Author(s): Butrym, Marta; Byvald, Fabian; Blanter, Marfa; Ringqvist, Emma E.; Vasylovska, Svitlana; Marjomäki, Varpu; Lau, Joey; Stone, Virginia M.; Flodström-Tullberg, Malin

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Vemurafenib inhibits the replication of diabetogenic enteroviruses in intestinal epithelial and pancreatic beta cells

Marta Butrym [a], Fabian Byvald [a], Marfa Blanter [a], Emma E. Ringqvist [a], Svitlana Vasylovska [b], Varpu Marjomäki [c], Joey Lau [b], Virginia M. Stone [a], Malin Flodström-Tullberg [a,*]

[a] Center for Infectious Medicine, Department of Medicine Huddinge / ANA Futura, Karolinska Institutet, Alfred Nobels Allé 8, 141 52, Stockholm, Sweden
[b] Department of Medical Cell Biology, Uppsala University, BOX 571, 751 23, Uppsala, Sweden
[c] Department of Biological and Environmental Science, University of Jyväskylä, P.O. Box 35, FI-40014, Jyväskylä, Finland

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ABSTRACT

Enteroviruses, which infect via the gut, have been implicated in type 1 diabetes (T1D) development. Prolonged faecal shedding of enterovirus has been associated with islet autoimmunity. Additionally, enteroviral proteins and viral RNA have been detected in the pancreatic islets of individuals with recent-onset T1D, implicating their possible role in beta cell destruction. Despite this, no approved antiviral drugs currently exist that specifically target enterovirus infections for utilisation in disease interventions.

Drug repurposing allows for the discovery of new clinical uses for existing drugs and can expedite drug discovery. Previously, the cancer drug Vemurafenib demonstrated unprecedented antiviral activity against several enteroviruses. In the present study, we assessed the efficacy of Vemurafenib and an analogue thereof in preventing infection or reducing the replication of enteroviruses associated with T1D. We tested Vemurafenib in intestinal epithelial cells (IECs) and insulin-producing beta cells. Additionally, we established a protocol for infecting human stem cell-derived islets (SC-islets) and used Vemurafenib and its analogue in this model.

Our studies revealed that Vemurafenib exhibited strong antiviral properties in IECs and a beta cell line. The antiviral effect was also seen with the Vemurafenib analogue. SC-islets expressed the viral receptors CAR and DAF, with their highest expression in insulin- and glucagon-positive cells, respectively. SC-islets were successfully infected by CVBs and the antiviral activity of Vemurafenib and its analogue was confirmed in most SC-islet batches.

In summary, our observations suggest that Vemurafenib and its analogue warrant further exploration as potential antiviral agents for the treatment of enterovirus-induced diseases, including T1D.

1. Introduction

Type 1 diabetes (T1D) is an autoimmune disease where the insulin-producing beta cells in the pancreas are destroyed (DiMeglio et al., 2018). Since the 1980s, T1D cases have quadrupled and the incidence continues to increase (Tedros, 2021). Genetic factors account for approximately 50 % of the risk for T1D, suggesting that environmental factors are involved (Noble, 2015). Numerous reports indicate that infections with *Enterovirus B* species (e.g., Coxsackie B viruses; CVBs) contribute to T1D (Carré et al., 2023) and two main hypotheses exist regarding this connection. The first hypothesis suggests an enterovirus (EV) infection contributes to the initiation of pancreatic islet autoimmunity and is based on studies reporting temporal associations between EV infections and islet autoantibody development (Laitinen et al., 2014; Oikarinen et al., 2011; Salminen et al., 2003; Vehik et al., 2019). In the second hypothesis it is postulated that an EV infection accelerates clinical disease onset in autoantibody positive individuals, as described in the DAISY study (Stene et al., 2010).

Enteroviruses (EVs) are common human viruses belonging to the *Picornaviridae* family and the genus includes poliovirus and clinically

* Corresponding author. Center for Infectious Medicine, Department of Medicine, Karolinska Institutet, Karolinska University Hospital Huddinge, Alfred Nobels Allé 8, Stockholm, SE-141 52, Sweden.

E-mail addresses: marta.butrym@ki.se (M. Butrym), fabian.byvald@ki.se (F. Byvald), m.l.blanter@lumc.nl (M. Blanter), emma.ringqvist@ki.se (E.E. Ringqvist), svitlana.vasylovska@mcb.uu.se (S. Vasylovska), varpu.s.marjomaki@jyu.fi (V. Marjomäki), joey.lau@mcb.uu.se (J. Lau), virginia.stone@ki.se (V.M. Stone), malin.flodstrom-tullberg@ki.se (M. Flodström-Tullberg).

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relevant non-polio enteroviruses (NPEs) such as Coxsackie-, echo-, EV71-, and rhinoviruses. Infections with EVs are often mild or asymptomatic, but sometimes they can cause severe diseases including acute flaccid myelitis, encephalitis and myocarditis. The transmission of EVs mainly occurs via the gastrointestinal (GI) tract, although some species, e.g. rhinoviruses and EV-D68, are transmitted through the respiratory system. Cells in the intestinal epithelium can be infected by EVs that enter through the GI tract which may eventually allow viral dissemination to other organs. EVs display different tissue tropisms thanks to their target receptors; for example, CVBs bind the coxsackie and adenovirus (CAR) receptor which is expressed in the heart and pancreas (Ifie et al., 2018; Pallansch and Roos, 2007), while many rhinoviruses bind to ICAM-1, which has high expression in the lung (Greve et al., 1989).

