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Design and cellular kinetics of dansyl-labeled CADA derivatives with anti-HIV and CD4 receptor down-modulating activity

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ABSTRACT

A new class of anti-retrovirals, cyclotriazadisulfonamide (CADA) and its derivatives, specifically down-regulate CD4, the main receptor of HIV, and prevent HIV infection in vitro. In this work, several CADA derivatives, chemically labeled with a fluorescent dansyl group, were evaluated for their biological features and cellular uptake kinetics. We identified a derivative KKD-016 with antiviral and CD4 down-modulating capabilities similar to those of the parental compound CADA. By using flow cytometry, we demonstrated that the dose-dependent cellular uptake of this derivative correlated with CD4 down-modulation. The uptake and activity of the dansyl-labeled compounds were not dependent on the level of expression of CD4 at the cell surface. Removal of the CADA compounds from the cell culture medium resulted in their release from the cells followed by a complete restoration of CD4 expression. The inability of several fluorescent CADA derivatives to down-modulate CD4 was not associated with their lower cellular uptake and was not reversed by facilitating their cell penetration by a surfactant. These results prove the successful integration of the dansyl fluorophore into the chemical structure of a CD4 down-modulating anti-HIV compound, and show the feasibility of tracking a receptor and its down-modulator simultaneously. These fluorescent CADA analogs with reversible CD4 down-regulating potency can now be applied in further studies on receptor modulation, and in the exploration of their potentials as preventive and therapeutic anti-HIV drugs.

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Abbreviations: HIV, human immunodeficiency virus; CADA, 9-benzyl-3methylene-1,5-di-*p*-toluenesulfonyl-1,5,9-triazacyclododecane (cyclotriazadisulfonamide); Dansyl, 5-dimethylamino-1-naphtalenesulfonyl; Tosyl, toluenesulfonyl; MFI, median fluorescence intensity; DMSO, dimethylsulfoxide; SI, selectivity index

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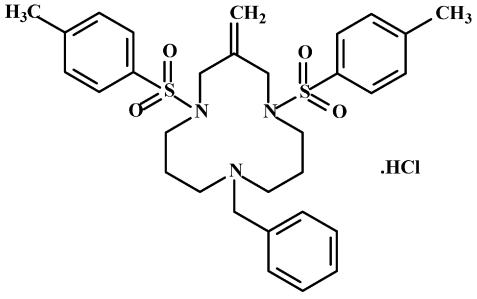
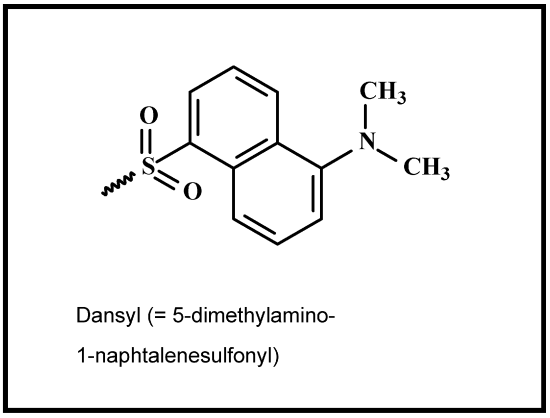
1. Introduction

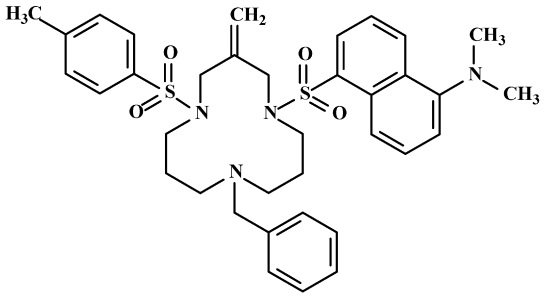
With an incidence of about 4 million new infections per year, the world urgently needs novel classes of safe and effective anti-retroviral agents targeting various stages of the replication of the human immunodeficiency virus type 1 (HIV-1). The infectious cycle of HIV-1 is typically initiated by the sequential interaction of the viral envelope glycoprotein gp120 with the cell surface receptor CD4 [1,2] and a cellular co-receptor belonging to the chemokine receptor family [3,4]. Based on the usage of the chemokine receptor CXCR4 and CCR5, HIV isolates are classified as X4- and R5-tropic, respectively [5]. To date, a number of agents that interfere with CD4 receptor attachment, including humanized, non-immunosuppressive IgG4 monoclonal antibody TNX-355 [6], have been credited with potent anti-HIV activity (reviewed in [7]). Cellular CD4 receptor density plays a significant role in the efficiency of viral infectivity [8,9] and can be considered as an important target for therapeutic intervention [10].

The small molecule cyclotriazadisulfonamide (CADA, Fig. 1) has been reported to be a novel promising HIV entry inhibitor [11,12]. The compound significantly decreases the amount of cell surface CD4 in MT-4 cells, without altering the expression of any other cellular receptor examined, including the HIV co-receptors. Moreover, the anti-HIV activity of CADA correlated with its ability to down-modulate CD4 receptor expression in human T cells [11]. Similar down-regulation of CD4 was observed in other T-cell lines, in freshly isolated

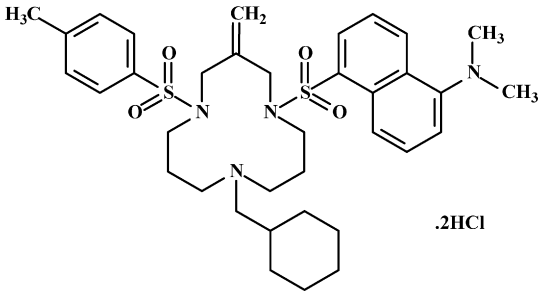


CADA

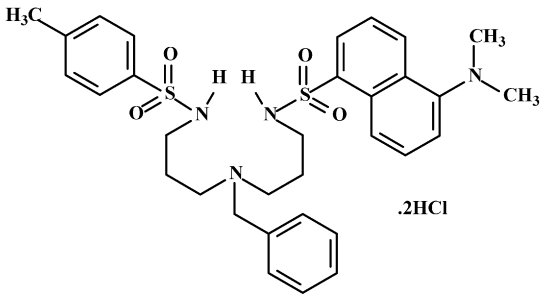




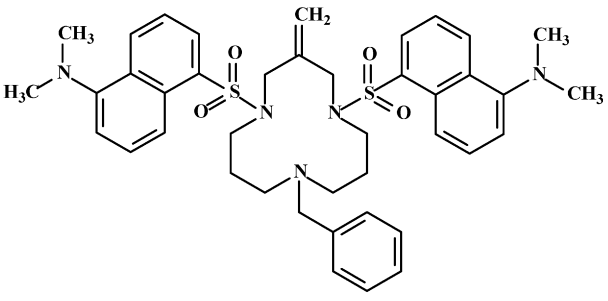
KKD-015



KKD-016



KKD-014



ES-KKD-024

Fig. 1 – Chemical structures of cyclotriazadisulfonamide (CADA) and its dansyl-labeled derivatives.

blood lymphocytes and monocytes, and in *ex vivo* human lymphoid tissue, resulting in consistent antiviral activity against laboratory adapted and primary clinical isolates of HIV-1 [11–14]. CD4 mRNA levels are not affected by CADA, suggesting that CD4 expression is not inhibited at a transcriptional level. CADA was evaluated in initial, preclinical anti-HIV drug development studies and was found to be soluble in human, rat and mouse plasma at 1.9–3.1 μM, to be stable in plasma (>200 h), and to be detectable in the bloodstream up to 2 h after intravenous injection in mice.

In spite of these promising results, the exact mechanism by which CADA down-regulates CD4 and inhibits HIV infection remains to be understood. In order to examine the distribution of these synthetic compounds in cells and their interactions with specific protein expression and secretion pathways, we developed fluorescent analogs that can be utilized in microscopic and flow cytometric studies. To preserve chemical activity of the compounds and to avoid steric hindrance, the fluorescent derivatives are best prepared by incorporation of relatively small fluorophores, like 1-dimethylaminonaphthalene-5-sulfonyl (=dansyl). Dansyl chloride has been used in structural studies [15,16] and in quantitative analyses of complex molecules that contain primary, secondary and tertiary amino and carboxylated aromatic groups [17]. Via dansylation we generated fluorescent derivatives that are excited with UV light and detected by means of their visible emission with great sensitivity.

