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FULL PAPER

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Probing chemical space of tick-borne encephalitis virus reproduction inhibitors with organoselenium compounds

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Abstract

Tick-borne encephalitis virus (TBEV), a member of the genus *Flavivirus*, is the leading cause of arboviral neuroinfections in Europe. Only a few classes of the nucleoside and non-nucleoside inhibitors were investigated against TBEV reproduction. Paving the way to previously unexplored areas of anti-TBEV chemical space, we assessed the inhibition of TBEV reproduction in the plaque reduction assay by various compounds derived from cyanothioacetamide and cyanoselenoacetamide. Compounds from seven classes, including 4-(alkylthio)-2-aryl-3-azaspiro[5.5]undec-4-ene-1,1,5-tricarbonitriles, 3-arylamino-2-(selenazol-2-yl)acrylonitriles, ethyl 6-(alkylseleno)-5-cyano-2-oxo-1,2-dihydropyridine-3-carboxylates, 6-(alkylseleno)-2-oxo-1,4,5,6-tetrahydropyridine-3-carbonitriles, 2-(alkylseleno)-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carbonitriles, 8-selenoxo-3,5,7,11-tetraazatricyclo[7.3.1.0^{2,7}]tridec-2-ene-1,9-dicarbonitriles, and selenolo[2,3-*b*]quinolines, inhibited TBEV reproduction with EC_{50} values in the micromolar range while showing moderate cytotoxicity and no inhibition of enterovirus reproduction. Thus, new scaffolds with promising anti-TBEV activity were found.

KEYWORDS
antivirals, *Flavivirus*, organoselenium compounds, tick-borne encephalitis virus

1 | INTRODUCTION

The viruses belonging to genus *Flavivirus* (family *Flaviviridae*), such as dengue virus (DENV), yellow fever virus (YFV), West Nile virus (WNV), Zika virus (ZIKV), tick-borne encephalitis virus (TBEV), etc., pose a serious threat to public health. They are capable of causing a variety of human diseases: from uncomplicated fevers to meningitis and encephalitis.[1] Only the infections caused by Japanese encephalitis virus (JEV), YFV, and TBEV are vaccine-preventable.[2] There is no specific therapy approved for infections caused by flaviviruses, and only supportive treatment is usually used.[2]

Flaviviruses are transmitted by arthropods: mosquitoes or ticks. Mosquito-borne flaviviruses are more common for South and South-East Asia, South and Central America, Africa, while the tick-borne ones are distributed in Europe, Siberia, and North America. One of the most medically important flaviviruses for Europe is TBEV, causing about 10000 cases of tick-borne encephalitis annually.[3] TBEV vaccination coverage is considered to be not quite sufficient.[4] Thus, the search for the small molecule therapeutics with anti-TBEV activity is necessary.

The TBEV virion is a spherical particle (diameter 50 nm) bearing 11 kb (+)ssRNA encapsulated in a lipid-protein shell.[1] The viral RNA

genome contains a single open reading frame and encodes three structural and seven non-structural proteins. Structural proteins are responsible for the virion structure and stability, virus entry, virion assembly and exit. Several studies showed the possibility to suppress TBEV reproduction in cells via interfering with the entry process.[5–8] Non-structural proteins mainly orchestrate the process of virus replication. One of the most promising targets for anti-TBEV drug design among the non-structural proteins is NS5 protein containing RNA-dependent RNA polymerase (RdRp) and methyltransferase (MTase) domains. Numerous nucleoside inhibitors that suppress TBEV replication in cell-based assays may interact with NS5.[9–11] Nonetheless, the chemical diversity of compounds tested for anti-TBEV activity is relatively low as compared to DENV and well-studied human immunodeficiency virus (HIV), or hepatitis C virus (HCV), resulting in the need for new scaffolds.

Antiviral activity is usually assessed for compounds composed of major biogenic elements: C, N, O, S, and P. The exploration of other elements' chemistry is a viable strategy for the discovery of new antiviral classes. One of the bio-essential elements is selenium, which plays an important role in several biological processes, including protection of cells from oxidative stress and deiodination of thyroid hormones.[12] Selenium deficiency is also associated with virulence and pathogenesis of a number of RNA viruses.[13] Selenium atom is often considered as a good bioisosteric replacement of sulfur and oxygen atoms in organic molecules.[14] Its radius and electronegativity are close to those of the sulfur atom.[14] The biological activity spectrum of organoselenium compounds is wide: from compounds with antioxidative properties to anti-inflammatory, antiproliferative, antimicrobial, and antiviral activities. For detailed reviews of biological activity of organoselenium compounds the reader is referred to Refs. [15,16].

