Pharmacokinetics and Therapeutic Uses of Mesna

Murray J. Cutler, The University of Western Ontario

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Pharmacology and Toxicology
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PHARMACOKINETICS AND

THERAPEUTIC USES OF MESNA

(Spine Title: Pharmacokinetics and Therapeutic Uses of Mesna)

(Thesis Format: Integrated-Article)

By

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Graduate Program

In

Pharmacology and Toxicology

Submitted in partial fulfillment

of the requirement for the degree of

Doctor of Philosophy

School of Graduate and Postdoctoral Studies

The University of Western Ontario

London, Ontario

November, 2010

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The thesis by

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entitled:

Pharmacokinetics and Therapeutic Uses of Mesna

is accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Date__________________________

Chair of the Thesis Examination Board
ABSTRACT

In the early 1980s, significant advancement in the safety of ifosfamide therapy was achieved by co-administrating mesna (sodium 2-mercaptoethane sulfonate) to prevent dose-limiting hemorrhagic cystitis. Mesna exerts its protective effect within the urine, where its free sulfhydryl group is able to conjugate cytotoxic metabolites. Within the circulation, however, mesna exists primarily as its inactive disulfide, dimesna. Dimesna is currently undergoing clinical development as a prodrug (BNP7787) to treat cisplatin-induced nephrotoxicity. Remarkably, chemoprotection is achieved without attenuation of efficacy of co-administered anti-cancer agents. This is widely attributed to the kidney-specific disposition and stability of dimesna.

We sought to evaluate the role of drug transporters in the disposition of dimesna. In vitro screens of uptake and efflux transporters identified putative mechanisms of apical and basolateral uptake of dimesna and subsequent secretion of mesna into renal tubules. Administration of the renal drug transporter inhibitor probenecid to healthy subjects significantly increased combined mesna and dimesna plasma exposure while decreasing the renal clearance due to secretion and steady-state volume of distribution.

Chemical reduction of dimesna to mesna is essential for the mitigation of ifosfamide- and cisplatin-induced toxicities. In vitro, reduction of dimesna was facilitated by redox enzymes of the thioredoxin and glutaredoxin systems and also by non-enzymatic thiol-disulfide exchange with cysteine and glutathione. These findings supported the further investigation of mesna as a thiol exchange agent to lower the toxic endogenous thiol amino acid homocysteine (Hcy).
Increased plasma total homocysteine (tHcy) is a graded, independent risk factor for the development of atherosclerosis and thrombosis. Over 90% of patients with end-stage renal disease (ESRD) have elevated plasma tHcy. Previous studies have expanded the use of mesna to exchange with albumin-bound Hcy, thereby enhancing its dialytic clearance. Although an initial pilot study of 12 mg/kg intravenous mesna administered predialysis caused a significant decrease in plasma tHcy compared to placebo, prolonged treatment had no effect on plasma tHcy.

Successful therapeutic uses of mesna and dimesna are likely due to their unique disposition by renal drug transporters and thiol-disulfide redox equilibrium. Loss of renal transporter function due to disease, drug-drug interactions, or genetic variability may decrease their therapeutic efficacy.

**KEYWORDS**

Mesna, Dimesna, Transporters, Thiol-disulfide exchange, Homocysteine, End-Stage Renal Disease, Pharmacokinetics
CO-AUTHORSHIP

Versions of chapters two and three of this thesis are undergoing revision prior to submission for publication. Chapter four has been published as:


Murray J. Cutler wrote the initial draft of all manuscripts, but received considerable aid with the experimental work and final manuscript preparation from colleagues and supervisors as follows.

Drs. Urquhart and Meyer zu Schwabedissen aided in experimental design and execution, and manuscript preparation. Thomas Velenosi helped with drug analysis. Dr. Dresser provided physicals to all subjects. Drs. Freeman, Kim, and Tirona supervised the project and aided in manuscript preparation. Erin Brouwer is credited with the artistic production of Figure 2.6.

CHAPTER 3: Murray J. Cutler, Thomas J. Velenosi, David J. Freeman.
ENZYMATIC AND NON-ENZYMATIC MECHANISMS OF DIMESNA METABOLISM
Thomas Velenosi aided in experimental design and execution. Dr. Freeman supervised the project and aided in manuscript preparation.

CHAPTER 4: Murray J. Cutler, Bradley L. Urquhart, David J. Freeman, J. David Spence, Andrew A. House. MESNA FOR THE TREATMENT OF HYPERHOMOCYSTEINEMIA IN HEMODIALYSIS PATIENTS
Dr. House recruited all patients and aided in experimental design and manuscript preparation. Drs. Spence and Urquhart aided in experimental design and manuscript preparation. Dr. Freeman supervised the project and aided in experimental design and manuscript preparation.
DEDICATION

To Grandpa
ACKNOWLEDGEMENTS

Over the past decade I’ve been exceedingly fortunate to share fun, food, and research with members, both past and present, of ‘Riederville’ and the ‘Kim Lab’. Michael Knauer, you’ve got all the talents to be a great PI, and your golf game is already ‘pretty OK’. Marianne DeGorter, I’ve no doubt that your hard work and intellect will continue to elevate you to scientific excellence.

To my friends of the Society of Automotive Engineers for giving me a new perspective on problem solving. ERTW.

Thanks to Dr. Jane Tucker for having the patience to teach me the foundations of cellular biology. Dr. Michael Rieder, I’ll always carry your erudite teachings of the ways of (scientific) war and victory in Grad School. Veni, vidi, vici. Dr. Jack Bend, you are a model of professionalism; I’m truly privileged to count you amongst my advisors. Dr. Peter Chidiac, you’ve always given me thoughtful insight into the art of scientific methods and communication. Dr. Andrew House, your kind support and enthusiasm for scientific investigation have been invaluable to me, both in the clinic, and in the lab.

Timely completion of this work would not have been possible, and not nearly as much fun, had it not been for the dedication of young pharmacologists Thomas Velenosi and Ankur Bodalia. Tom, I’ve no doubt your keen sense of humour and understanding of the scientific method will bring you great success in Dr. Urquhart’s lab and beyond. Ankur, you’re a valuable asset to the Freeman Lab; your curiosity and tenacity will lead you to a bright future in science.
To my best friend, Derek Bone, thanks for your collegiality and commiserations; without you Grad School would have been a lonely journey. I wish I could quit you. Here’s to many years of collaboration to come!

Thanks to everyone who has made our clinical research possible. To Linda Asher, for your pragmatic approach to clinical research. I’m grateful, both as a researcher and volunteer, for your thoughtfulness and compassion in the clinic. To Ruth Bullas, MaryJeanne Edgar, and the rest of the Nephrology Clinical Research Group for your help and dedication. To David Demelo for your ingenuity and willingness to always lend a hand, even while keeping an entire dialysis unit running!

To Dr. Edna Choo, I can’t thank you enough for the opportunities you gave me during my time at Genentech. I’m incredibly grateful for your depth of scientific knowledge, keen management, gracious hospitality, and above all, friendship. My time spent under your management was some of the best of my graduate career. Part of me will always long to return to San Francisco. Jason Boggs, your honesty, friendship, and perspective helped me choose my path as a scientist. I still can’t believe Michael Jackson’s dead. Emile Plise, you showed me the value of “just givin’ it a shot”. You are the embodiment of professional commitment; Genentech will continue to take care of you, too.

To Dr. Henriette Meyer zu Schwabedissen, you are the most brilliant molecular biologist I have ever met; I’m exceptionally lucky to have learned under your tutelage. Dr. Ute Schwarz, Cindy and I will miss your kindness and openness to share your time and knowledge. You’ll make a great Canadian, but don’t ever lose your German resolve! Dr. Rommel Tirona you’re a humble scientific genius who is never too busy to share your
wisdom, and for that I’ll always be indebted. How does it feel to do science with mortals? Dr. Richard Kim, thanks for all of the opportunities you’ve given me and for welcoming me as part of the Kim Lab. I have no doubt that through your vision and affable leadership Clinical Pharmacology will continue to grow to world renown.

Dr. David Bailey, our many conversations have imparted your wisdom as a scientist, father, and grandfather, and for that I’ll always be thankful. Lt. Scott Bailey, thanks for your bright technical support and shared ‘nerditry’. Your camaraderie within the lab has been missed these past few years, but I’m grateful for your service. Pro Patria.

Above all else, I’m most grateful for the guidance of Drs. Brad Urquhart and David Freeman. Brad, you’ve been a role model, mentor, and friend. You have always stirred in me aspirations of success in the lab, in the classroom, and on the ice! Dr. David Freeman, through wisdom and collegiality, you are universally regarded as the epitome of the very best of Western’s supervisors, mentors, and teachers. It has been a privilege and pleasure to work under your supervision. Your hands-on approach has continuously demonstrated that an open mind driven by passionate curiosity is at the heart of being a scientist. Our Coffee Time discussions, musings, and philosophies will be sorely missed.

To my fiancée Cindy Yip, for your support in the face of adversity, and never letting failure become an option; because this is just the beginning.
# TABLE OF CONTENTS

CERTIFICATE OF EXAMINATION................................................................................ii
ABSTRACT.......................................................................................................................iii
KEYWORDS......................................................................................................................iv
CO-AUTHORSHIP.............................................................................................................v
DEDICATION....................................................................................................................vi
ACKNOWLEDGEMENTS...............................................................................................vii
TABLE OF CONTENTS....................................................................................................x
LIST OF TABLES............................................................................................................xvi
LIST OF FIGURES.........................................................................................................xvii
LIST OF APPENDICIES...............................................................................................xxiii
LIST OF ABBREVIATIONS, SYMBOLS, NONMENCLATURE......................................xxiv

## CHAPTER 1: INTRODUCTION................................................................................1

1.1 Mesna Background...............................................................................................1

1.2 Mesna for Cancer Chemotherapy.......................................................................2
  1.2.1 Mesna............................................................................................................2
  1.2.2 Mesna Preclinical Development....................................................................3
    1.2.2.1 Pharmacodynamics..................................................................................4
    1.2.2.2 Pharmacodynamic Drug Interactions.......................................................7
    1.2.2.3 Safety.......................................................................................................8
  1.2.3 Clinical Development......................................................................................8
    1.2.3.1 Efficacy....................................................................................................9
    1.2.3.2 Standard Ifosfamide-Mesna IV Therapy..................................................11
    1.2.3.3 High-Dose Ifosfamide IV Therapy.............................................................12
    1.2.3.4 Oral Mesna Therapy................................................................................13
2.2 Methods..................................................................................................................98

2.2.1 In vitro Screen of Drug Transporters Capable of Dimesna Transport.................................................................98

2.2.2 In vitro Data Fitting and Statistical Analysis.................................................................................................................99

2.2.3 In vitro Determination of Efflux Transporters Capable of Mesna Export.........................................................................................99

2.2.4 Study Design and Volunteer Subjects.............................................................................................................................100

2.2.5 Total Mesna Analysis..........................................................................................................................................................101

2.2.6 Probenecid Analysis..............................................................................................................................................................102

2.2.7 Pharmacokinetic Analysis......................................................................................................................................................103

2.2.8 Estimation of Renal Clearance in the Presence of Probenecid.............................................................................................104

2.3 Results..................................................................................................................................................................................105

2.3.1 OATs are Responsible for Kidney-Specific Uptake of Dimesna.............................................................................................105

2.3.2 Differential Efflux of Dimesna and Mesna by ATP-Binding Cassette (ABC) Transporters and MATE1.........................106

2.3.3 Secretion of Mesna by Proximal Tubule Cells..........................................................................................................................107

2.3.4 In vivo Effect of Probenecid on the Disposition of Mesna....................................................................................................107

2.4 Discussion..............................................................................................................................................................................126

2.5 References............................................................................................................................................................................135

CHAPTER 3: ENZYMATIC AND NON-ENZYMATIC MECHANISMS OF DIMESNA METABOLISM.................................................................141

3.1 Introduction...........................................................................................................................................................................142
3.2 Materials and Methods

3.2.1 Materials

3.2.2 Enzymatic Activity of Purified Glutaredoxin and Thioredoxin Systems

3.2.3 Enzymatic Activity of Tissue Homogenates and Cell Lysates

3.2.4 Spectrophotometric Assay of Enzymatic Activity

3.2.5 Reduction of Dimesna by Non-Enzymatic Thiol-Disulfide Exchange and Cell Lysates

3.2.6 Thiol Analysis

3.2.7 Determination of Non-Enzymatic Thiol-Disulfide Exchange Micro-Rate Constants

3.2.8 Modeling of Non-Enzymatic Reduction of Dimesna in HeLa cell Incubations

3.2.9 Calculation of Dimesna/Mesna Equilibrium Constant

3.2.10 Calculation of Dimesna/Mesna Half-Cell Potential

3.2.11 Data Analysis and Statistics

3.3 Results

3.3.1 Non-Enzymatic Reduction of Dimesna

3.3.1.1 Estimation of Reaction Order and Macro-Rate Constants

3.3.1.2 Estimation of Micro-Rate Constants

3.3.1.3 Calculation of Dimesna Redox Equilibrium Constants and...
3.3.2 Enzymatic Reduction of Dimesna...............................................154
  3.3.2.1 Direct Reduction of Dimesna by Thioredoxin System........155
  3.3.2.2 Indirect Reduction of Dimesna by Glutaredoxin System....155
  3.3.2.3 Enzymatic Reduction of Dimesna by Tissue and Cell
           Homogenates........................................................................155

3.3.3 Contribution of Enzymatic and Non-Enzymatic Mechanisms of
           Reduction of Dimesna in HeLa Cell Lysates.........................156
  3.3.3.1 Reduction of dimesna by HeLa Cell Lysate Before and After
           Denaturation........................................................................156
  3.3.3.2 Modeling of Sulfhydryl and Disulfide Species in HeLa Cell
           Lysate..................................................................................157

3.4 Discussion................................................................................................179

3.5 References................................................................................................191

CHAPTER 4: MESNA FOR THE TREATMENT OF HYPERHOMOCYSTEINEMIA
           IN HEMODIALYSIS PATIENTS.........................................................198

4.1 Introduction..........................................................................................199

4.2 Methods..................................................................................................205
  4.2.1 Subjects.........................................................................................205
  4.2.2 Design of Studies...........................................................................205
  4.2.3 Dialysate Collection.......................................................................206
  4.2.4 Sample Analysis & Statistical Methods........................................207

4.3 Results.....................................................................................................210
4.3.1 One Week Study.................................................................210
4.3.2 Four Week Study...............................................................210
4.4 Discussion..............................................................................218
4.5 References............................................................................227

CHAPTER 5: GENERAL DISCUSSION.............................................235

5.1 Summary and Future Areas of Research.................................236
5.1.1 \textit{In vitro} and \textit{In vivo} Assessment of the Role of Organic Anion Transporters in the Disposition of Mesna and Dimesna........236
5.1.2 Enzymatic and Non-Enzymatic Mechanisms of Dimesna Metabolism.................................................................240
5.1.3 Mesna for the Treatment of Hyperhomocysteinemia in Hemodialysis Patients.........................................................242

5.2 Conclusions............................................................................245
5.3 References............................................................................246

APPENDICES.............................................................................252

CURRICULUM VITA................................................................259
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table 1.1</th>
<th>Cancer chemotherapy substrates and expression patterns of drug uptake transporters of the SLC22A and SLCO family in healthy and cancerous human tissues</th>
<th>36</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 2.1</td>
<td>Baseline characteristics of healthy volunteers of clinical mesna-probenecid interaction study</td>
<td>110</td>
</tr>
<tr>
<td>Table 2.2</td>
<td>Non-compartmental pharmacokinetic parameters of plasma total mesna concentration-time curves of clinical mesna-probenecid interaction study</td>
<td>111</td>
</tr>
<tr>
<td>Table 3.1</td>
<td>Estimated second-order micro-rate constants of S_N2 reaction scheme in Figure 3.5, n = 12, P-value calculated non-parametrically by Mann-Whitney U test</td>
<td>158</td>
</tr>
<tr>
<td>Table 4.1</td>
<td>Baseline Patient Characteristics; one-week study</td>
<td>212</td>
</tr>
<tr>
<td>Table 4.2</td>
<td>Baseline Patient Characteristics; four-week study</td>
<td>213</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure 1.1  Metabolism of ifosfamide and mesna. Ifosfamide is a chiral prodrug that predominantly undergoes autoinducible CYP3A4-mediated metabolism, yielding the reactive metabolite 4-hydroxyifosfamide. 4-Hydroxyifosfamide spontaneously forms its isomer aldoifosfamide, which subsequently breaks-down to active ifosfamide mustard and the potent urotoxin acrolein. Alternatively, ifosfamide may be metabolized by CYP2B6 to isomers (S)-2-dechloroethylifosfamide or (S)-3-dechloroethylifosfamide along with the nephrotoxin chloroacetaldehyde. Mesna exerts its protective effect within the urine, where its free sulfhydryl group covalently binds 4-hydroxyifosfamide forming inactive 4-sulfoethylthioifosfamide. Upon entering the circulation mesna undergoes metal-catalyzed autooxidation to dimesna which may be reduced to mesna via thiol exchange........................................6

Figure 1.2  Conjugation of cisplatin and metabolites by mesna. Cisplatin is readily hydrated by displacement of chlorine with water to form active monohydroxo and monoaqua metabolites. As a disulfide, dimesna cannot conjugate cisplatin or its hydrated reactive metabolites. Upon entry to organ parenchyma, dimesna undergoes thiol exchange with GSH and cysteine yielding active mesna which may form an inactive adduct with intracellular cisplatin or its metabolites........................................................16

Figure 2.1  Dimesna transport activity of human uptake transporters following heterologous expression in HeLa cells. [3H]Dimesna (552 nM) was used for all experiments. Cellular accumulation was measured after incubation at 37°C for 10 minutes. The data are expressed as percent of vector control (mean ± SE, n = 3). Differences between transfectants determined by ANOVA, *, ** P < 0.05).................................................................112

Figure 2.2  Kinetics of dimesna uptake by OAT1 (a), OAT3 (b), OAT4 (c) following heterologous transporter expression in HeLa cells. Transporter-mediated uptake was calculated as the difference between OAT-expressing cells and vector controls treated with dimesna of the same specific activity. Accumulated dimesna was assessed after incubation at 37°C for 10 min. Data represent mean ± SE (n = 3).................................................................114

Figure 2.3  Kinetics of dimesna uptake by OAT1 (a), OAT3 (b), OAT4 (c) following heterologous transporter expression in HeLa cells. Transporter-mediated uptake was calculated as the difference between OAT-expressing cells and vector controls treated with dimesna of the same specific activity. Inhibitory effect of probenecid is expressed as % vector control. Accumulated dimesna was assessed after incubation at 37°C for 10 min. Inhibition constant (K_i) was calculated assuming reversible one-site
competitive binding, [3H]dimesna concentration of 467.5 nM, and the previously determined $K_M$. Data represent mean ± SE (n = 4).

Figure 2.4  Dimesna transport activity of human efflux transporters following recombinant vaccinia-mediated expression in HeLa cells. [3H]Dimesna (552 nM) was used for all experiments. Cellular accumulation was measured after incubation at 37°C for 10 minutes. The data are expressed as percent of vector control (mean ± SE, n = 5). Differences between transfectants determined by ANOVA, *, ** $P < 0.05$.

Figure 2.5  Export of mesna by efflux transporters following incubation with dimesna. HeLa cells transfected with either efflux transporter alone (open circles) or uptake transporter OAT4 and efflux transporter (open triangles) by recombinant vaccinia. Following incubation with [3H]dimesna (1 µM) at 37°C for 60 minutes, [3H]mesna present in the supernatant was derivatized with monobromobimane and separated by liquid chromatography and fractions detected by liquid scintillation counting. (a) [3H]dimesna standards with and without chemical reduction to [3H]mesna prior to derivatization ([3H]mesna retention time ~12 min). (b) pEF (empty vector, open circles) and OAT4 (uptake transporter, open triangles), only. (c) - (i) efflux transporter and uptake-efflux transporter pairs. BCRP, breast cancer resistance protein; dpm, disintegrations per minute; MATE1, multidrug and toxin extrusion protein 1; MRP1, 2, 4, 5, multidrug resistance protein 1, 2, 4, 5; Pgp, P-glycoprotein.

Figure 2.6  Localization of identified dimesna and mesna transporters in the proximal tubule cell. At the basolateral membrane dimesna may be taken up from the blood via OAT1 and OAT3. Following filtration at the glomerulus (a), dimesna may also be taken up into proximal tubule cells at the luminal (apical) membrane via OAT4 (b). Intracellularly, dimesna undergoes reduction to active mesna capable of conjugating toxic metabolites of cisplatin and ifosfamide. Intracellular dimesna may be actively exported to the urine across the apical membrane via MRP4, or may be reduced and subsequently actively exported as mesna by Pgp, MRP2, or in exchange for protons via MATE1. ATP, adenosine triphosphate; MATE1, multidrug and toxin extrusion protein 1, MRP2, 4, multidrug resistance protein 2, 4; OAT1, 3, 4, organic anion transporter 1, 3, 4; Pgp, P-glycoprotein.

