Interaction of Probenecid with Methotrexate Transport and Release in the Isolated Rat Hepatocyte in Suspension

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ABSTRACT

Probenecid has been shown to delay the plasma clearance of methotrexate in the rat and to reduce both hepatic and renal excretion of methotrexate in this animal model. In order to probe the mechanism by which probenecid alters hepatic excretion of the antifolate, studies assessed the effects of probenecid on transport, accumulation, distribution, and release of methotrexate in the rat hepatocyte in suspension. Probenecid was found to effectively inhibit methotrexate influx with a Kᵢ of approximately 100 μM. Inhibition of methotrexate influx was accompanied by a reduction in methotrexate accumulation; with 200 μM probenecid, the levels of exchangeable and nonexchangeable intracellular methotrexate were reduced by 43.4 ± 2.4 (S.E.) and 41.8 ± 7.7%, respectively. As a consequence of reduced accumulation of the methotrexate substrate, the formation of cellular polyglutamate derivatives of methotrexate was likewise reduced. Concentrations of probenecid which inhibited methotrexate influx and accumulation by 70 to 80% did not markedly alter methotrexate efflux under conditions where efflux was effected by a washout procedure or by the presence of inducing agents, such as N\,O\,O'-dibutyryl cyclic adenosine 3':5'-monophosphate or α-agonists. These studies suggest that the inhibition of hepatic methotrexate secretion by probenecid in vivo is likely to be a consequence of interference with hepatic uptake of the antifolate rather than an interaction of probenecid and methotrexate at a hepatic "secretory" site.

INTRODUCTION

The organic acid, probenecid, delays clearance of the antifolate, MTX,
from the circulation by interfering with secretion of MTX by the renal tubules (4). Studies in the rat have demonstrated that probenecid delays clearance of MTX by the hepatic route as well (19, 20), contributing to prolonged elevation of circulating MTX levels. This delay in the hepatic clearance of MTX and the accompanying reduction in secretion of MTX into the bile is thought to arise from inhibition of hepatic secretion of MTX in the presence of probenecid (20); however, a direct interaction between probenecid and MTX at hepatic secretory sites has not been established.

Previous studies in our laboratory have demonstrated that negatively charged species, such as bile salts and organic anions, reduce influx and accumulation of MTX in isolated rat hepatocytes in suspension (11); it therefore appeared likely that the negatively charged probenecid molecule might likewise interfere with MTX uptake into the liver cell, thereby reducing both hepatic clearance of MTX and its secretion into the bile. The present studies were designed to evaluate the effects of probenecid on MTX influx, accumulation, and release from the isolated hepatocyte in suspension in order to identify the primary sites at which probenecid modifies the hepatic clearance and secretion of MTX.

MATERIALS AND METHODS

Preparation of Hepatocytes. Hepatocytes in suspension were prepared by a modification of the method of Berry and Friend (3) as described previously (13). Briefly, the livers of male Sprague-Dawley rats weighing between 225 and 275 g were perfused with a series of buffers (at 37°, pH 7.4, gassed with 95% O₂-5% CO₂) designed to deplete hepatic blood and chelate endogenous calcium at hepatic desmosomes. Subsequently, the liver was perfused with buffer containing 0.045% collagenase and 2 μM CaCl₂ for 20 min, and the tissue was excised, minced, suspended in a buffer with dilute collagenase, and further dispersed by gentle rotation in a 37° water bath. After filtration through gauze to remove undisgested tissue, the cell suspension was centrifuged at 50 to 60 × g for 2 min and washed 3 times with calcium-rich buffer containing 0.25% gelatin.

Transport Assay. Measurement of [³H]MTX uptake was described previously (14, 15). The hepatocyte pellet was gently dispersed in Krebs-Henseleit buffer. The cell suspension was added to flasks, stirred continuously with a Teflon paddle, and gassed with warmed and humidified 95% O₂-5% CO₂. [³H]MTX was added, following which portions of the cell suspension were injected into 10 volumes of 0.85% NaCl solution (saline) at 0° to halt MTX uptake. Cells were separated by centrifugation (150 x g for 1 to 2 min) and then washed twice in 0.85% NaCl solution at 0°. The washed pellet was aspirated into the tip of a Pasteur pipet, extruded onto a polyethylene sheet, and dried overnight at 60°. The dried pellets were pooled off the polyethylene, weighed directly on a Cahn Model 4700 electrobalance, and then digested in 0.2 ml of 1 M KOH for 1 hr at 60°. After cooling, the digest was neutralized with 0.2 ml of n HCl, then dissolved in 3 ml of Triton scintillation fluid, and counted in a Beckman LS-230 scintillation spectrometer. Discrimination between exchangeable and nonexchangeable intracellular MTX is based on the loss of a cell [³H] over the course of a 1-hr incubation after removal of medium [³H] by centrifugation of the pellet and washing with 0° buffer, followed by resuspension into MTX-free buffer at 37° (14).

