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Antiviral activity spectrum of phenoxazine nucleoside derivatives

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ABSTRACT

The phenoxazine scaffold is widely used to stabilize nucleic acid duplexes, as a part of fluorescent probes for the study of nucleic acid structure, recognition, and metabolism, etc. Here we present the synthesis of phenoxazine-based nucleoside derivatives and their antiviral activity against a panel of structurally diverse viruses: enveloped DNA herpesviruses varicella zoster virus (VZV) and human cytomegalovirus, enveloped RNA tick-borne encephalitis virus (TBEV), and non-enveloped RNA enteroviruses. Studied compounds were effective against DNA and RNA viruses reproduction in cell culture. 3-(2′-Deoxy-β-D-ribofuranosyl)-1,3-diaza-2-oxophenoxazine proved to be a potent inhibitor of VZV replication with superior activity against wild type than thymidine kinase deficient strains (EC_{50} 0.06 and 10 μM, respectively). This compound did not show cytotoxicity on all the studied cell lines. Several compounds showed promising activity against TBEV (EC_{50} 0.35–0.91 μM), but the activity was accompanied by pronounced cytotoxicity. These compounds may be considered as a good starting point for further structure optimization as antiherpesviral or antiflaviviral compounds.

1. Introduction

Natural nucleosides are unique starting points for design and development of new therapeutic agents because they are involved in all biological processes and are necessary building blocks of DNA and RNA. Modified nucleosides form an important class of compounds that are widely used for specific modulation of processes involving nucleosides. Currently more than 30 nucleoside/nucleotide analogues are approved for the treatment of cancer, viral, parasitic and bacterial infections (De Clercq and Li, 2016; Lapponi et al., 2016; Maffioli et al., 2017).

Nucleoside and acyclic analogues with expanded aromatic systems attract great attention, because of their unique physico-chemical properties and wide range of biological activity. There are several groups of such compounds (Fig. 1): (1) hetero-expanded purine nucleosides **1** and **2** with increased aromaticity and polarizability, inhibiting the replication of hepatitis C virus (Seley-Radtke et al., 2008; Zhang et al., 2008; Wauchope et al., 2012; Chen et al., 2015); (2) etheno compounds, among which the tricyclic derivative of acyclovir **3** (TACV) shows an inhibitory effect on herpes simplex viruses type 1 and 2 (HSV-1 & HSV-2) *in vitro* at concentrations of 2.7–7.6 μM, being less potent than acyclovir, but more selective (Boryski et al., 1988). Another representative of this series, N^2-etheno compound **4** with 3-(1,3-dihydroxy-2-propoxy)-methyl chain of the ganciclovir type, showed antiviral activity in the range of 0.05–0.7 μM for HSV-1 and HSV-2 and also inhibited other herpesviruses, e.g., varicella zoster virus (VZV) and cytomegalovirus (CMV) (Boryski et al., 1991; Golankiewicz et al., 1994); (3) furano[2,3-*d*]pyrimidine and pyrrolo[2,3-*d*]pyrimidine nucleosides, of which furano[2,3-*d*]pyrimidine 2′-deoxyribonucleosides **5a-b** with C8 or C10 alkyl substituent in the 6-position were the most potent and selective inhibitors of VZV to date (McGuigan et al., 1999; De Clercq, 2004; Andrei et al., 2005).

2-Oxo-1,3-diazaphenoxazine is a well known analog of cytosine with expanded heterocyclic ring system that have already been used to stabilize nucleic acid duplexes (Lin and Matteucci, 1998; Holmes et al., 2003; Ortega et al., 2007; Brown et al., 2014; Varizhuk et al., 2017; Kishimoto et al., 2017) and i-motif (Tsvetkov et al., 2018), to recognize

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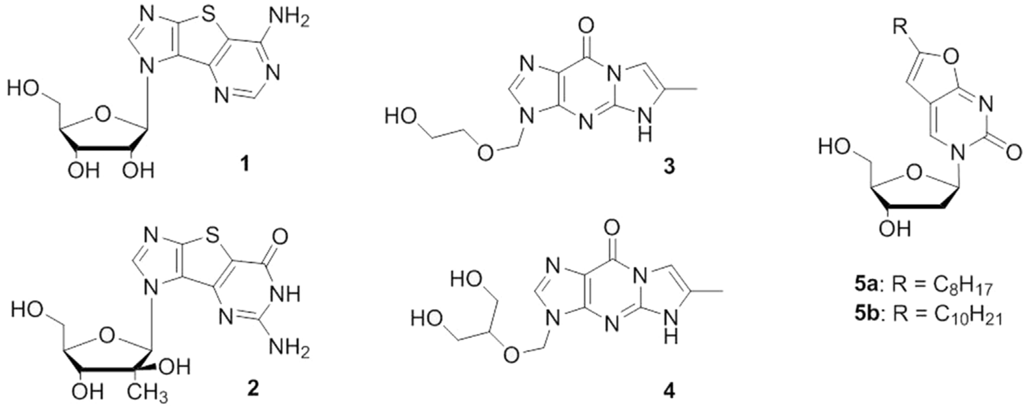


