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4,6-Dibenzamidopyrazolo[3,4-*d*]pyrimidine is a highly selective inhibitor of cytomegalovirus adsorption to cells

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Summary

The heterocycle, 4,6-dibenzamidopyrazolo[3,4-*d*]pyrimidine (DBAPP), inhibited cytopathology induced by human, mouse, and vervet monkey cytomegaloviruses (CMV) in vitro at 0.2 to 0.5 μM, but did not inhibit cell replication at ≤30 μM. Herpes simplex viruses were unaffected by the inhibitor. The antiviral agent ganciclovir was effective against these CMVs at 3–10 μM in parallel assays. DBAPP and ganciclovir were synergistic inhibitors when used in combination. The heterocycle was only active if applied to cells before virus replication, indicating that it inhibited virus adsorption. Cells pre-treated 1 h with 30 μM DBAPP, then extensively rinsed, were resistant to infection by mouse CMV even 3 days after removal of the inhibitor. Human and monkey CMVs were able to infect cells and replicate within 24 h of drug removal. When virus and DBAPP were combined together then dialyzed to remove the compound, mouse CMV infectivity was decreased 1.7 logs, whereas human CMV and monkey CMV infectivity titers were relatively unaffected. Treatment of mice with DBAPP twice a day for 7 days starting 6 h after mouse CMV inoculation caused a moderate increase in number of survivors at 30 mg/kg. Cell to cell spread of the virus may account for poor efficacy of the compound when added after virus infection. DBAPP may serve as a tool to explore aspects of CMV adsorption or to characterize the cellular component of the CMV receptor.

Human CMV; Mouse CMV; Monkey CMV; CMV receptor

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Introduction

The incidence of cytomegalovirus (CMV) infections has increased dramatically as a consequence of the acquired immune deficiency syndrome (AIDS) epidemic (Macher et al., 1983). At present, ganciclovir is the only compound that may have efficacy against CMV disease in AIDS patients (Laskin et al., 1987), and clinical trials are still in progress. Other acyclic nucleosides or nucleotides of guanine and cytosine also are active against human CMV in cell culture (Beauchamp et al., 1988; Duke et al., 1986; Snoeck et al., 1988) and in mice (Duke et al., 1986). From our own laboratory we recently reported various 2′-deoxyribosyl and arabinosyl analogs of sangivamycin that inhibited CMV, but also were cell-inhibitory at low concentrations (Smee et al., 1988). All of the above mentioned antiviral compounds probably inhibit the CMV-specific DNA polymerase, as was documented for ganciclovir (Duke et al., 1986; Mar et al., 1985). Other investigators have reported a different class of CMV inhibitors, sea algae extracts (Richards et al., 1978) and sulfated polysaccharides (Baba et al., 1988), that are active by virtue of blocking virus adsorption to cells.

Ganciclovir may be limited in its clinical use because it causes neutropenia in a large percentage of patients (Laskin et al., 1987). Also, ganciclovir-resistant strains of human CMV have been isolated from AIDS patients who were refractory to drug treatment (Erice et al., 1989). Because of these findings, the need to discover other therapies against CMV diseases is apparent. In our antiviral screening program, we identified a unique low molecular weight inhibitor of CMV replication, 4,6-dibenzamidopyrazolo[3,4-*d*]pyrimidine (DBAPP, Fig. 1), which is neither a nucleoside analog nor a polysaccharide, although the de-benzylated form of the heterocycle when ribosylated is a nucleoside analog. The effects of DBAPP on different strains of CMV, its mode of action, and its activity against mouse CMV in vivo are communicated here.

Materials and Methods

Antiviral compounds

Ganciclovir was generously provided by Syntex Research, Palo Alto, CA. 4,6-Dibenzamidopyrazolo[3,4-*d*]pyrimidine (DBAPP) was synthesized in our labora-

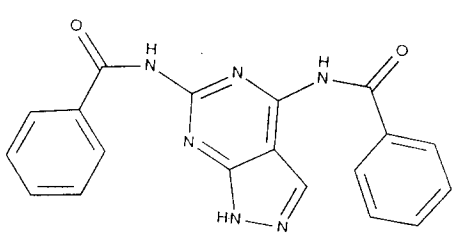

Fig. 1. Structure of 4,6-dibenzamidopyrazolo[3,4-*d*]pyrimidine (DBAPP).

tory by a published procedure (Davoll and Kerridge, 1961). Because the solubility of DBAPP was low, it was made up as an 8 mM solution in 100% DMSO. It was then rapidly diluted to 100 μM or less in Eagle's medium. For biological assays, drug-free controls were used containing equivalent amounts of DMSO. Ganciclovir was soluble in cell culture medium at 4 mM.

