Profound anti-HIV-1 activity of DAPTA in monocytes/macrophages and inhibition of CCR5-mediated apoptosis in neuronal cells

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Monocytes/macrophages (M/M) are strategic reservoirs of HIV-1, spreading the virus to other cells and inducing apoptosis in T-lymphocytes, astrocytes and neurons. M/M are commonly infected by R5 HIV-1 strains, which use the chemokine receptor CCR5. D-Ala-peptide T-amide (DAPTA), or Peptide T, named for its high threonine content (ASTTTNYT), is a synthetic peptide comprised of eight amino acids (185–192) of the gp120 V2 region and functions as a viral entry inhibitor by targeting selectively CCR5. The anti-HIV-1 activity of DAPTA was evaluated in M/M infected with R5 HIV-1 strains. DAPTA at 10^{-9} M inhibited HIV-1 replication in M/M by >90%. PCR analysis of viral cDNA in M/M showed that DAPTA blocks HIV entry and in this way prevents HIV-1 infection. Moreover, DAPTA acts as a strong inhibitor and was more active than the non-peptidic CCR5 antagonist TAK-779 in inhibiting apoptosis (mediated by R5 HIV-1 strains produced and released by infected M/M) on a neuroblastoma cell line. Our results suggest that antiviral compounds which interfere with receptor mechanisms such as CCR5 could be important, either alone or in combination with other antiretroviral treatments, in preventing HIV infection in the central nervous system and the consequential neuronal damage that leads to neuronal AIDS.

Keywords: apoptosis, CCR5, entry inhibitors, HIV-1, monocytes/macrophages

Introduction

Monocytes/macrophages (M/M) have an important role in all phases of human immunodeficiency virus type 1 (HIV-1) infection, acting as a vehicle for virus dissemination in the body and representing the major reservoir for long-term persistence of HIV-1 during highly active antiretroviral therapy (HAART) (Aquaro *et al.,* 2002; Perelson *et al.,* 1997; Sharkey *et al.,* 2000). Microglia, local differentiated M/M, are the main source of virus in the brain and their pathogenic secretory products cause neuronal AIDS (Guan *et al.,* 2002; Kazmierski *et al.*, 2003). HIV-1 entry into cells occurs after binding of the viral envelope glycoprotein gp120 to specific chemokine receptors in conjunction with the CD4 receptor (Kaul *et al.,* 2001; Martin-Garcia *et al.,* 2002). CCR5 in particular is the principal co-receptor for the HIV-1 strains that are most commonly transmitted between individuals and which predominate during the early years of infection. These strains prevail in the brain where they cause the manifestation of neuronal AIDS through the infection of CCR5-expressing monocytes and microglia (Baba *et al.,* 1999; Douek *et al.,* 2003; Polianova *et al.,* 2005). M/M and microglia are infected primarily by R5 HIV-1 strains, which use the β-chemokine receptor CCR5 (Kaul *et al.,* 2001). The CCR5 binding site seems to be masked until gp120 interacts with the CD4 receptor. This interaction triggers a conformational change in gp120, which then exhibits a high-affinity binding site for the co-receptor (Martin-Garcia *et al.,* 2002). The clinical relevance of the predominant use of CCR5 by HIV-1 is demonstrated by the effect of a naturally occurring CCR5 mutation, CCR5-Δ32, which generates a non-functional co-receptor (Baba *et al.,* 1999; Dean *et al.,* 1996; Liu *et al.,* 1996; O'Brien & Moore, 2000; Samson *et al.,* 1996; Wu *et al.,* 1997). Individuals who are homozygous for this mutation are strongly protected against HIV-1 infection, whereas infected, heterozygous individuals progress less rapidly to disease and death (Cartier *et al.,* 2003; Trkola

et al., 2001; Van Rij *et al.,* 1999). Individuals lacking functional CCR5 do not show obvious adverse effects on health, although they can be more vulnerable to West Nile Virus. In addition, the use of CCR5 inhibitors, such as vicriviroc, could be correlated with an increased incidence of cancer in HIV-1-infected patients. The natural ligands for the chemokine receptor CCR5, such as LD78β (a major variant of human MIP-1α), RANTES (Regulated upon Activation Normal T-cell Expressed and Secreted) and MIP-1α (macrophage inflammatory protein 1α), reduced virus entry and subsequently replication in M/M (Aquaro *et al.,* 2001). Therefore, blocking CCR5 on the M/M surface prevents HIV-1 infection of these cells (a crucial event during the initial spread and early phases of infection) and consequently limits the spread of infectious viral particles during the whole course of the disease (Aquaro *et al.,* 2002; Martin-Garcia *et al.,* 2002; Ruff *et al.,* 2001; Ruff *et al.,* 2003; Trkola A *et al.,* 2001). CCR5 is also expressed on neurons and astrocytes in the brain and, although neuronal cells are usually not productively infected by HIV-1, *in vitro* studies have shown that natural ligands of CCR5 protect neurons from gp120-mediated apoptosis (Brenneman *et al.,* 1999; Cartier *et al.,* 2003; Cocchi *et al.,* 1995; Dragic *et al.,* 2000; Kaul & Lipton, 1999; Kaul *et al.,* 2001; Meucci *et al.,* 1998; Trkola *et al.,* 2001). Taken together, CCR5 is an attractive target both for inhibition of CCR5-mediated HIV entry in M/M (and consequently for virus transmission of infected particles in the body), and for the prevention of gp120-induced apoptosis in neuronal cell lines. The identification of new CCR5-targeting antibodies, chemokines, chemokine analogues, small molecules and peptides, is therefore an important step in the development of new antiviral drugs that target virus entry (Doms, 2000) through mechanisms that differ from those of existing HAART drugs. Several different types of inhibitor for CCR5-mediated HIV-1 entry have now been identified and are in pre-clinical or clinical development as drug candidates (De Clercq, 2002; Horuk, 2003; Kazmierski *et al.,* 2003; LaBranche *et al.,* 2001; Michael & Moore, 1999; Moore & Stevenson, 2000; O'Hara & Olson, 2002; Schwarz & Wells, 2002). In particular we focused our attention on D-Ala-peptide T-amide (DAPTA), or Peptide T, named for its high threonine content (ASTTTNYT). DAPTA is a synthetic peptide comprised of eight amino acids (185–192) of the gp120 V2 region and functions as a viral entry inhibitor by targeting selectively CCR5 (Polianova *et al.,* 2005; Ruff *et al.,* 2001; Ruff *et al.,* 2003). This non-toxic small peptide suppresses the infection of peripheral blood monocytes, and thereby would prevent and reduce the population of infected differentiated M/M (Ruff *et al.,* 2003). Recently, in a small clinical trial, DAPTA has shown promising antiviral and immune benefits. Moreover, the peptide caused improvements in cognition in humans with HIV-1 infection, also suggesting its penetration into the central nervous system (CNS) (Heseltine *et al.,* 1998; Polianova *et al.,* 2005). A blind analysis of frozen stored plasma samples conducted by the National Institute of Mental Health (NIMH) in the early 1990s from the randomized, double-blind placebo-controlled trial of DAPTA for HIV-associated cognitive impairment (Heseltine *et al.,* 1998) found a significant reduction (0.54 $\log_{10}$, P= 0.037) in viral load between baseline and month 6 (Goodkin *et al.,* 2006). To better define potency and the potential mechanisms of action of DAPTA, we studied its inhibitory effect on CCR5 binding to gp120 in M/M and its effect on HIV-1-induced apoptosis in neuronal cell lines. The results indicate that DAPTA efficiently binds CCR5 and is able to inhibit HIV-1 entry, thus preventing HIV infection of M/M. In addition, DAPTA blocks HIV-1-M/M CCR5-mediated apoptosis in a neuronal cell line.

