Anti-CyHV-3 effect of fluorescent, tricyclic derivative of acyclovir 6-(4-MeOPh)-TACV *in vitro*

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Abstract

Introduction: *Cyprinid herpesvirus 3* (CyHV-3) is a virus infecting carp with disease symptoms of gill necrosis, fish discoloration, sunken eyes, and mortality reaching 90%. Several research groups have examined how to potentially abate the consequences of viral activity. Recently we showed that acyclovir inhibits CyHV-3 replication *in vitro* and in the present study we examined the anti-CyHV-3 activity of the tricyclic derivative of acyclovir 6-(4-MeOPh)-TACV (T-ACV), a fluorescent molecule known for higher lipophilicity than acyclovir, and therefore potentially better candidate for application *in vivo*. **Material and Methods:** CCB and KF1 cell lines were incubated with T-ACV at concentrations of 0, 66.67, and 133.33 µM for three days and toxicity examined with MTT and CV assays. To investigate the antiviral activity of T-ACV, the lines were infected with CyHV-3 or mock infected and incubated for three days with the drug at concentrations of 0 or 66.67 µM. The activity of T-ACV was evaluated by plaque assay and TaqMan qPCR. **Results:** T-ACV at a concentration of 66.67 µM displayed low toxicity and inhibited CyHV-3 activity by 13–29%, varying by cell line and method. **Conclusion:** The low anti-CyHV-3 activity of T-ACV indicates that it would be reasonable to screen several tricyclic derivatives of acyclovir for such activity.

Keywords: carp, *Cyprinid herpesvirus 3*, acyclovir, tricyclic derivative of acyclovir.

Introduction

Cyprinid herpesvirus 3 (CyHV-3) is a highly contagious virus transmitted among common and koi carp, and a member of the *Cyprinivirus* genus of the *Alloherpesviridae* family in the *Herpesvirales* order. CyHV-3, also known as koi herpes virus (KHV), is responsible for mortality as high as 80–95% in carp populations (22). The pathogen can enter a fish's body *via* the skin or pharynx, get into white blood cells, and easily spread to other organs, persisting even in fish that survive CyHV-3 infection in a latent form and harbouring the potential to reactivate from this latency. Reactivated virus can be transmitted to naïve fish and persist in carp populations for many years (25, 26).

Although there have been several approaches proposed to diminish the consequences of CyHV-3 infection, the problem has not been solved. The costs as well as the advantages and disadvantages of individual approaches have to be considered (1, 4, 11, 23). Oral administration of the anti-CyHV-3 remedy would be the most convenient treatment that could be applied against CyHV-3. Recently we discovered that acyclovir (9-((2-hydroxyethoxy)methyl)guanine, ACV) can inhibit CyHV-3 replication in the common carp brain (CCB) and Koi carp fin (KF1) cell lines (24). This nucleoside analogue is an inhibitor of human herpesviral DNA polymerase widely applied for treatment of infection with *Herpes simplex* viruses 1 and 2 (HSV-1 and 2) in humans. The molecule is activated (specifically, it is monophosphorylated) by herpesviral thymidine kinase much more readily than by cellular enzymes, therefore the activation step occurs mainly in virus-infected cells. Cell DNA-encoded kinase phosphorylates acyclovir monophosphate (ACV-MP) to acyclovir triphosphate (ACV-TP). The latter has much higher affinity to viral DNA polymerase than to cellular DNA polymerase.

Indeed ACV-TP is a substrate, inhibitor and inactivator of viral DNA polymerase, and therefore terminates replication of viral DNA (5, 6, 7, 14). We assumed that the anti-CyHV-3 activity of acyclovir in carp cell lines might be similar (24).

In the present study we examined a tricyclic derivative of ACV, namely 3,9-dihydro-3-((2-hydroxyethoxy)methyl)-6-(4-methoxyphenyl)-9-oxo-5 *H*-imidazo-(1,2-*a*)purine (originally named 6-(4-MeOPh)-TACV and abbreviated to T-ACV in this paper). This tricyclic molecule displays higher lipophilicity than ACV. The octanol–water partition coefficients P for T-ACV and ACV are 2.43 and 0.0363, respectively (28). It was already proved that when the P (also referred to as Do/b) value is lower than 3.5, the transepithelial permeability coefficient increases with the lipophilicity, therefore the higher lipophilicity of T-ACV than ACV might improve the absorption of the molecule from the digestive tract (27). Another interesting property of T-ACV is fluorescence (emission λ_{max} 359 nm). This phenomenon enables T-ACV molecules to be tracked in fluids and tissues (9). The quantification of this molecule is not affected by other UV-absorbing non-fluorescent plasma constituents, so the monitoring of T-ACV could be more straightforward than that of ACV (3). T-ACV was found to possess similar antiherpetic potency to ACV against HSV-1 and HSV-2 (10). Its desirable properties of lipophilicity and fluorescence could make this molecule a better candidate for application *in vivo* than ACV, therefore we examined T-ACV for anti-CyHV-3 activity in *in vitro* experiments with CCB and KF1 cell lines.

