Potent Bile Salt and Organic Anion Inhibition of Methotrexate Uptake and Accumulation in the Freshly Isolated Rat Hepatocyte

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ABSTRACT

Influx of [3H]methotrexate into freshly isolated hepatocytes is mediated by high- and low-affinity processes, both of which are inhibited by the bile salts, cholate, taurocholate, and deoxycholate, and the organic anions, bromosulfophthalein and rose bengal. At 100 μM concentrations, these compounds inhibit 1 μM [3H]methotrexate influx by 70 to 90%. Dixon plots established a similar Kᵢ (~20 μM) for taurocholate inhibition of the high- and low-affinity influx routes for methotrexate, although the kinetics of this inhibition has not, as yet, been established. Bile salt inhibition of methotrexate influx requires the simultaneous presence of the bile salt and methotrexate at the cell membrane. Hence, pretreatment of hepatocytes with taurocholate, with subsequent removal of the bile salt from the extracellular compartment, does not alter methotrexate influx. Methotrexate at high concentration was found to inhibit the influx of [3H]taurocholate, suggesting the possibility of shared transport site(s) for bile salts and methotrexate. Influx of the naturally occurring folate, 5-methyltetrahydrofolate, is also inhibited by the bile salts and bromosulfophthalein, although to a lesser extent than influx of methotrexate. Cholate and taurocholate reduce not only methotrexate influx but also the net level of intracellular methotrexate accumulation, with a proportional reduction in the synthesis of methotrexate polyglutamate derivatives. Deoxycholate and bromosulfophthalein inhibit methotrexate polyglutamate synthesis to a greater extent than inhibition of total drug uptake. The presence of albumin in the buffer markedly reduces bromosulfophthalein inhibition of MTX influx, but has a much lesser effect on taurocholate inhibition of methotrexate influx, presumably related to the much higher affinity of albumin for bromosulfophthalein than for taurocholate.

These studies suggest that the level of bile salts in the hepatic sinusoids under physiological conditions may influence uptake and accumulation of methotrexate and its polyglutamate derivatives in hepatic parenchymal cells and may, therefore, influence the potential of this agent for producing hepatotoxicity in chemotherapeutic regimens. This raises the possibility that alterations in this interaction, in terms of regulation of bile salt pool size or time of administration of methotrexate relative to meals, may yield useful approaches for reducing methotrexate accumulation and metabolism in the liver and thereby minimize methotrexate hepatotoxicity especially in long-term low-dose regimens.

INTRODUCTION

Influx of the folate analog MTX into suspensions of freshly isolated hepatocytes is mediated by 2 routes that differ primarily on the basis of their markedly different affinities for this agent (19). While, unlike other mammalian cells, the MTX influx process appears to be distinct from that of the naturally occurring folates (19, 27), there is evidence that transport of MTX and organic anions is related in that BSP and cholic acid inhibit uptake of MTX by the isolated perfused liver (41). Since the liver is responsible for the uptake from the plasma and secretion into the bile of a variety of organic acids and anions (3, 24) and since MTX is a bivalent anion subject to enterohepatic circulation (39, 40), studies were undertaken to examine the relationship among transport of bile salts, organic anions, and MTX in the freshly isolated hepatocyte system.

These studies demonstrate that bile salts and organic anions transported by the liver are potent inhibitors of both components of MTX influx as well as net MTX accumulation in the rat hepatocyte. These findings raise the possibility that hepatic sinusoid levels of bile salts during MTX chemotherapy may modify MTX uptake by the liver and may be a factor in determining drug hepatotoxicity (12, 23, 34, 43).

MATERIALS AND METHODS

Preparation of the Hepatocyte Suspension. Hepatocytes in suspension were prepared from male Sprague-Dawley rats by the collagenase perfusion method of Berry and Friend (7) except that hyaluronidase was excluded from the perfusion medium and the rate of perfusion was reduced to 30 ml/min. Cell membrane integrity, as determined by trypan blue exclusion, was greater than 85%.

Cells were washed in the buffer utilized for the uptake studies. This buffer contained 136 mM NaCl, 4.4 mM KCl, 16 mM NaHCO₃, 1.1 mM KH₂PO₄, 1 mM MgCl₂, and 1.9 mM CaCl₂, pH 7.4. Cells were stored on ice for no more than 45 min before experimentation.

