Evidence for the cellular uptake of anti-HIV-1 double drug KNI-1039

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Key Words: AZT; Protease; RT inhibitor; Double-Drug.

ABSTRACT

Combination therapy, or highly active antiretroviral therapy (HAART) comprising at least three anti-HIV drugs, nucleoside/nucleotide reverse transcriptase (RT) inhibitors, non-nucleoside reverse transcriptase inhibitors and protease (PR) inhibitors has become the standard treatment for AIDS, or HIV infected patients. This article reports the uptake and degradation of an anti-HIV double drug KNI-1039, a linked product of a PR inhibitor KNI-727 with an RT inhibitor AZT. Liquid chromatography/mass spectrometric analysis demonstrated that lymphocytes were able to uptake double drug KNI-1039 and that KNI-1039 reverted back to the parent drugs KNI-727 and AZT within lymphocytes. The uptake and dissociation of KNI-1039 released the parent drugs KNI-727 and AZT within the cell where both RT and PR inhibition were demonstrated. This anti-HIV effect of KNI-1039 was revealed by HIV-1 RT and PR inhibition studies in HIV-1-infected lymphocytes. This ‘double drug’ strategy involving the combination of two different classes of inhibitors together may enhance the anti-HIV efficacy synergistically, and help overcome the physiochemical, biological and anti-viral limits of the parent compounds.

Introduction

Human immunodeficiency virus type 1 (HIV-1) protease (PR) and reverse transcriptase (RT) are major targets for anti-HIV-1 therapy (HURWITZ et al., 2002; KAPLAN et al., 1993; TANG et al., 2003). Current anti-HIV-1 treatment regimens include combination chemotherapy comprising nucleo-
side, non-nucleoside RT inhibitors and PR inhibitors (DECLERQ, 2002; GOTO et al., 2001), which provides sufficient anti-viral effect and prevents the emergence of drug resistant mutants (DECLERQ., 2002; HURWITZ et al., 2002). This regimen also called highly active anti-retroviral therapy (HAART) has become the standard treatment for HIV-1 infected patients, and has shown ability to reduce viral loads and prolong survival of the patients (HURWITZ et al., 2002; STRANFORD et al., 2001). The concept of HAART is multi-targeting inhibition of viral functions by a cocktail of two or more drugs (DECLERQ E., 2002; STRANFORD et al., 2001). Though HAART has many advantages as HIV-1 is inhibited at various stages of the viral life cycle, it poses problems in clinical situations (PAPADOPOLOUS-ELEOPULOS et al., 1999; STRANFORD et al., 2001). The disadvantage of HAART may be eliminated or reduced if multi-targeting efficacy can be obtained in a single compound.

Matsumoto et al., (MATSUMOTO et al., 2001 a; MATSUMOTO et al., 2000) have synthesized and reported a group of compounds which showed inhibition properties against HIV-1 PR and RT. KNI-1039 synthesized by Matsumoto et al. (2001 a, 2000) represents a new class of drugs which uses a double drug strategy. KNI-1039 is a compound prepared by linking a PR inhibitor KNI-727 with an RT inhibitor AZT into a single molecule. In KNI-1039, AZT and KNI-727 are linked together by the esterification of the hydroxyl groups with a glutaryl glycine linker (MATSUMOTO et al., 2001 a; MATSUMOTO et al., 2001b; MATSUMOTO et al., 2000). Since the formation of double drug KNI-1039 involves the linking of free hydroxyl groups, the toxicity (PAPADOPOLOUS-ELEOPULOS et al., 1999) involved with these hydroxyl groups may be eliminated (MATSUMOTO et al., 2001a; MATSUMOTO et al., 2001b; MATSUMOTO et al., 2000).

