Uptake of Methotrexate, Aminopterin, and Methasquin and Inhibition of Dihydrofolate Reductase and of DNA Synthesis in Mouse Small Intestine

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SUMMARY

The lethal potencies of aminopterin, methasquin (MQ), and methotrexate (Mtx) in mice given single i.v. injections are in the respective order of 14:5:1. Doses causing (at 1 hr after injection) 50% inhibition of the incorporation of deoxyuridine into DNA of mouse small intestine are in a different order, namely, 2:0.6:1 for 0.06, 0.20, and 0.12 mg/kg, respectively. These minute doses are close to the calculated amount of each drug that would be just sufficient to bind and inhibit all of the folate reductase in the entire animal. Since intestinal degeneration is a probable cause of death in mice given lethal doses of the antifolates, the differing cytotoxic potencies of the agents must be related to factors other than the extent of the initial inhibition of the methylation of deoxyuridylic acid. Duration of action may be the prime factor. The inhibition caused by Mtx is more reversible than that caused by either aminopterin or MQ. For maintenance of 50% inhibition for 18 hr, 30 mg Mtx per kg must be given, whereas aminopterin and MQ would have the same effect at 0.9 and 0.4 mg/kg. Comparisons of the kinetics of uptake of the agents by small intestine are qualitatively similar to those observed previously in L1210 cells in vitro. Thus, aminopterin is more rapidly cumulated than is Mtx, while the uptake of MQ is slow by contrast with both pteridine derivatives. The rate loss of MQ from intestinal tissue is also slower than that of aminopterin or Mtx. Moreover, free MQ circulates in serum for appreciably longer times than either aminopterin or Mtx. The differences in potency and duration of action of MQ and Mtx may be related to differences in their respective rates of entry into and egress from susceptible cells; however, the greater potency of aminopterin remains inexplicable.

INTRODUCTION

Previous studies of the 2,4-diaminoquinazoline antifolate, MQ, have shown the agent to be a more potent inhibitor of L1210 leukemia than Mtx (7) and also more toxic to normal proliferating tissues such as the small intestine (12). In species such as the dog and man, the toxic activity is comparable to that of the highly potent antifolate aminopterin (5, 12, 13). Explanations for the high in vivo activity of MQ are not apparent. Studies in vitro indicate that MQ has greater affinity for microbial and mammalian folate reductase than does Mtx (1, 2, 8) but significantly less affinity for the carrier involved in active transport into L1210 cells (17).

The purpose of this work is to compare MQ and Mtx in a susceptible proliferating tissue in vivo, the crypt epithelium of mouse small intestine, by means described in recent work with Mtx (10). The comparison includes studies of uptake by small intestine, of duration of unbound drug in serum, of inhibition of the methylation of deoxyuridylic acid as measured by changes in the incorporation of UdR-3H into intestinal DNA, and of capacity to combine with intestinal folate reductase. To provide more meaningful data, we also compare both antifolates with aminopterin. Hopefully, these studies may provide further understanding of the mechanisms involved in the selective effects of antifolates in vivo.

MATERIALS AND METHODS

The animals used were male Swiss mice of the CD/1 line (Charles River Breeding Laboratories, Wilmington, Mass.), 5 to 6 weeks old and weighing 24 to 30 g. The procedures used for i.v. injection and for removing the entire small intestine, rinsing the tissue to remove luminal contents, homogenizing in 0.25 M sucrose, and centrifuging to prepare supernatants for enzyme and drug assays have been described (10). Blood for serum was obtained by severing the brachial plexus of etherized animals. The blood was allowed to clot for 30 min at room temperature and for an additional 60 to 90 min in ice before centrifugation. The measurement of the incorporation of TdR-3H or of UdR-3H into intestinal DNA has also been described (10).

Mtx and aminopterin were generously provided by the Lederle Laboratories, Pearl River, N. Y., and MQ was provided by the Drug Development Branch, Drug Research and...
Development, Chemotherapy, National Cancer Institute, Bethesda, Md. The drugs were used as supplied, for injection into mice. The purity of Mtx and MQ was equivalent to that of samples used in earlier studies (10, 12). The aminopterin, however, was a relatively impure preparation which was found by dihydrofolate reductase titration (see below) to have 59% of the inhibitory activity of chromatographically purified material. An identical percentage was recovered in the inhibitory fraction obtained by chromatography. Moreover, in preliminary studies, column eluates containing the inhibitory fraction were injected i.p. into mice daily for 5 successive days and were found to be about twice as toxic as the impure preparation. Attempts to obtain purified aminopterin by lyophilizing chromatographic fractions were unsuccessful, since the dried material was found to be contaminated with folic acid. Interestingly, folic acid is the major impurity detected by chromatography in samples of aminopterin supplied by the manufacturer. In view of such findings it was decided to use the crude sample directly in mice and to adjust doses to contain the amounts of aminopterin that are reported below.

