Chemistry SELECT

Supporting Information

Selective Inhibition of *Enterovirus A* Species Members' Reproduction by Furano[2,3-*d*]pyrimidine Nucleosides Revealed by Antiviral Activity Profiling against (+)ssRNA Viruses

Liubov I. Kozlovskaya, Anastasia D. Golinets, Anastasia A. Eletskaya, Alexey A. Orlov, Vladimir A. Palyulin, Sergey N. Kochetkov, Liudmila A. Alexandrova, and Dmitry I. Osolodkin*

Experimental Section

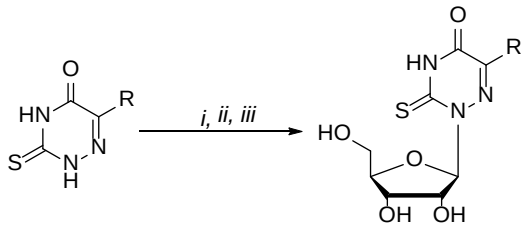
Chemistry

Previously published synthetic procedures were used for compounds **1**[s1], **2**[s2], **3**[s3], **6**-**10**[s4]. Commercial reagents of the companies Fluka (Germany), Aldrich, Sigma (United States), and Acrus (Belgium) were used. Solvents were purified by standard methods.

Column chromatography was carried out using Kieselgel 60 silica gel (40—63 μm) (Merck, Germany). NMR spectra (δ, ppm, spin—spin coupling constant, Hz) were recorded in DMSO-d_6 on an AMX III-400 spectrometer (Bruker, United States) with the working frequency of 400 MHz for ^{2}H NMR (the internal standard Me4Si) and 101 MHz for ^{13}C NMR (the internal standard methanol). UV spectra were recorded on a UV-2401P spectrophotometer (Shimadzu, Japan) in methanol. High-resolution mass spectra were recorded on a Bruker Daltonics micrOTOF-Q II device by electrospray ionization mass spectrometry (ESI-MS). Measurements were carried out in positive ion mode under following conditions: the spray capillary voltage 4500 V; the mass scanning range *m/z* 100–3000 Da; external calibration (Electrospray Calibrant Solution, Fluka); the nebulizer pressure 0.8 bar; flow rate 3 μL/min; nebulizer gas nitrogen (4 L/min); and interface temperature 190°C. Samples were delivered to the spray chamber of the m were delivered to a HPLC chromatograph from an acetonitrile—water solution 1:1 (5 μL).

*General method for obtaining compounds **4** and **5***

2-Thio-5-(tert-butyl)-6-azauracil **4** and 2-thio-5-phenyl-6-azauracil **5** were obtained using a previously published scheme[s5].



Scheme S1. *i*: BSA, 1,2-dichloroethane, 82 °C, 15 min; *ii*: 1,2,3,5-Tetra-O-acetyl-β-*D*-ribofuranose, $Me_3SiOTfl$, 1,2-dichloroethane, 82 °C, 45 min; *iii*: 24% aqua ammonia, dioxane, r.t., 21 h. R = *tert*-butyl (for **4**) or phenyl (for **5**).

A proper nucleic base (1 mmol) was suspended in 2-dichloroethane (10 mL) and then *N,O*-bis(trimethylsillyl)acetamide (0.7 mL, 2.7 mmol) was added. The mixture was stirred at 82 °C for 15 min, evaporated *in vacuo*, coevaporated with toluene (2 × 10 mL) and dissolved in 1,2-dichloroethane (10 mL). 1,2,3,5-Tetra-*O*-acetyl-β-D-ribofuranose (159 mg, 0.5 mmol) and a solution of $Me_3SiOTfl$ (0.1 mL, 0.6 mmol) in dichloroethane (5 mL) was added. The mixture was refluxed for 45 min, cooled to r.t., neutralized with pyridine (5 mL) and diluted with 20 mL of CH_2Cl_2. The organic layer was washed successively with water (3 × 10 mL) and a saturated NaCl solution (10 mL), dried over Na_2SO_4, and evaporated in vacuo. The residue was dissolved in dioxane (5 mL), and 24% aqueous ammonia (5 mL) was added. After 21 h, the solution was evaporated, and the residue was purified by column chromatography by column chromatography on silica gel (1.2 × 14 cm); elution was in a gradient of ethanol concentraions in CHCl3 (1 → 10%). Fractions containing target compounds were combined, evaporated *in vacuo*, dissolved in dioxane, and liophylized.

