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ANTIVIRAL PROPERTIES OF VERDAZYLS AND LEUCOVERDAZYLS AND THEIR ACTIVITY AGAINST GROUP B ENTEROVIRUSES

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Abstract. Enteroviruses are non-enveloped viruses of *Enterovirus* genus, *Picornaviridae* family, causing a variety of human diseases: from acute respiratory and intestinal infections to more severe pathologies including poliomyelitis, encephalitis, myocarditis, pancreatitis. Currently, no approved direct-acting antiviral drugs for treatment of enterovirus infections exists, whereas vaccination is available only for prevention of poliomyelitis and enterovirus 71 infection. Therefore, it is promising to conduct a search for inhibitors of enteroviruses life cycle in drug development to treat enterovirus infections. Here, antiviral properties of stable free radicals, verdazyls, and their precursors, leucoverdazyls, were investigated. It has been shown that leucoverdazyls vs verdazyls increased the survival of permissive cell culture infected with coxsackievirus. The activity range of the lead leucoverdazyl against RNA-containing and DNA-containing human viruses (in the viral yield reduction assay) and its proposed mechanism of action (time of addition assay) was studied. The lead compound suppressed reproduction of group B enteroviruses *in vitro*, with modest activity against influenza A virus and no activity against herpes virus type 1 and adenovirus type 5. The maximum decrease in viral titers was observed upon its addition to infected cells during early and middle stages of the virus life cycle. Thus, we concluded that the studied compound has a pronounced inhibitory activity against group B enteroviruses not belonging to the class of capsid binder inhibitors, without virucidal properties. Previously, we described antioxidant properties of leucoverdazyls. It is known that many viral infections are accompanied by production of reactive oxygen species and oxidative stress, and some compounds with antioxidant properties exhibit antiviral potential. Targeted chemical modifications of leucoverdazyls and further studies of leucoverdazyl mechanism of action as well as *in vivo* animal studies are needed. However, the results obtained may be useful for future development of new antiviral drugs to treat enteroviral infections.

Key words: *Enteroviruses, enteroviral infection, Coxsackievirus, verdazyles, leucoverdazyles, antiviral activity, antioxidants.*

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ПРОТИВОВИРУСНЫЕ СВОЙСТВА ВЕРДАЗИЛОВ И ЛЕЙКОВЕРДАЗИЛОВ И ИХ АКТИВНОСТЬ В ОТНОШЕНИИ ЭНТЕРОВИРУСОВ ГРУППЫ В

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Резюме. Энтеровирусы — группа безоболочечных вирусов рода *Enterovirus* семейства *Picornaviridae*, вызывающих разнообразные заболевания человека: от острых респираторных и кишечных до более тяжелых, включая полиомиелит, энцефалит, миокардит, панкреатит. На сегодняшний день отсутствуют зарегистрированные противовирусные препараты прямого действия для терапии энтеровирусных инфекций, вакцинация доступна только для профилактики полиомиелита и инфекции, вызванной энтеровирусом 71. Перспективен поиск молекул — ингибиторов жизненного цикла энтеровирусов для разработки новых лекарственных средств для терапии энтеровирусных инфекций. В данной работе были исследованы противовирусные свойства стабильных свободных радикалов — вердазилов, и их предшественников — лейковердазилов. Было показано, что лейковердазилы, в отличие от вердазилов, способны повышать выживаемость пермиссивной клеточной культуры при инфицировании вирусом Коксаки. Был исследован спектр активности соединения-лидера в отношении РНК-содержащих и ДНК-содержащих вирусов человека (методом снижения титра вирусного потомства) и его предполагаемый механизм действия (в тесте на время добавления исследуемого соединения). Соединение-лидер мощно подавляло репродукцию энтеровирусов группы В *in vitro*, обладало слабой активностью в отношении вируса гриппа А, при этом активность в отношении вируса герпеса 1 типа и аденовируса 5 типа отсутствовала. Наблюдалось максимальное снижение вирусных титров при добавлении этого соединения к инфицированным клеткам на ранних и средних стадиях жизненного цикла вируса. Таким образом, заключили, что исследованное соединение обладает выраженной ингибирующей активностью в отношении энтеровирусов группы В, при этом оно не относится к классу ингибиторов связывания капсида (в отличие от вещества сравнения плеконарила) и не проявляет вирулицидных свойств. Ранее были описаны антиоксидантные свойства лейковердазилов. Известно, что многие вирусные инфекции сопровождаются образованием активных форм кислорода и окислительным стрессом, а ряд соединений с антиоксидантными свойствами обладают противовирусным потенциалом. Необходимо расширить библиотеку лейковердазилов за счет направленных химических модификаций, выполнить дальнейшие исследования механизма действия лейковердазилов и исследования *in vivo* на животных моделях энтеровирусных инфекций. Тем не менее результаты исследования могут быть полезными для будущей разработки новых противовирусных препаратов для терапии энтеровирусной инфекции.

Ключевые слова: *энтеровирусы, энтеровирусная инфекция, коксакивирус, вердазилы, лейковердазилы, противовирусная активность, антиоксиданты.*

Introduction

Enteroviruses (EV) are a diverse group of small icosahedral non-enveloped viruses with single-stranded non-segmented positive RNA genome belonging to the *Picornaviridae* family. Currently genus *Enterovirus* encompasses 15 species: Enterovirus A-L and Rhinovirus A-C [29]. They are able to survive harsh environments and can cause both asymptomatic infections and more severe diseases: poliomyelitis, myocarditis, pancreatitis, encephalitis. Enteroviral infections (EVI) are an important problem of public health worldwide. The main manifestations of enteroviral infection include hand-foot-mouth disease, acute hemorrhagic conjunctivitis, herpangina [2].