**Drug Assay.** Total drug was determined in sera and in intestinal supernatants deproteinized by heat denaturation by a titration procedure measuring the inhibition of dihydrofolate reductase (20). The titrating enzyme was a partially purified reductase obtained from a strain of *Diplococcus pneumoniae* that is highly resistant to antifolates (18). Reference samples of aminopterin and Mtx for use as standards were purified chromatographically (16). In the present assay, the details of which have recently been described, the 3 drugs are stoichiometrically equivalent in potency between 0 and 80% inhibition of enzyme activity (17).

**Enzyme Assay.** Folate reductase in intestinal supernatants from untreated mice was titrated with drugs in a manner similar to that described by Werkheiser et al. (20). Endogenous isocitrate dehydrogenase required for regeneration of TPNH was activated by MnCl2, and nicotinamide was added to inhibit TPNH oxidase. Each assay tube in ice received 0.1 ml of 1 M 3,3-dimethylglutarate buffer, pH 6.1; 0.1 ml of 0.05 M DL-isocitrate (trisodium salt, type 1, Sigma Chemical Co., St. Louis, Mo.); 0.1 ml of 0.1 M nicotinamide; 0.02 ml of 1 M MnCl2, 0.02 ml of 2 mM TPNH; and 0.1 or 0.2 ml of intestinal supernatant. The mixture was brought to 0.8 ml with drug solution and distilled H2O and then was incubated for 5 min at 37°. After the tubes were returned to an ice bath, 0.2 ml of 0.4 mM folate was added, and the tubes were reincubated at 37° for 45 min. The presence of tetrahydrofolate was measured as diazotizable amine by the Bratton-Marshall reaction.

Free enzyme activity in intestines of treated mice was compared with that of control mice by incubation of 0.1 ml of supernatant without drug for 45 min. Under these conditions, the production of tetrahydrofolate was directly proportional to the amount of free enzyme present.

**RESULTS**

**Drug Toxicity.** We compared the 3 agents simultaneously by giving different drug doses to groups of 4 to 5 mice and replicating the experiment until 12 to 17 animals had been tested per dose. The median lethal doses of aminopterin, MQ, and Mtx were calculated to be 30, 83, and 425 mg/kg, respectively (Chart 1). None died earlier than 3 days after injection, and all but 2 deaths occurred between 3 and 7 days after injection. With respect to time of death, appearance of diarrhea, and weight loss, the course of intoxication was similar to that in recent descriptions of mice that received Mtx (10) or MQ (12).

**Intestinal DNA Synthesis.** In previous work, Mtx was found to inhibit UdR-3H incorporation into intestinal DNA maximally within 1 hr after its injection. The duration of maximal inhibition was dose dependent; recovery began within 2 to 4 and 4 to 6 hr after doses of 0.5 and 5 mg/kg, respectively (10). Preliminary studies with MQ also showed that inhibition of UdR-3H incorporation was maximal within 1 hr after administration of doses as low as 0.4 mg/kg; however, by contrast with the effects of comparable doses of Mtx, there was no recovery during the 1st 6 hr in mice given MQ. It appeared that MQ might be a more potent inhibitor or have more prolonged action.

The experiment of Chart 2 was done to distinguish between these possibilities. Various doses of Mtx, MQ, and aminopterin were injected at 0 time, and their effects on UdR-3H incorporation were determined 1, 6, or 18 hr later. From the data obtained, ID50 values were approximated for each drug at each time. At 1 hr after injection, the respective values for aminopterin, Mtx, and MQ were found to be 0.06, 0.12, and 0.2 mg/kg. At 6 hr, the ID50 values for aminopterin and Mtx were higher (0.15 and >0.4 mg/kg), while that for MQ (0.15 mg/kg) was somewhat less than at 1 hr. Much larger doses of Mtx were required to maintain inhibition for 18 hr. At this time, the ID50 for Mtx was approximately 30 mg/kg, while those for MQ and aminopterin were 0.4 and 0.9 mg/kg, respectively. It is evident (Chart 2) that Mtx and MQ are roughly equipotent when compared at 1 hr after injection, but that the 2 agents differ significantly in duration of action. Thus, to maintain a 50% inhibition for 18 hr, Mtx must be given in a 75-fold greater dose than MQ (that is, 30 versus 0.4 mg/kg). Similarly, aminopterin has a more prolonged action than Mtx and, in addition, is somewhat more potent at 1 hr.

