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In vitro effect of acyclovir and other antiherpetic compounds on the replication of channel catfish virus

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Summary

The antiviral effect of acyclovir (ACV; 9-(2-hydroxyethoxymethyl)guanine) on the replication of channel catfish virus (CCV), a poikilothermic herpesvirus, in brown bullhead cells (BB) was studied in vitro. Acyclovir at concentrations of 2 μM and 10 μM produced a 95 to 99% reduction in plaque numbers, respectively. At 10 μM ACV did not affect the growth of uninfected BB cells which could even be subcultured for four passages in the presence of the inhibitor. To be effective ACV had to be added early in the infectious cycle and was progressively less effective when added at later times after infection. Similar to homeothermic herpesviruses, the inhibition could be reversed with the addition of excess thymidine. The effects of three other antiviral agents, adenine-β-D-arabinofuranoside (ara-A), phosphonoacetic acid (PAA), and phosphonoformic acid (PFA) on CCV replication were evaluated either individually or in combination with ACV. Although the other agents were found to be less effective in inhibiting CCV replication than ACV, none of the agents interacted synergistically with ACV. The results indicated that interactions with ACV with ara-A, PFA and PAA were primarily additive. Mutants of CCV resistant to ACV were obtained and were found to be somewhat more resistant to PFA than was the stock CCV.

channel catfish virus; acyclovir; antiviral activity

Introduction

The number and size of fish aquaculture operations throughout the world have greatly increased and the viral diseases affecting these fishes have become economically important and prevalent. However, there remains yet to be developed effective antiviral therapy for the viral diseases of fish in aquaculture.

One virus of considerable economic importance in the United States is channel catfish virus (CCV), a herpesvirus [22] which has been reported to be responsible for mass mortalities of catfish fingerlings [9]. Mortality rates from CCV epizootics commonly approach 80 to 90% [18]. No effective antiviral chemotherapy is presently available against CCV.

A highly effective and specific antiviral compound against homeothermic herpesviruses is acyclovir (ACV) or 9-(2-hydroxyethoxymethyl)guanine [6]. The guanine derivative is phosphorylated to the monophosphate (acyclo-GMP) by viral thymidine kinase (TK) and subsequently converted to the di- and triphosphate forms (acyclo-GPD and acyclo-GTP, respectively) by cellular enzymes [17]. Uninfected cells convert significantly less ACV to the phosphorylated derivatives compared to virus-infected cells [6,11]. Acyclo-GTP is a potent inhibitor of HSV DNA polymerase, whereas the DNA polymerase of host cells is markedly less sensitive to inhibition [6,11,21]. In this study the effect of ACV on the replication of CCV in brown bullhead cells (BB) was investigated. In corollary studies the effect of other antiherpetic agents of different modes of action, such as adenine-β-D-arabinofuranoside (ara-A), phosphonoacetic acid (PFA), and phosphonoformic acid (PFA) was evaluated either individually or in combination with ACV. Our results confirm and extend the observations made by other investigators [13,14].

Materials and Methods

Virus and cells

Channel catfish virus, Auburn 1 (ATCC VR No. 665), from the American Type Culture Collection was propagated in a continuous line of brown bullhead catfish (BB) cells (CCL 59). The BB cells were obtained from the American Type Culture Collection Cell Repository at passage level #104 and were cultured as monolayers in MEM (minimal essential media with Hank's salts, plus L-glutamine) supplemented with 10% fetal calf serum (10 FCS).

Antiviral agents

Acyclovir, Wellcome Laboratories was kindly provided by G.B. Elion. The inhibitor was prepared in NaOH [4] as was adenine-β-D-arabinofuranoside (ara-A) (Sigma). Stock solutions of phosphonoformic acid (PFA) (Sigma and Calbiochem) and phosphonoacetic acid (PAA) (Calbiochem) were dissolved in distilled water. All stock solutions were sterilized by filtration through a 0.22 μm membrane filter and stored at -20°C.

