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Short Communication

# Towards the design of combination therapy for the treatment of enterovirus infections

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ABSTRACT

We report here on a comparative study of the activity of 10 enterovirus inhibitors against poliovirus 1, enterovirus 71 and human rhinovirus 14. Three of the selected molecules (Pleconaril, BTA-798 and V-073) are in clinical development. The in vitro antiviral activity of pairwise combinations of inhibitors indicated that most combinations resulted in an additive to slightly synergistic antiviral activity. However, the combination of ribavirin with a nucleoside polymerase inhibitor resulted in a pronounced antagonistic effect.



Most infections with enteroviruses have a mild or asymptomatic course. However, especially in neonates and infants, enterovirus infections can result in severe and life threatening conditions including myocarditis, pancreatitis and meningitis (Sawyer, 2002). In recent years, enterovirus 71 (EV71) has repeatedly caused epidemics in children, particularly on the Asian continent, resulting in high numbers of brain stem encephalitis and fatal cases (Chang, 2008). The EV71 epidemic is cautiously monitored and new cases are reported on a regular basis. There is obviously a need for potent drugs against EV71. Another member of the enterovirus genus that has regained interest is poliovirus (PV). Despite the fact that polio has been officially eradicated in Europe and the Americas, the virus continues to pose a threat in a number of African and Asian countries. Experts claim that global eradication of polio, which was initially foreseen by the year 2000, can only be achieved if at least two different anti poliovirus drugs (preferably with a different mode of action) be developed (Couzin, 2006). These drugs could be used to treat infected individuals, to contain outbreaks once vaccination has ceased, or to clear persistently infected patients (Collett et al., 2008). Human rhinoviruses (HRVs) comprise a large group within the enterovirus genus, consisting of more than 100 different serotypes, that in healthy individuals are associated with the development of common cold symptoms. Therefore, these viruses usually do not pose a menace to human health, although they have a large economical and social impact as lost school and working days (Patick, 2006). In patients with asthma or chronic obstructive pulmonary disease (COPD), rhinoviruses can have a more serious impact as increasing evidence confirms that exacerbations of COPD are triggered by infections with a respiratory virus, mostly a rhinovirus (Hershenson and Johnston, 2006). In this context, the development of a potent anti-rhinovirus agent is urgently needed. Not only is COPD predicted by the World Health Organization to become the third leading cause of death worldwide by 2030, one also has to keep in mind that vaccine development to prevent rhinovirus infections is technically unfeasible due to the high number of different serotypes. Hence, antiviral therapy remains the only specific treatment option.

In the past, many classes of molecules have been identified and reported that selectively inhibit the replication of enteroviruses (De Palma et al., 2008b). Several compounds were brought to clinical trials, mostly for the treatment of rhinovirus-induced colds, but eventually no drug was ever approved for further development (Senior, 2002). Three anti-enterovirus compounds are currently in clinical development. Pleconaril is being developed as an oral formulation for the treatment of enteroviral sepsis syndrome in neonates, whereas oral BTA798 is in a phase 2 study treating asthmatic adults with symptomatic human rhinovirus infection (De Palma et al., 2008b). Moreover, V-073 is being investigated for the treatment of poliovirus infections. We here report a comparative study on the in vitro antiviral activity of 10 known enterovirus inhibitors

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0166-3542/$ - see front matter © 2011 Elsevier B.V. All rights reserved.
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against enterovirus 71, poliovirus 1 and rhinovirus 14. An aspect that has to be considered with caution in the development of antivirals for enteroviruses is the risk of drug resistance development due to the high mutation rate of RNA viruses (Vignuzzi et al., 2006). To reduce the risk of resistance development, the combined use of antivirals with a different mode of action has proven a valuable strategy for other RNA viruses, including HCV and HIV (Cooper, 2005). We therefore wanted to explore the combined in vitro antiviral activity of combinations of enterovirus inhibitors.