The 1-hr ID50 values shown in Chart 2 are close to the estimated dose of Mtx that would be required to inactivate completely the folate reductase of small intestine and liver, i.e., 0.03 mg/kg. The latter value is derived from previous work with male CD/1 mice, in which the enzyme content of the 2 organs in titrating equivalents of Mtx was found to be 156 and 417 ng/g (10), and from the fact that each organ is about 5% of body weight. From what is known of other mammalian organs (3), it is likely that twice the dose of 0.03 mg/kg would be sufficient to inactivate total reductase in all tissues. The ID50 values for aminopterin, Mtx, and MQ at 1 hr are only about 2, 4, and 7 times greater, respectively, than the "titrating" dose of Mtx for small intestine and liver.

The values for the ID50 of aminopterin and Mtx at 18 hr
are in fair agreement with previous estimates of minimal doses required to free folate reductase in intestinal mucosa of mice to negligible quantities at 24 hr after injection (19); these minimal doses were, respectively, about 3 and 100 mg/kg or, with each agent, somewhat more than 3 times the 18-hr ID₅₀.

The inhibitions of UdR-3H incorporation (Chart 2) are probably due primarily to specific action of the agents on the conversion of deoxyuridylic to thymidylic acid. This assumption is supported by an experiment in which large doses of aminopterin and MQ were shown to have relatively little effect on the incorporation of TdR-3H into DNA. For this purpose, mice received i.v. injections of various doses of the agents or 0.9% NaCl solution and were killed 1 or 18 hr later. Ten min before being killed, they received TdR-3H (2 μmoles/kg; 10 μCi/μmole). The mean specific activity ± S. D. of intestinal DNA in 8 controls killed at 1 hr was 3020 ± 510 dpm/μmole DNA deoxyribose and in 8 other mice killed at 18 hr it was 2410 ± 450. In 10 mice given injections of MQ (3 at 1 hr after receiving 40 mg/kg, 3 at 1 hr after receiving 4 mg/kg, and 4 at 18 hr after receiving 4 mg/kg), the mean specific activity was 2090 dpm/μmole DNA deoxyribose, with individual values ranging from 1270 to 2750. In 8 mice given aminopterin (2 at 1 hr after receiving 40 mg/kg, 2 at 1 hr after receiving 4 mg/kg, and 4 at 18 hr after receiving 4 mg/kg), the mean specific activity was 2320 and the range was 1440 to 3150. Large doses of Mtx have also been found to have relatively little effect on thymidine incorporation in mouse intestine (10).

In previous work, cytotoxic doses of Mtx were found to cause significant losses of total DNA in the small intestine of CD/1 mice as the result of mitotic inhibition, necrosis, and atrophy, of crypt epithelium (10). By 24 hr after administration of 50 mg/kg, the total loss was about 25%, but it was negligible after they were given 5 mg/kg (10). In this work, the Mtx-treated mice (Chart 2) had little change in intestinal DNA by 18 hr. Mean values ± S. D. in animals that received 50, 15, and 5 mg/kg were found to be respectively equivalent to 31.5 ± 3.8, 30.9 ± 2.7, and 31.2 ± 1.6 μmoles deoxyribose per intestine. The 18-hr controls (Chart 2) had a mean DNA content of 32.1 ± 2.5. MQ and aminopterin, by contrast, caused significant losses. At 18 hr, the mice (Chart 2) given MQ in doses of 1.2, and 0.4 mg/kg had, respectively, 24.4 ± 3.3, 26.5 ± 3.2, and 28.0 ± 2.2 μmoles deoxyribose per intestine, and those given the same doses of aminopterin had 25.2 ± 2.2, 26.4 ± 2.5, and 30.8 ± 2.6. Thus, the higher activity of MQ and aminopterin in causing prolonged inhibition of deoxyuridine incorporation into DNA is associated with higher cytotoxic potency.

**Serum Concentrations.** Free Mtx disappears rapidly from the circulation due to renal and biliary excretion and, after low doses, due to binding to folate reductase in enzyme-rich tissues such as liver and intestine (11). Chart 3 shows that the rate of disappearance of aminopterin from serum was similar to that of Mtx over the 100-fold range of doses tested but that MQ was lost from the circulation more slowly. This result is in keeping with findings that, in rats, the urinary and biliary excretion of MQ appeared less rapid than that of Mtx (14). All 3 agents, however, reach negligible concentrations in mouse serum within 1 to 2 hr after injection of 0.1 mg/kg, an amount only slightly in excess of the folate reductase content of liver and intestine (see above).