Linking of a protease inhibitor and an RT inhibitor in the form of KNI-1039 into a single molecule not only includes the advantage of dual targets of attack into a single delivery system but also allows high accumulation of the parent drugs into the cells. This is because PR inhibitors which are normally insoluble in water become water soluble on conjugation (MATSUMOTO et al., 2001a; MATSUMOTO et al., 2001b; MATSUMOTO et al., 2000) allowing them to reach the target cell and enter it in a water soluble form. Since KNI-1039 is a linked form of AZT and a protease inhibitor KNI-727, every molecule of AZT taken up actively by the cell will result in a molecule of KNI-727 being taken up with it. This novel idea may help overcome the low cell permeability of PR inhibitors observed up until now (ARMBRUSTER et al., 2001; HOGGARD et al., 2003; MATSUMOTO et al., 2001a; MATSUMOTO et al., 2001b; MATSUMOTO et al., 2000; MEADEN et al., 2002).

Although KNI-1039 is shown to be cleaved into its parent drugs in a cell free system, and to inhibit the cytopathic effect of HIV-1 in cell culture (MATSUMOTO et al., 2001c) the uptake into Molt-4 cells and the cleavage of KNI-1039 within these cells has not been studied in detail. In this paper an attempt has been made to see if the uptake and cleavage occurs inside Molt-4 lymphocytes and if these cleaved products act as PR and RT inhibitors within Molt-4 lymphocytes.

Materials and Methods

Cells, Virus and Drugs

Cells used were human T cell line Molt-4 cells. The cells were cultured on a regular basis prior to experimentation, in RPMI-1640 medium supplemented with 10% fetal bovine serum. For the experiment the cell count was adjusted to 1 x 10⁵ cells which were seen to be the average cell count. The virus stock used in this experiment was HIV-1/LAV, which was re-cultured in Molt-4 cells, harvested and aliquoted into 1 ml freeze bottles and frozen at -80°C till ready for use in the experiment. The virus stock was then added to Molt-4 cells to infect them for use in the experiments.

Drugs used were AZT an RT inhibitor, KNI-727 a PR inhibitor and KNI-1039 a conjugate of KNI-727 and AZT. A mix of AZT and KNI-727 added together as separate drugs was also used. KNI-1039 and KNI-727 were synthesized at the Department of Medicinal Chemistry, Kyoto Pharmaceutical University. The drugs were dissolved in dimethyl sulphoxide (DMSO) and stored in 1 mM 5 ml aliquots. The drugs were diluted into the required intended experimental concentration of 100 μM as follows: 100 μl of drug was added to 900 μl of RPMI to get 100 μM of KNI-1039, AZT, KNI-727 and a mixture of AZT and KNI-727 added together as separate drugs. Drugs were then frozen at -80°C.

LC/MS analysis

Liquid chromatography/mass spectrometry (LC/MS) analysis was performed on the cell extracts of KNI-1039, KNI-727, AZT, and a
mixture of KNI-727 and AZT added together as separate drugs treated cells. Briefly 1x 10^6 Molt-4 cells were incubated for a period of 18 h at 37°C up to a maximum concentration of 30 μM (initial concentration) but not exceeding the 7 day cytotoxic concentration of KNI-1039, AZT, KNI-727 and a mixture of KNI-727 and AZT added together as separate drugs. Control non-drug treated flasks were also set up. The cells were then centrifuged at 200 x g for 10 minutes and resuspended in RPMI so as to wash the cells. This wash step was repeated 3 times after which the cells were centrifuged again and resuspended in 0.5 ml ice cold distilled water. The suspension was kept on ice till the suspension showed signs of cell lysis, and was homogenised occasionally to promote lysis. After total cell lysis, the suspension was ultracentrifuged at 140,000 x g for 30 min. The supernatant was collected, stocked and frozen, while the precipitate was re-suspended in distilled water and frozen.

The LC/MS system used in this study was Hitachi La Chrom HPLC system consisting of an L-7100 low-pressure gradient pump, L-7200 auto sampler, L-7300 column oven, L-7450 UV detector (Hitachi High-technologies, Tokyo, Japan) and a Hitachi M-8000 three dimensional quadruple mass spectrometry (3DQMS) with a sonic spray ionization (SSI) interface (Hitachi High-technologies, Tokyo, Japan).