Cells and viruses

Continuously passaged African green monkey (Vero), human embryonic lung (MRC-5) and mouse mammary tumor (C127I) cells were bought from the American Type Culture Collection (ATCC), Rockville, MD. The cells were maintained in Eagle's medium plus 10% FBS. Serum concentrations were reduced to 2% for experiments with confluent monolayers, and 50 μg gentamicin/ml was added to maintain sterility. The cell stocks were mycoplasma-free as determined by periodic testing using the Gen-Probe mycoplasma detection kit (Fisher Scientific Co.).

Herpes simplex virus type 1 (HSV-1) (KOS strain) was obtained from James North, Brigham Young University, Provo, UT. Herpes simplex virus type 2 (HSV-2) (MS strain), human cytomegalovirus (HCMV) (AD-169, Davis and Towne strains), mouse cytomegalovirus (MCMV) (Smith strain), and vervet monkey cytomegalovirus (VCMV) (CSG strain) were obtained from ATCC. For virus assays in culture, the herpes viruses were propagated in Vero cells (Smee et al., 1983), strains of HCMV and VCMV in MRC-5 cells (Freitas et al., 1985), and MCMV in C127I cells (Smee et al., 1989).

Antiviral and cytotoxicity assays

DBAPP was evaluated in 24-well microplates by cytopathic effect (CPE) inhibition assays (Smee et al., 1987) to determine its inhibitory activity against cytomegalo- and herpes viruses. Half-$\log_{10}$ dilutions of each compound were evaluated from 0.01 to 30 μM. The plates were evaluated when 100% virus-induced CPE was achieved, which was generally in 3 days for herpes viruses, 4–5 days for MCMV and VCMV, and 7–8 days for the strains of HCMV. Because of the length of time for assay of HCMV, the culture medium containing test compounds was replaced at 4 days. For each experiment, concentrations inhibiting CPE by 50% (ED_{50}) were determined to the nearest half-log. ED_{50} values reported in Table 1 were averages from 3–5 experiments.

Combination chemotherapy using DBAPP and ganciclovir was performed using half-log concentrations of each compound alone or in combination against HCMV, using the CPE inhibition methods described above. The concentrations selected were varied at or below the ED_{50} of each inhibitor. Determinations of additive or synergistic drug interactions were made by isobologram plotting after determining fractional inhibitory concentrations (FIC), as was described by Allen et al. (1982).

Virus plaque reduction assays were conducted according to a previous method (Freitas et al., 1985). Briefly, virus was adsorbed for 1.25 h, then medium containing 0.5% SeaPlaque Agarose (FMC Corp., Rockland, ME), was applied for

TABLE 1

Antiviral activity of DBAPP[a] against herpes simplex and cytomegaloviruses

Virus (strain)	Cell	50% Effective dose (μM)	
		Pre-treated[b]	Post-treated[c]
HSV-1 (KOS)	Vero	>30	>30
HSV-2 (MS)	Vero	>30	>30
HCMV (AD-169)	MRC-5	0.2	>30
HCMV (Davis)	MRC-5	0.5	>30
HCMV (Towne)	MRC-5	0.2	>30
MCMV (Smith)	C127I	0.3	30
VCMV (CSG)	MRC-5	0.3	>30

[a]Determined by cytopathic effect inhibition assays.
[b]DBAPP added to cells 15 min prior to virus, and was left on throughout the assay.
[c]Virus was adsorbed 1 h to cells, then the medium was removed and replaced with fresh medium containing DBAPP at various concentrations. The inhibitor was present throughout the assay.

4–5 days. Plates of MCMV and VCMV were fixed with 10% buffered formalin, stained with 1% crystal violet in 20% ethanol (after removing the agar overlay), and washed. HCMV plaques took longer to develop so were overlayed a second time with agar at 4 days and were incubated at 37°C for 3 more days. Then these plaques were overlayed with 0.002% (final concentration) neutral red dye for another day. Plaques were counted at 17.5X using a plaque viewer (Bellco Glass Co., Vineland, NJ).