Materials and methods

Cells

M/M were prepared and purified as described in published procedures (Aquaro & Perno, 2005). Briefly, peripheral blood mononuclear cells (PBMCs) were obtained from the blood of healthy HIV-seronegative donors. PBMCs were separated by Ficoll-Hypaque gradient centrifugation and seeded in plastic 48-well plates (Costar, Cambridge, MA, USA) at a density of 1.8×10^6 cells/ml in RPMI 1640 (Gibco, Gaithersburg, MD, USA) supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine and 20% heat-inactivated, mycoplasma- and endotoxin-free fetal calf serum (HyClone, Logan, UT, USA) (complete medium). Cell cultures were incubated in a humidified atmosphere with 5% CO_2 at 37°C. Non-adherent cells were removed 6 days after seeding by repeated gentle washing with warmed RPMI 1640, leaving a monolayer of adherent cells, which were incubated in complete medium as previously described. Adherent cells obtained using this technique consisted of >95% differentiated M/M. The neuroblastoma cell line SK-N-SH (Koenig *et al.*, 1986) was obtained from American Type Culture Collection (ATCC HTB-11) and maintained in RPMI 1640 supplemented with 10% fetal calf serum, 50 U/ml penicillin, 50 μg/ml streptomycin and 2 mM L-glutamine. Human T-lymphocytic C8166 cells were obtained from the American Type Culture Collection (Manassas, VA, USA).

Virus

The R5 HIV-1 strains BaL and 81A, for which the characteristics and genomic sequences have been previously described, were used (Cenci *et al.*, 1997; Perno *et al.*,

1998). HIV-1 NL4.3 was obtained through the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH, USA). Before use, the viruses were ultracentrifuged for 2 h at 22,000 rcf at 4°C.

Compounds

DAPTA was synthesized under GMP conditions and obtained from Bachem (Torrence, CA, USA). A stock solution, diluted in sterile water, was made fresh for each experiment. The CCR5 antagonist TAK-779 (*N,N*-dimethyl-*N*-(4-[[[2-(4-methylphenyl)-6,7-dihydro-5*H*-benzocycloheptenyl]carbonyl]amino]benzyl)-tetrahydro-2*H*-pyran-4-amonium chloride; Mr=531.13) (Baba *et al*., 1999) was obtained from Takeda Chemical Industries (Osaka, Japan). The anti-CCR5 monoclonal antibody (mAb) (clone 2D7) was purchased from BD Pharmingen (San Diego, CA, USA).

HIV-1 infection

Two days after isolation (that is, 7 days after plating) M/M were exposed to various concentrations of DAPTA (10^{-14}, 10^{-13}, 10^{-12}, 10^{-11} and 10^{-10} M) for 20 min, and then challenged with the R5 HIV-1 strains BaL or 81A (2,000 pg/ml of p24 gag). After 2 h incubation at 37°C in a humidified atmosphere supplemented with 5% CO_2, M/M were extensively washed with warm RPMI 1640 to remove the excess virus and then cultured in the presence of DAPTA. Supernatants were collected 7 days later and M/M were extensively washed with warm RPMI 1640; complete medium containing the same concentration of DAPTA was added. At the end of the experiment (21 days after infection), supernatant was collected and stored at -20°C. The experiment was run in triplicate, with six positive controls for each experiment.

Antiviral activity of DAPTA

Supernatants of acutely infected M/M collected at 14 and 21 days were assessed to determine virus production in the presence or absence of DAPTA by measurement of HIV p24 gag production using a commercially available HIV antigen ELISA kit (Biorad, France). The experiments were carried out in triplicate, with six positive controls (untreated virus-infected cells) for each experiment, and the results are presented as mean value with standard deviation. Moreover, we tested the antiviral activity of DAPTA (ranging from 10^{-9}–10^{-15} M) in C8166 infected by 1,000 pg/ml of CXCR4-using (X4) strains (HIV-1 NL4.3) by analysis of the cytophatic effect at days 3, 4 and 5 after infection.

