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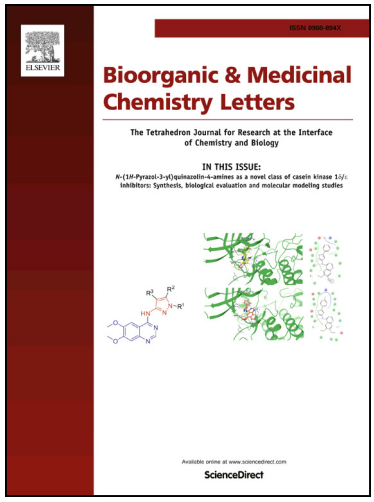

Virtual screening, synthesis and biological evaluation of DNA intercalating antiviral agents

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Virtual screening, synthesis and biological evaluation of DNA intercalating antiviral agents

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Abstract: This paper describes computer-aided design of new anti-viral agents against *Vaccinia virus* (*VaV*) potentially acting as nucleic acid intercalators. Earlier obtained experimental data for DNA intercalation affinities and activities against *Vesicular stomatitis virus* (*VSV*) have been used to build, respectively, pharmacophore and QSAR models. These models were used for virtual screening of a database of 245 molecules generated around typical scaffolds of known DNA intercalators. This resulted in 12 hits which then were synthesized and tested for antiviral activity against *VaV* together with 43 compounds earlier studied against *VSV*. Two compounds displaying high antiviral activity against *VaV* and low cytotoxicity were selected for further antiviral activity investigations.

Keywords: antiviral activity, vaccinia virus, structure-activity modelling, virtual screening, DNA affinity

Highlights:

virtual screening to discover potential nucleic acids intercalators

two hits active against Vaccinia virus were found

intercalating mechanism was proposed for hits

Viral diseases have a severe negative impact on human life worldwide[1,2] which motivates researchers to develop new antiviral drugs. Most of known target-specific antiviral compounds inhibit certain viral proteins, e.g. protease or polymerase [3]. Such compounds are rather selective, have low toxicity and the reduced risk of adverse effects. Corresponding drug discovery projects are frequently supported by different chemionformatics tools. Thus, a combination of QSAR and docking methods were used to identify a novel influenza virus neuraminidase inhibitor which is more potent than the commercialized drug Oseltamivir[4]. The virtual screening procedure involving similarity search, shape-based and pharmacophore models was used to discover HIV-1 reverse transcriptase dual inhibitors[5].

Broad spectrum antiviral agents may, however, be more advantageous than target-specific compounds in controlling multiple emerging pathogens [6]. There exist several major groups of broad-spectrum antivirals. One of them includes interferon and interferon inducers. Interferon is a protein produced as an immune response, inducing synthesis of protein kinase which phosphorylates initiation factor of translation and, therefore, prevents synthesis of viral proteins. The second group includes nucleotide analogs, i.e., substances which resemble DNA or RNA nucleotide but have an inappropriate nitrogenous base. Being captured by proteins or tRNA involved in the virus reproduction processes; they may lead to the synthesis of a non-coding sequences in viral nucleic acids.[7] The third group includes nucleic acid intercalators which may entry between the parallel pairs of bases in double helix of DNA or RNA. [8] To our knowledge, *in silico* approaches are rarely used in the design of broad spectrum antivirals and no computer-aided design of intercalators was reported so far.

In this study, we performed ligand-based virtual screening of new promising nucleic acid intercalators using Quantitative Structure-Activity Relationships (QSAR) and pharmacophore models. Selected hits were synthesized and tested experimentally against Vaccinia virus (VaV), which is a double-stranded DNA virus of the Poxviridae family similar to potential biothreat variola virus. It is widely used in the laboratory as a model system to study various aspects of viral biology and virus-host interactions. [23] Vaccinia viruses' reproduction takes place entirety in the cytoplasm that might facilitate the interaction of organic molecules with viral DNA leading to higher compounds' bio-performance.

