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Inhibition of Histamine-Metabolizing Enzymes and Elevation of Histamine Levels in Tissues by Lipid-Soluble Anticancer Folate Antagonists

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SUMMARY

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Potent inhibitors of dihydrofolate reductase can differ widely in the extent to which they inhibit histamine *N*-methyltransferase. This difference in activity provided a basis for selecting drugs as candidate anticancer agents which would have minimal interference with histamine metabolism. The drugs were studied *in vitro* for their effects on histamine *N*-methyltransferase and diamine oxidase (histaminase), and *in vivo* for their effects on histamine levels. Of the drugs studied, a trimethoxybenzylaminoquinazoline (TMQ, JB-11) and two diaminopyrimidines, 2,4-diamino-5-(1-adamantyl)-6-methylpyrimidine and metoprine, were found to be the most potent inhibitors of histamine *N*-methyltransferase *in vitro*, having K_i values of 7, 50, and 100 nM, respectively. TMQ and methasquin were potent inhibitors of diamine oxidase, having K_i values of 4.1 and 3.7 μM, respectively. *In vivo,* TMQ produced significant elevation of histamine levels in rat brain and kidney. A dimethoxybenzylpyridopyrimidine (BW 301U), a novel lipid-soluble antifolate being advanced for study as an antineoplastic agent, was as potent as methotrexate as an inhibitor of dihydrofolate reductase and had minimal effects on histamine levels *in vivo.* The evidence presented suggests that in the design of novel antifolates the effects of the drugs on histamine metabolism should be determined. The same approach is applicable to other classes of drugs.

INTRODUCTION

Drug toxicity is frequently a limiting factor in the use of chemotherapeutic drugs. The toxicity of antifolates used for cancer chemotherapy is manifest primarily through effects on the rapidly dividing cells of the bone marrow and gastrointestinal tract. This toxicity of antifolates is related to their properties as inhibitors of dihydrofolate reductase, leading to depletion of the reduced folate cofactors necessary for the synthesis of DNA and other metabolic functions (1). The folate-related toxicity of these drugs can be reversed by administration of calcium leucovorin. However, early clinical trials of several antifolates indicated that these compounds also possessed a significant degree of toxicity, which may not be folate related.

Clinical evaluation of metoprine as an anticancer agent indicated that this compound produced CNS,[1] cutaneous, and gastrointestinal toxicities (2–4). These toxicities suggested to us that metoprine could be acting as an inhibitor of histamine (HA) metabolism. Subsequent work (5, 6) has shown that metoprine is a potent competitive inhibitor of histamine *N*-methyltransferase (HMT) *in vitro* which acts competitively with respect to HA. This lipid-soluble diaminopyrimidine can cause complete inhibition of HMT *in vitro* at concentrations which can be attained in body fluids and tissues by doses in the range used for chemotherapy tests (2, 7). Additionally, a single oral dose of metoprine caused substantial prolonged elevations of HA levels *in vivo*, producing a 2.5- to 5-fold increase in rat brain and a 35- to 45-fold increase in rat kidney. During clinical studies of the anticancer agent triazinate, a diamino-*s*-triazine, CNS, and cutaneous toxicities similar to those seen with metoprine were observed (8, 9). Triazinate was also shown to be a competitive inhibitor of HMT *in vitro* (5).

In the central nervous system of mammalian species, HA is metabolized primarily by methylation of the nitrogen in the imidazole ring (10) in an *S*-adenosylmethionine-dependent reaction catalyzed by HMT. Interference with this inactivation process by a chemotherapeutic

[1] Abbreviations used: CNS, central nervous system; HA, histamine; HMT, histamine *N*-methyltransferase; DAO, diamine oxidase; SAM, *S*-adenosyl-L-methionine; TMQ, 2,4-diamino-5-methyl-6-[(3,4,5-trimethoxyanilino)methyl]quinazoline; DAMP, 2,4-diamino-5-(1-adamantyl)-6-methylpyrimidine.