Surveillance studies consistently show that EVs are prevalent worldwide. Over the past few decades there have been several EV outbreaks and epidemics, as reviewed in (Jartti et al., 2024). EV infections are known to cause chronic dilated cardiomyopathy and combined with their T1D link and high prevalence, this emphasises the critical need for preventative strategies including vaccines and effective antiviral treatments against EVs. Currently, no clinically approved therapeutics exist that specifically target NPEs aside from EV-A71 vaccines (approved exclusively in Asia). Notably, novel CVB vaccines have been produced (Hankaniemi et al., 2017; Stone et al., 2018, 2020, 2021a) which provided the basis for a clinical vaccine recently tested in phase I clinical trial (Hyoty et al., 2024).

Vaccines generally provide protection against the virus serotypes they were designed for whereas antiviral drugs can act more broadly. Antivirals may target a critically important cellular mechanism in the viral replication cycle thereby blocking the replication of all virus types within a virus species. Several drugs have been developed as possible antiviral treatments for EV infections (e.g. plecornaril) and others have been identified in drug repurposing screens (e.g. Itraconazole and Vemurafenib). Some have reached clinical trial, but most have not been approved for clinical practice due to safety issues, toxicity concerns, insufficient antiviral effects and/or non-beneficial interactions with the oral polio vaccine (Anasir et al., 2021; Baggen et al., 2018; Benschop et al., 2015). Clinically available drugs developed for other treatments, including cancer, can target host cell signalling and metabolic pathways. Repurposing these thoroughly tested molecules for a new manifestation as an antiviral therapy is an economically advantageous approach for identifying and producing novel antiviral drugs with a short path to clinical use. Vemurafenib, which is used to treat certain types of melanomas, was recently identified in a drug repurposing screen as a drug with potential anti-enteroviral properties (Laajala et al., 2023). A few recent studies have addressed the antiviral effect of Vemurafenib *in vitro* (Holzberg et al., 2017; Hu et al., 2022; Ianevski et al., 2020, 2022, 2024; Laajala et al., 2023), but its potential to block EV replication in cells located in the gut and human insulin-producing beta cells has not been tested.

The aim of the present study was to investigate the efficacy of Vemurafenib and an analogue thereof in attenuating or preventing the replication of EVs linked to the development of T1D in relevant cell types, namely intestinal epithelial cells (IECs) and insulin-producing beta cells.

2. Materials and methods

2.1. Drugs and other reagents

Vemurafenib, the analogue PLX7904 and Itraconazole were dissolved in 100% DMSO. Aliquots, including aliquots of plain DMSO (vehicle control), were stored at −80 °C. Working dilutions were prepared in complete medium for the corresponding cell type. Sterile filtering was performed using a 0.2 μm filter. DMSO diluted in complete medium was used as control. Diluted aliquots of the drugs were not reused. See Table S1 for more information.

2.2. Viruses

CVB3eGFP (Feuer et al., 2002), CVB3 Nancy and CVB4-E2 were propagated in HeLa cells. CVB3-V13 (Laitinen et al., 2014) was produced in RD cells. Sources in Table S1.

2.3. Cell lines

HeLa cells, INS-1832/13 cells (rat beta cell line) (Hohmeier et al., 2000), HT-29 cells (HTB-38) and Caco-2 cells were cultured at 37 °C and 5 % CO_2 and were passaged 2–3 times per week. All cell lines were mycoplasma negative. See Table S2 for more information.

2.4. Generation of stem cell-derived islets (SC-islets)

Human embryonic stem cells (hESC) (H1 cell line, Wicell®) were propagated, seeded and a seven-stage differentiation protocol was used to form SC-islets as previously described (Balboa et al., 2022). See Supplementary Methods S1.1. and Table S3 for further information.

2.5. Cytotoxicity studies

HeLa and INS-1 cells were treated with Vemurafenib, PLX7904 or vehicle (DMSO) for 6 h (INS-1) or 20 h (INS-1 and HeLa). Cell count and viability were determined by trypan blue exclusion assay. See Supplementary Methods S1.2. for more details.

2.6. Flow cytometry

Single cell suspensions were used for surface staining of CAR and DAF and for intracellular staining of dsRNA, insulin and glucagon. Cells were acquired on a BD Accuri or LSR Fortessa (BD Biosciences) and analysed in FlowJo v10 (BD Biosciences). See Supplementary Methods S.1.3. and Table S4.

2.7. Analysis of scRNA-seq data

Gene expression data of CD55 (DAF) and CXADR (CAR) in SC-derived islets produced from H1 stem cells (Balboa et al., 2022) and adult primary islets (Xin et al., 2018) were downloaded from https://singlecell.broadinstitute.org/single_cell/study/SCP1526 and the percentage of total beta cells or total alpha cells expressing each receptor was calculated for each origin of cells. See Supplementary Methods S1.4. for further information.

2.8. Virus infections in the presence or absence of drugs

HeLa and INS-1 cells were pre-treated with Vemurafenib, PLX7904 or vehicle at the indicated concentrations and then infected with CVB3eGFP (HeLa) or CVB4-E2 (INS-1). The cells were then cultured in the presence of drug/vehicle and after 4 h (HeLa) and 6 h or 20 h (INS-1) the cells were analysed by flow cytometry (section 2.6.). HeLa cells cultured for 20 h were assessed by standard plaque assay (section 2.10.). For the Itraconazole studies, HeLa and INS-1 cells were infected with CVB3 (HeLa) or CVB4 (both cell lines), cultured in the presence of Itraconazole (0–10 μM) for 6 h or 20 h and then analysed by flow cytometry (section 2.6.).

