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Regulatory effects of deoxyribonucleosides on the activity of 5-methoxymethyl-2′-deoxycytidine: modulation of antiherpes activity by deoxyguanosine and tetrahydrodeoxyuridine

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Summary

The effect of purine and pyrimidine deoxyribonucleosides on the activity of 5-methoxymethyl-2′-deoxycytidine (MMdCyd) against herpes simplex virus type 1 (HSV-1) was investigated. The antiviral activity of MMdCyd was decreased by deoxythymidine, deoxyuridine and deoxycytidine. Deoxyadenosine had no effect at concentrations up to 500 μM. In contrast, deoxyguanosine (dGuo) potentiated MMdCyd activity. The mean ED_{50} (1 5 μM) for the combination (MMdCyd plus 100 μM dGuo) was approximately 20-fold lower than that of MMdCyd (ED_{50} 26 μM) When tetrahydrodeoxyuridine (H_4dUrd, 540 μM) was added along with MMdCyd and dGuo, anti-HSV-1 activity of MMdCyd was further potentiated by 25-fold (ED_{50} 0.06 μM). The inhibition of virus replication, as determined by the plaque reduction assay, was further confirmed by virus yield studies and by parallel observations on virus-induced cytopathogenicity. The order of decreasing effectiveness for reducing the production of infectious virus particles (virus yield) by different treatments was: MMdCyd + dGuo + H_4dUrd > MMdCyd + dGuo > MMdCyd + H_4dUrd > MMdCyd > dGuo + H_4dUrd > dGuo > H_4dUrd

The effect of dGuo and dGuo in combination with H_4dUrd on deoxyribonucleoside triphosphate (dNTP) pools was determined in Vero cells infected with multiplicity of infection of 5 PFU/cell In the presence of 100 μM dGuo, there was approximately a 3-fold, 2-fold and 12-fold increase in dCTP, dTTP and dGTP pool

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sizes respectively, as compared to control (untreated) cells Treatment with H_4dUrd (1 06 mM) in combination with dGuo (100 μM), resulted in an increase of the dCTP pool and a marked fall in the dTTP and dGTP pool The possible mechanisms for potentiation of MMdCyd activity by dGuo and H_4dUrd are discussed

5-Methoxymethyl-2′-deoxycytidine, Tetrahydrodeoxyuridine (deaminase inhibitor), Deoxyguanosine potentiation; Antiherpes activity, dNTP pool, HSV-infected cell

Introduction

The antimetabolite, 5-methoxymethyl-2′-deoxycytidine (MMdCyd), is a selective antiherpes agent with low cytotoxicity (Aduma et al., 1990d; Gupta et al., 1989) The specificity of MMdCyd is more marked towards herpes simplex virus type 1 (HSV-1), and its antiviral activity is influenced by the cytidine/deoxycytidine (Cyd/dCyd) deaminase and deoxycytidylate (dCMP) deaminase activities of the cell lines used for antiviral assays (Aduma et al , 1990d, Gupta et al., 1989). When deamination is prevented, MMdCyd is a potent antiherpes agent MMdCyd requires metabolic activation by the pyrimidine nucleoside kinase of HSV as evidenced by its lack of activity against an HSV-1 mutant virus lacking thymidine kinase activity. In this respect, its behavior is similar to that of other nucleoside analogs with selective antiherpes activity (Chen et al., 1979, Cheng et al , 1981, Gupta et al, 1987) 5-Methoxymethyl-2′-deoxycytidine- 5′-monophosphate (MMdCMP) is likely processed by enzymes of the dCyd kinase pathway to its triphosphate, MMdCTP, which is the 'active' form of the drug responsible for its antiviral activity Studies using purified DNA polymerases provided support for this hypothesis (Aduma et al., 1990a).

The pyrimidine nucleoside kinase of HSV-1 has been shown to possess distinct thymidine and deoxycytidine phosphorylating activities which are regulated differently by metabolic end products (Jamieson and Subak-Sharpe, 1974; Jamieson et al., 1974; Leung et al, 1975) Studies were undertaken to examine the effects of naturally occurring purine and pyrimidine deoxyribonucleosides on the activity of MMdCyd These investigations are important from the point of view of understanding of regulation of MMdCyd metabolism in situ.