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agent could lead to accumulation of HA in the brain with consequent headache or other adverse effects. In other tissues and organs, HA which might accumulate because of inhibition of this pathway can be disposed of by diamine oxidase (DAO) which is present in many tissues (11). Since drugs which can inhibit DAO and/or HMT may have unwanted effects, biochemical screening was used to select folate antagonists having little or no interference with these HA-metabolizing enzymes. The same approach is applicable to other classes of drugs.

Inhibition of HMT has been reported for individual members of several classes of drugs including antimalarials, anticancer agents, antihistamines, diuretics and local anesthetics (5, 6, 12–14). Drugs within each class vary considerably in potency as measured by HMT enzyme assay. However, their effects on HA levels *in vivo* were transient or negative (15, 16). Consequently, it seemed advisable to examine several types of heterocyclic compounds known to be potent inhibitors of dihydrofolate reductase in order to select those compounds likely to have minimal interference with HA metabolism.

The present report shows that several antifolates, in addition to being potent inhibitors of dihydrofolate reductase, also possess the property of being inhibitors of HA metabolism and as a result may produce unwanted side effects. This property is not characteristic of all lipid-soluble antifolates since BW 301U, a potent lipid-soluble folate antagonist (17), retains high activity as an inhibitor of dihydrofolate reductase yet has minimal activity as an inhibitor of HA metabolism. A preliminary report of this work has been presented (18).

MATERIALS AND METHODS

Chemicals. [*methyl*-^{14}C]SAM (sp act, 57.8 mCi/mmol), [*methyl*-^{3}H]SAM (sp act, 10.5 Ci/mmol), and [1,4-^{14}C]-putrescine (sp act, 96.4 mCi/mmol) were purchased from New England Nuclear, Boston, Massachusetts; histamine, putrescine, aminoguanidine, *S*-adenosylmethionine, and methotrexate (*N*-{*p*-{[(2,4-diamino-6-pteridinyl)methyl]-methylamino}benzoyl}glutamic acid, NSC 740) were obtained from Sigma Chemical Company, St. Louis, Missouri. Aminopterin (*N*-{*p*-[(2,4-diamino-6-pteridinylmethyl)amino]benzoyl}glutamic acid, NSC 739), triazinate (α-2-chloro-4-[4,6-diamino-2,2-dimethyl-*s*-triazine-1-(2H)-yl]phenoxyl)-*N*,*N*-dimethyl-*m*-toluamideethanesulfonic acid, NSC 139105), methasquin (*N*-{*p*-[{2,4-diamino-5-methyl-6-quinazolinyl)methyl}amino]-benzoyl}aspartic acid, NSC 122870), and TMQ, 2,4-diamino-5-methyl-6-[(3,4,5-trimethoxyanilino)methyl]quinazoline, NSC 249008), were provided by the Drug Development Branch of the National Cancer Institute, Bethesda, Maryland. 2,4-Diamino-5-(1-adamantyl)-6-methylpyrimidine (DAMP) was a gift from Dr. S. F. Zakrzewski, Roswell Park Memorial Institute, Buffalo, New York. Metoprine (2,4-diamino-5-(3,4-dichlorophenyl)-6-methylpyrimidine, NSC 19494) and BW 301U (2,4-diamino-5-methyl-6-(2,5-dimethoxybenzyl)pyridopyrimidine) are Burroughs Wellcome Company (Research Triangle Park, N.C.) compounds.

