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Short communication

Experimental study on the prevention and treatment of murine cytomegalovirus hepatitis by using allitridin

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Abstract

Allitridin (diallyl trisulfide), a main effective compound of *Allium sativum* (garlic), was previously shown to inhibit the expression of immediate-early antigens and viral proliferation of human cytomegalovirus (HCMV) in vitro. Here we have examined the prophylactic and therapeutic efficacy of allitridin in a non-lethal murine cytomegalovirus (MCMV) hepatitis in methylprednisolone-immunosuppressed BALB/c mice. Allitridin was administered at 25 mg/kg per day (equal to the mean human dose) and 75 mg/kg per day in two regimens: prophylaxis plus therapy beginning at 2 days before infection and lasting for 18 days, and therapy lasting for 14 days initiated at 2 days after infection. Ganciclovir (GCV)-treated, infected, and non-infected mice served as controls. MCMV DNA load in the liver, plasma alanine aminotransferase (ALT) level and Knodell's histological activity index (HAI) score of liver section were evaluated. We found that MCMV DNA load was significantly decreased in all allitridin- and GCV-treated mice, compared with infected controls. Concomitantly, histopathological lesions in the liver and plasma ALT levels were reduced. Statistically, no significant differences were detected between the combined allitridin prophylaxis plus therapeutic and therapeutic groups regardless of dose and the GCV groups. Our results demonstrate the therapeutic efficacy of allitridin in mouse models with MCMV hepatitis.

Keywords: *Allium sativum*; Diallyl trisulfide; Murine cytomegalovirus; Hepatitis; Treatment

Human cytomegalovirus (HCMV) is a major pathogen causing severe disseminated infections in immunocompromised patients, hearing loss, eye damage, and mental retardation in congenitally infected neonates (Jacobson and Mills, 1988; Pass, 2002). In China, HCMV seropositive rate in pregnant women reached 90–96% (Fang et al., 1995a; Wen et al., 1996). For the infants born to seropositive mothers, up to 85% had HCMV infection by the end of the first year of their life, and half of victims suffered from liver impairment ranging from mild-hepato- or hepatospleenomegaly to hepatitis with or without hyperbilirubinemia in a perspective study (Fang et al., 1995b). Hepatitis is also often observed following solid organ transplantation, especially after liver transplantation, and in AIDS patients (Drew, 1988; Pollard, 1988). The drugs currently used for the treatment of HCMV infections are ganciclovir (GCV), foscarnet (PFA), and cidofovir. However, drug-resistant strains of HCMV in GCV- or PFA-treated patients have been frequently reported (Scholz et al., 2001). Therefore, there is a need to develop new anti-HCMV drugs.

Allium sativum (garlic) has been traditionally used in the treatment of fungal and bacterial infections of digestive tract. Many potent biological and pharmacological effects of several garlic preparations have been reported experimentally and clinically, including a reduction of the risk of cardiovascular diseases, to play a role in cancer prevention, and to be a promising candidate to maintain immunologic homeostasis (Ghazanfari et al., 2002; Moon et al., 2000). *Allium sativum* has also been found to be a potent antiviral drug (Nagai, 1973; Tsai et al., 1985). The activity against HCMV of garlic extract was evaluated in vitro by plaque assay and early antigen detection and displayed an inhibitory dose-dependent effect (Guo et al., 1993). Allitridin (diallyl trisulfide; molecular formula: $CH_2{=}CH{-}CH_2{-}S{-}S{-}S{-}CH_2{-}CH{=}CH_2$), a main organic compound of *A. sativum*, is responsible for its antimicrobial activities and is currently used for the treatment of fungal and bacterial infections by intravenous infusion in China. In a previous study, therapeutic effects of allitridin in mice with murine cytomegalovirus (MCMV) hepatitis were observed

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(Fang et al., 1999). In this study, we aim at demonstrating the prophylactic and therapeutic effects of allitridin in a MCMV hepatitis model and comparing them with those of GCV.

