Antiviral Research, 23 (1994) 203–218

AVR 00682

Antiviral Research

Cyclocreatine (1-carboxymethyl-2-iminoimidazolidine) inhibits the replication of human herpes viruses

James W. Lillie[a,1], Donald F. Smee[b], John H. Huffman[b], Lori J. Hansen[a], Robert W. Sidwell[b] and Rima Kaddurah-Daouk[a,*]

[a]*AMIRA, Inc., 83 Rogers Street, Cambridge, MA 02142, USA and* [b]*Utah State University, College of Agriculture, Department of Animal, Dairy and Veterinary Sciences, Logan, UT 84322, USA*

(Received 21 September 1993; revised manuscript accepted 2 December 1993)

Summary

The creatine kinase/creatine phosphate (CK/CrP) system plays an important role in cellular energy homeostasis. CK isoenzymes, which reversibly generate ATP from CrP, are compartmentalized at cellular sites where energy is produced or utilized. It has been noted that the expression of CK is induced in cells infected by several DNA viruses, implicating a role for cellular energy modulation as an important step for efficient viral replication. A CK substrate analog, 1-carboxymethyl-2-iminoimidazolidine (cyclocreatine; CCr), was tested in vitro for antiviral activity against a variety of herpes viruses and RNA viruses. Several members of the human herpes virus family were found to be sensitive to CCr, including herpes simplex types 1 and 2 (HSV-1 and HSV-2), varicella-zoster virus, and cytomegalovirus. When administered to mice infected vaginally with HSV-2, CCr significantly reduced mortality, reduced vaginal lesion scores, and lowered the titers of recoverable virus. This treatment combined with acyclovir appeared to enhance the antiviral effects of acyclovir. In a second model, mice infected intraperitoneally with HSV-2 and treated with CCr showed a significant increase in survival compared to placebo. We conclude that CCr is the first example of a new class of antiviral compounds that target the CK/CrP system.

Creatine kinase; Cyclocreatine; Herpes virus; Human cytomegalovirus; Antiviral agent

*Corresponding author. Fax: +1 (617) 3493440.
[1]Present address: ScripTech Pharmaceuticals, Inc., Joslin Diabetes Center, Boston, MA 02215, USA.

SSDI 0166-3542(93)E0068-5

Introduction

In naturally occurring infections, human DNA viruses normally encounter noncycling, terminally differentiated epithelial cells. To maximize the number of cells able to replicate viral DNA and the time spent in DNA replication, it is thought that some viruses alter cell cycle controls and the expression of specific cellular proteins (Braithwaite et al., 1983). Human cytomegalovirus (HCMV) and adenovirus infections have been shown to transcriptionally activate cellular genes, and both viruses induce the brain form of creatine kinase (CKB; Colberg-Poley and Santomenna, 1988; Kaddurah-Daouk et al., 1990; Colberg-Poley et al., 1992). In addition, the HCMV IE2 and adenovirus E1A gene products have been shown to transactivate the CKB gene by up to 11-fold in cotransfection experiments (Colberg-Poley et al., 1992; Kaddurah-Daouk et al., 1990).

The creatine kinase/creatine phosphate (CK/CrP) enzyme system plays a complex, multi-faceted role in cellular energy homeostasis (reviewed in Wallimann et al., 1992). Tissues of fluctuating high energy demand, such as the brain, heart and skeletal muscle utilize the natural phosphagen creatine phosphate (CrP), as a source for rapid and efficient regeneration of ATP. High concentrations of CrP are present in these organs, typically in the 5–30 mM range (Walker, 1979). Creatine kinase (CK) reversibly catalyzes the transfer of a high energy phosphate bond from CrP to ADP to generate ATP:

$$MgADP^{-} + CrP^{2-} + H^{+} \rightleftharpoons MgATP^{2-} + Cr$$

One of the key functions of the CK/CrP system is to provide the appropriate local ATP/ADP ratios at subcellular sites where CK is functionally coupled to ATP-consuming enzymes or processes (reviewed in Walliman et al., 1992). Another important aspect is the regulation of cellular ADP levels which has important metabolic consequences, such as control of mitochondrial respiration, prevention of inactivation of cellular ATPases, and prevention of net loss of cellular adenine nucleotide pools (Iyengar et al., 1982; Iyengar, 1984). These functions, regulated by the CK/CrP system, could be important for efficient viral replication.

Increased expression of the brain isoenzyme of CK (CKB) is associated with many examples of cellular proliferative processes, including response to steroid hormones such as estrogen and testosterone, and signal transducers such as phospholipase C and protein kinase C (Chida et al., 1990a; Chida et al., 1990b; Reiss and Kaye, 1981; Somjen et al., 1987; Somjen et al., 1989). Overexpression of CKB has been found in many types of tumors, such as small cell lung carcinoma, breast cancer and prostate cancer (Carney et al., 1984; Feld and Witte, 1977; Gadzar et al., 1981; Homburger et al., 1980; Ishiguro et al., 1990; Thompson et al., 1980). It has been recently shown that an analog of creatine, 1-carboxymethyl-2-iminoimidazolidine (Fig. 1; cyclocreatine; CCr; Rowley et al., 1971), can selectively inhibit the growth of tumor cells in vitro and in vivo

Creatine Cyclocreatine

Fig. 1. Chemical structures of creatine and cyclocreatine.

