Synthesis and anti-CVB 3 evaluation of substituted 5-nitro-2-phenoxybenzonitriles

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ABSTRACT

The synthesis and SAR of a series of 60 substituted 2-phenoxy-5-nitrobenzonitriles (analogues of MDL-860) as inhibitors of enterovirus replication (in particular of coxsackievirus B3 (CVB 3)) are reported. Several of the analogues inhibited CVB 3 and other enteroviruses at low-micromolar concentrations.

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Enteroviruses are non-enveloped, single-stranded (+) RNA viruses belonging to the picornavirus family. This large family harbours several pathogens that are implicated in a wide range of clinical manifestations, affecting humans as well as animals.[1] It is estimated that enteroviruses cause each year 10–15 million (or more) symptomatic infections.[2] Although often mild and self-limiting, enteroviruses may also be involved in more serious conditions, which can be life-threatening, such as pancreatitis, meningitis, encephalitis or myocarditis.[2] Coxsackieviruses, and in particular CVB 3, have often been associated with the development of myocarditis, which may lead to sudden death in young adults or progress to dilated cardiomyopathy.[3–5]

In the past, several compounds have been reported to be selective inhibitors of enteroviruses, some of which have entered clinical trials.[6] Pleconaril is the prototype of a series of broad-spectrum picornavirus inhibitors, known as 'WIN-compounds'.[7] These compounds block replication by inhibiting host cell attachment and/or uncoating, as a result of binding into a pocket, located underneath the canyon on the virion surface.[8] Despite its proven efficacy in several studies,[9,10] pleconaril was not approved by the FDA for the treatment of the common cold (due to rhinovirus infections),[11] but is still being studied on a compassionate base for the treatment of life-threatening infections in children, such as meningitis or encephalitis, but on the other hand it is also being developed at

Schering-Plough, under license of Viropharma, for the treatment of rhinovirus infections in high-risk asthma and COPD (chronic obstructive pulmonary disease) patients. Pleconaril is inactive against the cardiotropic coxsackievirus B3 (Nancy strain).[12,13] Although several research groups are still working on the synthesis of antivirals structurally related to pleconaril,[14–16] the search for novel broad-spectrum inhibitors of enteroviruses replication remains compulsory.

MDL-860 (2-(3,4-dichlorophenoxy)-5-nitrobenzonitrile, **1**)[17–20] was previously reported to possess broad-spectrum in vitro activity against picornaviruses by inhibiting an early event in virus replication, after initial uncoating.[19] This compound also elicited in vivo efficacy in a model of coxsackievirus B3-induced myocarditis.[18] However, MDL-860 was never further developed. To gain insight into the structural features that might contribute to a potential improvement in antiviral activity, 60 analogues of this lead compound were synthesized[21] (Scheme 1) and evaluated[22] for inhibition of coxsackievirus replication.
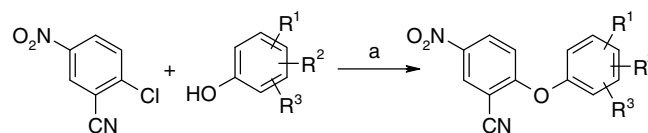
Scheme 1. Reagent and condition: (a) dry DMF, anhydrous K_2CO_3, room temperature, 24 h.

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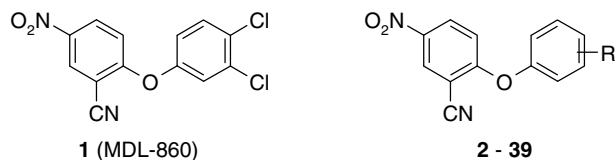
Figure 1. Structures of compounds **1** and **2**–**39**.

Figure 2. Structures of compounds **40**–**61**.

Table 1
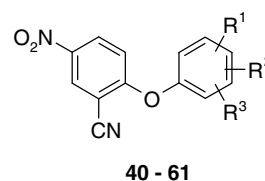Anti-CVB 3 activities and cytotoxicities for compounds **1** and **2**, and monosubstituted analogues **3**–**39**