**PCR analysis**

PCR was used for the detection of HIV-1 DNA in the cells. Drug treated cells (up to a maximum concentration of 30 μM but not beyond the cytotoxic concentration limit) were lysed in 50 μl of a lysis solution (final concentration of 10 mM Tris-HCl at pH 8.3, 2.5 mM MgCl2, 50 mM KCl, 0.5% Tween 20, 0.5% Nonidet-P40 and 120 μg/ml proteinase K) at 56°C for 1 h. Proteinase K was then inactivated at 95°C for 1 min and a final 10 min extension step at 72°C. A portion of the amplified PCR products was subjected to gel-electrophoresis, and the gel was stained with ethidium bromide.

**Electron Microscopy**

For ultrathin-section electron microscopy (EM) analysis, control treated HIV-1 persistently infected cultures and KNI-1039, KNI-727, AZT and a mixture of KNI-727 and AZT added together as separate drugs were processed as described previously (GOTO et al., 2001). In brief, cell pellets were obtained by low speed centrifugation of the cell cultures. The pellets were fixed with 2% glutaraldehyde in 0.01 M cacodylate buffer (pH 7.2) for 2 h at 4°C then post fixed with 1% osmium tetroxide in the same buffer for 2 h and treated with 1% tannic acid. After dehydration in ethanol, the specimens were embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Hitachi H-7100 electron microscope.

**Results**

**Liquid Chromatography/Mass Spectrometry (LC/MS) analysis for standardization of parent drug KNI-727 and AZT ionization peaks in Molt-4 cells.**

To determine uptake of KNI-1039 into lymphocyte and the de-linking of the parent drugs, molecular ions corresponding to the drugs in Molt-4 cells were examined in the LC/MS graph.

To determine the peaks corresponding to the drugs tested, cells were examined by LC/MS after treatment separately with KNI-727, AZT, and a mixture of KNI-727 and AZT added together as separate drugs. KNI-727 treated cells showed the presence of KNI-727 at the expected molecular ion peak (molecular mass) m/z 556 and 578 (Fig.1a). Cells only treated with AZT showed a presence of the expected molecular ions at a peak of (molecular mass) m/z 223 and 266 (Fig.1b). Molt-4 cells were further treated with a mixture of AZT and KNI-727 added together as separate drugs to see the presence of each drug in the cell. Peaks corresponding to KNI-727 (Fig.1c) and AZT (Fig.1d) were detected in the cells.

**Liquid Chromatography/Mass Spectrometry (LC/MS) analysis for the uptake of KNI-1039 and its de-linking into parent compounds KNI-727 and AZT**

Similarly to detect KNI-1039 and the parent drugs KNI-727 and AZT the LC/MS parameters were standardized for the expected KNI-1039.
Fig.1. LC/MS spectrographs of KNI-727, AZT and a mixture of AZT and KNI-727 added together as separate drugs treated Molt-4 cells.

(a) **KNI-727 treated Molt-4 cells.** The detection parameters of LC/MS were optimised for KNI-727. There is a clear presence of KNI-727, shown by a peak at (molecular mass) $m/z$ 578 marked with arrows.

(b) **AZT treated Molt-4 cells.** The detection parameters of LC/MS were optimised for AZT. There is a clear presence of AZT, shown by a peak at (molecular mass) $m/z$ 266. This peak is much smaller than the AZT peak of KNI-1039 treated cells marked with arrows.

(c) **AZT and KNI-727 added together as separate drugs treated Molt-4 cells.** The detection parameters were optimised for KNI-727 in this spectrograph. There is a clear peak of KNI-727 present at (molecular mass) $m/z$ 578 marked with arrows.

