Development of Water-Soluble Prodrugs of the HIV-1 Protease Inhibitor KNI-727: Importance of the Conversion Time for Higher Gastrointestinal Absorption of Prodrugs Based on Spontaneous Chemical Cleavage

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We designed and synthesized a series of water-soluble prodrugs of the HIV-1 protease inhibitor KNI-727 (**1**), which is a sparingly water-soluble drug with a water solubility of 5.5 μg/mL. These prodrugs, which contain a water-soluble auxiliary with two tandem-linked units, i.e., a self-cleavable spacer and a solubilizing moiety with an ionized amino function, exhibited a marked increase in water solubility ($>10^4$-fold) compared with the parent drug **1**. The mechanism of conversion to the parent drug **1** is not enzymatic but through a chemical cleavage at the spacer via an intramolecular cyclization−elimination reaction through an imide formation under physiological conditions. To diversify the conversion time for the parent drug regeneration, chemical modification of the auxiliary was carried out focusing on the introduction of cyclic tertiary amines, which can modify the basicity and/or conformational flexibility of the terminal amino function at the solubilizing moiety, and the change in bond length, which can attenuate the five-membered ring intermediate formation in the cleavage. These newly synthesized water-soluble prodrugs exhibited a practical water solubility with values greater than 50 mg/mL and enabled the constant regeneration of the parent drug **1** with diversified conversion times ranging from 4 min to 34 h as $t_{1/2}$ values under physiological conditions. All the water-soluble prodrugs tested regenerated the parent drug **1** in vivo as well as in vitro. A clear increase in the gastrointestinal absorption was observed in prodrugs **8**, **12**, and **13** with bioavailability (BA) values of 23%, 26%, and 29%, respectively. These BA values were 1.5−1.9-fold higher than that in the administration of the parent drug **1** alone. Other prodrugs showed only a similar or decreased BA compared to the parent drug **1**. From these results, we found that not only a high water solubility but also an appropriate conversion time of the prodrug with a relatively narrow limit of around 35 min via intraduodenal administration was necessary for significant improvement of the gastrointestinal absorption in water-soluble prodrugs based on the spontaneous chemical cleavage. This is the first successful water-soluble prodrug that suggests an increased BA value greater than the parent drug in HIV-1 protease inhibitors and is the first study to show the importance of optimal conversion time in water-soluble prodrugs. Consequently, a water-soluble strategy that can control the conversion time would be extensively applicable to improve the gastrointestinal absorption of sparingly water-soluble drugs. The present information is an intriguing discovery and is one of the key factors that will contribute to the future design of practical water-soluble prodrugs.

Introduction

The introduction of inhibitors of aspartic protease encoded by the human immunodeficiency virus type-1 (HIV-1) has revolutionized the treatment of HIV-1 infection and AIDS and markedly improved the life span and lifestyle of HIV-1-infected patients.[1−22] In particular, the combination of one protease inhibitor with two kinds of reverse transcriptase inhibitors (highly active antiretroviral therapy, HAART) has become a standard front-line therapy in the developed world.[23−30] More recently, treatment with two kinds of protease inhibitors (double protease therapy) was studied and showed clinical effectiveness in preventing development of tolerance by HIV-1 in clinical practice.[31] However, despite the hope and promise that these agents have engendered, very real problems exist with the current antiviral armamentarium, especially with protease inhibitors: the serious threat posed by the resistant strains of HIV-1[32−36] and the side effects such as lipodystrophy and toxicity.[21] The sparing water solubility and insufficient bioavailability are also serious problems of protease inhibitors.[37−41] Since most HIV-1 protease inhibitors have to penetrate the infected cells or virions to attack the protease therein and the catalytic domain in HIV-1 protease is hydrophobic, inhibitors that have a highly lipophilic property are required, and this affords an undesirable sparing water solubility to the inhibitors. However, for orally administered drugs, drug dissolution generally precedes gastrointestinal absorption and systemic availability. Namely, the rate of drug dissolution often determines the drug absorption.[42−44] Thus, the present sparingly water-soluble HIV-1 protease inhibitors are administered with a high percentage of additives in the formulation to facilitate gastrointestinal

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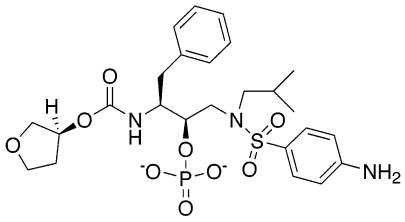
Chart 1. The Structure of Fosamprenavir[40,41]



dissolution. However, such a large amount of additives results in a high pill burden for patients, which leads to poor-adherence and deterrence to increase the amount of the drug administered per day to patients.[40,41] Therefore, water-soluble inhibitors, which do not need any additives, would improve the drug load per unit dose and enable increased bioavailability through dissolution in gastrointestinal fluids.

To overcome the sparing water solubility while retaining high lipophilicity, derivatization to water-soluble prodrugs is expected to be one of the most effective strategies.[44] This prodrug involves a covalently attached solubilizing moiety that is ionized at physiological pH and can be maintained as a soluble form in gastrointestinal fluids and then is gradually converted to the lipophilic parent drug without precipitation. The increased solubility of the prodrug and high membrane permeability of the well-dispersed and lipophilic parent drug will provide a higher driving force to be readily absorbed via the intestinal lumen.

To date, although there have been many studies of water-soluble prodrugs in the fields of antibacterial,[45,46] antiviral,[47] antihypertensive,[48−50] antiemetic,[51,52] and anticancer drugs,[53−63] successful in vivo results have been limited.[42] In the case of the HIV-1 protease inhibitors, Vertex Pharmaceuticals have developed fosamprenavir[40,41] (in a phase III clinical study; Chart 1), a water-soluble prodrug of an approved HIV-1 protease inhibitor, amprenavir.[7] In this prodrug, the hydroxyl group of amprenavir was converted to a phosphate ester to achieve a high water solubility and the use of organic additives causing a high pill burden could be avoided. However, total exposure to amprenavir was slightly reduced with a relative bioavailability of 90% compared with amprenavir itself in rats. Thaisrivongs et al. also reported a series of highly water-soluble phosphate prodrugs of U-75875 derivatives, while no evaluation of oral bioavailability was reported.[37] Consequently, outstanding successes with improved bioavailability in water-soluble prodrugs are few in HIV-1 protease inhibitors. Hence, new strategies toward water-soluble prodrugs are required.

Our strategy for water-soluble prodrugs is dependent on the intramolecular chemical reaction. Namely, the prodrugs have a general auxiliary containing two covalently and tandem-linked units through the hydroxyl group of the parent HIV-1 protease inhibitors. These units are composed of a self-cleavable spacer and a hydrophilic solubilizing moiety (Figure 1) with the following characteristics: (1) an ammonium ion is introduced into the solubilizing moiety to provide an effective water solubility, (2) the parent drug is released via a constant intramolecular cyclization reaction leading to the imide formation of the spacer moiety, and (3) the rate of the cyclization reaction can be controlled by

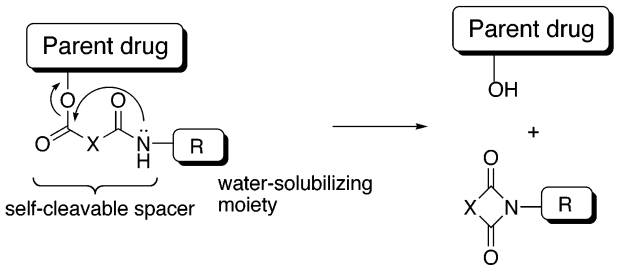


Figure 1. Water-soluble prodrug strategy that can release a parent drug based on the chemically triggered intramolecular cyclization reaction through imide formation.

