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Short Communication

# Persistent infection of human pancreatic cells with Coxsackievirus B4 is cured by fluoxetine

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ABSTRACT

Group B Coxsackieviruses (CVB) are involved in various acute clinical features and they can play a role in the development of chronic diseases like type 1 diabetes. The persistence of CVB has been described in vitro and in vivo in various models. Fluoxetine was reported to inhibit the replication of CVB1–3, which prompted us to study the in vitro antiviral activity of fluoxetine against CVB4 in models of acute infection. In addition we took advantage of a chronically CVB4-infected Panc-1 cell line to evaluate the antiviral effect of fluoxetine in a model of persistent CVB4 infection.

An inhibition of the CVB4 replication was obtained when fluoxetine was added at 5.48 μM to Hep-2 cell cultures. No inhibitory effect was observed when CVB4 was mixed with fluoxetine for 2 h and filtered to eliminate fluoxetine before inoculation to cells, or when cells were treated up to 96 h and washed before viral inoculation. Fluoxetine (5.48 μM) reduced viral replication by more than 50% in acutely infected Panc-1 cell cultures. A dramatic decrease of infectious particles levels in supernatants of Panc-1 cells chronically infected with CVB4 was obtained a few days after treatment with fluoxetine and no infectious viral particle was found as soon as day 21 of treatment, and intracellular enteroviral RNA was undetectable by RT-PCR after three weeks of treatment.

These data display that fluoxetine can inhibit the replication of CVB4 and can cure Panc-1 cells chronically infected with CVB4.



Human enteroviruses (HEVs) are small non-enveloped RNA viruses that belong to the *Picornaviridae* family. The genus enterovirus currently includes 7 species involved in human diseases (human enterovirus A–D and human rhinovirus A–C), and over 250 serologically distinct viruses among which several important human pathogens, e.g. poliovirus, coxsackievirus, echovirus, rhinovirus and enterovirus 71 (Knowles et al., 2012; Tapparel et al., 2013). Some of the non-polio enteroviruses can be involved in many severe acute clinical features such as meningitis, myocarditis or fulminate sepsis in newborns (Romero, 2008; Tapparel et al., 2013) while others especially type B Coxsackieviruses (CVB) are also reported to play a role in the development of chronic diseases like type 1 diabetes (Hober and Alidjinou, 2013; Hober and Sauter, 2010). In the pathogenesis of these diseases the persistence of CVB in host tissues is thought to be involved (Jaïdane and Hober, 2008; Jaïdane et al., 2010). The persistence of CVB4 in human pancreatic cell cultures, resulting in an impaired formation and viability of islet-like cell aggregates has been reported (Sane et al., 2013).

The wide range of antivirals currently marketed has not shown any significant activity against enteroviruses. Many compounds have been screened or are under investigation for their antiviral activity against enteroviruses but no antienterovirus drug is currently licensed worldwide (De Palma et al., 2008; Thibaut et al., 2011). Recently, fluoxetine, a selective serotonine reuptake inhibitor (SSRI) has been reported as an inhibitor of enterovirus replication in vitro. This medicine formerly used for the treatment of depression or other mental disorders, has a significant antiviral activity against CVB1–3 in vitro (Ulferts et al., 2013; Zuo et al., 2012).

In the present study the antiviral activity of fluoxetine against CVB4 in cell cultures acutely infected and in a model of in vitro persistent infection has been investigated.

The diabetogenic CVB4 E2 strain, kindly provided by Ji-Won Yoon (Julia McFarlane Diabetes Research Center, Calgary, Alberta, Canada) was propagated in Hep-2 cells. Hep-2 cells (BioWhittaker) were grown in minimum essential medium supplemented with 10% of fetal calf serum (FCS), 1% of L-glutamine, 1% of non-essential amino acids and 1% of penicillin and streptomycin. The human

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ductal cell line Panc-1 (ATCC) was cultured in Dulbecco's modified Eagle's medium supplemented with 10% of FCS, 1% of L-glutamine and 1% of penicillin and streptomycin. A model of persistent infection of Panc-1 cells with CVB4 has been previously reported by our team (Sane et al., 2013).

Fluoxetine chlorhydrate (Lilly France, France) was dissolved in DMSO. A concentration of 21.9 μM or below was shown to have no cytotoxicity on both Hep-2 and Panc-1 cells. To evaluate the antiviral activity of fluoxetine, cells were seeded in a 96 wells cell culture plate at $1.25 \times 10^4$ cells per well. Cells were inoculated with the virus at a MOI of 0.01 mixed with various dilutions of fluoxetine or DMSO. The plates were incubated at 37 °C, and the cell cultures were observed every day. The cytopathic effect (CPE) in the wells with fluoxetine was recorded when a CPE of 100% was reached in mock-treated wells inoculated with CVB4.

The viral titers in supernatants of chronically-infected cells were assessed using the end-point dilution assay, and the Spearman-Karber statistical method was used to determine the tissue culture 50% infectious dose (TCID50). Total RNA was extracted from cells using RNeasy kit (Qiagen). The level of intracellular viral RNA in acutely-infected cells was quantified by using a two-step real time PCR as described elsewhere (Alidjinou et al., 2013).

The presence of intracellular enterovirus RNA in persistently-infected cells was investigated by RT-PCR and semi-nested PCR using respectively the SuperScript® one-step RT-PCR and the Platinum® PCR Supermix kits (Invitrogen) as previously described (Sane et al., 2013).

The antiviral activity of fluoxetine has been assessed in Hep-2 and Panc-1 cell cultures inoculated with CVB4. Serial 2-fold dilutions starting from 21.9 μM fluoxetine were tested on both cells. A CPE of 100% was observed on day 2 post-infection (p.i.) in Hep-2 cell cultures. As shown in Fig. 1a, fluoxetine inhibited the CVB4-induced CPE and the production of infectious particles in Hep-2 cells at a concentration of 5.48 μM. The intracellular viral RNA was also significantly reduced (Fig. 1b).