(Lillie et al., 1993; Miller et al., 1993). These studies have suggested that the CK/CrP system is a potential target for chemotherapy.

CCr is phosphorylated efficiently in vitro and in vivo by CK to yield cyclocreatine phosphate (CCr~P; Annesley and Walker, 1977; Griffiths and Walker, 1976). Accumulation of CCr~P creates a new synthetic phosphagen pool, which partially replaces CrP, the natural phosphagen (Griffiths and Walker, 1976). CCr~P exhibits kinetic and thermodynamic properties distinct from those of CrP (Annesley and Walker, 1977). Although CCr~P is structurally similar to CrP, the phosphorous-nitrogen bond of CCr~P is more stable than that of CrP (Herriott and Love, 1968; Mendel and Hodgkin, 1954; Phillips et al., 1979). Using the V_{max}/K_m ratio as a measure of substrate quality, CCr~P is turned over 160-fold less efficiently than CrP (Annesley and Walker, 1977). Hence, the rate of ATP generation through the CK system is significantly reduced when CCr~P is used as the substrate.

In the current studies, we have evaluated the effect of CCr in vitro against a panel of RNA and herpes viruses. Antiviral activity was exhibited against the herpes viruses, including human cytomegalovirus (HCMV), varicella zoster virus (VZV), and HSV-1 and HSV-2. Millimolar levels of CCr were needed to reach the ED_{50} in vitro. This level is easily achievable in vivo by administration of CCr as 1% of the diet of laboratory animals (Walker, 1979). The efficacy of CCr was tested against HSV-2-induced vaginitis and encephalitis in mice. This report describes these antiviral studies, which indicate that CCr exhibits significant antiviral activity against herpes viruses.

Materials and Methods

Compounds

Cyclocreatine (CCr) was synthesized according to published protocols (Griffiths and Walker, 1976). Acyclovir (ACV) was purchased from a local pharmacy (added excipients were taken into consideration before administration).

Cell lines

The following cell lines were obtained from the American Type Culture Collection (ATCC; Rockville, MD): Maden Darby canine kidney (MDCK); human diploid embryonic lung (MRC-5); newborn human foreskin cells (Hs68); epidermoid carcinoma of the larynx (HEp-2); African green monkey

kidney cells (Vero); and contact-inhibited Swiss mouse fibroblasts (3T3). Guinea pig embryo cells (GPE) were obtained from Whittaker M.A. Bioproducts (Walkersville, MD). Cell lines obtained from the ATCC were grown as suggested (Hay et al., 1992). The GPE cells were grown in Eagle's minimum essential medium (Gibco BRL) supplemented with 10% fetal bovine serum and 0.05% $NaHCO_3$.

Viruses

The following viruses were obtained from the ATCC: human cytomegalovirus (HCMV), strain AD-169; influenza A (flu A), strain Port Chalmers/1/73 (H_3N_2); vesicular stomatitis virus (VSV), Indiana Lab strain; herpes simplex virus type 1 (HSV-1), strain F; herpes simplex virus type 2 (HSV-2), strain MS; murine cytomegalovirus (MCMV), strain Smith MSGV; and simian cytomegalovirus (SCMV), strain CSG. Influenza B (flu B), Hong Kong/5/72 strain, was provided by Marion T. Coleman of WHO International Influenza Center for the Americas, National Communicable Disease Center (Atlanta, Georgia). HSV-1, strain BW10168 was provided by M. Nixon Ellis of Burroughs Wellcome Co. (Research Triangle Park, NC). HSV-2, strain E194 was obtained from Milan Fiala of Harbor General Hospital (Torrence, CA). A set of 3 patient isolates of HCMV, including 1 [9-(1,3-dihydroxy-2-propoxymethyl)guanine; DHPG]-sensitive strain [C8708 (early DHPG therapy)] and 2 DHPG-resistant strains [C8807-37 (mid-DHPG therapy) and C8704 (late DHPG therapy)] were provided by Karen Biron of Burroughs Wellcome Co. (Research Triangle Park, NC). Guinea pig cytomegalovirus (GPCMV), strain 22122, was provided by Brigitte Griffith of the Veteran's Administration Medical Center (New Haven, CT). Varicella-zoster virus (VZV), strain Oka, was obtained from Diagnostic Hybrids, Inc. (Athens, OH). Parainfluenza virus type 3 (PIV-3), strain C243 was obtained from the Southern Research Institute (Birmingham, AL). Pseudorabies virus (PRV), strain Aujeszky, was provided by Albert Kaplan of the Albert Einstein Medical Center (Philadelphia, PA).

