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Synthesis and Evaluation of 2-Amino-6-fluoro-9-(2-hydroxyethoxymethyl)purine Esters as Potential Prodrugs of Acyclovir

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Abstract—2-Amino-6-fluoro-9-(2-hydroxyethoxymethyl)purine (**2**) and its ester derivatives **4a–d** were synthesized as potential prodrugs of acyclovir, and were evaluated for their oral acyclovir bioavailability in rats and in vivo antiviral efficacy in HSV-1-infected mice. Treatment of 2-amino-6-chloro-9-(2-hydroxyethoxymethyl)purine (**3**) with trimethylamine in THF/DMF (4:1) followed by a reaction of the resulting trimethylammonium chloride salt **5** with KF in DMF gave **2** in 78% yield. Esterification of **2** with an appropriate acid anhydride (Ac_2O, $(EtCO)_2O$, $(n\text{-}PrCO)_2O$, or $(i\text{-}PrCO)_2O$) in DMF in the presence of a catalytic amount of DMAP at room temperature produced the esters **4a–d** in 90–98% yields. Of the prodrugs tested in rats, the isobutyrate **4d** achieved the highest mean urinary recovery of acyclovir (51%) that is 5.7-fold higher than that of acyclovir (9%) and comparable to that of valacyclovir (50%). The prodrug **4d** protected dose-dependently the mortality of HSV-1-infected mice, and the group treated with **4d** at a dose of 400 mg/kg showed the longest mean survival day (14.6 ± 3.1 days) (mean ± S.D.).

Introduction

An acyclonucleoside 9-(2-hydroxyethoxymethyl)guanine (acyclovir) is a potent and highly selective inhibitor of the replication of herpesviruses including herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2), varicella-zoster virus (VZV), and Epstein-Barr virus (EBV) in cell cultures and in animals.[1] The efficacy of acyclovir in the treatment and suppression of HSV-1, HSV-2, and VZV infections has been extensively reviewed,[1] and acyclovir has been shown to play a role also in the suppression of cytomegalovirus (CMV) infections.[2,3] In humans, the average oral bioavailability of acyclovir is limited to approximately 20%, and this value decreases with increasing doses.[4] This degree of absorption is adequate to treat herpes simplex virus infections effectively, however greater absorption is necessary to treat the less susceptible VZV and CMV infections.[3,5,6] Therefore, much effort has been devoted over the past decade in attempts to find a prodrug of acyclovir that would retain the safety and efficacy profiles of acyclovir while greatly improving the oral bioavailability. Schaeffer et al. have developed two promising prodrugs of acyclovir, 6-deoxyacyclovir[7] and 2,6-diamino-9-(2-hydroxyethoxymethyl)purine (**1**).[8] 6-Deoxyacyclovir was well absorbed after oral administration and extensively oxidized to acyclovir by xanthine oxidase.[7] The 6-deoxy-6-amino congener of acyclovir, **1**, was readily deaminated to acyclovir by adenosine deaminase, an enzyme that is abundantly present in intestine and most other mammalian tissues.[9] Oral administration of **1** in dogs and rats resulted in peak plasma concentrations and total urinary recoveries of acyclovir greater than those observed after equivalent oral doses of acyclovir.[8] However, neither compound has a chronic toxicity profile in experimental animal models as favorable as that of acyclovir itself.[10,11] The L-valyl ester of acyclovir (valacyclovir), recently approved by FDA as a prodrug of acyclovir, is also rapidly and extensively converted to acyclovir after oral administration; the resulting plasma levels of acyclovir in rats and humans are 3 to 5 times