2-Thio-5-(tert-butyl)-6-azauridine (4) was obtained as described above from 2-thio-5-(tert-butyl)-6-azauracil (185 mg). Yield: 109 mg (69%). ^{1}H NMR (DMSO-d6): δ 1.27 (9H, s, t-Bu), 3.37-3.42 (1H, dd, *J* = 6 and 11 Hz, H-5'$_a$), 3.56-3.59 (1H, d, *J* = 11 Hz, H-5'$_b$), 3.78-3.82 (1H, dd, *J* = 6 and 10 Hz, H-4'), 4.16 (1H, s, H-3'), 4.22 (1H, s, H-2'), 4.62 (1H, s, OH-3'), 4.98 (1H, s, OH-5'), 5.23 (1H, s, OH-2'), 6.83-6.83 (1H, d, *J* = 7 Hz, H-1'). ^{13}C NMR (DMSO-d6): 27.22 ((CH$_3$)$_3$- (t-Bu)), 37.08 (C- (t-Bu)), 61.62 (C-5'), 70.11 (C-2'), 73.40 (C-3'), 84.53 (C-4'), 93.78 (C-1'), 150.81 (C=O), 155.49 (C=N), 174.43 (C=S). UV: λmax 272.1 nm (ε 15035). MS (ESI): calcd for $C_{12}H_{19}N_3O_5S$ 318.1118 $[M+H]^+$, found 318.1114.
2-Thio-5-phenyl-6-azauridine (5) was obtained as described above from 2-thio-5-(phenyl)-6-azauracil (205 mg). Yield: 123 mg (73%). ^{1}H NMR (DMSO-d6): δ 3.44-3.48 (1H, dd, *J* = 5 and 12 Hz, H-5'$_a$), 3.61-3.64 (1H, dd, *J* = 3 and 12 Hz, H-5'$_b$), 3.85-3.89 (1H, dd, *J* = 5 and 10 Hz, H-4'), 4.22-4.25 (1H, t, *J* = 5 Hz, H-3'), 4.30-4.31 (1H, m, H-2'), 4.67 (1H, s, OH-3'), 5.05 (1H, s, OH-5'), 5.33 (1H, s, OH-2'), 6.93-6.93 (1H, d, *J* = 7 Hz, H-1'), 7.46-7.53 (3H, m, H-Ph (o-, p-)), 7.95-7.97 (1H, m, H-Ph (m-)). ^{13}C NMR (DMSO-d6): 61.44 (C-5'), 70.11 (C-2'), 73.68 (C-2'), 84.62 (C-4'), 93.98 (C-1'), 128.20 (C-Ph (o-)), 128.40 (C-Ph (m-)), 130.41 (C-Ph (p-)), 131.40 (C-Ph (C-1)), 145.86 (C=N), 151.60 (C=O), 174.22 (C=S). UV: λmax 282.1 nm (ε 24000). MS (ESI): calcd for $C_{14}H_{15}N_3O_5S$ 338.0805 $[M+H]^+$, found 338.0807.

Biology

Cells and viruses

Porcine embryo kidney (PEK) and rhabdomyosarcoma (RD) cell lines were from Chumakov FSC R&D IBP RAS. The latter originated from NIBSC (UK).

All viruses used in the present work were obtained from Chumakov FSC R&D IBP RAS working collection of viruses.

Tick-borne encephalitis virus strain Absettarov (GenBank accession no. KU885457) was isolated from blood of the patient with acute TBE in 1951 in Russia.

Enterovirus A 71 isolate 46973 (GenBank accession no. KJ645808) was isolated from patient with acute flaccid paralysis in 2013 in Russia.

Enterovirus A Coxscakievirus A16 isolate 49360 was isolated from patient enteroviral infection in 2014 in Russia.

Enterovirus B Coxscakievirus B1 isolate 48461 was isolated from patient with enteroviral meningitis in 2013 in Russia.

Enterovirus B Coxscakievirus A9 isolate 46721 was isolated from patient with enteroviral meningitis in 2013 in Russia.

