Identification of antiviral compounds against equid herpesvirus-1 using real-time cell assay screening: Efficacy of decitabine and valganciclovir alone or in combination

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Abstract

Equid herpesvirus-1 infections cause respiratory, neurological and reproductive syndromes. Despite preventive treatments with vaccines, resurgence of EHV-1 infection still constitutes a major threat to equine industry. However, no antiviral compound is available to treat infected horses. In this study, 2,897 compounds were screened against EHV-1 using impedance measurement. 22 compounds were identified effective *in vitro* against EHV-1. (val)ganciclovir, decitabine, aphidicolin, idoxuridine and pritelivir (BAY 57-1293) are the most effective compounds and their antiviral potency were demonstrated on E. Derm, RK13 and EEK cells and against 3 different field strains of EHV-1 (ORF30 2254A/G/C). Valganciclovir and decitabine are the only combination tested that showed a synergistic effect *in vitro* using MacSynergy II, isobologramm and Chou-Talalay methods. Finally, this study demonstrated that decitabine needs to be phosphorylated by deoxycytidine kinase in order to be active against EHV-1. Deoxycitidine analogues, like decitabine, is a family of compounds identified for the first time with promising antiviral efficacy against herpesviruses.

Keywords

Real-time cell assay; Chemical library screening; Antiviral; Equid herpesvirus-1; ganciclovir; decitabine; synergism

1. Introduction

Herpesviruses (order *Herpesvirales*, family *Herpesviridae*) are enveloped viruses with a linear, double-stranded DNA genome of 125-290 kb. Among the five equid herpesviruses (EHV-1 to 5) frequently isolated in horses, EHV-1 is the most pathogenic and is endemic worldwide. EHV-1 infection in horses is associated with several clinical signs of disease, from usually mild respiratory distress, cough and discharge, to more severe secondary forms of diseases such as abortion, neonatal foal death and equine herpes myeloencephalopathy (EHM) (Allen, 2002). The prevalence of latent EHV-1 is estimated to be greater than 60% in horse population (Lunn et al., 2009).

Several vaccines are available against EHV-1. Their use reduce clinical signs of respiratory disease and virus shedding, which limits the extent of outbreaks. However, the protection provided against the secondary forms of the disease presents some limitations. While EHV-1 induced abortion storms have been prevented since the introduction of vaccination three decades ago, none of the commercially available EHV-1 vaccines have demonstrated its efficacy to prevent EHM. EHV-1 vaccine coverage is often too low to provide effective herd immunity. In this context, outbreaks still occur worldwide in horse populations. A recent outbreak reported in France in 2018 (Sutton et al., 2019), led to the cancellation of more than 200 horse competitions, thus generating large economic losses for the French equine industry. To complement prevention measures, such as vaccination and biosecurity, the use of antiviral treatment is sometimes considered to prevent severe forms of EHV-1 induced disease,

especially EHM. The occasional use of aciclovir during EHV-1 outbreaks has been reported (Friday et al., 2000; Henninger et al., 2007; Murray et al., 1998) but the therapeutic efficacy of this compound is difficult to assess in the absence of untreated animals as a control. Two experimental infections in horses treated with valaciclovir, an aciclovir pro-drug, have also shown divergent results (Garre et al., 2009; Maxwell et al., 2008).

EHV-1 is an alphaherpesvirus genetically closely related to herpes simplex virus type 1 (HHV-1) and varicella zoster virus (HHV-3) for which antiviral therapies are available. However, the emergence of human herpesvirus strains resistant to antiviral treatments, such as aciclovir, has motivated researches for new antiviral therapies (Jiang et al., 2016) and helicase primase inhibitors seem to be good candidates (James et al., 2015; Kleymann et al., 2002). Over the last two decades, drug repositioning has proven to be an effective strategy to meet therapeutic needs with nearly a hundred drugs repositioned since (Jourdan et al., 2020). Even in absence of approved EHV-1 antiviral treatment for practitioners, few antiviral molecules have been studied against EHV-1 *in vitro* and correspond to those already used in human medicine against herpesviruses such as aciclovir, ganciclovir, cidofovir and penciclovir (Maxwell, 2017; Vissani et al., 2016). Other compounds such as aphidicolin (Goodman et al., 2007), A-5021 (Glorieux et al., 2012), quercetin (Ferreira et al., 2018; Gravina et al., 2011) and the histone demethylase inhibitor OG-L002 (Tallmadge et al., 2018) have been studied against EHV-1 in different cell culture models. However, these molecules have never been tested in a standardised cellular model allowing proper comparisons.

We have recently developed a standardised Real-Time Cell Analysis (RTCA) model for evaluating the effect of antiviral compounds against EHV-1. This system relies on the measurement of cellular impedance in culture wells, which reflects cellular adhesion and proliferation. Results are expressed as Cell Index (CI) that enables a standardised and accurate analysis of EHV-1 cytopathic effects. This system has proven successful to determine the

efficacy of molecules against EHV-1 such as spironolactone (Thieulent et al., 2019). In the present work, a chemical library of 2,897 compounds comprising new chemical entities and FDA-approved drugs has been screened by impedancemetry to identify compounds against the EHV-1 Kentucky D (KyD) reference strain. As some associations between a DNA polymerase (ORF30) genotype (G/A at position 2254) and the type of disease have been reported by several studies (Goodman et al., 2007; Lunn et al., 2009; Nugent et al., 2006; Pronost et al., 2010), active molecules identified were subsequently tested against a panel of EHV-1 strains (A_{2254} or G_{2254}), including the newly identified EHV-1 ORF30 variant (C_{2254}) (Paillot et al., 2020). Decitabine was one of the most effective molecules identified, and the mode of action of this cytidine analogue has been further investigated.

2. Materials and methods

2.1. Cell lines

Equine dermal fibroblasts (E. Derm, NBL-6 ATCC® CCL-57, Manassas, VA), equine embryonic kidney cells (EEK, kindly provided by Merial, France) and rabbit kidney cells (RK13, ATCC® CCL-37™) were used in this study. E. Derm cells were maintained in Eagle's Minimum Essential Medium (ATCC®) and seeded at 1.2×10^4 cells/well in 96-well plates. EEK cells were maintained in MEM Alpha (Biowest, Nuaillé, France) supplemented with 2% Lactalbumin hydrolysate (Sigma, St. Quentin Fallavier, France), 1% L-glutamine (Eurobio, Courtaboeuf, France), 0.5% D-Glucose (Sigma) and seeded at 1.2×10^4 cells/well in 96-well plates. RK13 cells were maintained in EMEM with Earle's salts (Eurobio) supplemented with 1% L-glutamine (Eurobio) and seeded at 4.8×10^4 cells/well for 96-well plates. All media contained 10% fetal bovine serum (Eurobio), 100 IU/mL penicillin, 0.1 mg/mL streptomycin and 0.25 µg/mL amphotericin B (Eurobio) and were cultivated at 37 °C and 5% CO_2.

2.2. EHV-1 strains

The EHV-1 Kentucky D (KyD) strain (ATCC® VR700™) was used as the EHV-1 reference strain for compound screening and subsequently to confirm the antiviral effect of selected hits in the different cell lines. In addition, three French EHV-1 strains were also used in this study, including the ORF30 G_{2254} EHV-1 strain (FR-38991) isolated in 2009 from a horse with neurological disorders (LABÉO, France; nasal swab), the ORF30 A_{2254} EHV-1 strain (FR-6815) isolated in 2013 from lung biopsies of an aborted foetus (LABÉO, France) and the ORF30 C_{2254} EHV-1 strain (FR-56628) isolated in 2018 from PBMC of a horse with respiratory disorders (LABÉO, France) (Paillot et al., 2020). E. Derm and RK13 cells were infected with the KyD strain at MOIs of 0.01 and 0.04, respectively. EEK cells were infected with the four different EHV-1 strains at a MOI of 0.05.