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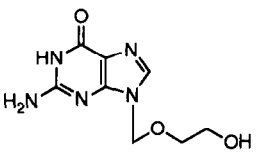
higher than those attainable with oral acyclovir itself.[11,12] When adjusted to equivalent plasma levels, valacyclovir showed the same safety profiles as acyclovir in a variety of subchronic and chronic toxicity studies in laboratory animals and humans.[11] In a previous communication, we prepared a 6-fluoropurine acyclonucleoside, 2-amino-6-fluoro-9-(2-hydroxyethoxymethyl)purine (**2**) as a potential prodrug of acyclovir and demonstrated that **2** was readily converted to acyclovir in the presence of calf intestinal mucosal adenosine deaminase in phosphate buffer solution.[13] From enzyme kinetic studies, it was found that **2** was 11.6 and 10.6 times more efficient substrate for adenosine deaminase in terms of V_{max}/K_m than the corresponding 6-aminopurine acyclonucleoside **1** and 6-chloropurine acyclonucleoside **3**, respectively.[13] Therefore, in this report, we prepared the acyl esters of **2**, **4a–d**, to maximize its oral bioavailability and evaluated for their potential as prodrugs of acyclovir.

Chemistry

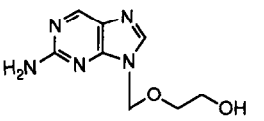
Target compounds, **4a–d** were synthesized in two steps from 2-amino-6-chloro-9-(2-hydroxyethoxymethyl)purine (**3**) as shown in Scheme 1. In our earlier work, **3** was treated with excess anhydrous trimethylamine in DMF at room temperature, and the isolated trimethylammonium salt **5** was further reacted with excess anhydrous KF in DMF to afford the desired **2** in a somewhat low yield (49%).[13] This rather inefficient conversion was attributed to the formation of a major by-product, 6-deoxy-6-dimethylaminoacyclovir, which was mainly produced from step a (Scheme 1) probably via intramolecular S_N-2 displacement by chloride. Thus, it was reasoned that this side reaction might be suppressed if the trimethylammonium chloride salt could be precipitated out completely from the reaction medium by using less polar solvents than DMF. A mixed solvent system, a 4:1 mixture of THF and DMF, served this purpose pretty well, showing only small amount of the 6-dimethylamino by-product by TLC. After the complete salt formation, the solvents were decanted, and the residual solvents were removed in vacuo. Resulting crude salts were subsequently treated with excess anhydrous KF in DMF at 80 °C to afford **2** in an improved yield of 78%. Reactions of **2** with an appropriate acid anhydride (Ac_2O, $(EtCO)_2O$, $(n\text{-}PrCO)_2O$, or $(i\text{-}PrCO)_2O$) in DMF in the presence of a catalytic amount of DMAP at room temperature produced the esters **4a–d** in 90–98% yields.

Results and Discussion

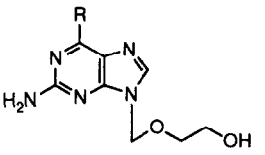
Among the ester derivatives **4a–d**, **4d** was the most soluble in H_2O (14.1 mg/mL) at 25 °C, showing a remarkable increase in aqueous solubility compared with those of the parent compound **2** (3.8 mg/mL) and acyclovir (1.5 mg/mL). The **4a** (4.8 mg/mL) and **4b** (5.6 mg/mL) also showed fair increases in water solubility. The aqueous stability of **2** and **4a–d** were examined at pH 1.2, 6.0, 7.4, and 8.0 at 37 °C, and the calculated half-lives ($t_{1/2}$) are shown in Table 1. The prodrugs **4a–d** were found to be quite stable at pH 6.0, 7.4, and 8.0, and among them, **4d** was the most stable with the $t_{1/2}$ values of 72 days, 10 days, and 10 days at pH 6.0, 7.4, and 8.0, respectively. The parent compound **2** was more stable at these buffer solutions than the ester derivatives **4a–d**. However, the prodrugs **2** and **4a–d** were relatively unstable at pH 1.2 ($t_{1/2}$ = 58–64 min), indicating that the fluoro atom at the C-6 of the purine ring is relatively



