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# Triterpene derivatives that block entry of human immunodeficiency virus type 1 into cells

**(betulinic acid derivatives/antiviral drug/envelope/syncytium formation/virus–cell fusion)**

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**ABSTRACT** **A series of triterpene compounds characterized by a stringent structure–activity relationship were identified as potent and selective inhibitors of human immunodeficiency virus type 1 (HIV-1) replication. Currently studied betulinic derivatives have 50% inhibitory concentrations ($IC_{50}$) against HIV-1 strain IIIB/LAI in the 10 nM range in several cellular infection assays but are inactive against HIV-2. These compounds did not significantly inhibit the *in vitro* activities of several purified HIV-1 enzymes. Rather, they appeared to block virus infection at a postbinding, envelope-dependent step involved in the fusion of the virus to the cell membrane.**

A survey of the agents that have been described to have activity against human immunodeficiency virus (HIV) reveals that only a limited number of the potential viral targets have been tackled, the most prominent being reverse transcriptase (RT) and protease (1–4). In particular, the designing and screening of agents that inhibit virus entry into cells have met with limited success. Despite high initial hopes, several candidate antagonists based on soluble CD4 have failed either *in vitro* or *in vivo* (5, 6). On the other hand, polyanionic compounds that interfere with virus binding or penetration, in particular sulfated polymers, are likely to be ineffective because their specificity and bioavailability are poor (7). Here we report on a class of hemisynthetic compounds derived from a natural triterpene which is targeted at HIV-1 entry into cells.

## MATERIALS AND METHODS

**Cell Lines and Virus.** Cell lines C8166 (ADP013), U-937 (ADP012), and CEM-4 (ADP006) were obtained from the Medical Research Council AIDS Directed Programme Reagent Project, National Institute for Biological Standards and Control, Hertfordshire, U.K. CEM-4 is a subclone enriched in CD4 antigen derived from the CEM T-lymphoblastoid tumor cell line. The H9 cell line (HTB176) was obtained from the American Type Culture Collection. The origins of the virus strains were as follows: LAI, ELI, and ROD were from L. Montagnier (Institut Pasteur, Paris); NDK (8); MN was from the Medical Research Council AIDS Reagent Project; IIIB and RF were from R. C. Gallo (National Institutes of Health); AP0595 and 2750 M were isolated from European patients. HIV-1 (LAI) batches used for screening were obtained from filtered (0.45 μm) supernatants of CEM clone 11 cells (Pasteur-Mérieux Sérums et Vaccins, Marnes-la-Coquette, France). Titers of these preparations were about $10^4$ $CCID_{50}$ (50% cell culture infective doses)/ml.

**Cellular Assays for HIV Inhibition.** CEM cells were usually grown at 37°C (5% $CO_2$) in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and Polybrene (Sigma, 2 μg/ml). In the routine CEM test, 25-μl samples of each compound dilution were distributed in triplicate in 96-well tissue culture microplates. Cells (125 μl; $5 \times 10^4$ per ml) were then added and suspensions were incubated for 1 hr at 37°C. Cells were infected with 100 μl of virus suspension (100–200 $CCID_{50}$) and cultured for 5 days. Cell viability was assessed by the spectrophotometric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-based assay (9). Mock-infected cultures were carried out in parallel to determine the degree of cytotoxicity of the compounds. p24 core antigen in culture supernatants was determined by ELISA (NEK060B kit from New England Nuclear). RT activity was measured by a poly(rA) $^3$H SPA scintillation proximity assay (NK9020 from Amersham). The antiviral assay using MT-4 cells was as described (10). The effect of compounds on the kinetics of viral production in human peripheral blood mononuclear cells (PBMCs) was also assayed. PBMCs from seronegative donors were isolated by Ficoll/Hypaque (Pharmacia) and stimulated for 3 days with phytohemagglutinin (2.5 μg/ml; Difco). Cells were then washed three times with medium, pelleted, and incubated with 50–100 $CCID_{50}$ of virus stock per ml. After 1 hr at room temperature, cells were resuspended in RPMI 1640 medium containing 10% fetal bovine serum, 10% T-cell growth factors (Lymphocult, Biotest Diagnostics, Danville, NJ), and anti-interferon α neutralizing antibody (Bayer, Wuppertal, F.R.G.) at 80 units/ml. Cells were plated at $10^6$ per ml in six-well culture plates (Costar) in the presence of the compound to be tested. At 3, 7, 10, and 14 days after infection, cultures were harvested and viable cells were examined by trypan blue exclusion. Aliquots of supernatants were frozen at −70°C until tested for p24 antigen, and cells were resuspended at the same density in complete fresh medium with the same concentration of the tested drug.

