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Short communication

R75761, a lead compound for the development of antiviral drugs in late stage poliomyelitis eradication strategies and beyond

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Abstract

In this study the antiviral activity of a panel of 18 out of 240 pyridazinamine analogues was evaluated against the Sabin strains of the three poliovirus types. We found one compound, R75761 which had a comparable 50% effective concentration (EC_{50}) value against all three poliovirus Sabin strains. Virus multiplication was reduced by $10^{4.0}$-fold, $10^{6.2}$-fold and $10^{6.6}$-fold for poliovirus type 1, type 2 and type 3, respectively. R75761 could be considered as a lead compound for development of anti-poliovirus drugs to be used during the late stage of poliovirus eradication and beyond.

Keywords: Anti-poliovirus compound; Polio eradication; Pyridazinamine analogue; Picornavirus; Poliovirus

Nobody would have thought 20 years ago when the World Health Assembly adopted the resolution of global polio eradication (World Health Assembly, 1988), that the "endgame" of this program would be so complex. The basic idea was to eliminate polio worldwide by the year 2000 using large-scale vaccination with the oral polio vaccine (OPV). The program of polio eradication was immediately very successful and has led to a dramatic decrease from an estimated 350,000 paralytic polio cases occurring in more than 125 endemic countries on 5 continents in 1988 (World Health Organization, 2002) to 1997 polio cases, restricted to only a few countries in 2006.[1] However, the ultimate success of the program to obtain a permanently poliovirus-free world without protective antibodies is a more daunting task than ever expected. When the current strategy for the "endgame" of poliovirus would be implemented by cessation of OPV usage, this strategy would result in the rapid increase of non-immune human populations (Agol, 2006). Therefore, a planned cessation of OPV is associated with a very high risk of polio outbreaks which can be caused by: (i) genetically unstable Sabin OPV strains which may lead to sufficient genetic changes transforming them into circulating highly divergent vaccine-derived polioviruses (VDPV) to assume characteristic wild poliovirus neurovirulence and transmissibility in vaccine recipients and contacts (Kew et al., 2002); (ii) immunocompromised individuals who may cryptically harbour polioviruses for many years (up to 10 years and more) after receiving OPV and pose a very great threat because of their ability to excrete and spread VDPV going unnoticed for years (Halsey et al., 2004); (iii) frequent genetic exchanges of Sabin strains with other species C enteroviruses and (vaccine-derived) polioviruses (Kew et al., 2002); (iv) reintroduction of wildtype polioviruses and vaccine strains in the community either unintentionally (from existing crypting sources) or on purpose (by bioterrorists); and (v) emerging of a poliovirus-like agent by mutation from contemporary C-cluster Coxsackie A virus (Jiang et al., 2007).

Consequently, it has been suggested by the Centers for Disease Control and Prevention and the World Health Organization

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[1] http://www.polioeradication.org/casecount.asp (Data as at 10 October 2007).

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that if, as currently planned, universal vaccination with OPV is discontinued, it would be extremely useful and may, in fact, be essential to have additional tools to control outbreaks of poliomyelitis in an increasingly susceptible world (Committee on Development of a Polio Antiviral and its Potential Role in Global Poliomyelitis Eradication, 2006). Candidate poliovirus antivirals and public health strategies might enhance the flexibility of outbreak response options in the post-OPV era by protecting recipients from infection, limiting spread through prophylaxis of susceptible contacts and reducing virus shedding of infected persons until immunity can be assured and helping to clear poliovirus from persistently infected individuals. To develop a successful antiviral drug to prevent poliovirus transmission, the identification and optimisation of active agents are required (Committee on Development of a Polio Antiviral and its Potential Role in Global Poliomyelitis Eradication, 2006).

So far, a relative extensive number of compounds have been shown to be potent inhibitors of the replication of several picornaviruses in vitro (for a recent review, see Barnard, 2006). A series of these anti-picornavirus antivirals have been tested in a comparative study for their ability to inhibit the in vitro replication of the three poliovirus types. A striking conclusion was that the activity of each of the tested compounds was always lower towards poliovirus type 1 than both other types with the exception of the pyridazinamine analogues and the protease inhibitors rupintrivir and Compound **1** which had potent and equivalent activities against all three poliovirus types (De Palma et al., 2008). One of the most potent inhibitors of in vitro poliovirus replication in this study was a pyridazinamine analogue. These pyridazinamine analogues are known to exert an antiviral activity on the early picornaviral replication steps (i.e., attachment, entry and/or uncoating) through their binding into hydrophobic pockets within the viral capsids and are commonly known as capsid-binding compounds (Andries et al., 1989). In addition to their antiviral activity, these compounds also stabilize the viral capsid against thermodenaturation and thereby preventing the loss of antigenicity of the heat-sensitive Sabin strains used in OPV (Rombaut et al., 1996).