The above plaque assay methods were used to explore aspects of the mode of action of the compound. DBAPP was applied to cells for 1 h, cells were extensively rinsed 6×, then virus added at different times after the final rinse. In another set of experiments undiluted stock viruses were combined with compound or DMSO control in medium containing 2% serum. After 5 min the 1 ml samples were placed in dialysis tubing and dialyzed 2 h in 1 l of 20 mM phosphate buffered saline, pH 7.4, at 4°C. This treatment plus dilution of the virus for plaque assays effectively dropped the concentration of DBAPP in these virus preparations by 10000-fold.

DBAPP was evaluated for its ability to inhibit cell proliferation of subconfluent monolayers. Twenty-four-well microplates were seeded with 5×10^3 cells/well and were fed medium containing 10% FBS and 50 μg gentamicin/ml. Five half $\log_{10}$ dilutions of compound were added to each well 1 day later. The cells were incubated 4–5 days at 37°C in 5% CO_2 until monolayers were approaching confluency. Then, the medium was replaced with MTT [3-(4,4-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (ICN Biochemicals, Cleveland, OH) at 4 mg/ml in culture medium. After 2 h incubation at 37°C, the medium was replaced with isopropanol containing 0.04 M HCl to elute out the formazen product. The medium at each inhibitor concentration was transferred to a 96-well plate so that absorbance (at 580 nm) could be read with an ELISA plate reader. The absorbance was proportional to the number of cells in each well. The absorbance was converted to percent of control values, which were plotted on semilog paper to estimate fifty

percent inhibitory concentrations (ID_{50}). These methods using MTT have been described previously (Smee et al., 1989).

Animal experiments

Swiss Webster female mice (Charles River Labs, Wilmington, MA) weighing 9–11 g each were inoculated intraperitoneally (i.p.) with 10 50% lethal doses of mouse-passaged MCMV (Freitas et al., 1985). Mice so infected die within 5–6 days of systemic infection, with the liver being the major target organ affected that leads to death (Overall et al., 1976). DBAPP was prepared as a sonicated suspension in saline, and was inoculated i.p. once into mice 6 hours after virus inoculation, then twice a day thereafter (at 7 a.m. and 4 p.m.) for 4 more days.

Results

The antiviral activity of DBAPP was determined in vitro against herpes simplex and various types of cytomegaloviruses (Table 1) using a cytopathic effect (CPE) inhibition assay. The herpes simplex viruses were unaffected by the compound, whereas 50% inhibition of human, monkey and mouse CMVs occurred at 0.2–0.5 μM concentrations. For comparative purposes the antiviral agent ganciclovir inhibited these CMVs in parallel assays at 3–10 μM. These results with DBAPP could only be achieved if the compound was added before virus adsorption, however. Delaying treatment until immediately after virus adsorption resulted in a loss of activity. DBAPP was a highly selective antiviral substance causing no impairment of MRC-5 and C127I cell proliferation at ≤30 μM.

Because DBAPP was only active if applied before virus exposure, it was sur-

TABLE 2

Inability of cytomegaloviruses to plaque titrate following a 1 h exposure of DBAPP to cells[a]

Hours after DBAPP treatment	Percent of control PFU[b]		
	MCMV	VCMV	HCMV[c]
2	0	100[d]	0
4	0	100	0
8	0	100	5
12	0	100	39
24	0	100	67
48	0	100	100
72	0	100	100

[a]After exposure, cells were rinsed and aspirated 6 times with drug-free culture medium. They were incubated until the indicated times of virus infection.

[b]PFU, plaque forming units. Assays were conducted in C127I (MCMV) or MRC-5 cells (VCMV and HCMV). Data are from a single representative experiment. Each virus was analyzed twice on separate occasions.

[c]AD-169 strain of virus.

[d]Values of 100 are ± 10% of untreated control.

mised that its mode of action involved blocking virus adsorption. Several studies were performed to explore whether the effect of the compound was on the virus, on the cells, or both. In a follow-up to studies presented in Table 1, we applied DBAPP to cells for 1 hour, extensively rinsed it off, then added virus. Surprisingly, the same degrees of inhibition of human, monkey, and mouse CMVs were achieved as those presented in Table 1, suggesting that DBAPP interacted with the cells to block virus adsorption. Using plaque reduction methods instead of the CPE inhibition assay, we determined that a 5 min exposure of DBAPP to cells (followed by extensive cell rinses) reduced HCMV (AD-169 strain), VCMV, and MCMV plaque numbers by 50% at 1, 1, and 0.5 μM respectively compared to untreated controls. Later an experiment was performed to determine how long DBAPP-treated cells were resistant to virus infection. For this study, cells were exposed to 30 μM DBAPP for 1 h and the cells were thoroughly rinsed. The CMVs were added at various times after removal of the compound, and cells were overlayed with agar. This treatment completely blocked the infectivity of MCMV when virus was applied up to 3 days after removal of the inhibitor (Table 2). In contrast, by 24 h the effects of DBAPP had mostly worn off allowing HCMV to plaque. VCMV efficiently plaqued when virus was adsorbed two hours after DBAPP removal.

