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ScienceDirect
Antiviral Research 72 (2006) 68–74
Antiviral Research
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Experimental study on the action of allitridin against human cytomegalovirus in vitro: Inhibitory effects on immediate-early genes

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Received 26 January 2006; accepted 28 March 2006

Abstract

Garlic (*Allium sativum*) extraction has been reported having anti-HCMV efficacy. This study was aimed to investigate the effect of allitridin (diallyl trisulfide, a compound from *A. sativum* extraction) on the replication of HCMV and the expression of viral immediate-early genes. In HCMV plaque-reduction assay, allitridin appeared a dose-dependent inhibitory ability with EC_{50} value of 4.2 μg/ml (selective index, SI = 16.7). Time-of-addition and time-of-removal studies showed that allitridin inhibited HCMV replication in earlier period of viral cycle before viral DNA synthesis. Both immediate early gene (ie1) transcription and IEA ($IE_1$72 and $IE_2$86) expression was suppressed by allitridin, but not by GCV in a single HCMV cycle format. In addition, allitridin appeared stronger inhibition on $IE_2$86 than on $IE_1$72. Decrease of viral DNA load in infected cells was also detected under allitridin treatment, probably due to an indirect consequence of the reduction in ie gene transcription. In summary, this study indicated that allitridin has anti-HCMV activity and the mechanism is associated with suppression of ie gene transcription.

Keywords: Cytomegalovirus; *Allium sativum*; Diallyl trisulfide; Cytomegalovirus; Immediate-early gene

1. Introduction

Human cytomegalovirus (HCMV; human herpes virus 5) is a widespread pathogen that has minor clinical impact on healthy individuals and is able to cause organ diseases in immunocompromised individuals such as AIDS patients (Springer and Weinberg, 2004; Yust et al., 2004) or organ transplant recipients (Paya et al., 2004).

HCMV genes' transcription during productive infection is regulated in a temporal cascade. Immediate-early (IE or α), early (E or β), and late (L or γ) proteins are synthesized sequentially. The products of ie genes including $IE_1$72 and $IE_2$86, which are initially expressed 0–4 h after infection, are strong trans-activators and regulate transcription of viral subsequently expressed genes as well as host cell genes. The ie gene expression plays such a key role in both HCMV replication and pathogenesis that inhibition of ie gene expression was considered as a therapeutic option (Scholz et al., 2001). However, the drugs currently used for the treatment of HCMV infections, including ganciclovir (GCV), foscarnet (PFA) and cidofovir, inhibited the activity of viral DNA polymerase expressed after the immediate-early phase of infection and had no effect on the expression of ie genes (Scholz et al., 2001).

Allium sativum (garlic) has been used for thousands years as a traditional Chinese medicine. It was found that garlic had many pharmacological effects, including antimicrobial, anti-hyperlipidemic, anti-oxidation and antineoplastic actions (Leuschner and Ielsch, 2003; Durak et al., 2004; Sengupta et al., 2004; Thomson and Ali, 2003). The activity of garlic extract against HCMV was firstly identified using a plaque-reduction assay in 1993 (Guo et al., 1993). Moreover, the injection of a main organic compound of *A. sativum*, namely allitridin (diallyl trisulfide; molecular formula: CH_2=CH–CH_2–S–S–S–CH_2–CH=CH_2; molecular weight: 178.3) had been found to be a potent drug to treat HCMV

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doi:10.1016/j.antiviral.2006.03.017

enteritis and pneumonitis in China (Xu et al., 2001; Lu, 1994). Our previous experiment in vitro also showed that allitridin could obviously inhibit the plaque formation of HCMV AD169 and 7 clinical insolated strains (Fang et al., 1999). The therapeutic efficacy of allitridin in vivo was demonstrated in a study of mouse model with murine cytomegalovirus (MCMV) hepatitis (Liu et al., 2004). Their study showed that the MCMV DNA load in liver tissue was significantly reduced and histopathological lesions and functions of liver was obviously improved after 14 days of allitridin treatment. Both mean dosage (equal to mean dosage of human being, 2 mg/kg per day) and high dosage (three-fold mean dosage) of allitridin treatments achieved the similar potency on reduction of viral DNA load with GCV therapy (Liu et al., 2004). Although allitridin is suggested as an alternative substance for the treatment of HCMV diseases, the mechanism of this chemical against HCMV remains unknown. In present study, we aimed to investigate the inhibitory action of allitridin on the replication of HCMV and the expression of viral immediate-early genes in human embryo lung fibroblast (HEL) cell-based system.

