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'Double-Drugs'— A New Class of Prodrug Form of an HIV Protease Inhibitor Conjugated with a Reverse Transcriptase Inhibitor by a Spontaneously Cleavable Linker

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Abstract—We designed and synthesized a new series of prodrug-type anti-HIV agents consisting of a peptidomimetic HIV protease inhibitor conjugated with a nucleoside reverse transcriptase inhibitor in an effort to enhance the antiviral activity. For the conjugation, a series of linkers that conjoin the two different classes of inhibitors have been investigated. Conjugates using a succinyl amino acid linker were shown to release the parent components via the spontaneous imide formation at a faster rate compared to conjugates using a glutaryl amino acid linker, as expected from the energetically favorable cyclization to the five-membered ring. Herein, we report a new 'double-drug' **4b** (KNI-1039) with a glutarylglycine linker, which exhibited extremely potent anti-HIV activity compared with that of the individual components.

Introduction

HIV-1 protease (HIV PR) and reverse transcriptase (RT) are major targets for anti-HIV drug discovery, and their inhibitors are widely used in the chemotherapy of AIDS.[1] Combination of HIV PR inhibitors and RT inhibitors has become the standard clinical practice to maintain the antiviral effect and to prevent emergence of a drug-resistance virus.[2,3] We have studied the structure–activity relationship of dipeptide-based HIV PR inhibitors.[4] Among them, KNI-413 (Fig. 1) and KNI-727 (Fig. 2) exhibited potent HIV PR inhibition but poor antiviral activity.[4–6]

The antiviral efficacy of inhibitors depends not only on the enzyme inhibitory activity, but also on their intracellular concentrations closely related to the membrane permeability.[7] The low antiviral activities of KNI-413 and KNI-727 were probably due to their insufficient cell membrane permeability. These findings prompted us to introduce the 'double-drug' strategy that combines an HIV PR inhibitor and a nucleoside RT inhibitor in a single molecule, taking into consideration the affinity of nucleosides towards cell membrane.[8] Previously, based on this 'double-drug' strategy, we had developed potent prodrug-type anti-HIV agents,[9,10] KNI-684 and KNI-694, in which the carboxyl group of HIV PR inhibitors, KNI-413 and KNI-852 was directly esterified with the 5′-hydroxyl group of a nucleoside RT inhibitor, 3′-azido-3′-deoxythimidine (AZT) (Fig. 1).[11] The anti-HIV activities of prodrug-type conjugates, KNI-684 and KNI-694 were found to be more potent than that of AZT and the parent protease inhibitors.[9,10] These results suggested the following: (a) the 'double-drug' strategy involving the combination of two different classes of inhibitors together would enhance the anti-HIV efficacy synergistically, and (b) also be effective in the improvement of its physicochemical characteristics. However, this 'double-drug' strategy using the direct esterification method was limited to inhibitors containing free carboxylic acid.

The objective of the present study was to apply the concept of 'double-drug' strategy to another dipeptide HIV PR inhibitor, KNI-727 which is deficient of a carboxyl group. The poor water solubility due to the high lipophilic nature of KNI-727 might restrict its penetration across the cell membrane, thus resulting in poor

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anti-HIV activity. To enhance the anti-HIV activity and improve the physicochemical characteristics of KNI-727, we designed and synthesized new hybrid-type prodrugs of KNI-727 conjugated with AZT by a series of linkers (Fig. 2). Here we can report a new 'double-drug' **4b** (KNI-1039) with a glutarylglycine linker (Fig. 3), which exhibits 920 and 62 times more potent anti-HIV activity than KNI-727 and AZT, respectively.