CPE inhibition experiments

Experiments to determine inhibition of viral cytopathic effect (CPE) were performed according to standard procedures (Sidwell and Huffman, 1971) in 96-well tissue culture plates (Corning Glass Works, Corning, NY). CCr was prepared and diluted in the appropriate medium. Viruses, diluted in test medium (VSV, HSV-1 [strain BW10168], GPCMV, PRV, flu A, flu B, and MCMV), were added to all compound test wells containing monolayers of the appropriate indicator cells (3 wells/dilution) and to 9 virus control wells. Test medium containing CCr without virus was added to all toxicity control wells. The activity of CCr against HSV-1 (strain F) and HSV-2 (strain MS) was tested as described above, except that the Vero cells were serum-starved prior to addition of the virus. The plates were incubated at 37°C until virus control wells had CPE readings near 100%. Cells were then examined microscopically and graded on a scale of 0–4, with 0 being no effect and 4 being 100% cell

death. The toxicity controls (medium containing CCr without virus) were graded for morphological changes due to cytotoxicity. Effective dose, 50% endpoints (ED_{50}) and cytotoxic dose, 50% endpoints (CD_{50}), were calculated by regression analysis from the CPE data and the toxicity control data, respectively. The therapeutic index (TI) for each substance tested was calculated by the formula: $TI = CD_{50}/ED_{50}$. Standard positive control drugs were used in each experiment (Ribavirin for PRV, PIV-3, VSV, flu A, and flu B; ACV for HSV-1, HSV-2, and MCMV; DHPG for GPCMV and SCMV).

Plaque reduction experiments

Plaque assay reduction experiments were performed as previously described by standard methods (Barnard et al., 1992). CCr was prepared at concentrations of up to 10 mg/ml in BME supplemented with 10% FBS and 0.035% $NaHCO_3$. Growth medium was aspirated from established monolayers of MRC-5 cells in 24-well tissue culture plates (Corning), and HCMV or VZV was added to each well except those to be used for cell controls. After the virus was adsorbed (HCMV by centrifugation at 2200 rpm for 30 min at room temperature, and VZV for 1 h at 37°C), the medium was aspirated and the appropriate drug dilutions were placed in test wells (2 wells/dilution). Tissue culture medium without CCr was added to cell control (− virus) and virus control wells (+ virus). DHPG was used as a positive control in each experiment. All plates were incubated until virus plaques were evident in virus control wells, then the cell monolayers were fixed and stained with 0.2% crystal violet in 10% buffered formalin. Plaques were counted using a dissecting microscope.

Growth rate in presence of CCr

The effect of CCr on cell proliferation was assessed by evaluating the growth rates for Hs68, MRC-5, and Vero cells in the presence or absence of CCr. For each cell line, approximately 2×10^4 cells were plated in triplicate into 24-well tissue culture plates (Corning) and incubated with various concentrations of CCr (0, 4.4, 8.9, 17.7, 35.5, and 70.9 mM) in the appropriate cell medium. Cell monolayers were harvested on days 0, 1, 2, and 3 by trypsinization, and the cell numbers were determined using a Coulter counter (Coulter Electronics, Inc., Hialeah, Florida).

In vivo antiviral experimental procedures

Animals

Female Swiss Webster mice weighing approximately 18–20 g were obtained from Simonsen Laboratories (Gilroy, CA). Until used in antiviral experiments, the animals were fed standard laboratory chow and tap water ad libitum.

HSV-2 vaginitis

Two experiments were carried out to study the effects of CCr treatment

against HSV-2-induced vaginitis. In Experiment 1, treatment with CCr was used alone, and in Experiment 2, CCr was used in combination with ACV. Mice were infected intravaginally with HSV-2 (strain E194) as described previously (Sidwell et al., 1990). Toxicity controls were similarly handled and sham infected. CCr was administered in the feed by pulverizing the drug with the laboratory chow (Farmers Exchange #5010 Picolab Mouse Chow) in a ratio of 1:100. The mixture was given to the animals in open cups or feeders designed for handling powdered food. Placebo control animals received powdered food for the same duration as the CCr-treated mice. Positive control animals received ACV by oral gavage (p.o.), and were fed standard mouse chow ad libitum. Lesion scores of 0 (normal, uninfected) to 4 (maximal infection) were assigned daily, on a masked basis, on days 3–14 of the infection as described previously (Sidwell et al., 1990). Animal deaths were noted daily through 21 days. In each experiment, 5 uninfected mice were used as toxicity controls for each drug used alone and in combination. Toxicity control animals were weighed on days equivalent to -3 and $+8$ in the infection in Experiment 1, and on days equivalent to 0 and $+15$ days in Experiment 2.