In order to build the models we collected a data set of 167 intercalators previously synthesized and tested at A.V. Bogatsky Physico-Chemical Institute [9] (BPCI), see Table A1 in Supplementary Material. All compounds have similar motifs: a polycyclic planar fragment (scaffold) linked to a basic amino group (Figure 1). According to the type of the scaffold, the data set was divided on seven classes of compounds, as it is shown in Figure 1. The binding constants (K_i) values measured by substitution of ethidium bromide in DNA [10] were reported for all compounds of this data set. For 117 out of 167 compounds another kind of a biological response - maximum antiviral effect, E_{max} (%), against *Vesicular stomatitis virus* (VSV) within 0.2 - 620 μM concentration range was measured as described in [9]. We believe that in the context of development of broad-spectrum antiviral agents, the models obtained for anti-VSV activity can be used to discover molecules active against VaV. This hypothesis looks reasonable assuming that both antiviral activities are related to intercalation of small molecules into viral nucleic acids.

The pharmacophore model has been built with the LigandScout[11] software on a subset of 161 compounds with $lg(K_i) \geq 4$ recognized as reasonable DNA intercalators. ZINC database[12] was used as a source of decoys for validation of pharmacophore models. Three best pharmacophore models (Figure 2 and Table A3) having the highest recall and precision values (0.66-1.00) were selected for virtual screening workflow. The classification model able to distinguish compounds with higher ($E_{max} \geq 50\%$) and lower ($E_{max} < 50\%$) maximum antiviral effect against VSV has been built using the Random Forest method[13] and simplex descriptors[14] Earlier, similar technique was successfully used in QSAR modeling of various antiviral activities. [15-17] The model's applicability domain was assessed with Euclidean distance-based method. [18] The model was trained on 94 compounds randomly selected from the PCI dataset. It performed well on the test set containing remaining 23 compounds (balanced accuracy BA = 0.78). More details about model development are given in the Section 2 of the Supplementary Materials.

Developed models have been applied to screen a library of 245 virtual compounds generated as a combination of scaffolds and some typical fragments available as building blocks at BPCI. This library was, at first, screened with the pharmacophore models retaining 42 compounds. Screening of remaining compounds with the classification model resulted in 12 hits which then were synthesized in BPCI, see synthesis description in Section 3 of Supplementary Material.

In view of development of broad-spectrum antiviral agents, the molecules discovered in virtual screening were completed by 43 active compounds earlier studied in BPCI against VSV. Resulting set of 55 compounds was screened with previously developed QSPR model [19] assessing aqueous solubility and with the PASS software [20] assessing affinities to the wide spectra of biological targets. According to these calculations, all 55 compounds didn't display any predicted side effects, toxicity and mutagenicity and had predicted solubility in water was larger than 10^{-5} mol/l.

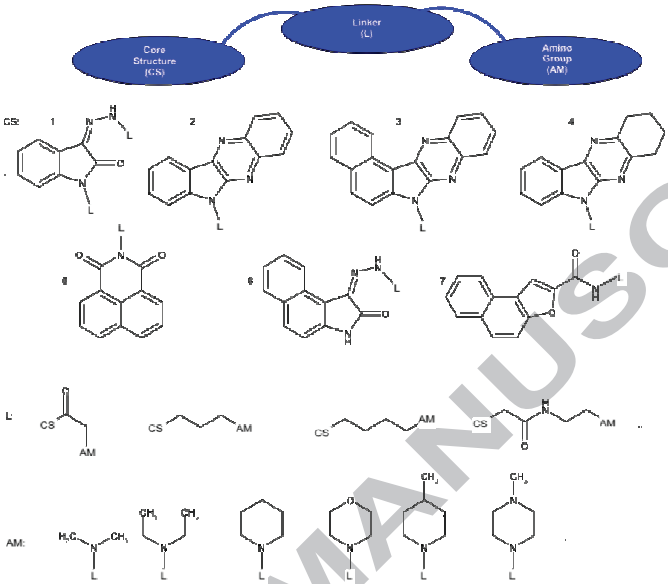


Figure 1. Seven classes of compounds according to core structures (CS) present in the modeling dataset.