Though enteroviruses infect patients from different age groups, the most vulnerable categories of the population are newborns and children, who have a greater chance of developing complications. According to a recent systematic review of clinical characteristics of severe neonatal enterovirus infection, in newborns hepatitis and myocarditis were the most common severe complications, while the highest lethality rate (38.6%) was observed in neonates with myocarditis [34].

Enteroviruses are distinguished by their ability to cause outbreaks often in organized children's groups. Disease activity is seasonal (typically in the summer and early fall in temperate climate) and the emergence of leading serotypes responsible for seasonal infection incidence rising is unpredictable. According to CDC (Center for disease control) large outbreaks of EVI caused by EV68 in the form of SARS associated with acute flaccid myelitis were noted in the USA in 2014 and 2016 in children [8]. By the 1990s, Enterovirus A71 became endemic in the Asia-Pacific region, causing major outbreaks every 3–4 years [26]. In Russia the rise in the incidence of enterovirus infections in different periods was caused by various non-polio enteroviruses, and the spectrum of enteroviruses isolated from patients with enterovirus infection varies [3, 4].

The tendency to genetic recombination with the emergence of new strains, as well as high resist-

ance to environmental conditions provides their long-term persistence outside host organisms. Population mobility and labor migration are important factors of enteroviruses spreading to new territories in the modern world. The formation of carriage of viruses, accompanied by prolonged release of enteroviruses into the external environment, contributes to the persistence of enteroviruses in the human population.

Treatment of EVI is mainly supportive, focusing on the management of the most severe symptoms. Despite some prominent success in the field, vaccine development for the prevention of non-polio enterovirus infections is complicated due to their high phenotypic diversity. Currently only a limited number of vaccines were approved to combat EVI including three vaccines against EV71 that have been licensed by National Medical Products Administration of China [22].

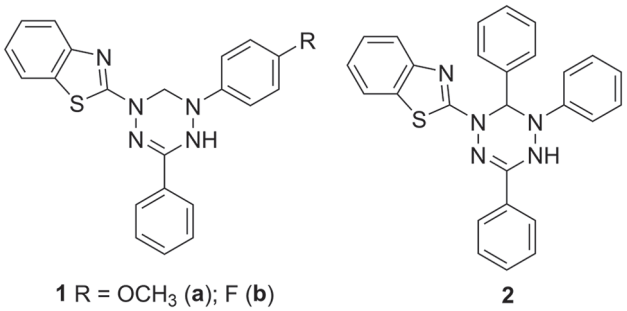
Much effort has been put into the development of antiviral drugs for the treatment of enteroviral infections. The following categories of direct enterovirus inhibitors targeting viral proteins were reported earlier: capsid binders (pleconaril and its derivatives), viral 3C protease inhibitors (rupintrivir and its analogs), drugs targeting viral replicative apparatus (both nucleoside analogs and non-nucleoside inhibitors) [1, 6]. Despite their activity in preclinical studies, none of them have been registered for EVI therapy due to lack of efficacy, poor pharmacokinetic profile, and/or adverse effects in clinical studies. Cellular proteins implicated in the viral life cycle may represent promising options for drug design [7, 33]. Drug repurposing screens also contributed to some progress in the field (for example, amiloride, fluoxetine) [25, 32]. However, it should be taken into account, that the potential application of repurposed drugs (for example, fluoxetine is an antidepressant, amiloride is a diuretic) can possibly cause unpredictable emergence of resistant strains if their intended use coincides with an asymptomatic EVI in a patient. Thus, there are no approved direct antiviral drugs for EVI treatment. The use of antiviral drugs will not only alleviate the disease in a particular patient, but will also reduce the release of the virus into the external environment. Therefore, there is a need for further search and development of novel antiviral drugs in view of clinical significance of enteroviral infection outbreaks.

We have recently reported new stable free radicals heterocyclic verdazyls containing a benzothiazole substituent [13, 14, 16] and their precursors — leucoverdazyls [15]. It should be noted that leucoverdazyls possess antioxidant activity, which suggests the presence of other types of activity.

In the present study, for the first time we investigated the antiviral activity of a small library of verdazyls and leucoverdazyls against group B enteroviruses. It was shown that leucoverdazyls (in contrast to verdazyls) potently suppress the reproduction of viral progeny *in vitro*. At the same time, the lead compound demonstrated modest activity towards influenza A virus and no activity against herpes simplex virus and adenovirus. For the lead compound studies of the putative mechanism of action were performed. Our study clearly demonstrated that leucoverdazyls are a promising group of novel heterocyclic compounds in view of EVI management. The results obtained can be further used for the development of antiviral drugs for the treatment of enterovirus infections.