2. Materials and methods

2.1. Compounds

Allitridin injection (containing 15 mg/ml of diallyl trisulfide) was purchased from Harvest Pharmaceutical Company (Shanghai, PR China; cat. no. 010501). Ganciclovir (GCV) injection was acquired from Ke-yi Pharmaceutical Ltd. (Hubei, PR China; cat. no. 010922).

2.2. Cell culture and virus

HEL cells were maintained in Dulbecco's modified Eagle's medium (Gibco BRL, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U of penicillin per ml, and 100 μg of streptomycin per ml. The AD169 strain of HCMV was provided by the Institute of Virology, Chinese Academy of Preventive Medical Sciences. The mean infective viral amount of stock was about 5×10^6 PFU/ml.

2.3. Cytotoxicity assay

The cytotoxicity of allitridin for HEL cells was determined based on the cell viability and measured by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mossmann, 1993). Briefly, HEL cells (10^4 per well) were cultured on a 96-well plate in the absence or presence of various concentrations of allitridin. Four wells of cells were used for each concentration and incubated at 37 °C and 5% CO_2 for 72 h. The plates were then read at 490 nm in a plate reader (Bio-Tek, USA). Every test was repeated twice. The percentage of survival cells of each tested group to that of the no-drug control was then calculated and used to determine the maximal tolerable concentration (MTC) and the median cytotoxic concentration (IC_{50}) for HEL cells of allitridin.

2.4. Plaque-reduction assay

Subconfluent HEL cells were infected with 0.5 ml of DMEM–2% FBS containing approximately 60 PFU of HCMV per ml. After an adsorption at 37 °C for 2 h, the inoculum was removed and the monolayer was overlaid with 1 ml of DMEM containing 2% FBS, 0.8% agar and the tested compounds at different concentrations and incubated at 37 °C. The medium was renewed every 3 days. After 7 days of culture, the cells were fixed with formaldehyde, stained with crystal violet, and the numbers of plaques were counted. The plaque reduction rate was calculated based on the mean plaque number of no-drug controls. The median efficacious concentration (EC_{50}) for antiviral activity was determined from dose–response curve using five concentrations of each drug in quadruplicate. The IC_{50} value of allitridin for HEL cells was divided by the appropriate EC_{50} value to obtain a selective index (SI).

2.5. Time-of-addition and removal studies

To understand which step or steps in the HCMV cycle the allitridin was interfering with, time-of-addition and time-of-removal experiments in a single cycle format were performed. Subconfluent monolayers of HEL cells were challenged with HCMV at MOI of 2.5. Viruses were adsorbed for 1 h and the end of viral adsorption was considered as zero point of time course. At −2, 0, 2, 4, 8, 12, 24, 36, 48, 60 h postinfection (p.i.), drug was added to quadruplicate wells to achieve a final allitridin concentration of 3, 6 and 9 μg/ml, respectively. The cultures were terminated at 72 h p.i. Following two cycles of freezing at −76 °C and thawing at 37 °C, the supernatant of lysates was collected and the amount of infectious viruses was determined by plaque assay on new cultures of HEL cells.

In time-of-removal studies, allitridin was added at −1 h p.i. At 0, 2, 4, 8, 12, 24, 36, 48 or 60 h p.i., the medium containing drugs was removed and the drug-free medium was added after three times washing with D-Hanks' balanced salt solution. As described above, all cultures were harvested at 72 h p.i. and the amount of virus in the supernatant was determined by plaque assay.