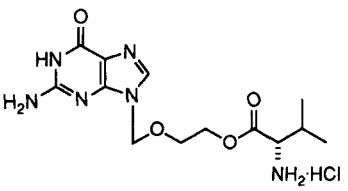
Acyclovir



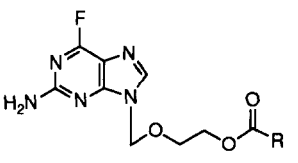
6-Deoxyacyclovir



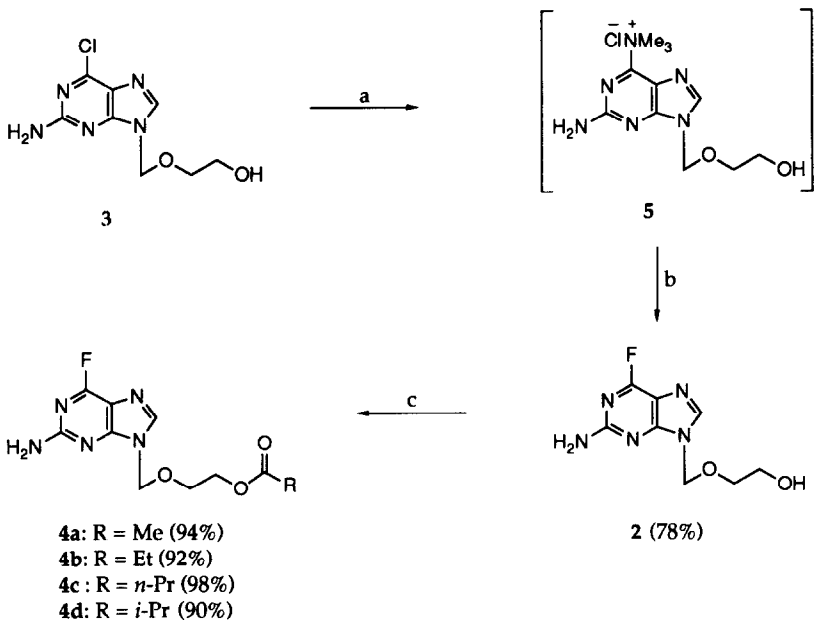
1: R = NH_2
2: R = F
3: R= Cl



Valacyclovir

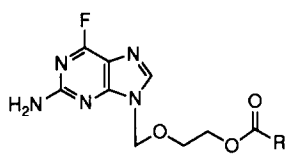


4a: R = Me
4b: R = Et
4c: R = *n*-Pr
4d: R = *i*-Pr



Scheme 1. (a) NMe_3 (equiv.), DMF/THF(1/4), −78 °C to rt, 5 days; (b) KF (10 equiv.), DMF, 80 °C, 3 h; (c) $(RCO)_2O$ (3 equiv.), DMAP (0.1 equiv.), DMF, rt, 20 min.

Table 1. Solubility and stability in aqueous solution, and oral bioavailability in rats of 2-amino-6-fluoro-9-(2-hydroxyethoxymethyl)purine esters **4a–d**



4a–d

Compound	R	Solubility in H_2O[a] (mg/mL, 25 °C)	Half-life[b] (37°C)				Urinary recovery of acyclovir (% dose)[e,f]
			pH 1.2[c]	pH 6.0[d]	pH 7.4[d]	pH 8.0[d]	
2		3.8[g]	64 min	89 days	56 days	36 days	38
4a	Me	5.8	64 min	59 days	10 days	5 days	44
4b	Et	5.6	62 min	39 days	6 days	6 days	46
4c	*n*-Pr	1.5	58 min	28 days	5 days	8 days	43
4d	*i*-Pr	14.1	61 min	72 days	10 days	10 days	51
Acyclovir		1.5					9
Valacyclovir							50

[a]Determined by the comparison of the UV absorbance of the saturated solution of each compound at 245 nm with that of the standard curve.
[b]Determined by HPLC using a C_{18} reversed-phase column.
[c]HCl/NaCl buffer.
[d]Sodium phosphate buffer.
[e]A single oral dose of the test compound (0.1 mmol/kg) was administered to two male SD rats. The total amount of acyclovir recovered in the urine over a 48 h period was determined by HPLC using a C_{18} reversed-phase column.
[f]Values are the mean of two independent experiments run.
[g]Ref.[13].