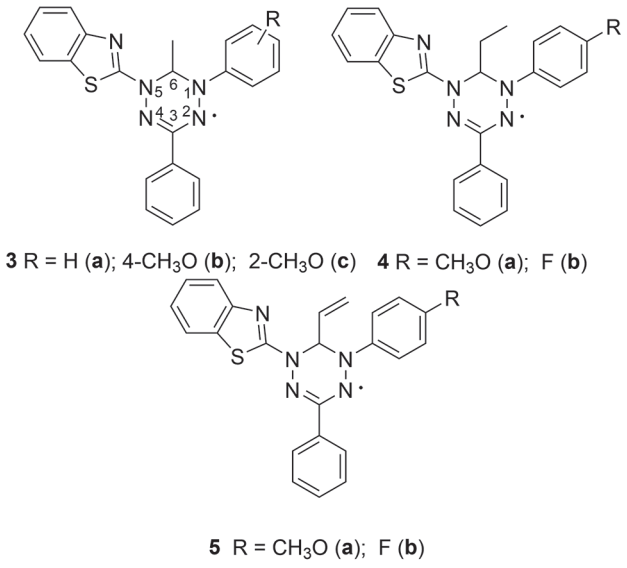
Materials and methods

Objects of the study. The leucoverdazyls 2-(1-aryl-3-phenyl-5,6-dihydro-4H-1,2,4,5-tetrazin-1-yl)-1,3-benzothiazoles (Fig. 1) were synthesized by alkylation of 1-aryl-5-(1,3-benzothiazol-2-yl)-3-phenylformazans with haloalkanes in alcoholic alkali, followed by cyclization of N-alkyl derivatives, according to the procedure described previously [15].



1 R = OCH_3 (**a**); F (**b**) **2**

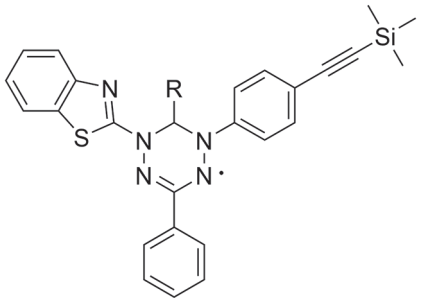
Figure 1. Structures of 2-(1-aryl-3-phenyl-5,6-dihydro-4H-1,2,4,5-tetrazin-1-yl)-1,3-benzothiazoles used in the study



3 R = H (**a**); 4-CH_3O (**b**); 2-CH_3O (**c**) **4** R = CH_3O (**a**); F (**b**)

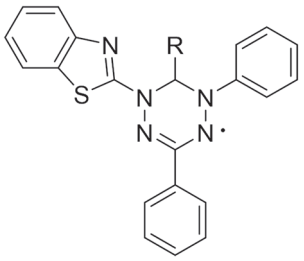
5 R = CH_3O (**a**); F (**b**)

Figure 2. Structures of 6-alkyl-5-aryl-1-(benzo[d]thiazol-2-yl)-3-phenylverdazyls and 5-aryl-1-(benzo[d]thiazol-2-yl)-3-phenyl-6-vinylverdazyls used in the study



6 R = CH_2=CH- (**a**); Ph (**b**)

Figure 3. Structures 5-(1,3-Benzothiazol-2-yl)-3-pheny-1-[4 phenylethynyl) phenyl] verdazyls used in the study



7 R = H (**a**); CH_3 (**b**)

Figure 4. Kuhn verdazyls used in the study

The synthesis of verdazyls (Fig. 2) were performed according to a previously published method [14]. The starting formazans were alkylated with various alkylating agents in EtOH for 15 min. After removing EtOH and the excess of alkylating agent, the respective N-alkylformazans were cyclized in heptane. Heptane was removed by distillation, and the obtained leucoverdazyls were oxidized using a 12-fold excess of PbO_2 in Me_2CO. Verdazyls were isolated in 35–50% yields after purification by column chromatography.

The Ethynyl derivatives (Fig. 3) were synthesized from 5-(benzothiazol-2-yl)-1-(4-iodophenyl)-3-phenyl-6-vinyl(phenyl)verdazyls using Sonogashira cross-coupling reactions carried out in two stages according to a previously published method [16].

Kuhn verdazyls (Fig. 4) were synthesized by the known procedure [21].

Reference compound pleconaril was generously provided by Dr. V.A. Makarov (Federal Research Center, Fundamentals of Biotechnology, Russian Academy of Sciences, Moscow, Russia).

Viruses and cells. Influenza A virus (IAV, strain A/Puerto Rico/8/1934 H1N1), Coxsackievirus 3 (CVB3, strain Nancy), Coxsackievirus 4 (CVB4, strain Powers), Herpes simplex virus type 1 (HSV1) and Human adenovirus 5 (Ad5) were obtained from the collection of viruses of the Pasteur Institute (St. Petersburg, Russia). Clinical isolate of Coxsackievirus 5 (CVB5) was kindly provided by the Regional Centre for Epidemiological Surveillance of Poliomyelitis (St. Petersburg, Russia). The following permissive cell lines were used in the studies: MDCK (ATCC #CCL-34), Vero (ATCC #CCL-81), A549 (ATCC #CCL-185). Infectious titers (in 50%-tissue culture infection dose, $TCID_{50}$) were determined in MDCK for IAV, in Vero cells for CVB3, CVB4, CVB5, HSV1 and in A549 cells for Ad5 by endpoint dilution assay using the following procedure. Permissive cells were seeded into 96-well plates in Eagle minimal essential medium (MEM, Paneco, Russia) supplemented with 5% fetal bovine serum (FBS, Gibco, USA). After 24 h, the media was aspirated, the wells were washed with saline, fresh MEM without FBS was added to the wells and the cells were infected with serial tenfold dilutions of virus stocks (100 μL per well, 4 wells for each dilution). The plates were incubated at +37°C in 5% CO_2 and observed daily for cytopathic effect (CPE). After 72 h (for enteroviruses and IAV) or 96 h (for HSV1 and Ad5), the viral titer was calculated in $TCID_{50}$ using the method of Spearman–Karber.