The early reports about antiviral activity of organoselenium compounds encompass mainly the investigation of Se-containing nucleoside analogs (Figure 1). Selenazofurin, the analog of broad-spectrum antiviral drug ribavirin, was studied against multiple viruses and showed activity comparable to ribavirin in cell-based assays.[17–21] Guanosine analog 7-methyl-8-selenoguanosine (7-Me-8-SeG) protected 58% of mice from death caused by (+)ssRNA Semliki Forest virus infection.[22] Analog of well-studied nucleoside inhibitor of HIV-1 and hepatitis B virus (HBV) (viruses with reverse transcription in the life cycle) replication β-(±)-l-(2-hydroxymethyl-1,3-oxaselenolan-5-yl)-5-fluorocytosine (β-Se-FddC) was shown to be active against these viruses in micromolar range.[23,24] On the contrary, 2′,3′-dideoxy-4′-selenonucleosides, e.g., β-4-Se-ddU, were completely inactive against HIV-1 up to 100 μM concentration.[25] Acyclic pyrimidine nucleosides (e.g., 6-PhSe-U) showed selective antiviral activity against HIV-1 and HIV-2 in cells but did not inhibit HIV-1 reverse transcriptase.[26] Non-nucleoside organoselenium antivirals include 1,2,3-selenadiazole thioacetanilides (STAs) showing anti-HIV activity probably realized through reverse transcriptase.[27] The mechanism of action of ebselen and its analogs[28–31] and diselenides[28,29] remains to be investigated in detail.

The data on antiviral activity of organoselenium compounds in major free repositories of bioactivity data, PubChem BioAssay[32] and ChEMBL,[33] are scarce. There are 1631 compounds in ChEMBL and 1856 compounds in PubChem BioAssay containing Se atom. This chemical space consists of acyclic Se containing compounds, Se

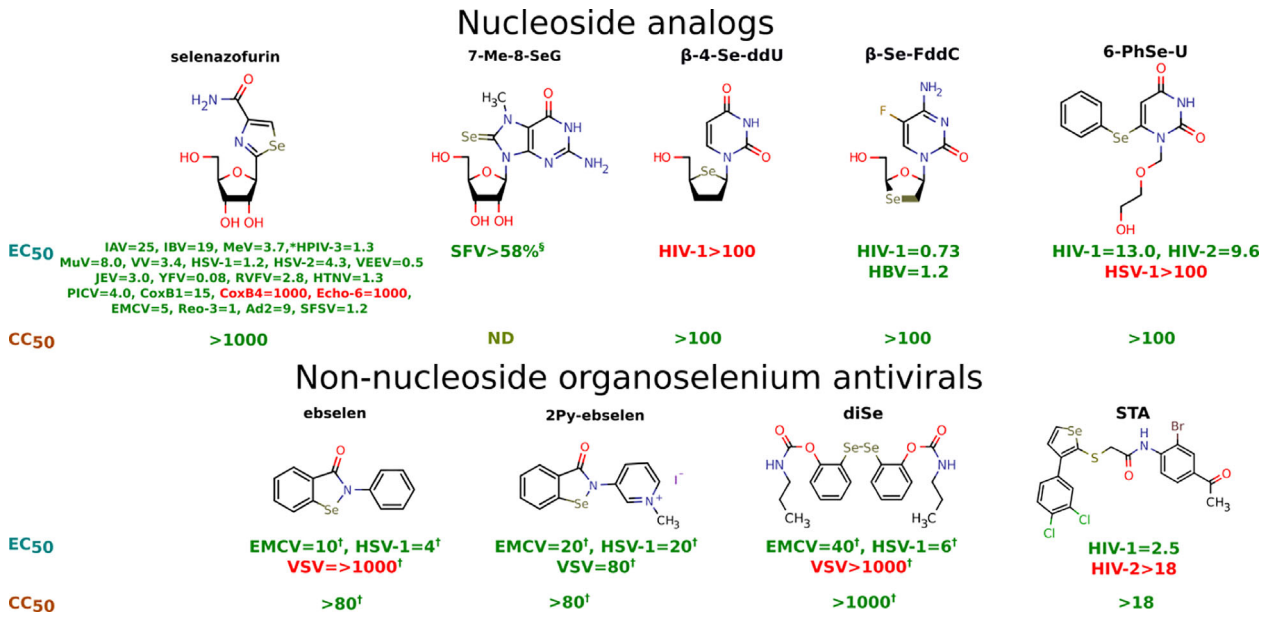


FIGURE 1 Several examples of organoselenium antivirals. EC_{50} and CC_{50} values are given in μM. †EC_{50}, CC_{50} in μg/mL; §% of survived mice. Abbreviations: IAV, influenza A virus; IBV, influenza B virus; MeV, measles virus; HPIV-3, human parainfluenza virus 3; MuV, mumps virus; VV, vaccinia virus; HSV, herpes simplex virus; VEEV, Venezuelan equine encephalitis virus; JEV, Japanese encephalitis virus; RVFV, Rift Valley fever virus; HTNV, Hantaan virus; PICV, Pichinde virus; CoxB1, Coxsackie virus B1; CoxB4, Coxsackie virus B4; Echo-6, echovirus type 6; EMCV, encephalomyocarditis virus; Reo-3, reovirus type 3; Ad2, adenovirus type 2; SFSV, sandfly fever Sicilian virus; SFV, Semliki Forest virus; HBV, hepatitis B virus; VSV, vesicular stomatitis virus

containing heterocycles, diselenides, and nucleoside analogs. New scaffolds investigated in this work enrich the chemical diversity of this space.

In the current paper, we present an investigation of antiviral activity of several organoselenium and organosulfur compound series derived from cyanothioacetamide and cyanoselenoacetamide. We found that compounds from seven series suppressed TBEV reproduction with EC_{50} values in micromolar range and showed moderate toxicity in porcine embryo kidney (PEK) cell line. To assess specificity of the compounds, we tested several of them against enteroviruses belonging to species *Enterovirus A*, *Enterovirus B*, and *Enterovirus C*. The compounds did not inhibit cytopathic effect induced by enteroviruses. We searched for the active compound scaffolds in ChEMBL and PubChem BioAssay to reveal similar compounds possessing antiviral activity realized through known mechanisms. Several close analogs were identified that allowed us to suggest possible mechanisms of action for our compounds.

