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Resistance to a Drug Blocking Human Immunodeficiency Virus Type 1 Entry (RPR103611) Is Conferred by Mutations in gp41

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A triterpene derived from betulinic acid (RPR103611) blocks human immunodeficiency virus type 1 (HIV-1) infection and fusion of $CD4^+$ cells with cells expressing HIV-1 envelope proteins (gp120 and gp41), suggesting an effect on virus entry. This compound did not block infection by a subtype D HIV-1 strain (NDK) or cell-cell fusion mediated by the NDK envelope proteins. The genetic basis of drug resistance was therefore addressed by testing envelope chimeras derived from NDK and a drug-sensitive HIV-1 strain (LAI, subtype B). A drug-resistant phenotype was observed for all chimeras bearing the ectodomain of NDK gp41, while the origins of gp120 and of the membrane anchor and cytoplasmic domains of gp41 had no apparent role. The envelope gene of a LAI variant, fully resistant to the antiviral effect of RPR103611, was cloned and sequenced. Its product differed from the parental sequence at two positions in gp41, with changes of arginine 22 to alanine (R22A) and isoleucine 84 to serine (I84S), the gp120 being identical. In the context of LAI gp41, the I84S substitution was sufficient for drug resistance. Therefore, in two different systems, differences in gp41 were associated with sensitivity or resistance to RPR103611. Modifications of gp41 can affect the quaternary structure of gp120 and gp41 and the accessibility of gp120 to antiviral agents such as neutralizing antibodies. However, a direct effect of RPR103611 on a gp41 target must also be envisioned, in agreement with the blocking of apparently late steps of HIV-1 entry. This compound could be a valuable tool for structure-function studies of gp41.

Human immunodeficiency virus (HIV) types 1 and 2 (HIV-1 and HIV-2) enter cells by a membrane fusion process mediated by their envelope glycoproteins, gp120 and gp41, which form a noncovalent complex (reviewed in references 16 and 33). The initial step of the HIV entry process is the interaction of the surface subunit gp120 with two cell surface molecules, CD4 and a chemokine receptor, recently shown to behave as HIV coreceptors. Although other chemokine receptors can mediate HIV entry, the principal coreceptors seem to be CCR-5 and CXCR-4, which are used by primary strains with a non-syncytium-inducing (NSI) phenotype and by cell line-adapted strains, respectively (reviewed in references 12 and 17). Primary strains with a syncytium-inducing (SI) phenotype can use either CXCR-4 or CCR-5 as their coreceptor (34). The interaction of gp120 with these components induces conformational changes in the gp120-gp41 complex and in some cases its dissociation (16, 33). By analogy with the influenza virus hemagglutinin model (2, 4), the role of these conformational changes might be to unmask the hydrophobic amino-terminal domain (fusion peptide) of the transmembrane envelope protein (gp41), allowing its interaction with the target cell membrane. These conformational changes seem to require interchain and intrachain hydrophobic interactions involving two α-helix domains of gp41, located immediately downstream from the fusion peptide (proximal helix) and upstream from the membrane anchor domain (distal helix) (13). Recent crystallographic studies have revealed that the proximal helix forms coiled-coil trimers, which in turn interact with the distal helix (5, 38). These interactions are probably critical for gp41 to reach its fusion-active conformation, thus explaining the antiviral activity of peptides derived from the proximal and distal α-helices (7, 14, 39, 40).

Antiviral agents blocking HIV-1 entry represent useful tools to study the function of viral envelope proteins. Most compounds have been shown, or proposed, to target gp120. For example, aurintricarboxylic acid (ATA) can block the CD4-gp120 interaction (31), while other polyanions, such as dextran sulfate, have a variable effect on this interaction and seem rather to interact with the third variable domain (V3) of gp120 (1, 20, 42). The V3 domain is a target of anti-HIV-1 neutralizing antibodies (29) and was recently found to participate in the interaction of gp120 with the CCR-5 or CXCR-4 coreceptor (24, 37, 41). Interference with the function of V3 could also play a role in the antiviral activity of the amphotericin B derivative MS8209 (23, 25) and other compounds such as ALX-40-C (19) and bicyclams (8). To our knowledge, there are no low-molecular-weight drugs blocking HIV-1 entry by action on a gp41 target.

We have studied the mechanism of action of RPR103611, a triterpene derived from betulinic acid which was previously found to inhibit HIV-1 replication at micromolar concentrations in different cell types (15). Since it blocked the formation of syncytia between $CD4^+$ and HIV-infected cells, with no apparent effect on the CD4-gp120 interaction, the RPR103611 compound was proposed to be an inhibitor of membrane fusion. It neutralized several cell line-adapted HIV-1 strains from genetic subtype B, in particular $HIV\text{-}1_{LAI}$ (LAI), as well as primary HIV-1 strains with an NSI phenotype (15). Therefore, its antiviral activity seemed to be independent from the type of coreceptor, CXCR-4 or CCR-5, used by the viral strains. In contrast, RPR103611 did not neutralize infection by

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HIV-2 or by HIV-1 strains isolated from African patients, such as HIV-1_{NDK} (subtype D) (NDK). This strain specificity was used to define the genetic basis of the sensitivity or resistance of HIV-1 strains to neutralization by RPR103611 and to further address the mechanism of its antiviral effect.