Figure 2.7  Mean plasma probenecid concentration Sessions 2-3. Healthy volunteers were given 0.4 g/m² oral mesna (Session 1), 1 g oral probenecid (Session 2) followed by 1 g probenecid twice-daily for 6 days, and finally 0.4 g/m² oral mesna (Session 3). Data fitted simultaneously to a two-compartment model with non-linear elimination (mean ± SD, n = 12).
Figure 2.8  Inhibition of total mesna clearance by probenecid. Healthy volunteers were given 0.4 g/m² oral mesna (Session 1), 1 g oral probenecid (Session 2) followed by 1 g probenecid twice-daily for 6 days, and finally 0.4 g/m² oral mesna (Session 3). Mean plasma total mesna (a, b) and total mesna excreted (c) during Session 1 (mesna only, ◊) and Session 3 (mesna + probenecid, □). Data represent mean ± SD, n = 12. ........................................................................................................... 124

Figure 2.9  Inhibition of total mesna clearance by probenecid. Healthy volunteers were given 0.4 g/m² oral mesna (Session 1), 1 g oral probenecid (Session 2) followed by 1 g probenecid twice-daily for 6 days, and finally 0.4 g/m² oral mesna (Session 3). Fraction of renal clearance due to secretion with and without probenecid (* P < 0.001). Data represent mean ± SD, n = 12. ........................................................................................................... 125

Figure 3.1  Production of mesna by bimolecular nucleophilic substitution (SN₂) reactions whereby a nucleophilic thiol (e.g. Cys or GSH) first displaces a mesna moiety of dimesna yielding mesna and a mixed disulfide, followed by substitution of the mesna moiety of the mixed disulfide, producing a second molecule of mesna and a homogeneous disulfide. Rate constants k₁, k₂, k₃, k₄ and species R, MM, RM, RR, and M denote parameters and variables (respectively) utilized to describe equilibrium kinetics in Equations 3.1 – 3.6. ........................................................................................................... 152

Figure 3.2  Non-enzymatic reduction of 1 mM dimesna by cysteine (a, b) and glutathione (c, d) mediated thiol exchange in PBS, 1 mM EDTA at 37°C, pH = 7.0. Free thiol was measured by MBB derivatization followed by liquid chromatography with fluorescence detection. Fitted curves (solid lines) correspond to one-phase exponential association and one-phase exponential degradation of mesna production and thiol (Cys or GSH) loss. Starting thiol concentrations are in micromolar. Data represented as mean ± SE, n = 3. ........................................................................................................... 160

Figure 3.3  Non-enzymatic reduction of dimesna by 1 mM cysteine (a, b) and 1 mM glutathione (c, d) mediated thiol exchange in PBS, 1 mM EDTA at 37°C, pH = 7.0. Free thiol was measured by MBB derivatization followed by liquid chromatography with fluorescence detection. Fitted curves (solid lines) correspond to one-phase exponential association and one-phase exponential degradation of mesna production and thiol (Cys or GSH) loss. Starting dimesna concentrations are in micromolar. Data represented as mean ± SE, n = 3. ........................................................................................................... 162

Figure 3.4  Initial velocities of mesna production by dimesna with excess Cys (a), Cys with excess dimesna (b), dimesna with excess GSH (c), and GSH with excess dimesna (d). Slope of the lines indicate thiol exchange of dimesna
is first order for each of the reactants. Data represented as mean ± SE, n = 3.

Figure 3.5  Observed first-order rate constants (k_{obs}) of mesna production in the presence of excess Cys (a) and GSH (b) versus starting dimesna concentrations. Slope of the line indicates second-order rate constant for the reduction of dimesna by cysteine (a) and GSH (b). Data represented as mean ± SE, n = 3.

Figure 3.6  Reduction of dimesna by purified recombinant thioredoxin (500 nM) and thioredoxin reductase (11.75 nM) in PBS, 1 mM EDTA at 37°C, pH = 7.0. Enzyme velocities were measured by analysis of NAD formed spectrophotometrically at 340 nm. Data represented as mean ± SE, n = 3. Differences between incubations determined by ANOVA, *, **, *** P < 0.01.

Figure 3.7  Scheme of dimesna (MSSM) reduction by the thioredoxin system. The N-terminal active site Cys residue of thioredoxin(SH)_{2} reduces protein disulfides by forming a transient mixed disulfide followed by fast thiol-disulfide exchange oxidizing a pair of reactive Cys at the active site producing thioredoxin(S)_{2}. Alternatively, reduced thioredoxin(SH)_{2} may directly reduce dimesna to form two mesna moieties and thioredoxin(S)_{2}. Reduction of thioredoxin(S)_{2} is facilitated by the flavoprotein thioredoxin reductase via electron transfer from NADPH.

Figure 3.8  Reduction of dimesna by purified recombinant glutaredoxin (14.15 nM) and glutathione reductase (80.59 nM) in PBS, 1 mM EDTA at 37°C, pH = 7.0. Enzyme velocities were measured spectrophotometrically at 340 nm. Data represented as mean ± SE, n = 3. Differences between incubations determined by ANOVA, * P < 0.01.

Figure 3.9  Scheme of dimesna (MSSM) reduction by the glutaredoxin system. Similar to thioredoxin, the N-terminal active site Cys residue of glutaredoxin(SH)_{2} reduces protein disulfides by forming a transient mixed disulfide followed by fast thiol-disulfide exchange oxidizing a pair of reactive cysteines at the active site producing oxidized glutaredoxin(S)_{2}. Additionally, glutaredoxin(SH)_{2} specifically reduces S-glutathionylated proteins and mixed disulfides, yielding a glutaredoxin-GSH conjugate. Dimesna (MSSM), although not a substrate of glutaredoxin, can first undergo non-enzymatic thiol exchange with GSH to produce the mixed mesna-glutathione disulfide (MSSG). MSSG can subsequently be reduced by glutaredoxin producing mesna and a glutaredoxin-GSH conjugate. The glutaredoxin-GSH conjugate can be reduced to glutaredoxin(SH)_{2} by an additional molecule of GSH, forming oxidized glutathione (GSSG). Glutathione reductase recycles GSSG to two molecules of GSH via electron transfer from NADPH.
Figure 3.10 Enzymatic activity of mouse kidney and liver homogenates, and HeLa cell lysates in the presence of 1 mM NADPH, 1 mM oxidized glutathione (GSSG) and/or 1 mM dimesna (MSSM). Data represented as mean ± SE, kidney and liver; n = 5, HeLa; n = 3, *; ** P < 0.01

Figure 3.11 Enzymatic activity of mouse kidney (○) and liver (□) homogenates incubated with 1 mM NADPH, 1 mM dimesna, and increasing concentrations of reduced glutathione (GSH). Data represented as mean ± SE, n = 5

Figure 3.12 Mesna (a), Cys (b), and GSH (c) concentrations following incubation of HeLa cell lysate (1 mg/mL in PBS, 1 mM EDTA) with 1 mM dimesna at 37°C before (○) or after (□) protein denaturation. Data represented as mesna ± SE, n = 3

Figure 3.13 Predicted concentrations of thiol species due to non-enzymatic thiol exchange as described by Equations 3.1-3.6 using second-order rate constants listed in Table 3.1 and basal endogenous thiol concentrations of HeLa lysate before (a) or after (b) denaturation. Observed mesna (Δ), Cys (○), and GSH (□) concentrations presented in Figure 3.12 are plotted as reference

Figure 4.1 Methionine pathway. Enzymes and vitamin cofactors indicated by open and shaded ellipses, respectively. Abbreviations used: 5,10-MTHF, 5,10-methylenetetrahydrofolate; 5-MTHF, 5-methyltetrahydrofolate; AdoHcy, S-adenosylhomocysteine; AdoMet, S-adenosylmethionine; BHMT, betaine-homocysteine methyltransferase; CBS, cystathionine ß-synthase; Cys, cysteine; Cysta, cystathionine; DHF, dihydrofolate; DHFR, dihydrofolate reductase; DMG, dimethylglycine; GGCS, γ-glutamylcysteine synthase; γ-GluCys, γ-glutamylcysteine; GS, glutathione synthase; GSH, glutathione; Hcy, homocysteine; MAT, methionine adenosyl transferase; Met, methionine; MS, methionine synthase; MTHFR, methylenetetrahydrofolate reductase; SAHH, S-adenosylhomocysteine hydrolase; SHMT, serine hydroxymethyltransferase; THF, tetrahydrofolate; TS, thymidylate synthase

Figure 4.2 Predialysis plasma total mesna concentrations of seven subjects administered 12 mg/kg of mesna thrice weekly for four weeks and twelve days after the last dose. Results are expressed as mean ± SD, n = 7

Figure 4.3 Predialysis plasma tHcy concentrations of seven subjects administered 12 mg/kg of mesna (Δ) and an equal volume of saline (□) thrice weekly for four weeks and twelve days after the last dose. Results are expressed as mean ± SD, n = 7
Figure 4.4 Pre- and postdialysis plasma tHcy concentrations following the first (day 0) and last (day 28) doses of placebo and mesna. Results are expressed as mean ± SD, n = 7, *, ** P < 0.05.............................................................216

Figure 4.5 tHcy excreted by three subjects administered 12 mg/kg of mesna (Δ) and an equal volume of saline (□) during the first dialytic session of each treatment arm. Results are expressed as mean ± SD, n = 1-3.................217
**LIST OF APPENDICES**

<table>
<thead>
<tr>
<th>Appendix</th>
<th>Description</th>
<th>Page</th>
</tr>
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CHAPTER 1: INTRODUCTION

1.1 Mesna Background

Mesna (sodium 2-mercaptoethane sulfonate, Mistabron®) amongst other thiolate compounds N-acetylcysteine (NAC) and S-carboxymethylcysteine, was first developed as a potential mucolytic for treatment of impaired mucociliary clearance in diseases such as asthma, chronic bronchitis, and cystic fibrosis. As a low molecular weight compound with a free sulfhydryl group, it was believed that mesna could reduce disulfide bonds of glycoproteins that constituted mucus, thereby reducing its viscosity and easing expectoration by patients (Houtmeyers et al., 1999). Despite promising in vitro results showing significant sputum-thinning (i.e. mucolytic) activity (Hirsch et al., 1966; Hirsch et al., 1969), and case reports of application in intensive care (Van De Walle et al., 1976), a clinical trial of aerosolized mesna failed to improve sputum clearance in patients with chronic bronchitis compared to isotonic saline aerosol (Clarke et al., 1979). In comparison, NAC remains the well-tolerated mucolytic of choice for management of sputum in asthma, cystic fibrosis, chronic obstructive pulmonary disease (Rogers, 2007).

Only after its chemical synthesis was mesna identified as coenzyme M in the methanogenic archaea methanobacterium hermoautotrophicum (Taylor & Wolfe, 1974). Methylated coenzyme M (2-(methylthio)ethanesulfonic acid) is a cofactor for the methylcoenzyme M methylreductase system which acts to reduce carbon dioxide (CO$_2$) to methane (CH$_4$) (Rouviere & Wolfe, 1988; Balch & Wolfe, 1979). Coenzyme M is essential to this hallmark process allowing methanogens to use CO$_2$ as an electron acceptor during metabolism (Fahey, 2001). Later screening revealed that the gram-negative bacterium Xanthobacter strain Py2 and gram-positive bacterium Rhodococcus
*rhodochrous* strain B276 also possess coenzyme M, where it functions as a cofactors in short-chain alkene metabolism (Allen *et al.*, 1999; Krum & Ensign, 2000). Thus, it would seem that through their own endogenous production, the methanogens and their recent evolutionary prokaryotic relatives were the first to exploit the nucleophilicity and redox properties of mesna as coenzyme M.

### 1.2 Mesna for Cancer Chemotherapy

#### 1.2.1 Mesna

Cancer treatment requires effective management of not only the disease but also the cancer therapy itself. Chemotherapy-induced toxicities frequently prohibit effective quantity and duration of administered doses. Various drugs have been developed to control side effects associated with chemotherapy, including erythropoietics for anemia (epogen, darbepoetin), antiemetics for nausea and vomiting (e.g. ondansetron, aprepitant), and immunostimulators for neutropenia (pegfilgrastim). Failure to provide adequate chemoprotection during regimen initiation may mandate withdrawal and postponement of treatment (i.e. dose delay), lowering the probability of curative therapy. In addition, sustained toxicity can lessen both short- and long-term quality of life of cancer survivors (Hensley *et al.*, 1999; Hogle, 2007).

Ifosfamide is a broad spectrum antineoplastic agent approved for first line treatment of cervical cancer, soft tissue sarcomas and second line treatment of pancreatic cancer. In addition, ifosfamide has been widely used in the treatment of refractory germ cell tumors and malignant lymphomas (Higgs *et al.*, 1989; Wagner, 1994). Although effective against a variety of sarcomas, the initial use of ifosfamide was limited by
hemorrhagic cystitis (Brade et al., 1985; Brock et al., 1979b; Cox, 1979), a severe urotoxic complication characterized by edema and hematoma of the bladder epithelium potentially leading to death (Gray et al., 1986; Watson & Notley, 1973; Philips et al., 1961).

Significant advancement in oxazaphosphorine therapy was achieved by the introduction of mesna as a chemoprotectant in the late 1970s and early 1980s. Concurrent mesna dosing significantly reduced the incidence of hemorrhagic cystitis, the previously dose-limiting side effect of the oxazaphosphorines, ifosfamide and cyclophosphamide (Andriole et al., 1987; Antman et al., 1985; Fukuoka et al., 1991; Kemeny et al., 1987; Scheef et al., 1979; Scheulen et al., 1983; Stuart-Harris et al., 1983).

1.2.2 Mesna Preclinical Development

Development of mesna for uroprotection was undertaken by the Pharmacology Department of the German Bielefeld-based Asta-Werke A.G. (now Frankfurt-based ASTA Medica A.G.) in the late 1970s, concurrently with an ongoing research program to synthesize new alkylating nitrogen mustards (Brock et al., 1981b; Brock, 1989). With little success in improving the tumor-specific targeting of new nitrogen mustard phosphoramidate esters (i.e. oxazaphosphorines), an alternate strategy of combating undesired toxicity with adjuvant pharmacological therapies was pursued (Brock et al., 1981b; Brock, 1989). Mesna emerged as a potent bladder-specific chemoprotectant without detriment to the activity of concomitant anticancer drugs (Brock et al., 1982; Brock et al., 1979a; Brock, 1978). The successful preclinical development of this “pharmacologically unremarkable” compound led to Asta-Werke A.G. becoming wholly
acquired by one of Germany’s largest chemical companies, Degussa-Hüls A.G. in 1983 (now Degussa GmbH of Evonik Industries A.G.) (Brock et al., 1982; Brock, 1989; Wolf, 1989).

A variety of pharmacological and ambulatory measures have been used for the prophylaxis of renal toxicities. Forced diuresis by intravenous hydration, administration of diuretics, and alkalization remains in practice today (Hensley et al., 2008), while organ-specific chemical inactivation of toxic metabolites proved more difficult (Brock et al., 1981b). Compounds possessing nucleophilic free sulfhydryl functional groups rapidly covalently bind to hydroxylated oxazaphosphorine rings, similar to endogenous thiols cysteine (Cys) and glutathione (GSH) (Brock et al., 1988; Hospers et al., 1999; Kurowski & Wagner, 1997). However, like these endogenous thiols, early thiol-containing compounds such as NAC showed wide tissue distribution (Connors, 1966). As a result, not only was renal toxicity attenuated, so too was the effectiveness of anticancer therapy (Brock et al., 1979a; Connors, 1966; ISHIDATE et al., 1953). Direct instillation of NAC to the bladder via catheterization protected against bladder toxicity, but was clinically impractical and was ineffective at protecting the remaining urinary tract (Creaven et al., 1976; Primack, 1971).

1.2.2.1 Pharmacodynamics

During their work to develop new alkylating agents with greater anticancer efficacy and lower toxicity, the Pharmacology Department of Asta-Werke catalogued the urotoxicity observed following dosing of each new compound to Sprague-Dawley rats. This meticulous work helped determine whether the active alkylating metabolites or other breakdown products were responsible for urotoxicity. Direct administration of alkylating
agents (e.g. mustards) did not induce renal or urothelial damage, whereas oxazaphosphorines capable of forming acrolein (e.g. ifosfamide) induced swelling and hemorrhagic foci. Furthermore, direct instillation of metabolites showed only 4-hydroxyifosfamide and acrolein to be potently urotoxic, providing theoretical targets for covalent binding and inactivation by thiols (Brock et al., 1981b; Brock et al., 1979b). Through the use of modern enzyme overexpression systems the complete metabolic fate of ifosfamide has since been identified (Kerbusch et al., 2001a).

Ifosfamide is a chiral prodrug that undergoes autoinducible hepatic cytochrome P-450 (CYP) metabolism to its active mustard (Figure 1.1) (Kerbusch et al., 2001a; Lind et al., 1989). Ifosfamide may be metabolized by CYP2B6 to isomers (S)-2-dechloroethylifosfamide or (S)-3-dechloroethylifosfamide along with the nephrotoxin chloroacetaldehyde (Granvil et al., 1999). However, ifosfamide is believed to predominantly undergo CYP3A4-mediated metabolism, yielding the reactive metabolite 4-hydroxyifosfamide. 4-Hydroxyifosfamide spontaneously forms its isomer aldoifosfamide which subsequently breaks-down to active ifosfamide mustard and the potent urotoxin acrolein (Chang et al., 1993; Walker et al., 1994).
Figure 1.1 Metabolism of ifosfamide and mesna. Ifosfamide is a chiral prodrug that predominantly undergoes autoinducible CYP3A4-mediated metabolism, yielding the reactive metabolite 4-hydroxyifosfamide. 4-Hydroxyifosfamide spontaneously forms its isomer aldoifosfamide, which subsequently breaks-down to active ifosfamide mustard and the potent urotoxin acrolein. Alternatively, ifosfamide may be metabolized by CYP2B6 to isomers (S)-2-dechloroethylifosfamide or (S)-3-dechloroethylifosfamide along with the nephrotoxin chloroacetaldehyde. Mesna exerts its protective effect within the urine, where its free sulfhydryl group covalently binds 4-hydroxyifosfamide forming inactive 4-sulfoethylthioifosfamide. Upon entering the circulation mesna undergoes metal-catalyzed autooxidation to dimesna which may be reduced to mesna via thiol exchange.
Using previously established methods of quantitation of urotoxicity in the rat, the ability of an average of three doses of 29 compounds to lower ifosfamide-induced urotoxicity was examined. From this screen, only mesna demonstrated the desired pharmacological and pharmacokinetic properties. Doses of 6.81 to 21.5 mg/kg IV mesna provided significant uroprotection, rapid renal excretion ($t_{1/2} = 1.4 \text{ h in rats}$), and little extra-renal tissue distribution (Brock et al., 1981c), consistent with prior observations of $[^{35}\text{S}]$mesna during its development as a mucolytic (Gobert et al., 1971). Mesna exerts its protective effect within the urine, where its free sulfhydryl group covalently binds 4-hydroxyifosfamide forming inactive 4-sulfoethylthioifosfamide. Conjugation with mesna may therefore prevent not only the spontaneous formation of urotoxic acrolein, but also active ifosfamide mustard (Figure 1.1) (Kurowski & Wagner, 1997; Manz et al., 1985).

**1.2.2.2 Pharmacodynamic Drug Interactions**

To ensure mesna would not interfere with the efficacy of ifosfamide and cyclophosphamide at doses required for adequate uroprotection in the rat and mouse, the drugs were tested in combination in a variety of tumour models. When used in molar ratios of 0.5:1 up to 21.5:1 (mesna:oxazaphosphorine) and given either simultaneously or one hour after oxazaphosphorine administration, mesna unequivocally had no effect on the survival time of leukaemia L5222, Yoshida’s sarcoma (colorectal cancer), and solid DS carcinosarcoma bearing rats or leukaemia L1210 bearing mice. With the increase in use of combination chemotherapy, the effect of systemic mesna was also tested against other anticancer drugs to ensure it would not reduce their antineoplastic effects. Mesna (100 mg/kg administered simultaneously) did not affect survival times conferred by 5-fluorouracil, cisplatin, doxorubicin, methotrexate or vincristine (Brock et al., 1982).
Brock et al. hypothesized that this unique pharmacological profile may be due to its hydrophilicity and rapid oxidation to its dimer, dimesna (Brock et al., 1981a; Brock et al., 1981c). However the underlying mechanisms of mesna’s disposition remained to be investigated.

1.2.2.3 Safety

Following promising preclinical pharmacodynamic results establishing mesna as a lead compound, more time consuming and expensive toxicological studies of mesna were completed. Mesna demonstrated very low toxicity in mice (LD$_{50}$ = 1968 mg/kg IV), rats (LD$_{50}$ = 1891 mg/kg IV), and beagle dogs (only vomiting and diarrhea at doses 100 - 316 mg/kg IV). No evidence of embryotoxicity was found in either rats given oral doses up to 2000 mg/kg or rabbits with oral doses up to 1000 mg/kg. Mesna did not induce reversion of growth of Salmonella typhimurium in histidine-free medium (Ames’ test) and thus has no detectable mutagenic potential (Brock et al., 1982). Most importantly, the dose required for complete prophylaxis of urotoxicity in the rat (21.5 mg/kg, (Brock et al., 1981c)) is approximately only 1% of the LD$_{50}$ (1891 mg/kg (Brock et al., 1982)) and indicative of a wide therapeutic index.

1.2.3 Clinical Development

The clinical efficacy of mesna (ASTA D-7093 now branded Uromitexan™ and Mitexan™) and favourable toxicity profile quickly became apparent in a series of clinical trials evaluating ifosfamide therapy with mesna. Prior to the addition of mesna to ifosfamide therapy, hematuria (blood in the urine; a useful marker of urotoxicity) occurred in almost all patients and dose-limiting hemorrhagic cystitis occurred in 18 –
40% of patients (Teufel & Pfleiderer, 1976; Czownicki & Utracka-Hutka, 1981; Van Dyk et al., 1972; Creaven et al., 1976).