2 | RESULTS AND DISCUSSION

2.1 | Chemistry

All compounds except **11c** were synthesized by us earlier and characterized in the previous papers. 4-(Alkylthio)-2-aryl-3-azaspiro-[5.5]undec-4-ene-1,1,5-tricarbonitriles **2** were prepared in three steps[34] from cyanothioacetamide **1** (Figure 2). All other compounds were synthesized starting from cyanoselenoacetamide **3**.[35] First, 3-arylamino-2-(selenazol-2-yl)acrylonitriles **4** were prepared by fusing selenoamide **3** with anilines and triethyl orthoformate, followed by the Hantzsch-type cyclization with α-bromoacetophenones.[36] Synthesis of ethyl 2-oxo-1,2-dihydropyridine-3-carboxylates **5** was described in our previous paper.[37] 2-(Alkylseleno)-1,4,5,6-tetrahydropyridine-3,5,5-tricarbonitriles **6** were prepared by a similar procedure to that for compounds **2**.[34] Tetrahydropyridines **7** (R^1 = CN) were prepared by Guareschi-type reaction of 1-(cyanoacetyl)-3,5-dimethylpyrazole[38] with 2-cyanoselenoacrylamides obtained by the reaction of **3** with aldehydes, followed by Se-alkylation.[39] Tetrahydropyridines **7** (X = H) were synthesized according to the procedure described in Ref. [40], starting from Meldrum's acid. Next, 2-(alkylseleno)-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carbonitriles **8** were prepared by the reaction of selenoamide **3** with aromatic aldehydes, cyclohexane-1,3-dione and *N*-methylmorpholine, followed by the treatment with alkyl halides.[41] Pyrimido[4,3-*b*][1,3,5]selenadiazines **9** were synthesized in two steps by the reaction of selenoamide **3** with aromatic aldehydes and a subsequent reaction of the formed 3-aryl-2-cyanoselenoacrylamides with RNH_2 and HCHO.[45,46] Other Mannich-type products, 3,7-diazabicyclo[3.3.1]nonanes **10**, have been synthesized by aminomethylation of triethylammonium 3,5-dicyano-6-oxo-4-(2-thienyl)-1,4,5,6-tetrahydropyridine-2-selenolate[39] with amines and an excess of formalin.[44] The tricyclic Mannich-type compounds **11a**,**b** were prepared by reacting 6-amino-3,5-dicyano-1,4-dihydropyridine-2-selenolates with primary amines and HCHO according to the reported procedures.[45–47] The compound **11c** was prepared in two steps by analogy with these procedures as shown in Scheme 1. Finally, selenolo

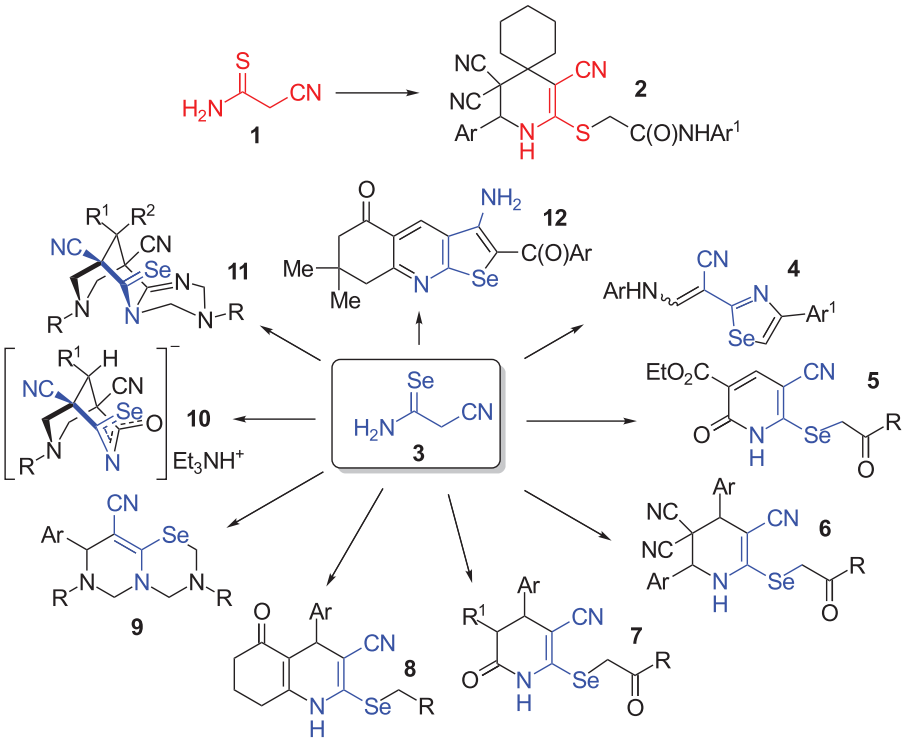
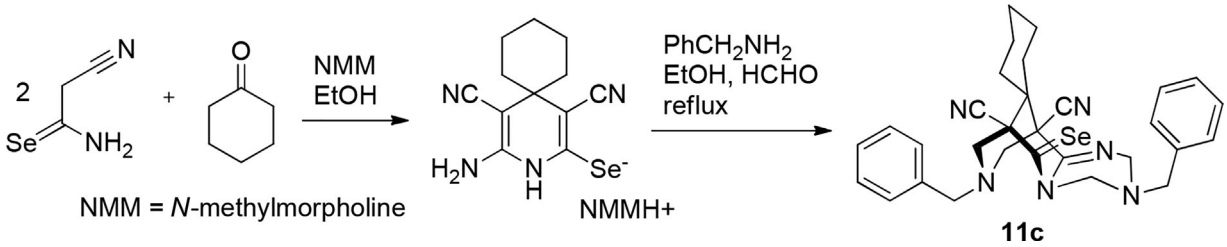


