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Inhibition of HIV-1 replication by newly developed adamantane-containing polyanionic agents

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Abstract

Newly developed antiviral compounds consisting of an adamantane derivative chemically linked to a water-soluble polyanionic matrix were shown to inhibit HIV-1 infection in lymphoblastoid cells, HeLa CD4+ β-galactosidase (MAGI) cells and macrophages. The effect of the compounds was recorded by measuring viral reverse transcriptase activity and p24 by ELISA in culture supernatant and by immunoblotting of cell lysates. In this paper we describe the data obtained with one of the most promising compounds, Amant. Amant was not toxic for the host cells at concentrations as high as 1 mg/ml. The inhibition of HIV-1 replication in MT-4 and MAGI cells was observed when Amant was added either before infection or with the virus (0 h of infection), and was expressed even when the compound added at 0 h was removed 1.5 h after infection. Its inhibitory concentration (IC_{50}) against HIV-1 and HIV-2 replication was 2–6 and 93 μg/ml, respectively. The anti-HIV-1 effect of the compound was gradually decreased when it was added 1 and 2 h post infection, and no inhibition was observed when the compound was added 4 h after infection, suggesting that the compound as a membranotropic drug blocks an early step of replication. It completely prevented the transport of Gag proteins into the nuclei. Pretreatment of the virus with Amant did not reduce its infectious activity. The classical adamantane derivatives amantadine and rimantadine hydrochloride did not inhibit HIV replication.

Keywords: Adamantane derivatives; Amant; Anti-HIV-1; Polyanionic matrix

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1. Introduction

The targets for antiviral therapy of HIV infection that can be blocked selectively by inhibitors are usually viral enzymes such as reverse transcriptase (RT) and protease. Viral and cellular membranes are actively involved in the replicative cycle, including such crucial steps as cell entry, transport of viral components, formation of mature virus progeny and budding. These events are excellent targets for specific inhibitors possessing membranotropic properties. To this end, we have created a new group of membranotropic agents, namely adamantane-containing polycarboxylic compounds, that are highly active against HIV-1. The newly developed compounds consist of three components: (1) the adamantane derivative; (2) a nontoxic anionogenic water-soluble polycarboxylic matrix; and (3) intermediate spacer groups of different length, structure and conformation (Fig. 1).

The classical adamantane derivatives amantadine and rimantadine hydrochloride are well-known inhibitors of influenza virus infection (Lubeck et al., 1978; Oxford and Galbraith, 1980); they block hydrophobic sites of viral protein M2 in the ionic channels, thus preventing viral uncoating (Hay et al., 1985; Sugrue et al., 1990; Helenius, 1992; Bukrinskaya et al., 1982a,b). However, the non-polar hydrocarbon nature of adamantane which interacts only with hydrophobic sites limits its antiviral specificity and may facilitate the appearance of resistant variants of the virus (Kiselev et al., 1993). We have introduced an additional component into the molecule, namely the polycarboxylic matrix. This component was used to enhance membranotropic affinity and amplify the target effect of the compound, since multiple anionogenic groups (COOH) are able to interact with a wide spectrum of hydrophilic sites. Spacer groups connecting the adamantane derivative with the polycarboxylic matrix were introduced as well.

In this paper we describe the antiviral effect of one of the compounds, Amant, on HIV-1 replication in MT-4 cells, MAGI (multinucleate activation of galactosidase indicator) cells and macrophages. Our data show that Amant is a low-toxic, selective inhibitor of HIV-1 infection.

2. Materials and methods

2.1. Compounds

The compounds contained approximately 12–35 wt.% of adamantane rings. The polyanionic matrix had 40–200 carboxylic groups with a molecular mass of 3–15 kDa, which is significantly lower than the clearance barrier of human kidneys (50–60 kDa). Spacers contained one to three groups of $-CH_2$ (see Fig. 1). Amant, one of the most promising antiviral compounds with a molecular mass of about 5 kDa, contained 60 carboxylic groups, 12 wt.% of adamantane and two dimethylenic bridges as spacer.