1.2.3.1 Efficacy

The efficacy of mesna to significantly lower the incidence of hematuria was first demonstrated in a randomized, open-label phase II study of IV mesna alone versus standard prophylaxis (approximately 4 L/d IV fluids, furosemide, alkalinization of the urine). Although mesna had previously been used as an aerosol for its mucolytic properties (Clarke et al., 1979; Van De Walle et al., 1976), the safe limit of systemic exposure in humans was unknown. In the absence of FDA guidelines for first-dose in human trials, mesna doses were arbitrarily initiated at 66% of total oxazaphosphorine dose and decreased to 33% and 17% in subsequent chemotherapy cycles until severe hematuria (> 50 rbs/µL urine) appeared. Three mesna doses of 17% and 33% were required to eliminate the occurrence of severe hematuria in two separate groups of four patients. One patient required three mesna doses of 66%. All nine patients receiving standard prophylaxis experienced severe hematuria. Although a dose greater than 17% was required in 5 of 9 patients to eliminate severe ifosfamide-induced hematuria, the authors concluded by recommending that mesna be administered in three doses of 20% of the daily oxazaphosphorine dose (equal to 60% of the daily oxazaphosphorine dose); immediately prior and at two and four hours after the start of oxazaphosphorine dosing. No differences in tumor response rates were observed between groups (Scheef et al., 1979). Although this trial design effectively identified the minimally effective dose (51% of daily oxazaphosphorine dose) such “dose-reduction” protocols would be considered
unethical today, and have subsequently been replaced by pharmacokinetic and allometric estimation of the minimally effective dose followed by dose-escalation.

A daily mesna dose of 60% of the daily oxazaphosphorine dose (administered in three equal doses at 0, 4, and 8 hours) recommended by the previous study (Scheef et al., 1979), was tested again in a small single-blind crossover trial of eight patients with bronchogenic carcinoma. Seven of eight patients receiving 2 g/m²/day ifosfamide alone experienced hematuria or symptoms of bladder irritation. Only one patient had hematuria during concomitant mesna administration. Five patients also completed fifteen courses of increased ifosfamide doses of 4 to 8 g/m²/day with mesna. Remarkably, hematuria was not seen until after three courses of ifosfamide, at doses known to cause severe toxicity in 18 of 25 patients and 25% mortality (Bryant et al., 1980; Van Dyk et al., 1972). This finding suggested mesna may greatly enhance the feasibility of “high-dose” ifosfamide (>2.5 mg/m²/day) (Bryant et al., 1980). Concurrent plasma concentration-time analysis revealed mesna had no effect on ifosfamide or mustard pharmacokinetics (Bryant et al., 1980), consistent with animal studies (Brock et al., 1982).

An early retrospective study of 748 patients who received 50 mg/kg/day ifosfamide for five days provided a well-powered comparison of mesna (administered at 60% of the ifosfamide dose divided between 0, 4, and 8 hours) versus standard prophylaxis of IV hydration, diuretics and alkalinization. Amongst patients receiving mesna, only 3.5% experienced urotoxicity compared to 38% of patients receiving standard care (Scheef & Soemer, 1980). Since this study, numerous phase I/II studies have consistently demonstrated decreased incidence of oxazaphosphorine-induced
urotoxicity with the use of mesna (Andriole et al., 1987; Antman et al., 1985; Kemeny et al., 1987; Scheulen et al., 1983; Stuart-Harris et al., 1983).

Most convincingly, the efficacy of this mesna dosing regimen in addition to standard prophylaxis has also been demonstrated in a large double-blind randomized, placebo-controlled study involving 91 patients receiving 2 g/m²/day dose ifosfamide. All patients received 2 L/day IV fluid and forty-five patients received IV mesna (20% of the ifosfamide dose administered at 0, 4, and 8 hours). The incidence of hematuria was significantly lower in the mesna group compared to placebo (6.7% with mesna versus 32.6% with placebo). In a blinded survey of patients, 80% judged receiving mesna to be “useful” compared to 34.8% of patients that believed receiving placebo to be beneficial (Fukuoka et al., 1991).

1.2.3.2 Standard Ifosfamide-Mesna IV Therapy

In light of current research, the American Society of Clinical Oncology (ASCO) has recommended guidelines for the use of intravenous mesna with ifosfamide therapy. Today mesna and ifosfamide are administered as bolus doses or together in either short (0.5 – 8 hours) or continuous (≥24 hours) infusions, typically as a five day regimen or “cycle”. During bolus dosing, mesna is administered 15 minutes prior to the start of ifosfamide infusion, and again 4 and 8 hours following ifosfamide (Hensley et al., 2008). Numerous clinical trials over the past three decades have empirically set the total daily intravenous mesna dose to 60% of the daily ifosfamide dose. Despite clinically effective dosing protocols, the incidence of moderate (hematuria) to severe (renal failure) ifosfamide-induced urotoxicity remains 3.5% to 6.7% per chemotherapy cycle, resulting in 19.2% to 34.0% of patients experiencing dose-limiting toxicity over the course of
treatment of six cycles (Hensley et al., 1999; Hensley et al., 2008; Schuchter et al., 2002).

Given the typical ifosfamide dose of 1 to 2.5 g/m², a single IV bolus dose of mesna at 20% of the ifosfamide dose would equate to 0.2 to 0.5 g/m² or roughly 5 to 12 mg/kg (Links & Lewis, 1999; Kerbusch et al., 2001a). Mesna doses may safely be increased. In humans, doses of 70 to 100 mg/kg IV were free of bone marrow, hepatic, and renal toxicities and did not impair CNS functions (Brade, 1987). Vomiting and diarrhea were only seen following IV doses greater than 80 mg/kg (Klein et al., 1983).

Although higher doses and various dosing intervals have been suggested (Brock et al., 1988), the efficacy of mesna doses in excess of 66% (16 to 40 mg/kg) at a frequency greater than four hours remains unknown, and thus optimal dosing schedules remain to be determined.

### 1.2.3.3 High-Dose Ifosfamide IV Therapy

The antineoplastic potency of ifosfamide is maintained by dosages up to 16 g/m²/day (Cerny et al., 1999; Frei, III et al., 1985; Verweij, 1998). To take advantage of the steep dose-response relationship high-dose continuous infusion regimens of ifosfamide are of growing interest (Kerbusch et al., 2001a; Anderson et al., 2008).

Continuous infusion avoids “spikes” in plasma concentrations, offering patients lower haematological toxicity with less nausea and emesis, compared to short infusions or bolus doses. It has been theorized that continuous infusion therapy may also increase efficacy of ifosfamide, by virtue of its autoinduction of metabolism to its active mustard (Kerbusch et al., 2000; Kerbusch et al., 2001b; Lewis et al., 1990).
Evaluation of the standard mesna dosing regimen for ifosfamide doses of 4 – 16 g/m²/day yielded urotoxicity in 10 – 27% of patients; much higher despite maintaining the daily mesna dose proportionality of 60% per day (Elias et al., 1990; Le Cesne et al., 1995; Sakurai et al., 1986). As previously highlighted in Figure 1.1, ifosfamide is activated to its cytostatic mustard and urotoxic metabolites through a combination of spontaneous and autoinducible enzymatic reactions (Kurowski & Wagner, 1993; Lind et al., 1989; Wagner, 1994). Whereas the proportion of toxic metabolites increases during the chemotherapeutic cycle (Kerbusch et al., 2001b), the amount of mesna administered remains a fixed percentage of the original ifosfamide dose. This divergence from linearity may account for the increased incidence of urotoxicity observed during “high-dose” continuous infusion ifosfamide (Elias et al., 1990; Le Cesne et al., 1995; Sakurai et al., 1986). However, as of yet, no guidelines for mesna doses or dosing schedules to accompany continuous infusion of ifosfamide doses in excess of 2.5 g/m²/day have been provided by the ASCO, stating insufficient research (Hensley et al., 2008).

The appropriateness of providing mesna as a percentage of daily ifosfamide depends on the assumption that the metabolism and disposition of ifosfamide and mesna are proportional, yet ifosfamide and mesna follow different biological fates. Unfortunately, there is a dearth of data regarding the mechanisms of mesna disposition, and so clinical administration of mesna remains heuristic (Hensley et al., 1999; Hensley et al., 2008; Schuchter et al., 2002).

1.2.3.4 Oral MesnaTherapy

To facilitate outpatient care during short-infusion chemotherapy, the intravenous solution of mesna can be administered orally in Canada, the United States, Germany, and
the United Kingdom (Goren, 1996). The development of both continuous infusion and oral dosing regimens would enable mesna to be provided as outpatient care, allowing patients to spend more time at home, alleviating not only patient stresses of hospitalization, but also associated costs of prolonged patient monitoring in clinics. Since their approval by the Food and Drug Administration (FDA) in 2002, mesna tablets have provide increased patient convenience, with the same bioavailability as IV solution (Goren et al., 1998b), while circumventing the poor palatability of the IV solution, likely increasing compliance during outpatient care (Cohen et al., 2002). Despite administering mesna orally since the early 1980’s (Araujo & Tessler, 1983; Bryant et al., 1980), it is widely recognized that an optimal oral mesna dosing regime has yet to be established (Cohen et al., 2002; Goren, 1996; Goren et al., 1997; Goren et al., 1998a; Links & Lewis, 1999; Olver et al., 2005). As a result, in its most recent clinical practice guidelines, the ASCO does not provide dosing recommendations for oral mesna in combination with ifosfamide infusions exceeding four hours (Hensley et al., 2008).

1.3 Dimesna for Cancer Chemotherapy

1.3.1 Dimesna

Similar to the nucleophilic sulfhydryls GSH and NAC, mesna may theoretically be beneficial for the mitigation of renal toxicities associated with other anticancer agents. Platinum-based anticancer drugs cisplatin (cis-diaminedichloroplatinum), carboplatin (cis-diammine(1,1-cycllobutane-dicarboxylato)platinum), and oxaliplatin (1,2-cyclohexane-1,2-diamine(ethanedioato)platinum) are broad spectrum DNA cross-linking agents approved for use against colorectal, non-small-cell lung, ovarian, prostate, and
testicular cancers. Although newer platinum-based drugs exhibit some nephrotoxicity, cisplatin is the most potent platinum-containing cytostatic agent and hence remains in use today despite its dose-limiting nephrotoxicity (Kelland, 2007; Rosell et al., 2002). Aggressive prehydration techniques have been developed to combat the nephrotoxicity associated with cisplatin, however it remains a significant cause of dose delay (Cvitkovic et al., 1977; Kelland, 2007).

So-called “first-generation” chemoprotectants sodium thiosulfate, diethyldithiocarbamate and amifostine, readily conjugate nephrotoxic monohydroxy- and monoaquo-monochloro species of cisplatin. However, the thiolate of each of these chemoprotectants may also covalently bind cisplatin, rendering it inactive prior to tumor penetration (Berry et al., 1990; Hausheer et al., 1998; Hospers et al., 1999; Qazi et al., 1988; Schuchter et al., 1992; Wagner et al., 1988). For this reason cisplatin is incompatible with sulfhydryls and must be administered separately, unlike ifosfamide and mesna which can be easily infused from the same IV drip (Brock et al., 1988). Mesna has previously been used to lessen cisplatin-induced nephrotoxicity in mice with mixed results (Kempf & Ivankovic, 1987; Millar et al., 1985).

To circumvent conjugation of parental cisplatin and lessen the gastrointestinal side effects associated with sulfhydryl compounds, mesna may be instead administered as its dimer, dimesna. As a disulfide, dimesna cannot conjugate cisplatin or its hydrated reactive metabolites monohydroxo- and monoaqua-cisplatin. Upon entry to organ parenchyma, dimesna may be chemically reduced by endogenous thiols (i.e. thiol exchange) to active mesna which may form an inactive adduct with intracellular cisplatin or its metabolites (Figure 1.2).
**Figure 1.2** Conjugation of cisplatin and metabolites by mesna. Cisplatin is readily hydrated by displacement of chlorine with water to form active monohydroxo and monoaqua metabolites. As a disulfide, dimesna cannot conjugate cisplatin or its hydrated reactive metabolites. Upon entry to organ parenchyma, dimesna undergoes thiol exchange with GSH and Cys yielding active mesna which may form an inactive adduct with intracellular cisplatin or its metabolites.
1.3.2 Dimesna Preclinical Development

BioNumerik Pharmaceuticals, Inc. in collaboration with the Vrije Universiteit Medical Centre in Amsterdam, Holland have led the development of dimesna (disodium-2',2'-dithio-bis-ethane sulfonate) as BNP7787, Tavocept™, for the mitigation of cisplatin-associated toxicities. The ongoing goal of their project is to find a patentable method to prevent or mitigate common toxicities of anticancer agents without preventing their efficacy thereby increasing the therapeutic index of chemotherapy (Hausheer et al., 2003).

Dimesna exerts significantly less toxicity than its reduced sulfhydryl moieties. Dimesna doses of up to 4000 mg/kg were nonlethal to rats and dogs. When compared to established LD$_{50}$s of mesna, dimesna demonstrates dose safety ratios in rats and dogs of > 2.7-fold and > 10-fold, respectively (Brock et al., 1982; Hausheer et al., 1998).

1.3.2.1 Pharmacodynamic Drug Interactions

*In vitro* measurements of the degradation half-lives of cisplatin with dimesna and mesna revealed dimesna reacted 5.5-fold slower than mesna. To ensure dimesna would not interfere with the efficacy of platinum-based drugs *in vivo*, dimesna was administered in combination with cisplatin and its analogue carboplatin to xenograft-bearing nude mice, a standard preclinical model of efficacy in cancer research. Efficacy of bi-weekly doses of cisplatin and carboplatin to slow human ovarian cancer cell-derived (OVCAR-3) xenografts were unaffected by 1000 mg/kg IV dimesna administered 5 min prior to platinum drugs (dimesna:cisplatin molar ratio of 184:1). Cisplatin administered with 1000 mg/kg IV mesna (mesna:cisplatin molar ratio of 368:1) was slightly, but not significantly less effective at slowing tumour growth, however animals receiving mesna
experienced greater weight loss than those receiving dimesna (Boven et al., 2002). Mixed results of previous studies evaluating the effect of mesna on cisplatin activity in tumour-bearing rodents have also been observed (Brock et al., 1982; Kempf & Ivankovic, 1987; Millar et al., 1985). Half of the mice receiving mesna and cisplatin suffered toxicity-associated death, whereas no death occurred in mice receiving dimesna (Boven et al., 2002). The corollary of this finding suggests dimesna may facilitate increased cisplatin dosing, consistent with observations that cisplatin dose may be safely increased from 6 to 9 mg/kg when preceded by 750 or 1500 mg/kg dimesna in rats bearing WARD colon tumours (Hausheer et al., 1998).

1.3.2.2 Mechanism of Action

Cisplatin exerts its nephrotoxic effects through toxification by the $\gamma$-glutamyltranspeptidase (GGT) xenobiotic metabolism pathway. Extensive work by Hanigan et al. has determined that cisplatin is glutathionylated both non-enzymatically by GSH and enzymatically by glutathione-S-transferase. Glutamate, glycine, and Cys residues are then sequentially hydrolyzed from cisplatin-GSH conjugates by GGT, aminopeptidase (APN), and finally cysteine-S-conjugate-β-lyase (CCBL) to form reactive thiolate-platinum species (Hanigan et al., 1994; Hanigan et al., 1996; Hanigan et al., 2001; Townsend et al., 2003). Recent *in vitro* work by BioNumerik has presented multiple mechanisms by which dimesna may prevent formation of these reactive nephrotoxins. Mesna, formed by the intracellular reduction of dimesna, may directly bind to cisplatin and hydrated metabolites forming conjugates that are unsusceptible to metabolism by either GGT or APN. In addition to direct conjugation, findings indicate that mesna-disulfide hetero conjugates that contain a terminal $\gamma$-glutamate moiety, such
as mesna-GSH and mesna-cysteinylglutamate, inhibit GGT and APN preventing the toxification of cisplatin leading to nephrotoxicity (Hausheer et al., 2010a; Hausheer et al., 2010b).

In addition to dimesna’s application in treating cisplatin-induced nephrotoxicity, dimesna may also be useful in the treatment of taxane-induced neurotoxicity. Dimesna has been shown to normalize tubulin hyperpolymerization induced by paclitaxel in vitro, suggesting dimesna may be useful in preventing unwanted microtubulin dysfunction (Hausheer et al., 2000), believed to be a major cause of taxane-induced neuropathy (Lee & Swain, 2006).

1.3.3 Dimesna Clinical Development

1.3.3.1 Phase I/II

In a phase I study dimesna was administered in escalating doses of 4.1 – 41 g/m² to 25 patients prior to cisplatin treatment for advance solid tumors. Side effects of dimesna were only observed at the highest dose (41 g/m²) and included nausea, vomiting, and dysgeusia. Cisplatin pharmacokinetics were not altered by dimesna. Although this was a relatively small study, patients receiving > 18.4 g/m² dimesna required significantly less IV hydration, suggesting dimesna may be chemoprotective (Boven et al., 2005; Verschraagen et al., 2003a). The efficacy of dimesna with cisplatin and docetaxel was further elucidated in a randomized phase II study in 151 patients with advanced non-small-cell lung cancer. Patients received 75 mg/m² cisplatin and 75 g/m² docetaxel supported with darbepoetin and pegfilgrastim preceded with or without a fixed dose of 40 g dimesna every other week for up to six cycles. The dimesna dose was chosen was determined by the average body surface area of patients plus one standard
deviation multiplied by the 18.4 g/m² used by Schilsky et al. (Miller et al., 2008; Schilsky et al., 2003). No difference between groups was observed in incidence of treatment delays or completion, or objective response rates, however median progression-free/overall survival times were increased with the addition of dimesna (Miller et al., 2008). Similar to mesna, it has been hypothesized that dimesna may potentiate the efficacy of anticancer agents by depletion of plasma GSH, without altering leukocyte GSH (Pendyala et al., 2000; Pendyala et al., 2003). Despite positive in vitro data (Hausheer et al., 2000), dimesna was ineffective at reducing neuropathy (Miller et al., 2008). A second phase I trial of dimesna in patients with non-small-cell lung cancer completed by BioNumerik in collaboration with Tokyo-based Grelan Pharmaceutical Co., Ltd. (a subsidiary of Osaka-based Takeda Pharmaceutical Co., Ltd.) has recently been published. Twenty-one patients received 75 mg/m² cisplatin and 175 mg/m² paclitaxel every three weeks for up to 4 cycles preceded by escalation doses of dimesna (4.1 - 41 g/m²). Although dose-limiting toxicities of dimesna were not reached, doses exceeding 18.4 g/m² produced much greater frequency of IV site discomfort, facial flushing and dysgeusia (Masuda et al., 2010). A dose of 18.4 g/m² provides a dimesna:cisplatin molar ratio of approximately 226:1, over 4-fold greater than the minimum molar ratio of 50:1 required for nephroprotection in rats (Hausheer et al., 1998). Thus, this lead the authors to suggest dose of 18.4 g/m² be used for future efficacy studies (Masuda et al., 2010).

1.3.3.2 Phase III

BioNumerik in alliance with German-based Baxter Oncology GmbH (a division of Baxter International) has recently completed a large randomized, controlled, phase III
trial of paclitaxel and herceptin in combination with dimesna to reduce the incidence of neuropathy in 764 patients with metastatic breast cancer, however the results have not yet been released (ClinicalTrials.gov ID# NCT00039780). Perhaps because of the success of this first efficacy trial, recruitment for a second phase III trial of dimesna is currently underway to determine its efficacy in combination with cisplatin and taxane drugs docetaxel and paclitaxel in patients with advanced primary adenocarcinoma of the lung (ClinicalTrials.gov ID# NCT00966914). As the populations of patients administered dimesna grows, understanding the underlying biology and potential causes of variations within populations will aid in the effective application of this new chemoprotectant.

1.4 Pharmacokinetics of Mesna and Dimesna

Although mesna and dimesna are unique molecular entities and regulated as two separate drugs, each could be considered a metabolite of the other, and thus their dispositions are inextricably linked. By convention, due to the technical difficulties of stabilizing and distinguishing low-molecular weight disulfides of mesna, its metabolites are often quantified collectively, which can make interpretation of results difficult. The metabolites mesna-mesna (i.e. dimesna), mesna-Cys, mesna-homocysteine, mesna-cysteinylglutamate, mesna-cysteinylglycine, and mesna-GSH have been collectively termed “dimesna” in some studies (Ormstad & Uehara, 1982; James et al., 1987; Shaw & Weeks, 1987) while others refer to the mixed disulfides containing a single mesna moiety as “mesna”, quantifying dimesna (i.e. mesna-mesna) separately (Goren et al., 1998b; Verschraagen et al., 2003c). Because of this disparity in the literature, and reversible redox of sulfhydryls and disulfides, analysis of “total” mesna as the sum of all mesna
moieties (reduced, low-molecular weight, and protein bound) has been recommended as an acceptable measurement of its pharmacokinetics (Goren et al., 1998b). However, these conventions will likely be obviated by growing accessibility of mass spectrometry equipment to academic laboratories.

