MOLECULAR PHARMACOLOGY, 18, 521–528

MOL PHARM

Biochemical Pharmacology of Lipophilic Diaminopyrimidine Antifolates in Mouse and Human Cells *in Vitro*

WILLIAM R. GRECO AND MAIRE T. HAKALA

Department of Experimental Therapeutics, Roswell Park Memorial Institute, Buffalo, New York 14263, and Department of Pharmacology and Therapeutics, State University of New York at Buffalo, Buffalo, New York 14214

Received April 16, 1980; Accepted June 10, 1980

SUMMARY

GRECO, W. R., AND M. T. HAKALA. Biochemical pharmacology of lipophilic diaminopyrimidine antifolates in mouse and human cells *in vitro*. *Mol. Pharmacol.* **18**: 521–528 (1980).

Four lipophilic 2,4-diaminopyrimidine antifolates, 2,4-diamino-5-(1-adamantyl)-6-ethylpyrimidine (DAEP), 2,4-diamino-5-(1-adamantyl)-6-methylpyrimidine (DAMP), 2,4-diamino-5-(3′,4′-dichlorophenyl)-6-methylpyrimidine (DDMP), and 2,4-diamino-5-(*p*-chlorophenyl)-6-ethylpyrimidine (PRM), were examined in cultures of several mouse and human cell lines and compared with methotrexate (MTX). Unlike MTX, the diaminopyrimidines not only inhibited dihydrofolate reductase, but were found to have a second, folate-independent site of action as judged by growth inhibition in a medium supplemented with the products of folate-dependent reactions (hypoxanthine, thymidine, and glycine). With respect to this site of inhibition, DAEP, DAMP, and DDMP were equipotent, with an ID_{50} for all cells in the range of 5–50 μM. The second site did not concern DNA polymerases α, β, or γ (EC 2.7.7.7), and excess methionine did not alleviate the inhibition. When folates in the medium were present at concentrations adequate for optimal growth (folic acid and N^5-methy-H_4 folic acid at 1 μM, folinic acid at 0.01 μM), the growth inhibitory potency of DAMP was the same. Increased concentrations of folinic acid protected the cells against DAMP to a certain degree, but not as well as hypoxanthine and thymidine. This is unlike MTX, which is competitively antagonized by folinic acid. Folinic acid and the products could also, to a limited degree, rescue cells preexposed to DAMP. Under folate-dependent conditions the growth of human cells was, on the average, four to eight times less sensitive to inhibition by DAEP, DAMP, and DDMP than that of mouse cells. This is unlike MTX. Neither the velocity or extent of cellular uptake of diaminopyrimidines nor the level of dihydrofolate reductase (EC 1.5.1.3) or the activity of TMP synthetase (EC 2.1.1.6) correlated with these sensitivities. No cellular metabolism of DAMP or DDMP was detected. The apparent affinities of the diaminopyrimidines to human and mouse dihydrofolate reductases may help explain the different sensitivities of cells to growth inhibition. While DAEP was the most potent growth inhibitor, with an ID_{50} in the 1 to 40 nM range for all cells examined, the average ID_{50} values for DAEP, DAMP, DDMP, and PRM related as 1:4:13:300. The true K_i values for DAEP, DAMP, and DDMP were 0.14, 0.68, and 2.1 nM, respectively, for the dihydrofolate reductase purified from MTX-resistant S-180 cells and 0.14, 0.43, and 1.0 nM, respectively, for the enzyme of MTX-resistrant KB cells. There was a positive correlation between the ID_{50} values for growth inhibition by the various compounds and the K_i values for dihydrofolate reductase.

INTRODUCTION

Lipophilic diaminopyrimidine antifolates comprise a group of antimetabolites which have been developed for use in antimalarial and cancer chemotherapy (1–4). Like MTX[1] these drugs are inhibitors of DHFR (EC 1.5.1.3),

This study was supported in part by USPHS Grant CA-04175.

[1] Abbreviations used: DHFR, dihydrofolate reductase; MTX, methotrexate; DAEP, 2,4-diamino-5-(1-adamantyl)-6-ethylpyrimidine; DAMP, 2,4-diamino-5-(1-adamantyl)-6-methylpyrimidine; DDMP, 2,4-diamino-5-(3′,4′-dichlorophenyl)-6-methylpyrimidine; PRM, 2,4-diamino-5-(*p*-chlorophenyl)-6-ethylpyrimidine (pyrimethamine); DDEP, 2,4-diamino-5-(3′,4′-dichlorophenyl)-6-ethylpyrimidine; dUrd, deoxyuridine; H_2folate, dihydrofolate; H_4folate, tetrahydrofolate; BSA, bovine serum albumin; KB/MTX, an MTX-resistant subline of KB cells; AT/174 and AT/3000, MTX-resistant sublines of S-180 cells; FGAR, 5′-phosphoribosyl-*N*-formylglycinamide; ID_{50}, concentration required to inhibit the growth of cells by 50%; Hyp, hypoxanthine; dThd, thymidine; V_i, reaction velocity in the presence of inhibitor; V_0, reaction velocity in the absence of inhibitor; m_e, molar equivalency of enzyme; E, amount of enzyme in arbitrary units; I_t, total inhibitor concentration; E_t total enzyme concentration; I_{50}, concentration required to inhibit enzyme velocity by 50%; K_m, Michaelis constant; S, substrate concentration.

0026-895X/80/060521-08$02.00/0

Copyright © 1980 by The American Society for Pharmacology and Experimental Therapeutics.
All rights of reproduction in any form reserved.

MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 6, 2012

but they differ from MTX by lacking a negative charge and being lipophilic. These properties largely account for the far more rapid and greater extent of cellular uptake of these compounds when compared with MTX (5).

The structures of the diaminopyrimidines, DAEP, DAMP, DDMP, and PRM, are compared with that of MTX in Fig. 1. Some of these compounds have previously been examined *in vitro* with respect to their cellular uptake (5, 6) and interaction with DHFR (7) and *in vivo* with respect to their metabolism (3), pharmacokinetics (3), and antitumor activity (4, 8, 9). The purpose of the present study was to examine the four diaminopyrimidines with respect to their mechanism of action and to explore the basis for the varied sensitivity of cells to these compounds. Preliminary reports on this work have been presented (10, 11).

