA to each drug approached the IC_{50}:IC_{50} ratio for the two drugs. For the second ratio, CADA (at the same concentration as in the first ratio) was mixed in combination with the appropriate drug at one-third the concentration as in the first ratio. For the third ratio, one-third the concentration of CADA was mixed with the other compound at the same concentration as in the first ratio. The result was that CADA was compared with each antiviral drug at three different ratios. Each experiment with drug combinations was performed three to five times.

After incubation with HIV-1, the cell viability was assessed as described in the antiviral assay above. The percentage of viable cells was calculated as a mean of duplicate infections (duplicate wells). The fraction affected, which equals the percentage of viable cells, at each dilution was calculated for CADA, for the drug to be tested, and for each ratio of CADA and drug. The combination index (CI) was calculated according to the method of Chou and Talalay [14], using the CalcuSyn for Windows software package (Biosoft, Cambridge, UK). A mutually exclusive model of analysis was used. The CI values are estimated from the data and reflect the nature of the interaction between drugs: < 1,

synergistic activity; 1, additive; > 1, antagonism. Thus, the value of CI is inversely proportional to the degree of synergy in the combination regimen. For ease of interpretation, the CI values for the calculated IC_{50}, concentration giving 75% inhibition (IC_{75}), and 95% inhibition (IC_{95}) values of only the first ratio (equipotent ratio) are reported here.

Results

Figure 1 shows the structure of the synthetic macrocycle cyclotriazadisulfonamide CADA.

Antiviral activity of CADA against primary isolates

In previous reports, the antiviral activity of CADA against different laboratory strains of HIV-1 and HIV-2 were demonstrated [6]. To evaluate the anti-HIV activity of CADA further against clinical isolates, multiple experiments were performed in PBMC in which CADA was tested against a set of R5-, R5/X4- and X4-using primary isolates. Viral replication of all isolates was found to be inhibited by the drug at a dose of 3.2 μmol/l, as evident from the low p24 antigen production in the supernatant (Fig. 2, left panel). In fact, complete protection could be observed for most isolates at this dose of CADA. Treatment with 0.64 μmol/l CADA resulted, on average, in 32% inhibition of viral replication, whereas a dose of 0.13 μmol/l had little or no protective effect (viral replication was, on average, 103% of control) (Fig. 2). Generally, the IC_{50} value of CADA was almost constant for the different clinical isolates (mean IC_{50} values were 1.04, 0.97 and 1.27 μmol/l for R5, R5/X4 and X4 isolates, respectively) and so did not depend on the coreceptor usage of the virus. Analysis of surface CD4 expression in uninfected control cells showed a significant downregulation at a dose of 3.2 μmol/l CADA (mean fluorescence intensity 12 versus 78 for untreated cells) (Fig. 2, right panel). The CD4 receptor expression gradually increased at lower concentrations of the compound (mean fluorescence intensity: 30 and 60 at 0.64 and 0.13 μmol/l, respectively).