Among the molecules included in this study, pleconaril was one of the earliest anti-rhinovirus drugs known as "capsid binders". These compounds prevent uncoating and/or attachment of the virus to the host cell by binding into a hydrophobic pocket underneath the floor of a canyon on then viral capsid (Smith et al., 1986). Pirodavir (Andries et al., 1992), the metabolically more stable analogue BTA798 (Watson et al., 2003) and V-073 (Buontempo et al., 1997) show a similar mode of action as pleconaril. Rupintrivir (AG-7088), an inhibitor of the viral 3C protease is a highly potent inhibitor of HRV replication that was shown to be well tolerated in man but for which clinical development was halted (Patick, 2006). Enviroxime is a benzimidazole derivative that is presumed to interfere with some function of the viral protein 3A, although its exact mode of action remains to be elucidated (Hsu et al., 2010; Heinz and Vance, 1995). Akin to enviroxime, TTP-8307 is a compound that was shown to induce resistance mutations in the non-structural protein 3A (De Palma et al., 2009). Ribavirin is a broad-spectrum antiviral with activity against several RNA viruses and 2′-*C*-methylcytidine is a nucleoside analog initially developed as an inhibitor of HCV replication but that is also active against other +ssRNA viruses, such as the foot-and-mouth disease virus (Goris et al., 2006; Coelmont et al., 2006). MDL-860 was previously reported to have broad-spectrum antiviral activity against enteroviruses with a yet undetermined mode of action (Powers et al., 1982).

The antiviral activity of compounds (alone or combined) was determined using a colorimetric cytopathic effect (CPE) reduction assay and data were expressed as $EC_{50}$, being the concentration of compound that inhibits virus-induced CPE formation by 50%. Cells, grown to confluency in 96-well plates, were infected with 100 $CCID_{50}$ (50% cell culture infective dose) and treated with serial dilutions of the compound. The cultures were incubated at 37 °C (35 °C for rhinovirus) for 3 days, until complete CPE was observed in the infected and untreated virus control (VC). Following incubation with MTS/PMS the optical density of each well was read at 498 nm in a microplate reader. CPE values were calculated as follows: % CPE = $[OD_{CC} - OD_{Virus+Compound}]/[OD_{CC} - OD_{VC}]$. In these formulae, $OD_{CC}$ corresponds to the optical density of the uninfected and untreated cell cultures (cell control), $OD_{VC}$ represents the infected and untreated cell cultures (virus control) and $OD_{Virus+Compound}$ are virus-infected cell cultures, treated with a given concentration of compound. The effect of drug–drug combinations were evaluated with the Mac Synergy II software developed by Prichard and Shipman, analyzing data for synergism, antagonism, or additive effects (Prichard and Shipman, 1990). When a combination is additive, data points form a horizontal surface that equals the zero plane. A surface that lies above the zero plane indicates a synergistic effect of the combination, a surface below the zero plane indicates antagonism. For each combination, a checkerboard with eight different concentrations (2-fold dilution) was studied. The graphs in Figs. 1 and 2 show the surfaces corresponding to the range of relevant concentrations, i.e. the concentrations where synergism or antagonism was present. Four independent experiments were carried out and average data are presented.

The antiviral activity of the selected compounds against poliovirus 1, rhinovirus 14 and enterovirus 71 is presented in Table 1. Enviroxime exerted more or less equipotent activity against all three viruses (with $EC_{50}$ values below 1 μM). Human rhinovirus 14 was inhibited most potently by the capsid binders pleconaril, pirodavir and BTA798 as well as by the viral protease inhibitor rupintrivir. The capsid binder V-073 appeared to be the most potent inhibitor of poliovirus replication with $EC_{50}$ values in the low nanomolar range but the compound had no effect on enterovirus 71. This latter virus was most potently inhibited by BTA798 with an $EC_{50}$ value of 200 nM. The other capsid binders resulted either in relatively potent anti-enterovirus 71 activity (pirodavir,