Enterovirus B Echovirus 6 isolate 42582 was isolated from sewage in 2012 in Russia.

Enterovirus B Echovirus 30 isolate 47783 was isolated from patient with enteroviral meningitis in 2013 in Russia.

Enterovirus C reference vaccine strain Sabin 1 of poliovirus type 1 (GenBank access no. V01150) is from Moscow RRL Polio collection originated from NIBSC (UK).

Cell toxicity assay (PEK cells)

A cytotoxicity test in PEK cells was performed as described previously[s6]. In brief, stock 5 mM DMSO solutions of the compounds were serially diluted two-fold in medium 199 in Earle solution (Chumakov FSC R&D IBP RAS, Russia) to obtain final concentrations starting from 50 μM. Equal volumes of compound dilutions were added in two replicates to the cell monolayers. Control cells were treated with corresponding sequential concentrations of DMSO in two replicates. After incubation at 37 °C on days 1 and 7, cells morphology and vitality was assessed via microscope. CC_{50} was calculated according to the Karber method.

Cell toxicity assay (RD cells)

Two-fold dilutions of compounds (concentration 5 mM) were prepared in Eagle minimal essential medium with doubled amino acids and vitamins (2×EMEM, Chumakov FSC R&D IBP RAS, Russia) to obtain final concentrations starting from approx. 104 μM. Equal volumes of compound dilutions were added in four replicates to the 96-well plates and covered with RD cell suspension (approx. 10^5 cells per well) in 2×EMEM with 5% FBS (Invitrogen, South America). Control cells were treated with the same sequential concentrations of DMSO, as in compound dilutions, in four replicates. After incubation at 37 °C on days 1 and 7, cells morphology and vitality was assessed via microscope. CC_{50} values were calculated according to the Karber method.

TBEV plaque reduction test

Plaque reduction test was performed as previously described[s6]. In brief, stock 5 mM DMSO solutions of the compounds were diluted in medium 199 in Earle solution (Chumakov FSC R&D IBP RAS, Russia) and added to the cells simultaneously with the virus (20-40 PFU/well) at final concentration of 50 μM in each well in 8 replicates. Virus with corresponding DMSO dilution was used as a control. Cells were incubated at 37 °C for 1 h for infectious virus adsorption. Then, each well was overlaid with 1 mL of 1.26% methylcellulose (Sigma) containing 2% FBS (Invitrogen, South America). After incubation at 37 °C for 6 days, the cells were fixed with 96% ethanol. Plaques were stained with 0.4% gentian violet and counted. EC_{50} were calculated according to the Reed-and-Muench method.

EV cytopathic effect inhibition test

Cytopathic effect inhibition test against representatives of Enterovirus genus was performed as described previously[s7]. In brief, eight 2-fold dilutions of stock solutions of the compounds in 4 replicates were prepared in 2×EMEM (Chumakov FSC R&D IBP RAS, Russia) to obtain a final concentration series starting from approx. 104 μM. Compound dilutions were mixed with equal volumes of the enterovirus suspension containing 100 $TCID_{50}$ (50% tissue culture infectious dose). Control cells were treated with the same sequential concentrations of DMSO as in compound dilutions. After 1 h incubation at 36.5 °C the RD cell suspension (approx.10^5 cells per well) in 2×EMEM containing 5% FBS (Invitrogen, South America) was added to experimental mixtures. Each experiment contained virus dose titration in the inoculate to assure the acceptable dose-range. After a 5-day incubation at 37 °C, cytopathic effect (CPE) was visually assessed via microscope. EC_{50} values were calculated according to the Karber method.

Time-of-addition test

There were several specifications of the experiment described in the Results and Discussion. Cells were incubated with NHC or DMSO for 1h, then virus pre-incubated with NHC or DMSO was added to the cells and left for 1 h for sorption and entry at 37 °C, and then 2×EMEM was added. Cells were washed after every incubation. Virus was harvested after a single replication cycle (main time points and 1 cycle length of used EVs replication cycle were determined in a separate experiment) and total virus yields were determined in RD cells as 50% tissue cell infectious dose ($TCID_{50}$). Experiments were performed in 2 replicates.

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