**Determination of Cytoplasmic Proviral DNA.** Shortly after infection, the proviral DNA obtained from a selective extraction was quantified by PCR amplification. H9 cells were preincubated in the presence or absence of the drug to be tested for 1 hr, infected with HIV-1 (LAI) at 500 $CCID_{50}$/ml for 1 hr, and grown in culture medium for another hour. Cells were then washed intensively, and cytoplasmic proviral DNA was extracted by a digitonin/proteinase K/RNase

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Abbreviations: AZT, 3′-azido-3′-deoxythymidine; HIV, human immunodeficiency virus; PBMC, peripheral blood mononuclear cell; sCD4, soluble CD4; RT, reverse transcriptase.

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protocol (11). A 152-bp fragment of the *gag* gene of HIV-1 and a 237-bp segment of the mitochondrial cytochrome *b* gene as a control were amplified by PCR. The absence of nuclear genomic DNA was checked on a 336-bp fragment of the β-globin gene. PCR products were visualized directly by fluorescence after electrophoresis in a 3% NuSieve agarose gel (FMC) and by autoradiography after transfer and hybridization with specific internal oligonucleotide probes. Amplification products were quantitated with a digital autoradiography detector (Packard), and the percentage of proviral DNA was estimated by taking the ratio of HIV-1 *gag* product to cytochrome *b* product and comparing it with that obtained from untreated control cells. 3′-Azido-3′-deoxythymidine (AZT) was used as a reference compound.

**Assays of Viral Enzymes.** In the exogenous RT assay (12), the reaction mixture (50 μl) contained 50 mM Tris·HCl (pH 8.4), 10 mM $MgCl_2$, 100 mM KCl, 2.2 mM dithiothreitol, 2.5 μM dGTP, and 0.05% (wt/vol) Triton X-100. The template poly(C) or poly(dC) and the primer $(dG)_{12-18}$ (Pharmacia) were used at concentrations of 40 and 6 μg/ml, respectively. Recombinant HIV-1 RT was used at 1 nM. HIV-1 integrase assays were carried out essentially as described (13). In brief, reaction mixtures containing 20 mM Hepes (pH 7.0), 10 mM $MnCl_2$, 50 mM NaCl, 10% (vol/vol) glycerol, 10 mM dithiothreitol, bovine serum albumin at 0.1 mg/ml, 0.5 pmol of $^{32}$P-labeled HIV-1 U5-end oligonucleotide substrate, and 5 pmol of purified recombinant HIV-1 integrase (14) were incubated at 30°C for 1 hr. Autointegration products were visualized and quantified by autoradiography after electrophoresis in a 20% polyacrylamide DNA-sequencing gel. For another protocol, 15 ng of plasmid DNA (3 kb) was included in the reaction mixture as a heterologous integration target: in this case, a 1.2% agarose gel was used (15). The assay for HIV-1 protease inhibition was as described (16).

**Time-of-Addition Experiment.** Delineation of the drug-sensitive phase was determined with MT-4 cells infected at a high multiplicity of infection, >1, with HIV-1 strain IIIB. Compounds were added, at a concentration 50-fold to 100-fold higher than their $IC_{50}$ value in the standard MT-4/3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay, to parallel cultures (37°C) in microtiter plates at the indicated time after the addition of virus to the cells. After the last addition, all cells were washed to remove unbound virus, resuspended in the same concentration of compound, and further incubated for 24 hr. HIV-1 p24 core protein was then quantitated by a sandwich ELISA (DuPont).

