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Antiviral activity of an immunomodulatory lipophilic desmuramyl dipeptide analog

R.W. Sidwell [a,*], D.F. Smee [a], J.H. Huffman [a], K.W. Bailey [a], R.P. Warren [a], R.A. Burger [a], C.L. Penney [b]

[a] *Institute for Antiviral Research, Utah State University, Logan, UT 84322-5600, USA*
[b] *Biochem Therapeutic, Inc., Laval, Quebec, Canada*

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Abstract

BCH-527, the lipophilic hydrochloride salt of octadecyl D-alanyl L-glutamine, was evaluated for efficacy against experimentally induced murine cytomegalovirus (MCMV), influenza A (H1N1) (IV-A), and Punta Toro virus (PTV) infections in mice. The compound was administered i.p. every other day for a total of 4 injections commencing 24 h previrus exposure. Doses ranged from 12.5 to 200 mg/kg per injection in the various experiments. The MCMV infection was significantly inhibited in two experiments by doses of 25–200 mg/kg, as manifested by increased numbers of survivors and decreased titers of virus recoverable from tissues. The IV-A infection was weakly inhibited, with antiviral activity seen in lowered lung scores and lung weights and less decline in arterial oxygen saturation values. The PTV infection was not inhibited. BCH-527 was stimulatory to cytotoxic T-cells, natural killer (NK) cells, macrophages, and splenic B-cells. The highest dose tested, 200 mg/kg, was inhibitory to cytotoxic T-cell activity and to some extent to NK cell and macrophage activity. These data suggest BCH-527 functions as an immune modulator in exerting the observed antiviral activity.

Keywords: BCH-527; Murine cytomegalovirus (MCMV); Influenza A (H1N1) virus; Punta Toro virus (PTV)

1. Introduction

The use of immunomodulating substances, including both synthetic products and cytokines (Werner and Zerial, 1984; Warren and Sidwell, 1994) has received continued attention for the control of significant virus disease of man.

* Corresponding author. Fax: +1 (801) 797 3959.

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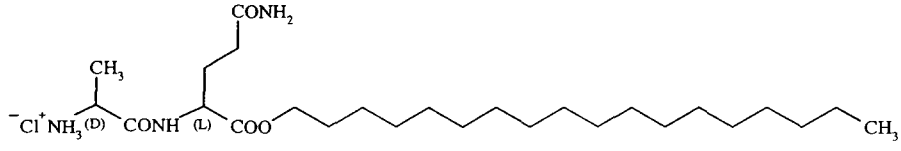

MW = 506.2
MP = 190° - 192°C

Fig. 1. Structure of the immunomodulator BCH-527: the hydrochloride salt of octadecyl D-alanyl L-glutamine.

One well-known immunomodulator is muramyl dipeptide (MDP; *N*-acetylmuramyl L-alanyl D-isoglutamine). MDP is a B-cell mitogen and macrophage activator (Warren and Chedid, 1988). Thus, it is effective in enhancing humoral immunity, even if given orally. The material has been reported to have moderate influenza virus-inhibitory effects when administered to mice prior to virus infection (Masihi et al., 1983). However, MDP induces a poor cell-mediated immune response unless it is administered in a hydrophobic medium (oil, liposomes) or made hydrophobic by chemical modification (lipophilic analogs) (Adam, 1985). Further, MDP is toxic e.g., causing pyrogenicity, transitory lymphopenia, and is associated with the induction of certain autoimmune diseases (Adam, 1985). Thus, there has been considerable effort directed towards the synthesis of lipophilic analogs of MDP. One promising compound, for example, is muroctasin, which contains MDP linked to long chain *N*-stearoyl lysine (reviewed by Azuma, 1992). However, muroctasin, and the majority of the MDP analogs synthesized to date retain the complex *N*-acetyl muramic acid portion. Few lipophilic desmuramyl dipeptide analogs of MDP have been described in the literature.

A new immunomodulator, the long-chain lipophilic desmuramyl MDP analog octadecyl D-alanyl L-glutamine (BCH-527, Fig. 1) has been shown by us in preliminary studies to be a significant stimulator of NK cell activity as well as to activate macrophage function and increase the number of murine splenic B-cells. This immunostimulatory activity suggested BCH-527 may inhibit certain viral infections, and a series of experiments was subsequently run to determine the antiviral efficacy of this immunomodulator against a spectrum of experimentally induced virus infections. Those studied were murine cytomegalovirus (MCMV), type A influenza virus (IV-A), and Punta Toro virus (PTV) infections in mice. This report describes the results of these antiviral experiments and concomitant immunological studies aimed at characterizing the immunological properties of this compound in the infected and uninfected animals.

