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New classes of anti-HIV-1 compounds active at different stages of infection

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Abstract

It is widely recognized that monocytes/macrophages (M/M) represent a crucial target of HIV-1 in the body and play a pivotal role in the pathogenic progression of the HIV-1 infection. This strongly supports the clinical relevance of therapeutic strategies able to interfere with HIV-1 replication in M/M. The important role of M/M in HIV-1 transmission, dissemination of infectious virus throughout the body, and in virus persistence, even in patients treated successfully with HAART therapy, suggests the necessity to identify new treatments against HIV-1 replication active at different stages of virus infection. HIV-1 cellular entry inhibitors are a promising class of potential anti-HIV-1/AIDS drugs. By interacting with the viral envelope glycoproteins (gp120 or gp41), and/or with CD4 or the coreceptors, these inhibitors block different steps in the complex sequence of events leading to virus–cell fusion, counteracting in this way the HIV-1 infection of the target cells. We focused on two CCR5 inhibitors, DAPTA and TAK-779, both able to inhibit the R5 HIV-1 replication in M/M. Our results indicate that DAPTA and TAK-779 are potent anti-HIV-1 compounds able to block the virus entry of R5 HIV-1 strains in M/M, suppressing viral replication in the cells. In particular, DAPTA proved to be able to inhibit the virus replication at extremely low drug concentrations. The use of coreceptor inhibitors, such as DAPTA and TAK-779, could be important to contribute to a possible synergism with other antiretroviral treatments. Another class of compounds able to act before HIV-1 entry, is represented by carbohydrate-binding agents (CBAs). These agents are recently proposed as innovative anti-HIV compounds selectively targeting the glycans of the HIV-1 envelope glycoprotein gp120 and preventing DC-SIGN-directed HIV capture by dendritic cells (DC) and subsequent transmission of the virus to CD4+ T-lymphocytes. We now found that CBAs also efficiently prevent R5 HIV-1 infection of human primary M/M that do not measurably express DC-SIGN but markedly express the macrophage mannose-binding receptor (MMR). We observed also that pre-exposure of X4 HIV-1 to CBAs is able to prevent efficient virus capture by M/M and subsequent syncytia formation in co-cultures of uninfected CD4+ T-lymphocyte C8166 cells and CBA-X4 HIV-1 exposed M/M. The potential of CBAs to impair M/M in their capacity of hosting virus replication and chronic production of new virus particles, but also preventing M/M to efficiently capture and transmit HIV to T-lymphocytes might be an important property to be taken into consideration in the eventual choice to select microbicide candidate drugs for clinical investigation. Since M/M represent chronically infected cells, it will be also interesting to study new drugs acting at a post-integration stage in the replication cycle of HIV-1. A unique class of drugs that may contribute to the control of the latent HIV-1 reservoir includes the quinolone derivatives, first reported as an important class of broad-spectrum antibacterials. Two novel 6-desfluoroquinolone derivatives (6-DFQs), HM-12 and HM-13, were evaluated for their anti-HIV activity in acutely, chronically and latently HIV-1-infected cell cultures (including M/M) and found to behave as potent HIV-1 transcription inhibitors. Interestingly, in a murine *in* vivo model in which mice are inoculated with latently HIV-1-infected human cells, 6-DFQs were shown to efficiently prevent virus activation upon TNFα triggering. Thus, these compounds are able to slow down virus replication, and should be interesting candidate drugs to be combined with entry, integrase or reverse transcriptase inhibitors that acts prior to the proviral integration in the treatment of HIV-1 infections. Because it is known that HIV-1 infection induces a significant perturbation of the oxidative status of M/M, it can be interesting also to study new drugs able to counteract the cell damage correlated with this oxidative condition. In particular, we studied MnTBAP (Mn(III)tetrakis(4-benzoic acid)porphrin chloride), a synthetic peroxynitrite decomposition catalyst, able to reduce oxidative stress subsequent to peroxynitrite generation in HIV-1-infected M/M and found the compound efficient in inhibiting HIV-1 replication in M/M. In summary, the inherent properties of HIV-1 infection of M/M should be taken into account to design therapeutic strategies aimed at achieving an optimal therapeutic effect in all tissue compartments where the virus hides and replicates. We have investigated four possible new drug classes of compounds that represent interesting candidate drug leads for further (pre)clinical studies.

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1. Introduction

Although the introduction of highly active antiretroviral therapy (HAART) has led to a dramatic decrease of both the morbidity and the mortality of patients with Human Immunodeficiency Virus type 1 (HIV-1) infection (Murphy et al., 2001; Palella et al., 1998), the eradication of HIV-1 infection is not yet achievable and the main reason is the presence of virus reservoirs in infected patients. Monocytes/ macrophages (M/M) are a strategic reservoir of HIV-1 during the whole course of the infection, even in patients receiving HAART (Sonza et al., 2001). M/M play an important role in all phases of human immunodeficiency virus type 1 (HIV-1) infection. Once infected by HIV-1, M/M survive and produce large amounts of infectious viral particles (an average of ~ 400 virions released daily by each infected cell) and this for a long time period (Aquaro et al., 2002). They are widely recognized as the second cellular target of HIV-1, and represent a vehicle for virus dissemination in the body and the major reservoir for long term persistence of HIV-1 during HAART (Aquaro et al., 2002; Perelson et al., 1997; Sharkey et al., 2000). HIV-1 infected M/M are widely distributed in all tissues and organs (Koenig et al., 1986; McElrath et al., 1989; Tschachler et al., 1987), including the central nervous system (CNS) where they represent the majority of cells infected by HIV-1 (Gabuzda et al., 1986; Tyor et al., 1993). It was in fact demonstrated that M/M are highly efficient to enter in CNS by blood brain barrier (BBB) (they are 20 folds more efficient than non infected) and at this level they can free virions in outside environment, acting as a Troy horse (Kim et al., 2003; Verani et al., 2005). Microglia, local differentiated M/M, are the main source of virus in the brain, whose pathogenic secretory products cause neuro-AIDS (Guan et al., 2002; Kazmierski et al., 2003). HIV-1 enters into the target cells after binding of the viral envelope glycoprotein gp120 to the CD4, as principal receptor, and CCR5 (R5) (whose ligands are RANTES, MIP-1α, MIP-1β) and CXCR4 (R4) (whose ligand is SDF-1), as coreceptors (called R5-using and X4-using tropic strains, respectively) (Cohen, 1996, Deng et al., 1996, Dragic et al., 1996, Schols, 2004). HIV-1 can penetrate in the CNS rapidly (hours or days after infection), the strains detectable at this level are often different from plasma strains either

genotypically and phenotypically: in addiction, they are in general macrophage tropic (M-tropic) (Gorry et al., 2001). However, quite recently some authors have described in CNS presence of X4- or dual-tropic strains which seem to be able to have access granted and replicate efficiently in M/M of HIV-related damage and that are thought to have a role in pathogenesis (Spudich et al., 2005, Yi et al., 1999, 2003, 2004).

HIV-1 replication in M/M is a crucial pathogenic event during the progression of HIV-1 infection. In fact, productively-infected M/M can fuse with CD4+lymphocytes and transfer the virus to these cells in the context of the antigen presentation and the immune response (Crowe et al., 1990); in addition infected M/M release cytotoxic factors that can mediate the activation of programmed cell death on bystander cells such as CD4+ and CD8+lymphocytes (Badley et al., 1997; Herbein et al., 1998; Garaci et al., 2003), neurons and astrocytes even without a direct infection of these cells (Aquaro et al., 2000; Mollace et al., 2002; Shi et al., 1996). In agreement with this result, as few as 500 HIV-1 infected M/M have been demonstrated to be able to completely deplete millions of autologous CD4+lymphocytes in a SCID mouse model (Garaci et al., 2003). HIV-1 infection in M/M is characterized by viral dynamics substantially different from that of CD4+lymphocytes. In fact, activated CD4+lymphocytes can sustain a rapid and exponential viral production followed by massive cell death (Bagnarelli et al., 1996). In contrast, M/M are resistant to the cytopathic effect of HIV-1 (Gendelman et al., 1988; Orestein et al., 1988) and produce virus over a prolonged period, with dynamics that increases linearly during the first 1-2 weeks of infection, followed by a plateau of high level of replication ($>10^8$ copies of unspliced/spliced RNA produced) lasting at least up to 60 days after infection (Aquaro et al., 2002). M/M can survive HIV-1 infection for long periods of time. This is mainly related to autocrine secretion of the nerve growth factor (NGF) associated with enhanced expression of the high-affinity NGF receptor p140 trkA on their surface. This complex interaction enhances the ability of M/M to cope with HIV infection, thus transforming them in a powerful, long-term infected, viral reservoir (Garaci et al., 1999). This supports the role of M/M as an important source of HIV-1 and as a real cellular reservoir able to challenge the attempts to

eradicate the virus from patients (Orenstein et al., 1997; Perelson et al., 1997; Schrager and D'Souza, 1998). The dynamics of virus replication, quite different in M/M and CD4+lymphocytes, may suggest that anti-HIV-1 drugs act differently in these cells. The important role of M/M in HIV-1 transmission, dissemination of infectious virus throughout the body, and in virus 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.

At present, treatment of HIV-infected individuals is based on combination therapy with HIV-1 reverse transcriptase (RT) and/or protease and/or gp41 inhibitors. Despite the notable success of HAART in reducing plasma viral loads to undetectable levels during HIV infection and slowing down clinical progression to AIDS, HAART fails to completely eradicate the virus in HIV-infected individuals. Additionally, emergence of multidrug-resistant viruses have increasingly been reported in patients receiving HAART, urging the need for new anti-HIV treatment strategies. On this basis, it is interesting study new and innovative anti-HIV-1 compounds working by mechanisms different from those of the existing HAART drugs and able to prevent or counteract the M/M infection and consequently the related cellular damages or to counteract the HIV infection in chronically infected M/M.

2. Entry inhibitors

2.1. CCR5 Inhibitors

HIV-1 enters into M/M after binding of the viral envelope glycoprotein gp120 to specific chemokine/HIV-1 coreceptors in conjunction with the CD4 receptor (Kaul et al., 2001; Mack et al., 1998). For this reason HIV-1 entry inhibitor are a promising new class of potential anti-HIV-1 drugs able to block different steps in the complex sequence of events leading to virus-cell fusion (De Clercq, 2002; LaBranche et al., 2001; Michael and Moore, 1999; Moore and Stevenson, 2000). In particular, M/M and microglia are infected primarily by HIV-1 strains that use the β-chemokine receptor CCR5 (R5 strains) (Albright et al., 1999; Choe et al., 1996; Ghorpade et al., He et al., 1997; 1998; Rana et al., 1997), and which predominant during the asymptomatic stages of HIV-1 infection (Baba et al., 1999; Connor et al., 1997). The clinical relevance of the CCR5 by HIV-1 is demonstrated by the impact of a naturally occurring CCR5 mutation, CCR5-Δ32, that generates a non-functional coreceptor (Berger et al., 1999; Liu et al., 1996; O'Brien and Moore, 2000; Seibert et al., 2006). Individuals who are homozygous for this mutation are significantly protected against HIV-1 infection and transmission, while infected, heterozygous individuals progress less rapidly to disease and death (Dragic et al., 2000; Seibert et al., 2006). Blocking CCR5 on the M/M surface with natural ligands for the CC-chemokine receptor (such as LD78β, RANTES or MIP-1 β), prevents HIV-1 infection of these cells (a crucial event during 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, 2003; Trkola 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 et al., 1999, 2001; Trkola et al., 2001). So, 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 prevention of gp120-

induced apoptosis in neuronal cell lines. For this reason, the identification of new CCR5-targeting antibodies, chemokines, chemokine analogs, small molecules and peptides, is an important step in the development of new antiviral drugs acting with different mechanism(s) of action, to synergistically control HIV-1 replication and damage directly or indirectly induced by the virus. Several different types of inhibitors 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; Kaul et al., 2001; LaBranche et al., 2001; Michael and Moore 1999; Moore and Stevenson 2000; O'Hara and Olson 2002; Schwarz and Wells, 2002).

2.1.1. D-Ala-Peptide T-amide (DAPTA)

D-Ala-Peptide T-amide (DAPTA), or Peptide T, named for its high threonine content (ASTTTNYT), is a synthetic non-toxic peptide comprised of eight amino acids (185-192) of the gp120 V2 region, that functions as a viral entry inhibitor by targeting selectively CCR5 (Polianova et al., 2005 ; Ruff et al., 2001, 2003). Recently, in a small clinical trial, DAPTA has shown promising antiviral and immune benefits, and caused improvements in cognition in humans with HIV-1 infection, suggesting also its penetration into CNS (Heseltine et al., 1998; Polianova et al., 2005). A blind analysis of frozen stored plasma samples conducted by the NIMH in the early-1990's 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). In order to better define potency and potential mechanisms of action of DAPTA, we studied its effect on the inhibition of binding CCR5–gp120 in M/M, and 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, and in addition blocks HIV-1-M/M CCR5-mediated apoptosis in a neuronal cell line.

2.1.1.2. 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 the figure 1. At the day 14 after HIV-1 infection, the p24 gag antigen production in the supernatants of HIV-1 BaL infected M/M was drastically dose-dependently reduced in the presence of DAPTA, ranging from 10^{-9} to 10^{-15} M. In the figure 1A we can see the results, expressed as a percentage compared to positive control, in which M/M were HIV-1 infected without DAPTA treatment (100%). The maximal viral inhibition observed was around 90% with 10^{-9} M DAPTA concentration. With another CCR5-using HIV-1 strain, 81A, we obtained comparable results. In 81A HIV-1-infected M/M, DAPTA 10^{-9} M reaches viral inhibition around 97% at day 14 after the infection (Figure 1B). Comparable results were confirmed at day 21 after infection (data not shown).

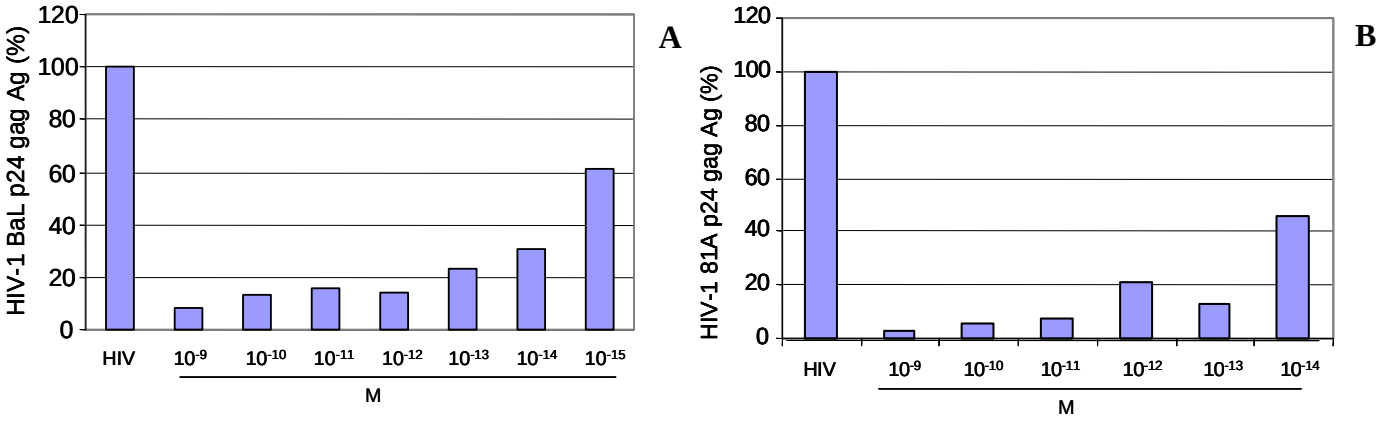


Figure 1: Antiviral activity of DAPTA in acutely R5 HIV-1 BaL (Panel A) and 81A (Panel B) infected M/M

The protective effect of DAPTA was confirmed by figure 2. As we can see, the presence of DAPTA (Panel A) is able to reduce the cytopathic effect, with syncytia formation and aggregation of cells, induced by R5 HIV-1 in M/M after 14 days of infection (Panel B).

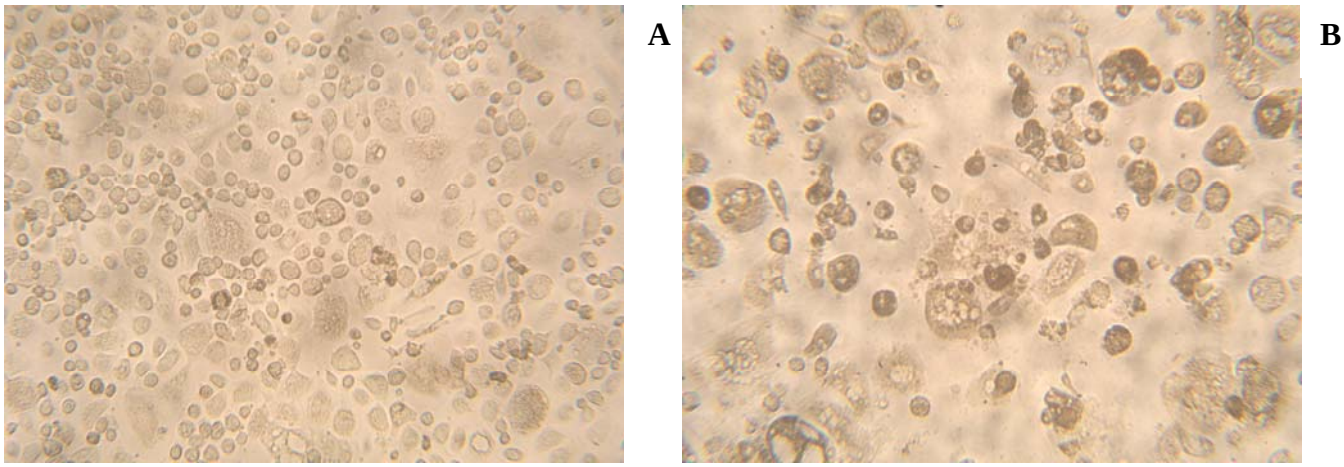


Figure 2: Light microscopic pictures of M/M after 14 days of R5 HIV-1 infection in presence (Panel A) or in absence of DAPTA (Panel B).

Moreover, we tested the HIV-1 p24 antigen production in cells infected by CXCR4-using (X4) strains such as HIV-1 NL4.3. In particular we used C8166 infected with 1000 pg/ml of HIV-1 NL4.3 and treated, where requested, with several doses of DAPTA (ranging from 10^{-19} to 10^{-15} M), and analyzed the cytophatic effect starting by 3 days after the infection. We observed that DAPTA was not able to prevent the syncytia formation, due to the X4 HIV-1 infection of T cells. So, we can conclude, that DAPTA showed a potent antiviral activity against R5 strains but not against X4 strains of HIV-1.

2.1.1.3. DAPTA reduces levels of HIV-1 DNA in macrophages

To further demonstrate that DAPTA blocks virus infection, M/M were analyzed for HIV-1 DNA formation. Eighteen hours post-infection, genomic DNA was extracted and two-fold dilution of cell equivalents (range 1 x 10^6-1.25 x 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 3A). We observed that HIV-1 DNA per 2.5 x 10^5 cells declined with 64% in the presence of peptide DAPTA (10^{-7} M) and with 70% in the presence

of 10^{-10} M peptide DAPTA, compared with not-treated cells. Control infected cultures (HIV-1BaL), in the absence of peptide DAPTA or 2D7 mAb, had approximately 1 x 10^4 HIV-1 copies per 10^5 M/M (i.e. 0.1 copy x 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 ug/ml was approximately 39% (Figure 3B). These data indicate that peptide 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.

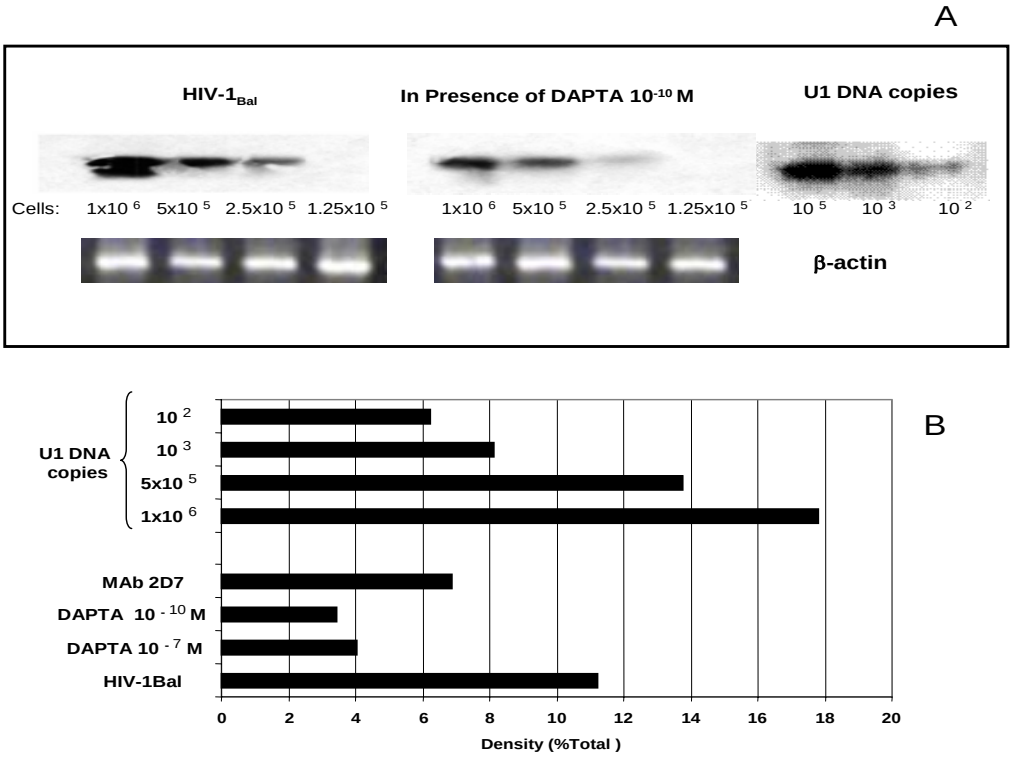


Figure 3: Reduction of HIV-1 DNA formation in M/M in presence of DAPTA. **A,** M/M were infected with HIV-1 BaL in presence or in absence of DAPTA at 10^{-7} M–10^{-9} M doses and mAb 2D7 (3000 pg/ul). HIV-1 DNA was extracted from M/M 18 h after infection and 1 x 10^6-1.25 x 10^5 cell equivalents were detected by Southern hybridization. HIV-1 DNA extracted from U1 cells, containing two integrated HIV copy, was used as positive standard. **B,** Band density was measured by UN-SCAN-IT (Silk Scientific Inc).

2.1.1.4. DAPTA inhibits CCR5 binding in macrophages

To confirm that DAPTA binding is specific for CCR5, a competition experiment between CCR5-FITC antibody 2D7 and DAPTA in M/M was done. 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 are CCR5+ positive. DAPTA treatment cells showed reduced binding of the 2D7 mAb to CCR5 in a dose dependent manner, with maximal reduction of CCR5 expression (9%) occurring at 10^{-12} M. (Figure 4) ($p \leq 0.001$). Overall, the inhibition of CCR5-binding by several DAPTA doses is about 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.

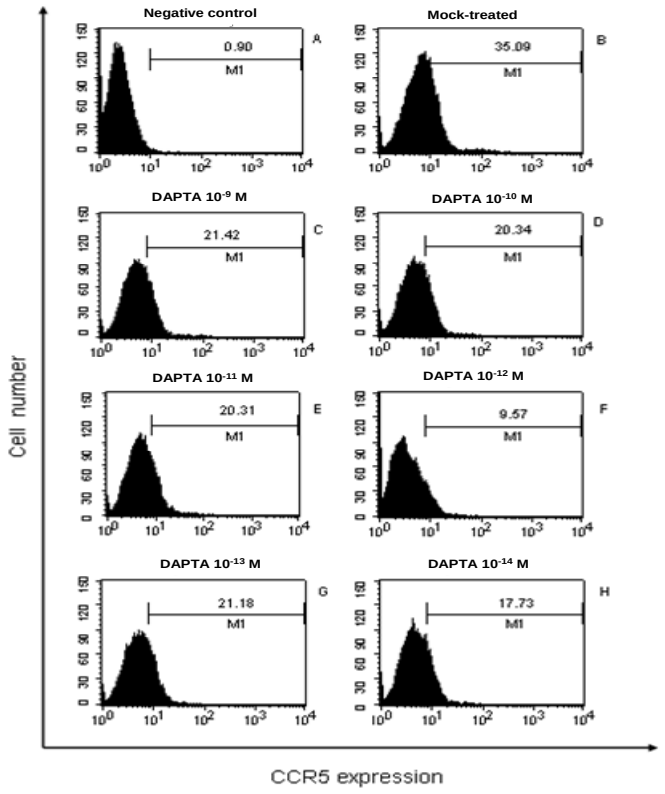


Figure 4: Binding of DAPTA to CCR5 in M/M. M/M were incubated with DAPTA for 30 min at 4°C. Surface CCR5 was detected with CCR5-FITC mAb (2D7) and the cells were analyzed with a FACScan flow cytometer. M1 represents the % of CCR5 positive cells.

2.1.2. TAK-779

One of the first CCR5 inhibitors described is TAK-779, a non-peptidic compound with small molecular weight (Mr 531.13), known to interact mainly with CCR5 (Baba et al., 1999; Dragic et al., 2000; Shiraishi et al., 2000; Takashima et al., 2001). TAK-779 acts as antagonist and binds predominantly within a cavity formed between the transmembrane helices 1, 2, 3 and 7 (Dragic et al., 2000). Binding interactions of TAK-779 to the CCR5 receptor probably induce conformational changes of the second extracellular loop and further obstruct the interaction between gp120 and the coreceptor (Baba et al., 1999; Dragic et al., 2000). The anti-HIV-1 activity in PBMCs and CCR5-transfected cell lines are described now in several papers, but no data are available yet in M/M. Here, we wanted to study the anti-HIV-1 activity of TAK-779 in M/M and, in order to apply these concepts to an in vivo model, we used severe combined immunodeficient (SCID) mice, engrafted with human peripheral blood lymphocytes (hu-PBL-SCID mice) in which immunological and viral parameters can be easily monitored. These mice represented a reliable system to study the pathogenesis of HIV-1 infection that may shed light on events not yet highlighted in primate models and in humans (Garaci et al., 2003).