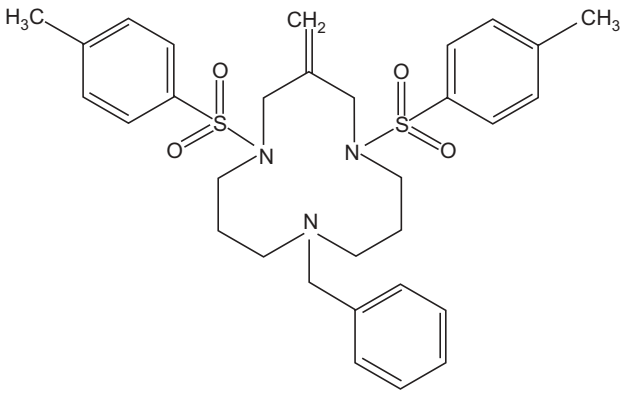


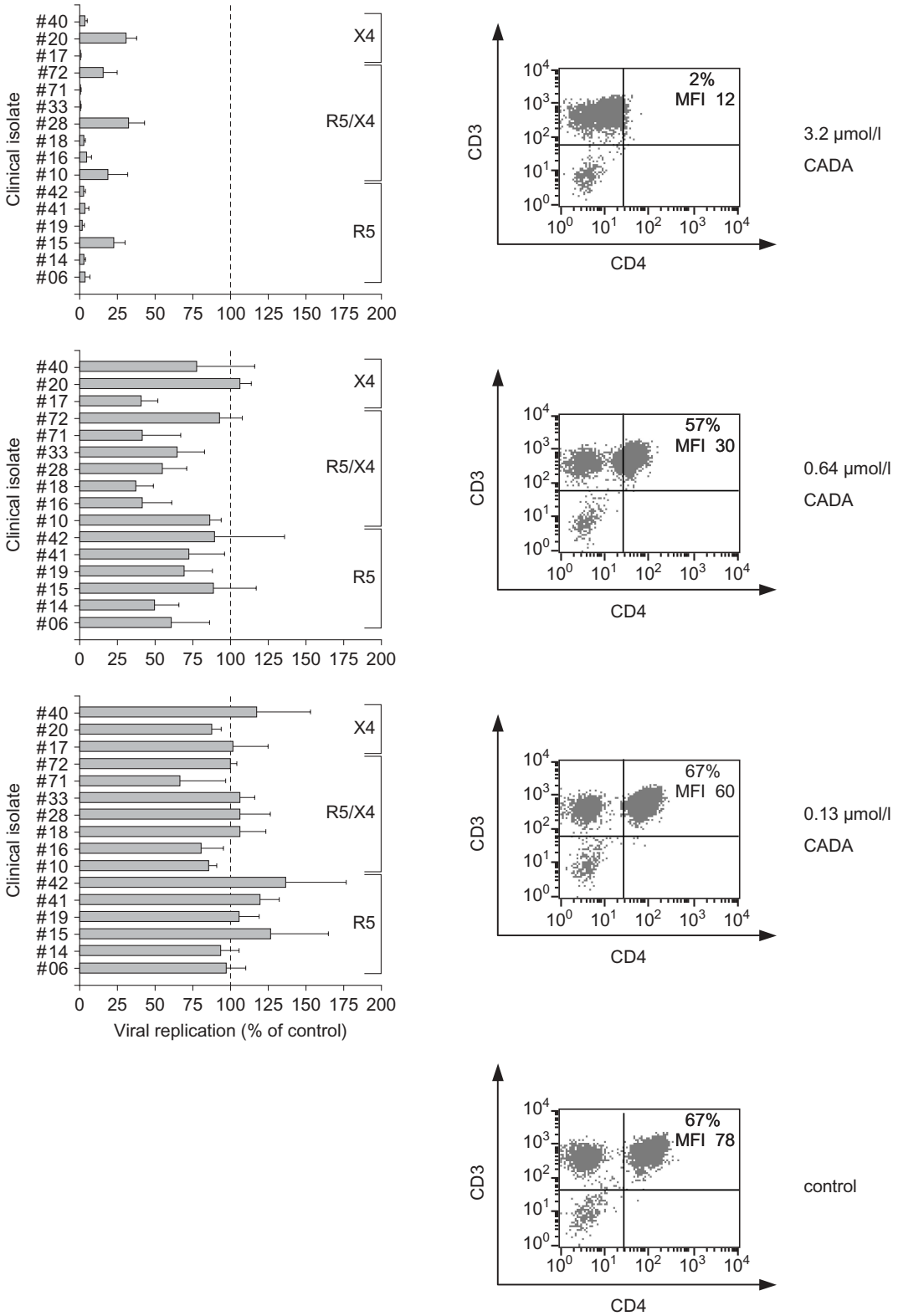
Fig. 1. Chemical structure of CADA (9-benzyl-3-methylene-1,5-di-*p*-toluenesulfonyl-1,5,9-triazacyclododecane).

