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A basis for the difference in toxicity of methotrexate, aminopterin and methasquin in mice

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The folate analogs, methotrexate (MTX), methasquin (MQ) and aminopterin (AM), vary considerably in their toxic potency in animals [1–10]. The basis for this difference is still not understood. All three derivatives affect DNA synthesis via inhibition of dihydrofolate reductase [10–16]. This inhibition is nearly stoichiometric in each case [10], and any differences shown to occur among these analogs are insignificant in respect to any overt manifestation on toxicity.

In mice, MTX, MQ and AM have a similar qualitative effect [2, 9, 10] on the proliferating crypt epithelium of the small intestine, the most drug-sensitive tissue in this species. The LD_{10} or LD_{50} for MTX, MQ and AM after a single dose, however, is roughly in the order of 18:3:1. Part of the increased toxicity associated with MQ when compared to MTX may be attributed to a lower rate of clearance from plasma [10]. The rate of clearance of MTX and AM, however, are essentially the same [10]. Previous work [10, 17] has also shown that the degree of lethality observed at varying doses of MTX is proportional to the duration of inhibition of DNA synthesis in the small intestine. The duration of inhibition by MTX in this tissue has subsequently been found [18] to depend upon the extent to which drug persists at levels above the dihydrofolate reductase content. In the present study, we have examined the extent of uptake and persistence of free (unbound to enzyme) MTX, MQ and AM in mouse small intestine at the approximate equimolar, equitoxic and therapeutically optimum doses. Our results suggest, as a basis for the relative toxicity of these agents, differences in the degree of persistence of each in this highly susceptible tissue.

BDF_1 (C57BL/6 × DBA_2 male) mice were injected intraperitoneally with drug. At varying times thereafter, animals were sacrificed by cervical dislocation, and the small intestine was surgically removed. The methods used for washing and homogenizing the tissue and extracting drug have been described [10, 17, 18]. The drug content of the heated tissue supernatant was determined by a titration assay with a microbial dihydrofolate reductase [19]. Values given in the results are averages based on determinations with two to four animals. The dihydrofolate reductase content in unheated tissue extracts was obtained by titration with antifolate [13, 19]. MTX and AM were generously supplied by Lederle Laboratories. MQ was obtained as a gift from Parke Davis & Co. When analyzed by enzyme titration, samples of MTX and MQ were essentially pure. The AM sample was only 59 per cent pure as determined by the same assay. An identical percentage was recovered in the inhibitory fraction obtained by chromatography on DEAE-cellulose. AM dosages are expressed as the equivalence in pure drug.

The uptake and loss of MTX, MQ and AM in mouse small intestine after the administration of 3 mg/kg i.p. are

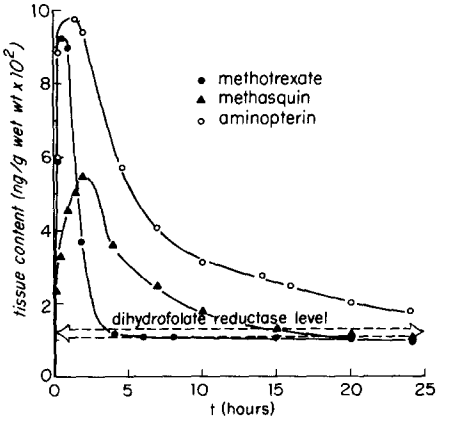

Fig. 1. Drug content of mouse small intestine at varying times after the administration of MTX, MQ or AM at 3 mg/kg i.p. Each point represents an average of two to four animals in two separate experiments.

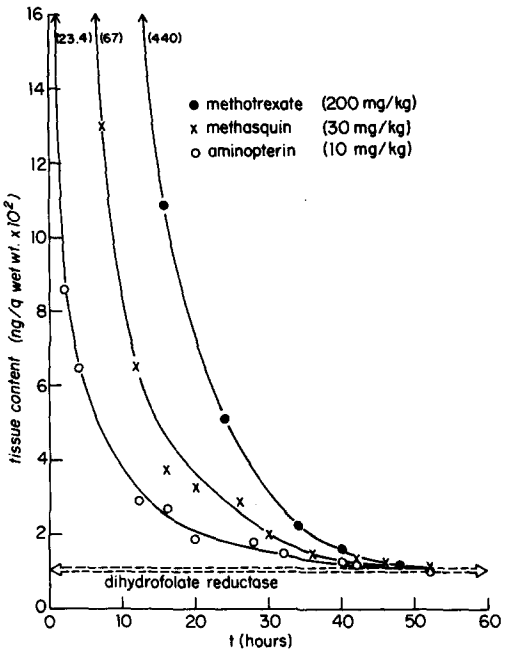

Fig. 2. Drug content of mouse small intestine at varying times after the administration of the approximate LD_{10} dose of MTX (200 mg/kg), MQ (30 mg/kg) and AM (10 mg/kg). Each point represents an average of two to four animals in two separate experiments.

shown in Fig. 1. The accumulation of MTX and AM was somewhat more rapid than that of MQ (maximum accumulation of MTX and AM occurred within 1 hr, but only after 2 hr with MQ). The tissue level of MTX and AM accumulated was about 9-fold the dihydrofolate reductase content (1·0 to 1·2 × 10^2 ng/g wet wt), but only 4- to 5-fold for MQ. Free MTX persisted intracellularly for only 3–4 hr after injection. However, free MQ persisted for about 16 hr and free AM for greater than 24 hr. Prior studies [5, 8] have shown

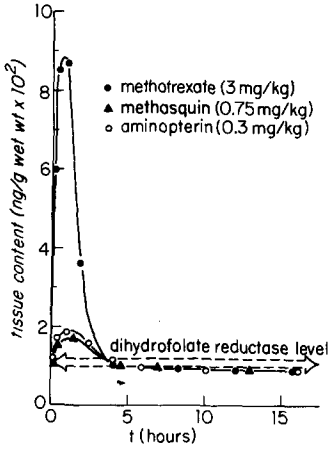

Fig. 3. Drug content of mouse small intestine at varying times after the administration of MTX (3 mg/kg), MQ (0·75 mg/kg) and AM (0·3 mg/kg) i.p. Each point represents an average of two to four animals in two separate experiments.

that at multiple daily doses, 3 mg/kg of MQ and AM are toxic, but MTX is not.