2.6. Analysis of the translation and transcription of HCMV immediate-early genes

2.6.1. Western blot analysis

HEL monolayers were infected with appropriate amount of HCMV (MOI of 0.25 or 2.5) and then treated with medium containing the testing compounds, allitridin or GCV (served as controls) at the concentration of EC_{50} or MTC. The cells were harvested at 24 or 72 h p.i. 50 μg proteins from each isolate were separated on 10% SDS-PAGE and transferred to nitrocellulose filter. The filters were first incubated with mouse anti-HCMV IEAs (kindly provided by Prof. Britt WJ, UAB hospital, USA) and rabbit anti-human β-actin antibodies (Santa Cruz Biotechnologies, USA), and then reacted with alkaline phosphatase-linked goat anti mouse IgG and horse

anti rabbit IgG (Zhongshan Biotech Lit, Beijing, PR China). Afterwards, BCIP/NBT was added to develop the desired bands.

2.6.2. Northern blot analysis

ie1 mRNA levels was detected by the method of Northern blot. HEL cells were infected with HCMV and treated with allitridin or GCV as described in Section 2.6.1. The cells were harvested at 4 h after infection. About 20 μg of total RNA from each isolate were analyzed according to the manufacturer's instructions of the North2South Direct HRP Labeling and Detection Kit (Pierce Biotechnology Inc., Rockford, IL). The cDNA probe for HCMV ie1 gene was generated from plasmid pSG5-IE72 (gift from the Institute of Virology, Chinese Academy of Preventive Medical Sciences), which contained the 474-nt Bgl II-Bam HI fragment from exon4 of HCMV ie1 gene. DNA probe for human β-actin was prepared by RT-PCR method.

2.7. Southern blot analysis for HCMV DNA replication

The suppression of allitridin on HCMV genome replication was analyzed by Southern blot assay. HEL cells were infected with HCMV and treated with allitridin or GCV as described in Section 2.6.1 and the cells were harvested at 72 h p.i. when maximal levels of progeny DNA synthesis occur. About 20 μg genomic from each isolate were analyzed with the DIG-labeled HCMV ie1 or human actin probes according to the manufacturer's instructions of DIG DNA Labeling and Detection Kit (Roche Diagnostics Corporation, USA).

2.8. Statistical analysis

All blotting assays were performed in triplicate, and the bars in figures denote standard errors (S.E.). The images were scanned and analyzed with TJTY300 Image Analysis System (Shanghai, China). The integral photo-density ratio (=desired band/β-actin band) was served as semi-quantitative parameter and used to determine the inhibiting rate for assessing the potency of drugs. Differences between experimental groups were assessed by analysis of variance. *P*-value less than 0.05 was considered as statistical significance.

3. Results

3.1. Cytotoxicity of allitridin on HEL cells

The direct cytotoxicity of allitridin on HEL cells was measured by MTT method. No appreciable effect of allitridin on the viability of cultured HEL cells was found under treatments of up to 9.6 μg/ml of the drug. When the concentration was higher than 9.6 μg/ml, allitridin showed some toxicity to HEL cells. Thus, the maximum tolerant concentration (MTC) of allitridin for HEL cells was 9.6 μg/ml, and the medial cytotoxic concentration (IC_{50}) was 70 μg/ml (95% confidence interval: 53–87 μg/ml).

3.2. Allitridin inhibits HCMV plaque formation in dose-dependent manner

To establish whether allitridin has dose-dependent inhibitory effect against HCMV, allitridin at serial concentrations ranging from 0.6 to 9.6 μg/ml was used to perform plaque-reduction assay. The results showed that the stronger inhibition of the viral plaque formation was obtained while higher concentration of allitridin was used. The MTC of allitridin (9.6 μg/ml) effectively blocked HCMV plaque formation by 69.81% and the EC_{50} value of allitridin for plaque inhibition was calculated as 4.2 μg/ml. Therefore, allitridin had a selective index (SI) of 16.7 ($IC_{50}/ED_{50} = 70/4.2$) here. GCV also effectively inhibited HCMV plaque formation as expected with an EC_{50} value of 1.03 μg/ml.