2. Materials and methods

2.1. Viruses

The Smith strain of MCMV, influenza A/NWS/33 (H1N1) virus, and the Adames strain of PTV were used. The MCMV was obtained initially from the American Type Culture Collection (ATCC, Rockville, MD). A mouse salivary gland preparation of MCMV was used in these studies. The IV-A was provided by K.W. Cochran of the

University of Michigan (Ann Arbor, MI). A pool was prepared in Madin Darby canine kidney cells. The PTV, obtained from the U.S. Army Medical Research Institute for Infectious Diseases (Fort Detrick, Frederick, MD), was prepared in rhesus monkey kidney cells. All viruses were pretitrated in mice before use in this study.

2.2. Mice

Three-week-old female Swiss Webster, BALB/c, and C57BL/6 mice weighing 9–11 g were obtained from Simonsen Laboratories (Gilroy, CA). They were fed standard mouse chow and tap water ad libitum for the duration of the studies. The animal studies were run following approval by the Utah State University Institutional Animal Care and Use Committee, and were carried out in the University's Laboratory Animal Research Center, which is an American Association for the Accreditation of Laboratory Animal Care (AAALAC)-accredited facility.

2.3. Compounds

BCH-527, the hydrochloride salt of octadecyl D-alanyl L-glutamine (Fig. 1), having a melting point of 190–192°C and a molecular weight of 506.2, was synthesized by modification of the approach described for other octadecyl dipeptides (Penney et al., 1993). Briefly, the octadecyl ester of *tert*-butyloxycarbonyl (BOC) L-glutamine was prepared by reaction of BOC-glutamine with octadecanol in tetrahydrofuron, in the presence of 1,1'-carbonyldiimidazole. After purification by flash silica gel chromatography, the BOC protecting group was removed by treatment of the product dissolved in methylene chloride with hydrogen chloride gas. To octadecyl L-glutamine hydrochloride, suspended in chloroform, was added triethylamine, BOC D-alanine and the coupling agent 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ). The protected octadecyl dipeptide was purified by crystallization (methylene chloride : ether), and the BOC group removed by treatment of the product with hydrogen chloride gas. The white hydrochloride salt of BCH-527 was greater than 99% pure, as determined by HPLC. Product structure was confirmed by NMR and mass spectroscopy.

The compound was suspended in 0.4% carboxymethylcellulose (CMC) when injected into mice. Ganciclovir (9-[1,3-dihydroxy-2-propoxymethyl]guanine) (Syntex Corp. Palo Alto, CA), used as a positive control for the MCMV experiments, was obtained from a local pharmacy. Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide), used as the positive control for the IV-A and PTV experiments, was obtained from ICN Pharmaceuticals, Inc. (Costa Mesa, CA). The positive control compounds were dissolved in physiological saline for use in mice.

2.4. Assay for tissue virus

Homogenates of the appropriate tissues were processed through a series of 10-fold dilutions and assayed in susceptible cells using viral-induced cytopathic effect as we have previously described (Sidwell et al., 1985, 1988, 1993). Fifty percent end points were calculated by the method of Reed and Muench (1938). Virus titers were expressed as $\log_{10}$ 50% cell culture infectious doses ($CCID_{50}$) per gram of tissue.

2.5. Natural killer (NK) cell activity

Splenic cells from treated mice were assayed for their ability to lyse YAC-1 tumor cells in a conventional 4 h chromium release assay as an indicator of NK cell activity (Morrey et al., 1990). In this assay, the spleen from each mouse was processed in a Lab Blender 80 stomacher (Seward Medical, London, UK) to obtain a single cell suspension; the cells were added to water to lyse the red blood cells, with the reaction stopped using 10 × phosphate-buffered saline. The cells were then washed twice in RPMI 1640 medium and resuspended in the same medium containing 10% fetal bovine serum (FBS). A total of 1×10^6 effector cells and 1×10^4 target cells were used; three replicates were run per sample. The YAC-1 cells (8×10^6/ml) were incubated for 90 min in 1000 μCi ^{51}Cr/ml, washed three times in RPMI 1640 medium with 10% FBS, rested 30 min, washed, then used in the assay. Each spleen was assayed separately. Ratios of splenic cells to tumor cells were 12.5 : 1, 25 : 1, 50 : 1, and 100 : 1. Cytotoxicity was expressed as percent chromium release by the formula:

$$\frac{\text{experimental counts per minute (CPM)} - \text{background CPM}}{\text{maximum CPM} - \text{background CPM}} \times 100.$$

2.6. Macrophage function assay

Macrophage function was assayed by interleukin-1 (IL-1) measuring in vitro production utilizing an ELISA kit (Genzyme Corp., Cambridge, MA) for murine IL-1. Splenocytes (5×10^6/well) in 24-well plates were incubated 1 h at 37°C to allow monocytes to adhere to the plate. Non-adherent cells were removed with 1 ml of RPMI 1640 medium. The same medium containing 10% FBS was then added to each well with 20 mg/ml lipopolysaccharide (Sigma Chemical Co., St. Louis, MO). The plates were incubated 24 h, after which the supernates were removed and analyzed by ELISA.