2.3. Compounds

This study includes 2,897 compounds from three different libraries: i) 1,200 compounds from the Prestwick® Chemical Library, containing mostly US Food and Drug Administration approved drugs (Prestwick Chemical, Illkirch, France) provided at 2 mg/mL in DMSO; ii) 1,660 compounds from the Centre d'Etudes et de Recherche sur le Médicament de Normandie (CERMN, Caen, France) provided at 10 mM in DMSO; iii) 37 compounds (called herein antiviral library) selected for their effects against different human viruses and dissolved at 10 mM in DMSO (Supplementary Table 1). RG108 (MedChemExpress) was dissolved at 20 mM in DMSO. All compounds were stored at -20°C before used.

2.4. Screening of compound libraries using the RTCA system

The screening by impedancemetry was performed with EHV-1 KyD-infected E. Derm cells using the RTCA MP system (ACEA Biosciences, Montigny le Bretonneux, France) as previously described (Thieulent et al., 2019). Controls cells were treated with 0.5% DMSO in

presence or absence of the virus. The screening was performed under blind conditions and 80 compounds were tested by plate at a final concentration of 10 µg/mL (Preswitck® Chemical Library), 10 µM (CERMN library) or 50, 10, 2 and 0.4 µM (antiviral library) in 0.5% DMSO. Each plate includes the controls required for calculation of the Z'-factor (Zhang et al., 1999). Only plates with a Z' factor upper than 0.5 were considered for further analysis as previously described by Thieulent et al. (2019). For each compound, the area under normalised Cell Index (CI) curves was calculated from 0 to 96 hours post-infection (hpi) (AUCn; (Pan et al., 2013). The time required for the CI to decrease by 50% after virus infection was also determined (CIT_{50}; (Fang et al., 2011), and compared with controls. Any increase in these two parameters reflects some protection of E. Derm cells from EHV-1 induced cytopathic effects. The cut-off determined for a molecule to be considered with an antiviral potential were (i) the AUCn increasing by 25%, and (ii) the CIT_{50} being delayed by >6 h as compared to non-treated cells (Thieulent et al., 2019).

2.5. Viral quantitation by qPCR assay

Cells were seeded and treated in 96-well plates as described in part 2.4. At 48 hpi, plates were frozen at -20°C to allow virus load quantitation in culture wells. After one cycle of freeze/thaw, nucleic acids were extracted using the QIAamp® Viral RNA Mini Kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions and stored at -20 °C until used. Quantitative PCR for EHV-1 was processed as previously described (Thieulent et al., 2019). Each thermal cycling was performed on a QuantStudio™ 12 K Flex Real- Time PCR System (Life Technologies).

2.6. Toxicity measurement

Cells were seeded in white opaque 96-well plates and after 24 h of culture, were treated with compounds. Cell viability was measured at 48 h post-treatment (hpt) by impedancemetry and ATP measurement using the CellTiter Glo® Luminescent Cell Viability Kit (CTG; Promega,

Charbonnière-les-bains, France), according to the manufacturer's instructions. Luminescence signal was acquired using an Infinite® M200 luminometer (Tecan, Lyon, France).

2.7. Research of synergistic effects between compounds against EHV-1

Drug combinations were tested on EHV-1 KyD-infected E. Derm cells using impedancemetry as a read out. For each combination, the two selected drugs were prepared separately by 2-fold serial dilution and mixed in 96-well plates to create an 8 by 10 matrix of single and combined diluted drugs. For each compound, the dilution range was designed to have the IC_{50} in the middle of the range, and the highest concentration inferior to the IC_{90}. In each plate, infected and non-infected cells with 1% DMSO were used as positive and negative controls, respectively. Synergistic or antagonistic effects were determined with the MacSynergy II program using first the Bliss independence model (Prichard and Shipman, 1990) applied on AUC_n values. This software calculated the volume of synergy/antagonism produced by the drug combination in a 95% confidence interval. Volumes were given as the area under a dose-response curve in the two dimensional situation (μM^2 %) and interpretation was made as previously described by Prichard et al. (1990). Values of 0-25, 25-50, 50-100, and >100 μM^2 % in either a positive or negative direction were defined as additive, minor synergy or antagonism, moderate synergy or antagonism, and strong synergy or antagonism, respectively.

Isobologram analysis and the Chou-Talalay method using the Loewe additivity model were used to confirm synergistic effect firstly observed by MacSynergy II program. Isobolograms were built as previously described by Feng et al. (2009) from IC_{50} values obtained by impedance measurement. The Chou-Talalay method is based on the median-effect equation and computed by CompuSyn software version 1.0 (ComboSyn, Inc., Paramus, New Jersey) (Chou and Talalay, 1984). The software extrapolated a combination index representing the interaction between two drugs from the percentage of inhibition of $\log_{10}$ viral genome copies

number produce in presence of each drug alone and in combination. The weighted average combination index (CI_{wt}) value was calculated as previously described (Drouot et al., 2016).

2.8. Statistical analysis

IC_{50} and CC_{50} values were calculated using a non-linear regression dose response inhibition curve (GraphPad Prism® software 6.0; La Jolla, CA, USA). The Selectivity Index (SI) was determined for each compound using the following formula: $SI = CC_{50}/IC_{50}$. IC_{50} values were compared by ANOVA with Tukey post hoc test.

3. Results

3.1. Screening and selection of the most effective compounds against EHV-1

The three libraries (*i.e.* Prestwick chemical, CERMN and antiviral library) were screened against EHV-1 KyD as described in the Materials and Methods section. Z'-factor values of the 38 screening 96-well plates were between 0.52 to 0.91, with a median of 0.71, insuring the robustness of our assay. From the 2,897 compounds tested, 25 were identified with a potential antiviral activity (hit detection rate of 0.9%) against EHV-1 (14, 1 and 10 in the three chemical libraries, respectively).

These molecules were then evaluated in dose-response assays on E. Derm cells by two-fold serial dilution (50 to 0.1 µM) by qPCR assay and impedance measurement. Antiviral properties were confirmed for 22 out of the 25 compounds (Table 1). Among these compounds, eight were selected as they complied with more stringent criteria: (i) the absence of toxicity on E. Derm cells at all the concentrations tested (CC_{50} > 50µM) and (ii) IC_{50} values below 50 µM at all time points between 48 and 120 hpi (Figure 1). Three of these compounds were acyclic guanosine analogues (aciclovir, ACV; ganciclovir, GCV; and ganciclovir prodrug valganciclovir, VGCV) inhibitors of the viral DNA polymerase, two were

deoxycytidine analogues (decitabine, DTB; gemcitabine, GTB) inhibitors of cancers by cellular DNA incorporation, and one was a deoxyuridine analogue (idoxuridine, IDU) also an inhibitor of the viral DNA polymerase. Aphidicolin (APD), a tetracyclic diterpene antibiotic, and pritelivir (BAY 57-1293), an inhibitor of the HHV-1 helicase-primase complex, were also selected.

3.2. Efficacy of eight selected compounds on different EHV-1 strains and cell lines

The antiviral activity of the eight selected compounds was further studied against EHV-1 KyD using two other cellular models: an equine cell line (EEK) and a rodent cell line (RK13) and results were compared with data obtained on E. Derm cells (Table 2). Gemcitabine did not show any antiviral effect at tested concentrations on EEK and RK13 cells (IC_{50} > 50 µM). The weak antiviral activity of aciclovir obtained on RK13 cells (31.0 µM) was comparable with the result obtained on E. Derm cells (30.1 µM) and showed no effect on EEK cells (IC_{50} > 50 µM). The six other compounds (aphidicolin, pritelivir, decitabine, idoxuridine, ganciclovir and valganciclovir) showed a good efficacy on the three cell lines.