Uptake Measurements. Measurement of initial uptake rates and net fluxes utilizing [3H]MTX, 5-[14C]CH₃-H₄-folate, [3H]AIB, and [3H]taurocholate used techniques described in detail previously (17, 22), and in the legends to the charts. The hepatocyte pellet was suspended gently in buffer, placed in incubation flasks, and stirred continuously with a Teflon paddle. The suspension was gassed with warmed and humidified 95% O₂-5% CO₂. Radiolabeled compounds were added, following which portions of the cell suspension were injected into 10 ml of a 0° 145 mM NaCl at pH 7.4 (to be referred to as the 0° NaCl solution), which results in cessation of net uptake (18, 19). For studies of influx kinetics for MTX and the initial uptake of [3H]taurocholate, labeled compounds were added to conical centrifuge tubes, and uptake was initiated by the addition of glutamic acid; BSP, bromosulfophthalein; 5-methyltetrahydrofolic acid; AIB, α-aminoisobutyric acid.

Received November 5, 1979; accepted February 19, 1980.

1 Supported by Grants CA-16906 and AM-18976 from the NIH.
2 Supported by Training Grant HL-07110 from the NIH.
3 To whom requests for reprints should be addressed.

The abbreviations used are: MTX, methotrexate (4-amino-10-methylpteroyl-glutamic acid); BSP, bromosulfophthalein; 5-methyltetrahydrofolic acid; AIB, α-aminoisobutyric acid.
the hepatocyte suspension. Uptake was terminated by the rapid injection of 10 ml of the 0° NaCl solution. For measurement of intracellular radiolabel, cells were separated by centrifugation at 1000 x g for 30 to 60 sec and then washed in the 0° NaCl solution. The washed pellet was aspirated into the tip of a Pasteur pipet, extruded onto a polyethylene sheet, dried overnight, weighed on a Cahn Instruments, Paramount, Calif., and then digested in 0.25 ml of 1 M KOH (1 hr at 60°). The digest was neutralized with 0.25 ml of 1 n HCl and dissolved in 3 ml of Readi-Solv scintillation fluid (Beckman Instruments, Fullerton, Calif.), and radioactivity was measured in a liquid scintillation spectrometer.

Determination of the ratio of intracellular water to dry weight [1.97 ± 0.41 (S.E.)] and the concentration of cell radiolabel in terms of µmol/liter of intracellular water has been described previously (19).

Analysis of Polyglutamate Derivatives of MTX. MTX and its polyglutamate derivatives were extracted by boiling with subsequent sonication of the washed cell pellet and then separated by DEAE-cellulose ion-exchange chromatography (18). Recovery of radioactivity from the ion exchange column was 87.2 ± 4.4% (n = 9).

Chemicals and Purification. [3',5',9-3H]MTX with an initial specific activity of 30 Ci/mmol was obtained from Amersham/Searle Corporation (Arlington Heights, Ill.), and both labeled and unlabeled MTX were purified by DEAE-cellulose column chromatography (18). 4-Amino-10-methylpteroylglutamyl-γ-gluatamyl-γ-glutamic acid was provided by Dr. John Montgomery, Southern Research Institute, Birmingham, Ala., and 4-amino-10-methylpteroylglutamyl-γ-glutamyl-γ-glutamic acid was provided by Dr. C. M. Baugh, University of South Alabama, Mobile, Ala. [3H]Taurocholic acid (specific activity, 3.39 Ci/mmol) and [methyl-3H]AlB (specific activity, 10 Ci/mmol) were obtained from New England Nuclear, Boston, Mass. dl-L-5-[14C]CH3-H4-folate was obtained from Hynson, Westcott, and Dunning, Inc., Baltimore, Md. Rose bengal was obtained from Fisher Scientific Company, Fairlawn, N. J.

RESULTS

Chart 1 shows the potent inhibitory effect of 100 µm concentrations of bile salts (Chart 1 A) and organic anions (Chart 1 B) secreted by the liver on influx of 1 µM [3H]MTX into freshly isolated hepatocytes in suspension. The degree of inhibition by the bile salts was comparable (70 to 80%), while BSP and rose bengal were slightly more potent inhibitors (~90% inhibition; see Table 1). Since, at this concentration of MTX, approximately 60% of the drug enters by a high-affinity route and 40% by a low-affinity route (19), the extent of inhibition by these agents indicates that both MTX influx routes must be suppressed. This is substantiated by further studies quantitating the effects of a representative bile salt, taurocholate, on both MTX influx routes. As indicated in Chart 2 by Dixon analysis (13), the K for the high-affinity MTX route (Chart 2 A) was 21.4 µM while the K for the low-affinity MTX influx route was 18.9 µM (Chart 2 B). The kinetics of inhibition could not, however, be clarified either by Dixon or Lineweaver-Burk analyses because of the accentuation of the variability in the data that occurs in the difference analysis used for the discrimination of the 2 influx routes (19).