3.3. Allitridin was effective when it presented during early period of HCMV replication

Time-of-addition and removal studies in a single viral cycle format at MOI of 2.5 were performed to determine when allitridin acts in the HCMV replication cycle. Marked inhibiting effects on viral titers were observed when allitridin was added at −2 and 0 h. In contrast, almost no effects were noted when the chemical was added after 8 h p.i. (Fig. 1). Our results also showed that inhibition of viral replication by allitridin was in a dosage-dependent way when it was added within first 8 h of infection. Results of time-of-removal studies were consistent with those of time-of-addition. Removal of allitridin at 0 h p.i. had non-effect on viral replication (Fig. 2). However, partial

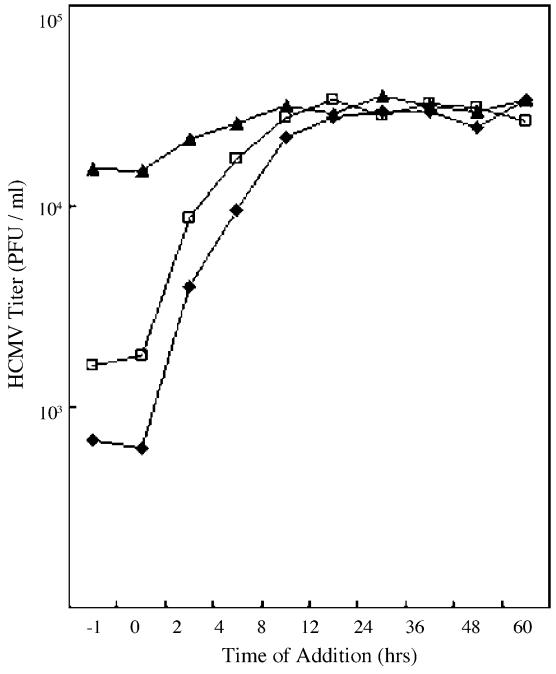

Fig. 1. Effect of time-of-addition of allitridin on HCMV yield. Subconfluent monolayers of HEL cells were infected with HCMV at MOI of 2.5 and treated with 3 μg/ml (▲), 6 μg/ml (□), 9 μg/ml (♦) allitridin at the indicated times. All plates were incubated for a total of 72 h, and progeny viral titers were quantified by plaque-formation assay.

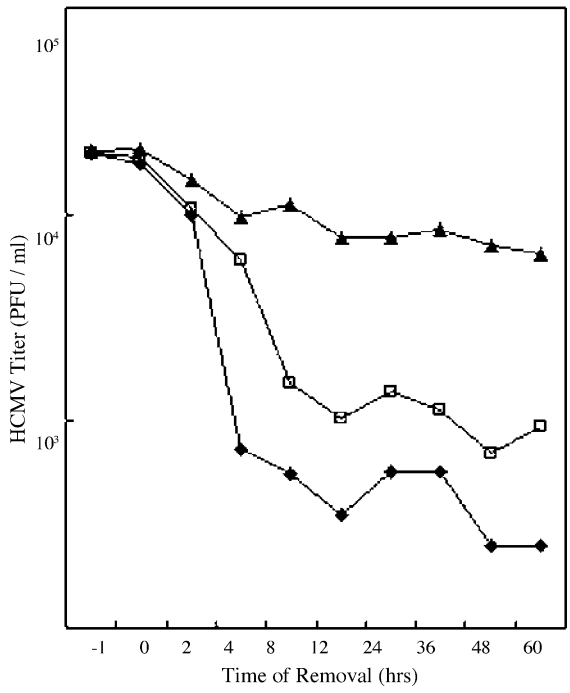

Fig. 2. Effect of time-of-removal of allitridin on HCMV yield. Subconfluent monolayers of HEL cells were infected with HCMV at MOI of 2.5 and were treated with 3 μg/ml (▲), 6 μg/ml (□), 9 μg/ml (♦) allitridin instantaneously. At the indicated times, the cells were rinsed to removal drug and fresh medium was added. Incubation was continued through 72 h, and progeny viral titers were determined.

inhibition was observed when allitridin was removed at 2 or 4 h p.i. (Fig. 2). The results showed that allitridin acted in the earlier time of HCMV cycle before viral DNA synthesis. In addition, significant inhibition was still noted when the drug was removed at 8–60 h p.i., which indicated that the early inhibition was not reversible.