The efficacy of the eight selected compounds was tested against three different EHV-1 strains with distinct ORF30 genotypes ($A/G/C_{2254}$) on EEK cells (Table 3). This study confirmed the low activity of gemcitabine (IC_{50} > 50 µM). Aciclovir showed a lower activity against the FR-38991 strain (G_{2254}: 36.0 µM) when compared with its activity against FR-6815 (A_{2254}: 14.2 µM) or FR-56628 (C_{2254}: 7.9 µM). Quite similarly, pritelivir and idoxuridine showed a lower activity against FR-38991 strain (2.7 µM and 5.7 µM, respectively) when compared with its activity against FR-6815 (0.9 µM and 1.5 µM, respectively) or FR-56628 (1.0 µM and 1.5 µM, respectively). Consistent results were obtained with the other molecules across all EHV-1 strains.

3.3. Research of synergistic effect and antiviral activity of the valganciclovir/decitabine combination

Dual-combination were tested between valganciclovir, one of the best candidate and four other compounds (aphidicolin, pritelivir, decitabine and idoxuridine) for synergistic or antagonistic effects against EHV-1. Using MacSynergy II analysis, only the valganciclovir/decitabine combination showed a synergistic effect that is illustrated by the strong signal above additive effects in the matrix of drug interactions (Figure 2A). The synergy volume of 63.24 µM² % obtained supports a moderate synergy (Table 4). The peak of synergy was reached when both compounds were used at 0.63 µM (1:1 ratio). Likewise, evaluation of the combination valganciclovir/decitabine by the isobologram method indicated synergy with ADA values of -0.30 ($p < 0.001$) (Figure 2B). Results obtained by impedancemetry were also confirmed by viral genome copy number measurement at 48 hpi and median-effect analysis for concentrations of valganciclovir and decitabine used alone or in combination at a 1:1 ratio. A synergistic effect was observed for the valganciclovir/decitabine combination as assessed by a weighted average combination index (CI_{wt}) of 0.20 (Figure 2C). The three other combinations (valganciclovir/APB, valganciclovir/ pritelivir, valganciclovir/ idoxuridine) tested were additive when measured by MacSynergy II method (Table 4) and were not tested by isobologram nor median-effect analysis. No cytotoxicity was observed at the maximal drug combinations tested for the four different combinations (Supplementary Figure 1).

3.4. Decitabine pre-treatment did not confer cell resistance to EHV-1 replication.

Although valganciclovir was developed as an antiviral against herpesviruses in the first place, this is not the case of decitabine. Indeed, decitabine is an anticancer agent which induces hypomethylation after integration in cellular DNA (Liu et al., 2007). To evaluate whether decitabine integration in target cell DNA provides protection from EHV-1 infection, cells

were treated overnight with decitabine before infection and/or just after infection. Both results obtained by cell impedance measurement (Figure 3A) and EHV-1 viral load measurement (Figure 3A) showed that decitabine pre-treatment did not protect cells from CPE formation and virus replication. A post-infection decitabine treatment was required to observe some significant inhibition of EHV-1 replication. The effect of RG108, another well know DNA methyltransferase inhibitor, was then tested against EHV-1 on E. Derm cells. RG108 did not show any antiviral effect when assessed by impedance measurement (Figure 4A) or virus load quantitation (Figure 4B). Altogether, this suggests that cellular DNA hypomethylation does not account for the inhibition of EHV-1 by decitabine.

3.5. Deoxycytidine competitively inhibits the antiviral effect of decitabine

decitabine is a deoxycytidine analogue and a pro-drug that must be phosphorylated by the deoxycytidine kinase (DCK) to be integrated in target cell DNA (Stresemann and Lyko, 2008). Interestingly, it has been shown that high levels of deoxycytidine can reverse the anticancer activity of gemcitabine, another deoxycytidine analogue, by competition for DCK-mediated phosphorylation (Halbrook et al., 2019). EHV-1-infected E. Derm cells were treated with decitabine in the presence of high concentrations of deoxycytidine (dC) or other nucleosides including cytidine, uridine, adenosine, guanosine (Figure 5A). Of all tested nucleosides, only dC blocked the antiviral activity of decitabine. This result was confirmed by microscopic observations and impedancemetry as dC reversed the cell-protective effect of decitabine against EHV-1 (Figure 5B and C).

4. Discussion

In this study, 2,897 compounds were screened against EHV-1 by impedancemetry as previously described (Thieulent et al., 2019), and 22 compounds were identified for their antiviral properties against this virus. AUC_n values coupled to CIT_{50} calculation were the two major criteria for filtering raw data and identify hits. The antiviral effect of selected

compounds was confirmed by dose-response assay using both impedancemetry and viral load quantitation. As the readouts were not the same, IC_{50} values obtained with these two methods differed as previously reported (Piret et al., 2016; Thieulent et al., 2019). Among the 22 compounds effective against EHV-1, eight molecules were selected using stringent criteria, including IC_{50} values below 50 µM over time and lack of cytotoxicity when used at 50 µM.

Ganciclovir and aciclovir were previously shown to be effective against EHV-1 *in vitro* (Garre et al., 2007; Thieulent et al., 2019). valganciclovir, the pro-drug and valine ester of ganciclovir, presents here an antiviral activity against EHV-1 similar to ganciclovir. Idoxuridine is a well know antiviral compound against herpesviruses in human such as HHV-1 and HHV-2 and also different animal species such as feline herpesvirus type-1 (De Clercq and Li, 2016; Maggs and Clarke, 2004). Pritelivir is an inhibitor of the helicase-primase complex of herpesviruses discovered in 2002 (Kleymann et al., 2002). It does not require any activation step unlike other nucleoside analogues such as ganciclovir. The antiviral effect of pritelivir was previously reported against HHV-1 and HHV-2 (Betz et al., 2002). However, this study is the first demonstrating the antiviral effect of this molecule against EHV-1. Decitabine and gemcitabine are two deoxycytidine analogues used in the treatment of acute myeloid leukemia (He et al., 2017) and recurrent ovarian cancer (Berg et al., 2019), respectively. To our knowledge, this is the first report showing that deoxycytidine analogues inhibit the replication of a herpesvirus. In this study, we have discovered that decitabine, gemcitabine and cytarabine are all effective against EHV-1 infection, at least *in vitro*. Of these three compounds, cytarabine has the lowest activity with an IC_{50} of 4.1 µM as determined by qPCR assay on E. Derm cells. Decitabine and gemcitabine were more potent EHV-1 inhibitors in this cellular model with IC_{50s} of 1.1 µM and 0.7 µM, respectively.

Quite surprisingly, brivudine and maribavir did not show any antiviral effect against EHV-1. Brivudine is one of the three deoxyuridine analogues, together with idoxuridine and

trifluridine, that have been used for decades against herpes simplex viruses (De Clercq and Li, 2016). Idoxuridine and trifluridine have showed a good efficacy against EHV-1 without toxicity in our cellular model, whereas brivudine was inactive (data not shown). Brivudine is the only one that needs to be specifically phosphorylated by viral thymidine kinase (TK) to become active (De Clercq and Li, 2016), suggesting that EHV-1 TK is unable to phosphorylate brivudine, which is in line with previous reports (De Clercq, 1984; Kit et al., 1987). Maribavir is a new antiviral drug in development against human cytomegalovirus (HHV-5), a betaherpesvirus (Price and Prichard, 2011). This compound showed no antiviral effect against EHV-1 in our model (data not show), in line with the lack of activity against the alphaherpesviruses HHV-1, HHV-2 and HHV-3 (Williams et al., 2003).