Compound	R	IC$_{50}$[a] (µM)	CC$_{50}$[a] (µM)	S.I.[b]
1	–	6.5 ± 4.9	>324	>49.8
2	H	8.7 ± 1.5	>416	>47.8
3	2-Cl	6.9 ± 3.1	>364	>52.8
4	3-Cl	4.9 ± 0.9	>364	>74.3
5	4-Cl	5.0 ± 4.1	>364	>72.8
6	2-CH$_3$	57 ± 15	>393	>6.9
7	3-CH$_3$	6.4 ± 4.6	>393	>61.4
8	4-CH$_3$	6.2 ± 2.2	>393	>63.4
9	2-Br	>143	>269	1.9
10	3-Br	5.0 ± 1.9	>313	>62.6
11	4-Br	5.7 ± 4.3	>313	>54.9
12	2-OCH$_3$	54 ± 6.5	>370	>6.9
13	3-OCH$_3$	12 ± 2.2	>370	>30.8
14	4-OCH$_3$	46 ± 3.6	>370	>8.0
15	2-CN	11 ± 2.7	>377	>34.3
16	3-CN	7.4 ± 1.5	>377	>50.9
17	4-CN	4.1 ± 2.6	>377	>92.0
18	3-Isopropyl	40 ± 37	>354	>8.9
19	3-N(CH$_3$)$_2$	31 ± 15	>353	>11.4
20	3-NH$_2$	241 ± 52	>392	>1.6
21	4-NH$_2$	106 ± 68	>392	>3.7
22	3-COOH	>352	>352	n.a.
23	4-COOH	>352	>352	n.a.
24	3-COCH$_3$	57 ± 4.6	>354	>6.2
25	4-COCH$_3$	35 ± 2.3	>354	>10.1
26	3-NO$_2$	38 ± 22	>351	>9.2
27	4-NO$_2$	3.3 ± 0.4	>351	>106.4
28	3-NHCOCH$_3$	121 ± 26	>336	>2.8
29	4- NHCOCH$_3$	>336	>336	n.a.
30	3-I	5.7 ± 1.9	>273	>47.9
31	4-I	5.7 ± 3.1	>273	>47.9
32	3-NH-CO-NH$_2$	>335	>335	n.a.
33	3-NH-CS-NH$_2$	234 ± 40	>318	>1.4
34	4-SCH$_3$	93 ± 34	>349	>3.8
35	4-SOCH$_3$	205 ± 9.2	>331	>1.6
36	4-SO$_2$CH$_3$	174 ± 16	>314	>1.8
37	4-*tert*-Butyl	>338	>338	n.a.
38	4-CONH$_2$	>353	>353	n.a.
39	4-CH$_2$OH	121 ± 91	>370	>3.1

[a] Values are means of three independent experiments ± standard deviation.
[b] In vitro selectivity index (CC$_{50}$/IC$_{50}$).

Table 2
Anti-CVB 3 activities and cytotoxicities for di- and trisubstituted analogues **40**–**61**

Compound	R^1, R^2, R^3	IC$_{50}$[a] (µM)	CC$_{50}$[a] (µM)	S.I.[b]
40	2,3-Cl$_2$	4.9 ± 3.1	>324	>66.1
41	2,4-Cl$_2$	13 ± 9.6	>324	>24.9
42	2,5-Cl$_2$	3.8 ± 2.2	>324	>85.3
43	3,5-Cl$_2$	>81	81	n.a.
44	2-Cl-4-NO$_2$	39 ± 9.0	>313	>8.0
45	2-Cl-4-CN	7.3 ± 4.3	>334	>45.8
46	2-Cl-4-Br	3.7 ± 2.7	>283	>76.5
47	2-Br-4-Cl	3.7 ± 2.6	>283	>76.5
48	2-NH$_2$-4-Cl	>53	53	n.a.
49	3-Cl-4-Br	6.7 ± 0.4	>283	>42.2
50	2-CH$_3$-4-Cl	11 ± 9	>346	>31.5
51	3-Me-4-Cl	2.0 ± 1.6	>346	>173
52	3-Et-4-Cl	>330	>330	n.a.
53	2,4-Br$_2$	6.2 ± 0.1	>251	>40.3
54	3,4-Me$_2$	99 ± 71	>346	>3.5
55	3-Me-4-Br	2.6 ± 0.2	>300	>115.4
56	3-Me-4-NO$_2$	11 ± 2.2	>334	>30.4
57	3-NO$_2$-4-NH$_2$	10 ± 9.0	>333	>33.3
58	3,5-Me$_2$	>324	>324	n.a.
59	2,3,4-Cl$_3$	>146	146	n.a.
60	2,4,5-Cl$_3$	59 ± 27	>291	>4.9
61	2,4,6-Cl$_3$	72 ± 20	>291	>4.0

[a] Values are means of three independent experiments ± standard deviation.
[b] In vitro selectivity index (CC$_{50}$/IC$_{50}$).

better than that of the lead compound against CVB 3. In particular, 2,3-, 2,4- and 3,4-disubstitution with halogens (or in part with other substituents such as methyl, cyano or nitro) were beneficial for anti-CVB 3 activity. Most potent activities were measured for the 4-chloro-3-methyl analogue **51** and for the 4-bromo-3-methyl analogue **55**. Interestingly, formal replacement of the methyl group in compound **51** by an ethyl group resulted in an inactive analogue (**52**). Also, replacement of the chlorine in compound **51** by a second methyl group resulted in reduced anti-CVB 3 activity (**54**). Similarly, 3,5-disubstitution with chlorine (**43**) or methyl (**58**), or trisubstitution with chlorine (compounds **59**–**61**) resulted in—more or less—inactive analogues.

