Development and Preclinical Studies of Broad-Spectrum Anti-HIV Agent (3′*R*,4′*R*)-3-Cyanomethyl-4-methyl-3′,4′-di-*O*-(*S*)-camphanoyl-(+)-*cis*-khellactone (3-Cyanomethyl-4-methyl-DCK)

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In prior investigation, we discovered that (3′*R*,4′*R*)-3-cyanomethyl-4-methyl-3′,4′-di-*O*-(*S*)-camphanoyl-(+)-*cis*-khellactone (**4**, 3-cyanomethyl-4-methyl-DCK) showed promising anti-HIV activity. In these current studies, we developed and optimized successfully a practical 10-step synthesis for scale-up preparation to increase the overall yield of **4** from 7.8% to 32%. Furthermore, compound **4** exhibited broad-spectrum anti-HIV activity against wild-type and drug-resistant viral infection of CD4+ T cell lines as well as peripheral blood mononuclear cells by both laboratory-adapted and primary HIV-1 isolates with distinct subtypes and tropisms. Compound **4** was further subjected to in vitro and in vivo pharmacokinetic studies. These studies indicated that **4** has moderate cell permeability, moderate oral bioavailability, and low systemic clearance. These results suggest that **4** should be developed as a promising anti-HIV agent for development as a clinical trial candidate.

As of February 2008, 32 anti-HIV drugs have been licensed by the U.S. Food and Drug Administration (FDA) (http://www.fda.gov/oashi/aids/virals.html). These compounds include 11 HIV protease inhibitors, 17 nucleoside and nonnucleoside reverse transcriptase (RT) inhibitors, 1 fusion inhibitor, 1 entry inhibitor (CCR5 coreceptor antagonist), 1 integrase inhibitor, and 1 multiclass combination product. Clinical combinations of these drugs, known as highly active antiretroviral therapy (HAART), have significantly reduced the morbidity and mortality of AIDS. However, increasing numbers of HIV/AIDS patients on HAART regimens fail to respond to current antiretroviral drugs becasue of the emergence of drug-resistant HIV mutants.[1] Therefore, it is essential to develop additional potent anti-HIV drugs with novel mechanisms of action or resistance profiles different from those of current anti-HIV therapeutics.

In our prior studies, 3′,4′-di-*O*-(*S*)-camphanoyl-(+)-*cis*-khellactone (**1**, DCK, Figure 1)[2] and its analogues were identified as a novel class of anti-HIV agents with potent activity in H9 lymphocytes. Systematic modification of **1** provided more than 150 khellactone derivatives, including mono-, di-, and trisubstituted **1** analogues, and their SAR study results have been published.[3−6] Notably, mechanistic studies have demonstrated that **1** and its analogues do target HIV-1 RT; however, they do not interfere with its RNA-polymerase activity but instead inhibit its DNA-dependent DNA polymerase activity. Thus, **1** analogues suppress the production of double-stranded viral DNA from a single-stranded DNA intermediate,[7] in stark contrast to current HIV-1 RT inhibitors that block the generation of single-stranded DNA from a RNA template. This unique mechanism of action provides an opportunity to discover a novel NNRTI that remains effective against HIV-1 RT multidrug resistant strains. Thus, we were strongly prompted to develop additional potent **1** analogues as potential clinical trial candidates.

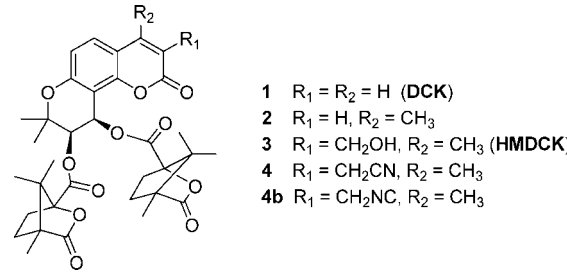

Figure 1. Structures of DCK analogues.

Because of its high potency and easy synthesis, 4-methyl-DCK (**2**) was chosen as the first drug candidate for preclinical studies. However, the poor bioavailability of **2** has limited its further development. Subsequently, another promising candidate 3-hydroxymethyl-4-methyl-DCK (**3**, HMDCK) showed moderate bioavailabity but lacked activity in a drug resistant strain.[8] Guided by SAR and 3D-QSAR results,[9] our research efforts next focused on the development of drug candidates with better pharmacokinetic profiles and potent anti HIV-1 drug-resistant activity. A cyano group shows good metabolic stability under most conditions. Moreover, it is also a good H-bond acceptor and can favorably interact with Tyr or Ser amino acid residues on the NNRTI binding site surface, which are critical determinants for several NNRTIs affinities.[10] In addition, reported evidence suggests that by introducing a cyano group, such as seen with the potent anti-HIV drug etravirine, the binding

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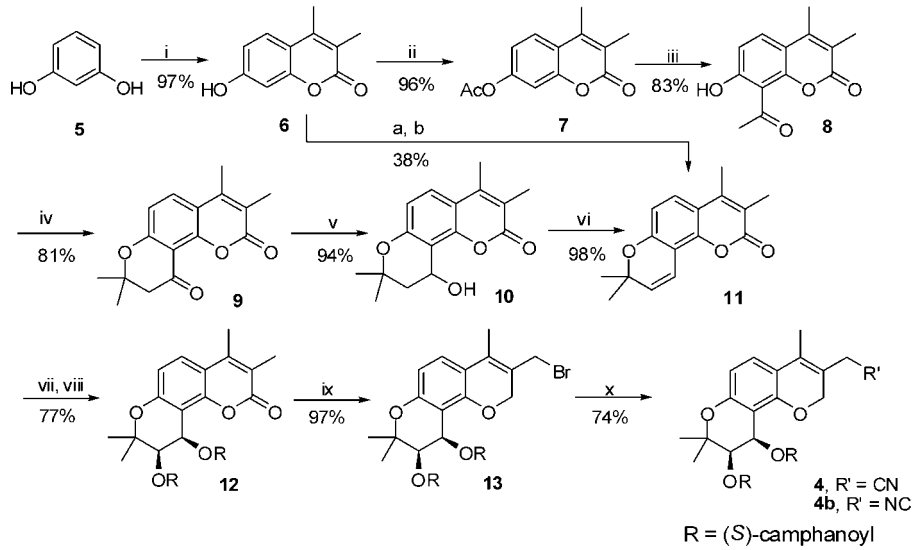
Scheme 1. Synthesis of Compound **4**[a]



[a] (i) Ethyl 2-methylacetoacetate, H_2SO_4; (a) 3-chloro-3-methyl-1-butyne, DMF, K_2CO_3, KI; (b) reflux in *N*,*N*-diethylaniline; (ii) Ac_2O; (iii) $AlCl_3$, high temperature; (iv) acetone, pyrrolidine; (v) $NaBH_4$, MeOH; (vi) TsOH, benzene, reflux; (vii) $K_3Fe(CN)_6$, K_2CO_3, $(DHQ)_2$-PYR, $K_2Os_2(OH)_4$, *t*-BuOH, H_2O (v/v 1:1), 0 °C; (viii) (*S*)-camphanic chloride, CH_2Cl_2, Py; (ix) NBS, benzene, reflux; (x) NaCN; see Table 2 for reaction conditions and products.