The antiviral activity of the 8 most efficient molecules was also validated in three cell lines and against different strains of EHV-1. E. Derm cells and EEK cells are both equine cell lines and most adapted to identify new antiviral compounds in equid species, especially EEK that was derived from a horse foetus that is one of the target of EHV-1 (Léon et al., 2008; Smith et al., 2010). Even though RK13 cells are not equine cells, they have been most frequently used in EHV1 antiviral studies (Azab et al., 2010; de la Fuente et al., 1992; Gibson, 1992; Rollinson, 1987). All compounds except aciclovir and gemcitabine showed some consistent antiviral activity in the three different cell lines. Aciclovir is the least active of the eight selected compounds, and was inactive when used with EEK cells. More surprisingly, although gemcitabine is very effective on E. Derm cells, it has no antiviral activity on RK13 and EEK cells. This suggests that gemcitabine is not properly phosphorylated in RK13 and EEK cells. In line with this hypothesis, decitabine, which is structurally very close to gemcitabine and also needs to be phosphorylated is less active on EEK and RK13 cells. Nevertheless, decitabine still exhibits a good efficacy in all three cellular models. The antiviral activity of selected molecules was also evaluated on EEK cells infected with three different EHV-1

strains isolated during outbreaks in France. Each strain exhibits different nucleotide (A/G/C) at position 2254 of ORF-30 (DNA polymerase). IC_{50} values of the compounds were close, independently of the strain used. This suggests that the mutations in the palm domain did not affect the effect of the selected molecule. This result was concordant with a previous report when comparing strains A and G at position 2254 of ORF-30. (Garre et al., 2007; Thieulent et al., 2019). Only aciclovir, pritelivir and idoxuridine were slightly less efficient on the FR-38991 (A_{2254}) strain. Surprisingly, no difference of susceptibility was observed between the three strains for aphidicolin treatment. This result differs from a previous report showing that a strain with the G_{2254} genotype is more sensitive to aphidicolin than a strain with the A_{2254} genotype (Goodman et al., 2007).

Ganciclovir is the most potent of the few compounds that were already known to be active against EHV-1 infection *in vitro* (Garre et al., 2007; Thieulent et al., 2019). In this study, valganciclovir was compared with ganciclovir in a standardized *in vitro* assay, and results showed similar activities against EHV-1. The pharmacokinetic of valganciclovir was previously studied in horse (Carmichael et al., 2013), showing 40% bioavailability after oral administration. Actually, this positions the valganciclovir as the best candidate for therapy of EHV-1 infected horses even if the cost of the molecule could be a limitation. In order to enhance the effect of valganciclovir, combination of valganciclovir with aphidicolin, pritelivir, decitabine and idoxuridine were analysed. Only the valganciclovir/decitabine combination showed a synergic effect. Synergy was confirmed by three different methods. In addition, this is the first analysis of drug combinations against EHV-1. Some studies have previously documented synergic effects between ganciclovir and other compounds against HHV-5 (Chou et al., 2018; Drew, 2006). Combinations of valganciclovir with idoxuridine and pritelivir were additive. Combination of ganciclovir with aphidicolin was never tested before. However, aciclovir acted synergistically with aphidicolin against HHV-1 when used at a 1:1

molar ratio (Michaelis et al., 2011). As decitabine acts in synergy with valganciclovir and was never tested against herpesviruses to our knowledge, the mechanism of action of this compound was further investigated. The pre-treatment of cells with decitabine did not provide antiviral effects against EHV-1, suggesting that decitabine incorporation in cell DNA did not mediate the antiviral effect observed when decitabine was administrated to the cell culture after EHV-1 infection. As decitabine is known to induce cellular DNA hypomethylation (Atallah et al., 2007; Schmelz et al., 2005), the antiviral effect of RG108, another DNA methyltransferase inhibitor, was also tested against EHV-1. The absence of RG108 activity against EHV-1 suggests that the inhibitor effect of decitabine against EHV-1 was not mediated by viral or cellular DNA hypomethylation.

Finally, decitabine is a drug that requires to be phosphorylated to act as an anticancer agent alike gemcitabine. This phosphorylation is dependent on deoxycytidine kinase (DCK) (Stresemann and Lyko, 2008). The use of deoxycytidine (dC) inhibited decitabine antiviral activity, probably by preventing decitabine phosphorylation by DCK through molecular competition. As the initial phosphorylation of decitabine by DCK is required for its activity, it is assumed that the decitabine active form in our model is the triphosphate form. Under this form, decitabine is probably integrated in EHV-1 DNA during viral replication, thus inhibiting viral growth. As opposed to ganciclovir that needs activation by viral TK (Sullivan et al., 1992), decitabine phosphorylation only relies on cellular kinase to be activated and may represent a good candidate against viral strains resistant to ganciclovir.

In conclusion, the antiviral effect of ganciclovir /valganciclovir and aphidicolin was confirmed against EHV-1. Most importantly, new EHV-1 inhibitors were identified, including idoxuridine, pritelivir and decitabine. The synergy observed between valganciclovir and decitabine is particularly interesting due to the complementarity of their mode of actions, and further investigations *ex vivo* and *in vivo* are warranted.

Conflict of interest

The authors declare no competing interests.

Acknowledgments

This work was supported by LABÉO, IFCE (Institut Français du Cheval et de l'Equitation, project AMIE), Fonds Eperon (project N87-2014, N07-2015, N07-2016, N13-2017 and N62-2017), Région Normandie (CPER R25 P3) and CENTAURE European project co-funded by Normandy County Council, European Union in the framework of the ERDF-ESF operationnal programme 2014-2020. We would like to thank Laurent Lemaitre from Boehringer Ingelheim, France who kindly provided us with EEK cells. We thank Christophe Denoyelle and Emilie Brotin from ImpedanCELL, Normandie Univ, UNICAEN, Caen, France for interacting and for their technical assistance with RTCA technology. We also thank all collaborators of the SAVE project.

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Table 1: List of compounds presenting an antiviral effect against EHV-1 Kyd strain on E. Derm cell.

Data presented in this table are the mean (S.D.) of three independent experiments. IC_{50}: IC_{50}[a] half maximal effective concentration measured by impedance using Real-Time Cell Analysis (RTCA) system or IC_{50}[d] qPCR assay.

CC_{50}: CC_{50}[b] half maximal cytotoxic concentration measured by impedance using RTCA system or CC_{50}[e] CellTiter-Glo (CTG) method. "CC_{50} > 50" means that the compound did not show toxicity at the highest concentration tested (50 µM).

SI: Selectivity Index is the ration of CC_{50} obtained by RTCA to IC_{50} obtained by RTCA[c] or CC_{50} obtained by CTG to IC_{50} obtained by qPCR[f]. If "CC_{50} > 50", an arbitrary value of SI is calculated with 50 µM, but it is probably underestimated.

Bold compounds are the selected compounds in part 3.1 of results.

Table 2: Combination analysis of compounds against EHV-1 KyD strain on E. Derm cells.

[a]Mean volumes of synergy or antagonism are presented based on 95% confidence levels using MacSynergy II method.

Values determined using MacSynergy II software (Prichard and Shipman, 1990) via area under normalised curves (AUC_n) data from 0 to 96 hours post-infection using impedance measurement. Results are obtained from three independently experiments.

Table 3: Susceptibility of EHV-1 KyD strain to the 8 selected antiviral compounds on three different cell line: E Derm, EEK and RK13.

[a]Significant difference between E. Derm and EEK cells ($^ap < 0.05$).

[b]Significant difference between E. Derm and RK13 cells ($^bp < 0.01$).