2.2. Cells and virus

The antiviral effect of Amant was determined in acutely infected MT-4 cells, MAGI cells and macrophages and in chronically infected Jurkat T cells. MT-4 cells and Jurkat T cells were grown in RPMI-1640, and MAGI cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum at 37°C in a CO_2 atmosphere. Human monocytes were isolated from the blood of healthy donors by adhesion to a plastic base and allowed to differentiate for 7 days. This standard procedure gives a 98% pure cell population, as determined by non-specific esterase activity (Bukrinsky et al., 1996).

HIV-1 virus, strains H9/IIIB (Muesing et al., 1985) and MFA (Stevenson et al., 1990), and HIV-2 (strain ROD) were used for infection at a

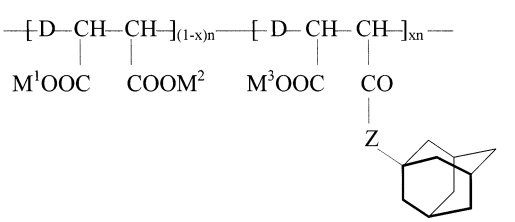

Fig. 1. Structure of adamantane-containing polymeric compounds. D, oxygen-containing monomer (divinyl ether, furan, 1,4-di-isopropoxybutene-2, etc.); M_1, M_2, M_3 = H and/or Na, K, NH_4 etc.; $n = 15–90$ (degree of polymerization); $x = 0.05–0.90$ (degree of modification); Z, spacer group containing $(-CH_2-)_m$, $m > 0$.

multiplicity of infection of about 0.1–0.5 syncytium-forming unit per cell. Human monocytes were inoculated with the ADA strain of the virus (2×10^5 cpm of RT activity per 10^6 cells). The time of virus addition was taken as time 0.

The cells were either pretreated with the compound for 2 h and the compound was present during infection, or the compound was added with the virus or at some intervals after infection. Culture supernatants were tested for virus production by ELISA using antibodies to HIV-1 Gag p24 antigen and by measuring RT activity of the virus. Cell lysates were obtained by the treatment of the cells with 1% Triton X-100 48 h post infection and tested for virus-specific proteins by immunoblotting.

2.3. Virus labeling with [^{3}H]myristic acid

HIV-1 was labeled for 24 h with [^{3}H]myristic acid (2–5 μCi/ml) as described by Bukrinskaya et al. (1996). The virus was purified by pelleting through 20% sucrose in phosphate-buffered saline (PBS).

2.4. Subcellular fractionation

Subcellular fractions of HIV-1-infected MT-4 were prepared as described (Bukrinskaya et al., 1996). Briefly, the cells were incubated in hypotonic buffer for 15 min, then homogenized in a Dounce homogenizer by 20 strokes. The nuclei were pelleted at $800 \times g$ for 3 min and purified by treatment with NTENT buffer [150 mM NaCl, 10 mM Tris–HCl pH 7.2, 1 mM EDTA, 3 μM/ml aprotinin, 0.1 mM phenylmethylsulphonyl fluoride (PMSF), 1% Triton X-100]. The supernatant was centrifuged at 14 000 rpm for 30 min to pellet membranes and cytoskeleton. The pellet was treated with NTENT to dissolve membranes and centrifuged again at 14 000 rpm for 30 min. The supernatant was designated the membrane fraction. The proteins from the membrane fraction were precipitated by ethanol. The membrane and nuclear fractions were analysed by immunoblotting for the presence of viral proteins.

2.5. Immunoblotting

The proteins were resolved on 15% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) gels, transferred to Highbond-C extra (Amersham) and analysed by Western blotting using seropositive sera from AIDS patients. Bound antibodies were visualized with peroxidase anti-Ig followed by enhanced chemiluminescence (Amersham).

2.6. Cytotoxicity of the compound

Non-infected MT-4 cells were treated with different concentrations of the compound (25–1600 μg/ml) for 24 h. The cytotoxic effect of the compound was tested either by determination of the viable cells after staining of the cells with 0.4% Trypan blue solution (CC_{50}) or by counting [^{3}H]thymidine incorporation into the acid-insoluble fraction. In that case [^{3}H]thymidine (4 μCi per 10^5 cells) was added for 1 h, then the cells were washed with PBS, lysed by 1% Triton X-100, and the radioactivity in the acid-insoluble fraction was counted.