1.4.1 Absorption

Early studies of orally administered IV preparations of mesna demonstrated that mesna moieties could be adequately absorbed to allow significant excretion of mesna into the urine (Brock et al., 1984). The appearance of both mesna and dimesna in the medium of inverted isolated small intestine injected with $[^{14}\text{C}]$dimesna (Ormstad et al., 1983) suggests the intestine possesses a reductive capacity and supports the clinical observation of the absorption of dimesna following oral administration (James et al., 1987; Shaw & Weeks, 1987). Although the small intestine is able to reduce dimesna to mesna, theoretically enhancing its efficacy, this newly reduced mesna rapidly oxidizes upon entering the circulation (Brock et al., 1981a; Ormstad et al., 1983). A lack of biliary excretion by isolated perfused rat liver suggests total mesna does not undergo enterohepatic circulation, allowing the fraction absorbed to be equated to bioavailability (Ormstad et al., 1983; Goren et al., 1998c). Mesna and dimesna are exclusively renally excreted (Brock et al., 1984; Kurowski & Wagner, 1997; Ormstad et al., 1983), allowing for estimation of bioavailability by analysis of cumulative urinary excretion following a single oral dose, in addition to the standard comparison of the area under the concentration-time curves (AUCs) following IV and oral doses (Rowland & Tozer, 1995). Analysis of urine data of healthy volunteers and patients undergoing ifosfamide
therapy yielded mean total mesna bioavailabilities of 0.47 and 0.33, respectively, but were not statistically different (Burkert et al., 1984).

A comparison of AUCs of total mesna following IV and oral 800 mg doses of mesna in an early pharmacokinetic study yields a bioavailability of 0.47, a close approximation of the bioavailability of 0.49 derived from 24 h urinary excretion data (James et al., 1987). Bioavailability of total mesna has now been established as 0.5, allowing oral doses of mesna for outpatient therapy to be simply administered at twice the IV dose (Baxter Healthcare Corporation, 2002). Although the uptake transporters responsible for the intestinal absorption of mesna and dimesna remain to be determined, mesna’s moderate bioavailability makes it an unlikely candidate for adverse intestinal drug-transporter interactions.

1.4.2 Non-Enzymatic Metabolism

Early studies of mesna’s in vivo fate reported complete spontaneous metal-catalyzed oxidation of mesna within the circulation to its dimer, dimesna (Brock et al., 1981c; Brock et al., 1981a; Ormstad et al., 1983). Similarly, mesna can also disulfide bond to low-molecular weight endogenous thiols Cys, GSH, homocysteine (Hcy), or cysteinyl-glycine. Mesna is moderately protein-bound, the majority of which binds covalently via a disulfide with the single non-intramolecular disulfide forming Cys residue of albumin, Cys$^{34}$, displacing endogenous thiols (Stofer-Vogel et al., 1993; Urquhart et al., 2006). The disulfide bonding of mesna moieties within the oxidative environment of the plasma is believed to be responsible for mesna’s inability to inactivate circulating chemotherapeutics (Brock et al., 1982).
However, plasma thiols exist as an equilibrium of sulfhydryl, protein disulfides, and low-molecular weight disulfides (Sengupta et al., 2001), thus, a proportion of mesna is expected to remain as an active sulfhydryl. This proportion likely went undetected in earlier studies using less sensitive methods of detection available today. More recent studies of IV dimesna pharmacokinetics show a dose-dependent increase in mesna exposure of 6% to 8% of dimesna AUC (Verschraagen et al., 2003a; Masuda et al., 2010). Whether the production of mesna within the circulation is due to mesna moieties reaching equilibrium with other circulating thiols as well as secretion of mesna by tissues remains to be determined.

In contrast to the circulation, intracellular dimesna concentrations have been hypothesized to be less than 1% of the mesna concentration (Hausheer et al., 2003). Similar to the reduction of glutathionylated molecules and inter- and intramolecular protein disulfide bonds, mesna disulfides may freely undergo thiol-disulfide exchange with endogenous thiols GSH and Cys to yield sulfhydryl mesna (Iversen et al., 2010; Urquhart et al., 2006; Verschraagen et al., 2004b). Although the kinetics of these reactions remain to be determined, they are hypothesized to proceed via a bimolecular nucleophilic substitution (S₂) reaction (Hausheer et al., 2003).

### 1.4.3 Enzymatic Metabolism

In addition to non-enzymatic (i.e. chemical) thiol exchange, thioredoxin and glutaredoxin systems of the thioredoxin family of thiol-disulfide oxidoreductases may also be involved in the reduction of dimesna (Verschraagen et al., 2004b). The thioredoxin and glutaredoxin systems are highly conserved, ubiquitous NADPH-dependent protein complexes of eukaryotic cells responsible for maintaining the balance
between oxidizing and reducing conditions (Berndt et al., 2007; Holmgren, 1985). These redox enzymes attenuate the signaling of reactive oxygen species (ROS) second messengers (e.g. hydrogen peroxide) by reducing ROS-induced oxidative modifications such as inter- and intramolecular disulfides (R-S-S-R), glutathionylation (R-S-SG), sulfenation (R-SOH), sulfination (R-SO₂H), sulfonation (R-SO₃H), and S-nitrosylation (R-S-NO) (Meyer et al., 2009; Berndt et al., 2007).

Reduction of dimesna by purified thioredoxin system consisting of the 12 kDa *E. coli* protein thioredoxin (*trxA*; Trx1, UniProtKB ID# P0AA25) and 55 kDa bovine thioredoxin reductase (*TXNRD1*; TR1, UniProtKB ID# O62768, EC 1.8.1.9) has been reported (Verschraagen et al., 2004b). Thioredoxin functions as an electron donor to disulfide-forming enzymes ribonucleotide reductase (Engstrom et al., 1974), methionine sulfoxide reductases (Brot & Weissbach, 1983), and peroxiredoxins (Rhee et al., 2005). Thioredoxin and glutaredoxin share a dicysteine active site Cys-X-X-Cys motif called the ‘thioredoxin-fold’ (Holmgren et al., 1975; Martin, 1995). The N-terminal active site Cys residue of the thioredoxin-fold in thioredoxin(SH)₂ reduces protein disulfides by forming a transient mixed disulfide followed by fast thiol-disulfide exchange oxidizing a pair of reactive Cys at the active site producing thioredoxin(S)₂ (Holmgren, 1995). Alternatively, reduced thioredoxin(SH)₂ may directly reduce dimesna to form two mesna moieties and thioredoxin(S)₂. Reduction of thioredoxin(S)₂ is facilitated by electron transfer from NADPH via thioredoxin reductase. Thioredoxin reductase is a flavin adenine dinucleotide (FAD)-containing homodimeric enzyme containing a selenocysteine (SeCys) as the penultimate residue at its C-terminal end, forming a Gly-Cys-SeCys-Gly
active site responsible for the recycling of oxidized thioredoxin(S)\textsubscript{2} to reduced thioredoxin(SH)\textsubscript{2} (Zhong \textit{et al.}, 2000).

The cytosolic glutaredoxin system consists of 12 kDa glutaredoxin (\textit{GLRX1}; thioltransferase-1, GRX1, UniProtKB ID#P35754, EC 1.8.4.2) and 56 kDa glutathione reductase (\textit{GSR}; GR, UniProtKB ID#P00390, EC 1.8.1.7). Glutaredoxin is primarily recognized to facilitate the GSH-dependent transfer of electrons to ribonucleotide reductase for the synthesis of deoxyribonucleotides (Holmgren, 1976). It has since been discovered that glutaredoxin also regulates dehydroascorbate reduction (Wells \textit{et al.}, 1990), apoptosis (Chrestensen \textit{et al.}, 2000; Daily \textit{et al.}, 2001), cellular differentiation (Takashima \textit{et al.}, 1999), and transcription factor signaling (Bandyopadhyay \textit{et al.}, 1998; Hirota \textit{et al.}, 2000; Nakamura \textit{et al.}, 1999). Similar to thioredoxin, the thioredoxin-fold of glutaredoxin(SH)\textsubscript{2} reduces protein disulfides by forming a transient mixed disulfide followed by thiol-disulfide exchange producing oxidized glutaredoxin(S)\textsubscript{2} (Bushweller \textit{et al.}, 1992). However, unlike thioredoxin, glutaredoxin(SH)\textsubscript{2} specifically reduces glutathionylated proteins and mixed disulfides, yielding a glutaredoxin-GSH conjugate (Holmgren, 1989). Thus, it has been proposed that dimesna undergoes thiol-disulfide exchange with GSH forming a mesna-GSH mixed disulfide which can then be acted upon by glutaredoxin(SH)\textsubscript{2}, forming glutaredoxin-GSH and sulphydryl mesna (Verschraagen \textit{et al.}, 2004b). Glutaredoxin-GSH conjugate can then be reduced to glutaredoxin(SH)\textsubscript{2} by an additional molecule of GSH, forming oxidized glutathione (GSSG). The homodimeric flavoprotein glutathione reductase then recycles GSSG to two molecules of GSH via electron transfer from NADPH (Fernandes & Holmgren, 2004; Meister & Anderson, 1983). Thus far, glutaredoxin has been shown to only bind GSH moieties of disulfides.
(Berndt et al., 2007; Holmgren, 1989), therefore it is unlikely that dimesna is a substrate for glutaredoxin.

Reduction of dimesna has been proposed to occur by enzymatic and non-enzymatic mechanisms of thiol exchange; (Ormstad et al., 1983; Verschraagen et al., 2004b) however, the reaction orders and rates of reduction relative to endogenous substrates remains unknown. The rapid reduction of dimesna and its mixed disulfides by tissue homogenates likely proceeds by multiple parallel pathways of thiol disulfide exchange, however the relative contribution of these pathways have yet to be determined.

1.4.4 Distribution

_In vitro_, both liver and kidney lysates can reduce dimesna to sulfhydryl mesna by GSH dependent mechanisms (Verschraagen et al., 2004b; Ormstad et al., 1983). However, _in vivo_, dimesna undergoes no hepatic reduction and known kidney selectivity (Ormstad et al., 1983; Verschraagen et al., 2004a). In a series of isolated perfused organ experiments, significant accumulation of mesna was observed following perfusion of isolated rat kidney with $[^{14}C]$dimesna, while no accumulation of either dimesna or mesna was seen in perfused liver tissue (Ormstad et al., 1983). This was corroborated by a later study of perfused rat liver by Goren et al. Neither mesna nor dimesna accumulated within the liver or were excreted in the bile. However, a significant increase in sulfhydryl mesna was observed in liver effluent perfusate. This observation is likely due to the increased sensitivity of electrochemical detection utilized by Goren et al. compared to analysis of total free thiol with Ellman’s reagent performed by Ormstad et al. (Goren et al., 1998c; Ormstad et al., 1983).
Differences in organ exposure also were seen following injection of WARD colorectal tumour-bearing rats with either mesna or dimesna. Liver, tumour, and red blood cells showed little or no exposure (as measured by the AUC of mesna and dimesna per gram tissue) while the kidney AUC exceeded that of plasma (Verschraagen et al., 2004a).

Ascites is an accumulation of fluid in the peritoneum that commonly occurs with malignant cancers due to reduced lymphatic drainage as a result of metastases to the peritoneal space (Tamsma et al., 2001). This fluid may represent an additional compartment of drug distribution, potentially altering a drugs pharmacokinetics. However, a case report of mesna and dimesna pharmacokinetics in a cancer patient receiving cisplatin therapy found only 4% of the dimesna dose was present in ascetic fluid and only 0.02% of this was present as mesna. The authors conclude that dose adjustments are not necessary in patients with ascites because its presence is unlikely to alter dimesna pharmacokinetics (Verschraagen et al., 2003b).

An early and comprehensive study of mesna pharmacokinetics, still cited in its current Compendium of Pharmaceuticals and Specialities monograph (Canadian Pharmacists Association, 2009), was conducted in six healthy subjects by James et al. The authors reported an apparent volume of distribution of the central compartment \(V_c\) and whole body clearance of mesna equal to 0.652 L/kg and 1.23 L/kg/h, respectively; approximately 45.6 L/h and 86.1 L/h for a 70 kg subject (James et al., 1987). However, this rapid clearance of mesna (half-life of approximately 22 minutes) was derived from only the initial (i.e. alpha) phase and thus likely represents the loss of sulfhydryl mesna due to oxidation in the plasma, not necessarily loss from the volume of distribution. This
observation is consistent with an earlier study that reported a half-life of 18 minutes by measurement of total sulfhydryl oxidation in plasma using Ellman’s reagent (Pohl et al., 1981). In contrast, a time-averaged renal clearance of 0.413 L/h/kg (approximately 28.9 L/h for a 70 kg subject) was calculated from four hour urine collection data; likely representing a closer estimation of whole body clearance of total mesna. Dimesna, which is both a metabolite and prodrug of mesna, had a half-life of 1.17 h (77 minutes) (James et al., 1987).

A study of combined IV and oral dosing of mesna involving ten patients receiving mesna during ifosfamide chemotherapy reported pharmacokinetic parameters of mesna and dimesna from single IV doses (Goren et al., 1997). The observed steady-state volume of distribution ($V_{ss}$) of mesna of 30 L is less than the $V_c$ reported by James et al. (45.6 L) (James et al., 1987). This difference is contrary to the definitions of volumes of distribution and their methods of calculation, which necessitates that $V_c \leq V_{ss} \leq V_\beta$, assuming the distribution of drug into plasma is faster than its distribution into tissues (Berezhkovskiy, 2007). The volume of the central compartment ($V_c$) is calculated as the ratio of the IV dose (i.e. amount of mesna in the body) and extrapolated initial concentration ($C_0$), prior to equilibration with sites of distribution outside of plasma water, and is thus an underestimation of drug’s volume at steady-state. At the other extreme, the volume of distribution estimated by terminal rate coefficient ($V_\beta$) is calculated as the ratio of the whole body clearance (CL) and terminal elimination rate constant (i.e. $k_{el}$, $k_\beta$, or $\lambda_z$). However, $V_\beta$ is an overestimation of a drug’s distribution because of the greater rate of change of the plasma concentration than the amount of drug in the body during the terminal phase. At steady-state, however, the rates of change of
observed plasma concentration and amount of drug in the body are equal, hence $V_{ss}$ calculated as the product of the CL and mean residence time (MRT) is considered a more accurate measure of a drug’s distribution (Berezhkovskiy, 2007). Thus, the disparity between these two small studies is likely due to population variance or pathological causes.

Goren et al. also provided an estimation of the $V_{ss}$ of dimesna. By assuming the amount of dimesna administered was equal to half of the IV mesna dose the authors calculated the $V_{ss}$ of dimesna to be 36 L (Goren et al., 1997). The equality of the apparent volumes of mesna and dimesna is likely due to the reversible redox cycling of mesna moieties as disulfides within a single compartment. For this reason, measurement of total mesna has been suggested to provide more therapeutically relevant pharmacokinetic parameters. The subsequent total mesna $V_{ss}$ was 21 L (Goren et al., 1997).

A study of mesna pharmacokinetics in nine patients receiving immunosuppressive cyclophosphamide therapy during bone marrow transplantation also reported parameters of mesna and dimesna. Mesna $V_{ss}$ and CL parameters were 1.09 L/kg (approximately 76.3 L for a 70 kg subject) and 0.244 L/kg/h (approximately 17.1 L/h for a 70 kg subject), respectively. Dimesna, measured as the sum of low-molecular weight disulfides, had a CL of 0.157 L/kg/h (approximately 11 L/h for a 70 kg subject). However, these parameter values reported by el Yazigi et al. are not widely referenced due to the sparse sampling (i.e. 2-3 data points) during the terminal phase (el Yazigi et al., 1997).

Direct measurement of dimesna and mixed disulfide pharmacokinetics with and without cisplatin in 25 patients enrolled in a phase II trial was presented in detail by Verschraegen et al. (Verschraegen et al., 2003a). Clinical pharmacodynamic data of this
trial was later reported by Boven et al. (Boven et al., 2005). Dimesna AUCs showed excellent linearity across all six dose levels ranging from 4.1 to 41 g/m². $V_{ss}$ and CL parameters of dimesna ranged from 0.23-0.26 L/kg (approximately 17.2 L for a 70 kg subject) and 8.1-9.2 L/h/1.73 m², respectively, across all dose levels with and without cisplatin (Verschraagen et al., 2003a). The half-life of dimesna (measured as the sum of mixed disulfides) was 1.4 h (Verschraagen et al., 2003a), closely approximating the dimesna half-life estimated following mesna administration (1.17 – 1.29 hours) (el Yazigi et al., 1997; James et al., 1987). Sulphydryl mesna reached a $C_{max}$ at 1.4 hours with an AUC of approximately 8% of dimesna, across all dose levels. $V_{ss}$ and CL of mesna could not be determined without deconvolution of the rate of mesna production. However the reported terminal half-life of 2.75 hours is significantly longer than previous half-lives calculated during the initial phase (18 – 22 minutes) of non-enzymatic oxidation (Verschraagen et al., 2003a; Pohl et al., 1981; James et al., 1987).

In a phase I trial reported by Masuda et al., dimesna pharmacokinetics were measured by liquid chromatography-mass spectrometry in 22 patients (Masuda et al., 2010). Doses levels were identical to Verschraagen et al.; ranging from 4.1 – 41 g/m² (Verschraagen et al., 2003a). Concurrent cisplatin and paclitaxel administration had no effect on mesna or dimesna parameters. Mean dimesna parameters $V_{ss}$, CL, and half-life across all dose levels were 14.0 L, 10.9 L/h, and 1.0 hour, respectively. AUC of sulphydryl mesna was approximately 6.2% of dimesna (Masuda et al., 2010). Interestingly, these values are similar to those previously reported by Verschraagen et al. using electrochemical detection following sodium borohydride reduction to quantify dimesna as the sum of all mixed disulfides (Verschraagen et al., 2003a). Although not
presented in the reports, a comparison of dose normalized dimesna $C_{\text{max}}$ values obtained by the two analytical methods allows for estimation of the proportion of low-molecular weight disulfides to be 73.3% dimesna. The remaining 26.7% is likely made up of (in order of their endogenous concentrations): mesna-Cys, mesna-GSH, mesna-Hcy, mesna-cysteinylglycine, and mesna-glutamylcysteine.

1.4.5 Excretion

As previously noted, mesna and dimesna are exclusively renally excreted at a clearance rate greater than glomerular filtration (Brock et al., 1984; Urquhart et al., 2007a; James et al., 1987; Kurowski & Wagner, 1997; James et al., 2007; Ormstad et al., 1983). Theoretically, the magnitude of chemoprotection is proportional to the fraction of mesna moieties excreted as active sulfhydryls (Kurowski & Wagner, 1997; Manz et al., 1985).

James et al. reported that the percentage of mesna excreted as a sulfhydryl from equal IV and oral doses are 31.8% and 17.6%, respectively (James et al., 1987). A similar result of 35.9% urinary sulfhydryl mesna following IV dosing was reported in an earlier study which also utilized sulfhydryl mesna-specific analysis (Ikeuchi & Amano, 1985). Not surprisingly, roughly half as much mesna was excreted following oral dosing given mesna’s bioavailability of 0.5. In a comparison of IV and oral dosing, Goren et al. reported the percentage of sulfhydryl mesna excreted following IV dosing was equivalent to oral administration of twice the IV dose (41% IV versus 37% oral) consistent with the observations of James et al. (Goren et al., 1997; James et al., 1987). Despite low (approximately 8%) circulating sulfhydryl mesna, the percent urinary sulfhydryl mesna is approximately 8-fold greater, thus secreted low-molecular weight mesna disulfide may undergo reduction within the renal epithelia. In addition the renal clearances of mesna
and dimesna in excess of glomerular filtration and excretion imply uptake and active renal secretion by renal parenchyma.

To date, only a single study has examined the saturable uptake of dimesna by renal tissue. Using freshly isolated rat renal epithelial cells, the uptake of 100 µM [14C]dimesna was temperature dependent and inhibitable by the adenosine triphosphate (ATP) inhibitor para-trifluoromethoxyphenylhydrazide (FCCP), suggesting dimesna accumulation occurs by either primary or secondary active protein-dependent transport. Kinetic analysis by Lineweaver-Burk plot revealed a $K_M$ and $V_{max}$ of 22 µM and 1.4 nmol/10^6 cells, respectively. However, only four concentrations of dimesna were tested, and without the use of an impermeable tracer (e.g. mannitol or Lucifer yellow) to control for increased permeability of the artificial system, passive transport of dimesna across the perfused vasculature could not be accounted for. Thus, observed kinetic parameters can only be considered approximations. The rate of dimesna uptake was not inhibited by 1 mM GSSG, cystine (CySSyC), Cys, NAC, or mesna, and only slightly decreased in the presence of 1 mM GSH, however the significance was not determined. Unlike the rate of reduction of dimesna, the rate of [14C]dimesna uptake was independent of cellular GSH concentration. Hence GSH could be considered a cofactor of dimesna metabolism, but not its uptake. Accumulation of [14C]dimesna was approximately 100-fold greater in isolated perfused rat kidneys with functioning glomeruli, compared to kidneys whose filtration capability had been diminished by perfusion with 10% albumin. This lead the authors to conclude that the accumulation of dimesna is dependent on filtration and occurs primarily as a reabsorptive process across the apical (luminal) membrane of the renal tubules (Ormstad & Uehara, 1982).
1.5 Drug Uptake Transporters in Cancer

Although able to bind reactive metabolites, mesna and dimesna do not interfere with the cytotoxic effects of cisplatin or ifosfamide (Araujo & Tessler, 1983; Boven et al., 2002; Brock et al., 1982; Millar et al., 1983; Scheef et al., 1979; Scheulen et al., 1983). This has been attributed to the rapid oxidation of mesna to inactive dimesna and the perception that such a polar substance would be unable to readily cross cell membranes (Brock et al., 1981a). However, Ormstad et al. clearly demonstrated the presence of transporter-mediated dimesna uptake by the kidney (Ormstad & Uehara, 1982). With the identification and characterization of a multitude of drug transporting proteins able to transport anionic, cationic, and zwitterionic molecules, the disposition of many chemotherapeutic drugs is now understood to be predominantly protein dependent (Dobson & Kell, 2008; Brock et al., 1981a; Ho & Kim, 2005).