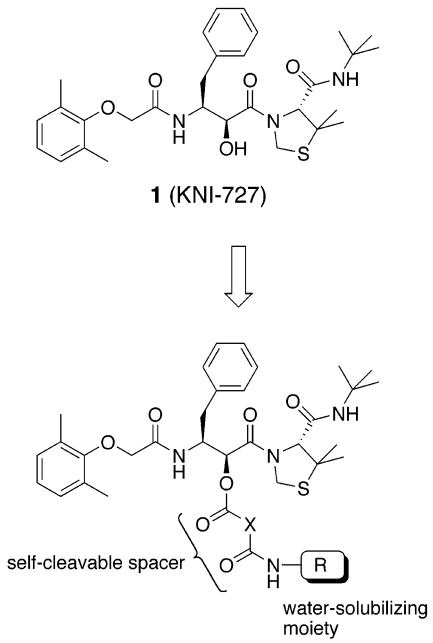


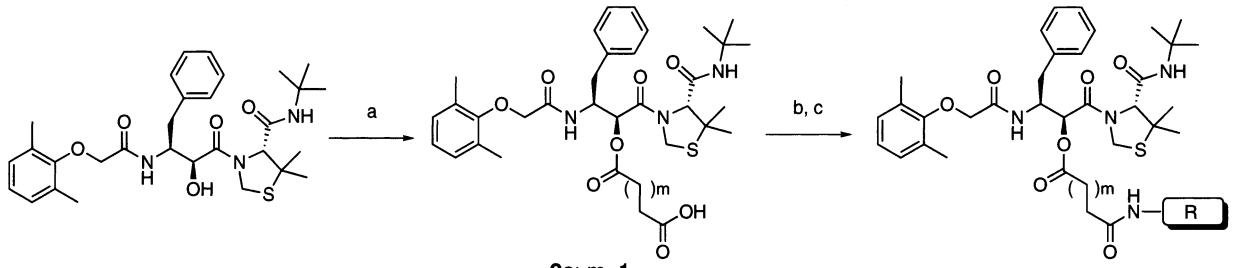
Figure 2. Application of the water-soluble prodrug strategy to a sparingly water-soluble drug **1** (KNI-727).

the structures of both the spacer and the solubilizing moieties and the pH of the media, but it is not affected by the individual difference in enzymatic activity.

Along these lines, we previously synthesized water-soluble prodrugs of the HIV-1 protease inhibitor, KNI-727 (**1**, Figure 2),[64] which is a potent peptidomimetic inhibitor containing an allophenylnorstatine (Apns, (2*S*,3*S*)-3-amino-2-hydroxy-4-phenylbutanoic acid) residue with a hydroxymethylcarbonyl (HMC) isostere derived from the structure of a natural scissile peptide sequence "Phe-Pro".[12,14,15] Inhibitor **1**, like other HIV-1 protease inhibitors, exhibited sparing water solubility in physiological media. The biological evaluation of several synthetic water-soluble prodrugs of **1** suggested that our strategy afforded not only a high water solubility but also a promising ability that the conversion time could be controlled by the auxiliary structures.

We herein describe the development of new auxiliaries that exhibited a wide range of constant conversion times, and the in vivo evaluation of prodrugs with those auxiliaries from a practical standpoint. On the basis of the structure−conversion time relationship among these auxiliaries, we developed a series of water-soluble prodrugs that could constantly produce the parent drug **1** with a variety of conversion times in vitro, in addition to a high water solubility, and discovered that some of

Scheme 1[a]



Compd	m	R	Compd	m	R
5	1	—Pr	**10**	2	—$(CH_2)_2$—N⟨O⟩ ·HCl
6	1	—$(CH_2)_2$—N⟨⟩ ·HCl	**11**	1	—CH_2—⟨N⟩ ·HCl
7	1	—$(CH_2)_2$—N⟨⟩ ·HCl	**12**	1	—CH_2—⟨N⟩ ·HCl
8	1	—$(CH_2)_2$—N⟨O⟩ ·HCl	**13**	1	—CH_2—⟨N⟩ ·HCl
9	1	—$(CH_2)_3$—N⟨O⟩ ·HCl			

[a] Reagents: (a) succinic anhydride (for **2a**) or glutaric anhydride (for **2b**), DCHA, THF−ether (1:2); (b) corresponding amine, EDC·HCl, HOBt, DMF; (c) preparative HPLC (linear gradient of CH_3CN in 12 mM aqueous HCl).

these prodrugs (**8**, **12**, and **13**) with a constant drug conversion time significantly increased the gastrointestinal absorption of the parent drug **1** in vivo. This result suggested a crucial aspect of the water-soluble prodrug strategy, namely, that the parent drug absorption was largely attributable to the conversion time of the prodrugs. Thus, the prodrugs that can realize the appropriate conversion time in addition to the high water solubility would improve systemic bioavailability.

Chemistry

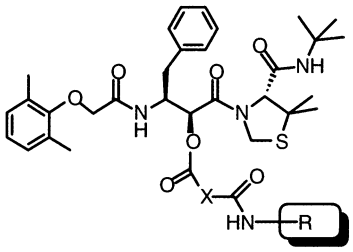
The synthesis of the prodrugs is shown in Scheme 1. The parent drug **1**, which was synthesized by the previously described method,[15] was treated with two kinds of dicarboxylic anhydrides in the presence of dicyclohexylamine (DCHA) in THF−ether (1:2) to afford the corresponding half-esters **2a** and **b**.[65,66] Next, the condensation of these half-esters with a series of amines such as ethylenediamine derivatives and aminomethylpyridines using the EDC−HOBt method[67,68] (EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HOBt, 1-hydroxybenzotriazole) in DMF afforded prodrugs **5**−**13**.

All synthetic compounds were purified and converted to the corresponding HCl salts by HPLC using a binary solvent system in a linear gradient of CH_3CN in 12 mM aqueous HCl, and the white fluffy powder of each purified prodrug obtained after lyophilization was used for the biological evaluation.

Results

In Vitro Evaluation of Water-Soluble Prodrugs of 1. In our preliminary study,[64] we synthesized water-soluble prodrugs **3** and **4** of the parent drug **1**. These prodrugs contained an auxiliary composed of a dicarboxylic acid-based chemically cleavable spacer and an ethylenediamine-based solubilizing moiety. As shown in Table 1, detailed statistical evaluation indicated that **3** and **4** showed practical water solubility with values greater than 50 mg/mL in comparison with that of the parent drug **1**, the water solubility of which was 5.5 μg/mL. The half-life ($t_{1/2}$) of **3** with a succinic acid-based spacer was short, with a value of 12.7 min, whereas the $t_{1/2}$ of **4** with a glutaric acid-based spacer was quite long, with a value of 20.7 h in phosphate-buffered saline (PBS, pH 7.4) at 37 °C. Since these values were the extremes, auxiliaries with diverse conversion times were developed by chemical modifications focused on the water-solubilizing moiety. Prodrug **5**, which had a methyl substituent in place of the $-NH_2$·HCl group of **3**, exhibited about 70-fold slower conversion, despite the presence of the same succinic acid-based spacer. These results suggested that the ionized amino group of **3** at the water-solubilizing moiety had an important role in the rapid conversion of the prodrug. Hence, four kinds of bulky cyclic tertiary amine structures, i.e., pyrrolidine, piperidine, morpholine, and pyridine, were newly introduced to the water-solubilizing moiety, and the $t_{1/2}$ values of the synthetic prodrugs in PBS (pH 7.4) at 37 °C were measured by RP-HPLC analysis (Table 1). Prodrugs **6** (pyrrolidine) and **7** (piperidine) exhibited shorter $t_{1/2}$ values (7.9 and 3.6 min, respectively) than that of the original ethylenediamine derivative **3**. The $t_{1/2}$ value of prodrug **6** was about 2-fold longer than that of **7**. On the other hand, when a morpholine structure was introduced instead of the piperidine structure in **7**, 11-fold extension of the $t_{1/2}$ value (40.3 min) was observed in prodrug **8**. In addition, the introduction of an *n*-propylmorpholine in prodrug **9** instead of the ethylmorpholine in **8** showed a further extension of the $t_{1/2}$ value to 79.5 min. Moreover, prodrug **10**, which is a