2.7. Infectivity assay

Hela-CD4-LTR/β-gal MAGI cells, which are HeLa cells that express CD4 and contain an integrated copy of a β-galactosidase gene under the control of LTR (Kimpton and Emerman, 1992), were plated in 96-well microtiter plates (2×10^4 cells/well). At 48 h post-infection, cells were harvested for quantitation of β-galactosidase production after hydrolysis of X-gal.

3. Results

Amant at concentrations up to 1000 μg/ml did not reduce the amount of ^{3}H-radioactivity incorporated after exposure of the cells to [^{3}H]thymidine (not shown). Counting the viable cells in the samples stained with Trypan blue showed that cytotoxicity of Amant was several-fold lower than that of amantadine and rimantadine hydrochloride ($CC_{50} = 1200$, 225 and 160

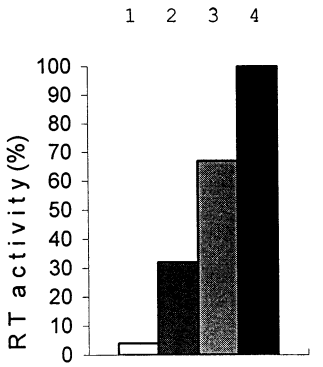

Fig. 2. Effect of Amant on HIV-1 replication in MT-4 cells measured by RT in supernatant fluid of MT-4 cells. Amant (10 μg/ml) was added simultaneously with the virus, 1 and 2 h after infection (columns 1, 2 and 3, respectively). Column 4, control untreated cells.

μg/ml, respectively). Thus, the design of the new adamantane-containing polyanionogenic compound sharply decreased its cytotoxic properties.

The antiviral activity of Amant against HIV-1 (strains H9/IIIB and MFA) was measured by cell viability, by syncytium formation and also by RT activity in the supernatant of infected MT-4 cells 24–48 h after infection. The IC_{50} was 2–6 μg/ml when the compound was added 2 h before infection or at 0 time of infection. The selectivity index (SI) was 200–600. The compound demonstrated very weak inhibition against HIV-2 (ROD strain) ($IC_{50} = 93$ μg/ml, $SI = 13$). The compound at a concentration 10 μg/ml strongly inhibited virus production (determined by RT activity) when added together with the virus (0 time). The effect was gradually decreased when the compound was added 1 and 2 h after infection (Fig. 2).

The same results were obtained when viral proteins were analysed by immunoblotting. Gag polyprotein p55, intermediate precursors p41 and p39 (Mervis et al., 1988) and the final product of protein cleavage, the mature core protein p24, could be readily visualized in the untreated cell controls. The other products of p55 cleavage, p17 and p7/p6, were not detected under these conditions of electrophoresis. It is seen in Fig. 3A that Amant strongly inhibited synthesis of virus-specific proteins when added 2 h before infection or simultaneously with the virus (0 h), but an antiviral effect was not observed when the compound was added at 2 h post-infection or later. When Amant was added at 0 time, and at 1.5 h the medium was removed and fresh medium without Amant was added, the inhibition of HIV-1 replication was still expressed (Fig. 3B).

The HIV-1 inhibitory effect of Amant could be ascribed to the whole molecular structure and not solely to the polycarboxylic matrix: concentrations of the matrix equivalent to those of Amant, when added 2 h before infection, did not inhibit HIV-1 replication, unless the concentation was higher than 100 μg/ml (Fig. 3C).

The efficacy of Amant was also compared to that of the adamantane derivatives amantadine and rimantadine hydrochloride. As seen in Fig. 3D, amantadine at a concentration up to 100 μg/ml did not produce any effect on viral replication even when the cells were pretreated with the drug and when it remained present during infection. The same results were obtained with rimantadine (data not shown).