Our results indicate that TAK-779 efficiently binds to CCR5, preventing in this way, the R5 HIV-1 infection in M/M and, importantly, the treatment of M/M with TAK-779 is able to prevent the CD4+T-lymphocytes depletion.

2.1.2.1. TAK-779 inhibits intracellular calcium signalling

To address the receptor specificity of TAK-779, we evaluated its ability to inhibit chemokine-induced intracellular calcium signalling mediated through CCR5. We performed calcium flux experiments with receptor-specific chemokine ligands and cells expressing the corresponding chemokine receptor, CCR5-transfected U87.CD4 cells and primary monocytes, respectively. After preincubation with or without TAK-779 at dose-dependent concentrations, the cells were stimulated with a CCR5-binding chemokine and the changes in intracellular calcium concentration were recorded by the use of the FLIPR system.

We observed that TAK-779 dose-dependently inhibited the calcium signalling induced by the CC-chemokine MIP-1α and RANTES, in monocytes and in CCR5-transfected U87.CD4 cells, respectively (EC50: 4-40 ng/ml) (Figure 5). These results show that TAK-779 potently blocked the CCR5-mediated Ca2+-signaling, not only in CCR5-transfected cells (Figure 5; Baba et al., 1999), but also in M/M.

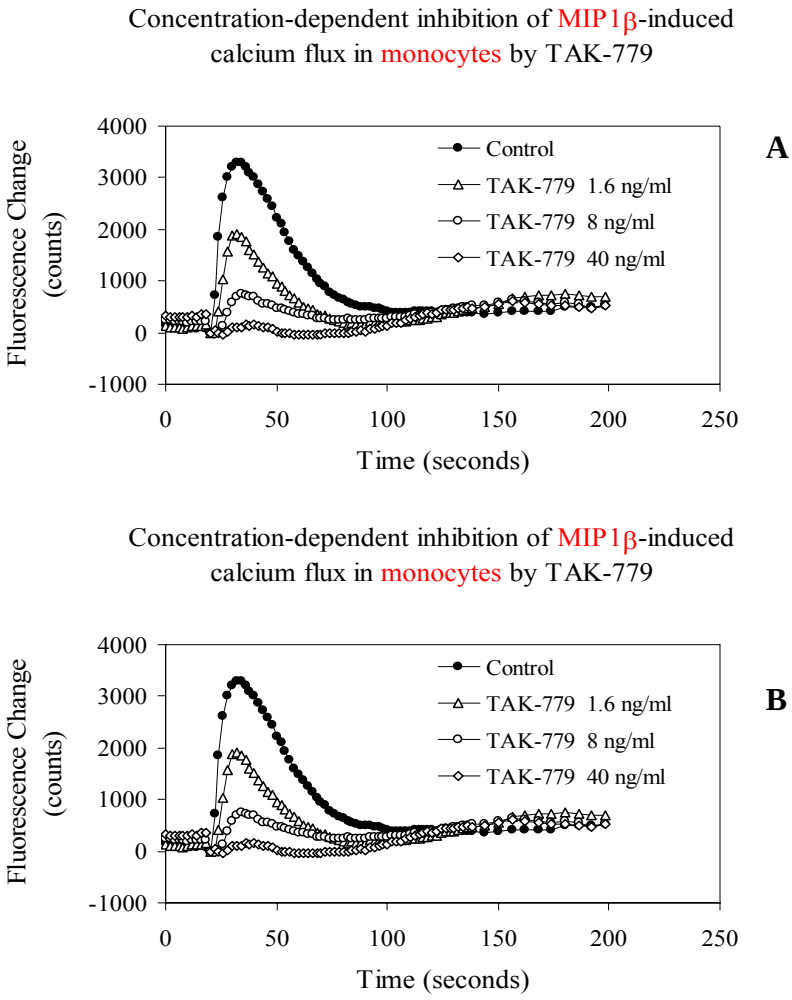


Figure 5: Dose-dependent inhibition of RANTES-induced calcium flux in U87.CD4.CCR5 cells by TAK-779 (Panel A) and dose-dependent inhibition of MIP 1β-induced calcium flux in monocytes by TAK-779 (Panel B).

2.1.2.2. TAK-779 potently inhibits HIV-1 replication in macrophages

Anti-HIV-1 activity of TAK-779 was evaluated in M/M obtained from the blood of several healthy donors and infected in vitro by different R5 viruses (HIV-1 BaL and ADA) 14 days after infection for p24 antigen production. TAK-779 showed a potent and dose-dependent antiviral activity against HIV-1 BaL and ADA, and also against an R5 clinical isolate (#5), with an average EC50 of 50 ng/ml. The results indicate that TAK-779 suppresses the viral replication in a dose dependent fashion, revealed by the profound inhibition of HIV-1 p24 gag Ag production. This inhibition reached the value of about 99% at 2 ug/ml concentration of TAK-779 (Figure 6). Comparable anti-viral potency was still achieved even after 21 days after the start of the experiment.

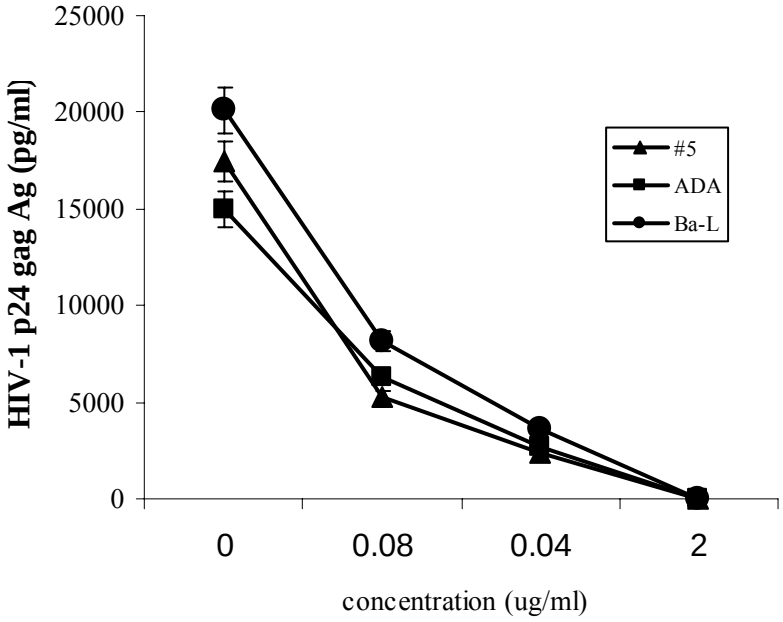


Figure 6: Potent antiviral activity of TAK-779 against two R5 strains of HIV-1 (BaL and ADA) and clinical isolate #5. Supernatants were collected at day 14 and tested for p24 antigen production.

2.1.2.3. TAK-779 prevents syncytia formation in HIV-1 infected macrophages

We also evaluated the effect of TAK-779 on syncytia formation in HIV-1-infected M/M. In our experiments we observed that the infection by R5 viruses (300 TCID50/ml BaL and ADA) induced the formation of syncytia in M/M at day 14, with aggregation of cells, often with circular arrangements of the corresponding nuclei and an evident cytopathic effect compared to uninfected M/M. M/M infected with the same amount of BaL and ADA, but treated with TAK-779 (1 ug/ml), clearly showed a reduced cytopathic effect and much less syncytia (Figure 7). These results also show that TAK-779 profoundly inhibited the syncytia formation and cytopathic effects in R5 HIV-1-infected M/M.

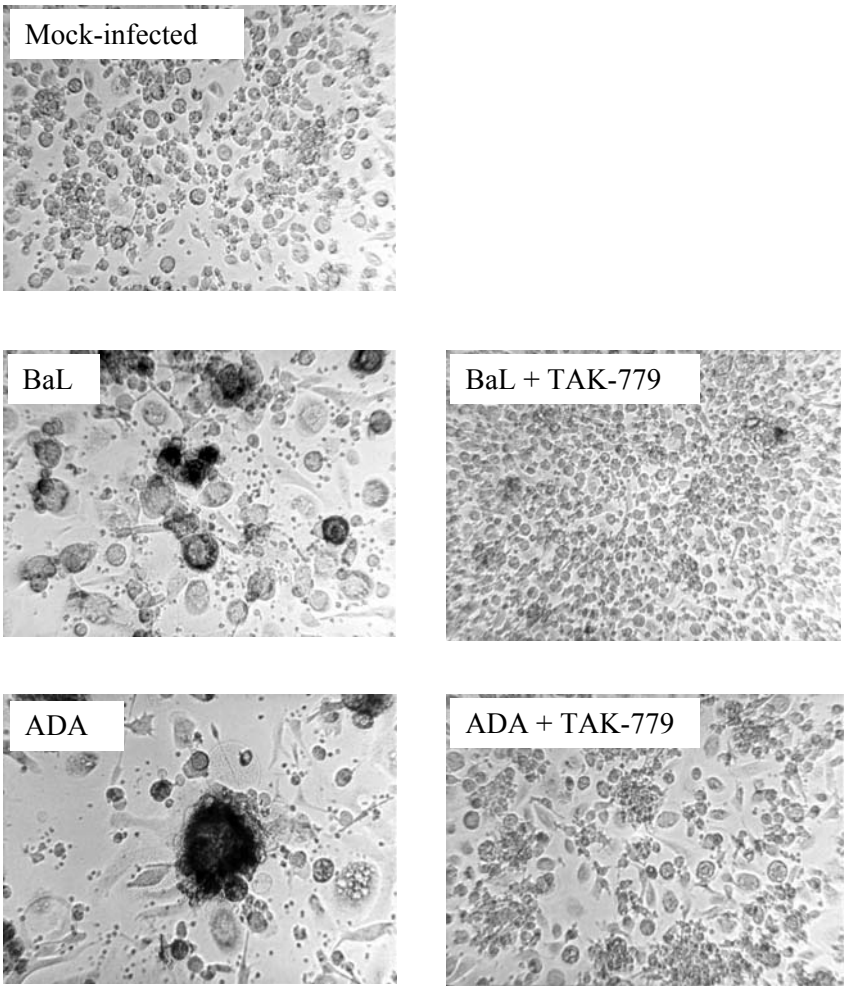


Figure 7: Light microscopic pictures of M/M after 14 days of R5 HIV-1 (BaL and ADA) infection in presence or in absence of TAK-779 (1 ug/ml).

2.1.2.4. HIV-1-infected macrophages induce severe CD4+ T cell depletion in hu-PBL-SCID mice

To apply these concepts to an in vivo model, we used SCID mice, engrafted with hu-PBL-SCID mice. These mice represented a reliable system to study the pathogenesis of HIV-1 infection that may shed light on events not yet highlighted in primate models and in humans. In this model the hu-PBL-SCID mice can be successfully infected with HIV-1 and the immunological and viral parameters of which can be monitored. We known that mice, reconstituted with PBMC from healthy donors and inoculated with HIV-infected M/M showed a dramatic depletion of CD4+ T-lymphocytes, with the presence of HIV-DNA in spleen and lymph nodes, and both an overwhelming infection and a sustained plasma viremia (Garaci et al., 2003). The ability of M/M to communicate with and transfer HIV-1 to, CD4+ lymphocytes is based on their anatomical location in lymph nodes and tissues and on their physiological interaction with CD4+ lymphocytes in the context of antigen presentation and immune response (Garaci et al., 2003). In this model, we wanted to evaluate the effect of TAK-779 on HIV-1 infection of M/M, injected i.p. in hu-PBL-SCID mice. We observed that inoculation in hu-PBL-SCID mice of 5000 untreated R5 HIV-1 infected M/M, after one week of infection, resulted in a dramatic depletion of 93% of the CD4+ human T-lymphocytes. In sharp contrast, the CD4+ T-lymphocytes were only partially depleted when the mice received 5000 R5 HIV-1-infected M/M treated with TAK-779 (2 ug/ml), reaching a percentage of depletion of 60% compared to the mice inoculated with uninfected M/M (Figure 8).

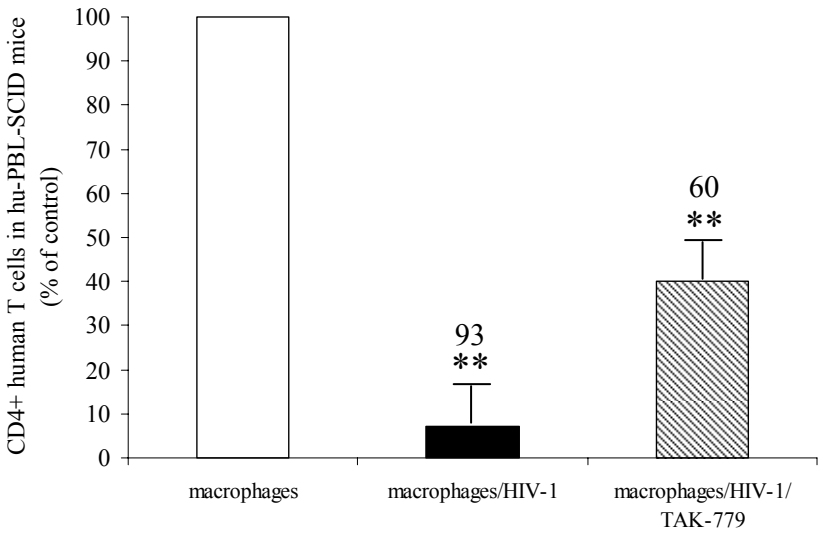


Figure 8: TAK-779 inhibits CD4+ T- lymphocytes depletion induced by HIV-1 infected M/M in hu-PBL-SCID mice. Percentages of CD4+T-lymphocytes depletion was measured in hu-PBL-SCID mice at day 14 after challenge with 5000 HIV-infected M/M, 5000 TAK-779-treated HIV-1 infected M/M, and 5000 mock-infected M/M; (** $p < 0.001$; * $p=0.001$) when compared with mice challenged with uninfected M/M.

2.1.2.5. Effects of DAPTA and TAK-779 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 presence or in absence of DAPTA (at different doses) and TAK-779. The SK-N-SH line has the potential of differentiating to neural cells in the presence of retinoic acid, and it has been used as a model of primary neurons (Speth C 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 9); indeed an inhibition of CCR5 expression of 68.5% and 72% in presence of 10^{-13} M and 10^{-12} M DAPTA concentration respectively was observed in comparison with unexposed SK-N-SH ($p<0.001$). In the presence of TAK-779 (1.8×10^{-6} M) the inhibition is about 61%.

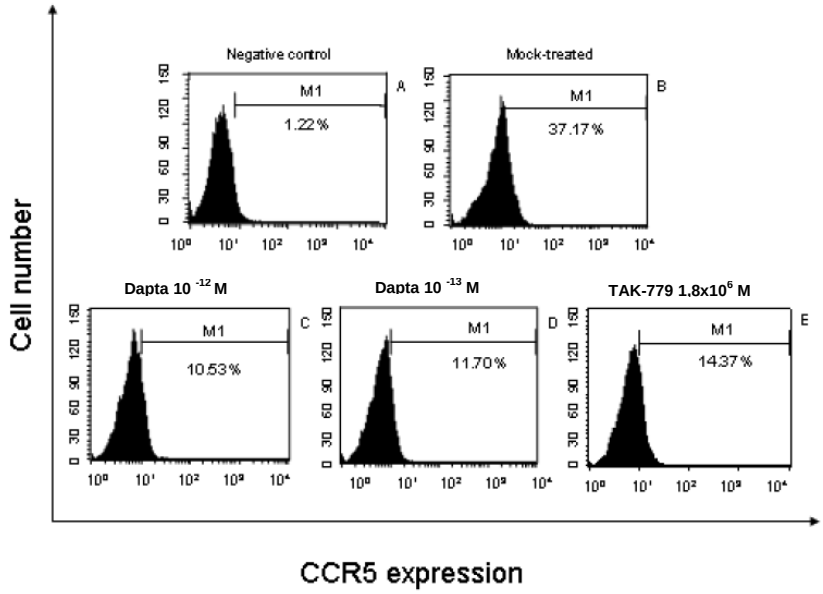


Figure 9: Binding of DAPTA to SK-N-SH cells. Differentiated SK-N-SH cells were incubated with DAPTA for 30 min at 4°C. Surface CCR5 was detected with CCR5-FITC mAb (2D7) and the cells were analyzed with a FACScan. Negative control of SK-N-SH not stained with CCR5-FITC antibody (A). 2D7 stained untreated cells (B). SK-N-SH treated with 10^{-12} M (C) or with DAPTA 10^{-13} M (D) or with TAK-779 1.8 x 10^{-6} M (E). The percentage of CCR5 positive cells are indicated in each histogram.

Finally, we exposed differentiated SK-N-SH cells to the R5 HIV-1 BaL, in the presence or absence of DAPTA and TAK-779, 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. These results shown at day 5 (Figure 10). 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 released from infected M/M, was mainly related to apoptosis. Indeed, flow cytometric analysis showed apoptosis in 60% of SK-N-SH cells exposed to HIV-1 BaL compared to 28% and 26% observed in DAPTA 10^{-13} M and 10^{-12} M treated cells, respectively. Moreover, SK-N-SH cells treated with 1.8 x 10^{-6} M TAK-779 (a concentration able to strongly inhibit virus replication in M/M) resulted in a 30% inhibition of apoptosis compared to the cells not treated with TAK-779 (Figure 10). These data show that DAPTA potently blocks R5 gp120-mediated neuronal apoptosis, and DAPTA is even more potent in preventing the neuronal apoptosis compared to TAK-779 and provide a rationale

for DAPTA to be evaluated as a potential therapeutic agent for the neuropsychiatric and neurological sequelae of AIDS.

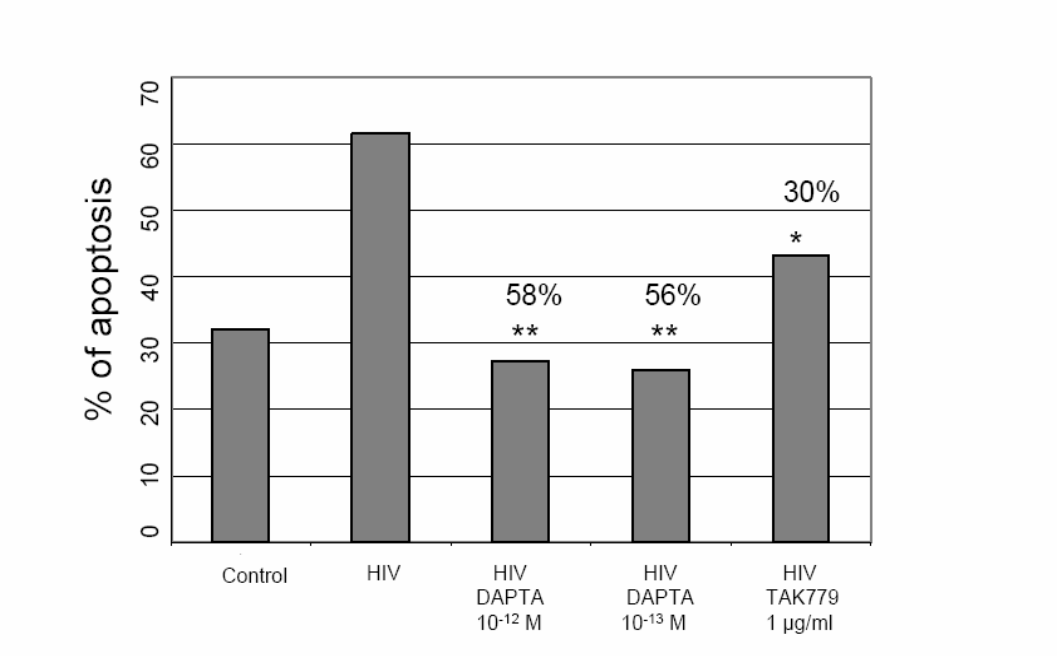


Figure 10: Levels of apoptosis in R5 HIV-1 BaL exposed and DAPTA/TAK-779 treated SK-N-SH cells. The apoptotic cells were stained with PI and analyzed with a FACScan flow cytometer. Statistical analysis was by $\chi 2$ Test (** $p < 0.001$;* $p=0.001$), for DAPTA or TAK-779 vs. control of infected but not drug-treated cells. Numbers over bars represent the % of apoptosis inhibition.

2.1.3. Discussion

The important role of M/M in HIV-1 transmission, dissemination of infectious virus throughout the body, and in virus persistence, even in patients treated successfully with HAART therapy, suggests the necessity to identify new treatments that can act working by mechanism different than those of existing HAART drugs against HIV-1 replication and related cellular damage caused by these cells. Since HIV-1 needs coreceptors after CD4 binding to entry in M/M, it is clear that these receptors became obvious targets for antiviral drug development. CCR5 and CXCR4 are the principal HIV-1 coreceptors for HIV-1 entry in target cells (Berger et al., 1999; Dragic et al., 1996; Feng et al., 1996). M/M, in whose HIV-1 infection is persistent and productive for long periods of time, are infected primarily by HIV-1 strains

that use CCR5 as coreceptor (Kaul et al., 2001). Actually, several types of CCR5 inhibitors have 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 and Moore, 1999; Moore and Stevenson, 2000; O'Hara and Olson, 2002). The present study aimed to determine the effects of DAPTA, a synthetic peptide comprised of eight amino acids of the gp120 V2 region (Polianova et al., 2005; Ruff et al., 2001, 2003), and of TAK-779, a non peptidic and antagonistic compound of CCR5 (Baba et al., 1999; Dragic et al., 2000), proposed to function as a viral entry inhibitor selectively targeting the chemokine receptor CCR5.

Both with DAPTA as with TAK-779 we observed an inhibition of R5 HIV-1 replication in M/M. This is probably correlated with a reduction of viral entry in the cells, due to a binding and obstruction of CCR5 by the coreceptor inhibitors.

DAPTA showed a potent antiviral activity against HIV-1 R5 strains (a dose response anti-HIV-1 activity was observed in a low dose range both with BaL as with 81A, Figure 1) but not X4 strains. This effect is here shown to be mediated in part by a down-expression of CCR5-coreceptor by DAPTA, in line with previous studies demonstrating that binding of HIV-1 coreceptors by their natural ligands contribute 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 and thus inhibit the entry of HIV-1 in host cells.

TAK-779 also interacted with a dose-dependent antiviral activity against R5 HIV-1 strains, both against R5 isolates (BaL and ADA) as against clinical R5 isolates (#5) in M/M. Binding interactions of TAK-779 to the CCR5 receptor probably induce conformational changes of the second extracellular loop and further obstruct the interaction between gp120 and the coreceptor (Baba et al., 1999; Dragic et al., 2000) and consequently the viral entry in the cells, inhibiting, in this way, R5 HIV-1 replication in M/M.

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). Under these circumstances of chronic viral production, the activity of reverse transcriptase inhibitors is absent in persistently-infected M/M, and that of protease inhibitors is limited (Aquaro et al., 1997, 1998). The inhibition of entry of HIV-1 in M/M is then particularly relevant, and may represent a better way to control the progression of the disease.

It is known that M/M, not only sustain a long-term persistent virus replication, but also play a role in the profound and selective death of CD4+ T lymphocytes. M/M, in fact, transfer virus particles and produce factors that can mediate, directly or indirectly, the activation of programmed cell death on bystander cells both in vitro and in vivo (Aquaro et al., 2000; Badley et al., 1997; Garaci et al., 2003). In our experiments we observed that the pre-treatment with TAK-779 (2 ug/ml) of R5 HIV-1 infected M/M dramatically reduces the depletion of CD4+ T-lymphocytes in hu-PBL-SCID mice. So, the impediment of gp120 binding to CCR5 can prevent the entry of HIV-1 in M/M, the viral reservoir in which HIV-1 replicate and survive for long time, and thus to control the progression of the disease, particularly if used at early stages of the disease, when virus spreading is still limited. Moreover, because M/M can recruit lymphocytes and trigger their cell death through the release of virus proteins, chemokines and other factors (Garaci et al., 2003; Kaul et al., 2001) the use of entry inhibitors, as TAK-779, can prevent the bystander phenomenon that leads to the death of the majority of lymphocytes.

Moreover, the HIV-1 entry mediated by M/M in the brain, where these cells are key elements in the pathogenesis of HIV-encephalitis, causes several pathological abnormalities that can have a clinical appearance in AIDS Dementia Complex (ADC), also because in this anatomical privileged site HAART drugs are not able to enter the brain. The availability of binding inhibitors is therefore even more important, since it can prevent the bystander phenomenon that leads to death of the majority of lymphocytes and neurons even if those cells are not directly infected.

Indeed, CCR5 expression in neurons is responsible to 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). CCR5-binding by inhibitors, 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, of neuronal cell lines apoptosis

induced by HIV-1 R5 strains. DAPTA, likely binds CCR5 at a site different than TAK-779, whose direct antiviral effect is clearly more pronounced than that induced by DAPTA. Due to the putative differences in CCR5-binding, it is conceivable that the phenomenon 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 to apoptosis of chemokine receptor targeted drugs. Recent tests in an animal model revealed that DAPTA, currently in phase II trials for HIV-1 disease, is able to block the release of the pro-inflammatory cytokines TNF-α and interleukin-1, counteracting in this way, the inflammatory state in the brain associated with HIV-1 infection in ADC (Tuttle et al., 2001). This may 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, may provide a major antiviral effect, particularly if used at early stages of the disease, when virus spreading is still limited.

In conclusion, the usage of coreceptor inhibitors, as DAPTA or TAK-779, may synergistically contribute to the control of HIV-1 replication and to the damage directly or indirectly induced by this virus.