Table 1. Water Solubility and Converting Time of Prodrugs



compd	X	R	water solubility[a] mg/mL	water solubility[a] ratio	$t_{1/2}$ [a,b] (min)
1			$(5.5 \pm 0.3) \times 10^{-3}$		
3	$-CH_2CH_2-$	$-(CH_2)_2NH_2 \cdot HCl$	77.1 ± 4.5	14018	12.7 ± 0.7
4	$-CH_2CH_2CH_2-$	$-(CH_2)_2NH_2 \cdot HCl$	50.3 ± 4.4	9145	1244 ± 35.4
5	$-CH_2CH_2-$	$-CH_2CH_2CH_3$	$<(5.5 \pm 0.3) \times 10^{-3}$		922 ± 17.8
6	$-CH_2CH_2-$	$-(CH_2)_2$-pyrrolidinyl·HCl	93.8 ± 7.0	17055	7.9 ± 0.2
7	$-CH_2CH_2-$	$-(CH_2)_2$-piperidinyl·HCl	47.8 ± 6.0	8691	3.6 ± 0.1
8	$-CH_2CH_2-$	$-(CH_2)_2$-morpholino·HCl	53.2 ± 1.8	9673	40.3 ± 0.9
9	$-CH_2CH_2-$	$-(CH_2)_3$-morpholino·HCl	73.6 ± 9.3	13382	79.5 ± 2.0
10	$-CH_2CH_2CH_2-$	$-(CH_2)_2$-morpholino·HCl	57.1 ± 6.0	10382	2056 ± 48.7
11	$-CH_2CH_2-$	$-CH_2$-2-pyridyl·HCl	31.9 ± 9.5	5800	48.5 ± 0.2
12	$-CH_2CH_2-$	$-CH_2$-3-pyridyl·HCl	30.3 ± 5.8	5509	35.4 ± 2.3
13	$-CH_2CH_2-$	$-CH_2$-4-pyridyl·HCl	91.5 ± 2.5	16636	34.9 ± 0.6

[a] Values are means ± SEM of three experiments. [b] $t_{1/2}$ is the time required for 50% release of parent drug **1** at 37 °C in phosphate-buffered saline (pH 7.4).

glutaric acid derivative of **8** at the spacer moiety, exhibited the longest $t_{1/2}$ value (34.3 h). Then, the introduction of the pyridine structures, i.e., 2-, 3-, and 4-aminomethylpyridines in **11**, **12**, and **13**, yielded $t_{1/2}$ values of 48.5, 35.4, and 34.9 min, respectively, which are 3−4-fold longer than that of **3**.

As shown in Table 1, HCl salts of these newly synthesized prodrugs **6**−**13** exhibited a similar water solubility to **3** and **4**. Those were practical values ranging from 30.3 to 93.8 mg/mL, which correspond to 5500−17000-fold higher water solubility than that of the parent drug **1**.

In Vivo Experiment. The effect of water-soluble prodrugs on gastrointestinal absorption of the parent drug **1** was examined in rats. **1** and eight prodrugs, **3**, **4**, **6**, **8**−**10**, **12**, and **13**, were administered intraduodenaly (id.) at a dose equivalent to 10 mg/kg of **1**. The parent drug **1** was also administered intravenously (iv) at a dose of 1 mg/kg to calculate the area under the concentration curve from 0 to 8 h (AUC_{0-8}) and the bioavailability (BA). Blood samples were collected periodically through the femoral arterial cannula for 8 h after administration, and plasma concentrations of the parent drug **1** were measured by RP-HPLC. The maximum plasma drug concentration (C_{max}) and the time to the maximum plasma drug concentration (T_{max}) were also determined. Although the water solubility of prodrugs was sufficient for id. dosing, prodrugs were administered in the same cosolvent vehicle as the parent drug **1** to allow direct comparison.

As shown in Figure 3A and Table 2, when the parent drug **1** was administered, the plasma concentration of **1** reached a peak at 5 min (T_{max}) with the C_{max} value of 1.23 μg/mL, and the calculated AUC_{0-8} and BA values were 215 μg min/mL and 15.8%, respectively. These values were used as the standard for subsequent evaluation of the synthesized prodrugs.

In the administration of prodrug **3**, the mean plasma concentration profiles of **1** showed reduced C_{max} (0.65 μg/mL) and similar BA (17.5%) values in comparison with those of **1** administered alone. This was due to the broad absorption profile of **1** with two gentle absorption peaks at 15 and 240 min, in which a relatively constant plasma concentration was maintained for 5 h (Figure 3B). The prodrug **3** was not detected in plasma after administration (data not shown). On the other hand, prodrug **4** showed significantly low C_{max} and BA values (0.46 μg/mL and 9.4%, respectively) with a slow onset of the absorption (T_{max} = 60 min).

Table 2. Pharmacokinetic Parameters of the Parent Drug **1** after Intraduodenal Administration of **1** or Its Water-Soluble Prodrugs

compd	*n*	C_{max} [a,b] (μg/mL)	T_{max} [c] (min)	AUC[a] (μg min/mL)	BA[a,d] (%)
1	5	1.23 ± 0.26	5	215 ± 41.2	15.8 ± 3.0
3	3	0.65 ± 0.08[e]	240[e]	239 ± 36.2	17.5 ± 2.7
4	3	0.46 ± 0.31	60	128 ± 43.6	9.4 ± 3.2
6	3	0.84 ± 0.14	15	219 ± 7.00	16.1 ± 0.5
8	3	1.89 ± 0.41	30	315 ± 88.0	23.1 ± 6.4
9	3	0.41 ± 0.05	30	94 ± 37.6	6.9 ± 2.8
10	3	0.48 ± 0.14	30	74 ± 23.6	5.4 ± 1.8
12	3	1.37 ± 0.12	30	360 ± 34.0	26.4 ± 2.5
13	3	1.62 ± 0.26	30	394 ± 50.7	29.0 ± 3.7

[a] Values are means ± SEM of five or three experiments. [b] C_{max} values are defined as the maximum mean plasma concentration achieved at each time point. [c] T_{max} values are defined as the time to the maximum mean plasma concentration achieved at each time point. [d] BA was determined by comparing the mean areas under the concentration curves (AUC) (×10) for 8 h after intravenous administration of **1** (1362.1 μg min/mL) and intraduodenal administration of **1** or prodrugs. [e] Second peak.

To examine the influence of the solubilizing moiety structure and conversion time more precisely on gastrointestinal absorption, the prodrugs **6**, **8**−**10**, **12**, and **13**, which have diverse $t_{1/2}$ values ranging from 7.9 min to 34 h, were administered in the same manner. Prodrug **6**, with a pyrrolidine substituent and a $t_{1/2}$ value of 7.9 min, showed C_{max}, T_{max}, and BA values of 0.8 μg/mL, 15 min, and 16.1%, respectively. This absorption profile was almost similar to that of prodrug **3** (Figure 3B and Table 2).

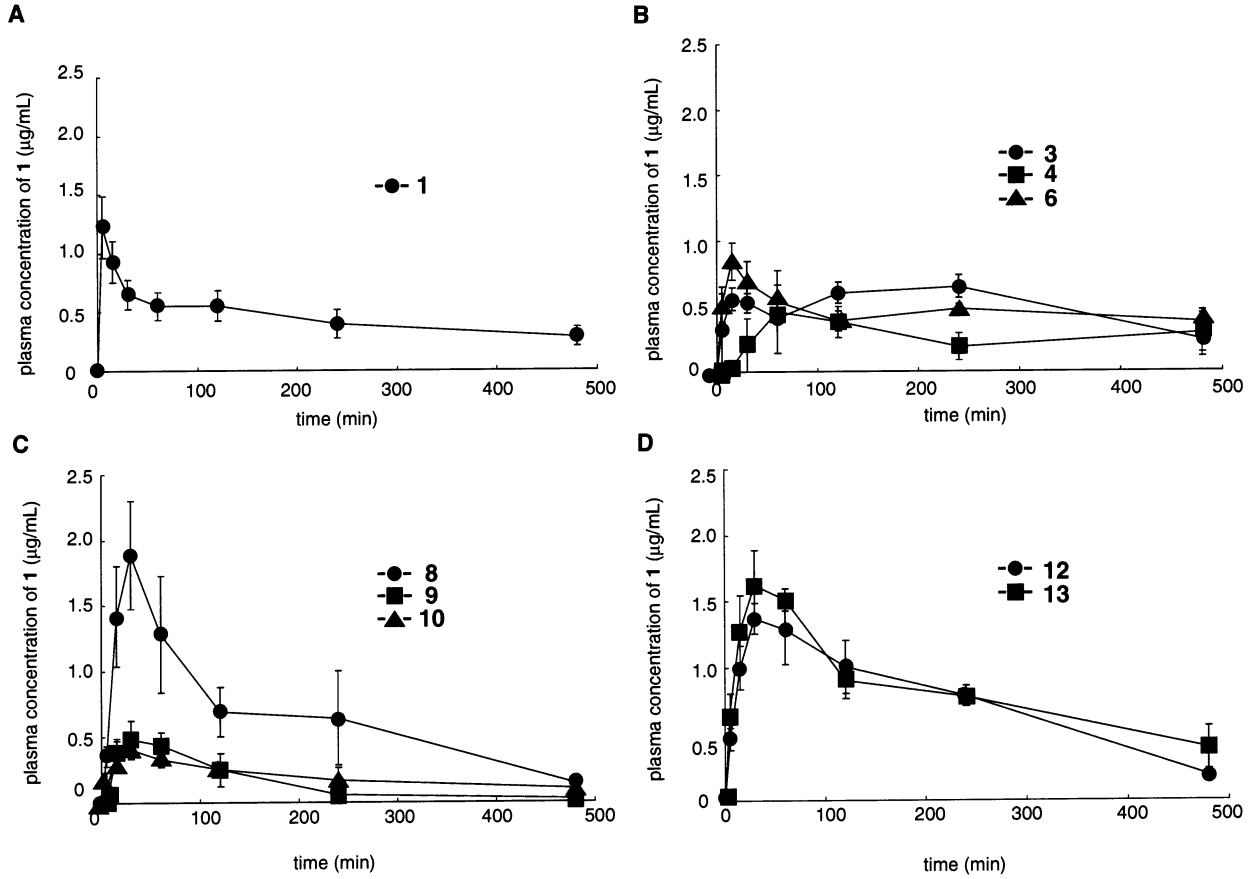


Figure 3. Pharmacokinetic study of the parent drug **1** and its water-soluble prodrugs.