MATERIALS AND METHODS

Viral strains and cell lines. HIV-1 strain LAI was produced by transfection of HeLa cells with a reconstructed provirus (21). HIV-1 strain NDK (36) and HIV-2_{EHO} (EHO) (27) were produced by chronically infected and acutely infected CEM cells, respectively. The HeLa-Env/LAI, HeLa-Env/LAI(V3-NDK), and HeLa-Env/LAI(V3-ROD) cell lines stably expressing wild-type or chimeric envelope (Env) proteins from LAI have been described elsewhere (24, 32). The HeLa-Env/NDK cell line stably expresses a chimeric Env protein, consisting of the gp120 and gp41 ectodomain from NDK and the membrane anchor and cytoplasmic domains of gp41 from LAI. The expression vector was obtained by substituting an *Eco*RI-*Xba*I NDK fragment and an *Xba*I-*Bam*HI fragment from the LN-3 chimera (see below and Fig. 4) for the *Eco*RI-*Bam*HI fragment of the MA243 vector (32), a LAI provirus with deletion of the *gag*, *pol*, *vif*, *vpr*, and *nef* genes and replacement of *nef* by the dihydrofolate reductase gene (*dhfr**) conferring resistance to methotrexate. The Env/ROD expression vector will be described in detail elsewhere (35). Briefly, it was derived from an HIV-2_{ROD} provirus (28) by deletion of a *Hin*dIII fragment within *gag-pol* and insertion of *dhfr** downstream of *env*. The Env expression vectors were stably transfected into HeLa cells by calcium phosphate precipitation, and clones were selected in medium containing 2 μM methotrexate, as described previously (32). Cell clones were tested for Env expression by syncytium formation assays with the $CD4^+$ HeLa-P4 cell line (6). The adherent cell lines (HeLa derivatives) were propagated in Dulbecco's modified Eagle's medium, and the T-cell line CEM was propagated in RPMI 1640 medium. All culture media were supplemented with antibiotics, glutamine, and 10% fetal calf serum.

Chimeric envelope proteins. The Env-NDK expression vector was derived from a cloned NDK provirus (36) by deletion of internal *Eco*RI fragments corresponding to nucleotides (nt) 386 to 5278 (the numbering for the LAI and NDK sequences is according to reference 18). All chimeric LAI-NDK *env* genes (LN-1 to LN-9) except for LN-2 and LN-3 were cloned in the NS105 vector, differing from MA243 by the presence of *Mlu*I (nt 6706), *Sma*I (nt 6818), and *Eco*RV (nt 7834) sites (see Fig. 4). The *Mlu*I site does not modify LAI Env. The S339T and N679D mutations created by *Sma*I and *Eco*RV, respectively, have no apparent effect. The LN-1 chimera was obtained by subcloning a PCR fragment of NDK *env* between the *Mlu*I and *Sma*I sites of NS105, as described previously (24). LN-2 was obtained by subcloning a fragment from *Eco*RI (nt 5325 in LAI) to *Cla*I (nt 6664 in NDK) from LN-1 into the Env-NDK vector. LN-3 was obtained by subcloning an *Xba*I-*Eco*RV PCR fragment from NDK *env* (nt 7582 to 7748) and an *Eco*RV-*Xho*I fragment from NS105 into the *Xba*I and *Xho*I sites of LN-2, respectively. The NDK fragment was amplified with primers NDK-*Bgl*II (5′ GGGAGATCTAAAAGAGCAATAGGA, plus strand; *Bgl*II site underlined) and NDK-*Eco*RV (5′ ATCAAACCAATTCCACAAACTTGC, minus strand; *Eco*RV site underlined) and digested with *Xba*I. LN-4 was obtained by ligation of a PCR fragment amplified from LN-3 with the primers NDK-*Sma*I (5′ GGGCAGAATGGAATAAAGCTTTA, plus strand; *Sma*I site underlined) and LAI-*Bam*HI (5′ GATCGTCCCAGATAAGTGCTAAGGATCC, minus strand; *Bam*HI site underlined) between the *Sma*I and *Bam*HI sites of NS105. LN-5 was obtained by ligating an *Mlu*I-to-blunt-end PCR fragment from NDK (nt 6632 to 7265) and a blunt-end-to-*Bam*HI PCR fragment from LAI (nt 7355 to 8068) between the *Mlu*I and *Bam*HI sites of NS105, reconstructing a *Pvu*I site at the gp120-gp41 junction. The *Pvu*I site creates a V2I substitution in LAI gp41. The NDK fragment was amplified with NDK-*Mlu*I (5′ GGGACGCGTCCCTA CAAATATACAAG, plus strand; *Mlu*I site underlined) and NDK-*Pvu*I (5′ TCG CTCTTTTTTCTCTTTCCACCAC, minus strand; *Pvu*I site underlined); the LAI fragment was amplified with LAI-*Pvu*I (5′ TCGGAATAGGAGCTTTGTT CCTTGGG, plus strand; *Pvu*I site underlined) and LAI-*Bam*HI. LN-6 was obtained by ligation of a PCR fragment amplified from LN-5 with the NDK-*Sma*I and LAI-*Bam*HI primers, between the *Sma*I and *Bam*HI sites of NS105. LN-7 was obtained by ligating an *Mlu*I-to-blunt-end PCR fragment from LAI (nt 6706 to 7354) and a blunt-end-to-*Bam*HI PCR fragment from LN-3 (nt 7266 of NDK to nt 8068 of LAI) between the *Mlu*I and *Bam*HI sites of NS105. The LAI fragment was amplified with LAI-*Mlu*I (5′ GGGACGCGTCCCACAACAATA CAAG, plus strand; *Mlu*I site underlined) and LAI-*Pvu*I (5′ TCGCTCTTTTT TCTCTCTGCACCAC, minus strand; *Pvu*I site underlined), and the LN-3 fragment was amplified with NDK-*Pvu*I (5′ TCGGACTAGGAGCTGTGTTCCTT GGG, plus strand; *Pvu*I site underlined) and LAI-*Bam*HI. LN-8 and LN-9 were obtained by creating *Apa*LI and *Xba*I sites in NS105 at nt 7462 and 7669 of LAI, respectively, and substituting the *Apa*LI-*Bam*HI or the *Xba*I-*Bam*HI fragment from LN-3.