Enzyme assays. HMT was partially purified from the cerebral cortex of bovine brain and assayed as previously described (5, 6). DAO was partially purified from rat cecum. Ceca from 20 rats were removed and washed in physiological saline. The ceca were homogenized in 4 vol of 0.05 M sodium phosphate, pH 7.2, using a Polytron homogenizer and centrifuged at 26,000*g* for 20 min. The supernatant was fractionated with ammonium sulfate and the fraction precipitating between 25 and 60% ammonium sulfate was retained for further purification. Following dialysis of the ammonium sulfate precipitate, the preparation was charged onto a 2.5 × 25-cm column of DEAE-cellulose which had been equilibrated with 0.01 M sodium phosphate, pH 7.2, washed with the same buffer, and eluted with a linear gradient from 0.01 M sodium phosphate, pH 7.2, to 0.01 M sodium phosphate, pH 7.2, containing 0.5 M NaCl. The active fractions were pooled, concentrated, and used as the source of enzyme. DAO was assayed using the method of Okuyama and Kobayashi (19). Incubation mixtures consisted of 100 μmol sodium phosphate buffer, pH 7.2, 0.2 μmol putrescine (0.1 μCi [1,4-^{14}C]putrescine), enzyme, and where appropriate antifolate in a total volume of 1 ml. Incubations were for 20 min at 37°C. The reactions were terminated by adding 1 ml of 0.5 M borate buffer, pH 10.5, and 20 μl of 0.01 M aminoguanidine, and the radioactive product was extracted with 6 ml of toluene. Four milliliters of the toluene extract was counted in 10 ml of an Omnifluor-toluene scintillation mixture.

Kinetic analysis. Kinetic analysis was performed using the method of Lineweaver and Burk (20). K_i values were calculated from replots as described by Segal (21).

Histamine levels. The effects of drugs on histamine levels *in vivo* were determined in male Sprague–Dawley rats as described previously (5, 6).

RESULTS

The antifolates used in this study are all potent inhibitors of dihydrofolate reductase and several have been studied clinically as antitumor agents. The results presented in Table 1 show that these antifolates, in addition to being potent inhibitors of dihydrofolate reductase, also inhibit the methylation of HA *in vitro*. The most potent inhibitors were metoprine, which has also been shown to be an effective inhibitor of HA metabolism *in vivo* (5, 6), TMQ, and DAMP, novel antifolates being advanced for study as chemotherapeutic agents (22–24). Only metho-

TABLE 1

Inhibition of histamine N-methyl transferase by antifolates

Drug	Type of diamino heterocycle	Inhibition (%)				
		10^{-8} M	10^{-7} M	10^{-6} M	10^{-5} M	10^{-4} M
Metoprine	Pyrimidine[a]	0	30	69	93	98
DAMP	Pyrimidine	—	34	73	96	98
Triazinate	*s*-Triazine	0	9	46	82	97
Methasquin	Quinazoline	—	—	0	55	94
TMQ	Quinazoline	26	80	96	99	—
BW 301U	Pyridopyrimidine	—	—	0	11	91
Methotrexate	Pteridine	—	—	0	0	0
Aminopterin	Pteridine	—	—	0	0	0

[a] Trimethoprim, an antibacterial diaminopyrimidine, was inactive at 10^{-4} M as an inhibitor of HMT (5).

trexate and aminopterin showed no inhibition of HMT at concentrations up to 1×10^{-4} M.

In tissues other than brain, HA can also be metabolized by DAO. The results illustrated in Table 2 show that the same antifolates which are capable of inhibiting HMT can also inhibit DAO. In this case, however, methasquin and TMQ are the most potent inhibitors of the enzyme, whereas metoprine and DAMP, which were good inhibitors of HMT, are relatively weak as an inhibitors of DAO. Methotrexate and aminopterin, two antifolates which were inactive as inhibitors of HMT and which clinically do not exhibit side effects indicative of inhibition of HA metabolism, were also inactive as inhibitors of DAO.

Kinetic analysis of the inhibition of HMT and DAO by the antifolates most active against these enzymes, illustrated in Fig. 1 for TMQ, showed that these compounds were competitive inhibitors of HMT, with respect to HA, and noncompetitive inhibitors of DAO with respect to putrescine. The K_i values illustrated in Table 3 show that methasquin and TMQ are potent inhibitors of both HMT and DAO. In fact, TMQ, with a K_i value of 7 nm, is more than 10-fold more active as an inhibitor of HMT than is metoprine, a compound shown previously (5, 6) to be one of the most active inhibitors of this enzyme *in vitro*. Metoprine and triazinate, though both quite potent inhibitors of HMT, were relatively weak inhibitors of DAO. BW 301U, another novel antifolate also being developed for use as an antineoplastic agent (17), had weak activity as an inhibitor of both HMT and DAO.

