ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 2010, p. 2017–2022 Vol. 54, No. 5
0066-4804/10/$12.00 doi:10.1128/AAC.01186-09

Antiviral Properties, Metabolism, and Pharmacokinetics of a Novel Azolo-1,2,4-Triazine-Derived Inhibitor of Influenza A and B Virus Replication[▽]

Inna Karpenko,[1] Sergey Deev,[2] Oleg Kiselev,[3] Valerey Charushin,[2] Vladimir Rusinov,[2] Eugeney Ulomsky,[2] Ella Deeva,[3] Dmitry Yanvarev,[1] Alexander Ivanov,[1] Olga Smirnova,[1] Sergey Kochetkov,[1] Oleg Chupakhin,[2] and Marina Kukhanova[1]*

Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow,[1] Postovsky Institute of Organic Synthesis, Ural Division of the Russian Academy of Sciences, Ekaterinburg,[2] and Research Institute of Influenza, Russian Academy of Medical Sciences, St. Petersburg,[3] Russia

Received 21 August 2009/Returned for modification 29 November 2009/Accepted 10 February 2010

Influenza viruses of types A and B cause periodic pandemics in the human population. The antiviral drugs approved to combat influenza virus infections are currently limited. We have investigated an effective novel inhibitor of human influenza A and B viruses, triazavirine {2-methylthio-6-nitro-1,2,4-triazolo[5,1-c]-1,2,4-triazine-7(4Í)-one} (TZV). TZV suppressed the replication of influenza virus in cell culture and in chicken chorioallantoic membranes, and it protected mice from death caused by type A and B influenza viruses. TZV was also effective against a rimantadine-resistant influenza virus strain and against avian influenza A virus H5N1 strains. The pharmacokinetic parameters and bioavailability of TZV were calculated after the administration of TZV to rabbits. The TZV metabolite AMTZV {2-methylthio-6-amino-1,2,4-triazolo[5,1-*s*]-1,2,4-triazin(e)-7(4Í)-one} was discovered in ÍÅK 293T and Huh7 cell cultures, a liver homogenate, and rabbit blood after intragastric administration of TZV. AMTZV was nontoxic and inactive as an inhibitor of influenza virus in cell culture. Most likely, this metabolite is a product of TZV elimination.

The design of effective new drugs against influenza viruses is one of the most socially important goals of modern medicine, due to the epidemic character of the disease, the high variability of the virus, and the development of serious complications, occasionally resulting in patient death. During seasonal epidemic outbreaks, 10 to 20% of the population suffers from virus infections, and pandemic outbreaks can increase this number 4 to 5 times (3, 12, 13, 14). The spectrum of drugs inhibiting the pathogen is limited. Recent studies of avian influenza A H5N1, H7N7, and H9N2 viruses and of the H1N1 swine flu virus showed that the mutability of viruses is very high and that they can overcome interspecies barriers and infect humans (7, 17, 22, 23). These facts stimulated intensive studies of new agents capable of inhibiting influenza viruses of different origins (1, 10, 12, 21, 22, 27).

At present, two types of drugs are used in clinical practice. The first type is represented by the adamantane derivative amantadine and its methyl derivative rimantadine (5, 6, 8). These drugs inhibit viral replication during the early stage of infection by blocking the ion channels that are formed in the envelopes of influenza virus particles by the M2 protein. The channels are required for the maintenance of low pH in the virion and the release of viral ribonucleoprotein from the M1 protein, followed by the initiation of transcription of the viral genome (9, 28, 34). Adamantane-based drugs have been useful only in the treatment of influenza A infection, because only the A strains of influenza virus have M2 ion channel proteins. Although drugs of this type are effective against influenza A virus infection, they may cause serious adverse effects and have given rise to the rapid emergence of drug-resistant viral strains (8, 19, 25, 29, 31). However, despite these drawbacks, adamantane-derived drugs remain the key drugs in influenza therapy. The second type of anti-influenza drugs is represented by zanamivir and oseltamivir, the inhibitors of a viral membrane enzyme, α-neuraminidase, that plays a key role in the life cycle of influenza viruses. The enzyme destroys the sialic-acid-containing receptor on the surfaces of infected cells and is thus involved in the release of new virions from the host cell surface to begin a new round of infection (15, 16, 20, 35). Both zanamivir and oseltamivir have advantages over the M2 protein inhibitors with respect to efficacy, resistance pattern, and antiviral spectrum (in that they inhibit both influenza virus types A and B). However, some fatalities and neuropsychiatric events have been reported with the use of oseltamivir, especially in pediatric cases (2, 4, 18). Recently it has been shown that agents synthesized on an azolo-1,2,4-triazine base possessed high activities against both A and B influenza viruses (1).

Here a novel effective inhibitor of influenza A and B viruses, triazavirine {2-methylthio-6-nitro-1,2,4-triazolo[5,1-c]-1,2,4-triazine-7(4Í)-one} (TZV), is described. The structure of TZV is presented in Fig. 1. We show that TZV inhibited virus replication in cell cultures and in chicken chorioallantoic membranes (CAM) and that it protected mice infected with influenza A or B virus serotypes from death. TZV was also active against a rimantadine-resistant strain and a highly pathogenic avian influenza A virus H5N1 strain. The metabolism of TZV

* Corresponding author. Mailing address: Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow, Russia. Phone: 7 (499) 1352255. Fax: 7 (499) 1351405. E-mail: kukhan86@hotmail.com.

▽ Published ahead of print on 1 March 2010.