A selection of six compounds was evaluated against the other five coxsackie B viruses (Table 3). All of the analogues, with the exception of **51**, which was inactive against CVB 4 and 6, had broad-spectrum activity with analogues **3** and **40** being the most potent ones.

Table 3
Activities (on Vero cells, µM)[a] of selected compounds against CVB 1, 2, 4, 5 and 6

Compound	CVB 1	CVB 2	CVB 4	CVB 5	CVB 6
1	15 ± 6.9	15 ± 11	17 ± 9.9	18 ± 9.4	12 ± 5.2
2	6.9 ± 3.3	17 ± 6.9	26 ± 3.3	10 ± 4.8	19 ± 4.2
3	5.6 ± 1.4	6.2 ± 0.7	14 ± 7.3	3.1 ± 2.1	8.1 ± 0.5
7	8.9 ± 3.1	11 ± 3.3	22 ± 1.0	13 ± 6.1	22 ± 4.4
40	3.3 ± 1.7	8.6 ± 6.3	17 ± 0.6	4.8 ± 2.2	10 ± 4.2
51	4.8 ± 3.4	5.2 ± 0.8	>346	2.1 ± 0.5	>346

[a] Values are means of three independent experiments ± standard deviation.

First, the unsubstituted analogue **2** and a set of 38 monosubstituted MDL-860 analogues (**3**–**39**) (Fig. 1) were synthesized and tested against CVB 3 (Table 1). Several compounds exhibited activities and selectivities comparable to that of the lead compound (**1**), in particular when substituted with a halogen, a cyano group or a 4-nitro group. Introduction of polar and/or bulkier substituents seemed to result in reduced antiviral activities. There also appeared a preference—at least for some substituents—for positions 3 and 4.

Recently, it has been proposed that the anti-human rhinovirus 2 (HRV-2) activity of MDL-860 and its analogues is highly dependent on hydrophobicity.[23] This may possibly also be applied for the activities against enteroviruses within this class of inhibitors.

Next, a set of 22 di- and trisubstituted MDL-860 analogues (**40**–**61**) was prepared (Fig. 2, Table 2). Again, several of these compounds had activities and selectivities comparable to or slightly

Table 4
Activities (on MRC-5 cells, μM)[a] of selected compounds against selected non-CVB picornaviruses

Compound	CVA 24	Echo 9	Echo 11	Entero 68
1	1.8 ± 0.9	>324	6.7 ± 3.7	12 ± 9.0
2	>240	>416	>416	>416
3	23 ± 7.7	35 ± 18	35 ± 15	22 ± 7
5	>364	>364	>364	>364
7	19 ± 9.4	>393	>393	76 ± 28
27	>350	>350	>350	>350
40	>309	>323	>323	>323
42	>323	>323	>323	>323
51	234 ± 70	>346	>346	>346

[a] Values are means of three independent experiments ± standard deviation.

Nine compounds were selected for evaluation against a set of 4 non-CVB enteroviruses (Table 4). This selection was based on the activities against CVB 3 and on diversity with respect to the substitution pattern of the phenoxy substructure. The lead compound MDL-860 (**1**) proved active against CVA 24, echovirus 11 and enterovirus 68, but was inactive against echovirus 9. Only the 2-chloro analogue (**3**) proved active against all four non-CVB enteroviruses, and five analogues (**2**, **5**, **27**, **40** and **42**) were inactive against any of the four viruses. Compound **40**, therefore, seemed to be a selective inhibitor of coxsackie B virus replication. Compound **7** had moderate activity against CVA 24 and enterovirus 68, whereas the 3-methyl-4-chloro analogue **51** exhibited only weak activity against CVA 24.

The exact mode of action of this class of compounds against enteroviruses has yet to be figured out. Without knowing the target (and the structural differences of the target within the different enteroviruses), it is very difficult to fully understand the obtained structure–activity relationships (SARs).