FIGURE 2 Diversity of tested compounds[34–48]



SCHEME 1 Synthesis of compound **11c**

[2,3-*b*]quinolines **12** were prepared in three steps as described elsewhere.[48]

2.2 | Antiviral activity screening

Compounds bearing 10 different scaffolds: 4-(alkylthio)-2-aryl-3-azaspiro[5.5]undec-4-ene-1,1,5-tricarbonitriles **2**, 3-arylamino-2-(selenazol-2-yl)acrylonitriles **4**, ethyl 6-(alkylseleno)-5-cyano-2-oxo-1,2-dihydropyridine-3-carboxylates **5**, 6-(alkylseleno)-2,4-diaryl-1,2,3,4-tetrahydropyridine-3,3,5-tricarbonitriles **6**, 6-(alkylseleno)-2-oxo-1,4,5,6-tetrahydropyridine-3-carbonitriles **7**, 2-(alkylseleno)-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carbonitriles **8**, pyrimido[4,3-*b*][1,3,5]selenadiazines **9**, 3,7-diazabicyclo[3.3.1]nonanes **10**, 8-selenoxo-3,5,7,11-tetraazatricyclo[7.3.1.0^{2,7}]tridec-2-ene-1,9-dicarbonitriles **11**, and selenolo[2,3-*b*]quinolines **12** were screened for cytotoxicity in PEK cells in the preliminary test. All compounds from series **6** and **9**, along with several compounds from other series, were clearly toxic and were not investigated further (all data for them in Table 1 are shown as ND).

Low-toxic compounds from other series were assessed as inhibitors of TBEV reproduction in the plaque reduction test, and their EC_{50} values were determined (Table 1). Compounds were pre-incubated with the virus to take into account the possibility of realizing their activity through interactions with the virion at the stage of virus attachment and entry. The only moderately toxic compound **10b** from series **10** was not active at 50 μM concentration. Seven series thus remained to be further investigated: **2**, **4**, **5**, **7**, **8**, **11**, and **12**. Compounds **2b**,**c**, **4a–c**, **7b**,**g**,**h**, **11a**,**c**, and **12a**,**b** suppressed the reproduction of TBEV in micromolar concentrations with no signs of cytotoxicity at 50 μM.

To assess the specificity of compounds to TBEV, compounds **2c**, **4a–c**, **7h**, **8c**, **11c**, and **12a** were tested for inhibition of cytopathic effect induced by enteroviruses (*Enterovirus A*, *Enterovirus B*, *Enterovirus C*). None of them showed activity at 62.5 μM.

2.3 | Structure–activity relationships and literature analogs

The compounds tested in this study represent one of the few examples of systematic investigation of antiviral activity for organoselenium compounds. To initiate the mechanism of action studies, we started with a thorough analysis of already published studies in the hope that reasonable starting hypotheses may be derived from this amount of data. The diverse chemical nature of the compounds makes it necessary to consider them separately, grouped by scaffolds.

2.3.1 | Scaffold 2

Organosulfur compounds from series **2** were poorly soluble in cell culture medium even at the 50 μM concentration. All of them were non-toxic, but only **2b** and **2c** inhibited TBEV reproduction. Activity decrease of **2a** compared to **2b**,**c** is associated with the replacement of the phenyl at position 2 of tetrahydropyridine core with 2-furyl. A similar organosulfur compound **13** (Figure 3) with a keto group in the corresponding position inhibited nuclear factor (erythroid-derived 2)-like 2 (Nrf2) in a qHTS assay.[49] Nrf2 plays an important role in antiviral and cell death responses to the DENV infection.[50,51] It was shown that DENV activates Nrf2 through PERK under endoplasmic reticulum stress to enhance TNF-α production through transcriptionally upregulating CLEC5A in mononuclear phagocytes.[50] On the other hand, DENV activated antioxidant pathways regulated by Nrf2, contributing to the regulatory control of antiviral and apoptotic responses by maintaining redox homeostasis.[51] Thus, Nrf2 is a host target for anti-flavivirus compound design that is worth being investigated further. Compound **13** was also a hit against *Vaccinia virus* in an HTS campaign (Figure 3),[52] thus supporting the hypothesis of compound activity realized through the interaction with the host targets. 1,4,5,6-Tetrahydropyridine ring was the core of the scaffold of anti-TBEV compound **14** and its analogs discovered previously in our lab (Figure 2).[5]

2.3.2 | Scaffold 4

All compounds bearing scaffold **4** showed the same EC_{50} values of 7–8 μM and no cytotoxicity at 50 μM concentration due to small difference between them, of just one methyl. There was no information available about antiviral activity of similar organoselenium compounds, but sulfur analog **15** showed micromolar activity against HCV in a high-throughput cell-based assay (Figure 3).[53] Further investigation of its mechanism of action was not reported.