In this study a panel of 18 out of 240 pyridazinamine derivatives, which were originally assayed for their ability to stabilize the OPV, were selected on basis of a previous study in which they showed antiviral activity (Rombaut et al., 1996). The cytotoxicity and the antiviral activity expressed as, respectively, 50% cytotoxic concentration (CC_{50}) and 50% antivirally effective concentration (EC_{50}) of the selected compounds were determined for each of the three Sabin strains (Table 1) using the same method originally described by Andries et al. (1992). Please note that different endpoints were used to measure the cytotoxicity and the antiviral activity (both tests are described in the caption of Table 1). The antiviral activity of these compounds is here also lower towards poliovirus type 1 than both other types, except for two compounds, namely R75761 and R78206, which had the lowest EC_{50} values against all three poliovirus Sabin strains. Selectivity indices towards the three Sabin strains of $\geq$9583 and $\geq$1944, respectively, were calculated. R75761 and R78206 were

Table 1
Comparison of cytotoxic and antiviral activities of selected compounds against the three types of poliovirus in HeLa cells

Compound no.	CC_{50}[a] (μM)	EC_{50} (μM)[b]			Selectivity index[c]		
	HeLa cells	Sabin type 1	Sabin type 2	Sabin type 3	Sabin type 1	Sabin type 2	Sabin type 3
75761	288	0.030	0.003	0.015	9583	115,000	19,167
77089	118	0.103	0.028	0.020	1146	4,273	5,875
77805	28	0.200	0.083	0.023	138	333	1,222
77975	>125	2.263	0.668	0.123	>55	>187	>1,020
78206	88	0.045	0.005	0.008	1944	17,500	11,667
79083	33	1.158	0.220	0.118	28	148	277
79654	348	2.425	0.085	0.070	143	4,088	4,964
79892	278	9.873	2.278	2.875	28	122	97
80619	115	1.878	0.355	0.410	61	324	280
80671	>125	2.723	0.083	0.120	>46	>1,515	>1,042
81586	95	16.118	0.205	0.263	6	463	362
81974	118	0.100	0.010	0.030	1175	11,750	3,917
82461	>125	17.613	14.523	2.333	>7	>9	>54
83774	88	0.273	0.110	0.018	321	795	5,000
84028	125	0.295	0.158	0.018	424	794	7,143
84199	110	18.110	3.280	1.000	6	34	110
84736	273	0.613	0.908	0.085	445	300	3,206
88597	173	2.275	0.570	0.075	76	303	2,300

[a] CC_{50} is defined as the 50% inhibitory concentration for cytotoxicity. The assay is described fully elsewhere (Andries et al., 1992). Briefly, HeLa cells were treated with various concentrations of the antiviral compounds and the number of living cells present in triplicate cultures were determined at the time of addition and every 24 h for 3 days. Following trypsinization, the number of viable cells was counted in triplicate with a Coulter Counter (model ELT8; Ortho).

[b] EC_{50} is defined as the 50% antivirally effective concentration to inhibit cytopathicity. The assay is described fully elsewhere (Andries et al., 1992). Briefly, 100 $CCID_{50}$ doses of virus were mixed with serial dilutions of the antiviral compound. After incubation, the mixtures were added to susceptible cells, and after further incubation, the viability of the infected cells were quantified spectrophotometrically by a tetrazolium colorimetric method.

[c] Selectivity index: $[CC_{50}]/[EC_{50}]$.

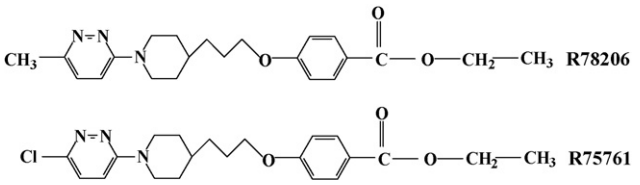

Fig. 1. Chemical structure of R78206 and R75761.

selected for further study and their chemical structure is depicted in Fig. 1. The cell number used to determine the cytotoxicity is a rather insensitive measure and therefore cytotoxicity may be underestimated.