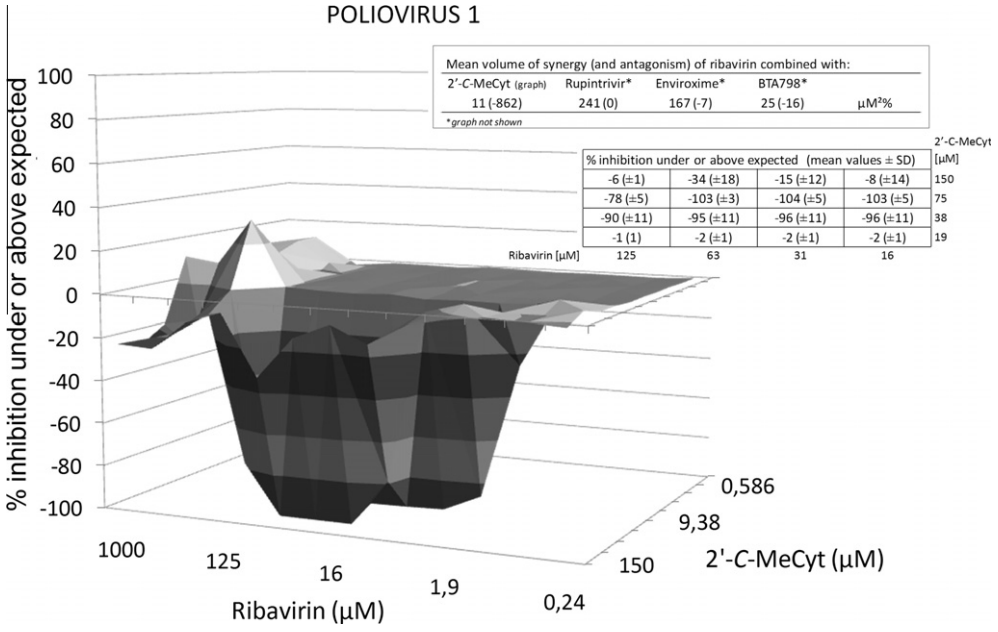



**Fig. 1.** In vitro anti-poliovirus activity of ribavirin combined with 2′-*C*-methylcytidine (graph + table) or with rupintrivir, enviroxime and BTA-798 (table only). Values under the zero plane indicate antagonistic activity, values in the zero plane indicate additive activity, and values above the zero plane indicate synergistic activity. All data points are averages of four independent experiments. Mean volumes of synergy (antagonism) are presented based on 95% confidence values.