MTX Polyglutamate Formation. Extraction of cells [³H]MTX and [³H]-polyglutamate derivatives was performed after [³H]MTX-loaded hepatocytes had been washed 3 times with 0° saline. The cell pellet was lysed by suspension in 4 ml of boiling water with continuous heating for 5 min. The cell extract was subsequently frozen, thawed, and sonicated for 5 min in a bath sonicator to ensure complete extraction of cell [³H]. The precipitated protein was removed by sequential centrifugation at 15,000 × g for 15 min in a refrigerated Beckman R2B centrifuge and filtration of the cell extract through Aminon Centriflo cones (exclusion limit, M₀ ~25,000).

Resolution of MTX and MTX polyglutamate derivatives was accom-

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3 The abbreviations used are: MTX, methotrexate; dibutyryl cyclic AMP, N\,O\,O'-dibutyryl cyclic adenosine 3':5'-monophosphate.

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plished utilizing reverse-phase high-pressure liquid chromatography with Gilson Model 302 pumps and a Spherisorb ODS 5-μm reverse-phase column obtained from Thomson Instrument Co., Newark, DE. Resolution was accomplished in 9 min at a flow rate of 2 ml/min using a gradient of 7.5 to 9% acetonitrile in 0.1 M acetate buffer, pH 5.5, as the mobile phase (8).

Materials. [3',5',9-3H]MTX was obtained from Moravek (City of Industry, CA) and purified by high-pressure liquid chromatography (8). Collagenase was obtained from Sigma Chemical Co., St. Louis, MO. All other chemicals utilized were reagent grade. Authentic standards of 4-amino-10-methylpteroyldiglutamic acid and 4-amino-10-methylpteroyltrimethylglutamic acid were kindly provided by Dr. C. M. Baugh.

RESULTS

Effects of Probenecid on MTX Influx. Chart 1 shows the uptake of 1 μM [3H]MTX into isolated hepatocytes in suspension in the presence of increasing concentrations of probenecid. Uptake of 1 μM [3H]MTX is a linear function of time, indicating that this cellular accumulation represents unidirectional MTX transport, undisturbed by efflux, binding, or metabolism. Chart 1 demonstrates that MTX influx is very sensitive to the presence of probenecid, as a probenecid concentration of 10 μM reduces MTX influx to 75% of its control value. As the probenecid concentration is raised, MTX influx is further reduced, so that at 200 μM probenecid, influx has been inhibited by 63.9 ± 5.1% (6 experiments).

The kinetics for inhibition of MTX transport by probenecid was determined by a Dixon plot analysis utilizing varied concentrations of [3H]MTX (1, 2, and 5 μM) and increasing concentrations of probenecid (10 to 200 μM). Chart 2 demonstrates that the apparent Kₐ for inhibition of MTX influx was 100 μM; the intersection of the extrapolated lines above the abscissa suggests that inhibition of MTX influx by probenecid results from a competitive interaction between the 2 anionic species.

Effects of Probenecid on the Cellular Accumulation of MTX. Accumulation of MTX in the liver cell may contribute to the early phase of MTX clearance from the circulation (17). The effects of increasing concentrations of probenecid on accumulation of [3H]MTX in the rat liver cell are presented in Table 1. As expected from the potent inhibition of MTX influx by probenecid, net MTX accumulation is likewise reduced by probenecid. Concentrations of probenecid as low as 10 μM are effective in reducing MTX accumulation, while 200 μM probenecid reduces MTX accumulation by 62.5 ± 3.76%.