Fig. 1. Nucleoside and acyclic analogues with an expanded aromatic system.

or detect 8-oxo-dG or a damaged nucleotide within oligonucleotides (Nakagawa et al., 2007; Nasr et al., 2009; Li et al., 2010; Taniguchi et al., 2014), and covalently capture 8-nitroguanosine (Fuchi and Sasaki, 2014, 2015) and 8-thioguanosine (Fuchi et al., 2015). In addition, these derivatives are considered as fluorescent probes for the study of nucleic acid structure, recognition, and metabolism (Gardarsson et al., 2011; Rodgers et al., 2014). Given the ready incorporation of phenoxazine nucleosides into a nucleic acid chain (Edwards et al., 2011), they may impair the replication and transcription of cells and viruses, affecting the efficacy of DNA and RNA synthesis by cellular or viral polymerases. Nevertheless, despite a large number of applications in nucleic acid chemistry and molecular biology, antiviral properties of phenoxazine nucleosides have not been properly characterized yet. In the same time, phenoxazines without a sugar moiety have been reported to possess strong antiviral activity against CMV and moderate inhibition of HSV-1 and HSV-2 reproduction (Hayashi et al., 2008), thus showing possibility for further analysis.

Here we report the synthesis of new and known phenoxazine nucleoside derivatives bearing a 2′-deoxyribose or ribose moiety and their antiviral activity against a panel of structurally diverse DNA and RNA viruses, comprising VZV (*Human alphaherpesvirus 3*) wild-type and thymidine kinase deficient (TK^{-}) strains, CMV (*Human betaherpesvirus 5*), TBEV (*Tick-borne encephalitis virus,* a member of the *Flavivirus* genus), enterovirus A71 (EVA71, *Enterovirus A*), coxsackievirus B1 (CVB1, *Enterovirus B*), and poliovirus 1 (PV1, *Enterovirus C*). Herpesviruses are enveloped dsDNA viruses, while TBEV and enteroviruses represent (+)ssRNA viruses, enveloped and non-enveloped, respectively. Flavi- and herpesviruses were suppressed by the compounds of the series, and in most cases antiviral activity was accompanied by a pronounced cytotoxicity, thus limiting direct applicability of the compounds. None of the compounds inhibited the reproduction of enteroviruses. Phenoxazine 2′-deoxyriboside **7a** potently inhibited VZV without signs of cytotoxicity on all the studied cell lines and may be considered as a good starting point for the further structure optimization.

2. Materials and methods

2.1. Chemical synthesis

Detailed chemical procedures are reported in the Supplemental information file.

2.2. Biological assays

2.2.1. Cells and viruses

Human embryonic lung (HEL) fibroblasts were purchased from ATCC [HEL 299 (ATCC® CCL-137™).

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

Herpes simplex virus type 1 (HSV-1) strain KOS (ATCC® VR-1493™), thymidine kinase-deficient (TK^{-}) HSV-1 KOS strain resistant to ACV (ACV^{r}) was selected *in vitro* under increasing concentrations of ACV, herpes simplex virus type 2 (HSV-2) strain G (ATCC® VR-734™); varicella-zoster virus (VZV) strain OKA (ATCC VR-795), TK^{-} VZV strain 07-1 (kindly provided by Shiro Shigeta, Fukushima Medical Center, Japan; human cytomegalovirus (CMV) strains AD-169 (ATCC® VR-538™) and Davis (ATCC® VR-807™) were used at the Rega Institute for Medical Research.

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

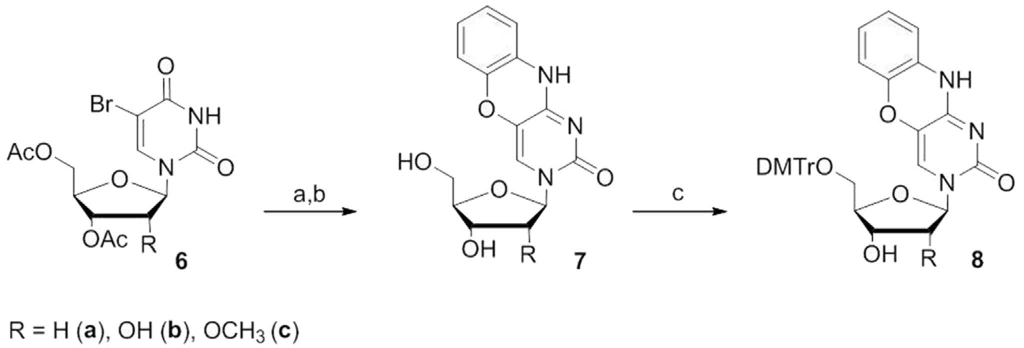
Enterovirus A71 isolate 46973 (GenBank accession no. KJ645808) was isolated from patient with acute flaccid paralysis in 2013 in Russia. *Enterovirus B* Coxsackievirus B1 isolate 48461 was isolated from patient with enteroviral meningitis in 2013 in Russia. *Enterovirus C* reference vaccine strain Sabin 1 of poliovirus type 1 (GenBank access no. V01150) originated from NIBSC (UK).