The effect of Amant on HIV-1 replication was determined in chronically infected Jurkat T cells. The production of the viral proteins was measured by ELISA in cell lysates and in culture supernatants 72 h after the treatment of the cells

Fig. 3. Virus-induced proteins in MT-4 cells analysed by immunoblotting. (A) Amant was added 1 h before infection (lane 2), simultaneously with the virus (lane 3) and 1, 2, 3 and 4 h (lanes 4–7 respectively) post-infection. Lane 1, control infected cells. Virus-induced proteins were detected in cell lysates 48 h post-infection using seropositive serum from AIDS patients. (B) Amant was added simultaneously with the virus and removed 1.5 h post-infection (lane 1) or was not removed (lane 2). Lane 3, control untreated cells. The viral proteins were revealed in cell lysates by immunoblotting as described in Fig. 2. (C) Polycarboxylic matrix was added at a concentration of 100 or 200 μg/ml (lanes 2 and 3, respectively) 2 h before infection and was present during infection. Lane 1, control infected cells. (D) Amantadine was added at a concentration of 2, 5, 10, 30 or 100 μg/ml (lanes 2–6, respectively) 2 h before infection and was present during infection. Lane 1, control infected cells. (a) The proteins in cell lysates were detected by immunoblotting 48 h after infection, as described in Fig. 2A. (b) Results of immunoblotting expressed quantitatively. Grey columns, p24; black columns, p55.

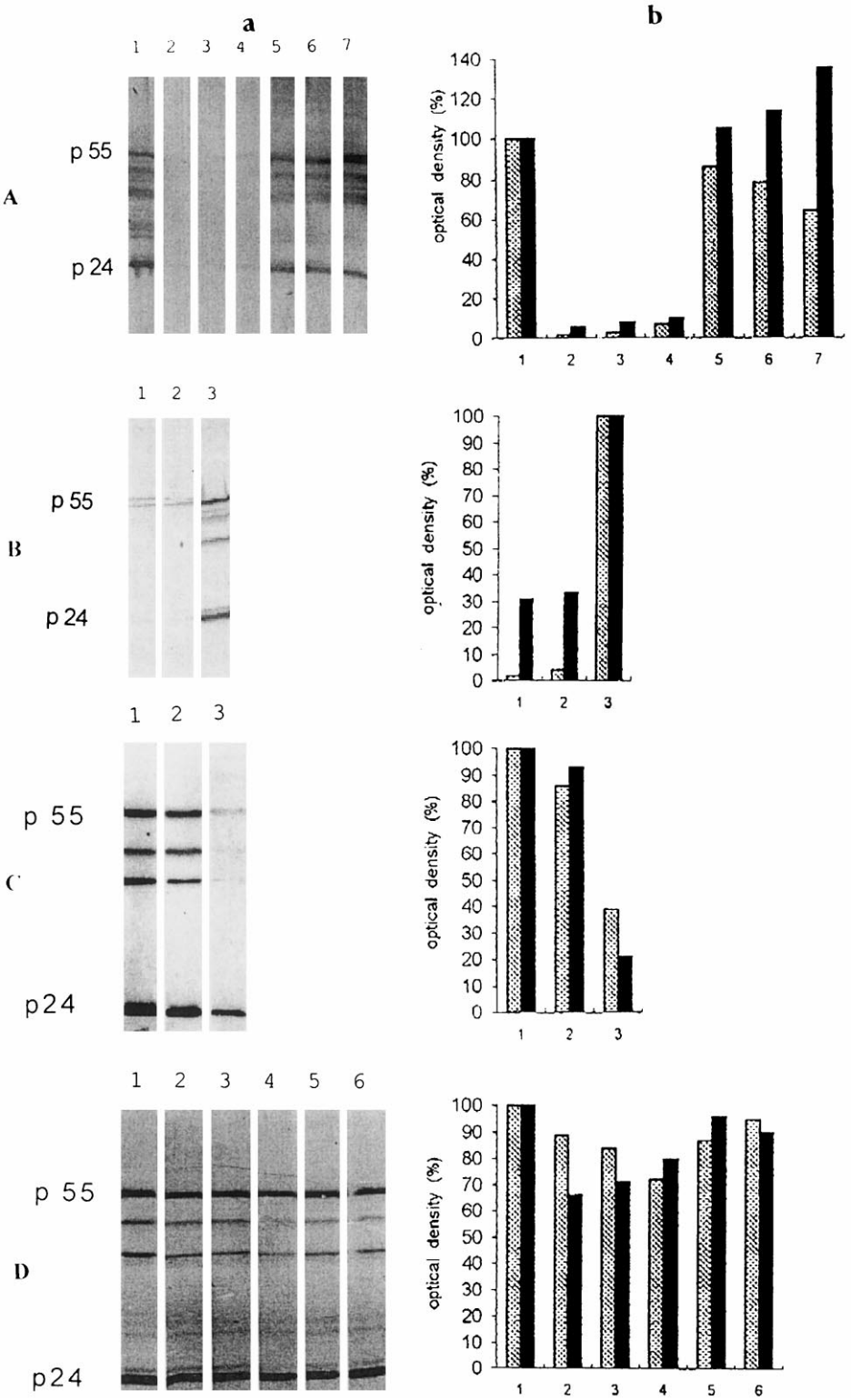
a
b
A
B
C
D
p 55
p 24
1 2 3 4 5 6 7
1 2 3
1 2 3 4 5 6
optical density (%)
0 10 20 30 40 50 60 70 80 90 100 120 140