A single dose of metoprine is capable of producing a significant elevation of HA levels in both rat brain and kidney (5, 6). *In vitro,* TMQ is a more potent inhibitor of both HMT and DAO than is metoprine. The results presented in Fig. 2 show that *in vivo* TMQ is also capable of producing elevations of HA levels in rat brain and kidney. Following a single intraperitoneal dose of 10 mg/kg, TMQ caused a twofold increase in brain HA levels. Brain levels were maximally elevated 8 h after administration of drug. Kidney levels rose sevenfold, were maximal 1 h after administration, and approached control levels at 8 h. Thus TMQ, like metoprine, is capable of elevating HA levels *in vivo*. In contrast to TMQ, administration of BW 301U at the same dose produced no elevation of HA levels in brain and less than a threefold elevation of HA levels in kidney which was of short duration. HA levels returned to control values 2 h after administration of the drug. In additional studies not shown in the present report, BW 301U did not increase HA levels in rat brain and had even less effect on HA levels in kidney than in the studies presented above.

TABLE 2

Inhibition of diamine oxidase by antifolates

Drug	Inhibition (%)		
	10^{-6} M	10^{-5} M	10^{-4} M
Metoprine	0	14	30
DAMP	0	29	72
Triazinate	0	14	43
Methasquin	15	68	92
TMQ	19	66	88
Methotrexate	0	0	16
Aminopterin	0	0	0

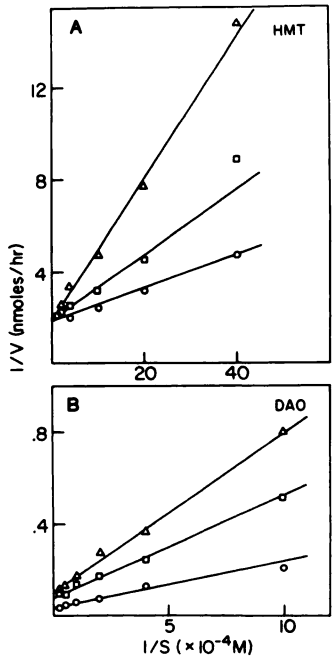

FIG. 1. *Kinetics of inhibition of histamine metabolizing enzymes by TMQ.*

(A) Inhibition of HMT by TMQ. Key: ○, no inhibitor; □, 1×10^{-8} M TMQ; △, 2.5×10^{-8} M TMQ. (B) Inhibition of DAO by TMQ. Key: ○, no inhibitor; □, 4×1^{-6} M TMQ; △, 8×10^{-6} M TMQ. Values are the means of duplicates for each point.

DISCUSSION

Histamine has been shown to produce a large number of adverse reactions whether administered directly or released endogenously. Elevation of HA levels can cause intense headache, pain, and itch, decrease in blood pressure, increase in gastric acid secretion, bronchospasm, dyspnea, metallic taste, vomiting, and diarrhea (25). During clinical trials with metoprine and triazinate, it was found that the toxicities produced by these drugs included headache, respiratory depression, fatigue, weak-

TABLE 3

K_i values of antifolates as inhibitors of histamine metabolism

	K_i ($\times 10^6$ M)	Type[a]
HMT		
Metoprine	0.1	C
DAMP	0.05	C
Triazinate	0.6	C
Methasquin	2.3	C
TMQ	0.007	C
BW 301U	1.8	C
DAO		
Methasquin	3.7	NC
TMQ	4.1	NC
BW 301U	32	NC

[a] C designates competitive inhibition and NC, noncompetitive inhibition.