[c]Significant difference between E. Derm and EEK cells ($^cp < 0.05$).

[d]Significant difference between E. Derm and RK13 cells ($^dp < 0.01$).

[e]Significant difference between EEK and E. Derm cells ($^ep < 0.01$).

[f]Significant difference between EEK and RK13 cells ($^fp < 0.05$).

[g]Significant difference between RK13 and E. Derm cells ($^gp < 0.01$).

[h]Significant difference between RK13 and EEK cells ($^hp < 0.01$).

Table 4: Susceptibility of French isolates of EHV-1 to the 8 selected antiviral compounds: EC50 value were obtained by qPCR on EEK cells.

[#]Mutation of amino-acid at position 2254 of the ORF30 (Nugent et al., 2006). G_{2254} means a guanine (Aspartic Acid (D_{752})) in position 752 of the protein, A_{2254} means an adenine (Asparagine (N_{752})) and C_{2254} means a cytidine Asparagine (Histidine (H_{752})).

[a]Significant difference between strains FR-38991 and FR-56628 ([a]$p < 0.05$).

[b]Significant difference between strains FR-38991 and FR-6815 ([b]$p < 0.01$).

[c]Significant difference between strains FR-38991 and FR-56628 ([c]$p < 0.01$).

[d]Significant difference between strains FR-38991 and FR-6815 ([d]$p < 0.01$).

[e]Significant difference between strains FR-38991 and FR-56628 ([e]$p < 0.01$).

Figure 1: Antiviral effect of selected compounds against EHV-1 KyD strain on E. Derm cells at different times post-infection using impedance measurement. Results are obtained from three independent experiments. IC_{50}: half maximal effective concentration. Dotted line represent the cut off IC_{50} value.

Figure 2: Synergistic inhibition of EHV-1 KyD strain replication in E. Derm cells by combination of valganciclovir/decitabine. (**A**) Analysis of interaction of VGCV and DTB using impedance measurement with MacSynergy II software. Peaks of statistically significant (95% confidence level) synergy are shown above the plane in colours from grey to blue, with dark blue indicating a strong synergy. The volume of synergy for this interaction is 63.24, which is interpreted as moderate synergy. Results are obtained from three independently experiments performed using impedance measurement. (**B**) Isobologram analysis of the interaction of VGCV and DTB using impedance measurement. The diagonally dotted line in red represents additivity. Values below and above this line are interpreted as synergy or antagonist, respectively. The ADA value for this interaction is -0.30 ($p < 0.001$) which is interpreted as synergy. Results are obtained from three independently experiments performed using impedance measurement. (**C**) Median-effect analysis table representing the interaction of VGCV and DTB at 1:1 ratio using qPCR assay. Combination Index (CI) was calculated using the Chou and Talalay equation (Chou & Talalay, 1984). $CI < 1$, $CI = 1$ and $CI > 1$ indicate synergism, additive and antagonism, respectively. The weighted CI is calculated as

follows: $CI_{wt} = (CI_{50} + 2CI_{75} + 3\ CI_{90} + 4CI_{95})/10$. The CI_{wt} value for this interaction is 0.20 which is interpreted as synergy. Results are obtained from three independently experiments performed using qPCR assay.

Figure 3: Decitabine (5 µM) pre-infection or post-infection treatment after infection by EHV-1 KyD strain of E. Derm cells measured at 48 hpi by (**A**) impedance measurement and (**B**) viral genome copies number quantitation. Results are obtained from three independents experiments ($^{***}p < 0.001$).

Figure 4: Effect of the DNA methyltransferase inhibitor, RG108 and decitabine on E. Derm cells infected by EHV-1 KyD strain measured at 48 hpi by (**A**) impedance measurement and (**B**) viral load quantitation. Results are obtained from three independents experiments.

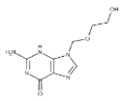
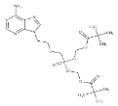
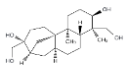
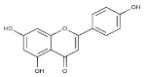
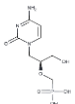
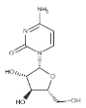
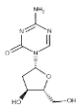
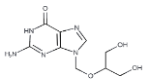
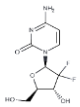
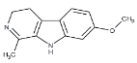
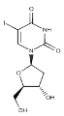
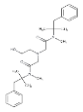
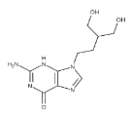
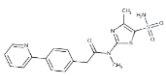
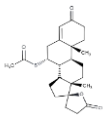
Figure 5: Effect of deoxycytidine (dC) on the antiviral effect of decitabine (DTB) against EHV-1. (**A**) Viral genome copies number produces in the cell culture supernatant at 48 hpi in absence (0 µM) or presence (1.6 and 6.4 µM) of DTB with 100 µM of cytidine, uridine, adenosine, guanosine and dC. Results are obtained from five independents experiments ($^{*}p < 0.05$) (**B**) Microscopic observation at 48 hpi of E. Derm cells infected or not by EHV-1 KyD strain in presence of DTB (6.4 µM) with or without dC (100 µM) treatment. (**C**) Impedance measurement at 48 hpi of E. Derm cells treated with increased concentrations of DTB in the presence of the indicated concentration of dC. Results are obtained from three independents experiments.

Supplementary Table 1: Library of selected compounds for their antiviral effects on different family of virus.

Supplementary Figure 1: Cytotoxicity assay of combinations on equine dermal cells. Toxicity evaluation is measured by luminescence assays using CellTiter Glo® kit. Histogram

represents the percentage viability of E. Derm cells treated with the highest concentration of compounds compared to mock-treated cells. Each data corresponds to mean ± SD of three independent experiments.

Table 1

			Impedance measurement using Real Time Cell Analysis (RTCA) system			qPCR assay	CellTiter Glo (CTG)		
Class of compounds	Compounds	Structure	IC_{50} [μM (S.D.)][a]	CC_{50} [μM (S.D.)][b]	SI[c]	IC_{50} [μM (S.D.)][d]	CC_{50} [μM (S.D.)][e]	SI[f]	Supplier
Acyclic guanosine analogue	**Aciclovir (ACV)**		**30.1 (13.3)**	**>50**	**>1.7**	**49.4 (3.7)**	**>50**	**>1.01**	**Prestwick Chemical**
Acyclic cytidine monophosphate analogue	Adefovir dipivoxil (ADV)		0.8 (0.3)	3.2 (1.4)	4.0	0.1 (0.0)	3.7 (2.1)	37.0	TargetMol
Diterpene	**Aphidicholin (ADP)**		**3.4 (3.3)**	**>50**	**>14.7**	**1.4 (0.2)**	**>50**	**>35.7**	**MedChem express**
Flavonoid	Apigenin (APG)		>50	>50	N.D.	31.0 (22.5)	>50	>1.6	Prestwick Chemical
Acyclic cytidine monophosphate analogue	Cidofovir (CDV)		20.7 (4.7)	>50	>2.4	23.3 (10.0)	>50	>2.1	Prestwick Chemical
Deoxycytidine analogue	Cytarabine (CTB)		24.3 (7.6)	31.2 (2.6)	1.3	4.1 (1.3)	>50	>12.2	TargetMol
Deoxycytidine analogue	**Decitabine (DTB)**		**0.5 (0.1)**	**>50**	**>100**	**1.1 (0.5)**	**>50**	**>45.5**	**TargetMol**
Acyclic guanosine analogue	**Ganciclovir (GCV)**		**2.7 (1.3)**	**>50**	**>18.5**	**2.8 (0.9)**	**>50**	**>17.9**	**Prestwick Chemical**
Deoxycytidine analogue	**Gemcitabine (GTB)**		**12.0 (3.4)**	**>50**	**>4.2**	**0.7 (0.3)**	**>50**	**>71.4**	**TargetMol**
Alkaloid	Harmine (HAR)		>50	>50	N.D.	13.6 (7.6)	>50	>3.7	Prestwick Chemical
Deoxyuridine analogue	**Idoxuridine (IDU)**		**4.9 (1.5)**	**>50**	**>10.2**	**4.7 (1.6)**	**>50**	**>10.6**	**Prestwick Chemical**
Pseudopeptide	Oxethacaine (OXT)		34.9 (20.2)	>50	>1.4	19.3 (6.7)	21.05 (4.8)	1.1	Prestwick Chemical
Acyclic guanosine analogue	Penciclovir (PCV)		9.8 (6.1)	>50	>5.1	>50	>50	N.D.	TargetMol
Phenylpyridine	**Pritelivir (BAY 57-1293)**		**12.6 (1.8)**	**>50**	**>4.0**	**6.6 (0.7)**	**>50**	**>7.6**	**MedChem express**
Steroid lactone	Spironolactone (SPR)		36.0 (1.3)	>50	>1.4	27.7 (6.8)	>50	>1.8	Prestwick Chemical