3.4. Allitridin inhibits the expression of ie genes

We next examined the inhibitory effect of allitridin on the expression of viral IE proteins. As shown in Fig. 3, the expression of $IE_1$72 (inhibiting rate, 42.6–64.9%, Fig. 3A) and $IE_2$86 (inhibiting rate, 50.7–70.6%, Fig. 3B) was suppressed after allitridin treatment for 24 h, no matter high or low MOI was used. At this time point, GCV did not affect the expression of IE proteins as expected (Fig. 3A and B). Suppression of IEA was also observed in second viral cycle in allitridin treated cells (Fig. 3C and D). At 72 h p.i., $IE_1$72 protein level was suppressed by 36.4–49.3% (Fig. 3C) while $IE_2$86 was strongly suppressed by 77.9–87.7% (Fig. 3D). Due to second cycle effects, GCV also had some inhibitory effect on IE proteins (Fig. 3C and D). However, although the inhibition on $IE_1$72 by both drugs was comparable, the suppression on $IE_2$86 expression by GCV was weaker than that by allitridin. With ED_{50} of allitridin, inhibitory rate of $IE_2$86 expression reached 77.9–82.2%, approaching to those of MTC ($2.23 \times ED_{50}$) allitridin treating (79.3–87.7%) and $2.23 \times ED_{50}$ GCV treatment (73.9–79.0%), and 1.3–1.7 times as high as that of ED_{50} GCV treated (45.7–62.3%).

The transcription of ie1 gene was also assessed for inhibitory efficacy of allitridin (Fig. 4). In both high and low MOI group, a lower level of ie1 transcript was observed in allitridin

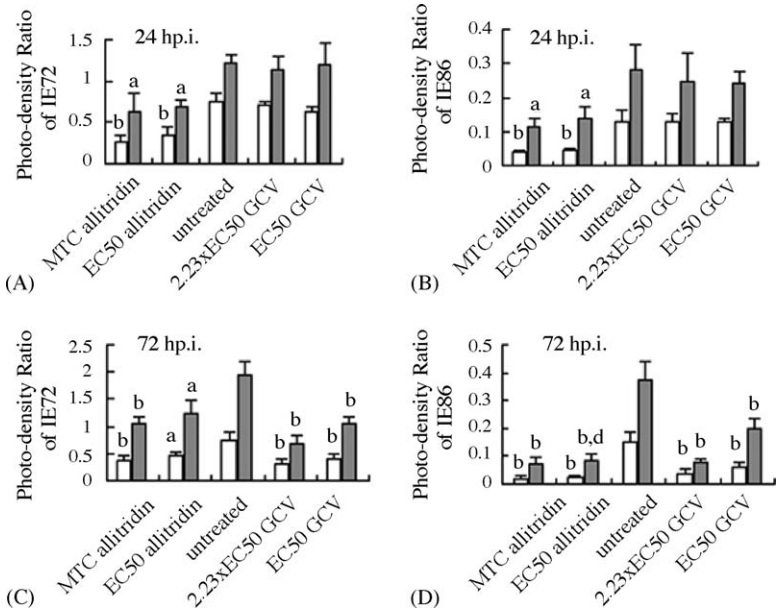

Fig. 3. Effects on $IE_1$72 and $IE_2$86 expression of allitridin and GCV. HEL cells infected with HCMV at MOI of 0.25 (□) or 2.5 (■) and treated for 24 and 72 h with allitridin or GCV. IE proteins were semi-quantified by the method of Western blot (Section 2.6.1). The integral photo-density ratio (=desired band/β-actin band) denoted the quantitative analysis of viral IE protein expression. The results obtained (mean values and standard error from experiments replicated for three times) are represented graphically. (A) photo-density ratio of IE72 band at 24 h postinfection; (B) photo-density ratio of IE86 band at 24 h postinfection; (C) photo-density ratio of IE72 band at 72 h postinfection; (D) photo-density ratio of IE86 band at 72 h postinfection. $^{a}P < 0.05$, $^{b}P < 0.01$ vs. untreated control. $^{d}P < 0.01$ vs. EC_{50} GCV group.