2.3.3 | Scaffold 5

Both compounds with scaffold **5** were rather toxic, although **5b** inhibited TBEV reproduction with EC_{50} two-fold lower than CC_{50}.

TABLE 1 Cytotoxicity and anti-TBEV activity of the compounds

	Substituents						
#	R	R^1 or Ar^1	R^2 or Ar	CC_{50} (24 h)	CC_{50} (7 d)	EC_{50}	Ref.[a]
2a	–	4-MeC_6H_4	2-Furyl	>50	>50	>50	[34]
2b	–	4-MeC_6H_4	Ph	>50	>50	8 ± 1	[34]
2c	–	4-Me-3-ClC_6H_3	Ph	>50	>50	4.7 ± 0.3	[34]
4a	–	Ph	Ph	>50	>50	8 ± 1	[36]
4b	–	Ph	4-MeC_6H_4	>50	>50	7 ± 1	[36]
4c	–	4-MeC_6H_4	Ph	>50	>50	7 ± 2	[36]
5a	4-MeC_6H_4	–	–	<50	<50	ND[b]	[37]
5b	4-MeC_6H_4NH	–	–	18	35	9 ± 3	[37]
6a	4-MeC_6H_4NH	–	2-Thienyl	ND	ND	ND	[34]
6b	4-MeC_6H_4	–	2-Furyl	ND	ND	ND	[34]
7a	2-Thienyl	CN	2-Thienyl	<50	<50	ND	[39]
7b	OMe	CN	2-Furyl	>50	>50	9 ± 7	[39]
7c	Ph	CN	2-Furyl	>50	<50	ND	[39]
7d	4-MeC_6H_4	CN	2-Furyl	>50	18	ND	[39]
7e	PhNH	CN	2-Furyl	<50	<50	ND	[39]
7f	4-MeC_6H_4NH	CN	2-Furyl	ND	ND	ND	[39]
7g	OMe	CN	Ph	>50	>50	14 ± 13	[39]
7h	OEt	CN	Ph	>50	>50	14 ± 9	[39]
7i	Ph	CN	Ph	>50	<6.25	3	[39]
7j	4-MeC_6H_4	CN	Ph	76	52	5.34 ± 0.18	[39]
7k	PhNH	CN	Ph	<50	<50	ND	[39]
7l	4-MeC_6H_4NH	CN	Ph	ND	ND	ND	[39]
7m	Ph	CN	2-$MeOC_6H_4$	>50	7	4 ± 3	[39]
7n	4-MeC_6H_4	H	2-FC_6H_4	35	35	>50	[40]
7o	PhNH	H	2-FC_6H_4	>50	>50	>50	[40]
7p	4-MeC_6H_4NH	H	2-FC_6H_4	>50	>50	>50	[40]
8a	H	–	2-Furyl	>50	>50	>50	[41]
8b	Ph	–	2-Furyl	>50	28	24 ± 3	[41]
8c	C(O)Ph	–	2-Furyl	35	31	6 ± 2	[41]
8d	4-Me-3-$ClC_6H_3NHC(O)$	–	2-Furyl	<50	<50	ND	[41]
8e	PhNHC(O)	–	4-MeC_6H_4	>50	~50	11 ± 7	[41]
8f	4-$MeC_6H_4NHC(O)$	–	4-MeC_6H_4	ND	ND	ND	[41]
9a	4-MeC_6H_4	–	2-Thienyl	<50	<50	ND	[42]
9b	4-FC_6H_4	–	2-Thienyl	<50	<50	ND	[43]
9c	$PhCH_2$	–	2-Thienyl	<50	<50	ND	[42]
9d	4-MeC_6H_4	–	Ph	<50	<50	ND	[43]
10a	2-Thienyl	$PhCH_2$	–	ND	ND	ND	[44]
10b	2-Thienyl	4-MeC_6H_4	–	>50	35	>50	[44]
11a	CH_3	H	Ph	>50	>50	3 ± 0	[47]

(Continues)

TABLE 1 (Continued)

	Substituents						
#	R	R^1 or Ar^1	R^2 or Ar	CC_{50} (24 h)	CC_{50} (7 d)	EC_{50}	Ref.[a]
11b	CH_2Ph	H	2-$EtOC_6H_4$	>50	35	4.90 ± 0.26	[47]
11c	CH_2Ph	$R^1 + R^2 = (CH_2)_5$		>50	>50	2.34 ± 0.23	–
12a	–	–	Ph	>50	>50	3 ± 1	[48]
12b	–	–	4-MeC_6H_4	>50	>50	0.436 ± 0.016	[48]

All values in μM.
[a]References to the papers where compounds were described.
[b]ND, not determined.

Such a therapeutic index is not attractive for further development, thus investigation of this scaffold was not performed.

2.3.4 | Scaffold 7

The compounds with scaffold **7** were more toxic with aryl R groups than with the alkyl ones (e.g., **7b** vs. **7c–f**, **7g**,**h** vs. **7i–l**). Similar in shape organosulfur molecule **16** inhibited Nrf2 in the qHTS assay.[49] Thus, the mechanism of action for scaffold **7** compounds may be related to host cell proteins.