2.1.4. *Materials and Methods of the chapter*

2.1.4.1. *Cells*

M/M were prepared and purified as described in published procedures (Aquaro and 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, Mass.) at a density of 1.8×10^6 cells/ml in RPMI 1640 (Gibco, Gaithersburg, Md.) supplemented with 50 U of penicillin/ml, 50 ug of streptomycin/ml, 2 mM

L-glutamine, and 20% heat-inactivated, mycoplasma- and endotoxin-free fetal calf serum (HyClone, Logan, Utah) (complete medium). Cell cultures were incubated in a humidified atmosphere with 5% CO2 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 are incubated in complete medium as previously described. Adherent cells obtained with this technique consisted of >95% differentiated M/M.

Peripheral blood lymphocytes (PBL) were purified from PBMC by repeated adherences to remove monocytes, and then cultured with the same medium as M/M, supplemented with 2 ug/ml phytohemagglutinin (PHA). Stimulation was carried out for 72 hours; afterward, the medium was discarded, cells were washed three times with RPMI 1640 and the concentration was adjusted to 5 x 10^5 cells per ml of medium supplemented with 50 U/ml recombinant interleukin-2 (IL-2).

The neuroblastoma cell line SK-N-SH (Koenig et al., Science 1986) was obtained from American Type Culture Collection (ATCC HTB-11) and maintained in RPMI 1640 supplemented with 10% fetal calf serum, 50 U of penicillin/ml, 50 ug of streptomycin/ml, 2 mM L-glutamine. To differentiate these cells to a neural cell phenotype they were exposed to 1 M retinoic acid (Sigma, Chemical Co., St. Louis, MO) for 4 days.

CCR5-transfected human astroglioma U87.CD4 cells were kindly provided by Dr. Dan R. Littman (Skirball Institute of Biomolecular Medicine, New York, NY, USA) and were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Paisley, UK) containing 10% heat-inactivated fetal bovine serum (FBS) (BioWhittaker Europe, Verviers, Belgium), 0.01 M HEPES buffer (Life Technologies), 0.2 mg/ml geneticin (G-418 sulfate) (Life Technologies) and 1 ug/ml puromycin (Sigma-Aldrich, St. Louis, MO, USA).

2.1.4.2. *Virus*

Different R5 viral strains, BaL, 81A and ADA whose characteristics and genomic sequence have been previously described, were used (Cenci et al., 1997; Gartner et al., 1986; Popovic et al., 1984). The

clinical R5 HIV-1 # 5 isolate was isolated from the plasma of an HIV-1-infected patient and passaged only once in PBMC. The viruses were expanded in M/M, supernatants were collected, ultracentrifuged for two hours at 22 000 rcf at 4°C, and stored at -80°C before use (Perno et al., 1993). Characteristics of viral stocks used for this study were $2.1x10^8$ HIV-RNA genomes/ml (corresponding to 35 ng of p24 antigen) and 5 x 10^3 tissue culture infectious doses 50% per ml (TCID50/ml) as assessed by virus titration in other primary M/M cultures.

2.1.4.3. *Compounds*

D-Ala-Peptide-T-Amide (DAPTA) was synthesized under GMP conditions and obtained from Bachem (Torrence, CA). 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-5Hbenzocycloheptenyl]carbonyl]amino]benzyl)-tetrahydro-2H-pyran-4-amonium chloride; Mr=531.13) (Baba M et al., PNAS 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).

2.1.4.4. *Assessment of antiviral activity of DAPTA in acutely infected macrophages*

Two days after isolation (i.e. 7 days after plating) M/M were exposed to various concentrations of DAPTA (10^{-15}, 10^{-14}, 10^{-13}, 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9} M) for 20 min, and then, without washing out the drug, challenged with the R5 HIV-1 strains BaL or 81A (3000 pg/ml of p24 gag Ag). For the TAK-779 experiments M/M were treated with the compound (0.08, 0.4 and 2 ug/ml of TAK-779) for 20 min, and then they were challenged with 300 TCID50/ml, a virus dose affording a maximal virus production from M/M, HIV-1 BaL , HIV-1 ADA and HIV-1 R5 clinical isolate # 5. Two hours after virus challenge at 37°C in a humidified atmosphere supplemented with 5% CO2, M/M were washed extensively with warm RPMI 1640 to remove the excess virus and complete medium containing the appropriate drugs (DAPTA or TAK-779) was replaced. Fresh complete medium and drugs were added at 7 day. Supernatants were collected at different time points and at day 14 assessed for virus production by

analysis of HIV-1 p24 gag Ag production with a commercially ELISA kit (Biorad, France). Moreover, we analyzed by light microscopy the cytopathic effect in M/M at day 14 after infection. The experiment was run in triplicate, with six positive controls for each experiment. The geometric mean of p24 gag Ag production of replicates in each experiment was used to determine the effective drug concentration where 50% and 90% of viral replication is inhibited (EC50 and EC90, respectively). Statistical analysis was performed by $\chi 2$ Test (** $p < 0.001$; * $p=0.001$), for drug treated vs. control of infected but not drug-treated M/M.

2.1.4.5. *HIV-1 DNA analysis in 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 presence or in absence of DAPTA (10^{-9} M and 10^{-7} M doses) and anti-CCR5 mAb 2D7 (3000 pg/ul). For HIV-1 proviral integration analysis genomic (total) DNA was isolated in from M/M after 18 h of HIV-1 infection (Qiagen DNA isolation and purification kit) and 1 x 10^6-1.25 x 10^5 cell equivalents were amplified in an inverse/nested PCR specific for a conserved region within gag gene (primers pair SK39/SK38; Gene Bank accession numbers A24318/A26625, synthesized by Gibco BRL/Invitrogen Life Technologies Carlsbad, CA). The 115-bp PCR products were detected by oligomer hybridization using 3'-fluorescein labelled probes SK19 (Gene Bank accession number A24328). The probe was labelled with Gene Images 3'-oligolabelling Module (Amersham, Piscataway, NJ) according manufacturer's procedure and specific target sequences immobilized on the Hybon-N nylon membrane (Amersham, Piscataway, NJ) were detected by Gene Images ECL Detection Kit (Amersham, Piscataway, NJ) by exposing to blue-light sensitive X-ray film (Pegasus Scientific Inc., Burtonsville, MD). Amplification of β-actin housekeeping gene was utilised 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, Utah, USA).

2.1.4.6. *Interaction of DAPTA with CCR5*

To verify the specificity of DAPTA-CCR5 binding we assessed the percentage of CCR5 expression in 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 1600 rpm for 10 min, counted and resuspended at a density of 2 x 10^5 cells /ml in complete medium. The M/M were then incubated with DAPTA, at several doses, for 20 min at 4°C or at 37°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 PBS and analyzed with a FACScan flow cytometer (Becton Dickinson). Ten thousand events were collected for each sample. The data were acquired and analyzed by the Lysis II program (Becton Dickinson). The same staining procedure was repeated for the neuronal cell line SK-N-SH.

2.1.4.7. *Measurement of intracellular calcium concentrations*

Adherent CCR5-transfected U87.CD4 cells and freshly isolated primary monocytes were seeded in 0.1% gelatin-coated 96-well black-wall microplates (Costar, Cambridge, MA, USA) at 2×10^4 cells per well for U87.CD4 cells and at 2×10^5 cells per well for primary monocytes on the day prior to the experiment. On the day of the experiment, the plated monolayers were loaded with the fluorescent calcium indicator Fluo-3 acetoxymethyl (Molecular Probes, Leiden, The Netherlands) at 4 uM for 45 min at 37°C. After thorough washing with calcium flux assay buffer (Hanks' balanced salt solution with 20 mM HEPES buffer and 0.2% bovine serum albumin (BSA), pH 7.4), the cells were pre-incubated for 15 min at 37°C with TAK-779 at the indicated concentrations in the same buffer. Then, the intracellular calcium mobilization in response to the appropriate chemokine (RANTES or MIP-1α) was measured at 37°C by monitoring the fluorescence as a function of time simultaneously in all the wells using a Fluorometric Imaging Plate Reader (FLIPR) (Molecular Devices, Sunnyvale, CA, USA) (Princen et al., 2003).

2.1.4.8. *hu-PBL-SCID mouse model*

M/M, infected with R5 HIV-1 in the presence or in the absence of TAK-779, were gently scraped from plastic plates, suspended in PBS, and washed twice. After counting, M/M were resuspended in RPMI medium 1640 and injected i.p. in SCID mice, engrafted with human peripheral blood lymphocytes (hu-PBL-SCID mice) at different concentrations. For the experiment were used CB17 scid/scid female mice at 4 weeks of age and were kept under specific pathogen-free conditions. These SCID mice were housed in microisolator cages; all food, water, and bedding were autoclaved before use.

The hu-PBL-SCID mice were killed 14 days after the injection with the M/M, and cells were collected from the peritoneal cavity. At each time, a two-step peritoneal lavage was done. The first washing was performed with 1 ml of cold RPMI medium 1640. The recovered volume was centrifuged, and the supernatant was stored at -20°C while the cells were pooled with those obtained from a second 4-ml washing as described (Rizza et al., 1996). Cells recovered from the peritoneum of the hu-PBL-SCID mice were resuspended in PBS and incubated with the appropriate fluorochrome-conjugated mAbs for 30 min. The cells were then washed with a mixture of PBS, 2% FCS, and 0.1% sodium azide and fixed with 2.5% paraformaldehyde. Three-color flow cytometry was performed with a FACScan fluorescence-activated cell-sorter cytometer (Becton Dickinson) and cells were analyzed with CellQuest (Becton Dickinson) software. A total of 20000 events per sample were collected. Cells were analyzed according to forward and side scatter properties to gate the live cell populations. The mAbs used were directly-labeled anti-human CD45-PerCp, CD4-FITC and CD8-PE (all obtained from Becton Dickinson).

2.1.4.9. *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 60 000 cells/ wells 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 8000 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% CO2 for 5 days. On the day of analysis, the cells were gently detached with trypsin-EDTA (0.02%) and centrifuged at 1600 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 1500 rpm for 10 min, the pellets were washed with PBS, incubated with Propidium Iodide (PI; 100 ug/ml) and RNase (250 ug/ml Qiagen) at 4°C for 2 hours in the dark. Then the samples were washed twice with PBS and PI-stained cells were analyzed by monitoring the incorporation of PI intracellular with a FACScan flow cytometer. Ten thousand events were collected for each sample. Data were acquired and analyzed by the Lysis II program (Becton Dickinson). Statistical analysis was performed by $\chi 2$ Test (** $p < 0.001$; * $p=0.001$), for DAPTA vs. control of not drug-treated neuronal cell lines.

2.2. Carbohydrate-Binding Agents (CBAs)

HIV-1 is an enveloped virus whose surface glycoprotein gp120 binds CD4 and a co-receptor on the target cell membrane to initiate infection. Many of the gp120 glycosylation sites are terminally mannosylated, a pattern common to many pathogens (Nguyen and Hildreth, 2003). Carbohydrate-binding agents (CBA) have been recently proposed as innovative anti-HIV compounds selectively targeting the glycans of the HIV-1 envelope glycoprotein gp120. Short pre-exposure of HIV-1 to CBAs prevents the DC to efficiently bind HIV-1 and no syncytia formation occurs upon subsequent co-cultivation with T-lymphocytes. Thus, the mannose-specific CBAs (i.e. the plant lectins HHA, GNA, NPA and CA; the procaryotic cyanovirin-N (CV-N)) and the GlcNAc-specific (i.e. the plant lectin UDA) but not other entry inhibitors, or polyanionic compounds, efficiently abrogate the DC-SIGN-directed HIV-1 capture and subsequent transmission to T-lymphocytes. Such compounds should have the potential to impair the ability of DC to capture HIV and to transmit HIV to T lymphocytes (Balzarini et al., 2007). The aim of our study is to demonstrate the ability of CBAs to inhibit HIV-1 capture also in M/M, one of the major cellular targets for HIV-1 infection and virus reservoir, and subsequent virus transmission to CD4+ T-lymphocytes. M/M contribute to the transmission and the pathogenesis of HIV-1 infection throughout the progression of HIV-1 infection especially at late stages when CD4+ T lymphocytes have been extensively depleted (Williams et al., 2002; Tomkowicz et al., 2006; Herbein et al., 2002). In fact, productively-infected M/M can fuse with uninfected CD4+ T lymphocytes and transfer the virus to these cells, thus further contributing to depletion of CD4+ T lymphocytes (Crowe S et al., 1990); in addition, HIV-1 infected M/M may induce the apoptosis on bystander uninfected cells, such as CD4+ and CD8+ T lymphocytes, neurons and astrocytes by releasing cytotoxic factors (Aquaro et al., 2000; Badley et al., 1997; Herbein et al., 1998; Mollace et al., 2002; Shi et al., 1996). Recently, it has been demonstrated that as few as 500 HIV-exposed M/M cause complete depletion of several millions of autologous CD4+ T-lymphocytes, sustained HIV-viremia and spreading of HIV-1-DNA in mouse lymphoid organs (Garaci et al., 2003). Therefore, M/M sustain persistent and continuously productive HIV infection (Li et al., 1999). We have looked at the ability of Macrophage Mannose

Receptor (MMR) on M/M, that lack expression of DC-SIGN, a a dendritic cell-specific ICAM-3 grabbing receptor/HIV-1-binding protein responsible of the adhesion between DC cells and resting T-cells (Geijtenbeek et al., 2000), to bind to HIV and to enable the transmission to T-cells in co-culture. The MMR is a 175-kDA transmembrane glycoprotein containing three types of characterizing domains, two of which have distinct carbohydrate recognizing properties. The amino-terminal cystein-rich domain plays a critical role in binding sulphated glycoproteins. The C-type lectin domains facilitate carbohydrate-dependent uptake of mannosylated protein antigens on micro-organisms including bacteria, yeast, enveloped viruses and protozoans (Reading et al., 2000). The MMR shows high affinity for mannose and fucose, intermediate affinity for N-acetylglucosamide and glucose and low affinity for galactose (Stahl et al., 1998). Because these terminal sugars are rarely found on mammalian cell-surface, the MMR could be responsible for recognition of self and nonself antigens (Engering et al., 1997). It also plays a key role in pathogen-related acquired host defence by mediating antigen internalization and delivery to MHC class II compartments for antigen presentation (Engering et al., 1997; Tan et al., 1997). Our data show that the HIV-1-association with M/M, that lack expression of DC-SIGN, is MMR mediated, as evidenced by inhibition with soluble mannose-binding lectin and with MMR antibody.

The outcome of this type of studies would be very helpful to guide the choice of potential candidate microbicide drugs (Balzarini et al., 2004; Balzarini and Van Damme, 2007).

2.2.1. Capture of various HIV-1 strains by B-Lymphocyte Raji/DC-SIGN cells and macrophages

Exponentially growing DC-SIGN-expressing B-lymphoblast Raji cells were suspended in cell culture medium at 6 x 10^6 cells/400 ul. M/M were obtained by Ficoll-Hypaque. Raji-DC-SIGN and M/M were exposed to HIV-1 for 1 or 2 hours, respectively. Then, the unabsorbed virus was carefully removed by several washing steps. It was calculated that after the serial washing steps, we removed the HIV-1 p24 remained in the supernatant.

Then we analyzed the HIV-1 capture by Raji cells and M/M (previously detached from the plates) by a HIV-1 p24 ELISA.

The results represented in the figure represent the percentage of captured HIV-1 p24 calculated compared with the input of HIV-1 24. HIV-1 p24 associated with Raji/DC-SIGN cells and M/M could be reliably measured. HIV-1 NL4.3 was most efficiently captured (10% of input virus), whereas HIV-1 IIIB was least efficiently captured (1% of input virus). For each individual strain, similar capture efficiency was observed for DC-SIGN-expressing cells and for M/M.

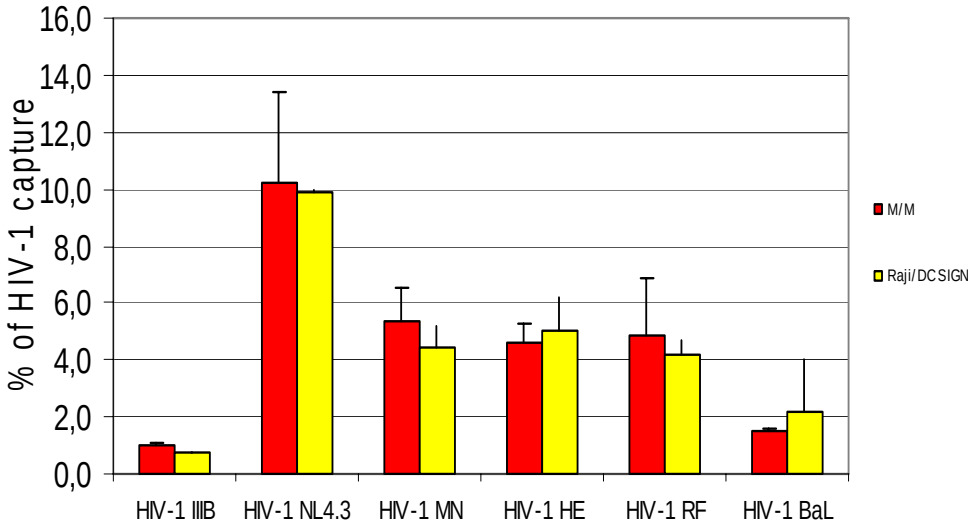


Figure 11: Capture HIV-1 p24 by M/M and Raji/DC SIGN. Raji/DC-SIGN and M/M were exposed to several strains of HIV-1. After the time of infection the cells were washed to be sure to remove the unbound virus. The Raji/DC-SIGN cell cultures and M/M were then analysed for p24 content by a p24 ELISA.

2.2.2 Inhibitory effect of CBAs on the ability of macrophages to capture HIV-1 particles

High amounts of HIV-1 particles (100 ul; 67000 pg/ml) were exposed to serial dilutions of the test compounds for 30 min. Then, the drug-exposed virus suspensions were added to M/M (100 ul/ well) for 2 hours at 37°C after which the cells were thoroughly washed four times with 1 ml RPMI 20% as described above. M/M were then detached by ELISA analysis for p24 content (Panel A). The analysis of the HIV-1 p24 Ag from the last wash was negative and this revealed the there were not virus particle in the medium and that the HV-1 p24 Ag revealed was only for the virus captured from M/M. The percentage of captured p24 Ag HIV-1 (pg/ml) calculated compared with beginning p24 Ag HIV-1 (pg/ml) .

The results show that the CBAs dose-dependently inhibited capture of HIV-1 by the M/M

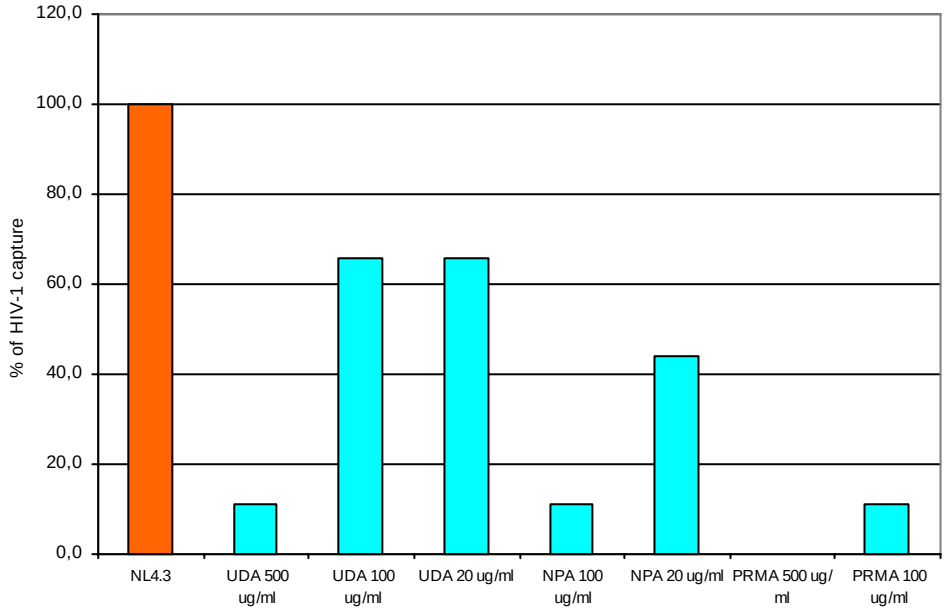


Figure 12: Inhibitory effect of CBAs on the ability of to capture HIV-1 particles. HIV-1 (NL4.3) particles were exposed to various dilutions of the test compounds (30 min) prior to administration to M/M for 2 hours. After removal of unbound virus by several washing steps, cell-associated virus was qualified by p24 ELISA.

2.2.3. Cocultivation of T Lymphocyte C8166 Cells and HIV exposed macrophages

Because M/M can capture HIV-1 particles and transmit HIV-1 to T-cells we wanted to see if CBAs are able to prevent the transmission of the virus particles to C8166 T lymphocytes (cocultivation) by HIV-1 NL4.3-exposed M/M (200000/ well; 1 ml). As described for the capture, 67000 pg/100 ul of HIV-1 were exposed to the serial dilutions of the test compounds for 30 min. CBA-exposed virus suspensions were added to M/M (100 ul/ well) for 2 hours at 37°C after which the cells were thoroughly washed four times with RPMI 20% to be sure to remove unabsorbed virus particles. Then C8166 (200.000/ well; 1 ml) were added to M/M and cocultivated for 3-4 days. The supernatant analysis of the HIV-1 p24 from the last wash was negative and this revealed the absence of virus particles in the medium able to infect the T cells. The coculture of the CBA/ virus-exposed M/M and C8166 did not result in increased p24 amounts in the cell cultures after 3 and 4 days. The percentage of HIV-1 p24 (pg/ml) transmission was expressed as a percent of the value of the positive control (100%), in which M/M were incubated with HIV-1 NL4.3 without the CBAs. As we can see in the figure 13 the CBAs, both UDA (Panel A) as NPA (Panel B), are able to prevent the transmission of HIV-1 from M/M to T cells at 3 and 4 days.

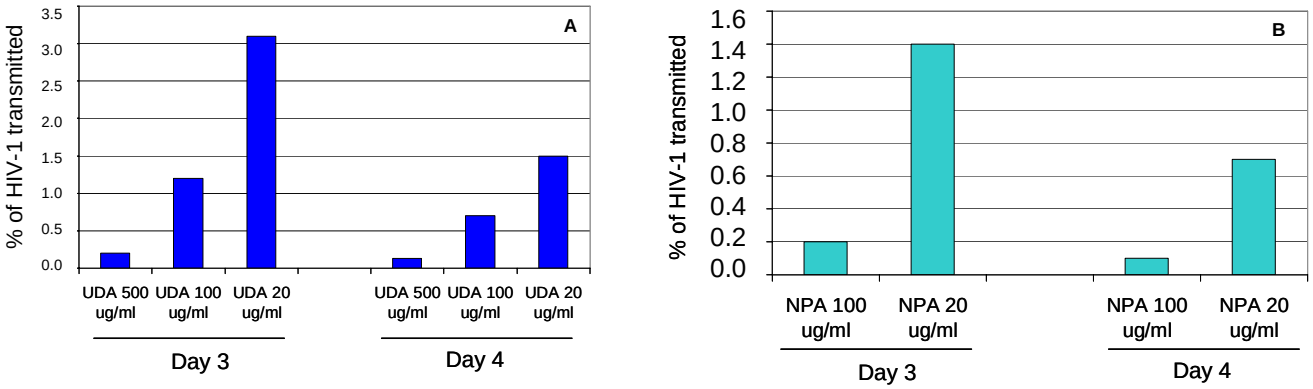


Figure 13: Inhibitory Effect of CBAs UDA (Panel A) and NPA (Panel B) on the Ability of M/M to Transmit captured HIV-1 Particles. HIV-1 (NL4.3) particles were exposed to various dilutions of the test compounds (30 min) prior to administration to M/M for 2 hours. After removal of unbound virus by several washing steps M/M were cocultivated with C8166 cells. The virus production was qualified by p24 ELISA after 3 and 4 days of coculture

Indeed, we observed in figure 14 that in cocultures of uninfected C8166 cells and HIV-1–exposed M/M giant cells appear at days 3 and 4 after the cocultivation (Panel A). On the contrary, no syncytia were microscopically observed to appear in the C8166 coculture with uninfected M/M (Panel B). Interestingly, uninfected C8166 cells were predominantly clustered at M/M locations.

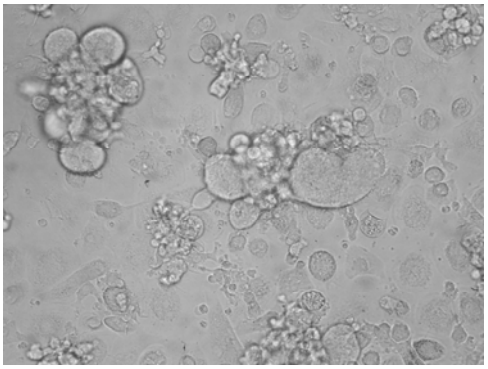
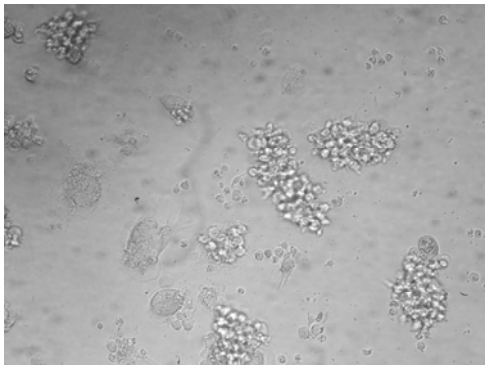
A   B

Figure 14: cytophatic effect induced by HIV-1 NL4.3 exposed M/M cocultured with T cells. **Panel A**: 3 days cocultures of uninfected C8166 cells + HIV-1-exposed M/M; **Panel B**: 3 days coculture of uninfected C8166 cells + M/M.

