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Communication

Effect of Chemotherapeutic Agents on the Uptake and Excretion of Amethopterin by the Isolated Perfused Rat Liver[1]

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ABSTRACT

The effect of a variety of chemotherapeutic agents on the hepatic transport of amethopterin was studied in the isolated perfused rat liver. Vincristine (1, 10, and 100 μM), dactinomycin (10 and 50 μM), and cyclophosphamide (1, 2.5, and 5 mM), when added individually to the perfusion medium, produced a dramatic decrease in the rate of amethopterin (1 μM) uptake and biliary excretion during a 2-hr perfusion. The inhibitory effect on uptake and bile excretion of amethopterin increased as the concentration of added drug increased. 5-Fluorouracil (10 mM) and cycloheximide (0.1 mM) had no effect on the uptake or excretion of amethopterin. The hepatic tissue:medium ratio ranged from 1.3 to 15, with the highest concentrations observed in the presence of dactinomycin. Parallel studies on the hepatic synthesis of albumin demonstrated a depression in the rate of albumin synthesis by the presence of vincristine, dactinomycin, cyclophosphamide, or cycloheximide. Bile flow was decreased only in the presence of vincristine and dactinomycin. These studies indicate that hepatic uptake and biliary excretion of amethopterin are inhibited by vincristine, dactinomycin, and cyclophosphamide, that dactinomycin produces a greater than expected degree of intracellular accumulation of amethopterin, and that the mechanism of hepatic amethopterin transport does not appear to be dependent on protein synthesis.

INTRODUCTION

Studies from 2 laboratories using *in vitro* rat liver systems have shown that amethopterin is transported into the liver via a saturable, energy-dependent, carrier-mediated, and low-affinity (K_m = 1.3 to 2.3 mM) uptake mechanism (5, 9) followed by excretion into bile against a high-concentration gradient (9). During exit from the cell, amethopterin achieves concentrations in the bile 70- to 120-fold more than that in the perfusion medium (9). Inhibitors of anaerobic and aerobic metabolism, which enhance amethopterin uptake in L1210 murine leukemia cells and Ehrlich ascites tumor cells (2, 3, 8), lead to inhibition of amethopterin uptake in the liver (5, 9). The effect of vincristine, which also enhances amethopterin uptake in L1210 murine leukemia cells and Ehrlich ascites tumor cells (1, 10) and other chemotherapeutic agents commonly used in combination with amethopterin on the hepatic transport of amethopterin, has not been determined. This study was designed to determine whether treatment with vincristine, dactinomycin, cyclophosphamide, or 5-fluorouracil would influence the hepatic uptake or biliary excretion of amethopterin and thus possibly change the pharmacological behavior of amethopterin during combination chemotherapy with these agents.

MATERIALS AND METHODS

The following were obtained commercially: [3,5-^{3}H]amethopterin (12.4 Ci/nmol) (Amersham/Searle Corp., Arlington Heights, Ill.) [the radiochemical purity of [^{3}H]amethopterin was greater than 98%, as determined by ion-exchange chromatography as described previously (9)]; tritiated water, L-[^{14}C]leucine (0.25 mCi/0.11 mg), and 5-fluorouracil (Calbiochem, La Jolla, Calif.); cycloheximide (ICN Pharmaceuticals, Inc., Cleveland, Ohio); No. 13255 Chromagram sheets (Fisher Scientific Co., Pittsburgh, Pa.). The remaining chemotherapeutic agents were gifts: vincristine sulfate, Eli Lilly & Co. (Indianapolis, Ind.); dactinomycin, Merck Sharp and Dohme (Rahway, N. J.); cyclophosphamide monohydrate, Meade Johnson (Evansville, Ind.); and amethopterin, Lederle (Pearl River, N. Y.).

Adult male Sprague-Dawley rats, 250 to 400 g, served as liver donors. The perfusion technique and apparatus have been described previously (6, 9). [^{3}H]Amethopterin (1 μM) and the desired chemotherapeutic agent were added to the perfusion medium at the outset. Each drug was tested at least twice at 3 to 6 different concentrations. Perfusions were continued for 2 hr; samples (1 to 2 ml) of the perfusate were withdrawn at Time 0 and at 10- or 20-min intervals thereafter. One ml of the sample was dissolved in Aquasol for radioactive counting, and the remainder was assayed for albumin concentration. Amethopterin uptake was determined by measuring the disappearance of [^{3}H]-amethopterin from the perfusion medium. Studies with dactinomycin were corrected for quenching by using tritiated water as an internal standard. Rat albumin in the perfusate was measured by the radial immunodiffusion technique (7). Prior studies confirmed that albumin was being newly synthesized by demonstrating that [^{14}C]leucine was incorporated in the antibody-specific albumin precipitate linearly between 1 and 5 hr. The bile was collected at