2.2.2. Cell toxicity evaluation and cytostatic assay in HEL cells

Cytotoxicity of the tested compounds was expressed as the minimum cytotoxic concentration (MCC) or the compound concentration that caused a microscopically detectable alteration of cell morphology. Alternatively, the cytostatic activity of the test compounds was measured based on inhibition of cell growth. HEL cells were seeded at a rate of 5×10^3 cells/well into 96-well plates and allowed to proliferate for 24 h. Then, medium containing different concentrations of the test compounds starting at 100 μM was added. After 3 days of incubation at 37 °C, the cell number was determined with a Coulter counter. The cytostatic concentration was calculated as the CC$_{50}$ (compound concentration required to reduce cell proliferation by 50%).

2.2.3. Cell toxicity assay in PEK cells

A cytotoxicity test in PEK cells was performed as described previously (Orlov et al., 2016). Stock solutions of the compounds in DMSO (concentration of approximately 5–20 mM) were serially diluted twofold in medium 199 in Earle solution (FSBSI “Chumakov FSC R&D IBP RAS”, Russia) to obtain final concentrations (stock/100 μM). Equal volumes of compound dilutions were added in two replicates to the cell monolayers. Control cells were treated with corresponding sequential concentrations of DMSO in two replicates. After incubation at 37 °C on days 1 and 7, cells morphology and vitality was assessed via microscope. CC$_{50}$ was calculated according to the Karber method (Karber, 1931).



Scheme 1. The preparation of the 5′-OH and 5′-O-DMTr phenoxazine derivatives. Reagents and conditions: (a) PPh_3, CCl_4, CH_2Cl_2, reflux; then 2-aminophenol, DIPEA, rt; (b) DIPEA, C_2H_5OH, reflux, then aq NH_3, rt; (c) DMTr-Cl, Py, rt.

2.2.4. Cell toxicity assay in RD cells

Two-fold dilutions of compounds (concentration 5–20 mM) were prepared in Eagle minimal essential medium with doubled amino acids and vitamins (2 × EMEM, FSBSI “Chumakov FSC R&D IBP RAS”, Russia) to obtain final concentrations starting from approx. stock/24 μM. Equal volumes of compound dilutions were added in four replicates to the 96-well plates and covered with RD cell suspension (approx. 10^5 cells per well) in 2 × EMEM with 5% FBS (Invitrogen, South America). Control cells were treated with the same sequential concentrations of DMSO, as in compound dilutions, in four replicates. After incubation at 37 °C on days 1 and 7, cells morphology and vitality was assessed via microscope. CC_{50} values were calculated according to the Karber method (Karber, 1931).

2.2.5. Herpesviruses cytopathicity or plaque reduction test

Confluent HEL cell cultures in 96-well plates were inoculated with 100 $CCID_{50}$ of virus ($CCID_{50}$ is a virus dose to infect 50% of the cell cultures) (CMV) or with 20 plaque forming units (PFU) (VZV). Following a 2 h adsorption period, viral inoculum was removed and the cell cultures were incubated in the presence of varying concentrations of the test compounds starting at 100 μM. Viral cytopathicity (CMV) or plaque formation (VZV) was recorded as soon as it reached completion in the control virus-infected cell cultures that were not treated with the test compounds. Antiviral activity was expressed as the EC_{50} [compound concentration required to reduce virus-induced cytopathicity (CMV) or viral plaque formation (VZV) by 50%].

2.2.6. TBEV plaque reduction test

Plaque reduction test was performed as previously described (Orlov et al., 2016). In brief, stock solutions of the compounds in DMSO were diluted in medium 199 in Earle solution (FSBSI “Chumakov FSC R&D IBP RAS”, Russia) and added to the cells simultaneously with the virus (20–40 PFU/well) at final concentration of approximately 50–200 μM in each well in 8 replicates. Virus with corresponding DMSO dilution was used as a control. Cells were incubated at 37 °C for 1 h for infectious virus adsorption. Then, each well was overlaid with 1 mL of 1.26% methylcellulose (Sigma) containing 2% FBS (Invitrogen, South America). After incubation at 37 °C for 6 days, the cells were fixed with 96% ethanol. Plaques were stained with 0.4% gentian violet and counted. EC_{50} were calculated according to the Reed-and-Meunch method (Reed and Muench, 1938).

2.2.7. Enteroviruses cytopathic effect inhibition test

Cytopathic effect inhibition test against representatives of *Enterovirus* genus was performed as described previously (Kozlovskaya et al., 2018). In brief, eight 2-fold dilutions of stock solutions of the compounds in 4 replicates were prepared in 2 × EMEM (FSBSI “Chumakov FSC R&D IBP RAS”, Russia) to obtain a final concentration series starting from approx. stock/24 μM. Compound dilutions were mixed with equal volumes of the enterovirus suspension containing 100 $TCID_{50}$ (50% tissue culture infectious dose). Control cells were treated with the same sequential concentrations of DMSO as in compound dilutions. After 1 h incubation at 36.5 °C the RD cell suspension (approx.10^5 cells per well) in 2 × EMEM containing 5% FBS (Invitrogen, South America) was added to experimental mixtures. Each experiment contained virus dose titration in the inoculate to assure the acceptable dose-range. After a 5-day incubation at 37 °C, cytopathic effect (CPE) was visually accessed via microscope. EC_{50} values were calculated according to the Karber method (Karber, 1931).