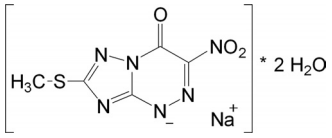

FIG. 1. Structure of TZV.

was studied in kidney (HEK 293T) and hepatic (Huh7) cell cultures. The pharmacokinetic parameters and bioavailability of TZV were calculated following intragastric (i.g.) and intravenous (i.v.) administration to rabbits. A TZV metabolite {2-methylthio-6-amino-1,2,4-triazolo[5,1-s]-1,2,4-triazine-7(4H)-one} (AMTZV), formed in the process of reduction of the TZV nitro group to an amino group, was found in HEK 293T and Huh7 cell cultures, in a liver homogenate after incubation with TZV, and in rabbit blood after i.g. administration. The resulting metabolite was neither active nor toxic in cell cultures infected with influenza viruses and most likely was a TZV elimination product.

MATERIALS AND METHODS

^{1}H nuclear magnetic resonance (NMR) spectra were registered on an Avance DRX 400 spectrometer (Bruker Corporation) with a working frequency of 400 MHz. Liquid chromatography-mass spectrometry was performed on an LCMS Shimadzu 2010 mass spectrometer (Shimadzu Corporation, Japan) using a Supelco LC-18 column (4.6 mm by 250 mm) at 60°C with a mobile phase of acetonitrile-water (1:1) and with chemical ionization at normal pressure (atmospheric-pressure chemical ionization [APCI]). The analysis was performed in the selected ion monitoring (SIM) mode. UV spectra were recorded on a Shimadzu UV-2401PC spectrophotometer (Shimadzu Corporation, Japan). Centrifugation was performed on an Eppendorf centrifuge, model 5415 (Eppendorf AG, Germany). For sample concentration, an Automatic SpeedVac concentrator, model AS260 (Savant), was used. High-performance liquid chromatographic (HPLC) analysis of TZV and its metabolites was performed on a Nucleosil 100 C_{18} column (5 μm; 4 mm by 150 mm) with a C_{18} precolumn (4 by 8 mm) using a Gilson chromatograph (France) supplied with a digital GSIOC 506 controller (Gilson) and a Gilson-315 UV detector with varied wavelengths.

Sample analysis. HPLC conditions for sample analysis were as follows. Solution A was 0.1% heptafluorobutyric acid [$CF_3(CF_2)_2COOH$] (pH 3), and solution B was 80% ethyl alcohol (EtOH). The gradient for solution B was 0% for 5 min, from 0% to 12% for 20 min, from 12% to 14% for 5 min, from 14% to 40% for 8 min, and from 40% to 100% for 2 min. A λ of 360 nm was registered for TZV and a λ of 321 for the TZV metabolite. The rate of elution was 0.5 ml/min.

Influenza virus strains. Virus strains A/Aichi/2/68 (H3N2), A/Victoria/35/72 (H3N2), B/Lee/40, and B/Samara/253/99 were received from the collection of the Research Institute of Influenza, Russian Academy of Medical Sciences, St. Petersburg, Russia. The rimantadine-resistant A/PR/8/34 (H0N1) and A/NIBRG-14 (H5N1) (A/Vietnam/1194/2004 plus A/PR/8/34) strains were a kind gift from the National Institute for Biological Standards and Control, London, United Kingdom. The A/Duck/Potsdam/1402-6/86 (H5N2), A/Mallard/NT/12/02 (H7N3), and A/Hong Kong/1073/99 (H9N2) strains were from the Centers for Disease Control and Prevention, Atlanta, GA.

Cell lines and animals. Madin-Darby canine kidney (MDCK) cells were from the Institute of Influenza Viruses, St. Petersburg, Russia. Human HEK 293T kidney cells and Huh7 hepatic cells were from the Engelhardt Institute of Molecular Biology. Mice of the CBA line, weighing 16 to 18 g, were received from the "Rappolovo" laboratory animal nursery, Leningradskaya region, Russia. Male Chinchilla rabbits (body weight, 3.0 ± 0.5 kg) were from the Manikhino hatchery (Moscow region, Russia).

Chemistry. The synthesis of TZV has been reported previously (1). ^{1}H NMR spectrum (dimethyl sulfoxide [DMSO]-d_6, δ, ppm): 2.52 (3H, s, SCH_3), 4.76 (4H, broad s, $2H_2O$). Element analysis: C 20.83, H 2.62, N 29.21, S 11.20%. UV (H_2O): λ_{max} 257, ε = 17,172; λ_{max} 360, ε = 12,520. Mass: 227 (100.0%) [M-Na]$^-$, 228 (10.8%) [M + 1–Na]$^-$, 199 (7.4%) [M + 2–Na]$^-$.

2-Methylthio-6-amino-1,2,4-triazolo[5,1-c]-1,2,4-triazine-7(4H)-one (AMTZV) was synthesized by a method similar to that described in reference 32. Briefly, triazavirine (0.5 g; 1.7 mmol) was suspended in water (5 ml), and a solution of sodium dithionite (1 g) in water (15 ml) was added. The reaction mixture was stirred at room temperature for 1 h, and the resulting precipitate was filtered and dried on air to give 0.225 g (45%) of the product. ^{1}H NMR spectrum (DMSO-d_6, δ, ppm): 2.52 (3H, s, SCH_3), 6.35 (2H, br. s, NH_2), 13.19 (1H, br. s, NH). Mass: 197 (100.0%) [M-H]$^-$, 198 (9.4%) [M + 1–H]$^-$, 199 (3.3%) [M + 2–H]$^-$. UV (water): λ_{max} 249, ε = 29 218; λ_{max} 321, ε = 6 424.