Plaque formation assay

The liquid overlay plaque formation assay was used to determine the antiviral activity of the various chemical inhibitors evaluated [3]. Monolayers of BB cells in either 25-cm^2 plastic tissue culture flasks or 2 oz. glass prescription bottles were incubated with 1 ml inoculum of the appropriate virus dilution. After adsorption for 90 min at room temperature (25°C), the monolayers were rinsed twice with MEM and

an aliquot of MEM plus dialyzed 5 FCS either with or without inhibitor was added. After incubation at 30°C for 36 h, the medium was decanted and the cell monolayers stained with 0.5% crystal violet in 0.1 M citric acid for approximately 1 min. The monolayers were then rinsed and scored for plaques. The reduction from untreated infected controls was determined and used to calculate the dose to yield a 50%, 90% and 99% plaque reduction.

Single-cycle growth experiments

Replicate monolayers of BB cells were infected with virus at a multiplicity of infection (m.o.i.) of 5. After adsorption (90 min) at room temperature the cultures were washed twice with MEM and overlaid with maintenance media containing either the viral inhibitor or none. At various times post-infection (p.i.) flasks from each experimental group were frozen at -80°C for subsequent assay of virus. Prior to titration each experimental group was briefly sonicated and clarified of cell debris by low speed centrifugation. The virus samples were then dialysed against MEM and the total yield of virus determined by plaque assay. In certain experiments the effect of the addition of excess thymidine to reverse the inhibition of CCV replication by ACV was examined.

Viral DNA synthesis

The synthesis of CCV-DNA in BB cells in the presence or absence of ACV was analyzed by the method of Roizman and Roane [19]. Viral DNA was prepared by the method of Green et al. [12] and the extracted DNA was centrifuged to equilibrium in CsCl (starting density 1.70 g/cm^3) at 40 000 rpm for 36 h in a SW 50.1 rotor at 15°C and the amount of TCA-precipitated radioactivity in the band of DNA was determined.

Interaction of viral inhibitors

A dose-response curve based on yield of infectious virus was established for each inhibitor tested. The agents were then tested in combination and the results plotted and analyzed by the isobologram method [2,7]. In brief, the concentration of each inhibitor used in combination to achieve a given reduction was divided by the concentration of the respective inhibitor that alone would produce the same reduction. This number represents the fractional inhibitory concentration (FIC). The calculated FICs for each pair of inhibitors evaluated were then plotted as the x,y coordinates of the isobologram. Rather than deal with additive, slightly additive, indifferent and many other subdivisions of interaction, only additive, synergistic, and antagonistic categories were established. Where $0.75 \leqslant FIC_x + FIC_y \leqslant 1.25$, the overall interaction for that particular combination was considered additive. If the sum of the paired values was greater than 1.25, the interaction was considered antagonistic, and if it was less than 0.75, it was considered synergistic.

Results

Plaque reduction by ACV

Preliminary experiments were made to determine the minimum concentration of the

inhibitor needed to give a clear-cut reduction in the number of plaques formed. When added at the time of infection ACV at a concentration of 2 μM produced a 95% (1–2 log) reduction in plaque numbers (Fig. 1). A 10 μM concentration of the inhibitor which produced a greater than 99% plaque reduction did not visibly affect the growth of uninfected BB cells. Such BB cells did not show any visible cytotoxic effect or loss of growth ability even when subcultured for four passages under 10 μM ACV. In order to obtain a similar reduction in plaque number, the other inhibitors tested required the following concentrations: 20 μM ara-A, 3000 μM PFA or 2500 μM PAA.

Effect on virus replication

Once the useful range of ACV was established, its effect on the single cycle growth curve of CCV was examined. When 2 μM ACV was added at the time of infection, the yield of infectious virus was reduced by approximately 2 logs (Fig. 2). This reduction is apparent by 12 h p.i. and is maintained throughout the course of the infection. Despite the marked difference in production of virus, infected monolayers of BB cells (m.o.i. = 5) whether in the presence or absence of 2 μM ACV were both completely destroyed by 36 h p.i.