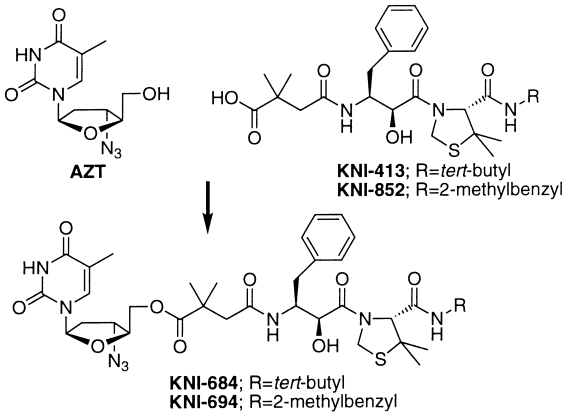


Figure 1. Structures of dipeptide HIV PR inhibitors, KNI-413 and KNI-852, and the conjugates, KNI-684 and KNI-694.

Conjugation Strategy

As for the linkers, dicarboxylic acid derivatives were used in the study to covalently link both the hydroxyl groups of KNI-727 and AZT. At first, we incorporated succinic acid as a linker, but the resulting compound KNI-935 (Fig. 4) was ineffective ($EC_{50} = 188.9$ nM, HIV-1_{IIIB}/CEM-SS) in the anti-HIV assay. The probable reason could be due to the high stability of the succinyl ester linkage towards enzymatic cleavage as described by Calenbergh and Nair,[12] and hence the parent drugs were not regenerated inside the cell. The major consideration in the design of these prodrugs is focused not only on the transportation across the cell membrane, but also on its reversion to the parent compounds in the cytoplasm to act on their respective targets.[13] The essential criteria in the design of such prodrugs are that (i) the 'double-drugs' should contain a linker that is stable outside the target cell and (ii) once in the cytoplasm, it should regenerate the parent compounds. Based on these criteria, a series of linkers which have the ability to cleave spontaneously in physiological environment were studied (Fig. 3).

As the esters of succinamic acid are easily hydrolysed via succinimide formation under mild alkaline conditions,[14]

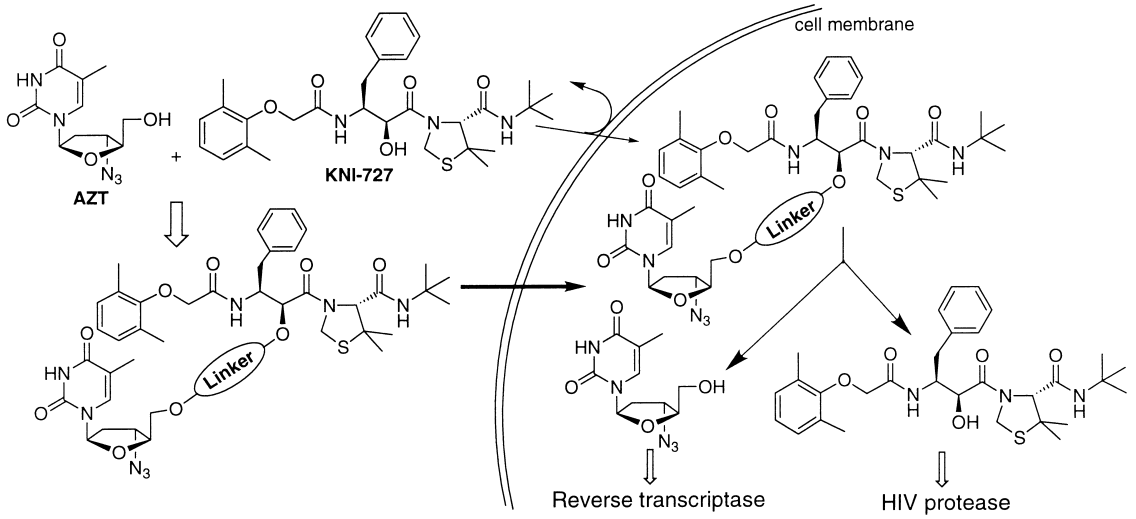


Figure 2. Design and proposed mechanism of hybrid-type Anti-HIV agents.