In this paper, we describe a series of fluorescent analogs of CADA bearing the dansyl fluorophore and characterize their biological and pharmacokinetic properties. These fluorescent compounds proved to be valuable tools for flow cytometric studies to evaluate compound uptake and to decipher mechanisms of receptor down-modulation.

2. Materials and methods

2.1. Synthesis of the dansyl-labeled CADA derivatives

The compounds CADA·HCl, KKD-014·2HCl, KKD-015 and KKD-016·2HCl were synthesized as described previously [18] and used in the free base or salt forms as listed. Analog ES-KKD-024 was synthesized by analogous methods from bis(3-aminopropyl)benzylamine [18] in the following two steps. A solution of 2.09 g (9.5 mmol) of bis(3-aminopropyl)benzylamine, 14.5 ml of 2 N aqueous NaOH and 27 ml of saturated aqueous NaCl was added over 9 h to a stirred solution of 5 g (18.5 mmol) of 5-dimethylamino-1-naphthalenesulfonyl chloride in 22 ml of ether, then the reaction mixture was stirred for another 12 h at room temperature. The supernatant liquid was poured into a separatory funnel, and the upper two layers were poured into a 200 ml round bottomed flask. A solution of the residual solid in the reaction flask in 20 ml of $CHCl_3$ was extracted with 5 ml of 2 N aqueous NaOH. The chloroform extract was combined with the upper two layers from the previous separation, dried ($MgSO_4$), filtered, and concentrated by rotary evaporation. The resulting fluorescent yellowish-green crystals were dried in vacuo (0.5 mm, 45 °C), yielding 5.55 g (86%) of *N,N′*-bis(5-dimethylamino-1-naphthalenesulfonamido)bis(3-aminopropyl)benzylamine, mp 69–72 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.51 (d, J = 9 Hz, 2 H, 2-dns), 8.26 (d, J = 9 Hz, 2 H, 4-dns), 8.18 (d, J = 7 Hz, 2 H, 8-dns), 7.5 (m, 4 H, 3,7-dns), 7.2 (m, 7 H, 6-dns, Bn), 5.71 (s, 2 H, NH), 3.32 (s, 2 H, CH_2Ph), 2.87 (s, 16 H, NCH_3, H3), 2.22 (m, 4 H, H1), 1.46 (m, 8 H, H2). ^{13}C NMR (400 MHz, $CDCl_3$) δ 152.2, 138.2, 135.3, 130.5, 130.1, 129.9, 129.6, 129.4, 128.7, 128.5, 127.5, 123.4, 119.3, 115.4, 58.8, 52.1, 45.6, 42.6, 26.3. MS (FAB) m/z 688 (MH^+). Anal. Calcd. for $C_{37}H_{45}N_5O_4S_2$: C, 64.60; H, 6.59; N, 10.18; S, 9.32. Found: C, 64.74; H, 6.79; N, 10.09; S, 9.64. A solution of 2.5 g (3.65 mmol) of *N,N′*-bis(5-dimethylamino-1-naphthalenesulfonamido) bis(3-aminopropyl)benzylamine in 10 ml of anhydrous *N,N*-dimethylformamide (DMF) was added slowly to a stirred mixture of NaH (0.36 g of a 60% (w/w) slurry in mineral oil, 0.22 g NaH, 91 mmol, washed with hexane under N_2) and 40 ml of anhydrous DMF at 75 °C. A solution of 0.5 g (3.65 mmol) of 3-chloro-2-chloromethyl-1-propene in 10 ml of anhydrous DMF was added over 8 h by means of a syringe pump. The reaction mixture was stirred at room temperature for 12 h and the solvent was removed by rotary evaporation. A solution of the residue in 50 ml of $CHCl_3$ was washed with water (3 × 25 ml) then concentrated to dryness by rotary evaporation. Chromatography on silica gel, eluting with 2:8 ethyl acetate/hexane (v/v) gave 0.4 g (15%) of ES-KKD-024 as a light yellow solid, which was converted to its HCl salt as described for CADA·HCl (Bell et al. [18]) and purified by sonication of a suspension in ether/CH_2Cl_2 for 1 h at room temperature. Anal. Calcd. for $C_{41}H_{49}N_5O_4S_2{\cdot}3HCl{\cdot}5{\cdot}25H_2O$: C, 52.17; H, 6.62; N, 7.42. Found: C, 51.89; H, 6.12; N, 7.16. A solution of 0.1 g of ES-KKD-024·3HCl in 5 ml of CH_2Cl_2 was stirred with 5 ml of 2 M aqueous NaOH and 5 ml of saturated aqueous NaCl at room temperature for 2 h. The organic layer was washed with water, followed by 5 ml of saturated aqueous NaCl, dried (Na_2SO_4), and concentrated by rotary evaporation. The residue was dried in vacuo to give 0.08 (92%) of ES-KKD-024 free base, mp 100–112 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.54 (d, J = 8 Hz, 2 H, 2-dns), 8.32 (d, J = 8 Hz, 2 H, 4-dns), 8.18 (d, J = 8 Hz, 2 H, 8-dns), 7.51 (m, J = 8 Hz, 4 H, 3,7-dns), 7.19 (m, 5 H, Bn), 7.10 (d, J = 8 Hz, 2 H, 6-dns), 5.15 (s, 2 H, C = CH_2), 3.95 (s, 4 H, H2, H4), 3.34 (s, 2 H, CH_2Ph), 3.23 (t, J = 6 Hz, 4 H, H6, H12), 2.89 (s, 12 H, NCH_3), 2.22 (t, J = 6 Hz, 4 H, H8, H10), 1.56 (quintet, J = 6 Hz, 4 H, H9, H11). ^{13}C NMR (75 MHz, $CDCl_3$) δ 152.0, 139.5, 138.9, 134.7, 130.7, 130.4, 130.3, 129.0, 128.4, 128.3, 127.1, 123.4, 119.7, 116.8, 115.5, 59.3, 51.0, 49.8, 45.7, 43.8, 25.3. IR (KBr, cm^{-1}): 3424(br), 3028(w), 2944(w), 2593(br), 2420(br), 1604(w), 1471(m), 1314(m), 1163(s), 1041(w), 896(w), 790(s), 583(s). MS(MALDI) m/z 740 (MH^+). Nuclear magnetic resonance (NMR) chemical shifts (δ) are reported in ppm units, relative to tetramethylsilane (TMS) as an internal standard (δ = 0.00). The structures of the CADA derivatives are shown in Fig. 1. Stock solutions of each compound were prepared by dissolving 10 mg in 10 ml of dimethylsulfoxide (DMSO) (VWR International, Leuven, Belgium) and were stored at RT in the dark. For the experiments with a surfactant, pluronic F-127 (Life Technologies, Paisley, UK) was used as a 20% (w/v) solution in DMSO. The stock solution of the CADA compounds (in DMSO) was mixed at a 1:1 ratio with pluronic F-127 (10 μl of each). In parallel (as a negative control), 10 μl of the stock solution was mixed with 10 μl of DMSO. The mixtures were kept at RT for 10 min and immediately used for preparing the different dilutions of the compounds in culture medium.

2.2. Viruses and cell cultures

The HIV-1 T-tropic (X4) molecular clone NL4.3 ($X4_{NL4.3}$) was obtained from the National Institute of Allergy and Infectious Disease AIDS reagent program (Bethesda, MD). The HIV-1 stock was obtained from the culture supernatant of HIV-infected MT-4 cells. 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 HEK 293T-cell line was cultured in Dulbecco's modified Eagle medium (Life Technologies) containing 10% FBS and 0.01 M HEPES buffer (Life Technologies). All cell cultures were maintained at 37 °C in a humidified, CO_2-controlled atmosphere, and subcultivations were done every 2–3 days.

