Reduction of Dimesna to Mesna by the Isolated Perfused Rat Liver

Marshall P. Goren, Li C. Hsu, and Jackie T. Li

Departments of Pathology and Laboratory Medicine [M. P. G., L. C. H., J. T. L.] and Hematology-Oncology [M. P. G.], St. Jude Children's Research Hospital, Memphis, Tennessee 38105-2794

ABSTRACT

Mesna is administered with ifosfamide and cyclophosphamide to reduce the incidence of hemorrhagic cystitis. In the present model of mesna metabolism and disposition, mesna is rapidly and irreversibly oxidized to dimesna in the plasma, passes unchanged through the liver, and is then reduced by the kidney and excreted. Our detection of a high ratio of mesna to dimesna in the plasma of clinical samples led us to reinvestigate the hepatic metabolism of mesna and dimesna. We perfused isolated rat livers from female Sprague Dawley rats with protein-free buffer solution containing dimesna at concentrations observed during therapy. In single-pass perfusions, each liver was perfused with up to three dimesna concentrations during consecutive 20-min periods. Recirculating perfusions were used to study single supratherapeutic concentrations of dimesna or mesna. Mesna and dimesna concentrations were measured by specific chromatographic procedures. Dimesna reduction, adjusted by the effluent flow rate and liver weight (0.4–58.5 nmol/min/g liver), correlated closely by linear regression (r = 0.98; n = 36) to the perfused dimesna concentration (4.2–249 μM), indicating a clearance of 0.20 ml/min/g liver. The concentration of dimesna that entered the liver closely matched the summed concentration of mesna and dimesna emerging in the effluent perfusate (single-pass experiments: slope, 0.98; intercept, –0.30; r = 1.00; n = 31). Only trace amounts of unidentified thiols were detected in the bile during recirculation of perfusates with 1 mM mesna or 250 μM dimesna. The effluent mesna concentration correlated inversely with the flow rate, which was consistent with a low extraction ratio in the perfusion model. These data suggested that the dimesna reduction rate was limited by hepatic uptake. Dimesna reduction was decreased by agents that deplete glutathione. Pretreatment of rats with up to 100 mg/kg ifosfamide did not impair hepatic dimesna reduction. In control experiments, dimesna was not reduced during recirculation through the apparatus without a liver. Mesna was oxidized to dimesna during oxygenation of the perfusate in the reservoir, but mesna injected directly into the perfusate just before entry into the liver passed unchanged into the effluent. Extrapolation of the dimesna clearance data from the perfusion model to humans suggests that hepatic dimesna reduction may counterbalance the rapid oxidation of mesna in plasma. The proposed equilibrium is consistent with clinical observations and suggests a new model for mesna metabolism and disposition.

INTRODUCTION

Mesna [2-mercaptoethanesulfonate sodium (HS-CH₂-CH₂SO₃⁻-Na⁺)] is administered to ifosfamide- and cyclophosphamide-treated patients to reduce the incidence of hemorrhagic cystitis (1). In the present model of mesna metabolism and disposition, mesna is rapidly oxidized in plasma from a free thiol to a disulfide (dimesna), which is physiologically inert and is the principal metabolite in the blood and urine (1–7). Relatively little mesna (10%) is protein-bound (6, 7). Plasma mesna and dimesna are confined to the vascular compartment and do not penetrate malignant or normal tissues with the exception that dimesna is absorbed by renal tubular cells, enzymatically reduced to mesna, and excreted into the renal tubular lumen (4, 5). Mesna passes into the urine where it protects the surface of the bladder by neutralizing the toxic ifosfamide metabolite acrolein (8, 9). Because of its limited metabolism, mesna protects the urinary tract, but does not impair anticancer therapy (2, 3).