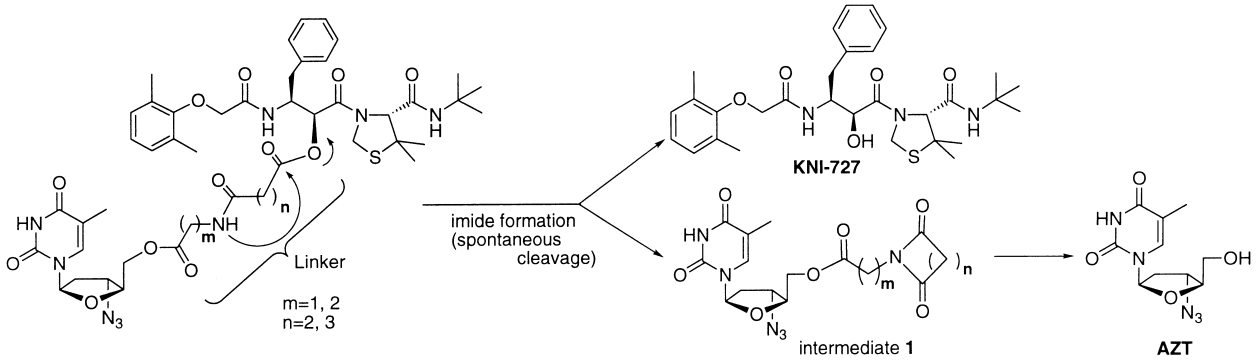


Figure 3. Conversion of double-drugs to KNI-727 and AZT.

we employed succinylglycine as a linker. The compound with this linker could disintegrate spontaneously to release KNI-727 and the ester intermediate **1**. The enzymatic hydrolysis of the ester intermediate **1** within the cell could release AZT. In order to study the spontaneous cleavage rate, conjugates with various linkers such as glutarylglycine, succinyl-β-alanine, and glutaryl-β-alanine were synthesized and analyzed.

Chemistry

The synthesis of these prodrugs is summarized in Scheme 1. Briefly, AZT (1 mmol, 267 mg) was coupled with Boc-Gly-OH (1.1 mmol, 193 mg) using DCC (1.1 mmol, 227 mg) in the presence of dimethylaminopyridine (DMAP) (0.1 mmol, 12 mg). The product was purified by column chromatography and later deprotected with 4 N HCl/dioxane to yield the corresponding ester of AZT (**2a**) (90% yield). KNI-727 (0.18 mmol, 100 mg) was coupled with succinic anhydride (0.2 mmol, 26 mg) in THF-ether in the presence of dicyclohexylamine (DCHA) (0.2 mmol, 43 μL) to yield the half-ester of KNI-727 (**3a**) (90% yield). Condensation of **3a** (0.15 mmol, 100 mg) and **2a** (0.3 mmol, 108 mg) using 1-ethyl-3-(3-(dimethylamino) propyl)carbodiimide hydrochloride (EDC·HCl) (0.15 mmol, 29 mg) in the presence of 1-hydroxybenzotriazole (HOBt) (0.17 mmol, 25 mg) resulted in the prodrug (**4a**) which was finally purified by silica gel chromatography (70% yield). The other prodrugs (**4b**, **4c**, **4d**) were also synthesized in a similar manner.