Drug-resistant LAI. PCR was performed on HeLa-P4 cells 24 h after infection with the RPR103611-resistant LAI population (LAI-R*), using primers 5′ GAG CAGAAGACAGTGGCAATG (plus strand; *env* ATG underlined) and LAI-*Bam*HI. The resulting fragment (nt 5785 to 8073) was digested with *Kpn*I (nt 5925) and *Bam*HI (nt 8068) and subcloned for restriction mapping and sequence analysis. Four inserts were subcloned between the *Kpn*I and *Bam*HI sites of NS105. The resulting vectors (R1 to R4) expressed functional and drug-resistant Env proteins. Automated sequencing of the entire *Kpn*I-*Bam*HI fragment of R1 revealed two differences with wild-type LAI *env*, at nt 7415 (G-to-C change) and 7601 (T-to-G change), resulting in two amino acid differences in gp41, R22A and I84S. The same mutations were found in R2, R3, and R4, but only this region of *env* was sequenced. The *Kpn*I-*Bam*HI fragment of R1 was subcloned into the LAI provirus to derive LAI-R. The LAI R22A Env vector was obtained by inserting a *Kpn*I-*Hga*I fragment (nt 5925 to 7422) from R1 *env* into the same sites of NS105. The LAI I84S Env vector was derived from NS105 by site-directed mutagenesis with the oligonucleotide 5′ TCCAGAGCAACCCCAACTCCCAG CAGCTG (*Pvu*II site underlined).

Assays for antiviral activity. The betulinic acid derivative RPR103611 was obtained from Rhône-Poulenc Rorer as a dry powder and diluted in dimethylformamide (10 mM stock solution). Azidothymidine and ATA were obtained from Sigma (St. Louis, Mo.). MS8209 was obtained from Mayoly-Spindler (Chatou, France). Viral titrations and syncytium formation assays using HeLa-P4 cells were performed essentially as described previously (23). For syncytium formation assays based on the transient expression of Env, HeLa cells were transfected by calcium phosphate precipitation (overnight contact with DNA) in six-well trays. The cells were detached with trypsin 36 to 40 h after transfection. About 2×10^5 cells were used to seed six-well trays containing an equivalent number of HeLa-P4 cells in medium containing 10 μM RPR103611 or the same volume of solvent. After overnight coculture, adherent cells were fixed in 0.5% glutaraldehyde, washed in phosphate-buffered saline (PBS), and stained with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (23).

RESULTS

Activity of RPR103611 on HIV-1 entry. The antiviral activity of RPR103611 had been studied in assays measuring HIV-1 production after several replicative cycles (15), which did not allow precise determination of the step of the retroviral cycle that was affected by the compound. Here, we have used the target cell line HeLa-P4, in which the HIV infectious titers can be determined as soon as 20 h after virus-cell contact, thus, after a single cycle of reverse transcription (6, 23). In these cells, expression of the HIV transactivating protein Tat, following infection or fusion with Tat^+ cells, results in the upregulation of β-galactosidase expression. Infected cells or syncytia can therefore be stained blue after incubation with the X-Gal substrate and easily scored. In this assay, the infection of HeLa-P4 cells by HIV-1 strain LAI was abolished when the inoculum contained RPR103611 (10 μM), whether or not the drug was present in the culture medium after the 1-h virus-cell contact (Fig. 1). An almost complete antiviral effect was also observed when the virus-cell contact step was performed in the absence of drug and RPR103611 (10 μM) was added to the culture medium either immediately after the inoculum was washed off or 1 h later (Fig. 1). Unlike reverse transcriptase inhibitors, the RPR103611 compound had little or no effect on HIV-1 infectivity when it was added 2 h after the end of the virus-cell contact step (Fig. 1 and other data not shown). In agreement with previous observations (15), pretreatment of HeLa-P4 cells with RPR103611, or cell-free incubation of LAI with RPR103611, had no effect on infectivity (data not shown). These experiments were consistent with the blocking of HIV-1 entry by RPR103611, probably at a relatively late step. Indeed, in this type of assay, other compounds, such as MS8209, ATA, or bicyclams, blocked infection when they were present during virus-cell contact but had only a partial effect or no effect when they were added afterwards (22, 23).

