CADA, a Potential Anti-HIV Microbicide that Specifically Targets the Cellular CD4 Receptor

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Abstract: The cyclotriazadisulfonamide (CADA) compounds are a new class of specific CD4-targeted HIV entry inhibitors. The *in vitro* anti-HIV activity of CADA was shown to correlate with its ability to specifically downmodulate cell surface expression of the CD4 receptor in human cells. Here, we evaluated its potential as an anti-HIV microbicide. CADA exerted a clear CD4 receptor downregulating effect in dendritic cells (DC) and subsequently inhibited HIV-1_{BaL} replication in DC/T cell co-cultures. The compound proved to be active against a variety of clinical isolates belonging to the HIV-1 subtypes A, B, C, D, F, G, H, AE and O. Furthermore, it prevented human T cells from being infected with the laboratory-adapted strains X4 HIV-$1_{NL4.3}$ and R5 SIV_{mac251}. Flow cytometric analysis demonstrated a significant and dose-dependent downregulation of CD4 on macaque PBMCs. In addition, the compound exerted a marked anti-SIV_{mac251} activity in these cells from simian origin. The combination of CADA with cellulose acetate phthalate (CAP) resulted in a synergistic inhibition of HIV-1 and SIV infection. Finally, gel formulated CADA proved to preserve the CD4 downmodulating and antiviral activity of this compound when formulated as a microbicide gel. Thus, our data suggest that CADA may have potential as a broad-spectrum anti-HIV microbicide drug candidate. The preservation of the activity of gel formulated CADA will make it now feasible for testing this unique entry inhibitor in non-human primates, not only as a single drug but also in a synergistic conjunction with other anti-HIV compounds.

Keywords: Cyclotriazadisulfonamide (CADA), CD4 receptor, microbicide, HIV entry, SIV, broad spectrum.

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INTRODUCTION

In the effort to halt the spread of HIV, great attention has been given to the development of effective microbicides [1]. Most HIV transmission occurs on the mucosal surfaces of the cervicovaginal and gastrointestinal tracts during unprotected vaginal or rectal sexual intercourse. In order to prevent HIV entering the body through the surfaces lining the vagina, cervix, colon or rectum, topical microbicides can be applied as a practical and cost-effective method to block HIV transmission. This prevention strategy should empower women to protect themselves, especially in sub-Saharan Africa where young women are at high risk of HIV-1 infection [2].

A microbicide must be safe, acceptable, efficacious, and affordable in order to be useful for slowing the global spread of HIV-1 [1, 3]. Intensive research worldwide has resulted in more than 60 candidate microbicides in development, and few of them were already in phase III clinical trial stage at the beginning of 2006. The spermicide nonoxynol-9 was the first compound evaluated for its potential as a microbicide. Although it showed *in vitro* activity against HIV-1 and other sexually transmitted infections and prevented simian immunodeficiency virus (SIV) infections in macaques [4-9], nonoxynol-9 failed to exert protection against HIV-1 transmission in women. In clinical trials it caused toxic side-effects and even enhanced HIV-1 infection and transmission, so that it was no longer pursued as a potential HIV-1 preventive agent [10]. The microbicide cellulose sulfate (CS) was found save in a phase I safety study [11]. Unfortunately, in early 2007, the International AIDS Society (IAS; www.ias2007.org) announced the discontinuation of the phase III clinical trial because preliminary results indicated an increased risk of HIV transmission/infection in CS-treated women, resulting in another failure of efficacy clinical trials of microbicides. However, based on the promising results of Carraguard and PRO 2000 [12, 13], assessment of other potential microbicides should continue, but with great caution and with more emphasis on preclinical demonstration of efficacy and safety.

The small molecule cyclotriazadisulfonamide (CADA) has been reported to be a novel promising HIV entry inhibitor [14, 15]. The compound significantly decreases the amount of cell surface CD4 - the main receptor for HIV - without altering the expression of any other cellular receptor examined so far. This specific CD4 receptor downmodulating compound inhibited the infection of different HIV-1 laboratory strains and subtype B clinical isolates, irrespective of coreceptor usage [14-16]. Moreover, for this class of entry inhibitors a clear correlation was recorded between the CD4 downregulating and antiviral activity [17, 18]. In addition, CADA exerted a synergistic anti-HIV activity when com-

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bined with different reverse transcriptase, protease and entry inhibitors, such as fuzeon [15].

In this paper, we further investigated the microbicidal potential of CADA. The prevention of HIV-1 transmission in co-cultures of dendritic cells and T lymphocytes, and the broad spectrum antiviral property of CADA against several HIV subtypes was investigated *in vitro*. Also, in view of further testing in an *in vivo* model, the preservation of CADA's biological activity in macaque cells was examined, as well as the successful formulation of this entry inhibitor in a microbicidal gel.

MATERIALS AND METHODS

Compounds

The compound CADA.HCl was synthesized as described previously [19] and used in the salt form. Cellulose acetate phthalate (CAP) and polyethylene glycol 400 were obtained from Acros Organics (Geel, Belgium). Hercules (Wilmington, DE, USA) supplied Natrosol 250 HHX Pharm (hydroxyethylcellulose). 2-Hydroxypropyl-β-cyclodextrin (HPβ CD, Fluka), methyl- and propylparaben were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Viruses and Cell Cultures

The HIV-1 T-tropic (X4) molecular clone NL4.3 (HIV-$1_{NL4.3}$) and the EHO strain of HIV-2 (HIV-2_{EHO}) were obtained from the National Institute of Allergy and Infectious Disease AIDS Reagent Program (Bethesda, MD). Their viral stocks were collected from the culture supernatant of HIV-infected MT-4 cells. The HIV-1 M-tropic (R5) molecular clone BaL ($R5_{BaL}$) was kindly provided by the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program (Rockville, MD) and its viral stock was collected from the culture supernatant of infected PHA-preactivated PBMCs. Primary clinical isolates representing different HIV-1 clades were all kindly provided by L. Lathey from BBI Biotech Research Laboratories, Inc., Gaithersburg, MD, and their co-receptor use (R5 or X4) was determined by us on the astroglioma U87.CD4 cell line transfected with either CCR5 or CXCR4. Viral stocks of the clinical isolates were generated by coculture of PBMCs from healthy donors with lymphocytes from an HIV-1 infected person. The pathogenic SIV virus strain SIV_{mac251} was kindly provided by R. Le Grand (CEA, Fontenay aux Roses, France) [20]. The $CD4^+$ T-cell lines MT-4 and SupT1 were obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured in RPMI-1640 medium (Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (FBS; BioWhittaker Europe, Verviers, Belgium) and 2 mM L-glutamine (Life Technologies). The PM1 cell line was obtained from the National Institute of Allergy and Infectious Diseases AIDS Reagent Program (Bethesda, MD) and maintained in RPMI 1640 supplemented with 10% fetal bovine serum. All cell cultures were maintained at 37°C in a humidified, CO_2-controlled atmosphere, and subcultivations were done every 2 to 3 days.

