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Anti-enteroviral activity of new MDL-860 analogues: Synthesis, *in vitro/in vivo* studies and QSAR analysis

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ABSTRACT

A series of 60 nitrobenzonitrile analogues of the anti-viral agent MDL-860 were synthesized (50 of which are new) and evaluated for their activity against three types of enteroviruses (coxsackievirus B1, coxsackievirus B3 and poliovirus 1). Among them, six diaryl ethers (**20e**, **27e**, **28e**, **29e**, **33e** and **35e**) demonstrated high *in vitro* activity (SI > 50) towards at least one of the tested viruses and very low cytotoxicity against human cells. Compound **27e** possesses the broadest spectrum of activity towards all tested viruses in the same way as MDL-860 does. The most active derivatives (**27e**, **29e** and **35e**) against coxsackievirus B1 were tested *in vivo* in newborn mice experimentally infected with 20 MLD_{50} of coxsackievirus B1. Compound **29e** showed promising *in vivo* activity (protection index 26% and 4 days lengthening of mean survival time). QSAR analysis of the substituent effects on the *in vitro* cytotoxicity (CC_{50}) and anti-viral activity of the nitrobenzonitrile derivatives was carried out and adequate QSAR models for the anti-viral activity of the compounds against poliovirus 1 and coxsackievirus B1 were constructed.

1. Introduction

Enteroviruses are members of the *Picornaviridae* family, comprising small non-enveloped viruses with single stranded positive sense RNA genome. They are usually agents of mild infections but also cause encephalitis, myocarditis, poliomyelitis, acute heart failure, and diabetes mellitus. Enteroviruses are subject to significant changes over time because of errors introduced during genome replication. Intraspecies recombination between enteroviruses is also common, further promoting genetic diversity. This genetic plasticity allows for widespread epidemics and sporadic outbreaks to occur. Enteroviruses are now classified into 15 distinct species. Among them are polioviruses (causal agents of poliomyelitis in humans and nonhuman primates), coxsackie A viruses (associated with herpangina, human central nervous system disease, and flaccid paralysis in suckling mice), coxsackie B viruses (human central nervous system and cardiac disease, diabetes, spastic paralysis in mice), and the echoviruses (nonpathogenic in mice, and not initially linked to human disease). New strains of coxsackievirus B1 (CVB1), enterovirus-A71 (EV-A71), and enterovirus-D68 (EV-D68) have emerged as causes of recent outbreaks in the United States, South-Eastern Asia, and other countries, including more severe disease manifestations than previously described. A recent outbreak of CVB1 has once again demonstrated the epidemic potential of enteroviruses. In mid-2007, cases of severe neonatal disease due to CVB1 were recognized nearly simultaneously in several USA cities. In general, this virus was recognized as one of the most commonly circulating enteroviruses in USA between 2009 and 2013 [1].

Significant progress has been made in the global effort to interrupt poliovirus transmission and eradicate polio. However, attempts to eliminate poliovirus 1 (PV1) circulation are still running in countries like Afghanistan, Pakistan and Tadjikistan, but progress has been delayed by factors that have made vaccination unavailable for approximately 5–25% of children in the region [1]. In addition, to the best of our knowledge, an efficient and approved chemotherapy against

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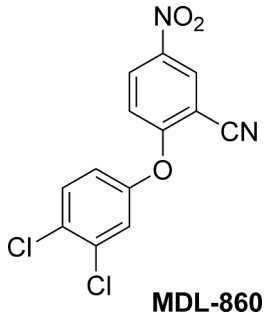


Fig. 1. Structure of MDL-860.

polioviruses (as well as against coxsackieviruses) does not exist [2]. Such therapy could be complementary to vaccination, especially in problematic regions worldwide.

The evolution of drug resistance is a challenge for successful development of new anti-enteroviral agents with promising activity and potential for further development [3]. Nevertheless, few compounds have been in clinical trials so far – disoxaril, pleconaryl, pirodavir and its analogues, some isoxazoles, imidazolidinones, chalcones and flavanes [4]. Apart from drug-resistance, some additional issues were observed – drug-drug interactions, low *in vivo* activity, side effects etc. [1,5]. Recent studies have shown reborn interest in the synthesis and *in vitro* evaluation of new small molecules active against enteroviruses (PV1, coxsackievirus B3 (CVB3) and coxsackievirus B5) [6,7]. Diarylether MDL-860 (2-(3,4-dichlorophenoxy)-5-nitrobenzonitrile, also known as DNB) (Fig. 1) was firstly reported in the 1980's. Later on, it was recognized that MDL-860 and its analogues possess a broad-spectrum of *in vitro* activity against picornaviruses along with very low cytotoxicity toward human cells [8,9].

Our recent studies were focused on the mechanism of action of MDL-860 [10] and further development of new MDL-860 analogues (Fig. 2) active against CVB1, CVB3 and PV1 [11]. In the light of current research [8–11], it is clear that most promising is the development of new MDL-860 analogues bearing unaltered 2-cyano-4-nitro ring. In our previous work, initial screening of twelve MDL-860 analogues resulted in three compounds (A1, A7 and A8) with high activity against PV1 and CVB1 and only two compounds (A2 and A3) with moderate activity towards CVB3. In addition, compounds A1, A7 and A8 exhibited activity towards CVB1 experimental neuroinfection in newborn mice [11].

The aim of the present study is the synthesis of new MDL-860 analogues possessing unaltered 2-cyano-5-nitro substituted benzene ring as a common fragment, in order to prove limits of possible variations in the other ring of MDL-860, leading to improved antiviral activity.

2. Results and discussion

2.1. Chemistry

Four series of MDL-860 analogues were synthesized and evaluated for anti-viral activity. Detailed synthetic procedures and analytical data are presented in Supplementary data. The synthesis of non-commercial starting compounds was also described in Supplementary data. All target compounds were obtained through simple one-step nucleophilic aromatic substitution reactions of series of phenols (**1a-36a**), thiols (**1b-9b**), amines (**1c-11c**) and *N*-heterocycles (**1d-3d**) with 2-chloro-5-nitrobenzonitrile (**1**) in presence of a base. Due to the electron-deficient nature of **1**, chlorine substitution was performed in relatively mild conditions with no catalyst needed [12]. All compounds were purified by column chromatography and/or recrystallization.