3. Results

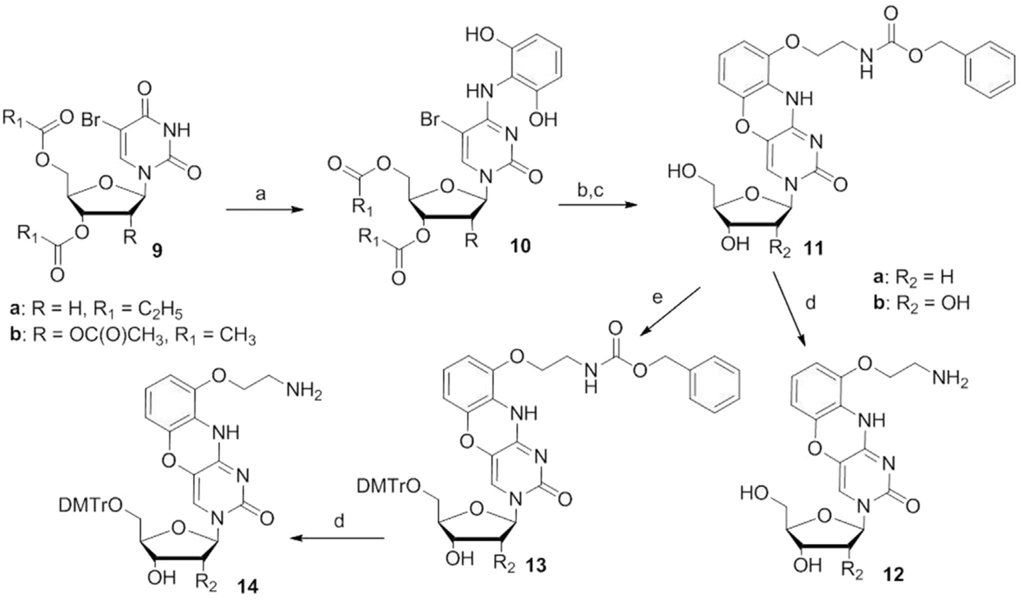
3.1. Chemistry

Twelve of 18 tested compounds were synthesized earlier and described in scientific or patent literature. Of these known derivatives, compounds **7a**, **8a**, and **11a** were prepared by a method developed by Matteucci group (Lin et al., 1995; Lin and Matteucci, 1998). The structures of **7b, 11b**, and **12b** were covered by patents without synthesis details (Manoharan and Rajeev, 2009a, 2009b; Manoharan, 2009; Manoharan et al., 2010). The synthesis of **7c**, **8b-c**, and **13b** was described in patents (Manoharan and Rajeev, 2009a, 2009b; Manoharan, 2009; Manoharan et al., 2010; Lin and Matteucci, 1997). Compound **12a** was an intermediate in the synthesis of G-clamp H-phosphonate/phosphoramidite, used without isolation and characterization (Lin and Matteucci, 1998; Ortega et al., 2007), and was also described without synthetic details in patents (Lin and Matteucci, 1997; Bodepudi et al., 2016). Finally, the preparation of 1,3-diaza-2-oxophenoxazine **17** was described earlier (Edwards et al., 2011). Compounds **10b, 13a, 14a-b, 15**, and **16** were newly synthesized.

In general, a well-developed methodology with small changes was followed for the synthesis (Lin et al., 1995; Lin and Matteucci, 1998; Ortega et al., 2007). The preparation of the 5′-OH and 5′-O-DMTr phenoxazine nucleosides **7a-c** and **8a-c** starting from corresponding 5-bromouracil derivatives **6a-c** (Scheme 1) is given in full detail in the Supplementary Material.

G-clamp-based derivatives **12a-b** and **14a-b** was prepared as described (Scheme 2, see also Supplementary Material for details).

To prepare new N^{10}-alkylated derivatives, **8a** was alkylated with iodoacetamide in the presence of DBU, yielding **15,** which, in turn, was 5′-O-deprotected for the preparation of **16** (Scheme 3). 1,3-Diaza-2-oxophenoxazine **17** was prepared earlier starting from 5-bromouracil (Edwards et al., 2011). For the synthesis of **17**, an alternative method was developed using glycosidic bond cleavage of **7a** in the presence of $HCl_{aq.}$ (Scheme 3).



Scheme 2. The preparation of the 5′-OH and 5′-O-DMTr G-clamp-based derivatives. Reagents and conditions: (a) PPh_3, CCl_4, CH_2Cl_2, reflux; then 2-aminoresorcinol, DBU, rt; (b) benzyl-N-(2-hydroxyethyl)carbamate, DEAD, PPh_3, CH_2Cl_2, rt; (c) DIPEA, C_2H_5OH, reflux, then aq NH_3, rt; (d) H_2/Pd, CH_3OH, rt; (e) DMTr-Cl, Py, rt.