**Titration of Intestinal Folate Reductase.** Equivalent end points were obtained when intestinal folate reductase was titrated at pH 6.1 with either Mtx, aminopterin, or MQ. In the titration illustrated in Chart 4, the end points values for the 3 drugs were essentially the same; the single end point depicted...
Chart 3. Drug concentrations in serum of mice given different i.v. doses of aminopterin, Mtx, and MQ. ●, 10 mg/kg; ○, 1 mg/kg; ▲, 0.1 mg/kg. Each point is the result from a single animal. The lines connect average values obtained at different times after different doses. Serum concentrations < 1 ng/ml are plotted arbitrarily just above the abscissa.

Chart 4. Titration of folate reductase with aminopterin, Mtx, and MQ in supernatant prepared from the small intestine of untreated mice. The amount of drug added to each reaction tube is expressed as ng/g of intestinal wet weight. In the chart was 161 ng/g. In a replicate determination with supernatant prepared from a 2nd pool of control intestines, the end point was 143 ng/g. These values are similar to the previously reported mean ± S. D. of 156 ± 19 ng Mtx per g for the intestinal enzyme content of control CD/1 mice (10).

Intestinal Drug Concentrations. The uptake of the 3 antifols by intestinal tissue was studied after the administration of doses that spanned the range of the 1-hr ID₅₀ values of Chart 2 and were equivalent to 1.7 and 6.7 times the total folate reductase content of liver and small intestine (see above). The results are shown in Chart 5. After the injection of aminopterin, drug uptake was rapid and maximal within 15 min. Slower rates of cumulation were evident in animals given the 2 lower doses of Mtx, although the final concentrations at 2 hr were similar to values obtained after the administration of equivalent amounts of aminopterin. The uptake of MQ was considerably less than that of aminopterin or Mtx after each of the 3 doses tested, although the drug was cumulated maximally within 15 to 30 min after injection. The maximal concentrations obtained with each drug varied in a dose-dependent manner. After Mtx and aminopterin were given in doses of 0.05 and 0.1 mg/kg, the maxima were less than the folate reductase content of intestine [i.e., 156-ng equivalents of Mtx per g (10)] while, after either drug was given at a dose of 0.2 mg/kg, the values initially exceeded and then decreased to enzyme levels by 1 to 2 hr. The maximal concentration achieved after MQ was given at a dose of 0.2 mg/kg was equivalent to about one-half of enzyme content. Between 2 and 6 hr after injection of 0.2 mg/kg, aminopterin and Mtx concentrations slowly decreased while MQ levels remained constant.

The data of Chart 5 suggest that after treatment with MQ there is less drug bound to intestinal folate reductase and, therefore, more free enzyme activity than after equivalent doses of aminopterin and Mtx. Further evidence for this was obtained in the experiment (Chart 6) in which total drug concentration and free enzyme activity were compared in intestinal supernatants after injection of 0.2 mg/kg. As in the animals described in Chart 5, the mice given Mtx and aminopterin had initial drug concentrations at 1 hr that were nearly equivalent to intestinal enzyme content. Free enzyme activity was less than 5% of that in untreated controls. Between 1 and 6 hr, the drug levels fell steadily, while there were simultaneous increases in free enzyme. In the animals given MQ, the drug content remained constant between 1 and 6 hr at approximately one-half of intestinal enzyme content. Correspondingly, free enzyme activity remained essentially constant in between 30 and 40% of untreated controls. Losses of drug from intestines of mice given Mtx and aminopterin were also evident during the period between 4 and 24 hr when

Chart 5. Drug concentrations in intestine of mice given different i.v. doses of aminopterin, Mtx, and MQ. ●, aminopterin; ○, Mtx; ×, MQ. Values are expressed in ng/g, wet weight. Each value is the mean ± S.D. for 3 mice except that, at 1 to 6 hr after administration of 0.2 mg/kg, there are 6 to 9 mice per point.
they averaged 35 and 45 ng/g, respectively. During the same period, the average loss of MQ was 17 ng/g. Average increases in free enzyme activity were also greater between 4 and 24 hr after injection, in mice given Mtx and aminopterin than in those given MQ, namely, 43 and 39 versus 28%. The results with Mtx (Chart 6) are in agreement with previous studies of CD/1 mice given doses of 0.5 and 5 mg/kg; in those studies, substantial increases occurred between 8 and 24 hr after injection (10).