The synthesis of aryl ethers **1e-36e** (Table 1) and aryl thioethers **1f-10f** (Table 2) from **1** and the corresponding phenols (**1a-36a**) and thiophenols (**1b-9b**) was performed at 80 °C in dry DMSO generally using powdered KOH as a base. In some cases NaOH or K_2CO_3 were used instead (**15e**, **20e**, **21e**, **36e**, **5f** and **8f**). Reaction progress was monitored by TLC. Compound **11e** was obtained from **1** and *in situ* generated CF_3CH_2ONa (from dry CF_3CH_2OH and NaH) in refluxing CF_3CH_2OH. Similar procedure was applied for the preparation of benzyl ether **12e**. Compound **10f** was obtained as a single product through spontaneous cyclisation during the reaction between **1** and mercaptobenzimidazole (**9b**) [13]. The aryl thioether **9f** was therefore not isolated. It should be mentioned that the preparation of compound **36e** (2-(2,5-diiodophenoxy)-5-nitrobenzonitrile) was initially not aimed. Instead, we had planned to obtain a 3,4-diiodo-analogue of MDL-860. Unfortunately, the synthesis of this derivative from 3-iodophenol turned out to be quite challenging. All attempts led to isolation of 2,5-diiodophenol (**36a**) with ca. 95% purity. It was not possible to further purify compound **36a**, thus it was used for the synthesis of **36e** as it was. The purification of compound **36e** was a serious challenge as well. Column chromatography purification followed by several successive recrystallizations led to isolation of **36e** with ca 95% purity. According

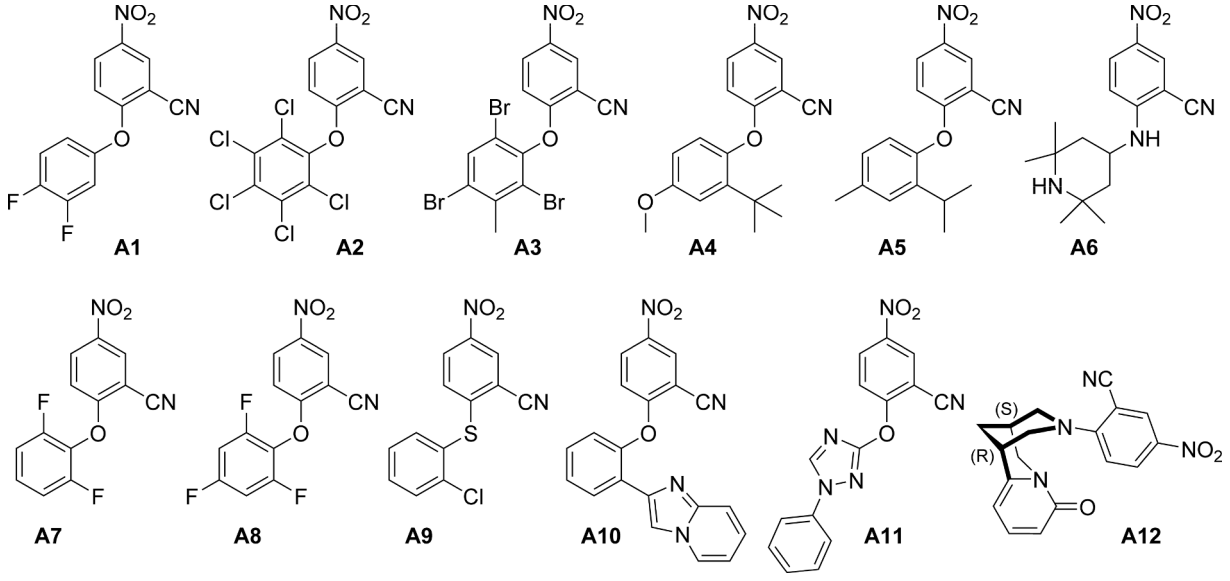
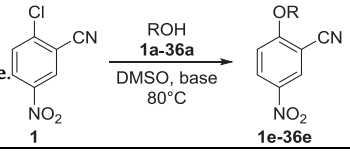
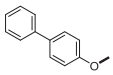
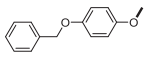
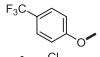
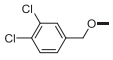


Fig. 2. Recently published analogues of MDL-860.

Table 1

Synthesis of diaryl ethers **1e-36e**.

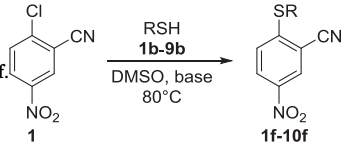


ROH	RO–	Ether	ROH	RO–	Ether	ROH	RO–	Ether
1a		**1e**	**13a**		**13e**	**25a**		**25e**
2a		**2e**	**14a**		**14e**	**26a**		**26e**
3a		**3e**	**15a**		**15e**	**27a**		**27e**
4a		**4e**	**16a**		**16e**	**28a**		**28e**
5a		**5e**	**17a**		**17e**	**29a**		**29e**
6a		**6e**	**18a**		**18e**	**30a**		**30e**
7a		**7e**	**19a**		**19e**	**31a**		**31e**
8a		**8e**	**20a**		**20e**	**32a**		**32e**
9a		**9e**	**21a**		**21e**	**33a**		**33e**
10a		**10e**	**22a**		**22e**	**34a**		**34e**
11a		**11e**	**23a**		**23e**	**35a**		**35e**
12a		**12e**	**24a**		**24e**	**36a**		**36e**[a]

[a] Compound **36e** contains ca. 5% of the 3,4-diiodo isomer.

Table 2

Synthesis of thioethers **1f-10f**.



RSH	RS–	Thio-ether	RSH	RS–	Thio-ether	RSH	RS–	Thio-ether
1b		**1f**	**4b**		**4f**	**7b**		**7f**
2b		**2f**	**5b**		**5f**	**8b**		**8f**
3b		**3f**	**6b**		**6f**	**9b**		**9f**[a]
								10f

[a] Not isolated.

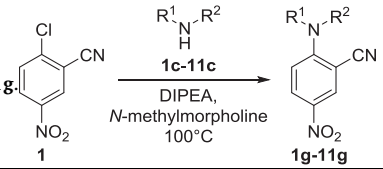
to X-ray data obtained (see Section 2.2) on crystal grown form that mixture compound **36e** was found to contain 5% of its 3,4-diiodo substituted analogue, thus indicating the presence of 3,4-diiodophenol in **36a**.

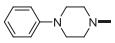
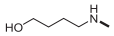
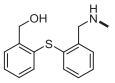
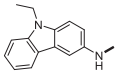
A series of arylamines **1g-11g** (Table 3) were synthesized through reaction of **1** with amines **1c-11c** at 100 °C in a mixture of dry *N*,*N*-diisopropylethylamine (DIPEA) and *N*-methylmorpholine (NMM). Amine **1g** was synthesized from **1** and dry DMF (as convenient *in situ* source of dimethylamine) at 130 °C according to a described procedure [14]. In some cases (**6g**, **7g** and **8g**) decomposition products (black tar) and unreacted **1** were observed. *N*-arylation of heterocycles **1d-3d** applying common conditions (NaH/dry DMSO) afforded the corresponding compounds **1h-3h** in good to excellent yields (Scheme 1). A trifluoromethyl substituted analogue (**4**) of MDL-860 was prepared from 1-fluoro-4-nitro-2-(trifluoromethyl)benzene (**2**) and 3,4-dichlorophenol (**3**) in DMSO (Scheme 2).

It should be pointed out that most of the compounds obtained in this study were synthesized for the first time. The synthesis of compounds **4e** [15], **11e** [16–20], **1g** [12,14,21–25], **8g** [12,26], **10g** [27] and **1h** [25] was described elsewhere in different context studies, not being related to the present biological investigations. The anti-viral activity of MDL-860 [28], **16e** [28], **17e** [28], **23e** [8,28] and **1f** [28] against other enteroviruses is discussed in Section 2.3.