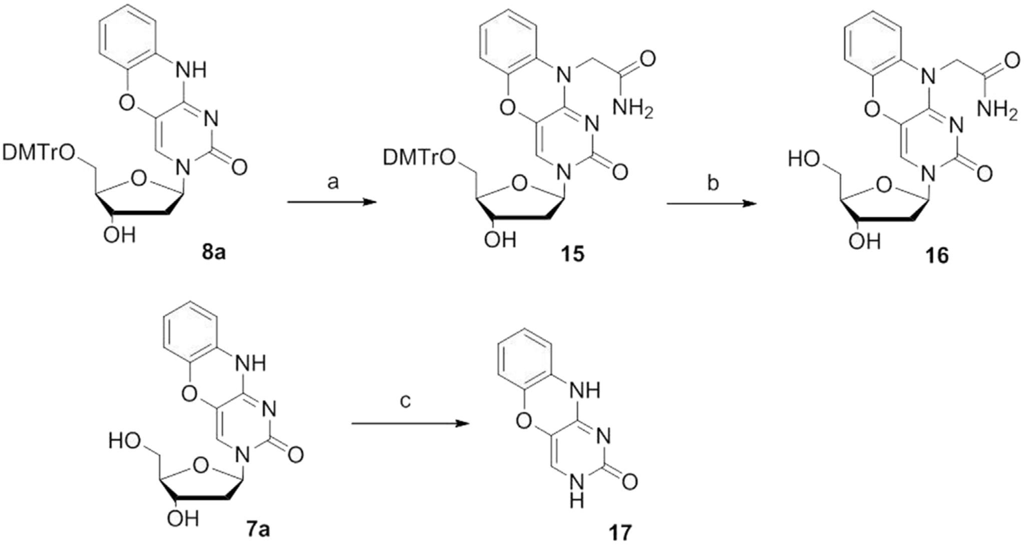
3.2. Biology

All synthesized final compounds together with some intermediates (Fig. 2) were tested for their antiviral activities against the human pathogenic VZV, CMV, TBEV and enteroviruses of A, B, and C species.

Inhibition of dsDNA herpesviruses was measured by viral cytopathic effect (CPE) reduction assay (CMV) or plaque reduction assay (VZV) in human embryonic lung (HEL) cell monolayers. Wild type [OKA (TK^+)] and thymidine kinase deficient [07-1 (TK^-)] VZV strains, and AD-169 and Davis CMV strains were used. Antiviral activity is expressed as the 50% effective concentration (EC_{50}) or concentration required to reduce viral CPE (CMV) or viral plaque formation (VZV) by 50%. Cytotoxicity in the same cell line was assessed by two parameters: minimal cytotoxic concentration (MCC) that caused a microscopically detectable alteration of cell morphology in confluent monolayers and by cytostatic activity, i.e. 50% cytostatic concentration (CC_{50}) or concentration required to reduce cell growth by 50% on replicating growing cells. The results are given in Table 1.

Compounds **7a, 8a, 8b, 12a, 14a, 15** showed activity against VZV. Compound **7a** emerged as the most active one against VZV [OKA (TK^+)] strain $EC_{50} = 0.06\,\mu M$], but it was about 170 times less active against the 07-1 (TK^-) strain. Compared to the reference anti-VZV drugs, **7a** was 42-fold more active than acyclovir and about 3-fold less active than brivudine against the OKA strain and similar to the reference drug, compound **7a** had decreased activity against TK deficient VZV. Interestingly, **7a** was not cytotoxic or cytostatic for HEL cells at the maximum concentration tested (i.e. 100 μM), resulting in selectivity indices (ratio CC_{50}/EC_{50}) of 1667 (OKA strain) and 10 (07-1 strain).

Compounds **8a, 8b, 12a, 14a, 15** showed anti-VZV activity in the micromolar range and proved equally active against VZV TK^+ and TK^- strains, their EC_{50}s being comparable to the ones of the reference compound acyclovir against the OKA strain. In contrast to **7a**, compounds **8a, 8b, 12a, 14a, 15** proved cytostatic for HEL cells (CC_{50} 9.7–37 μM) and thus poorly selective. These compounds were not only



Scheme 3. The preparation of the N^{10}-alkylated derivatives and 1,3-diaza-2-oxophenoxazine. Reagents and conditions: (a) iodoacetamide, DBU, CH_2Cl_2, rt; (b) acetic acid/H_2O, 50 °C; (c) conc. HCl/H_2O, 60 °C.