MATERIALS AND METHODS

Chemicals. DAMP, DAEP, and [^{3}H]DAMP (27 μCi/μmol) were used as their ethylsulfonate salts and were kindly provided by Dr. Sigmund Zakrzewski of this department. Unlabeled DDMP and [2-^{14}C]DDMP (13.8 μCi/μmol) were generous gifts of Dr. C. A. Nichol, Wellcome Research Laboratories, Research Triangle Park, N. C. [2-^{14}C]Glycine (56 μCi/μmol) was obtained from the Amersham/Searle Co., Arlington Heights, Ill., and [2-^{14}C]dUrd (54.5 μCi/μmol) from Schwarz-Mann, Orangeburg, N. Y. PRM was purchased from the Burroughs Wellcome Co., N.C.; folic acid, NADPH, BSA, and unlabeled dUrd from Sigma Chemical Co., St. Louis, Mo.; MTX from Nutritional Biochemicals, Cleveland, Ohio; L-azaserine from Calbiochem Co., La Jolla, Calif.; and horse serum, fetal calf serum, and powdered 1640 medium from Grand Island Biological Co., Grand Island, N.Y. *dl*-Folinic acid was a gift from Lederle Laboratories, Pearl River, N. Y. The concentrations refer to the natural diastereoisomer. *dl*-N^5-Methyl-H_4 folate was obtained as a Ba salt from Sigma Chemical Co. and was 90–95% pure. H_2Folic acid was prepared and recrystallized by the method of Blakely (12).

Purity of labeled compounds. The purities of [^{3}H]-DAMP, [2-^{14}C]DDMP, and [2-^{14}C]PRM were assessed by thin-layer chromatography according to the procedures of Zakrzewski *et al.* (3) and DeAngelis *et al.* (13). The purities of DDMP and PRM were >99%, and that of DAMP was >98%.

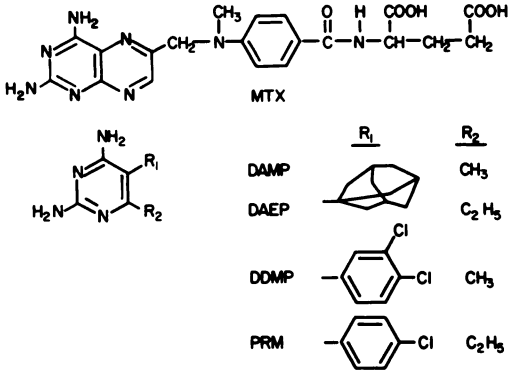

FIG. 1. *Structures of methotrexate and the diaminopyrimidine antifolates*

Cells. The origin and maintenance of the cells have previously been described (14). The mouse cell ines used were: sarcoma, S-180; leukemia, L-1210; kidney, MUK; adenocarcinomas, RAG and TA3; fibroblast, LM; mammary tumor, MMT; and Ehrlich ascites carcinoma, EAC. The human cells were: fibroblasts, U cells; lymphoma, BL; nephroma, Wilms; myeloma, Myel; and carcinomas, HeLa, KB, and Hep-2. The MTX-resistant subline of KB cells, KB/MTX, was developed from the parent line by gradually increasing the concentration of MTX from 5 × 10^{-8} to 5 × 10^{-5} M over a period of 8 months. The development was carried out in 1640 medium supplemented with 5% fetal calf serum and 30 μM thymidine. KB/MTX cells were maintained in 1640 medium in the presence of 50 μM MTX and 30 μM thymidine. To free the cells of bound MTX, they were grown for at least 2 weeks in folate-free 1640 medium supplemented with 100 μM hypoxanthine, 30 μM thymidine, and 100 μM glycine.

The cells were monitored weekly for the absence of mycoplasma (15).

Growth inhibition studies. These were conducted as described previously (14). The medium used was either that of Eagle supplemented with 5% horse serum (Figs. 5 and 6) or 1640 supplemented with 5% fetal calf serum (Figs. 2–4) appropriately modified as explained in the individual legends.

Purified DHFR. Human and mouse DHFR, isolated from KB/MTX and AT/3000 cells, respectively, and purified by MTX-affinity chromatography (16), were kindly provided by Mrs. Barbara Domin and Dr. Y-C. Cheng of this department.

DHFR assay. DHFR activity in the presence or absence of the diaminopyrimidines was measured at 36°C and pH 7.5 by following the decrease in absorbance at 340 nm caused by the oxidation of NADPH to $NADP^+$ and the reduction of H_2folic acid to H_4folic acid using a Cary 118 recording spectrophotometer (Varian Instrument Division, Palo Alto, Calif.). The experimental conditions were those of Domin *et al.* (16). The reaction mixture in a final volume of 1 ml contained 50 mM K-phosphate, pH 7.5, 100 μM NADPH, 150 mM KCl, 10 mM mercaptoethanol, 50 to 75 μM H_2folic acid, and the purified enzyme. All components, except H_2folic acid, were combined and incubated for 15 min at 36°C. H_2Folic acid was then added to start the reaction. The blank was devoid of H_2folate. These reaction conditions were used for the determination of the K_i values.

DHFR titration. The content of DHFR in cell extract was determined by titrating with MTX. This was done by following the decrease in absorbance at 340 nm, at 30°C using a Cary 118 spectrophotometer. The assay mixture contained 150 mM KCl, 100 mM Na-citrate buffer, pH 5.9, 10 mM mercaptoethanol, 75 μM NADPH, 75 μM H_2folate, and cell extract. The results are expressed in molar equivalents of MTX-binding sites per milligram of cell extract protein.

Incorporation of [2-^{14}C]deoxyuridine. Monolayers of cells in T-15 flasks were preincubated at 36°C for certain periods of time with or without varied concentrations of the inhibitors in 2 ml of Eagle's medium containing 1 μM folate and supplemented with 30 μM thymidine, 1 mM hypoxanthine, 100 μM glycine, and 5% dialyzed horse

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 6, 2012

serum. After the preincubation the medium was poured off and the cell layer rinsed three times with 2 ml of warm, serum- and folate-free Eagle's medium. Two milliliters of serum- and folate-free Eagle's medium, supplemented with 5 μM [2-^{14}C]dUrd (about 500,000 dpm/ml), was then added and duplicate samples were incubated for 30 and 60 min, during which time the incorporation was linear with time. Acid-soluble material was removed and the remaining cell layer was analyzed for protein and radioactivity. In S-180 cells 90% of the acid-insoluble radioactivity derived from dUrd was found in DNA and 10% in RNA.