Materials and Methods

Cell cultures

Vero cells were grown in Eagle's Minimum Essential Medium (MEM) containing 10% fetal bovine serum (FBS) as previously described (Aduma et al., 1990b; Ayisi et al , 1980, Babiuk et al , 1975) Confluent monolayers were prepared by

seeding 5×10^4 cells into each well of a microtiter (96-well) plate or 2.5×10^5 cells into each well of a 12-well tissue culture plate. The cultures were incubated at 37°C in a humidified CO_2 (5%) atmosphere. Vero cells, extensively used in these studies, were checked at regular intervals for mycoplasma infections and were free from mycoplasma contamination. Tissue culture supplies were purchased from Flow Laboratories, McLean, Virginia

Viruses

Stocks of HSV-1 strains KOS and 76 were prepared and titrated as described previously (Aduma et al., 1990b; Ayisi et al., 1980). HSV-1 strain KOS was provided by Dr. Misra, and strain 76 was originally isolated from a human labial lesion by Dr. Babiuk, Veterinary Microbiology, University of Saskatchewan. Antibodies specific to HSV-1 were prepared according to published procedures (Ayisi et al., 1980)

Chemicals and biologicals

MMdCyd (Zongchao et al., 1990) was synthesized as described by Gupta et al. (1989). H_4dUrd was obtained from Raylo, Edmonton, Alberta. Deoxyribonucleosides (dCyd, dUrd, dThd, dAdo and dGuo) and deoxyribonucleoside-5′-triphosphates (dATP, dCTP, dGTP and dTTP) were purchased from Sigma Chemical Company, St. Louis, MO. Radiolabelled deoxynucleotides, [*methyl*-^{3}H]thymidine-5′-triphosphate ([^{3}H]dTTP, specific activity 46 Ci/mmol) and deoxy[5-^{3}H]cytidine-5′-triphosphate ([^{3}H]dCTP, specific activity 24 Ci/mmol) were obtained from Amersham Corporation, Oakville, Ontario.

Antiviral activity

Plaque reduction assay

The assay conditions were similar to those described earlier (Aduma et al., 1990b; Ayisi et al., 1980). Briefly, confluent monolayers were infected with either 50 or 100 plaque forming units (PFU) of virus per well and incubated at 37°C. After one hour the unadsorbed virus was removed by washing with MEM. Each compound at the appropriate concentration dissolved in maintenance medium (MEM containing 4% FBS) was added. HSV-1 antibody (1 or 2 neutralizing units) was also added to minimize the production of secondary plaques. Incubation was carried out at 37°C in a humidified 5% CO_2 atmosphere for 72 h. The plaques were stained and enumerated. In each experiment, toxicity controls (containing medium only) and virus controls (containing virus and medium only) were run simultaneously.

Effect of deoxyribonucleosides on MMdCyd activity The effect of pyrimidine-2′-deoxyribonucleosides (dCyd, dThd and dUrd) and purine-2′-deoxyribonucleosides (dAdo and dGuo) on the activity of MMdCyd was studied using the plaque reduc-

tion assay. Experimental protocols used were (1) each deoxyribonucleoside was added at a fixed concentration with increasing concentrations of MMdCyd (1 8–945 μM) to Vero cells infected with HSV-1, dose-response curves were constructed and the ED_{50} for MMdCyd was determined, (2) each deoxyribonucleoside at 0, 1, 10, 25, 50, 100 and 500 μM was added to a fixed amount of MMdCyd (30 μM), from dose-response curves, the ED_{50} of MMdCyd in the presence of different concentrations of each nucleoside were calculated, and (3) the effect of simultaneous addition of equimolar amounts of dCyd on MMdCyd activity was determined.