In a study to determine the bioavailability of mesna in healthy human subjects, we detected more mesna than dimesna in nearly all plasma samples at all times after both i.v. and oral doses (10). This mesna is unlikely to be the remnant of unchanged drug because of its initial rapid clearance (7, 10–14). One suggestion to explain the persistence of mesna in the blood has been the slow release of sequestered mesna from erythrocytes (7). An alternative explanation might be the organ-mediated uptake and reduction of dimesna, followed by the reintroduction of mesna into the circulation. Earlier studies in rats treated with radiolabeled mesna and dimesna led authors to discount this possibility (4–7). The radioactivity detected in the individual organs of rats could be accounted for by residual blood within the organ, which suggested negligible uptake of the drug. The one exception was the accumulation of radioactivity in rat kidneys, which was consistent with the renal tubular reduction of mesna and the excretion of mesna into the urine. Perfusion of isolated rat kidneys with dimesna showed that mesna was reduced and excreted into the urine as mesna, but that only traces of mesna were reintroduced into the effluent perfusate (4, 5). Studies of isolated hepatocytes and of the isolated perfused liver led to the conclusion that the liver was totally inactive in the whole-body handling of mesna and dimesna in the rat (4).

Our clinical data (10–12) prompted us to reinvestigate the possibility of organ-mediated regeneration of mesna from dimesna. We studied the liver because it is a net exporter of glutathione (15), and glutathione seems to be involved in the reduction of dimesna (4). We perfused isolated rat livers with dimesna and looked for mesna in the effluent. Some rats were pretreated with agents that modulate hepatic glutathione (16). Our results show glutathione-dependent hepatic reduction of dimesna to mesna that is proportional to dimesna concentrations over a therapeutically relevant range.

MATERIALS AND METHODS

Chemicals. Dimesna (ASTA Medica AG, Frankfurt, Germany) contained 0.27% mesna; mesna (Bristol-Myers Squib, Princeton, NJ) was determined to contain 0.6% dimesna as an impurity.

Isolated Liver Perfusion. Female Sprague Dawley rats (weights, 189–368 g; Harlan Sprague Dawley, Indianapolis, IN) were anesthetized by i.p. injection with a saline solution (0.1 ml/100 g rat weight) of 100 mg/ml ketamine and 20 mg/ml xylazine. After i.p. injection of 400 units of heparin, the rat liver was freed from the surrounding connective tissue, and the bile duct, the portal vein, and the inferior vena cava were cannulated in situ within a 20-min period. The liver was transferred onto the warmed organ tray of a Perfuser twotoen Extracorporeal Perfusion System (MX International, Inc., Aurora, CO). The perfusate (37.0°C; SD ± 0.5°C) was introduced through the portal vein, and the effluent exited from the vena cava. The perfusate was a modified Krebs-Henseleit solution containing 118 mM NaCl, 4.8 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 2.5 mM CaCl₂, 25 mM NaHCO₃, 25 mM HEPES, and 10 mM glucose at pH 7.4 (17). The perfusate was supplemented in some instances with 50 μM taurocholate to enhance the flow of bile (18), which was collected into tubes containing 50 μl of 1 N HCl and 5 μl of 10% EDTA. The erythrocyte-
free protein-free buffer was continuously aerated (60 ml/min) for two 500-ml reservoir chambers) with a mixture of 95% O₂ and 5% CO₂. Rat livers were perfused at a targeted effluent flow rate of 4 ml/min/g liver (19) for up to 2 h. To assess the viability of the liver, the effluent flow rates and the perfusate concentrations of the enzymes lactate dehydrogenase, alanine aminotransferase, and aspartate aminotransferase were monitored during perfusions. The constancy of the pH, temperature, and oxygen-flow rate also were monitored because of their potential effect on the spontaneous oxidation rate of the free-thiol mesna. The liver was weighed after the perfusion.

**Perfusion Mode and Computations.** In recirculating perfusions, the effluent emptied back into the reservoir, which had a starting volume of 500 ml. The effluent and reservoir were sampled (1–2 ml) at frequent intervals during the perfusion. The rate of dimesna reduction was computed from the rate of mesna accumulation in the perfusate. This was determined from the product of the reservoir volume adjusted by the volume removed for sampling, and the mesna concentrations at 5, 10, 15, and 20 min. The calculated rate of increase in the moles of mesna was divided by one-half to obtain dimesna equivalents and expressed relative to the weight of the liver.