**Virus Binding and Syncytium Formation.** The interaction between recombinant soluble CD4 (sCD4) (a gift from D. Klatzmann, Hôpital de la Pitié, Paris) and HIV-1 envelope glycoprotein gp120 (Repligen) was analyzed with either a CD4–gp120 ELISA kit (DuPont) or the recently developed biosensor technology (BIAcore, Pharmacia) (17). The binding of HIV-1 virion particles to $CD4^+$ cells (CEM) was evaluated by flow cytometry (18) on an ATC 3000 fluorescence-activated cell sorter (ODAM, Wissembourg, France) with anti-gp120 (NEA 9301, DuPont) or anti-CD4 (OKT4, Ortho Diagnostics) monoclonal antibodies. The binding of these mouse monoclonal antibodies was revealed with a fluorescein-conjugated goat anti-mouse IgG antibody (Immunotech, Luminy, France). In these experiments, aurintricarboxylic acid or sCD4 was used as reference compound and AZT as a negative control. The inhibition of syncytium formation was quantified by using a HeLa $CD4^+$ LTR (long terminal repeat)–*lacZ* indicator cell line, called P4. The cytoplasm of these cells turns blue upon treatment after infection by cell-free virus or after fusion with cells expressing HIV-1 Env and Tat proteins (19, 20). In brief, indicator cells ($10^4$ per well) were incubated for 24 hr in a microtiter plate, and pretreated for 1 hr at the desired concentration of compound. H9/IIIB chronically infected cells or HL2/3 cells, harboring a defective provirus but expressing high levels of HIV-1 proteins (21), were then added ($5 \times 10^3$ per well) in the presence of the drug. After 24 hr at 37°C, cells were washed, fixed in 1% formaldehyde/0.2% glutaraldehyde for 5 min, washed again, and incubated in with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside as described (20). Blue giant cells were counted by microscopic examination at ×40 magnification. In other experiments, CEM cells ($10^6$ per ml) were infected with recombinant vaccinia virus expressing either the wild-type HIV-1 LAI envelope (VV.TG.9-1/gp120-41) or an envelope mutated in the cleavage site (VV.TG.1139/gp160), at a multiplicity of infection of 2–3 for 1 hr at room temperature (23). Vaccinia-infected cells were then grown for 20–24 hr at 37°C in the presence or absence of the tested drug. Observations by light interference contrast microscopy indicated that VV.TG.9-1 efficiently induced the formation of syncytia, whereas VV.TG.1139 produced only rosettes characteristic of cellular aggregation mediated by CD4–gp160 binding. OKT4a monoclonal antibody, which efficiently blocks both events, was used as a reference compound.

## RESULTS AND DISCUSSION

Certain amide derivatives of betulinic acid, a triterpene extracted from plane tree bark (Fig. 1), were initially found to be modest inhibitors of both HIV-1 protease *in vitro* and HIV-1 infection in cellular assays. However, in the course of examining compounds having subsequent chemical modifications, we unexpectedly detected a derivative bearing an 11-aminoundecanoic lateral chain (RP70034; Fig. 1), which had lost all measurable antiprotease activity but was at least 10-fold more potent against HIV-1 (LAI) in cellular infection

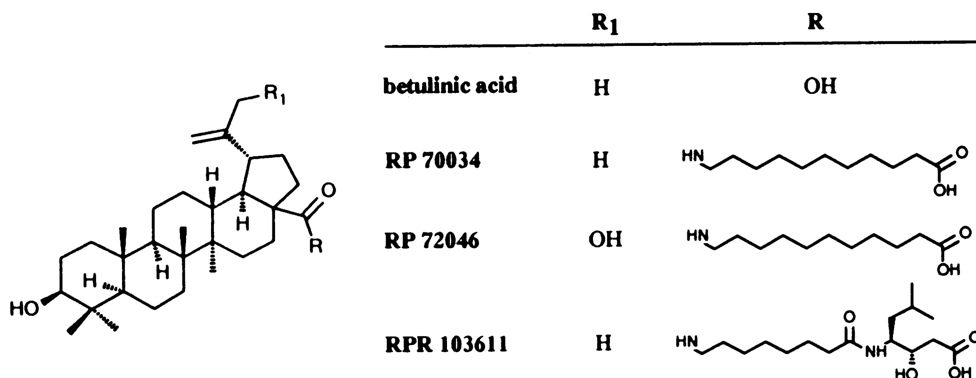


FIG. 1. Structures of betulinic acid derivatives. Compound RPR 103611, *N*′-{*N*-[3*b*-hydroxyl-20(29)-ene-28-oyl]-8-aminooctanoyl}-L-statine (molecular weight, 755.14; melting point, 158°C) was synthesized by a five-step procedure starting from betulinic acid, with an overall yield of 31% (22).

