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Cycluridine: A novel antiviral effective against flaviviruses

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Abstract

This review describes the contemporary state of research for antivirals effective against flaviviruses, especially focusing on inhibitors of the pestivirus causative agent of bovine viral diarrhoea virus. We highlight cycluridine, an originally synthesized Mannich's base [a tetrahydro-2(1H)-pyrimidinones derivative], as a highly effective antiviral possessing a strong inhibitory effect on bovine viral diarrhoea virus replication. Cycluridine was active against replication of a wide variety of bovine viral diarrhoea virus strains in cell cultures. The drug-sensitive period in the bovine viral diarrhoea virus replication cycle included the latent period and the exponential phase; a 90-min delay in the peak of viral RNA synthesis was observed. Cycluridine administered orally manifested a pronounced protective effect in calves with natural mucosal disease/viral diarrhoea and calves experimentally infected with bovine viral diarrhoea virus. Its magnitude of activity and selectivity places cycluridine in the lead among all known substances with anti- bovine viral diarrhoea virus activity. Additionally, cycluridine applied subcutaneously showed anti-tick-born encephalitis virus activity, manifesting a marked protective effect in mice infected with tick-born encephalitis virus. Cycluridine could be a prospective antiviral in veterinary and medical practice for the treatment of bovine viral diarrhoea virus and other flavivirus infections.

Keywords

Cycluridine, bovine viral diarrhoea virus, flaviviruses, antivirals

Antivirals against *Flaviviridae*

The *Flaviviridae* family consists of four genera: *Flavivirus* (including yellow fever virus, West Nile virus, dengue virus, zika virus, tick-borne encephalitis virus, etc.), *Hepacivirus* (hepatitis C virus and GB virus B), *Pegivirus* (GB virus A, GB virus C, and GB virus D) and *Pestivirus* (bovine viral diarrhoea virus, classical swine fever virus, and border disease virus).

The antiviral field made remarkable advances during the last years with the development of effective antivirals against hepatitis C virus.[1–3] Several drugs were found, and they now occupy a noteworthy place in clinical practice for the treatment of hepatitis C acute and chronic infection, which is a major problem worldwide.[2,4,5] This was possible largely because of Bartenschlager's[6] technology for quantitative assessment of hepatitis C replication using a replicon-based high-throughput assay.

This methodology contributed significantly as a step in several anti-flavivirus screening programs. An excellent example of this connection is the identification of yellow fever virus (YFV) replication inhibitors resulting from the use of YFV replicon construct, YE-R.luv2A-RP, which expressed a *Renulla* luciferase gene in a replication-dependent manner.[7]

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The problem with antivirals, directed even aginst the very actual zika virus infection, is far from a resolution. Currently, there are no antiviral drugs that can save human populations from potential pandemic threats by this virus.[8] Recently there has been data on the antiviral activity of xiyanping (9-dehydroi-17-hydro-andrographolide + sodium 9-dehydro-17-andrographolide-19-yl sulfate) towards this virus.[9]

Antiviral research and development against dengue virus is in a similar state.[10] A lot of work has been done on the formulation of possible targets for effective antivirals. Nevertheless, there are presently no drugs against dengue in the clinic. It was found that castanospermine inhibited replication of all four dengue virus serotypes *in vitro* and efficiently protected mice against a lethal dengue virus type 2 challenge.[11] In addition, siRNAs could be considered effective anti-dengue virus agents *in vitro*,[12] but the road to their use in the clinic looks very long.

Novel peptide hybrids based on 2,4-thiazolidinedione scaffolds containing non-polar groups that attack West Nile virus (WNV) protease manifested a strong inhibitory effect on WNV and dengue virus replication *in vitro*. Experimental results support the hypothesis that a non-polar group in the scaffold is important for binding these inhibitors in the hydrophobic pockets of WNV and dengue virus NS2B-NS3 serine proteases.[13] However, castanospermine did not manifest activity toward WNV infections in mice.[11]