The dose-dependent antiviral activity of R75761 and R78206 was further evaluated on multiple rounds of virus replication by means of cytopathic effect (CPE) reduction and infectious virus yield reduction assays. R75761 and R78206 inhibited viral replication of poliovirus type 2 and 3 by 100% as measured by CPE inhibition formation at concentrations that had no effect on infected host cells (Fig. 2). The maximum yield reduction achieved for poliovirus type 3 by R75761 and R78206 was,

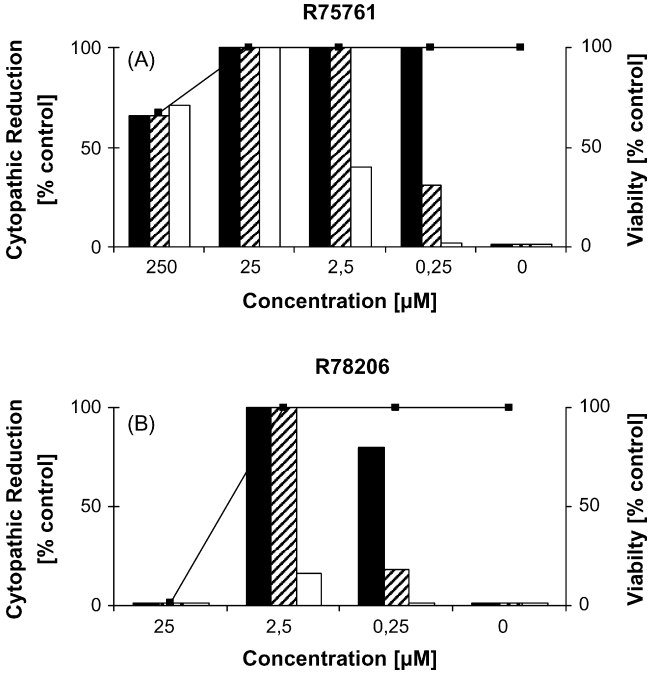

Fig. 2. Effect of R75761 (A) and R78206 (B) on cytopathic effect (bars) induced by poliovirus type 1 Sabin (open bars), poliovirus type 2 Sabin (shaded bars) or poliovirus type 3 Sabin (black bars) and cytotoxicity (lines) in HeLa cells. The CellTiter-Blue® Cell Viability Assay (Promega, The Netherlands) was used to monitor cell viability of infected or uninfected cells in order to calculate CPE reduction and cytotoxicity. For CPE values, HeLa cells (4×10^4 cells) were infected with 100 PFU of virus together with compound. Stock solutions of both compounds of 25 mM were prepared in dimethylsulfoxide (DMSO) and diluted in PBS. Cultures were incubated for 3 days until complete CPE was observed in infected cells without compound but with DMSO (control cultures). After removal of the medium, CellTiter-Blue® Reagent was added to each well and the cells were incubated for 2 h at 37 °C prior to recording fluorescence ($560_{EX}/590_{EM}$). Data are expressed as percent viability compared to the control cultures. Cytotoxicity was determined by the same-experimental set-up as for CPE reduction except that uninfected cultures were used. Data are expressed as percent viability compared to uninfected and untreated cell cultures.

