Rapid propagation of low-fitness drug-resistant mutants of human immunodeficiency virus type 1 by a streptococcal metabolite sparsomycin

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Here we report that sparsomycin, a streptococcal metabolite, enhances the replication of HIV-1 in multiple human T cell lines at a concentration of 400 nM. In addition to wild-type HIV-1, sparsomycin also accelerated the replication of low-fitness, drug-resistant mutants carrying either D30N or L90M within HIV-1 protease, which are frequently found mutations in HIV-1-infected patients on highly active antiretroviral therapy (HAART). Of particular interest was that replication enhancement appeared profound when HIV-1 such as the L90M-carrying mutant displayed relatively slower replication kinetics. The presence of sparsomycin did not immediately select the fast-replicating HIV-1 mutants in culture. In addition, sparsomycin did not alter the 50% inhibitory concentration (IC50) of anti-retroviral drugs directed against HIV-1 including nucleoside reverse transcriptase inhibitors (lamivudine and stavudine), non-nucleoside reverse transcriptase inhibitor (nevirapine) and protease inhibitors (nelfinavir, amprenavir and indinavir). The IC50s of both zidovudine and lopinavir against multidrug resistant HIV-1 in the presence of sparsomycin were similar to those in the absence of sparsomycin. The frameshift reporter assay and Western blot analysis revealed that the replication-boosting effect was partly due to the sparsomycin’s ability to increase the –1 frameshift efficiency required to produce the Gag–Pol transcript. In conclusion, the use of sparsomycin should be able to facilitate the drug resistance profiling of the clinical isolates and the study on the low-fitness viruses.

Keywords: drug resistant mutants, enhancement of replication, HIV-1, low-fitness mutants, sparsomycin

Introduction

Highly active antiretroviral therapy (HAART) has been successful in controlling the progression of AIDS caused by HIV-1. However, HAART has accelerated the emergence and spread of multidrug-resistant HIV-1. Once drug-resistant HIV-1 occurs in a HIV-1-infected patient, the success rate of HAART drops substantially. Resistance testing has been shown to be valuable to optimize HAART against HIV-1 infection (Hirsch et al., 2000; Rodriguez-Rosado et al., 1999). Profiling drug resistance might be necessary even before the initiation of HAART because of the spread of drug-resistant HIV-1 (Boden et al., 1999; Gehringer et al., 2000; Verly et al., 1999).

Genotypic and phenotypic resistance testing are the two major ways to determine the drug resistance of clinical HIV-1 isolates. For genotyping, the HIV-1 genome isolated from the infected individuals is sequenced. This HIV-1 genome is then cross-referenced with a database and we are able to predict the drug resistance profile of HIV-1. However, it is impossible to predict the phenotype when we encounter a combination of mutations that has never been documented. This may raise a concern when a new drug is released in the market. Another problem in the genotyping is the presence of genotype-phenotype discordance (Parkin et al., 2003; Sarmati et al., 2002).

Alternatively, for the phenotypic resistance testing, the drug resistance profiles are measured by many biological/virological assay systems (Hertogs et al., 1998; Iga et al., 2002; Jarmy et al., 2001; Kellam & Larder, 1994; Menzo et al., 2000; Walter et al., 1999). Phenotypic resistance testing is powerful because the diagnosis is based on experimental observations. Among the systems, ones that depend on the multi-round HIV-1 replication seemed to provide the best drug resistance data reflecting the in vivo condition. However, many drug-resistant mutants have lower replication capabilities than wild-type (wt) HIV-1, which makes the phenotypic resistance testing difficult and time-consuming. In order to overcome these problems, it would be useful to develop a technique to make HIV-1
replicate faster without altering the effectiveness of antiretroviral compounds.

During our search for an inhibitor of HIV-1 replication, we found sparsomycin, a metabolite from Streptomyces sparsogenes, which reproducibly enhanced the replication of HIV-1. Therefore, we tested whether sparsomycin merits phenotypic drug resistance profiling studies on low-fitness HIV-1 isolates.