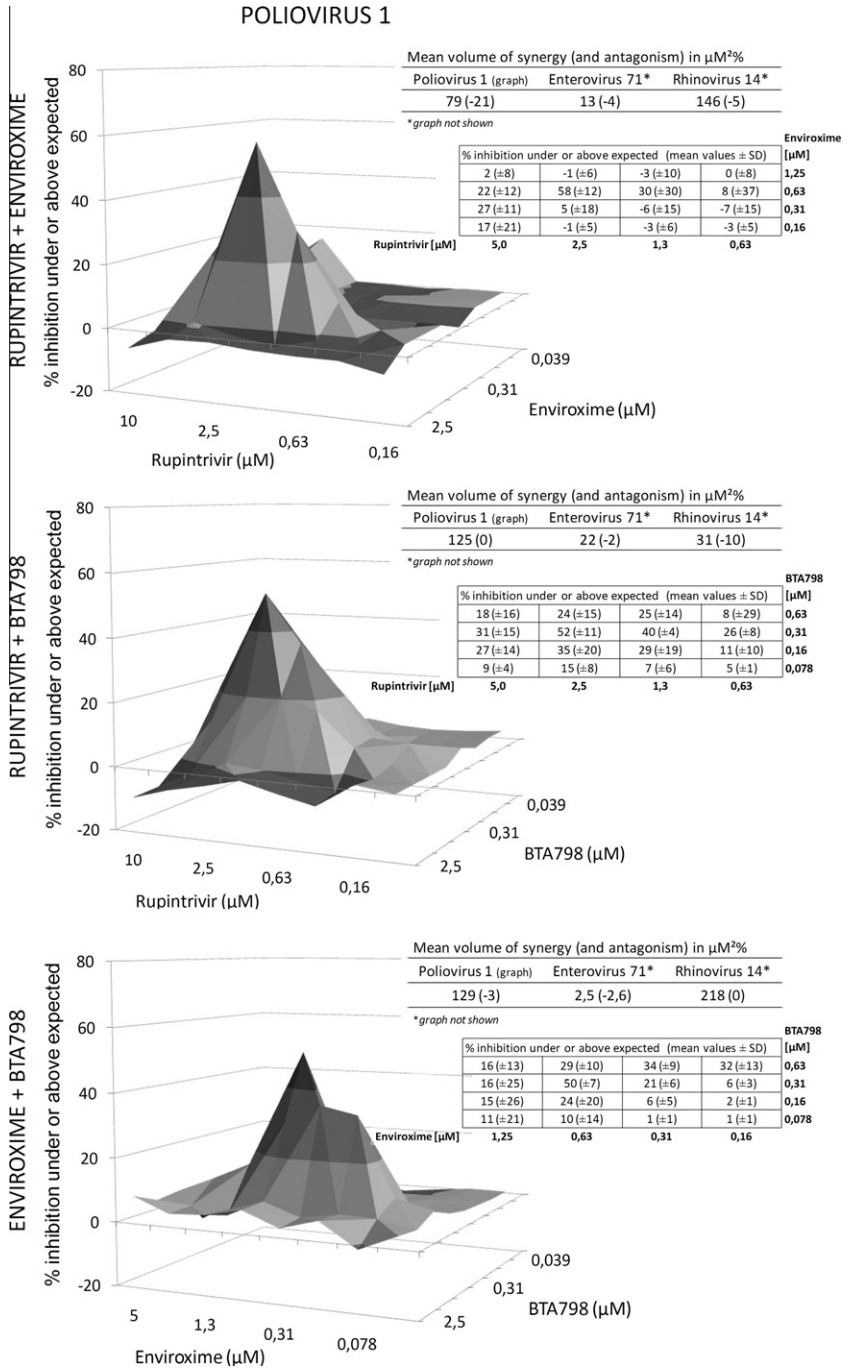
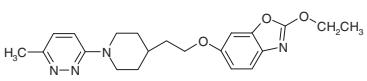
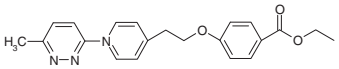
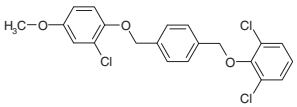
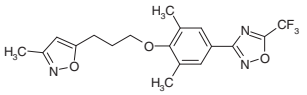
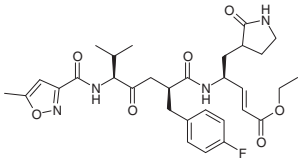
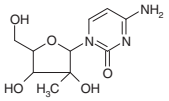
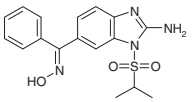
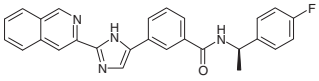
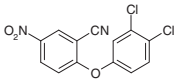
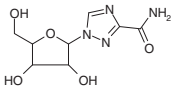



**Fig. 2.** Combined antiviral activity of BTA-798, enviroxime and AG7088 against poliovirus Sabin 1 (graph + table) or enterovirus 71 and human rhinovirus 14 (table only). Values under the zero plane indicate antagonistic activity, values in the zero plane indicate additive activity, and values above the zero plane indicate synergistic activity. All data points are averages of four independent experiments. Mean volumes of synergy (antagonism) are presented based on 95% confidence values.

$EC_{50}$ = 0.44 µM) or proved totally inactive against enterovirus 71 (pleconaril and V-073, $EC_{50}$ > 300 µM). Marked differences in susceptibility of enteroviruses to capsid binders are thus observed. Rupintrivir inhibited replication of poliovirus 1 and enterovirus 71, but did so less efficient than for human rhinovirus 14. The anti-poliovirus activity of rupintrivir in BGM cells was somewhat less pronounced than in HeLa cells. Indeed, in HeLa cells the anti-poliovirus activity was at least 10-fold more potent than in BGM cells (De Palma et al., 2008a).