Experiment 1. Ten infected mice were fed 1% CCr, beginning 3 days prior to virus inoculation and continuing ad libitum for 8 days post-inoculation (after which time they received standard mouse chow). Twenty infected animals received only powdered feed without CCr ad libitum (placebo controls). As positive controls in this experiment, 10 additional infected mice were treated with ACV p.o. twice daily (200 mg/kg/day) for 7 days beginning 1 h prior to inoculation.

Vaginal virus titers were determined from swabs taken 2 days after virus inoculation. Mice were swabbed intravaginally (5 mice per treatment group) with a cotton swab moistened in cell culture medium, which was then placed in 1 ml of medium and frozen at $-80°C$. Titrations of the virus were conducted in 96-well plates seeded with Vero cells using $\log_{10}$ dilutions, using CPE as the endpoint. Each virus titration was done in quadruplicate.

Experiment 2. Ten infected animals received 1% CCr in the diet, beginning 3 days prior to virus inoculation and continuing for 14 days post-infection. Ten infected mice received ACV (100 mg/kg/day) p.o. twice daily for 5 days starting 24 h post-inoculation. Ten infected mice received a combination of CCr and ACV in the same manner as described for each drug given alone. Twenty mice received powdered feed without CCr (placebo controls).

HSV-2 encephalitis

Ten mice were fed 1% dietary CCr ad libitum beginning 14 days prior to virus inoculation and continued for 8 days post-infection. The placebo group consisted of 19 mice fed ground chow without CCr ad libitum 14 days prior to virus challenge and continued for 8 days post-infection. Ten positive control mice were treated twice daily with ACV p.o. (200 mg/kg/day) for 8 days

starting 1 h prior to virus inoculation. After the treatments, mentioned above, the animals were fed standard mouse chow. The mice were infected intraperitoneally with 1×10^5 pfu/mouse of HSV-2 (MS strain). This was a 90% lethal dose in these animals. Two groups of 5 uninfected mice were treated using the same regimens as were done for the virus-infected animals to serve as toxicity controls. The toxicity control mice were weighed on days equivalent to day -14 and $+8$ of the infection. Deaths were recorded daily for 21 days.

Statistics

Survival increases were analyzed using the Fisher exact test. The Mann Whitney U test was used for mean day to death and virus titer reductions. Average daily vaginal lesion score reductions were evaluated using the Student's *t* test. The grand average lesion scores were analyzed by the analysis of variance (ANOVA).

TABLE 1

In vitro antiviral activity of CCr

Virus	Cell line	Assay[a]	ED_{50}[b] (mM)	CD_{50}[c] (mM)	TI[d]
Herpesviruses					
HCMV (AD169)[e]	MRC-5	PR	2.3	>50	>21.8
HCMV (C8708)	MRC5	PR	9.8	>50	>5.1
HCMV (C8805-37, $DHPG^R$)	MRC-5	PR	8.5	>50	>5.9
HCMV (C8704, $DHPG^R$)	MRC-5	PR	10.3	>50	>4.9
VZV	MRC-5	PR	4.0	>50	>12.5
SCMV	MRC-5	PR	5.9	>50	>8.5
HSV-1 (F)	Vero	CPE	8.6	>50	>5.8
HSV-1 (BW10168, TK−)	Vero	CPE	6.3	>50	>7.9
HSV-2	Vero	CPE	12.4	>50	>4.0
MCMV	3T3	CPE	16.1	>50	>3.1
PRV	MRC-5	CPE	16.2	>50	>3.1
GPCMV	GPE	CPE	23.2	>50	>2.2
RNA viruses					
flu A	MDCK	CPE	>28.4	>30	N.D.[f]
flu B	MDCK	CPE	28.4	>30	>1.0
PIV-3	HEp-2	CPE	>28.4	>30	N.D.
VSV	Vero	CPE	>28.4	>50	N.D.

[a]CPE: inhibition of cytopathic effect; PR: plaque reduction.
[b]ED_{50}: effective dose, 50%.
[c]CD_{50}: cytotoxic dose, 50%.
[d]TI: therapeutic index (TI = ED_{50}/CD_{50}).
[e]Virus abbreviations: HCMV, human cytomegalovirus; VZV, varicella-zoster virus; SCMV, simian cytomegalovirus; HSV-1, herpes simplex type 1; HSV-2, herpes simplex type 2; MCMV, murine cytomegalovirus; PRV, pseudorabies virus; GPCMV, guinea pig cytomegalovirus; flu A, influenza A; flu B, influenza B; PIV-3, parainfluenza virus type 3; VSV, vesicular stomatitis virus.
[f]N.D.: not determined.