sensitive in the acidic buffer solution to undergo hydrolysis. The bioavailability of acyclovir after a single oral administration (0.1 mmol/kg) of **2** and **4a–d** was estimated by determining the total amount of acyclovir recovered in the urine over a 48 h period and was compared with those of acyclovir and valacyclovir. The isobutyrate **4d** achieved the highest mean urinary recovery of acyclovir (51%) that is 5.7-fold higher than that of acyclovir (9%) and comparable to that of valacyclovir (50%). The propionate **4b** (46%), the acetate **4a** (44%), the butyrate **4c** (43%), and the parent compound **2** also showed approximately 4–5-fold higher mean urinary recovery of acyclovir compared with that of acyclovir. The antiviral efficacy of **4d** on HSV-1 induced mortality in BALB/c mice was evaluated and was compared with those of acyclovir and valacyclovir (Table 2). HSV-1-infected mice receiving no antiviral therapy developed a wasting syndrome by day 2 postinfection, started to die, and showed a mean survival day of 6.7 ± 1.4 days (mean ± S.D.). And, no mice were surviving on the last day of evaluation (day 21). However, following twice-daily 4-day consecutive treatment, both **4d** and valacyclovir protected dose-dependently the mortality of HSV-1-infected mice. The survival rates of the groups treated with **4d**, valacyclovir, or acyclovir at doses of 200 or 400 mg/kg were 20 and 30%, 10 and 40%, and 10 and 10%, respectively. All of the treated groups caused increses in the mean survival day, as compared with those of untreated group (6.7 ± 1.4 days), among them, the group treated with **4d** at a dose of 400 mg/kg showed the longest mean survival day (14.6 ± 3.1 days). In conclusion, 2-amino-6-fluoro-9-(2-isobutyryloxyethoxymethyl)purine (**4d**) achieved the highest oral mean urinary recovery of acyclovir in rats that was comparable to that of valacyclovir, showed the comparable in vivo antiviral efficacy to valacyclovir, and was found to be quite stable at pH 6.0, 7.4, and 8.0 buffer solutions and sufficiently soluble in the aqueous solution. On the basis of these results, the extensive studies on pharmacokinetics, enteric-coating formulation, and toxicology of **4d** are presently under way in our laboratory.

Table 2. Effect of 2-amino-6-fluoro-9-(2-isobutyryloxyethoxymethyl)purine (**4d**), acyclovir, and valacyclovir on HSV-1 induced mortality in BALB/c mice[a]

Compound	Dose (μmol/kg)	% Survival	Mean survival[b] (days)
4d	100	0	7.7 ± 1.0[d]
	200	20	10.1 ± 2.2[d]
	400	30	14.6 ± 3.1[d]
Acyclovir	100	0	8.7 ± 0.8[d]
	200	10	10.2 ± 2.1[d]
	400	10	12.9 ± 1.5[d]
Valacyclovir	100	0	11.0 ± 3.2[d]
	200	10	11.9 ± 3.0[d]
	400	40	12.0 ± 5.7[c]
Untreated		0	6.7 ± 1.4

[a]Four-week-old male BALB/c mice (10 mice/treated group and 20 mice/untreated group) were infected intraperitoneally with 10^4 PFU of HSV-1 (KOS strain), and each compound was administered orally twice-daily (at 10 a.m. and 6 p.m.) starting 18 h after virus inoculation for 4 days.

[b]Each value represents the mean ± S.D.

[c]Significantly different from untreated group ($P < 0.05$) by the two-tailed Mann–Whitney *U*-test.

[d]Significantly different from untreated group ($P < 0.01$) by the two-tailed Mann–Whitney *U*-test.