2.3. Construction of stably CD4 transfected cell line

The pcDNA3 expression vector (Life Technologies) encoding the wild-type CD4 receptor (kindly provided by Dr. O. Schwartz, Institut Pasteur, Paris, France) was co-transfected with the pPUR selection vector encoding puromycin resistance (CLONTECH Laboratories, Palo Alto, CA) into HEK 293T cells by the use of FuGENE 6 Transfection Reagent (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions. Puromycin (1 μg/ml) selection was started after 18 h. After approximately 3 weeks, a nicely growing puromycin-resistant cell culture, containing about 20% $CD4^+$ cells (as determined by flow cytometry), was established. An enrichment of the $CD4^+$ cells was obtained as follows. Approximately 4×10^6 cells in 500 μl of PBS containing 2% FBS were incubated with 20 μl of nonconjugated mouse anti-human CD4 antibody clone SK3 (BD PharMingen, San Diego, CA) for 30 min at room temperature. After two washing steps, the cells were incubated with 2×10^6 sheep anti-mouse IgG-conjugated Dynabeads M450 (Dynal, Oslo, Norway) in 8 ml of PBS with 2% FBS for 1 h at 4 °C under continuous gentle rotation. Thereafter, the $CD4^+$ cells (which had bound magnetic beads at their surface) were isolated from the cell suspension by magnetic separation and were thoroughly washed with PBS and transferred to puromycin-containing growth medium.

2.4. Antiviral assay

MT-4 cells were infected with the HIV-1 strain $X4_{NL4.3}$. Briefly, 5-fold dilutions of the compounds (in 100 μl) 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 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). 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.

2.5. Fluorescence spectroscopy of dansyl

Fluorescence excitation and emission spectra were recorded on a LS50B Luminescence Spectrometer (PerkinElmer, Wellesley, MA) and analyzed with the FL WinLab software. The different concentrations of KKD-016 (i.e., 2.70, 1.35 and 0.68 μM) were made in PBS (pH 7.4).

2.6. Flow cytometry

To study the effect of the CADA derivatives on surface CD4 antigen expression, MT-4, SupT1 and $CD4^+$ HEK 293T cells were incubated with a serial 5-fold dilution of the compounds or medium at 37 °C. CD4 expression was analyzed at specific time points as indicated in the text and in the legends to the figures. Briefly, after washing with phosphate-buffered saline containing 2% FBS, cells were incubated with the anti-CD4 mAb (FITC, PE-Cy7 or APC-Cy5.5 labeled; from Caltag Laboratories, Burlingame, CA) for 30 min at RT. Then the cells were washed and analyzed immediately. Data were acquired with a LSRII flow cytometer (BD Biosciences, San Jose, CA) using 355, 407, 488, 532 and 638 laser lines and the Diva 4.12 software. Data were analyzed with the FLOWJO software (Tree Star, San Carlos, CA). Down-regulation 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 down-modulation, 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} values for CD4 down-modulation of the compounds were calculated as the concentration of the compounds required for 50% decrease of the expression of CD4 at the cell surface. For the evaluation of the amount of dansyl-labeled CADA compounds in the cells, cells were incubated with a serial 5-fold dilution of the compounds at 37 °C. Then, at the indicated time points, the cells were washed (and in the combination experiments stained with anti-CD4 mAb as described above) and analyzed immediately.

2.7. Cytotoxicity assay

Cellular toxicity of the compounds was measured after 3 days of incubation of the cells with 5-fold dilutions of the compounds. The toxicity was determined by trypan blue exclusion and also by propidium iodide and flow cytometry. The CC_{50} values of the CADA analogs correspond to the concentrations (micromolar) required to reduce the viability of the cells by 50%.

2.8. Immunocytochemistry

Coverslips were coated with poly-L-lysine (Sigma Chemical Co., Bornem, Belgium) as follows. The coverslips were first sonicated in acetone for 15 min at RT, washed profoundly in distilled water and dried at air. Then, drops of polylysine

(0.1 mg/ml) solution were spread on the coverslip surface and left at RT until dry. For the staining with anti-CD4, MT-4 cells were pre-treated with the CADA compounds for 48 h, washed in PBS and resuspended in culture medium (1×10^5 cells/ml). Cells were plated on the coverslip and incubated at 37 °C for 30 min. The coverslips were then washed gently with PBS, and transferred to a 4% paraformaldehyde (Sigma) solution in PBS for fixation (15 min RT). Samples were washed 3 times in PBS, and 500 μl of antibody dilution (Alexa Fluor 488 labeled anti-CD4 mAb diluted 1:10 in PBS containing 10% mouse serum; Caltag) was added to the coverslips and incubated for 1 h at RT. Humidity was maintained by placing a moist strip of cut sponge at one end of the coverslip. Next, samples were washed 3 times in PBS (3×10 min) and excess of PBS was removed. On a clean dry slide, 20 μl of mounting media was applied before the coverslip was put on the slide. Imaging of control and CADA-treated cells was performed on a Zeiss LSM 510 upright two-photon microscope equipped with an Argon 488 nm laser and a Chameleon laser (tunable from 720 to 930 nm), using an Achroplan 63×/0.9 W objective and BP 500–550 IR and BP 435–485 IR filters. For the excitation of the dansyl fluorophore, the multi-stage mode-locked Chameleon laser was set at 720 nm. Images were generated with the Carl Zeiss Laser Scanning Systems LSM 510 software. The pictures of control and treated cells were taken under the same experimental conditions.

2.9. 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 for each set of experiments for CD4 down-modulation and anti-HIV. For the correlation, the Pearson's linear correlation coefficient (r) was calculated. Statistical analysis was performed with FigSys statistical software (Biosoft, Cambridge, UK).

3. Results

3.1. Anti-HIV activity of dansyl-labeled CADA derivatives

To identify the most active anti-HIV drugs, different derivatives of the lead compound CADA have been synthesized [18]. New analogs have been labeled with a dansyl group in their side chain (Fig. 1). We tested the antiviral activity against $X4_{NL4.3}$ of the dansyl-labeled CADA analogs in the MT-4 $CD4^+$ T-cell line (Table 1). KKD-015 is a CADA derivative in which a dansyl group substituted for one of the tosyl sidearms (Fig. 1). This modification in the chemical structure had a detrimental effect on the antiviral activity resulting in a 7-fold decrease (EC_{50} were 1.3 and 9.5 μM for CADA and KKD-015, respectively). If the second tosyl arm was also replaced by a dansyl group, the resulting CADA analog (i.e., compound ES-KKD-024) had no detectable antiviral activity anymore ($EC_{50} > 75$ μM). Replacement of the benzyl tail of KKD-015 by a cyclohexylmethylene group, as in analog KKD-016, resulted in an improvement of the anti-HIV-1 activity. Importantly, the dansyl-labeled CADA derivative KKD-016 inhibited viral infection dose-dependently (Fig. 2) and exhibited an antiviral activity within the range of that of the unlabeled CADA (EC_{50} were 1.8 and 1.3 μM, respectively; $P = 0.29$; $n = 3$). Finally, modification of the cyclododecane ring structure, such as the open ring in compound KKD-014, resulted in a loss of the anti-HIV activity ($EC_{50} > 35$ μM).

Table 1 – Anti-HIV-1 activity and cellular toxicity of CADA and several of its dansyl-labeled analogs

Compound	Anti-HIV-1 activity EC_{50} (μM)	Cellular toxicity CC_{50} (μM)
CADA	1.32 ± 0.48	42 ± 8
KKD-014	>35	35 ± 5
KKD-015	9.49 ± 0.72	>75
KKD-016	1.80 ± 0.24	51 ± 10
ES-KKD-024	>75	>75

The compounds were used in the free base or salt forms as listed under Section 2. The EC_{50} for HIV-1 (NL4.3) infection in MT-4 cells was the concentration of the compound required to reduce viral HIV-1 replication by 50% as measured by the p24 Ag ELISA. The CC_{50} was the 50% cytotoxic concentration, i.e., the concentration of the compound required to reduce the viability of the cells by 50%. Data represent mean values ± S.E.M. from three independent experiments.

The biological activity of the CADA compounds was not correlated with an increased cellular cytotoxicity (Table 1): the same range of CC_{50} values were measured for the active (i.e., CADA, KKD-015 and KKD-016) and inactive (i.e., KKD-014 and ES-KKD-024) analogs. Calculation of the selectivity index (SI) showed for both CADA and KKD-016 high therapeutic indexes of 32 and 28, respectively.