2.7. Cytotoxic T-cell (CTL) assay

Effector cells were prepared from spleens of IV-A-infected BALB/c mice by mincing the spleens and suspending the cells in RPMI 1640 medium supplemented with defined 10% fetal calf serum (HyClone, Logan, UT). In a 1 : 1 ratio, the effector cells were stimulated with normal mouse splenocytes exposed to the same IV-A used in the antiviral study. The effector/stimulator cell mixture was incubated at 37°C for 5 days, washed and added to P815 target cells at ratios of 7.5 : 1, 15 : 1, 30 : 1 and 60 : 1 in 96-well tissue culture plates. The target cells were prepared by constant exposure to influenza virus and to ^{51}Cr-containing medium for 1 h at 37°C, washed, and allowed to incubate 2 h before use. As controls, target cells were mixed with either detergent or medium and the total ^{51}Cr release determined. The plates were incubated 4 h at 37°C, centrifuged and the supernatants assayed for ^{51}Cr release in CPM. The percent specific lysis was determined according to the formula:

$$\frac{\text{CPM of target cells + effector cells} - \text{CPM of target cells in medium}}{\text{CPM of detergent-treated cells} - \text{CPM of target cells in medium}} \times 100.$$

2.8. Determination of serum alanine aminotransferase and aspartate aminotransferase (ALT, AST)

These liver enzymes, quantitated as an assay of toxicity of BCH-527 and as a determination of liver disease in the PTV experiment, were determined using colorimetric kits from Sigma Chemical Co. (St. Louis, MO) as described previously (Sidwell et al., 1988).

2.9. Splenic cell enumeration assay

Dispersed splenocytes were reacted with fluoroscein isothiocyanate-labeled murine monoclonal antibody anti-Ly5 for B-cell enumeration and phycoerythrin-labeled monoclonal antibody anti-Thy 1.2 for the T-cell phenotype. The labeled cells were then analyzed with a flow cytometer (EPICS-C, Coulter Corp., Hialeah, FL).

2.10. Experiment design

2.10.1. Immunology studies

Experiments were run in both BALB/c and C57BL/6 mice to determine effects of BCH-527 on selected immune parameters in the animals. BALB/c mice infected intranasally (i.n.) with an 80% lethal dose of IV-A and a similar number of uninfected animals were treated i.p. with 50, 100, or 200 mg/kg of BCH-527 or with CMC only; 24 h later, 5 mice in each group of uninfected mice were killed and their spleens assayed for NK cell activity, CTL response, and macrophage function. Treatment continued with the compound administered every other day for three more treatments; 5 infected and uninfected mice at each dosage as well as the same number of untreated animals were killed 24 h after the last treatment and again their spleens assayed for the same immunologic parameters.

C57BL/6 mice were treated i.p. every other day with 4 injections of a 50 mg/kg dose of BCH-527. Five of these animals and 5 normal control mice were killed 24 h after the final treatment and their spleens removed and the splenocytes assayed for NK cell activity, macrophage function, and T- and B-cell enumeration.

2.10.2. Antiviral studies

MCMV experiments.

Two experiments were run to evaluate the MCMV-inhibitory activity of BCH-527. In one experiment, BALB/c mice were infected intraperitoneally (i.p.) with a 90% lethal dose (approximately 10^6 cell culture 50% infectious doses) of MCMV. BCH-527, in doses of 12.5 and 25 mg/kg, was injected i.p. once daily on days -1, $+1$, $+3$, and $+5$ relative to virus challenge. One group also received the compound per os (p.o.) by the same schedule in a dose of 100 mg/kg. Placebo controls received CMC i.p. by the same treatment schedule. Ten mice in each group and 20 in the placebo control group

were observed daily for 21 days for occurrence of death; 5 additional infected, treated mice in each group were killed on day 5 and their livers, lungs, and spleens assayed for virus titer. Five uninfected mice were treated in parallel with each dose of BCH-527 to serve as toxicity controls.