Table 1. Yields in Original and New Synthetic Routes for Target Compound **4**

steps	improved yield (%)	original yield (%)[3,6]
5 → **6**	97	87
6 → **11**	60(fivesteps)	38 (twosteps)
11 → **12**	77	64
12 → **13**	97	75
13 → **3**	74	49
total	32	7.8

affinity of a quinazoline inhibitor to its target (containing Tyr30 and Tyr50) increased dramatically by 30000-fold because of hydrogen bond formation between the cyano nitrogen and two phenol hydroxyls of two tyrosines.[11] On the basis of the aforementioned evidence, the 3-cyanomethyl moiety was selected to replace the 3-hydroxymethyl group of **3**. As we expected, (3′*R*,4′*R*)-3-cyanomethyl-4-methyl-DCK (**4**) discovered through this study not only exhibited promising potency against wild-type HIV-1 replication in H-9 lymphocytes (Table 4)[6] but also showed activity against several resistant strains. In this paper, we report the broad-spectrum anti-HIV potency of **4** against wild-type and drug resistant viral stains, its pharmacokinetics, and a practical synthetic protocol for scale-up synthesis of this promising compound.

Chemistry

As stated above, because **4** is a good potential drug candidate for preclinical studies, a practical synthesis for scale-up of **4** is merited and has been developed. The total yield of **4** was significantly increased from 7.8% in the original 7-step synthesis[6] to 32% in the new 10-step synthesis with optimized conditions, as shown in Scheme 1 and Table 1. The key intermediate 3,4-dimethylseselin (**11**) was originally prepared from 3,4-dimethyl-7-hydroxycoumarin (**6**) by a two-step reaction (see Scheme 1, steps a and b): (a) nucleophilic substitution with 3-chloro-3-methyl-1-butyne followed by (b) Claisen rearrangement and cyclization in *N*,*N*-diethylaniline at reflux temperature (>200 °C). However, formation of a linear pyranocoumarin isomer was unavoidable, resulting in lower yields and difficult purification in scale-up synthesis. Thus, the following alternative synthetic route was developed to synthesize intermediate **11** exclusively and efficiently. Acetylation of the 7-hydroxyl of **6** with refluxing acetic anhydride provided 7-acetoxycoumarin **7** in 96% yield. When $AlCl_3$ was used and the mixture was slowly heated to 180 °C, **7** underwent Fries arrangement providing 8-acetyl-7-hydroxycoumarin (**8**), which was easily purified by crystallization from EtOH, in an 83% yield. Meanwhile, we separated the filtrate by column chromatography to obtain a small amount of 6-acetylated isomer. The structures of **8** and its 6-acetylated isomer were identified by spectroscopic analysis, and HPLC showed that the crystalline **8** was pure, with no 6-acetylated isomer being detected. Condensation of **8** with acetone in the presence of pyrrolidine yielded the fused angular pyranocoumarin **9** in an 81% yield. Reduction of the 4′-carbonyl of **9** with $NaBH_4$ in MeOH yielded **10**. Without purification, subsequent dehydration of **10** with TsOH exclusively generated key intermediate 3,4-dimethylseselin **11**, which was readily purified by crystallization from EtOH. This current five-step preparation of **11** from **6** has a total yield of 59%, which is much higher than the 38% yield obtained in the original two-step synthesis, even though it is three steps longer. Additionally, this modified route to the key intermediate **11** has additional advantages for scale-up synthesis: it avoids production of the linear isomer, does not require chromatographic purification, and eliminates use of the costly reagent 3-chloro-3-methylbutyne-1 and the environmental contaminant *N*,*N*-diethylaniline.

Sharpless asymmetric dihydroxylation of **11** yielded (3′*R*,4′*R*)-3,4-dimethyl-(+)-*cis*-khellactone, which was dried in vacuo and used directly, without further purification, in the acylation step with (*S*)-camphanic chloride in pyridine and CH_2Cl_2. (3′*R*,4′*R*)-3,4-Dimethyl-DCK (**12**) was produced in a two-step yield of ~77%, with less than 5% of the (3′*S*,4′*S*)-isomer, as measured by HPLC. Compound **12** was treated with *N*-bromosuccinimide at a mole ratio of 1:1.2 in refluxing benzene for 4 h to produce exclusively 3-bromomethyl-4-methyl-DCK (**13**)[4,6] in 97% yield. Even though both 3- and 4-methyl groups were present, bromination occurred regioselectively only at the 3-methyl. In addition to ^{1}H NMR shifts, the position of bromination was verified using NOE (nuclear Overhauser effect) measurement, in which the signals of a methyl singlet (2.41 ppm, s) and 6-proton (6.80 ppm, d, $J = 8.8$ Hz) showed obviously increased

Table 2. Solvent Impact on Preparing DCK Nitrile (**4**) and Isonitrile (**4b**) Analogues

entry	solvent	catalyst	temp (°C)	time (h)	product(s)	yield (%)
1	DMF		room temp	24		
2	CH_3CN		room temp	24		
3	DMF + H_2O		room temp	6	nitrile/isonitrile 3.5/1	75
4	$CHCl_3/H_2O$	$Bu_4N^+I^-$	room temp	6	nitrile	60
5	$CHCl_3/H_2O$	$Bu_4N^+I^-$	60 °C	6	isonitrile	60
6	DMF	$Bu_4N^+I^-$	room temp	12	nitrile	56
7	CH_3CN	$Bu_4N^+I^-$	room temp	12	isonitrile	53
8	acetone	$Bu_4N^+I^-$	room temp	12	isonitrile	38
9	THF	$Bu_4N^+I^-$	room temp	24	isonitrile	28
10	EtOH + H_2O	$Bu_4N^+I^-$	room temp	12	nitrile	62
11	EtOH (anhydrous)	$Bu_4N^+I^-$	room temp	12	nitrile	65
12	95% EtOH		room temp	12	nitrile	60
13	EtOH (anhydrous)		room temp	12	nitrile	62
14	95% EtOH + DMF (v/v 3:2)		room temp	1	nitrile	74

Table 3. Spectroscopic Data Differences between Nitrile (**4**) and Isonitrile (**4b**)

	nitrile (**4**)		isonitrile (**4b**)	
spectral technique				
^{1}H NMR (δ ppm)	3.72	3-C**H$_2$**CN	5.27	3-C**H$_2$**NC
^{13}C NMR (δ ppm)	15.7	3-**C**H$_2$CN	59.5	3-**C**H$_2$NC
IR (cm^{-1})	2972	2935	2341	2360 (NC)
MS (ESI) *m/z* (%)	676.5	([M + 1]$^+$, 100)	676.5	649.(M − NC, 10) ([M + 1]$^+$, 5),
HPLC[a] t_R (min)	10.9		5.6	

[a] HPLC: column, C18 Axxion, 15 cm × 0.4 cm; mobile phase, 60% acetonitrile plus 25 mM HOAc/H_2O; flow rate 1.5 mL/min; λ = 320 nm.

response of about 14% and 12%, respectively, when the 5-proton (7.61 ppm, d, J = 8.8 Hz) was irradiated. This fact indicated that the 3-methyl has higher reactivity than the 4-methyl during free radical type bromination.