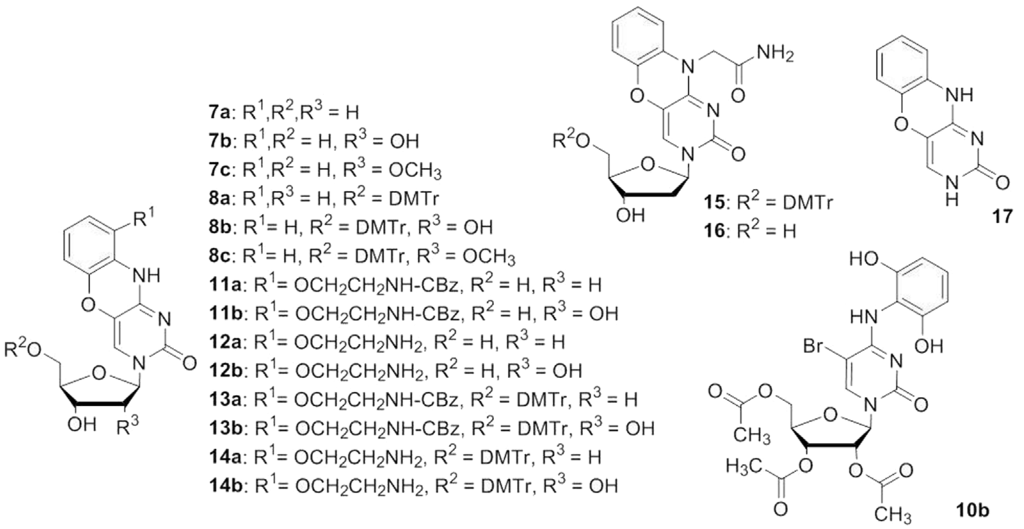


Fig. 2. Phenoxazine derivatives tested for their antiviral activities against the human pathogenic VZV, CMV, TBEV and enteroviruses of A, B, and C species.

Table 1
Anti-herpesvirus activity and corresponding cytotoxicity of phenoxazine nucleosides.

Cmpd #	VZV EC_{50} (μM)		CMV EC_{50} (μM)		Human embryonic lung (HEL) cells Cytotoxicity (μM)	
	OKA strain (TK+)	07-1 strain (TK−)	AD-169 strain	Davis strain	Cell morphology (MCC)	Cell growth (CC_{50})
7a	0.06 ± 0.01	10 ± 5	n/a[a]	n/a	> 100	> 100
7b	n/a	n/a	n/a	n/a	> 100	ND[b]
7c [c]	55	47	n/a	49	> 100	ND
8a	1.5 ± 0.3	1.2 ± 0.5	4	4	20	12.2 ± 0.8
8b	2.7 ± 1.3	1.8	n/a	n/a	9 ± 5	9.7 ± 1.5
8c [c]	n/a	n/a	n/a	2.9	20	ND
10b	n/a	n/a	n/a	n/a	> 100	ND
11b	n/a	n/a	n/a	n/a	> 100	ND
12a [c]	2.23	1.46	n/a	n/a	4	ND
12b [c]	n/a	3.57	n/a	2.19	20	ND
13b [c]	n/a	n/a	n/a	10.9	60 ± 40	ND
14a	1.7 ± 0.1	n/a	n/a	1.27	9 ± 5	14.4 ± 0.7
14b	n/a	n/a	n/a	n/a	4	ND
15 [c]	8.94	4.63	n/a	4	47 ± 27	37 ± 5
16	n/a	n/a	n/a	n/a	> 100	ND
17 [c]	67	n/a	n/a	n/a	> 100	ND
Reference drugs	acyclovir	acyclovir	ganciclovir	ganciclovir	> 350	> 440
	2.5 ± 0.4	39 ± 9	5.99	1.06		
	brivudin 0.022 ± 0.008	brivudin	cidofovir	cidofovir	> 300	> 300
		1.9 ± 0.5	0.51	0.3		

EC_{50} – Effective concentration required to reduce virus plaque formation by 50% (20 PFU VZV) or viral CPE (CMV).
MCC – Minimal cytotoxic concentration that causes a microscopically detectable alteration of cell morphology.
CC_{50} – Cytotoxic concentration required to reduce cell growth by 50%.
[a] n/a – Not active against the respective virus at non-toxic concentrations.
[b] ND – Not determined.
[c] Experiments for low active compounds were performed once in 2 replicates.

cytostatic, but they were also able to alter cell morphology, when studied on confluent HEL cells.

8a, 8c, 12b, 13b, 14a, and **15** showed only minor activity against CMV strain Davis with EC_{50}'s in the micromolar range, comparable to ganciclovir, but less effective than cidofovir. However, none of the compounds (except **8a**) showed inhibitory effect against CMV strain AD-169 in the studied range of concentrations. Considering their cytostatic and cytotoxic effects on HEL cells, and the lack of activity for the AD-169 CMV strain, **8a, 8c, 12b, 13b, 14a, 15** cannot be considered as CMV leads. Additionally, none of the compounds showed activity when tested against HSV-1 and HSV-2 by CPE reduction assay in HEL cells.

Inhibition of reproduction of (+)ssRNA viruses was measured using 50% plaque reduction assay for TBEV in porcine embryo kidney (PEK) cells monolayer and 50% cytopathic effect inhibition assay for enteroviruses in growing human rhabdomyosarcoma (RD) cells. Acute and chronic cytotoxicity in the same cell lines were also assessed as cytotoxic concentration (CC_{50}) required to cause a microscopically detectable alteration of cell morphology in 50% cells in confluent PEK cells and cytotoxic concentration (CC_{50}) required to reduce cell growth by 50% on replicating growing RD cells. The results are given in Table 2. None of the tested compounds inhibited the reproduction of enteroviruses, while most of them were substantially toxic for growing RD cells, especially when the cumulative effect was estimated as chronic cytotoxicity. On the other hand, the compounds did not show signs of acute toxicity on PEK cells, but chronic toxicity was pronounced as well for series **8** and **12**. Potent inhibition of TBEV reproduction was shown by DMTr protected molecules (EC_{50}

Table 2
Inhibition of (+)ssRNA viruses' reproduction by phenoxazine nucleosides and corresponding cytotoxicity.

