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Exploration of the anti-enterovirus activity of a series of pleconaril/pirodavir-like compounds

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Abstract

Background: The *Enterovirus* genus of the Picornaviridae is represented by several viral pathogens that are associated with human disease, namely Poliovirus 1, Enterovirus 71 and Rhinoviruses. Enterovirus 71 has been associated with encephalitis, while Rhinoviruses are a major cause of asthma exacerbations and chronic obstructive pulmonary disease. Based on the structure of both pleconaril and pirodavir, we previously synthesized some original compounds as potential inhibitors of Rhinovirus replication.

Methods: These compounds were explored for in vitro antiviral potential on other human pathogenic Enteroviruses, namely Enterovirus 71 on rhabdo-myosarcoma cells, Coxsackievirus B3 on Vero cells, Poliovirus 1 and Echovirus 11 on BGM cells.

Results: Activity was confirmed for compound against Rhinovirus 14. Furthermore, few compounds showed a cell-protective effect on Enterovirus 71, presented a marked improvement as compared to the reference drug pleconaril for inhibitory activity on both Enterovirus 71 and Poliovirus 1. The most striking observation was the clear cell protective effect for the set of analogues in a virus-cell-based assay for Echovirus 11 with an effective concentration (EC_{50}) as low as 0.3 μM (Selectivity index or SI = 483), and selectivity indexes greater than 857 ($EC_{50} = 0.6$ μM) and 1524 ($EC_{50} = 0.33$ μM).

Conclusion: Some of the evaluated compounds showed potent and selective antiviral activity against several enterovirus species, such as Enterovirus 71 (EV-A), Echovirus 11 (EV-B), and Poliovirus 1 (EV-C). This could be used as a starting point for the development of other pleconaril/pirodavir-like enterovirus inhibitors with broad-spectrum activity and improved effects as compared to the reference drugs.

Keywords

Enterovirus, antivirals, pleconaril, pirodavir, early stage

Introduction

The genus *Enterovirus* of the family Picornaviridae is divided into 12 species (enterovirus EV-A to-J, and rhinovirus HRV-A to -C), each of which contains multiple (sero) types, which in turn group a wide variety of strains. Human pathogens like poliovirus (PV), enterovirus (EV), Coxsackievirus (CV) and rhinovirus (HRV) are well-known representatives.[1,2] In general, enterovirus infections are sub-clinical or very mild, such as the common cold caused by rhinoviruses. However, PV, Enterovirus 71 (EV71) and several other members of this virus genus are known to cause inflammation of the central nervous system,[3,4] and rhinovirus infections have been associated with asthma exacerbation and chronic obstructive pulmonary disease.[5,6]

Enteroviruses are un-enveloped, icosahedral virions that contain a single-stranded positive-sense RNA genome which encodes, amongst others, four structural proteins (VP-1 to -4). The hydrophobic pocket beneath the groove in the VP1 capsid protein is a well-known

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target for the development of antivirals, which are also known as 'capsid binders': interference with virus entry and uncoating is brought about through interaction with the VP1 capsid protein.[7,8] Pleconaril (Schering Plough) and pirodavir (R77975, Janssen) are two of the best studied capsid binders. Pleconaril has broad-spectrum antiviral activity against several entero- and rhinoviruses, and positive clinical outcomes of naturally acquired rhinovirus infections have been reported after pleconaril treatment.[9] However, the side effects of pleconaril treatment (CYP induction) have not outweighed the burden of disease, and the drug has not been approved by the FDA.[10] Pirodavir is a potent *in vitro* inhibitor of both HRV-A and HRV-B.[7] Despite this *in vitro* activity, treatment of naturally occurring rhinovirus infections has not produced any clinical benefits and has been associated with side effects, including nasal dryness and an unpleasant taste.[11,12] Recently, a clinical phase II trial with vapendavir (BTA798, Biota Holdings), a pirodavir derivative, was successfully completed in asthma patients: treatment with vapendavir significantly reduced the respiratory symptoms caused by naturally acquired rhinovirus-induced asthma exacerbations.[13]

We have previously described a panel of novel compounds **[6c–10c]**, which were developed based on the core structure of both pleconaril and pirodavir with substantial modifications in the central hydrocarbon chain and the pyridazinyl-piperidinyl moiety (see supplemental material for chemical data).[14]