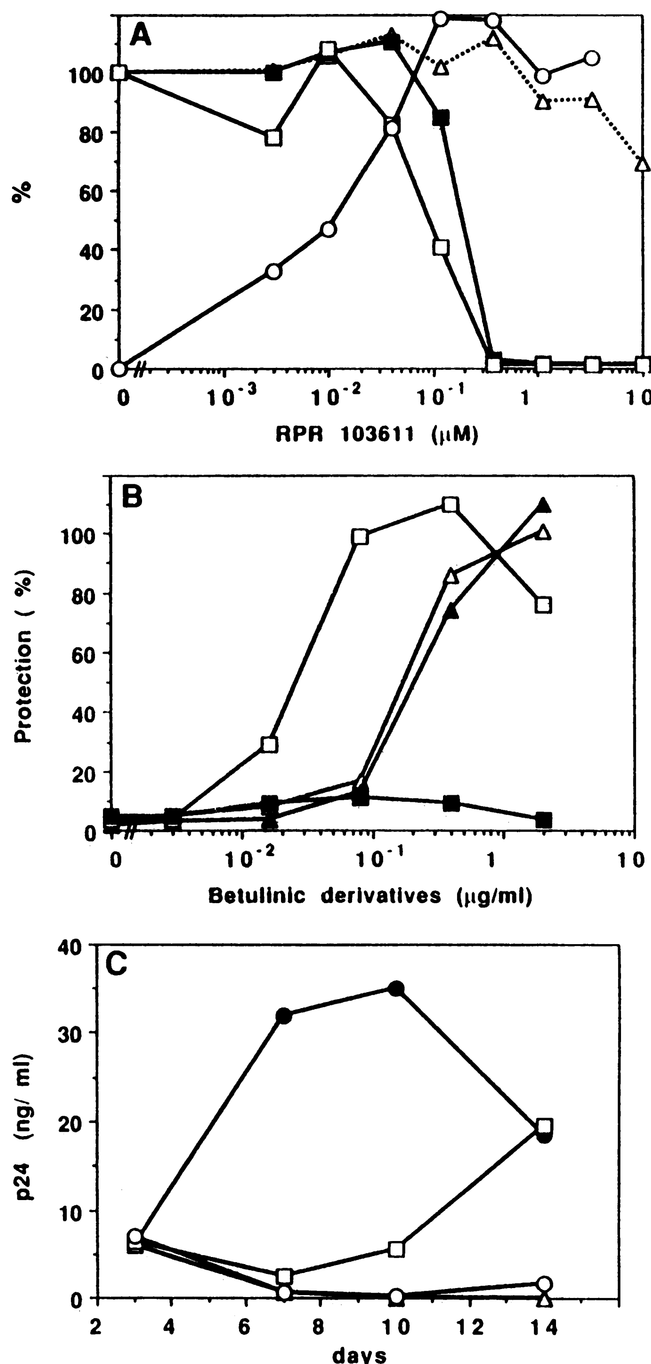


FIG. 2. Anti-HIV-1 activity of betulinic acid derivatives in cellular assays. (*A*) Activity against the LAI strain of HIV-1 grown in CEM-4 cells. The index of protection from HIV-1 cytopathic effect (○) is shown together with the inhibition of viral production in the supernatant as estimated from both p24 (□) and RT activity (■) measurements. The effect of the product on cell viability is indicated (△). (*B*) Comparative activities in the MT-4 antiviral assay as estimated from the protection index against HIV-1 (IIIB) cytopathic effect for RP 70034 (△), RP 72046 (▲), and RPR 103611 (□). The activity of RPR 103611 against HIV2 (ROD) in the same assay (■) is shown for comparison. As in *A*, the 100% protection level corresponds to the value observed without any compound or virus added to the culture. (*C*) Activities of betulinic derivatives against acute HIV-1 (LAI) infection in PBMCs. The kinetics of viral expression measured by the production of p24 antigen in the supernatant are indicated in the absence (●) or presence of 1 μM RP 72046 (□), RPR 103611 (○), or AZT (△).

assays ($IC_{50} \approx 0.3$ μM) than the original compounds. Structure–activity relationships for this series of derivatives suggested a rather stringent specificity. In particular, analogous derivatives of other tested natural triterpenes were inactive, an optimal length of the lateral chain was needed, and several positions on the molecule could not be modified, even slightly, without significant loss of activity (unpublished work). Chemical optimization of the lateral chain resulted in more potent compounds, represented by RPR 103611 (Figs. 1 and 2*B*), the mechanism of which was further studied.