Data obtained in a similar experiment employing the approximate LD_{10} dose for MTX (200 mg/kg), MQ (30 mg/kg) and AM (10 mg/kg) are shown in Fig. 2. In approximately 1 hr, AM accumulated to about 20 times the dihydrofolate reductase level. The maximum accumulation of MQ was nearly 70-fold and MTX was greater than 400-fold enzyme level in the same time. Loss of drug was most rapid in the case of MTX and least rapid for AM, so that the concentration of all three drugs in tissue reached the level of the dihydrofolate reductase content at about the same time (50 hr).

The uptake and loss of drug in mouse small intestine were also measured after the single i.p. administration of the optimal, every other day, anti-leukemic dose. For MTX, MQ and AM this was 3·0, 0·75 and 0·3 mg/kg respectively. The results are given in Fig. 3. In this experiment, the maximum uptake of MTX (8-fold enzyme level) occurred in 1 hr and the drug content of tissue was reduced to enzyme level in about 4 hr. The accumulation of MQ and AM was maximum within 1 hr, but in both cases accumulation did not reach 2-fold enzyme level. Like MTX, the tissue content was reduced to enzyme level in about 4 hr. At these doses given every other day, no toxicity is observed with any of the three agents [5, 8]. Data in Fig. 3 also show that the loss of MTX, MQ and AM bound to dihydrofolate reductase in mouse small intestine occurred at essentially the same low rate. This is in agreement with the similar degree of titration inhibition of this enzyme obtained with all three drugs [10].

In other studies [10], measurements of initial drug uptake in the small intestine made at very low doses (0·05–0·2 mg/kg i.v.) revealed a very rapid rate of uptake of AM, but a very low rate of uptake of MQ, when compared to MTX uptake. In L1210 leukemia cells [19, 20], the rate of influx *in vitro* of AM as compared to MTX and MQ was also very rapid. Consequently, steady-state levels in L1210 cells of AM were significantly higher than those attained with MTX. Although the rate of influx of MQ in these cells was also very low, steady-state levels of MQ were higher than with MTX. This can be attributed [19, 20] to the extremely low rate of efflux of this drug when compared to both MTX and AM. Similar relationships between the influx and efflux of MTX, MQ and AM might also explain the difference in persistence of free drug in the small intestine at a time when plasma levels are falling to a low level. In addition, a greater persistence of MQ in the small intestine in comparison to MTX might be due in part to the lower rates of clearance from plasma also observed in mice [10]. Differences in the rate of entry into tissue were suggested by Werkheiser [21] as a possible explanation for the greater toxicity of AM as compared to MTX.

Some correlation has been demonstrated [22, 23] between the relative rates of oxidation of antifolates by liver aldehyde oxidase and toxicity in different animal species. Aldehyde oxidase from rabbit and guinea pig, both relatively resistant species, readily metabolizes both MTX and AM, but enzymes from the highly susceptible mouse and rat are only poorly active [24]. Although some difference could also be shown in the oxidation of MTX and AM by mouse and rat liver enzymes, any relevance to the relative toxicity of these two agents in these species is questionable, since the overall level of activity of these enzymes is so low. In fact, when given to mice, very little MTX is actually metabolized, since almost none of the drug is recovered in urine in an oxidized form [25].

Appreciable metabolism of MTX has been shown to occur in the lumen of mouse small intestine by microbial action [26, 27]. The net result demonstrated was a reduction in the absorption of drug and in the amount cleared from plasma via the kidney. Since parentally administered drug (as in the present study) enters the gut via the biliary secretions, it is conceivable that some difference in metabolism of MQ, AM and MTX might contribute to the differences observed in the pharmakinetics of these agents. This appears unlikely, however, for the following reasons. In the first place, the absorption of MQ from the gut in both rats and dogs has been shown to be negligible [28]. One would expect that a similar low level of absorption occurs in mice. This is in agreement with the generally low rate of penetration of this agent also found in mouse intestinal tissue [10] and murine tumor cells [18–20]. Second, any appreciable difference in the metabolism of AM and MTX, which would affect the amount of absorption, should be reflected in a difference in plasma levels, especially during the elimination phase [29]. Plasma pharmakinetics for each agent were, in fact, found [10] to be essentially identical in mice during an interval encompassing a portion of the elimination phase [10].

In the main, the difference in the uptake and persistence of free MTX, MQ and AM in mouse small intestine shown here agrees with data reported previously [10] on the onset and duration of the inhibition of DNA synthesis in this organ at comparable doses. The correlation between the drug data presented here and the biochemical effects in susceptible proliferating tissue on the one hand and between the relative toxicity on the other hand strongly suggests the importance of membrane-transport phenomena in mediating lethal or toxic effects of this class of drug.

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