In single-pass perfusions, each liver was treated with up to three dimesna concentrations during consecutive 20-min exposures. The mesna reduction rates at 5, 10, 15, and 20 min during steady state were computed from the product of one-half of the effluent mesna concentration exiting the liver (minus any detectable mesna in the reservoir) and the effluent flow rate; the mean of the rates at the four sample times for each perfused dimesna concentration was divided by the liver weight.

**Glutathione Modulation.** Buthionine sulfoximine, dissolved in perfusion buffer, was injected i.p. into fasting rats at a dose of 4 mmol/kg at 180 min and at 90 min before surgery (20). Ethacrynic acid (2 mg/kg and 5 mg/kg), dissolved in perfusion buffer and adjusted to pH 7.4, was injected i.p. at 90 min before surgery (16). Ethacrynic acid (2 mg/kg) was coadministered with buthionine sulfoximine in one rat. Diethyl maleate (3 mmol/kg, dissolved in olive oil (3–4 parts v/v), was injected i.p. at 1 h or at 5 h before surgery; Refs. 16 and 21). L-2-oxothiazolidine-4-carboxylate (6.5 mmol/kg) in 0.9% saline was adjusted to physiological pH and injected i.p. at 4 h before surgery (16). Drugs were sterilized by filtration before injection.

**Analytical Procedures.** Mesna and dimesna concentrations were stabilized by HCl (0.18 N, final concentration) and EDTA (0.09%), and aliquots were stored below −70°C until analysis. Mesna and dimesna in acidified buffer were separated with high performance liquid chromatography by ion-pairing on a reverse-phase column (Beckman System Gold) or by gradient elution with 1 M NaCl on an anion exchange column (Hewlett Packard 1090 L system) and then quantitated by postcolumn sulfitolysis and reaction with 2-nitro-5-thiosulfobenzoate, an agent that reacts specifically with thiols and disulfides. The liver was weighed after the perfusion.

**Liver Viability.** Lactate dehydrogenase concentrations usually increased by 40–60 min, whereas aspartate aminotransferase and alanine aminotransferase levels were usually stable for 2 h. However, even several-fold increases in these indicators of hepatocellular damage did not predict for impaired reductive capacity. No data were excluded due to changes in enzyme release, pH, or perfusion flow rate.

**Mesna Perfluations.** To determine the rate of spontaneous oxidation of mesna to dimesna in our system, 1 mM mesna in the erythrocyte-free and protein-free buffer was recirculated through the apparatus without a liver. Fig. 1 shows a steady decline over 4 h in mesna concentrations accompanied by the appearance of dimesna. The sum of the mesna and twice the dimesna concentration remained unchanged, which suggested that mesna formed only dimesna. The rate of oxidation under the study conditions was 0.27%/min as mesna concentrations declined from 1014 to 360 μM. When the experiment in Fig. 1 was repeated with an oxygen flow rate that was halved to 30 ml/min, the rate of dimesna formation was reduced to 0.17%/min (data not shown).

To determine whether mesna passes unchanged through the hepatic vasculature, mesna was infused by use of a syringe pump directly into the perfusate (single pass) just before it entered the liver. To obtain a final 1 mM mesna concentration, the flow rate of the syringe pump was calibrated to deliver 0.51 ml/min of 40.1 mM mesna into perfusate that was pumped at a flow rate of 20 ml/min into a 9.9-g liver. Effluent samples were collected every minute for 10 min and then at 5-min intervals up to 30 min. The effluent mesna concentration attained equilibrium by min 4 (data not shown), which suggested that a 5-min interval should be sufficient to attain equilibrium in single-pass experiments. The dimesna as a percentage of the mesna in the effluent was not different from that in the syringe, which suggested no organ-mediated oxidation.