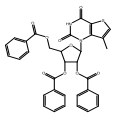
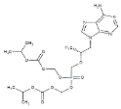
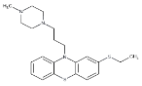
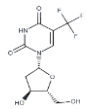
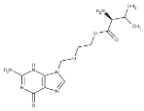
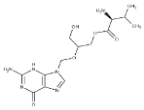
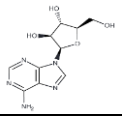
Nucleoside analogue	sr6362		8.4 (4.5)	>50	>6.0	>50	>50	N.D.	CERMN
Acyclic cytidine monophosphate analogue	Tenofovir disoproxil (TDF)		>50	17.7 (3.7)	N.D.	3.0 (1.4)	35.8 (15.5)	11.9	TargetMol
Phenothiazine	Thiethylperazine dimaleate (THT)		>50	17.6 (2.4)	N.D.	10.3 (2.8)	10.1 (0.9)	1.0	Prestwick Chemical
Deoxyuridine analogue	Trifluridine (TFT)		21.6 (10.9)	>50	>2.3	5.3 (1.6)	>50	>9.4	Prestwick Chemical
Acyclic guanosine analogue	Valaciclovir (VACV)		34.7 (4.3)	>50	>1.4	23.5 (3.6)	>50	>2.1	TargetMol
Acyclic guanosine analogue	**Valganciclovir (VGCV)**		**1.7 (0.3)**	**>50**	**>29.4**	**1.8 (0.3)**	**>50**	**>27.8**	**TargetMol**
Adenosine analogue	Vidarabine (VDR)		12.9 (6.2)	>50	>3.9	8.3 (0.7)	>50	>6.0	Prestwick Chemical

Table 2

	IC_{50} values measured by qPCR [µM (S.D.)] against EHV-1 KyD strain							
Cells	ACV	APD	BAY 57-1293	DTB	GCV	GTB	IDU	VGCV
E. Derm	30.1 (13.3)	3.4 (3.3)	6.6 (0.7)	1.1 (0.5)[a,b]	2.8 (0.9)[c,d]	0.7 (0.3)	4.7 (1.6)[e]	1.8 (0.3)[g]
EEK	>50	0.1 (0.0)	11.4 (1.4)	8.7 (3.4)[a]	1.2 (0.1)[c]	>50	16.2 (1.2)[e,f]	1.8 (0.3)[h]
RK13	31.0 (9.3)	1.9 (0.7)	10.1 (2.3)	12.5 (2.1)[b]	0.6 (0.2)[d]	>50	9.3 (1.7)[f]	1.0 (0.2)[g,h]

Table 3

Strains (ORF30 profil)[#]	EC_{50} values measured by qPCR [µM (S.D.)] on EEK cells							
	ACV	APD	BAY 57-1293	DTB	GCV	GTB	IDU	VGCV
FR-6815 (A_{2254})	14.1 (5.3)	0.1 (0.0)	0.9 (0.1)[b]	2.4 (0.6)	0.8 (0.4)	>50	2.1 (0.6)[d]	1.2 (0.6)
FR-38991 (G_{2254})	36.0 (11.0)[a]	0.1 (0.0)	2.7 (0.1)[b,c]	5.8 (2.5)	0.7 (0.4)	>50	5.7 (1.1)[d,e]	1.1 (0.5)
FR-56628 (C_{2254})	7.9 (1.0)[a]	0.1 (0.0)	1.0 (0.3)[c]	6.8 (6.2)	0.6 (0.2)	>50	1.5 (0.6)[e]	1.0 (0.3)

Table 4

Conbinations	MacSynergy II analysis[a]		
	Synergy (µM² %)	Antagonism (µM² %)	Predicted interaction
VGCV + APD	0.16	-17.95	Additif
VGCV + BAY 57-1293	4.56	-1.27	Additif
VGCV + DTB	63.24	0	Moderate synergy
VGCV + IDU	1.5	0	Additif

Figure 1

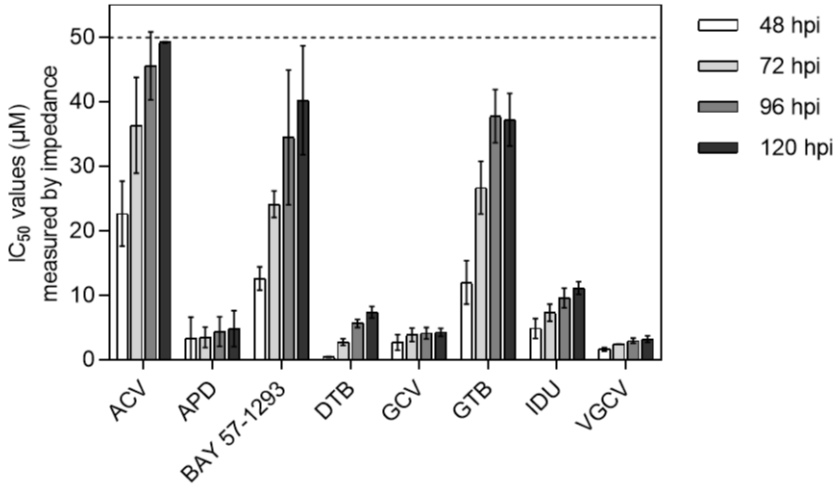
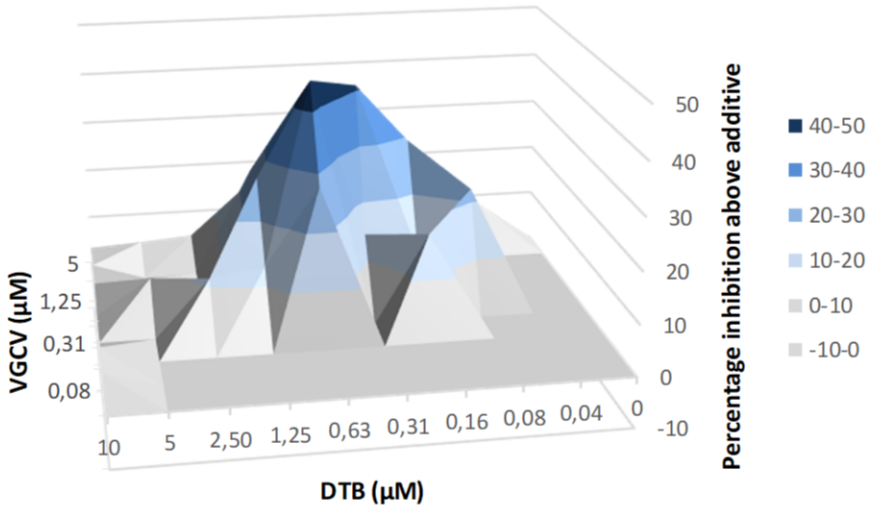
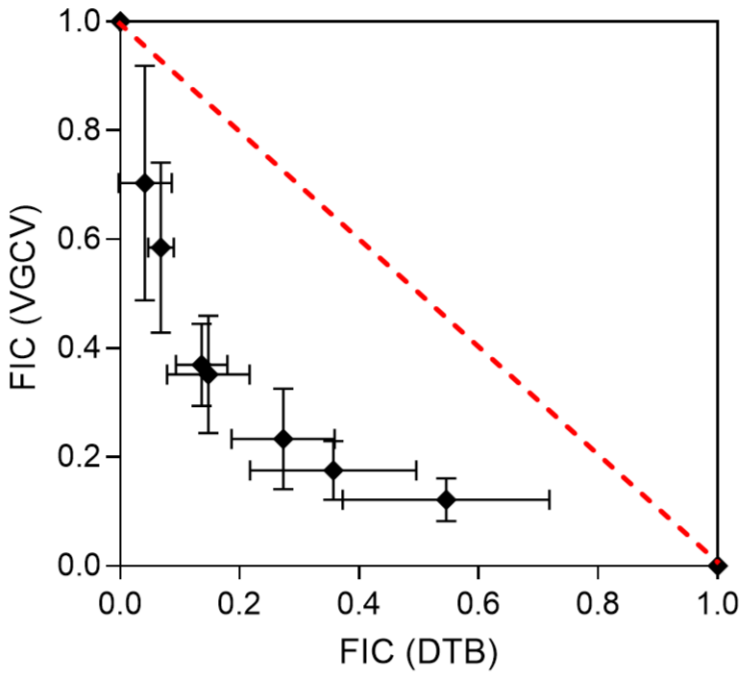