To further evaluate the mechanism of bile salt inhibition of MTX influx, hepatocytes in suspension were first incubated with 100 µm taurocholate, washed free of the bile salt with

| Table 1 |
|-----------------|-----------------|
| **METX influx** | **5-CH3-H4-folate influx** |
| (% of inhibition) | (% of inhibition) |
| Taurocholate | 73.88 ± 2.43² | 43.73 ± 13.77² |
| Cholate | 72.33 ± 3.03 | 61.64 ± 7.35² |
| Cholic acid (in ethanol) | 79.61 ± 1.44 | 54.36 ± 7.86 |
| Ethanol | 91.79 ± 1.08 | 44.52 ± 13.68 |
| BSP | 91.79 ± 1.08 | 44.52 ± 13.68 |
| Rose bengal | 94.73 ± 2.27 | 54.36 ± 7.86 |

² Mean ± S.E.

³ Numbers in parentheses, number of experiments.

⁴ Inhibition of 5-CH3-H4-folate influx is significantly different than inhibition of MTX influx, p < 0.05.

⁵ Inhibition of 5-CH3-H4-folate influx is significantly different than inhibition of MTX influx; p < 0.01.
buffer at room temperature, and then exposed to 1 μM [3H]-
MTX. There was negligible inhibition of MTX influx. Hence, bile
salt inhibition of MTX influx required the simultaneous presence
of the bile compound with MTX at the outer cell membrane,
indicating that the effects of bile salts cannot be attributed to
an irreversible alteration of the hepatocyte membrane.

To assess the specificity of transport inhibition by the bile
compounds and organic anions, the effects of these agents on
transport of 5-CH3-H4-folate and AIB was evaluated. Despite
evidence that 5-CH3-H4-folate and MTX utilize different trans-
port routes (19, 27), the bile compounds and organic anions
inhibited influx of 5-CH3-H4-folate, although to a lesser extent
than they inhibited MTX influx (Table 1). However, this is not a
generalized inhibitory effect on membrane transport processes
since 100 μM taurocholate was virtually without effect on 1 mM
AIB influx (8.5 ± 4.2% inhibition; p > 0.1 in 5 experiments),
and 100 μM BSP inhibited 1 mM AIB influx by only 20.3 ± 4.3%
(p < 0.02 in 5 experiments). Likewise, influx of [3H]AIB over a concentration range of 25 to 750 μM was not inhibited
by 100 μM taurocholate. Further, the transmembrane gradient
achieved for AIB is only slightly reduced by taurocholate and
BSP, while ouabain eliminates the gradient completely (Chart
3).

MTX transport in a variety of cells is influenced by the
extracellular anionic composition (20, 21, 29). However, the
inhibitory effect of BSP and bile salts secreted by the liver is
considerably more potent than that of other inorganic or
organic anions in rat hepatocytes. Hence, at a concentration of
100 μM, inhibition by ATP, ADP, or AMP is negligible, phos-
phate and sulfate have no effect at all, and nitrate is slightly
stimulatory (Chart 4), while BSP and bile salts markedly inhibit
influx of MTX at comparable concentrations (Chart 1). Since
some albumin is present in the hepatic interstitium and may
influence the interaction among the bile salts, anions, and MTX
at the hepatocyte membrane, studies evaluated the influence
of albumin on the inhibitory effect of BSP or taurocholate on
MTX influx (Chart 5). Inhibition of MTX influx by taurocholate
remained quite potent (71.1 ± 2.1% inhibition in the absence of
albumin; 59.1 ± 3.5% inhibition in the presence of albumin,
4 g/100 ml). However, the degree of inhibition of MTX influx by
BSP was reduced from 89.8 ± 0.59% to 24.8 ± 5.5% in 4
experiments. MTX influx was essentially unaltered by albumin
(84.5 ± 17.3% of control in 4 experiments; p > 0.2).