2.2.4. Antiviral activity of CBAs in HIV-1 BaL infected macrophages

M/M preincubated with several doses of CBAs (Panel A: HHA, Panel B: NPA, Panel C: UDA, Panel D: PRM-A) were infected with HIV-1 BaL (3000 pg/ml) and supernatants were collected at day 7 and 14. The HIV-1 p24 analysis by ELISA at day 14 revealed that CBAs are able to prevent the HIV-1 BaL replication in M/M, perhaps blocking the virus glycosylation sites responsible of the binding and, consequently, the virus entry and infection of M/M. Results are expressed as a percent of the value of the positive control, in which M/M were incubated with HIV-1 BaL without the CBAs. The values of EC50 (ug/ml) and EC90 (ug/ml) were shown in the table. The geometric mean of p24 gag production was used to determine the effective drug concentration where 50% and 90% of viral replication is inhibited (EC50 and EC90, respectively), by linear regression of the log of the percent HIV-1-p24 production (compared to untreated controls) versus the log of the drug concentration.

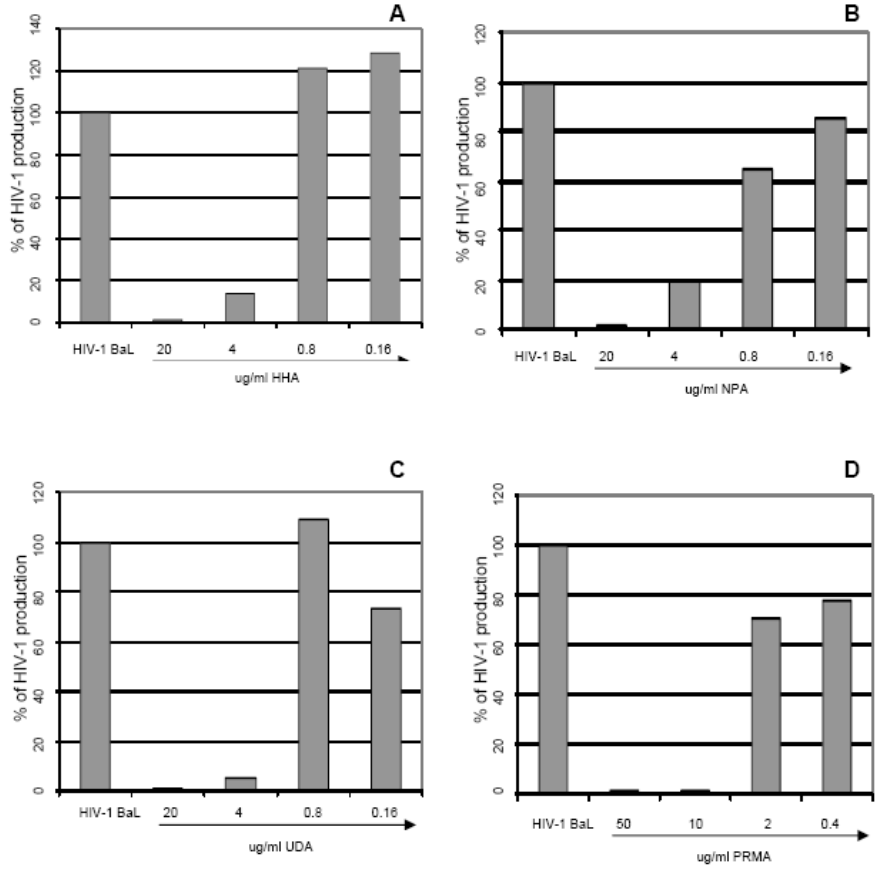


Figure 15: Antiviral activity of CBAs in HIV-1/ BaL infected M/M. The virus production was qualified by p24 ELISA at day 14 after the infection.

CBA	EC_{50}[a] (uM)	EC_{90}[b] (uM)
HHA	0.040	0.132
GNA	0.038	0.666
NPA	0.026	0.174
CA	0.01	0.10
UDA	0.207	0.415
CN-V	0.0005	0.002
PRMA	3.8	9.5

Table 1: Inhibitory activity of CBAs on the HIV-1 BaL infection of M/M

[a]50% Effective concentration.

[b]90% Effective concentration.

2.2.5. DC-SIGN and MMR expression in macrophages

We examined the expression of DC-SIGN and MMR receptors in M/M by flow cytometry. We observed that M/M don't express DC-SIGN (0.8% positive), indicating that this protein doesn't contribute to HIV-M/M binding, while M/M express MMR (11.5% positive).

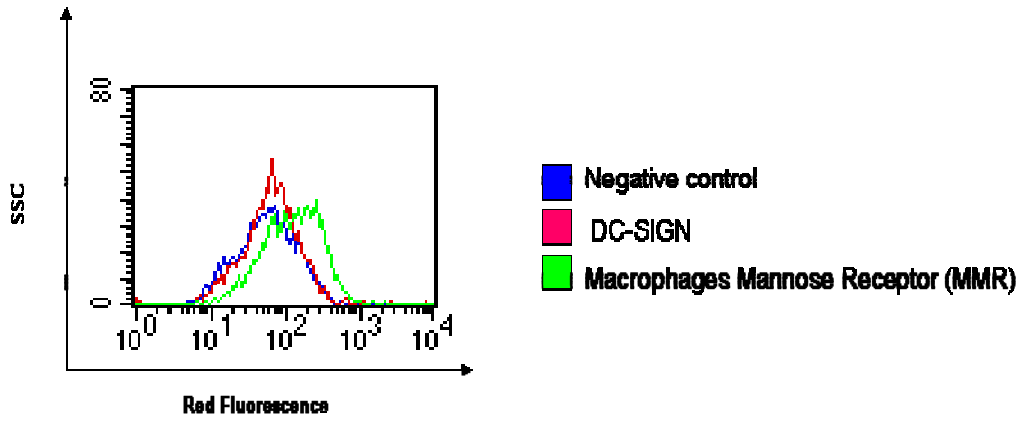


Figure 16: Cell surface expression of the receptors DC-SIGN and MMR on M/M.

2.2.6. Role of Mannose Receptor, DC-SIGN and CD4 on HIV-1 capture and transmission in macrophages

To study the role of MMR in the capture and transmission of HIV-1, we compare the effect of MMR antibody with that of several receptors inhibitors. M/M were incubated with MMR antibody (10 ug/ml), DC-SIGN antibody (10 ug/ml), for 15 min at 37°C. HIV NL4.3 (67000 pg/100 ul) was then added and incubated 2 hours at 37°C. Cells were then washed four times and detach for the HIV-1 p24 analysis (capture analysis) (Figure 17 Panel A). For the transmission experiments C8166 were added to M/M and the analysis of HIV-1 p24 was done at day 3 and 4 after coculture (Figure 17 Panel B). The percentage of captured or transmitted p24 HIV-1 was expressed as a percent of the value of the positive control, in which M/M were incubated with HIV-1 and without CBAs.

The results show that MMR antibody is able to prevent in M/M both the HIV-1 particles capture as the transmission to C8166, while CD4 and DC-SIGN antibodies don't prevent these effects. This shows the relevance of MMR and not of DC-SIGN or CD4 in the capture and in the consequently transmission of HIV-1 particles from M/M to T cells.

So, the ability of anti-MMR antibody to inhibit the capture and the transmission to T cells further supported the idea that the mannose-dependent association of HIV-1 with M/M is MMR mediated.

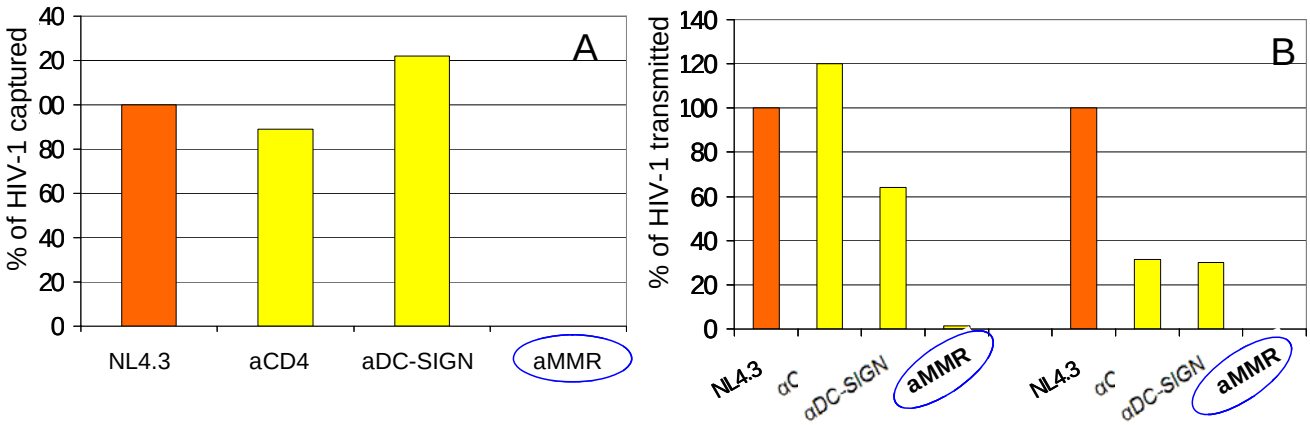


Figure 17: Role of the Mannose Receptor, DC-SIGN and CD4 on HIV-1 capture and transmission in M/M

Same results are shown in the figure 18. In the presence of the MMR antibody, in fact, the syncytia formation induced by HIV-1 to C8166 after 4 days of coculture, is prevented, while the presence of CD4 or DC-SIGN antibodies do not prevent this cytopathic effect.

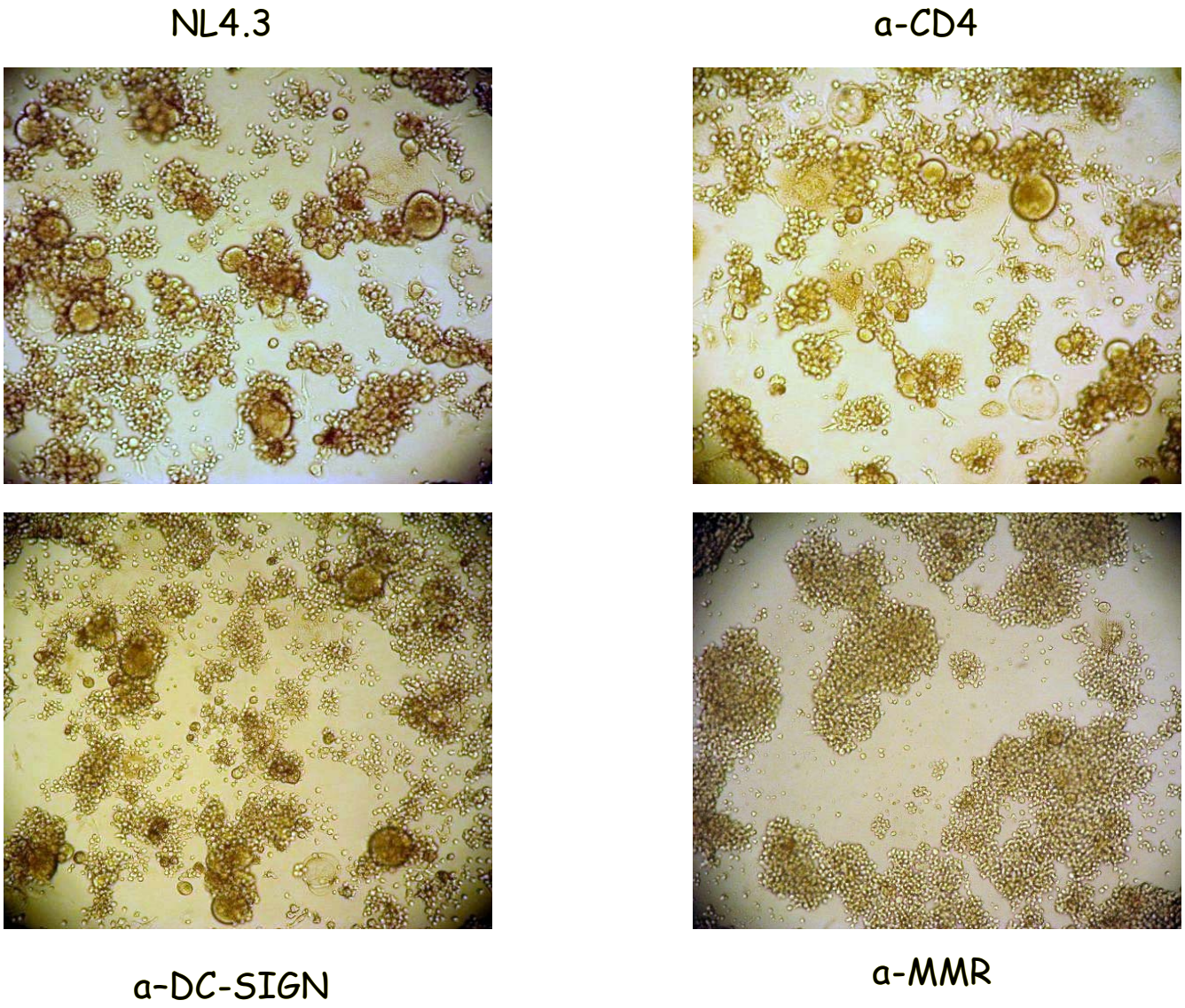


Figure 18: Mannose Receptor, but not DC-SIGN and CD4, can prevent the syncytia formation induced by HIV-1 NL4.3 exposed M/M to T cells

2.2.7. Inhibitory effect of mannan on the ability of macrophages to capture HIV-1 and transmit to T cells

To confirm the role of the MMR in HIV capture by M/M we used mannan (MMR inhibitor) (at 2.5 mg/ml). In this experiment we used two different procedures:

- Mannan 2.5 mg/ml was added to M/M for 15' at 37°C and then was added the virus (67000 pg/ 100 ul) for two hours at 37°C
- Mannan 2.5 mg/ml was mixed with virus (67000 pg/ 100 ul) for 15' at 37°C and then the mix was put on M/M for two hours at 37°C.

The cells were thoroughly washed four times with RPMI 20% as described above. M/M were then detached for ELISA p24 analysis. The analysis of the HIV-1 p24 from the last wash was negative and this revealed the there were not virus particle in the medium and that the HV-1 p24 revealed was only for the virus captured from M/M (Figure 19 Panel A).

For the transmission after the last wash, C8166 were added to M/M and at day 3 and 4 after coculture the analysis of HIV-1 p24 was done (Figure 19 Panel B). Results are expressed as a percent of the value of the positive control, in which M/M were incubated with HIV-1 NL4.3 without the CBAs.

Mannan partially inhibited HIV-1 capture by M/M and subsequent transmission to T cells.

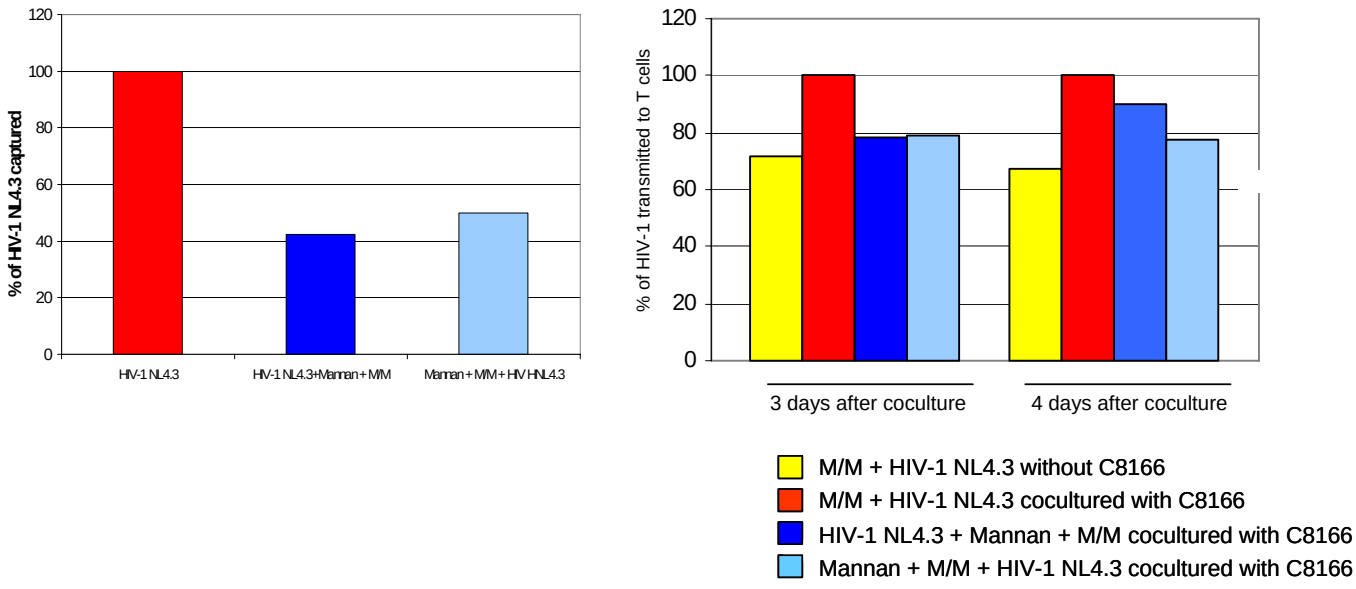


Figure 19: Inhibitory Effect of Mannan on the Ability of M/M to Capture HIV-1 and Transmit to T cells

2.2.8. Discussion

The characterization of M/M as infected cells able to spread virus to bystander cells, and to interfere with the homeostasis of the immune system and of the neural compartment, strongly supports the importance of inhibiting virus replication in such cells. Generally, the relevance of M/M in the pathogenesis of HIV infection underlines the importance of testing the antiviral efficacy of new compound inhibitors of different stages of the virus life-cycle (inhibitors of entry, integrase, nuclear transport, etc.) in M/M, early in development.

CBAs have been recently proposed as innovative anti-HIV compounds targeting selectively the glycans of the envelope glycoprotein gp120 (Balzarini et al., 2005). Recent studies showed that the CBAs have a remarkable antiviral activity against a variety of HIV-1 clade isolates, laboratory HIV-1 strains, and proved to be not toxic in mammalian cell models (Balzarini et al., 2005). These compounds were found very active against CCR5-using HIV-1 strains in M/M (Balzarini et al., 2004). In fact, the CBAs are able to prevent the HIV-1 replication in M/M infected with HIV-1 BaL, as we can see by analysing the p24 gag Ag production at day 14 after the infection. It is also known that M/M can interact with CD4+ T-lymphocytes and transfer the virus to these cells. A short pre-exposure of HIV-1 to CBAs can prevent the capture of the virus by M/M, and the subsequent transmission of virus particles captured to T cells. So, we can conclude that CBAs are able to inhibit the HIV-1 infection of M/M, interacting with the glycans of the gp120 HIV-1, and so counteracting the binding between HIV-1 and M/M. CBAs can also prevent the cell-virus interactions and subsequent HIV-1 transmission to T cells.

The potential of CBAs to impair M/M in their capacity of capture and transmission of HIV to T-lymphocytes might be an important property to be taken into consideration in the eventual choice to select microbicide candidate drugs to the clinical setting. For these reasons, CBAs represent promising compounds able to compromise the infectivity and transmission of HIV by M/M.

Moreover, resistance to carbohydrate-binding lectins is mediated by the selection of N-linked glycosylation site deletions in HIV-1 gp120. This represents a concept fundamentally different from all

the currently available therapeutic approaches. In fact, the loss of the carbohydrate shield may determine the exposure of new epitope targets of the neutralizing antibodies, thus enhancing immune response against HIV-1. For these reasons, CBAs represent promising compounds able to compromise the viability and infectivity of HIV by a synergistic action of drug-treatment and immune surveillance (Balzarini et al., 2005).

CBA will attach to the glycans on HIV gp120, preventing the virus from efficiently entering into its target cells. By doing this, CBA will exert pressure on the virus to escape its inhibitory action by selecting for mutations in its envelope glycoprotein. This will predominantly result in deletion of enveloped glycans as already demonstrated to occur in CBA-exposed HIV-1-infected cell cultures (Balzarini, 2007).

Instead, they may allow to trigger an efficient immune response directed against the mutated virus. By doing this, the CBAs put HIV for a dilemma: when the virus wants to efficiently escape CBA drug pressure, it needs to delete its *env* glycosylation sites. However, this will result in exposure of previously hidden immunogenic epitopes. This will in turn trigger the immune system by producing neutralizing antibodies and provoke a cellular immune response. When the virus wants to escape the immune pressure, it needs to restore the glycans on its *env* to hide again the immunogenic epitopes. However, this will result in inhibition of the virus by the CBAs again. Thus, the CBA concept aims to put the virus in a corner of no return and to force it to choose between two evils: to be inhibited by the CBA preventing it from entering its target cells and/or to be suppressed by the immune system. We believe that with the CBA concept, we may hit the Achilles heel of enveloped viruses such as HIV that need the presence of a dense glycan shield on their envelope to be efficiently infectious and to continuously circumvent the immune surveillance.

2.2.9. *Materials and Methods of the chapter*

2.2.9.1. *Compounds*

The mannose-specific plant lectins from Hippeastrum hybrid (HHA), Narcissus pseudonarcissus (NPA) and the GlcNAc-specific plant lectin from Urtica dioica (UDA) were derived and purified from these plants, as described before (Van Damme et al., 1987, 1988, 1991). Pradimicin A (PRM-A) (M.W. 838) was isolated and purified from the fermentation broth of Actinomadura hibisca. Cyanovirin (CN-V) was kindly provided by Dr. JB McMahon (National Institutes of Health, Bethesda, MD) and Dr. A Bolmstedt (Göteborg, Sweden). Mannan (from Saccharomyces cerevisiae) is from Sigma (M7504).

2.2.9.2. *Cells*

Human T-lymphocytic C8166 cells were obtained from the American Type Culture Collection (Manassas, VA). The Raji/DC-SIGN cells were constructed by Geijtenbeek et al. (2000). Because HIV infection studies and cultivation of immature DCs are tedious and labor-intensive, other approaches have been introduced to study the role of DC-SIGN in HIV transmission, including Raji cells stably transfected with DC-SIGN (Geijtenbeek TBH et al., 2000). It was demonstrated that such Raji/DC-SIGN cells capture and transmit HIV at efficiencies comparable with those of monocyte-derived dendritic cells (Baribaud et al., 2002; Geijtenbeek et al., 2000; Trumpfheller et al., 2003). DC-SIGN-expressing Raji/DC-SIGN cells were kindly provided by Dr. L Burleigh (Institut Pasteur, Paris, France). All cell lines mentioned were cultivated in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (BioWittaker Europe, Verviers, Belgium), 2 mM L-glutamine and 0.075 M NaHCO3.

Primary M/M were obtained as described in the chapter 2.1.4.1.

2.2.9.3. *Viruses*

HIV-1 (IIIB and BaL) was provided by RC Gallo and M Popovic (at that time at the National Cancer Institute, National Institutes of Health, Bethesda, MD). HIV-1(HE) is a clinical isolate, derived from a Belgian AIDS patient in 1987 and later propagated in MT-4 cells. HIV-1 NL4.3, MN, RF, were obtained through the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH) (Adachi et al., 1986).

2.2.9.4. *HIV-1-capture by Raji/DC-SIGN cells and macrophages*

Exponentially growing B-lymphoblastoid DC-SIGN-expressing Raji/DC-SIGN cells were suspended in cell culture medium at 6 x 10^6 cells/400 ul. 0.4 ml-suspensions of Raji cells were exposed to 0.6 ul HIV-1 for 60 min, after which 39 ml culture medium was added to the virus-infected cell culture. The cells were centrifuged at 1,250 rpm for 10 min, 39.9 ml supernatant carefully removed and the virus-exposed cells were resuspended in 40 ml medium. After a second centrifugation step, 39.9 ml supernatant was again removed, and the remaining 0.1 ml cell suspension was 10-fold diluted in cell culture medium to 1 ml. Under these experimental (washing) conditions, a maximum of 8 pg HIV-1 p24 could have remained in the 1 ml supernatant (or 0.4 pg in 50 ul). The Raji/DC-SIGN cell cultures were then analyzed for p24 content by a p24 ELISA (Perkin Elmer, Boston, MA).

M/M obtained by Ficoll-Hypaque from the blood of seronegative donors, were exposed to 100 ul of HIV-1 for two hours after which 4 washes with RPMI 20% were done to remove the unbound virus; to confirm this we collected 100 ul from the last wash to be analyzed by ELISA test. The percentage of captured p24 HIV-1 (pg/ml) was calculated compared with beginning p24 HIV-1 (pg/ml).

2.2.9.5. *Effect of short exposure of HIV-1 to test compounds on HIV-1-capture by macrophages*

HIV-1 was pre-exposed, where requested, to several dilutions of the test compounds for 15 min at 37°C. Then drug-exposed virus suspensions (HIV-1 NL4.3 67000 pg in 100 ul) were added to M/M

for 2 hours at 37°C. Then, the virus dilution were removed and the cells were carefully washed for four times with RPMI 20%, to be sure to remove all the virus in the well; to confirm this we collected 100 ul from the last wash to be analyzed by p24 ELISA (Perkin Elmer, Boston, MA). Then the cells were detached from the wells and collected to analyze the HIV-1 p24 -capture by ELISA test.

2.2.9.6. *Effect of short exposure of HIV-1 to test compounds on HIV-1-transmission from macrophages to T-cells*

Like the capture procedure, we pre-exposed at 37 °C (15 min) HIV-1 (NL4.3 67000 pg in 100 ul) to several CBA dilutions, where requested. Then we added the HIV/CBA to M/M. After 2 hours of infection at 37 °C, we removed the virus dilution and washed the cells for four times with RPMI 20%. We collected 100 ul from the last wash for p24 ELISA analysis to be sure that we removed all the virus from the medium. Then we added 100000 C8166 cells/well (1ml RPMI 20%) to the M/M. We then determined the HIV-1 transmission from M/M to C8166 analyzing the HIV-1 p24 production in the supernatants from the cocultures at 3 and 4 days (ELISA analysis).