Primary Cells

Buffy coat preparations from healthy donors were obtained from the Blood Bank in Leuven, Belgium. Human PBMC were first isolated by density gradient centrifugation over Lymphoprep (d = 1.077 g/ml) (Nycomed, Oslo, Norway). Monocytes were isolated from peripheral blood by positive selection using CD14 magnetic beads (Miltenyi Biotech GmbH, Gladbach, Germany) according to the manufacturer's instructions, with the exception of the use of cold (4°C) phosphate buffered saline supplemented with 1% human serum and 2 mM EDTA (Sigma). Direct sorting yielded purities of ≥98% $CD14^+$ monocytes as assessed by flow cytometry (CD3 and CD14 staining). Monocytes were converted to immature monocyte-derived DCs (MO-DCs) by upright culturing in T-25 or T-75 culture flasks (Becton Dickinson, San Jose, CA) at concentrations of 1 x 106 cells/ml in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (BioWhittaker, Walkersville, MD), 400 U/ml granulocyte-macrophage colony-stimulating factor (GM-CSF), and 500 U/ml interleukin-4 (IL-4) (both from PeproTech, Rocky Hill, NJ). After 6 days of culturing without additional feeding, DC populations have the characteristic surface phenotype of high levels of CD206, CD209, and CD1a and negligible CD25, CD80, CD83 and CD14. At this time point, half of the culture medium was replaced with fresh IL-4-GM-CSF medium, and MO-DCs were used only within a time frame of 3 days after this time point. In parallel, the lymphocyte fraction was frozen in liquid nitrogen and thawed on the day of infection. $CD4^+$ T cells were purified by positive selection, using a $CD4^+$ isolation kit (Dynal, Oslo, Norway) and yielded purities of > 95% $CD4^+$ and $CD3^+$.

Isolation of PBMC from the Blood of Macaques

Blood was collected from cynomolgus macaques (*Macaca fascicularis*) as described elsewhere [21] and blood mononuclear cells were obtained by centrifugation on a Ficoll density gradient. Red blood cells were then lysed by exposure for 5 min to hypotonic shock, and PBMC were washed twice in PBS. Cells were then frozen in liquid nitrogen and thawed on the day of the experiment.

DC/T Cell Co-Culture Assay

MO-DCs were suspended at 2 X 10^6 cells/ml in complete medium. Fifty µl of DCs was dispensed in a 96-well plate and 50 µl of either medium (Fig. **1c**) indicated as 'DC') or resting autologous $CD4^+$ T cells at 2 X 10^6 cells/ml (Fig. **1c**) labeled as 'DC/T cells') was added. Then, CADA (100 µl), prepared in a 10-fold dilution series in complete medium was administered and cells with compound were incubated for 24 h. Next, HIV-1_{BaL} at a multiplicity of infection (MOI) of 10^{-3} was added and cells with virus were incubated for 2 h at 37°C. Afterwards, cells were washed at least six times to remove unbound virus and in the case of DC alone, at this moment autologous $CD4^+$ T cells were added, and the co-cultures were incubated with the same concentration of CADA for 24 h. Then, cells were washed again, and cultured in the presence of medium (Fig. **1c**) indicated as '1 d') or compound (Fig. **1c**) indicated as '14 d') for 2 weeks. At the end of the experiment HIV-1 p24 antigen was measured in the culture supernatant.

Antiviral Assay

MT-4 cells were infected with the HIV-1 strain $X4_{NL4.3}$. Briefly, 5-fold dilutions of the compounds or gels (in 100 µl) were added to 96-well flat-bottomed plates (International

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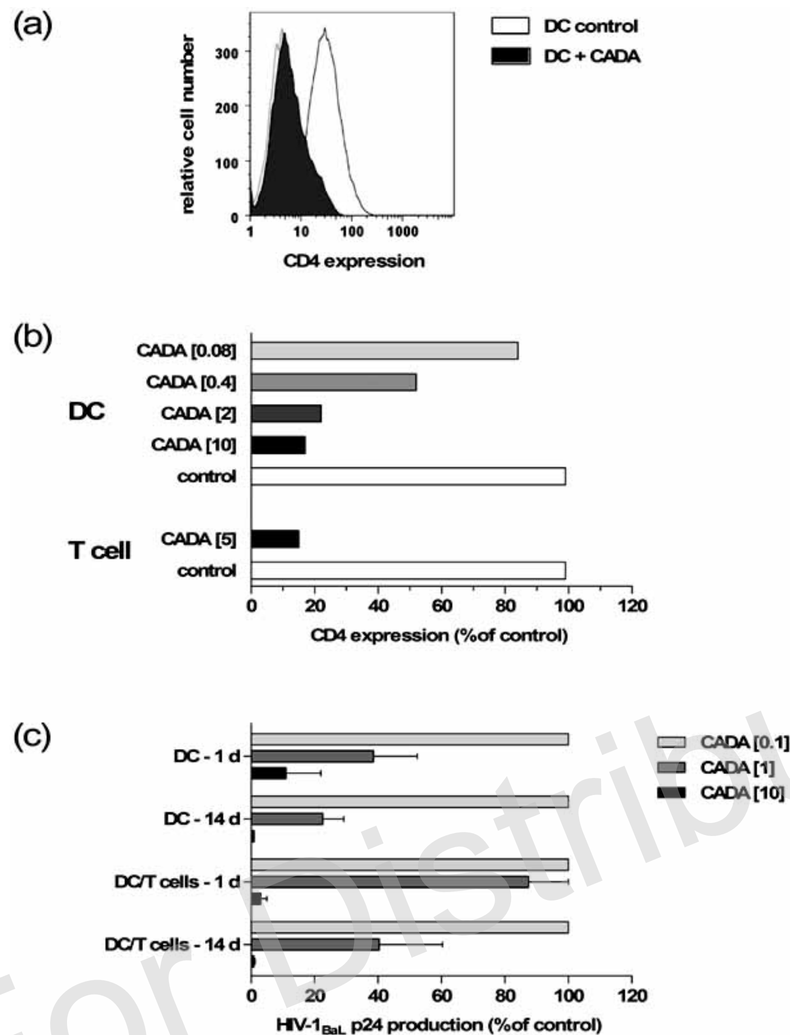

