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Original research

Combination of dasabuvir and PSI-6206 for the treatment of coxsackievirus B3 infection

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ABSTRACT

Coxsackievirus B3 (CVB3) infections may cause life-threatening diseases and have no approved specific treatment. Some promising approaches to treat viral diseases include drug repurposing and combination therapy. We have selected in this study dasabuvir, an approved antiviral drug, and PSI-6206, an experimental drug and determined their individual and combined antiviral activity against CVB3 replication *in vitro*. Our results show that the individual drugs inhibited CVB3 infection in a dose-dependent manner, at a selective index >10 with a strong synergetic antiviral effect of the two compounds. Given that dasabuvir has already been approved for the treatment of hepatitis C virus infection, treatment of CVB3-related disease with this drug may represent a promising treatment strategy.

1. Introduction

Coxsackievirus B virus 3 (CVB3) infection is a major cause of viral-induced dilated myocarditis among children and young adults.[1] There is no specific prophylactic vaccine or treatment and although there are efforts to develop effective anti-CVB3 drugs, few have been tested in clinical trials and none have been licensed for this indication.[2]

One of the approaches for the treatment of viral infections involve the repurposing of promising existing drugs or drug candidates, such as in the case of influenza, Ebola and the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).[3] We have therefore examined potential drugs for repositioning in the treatment of CVB3-related infection. To this end, we first tested the individual antiviral activity of dasabuvir (ABT-333), and PSI-6206 (RO-2433, GS-331007). Dasabuvir, which is sold under the brand name of Exviera®, is a direct-acting non-nucleoside inhibitor of the RNA-dependent RNA polymerase (RdRp), approved for the treatment of hepatitis C virus (HCV) infections.[4] It is often used as part of combination therapy including ombitasvir/paritaprevir/ritonavir for HCV genotype 1 infection because of a good safety and tolerability profile.[5] PSI-6206 is an experimental drug currently being developed, as a potent inhibitor of RdRp, also for the treatment of HCV infection. It is a nucleoside analogue, competitive inhibitor of native RNA synthesis.[6]

Antiviral drug combination is associated with therapeutic success using lower drug dose and decreased risk of drug resistance.[7] Thus, we also examined the efficacy of the two-drug combination of dasabuvir and PSI-6206 against CVB3 infection, using commonly used reference models to analyse drug interactions.[8]

2. Materials and methods

2.1. Virus, cell lines and compounds

Coxsackievirus B3 Woodruff strain was used in our experiments. Vero and HeLa cells lines were cultured in Dulbecco Modified Eagle Medium (DMEM) (HyClone Laboratories, Inc, South Logan, Utah) with 10% foetal bovine serum (FBS) at 37 °C with 5% CO_2. Dasabuvir (ABT-333), and PSI-6206 (RO-2433, GS-331007) (Selleck, USA) supplied in powder form were dissolved in dimethyl sulfoxide (DMSO) and kept at −80 °C.

2.2. Cytotoxicity and effective concentration assay

Cytotoxicity (CC) and effective concentrations (EC) of both compounds were determined by a cytopathic effect (CPE)-based assay as previously described,[9] using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. The drug concentration that resulted in a 50% decrease of cell viability was defined as 50% cytotoxicity (CC_{50}) and the 50% effective concentration (EC_{50}) the one required to achieve the half maximal of the CPE inhibition effect. The

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selective index (SI) was calculated as SI = CC_{50}/EC_{50}.

2.3. Virus susceptibility testing

Virus susceptibility was determined by a virus yield-reduction assay and defined as fold-reduction of virus produced after treatment, compared with untreated cells. Virus titre was determined by a standard 50% tissue culture infective dose ($TCID_{50}$)[10] and calculated as previously described.[11]

2.4. Time-of-addition assay

To identify the time point at which the compounds inhibited CVB3 infection, we have analysed them in a time-of-addition assay. Briefly, CVB3-infected HeLa cells were treated with dasabuvir (18 μM) or PSI-6206 (125 μM) at different time points with interval of 1 hpi. Total protein and cell supernatants were assayed by western blotting and the TCID50 assay, respectively.

2.5. Antiviral drug combination matrices

Briefly, HeLa cells were seeded in a 96-well plate (making 10 by 10 matrix) and incubated overnight, prior to virus infection. Five microliters of 2-fold serial dilutions of each compound was added to the virus-infected (MOI = 0.01) cells and the total volume in each well was brought to 100 μM. After 48 hpi, anti-CVB3 activity of the drug combination was determined by a CPE-based assay.