Another major improvement was the cyanation of **13**. In our original synthesis, compound **13** was reacted with sodium cyanide in DMSO at 60 °C to give **4** in a 49% yield.[6] However, DMSO is an environmental contaminant and not a suitable solvent for scale-up synthesis of a drug candidate, especially when used in the last step. Nitriles are generally prepared by reacting primary alkyl halides with inorganic metal cyanide salts; thus, a suitable solvent system in which both kinds of reagents are soluble is very crucial. We investigated the impact of various solvents and phase-transfer catalysts as shown in Table 2 to find a better solvent system. Accordingly, we tried first to use the polar aprotic solvent anhydrous DMF or acetonitrile rather than DMSO (entries 1 and 2, respectively). After the mixtures were stirred at room temperature for 24 h, both reactions failed because the inorganic sodium cyanide was barely soluble in these solvents. Adding a small amount of water to the DMF (DMF/water 60:1 v/v) greatly increased the solubility of sodium cyanide, resulting in a 75% yield of product (entry 3). However, the ^{1}H NMR spectrum of the product showed two different proton signals (δ 3.72 and 5.27 ppm) for the 3-CH_2 in a ratio of about 3.5:1, which indicated that the product was a pair of isomers. Each isomeric product could be obtained exclusively by using two-phase $CHCl_3/H_2O$ in the presence of tetrabutylammonium iodide ($Bu_4N^+I^-$) when the reaction was carried out at room temperature (entry 4) or reflux (entry 5, 60−70 °C). The former conditions provided only one product (3-CH_2 signal at δ 3.72 ppm), and the latter conditions afforded the other product (3-CH_2 signal at δ 5.27 ppm) in the ^{1}H NMR spectrum. Additional spectroscopic evidence (^{13}C NMR, MS, and IR spectra) confirmed that the former product was the nitrile **4** and the latter compound was the isonitrile isomer **4b**. Their spectroscopic differences are shown in Table 3. Products **4** and **4b** had HPLC retention times of 10.9 and 5.6 min, respectively.

Table 4. Anti-HIV Activity Comparison of DCK Analogue Candidates **2**, **3**, and **4** in H9 Lymphocytes in Previous Publications[a,4,6]

DCK analogue	IC_{50} (μM)	EC_{50} (μM)	TI
2	23.6	0.0015[4]	15733
3	24.9	0.004[4]	6225
4	>37.0	0.0024[6]	15417

[a] Data presented are averages of at least two separate experiments performed by Panacos Pharmaceuticals, Inc. IC_{50}: concentration that inhibits uninfected H9 cell growth by 50%. EC_{50}: concentration that inhibits viral replication by 50%. TI = IC_{50}/EC_{50}. For assay method, see refs 4 and 6.

Other phase-transfer catalysts, including benzyltriethylammonium chloride, methyltriethylammonium chloride, dibenzo-18-crown-6, and cetyltrimethylammonium chloride, were also investigated in the two-phase reaction system (data not shown), but $Bu_4N^+I^-$ gave better results. Addition of $Bu_4N^+I^-$ directly into DMF (entry 6) or acetonitrile (entry 7) improved the solubility of sodium cyanide and gave moderate yields of nitrile and isonitrile, respectively, compared with failed entries 1 and 2. Furthermore, the effects of other polar aprotic or protic solvents with or without $Bu_4N^+I^-$ (entries 8−13) were investigated. Different solvents afforded either nitrile **4** or isonitrile **4b** exclusively. EtOH [containing water or anhydrous, with or without Bu4N$^+$I$^-$ (entries 10−13)] showed high yields of nitrile **4**. Because EtOH is safe and inexpensive, it is usually a good solvent choice for scale-up synthesis. However, compound **13** had limited solubility in EtOH when the reaction scale was increased. Thus, a mixed solvent system of 95% EtOH/DMF (3:2 v/v) (entry 14) was developed. Alkyl bromide **13**, which is soluble in DMF, was added slowly to an EtOH solution of sodium cyanide over a 10−15 min period. After 2 h of stirring at room temperature, this reaction produced only the expected cyanide **4** in a 74% yield. The [α] value of pure **4** is +22.81° in chloroform, in which the 3′*S*,4′*S* stereoisomer of **4** was measured to be less than 0.5% by HPLC.

Results and Discussion

Broad-Spectrum Anti-HIV Activity. In our previous studies, compound **4** exhibited similar potency to parent compounds **2** and **3** but lower toxicity in HIV-1_{IIIB} infected H9 lymphocytes assay (Table 4).[4,6] In the current study, we further tested compound **4** in other different assays, including laboratory-adapted, primary isolates and drug-resistant strains (Tables 5−7). It is noted that because of different protocols, the current assay is less sensitive. Further screening results indicate that compound **4** exhibited potent inhibitory activity against infection of CD4+ T cell lines and peripheral blood mononuclear cells (PBMCs) by laboratory-adapted and primary HIV-1 isolates. The inhibitory activity of **4** on infection of CD4+ T cells and of MT-2

Table 5. Antiviral Data of **4** against Infection by Laboratory-Adapted HIV-1 Strains in CD4+ T Cell Lines

virus	tropism	cell line	CC_{50} (μM)[a]	EC_{50} (μM)[b]	SI
HIV-1 IIIB	X4	MT-2	40.83 ± 3.59[c]	0.47 ± 0.09	86.14
HIV-1 Lai	X4	MT-4	>100	1.51 ± 0.11	>66

[a] XTT assay was used to determine the 50% cytotoxic concentration (CC_{50}) of **4**. [b] p24 ELISA was used to determine 50% effective concentration (EC_{50}) of compound **4** against HIV-1 strains. [c] Compound **4** was tested in triplicate, and the data are presented as the mean ± SD.