Fig. (1). Dose-dependent CD4 downmodulating activity of CADA in dendritic cells and subsequent inhibition of HIV-1 transmission to T cells. (**a**) $CD14^+$ monocytes were isolated from the blood of healthy donors and stimulated with GM-CSF and IL-4 to differentiate into immature monocyte-derived dendritic cells (MO-DC) as described in Materials and Methods. The MO-DC were then treated with CADA (5 µg/ml) for 24 hrs and stained with an anti-CD4 mAb. The cell surface CD4 expression of control cells (white histogram; MFI = 29.2) and CADA-treated cells (black histogram; MFI = 5.4) is shown. Fluorescence is plotted on a logarithmic scale. The peak for the corresponding non-specific background fluorescence measured with identical instrument settings (given by the white histogram in grey line) coincides with that of the CADA treated cells and is almost invisible on this picture (MFI = 4.6). These are data from one representative experiment, which was repeated several times with similar results. (**b**) MO-DC were treated with different concentrations of CADA (as indicated between brackets) for 24 hrs and subjected to CD4 staining. In parallel, the T cell lymphoma PM1 cells were treated with 5 µg/ml of CADA for 2 days before CD4 staining. The CD4 expression is given as percentage of that measured in the untreated cells. (**c**) MO-DCs were pre-incubated with CADA at the indicated concentrations during 24 h before exposure to HIV-1_{BaL} for 2 h. Next, MO-DC were extensively washed, mixed with autologous $CD4^+$ T cells and incubated with the compound during 24 h. Then co-cultures were washed and further cultured in the presence of medium (DC – 1 d) or compound (DC – 14 d) for 2 weeks. In addition, co-cultures of MO-DC and autologous $CD4^+$ T cells were pre-incubated with CADA at the indicated concentrations during 24 h, infected with HIV-1_{BaL} for 2 h, washed, incubated with CADA for 24 h, washed and further cultured in the presence of medium (DC/T cells – 1 d) or compound (DC/T cells – 14 d) for 2 weeks. At the end of the experiment, the amount of p24 was measured in the co-culture supernatant. Bars represent mean ± SEM of 2 (DC) or 4 (DC/T cells) independent experiments.

Medical, Brussels, Belgium). Then, to each well, 7.5 X 10^4 MT-4 cells were added in 50 µl of medium, followed by 50 µl (500 pg/ml p24 Ag) of diluted HIV-1 stock. Cytopathic effect (CPE) induced by the virus was checked microscopically at regular times. When strong CPE was observed (mostly after 4 or 5 days of incubation) in untreated HIV-infected cells, the supernatant of all samples was collected simultaneously and stored at – 20°C. We assessed productive HIV-1 infection by measuring p24 Ag concentration in culture medium using a p24 Ag ELISA commercial kit (Perkin Elmer, Boston, MA). For HIV-2 p27 Ag detection, the INNOTEST from Innogenetics (Temse, Belgium) was used. Finally, the EC_{50} value of the compounds (i.e., the concentration of the compound required for 50% reduction in HIV replication as measured by the p24 antigen production) was calculated.

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For the antiviral activity of CADA against the HIV-1 clinical isolates or SIV_{mac251}, PBMC were first stimulated with 2 µg/ml phytohemagglutinin (PHA) (Sigma Chemical, Bornem, Belgium) for 3 days at 37° C and washed prior infection. Five-fold dilutions of the compound (in 250 µl) were added to 48-well flat-bottomed plates (Iwaki Glass, Iwaki, Japan). Then, to each well, 5 X 10^5 PHA-blasts were added in 200 µl of medium supplemented with 1 ng/ml IL-2, followed by 50 µl (1000 pg/ml p24 or p27 Ag) of diluted HIV-1 stock. After 4 days, 100 µl fresh medium with IL-2 was added. The supernatant of each sample was collected after 10 days of incubation, stored at – 20° C and analyzed for its p24 or p27 content.

Thymocytes from cats were treated with different concentrations of CADA and infected with the cell culture-adapted feline immunodeficiency virus (FIV) isolate Petaluma as described in detail elsewhere [22].

Flow Cytometry

To study the effect of CADA on surface CD4 antigen expression, cells were incubated with a serial 5-fold dilution of CADA or medium at 37°C. CD4 expression was analyzed at 1 day (MO-DC) or 3 days (macaque PBMC) after the start of CADA treatment. Briefly, after washing with phosphate-buffered saline containing 2% FBS, cells were incubated with an anti-CD4 mAb for 30 min at RT. Then the cells were washed, fixed in 1% formaldehyde and analyzed immediately. The following mAbs were used: PE-labeled anti-human CD4-specific mAb clone SK3 (BD Biosciences, San Jose, CA); FITC-labeled anti-human CD4-specific mAb clone MT310 (DakoCytomation, Glostrup, Denmark); FITC-labeled anti-monkey CD3-specific mAb clone FN-18 (BioSource Europe, S.A., Nijvel, Belgium); PE-labeled anti-human CD8-specific mAb clone DK25 (DakoCytomation). Data were acquired with a FACSCalibur flow cytometer (BD Biosciences) using the 488 laser line and the CellQuest software (BD Biosciences). Data were analyzed with the FLOWJO software (Tree Star, San Carlos, CA). Downregulation of CD4 was evaluated by the downshift of fluorescence intensity of the CADA treated cells stained for CD4, relative to matched untreated cells stained for CD4. For the calculation of the CD4 receptor downmodulation, the median fluorescence intensity (MFI) for CD4 of each sample was expressed as percentage of the MFI of control cells (after subtracting the MFI of the unstained control cells). Finally, the EC_{50} value for CD4 downmodulation of CADA was calculated as the concentration of the compound required for 50% decrease of the expression of CD4 at the cell surface.

Gel Formulation of CADA

A gel formulation of CADA.HCl (final concentration 0.05%) was developed using hydroxyethylcellulose (HEC, 1.5%) as the gelling polymer, methyl- (0.18%) and propylparaben (0.02%) as preservatives and polyethylene glycol 400 (PEG 400, 5%), ethanol (3%) and 2-hydroxypropyl-β-cyclodextrin (HPβCD, 20% or 10%) as solubilizing excipients. First, CADA.HCl (6 mg/ml) was dissolved in a mixture of PEG 400 and ethanol (5:3). Subsequently, this mixture was added to a solution containing appropriate amounts of parabens and HPβCD in aqueous buffer (KH_2PO_4 20 mM, pH 4.7) to reach a final concentration of CADA.HCl of 0.05%. The resulting solution was added to HEC powder; upon mixing, a clear and homogeneous gel was formed. The pH of the gel amounted to 4.7. Corresponding placebo gels were prepared in a similar way, without inclusion of CADA.