To establish whether SC-islets are susceptible to CVB infection, SC-islets were infected with different PFUs of CVB3 Nancy (4×10^3 to 4×10^5 PFU/islet) and then cultured for 48 h. In the antiviral drug studies, SC-islets were pre-treated with Vemurafenib, PLX7904 or vehicle for 1 h, infected with CVB3 (4×10^3 PFU/islet) and then incubated in the presence of drug/vehicle for 48 h. In both studies, after 48 h, replicating virus was assessed by standard plaque assay (section 2.10.).

See Supplementary Methods S1.5. for more detail.

2.9. Cytopathic effect (CPE)

HT-29 or Caco-2 cells were pre-treated with Vemurafenib (5 μM and 10 μM) or vehicle, infected with CVB3-V13 and then cultured in the presence of drug or vehicle for 48 h. Cells were then fixed, stained with crystal violet and optical density was measured at 595 nm. See Supplementary Methods S1.6. for further information.

2.10. Plaque assay

Viral titres in culture media were measured by standard plaque assay using HeLa cells. See Supplementary Methods S1.7. for more information.

2.11. Statistical analysis

Statistical analysis was performed using GraphPad Prism 10.2.3 (San Diego, USA). Multiple comparisons between groups were performed using one-way ANOVA with Šídák's multiple comparisons test or with the non-parametric Friedman test with Dunn's multiple comparisons. *p < 0.05; **p < 0.01, ***p < 0.001, ****p < 0.0001.

3. Results

3.1. Vemurafenib has antiviral activity against CVB3 infection without affecting cell viability

To address whether Vemurafenib or the analogue PLX7904 have antiviral activity against EVs commonly linked to T1D (Carré et al., 2023), we first performed a cytotoxicity assay to examine drug effects on cell survival and replication in HeLa cells. After 20 h treatment neither