HIV-1 DNA analysis in the presence of DAPTA

PCR analysis of integrated HIV-1 proviral DNA was performed on differentiated M/M. M/M were obtained from peripheral blood by adherence, cultured for 5 days and infected with HIV-1 BaL (30 pg/ml) in the presence or absence of DAPTA (10^{-9} M and 10^{-7} M doses) and anti-CCR5 mAb 2D7 (3,000 pg/µl). For HIV-1 proviral integration analysis, genomic (total) DNA was isolated from M/M after 18 h of HIV-1 infection (Qiagen DNA isolation and purification kit; Qiagen, Valencia, CA, USA) and 1×10^{6}–1.25×10^{5} cell equivalents were amplified in an inverse/nested PCR specific for a conserved region within the *gag* gene (primer pair SK39/SK38; GenBank accession numbers A24318/A26625, synthesized by Gibco BRL/Invitrogen Life Technologies Carlsbad, CA, USA). The 115 bp PCR products were detected by oligomer hybridization using 3′-fluorescein-labelled probes SK19 (GenBank accession number A24328; http://www.ncbi.nlm.nih.gov). The probe was labelled with Gene Images 3′-oligolabelling Module (Amersham, Piscataway, NJ, USA) according to the manufacturer's procedure and specific target sequences immobilized on the Hybon-N nylon membrane (Amersham, Piscataway, NJ, USA) were detected by Gene Images ECL Detection Kit (Amersham, Piscataway, NJ, USA) by exposing to blue-light sensitive X-ray film (Pegasus Scientific Inc., Burtonsville, MD, USA). Amplification of the β-actin housekeeping gene was used to evaluate the efficiency of the extraction procedure and to estimate the concentration of isolated DNA. DNA isolated from U1 cells in which two HIV-1 proviral copies are integrated in each cell genome was used as a positive control and semi-quantitative analysis. Band density was measured by software program UN-SCAN-IT-gel software (Silk Scientific Inc., Orem, UT, USA).

Interaction of DAPTA with CCR5

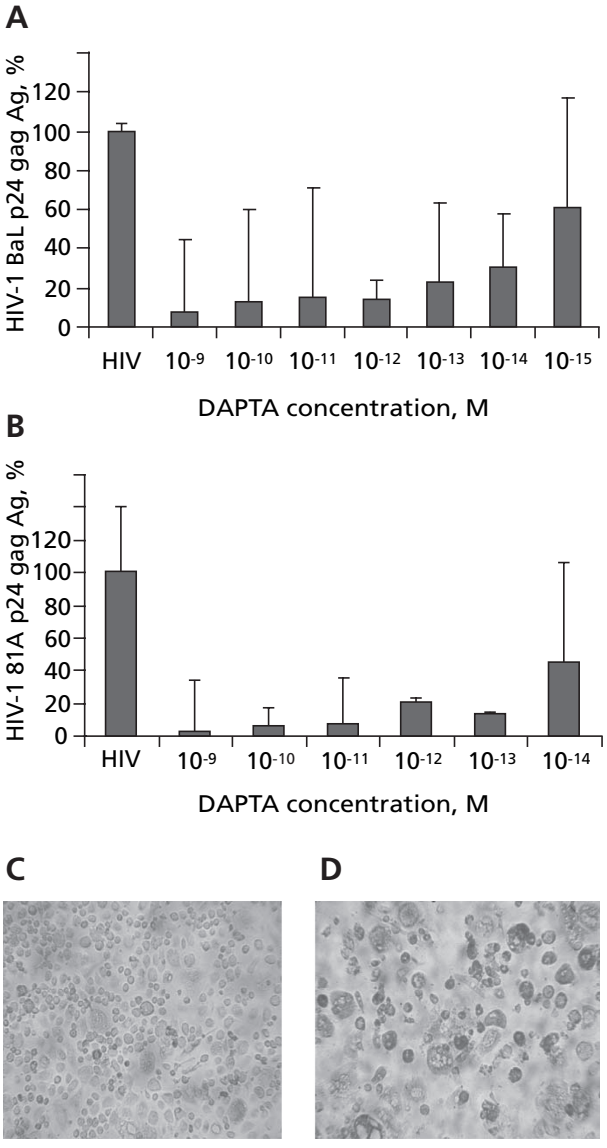
To verify the specificity of DAPTA–CCR5 binding we assessed the percentage of CCR5 expression in the presence of DAPTA in M/M and in the differentiated neuronal cell line SK-N-SH. M/M were detached gently from the plates with trypsin/EDTA (0.02%), centrifuged at 1,600 rpm for 10 min, counted and resuspended at a density of 2×10^{5} cells /ml in complete medium. The M/M were then incubated with DAPTA, at several doses, for 20 min at 4°C and then stained with FITC-labelled anti-CCR5 mAb (2D7, BD Pharmingen) for 30 min at 4°C in the dark. After incubation, stained cells were washed with phosphate-buffered saline (PBS) and analysed with a FACScan flow cytometer (Becton Dickinson; Becton Drive, Franklin Lakes, NJ, USA). Ten thousand events were collected for each sample. The data were acquired and analysed by the Lysis II program (Becton Dickinson). The same staining procedure was repeated for the neuronal cell line SK-N-SH.

Flow cytometric analysis of neuronal apoptosis

We assessed neuronal apoptosis in the neuroblastoma cell line, SK-N-SH, after retinoic-acid-induced differentiation.