Combination Experiment and Evaluation of Synergy

The antiviral activity of CADA in combination with CAP was tested. Assays were designed so that the EC_{50} value of the test compound would occur in the middle of the dilution range (3-fold dilutions). The combinational activity was assayed in a single 96-well plate as described in detail elsewhere [15]. The combination index (CI) was calculated according to the method of Chou and Talalay [23], 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 EC_{50}, concentration giving 75% inhibition (EC_{75}), and 95% inhibition (EC_{95}) values of the equipotent ratio are reported here.

Statistical Analysis

Statistical analysis performed on the results included the calculation of the mean, standard error of the mean (SEM) and P values by use of a paired Student t test. The significance level was set as $P = 0.05$, and the actual P values are indicated. Statistical analysis was performed with GraphPad Prism statistical software (GraphPad Software, Inc., San Diego, CA).

RESULTS

CADA Dose-Dependently Downmodulates the CD4 Expression in Dendritic Cells

Previously, we have demonstrated the specific CD4 receptor downregulating and anti-HIV-1 activity of CADA in human T cells [14, 15]. Here, we tested the potency of CADA in downmodulating CD4 on dendritic cells. Immature monocyte-derived dendritic cells (MO-DC) were treated with CADA for 24 hrs and stained with an anti-CD4 mAb. As shown in Fig. **1a**, treatment of dendritic cells with CADA resulted in a marked decline in cell surface CD4 expression. The median fluorescence intensity (MFI) for CD4 in CADA-treated cells was decreased by 1 log compared to that in untreated cells (MFI were 5.5 and 29.2, respectively; Fig. **1a**). Furthermore, the CD4 downmodulating effect of CADA in MO-DC was dose-dependent (Fig. **1b**). At a concentration of 0.4 µg/ml of CADA, a 50% reduction in CD4 expression was obtained (EC_{50} = 0.4 µg/ml). Treatment of MO-DC with 10 µg/ml of CADA resulted in 83% downregulation of cell surface CD4, an effect that was similar to that observed for CADA treatment of $CD4^+$ T cells (i.e., 85% CD4 downmodulation; Fig. **1b**).

CADA Inhibits HIV-1 Infection of MO-DC/T Cell Co-Cultures

We further investigated if the CD4 downregulating activity of CADA on dendritic cells was reflected by a prevention of HIV infection of MO-DC and the subsequent viral transmission to T cells. As expected, a very short exposure of dendritic cells or virus stock to CADA (i.e., 30 min pre-

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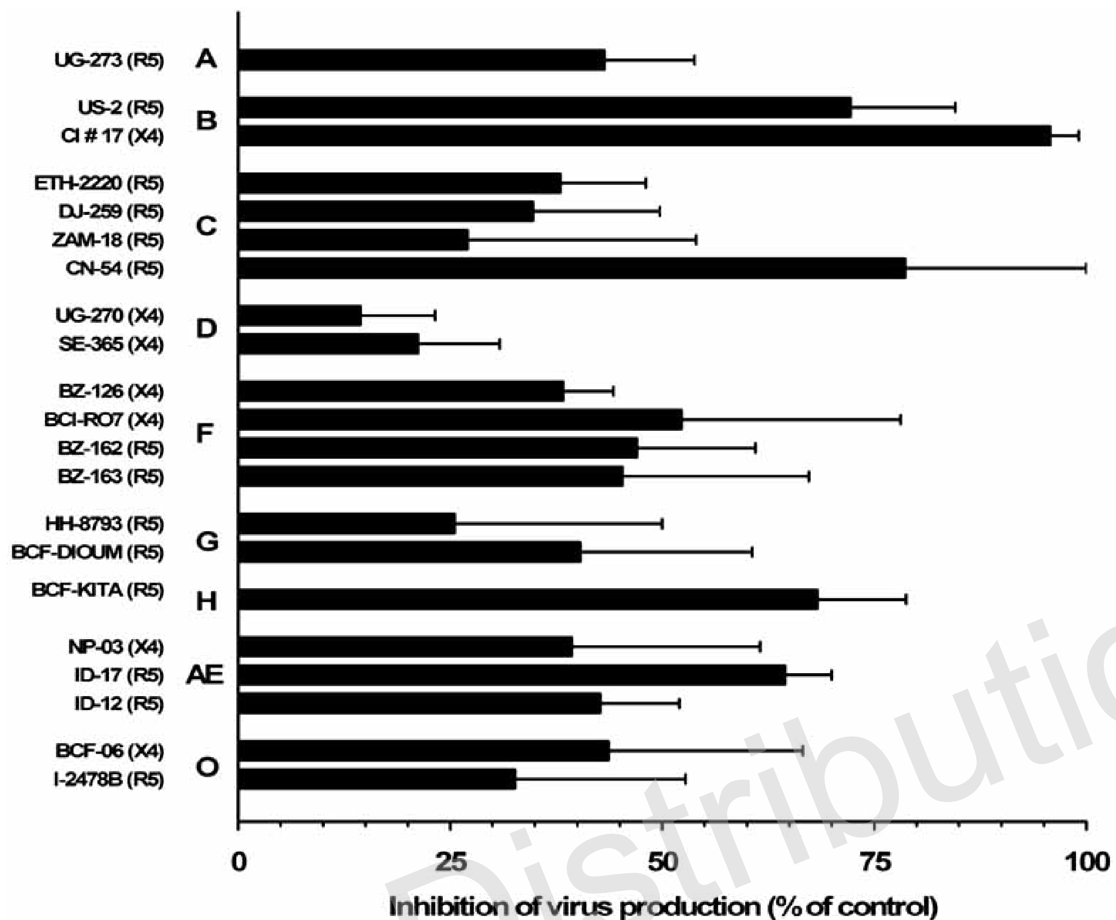

Fig. (2). Broad spectrum anti-HIV-1 activity of CADA against clinical isolates of different subtypes. Human PBMCs were exposed to 21 HIV isolates belonging to different clades (A, B, C, D, F, G, H and AE of group M and 2 isolates of group O) and treated with the CADA-compound at different concentrations. CADA-treatment was sustained throughout the experiment (2 weeks) and at the end of the experiment, the amount of p24 or p27 was measured in the supernatant. For each isolate, the experiment was performed several times with cells from a different donor. The figure summarizes the data obtained from experiments with PBMCs of up to 16 donors treated with 10 µg/ml of CADA. Bars represent mean ± SEM of 2 to 6 independent experiments.