2.3.5 | Scaffold 8

These compounds showed a moderate anti-TBEV activity while being quite toxic for PEK cells. Substituents in aromatic ring in the position 4 of 3-cyano-5-oxo-1,4,5,6,7,8-hexahydroquinoline scaffold did not influence the activity significantly. On the contrary, enlargement of R substituents led to the increase of both activity and toxicity. For instance, **8a** was non-toxic and inactive, while **8c** showed micromolar activity and was moderately toxic. Decoration of Ph ring in R led to higher toxicity and did not improve potency of the compounds. To the best of our knowledge, biological activity of similar organoselenium compounds was never studied, but organosulfur analogs were studied in different biological assays. E.g., **8c** analog **17** (Figure 3) inhibited Marburg virus entry in a high-throughput assay.[54] Another compound very similar to **8e**, **18**, was selected in a virtual screening campaign targeting DENV envelope protein E.[55] However, **18** showed no activity against DENV in the plaque reduction assay.

2.3.6 | Scaffold 11

Tricyclic compounds from series **11** represent a totally new class of efficient and moderately toxic anti-TBEV compounds. Such scaffolds were never studied previously in any biological assays. To date, the only known approach to the construction of the tricyclic core of **11**-like 3,5,7,11-tetraazatricyclo[7.3.1.0^{2,7}]tridec-2-enes is based on

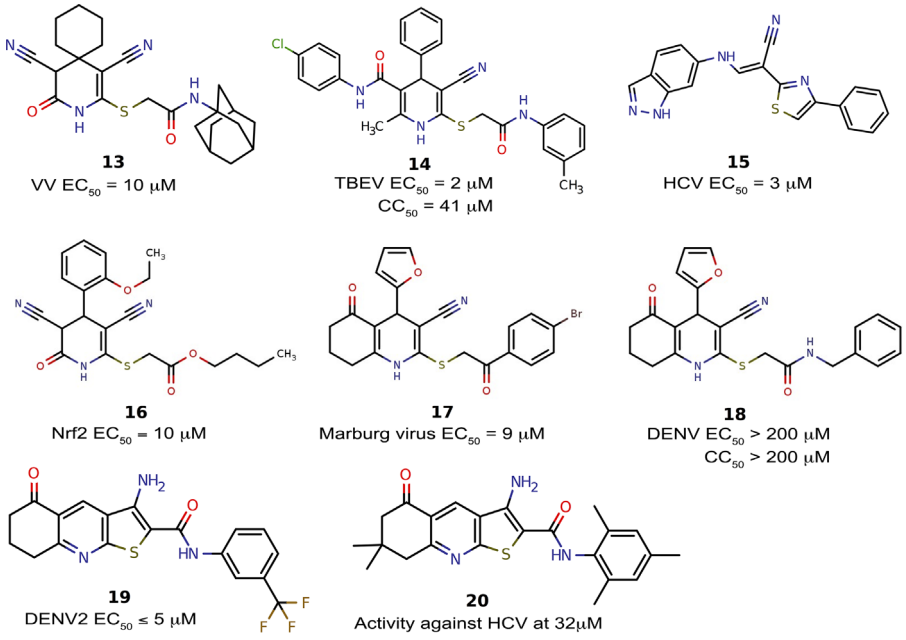


FIGURE 3 Structural analogs of the tested compounds

the one-step protocol including the treatment of C(3),C(5),N(1),C(6) NH_2 polynucleophilic 6-amino-1,4-dihydro- or 6-amino-1,2,3,4-tetrahydropyridine substrates[45–47,56–62] with primary amines and an excess of HCHO. Despite the complexity of the tricyclic structure, the synthetic procedure is simple and efficient, giving rise to 3,5,7,11-tetraazatricyclo[7.3.1.$0^{2,7}$]tridec-2-enes in good yields. The crystal structure of tetraazatricyclo[7.3.1.$0^{2,7}$]tridec-2-enes was determined by X-ray diffraction[46,59–61]; the study revealed that the conformations of 1,3,5-triazine ring and the neighboring piperidine ring are intermediate between a *half-chair* and a *half-boat*, while another piperidine cycle has a *chair* conformation. Thus, the compounds like **11** could be considered as 1,3,5-triazine-fused analogs of the long-known bispidines (3,7-diazabicyclo[3.3.1]-nonanes) with reported biological activity. Further studies are currently underway to determine the range of biological activity of tricycles **11**.

2.3.7 | Scaffold 12

Selenolo[2,3-*b*]quinolines **12a** and **12b** are among the most potent TBEV reproduction inhibitors. Their organosulfur congeners (e.g., **19**) are potent inhibitors of DENV reproduction[63] acting through the interaction with the capsid protein C.[64,65] This protein is a rather conserved one among flaviviruses, so the same mechanism of action may be hypothesized for selenolo[2,3-*b*]quinolines **12**. Similar compound **20** showed the inhibitory activity (at the highest concentration only) against Nrf2 and HCV in HTS campaigns[49,66] (Figure 3). Thus, the mechanism of action of these compounds may be partially related to the interaction with the host cell targets.