Material and Methods

Cell cultures. Common carp brain cells (CCB, ECACC 10072802) were purchased from the European Collection of Authenticated Cell Cultures. Koi carp fin (KF1, ECACC 10072801) cells were kindly provided by Professor Dieter Steinhagen and Doctor Mikołaj Adamek from the Fish Disease Research Unit of the University of Veterinary Medicine in Hannover. The cells were maintained under the same conditions as we described previously (24).

Tricyclic derivative of ACV. T-ACV was synthesised as has been described (10) and was kindly provided by Doctor Tomasz Ostrowski from the Institute of Bioorganic Chemistry of the Polish Academy of Sciences. The structural formula is depicted in Fig. 1.

Cell viability and rate of metabolism evaluation. The cell viability and the rate of metabolism were evaluated with crystal violet (CV) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays as has been described (15, 19, 21) with modifications that we recently explained in detail (24). The optimal densities of seeded cells in this study were

0.9×10^5 cells/cm^2 and 1.2×10^5 cells/cm^2 for CCB and KF1 cells, respectively. The cells seeded at this density were in the phase of exponential growth on the day of the cytotoxicity assay. The toxicity of T-ACV at the concentrations 0, 66.67, and 133.33 μM was determined after three days of incubation. The culture medium did not contain phenol red and was supplemented with 2% dimethyl sulphoxide (DMSO). For each experimental condition (66.67 and 133.33 μM of T-ACV) the percentage of control (0 μM of T-ACV) was calculated and used for further analysis. Each repetition of the experiment was performed in three technical replicates.
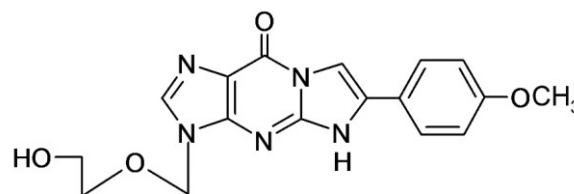
Fig. 1. The tricyclic derivative of acyclovir 3,9-dihydro-3-((2-hydroxyethoxy)methyl)-6-(4-methoxyphenyl)-9-oxo-5H-imidazo (1,2-*a*)purine T-ACV

Virus. *Cyprinid herpesvirus 3* (CyHV-3) was isolated in the Department of Fish Diseases at the National Veterinary Research Institute in Puławy, Poland in 2005 from infected common carp (16). The virus was propagated in CCB cell culture, divided into aliquots, and stored at −80°C until use. The tissue culture infective dose (TCID$_{50}$/ml) for CCB cells was estimated by the Spearman–Kärber method (17) and was 5.21×10^5 TCID$_{50}$/ml.

Infection and experimental set-up. CCB and KF1 cells were seeded at 2×10^5 cells/cm^2 on six-well plates (Orange Scientific, Belgium). After 24 h incubation, the cells were infected with CyHV-3 virus at 216 PFU/well for 2 h, then the medium was replaced with 2 ml of the culture medium supplemented with 2% DMSO alone (controls) or containing 66.67 μM of T-ACV. The control for calculations constituted CyHV-3 infected cells kept in the culture medium with 2% DMSO but 0 μM of T-ACV. Additional mock-infected controls were incorporated. This protocol was applied in cytopathic effect (CPE) experiments (in which the effect was determined by plaque assay) and in experiments evaluating viral load by TaqMan qPCR. The media were supplemented with 2.4% carboxymethylcellulose (CMC – Sigma, Germany) for the plaque assay.

Plaque assay. The plaque assay was performed by the CV method (21) with modifications (24). The CyHV-3 virus induced a CPE that was displayed as plaques. The plaques were observed on the 7th day post infection (dpi). The number of plaques was calculated in CyHV-3–infected and T-ACV–treated cells. The value was divided by the number of plaques in the

control and shown as a percentage of the control. Additional mock-infected controls were performed to monitor proper performance of the experiment. The experiments were repeated 10 (CCB) or 8 (KF1) times.