To avoid overgrowth, SK-N-SH were seeded in Petri plates at a density of 20,000 cells/ml in RPMI 10% medium and exposed, at day 1 after seeding, to 1 M retinoic acid for 4 days. After differentiation, culture medium containing retinoic acid was completely removed from the Petri plates and replaced with fresh RPMI 10% medium containing 8,000 pg/ml of p24-gag of HIV-1 BaL grown in M/M cultures and DAPTA (at 10^{-13} and 10^{-12} M). As control, we used the CCR5 antagonist TAK-779 (1.8×10^{-6} M). The cells were then incubated at 37°C in humidified air containing 5% CO_2 for 5 days. On the day of analysis, the cells were gently detached with trypsin/EDTA (0.02%) and centrifuged at 1,600 rpm for 10 min. Pellets were washed with phosphate buffered saline (PBS), placed in ice, and permeated with ice-cold 70% ethanol for 30 min. The aliquots were centrifuged at 1,500 rpm for 10 min, the pellets were washed with PBS, incubated with propidium iodide (PI; 100 µg/ml) and RNase (250 µg/ml Qiagen) at 4°C for 2 h in the dark. Samples were then washed twice with PBS and PI-stained cells were analysed by monitoring the incorporation of intracellular PI with a FACScan flow cytometer. Ten thousand events were collected for each sample. Data were acquired and analysed by the Lysis II program (Becton Dickinson). Statistical analysis was performed by χ^2 test for DAPTA-treated cells versus a control of untreated neuronal cell lines.

Figure 1. Antiviral activity of DAPTA in monocytes/macrophages



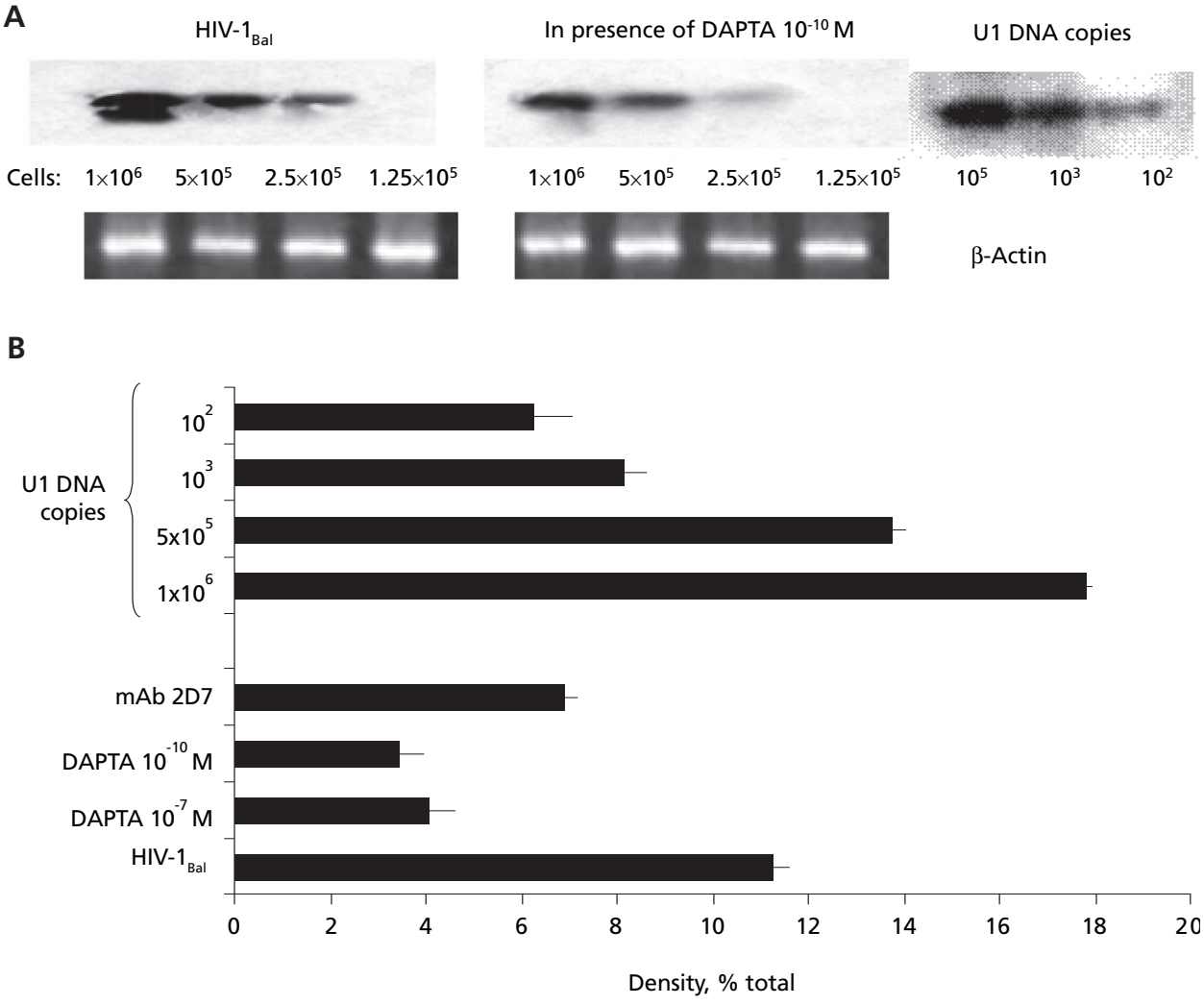
Monocytes were cultured for 6 days to generate monocytes/macrophages (M/M) that were infected with **(A)** HIV-1 BaL or **(B)** HIV-1 81A in the presence of D-Ala-peptide T-amide (DAPTA) at the concentrations shown. Supernatants were collected at day 14 and tested for p24 gag antigen (Ag) production. Each experiment was run in triplicate. The results are presented as mean value with standard deviation. Values are given as the percentage of virus inhibition. The cytopathic effect of DAPTA as assessed by light microscopy after 14 days of HIV-1 BaL infection in **(C)** the presence of DAPTA (10^{-9} M dose) or **(D)** the absence of DAPTA.