The data shown in Chart 1 and Table 1 indicate that increasing the concentration of probenecid in the incubation medium results in a concentration-dependent reduction of MTX influx and net MTX accumulation. This observation suggests that reduction of net MTX accumulation in the rat hepatocyte by probenecid is a direct consequence of inhibition of MTX influx; hence, MTX efflux would appear to be undisturbed by probenecid. In order to confirm that MTX efflux is relatively insensitive to the presence of probenecid, studies were designed to evaluate the effects of probenecid on MTX efflux induced by a washout procedure, by exposure to dibutyryl cyclic AMP or to an α-adrenergic stimulus.

Effects of Probenecid on MTX Efflux. Studies with murine tumor cells in vitro have demonstrated marked inhibition of MTX efflux by probenecid, with a resultant elevation of steady-state levels of intracellular drug (25). However, the reduction in intracellular accumulation of MTX in hepatocytes in the presence of probenecid (Table 1) suggests minimal effects of probenecid on MTX efflux from liver cells. Chart 3 shows the efflux of 3H from [3H]MTX-loaded cells which have been washed and resuspended into MTX-free buffer in the absence or presence of 200 μM probenecid. A similar level of intracellular (nonexchangeable) drug was achieved in the absence as well as the presence of probenecid; the parameters of drug efflux, although not amenable to a strict kinetic analysis, did not change appreciably in the presence of probenecid.

Previous studies have demonstrated that agents as diverse as the phosphodiesterase inhibitors, dibutyryl cyclic AMP and isobutylmethylxanthine (12), as well as adrenergic catecholamines and vasopressin (13), are effective in the induction of MTX release from the isolated hepatocyte in suspension, a
phenomenon that may prove to be related to the induction of hepatic secretory processes in vivo. Charts 3, b and c, respectively, demonstrates the effects of 200 μM probenecid on MTX efflux induced by dibutyryl cyclic AMP or the α-adrenergic stimulus of epinephrine plus isobutylmethylxanthine. As in studies where efflux was induced by simple alteration of the transmembrane concentration gradient for MTX (Chart 3a), there was no discernible effect of this concentration of probenecid on the kinetics of MTX efflux or the level of drug remaining within the cell.

**Distribution of Intracellular [3H]MTX in the Presence of Probenecid.** In the hepatic cell, MTX is distributed in both exchangeable and nonexchangeable compartments (14). Exchangeable drug is experimentally defined as that portion of intracellular MTX which exits from the cell upon washing and resuspension of cells into MTX-free buffer. Nonexchangeable MTX consists of drug bound to dihydrofolate reductase, drug bound to other intracellular binding sites, as well as MTX polyglutamate derivatives. The intracellular distribution of MTX was compared in control hepatocytes and in hepatocytes exposed to probenecid by subjecting the [3H]MTX-loaded cells to a washout procedure (Chart 4). In the presence of 200 μM probenecid, the levels of exchangeable drug were reduced by 43.37 ± 2.43%, while the levels of nonexchangeable drug were similarly reduced by 41.76 ± 7.76% (7 experiments).

In studies with murine tumor cells, elevation of intracellular MTX levels by probenecid resulted in enhanced formation of polyglutamate derivatives as a result of increased levels of substrate and prolonged intracellular retention of MTX (9). In the present studies, inhibition of MTX uptake into the hepatocyte by probenecid resulted in a concomitant reduction in the absolute levels of intracellular MTX polyglutamate derivatives formed; the level of MTX polyglutamates as a percentage of total cell 3H in control and probenecid-treated cells as determined by the Student t test (Table 2).

**Reversibility of Probenecid Effects on MTX Influx.** In order to establish that probenecid does not damage the hepatocyte membrane or membrane transport processes, hepatocytes were incubated with probenecid for 10 min at 37° and washed twice
with incubation buffer at 37° before exposure of the cells to \[^3H\]MTX. The subsequent influx of \[^3H\]MTX was not significantly different from that in control cells (data not shown), indicating that membrane integrity was maintained after exposure to probenecid.

**DISCUSSION**

In studies with murine tumor cells *in vitro*, probenecid has been shown to elevate intracellular \[^3H\]MTX levels by preferential inhibition of MTX efflux (25); as a consequence of the elevation of cellular MTX levels, the formation of MTX polyglutamate derivatives is increased (9). Sirotnak *et al.* (24) have also demonstrated that probenecid elevates MTX levels in tumor cells *in vivo* and enhances the therapeutic effectiveness of MTX in tumor-bearing animals.