Effect of dGuo and dGuo + H_4dUrd on MMd Cyd activity Vero cell (monolayers) were infected with HSV-1 After 1 h the unadsorbed virus was removed by washing with MEM, H_4dUrd (540 μM)† was added and monolayers were incubated at 37°C for 2 h. The monolayers were then washed with MEM to remove residual amounts of H_4dUrd from the surface of the cells and MMdCyd plus dGuo (100 μM) were added In another series of experiments, after virus adsorption, monolayers were overlaid simultaneously with MMdCyd, 100 μM dGuo and 540 μM H_4dUrd. The concentration of MMdCyd varied from 0 04–40 μM The microtitre plates were incubated for 72 h and plaques were enumerated

Virus yield studies

Vero cell monolayers in 60-mm Petri dishes were infected with 0 001 PFU/cell (250 PFU) of HSV-1 strain KOS per dish. After adsorption for 1 h, the residual virus was removed, monolayers were washed with MEM and the cells were overlaid with compounds. Treatment schedules consisted of. dGuo, H_4dUrd, dGuo + H_4dUrd, MMdCyd, MMdCyd + dGuo, MMdCyd + H_4dUrd and MMdCyd + dGuo + H_4dUrd The final concentration of each compound was dGuo (100 μM), H_4dUrd (540 μM) and MMdCyd (30 μM) Antiserum was not included in the overlay The cell cultures were incubated at 37°C in a 5% CO_2 humidified atmosphere At periodic intervals (1, 12, 24, 48 and 72 h) post infection, the samples which had received similar treatment were pooled and titrated using Vero cells to determine the amount of virus present.

The ability of virus present intracellularly to resume replication after removal of drug was determined using the following procedure. Monolayers were washed with MEM, cells were overlaid with growth media and incubated for 72 h. Intracellular virus was harvested by two cycles of freezing (–70°C) and thawing (22°C) of cells, and the supernatant fluid from samples which had received similar treatment was pooled and the virus titer was determined In each experiment, cell controls (containing medium only), virus controls (containing virus and medium only), toxicity controls (containing test compound and medium only) and inhibitor controls

† The optimum concentration of inhibitor for cell culture experiments determined from dose response curves of antiherpes activity of MMdCyd in the presence of increasing amounts of H_4dUrd (Aduma et al , 1990d)

(infected cells overlaid with 540 μM H_4dUrd or 100 μM dGuo) were run simultaneously. Each experiment was repeated at least three times.

Cytotoxicity and cell viability

To ascertain that the enhanced potency of MMdCyd by dGuo was not due to toxic effects on cells, the effect of increasing concentrations of dGuo (25–1000 μM) and 100 μM dGuo in combination with H_4dUrd (1.06 mM) on cell viability was assessed by the trypan blue dye exclusion and the cell proliferation assays after the cells had been reseeded for 6 more days. Briefly, cells were seeded in 60-mm Petri dishes and allowed to grow to 100% confluency (18–24 h) Each compound at the appropriate concentration dissolved in maintenance medium (MEM plus 4% FBS) was added the next day. At this time, the cell density was determined by trypsin-versenizing and counting cells from one dish. Controls (cells plus media only) were run simultaneously After 72 h incubation, cells were examined for microscopic toxicity (increased granularity, vacuolation and destruction of monolayers), trypsinized, resuspended in fresh growth MEM, and an aliquot was counted using a model FN Coulter Counter (Coulter Electronics, Inc., Hialeah, FL, U.S.A.). Remaining cells were serially diluted, reseeded in new 60-mm Petri dishes and incubated again for four days At the end of this period, cells were trypsinized and counted. Cells in parallel dishes were stained with trypan blue and the number of colonies was determined. The cytotoxicity against rapidly dividing cells was determined in a similar manner except that each compound at appropriate concentrations was added within 10 min of seeding the cells

Effect of deoxyguanosine on deoxynucleotide pools

The effect of dGuo on deoxyribonucleoside triphosphate (dNTP) pools of uninfected and HSV- infected Vero cells was determined. HSV-1 strain KOS was used at multiplicity of infection of 5 PFU per cell. The amount of dGuo used varied from 25 to 1000 μM. The procedures for cell infection, extraction and enzymatic analysis of dNTP pools have been described (Aduma et al., 1990b).