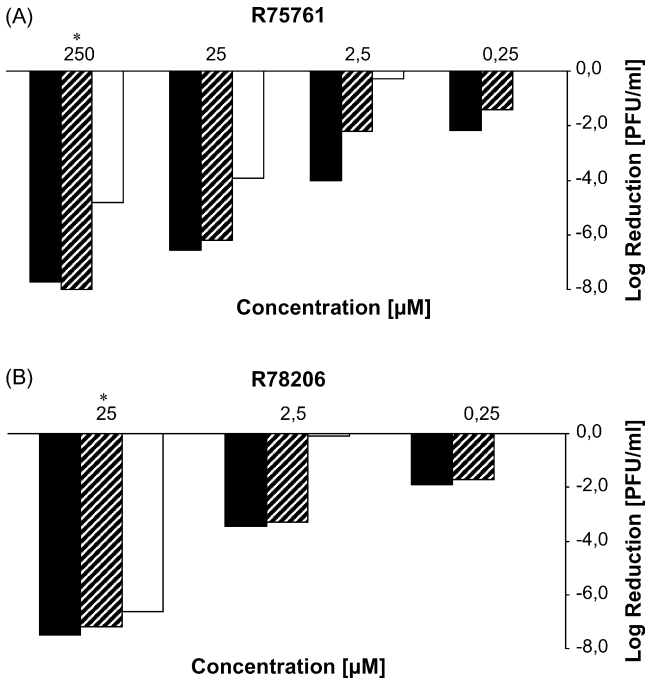

Fig. 3. Effect of R75761 (A) and R78206 (B) on infectious virus production in infected HeLa cell cultures by poliovirus type 1 Sabin (open bars), poliovirus type 2 Sabin (shaded bars) or poliovirus type 3 Sabin (black bars). HeLa cells (4×10^4 cells) were infected with 100 PFU of virus together with compound. Stock solutions of both compounds of 25 mM were prepared in dimethylsulfoxide (DMSO) and diluted in PBS. Cultures were incubated for 3 days until complete CPE was observed in infected cells without compound but with DMSO (control cultures). Virus yield of the supernatant was measured by a standard plaque assay. Data are expressed as the log reduction in virus yield compared to control cultures. *Cytotoxic concentration as determined in Fig. 2.

respectively, $10^{6.6}$-fold and $10^{3.4}$-fold and for poliovirus type 2, respectively, $10^{6.2}$-fold and $10^{3.3}$-fold at concentrations that showed no signs of cytotoxicity (Fig. 3A and B). For poliovirus type 1, only R75761 was able to fully block CPE formation at a concentration of 25 μM where no cytotoxicity for the infected host cells could be observed (Fig. 2A) and virus yield was 10^4-fold reduced compared with a control culture (Fig. 3A). However, R78206 never reached a level of 100% protection for CPE formation by poliovirus type 1 (Fig. 2B) and had little or no effect on poliovirus type 1 yield reduction (Fig. 3B) ($10^{0.1}$-fold reduction at 2.5 μM) at non-cytotoxic concentrations. Similar results for CPE reduction and infectious virus yield reduction by R75761 and R78206 were obtained for poliovirus type 1 Mahoney strain (data not shown). Poliovirus type 2 and 3 were reduced by R78206 in a same dose-dependent manner as by R75761 (Fig. 3A and B).

Although R75761 and R78206 have similar EC_{50} values towards poliovirus type 1 (as determined in a single replication cycle), they have different CPE reduction values (as determined in multiple replication rounds). R78206 has serious shortcomings to protect cells against poliovirus type 1 and this decreases the interest for R78206 to be used as a potential lead compound for the development of a polio antiviral. R75761, on the other hand, has the most potent activity to reduce CPE formation and virus yield of poliovirus type 1 in multiple repli-

cation rounds (both tests are described in detail in the legend of Figs. 2 and 3). The same is true about the antiviral activity of R75761 against the different poliovirus types. Although R75761 has similar EC_{50} values towards the three types of poliovirus, the antiviral activity of R75761 against poliovirus type 2 and 3 is more pronounced then against poliovirus type 1 in an assay with multiple replication rounds. Nonetheless R75761 reduced poliovirus type 1 yield by 10^4-fold. Expressing the antiviral activity of a compound only based on a EC_{50} value is therefore not a good indication and could be misleading. It would be better that additional tests based on multiple replication rounds are performed next to the EC_{50}. These multiple replication rounds assays reflect better the natural replication of the virus.

One drawback for the use of R75761 and other pyridazinamine derivates is the presence of an ester functionality in their structure which undergoes facile hydrolysis to an inactive acidic form in vivo resulting in a poor pharmacokinetic profile (Andries, 1993). A new class of oxime ether derivatives of the pyridazinamine analogue pirodavir, however, has been developed. These derivatives have been shown to have, besides antirhinovirus activity, a potent antiviral activity against poliovirus type 1 but, having a limited activity towards both other types (Barnard et al., 2004); they also appear to be orally bioavailable in animal models (Watson et al., 2003).

In conclusion: (i) the compound R75761 has antiviral activity towards all three poliovirus types; (ii) R75761 is active against vaccine derived and wildtype poliovirus; (iii) the EC_{50} of R75761 lies in the low micromolar range. This taken together with the facts that (i) capsid-binding compounds, in general, interrupt critical replication functions, (ii) the likelihood that these compounds may thwart the emergence of resistance in combination with other antiviral compounds acting on a different target (Committee on Development of a Polio Antiviral and its Potential Role in Global Poliomyelitis Eradication, 2006) and (iii) pyridazinamine analogues are well studied (McKinlay et al., 1992), a synthesis approach is now in progress leading to novel (pyridazinyl oxime ether) compounds that are related to the structure of R75761 and are orally available which in turn then can be used in the final stages of polio eradication and beyond to guarantee a safe discontinuation of OPV vaccination, to protect vaccinated individuals, to restrict possible outbreaks and to prevent possible virus shedding by infected individuals.

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