Table 3

Synthesis of arylamines **1g**-**11g**.



Amine	R^1R^2N-	Aryl-amine	Amine	R^1R^2N-	Aryl-amine	Amine	R^1R^2N-	Aryl-amine
1c		**1g**	**5c**		**5g**	**9c**		**9g**
2c		**2g**	**6c**		**6g**	**10c**		**10g**
3c		**3g**	**7c**		**7g**	**11c**		**11g**
4c		**4g**	**8c**		**8g**			

2.2. X-ray

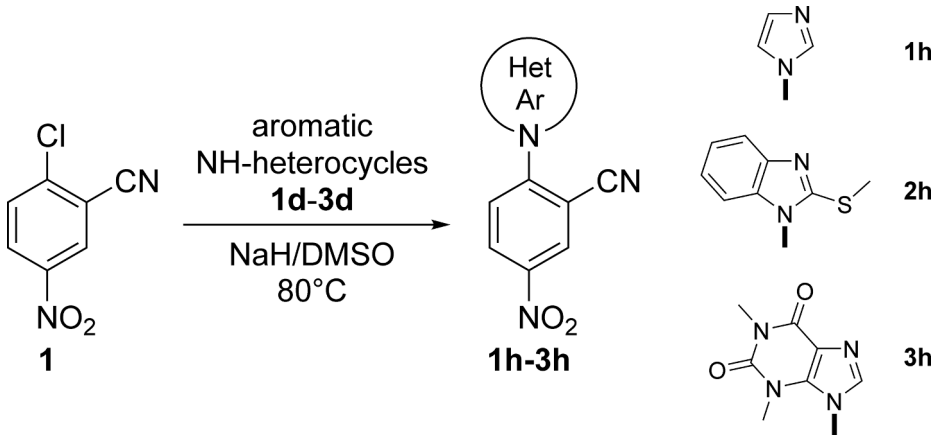
Application of single crystal X-ray diffraction was used in this study. This method was necessary in order to confirm the structure of **36e**, on the other hand the results obtained were useful to elucidate the structure of the impurity in **36e**. Thus, the crystal structure of compound **36e** was elucidated by single crystal X-ray diffraction (Fig. 3). Single crystals were obtained by slow evaporation of a concentrated solution of **36e** in isopropanol. The most important crystallographic data and refinement parameters for **36e** are shown in Table 4, while bond distances, angles, torsion angles and other details about structure solution and refinement are listed in Supplementary data (Tables S1 and S2, Figs. S1 and S2). The crystal structure revealed the presence of impurity (ca. 5%) of 2-(3,4-diiodophenoxy)-5-nitrobenzonitrile. It is interesting to note that *I4* shifts from its "original" I2 position and the distance between iodines I3 and *I4* from the minor component is 3.918 Å The hypothetical I2…I3 distance being 3.232 Å. The tow ring systems (diiodophenoxy and nitrobenzonitrile) are essentially planar (*rmsd* of 0.01 Å for both) though the angle between their mean planes is 81.5° e.g. the bridging O1 allows rotation of the ring systems along C—O1 bond.

2.3. Virology

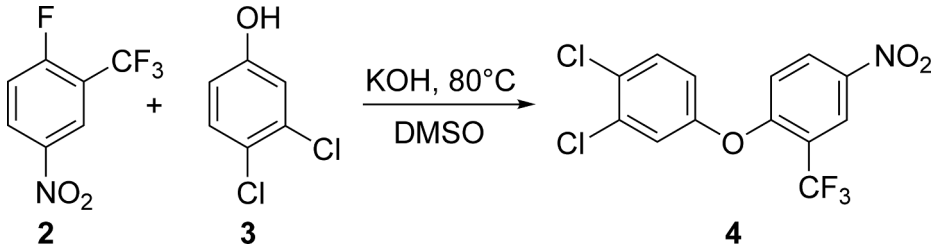
The newly synthesized MDL-860 derivatives (**1-36e**, **1-10f**, **1-11g**, **1-3h** and **4**) were subjected to *in vitro* screening study for activity towards PV1, CVB1 and CVB3 (Table 5). The CPE inhibition test was used, following the procedure of *Borenfreund* and *Puerner* [29]. Previously published data for compounds **A1-A12** [11] are presented for comparison. It was demonstrated that compound **27e** (by analogy with MDL-860) possesses the broadest spectrum of activity in this study (against PV1, CVB1 and CVB3). Compound **35e** was active against CVB1 and CVB3. Significant activity towards PV1 and CVB1 demonstrated by **28e**. Compounds **20e** and **29e** were effective only against CVB1 and **22e**, **30e** and **33e** – only against PV1. Moderate activity against PV1 and CVB1 was demonstrated by compound **4e**; against PV1 – only by **17e**, **18e**, **24e** and **31e**; compound **13e** was active against CVB1 and **14e** – against CVB3. It should be mentioned that the antiviral activity of **16e**, **17e**, **23e** and **1f** was investigated in the early 1980's against rhinoviruses and Coxsackie A21 virus [8,28]. Their activities were generally higher compared to those observed against PV1, CVB1 and CVB3 in this study (Table 5). Only MDL-860 could be considered to possess wide spectrum of activity – it demonstrated high *in vitro* activity against all tested rhinoviruses and coxsackie viruses.

Among the thioethers, **3f** and **8f** demonstrated weak activity against CVB1. The main disadvantage of **8f** appears to be its high cytotoxicity (CC_{50} = 18.7 μM). Since thioethers are able to oxidize easily in biological media, it is not clear whether **3f** and **8f** are the active compounds or just prodrugs. Thus, further investigation of sulfone analogues of **3f** and **8f** is necessary.

The data presented in Table 5 unambiguously show that even small changes in the MDL-860 molecule dramatically influence the *in vitro* activity. Interestingly, new active compounds could be found exclusively among the diarylethers. Exploring in more detail the group of 2-cyano-5-nitro substituted ethers (compounds **A1-A5**, **A7**, **A8**, **A10**, **A-**



Scheme 1. Synthesis of *N*-arylated heterocycles **1h**-**3h**.



Scheme 2. Synthesis of $-CF_3$ derivative **4**.