Results

Regulatory effects of deoxyribonucleosides on MMdCyd activity

The results of studies with MMdCyd and dCyd added at equimolar concentrations to HSV-infected Vero cells are shown in Fig. 1. The antiviral activity of MMdCyd decreased as the concentration of dCyd increased in the medium. The effect of increasing concentrations of dThd, dUrd and dCyd on the antiviral activity of HSV-1 in Vero cells is shown in Fig. 2. Reversal of antiviral activity in the presence of exogenously added nucleosides, dThd, dUrd and dCyd, started to occur at 1, 10 and 25 μM respectively. The difference between dUrd and dCyd was

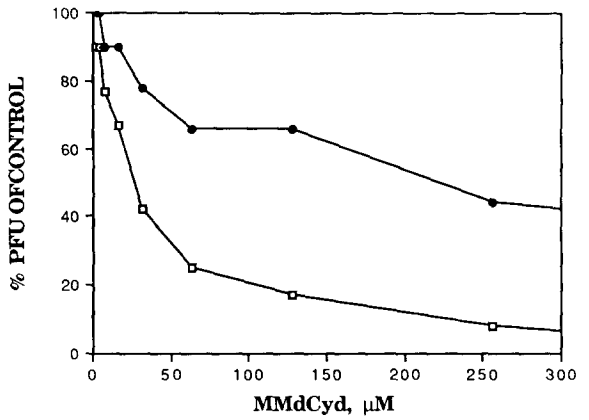

Fig 1 Effect of equimolar concentrations of deoxycytidine (dCyd) on the antiviral potency of MMdCyd ⊡—⊡, MMdCyd alone, ●—● MMdCyd + equimolar concentrations of dCyd Antiviral assays were carried out using Vero cells Cells were infected with HSV-1 (KOS strain), virus input was 50 PFU per well Values reported are means of four determinations

not statistically significant at higher concentration ($P < 0.05$) The effects of purine nucleosides on the antiviral activity of MMdCyd are shown in Fig. 3. Deoxyadenosine (up to 500 μM) had no effect on the activity of MMdCyd. Co-administration with dGuo also had no effect on the antiviral activity at 1 and 10 μM However, interestingly and unexpectedly, as dGuo concentration was increased to 25, 50 and 100 μM, there was a marked potentiation of MMdCyd activity The ED_{50} for the combination of MMdCyd and 100 μM dGuo was in the range of 1 1–2.2 μM. Therefore, additional combination chemotherapy experiments using dGuo, H_4dUrd

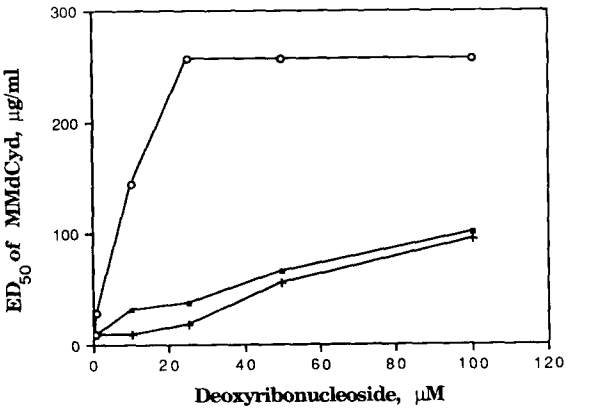

Fig 2 Reversal of the antiviral activity of MMdCyd by pyrimidine deoxyribonucleosides ○—○, MMdCyd + deoxythymidine (dThd), ■—■, MMdCyd + deoxyuridine (dUrd), +—+, MMdCyd + deoxycytidine (dCyd) MMdCyd 30 μM (8 5 μg/ml) was used in all experiments At this concentration, MMdCyd caused 50% reduction in plaque formation (ED_{50}) Antiviral assays were carried out using Vero cells Cells were infected with HSV-1 (KOS strain), virus input was 50 PFU per well The curves originate from the ED_{50} activity of MMdCyd in the absence of nucleosides Values reported are means of four determinations