FGAR biosynthesis. This was measured by determining the incorporation of [^{14}C]glycine into cellular FGAR by the method of Henderson (17), modified as follows. The preincubation with diaminopyrimidines was carried out as above except in this case, larger cultures in T-30 flasks with 5 ml of medium were used. The rinsed cell layers were then incubated in duplicate for 10 min with 4 ml of folate- and serum-free medium containing 12 μM L-azaserine. One milliliter of similar medium containing 5 mM [2-^{14}C]glycine (1 μCi/μmol) was then added and the incubation continued for 1 and 2 h. The medium was poured off and the cell layer rinsed twice with 5 ml of ice-cold, serum-free medium. The cell layer was then extracted with 4 ml of 5% trichloroacetic acid. After removal of trichloroacetic acid with ether, duplicate 1.3-ml samples of the extract were chromatographed on Dowex-1-formate columns and fractions containing FGAR were counted for ^{14}C. The remaining cell layer was dissolved into 0.2 N NaOH and analyzed for protein. The results were calculated per milligram of total cellular protein and are expressed as percentage of control.

Protein assay. Protein was determined by the method of Lowry using BSA as the standard.

Cellular uptake of diaminopyrimidines. Almost confluent monolayers of cells in Falcon T-25 flasks (Becton, Dickinson and Co., Oxnard, Calif.) were incubated with 5 ml of serum- and folate-free Eagle's medium (prewarmed to 36°C) supplemented with 10 μM labeled diaminopyrimidine. Constant temperature was maintained by a circulating water bath (GCA/Precision Scientific). After incubation at 36°C, the medium was poured off, and the cell layer rinsed three times with 5 ml of ice-cold, serum- and folate-free Eagle's medium. Zero-time points were obtained by exposing the cultures on ice to ice-cold experimental medium for less than 1 s and by rinsing as above. The cell layers were dissolved in 4 ml of 2 N NaOH, and aliquots assayed for protein and radioactivity. The uptake at time *t* was corrected for the zero-time uptake and is expressed as moles of drug taken up per milligram of total cell protein.

Cellular metabolism of diaminopyrimidines. Duplicate monolayer cultures in T-75 flasks containing cells in log phase were incubated with 10 μM radiolabeled DDMP or DAMP in 10 ml of serum-free medium for 24 h at 36°C. A 24-h exposure to 10 μM concentration of these drugs in Eagle's medium would kill the cells. To prevent this, the medium was supplemented with 100 μM hypoxanthine, 30 μM thymidine, and 100 μM glycine. The medium was poured off, and the cell layer was rinsed three times with 10 ml of ice-cold, serum-free medium. The cells on ice were then extracted with 5 ml of ice-cold absolute ethanol for 5 min. The ethanol extract, which contained 93 to 98% of the total radioactivity present in the cells initially, was centrifuged at 300 rpm for 10 min, and the supernatant was evaporated under a stream of nitrogen in the dark. The residue was dissolved in 1 ml of 0.1 N HCl and chromatographed as described by Zakrzewski *et al* (3).

Statistical analyses. Nonlinear regression was performed using MLAB software (18) from a PROPHET computer terminal located at the Research Institute on Alcoholism, Buffalo, N. Y.

RESULTS

Inhibition of folate-dependent growth. Figures 2 and 3 show the concentrations (ID_{50}) of DAEP, DAMP, DDMP, PRM, and MTX which were required to cause 50% inhibition of growth of a number of mouse and human cell lines in RPMI-1640 medium containing 2 μM folic acid. Among the diaminopyrimidines, DAEP was the most potent inhibitor, with an ID_{50} in the range of 1 to 40 nM for all the cell lines tested. The general order of potency was DAEP > DAMP > DDMP > PRM so that the average ID_{50} values for these compounds related as 1:4:13:300, respectively. On the average, human cells required four to eight times more of DAEP, DAMP, and DDMP for inhibition than mouse cells, but only 50% more of PRM. No such differences were noted for MTX.

When the individual cell lines are compared, S-180

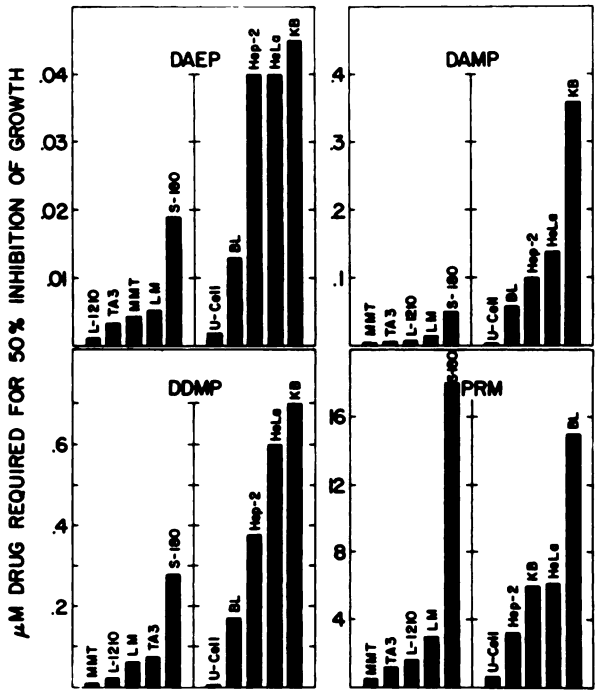

FIG. 2. *Growth inhibitory potency of diaminopyrimidines against mouse and human cells in 1640 medium supplemented with 5% heat-inactivated fetal calf serum*

The ordinate indicates the concentration of the compound which caused 50% inhibition of growth when it was present continuously for 3 to 6 days, depending on the cell line. The values are averages from one to eight separate experiments, each carried out in triplicate. The details of the methodology have been published (14).