Tick-borne encephalitis represents one of the most serious arbovirus neuroinfections in Europe and northern Asia. No antivirals are available at present, and the literature even states that there is no urgent need for efficient drugs to treat patients with TBEV infection.[14] Nevertheless, exhaustive research using standardized *in vitro* assay systems found three nucleoside analogues, namely 7-deaza-2′C-methyladenosine (7-deaza-2′-CMA), 2′-C-methyladenosine (2′-CMA), and 2′-C-methylcytidine (2′-CMC) to be inhibitors of TBEV replication *in vitro*. High antiviral activity and low toxicity characterize 7-deaza-2′-CMA as a potential therapeutic agent in treating TBEV infection. TBEV polymerase is considered a target of this compound.[15]

Intensive work has been completed in the search for antivirals effective against the pestivirus bovine virus diarrhoea virus (BVDV). Presented here are the most impressive studies in that direction. First, we should mention inhibitors of the enzyme inosine-5′-monophosphate dehydrogenase (IMPDH) (E.C.1.1.1.205), a series of analogues of mycophenolic acid and ribavirin. These were tested in MDBK cells against a cytopathic strain of BVDV. Five of these compounds manifested a marked activity in a cell-free system versus the IMPDH enzyme. Among them, a higher effect was manifested by mizoribin.[16] Mizoribin, a nucleoside analogue clinically used as an immunosuppressant, was found to be active against BVDV replication in cell cultures. Experimentally, a synergistic anti-BVDV effect *in vitro* was demonstrated for the combination of mizoribin or ribavirin with IFN-α.[17]

A large scale *in vitro* screening program of 93 aromatic cationic molecules against a noncytopathic BVDV strain replicated in MDBK cells selected five compounds, two of which manifested a pronounced activity, the monocationic benzimidazole-substituted aryl furans DB771 and DB772.[18] As a next step, the compound 2-2(2-benzimidazolyl)-5-[4-(2-imidazolino)-phenyl]furan dihydrochloride (DB772) was tested in calves.[19] The compound was administered in one BVDV-free calf, once, at a dose of 1.6 mg/kg intravenously; another BVDV-free calf was treated three times a day for six days at 9.5 mg/kg intravenously. Subsequently, four calves were treated intravenously with 12 mg/kg DB772 three times a day for six days, and two calves were given the diluent (placebo). BVDV inhibition was established for 14 days in one calf and for at least three days in three calves. The virus isolated from these three calves was DB772 resistant *in vitro*. No adverse effects of DB772 were found.[19]

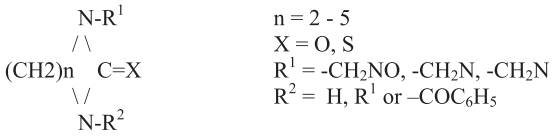
Substances suppressing BVDV entry in the cell (through a clathrin-dependent endocytic pathway), such as genistein (a tyrosine kinase inhibitor), inhibited BVDV replication.[20] 5-[(4-bromophenyl)methyl]-2-phenyl-5H-imidazo[4,5-c]pyridine (BPIP) was characterized as a highly selective inhibitor of BVDV replication *in vitro* (SI ~ 2,000). This substance markedly inhibits viral RNA synthesis and the production of infectious virus, targeting viral RNA-dependent RNA polymerase. It was inactive against hepatitis C virus (HCV) subgenomic replicons and yellow fever virus replication and weakly effective against GB virus C (formerly known as hepatitis G virus), a member of the *Pegivirus* genus.[21]

Another substance active toward BVDV is thiosemicarbazone derived from 5,6-dimethoxy-1-indanone (TSC). Evidence shows that this compound inhibits BVDV replication in MDBK cells, acting at a point of time that coincides with the onset of viral RNA synthesis. Thus, the substance inhibits the activity of BVDV replication complexes. It was determined that TSC binds to the finger domain of the viral RNA-dependent RNA polymerase.[22]

Antiviral spectrum of cyclic ureas

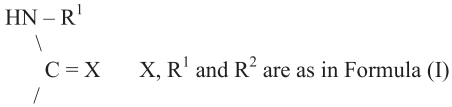
Planned synthesis based on our previous work on N, N′-disubstituted ureas and thioureas with antiviral effects[23–25] resulted in a series of cyclic urea and thiourea

derivatives and related compounds: tetrahydro-2(1H)-pyrimidinones and tetrahydro-2(1H)-pyrimidinethions[26] (I, II):



where X is O or S

(I)