Experimental

Melting points were determined on a Mettler (FP 62) melting point apparatus and are uncorrected. Infrared spectra were recorded on a Perkin–Elmer 1600 FTIR spectrophotometer and UV spectra on a Hewlett–Packard 8452A spectrometer. ^{1}H NMR spectra were recorded on a Varian Unity 300 spectrometer. The chemical shifts are reported in parts per million (ppm) relative to internal tetramethylsilane in $CDCl_3$ or DMSO-d_6. ^{1}H noise-decoupled ^{13}C NMR spectra were recorded on a Varian Unity 300 spectrometer at 75.4 MHz. When $CDCl_3$ or DMSO-d_6 was used as solvent, it served as the internal standard at δ 77.0 or 39.5, respectively. Electron impact mass spectra (EI-MS) were obtained on a VG Quattro mass spectrometer. Analytical thin-layer chromatography (TLC) was performed on Merck silica gel 60F-254 glass plates. Flash column chromatography was performed using Merck silica gel 60 (230–400 mesh). Elemental analyses were performed on a Carlo Erba 1106 elemental analyzer.

An improved synthesis of 2-amino-6-fluoro-9-(2-hydroxyethoxymethyl)purine (2). Anhydrous trimethylamine (32 mL) was condensed at −78 °C and was added dropwise to a cooled suspension of 2-amino-6-chloro-9-(2-hydroxyethoxymethyl)purine (**3**) (8.50 g. 43.91 mmol) in a mixture of anhydrous THF (520 mL) and DMF (130 mL) at −78 °C under nitrogen atmosphere via a cannula. The resulting suspension was warmed to room temperature immediately after addition of trimethylamine, and was stirred under nitrogen atmosphere at room temperature for 5 days. The reaction mixture was allowed to be settled down, the supernant solvent was decanted off from the white precipitates, and the residual volatiles were removed completely in vacuo to afford the corresponding ammonium salt **5** as a white solid. The resulting ammonium salt was treated with anhydrous KF (20.3 g, 0.35 mol) in anhydrous DMF (500 mL) at 80 °C for 3 h while the gaseous

trimethylamine by-product was being removed from the reaction medium under reduced pressure using an aspirator (about 40 mmHg). The reaction mixture was cooled to room temperature, filtered through a glass filter, and the filtrate was evaporated to dryness in vacuo to give a yellow solid. The crude product was purified by column chromatography on silica gel (gradient elution: 5% MeOH in $CHCl_3$, 10% MeOH in $CHCl_3$, and 20% MeOH in $CHCl_3$), and the slightly impure product was triturated once from cold MeOH to afford pure **2** (6.22 g, 78%) as a white powder. Spectroscopic data and physical properties were identical to those reported in the literature.[13]

Synthesis of 4a–d: General procedure. To a solution of **2** (400 mg, 1.76 mmol) and a catalytic amount of DMAP (22 mg, 0.18 mmol) in anhydrous DMF (7 mL) was added an appropriate acid anhydride (acetic anhydride, propionic anhydride, butyric anhydride or isobutyric anhydride) (5.28 mmol) at room temperature under nitrogen atmosphere, and the mixture was stirred for 20 min at room temperature. MeOH (5 mL) was added, and the reaction mixture was evaporated to dryness under reduced pressure. The residue was partitioned between $CHCl_3$ (3×25 mL) and saturated aqueous $NaHCO_3$ solution, and the organic layer was dried ($MgSO_4$), filtered, and evaporated to dryness under reduced pressure to give a yellow solid. The crude product was purified by column chromatography on silica gel (5% MeOH in $CHCl_3$) to afford the corresponding ester as a white solid. Analytically pure material was obtained by recrystallization from the solvents indicated below.