**DISCUSSION**

Although the 3 antifolates have been shown to be qualitatively similar in their cytotoxicity for the proliferating crypt epithelium of intestine, aminopterin and MQ are significantly more potent than Mtx in inducing intestinal lesions (12, 13). In this work, we have studied the incorporation of deoxyuridine into DNA to estimate the functional disturbance induced by each drug in intestinal DNA synthesis. Presumably, decreases in incorporation are due mostly to inhibition of the methylation of deoxyuridylic acid by thymidylate as the result of primary inactivation of folate reductase and depletion of tetrahydrofolate derivatives (3). We have shown that the 3 drugs do not differ greatly in potency when their capacity to inhibit DNA synthesis is measured at an early time after injection (Chart 2). However, when they are compared for prolonged action, it becomes evident that Mtx is significantly less potent than either aminopterin or MQ. Thus, Mtx must be given in doses that exceed those of aminopterin or MQ by more than an order of magnitude if significant inhibition is to be maintained for at least 18 hr (Chart 2). Previous work has shown that pathological changes occur in the small intestine of mice only after the administration of doses of Mtx that cause persistent inhibition of DNA synthesis. Presumably, decreases in free enzyme activity were also greater between 4 and 24 hr after injection (10). The greater reversibility of the primary biochemical action of Mtx would appear to account for the fact that it must be given in higher doses than MQ or aminopterin in order to induce equivalent cytotoxic and lethal effects.

Two factors seemingly contribute to the longer duration of action of MQ. When given in doses that approach intoxicating levels, MQ disappears from the circulation at slower rates than Mtx [compare Mtx and MQ in serum after doses of 1 and 10 mg/kg (Chart 3)]. MQ also persists in intestinal tissue, presumably bound to folate reductase, for longer periods than does Mtx (Charts 5 and 6). Neither of the above factors accounts for the longer duration of action of aminopterin. Its rate of disappearance from serum is not appreciably different from that of Mtx (Chart 3), and it escapes from intestinal tissue about as rapidly (Charts 5 and 6). However, the similarity in rates of loss of Mtx and aminopterin appears to be true only after the administration of low doses of an order of magnitude equivalent to the total folate reductase content of the small intestine of mice given aminopterin, Mtx, and MQ (0.2 mg/kg, i.v.). , aminopterin; o, Mtx; X, MQ. Drug concentrations are expressed in ng/g; wet weight; enzyme activity is expressed in percentage of controls given 0.9% NaCl solution i.v. The points at 1, 2, 4, and 24 hr are means ± S.D. for 5 mice; the points at 6 hr are for individual mice.

There are discrepancies between the results of Charts 2 and 5 in the relationship between the amount of drug in the tissue and the extent of the inhibition of intestinal DNA synthesis. For example, at 1 hr after injection of MQ and Mtx (0.2 mg/kg), there are nearly equivalent inhibitions of UdR-3 H incorporation (Chart 2). However, Chart 5 shows that at 1 hr Mtx is present in intestinal tissue of mice given 0.2 mg/kg in an amount equivalent to or in excess of folate reductase content, while there is only one-half as much MQ. At 6 hr the persistence of Mtx itself is increased when the drug is given in doses with significant pathological effects (10). The difference between low and high doses raises the possibility that there are enzymatic sites of action in susceptible cells, other than folate reductase, for which aminopterin may have greater affinity than does Mtx. These could be affected when nonreductase-bound drug is present in high concentration as the result of high levels of cumulation after large doses. Levels of accumulation of aminopterin and Mtx above the folate reductase content of intestine are shown in Chart 5 after doses of 0.2 mg/kg. Even higher amounts have been reported following larger doses of Mtx (10). Uptake in excess of folate reductase content also has been demonstrated in L1210 cells in vitro (17). Suggestions of other sites of enzyme inhibition such as thymidylate synthetase have already been advanced by others (4, 15).

Charts 3 and 5 show that aminopterin and Mtx and possibly MQ are concentrated by intestinal tissue to an extent that exceeds levels of circulating free drug. There is a remarkable similarity in uptake by intestine in vivo with that shown by L1210 cells in vitro; aminopterin is accumulated more rapidly than Mtx, and the uptake of both pteridines exceeds that of MQ (17). It is reasonable to propose that intestinal tissue has a carrier-mediated transport system for the antifolates with properties similar to that of the leukemic cells (6, 17).
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concentration of MQ remains the same as at 1 hr and is lower than that in mice given the same dose of aminopterin; yet the inhibition caused by MQ is equivalent to or greater than that induced by aminopterin. Such findings suggest that folate reductase may be distributed among proliferating and nonproliferating components of the intestinal epithelium and that MQ is preferentially accumulated by cells in the former compartment. If so, its binding to a smaller fraction of the total tissue enzyme would result in equivalent inhibitions of DNA synthesis.

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