Antiviral activity on an MDCK cell culture. The cells were grown in Dulbecco's modified minimal essential medium (MEM)/E (Sigma-Aldrich, Germany) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate. A suspension of MDCK cells was cultivated at 37°C under 5% CO_2 until a monolayer was formed. Cell viability was determined by a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. The starting allantoic liquid containing the A/H3N2, A/H5N1, A/H5N2, A/H7N3, or A/H9N2 virus was diluted within the range of 10^{-1} to 10^{-7} and was added to wells containing confluent cell monolayers. Virus titers were estimated by hemagglutinin titration and plaque assays according to standard procedures (24, 33). After virus adsorption for 1 h, the water solutions of target compounds were added at various concentrations. The virus yield was determined after 48 h by a hemagglutination test with a 0.5% chicken erythrocyte suspension according to the method of reference 26. Each compound concentration was tested in duplicate.

***In vitro* study of antiviral activity in CAM.** Chorioallantoic membranes (CAM) from 11- to 13-day-old chick embryos were minced to fragments of about 1 mm, which were suspended in Hanks' salt solution containing penicillin and streptomycin sulfate. The starting allantoic liquid containing viruses was diluted within the range of 10^{-1} to 10^{-7} and was added to wells with confluent cell monolayers. Each suspension was incubated at 37°C for 1 h, and water solutions of TZV at various concentrations were added to the plates. Antiviral activity was estimated by hemagglutinin titration and plaque assays after 48-h incubations at 36 to 37°C according to the method of reference 26.

TZV stability in a rabbit liver homogenate. The rabbit liver homogenate was obtained by a procedure similar to that described in reference 30. The reaction mixture containing 25 mg/ml protein and 500 μM TZV was incubated at 37°C. After certain time intervals, aliquots were taken, and the reaction was terminated by the addition of cool methanol up to 66% (vol/vol). The precipitate formed was pelleted by centrifugation for 4 min at 10,000 × *g*. The supernatant was dried in a SpeedVac freeze dryer; the residue was dissolved in water; and the products were analyzed by HPLC as described above. The compound concentrations were calculated by peak areas.

TABLE 1. Anti-influenza virus activities of TZV and rimantadine in MDCK cell cultures

Compound and concn (μM)	Log reduction in virus titer[a]				
	A/H3N2 (A/Victoria/35/72)	A/H5N1 (A/Vietnam/1194/2004 + A/PR/8/34)	A/H5N2 (A/Duck/Potsdam/1402-6/86)	A/H7N3 (A/Mallard/NT/12/02)	A/H9N2 (A/HongKong/1073/99)
Triazavirine					
385	1.00 ± 0.15	2.00 ± 0.20	0.50 ± 0.06	0.25 ± 0.07	0.00[b]
960	1.00 ± 0.12	2.25 ± 0.18	1.60 ± 0.15	3.50 ± 0.20	0.50 ± 0.10
Rimantadine (502)	5.00 ± 0.80	1.00 ± 0.50	2.00 ± 0.30	3.00 ± 0.25	0.75 ± 0.08

[a] Values are means ± SD from two independent experiments.
[b] TZV was inactive at this concentration.

TABLE 2. Log reductions in titers of influenza A and B viruses in the presence of TZV and rimantadine in CAM fragments

Compound and concn (μM)	Log reduction in virus titer[a]						
	B (B/Samara/253/99)	A/H3N2 (A/Victoria/35/72)	A/H0N1 (A/PR/8/34)	A/H5N1 (A/Vietnam/1194/2004 + A/PR/8/34)	A/H5N2 (A/Duck/Potsdam/1402-6/86)	A/H7N3 (A/Mallard/NT/12/02)	A/H9N2 (A/HongKong/1073/99)
Triazavirine							
62	2.5 ± 0.2	2.50 ± 0.30	2.0 ± 0.4	3.0 ± 0.2	2.5 ± 0.3	2.5 ± 0.2	1.5 ± 0.1
124	2.8 ± 0.4	2.75 ± 0.15	2.5 ± 0.5	4.0 ± 0.2	3.5 ± 0.4	3.5 ± 0.3	3.0 ± 0.2
310	2.9 ± 0.4	3.25 ± 0.20	2.7 ± 0.8	4.0 ± 0.2	3.5 ± 0.4	3.4 ± 0.3	2.9 ± 0.1
Rimantadine (110)	0[b]	5.00 ± 0.45	1.0 ± 0.2	0.5 ± 0.3	3.0 ± 0.2	3.0 ± 0.2	1.5 ± 0.1

[a] Values are means ± SD from three independent experiments.
[b] Rimantadine was not active at the concentration given.

TZV metabolism in cell cultures. A TZV water solution was added to petri dishes containing monolayers of 6×10^6 HEK 293T kidney cells or 6×10^6 Huh7 liver cells to a final concentration of 1 mM. The cell culture was incubated with the test compound for 1.5 h or 24 h. The cells were washed three times with phosphate-buffered saline (PBS), suspended in an equal volume of PBS, and broken three times by cryolysis. An equal volume of 6% trifluoroacetic acid was added to the suspension, which was then centrifuged, and the precipitate was separated. The supernatants were adjusted to a neutral pH by the addition of saturated Na_2CO_3 and were analyzed by HPLC as described above.