In addition, when CADA was administered to MT-4 cells infected with the laboratory-adapted X4 strain HIV-$1_{NL4.3}$, a comparable IC_{50} value was obtained (mean IC_{50} 1.09 μmol/l), indicating that CADA has the same anti-HIV activity for the laboratory strain as for the primary isolates. The use of a tetrazolium-based colorimetric assay [13] for the determination of the antiviral activity of CADA in MT-4 cells resulted in similar IC_{50} values as found using the p24 antigen ELISA data [8]. Therefore, the more convenient colorimetric assay was used in the MT-4/HIV-$1_{NL4.3}$ combination studies of CADA with other antiretroviral drugs.

Combination of CADA with reverse transcriptase inhibitors

The antiviral activity of CADA was evaluated in combination with drugs currently approved by the US Food and Drug Administration (FDA) within the classes nucleoside reverse transcriptase inhibitors (NRTI) and nucleotide reverse transcriptase inhibitors (NtRTI). MT-4 cells, infected with HIV-$1_{NL4.3}$, were incubated with increasing concentrations of CADA alone, RT inhibitor alone, or the 1:1 equipotent fixed ratio combination as described above. No cytotoxicity

Fig. 2. Antiviral and CD4-downregulating activity of CADA in peripheral blood mononuclear cells (PBMC). (Left panels) PBMC were treated with increasing concentrations of CADA and infected with six R5, seven R5/X4 or three X4 primary isolates. After 8 days of infection, supernatant was collected; p24 antigen content in the supernatant was measured and compared with that of untreated infected cells in order to determine the percentage viral replication. Bars represent mean ±SEM of at least three different experiments. (Right panels) In parallel, CD4 expression in uninfected PBMC was determined to show the CD4-downmodulating activity of CADA after 3 days of treatment. Dot plots represent the flow cytometric analysis of CD4 and CD3 receptor expression on the cell surface of lymphocytes by staining with the fluoroscein isothiocyanate-labeled CD4-specific monoclonal antibody (clone SK3) and the phycoerythrin-labeled CD3-specific monoclonal antibody (clone Leu-4). The percentage of CD4 T cells and the mean fluorescence intensity (MFI) of CD4 fluorescence is indicated in each dot plot. The data shown are from one representative experiment, which was repeated four times with comparable results by using PBMC from four different donors.



was observed in uninfected MT-4 cells at the highest concentration of drug used (data not shown). The IC_{50} values (mean ±SD of three to four independent experiments) for CADA, the NRTI/NtRTI and their combinations were determined (Table 1). The IC_{50} for CADA as single treatment ranged from 0.9 to 1.3 μmol/l and significantly fell to 0.3 μmol/l (on average) when used in combination with each NRTI/NtRTI ($P < 0.05$ according to Student's t-test). For the NRTI/NtRTI, there was a 1.8- to 2.9-fold reduction of the IC_{50} values when used in combination with CADA, which was significant for zidovudine, stavudine, lamivudine and abacavir ($P < 0.05$).

CADA was then tested in combination with non-nucleoside reverse transcriptase inhibitors (NNRTI). In the combination experiments with nevirapine, delavirdine and efavirenz (Table 1), IC_{50} values (mean of three to four independent experiments) for single CADA treatment were 1.3, 1.1 and 1.1 μmol/l, respectively, and significantly decreased to 0.3, 0.2 and 0.5 μmol/l when tested in a two-drug combination ($P < 0.05$). For the NNRTI, combination with CADA resulted in a 2.3-fold reduction of the IC_{50} for nevirapine ($P < 0.001$), a 3.1-fold reduction for delavirdine ($P < 0.05$) and a 1.8-fold reduction of the IC_{50} for efavirenz (Table 1).

Synergistic action of CADA and reverse transcriptase inhibitors

CI values were determined to investigate possible synergistic interactions between CADA and RT inhibitors. The combination of CADA with the NRTI/NtRTI and the NNRTI are illustrated in Table 2. CI values at the 1:1 fixed drug ratio [i.e., the equipotent ratio (IC_{50}/IC_{50}) CADA/RT inhibitor] at the calculated IC_{50}, IC_{75} and IC_{95} concentrations, were all < 1, indicating, according to the method of Chou and Talalay [14], a synergistic interaction between CADA and the RT inhibitors (Table 2). Synergism was seen (CI, 0.3–0.7) when CADA was combined at the calculated IC_{95} concentration with zidovudine, lamivudine, zalcitabine, didanosine, abacavir, tenofovir or delavirdine; moderate synergism (CI, 0.7–0.85) was observed for stavudine, nevirapine and efavirenz. Comparable CI values were also obtained at the 3:1 and 1:3 fixed drug ratio, which confirmed the synergistic interaction between CADA and the RT inhibitors (data not shown).