(d) **AZT and KNI-727 added together as separate drugs treated Molt-4 cells.** The detection parameters were optimised for AZT in this spectrograph. There is small peak of AZT present at (molecular mass) $m/z$ 266 marked with arrows. This peak is much smaller compared to the peak of AZT in KNI-1039 treated cells.
molecular ions 976 and 998. A clear LC/MS peak was observed in KNI-1039 treated cells at (molecular mass) m/z 976 and 998 which confirmed the presence of KNI-1039 inside the cells (Fig.2a). When the LC/MS parameters were standardized for detecting KNI-727 in KNI-1039 treated cells, a clear peak of molecular ions corresponding to KNI-727 was observed at (molecular mass) m/z 556 and 578 (Fig.2b). Finally when the LC/MS parameters were standardized for detecting AZT in KNI-1039 treated cells; two clear peaks confirming the presence of AZT were detected at (molecular mass) m/z 223 and 266 (Fig.2c). AZT detected in KNI-1039-treated Molt-4 cells was the highest compared to both AZT treated cells and KNI-727 and AZT added together as separate drugs treated cells. Since KNI-1039-treated cells showed the presence of KNI-1039, KNI-727 and AZT, KNI-1039 is taken up by the cell and successfully de-links into its parent drugs KNI-727 and AZT (Fig.3). Control cells not treated with any drug failed to show the same peaks as did the wash buffer obtained after washing the cells (data not shown) thus confirming the above drugs were found inside the cells.

fig.2. LC/MS data spectrographs for KNI-1039 treated Molt-4 cells

(a) KNI-1039 treated Molt-4 cells. A clear peak of KNI-1039 at (molecular mass) m/z of 976 marked with arrows is visible in the spectrograph. This shows that KNI-1039 was able to enter the cell. Spectrometric conditions were optimised for KNI-1039.

(b) KNI-1039 treated Molt-4 cells detection parameters optimised for KNI-727. To see that KNI-1039 degrades into KNI-727 and AZT into the cell, LC/MS was performed and optimised for KNI-727. The spectrograph shows a clear peak for KNI-727 at an (molecular mass) m/z of 578 marked with arrows. This shows that KNI-1039 was able to breakdown and release KNI-727.

(c) KNI-1039 treated Molt-4 cells detection parameters optimised for AZT. To see that KNI-1039 degrades into AZT in the cell, LC/MS was performed and optimised for AZT. The spectrograph shows a clear peak for AZT at an (molecular mass) m/z of 266 marked with arrows. This shows that KNI-1039 was able to breakdown and release AZT. The peak detected in AZT from KNI-1039 was much larger than the peak in only AZT treated cells and KNI-727 and AZT mix treated cells.
Effect of KNI-1039 on HIV-1 RT in acute infection system

The effect of KNI-1039 on HIV-1 RT was determined in acutely infected cells by PCR analysis for proviral DNA. PCR analysis of the cells treated up to a maximum initial concentration of 30 μM but not exceeding the cytotoxic concentration of KNI-1039 showed a total absence of viral DNA at a weight of 141 bp corresponding to the HIV-1 LTR primer sequence (Fig.4). The same result was also observed with 30 μM (initial concentration not exceeding cytotoxic concentration) of AZT which also showed a negative result for cells treated with AZT. AZT and KNI-727 added together as separate drugs also showed no band production. Thirty micromolar (initial concentration not exceeding cytotoxic concentration limit) of KNI-727 showed a slight band production but this was expected and will be discussed further in the discussion section. These findings indicate that KNI-1039 blocks reverse transcription in the HIV-1 life cycle.

Effect of KNI-1039 on HIV-1 PR in a persistent infection system

The effect of KNI-1039 on HIV-1 PR was determined in persistently infected cells morphologically by electron microscopic analysis. In HIV-1 infected Molt-4 cells treated with KNI-1039 there was a total absence of mature virus formation (Fig.5a). KNI-727, along with a mixture of KNI-727 and AZT added together as separate drugs also showed a total absence of mature virus formation (Fig.5b, 5d). AZT showed a maximum number of mature viral particles in this experiment (Fig.5c).
Fig. 5. Electron micrographs of KNI-1039, KNI-727 and AZT treated Molt-4 HIV-1 persistently infected cells.

(a) KNI-1039 double drug treated HIV-1 persistently infected cells. The slide shows a total absence of mature HIV-1 virions, showing the drugs ability to inhibit protease and viral maturation during the persistent stage of infection. Most of the virus particles seen are immature particles interspersed with a few aberrant particles (marked with arrows). Aberrant particles were defined as showing neither mature nor immature characteristics.