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 6, 2012

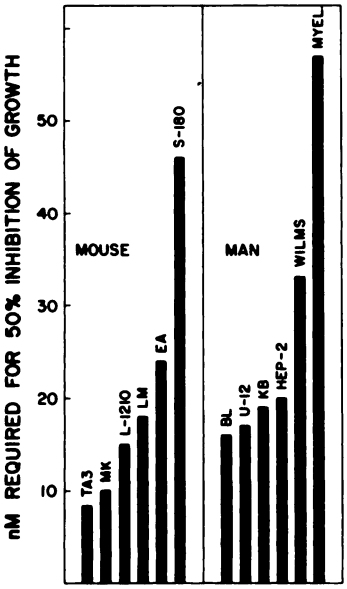

FIG. 3. *Growth inhibitory potency of MTX against mouse and human cells in vitro; conditions as in Fig. 2*

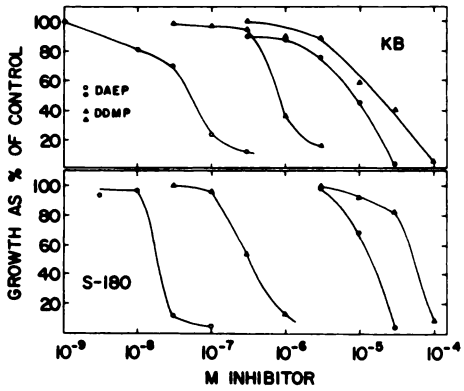

FIG. 4. *Growth inhibition of human KB and mouse S-180 cells by DAEP and DDMP under folate-dependent (1640 medium; open symbols) and folate-independent conditions (1640 supplemented with 100 μM hypoxanthine, 30 μM thymidine, and 100 μM glycine; solid symbols)*
The cells were grown for 5 days in the presence of the inhibitors and the medium was changed on the third and fourth days. Each point is an average of triplicates. The growth in controls was 15 to 20 times the inoculum.

cells are clearly the least sensitive among mouse cells, not only to the diaminopyrimidines, but to MTX as well. The fibroblasts (U cells) are the most sensitive among human cells to the four diaminopyrimidines, while Burkitts lymphoma cells are more sensitive than the three carcinoma cell lines to DAEP, DAMP, and DDMP.

Inhibition of folate-independent growth. The growth of cells can be made independent of folate metabolism (19) by providing them with the products of folate-dependent reactions (hypoxanthine, thymidine, and glycine). Under these conditions 200 and 1000 times more DAEP and 20 and 200 times more DDMP were required to inhibit the growth of human KB and mouse S-180 cells, respectively, than under folate-dependent conditions (Fig. 4). This inhibition of growth shows that diaminopyrimidines, unlike MTX, have a folate-independent site of action. Table 1 lists the ID_{50} concentrations of these compounds under folate-independent conditions and reveals their quite similar potency toward this second site. Attempts were made to explore the second site of action. Up to 1 mM DAMP had no effect on DNA polymerases α, β, and γ (EC 2.7.7.7), partially purified from the leukocytes of a patient with acute lymphocytic leukemia.[2] Tetrahydropteroylglutamate methyltransferase (EC 2.1.1.13) was also considered as a possible candidate for the action of DAMP. However, raising the concentration of L-methionine from 0.1 to 1.1 mM had no effect on the potency of DAMP as growth inhibitor of KB cells in a medium supplemented with hypoxanthine, thymidine, and glycine (not shown).

Inhibition of folinic acid-dependent growth. Earlier work had shown that 10^{-8} M folinic acid or 10^{-6} M folic acid is capable of supporting optimal growth of S-180 cells. In those studies the requirement of folate-depleted cells (cells grown for a week in folate-free medium supplemented with hypoxanthine, thymidine, and glycine) for half-maximal growth was shown to be 1.5 nM folinic acid (natural stereoisomer) or 150 nM folic acid (20). The corresponding requirement of S-180 cells for N^5-methyl-H_4 folic acid was equal to that of folate, i.e., a 150 nM concentration of the natural stereoisomer (data not shown).[3]

The growth inhibitory potency of DAMP was similar in folic acid, folinic acid, or N^5-methyl-H_4 folic acid medium (Fig. 5). However, by increasing the concentration of folinic acid, S-180 cells could be protected against DAMP, but only to a limited extent. The products (hypoxanthine and thymidine) provided a four to five times better protection of S-180 cells against DAMP than the maximally protective concentration of folinic acid (Fig. 5). In the presence of folinic acid, 10,000 times above the optimal level, 60 times more DAMP was required for growth inhibition and the relationship between DAMP and folinic acid was not a competitive one. This is in contrast to the relationship between MTX and folinic acid, where the competitive ratio was 9.0 for the parent and 600 and 1500 for two MTX-resistant sublines of S-180 cells (20).

Rescue with folinic acid or products. Figure 6 shows the rescue of S-180 cells after 16 h of preincubation with DAMP. When cells after 16 h of preincubation with 1 μM DAMP were transferred into drug-free medium containing 1 μM folate, only 20% of control growth was observed, while folinic acid at or above 0.1 μM supported growth equal to controls. If preincubated with 30 μM DAMP, neither 10 μM folinic acid nor the products could fully rescue the cells and concentrations of DAMP above that level were irreversibly lethal to the cells.

Effect on incorporation of deoxyuridine and on synthesis of FGAR. The incorporation of deoxyuridine into cellular DNA was examined after 6 h of preincubation of

[2] These tests were kindly carried out by Dr. R. DiCioccio of this department.

[3] Later studies have revealed that in a medium supplemented with 100 μM Na-ascorbate the requirement of cells for N^5-methyl H_4 folic acid is equal to that for folonic acid.

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 6, 2012

TABLE 1

Inhibition of folate-independent growth of mouse and human cells by diaminopyrimidines

Cell line	ID_{50} (μM)[a]			
	DAEP	DAMP	DDMP	PRM
Mouse				
S-180	11.5	12	46	65
L cells	10	6.6	52	100
TA3	32	29	—	—
MMT	13	37	7.0	30
L-1210	11	12	20	>100
Human				
U cells	12	11	15	30
KB	7.5	10	13	19
Hep-2	8.6	13	4.5	33
HeLa	12	12	26	38

[a] Cells were grown in monolayer in the presence of the inhibitors in 1640 medium supplemented with 500 μM hypoxanthine, 30 μM thymidine, 100 μM glycine, and 5% fetal calf serum for 5 or 6 days involving two or three changes of the medium. Growth in the drug-free controls was 10 to 20 times the inoculum. ID_{50} indicates the concentration of the compound causing 50% inhibition of growth.

cells with varied concentrations of DAMP and DAEP and this was compared with the incorporation of glycine into FGAR (Fig. 7). DAEP was found to be about three times more potent than DAMP in causing inhibition of both processes. This difference correlates well with the greater potency of DAEP as an inhibitor of growth (Fig. 2). Figure 7 also reveals that the incorporation of deoxyuridine into DNA is 10 times more sensitive to DAMP and DAEP than the synthesis of FGAR.