However, when prodrug **8** ($t_{1/2}$ = 40.3 min) with an ethylmorpholine substituent was administered, a significantly higher plasma concentration of **1** was observed with a C_{max} of 1.89 μg/mL, T_{max} of 30 min, and BA of 23.1% (Figure 3C and Table 2). Both the C_{max} and the BA values were approximately 1.5-fold higher than those of the parent drug **1** administered alone.

To study the effect of the morpholine structure on gastrointestinal absorption of **1**, prodrug **9**, in which an *n*-propylmorpholine substituent was introduced as a solubilizing moiety, and prodrug **10**, in which the succinic acid spacer in **8** was replaced with glutaric acid, were examined. Prodrug **9**, which exhibited a 2-fold longer $t_{1/2}$ value (80 min) than **8**, showed a significant decrease in C_{max} and BA with values of 0.41 μg/mL and 6.9%, respectively. The BA value was 3.3-fold less than that of **8**. On the other hand, prodrug **10**, having quite long $t_{1/2}$ value of 34 h, also exhibited significantly a lower BA value of 5.4%. These findings suggested that the conversion time, not the morpholine structure, was important for higher gastrointestinal absorption.

Next, to confirm the importance of conversion time in gastrointestinal absorption, prodrugs **12** and **13**, which have a similar $t_{1/2}$ value to **8** but a different chemical structure at the solubilizing moiety, were also studied. Prodrugs **12** ($t_{1/2}$ = 35.4 min) and **13** ($t_{1/2}$ = 34.9 min) had 3- and 4-pyridyl substituents instead of the morpholine substituent in **8** (Figure 3D and Table 2). Both prodrugs also exhibited a higher absorption profile of the parent **1**. The observed C_{max}, T_{max}, and BA values for **12** were 1.37 μg/mL, 30 min, and 26.4%, respectively, and this BA value was 1.7-fold higher than that of the parent drug **1** administered alone. Similarly, the observed C_{max}, T_{max}, and BA values in **13** were 1.62 μg/mL, 30 min, and 29.0%, respectively, showing 2-fold higher C_{max} and BA values than that of the parent drug **1** administered alone. The late onset of absorption was also observed in both cases as well as other prodrugs. Interestingly, almost one-third of prodrugs **12** and **13** versus the produced parent drug **1** were also detected intact in plasma without any conversion with AUC values of 134.5 ± 12.9 and 139.0 ± 33.7 μg min/mL, respectively, while **3**, **4**, **6**, and **8**−**10** were not observed, suggesting that prodrugs **12** and **13** underwent partially direct absorption without converting to the parent drug **1**.

Since the PEG solution was used in all in vivo experiments to allow for direct comparison with solubilized parent drug **1** at the administration point, we examined id. administration of parent drug **1** and the most effective prodrug **13** in a saline solution instead of in a 50% PEG solution, to rule out any excipient effect of PEG on gastrointestinal absorption. The C_{max}, T_{max}, AUC_{0-8}, and BA values of **1** on administration of **13** were 1.25 μg/mL, 30 min, 472.2 μg min/mL, and 34.6%, respectively (Table 3). The observed BA value was slightly higher for prodrug **13** administered in saline than for prodrug **13** with a 50% PEG solution (BA = 29.0%), and 2.2-fold higher than the case when **1** was administered alone with a 50% PEG solution (BA = 15.8%). In the administration with saline, $^1/_{50}$ of intact **13** versus the produced parent drug **1** was detected in the plasma with an AUC value of 9.8 ± 3.7 μg min/mL, while $^1/_3$ of **13** with an AUC value of 139.0 ± 33.7 μg

Table 3. Pharmacokinetic Parameters of the Parent Drug **1** after Intraduodenal Administration of **1** or Prodrug **13** as a Saline Solution

compd	*n*	C_{max}[a,b] (μg/mL)	T_{max}[c] (min)	AUC[a] (μg min/mL)	BA[a,d] (%)
1	3	0.14 ± 0.07	15	1.7 ± 0.88	0.13 ± 0.07
13	3	1.25 ± 0.07	30	472.2 ± 17.6	34.6 ± 1.3

[a] Values are means ± SEM of three experiments. [b–d] See Table 2.

min/mL was detected when administered with a 50% PEG solution. On the other hand, parent drug **1** was almost not dissolved in saline, and the BA of **1** when administered in this suspension was quite low, with a value of 0.13% (Table 3).

Discussion

In the previously reported water-soluble prodrugs, the strategy of using endogenous enzymes such as esterases, peptidases, and phosphatases was mostly employed for the cleavage of a water-soluble modifier to regenerate a parent drug.[37,40−48,50−63] This biocleavage is effective only where the active enzymes exist such as at the intestinal brush border membrane, and a prodrug is safely distributed therein with no undesired degradation. In contrast, in the strategy using the chemical cleavage, the conversion occurs anywhere under physiological conditions immediately following administration. Therefore, in this type of prodrug, an appropriate conversion time in addition to higher water solubility was suggested to be necessary to show effective intestinal absorption to enable proper and full-dose supply of a parent drug in solution to the gastrointestinal brush border membrane through the intestinal fluids. However, there has been no precise information on the optimal conversion time in water-soluble prodrugs for effective gastrointestinal absorption. Also, it appeared difficult to consider the conversion time using prodrugs based on the enzyme cleavage, because of their extremely complex cleavage processes, including different tissue distribution and individual difference in cleavage enzymes, and the existence of many similar enzymes or isozymes. It is suggested that the conversion time of our prodrugs was more suitable to obtain information regarding the gastrointestinal absorption, because it has a simpler mechanism based on a spontaneous chemical reaction leading to a constant conversion time. In addition, it was suggested that the conversion time could be controlled by modification of the chemical structure of the auxiliary units. Hence, in the present study, we prepared a series of prodrugs whose auxiliary units were chemically modified to create diversified conversion times. And, the synthesized prodrugs were evaluated in vitro and in vivo to examine the correlation between the conversion time of prodrugs and gastrointestinal absorption of the parent drug.

Development of Water-Soluble Prodrugs with Diversified Conversion Time. From the findings that the terminal amino group of the auxiliary unit in prodrug **3** was important for rapid conversion, it was postulated that the effect of this amino group on the accelerated imide formation could be explained by the neighboring-group participation illustrated in Figure 4A. Namely, the electron-donating effect of this group could activate the amide nitrogen atom through a five-membered ring intermediate and then result in the imide formation through an alternative five-membered ring intermediate for amidolysis of the ester bond (Figure 4A-b). A similar observation was also reported as a side reaction in peptide synthesis.[69]

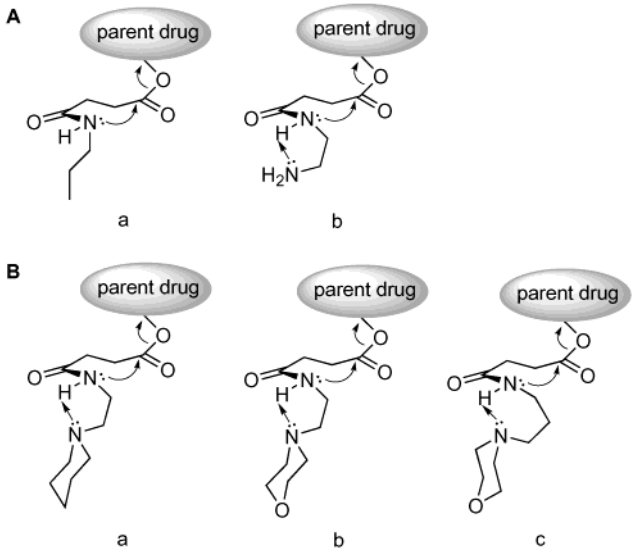


Figure 4. Proposed conversion mechanism of the water-soluble prodrugs.