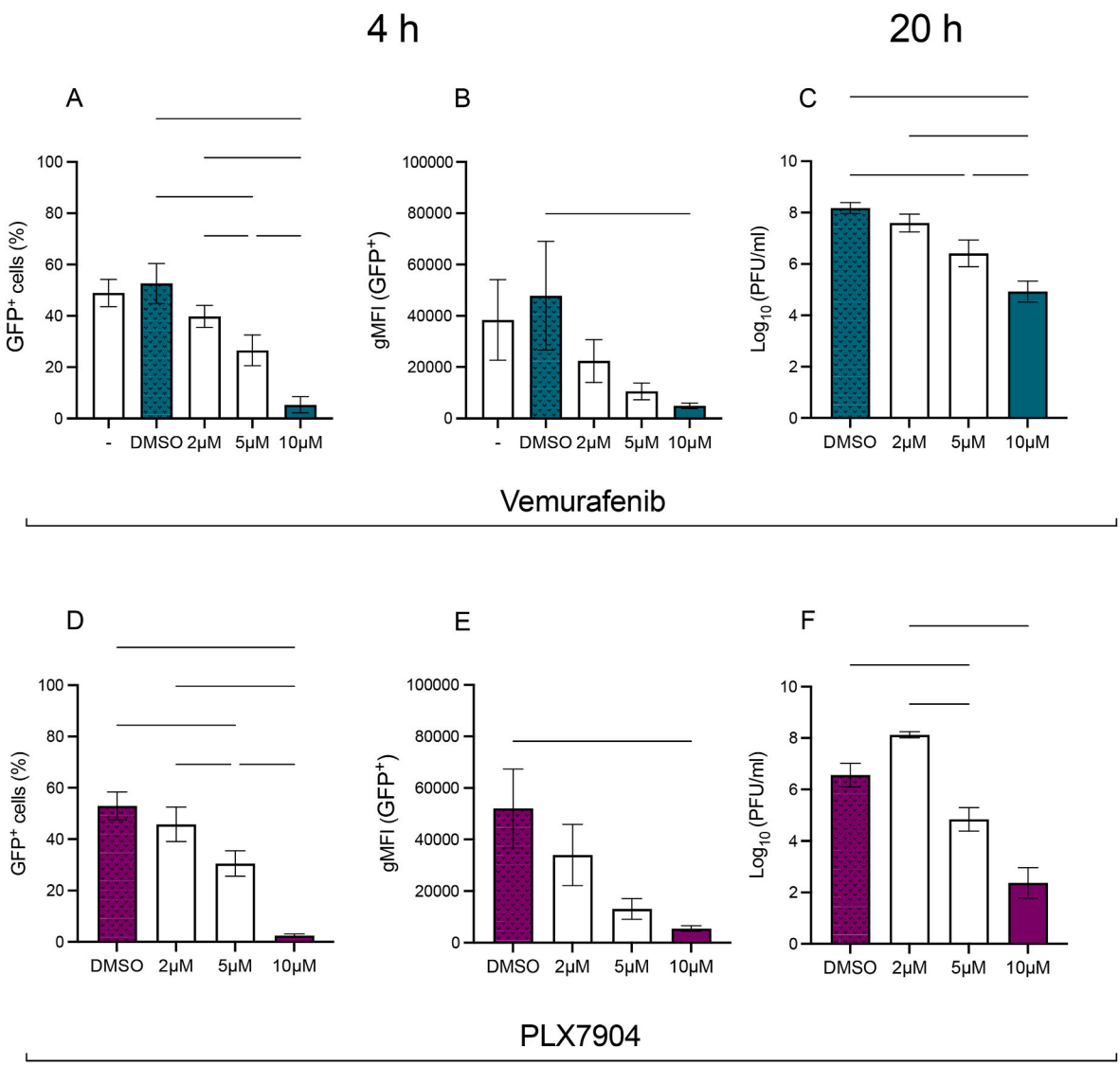

Fig. 1. Vemurafenib and the analogue PLX7904 attenuate CVB3 replication in HeLa cells. HeLa cells were untreated (−), pre-treated with Vemurafenib (A, B, C; teal bars) or PLX7904 (D, E, F; purple bars) or vehicle (DMSO, Vemurafenib, 0.5 %; PLX7904, 2 %) for 1 h and infected with CVB3eGFP (4 h, MOI 10; 4 h or 20 h, MOI 0.1) then cultured for 4 h (n = 4; A, B, D, E) or 20 h (Vemurafenib, n = 4; PLX7904, n = 3; C, F). Vehicle, Vemurafenib and PLX7904 were present during the infection and the incubation period. Virus infection (A, D) and propagation (B, E) were assessed at 4 h post infection using flow cytometry by measuring the percentage (%) of GFP positive cells and geometric mean fluorescence intensity (gMFI), respectively. At 20 h post infection titres of replicating virus particles in culture supernatants were measured by plaque assay. Data are shown as means ± SD. Statistical analysis was performed using one-way ANOVA with Šídák's multiple comparisons test in A, B, C, F. Non-parametric Friedman test with Dunn's multiple comparisons was used to analyse B and E (*p < 0.05; **p < 0.01).

drug had a cytotoxic effect at the concentrations used, although there was a small but significant decrease in the total number of cells after treatment with 10 μM Vemurafenib (Fig. S1.).

Next, we examined the efficacy of Vemurafenib in attenuating virus replication after one or several virus replication cycles. HeLa cells were pre-treated with varying Vemurafenib concentrations or with vehicle and infected with a GFP expressing recombinant CVB3 virus (CVB3eGFP) (Feuer et al., 2002) for 4 h (one cycle) or 20 h (several cycles). At 4 h post infection, 40–60 % of CVB3eGFP infected cells in the control group were GFP positive. The mean percentage of infected (GFP^+) cells in the 5 and 10 μM Vemurafenib groups was significantly lower compared to cells treated with vehicle (Fig. 1A). Geometric fluorescent mean (gMFI) measurements reflect viral protein expression. Reduced gMFI measurements, and thereby protein levels, were seen with all Vemurafenib concentrations which reached statistical significance at 10 μM (Fig. 1B). A statistically significant decrease in the production of infectious virus particles measured by plaque assay was also seen after 20 h of treatment with 5 μM or 10 μM Vemurafenib (Fig. 1C). Similar results were observed with the Vemurafenib analogue PLX7904 (Fig. 1D–F). Furthermore, the antiviral effect was similar to or better than treatment with another repurposed drug with anti-enteroviral activity, Itraconazole (Fig. S2). Taken together, these studies show that both Vemurafenib and PLX7904 reduce CVB3 replication in HeLa cells highlighting their antiviral activity against enteroviruses.

3.2. Vemurafenib protects intestinal epithelial cell lines from CVB3 infection

To assess whether Vemurafenib protects against viruses at the primary site of infection we used the human IEC cell lines Caco-2 and HT-29, which are susceptible to CVB3-V13 infection (Stone et al., 2021b). CVB3-V13 was selected as it infects cells through the CVB receptor, decay acceleration factor (DAF). The selected cell lines express CAR and DAF (Stone et al., 2021b), although DAF is more accessible to the virus than CAR (Cohen et al., 2001; Pan et al., 2011; Shieh and Bergelson, 2002).

After Vemurafenib or vehicle pretreatment, CVB3-V13 infection and 48 h of culture in the presence of drug, optical density (O.D.) of the cell monolayers was measured. A significant reduction in the density of both Caco-2 and HT-29 cells was seen following CVB3-V13 infection. Contrastingly, IECs treated with Vemurafenib exhibited markedly better survival rates after infection (Fig. 2A and B). Notably, Vemurafenib had a growth-inhibitory effect on HT-29 cells. However, when added to CVB3-V13-infected HT-29 cells, cell confluency increased to the same level as measured in cells treated with Vemurafenib alone. Collectively, these findings suggest that Vemurafenib protects cells found at the first site of infection, namely IECs, from CVB infection.

3.3. Exposure to Vemurafenib attenuates CVB replication in insulin-producing beta cells

Using INS-1 cells (a rodent beta cell line) we next investigated whether Vemurafenib protects insulin-producing beta cells from CVB4 infection (previous studies indicated that INS-1 cells are not susceptible to CVB3; unpublished data). Cells were infected in the presence of Vemurafenib or vehicle (DMSO) for 6 h or 20 h and double-stranded RNA (dsRNA; a replication intermediate) molecules were measured by flow cytometry. At both time points post infection, around 20–45 % of the infected INS-1 cells were positive for dsRNA (Fig. 3A and B). A dose dependent reduction in dsRNA positive cells was seen in the Vemurafenib treated groups at both 6 h and 20 h post infection (Fig. 3A and B). Exposure to Vemurafenib alone for 6 h or 20 h had no noticeable cytotoxic effects on the cells (Fig. S3), and the antiviral activity of Vemurafenib was more potent than that observed with Itraconazole (Fig. S4).