Strain specificity. The effect of RPR103611 on infectivity was compared in HeLa-P4 cells for two genetically divergent HIV-1 strains, LAI (subtype B) and NDK (subtype D), and one HIV-2 strain, EHO. Parallel assays showed no apparent effect of RPR103611 on cell viability (as determined by trypan blue exclusion) or proliferation during the time course of the assay (20 h), even at the higher concentration (30 μM) (data not shown). The infectious titer of LAI was markedly reduced when infections were performed in the presence of 1 or 3 μM

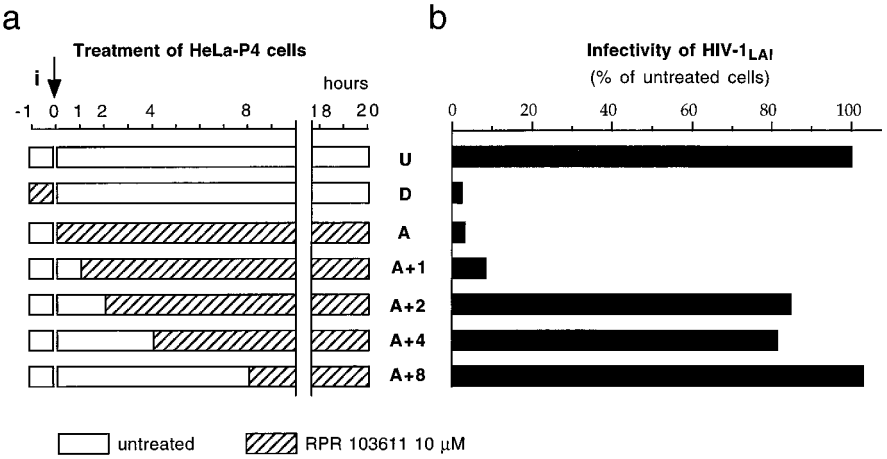

FIG. 1. Time requirement for the inhibition of LAI infection by RPR103611. Subconfluent monolayers of HeLa-P4 cells in 12-well trays were treated with RPR103611 either during the 1-h infection step (i) or at different times after this step, as indicated (a). The infection step was ended by washing the cells twice with PBS (arrow), and the cells were grown for 20 h before fixation. Infectivity was measured by scoring blue cells after staining with X-Gal (b). Results are shown relative to those for untreated cells (U). The virus inoculum yielded about 200 blue-stained cells per well in the absence of drug. The means of two experiments are shown. Treatment with drug was performed during virus-cell contact (D), immediately afterward (A), or from 1 h (A+1) to 8 h (A+B) afterward.

RPR103611, and infectivity was abolished at 10 or 30 μM. In contrast, there was no significant reduction of the infectious titers of NDK and EHO at these concentrations (Fig. 2), in agreement with other experiments (15).

Strain-specific effects of RPR103611 were also observed in cell-cell fusion assays between HeLa-P4 cells and effector cells stably expressing wild-type HIV-1 or HIV-2 Env and Tat. The fusion of HeLa-P4 and HeLa-Env/LAI cells, expressing the wild-type LAI Env, was totally blocked when coculture was performed in the presence of 10 μM RPR103611 (Fig. 3). In contrast, the numbers of syncytia formed with effector cells expressing Env from strain NDK or from the drug-resistant HIV-2 strain ROD were reduced by only 20 and 35%, respectively (Fig. 3). Although cell fusion mediated by Env from strains NDK and ROD was not totally resistant to RPR103611, this assay showed a clear-cut phenotypic difference from LAI Env. It was therefore used in subsequent experiments to map the Env domains supporting sensitivity or resistance to RPR103611.

Fusion of HeLa-P4 cells with effector cell lines expressing chimeric LAI Env with the V3 loop from strain NDK or ROD was totally blocked by 10 μM RPR103611 (Fig. 3), indicating that the V3 domain of gp120 was not sufficient to confer a drug-resistant phenotype in the context of LAI envelope proteins.

Chimeric envelope proteins. Chimeric *env* genes were constructed and expressed in HeLa cells by transient transfection, using vectors also expressing HIV-1 Tat (see Materials and Methods). All the chimeric Env proteins depicted in Fig. 4 allowed fusion of transfected HeLa cells with HeLa-P4 cells with similar efficiencies. Of particular interest was the functionality of the LN-7 chimera, showing that the gp120 and gp41 of two relatively distant HIV-1 strains can form a functional complex. When syncytium formation assays were performed in the presence of 10 μM RPR103611, two types of effects were

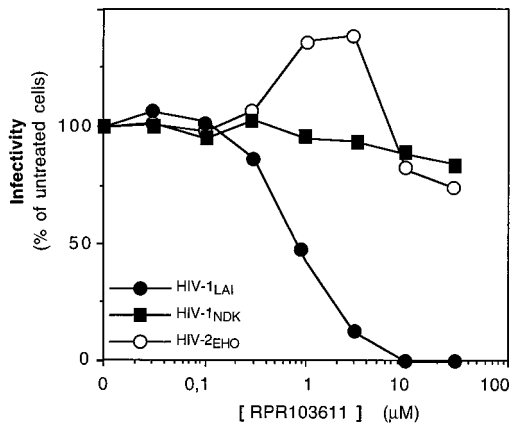

FIG. 2. Strain-specific activity of RPR103611. HeLa-P4 cells were infected in 96-well plates with LAI, NDK, or EHO by replacing the culture medium with fresh medium containing the inoculum and RPR103611 at the indicated concentration. The cells were fixed and stained with X-Gal 20 h after infection. Results are shown relative to those for untreated cells (means of triplicate wells).