3.2. CD4 receptor down-modulation by dansyl-labeled CADA analogs

Since the anti-HIV activity of the CADA compounds correlated with their ability to down-modulate CD4 [19], we evaluated the

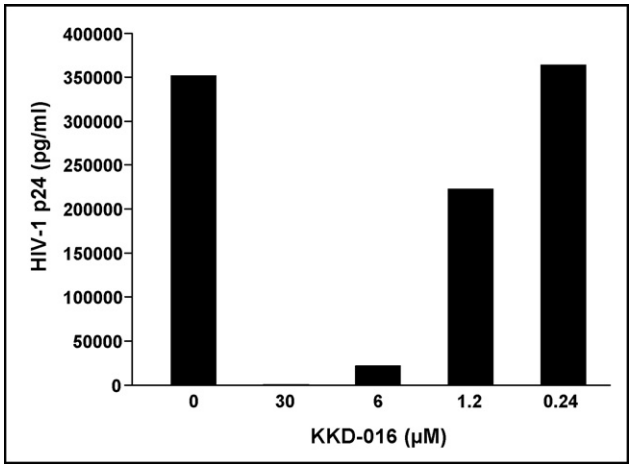


Fig. 2 – Dose-dependent anti-HIV-1 activity of the dansyl-labeled CADA analog KKD-016. MT-4 cells were infected with HIV-1 (NL4.3) in the presence of different doses of KKD-016. After 5 days, supernatant was collected and analyzed for its p24 content. One representative experiment of two is shown.

CD4 down-modulating ability of the dansyl-labeled CADA analogs using MT-4 cells. After 24 h of incubation of the cells with the compounds, the expression of CD4 on the cell surface was measured by means of flow cytometry. As depicted in Fig. 3A (left panel), treatment of MT-4 cells with CADA resulted in a marked dose-dependent decline in the expression of CD4 on the cell surface. A similar dose-dependent CD4 down-modulation was observed for the compounds KKD-015 and KKD-016 (right panel), i.e., the two analogs that were active against HIV-1 infection. The EC_{50} values (mean ± S.E.M.; $n = 3$) for CD4 down-modulation in MT-4 cells were 0.55 ± 0.09 μM, 1.72 ± 0.25 μM and 0.97 ± 0.13 μM for CADA, KKD-015 and KKD-016, respectively. A comparable decrease in CD4 expression was observed in the T-cell line SupT1: EC_{50} was 0.73 ± 0.05 μM for CADA, 5.61 ± 3.42 μM for KKD-015 and 0.80 ± 0.14 μM for KKD-016 ($n = 2$). The KKD-014 and ES-KKD-024 derivatives did not affect the expression of CD4 (Fig. 3A). Again, compound KKD-016 proved to be the most active dansyl-labeled CADA analog with a CD4 down-modulating potency that was similar to that of the unlabeled lead compound CADA ($P = 0.78$ and 0.07 for the comparison of the mean EC_{50} values in SupT1 and MT-4, respectively).

The effect of the CADA-derivatives on the expression of CD4 was confirmed microscopically. MT-4 cells were incubated with CADA and KKD-016 for 2 days. Then, the cells were plated on a polylysine-coated coverslip and stained with Alexa488-labeled anti-CD4 antibodies. As shown in Fig. 3B the fluorescence intensity at the surface of the cells treated with CADA is markedly lower compared to the intensity of control cells stained for CD4 under the same conditions. Also, a similar down-regulation of CD4 expression was observed when the cells were incubated with KKD-016.

3.3. Flow cytometric detection of the dansyl analogs

We used the dansyl fluorophore for flow cytometric evaluation of the cellular distribution of CADA derivatives. Towards this goal we evaluated by means of spectrometry the fluorescence spectrum of the dansyl-tagged CADA analog KKD-016 (Fig. 4A). The dansylated compound in solution has an excitation peak at 345 nm, whereas maximum emission fluorescence was noted at 485 nm. We used these wavelengths for flow cytometry of MT-4 cells incubated for 48 h with different concentrations of the compounds. The histogram analyses of the cells treated with the dansyl analogs and excited with UV are shown in Fig. 4B. Whereas the cells treated with unlabeled CADA exhibited no detectable fluorescence (data not shown), incubation of MT-4 cells with each of the dansyl-tagged CADA derivatives resulted in a strong dose-dependent fluorescence. The MFI of the cells treated with 15 μM of the active compounds KKD-015 and KKD-016 were 1010 and 1709, respectively (Fig. 4B). The loading of the cells with the inactive analog KKD-014 was more efficiently as evidenced by the higher fluorescence signal of loaded cells (MFI for 15 μM was 2385). In contrast, we observed a considerable lower uptake of the inactive analog ES-KKD-024 (MFI for 15 μM was 672; Fig. 4B). Also, in $CD4^+$-transfected HEK 293T cells similar dose-dependent uptake of dansyl-labeled CADA derivatives was observed.

3.4. Kinetics of compound uptake and influences on the expression of CD4

Using flow cytometry, we measured the amount of compound at different time points after exposure. Fig. 5A shows the kinetics of compound uptake in MT-4 cells. The inactive derivative KKD-014 is taken up by the cells more rapidly, reaching its saturation level already after 5 min. In contrast, the uptake of the analog KKD-016 is slower but steady. A substantial accumulation of KKD-016 was observed in the first 4 h of incubation (MFI for dansyl fluorescence was 646) that saturated during longer exposure of the cells to the drug (after 24 h the MFI was 715) (Fig. 5C). Comparable kinetics was observed for the uptake of these compounds in $CD4^+$-transfected HEK 293T cells (data not shown). As demonstrated in Fig. 5B, compound KKD-016 was taken up by the $CD4^+$ 293T cells with the same efficiency irrespective of the amount of CD4 on the cell surface. The dansyl fluorescence of cells expressing a low CD4 amount was similar to that of those expressing a high CD4 amount (dansyl MFI was 990 and 992, respectively). Furthermore, the uptake of KKD-016 by $CD4^-$ cells was identical to that of $CD4^+$ cells (Fig. 6).

Removal of the CADA analogs from the culture medium by extensive washing of the cells resulted in a rapid decline in dansyl fluorescence (Fig. 5C, dotted line). Measurement of the CD4 expression in MT-4 cells after exposure to CADA revealed a time dependent down-modulation of CD4 (Fig. 5C, solid line) that reached its maximum after 16 h of incubation, and was sustained for longer treatment. A 6 h exposure to this compound resulted in a 50% reduction in the surface expression of CD4 in treated MT-4 cells. Also, in $CD4^+$ HEK 293T cells, a time dependent CD4 down-regulation was observed after treatment with the CADA analog, showing approximately 50% decrease in CD4 expression after 4 h of incubation (the MFI for CD4 decreased from 5950 to 3073). Importantly, removing the compound from the medium resulted in a time-dependent re-expression of CD4 on the cell surface, which was completed within 24 h (Fig. 5C, right).