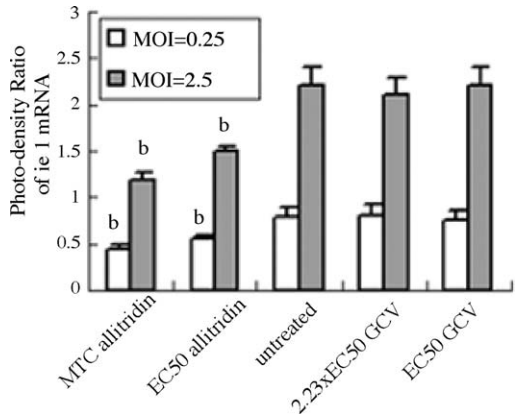

Fig. 4. Effects on ie1 transcription of allitridin and GCV. HEL cells infected with HCMV at multiplicity of 0.25 or 2.5 PFU and treated for 4 h with allitridin or GCV. Total RNAs of cells were extracted and ie1 mRNA level were semi-quantified by the method of Northern blot (Section 2.6.2). The integral photo-density ratio (=desired band/β-actin band) denoted the quantitative analysis of ie1 mRNA level. And the results obtained (mean values and standard error from experiments replicated for three times) are represented graphically. $^{b}P < 0.01$ vs. untreated control.

treated cells (4 h) than in untreated control ($P < 0.01$). With EC_{50} and MTC allitridin, ie1 mRNA level were suppressed by 30.38–32.13% and 44.30–46.61%, respectively. No significant differences were noted between GCV treatment group and untreated control ($P > 0.05$).

3.5. Allitridin decreases HCMV DNA loads in infected HEL cells

The viral DNA load present in the treated cells at 72 h p.i. was semi-quantified by Southern blot. Fig. 5 shows a typical change of HCMV DNA loads. About 4.2 and 9.6 μg/ml of allitridin caused a statistically significant reduction of viral DNA load by

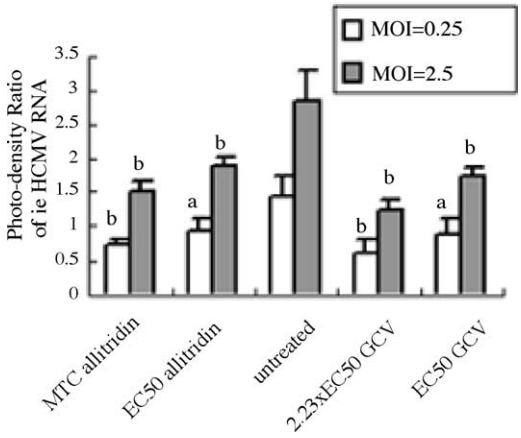

Fig. 5. Effects on HCMV DNA load of allitridin and GCV. HEL cells infected with HCMV at multiplicity of 0.25 or 2.5 PFU and treated for 72 h with allitridin or GCV. Total DNA of cells was extracted and HCMV DNA loads in infected cells were semi-quantified by the method of Southern blot (Section 2.7). The integral photo-density ratio (=desired band/β-actin band) denoted the quantitative analysis of viral DNA loads. And the results obtained (mean values and standard error from experiments replicated at three times) are represented graphically. $^{a}P < 0.05$, $^{b}P < 0.01$ vs. untreated control.

32.98–33.80% and 46.32–49.30% ($P < 0.05$). Moreover, there were no statistical differences between allitridin and GCV treatment groups ($P > 0.05$), although the inhibiting rates of GCV treatment group were slight higher and reached 36.62–38.95% and 54.93–56.14%.

4. Discussion

The time-of-addition and removal studies gave us clues about when allitridin acted in the HCMV replication cycle. Although viral replication was obviously inhibited when allitridin was existed in early period of viral cycle, almost no inhibition was noted while it was added in the period of viral DNA replication (after 8 h p.i.). This result indicated that allitridin did not act in L genes' transcription, but in the early period of viral cycle like viral adsorption and penetration, or expression of IE and E genes. Blocking of viral adsorption and penetration should be excluded based on our results that no obvious inhibition of viral replication was observed while the drug was removed from the culture immediately after viral inoculation. Our previous study also showed that allitridin has little effect on HCMV absorption by performing viral adsorption blocking experiments (Shu et al., 2003). Since inhibition of viral replication was observed when allitridin was removed at 2 or 4 h p.i., immediate-early gene should be considered as the possible replication target in the mechanism of action.