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treatment) had no antiviral effect (not shown). However, CADA clearly inhibited HIV-1_{BaL} infection of DC/T cell co-cultures when the cells were pre-treated for 24 h prior virus exposure (Fig. **1c**), a time frame needed for CADA to bring about a maximum CD4 downmodulating effect in human cells [14, 18]. Administration of the compound to the DC subpopulation only was sufficient to result in a marked decrease in virus production in the co-culture supernatant (Fig. **1c**); DC – 1 d). The mean EC_{50} ± SEM for CADA was 0.8 ± 0.4 µg/ml, indicating a clear inhibitory activity of the compound for DC infection and subsequent HIV-1 transmission to autologous $CD4^+$ T cells. This antiviral effect of CADA was enhanced when the treatment was sustained for 2 weeks post virus challenge (mean EC_{50} ± SEM was 0.5 ± 0.1 µg/ml). Also, when the DC - T cell mixture was subjected to CADA pre-treatment (24 h) prior virus challenge, a significant antiviral effect of CADA was observed: at a concentration of 10 µg/ml of CADA the viral p24 production was almost at an undetectable level (*P* value = 0.0102, Student's *t*-test for comparison with untreated control). When CADA treatment of the co-culture was sustained for 2 weeks post virus challenge (Fig. **1c**), DC/T cells – 14 d), the anti-HIV activity of CADA was more pronounced as evidenced by lower p24 values measured at 1 and 10 µg/ml (mean EC_{50} ± SEM for CADA were 2.7 ± 0.6 and 1.1 ± 0.7 µg/ml, for a post infection treatment of 1 and 14 days, respectively).

Broad Spectrum Anti-HIV Activity of CADA Against Strains of Different Clades

Next, we evaluated the antiviral activity of CADA against 21 clinical isolates belonging to different clades of HIV-1. Human PBMCs were exposed to various HIV isolates and treated with the CADA-compound, and treatment was sustained throughout the experiment. For each isolate, the experiment was performed several times with cells from a different donor. In Fig. **2**, the data collected from experiments with PBMCs from 16 donors are summarized. In accordance to our earlier report [15], CADA inhibited viral replication of clade B clinical isolates, irrespective of co-receptor usage (EC_{50} were 0.9 and 2.8 µg/ml for X4 and R5, respectively). Furthermore, CADA exerted an antiviral activity against the panel of clinical isolates belonging to different subtypes: it proved to be active against viruses from clades A, B, C, D, F, G, H, AE and O. For many isolates we observed some donor-dependent variation in the antiviral potency of CADA (Fig. **2**). However, in overall, the compound

proved to prevent virus entry of all clades, resulting in a reduction of virus production for most isolates (i.e., on average approximately 50% decrease in virus production).

CD4 Downmodulation in Macaque PBMC

In view of our investigation of CADA's potential as a microbicide in an *in vivo* model, we first tested its CD4 downmodulating potency in cells of macaque origin. Cells were isolated from the blood of macaques, stimulated with PHA and IL-2, and then treated with CADA at different concentrations for 3 days. The flow cytometric analysis of the experiments with PBMCs from two different macaques is summarized in Fig. **3**. In both experiments, treatment of the cells with 10 and 2 µg/ml of CADA clearly diminished the expression of CD4 on the cell surface of T lymphocytes, whereas the expression of the CD3 and CD8 receptors on these cells was unaffected. Calculation of the EC_{50} concentration for CD4 down-modulation by CADA resulted in similar values in both experiments (EC_{50} were 0.65 and 0.69 µg/ml for donor 1 and 2, respectively).

Antiviral Activity of CADA Against Simian Immunodeficiency Virus Infection

For the investigation of the antiviral activity of CADA against SIV infection *in vitro*, human MT-4 T-cells were treated with CADA and infected with SIV_{mac251}. As shown in Table **1**, CADA prevented MT-4 cells from HIV-1 and SIV infection (E_{50} were 0.7 and 1.2 µg/ml, respectively). Also, CADA proved to be active in cells from macaque origin. The antiviral activity of CADA against SIV was similar for simian and human cells (EC_{50} were 1.0 and 1.2 µg/ml, respectively; Table **1**). As expected, CADA had no antiviral activity against FIV infection of PBMC from cats ($EC_{50} > 50$ µg/ml), a virus that is known not to depend on the cellular CD4 receptor for its entry [22, 24].

Preservation of the Activity of Gel Formulated CADA

A gel formulation containing the HCl-salt of CADA was developed and tested for the preservation of its CD4 downmodulating and anti-HIV activity *in vitro*. Preliminary experiments (data not shown) revealed the need for excipients to enhance the solubility and activity of the poorly water-soluble CADA upon formulation. Using the solubilizing excipients HPβCD, PEG 400 and ethanol, CADA.HCl could be included in a HEC-based gel at a concentration of 0.05%. The resulting gels were clear and homogeneous. Table **2** reports the CD4 down-modulating and anti-HIV activity of two CADA-gels, containing 20% (gel 1) and 10% (gel 2) of HPβCD, as compared to the activity of placebo gels. Upon incubating the CADA gels on MT-4 and SupT1 cells, we observed a marked CD4 downregulation, whereas the placebo gels did not affect the CD4 expression at all (Table **2**). However, we noted a slight decrease in the CD4 downregulating activity of gel formulated CADA compared to the native compound (4.4- and 3.3-fold decrease for gel 1 in MT-4 and SupT1, respectively). An increase in the concentration of the solubilizing excipient HPβCD was reflected by an improvement of the activity of the gel: in MT-4 the EC_{50} for CD4 downmodulation decreased from 22.3 to 3.2 mg/ml upon increasing the HPβCD concentration from 10 to 20% (Table **2**). Comparable results were obtained in SupT1 cells: EC_{50} were 2.3 and 19.8 mg/ml for gel 1 and gel 2, respectively. In MT-4 cells, the CADA-induced CD4 downmodulation correlated with a clear anti-HIV-1 effect, as evidenced by the similar EC_{50} values obtained with native compound (i.e., EC_{50} were 0.36 and 0.32 µg/ml for CD4 downmodulation and anti-HIV-1, respectively; Table **2**). Accordingly, for gel 1 the CD4 downmodulating potency corresponded to its antiviral activity (EC_{50} were 3.2 and 2.5 mg/ml, respectively), as was also the case for gel 2, although to a lesser content (EC_{50} were 22.3 and 12.3 mg/ml, respectively). In parallel, we observed a weak anti-HIV-1 effect in MT-4 cells with the placebo gels, which is presumably due to their HPβCD content. However, in PBMCs the placebo gels did not affect viral replication (Table **2**). In contrast, both CADA gels exerted an antiviral activity against the infection of PBMC with a clade B isolate, although with reduced efficacy compared to $HIV\text{-}1_{NL4.3}$ infection in MT-4 cells (9- and 5-fold reduction for gel 1 and 2, respectively).