During the course of this investigation it was observed that the cell density of the BB monolayers affected plaque formation even when inoculated with the same dilution of virus. Those with especially dense (5-day-old) monolayers showed fewer plaques than those that were less dense (3-day-old). Furthermore, plaque reduction assays conducted in the presence of 2 μM ACV showed a greater reduction in plaque number in densely packed monolayers than in less dense monolayers. However, infectivity assays revealed no difference in virus yield between the two kinds of monolayers. Dense and light monolayers of BB cells produced similar yields of CCV when inoculated with the same stock of virus. In each case they showed a similar inhibition of virus production when treated with 2 μM ACV.

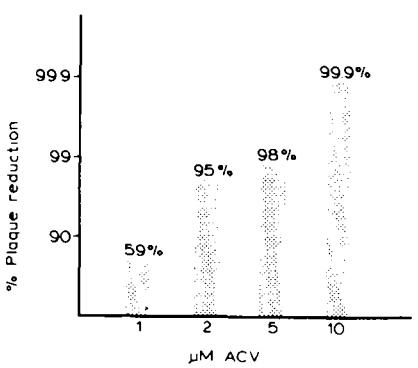

Fig. 1. Effect of dose concentration of acyclovir (ACV) on CCV replication. Serial dilutions of CCV stock were used to infect monolayer cultures of BB cells. After a 90 min adsorption, the cell layer was washed and medium containing various concentrations of ACV added. The cultures were then incubated for 30 h at 30°C, stained and the plaques enumerated. The number of plaques per ml of stock in the presence of ACV divided by the number of plaques in the absence of ACV times 100% determined the plaque reduction.

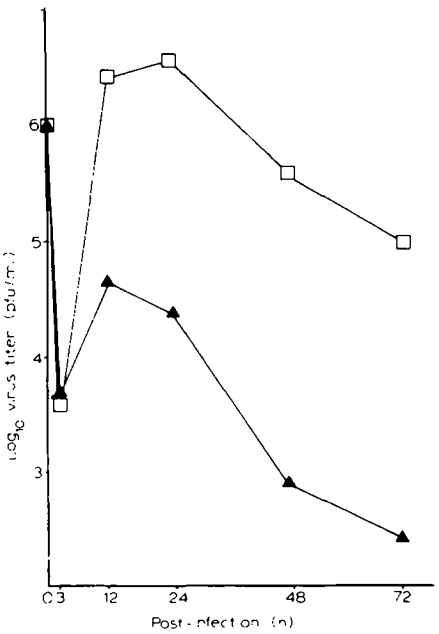

Fig. 2. Single cycle growth curve of CCV at 27°C. The BB cells were infected at an m.o.i. of 5 with CCV and at various times p.i. replicate cultures frozen (−20°C) for later assay of virus. □, no acyclovir present; ▲, 2 μM acyclovir present from 0 h p.i.

Effect of time of addition of ACV

The pretreatment of cells with ACV at concentrations (2 μM) which normally inhibited virus replication, prior to infection with CCV (m.o.i. = 5) did not enhance the ability of the inhibitor added at the time of infection to suppress viral replication, and pretreatment alone with ACV was ineffective (Table 1). It is apparent that ACV is effective only when added early in the infectious cycle of the virus and is progressively less effective when added at later times after infection.

Reversal of inhibition by ACV

The effect of the addition of excess thymidine to reverse the inhibition of CCV replication by ACV was examined. It is apparent from Fig. 3 that 4 μM, 8 μM and 10 μM thymidine when added at 0 h p.i. reversed the inhibitory action of 2 μM ACV. The higher concentrations of thymidine produced the greater reversals. When a constant amount of thymidine is added at later times after the addition of ACV to infected cells, there is a decrease in the production of virus indicating a lesser ability to reverse the inhibitory effects of ACV (results not shown).