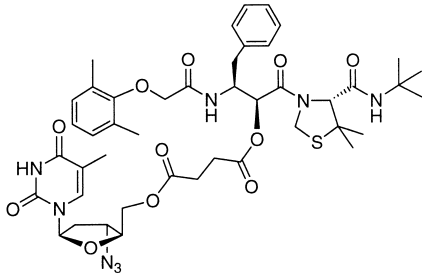
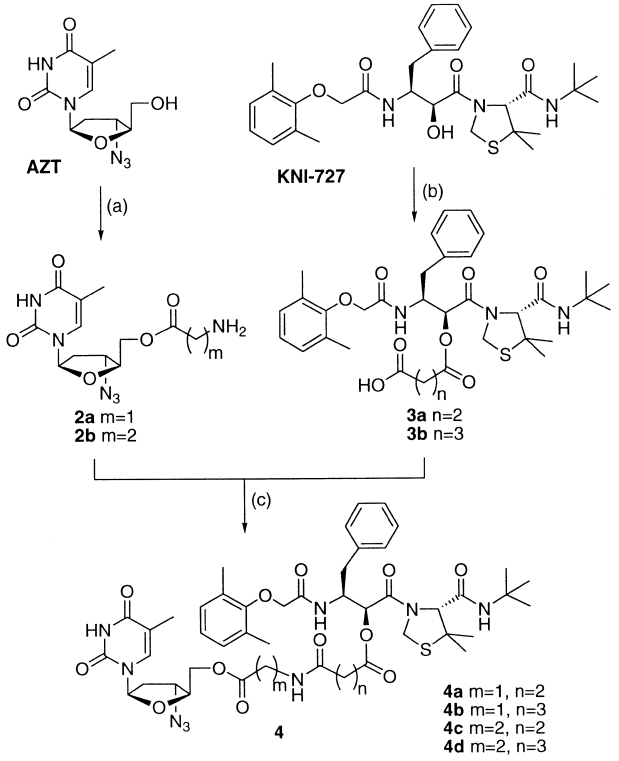


Figure 4. Structure of the conjugate KNI-935 using a succinic acid linker.



Scheme 1. Reagents and conditions: (a) i. Boc-Gly-OH or Boc-β-Ala-OH, DCC, DMAP, DMF; ii. 4N HCl/ dioxane; (b) Succinic anhydride or glutaric anhydride, DCHA, THF-ether (1:2). (c) EDC·HCl, HOBt, DMF.

Biological Evaluation and Discussion

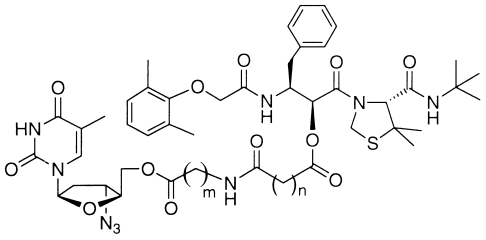
The inhibitory potencies of these prodrug-type conjugates against HIV PR were evaluated as reported.[6] All of the conjugates exhibited poor enzyme inhibition, whereas the parent HIV PR inhibitor, KNI-727, had a good enzyme inhibitory activity (Table 1). This could be attributed to the blockage of the hydroxyl moiety in KNI-727, which plays a significant role in the interaction with the catalytic aspartyl residues of the HIV PR.[4]

The anti-HIV activities of the prodrug-type conjugates were evaluated in in vitro conditions. Briefly, HIV-1_{LAI} (100 $TCID_{50}$) and each 4-fold diluted prodrug were inoculated to 5000/well Molt-4 cells in a microplate and cultured for 7 days. EC_{50} values were calculated from the ratios of surviving cells which were quantitated by WST-8 assay kit[15] (Dojin Lab., Kumamoto, Japan). All of the prodrug-type conjugates were found to be more potent than the parent HIV PR inhibitor, KNI-727.

The key feature of these prodrugs are the linkers which disintegrate spontaneously in the aqueous media to their parent compounds once inside the cell (Fig. 3). The disintegration behavior of these prodrugs in phosphate buffered saline (PBS, pH 7.4, at 37 °C) was determined by HPLC. For all the prodrugs studied, the spontaneous imide formation with the release of KNI-727 and ester intermediate (**1**) were observed. The releasing capability of KNI-727 varied depending upon the linkers. Compound **4a** conjugated by a succinylglycine linker had the faster releasing rate ($t_{1/2}$ = 1.4 h) compared with compound **4b** containing a gutarylglycine linker ($t_{1/2}$ = 23.3 h), as expected from the energetically favorable cyclization to the five-membered ring. The $t_{1/2}$ of compounds (**4c**, **4d**) containing β-alanine in the linker were longer than those of compounds (**4a**, **4b**) which contain glycine.