Anti-influenza activity of TZV in animal models. Animal experiments were carried out in accordance with guidelines set forth in the *Guide for the Care and Use of Laboratory Animals* (21a). Antiviral activity in CBA mice infected with the A/Aichi/2/68 (H3N2) or B/Lee/40 influenza virus was assessed. Each group of mice included 20 animals. The virus was administered intranasally at 1 and 10 50% lethal doses (LD_{50}) under slight ether anesthesia. A TZV water solution (0.2 ml) was administered by the intragastric (i.g.) route according to one of three schemes: the treatment-and-prophylactic scheme (24 and 1 h before infection [−24 and −1 h] and 24, 48, and 72 h after infection [+24, +48, and +72 h]), the prophylactic scheme (−24 h and −1 h), or the treatment scheme (+24 h, +48 h, and +72 h). Rimantadine (Aldrich Chemical Co., Milwaukee, WI) was used as a control. The animals were watched for 14 days, and deaths in the control and experimental groups were reported every day. Based on these data, the degree of animal protection was calculated for TZV and compared with that of rimantadine.

Pharmacokinetics in rabbits. For a single i.g. administration of TZV to rabbits, the animals ($n = 4$) were anesthetized with a 10:1 ether-Fluorothane mixture, and a polyurethane gastrointestinal tube was introduced 15 cm deep. TZV was administered as a water solution (12 ml) at a dose of 105 mg/kg of body weight. For i.v. administration to rabbits ($n = 4$), TZV (4.3 mg/kg of body weight) was injected into the vena auricularis marginalis in physiological solution (1 ml) for 1 min according to the method of reference 11. At predetermined time points up to 24 h postdosing, blood samples (1 ml on average) were collected from the vena auricularis marginalis in a self-flowing manner into microtubes containing 5 μl of heparin (5,000 U/ml). The tubes were shaken, and 0.5-ml aliquots were taken out, mixed with methanol (1 ml), and stored at −24°C. The control blood samples were taken before administration of the test compounds. Prior to HPLC analysis, the samples were centrifuged at $1,500 \times g$ for 10 min; the supernatant was evaporated in a vacuum; and the residue was dissolved in water (100 μl). The samples were then analyzed by HPLC under the conditions described above.

Pharmacokinetic parameters were calculated using the Kinetica program (version 4.4.1; Thermo Electron Corporation). Pharmacokinetics following i.g. administration was studied by the extravascular noncompartmental model of the Thermo Kinetica program. For i.v. administration, the noncompartmental i.v. infusion model was used. The following parameters were determined: the total area under the plasma concentration-versus-time curve (AUC_{tot}), the apparent elimination half-life ($T_{1/2}$), the maximum concentration of the compound in plasma (C_{max}), the time to C_{max} (T_{max}), and the mean residence time (MRT). The i.g. bioavailability (F) of the compound was calculated as $(AUC_{i.g.}/dose_{i.g.})/(AUC_{i.v.}/dose_{i.v.})$, where $AUC_{i.g.}$ is the AUC of the compound after i.g. administration, $dose_{i.g}$ is the i.g. dose, $AUC_{i.v}$ is the AUC after i.v. administration, and $dose_{i.v.}$ is the i.v. dose. Total clearance (CL) from plasma was calculated as dose/AUC. The volume of distribution (V_{ss}) of TZV at steady state was determined as CL × MRT.

Statistical analysis. The statistical significance of comparisons between treated groups was assessed by a paired Student t test. All values are expressed as means ± standard deviations (SD). Each pharmacokinetic experiment was repeated at least three times. The lower limits of detection of TZV and its metabolite were 0.01 μg/ml, with a signal/noise ratio of ≥10. P values of <0.05 were considered statistically significant.

RESULTS

The antiviral activities of TZV against influenza A and B viruses were studied with various model systems, including cell cultures, CAM fragments, and experimental animals.

Anti-influenza activities of TZV in MDCK cells and CAM fragments. The antiviral effect of TZV was investigated in MDCK cell cultures and CAM fragments after infection with different strains of influenza A and B viruses. MDCK cell cultures or CAM fragments infected with viruses were treated with different concentrations of TZV immediately after the virus adsorption period, and virus titers were determined at 48 h postinfection. Table 1 presents comparative data on log reductions in the infective potentials of viruses in MDCK cells in the presence of TZV. Rimantadine was used as a control. Except for the A/H3N2 virus strain, the activity of TZV against all influenza A viruses was similar to that of rimantadine. TZV was nontoxic at the effective antiviral concentration for uninfected cells (50% cytotoxic concentration [CC_{50}], >8 mM).

The model of TZV antiviral activity against influenza A and B viruses in CAM fragments is presented in Table 2. The data show that TZV inhibits influenza viruses of both the A and B serotypes, including the rimantadine-resistant A/PR/8/34

TABLE 3. Protection of mice from death caused by type A and B influenza viruses with the treatment-and-prophylactic scheme

Compound	Dose (mg/kg)	No. of animals surviving/total no. of animals (% protection)[a]	
		A/Aichi/2/68 (H3N2)	B/Lee/40
TZV	150	15/20 (75)	13/20 (65)
	100	14/20 (70)	13/20 (65)
Rimantadine	50	16/20 (80)	3/20 (15)
	100	17/20 (85)	4/20 (20)
Control		0/20 (0)	0/20 (0)

[a] Values are mean data from three independent experiments.