Table 6. Inhibitory Activity of **4** against HIV-1 RT Resistant Strains in MT-2 Cell Line[a]

multi-RT resistant HIV-1 strains	EC_{50} (μM)	
	4	nevirapine
8605MR[a]	1.81 (8.96-fold)[b]	>14.80 (>51.21-fold)[b]
6005MR[c]	0.43 (2.13-fold)[b]	>14.80 (>51.21-fold)[b]
7324-1[d]	3.93 (14.41-fold)[b]	>14.80 (>51.21-fold)[b]
wild type -HIV-1_{IIIB}	0.20	0.29

[a] 8605MR: 41 L, 67N, 210W, 215Y, 184V, 103N. [b] Compared with EC_{50} of wild-type virus. [c] 6005MR: 41L, 74V, 184V, 210W, 215Y, ins SS, 98G. [d] 7324-1: multidrug resistant HIV-1 reverse transcriptase (RT) clone.

Table 7. Antiviral Activity of **4** against Infection of PBMCs by Primary HIV-1 Isolates of Distinct Subtypes and Tropisms

isolates	subtype	tropism	EC_{50} (μM)[a]	EC_{90} (μM)
94UG103	A	X4R5	0.84 ± 0.07	1.63 ± 0.30
92US657	B	R5	5.09 ± 0.58	9.22 ± 1.02
93MW959	C	R5	0.87 ± 0.079	3.06 ± 0.68
92UG001	D	X4R5	0.98 ± 0.35	5.23 ± 2.44
93BR020	F	X4R5	2.18 ± 0.04	3.76 ± 0.13
RU570	G	R5	3.27 ± 0.17	8.93 ± 0.19
BCF02	group O	R5	9.92 ± 1.15	18.1 ± 2.3

[a] Tests were done in triplicate, and the data are presented as the mean ± SD. The CC_{50} value of **4** to PBMCs is >150 μM.

and MT-4 cells by laboratory-adapted HIV-1 strains IIIB and Lai (X4-tropic virus) and Bal (R5-tropic virus) was determined as previously described.[12,13] The in vitro cytotoxicity of **4** was assessed using a XTT assay.[14] As shown in Tables 5 and 7, compound **4** significantly inhibited infection by both X4- and R5-tropic viruses with EC_{50} values at submicromolar levels and selective index (SI) values ranging from 66 to 182. Subsequently, we sought to determine whether **4** is also effective against HIV-1 variants resistant to RTIs. As shown in Table 6, compound **4** had an EC_{50} of approximately 4 μM for inhibiting infection by HIV-1 7324-1, which is a panel of 14 prototypical infectious multidrug resistant HIV-1 reverse transcriptase (RT) clones, including those with each of the published nucleoside analogue RT mutations in the combinations that occur most frequently in HIV-infected individuals.[15] This value is about 8-fold higher than that against infection by wild-type HIV-1 IIIB. This result suggests that the multidrug resistant HIV-1 variant is only moderately resistant to **4**, confirming that the newly synthesized **4** is an RTI with better resistance profiles than other RTIs.

$CD4^+$ T lymphocytes in PBMCs are the primary target cells for HIV-1 infection. On the basis of the HIV-1 envelope gene sequences, primary HIV-1 isolates can be classified into different genotypes, i.e., group M subtypes A−H and group O.[16−18] A, C, D, and G subtypes predominate in sub-Saharan Africa,[19] and E subtype predominates in Thailand[20] and B subtype in the U.S. and Western Europe.[21] HIV-1 isolates can be further classified into R5-, X4-, and dual (R5X4)-tropic viruses, which use CXCR4, CCR4, and both coreceptors, respectively.[17,22,23] We tested the inhibitory activity of **4** on infection by a series of primary HIV-1 isolates. As shown in Table 7, compound **4** significantly inhibited infection of PBMCs by all primary HIV-1 isolates tested, including subtypes A, B, C, D, G, and group O with R5- and X4R5 dual-tropisms. The EC_{50} values for inhibition of HIV-1 infection in PBMCs ranged from 0.8 to 10 μM. These results suggest that **4** has potent antiviral activity against a broad-spectrum of HIV-1 isolates.

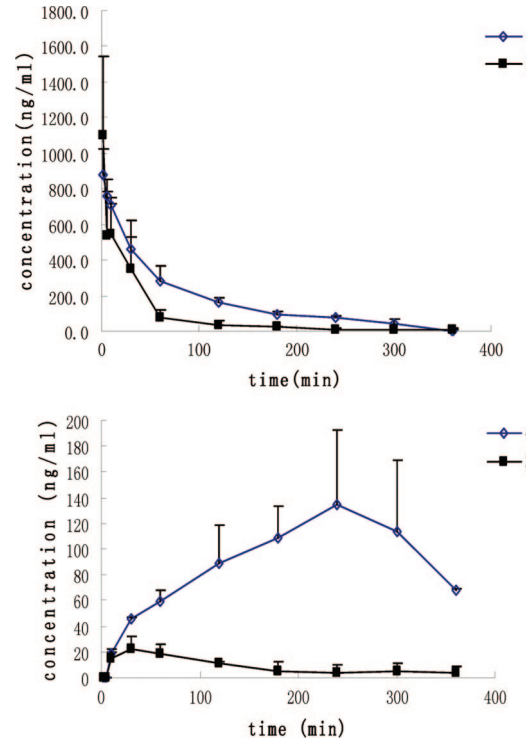


Figure 2. Mean plasma concentration-versus-time curves of **3** and **4** with CMC after iv administration at 2 mg/kg (above) and ig administration at 20 mg/kg (bottom) to SD rats (n = 3), respectively.

Pharmacokinetic Studies. To be a useful anti-HIV drug candidate, a compound must be able to penetrate the cell membrane in order to interfere with replication steps inside the cell. In a colonic adenocarcinoma cell (Caco-2) assay, **4** had an apparent permeability coefficient (P_{app}) of 5.16×10^{-6}, indicating that it can readily penetrate the cell membrane.

In in vivo studies, compound **4** was administered via intravenous (iv, 2 mg/kg) and oral (ig suspension, 20 mg/kg) routes to adult male Sprague−Dawley rats (180−200 g). Nine blood samples (0.4 mL) were collected sequentially over an 8 h period and immediately centrifuged. The separated plasma samples were stored at −20 °C for analysis. Pharmacokinetic parameters were calculated by noncompartmental analysis by using DAS, version 2.0. Initially, compounds **3** and **4** were administered as carboxymethylcellulose (CMC) suspensions, and although both compounds showed fairly low absorption in vivo, **4** was clearly better than **3** (Figure 2). To improve solubility and thus increase absorption in vivo, compound **4** was then administered in 10% poly(ethylene glycol) (PEG) 400 to rats via intravenous and oral routes. With this administration vehicle, **4** exhibited moderate oral bioavailability (F = 17.8%) in the rat, as shown in Table 8 and Figure 3. Meanwhile, compound **4** also showed low systemic clearance (0.052 (L/min)/kg) and a moderate half-time (167 min). Thus, current results indicate that the presence of the cyano moiety resulted in improved molecular pharmaceutical properties and provided lower lipophilicity, low systemic clearances, additional absorption, and longer half-life, subsequently enhancing oral bioavailability.