Materials and methods

Cells and viruses
Human embryonic kidney (HEK) 293T cells were maintained in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), penicillin and streptomycin (Invitrogen, Carlsbad, CA, USA). H9, Jurkat, SupT1 and HPB-Ma cells were maintained in RPMI1640 (Sigma-Aldrich) supplemented with 10% FBS, penicillin and streptomycin. All the cell lines were incubated at 37°C in a humidified 5% CO2 atmosphere. As previously described, HIV-1 (HXB2) was produced by transfecting proviral DNA into 293T cells and collecting the culture medium 3 days post-transfection (Komano et al., 2004). The replication-incompetent HIV-1 (HXB2 Δenv, Δrev, Δenv, Δnef) was produced by transfecting the proviral DNA carrying renilla luciferase with the nef open reading frame into 293T cells, along with the expression plasmid for env, tat, rev and nef (pIIIex) as described previously in Komano et al. (2004). As previously described, the D30N, L90M, and D25N protease mutants of HIV-1 were generated by the site-directed mutagenesis (Sugiura et al., 2002). The multidrug-resistant HIV-1 DR3577 was a clinical isolate from a patient on HAART in which reverse transcriptase carried the following mutations M41L, D67N, K70R, V75M, K101Q, T215F and K219Q; and protease carried the following mutations L10I, K20R, M36I, M46I, L63P, A71V, V82T, N88S and L90M. For the generation of replication-incompetent murine leukemia virus (MLV) vector expressing firefly luciferase, pCMMP luciferase was transfecting into 293T cells along with gag/pol and VSV-G expressing plasmids as described previously (Komano et al., 2004).

Chemical compound
Sparsomycin was either purchased from Sigma-Aldrich (cat. S1667) or obtained from Dr Nakajima (Toyama Prefectural University, Toyama, Japan). Sparsomycin was dissolved in 2mM dimethyl sulphoxide and stored at −20°C until use.

Monitoring HIV-1 replication
For HIV-1 infection, 1×10^6 cells were incubated with the culture supernatant containing approximately 10 ng of p24. Alternatively, wt HIV-1, or D30N and L90M mutants were introduced into cells either by electroporation or DEAE-dextran-mediated protocol as previously described (Matsuda et al., 1993; Miyauchi et al., 2005). The culture supernatants were collected every time the infected cells were split until they ceased to proliferate. The amount of p24 antigen of HIV-1 in the culture supernatants was quantified by using Retro TEK p24 antigen ELISA kit according to the manufacturer’s protocol (Zepro Metrix, Buffalo, NY, USA). The signal was detected by Vmax ELISA reader (Molecular Devices, Palo Alto, CA, USA).

Determining 50% inhibitory concentrations (IC50)
IC50 was calculated by using a reporter cell line, MARBLE, developed by Sugiura et al. (personal communication). In brief, a clone of HPB-Ma carrying the long terminal repeat (LTR)-driven firefly luciferase cassette integrated in its genome was infected with HIV-1 and incubated in the presence of varying concentrations of antiretroviral compounds for a week. The cells were then lysed to measure the firefly luciferase activity, which represented the propagation of HIV-1 in culture. The firefly luciferase activity was normalized by constitutively-expressed renilla luciferase activity. The dual luciferase assay was performed according to the manufacturer’s protocol (Promega, Madison, WI, USA). Chemiluminescence was detected by Lmax (Molecular Devices).