Cmpd #	TBEV EC_{50} (μM)	Enteroviruses EC_{50} (μM)			PEK cells CC_{50} (μM)		RD cells CC_{50} (μM)	
	Absettarov strain	EVA71	CVB1	PV1	24 h	7 d	24 h	7 d
7a	> 25	n/a[a]	n/a	n/a	> 100	> 100	> 100	> 100
7b	> 25	n/a	n/a	n/a	> 100	> 100	> 100	> 100
7c	47 ± 1	n/a	n/a	n/a	> 100	> 100	> 100	> 100
8a	3.4 ± 1.4	n/a	n/a	n/a	23 ± 14	< 12.5	4.8 ± 0.9	4.8 ± 0.9
8b	0.9 ± 0.3	n/a	n/a	n/a	> 100	< 12.5	10 ± 2	4.8 ± 0.9
8c	1.44 ± 0.01	n/a	n/a	n/a	> 50	28 ± 11	10 ± 2	4.8 ± 0.9
10b	> 75	n/a	n/a	n/a	ND[b]	ND	77 ± 15	19 ± 4
11a	n/a	ND	ND	ND	> 50	> 50	ND	ND
11b	n/a	n/a	n/a	n/a	> 80	> 80	> 100	> 100
12a	12 ± 1	n/a	n/a	n/a	> 100	< 12.5	4.8 ± 0.9	1.2 ± 0.2
12b	14.1 ± 1.9	n/a	n/a	n/a	> 100	< 16	10 ± 2	3.4 ± 0.7
13a	0.7 ± 0.5	ND	ND	ND	> 50	> 50	ND	ND
13b	0.4 ± 0.3	n/a	n/a	n/a	> 40	> 40	19 ± 4	16 ± 3
14a	0.9 ± 0.3	n/a	n/a	n/a	> 50	32 ± 11	10 ± 2	9.6 ± 1.9
14b	2.40 ± 0.02	n/a	n/a	n/a	> 60	35 ± 12	6.8 ± 1.3	8.1 ± 1.6
15	2.0 ± 0.5	n/a	n/a	n/a	> 50	> 50	10 ± 2	10 ± 2
16	n/a	n/a	n/a	n/a	121 ± 43	> 90	> 100	> 100
17	107.9 ± 0.4	n/a	n/a	n/a	> 180	ND	> 100	> 100
Reference compounds	N^6-(9-Anthrylmethyl)-adenosine	N^6-Benzyladenosine			N^6-(9-Anthrylmethyl)-adenosine		N^6-Benzyladenosine	
	7.8 ± 0.4	2.5 ± 0.5	8 ± 3	10 ± 1	> 50	> 50	10 ± 2	10 ± 2

EC_{50} – Effective concentration required to reduce virus plaque formation by 50% (20–40 PFU TBEV) or to reduce cytopathic effect by 50% (enteroviruses).
CC_{50} – Cytotoxic concentration required to reduce cell viability by 50.
[a] n/a – Not active against respective virus at non-toxic concentrations.
[b] ND – Not determined.

0.35–10.7 μM).

The compounds have also been evaluated against other RNA viruses: enveloped (−)ssRNA vesicular stomatitis virus (family *Rhabdoviridae*) and respiratory syncytial virus (family *Pneumorviridae*) (in HeLa cells), non-enveloped dsRNA reovirus-1 (family *Reoviridae*), enveloped (+)ssRNA Sindbis virus (family *Togaviridae*) and yellow fever virus (family *Flaviviridae*), enveloped (−)ssRNA Punta Toro virus (in Vero cells) and enveloped (−)ssRNA influenza virus A and influenza virus B (in MDCK cell) by CPE reduction assay, but no antiviral activity was found at the highest compound concentration (100 μM) studied (Głowacka et al., 2014).

4. Discussion

The phenoxazine scaffold is widely used to stabilize nucleic acid duplexes, as potential fluorescent probes for the study of nucleic acid structure, recognition, and metabolism,etc. Here we presented the synthesis of phenoxazine-based nucleoside derivatives and their antiviral activity against a panel of structurally diverse human-pathogenic viruses VZV, CMV, TBEV and enteroviruses of A, B, and C species. Exploration of structure-activity and structure-toxicity relationships for phenoxazine derivatives employed three variation points: pattern of phenoxazine moiety modification (**7**, **8** vs. **11**, **13** vs. **12**, **14** vs. **15**, **16**), substitution of pentose 2′ position (**a** vs. **b** vs. **c**), and 5′-*O*-dimethoxytritylation of pentose (**7**, **11**, **12**, **16** vs. **8**, **13**, **14**, **15**).