Enzyme activities versus sensitivity to MTX and DAMP. Figure 2 had shown how mouse cells in general were more sensitive than human cells to growth inhibtion by DAEP, DAMP, and DDMP. The possibility was explored that these differences might correlate with enzyme activities. Table 2 compares DHFR levels, TMP synthetase activities, and ID_{50} values of MTX and DAMP for a number of mouse and human cells. No correlation was noted between the sensitivity to DAMP

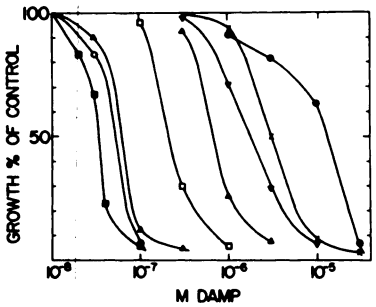

FIG. 5. *Partial protection of S-180 cells with folinic acid or products against growth inhibition by DAMP*

The basic medium was folate-free Eagle's medium supplemented with 5% horse serum and either 1 μM folic acid (■), 1 μM 5-methyl-H_4 folic acid (▲), or folinic acid at the following concentrations: 10^{-8} (○), 10^{-7} (□), 10^{-6} (△), 10^{-5} (▽), or 10^{-4} M (×). Product reversal study (●) was carried out in folate medium supplemented with 100 μM hypoxanthine, 30 μM thymidine, and 100 μM glycine. Growth was continued for 6 days in the continuous presence of DAMP by changing the medium on the third, fourth, and fifth days.

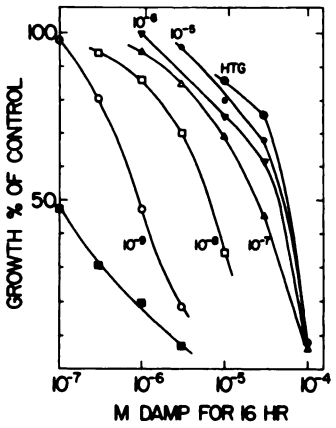

FIG. 6. *Rescue of S-180 cells with folinic acid or products after 16 h of preincubation with DAMP*

Monolayers of S-180 cells (450,000 cells/T-15 flask) were preincubated for 16 h with varied concentrations of DAMP in Eagle's medium containing 1 μM folate and supplemented with 1 mM hypoxanthine, 30 μM thymidine, 100 μM glycine, and 5% dialyzed horse serum. At the end of incubation, the medium was removed, the cell layer was rinsed with folate-free medium, and 2 ml of growth medium was added. Growth medium consisted of folate-free Eagle's medium supplemented with 5% dialyzed horse serum and varied molarities of folinic acid as indicated in the figure or hypoxanthine, thymidine, and glycine (HTG) as in Fig. 5. The solid squares show growth in medium free of folinic acid but supplemented with 1 μM folate. The growth was allowed to proceed for 3 days, with daily changes of the medium. Growth in the controls was nine times the inoculum.

or MTX and the TMP synthetase activity. In fact, the very low TMP synthetase activity in the MTX-resistant subline of S-180 cells (AT/174) and its increased resistance to both inhibitors provide evidence to the contrary.

Evidence for a direct correlation between DHFR levels and MTX-resistance has been shown to exist in the S-180 cell system, where the kinetic properties of the enzyme and cellular uptake of MTX were unchanged (21). It is of interest that AT/174 cells, which contain 68 times more DHFR than the parent S-180 cells, required for

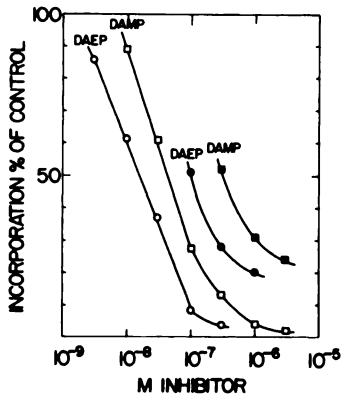

FIG. 7. *Effect of 6 h of incubation of S-180 cells with DAEP and DAMP on the subsequent incorporation of deoxyuridine into DNA (open symbols) and on the synthesis of FGAR (solid symbols)*

The experimental details are described in Materials and Methods. The untreated controls incorporated deoxyuridine 860 ± 250 pmol/30 min/mg total cellular protein. The incorporation of glycine into FGAR in the controls was 3.7 ± 0.66 nmol/30 min/mg total cellular protein. The points are averages of four separate experiments, each carried out in duplicate.

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 6, 2012

TABLE 2

Dihydrofolate reductase and TMP synthetase activities versus growth inhibition by MTX and DAMP

Cell line	ID_{50} (nM)[a]		DHFR level[b]	TMP synthetase[c]
	MTX	DAMP		
			pmol/mg prot ± SE	*pmol/mg prot/min ± SE*
Human				
U cell	17	2.2	—	111 ± 6.9
Hep-2	20	100	7.6 ± 1.2	109 ± 9.5
HeLa	17	140	13 ± 0.46	162 ± 3.1
KB	19	360	7.9 ± 0.76	156 ± 5.8
KB/MTX	140,000	7,000	440 ± 38	510 ± 23
Mouse				
MMT	—	5.0	9.4 ± 1.6	76 ± 7
LM	18	14	12 ± 1.6	131 ± 6.0
S-180	50	49	13 ± 0.05	36 ± 4.8
AT/174	8,000	370	862	1.5

[a] Inhibition of folate-dependent growth. See the legends to Figs. 2 and 3.

[b] Determined by titration with MTX at pH 5.9 and 30°C using H_2folate as substrate. Expressed in molar equivalents of MTX binding sites per milligram of protein in cell extract.

[c] From R. M. Evans, J. D. Laskin and M. T. Hakala, in press.

growth inhibition 160 times more MTX, but only 9 times more DAMP than the parent cells. This indicates superiority of the diaminopyrimidines over MTX as inhibitors of cells resistant to MTX when the resistance is due to increased levels of DHFR. The factors, which might be involved in allowing the KB/MTX cells to tolerate 7400 times more MTX than the parent cells have not been explored in detail. One of these factors is the DHFR level, which is elevated 55 times over that in the parent KB cells. This relatively small difference in DHFR level as compared with the high degree of resistance to MTX (7400-fold) suggests that, unlike in the S-180 cell system, additional factors may contribute to MTX resistance. The 3-fold increase in TMP synthetase activity in KB/MTX cells is interesting in light of the opposite relationship in AT/174 cells. Only 17 times more DAMP was required to inhibit the growth of KB/MTX than that of the parent. The small degree of resistance to DAMP results not only from the relatively low sensitivity of the parent KB to DAMP (ID_{50} 360 nM) but also from the fact that in KB/MTX cells the inhibition of the second, folate-independent site becomes growth limiting at 7000 nM DAMP. This was shown by a complete overlap of the DAMP growth inhibition curves of KB/MTX whether the cells were grown under folate-dependent or -independent conditions (data not shown). When one compares the cell lines, never exposed to MTX, with respect to their sensitivity to DAMP, one finds no correlation between the sensitivities and the levels of DHFR (Table 2).