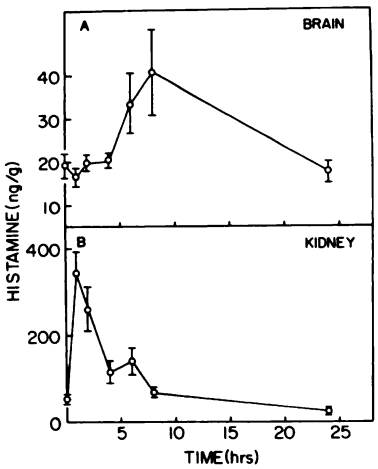

FIG. 2. *Effect of TMQ on histamine levels in rat tissue*
TMQ, 10 mg/kg, was administered intraperitoneally as a suspension in 0.5% Tween 80. (A) Rat brain; (B) rat kidney. Each point represents the mean ± SE from five animals.

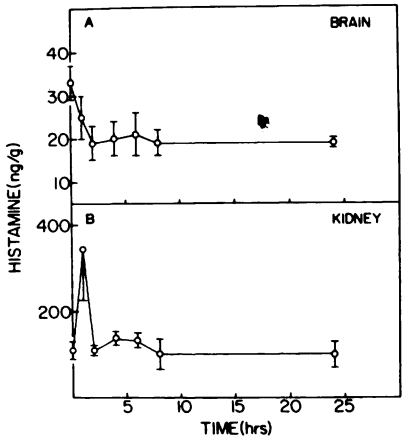

FIG. 3. *Effect of BW 301U on histamine levels in rat tissue*
BW 301U, 10 mg/kg, was administered intraperitoneally as a suspension in 0.5% Tween 80. (A) Rat brain; (B) rat kidney. Each point represents the mean ± SE from five animals.

ness, epigastric warmth, dermatological reactions, and metallic taste (2–4, 8, 9). Although histamine levels in plasma or cerebrospinal fluid were not measured during the clinical studies, the symptoms described are consistent with an elevation of HA levels in these patients. It is a difficult problem, however, to determine the extent to which CNS effects are associated with an accumulation of HA or with a drug-induced impairment of folate metabolism. These observations combined with a literature report on antimalarial agents which are inhibitors of HMT (12) led to our investigation of the effects of these drugs on the metabolism of HA.

Metoprine was found to be a potent competitive inhibitor of HMT *in vitro* and was also capable of producing marked elevation of HA levels *in vivo*. In the rat, metoprine produced a 2.5- to 5-fold elevation of brain HA levels and a 35- to 45-fold elevation of kidney HA levels. In the same study, triazinate was also shown to be a potent competitive inhibitor of HMT *in vitro* (5, 6).

The present study on the effects of other antifolates used in the treatment of neoplastic diseases and on HA metabolism *in vitro* and *in vivo* extends the previous investigations and shows that several of these drugs are potent inhibitors of HA metabolism. TMQ, metoprine, DAMP, and triazinate were the most potent inhibitors of HMT *in vitro*. Methasquin and BW 301U exhibited less activity, and methotrexate and aminopterin were ineffective as inhibitors. Since HA can also be metabolized by oxidative deamination, the effects of these drugs on the activity of DAO were also examined. Against this enzyme, TMQ and methasquin were the strongest inhibitors, while metoprine, DAMP, triazinate, and BW 301U were considerably less active. Several other quinazoline compounds which were tested were found to be potent inhibitors of both HMT and DAO. Further studies are needed to determine whether this inhibition is a common property of compounds having a quinazoline ring. As with HMT, methotrexate, and aminopterin were inactive as inhibitors of DAO.