To determine whether DBAPP had virus-inactivating properties, the compound was combined with each type of CMV, then the inhibitor was removed by dialysis. The results in Table 3 show a differential effect depending upon the virus. HCMV and VCMV were largely unaffected by extracellular DBAPP treatment, whereas MCMV dropped 1.7 logs in titer. Similar results with MCMV were achieved in a subsequent experiment.

Since DBAPP appears to have a different mode of action than ganciclovir, combination chemotherapy experiments were conducted in cell culture to verify this point. A synergistic antiviral effect can be anticipated when two compounds operate by different modes of action. In this experiment presented as an isobologram plot (Fig. 2), additive drug interactions occur near or slightly to the left of the dotted line of the figure. The shape of the hyperbola indicates a marked synergistic antiviral interaction between the two compounds. The fractional inhibitory concentration (FIC) values are less than 0.5, which are mathematical indications of synergy (Allen et al., 1982).

TABLE 3

Virus-inactivating properties of DBAPP against cytomegaloviruses

DBAPP Conc. (μM) before dialysis[a]	Plaque forming units/ml[a]		
	HCMV	VCMV	MCMV
0	6.2	5.5	5.4
30	6.0	4.9	3.7

[a]Undiluted virus and DBAPP/DMSO or DMSO were combined for 5 minutes, then were subjected to 2 h of dialysis prior to plaque assay. HCMV and VCMV were plaque titrated in MRC-5 cells. MCMV was titrated in C127I cells. Values represent individual samples titrated in triplicate.

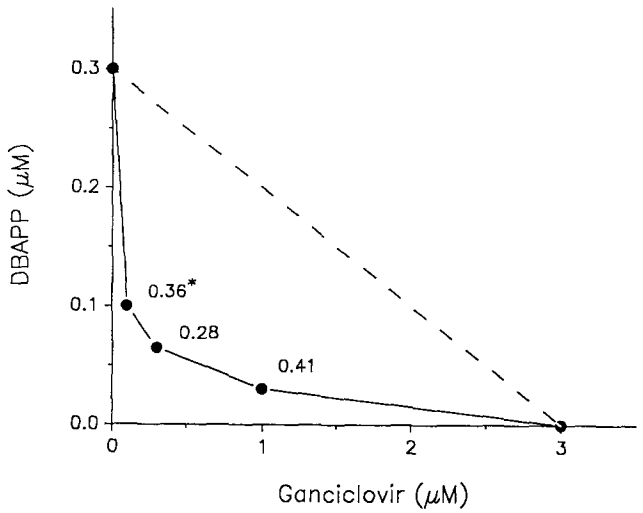

Fig. 2. Synergistic antiviral activity of DBAPP combined with ganciclovir against HCMV (AD-169 strain); *fractional inhibitory concentration (FIC) values.

In a final experiment, the activity of DBAPP was determined against a MCMV infection in weanling mice (Table 4). The mice received their first dose of compound 6 hours after virus adsorption to eliminate the possibility of inactivating input virus prior to infection. A moderate effect may have been achieved at 30 mg/kg, possible toxicity may have occurred at 100 mg/kg, and no activity was evident at 10 mg/kg.

Discussion

We have identified a potent inhibitor of cytomegaloviruses that appears to act by virtue of inhibiting virus adsorption. DBAPP can accomplish this by direct in-

TABLE 4

Effects of DBAPP on a murine cytomegalovirus infection in weanling mice

Dose[a] (mg/kg)	Survivors/total (%)	Mean survival time[b] (days)
0[c]	6/16 (38)	5.6 ± 0.5[d]
10	7/16 (44)	5.9 ± 1.2
30	12/16 (75)	7.0 ± 2.2
100	4/16 (25)	6.1 ± 0.7

[a]A half-daily intraperitoneal (i.p.) dose was administered 6 h after i.p. virus inoculation. Then half-daily doses were administered twice a day for 4 days starting 24 h after virus challenge.
[b]Of mice that died. Survivors lived through 21 days.
[c]Saline served as the placebo and as diluent for DBAPP.
[d]Standard deviation.