Fig. 3.

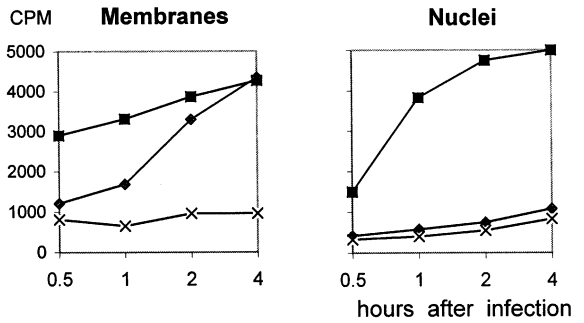

Fig. 4. Effect of Amant and DS on virus adsorption and nuclear transport. Amant (10 (μg/ml; grey squares) and DS (20 μg/ml; ×) were added to MT-4 cells with the virus which was prelabeled with [^{3}H]myristic acid. The samples of the cells were removed at intervals, fractionated and ^{3}H-radioactivity was counted in cell fractions. ■, untreated cells control.

with the compound. Amant did not inhibit HIV-1 replication in Jurkat T cells, nor did it prove effective in MAGI cells even at a concentration of 50–100 μg/ml (not shown). This is in accord with an action targeted at an early step of the acute viral infection.

To examine if Amant interacts with virions themselves, the virions were pretreated with the compound for 1 h at 37°C; then the virus particles were pelleted and resuspended virus was analysed for its ability to adsorb to the cells in the absence of the compound. There was no difference in adsorption on the cells of untreated and Amant-pretreated virions. Similarly, no difference was revealed in the synthesis of viral proteins 48 h after infection induced by pretreated and untreated viruses (not shown). Thus, the virions pretreated by Amant did not lose the ability to infect MT-4 cells. This suggests that the compound does not modify the virions.

We tried to determine which step of HIV-1 replication was blocked by the compound. The effect of Amant was compared to that of dextran sulphate (DS) (Baba et al., 1988; Mitsuya et al. 1988). DS is a polyanion (polysulphate) that is similar to the polyanionic matrix (polycarboxylate) of Amant. The virus prelabelled with [^{3}H]myristic acid was used in that experiment. As shown earlier, matrix protein p17 and Gag precursor p55, both possessing myristic acid at their N-terminal, were labelled with the isotope and found in cell nuclei soon after infection (Bukrinskaya et al., 1996). In the experiment shown in Fig. 4, Amant and DS were added to the cells together with the labelled virus and the samples of the cells were taken at intervals, fractionated as described in Section 2 and ^{3}H-radioactivity was determined in membrane and nuclear fractions. As seen in Fig. 4, DS almost completely blocked virus adsorption on cell membranes. Amant decreased adsorption of the virus during the first hour up to 50%, but later on the amount of adsorbed virus reached the control level. Even higher concentrations of Amant such as 50 μg/ml did not block adsorption by more than 50% (not shown). Both drugs prevented the transport of viral proteins into the nuclei. It follows that, while the antiviral effect of DS was related to the inhibition of virus adsorption, Amant only slowed down adsorption during the first hour, but fully prevented the transport of viral proteins to the nuclei by blocking some post-adsorption events.