3.5. Increased uptake of CADA analog after surfactant administration

To find out whether the lack of CD4 receptor down-modulating potency of ES-KKD-024 is a consequence of its poor entry into cells, we improved its uptake. We formulated the analogs with a lipophilic adjuvant, a non-ionic surfactant, and assessed if this could modify their uptake by $CD4^+$ cells. Pluronic F-127 is a non-ionic polyol used in several studies to facilitate the solubilization and uptake of water-insoluble dyes [20,21]. A stock solution of the dansyl-labeled analog was prepared as a 1:1 mixture of pluronic F-127 and DMSO, and appropriate dilutions of the compound were evaluated for uptake and biological properties. As shown in Fig. 7, the use of pluronic F-127 affected the uptake of the CADA analogs differently with the length of the incubation time. A 1 h incubation (Fig. 7A) of the surfactant mixture with the active compound KKD-016 resulted in an increased uptake of the latter (2.5-fold increase). While the surfactant was ineffective for the inactive compound KKD-014, as evidenced by the similar MFI values, the efficiency of ES-KKD-024 uptake by the cells was 13 times

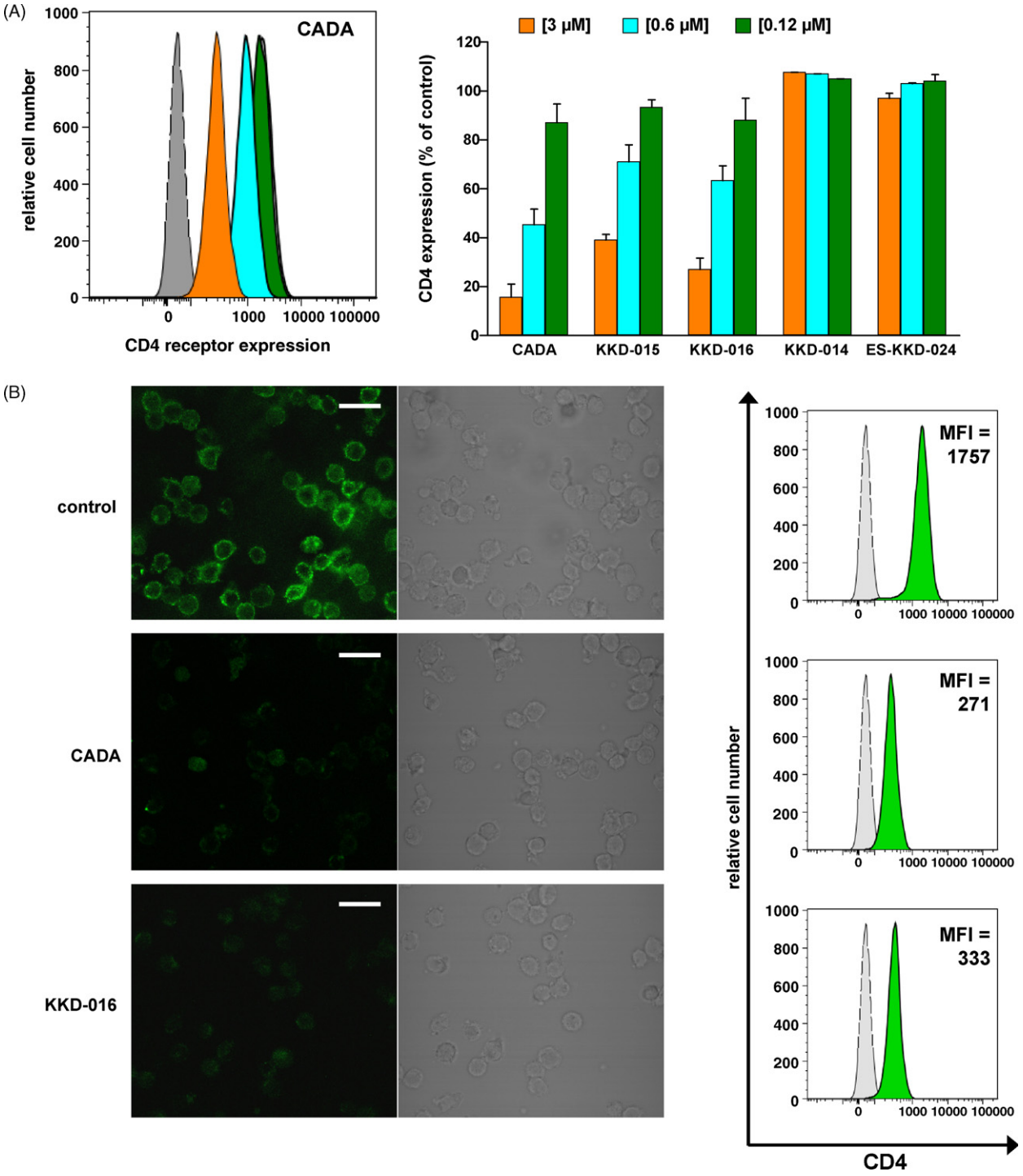


Fig. 3 – (A) Dose-dependent CD4 down-modulation in MT-4 cells after exposure to CADA analogs. The left panel shows the cell surface CD4 expression after single staining of MT-4 cells with PE-Cy5.5 labeled anti-CD4 mAb. Cells were treated with different concentrations of CADA for 24 h (3 μM = orange histogram, 0.6 μM = blue histogram and 0.12 μM = green histogram). Fluorescence is plotted on a logarithmic scale. The histogram in grey shows the corresponding non-specific background fluorescence measured with identical instrument settings. The CD4 expression of untreated control cells (peak in dotted line) coincides with the green histogram of CADA-treated cells and is not visible on this picture. These are data from one representative experiment, which was repeated 3 times with similar results. Right panel: CD4 receptor expression in MT-4 cells after treatment with the dansyl-labeled derivatives for 24 h at 3 different concentrations (μM) as indicated. As a positive control, the lead compound CADA was also included. The bars represent the CD4 receptor expression after staining with PE-Cy5.5 labeled anti-CD4 mAb as percentage median fluorescence intensity (MFI) of the untreated control cells. Data represent mean values ± S.D. for 3 independent experiments. (B) Fluorescent microscopic view of the CD4 expression in MT-4 cells after treatment with CADA compounds. The cells were incubated for 48 h with control medium or