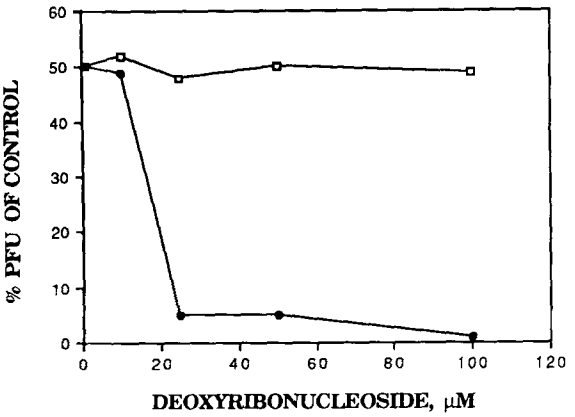

Fig 3 Effect of deoxyadenosine (dAdo) and deoxyguanosine (dGuo) on the antiviral potency of MMdCyd ⊡–⊡, MMdCyd + dAdo, ●—●, MMdCyd + dGuo Antiviral assays were carried out using Vero cells Cells were infected with HSV-1 (KOS strain), virus input was 50 PFU per well MMdCyd 30 μM (8 5 μg/ml) was used in all experiments At this concentration MMdCyd caused 50% reduction in plaque formation (ED_{50}) The curves originate from the ED_{50} activity of MMdCyd in the absence of nucleosides Values reported are means of four determinations

TABLE 1

Antiviral activity[a] and enhancement of the selectivity of MMdCyd by tetrahydrodeoxyuridine (H_4dUrd, 540 μM) and deoxyguanosine (dGuo, 100 μM)

Compound	ED_{50} (μM)[b]	MTC (μM)[c]	AI[d]
MMdCyd	26	> 3000	> 115
MMdCyd + H_4dUrd	1 5	> 3000	> 2 000
MMdCyd + dGuo	1 5	> 3000	> 2 000
MMdCyd + H_4dUrd + dGuo	0.06	> 3000	> 50,000

[a]Antiviral assays were carried out using Vero cells All compounds were added immediately after virus infection HSV-1 strains KOS and 76 were used Virus input was 50 or 100 plaque forming units (PFU) per well used

[b]ED_{50} = concentration required to reduce plaque formation by 50% Mean values of 12 determinations

[c]Minimum toxic concentration (MTC) required to produce definite evidence of microscopic toxicity on a monolayer of Vero cells

[d]Antiviral index (AI) was determined by dividing MTC by ED_{50}

(540 μM) and dGuo (100 μM) plus H_4dUrd (540 μM) with MMdCyd were carried out to determine the effect of different treatments on the potency of MMdCyd using the plaque reduction assay. Results, summarized in Table 1, indicate that dGuo and H_4dUrd strongly potentiate the anti-HSV-1 activity of MMdCyd: ED_{50}s were 19–39 μM (when used alone), 1.1–2.2 μM (with dGuo), 0 03–0.1 μM (with dGuo + H_4dUrd) and 0 12 μM (with dGuo after a 2-h pre-incubation with H_4dUrd). Consequently, there was a very marked improvement in the selectivity of MMdCyd as shown by an increase in the antiviral index. Both dGuo (up to 500 μM) and H_4dUrd (up to 2.12 mM) were devoid of antiviral activity. The antiviral activity of 5-methoxymethyl-2′-deoxyuridine (MMdUrd, corresponding deoxyuridine analog) was not enhanced in the presence of 1.06 mM H_4dUrd.

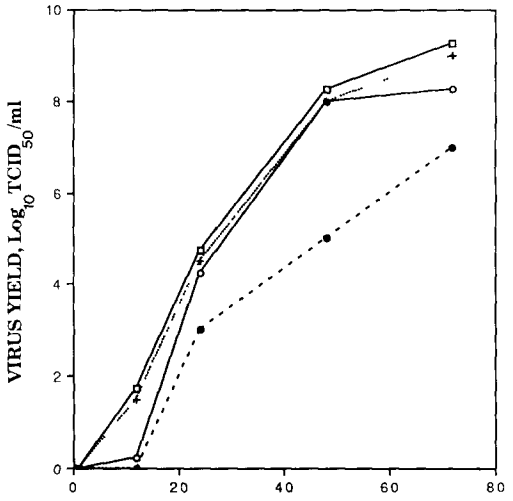

Fig 4 Effect of deoxyguanosine (dGuo), tetrahydrodeoxyuridine (H_4dUrd) and dGuo plus H_4dUrd on the production of infectious virus particles at different times of the infection process Total virus yield (supernatant + intracellular) control (untreated), ⊡—⊡, dGuo, o—o, H_4dUrd, + +, dGuo + H_4dUrd, ● ● Vero cells in 60-mm Petri dishes were infected with HSV-1 strain KOS Virus input was 0 001 PFU/cell (250 PFU/well) Concentration of each compound was dGuo (100 μM) and H_4dUrd (540 μM)