Results

In vitro antiviral activity of CCr

The results of CCr antiviral activity against a panel of RNA viruses and herpes viruses in vitro are summarized in Table 1. The four RNA viruses tested were essentially not inhibited by CCr, with ED_{50} values equal to or greater than 28.4 mM (the highest concentration tested for these viruses). The viruses most sensitive to CCr included HCMV, VZV and SCMV. The ED_{50} values for the various herpes viruses tested ranged from 2.3–23.2 mM. CCr appeared to be more active against primate viruses (e.g., HCMV, VZV, and SCMV) than against the mouse (MCMV), guinea pig (GPCMV), and pig (PRV) herpes viruses. The activity demonstrated against HSV-1 and HSV-2 was somewhat intermediate, being neither the least nor the most sensitive of the herpes viruses to CCr in vitro.

Analysis of the antiviral activity of CCr against HCMV in MRC5 cells in a single-cycle virus yield experiment demonstrated an ED_{50} value of 4 mM (data not shown), which is consistent with the value obtained by plaque reduction (2.3 mM). We have also examined the activity of CCr against HCMV, HSV-1 and HSV-2 in different cell lines and did not see a substantial difference in its activity (data not shown). For example, the ED_{50} for CCr against HCMV in Hs68 (human foreskin fibroblast) cells was 4.6 mM, which is very similar to that seen in MRC-5 (normal human lung) cells. HSV-1 and HSV-2 were also examined in BALB/c (normal mouse fibroblast), DU145 (human prostate carcinoma), and A549 (human lung carcinoma) cells, and showed similar results to those obtained in Vero (monkey kidney) cells.

Judged by visual examination for toxicity to the cell monolayers, CCr had CD_{50} values of greater than 50 mM in most cell lines (Table 1). To confirm the lack of cytotoxicity of CCr, the effects of this material on cell growth and cellular DNA synthesis were determined in Vero, Hs68 and MRC-5 cells. Growth rate analysis (Fig. 2) demonstrated that Hs68 cells were moderately sensitive to the highest dose (70.9 mM CCr), but did not reach a 50%

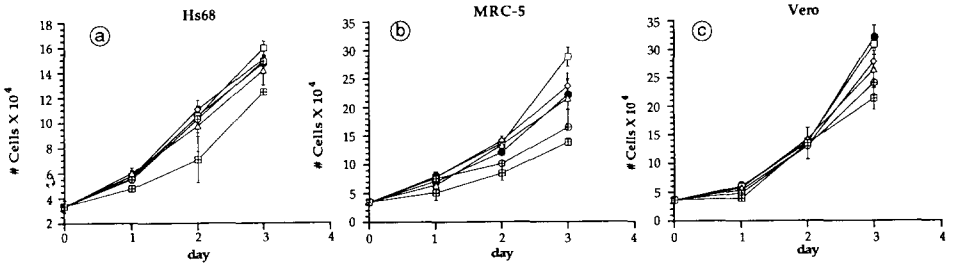

Fig. 2. Effect of CCr on the growth of (a) Hs68, (b) MRC-5, and (c) Vero cell lines in vitro. Cells were incubated with increasing concentrations of CCr (assayed in triplicate). After 1, 2, and 3 days in the presence of CCr, the cells were harvested and the number of cells/well were determined. Symbols: (concentration of CCr); 0 mM, ●; 4.4 mM, □; 8.9 mM, ◇; 17.7 mM, △; 35.5 mM, ⊕; 70.9 mM, ⊞.

inhibitory concentration (IC_{50}) when exposed to the drug for up to 3 days. MRC-5 cells exhibited sensitivity to 35.5 mM and 70.9 mM CCr, with an approximate IC_{50} reached with 70.9 mM CCr by day 3. Vero cells were not sensitive to CCr until the third day of the analysis, at which time the highest dose of CCr (70.9 mM) approached the IC_{50} value. An additional experiment which analyzed the effect of CCr on DNA synthesis in dividing cells, by tritiated thymidine incorporation, was carried out. In this experiment, the IC_{50} values calculated for CCr in Hs68 and MRC-5 cells were 56.8 and 47.6 mM, respectively, while an IC_{50} value for Vero cells was not attained with up to 70.9 mM CCr (data not shown). Together with the CD_{50} values obtained by analyzing CPE, these results demonstrate that the observed antiviral effect of CCr at low mM levels is not due to inhibition of cellular growth.

Inhibition of drug-resistant strains of HSV and HCMV

The activity of CCr was tested against a set of three viruses isolated from two AIDS patients at different stages of DHPG therapy. HCMV strains C8708 and C8704 were isolated early (day 3) and late (day 131) in the course of DHPG therapy of a patient with HCMV hepatitis, respectively, while C8805-37 was isolated mid-therapy (day 60) from a patient with HCMV colitis (Erice et al., 1989; Stanat et al., 1991). These isolates have been documented to have a correlation of increasing resistance to DHPG with length of treatment, as measured by a plaque reduction assay (Stanat et al., 1991). In our experiments, C8708 showed equal sensitivity, C8805-37 was 3 times more resistant, and C8704 was 9 times more resistant to DHPG, all relative to AD169 (data not shown), which is consistent with the data of Stanat et al. (1991). The results of the experiments testing these isolates for sensitivity to CCr (Table 1) indicate that CCr inhibits the DHPG-resistant HCMV strains with the same ED_{50} as the DHPG-sensitive strain of HCMV (C8708). These strains were all 3- to 4-fold less sensitive to CCr as compared to AD169, a common laboratory strain of HCMV. However, while the strains became increasingly resistant to DHPG with an increase in the length of therapy, no increase in resistance to CCr was observed.