Compound **4b** (KNI-1039) containing a glutarylglycine linker exhibited a remarkable anti-HIV activity (EC_{50} = 0.1 nM) and low cytotoxicity (therapeutic index >2000). This antiviral activity was 920 and 62 times more potent than KNI-727 and AZT, respectively. This result suggested that the conjugates would penetrate the cell membrane and then the two classes of enzyme inhibitors generated could attack different targets in the infected cells, thus

Table 1. Anti-HIV activities and physicochemical characteristics of 'double-drugs'.



Compound	m	n	Inhibition of HIV protease[a]	EC_{50} HIV-1_{LAI}/ Molt-4	Relative potency	$t_{1/2}$ (h)[b]	Water solubility (μg/mL)
4a (KNI-1038)	1	2	37%	5.3 nM	1.2	1.4	290
4b (KNI-1039)	1	3	55%	0.1 nM	62	23.3	371
4c (KNI-1046)	2	2	7%	1.0 nM	6.2	3.8	373
4d (KNI-1047)	2	3	12%	3.4 nM	1.8	40.0	235
KNI-727			100%	92.0 nM	0.1	—	91
AZT			—	6.2 nM	1.0	—	—

[a]% of HIV-1 protease inhibition in the presence of 5 μM of inhibitors.
[b]$t_{1/2}$ is the time required for 50% release of KNI-727 at 37 °C in phosphate buffered saline (pH 7.4).

exhibiting synergistic anti-HIV activity. On the other hand, compound **4a** (KNI-1038) containing a succinylglycine linker showed similar antiviral activity as that of AZT, which might be due to the instability of compound **4a** ($t_{1/2} = 1.4$ h). Compound **4a** might disintegrate outside the cell, resulting in the release of AZT intermediate which may penetrate into the cell to inhibit RT. The $t_{1/2}$ of **4c** (KNI-1046) was longer than that of **4a**, that is, the anti-HIV activity of **4c** was 5 times more potent than that of **4a**. Among all the prodrugs, compound **4d** (KNI-1047) exhibited longer half life ($t_{1/2} = 40$ h), however, its antiviral activity was poor. The poor antiviral activity of **4d** may be attributable to the high stability which prevents the disintegration to its parent compounds. These results show that the antiviral activity may be influenced by the stability of conjugates.[16] The antiviral efficacy of conjugated compounds depends on many factors, such as enzyme inhibition, cell membrane permeability, extracellular stability, intracellular disintegration, and the correlation between them is very complicated.

The water solubility of these prodrugs was determined by RP-HPLC. Aliquots of these prodrug solutions were injected and the concentrations were determined by comparison of the peak area (Table 1). All the prodrugs exhibited better water solubilities (290–373 μg/mL) compared to the parent compound KNI-727 which was less water-soluble (91 μg/mL). These results indicate that the prodrug-type conjugates satisfied one of the essential criteria in improving the physicochemical characteristics of the compounds with a subtle balance between hydrophilicity and lipophilicity, and thus would enhance the cell membrane permeability.

In conclusion, based on the prodrug concept, novel hybrid-type anti-HIV agents were developed. Among the prodrugs studied, compound **4b** (KNI-1039), a 'double-drug' of an HIV PR inhibitor KNI-727 conjugated with a nucleoside RT inhibitor AZT by a glutarylglycine linker, exhibited a remarkable anti-HIV activity. This antiviral activity was 920 and 62 times more potent than that of KNI-727 and AZT, respectively. We believe that this 'double-drug' approach in the field of medicinal chemistry would pave the way in the development of more potent drugs. The extension of this 'double-drug' strategy to other compounds is in progress.

Acknowledgements

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