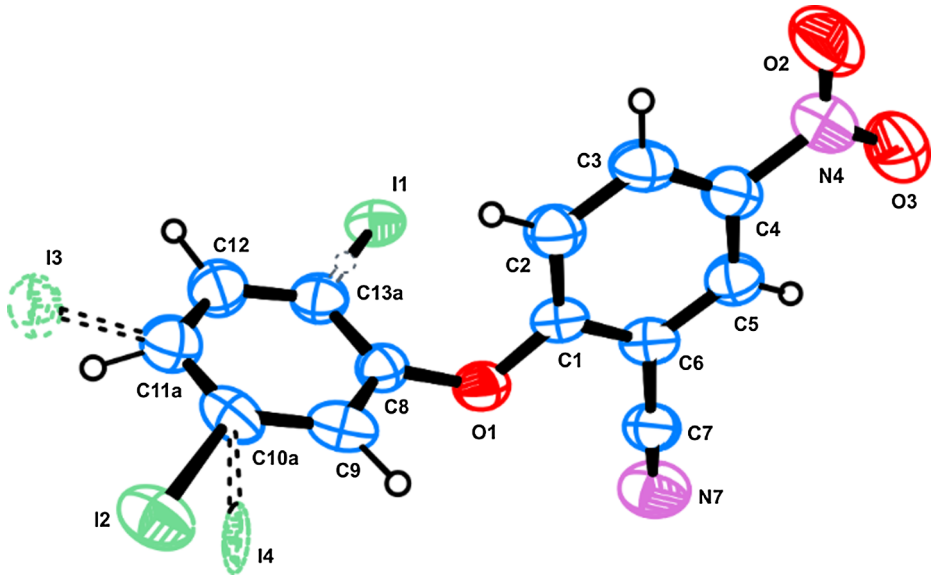


Fig. 3. ORTEP drawing of compound **36e** showing the atomic numbering system and the observed disorder of the iodine's (minor component of 5.58% is shown as dashed lines).

Table 4
Most important crystallographic and data refinement parameters for compound **36e**.

Empirical formula	$C_{13}H_6I_2N_2O_3$
Formula weight	492.00
Temperature/K	290
Crystal system	Monoclinic
Space group	$P2_1/c$
a/Å	21.1900(7)
b/Å	5.60500(10)
c/Å	13.2171(4)
α/°	90
β/°	106.026(4)
γ/°	90
Volume/Å^3	1508.79(8)
Z	4
ρ_{calc} g/cm^3	2.166
μ/mm^{-1}	32.829
F(0 0 0)	912.0
Crystal size/mm^3	0.3 × 0.25 × 0.12
Radiation	Cu Kα (λ = 1.54184)
2Θ range for data collection/°	8.684–148.524
Index ranges	$-22 \le \mathbf{h} \le 26$, $-6 \le k \le 6$, $-15 \le l \le 16$
Reflections collected	9013
Independent reflections	3015 [R_{int} = 0.0544, R_{sigma} = 0.0431]
Data/restraints/parameters	3015/0/202
Goodness-of-fit on F^2	1.026
Final R indexes [$I > = 2\sigma$ (I)]	R_1 = 0.0511, wR_2 = 0.1304
Final R indexes [all data]	R_1 = 0.0706, wR_2 = 0.1456
Largest diff. peak/hole/e Å^{-3}	1.11/−1.32
CCDC number	1,876,618

11 and **1e-36e**), it is clear that the presence of two and three halogen substituents at different positions in the secondary benzene ring is optimal for high activity. On the other hand, poly-halogenated ethers (**A2**, **15e-17e**) are inactive. Most of the diarylethers (with a few exceptions: **12e**, **15e**, **18e**, **24e**, **32e**) are showing very low toxicity. In the light of the recently discovered mechanism of action of MDL-860 [10], one could argue that all active diarylethers have a similar mechanism of action, namely, an irreversible covalent modification of phosphatidylinositol-4 kinase III beta (PI4KB). PI4KB is one of the most important enzymes in mammals, responsible for replication of enteroviruses in the host cells [30]. It could be assumed that alteration of the halogen substituents in diarylethers is important. This may cause small changes in the shape and geometry of the molecules but may impact significantly the PI4KB modification and *in vitro* activity, respectively.

Other type of substituents (e.g. **A4**, **A5**, **1e-3e**, **5e**, **6e**, **13e**, **14e**), or the presence of heterocyclic moieties (**A10**, **A11**, **4e**, **7e-10e**) instead of benzene ring, generally led to lack of activity. Other series of compounds (**1f-10f**, **1g-11g** and **1h-3h**, containing different bridge heteroatoms, i.e. *S*, *N*) were completely inactive (except for **8f**). It is noteworthy that even very close isosteric analogues of MDL-860 (like thioether **1f** or ethers **4** and **12e**) are also inactive. Probably these compounds are not able to modify PI4KB or they undergo biochemical transformations before reaching the enzyme.

Compound **4** is the only MDL-860 analogue in this study, possessing a different substituent in the primary aromatic ring ($-CF_3$ instead of −CN). The role of the substituents in this ring is still unclear and further studies of such series of compounds is necessary. For example, some published results [8] show that replacement of the cyano group with carboxyl group in MDL-860 leads to carboxylic acid with good antiviral activity. Moreover, this replacement automatically allows improvement

Table 5
In vitro screening data for anti-enteroviral activity.

Compound[a]	Cytotoxicity CC_{50} (μM)	PV1		CVB1		CVB3	
		IC_{50} (μM)	SI	IC_{50} (μM)	SI	IC_{50} (μM)	SI
MDL-860	493.0	6.8	72.5	0.8	586.9	2.7	182.0
A1	320.0	2.7	118.0	0.8	405.0	NA	–
A2	119.1	NA	–	10.9	10.9	5.8	20.5
A3	570.3	NA	–	256.1	2.2	29.6	19.6
A4	22.0	NA	–	NA	–	NA	–
A5	94.3	NA	–	16.2	5.8	NA	–
A6	675.0	NA	–	NA	–	426.0	1.5
A7	355.0	6.8	52.2	0.7	507.1	NA	–
A8	517.5	2.7	191.6	0.75	690.0	NA	–
A9	53.8	NA	–	NA	–	9.9	5.4
A10	718.0	NA	–	NA	–	NA	–
A11	570.8	NA	–	190.1	3.0	NA	–
A12	492.2	234.0	2.1	152.2	3.2	NA	–
1e	123.0	NA	–	NA	–	NA	–
2e	175.0	NA	–	NA	–	NA	–
3e	123.0	NA	–	NA	–	NA	–
4e	332.0	30.6	11.5	22.8	14.5	NA	–
5e	346.6	NA	–	NA	–	NA	–
6e	280.0	NA	–	NA	–	NA	–
7e	450.0	NA	–	NA	–	NA	–
8e	576.0	NA	–	NA	–	NA	–
9e	423.0	NA	–	NA	–	NA	–
10e	367.0	NA	–	142.0	2.5	NA	–
11e	165.0	NA	–	NA	–	NA	–
12e	10.1	NA	–	NA	–	NA	–
13e	291.0	NA	–	12.7	22.9	NA	–
14e	680.0	NA	–	NA	–	54.0	12.5
15e	47.1	NA	–	NA	–	NA	–
16e	195.0	79.0	2.4	NA	–	NA	–
17e	287.0	22.8	12.6	NA	–	NA	–
18e	18.7	1.0	18.7	NA	–	NA	–
19e	95.0	11.0	8.6	NA	–	NA	–
20e	199.0	32	6.2	2.1	95	NA	–
21e	572.0	NA	–	NA	–	NA	–
22e	219.0	6.8	32.2	NA	–	NA	–
23e	272.0	NA	–	NA	–	NA	–
24e	30.7	1.8	17.0	NA	–	NA	–
25e	92.0	NA	–	NA	–	NA	–
26e	547.0	NA	–	NA	–	NA	–
27e	785.0	11.0	71.3	6.4	122.6	6.8	115.4
28e	234.0	2.7	86.6	6.1	38.3	NA	–
29e	342.0	NA	–	2.9	117.9	NA	–
30e	200.0	4.3	46.0	NA	–	NA	–
31e	155.0	10.0	15.5	NA	–	NA	–
32e	30.5	5.2	5.8	NA	–	NA	–
33e	107.0	1.0	107	NA	–	NA	–
34e	215.0	NA	–	NA	–	NA	–
35e	493.0	NA	–	3.7	133.2	1.0	493.0
36e	273.0	NA	–	NA	–	NA	–
1f	132.0	NA	–	NA	–	NA	–
2f	211.0	NA	–	NA	–	NA	–
3f	187.5	NA	–	24.9	7.5	NA	–
4f	161.3	NA	–	NA	–	NA	–
5f	13.6	NA	–	NA	–	NA	–
6f	14.6	NA	–	NA	–	NA	–
7f	16.5	NA	–	NA	–	NA	–
8f	18.7	NA	–	3.1	6.0	NA	–
10f	349.2	NA	–	NA	–	NA	–
1g	651.0	NA	–	NA	–	NA	–
2g	332.6	NA	–	NA	–	NA	–
3g	336.7	NA	–	NA	–	NA	–
4g	495.4	NA	–	NA	–	NA	–
5g	617.0	255.0	2.4	NA	–	NA	–
6g	12.6	NA	–	NA	–	NA	–
7g	346.6	NA	–	NA	–	NA	–
8g	199.0	NA	–	NA	–	NA	–
9g	55.4	NA	–	NA	–	NA	–
10g	336.4	NA	–	NA	–	NA	–