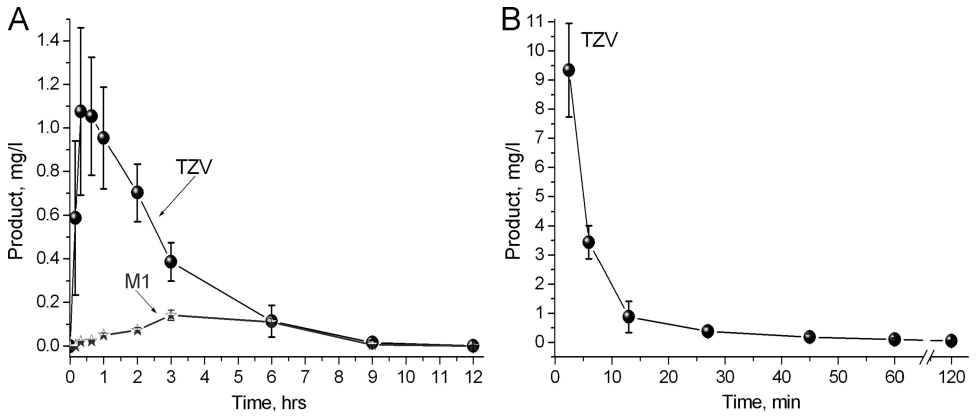

FIG. 2. Time-concentration profiles of TZV and its metabolite (M1) in rabbit plasma after either i.g. administration of a 105-mg/kg dose (A) or i.v. administration of a 4.3-mg/kg dose (B). Datum points represent four animals. The concentrations of products were calculated after HPLC analysis of rabbit blood samples. The lower limit of detection was 0.01 μg/ml.

strain. As can be seen, TZV reduced the titer of an influenza B virus (B/Samara/253/99) by 2.5 to 3 log units, while rimantadine inhibited only influenza A virus replication. In addition, the activity of TZV against avian influenza virus A/H5N1 was four to eight times higher than that of rimantadine.

Anti-influenza activities of TZV in animal models. As shown in Table 3, when administered according to the treatment-and-prophylactic scheme (24 and 1 h before infection and 24, 48, and 72 h after infection), TZV protected mice from death caused by influenza viruses of types A and B. TZV was administered by the i.g. route in the dose range of 1 to 200 mg/kg of body weight. Optimal effective doses were determined to be 50 to 100 mg/kg of body weight. As is evident from the data, TZV and rimantadine provided similar levels of protection for mice infected with a serotype A influenza virus (A/Aichi/2/68 [H3N2]), but the survival rates of animals infected with type B (B/Lee/40) and treated at about the same dosage were three to four times higher with TZV than with rimantadine. Therefore, TZV could protect 65 to 75% of mice infected with either A or B viruses. TZV was also effective when a treatment (+24 h, +48 h, and +72 h) or prophylactic (−24 h and −1 h) schemes of TZV administration were applied (data not shown). It is also noteworthy that the toxicity of TZV is low: following intraperitoneal administration to mice, the LD_{50} of TZV was 1,400 ± 120 mg/kg of body weight, and following i.g. administration, the LD_{50} was 2,200 ± 96 mg/kg of body weight. The essential characteristics of potential antiviral drugs are their stability, metabolic transformation, pharmacokinetics, and bioavailability.

TZV pharmacokinetic parameters following single-dose i.v. or i.g. administration to rabbits. To determine the i.g. bioavailability of TZV, the compound was administered to rabbits ($n = 4$) by the i.g. and i.v. routes. At predetermined time points up to 24 h postdosing, blood samples were collected and analyzed by HPLC. For 10 min after i.g. administration, the TZV peak, with a retention time (T_{ret}) of 22.5 min, was the only one observed in rabbit blood, whereas 2 h later, a new peak (M1) appeared, increasing with time, with a T_{ret} of 27.5 min. The concentrations of TZV and M1 in rabbit blood within 12 h after dosing (105 mg/kg of body weight) are shown in Fig. 2A. The C_{max} of TZV (1.1 mg/liter) was achieved in 0.40 ± 0.16 h, and the half-time of elimination was 1.1 h. The CL of TZV was 37.0 ± 11.2 liters/h · kg, and the V_{ss} was 83.5 ± 19.2 liters/kg. The M1 concentration increased for 3 h and then decreased insignificantly over the next 5 h. Following i.v. administration of a 4.3-mg/kg dose of TZV to rabbits, the concentration of TZV in plasma fell quickly, with a $T_{1/2}$ of 0.9 h (Fig. 2B). In contrast to the findings for i.g. administration, the metabolite M1 was not detected. The only product observed during the whole experimental time (24 h) was TZV. A summary of the values of the pharmacokinetic parameters of TZV and its metabolite is provided in Table 4. The i.g. bioavailability (F) of TZV in rabbits was calculated as 12.5%.

Formation of the TZV metabolite (M1) in a rabbit liver homogenate. Most likely the TZV metabolite was formed in the liver or kidney. To confirm this assumption, TZV was incubated with a rabbit liver homogenate. Figure 3 shows the HPLC analysis of the liver homogenate after incubation with 500 μM TZV. As can be observed, a new peak, increasing with time, was observed after 10 min of incubation. The retention time of this peak agreed well with that of the metabolite formed in rabbit blood following i.g. administration of TZV.

TABLE 4. Values of pharmacokinetic parameters for TZV and its metabolite (M1) following a single i.g. or i.v. administration of TZV to rabbits ($n = 4$)[a]

Compound, route (dose [mg/kg])	C_{max} (mg/liter)	T_{max} (h)	AUC_{tot} (mg · h/liter)	$T_{1/2}$ (h)	MRT (h)	CL (liters/h · kg)	V_{ss} (liters/kg)
TZV, i.g. (105)	1.1 ± 0.1	0.40 ± 0.16	3.10 ± 0.8	1.1 ± 0.1	2.3 ± 0.2	37.0 ± 11.2	83.5 ± 19.2
AMTZV	0.14 ± 0.02	3.00 ± 0.0	0.69 ± 0.11	0.97 ± 0.05	4.14 ± 0.02	44.4 ± 7.6	58.5 ± 10.3
TZV, i.v. (4.3)			1.2 ± 0.3	0.50 ± 0.09	0.32 ± 0.03	14.0 ± 3.7	1.2 ± 0.3

[a] Values are means ± standard deviations for four animals.