DNA isolation. The isolation of the DNA was performed by means of the salt method (2), with modifications (24).

TaqMan qPCR quantification of CyHV-3 DNA copy number (viral load). The number of CyHV-3 DNA copies was evaluated by means of TaqMan qPCR with the primers and probes shown in Table 1 (8). The method was modified as we described in our previous study (24). The number of CyHV-3 copies was divided by the number of cellular DNA copies and the result accepted as the viral load. The viral load of the experimental group was divided by the viral load in the control group and the result expressed as a percentage of the control viral load. The experiments were repeated 14 (CCB) or 9 (KF1) times.

Statistical data analysis. The data were analysed by paired *t*-test in GraphPad Prism 8, (GraphPad Software, USA). The differences were considered significant at p ≤ 0.05.

Results

Cytotoxicity of T-ACV. The cytotoxicity of T-ACV was determined by the MTT and CV methods.

The T-ACV did not decrease cell viability in CCB or KF1 cell lines when examined with an MTT assay. The CV assay revealed low toxicity of T-ACV at p ≤ 0.05 at a concentration of 66.67 µM in the KF1 cell line. In the CCB cell line, the highest concentration of T-ACV (133.33 µM) also displayed low toxicity at p ≤ 0.05, while in KF1 cells this concentration had more pronounced toxicity at p ≤ 0.01 (Fig. 2). The concentration of 66.67 µM was adopted for use in further study since the cytotoxicity of this concentration was low.

Anti-CyHV-3 effect of T-ACV. The antiviral activity of T-ACV at the concentration of 66.67 µM was evaluated by plaque assay on the 7th dpi and by TaqMan qPCR on the 3rd dpi. In CCB cells that were treated with T-ACV the number of plaques was lower by about 13.31% ± 19.54% when compared to the control but the difference did not reach statistical significance. The inhibition of CPE was significant (p ≤ 0.01) in KF1 cell culture and was 29.34% ± 17.32% (Fig. 3). The inhibitory effect of T-ACV on virus replication was confirmed with TaqMan qPCR. Although the slight reduction of viral load in the CCB cell line was not significant (28.34% ± 54.55%), in the KF1 cell line the viral load was reduced by 24.95% ± 19.71% and statistical significance was obtained at p ≤ 0.01 (Fig. 4).
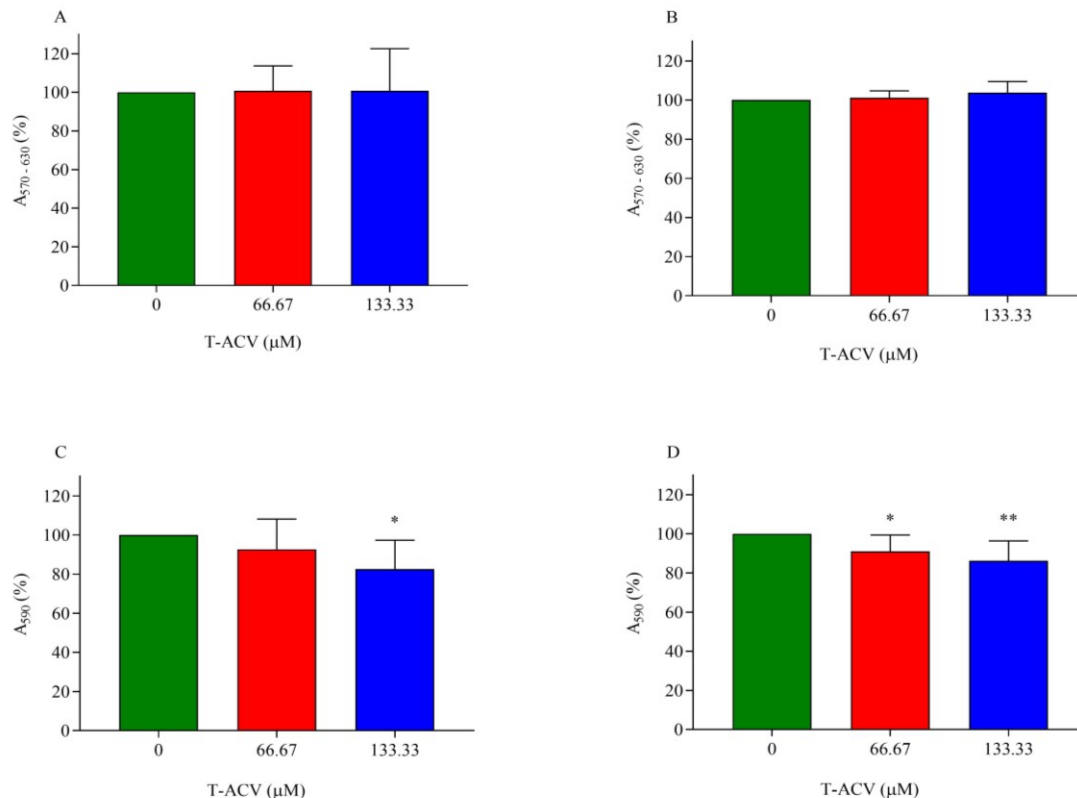
Fig. 2. The cytotoxicity of T-ACV at concentrations of 66.67 and 133.33 µM in CCB and KF1 cell lines at the phase of exponential growth. The graphs depict results yielded by an MTT assay in CCB, n = 9 (A) and KF1, n = 8 (B) and by CV assay in CCB, n =8 (C) and KF1, n = 8 (D). The data are expressed as a percentage of absorbance of the control group (0 µM T-ACV) and presented as mean ± SD. The differences in a paired *t*-test between the control group and T-ACV group were significant at p ≤ 0.05* or p ≤ 0.01**