2.2.9.7. *Antiviral effect of CBAs on HIV-1 BaL production*

M/M, obtained from blood of seronegative donors, were pre-treated for 15 min with several doses of CBAs, and infected with 3000 pg/ml of HIV-1 BaL. After two hours of infection, we removed the medium and washed the cells for four time with RPMI 20%, and cultured with CBAs, where requested. At day 7, the supernatants were collected and the CBAs were replaced to the new medium. At 14 days, the supernatants were collected and tested for p24 antigen production by p24 ELISA (Perkin Elmer, Boston, MA). Each experiment was run in triplicate. The geometric mean of p24 production of replicates in each experiment was used to determine the effective drug concentration where 50% and 90% of viral replication is inhibited (EC50 and EC90, respectively), by linear regression of the log of the percent HIV-1-p24 production (compared to untreated controls) versus the log of the drug concentration.

2.2.9.8. *Flow cytometry analysis*

To analyze the dendritic cell-specific ICAM-3 grabbing non integrin (DC-SIGN) and Macrophages Mannose Receptor (MMR) expression on M/M we stained the cells with anti-DC-SIGN (DCN46-FITC; BD Biosciences) and anti-MMR (CD206-PE; BD Biosciences) and we processed for flow cytometry as described previously (Vermeire K et al., 2003).

2.2.9.9. *Effects of CD4, DC-SIGN, MMR antibodies and mannan on HIV-1 capture and transmission to T-lymphocytes*

M/M were pre-incubated with inhibitors (mannose receptor antibody CD206 BD Pharmingen 10 ug/ ml; DC-SIGN antibody BD Pharmingen 10 ug/ml; CD4 antibody BD Pharmingen 10 ug/ ml) for 15 min at 37°C. HIV NL4.3 (67000 pg/ 100 ul) was then added and incubated for 2 hours at 37°C.

For the mannan we used two different procedures:

1) mannan 2.5 mg/ml was added to M/M for 15' at 37°C after which the virus (67000 pg/ 100 ul) was added for two hours at 37°C

2) mannan 2.5 mg/ml was mixed with virus (67000 pg/ 100 ul) for 15' at 37°C and then the mix was put on M/M for two hours at 37°C.

Cells were then washed four times with RPMI 20%, to be sure to remove all the virus in the well; to confirm this we collected 100 ul from the last wash to be analyzed by p24 ELISA (Perkin Elmer, Boston, MA). Then the cells were detached from the wells and collected to analyze the HIV-1 p24 by ELISA for the capture experiment; for the HIV-1 transmission experiment, M/M were co-cultured with C8166 for 3 or 4 days and then supernatants was analyzed to evaluate the virus transmission.

3. HIV-1 transcription inhibitors

3.1. New 6-desfluoroquinolone derivatives (6-DFQs)

The reversion of productively infected $CD4^+$ T cells to a resting memory state, induces a post-integration latency in which viral transcription is minimal and limited to the production of short, abortive HIV-1 transcripts (Hermankova et al., 2003; Lassen et al., 2004). This population of cells with a memory phenotype has a relatively long half-life and serves as a potential source of reactivation of viral replication, thereby creating a major obstacle for the eradication of the virus from HIV-infected patients. Since HAART fails to cure the infection and considering the fact that the viral transactivator Tat protein has been shown to upregulate HIV-1 gene expression in PBMCs from patients on HAART (Lin et al., 2003), a new anti-HIV treatment strategy may arise based upon HIV-1 transcription inhibitors (Stevens et al., 2006).

A unique class of drugs that may contribute to the control of the latent HIV-1 reservoir includes the quinolone derivatives. Quinolones were first reported as an important class of broad-spectrum antibacterials based on the inhibition of prokaryotic type II topoisomerases, namely DNA gyrase and, in a few cases, topoisomerase IV (Richter et al., 2004). In addition to the antibacterial properties, the quinolones have been shown to inhibit HIV-1 replication *in vitro* in both acutely and chronically HIV-infected cell lines by interfering with Tat-mediated transcription (Baba et al., 1997, 1998; Cecchetti et al., 2000; Parolin et al., 2003 Richter et al., 2004; Richter et al., 2005; Stevens et al., 2005; Tabarrini et al., 2004). The mechanism of anti-HIV action could be ascribed to the interaction of the quinolone with the bulge of the HIV-1 TAR RNA element resulting in the inhibition of the Tat-TAR complex formation (Richter et al., 2004).

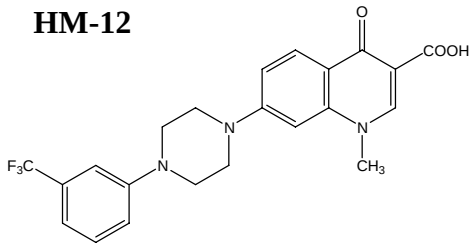
In order to evaluate the potential of HIV-1 transcription inhibitors such as the quinolone derivatives, we developed a novel in vivo model of HIV-1 latency that can be reactivated by the administration of human tumor necrosis factor-α (TNF-α). This study represents the first proof of concept that

quinolone-based drugs are inhibitory to HIV-1 replication in vivo, and may prevent the latent viral HIV-1 reservoir from reactivation.

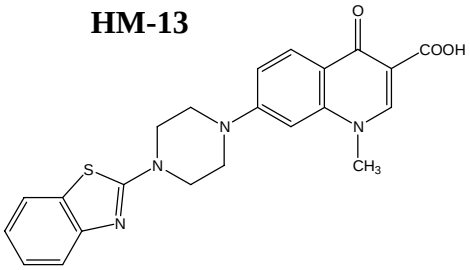
3.1.1. The 6-DFQ derivatives HM-12 and HM-13 inhibit HIV-1 replication in acutely and chronically HIV-1-infected macrophages

Indeed, two novel 6-desfluoroquinolone derivatives HM-12 and HM-13 were examined for their inhibitory effects on both acutely and chronically HIV-1-infected M/M.

HM-12



HM-13



The EC_{50} and EC_{90} values were calculated by comparing p24 antigen concentrations in supernatants of treated, infected cells with those of untreated, infected cells at day 14 post-infection. A significant dose-dependent antiviral activity of HM-12 and HM-13 was achieved in acutely virus-infected M/M (i.e. treated with drugs prior to virus challenge) with EC_{50} values of 0.050 ug/ml and 0.025 ug/ml, respectively (Table 2). To study the activity of HM-12 and HM-13 in chronically HIV-infected M/M, antiviral treatment was started 10 days after infection, when both HIV-1 p24 and genomic HIV-RNA released in the supernatants showed stable virus production. In chronically infected M/M we observed EC_{50} values of 0.050 ug/ml for HM-12 and 0.011 ug/ml for HM-13, respectively (Table 2).

Compound	M/M acute infection (ug/ml)		M/M chronic infection (ug/ml)	
	EC_{50}[a]	EC_{90}[b]	EC_{50}[a]	EC_{90}[b]
HM-12	0.050 ± 0.01	1.0 ± 0.02	0.050 ± 0.005	1.0 ± 0.03
HM-13	0.025 ± 0.005	0.20 ± 0.01	0.011 ± 0.008	0.16 ± 0.01

[a] 50% effective concentration, or compound concentration required to achieve 50% reduction of p24 production in HIV-1-infected M/M cells.

[b] 90% effective concentration, or compound concentration required to achieve 90% reduction of p24 production in HIV-1-infected M/M cells.

Data represent the average values (± SD) for at least three independent experiments each run in triplicate (1 ug/ml HM-12 equals 2.32 uM; 1 ug/ml HM-13 equals 2.38 uM).

Both quinolones were also examined for their inhibitory effects on the reactivation of HIV-1 from a latent viral reservoir in two different M/M lineages. Latently HIV-1-infected promyelocytic cells (OM-10.1) and latently HIV-1-infected promonocytic cells (U1) in which the virus remains in a dormant state were used. Upon hTNF-α or PMA exposure, a dramatic (~1000-fold) increase in HIV-1 expression occurred in both M/M cell lineages, which was set as hundred percent (Figure 20). In the presence of varying concentrations of quinolone derivatives HM-12 and HM-13 a dose-dependent inhibition of viral p24 production was observed in both latently HIV-1-infected cell lines. Similar inhibition was noted irrespective of the stimulation conditions (hTNF-α or PMA) at compound concentrations far below cytotoxicity. HM-12 and HM-13 inhibited HIV-1 production in OM-10.1 cell cultures by 50% (EC_{50}) at a concentration of 0.054 ug/ml and 0.045 ug/ml, respectively, when stimulated with hTNF-α, while stimulation with PMA resulted in comparable EC_{50} values of 0.039 ug/ml and 0.04 ug/ml, respectively. The 50% cytotoxic concentration (CC_{50}) of HM-12 and HM-13 in OM-10.1 cells was around 5 ug/ml. Evaluation of both quinolones in

latently HIV-1-infected U1 cells upon hTNF-α and PMA stimulation resulted in a similar concentration-dependent inhibition of virus production, though with slightly increased cytotoxicity.

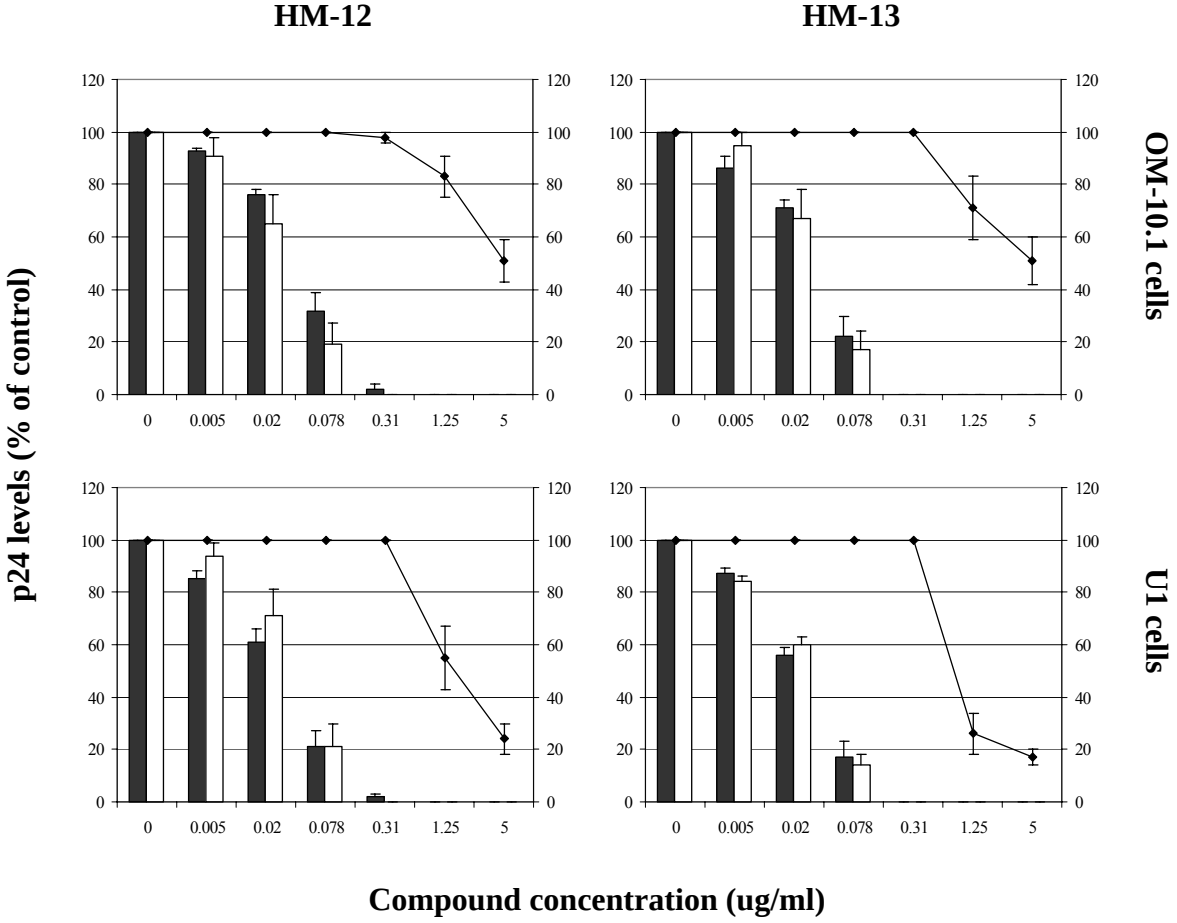


Figure 20: Inhibitory effect of HM-12 and HM-13 on hTNF-α- and PMA-induced expression of HIV-1 in OM-10.1 and U1 cells. The cells were incubated with the compounds for 2 h, stimulated with either 1 ng/ml hTNF-α (black bars) or 0.02 uM PMA (white bars) and further incubated for 48 h. Supernatants were then collected for p24 antigen quantification and the p24 levels are expressed as the percent of control (no compound).

3.1.2. The 6-DFQ derivatives HM-12 and HM-13 inhibit HIV-1 transcription in latently infected macrophages

In order to investigate the effect of the quinolones on HIV-1 transcription, we quantified the full-length viral transcripts produced upon stimulation of latently HIV-1 infected cells with hTNF-α or PMA. In this context, we made a construct that contains a 93 bp fragment from the RT gene of HIV-1 that served as standard for the quantification of the population of full-length viral mRNAs. The low amount of viral transcripts quantified under unstimulated conditions were subtracted from the amount measured in all stimulated samples once all data were normalized using the quantification results obtained for the endogenous control GAPDH. Following hTNF-α and PMA stimulation of the latently HIV-1-infected OM-10.1 and U1 cells, we observed a 1000-fold increase in viral mRNA production. The samples representing the stimulation condition in the absence of inhibitor were set as one hundred percent and used as reference to express the effect of the quinolones HM-12 and HM-13 on HIV-1 transcription in the latently HIV-1-infected cells. As shown in figure 21, both compounds were endowed with remarkable suppressive effects on HIV-1 transcription in OM-10.1 and U1 cells upon stimulation with either hTNF-α or PMA. HM-12 and HM-13 inhibited viral mRNA production in OM-10.1 cell cultures by 50% at a concentration of 0.067 ug/ml and 0.060 ug/ml, respectively, when stimulated with hTNF-α, while stimulation with PMA resulted in 50% inhibitory values of 0.032 ug/ml and 0.040 ug/ml, respectively. The dose-dependent decrease of HIV-1 mRNA production observed in the OM-10.1 and U1 cell cultures upon quinolone treatment closely correlated with their inhibitory effect on viral p24 production from the latently HIV-1-infected cells upon stimulation with hTNF-α and PMA.

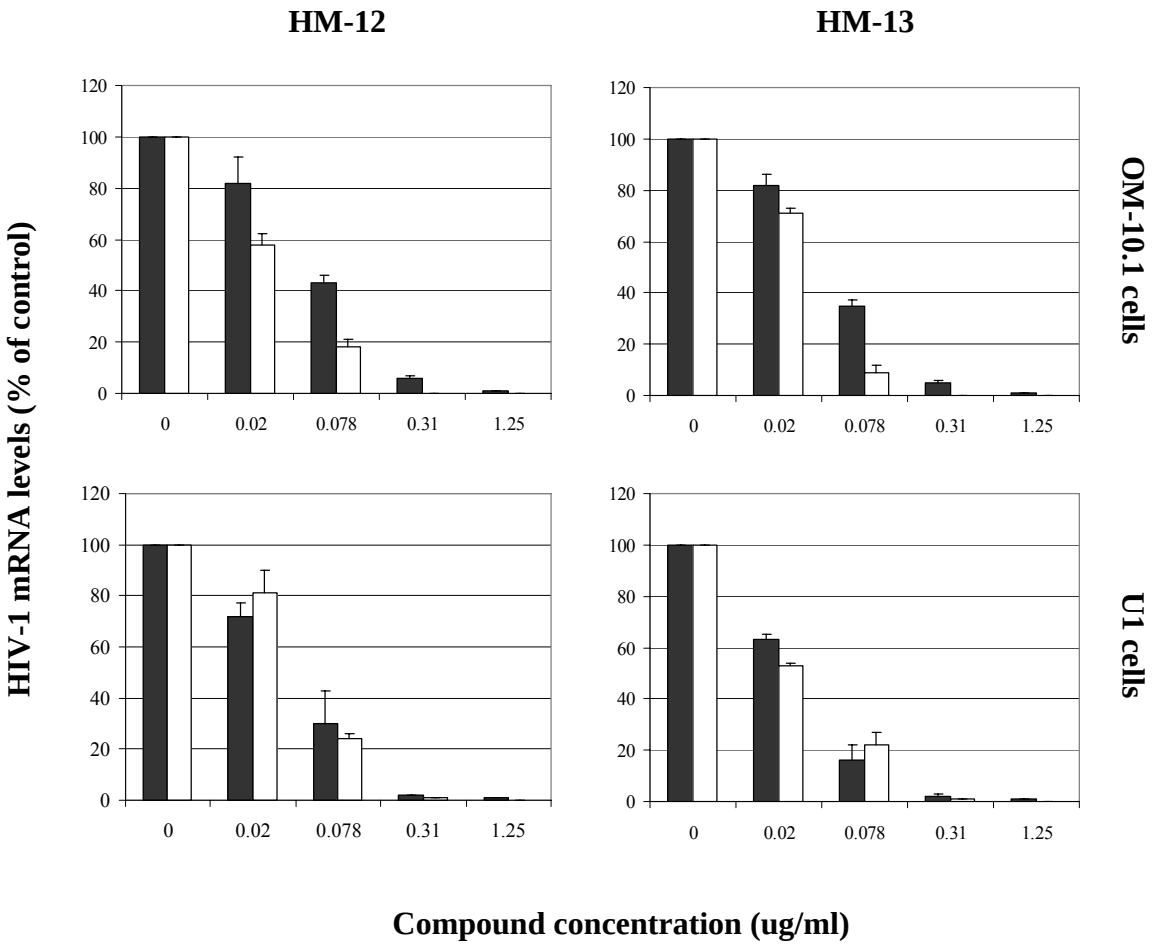


Figure 21: Inhibitory effect of HM-12 and HM-13 on HIV-1 RNA transcription in latently HIV-1-infected OM-10.1 and U1 cell lines after stimulation with 1 ng/ml hTNF-α (black bars) and 0.02 uM PMA (white bars). Total RNA was isolated from the cells by the TRIzol RNA extraction method. Quantification of full length viral RNA was assessed by real-time PCR amplifying a 93 bp fragment of the HIV-1 RT gene using the TaqMan technology. In order to obtain HIV-1 RNA amounts, correction was included using GAPDH as endogenous control.

3.1.3. Effects of the 6-DFQ derivatives HM-12 and HM-13 on viral reactivation in an artificial *in vivo* model of HIV-1 latency

We have developed an artificial xenochimeric model of HIV-1 latency in order to determine whether the quinolone derivatives can also control the latent HIV-1 reservoir *in vivo*. Male SCID mice of reproductive age (4-6 weeks old) were inoculated intraperitoneally with 10^7 latently HIV-1-infected OM-10.1 cells and found to develop malignant tumours around the pancreas, and the small and large intestinal canal. Four weeks after inoculation, the cancer became fatal to the mice. If mice were treated with 40 ug hTNF-α three weeks after inoculation with OM-10.1 cells, plasma viral load was increased 10- to 100-fold compared to the untreated mice without any visible signs of agony or fatality (Figure 22). As shown in Figure 22, plasma viral load was monitored by HIV-1 p24 antigen (panel A) and HIV-1 RNA (panel B). It was found that both parameters (p24 concentration and number of HIV-1 RNA copies) correlated well with one another. Therefore, both parameters were used to evaluate the effect of the quinolone derivatives on the hTNF-α-induced virus production *in vivo*. Initially, we investigated HM-13 by intraperitoneal administration at a drug dose of 50 mg/kg once daily for a period of three days prior to hTNF-α administration. Approximately 18 hours after hTNF-α stimulation, viral load was analyzed and found to contain as much HIV-1 as found in the absence of stimulation. This result was repeated in an independent experiment and confirmed the complete suppressive effect of HM-13 on viral reactivation in this artificial *in vivo* model (Figure 22, panel A and B). Additionally, the quinolone derivative HM-12 at a dose of 50 mg/kg/day administered for a period of three days was also found to be endowed with a pronounced inhibitory effect on viral reactivation *in vivo*. In order to obtain insights in the hTNF-α plasma levels present at 18 hours after intraperitoneal administration to the mice, we quantified, in parallel with the viral p24 levels, the hTNF-α levels in the plasma and observed values ranging between 900 and 1900 pg/ml under all tested conditions in the absence or presence of HM-12 or HM-13 (Figure 22, panel C).

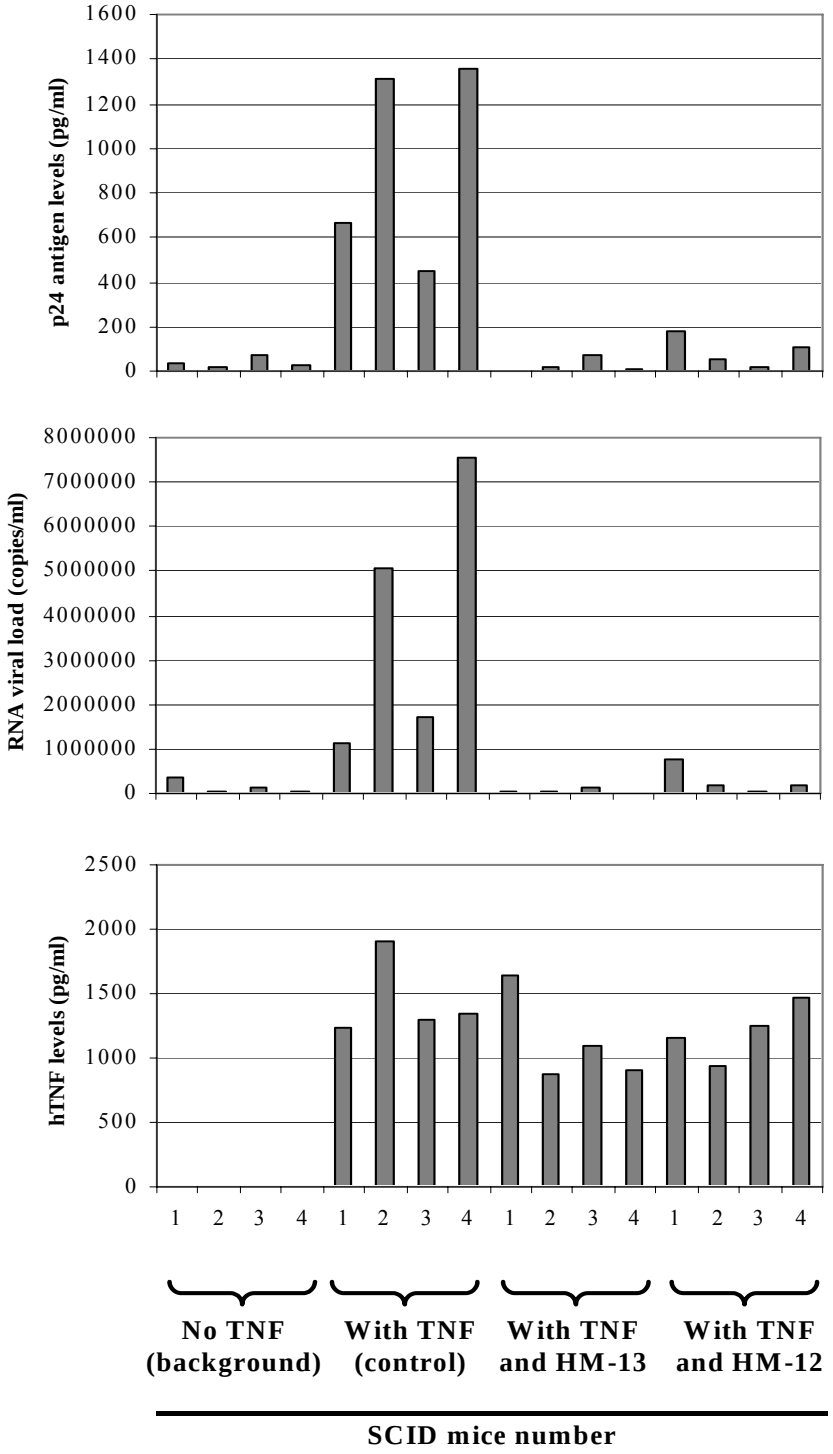


Figure 22: Evaluation of the quinolone derivatives on viral reactivation in a novel established artificial in vivo model of HIV-1 latency upon hTNF-α stimulation. Viral load was measured in the blood samples by analysis of HIV-1 p24 antigen and RNA viral load determination. In parallel, hTNF-α levels were quantified in plasma.

3.2. Discussion

Previously, it has been shown that the quinolone derivative, bearing a methyl substituent at the N-1 position, an amino group at the C-6 position and a 4-(2-pyridyl)-1-piperazine moiety at the C-7 position (WM5) can inhibit the Tat-mediated transcription process (Parolin et al., 2003). In particular, WM5 binds with high affinity to the bulge of the TAR element which results in an inhibition of the Tat-TAR complex formation (Richter et al., 2004). Subsequent structural investigations on a number of analogues of the lead compound WM5 has allowed us to obtain insights in their structure-activity relationship (SAR) (Tabarrini et al., 2004). We successfully eliminated the amino group from the C-6 position and positioned a suitable 4-arylpiperazine at the C-7 position resulting in the development of a new series of potent 6-DFQs. Indeed, we found that substitution at the *N*-4 piperazine core with a *m*-trifluoromethylphenyl (HM-12) or a benzothiazole (HM-13) resulted in pronounced anti-HIV properties, particularly in M/M cell lineages, with EC_{50} values in the ng/ml range. We observed not only in the acute infection model but also in the chronic and latent infection model pronounced antiviral activities for both quinolones HM-12 and HM-13 at drug concentrations as low as 40 ng/ml. In order to confirm that the inhibitory effects of both quinolones were located at the level of HIV-1 transcription as previously reported for the parent WM5 compound, we quantified the viral transcripts in latently HIV-1-infected cells upon reactivation of the provirus with hTNF-α and PMA. Since we found that HM-12 and HM-13 inhibited the production of viral mRNAs at concentrations in the ng/ml range, we proved that both quinolone compounds act at a post-integrational step in the HIV-1 replication cycle by inhibiting HIV-1 transcription.