3 | CONCLUSIONS

Phenotypic screening of 10 organoselenium and 1 organosulfur compound classes revealed a specific inhibition of TBEV reproduction. Seven classes showed micromolar activity while being moderately cytotoxic. The inhibitory activity of these compound series is probably realized by different mechanisms including inhibition of the virus entry process through acting on the virus envelope proteins, capsid protein or interfering with the host cell targets thus preparing the ground for the development of new anti-TBEV compounds classes. The detailed mechanism of action of these compounds as well as the role of selenium atom will be investigated in the further studies.

4 | EXPERIMENTAL

4.1 | Data analysis

The search for structural analogs was done via the ChEMBL web interface (version 22, November 2016). Substructure search and similarity search with threshold similarity percentage of 70% were used, and retrieved ChEMBL structures were visually analyzed. Compounds containing "Se" element symbol were retrieved from MySQL version of ChEMBL database (version 23, August 2017) and PubChem web interface (accessed in October 2017). Numbers of individual *molregno* and CID entries were calculated.

4.2 | Chemistry

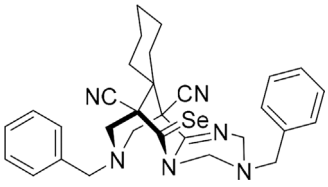
IR spectra were recorded in vaseline oil on an IKS-29 spectrophotometer (LOMO, Russia). NMR spectra were recorded on a Varian Gemini spectrometer (200 MHz) in DMSO-d_6, with TMS as an internal standard. ^{13}C NMR spectrum of compound **11c** was recorded on a Bruker DRX-400 (100 MHz) spectrometer in CCl_4 + DMSO-d_6 (1:1) with TMS as an internal standard. Elemental analysis was performed on a Carlo-Erba 1106 Elemental Analyzer with ±0.40% error. The melting points were determined on a Koefler hot bench and were not corrected. The purity of the synthesized compounds was monitored by TLC on Silufol UV-254 plates in the 1:1 acetone–hexane system with visualization by iodine vapor and a UV detector.

The InChI codes of the investigated compounds together with some biological activity data are provided as Supporting Information.

4.2.1 | *N*-Methylmorpholinium 4-amino-1,5-dicyano-3-azaspiro[5.5]undeca-1,4-diene-2-selenolate

A mixture of 1.41 mL (13.6 mmol) freshly distilled cyclohexanone, 2.00 g (13.6 mmol) of cyanoselenoacetamide, and three drops of *N*-methylmorpholine in absolute EtOH (30 mL) was stirred under a constant stream of argon for 15 min, then 2.00 g (13.6 mmol) of cyanoselenoacetamide and 2.0 mL (20.4 mmol) of *N*-methylmorpholine were added. The solution formed was stirred under argon atmosphere for another 10 min, filtered through a paper filter to remove traces of solid matter (selenium), and left to stand under argon for 24 h. The crystalline solid was filtered off, washed with cold EtOH, acetone, and Et_2O to give 2.07 g (43%) of desired selenolate. The spectral data are the same as reported.[67]

4.2.2 | 5,11-Dibenzyl-8-selenoxo-3,5,7,11-tetraazaspiro[cyclohexane-1′,13-tricyclo[7.3.1.$0^{2,7}$]-tridec[2]ene]-1,9-dicarbonitrile (11c)



A mixture of *N*-methylmorpholinium 4-amino-1,5-dicyano-3-azaspiro[5.5]undeca-1,4-diene-2-selenolate (400 mg, 1.1 mmol), 2.4 mmol of benzylamine and an excess (1.5 mL, 17.0 mmol) of 37% aq. HCHO free from paraformaldehyde in EtOH (40 mL) was stirred under a constant stream of argon until complete dissolution of the starting selenolate occurred (5–10 min). Then the solution was heated

under reflux in argon atmosphere for 2–3 min, filtered through a paper filter, and left to stand at ambient temperature for 24 h. The crystalline solid was filtered off, washed with EtOH to give pure **11c**. Yield 31%, mp 204–206°C. IR-spectrum, ν, cm^{-1}: 2237 (2 C≡N), 1670 (C═N). NMR ^{1}H spectrum, δ, ppm (*J*, Hz): 1.23–2.00 (10H, m, $(CH_2)_5$); 2.75 (1H, d, ^2J = 13.1, H-12 or H-10); 3.29 (1H, d, ^2J = 13.1, H-10 or H-12); 3.73–3.82 (6H, m, H-12, H-10 and 2 C$\underline{H}_2$Ph overlapped); 3.95 (2H, dd, ^2J = 13.4, 2 H-6); 4.26 (2H, m, 2 H-4); 7.24–7.36 (10H, m, 2 Ph). NMR ^{13}C spectrum, δ, ppm (*J*, Hz): 19.6 (2 CH_2), 25.9 (CH_2), 26.1 (CH_2), 26.2 (CH_2), 35.5 (C_{quat}), 38.2 (C_{quat}), 39.0 (C_{quat}), 50.5 (NCH_2), 51.8 (NCH_2), 52.3 (NCH_2Ph), 53.3 (NCH_2Ph), 58.9 (NCH_2N), 60.4 (NCH_2N), 114.7 (C≡N), 115.6 (C≡N), 127.55 (CH_{Ar}), 127.57 (CH_{Ar}), 128.2 (CH_{Ar}), 128.3 (CH_{Ar}), 129.7 (CH_{Ar}), 129.9 (CH_{Ar}), 138.5 (C-1 Ph), 138.8 (C-1 Ph), 169.6 (C═N), 197.0 (C═Se). Found (%): C 64.70; H 5.90; N 15.05. $C_{30}H_{32}N_6Se$ (*M* = 555.59). Calculated (%): C 64.86; H 5.81; N 15.13.