TMQ, like metoprine, was also capable of elevating HA levels *in vivo*. Following administration of a single intraperitoneal injection of TMQ, HA levels were significantly elevated in both rat brain and kidney. Kidney levels were maximally elevated 1 h after drug administration, the earliest time point examined, and did not return to control values until 8 h after the drug was given. Peak drug levels in kidney corresponded with peak HA levels.[2] Drug concentrations at this time were approximately 1.7×10^{-5} M, a concentration high enough to completely inhibit HMT *in vitro*. However, HA levels in brain were not maximally elevated until 8 h after drug administration. This differential time course of elevation may be due to the slower accumulation of drug in brain tissue. Metoprine, which is considerably more lipid-soluble than TMQ (log *P*, the log of the octanol–water partition coefficient and a measure of the lipid solubility, is 2.82 for metoprine and 0.88 for TMQ), requires 5 h to reach peak concentrations in brain. HA levels are also maximally elevated at this time (5, 6). Thus the 8-h time period required for the maximal elevation of HA levels in the brains of animals treated with TMQ, a drug less lipid-soluble than metoprine, may reflect the time required to accumulate a concentration of TMQ sufficient to cause the observed increase in HA levels in brain. Due to technical problems in the extraction of TMQ from brain, drug levels have not been determined satisfactorily in this tissue. However, levels of HA in various brain regions are several orders of magnitude lower than the K_m value of HA for HMT (6). Thus, with a competitive inhibitor having such a low K_i for HMT, significant inhibition of enzyme activity could be expected at these substrate levels in the presence of low concentrations of TMQ. In contrast to TMQ, BW 301U produced no elevation of HA levels in the CNS. In fact, following administration of BW 301U the levels of HA in rat brain decreased relative to controls. The reason for this decrease is not known, but similar observations have been made in other studies with this drug, as well as with other pyridopyrimidines. In the data presented here as well as in studies not reported, BW 301U produced only a transient increase (0.5- to 3-fold) increase in HA levels

[2] Carl W. Sigel, unpublished observations.

in rat kidney. HA levels returned to control values by 2 h.

This inhibition of the methylation of HA could be of considerable importance in man since it has been shown that this pathway is the primary route of metabolism of the amine (26, 27). During clinical studies with metoprine, drug levels in plasma reached concentrations of 10^{-5} to 10^{-6} M within a few hours after oral administration of the drug (2). Single intravenous doses of triazinate resulted in serum levels of 10^{-5} M or higher for 8 h, and with repeated doses these serum levels could be maintained (8). Both metoprine and triazinate are quite lipid-soluble and readily enter the CNS. Following oral administration of 10 mg/kg metoprine to rats, concentrations of the drug in brain were approximately 2×10^{-5} M (7). Even higher concentrations of triazinate were found in the brain of a patient who underwent craniotomy for a brain tumor following a dose of 60 mg/m^2 (8). Thus, both compounds are capable of reaching concentrations *in vivo* which are more than sufficient to produce a significant inhibition of HMT activity *in vitro*.

BW 301U and TMQ are also moderately lipid soluble and would be expected to readily enter brain and other tissues. The elevation of HA levels in rat brain following a single intraperitoneal dose of TMQ indicates that this compound does enter the CNS. BW 301U, which is a much weaker inhibitor of HMT and DAO than TMQ, does not elevate brain HA levels and has only slight and transitory effects on HA levels in the kidney. Although methasquin, like methotrexate and aminopterin, is a polar compound and enters the cells by way of the folate transport system (28), the long intracellular half-life of the drug relative to methotrexate and aminopterin (29) may be important in the expression of nonfolate related toxicities, in this case, the inhibition of HA metabolism.

BW 301U and TMQ are each as potent as methotrexate as inhibitors of dihydrofolate reductase. However, BW 301U would appear to have an advantage over TMQ by virtue of its significantly lower inhibition of HA metabolism. Moreover, in those cases where methotrexate entry is limited, BW 301U would be useful because its entry into the cell (17, 30), like that of metoprine and triazinate, appears to be by diffusion rather than by the use of the carrier-mediated folate transport system.

In contrast to inhibitors of HA metabolism which have been previously studied (15, 16), the lipid-soluble inhibitors of dihydrofolate reductase can rapidly diffuse across the blood–brain barrier and reach high concentrations in brain. Independent of their chemotherapeutic potential for the treatment of brain tumors, those compounds which are potent inhibitors of HA metabolism now provide a means of elevating brain levels of HA which may be useful in assessing the function of HA in the CNS.

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