Since these results and all previous data concerning the quinolones were obtained *in vitro*, we aimed at establishing the anti-HIV potency of these HIV-1 transcription inhibitors *in vivo*. For this purpose, we developed an artificial but novel xenochimeric model of HIV-1 latency in which HIV-1 could be recovered from the blood stream within 18 hours post-exposure of the animals with hTNF-α. Virus production was quantified by p24 as well as RNA viral load in plasma. It was found that

both parameters closely correlated with each other. Using this animal model, we provide the first evidence of a potent anti-HIV activity of quinolone-based drugs *in vivo*. Initially, we evaluated HM-13 for its effect on the reactivation of HIV-1 from the artificial viral reservoir and found that the compound had a pronounced suppressive effect on viral reactivation *in vivo* under experimental conditions where no visible signs of drug toxicity could be detected before and after the SCID mice were sacrified. The quinolone analogue HM-12 was also endowed with a marked anti-HIV activity in this artificial SCID mouse model of HIV-1 latency. Since both quinolones represent lead compounds, we plan to search and test for a larger number of compounds with optimal antiviral/cytotoxic properties in order to find clinically effective agents.

The quinolone derivatives are known as an important class of broad-spectrum antibacterials. The molecular mechanism of antibacterial action [i.e. prokaryotic DNA gyrase (topoisomerase II) and topoisomerase IV] is clearly different from the mechanism of anti-HIV action. Therefore, it is obvious that optimisation of quinolone derivatives must show pronounced antiviral activity in the absence of appreciable antibacterial activity. This is important in view of the risk of potential resistance development in quinolone-exposed bacteria upon frequent or continuous drug exposure. Since the quinolones act at a post-integration stage in the replication cycle of HIV, they markedly slow down virus replication, and should be interesting candidate drugs to be combined with entry, integrase or reverse transcriptase inhibitors. It would also be interesting to find out whether the quinolone derivatives act synergistically when combined with HIV protease inhibitors that act at a late stage in the virus infection cycle.

In conclusion, we have shown that quinolone-based drugs are inhibitory to viral reactivation from an artificial latent HIV-1 reservoir both *in vitro* and *in vivo*. Since this study is the first to demonstrate anti-HIV activity of quinolones *in vivo*, it certainly prompts further investigations in the potential of the quinolones in the treatment of HIV-1 infections.

3.3. *Materials and Methods of the chapter*

3.3.1. *Compounds and plasmid constructs*

The 6-desfluoroquinolone derivatives HM-12 and HM-13 synthesis will be reported elsewhere (Tabarrini et al., 2005). The construct pHIV-RT-Q was made as follows. After a reverse transcription reaction on total RNA isolated from OM-10.1 cells to cDNA, a PCR was performed with forward primer HIV-RT-F CATTCCTTTGGATGGGTTATGAA and reverse primer HIV-RT-R TGTCATTGACAGTCCAGCTGTCT in order to amplify a 93 bp fragment from the HIV-1 RT gene for cloning into the TOPO cloning vector (Invitrogen, Merelbeke, Belgium). The construct was verified by sequencing and used as standard for quantitative real-time PCR. pGAPDH-Q was used as internal control in the quantitative real-time PCR reaction and was constructed by cloning the complete GAPDH gene from the cDNA mix with forward primer GAPDH-F GACAGTCAGCCGCATCTTAT and reverse primer GAPDH-R CTTCCTCTTGTGCTCTTGCTG into the TOPO cloning vector. This construct was also verified by sequencing.

3.3.2. *Cells and viruses*

The latently HIV-1-infected promyelocytic OM-10.1 cell line (Butera et al., 1991) and the latently HIV-1-infected promonocytic U1 cells (Folks et al., 1987) were maintained in RPMI-1640 medium (Life technologies, Merelbeke, Belgium), supplemented with 10% heat-inactivated fetal calf serum (FCS, Integro, Zaandam, The Netherlands), 2 mM L-glutamine (Life technologies) and 0.1% $NaHCO_3$ (Life technologies), and incubated at 37 °C in a humidified CO_2-controlled atmosphere. Human M/M were prepared as described in chapter 2.1.4.1. The monocytotropic HIV-1 strain was used in this study involving primary M/M. Characteristics and genomic sequence of this strain have been previously described (Cenci et al., 1997; Gartner et al., 1986; Perno et al., 1993; Popovic et al., 1984). The virus was incubated in M/M, supernatants were collected, filtered and stored at -80°C before use (Perno et al., 1993). Characteristics of viral stocks used for this study were $2.1x10^8$ HIV-

RNA genomes/ml (corresponding to 35 ng/ml of p24 antigen) and 5 x 10^3 tissue culture infectious doses 50% per ml ($CCID_{50}$/ml), as assessed by virus titration in other primary M/M cultures.

3.3.3. *Assessment of antiviral drug activity in acutely infected macrophages*

One day after separation (i.e. 6 days after plating), M/M were treated with various concentrations of drugs (HM-12, HM-13), and then exposed to 2000 pg/ml of HIV-1 (BaL). Two hours after virus challenge, M/M were washed to remove the viral inoculum, and complete medium containing the appropriate drugs was replaced. M/M were then cultured for the duration of the experiments by replenishing them with fresh complete medium and drugs every 7 days. Supernatants were collected at different time points (7 and 14 days) for assessment of virus production by analysis of HIV-1 p24 production with a commercially available kit (Bio-Rad). The p24 antigen evaluation was repeated at later time points in selected experiments; the geometric mean of p24 gag Ag production of replicates in each experiment was used to determine the effective drug concentration where 50% and 90% of viral replication is inhibited (EC_{50} and EC_{90}, respectively), by linear regression of the log of the percent HIV-1-p24 production (compared to untreated controls) *versus* the log of the drug concentration.

3.3.4. *Assessment of antiviral drug activity in chronically infected macrophages*

M/M were defined chronically infected when no new rounds of infection occurred in *in vitro* cultures and the p24 production remained stable. Our previous experience demonstrated that such status of chronical infection starts from day 10 after virus challenge. For this purpose, M/M were challenged with 2000 pg/ml of HIV-1 BaL (in the absence of any drug) at day 0. At the time chronic infection was established, M/M were carefully washed at least twice to remove any virus present in the supernatants, replenished with fresh complete medium containing HM-12, or HM-13 at the same doses as used for the acute treatment, and cultured under the same conditions as described before. Each drug concentration was run in triplicate or quadruplicate while positive controls were run in sextuplicate. The supernatants of HIV-1-infected M/M were collected at days

13, 17 and 20, and the drugs were re-added at these time points at the appropriate concentrations. The supernatants of the cell cultures taken at 17 and 20 days were assessed to determine virus production in the presence or in the absence of drugs by measuring HIV p24 production.

3.3.5. *Assessment of antiviral drug activity in latently infected macrophages*

The activity values of the quinolone derivatives against latent HIV-1 infection were based on the inhibition of p24 antigen production in OM-10.1 and U1 cells after stimulation with hTNF-α (Roche Diagnostics Belgium) and PMA (Sigma Chemical Co., Bornem, Belgium). Briefly, OM-10.1 and U1 cells (500.000 cells/ml) were incubated in the presence or absence of the compounds for 2 h in 48-well plates. After this short incubation period, the cell cultures were stimulated with 1 ng/ml of hTNF-α or 0.02 uM of PMA followed by transfer of 2 times 200 ul to a 96-well plate for cytotoxicity evaluation. After a 2-day incubation period at 37 °C, the cell culture supernatants were collected from the 48-well plates and examined for their p24 antigen levels with the HIV-1 p24 ELISA kit (NEN, Brussels, Belgium). Cytotoxicity of the compounds for both latently HIV-1-infected OM-10.1 and U1 cell lines in the 96-well plates were based on the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability staining as previously described (Pauwels et al., 1988).

3.3.6. *Quantitative real-time PCR*

The quantitative determination of full length viral RNA in latently HIV-1-infected cell lines OM-10.1 and U1 was assessed by real-time detection based on the TaqMan technology using the plasmid pHIV-RT-Q as standard and pGAPDH-Q as internal control. Cells were seeded in 24-well plates at a density of 750.000 cells per well and were exposed to different concentrations of the compounds for 1 h. Then, 1 ng/ml of hTNF-α or 0.02 uM of PMA was added to the cell cultures and further incubated overnight at 37 °C. The next day, cells were collected and RNA was extracted using the TRIzol method (Invitrogen, Merelbeke, Belgium). The PCR reactions were performed in 96-well optical reaction plates with final volumes of 25 ul per well. The PCR reaction contained 5

ul RNA sample added to a mixture of 12.5 ul of TaqMan one-step RT-PCR master mix, 1.25 ul endogenous GAPDH control mixture, 600 nM of each primer HIV-RT-F and HIV-RT-R, and 250 nM of the TaqMan HIV-RT probe ATAAATGGACAGTACAGCCTATAGTGCTGCCAGA with the reporter dye 6-carboxyfluorescein (FAM) at the 5'-end and the quencher dye 6-carboxytetramethylrhodamine (TAMRA) at the 3'-end. The real-time PCR reaction on the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) was as follows: 30 min. at 48 °C for reverse transcription, 10 min. at 95 °C for enzyme activation, 45 cycles of amplification (15 s at 95 °C and 1 min. at 60 °C each) with measurement of fluorescence at the end of each elongation step. All assays included two negative controls (water) and a dilution series of the plasmid pHIV-RT-Q that contained a 93 bp fragment from the RT gene of HIV-1, as well as the endogenous control plasmid pGAPDH-Q containing the complete gene of human GAPDH. The detection of GAPDH RNA was conducted using a TaqMan Pre-Developed Assay Reagent containing a VIC/TAMRA-labeled TaqMan probe (Applied Biosystems, Foster City, CA). The standard curve of the threshold cycle (C_T) values was constructed for each PCR assay in order to automatically calculate the sample quantities using the software for data analysis (Livak et al., 2001). All samples were performed in duplicate.

3.3.7. *Animal experiments*

Male SCID mice of reproductive age (4-6 weeks old) were bred at the Rega Institute under specific pathogen-free conditions and used throughout the experiments. SCID mice were inoculated intraperitoneally with 10^7 latently HIV-1-infected OM-10.1 cells. Four animals were used for each experimental condition. On day 19, 20 and 21 post-inoculation (p.i), two groups of 4 SCID mice were injected, intraperitoneally, once daily with the DMSO-dissolved quinolones HM-12 and HM-13 at doses of 50 mg/kg/day. Then, one hour later on day 21 p.i., drug-treated as well as untreated SCID mice were injected intraperitoneally with 40 ug of hTNF-α that was obtained from Dr. Pieter Rottiers of the Department for Molecular Biomedical Research (VIB/UGent). All mice were

anesthetized on day 22 p.i., blood samples were collected by cardiac puncture and the mice were sacrificed. Viral load was measured by analysis of HIV-1 p24 antigen and RNA viral load determination in the serum. Additionally, hTNF-α levels were quantified using the hTNF-α ELISA kit (Roche Diagnostics Belgium). Finally, different vital tissues of the mice were histologically examined.

4. Antioxidants compounds

4.1. MnTBAP

Evidence exists suggesting that enhanced oxidative stress may be involved in the pathogenesis of HIV infection and HIV-1-infected patients are under chronic oxidative stress (Aukrust et al., 2003; Dröge, et al., 1994; Olinski et al., 2002). The activation of $CD4^+$ T-lymphocytes and M/M, which occurs during HIV infection, is most likely due to increased production of free radicals such as superoxide anion, peroxynitrite (the by-product of super oxide and nitric oxide, NO) and hydroxyl radical which is generated by peroxynitrite decomposition (Mollace et al., 2001, 2002). In addition, elevated serum levels of hydroperoxides and malondialdehyde (MDA), which are recognized as markers of lipid peroxidation subsequent to free radical overproduction, have also been found in asymptomatic HIV-1-infected patients early in the course of the disease (Palamara et al., 1996). Under normal circumstances, in healthy individuals, the free radicals burden is highly regulated by the endogenous antioxidant systems (i.e. superoxide dismutase enzymes, SOD) and glutathione peroxidase. Previous studies suggest that oxidative stress plays a crucial role during HIV-1 pathogenesis, including viral replication, inflammatory response, decreased immune cell proliferation, loss of immune function, chronic weight loss and increased sensitivity to drug toxicity. In particular, the disruption of oxidative status contributes to the cell damage observed during HIV-1 infection, yet it is worth stressing that such alteration is particularly relevant in M/M (Garaci et al., 1997; Palamara et al., 1996). The alteration of the homeostasis induced by HIV-1 infection in M/M, with consequent production of toxic factors, is claimed to be the main cause of neuronal damage during AIDS (Mollace et al., 2001). In particular, the release of some coating component of HIV-1, such as gp120 glycoprotein by HIV-1-infected M/M produces both direct and indirect effects in the CNS and in the systemic compartment (Nottet et al., 1995; Price et al., 1996). Furthermore, HIV-1 infection induces a significant perturbation of oxidative status of M/M associated with an increased production of MDA and a decreased synthesis of endogenous

glutathione (Palamara et al., 1996). In addition, the HIV regulatory proteins induce a reduction of expression and activity of MnSOD, the mitochondrial isoform of the enzyme (Weisiger & Fridovich 1973), leading to a sustained generation of superoxide and peroxynitrite and in turn imbalance of the cellular homeostasis (Flores et al., 1993; Westendorp et al., 1995). The associations of HIV infection with the formation of free radical species led us to hypothesize that superoxide and peroxynitrite may represent important mediators of M/M-HIV replication. To test this, we employed MnTBAP (Mn(III)tetrakis(4-benzoic acid)porphrin chloride), a synthetic peroxynitrite decomposition catalyst proven to reduce oxidative stress subsequent to peroxynitrite generation (Muscoli et al., 2003; Swingler et al., 1999).

4.1.2. Antiviral activity in acutely-infected macrophages

A significant dose dependent antiviral activity of MnTBAP was achieved in acutely-infected M/M (i.e. treated with drugs prior to virus challenge). In particular, the 1.2 uM dose of MnTBAP led to a reduction of p24 gag Ag production down to about 15% (Figure 23). Indeed, concentration of 30 μM completely inhibited HIV-1 replication up to day 14, end of experiment, affording undetectable levels of p24 gag Ag production (Figure 23).

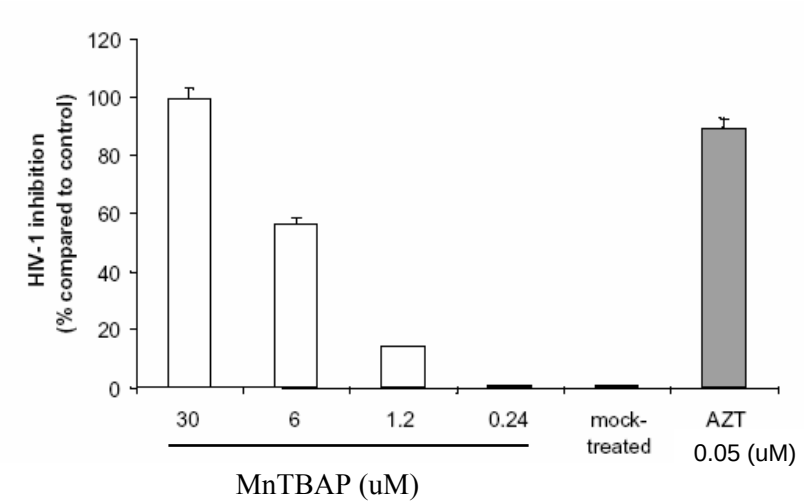


Figure 23: Antiviral activity of MnTBAP in acutely HIV-1 infected M/M.

Interestingly, virus inhibition remained constant for all concentrations tested up to day 14 (data not shown). Therefore, neither major breakthrough nor cumulative inhibition of virus replication occurred in acutely-infected M/M at least up to 14 days after infection. EC_{50} and EC_{90} were then calculated and found to be 3.7 (±0.05) μM and 19.5 (±0.5) μM, respectively (Table 3).

Table 3. Anti-HIV-1 activity of MnTBAP in macrophages and lymphocytes.

Cells	EC_{50} (μM)	EC_{90} (μM)	TC_{50} (μM)
Macrophages			
Acutely infected	3.7 (±0.05)	19.5 (±0.5)	60
Chronically infected	6.3 (±0.003)	30 (±0.6)	60
Lymphocytes			
Acutely infected	7.4 (±0.06)	21.3 (±0.6)	50

The geometric mean of p24 gag Ag production of replicates in each experiment was used to determine the effective drug concentration where 50% and 90% of viral replication is inhibited (EC50 and EC90, respectively), by linear regression of the log of the percent HIV-1 p24 production (compared to untreated controls) versus the log of the drug concentration. TC50: toxic concentration 50%.

4.1.3. Antiviral activity in chronically-infected macrophages

To study the activity of MnTBAP in chronically-infected M/M, antiviral treatment was started 10 days after infection, when both HIV-1 p24 gag Ag (Figure 24) and genomic HIV-RNA (data not shown) released in the supernatants show a stable virus production. Like a control of chronic inhibition we used the protease inhibitor Amprenavir (4 uM). A decrease in the release of mature proteins, compared to control, was already detectable by day 5 after drug treatment with the highest concentrations of MnTBAP (30 uM and 6 uM) (Figure 24), and become more pronounced at day 10 (30 uM). Starting from day 5 of drug treatment, and until the end of the experiment, a quasi-stable and substantial inhibition of the release of HIV-1 p24 gag Ag was detected with concentrations of MnTBAP of 6 and 30 uM (about 50% and 90% at day 5, respectively) up to day 10 after treatment. No complete inhibition of virus replication could be achieved at the highest non-toxic concentrations tested (Figure 24).

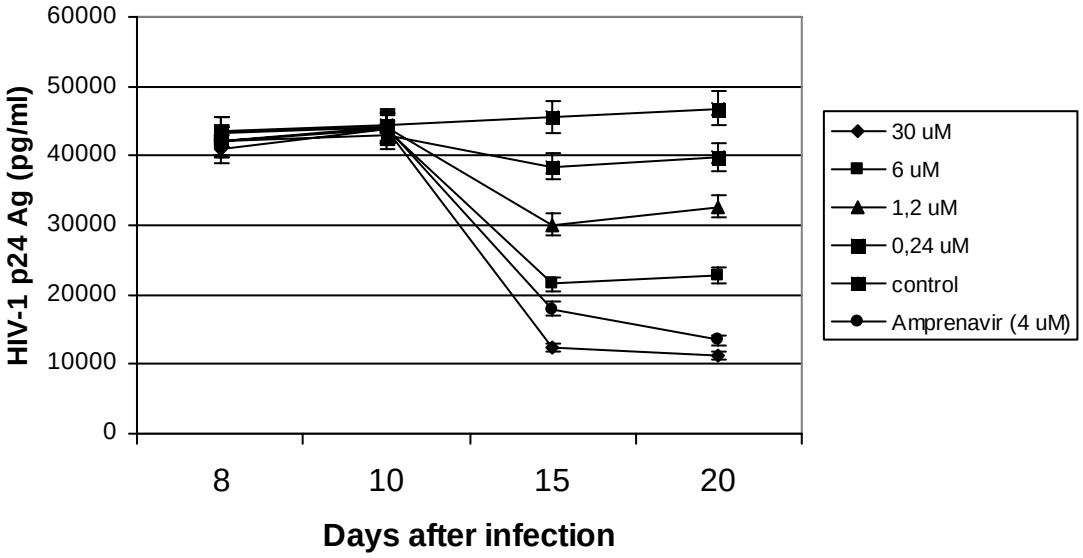


Figure 24: Antiviral activity of MnTBAP in chronically HIV-1 infected M/M

Based on these data, EC_{50} and EC_{90} of MnTBAP were 6.3 (±0.003) uM and 30 (±0.6) uM, respectively (Table 3). Treatment with 10 uM of AZT (about 200-fold greater than its EC_{90} in HIV-1-acutely infected M/M) was not able to reduce the production of HIV-1-p24 gag Ag in chronically

infected M/M (data not shown). This further confirms the absence of new rounds of replication in these cells after day 10 of infection (Aquaro et al., 2002; Perno et al., 1998).

4.1.4. Acutely-infected PBL

We wished to compare these results with those obtained using protease inhibitors in PBL. MnTBAP has shown a stable antiviral activity in acutely infected PBL until the end of the experiment (day 10 after infection), with an EC_{50} of 7.4 (±0.06) uM and an EC_{90} of 21.3 (±0.6) uM (Table 3). These EC_{50} and EC_{90} are and in the range (or lower in the case of EC_{90}) of those determined in acutely infected M/M (Table 3).

4.1.5. Drug toxicity

Treatment of M/M and PBL with concentrations of MnTBAP showed no decrease in cell number, thus suggesting the absence of major toxicity (Table 3). Thus, the antiviral activity observed in these experiments can be attributed solely to the MnTBAP inhibitory effect.

4.1.6. HIV-1 p25 and p55 gag proteins analysis

The western blots of lysates of acutely and chronically infected M/M treated with MnTBAP are shown in Figure 25. When the M/M lysates were examined, the inhibition of HIV-1-p24 antigen release into the supernatants correlated with the disappearance of the p24 band in the immunoblots in both acutely and chronically infected M/M. Interestingly, HIV-1 p55 antigen was also inhibited by MnTBAP treatment (Figure 25).

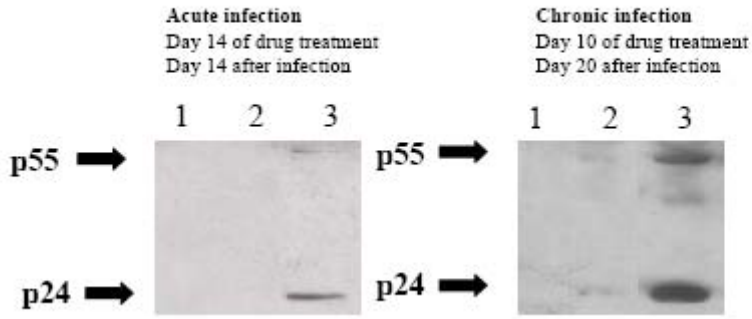


Figure 25: MnTBAP reduces p24 and p55 expression in HIV-infected macrophages. Western blots of lysates of acutely and chronically infected M/M.

Line 1: Mock-infected macrophages

Line 2: macrophages HIV-1 BaL infected and treated with MnTBAP (30 υM)

Line 3: macrophages HIV-1 BaL infected

4.1.7. Selective inactivation of peroxynitrite in HIV-1 infected macrophages

Furthermore, HIV-1 replication was associated with an increase of nitrotyrosine staining indicating the HIV-related peroxynitrite formation (Figure 26 A) as evaluated 14 days after HIV-infection. Treatment of acutely-infected M/M with MnTBAP (30 uM) inhibited the nitrotyrosine staining observed during HIV-1 replication (Figure 26 D) by removing the superoxide and peroxynitrite formation. As a control and consistent with previously published data, 0.05 uM AZT induced about 90% inhibition of virus replication in these acutely infected M/M (Figure 23), but did not affected the nitrotyrosine staining (Figure 26 C).

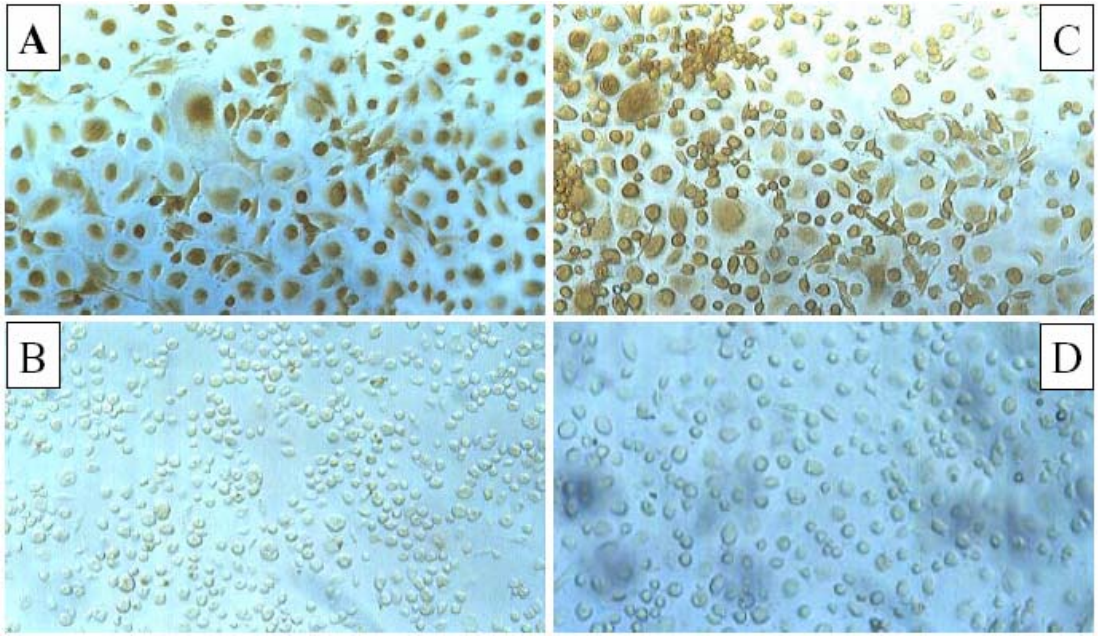


Figure 26: MnTBAP inhibits nitrotyrosine formation in HIV-infected M/M.

Photomicrographs (optical microscopy) of nitrotyrosine staining in HIV-1-infected M/M. HIV-1 infection enhance the immunocytochemical expression of nitrotyrosine (Panel A) in compared to mock-infected macrophages (Panel B), indicating an increased production of peroxynitrite. Acute treatment with MnTBAP (30 uM) (Panel D), but not with AZT (0.05 uM) (Panel C) is able to inhibit in macrophages HIV-related peroxynitrite formation.