To detect the effect of the liver on the spontaneous rate of mesna oxidation in the buffer during a recirculating perfusion, 1 mM mesna in 500 ml of perfusate in the reservoir was pumped through a 9.6-g rat liver using conditions that were otherwise identical to those of Fig. 1; the effluent was sampled at 5-min intervals. By 90 min, the mesna decreased by 16% in the buffer as it recirculated through the liver (data not shown), as compared with the 21% decrease in mesna observed at 90 min without a liver (Fig. 1). There was a corresponding increase in dimesna, and the sum of mesna and dimesna remained steady.

Taken together, these control experiments showed that mesna spontaneously oxidized in the buffer, but passed unchanged through the hepatic vasculature.

**Recirculating Dimesna Perfluations.** In a control experiment, 1 mM dimesna was not reduced when recirculated through the apparatus without a liver (data not shown). We recirculated dimesna at supratherapeutic concentrations through livers to detect any reduction to mesna. Fig. 2 shows that dimesna was continuously reduced to mesna over a 2-h period. After 60 min, the dimesna-reducing activity declined in two of the three livers. The activity of the third liver remained steady for 2 h (Fig. 2, O), by which time 35% of the dimesna in the 500-ml reservoir was reduced to mesna.
and Flow Rate. Mesna averaged 4.5% of the total drug emerging in computed from these single-pass experiments, and from those of five dimesna concentrations ranging from 5-250 μM (based on > 100-μM experiments, the mesna concentration at 5 min was less than half of concentration/g liver correlated strongly but inversely with flow rate. perfusions performed with 50 μM dimesna (Fig. 4), the effluent mesna concentrations that might be observed during mesna therapy, including those instances because the system had not attained steady state. thus, it was possible to evaluate up to three drug concentrations during consecutive 20-min periods with each liver preparation. In a representative experiment (Fig. 3), steady-state concentrations were attained by 5 min after initiating each new drug concentration. In other experiments, the mesna concentration at 5 min was less than half of the value at 10, 15, and 20 min for 16% of the tested drug concentrations; so, the rate at 5 min was not included in the mean value in those instances because the system had not attained steady state.

To determine whether the reduction rate was proportional to dimesna concentrations that might be observed during mesna therapy, we perfused 15 livers (5.1–9.2 g) with a single pass of perfusate (range of flow rates, 1.7–5.5 ml/min/g liver) containing nominal dimesna concentrations ranging from 5–250 μM (based on a >100-μM value in a patient with a nephrectomy; Ref. 11). The reduction rates computed from these single-pass experiments, and from those of five recirculating perfusions are shown in Fig. 4. The rate of dimesna reduction (0.4–58.5 nmol/min/g liver) correlated closely by linear regression to the measured dimesna in the perfusate (4.2–249 μM).

Inverse Correlation between Effluent Mesna Concentration and Flow Rate. Mesna averaged 4.5% of the total drug emerging in the effluent at a mean flow rate of 4.0 ml/min/g liver. The percentage of the reduction was similar (means, 4.1–5.3%) for each of the six dimesna concentration groups (5–250 μM). However, individual values ranged from 2.2–8.4% in relationship to the flow rate. For the 10 perfusions performed with 50 μM dimesna (Fig. 4), the effluent mesna concentration/g liver correlated strongly but inversely with flow rate (r = −.92), declining by half as the effluent flow rate increased from 22 to 36 ml/min. This inverse correlation might be explained by limited hepatic uptake of dimesna such that hepatocytes were bathed in the same 50 μM dimesna regardless of the flow rate; by contrast, the amount of mesna secreted by hepatic cells was constant and was diluted to lower concentrations at higher effluent flow rates.