(*continued on next page*)

Table 5 (*continued*)

Compound[a]	Cytotoxicity CC_{50} (μM)	PV1		CVB1		CVB3	
		IC_{50} (μM)	SI	IC_{50} (μM)	SI	IC_{50} (μM)	SI
11g	454.0	NA	–	NA	–	NA	–
1h	332.8	NA	–	NA	–	NA	–
2h	94.2	NA	–	NA	–	NA	–
3h	339.9	NA	–	NA	–	NA	–
4	22.8	NA	–	NA	–	NA	–

NA – not active.

[a] MDL860 was used as a reference compound; compounds **A1**-**A12** were already published in respect of their synthesis and activity against PV1, CVB1 and CVB3 [11].

Table 6
Study of the *in vivo* activity of compounds **27e**, **29e**, and **35e** against CVB1 experimental neuroinfection in newborn mice. Data are from three independent experiments (average).

Compound/Dose	Survivors/ Total	MST ± SD, days[a]	Δ, days	Mortality, %	PI, %
27e/25 mg/kg	0/16	3.2 ± 0.5^{ns}	0.2	100	0
27e/50 mg/kg	3/21	5.4 ± 0.5^{ns}	+2.4	86	14.2
29e/25 mg/kg	6/23	7.0 ± 1.0**	+4.0	74	26
29e/50 mg/kg	0/26	3.1 ± 0.4^{ns}	+0.1	100	0
35e/25 mg/kg	0/17	6.1 ± 0.8*	+3.1	100	0
35e/50 mg/kg	1/23	4.6 ± 0.6^{ns}	+1.6	96	4.3
MDL-860	0/27	6.1 ± 0.9*	+3.1	100	0
Placebo	0/16	3.0 ± 0.3	–	100	0

[a] One-way ANOVA (Bonferroni's multiple comparison post-test); MST – mean survival time; PI – protection index; SD – standard deviation.

** $p < 0.01$vs. placebo group.

* $p < 0.05$ vs. placebo group; ns – not significant.

of water solubility through possible formation of salts.

The most active derivatives (**27e**, **29e** and **35e**) against CVB1 were tested for *in vivo* activity in newborn mice experimentally infected with 20 MLD_{50} CVB1. Compounds **27e**, **29e** and **35** were administered subcutaneously as daily doses of 25 and 50 mg/kg following 12-days course since the day of viral inoculation. The results obtained showed moderate protective effects of **27e** and **29e**. A marked lengthening of the mean survival time was observed for **29e** (25 mg/kg) and **35e** (25 mg/kg) (Table 6 and Fig. 4). Taking into account this lack of activity, along with the previously reported promising results for compounds **A1** (PI 50%), **A7** (PI 33%) and **A8** (PI 11%) [11], it could be suggested that there is no correlation between *in vitro* and *in vivo* activity. Since the pharmacological properties and especially the mechanisms of transport across the cell membranes for these diaryl ethers are unknown, it is difficult to explain these results. Moreover, the diaryl ether structures imply extremely poor solubility in water. The nature of the substituents does not allow chemical modification of the active compounds in order to improve solubility and/or membrane transport (e.g. conversion to prodrugs – salts, esters, etc.). Further formulation of the *in vitro* active compounds through preparation of nanoparticles or complexes with water soluble polymers could increase significantly the *in vivo* effects.

2.4. QSAR analysis

QSAR analysis of substituent effects on the *in vitro* cytotoxicity (CC_{50}) and anti-viral activity (against PV1, CVB1, CVB3) of the 5-nitrobenzonitrile derivatives was carried out. A dataset consisting of 72 5-nitrobenzonitrile derivatives and one 3-trifluoromethylnitrobenzene derivative was used in this study. Structural descriptors for all investigated compounds were calculated using the simplex representation of molecular structure (SiRMS) approach [31,32]. The calculation of descriptors was carried out at the 2D level of molecular structure representation using the Dragon program. In this case only molecular topology is taken into account, i.e. all information is extracted from the structural formula. It should be noted that 2D-QSAR models are the most popular in structure-property studies [31,32]. The efficiency of such models is due to the fact that the topological model of the molecular structure implicitly contains information about the possible conformations of the molecule.

The simplex approach is based on isolating and counting the number of molecular fragments (pairs, triples, quadruples of atoms) in which a certain sequence of changes in some property is observed. That is, in the framework of SiRMS, any molecule can be represented as a system of different specific fragments (simplexes) of fixed composition and topology. Various atomic characteristics can be used for the vertex differentiation in the simplex, such as the uniqueness of the atom (atom nature or a more detailed type), partial charge, lipophilicity,

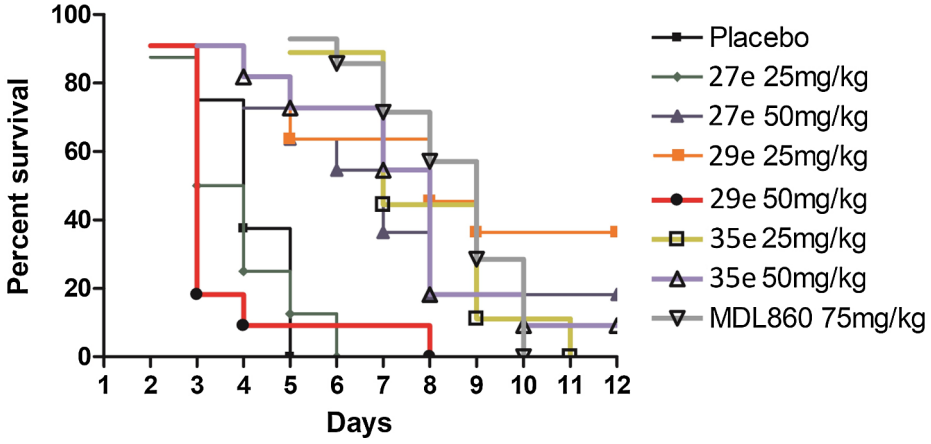


Fig. 4. Individual effects of compounds **27e**, **29e**, **35e** and MDL-860 in experimental neurotropic infection with Coxsackievirus B1 in newborn mice.