2.6. Statistical analysis of the efficacy of the drug combination

The efficacy of the two-drug combinations was determined by the Bliss Independence Model, using MacSynergy II.[8] The model was used to calculate the synergy, antagonism and additive volumes of the antiviral effects of the two drugs combination at 95% confidence interval. Dataset from at least three independent experiments were used to calculate the means of the drug combination. Volumes (μM^2%) greater than +100 or less than −100 were considered as strongly synergistic or antagonistic, respectively. Volumes between +50 and + 100 or between −50 and −100 were considered moderately synergistic and antagonistic, respectively. Whereas, volumes between - 25 and + 25 were considered as additive antiviral effect.[12]

3. Results

3.1. Dasabuvir and PSI-6206 are potent inhibitors of CVB3 replication

To investigate whether dasabuvir and PSI-6206 can inhibit CVB3 replication, we initially determined the CC_{50} and EC_{50} of both compounds in a CPE-based assay. Our result show that the two compounds inhibited CVB3-induced CPE with minimal cytotoxicity. Dasabuvir and PSI-6206 possess anti-CVB3 activity with an EC_{50} of approximately 0.73 μM and 34.6 μM, respectively. Both compounds selectively inhibited CVB3 replication with an SI > 10 (Fig. 1A). To validate this anti-CVB3 activity of both inhibitors, virus-yield-reduction assay was performed as described above. Results show that dasabuvir and PSI-6206 reduced viral titre by approximately 3.3 and 1.8-fold (at the highest concentration tested), respectively, as compared to untreated virus-infected cells (Fig. 1B). Importantly, viral protein and RNA levels were significantly reduced in cells treated with these inhibitors (Fig. 1C and D).

3.2. Antiviral activity of a two drug combination

To demonstrate the antiviral activity of the drug combination, we first examine the combined cytotoxicity of the two drugs. To this end, HeLa cells were treated with two-fold serial dilutions of the compounds and cell viability was examined by the MTT assay. As shown in Fig. 2A, the combination of both dasabuvir and PSI-6206 did not cause significant synergetic cytotoxicity. Cytotoxicity as measured by the percentage difference between treated and control cells showed percentages of cell viability above 80%. This was considered as an absence of cytotoxicity.[13]

Following this, to examine whether the drug combination was synergetic, antagonistic, or only had an additive antiviral effect, both compounds were titrated in a checkerboard manner so that antagonism or synergism could be determined in the same experiment. The MacSynergy II program, based on the Bliss Independence Theor,y was used