3.4. Human stem cell-derived islets express entry receptors for CVBs

Stem cell-derived islets (referred to as SC-islets) were used to study the impact of Vemurafenib on insulin-producing cells of human origin. They resemble primary human islets in their makeup and consist of alpha- and beta cells (Balboa et al., 2022), and SC-islets are also a more accessible source. While successful CVB infection of SC-islets has been

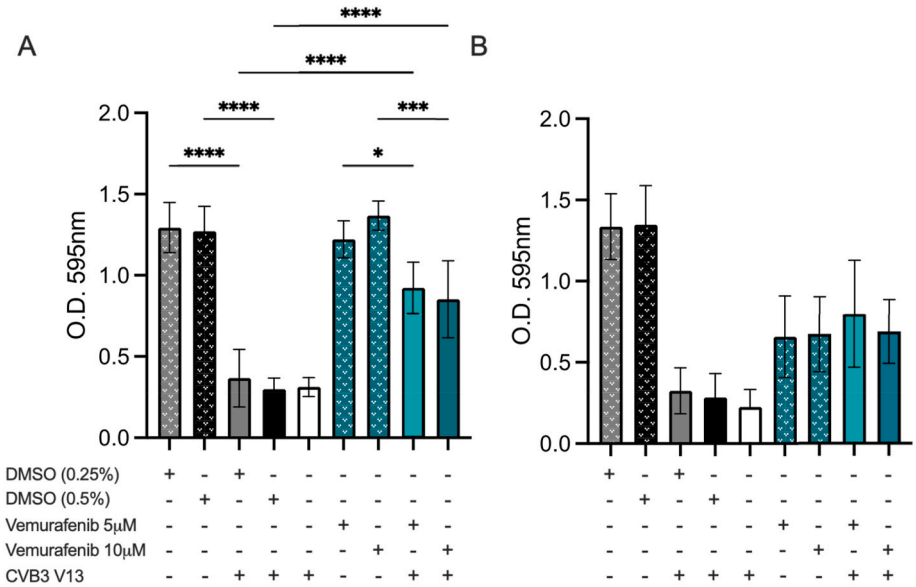

Fig. 2. Vemurafenib protects intestinal epithelial cells from CVB3 induced cytopathic effect (CPE). A-B. Caco-2 (A) and HT-29 (B) cells were pre-treated with Vemurafenib (5 or 10 μM; teal bars) or vehicle (DMSO, 0.25 % or 0.5 %; grey scale bars) for 1 h, infected with CVB3-V13 (Caco-2 MOI 10^{-3}; HT-29 MOI 10^{-4}) and cultured for 48 h in the continuous presence of drug or vehicle at equimolar concentrations. After 48 h the cells were fixed with Carnoy's reagent and stained with crystal violet. Protection from infection-induced CPE was assessed by optical density (O.D.) measurements. Data shown represents mean ± SD; A, n = 4 biological replicates with an average of 5–6 technical replicates in each; B, n = 3 biological replicates with an average of 3–6 technical replicates in each. Statistical analysis was performed using one-way ANOVA with Šídák's multiple comparisons test in A (*$p < 0.05$; ***$p < 0.001$; ****$p < 0.0001$).

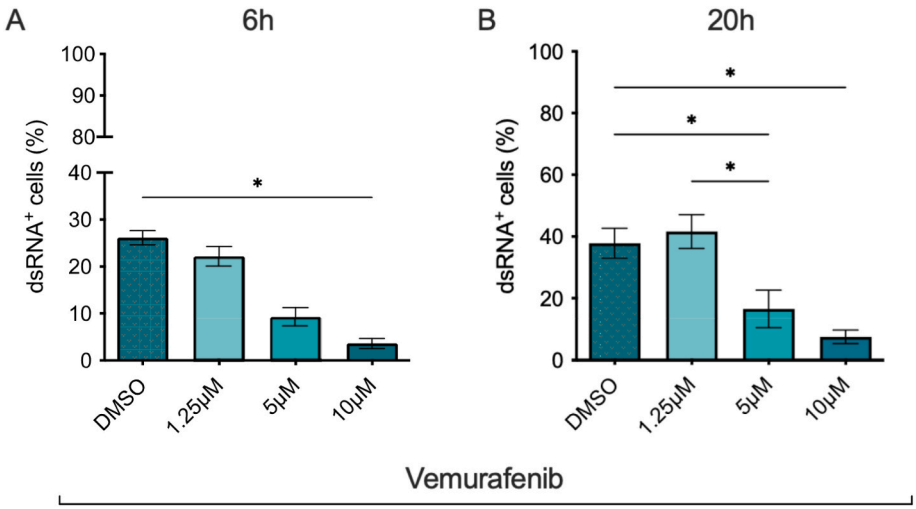

Fig. 3. Vemurafenib treatment reduces CVB replication in insulin-producing cells. INS-1832/13 cells were treated with vehicle (DMSO at a concentration that corresponds to the 10 μM Vemurafenib treatment) or increasing concentrations of Vemurafenib (1.25 μM, 5 μM, 10 μM), infected with CVB4-E2 (MOI 150) and cultured for 6 h (A) or 20 h (B) in the continuous presence of Vemurafenib or vehicle (n = 3). Infection was assessed by analysing intracellular dsRNA by flow cytometry. The percentage of dsRNA positive cells is shown as means ± SD. *p < 0.05, one-way ANOVA, with Šídák's multiple comparisons test.

documented (Böhnke et al., 2021; Nyalwidhe et al., 2020), the expression of CVB entry receptors on SC-islet cell surfaces remained unexplored. We initially examined whether SC-islets express the CVB entry receptors. Eight separate batches of SC-islets were dissociated into single cells and stained for the surface expression of CAR and DAF/CD55. In six out of eight batches, most cells were DAF positive (range 80–98 %), and 25–55 % were also positive for CAR (Fig. 4A and B). In another batch there were high proportions of CAR single positive cells and double negative cells, indicating occasional batch variation. Insulin and/or glucagon staining was included in the analysis of a few batches and we found that insulin positive (beta) cells were only highly positive for CAR (Fig. 4C). In batches of SC-islets stained for glucagon, glucagon positive