4.3 | Biology

4.3.1 | Cells and viruses

Porcine embryo kidney (PEK) and rhabdomyosarcoma (RD) cell lines were from Chumakov FSC R&D IBP RAS. Cell lines were maintained at 37 °C in medium 199 or 2 × EMEM (Chumakov FSC R&D IBP RAS, Russia), respectively, supplemented with 5% fetal bovine serum (Invitrogen). All viruses used in the present work were obtained from Chumakov FSC R&D IBP RAS working collection of viruses. Tick-borne encephalitis virus strain Absettarov (GenBank accession no. KU885457), Enterovirus A 71 isolate 46973 (GenBank accession no. KJ645808), Enterovirus B Coxscakievirus B1 isolate 48461, and Enterovirus C reference vaccine strain Sabin 1 of poliovirus type 1 (GenBank accession no. V01150) were obtained from Chumakov FSC R&D IBP RAS collection of viruses. RD cell line and strain Sabin 1 originated from NIBSC (UK).

4.3.2 | 50% Plaque reduction test

Plaque reduction test protocol was adopted from Ref. [7,68]. In brief, fourfold dilutions of the compounds were preincubated with the virus (20–40 PFU) in 96-well plates at 37°C in CO_2-incubator and then added to PEK cell monolayers in 24-well plates (seeded and incubated for 72 h at 37°C). Virus control was treated with the same sequential concentrations of DMSO, as it was in compounds dilutions. The plates were incubated for 1 h and overlaid with 1.26% methylcellulose. After 6 days, cells were fixed with ethanol and stained with 0.4% gentian violet. EC_{50} values were calculated according to the Reed-and-Muench method.[69]

4.3.3 | Preliminary cell toxicity test

Preliminary cell toxicity test was performed as previously described.[5] In brief, PEK cells were seeded and incubated for 72 h at 37°C. Stock solutions of the compounds with concentration in the range of 5–25 mM (depending on compound solubility in DMSO) were prepared in 100% DMSO (Sigma). Twofold dilutions of compounds were prepared in medium 199 on Earle solution (Chumakov FSC R&D IBP RAS) in 96-well plates to obtain final concentrations of 50–250 μM. Equal 100 μL aliquots of compound dilutions were added in two parallels to each well of 96-well plates with the cells. Cell control was treated with the same sequential concentrations of DMSO, as it was in compounds dilutions. After incubation at 37°C in CO_2-incubator on days 1 or 7 the cultural supernatant was gently removed from the cells. A total of 0.0002% solution of neutral red in PBS (Sigma) was added to the washed cells, and cells were incubated for 30 min at 37°C in CO_2-incubator that vital cells would absorb the dye. Afterwards cells were gently washed with PBS twice and 100 μL of 96% ethanol was added. Absorption was counted in MultiScan FC (Thermo) at 450 nm. CC_{50} was calculated according to Reed-and-Muench method.[69]

4.3.4 | Cell toxicity assay

A protocol for cytotoxicity test in PEK cells was adopted from Ref. [7]. In brief, PEK cells were seeded and incubated for 72 h at 37°C. Twofold dilutions of compounds (concentration 5 mM) were prepared in medium 199 in Earle solution (Chumakov FSC R&D IBP RAS) to obtain final concentrations starting from 50 μM. Equal volumes of compound dilutions were added in four replicates to the cells. 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 or 7, cells morphology and vitality was assessed via microscope. CC_{50} values were calculated according to the Karber method.[70]

4.3.5 | EV cytopathic effect inhibition test

Cytopathic effect inhibition test against members of *Enterovirus* genus was performed as described previously.[6,68] In brief, eight twofold dilutions of stock solutions of the compounds in four replicates were prepared in 2 × EMEM medium (Chumakov FSC R&D IBP RAS) to obtain a final concentration series starting from 62.5 μM. Compound dilutions were mixed with equal volumes of the enterovirus suspension containing 100 TCID50 (50% tissue culture infective dose). Control cells were treated with the same sequential concentrations of DMSO. After 1 h incubation at 36.5°C the RD cell suspension in 2 × EMEM medium containing 5% FBS was added to experimental mixtures. Each experiment contained virus dose titration in the inoculate to assure the acceptable dose-range. After a 5-day incubation at 37°C, cytopathic effect (CPE) was visually assessed via microscope. EC_{50} values were calculated according to the Karber method.[70]

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AUTHORS' CONTRIBUTIONS

DIO, VVD, LIK, and VAP initiated and designed the study. KAF, SGK, and VVD performed the compound synthesis and characterization. AAE, AAO, ADG, and LIK performed cell toxicity and antiviral activity study. AAO, AAE, LIK, VVD, and DIO analyzed the data. DIO, LIK, VVD, and VAP coordinated and supervised the study. AAO, DIO, LIK, and VVD wrote the paper. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

The authors declare no competing financial interest.

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SUPPORTING INFORMATION

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