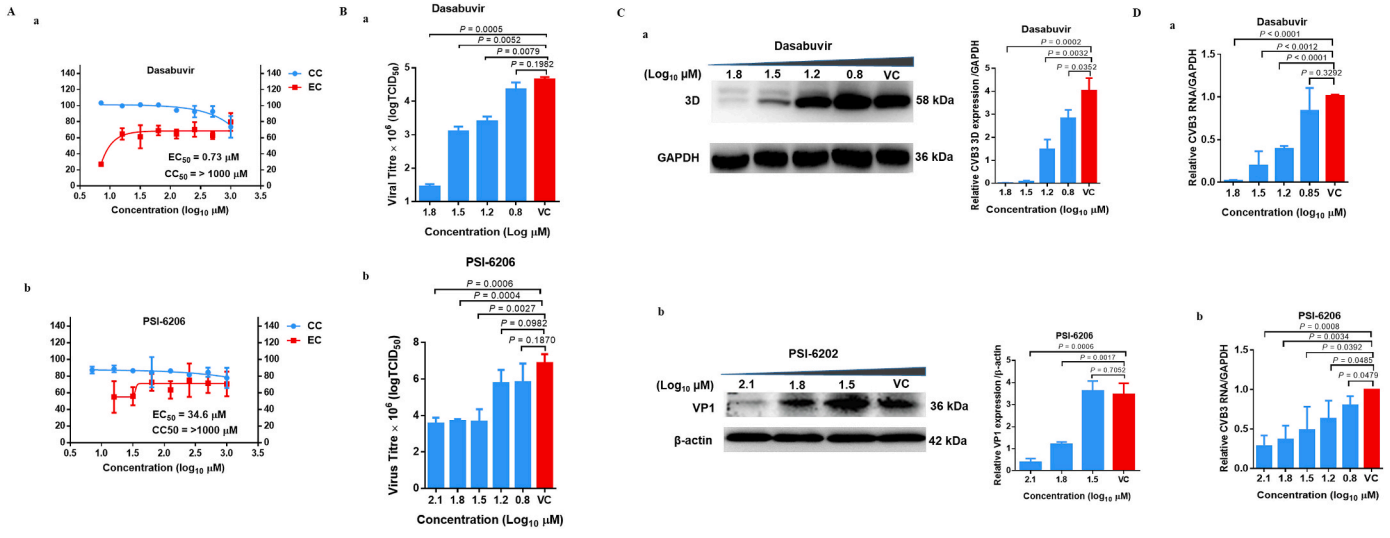

Fig. 1. Dasabuvir and PSI-6206 inhibit CVB3 replication in a dose-dependent manner. (A) The CC_{50} and EC_{50} of dasabuvir and PSI-6206 were determined using the CPE assay. Two-fold serial dilutions of the compounds were added to HeLa cells at the indicated concentration. For the EC_{50} assay, cells were infected with CVB3 (MOI = 0.01) and CPE was determined at 72 hpi by the MTT assay. The EC_{50} and CC_{50} were analysed by nonlinear regression (GraphPad Prism Version 6.02). **(B)** Virus yield-reduction assay. CVB3-infected Vero cells (MOI = 1) were treated with dasabuvir and PSI-6206 at the indicated concentration, respectively. Virus supernatants were collected at 24 hpi and virus titres were determined by the TCID50 assay. **(C and D)** Dasabuvir and PSI-6206 effectively inhibit CVB3 replication. Virus-infected (MOI = 0.1) HeLa cells were treated with two-fold serial dilutions of the test compounds at the indicated concentration. Total proteins and RNAs were extracted at 18 hpi and analysed by western blotting and RT-qPCR, respectively. Error bars represent SD, $n = 3$, *t*-test.

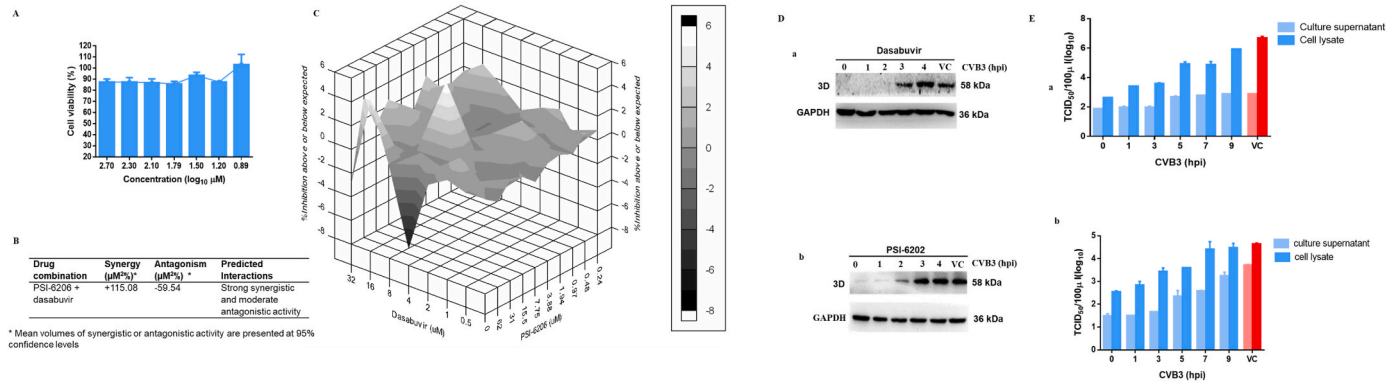

Fig. 2. Analysis of a two-drug combination of dasabuvir and PSI-6206. (A) HeLa cells were treated with both dasabuvir and PSI-6206 at the indicated concentrations. Cell viability was determined by the MTT assay. **(B and C)** Mean volumes of the average of three independent experiments are presented at 95% confidence levels. **(D and E)** Time-of-addition assay. HeLa cells were infected with CVB3 (MOI = 0.1) and cultured in medium containing dasabuvir (15 μMh) and PSI-6206 (125 μM) at the indicated time points. **(D)** Total proteins extracted from the cells at 9 hpi were analysed by western blotting. **(E)** Virus supernatants were collected and cells lysed by freeze thawing. Viral titre was determined by the TCID50 assay. Error bars represent SD, $n = 3$, *t*-test.

to evaluate three independent raw experimental data. The combination of the two inhibitors showed a strong synergetic and moderate antagonistic antiviral activity against CVB3 replication. Corresponding numerical values of the mean volume of synergy and antagonism at 95% confidence level are shown in Fig. 2B. Graphic presentation of the results that display synergy and antagonism as peaks above or below a predicted additive plane in a three-dimensional graph was plotted using Delta Graph 7 (Fig. 2C). These results suggest that the combination of dasabuvir and PSI-6206 have enhanced antiviral effect against CVB3 replication.