Cytoprotection assay. Vero cells were seeded in 96-well plates 24 h before the assay. Cells were washed with saline and serial dilutions of tested compounds in MEM without FBS were added to cells. No compounds were added to virus control wells. The plates were incubated for 1 h at 37°C at 5% CO_2. Then the cells were infected with CVB3 Nancy (m.o.i range of 1–0,001) and incubated for 72 h at 37°C at 5% CO_2. No virus was added to cytotoxicity control wells. Thereafter cell viability was assessed by MTT test using the following procedure. The cells were washed with saline, and a solution of Thiazolyl blue tetrazolium bromide (AppliChem, USA) (0.5 μg/mL) in MEM was added to the wells (100 μL per well). After 2 h of incubation at 37°C in 5% CO_2, the supernatant from wells was discarded, and the formazan residue was dissolved in DMSO (100 μL per well). The optical density of cells was then measured on a Multiskan multifunctional reader (ThermoScientific, Shanghai, China) at a wavelength of 540 nm.

The cytoprotective activity of compounds was considered as their ability to increase the values of OD compared to control wells (with virus only). Based on the results obtained, the values of EC_{50}, i.e. the concentration leading to 50% cytoprotection after infection of cells, was calculated using GraphPad Prism 6.01 software.

Cytotoxicity assay. MTT test was used to study the cytotoxicity of the compounds. The permissive cells were seeded in 96-well plates in Eagle minimal essential medium (MEM) supplemented with 10% FBS. After 24 h, the media was removed, and the wells were washed with saline. Compounds were dissolved in DMSO, and a series of two-fold dilutions of each compound (1000–4 μg/mL) in MEM without FBS were prepared and added to the cells in triplicates (200 μL per well). The maximal concentra-

tion of DMSO was 0.5%, MEM with 0.5% DMSO was added to cell control wells. Cells were incubated for 24 h or 48 h at 37°C in 5% CO_2 in the presence of the dissolved compounds. Thereafter the MTT was performed as described above (see "Cytoprotection assay" item). The optical density of cells was then measured on Multiskan multifunctional reader (ThermoScientific, Shanghai, China) at a wavelength of 540 nm and plotted against the concentration of the compounds to generate the dose–response curve. The 50% cytotoxic dose (CC_{50}) of each compound (i.e., the compound concentration that causes the death of 50% of cells in a culture, or decreases the optical density twice as compared to the control wells) was calculated using four-parameter logistic nonlinear regression model (GraphPad Prism 6.01).

Virus yield reduction assay. Antiviral activity of the compounds was further evaluated using viral yield reduction assay. The corresponding permissive cell lines were seeded in MEM supplemented with 5% FBS in 24-well plates. When the cells confluence reached 100%, the compounds tested were dissolved in DMSO, and a series of three-fold dilutions of each compound in MEM without FBS was added to the cells and incubated at 37°C in 5% CO_2. After 1 h, the medium was discarded, equal volumes of fresh serial dilutions of each compound and viral suspension in MEM without FBS at MOI 0.01 was added to all the wells of the plate. In cell control wells only MEM without FBS was added. In virus control wells no compounds were added. The plates were incubated at 4°C for 1 h. Thereafter, the unbound virus was washed away and three-fold dilutions of each compound in MEM without FBS was added to the wells (1 mL). After 24 h (for enteroviruses and IAV) of 48 h (for HSV1 and Ad5) of incubation at 37°C in 5% CO_2, the infectious titer of viral progeny in cell supernatant (in $TCID_{50}$) for each compound concentration, cell control, and virus control wells were determined in permissive cell lines by endpoint dilution assay.

The titer of virus progeny (% relative to the virus control) was plotted against log concentration of the compounds used to generate the dose–response curve. The 50% virus-inhibiting concentration (IC_{50}) of each compound tested (the concentration leading to 50% inhibition of virus reproduction after infection of cells) was calculated using four-parameter logistic nonlinear regression model (GraphPad Prism 6.01). Selectivity index (SI) was calculated as a ratio of CC_{50} to IC_{50} values.

Virucidal assay. For virucidal assay a serial dilution of the leader compound in serum-free MEM was mixed with equal volume of 10^4 $TCID_{50}$ of Coxsackievirus B4 (Powers) in sterile test tubes and incubated at room temperature for 1 h. 96% ethanol was used as a control. In 1 h of incubation, the infectious titer of CVB4 was evaluated by endpoint dilution assay in Vero cells as described above (see "Viruses and cells" item).

Time-of-addition assay. Time of addition assay was performed according to the recommendation described in [11]. Vero cells were seeded in 24-well plates in 24 h before the beginning of the assay to reach a confluence of 90%. The leader compound was sequentially added in concentration of 25 μM in the following time points to the respective individual wells in the plate: (–2), (–1), 0, 2, 4, 6, where (–2) means 1 h before addition of the virus, (–1) — addition of the virus, 0 — completion of viral sorption on the cell surface, 2, 4, 6 — 2, 4 or 6 h after of virus sorption and washout of non-adsorbed virus. At (–1) suspension of 10^6 $TCID_{50}$ of CVB4 was added to all the wells except cell control and the plate was incubated at +4°C for 1 h in order to synchronize the infection in all conditions, the unbound virus was washed thereafter and the plate was returned to +37°C. Capsid-binder pleconaril was used as a control. In 8 h after the completion of virus sorption the experiment was stopped and the infectious viral titer was measured in each well using end-point titration in Vero cells.

Statistics. All *in vitro* experiments were repeated three times. The results were analyzed using GraphPad Prism 6.01. The values of CC_{50}, EC_{50} and IC_{50} were represented as mean±SD.