Reporter assay
The −1 frameshift reporters, pLuc (−1) and pLuc (0), were kindly provided by Dr Brakier-Gingras (Dulude et al., 2002). The renilla luciferase expression vector pRL/CMV was purchased from Promega. pLTR Luc encoded GFP-luciferase under the regulation of HIV-1’s LTR promoter (Komano et al., 2004). pLTRanfelLuc encoded renilla luciferase by substituting nef in the proviral context of HXB2 (Komano et al., 2004). Plasmids were transfected into 293T cells by Lipofectamine 2000 plus reagent in accordance with the manufacturers’ protocol (Invitrogen). For the detection of luciferase activities, the dual glo luciferase assay was performed at 2–3 days post-transfection or post-infection according to the manufacturers’ protocol (Promega). The signal was detected by Vmax ELISA reader (Molecular Devices).

Western blot analysis
COS-7 cells were transfected with Lipofectamine 2000 (Invitrogen) or FuGEN6 (Roche, Basel, Switzerland) according to the manufacturer’s protocol with proviral DNA encoding the D25N protease mutant. At 48 h post-transfection, cells were washed with PBS and lysed in a buffer containing 4% SDS, 100 mM Tris-HCl (pH 6.8), 12% 2-ME, 20% glycerol and bromophenol blue.
Samples were boiled for 10 min. Protein lysates approximately equal to 5×10⁴ cells were separated in 5–20% SDS-PAGE (Perfect NT Gel, DRC, Tokyo, Japan), transferred to a polyvinylidene fluoride (PVDF) membrane (Immobilon-P®, Millipore, Billerica, MA, USA), and blocked with 5% dried non-fat milk (Yuki-Jirushi, Tokyo, Japan) in PBS. For the primary antibody, we used rabbit anti-Gag polyclonal antibody or mouse anti-Gag monoclonal antibody. For the secondary antibody, either a biotinylated anti-rabbit antibody or a biotinylated antimouse goat antibody (GE Healthcare Bio-Science, Piscataway, NJ, USA) was used. For the tertiary probe, a horseradish peroxidase-conjugated streptavidin (GE Healthcare Bio-Science) was used. Signals were developed by incubating blots with a chemiluminescent horseradish peroxidase-conjugated streptavidin (GE Healthcare Bio-Science) and detected by using Lumi-Imager F1 (Roche).

Results

The structure of sparsomycin, a metabolite from Streptomyces sparsogenes, is unique in that it comprises two unusual entities, a monooxidithioacetel moiety and a uracil acrylic acid moiety (Figure 1A). H9 cells were infected with HIV-1 and then maintained in the presence of varying concentrations of sparsomycin. Dimethyl sulphoxide was added in the absence of sparsomycin throughout this study. At 7 days post-infection, a massive syncytial formation was found in the presence of sparsomycin (Figure 1B). The higher the concentration of sparsomycin, the faster p24 accumulated in the culture supernatants (Figure 1C).

Similar observations were made in Jurkat, SupT1 (Figures 1D and E), and HPB-Ma cells although the speed of p24 accumulation appeared different among the cell lines. On the other hand, sparsomycin did not show any detectable effect on the cell growth under concentrations of 500 nM.

These results could be due to sparsomycin’s ability to either boost HIV-1 replication or select a mutant that replicated substantially faster than the wt HIV-1. To differentiate these possibilities, we recovered the virus-containing culture supernatants from the H9 cell culture at the peak of HIV-1 replication in the presence of 400 nM sparsomycin (asterisk in Figure 1F). Then fresh H9 cells were infected with the recovered virus, the cells were split into two samples and 400 nM of sparsomycin was added to each sample. If sparsomycin selected fast-growing mutants, the replication profiles of HIV-1 should resemble the original sample with sparsomycin (solid circle, Figure 1F) regardless of sparsomycin’s presence. However, the replication profile in the presence of sparsomycin shifted leftward (Figure 1G), suggesting that it was unlikely that sparsomycin selected the fast-replicating viral mutants. Therefore, it is likely that sparsomycin boosted HIV-1 replication.