4.1.8. Effects of MnTBAP upon virus infectivity

We investigated the production of infectious virus particles by both acutely and chronically infected M/M. Supernatants of HIV-1 acutely and chronically infected M/M previously treated with MnTBAP 6 uM and 30 uM, respectively, were titered in cultures of M/M and compared with the infectivity of not treated HIV-1 infected M/M supernatants taken at the same time-point. The infectivity of supernatants of not treated HIV-1 acutely infected M/M had a titre of 6.57×10^3 $TCID_{50}$/ml (Figure 27 A), while supernatants from MnTBAP (6 uM) treated HIV-infectd M/M showed a not detectable infectivity (Figure 27 A). Similarly, the infectivity of supernatants of not treated HIV-1 chronically infected M/M had a titre of 5.62×10^3 $TCID_{50}$/ml (Figure 27 B), while

supernatants from MnTBAP (30 uM) treated HIV-infected M/M had a titer of 4.21 x 10^2 $TCID_{50}$/ml (Figure 27 B), that means a reduction of more than 92% of infectivity.

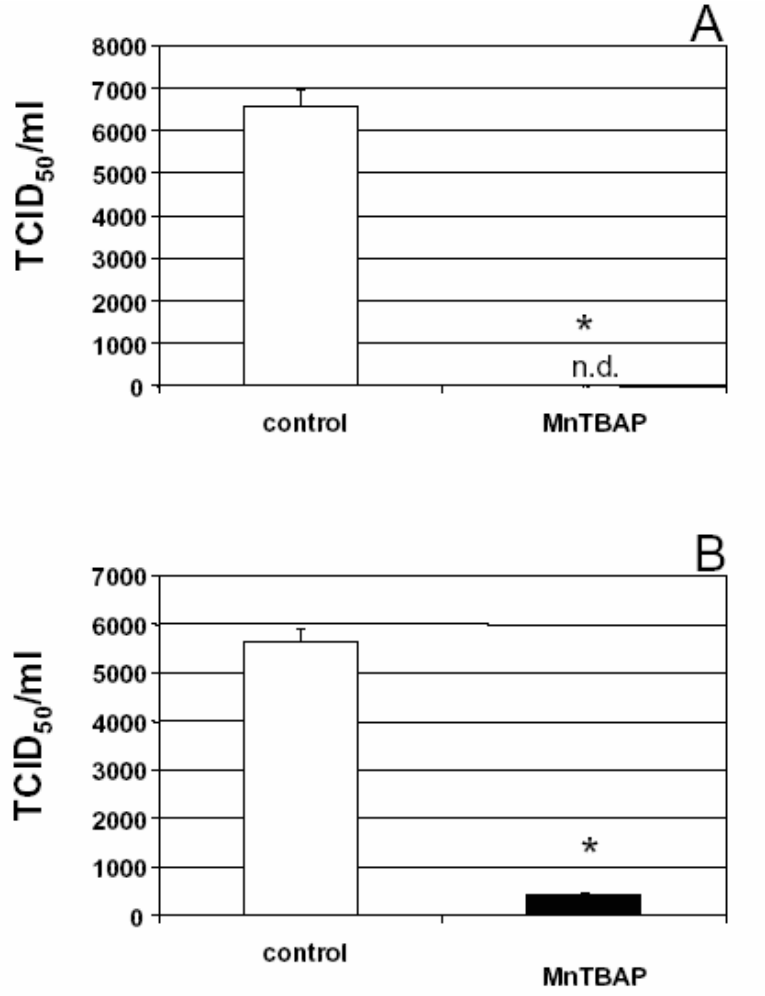


Figure 27: Analysis of infectivity of virus particles produced by M/M exposed to serial dilution of supernatants from MnTBAP treated or not-treated HIV-1-infected M/M. The TCID50/ml was calculated according to Reed and Muench method. MnTBAP reduces TCID50 about a log both in acutelly (Panel A) as in chronically (Panel B) HIV-1-infected M/M compared to infected and non treated M/M.

4.1.9. Ultrastructural analysis of acutelly-infected macrophages treated with MnTBAP

In order to better understand the mechanism through which the removal of peroxynitrite was acting on HIV-1 acutely infected M/M, electron microscopy was performed at day 14 after treatment.

MnTBAP at concentration of 6 uM dramatically reduced the presence of HIV-1 particles inside cytoplasmic vacuoles and in the extracellular compartment (Figure 28). Moreover, the number of these cytoplasmic vacuoles was diminished by MnTBAP treatment in HIV-1-infected M/M, compared to untreated HIV-1-infected M/M. These observations might contribute to the profound infectivity reduction induced by MnTBAP treatment.

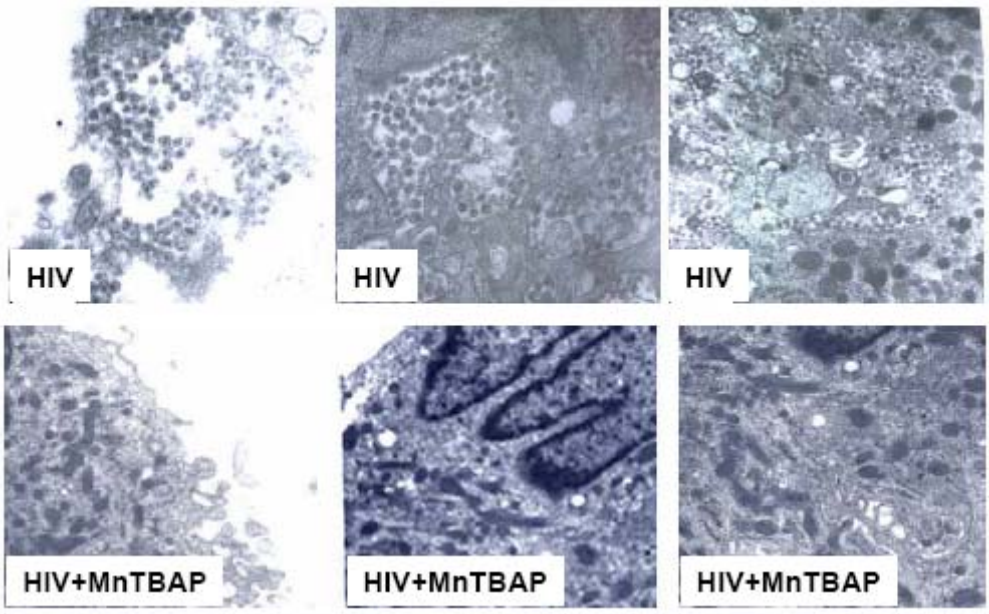


Figure 28: Electron microscopy of untreated or MnTBAP-treated HIV-1 infected M/M. Untreated M/M show accumulation of many mature particles, at different stages of maturation, in cytoplasmatic vacuoles and in the extracellular space. By contrast in MnTBAP 30 uM) treated M/M no viral particles are found. This observation support the hypothesis that MnTBAP treatment is able to prevent enveloped and unenveloped virions production.

4.2. Discussion

The design of the this study was based on the crucial importance of infected M/M in the pathogenesis and progression of HIV-1 infection. During HIV-1 infection the imbalance of the

intracellular redox status due to inflammatory stress have been previously reported (Kalebic et al., 1991). Indeed, free radicals are generated following HIV infection of M/M and microglia (Garaci et al., 2003; Mollace et al., 2001; Sonza et al., 2001). In particular, HIV-1 replication is enhanced under oxidative condition in vitro (Nabel et al., 1987). For example, *in vitro* HIV-1 infection of M/M resulted in superoxide and peroxynitrite production (Boven et al., 1999, 2000). Indeed, our findings have shown that immunohistochemical staining for nitrotyrosine, the footprint of peroxynitrite, showed extensive immunoreactivity in HIV-1 M/M cytoplasm and this overproduction was counteracted by MnTBAP, but not by AZT treatment.

Furthermore, significant and sustained inhibition of HIV-1 replication was obtained in both acutely and chronically infected M/M by removing the overt production of peroxynitrite. The effect of MnTBAP, a peroxynitrite decomposition catalyst, upon both the core protein p24 and its precursor p55 suggests that that free radicals may interfere with HIV-1 protein expression in both acute and chronic infection thus suggesting the important role played by the oxidative status in HIV-1 replication. This is in agreement with previous observations which point out the role of oxidative stress in HIV-1 proteins maturation and folding in both lymphocytes and M/M (Arp et al., 2005; Davis et al., 1999; Gulow et al., 2005). To better investigate this point, we evaluated the presence of HIV mature particles in M/M by electron microscopy. Indeed, electron microscopic analysis of intracellular compartments in HIV-1 infected M/M has shown that the number of mature virus particles was dramatically decreased by inactivation of peroxynitrite. Moreover, virus particles budding was also fully inhibited in agreement with previous studies where restoration of the oxidative status homeostasis led to the inhibition of HIV-1 budding in M/M.

Lack of HIV-1 maturation is correlated to a dramatic reduction of virus infectivity. The production of infectious virus particles by both acutely and chronically infected M/M was strongly counteracted (a reduction of about 4 and 2 log, respectively) by MnTBAP treatment. Nevertheless, complete inhibition of HIV replication was not achieved. This is not surprising since all the HIV

inhibitors are less (or even not) active in chronically-infected when compared with acutely-infected M/M (Aquaro et al., 2002; Perno et al., 1993, 1994, 1998).

It can be hypothetized that the dramatic virus inhibition obtained by the employment of peroxynitrite decomposition catalyst is due to, at least in part, an indirect mechanism on NF-kB pathway. Indeed, it is well known that reactive oxygen species activate NF-kB that, in turn, is an obligatory step for HIV-1, together with several viruses, replication (Aquaro et al., 2001; Brach et al., 1992, although further experiments are needed to confirm this hypothesis). However, other than this hypothesis, overall data presented in this article suggest that the inhibition of virus maturation and release can be related to a block of post-transcriptional/post-translational events of the virus life cycle. In fact, MnTBAP treatment substantially modify the expression of virus proteins in chronically (better in acutely) infected M/M. This structural proteins are crucial for the infectivity of HIV-1. Nevertheless, we cannot exclude that other factors related to peroxynitrite inactivation influence the virus replication. In conclusion our results highlight the role of peroxynitrite generation in HIV replication in human M/M and show that the removal of peroxynitrite by selective antioxidants such as peroxynitrite decomposition catalysts contribuits to the inhibition of HIV replication in M/M, the cells acting as a reservoir of the virus. Furthermore, data here reported suggest the potential usefulness of these compounds alone or in association with other antiretrovirals and may represent the basis for alternative and efficient strategies for the treatment of HIV-1 infection.

4.3. *Materials and Methods of the chapter*

4.3.1. *Compounds*

The peroxynitrite decomposition catalyst MnTBAP was purchased from Alexisis Biochemicals (Switzerland). 3'-azido-2', 3'-dideoxythymidine (AZT), an inhibitor of HIV replication, was used as control at concentrations known to be active against HIV-1 replication. All compounds and reagents

(with the exception of MnTBAP) were obtained from Sigma (St. Louis, USA). The nucleoside analogue reverse transcriptase inhibitor AZT was diluted in PBS and stored at –80°C before using.

4.3.2. *Cell cultures*

Primary M/M and PBL were obtained as described in chapter 2.1.4.1

4.3.3. *HIV-1 isolates*

The virus strains used in this study are HIV-1 BaL and IIIB (chapter 2.2.9.3).

4.3.4. *Drug toxicity*

M/M and PBL were treated for 14 to 21 days in the presence of different concentrations of MnTBAP. Cell viability of M/M and PBL was visually assessed (and compared to untreated controls) using the trypan blue exclusion method. Briefly, cells were exposed to dye, and then visually examined to determine whether cells take up or exclude dye. The live cells that possess intact cell membranes exclude trypan blue, whereas dead cells do not. Drug toxicity was assessed in the absence of viral infection.

4.3.5. *Assessment of drug activity in acutely infected macrophages*

One day after separation (i.e. 6 days after plating), M/M were treated with various concentrations of drugs (MnTBAP, 0.24, 1.2, 6 and 30 uM; AZT, 0.05 uM), and then exposed to 300 $TCID_{50}$/ml of HIV-1 BaL (a virus dose affording a maximal virus production from M/M). Two hours after virus challenge, M/M were washed to remove the viral inoculum, and complete medium containing the appropriate drugs was replaced. M/M were then cultured for the duration of the experiments by refunding them with fresh complete medium and drugs every 2 days. Supernatants were collected at different time points for assessment of virus production by analysis of HIV-1 p24 gag Ag production with a commercially available kit (Abbott labs, Pomezia, Italy) as described before. The p24 gag Ag evaluation was repeated at later time points in selected experiments; the geometric mean of p24 gag Ag production of replicates in each experiment was used to determine

the effective drug concentration where 50% and 90% of viral replication is inhibited (EC_{50} and EC_{90}, respectively), by linear regression of the log of the percent HIV-1-p24 production (compared to untreated controls) versus the log of the drug concentration.

The differences in the EC_{50} and EC_{90} in different cell populations and under different conditions of infection were assessed using the Student's t test.

4.3.6. *Assessment of antiviral drug activity in chronically infected macrophages*

M/M were defined chronically infected when no new rounds of infection occur in *in vitro* cultures and the p24 production remains stable. Our previous experience demonstrated that such status of chronical infection occurs starting from day 10 after virus challenge. For this purpose, M/M were challenged with 300 $TCID_{50}$/ml of HIV-1 BaL (in the absence of any drug) at day 0, and p24 gag Ag analysis was carried out from day 6 up to the point when at least two consecutive determinations showed stable production. At the time of chronical infection (hereinafter called day 0 for these experiments with chronically-infected M/M), M/M were carefully washed at least twice to remove any virus present in the supernatants, replenished with fresh complete medium containing various concentrations of MnTBAP (0.24-30 uM) or AZT (10 uM), and cultured under the same conditions as described before. Each drug concentration was run in triplicate or quadruplicate while positive controls were run in sextuplicate. Therefore, unless differently stated, drugs were then added at the time of chronical infection (i.e. day 10), and replaced each time of medium change (i. e. every 2 days).

4.3.7. *Assessment of drug activity in acutely-infected PBL*

PBL were plated in 48-well plates in the presence or absence of various concentrations of drugs, and challenged 30 minutes later with 300 $TCID_{50}$/ml of HIV-1 IIIB. After 2 hours cells were washed, counted, and plated with complete medium containing the appropriate drugs concentrations. Assessment of virus replication was performed by HIV-1-p24 ELISA.

4.3.8. *Virus infectivity*

Infectivity of virus particles produced by HIV-1-infected M/M was evaluated on M/M obtained from a different seronegative donor exposed to serial dilution of supernatants from drug treated or not-treated HIV-1-infected M/M. The $TCID_{50}$/ml was calculated according to Reed and Muench method.

4.3.9. *Western blot analysis*

After cells were washed with phosphate-buffered saline (PBS, BioWhittaker, Walkersville, MD), they were lysed with 0.75% Triton X-100 lysis buffer containing 300 mM NaCl, 50 mM Tris-HCl, pH 7.4, 2 uL/mL DMSO, and a cocktail of protease inhibitors containing 10 ug/mL Leupeptin, 20 ug/mL Aprotinin, 25 uM *p*-nitrophenyl guanidinobenzoate (pNGb). After a ten minute incubation in lysis buffer at 4°C, the cell lysate was clarified by centrifugation for ten minutes at 10,000 rpm. Total protein concentration was determined using the BCA Assay (Pierce, Rockford, IL). Cell lysates were resuspended in SDS sample buffer containing 50 mM dithiotreitol (DTT). Cell lysates (2 ug) were then loaded in a 10% Bis-Tris polyacrylamide gel (Novex, San Diego, CA), after separation by SDS/PAGE, proteins were transferred electrophoretically to nitrocellulose membranes and detected with a monoclonal mouse antibody to HIV-1-p24 (Intracel, Cambridge, MA).

4.3.10. *Immunocytochemical Staining*

Immunocytochemical staining for nitrotyrosine was performed on treated or not treated M/M. M/M were fixed with 4% paraformaldeyde dissolved in 0.1% phosphate buffer (pH 7.4). Nonspecific staining was blocked with 3% normal goat serum in 0.5 M Tris-HCl, pH 7.4 containing 0.2% Tween 20 for 1 h at room temperature. All subsequent incubations were carried out in this buffer. For detection of nitrotyrosine immunoreactivity, cells were incubated overnight at 4°C with an anti-nitrotyrosine monoclonal Ab (Cayman, 1:500). Treatment with secondary antibody, A/B complex, and DAB were performed by the manufacturer's instructions (Vector ABC Elite Kit, Vector Laboratories).

4.3.11. *Ultrastructural studies*

Cells for electron microscopy were fixed in 2.5% glutaraldehyde in PBS pH7.4 at 4 °C and then washed for 2 hours in PBS and post fixed in osmium tetroxide 1.33% for 2 hours at 4 °C. After several washes in PBS, the cells were dehydrated in graded alcohol, transferred into toluene, and embedded in Epon 812 resin. The resin was allowed to polymerize in a dry oven at 60 °C for 24 hours. Thin sections were cut with a glass knife Reichert microtome, stained with toluidine blue and examined on Axioscope microscope. Ultra-thin sections were cut on a Reichert microtome using a diamond knife, stained with uranyl-acetate-lead-hydroxide and evaluated and photographed on a Philips electron microscope CM 10 (Philips).

5. Conclusions

The relevant role of M/M, as a "Trojan horse" in which HIV-1 can survive and replicate for a long period of time, and the characterization of M/M as infected cells able to spread virus to bystander cells, causing damage in the immune and neural systems, strongly supports the importance of the development of modalities to inhibit virus replication in such cells. Generally, the relevance of M/M in the pathogenesis of HIV infection underlines the importance of testing the antiviral efficacy of new compound inhibitors of different stages in the virus life-cycle of M/M, early in the development (such as entry inhibitors, transcription inhibitors, antioxidants, etc.). Our studies investigated four possible new drug classes of compounds, such as CCR5-inhibitors, CBAs, transcription inhibitors and oxidative stress inhibitors that can be candidate drug leads for further (pre)clinical studies.

References

1. Amara A, Gall SL, Schwartz O, Salamelo J, Montes M, Loetscher P, Maggiolini M, Virelizier JL, Arenzana-Seisdedos F: HIV coreceptor downregolation as antiviral principle: SDF-1α-dependent internalization of the chemokine receptor CXCR4 contributes to inhibition of HIV replication. J Exp Med 1997,186:139-146.

2. Aquaro S, Perno CF, Balestra E, Balzarini J, Cenci A, Francescani M, Panti S, Serra F, Villani N, Caliò R: Inhibition of replication of HIV in primary M/M by different antiviral drugs and comparative efficacy in lymphocytes. J Leukoc Biol 1997,62:138-143.

3. Aquaro S, Caliò R, Balestra E, Bagnarelli P, Cenci A, Bertoli A, Gavazzi B, Di Pierro D, Francescani M, Abdelahad D, Perno CF: Clinical implications of HIV dynamics and drug resistance in macrophages. J Biol Regul Homeost Agents 1998,12:23-27.

4. Aquaro S, Panti S, Caroleo MC, Balestra E, Cenci A, Forbici F, Ippolito G, Mastino A, Testi R, Mollace V, Calio R, Perno CF: Primary macrophages infected by human immunodeficiency virus trigger CD95-mediated apoptosis of uninfected astrocytes. Journal of Leukocyte Biology 2000,68:429-435.

5. Aquaro S, Bagnarelli P, Guerci T, De Luca A, Clementi M, Balestra E, Caliò R, Perno CF: Long-term survival and virus production in human primary macrophages infected by human immunodeficiency virus. J Med Virol 2002,68:479-488.

6. Aquaro S, Caliò R, Balzarini J, Bellocchi MC, Garaci E, Perno CF: Macrophages and HIV infection: therapeutical approaches toward this strategic virus reservoir. Antiviral Res 2002,55:209-225.

7. Aquaro S, Perno CF: Assessing the Relative Efficacy of Antiretroviral Activity of different Drugs on Macrophages. In Human Retrovirus Protocols. Edited by Zhu T. Human Press, Totowa, NJ; 2005: 445-453.

8. Arp J, Rieder MJ, Urquhart B, Freeman D, Tucker MJ, Krizova A, Lehmann D, Dekaban GA: Hypersensitivity of HIV-1-infected cells to reactive sulfonamide metabolites correlated to expression of the HIV-1 viral protein tat. J Pharmacol Exp Ther 2005,314:1218-1225.

9. Aukrust P, Muller F, Svardal AM, Ueland T, Berge RK, Froland SS: Disturbed glutathione metabolism and decreased antioxidant levels in human immunodeficiency virus-infected patients during highly active antiretroviral therapy—potential immunomodulatory effects of antioxidants. J Infect Dis 2003,188:232-238.

10. Baba M, Okamoto M, Makino M, Kimura Y, Ikeuchi T, Sakaguchi T, Okamoto T: Potent and selective inhibition of Human Immunodeficiency Virus Type 1 transcription by piperazinyloxoquinoline derivatives. Antimicrob Agents Chemother 1997,41:1250-1255.

11. Baba M, Okamoto M, Kawamura M, Makino M, Higashida T, Takashi T, Kimura Y, Ikeuchi T, Tetsuka T, Okamoto T: Inhibition of Human Immunodeficiency Virus Type 1 replication and cytokine production by fluoroquinoline derivatives. Mol Pharmacol 1998,53:1097-1103.

12. Baba M, Nishimura O, Kanzaki N, Okamoto M, Sawada H, Iizawa Y, Shiraishi M, Aramaki Y, Okonogi K, Ogawa Y, Meguro K, Fujino M: A small-molecule, nonpeptide CCR5 antagonist with highly potent and selective anti-HIV-1 activity. Proc Natl Acad Sci USA 1999,96:5698-5703.

13. Badley AD, Dockrell D, Simpson M, Schut R, Lynch DH, Leibson P, Paya CV: Macrophage-dependent apoptosis of CD4+ T lymphocytes from HIV-infected individuals is mediated by FasL and tumor necrosis factor. Journal of Experimental Medicine 1997,185:55-64.

14. Balzarini J : Targeting the glycans of gp120: a novel approach aimed at the Achilles heel of HIV. Lancet Infect Dis 2005,5:726-731.

15. Balzarini J, Hatse S, Vermeire K, Princen K, Aquaro S, Perno CF, De Clercq E, Egberink H, Vanden Mooter G, Peumans W, Van Damme E, Schols D: Mannose-specific plant lectins from the Amaryllidaceae family qualify as efficient microbicides for prevention of human immunodeficiency virus infection. Antimicrob Agents Chemother. 2004,48:3858-3870.

16. Balzarini J, Van Damme L. Lancet: Microbicide drug candidates to prevent HIV infection. 2007,369:787-797.

17. Balzarini J, Van Herrewege Y, Vermeire K, Vanham G, Schols D: Carbohydrate-binding agents efficiently prevent dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN)-directed HIV-1 transmission to T lymphocytes. Mol Pharmacol. 2007,71:3-11.

18. Balzarini J, Van Laethem K, Hatse S, Vermeire K, De Clercq E, Peumans W, Van Damme E, Vandamme AM, Bolmstedt A, Schols D: Profile of resistance of human immunodeficiency virus to mannose-specific plant lectins. J Virol 2004,78:12724.

19. Balzarini J, Van Laethem K, Hatse S, Froeyen M, Peumans W, Van Damme E, Schols D: Carbohydrate-binding agents cause deletions of highly conserved glycosylation sites in HIV GP120: a new therapeutic concept to hit the achilles heel of HIV. J Biol Chem 2005,280:41005-41014.

20. Berger EA, Murphy M, and Faber JM: Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism and disease. Annu Rev Immunol 1999,17:657-700.

21. Boven LA, Gomes L, Hery C, Gray F, Verhoef J, Portegies P, Tardieu M, Nottet HS: Increased peroxynitrite activity in AIDS dementia complex: implications for the neuropathogenesis of HIV-1 infection. J Immunol 1999,162:4319-4327.

22. Boven LA, Middel J, Verhoef J, De Groot CJ, Nottet HS: Monocyte infiltration is highly associated with loss of the tight junction protein zonula occludens in HIV-1-associated dementia. Neuropathol Appl Neurobiol 2000,26:356-360.

23. Brach MA, De Vos S, Arnold C, Gruss HJ, Mertelsmann R, Herrmann F: Leukotriene B4 transcriptionally activates interleukin-6 expression involving NK-chi B and NF-IL6. Eur J Immunol 1992,22:2705-2711.

24. Brenneman DE, Hauser J, Spong CY, Phillips TM, Pert CB, Ruff M: VIP and D-ala-peptide T-amide release chemokines which prevent HIV-1 GP120-induced neuronal death. Brain Res 1999,838:27-36.

25. Butera ST, Perez VL, Wu BY, Nabel GJ, Folks TM: Oscillation of the human immunodeficiency virus surface receptor is regulated by the state of viral activation in a $CD4^+$ cell model of chronic infection. J Virol 1991,65:4645-4653.

26. Cartier L, Dubois-Dauphin M, Hartley O, Irminger-Finger I, Krause KH: Chemokine-induced cell death in CCR5-expressing neuroblastoma cells. J Neuroimmunol 2003,145:27-39.

27. Cecchetti V, Parolin C, Moro S, Pecere T, Filipponi E, Calistri A, Tabarrini O, Gatto B, Palumbo M, Fravolini A, Palù G: 6-Aminoquinolones as new potential anti-HIV agents. J Med Chem 2000,43:3799-3802.

28. Cenci A, Perno CF, Menzo S, Clementi M, Erba F, Tavazzi B, Di Pierro D, Aquaro S, Caliò R: Selected nucleotide sequence of the pol gene of the monocytotropic strain HIV type 1 BaL. AIDS Res Hum Retroviruses 1997,13:629-632.