A thymidine kinase (TK) mutant of HSV-1 (BW10168) was also evaluated for sensitivity to CCr (Table 1). Because the HSV TK phosphorylates ACV, which is required for activation, this strain is resistant to ACV (Field et al., 1980; Fyfe et al., 1978). In our experiment, this strain was approximately 10-fold more resistant to ACV as compared to wildtype HSV-1 (data not shown). BW10168 did not demonstrate altered sensitivity to CCr compared to the wildtype HSV-1 (strain F).

In vivo activity against HSV-2 vaginitis

The antiviral activity against HSV-2 was assessed in a mouse HSV-2-induced vaginitis model. In this experiment, mice were pretreated with 1% CCr in feed for 3 days prior to viral inoculation, in order to build up CCr to mM levels in the serum and tissues. The effects of dietary CCr on HSV-2 vaginal infections

TABLE 2

Effect of CCr and ACV on HSV-2-induced vaginitis in mice

Treatment	Infected, treated mice				Uninfected controls	
	Survivors/ total	Mean day to death[a]	Mean vag virus titer (log_{10})[b]	Grand avg. lesion score[c]	Survivors total	Weight gain[d]
CCr[e]	$6/10^{**}$	$9.2 \pm 2.5^{**}$	$3.1 \pm 1.6^{*}$	$1.2 \pm 0.5^{***}$	5/5	6.5
ACV[f]	$6/10^{**}$	$13.0 \pm 4.8^{***}$	$2.9 \pm 1.6^{*}$	$0.4 \pm 0.1^{***}$	5/5	2.9
placebo[g]	2/20	6.4 ± 1.6	5.7 ± 1.7	3.0 ± 1.0	5/5	2.5

[a]Mice that died on or before day 21.

[b]Vaginal virus titers were determined from swabs taken on day 2.

[c]Average of lesion scores obtained on days 3–14 post-inoculation.

[d]Difference between animal weights on day −3 and day +8.

[e]CCr treatment (1% in powdered food) began 3 days prior to virus inoculation and continued for 8 days after infection.

[f]ACV treatment (200 mg/kg/day, administered p.o.) began 1 h prior to virus inoculation and continued twice daily for 7 days.

[g]Placebo treatment (powdered food) began 3 days prior to virus inoculation and continued for 8 days after infection. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

in mice are summarized in Table 2. CCr significantly reduced the average lesion score of mice infected intravaginally with HSV-2. The grand average (days 3–14) of the vaginal lesion scores for CCr treated animals was 2.5 times lower than the placebo controls ($P<0.001$) but was higher than the grand average for ACV (1.2 vs. 0.4).

Mortality and recoverable virus titers were also reduced by CCr treatment, relative to placebo controls (Table 2). Mice in the CCr treatment group had a 60% survival rate for the 21 day experiment, as compared to 10% of those in the placebo group ($P<0.01$), and 60% in the ACV treatment group. The mean day to death of the four animals that died in the CCr treatment group was significantly increased compared to the control group ($P<0.01$). Treatment with CCr significantly reduced the recoverable vaginal virus titer relative to the placebo group, by approximately three logs ($P<0.05$). The low vaginal virus titer for CCr-treated animals was comparable to that achieved by the ACV treatment.

All uninfected toxicity control animals survived treatment with 1% dietary CCr and exhibited no obvious adverse affects, such as ruffled fur, tremors, or prostration. All uninfected toxicity control animals gained weight. The mice in the CCr treatment group gained more weight than the ACV treatment group and the placebo controls.

Effect of combination of CCr and ACV on HSV-2 vaginitis

The effects of the combined treatment of 1% CCr in the food and p.o.-administered ACV on HSV-2-induced vaginitis in mice are summarized in Table 3. CCr single-agent therapy again reduced the severity of vaginal lesions, as illustrated in Fig. 3. The grand average (days 3–14) vaginal lesion score for

TABLE 3

Effect of CCr used in combination with ACV on HSV-2-induced vaginitis in mice

Treatment	Infected, treated mice			Uninfected controls	
	Survivors/ total	Mean day to death[a]	Grand avg. lesion score[b]	Survivors/ total	Weight gain (g)[c]
CCr[d]	8/10	6.0 ± 0.0	0.8 ± 0.2***	5/5	7.8
ACV[e]	8/10	12.0 ± 1.4	0.7 ± 0.4***	5/5	3.5
CCr + ACV[f]	10/10*	>21**	0.3 ± 0.2***	5/5	7.0
placebo[g]	8/20	9.2 ± 3.2	1.8 ± 0.7	5/5	5.1