Table 1. Median inhibitory concentrations (IC_{50})[a] of CADA and clinically available anti-HIV drugs as single drug treatment and in combination at equipotent ratio.

	IC_{50} drug alone[b]		IC_{50} combination[c]	
Inhibitor	CADA (μmol/l)	Inhibitor	CADA (μmol/l)	Inhibitor
NRTI/NtRTI				
Zidovudine (nmol/l)	1.26 ± 0.14[d]	11.34 ± 0.60	0.34 ± 0.10*	5.50 ± 0.86*
Stavudine (μmol/l)	0.98 ± 0.21	0.32 ± 0.08	0.36 ± 0.12*	0.15 ± 0.04*
Lamivudine (μmol/l)	0.88 ± 0.03	1.29 ± 0.43	0.29 ± 0.10**	0.64 ± 0.19*
Zalcitabine (μmol/l)	1.01 ± 0.13	0.95 ± 0.45	0.26 ± 0.16**	0.33 ± 0.16
Didanosine (μmol/l)	1.08 ± 0.16	5.20 ± 1.08	0.29 ± 0.03*	2.91 ± 0.30
Abacavir (μmol/l)	1.19 ± 0.03	1.41 ± 0.05	0.31 ± 0.11*	0.72 ± 0.17*
Tenofovir (μmol/l)	1.11 ± 0.28	1.70 ± 0.79	0.27 ± 0.06*	0.68 ± 0.18
NNRTI				
Nevirapine (nmol/l)	1.27 ± 0.30	28.24 ± 3.32	0.34 ± 0.17*	12.14 ± 2.47**
Delavirdine (nmol/l)	1.06 ± 0.26	13.62 ± 1.32	0.24 ± 0.02*	4.40 ± 0.41*
Efavirenz (nmol/l)	1.07 ± 0.22	1.30 ± 0.50	0.46 ± 0.11*	0.73 ± 0.30
Protease inhibitors				
Lopinavir (nmol/l)	0.97 ± 0.24	9.31 ± 2.79	0.28 ± 0.06*	4.33 ± 0.99*
Saquinavir (nmol/l)	1.24 ± 0.11	12.27 ± 2.70	0.29 ± 0.05*	6.32 ± 1.05*
Amprenavir (nmol/l)	1.26 ± 0.10	28.63 ± 7.13	0.55 ± 0.12*	16.00 ± 3.39
Indinavir (nmol/l)	0.94 ± 0.24	24.30 ± 5.42	0.45 ± 0.12*	10.66 ± 2.95*
Nelfinavir (nmol/l)	1.01 ± 0.22	18.35 ± 6.94	0.28 ± 0.07*	9.49 ± 2.39
Ritonavir (nmol/l)	1.32 ± 0.03	64.13 ± 16.56	0.38 ± 0.11*	13.93 ± 4.06*
Entry inhibitors				
AMD3100 (nmol/l)	1.02 ± 0.17	20.97 ± 4.41	0.41 ± 0.13*	8.18 ± 2.52*
Enfuvirtide (ng/ml)	0.95 ± 0.28	41.86 ± 19.74	0.46 ± 0.13	11.59 ± 3.15
Lectin GNA (ng/ml)	1.11 ± 0.41	135.63 ± 48.32	0.48 ± 0.14	47.84 ± 14.14
Lectin HHA (ng/ml)	0.96 ± 0.31	106.60 ± 37.39	0.38 ± 0.15	37.91 ± 14.89

NRTI, nucleoside reverse transcriptase inhibitor; NtRTI, nucleotide reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor.
[a]The median inhibitory concentration for NL4.3 infection in MT-4 cells.
[b]Single drug treatment (CADA alone or the inhibitor alone); mean ±SD from at least three independent experiments.
[c]Two-drug combinations at the equipotent ratio (1:1 or IC_{50} CADA/IC_{50} inhibitor); mean ±SD from at least three independent experiments.
*$P < 0.05$; ** $P < 0.001$ (Student's t-test for unequal variances) compared with single drug treatment.