29. Chun T-W, Stuyver L, Mizell SB, Ehler LA, Mican JAM, Baseler M, Lloyd AL, Nowak MA, Fauci AS: Presence of an inducible HIV-1 latent reservoir during highly active antiretroviral therapy. Proc Natl Acad Sci USA 1997,94:13193-13197.

30. Cocchi F, De Vico AL, Garzino-Demo A, Arya SK, Gallo RC, Lusso P: Identification of RANTES, MIP-1 α, and MIP-1 β, major HIV-suppressive factors produced by CD8+T cells. Science 1995,270:1811-1815.

31. Cohen J: Likely HIV cofactor found. Science 1996,272:809-810.

32. Crowe SM, Mills J, Kirihara J, Boothman J, Marshall JA, Mc Grath MS: Full-length recombinant CD4 and recombinant gp120 inhibit fusion between HIV infected macrophages and uninfected CD4-expressing T-lymphoblastoid cells. AIDS Res Hum Retrovir 1990,6: 1031-1037.

33. Davis DA, Yusa K, Gillim LA, Newcomb FM, Mitsuya H, Yarchoan R: Conserved cysteines of the human immunodeficiency virus type 1 protease are involved in regulation of polyprotein processing and viral maturation of immature virions. J Virol 1999,73:1156-1164.

34. De Clercq E: New developments in anti-HIV chemotherapy. Biochim Biophys Acta 2002,1587:258–275.

35. Deng H, Liu R, Ellmeier W, Choe S, Unutmaz D, Burkhart M, Di Marzio P, Marmon S, Sutton RE, Hill CM, Davis CB, Peiper SC, Schall TJ, Littman DR, Landau NR: Identification of a major co-receptor for primary isolates of HIV-1. Nature 1996,381:661-666.

36. Douek DC, Picker LJ, Koup RA: T cell dynamics in hiv-1 infection. Annu Rev Immunol 2003,21:265–304.

37. Dragic T, Litwin V, Allaway GP, Martin SR, Huang Y, Nagashima KA, Cayanan C, Maddon PJ, Koup RA, Moore JP, Paxton WA: HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. Nature 1996,381:667-673.

38. Dragic T, Trkola A, Thompson DAD, Cormier EG, Kajumo FA, Maxwell E, Lin SW, Ying W, Smith SO, Sakmar TP, Moore JP: A binding poket for a small molecule inhibitor of HIV-1 entry within the transmembrane helices of CCR5. Proc Natl Acad Sci USA 2000,97:5639-5644.

39. Dröge W, Eck HP, Mihm S: Oxidant-antioxidant status in human immunodeficiency virus infection. Methods Enzymol 1994,233:594-601.

40. Engering AJ, Cella M, Fluitsma DM, Hoefsmit EC, Lanzavecchia A, Pieters J. Mannose receptor mediated antigen uptake and presentation in human dendritic cells. Adv Exp Med Biol 1997,417:183–187.

41. Feng Y, Broder C.C. , Kennedy PE and Berger EA: HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. Science 1996,272:872-877.

42. Finzi D, Hermankova M, Pierson T, Carruth LM, Buck C, Chaisson RE, Quinn TC, Chadwick K, Margolick J, Brookmeyer R, Gallant J, Markowitz M, Ho DD, Richman D, Siliciano RF: Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. Science 1997,278:1295-1300.

43. Flores SC, Marecki JC, Harper KP, Bose SK, Nelson SK, McCord JM: Tat protein of human immunodeficiency virus type 1 represses expression of manganese superoxide dismutase in HeLa cells. Proc Natl Acad Sci USA 1993,90:7632-7636.

44. Folks TM, Justement J, Kinter A, Dinarello CA, Fauci AS: Cytokine-induced expression of HIV-1 in a chronically infected promonocyte cell line. Science 1987,238:800-802.

45. Gabuzda DH, Ho DD, de la Monte SM, Hirsch MS, Rota TR, Sobel RA: Immunohistochemical identification of HTLV-III antigen in brains of patients with AIDS. Ann Neurol 1986,20:289-295.

46. Garaci E, Palamara AT, Circolo MR, D'Agostini C, Abdel-Latif MS, Aquaro S, Lafavia E, Rotilio G: Intracellular GSH content and HIV replication in human macrophages. J Leukoc Biol 1997,62:54-59.

47. Garaci E, Aquaro S, Lapenta C, Amendola A, Spada M, Covaceuszach S, Perno CF, Belardelli F: Anti-nerve growth factor Ab abrogates macrophage-mediated HIV-1 infection and depletion of CD4+ T lymphocytes in hu-SCID mice. Proc Natl Acad Sci USA 2003,100:8927-8932.

48. Gartner S, Markovits P, Markovits DM, Kaplan MH, Gallo RC, Popovic M: The role of mononuclear phagocytes in HTLV-III/LAV infection. Science 1986,233:215-219.

49. Goodkin K, Vitiello B, Lyman WD, Asthana D, Atkinson JH, Heseltine PN, Molina R, Zheng W, Khamis I, Wilkie FL, Shapshak P: Cerebrospinal and peripheral human immunodeficiency virus type 1 load in a multisite, randomized, double-blind, placebo-controlled trial of D-Ala1-peptide T-amide for HIV-1-associated cognitive-motor impairment. J Neurovirol 2006,12:178-189

50. Greenhead P, Hayes P, Watts PS, Laing KG, Griffin GE, Shattock RJ: Parameters of human immunodeficiency virus infection of human cervical tissue and inhibition by vaginal virucides. J Virol 2000,74:5577–5586

51. Guan E, Wang J, Roderiquez G, Norcross MA: Natural truncation of the chemokine MIP-1 beta /CCL4 affects receptor specificity but not anti-HIV-1 activity. J Biol Chem 2002,277:32348-32352.

52. Gulow K, Kaminski M, Darvas K, Suss D, Li-Weber M, Krammer PH: HIV-1 trans-activator of transcription substitutes for oxidative signaling in activation-induced T cell death. J Immunol 2005,174:5249-5260.

53. Herbein G, Mahlknecht U, Batliwalla F, Gregersen P, Pappas T, Butler J, O'Brien WA, Verdin E: Apoptosis of CD8+ T cells is mediated by macrophages through interaction of HIV gp120 with chemokine receptor CXCR4. Nature 1998,395:189-194.

54. Herbein G, Coaquette A, Perez-Bercoff D, Pancino G: Macrophage activation and HIV infection: can the Trojan horse turn into a fortress? Curr Mol Med 2002,2:723-738.

55. Hermankova M, Siliciano JD, Zhou Y, Monie D, Chadwick K, Margolick JB, Quinn TC, Siliciano RF: Analysis of Human Immunodeficiency Virus Type 1 gene expression in latently infected resting $CD4^+$ T lymphocytes in vivo. J Virol 2003,77:7383-7392.

56. Heseltine PN, Goodkin K, Atkinson JH, Vitiello B, Rocon J, Heaton RK, Eaton EM, Wilkie FL, Sobel E, Brown SJ, Feaster D, Schneider L, Goldschmidts WL, Stover ES:

Randomized double-blind placebo-controlled trial of peptide T for HIV-associated cognitive impairment. Arch Neurol 1998,55:41-51.

57. Horuk R: Development and evaluation of pharmacological agents targeting chemokine receptors. Methods 2003,29:369–375.

58. Kalebic T, Kinter A, Poli G, Anderson ME, Meister A, Fauci AS: Suppression of human immunodeficiency virus expression in chronically infected monocytic cells by Glutathione, Glutathione Ester, and N-Acetylcysteine. Proc Natl Acad Sci USA 1991,88:986-990.

59. Kaul M, Lipton SA: Chemokines and activated macrophages in HIV gp120-induced neuronal apoptosis. Proc Natl Acad Sci USA 1999,96:8212-8216.

60. Kaul M, Garden GA, Lipton SA: Pathways to neuronal injury and apoptosis in HIV-associated dementia. Nature 2001,410:988-994.

61. Kazmierski W, Bifulco N, Yang H, Boone L, DeAnda F, Watson C, Kenakin T: Recent progress in discovery of small-molecule CCR5 chemokine receptor ligands as HIV-1 inhibitors. Bioorg Med Chem 2003,11:2663–2676.

62. Kim WK, Corey S, Alvarez X, Williams K: Monocyte/macrophage traffic in HIV and SIV encephalitis. J Leukoc Biol 2003,74:650-656.

63. Koenig S, Gendelman HE, Orenstein JM, Dal Canto MC, Pezeshkpour GH, Yungbluth M, Janotta F, Aksamit A, Martin MA, Fauci AS: Detection of AIDS virus in macrophages in brain tissue from AIDS patients with encephalopathy. Science 1986,233:1089-1093.

64. LaBranche CC, Galasso G, Moore JP, Bolognesi DP, Hirsch MS, Hammer SM: HIV fusion and its inhibition. Antiviral Res 2001,50:95–115.

65. Lassen KG, Bailey JR, Siliciano RF: Analysis of Human Immunodeficiency Virus Type 1 transcriptional elongation in resting $CD4^+$ T cells in vivo. J Virol 2004,78:9105-9114.

66. Li S, Juarez J, Alali M, Dwyer D, Collman R, Cunningham A, Naif HM: Persistent CCR5 utilization and enhanced macrophage tropism by primary blood human immunodeficiency virus type 1 isolates from advanced stages of disease and comparison to tissue-derived isolates. J Virol 1999,73:9741-9755.

67. Lin X, Irwin D, Kanazawa S, Huang L, Romeo J, Yen TS, Peterlin BM:. Transcriptional profiles of latent Human Immunodeficiency Virus in infected individuals: effects of Tat on the host and reservoir. J Virol 2003,77:8227-8236.

68. Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. Methods 2001,25:402-408.

69. Mack M, Luckow B, Nelson PJ, Cihak J, Simmons G, Clapham PR, Signoret N, Marsh M, Stangassinger M, Borlat F, Wells TN, Schlondorff D, Proudfoot AE: Aminooxypentane-RANTES induces CCR5 internalization but inhibits recycling: a novel inhibitory mechanism of HIV infectivity. J Exp Med 1998,187:1215-1224.

70. Martin-Garcia J, Kolson DL, Gonzalez-Scarano F: Chemokine receptors in the brain: their role in HIV infection and pathogenesis. AIDS 2002,16:1709-1730.

71. McElrath MJ, Pruett JE, Cohn ZA: Mononuclear phagocytes of blood and bone marrow: comparative roles as viral reservoirs in human immunodeficiency virus type 1 infections. Proc Natl Acad Sci USA 1989,86:675-679.

72. Meucci O, Fatatis A, Simen AA, Bushell TJ, Gray PW, Miller RJ: Chemokines regulate hippocampal neuronal signalling and gp120 neurotoxicity. Proc Natl Acad Sci USA 1998,95:14500-14505.

73. Michael NL, Moore JP: HIV-1 entry inhibitors: evading the issue. Nat Med 1999,5:740–742.

74. Mollace V, Nottet HSLM, Clayette P, Turco C, Muscoli C, Salvemini D, Perno CF: Oxidative stress and neuroAIDS: triggers, modulators and novel antioxidants. Trends Neurosci 2001,24:411-416.

75. Mollace V, Salvemini D, Riley DP, Muscoli C, Iannone M, Granato T, Masuelli L, Modesti A, Rotiroti D, Nistico R, Bertoli A, Perno CF, Aquaro S: The contribution of oxidative stress in apoptosis of human-cultured astroglial cells induced by supernatants of HIV-1-infected macrophages. J Leukoc Biol 2002,71:65-72.

76. Moore JP, Stevenson M: New targets for inhibitors of HIV-1 replication. Nat Rev Mol Cell Biol 2000,1:40–49.

77. Murphy EL, Collier AC, Kalish LA, Assmann SF, Para MF, Flanigan TP, Kumar PN, Mintz L, Wallach FR, Nemo GJ: Highly active antiretroviral therapy decreases mortality and morbidity in patients with advanced HIV disease. Viral Activation Transfusion Study Investigators. Ann Intern Med 2001,135:17-26.

78. Muscoli C, Cuzzocrea S, Riley DP, Zweier JL, Thiemermann C, Wang ZQ, Salvemini D: On the selectivity of superoxide dismutase mimetics and its importance in pharmacological studies. Br J Pharmacol 2003,140:445-460.

79. Nabel G, Baltimore D: An inducible transcription factor activates expression of human immunodeficiency virus in T cells. Nature 1987,326:711-713.

80. Nguyen DG, Hildreth JE. Involvement of macrophage mannose receptor in the binding and transmission of HIV by macrophages. Eur J Immunol 2003,33:483-493.

81. Nottet HS, Gendelman HE: Unraveling the neuroimmune mechanisms for the HIV-1-associated cognitive/motor complex. Immunol Today 1995,16:441-448.

82. O'Hara BM, Olson WC: HIV entry inhibitors in clinical development. Curr Opin Pharmacol 2002,2:523–528.

83. Olinski R, Gackowski D, Foksinski M, Rozalski R, Roszkowski K, Jaruga P: Oxidative DNA damage: assessment of the role in carcinogenesis, atherosclerosis, and acquired immunodeficiency syndrome. Free Radic Biol Med 2002,33:192-200.

84. Palamara AT, Perno CF, Aquaro S, Buè MC, Dini L, Garaci E: Glutathione inhibits HIV replication by acting at late stages of the virus life cycle. AIDS Res Hum Retrovir 1996,12:1537-1541.

85. Palella FJ Jr, Delaney KM, Moorman AC, Loveless MO, Fuhrer J, Satten GA, Aschman DJ, Holmberg SD: Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. HIV Outpatient Study Investigators. N Engl J Med 1998,338:853-60.

86. Parolin C, Gatto B, Del Vecchio C, Pecere T, Tramontano E, Cecchetti V, Fravolini A, Masiero S, Palumbo M, Palù G: New anti-Human Immunodeficiency Virus Type 1 6-aminoquinolones: mechanism of action. Antimicrob Agents Chemother 2003,47:889-896.

87. Pauwels R, Balzarini J, Baba M, Snoeck R, Schols D, Herdewijn P, Desmyter J, De Clercq E: Rapid and automated tetrazolium-based colorimetric assay for the detection of anti-HIV compounds. J Virol Methods 1988,20:309-321.

88. Perelson AS, Essunger P, Cao Y, Vesanten M, Hurley A, Saksela K, Marokovitz M, Ho D: Decay characteristics of HIV-1 infected compartments during combination therapy. Nature 1997,387:188-191.

89. Perno CF, Bergamini A, Pesce CD, Milanese G, Capozzi M, Aquaro S, Thaisrivongs S, Tarpley WG, Zon G, D'agostini C, Rocchi G, Garaci E, Caliò R: Inhibition of the protease of human immunodeficiency virus blocks replication and infectivity of the virus in chronically infected macrophages. J Infect Dis 1993,168:1148-1156.

90. Perno CF, Aquaro S, Rosenwirth B, Balestra E, Peichl P, Billich A, Villani N, Caliò R: In vitro activity of inhibitors of late stages of the replication of HIV in chronically infected macrophages. J Leukoc Biol 1994,56:381-386.

91. Perno CF, Newcomb FM, Davis DA, Aquaro S, Humphrey RW, Caliò R, Yarchoan R: Relative potency of protease inhibitors in monocytes/macrophages acutely and chronically infected with human immunodeficiency virus. J Infect Dis 1998,178:413-422.

92. Polianova MT, Ruscetti FW, Pert CB, Ruff MR: Chemokine receptor-5 (CCR5) is a receptor for the HIV entry inhibitor peptide T (DAPTA). Antiviral Res 2005,67:83-92.

93. Popovic M, Sarnagadharan MG, Read E, Gallo RC: Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. Science 1984,224:497-500.

94. Princen K, Hatse S, Vermeire K, Bridger GJ, Skerlj RT, De Clercq E, Schols D: The antiviral activity of the CXCR4 antagonist AMD3100 is independent of the cytokine-induced CXCR4/HIV coreceptor expression level. AIDS Res Hum Retroviruses 2003,19:1135-1139.

95. Price RW: The cellular basis of central nervous system HIV-1 innfectio and AIDS dementia complex. J Neuro-AIDS 1996,1:1-20.

96. Reading PC, Miller JL, Anders EM. Involvement of the mannose receptor in infection of macrophages by influenza virus. J Virol 2000;74: 5190–5197.

97. Richter S, Gatto B, Tabarrini O, Fravolini A Palombo M: Antiviral 6-amino-quinolones: molecular basis for potency and selectivity. Bioorg Med Chem Lett 2005,15:4247-4251.

98. Richter S, Parolin C, Gatto B, Del Vecchio C, Brocca-Cofano E, Fravolini A, Palù G, Palombo M: Inhibition of Human Immunodeficiency Virus Type 1 Tat-*trans*-activation-responsive region interaction by an antiviral quinolone derivative. Antimicrob. Agents Chemother 2004,48:1895-1899.

99. Richter S, Parolin C, M Palumbo, Palù G: Antiviral properties of quinolone-based drugs. Curr Drug Targets Infect Disord 2004,4:111-116.

100. Rizza P, Santini SM, Logozzi MA, Lapenta C, Sestili P, Gherardi G, Lande R, Spada M, Parlato S, Belardelli F, Fais S. T-cell dysfunctions in hu-PBL-SCID mice infected with human immunodeficiency virus (HIV) shortly after reconstitution: in vivo effects of HIV on highly activated human immune cells. J Virol. 1996,70:7958-7964.

101. Ruff MR, Melendez-Guerrero LM, Yang Q, Ho WZ, Mikovits JW, Pert CB, Ruscetti FA: DAPTA inhibits HIV-1 infection mediated by the chemokine receptor-5 (CCR5). Antiviral Res 2001,52:63-75.

102. Ruff MR, Polianova M, Yang Q, Leoung GS, Ruscetti FW, Pert CB: Update on D-Ala-DAPTA-Amide (DAPTA):A viral entry inhibitor that blocks CCR5 chemokine receptors. Curr HIV Res 2003,1:51-67.

103. Schols D: HIV co-receptors as targets for antiviral therapy. Curr Top Med Chem 2004,4:883-893.

104. Schwarz MK, Wells TNC: New therapeutics that modulate chemokine networks. Nat Rev Drug Discov 2002,1:347–358.

105. Sharkey ME, Teo I, Greenough T, Sharova N, Luzuriaga K, Sullivan JL, Bucy RP, Kostrikis LG, Haase A, Veryard C, Davaro RE, Cheeseman SH, Daly JS, Bova C, Ellison RT, Mady B, Lai KK, Moyle G, Nelson M, Gazzard B, Shaunak S, Stevenson M: Persistence of episomal HIV-1 infection intermediates in patients on highly active anti-retroviral therapy. Nat Med 2000,6:76-81.

106. Shi B, De Girolami U, He J, Wang S, Lorenzo A, Busciglio J, Gabuzda D: Apoptosis induced by HIV-1 infection of the central nervous system. Journal of Clinical Investigation 1996,98:1979-1990.

107. Sonza S, Multimer HP, Oelrichs R, Jardine D, Harvey K, Dunne A, Purcell FD, Birch C, Crowe MS: Monocytes harbour replication-competent, non latent HIV-1 in patients on highly active antiretroviral therapy. AIDS 2001,15:17-22.

108. Speth C, Joebstl B, Barcova M, Dierich MP: HIV-1 envelope protein gp41 modulates expression of interleukin-10 and chemokine receptors on monocytes, astrocytes and neurones. AIDS 2000,14:629-636.

109. Spudich SS, Huang W, Nilsson AC, Petropoulos CJ, Liegler TJ, Whitcomb JM, Price RW HIV-1 Chemokine Coreceptor Utilization in Paired Cerebrospinal Fluid and Plasma Samples: A Survey of Subjects with Viremia. J Infect Dis 2005,191:890-898.

110. Stahl PD, Ezekowitz RA. The mannose receptor is a pattern recognition receptor involved in host defense. Curr Opin Immunol 1998;10: 50–55.

111. Stevens M, Balzarini J, Tabarrini O, Andrei G, Snoeck R, Cecchetti V, Fravolini A, E De Clercq, Pannecouque C: Cell-dependent interference of a series of new 6-aminoquinolone derivatives with viral (HIV/CMV) transactivation. J Antimicrob Chemother 2005,56:847-855.

112. Stevens M, E De Clercq, Balzarini J: The regulation of HIV-1 transcription: molecular targets for chemotherapeutic intervention. Med Res Rev 2006,26:595-625.

113. Swingler S, Mann A, Jacque J, Brichacek B, Sasseville VG, Williams K, Lackner AA, Janoff EN, Wang R, Fisher D, Stevenson M: HIV-1 Nef mediates lymphocyte chemotaxis and activation by infected macrophages. Nat Med 1999,5:997-1003.

114. Tabarrini O, Stevens M, Cecchetti V, Sabatini S, Dell'Uomo M, Manfroni G, Palumbo M, Pannecouque C, De Clercq E, Fravolini A: Structure modifications of 6-aminoquinolones with potent anti-HIV activity. J Med Chem 2004,47:5567-5578.

115. Tan MC, Mommaas AM, Drijfhout JW, Jordens R, Onderwater JJ, Verwoerd D, et al. Mannose receptor mediated uptake of antigens strongly enhances HLA-class II restricted antigen presentation by cultured dendritic cells. Adv Exp Med Biol 1997;417: 171–174.

116. Tyor WR, Power C, Gendelman HE, Markham RB: A model of human immunodeficiency virus encephalitis in scid mice. Proc Natl Acad Sci USA 1993,90:8658-8662.

117. Tomkowicz B, Lee C, Ravyn V, Cheung R, Ptasznik A, Collman R: The Src kinase Lyn is required for CCR5 signaling in response to MIP-1{beta} and R5 HIV-1 gp120 in human macrophages. Blood 2006,107:4234-4239.

118. Trillo-Pazos G, McFarlane-Abdulla E, Campbell IC, Pilkington GJ, Everall IP: Recombinant nef HIV-IIIB protein is toxic to human neurons in culture. Brain Res 2000,864:315-326.

119. Trkola A, Ketas TJ, Nagashima KA, Zaho L, Cilliers T, Morris L, Moore JP, Maddon PJ, Olson WC: Potent broad-spectrum inhibition of human immunodeficiency virus type 1 by the CCR5 monoclonal antibody PRO140. J Virol 2001,75:579-588.

120. Tschachler E, Groh V, Popovic M, Mann DL, Konrad K, Safai B, Eron L, Di Marzo Veronese F, Wolff K, Stingl G: Epidermal Langerhans cells a target for HTLV-III/LAV infection. J Invest Dermatol 1987,88:233-237.

121. Tuttle DL, Harrison JK, Anders C, Sleasman JW, Goodenow MM: Expression of CCR5 increases during monocyte differentiation and directly mediates macrophage susceptibility to infection by human immunodeficiency virus type 1. J Virol 1998,72:4962-4969.

122. Van Rij RP, Hallaby PT, Lange JM, Visser J, Husman AM, Van't Wout AB, Schuitemaker H: Reduced prevalence of the CCR5 32 heterozygous genotype in human immunodeficiency virus-infected individuals with AIDS dementia complex. J Infect Dis 1999,180:854-857.

123. Verani A, Gras G, Pancino G. (2005) Macrophages and HIV-1: dangerous liaisons. Mol Immunol.;42:195-212.

124. Weisiger & Fridovich: Superoxide dismutase. Organelle specificity. J Biol Chem 1973,248:3582-3592.

125. Westendorp MO, Frank R, Ochsenbauer C, Stricker K, Dhein J, Walczak H, Debatin KM, Krammer PH: Sensitization of T cells to CD95-mediated apoptosis by HIV-1 Tat and gp120. Nature 1995,375:497-500.

126. Williams KC, Hickey WF: Central nervous system damage, monocytes and macrophages, and neurological disorders in AIDS. Annu Rev Neurosi 2002,25:537-562.

127. Wong JK, Hezareh M, Günthard HF, Havlir DV, Ignacio CC, Spina CA, Richman DD: Recovery of replication-competent HIV despite prolonged suppression of plasma viremia. Science 1997,278:1291-1295.

128. Yeung MC, Geertsma F, Liu J, Lau AS: Inhibition of HIV-1 gp120-induced apoptosis in neuroblastoma SK-N-SH cells by an antisense oligodeoxynucleotide against p53. AIDS 1998,12:349-354.

129. Yi Y, Lee C, Liu QH, Freedman BD, Collman RG: Chemokine receptor utilization and macrophage signaling by human immunodeficiency virus type 1 gp120: Implications for neuropathogenesis. J Neurovirol 2004,10:91-96.

130. Yi Y, Chen W, Frank I, Cutilli J, Singh A, Starr-Spires L, Sulcove J, Kolson DL, Collman RG: An unusual syncytia-inducing human immunodeficiency virus type 1 primary isolate from the central nervous system that is restricted to CXCR4, replicates efficiently in macrophages, and induces neuronal apoptosis. J Neurovirol 2003,9:432-441.

131. Yi Y, Isaacs SN, Williams DA, Frank I, Schols D, De Clercq E, Kolson DL, Collman RG. (1999) Role of CXCR4 in cell-cell fusion and infection of monocyte-derived macrophages by primary human immunodeficiency virus type 1 (HIV-1) strains: two distinct mechanisms of HIV-1 dual tropism. J Virol. 73:7117-25.

List of abbreviations

HIV-1, Human Immunodeficiency Virus type 1

HAART, highly active antiretroviral therapy

M/M, monocyte/macrophages

PBMC, peripheral blood mononuclear cells

DC, dendritic cells

CNS, central nervous system

BBB, blood brain barrier

DAPTA, D-Ala-Peptide T-amide

hu-PBL-SCID mice, human peripheral blood lymphocytes

TCID50, Tissue Culture Infective Dose 50

R5, CCR5

X4, CXCR4

CBA, carbohydrate-binding agents

PRM-A, pradimicin A

GlcNAc, N-acetylglucosamine

UDA, Urtica dioica

NPA, Narcissus pseudonarcissus

HHA, Hippeastrum hybrid agglutinin

CV-N, cyanovirin

MMR, mannose-binding receptor

AZT, zidovudine

6-DFQs, 6-desfluoroquinolone derivatives

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