Materials and methods

The purpose of the present study was to explore the antiviral potential of the above-mentioned compounds together with an additional set of similar substances (compounds **[6002–6702]**)[15] on the replication of a selection of other human pathogen enteroviruses, namely EV71, poliovirus 1 (PV1), coxsackievirus B3 (CVB3), and echovirus11 (ECHO11). The synthesis and chemical properties of the molecules of the series **[6c-10c]** were previously described by Bernard et al.,[14] and also by Laconi et al.[15] for those of series **[6002–6702]**. Pleconaril was kindly provided by V. Makarov (RAS, Institute of Biochemistry, Russia). For independent confirmation of the anti-rhinovirus activity observed for some of the compounds,[14] a multi-cycle, virus-cell-based cytopathogenic effect (CPE) reduction assay for rhinovirus HRV-A2 and HRV-B14 was performed in HeLa cells. HeLa Rh cells (a HeLa subclone, highly susceptible to rhinovirus-induced CPEs, kindly provided by K. Andries (Janssen Pharmaceutica, Belgium)), Buffalo green monkey (BGM, ECACC 90092601) cells, Vero cells (ATTC CCL-81) and human rhabdomyosarcoma (RD, ECACC 85111502) cells were grown in MEM Rega3 medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Integro), 2 mML-glutamine (Gibco) and 0.075% NaHCO3(Gibco) at 378°C and 95–99% relative humidity in a 5% CO_2 incubator. Both rhinovirus serotypes HRV-2 and HRV-14 were kindly provided by K. Andries, and were cultivated on HeLa Rh cells in the presence of 30 mM $MgCl_2$. Enterovirus 71 strain BrCr (EV71 BrCr), a gift from F. van Kuppeveld (Universiteit Utrecht, The Netherlands), was grown on RD cells. PV 1 strain Sabin (BGM cells), derived from infectious clone pT7/S1F, was obtained from A J Macadam.[16] Echovirus 11 (ECHO11) strain Gregory was obtained from K. Andries and Coxsackievirus B3 (CVB3, strain Nancy) was derived from plasmid p53CB3/T7,[17] both were propagated on BGM cells.

The antiviral activity of the compounds was evaluated in an MTS (3 -(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)-based assay. Briefly, HeLa Rh cells were seeded at a density of 1.8×10^4 cells per well (100 mL) in 96-well cell culture plates (Falcon, BD Biosciences). Rhinovirus assays were performed using MEM Rega3 medium supplemented with 2% FBS, 2 mM-glutamine, 0.075% $NaHCO_3$ and 30 mM $MgCl_2$. For the remaining picornaviruses, assays were performed in the same medium in the absence of $MgCl_2$. Cells were allowed to adhere overnight, after which serial dilutions of the compound and virus inoculum (multiplicity of infection (moi) optimized for each virus strain) were added. The cultures were subsequently incubated for three days at 35 or 37°C, for rhinovirus and the other picornaviruses, until complete virus-induced CPEs were observed in the untreated and infected virus control conditions (VC). After removal of the medium, 100 mL Phenol Red-free MEM (Invitrogen) containing 5% MTS-phenazine (Promega) were added. Following a 1 h incubation period at 35 or 37°C, the optical density of each well at 498 nm (OD 498) was determined using a microplate reader (Safire 2, Tecan). The optical density values were converted to control percentages and logarithmic interpolation was used to calculate the 50% effective concentration (EC_{50}) as the compound concentration resulting in a 50% protective effect against virus-induced CPE. In addition, the cell morphology of each compound condition was evaluated microscopically in a multi-cycle assay on a confluent cell layer for minor signs of CPE or for adverse effects caused by the compound. CC_{50} = 50% cytotoxic concentration, selectivity index (SI), EC_{50}/CC_{50}.

Results

The activity of compound **8c**[8c] against HRV-B14 was confirmed.[15] An average EC_{50} of 34 μM was obtained, which is higher than the published value of 1.4 μM (Table 1). The double oxygen in the central aliphatic

Table 1. Antiviral activity[a] against HRV-A2 and HRV-B14.