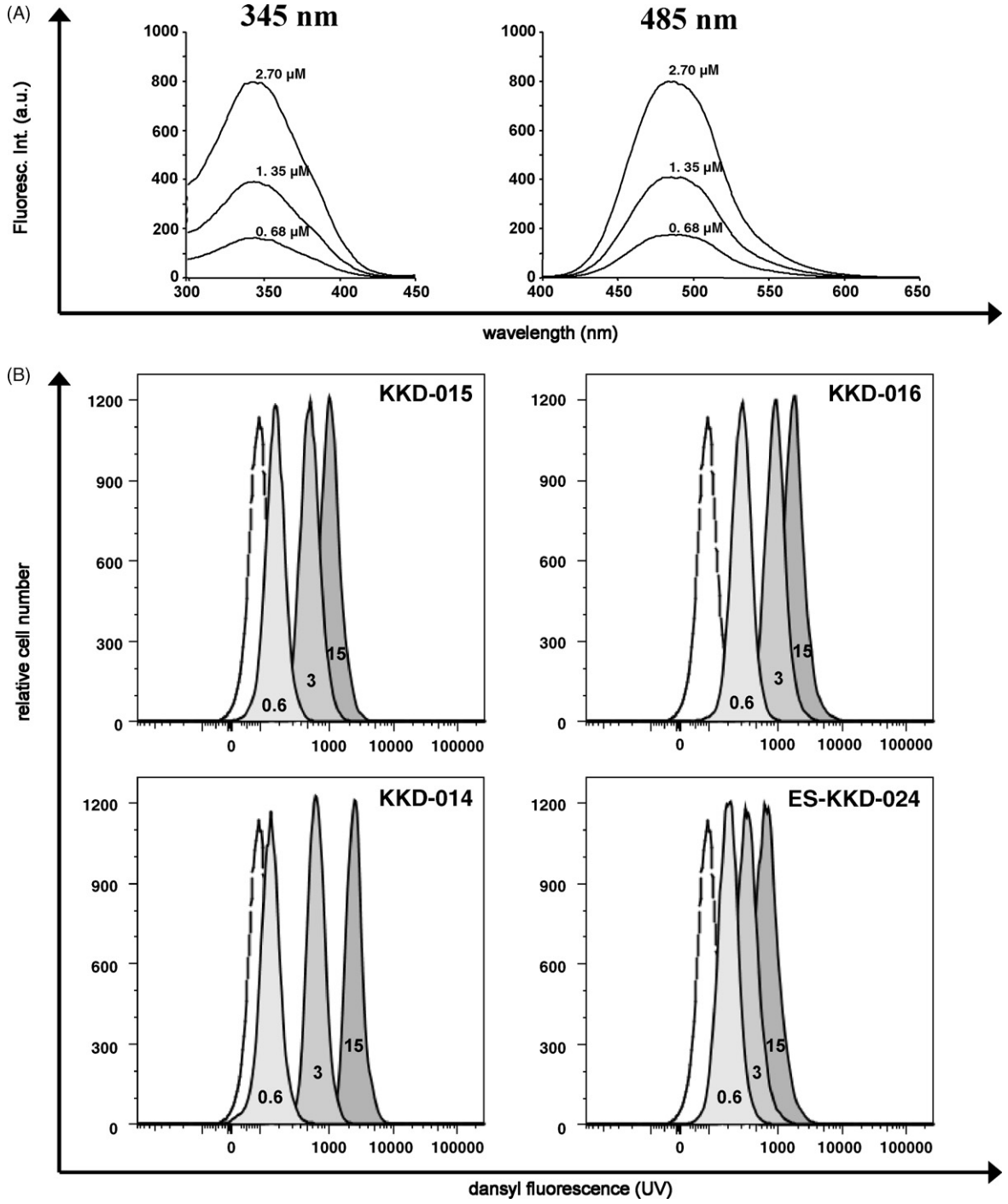


Fig. 4 – (A) Fluorescence excitation (left) and emission (right) spectra of KKD-016 at 3 different concentrations (2.7, 1.35 and 0.68 μM) in PBS buffer showing excitation maximum at 345 nm and emission maximum at 485 nm. (B) Dose-dependent flow cytometric measurement of dansyl fluorescence in MT-4 cells treated with increasing concentrations (0.6, 3 and 15 μM) of dansyl-labeled CADA analogs for 48 h. The histograms show the fluorescence intensity after UV excitation of the dansyl fluorophores. Fluorescence is plotted on a logarithmic scale. The white histogram in dashed line shows the corresponding non-specific background fluorescence as measured with identical instrument settings. These are data from one representative experiment, which was repeated 3 times with comparable results.

15 μM of CADA or KKD-016. Then, the cells were plated on a polylysine-coated coverslip, stained with Alexa488-labeled anti-CD4 antibodies and subjected to microscopy (left panels). The pictures were taken under the same experimental conditions. Scale bars, 20 μm. In parallel, the CD4 expression was measured by flow cytometry (right panels). In each histogram panel, the MFI for CD4 receptor expression is given.

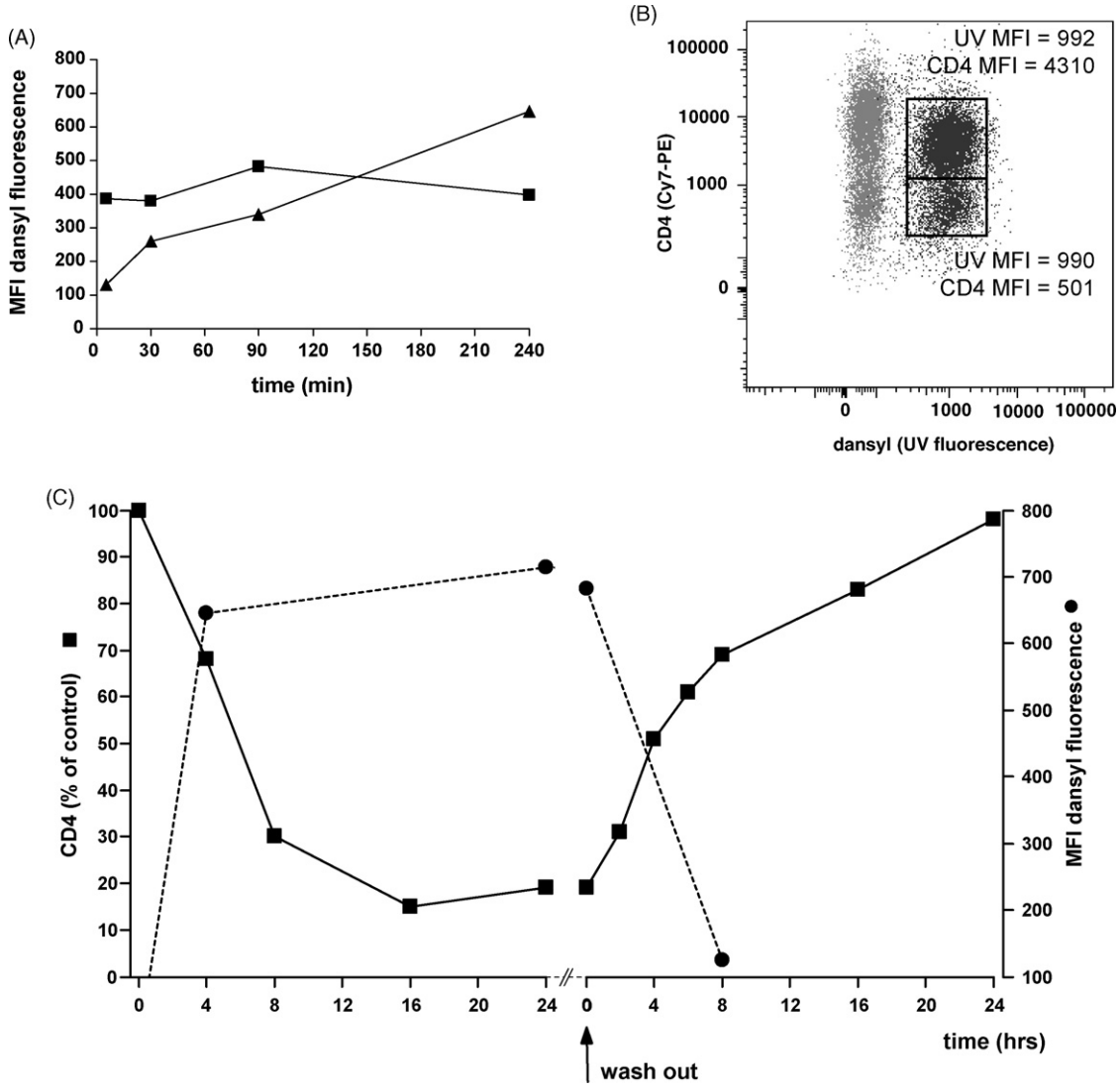


Fig. 5 – (A) Different capture of active (KKD-016) and inactive (KKD-014) dansyl-tagged CADA derivatives by MT-4 cells. The figure shows the kinetics of compound uptake in MT-4 cells. Cells were exposed to 3 μM of KKD-016 (triangle) or KKD-014 (square), and UV fluorescence was measured at the indicated time points by flow cytometry. Comparable kinetics was noted with 15 μM of compound. (B) Dansyl-labeled CADA compound uptake is independent of the level of CD4 expression. The dot plot represents the flow cytometric analysis of dansyl and CD4 fluorescence in $CD4^+$-transfected HEK 293T cells. Cells were treated with 15 μM of KKD-016 (black dots) or control medium (grey dots) for 4 h, stained with CD4 mAb (PE-Cy7 labeled) and subjected to flow cytometry. On the plot, the KKD-016 treated 293T cells are gated in two groups according to their CD4 expression level. The MFI values for dansyl and CD4 fluorescence of the corresponding gated cells are given. (C) Time dependent reversible CD4 down-modulation (square) in MT-4 cells treated with the CADA compounds. When a maximum CD4 down-regulation was reached in the cells after exposure to the compound (3 μM), cells were washed 3 times with PBS and incubated in fresh medium without compound (indicated by arrow). The CD4 expression at the indicated time points is given as the percentage of the MFI of untreated control cells stained with CD4-PE, showing a complete restoration of the CD4 expression. In parallel, the MFI of dansyl KKD-016 fluorescence of the cells (circle) is given. After washing of the cells (arrow), the amount of dansyl compound in the cells is dramatically declining as measured by flow cytometry.

higher when the compound was dissolved in a surfactant mixture as compared to the DMSO solution of ES-KKD-024 (MFI were 1870 and 142, respectively; Fig. 7A). Also, a longer incubation (48 h) resulted in a major dose-dependent uptake of ES-KKD-024 when administered as a surfactant mixture (Fig. 7B, bottom panels). Although in the presence of pluronic F-127 ES-KKD-024 was markedly more taken up by the cells and reached a loading level comparable to that of KKD-016, ES-KKD-024 was not able to substantially decrease the expression of CD4. Only a modest down-modulating effect on CD4 receptor expression was observed at 3 and 15 μM of ES-KKD-024 (Fig. 7B). In contrast, for KKD-016 a 48 h incubation with the pluronic mixture did not increase the accumulation of the compound in the cells: a similar dansyl signal was