Replication-enhancing effects were also seen by using the chemically-synthesized derivatives of sparsomycin (unpublished data; Nakajima et al., 2003). The replication-boosting effect levelled-out at 500 nM, an approximately 20-fold lower concentration than the 50% toxic dose (TD₅₀) of sparsomycin (Ash et al., 1984).

To demonstrate the usefulness of sparsomycin in HIV-1 research, we have examined whether sparsomycin can also boost the replication of drug-resistant low-fitness isolates. The D30N and L90M are common drug-resistant mutations found within HIV-1 protease in HIV-1-infected patients on HAART (Devereux et al., 2001; Kantor et al., 2002; Pellegrin et al., 2002; Sugira et al., 2002). We introduced proviral DNA carrying the D30N or L90M mutation into H9, Jurkat, and SupT1 cells. HIV-1 replication was then monitored in the presence of 400 nM of sparsomycin. The replication of both viral mutants was substantially enhanced in the presence of sparsomycin in H9 cells (Figures 2A and B). The replication of the L90M-carrying mutant was also enhanced in Jurkat and SupT1 cells (Figures 2C and D). Of note, the replication enhancement appeared profound when HIV-1 displayed relatively slower replication kinetics (for example, the replication of D30N-carrying mutant versus the wt HIV-1 in H9 cells or the replication of HIV-1 in SupT1 versus H9 cells).

Considering the use of sparsomycin in the phenotypic resistance testing, it is critical to know whether sparsomycin affects HIV-1’s sensitivity to the antiretroviral drugs. The respective IC₅₀ of representative antiretroviral drugs in the absence and the presence of 400 nM sparsomycin were as follows: reverse transcriptase inhibitors; lamivudine, 13.7 and 10.4 nM, and stavudine, 6.3 and 17.0 nM; an non-nucleoside reverse transcriptase inhibitor, nevirapine, 78.2 and 146.4 nM; and protease inhibitors, nelfinavir, 2.8 and 1.0 nM, indinavir, 4.2 and 3.0 nM, and amprenavir, 3.4 and 3.3 nM. Then, we examined whether the presence of sparsomycin affected the IC₅₀ of both zidovudine (AZT) and lopinavir (LPV) against a multidrug-resistant HIV-1 isolate, DR3577. The magnitude of both AZT and LPV-resistance of DR3577 was in the order of 2 log (data not shown). The IC₅₀ of AZT in the presence and absence of 400 nM sparsomycin were 14.0 and 36.7 nM, respectively, and for LPV they were 103.1 and 78.9 nM, respectively. These data suggested that the presence of sparsomycin did not significantly influence the IC₅₀ of antiretroviral drugs on the replication of both wt and drug-resistant HIV-1.