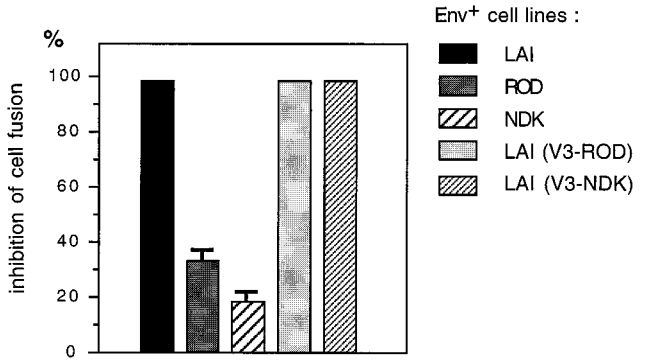

FIG. 3. Inhibition of Env-mediated fusion by RPR103611. HeLa-P4 cells were cocultured with HeLa cells stably expressing the Env of strain LAI, NDK, or HIV-2_{ROD} or chimeric LAI Env with the gp120 V3 domain of ROD or NDK. The Env^+ cells also express Tat, and their fusion with HeLa-P4 cells results in transactivation of the LTR*lacZ* transgene. Six-well trays were seeded with about 2×10^5 cells of each type, in the absence or presence of 10 μM RPR103611. The cells were stained with X-Gal after overnight coculture, and blue-stained foci were counted. From 5,000 to 10,000 syncytia per well were scored in the absence of drug, depending upon the type of Env^+ cells. Bars represent the inhibition of cell fusion by RPR103611, relative to that of untreated cocultures (means of at least three experiments).

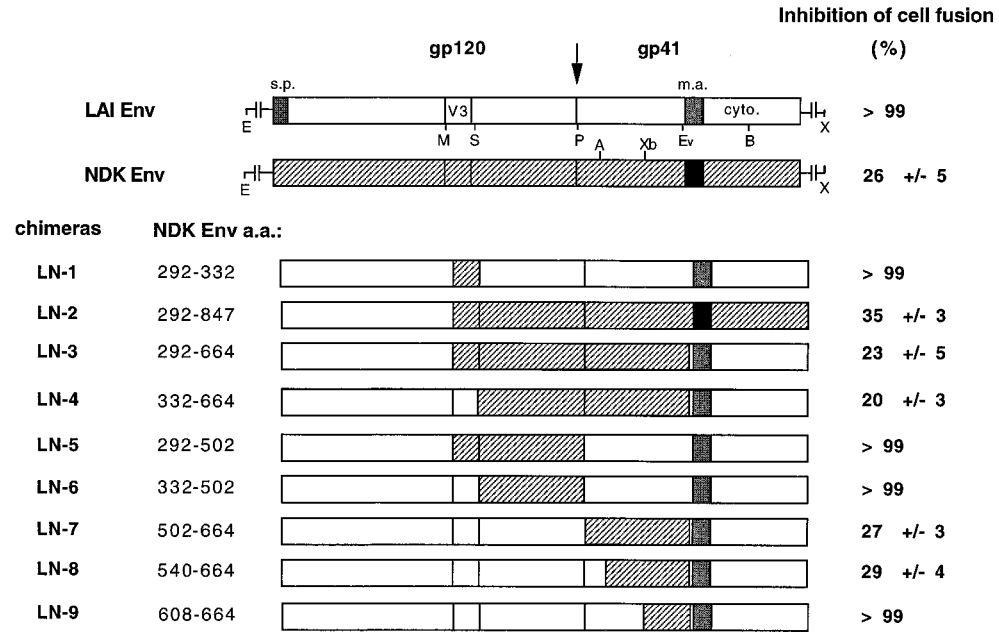

FIG. 4. Effect of RPR103611 on chimeric LAI-NDK Env. Cocultures were performed in the presence or absence of 10 μM RPR103611 with HeLa-P4 cells and HeLa cells expressing the wild-type LAI or NDK Env or with the LN-1 to LN-9 Env chimeras. The Env^+ cells were harvested 36 to 40 h after transfection, and coculture was performed as described for Fig. 3. Results are the means of at least three experiments. The *Mlu*I (M), *Sma*I (S), *Pvu*I (P), and *Eco*RV (Ev) restriction sites used for construction of the chimeras have been created in the NS105 Env expression vector (see Materials and Methods). *Bam*HI (B) is a natural site in LAI *env*, while *Apa*LI (A) and *Xba*I (Xb) are natural sites in NDK *env*. The *Eco*RI (E) and *Xho*I (X) sites outside *env* are conserved in strains LAI and NDK. Also shown are the signal peptide (s.p.) and V3 domain of gp120 and the membrane anchor (m.a) and cytoplasmic (cyto.) domains of gp41. a.a., amino acids.

observed. Cell fusion mediated by the wild-type LAI Env and by the LN-1, LN-5, LN-6, and LN-9 chimeras was totally blocked, while fusion mediated by the wild-type NDK Env and by other chimeras was reduced by 20 to 35% but not abolished (Fig. 4). As previously observed, the substitution of the NDK V3 domain into LAI Env (LN-1) did not confer drug resistance, and the same was observed upon substitution of other domains of NDK gp120 (e.g., LN-5). In summary, the drug blocked cell-cell fusion mediated by all chimeric Env proteins in which gp41 was derived from LAI but had a limited effect on fusion mediated by chimeras with NDK gp41. The results with the LN-7 and LN-8 chimeras indicated that the gp120 of NDK and certain regions of NDK gp41 had no apparent role in the drug-resistant phenotype. The structure of the LN-8 chimera and the existence of amino acid identities between the LAI and NDK proteins allowed the conclusion that the first 38 residues of NDK gp41 and the region of gp41 extending beyond residue 148 were not required for resistance to RPR103611. These regions of gp41 correspond to the fusion peptide and domain preceding the proximal helix (region from amino acids 1 to 38) and to a part of the distal helix, to the membrane anchorage and cytoplasmic domains (residues beyond 148). In addition, the results obtained with the LN-9 chimera indicate that the region of NDK gp41 extending beyond position 109 was not sufficient to confer drug resistance.