The efficacy of cancer chemotherapy is dependent on the accumulation of the drug at the site of action and presence of the target. Many cancers circumvent susceptibility by innate and adaptive mechanisms such as increased drug detoxification, increased drug target expression, mutations of drug targets, alterations in apoptosis and cell cycle, decreased facilitative drug uptake, and increased drug efflux (Bush & Li, 2002; Desoize & Jardillier, 2000; Donnenberg & Donnenberg, 2005; Moscow et al., 1995).

The most studied, and perhaps most common, mechanism of drug resistance is the expression of membrane proteins of the energy-dependent ATP-binding cassette (ABC) transporter family. Prototypical drug efflux transporters Pgp (MDR1; ABCB1), BCRP
(ABCG2), MRP1 (ABCC1) were first discovered in cancer cell lines, and thus their role to facilitate drug resistance in cancer was immediately apparent (Cole et al., 1992; Dano, 1973; Doyle et al., 1998; Juliano & Ling, 1976; McGrath & Center, 1988). The ability of the family of ABC transport proteins to confer drug resistance has been extensively reviewed (Szakacs et al., 2006).

Conversely, prototypical drug uptake transporters of the SLC22 and SLCO superfamilies, OCT1 (SLC22A1) (Gorboulev et al., 1997d), OAT1 (SLC22A6) (Lopez-Nieto et al., 1997b), OATP1A2 (SLCO1A2) (Kullak-Ublick et al., 1995) were first cloned from kidney and liver; tissues responsible for the clearance of drugs, and thus their roles have largely been studied in the context of drug absorption, distribution, metabolism, and excretion (ADME) (Hagenbuch, 2010). Hence members of the SLC22 and SLCO superfamilies represent ideal candidates of the putative drug transporter(s) proposed by Ormstad et al (Ormstad & Uehara, 1982). Localization of these drug uptake transporters in cancerous and healthy human tissues along with known substrates commonly administered during chemotherapy are listed in Table 1.1.
Table 1.1  Cancer chemotherapy substrates and expression patterns of drug uptake transporters of the *SLC22A* and *SLCO* family in healthy and cancerous human tissues.

<table>
<thead>
<tr>
<th>Transporter (Gene)</th>
<th>Chemotherapy Substrates</th>
<th>Healthy Tissue Localization</th>
<th>Cancerous Tissue Localization (change in expression*)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SLC22A family</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OCT1 (<em>SLC22A1</em>)</td>
<td>bamet-R2 (Briz <em>et al</em>., 2002j)</td>
<td>adrenal gland <em>a</em> (Nies <em>et al</em>., 2009h)</td>
<td>colon polyp (no change) <em>a</em> (Ballestero <em>et al</em>., 2006m)</td>
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<td></td>
<td>bamet-UD2 (Briz <em>et al</em>., 2002i)</td>
<td>breast mammary epithelium <em>a</em> (Alcorn <em>et al</em>., 2002h; Kwok <em>et al</em>., 2006d)</td>
<td>colon cancer (no change) <em>a</em> (Ballestero <em>et al</em>., 2006l; Zhang <em>et al</em>., 2006h)</td>
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<td></td>
<td>cDPCP (Lovejoy <em>et al</em>., 2008a)</td>
<td>kidney <em>a</em> (Verhaagh <em>et al</em>., 1999f; Motohashi <em>et al</em>., 2002e; Jung <em>et al</em>., 2008i)</td>
<td>leukemia <em>a</em> (Wang <em>et al</em>., 2008b; Thomas <em>et al</em>., 2004b)</td>
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<td></td>
<td>cisplatin (Yonezawa <em>et al</em>., 2006e)</td>
<td>liver; basolateral &amp; cholangiocytes <em>ab</em> (Nies <em>et al</em>., 2009g; Zhang <em>et al</em>., 1997)</td>
<td>non-small cell lung cancer (no change) <em>a</em> (Monks <em>et al</em>., 2007)</td>
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<td></td>
<td>oxaliplatin (Zhang <em>et al</em>., 2006i)</td>
<td>lung <em>a</em> (Monks <em>et al</em>., 2007); Nies <em>et al</em>., 2009f</td>
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<td></td>
<td>imatinib (Hu <em>et al</em>., 2008e; Thomas <em>et al</em>., 2004a; Wang <em>et al</em>., 2008c)</td>
<td>ovary <em>a</em> (Jung <em>et al</em>., 2008h)</td>
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<td></td>
<td>meta-iodobenzyl-guanidine (Bayer <em>et al</em>., 2009b)</td>
<td>prostate <em>a</em> (Jung <em>et al</em>., 2008g)</td>
<td></td>
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<tr>
<td><strong>OCT2 (<em>SLC22A2</em>)</strong></td>
<td>bamet-R2 (Briz <em>et al</em>., 2002h)</td>
<td>brain <em>ab</em> (Busch <em>et al</em>., 1998)</td>
<td>colon cancer (increased) <em>a</em> (Zhang <em>et al</em>., 2006f)</td>
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<tr>
<td></td>
<td>bamet-UD2 (Briz <em>et al</em>., 2002g)</td>
<td>colon <em>a</em> (Jung <em>et al</em>., 2008)</td>
<td>non-small cell lung cancer (decreased) <em>a</em> (Monks <em>et al</em>., 2007)</td>
</tr>
<tr>
<td></td>
<td>cDPCP (Lovejoy <em>et al</em>., 2008b)</td>
<td>heart <em>a</em> (Jung <em>et al</em>., 2008e)</td>
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<tr>
<td><strong>OCT3 (SLC22A3)</strong></td>
<td><strong>OCTN1 (SLC22A4)</strong></td>
<td><strong>OCTN2 (SLC22A5)</strong></td>
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<td>meta-iodobenzyl-guanidine (Bayer et al., 2009a)</td>
<td>doxorubicin (Okabe et al., 2008e)</td>
<td>imatinib (Hu et al., 2008d)</td>
<td></td>
</tr>
<tr>
<td>oxaliplatin (Yonezawa et al., 2006c; Zhang et al., 2006e)</td>
<td>mitoxantrone (Okabe et al., 2008d)</td>
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<tr>
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<td>bone marrow a (Tamai et al., 1997c)</td>
<td>breast mammary epithelium a,b (Alcorn et al., 2002e; Kwon et al., 2006e)</td>
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<td>prostate a (Tamai et al., 1997b)</td>
<td>skeletal muscle a (Tamai et al., 1997a)</td>
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<td>lung a (Nies et al., 2009a)</td>
<td>small intestine a (Glaeser et al., 2007b)</td>
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<td>skeletal muscle a (Verhaagh et al., 1999g)</td>
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<td>non-small cell lung cancer (no change) a (Monks et al., 2007u)</td>
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<td>Name/Location</td>
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<td>Target Tissues/Cells</td>
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<td>bone marrow * (Okabe <em>et al.</em>, 2005b; Gong <em>et al.</em>, 2002i), peripheral blood leukocytes * (Gong <em>et al.</em>, 2002h)</td>
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<td>acute myelogenous leukemia * (Okabe <em>et al.</em>, 2005b; Gong <em>et al.</em>, 2002a)</td>
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<td>chronic myeloid leukemia * (Gong <em>et al.</em>, 2002b)</td>
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<td>ovarian cancer * (Ota <em>et al.</em>, 2007b)</td>
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<td>methotrexate (Sekine <em>et al.</em>, 1997a; Takeda <em>et al.</em>, 2002)</td>
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<td>brain; apical (choroid plexus) * * (Cihlar <em>et al.</em>, 1999a; Alebouyeh <em>et al.</em>, 2003b)</td>
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<td>kidney; basolateral * (Motohashi <em>et al.</em>, 2002c; Hosoyama <em>et al.</em>, 1999; Cihlar <em>et al.</em>, 1999c; Sun <em>et al.</em>, 2001g)</td>
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<td>lung * (Monks <em>et al.</em>, 2007t)</td>
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<td>skeletal muscle * (Cihlar <em>et al.</em>, 1999b)</td>
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<td><strong>OAT2 (SLC22A7)</strong></td>
<td>5-fluoruracil (Kobayashi <em>et al.</em>, 2005b); methotrexate (Sun <em>et al.</em>, 2001f); paclitaxel (Kobayashi <em>et al.</em>, 2005a)</td>
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<td>liver * (Shin <em>et al.</em>, 2010; Sun <em>et al.</em>, 2001d)</td>
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<td>lung * (Monks <em>et al.</em>, 2007t)</td>
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<td>methotrexate (Cha <em>et al.</em>, 2001c; Takeda <em>et al.</em>, 2002)</td>
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<td>adrenal tissue * (Asif <em>et al.</em>, 2005b)</td>
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<td>brain; apical (choroid plexus) * * (Alebouyeh <em>et al.</em>, 2003a)</td>
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<td>kidney; basolateral * (Motohashi <em>et al.</em>, 2002b; Cha <em>et al.</em>, 2001b; Sun <em>et al.</em>, 2001c)</td>
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<td>lung * (Monks <em>et al.</em>, 2007p)</td>
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<td>adrenal cancer * (Asif <em>et al.</em>, 2005a)</td>
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<td>non-small cell lung cancer (no change) * (Monks <em>et al.</em>, 2007o)</td>
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<td><strong>OAT4 (SLC22A11)</strong></td>
<td>methotrexate (Takeda et al., 2002)</td>
<td>adrenal tissue&lt;sup&gt;a&lt;/sup&gt; (Asif et al., 2005c)</td>
<td>kidney; apical&lt;sup&gt;a,b&lt;/sup&gt; (Cha et al., 2000; Babu et al., 2002)</td>
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**SLCO family**

|**OATP1A2 (SLCO1A2)** | bamet-R2 (Briz et al., 2002f) | brain capillary endothelia; basolateral (abluminal)<sup>a,b</sup> (Lee et al., 2005c; Bronger et al., 2005a; Gao et al., 2000) | liver; apical<sup>b</sup> (Lee et al., 2005b) | kidney; apical<sup>b</sup> (Lee et al., 2005a) | lung<sup>a</sup> (Monks et al., 2007l) | small intestine; apical<sup>a,b</sup> (Glaeser et al., 2007f) | eye pars plana ciliary body epithelium; basolateral<sup>b</sup> (Gao et al., 2005e) | breast mammary epithelium<sup>a</sup> (Alcorn et al., 2002d) | colon polyp (decreased)<sup>a</sup> (Ballestero et al., 2006k) | colon cancer (decreased)<sup>a</sup> (Ballestero et al., 2006j) | brain glioma<sup>a</sup> (Bronger et al., 2005b) | breast cancer (increased)<sup>a,b</sup> (Meyer zu Schwabedissen et al., 2008d; Miki et al., 2006a) | non-small cell lung cancer (no change)<sup>a</sup> (Monks et al., 2007k) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | bamet-UD2 (Briz et al., 2002e) | imatinib (Hu et al., 2008c) | methotrexate (Badagnani et al., 2006b) | colon polyp (increased)<sup>a</sup> (Ballestero et al., 2006i) | colon cancer (increased)<sup>a</sup> (Ballestero et al., 2006h) | hepatocellular carcinoma (no change)<sup>a,b</sup> (Vavricka et al., 2004d) | hepatocellular carcinoma<sup>b</sup> (Cui et al., 2003a) |

<p>|<strong>OATP1B1 (SLCO1B1)</strong> | BNP1350 (Oostendorp et al., 2009a) | gimatecan (Oostendorp et al., 2009b) | lapatinib (Polli et al., 2008) | methotrexate (Abe et al., 2001f; Sasaki et al., 2004a) | SN-38 (Nozawa et al., 2005a; Oostendorp et al., 2009c) | bamet-R2 (Briz et al., 2002d) | bamet-UD2 (Briz et al., 2002c) | liver; basolateral&lt;sup&gt;b&lt;/sup&gt; (Hsiang et al., 1999; Konig et al., 2000a) | small intestine&lt;sup&gt;a&lt;/sup&gt; (Glaeser et al., 2007d) | colon polyp (increased)&lt;sup&gt;a&lt;/sup&gt; (Ballestero et al., 2006i) | colon cancer (increased)&lt;sup&gt;a&lt;/sup&gt; (Ballestero et al., 2006h) | hepatocellular carcinoma (no change)&lt;sup&gt;a,b&lt;/sup&gt; (Vavricka et al., 2004d) | hepatocellular carcinoma&lt;sup&gt;b&lt;/sup&gt; (Cui et al., 2003a) |</p>
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<th>methotrexate (Abe et al., 2001e)</th>
<th>docetaxel (Smith et al., 2005c)</th>
<th>paclitaxel (Smith et al., 2005b)</th>
<th>SN-38 (Yamaguchi et al., 2008c)</th>
<th>liver; basolateral (^{ab}) (Abe et al., 2001d; Lee et al., 2008d; Konig et al., 2000b)</th>
<th>small intestine (^a) (Glaeser et al., 2007a)</th>
<th>colon (no change) (^a) (Ballestero et al., 2006g)</th>
<th>colon cancer (no change) (^a) (Ballestero et al., 2006f)</th>
<th>colon cancer (increased) (^{ab}) (Lee et al., 2008c)</th>
<th>colon cancer (^{ab}) (Abe et al., 2001c)</th>
<th>gastric cancer (^{ab}) (Abe et al., 2001b)</th>
<th>hepatocellular carcinoma (decreased) (^{ab}) (Vavricka et al., 2004c)</th>
<th>hepatocellular carcinoma (^b) (Narita et al., 2009; Cui et al., 2003b)</th>
<th>non-small cell lung cancer (increased) (^a) (Monks et al., 2007j)</th>
<th>pancreatic cancer (^{ab}) (Abe et al., 2001a)</th>
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<td>lung (^a) (Monks et al., 2007i)</td>
<td>testis; Leydig cells (^{ab}) (Pizzagalli et al., 2002a)</td>
<td>brain glioma (^a) (Bronger et al., 2005c)</td>
<td>non-small cell lung cancer (no change) (^a) (Monks et al., 2007h)</td>
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<td>breast (^a) (Wlcek et al., 2008p)</td>
<td>colon (^a) (Lu et al., 1996h)</td>
<td>eye (^a) (Schuster et al., 1997)</td>
<td>heart (^a) (Lu et al., 1996g)</td>
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<td>liver (^a) (Lu et al., 1996c)</td>
<td>lung (^a) (Monks et al., 2007g; Lu et al., 1996d)</td>
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<th>breast (^a) (Wlcek et al., 2008i)</th>
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| OATP5A1 (SLCO5A1) | breast \(^a\) (Wlcek et al., 2008g) | breast cancer (no change) \(^a\) (Wlcek et al., 2008f) |
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| OATP6A1 (SLCO6A1) | testis \(^a\) (Lee et al., 2004a; Suzuki et al., 2003) | bladder cancer \(^a\) (Lee et al., 2004b) | oesophageal cancer \(^a\) (Lee et al., 2004c) | lung cancer \(^a\) (Lee et al., 2004d) |

* Relative to paired healthy surrounding tissue if available

\(^a\) mRNA by qRT-PCR or Northern blot

\(^b\) protein by Western blot or immunohistochemistry

Excellent large-scale screens of relative-mRNA expression of uptake transporter can be found elsewhere (Bleasby et al., 2006; Hilgendorf et al., 2007; Okabe et al., 2008c)
1.5.1 Organic Cation Transporters

The organic cation transporters (OCTs) and organic cation/carnitine transporters (OCTNs) are polyspecific transporters that facilitate diffusion of cationic molecules less than 400 Da into the cell, with the electrochemical gradient (Koepsell et al., 2007a). The first OCT cloned, Oct1 (Slc22a1), was isolated from rat kidney based on N-methylnicotinamide-inhibitable uptake of the cationic compound tetraethylammonium (TEA) (Grundemann et al., 1994). The first human OCT1 orthologue was later identified and found to be predominately expressed in liver (Gorboulev et al., 1997b). Current human cation transporters of the SLC22A superfamily include OCT1-3 (SLC22A1-3), OCTN1-2 (SLC22A4-5), and OCT6 (SLC22A16) (Koepsell et al., 2007c).

OCT2 is predominantly expressed at the basolateral membrane of proximal and distal tubules of the kidney to facilitate uptake of prostaglandin E\textsubscript{2} and prostaglandin F\textsubscript{2a} (Kimura et al., 2002; Motohashi et al., 2002a; Gorboulev et al., 1997a). OCT1 and OCT3 are expressed at the sinusoidal membrane of hepatocytes where they likely facilitate the uptake of corticosterone, β-estradiol, and testosterone from the blood (Koepsell et al., 2007b; Wu et al., 1998b). Although OCT3 is also referred to as the extraneuronal monoamine transporter due to its ubiquitous mRNA expression and affinity for dopamine and norepinephrine, each of the OCTs likely contributes to the clearance of endogenous monoamines because of their shared affinity for biogenic amines such as dopamine, epinephrine, norepinephrine, histamine, and serotonin (Eisenhofer, 2001; Wu et al., 1998c).

Expression of OCT1 has been linked to accumulation of the tyrosine kinase inhibitor imatinib, but not nilotinib in mononuclear cells. Imatinib is indicated for first-
line therapy of chronic myeloid leukemia (CML) and produces complete cytogenetic remission in approximately 80% of patients. Investigations into the potential causes of optimal response revealed the enabling role of OCT1 in imatinib therapy. Inhibition of OCT1, but not OCT2 and OCT3, prevented imatinib accumulation in a human T-lymphoblastoid cell line derived from a patient with acute lymphoblastic leukemia (Thomas et al., 2004c). To observe if a clinically relevant trend exists between OCT1 mRNA expression and imatinib response, 30 patients with CML were classified as either responders, if they achieved complete cytogenetic remission, or non-responders. Bone marrow mononuclear cell OCT1 mRNA levels of non-responders were significantly lower than those of responders (Crossman et al., 2005). A trend was observed between OCT1 mRNA levels and intracellular retention of imatinib; however given the small sample size of 19 subjects the relationship was not significant (White et al., 2006). A wider screen of uptake transporters revealed that imatinib is a substrate for OCTN2, OATP1A2, OATP1B3, and only a weak substrate for OCT1. OCT1 expression may only be a marker for mononuclear cell imatinib accumulation by other transporters (Hu et al., 2008b). In a relatively large cohort of 70 patients, OCT1 mRNA levels correlated with complete cytogenetic remission, progression-free survival, and overall survival, demonstrating OCT1 to be an important predictor to patient response (Wang et al., 2008a). Supporting prior work suggesting imatinib is a substrate of OCT1, functional activity of OCT1 in mononuclear cells of patients with CML prior to imatinib treatment strongly correlates with successful therapy. Patients with higher OCT1-mediated imatinib uptake had a greater rate of complete molecular remission at five years, while low OCT activity correlated with lower event-free and overall survival rates and higher kinase
domain mutation rate. These clinical findings led the authors to suggest that measurement of OCT1 activity in patient mononuclear cells may be used to personalize imatinib dosing (White et al., 2010).

Uptake transporters may also mediate adverse drug-drug interactions by either preventing clearance leading to intolerable toxicity or restricting permeability to target tumors. Such interactions are likely to become a greater concern as new therapies are given in-addition-to current standards of care rather than monotherapy. For example, although not a substrate, gefitinib has been shown to inhibit OCT1 and OCT2 activities (Galetti et al., 2010). Screening the new Src family kinase inhibitor saracatinib with current first-line therapies demonstrated attenuation in oxaliplatin efficacy in colorectal cancer cells. Transport studies revealed saracatinib-dependent antagonism of OCT2-mediated oxaliplatin uptake (Morrow et al., 2010).

Arguably the most widely used anti-cancer substrates of OCTs are the platinum-based drugs cisplatin and oxaliplatin. While consensus exists that OCT1, OCT2, and OCT3 are capable of transporting oxaliplatin, but not carboplatin, variable transport of cisplatin by OCT-expressing mammalian cell lines has been reported (Burger et al., 2010a; Yokoo et al., 2008b; Yonezawa et al., 2006d; Zhang et al., 2006d). OCTs may not be the only uptake transporters contributing to the differential efficacy of oxaliplatin (Holzer et al., 2006). Intracellular accumulation of oxaliplatin is also less susceptible than either cisplatin or carboplatin to copper transporter 1 (CTR1) which works to sequester platinum moieties to an intracellular compartment, preventing the formation of DNA adducts. More convincingly, expression of OCT1 and OCT2 has been shown to sensitize Madin-Darby canine kidney (MDCK) cells to oxaliplatin, but not cisplatin or carboplatin
cytotoxicity (Zhang et al., 2006c). Cisplatin was the first of these therapies to be approved by the FDA and is effective against a variety of solid tumors including testicular and ovarian cancers, but suffers from nephrotoxicity and acquired drug resistance (Raymond et al., 2002a). The later analogue carboplatin incited less nephro- and gastrointestinal toxicity, but suffered from a similar drug resistance profile, and thus was of little use against cisplatin-refractory tumors (Raymond et al., 2002c). In comparison, oxaliplatin showed greater efficacy against tumors previously resistant to platinum-based drugs (Raymond et al., 1998; Rixe et al., 1996). Subsequently oxaliplatin has been indicated for first- or second-line therapy with 5-fluorouracil (5-FU) and leucovorin against advanced colorectal cancer (Raymond et al., 2002b). Other anti-cancer agents melphalan, irinotecan, and vincristine are also likely substrates of OCT3, given its overexpression sensitizes cells in culture while inhibiting uptake of the organic cation 4-methyl-pyridinium iodide (MPP) (Shnitsar et al., 2009b).