Results

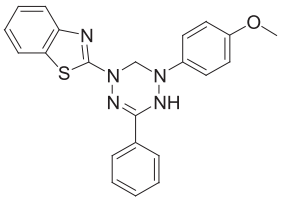
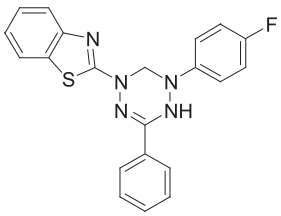
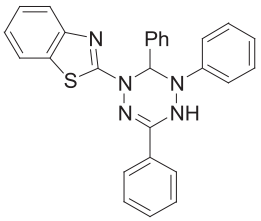
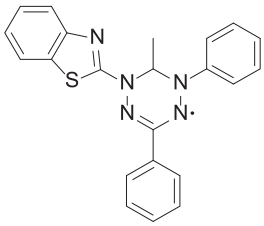
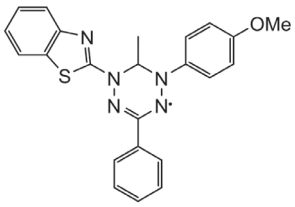
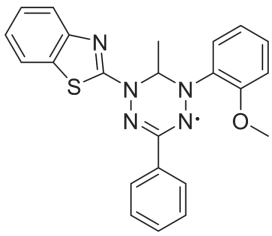
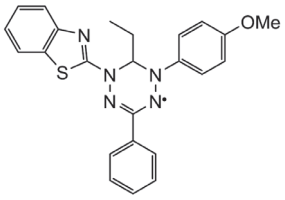
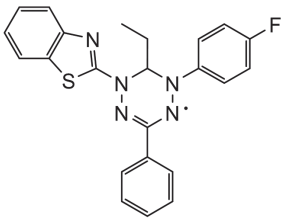
Leucoverdazyls exhibit cytoprotective and virus inhibiting activity in Coxsackievirus B3 infection *in vitro* at low micromolar concentrations

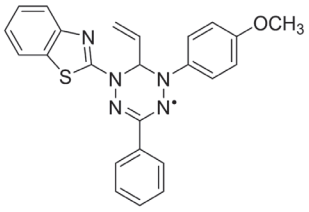
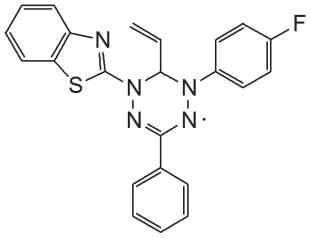
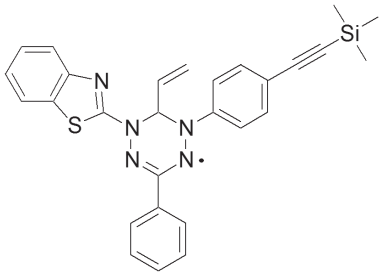
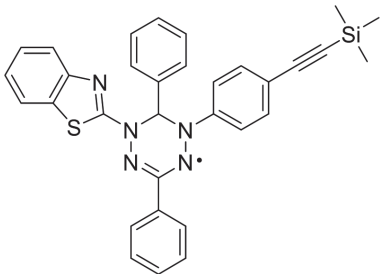
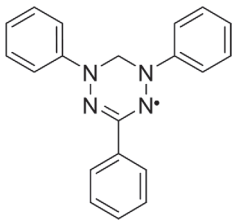
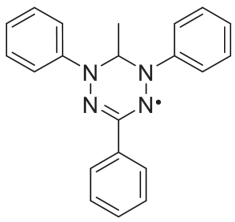
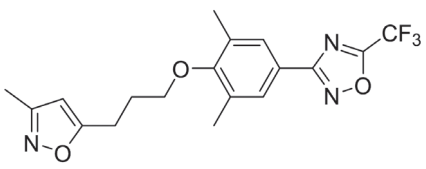
In the beginning of our study, cytoprotective properties of first 3 leucoverdazyls and 11 verdazyls against pleconaril-resistant strain Coxsackievirus B3 Nancy were determined using cytoprotection assay [28]. This assay allows to evaluate cell survival depending on concentration of tested compounds after infection with a virus. Cell survival is assessed by MTT assay based on the measurement of cell respiration activity. Pleconaril was used as a reference drug. The results of cytoprotection assay are presented in the Table 1 below.

As can be seen from Table 1 verdazyls had no cytoprotective effect *in vitro* upon CVB3 infection. In contrast, leucoverdazyl 1a (1 out of 3 tested) was able to increase cell viability in comparison to virus control. Its selectivity was comparable with the reference drug pleconaril. We, therefore, focused our further investigations on this compound, 1a.

To confirm virus-inhibition activity of 1a, we performed a viral yield reduction test. As described above, this test measures the titer of viral progeny in the supernatant of the infected culture depending on the compound concentration. Therefore, it is possible to evaluate antiviral activity of the compounds. The results of viral yield reduction assay are summarized in the Fig. 5 below.

Table 1. Results of cytoprotection assay against CVB3 Nancy

Number	Structure	CC_{50}, μM *	EC_{50}, μM **	SI ***
1a		150.7±20.2	< 9	> 16
1b		12.8±2.1	10 ±1.7	> 1
2		> 671	> 671	> 1
3a		> 781	> 781	> 1
3b		> 233	> 233	> 1
3c		> 241	> 241	> 1
4a		> 233	> 233	> 1
4b		> 240	> 240	> 1

Number	Structure	CC_{50}, μM *	EC_{50}, μM **	SI ***
5a		> 234	> 234	> 1
5b		> 724	> 724	> 1
6a		> 203	> 203	> 1
6b		> 184	> 184	> 1
7a		> 319	159.2±16.5	< 1
7b		> 305	33.5±4.1	< 1
Pleconaril		57.4±6.1	2.6±0.3	22

Notes. *CC_{50} is the cytotoxic concentration, the concentration resulting in death of 50% cells; CC_{50} were evaluated after 72 h of incubation of Vero cells with compound alone. **EC_{50} is the 50% cytoprotective concentration, the concentration leading to 50% cytoprotection after infection of cells with CVB3 (at m.o.i 0.001); ***SI is the selectivity index, the ratio of CC_{50}/EC_{50}. The data presented were obtained from three independent experiments and CC_{50} and EC_{50} are shown as mean ± SD. < indicates lower than minimal tested concentration, > indicates higher than the maximum tested concentration.