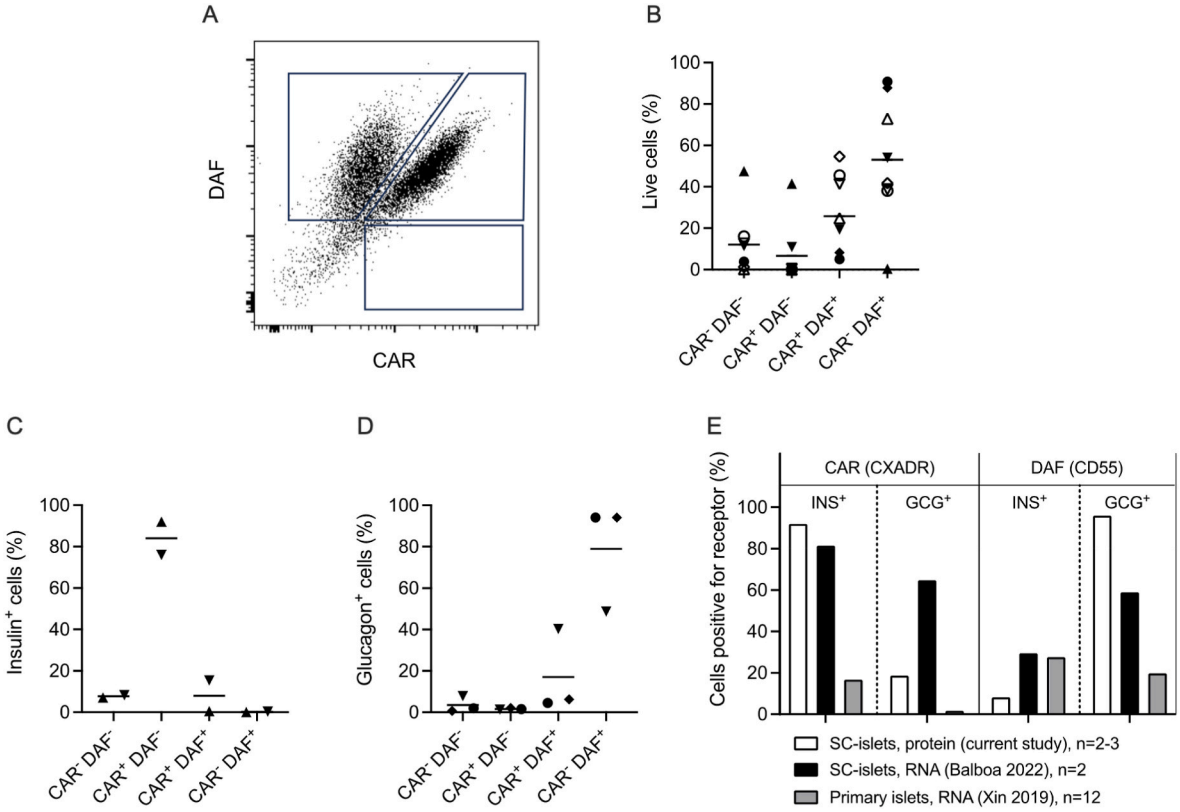

Fig. 4. Human SC-islets express CVB3 entry receptors. A-D. SC-islets (n = 8 separate batches) were dispersed into a single cell solution, stained for surface CAR and DAF receptor expression, and analysed by flow cytometry. Dot-plot (A) shows data from one representative batch and (B) shows the percentage of single cells expressing CAR and DAF in individual batches (n = 8; separate batches represented by different shapes). C and D. Summary of the percentage of cells positive for both CAR and/or DAF and insulin (INS^+; C) or glucagon (GCG^+; D) in SC-islets (n = 2–3, separate batches). E. Comparison of CAR, DAF, insulin and glucagon positivity at the protein level with published RNA-seq data for SC-islets (n = 2) and primary islets (n = 12).

(alpha) cells were DAF positive regardless of CAR expression (Fig. 4D). We compared our protein level data with previously published single cell RNA-seq data from SC-islets and primary islets (Balboa et al., 2022; Xin et al., 2018) (Fig. 4E) which confirmed that beta cells are preferentially CAR positive and alpha cells predominantly express DAF. Isolated primary islets have a much lower fraction of cells positive for either receptor at the RNA level when compared to SC-islets.

3.5. CVB replication in human SC-islets is inhibited by Vemurafenib and PLX7904

Next, we tested whether SC-islets are permissive to CVB infection. SC-islets were infected with CVB3 and titres of replicating virus were measured after 48 h. CVB3 replicated in all SC-islet batches (Fig. 5A). A clear dose response was seen with higher titres of replicating virus particles in the supernatant from SC-islets infected with a high CVB3 multiplicity of infection (MOI) compared to SC-islets infected with 10 or 100-fold lower MOIs of virus, highlighting the relationship between virus dose and the degree of virus-propagation in SC-islets (Fig. 5B). Based on our observations, we selected the MOI 4×10^3/islet for further studies.

To determine whether Vemurafenib and its analogue PLX7904 protect SC-islets from CVB3 infection, we treated different batches of SC-islets with Vemurafenib, PLX7904 or vehicle (DMSO), infected the islets, and cultured them for 48 h in the continued presence of drug. Culture supernatant viral titres were measured and in most of the SC-islet batches, Vemurafenib and PLX7904 lowered viral titres compared to those seen in the vehicle groups (Fig. 5C). Taken together, these studies suggest that Vemurafenib and PLX7904 attenuate CVB infection in human islet cells.