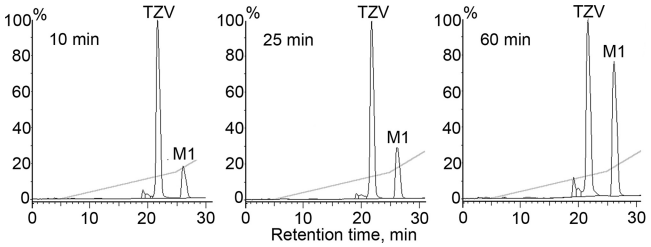

FIG. 3. HPLC analysis of a rabbit liver homogenate after incubation with 500 μM TZV.

TZV metabolism in HEK 293T kidney and Huh7 liver cell cultures. HEK 293T or Huh7 cell cultures were incubated with 500 μM TZV for different times. HPLC analysis of cellular extracts revealed the presence of both TZV and the metabolite, whose retention time agreed with those of the M1 metabolite formed in a rabbit liver homogenate and the metabolite detected in rabbit blood following i.g. TZV administration (Fig. 4).

TZV metabolite structure. The identities of the metabolites found in cell cultures and in rabbit blood following i.g. administration and upon incubation of TZV in a rabbit liver homogenate were confirmed by comparison of retention times using data from HPLC analysis, UV spectra, and mass spectra. The patterns of UV spectra for TZV and the metabolite formed upon incubation of TZV with a rabbit liver homogenate are shown in Fig. 5. The patterns for TZV and M1 looked obviously different: TZV had two λ_{max}, one at 257 nm and one at 360 nm, while the λ_{max} for M1 were at 249 nm and 320 nm. We assumed that the TZV nitro group could be reduced to give 2-methylthio-6-amino-1,2,4-triazolo[5,1-c]-1,2,4-triazine-7(4H)-one (AMTZV). AMTZV was synthesized, and we showed that the UV pattern of AMTZV was identical to that of M1. For verification of the structure of the M1 metabolite formed in the liver homogenate, its mass spectrum was compared with that of the synthesized compound (Fig. 6A and B). A major ion was $[M\text{-}H]^-$, with a molecular mass of 197, which agrees with that of AMTZV. Also, a peak of the TZV $[M\text{-}H]^-$ ion 227 was present. For ions 197 and 227, the respective satellite ions corresponding to 198 $[M + 1\text{–}H]^-$ and 199 $[M + 2\text{–}H]^-$, as well as 228 $[M +$

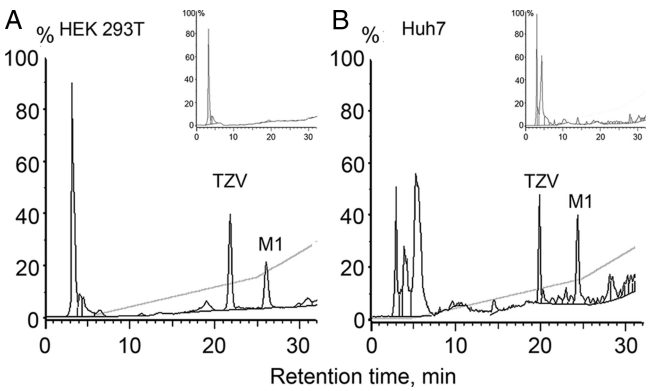

FIG. 4. HPLC analyses of HEK 293T (A) and Huh7 (B) cell lysates after incubation with 500 μM TZV for 5 h. (Insets) Chromatograms of control samples of intact cell lysates.

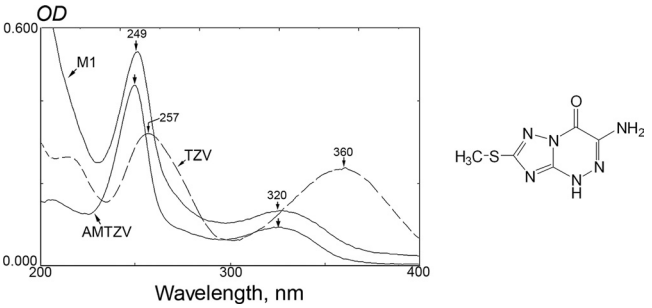

FIG. 5. (Left) UV spectra of TZV, the metabolite (M1) formed after incubation with a rabbit liver homogenate, and AMTZV, obtained by chemical synthesis. OD, optical density. (Right) Structural formula of AMTZV.

1–H]$^-$ and 229 $[M + 1\text{–}H]^-$, were observed. The ion structure corresponding to peak 213 is obscure. Peak 167 may relate to a metabolite degradation product, which was confirmed by mixing [M1 + AMTZV] and [M1 + ^{15}N-AMTZV] (data not shown).

Based on the results obtained, we assumed that the TZV metabolite discovered in rabbit blood serum after i.g. administration or in the extracts of Huh7 and HEK 293T cell cultures is a product of the reduction of the nitro group to an amino group. AMTZV displayed no activity against an H3N2 strain (A/Victoria/35/72) in cell culture at concentrations as high as 1,000 μg/ml. Reductions in the virus titer did not exceed 0.75 log unit (data not shown).