Table 2. Combination indices for two-drug combinations of CADA with reverse transcriptase inhibitors.

	CI at varying HIV-1 inhibition[b]			
Drug[a]	50%	75%	95%	Synergy[c]
NRTI/NtRTI				
Zidovudine	0.88 ± 0.20	0.67 ± 0.18	0.49 ± 0.19	+++
Stavudine	0.83 ± 0.15	0.77 ± 0.20	0.72 ± 0.24	++
Lamivudine	0.83 ± 0.10	0.64 ± 0.13	0.51 ± 0.13	+++
Zalcitabine	0.69 ± 0.12	0.49 ± 0.17	0.34 ± 0.20	+++
Didanosine	0.84 ± 0.07	0.69 ± 0.16	0.58 ± 0.20	+++
Abacavir	0.75 ± 0.04	0.66 ± 0.05	0.58 ± 0.06	+++
Tenofovir	0.68 ± 0.20	0.56 ± 0.24	0.49 ± 0.28	+++
NNRTI				
Nevirapine	0.83 ± 0.15	0.78 ± 0.13	0.72 ± 0.12	++
Delavirdine	0.57 ± 0.15	0.45 ± 0.22	0.37 ± 0.25	+++
Efavirenz	0.96 ± 0.03	0.87 ± 0.08	0.82 ± 0.17	++

NRTI, nucleoside reverse transcriptase inhibitor; NtRTI, nucleotide reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; CI, combination index.
[a]Two-drug combinations of CADA with the indicated inhibitor. For each CADA–inhibitor combination, a 1:1 fixed drug ratio was used based on the median inhibitory concentrations (IC_{50}) of each drug alone and represents the equipotent ratio (IC_{50} CADA/IC_{50} drug).
[b]CI (mean ±SD from at least three independent experiments): < 1, synergism; 1, additive effect; > 1, antagonism.
[c]Synergy at the calculated concentration giving 95% inhibition (IC_{95}): +, slight synergism (CI, 0.85–0.90); ++, moderate synergism (CI, 0.7–0.85); +++, synergism (CI,: 0.3–0.7); ++++, strong synergism (CI , 0.1–0.3).

Synergistic action of CADA and protease inhibitors

A second important class of currently FDA-approved antiretroviral drugs are the protease inhibitors. In our anti-HIV-1 single drug test system, all protease inhibitors had antiviral activity in the lower nanomolar range, whereas the IC_{50} values for CADA were 1.0–1.3 μmol/l (Table 1). Combining CADA with the protease inhibitors resulted in a significant reduction of the IC_{50} for CADA, lopinavir, saquinavir, indinavir and ritonavir ($P < 0.05$); for amprenavir and nelfinavir, respectively, a 1.8- and 1.9-fold reduction of the IC_{50} was noted (Table 1). The two-drug treatment CADA/ritonavir proved to be the most effective combination, as a 3.5- and 4.6-fold decrease of the IC_{50} for CADA and ritonavir, respectively, was observed.

The CI value for CADA in combination with protease inhibitors are given in Table 3. A marked synergistic interaction (CI, < 0.6) could be observed when CADA was combined with ritonavir at all inhibitory concentration levels. At the calculated IC_{95}, CADA appeared to be potently synergistic (CI, < 0.65) with all protease inhibitors tested with the exception of amprenavir, for which a moderate synergistic interaction was noted. In general, the 1:1 combination of CADA with each protease inhibitor resulted in synergism (CI, < 1) at all calculated inhibitory concentra-

Table 3. Combination indices for two-drug combinations of CADA with protease inhibitors.