		HRV-A2			HRV-14		
Compound	$CC_{50} \pm SD$ (μM)	EC_{50} (μM)	EC_{90}	SI	EC_{50} (μM)	EC_{90}	SI
6b	213 ± 84	>213	–	–	>213	–	–
6c	>659	>330	–	–	>247	–	–
7a	49 ± 13	>49	–	–	>49	–	–
8a	>591	>296	–	–	>296	–	–
8b	>560	>280	–	–	>280	–	–
8c	>507	>281	–	–	34 ± 11	68.2	>15
9c	>636	>318	–	–	>318	–	–
9d	81 ± 21	>81	–	–	>81	–	–
10c	236 ± 178	>236	–	–	>236	–	–
6002	5.6 ± 0.9	>5.6	–	–	>5.6	–	–
6005	34 ± 6	>34	–	–	>34	–	–
6230	24 ± 5	>24	–	–	>24	–	–
6232	>553	>277	–	–	>277	–	–
6273	285 ± 21	>285	–	–	>285	–	–
6373	213 ± 28	>213	–	–	>213		–
6473	>63	>63	–	–	>63	–	–
6501	11 ± 4	>11	–	–	>11	–	–
6502	12 ± 5	3.6 ± 1.3	4.7	2.4	4.1 ± 0.4	6.16	3
6702	>68	>68	–	–	>68	–	–
Pleconaril[b]	>131	0.1 ± 0.1	–	>1310	0.3 ± 0.2	–	>437

–: not tested; SI: selectivity index, calculated as CC_{50}/EC_{50}.

[a]Antiviral activity was assessed in a virus-cell-based CPE reduction assay with MTS read-out in HeLa cells (Rh strain). CC_{50} = the calculated concentration of compound that induces 50% reduction in cell viability (on confluent cell monolayer), as compared to an untreated, uninfected control condition. EC_{50} = the calculated concentration of compound that induces a 50% cell protective effect in treated, infected cells. Values are median ± median absolute deviation, calculated using the values derived from dose response curves of ≥four experiments of which at least two were set up independently.

[b]100% inhibition of virus-induced cytopathic effect that can be achieved with this compound (as determined by microscopic inspection).

moiety coupled to the acetyl-phenyl group seemed determinant for the anti-rhinovirus activity of compound **8c**[8c]. Similar activity against both HRV-A2 and HRV-B14 was only shown by the other heterocyclic derivative **6502**[6502], although with a rather small selectivity index (SI = 2.4 and 3, respectively). In this case, the central amino-pentyloxy-benzoate group played a key role for 6502[6502] activity. Subsequently, the antiviral activity of the compounds was assessed against four prototype enterovirus strains. The EV-A clade was represented by EV71, EV-B by CVB3 and ECHO11 and EV-C by PV1. For PV1 and ECHO11, the multi-cycle, virus-cell-based CPE reduction assays were performed using Buffalo Green Monkey (BGM) cells, while Vero cells were used for CVB3 and RD cells for EV71. Compounds **6230**[6230], **6273**[6273] and **6502**[6502] induced a cell protective effect in the EV71 (EV-A) assay with a 50% effective concentration (EC_{50}) of, respectively 15, 11 and 14 μM (Table 2). Taking into account the adverse effect of the compounds on uninfected cells (CC_{50}, 50% adverse effect on cell metabolism), it was possible to calculate a maximum selectivity index ($SI = CC_{50}/EC_{50}$) of >52 for compound **6273**[6273]. The majority of compounds were not active in the assay for CVB3 (EV-B): an EC_{50} of 11 μM with a marginal SI = 1 was only obtained for compound **6501**[6501], (Table 2). In addition, only compound **6273**[6273] was found to exhibit a limited cell protective effect in the assay for PV1 (EV-C) (Table 3). However, as compared to pleconaril, compound **6273**[6273] also showed a clear improvement in anti-PV1 activity, with an EC_{50} of 11 ± 1, and a SI of >47 vs. 0 of the reference drug; the EC_{90} was not determined for compound solubility problems.

In sharp contrast, a cell protective effect was observed for all but one of the compounds in the assay for ECHO11 (EV-B), with EC_{50}s in the range between 0.3 and 108 μM (SI ranging from 3 to >857 and 1524) (Table 3). It is interesting to note that at least one concentration of seven of these compounds was able to completely inhibit the production of virus-induced

Table 2. Antiviral activity against EV71 (EV-A clade) and CVB3 (EV-B).