A second experiment utilized Swiss Webster mice infected with the same concentration of MCMV used in the above study, and treated as above with BCH-527 at doses of 50, 100, and 200 mg/kg. In this study, ganciclovir was included as a known positive control, administered i.p. at a dose of 25 mg/kg/day once daily for 5 days beginning on day +1. In this experiment, 10 mice in each infected, drug-treated group and 20 infected, placebo control animals were observed for death daily for 21 days and 5 additional mice in each group were killed on days +3 and +6 for assay of virus titers in livers, lungs, spleens, and salivary glands. Five toxicity control mice were again run in parallel with each infected drug-treated group.

IV-A experiments.

The animals were infected intranasally (i.n.) with an 80% lethal dose of virus. Groups of 15 infected mice were treated with BCH-527 at doses of 50, 100 and 200 mg/kg; treatments were i.p. on days −1, +1, +3, +5 and +7 relative to virus exposure. As a positive control group, 15 mice received 75 mg/kg/day of ribavirin administered i.p. twice daily for 6 days beginning 4 h after virus exposure. Placebo (CMC) was administered to 25 infected mice using the BCH-527 treatment schedule. Ten mice in each infected, drug-treated group and 20 animals receiving placebo were assayed on days 3–10 for arterial oxygen saturation (SaO_2) using a pulse oximeter as previously described (Sidwell et al., 1992). Deaths were noted in these groups daily for 21 days. On days 2, 4, 6, 8 and 10 of the infection, 5 additional infected, treated mice in each group were killed and their lungs removed for determination of lung consolidation as assayed by lung weight and scores of 0 (normal), 1 (25% consolidation), 2 (50% consolidation), 3 (75% consolidation) and to 4 (100% consolidation) and for virus titer. Toxicity controls (5 mice/dose) and a similar number of normal controls were run in parallel; host weight differences in these groups were determined 18 h after termination of treatment. An additional 5 toxicity control mice were killed on day 8 to assay for levels of ALT and AST.

PTV experiment.

C57BL/6 mice were infected subcutaneously (s.c.) with an 80% lethal dose of PTV; treatments with BCH-527 were given i.p. on days −1, +1, +3 and +5 of the infection, using doses of 1.6, 5, 16, and 50 mg/kg per injection. Ribavirin at a dose of 75 mg/kg/day was given i.p. twice daily for 3 days beginning 4 h postvirus exposure. Fifteen infected mice were included in each group, with 30 animals used as placebo controls. Ten mice were observed for death daily for 21 days. The remaining animals in each group were killed on day 4 and their livers removed and assigned a hepatic icterus score of 0 (normal) to 4 (maximal discoloration). The livers and sera were assayed for infectious virus titers. The serum was also assayed for ALT and AST as a measure of hepatic disease. Toxicity and normal control animals (5/group) were run in parallel, with deaths and host weight gain determined.

2.11. Statistical analysis

Increases in survivors compared with placebo controls were evaluated using χ^2 analysis with Yates' correction. The *t*-test was employed to analyze differences in mean survival times and decreases in virus titers in tissues. Ranked sum analysis (Wilcoxon test) was used to compare score determinations. The immunological data were evaluated by *t*-test and computer-derived standard deviations determined.

3. Results

3.1. Antiviral studies

3.1.1. MCMV experiments

In the initial experiment run in BALB/c mice, only the higher i.p. dose used, 25 mg/kg, prevented deaths in the mice, with 5 of 10 infected mice surviving compared to 1 of 10 placebo-treated controls. Moderate virus titer reductions (approximately 1 $\log_{10}$, $P = 0.5–0.7$) were seen in the liver, lungs and spleens using this dose. The lower dose of 12.5 mg/kg was not effective in this experiment. Oral treatment with this compound was not effective, even at the relatively high (100 mg/kg) dose used. All toxicity control animals survived treatment and showed no signs of adverse effects. This experiment was repeated using higher BCH-527 doses (Table 1), and in this experiment significant efficacy was seen at all doses. Virus titer reductions were particularly manifested on day 3 in the liver, lungs and spleens, and in salivary glands on day 6. Ganciclovir treatment prevented deaths from occurring in all infected mice, and significantly reduced virus titers in the liver on day 3, but not in the other tissue, at either sampling time. All BCH-527 toxicity control mice survived treatments and gained 6.9–9.0 g compared to 9.2 g in the normal controls.