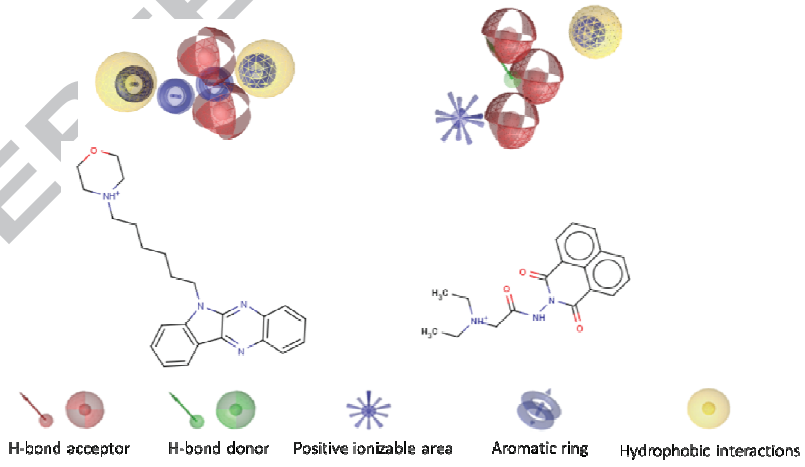


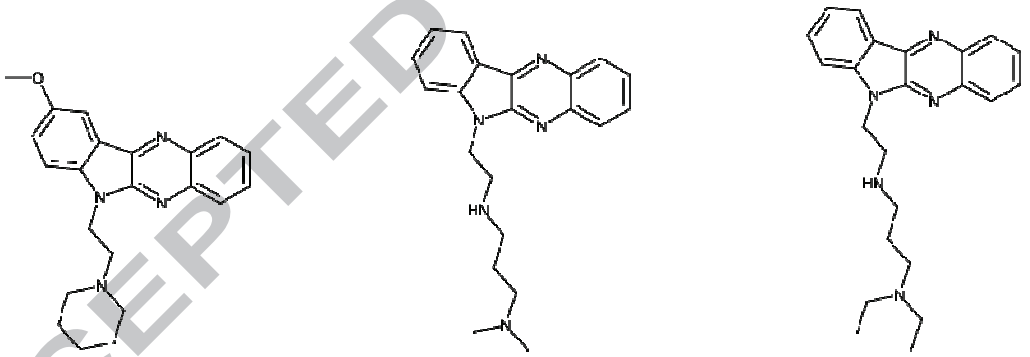
Figure 2. Examples of retrieved hits by pharmacophore models

At the next step, all these compounds have been tested for their cytotoxicity via MTT assay and Real-time cell analysis[21]. Antiviral activity against *Vaccinia virus* was determined using GFP expression quantitation and plaque forming units' assay[23]. Interferon inducing capacity was assessed by decrease of a cytopathic effect caused by virus. The detailed description of experiments and experimental values for all compounds are provided in the Section 4 of the Supplementary materials.

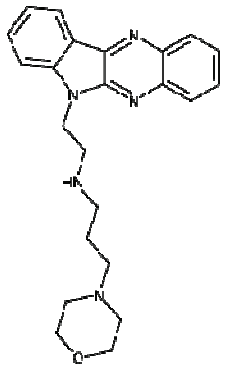
15 out of 55 compounds were not soluble enough in 20% aqueous DMSO in order to complete sample preparation. The cytotoxicity of 40 compounds with respect to CV-1 cells was measured at samples concentration 0.1, 10, 100 μM. Cell viabilities observed for the most active compounds at concentration 10 μM are listed in Table 1. The values of CC_{50} (half-maximal cytotoxicity concentration) were evaluated for each sample at time point 24 h. Based on these data concentration 10 μM was chosen for screening of antiviral activity of the compounds because at this concentration we observed no or slight cytotoxicity for most of them.