Selection of a drug-resistant HIV-1 strain. To complete this study, we sought to select a drug-resistant LAI isolate by infecting CEM cells (a $CD4^+$ T lymphoid cell line) in the presence of 10 μM RPR103611. Virus production was markedly delayed compared to that in untreated cells but was eventually observed after some 30 days (Fig. 5a). Virus harvested at this time (designated LAI-R*) was used to infect fresh CEM cells in the presence or absence of drug. Virus production was observed in both cases, and similar levels of p24 antigen were measured at days 8 and 12 in the supernatants (Fig. 5b), indicating that the inoculum was fully resistant to the antiviral activity of RPR103611. There was no virus production when CEM cells were infected with LAI-R* in the presence of azidothymidine, as expected, or ATA (Fig. 5b).

In order to obtain a drug-resistant *env* gene, PCR was performed on DNA from HeLa-P4 cells infected with LAI-R* in the presence of 10 μM RPR103611. The primers allowed amplification of an *env* fragment encompassing most of gp120 and all of the ectodomain of gp41, which was directly subcloned into an Env expression vector. Four independent clones were tested by transient transfection of HeLa cells and syncytium formation assays with HeLa-P4 cells. The efficiency of cell-cell fusion mediated by these Env proteins was similar to that of wild-type LAI Env, but it was not blocked by RPR103611 (Fig. 6 and other data not shown). The *env* gene of one clone was substituted into a LAI provirus. Virus produced by transfection of this construct into HeLa cells (LAI-R) was fully resistant to the antiviral effect of RPR103611, but infection was neutralized by two other inhibitors of HIV-1 entry, MS8209 and ATA (Fig. 5c).

Role of gp41 mutations. The LAI-R *env* gene was completely sequenced. Its product differed from the parental LAI Env by two amino acid substitutions in gp41, changing arginine to alanine at position 22 (R22A) and isoleucine to serine at position 84 (I84S) (Fig. 7). The gp120 proteins of LAI and LAI-R were identical. Mutations in gp41 were therefore responsible for the drug-resistant phenotype of LAI-R, consistent with previous results with the LAI-NDK Env chimeras.

The R22A and I84S mutations were separately introduced into LAI Env, and their effects were tested in syncytium formation assays. The I84S mutation was both necessary and sufficient for resistance to RPR103611, while the R22A mutation apparently had no role (Fig. 6). Accordingly, an A22