3.3. *Dasabuvir and PSI-6206 target CVB3 replication at the early stage of virus infection*

To gain further insight into the mechanism of the compound anti-CVB3 activity, a time-of-addition assay was performed. As shown in Fig. 2D, the maximum inhibition of CVB3 replication was achieved when the compounds were added in the early hours of infection. When added earlier than 2 hpi, the expression of the viral 3D protein was almost completely absent. Likewise, the CVB3 virion titre gradually increased in a time-dependent manner (Fig. 2E), suggesting that both compounds inhibited the CVB3 replication cycle at the early stage of infection.

4. Discussion

This study provides experimental evidence of a potential new use for two drugs. We show that individual drug treatment of virus-infected cells with dasabuvir and PSI-6206 has a potent antiviral activity against CVB3 replication. Likewise, *in vitro* pharmacological evaluation of the dasabuvir and PSI-6206 combination therapy against CVB3 infection show a strong synergistic interaction, suggesting that future studies should further investigating the *in vivo* antiviral activity of these CVB3 inhibitors.

Several antiviral agents targeting different stages of the CVB3 replication cycle have been suggested as potential drug candidates, yet none has yet been approved. This is mostly due to concerns about safety (i.e. cytotoxicity) and unsatisfactory antiviral outcomes, as seen with pleconaril and rupintrivir developed for the treatment of picornavirus-related infections.[14]

Our results show that dasabuvir and PSI-62016, the two tested compounds, inhibit CVB3-induced CPE without significant cytotoxicity, in a dose-dependent manner. They inhibit CVB3-induced CPE with an EC_{50} of 0.73 μM and 34.6 μM, respectively. These results strongly suggest that both compounds might be useful for the treatment of CVB3-related infections. However, compared with CVB3 infection, dasabuvir seems to be a more potent inhibitor of HCV infection with an IC_{50} of 2.2 nM.[15] Furthermore, the result of cytotoxicity revealed that both compounds do not cause significant cytotoxicity in treated cell lines, with an estimated CC_{50} value > 1000 μM resulting in SI values > 10. This may likely mean that both compounds are less toxic and can be well-tolerated since antiviral agents with SI value ≥ 4 can be considered relatively safe and suitable for therapeutic purposes.[16] However, cytotoxicity and antiviral activities of both compounds should be carefully tested in animal model to ensure their activity and safety *in vivo*.

Importantly, dasabuvir and PSI-6206 effectively inhibited CVB3 replication in the infectious virus assays. The antiviral activity of the two inhibitors in both cell-based qRT-PCR and western blotting assays clearly demonstrated that these compounds are potent inhibitor of CVB3 replication. In the confirmatory viral yield-reduction assay, dasabuvir and PSI-6206 monotherapy showed effective antiviral activity against CVB3 infections. They could therefore be potential promising drug for a repositioning strategy.

Notably, the results of the drug combination assay from this study demonstrated that the treatment of virus-infected cells with two-drug combination of dasabuvir and PSI-6206 produced a strong synergetic anti-CVB3 activity. Although dasabuvir and PSI-6206 belong to the same class of RdRp inhibitor, they target different virus replication factors. Dasabuvir is a direct-acting antiviral, whereas PSI-6206 is a nucleoside analogue inhibitor of RdRp activity. This could contribute to a multi-target strategy for the treatment of CVB3 infection.[17] It is likely that the two molecules complement each other to interact with viral RdRp activity, resulting in strong synergitic antiviral effect as observed. Similarly findings by Nikolaeva-Glomb, L. and A.S. Galabov, have shown that the combination of anti-enterovirus agents with different mechanism of action produces a synergistic antiviral effect.[18]

The result of our time-of-addition assay clearly showed that dasabuvir and PSI-6206 exert their antiviral activities when the compounds were added earlier than 3 hpi. This is consistent with the high level of 3D protein observed when cells are treated later than 3 hpi. Consequently, it seems that dasabuvir and PSI-6206 do not block the assembly and release of new viral progeny, but rather, inhibit event(s) in the early stage of CVB3 replication. Further studies are required to determine which specific events in the early stage in the CVB3 replication cycle are blocked by these compounds.

In conclusion, this study shows that dasabuvir and PSI-6206 are potent inhibitors of CVB3 infection *in vitro*. Our findings further demonstrate that the combinations of dasabuvir and PSI-6206 has strong synergistic antiviral effects against CVB3 infection. We hope to further evaluate the *in vivo* antiviral activity of both inhibitors and to further

elucidate the specific mechanisms of their antiviral activity in CVB3 infection.

Author contributions

O. I. O. was involved in laboratory testing and writing of the manuscript, Z. Z. was involved in the conceptualization and methodology of this study.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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