Results

DAPTA inhibits R5 HIV-1 replication in macrophages

Viral replication and production in HIV-1-infected M/M treated with DAPTA was assessed 14 and 21 days after infection for p24 antigen production. A representative experiment is shown in Figure 1. Fourteen days after HIV-1 infection, p24 gag antigen production in the supernatants of HIV-1 BaL infected M/M was found to be drastically reduced in a dose-dependent manner in the presence of DAPTA, at concentrations ranging from 10^{-9}–10^{-15} M. Figure 1A shows the results, expressed as a percentage compared with the positive control, in which M/M were infected with HIV-1 without DAPTA treatment (100%). The maximal viral inhibition observed was ~90% with 10^{-9} M DAPTA concentration. With another R5 HIV-1 strain, 81A, we obtained comparable results. In 81A HIV-1-infected M/M, 14 days after infection ~97% viral inhibition is reached using 10^{-9} M DAPTA (Figure 1B). Comparable results were confirmed at day 21 after infection (data not shown). The protective effect of DAPTA was confirmed by light microscopic pictures. As we can see, the presence of DAPTA (Figure 1C) is able to reduce the cytopathic effect, with a decrease in syncytia formation and aggregation of cells induced by R5 HIV-1 in M/M after 14 days of infection (Figure 1D). By contrast, in C8166 infected with 1,000 pg/ml of HIV-1 NL4.3 and treated with DAPTA, the peptide was unable to prevent syncytia formation owing to the X4 HIV-1

Figure 2. Reduction of HIV-1 DNA formation in monocytes/macrophages in the presence of DAPTA



(A) Monocytes/macrophages (M/M) were infected with HIV-1 BaL in the presence or absence of D-Ala-peptide T-amide (DAPTA) at 10^{-7}–10^{-10} M doses and the anti-CCR5 monoclonal antibody (mAb 2D7; 3,000 pg/μl), as described. HIV-1 DNA was extracted from *in vitro* differentiated M/M 18 h after infection and 1×10^6–1.25×10^5 cell equivalents were detected by Southern hybridization. HIV-1 DNA extracted from U1 cells, containing two integrated HIV copies/cell, was used as positive standard. **(B)** Band density was measured by UN-SCAN-IT (Silk Scientific Inc). The results are from one representative experiment of two.

infection of T-cells (data not shown). We can conclude that DAPTA showed a potent antiviral activity against R5 strains, but not against X4 strains of HIV-1.

DAPTA reduces levels of HIV-1 DNA in human primary macrophages

To further demonstrate that DAPTA blocks virus infection, M/M were analysed for HIV-1 DNA formation. Eighteen hours post-infection, genomic DNA was extracted and twofold dilution of cell equivalents (range 1×10^6–1.25×10^5) were amplified in an inverse/nested PCR specific for a conserved gag region of the viral genome. Semi-quantitative analyses of HIV-1 DNA in M/M were performed by comparison of DNA amplification products from infected cells, standardized by PCR for β-actin, to standards of amplified U1 DNA copies and cell numbers. The UN-SCAN IT-gel software (Silk Scientific Inc.) was used to determine band densities (Figure 2A). We found that HIV-1 DNA per 2.5×10^5 cells declined in the presence of DAPTA when compared with untreated cells: DNA levels were reduced to 64% in the presence of 10^{-7} M DAPTA and 70% in the presence of 10^{-10} M DAPTA. Control infected cultures (HIV-1 BaL), in the absence of DAPTA or 2D7 mAb, had ~1×10^4 HIV-1 copies per 10^5

M/M (that is, 0.1 copy per M/M). The inhibition of HIV-1 DNA formation detected in M/M in the presence of mAb 2D7 at the maximum amount of 3 μg/ml was ~39% (Figure 2B). These results are in agreement with published data (Tuttle *et al.*, 1998). These data indicate that DAPTA inhibits productive infection in M/M by blocking specifically the CCR5-dependent entry with a potency greater than that of the specific anti-CCR5 antibody 2D7.

DAPTA reduced CCR5 mAb binding in human primary macrophages

To confirm that DAPTA binding is specific for CCR5, a competition experiment was performed between CCR5-FITC antibody 2D7 and DAPTA in M/M. 2D7 antibody recognizes a conformation-dependent epitope in the second extracellular loop of CCR5, and is a potent inhibitor of R5 virus cell entry. Flow cytometric analysis showed that 35% of mock-treated M/M were CCR5-positive. DAPTA treatment of cells showed reduced binding of the 2D7 mAb to CCR5 in a dose-dependent manner, with maximal reduction of CCR5 detection (9%) occurring at 10^{-12} M (Figure 3) (P=0.001). Overall, the inhibition of CCR5 binding by several DAPTA doses is ~43% and reaches a maximum of 73% with 10^{-12} M. These results suggest that DAPTA reduced the CCR5 antibody binding to the receptor in M/M by masking the binding site.

Effects of DAPTA on CCR5 binding and gp120-induced apoptosis in neuronal cell lines