This hypothesis afforded the idea that the modification of the terminal amino group could attenuate the fast cyclization reaction of **3** and provide the diversified conversion times to the prodrugs. Therefore, modifications that can modulate the neighboring-group participation, such as enhancement of the steric effect that prevents the effective formation of the five-membered ring intermediate and attenuation of the electron-donating effect at the terminal amino group, were considered. Namely, we introduced sterically hindered cyclic tertiary amines with different electronic states such as pyrrolidine, piperidine, morpholine, and pyridine structures to the solubilizing moiety. However, the substitution with pyrrolidine and piperidine in prodrugs **6** and **7** accelerated the conversion with the resultant shorter $t_{1/2}$ values of 7.9 and 3.6 min, respectively, than that of **3** with an original amino group ($t_{1/2}$ = 12.7 min). These results were probably because bulky cyclic amine structures help to restrict the *tert*-amino group to the conformation suitable for the neighboring-group participation rather than affording steric hindrance (Figure 4B-a). In contrast, the 10-fold increase in the $t_{1/2}$ value in the case of **8**, which has a morpholine substituent as opposed to the piperidine substituent of **7**, could be explained as an electron-inductive effect by an oxygen atom at the 1-position of the morpholine structure (Figure 4B-b). Moreover, the observed further extension of the $t_{1/2}$ value (79.5 min) by the introduction of propyl morpholine in **9** was probably due to the reduced electron-donating effect of the *tert*-amino group through an energetically less favorable six-membered ring intermediate formation (Figure 4B-c). The longest $t_{1/2}$ value (34 h) of **10**, which had the glutaric acid derivative of **8** at the spacer moiety, may be also due to the energetically less favorable six-membered ring intermediate formation at the spacer moiety.[70] This revealed that the enthalpic effect of the cyclic intermediate formation on the conversion time exerted a potent

influence on the spacer moiety rather than the water-solubilizing moiety, and the modification at the water-solubilizing moiety was suitable for fine-tuning of the conversion time. A similar extension of the $t_{1/2}$ value comparing to **8** was observed in the introduction of a weaker base, i.e., pyridines, in prodrugs **11**−**13**, although the reason was unclear. However, it appears that the introduction of a weaker base such as morpholine or pyridine was effective to slightly elongate the conversion time in this type of water-soluble prodrug.

From these findings, a series of prodrugs of **1** containing practical water solubility and diversified conversion times ranging from 4 min to 34.3 h in vitro could be developed by modifications focused on the chemical structure of the solubilizing moiety.

Effect of Conversion Time on Gastrointestinal Absorption. To understand the effect of the synthesized water-soluble prodrugs on the gastrointestinal absorption of the parent drug, an id. administration of the above-mentioned prodrugs was carried out in rats. This administration route was selected to eliminate other influences, such as low gastric pH conditions and gastric emptying rate in oral administration. All the prodrugs tested in vivo regenerated the parent drug **1** with a late onset of the absorption (Table 2 and Figure 3), and not the prodrugs but only the parent drug **1** appeared in rat plasma in most cases, except for **12** and **13**, having the pyridine structure. This late onset was probably due to the existence of a time lag for the conversion to the parent drug **1**. From the analysis of the plasma concentration of the parent drug **1** for 8 h after administration, a clear increase in the gastrointestinal absorption was observed in prodrugs **8**, **12**, and **13** with 1.5−1.9-fold higher BA values in comparison with the administration of the parent drug **1** alone, and other prodrugs showed similar or decreased BA values to the parent drug **1**. These results suggest that, despite all prodrugs used in this study showing practical water solubility as shown in Figure 5A, there was no correlation between the water solubility and BA values, suggesting that high water solubility alone is insufficient for improving gastrointestinal absorption. Similar observations were also reported in other drugs.[42]

The increased plasma concentration of the parent drug **1** and the nonappearance of the prodrug within plasma after the administration of the prodrug **8** suggested that **8** was efficiently provided to the intestinal brush border membrane and converted to **1** therein, resulting in higher gastrointestinal absorption of **1**. Meanwhile, the ionized hydrophilic primary amine structure of the auxiliary unit in prodrugs **3** and **4** may interfere in the intestinal absorption. Hence, the amphipathic nature of morpholine in **8** could increase the penetration of the molecule to gastrointestinal cell membrane.[71] However, since similar analogues with the morpholine structure such as **9** and **10**, which have longer conversion times, showed significant decreases in the BA values, it is suggested that the amphipathic morpholine structure is not a critical factor for increasing the BA. On the other hand, the finding that prodrugs **12** and **13** having the pyridine structure were significantly absorbed without the conversion to the parent drug was an interesting novel observation in

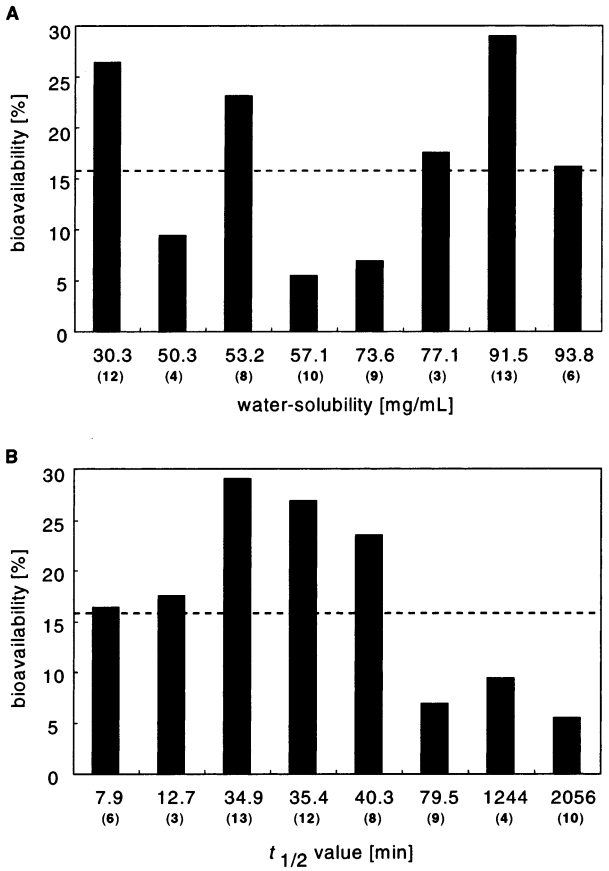


Figure 5. Effects of (A) water solubility and (B) $t_{1/2}$ value of prodrugs on the bioavailability (BA) of **1**. The dotted line exhibits the BA of **1** when the parent drug **1** was administered alone (15.8%). The compound numbers are in parentheses.

water-soluble prodrugs. This may contribute to the observed slightly higher BA in **12** and **13** than in **8**, and one possible explanation of this direct-absorption is a passive transport dependent on the amphipathic nature of the pyridine structure, although the participation of active transporters was unclear.

In the meantime, the enzymatic regeneration of the parent drug **1** in these prodrugs appeared to be negligible, since, in our previous study, prodrug **3** was stable in the treatment of porcine liver esterase in vitro.[64] In addition, this is also supported by the result that the structurally similar prodrugs **8** and **9**, which would be similarly recognized by endogenous esterases, showed clear differences in BA.

In the relationship between conversion time and gastrointestinal absorption, prodrugs **8**, **12**, and **13**, which showed improved gastrointestinal absorption, had almost the same conversion time of approximately 35 min, although the solubilizing moieties among these prodrugs had different structures, either morpholine or pyridine. In contrast, the prodrugs with conversion times of either less than 10 min or greater than 80 min did not show any improvement in BA, suggesting that the gastrointestinal absorption of the parent drug **1** was clearly dependent on the conversion time of the prodrugs, and the conversion time for favorable gastrointestinal absorption is limited within a relatively narrow range (Figure 5B). It follows that the prodrugs with shorter $t_{1/2}$ values distribute the parent drug in the gastrointestinal fluid insufficiently for an effective

absorption, and prodrugs with longer $t_{1/2}$ values fail to carry the formed parent drug to the proper place where the parent drug is absorbed effectively. Therefore, both a timely and sufficient supply of the parent drug to the gastrointestinal brush border membrane through intestinal fluids is critical in water-soluble prodrugs based on the chemical cleavage, and this kind of water-soluble prodrug should be designed in consideration of not only the water solubility but also the optimal conversion time.