	CI at varying HIV-1 inhibition[b]			
Protease inhibitor[a]	50%	75%	95%	Synergy[c]
Lopinavir	0.77 ± 0.05[d]	0.59 ± 0.04	0.46 ± 0.03	+++
Saquinavir	0.79 ± 0.10	0.62 ± 0.10	0.49 ± 0.12	+++
Amprenavir	0.99 ± 0.05	0.88 ± 0.02	0.80 ± 0.04	++
Indinavir	0.88 ± 0.07	0.75 ± 0.10	0.64 ± 0.13	+++
Nelfinavir	0.84 ± 0.24	0.69 ± 0.30	0.59 ± 0.35	+++
Ritonavir	0.59 ± 0.20	0.46 ± 0.17	0.37 ± 0.14	+++

CI, combination index.
[a]Two-drug combinations of CADA with the indicated protease inhibitor. For each drug combination, a 1:1 fixed drug ratio is used based on median inhibitory concentrations (IC_{50}) of each drug alone and represents the equipotent ratio (IC_{50} CADA/IC_{50} drug).
[b]CI (mean ±SD from at least three independent experiments): < 1, synergism; 1, additive effect; > 1, antagonism.
[c]Synergy at the calculated concentration giving 95% inhibition (IC_{95}): +, slight synergism (CI, 0.85–0.90); ++, moderate synergism (CI, 0.7–0.85); +++, synergism (CI,: 0.3–0.7); ++++, strong synergism (CI , 0.1–0.3).

tions (Table 3), which was also the case at the 3:1 and 1:3 fixed drug ratio (data not shown).

Synergistic action of CADA and entry inhibitors

Finally, combinations of CADA with other inhibitors of HIV-1 entry–fusion were evaluated. As the fusion inhibitor T-20 (enfuvirtide) is the only FDA-approved entry inhibitor to date, other agents that target viral entry were also included. The bicyclam AMD3100 is a specific antagonist for the HIV coreceptor CXCR4 [15,16]. The two mannose-specific plant lectins GNA and HHA have been reported to suppress HIV infection and HIV transmission by preventing entry of HIV into its target cells via targeting the heavily glycosylated gp120 envelope glycoprotein [17,18]. The combination of CADA with AMD3100 resulted in a 2.5-fold reduction of the IC_{50} value for both drugs ($P < 0.05$) (Table 1). When CADA was administered together with enfuvirtide, there was a 2- and 3.6-fold decrease of the IC_{50} value for CADA and enfuvirtide, respectively. For both gp120-interacting lectins, the combination with CADA induced a 2.8-fold fall in their IC_{50} values.

As summarized in Table 4, CADA exhibited potent synergistic interactions with the viral entry inhibitors AMD3100, T-20, GNA and HHA at the 1:1 equipotent ratio (all CI values, < 0.8). However, when CADA was combined with each entry inhibitor at the 3:1 or 1:3 fixed ratio, moderate synergistic (i.e., CI, 0.7–0.85) to nearly additive (i.e., CI, 0.90–1.10) interactions were observed (data not shown).

Discussion

Cyclotriazadisulfonamides, of which CADA (Fig. 1) can be considered as a lead compound, represent a novel class of anti-HIV drugs with specific CD4 receptor downmodulating activity [6–8]. Their mechanism of action has not been completely elucidated; however, it has been demonstrated that CADA specifically decreases the expression of surface CD4 without affecting other cellular receptors. CADA does not bind directly to the extracellular part of cell surface CD4 with subsequent receptor internalisation, nor does it act at the transcriptional level [6]. An interesting feature of the cyclotriazadisulfonamides is the reversible nature of their CD4-downregulating activity: that is, CD4 expression on the cells is rapidly restored to normal levels after removal of the drug [6]. Administration of CADA to CD4 cells results in a quantitative decrease (almost 90%) of the CD4 receptor expression and, thus, in a reduction of the CD4 receptor density to below the level that is required for efficient HIV infection. What the impact is of a diminished CD4 receptor expression on the complex interplay between the immune cells, and if the residual CD4 expression on the surface of CADA-treated immunocompetent cells is still sufficient to elicit the desired immune responses, is currently under investigation.