Virus yield studies

The production of infectious virus particles (virus yield) on a time-course basis post infection of HSV-infected Vero cells after different treatments is shown in Figs 4 and 5 At 12 h post infection, there was a noticeable difference in the production of infectious virus particles in untreated cells and HSV-infected Vero cells The virus yield (in log units) was 1 75 (untreated), 1 5 (H_4dUrd) and 0.25 (dGuo) During the same period no infectious virus particles were produced in Vero cells treated with MMdCyd, MMdCyd + dGuo, dGuo + H_4dUrd, and MMdCyd + H_4dUrd The combination MMdCyd + dGuo + H_4dUrd inhibited completely production of virus particles up to 24 h post infection. The order of decreasing effectiveness for the different treatments after 72 h was MMdCyd + dGuo + H_4dUrd > MMdCyd + dGuo > MMdCyd + H_4dUrd > MMdCyd > dGuo + H_4dUrd > dGuo > H_4dUrd > untreated controls These findings were consistent with parallel observations on virus-induced cytopathogenicity (CPE). In these experiments, virus CPE was markedly less pronounced at 12, 24, and 48 h in cells treated with dGuo + H_4dUrd as compared to MMdCyd, dGuo, H_4dUrd or controls. However, at 72 h virus CPE in the dGuo + H_4dUrd treated cells was approaching the control values indicating that somehow the blockage had been overcome.

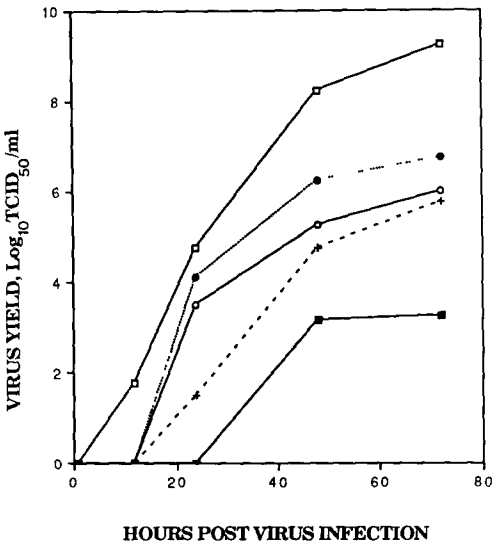

Fig 5 Effect of MMdCyd and MMdCyd in combination with deoxyguanosine (dGuo) and tetrahydrodeoxyuridine (H_4dUrd) on the production of infectious virus particles at different times of the infection process Total virus yield (supernatant + intracellular) control (untreated), ⊡—⊡, MMdCyd ● ●, MMdCyd + dGuo, + +, MMdCyd + H_4dUrd ○—○, MMdCyd + dGuo + H_4dUrd, ■—■ Vero cells in 60-mm Petri dishes were infected with HSV-1 strain KOS Virus input was 0 001 PFU/cell (250 PFU/well) Concentration of each compound was MMdCyd (30 μM), dGuo (100 μM) and H_4dUrd (540 μM)

Cytotoxicity

To ensure that the mechanism of potentiation by dGuo was not due to an inhibitory action on cell growth, the effects of various concentrations of dGuo on monolayers and rapidly growing Vero cells were examined. dGuo was devoid of cytotoxicity at concentrations up to 250 μM. However, at 500 μM and 1 mM dGuo, the percentage cell survival was 88% and 63% respectively as compared to controls. H_4dUrd was devoid of cytotoxicity at concentrations up to 2.12 mM (highest concentration tested). After exposure to H_4dUrd (1.06 mM) plus dGuo (100 μM), the cell viability was reduced to 87% as compared to untreated Vero cells. The minimum toxic concentration (MTC) of MMdCyd for monolayers and rapidly dividing Vero cells was greater than 3 mM (highest concentration tested).