Bile and Other Metabolites. Bile was studied only during recirculating perfusions because an hour or more was required to collect a sample of sufficient size for analysis at bile flow rates ranging from 0.2–1.0 μl/min/g liver. Chromatographic peaks at the limit of quantitation near the retention time for dimesna were detected in bile after perfusion with 1 mM mesna, and several additional unidentified peaks were observed after perfusion with high dimesna concentrations (250, 500, and 1000 μM). These, presumably, were sulfides because the bile was alkaline (about pH 8.4) before preservation. Considering the low volume of bile and the small peak size, the amount of any mesna-associated thiol extracted from the perfusate and excreted into bile was negligible.

The sum of the concentration of dimesna and half that of mesna in the effluent during the single-pass experiments represented in Fig. 4 closely approximated the reservoir dimesna concentration (slope, 0.98; intercept, −0.30; r = 1.00; n = 31). Small chromatographic peaks in the perfusate were detected by the end of some recirculating experiments, which may reflect the release of endogenous thiols (15) and the formation of mixed mesna disulfides in trace amounts. Thus, essentially all perfused dimesna could be accounted for in the effluent as unchanged dimesna or as mesna, which was consistent with the negligible extraction into bile, and the reported (4) negligible accumulation of radiolabeled drug in liver.

Glutathione Modulation. Agents that deplete glutathione impaired the hepatic reduction of dimesna in comparison with the mean (8.6 ± 2.0 SD nmol/min/g liver) and range (5.7–11.4 nmol/min/g liver) of the 50 μM control group shown in Fig. 4. The livers of rats that were pretreated with ethacrynic acid, buthionine sulfoximine, or a combination of the two drugs showed a mean hepatic reduction rate of 5.5 ± 0.4 nmol/min/g liver (range, 5.3–5.8; n = 4). Reduction of dimesna by the isolated perfused livers of rats obtained at 1 h (4.9 nmol/min/g; n = 2) and at 5 h (6.6 nmol/min/g; n = 2) after treatment with diethyl maleate were consistent with the acute depletion of...
determined in 15 single-pass perfusion experiments (C) from the effluent concentration in the reservoir. The reduction rates in the five recirculating experiments (•) were computed from the initial slope (5-20 min) of the mesna values (see Fig. 2). An additional 31 reduction rates were determined in 15 single-pass perfusion experiments (C) from the effluent mesna concentrations (see Fig. 3). Regression statistics: rate = 0.20x - 1.08; r = 0.98; n = 36. The inset highlights data within the therapeutically relevant range (rate = 0.17x + 0.03; r = 0.89; n = 17); four overlapping data points were adjusted to permit their visual resolution.

The inset highlights data within the mesna concentrations (see Fig. 3). Regression statistics: rate = 0.20x - 1.08; r = 0.98; n = 36. The inset highlights data within the therapeutically relevant range (rate = 0.17x + 0.03; r = 0.89; n = 17); four overlapping data points were adjusted to permit their visual resolution.

**DISCUSSION**

Hepatic dimesna reduction by the isolated perfused rat liver was proportional over the range of dimesna concentrations previously observed in patients. All of the perfused dimesna could be accounted for as dimesna and mesna in the effluent. Only traces of thiols were detected in the bile after perfusion with mesna or dimesna. Effluent mesna concentrations correlated inversely with the perfusate flow rate. Taken together, these data suggest that the rate of dimesna reduction was limited by hepatic uptake. Reduction seemed to be glutathione-dependent. Ifosfamide doses up to 100 mg/kg did not impair hepatic dimesna reduction.

Extrapolation of data from the isolated perfused rat liver to humans suggests a potentially substantial hepatic contribution to the metabolism and disposition of mesna. Dimesna reduction is catalyzed by cytosolic glutathione-associated enzymes (4) that show similar activity in the isolated hepatocytes of human and rat liver (25, 26). If the assumption is reasonable that rat and human liver absorb (27) and reduce dimesna at a similar rate, then the rat liver dimesna clearance rate of 0.20 ml/min/g liver (Fig. 4) predicts a corresponding plasma clearance of 368 ml/min for adult males with a median liver weight (28) of 1840 g. Considering that human blood volume is approximately 70 ml/kg, and that dimesna would occupy the 60% that is plasma, the volume of distribution would be ±3 liters. The corresponding half-life may, thus, be as short as 5.6 min [ln(2) • volume of distribution / clearance]. This half-life represents the predicted reduction rate of dimesna by the human liver.