Table 8. Pharmacokinetic Parameters of **4** in Male Sprague−Dawley Rats (180−200 g) by iv (2 mg/kg) and ig (20 mg/kg) Dosing in 10% PEG400[a]

compd	administration	CL ((L/min)/kg)	V_d (L/kg)	AUC(0−t) (μg/(L·min))	$t_{1/2}$ (min)	F (%)
4	iv	0.01 ± 0.003	2.34 ± 0.31	195270.08	97 ± 11	
	ig	0.058 ± 0.01	8.19 ± 2.60	348067.29	167 ± 30	17.8

[a] Compound **4** was tested in triplicate, and the data are presented as the mean ± SD (n = 3).

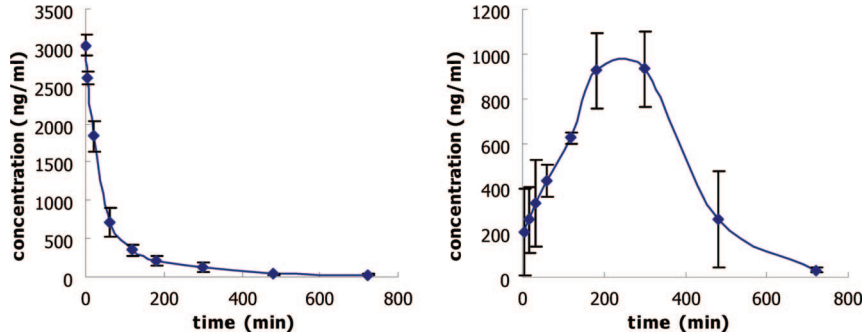


Figure 3. Mean plasma concentration-versus-time curves of **4** in PEG400 after iv administration at 2 mg/kg (left) and ig administration at 20 mg/kg (right) to SD rats (n = 3), respectively.

Therefore, compound **4** should be further developed as a potential drug candidate.

Conclusions

Drug resistance has become a critical problem in the development of new NNRTIs. However, further modification of **2** has led to the identification of **4** as a new promising lead for anti-HIV preclinical studies. It possesses not only potent antiviral activity against infection of CD4+ T cell lines and PBMCs by both laboratory-adapted and primary HIV-1 isolates with distinct subtypes and tropisms but also moderate efficacy against multidrug resistant HIV strains. In addition, a practical 10-step synthesis of **4** was successfully developed and optimized. In particular, the preparation of the key intermediate **11** and the cyanation in the final step were greatly improved. The modified synthesis gave a high overall yield of 32%, with minimized isomer formation, simplified purification, and elimination of high-cost reagents and toxic solvents, thus providing clear advantages for further scale-up synthesis. Pharmacokinetic studies also indicated that **4** has moderate oral bioavailability and cell permeability and a reasonable half-life. All current results suggest that **4** has potential for development into an anti-HIV clinical trials candidate.

Experimental Section

General Information. Melting points were measured on a RY-1 melting apparatus without correction. ^{1}H NMR spectra were measured on a JNM-ECA-400 spectrometer using TMS as the internal standard. The solvent used was $CDCl_3$ unless otherwise indicated. Mass spectra were measured on a Perkin-Elmer Sciex API-3000 mass with Turbo Ionspray ionization. HPLC (Agilent 1100) experimental conditions were as follows: C18 column (15 cm × 0.4 cm), 60% acetonitrile plus 25 mM HOAc/H_2O as mobile phase, 1.5 mL/min flow rate, UV detection at λ = 320 nm. The diastereoisomeric excess percentages were measured by HPLC. Silica gel (GF_{245} and 200−400 mesh) was purchased from Qingdao Haiyang Chemical Co., Ltd., for TLC, PTLC, and column chromatography. A Flash+ system from Biotage, Inc., was used for medium-pressure column chromatography. All other chemicals were obtained from Beijing Chemical Works or Aldrich, Inc.

3,4-Dimethyl-7-hydroxycoumarin (6). A mixture of resorcinol (**5**, 44.0 g, 0.4 mol) and ethyl 2-methylacetoacetate (66 mL) in a 1:1.15 molar ratio was quickly dropped into H_2SO_4 (98%, 120 mL) with vigorous stirring at ice-bath temperature for 10−15 min. The mixture was stirred at room temperature for 2−3 h, poured into ice−water, and allowed to stand overnight. The precipitated white solid was filtered, washed with water until neutral, and dried in vacuo to produce 73.7 g of **6** (97% yield): colorless crystals from EtOH, mp 220 °C dec; ^{1}H NMR (CD_3OD) δ 2.11 (3H, s, CH_3-3), 2.38 (3H, s, CH_3-4), 6.65 (1H, d, J = 2.4 Hz, H-8), 6.77 (1H, dd, J = 2.4 and 8.8 Hz, H-6), 7.57 (1H, d, J = 8.8 Hz, H-5).

7-Acetoxy-3,4-dimethylcoumarin (7). 3,4-Dimethyl-7-hydroxycoumarin (**6**) (19.0 g, 100 mmol) in acetic anhydride (50 mL) was heated to reflux for 1.5 h. After cooling to 50 °C, the reaction mixture was slowly poured into ice−water (300 mL) with vigorous stirring. The solid was filtered, washed with water until neutral, and recrystallized from 95% EtOH (500 mL) to yield **7** as light-yellow needles: 22.3 g, 96% yield, mp 164−5 °C; ^{1}H NMR δ 2.21 (3H, s, 3-CH_3), 2.34 (3H, s, 4-CH_3), 2.39 (3H, s, 7-CH_3CO), 7.05 (1H, dd, J = 2.4 and 8.4 Hz, 6-H), 7.08 (1H, d, J = 2.4 Hz, 8-H), 7.60 (1H, d, J = 8.4 Hz, 5-H).

8-Acetyl-3,4-dimethyl-7-hydroxycoumarin (8). 7-Acetoxy-3,4-dimethylcoumarin (**7**) (23.2 g, 100 mmol) and anhydrous aluminum chloride (53.4 g, 400 mmol) were mixed and ground to a fine powder. The mixture was placed in a flask (500 mL) and heated from 130 to 180 °C (in an oil bath) within 2 h and kept at 170−180 °C for another 2 h. After scraped ice (120 g) was added to the flask at room temperature, 5% aqueous HCl (300 mL) was slowly dropped into the flask with vigorous stirring. The mixture was then heated slowly to 110 °C and kept at the same temperature for 1 h to produce a suspension. The solid was filtered, washed until neutral, and then crystallized from EtOH (500 mL) to yield **8** (19.3 g): 83% yield, yellow crystals, mp 175−176 °C; ^{1}H NMR δ 2.21 (3H, s, 3-CH_3), 2.39 (3H, s, 4-CH_3), 2.97 (3H, s, 8-CH_3CO), 6.91 (1H, d, J = 8.8 Hz, 6-H), 7.69 (1H, d, J = 8.8 Hz, 5-H), 13.46 (1H, s, 7-OH). A small amount of 6-acetyl-3,4-dimethyl-7-hydroxycoumarin, an isomer of **8**, was obtained via flash column chromatography from the crystallization filtrate: mp 213−214 °C; ^{1}H NMR δ 2.21 (3H, s, 3-CH_3), 2.42 (3H, s, 4-CH_3), 2.72 (3H, s, 6-CH_3CO), 6.85 (1H, s, 8-H), 7.97 (1H, s, 5-H), 12.56 (1H, s, 7-OH).