Changes in dNTP pool sizes

As anticipated in uninfected cells, perturbation of dNTP pool sizes occurred on exposure to increasing dGuo concentration (Table 2). For example, at 100 μM dGuo, the levels of dCTP and dTTP pools were reduced; dATP pools were not affected and dGTP pools showed a 4-fold increase over controls. These effects were enhanced at higher concentrations of dGuo (500 μM). In HSV-infected Vero cells, treatment with 100 μM dGuo resulted in approximately a 3-fold, 2-fold and

TABLE 2

Effect of 2′-deoxyguanosine (dGuo) and H_4dUrd on deoxyribonucleoside triphosphate pools of uninfected and HSV-1-infected Vero cells

Treatment	Deoxyribonucleoside triphosphate (dNTP) (pmol/10^6 cells)[a]			
	dCTP	dTTP	dATP	dGTP
Uninfected Vero cells[b]				
Control	15	34	18	20
dGuo (μM)	–	–	–	–
25	13	17	18	42
50	9	17	19	88
100	11	13	19	76
250	8	13	19	102
500	7	6	19	146
HSV-infected Vero cells[c]				
Control	23	129	12	52
dGuo (μM)	–	–	–	–
25	23	110	12	290
50	33	118	12	373
100	64	214	12	600
250	78	233	14	558
500	60	247	25	738
dGuo (100 μM) + H_4dUrd (1 06 mM)	129	27	34	29

[a]Values are means of two duplicate determinations dNTP pools were determined 8 h post infection
[b]Vero cells were mock-infected for 1 h
[c]Vero cells were infected with HSV-1 strain KOS at an MOI of 5 PFU/cell for 1 h Treatment was given for 7 h

12-fold increase in dCTP, dTTP and dGTP pool size respectively, as compared to untreated cells; dATP pools remained unaffected Interestingly, exposure to higher concentrations of dGuo (up to 500 μM) had little additional effect on dCTP and dTTP pools High dCTP and dTTP pools indicate that reduction of CDP and UDP continued in the presence of high concentrations of dGTP.

When HSV-infected Vero cells were treated with a combination of dGuo and H_4dUrd, there was a marked rise in dCTP (129 pmol) and a dramatic fall in dTTP (27 pmol) pool sizes. There was also a very sharp decrease in the dGTP pool (29 pmol) as compared to dGuo treatment alone (dGTP 600 pmol) and an almost 3-fold expansion of the dATP pool (34 pmol)

Discussion

Nucleosides and their analogs penetrate cell membranes using a specific carrier system (Paterson et al., 1981) Thus the reversal of activity of MMdCyd by pyrimidine deoxyribonucleosides, at least in part, was due to competition with transport across the membrane. Our studies using dUrd and dihydrodeoxyuridine in relation to the antiviral activity of 5-bromovinyl-2′-deoxycytidine (BrVdCyd) and

MMdCyd lend support to this hypothesis (Aduma et al., 1990b,d). Bittlingmaier et al (1977) have shown that 5-bromodeoxyuridine (BrdUrd) and dThd uptake was significantly increased following infection with herpes viruses. Two types of uptake were identified: facilitated transport at low dThd concentration, and simple diffusion at high substrate concentration. In contrast, the increase in dCyd or dUrd uptake was only slight in comparison to that of dThd. Thus, variation in the uptake of dCyd, dUrd and dThd and the intracellular level achieved in HSV-infected Vero cells was likely a major reason for the differences observed in the ability of these nucleosides to reverse the activity of MMdCyd. Furthermore, deoxyribonucleosides (dCyd, dUrd and dThd) would compete with the phosphorylation of MMdCyd by the virus-induced pyrimidine nucleoside kinase, an enzyme whose activity is regulated in a complex manner by deoxyribonucleosides and deoxyribonucleoside triphosphates (Jamieson and Subak-Sharpe, 1974). For example, dThd competing with MMdCyd for phosphorylation would decrease the amount of MMdCMP formed In addition, dTTP arising in situ from dUTPase and DNAse activities along with dTTP formed by initial viral phosphorylation would inhibit phosphorylation of the analog allosterically Since dUrd serves as a precursor for dThd, its intracellular accumulation would cause reversal of activity in a similar manner by competing for phosphorylation and through its conversion to dTTP. Reversal of antiviral activity by dCyd was less than dThd or dUrd because dCyd was most likely deaminated to dUrd which then was responsible for reversing the activity of MMdCyd. Since the levels of deoxyribonucleosides may vary from tissue to tissue and various stressful conditions, the present study highlights the need to further understand how these changes affect the action of specific antiviral nucleosides. Equally important is knowledge of medium composition and cell types when evaluating antiviral potency of pyrimidine nucleoside kinase-activated compounds in vitro.