(b) KNI-727 protease inhibitor treated HIV-1 persistently infected cells. The slide shows a total absence of mature HIV-1 virions, showing the drugs ability to inhibit protease and viral maturation during the persistent stage of infection. Most of the virus particles seen are immature particles interspersed with a few aberrant particles (marked with arrows). Aberrant particles were defined as showing neither mature nor immature characteristics.

(c) AZT treated HIV-1 persistently infected cells. The slide shows the presence of abundant mature HIV-virions, along with very few immature and aberrant particles (marked with arrows). Aberrant particles were defined as showing neither mature nor immature characteristics. This slide shows that AZT was unable to prevent viral maturation or in other words was unable to inhibit the function of protease in HIV-1, this shows the inability of AZT to function as a protease inhibitor in the persistent stage of infection.

(d) Mixture of KNI-727 and AZT added together as separate drugs treated HIV-1 persistently infected cells. The slide shows a total absence of mature HIV-1 virions (marked with arrows), showing the ability of KNI-727 to inhibit protease and viral maturation during the persistent stage of infection. Most of the virus particles seen are immature particles interspersed with a few aberrant particles. Aberrant particles were defined as showing neither mature nor immature characteristics.
The viral particles were categorised as mature, immature and aberrant virus particles and then statistically analysed. Aberrant particles were defined as particles which could neither be categorised as mature nor immature (BAHMANI et al., 2000; GOTO et al., 2001). Table 1 shows the formation of mature virions corresponding to the drugs which were used in the experiment. KNI-1039 showed a total absence of mature virus particles at 0% and a presence of immature virus particles at 84.8% along with the formation of aberrant particles at 15.2%. KNI-727 along with KNI-1039 showed a total absence of mature particle formation at 0% followed by the presence of immature particles at 92.9% and aberrant particles at 7.1% (Table.1). A mixture of KNI-727 and AZT added together as separate drugs also showed a total absence of mature particles at 0% and the presence of 94.2% immature particles with a remainder 5.8% observed as aberrant particles. In contrast to KNI-1039, KNI-727 and a mixture of KNI-727 and AZT added together as separate drugs, AZT showed the presence of mature HIV-1 at 93.5% followed by immature particles at 1.6% and aberrant particles at 4.6% (Table.1). Thus cells treated with AZT showed a maximum production of HIV-1 virions highlighting AZT’s inability to inhibit viral protease. KNI-1039 and KNI-727 on the other hand showed success in inhibiting viral maturation induced by protease. Since the absence of mature particles was associated with inhibition of viral PR, these findings indicated that KNI-1039 inhibits viral protease.

Discussion

HIV-1 RT is vital to the transcription of the viral gene (MEADEN et al., 2002; PAPADOPOLOUS-ELEOPULOS et al., 1999), and HIV-1 PR is essential to viral maturation and functional protein formation (GOTO et al., 2001; KAPLAN et al., 1994). Since these functional enzymes are highly conserved during viral replication, they have become much sought after targets for anti-HIV therapy or HAART (DECLERQ et al., 2002; GOTO et al., 2001; HURWITZ et al., 2002; STRANFORD et al., 2001). Double-drug KNI-1039 is an attempt to improve the drug delivery and uptake of anti-HIV-1 compounds, AZT an RT inhibitor and KNI-727 a PR inhibitor, and is an attempt to combine the advantages of both an RT inhibitor and PR inhibitor into a single compound. In previous papers it has been shown that KNI-1039 has both the advantages of an RT inhibitor with a PR inhibitor while overcoming the disadvantages associated with their use singularly (MATSUMOTO et al., 2001b; MATSUMOTO et al., 2000). In this paper further aspects of drug delivery including drug uptake into the cell, cleavage into effector parent drugs and their corresponding anti-HIV-1 inhibitory effects have been examined.