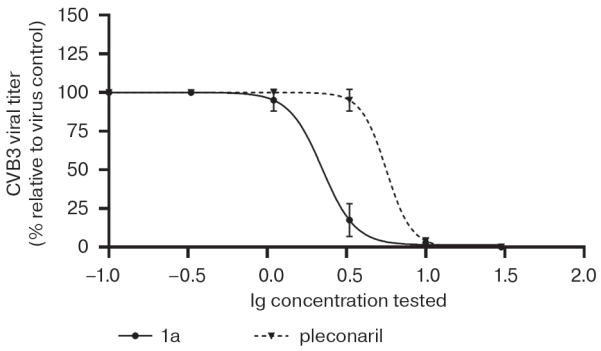


Figure 5. Virus-inhibiting properties of 1a against Coxsackievirus B3 (Nancy) in viral yield reduction assay

Notes. Virus-inhibiting activity of compound 1a against Coxsackievirus B3 (Nancy) based on viral yield assay results. The titer of viral progeny in Vero supernatants was evaluated relative to the titer in virus control and plotted against log compound concentration. Pleconaril was used as a reference compound. IC_{50} values for 1a and for pleconaril were as follows: 5.4±0.8 μM and 15.2±1.8 μM, respectively. IC_{50} is the 50% virus-inhibiting concentration, the concentration leading to 50% inhibition of virus reproduction after infection of cells (at m.o.i 0.01); The data presented as the mean of three independent experiments. Values are the mean ±SD of three independent experiments.

As can be seen from Fig. 5, the results of viral yield reduction assay supported the results of cytoprotection assay. Leucoverdazyl 1a demonstrated remarkable anti-enteroviral activity *in vitro* against pleconaril resistant CVB3 strain with micromolar IC_{50} value lower than that of pleconaril. Cytotoxicity of 1a and pleconaril was determined using MTT assay after 24-hour incubation of Vero cells with serial dilutions of the compounds tested. The following CC_{50} values were obtained: for 1a CC_{50} > 1347 μM and for pleconaril CC_{50} = 656±52 μM. The selectivity index (SI) values were calculated as the ratio of CC_{50}/IC_{50}: > 249 for 1a and 43 for pleconaril, respectively. 1a was less toxic than pleconaril and its selectivity towards CVB3 was superior to pleconaril.

Table 2. *In vitro* activity of 1a against some enteroviruses of group B

	CC_{50}, μM *	IC_{50}, μM**	SI***
1a			
CVB4	> 1347	0.72±0.21	> 1924
CVB5	> 1347	0.85±0.15	> 1587
Pleconaril			
CVB4	656±52	1.91±0.17	364
CVB5	656±52	< 0.1	> 6560

Notes. *CC_{50} is the cytotoxic concentration, the concentration resulting in death of 50% cells; CC_{50} were evaluated after 24 h of incubation of Vero cells with compound alone. **IC_{50} is the 50% virus-inhibiting concentration, the concentration leading to 50% inhibition of virus reproduction after infection of cells (at m.o.i of 0.01); ***SI is the selectivity index, the ratio of CC_{50}/IC_{50}. The data presented are the mean of three independent experiments. The CC_{50} and IC_{50} are presented as the mean ± error of the experiment. > indicates higher than the maximum tested concentration.

Table 3. Activity spectra of 1a towards RNA and DNA enveloped and non-enveloped viruses

	CC_{50}, μM*	IC_{50}, μM**	SI***
IAV	1246±180	50±6	25
HSV1	77±8	> 124	< 1
Ad5	623±7	> 124	< 6

Notes. *CC_{50} is the cytotoxic concentration, the concentration resulting in the death of 50% cells; CC_{50} were evaluated after 24 (MDCK cells for IAV) or 48 h (Vero cells for HSV1 and A549 cells for Ad5) of incubation with compound alone by using MTT test.**IC_{50} is the 50% virus-inhibiting concentration, the concentration leading to 50% inhibition of virus reproduction after infection of cells (at m.o.i 0.01); ***SI is the selectivity index, the ratio of CC_{50}/IC_{50}. The data presented as the mean of three independent experiments. The CC_{50} and IC_{50} are presented as the mean ± error of the experiment. > indicates higher than the maximum tested concentration. > indicates higher than the maximum tested concentration.

Activity spectra of 1a *in vitro*

In order to assess whether 1a possesses strain-specific or wider activity, we performed viral yield reduction assay using 1a and two pleconaril sensitive enteroviruses: Coxsackievirus B4 (strain Powers), Coxsackievirus B5 (clinical isolate). The results are presented in Table 2. Pleconaril was used as a reference drug.

The lead compound 1a showed prominent inhibitory activity against CVB4 and CVB5 *in vitro*, which is indicative of high anti-enteroviral potential, though its activity against CVB5 was lower than of pleconaril.