2-Amino-6-fluoro-9-(2-acetoxyethoxymethyl)purine (4a). yield 94%; mp 121.7–122.4 °C (EtOAc–hexane); UV (H_2O) λ_{max} 214 (ε 25500), 244 (9100), and 288 (6400) nm; IR (KBr) 3494, 3317, 3195, 1726, 1645, 1575, 1259 cm^{-1}; ^1H NMR (DMSO-d_6/TMS) δ 1.94 (s, 3 H, CH_3), 3.70 (t, J=4.7 Hz, 2 H, OCH_2), 4.08 (t, J=4.7 Hz, 2 H, CH_2OAc), 5.48 (s, 2 H, NCH_2O), 6.99 (br s, 2 H, NH_2), 8.24 (s, 1 H, H-8); EI-MS m/z 269 (M^+). Anal. calcd for $C_{10}H_{12}FN_5O_3$: C, 44.61; H, 4.49; N, 26.01. Found: C, 44.42; H, 4.63; N, 25.88.

2-Amino-6-fluoro-9-(2-propionyloxyethoxymethyl)purine (4b). yield 92%; mp 102.3–103.0 °C (EtOAc–hexane); UV (H_2O) λ_{max} 214 (ε 25100), 244 (9000), and 288 (6300) nm; IR (KBr) 3452, 3336, 3217, 3105, 1719, 1643, 1573, 1215 cm^{-1}; ^1H NMR (DMSO-d_6/TMS) δ 1.12 (t, J=7.6 Hz, 3 H, CH_2CH_3), 2.32 (q, J=7.6 Hz, 2 H, CH_2CH_3), 3.76 (t, J=4.7 Hz, 2 H, OCH_2), 4.23 (t, J=4.7 Hz, 2 H, CH_2OCO), 5.39 (br s, 2 H, NH_2), 5.54 (s, 2 H, NCH_2O), 7.89 (s, 1 H, H-8); EI-MS m/z 283 (M^+). Anal. calcd for $C_{11}H_{14}FN_5O_3$: C, 46.64; H, 4.98; N, 24.72. Found: C, 46.52; H, 5.08; N, 24.61.

2-Amino-6-fluoro-9-(2-butyryloxyethoxymethyl)purine (4c). yield 98%; mp 95.5–96.3 °C (ether–hexane); UV (H_2O) λ_{max} 214 (ε 23400), 244 (8200), and 288 (5700) nm; IR (KBr) 3426, 3331, 3225, 3119, 1724, 1645, 1576, 1221, 1177, 1097 cm^{-1}; ^1H NMR ($CDCl_3$/TMS) δ 0.93 (t, J=7.5 Hz, 3 H, CH_3), 1.63 (qt, J=7.5 Hz, J=7.4 Hz, 2 H, CH_2CH_3), 2.27 (t, J=7.4 Hz, 2 H, $COCH_2$), 3.76 (t, J=4.7 Hz, 2 H, OCH_2), 4.22 (t, J=4.7 Hz, 2 H, CH_2OCO), 5.37 (br s, 2 H, NH_2), 5.53 (s, 2 H, NCH_2O), 7.88 (s, 1 H, H-8); EI-MS m/z 297 (M^+). Anal. calcd for $C_{12}H_{16}FN_5O_3$: C, 48.48; H, 5.42; N, 23.56. Found: C, 48.53; H, 5.45; N, 23.38.

2-Amino-6-fluoro-9-(2-isobutyryloxyethoxymethyl)purine (4d). yield 90%; mp 85.3–87.1 °C (ether–hexane); UV (H_2O) λ_{max} 214 (ε 26400), 244 (9300), and 288 (6500) nm; IR (KBr) 3342, 3219, 1726, 1657, 1572, 1210, 1108 cm^{-1}; ^1H NMR ($CDCl_3$/TMS) δ 1.14 (d, J=6.9 Hz, 6 H, $CH(CH_3)_2$), 2.53 (septet, J=6.9 Hz, 1 H, $CH(CH_3)_2$), 3.76 (t, J=4.8 Hz, 2 H, OCH_2), 4.22 (t, J=4.8 Hz, 2 H, CH_2OCO), 5.34 (br s, 2 H, NH_2), 5.54 (s, 2 H, NCH_2O), 7.88 (s, 1 H, H-8); EI-MS m/z 297 (M^+). Anal. calcd for $C_{12}H_{16}FN_5O_3$: C, 48.48; H, 5.42; N, 23.56. Found: C, 48.24; H, 5.48; N, 23.44.