Allitridin and GCV were provided by Hefeng Pharmaceutical Company (Shanghai, China) and Keyi Pharmaceutical Ltd. (Hubei, China), respectively. The dosage for mice was determined by the following equation: $D_B = D_A \times R_B/R_A \times (W_A \times W_B)^{2/3}$, where W is the body weight, D the dosage, A denotes is human, B denotes mouse, and R is the build coefficient (Xu et al., 1991). Two dosages of allitridin, 25 mg/kg per day (equal to mean dosage of human being, 2 mg/kg per day) and 75 mg/kg per day (three-fold mean dose), and 60 mg/kg per day of GCV (corresponding to 5 mg/kg per day for human being) were given by intraperitoneal (i.p.) injection once a day in our experiments.

An immunosuppressed model of hepatitis was established. We first wanted to evaluate the possible toxic effects of steroid and allitridin in this model. Fifteen female BALB/c mice (4-week-old, 10–12 g, Purebred Animal Room, Academy of Medical Science, Hubei, China) were housed under a 12/12 h light/dark cycle and allowed unrestricted access to food and water. After housing for 1 week, mice were immunosuppressed by intramuscular injection with methylprednisolone sodium succinate (100 mg/kg, Pharmacia & Upjohn Co., Puurs, Belgium) every 4 days. Five mice were injected, i.p., with 75 mg/kg per day of allitridin for 18 days and the rest of 10 mice were given by 0.89% sodium chloride, i.p., injection for 14 days. Then, they were sacrificed. Their plasma specimens were measured for the level of alanine aminotransferase (ALT) by using the Olympus AU1000 Automated Chemistry Analyzer (Edward, Canada). The liver tissues were embedded in paraffin, and the pathological changes and Knodell's histological activity index (HAI; Knodell et al., 1981) of liver sections were assessed by a skilled pathologist who were unaware of the treatments. Both steroid plus allitridin-treated and only steroid-treated mice grew up normally during the period of experiment and had no pathological lesion in liver tissues and normal ALT levels (42.0 ± 13.00 and 40.7 ± 12.02 U/l, $t = 0.408$, $P > 0.05$), indicating that 75 mg/kg per day of allitridin and steroid that we used had no liver toxicity.

Sixty mice were immunosuppressed by intramuscular injection with methylprednisolone sodium succinate (100 mg/kg) every 4 days initiated at 2 days before MCMV infection. These steroid-treated 5-week-old mice were then inoculated intraperiton with 1×10^6 pfu of MCMV K181 (prepared from MCMV-infected murine embryo fibroblasts) 2 days after the first injection of steroid and were randomly divided into four groups: allitridin prophylaxis plus therapy group (prophylaxis/therapy group) with 18-day course beginning 2 days before infection; allitridin therapy group with 14-day course initiated 2 days after infection; GCV therapy group; and infected controls (administration of the same volume of vehicle, 0.89% sodium chloride). The first two groups were also divided into 75 and 25 mg/kg per day dosage subgroups and the last two groups had the same regimen as the allitridin therapy group. Because the sublethal dose of low virulence MCMV were used, no mouse died during our experiments. By the end of treatment, the animals were sacrificed for detection of plasma ALT levels and evaluation of HAI scores of liver sections. MCMV immediate-early (IE) DNA in liver tissues was examined by in situ hybridization assay, using a method modified from Sambiase et al. (2000). The probe for MCMV IE genes were provided by Institute of Virology, Academy of Preventive Medical Sciences, Beijing, China, and the Dig DNA labeling and detection kits were supplied by Roche Diagnostics Corporation (USA). Hybridization signal was analyzed by Olympus HPIAS-1000 True Color Image Analysis System (Beijing, China) and integral photo-density (IPD) served as the parameter to determine the load of MCMV DNA semi-quantitatively.