3.1.2. IV-A experiments

The data using BCH-527 in IV-A-infected mice are summarized in Table 2. Ribavirin treatment prevented death in all the infected mice, reduced lung consolidation, lowered lung virus titers, and prevented the usual infection-associated decline in SaO_2. Treatment with BCH-527 was marginally effective, as seen by a modest (1–2 day) delay in death, significantly decreased lung scores and lung weights, and a moderate ($P = 0.5–0.9$) inhibition of SaO_2 decline. For the purposes of brevity, Table 2 shows only the results of lungs taken on day 6, and SaO_2 readings taken on day 10, when the differences between drug- and placebo-treated values were greatest. Although no deaths occurred in the toxicity control mice, weight losses of 0.8 and 0.6 g were seen in the animals receiving 200 and 100 mg/kg doses, respectively, of BCH-527. No significant increases in liver enzymes (ALT, AST) were seen in the blood of toxicity control mice treated with this compound (data not shown). A weight loss of 0.3 g also was seen in the ribavirin-treated toxicity controls. The normal control mice gained 2.6 g in this same time period.

Table 1
Effect of BCH-527 treatment on MCMV infection in Swiss Webster mice

Compound	Dose (mg/kg/inj.)	Infected, treated		Mean virus titer ± S.D. [a]							
		Surv/total	Mean day to death	Liver		Lung		Spleen		Salivary gland	
				Day 3	Day 6	Day 3	Day 6	Day 3	Day 6	Day 3	Day 6
BCH-527	200	10/10 [c]	> 21.0 [c]	2.9 ± 0.4 [c]	< 2.5 ± 0.0	< 2.5 ± 0.0	3.1 ± 0.4 [b]	5.3 ± 0.4 [b]	3.7 ± 0.7	< 2.5 ± 0.0	3.5 ± 0.7
BCH-527	100	7/9	10.5 ± 6.3	2.9 ± 0.7 [c]	< 2.5 ± 0.0	< 2.5 ± 0.0	3.9 ± 0.8	4.7 ± 1.6	4.9 ± 0.6	3.0 ± 0.4	2.8 ± 0.3 [b]
BCH-527	50	9/10 [b]	4.0 ± 0.0	3.1 ± 0.7 [b]	3.1 ± 1.1	< 2.5 ± 0.0	3.4 ± 0.6	6.1 ± 0.6	4.3 ± 1.2	3.0 ± 0.4	2.7 ± 0.1 [b]
Ganciclovir	12.5	10/10 [c]	> 21.0 [c]	2.6 ± 0.2 [c]	3.3 ± 0.8	< 2.5 ± 0.0	3.5 ± 0.6	5.7 ± 0.3	3.6 ± 0.9	3.1 ± 0.6	3.3 ± 0.8
CMC	–	9/20	6.5 ± 2.5	4.7 ± 0.3	3.3 ± 1.5	3.1 ± 0.7	4.7 ± 1.2	6.3 ± 0.6	4.2 ± 1.4	< 2.5 ± 0.0	4.2 ± 1.3

Note: BCH-527 treatment was administered i.p. on days −1, +1, +3, +5, and +7 relative to virus inoculation.
[a] Log_{10} 50% cell culture infectious doses/g.
[b] $P < 0.05$, compared to CMC-treated controls.
[c] $P < 0.01$, compared to CMC-treated controls.

Table 2
Effect of BCH-527 treatment on IV-A infection in BALB/c mice

Compound	Dose (mg/kg/inj.)	Infected, treated					
		Surv/ total	Mean day to death	Mean lung score [a]	Mean lung wt. (mg) [a]	Mean lung virus titer (log_{10}) [a]	Mean SaO_2 (%) [b]
BCH-527	200	0/10	12.3±3.4	1.8±0.3 [c]	124±23 [c]	6.9±0.5	83.1±7.7
	100	2/10	13.3±2.6	1.8±0.8 [c]	136±39	6.6±0.3	83.9±2.9
	50	0/10	12.6±1.3	1.2±0.8 [c]	124±43	5.9±1.2	85.2±2.2
Ribavirin	75	10/10 [d]	>21.0±0 [d]	1.3±0.9 [c]	142±16	5.6±0.5 [c]	88.6±1.2 [c]
CMC	–	4/20	11.0±1.9	2.7±0.6	196±54	6.6±0.1	82.0±2.8

Note: BCH-527 treatment was administered i.p. on days −1, +1, +3, +5, and +7 relative to virus exposure.
[a] Scores of 0–4 assigned according to % visible consolidation. Samples taken on day +6.
[b] Arterial oxygen saturation as determined by pulse oximeter. Day +10 data.
[c] $P < 0.05$, compared to CMC-treated controls.
[d] $P < 0.01$, compared to CMC-treated controls.