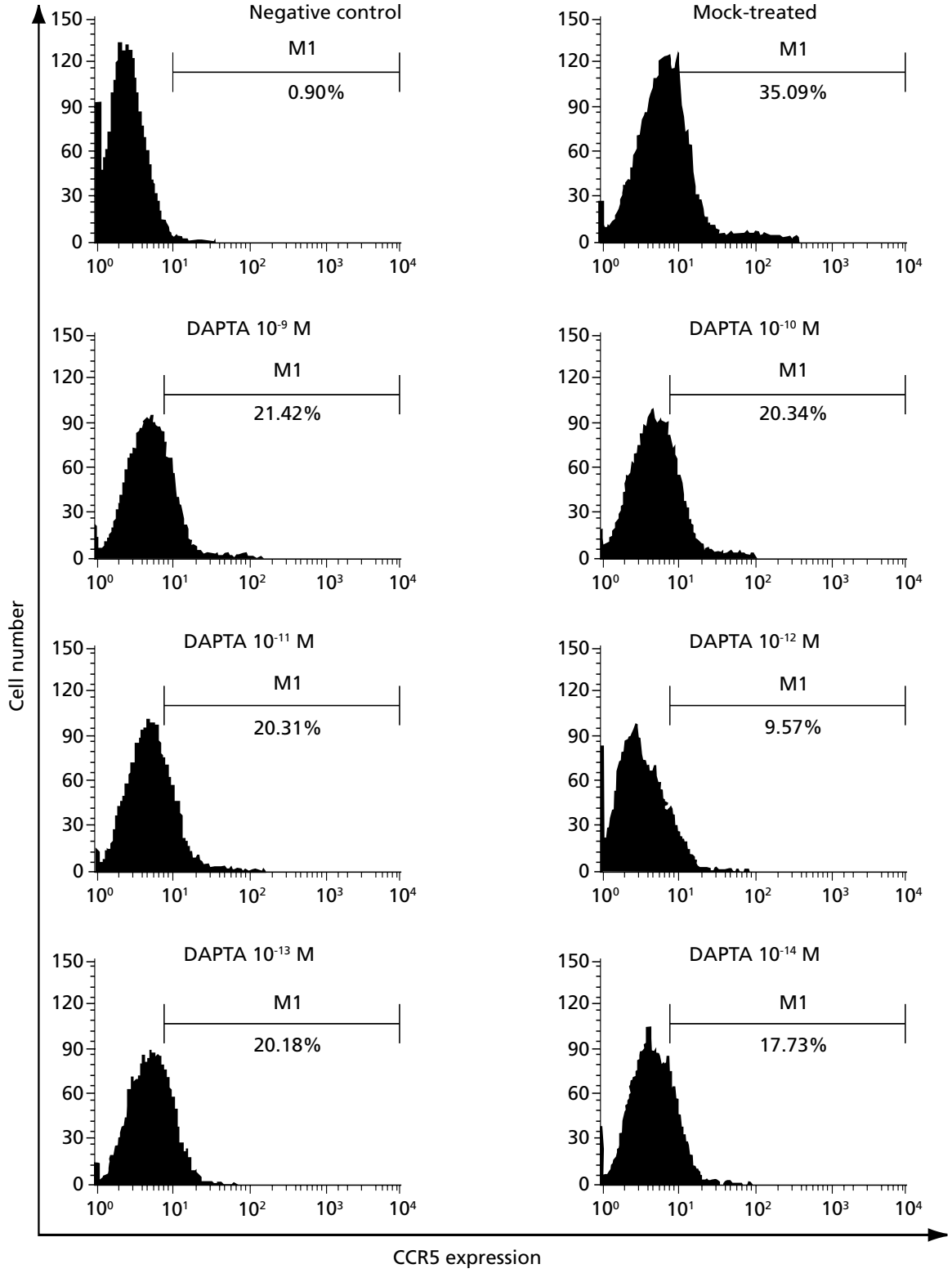
To assess CCR5 expression on the surface of a neuronal cell line, SK-N-SH cells were stained with 2D7 mAb in the presence or absence of DAPTA (at different doses) and TAK-779. The SK-N-SH line has the potential to differentiate to neural cells in the presence of retinoic acid, and it has been used as a model of primary neurons (Speth *et al.*, 2000; Trillo-Pazos *et al.*, 2000; Yeung *et al.*, 1998). The results indicate that CCR5 expression in these differentiated cells is limited and further reduced in the presence of DAPTA (Figure 4A); indeed an inhibition of CCR5 expression of 68.5% and 72% in the presence of 10^{-13} M and 10^{-12} M DAPTA, respectively, was observed in comparison with unexposed SK-N-SH cells (P<0.001). In the presence of TAK-779 (1.8×10^{-6} M) the inhibition is ~61%. Thus, DAPTA is more potent than TAK-779 in reducing CCR5 co-receptor expression in a neuronal cell line.

Finally, we exposed differentiated SK-N-SH cells to R5 HIV-1 BaL in the presence or absence of DAPTA, and assessed neuronal apoptosis. Time-course studies revealed that cell apoptosis in this cellular line occurred between 5 and 6 days after addition of the virus. Figure 4B shows the results at day 5. In particular, when SK-N-SH were incubated with HIV-1 BaL, a dramatic reduction of cell viability was seen by flow cytometric analysis. The cytopathic effect observed in SK-N-SH exposed to R5 HIV-1 was mainly related to apoptosis. Indeed, flow cytometric analysis in DAPTA-treated cells (10^{-13} M and 10^{-12} M) and exposed to HIV-1 BaL showed a complete reduction (100%) of apoptosis compared with a positive control, in which cells were exposed to HIV-1 BaL but untreated with DAPTA. To compare the anti-apoptotic effect of DAPTA with other CCR5-binding molecules, we also tested the CCR5 antagonist TAK-779. SK-N-SH cells treated with 1.8×10^{-6} M TAK-779 (a concentration able to strongly inhibit virus replication in M/M) resulted in only a 60% inhibition of apoptosis compared with the cells untreated with TAK-779 (Figure 4B). These data show that DAPTA potently blocks R5 gp120-mediated neuronal apoptosis and suggest that DAPTA is even more potent in preventing neuronal apoptosis than TAK-779. The results provide a rationale for DAPTA to be evaluated as a potential therapeutic agent for the neuropsychiatric and neurological sequelae of AIDS.