electronegativity, refraction, van der Waals interactions, H-bond donor/acceptor potential, etc. For atomic characteristics having real values (refraction, electronegativity, etc.) at the preliminary stage, the range of values is divided into a certain number of groups. The number of groups (G) is a tuning parameter of models and can vary (as a rule G = 3–7). In addition, electronegativity, refraction, molecular weight and octanol-water partition coefficient (LogP) are calculated as integral descriptors that describe the whole molecule.

Moreover, certain parameters calculated by Dragon program were also used [33]. Most of the Dragon descriptors are integral structural characteristics of the molecule, but many of them are difficult to interpret. Thus, simplex descriptors and Dragon descriptors were used for the development of models. A total of about 8000 structural descriptors were calculated for the evaluated molecules. The relationships between the calculated molecular descriptors and the investigated properties of these molecules were established by methods of partial least squares (PLS) [34] and random forest (RF) [35]. Primarily, mutually-correlated and constant parameters were eliminated. Then the procedure of Trend Vector [36] was used to form initial sets of molecular descriptors. The procedure of Genetic Algorithm was used for development of PLS-models [37].

In this study, QSAR model reflecting the structural influence of investigated compounds on their cytotoxicity was developed. In the preliminary model development it was found that compound **27e** is an outlier, so this molecule was excluded from the dataset. Thus, 72 molecules were included in the training set for model development. QSAR model was built using the PLS method with three latent variables (based on 25 descriptors in the final) and with the following statistical characteristics: determination coefficient for the training set $R^2 = 0.82$, coefficient of determination for cross-validation (leave one out) $Q^2 = 0.70$, standard error $S_{ts} = 84$, $S_{cv} = 110$ for training set and cross-validation, respectively. A "randomization" procedure (*Y-Scrambling*) was used to confirm the "non-randomness" of the developed QSAR model [31]. The statistical characteristics obtained using the *Y-Scrambling* procedure were lower in indices than in the final model: $R^2_{(scr)} = 0.21 \pm 0.02$, $Q^2_{(scr)} = 0.10 \pm 0.02$. Thus, the non-randomness of the established relationship between the structure of the studied compounds and their cytotoxicity (Fig. 5) can be stated. Nevertheless, even the approximated QSAR model is not of sufficient quality to predict cytotoxicity. We used it as an auxiliary for a qualitative assessment of the effect of substituents on cytotoxicity only (see Fig. 6).

The clear mechanistic interpretation is one of the advantages of SiRMS approach [31]. On the basis of developed QSAR models the influence of each atom over a particular property can be calculated. The contribution of each atom in the molecule can be defined as the ratio of the sum of PLS regression coefficients for all simplexes containing this atom to the number of atoms in the simplex. The atomic contribution depends on the number of simplexes that include this atom. The number of simplexes is not constant. It varies in different molecules and depends on other constituents. Thus, this contribution is non-additive. The analysis of such information allows selecting different fragments which have negative or positive influence on a considered property.

The relative influence of different substituents in the 2-position of the 5-nitrobenzonitrile moiety on cytotoxicity (CC_{50}) is shown in Fig. 6. It could be stated that the introduction of fluorine and chlorine atoms into the aromatic ring promotes greater cytotoxicity.

Further in this study, QSAR models reflecting the structural influence of the investigated compounds on the *in vitro* activity (IC_{50}) against poliovirus 1 (PV1) were developed. It was found that among the 73 compounds, only 20 exhibit anti-PV1 activities. Thus, the original activity values were coded as follows: 1-active and 0-inactive. In this case, RF method was used for decision of classification task. RF models were constructed according to the described original RF algorithm [35]. RF is an ensemble of single decision trees. Each tree has been grown as follows: (i) A bootstrap sample, which will be a training set for the current tree, is produced from the whole training set of *N* compounds. Compounds which are not in the current tree training set are placed in an out-of-bag (OOB) set (OOB set size is $\sim N/3$). (ii) The best split by

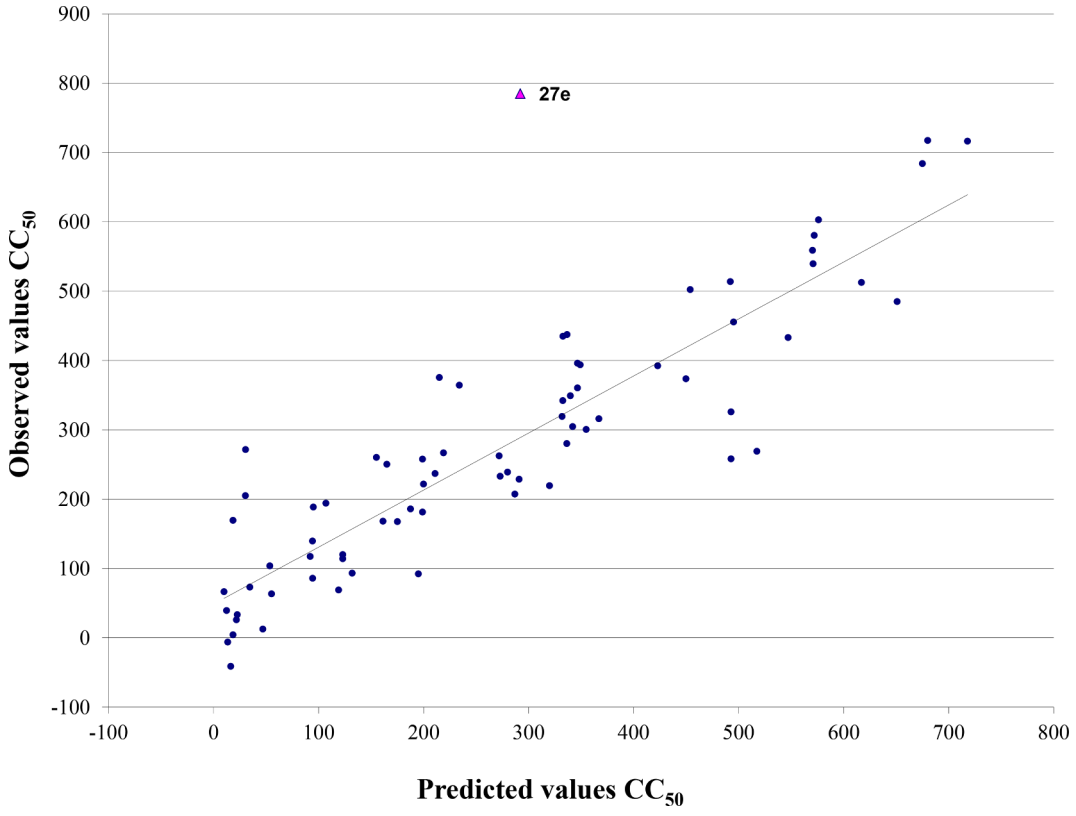


Fig. 5. Observed versus predicted diagram of cytotoxicity (CC_{50}) values for 73 molecules; compound **27e** is an outlier.