Next, we investigated whether 1a suppresses enteroviruses' life cycle only or is capable of inhibiting other genetically diverse negative strand RNA or DNA viruses, its antiviral activity towards IAV (ssRNA-negative enveloped virus) and HSV1 (dsDNA enveloped virus) and Ad5 (dsDNA non-enveloped virus) was further evaluated in viral yield reduction assay (for the results see Table 3).

The compound showed only modest activity against RNA virus — Influenza A — in contrast to its inhibitory potential against Coxsackieviruses. The activity against DNA-viruses — Ad5 and HSV1 — was negligible. Therefore, it was suggested that 1a is specific against EV.

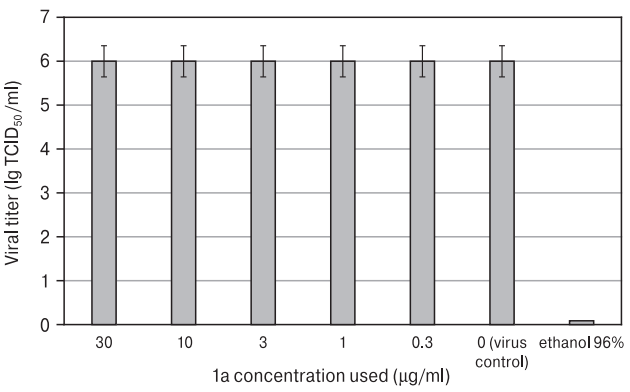


Figure 6. 1a shows no virucidal activity against Coxsackievirus B4 (Powers strain)

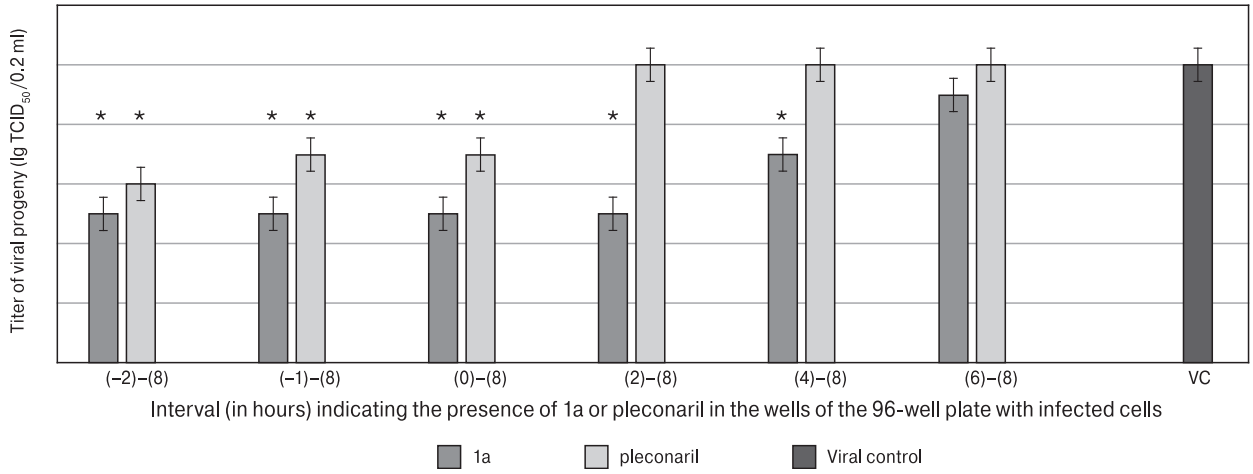


Figure 7. 1a inhibits early and middle stages of Coxsackievirus life cycle in Vero cells
Note. The activity of compound 1a against the Coxsackie B4 virus (Powers strain) depending on the time of addition to a permissive cell line upon CVB4 infection. Vero cells were infected with CVB4 (–1 h), 1a (25 μM) was added at the indicated time points (in hours) before the virus (–2 h), concomitantly with the virus (–1 h) and after (0, 2, 4, 6 h) infection, where 0 corresponds to the moment of completed virus absorption on the cell surface. The infectious activity of the viral progeny was evaluated by end-point titration in the Vero cells in lg $TCID_{50}$/0.2 ml. Pleconaril (25 μM) was used as a reference compound. Values are presented as the mean ± SD of three independent experiments. Asterisk indicates significance of difference in virus titer for 1a and pleconaril relative to the virus control, $p < 0.05$ by Mann–Whitney U-test.

1a inhibits early and middle stages of enterovirus life cycle

For 1a the possible mechanism of activity was studied in the following assays described below. Firstly, we investigated whether it has any virucidal activity. For this purpose, 1a was incubated with Coxsackievirus B4 (strain Powers) for 1 h and infectious titer was further determined in Vero cells using end-point titration assay (Fig. 6).

Thereafter, we focused on what stage of the viral cycle 1a displays the highest inhibiting activity. Using these results, it would be possible to deduce the viral or cellular proteins that could be a target for the compound. For this purpose, a time-of-addition assay was performed. We cultivated CVB4, adding and removing 1a at different time points regarding the infecting time. After one cycle of reproduction (8 hpi), the infectious titer of viral progeny was determined in end-point dilution assay (Fig. 7).

As can be seen from the data presented, the most pronounced effect of 1a was reached when it was present in the culture medium from –2 to 4 h post-infection (hpi). Inhibitory effect was absent after 4 hpi time point. Pleconaril used as a reference compound was mostly active from –1 point till 1 hpi as expected according to its capsid-binder characteristics. It belongs to the capsid binder group of antivirals which interact with various depressions on the surface of enteroviral capsid [5]. This interaction leads to stabilization of virus particles and prevents them from penetrating the cell.