The effect of betulinic derivatives in cellular infection assays is shown in Fig. 2. Efficient protection from the virus-induced cytopathic effect was observed in various cell lines. Similar efficiencies were observed with CEM cells, for which an important single-cell killing component is usually observed (9, 24), and with MT-4 cells transfected with proviral DNA of human T-lymphocytic virus type I, where the formation of syncytia contributes to the development of the cytopathic effect (10). Indeed, this effect is accompanied by a suppression of virus production, measured either as RT activity or as the concentration of p24 antigen in the culture supernatant (Fig. 2*A*). We also checked the activity of the compounds in several other cell lines, including the monocytic cell line U-937, and in peripheral blood lymphocytes (Table 1 and Fig. 2*C*). $IC_{50}$ values for RPR 103611 varied between 40 and 100 nM in most cell systems, with selectively indexes in excess of 100. [The precise assessment of cytotoxic concentrations ($CC_{50}$) for some betulinic derivatives can be hampered by the limited solubility of these compounds above 10 μM (formation of gels), depending on experimental conditions.] In addition to HIV-1 strain IIIB, betulinic derivatives were also found to be active against laboratory strains MN and RF, as well as against several HIV-1 clinical isolates. However, no significant activity could be found against HIV-2 (ROD and EHO isolates) when RPR 103611 was tested under the same conditions as those used for HIV-1 strain IIIB/LAI, showing that the activity of the betulinic derivatives tested is restricted to HIV-1 (Fig. 2*B*). Evidence that the specificity for these compounds could be restricted even within the HIV-1 family was supplied by the observation that most compounds active against IIIB/LAI were not active, or much less active, against two isolates of Zairian origin, NDK and ELI (Table 1). Thus, betulinic derivatives apparently interfere with a very specific molecular event of the replication cycle.

Table 1. Anti-HIV activity of RPR 103611 in various cell cultures

| Virus | Strain | Cell | $IC_{50}$, μM | SI |
|---|---|---|---|---|
| HIV-1 | LAI or IIIB | CEM-4 | 0.058 | >200 |
| | | MT-4 | 0.045 | >200 |
| | | H9 | 0.030 | >90 |
| | | C8166 | 0.27 | >10 |
| | | U-937 | 0.08 | >125 |
| | | PBMCs | <0.1 | >30 |
| | MN | MT-4 | 0.04 | >200 |
| | RF | MT-4 | 0.75 | >15 |
| | NDK | CEM-4, MT-4 | >5 | <1 |
| | ELI | CEM-4 | >5 | <1 |
| | APO 595 | PBMC | 0.2 | >50 |
| | 2750 M | MT-4 | 0.14 | >70 |
| HIV-2 | ROD | MT-4 | >10 | <1 |
| | EHO | MT-4 | >10 | <1 |

Data represent mean values for two to nine experiments. $IC_{50}$ represents the 50% inhibitory concentration for the cytopathogenicity of HIV (CEM-4, MT-4, C8166) or for the production of RT activity or p24 core antigen (H9, U-937, PBMCs). The selectivity index (SI) corresponds to the ratio $CC_{50}/IC_{50}$, where $CC_{50}$ is the 50% cytotoxic concentration for mock-infected cells. In the case of LAI in PBMCs, the lowest tested concentration was 0.1 μM and gave >50% inhibition.

Active derivatives were also examined for a possible inhibition of the *in vitro* activity of several purified HIV-1 enzymes. No inhibition of RT, integrase, or protease was detected at concentrations of the derivatives compatible with cellular activity (data not shown), suggesting that the corresponding steps were not involved in the inhibitory mechanism of these compounds. In fact, evidence was obtained from two different experiments that betulinic derivatives act at an early stage in the infectious cycle. First, we observed a clear dose-dependent inhibitory effect of compounds at micromolar concentrations on the synthesis of proviral cytoplasmic DNA, as measured by PCR only 2 hr after the onset of infection (data not shown). Second, we studied the influence of delaying the addition of compounds at various times soon after the exposure of MT-4 cells to HIV-1. Such an experiment showed (Fig. 3*A*) that postponing the addition of RPR 103611 for 1 hr was enough to cancel the inhibitory potency of this compound on the subsequent production of viral antigens. Betulinic derivatives behaved in this particular experiment very similarly to agents that interfere with the binding of virus to cells, such as sulfated polysaccharides, or to compounds proposed to interact with a viral uncoating event, such as bicyclams (25). In fact, that the betulinic derivatives affect virus binding was ruled out by several experiments showing that betulinic derivatives did not significantly inhibit either the binding of the virus to CD4 cells (Fig. 4*A*) or the binding of sCD4 to gp120 (Fig. 4*B*). Proof for the mechanism of action came from assays designed to monitor syncytium formation between uninfected CD4 cells and chronically HIV-1-infected cells or cells expressing the HIV-1 envelope. Betulinic derivatives efficiently blocked the formation of syncytia induced by HIV-1 strain IIIB (Fig. 3*B*) but not the formation of giant cells induced by the NDK strain (R.P., unpublished data; the lack of activity in HIV-1 strain NDK-dependent syncytia assays correlates well with the inefficacy of this compound against the NDK isolate in