Bile salt inhibition of MTX influx is also accompanied by a
reduction in net drug accumulation (Chart 6). As previously
shown (18), freshly isolated hepatocytes synthesize polyglu-
tamates of MTX. In these studies, approximately 40% of intra-
cellular [3H]extracted after exposure of cells to [3H]MTX for 75
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intercept was observed that has been attributed to surface binding (1, 37). Although taurocholate influx was unaltered by the simultaneous presence of up to 0.5 mM MTX (p > 0.2, 3 experiments), 5 mM MTX inhibited [3H]taurocholate influx 42.4 ± 7.5% (p < 0.01, 4 experiments). Hence, not only is MTX influx inhibited by taurocholate but also taurocholate influx is inhibited by high concentrations of MTX.

DISCUSSION

Previous studies from this laboratory indicate that MTX influx into freshly isolated hepatocytes is mediated by at least 2 routes which differ primarily in their affinities for this antifolate (19). Bile salts and the organic anions tested inhibit both transport routes for MTX. The observation that taurocholate inhibits both the high- and low-affinity routes for MTX with

![Chart 4. Effect of adenine nucleotides or inorganic anions on influx of MTX. Hepatocytes in suspension were simultaneously exposed to 1 μM [3H]MTX and 100 μM concentrations of the indicated compounds, and the time course of uptake was monitored for 200 sec. Mean of the uptake slopes from 4 experiments; bars, S.E.]

![Chart 5. Effect of albumin on the inhibition of MTX influx by BSP or taurocholate. MTX influx is indicated as a percentage of the control rate in 4 separate experiments; bars, S.E.]

min represent MTX polyglutamates. While taurocholate markedly reduces net uptake of MTX, the percentage of intracellular 3H that represents MTX polyglutamates is unchanged (Chart 7). On the other hand, not only do deoxycholate and BSP inhibit transport of MTX but they also decrease the percentage of polyglutamate derivatives formed.

In an attempt to further clarify the interaction between MTX and bile salts, the effect of MTX on [3H]taurocholate uptake was evaluated (data not shown). Uptake of 1 μM [3H]taurocholate is linear for 25 sec at a rate of 35.6 ± 2.2 μmol per liter cell water per min (7 experiments). A small positive ordinate intercept was observed that has been attributed to surface binding (1, 37). Although taurocholate influx was unaltered by the simultaneous presence of up to 0.5 mM MTX (p > 0.2, 3 experiments), 5 mM MTX inhibited [3H]taurocholate influx 42.4 ± 7.5% (p < 0.01, 4 experiments). Hence, not only is MTX influx inhibited by taurocholate but also taurocholate influx is inhibited by high concentrations of MTX.

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![Chart 6. The time course of uptake of 3H over 75 min of incubation of hepatocytes with 1 μM [3H]MTX. Effect of simultaneous exposure to 100 μM bile salts. The 3H level achieved after exposure to BSP (not shown) is similar to that in the presence of chenodeoxycholate.]

![Chart 7. Effect of 100 μM taurocholate, deoxycholate, or BSP on cell MT and MTX polyglutamates synthesized by hepatocytes in suspension 75 min after exposure to 1 μM [3H]MTX. Numbers in parentheses, number of experiments.]