Next we wanted to evaluate the combined effect of antivirals on the replication of enteroviruses. Our laboratory previously reported that ribavirin antagonizes the anti-hepatitis C virus activity of 2′-*C*-methylcytidine (Coelmont et al., 2006). Likewise we observed in the current study that the combination of these two molecules resulted in marked antagonistic antiviral effect against poliovirus. In fact, at certain concentrations the antiviral effect of the combination was 100% lower than the predicted antiviral effect if the activity of both compounds would be additive (−862 µM²%, Fig. 1). On the other hand, when ribavirin was combined with other molecules, such as for example BTA798, no antagonism was observed (Fig. 1). Instead, when ribavirin was combined with either rupintrivir or enviroxime, a slight synergistic activity was observed. Ribavirin has been

**Table 1**
Effect of selected anti-enterovirus compounds on the replication of three enteroviruses.

| | | Human Rhinovirus 14 | Poliovirus 1 | Enterovirus 71 |
|---|---|---|---|---|
| BTA798 |  | 0.020 ± 0.009 | 0.55 ± 0.14 | 0.20 ± 0.02 |
| Pirodavir |  | 0.027 ± 0.001 | 20 ± 2 | 0.44 ± 0.34 |
| V-073 |  | >10 | 0.026 ± 0.023 | >300 |
| Pleconaril |  | 0.061 ± 0.021 | >300 | >300 |
| Rupintrivir (AG-7088) |  | 0.011 ± 0.004 | 4.5 ± 0.36 | 0.90 ± 0.07 |
| 2′-*C*-Met-Cyt |  | 12 ± 2 | 27 ± 1 | 27 ± 1 |
| Enviroxime |  | 0.21 ± 0.09 | 0.79 ± 0.13 | 0.45 ± 0.12 |
| TTP-8307 |  | 2.4 ± 0.4 | 1.1 ± 0.8 | 2.6 ± 0.2 |
| MDL-860 |  | 0.71 ± 0.01 | 0.88 ± 0.09 | 3.8 ± 5.2 |
| Ribavirin |  | >2000 | 320 ± 46 | >2000 |

Data are expressed as $EC_{50}$ values (μM) and are means ± SD for at least three independent experiments.

reported to function as an RNA virus mutagen, forcing the virus into "error catastrophe" (Crotty et al., 2000). Although it is likely that ribavirin and 2′-*C*-methylcytidine interfere with each other's metabolism, the precise mechanism of the marked antagonism between both nucleoside analogs remains to be studied.

We next selected three compounds (i.e. the capsid binder BTA798, the protease inhibitor rupintrivir and the 3A-targeting compound enviroxime) that all have different mechanisms of action and for which (unlike the case with ribavirin and 2′-*C*-methylcytidine) it is expected that there will be no interference with each other's mechanism. For each possible combination of the three compounds, the vast majority of data points resulted in a more or less horizontal surface that equals the zero plane (data not shown). However, when we analyzed the combinations in "close-up" (i.e. in a more narrow concentration range) synergistic effects were noted (Fig. 2). In some instances, the calculated degrees of synergism were statistically significant [e.g. for rupintrivir + BTA798 against poliovirus (125 $\mu M^2\%$)], whereas in other instances they were not [(rupintrivir + BTA798 against enterovirus 71; (22 $\mu M^2\%$)]. However, as mentioned earlier, at most of the concentrations tested, all of these combinations resulted in an additive effect. We therefore believe that for this particular combination (i.e. rupintrivir + BTA798) it would be incorrect to state that the drugs work synergistically against poliovirus. Rather, one can conclude that both compounds act in an additive fashion and exhibit for some viruses (in this case poliovirus) activity at a narrow concentration range with slight synergistic activity. The most important conclusion from these combination studies remains, however, that the combinations studied (with the exception of ribavirin + 2′-*C*-methylcytidine) do not result in an antagonistic activity and that they can in theory be combined (in an attempt to have a more potent antiviral effect and to prevent the development of drug resistance). The comparative antiviral study reported here also provides a reference panel for future studies with anti-enterovirus compounds. Obviously, drugs developed against either rhino- or enterovirus infections should ideally have broad-spectrum activity so that they can be used (if needed off-label) for the treatment of often life-threatening enteroviral infections for which no drug(s) will be developed.

## Conflict of interest

The authors state to have no commercial or other conflicts of interest.

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