Table 1. Antiviral Activity of CADA Against Different Lentiviruses

CADA EC_{50} (µg/ml)			
HIV-1 $_{NL4.3}$	SIV $_{mac\ 251}$	SIV $_{mac\ 251}$	FIV $_{petaluma}$
MT-4[a]	MT-4	Macaque PBMC[b]	Feline thymocytes[c]
0.7 ± 0.1	1.2 ± 0.3	1.0 ± 0.3	> 50

[a]Human T cells (MT-4 T cell line) were treated with different concentrations of CADA and infected with the indicated virus. After 5 days of infection, supernatant was collected and its viral core antigen p24 (for NL4.3 infection) or p27 (for SIV infection) content was determined by ELISA to calculate the EC_{50} value. Values are mean ± SEM with n = 6 (NL4.3) and 2 (SIV).
[b]PBMC from the blood of macaques were stimulated with PHA (2 µg/ml) and IL-2 (1 ng/ml) for 4 days. Then PHA-blasts were washed, treated with CADA and infected with SIV. After 9 days of infection, supernatant was collected and p27 amount was determined to calculate the EC_{50} value. Value is mean ± SEM of 4 experiments from 4 different animals.
[c]Thymocytes from healthy cats were treated with different concentrations of CADA (up to 50 µg/ml) and infected with the feline immunodeficiency virus strain Petaluma.

Synergistic Activity of CADA in Combination with CAP

In view of the development of a more effective anti-HIV microbicide, we investigated the combination of CADA with cellulose acetate phthalate (CAP), a known pharmaceutical excipient with a long history of safe use in humans. We tested whether both microbicide candidates could inhibit viral infection synergistically. The 1:1 equipotent fixed ratio combination of CADA with CAP against HIV-1 infection in MT-4 cells resulted in a clear synergistic effect of both compounds: the combination index at the calculated EC_{50}, EC_{75} and EC_{95} were 0.86 ± 0.12, 0.67 ± 0.09 and 0.56 ± 0.08, respectively (mean ± SD from 3 independent experiments), indicating, according to the method of Chou and Talalay [23], a synergistic interaction. Almost identical CI-values were obtained for SIV_{mac251} infection in MT-4 cells, showing an enhanced antiviral effect for the CADA-CAP combination (CI at the calculated EC_{50}, EC_{75} and EC_{95} were 0.82 ± 0.10, 0.65 ± 0.12 and 0.63 ± 0.10, respectively; mean ± SD from 3 independent experiments). A HEC formulation of the combination of CADA and CAP is currently under investigation, but preliminary results demonstrated the feasibility of combining these 2 anti-HIV agents in a microbicide gel with preservation of their antiviral potency (not shown).

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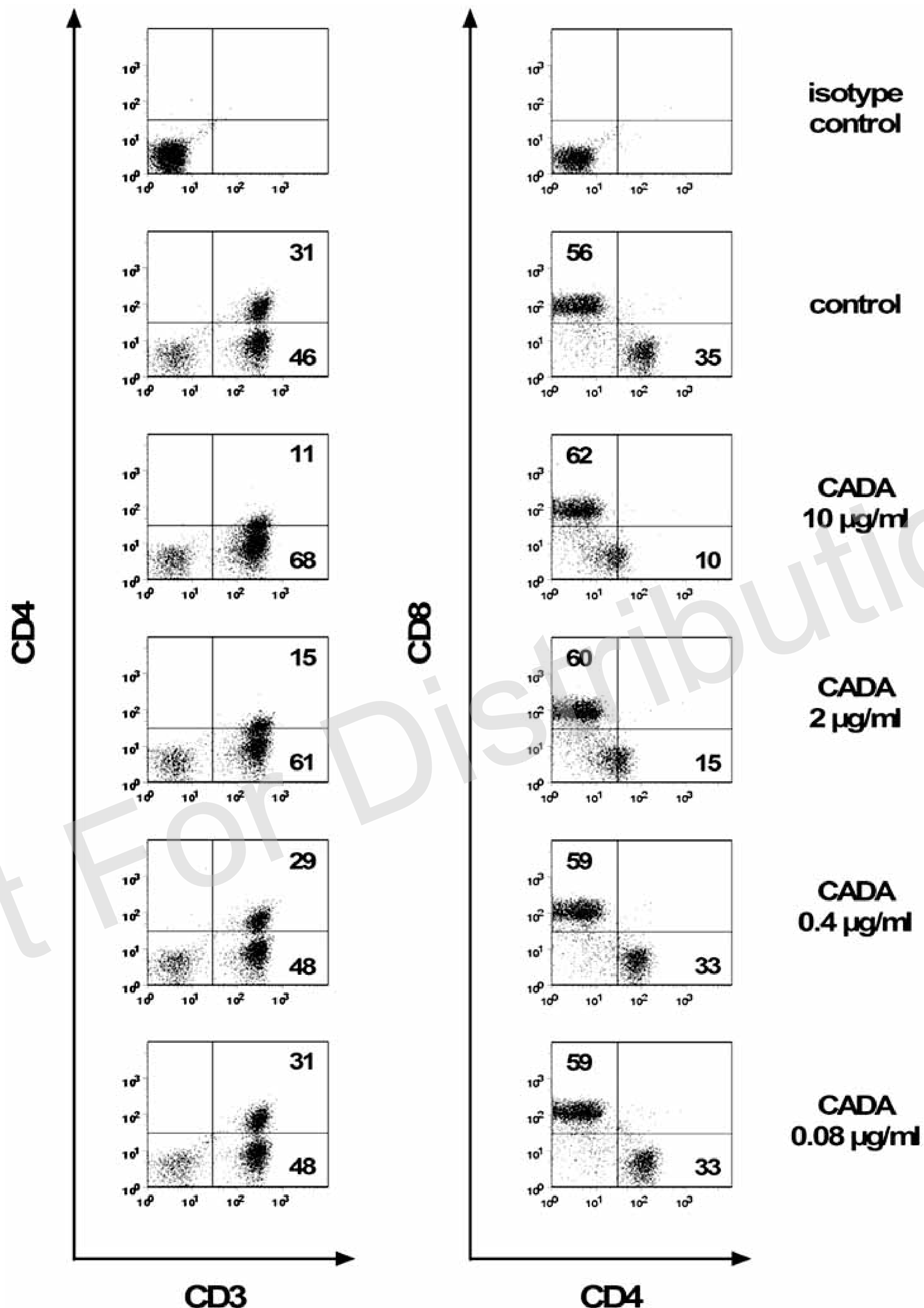

Fig. (3). CD4 downmodulating activity of CADA in peripheral blood mononuclear cells (PBMC) from macaques. CD4 expression in PHA-stimulated PBMC from macaques was determined to show the dose-dependent CD4 downmodulating activity of CADA after 3 days of CADA treatment. As a control, the expression of CD3 and CD8 was also evaluated to demonstrate that CADA only affects the CD4 receptor. The expression levels of CD3 and CD4 were measured simultaneously by labeling macaque lymphocytes from one animal with a FITC-conjugated anti-CD3 and a PE-conjugated anti-CD4 mAb (left panels), or by staining cells from another animal with a FITC-labeled anti-CD4 and a PE-labeled anti-CD8 mAb (right panels). The dot plots represent the flow cytometric analysis of CD3/CD4 or CD4/CD8 receptor expression on the cell surface of macaque lymphocytes after 3 days of CADA treatment. In each plot, the percentage of positive cells is indicated. The isotype control represents the corresponding non-specific background fluorescence measured with identical instrument settings.