Effect of ACV on CCV-DNA synthesis

The effect of inhibitory concentrations of ACV on the synthesis of CCV-DNA in BB cells was examined. The amount of ^{3}H-labeled DNA appearing in the supernatant of a

TABLE 1

Effect of time of addition of acyclovir on synthesis of CCV

Exposure to 2 μM acyclovir (ACV)	Time p.i. that replication is stopped (h)	Yield of virus (pfu/ml)	% inhibition of CCV
No ACV (CCV control)	12	4.1×10^5	0
	24	5.9×10^5	0
ACV present 12 h pre-infection and	12	5.6×10^5	0
removed at start of adsorption	24	5.2×10^5	0
ACV present 12 h pre-infection and	12	3.1×10^3	99
present post infection	24	4.2×10^3	99
ACV added at end of adsorption	12	3.3×10^4	90
(0 h p.i.)	24	6.0×10^4	90
ACV added 1.5 h after adsorption	12	1.0×10^5	75
(1.5 h p.i.)	24	1.1×10^5	81
ACV added 5 h after adsorption	12	6.0×10^5	0
(5 h p.i.)	24	6.5×10^5	0

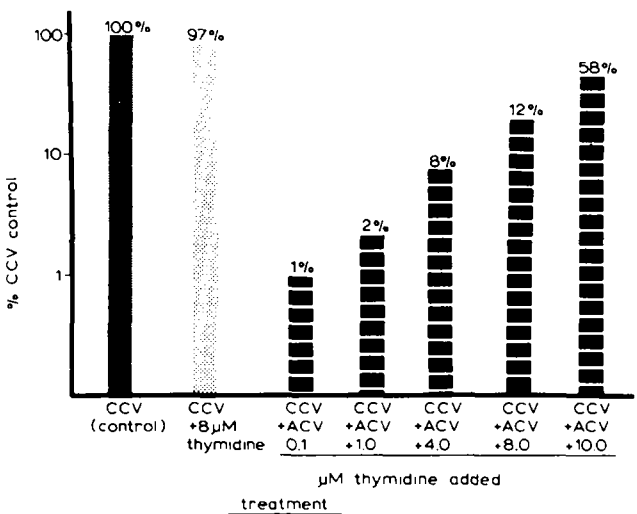

Fig. 3. Reversal of acyclovir-inhibition of CCV replication by thymidine. Monolayers of BB cells were infected with CCV (m.o.i. = 1), and at the end of adsorption (90 min) overlayed with medium containing 2 μM ACV and varying concentrations of thymidine. The cultures were then incubated 24 h at 27°C.

modified Hirt extraction from ACV-treated infected cells was markedly less than that found in the corresponding extracts derived from untreated infected cells (Table 2). Isopycnic banding of DNA from the Hirt supernatant in a CsCl gradient (Fig. 4), indicated that the decrease in acid-precipitable label in ACV-treated cells corresponded to a decrease in the production of viral DNA. The viral DNA was found to have a buoyant density of 1.70 g/cm^3 in contrast to cellular DNA which was 1.68 g/cm^3.

Replication of CCV in the presence of single and dual combinations of inhibitors

The effect of four antiviral agents on CCV replication was measured by infectivity assays. The data was used to plot dose-response curves (Fig. 5). In agreement with the plaque reduction assay, the results of the infectivity assay showed ACV to be the most effective agent tested. It should be noted that 500 μM PFA by Calbiochem appeared to enhance virus production although a similar concentration of PFA by Sigma did not produce the same effect. In fact, low levels (500 μM) of PFA from Calbiochem were consistently and repeatedly found to yield titers of virus in excess of the control.