Figure 2

A



B



C

		CI values extrapolated at % of virus inhibition			
		50	**75**	**90**	**95**
VGCV + DTB (1:1)	**Experience 1**	0.44	0.33	0.24	0.20
	Experience 2	0.39	0.24	0.15	0.11
	Experience 3	0.33	0.21	0.13	0.10
	Mean (S.D.)	0.39 (0.05)	0.26 (0.06)	0.18 (0.06)	0.14 (0.06)
	CI_{wt}		**0.20**		
Drug combinatory			Synergism		

Figure 3

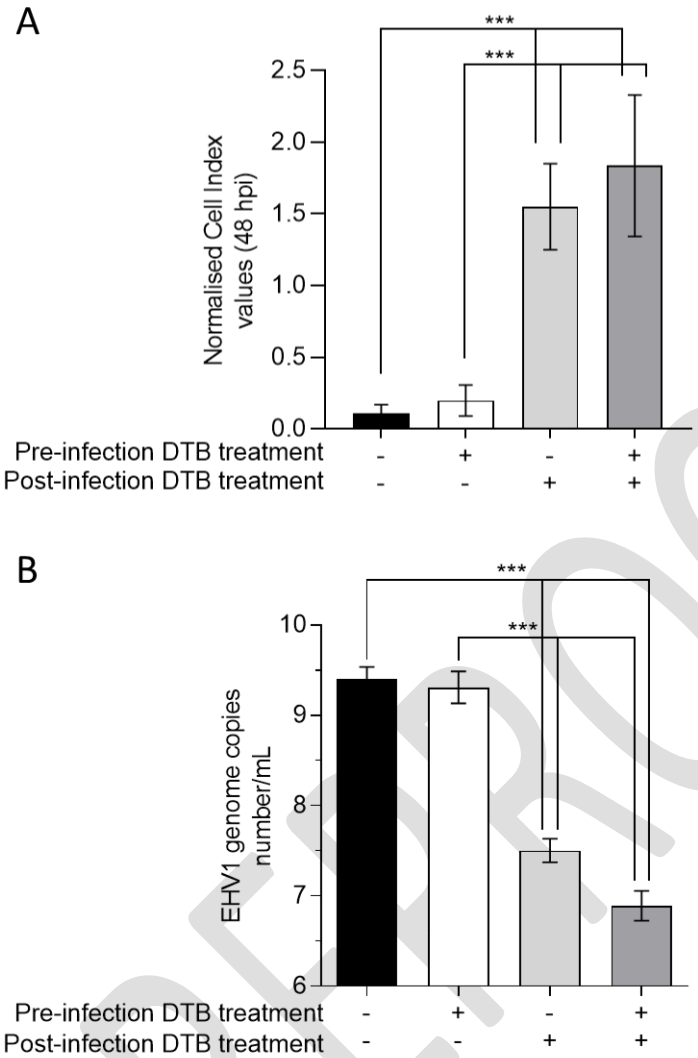


Figure 4

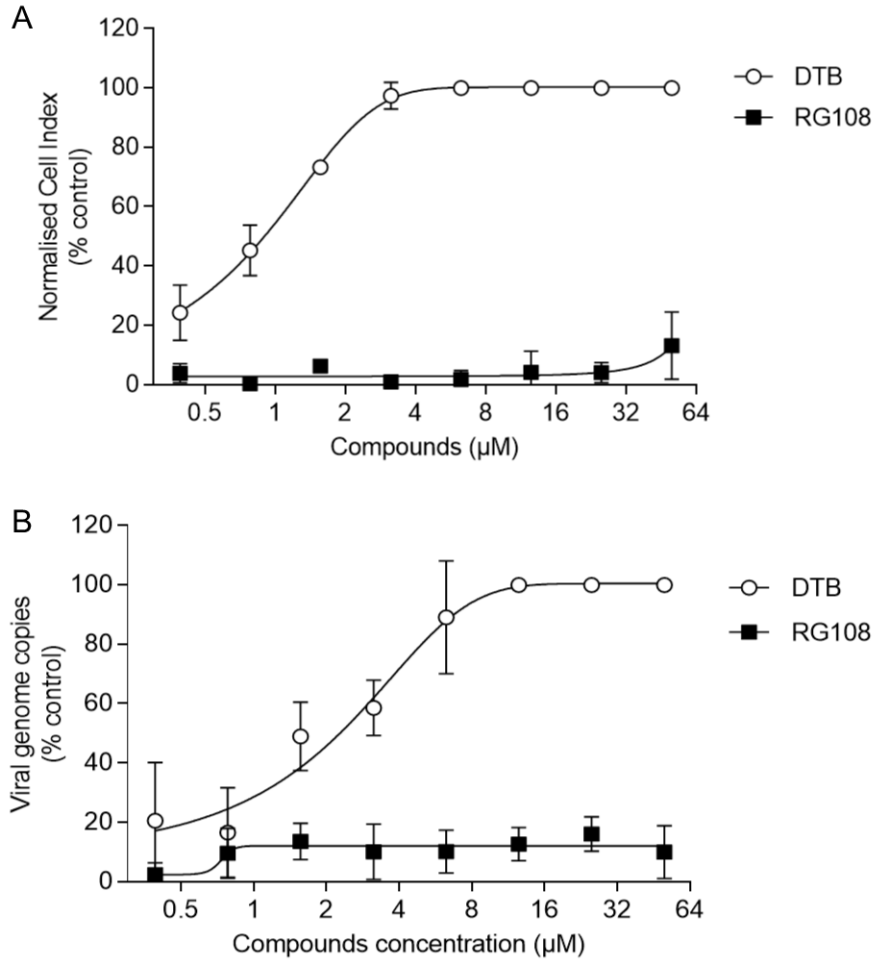
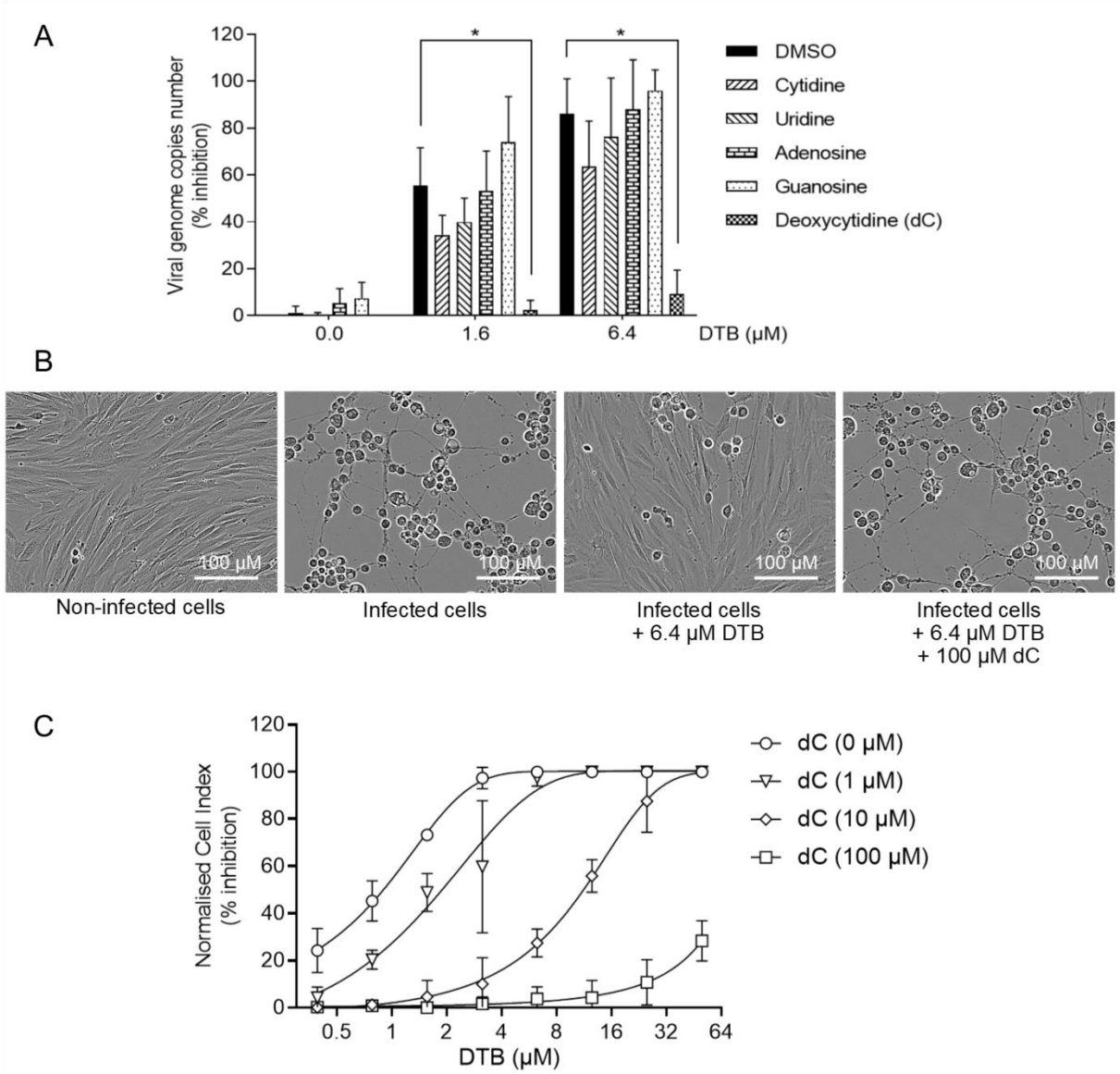

A
Normalised Cell Index
(% control)
120
100
80
60
40
20
0
0.5
1
2
4
8
16
32
64
Compounds (μM)
DTB
RG108
B
Viral genome copies
(% control)
120
100
80
60
40
20
0
0.5
1
2
4
8
16
32
64
Compounds concentration (μM)
DTB
RG108

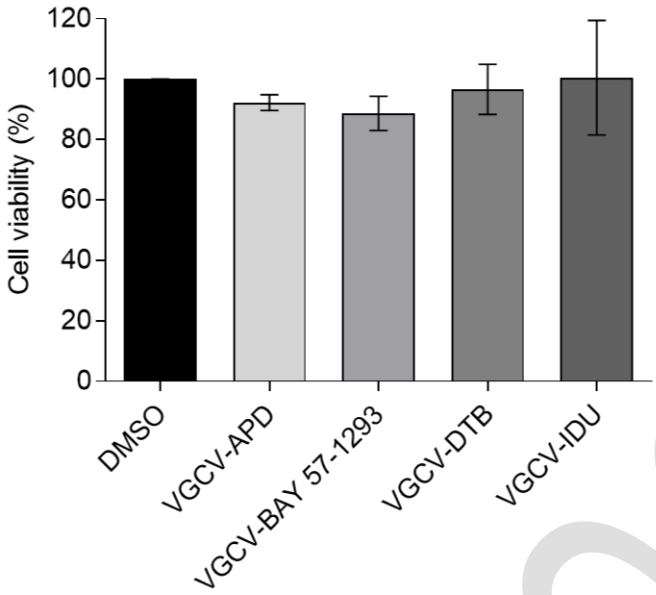
Figure 5


A
Viral genome copies number (% inhibition)
120
100
80
60
40
20
0
*
*
0.0
1.6
6.4
DTB (μM)
DMSO
Cytidine
Uridine
Adenosine
Guanosine
Deoxycytidine (dC)
B
100 μM
100 μM
100 μM
100 μM
Non-infected cells
Infected cells
Infected cells
+ 6.4 μM DTB
Infected cells
+ 6.4 μM DTB
+ 100 μM dC
C
Normalised Cell Index (% inhibition)