[a]Mice that died on or before day 21.
[b]Average of lesion scores obtained on days 3–14 post-inoculation.
[c]Difference between animal weights on day 0 and day 15.
[d]CCr treatment (1% in powdered food) began 3 days prior to virus inoculation and continued for 14 days after infection.
[e]ACV treatment (100 mg/kg/day, administered p.o.) began 24 h after virus inoculation and continued twice daily for 5 days.
[f]Combination treatment animals received both CCr and ACV treatments described above.
[g]Placebo treatment (powdered food) began 3 days prior to virus inoculation and continued for 14 days after infection. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

CCr treated animals was significantly better than the placebo group ($P<0.001$). In this experiment, the efficacy of CCr was not significantly different from ACV administered at 100 mg/kg/day ($P>0.05$). The animals receiving the combination of CCr and ACV had lower grand average lesion scores than using either drug alone. The combination treatment was significantly better than placebo group ($P<0.001$), and the ACV treatment ($P<0.01$).

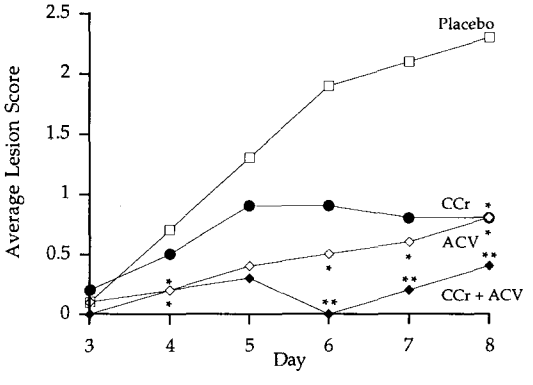

Fig. 3. Effect of combination treatment with CCr and ACV on severity of HSV-2-induced vaginal lesion scores. Treatments: 10 mice were administered 1% dietary CCr beginning three days prior to intravaginal virus inoculation and continued on for 14 days post-inoculation; 10 mice received ACV (100 mg/kg/day) p.o. for 5 days, beginning 24 h post-inoculation; 10 mice received both CCr and ACV as indicated for each single drug treatment; and 20 infected mice were given ground feed without CCr (placebo). The mice were evaluated and assigned vaginal lesion scores (0 to 4, with 0 = no lesion and 4 = most severe lesion). Shown are the averaged scores for the different treatment groups on days 3–8 post-infection. $^{*}P<0.05$, $^{**}P<0.01$.

TABLE 4

Effect of CCr and ACV on survival of mice with HSV-2-induced encephalitis

Treatment	Infected, treated Mice		Uninfected controls	
	Survivors/ total	Mean day to death[a]	Survivors/ total	Weight gain (g)[b]
CCr[c]	5/10**	10.8 ± 2.4	5/5	8.1
ACV[d]	7/10***	12.0 ± 3.5	5/5	6.1
placebo[e]	1/19	10.2 ± 2.3	5/5	4.8

[a]Mice that died on or before day 21.
[b]Difference between animal weights on day −14 and day +8.
[c]CCr treatment (1% in food) began 14 days prior to virus inoculation and continued for 8 days after infection.
[d]ACV treatment (200 mg/kg/day, administered p.o.) began 1 h prior to virus inoculation and continued twice daily for 8 days.
[e]Placebo treatment (powdered food) began 14 days prior to virus inoculation and continued for 8 days after infection. $^{**}P<0.01$; $^{***}P<0.001$.

Survivors doubled in the CCr and ACV treatment groups, from 40% in the placebo to 80% in both single-treatment groups. The combination of CCr and ACV was more effective in increasing survival than either compound alone. All animals receiving the combination treatment survived, which was significantly better than the placebo group ($P<0.05$), but not significantly better than either single treatment, due to the small sample size. The uninfected toxicity control animals treated with CCr showed no visible signs of toxicity and the mice gained slightly more weight than the placebo and ACV controls.

In vivo activity against HSV-2 encephalitis

An HSV-2 encephalitis model was also used to assess the in vivo antiviral activity of CCr. Feeding of 1% CCr began two weeks in advance of i.p. virus inoculation to allow the compound time to cross the blood brain barrier and to accumulate in the central nervous system. Oral gavage treatments with ACV were administered twice daily for 8 days starting 1 h prior to virus challenge. Death was used as a parameter to evaluate the infection and was recorded daily for 21 days. The results of the experiment indicate that CCr caused a significant ($P<0.01$) reduction in mortality relative to the placebo control (Table 4). The mice treated with CCr had a 50% survival rate vs. a 5% survival rate in the placebo controls. Mice treated with ACV had a 70% survival rate ($P<0.001$). Uninfected toxicity controls showed that both CCr- and ACV-treated animals gained more weight than the placebo controls.