The dose-response curves were used to calculate the FIC for each member of the pair of inhibitors at the concentrations required to produce a particular endpoint of

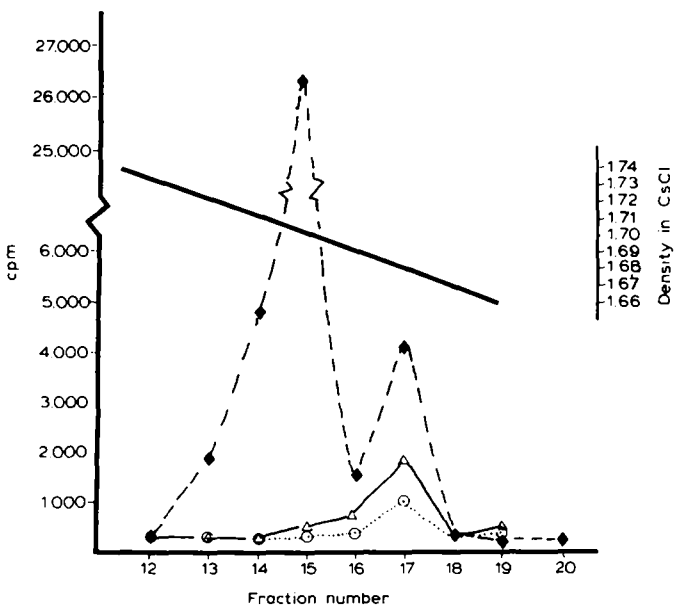

Fig. 4. CCV-DNA synthesis in the presence and absence of acyclovir. Monolayers of BB cells were infected with CCV and overlayed with medium containing [^{3}H]thymidine (4 μCi/ml) in the presence or absence of 5 μM ACV. The cultures were harvested after 18 h incubation at 27°C. The DNA recovered in the Hirt supernatant was isopycnically banded in CsCl by centrifugation in a preparative ultracentrifuge for 36 h at 40 000 rpm at 15°C. Fractions were collected from the bottom of the tube, spotted on filter paper, treated with cold 5% TCA and the relative amount of acid-precipitable label determined. ♦, CCV; △, CCV + 5 μM ACV; ○, no CCV (BB).

TABLE 2

Synthesis of CCV-DNA[a] in the presence of 5 μM acyclovir

Growth conditions	[^{3}H]Thymidine (cpm)
No CCV	32
(control)	42
CCV	18 846
	16 559
CCV +	683
5 μM ACV	409

[a] Acid-insoluble [^{3}H]thymidine-DNA from Hirt extract of CCV infected BB cells harvested at 18 h p.i.

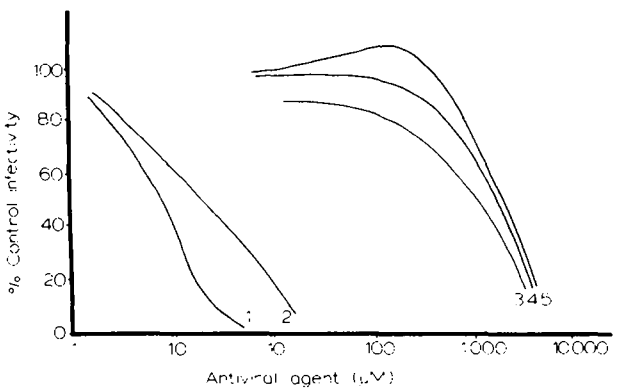

Fig. 5. Summary of dose-response curves to various antiviral inhibitors. Inhibitors were added at the end of 90 min virus adsorption. The cultures were incubated at 27°C, and assayed for infectivity 24 h p.i. The virus yield was plotted as the percent of control (no antiviral agent) vs. infectivity. 1 = acyclovir; 2 = ara-A; 3 = phosphonoacetic acid; 4 = phosphonoformic acid (Sigma); 5 = phosphonoformic acid (Calbiochem).

antiviral activity. If the two agents in combination produced a stronger antiviral effect than the sum of the individual activities ($FIC_x + FIC_y < 0.75$), the combination was considered synergistic. Likewise, if the two agents were antagonistic the observed effect would be less than the sum of the individual activities ($FIC_x + FIC_y > 1.25$). This is represented graphically by plotting each pair of FIC values on the x,y coordinates of an isobologram (Fig. 6). As can be seen, ACV interacts in an additive manner with ara-A, PPA and PFA, when combined with an inhibitory concentration of those agents. PFA (Calbiochem) at a low, noninhibitory concentration appeared to interact in an antagonistic manner with ACV, but at higher concentrations interacted in an additive manner (results not shown).