Although we adopted a 50% PEG solution for id. administration of both parent drug and prodrugs in order to solubilize parent drug **1** and allow for direct comparison, we used saline for id. administration to confirm the concept that it is possible to avoid adding large amounts of excipients to the drug formulation. The fact that the BA value of **1** in the administration of prodrug **13** with saline was slightly higher than the administration with a 50% PEG solution (Table 3) suggests that the excipient effect of PEG did not play a key role on high gastrointestinal absorption of prodrug **13**. This further suggests that use of this type of water-soluble prodrug can help patients avoid the high pill burden and poor adherence associated with the use of additives. The reason for the slightly higher absorption observed with saline remains unclear. The fact that most of the intact prodrug **13** was not detected in the plasma when administered in saline suggests that most of the prodrug was converted to parent drug **1** in the gastrointestinal fluid and subsequently absorbed, although PEG facilitated the absorption of intact **13**. Hence, these results appear to support the concept that the conversion time in prodrugs **12** and **13**, which both possess pyridine structures at the solubilizing moiety, contributes to their high bioavailability.

Therefore, this is the first study to introduce the importance of the conversion time in water-soluble prodrugs. The auxiliary developed here that can control the conversion time will contribute to the design of water-soluble prodrugs with high bioavailability for the sparingly water-soluble drugs such as HIV-1 protease inhibitors and other drugs with a hydroxyl group as an anchor and may help to solve the problems such as the erratic oral absorption profile, poor oral bioavailability, and the limitations in the availability of both oral and parenteral dosage leading to poor adherence.

Conclusion

On the basis of the modulation of neighboring-group participation at the terminal amino function of the self-cleavable auxiliary, we have developed a series of water-soluble prodrugs that realized constant regeneration of the parent drug **1** (KNI-727) with a variety of conversion times in vitro in addition to a high water solubility. From the correlation study between the conversion times of the prodrugs and the bioavailability of the parent drug, we found that not only high water solubility but also appropriate conversion time of the prodrug within relatively narrow limits of approximately 35 min after id. administration was necessary for significant improvement of the gastrointestinal absorption in the water-soluble prodrugs based on the spontaneous chemical cleavage of the water-soluble auxiliary. Thus, this study demonstrates the first successful in vivo evidence that the water-soluble prodrugs of an HIV-1 protease inhibitor could significantly increase the plasma concentration of the parent drug.

Experimental Section

General Methods. Reagents and solvents were obtained from Wako Pure Chemical Ind., Ltd. (Osaka, Japan), Nacalai Tesque (Kyoto, Japan), and Aldrich Chemical Co. Inc. (Milwaukee, WI) and used without further purification. Column chromatography was performed on Merck 107734 silica gel 60 (70−230 mesh). TLC was performed using Merck silica gel $60F_{254}$ precoated plates. Melting points were measured on a Yanagimoto micro-melting apparatus without correction. Analytical HPLC was performed using a C18 reverse phase column (4.6 × 150 mm; YMC Pack ODS AM302) with a binary solvent system: linear gradient of CH_3CN in 0.1% aqueous TFA at a flow rate of 0.9 mL/min, detected at UV 202 or 230 nm. Preparative HPLC was carried out on a C18 reverse phase column (20 × 250 mm; YMC Pack ODS SH343-5) with binary solvent system: linear gradient of CH_3CN in 12 mM aqueous HCl or 0.1% aqueous TFA at a flow rate of 5.0 mL/min, detected at UV 230 nm. Solvents used for HPLC were of HPLC grade. All other chemicals were of analytical grade or better. ^{1}H NMR spectra were obtained on a JEOL 300 MHz spectrometer with TMS as an internal standard. FAB-MS was performed on a JEOL JMS-SX102A spectrometer equipped with the JMA-DA7000 data system.

Animals. Male Sprague−Dawley rats, about 7 weeks old, were obtained from Nippon SLC Co. Ltd. (Hamamatsu, Japan). The rats were housed under controlled environmental conditions and fed commercial feed pellets. All rats had free access to food and water.

Water Solubility. The parent drug **1** and the prodrugs **3**−**13** were saturated in distilled water and shaken vigorously. The saturated solutions were sonicated for 15 min at 25 °C and passed through a centrifugal filter (0.45 nm filter unit, Ultrafree-MC, Millipore). The filtrate was analyzed using RP-HPLC.

Stability Studies of Prodrugs in PBS Buffer. The disintegration profiles of the prodrugs **3**−**13** were determined in phosphate-buffered saline (PBS, pH 7.4). To 1 mL of PBS (pH 7.4) was added 10 μL of prodrug solution (0.5 mM in DMSO) and the mixture was incubated at 37 °C in a water bath. At the desired time points whole samples (1 mL) were directly analyzed by RP-HPLC. HPLC was performed using a C18 (4.6 × 150 mm; YMC Pack ODS AM302) reverse-phase column with binary solvent system: linear gradient of CH_3CN (40−100%, 30 min) in 0.1% aqueous TFA at a flow rate of 0.9 mL/min, detected at UV 230 nm.

In Vivo Pharmacokinetics Studies Using Rats. In the id. administration studies, five or three male Sprague−Dawley rats weighing 240−300 g were fasted overnight with free access to water for at least 12 h. Rats were anesthetized with light diethyl ether and surgically prepared. At 30 min before administration, 250 μL of the blank blood sample was removed via a femoral artery cannula. Doses of the parent drug **1** and prodrugs **3**, **4**, **6**, **8**−**10**, **12**, and **13** were freshly prepared as a 50% PEG (8000) or saline solution immediately before administration. The administered volume of drugs was 1 mL/kg. All of these compounds were administered intraduodenally via a duodenal cannula at a dose equivalent to 10 mg/kg of **1**. Systemic blood was sampled via a femoral artery cannula (250 μL) at selected times (5 min, 15 min, 30 min, 1 h, 2, 4, and 8 h) after dosing. Blood samples were kept on ice, heparinized, and centrifuged, then the plasma was immediately stored at −80 °C until analysis by RP-HPLC.

The plasma samples were combined with 700 μL of ethyl acetate, vortexed vigorously for 1 min, and shaken for 1 h at room temperature followed by centrifugation at 2500*g* for 15 min. The organic layer (550 μL) was evaporated to dryness using a rotary evaporator (SC100, Savant), and then samples were reconstituted in 60 μL of methanol with vortexing. After centrifugal filtration (0.45 nm filter unit, Ultrafree-MC), the filtrate (30 μL) was analyzed using RP-HPLC.

In the iv dosing study ($n = 2$), the dose of the parent **1** was prepared in a manner similar to that described for id. dosing, except for the cosolvent (ethanol, 0.5 mL/kg) and dosing at 1 mg/kg. The prepared sample was intravenously administered via a femoral vein and systemic blood was sampled via a femoral artery cannula (100 μL) at selected times (1, 5, 15, and 30 min, 1, 2, 4, and 8 h) after dosing. Subsequent treatment of the plasma samples was also in a similar manner as described for the id. dosing. The AUC_{0-8} (×10) value in iv administration of **1** was 1362.1 μg min/mL.

The parent **1** and prodrugs were separated from plasma contaminants on a C18 reverse phase column (4.6 × 150 mm; YMC Pack ODS AM302) with a binary solvent system. The elution condition was a linear gradient of 45−55% acetonitrile in 0.1% TFA for 30 min at a flow rate of 0.9 mL/min with UV detection at 202 nm. The assays for **1** were linear (correlation coefficient > 0.998) over the concentration range of 0−3 μg/mL, and the detection limit of quantification was 0.07 μg/mL.

Pharmacokinetic Analysis. The C_{max} value was defined as the maximum mean plasma concentration, the T_{max} value was defined as the time to the maximum mean plasma concentration, and the mean area under the plasma concentration−time curve (AUC) was determined using the linear trapezoidal rule. Bioavailability (BA) was determined by comparing the AUC (×10) values after the intravenous administration of the parent drug **1** and intraduodenal administration of **1** alone or the prodrugs.