It has been shown previously that early events of HIV-1 replication are strongly dependent upon filamentous actin, and depolymerization of actin network by cytochalasin D impairs virus entry and transport of preintegration complex to the nucleus (Bukrinskaya et al., 1998). We compared the effect of Amant to that of cytochalasin D using a single cycle MAGI infectivity assay (Kimpton and Emerman, 1992). Cytochalasin D markedly impaired viral infectivity when added 2 h prior to virus infection and was present during infection (Fig. 5), but the inhibitory effect was sharply decreased when the drug was added with the virus (0 time of infection) in accordance with the data that a time period of about 1 h is required for depolymerization of the actin network (Brown and Spudich, 1981; Bukrinskaya et al., 1998).

Like cytochalasin D, Amant was also ineffective when added 2 h after infection (Fig. 2A,B). However, unlike cytochalasin D, Amant similarly inhibited viral infectivity when added 2 h before infection and at 0 time. In this respect the effect of Amant differed from that of cytochalasin D, suggesting that Amant as a membranotropic compound impairs another (early) step of the HIV-1 life-cycle.

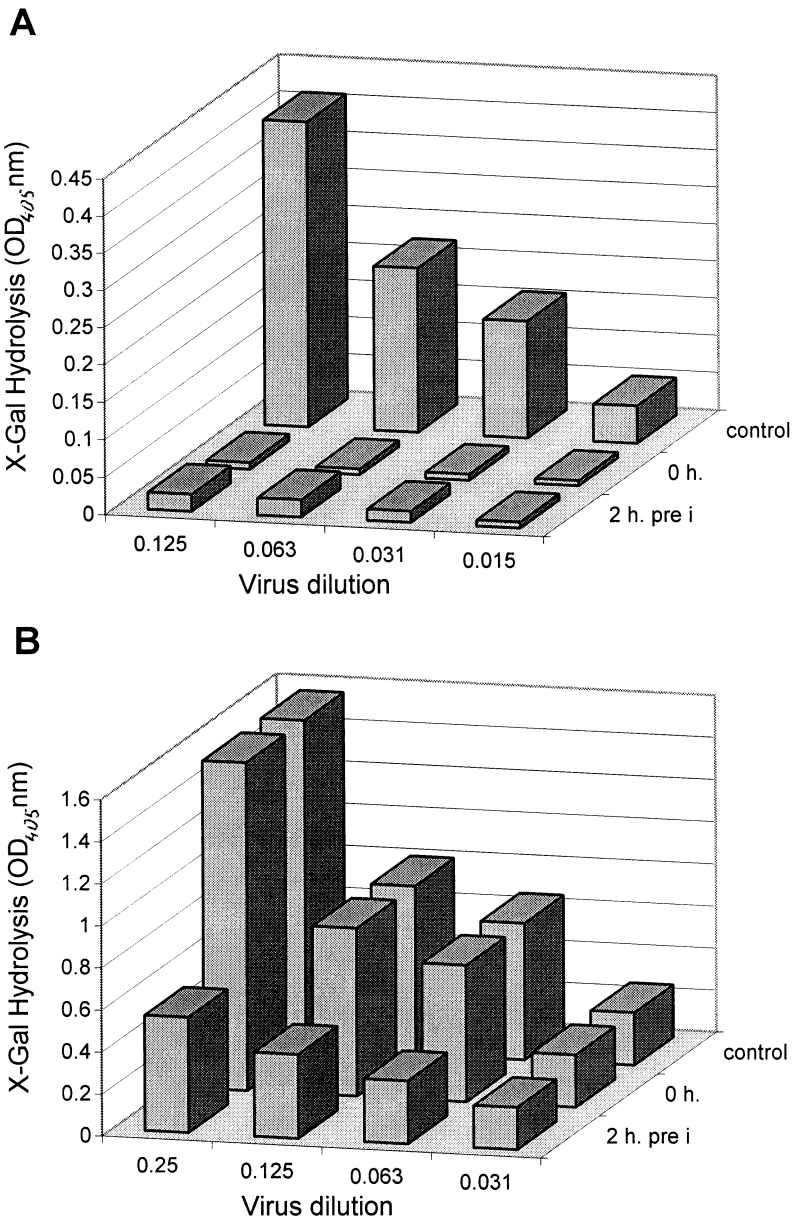

Fig. 5. Effect of Amant (A) and cytochalasin D (B) on HIV-1 infectivity determined by the single cycle MAGI infectivity assay. Both compounds were added at a concentration of 2 μM 2 h before infection or at 0 time. The effect was determined at 48 h after infection.