Three situations were tested in Hep-2 cell cultures to study the mode of action of fluoxetine. The molecule used at a final concentration of 5.48 μM was (i) added to cells inoculated with CVB4; (ii) mixed with CVB4 before inoculation to cells (iii) incubated with cells that were subsequently inoculated with CVB4. Fluoxetine inhibited completely the CVB4-induced CPE when added to cell cultures at 0, 2, and 4 h post infection. A residual CPE, 10% and 50%, was observed when fluoxetine was added to cell cultures at 20 and 24 h post infection, respectively (see Fig. 1c). In other experiments, CVB4 was mixed with fluoxetine for 2 h and the mixture was filtered on Illustra MicroSpin Columns® by centrifugation at 735 g for 1 min, and the filtrate was then inoculated to Hep-2 cell cultures. In parallel, Hep-2 cell cultures were also inoculated with filtered CVB4 or with the following mixtures: CVB4 added to filtered fluoxetine or fluoxetine added to filtered CVB4. The results are shown in Fig. 1d. When cells were inoculated with filtered virus, a CPE was observed. In contrast when the filtered virus was mixed with fluoxetine the CPE was inhibited, whereas no inhibition was observed in all conditions that include a step of filtration of fluoxetine. Finally, cell cultures were treated with fluoxetine for various time intervals ranging from 2 to 96 h, then after washings with culture medium, CVB4 was inoculated. No inhibition of the CVB4-induced CPE was observed in these conditions (data not shown).

Fluoxetine was also tested in acutely infected Panc-1 cells. A 100% CPE was observed in control wells on day 5 p.i. A concentration of 5.48 μM reduced the CPE and the viral titer by more than 50% and the level of intracellular enterovirus RNA was also decreased but to a lesser extent (see Fig. 2a and b). When treatment with fluoxetine at 5.48 μM was repeated after 48 h, the viral replication on day 5 p.i. was more extensively reduced up to 100% (data not shown).

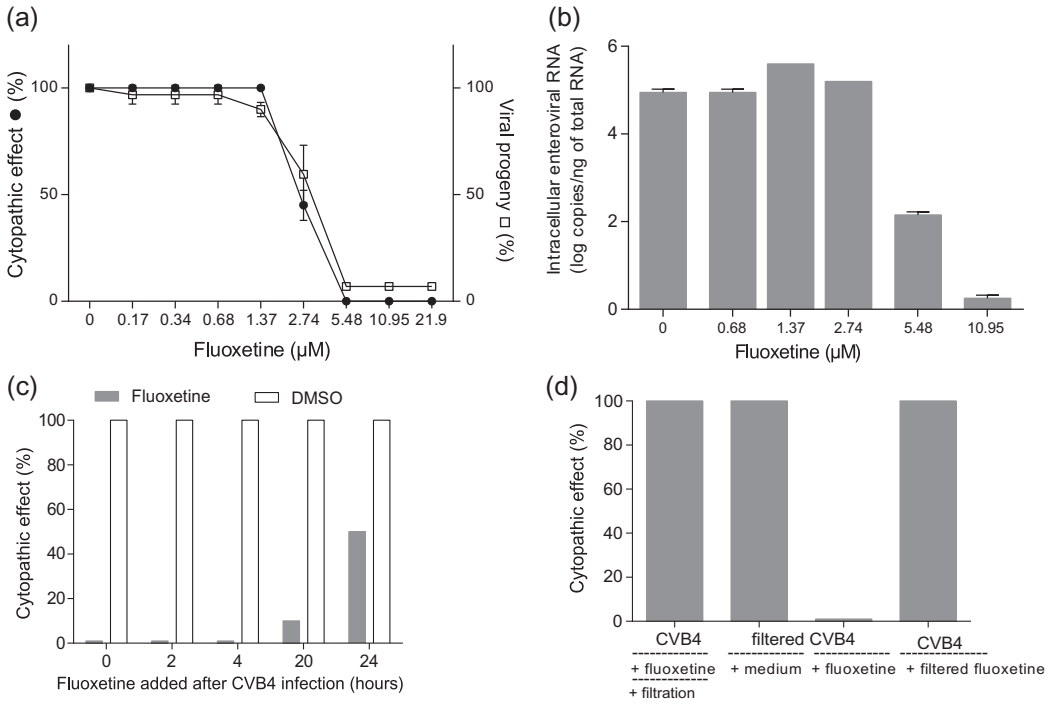


**Fig. 1.** Fluoxetine inhibits the CVB4 replication in Hep-2 cells. Hep-2 cells were seeded in 96 wells cell culture plates at $1.25 \times 10^4$ cells per well. CVB4E2, at MOI 0.01, mixed with various dilutions of fluoxetine was added to cell cultures (a–b). Fluoxetine at 5.48 μM was added to Hep-2 cell cultures at 0, 2, 4, 20 and 24 h post infection with CVB4E2 (c). CVB4E2 was mixed with fluoxetine at 5.48 μM for 2 h and the mixture was filtered and the filtrate was then inoculated to Hep-2 cell cultures. Hep-2 cell cultures were also inoculated with filtered CVB4E2, filtered CVB4E2 mixed with fluoxetine for 2 h, or CVB4E2 mixed for 2 h with filtered fluoxetine (d). The cytopathic effect in the wells with fluoxetine was recorded when it reached 100% in controls. The viral progeny was determined in supernatants using endpoint dilution assay and expressed as percentage of controls, and the level of intracellular enterovirus RNA was quantified by using real-time RT-PCR. The results are representative of two independent experiments.