3,4-Dimethyl-3′4′-dihydroseselin-4′-one (9). A mixture of **8** (23.2 g, 100 mmol), pyrrolidine (2 mL), acetone (20 mL, excess), and CH_2Cl_2 (100 mL) was stirred at room temperature for 48 h. The mixture was then poured into ice−water (50 mL), acidified with 5% aqueous HCl to pH 5−6, and extracted with CH_2Cl_2 three times. The organic phase was washed to neutral and dried over Na_2SO_4, and then the solvent was removed in vacuo. The residue was recrystallized from EtOH to produce **9** as light-yellow crystals: 25.8 g, 81% yield, mp 213−4 °C; ^{1}H NMR (DMSO) δ 1.43 (6H, s, 2′-CH_3 × 2), 2.07 (3H, s, 3-CH_3), 2.36 (3H, s, 4-CH_3), 2.83 (2H, s, 3′-CH_2CO), 6.95 (1H, d, J = 8.8 Hz, 6-H), 7.91 (1H, d, J = 8.8 Hz, 5-H).

3,4-Dimethyl-4′-hydroxy-3′,4′-dihydroseselin (10). $NaBH_4$ (11.4 g, 300 mmol) in water (50 mL) was dropped into a solution of **9** (27.2 g, 100 mmol) in MeOH (300 mL) at ice-bath temperature. Adding a few of drops of 1 M aqueous NaOH kept the reaction in a basic condition. The mixture was stirred at room temperature for 12 h until the reaction was complete (TLC, petroleum/EtOAc 4:1). The pH was adjusted to 5−6 with aqueous 5% HCl, and the mixture was extracted with EtOAc three times. The organic phase was washed with 5% $NaHCO_3$, water, and brine successively. After drying over anhydrous Na_2SO_4, the solvent was removed in vacuo to give crude **10** (25.5 g, 93% yield). The crude product could be used in the next reaction without further purification. It was also purified by a flash silica chromatography (eluant EtOAc/petroleum ether 0−30%) to give a light-yellow solid: mp 166−167 °C; ^{1}H NMR δ 1.41 and 1.47 (each 3H, s, 2′-CH_3), 2.13 (2H, d, J = 5.2 Hz, 3′-CH_2), 2.19 (3H, s, 3-CH_3), 2.38 (3H, s, 4-CH_3), 3.23 (1H, s, 4′-OH), 5.26 (1H, m, 4′-H), 6.78 (1H, d, J = 9.2 Hz, 6-H), 7.45 (1H, d, J = 9.2 Hz, 5-H).

3,4-Dimethylseselin (11). Compound **10** (27.4 g, 100 mmol) in anhydrous benzene (250 mL) in the presence of TsOH (2 g, catalytic amount) was heated to reflux for 15 min with TLC monitoring (petroleum ether/EtOAc 10:1). After the mixture was cooled to room temperature, more EtOAc was added to the mixture, and the organic phase was washed successively with 10% aqueous NaOH, water, and brine and dried over anhydrous Na_2SO_4, After removing solvent in vacuo, the residue was purified by a flash column (silica, eluant 0−10% EtOAc/petroleum ether) to give 25.0 g of **11**: 98% yield, colorless needle crystals from EtOH/H_2O, mp 135−136 °C (lit. 121−122 °C); ^{1}H NMR δ 1.47 (6H, s, 2′-CH_3 × 2), 2.18 (3H, s, 3-CH_3), 2.35 (3H, s, 4-CH_3), 5.71 (1H, d, J = 9.6 Hz, 3′-H), 6.72 (1H, d, J = 8.8 Hz, 6-H), 6.91 (1H, d, J = 9.6 Hz, 4′-H), 7.35 (1H, d, J = 8.8 Hz, 5-H).

3,4-Dimethyl-3′,4′-di-*O*-(*S*)-camphanoyl-(+)-*cis*-khellactone (12). A mixture of $K_3Fe(CN)_6$ (20 g, 60 mmol), K_2CO_3 (10 g, 72.5 mmol), 2,5-diphenyl-4,6-bis(9-*O*-dihydroquinyl)pyrimidine [$(DHQ)_2$-PYR] (531 mg, 0.6 mmol), and $K_2OsO_2(OH)_4$ (220 mg, 0.6 mmol) was solubilized in 260 mL of *t*-BuOH/H_2O (v/v, 1:1) at room temperature. Then the solution was cooled to 0 °C, and methanesulfonamide (1.9 g, 20 mmol) added under stirring. When the solution turned from light-yellow to orange, **11** (5.1 g, 20 mmol) was added. The mixture was stirred at 0 °C for 24 h. $Na_2S_2O_5$ (excess), water, and more $CHCl_3$ were added. After being stirred for 0.5 h at room temperature, the mixture was extracted with $CHCl_3$ three times. The combined organic layer was dried over $MgSO_4$, then solvent was removed in vacuo to give 3,4-dimethyl-(+)-*cis*-khellactone, which was acylated directly, without further purification, with (*S*)-(−)-camphanic chloride (13.0 g, 60 mmol) in pyridine/CH_2Cl_2 (100 mL with 3:5 v/v) for 24 h at room temperature monitored by TLC (cyclohexane/EtOAc 7:3). The mixture was diluted with EtOAc and washed successively with 10% aqueous HCl, water, and brine. The organic phase was dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo. The residue was separated by a flash column, eluting with a 0−50% gradiant of EtOAc/hexane to give 5.0 g of pure **12** (77% yield), as calculated from **11**, with 99.0% stereoselectivity for (3′*R*,4′*R*)-isomer measured by HPLC.

3-Bromomethyl-4-methyl-3′,4′-di-*O*-(*S*)-camphanoyl-(+)-*cis*-khellactone (13). A mixture of **12** (19.5 g, 30 mmol) and *N*-bromosuccinimide (NBS, 6.4 g, 36 mmol) in 50 mL of anhydrous benzene was heated under reflux for 4 h until the reaction was completed as monitored by TLC. The mixture was cooled at 4 °C overnight, the solid filtered, and the solvent removed in vacuo to give a 19.7 g of **13** (97% yield), which was sufficiently pure to use in the next reaction without further purification.