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Table 2. Preservation of the Activity of Gel Formulated CADA

Compound	Conc. Units	EC_{50} for CD4 Downmodulation[a]		EC_{50} for Anti-HIV-1 Activity[b]	
		CD4 MT-4	CD4 SupT1	HIV-$1_{NL4.3}$ MT4	HIV-$1_{CI\#17}$ PBMC
Native CADA	µg/ml cpd	0.36 ± 0.03[c]	0.35 ± 0.06	0.32 ± 0.01	0.86 ± 0.01
0.05% CADA gel 1 (HEC 1.5% + hp-β-CD 20%)	mg/ml gel	3.17 ± 0.29	2.31 ± 0.24	2.51 ± 0.20	22.94 ± 0.82
	µg/ml cpd	1.58 ± 0.14	1.16 ± 0.12	1.25 ± 0.10	11.47 ± 0.41
	gel dilution	316	433	398	44
Placebo gel 1 (HEC 1.5% + hp-β-CD 20%)	mg/ml gel	> 100	> 100	33.45 ± 10.92	> 100
	gel dilution	< 10	< 10	30	< 10
0.05% CADA gel 2 (HEC 1.5% + hp-β-CD 10%)	mg/ml gel	22.33 ± 1.20	19.77 ± 2.74	12.26 ± 0.66	57.89
	µg/ml cpd	11.18 ± 0.61	9.91 ± 1.40	6.13 ± 0.33	28.95
	gel dilution	45	51	82	17
Placebo gel 2 (HEC 1.5% + hp-β-CD 10%)	mg/ml gel	> 100	> 100	48.15 ± 7.24	> 100
	gel dilution	< 10	< 10	21	< 10

[a]The EC_{50} for CD4 downmodulation was the concentration of the native compound or the gel required for 50% reduction of cell surface CD4 expression, as measured by flow cytometry.
[b]The EC_{50} value for HIV-1 infection was the concentration of the native compound or the gel required to reduce viral HIV-1 replication by 50% as calculated from the p24 content in the supernatant of infected cells at the end of the experiment (day 3 for MT4 cells and day 10 for PBMCs).
[c]values are mean ± SEM from 2 to 6 different experiments.

DISCUSSION

Viral entry inhibitors are entering the arena of anti-HIV drugs for clinical use. Recently, the CCR5 receptor antagonist, maraviroc, has been approved by the FDA for antiretroviral therapy of HIV, and has become – besides the fusion inhibitor fuzeon – the second HIV entry inhibitor to reach the clinic to date. In addition, several new drugs are currently finding their way in the developmental process as novel anti-HIV compounds (reviewed by [25, 26]). The small synthetic molecules cyclotriazadisulfonamides, of which CADA can be regarded as the lead compound, represent such a new class of HIV entry inhibitors [14, 15]. It was demonstrated that CADA specifically decreases the expression of surface CD4 without affecting other cellular receptors [14]. The compound had consistent CD4 downmodulating activity in different human T cell types, which correlated with its antiviral potency against laboratory and subtype B clinical isolates of HIV-1 [15-17].

In the present study we tested CADA for its activity in dendritic cells, as they play a central role in the transmission of HIV. MO-DCs, although expressing CD4 to a lower extent, were found to exert equal sensitivity to the receptor downmodulating potency of CADA as T lymphocytes do (i.e., approximately 85 % reduction in CD4). This is in line with our earlier report showing a significant decrease of CD4 with CADA in monocytes and macrophages [27]. The reduced CD4 expression on DCs by CADA proved to be sufficient to prevent HIV infection of the co-culture of DCs with T lymphocytes. Studies have shown that HIV-1 can bind to and infect DCs and that these infected cells – even at very low incidence – are responsible for enhancing viral transmission to lymphocytes [28-31]. In our experiments the antiviral effect of CADA is presumably the result of preventing HIV entering the DC, rather than interfering with the capture of the virus *via* e.g. DC-SIGN or other C-type lectin receptors. This can also explain why a very short exposure of CADA to cells or virus has no antiviral effect, as the compound does not bind to the viral envelope or any extracellular part of CD4 (data on file). As shown in previous reports [14, 18], the decrease of CD4 at the cell surface becomes substantial only after several hours of incubation with CADA, presumably by an interruption in receptor synthesis or intracellular transport. A sustained CADA treatment of the co-culture after virus exposure improved its antiviral effect, most likely because also the entry of virus in $CD4^+$ T cells is prevented during later multiple replication rounds. Thus, the consistent CD4 downmodulating effect of CADA on all $CD4^+$ cells can act on different target cells involved in HIV transmission, and can, by reducing CD4 receptor density to below the level that is required for efficient HIV infection, result in a general antiviral effect. Recently, a clear anti-HIV effect of CADA in human lymphoid tissue was shown [16], pointing to its application *ex vivo*. Accordingly, it was demonstrated that blockade of only the primary receptor for HIV-1, CD4, was sufficient to inhibit localized HIV-1 infection of human cervical tissue in an *ex vivo* explant model [32].

The most common subtypes of HIV-1 globally are subtypes C and A [33], and especially in Africa, the prevalence of non-B clades is very high. Thus, it is important that viruses of all clades are included in any evaluation of potential virus-specific intervention strategy. In general, CADA could prevent human PBMCs from infection with isolates across different clades, including A, B, C, D, F, G, H, and AE, although it was clear that isolates from clade B were more

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prone to the CD4 downmodulating effect of CADA. This confirms the results of our previous study in which we demonstrated its antiviral activity against 16 isolates of clade B, irrespective of their coreceptor use [15]. Our observation that interference with the binding of gp120 to CD4 most efficiently affects isolates of mainly clade B has been complemented by reports of other groups [34-40]. Anti-gp120 mAb b12, directed against an epitope overlapping the CD4 binding site, exhibited greater activity against clade B than non-B viruses, and neutralised 50% of viruses, including some from almost every clade [34]. BMS-378806, a small gp120-binding molecule that specifically interferes with the attachment to CD4, has anti-HIV potency mainly against subtype B viruses although with great inter-clade variation ranging from 1 to > 10,000 nM, whereas isolates from clade A, C, D, E, F and G were remarkably less or even insensitive to the drug [35]. Also, primarily isolates of clade B can be profoundly neutralised by sCD4, whereas viruses of clades A, C and D are less prone to neutralisation by sCD4 or IgG1 b12 [36-39].