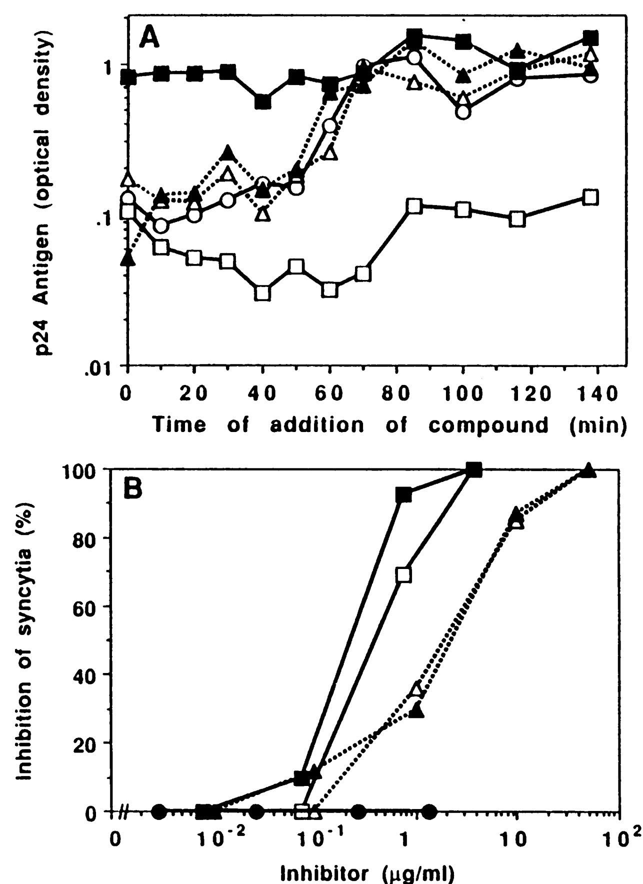


FIG. 3. Betulinic acid derivatives act at an early step of the virus infection cycle. (*A*) Variation of p24 antigen production with the time of addition of the inhibitory compound. Control infection was provided by the addition of compound-free medium (■). During the studied time frame, AZT (0.05 μg/ml; □) was always protective. In these conditions, RPR 103611 (1 μg/ml; ○) could not be distinguished from dextran sulfate (50 μg/ml; ▲) or from the bicyclam compound JM2763 (20 μg/ml; △): all three compounds lost their protective capacity after about 1 hr. (*B*) Inhibition of syncytium formation mediated by either H9/IIIB (open symbols) or HL2/3 (filled symbols) with the P4 indicator cell line. The effect of RPR 103611 (squares) is shown in comparison with that of dextran sulfate (triangles). In these conditions, AZT was completely inactive (circles), even with the chronically infected cells.