3-Cyanomethyl-4-methyl-3′,4′-di-*O*-(*S*)-camphanoyl-(+)-*cis*-khellactone (4). A solution of sodium cyanide (0.69 g, 14 mmol) in 40 mL of 95% EtOH was cooled in an ice bath. Compound **13** (7.29 g, 10 mmol) in 15 mL of DMF was added slowly to the above solution over a 15−20 min period. After 2 h of stirring at room temperature, the mixture was poured into ice−water and extracted three times with EtOAc. The combined organic phase was washed with brine, dried ($MgSO_4$), evaporated, and decolorized by passage through a short silica gel column (cyclohexane/acetone/CH_2Cl_2 7:2:1) to afford the pure desired nitrile **4** (5.0 g, 74%): white solid, mp 164−166 °C, 99.0% de (measured by HPLC), $[\alpha]_D$ + 22.81° (*c* 1.01, $CHCl_3$); ^{1}H NMR δ 1.08−1.12 (ms, 18H, 6 × CH_3), 1.46 and 1.49 (each s, 3H, 2′-CH_3), 1.66, 1.91, 2.18, and 2.51 (each m, 2 × CH_2), 2.52 (s, 3H, 4-CH_3), 3.72 (s, 2H, 3-CH_2), 5.40 (d, 1H, J = 4.8 Hz, 3′-H), 6.65 (d, 1H, J = 4.8 Hz, 4′-H), 6.89 (d, 1H, J = 8.8 Hz, 6-ArH), 7.62 (d, 1H, J = 8.8 Hz, 5-ArH); ^{13}C NMR δ ppm 9.6 and 9.9 (2 × CH_3), 15.7 (3-CH_2), 16.3, 16.4, 16.6, and 16.7 (4 × CH_3), 21.2 (2 × CH_3) and 26.1 (4-CH_3), 28.9, 29.0, 30.9, and 31.3 (4 × CH_2), 54.6, 54.7, 54.9, and 55.1 (4 × C), 61.5 and 72.1 (2 × CH), 112.8 (CN), 90.7, 91.3, 106.3, 113.3, 116.3 (6 × C), 114.9 and 127.2 (2 × CH), 150.6, 152.2, 156.7, 159.2, 167.0, 167.4 (6 × C), 177.4 and 178.6 (C═O); IR cm^{-1} (KBr) 2972, 2935; MS (ESI) *m*/*z* (%) 676.5 ($[M + 1]^+$, 100), 693.5 ($M + NH_4^+$,48), 698.5 ($M + Na^+$,75). Anal. ($C_{37}H_{41}O_{11}N\cdot$) C 66.08, H 6.25, N 1.64. Calculated: C 65.77, H 6.12, N 2.07.

Isonitrile 4b. A mixture of **13** (73 mg, 0.1 mmol), $Bu_4N^+I^-$ (18.4 mg, 0.05 mmol), and sodium cyanide (11 mg, 0.22 mmol) in 10 mL of CH_3CN was stirred at room temperature for 12 h. The mixture was then poured into ice−water and extracted with EtOAc three times. The combined organic phase was washed with brine and dried over $MgSO_4$. After removal of the solvent in vacuo, the residue was separated by PTLC (eluant cyclohexane/acetone 2:1) to give **4b** (36 mg, 53% yield); ^{1}H NMR δ 1.08−1.12 (ms, 18H, 6 × CH_3), 1.46 and 1.50 (each s, 3H, 2′-CH_3), 1.66, 1.91, 2.18, and 2.51 (each m, 2 × CH_2), 2.52 (s, 3H, 4-CH_3), 5.27 (s, 2H, 3-CH_2), 5.38 (d, 1H, J = 4.8 Hz, 3′-H), 6.65 (d, 1H, J = 4.8 Hz, 4′-H), 6.88 (d, 1H, J = 8.8 Hz, 6-ArH), 7.63 (d, 1H, J = 8.8 Hz, 5-ArH); ^{13}C NMR δ ppm 9.55 and 9.63 (2 × CH_3), 15.26, 16.26, 16.36, 16.60, 16.66, and 21.14 (6 × CH_3) and 26.13 (4-CH_3), 28.91, 28.96, 29.20, and 30.55 (4 × CH_2), 54.20, 54.60, and 54.89, 55.04 (4 × C), 59.74 (3-CH_2), 61.41 and 72.06 (2 × CH), 90.72 (NC), 91.07, 91.30, 106.06, 113.44, 116.39 (5 × C), 114.59 and 127.64 (2 × CH), 152.35, 152.70, 156.62, 159.35, 166.89, 167.25 (6 × C), 177.95, 178.01 and 178.51 (C═O); IR cm^{-1} (KBr) 2341, 2360 (NC); MS (ESI) *m*/*z* (%): 676.5 ($[M + 1]^+$, 5), 649.5 (M − NC,10).

Detection of HIV-1 Replication As Measured by p24 Antigen Production. MT-2 and MT-4 cells, laboratory-adapted HIV-1 strains, and primary HIV-1 isolates were obtained from the NIH AIDS Research and Reference Reagent Program. The inhibitory activity of compounds on HIV-1 infection was determined as previously described.[24] In brief, 1 × 10^4 MT-2 cells were infected with an HIV-1 strain (100 $TCID_{50}$) in 200 μL of RPMI 1640 medium containing 10% FBS in the presence or absence of a test compound at graded concentrations overnight. Then the culture supernatants were removed and fresh media containing no test compounds were added. On the fourth day postinfection, an amount of 100 μL of culture supernatants was collected from each well, mixed with equal volumes of 5% Triton X-100, and assayed for p24 antigen, which was quantitated by ELISA. Briefly, wells of polystyrene plates (Immulon 1B, Dynex Technology, Chantilly, VA) were coated with HIV immunoglobulin (HIVIG), which was prepared from plasma of HIV-seropositive donors with high neutralizing titers against $HIV\text{-}1_{IIIB}$, in 0.085 M carbonate−bicarbonate buffer (pH 9.6) at 4 °C overnight, followed by washes with washing buffer (0.01 M PBS containing 0.05% Tween-20) and blocking with PBS containing 1% dry fat-free milk (Bio-Rad Inc., Hercules, CA). Virus lysates were added to the wells and incubated at 37 °C for 1 h. After extensive washes, anti-p24 mAb (183−12H-5C), biotin labeled antimouse IgG1 (Santa Cruz Biotechnology, Santa Cruz, CA), streptavidin-labeled horseradish peroxidase (Zymed, S. San Francisco, CA), and the substrate 3,3′,5,5′-tetramethylbenzidine (Sigma Chemical Co., St. Louis, MO) were added sequentially. Reactions were terminated by addition of 1 N H_2SO_4. Absorbance at 450 nm was recorded in an ELISA reader (Ultra 386, TECAN, Research Triangle Park, NC). Recombinant protein p24 purchased from U.S. Biological (Swampscott, MA) was included for establishing standard dose response curves. Each sample was tested in triplicate. The percentage of inhibition of p24 production was

calculated as previously described.[25] The effective concentrations for 50% inhibition (EC_{50}) were calculated using a computer program, designated CalcuSyn,[26] kindly provided by Dr. T. C. Chou (Sloan-Kettering Cancer Center, New York, New York).