Cellular uptake versus sensitivity. The possible role of the velocity or extent of drug uptake in the sensitivity to DDMP was examined by comparing HeLa and MMT cells, which differ by 60-fold in sensitivity to DDMP (ID_{50} for MMT, 10 nM; for HeLa, 600 nM) (Fig. 8). If any difference were to be seen, it rather suggests that DDMP is taken up more rapidly by the less sensitive HeLa cells. While the plateau level of DDMP appears higher in the more sensitive MMT, the difference was not statistically significant. When similar studies were conducted with KB, KB/MTX, and Hep-2 cells, the patterns of DDMP uptake were similar to those seen in Fig. 8 (5).

Cellular metabolism of diaminopyrimidines. Metabolism of [2-^{14}C]-DDMP in MMT and KB cells was examined by incubating cells for 24 h with 10 μM drug. These cells differ 70-fold in their sensitivity to DDMP. No metabolic products were detected on chromatography; all the radioactivity was recovered as unaltered DDMP. Similar results were obtained with [^{3}H]DAMP in Hep-2 cells.

Affinity to DHFR. K_i values for DAEP, DAMP, and DDMP were determined using purified mouse and human DHFR. The mouse enzyme isolated from the MTX-resistant subline of S-180 cells has been shown to be identical to that of the parent cells (21). The human DHFR, isolated from the MTX-resistant subline of KB cells, has also been shown to have identical kinetic properties (K_m for NADPH and H_2 folate and K_i for MTX) to the enzyme from the parent KB cells (16).

On the basis of earlier studies (22) DAEP, DAMP, and DDMP appear to be relatively tight-binding inhibitors of DHFR. To accurately determine the K_i values of such inhibitors, both the inhibitor (I_t) and the enzyme concentration (E_t) must be varied (23). In this case, 4 to 6 levels of the enzymes and 5 to 8 levels of the inhibitors were used, resulting in 15 to 52 velocity measurements for each K_i value. The data were mathematically analyzed by nonlinear regression using Eq. [1], which was adapted from that of Morrison (24).

$$V_i = V_0/2m_eE\{m_eE - I_t - K_{i_{app}} + [(I_t + K_{i_{app}} - m_eE)^2 + 4K_im_eE]^{\frac{1}{2}}\}. \quad [1]$$

The superiority of this computer method has been discussed elsewhere (25). The resulting K_i values increased with increasing concentrations of the substrate, indicating a competitive relationship. In order to obtain the true K_i values, these apparent K_i values were divided with the term $(1 + S/K_m)$ (25). The K_m values used in these calculations were 0.67 μM for the human enzyme and 1.72 μM for the mouse enzyme (16). Under the conditions used the ratios of E_tK_m/K_iS for DAEP and

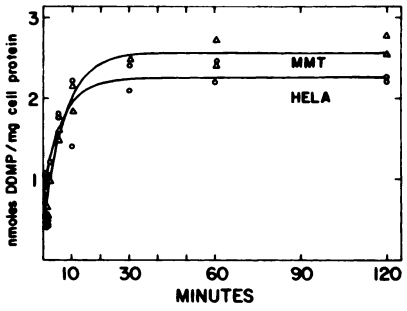

FIG. 8. *Uptake of 10 μM [2-^{14}C]DDMP by MMT and HeLa cells* Each point represents data obtained with a monolayer culture in one T-25 flask. The results are expressed per milligram of total cell protein. For details of methodology, see Materials and Methods.

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 6, 2012

MOL PHARM

MOLECULAR PHARMACOLOGY

DAMP varied between 0.4 and 2.7 and thus were within the limits of zone B (0.1 to 100) as defined by Goldstein for tight-binding, competitive inhibitors (26).

The apparent and true K_i values are listed in Table 3. A comparison of the three diaminopyrimidines DAEP, DAMP, and DDMP reveals a similarity in the relationship between the K_i values and the ID_{50} values for growth inhibition. For the mouse enzyme, the K_i values relate as 1:4:9, while the ID_{50} values for growth inhibition relate as 1:4:13 for DAEP, DAMP, and DDMP, respectively. The apparent K_i values are directly related to the I_{50} values for inhibition of the enzyme, as seen from Eq. [2] of Cha (23):

$$I_{50} = K_{i_{app}} + E_t/2. \quad [2]$$

To explore the basis for the differences in sensitivity between S-180 and KB cells to growth inhibition by DAEP, DAMP, and DDMP, the apparent K_i values of these compounds for DHFR of AT/3000 and KB/MTX cells were compared. All the apparent K_i values for the KB/MTX enzyme appeared to be greater than the corresponding K_i values for AT/3000 enzyme. However, the difference was statistically significant only in the case of DAEP ($P \leq 0.01$). The inherent variability in the enzyme assay precludes any far reaching conclusions. The correlation coefficient between the ID_{50} values for growth inhibition for the three compounds and the apparent K_i values for DHFR was 0.86 and significant at $P = 0.05$. The correlation coefficient on a log–log scale was 0.92 and was significant at $P = 0.01$. These relationships between K_i values and ID_{50} values provide evidence that the affinity of the different diaminopyrimidines to the target enzyme is the principal factor which determines their relative potencies as inhibitors of growth. There is also suggestive evidence to indicate that the affinity of a certain diaminopyrimidine to DHFR of the target cell is a factor which determines the different sensitivities of cells to growth inhibition by that diaminopyrimidine.