Insertion of the DNA sequence encoding GFP into the thymidine kinase (TK) gene of *Vaccinia virus* significantly improves tracking of the virus without interfering with its ability to replicate. *Vaccinia virus* strain LIVP-GFP expressed GFP under the control of the early-late VACV VV7.5 promoter which resulted in efficient GFP expression during all stages of viral infection so that one can easily monitor the development of viral infection by measuring the level of GFP. Compounds antiviral activity at 10 μM was evaluated in experiments with CV-1 cells infected with LIVP-GFP similarly to [24]. CV-1 cells were treated with the compounds (10 μM) in duplicate. After 4 h of incubation the medium was removed and cells were infected by LIVP-GFP at a multiplicity of infection (MOI) of 0.01. LIVP-GFP-infected cells were incubated for additional 24 h prior to being processed for flow cytometry. The Relative expression of GFP is used for the primary assessment of the antiviral activity since it shows a decline in viral proteins formation (Table 1). The screening performed showed that five compounds reduced GFP expression more than twice (Table 1) whereas other compounds lack GFP expression inhibition potency (see Table A5 of Supplementary Material). These five compounds were retained for further testing. Notice that one of them (**171**) was designed in this work whereas four others (**115, 114, 111** and **67**) were previously studied against VSV.

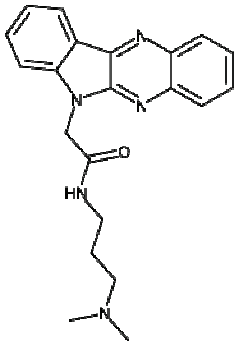
Incubation of the cells with compounds **171, 67, 115, 111** (Figure 3) at concentration 30 - 50 μM prior to infection results in 8 - 10 fold inhibition of GFP expression which reflects the strong antiviral activity of these compounds. Due to relatively high cytotoxicity of the compound **114** (CC_{50} ≈ 50 μM) its antiviral activity was evaluated at concentrations not exceeding 10 μM: even at this relatively low concentration **114** twice reduce GFP expression level, thus showing rather pronounced antiviral activity.



171 **115** **114**



111



67

Figure 3. Structures of the most active compounds identified in this study.

Table 1. CC_{50} and the antiviral activity measured by Flow cytometry analysis of GFP expression in the infected cells (Cells viability and Relative expression of GFP are given at concentration of 10 µM). K- (negative control) – untreated, uninfected cells (cells autoflurescence). K+ (positive control) – untreated, infected cells. Information on all 40 compounds GFP expression test results is given in Table A5.

Compound ID	Cells viability, %	CC_{50}, µM	Relative expression of GFP, %
K+			100
K-			8
171	79±10	≈100	23
114	74±8	≈50	28
67	84±16	100	42
115	88±9	≈50	45
111	79±6	≈50	48

We applied the plaque forming assay to analyze the effect of compounds **171, 67, 115, 111, 114** on viral infection development and infectious viral particles production in CV-1 cells. The virus titer was measured in the medium of infected cells pre-incubated for 4 h prior to infection with or without (control) above-mentioned compounds taken at different concentration (Table 2). In parallel real-time monitoring of cell viability using xCelligence Real-Time Cell Analyzer was

performed (see Figure A1 in Supplementary materials). Data obtained using xCelligence system are in a good agreement with the results of MTT test.

Analysis of the antiviral activity showed that pre-incubation of CV-1 cells with the compounds resulted in the decrease of virus titer in cell medium by 0.5 – 1 lg(PFU/ml) and these results are consistent with the data obtained by flow cytometry. There are two lead compounds which exhibit antiviral activity in a concentration dependent manner, namely **171** (Δ_{titre} = 0.9 lg PFU/ml) and **67** (Δ_{titre} = 0.8 lg PFU/ml); for other tested compounds the differences in the viral titter were less pronounced (Δ_{titre} = 0.5 – 0.7 lg PFU/ml). As for **114** (Δ_{titre} = 0.5 – 0.7 lg PFU/ml) no dependence of the antiviral activity on the compound concentration was observed together with stimulation of cell proliferation. Compounds **115** and **111** inhibited viral infection only at the highest concentration used (30 μM) by 0.5 – 0.6 lg PFU/ml and at this concentration 25% of CV-1 cells died. Based on these experiments, SI values for these compounds have been calculated (Table 3).