3.1.3. PTV experiment

The lethal hepatic disease induced by PTV was not significantly inhibited by BCH-527 therapy. In the experiment, a 75% death rate occurred in the placebo-control animals, with a mean day to death of 5.7 days. Infected mice treated with BCH-527 all died, with increases in mean day to death of only 0.5–0.7 days seen. No reductions in hepatic icterus score, ALT, AST, liver or serum virus titers occurred. Ribavirin treatment resulted in 80% of the animals surviving and significant decreases in all other disease parameters (data not shown).

3.2. Immunologic studies

Treatment with the 200 mg/kg dose of BCH-527 resulted in a highly significant inhibition of CTL activity on infection day 8 in the IV-A-infected mice (Table 3). A moderate stimulation of CTL was seen in mice receiving the 100 mg/kg dose of BCH-527. This increase was seen at all effector : target cell ratios, but was significant only at the 7.5 : 1 ratio. A slight, non-significant, CTL increase was also seen in mice

Table 3
Effect of BCH-527 treatment on cytotoxic T-cell activity in IV-A-infected BALB/c mice

Treatment group	Dosage (mg/kg/inj.)	% Specific lysis ± S.D. at effector : target ratios of:			
		7.5:1	15:1	30:1	60:1
BCH-527	200	0.2±0.1 [a]	1.3±0.6 [a]	6.8±1.9 [a]	18.0±2.2 [a]
	100	8.5±2.4 [a]	17.4±2.7	29.1±3.1	43.5±3.5
	50	4.6±1.7	13.1±1.9	24.2±2.1	37.9±2.4
CMC	–	3.2±4.0	14.1±1.2	22.4±5.1	35.8±5.8

Note: BCH-527 treatment was administered i.p. on days −1, +1, +3, and +5 relative to virus exposure, with splenocytes assayed 24 h after final injection.
[a] $P < 0.01$, compared to CMC-treated controls.

Table 4
Effect of i.p. BCH-527 treatment on natural killer cell activity in IV-A-infected and uninfected BALB/c mice

Treatment group	Dosage (mg/kg/inj.)	Mean ^{51}Cr release ± S.D. at effector : target ratios of:			
		12 : 1	25 : 1	50 : 1	100 : 1
Uninfected, 24 h postinjection 1					
BCH-527	200	20.4 ± 2.4	17.3 ± 1.4	11.3 ± 0.6	6.6 ± 0.6
	100	20.0 ± 2.0	16.6 ± 1.9	10.6 ± 1.8	5.9 ± 1.6
	50	21.2 ± 2.9	17.6 ± 1.1	10.8 ± 1.3	6.1 ± 1.2
CMC	–	21.5 ± 3.8	18.3 ± 0.6	12.0 ± 1.4	7.2 ± 1.3
Uninfected, 24 h postinjection 4					
BCH-527	200	18.2 ± 1.0 [a]	12.8 ± 0.7 [b]	8.6 ± 0.6 [a]	1.1 ± 0.5 [b]
	100	22.3 ± 2.0	16.4 ± 1.8	11.8 ± 1.7	3.6 ± 1.4
	50	23.3 ± 1.0 [b]	17.1 ± 0.6 [b]	12.7 ± 0.4 [b]	4.3 ± 0.3 [b]
CMC	–	20.1 ± 1.1	14.5 ± 0.5	9.8 ± 0.6	2.2 ± 0.4
Infected, 24 h postinjection 4					
BCH-527	200	17.6 ± 1.1	12.9 ± 0.9	9.6 ± 0.9	7.3 ± 0.3 [b]
	100	26.5 ± 1.6 [b]	20.8 ± 1.4 [b]	16.9 ± 1.3 [b]	12.1 ± 1.2 [b]
	50	23.9 ± 1.1 [b]	18.5 ± 1.0 [b]	14.8 ± 0.9 [b]	10.3 ± 0.8 [b]
CMC	–	16.6 ± 2.3	13.1 ± 2.1	7.5 ± 1.8	3.2 ± 1.6

Note: Treatment with BCH-527 was every other day; in infected mice, it was on days −1, +1, +3, and +5 relative to virus exposure.
[a] $P < 0.05$, compared to CMC-treated controls.
[b] $P < 0.01$, compared to CMC-treated controls.

receiving the 50 mg/kg/dose, which was apparent in 3 of 4 effector : target cell ratios. The utilization of P815 target cells, which are insensitive to NK cell activity, would indicate the observed activity was due to CD8–CTL effects.