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the end of each hr; bile flow was 0.26 to 1.2 ml/hr. An aliquot of bile was monitored for radioactivity. Prior studies have shown that radiolabel in bile represents unchanged amethopterin (9). Hepatic cell-free extracts were obtained following a 2-hr perfusion by homogenizing the entire liver with a Teflon-glass, motor-driven homogenizer, by centrifuging at 30,000 × *g* for 20 min at 2°, and by recovering the supernatant. The hepatic tissue:medium ratio was determined by dividing the amethopterin concentration per g liver in the cell-free extract by the final amethopterin concentration per ml in the perfusion medium. The radiochemical purity of amethopterin in the perfusion medium at the conclusion of the 2-hr perfusion was analyzed by thin-layer chromatography and a 1% K_2HPO_4 solvent system. Unlabeled amethopterin was added as a marker. Fluorescence quenching was detected with a fluorescent lamp, and radioactivity was determined by dissolving the radioactive material in Aquasol and monitoring the sample for ^3H.

RESULTS AND DISCUSSION

Hepatic uptake of amethopterin was markedly decreased when vincristine (10 μM), dactinomycin (50 μM), or cyclophosphamide (5 mM) was added to perfusion medium (Chart 1). The inhibitory effect increased as the concentration of the added drug increased (Table 1). 5-Fluorouracil (10 mM) and cycloheximide (0.1 mM) had no effect on the uptake of amethopterin by the liver under the same conditions (Table 1). The inhibitory effect is comparable to that observed with many structurally diverse compounds with different sites of action, including folate analogs (5, 9), dinitrophenol (5, 9), sulfhydryl inhibitors (5, 9), and 2-heptyl-4-hydroxyquinoline *N*-oxide (5).

These findings are of special interest when compared to the effect of *Vinca* alkaloids, azide, and other metabolic poisons on L1210 murine leukemia cells and Ehrlich ascites tumor cells in which these agents potentiate cellular uptake of amethopterin (1–4, 8, 10). The mechanism of action in these latter systems is thought to be an inhibition of cell metabolism with consequent inhibition of an energy-dependent process (unidirectional efflux), which controls amethopterin accumulation within the cell (1, 2); these effects may be regulated by endogenous cyclic adenosine 3′:5′-monophosphate (4). The mechanism of inhibition in the hepatic uptake process is not known and will require further study.

Bile excretion of amethopterin was reduced to a greater degree than was the reduction in hepatic amethopterin uptake in the presence of vincristine and dactinomycin, whereas cyclophosphamide led to a decrease in bile excretion of amethopterin, which was similar to the decrease in hepatic uptake (Table 1). As in the uptake studies, 5-fluorouracil and cycloheximide had no inhibitory effect on bile excretion of amethopterin. These studies suggest that vincristine and dactinomycin may have a separate effect on bile excretion of amethopterin.

Tissue amethopterin concentration was determined by measuring the accumulated ^3H in the cell-free extract at the end of a 2-hr perfusion. The tissue:medium ratio (range, 1.3 to 15) exceeded 1 for each of the conditions indicated in Table 1. Isolated hepatocytes, which lack biliary channels, also accumulate amethopterin at levels greater than those found in the medium (5). The higher tissue concentration of amethopterin observed in the presence of dactinomycin further suggests that this agent may have a direct effect on bile excretion.

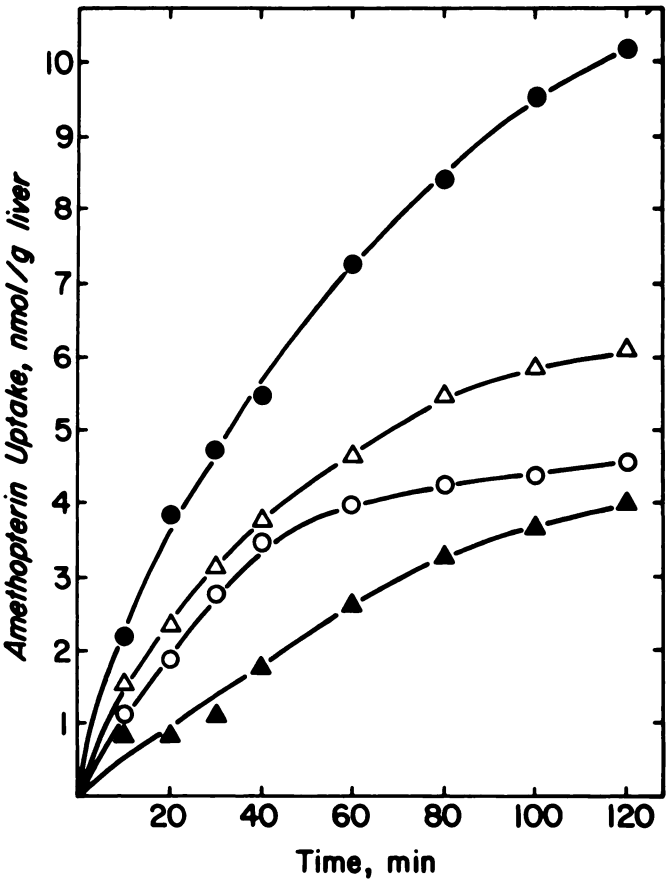

Chart 1. Effect of chemotherapeutic agents on amethopterin uptake during 2-hr liver perfusion. Uptake of amethopterin during the 2-hr liver perfusion was measured as described in "Materials and Methods." Initial concentration of [^3H]amethopterin, 1 μM; ●, no inhibitor; △, 10 μM vincristine; ○, 50 μM dactinomycin; ▲, 5 mM cyclophosphamide.