Discussion

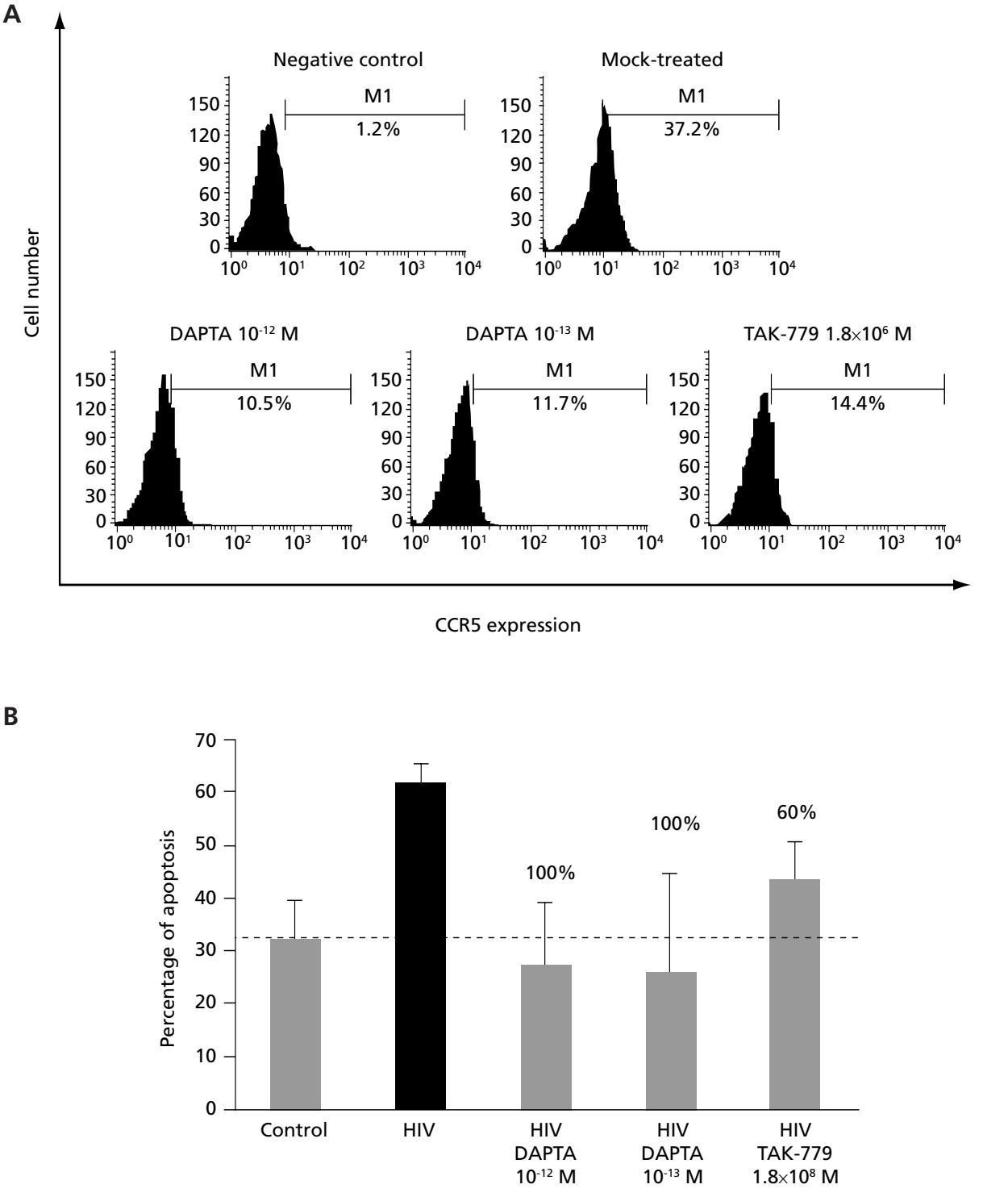
The important role of M/M in HIV-1 transmission, dissemination of infectious virus throughout the body, and in viral persistence, even in patients treated successfully with HAART therapy, suggests the necessity to identify new treatments against HIV-1 replication and related cellular damage caused by these cells. The development of new powerful drugs acting before HIV-1 infection represents a priority, particularly for cells such as M/M whose infection is persistent and productive for long periods of time. HIV-1 cellular entry inhibitors are a promising new class of potential anti-HIV-1/AIDS drugs (De Clercq, 2002; LaBranche *et al.*, 2001; Michael & Moore, 1999; Moore & Stevenson, 2000). By interacting with the viral envelope glycoproteins (gp120 or gp41), with CD4 or with co-receptors, these inhibitors block different steps in the complex sequence of events leading to virus–cell fusion (Cocchi *et al.*, 1995; Koenig *et al.*, 1986; Meucci *et al.*, 1998; Michael & Moore, 1999). The HIV-1 co-receptors are particularly attractive targets for entry inhibitors, because they belong to the protein superfamily of G-protein-coupled receptors (GPCRs) (Horuk *et al.*, 2003; Kazmierski *et al.*, 2003; Schwarz *et al.*, 2002; Seibert & Sakmar, 2004). The present study aimed to determine the effects of DAPTA, a synthetic peptide comprised of eight amino acids of the gp120 V2 region, which is proposed to function as a viral entry inhibitor by selectively targeting the chemokine receptor CCR5 (Polianova *et al.*, 2005; Ruff *et al.*, 2001; Ruff *et al.*, 2003). It is reported that DAPTA is 34 to 180 times more potent than Maraviroc, an allosteric CCR5 inhibitor, in inhibiting gp120–sCD4 complex binding to CCR5 (IC_{50} 0.06–0.32 nM versus 11 nM) (Polianova, 2005; Dorr *et al.*, 2005). DAPTA showed

Figure 3. Interaction of DAPTA with the chemokine receptor CCR5 in monocytes/macrophages



Monocytes/macrophages (M/M) were incubated with D-Ala-peptide T-amide (DAPTA) for 20 min at 4°C; mock-treated M/M were untreated with DAPTA. Surface CCR5 was detected with CCR5-FITC mAb (2D7) and the cells analysed by flow cytometry. M1 represents the percentage of CCR5-positive cells. One representative experiment out of three independent experiments is shown.