CCV mutants resistant to ACV

Channel catfish virus mutants resistant to acyclovir (ACV-RC) were obtained by repeated passage of the virus in the presence of 2 μM or 10 μM ACV with subsequent

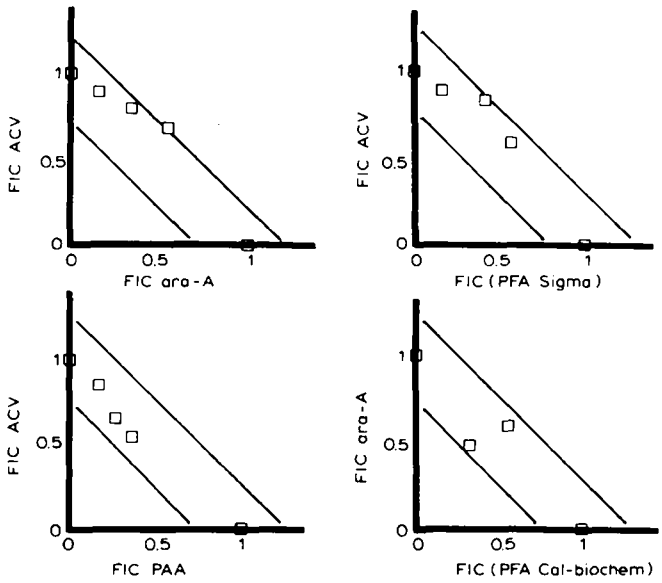

Fig. 6. Graphic representation of the effect of combination of inhibitors on the replication of CCV as tested by the infectivity assay. The plot of the fractional inhibitory concentration (FIC) of additive combinations of paired compounds should form a diagonal line ($FIC_x + FIC_y = 1$), within the outlined zone. Synergistic combinations ($FIC_x + FIC_y < 0.75$), should extend below the additive zone and antagonistic combinations ($FIC_x + FIC_y > 1.25$), should extend above the zone of additivity. The concentrations of the inhibitors were varied about the dose, which individually would inhibit virus production by 50%.

TABLE 3

Plaque reduction of CCV and ACV-RC

Virus[a]	% plaque reduction			
	2 μM ACV	1600 μM PFA	20 μM Ara-A	800 μM PAA
CCV	95	75	97	80
ACV10-RC	0	45	96	nd
ACV2-RC-1	71			
ACV2-RC-2	58			

[a] ACV10-RC = CCV mutant resistant to 10 M acyclovir; ACV2-RC-1 = first CCV mutant resistant to 2 M ACV; ACV2-RC-2 = second CCV mutant resistant to 2 M ACV.

[b] Plaque reduction: (virus yield without inhibitor – virus yield with inhibitor)/virus yield without inhibitor.

plaque purification. The two isolates (ACV2RC-1 and ACV2RC-2), selected in the presence of 2 μM ACV, exhibited partial and differing degrees of resistance to ACV. One isolate selected in the presence of 10 μM ACV (ACV10RC) was tested for its resistance to ACV after each passage. Although the presence of 2 μM ACV decreased

the titer of the second passage isolate by 40%, by the fourth passage there was no decrease in virus production in the presence of 2 μM ACV. Furthermore, the plaques from the ACV10RC isolate were found to be one half to two thirds the size of normal CCV plaques on the BB monolayers. The ACV10RC isolate when tested for resistance to other antiviral agents was found to be somewhat more resistant to PFA than was the stock CCV (Table 3).