Table 1. Primers and probes used for TaqMan qPCR quantification of CyHV-3 DNA copy number

Target	Sequence FP	Sequence RP	Probe	Primer sequence source
CyHV-3	KHV-86f GACGCCGGAGACCT TGTG	KHV-163r CGGGTTCTTATTTTT GTCCTTGTT	KHV-109p FAM-CTTCCTCTGCTCGGCGAGC ACG-BHQ	Gilad *et al.* (8)
Carp glucokinase	CgGluc-162f ACTGCGAGTGGAGA CACATGAT	CgGluc-230r TCAGGTGTGGAGCG GACAT	cgGluc-185p VIC-AAGCCAGTGTCAAAATGC TGCCCACT-MGF-NFQ	Gilad *et al.* (8)
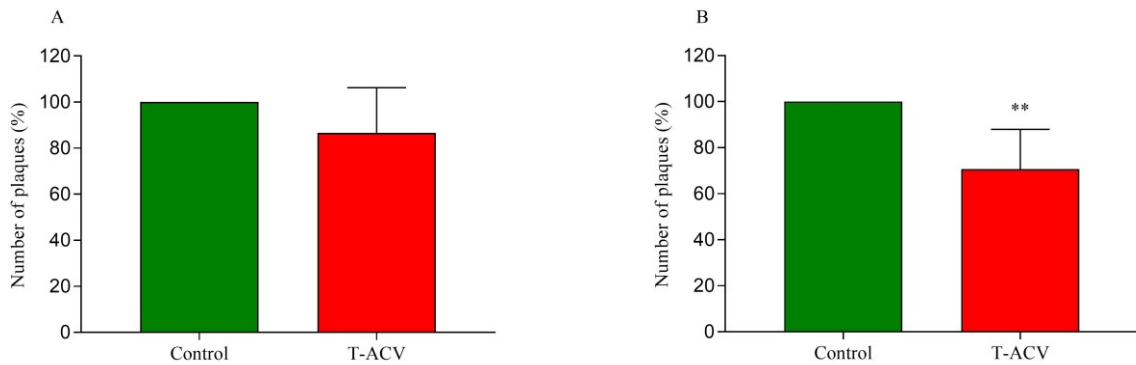
Fig. 3. The anti-CyHV-3 activity of T-ACV in CCB, n = 10 (A) and KF1, n = 8 (B) cell lines determined by plaque assay. The data are expressed as a percentage of plaques of the control group (CyHV-3 infected, 0 µM T-ACV) and presented as mean ± SD. The differences in a paired *t*-test between the control group and T-ACV group were significant at p ≤ 0.01**
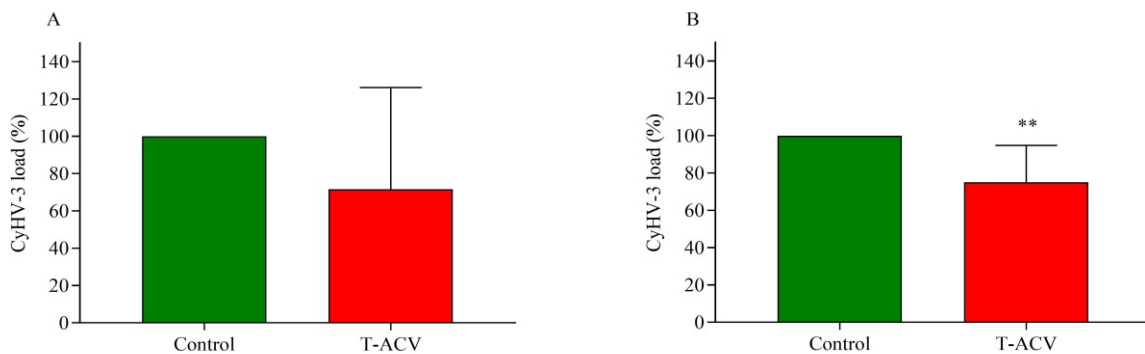
Fig. 4. The anti-CyHV-3 activity of T-ACV in CCB, n = 14 (A) and KF1, n = 9 (B) cell lines determined by TaqMan qPCR assay. The data are expressed as a percentage of viral load of the control group (CyHV-3 infected, 0 µM T-ACV) and presented as mean ± SD. The differences in a paired *t*-test between the control group and T-ACV group were significant at p ≤ 0.01**