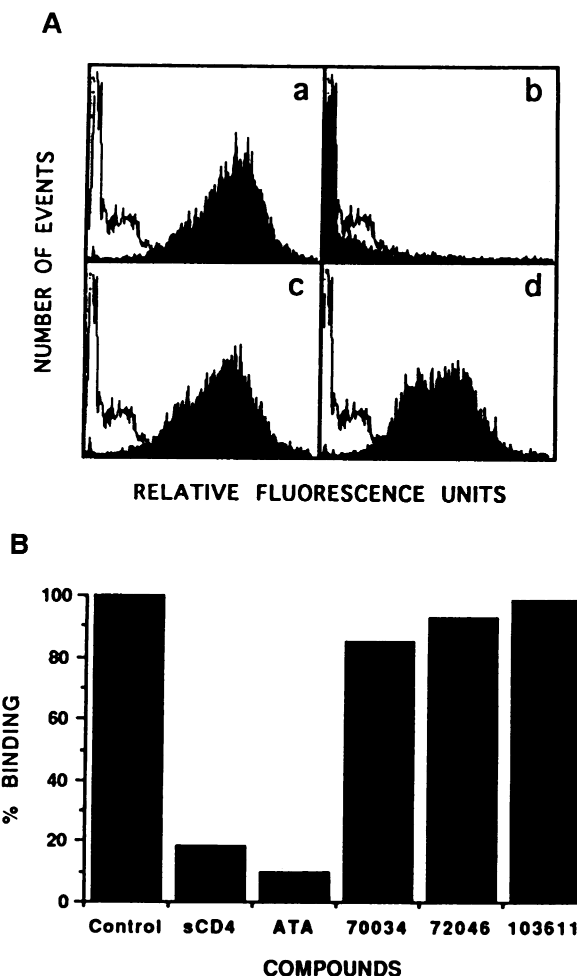


FIG. 4. Betulinic acid derivatives do not block virus binding. (*A*) Flow cytofluorimetric analysis of the binding of HIV-1 LAI virions to $CD4^+$ CEM cells. Open profile, fluorescence intensity of the negative control corresponding to CEM cells incubated with fluorescein-conjugated antibody only; filled profile, fluorescence in the presence of the specific anti-gp120 antibody. (*a*) Control without compound. (*b*) Inhibition of gp120 binding by aurintricarboxylic acid (100 μg/ml). (*c* and *d*) Lack of inhibition of gp120 binding by betulinic acid derivatives (1 μM) RP 72046 and RPR 103611, respectively. In the same conditions, sCD4 was active, whereas AZT had no effect (data not shown). (*B*) Quantification of binding of the human serum albumin (HSA)–CD4 fusion protein (26) to gp120 using biosensor technology. Briefly, HSA–CD4 binds to immobilized gp120 on the sensor chip and the signal can be further amplified by anti-HSA polyclonal antibody. The control (without compound) defines 100% binding. Soluble CD4 (2.2 μM) and aurintricarboxylic acid (ATA, 50 μM) inhibit the gp120–CD4 interaction whereas betulinic acid derivatives RP 70034, RP 72046, and RPR 103611 at 10 μM had no effect.

cellular infection assays). More specific syncytium assays were carried out by using recombinant vaccinia viruses expressing either the normal envelope from strain HIV-1 LAI or an uncleavable mutant gp160 protein (23). Active betulinic derivatives at submicromolar concentrations inhibited the envelope-dependent syncytium formation but did not block the formation of aggregates observed when the envelope variant was used (data not shown). Finally, the compounds at active concentrations did not exert a direct viricidal effect, since preincubation of the virus with the compounds did not modify its subsequent infective capacity.

Together, these results provide evidence that the betulinic derivatives studied block virus infection at a postbinding step necessary for virus–membrane fusion and that the target of these compounds is contained within, or interacts with, the HIV-1 envelope gp120/gp41. This is, to our knowledge, the first description of a nonpeptidic compound having this potential, since, until now, only monoclonal antibodies or peptides have been shown to selectively affect the HIV-1 membrane fusion step (27, 28). However, drugs that block fusion events induced by certain myxoviruses have been described—for example, amantadine in the case of influenza A (reviewed in ref. 29).

Unlike the relatively well-known gp120–CD4 binding, the postbinding molecular events leading to HIV-1 envelope-mediated membrane fusion are much less well understood but are likely to be characterized by major conformational changes of the whole gp120/gp41 complex, ultimately resulting in the productive exposure of the gp41 N-terminal fusogenic domain. This mechanism is important not only for the penetration of free virus into cells but also for cell fusion processes that can lead to syncytium formation, an important determinant of *in vitro* cytopathogenicity, as well as cell-to-cell viral dissemination (30, 31). Studies have mapped elements of the gp120 and gp41 glycoproteins essential for efficient membrane fusion (32); it will be important to determine which of these peptides is affected by the inhibitory mechanism of betulinic acid derivatives. Because of their mode of action, the potential clinical usefulness of these agents in treating HIV-1 disease deserves further investigation.

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