Water solubility. A standard solution was prepared by dissolving 1 mg of the test compound in 10 mL of H_2O at 20 °C. The standard solution was diluted with H_2O as necessary, and the diluted standard solutions were scanned by UV at 245 nm to obtain a standard curve. A saturated solution was prepared by vortex-mixing in H_2O for 1 min, ultrasonification for 1 min, vortex-mixing for 3 min, ultrasonification for 1 min, and finally vortex-mixing for 5 min in the presence of excess compound. After the saturated solution was filtered to remove excess compound using 0.45-μm Millipore filters, the solution was diluted with H_2O and then scanned by UV at 245 nm. Total solubility was then determined by the comparison of the absorbance of the saturated solution with that of the standard curve.

Aqueous stability. Fifty microliters of 2.5 mM stock solution of the test compound was added to sodium phosphate buffer (pH 6.0, pH 7.4, or pH 8.0) or HCl/NaCl buffer (pH 1.2) to give a final concentration of 125 μM. Immediately after the compound was mixed with the buffer, incubation of the solution was initiated at 37 °C. At various intervals, 100 μL of the sample was taken throughout the indicated incubation times and immediately mixed with 900 μL of 0.1 M phosphate buffer (pH 7.0). The sample was then eluted at a flow rate of 1 mL/min with the following three-step gradient; (step 1) a 10 min isocratic elution with 100% buffer A (0.1% phosphoric acid), (step 2) a 25 min linear gradient from 100% buffer A to 55% buffer A and 45% buffer B (80% MeCN in 0.1% phosphoric acid), (step 3)

a 4 min isocratic elution with 55% buffer A and 45% buffer B. A Waters Symmetry C_{18} column (15 cm by 3.9 mm ID) was equilibrated with 100% buffer A for 10 min before each sample injection. The UV absorbance of the column effluent was monitored at 248 nm. The half-life of the compound was calculated from the concentration of the parent compound.

Oral bioavailability. The bioavailability of the test compound was estimated by determining the total amount of acyclovir in the urine using HPLC. Urine was collected for 48 h in a metabolic cage after oral administration of a single 0.1 mmol/kg dose of the test compound to two male Sprague-Dawley rats (200–250 g). A 5% solution of sodium azide (0.4 mL per estimated 100 mL of urine) was added to each urine receptacle before collection to prevent bacterial growth. The collected urine was filtered (0.45-μm), and the acyclovir concentration was analyzed by HPLC as follows. A Waters Symmetry C_{18} column (15 cm by 3.9 mm ID) equipped with a compatible guard column was eluted at a flow rate of 1 mL/min with the following three-step gradient; (step 1) a 10 min isocratic elution with 100% buffer A (0.1% phosphoric acid), (step 2) a 25 min linear gradient from 100% buffer A to 55% buffer A and 45% buffer B (80% MeCN in 0.1% phosphoric acid), (step 3) a 4 min isocratic elution with 55% buffer A and 45% buffer B. Column was equilibrated with 100% buffer A for 10 min before each sample injection. The UV absorbance of the column effluent was monitored at 248 nm.

In vivo antiviral efficacy test. Four-week-old male specific pathogen-free (SPF) BALB/c mice (Charles River, Sekyo, Japan) (10 mice/treated group and 20 mice/untreated group) were infected intraperitoneally with 10^4 PFU of HSV-1 (KOS strain), and each compound dissolved or suspended in phosphate-buffered saline was administered orally twice-daily (at 10 a.m. and 6 p.m.) starting 18 h after virus inoculation for 4 days. Death and body weight change were recorded for 21 days after virus inoculation. The parameters used to assess in vivo antiviral efficacy include prevention of mortality and delay in mean survival day to death.

Statistical analysis. The two-tailed Fisher's exact test was used for determination of significant difference in survival rate, and two-tailed Mann–Whitney *U*-test was used for determination of significant difference in mean survival day. *p*-values of < 0.05 were considered statistically significant.

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