Data obtained from LC/MS experiments showed that double-drug KNI-1039 entered Molt-4...
cells as KNI-1039 and de-linked into its parent drugs within the cells. These results proved that KNI-1039 did not de-link outside the cell. Previous studies on effectivity/cytotoxicity (MATSUMOTO et al., 2000) have found that KNI-1039 has an EC_{50}/CC_{50} ratio higher than AZT and much higher than that of a mixture of KNI-727 and AZT added as separate drugs. The studies confirm that KNI-1039 does not de-link to release the parent compounds outside the cell, owing to the fact that if such an event had occurred the EC_{50}/CC_{50} values of KNI-1039 would not be higher than a mixture of KNI-727 and AZT added together as separate drugs.

Once entry of the double drug KNI-1039 into the cell and its de-linking into the parent compounds was confirmed by LC/MS experiments, further experiments were done to reconfirm the ability of the parent drugs forming the double drug KNI-1039 to revert back to their active forms as an anti-HIV-1 RT inhibitor and an anti-HIV-1 PR inhibitor. KNI-1039 showed the ability to inhibit HIV-1 RT by inhibiting the synthesis of proviral DNA as was observed by PCR analysis which showed a total absence of proviral DNA in KNI-1039 treated cells. AZT and a mixture of KNI-727 and AZT added together as separate drugs also showed the ability to inhibit proviral DNA production. These data prove that double drug KNI-1039 is de-linking and the PR inhibitor KNI-727 is separating to release AZT in its singular effector form as an RT inhibitor inside the cell and is capable of preventing the synthesis of proviral DNA in the same way as non-linked AZT.

Once reverse transcription of the viral gene and its integration into the cellular DNA has occurred, the only way of preventing further spread of the virus to other cells is by stopping the formation of mature HIV-1 particles which are highly infectious (IKUTA et al., 1988). At the persistent infection stage, the inhibition of PR by a protease inhibitor would interfere with viral maturation hence stop further cell infection. KNI-1039 was successful in inhibiting HIV-1 viral maturation as were KNI-727 and a mixture of KNI-727 and AZT added together as separate drugs but AZT was not successful. These results prove further that double drug KNI-1039 is de-linking to release KNI-727 in its singular effective form as a PR inhibitor inside the cell and is capable of preventing the maturation or synthesis of infectious HIV-1 virus particles in the same way as non-linked normal KNI-727.

Linkage of an RT inhibitor with a PR inhibitor into a single molecule has more than one advantage over the parent drugs administered separately while at the same time eliminating the disadvantages known to the practitioners of HAART. Double-drug KNI-1039 successfully delivers an RT inhibitor and a PR inhibitor into the cell. The double-drug was able to dissociate into its parent compounds an RT inhibitor and a PR inhibitor, and was able to inhibit HIV-1 at both the acute stage and persistent stage of infection.

In this manuscript the experiments were focused on drug uptake and the ability of the parent compounds AZT and KNI-727 to show known anti-HIV-1 activity within the cell. Experiments to determine the activation of AZT both, in its linked form as a part of KNI-1039 and its de-linked form were not examined here, therefore it was not possible to confirm whether KNI-1039 itself, in its linked double drug form inhibits HIV-1 RT directly within HIV-1 infected Molt-4 cells. Matsumoto et al. (MATSUMOTO et al., 2001a; MATSUMOTO et al., 2001b; MATSUMOTO et al., 2001c; MATSUMOTO et al., 2000) demonstrated that in a cell free system KNI-1039 revealed 55% PR inhibiting activity in its linked double drug form, however, in this manuscript experiments were not performed to confirm these findings within Molt-4 cells. Although further studies on KNI-1039 are needed, double-drug strategy is a novel approach to the usually prescribed administration of an RT inhibitor and a PR inhibitor separately.

In this study AZT prodrug and a PR inhibitor were linked together, however other double-drugs may be designed for the treatment of other diseases which require the administration of 2 or more drugs.

Acknowledgement

This study was supported in part by the Frontier Research Program of the Ministry of Education, Science and Culture of Japan, and grants from the Ministry of Education, Science and Culture of Japan, Japan Health Science Foundation and the Osaka Tuberculosis Research Foundation. We thank Mr. Yoshihiko Fujoka for his technical assistance in preparing the electron micrographs.
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Received April 15, 2005
Accepted May 9, 2005