Inhibitory activity of compound **4** against infection of PBMCs by a primary HIV-1 isolate was determined as previously described.[27] Briefly, PBMCs were isolated from the blood of healthy donors at the New York Blood Center by standard density gradient centrifugation using Histopaque-1077 (Sigma). The cells were cultured in 75 cm^2 plastic flasks at 37 °C for 2 h. The nonadherent cells were collected and resuspended at 5×10^6 cells in 10 mL of RPMI-1640 medium containing 10% fetal bovine serum (FBS), 5 μg/mL phytohemagglutinin (PHA), and 100 U/mL IL-2 (Sigma), followed by incubation at 37 °C for 3 days. The PHA-stimulated cells were infected with a primary HIV-1 isolate at a 0.01 multiplicity of infection (MOI) in the presence or absence of the test compound at graded concentrations. Culture media were changed on the second day and then every 3 days. The supernatants were collected 7 days postinfection and tested for p24 antigen by ELISA as described above. The percent inhibition of p24 production and EC_{50} values were calculated as described above.

Assessment of in Vitro Cytotoxicity. The in vitro cytotoxicity of compounds on MT-2 cells was measured by XTT assay.[15] Briefly, 100 μL of the test compound at graded concentrations were added to equal volumes of cells (5×10^5/mL) in wells of 96-well plates. After incubation at 37 °C for 4 days, 50 μL of XTT solution (1 mg/mL) containing 0.02 μM of phenazine methosulfate (PMS) was added. After 4 h, the absorbance at 450 nm was measured with an ELISA reader. The CC_{50} (concentration for 50% cytotoxicity) values were calculated using the computer program CalcuSyn.[28]

Pharmacokinetic Assessments. Chromatographic analyses were performed using a Gold ODS-C18 column (3 μm, 150 mm × 2.1 mm, Hypersil Thermo) kept at 20 °C. The mobile phase was composed of acetonitrile−water−formic acid in the ratio of 65:35:0.1 (v/v/v) at a flow rate of 0.2 mL/min. Acetonitrile was HPLC grade, and other chemical reagents and solvents were of analytical grade. A Thermo Finnigan TSQ tandem mass spectrometer equipped with ESI source (San Jose, CA) and Surveyor LC pump were used for LC−MS/MS analysis. Data acquisition and data processing were performed by using Xcalibur software and LCQuan 2.0 data analysis program (Thermo Finnigan), respectively. Optimized MS parameters were as below: 4500 V spray voltage, 12.6 psi sheath gas pressure, 3.3 psi auxiliary valve flow, and 280 °C of capillary temperature. The selected reaction monitoring (SRM) mode was used, and transitions selected for quantification were as follows: m/z 698.0 → m/z 292.8 for test compounds and m/z 285.1 → m/z 138.0 for internal standard (IS) synthesized by pharmaceutical laboratory. Optimized collision energy of 47 eV was used for test compounds and 21 eV for IS. Scan time was 0.5 s per transition. Solutions of test compounds and IS were infused into the mass spectrometer.

Preparation of Samples. By use of a simple protein precipitation method, a test compound was extracted from rat plasma. Plasma samples for standard curves were prepared by spiking 100 μL of rat plasma with 50 μL of various concentrations of each test compound ranging from 10 to 10 000 ng/mL and a constant volume (50 μL) of the internal standard (IS) (2 μg/mL in acetonitrile). The final concentrations of standards were set to 5, 10, 50, 100, 200, 1000, 2000, and 5000 ng/mL. To each tested plasma sample (100 μL), 50 μL of the same concentration of IS was added. The plasma was then mixed with 400 μL of acetonitrile. After the mixture was vortexed and centrifuged at 10000g for 10 min, the supernatant was transferred to autosampler vials, and only 5 μL of the supernatant was injected into the LC−MS/MS system for analysis. The calibration curve $y = ax + b$ was obtained by assigning the concentration of T and the peak area ratio of T to IS and to x and y, respectively. Subsequently, a $1/x$ weighted linear regression was performed. The amount of each test compound in the plasma samples was back-calculated using the standard curves.

In Vivo Animal Test. Three male Sprague−Dawley rats were used in each study. Each of these rats was dosed with a testing compound at 20 mg/kg for ig administration and 2 mg/kg for iv administration each in cmc. Blood samples were collected at 0, 5, 10, 30, 60, 120, 180, 240, 300, and 360 min (Figure 2) and were immediately centrifuged to separate the plasma fractions. When compound **4** was administrated in 10% PEG400, the blood samples were collected at 0, 5, 15, 30, 60, 120, 180, 300, 480, and 720 min (Figure 3). The plasma samples obtained were stored at −20 °C until analysis. Concentration-versus-time profiles were obtained for each analyte, and standard noncompartmental analysis was performed on the data using DAS, version 2.0, to recover the area under the curve (AUC) and other noncompartmental parameters. Bioavailability was estimated by dividing the dose-normalized AUC(0−t) resulting from oral administration by the AUC(0−t) resulting from intravenous administration (where t is the last time point with measurable drug concentrations in the study). Data for each pharmacokinetic parameter were averaged and reported as mean ± standard deviation. Calibration curves for testing compounds in plasma were linear in the concentration range of 5−5000 ng/mL, with correlation coefficients of ≥0.990 for all curves.

Caco-2 Cell Experiment. Caco-2 monolayers exhibiting a transepithelial electrical resistance (TEER) value of more than 300 Ω cm^2 were used within 21 days after seeding. The culture medium was removed from both apical (AP) and basolateral (BL) sides, and the monolayers were washed twice with warm phosphate buffered saline (PBS) with calcium and magnesium. The monolayers were preincubated at 37 °C for 30 min in a CO_2 incubator with prewarmed transport media. The transport medium consisted of Hanks balanced salt solution (HBSS), 10 mM HEPES buffer, and 25 mM D-glucose, and the pH was adjusted to 7.4. Testing compounds at final concentrations of 10 mM in HBSS were added to the apical compartment. The basolateral compartment contained only HBSS. All transport studies were conducted in the AP to BL direction. At selected times, ranging from 10 to 60 min, an amount of 50 μL of samples was removed from the BL compartment for analysis and replaced with an equal volume of HBSS. Samples were analyzed using liquid chromatography mass spectrometry (LC/MS). The initial flux of the drug (J) was determined from the slope of the linear plot of the cumulative amount of drug transported versus time. Permeability was estimated by calculating the apparent permeability coefficient (P_{app})[29] according to $P_{app} = J/[(AS)C_0]$, in which C_0 is the initial concentration in the donor compartment and AS is the surface area of the monolayer.

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