2 out of 40 compounds tested in the antiviral screening, namely **171** and **67**, display a prominent activity by approximately tenfold decrease in infectious viral particles production. Compound **67** is characterized additionally by somewhat lower cytotoxicity in comparison with **171** (under similar conditions 90 and 75% of cells remained viable for **67** and **171**, respectively).

Antiviral activity of the studied compounds could be a result of either direct inhibition of viral infection by virus life cycle disruption or by inducing interferon (IFN) production by the cells. In order to analyze whether compounds work as IFN-α/β inducers we estimated the level of IFN in murine fibroblasts, infected with murine encephalomyocarditis virus (EMCV) after treatment with the selected compounds. Studied compounds did not induce IFN-α/β production on a detectible level. Taking into account that efficacy of induction of IFN-α/β expression varied significantly in different cell lines we additionally tested induction of IFN-α in mouse spleen cells treated (stimulated) with the compounds **171**, **67**, **115**, and **111**. In these experiments no induction of IFN-α after the treatment of mouse spleen cells with compounds was observed. Noteworthy, Cycloferon used as a positive control, stimulated IFN expression both in murine fibroblasts and in the mouse spleen cells.

Thus, two most promising compounds **171** and **67** inhibit virus reproduction by at least 8 and 6 folds, respectively in considerably lower concentrations than their $CC_{50,}$ which makes them eligible candidates for further antiviral research. The discovered hits were tested for DNA affinity (K_i) according to the procedure reported previously [10]. They display reasonable intercalating activity: lg(K_i) = 6.03 and 5.20 for compounds **171** and **67**, respectively.

Table 2. Antiviral activity of potent compounds measured by classical plaque forming assay. n.d. – activity not determined. [1)] Virus titer in the infected cell incubated in the presence of 0.002, 0.02 or 0.1% of DMSO in the cell medium was 3.1 ± 0.3 PFU/ml, similar to K+.

Compound ID	C, μM	Viable cells, %	Virus titer, lg(PFU/ml)
K+[1)]		100±0.1	3.3±0.1
171	1	107.8±0.1	3.1±0.1
	10	96.9±0.2	2.8±0.1
	50	75.6±0.1	2.4±0.1

67	1	103.6±0.2	3.4±0.1
	10	131.4±0.1	2.8±0.1
	50	90.0±0.1	2.5±0.0
115	1	113.7±0.1	3.5±0.1
	10	110.3±0.1	3.3±0.0
	30	75±0.0	2.7±0.1
111	1	107.2±0.1	3.6±0.2
	10	110.1±0.1	3.3±0.1
	30	75±0.0	2.6±0.1
114	1	97.1±0.1	2.8±0.0
	5	125.3±0.1	2.8±0.1
	10	125±0.0	2.6±0.1
	50	17.2±0.0	n.d.

Table 3. Antiviral activity and cytotoxicity of the compounds tested

Compound ID	[a]CC_{50}, µM	[b]IC_{50}, µM	Selectivity Index(SI)[c]
171	170	5	34
67	500	10	50
115	92	22	4
111	81	19	4
114	30	<1	<30

[a] cytotoxic concentration 50%, compound concentration required to reduce cell viability by 50% assessed by the MTT assay.
[b] inhibitory concentration 50%, compound concentration producing 50% inhibition of virus replication assessed by the PFU assay.
[c] selectivity index: ratio between cytotoxic concentration (CC_{50}) and inhibitory concentration (IC_{50}).

In this work, several broad spectrum antiviral agents acting as nucleic acids intercalators were discovered. First, 12 compounds were selected in virtual screening with the help of developed pharmacophore and QSAR models and then synthesized. Together with previously studied 43 compounds, they were tested experimentally against Vaccinia virus. Out of these, 2 molecules displayed high antiviral activity, reasonable DNA affinity and low toxicity. The interferon induction capacity has not been detected for these compounds which supports our hypothesis about intercalation mechanism of their antiviral activity.

Supplementary Material

It contains information about the development and performance of the virtual screening tools, synthesis of hit compounds and the detailed description of the biological assays

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