4. Discussion

Vemurafenib is a cancer drug that has been shown to have antiviral activity against EVs (Laajala et al., 2023). EVs have been linked to the development of T1D and in this study we show that Vemurafenib reduces CVB replication in several clinically relevant cell types.

Vemurafenib is used to treat advanced malignant melanoma. It is an inhibitor of the mutated $BRAF^{V600E}$ which stimulates tumour cell proliferation via the RAF/MEK/ERK pathway (Bollag et al., 2010; Yang et al., 2010). Previous studies have shown that Vemurafenib has antiviral activity (Holzberg et al., 2017; Hu et al., 2022; Ianevski et al., 2022). Recently it was demonstrated that Vemurafenib's antiviral activity is independent of its effect on the RAF/MEK/ERK pathway and is linked to cellular phosphatidylinositol 4-kinase type IIIb (PI4KB) (Laajala et al., 2023). Here, we confirmed the recent study that also documented the antiviral activity of Vemurafenib against CVB viruses (Laajala et al., 2023). We observed a potent inhibition of CVB replication and the release of new infectious virus particles at drug concentrations that were not cytotoxic to the cells studied. Moreover, we confirmed, for the first time, the antiviral properties of PLX7904, a Vemurafenib analogue. PLX7904 also inhibits RAF but is expected to cause both fewer side effects and less paradoxical activation than Vemurafenib (Le et al., 2013). The antiviral effects of both Vemurafenib and PLX7904 exceeded that of the Itraconazole, another repurposed drug with anti-enteroviral activity.

In initial studies, increasing concentrations of Vemurafenib protected HeLa cells from CVB3 infection without significantly influencing cell viability. HeLa cells do not have the $BRAF^{V600E}$ mutation (Pappa et al., 2006), further confirming that the antiviral effect of the drug is uncoupled from its effect on $BRAF^{V600E}$ (Laajala et al., 2023). Given that the intestine is the primary site of infection for CVBs (Wells and Coyne, 2019), we investigated Vemurafenib's effect on human IECs. Our studies initially focused on Caco-2 cells and we observed that Vemurafenib provided protection from CVB3-induced CPE. Additionally, we utilised HT-29 cells which carry the $BRAF^{V600E}$ mutation and confirmed the growth inhibitory effect of the drug (i.e. treatment caused reduced optical density). While we cannot rule out the possibility that Vemurafenib's growth inhibitory effect may impact virus replication, the addition of Vemurafenib led to improved cell density in CVB3 infected HT-29 cell cultures. Importantly, these studies expand the known number of cell types where Vemurafenib exerts antiviral properties and includes IECs which are located at the primary infection site.

Given EV infections of the beta cell (especially CVBs) are associated with T1D development (Laitinen et al., 2014; Oikarinen et al., 2014; Richardson and Morgan, 2018; Salminen et al., 2003), we investigated whether Vemurafenib offers protection against CVB infections in beta cells. We initially employed a beta cell line (INS-1832/13) and observed that Vemurafenib dose-dependently protected against CVB infection. Motivated by this, we subsequently investigated insulin-producing cells of human origin.

SC-islets are becoming an increasingly accessible alternative to primary human islets. As with primary islets, SC-islets comprise of several distinct cell types, including alpha, beta, and delta cells (Balboa et al., 2022). Here, we first examined whether SC-islets express the entry receptors for CVBs (CAR and DAF), which had not been previously described. With a few exceptions, SC-islet cells expressed DAF with varying proportions of these cells co-expressing CAR. Only a small number of cells were single positive for CAR.

In some of the batches of SC-islets, we specifically analysed CAR and DAF expression in glucagon positive (alpha) and insulin positive (beta) cells. We confirmed existing data regarding CAR expression in the human endocrine pancreas (Hodik et al., 2016; Ifie et al., 2018), and more specifically beta cells (Ifie et al., 2018; Xin et al., 2018) and show

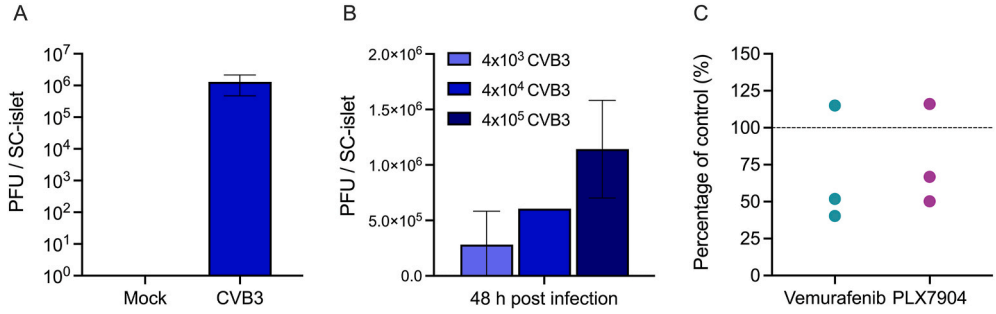

Fig. 5. Vemurafenib and its analogue can mitigate CVB3 infection in SC-islets. A and B. SC-islets were infected with CVB3 Nancy (A, 4×10^4 PFU/SC-islet, n = 2; B, 4×10^3, 4×10^4, 4×10^5 PFU/SC-islet, n = 1). Following 1 h infection, SC-islets were cultured in duplicates of 20 islets. 48 h later the number of infectious virus particles released into the culture media was measured using a standard plaque assay technique. Data is presented as plaque forming unit (PFU)/SC-islet. Mean ± SD. C. SC-islets were infected with CVB3 Nancy (4×10^3 PFU/SC-islet) and treated with vehicle alone (0.25 % DMSO), 5 μM of Vemurafenib (n = 3; teal dots) or 5 μM PLX7904 (n = 3; purple dots). Infectious virus particles released to the culture supernatant were measured by plaque assay at 48 h post infection. Data is presented as the percentage of the DMSO-treated CVB3-infected control.