(II)

A series of these compounds revealed pronounced antiviral effects against viruses belonging to three taxonomic groups: *Togaviridae, Flaviviridae*, and *Orthomyxoviridae*.[27–34]

Anti-pestivirus activity of CU

BVDV is the causative agent of mucosal disease/viral diarrhoea in Bovidae

The *Pestivirus* genus of *Flaviviridae* includes BVDV, classical swine fever virus (CSFV), previously known as hog cholera virus, and border disease virus of sheep (BDV).[35] There are some similarities in the pathology of all these virus infections. *In utero* transmission to the fetus can cause early embryonic losses, severe congenital abnormalities, and, particularly with BVDV, life-long persistent infections. [35–38] BVDV shares many similarities with HCV (a member of the *Hepacivirus* genus), yet it is more amenable to virologic and genetic analysis. For both BVDV and HCV, transition is initiated via an internal ribosome entry site.[39]

BVDV is the single stranded RNA virus responsible for two distinct disease entities in cattle: bovine virus diarrhea, which is characterized by high morbidity and low mortality, and mucosal disease, which is sporadic but highly fatal.[40]

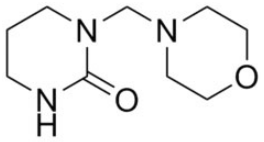
BVDV exists under two genotypes, BVDV 1 and BVDV 2.[41–44] Virus strains within both genotypes may occur as one of two biotypes, cytopathic or noncytopathic.[45] It was established that cytopathic BVDV arise through the mutation of noncytopathic virus and is known that p80 is the marker protein for cytopathic BVDV.[45] A highly fatal form of BVDV termed mucosal disease occurs when an animal persistently infected with noncytopathic BVDV becomes superinfected with cytopathic BVDV.[40] Pestiviruses represent the first RNA viruses for which recombination with cellular protein-coding sequences has been reported. As a result of such recombination, cytopathogenic BVDV can develop from noncytopathogenic strains.[40] So, cattle persistently infected with a noncytopathic BVDV strain can develop fatal mucosal disease after superinfection with a cytopathic strain.[44]

BVDV is also responsible for reproductive failure in cattle.[42,46] The virus can pass vertically from cow to fetus, causing abortion, birth of malformed calves, and calves born with persistent and life-long infections.[47] BVDV has also been reported in other animals: buffaloes, sheep, goats, camels, bison, deer, rabbits, and domestic cats.[48] Cell lines derived from animals (American Type Culture Collection) were found to be contaminated with BVDV.

In general, BVDV produces significant economic losses for the cattle industry,[45,36] and there are many reasons to consider antiviral chemotherapy as a reasonable means in the struggle against BVDV-induced diseases. Unfortunately, until now no effective inhibitors of BVDV replication have been found.

Here we present cycluridine as a strongly effective antiviral against BVDV infection.

Effect of cycluridine (CU) on BVDV replication in vitro



1-(4-morpholinomethyl)-tetrahydro-2(1H)-pyrimidinone [cycluridine (CU), DD-13, mopyridone]

The antiviral spectrum of CU is presented in Table 1. CU manifested a marked inhibitory effect on the replication of all BVDV strains tested – TVM-2 (cytopathic standard laboratory strain), C/Riems Island (cytopathic strain, supplied from Veterinary Institute, Insel Riems, Germany), Kableshkovo (cytopathic strain isolated in Bulgaria) and Oregon C24V (attenuated vaccinal strain) – in various cell cultures – primary calf kidney cell cultures, primary culture of calf trachea, and calf tracheal cell line (CTC) – in one-step growth and multicycle replication experimental designs. In the multi-cycle growth setup, CU's (at 30 μg/ml concentration) inhibitory effect ($\Delta\log_{10}$) was 3.0–3.8 120-h post virus inoculation at a low m.o.i. (0.0015).[49]

All tested BVDV strains demonstrated approximately identical susceptibilities to CU in the four different cell cultures mentioned above. When register the infectious

Table 1. Antiviral spectrum of tetrahydropyrimidinone derivatives (cycluridine included).