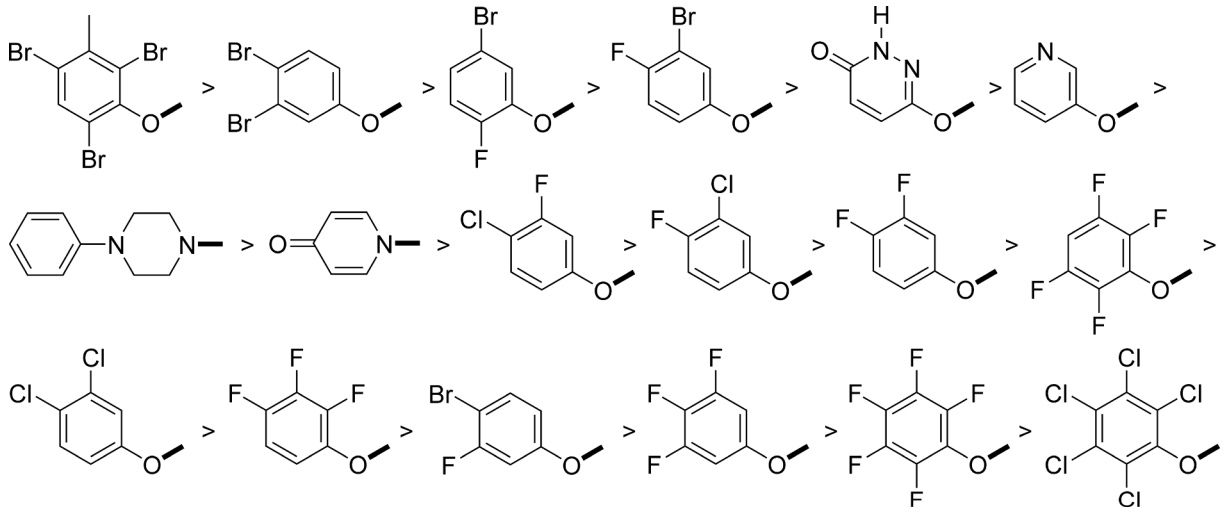


Fig. 6. Relative influence of different substituents in the 2-position of 5-nitrobenzonitrile on cytotoxicity (CC_{50}).

Table 7
Statistical parameters for classification models PV1.

No	OBB set	MCC	AC	SP	SE
1	20 + 53 = 73	0.58	0.84	0.91	0.65
2	20 + 20 + 53 = 93	0.90	0.95	0.91	1.0
3	20 + 20 + 20 + 53 = 113	0.90	0.95	0.89	1.0

Matthew's correlation coefficient: $MCC = (TP \times TN - FP \times FN)/((TP + FP) \times (TP + FN) \times (TN + FP) \times (TN + FN))^{1/2}$.
Accuracy: $AC = (TP + TN)/(TP + TN + FP + FN)$.
Specify: $SP = TN/(TN + FP)$.
Sensitivity: $SE = TP/(TP + FN)$.
TP = true positive; TN = true negative; FP = false positive; FN = false negative.

Table 8
Statistical parameters for classification models CVB1.

No	OBB set	MCC	AC	SP	SE
4	19 + 54 = 73	0.24	0.75	0.93	0.26
5	19 + 19 + 54 = 92	0.90	0.95	0.91	1.0
6	19 + 19 + 19 + 54 = 111	0.71	0.93	0.85	1.0

classification and regression tree (CART) algorithm [35] among the *m* randomly selected descriptors from whole set of *M* ones in each node is chosen. The value of *m* is just one tuning parameter for which RF models are sensitive. (iii) Each tree is grown to the largest possible extent (there is no pruning).

Since the dataset is unbalanced, i.e. the count of active and inactive molecules is significantly different, a special procedure for balance was used. The count of inactive molecules was constant (53 molecules) and the count of active ones was duplicated. In the first series, 20 active molecules (all of active and inactive – 73 molecules) were used; in the second series the count of active molecules was increased twofold, i.e. 40 active molecules (a total of 93 molecules); in the third series the count of active molecules was increased threefold – 60 molecules (a total of active and inactive – 113 molecules).

The resulting QSAR models for the training set showed an unmistakable classification. The predictive ability of the QSAR models was evaluated using the "out-of-bag" (OOB) procedure [35]. The quality of the classification models was assessed according to the following statistical characteristics (Table 7):

As it can be seen from Table 7, the balancing of models leads to a significant quality improvement. Model 2 could be considered as the most appropriate.

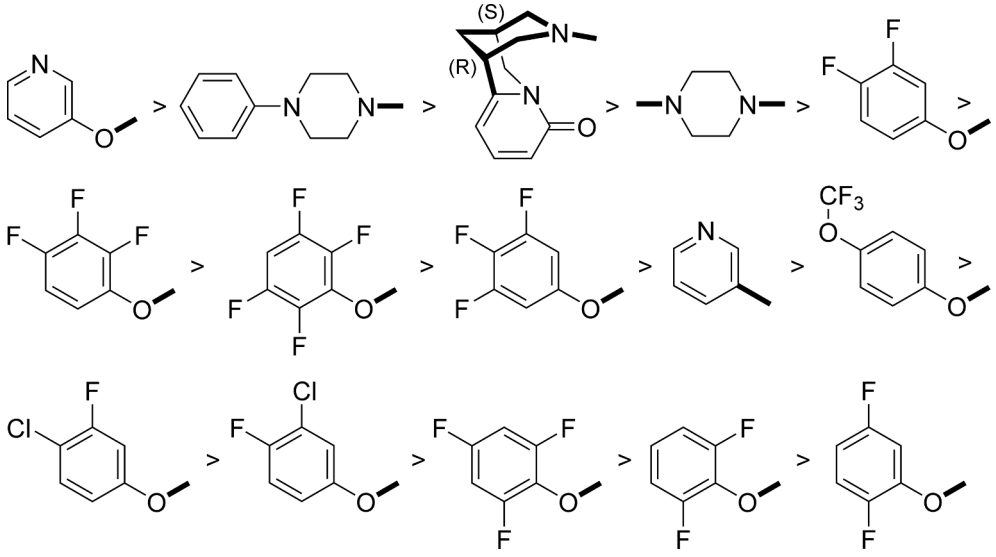


Fig. 7. Relative influence of different substituents in the 2-position of 5-nitrobenzonitrile on the activity against PV1.