DNA synthesis in infected cells tested by PCR is shown in Fig. 6. It is seen that early and late DNA synthesis was not detected in the cells treated with the compound at a concentration of 10 μg/ml and higher. No circular forms of DNA which are revealed only in the nuclei (Bukrinsky et al., 1993) were observed, confirming that the preintegration complex did not reach the nuclei in the presence of the compound.

It was important to establish whether Amant is able to inhibit HIV-1 replication in macrophages which are known to be crucial cells for HIV infection. As seen in Fig. 7A, a significant antiviral effect was observed at 12–21 days after infection of macrophages (viral replication monitored by RT activity). Amant inhibited HIV-1 replication in macrophages at a higher concentration than in T cells: the IC_{50} in macrophages was about 20 μg/ml, or approximately 10 times higher than in T cells. Fig. 7B shows that Amant, unlike amantadine, did not produce any cytotoxic effect in macrophages at a concentration up to 80 μg/ml.

4. Discussion

Our results show that Amant represents a virtually non-toxic ($CC_{50} = 1200$ μg/ml) and effective anti-HIV-1 agent ($IC_{50} = 2–6$ μg/ml). Strong inhibition of viral reproduction was observed when the compound was added to MT-4 cells before infection or simultaneously with the virus. Its SI

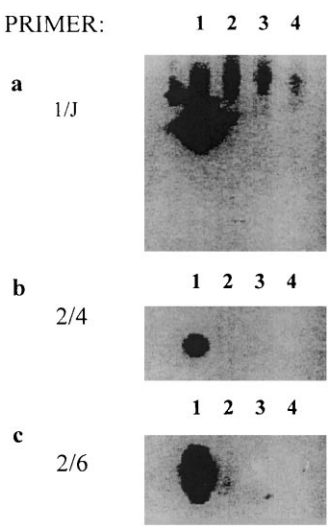

Fig. 6. Effect of Amant on HIV-1 DNA synthesis in infected cells as revealed by polymerase chain reaction (PCR). PCR was done as described by Bukrinskaya et al. (1996). a–c, PCR with the primers 1/J (primer revealing early DNA synthesis), 2/4 (primer revealing late DNA synthesis) and 2/6 (primer revealing circular DNA in nuclei), respectively. Lanes: 1, control; 2, 3 and 4, 10, 25 and 50 μg/ml Amant, respectively.