This theoretical hepatic dimesna reduction rate may counterbalance the rapid oxidation of mesna in human plasma. The concentration of mesna in oxygenated human plasma decreases with a half-life of about 5 min (4). Mesna oxidation in plasma is inhibited by EDTA, which is consistent with catalysis by metal-dependent enzymes (4, 7, 29). After oxygenation for 40 min at 37°C in a protein-free perfusate similar to ours, mesna concentrations were reported (4) to decrease by only 15% (0.38%/min, which is similar to our 0.27%/min oxidation rate; Fig. 1).

In volunteer subjects who received oral mesna, the measured ratio of mesna to dimesna in peripheral venous plasma was about 2.7, based on the area under the blood concentration versus time curves (10). Considering our predicted clearance of 368 ml/min dimesna for an average male and an approximate hepatic plasma flow rate of 870 ml/min (30), the mean dimesna extraction ratio (clearance / flow rate) for an adult male would be predicted to be 42%. This converts to a ratio of 1.5 moles of mesna for each mole of dimesna in the vena caval effluent [assuming complete reduction to mesna, (extraction ratio • 2)/ (1 − extraction ratio)]. Therefore, much of the dimesna in the blood may be reduced by the liver, contrary to the existing paradigm. The predicted mesna output is based on an assumption that only dimesna enters the liver. If one considers the additional circulating mesna that passes unchanged through the liver, then the predicted ratio of mesna to dimesna emerging from the liver may approach the actual ratio measured in human subjects. It is also possible that other organs contribute to the reduction of dimesna.

In addition, we underestimated the hepatic reductive activity/g liver in the rat model because we weighed the liver after the end of the perfusion. The liver is an expandable venous organ that readily enlarges in response to elevated arterial or venous pressure (30). Female Sprague Dawley rats have an average autopsied liver weight (6.77 ± 0.66 g; n = 10) that is 2.3% of body weight (292.4 ± 27.7 g) at 1 year of age (31). By contrast, we measured liver weights...
(8.3 ± 0.6 g) after perfusion that were 2.7% of the body weight (302 ± 12.5 g; range 282–320 g; n = 11). Our measured postperfusion liver weights were 17% higher than the expected weights because of the high buffer flow rate required to oxygenate the liver without blood cells. The resulting underestimate in the reduction rate/g liver would account for some of the difference between the mesna to dimesna ratio in the rat liver model and that observed in human subjects.

Ormstad et al. (4) reported that mesna and dimesna pass unchanged through the hepatic vasculature, are not taken up into liver cells, and are not excreted into bile. Our data support their conclusions that mesna and dimesna do not accumulate in liver cells and are not excreted into bile, and that mesna passes unchanged through the hepatic vasculature. Their evidence that dimesna reduction is glutathione-dependent is supported by our findings with agents that deplete glutathione. However, we detected significant hepatic dimesna reduction; this may stem from the increased specificity of our chromatographic procedure, because the conditions of liver perfusion seem to be otherwise similar.

In the present model of mesna disposition, mesna is irreversibly oxidized in the plasma to dimesna, which is the principal metabolite; and urinary mesna is derived by renal tubular reduction of filtered dimesna (3, 4). Our data support an alternative model in which plasma mesna is in equilibrium with dimesna. The liver recycles dimesna to mesna, but does not inactivate or extract either form for excretion into bile. Hepatic dimesna reduction is glutathione-dependent. Dimesna also might be reduced in plasma by reaction with endogenous thiols that were not present in this experimental model. A substantial proportion of the urinary mesna required to avert hemorrhagic cystitis may be derived by glomerular filtration of plasma mesna rather than by renal tubular reduction of dimesna.

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