		EV71				CVB3		
Compounds	CC_{50} (μM)	EC_{50} (μM)	EC_{90}	SI	CC_{50} (μM)	EC_{50} (μM)	EC_{90}	SI
6b	435 ± 82	>328	–	–	245 ± 20	>245	–	–
6c	>659	>330	–	–	>659	>330	–	–
7a	190 ± 4	>190	–	–	282 ± 185	>279	–	–
8a	>532	>296	–	–	>591	>296	–	–
8b	>560	>280	–	–	>560	>280	–	–
8c	>563	>281	–	–	>563	>281	–	–
9c	>636	>318	–	–	>636	>318	–	–
9d	481 ± 25	>344	–	–	223 ± 4	>223	–	–
10c	>609	>305	–	–	>34	>34	–	–
6002	>130	>130	–	–	12 ± 1	>12	–	–
6005	59 ± 0.3	>59	–	–	69 ± 7	>69	–	–
6230	>32	15 ± 11	>32	>2	21 ± 4	>21	–	–
6232	277	>277	–	–	277	>277	–	–
6273	>571	11 ± 0.4	19.8 ± 0.2	>52	>143	>143	–	–
6373	>136	>26	–	–	>122	>102	–	–
6473	>71	>53	–	–	>128	>107	–	–
6501	>72	>36	–	–	12 ± 0.4	11 ± 1	>12	1.0
6502	28 ± 0.7	14 ± 5	>28	2.0	20 ± 6	>20	–	–
6702	>272	>272	–	–	>290	>272	–	–
pleconaril	>131	15 ± 12	–	>9	62 ± 15	>62	–	–

–: not tested.
See also legend of Table 1. RD cells were used instead of HeLa cells for EV71. Vero cells were used for CVB3.

cytopathic effects, without causing any adverse effects on host cell and monolayer morphology, which was assessed by microscopic inspection. For substances **6002**[6002] to **6702**[6702], in general, only a limited adverse effect was observed on BGM cells (high CC_{50} values which are indicative of low cytotoxicity) with **6273**[6273] and **9d**[9d] showing the highest selectivity (SI >857 and 1524, respectively) against echovirus-induced cell death. A structure-activity analysis (SAR) on some of the most active compounds of the series **6002–6702**[6002-6702], namely **6273**[6273], **6473**[6473], **6373**[6373], revealed that they essentially differ in the composition of the central aliphatic chain, showing that –NH > –O > –S (SI = 857, 278, 188, respectively); moreover, the substance **6002**[6002] (SI = 483) also presents an amino group in the central chain that confirms a key role of this chemical group for the inhibition of the ECHO11 virus. For compounds **6b**[6b] to **10c**[10c], an adverse effect on BGM cells could only be observed at high concentrations. Only molecules **9d**[9d] and **8c**[8c] showed a significant window of antiviral activity (SI of 1524 and >134, respectively), which implies that the insertion of a double oxygen in the central aliphatic region, common to most of this series of molecules, does not give any advantage in terms of activity. However, the best results for both cytotoxicity and antiviral activity were obtained by molecule **9d**[9d] that shows a fluorine atom in the right phenyl group (SI = 1524); changing the –F atom with an acetylphenyl moiety, as in **9c**[9c], leads to an almost 50-fold decrease in inhibitory activity.

Discussion

Previously obtained data indicated that compound **8c**[8c] interferes with early steps of rhinovirus infection, which is in accordance with the known action mechanism of the parent compounds pleconaril and pirodavir.[14] The difference between the present values and those obtained in the previous work[14] can be largely attributed to the setup of the assay (HeLa cells Ohio strain and HRV strains from ATCC on sub-confluent cells were used in the previous mentioned paper). The fact that activity depends on the assay setup is a quite common observation for capsid-binding compounds. The majority of rhinoviruses (including HRV-B14) use the ICAM-1 receptor, while, to date, only 11 HRV strains (minor group, including HRV-A2) have been shown to use members of the LDL-receptor

Table 3. Antiviral activity against echovirus 11 (EV-B) and poliovirus 1 (EV-C).