In summary, several of the MDL-860 analogues are selective inhibitors of CVB replication with activities that are comparable to or slightly better than that of the lead compound. The SAR of this class of compounds for CVB 3 does, however, not parallel the SAR for other enteroviruses. At least one compound (**3**) was identified that had broad-spectrum enterovirus activity and that was—unlike the lead compound MDL-860—active against echovirus 9. Further exploration of this class of compounds should allow obtaining further insights into the SAR for inhibition of enterovirus replication. Also studies are underway to identify the molecular target of this class of compounds. Once this target has been identified, this information may help to rationally design analogues with improved antiviral activity.

Acknowledgments

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21. All compounds were characterized by ^{1}H NMR spectra, MS spectra, combustion analyses and melting points. All analogues except **33**, **35** and **36** were prepared by reaction of 2-chloro-5-nitrobenzonitrile with an appropriately substituted phenol (Scheme 1). Compound **33** was synthesized by reaction of **20** with potassium thiocyanate in a mixture of toluene and 6 N HCl (6 h at 90 °C). Compounds **35** and **36** were prepared by oxidation of compound **34** with metachloroperbenzoic acid (1 or 2.5 equiv, respectively) in dichloromethane at ambient temperature. Synthesis of MDL-860 (**1**) as an example: To a mixture of 2-chloro-5-nitrobenzonitrile (300 mg), 3,4-dichlorophenol (268 mg, 1 equiv) and anhydrous potassium carbonate (341 mg, 1.5 equiv) was added 3 mL of dry DMF, and the resulting suspension was stirred for 24 h at ambient temperature (tlc control: silica gel, eluent: light petroleum/ethyl acetate = 3:1 (v/v)). Then, water (50 mL) was slowly added and the resulting precipitate was filtered, washed with water and dried. The crude product was purified by recrystallization from a mixture of diisopropyl ether (20 mL) and ethyl acetate (7 mL); off-white crystals; mp: 156–158 °C (155–156 °C);[17] yield: 63%; ^{1}H NMR (200 MHz, DMSO-d_6) δ 8.88 (d, 1H, H6, *J* = 2.8 Hz), 8.43 (dd, 1H, H4, *J* = 9.3, 2.8 Hz), 7.84–7.78 (m, 2H, H2′/5′), 7.39 (dd, 1H, H6′, *J* = 8.8, 2.8 Hz), 7.19 (d, 1H, H3, *J* = 9.3 Hz); MS (CI): *m/z* calcd for $C_{13}H_6Cl_2N_2O_3$ (M$^+$) 308.0, found 307.9; Anal. ($C_{13}H_6Cl_2N_2O_3$): Calcd C, 50.51; H, 1.96; N, 9.06. Found C, 50.54; H, 2.09; N, 8.97.
22. *Antiviral assay.* The antiviral activity of the selected compounds was determined using an MTS-based cytopathic effect (CPE) reduction assay and was expressed as the 50% effective concentration (EC$_{50}$), or the concentration of compound that inhibits virus-induced cytopathic effect formation by 50%. Cells, grown to confluency in 96-well plates, were infected with 100 CCID$_{50}$ of virus, one CCID$_{50}$ being the 50% cell culture infective dose. After an adsorption period of 2 h at 37 °C, virus was removed and serial dilutions of the compounds were added. The cultures were further incubated at 37 °C for 3 days, until complete CPE was observed in the infected and untreated virus control (VC). After removal of the medium, 90 μl medium and 10 μl MTS/PMS (Promega, Leiden, The Netherlands) were added to each well. After an incubation period of 2 h at 37 °C, the optical density of each well was read at 498 nm in a microplate reader. CPE values were calculated as follows: % CPE = 100*[OD$_{CC}$ − OD$_{CVB3+Compound}$]/[OD$_{CC}$ − OD$_{VC}$]. In these formulae, OD$_{CC}$ corresponds to the optical density of the uninfected and untreated cell cultures, OD$_{VC}$ represents the infected and untreated cell cultures and OD$_{CVB3+Compound}$ are CVB3-infected cell cultures, treated with a given concentration of compound. *Cytotoxic assay.* The cytotoxicity of the compounds was evaluated by the MTS-method and the 50% cytotoxic concentration (CC$_{50}$) was calculated. Briefly, the same experimental set-up was used as for the antiviral assay, but for cytotoxicity determination, uninfected cultures were incubated with serial dilution of compound for three days at 37 °C. The cytotoxic activity was calculated using the following formula: % CPE = 100*[OD$_{CC}$−OD$_{Compound}$]/OD$_{CC}$, where OD$_{CC}$ corresponds to the optical density of the uninfected and untreated cell cultures and OD$_{Compound}$ are uninfected cultures, treated with compound.
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