Coxsackievirus lifecycle is relatively short (6–8 h) and its timing has been extensively studied previously in cell culture. According to the latest research viral genomic RNA is visible inside the cell until 1–2 hpi, the replication of the viral RNA starts 2–3 h after infection and the translation — at 3–4 hpi, viral progeny capsid proteins are detected after 4 hpi [27]. Therefore, the inhibitory effect of 1a spans the following stages of the virus life cycle: cell attachment, penetration, genomic RNA transcription, proteolytic processing and RNA replication.

Discussion

Enteroviral infection affects millions of people worldwide. Despite wide distribution of enteroviruses and economic damage from cases of temporary disability caused by enterovirus infection, vaccination option is available only for polioviruses and EV7. Besides, there are no approved antivirals to treat non-polio enteroviral infections.

In the present study for the first-time we investigated antiviral properties of novel heterocyclic compounds — free radicals heterocyclic verdazyls and their precursors leucoverdazyls — against coxsackieviruses including strain, resistant to well-known drug — pleconaril — used herein as reference compound. It was revealed that leucoverdazyls but not verdazyls possess potent virus inhibiting activity against group B enteroviruses without affecting life cycle of genetically distinct DNA viruses (herpes virus and adenovirus) while having slight activity against IAV virus (enveloped virus with negative segmented ss-RNA). The maximum decrease in viral titers upon addition of 1a was observed in the early and middle stages of coxsackievirus life cycle: if 1a was added be-

fore virus or up to 4 h post infection. Leucoverdazyl 1a doesn't belong to capsid binder class of inhibitors and has no virucidal activity against coxsackievirus.

Previously we described antioxidant properties of leucoverdazyls [15]. Oxidative stress is in general an imbalance between the production of reactive oxygen species (ROS) and their removal by endogenous antioxidant cellular enzymes [23]. An increase in oxidative stress is associated with changes in cell metabolism and physiology, and has a strong effect on viral infection. ROS can be produced through various endogenous mechanisms: mitochondria, NADPH (nicotinamide adenine dinucleotide phosphate) oxidases (NOX), cytochrome P450, endoplasmic reticulum, peroxisomes, lysosomes [24]. Mitochondria are regarded as the prime source of endogenous ROS due to their main role in oxidative ATP production.

Though virus-induced increased ROS levels trigger innate antiviral immunity, different viruses have elaborated various delicate strategies to manipulate mitochondrial respiratory and apoptotic functions to ensure a successful life cycle [17]. For example, adenovirus dampens mitochondria-dependent intrinsic apoptosis via adenoviral E1B 19K protein, which was one of earliest viral antagonists of Bcl-2 (a cellular protein that inhibits apoptosis) discovered. E1B 19K protein shares functional and structural homology with Bcl-2 and inhibits p53-dependent apoptosis. Influenza A virus infection causes an imbalance in the intracellular redox state, leading to depletion of glutathione (one of the most studied cell antioxidants) and an increase in NADPH oxidase 4 (NOX4)-mediated ROS production. This pro-oxidant state promotes viral replication including the folding of surface glycoproteins and the nucleus-cytoplasmic traffic of viral ribonucleoprotein [12]. EV71 infection also leads to increased ROS generation, accompanied by decreased levels of antioxidants (taurine and hypotaurine) [9]. It was shown, that enteroviral 2B protein interacts with mitochondrial voltage-dependent anion channel 3 (VDAC3) — one of the pore-forming protein localized to the outer membrane of mitochondria, and this interaction is essential to mitochondrial ROS generation and viral replication [10]. It is described that oxidative stress elicited by viruses can promote inflammation and subsequent tissue destruction [30, 19].

It has been published previously that compounds with antioxidant properties may possess antiviral effects. For example, mitochondria-targeted antioxidants MitoTEMPO and Mitoquinone (MitoQ, synthetic analogue of coenzyme Q10) substantially inhibited enteroviruses replication *in vitro* and *in vivo* [9, 20]. In murine pancreatitis model of coxsackievirus B4 infection application of dihydroquercetin, a flavonoid from larch wood, provided decrease of virus titer in pancreatic tissue and restored impaired antioxidant properties in the tissue [18]. In the murine model of IAV, intranasal delivery of MitoTEMPO, resulted in a reduction in airway/lung inflammation, neutrophil infiltration, viral titers as well as morbidity and mortality of animals [30]. Replication of influenza virus was inhibited by the following antioxidants: pyrrolidine dithiocabamate, N-acetyl-l-cysteine, glutathione, nordihydroguaiaretic acid, resveratrol, ascorbic acid, 5,7,4-trihydroxy-8-methoxyflavone, catechins, quercetin 3-rhamnoside, isoquercetin and oligonol [31]. Therefore, use of antioxidants either in monotherapy or in combination with various direct acting antivirals targeting different steps in viral life cycle can be useful for the treatment of viral infection.

Conclusion

Leucoverdazyls are potent inhibitors of group B enteroviruses *in vitro*. Further studies are needed to elucidate their precise mechanisms of action including assessment of its direct impact on intracellular ROS generation, resistant clone selection and mapping of resistance mutations. It may be feasible to test its activity against group A and C enteroviruses and other viruses with (+) ssRNA genome and perform *in vivo* studies on animal models of EVI. We plan to expand the library of leucoverdazyls through targeted chemical modifications in order to disclose its pharmacophore and improve their virus-inhibiting properties. Nevertheless, the results of the study can serve as a basis for future development of novel antivirals to use in monotherapy or in combinational treatment of EVI.

Acknowledgements

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