Susceptible viruses		
Togaviridae	*Alphavirus*	*SFV (CC, mice)*
		Sindbis (CC, mice)
		WEEV (CC, mice)
		VEEV (mice)
	Rubivirus	Rubella (CC)
Flaviviridae	*Flavivirus*	TBEV (mice)
		YFV (CC)
	Pestivirus	BVDV (CC, calves)
Orthomyxoviridae	Influenza virus A and B (CC, mice)	
Resistant viruses		
Picornaviridae		Polio 1 (CC)
Paramyxoviridae		NDV, Respiratory syncytial (CC, OC)
Rhabdoviridae		VSV (CC)
Retroviridae		ALV, BLV (CC)
Adenoviridae		CELO (CC)
Herpesviridae		PsRV (CC)
Poxviridae		Vaccinia (CC)

CC: cell culture; OC: organ culture; TBEV: anti-tick-born encephalitis virus; BVDV: bovine viral diarrhoea virus.

virus yield at 120th h post virus inoculation (m.o.i. 0.15) at a study of CU (30 μg/ml), Δlogs of 2.0, 2.3 and 2.3 were found for the strains of Kableshkovo, TVM and Oregon C24V, respectively.[49]

CU's effect against TVM strain replication in CTC was identical to that of ribavirin when tested by the CPE inhibition method ($MIC_{50} = 0.32$ μg/ml). However, the selectivity of CU was markedly higher because of its lower toxicity (Table 2).

Study of the mode of action of cycluridine (CU) against BVDV replication

Experiments were carried out with the cytopathic C Insel Riems strain, replicated in CT cells. Initially the CU dose–response curve was followed for compound concentrations ranging from 0.01 to 32 μg/ml, at Δ0.5 log dilutions (Figure 1). CU markedly inhibited BVDV growth: Δlogs > 2.0 $CCID_{50}$ at concentrations ≥ 1 μg/ml, attaining 4.0 logs at 3.2 μg/ml, 6 logs at 10 μg/ml, and > 7.0 logs at 32 μg/ml.

Then, a timing-of-addition study was conducted (Figure 2). Figure 2 shows the virus growth curves, with the latent period lasting until 10 h of post virus inoculation, the exponential phase at 12–18 h, and the plateau at 18–30 h. The compound (3 μg/ml) was added immediately after virus inoculation or at different times post infection. As seen in Figure 2, the addition of CU

Table 2. Effect of CU and ribavirin on replication of the TVM-2 strain of BVDV in CTC (CPE inhibition test).

Compound	MIC_{50} (μg/ml)	$CGIC_{50}$ (μg/ml)	SI
CU	0.32	51.5	156.1
Ribavirin	0.32	20.0	60.1

MIC_{50}: 50% minimal CPE inhibitory concentration; $CGIC_{50}$: 50% cell growth inhibitory concentration (concentration reducing the cell number by 50% in the cytotoxicity test, tracing the growth curve of the cell culture); SI: selectivity index ($CGIC_{50}/MIC_{50}$); BVDV: bovine viral diarrhoea virus; CTC: calf tracheal cell line; CU: cycluridine. Incubation time: 120 h.

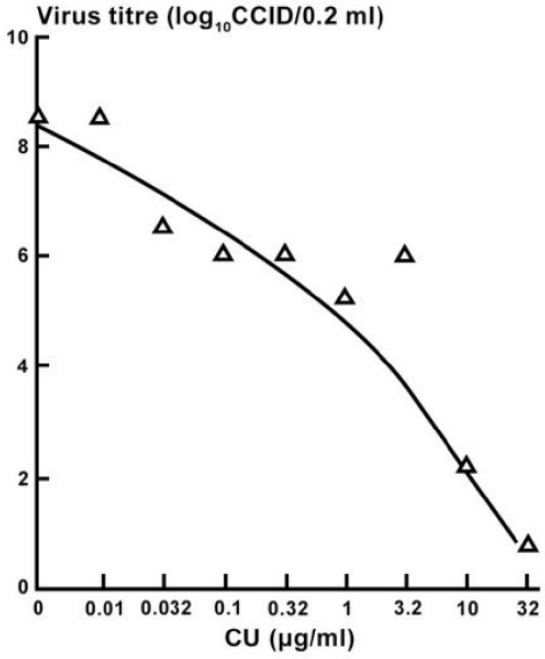


Figure 1. Effect of CU on bovine viral diarrhoea virus (cytopathic C Insel Riems strain) replication in calf tracheal cell line cell line: cycluridine dose–response curve. Calf tracheal cell line with growth medium: Eagle's MEM, Gibco with 2 mM L-glutamine, 10% inactivated calf serum preliminarily tested for bovine viral diarrhoea virus antibody, penicillin, and streptomycin. Maintenance medium: Eagle's MEM, Gibco with 2% fetal bovine serum. Incubation time: 120 h.