MMdCyd potency was significantly increased in the presence of 25 μM dGuo and maximal enhancement occurred at 100 μM dGuo. This finding is not only interesting, but also surprising. How can a natural constituent of cells potentiate the action of an antiviral compound? The combination of MMdCyd with H_4dUrd and dGuo was even more potent than MMdCyd + dGuo or MMdCyd +H_4dUrd. These results indicate that when MMdCyd is used in combination with H_4dUrd and dGuo, virus replication may be inhibited by interference at multiple sites. Studies on the yield of infectious virus particles and virus-induced cytopathogenicity substantiated the findings of the plaque reduction assays. The data on cytoxicity of dGuo shows that the potentiation of MMdCyd activity is not due to toxic effects on cells.

The mechanism of potentiation of MMdCyd activity by dGuo is an enigma. dGMP has been reported to be a potent inhibitor of dCMP deaminase (Mancini et al., 1983). Assuming that the potentiation of antiherpes activity by dGuo was due to inhibition of dCMP deaminase, then studies on dNTP pools of HSV-infected cells should provide some information on its mechanism of action. The dCTP, dTTP and dATP pools remained essentially the same as in the controls following treatment with dGuo (up to 50 μM) However, in the presence of 100 μM dGuo and above there was marked expansion of the dCTP and dTTP pools These results sug-

gest that neither virus-induced dCMP deaminase nor virus-induced ribonucleotide reductase was being inhibited Neither can it be said that these changes are due to activation of these enzymes HSV-induced ribonucleotide reductase is resistant to feed-back control by deoxyribonucleoside triphosphates (Averett et al , 1983) Thus, the increased levels of dCTP and dTTP triphosphates do not activate the viral ribonucleotide reductase. The accumulation of dCTP and dTTP, however, seems to indicate that their utilization for DNA synthesis is decreased When dGuo was used in combination with H_4dUrd, the increase in dCTP was almost 2-fold greater than was observed with 500 μM dGuo alone while the dTTP pool was diminished These results are consistent with the effect of H_4dUrd and H_4dUMP as inhibitors of dCyd deaminase and dCMP deaminase, respectively (Maley and Maley, 1971). Under similar conditions dGTP levels declined to 29 pmol, probably as a result of increased utilization of dGTP at a time when the dTTP level is low These perturbations in dNTP pools may be sufficient to cause inhibition of viral replication seen with H_4dUrd plus dGuo

The combination of MMdCyd, dGuo and H_4dUrd was extremely potent in inhibiting viral replication and decreasing virus yield We suggest the following mechanisms for potentiation of antiherpes activity of MMdCyd by dGuo and H_4dUrd H_4dUrd prevents conversion of dCyd to dUMP, which leads to diminished dTTP pools (Aduma et al , 1990b,c) MMdCyd is incorporated into viral DNA (Aduma et al., 1990a) dGuo, after conversion to dGTP, exerts an inhibitory effect on the utilization of dTTP and also facilitates incorporation of MMdCyd into viral DNA Thus, the combination of MMdCyd plus dGuo and H_4dUrd leads to diminished dTTP pools as well as failure to utilize dTTP for viral DNA synthesis As a result, viral DNA synthesis occurs at a reduced rate, and with MMdCTP being incorporated into specific sequences, the capacity of the newly formed DNA to serve as a template-primer is greatly reduced We are in the process of synthesizing radiolabelled MMdCyd, and studies on the mechanism of MMdCyd to test these hypotheses will be undertaken

In summary, based on the findings presented in this paper and with previously published studies from our laboratory on the antiherpes activity of MMdCyd (Aduma et al , 1990a,c,d, Gupta et al , 1989), it is reasonable to conclude that when deamination is prevented, MMdCyd is a potent and selective antiherpes agent. Tetrahydrodeoxyuridine and deoxyguanosine may be useful adjuncts in combination with MMdCyd for the treatment of HSV infections

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