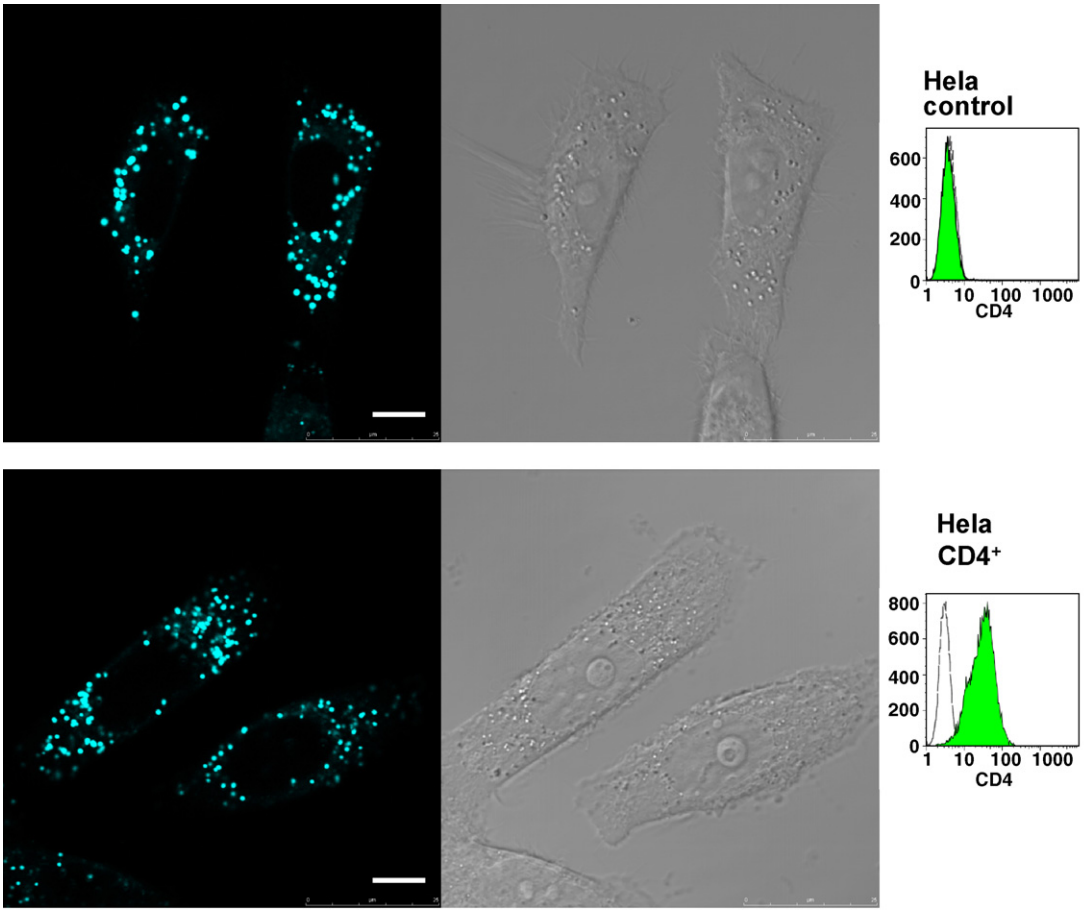


Fig. 6 – Similar uptake of the dansyl-labeled CADA compound KKD-016 by $CD4^-$ and $CD4^+$ cells. The pictures represent a fluorescent microscopic view of the accumulation of dansyl-labeled CADA compound in the cytosol of Hela cells (cyan). Control cells (top panels) and $CD4^+$ Hela cells (bottom panels) were treated with 15 μM of KKD-016 for 4 h and subjected to confocal microscopy. The pictures were taken under the same experimental conditions. Scale bars, 10 μm. In parallel, the CD4 expression (green histogram) of the cells was measured by flow cytometry just before KKD-016 administration (right panels).

observed for cells treated with KKD-016 in the presence or absence of pluronic F-127 (Fig. 7B, top panels). Accordingly, the CD4 down-modulating potency of KKD-016 was not increased by the surfactant. In fact, treatment with the surfactant only accelerated the loading of the cells with KKD-016: approximately the same amount of this dansyl-labeled CADA analog was observed when the cells were incubated for 1 h with the KKD-016 pluronic F-127 mixture as when they were treated for 4 h with KKD-016 in DMSO alone (MFI were 2194 and 1796, respectively).

4. Discussion

The small synthetic molecules cyclotriazadisulfonamides, of which CADA (Fig. 1) can be regarded as the lead compound, represent a new class of HIV entry inhibitors [11,12]. It was demonstrated that CADA specifically decreases the expression of surface CD4 without affecting other cellular receptors [11]. The compound had consistent CD4 down-regulating activity in different cell types, which correlated with its broad spectrum antiviral potency against laboratory and clinical isolates of HIV-1 and HIV-2 [11–14]. To better decipher the peculiar mechanism of action of this CD4 down-modulator, we constructed fluorescent CADA analogs. We opted for the small fluorophore dansyl since it could be integrated into the chemical synthesis of CADA. This fluorophore has been earlier used and proved useful in several studies on ligand-receptor interactions [22–26].

Here, we incubated cells with the dansyl-labeled CADA derivatives and analyzed them by flow cytometry. Cell incubation at different concentrations of these derivatives resulted in a bright dose-dependent fluorescent signal, suggesting that the compound distribution reached an equilibrium between culture medium and the intracellular compartment. The narrow Gaussian distribution of fluorescence in the cells indicates an equal uptake of the CADA compounds over the total cell population. This can explain the uniformity of the CD4 down-modulation within different cell types, such as T cells, monocytes and dendritic cells [11–14].

In order to select for a fluorescent analog with biological activity similar to or better than CADA, we first evaluated the