in the first 6 h post virus inoculation significantly inhibited virus replication (3–5 logs decrease of the virus titer). The addition of CU after 12 h was slightly effective. Evidently, BVDV replication was sensitive to the compound during the latent and exponential periods.

The effect of CU on the synthesis of BVDV RNA was studied by measuring of the acid-insoluble radioactivity after ^{3}H-uridine labeling of virus-infected CT cells as well as virus-infected and CU-treated cells. Infectious virus titer was determined in parallel.

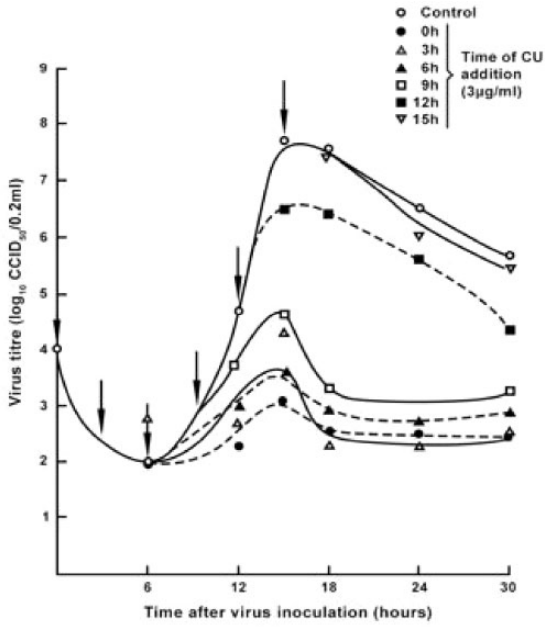


Figure 2. Effect of CU (3 μg/ml) on bovine viral diarrhoea virus (cytopathic C Insel Riems strain) replication in calf tracheal cell line in the one-step virus growth cycle setup: timing-of-addition study. The compound (3 μg/ml) was added immediately after virus inoculation (time 0 hr) or at 3, 6, 9, 12, or 15 h. Infectious virus samples were taken at 6, 12, 15, 18, 24, and 30 h.

Prior to these experiments, we found that CU does not suppress the synthesis of the cellular RNA at concentrations of 0.1–40 μg/ml (Table 3).

The viral RNA synthesis curve (Figure 3) reached its maximum at 8 h. CU added immediately after virus inoculation (time 0) did not influence the extent of viral RNA synthesis but only moved the height of RNA synthesis maximum to 90 min later. It is very important to mark the big contrast between CU's effect on the production of infectious virions and its effect on the synthesis of viral RNA. It is evident that the compound does not interact with viral RNA and most probably does not act on proteins involved in viral RNA synthesis (e.g. RNA polymerase). The target of CU may be another virus-specific protein that plays an important role in virion assembly.

Effect of CU on BVDV infection in calves

First, CU was tested in calves (100 kg weight) seronegative to BVDV.

Animals were infected intravenously with 7.3 log_{10} $CCID_{50}$ of BVDV Kableshkovo strain. The treatment course (oral administration with the milk) started on the day of virus inoculation and lasted six days (at 12-h dose intervals). The data from clinical observations of infected groups of calves treated with different doses of CU demonstrated the lack of any deviations from the normal physiological state. In the control group (placebo treated), moderate clinical symptoms (temperature increase, conjunctivitis, anorexia, and, in one animal, reduced leukocyte count) were observed. As Table 4 shows, the CU course markedly prevented the development of BVDV infection in experimentally infected calves, based on the clinical parameters recorded.

Table 3. Effect of CU on the synthesis of CTC cellular RNA.