We have previously shown the activity of CADA against laboratory HIV-1 strains and HIV-1 variants resistant to RT and entry inhibitors [6]. In the present study, we tested CADA for its antiviral potency against primary HIV-1 isolates, as this will be of interest if this class of CD4-interacting HIV inhibitors is used in a clinical setting. For the 16 different isolates examined, we found a consistent activity against R5-, R5/X4- and X4-using primary isolates, demonstrating a broad anti-HIV spectrum for CADA. The antiviral activity also correlated with the potency of CADA to decrease the surface CD4 expression in PBMC. Several reports have shown that the cell surface CD4 receptor density is extremely important for the efficiency of viral infection [19–25], especially for clinical isolates, which have a lower affinity for the CD4 receptor than do laboratory-adapted virus strains [21,22,24].

Table 4. Combination indices for two-drug combinations of CADA with entry inhibitors.

	CI at varying HIV-1 inhibition[b]			
Drug[a]	50%	75%	95%	Synergy[c]
Entry inhibitors				
AMD3100	0.79 ± 0.10	0.69 ± 0.09	0.61 ± 0.10	+++
Enfuvirtide (T-20)	0.79 ± 0.04	0.67 ± 0.07	0.58 ± 0.09	+++
Lectin GNA	0.80 ± 0.08	0.71 ± 0.05	0.64 ± 0.10	+++
Lectin HHA	0.74 ± 0.08	0.64 ± 0.15	0.56 ± 0.20	+++

CI, combination index.
[a]Two-drug combinations of CADA with the indicated entry inhibitor. For each drug combination, a 1:1 fixed drug ratio is used based on median inhibitory concentrations (IC_{50}) of each drug alone and represents the equipotent ratio (IC_{50} CADA/IC_{50} drug).
[b]CI (mean ±SD from at least three independent experiments): < 1, synergism; 1, additive effect; > 1, antagonism.
[c]Synergy at the calculated concentration giving 95% inhibition (IC_{95}): +, slight synergism (CI, 0.85–0.90); ++, moderate synergism (CI, 0.7–0.85); +++, synergism (CI,: 0.3–0.7); ++++, strong synergism (CI , 0.1–0.3).

Next, CADA was tested as a single agent and was also evaluated in combination with other antiretroviral compounds in order to determine if there were possible adverse effects arising from their interaction. CADA inhibited HIV-$1_{NL4.3}$ infection of T cells, either as a single drug or as part of a combination regimen. There was a clear decrease in IC_{50} of CADA and the other inhibitors when used in combination than when used alone. These data indicate that the same anti-HIV effect can be obtained with lower doses of the individual drugs and, thus, with less toxic side-effects. At the 95% inhibition level, synergism (CI, < 0.7) was observed in dual treatments with all RT inhibitors, except for stavudine, nevirapine and efavirenz, for which the combination could be best characterized as moderate synergistic (CI, 0.70–0.85). Besides the moderate synergism between CADA and amprenavir, the interaction of CADA with protease inhibitors proved also to be synergistic, especially with ritonavir, for which a significant synergism (CI, < 0.6) was observed at all inhibitory concentration values tested.

As CADA targets viral infection at the fusion–entry step, we wanted also to evaluate its interaction with other entry inhibitors. The combination of CADA with AMD3100, T-20, GNA or HHA at an optimal 1:1 equipotent fixed ratio did result in a synergistic inhibition (CI, < 0.8). Notably, when an optimal 1:1 ratio was used for CADA and each entry inhibitor (Table 4), a general stronger (synergistic) inhibition of HIV-1 replication could be observed compared with that for the 1:3 and 3:1 ratios (not shown). These data may suggest a need to optimize each antiviral drug 'cocktail' as CADA seems to augment the activity of other entry inhibitors for efficient blocking of virus replication when both agents are provided at sufficient amounts (i.e., at the 1:1 ratio). As the CD4 receptor is involved in attachment of the virus to the cell [1,2], as well as in the exposure of the coreceptor binding site on gp120 [26,27] and in the fusogenic conformation of gp41 [27,28], one can expect that a decrease in the CD4 receptor density will positively influence the activity of agents that target sequential steps in the fusion–entry process. Accordingly, Allaway *et al.* [29] reported that CD4-based molecules, which inhibit HIV attachment, act synergistically with anti-gp120 and anti-gp41 antibodies, which block HIV-1 fusion.