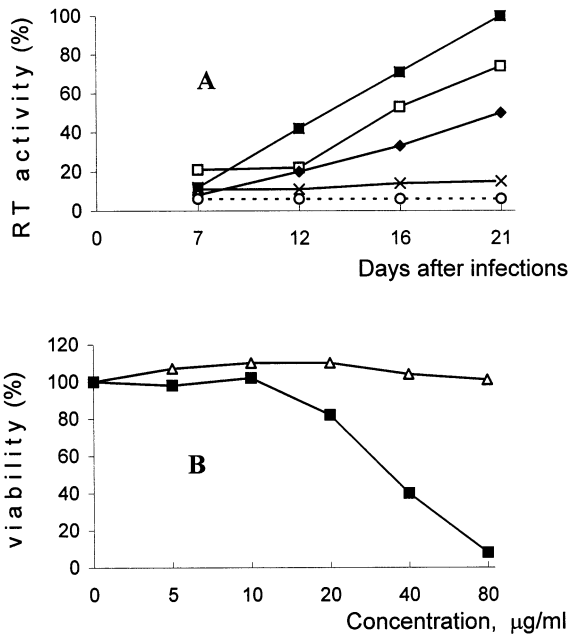

Fig. 7. Effect of Amant on HIV-1 replication in macrophages (A) and viability of the cells (B). (A) Amant at concentrations of 5 (open squares) 20 (grey squares) and 80 (×) μg/ml was added to the cells at time 0. RT activity was measured during 21 days of incubation. ■, infected not treated cells; ○, uninfected cells. (B) Amant (△) and amantadine (■) at different concentrations (5, 10, 20, 40 or 80 μg/ml) were added to the infected cells at time 0. The viability of the cells was measured by counting viable cells 21 days after infection.

was 200–600. Only slight inhibition of HIV-2 was observed at a high concentrations of Amant ($IC_{50} = 93$ μg/ml), suggesting that the compound affords rather a selective protection against HIV-1. This phenomenon might be explained by the different surface structures of HIV-1 and HIV-2 involved in interaction with the cell membrane (receptors). However, since only one HIV-2 strain was tested, it is not excluded that the compound could be more active against other HIV-2 strains.

Amant contains an adamantane group, which, however, is not sufficient for anti-HIV-1 activity since the classical adamantane derivatives amantadine and rimantadine hydrochloride are not effective against HIV infection. Other authors also showed that derivatives of adamantane are not active, or are only active at high concentrations (i.e. 115 and 158 μg/ml) against HIV-1, while at

4–6 μg/ml they inhibited influenza A replication (Kolocouris et al., 1994, 1996; Da Settimo et al., 1995).

Therefore, the rather potent anti-HIV-1 activity of Amant may be provided by the adamantane group coupled with the polyanionic component. The polycarboxylic matrix itself does not markedly inhibit HIV-1 replication but its anionogenic COOH groups could develop additional multi-point electrostatic interactions with hydrophilic sites at cellular membranes. Many polyanionic substances are known to inhibit adsorption and/or fusion of HIV-1 (Lederman et al., 1992; Jansen et al., 1993; Jagodzinski et al., 1994; Kuipers et al., 1996; Lorenz et al., 1997; Swart et al., 1997; for a review, see De Clercq, 1995a,b).

Amant does not directly interact with HIV-1 virions but impairs an early step of the viral life-cycle. It slows down virus adsorption during the first hour of infection. The delayed binding of the virus could be due to the some non-specific effect mediated by the charged nature of the compound. It is also possible that Amant induces some changes in the conformation of HIV-1 receptors (CD4 or/and CXCR4). However, the inhibition of virus replication may well result from the interaction with some post-adsorption step(s). Since Amant is effective only when added not later than 2 h after infection, these steps could represent very early events in virus life-cycle such as fusion of cellular and viral membranes, viral uncoating or import of Gag proteins into the nuclei.

The molecular target for Amant is of special interest. It has been shown for influenza type A virus that amantadine hydrochloride interacts with membrane-associated M2 viral protein inside ionic channels of the viral envelope and, as a consequence, virus uncoating is blocked (Bukrinskaya et al., 1982a,b; Hay et al., 1985; Helenius, 1992). Like amantadine, Amant effectively inhibits influenza type A virus, but it also inhibits influenza type B virus replication that is not sensitive to amantadine and rimantadine (Stotskaya et al., 1995), confirming that the conjunction of the adamantane derivatives with the polyanionic matrix essentially increases their antiviral activity spectrum. These adamantane–polyanionic matrix constructs deserve further attention as new membranotropic anti-HIV-1 agents.

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