CU concentration μg/ml	CPM × 10^3 of the acid insoluble precipitate
75	11.3
50	23.0
40	33.8
32	33.1
10	35.0
5	32.7
3.2	34.2
1.0	32.9
0.32	33.8
0.1	32.8
0.32	35.6

CTC: calf tracheal cell line; CU: cycluridine.

The data in Table 5, from our serological study using a virus-neutralizing reaction, reveal identical antibody responses in calves from different test groups (a 2–4 log_{10} increase in antibody titer on Day 15). Obviously, the CU treatment course did not prevent the development of immune response to the BVDV infection.

Next, two field trials were conducted to test the therapeutic effect of CU on calves with natural mucosal disease/viral diarrhea. The field trials were carried out in the Silistra Regional Veterinary Station on boxes containing premises prepared especially for the trial, situated in a territory guarded by policemen, in order to prevent the infection spread. The staff participating in the trial was previously educated.

The first trial consisted of three experimental treatment groups with six healthy calves each (3–4 months old, weighing 80–100 kg): Group I = 1 mg/kg CU; Group II = 10 mg/kg CU; Group III = placebo. The animal groups were placed in separate stalls, and three diseased animals with well-manifested clinical symptoms of mucosal disease/viral diarrhoea and serologically confirmed etiology were introduced into each stall. The CU compound was applied orally with the milk at doses of 10 (or 1) mg/kg daily (at 12-h dose intervals) for nine days.

In the second trial, two treatment groups of 10 calves each (15–20 days old, body weight = 50–60 kg) were

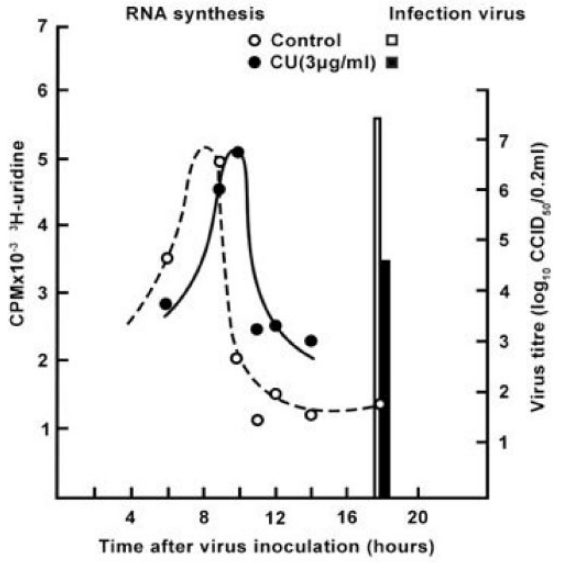


Figure 3. Effect of cycluridine (3 μg/ml) on bovine viral diarrhoea virus (cytopathic C Insel Riems strain) replication in the one-step virus growth cycle setup: virus-specific RNA synthesis curve. Cell cultures, grown 48 h in scintillation flasks, were infected with bovine viral diarrhoea virus (C strain) at m.o.i. 10–20 via 60 min adsorption at 0°C. Afterward, cell monolayers were rinsed with PBS, overlaid with EMEM with 2% bovine serum containing 3 μg/ml cycluridine, and incubated at 37°C. At various times post infection cells were treated 60 min with 0.5 μg/ml actinomycin D (Sigma) solution (100 μl) containing 5 μCi 3H-uridine, which was added to the culture medium. When labeling was completed, the medium was aspirated, and cells were rinsed with PBS and lysed with 2 ml of 5% SDS solution in PBS for 15 min at room temperature. Then, an equal volume (2 ml) of 10% cold (4°C) solution of trichloracetic acid (TCA) was added to each flask to precipitate macromolecules (both viral and cellular proteins and RNAs) for 60 min. The resulting suspension was filtered through nitrocellulose membrane (0.45 nm pore size; Millipore, USA), and each precipitate was washed three times with 5 ml ice-cold 5% TCA solution and twice with 10% ethanol. Filters were dried at room temperature, and then each filter was placed in 5 ml scintillation solution in a scintillation flask. The acid-insoluble radioactivity present on these filters was assayed by a standard method. The amount of retained radioactivity was determined in a liquid scintillation counter (Intertechnique SL-3000, Plaisir, France).

housed in separate stalls: Group I = 10 mg/kg CU and Group II = placebo. The infecting and therapeutic course was conducted as in the first field trial.