Finally, we investigated the possible mechanisms that sparsomycin enhanced the replication of HIV-1 and its mutants although the estimated magnitude of enhancement per single replication cycle was small. To do this, we used non-T cells to increase the sensitivity of assays. First, we examined if the early phase of HIV-1’s life cycle was
positively affected by sparsomycin. In the presence of increasing concentrations of sparsomycin, 293 CD4+ T-cells and NP2 CD4 CXCR4 cells were infected with either a replication-deficient HIV-1 vector enveloped with its own Env or a VSV-G-pseudotyped MLV vector. Two days post-infection, cells were lysed to measure the luciferase activities representing the efficiency of viral infection. Our results indicate that luciferase activities were not significantly increased at the replication-enhancing dose for both HIV-1 and MLV vectors (Figure 3A). Thus suggesting that the early phase of the retroviral life cycle was not detectably affected by sparsomycin.
Next, we examined the possible active role of sparsomycin in the late phase of HIV-1's life cycle. Sparsomycin has been reported to be a potential enhancer of the -1 frameshift (Dinman et al., 1997). Therefore, we tested whether sparsomycin could positively affect the efficiency of the translational -1 slip at HIV-1's frameshift signal using the reporter assay system established by Dulude et al. (2002). The -1 frameshift reporter was created by placing the firefly luciferase in the pol frame, pLuc(-1), whereas the control plasmid pLuc(0) has the luciferase in the gag frame after the frameshift signal (Figure 3B). In addition, HIV-1's LTR-driven luciferase reporter constructs were tested (pLTR Luc and pLTRΔnefLuc; Figure 3B). We transfected these reporter plasmids into 293T cells along with the renilla luciferase-expressing plasmid phRL/CMV (Promega) to measure the non-specific or toxic effects, if any, of sparsomycin. Cells were incubated in the presence of varying concentrations of sparsomycin for 3 days. Then the dual luciferase assay was performed. The pLuc(-1) behaved differently from the other groups in that the luciferase activities from the pLuc(-1) increased in a dose-dependent fashion. The magnitude of increase was 2.3-fold at the replication-enhancing dose (Figure 3C). The positive correlation between the relative luciferase activity and the concentration of sparsomycin was statistically significant (r=0.926, P<0.001, n=8, Student's t-test). In contrast, the luciferase activities from the other reporters, even the renilla luciferase plasmid...
co-transfected with the pLuc(-1) vector, remained unchanged (Figure 3C). These data suggested that sparsomycin positively affected the efficiency of HIV-1's –1 frameshift. It also suggested that sparsomycin did not enhance transcription from the viral promoter or the translation of proteins driven by the LTR promoter to enhance HIV-1 replication.

If the efficiency of –1 frameshift was increased, we would expect that the Gag–Pol to Gag ratio to increase. To test this, we transfected COS-7 cells with the HIV-1's proviral DNA carrying the D25N mutation in protease that produced catalytically inactive protease to increase the sensitivity of detecting Gag–Pol (Xie et al., 1999). When sparsomycin was added, the intensities of Gag–Pol gradually increased in relation to the reporter assay. The Gag–Pol to Gag ratio reached 1.3-fold at 400 nM sparsomycin when normalized with results produced in the absence of sparsomycin (Figure 3D). The average and standard deviation of the Gag–Pol to Gag ratio from four independent experiments were 1.29 ± 0.14 at the replication enhancing concentration of sparsomycin (1.29-, 1.48-, 1.16-, and 1.24-fold increase). Similar results were obtained by using

Figure 3. The possible mechanism of HIV-1 replication enhancement by sparsomycin

(A) The single round infection efficiencies of HIV-1 and murine lukaemia virus (MLV) vectors measured by the virally-encoded luciferase activities in 293 CD4+ T-cells and NP2 CD4 CXCR4 cells in the presence of varying concentrations of sparsomycin. (B) The schematic drawing of constructs used in the reporter assay. The HIV-1's frameshift signal (fs) was placed between the CMV promoter and the luciferase. The luciferase was placed in either the gag frame (pLuc(0)) or the pol frame (pLuc(-1)). The renilla luciferase expression vector phRL/CMV was used in parallel. The pLTR Luc encodes the GFP-luciferase driven by HIV-1's LTR promoter. The pLTR/nefLuc has the renilla luciferase substituting nef in the proviral context of HXB2. (C) The luciferase activities from the above reporter constructs without sparsomycin were set as 100% and the relative luciferase activities in the presence of sparsomycin were shown. The renilla luciferase activities from phRL/CMV were shown for the pLuc(-1) (solid circle) and pLTR/nefLuc (solid triangle) transfections in particular. The pLuc(-1) behaved differently from the other groups and the positive correlation between the relative luciferase activity and the concentration of sparsomycin was statistically significant (r=0.926, P<0.001, n=8, Student's t-test). The si was within 10% from the average. Shown are the representative data from two independent experiments. (D) Western blot analysis to measure the Gag–Pol and Gag ratio. Cell extracts were separated in the SDS-polyacrylamide gel and immunoblotted by using the rabbit polyclonal antibodies raised against p24. (lane 1, 0 nM; lane 2, 20 nM; lane 3, 200 nM; lane 4, 400 nM; lane 5, MOCK). The lower panel shows the Gag–Pol signal obtained from the long exposure of the same blot.
two different antibodies recognizing Gag. We were unable to detect a significant increase in the Env/Gag ratio (unpublished data), suggesting that the sparsomycin's effect on Gag–Pol/Gag ratio was specific. These data suggested that the translational efficiencies of viral proteins were not equally enhanced by sparsomycin. Altogether, it was strongly suggested that the sparsomycin's replication-boosting effect on HIV-1 was partly due to the enhancement of the –1 frameshift efficiency.