DISCUSSION

Influenza infection has been studied for more than 80 years. Despite considerable progress in treatment, influenza remains an uncontrolled global infection with unpredictable morbidity and mortality rates. In the last two or three decades, the dominating viruses of influenza epidemics belonged to serotype A, whereas recent years have witnessed epidemic episodes caused by viruses of both the A and B serotypes (3, 7, 17). Influenza virus A (H5N1) circulating in birds is a menace to humans and

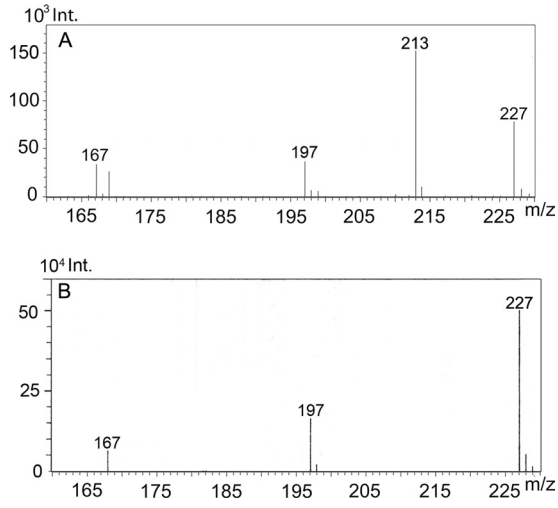

FIG. 6. Fragments of mass spectra of metabolite M1 (A) and synthetic AMTZV (B).

may result in an influenza virus pandemic (23). In light of the current pandemic threat and the inevitable emergence of resistance to anti-influenza drugs, the development of new anti-influenza drugs must be a high priority.

Here we describe a novel inhibitor of influenza viruses of different types. In contrast to the well-known anti-influenza A drug rimantadine, TZV inhibited the replication of a serotype B influenza virus in a CAM model and protected mice experimentally infected with influenza virus from death by use of either a preventive or a therapeutic scheme of treatment. TZV displayed higher activity against the pathogenic H5N1 strain than rimantadine. The pharmacokinetic parameters in rabbits following i.g. and i.v. administration showed that the bioavailability of TZV was 12.5%. After i.g. administration to rabbits, a TZV metabolite was found in rabbit blood as a result of the conversion of the TZV nitro group to an amino group {2-methylthio-6-amino-1,2,4-triazolo[5,1-c]-1,2,4-triazine-7(4H)-one} (AMTZV). Most likely the metabolite is formed in the liver or the kidney, or in both organs. This assumption is supported by the emergence of the metabolite in kidney and liver cell cultures and in a liver homogenate after incubation with TZV. The metabolite did not inhibit the replication of influenza virus type A (H3N2) in MDCK cell cultures at concentrations as high as 1,000 μM and did not show toxicity up to 5 mM. Presumably, the nontoxic and inactive compound AMTZV is a product of TZV modification ensuring the elimination of the latter from the organism. The mechanism of action of TZV on influenza virus replication should be elucidated, although TZV displayed no activity when it was tested as an inhibitor of viral neuraminidase. At present, TZV is under clinical development for the treatment of influenza A and B virus infections.

ACKNOWLEDGMENTS

This work was supported by the Russian Foundation for Basic Research, project 08-04-00552, and Presidium RAN "Molecular and Cellular Biology."

We are very thankful to K. Seley-Radtke (University of Maryland) for useful discussions and criticism.

REFERENCES

1. **Chupakhin, O. N., V. L. Rusinov, E. N. Ulomsky, V. N. Charushin, A. Y. Petrov, and O. N. Kiselev.** March 2007. 2-Methylthio-6-nitro-1,2,4-triazolo[5,1-C]-1,2,4-triazine-7(4H)-one. Sodium salt dihydrate possessing antiviral activity. Russian patent RU 2,294,936.
2. **Fuyuno, I.** 2007. Tamiflu side effects come under scrutiny. Nature **446:**358–359.
3. **Glezen, W. P.** 1996. Emerging infections: pandemic influenza. Epidemiol. Rev. **18:**64–76.
4. **Hama, R.** 2007. Fifty sudden deaths may be related to central suppression. BMJ **335:**59.
5. **Hay, A.** 1996. Amantadine and rimantadine—mechanisms, p. 44–58. *In* D. Richman (ed.), Antiviral drug resistance. John Wiley and Sons Ltd., Chichester, United Kingdom.
6. **Hay, A. J., A. J. Wolstenholme, J. J. Skehel, and M. H. Smith.** 1985. The molecular basis of the specific anti-influenza action of amantadine. EMBO J. **4:**3021–3024.
7. **Hayden, F.** 2004. Annex 5: considerations for the use of antivirals during an influenza pandemic, p.5–1–5–18. *In* WHO guidelines on the use of vaccines and antivirals during influenza pandemics. World Health Organization, Geneva, Switzerland. http://www.wpro.who.int/internet/resources.ashx/CSR/Publications/11_29_01_A.pdf.
8. **Hayden, F. G., and A. J. Hay.** 1992. Emergence and transmission of influenza A viruses resistant to amantadine and rimantadine. Curr. Top. Microbiol. Immunol. **176:**119–130.
9. **Jing, X., C. Ma. Y. Ohigashi, F. A. Oliveira, T. S. Jardetsky, L. H. Pinto, and R. A. Lamb.** 2008. Functional studies indicate amantadine binds to the pore of the influenza A virus M2 proton-selective ion channel. Proc. Natl. Acad. Sci. U. S. A. **105:**10967–10972.
10. **Johnston, S. L.** 2002. Anti-influenza therapies. Virus Res. **82:**147–152.
11. **Khandazhinskaya, A. L., D. V. Yanvarev, M. V. Jasko, A. V. Shipitsin, V. A. Khalizev, S. I. Shram, Y. S. Skoblov, E. A. Shirokova, and M. K. Kukhanova.** 2009. 5′-aminocarbonyl phosphonates as new zidovudine depot forms: antiviral properties, intracellular transformations, and pharmacokinetic parameters. Drug Metab. Dispos. **37:**494–501.
12. **Klenk, H. D., and R. Rott.** 1988. The molecular biology of influenza virus pathogenicity. Adv. Virus Res. **34:**247–280.
13. **Kuznetsov, O. K., and O. I. Kiselev.** 2003. Influenza viruses with pandemic potential and measures to prevent their emergence: facts, hypothesis. Med. Acad. J. **2:**112–121.
14. **Laver, W. G., N. Bischofberger, and R. G. Webster.** 1999. Disarming flu viruses. Sci. Am. **280:**78–87.
15. **Leneva, I. A., N. Roberts, E. A. Govorkova, O. G. Goloubeva, and R. G. Webster.** 2000. The neuraminidase inhibitor GS 4104 (oseltamir phosphate) is efficacious against A/Hong Kong/156/97 (H5N1) and A/Hong Kong/1074/99 (H9N2) influenza viruses. Antiviral Res. **48:**101–115.
16. **Liu, C., M. C. Eichelberger, R. W. Compans, and G. M. Air.** 1995. Influenza type A virus neuraminidase does not play a role in virus entry, replication, assembly, or budding. J. Virol. **69:**1099–1106.
17. **Mackay, W. G., A. M. van Loon, M. Niedrig, A. Meijer, B. Lina, and H. G. Niesters.** 2008. Molecular detection and typing of influenza viruses: are we ready for an influenza pandemic? J. Clin. Virol. **42:**194–197.
18. **Maxwell, S. R.** 2007. Tamiflu and neuropsychiatric disturbance in adolescents. BMJ **334:**1232–1233.
19. **Monto, A. S.** 2008. Antivirals and influenza: frequency of resistance. Pediatr. Infect. Dis. J. **27**(10 Suppl.)**:**S110–S112.
20. **Moscona, A.** 2005. Neuraminidase inhibitors for influenza. New Engl. J. Med. **353:**1363–1373.
21. **Mounts, A. W., H. Kwong, H. S. Izurieta, Y. Ho, T. Au, M. Lee, C. Buxton Bridges, S. W. Williams, K. H. Mak, J. M. Katz, W. W. Thompson, N. J. Cox, and K. Fukuda.** 1999. Case-control study of risk factors for avian influenza A (H5N1) diseases, Hong Kong, 1997. J. Infect. Dis. **180**(Suppl. 2)**:**505–508.