(1*S*,2*S*)-1-({(4*R*)-4-[(*tert*-butylamino)carbonyl]-5,5-dimethyl-1,3-thiazolan-3-yl}carbonyl)-2-{[2-(2,6-dimethylphenoxy)acetyl]amino}-3-phenylpropyl 4-Oxo-4-(propylamino)butanoate (5). To the solution of 100.0 mg (0.15 mmol) of **2a**[65,66] in DMF were added propylamine (18.8 μL, 0.23 mmol), HOBt (28.0 mg, 0.18 mmol), and EDC·HCl (44.0 mg, 0.23 mmol) at 0 °C and the mixture stirred for 18 h at room temperature. After removal of the solvent in vacuo, the residue was dissolved in EtOAc (30 mL), washed with 10% citric acid and saturated NaCl, dried over anhydrous Na_2SO_4, and concentrated in vacuo. Purification of the product by preparative HPLC and lyophilization of the corresponding fraction gave 56.2 mg of the title compound as a white foam: yield 53.0%; mp 78−80 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.21 (d, J = 9.0 Hz, 1H), 7.89 (t, J = 5.4 Hz, 1H), 7.63 (s, 1H), 7.38 (d, J = 6.9 Hz, 2H), 7.28−7.12 (m, 3H), 7.01−6.89 (m, 3H), 5.35 (d, J = 4.2 Hz, 1H), 5.09 (d, J = 8.9 Hz, 1H), 4.91 (d, J = 8.9 Hz, 1H), 4.52−4.47 (m, 2H), 4.21 (d, J = 14.4 Hz, 1H), 3.98 (d, J = 14.4 Hz, 1H), 3.01−2.84 (m, 4H), 2.69−2.59 (m, 2H), 2.50−2.39 (m, 2H, partially overlapped with DMSO peaks), 2.13 (s, 6H), 1.47 (s, 3H), 1.40 (s, 3H), 1.38−1.31 (m, 2H), 1.25 (s, 9H), 0.80 (t, J = 7.4 Hz, 3H); HRMS (FAB) m/z 697.3630 for $[M + H]^+$ (calcd 697.3635 for $C_{37}H_{53}N_4O_7S$). Anal. ($C_{37}H_{52}N_4O_7S\cdot H_2O$) C, H, N.

(1*S*,2*S*)-1-({(4*R*)-4-[(*tert*-butylamino)carbonyl]-5,5-dimethyl-1,3-thiazolan-3-yl}carbonyl)-2-{[2-(2,6-dimethylphenoxy)acetyl]amino}-3-phenylpropyl 4-Oxo-4-[(2-tetrahydro-1*H*-1-pyrrolylethyl)amino]butanoate Hydrochloride (6). Compound **6** was prepared from **2a** and 1-(aminoethyl)pyrrolidine in a manner similar to that described for compound **5**: yield 31.6%; mp 115−117 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.38 (br s, 1H), 8.31 (t, J = 5.4 Hz, 1H), 8.25 (d, J = 9.0 Hz, 1H), 7.67 (s, 1H), 7.39 (d, J = 6.9 Hz, 2H), 7.28−7.16 (m, 3H), 7.01−6.90 (m, 3H), 5.33 (d, J = 3.9 Hz, 1H), 5.09 (d, J = 8.7 Hz, 1H), 4.91 (d, J = 8.7 Hz, 1H), 4.53−4.47 (m, 2H), 4.22 (d, J = 14.3 Hz, 1H), 3.99 (d, J = 14.3 Hz, 1H), 3.54 (br s, 2H), 3.40−3.34 (m, 2H, partially overlapped with H_2O peak), 3.19−3.15 (m, 2H), 2.98−2.84 (m, 4H), 2.71−2.50 (m, 4H, partially overlapped with DMSO peaks), 2.14 (s, 6H), 1.96−1.85 (m, 4H), 1.47 (s, 3H), 1.40 (s, 3H), 1.24 (s, 9H); HRMS (FAB) m/z 752.4066 for $[M + H]^+$ (calcd 752.4057 for $C_{40}H_{58}N_5O_7S$). Anal. ($C_{40}H_{57}N_5O_7S\cdot HCl\cdot 2H_2O$) C, H, N.

(1*S*,2*S*)-1-({(4*R*)-4-[(*tert*-butylamino)carbonyl]-5,5-dimethyl-1,3-thiazolan-3-yl}carbonyl)-2-{[2-(2,6-dimethylphenoxy)acetyl]amino}-3-phenylpropyl 4-Oxo-4-[(2-piperidinoethyl)amino]butanoate Hydrochloride (7). Compound **7** was prepared from **2a** and 1-(aminoethyl)piperidine in a manner similar to that described for compound **5**: yield 23.9%; mp 105−107 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.13 (br s, 1H), 8.38 (t, J = 5.4 Hz, 1H), 8.25 (d, J = 8.7 Hz, 1H), 7.67 (s, 1H), 7.39 (d, J = 7.5 Hz, 2H), 7.28−7.16 (m, 3H), 7.01−6.90 (m, 3H), 5.33 (d, J = 3.9 Hz, 1H), 5.09 (d, J = 8.7 Hz, 1H), 4.90 (d, J = 8.7 Hz, 1H), 4.53 (S, 1H), 4.49−4.35 (m, 1H), 4.22 (d, J = 14.4 Hz, 1H), 3.99 (d, J = 14.1 Hz, 1H), 4.45−3.39 (m, 5H, partially overlapped with H_2O peak), 3.09−3.05 (m, 6H), 3.00−2.84 (m, 2H, partially overlapped with DMSO peaks), 2.74−2.60 (m, 2H), 2.14 (s, 6H), 1.76−1.65 (m, 4H), 1.47 (s, 3H), 1.40 (s, 3H), 1.25 (s, 9H); HRMS (FAB) m/z 766.4218 for $[M + H]^+$ (calcd 766.4213 for $C_{41}H_{60}N_5O_7S$). Anal. ($C_{41}H_{59}N_5O_7S\cdot HCl\cdot 2.5H_2O$) C, H, N.

(1*S*,2*S*)-1-({(4*R*)-4-[(*tert*-butylamino)carbonyl]-5,5-dimethyl-1,3-thiazolan-3-yl}carbonyl)-2-{[2-(2,6-dimethylphenoxy)acetyl]amino}-3-phenylpropyl 4-[(2-Morpholinoethyl)amino]-4-oxobutanoate Hydrochloride (8). Compound **8** was prepared from **2a** and 4-(2-aminoethyl)morpholine in a manner similar to that described for compound **5**: yield 49.3%; mp 126−128 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.89 (br s, 1H), 8.35 (br t, 1H), 8.25 (d, J = 8.7 Hz, 1H), 7.67 (s, 1H), 7.39 (d, J = 7.2 Hz, 2H), 7.28−7.16 (m, 3H), 7.02−6.90 (m, 3H), 5.33 (d, J = 3.9 Hz, 1H), 5.09 (d, J = 8.4 Hz, 1H), 4.91 (d, J = 8.4 Hz, 1H), 4.53 (s, 1H), 4.50−4.40 (m, 1H), 4.22 (d, J = 14.1 Hz, 1H), 4.00 (d, J = 14.1 Hz, 1H), 3.96−3.91 (m, 2H), 3.80 (br t, J = 11.4 Hz, 2H), 3.44−3.33 (m, 4H, partially overlapped with H_2O peak), 3.14−2.84 (m, 4H), 2.89−2.84 (m, 2H), 2.74−2.61 (m, 2H), 2.50−2.48 (m, 2H, partially overlapped with DMSO peaks), 2.14 (s, 6H), 1.50 (s, 3H), 1.40 (s, 3H), 1.25 (s, 9H); HRMS (FAB) m/z 768.4011 for $[M + H]^+$ (calcd 768.4006 for $C_{40}H_{58}N_5O_8S$). Anal. ($C_{40}H_{57}N_5O_8S\cdot HCl\cdot 3H_2O$) C, H, N.