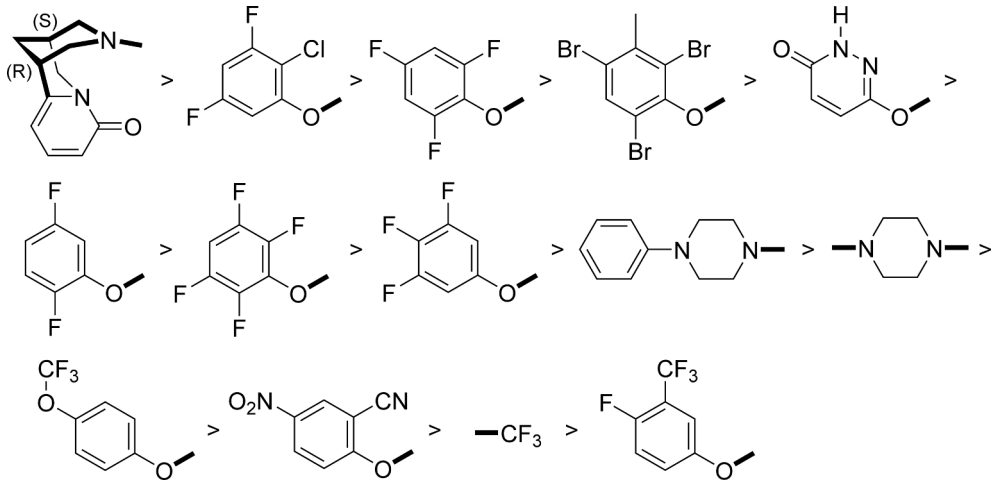


Fig. 8. Relative influence of different substituents in the 2-position of 5-nitrobenzonitrile on the activity against CVB1.

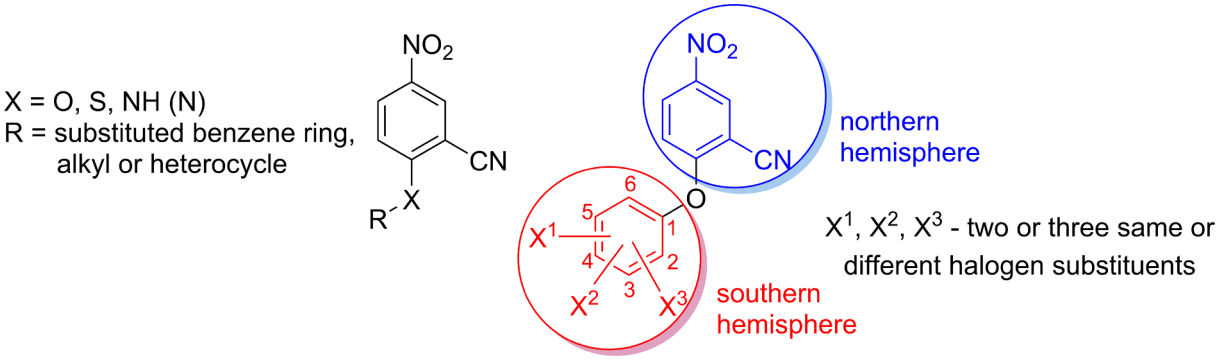


Fig. 9. General formula of all obtained compounds (left formula) and active 2-phenoxy-5-nitrobenzonitriles (right formula).

Interpretation analysis of the QSAR models allowed for estimation of the relative influence of the substituents in the 5-nitrobenzonitrile moiety on activity (Fig. 7). It can be seen, that nitrogen-containing substituents promote the given activity to a greater extent.

Further, classification QSAR models regarding the *in vitro* activity (IC_{50}) against coxsackieviruses B1 (CVB1) were developed. The model developments were carried out in a similar manner, except that in this case there were 19 active and 54 inactive molecules. This dataset was also balanced. RF method was used for decision of classification task.

Like the previous task, Model 5 is the best model with twice the number of active compounds (Table 8). The relative influence of substituents on antiviral activity is given in Fig. 8.

As it can be seen from this sequence, the character of the influence of substituents on anti-viral activity against CVB1 differs significantly from the similar influence on the anti-PV1 activity. It can be noted that the presence of cytisine moiety promotes both types of activity.

Unfortunately, there weren't adequate QSAR models for the anti-viral activity of the investigated compounds against CVB3. Obviously, this is due to the high imbalance in the training set which consists of only 8 active compounds out of 73.

Thus, classification QSAR models with adequate statistical characteristics were obtained to estimate the anti-viral activity of investigated compounds against poliovirus and coxsackiviruses. These models will be used in further studies for virtual screening and molecular design of new anti-viral agents corresponding to the "domain applicability" (DA) of developed QSAR models.

3. Conclusions

In summary, a series of 60 analogues of the anti-viral agent MDL-860 were synthesized and evaluated for activity against different types of enteroviruses. All compounds contain a 5-nitrobenzonitrile moiety bridged through a heteroatom (O, N and S) to a second aromatic ring bearing different substituents (Fig. 9, left formula). The compounds were subjected to an *in vitro* screening study for activity towards PV1, CVB1 and CVB3 viruses. The most active ones (6 compounds with SI > 50) were found among the diarylether derivatives (O as a bridge heteroatom). Moreover, the nature of the substituents in the secondary aromatic ring was found to be crucial for the anti-viral activity – only diarylethers containing two to three halogen substituents in the secondary benzene ring demonstrated promising anti-viral activity. Other type of substituents or the presence of heterocyclic moieties instead of a benzene ring generally led to lack of activity. The most active against CVB1 derivatives (**27e**, **29e** and **35e**) were tested for *in vivo* activity in newborn mice experimentally infected with 20 MLD_{50} of CVB1. Compound **29e** showed promising activity (protection index 26% and 4 days lengthening of mean survival time). Based on the experimental data obtained (including data from our previous study [11]), a generalization of the structure of the most active compounds against PV1, CVB1 and CVB3 viruses is shown in Fig. 9 (right formula).

QSAR analysis of substituent effects on the *in vitro* cytotoxicity (CC_{50}) and anti-viral activity (against PV1, CVB1, CVB3) of the nitrobenzonitrile derivatives was carried out, including data from our previous study [11]. The results obtained allowed to perform qualitative assessment of the effect of substituents on cytotoxicity of the compounds and to construct adequate QSAR models for anti-viral activity against PV1 and CVB1. These models could be useful for further virtual screening and molecular design of new anti-viral agents in accordance with the "domain applicability" (DA) of developed QSAR models.

Our study has revealed that the presented nitrobenzonitrile derivatives are promising class of anti-viral agents possessing high *in vitro* activity and selectivity toward PV1, CVB1 and CVB3 viruses accompanied with very low cytotoxicity. The poor water solubility of these compounds is a possible explanation for the absence of correlation between their *in vitro* and *in vivo* activity. Nevertheless, further *in vivo* experiments could be undertaken after an appropriate formulation of the active *in vitro* compounds. Thus, we have developed this class of compounds, including previously unexplored variation of the substituents in the southern hemisphere (Fig. 9). It could be concluded that within the current study, we exhausted all the possibilities for successful variations of the substituents in the southern hemisphere of MDL-860. It seems that changes in the northern hemisphere could be much more perspective for further investigations.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.02.020.

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