Discussion

Although tremendous gains have been made in recent years, in the successful treatment of viral infections with chemotherapeutic agents, very few studies have dealt with the applicability of these treatments to viral diseases of fish [13–15]. Two of the most effective antiherpetic agents developed for use in humans are ara-A and ACV. It appears [4,10] that ara-A like ACV is phosphorylated inside the cell to an active form which then inhibits the action of the DNA polymerase. Phosphonoacetic acid and PFA are also two other effective inhibitors of HSV and both act, presumably through noncompetitive interference, on the viral DNA polymerase [5,16]. The present study examines the ability of these four promising human antiherpetic agents to inhibit the replication of a fish herpesvirus.

The results presented here demonstrated that while 2 μM ACV did not cause any apparent cytotoxic effects in uninfected host cells it reduced CCV production by 90–99%. Although ACV and ara-A were as efficacious against CCV as they were reported to be against HSV [5], PAA was found to be about 10 times less effective against CCV than against HSV. This is in accord with the findings of Koment and Haines [15]. In the present study PFA was also found to be a rather poor inhibitor of CCV.

Although the other agents tested were found to be less effective in inhibiting CCV than was ACV, none of these agents interacted synergistically with ACV. It was apparent from the results that interactions of ACV with PFA, PAA and ara-A were primarily additive. Previous work with varicella-zoster virus in mammalian cell systems, had also shown the ACV/PAA interaction to be additive [2]. Acyclovir was also reported to have additive interactions with PFA and ara-A when tested for efficiency against HSV-1 [20]. While no synergistic interactions were seen, low levels of PFA (Calbiochem) were found to interact antagonistically with 1 μM ACV. It was not known why 500 μM PFA from Calbiochem, but not 500 μM PFA from Sigma, appeared to enhance virus production (Fig. 5). While higher levels, 1000–5000 μM, PFA of both brands were tested and found to inhibit CCV, levels of PFA lower than 500 μM were, unfortunately, not tested in CCV-infected cells. However, difference in inhibition of CCV replication might be due to the degree of purity of the two PFA preparations cannot be ruled out.

Although the present study does not directly indicate that the mechanism of inhibition by ACV in CCV-infected cells is similar to that seen in HSV-infected cells, the accumulated evidence strongly implies that an early viral dependent process such as, viral-DNA synthesis is affected. Acyclovir must be added at the time of infection for

maximal effect, although when ACV was added late at 4 h p.i., there was still a marked degree of inhibition. The uptake studies with [^{3}H]thymidine provided additional supportive evidence that, in CCV-infected fish cells as in HSV-mammalian systems, ACV primarily interfered with viral DNA synthesis. This strongly suggests that CCV like HSV very likely has a viral coded thymidine kinase (TK). In addition, the ability of excess thymidine to reverse the inhibition of CCV by ACV is similar to that reported for HSV where the ACV-GTP is known to competitively inhibit HSV-DNA synthesis [6]. This also suggests that ACV may inhibit CCV-DNA synthesis in a competitive manner. The ease with which ACV resistant mutants of CCV are obtained is consistent with CCV having a viral coded TK. In those viruses where a viral TK is required to convert ACV to its active form, any mutation which inactivates the viral TK gene will render the virus resistant to ACV [8]. However, the present study does not specifically demonstrate the existence of a CCV coded TK and the results could be explained by other less likely mechanisms. We are currently examining the phosphorylation of acyclovir in CCV-infected and uninfected BB cells. The formation of ACV-monophosphate and its subsequent conversion to ACV-triphosphate would provide strong evidence that a viral associated TK is involved in the inhibition mechanism.

Although this study has provided useful information to those who hope to better control CCV disease in the catfish through chemotherapy the effectiveness of ACV against CCV at the whole animal level remains yet to be determined.

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