The maintenance of expression of OCT1 and increase in OCT2 and OCT3 expression in colorectal cancer may contribute to the greater effectiveness of oxaliplatin against colorectal cancer than either cisplatin or carboplatin seen clinically (Ballestero et al., 2006e; Yokoo et al., 2008a; Zhang et al., 2006b). Structure-activity experiments have demonstrated the 1,2-diaminocyclohexane (DACH) moiety of oxaliplatin facilitates its interaction with OCT1 and OCT2 (Zhang et al., 2006a). A further increase in efficacy has been sought by synthesizing new platinum agents that are also substrates for OCTs (Briz et al., 2002b; Lovejoy et al., 2008c). Third-generation platinum agent picoplatin is a substrate for OCT1 and OCT2, but not OCT3. Interestingly, OCT1-expressing human embryonic kidney cell (HEK293) xenografts were more susceptible to picoplatin than
wild-type, however pharmacokinetic parameters of picoplatin in wild-type and OCT1 knock-out mice were not different (More et al., 2010). This suggests OCT1 may only control the tumor disposition of picoplatin and not its clearance.

Bile acid-cisplatin derivatives bamet-R2 \([\text{cis-diammine-chloro-cholylglycinate-platinum(II)}]\) and bamet-UD2 \([\text{cis-diammine-bisursodeoxycholate-platinum(II)}]\) showed greater accumulation in HEK293 cells expressing OCT1 or OCT2 compared to the cisplatin moiety alone (Briz et al., 2002a). Furthermore, no difference in accumulation of bamet-UD2 between \textit{Xenopus laevis} oocytes injected with RNA isolated from either healthy tissue or colorectal cancers and polyps, suggesting bile acid derivatives may not suffer from the same intrinsic resistance as cisplatin (Ballestero et al., 2006d). The cationic analogue \textit{cis-diammine(pyridine)-chloroplatinum(II)} (cDPCP) exploits the propensity of OCT1 and OCT2 to uptake monovalent cations, showing great accumulation in transfected cells. Moreover, cDPCP was less toxic to non-OCT expressing cells while maintaining potent transcription blockade and reduced susceptibility to mammalian excinuclease, the common mechanism of platinum-adduct repair (Lovejoy et al., 2008d).

OCTN1 and OCTN2 are expressed at kidney proximal tubules, lactating breast epithelium, and cardiac tissues to facilitate excretion of cations and the uptake of carnitine, an essential molecule for mitochondrial fatty acid metabolism and energy production (Grube et al., 2006a). Little is known regarding the expression of OCTN1 and OCTN2 in cancers, only the maintenance of mRNA expression in non-small cell lung cancer has been found (Monks et al., 2007c). Given that overexpression of OCTN1 sensitizes cells to mitoxantrone and doxorubicin by facilitating their cellular
accumulation, the expression of OCTN1 in common targets such as breast, ovarian, and prostate cancers should be investigated (Okabe et al., 2008b). OCTN2 has demonstrated modest uptake of imatinib, sorafenib, and sunitinib (Hu et al., 2008a; Hu et al., 2009). Given its expression in cardiac endothelia, organic cation transporters may also contribute to tyrosine kinase inhibitor-associated cardiac toxicity (Lenihan, 2008; Mann, 2006).

OCT6, unlike other organic cation transporters of the SLC22A family, is preferentially expressed in the testis and hematopoietic cell types, but absent in the kidneys and liver (Enomoto et al., 2002e; Gong et al., 2002c; Okabe et al., 2005i). Early work to characterize OCT6 identified it as a doxorubicin transporter expressed in a variety of leukemias (Gong et al., 2002d; Okabe et al., 2005j). OCT6 expression has since been identified in malignant breast, endometrial, and ovarian tissues (Sato et al., 2007b; Ota et al., 2007a). Expression of OCT6 varies throughout the menstrual cycle, peaking during the secretory phase. In vitro, mRNA expression of OCT6 by endometrial cancer cells is increased in response to progesterone (Sato et al., 2007a). Improved response rates to cyclophosphamide/doxorubicin/5-fluorouracil chemotherapy with the addition of medroxyprogesterone may be due to increased expression of doxorubicin transporter OCT6 by endometrial cancers (Ayoub et al., 1988).

A pharmacogenomic study of patients receiving doxorubicin therapy has suggested a clinical role of OCT6 polymorphisms in breast cancer therapy. The T1226C variant of OCT6 was associated with a greater incidence of dose delay (a surrogate marker of toxicity), while the A146G, T312C, and T755C SNPs decreased the incidence of dose delay; however no OCT6 allele had any effect on patient survival (Bray et al.,
Due to the complexity of doxorubicin disposition, patient outcome is likely to be best described by a composite of \textit{ABCB1}, \textit{CYP2B6}, and \textit{SLC22A16} haplotypes.

The localization of OCT6 in the testis, and ability to sensitize testicular cancer cells to first-line chemotherapeutic agent bleomycin, suggests the presence of OCT6 may drive germ cell cancer cure rates (Aouida \textit{et al.}, 2010a). Furthermore, the propensity of other organic cation transporters to transport platinum agents makes OCT6 an ideal candidate to explain the remarkable therapeutic efficacy of cisplatin and oxaliplatin in the treatment of the majority of testicular cancers. Conversely, screening for OCT6 expression prior to initiation of chemotherapy may help identify those patients requiring alternate chemotherapy regimens due to low uptake transporter expression.

\textbf{1.5.2 Organic Anion Transporters}

First cloned from rat kidney, renal organic anion transporter 1 (Slc22a6; Oat1, originally NKT) was identified as a probenecid-inhibitable \textit{para}-aminohippurate (PAH)/dicarboxylate exchanger (Sekine \textit{et al.}, 1997b; Lopez-Nieto \textit{et al.}, 1997a; Sweet \textit{et al.}, 1997). Human homologues include functionally characterized OAT1-4 (SLC22A6-9) and URAT1 (SLC22A12; urate transporter 1) (Ahn & Nigam, 2009c). Six of the eight known OATs exist as linked gene pairs within the genome; OAT1-OAT3, OAT4-URAT1, and OAT5-UST3 (unknown solute transporter 3) (Eraly \textit{et al.}, 2004). Coordinated transcription of these gene pairs is reflected in their pattern of tissue expression. OAT1 and OAT3 are expressed at the basolateral membranes of the proximal tubule and blood-brain barrier where they facilitate the first step in the clearance of uremic toxins such as indoxyl sulfate and neurotransmitter metabolites such as homovanillic acid (Sweet, 2005).
Renal OATs also play an important role in plasma urate homeostasis through its secretion by basolateral OAT1 and OAT3 and reabsorption by apical OAT4 and URAT1 (Bakhiya et al., 2003; Enomoto et al., 2002a; Hagos et al., 2007; Ichida et al., 2003; Islam et al., 2008b). In the proximal tubule cell the basolateral sodium/dicarboxylate cotransporter 3 (SLC13A3; NaDC3) with the aid of mitochondrial production, establish an intracellular-to-extracellular concentration gradient of α-ketoglutarate (Pritchard, 1995). Organic anion transporters 1 & 3 concentrate organic anions within the cell by exchanging an organic anion for the dicarboxylate anion α-ketoglutarate. Thus, OAT1 and OAT3 are believed to facilitate the first step in the secretion of anionic uremic toxins such as indoxyl sulfate and indole acetate (Ahn & Nigam, 2009d; Deguchi et al., 2004). At the apical membrane, OAT4 concentrates organic anions in exchange for dicarboxylate anions succinate or α-ketoglutarate (Rizwan & Burckhardt, 2007). In contrast to other subfamily members, OAT2 is most highly expressed at the basolateral membrane of hepatocytes. OAT2 has been proposed to regulate hepatic metabolism by uptake of short-chain fatty acids, but its role and mechanism of function remain controversial (Islam et al., 2008a; Ahn & Nigam, 2009b). Each of the OATs have also shown specificity for endogenous steroids 5-dehydroepiandrosterone (DHEAS), estrone-2-sulfate, and estradiol-17β-glucuronide, prostaglandins E2 and F2α, as well as cyclic nucleotides cAMP and cGMP, and thus likely play a role in hemodynamics and metabolism (Ahn & Nigam, 2009a; Islam et al., 2008c).

Like other members of the SLC family, our current knowledge of OAT expression in cancer is largely limited to cultured cell lines (Okabe et al., 2008a). A survey of solute carrier mRNA expression in 19 non-small cell lung carcinoma tumors
revealed a small decrease in OAT1 and little change in OAT2, OAT3, and OAT4 compared to paired healthy tissue (Monks et al., 2007). In comparison to normal renal cortical slices, uptake of the prototypical OAT substrate PAH was absent in renal cell carcinoma tissue and declined with severity of the tumor stage (Fleck et al., 2000b; Fleck et al., 1997). However, drug uptake activity could be stimulated by dexamethasone (Fleck et al., 2000a), and thus may provide a mechanism for improved efficacy during combination therapy for advanced renal cell carcinoma in addition to NF-κB inhibition (Arai et al., 2008; Schoffski et al., 2009).

The human OATs are known to interact with a wide variety of drugs, notably β-lactam antibiotics, non-steroidal anti-inflammatories (NSAIDs), anti-virals, diuretics, and angiotensin-converting enzyme (ACE) inhibitors, however reports of anti-cancer agents as substrates for OATs are limited (Sekine et al., 2006). With the exception of paclitaxel transport by OAT2 (Kobayashi et al., 2005c), anti-cancer agent specificity of OATs appears to be restricted to anti-metabolites. Although only human OAT2 has been shown to transport 5-fluoruracil (5-FU) (Kobayashi et al., 2005d), rodent Oat1 and Oat3 have exhibited uptake of the anti-purines 6-mercaptopurine and 5-FU (Kobayashi et al., 2004; Mori et al., 2004). Transport by in human orthologues remains to be confirmed.

A common substrate of the OATs is the anti-folate methotrexate (Cha et al., 2001a; Sekine et al., 1997c; Sun et al., 2001a; Takeda et al., 2002). Methotrexate is eliminated by a combination of glomerular filtration and OAT1- and OAT3-mediated tubular secretion (Shen & Azarnoff, 1978). During chemotherapy, inhibition of OAT-mediated clearance of methotrexate by NSAIDs or the uricosuric agent probenecid can lead to life-threatening myelosuppression and acute renal failure (Basin et al., 1991;
Ellison & Servi, 1985; Frenia & Long, 1992; Thyss et al., 1986). Patient response to this renally cleared folate analogue is likely determined by distribution by folate carriers RFC1 (reduced folate carrier 1; \textit{SLC19A1}) and PCFT (proton coupled folate transporter; \textit{SLC46A1}) and elimination by OATs (Zhao et al., 2008). The influence of renal OATs has been largely focused on the pharmacokinetics of anti-cancer agents, while expression of OATs in many cancers and the role of this subfamily on the potency of anti-cancer agents remain to be studied.

1.5.3 Organic Anion Transporting Polypeptides

Transporters of the organic anion-transporting polypeptide (OATP) superfamily facilitate polyspecific sodium-independent uptake of amphipathic anions, zwitterions, and some cations. The first member of the \textit{SLCO} (originally \textit{SLC21}) superfamily was cloned from rat liver as a sodium-independent sulfobromophthalein and bile salt uptake transporter and denoted Oatp1. Transport of substrates may be driven by exchange with taurocholate, bicarbonate, or GSH. Eleven human OATPs of six families (OATP1-6) and four subfamilies (A-D) have since been cloned. The tissue localization and substrate specificity of the OATPs have implicated them as important determinants of drug disposition, toxicity, and efficacy (Hagenbuch & Gui, 2008a).

Currently, well-characterized members include the widely expressed OATP1A2 (\textit{SLCO1A2}) and OATP2B1 (\textit{SLCO2B1}) and liver-specific OATP1B1 (\textit{SLCO1B1}) and OATP1B3 (\textit{SLCO1B3}). Prototypical substrates of OATPs are typically sterols such as the hormone estrone-3-sulfate (E1S) and bile acid taurocholate, but many members are capable of transporting common drugs such as ACE inhibitors, angiotensin receptor antagonists, antibiotics, and statins, hence interest in the effect of OATPs on
pharmacotherapy has continued to grow. OATPs fulfil a variety of physiological roles facilitating the metabolism and signalling of eicosanoids, estrogens, peptides, thyroid hormones, and their metabolites, in addition to mediating the first step in hepatic clearance of circulating bilirubin, bile acids and conjugates (Hagenbuch & Gui, 2008b).

Irinotecan (CPT-11) is approved as first-line therapy for the treatment of metastatic colorectal cancer; hence the role of intestinal OATPs in the accumulation of SN-38 by colorectal cancer is of great interest. Although the hepatic disposition of irinotecan is likely dependent on OCT3 (Shnitsar et al., 2009a), the potent metabolite SN-38 may be a substrate of intestinal OATP1B1 and OATP1B3 (Nozawa et al., 2005b; Oostendorp et al., 2009d; Yamaguchi et al., 2008b). Expression of OATP1B1 and OATP1B3 protein in addition to OATP1A2 and OATP4A1 mRNA has been detected in colorectal cancers (Abe et al., 2001g; Ancona et al., 2006; Ballestero et al., 2006c; Lee et al., 2008b). Both SN-38 and its glucuronide are excreted into the gut via the bile. Following cleavage from the glucuronide by gut microflora, SN-38 may be reabsorbed by the intestine (Takasuna et al., 1996). Uptake of SN-38 by OATP1B1 has been observed by different laboratories; however, evidence for SN-38 transport by OATP1B3 is inconsistent and likely due to differences in transporter expression systems (Nozawa et al., 2005c; Yamaguchi et al., 2008a). The increased expression of SN-38 uptake transporters by neoplastic tissue may contribute to irinotecan’s efficacy (Ballestero et al., 2006b; Lee et al., 2008a). Alternatively, overexpression of functional OATP1B3 confers apoptotic resistance in colon cancer cells by reduced transcriptional activity of p53 (Lee et al., 2008e).
Similar to their tissue of origin, OATP1B1 and OATP1B3 are expressed in hepatocellular carcinomas (Cui et al., 2003c; Narita et al., 2009; Vavricka et al., 2004b). Due to the heterogeneity of cancers, even from the same tissue of origin, transporter expression varies between classifications. A detailed histological study of benign liver tumours revealed decreased staining of OATP1B1/1B3 in hepatocellular adenomas, while strong expression patterns were observed in focal nodular hyperplasias (Vander et al., 2005). OATP1B1 and OATP1B3 were also detected in a large majority of hepatocellular carcinomas, while staining of cholangiocarcinomas and liver metastases of colorectal and pancreatic adenocarcinomas were negative (Cui et al., 2003d). Interestingly, in a study of 13 paired hepatocellular carcinoma and healthy tissue samples, a decrease in OATP8 protein expression was observed which correlated to an increase in the liver-enriched transcription factor hepatocyte nuclear factor 3β (HNF3β). In contrast, no significant change in OATPC expression was detected, suggesting a mechanism of differential regulation of expression in cancerous tissue (Vavricka et al., 2004a). Larger investigations into expression of these transporters are needed if the use of bile acid conjugates of anti-cancer drugs such as cisplatin is to be considered.

A multitude of OATP mRNAs are expressed in non-small cell lung cancer, notably an up-regulation of OATP1B3 (Monks et al., 2007). OATP1B3 has also been shown to uptake current lung cancer drugs paclitaxel and docetaxel (Smith et al., 2005a). Common single nucleotide polymorphisms of OATP1B3 may only play a limited role in paclitaxel pharmacokinetics (Smith et al., 2007). However the relative contribution of tumor volume to paclitaxel apparent distribution is small, and whether tumor specific expression correlates to patient outcome remains to be determined. To this end, the use of
uptake transporter-specific radiotracers may help identify susceptible tumors and aid in optimizing anti-cancer therapy to prevent early drug resistance (Narita et al., 2009; Tsuboyama et al., 2010).

Expression of uptake transporters may render tumors sensitive to hormonal growth stimuli. Uptake of E1S by many OATPs had lead to investigation into their role in hormone-dependent breast cancers. Many of the OATPs are expressed in breast cancers, including OATP1A2 (Meyer zu Schwabedissen et al., 2008c; Miki et al., 2006b), OATP1B3 (Muto et al., 2007b), OATP2A1 (Wlcek et al., 2008c), OATP2B1 (Al Sarakbi et al., 2006a), OATP3A1 (Wlcek et al., 2008d), OATP4A1 (Wlcek et al., 2008c), OATP4C1 (Wlcek et al., 2008b), and OATP5A1 (Wlcek et al., 2008a). The proliferation of hormone-dependent breast cancer MCF-7 cells can be controlled by OATP1B3-mediated uptake of E1S (Maeda et al., 2010; Nozawa et al., 2005d). Clinically, OATP1B3 may be expressed in up-to half of all breast carcinomas. OATP1B3 expression may also be a strong prognostic marker; correlating to low risk of recurrence in estrogen receptor-positive cases (Muto et al., 2007a).

OATP1A2 is significantly up-regulated at the protein level in breast cancer and may also play a pivotal role in pathogenesis (Meyer zu Schwabedissen et al., 2008b; Miki et al., 2006c). Expression of OATP1A2 can be induced through binding of the nuclear pregnane X receptor (PXR) to a promoter region of SLCO1A2 (Meyer zu Schwabedissen et al., 2008a). Increased OATP1A2, in turn, induces breast cancer proliferation through accumulation of estrogen precursors. Given that estrone and 17β-estradiol are activators of PXR (Jacobs et al., 2005; Mnif et al., 2007), the subsequent increase in hormone
accumulation results in feed-forward regulation of OATP1A2 expression and breast cancer hyperplasia (Meyer zu Schwabedissen et al., 2008e).

Gliomas are characterized by strong chemoresistance due to the low permeability of the blood-brain barrier. Unlike at epithelial layers of other tissues, uptake transporter expression may confer resistance at the blood-brain barrier by abluminal expression; facilitating the clearance of anti-cancer agents from the brain parenchyma (Deeken & Loscher, 2007). A screen of a diverse panel of 61 glioma specimens revealed mRNA expression of OATP1A2, OATP1C1, OATP2B1, and OATP4A1. However, only OATP1A2 and OATP2B1 protein were present at the blood-brain and blood-tumor barriers, and absent in endothelial and glioma cells of tumors (Bronger et al., 2005g). Despite an apparent dearth of drug uptake transporter expression by gliomas, expression of known neuronal OAT and OCT transporters remains to be determined.

Although relatively few studies have evaluated uptake of anti-cancer agents by OATPs, similar to the OATs, methotrexate may emerge as a common substrate (Abe et al., 2001h; Badagnani et al., 2006a; Mikkaichi et al., 2004c; Sasaki et al., 2004b). In vitro expression of OATP1B3 is capable of sensitizing cells to methotrexate (Abe et al., 2001i). Given the expression of OATP1B3 protein in many colorectal cancers (Abe et al., 2001j; Ballestero et al., 2006a; Lee et al., 2008f), its expression may contribute to the sensitization of tumors to anti-folate therapy. In conjunction with the growing list of anticancer substrates, future investigations will elucidate the role of OATPs in cancer biology and its treatment.
1.6 Focus of Thesis

The pharmacokinetics of drugs plays a crucial role in their clinical effectiveness. This is especially true of chemotherapeutics, where their distribution between cancerous and healthy tissue often determines a drug’s therapeutic index. Control of side effects associated with cancer chemotherapy is essential to provide adequate dosing of anticancer drugs. Mesna is a sulfhydryl drug that has been in use for the past thirty years to mitigate ifosfamide-induced hemorrhagic cystitis. Dimesna, the disulfide dimer and primary metabolite of mesna, is currently being evaluated as a prodrug in phase III trials for the treatment of cisplatin induced nephrotoxicity. Remarkably, mesna and dimesna specifically target the kidney without attenuation of the efficacies of ifosfamide or cisplatin.

Circulating dimesna must gain entry to the kidneys and urine as chemically reduced mesna in order to exert its protective effects. In chapter two we elucidate the renal drug transporters responsible for reabsorption of dimesna into the proximal tubule cells and subsequent efflux of active mesna into the urine. In addition, we identify novel transcellular pathways of transporter-mediated active secretion of mesna by the proximal tubule cells. The clinical importance of our findings is highlighted by a controlled drug interaction study demonstrating that transporter-mediated secretion represents a significant fraction of total mesna clearance.

In chapter three we examine in detail the kinetics of dimesna metabolism by non-enzymatic thiol-disulfide thiol exchange with abundant endogenous thiols Cys and GSH, and further elucidate the roles of members of the thioredoxin family of oxidoreductases in the metabolism of dimesna.
Glutathione is an endogenous antioxidant thiol that facilitates drug resistance by the detoxification of many anti-cancer agents (Gatti & Zunino, 2005). The depletion of GSH and its precursors, Cys and Hcy, in patients receiving mesna was originally investigated to provide a rationale for the potentiation of chemotherapy. The disulfide bonding of sulfhydryl (i.e. “free”) mesna to Cys34-albumin displaces endogenous thiols, increasing their free fraction and subsequent renal clearance (Urquhart et al., 2006; Urquhart et al., 2007a). Although mesna did indeed lower circulating GSH, Cys, and Hcy (Lauterburg et al., 1994; Pendyala et al., 2000; Stofer-Vogel et al., 1993), whether this contributes to the efficacy of co-administered ifosfamide remains to be determined.