21a. **National Institutes of Health.** 1986. Guide for the care and use of laboratory animals. NIH publication 86/609/EEC. National Institutes of Health, Bethesda, MD.

22. **Osterholm, M. T.** 2005. Preparing for the next pandemic. New Engl. J. Med. **352:**1839–1842.
23. **Pappaioanou, M.** 2009. Highly pathogenic H5N1 avian influenza virus: cause of the next pandemic? Comp. Immunol. Microbiol. Infect. Dis. **32:**287–300.
24. **Pica, F., A. T. Palamara, A. Rossi, A. De Marco, C. Amici, and M. Santoro.** 2000. Δ^{12}-prostaglandin J_2 is a potent inhibitor of influenza A virus replication. Antimicrob. Agents Chemother. **44:**200–204.
25. **Pielak, R. M., J. R. Schnell, and J. J. Chou.** 2009. Mechanism of drug inhibition and drug resistance of influenza A M2 channel. Proc. Natl. Acad. Sci. U. S. A. **106:**7379–7384.
26. **Reed, L., and H. Muench.** 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. **27:**493–497.
27. **Rossignol, J. F., S. La Frazia, L. Chiappa, A. Ciucci, and M. G. Santoro.** 28 July 2009. Thiazolides, a new class of anti-influenza molecules targeting viral hemagglutinin at the post-translational level. J. Biol. Chem. [Epub ahead of print.] doi:10.1074/jbc.M109.029470.
28. **Schnell, J. R., and J. J. Chou.** 2008. Structure and mechanism of the M2 proton channel of influenza A virus. Nature **451:**591–595.
29. **Schwartz, M., M. Patel, Z. Kazzi, and B. Morgan.** 2008. Cardiotoxicity after massive amantadine overdose. J. Med. Toxicol. **4:**173–179.
30. **Skoblov, Y., I. Karpenko, E. Shirokova, K. Popov, V. Andronova, G. Galegov, and M. Kukhanova.** 2004. Intracellular metabolism and pharmacokinetics of 5′-hydrogen phosphonate of 3′-azido-2′,3′-dideoxythymidine, a prodrug of 3′-azido-2′,3′-dideoxythymidine. Antiviral Res. **63:**107–113.
31. **Smith, E. J.** 2008. Amantadine-induced psychosis in a young healthy patient. Am. J. Psychiatr. **165:**1613.
32. **Ulomskii, E. N., E. V. Tsoi, V. L. Rusinov, O. N. Chupakhin, G. L. Kalb, and I. M. Sosonkin.** 1992. Reduction of nitro derivatives of azolo[5,1-*c*][1,2,4]triazines. Khimiya Geterotsiklicheskikh Soedinenii **5:**674–677. (In Russian.)
33. **Veeraraghavan, N., M. W. Kiritikar, and T. T. Sreevalsan.** 1961. Studies on the cultivation of influenza virus *in vitro*. Bull. W. H. O. **24:**711–722.
34. **Wang, C. K., L. H. Takeuchi, and R. A. Lamb.** 1993. Ion channel activity of influenza A virus M2 protein: characterization of the amantadine block. J. Virol. **67:**5585–5594.
35. **Ward, P., I. Small, J. Smith, and R. Dutkowski.** 2005. Oseltamivir (Tamiflu) and its potential for use in the event of an influenza pandemic. J. Antimicrob. Chemother. **55**(Suppl. 1)**:**i5–i21.