Discussion

CCr is a new potential antiviral agent, which appears to act through the CK/CrP pathway. In vitro results presented here suggest that CCr is a selective

antiviral agent with little cellular toxicity. Of the viruses tested to date, the antiviral activity of CCr appears to be limited to herpes viruses, particularly HCMV and VZV, with at least moderate activity also seen in the other herpes viruses evaluated. In addition, results from two HSV-2 mouse models demonstrate that CCr has antiviral activity against HSV-2 in vivo.

The evolution of drug-resistant viral strains has increased the need for drugs that work through alternative pathways. CCr was active against the drug-resistant strains of HCMV and HSV-1 tested in vitro. Somewhat higher concentrations of CCr were required to reach the ED_{50} values for the DHPG-resistant HCMV strains, relative to the laboratory strain tested. At this time, we do not know if this data is reflective of the activity of CCr against patient isolates in general, or if these isolates are coincidentally slightly less sensitive to CCr. However, while two of these clinical isolates (HCMV strains C8805-37 and C8704) showed a significant resistance to DHPG, no increase in resistance to CCr was observed as compared to the DHPG-sensitive isolate (C8708). The activity of CCr against the ACV-resistant strain of HSV-1 is equivalent to the activity against the wildtype HSV-1 strain, which indicates that the viral TK gene is not necessary for the action of CCr. Testing additional patient isolates and well-characterized mutants will enable us to more definitively determine the activity of CCr against patient isolates and whether or not it acts through a mechanism distinct from that of DHPG and ACV.

As demonstrated in the HSV-2 vaginitis model, combination treatment with CCr and ACV may also be beneficial. However, the single dose combination utilized in our experiment did not allow an intensive statistical evaluation of whether the enhanced antiviral activity seen by the drug combination was additive or synergistic. Further experimentation with drug combinations are currently being carried out.

The activity of CCr against responsive viruses required low mM levels in vitro. Previous studies involving CCr demonstrated that mM levels were achievable in vivo by feeding CCr at 1% of the diet to mice, rats, and chicks (reviewed in Walker, 1979). For example, chicks fed 1% CCr for 6 days establishes 0.9 mM CCr in the serum and up to 20–30 mM CCr in the heart and breast muscle, which are organs that express high levels of CK (Griffiths and Walker, 1976). These levels are comparable to the concentration of creatine that is normally found in these organs. In the HSV-2-induced vaginitis model, we pretreated the mice for 3 days with 1% dietary CCr before administering the virus in order to allow CCr to build up to mM levels in the blood and tissues. In our experiment, mice fed 1% CCr for 3 days achieved approximately 0.5 mM CCr in the serum (data not shown). A slower build up of CCr in the brain has been noted (Griffiths and Walker, 1976; Roberts and Walker, 1983). We therefore chose to administer CCr for 14 days before the inoculation of the virus in the HSV-2-induced encephalitis model, which results in the build up of CCr to approximately 5 mM in the brain (data not shown). It remains to be seen whether shorter periods of administration and treatment beginning after infection would also be effective. Other routes of administration of CCr, such

as i.p. and i.v., are being tested in animal models for antiviral efficacy. The observed antiviral activity of CCr against HCMV in vitro has led to the evaluation of its activity against HCMV-induced chorioretinitis in the rabbit model (Dunkel et al., 1993). Preliminary data suggests that i.v. administration of CCr, beginning 2–6 h post-infection, results in significant protection of the retina (E. Dunkel, unpublished data).

It is important to note that prolonged administration and achievement of mM levels of CCr in vivo has been well tolerated and appears to be nontoxic in mice, rats, chicks and dogs (Griffiths and Walker, 1976; Woznicki and Walker, 1980; Roberts and Walker, 1983; Lillie et al., 1993; our unpublished data). This is consistent with the view that the CK/CrP system is an auxiliary energy system. Recently, a null mutation of the muscle CK (CKM) gene was introduced into the germline of mice (van Deursen et al., 1993). The progeny were found to be viable, fertile, and exhibit no overt abnormalities. These mice did, however, display an altered performance of the skeletal muscle in regards to endurance and burst activity. These observations support the hypothesis that the CK/CrP system is important for maximal energy utilization in certain high-energy requiring processes such as those found in neuronal and muscle cells, but is dispensable in other cell types.

The mechanism by which CCr inhibits virus replication is unknown. The induction of CKB in some virus-infected cells suggests that there is a high energy requirement for viral replication. One possibility is that CCr could inhibit viral propagation by affecting the rate of ATP generation or utilization. Other functions regulated by the CK/CrP system, such as ATP/ADP ratios, intracellular levels of ADP and nucleotide pools, all represent targets for modulation by CCr that could affect efficient viral replication. Other mechanisms, such as immunomodulation, may also be involved in the in vivo activity seen. Experiments are underway to better understand the mechanism of action of this novel antiviral agent in vitro and in vivo.

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