Interestingly, the application of mesna to lower plasma tHcy may itself be therapeutically useful. The non-protein forming thiol amino acid Hcy is a graded, independent risk factor for atherosclerosis and thrombosis (Chao et al., 1999; Graham et al., 1997; Nygard et al., 1997; Vasan et al., 2003; Wald et al., 2002). In chapter four we further develop the recent novel clinical application of mesna to lower plasma tHcy in ESRD (Urquhart et al., 2007b; Urquhart et al., 2008). Plasma tHcy levels are elevated in over 90% of patients with ESRD despite B-vitamin supplementation (House & Donnelly, 1999; Spence et al., 1999; Wrone et al., 2004). Approximately 75% of Hcy is protein-bound, and thus unavailable for clearance by routine hemodialysis (House et al., 2000; Mansoor et al., 1993). The use of mesna may provide an additional strategy to lower Hcy, and in turn, the atherothrombotic burden of ESRD.
The global hypothesis of this thesis is that the pharmacological characteristics of mesna and dimesna are determined by drug transporters and thiol metabolism. The specific objectives are:

1) To identify the transporters responsible for the renal-specific disposition of mesna and dimesna.

2) To determine the in vivo contribution of renal drug transporters to the pharmacokinetics of total mesna.

3) To determine the enzymatic and non-enzymatic mechanisms of dimesna metabolism.

4) To measure the effect of mesna to lower plasma total homocysteine (tHcy) in end-stage renal disease (ESRD).

Mesna and dimesna have undergone continuous development for new indications throughout their history. Here we elucidate the biological characteristics of these compounds underlying their unique pharmacological properties and continue to expand the therapeutic application of mesna.
1.7 References


CHAPTER 2: *IN VITRO AND IN VIVO* ASSESSMENT OF THE ROLE OF
ORGANIC ANION TRANSPORTERS IN THE DISPOSITION OF MESNA AND
DIMESNA
2.1 Introduction

In the early 1980s, significant advancement in the safety of ifosfamide therapy was achieved by coadministration of mesna (sodium 2-mercaptoethane sulfonate) to reduce previously observed dose-limiting hemorrhagic cystitis (Andriole et al., 1987). Mesna exerts its protective effect within the urine, where its free sulfhydryl group is able to bind to, and thereby inactivate, reactive metabolites (Kurowski & Wagner, 1997). Within the circulation mesna exists primarily as its inactive dimer, dimesna (disodium 2,2-dithio-bis-ethane sulfonate), which is currently undergoing clinical development as a prodrug (BNP7787; Tavocept™) to treat cisplatin-induced nephrotoxicity (Boven et al., 2005; Verschraagen et al., 2003a).

Dimesna undergoes selective kidney reduction and elimination in excess of glomerular filtration (Ormstad et al., 1983; Urquhart et al., 2007; Brock et al., 1981; Verschraagen et al., 2004a). Remarkably, neither mesna nor dimesna interfere with the efficacy of coadministered antineoplastic agents (Millar et al., 1983; Verschraagen et al., 2003a). This has been attributed to the rapid oxidation of mesna to dimesna and its apparent inability to readily cross cell membranes (Brock et al., 1981). However, with the identification and characterization of drug transporting proteins, the organ-specific disposition of drugs is now understood to be much more complex (Ho & Kim, 2005).

Similar to drug metabolizing enzymes, the ability of drug transporters to influence the disposition of drugs raises the potential for transporter-mediated drug-drug interactions (DDIs) and polymorphisms of drug transporters to alter drug response (Giacomini et al., 2010; Maeda & Sugiyama, 2008). Loss of transport function, whether
due to genetic variation or coadministration of an inhibitor, may adversely alter the safety and efficacy of the drug by preventing its excretion or restricting access to its site of action. Amongst the renal drug transport proteins, uptake transporters OAT1 (*SLC22A6*) and OAT3 (*SLC22A8*) have been implicated as clinically relevant drug transporters that could potentially mediate DDIs of substrate drugs cleared by the kidney through active secretion. Indeed, labeling by the Food and Drug Administration of the antiviral cidofovir (Vistide®; Gilead) and the antihyperglycemic sitagliptin (Januvia™; Merck) has included information of interactions with OATs (Giacomini *et al*., 2010).

Given the tissue-specific disposition of mesna and dimesna, and previously hypothesized obligatory reduction of dimesna by the kidney parenchyma (Ormstad & Uehara, 1982), our initial objective was to measure the dimesna transport capacity of renal and hepatic drug transporters. We extended our *in vitro* results with a clinical pharmacokinetic study using the transport inhibitor probenecid to determine the *in vivo* contribution of transporter-mediated secretion of combined mesna and dimesna.

In light of our identification of uptake and efflux transporters of dimesna, we believe the organ specificity of dimesna, and therefore activation of dimesna to mesna within the proximal tubule cells, is the result of differential transport across epithelia. Polymorphisms of renal transport proteins or transporter-mediated drug-drug interactions may lower the efficacy of the chemoprotectants mesna and dimesna, potentially causing withdrawal from chemotherapy due to toxicity (i.e. dose delay).
2.2 Methods

2.2.1 In vitro Screen of Drug Transporters Capable of Dimesna Transport

Transport studies were performed in HeLa cells using a transient heterologous expression system as previously described (Cvetkovic et al., 1999; Kim et al., 1999). Human cervical carcinoma cell lines (HeLa cells) were seeded on 12-well plates and infected with recombinant vaccinia virus (vTF7-3, ATCC # VR-2153) in low serum Opti-MEM (Invitrogen, Carlsbad, CA) prior to transfection of either cDNA (packaged in either pEF6/V5-His-TOPO or pcDNA3.1/V5-His-TOPO) or empty plasmid (1 µg total) with Lipofectin reagent (Invitrogen). cDNA in a 1:4 ratio of uptake to efflux was used in the experiments requiring transfection of both uptake and efflux transporters. Cells were incubated at 37°C for 16 hours prior to transport experiments. Uptake of [3H]dimesna (1 mCi/mmol, > 98% purity, Moravek Biochemicals, Brea, CA) was determined in the presence and absence of probenecid (Sigma-Aldrich, St. Louis, MO) diluted in low serum Opti-MEM (Invitrogen) for transporter screens and Krebs-Henseleit bicarbonate (KHB) buffer for kinetic assays following 10 minute incubation at 37°C in a humidified 5% CO2 atmosphere. A [3H]dimesna accumulation-time course determined 10 min to be within the initial linear uptake phase for each of the OATs tested. Cells were washed three times with ice cold phosphate-buffered saline and lysed with 1% sodium dodecyl sulfate prior to liquid scintillation counting. During initial screening of uptake transports, taurocholate uptake into cells transfected with sodium-taurocholate cotransporting polypeptide (NTCP; SLC10A) cDNA was used as a positive control for transfection and expression efficiency, as previously described (Glaeser et al., 2007; Tirona et al., 2003). Dimesna
uptake by organic anion transporter 4 (OAT4; *SLC22A11*) was used as a positive control during kinetic experiments. Cold dimesna used to assess dimesna uptake kinetics was a kind gift from Dr. G. Koren (University of Toronto, Toronto, Canada).

### 2.2.2 *In vitro* Data Fitting and Statistical Analysis

Parameters representing the affinity of dimesna for OATs (K_M) and inhibition of transport by probenecid (K_i) were determined by nonlinear regression using Prism (GraphPad Software, Inc., San Diego, CA) assuming rapid equilibrium saturation kinetics and competitive inhibition. Calculated K_is of probenecid with OAT1 and OAT3 is within 2-3-fold of stably-transfected cell lines (Takeda *et al.*, 2001). Statistical analysis of dimesna uptake by screened uptake and efflux transporters was performed using one-way ANOVA followed by Tukey’s multiple comparison tests. *P*-values less than 0.05 were considered statistically significant. *In vitro* data fitting and statistical analysis was completed using Prism (GraphPad Software, Inc., San Diego, CA).

### 2.2.3 *In vitro* Determination of Efflux Transporters Capable of Mesna Export

HeLa cells transfected as outlined above were incubated with [³H]dimesna (1 µM in KHB buffer) at 37°C for 60 minutes. [³H]mesna present in the supernatant was derivatized with monobromobimane (EMD Biosciences, Gibbstown, NJ), and separated by liquid chromatography and fractions detected by liquid scintillation counting. Reduced [³H]mesna exported from transfected cells was derivatized by adding 100 µL of supernatant to 50 µL of 12.5 mM monobromobimane followed by 50 µL of 50 mM Tris buffer (pH = 9.0). The reaction mixture was then incubated at 37°C for 15 minutes and 50 µL of 4% acetonitrile: 96% 25 mM ammonium formate buffer (pH = 3.75) was added.
to stabilize labeled thiols. Derivatized samples were injected onto a Zorbax SB-C18 column (150 x 3.2 mm, 5 µm particle; Agilent Technologies, Santa Clara, CA) and eluted isocratically with 10% acetonitrile: 90% 25 mM ammonium formate: 0.1 % formic acid buffer containing 0.75 mM dibutylamine (pH = 3.75) at a flow rate of 0.6 mL/min by a Waters 510 LC pump (Waters, Milford, MA). Eluent was collected as one minute fractions directly into scintillation vials. Authentic labeled [³H]mesna was produced by chemical reduction of [³H]dimesna with NaBH₄ in 0.05 M NaOH (Sigma-Aldrich) prior to derivatization and chromatographic and liquid scintillation analysis. HeLa cells transfected with empty vector (pEF) or uptake transporter only (OAT4) were included as negative controls.

2.2.4 Study Design and Volunteer Subjects

Twelve healthy subjects (nine male; three female, mean age 31 years; range 22-54, mean weight 78.5 kg; range 54-108, mean body surface area 1.95 m²; range 1.60-2.36) were enrolled in a three session study. The study protocol was approved by The University of Western Ontario Health Sciences Research Ethics Board and all subjects provided written informed consent. Dimesna is currently an investigational new drug undergoing clinical development in the United States and has not been approved for use in Canada. However, the oral administration of intravenous mesna solution results in rapid oxidation of mesna to dimesna during absorption into the systemic circulation (Ormstad et al., 1983). Thus, we chose to evaluate the pharmacokinetics of total mesna following administration of the OAT inhibitor probenecid.
Glomerular filtration rate was estimated by the 4 variable Modification of Diet in Renal Disease Study equation using serum creatinine values obtained prior to initiation of the study (Levey et al., 2007). Creatinine analysis was conducted in the core laboratory of University Hospital using the Jaffé reaction method (Spencer, 1986). All subjects had eGFRs ≥ 102 mL/min/1.73 m².

During the first of three study sessions (Session 1), subjects received 0.4 g/m² mesna (Uromitexan®; Baxter Corporation, Mississauga, Canada) in 300 mL water. Following at least one week of washout, subjects received a single 1 g oral dose of probenecid during Session 2 and maintained a dosing regimen of 1 g oral probenecid (Benuryl®; Valeant Ltd., Montreal, Canada) twice daily, for six days. During Session 3, subjects returned to the clinical research unit on the seventh day to receive 1 g oral probenecid one hour prior to receiving 0.4 g/m² mesna in 300 mL of water. Peripheral venous blood samples (5 mL) were drawn just prior (0 h) and 1, 2, 3, 4, 5, 6, 7, 8, and 24 hours after dosing and immediately centrifuged at 2,000 g to obtain plasma which was stored at -20°C until analysis. Total urine output was collected during each of the 24 hour study sessions, from which 4, 8, and 24 hour aliquots were stored at -20°C until analysis.

2.2.5 Total Mesna Analysis

Total mesna analysis was conducted with slight modification to methodology previously described (Urquhart et al., 2006). Disulfide bonds were completely reduced by addition of 53 mM NaBH₄ in 0.05 M NaOH and incubation at 37°C for 5 minutes. Residual reducing agent was quenched by 1.2 M HCl (Sigma-Aldrich) and thiols were derivatized with 12.5 mM monobromobimane (25% acetonitrile, 3 mM EDTA) at 37°C.
for 15 minutes. Protein was precipitated by addition of 15% perchloric acid (Sigma-Aldrich) and centrifugation. Supernatant pH was raised to 4.0 with 0.5 M citrate: 2.5 M NaOH solution (Sigma-Aldrich). Mesna derivatized with monobromobimane was injected onto a Zorbax SB-C18 column (150 x 3.2 mm, 5 µm particle) maintained at 40°C in a Hewlett Packard 1090 LC (Agilent Technologies). Analytes were eluted with a gradient of 4% acetonitrile: 96% ammonium formate: 0.1% formic acid containing 0.75 mM dibutylamine (pH 3.75) to 17% acetonitrile: 83% ammonium formate: 0.1% formic acid containing 0.75 mM dibutylamine (pH 3.75) over 20 minutes at a flow rate of 0.5 mL/min. All solvents and reagents were of HPLC-grade or higher. Derivatized mesna was detected at 12.5 minutes by a Waters 474 scanning fluorescence detector (λ_{excitation} = 390 nm, λ_{emission} = 480 nm; Waters). The accuracy and precision of the assay were 1.3% and 2.0%, respectively.

### 2.2.6 Probenecid Analysis

Probenecid analysis was conducted by solid phase extraction followed by liquid chromatography and ultraviolet detection. Briefly, 100 µL of plasma was mixed with internal standard (n-propyl p-hydroxybenzoate; Sigma-Aldrich) and loaded onto 1 mL C18 solid phase extraction tubes (100 mg end-capped ODS packing, Agilent Technologies) conditioned to manufacturer’s specifications. Packing was washed with 1 mL of Milli-Q water and analytes eluted with methanol containing 0.1% formic acid, 0.1% triethylamine. Eluent was dried at 40°C under medical air and reconstituted with mobile phase prior to being injected onto a Novopak C18 column (50 x 3.9 mm, 5 µm particle; Waters) maintained at 40°C in a Hewlett Packard 1090 LC. Analytes were eluted
with a gradient of 25% methanol: 15% acetonitrile: 60% 10 mM potassium phosphate buffer: 0.08% triethylamine (pH = 3.0) to of 35% methanol: 35% acetonitrile: 30% 10 mM potassium phosphate buffer: 0.08% triethylamine (pH = 3.0) over six minutes at a flow rate of 0.5 mL/min. Probenecid and internal standard were detected with a Hewlett Packard 1050 variable wavelength ultraviolet detector set to 244 nm (Agilent Technologies). The accuracy and precision of the assay were 3.5% and 9.0%, respectively.

2.2.7 Pharmacokinetic Analysis

Prior studies have shown probenecid elimination to be saturable at the dose used in this study (1 g twice-daily) (Cunningham et al., 1981; Selen et al., 1982). Simultaneous fitting of mean probenecid plasma concentrations from first (Session 2) to last (Session 3) doses determined that probenecid plasma concentration-time profiles were best described by a two-compartment model with nonlinear elimination from the central compartment. Data were transformed by relative weighting (weighted 1/y^2) and least squares fitting of the model was performed using Scientist (Micromath Research, Salt Lake City, UT).

Non-compartmental analysis of total mesna data was completed using WinNonlin (version 5.2, Pharsight, Mountain View, CA). C_{max} and t_{max} were obtained directly. Observed AUCs for plasma and urine data were calculated by the linear trapezoidal method. V_{ss} was calculated as Dose*AUMC_{0–\infty}/AUC_{0–\infty}, CL_{u,renal} was calculated as A_{e 0–24}/(AUC_{0–24}*f_u). Net fraction of renal clearance due to secretion (CL_{sec}) was calculated as (CL_{u,renal} - f_uGFR)/CL_{u,renal}.\)
2.2.8 Estimation of Renal Clearance in the Presence of Probenecid

The estimated decrease in renal clearance by inhibition of multiple elimination pathways was calculated as:

$$\text{CL}_{u,\text{renal},i} = \text{CL}_{u,\text{renal}} \times ((0.5/(1 + I_{u,\text{max}}/ K_{i,OAT1})) +$$

$$\quad (0.5/(1 + I_{u,\text{max}}/ K_{i,OAT3}))) \quad \text{(Eq. 2.1)}$$

where $I_{u,\text{max}}$ is the maximum unbound concentration of probenecid obtained from fitting mean plasma data. $K_{i,OAT1}$ and $K_{i,OAT3}$ are inhibition constants for OAT1, and OAT3, respectively, obtained from in vitro data (Ito et al., 2005). In the absence of absolute quantitative expression data, both transporters were assumed to contribute equally to total mesna clearance.
2.3 Results

2.3.1 OATs are Responsible for Kidney-Specific Uptake of Dimesna

Saturable, kidney-specific, uptake of dimesna has previously been observed in experiments using perfused organs and isolated epithelial cells (Ormstad & Uehara, 1982; Ormstad et al., 1983), yet the identity of the responsible transporter(s) remained to be elucidated. To identify potential transporters we screened 12 human uptake transporters using a heterologous transporter expression system in HeLa cells (Figure 2.1). Following a 10 minute incubation with 552 nM $[^3]$Hdimesna, only the predominantly renally-expressed OAT1 (SLC22A6), OAT3 (SLC22A8), and OAT4 (SLC22A11) significantly increased dimesna accumulation in transfected cells. Interestingly, transporters of the hepatic OATP family did not increase dimesna accumulation over vector control, supporting the previously established lack of hepatic uptake of dimesna and mesna.

Using a heterologous over-expression system in HeLa cells the uptake of dimesna by OAT1, OAT3, and OAT4 was determined to be saturable with $K_M$ of 635.9 ± 279.6, 390.3 ± 292.5, and 589.8 ± 232.9 µM, respectively (Figure 2.2). Uptake of dimesna by specific OATs was further characterized using probenecid, the prototypical inhibitor of the OAT family. Using the previously measured $K_M$, the $K_i$ of probenecid for OAT1, OAT3, and OAT4 was calculated directly to be 26.0 ± 8.9, 32.3 ± 14.5, and 55.9 ± 20.8 µM, respectively (Figure 2.3).
2.3.2 Differential Efflux of Dimesna and Mesna by ATP-Binding Cassette (ABC) Transporters and MATE1

Following identification of members of the OAT family as dimesna uptake transporters, we aimed to identify which efflux transporters are capable of exporting dimesna and mesna, thereby facilitating urinary secretion. We compared [³H]dimesna accumulation in HeLa cells co-transfected with OAT4 and empty vector (conferring uptake only) to cells co-transfected with OAT4 and efflux transporter (conferring both uptake and potential efflux). An efflux transporter capable of exporting either [³H]dimesna or its metabolite [³H]mesna would cause a significant decrease in cellular accumulation of radioactivity compared to cells co-transfected with OAT4 and empty vector. HeLa cells transfected with vector control and efflux transporter were included as negative controls. Each of the efflux transporters screened, BCRP (ABCG2), MATE1 (SLC47A1), MRP1 (ABCC1), MRP2 (ABCC2), MRP4 (ABCC4), MRP5 (ABCC5), and Pgp (ABCB1) significantly reduced [³H]dimesna accumulation compared to cells only transfected with the uptake transporter OAT4 (Figure 2.4). Although indicative of active efflux transport activity, a simple decrease in radioactivity could not differentiate between efflux of dimesna or mesna. Therefore, to determine whether efflux transporters were capable of exporting mesna (following intracellular reduction) we utilized the thiol-specific reagent monobromobimane to derivatize free thiol present in the buffer following incubation of transfected cells with [³H]dimesna. Derivatization of [³H]mesna facilitated the separation from parent [³H]dimesna by liquid chromatography prior to detection by liquid scintillation counting. Screening each OAT4-efflux transporter pair and
corresponding negative controls revealed that only HeLa cells co-transfected with OAT4 and either MATE1, MRP2, or Pgp are capable of exporting [³H]mesna following incubation with [³H]dimesna (Figure 2.5).

2.3.3 Secretion of Mesna by Proximal Tubule Cells

The kidney has been implicated as the site of dimesna reabsorption, reduction to mesna, and subsequent secretion to the lumen. Here we propose that the luminal proximal tubule transporter OAT4 facilitates the reabsorption of dimesna, and therefore its reduction to mesna, whereas MATE1, MRP2, and Pgp facilitate mesna and/or dimesna efflux back into the lumen. Similarly, dimesna may be excreted unchanged by MRP4. Given the known localization of OAT1 and OAT3 at the basolateral side of proximal tubule cells and multiple efflux transporters at the luminal membrane, potential transcellular pathways may exist to permit the active secretion of both dimesna and mesna into the urine, following uptake of dimesna from the blood, thereby facilitating the conjugation of toxic metabolites of cisplatin and ifosfamide within the proximal tubule cells and urine (Figure 2.6).

2.3.4 In vivo Effect of Probenecid on the Disposition of Mesna

The urinary clearance of total mesna (the sum of all mesna moieties) exceeds glomerular filtration rate (Urquhart et al., 2007; James et al., 1987), furthermore the transfer of free (reduced) mesna to both the kidney parenchyma and urine is essential for its protective effect. In light of the identification of renal OAT transporters as responsible for the uptake of dimesna by proximal tubule cells, we aimed to determine the contribution of drug transporters to total mesna disposition in vivo. Probenecid has
proven to be a useful *in vivo* tool for the elucidation of the roles of OAT1 and OAT3 in the clearance of renally excreted drugs (Kim *et al.*, 2005), although it also inhibits MATE1 and members of the MRP family, albeit at lower affinity (Deeley *et al.*, 2006; Meyer zu Schwabedissen *et al.*, 2010).