Different susceptibility patterns were observed for DNA and RNA viruses, depending on the pentose hydroxylation state. Deoxyribose derivatives tend to inhibit multiplication of dsDNA viruses VZV and CMV more consistently compared with ribose derivatives. Unsubstituted compound **7a** showed the most potent anti-VZV activity in the series, whereas its ribose analogues **7b, c** were much less active. Most of the active compounds showed higher inhibition rate and range against VZV variants, than against CMV ones. The most potent VZV replication inhibitor **7a** is the deoxyribose derivative of unsubstituted phenoxazine that showed inhibition in micromolar range for both TK^+ and TK^- strains. This compound selectively inhibits the replication of the VZV OKA strain, and does not cause toxic or cytostatic effects in any of the studied cell lines under the studied conditions. Anti-VZV and anti-CMV activity of less specific compounds **8a** and **12a** is accompanied by substantial toxicity against all the studied cell lines, thus restricting their further development.

Substitution in the phenoxazine core was only partially sampled in our series. Introduction of 2-aminoethoxy moiety in position 9 (series **12**), that is responsible for additional hydrogen bonding with complementary guanine within duplexes, slightly decreased anti-VZV activity and introduced moderate anti-TBEV activity, but was associated with increased cytotoxicity. Protection of 2-aminoethoxy group with Cbz group eliminated the anti-herpesvirus activity. N^{10}-(2-Amino-2-oxoethyl)ation of phenoxazine also eliminated the antiviral activity of unsubstituted deoxyribosides. 2′-*O*-Methylation of ribose (**7c**) deteriorated the potency of the compound, but did not induce the cytotoxicity.

5′-*O*-Dimethoxytritylation rendered all of the compounds consistently active against TBEV replication *in vitro*, no matter the hydroxylation state of position 2′. This observation is in line with previous findings (Angusti et al., 2008; De Burghgraeve et al., 2013; Chatelain et al., 2013; Saudi et al., 2014; Vernekar et al., 2015; McGuigan et al., 2016; Orlov et al., 2017) and adds DMTr groups to the arsenal of hydrophobic substituents leading to anti-flaviviral activity of 5′-*O*-substituted nucleosides. Notably, VZV and CMV were also occasionally inhibited by these derivatives, making this substitution pattern efficient not only for ssRNA flaviviruses, but also for dsDNA herpesviruses. Although, activity was accompanied by pronounced toxicity. Nevertheless, compounds from series **13–15** were not toxic for PEK cells, and thus could be further pursued as flavivirus replication inhibitors.

The reason for the potent antiviral effect of 5′-O-substituted nucleosides is not completely understood (Eyer et al., 2018). Nevertheless, certain hypotheses may be drawn based on literature analysis. 5′-O-Tritylated nucleosides are efficient cell permeable inhibitors of host enzyme thymidine phosphorylase (Liekens et al., 2006). The role of this enzyme in the pathogenesis of flaviviruses and enteroviruses was never shown. Nevertheless, trytilated uridines inhibit flavivirus RNA-dependent RNA polymerase (De Burghgraeve et al., 2013). Besides, the thymidine phosphorylase plays an important role in the metabolism of nucleoside antivirals used for treatment of herpesviral infections (Guenther et al., 2002). Thus, one may suggest simultaneous action of the tritylated nucleosides onto the host and virus enzymes as the reason for improved antiviral activity compared to unsubstituted nucleoside analogues, which may be also less cell-permeable.

Another effect worth noticing is the increase in cytotoxicity of most of the compounds when tested on growing cells. The effect is expected, as any stabilization of DNA or RNA-DNA duplexes would affect most strikingly highly metabolically active growing and replicating cells. Stabilization of nucleic acid duplexes would make it harder for cellular machinery to unwind or separate the strands that would decrease DNA replication and transcription. During the experiments we observed that cells grown in confluent monolayers, where cellular growth was blocked (contact inhibition), were less affected and showed higher CC_{50} or MCC values in comparison with cytostatic effect of the same compound studied in growing cells: MCC values were substantially lower than CC_{50} values for HEL cells, and CC_{50} values for confluent PEK cells were higher for than the ones obtained on growing RD cells. Therefore, one can propose that fast replicating viruses, like enteroviruses, would be affected by nucleic acid stabilizing agents. Nevertheless, all studied compounds were inactive against three *Enterovirus* species.

5. Conclusion

The synthesis of phenoxazine-based nucleoside derivatives and their antiviral activity against a panel of structurally and replicatively diverse viruses with DNA and RNA genomes, consisting of varicella zoster virus (VZV), human cytomegalovirus (CMV), tick-borne encephalitis virus (TBEV), and a selection of enteroviruses was presented. 3-(2′-Deoxy-β-D-ribofuranosyl)-1,3-diaza-2-oxophenoxazine **7a** proved to be a potent inhibitor of VZV replication with higher activity against wild type than thymidine kinase deficient strains (EC_{50} 0.06 and 10 μM, respectively). This compound did not show cytotoxicity on all the studied cell lines and may be considered as a good starting point for the further structure optimization. Several compounds showed promising activity against TBEV (EC_{50} 0.35–0.91 μM), but the activity was accompanied with pronounced cytotoxicity. Nevertheless, compounds from similar series were not toxic for PEK cells, and thus could be further pursued as flavivirus replication inhibitors.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.01.010.

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