NK cell activity was monitored in uninfected animals after a single injection of BCH-527 and again after 4 injections (Table 4). Also shown in the table are data from IV-A-infected mice treated with 4 injections of the compound. The single treatment of uninfected mice did not stimulate NK cell activity at any dosage; indeed, mild decreases of up to 18% were seen. Multiple treatments of the uninfected animals continued to be suppressive at the highest dosage used (200 mg/kg), but significant stimulation occurred at the lower dosages used. In uninfected C57BL/6 mice, similar multiple treatments using 50 mg/kg of BHC-527 resulted in NK cell stimulation of 23–63% (data not shown). In the infected animals, however, marked stimulation of NK cell activity was seen at both 50 and 100 mg/kg dosages of BCH-527; the 200 mg/kg dosage was particularly stimulatory at the 50 : 1 and 100 : 1 effector : target cell ratios.

Macrophage activation data are summarized in Table 5. The same pattern of response seen with NK cell activation also occurred in the macrophage activation experiment, with a single BCH-527 injection of uninfected mice being ineffective or, at the 200 mg/kg dose, somewhat inhibitory, whereas multiple treatments in uninfected mice were moderately stimulating and in IV-A-infected mice strongly enhancing to macrophage activation as measured by IL-1 levels in the animal. In a similar experiment run in uninfected C57BL/6 mice using only 50 mg/kg of BCH-527, an 18% activation of macrophages occurred (data not shown).

Table 5
Effect of i.p. BCH-527 treatment on macrophage activation in IV-A-infected and uninfected BALB/c mice

Treatment group	Dosage (mg/kg/inj.)	IL-1 concentration ± S.D. (mg/ml)		
		Uninfected, 24 h postinjection (1)	Uninfected, 24 h postinjection (4)	Infected, 24 h postinjection (4)
BCH-527	200	16.6 ± 3.7 [b]	31.4 ± 3.4 [a]	29.1 ± 7.4 [b]
	100	32.6 ± 2.7	40.7 ± 8.2 [b]	179.3 ± 27.0 [b]
	50	34.5 ± 3.0	21.2 ± 3.3 [a]	46.4 ± 6.4 [b]
CMC	–	33.9 ± 5.0	26.5 ± 5.1	9.1 ± 1.6

Note: Treatment with BCH-527 was every other day; in infected mice, it was on days −1, +1, +3 and +5 relative to virus exposure.
[a] $P < 0.05$, compared to CMC-treated controls.
[b] $P < 0.01$, compared to CMC-treated controls.

Effects of BCH-527 on total splenic B- and T-cells were determined only in C57BL/6 mice using 50 mg/kg of the compound. One day after the fourth every-other day treatment, flow cytometric analysis indicated a 20% decrease in total T-cells and a 16% increase in B-cells (data not shown).

4. Discussion

These experiments indicate the immunomodulating agent BCH-527 has a moderate antiviral activity against MCMV infections, weak activity against IV-A infections and no apparent effect on PTV infections utilizing an every-other-day treatment regimen. It is possible that other treatment schedules for administering the compound would have yielded different results since the rate of stimulation, time of maximal stimulation, and time for the immune response to return to normal have not yet been fully defined. The related material, MDP, was reported to be quite inhibitory to influenza virus infections in mice, but only after a 3-week pretreatment (Masihi et al., 1983).

BCH-527 appeared reasonably well tolerated by the mice in these studies as determined by an absence of death, lack of increased ALT and AST, and general healthy appearance of the toxicity controls, although at doses of 100 and 200 mg/kg some weight loss did occur in these animals. Autopsy of a separate group of outbred mice 1 week after i.p. injection of the two highest doses of BCH-527 revealed that the weight loss may have arisen from irritation by BCH-527. Although little or no BCH-527 remained in the peritoneal cavity, some peritoneal lesions were observed in the abdominal cavity which were consistent with irritation and process of repair. Also, it was noted that the 200 mg/kg dose was sometimes immunosuppressive. However, suppression of the immune response at doses higher than observed for stimulation is typical of immunomodulating substances; e.g. the 'paradoxical' region of the dose–response curve (Spreafico, 1985).

The design of BCH-527 hypothesized that an important part of the immunomodulatory 'signal' present in MDP resides in the dipeptide portion of the molecule. It is possible that one function of the muramic acid is to anchor, and protect, the active

conformation of the dipeptide. This is supported by NMR studies (Fermandjian et al., 1987) which suggest the active conformation mimics a β-turn, facilitated by the alternating (D, L) stereochemistry. Synthesis and immunological evaluation of the octadecyl esters of the dipeptide portion of MDP, L-alanyl D-isoglutamine, as well as the stereoisomer of BCH-527, L-alanyl D-glutamine, did not reveal immune cell subset activation comparable to BCH-527. The reverse chiralty of BCH-527 may result in a bend of unique conformation and properties. Oligopeptides composed of D-alanyl L-glutamine have recently been reported to have a propensity for a bend in that they can spontaneously assemble into nanotubes (Ghadiri et al., 1993). With regard to the introduction of lipophilicity into BCH-527, the octadecyl chain was selected because of the properties it imparts to the dipeptide, as evidenced by the lipophilic immunoadjuvant octadecyl tyrosine (ST). ST has been well described in the literature (Penney et al., 1993) as an inert carrier of immunological activation signals provided by vaccine antigens, allergens, etc.