Elevation of MTX levels in murine tumor cells *in vivo* by probenecid may result from direct interaction of the probenecid with the cellular transport systems for MTX as well as reduced hepatic and renal clearance of MTX from the circulation. The present studies suggest that reduced hepatic clearance of MTX in the presence of probenecid is a consequence of inhibition of MTX uptake into the hepatic cell.

Probenecid is an effective inhibitor of MTX influx into the liver cell. The K_m of MTX influx is approximately 100 μM (at concentrations of MTX which enter the hepatocyte primarily by its high-affinity influx route). Inhibition of MTX influx by probenecid appears to be competitive by a Dixon plot analysis, suggesting that probenecid may bind directly to the same site on the transport “carrier” molecule as does MTX. However, the extrapolated lines intercept very close to the X-axis; it is therefore difficult to eliminate the possibility of a noncompetitive interaction where probenecid may alter the overall negative change of the carrier molecule, interfering with MTX influx. A comparison of the K_m for inhibition of MTX influx into the hepatocyte with that for inhibition of MTX influx in the L1210 leukemia cell (25) indicates that MTX transport in the liver is 10- to 20-fold more sensitive to the presence of probenecid. This is to be expected, as the liver cell appears to express specific anion and bile salt transport routes (2, 23); inhibition by probenecid of MTX uptake in tumor cells reflects a more general inhibition of MTX transport by anions (16, 18).

Probenecid reduces both MTX influx and the cellular accumulation of MTX. The levels of MTX are reduced equally in both exchangeable and nonexchangeable compartments. Formation of MTX polyglutamates is reduced, as would be expected when substrate for an enzymatic reaction is limited. However, the ratio between intracellular polyglutamates and total intracellular \(^3H\) is similar in controls and in probenecid-treated cells (as determined by a paired t test). This suggests that probenecid does not directly interfere with conversion of MTX to polyglutamate derivatives and that substrate levels in both control and probenecid-treated cells are significantly below the K_m for this enzymatic process (21).

Probenecid does not appear to alter the rate of MTX efflux or the final intracellular MTX levels achieved when efflux is induced by a simple washout procedure, or after exposure of hepatocytes to dibutyryl cyclic AMP or epinephrine plus isobutylmethylxanthine. This observation suggests that the effects of probenecid in delaying hepatic clearance of MTX *in vivo* result from inhibition of MTX uptake into the hepatic cell, not from inhibition of MTX efflux.

MTX remaining within the cells after the washout procedure is considered “nonexchangeable” drug and represents drug bound to dihydrofolate reductase, drug bound to other subcellular sites, as well as polyglutamate derivatives of MTX. The long-term disposition of this drug is not known. However, since probenecid does not interfere with release of exchangeable drug, it is suggested that release of nonexchangeable drug would similarly not be altered by the presence of probenecid.

There are a number of possible explanations for the lack of effect of probenecid on MTX efflux from the liver cell, while efflux is markedly inhibited in the tumor cell. While probenecid and MTX may share efflux routes in the hepatocyte, the level of probenecid achieved within the hepatic cell may be insufficient to inhibit efflux; intracellular probenecid levels were not assessed in the present or in previous studies, as radiolabeled probenecid is not readily available, and spectrophotometric assays are not sufficiently sensitive (6). Alternatively, high levels of probenecid may actually be achieved within the liver cell, but the K_e of efflux may also be exceptionally high, or the “carrier” may have a very high capacity for the simultaneous transport of both probenecid and MTX from the hepatic cell. Additional possibilities include that efflux of MTX from the hepatocyte does not occur via a discrete mediated pathway or that the carrier for MTX is very restrictive and does not recognize probenecid as a substrate. It is important to note that the parameters of hepatic secretion have not been studied extensively and that the importance of facilitated transport as a mechanism of drug exit from the hepatic cell into the bile remains controversial (7).

The conclusion to be derived from the present studies is that the effects of probenecid on the secretion of MTX into the bile (19, 20) do not appear to result from a direct interaction of probenecid with the secretory process itself but rather reflect inhibition of hepatic cell uptake of MTX. Probenecid may therefore prove to have clinical utility by virtue of reducing hepatic cell uptake and toxicity of MTX (5, 22); in fact, studies by Sirotnak show a transient decrease of hepatic accumulation of MTX by probenecid (24). These additional effects of probenecid would occur concomitantly with the elevation by probenecid of MTX levels in the plasma (1) and the tumor cell (9, 24, 25).

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