DISCUSSION

The lipophilic 2,4-diaminopyrimidines were designed to provide potent antifolates with improved capacity for cellular uptake (3). Indeed, their uptake is about 10,000 times more rapid than that of MTX (5). The present study reveals further differences from MTX. Thus, under folate-dependent conditions human cells were, on the average, less sensitive than mouse cells to growth inhibition by DAEP, DAMP, and DDMP (Fig. 2). No such differences were observed for MTX (Fig. 3). Furthermore, the cells could be protected against these compounds only to a certain degree with folinic acid. This is in contrast to MTX, which has a competitive relationship with folinic acid (20). Unlike MTX, the diaminopyrimidines revealed a folate-independent site of action which became growth limiting in medium supplemented with hypoxanthine and thymidine (Fig. 4 and Table 1) and undoubtedly also restricted the protection and rescue with folinic acid (Figs. 5 and 6).

Hitchings *et al.* (27) were the first to point out that diaminopyrimidines such as DDMP may have more than one site of action. Their studies with *Streptococcus faecalis* showed that DDMP inhibited not only the conversion of folate to a reduced form, but the utilization of folinic acid as well, the former being about 500 times more sensitive to DDMP. In 1972, Ho *et al.* (7) reported a folate-independent site of action for four diaminopyrimidines, one of them DAMP, in mouse TA3 cells. The ID_{50} values varied from 30 to 50 μM and were the same regardless of their growth inhibitory potency under folate-dependent conditions. The present study leaves no doubt that diaminopyrimidines have a second, folate-independent site of action in all mammalian cells tested so far. Since clinical applications of these compounds in cancer patients often depend on the use of folinic acid for protection against and rescue from toxicity (9), the limits of such protection should be understood. This must be of special concern considering that the second site is still relatively sensitive, with the ID_{50} concentrations falling to between 5 and 50 μM for DAEP, DAMP, and DDMP (Table 1).

The second site was not DNA polymerase, nor did it concern methionine deficiency. Histamine-*N*-methyltransferase (EC 2.1.1.8), partially purified from bovine brain, has been shown to be a sensitive target for DDMP, DDEP, and PRM, with K_i values of 0.1, 0.8, and 0.9 μM, respectively (28). Inhibition of this enzyme may account for the clinically observed toxicities of DDMP, but the presence of this enzyme in cells in culture is not known, nor is a role for such an enzyme in these cells easy to envision. DAMP and DAEP have been shown to inhibit human TMP synthetase (29), and the K_i value for the more potent DAMP was 175 μM. However, being a folate-dependent enzyme, TMP synthetase can hardly represent the second site of action, and in addition, the growth inhibition was not overcome by thymidine, the product of that reaction.

With regard to the folate-dependent growth the inhibitory potencies of the four diaminopyrimidines were very different, as were also the sensitivities of mouse and human cells. In this study the average ID_{50} values for DAEP, DAMP, DDMP, and PRM for all cells tested related as 1:4:13:400, respectively. Earlier studies had shown that DAEP was more potent than DAMP as a growth inhibitor of mouse TA3 cells and also as an

TABLE 3

Relationship between K_i values for DHFR and ID_{50} values for cell growth

Source of DHFR	Compound	$K_i \pm SE$[a]	$K_{iapp} \pm SE$[b]	ID_{50}[c]
		nM	*nM*	*nM*
AT/3000	DAEP	0.136 ± 0.025	4.33 ± 0.80	19
	DAMP	0.683 ± 0.23	21.8 ± 7.3	49
	DDMP	2.12 ± 0.19	67.6 ± 6.1	280
KB/MTX	DAEP	0.144 ± 0.016	10.7 ± 1.2	45
	DAMP	0.430 ± 0.049	32.1 ± 3.7	360
	DDMP	1.030 ± 0.054	76.9 ± 4.0	700

[a] K_i values determined at pH = 7.5 as described in Materials and Methods.

[b] Apparent K_i values were calculated from the true K_i values, assuming a uniform dihydrofolate concentration of 50 μM and using the equation $K_{iapp} = K_i (1 + S/K_m)$.

[c] For the determination of ID_{50} values of the parent S-180 and KB cells, see the legend to Fig. 2.

Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 6, 2012

inhibitor of partially purified DHFR of S-180 cells (7). In the present study, the determination of the K_i values was carried out with highly purified enzymes and the data were analyzed with the aid of nonlinear regression in a manner necessary for the study of tight-binding enzyme inhibitors (25). Evidence is presented here to suggest that the affinities to the target enzyme (DHFR) constitute the basis for the different potencies of these compounds in inhibiting cellular growth. The basis for the lesser sensitivity of human than of mouse cells to growth inhibition by diaminopyrimidines was also examined. This difference between mouse and human cells was not due to differences in DHFR levels, TMP synthetase activities, diaminopyrimidine uptake, or metabolism. There is suggestive evidence which indicates that the greater sensitivity of mouse cells to diaminopyrimidines is due to a greater affinity of these compounds to mouse DHFR as revealed by lower apparent K_i values. An analogous situation has been demonstrated in the case of MTX. Jackson *et al.* (30) compared the sensitivities of four cell lines to MTX and found that neither the level of DHFR, the activities of TMP synthetase, nor the drug uptake could explain the different sensitivities. The different sensitivities were due to different affinities of MTX to DHFR purified from each cell line.

In conclusion, the diaminopyrimidine antifolates form a group of compounds which in some respects resemble MTX, but differ from it enough to require consideration as a separate category of pharmacologically active compounds. This is warranted especially in view of the second, relatively sensitive, folate-independent site of action, which so far eludes a more accurate description.