Discussion

Since the late 1990s CyHV-3 has caused severe economic losses by affecting common carp populations in pond aquaculture and the ornamental koi carp bred for pleasure and for competitive exhibitions. The trade as well as holding fish in one tank during exhibitions accelerated the spread of the virus (13). Scientists are constantly trying to find the best remedy for CyHV-3 induced disease. The very efficient approaches of vaccination and natural immunisation of fish by incubation in high temperature (≈30ºC) (20) are too expensive for application in pond aquaculture. Simpler treatment seems to be the addition of certain antiviral substances to water or feed. Recently it was discovered that extract from *Clinacanthus nutans* L. is active against CyHV-3 in experiments *in vivo* (12). Exopolysaccharide from *Arthrospira platensis* L. also indicated anti-CyHV-3 activity in experiments *in vitro* (18). In addition, our recent study proved that acyclovir inhibits CyHV-3 replication in experiments *in vitro* (24).

This study addressed antiviral activity of a fluorescent tricyclic acyclovir derivative, a molecule that dissolves in the culture medium very weakly. Addition of DMSO increases the solubility of T-ACV, therefore we mixed DMSO at a concentration of 2%, far stronger than the 0.1% most commonly used in drug research. DMSO is only mildly toxic to the cells and

although the concentration of DMSO reaching 2% v/v appeared to be slightly toxic, it was still possible to examine the toxicity of T-ACV in CCB and KF1 cell lines. The presence of 2% DMSO in the culture medium made it possible to dissolve T-ACV at a concentration as high as 133.33 µM; application of T-ACV at higher concentration results in precipitation of the molecule.

According to the results obtained in one of the two cytotoxicity assays in the KF1 cell line, T-ACV indicates low toxicity (at $p \leq 0.05$) at a concentration equal to 66.67 µM, therefore this concentration was considered maximal and used in further experiments. Our previous study showed that ACV at a concentration of 66.67 µM decreased the CyHV-3 load by 76–88% (24). Contrastingly, with T-ACV we observed only a slight decrease in the CyHV-3 load (25%–28%). Although ACV and T-ACV were not examined in the same experiment, a similar effect would have been expected because the antiviral activities of ACV and T-ACV against HSV-1 and HSV-2 were similar in a mammalian system (9, 10). On the other hand, ganciclovir, another nucleoside analogue that differs from ACV by the presence of an additional CH_2OH moiety, was also inactive against CyHV-3 virus (18). Based on these findings, it seems probable that there might be very narrow specificity of CyHV-3-encoded DNA polymerase for ACV-based molecules. The weaker anti-CyHV-3 activity of T-ACV together with the emerging cytotoxicity at the concentration of 66.67 µM does not place T-ACV among the good candidates for further research on the treatment of CyHV-3 disease. Nevertheless, it seems reasonable to screen other tricyclic derivatives of ACV for anti-CyHV-3 activity because the outcomes have some potential to be more desirable.

Conflicts of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

Animal Rights Statement: Not applicable.

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