The results of the first field trial are presented in Table 6. As is well demonstrated, CU applied orally at a concentration of 10 mg/kg daily protected calves from developing the disease. The only symptom was a one-day increase in body temperature, registered in half the animals; no other symptoms were apparent. The treatment course with CU at a daily dose of 1 mg/kg was completely ineffective, with animals developing the characteristic clinical picture analogous to the placebo group, namely, elevated body temperature for 3–7 days, and conjunctivitis, anorexia, and bronchitis in the majority of calves.

Similar results were obtained during the second trial. None of the animals treated with CU (10 mg/kg daily) became sick. There were no cases of elevated temperature. All animals in the placebo-treated group demonstrated strongly manifested clinical symptoms of mucosal disease/viral diarrhoea and elevated temperature with a characteristic two-wave curve.

Some difference in the antiviral effect of CU was observed between the experimental infection and the natural mucosal disease. This could be connected to the degree of infection. The effective daily oral dose of CU for the experimental infection was 3 mg/kg, whereas for the natural infection, the effective CU dose was 10 mg/kg.

The CU toxicological tests on calves followed the pattern for determining acute toxicity (single-dose toxicity) and subacute toxicity (daily intake of the compound for 30 days). They demonstrated very good CU tolerability by calves as well as the complete safety of the compound's effective treatment course and the harmlessness of the therapeutic doses applied.

Effect of CU on viruses of the *Flavivirus* genus

We also studied CU's effect on the replication of two viruses belonging to another genus, *Flavivirus*, of the *Flaviviridae* family. These were tick-borne encephalitis virus and yellow fever virus.

Effect against tick-borne encephalitis virus

This study was conducted in the Laboratory of Comparative Virology of the Ivanovsky Institute of Virology, Russian Academy of Medical Sciences, Moscow. The compound was tested on an *in vivo* model, experimental infection with TBEV in mice (ICR line, 10–12 g weight, male). Mice were inoculated subcutaneously with TBEV Sophin strain (10 LD_{50}/0.2 ml). CU was applied subcutaneously at a 150 mg/kg dose according to three different protocols: (a) a single dose, 4 h prior to virus inoculation; (b) four doses, at 4, 24, 48, and 72 h after virus inoculation; and (c) as a course from day 1 to day 5 post virus inoculation, with two intakes per day. The control (placebo) group was included in the experimental setup.

The results presented in Table 7 show that CU manifested a marked protective effect when applied according to protocols (b) and (c).

Moreover, this antiviral effect of CU, registered in *in vivo* experiments, could be characterized with a

Table 4. Clinical symptoms in calves infected with BVDV and treated orally with CU.

Exp. Group	Calf No	Cmpd daily dose (mg/kg)	Fever (days)	Conjunc-tivitis	Anorexia	Decreased Leukocyte number
I	1	1	–	–	–	–
	2		–	–	–	–
	3		–	–	–	–
II	4	3	–	–	–	–
	5		–	–	–	–
	6		–	–	–	–
III	7	10	–	–	–	–
	8		–	–	–	–
	9		–	–	–	–
IV	10	Placebo	4	+	+	+[a]
	11		1	+	–	–
	12		2	+	+	–

BVDV: bovine viral diarrhoea virus; CU: cycluridine.
[a]Leukocyte count reduced by 3000 in 1 ml.

Table 5. Virus-neutralizing antibodies in sera of calves experimentally infected with BVDV (Kableshkovo strain) and treated with CU course.