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similar K's is compatible with a number of possibilities including: (a) an interaction between this bile salt and a single site on the hepatocyte membrane that affects both transport routes for MTX; or (b) taurocholate may have similar affinities for 2 carriers at different membrane interfaces ([i.e., sinusoidal, canalicular, or contiguous with other hepatocytes (14)]) that have markedly different affinities for MTX. In any event, bile acids and organic anions secreted by the liver are very potent inhibitors of the transport of MTX and, to a lesser extent, of the naturally occurring folate, 5-CH3-H4-folate. While the taurocholate K, for the site with which it interacts to inhibit MTX transport was quantitated, the data did not establish the kinetics of inhibition (competitive, noncompetitive, etc.). The data do indicate, however, that high levels of MTX can perturb transport of taurocholate, suggesting the proximity of the 2 routes or a shared process. While concentrations of MTX below 0.5 mM do not inhibit taurocholate influx, this does not exclude the possibility of an interaction at the level of a high-affinity process. If it is assumed that MTX and taurocholate share the same high-affinity route and that the taurocholate K, for inhibition of MTX influx via the high-affinity route is equal to its Km [a value that is, in fact, similar to the measured Km for taurocholate in other studies (4, 37)], then the expected influx of 1 μM [3H]taurocholate by this route can be calculated. Using the MTX Vmax of 1.5 μmol per liter cell water per min (19) and a Km of 21.4 μM, the expected influx of taurocholate would be 0.067 μmol per liter cell water per min. This represents less than 0.2% of the total measured [3H]taurocholate influx of 35.6 ± 2.2 μmol per liter cell water per min so that even complete inhibition of this component would not be perceptible experimentally. This indicates further that more than 99% of taurocholate influx must be mediated by a process distinct from that for the high-affinity MTX route. Since influx of taurocholate into hepatocytes in suspension is a heterogenous process with saturable and nonsaturable components (4), it is unclear as to which components of taurocholate influx are inhibited by MTX. Nonetheless, there are a number of similarities between bile salt and MTX influx which include: (a) sodium dependence (4, 5, 19, 26, 37); (b) sensitivity to metabolic inhibitors (4, 5, 19, 26, 37); (c) inhibition by organic anions such as BSP (37); and (d) a similar influx Q2737 of 5.3 for taurocholate (calculated from Ref. 37) versus 4.3 for MTX (19). On the other hand, while BSP is a potent inhibitor of MTX influx into hepatocyte suspensions, BSP influx is not influenced by metabolic inhibitors, extracellular sodium, or the presence of taurocholate in the extracellular fluid (38).

Potent inhibition of MTX transport by the bile compounds coupled with MTX inhibition of taurocholate influx suggests specific interactions among the transport carriers. That the effects of bile salts on MTX transport are not simply due to generalized alterations in membrane transport processes or inhibition of energy metabolism (8, 15, 32) is suggested by the observations that influx of AIB is minimally affected and that a transmembrane gradient for AIB is sustained in the presence of bile compounds. This is not unexpected since much higher concentrations of bile salts are required to inhibit oxidative metabolism (33). The observation that the effects of bile salts can be reversed after their removal from the extracellular compartment excludes a nonspecific detergent-like effect on the cell membrane (30). Finally, inhibition of MTX influx cannot be attributed solely to the anionic nature of bile salts, as reported for MTX transport in other cell systems (20, 21, 29), since, at similar concentrations, a variety of inorganic and organic anions have little or no inhibitory activity in this hepatocyte system.

Bile salts not only inhibit MTX influx, but also significantly reduce net drug accumulation and reduce the level of polyglutamate derivatives within the cell. In the case of deoxycholate, the reduction in the net level of polyglutamates was far out of proportion to the depression of net cellular accumulation of MTX. This effect of bile salts on MTX uptake and the formation of polyglutamates may have important clinical relevance. Bile salt levels in portal blood may influence MTX uptake into liver cells when the drug is administered either p.o. or i.v. The bile salts, at concentrations similar to those achieved physiologically in the rat (11, 35), reduce MTX influx into rat hepatocytes. In humans, the 2- to 4-g bile salt pool (25, 42) accumulates in the gall bladder during fasting, is released into the intestine during meals, and is followed by absorption into the portal blood, transport to the liver, and secretion into the bile. Bile salts traverse the enterohepatic circulation 2 to 3 times during each meal, and the concentration of the bile salts in the portal blood during fasting (~20 μM) (2) must increase substantially during meals. Assuming a comparable interaction between MTX and bile salts in the human liver, the bile salt content of the portal system could profoundly influence uptake of MTX into the hepatocyte. Hence, the interaction between MTX and bile salts in the hepatic sinusoid represents a phenomenon that may be an important factor in determining the extent of drug uptake into the liver and the accumulation of MTX polyglutamate derivatives in liver cells. This may be particularly relevant to MTX hepatotoxicity since the polyglutamate derivatives are a pharmacologically active form of the drug (28) that are retained within cells and can sustain suppression of dihydrofolate reductase activity, while the effects of MTX alone are rapidly reversible (19, 36). Hence, manipulation of this interaction, in terms of regulation of bile salt pool size, time of administration relative to meals, or coadministration of other agents, may provide useful approaches for reducing MTX hepatotoxicity, especially in long-term low-dose regimens (12, 23, 34, 43).

REFERENCES

8. The pharmacological activity of MTX polyglutamates is compatible with the observation that polyglutamates of the naturally occurring folates are often preferred substrates (9, 10, 16, 31).
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