Hepatocyte viability was determined by parallel studies in which the effect of amethopterin, vincristine, dactinomycin, cyclophosphamide, 5-fluorouracil, and cycloheximide on albumin synthesis and bile flow by the liver was measured during the 2-hr perfusion. These results are shown in Table 2. Albumin synthesis was depressed by dactinomycin, cyclophosphamide, and cycloheximide, as would be expected, since each compound directly inhibits protein synthesis; vincristine, an inhibitor of microtubular assembly, also depressed albumin synthesis. Amethopterin (1 μM and 1 mM) and 5-fluorouracil (10 mM) had no inhibitory effect on protein synthesis. Bile flow was reduced only by vincristine and dactinomycin; the mechanism for this effect is not known. The radiochemical purity of amethopterin in the perfusion medium after a 2-hr experiment was maintained, as demonstrated by thin-layer chromatography. In 5 experiments the entire chromatogram was examined, and radioactivity was found only in the single fluorescence quenching spot identified as unmetabolized amethopterin.

This study indicates that hepatic uptake of amethopterin can be inhibited by at least 3 chemotherapeutic agents,

Table 1

Effect of chemotherapeutic agents on hepatic uptake, biliary excretion, and tissue retention of amethopterin by the isolated perfused rat liver

Additions	Amethopterin transport, 60 min (nmol/g liver)		Tissue:medium ratio of amethopterin
	Uptake	Bile excretion	
Amethopterin, 1 μM	7.4 ±1.6[a] (4)[b]	3.2 ±1.9 (4)	4.6 ±1.6 (3)
Amethopterin, 1 μM			
Plus vincristine[c]			
1 μM	5.4	1.2	2.2
10 μM[d]	4.6	0.8	2.0
100 μM	2.2	0.3	1.4
Plus dactinomycin[c]			
10 μM	6.2	1.7	15.3
50 μM[d]	4.0	0.3	7.2
Plus cyclophosphamide[c]			
1 mM	5.8	3.6	4.8
2.5 mM	4.0	2.2	2.2
5.0 mM[d]	2.6	0.8	1.3
Plus 5-fluorouracil[c]			
10 mM	7.7	4.4	5.2
Plus cycloheximide[c]			
100 μM	7.6	4.2	4.2

[a] Mean ± S.D. (control).
[b] Number in parentheses, number of experiments.
[c] Data represent the mean of 2 or 3 different experiments for each concentration indicated.
[d] Data were obtained during the same experiments illustrated in Chart 1.

Table 2

Effect of chemotherapeutic agents on albumin synthesis and bile flow by the isolated perfused rat liver

Additions	Albumin synthesis (μg/g liver/hr)	Cumulative bile flow, 120 min (μl/g liver)
None	149 ± 50[a] (16)[b]	86 ± 11 (7)
Amethopterin, 1 μM	95.8	91
Amethopterin, 1 mM	104.6	118
Amethopterin, 1 μM		
Plus vincristine		
1 μM	102	54
10 μM[c]	49	23
100 μM	54	37
Plus dactinomycin		
10 μM	43	58
50 μM[c]	51	31
Plus cyclophosphamide		
1 mM	23	129
2.5 mM	56	104
5.0 mM[c]	55	102
Plus 5-fluorouracil, 10 mM	130	105
Plus cycloheximide, 100 μM	38	78

[a] Mean ± S.D. (control).
[b] Number in parentheses, number of experiments.
[c] Data were obtained during the same experiments illustrated in Chart 1.

which have differing mechanisms of anticancer action. The maintenance of hepatocyte viability, as demonstrated by continued albumin synthesis and bile flow, suggests that the effect is complex and involves more than a nonspecific arrest of cell metabolism. For a determination of whether amethopterin transport is dependent on protein synthesis, studies were performed with cycloheximide in the perfusion medium. This agent decreased protein synthesis, but it had no effect on amethopterin uptake or bile excretion, thereby reducing the likelihood that amethopterin transport is dependent on protein synthesis.

Although the concentrations of the chemotherapeutic agents required to decrease hepatic amethopterin transport in the rat might be considered high for the clinical analogy (10), demonstration of an inhibitory effect on amethopterin transport in human liver could have important implications regarding treatment protocols that use these agents. It is important to recognize that both increased antiproliferative activity and decreased biliary excretion of amethopterin, which could influence intestinal toxicity from this agent, may occur when these drugs are used in combination.

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