120
100
80
60
40
20
0
0.5
1
2
4
8
16
32
64
DTB (μM)
dC (0 μM)
dC (1 μM)
dC (10 μM)
dC (100 μM)

Supplementary table 1

Compounds	Suppliers
2’-C-methylcytidine	Ark Pharm, Inc.
25-hydroxycholesterol	Cayman Europe
Abacavir	TargetMol
Adefovir dipivoxil	TargetMol
Arbidol	TargetMol
Atorvastatin	Sigma
Brivudine	MedChemExpress
Capecitabine	TargetMol
Cidofovir	TargetMol
Cytarabine	TargetMol
Decitabine	TargetMol
Didanosine	TargetMol
DMXAA	TargetMol
Eflornithin (dfmo)	Sigma
Emtricitabine	TargetMol
Entecavir	TargetMol
Famciclovir	TargetMol
Favipiravir	TargetMol
Fluorouracile	AK Scientific, Inc.
Fluvastatin	Sigma
Gemcitabine	TargetMol
Lamivudine	TargetMol
Maribavir	TargetMol
Mercaptopurine	TargetMol
Nelarabine	AK Scientific, Inc.
Penciclovir	TargetMol
Pravastatin	Sigma
Pritelivir (BAY 57-2193)	MedChemExpress
Proguanil	BIONET / Key Organics Ltd.
Simvastatin	Sigma
Sofosbuvir	TargetMol
Stavudine	TargetMol
Telbivudine	TargetMol
Tenofovir disoproxil	TargetMol
Thioguanine	TargetMol
Valaciclovir	TargetMol
Valganciclovir	TargetMol

Supplementary figure 1



Graphical abstract