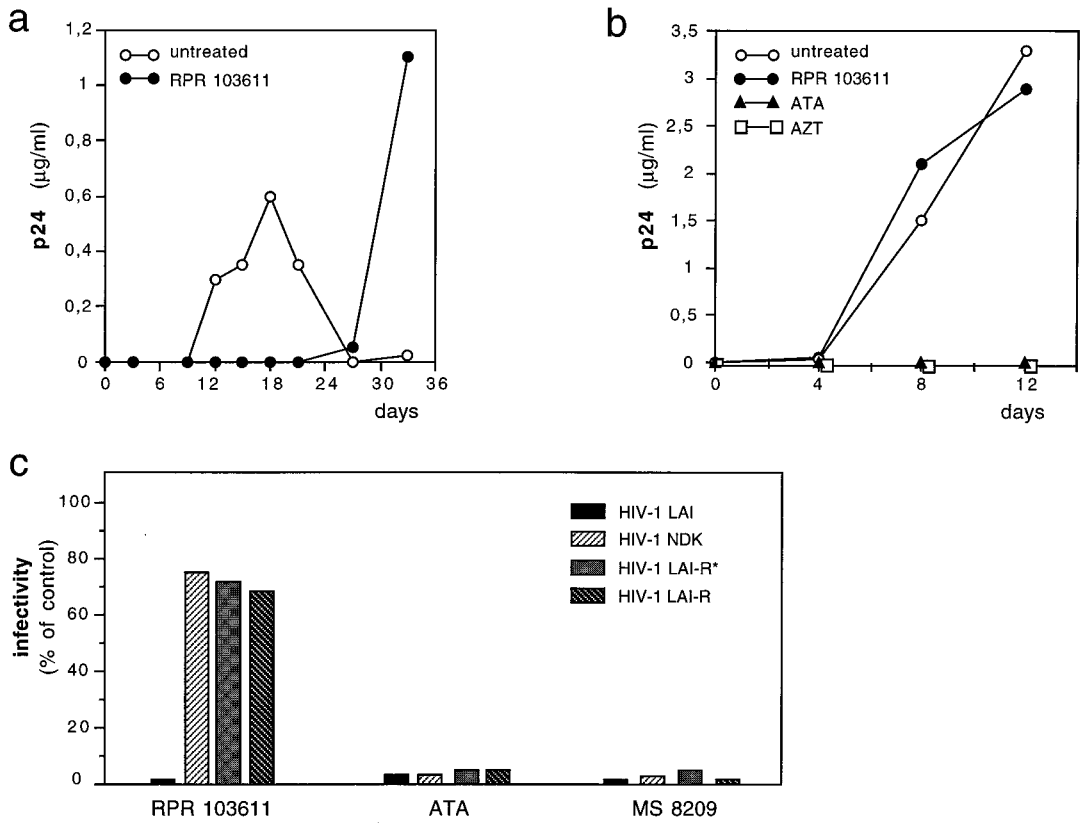

FIG. 5. Isolation and characterization of a drug-resistant LAI strain. (a) CEM cells were infected with LAI and grown in the presence or absence of 10 μM RPR103611. Virus production was monitored every 3 days by assaying the p24 concentration in the supernatant. Virus harvested at day 33 in treated cells was designated LAI-R*. (b) CEM cells were infected with LAI-R* and grown in the presence of either 10 μM RPR103611, 10 μM ATA, or 5 μM AZT or in the absence of drug, as indicated. (c) HeLa-P4 cells were infected in 12-well trays with HIV-1 strain LAI, NDK, LAI-R*, or LAI-R (produced by a recombinant LAI provirus bearing the R22A and I84S mutations in gp41 [see Materials and Methods]) in the absence or in the presence of 10 μM RPR103611, 10 μM ATA, or 10 μM MS8209. The last two compounds are inhibitors of HIV-1 entry. The cells were fixed and stained with X-Gal 20 h after infection. Bars represent the efficiency of infection relative to that of untreated cells (means of three experiments).

residue is found in the HXB2 molecular clone (26), a LAI variant. Virus produced by transfection of HXB2 was fully sensitive to the antiviral effect of RPR103611 (data not shown). Also, an A22 residue is found in a number of HIV-1 strains (18), irrespective of their sensitivity or resistance to this product (Fig. 7).

DISCUSSION

The betulinic acid derivative RPR103611 blocked HIV-1 replication and cell-cell fusion mediated by HIV-1 envelope proteins but had no apparent effect on the adsorption of HIV-1 to the cell surface or on the interaction of CD4 and gp120 in vitro, strongly suggesting that it acts as an inhibitor of HIV-1 entry (15). By using a target cell line (HeLa-P4) in which the infectivity of HIV-1 can be assayed after a single replicative cycle, we found that RPR103611 could block HIV-1 infection when it was present during a 1-h virus-cell contact step, like several inhibitors of HIV-1 entry, such as MS8209, ATA, or bicyclams (22, 23). However, unlike these compounds, RPR103611 could also block infection when it was added after virus-cell contact, and it still had a detectable antiviral activity when it was added 1 h later. Unlike reverse transcription inhibitors, it had little or no activity 2 h after virus-cell contact. These experiments confirmed the effect of the RPR103611 compound on HIV-1 entry and showed that its target was accessible later after virus adsorption than for MS8209, ATA, or bicyclams. These properties might suggest a dual activity of RPR103611, on both early and late steps of virus entry. Alternatively, it might be envisioned that RPR103611 acts only on a late step of HIV-1 entry, provided that two assumptions are made. One is that most virions of the inoculum adsorb rapidly to cells but do not complete entry in the first 2 h, hence they remain accessible to the antiviral agent. It must also be assumed that adsorbed virions are not detached by washing the cells with medium or PBS. This view seems consistent with previous studies of the kinetics of membrane fusion mediated by HIV-1 Env. Experiments based on fluorescent-dye redistribution (10, 11) or virus photoinactivation (9) have indeed shown that the early steps, corresponding to virus adsorption or to cell-cell contact, were very rapid and that membrane fusion itself did not occur before a lag varying from 15 min to hours (9).

The early steps of HIV-1 entry, or of cell-cell fusion, are essentially mediated by the surface envelope protein gp120, while conformational changes in gp120 and gp41 are required before the latter can reach a fusion-active conformation and initiate membrane fusion (16, 33). The compounds that need to be present during virus-cell contact to block HIV-1, such as ATA, bicyclams, and MS8209, seem to target gp120. Indeed, ATA blocks the gp120-CD4 interaction (31), while differences in gp120 seem responsible for the sensitivity or resistance of

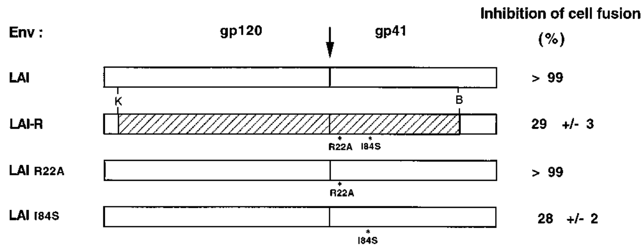

FIG. 6. gp41 mutations conferring drug resistance. Env expression vectors were transfected in HeLa cells, and coculture was performed with HeLa-P4 cells as described for Fig. 4, with or without 10 μM RPR103611. The LAI-R Env expression vector was obtained by substituting a PCR fragment (hatched bars) into the *env* gene of LAI using the natural sites *Kpn*I (K) and *Bam*HI (B).