Discussion

In the present study, we have demonstrated that sparsomycin is an enhancer of HIV-1 replication in many human T cell lines at concentrations between 400–500nM. Our preliminary observation suggested that HIV-1 replication was also enhanced in primary peripheral blood monocyte culture (data not shown). Sparsomycin should be able to accelerate the study on the low-fitness HIV-1 such as drug-resistant mutants. As sparsomycin did not alter the IC50 of multiple antiretroviral drugs on both wt and drug-resistant HIV-1, its usage should be able to facilitate the phenotypic resistance testing of clinical isolates and as a result, benefit HIV-1-infected patients. Our observation raised an immediate concern as to whether sparsomycin-producing Streptomyces species caused an opportunistic infection in humans, which influenced AIDS progression. However, we did not find any reports suggesting so.

Sparsomycin and puromycin are the only antibiotics that can inhibit protein synthesis in bacterial, archaeal and eukaryotic cells (Ottenheijm et al., 1986; Porse et al., 1999). Sparsomycin has the ability to enhance the –1 frameshift in mammalian cells as well as S. cervisiae (Dinman et al., 1997). The proposed molecular mechanism behind this ability was either through a higher affinity of the donor stem for the ribosome and slowing down the rate of the peptidyl transfer reaction, or a change in the steric alignment between donor and acceptor tRNA stems resulting in decreased peptidyl-transfer rates. Conversely, puromycin is not known to enhance the –1 frameshift in mammalian cells. At sub-toxic concentrations, puromycin was unable to enhance the HIV-1 replication (unpublished data). These data, along with the data provided in this paper, implied that the sparsomycin's unique ability to enhance the –1 frameshift might play a role in boosting the HIV-1 replication.

The maintenance of the –1 frameshift efficiency at the optimal range is critical for HIV-1 to replicate (Jacks et al., 1998). Therefore, limiting Gag–Pol production should lead to an inhibition of viral replication because pol encodes enzymes essential for viral replication (Levin et al., 1993). In contrast, it was also reported that increasing the Gag–Pol to Gag ratio by twofold resulted in a reduction of viral replication (Hung et al., 1998; Shehu-Xhilaga et al., 2001). Thus, a modest alteration of the –1 frameshift efficiency should markedly affect the replication capacity of HIV-1. Our data indicated that sparsomycin increased the efficiency of –1 frameshift by 1.3-fold, which produces a better replication capacity for HIV-1. As a result, we hypothesize that HIV-1 has a 'suboptimal' –1 frameshift efficiency. In theory, the 1.3-fold difference per one replication cycle becomes approximately 10-fold after 10 rounds of viral replication cycle because the effect accumulates exponentially. The difference should become larger when HIV-1 replicates with the slower kinetics and the replication profile is monitored over a longer time course. In fact, our experimental data were in good agreement with the above estimation. In nature, HIV-1 does not accumulate mutations within the frameshift signal to achieve the higher frameshift efficiencies. This implies that there are multiple and complex regulatory mechanisms that keep the efficiency of the –1 frameshift at suboptimum. Under these conditions, the best efficiency of HIV-1 survival in the host might be achieved. Altogether, one of the possible mechanisms that sparsomycin boosted the HIV-1 replication could be the enhancement of the –1 frameshift efficiency.

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References