The prophylactic and therapeutic efficacy of allitridin on MCMV hepatitis model is shown in Table 1. ALT levels, HAI scores, and IPD values of MCMV DNA in liver tissues were significantly lowered in allitridin prophylsxis/therapy, therapy and GCV groups than those of infected controls ($P < 0.05$), although they were still higher than those of non-infected mice ($P < 0.05$). Moreover, there were no statistical differences between all compound treatment groups irrespective of dose. These results suggest that allitridin with 14-day course of treatment in MCMV hepatitis models significantly improved their hepatic function and reduced histopathological lesions and the MCMV DNA load in liver tissues as well. Moreover, the efficacy of allitridin was similar to GCV at the dose employed. These findings are consistent with previous in vitro and in vivo studies (Fang et al., 1999; Guo et al., 1993) and now demonstrate the therapeutic effects of allitridin in MCMV hepatitis model. However, both administering a prophylactic dose and increasing the dosage of allitridin did not to enhance the efficacy in these MCMV hepatitis models. This was not in agreement with the dose-dependent effects of allitridin on HCMV-infected cells in vitro and the prophylactic effect of the drug in patients with bone marrow transplantation reported by Meng et al. (1992). The differences between the in vivo and in vitro studies could be explained by the complexity of the body, where drug metabolism and delivery to target organs are expected to be much more complicated and might be quite different from single cells. In addition, the prophylactic course of 2 days in this study might not be long enough to show the effects of prophylaxis. Further experiments need to consider with a wider range of doses and a longer course of prophylaxis.

The mechanism of allitridin against CMV is unclear. Our previous investigation showed that allitridin inhibited the expression of HCMV IE antigens in a dose-dependent way with the highest inhibition (89.3 and 90.5%) at 72 and 96 h after infection. Southern blot analysis also showed allitridin inhibition of the replication of HCMV IE genes by 36.3% (data not shown). Inhibition of CMV IE gene expression is considered to be a therapeutic option because of

Table 1
Effects of allitridin on MCMV hepatitis in immunosupressed mice

Group	ALT (U/l)	HAI	MCMV DNA (IPD)
Allitridin prophylaxis/therapy[a]			
75 mg/kg per day	53.3 ± 17.94 (36–85)[b]	2.8 ± 1.93*,[b]	118.84 ± 44.82*,[b]
25 mg/kg per day	61.8 ± 12.73 (45–86)[b]	3.2 ± 1.99*,[b]	134.84 ± 44.51*,[b]
Allitridin therapy[c]			
75 mg/kg per day	61.0 ± 13.87 (45–91)[b]	3.2 ± 1.62*,[b]	131.54 ± 94.60*,[b]
25 mg/kg per day	62.4 ± 13.38 (50–81)[b]	3.5 ± 1.65*,[b]	137.92 ± 66.45*,[b]
Ganciclovir therapy (60 mg/kg per day)[c]	62.0 ± 12.68 (39–81)[b]	2.6 ± 1.51*,[b]	134.62 ± 60.83*,[b]
Infected (vehicle-treated) control	97.0 ± 44.98 (58–201)	6.7 ± 2.83**	220.86 ± 108.00**
Non-infected (vehicle-treated) control	40.7 ± 12.02 (24–58)	0	0

The values in parentheses are the range. ALT: alanine aminotransferase; HAI: Knodell's histological activity index; IPD: integral photo-density. All the mice were immunosuppressed by the administration of methylprednisolone succinate every 4 days initiated at 2 days before MCMV inoculation. The results presented as the mean ± S.D. There were 10 mice in each group or subgroup.

[a] Eighteen days of treatment starting 2 days before infection.

[b] $P < 0.05$, compared with infected controls (variance analysis and Newman–Keuls test for group comparisons).

[c] Fourteen days of treatment starting 2 days before infection.

* $P < 0.05$, compared with non-infected controls.

** $P < 0.01$, compared with non-infected controls.

their important biological functions during CMV infection (Scholz et al., 2001). We also found that allitridin could inhibit HCMV-induced apoptosis and up-regulated bcl-2 mRNA expression and down-regulated fas mRNA expression in HCMV-infected cells in vitro, which might be one of the reasons by which allitridin reduced the HAI lesion score in the liver (Li et al., 1999). On the other hand, allitridin is able to activate T lymphocytes and enhance macrophages to produce hydrogen peroxide (Feng et al., 1994). We previously found that allitridin up-regulated the expression of Th1 cytokines, IL-2, and IFN-γ, and inhibited the expression of Th2 cytokine, IL-10 both in normal mice and immunosuppressed mice with MCMV infection after treatment of 14 days, indicating a Th1 dominant status (data is not shown). Apparently, the immunomodulatory activity of allitridin might play an indirect role in reducing MCMV hepatitis.

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