		ECHO11			PV1		
Compounds	CC_{50} (μM)	EC_{50} (μM)	EC_{90}	SI	EC_{50} (μM)	EC_{90}	SI
6b	435 ± 35	49 ± 12	–	9	>61	–	–
6c	>659	77 ± 14	171 ± 6.0	>9	>330	–	–
7a	374 ± 3	19 ± 2	51.2	20	>279	–	–
8a	>591	108 ± 20	218	>5	>296	–	–
8b[a]	>560	11 ± 2	24.2 ± 0.8	>51	>280	–	–
8c[a]	>563	4.2 ± 0.2	6.86 ± 0.8	>134	>281	–	–
9c	>636	22 ± 1	30.7 ± 3.1	>29	>318	–	–
9d[a]	503 ± 8	0.33 ± 0.04	0.73 ± 0.1	1524	>344	–	–
10c	107 ± 7	>107	>107	<1	>107	–	–
6002[a]	>145	0.3 ± 0.2	0.41 ± 0.2	483	>145	–	–
6005	60 ± 1	9.4 ± 1.8	12.8 ± 1.4	6	>60	–	–
6230	9 ± 1	2.3 ± 0.5	2.88 ± 0.1	4	>9	–	–
6232	>277	0.18 ± 3	2.88 ± 0.3	1538	>277	–	–
6273	>514	0.17 ± 0.2	0.38 ± 0.2	>857	11 ± 1	nd	>47
6373[a]	>245	1.3 ± 0.2	3.21 ± 0.6	>188	>204	–	–
6473[a]	>142	0.51 ± 0.02	0.83 ± 0.7	>278	>142	–	–
6501	11 ± 0.2	3.7 ± 0.5	4.06 ± 0.5	3	>287	–	–
6502	7 ± 0.5	1.8 ± 0.4	3.95 ± 0.7	4	7 ± 0.5	>7	–
6702[a]	> 544	35 ± 2.3	63.9 ± 9.1	16	>272	–	–
pleconaril	>131	1.7 ± 0.4	–	>77	>131	–	–

nd: EC90 value was not achievable at the highest concentration tested.
See also legend of Table 1. BGM cells were used instead of HeLa cells.
[a]100% inhibition of virus-induced cytopathic effect that can be achieved with this compound (as determined by microscopic inspection).

family for host cell binding and entry.[18] This difference in receptor usage may partly explain the difference in the antiviral activity of compounds **8c**[8c] and **6501**[6501] against HRV-B14 vs. HRV-A2. The capsid binders pirodavir and pleconaril have been shown to block interaction with the ICAM-1 receptor for some major HRV strains, while inhibition of other rhinovirus strains seems to be primarily associated with stabilization of the virion, which as such blocks virus entry and uncoating. Therefore, a mechanism involving increased virion stability may explain the almost equipotent activity of compound **6502**[6502] against both HRV-A2 and HRV-B14. Concerning compound **6273**[6273], it resulted almost five times less cytotoxic than pleconaril, but more than five times as active in inhibiting EV71 (SI >52 vs. >9, respectively). This improvement of antiviral activity could be due to the amino group in the central chain coupled to a methylisoxazol moiety, which is characteristic of this molecule. Moreover, the presence of a sulfur atom in the long central aliphatic chain differentiates compound **6501**[6501] from the others and may therefore be important for its inhibitory, albeit modest, activity against the virus CVB3. It would be interesting to synthesize more sulfur-containing **6501**[6501] derivatives in order to explore this property in the context of selective anti-CVB compounds.

In conclusion, some of the evaluated compounds show selective antiviral activity against several enterovirus species, such as EV71 (EV-A), ECHO11 (EV-B) and PV1 (EV-C). This could be used as a starting point for the development of other pleconaril/pirodavir-like enterovirus inhibitors with broad-spectrum activity and a different structure. Of particular interest is the observation of the activity of all the molecules against ECHO 11, with EC_{50s} that reach low μM and even high nM values. It is also worth noting that compound **6273**[6273] presents a marked improvement as compared to the reference drug pleconaril for inhibitory activity on EV71 and PV1.

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Conflict of interest

None declared.

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