The attachment of the HIV envelope to cellular CD4 represents an important target for new antiviral therapies. When used in HIV prophylaxis, drugs that interfere with the earliest events in the replication cycle may have an advantage over existing therapeutic approaches that target the viral enzymes RT and protease, as they may prevent virus entry into new target cells and subsequently reduce the number of latent reservoirs for HIV. To date, a number of agents that interfere with CD4 receptor attachment have been accredited with potent *in vitro* anti-HIV activity [30–34]. Although soluble CD4 therapy in patients with AIDS did not fulfill the high therapeutic expectations [35], probably because of activation of gp120 for subsequent interaction with the HIV coreceptor [36,37], several more recent reports have pointed to blocking of binding to CD4 receptor as a successful approach to suppress AIDS virus replication *in vivo* [38–44]. Furthermore, as several domains of the CD4 receptor are used by HIV to enter CD4 cells [45], a specific downmodulator of the complete CD4 receptor, such as CADA, may be considered as a more effective antiviral agent with activity against a wide variety of HIV strains and isolates.

Although CD4 is the primary receptor for HIV entry, several CD4-independent HIV strains have been reported [46–50]. These viruses appear to infect their target cells in the absence of the CD4 receptor by using a coreceptor, although they show higher infectivity and replicative ability when CD4 is expressed on the cell surface. CD4-independent HIV isolates can also be obtained from HIV-infected persons, but these viruses show enhanced sensitivity to antibody-mediated neutralization [47,50–52]. In addition, one can expect that treatment with CD4-downmodulating drugs will ultimately result in the selection of clinical isolates with reduced CD4 dependency; however, the ability of viruses to infect cells with low levels of CD4 seems to correlate with increased sensitivity to neutralization [53–54].

Our data clearly show that entry inhibitors are likely to act synergistically with current HIV therapies directed against post-entry steps, an observation that is in line with previously reported combination data. Antiviral synergy has been observed between naphthalene sulfonate polymers (PIC 024-4 and PRO 2000) – which bind to CD4 and block binding of gp120 – and zidovudine [32]. Also the CXCR4-binding chemokine Met-SDF-1β showed synergy to additivity with either zidovudine or nelfinavir [55]. The CCR5 antagonist SCH-C has been reported to exert synergistic interactions with several NRTI, NNRTI and protease inhibitors [56].

The approval of the fusion inhibitor T-20, currently renamed as enfuvirtide (Fuzeon), provides proof of principle for the development of entry inhibitors as practical and potent antiviral agents. The clinical investigation of T-20 has followed demonstration of its marked *in vitro* antiviral activity [57–59] and of synergism between T-20 and other antiretroviral drugs, such as the CD4–immunoglobulin fusion protein PRO 542 [60], the CCR5 antagonist SCH-C [56] and the CXCR4 antagonist AMD3100 [61]. The significant virological advantage of T-20 treatment in several clinical trials [62–64] further encourages the potential

use of entry inhibitors as a component of multidrug salvage therapy in patients highly experienced in antiretroviral therapy.

In conclusion, the results of this study demonstrate that the CD4-downmodulating compound CADA has a potent anti-HIV activity against a broad spectrum of primary isolates and a favorable interaction *in vitro* with several RT, protease and entry inhibitors. However, further studies are needed to address whether synergistic interactions also exist *in vivo* and to examine possible toxicity and pharmacokinetic issues. As an increasing number of drug-resistant HIV isolates is emerging, compounds that specifically interact with the CD4 receptor, such as the cyclotriazadisulfonamides, should deserve further attention as a novel class of potential antiretroviral drug.

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