To confirm the hypothesis, the IE gene transcription and protein expression under allitridin or GCV treatment was investigated. The results showed that allitridin, but not GCV exhibited significant inhibition of *ie*1 gene transcription and of IE72/86 protein translation in the end of one viral replication cycle (24 h p.i.). These results indicated that allitridin could act on inhibiting ie gene expression, unlike GCV which inhibit activity of viral DNA polymerase. At 72 h p.i., allitridin still show strong inhibition on the expression of IE72/86 proteins. Meanwhile, a decreased IE protein level was also detected in GCV treated cells at 72 h p.i. due to its suppression on viral DNA synthesis in the first round of viral replication. However, stronger suppression of $IE_2$86 was observed in allitridin rather than GCV treated cells. As expected, viral DNA replication were effectively inhibited by allitridin treatment probably due to an indirect consequence of low *ie* gene expression, which interfere its functions of regulating viral replication (Greaves and Mocarski, 1998; Gawn and Greaves, 2002; Marchini et al., 2001).

One interesting finding is that allitridin appeared to inhibit the expression of $IE_2$86 to a greater extent than $IE_1$72. Although the expression of both $IE_1$72 and $IE_2$86 is under the control of the same promoter, major immediate early promoter (MIEP), it is still not clearly defined about how MIEP differently modulates the two kinds of protein expression. Therefore, it is difficult to identify the precise mechanism underlying the inhibitory action of allitridin. Three sulfur atoms and two *S*-allyl groups allitridin contains make it possible to directly bind to protein, enzyme and DNA, and consequently to affect the function of them. It was recently reported that diallyl trisulfide activated by the versatile epoxide-forming oxidant dimethyldioxirane (DMDO) could prevent the binding of [3H]-labeled 17β-estradiol to DNA and

was able to directly inhibit the RNA polymerase (Yu et al., 2003). Whether allitridin acts be a similar mechanism against HCMV needs to be further determined.

The inhibitory efficacy of allitridin on IE proteins, especially on $IE_2$86, would be its superiority over GCV in the treatment of CMV infections, due to the important role of IE proteins play in HCMV pathogenesis (Scholz et al., 2001). As strong transactivators, IE proteins, especially $IE_2$86, stimulate the transcription of some host cell genes involving in cell cycle regulation and inflammatory immune responses, etc (Scholz et al., 2001; Margolis et al., 1995; Castillo and Kowalik, 2002; Zhu et al., 1995; Geist and Dai, 1996; Murayama et al., 2000). In a previous report we described, the use of allitridin could alleviate the disturbance of HEL cell cycle caused by HCMV infection (Nie et al., 2003). Considering the result of present study, the inhibition on IE proteins may play a role in that process.

Since allitridin exhibited some cytotoxicity in HEL cells, the drug concentrations used in this experiment were carefully selected. Allitridin under MTC had no effect on the viability of HEL cells basing on the MTT assay. EC_{50} (4.2 μg/ml, equivalent to 23 μM) and MTC (9.6 μg/ml, equivalent to 54 μM) used here was comparable with the concentration used in previously published reports on rat primary hepatocytes (Wu et al., 2004) and HEK 293T cells (Elango et al., 2004). Under the concentrations of allitridin we used here, activity of allitridin against HCMV was both dose-dependent and viral cycle specific.

Because of the cytotoxicity on HEL cells, the therapeutic window of allitridin for the antiviral effect is small in the present study. However, allitridin has been used successfully as an intravenous treatment in China, especially for cryptococcosis. And reports from the People's Republic of China indicate that the intravenous use of the drug is safe and relatively free of negative side-effects (Shen et al., 1996). It is difficult to compare the cytotoxicity of allitridin in our studies to in vivo studies because the concentration of allitridin in the blood is not known. Several studies reported that IC_{50} of allitridin against *Cryptococcus neoformans* was around 2.5–4 μg/ml (Davis et al., 1990, 1994), which is comparable to IC_{50} against HCMV in our study. Therefore, further studies may be worthwhile to determine whether allitridin have a place in the treatment of infections caused by HCMV.

Acknowledgements

This work was sponsored by grant (no. 30070928) from the National Natural Science Foundation of China (to Feng Fang).

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