REFERENCES

1. Hitchings, G. H., E. A. Falco, H. Vanderwerff, P. B. Russel and G. B. Elion. Antagonists of nucleic acid derivatives. VII. 2,4-Diaminopyrimidines. *J. Biol. Chem.* **199:** 43–56 (1952).
2. Cavallito, J. C., C. A. Nichol, W. D. Brenckman, Jr., R. L. DeAngelis, D. R. Stickney, W. S. Simmons and C. W. Sigel. Lipid-soluble inhibitors of dihydrofolate reductase. I. Kinetics, tissue distribution, and extent of metabolism of pyrimethamine, metoprine and etoprine in the rat, dog, and man. *Drug. Metab. Dispose.* **6:** 329–337 (1978).
3. Zakrzewski, S. F., C. Dave, L. H. Mead and D. S. Deluomo. Studies with a new antifolate 2,4-diamino-5-adamantyl-6-methylpyrimidine (DAMP). Tissue distribution and disposition of 2,4-diamino-5-adamantyl-6-methylpyrimidine and its metabolite. *J. Pharmacol. Exp. Ther.* **205:** 19–26 (1978).
4. Zakrzewski, S. F., C. Dave and F. Rosen. Comparison of the antitumor activity and toxicity of 2,4-diamino-5-(1-adamantyl)-6-methylpyrimidine and 2,4-diamino-5-(1-adamantyl)-6-ethylpyrimidine. *J. Nat. Cancer. Inst.* **60:** 1029–1033 (1978).
5. Greco, W. R., and M. T. Hakala. Cellular pharmacokinetics of lipophilic diaminopyrimidine antifolates. *J. Pharmacol. Exp. Ther.* **212:** 39–46 (1980).
6. Hill, B. T., L. A. Price and J. H. Goldie. Methotrexate resistance and uptake of 2,4-diamino-5-(3′4′-dichlorophenyl)-6-methylpyrimidine by L5178Y cells. Selective protection with folinic acid. *Eur. J. Cancer.* **11:** 545–553 (1975).
7. Ho, Y. K., M. T. Hakala and S. F. Zakrzewski. 5-(1-Adamantyl)-pyrimidines as inhibitors of folate metabolism. *Cancer Res.* **32:** 1023–1028 (1972).
8. Murphy, L. M., R. R. Ellison, D. A. Karnofsky and J. H. Burchenal. Clinical effects of dichloro- and monochlorophenyl analogues of diaminopyrimidines: Antagonists of folic acid. *J. Clin. Invest.* **33:** 1388–1396 (1954).
9. Price, L. A., B. T. Hill and J. H. Goldie. DDMP and selective folinic acid protection in the treatment of malignant disease: A further report. *Clin. Oncol.* **3:** 281–286 (1977).
10. Greco, W. R., and M. T. Hakala. On folate-dependent and -independent sites of action of diaminopyrimidine antifolates in human and mouse cells. *Proc. Amer. Assoc. Cancer Res.* **19:** 206 (1978).
11. Greco, W. R., L. N. Kenny, S. F. Zakrzewski and M. T. Hakala. Comparison of methotrexate (MTX) with two diaminopyrimidines in Sarcoma 180 (S-180) cells *in vitro*. *Fed. Proc.* **34:** 589 (1975).
12. Blakely, R. L. Crystalline dihydropteroylglutamic acid. *Nature* **188:** 231–232 (1960).
13. DeAngelis, R. L., W. S. Simmons and C. A. Nichol. Quantitative thin layer chromatography of pyrimethamine and related diaminopyrimidines in body fluids and tissues. *J. Chromatogr.* **106:** 41–49 (1975).
14. Slocum, H. K., and M. T. Hakala. Mechanism of natural resistance to N^6-(Δ^2-isopentenyl)adenosine in cultured cells. *Cancer Res.* **35:** 423–428 (1975).
15. Hakala, M. T., J. F. Holland and J. S. Horoszewicz. Changes in pyrimidine deoxyribonucleoside metabolism in cell culture caused by mycoplasma (PPLO) contamination. *Biochem. Biophys. Res. Commun.* **11:** 466–471 (1963).
16. Domin, B. A. *Human Dihydrofolate Reductase: Purification and Characterization of Dihydrofolate Reductase from Methotrexate Resistant KB Cells and Comparison with Enzyme from KB Parent Cells, Acute Myelogenous Leukemia Cells and Mouse S-180 AT/3000 Cells*, Ph.D. thesis. State University of New York at Buffalo (1980).
17. Henderson, J. F. Feedback inhibition of purine biosynthesis in ascites tumor cells. *J. Biol. Chem.* **237:** 2631 (1962).
18. Knott, G. D., and D. K. Reece. MLAB: A civilized curve-fitting system. Proceedings of the ONLINE '72 International Conference, Brunel University, England, Vol. 1, 497–526 (1972).
19. Hakala, M. T. Prevention of toxicity of amethopterin for Sarcoma-180 cells in tissue culture. *Science* **126:** 255 (1957).
20. Hakala, M. T., S. F. Zakrzewski and C. A. Nichol. Relation of folic acid reductase to amethopterin resistance in cultured mammalian cells. *J. Biol. Chem.* **236:** 952–958 (1961).
21. Hakala, M. T. Biochemical basis of resistance to folic acid antagonists. *FEBS Symposium, Vol. 16*, 31–47 (1969).
22. Jonak, J. P., L. H. Mead, Y. K. Ho and S. F. Zakrzewski. Effect of the substituent at C-6 on the biological activity of 2,4-diamino-5-(1-adamantyl)-pyrimidines. *J. Med. Chem.* **16:** 724–725 (1973).
23. Cha, S. Tight-binding inhibitors. I. Kinetic behavior. *Biochem. Pharmacol.* **24:** 2177–2185 (1975).
24. Morrison, J. F. Kinetics of the reversible inhibition of enzyme-catalyzed reactions by tight-binding inhibitors. *Biochim. Biophys. Acta* **185:** 269–286 (1969).
25. Greco, W. R., and M. T. Hakala. Evaluation of methods for estimating the dissociation constant of tight-binding enzyme inhibitors. *J. Biol. Chem.* **254:** 12104–12109 (1979).
26. Goldstein, A. The mechanism of enzyme inhibitor substrate reactions. *J. Gen. Physiol.* **27:** 529–580 (1944).
27. Hitchings, G. H., E. A. Falco, G. B. Elion, S. Singer, G. B. Waring, D. J. Hutchinson and J. H. Burchenal. 2,4-Diaminopyrimidines as antagonists of folic acid and folinic acids. *Arch. Biochem.* **40:** 479–481 (1952).
28. Duch, D. S., S. W. Bowers, W. D. Brenckman, Jr., and C. A. Nichol. Inhibition of brain histamine-N-methyltransferase (HMT) by diaminopyrimidine anticancer agents. *Pharmacologist* **18:** 205 (1976).
29. Dolnick, B. J., and Y-C. Cheng. Purification and characterization of thymidylate synthetase from the blast cells of patients with acute myelocytic leukemia. *Fed. Proc.* **36:** 774 (1977).
30. Jackson, R. C., L. I. Hart and K. R. Harrap. Intrinsic resistance to methotrexate of cultured mammalian cells in relation to the inhibition kinetics of their dihydrofolate reductases. *Cancer Res.* **36:** 1991–1997 (1976).

Send reprint requests to: Maire T. Hakala, Roswell Park Memorial Institute, New York State Department of Health, Grace Cancer Drug Center, 666 Elm Street, Buffalo, N. Y. 14263.

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 6, 2012