HIV strains to MS8209 (25) or bicyclams (8). Our different results showing that genetic differences in gp41, and not in gp120, support the sensitivity or resistance of HIV-1 strains to RPR103611 are therefore consistent with its effect on a late step of HIV-1 entry.

The role of gp41 was observed in two different contexts, for chimeric envelope proteins derived from HIV-1 strains which are sensitive (LAI) and resistant (NDK) to RPR103611, on the one hand, and for a drug escape variant of the LAI strain (LAI-R) on the other. Drug resistance was observed only for LAI-NDK Env chimeras that had the gp41 ectodomain of NDK, while the origin of gp120 made no difference. Then, the envelope proteins of LAI-R and LAI differed only in their gp41 subunits, while their gp120 subunits were identical. A single mutation in LAI-R gp41 (I84S) was actually sufficient for drug resistance. Mutations in gp41 can affect the stability of the gp120-gp41 complex (3) and could also possibly modify the access of a compound to a target on gp120. There is indeed a precedent for a single mutation in the proximal helix of gp41 (A71T in the HXB2 strain) associated with resistance to a neutralizing antiserum, that is, to a reagent targeting primarily the gp120 subunit (26). However, the fact that we observed resistance to RPR103611 both with a point mutant of LAI gp41 and with several chimeric LAI-NDK gp41 proteins, as well as the antiviral activity of RPR103611 1 h after viral adsorption, leads us to favor the view that the antiviral activity of RPR103611 is exerted directly on a step of HIV-1 entry mediated by the gp41 subunit.

In the context of LAI-NDK chimeras, the amino-terminal region of NDK gp41 (residues 1 to 38), including the fusion peptide, and the region of gp41 preceding the membrane-spanning domain were not required for drug resistance. The region of the NDK gp41 ectodomain that extended beyond residue 108, including the distal α-helix domain, was not by itself sufficient to confer drug resistance. However, we cannot rule out that it plays a role in the NDK phenotype, in association with another domain of gp41. Reciprocal exchanges and substitutions of smaller domains of gp41 will be needed to define the minimal domain allowing a phenotype switch.

In the context of LAI gp41, the I84S mutation was sufficient to confer resistance to RPR103611. The isoleucine could be part of the RPR103611 target, and its replacement by a serine could prevent a direct interaction with the compound. Alternatively, the I84S mutation might also have indirect effects on gp41 and prevent the access of RPR103611 to another site. The I84 residue is indeed extremely conserved (18) and is only rarely replaced by another hydrophobic residue, for example, a phenylalanine in the MN strain (Fig. 7). A polar amino acid at this position might therefore have effects on gp41 conformation. These hypotheses could be addressed by testing the binding of anti-gp41 monoclonal antibodies to their gp41 epitopes in the presence and in the absence of drug (30). The fact that an isoleucine is found at position 84 in the gp41 of drug-resistant HIV-1 strains such as NDK or ELI (Fig. 7) indicates that other residues of gp41 are important for the antiviral activity of RPR103611.

Drugs acting on a gp41 target might be interesting candidates for the chemotherapy of HIV infection, since gp41 is far more conserved than gp120 across HIV-1 strains and a priori less tolerant to drug escape mutations. The frequency of spontaneous resistance to RPR103611 among HIV-1 and HIV-2 strains and the relatively easy adaptation to drug resistance in vitro certainly limit the interest of this compound. However, it might be a valuable tool for study of the molecular mechanism

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                                            ApaL I
        <--   f.p.    -->                     |  <---    DP-107     60
LAI     AVGIGALFLGFLGAAGSTMGARSMTLTVQARQLLSGIVQQQNNLLRAIEAQQHLLQLTVW
LAI-R   ---------------------A--------------------------------------
MN      -A.------------------A-V-------L---------------------M------
NDK     -I-L--V--------------A-V---------M----H---------------------
ELI     -I-L--M----------------V---------M--------------------------

                                                       Xba I
           DP-107   --->                                 |         120
LAI     GIKQLQARILAVERYLKDQQLLGIWGCSGKLICTTAVPWNASWSNKSLEQIWNNMTWMEW
LAI-R   -----------------------S------------------------------------
MN      --------V--------------F-----------T------------DD--------Q-
NDK     --------V-------R------------RH---------S----R--DE--Q-------
ELI     ------------------------------H---------S----R--NE--Q-------

                                                   EcoR V
            <----         DP-178          ---->      |        <-TM 180
LAI     DREINNYTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWFNITNWLWYIKIFIMIVGG
LAI-R   ------------------------------------------------------------
MN      E---D------Y-L-K---T----------------------D-----------------
NDK     E---D---G--Y-------I------K---------------S--K------L-------
ELI     E---D---G--Y-------T------K---------------S--Q-----------I--
```

FIG. 7. Amino acid sequences of the gp41 ectodomains of HIV-1 strains sensitive or resistant to RPR103611. The MN strain (subtype B) is sensitive to RPR103611, while ELI (subtype D) is resistant (15). The *Apa*LI, *Xba*I, and *Eco*RV sites were used for construction of certain LAI-NDK chimeras. Also shown are the locations of the DP-107 and DP-178 peptides corresponding to the predicted α-helix domains of gp41, the fusion peptide (f.p.), and the boundary of the membrane anchor domain (TM).

of gp41-mediated membrane fusion, like peptides derived from the gp41 sequence (39, 40), and for the designing of future antiviral agents.

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