teraction with the virus (MCMV specifically), or by interaction with the cell surface (affecting human, monkey and mouse CMVs). The effect of a 1 h exposure on cells is long lasting for MCMV (up to at least 3 days) but less for HCMV and VCMV. This suggests that the heterocycle binds tightly to C127I cells but not as tightly to MRC-5 cells. It is surmised that DBAPP binds or interacts at or near the receptor required for CMV adsorption. Indirect evidence for this comes from the fact that the inhibitor is specifically active against CMVs and not other types of viruses. For example, DBAPP was inactive against herpes simplex viruses (Table 1), and many other enveloped (influenza A, parainfluenza type 3, Semliki Forest, and visna) and non-enveloped (adeno- and rhino-) viruses at $\leq$ 30 μM (unpublished results).

Several attempts were made to directly quantify the binding of MCMV to treated cells in order to differentiate between blocking of virus adsorption versus interference with internalization subsequent to receptor-virion complex formation. Using methods applicable to herpes simplex viruses for generating purified radioactive virions (WuDunn and Spear, 1989), we were unable to produce infectious virions with high enough specific radioactivity to develop a good assay. One problem was the tremendous loss (at least 2 logs) of infectivity titer associated with the purification method.

Combination studies with ganciclovir showed the two compounds were synergistic inhibitors of HCMV. Although the combination of the two inhibitors did not alter the morphology of uninfected stationary monolayers, an evaluation of possible increased toxicity to actively growing cells was not assessed. The antiviral effect of DBAPP primarily involves reducing input virus infectivity rather than exerting a biochemical effect inside the cell, whereas ganciclovir inhibits the intracellular CMV DNA polymerase (Duke et al., 1986).

Certain analogs of DBAPP have been synthesized and evaluated for antiviral activity against CMVs. DBAPP is a precursor of nucleoside analogs since the heterocycle differs from purine by the placement of one nitrogen in the imidazole ring. The de-benzylated diaminopyrazolopyrimidine heterocycle does not show anti-CMV activity, nor does the nucleoside analog 4,6-diaminopyrazolo-[3,4*d*]pyrimidine riboside or its 2′,3′,5′-triacetylated riboside form (unpublished results).

The unusual activity of DBAPP to specifically block CMV adsorption makes it different from sea algae extracts (Richards et al., 1978) and sulfated polysaccharides (Baba et al., 1988) that not only inhibit CMVs, but are inhibitory to a broad spectrum of enveloped viruses. In unpublished work one of us (DFS) performed with partially purified polysaccharides from *Constantinea simplex* sea algae extracts at another institution, the effect of these substances on cells was fully reversible (i.e., viruses would readily plaque when added immediately after cells were rinsed free of compound). DBAPP has some degree of persistent association with cells, especially C127I cells. Because of this property, the compound may be useful as a tool to explore aspects of the CMV adsorption process or to characterize the cellular component of the CMV receptor.

The results of animal studies show DBAPP to be a weak inhibitor of MCMV.

Extremely low water solubility could have adversely impacted on its efficacy in mice. Alternatively, DBAPP may have a poor metabolic disposition in vivo, but this has not been determined. Because the compound only shows antiviral activity in cell culture when added before virus adsorption (Table 1), once an infection is initiated the virus can spread from cell to cell escaping drug action. The degree to which cell to cell spread of MCMV contributes to pathogenesis relative to spread by viremia should be investigated. Overall and colleagues (1976) reported MCMV titers to be highest in the blood 1 day after virus inoculation, but on days 2 through 6 visceral organs had 2 to 3 logs more virus in them, suggesting that the viremic phase may not be a significant factor contributing to disease progression. Presumably DBAPP can only exert its effects in extracellular bodily fluids and would not affect virus replication in cells of organs and tissues.

Other inhibitors of CMV adsorption such as sea algaes were also found to be poorly active in animals against MCMV (Richards et al., 1978) and share the same property as DBAPP of only being active in cell culture when added before virus inoculation. Because of low in vivo activities of DBAPP and sea algae extracts, these data suggest that inhibitors which block virus adsorption are not viable candidates for antiviral therapy of CMV infections. Animal studies with sulfated polysaccharides such as dextran sulfate, pentosan polysulfate and carrageenans (Baba et al., 1988) should be conducted to verify this hypothesis.

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