In our study, the infection of PBMCs was abrogated by the CD4 downmodulating activity of CADA for virtually all the clinical isolates tested. Recently, the broad HIV-1 neutralising specificities of several patient sera were mapped to the functionally conserved gp120 CD4-binding site [41], proving that preventing gp120 to bind its main receptor has broad spectrum potency. Although for CADA a partial antiviral activity was noted against a number of isolates, we did not observe a lack of antiviral potency of CADA against a specific clade. This is in sharp contrast with our previous study of the broadly HIV-1 neutralizing antibody 2G12 and the carbohydrate-binding plant lectin GNA with microbicidal potential [42]. Based on the same set of isolates used in this work, 2G12 proved to be inactive against clade C, D, AE, F, G and O [42]. Also, GNA that significantly inhibited viral infection of the lab strain HIV-1_{IIIB} was shown to be remarkable less potent for clade B clinical isolates (50-fold reduction) and was inactive against isolates from clade C, D or G [42].

In our hands, we observed for each clinical HIV-1 isolate some inter-donor variation in the antiviral potency of the compound. This variation may be related to a different response of the cells to CADA in disturbing a biological process (i.e., CD4 synthesis and/or transport), which is distinct from the immediate steric hindrance created by a mAb. In our experiments, compound and virus are administered to PBMCs simultaneously, making it possible for some viruses to take advantage of the delay in CD4 downmodulation. Secondly, the variation can be attributed to a different density of the HIV receptors on the cells from various donors. As the PBMCs are stimulated with PHA, it is plausible that the activation of the cells results in different expression levels of CD4 and, especially, the chemokine receptors. It has been reported that CD4 and coreceptor concentration requirements for efficient infections by R5 virus are interdependent: cells with a large amount of CD4 required only a trace amount of CCR5 for maximal susceptibility to infection with diverse isolates, and vice versa [43-45]. This implies that for each HIV-1 isolate the requirement for CD4, and thus the sensitivity to CADA, will decrease when the coreceptor is expressed at saturating amounts. Accordingly, Blish *et al.* reported that one of the few clade A isolates that could be neutralised by sCD4 was markedly less sensitive to inhibition of the CCR5 receptor [37], demonstrating that for a successful intervention of virus entry both CD4 and coreceptor should be blocked. Furthermore, our data suggest that there is a tendency for CADA to more efficiently prevent infection of R5 than X4 viruses, which was also observed in human lymphoid tissues *ex vivo* [16]. Of note is that in our experimental set-up more R5 isolates were included than X4, resulting of course in an unequal amount of samples for R5 and X4 analysed. However, in terms of viral transmission it would be more preferable to prevent infection of R5 viruses.

Before moving the drug to a non-human primate model for preclinical testing, we wanted to ascertain that CADA is active in simian cells. When cells from macaques were evaluated for their sensitivity to CADA, we measured a CD4 downmodulation that was similar to that observed in human PBMCs [15]. Subsequently, these CADA-treated macaque cells were prevented from being infected by a pathogenic SIV strain, proving the feasibility of using the compound in an *in vivo* model. Several CCR5 inhibitors have already been successfully evaluated in the rhesus macaque model [46-49]. A notable example is also the activity of mAb IgG1 b12 in this model [50]. Furthermore, in the vaginal transmission model, BMS-378806 protected macaques from a vaginal SHIV challenge [46], demonstrating that targeting the attachment of gp120 to CD4 is an effective means of intervention *in vivo*.

An important criterion that is likely to be the key to the successful development of a candidate microbicide is the feasibility of its production on a large scale at low cost. As CADA is a small and stable chemical molecule (MW 618) [14, 19] it should be feasible to produce large amounts of this drug at an appropriate cost. In the context of a topical microbicide application for CADA, we explored the feasibility of formulating CADA in a gel with preservation of its activity. Because of its stability and safety [51], hydroxyethylcellulose was selected as the gelling polymer. Due to the low aqueous solubility of CADA, successful formulation required the use of solubilizing excipients. Cyclodextrins are widely used as pharmaceutical solubilizers for various applications, including vaginal gels [52, 53]. By using HPβCD at concentrations of 10 or 20%, we succeeded in formulating CADA.HCl at a concentration of 0.05%. From our data in MT-4 cells, it was clear that the CADA gel preserved the CD4 downmodulating and antiviral potency of the compound. Notably, in this T-cell line, but not in PBMCs, a partial anti-HIV effect was observed with the placebo gels, which can be attributed to their cyclodextrin contents as it can remove cholesterol and disrupt lipid raft regions involved in viral infection [54, 55]. Further optimisation of the CADA gels is in progress. In addition, as CADA requires continuous interaction with the target cells in order to significantly downmodulate CD4, it would be interesting to investigate its formulation in a vaginal ring system. By this means, a continuous release of the drug should be achieved locally, which could then effectively decrease the amount of CD4 molecules on the cell surface of the $CD4^+$ target cells.

Importantly, great attention has to be given to the development of a gel formulation in which different drugs are combined, acting on distinct levels in the viral replication cycle. In this context, the combination of the HIV-1 entry

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inhibitors BMS-378806, CMPD167 and C52L - acting on CD4 attachment, coreceptor binding and fusion, respectively - was reported to successfully protect macaques from vaginal SHIV challenge [46] and to be effective against HIV-1 isolates from multiple genetic subtypes [56]. Here, we observed a significant synergistic activity for the combination of CADA with cellulose acetate phthalate (CAP), a common pharmaceutical excipient with HIV-1 entry inhibitory activity [57-59]. Previously, we have shown the synergy of CADA with 10 reverse transcriptase inhibitors (RTI), 6 protease inhibitors and 4 entry inhibitors [15]. Also, the combination of CAP with the RTI UC781 had synergistic and complementary effects against HIV-1 infection [60]. Safety studies with a CAP gel formulation showed minimal toxicity in rhesus macaques, supporting its use as a candidate vaginal microbicide in humans [61].

In conclusion, the results of this study demonstrate that the CD4 downmodulating compound CADA may have potential as a broad-spectrum anti-HIV microbicide drug candidate with inhibitory activity on HIV transmission in DC/T cell co-cultures. Based on its prevention against SIV infection of simian cells, and the preservation of the biological activity of gel formulated CADA, it will now be feasible for testing this entry inhibitor in an *in vivo* model. Thus, small molecule CD4 downmodulators can provide valuable additions to the microbicide arsenal, not only as a single drug but especially in a synergistic conjunction with other anti-HIV compounds.

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