that CAR is highly expressed in insulin-positive cells. DAF expression in beta cells was generally low. Interestingly, glucagon-positive cells exhibited high DAF expression but had few CAR-positive cells. Although our study was limited by the number of SC-islet batches analysed and demonstrated that CAR and DAF expression has some variation between batches, the high expression of CAR by insulin-positive cells indicates the usefulness of SC-islets as a primary human beta cell model in EV experiments.

CVB3 effectively replicated in SC-islets, resulting in the production of new infectious virus particles in a dose-dependent manner. Using the collective observations regarding Vemurafenib effects on cell viability and number in the cell types studied, a 5 μM dose was selected for SC-islet studies. Virus particle production was reduced in 66 % (2/3) of CVB3 infected SC-islet batches treated with Vemurafenib or PLX7904. Why Vemurafenib and PLX7904 did not consistently provide protection against infection in all batches currently remains unknown however it could involve batch variations in the SC-islets. Future studies involving additional SC-islet batches should address these observations. Nevertheless, the reduction in virus replication observed in several batches suggests these drugs may be beneficial for human islet cells infected with CVBs.

For the treatment of malignancies, the recommended starting dose of Vemurafenib is 960 mg twice daily, administered orally, with dose adjustments allowed for significant adverse events. The *in vivo* concentration of Vemurafenib in treated patients can vary significantly. Using the aforementioned starting dose, the maximum plasma steady-state concentration (C_{max}) is approximately 62 mg/L, corresponding to a molar concentration of around 127 μM (Kichenadasse et al., 2020). Our study provides important evidence showing that Vemurafenib, even when used at concentrations more than 10-fold lower than those reached in cancer patients, protects IECs and insulin-producing cells from infection. As many EVs infect via the gastrointestinal tract, it is possible that oral administration of the drug results in high local drug concentrations in the intestine, which would have beneficial antiviral effects and prevent infection at this site. We recently showed that Vemurafenib can clear a persistent infection in pancreas-derived cells *in vitro* (Laajala et al., 2023) therefore Vemurafenib may also be beneficial for persistent infections of the gut. This has important implications as prolonged faecal shedding of enterovirus has been linked with the appearance of islet autoantibodies in children at genetic risk of developing T1D (Vehik et al., 2019).

Although Vemurafenib exhibited potent antiviral activity against CVBs at concentrations lower than those typically achieved in treated patients, its use may still be limited due to the risk of serious side effects (e.g. heart, liver and kidney problems). Here we highlight that the efficacy of an alternative $BRAF^{V600E}$ inhibitor, PLX7904, in blocking CVB infection is comparable with Vemurafenib. Notably, PLX7904 exhibits improved efficacy and a modified mode of action, potentially mitigating side effects (Zhang et al., 2015). Given that Vemurafenib's antiviral effect has been linked to PI4KB rather than $BRAF^{V600E}$ or MAPK signalling, there is substantial potential for developing drugs that specifically target EV replication while minimising effects on the original cellular target. Moreover, the combined use of two or more drugs with synergistic effects allows for the utilisation of lower drug concentrations, as recently demonstrated for Vemurafenib in *in vitro* studies (e.g. Vemurafenib combined with emetine, homoharringtonine, anisomycin, or cycloheximide against echovirus 1) (Ianevski et al., 2020, 2022). This may further minimise the side effects associated with individual drugs.

Cancer treatment with checkpoint inhibitors, such as PD-L1 inhibitors, can increase the risk of developing checkpoint inhibitor–associated autoimmune diabetes mellitus, a condition resembling T1D (Wu et al., 2023). In contrast, no associations have been documented between Vemurafenib and diabetes. T1D is an autoimmune condition where the immune system attacks insulin-producing beta cells. PD-L1 normally binds to PD-1 on T cells to regulate immune responses. PD-L1 inhibitors block this signal, enhancing the immune system's ability to attack cancer cells but they can potentially lead to a loss of immune tolerance. The mechanism of PD-L1 inhibitors differs from that of Vemurafenib, making it unlikely that Vemurafenib could trigger T1D.

In summary, our study presents compelling evidence that both Vemurafenib and its analogue have potent antiviral effects against CVB infections in clinically relevant cell types. These findings lay the groundwork for future developments involving these compounds, either individually or in combination with other drugs, to establish antiviral treatments aimed at preventing enterovirus-induced diseases, including T1D.

CRediT authorship contribution statement

Marta Butrym: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Fabian Byvald:** Writing – review & editing, Visualization, Investigation, Formal analysis, Data curation. **Marfa Blanter:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Emma E. Ringqvist:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Svitlana Vasylovska:** Writing – review & editing, Resources, Methodology. **Varpu Marjomäki:** Writing – review & editing, Resources, Funding acquisition, Conceptualization. **Joey Lau:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition. **Virginia M. Stone:** Writing – review & editing, Supervision, Investigation, Funding acquisition, Data curation. **Malin Flodström-Tullberg:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2024.106021.

Data availability

Data will be made available on request.

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