Figure 4. Effects of DAPTA on SK-N-SH cells



(A) Differentiated SK-N-SH cells were incubated with D-Ala-peptide T-amide (DAPTA) for 30 min at 4°C. Surface CCR5 was detected with CCR5-FITC mAb (2D7) and the cells were analysed by flow cytometry. Negative controls are SK-N-SH cells not stained with CCR5-FITC antibody. The percentage of CCR5-positive cells (M1) is indicated in each histogram. **(B)** Differentiated SK-N-SH cells were exposed to R5 HIV-1 BaL in the presence or absence of DAPTA at the indicated doses. The apoptotic cells were stained with propidium iodide and analysed by flow cytometry. Statistical analysis was by χ^2 test. The percentage of apoptotic cells is significantly lower ($P<0.001$) for DAPTA or TAK-779 versus control of infected but untreated cells. Numbers above the bars represent the percentage apoptosis inhibition.

potent antiviral activity against HIV-1 R5 strains, but not against X4 strains (Ruff *et al*., 2001). This effect was shown to be mediated, in part, by a downregulation of CCR5 co-receptor expression by DAPTA, in line with previous studies demonstrating that binding of HIV-1 co-receptors by their natural ligands contributes to the inhibition of viral replication (Amara *et al*., 1997; Mack *et al*., 1998). Inhibition of viral replication may be ascribed to high-affinity binding of DAPTA to CCR5 and subsequent impediment of gp120 binding to this receptor, thus inhibiting the entry of HIV-1 into host cells. A dose-response anti-HIV-1 BaL activity was observed in a low range from 10^{-10}–10^{-12} M (Figure 1A) and the maximum value of viral inhibition was observed at 10^{-12} M concentrations of DAPTA. These results confirmed that DAPTA acts at low doses, as published previously (Redwine *et al*., 1999; Ruff *et al*., 2001). We observed that the DAPTA inhibitory effect was often bi-phasic, as observed in previous experiments in which DAPTA was used to prevent monocyte chemotaxis induced by HIV-1-gp120 (Redwine *et al*., 1999) or to block neuronal toxicity by gp120 (Brenneman *et al*., 1999).

It is known that other peptides, such as vasoactive intestinal peptide (VIP), show this biphasic response suggesting that this is not an unusual mechanism of action (Brenneman *et al*., 1988). An explanation for this 'inverse U' dose effect is a ligand–ligand interaction (Guan *et al*., 2002; Ruff *et al*., 2001) or receptor signalling with desensitization at higher doses.

The effect of HIV-1 entry inhibition is also seen in the remarkable decrease of HIV-1 DNA detected in DAPTA-treated M/M. This is particularly relevant to the long-term persistent virus replication typical of infected M/M, which are able to sustain production of virus particles for weeks or months after virus integration (Aquaro *et al*., 2002). In these conditions of chronic infection with persistent viral production, the activity of reverse transcriptase inhibitors is absent in chronically infected M/M, and that of protease inhibitors is limited (Aquaro *et al*., 1997; Aquaro *et al*., 1998). The inhibition of entry of HIV-1 in M/M is then particularly relevant, and could represent a better way to control the progression of the disease. M/M can recruit lymphocytes and trigger their death, through several events, mediated by viral proteins (such as Nef and gp120), or by chemokines and other factors produced during infection (Garaci *et al*., 2003; Kaul *et al*., 2001). The same phenomena occur at the CNS level, where M/M are the key cells in the pathogenesis of HIV encephalitis. In fact, the HIV-1 entry mediated by M/M in the brain causes several pathological abnormalities that can have a clinical appearance in AIDS Dementia Complex (ADC). In fact, antiretroviral drugs can cross the blood brain barrier and enter into the brain, but are not able to completely eliminate the virus from this anatomical district. The availability of binding inhibitors is therefore even more important, as it can prevent the bystander phenomenon that leads to the death of the majority of lymphocytes and neurons even if those cells are not directly infected.

The mechanism(s) by which DAPTA inhibits virus replication are most probably linked to competitive binding with HIV gp120 on its natural receptor CCR5 (Polianova *et al*., 2005) (Figure 2). The ability of DAPTA to antagonize CCR5 (Redwine *et al*., 1999) could explain DAPTA-mediated inhibition of R5 gp120 induced apoptosis in neural cells. Indeed, CCR5 expression in neurons is responsible for apoptosis induced by gp120–CCR5 binding (Cartier *et al*., 2003; Cocchi *et al*., 1995; Dragic *et al*., 2000; Kaul *et al*., 2001; Trkola *et al*., 2001). DAPTA binding of CCR5 is able to significantly prevent apoptosis in neuronal cell lines exposed to HIV-1 R5 strains. DAPTA is a more potent inhibitor than TAK-779, a low molecular weight molecule able to bind to CCR5 and to induce a potent inhibition of virus replication. DAPTA probably binds CCR5 at a site different from that of TAK-779, the direct antiviral effect of which is clearly more pronounced than that induced by DAPTA. Because of the putative differences in CCR5 binding, it is conceivable that the phenomena of apoptosis induction and that of virus entry are mediated by different parts of HIV gp120 and, thus, differently contribute to the pathogenesis of HIV-1 infection. Additionally, signalling effects through CCR5 may differentially affect entry compared with apoptosis of chemokine receptor targeted drugs. Recent tests in an animal model revealed that DAPTA, currently in Phase II trials for HIV-1 infection, is able to block the release of the pro-inflammatory cytokines tumour necrosis factor-α and interleukin-1. In this way, DAPTA counteracts the inflammatory state in the brain associated with HIV-1 infection in ADC (Rosi *et al*., 2005). This might open the way to antiviral approaches that combine various inhibitors of HIV-1 entry, acting with different mechanism(s) of action, to synergistically control HIV-1 replication and damage directly or indirectly induced by the virus. Targeting such approaches to M/M, which are pivotal cells in the progression of HIV-related damage, could provide a major antiviral effect, particularly if used in the early stages of the disease when virus spread is still limited. Owing to its potency and combined effect against both HIV-1 replication and neuronal damage, DAPTA deserves future studies to evaluate its potential use in clinical settings.

In conclusion, DAPTA seems to be a promising agent for the treatment and prophylaxis of HIV-1 infection. It will be interesting to determine if this non-toxic peptide develops viral resistance and to find out if it is able to inhibit HAART-resistant HIV-1 strains.

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