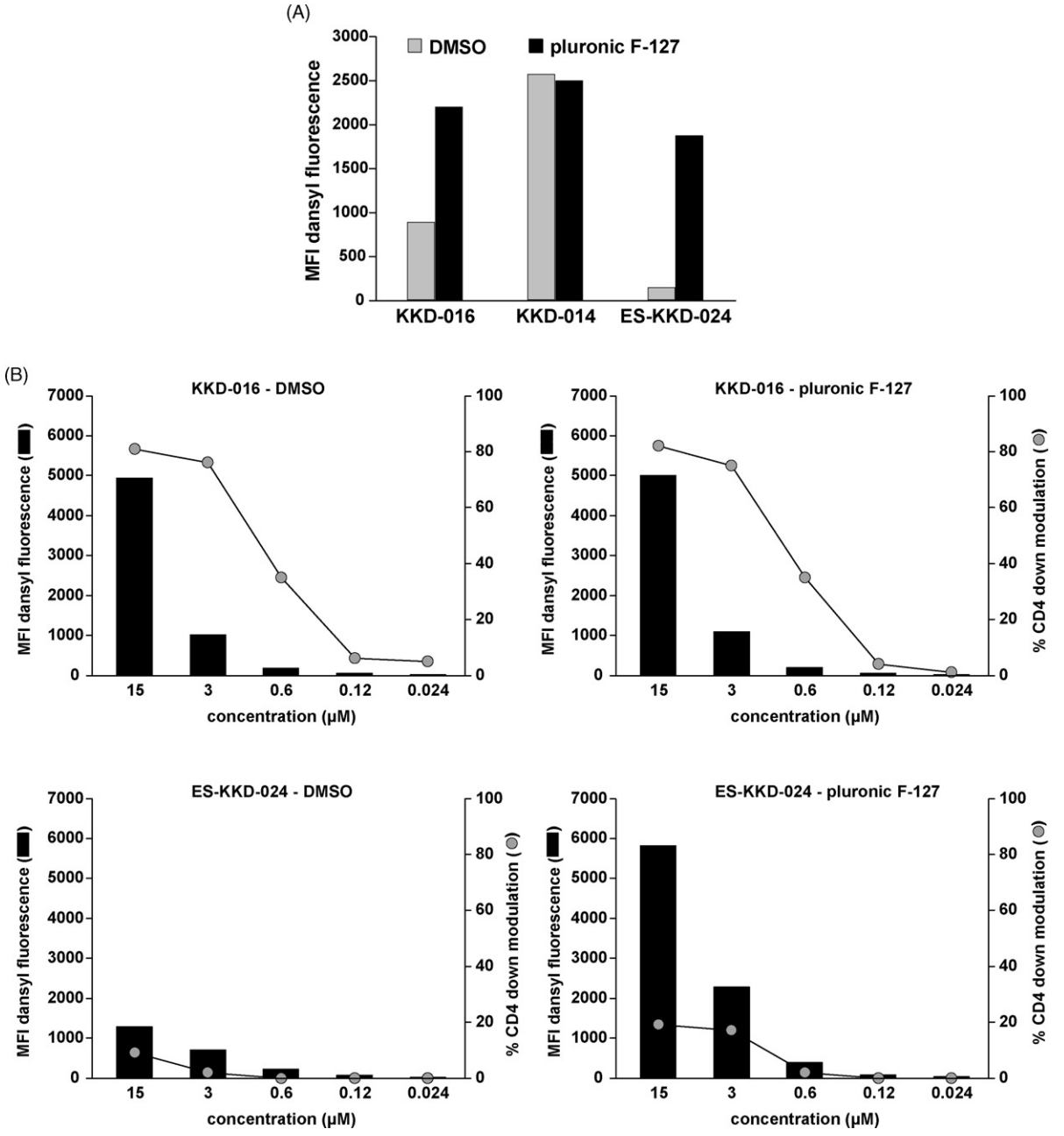


Fig. 7 – (A) Improvement of compound capture by administration of the surfactant pluronic F-127. Uptake of dansyl-labeled CADA analogs in CD4⁺ HEK 293T cells after 1 h of treatment with 3 μM of compound of which the stock solution was dissolved in DMSO only (grey histogram) or dissolved in a 1:1 mixture of DMSO and pluronic F-127 (black histogram). The MFI for dansyl fluorescence (UV) is given. Comparable data were obtained with 15 μM of compound. (B) Increased ES-KKD-024 uptake by the cells is not correlated with stronger CD4 down-modulation. Dansyl fluorescence and CD4 expression in MT-4 cells after 48 h treatment with KKD-016 (upper panels) or ES-KKD-024 (lower panels) of which the stock solution was prepared by dissolving the compound in DMSO only (left panels) or in a 1:1 mixture of DMSO and pluronic F-127 (right panels). The black histograms represent the MFI for dansyl fluorescence at the indicated concentration of compound. In parallel, the corresponding CD4 receptor cell surface expression at each concentration was measured and is given as the percentage CD4 receptor down-modulation (line).

antiviral and CD4 down-modulating potency of the dansyl-labeled derivatives. As shown earlier [19], small modifications in the chemical structure of CADA may significantly affect the activity of the derivatives. Changes in the sidearms were reported to have mostly a detrimental effect on the CD4 down-regulating activity. Accordingly, compound ES-KKD-024, in which both tosyl sidearms were substituted by dansyl, did not down-modulate CD4 and did not protect the cells from HIV-1 infection. This observation is in agreement with our computational three-dimensional quantitative structure-activity

relationship (QSAR) analyses [18], showing that increasing steric bulk of the sidearm regions correlates with a decrease in the CD4 down-modulation potency. However, derivative KKD-015, in which only one tosyl sidearm was replaced by dansyl, retained CD4 receptor down-modulating activity although the EC_{50} of this compound was substantially higher than of CADA. This indicates that the presence of at least one tosyl sidearm is crucial to the CD4 receptor down-modulating property. Also, comparing the structures of KKD-015 and KKD-016 led us to conclude that the benzyl tail plays an important role in the activity of these CADA derivatives as well. Indeed, exchange of the benzyl tail of KKD-015 for a cyclohexylmethylene group as in KKD-016 increased the CD4 down-modulating potency of the compound. This observation is in agreement with earlier reported higher activity of compound QJ-028, an analog in which a cyclohexylmethylene group replaced the benzyl group of CADA [19]. Thus, for KKD-016, the decrease in activity as a result of the dansyl sidearm was compensated by the exchange of the benzyl tail with a cyclohexylmethylene group, so that KKD-016 retained the potency of the parental inhibitor. Finally, the disruption of the triazacyclododecane ring structure (compound KKD-014) resulted in a complete loss of activity. The alteration of this ring, in particular of the three-carbon bridge between the tosyl and dansyl group, probably disturbs the distance between the two sidearms that seems to be of crucial importance for maintaining the biological activity of this class of compounds [18].

To test whether the CD4 down-modulating activity is related to the cellular uptake of these compounds, we compared distribution of active and inactive CADA derivatives in several cell populations. We found similar levels of dansyl fluorescence in cells incubated with inactive KKD-014 and active KKD-015. Thus, the inability of the former CADA derivative to down-regulate CD4 is not due to a shortage of compound in the cells. Moreover, even when the cell uptake of ES-KKD-024 was greatly increased by administration of the surfactant pluronic F-127, there was no CD4 down-modulation. However, it is conceivable that fine intracellular distribution of these compounds may be related to their differential activity. A more detailed study with, e.g. confocal microscopy is required to further define the exact location of the CD4 down-modulator in specific subcellular compartments.

Both active and inactive CADA compounds entered all the analyzed cell populations independently of their expression of CD4. Interestingly, for active compounds entering CD4-expressing cells, a clear relationship between their uptake and CD4 down-modulating activity was observed. This was demonstrated by flow cytometry that allowed simultaneous evaluation of both parameters in single cells. The slow uptake of the compound correlated with a gradual decrease in CD4. At a saturating level of CADA derivative, the low expression of CD4 was sustained as long as the compound was present in the culture medium. After removal of the CADA compounds from the culture medium, its amount in the cell dropped dramatically resulting in a complete restoration of CD4 expression. The reversibility of CADA-triggered CD4 down-regulation may be important if this drug is used in a clinical setting, e.g. as microbicide, since it may down-modulate CD4 only temporally at the time of the threat of HIV infection.

The above-described results with fluorescent CADA derivatives confirm our earlier conclusion [19] that down-modulation of the HIV receptor CD4 mediates the anti-HIV activity of CADA. Here, we found a strong correlation between EC_{50} values for CD4 down-regulation and suppression of HIV infection (correlation coefficient $r = 0.96$). This shows that removal of a large amount of the main HIV receptor from the cell surface or bringing this CD4 receptor density below a level that is required for efficient viral entry [8,9,11] is sufficient to inhibit HIV-1 infection of target cells.

In view of their potentials as preventive and therapeutic anti-HIV drugs, it is important that these CADA derivatives are not cytotoxic. Down-regulation of the CD4 receptor expression by the CADA compounds was not detrimental to the cell viability. This indicates that cellular cytotoxicity related to this class of drugs is not linked to specific interference with CD4 receptor expression as such. Therefore, CD4 down-modulation seems to be mediated by specific mechanisms rather than by decrease in general cell viability. This conclusion is further supported by our observations that CADA-treated cells remain fully immunocompetent, probably due to residual CD4 receptors that are expressed on their surface (manuscript in preparation). Another important advantage of CADA is that its uptake and activity do not depend on CD4 expression on the cell surface. Thus, CADA is effective even for cells with low level of CD4 expression, such as monocytes and dendritic cells [13], which are nevertheless susceptible to HIV infection and are important cells in HIV transmission [27–29]. CADA may therefore have its potential in the development of topical microbicides that specifically target mechanisms of HIV transmission and prevent the sexual spread of HIV [30].

In conclusion, the dansyl fluorophore was successfully integrated in the chemical structure of CADA with preservation of the CD4 down-modulating and anti-HIV activity. Application of flow cytometry in combination with the dansyl-labeled CADA derivatives enabled the study of their uptake and CD4 down-modulating activity in single cells simultaneously. We demonstrate that CADA compounds do not affect cell viability and that their uptake and activity are independent of the level of CD4 receptor expression. Also, we show that the effect of these CD4 down-modulators is reversible. Fluorescent CADA analogs can now be used to investigate in depth the specific mechanism of action of this novel class of anti-retrovirals which can provide new insights into the HIV entry process and can lead to the development of new anti-HIV drugs.

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