Exp. Group	Calf No.	CU daily dose (mg/kg)	Virus-neutralizing antibodies[a] in the blood samples harvested on	
			Day 0	Day 15
I	1	1	4	4/8[a]
	2		4	4
	3		4	8
II	4	3	4	8/16
	5		4	8/16
	6		4	4/8
III	7	10	4	8
	8		4	4/8
	9		4	8
IV	10	Placebo	4	8/16
	11		4	4/8
	12		4	4

BVDV: bovine viral diarrhoea virus; CU: cycluridine.
[a]Reciprocal of the end-point dilution of the serum sample with neutralization of 100 ID_{50} of BVDV (Kableshkovo strain).

marked selectivity. A detailed study on the acute (single-dose) toxicity of tetrahydro-2(1H)-pyrimidinones for mice weighing 17–20 g (males of ICR random-bred line), administered orally or intraperitoneally, manifested comparatively high values, approximately 12,800 and 3500 mg/kg, respectively. Similarly, low toxicity values were found for rats in a study of the single-dose toxicity and subacute (5 and 14 every day administration) toxicity, for an orally administered daily dose of 37.5 mg/kg.[50]

Effect against yellow fever virus

CU was tested against YFV (17D strain; infectious titer 6–7 log_{10}) replication in a cell line of human

Table 6. Clinical symptoms in 4-month-old calves with natural mucosal disease/viral diarrhoea, treated orally with CU (trial 1).

Trial Group	Calf no.	Cmpd daily (mg/kg)	Fever (days)	Conjunctivitis	Anorexia	Bronchitis
I	1	1	3[a]	+	+	−
	2		4	+	+	+
	3		6	+	+	−
	4		3	+	+	+
	5		4	+	+	−
	6		7	+	+	+
II	7	10	1	−	−	−
	8		−	−	−	−
	9		−	−	−	−
	10		−	−	−	−
	11		1	−	−	−
	12		1	−	−	−
III	13	Placebo	4	+	+	+
	14		5	+	+	+
	15		6	+	+	+
	16		4	+	+	−
	17		4	+	+	−
	18		5	+	+	+

CU: cycluridine.
[a]Number of days with a body temperature over 40°C.

Table 7. Effect of CU on TBEV (Sophin strain) infection in mice.

Treatment course	Survivors (%)*
(a) Single dose: −4 h	10.0[ns]
(b) Four doses: +4 h, +24 h, +48 h, +72 h	30.0*
(c) Course + Day 1–day 5 (two intakes daily)	40.0**
Placebo	0

Tests were done on 20 animals per experimental group. Time interval before or after virus inoculation were shown. Statistical analysis was done by the two-tailed Fischer's exact test: ns, not significant (to Placebo group); *$P = 0.02$ (to Placebo group); *$P = 0.003$ (to Placebo group). TBEV: tick-born encephalitis virus; CU, cycluridine.

adenocarcinoma. The compound was applied immediately after virus inoculation or at 24 h. Infectious virus yields were determined at 24 or 48 h, respectively (24-h yields), using a plaque method.

A small inhibitory effect of 30% was recorded when CU (30 μg/ml) was added simultaneously with the virus. CU added at 24 h (i.e. soon after the appearance of infectious viral particles or the initial CPE), even at high concentrations (50–100 μg/ml), had no effect.

Concluding remarks

The results obtained from the study of CU's anti-BVDV activity can be summarized as follows:

(i) CU is a strong inhibitor of BVDV replication in cell cultures.
(ii) The drug-sensitive period in the BVDV replication cycle occurred in the latent period and the exponential phase; a 90-min delay in the peaking of viral RNA synthesis was observed.
(iii) CU administered orally manifested a pronounced protective effect in calves with natural mucosal disease/viral diarrhoea or experimentally infected with BVDV.
(iv) CU applied subcutaneously showed anti-TBEV activity, manifesting a marked protective effect in mice infected with TBEV.
(v) CU exerted a weak inhibitory effect on replication of YFV in cell cultures.

Convincing evidence was manifested regarding the compound's very high activity against BVDV, in both *in vitro* and *in vivo* experiments. Moreover, CU demonstrated a significant efficiency in a treatment course applied in cattle with natural mucosal disease/viral

diarrhoea. The conditions of the two trials were very close or identical to those used in veterinary clinical practice; moreover, the trials could be considered as double-blind trials, keeping in mind the trials staffs' preparations and the administration procedures for the CU and the solvent in the treated and placebo groups.

The data presented here demonstrate and characterize CU to be the most effective anti-BVDV antiviral studied and described.

Antiviral activity of CU towards TBEV infection *in vivo* (in mice) is impressive, and it shows CU's potential as an anti-flavivirus antiviral.

Declaration of conflicting interests

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