(1*S*,2*S*)-1-({(4*R*)-4-[(*tert*-butylamino)carbonyl]-5,5-dimethyl-1,3-thiazolan-3-yl}carbonyl)-2-{[2-(2,6-dimethylphenoxy)acetyl]amino}-3-phenylpropyl 4-[(3-Morpholinoethyl)amino]-4-oxobutanoate Hydrochloride (9). Compound **9** was prepared from **2a** and 4-(2-aminopropyl)morpholine in a manner similar to that described for compound **5**: yield 47.0%; mp 112−114 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.72 (br S, 1H), 8.23 (d, 1H, J = 8.7 Hz), 8.15 (t, J = 5.7 Hz, 1H), 7.65 (s, 1H), 7.39−7.37 (m, 2H), 7.28−7.16 (m, 3H), 7.02−6.90 (m, 3H), 5.34 (d, J = 4.2 Hz, 1H), 5.08 (d, J = 8.7 Hz, 1H), 4.91 (d, J = 8.7 Hz, 1H), 4.53−4.47 (m, 2H), 4.22 (d, J = 14.4 Hz, 1H), 3.99 (d, J = 14.4 Hz, 1H), 3.95−3.91 (m, 2H), 3.81−3.72 (m, 2H), 3.14−2.84 (m, 8H), 2.73−2.63 (m, 2H), 2.54−2.41 (m, 3H, partially overlapped with DMSO peaks), 2.14 (s, 6H), 1.92−1.79 (m, 2H), 1.49 (s, 3H), 1.40 (s, 3H), 1.25 (s, 9H); HRMS (FAB) m/z 782.4166 for $[M + H]^+$ (calcd 782.4163 for $C_{41}H_{60}N_5O_8S$). Anal. ($C_{41}H_{59}N_5O_8S\cdot HCl\cdot 4H_2O$) C, H, N.

(1*S*,2*S*)-1-({(4*R*)-4-[(*tert*-butylamino)carbonyl]-5,5-dimethyl-1,3-thiazolan-3-yl}carbonyl)-2-{[2-(2,6-dimethylphenoxy)acetyl]amino}-3-phenylpropyl 5-[(2-Morpholinoethyl)amino]-4-oxopentanoate Hydrochloride (10). Compound **10** was prepared from **2b**[65,66] and 4-(2-aminoethyl)-morpholine in a manner similar to that described for compound **5**: yield 54.7%; mp 108−110 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.77 (br s, 1H), 8.27−8.20 (m, 2H), 7.69 (s, 1H), 7.39 (d, J = 6.9 Hz, 2H), 7.27−7.16 (m, 3H), 7.01−6.90 (m, 3H), 5.32 (d, J = 3.6 Hz, 1H), 5.12 (d, J = 8.7 Hz, 1H), 4.91 (d, J = 8.7 Hz, 1H), 4.55 (s, 1H), 4.53−4.49 (m, 1H), 4.22 (d, J = 14.1 Hz, 1H), 4.00 (d, J = 14.1 Hz, 1H), 3.98−3.92 (m, 2H), 3.78 (br t, J = 13.4 Hz, 2H), 3.46−3.42 (m, 4H), 3.16−3.04 (m, 4H), 2.98−2.84 (m, 2H), 2.44 (t, J = 7.2 Hz, 2H), 2.21 (t, J = 7.2 Hz, 2H), 2.13 (s, 6H), 1.87−1.79 (m, 2H), 1.47 (s, 3H), 1.40 (s, 3H), 1.25 (s, 9H); HRMS (FAB) m/z 782.4170 for $[M + H]^+$ (calcd 782.4163 for $C_{41}H_{60}N_5O_8S$). Anal. ($C_{41}H_{59}N_5O_8S\cdot HCl\cdot 3.5H_2O$) C, N; H: calcd, 7.66; found, 7.22.

(1*S*,2*S*)-1-({(4*R*)-4-[(*tert*-butylamino)carbonyl]-5,5-dimethyl-1,3-thiazolan-3-yl}carbonyl)-2-{[2-(2,6-dimethylphenoxy)acetyl]amino}-3-phenylpropyl 4-Oxo-4-[(2-pyridylmethyl)amino]butanoate Hydrochloride (11). Compound **11** was prepared from compound **2a** and 2-aminometh-

ylpyridine in a manner similar to that described for compound **5**: yield 62.7%; mp 124−126 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.91 (t, J = 5.6 Hz, 1H), 8.76 (m, 1H), 8.41 (t, J = 7.5 Hz, 1H), 8.26 (d, J = 8.7 Hz, 1H), 7.83 (br d, 2H), 7.68 (S, 1H), 7.36 (d, J = 6.9 Hz, 2H), 7.26−7.14 (m, 3H), 6.99−6.87 (m, 3H), 5.32 (d, J = 3.9 Hz, 1H), 5.08 (d, J = 8.7 Hz, 1H), 4.90 (d, J = 8.7 Hz, 1H), 4.61 (d, J = 5.4 Hz, 2H), 4.52−4.45 (m, 2H), 4.20 (d, J = 14.1 Hz, 1H), 3.97 (d, J = 14.1 Hz, 1H), 3.04−2.82 (m, 2H), 2.73−2.66 (m, 2H), 2.61−2.59 (m, 2H), 2.11 (s, 6H), 1.46 (s, 3H), 1.39 (s, 3H), 1.23 (s, 9H); HRMS (FAB) *m/z* 746.3592 for $[M + H]^+$ (calcd 746.3587 for $C_{40}H_{52}N_5O_7S$). Anal. ($C_{40}H_{51}N_5O_7S\cdot HCl\cdot 5H_2O$) C, H, N.

(1*S*,2*S*)-1-({(4*R*)-4-[(*tert*-butylamino)carbonyl]-5,5-dimethyl-1,3-thiazolan-3-yl}carbonyl)-2-{[2-(2,6-dimethylphenoxy)acetyl]amino}-3-phenylpropyl 4-Oxo-4-[(3-pyridylmethyl)amino]butanoate Hydrochloride (12). Compound **12** was prepared from compound **2a** and 3-aminomethylpyridine in a manner similar to that described for compound **5**: yield 50.7%; mp 119−121 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.69−8.66 (m, 3H), 8.24−8.18 (m, 2H), 7.81 (dd, J = 8.1, 5.6 Hz, 1H), 7.65 (s, 1H), 7.36 (d, J = 6.9 Hz, 2H), 7.26−7.15 (m, 3H), 6.99−6.88 (m, 3H), 5.34 (d, J = 3.9 Hz, 1H), 5.08 (d, J = 8.7 Hz, 1H), 4.91 (d, J = 8.7 Hz, 1H), 4.52−4.49 (m, 2H), 4.20 (d, J = 14.1 Hz, 1H), 3.97 (d, J = 14.1 Hz, 1H), 2.97−2.83 (m, 2H), 2.75−2.61 (m, 2H), 2.54−2.44 (br m, 2H, partially overlapped with DMSO peaks), 2.11 (s, 6H), 1.46 (s, 3H), 1.39 (s, 3H), 1.24 (s, 9H); HRMS (FAB) *m/z* 746.3593 for $[M + H]^+$ (calcd 746.3587 for $C_{40}H_{52}N_5O_7S$). Anal. ($C_{40}H_{51}N_5O_7S\cdot HCl\cdot 2H_2O$) C, H, N.

(1*S*,2*S*)-1-({(4*R*)-4-[(*tert*-butylamino)carbonyl]-5,5-dimethyl-1,3-thiazolan-3-yl}carbonyl)-2-{[2-(2,6-dimethylphenoxy)acetyl]amino}-3-phenylpropyl 4-Oxo-4-[(4-pyridylmethyl)amino]butanoate Hydrochloride (13). Compound **13** was prepared from **2a** and 4-aminomethylpyridine in a manner similar to that described for compound **5**: yield 24.6%; mp 115−117 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.83−8.76 (m, 3H), 8.25 (d, J = 8.7 Hz, 2H), 7.68 (s, 1H), 7.35 (d, J = 6.9 Hz, 2H), 7.26−7.16 (m, 3H), 7.00−6.89 (m, 3H), 5.35 (d, J = 4.2 Hz, 1H), 5.09 (d, J = 8.7 Hz, 1H), 4.93 (d, J = 8.7 Hz, 1H), 4.53−4.49 (m, 4H), 4.22 (d, J = 14.1 Hz, 1H), 3.97 (d, J = 14.1 Hz, 1H), 2.98−2.78 (m, 2H), 2.75−2.67 (m, 2H), 2.61−2.54 (m, 2H), 2.12 (s, 6H), 1.47 (s, 3H), 1.40 (s, 3H), 1.25 (s, 9H); HRMS (FAB) *m/z* 746.3580 for $[M + H]^+$ (calcd 746.3587 for $C_{41}H_{52}N_5O_7S$). Anal. ($C_{40}H_{51}N_5O_7S\cdot HCl\cdot 3H_2O$) C, H, N

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