A number of immunomodulating substances have been reported to exert significant MCMV disease-inhibitory effects. These include polyriboinosinic-cytidylic acid (poly IC) (Kern et al., 1978), poly IC complexes with lysine (poly ICLC) (Kern et al., 1978; Kunder et al., 1993a–c), bropirimine (Brideau and Wolcott, 1985), maleic anhydride divinyl ether (MVE-2, Kunder et al., 1993b,c), CL 246,738 (Kunder et al., 1993a,c), 7-thia-8-oxoguanosine (Smee et al., 1990), ImuVert (Sidwell et al., 1992), and recombinant murine interferon (IFN)-γ and IFN-α-B/D (Kunder et al., 1993c).

A review of the known immunomodulatory effects of these compounds reveals no obvious 'common thread' of immunologic effect which could be used as a target for developing anti-CMV therapies, although we are gaining a better understanding of what is required. Stimulation of NK cells and activation of cytotoxic T-cells appear to play a role based on reports that NK cells are important for the control of acute MCMV in mice (Bancroft et al., 1981; Ebihara and Minamishima, 1984) and, together with cytotoxic T-cells, aid in human recovery from CMV infections (Quinnan et al., 1982). However, recent published studies indicate NK cells are not always required for immunomodulator-induced protection against MCMV infections (Kunder et al., 1993c). In the latter report, NK cells in the host were depleted using anti-NK antibody and it was shown that high doses of IFN still rendered a protective effect. However, at lower doses, the activity of the IFN did appear dependent on NK cells. Poly ICLC, which is considered to act primarily as an IFN inducer (Levy et al., 1975), also restored the NK cell activity made deficient by MCMV infection in SCID mice (Kunder et al., 1993b). This latter study suggested that IFN induction coupled with subsequent NK cell enhancement appear to be potent mechanisms for consideration in selecting immunomodulators for use against MCMV infections in T- and B-cell deficient hosts. We have previously shown that the MCMV disease-inhibitory effects of ImuVert were the same in both normal animals and mice treated with anti-NK cell antibody, indicating that this immunomodulator was acting by a mechanism other than NK cell activation (Sidwell et al., 1993).

It is known that a primary immunological effect of bropirimine, CL 246,738, poly ICLC, and 7-thia-8-oxoguanosine is strong induction of IFN. How IFN brings about protection in the MCMV-infected host is still unclear; a direct antiviral effect may occur,

or the material may act indirectly through activation of effector cells such as macrophages and/or NK cells. MVE-2, shown to have significant MCMV disease inhibitory effects (Kunder et al., 1993b), is primarily known to be a macrophage inhibitor. It is a weak IFN inducer (Sidwell et al., 1994). It is pertinent to note that we observed BCH-527 to also induce macrophage activity in the present study.

Less is known concerning the effects of immunomodulators on influenza virus infections. Some immunomodulatory substances reported to be inhibitory to experimentally induced influenza virus infections are lentinan (Irinoda et al., 1992), MVE-2 (Carrano et al., 1984), tilorone (Megel and Gibson, 1984), and methionine-enkephalin (Sidwell et al., Abstract, 34th ICAAC, 1994). The effects of both lentinan and MVE-2 are linked to macrophage activation (Carrano et al., 1984; Irinoda et al., 1992), an activity also seen with BCH-527. The PTV, a *phlebovirus* in the Bunyaviridae family of viruses, is particularly sensitive to IFN; we have previously shown a wide variety of IFN stimulators as well as human recombinant IFN-α-A/D to markedly inhibit the hepatotropic disease induced by this virus. BCH-527 did not induce IFN in mouse L929 cells by a previously described (Sidwell and Huffman, 1971) method (data not shown).

Three strains of mice were used in these studies; positive anti-MCMV effects were observed in both BALB/c and Swiss Webster mice. The C57BL/6 mice used in the PTV study were shown to respond to the BCH-527 immunostimulation in a manner similar to the BALB/c animals. We conclude that all mouse strains used responded similarly to the immune modulatory effects of this compound.

Since BCH-527 did not bring about total cures in the antiviral studies performed to date, its value may be greatest if the material is used in combination with substances having a more direct antiviral effect.

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