To determine the *in vivo* contribution of transporter-mediated processes of the kidney to the disposition of total mesna, 12 healthy subjects with normal renal function (mean GFR = 7.3 L/h/1.73 m$^2$; Table 2.1) and free of concomitant drugs were enrolled in a three session clinical study. Baseline characteristics of healthy volunteers are listed in Table 2.1. Oral mesna (0.4 g/m$^2$) was given alone during Session 1. One week following Session 1, baseline probenecid profiles were established (Session 2) and subjects maintained on probenecid until receiving a second dose of mesna (Session 3), during which time both mesna and probenecid plasma concentrations were measured.

Simultaneous fitting of mean probenecid plasma concentrations from first (Session 2) to last (Session 3) doses to a two-compartment model with non-linear elimination estimated a peak plasma probenecid concentration of 356.9 µM, which correlated to a maximum unbound concentration of 28.6 µM (Figure 2.7). Modeling of the concentration-dependent elimination of probenecid estimated a $V_{\text{max},i}$ and $K_{M,i}$ of 63.1 mg/h and 445 mg/L, respectively.

As anticipated based on prior results (Urquhart *et al.*, 2007), time-averaged renal clearance of total mesna during Session 1 (mesna only) was significantly higher than GFR for each subject (7.29 ± 0.71 versus 10.6 ± 2.2 L/h/1.73 m$^2$; $P < 0.001$; Table 2.2 and Table 2.1). Probenecid significantly increased plasma total mesna $C_{\text{max}}$ 1.4-fold
(102.5 ± 30.2 versus 147.4 ± 45.6 µmol/L; \( P = 0.005 \); Table 2.2) and total mesna area under the concentration-time curve (AUC) 91 ± 34% (894 ± 200 versus 1689 ± 409 µmol·h/L; \( P < 0.001 \); Table 2.2) while decreasing average urinary excretion rate over 24 hours by 17 ± 23% (960.0 ± 187.2 versus 771.9 ± 184.2 mg/h; \( P = 0.021 \); Table 2.2; Figure 2.8). Following probenecid administration, total mesna renal clearance (\( \text{CL}_{u,\text{renal}} \)) decreased 55.1 ± 12.3% (10.6 ± 2.2 versus 4.7 ± 1.1 L/h/1.73 m²; \( P < 0.001 \); Table 2.2). In addition, the fraction of renal clearance due to secretion (\( \text{CL}_{\text{sec}} \)) decreased 67.0 ± 12.7% (0.80 ± 0.05 versus 0.26 ± 0.10; \( P < 0.001 \); Figure 2.9). Interestingly, the apparent steady-state volume of distribution (\( V_{\text{ss}}/F \)) decreased 45.2 ± 13.4% (59.0 ± 17.8 versus 31.3 ± 8.0 L; \( P < 0.001 \); Table 2.2), leaving the elimination half-life unaltered (7.3 ± 1.7 versus 7.7 ± 1.4; \( P = 0.53 \); Table 2.2). Estimation of renal clearance due to OAT1 and OAT3 in the presence of probenecid by inhibition of multiple elimination pathways (Equation 2.1), using the maximum unbound plasma probenecid concentration of 28.6 µM and \textit{in vitro} \( K_{\text{is}} \), predicted an unbound renal clearance of 5.33 L/h/1.73 m².
<table>
<thead>
<tr>
<th>Subject</th>
<th>Sex</th>
<th>Age</th>
<th>Weight (kg)</th>
<th>BSA (m²)</th>
<th>Dose (mg)</th>
<th>GFR (L/h/1.73 m²)</th>
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<td>1</td>
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<td>102.0</td>
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<td>19.1</td>
<td>0.26</td>
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<td>0.71</td>
</tr>
</tbody>
</table>

BSA, body surface area; GFR, glomerular filtration rate.
Table 2.2 Non-compartmental pharmacokinetic parameters of plasma total mesna concentration-time curves of clinical mesna-probenecid interaction study.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mesna</th>
<th>Mesna + Probenecid</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; (µmol/L)</td>
<td>102.5 ± 30.2</td>
<td>147.4 ± 45.6</td>
<td>0.005</td>
</tr>
<tr>
<td>t&lt;sub&gt;max&lt;/sub&gt; (h)</td>
<td>2.8 ± 1.6</td>
<td>3.4 ± 0.8</td>
<td>0.239</td>
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<tr>
<td>AUC&lt;sub&gt;0-24&lt;/sub&gt; (µmol·h/L)</td>
<td>806.8 ± 163.5</td>
<td>1,479.0 ± 337.6</td>
<td>&lt; 0.001</td>
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<tr>
<td>AUC&lt;sub&gt;0-∞&lt;/sub&gt; (µmol·h/L)</td>
<td>894.0 ± 199.7</td>
<td>1,688.7 ± 409.0</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>AUMC&lt;sub&gt;0-∞&lt;/sub&gt; (µmol·h²/L)</td>
<td>8,692.5 ± 3,352.4</td>
<td>18,856.9 ± 6,915.0</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>MRT&lt;sub&gt;0-∞&lt;/sub&gt; (h)</td>
<td>9.5 ± 1.8</td>
<td>11.0 ± 2.4</td>
<td>0.123</td>
</tr>
<tr>
<td>V&lt;sub&gt;ss&lt;/sub&gt;/F (L)</td>
<td>59.0 ± 17.8</td>
<td>31.3 ± 8.0</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Elimination half-life (h)</td>
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<td>7.7 ± 1.4</td>
<td>0.532</td>
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<tr>
<td>A&lt;sub&gt;e&lt;/sub&gt;&lt;sub&gt;0-24&lt;/sub&gt; (mg/1.73 m&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>379.8 ± 45.2</td>
<td>312.5 ± 62.2</td>
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</tr>
<tr>
<td>A&lt;sub&gt;e&lt;/sub&gt;&lt;sub&gt;0-24&lt;/sub&gt; (mg/h)</td>
<td>960.0 ± 187.2</td>
<td>771.9 ± 184.2</td>
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</tr>
<tr>
<td>CL&lt;sub&gt;u,renal&lt;/sub&gt; (L/h/1.73 m&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>10.6 ± 2.2</td>
<td>4.7 ± 1.1</td>
<td>&lt; 0.001</td>
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</tbody>
</table>

Data are shown as mean ± SD, P-values calculated by paired t-test. C<sub>max</sub>, peak concentration; t<sub>max</sub>, time to peak concentration; AUC, area under plasma concentration-time curve; AUMC<sub>0-∞</sub>, area under the first moment curve from 0 to infinity; MRT<sub>0-∞</sub>, mean residence time from 0 to infinity; V<sub>ss</sub>, volume of distribution at steady-state; F, bioavailability; A<sub>e</sub><sub>0-24</sub>, amount excreted in urine from 0 to 24 hours; CL<sub>u,renal</sub>, unbound renal clearance; GFR, glomerular filtration rate.
Figure 2.1 Dimesna transport activity of human uptake transporters following heterologous expression in HeLa cells. \([{}^3\text{H}]\text{Dimesna}\) (552 nM) was used for all experiments. Cellular accumulation was measured after incubation at 37°C for 10 minutes. The data are expressed as percent of vector control (mean ± SE, n = 3). Differences between transfectants determined by ANOVA, *, ** P < 0.05.
Figure 2.2  Kinetics of dimesna uptake by OAT1 (a), OAT3 (b), OAT4 (c) following heterologous transporter expression in HeLa cells. Transporter-mediated uptake was calculated as the difference between OAT-expressing cells and vector controls treated with dimesna of the same specific activity. Accumulated dimesna was assessed after incubation at 37°C for 10 min. Data represent mean ± SE (n = 3).
Figure 2.2

(a) $K_M = 635.9 \pm 279.6 \mu M$

(b) $K_M = 390.3 \pm 292.5 \mu M$

(c) $K_M = 589.8 \pm 232.9 \mu M$
Figure 2.3  Kinetics of dimesna uptake by OAT1 (a), OAT3 (b), OAT4 (c) following heterologous transporter expression in HeLa cells. Transporter-mediated uptake was calculated as the difference between OAT-expressing cells and vector controls treated with dimesna of the same specific activity. Inhibitory effect of probenecid is expressed as % vector control. Accumulated dimesna was assessed after incubation at 37°C for 10 min. Inhibition constant (K_i) was calculated assuming reversible one-site competitive binding, [3H]dimesna concentration of 467.5 nM, and the previously determined K_M. Data represent mean ± SE (n = 4).
Figure 2.3

(a) $K_i = 25.96 \pm 8.88 \mu M$

(b) $K_i = 32.27 \pm 14.53 \mu M$

(c) $K_i = 55.87 \pm 20.80 \mu M$
Figure 2.4 Dimesna transport activity of human efflux transporters following recombinant vaccinia-mediated expression in HeLa cells. $[^3]$H]Dimesna (552 nM) was used for all experiments. Cellular accumulation was measured after incubation at 37°C for 10 minutes. The data are expressed as percent of vector control (mean ± SE, n = 5). Differences between transfectants determined by ANOVA, *, ** P < 0.05).
Figure 2.5 Export of mesna by efflux transporters following incubation with dimesna.

HeLa cells transfected with either efflux transporter alone (open circles) or uptake transporter OAT4 and efflux transporter (open triangles) by recombinant vaccinia. Following incubation with $[^3\text{H}]$dimesna (1 µM) at 37°C for 60 minutes, $[^3\text{H}]$mesna present in the supernatant was derivatized with monobromobimane and separated by liquid chromatography and fractions detected by liquid scintillation counting. (a) $[^3\text{H}]$dimesna standards with and without chemical reduction to $[^3\text{H}]$mesna prior to derivatization ($[^3\text{H}]$mesna retention time ~12 min). (b) pEF (empty vector, open circles) and OAT4 (uptake transporter, open triangles), only. (c) - (i) efflux transporter and uptake-efflux transporter pairs. BCRP, breast cancer resistance protein; dpm, disintegrations per minute; MATE1, multidrug and toxin extrusion protein 1; MRP1, 2, 4, 5, multidrug resistance protein 1, 2, 4, 5; Pgp, P-glycoprotein.
Figure 2.5
**Figure 2.6** Localization of identified dimesna and mesna transporters in the proximal tubule cell. At the basolateral membrane dimesna may be taken up from the blood via OAT1 and OAT3. Following filtration at the glomerulus (a), dimesna may also be taken up into proximal tubule cells at the luminal (apical) membrane via OAT4 (b).

Intracellularly, dimesna undergoes reduction to active mesna capable of conjugating toxic metabolites of cisplatin and ifosfamide. Intracellular dimesna may be actively exported to the urine across the apical membrane via MRP4, or may be reduced and subsequently actively exported as mesna by Pgp, MRP2, or in exchange for protons via MATE1. ATP, adenosine triphosphate; MATE1, multidrug and toxin extrusion protein 1, MRP2, 4, multidrug resistance protein 2, 4; OAT1, 3, 4, organic anion transporter 1, 3, 4; Pgp, P-glycoprotein.
Figure 2.6

a

b
Figure 2.7  Mean plasma probenecid concentration Sessions 2-3. Healthy volunteers were given 0.4 g/m² oral mesna (Session 1), 1 g oral probenecid (Session 2) followed by 1 g probenecid twice-daily for 6 days, and finally 0.4 g/m² oral mesna (Session 3). Data fitted simultaneously to a two-compartment model with non-linear elimination (mean ± SD, n = 12).
Figure 2.8 Inhibition of total mesna clearance by probenecid. Healthy volunteers were given 0.4 g/m² oral mesna (Session 1), 1 g oral probenecid (Session 2) followed by 1 g probenecid twice-daily for 6 days, and finally 0.4 g/m² oral mesna (Session 3). Mean plasma total mesna (a, b) and total mesna excreted (c) during Session 1 (mesna only, ◊) and Session 3 (mesna + probenecid, □). Data represent mean ± SD, n = 12.
Figure 2.8

(a) Plasma Total Mesna Concentration (µM) over time (h).

(b) Plasma Total Mesna Concentration (µM) over time (h).

(c) Total Mesna Excreted in Urine (% Dose) over time (h).
Figure 2.9 Inhibition of total mesna clearance by probenecid. Healthy volunteers were given 0.4 g/m² oral mesna (Session 1), 1 g oral probenecid (Session 2) followed by 1 g probenecid twice-daily for 6 days, and finally 0.4 g/m² oral mesna (Session 3). Fraction of renal clearance due to secretion with and without probenecid (* $P < 0.001$). Data represent mean ± SD, n = 12.
2.4 Discussion

It has previously been hypothesized that the kidney specific disposition of the highly polar chemoprotectants mesna and dimesna is due to differential transport across the epithelia of various organs and tumors (Ormstad & Uehara, 1982; Ormstad et al., 1983; Verschraagen et al., 2004a). We screened a diverse panel of uptake and efflux transporters. We note that OAT4, in addition to OAT1 and OAT3 are capable of significantly increasing cellular dimesna accumulation. Our observation that members of the OAT family of uptake transporters are capable of dimesna transport is perhaps not surprising when the structural similarities between the sulfamoyl group of probenecid and sulfonyl groups of dimesna are considered. However, there remains a substantial knowledge gap between the structure-function of transporters and their substrate specificities (Hagenbuch, 2010).

In context of the known patterns of expression in the kidney, dimesna may be taken up by proximal tubule cells from either the apical (lumen) side via OAT4, or basolateral (blood) side via OAT1 and OAT3 (Ekaratanawong et al., 2004; Motohashi et al., 2002). The inability of the organic anion transporting polypeptides (OATPs) to uptake dimesna explains previous observations of dimesna’s poor hepatic permeability (Verschraagen et al., 2004a; Goren et al., 1998b; Ormstad et al., 1983). However, there exists conflicting data regarding the role of the liver in dimesna reduction (Goren et al., 1998b; Ormstad et al., 1983). In vitro, both liver and kidney lysates can reduce dimesna to its active form by glutathione dependent mechanisms (Verschraagen et al., 2004b). However, in vivo, dimesna undergoes selective reduction by the kidney and subsequent
elimination (Ormstad et al., 1983; Brock et al., 1981; Verschraagen et al., 2004a).

Previous experiments have shown significant accumulation of mesna following perfusion of isolated rat kidney with $^{[14]C}$-dimesna, while no accumulation of either dimesna or mesna was seen in perfused livers (Ormstad et al., 1983). Differences in organ exposure were also seen following injection of colorectal tumour-bearing rats with either mesna or dimesna. Liver, tumour, and red blood cells experienced little or no exposure, while the kidney AUC exceeded that of plasma (Verschraagen et al., 2004a). Clinically, the dearth of OAT expression by many cancers treated by cisplatin-dimesna and mesna-ifosfamide therapies may explain these discrepancies. It is important to note that although mesna is able to chelate reactive metabolites, dimesna and mesna are not tumor protective (Millar et al., 1983; Okabe et al., 2008; Verschraagen et al., 2003a).

Uptake of dimesna by OAT1, OAT3, and OAT4 was moderately inhibited by probenecid ($K_s = 26.0 - 55.9 \mu M$) and saturable with apparently low affinities ($K_M = 390.3 - 635.9 \mu M$) compared to reference substrates para-aminohippurate (PAH) and estrone-3-sulfate. However, both mesna and dimesna are typically given in gram quantities during chemotherapy, resulting in high circulating dimesna concentrations. For example, a single 0.8 g intravenous dose of mesna results in plasma and urine dimesna $C_{max}$ of approximately 183 $\mu M$ and 4,300 $\mu M$, respectively (James et al., 1987). Patients undergoing ifosfamide chemotherapy typically receive 0.6 – 1.5 g/m$^2$ of mesna intravenously over eight hours (Hensley et al., 2008). Due to the inherent stability of dimesna, it has been administered at doses up to 41 g/m$^2$, resulting in plasma dimesna concentrations of upwards of 4,584 $\mu M$ and urine concentrations of 84,900 $\mu M$. 
(Verschraagen et al., 2003a; Verschraagen et al., 2001). Since the $K_M$s measured here are near or below clinically relevant concentrations of dimesna, our data support a significant role of OATs in the clearance of dimesna and this pathway may represent the rate limiting step in reuptake and transcellular secretion (Hensley et al., 2008; James et al., 1987; Verschraagen et al., 2001; Verschraagen et al., 2003a).

The ability of various efflux transporters to lower dimesna accumulation was tested using HeLa cells double-transfected with both OAT4, to facilitate dimesna uptake, and the efflux transporter of interest. Consistent with the polyspecific nature of many efflux transporters, each of the transporters screened (BCRP, MATE1, MRP1, MRP2, MRP4, MRP5, and Pgp) significantly lowered residual intracellular radioactivity following [$^3$H]dimesna incubation, compared to transfection with OAT4 alone. To further characterize whether reduction in radioactivity was due to export of either [$^3$H]dimesna or additionally its metabolite, [$^3$H]mesna, we sought to label [$^3$H]mesna exported from transfected HeLa cell cultures prior to isolation by liquid chromatography and scintillation counting. Although each of the efflux transporters screened could lower accumulated radioactivity, only MATE1, MRP2, and Pgp could export mesna in its reduced form. It should be noted that our methodology cannot rule out the export of both dimesna and mesna by MATE1, MRP2, and Pgp. MATE1 has previously been described as an electroneutral H$^+$/organic cation exchanger (Otsuka et al., 2005). Our finding that this renal efflux transporter is capable of exporting the anionic drug mesna further expands its functional role within the kidney.
Despite the moderate protein binding of plasma total mesna (28%), its rate of renal excretion exceeds its filtration rate (Urquhart *et al.*, 2007; James *et al.*, 1987). In light of our identification of uptake and efflux transporters of dimesna, and given their known localization, the dimesna and mesna transporters outlined herein may represent both reabsorption-resecretion and transcellular secretion mechanisms of dimesna disposition (Figure 2.6). The apical uptake transporter OAT4 in concert with MATE1, MRP2, and Pgp may be responsible for the yet unidentified mechanism of dimesna reabsorption and subsequent secretion of mesna following intracellular reduction. Similarly, upon reabsorption of dimesna, it may be secreted back into the lumen via MRP4, or possibly MATE1, MRP2, and Pgp.

Similar to the coordination of renal organic cation transporter 2 (OCT2/SLC22A2) and MATE1 in the transport of metformin (Meyer zu Schwabedissen *et al.*, 2010), basolateral uptake transporters OAT1 and OAT3 in combination with apical efflux transporters MATE1, MRP2, MRP4, and Pgp represent a transcellular mechanism of mesna and/or dimesna excretion. Our results suggest that total urinary mesna not only originates from glomerular filtration, but also secretion facilitated by OAT1 and OAT3 of renal proximal tubule cells. Given that dimesna must gain access to the kidney parenchyma to exert its protective effect against cisplatin, and mesna must accumulate in the bladder to mitigate ifosfamide toxicity, inhibition of a combination of OAT1 and OAT3 has the potential to lower the tolerated dose or efficacy of such chemotherapeutic drugs.
The recognition that drug transporters can control the absorption, distribution, and excretion of drugs has led to a growing concern of the liability of genetic variability and transporter-mediated DDIs. Functional single nucleotide polymorphisms (SNPs) of many of the renal dimesna transporters have been previously identified. MATE1 SNPs c. 404T>C (p. 159T>M) and c. 1012G>A (p. 338V>A) result in loss of function of metformin and tetraethylammonium transport activity in vitro (Meyer zu Schwabedissen et al., 2010). SNPs of the 5’ untranslated region of MRP2, -24C>T and -1019A>G, result in decreased transcription and have been linked to clinical irinotecan- and methotrexate-induced toxicities (de Jong et al., 2007; Haenisch et al., 2007; Rau et al., 2006).

Recently, functional SNPs of the relatively highly conserved OAT family have also been identified (Erdman et al., 2006; Bleasby et al., 2005; Fujita et al., 2005; Shima et al., 2010). Although OAT1 SNPs do not appear to affect transport of prototypical substrate PAH (Fujita et al., 2005; Bleasby et al., 2005), SNP c. 728G>A (p. 50R>H) showed decrease affinity for nucleoside phosphonate analogs adefovir, cidofovir, and tenofovir (Bleasby et al., 2005). OAT3 SNP c. 913A>T (p. 305I>F) confers substrate specific decrease in transport activity (Erdman et al., 2006), and OAT4 SNPs c. 735C>T (p. 121R>C) and c. 837G>A (p. 155V>G) result in lower plasma member expression and thus reduced transport activity (Shima et al., 2010). Possession of these SNPs alone or in combination may contribute to incidence of nephro- and urotoxicities resulting in dose delay, however the functional significance of polymorphisms of renal transporters in dimesna and mesna transport remains to be determined.
With respect drug-induced loss of function, the International Transporter Consortium has provided decision trees to aid in determining when a compound may be at risk of a transporter-mediated DDI, and suggested the use of probenecid as a known \textit{in vivo} inhibitor of OAT1 and OAT3 for validation of \textit{in vitro} findings (Giacomini \textit{et al.}, 2010; Yasui-Furukori \textit{et al.}, 2005). Given our \textit{in vitro} evidence, we sought to determine the \textit{in vivo} contributions of renal drug transporters to the excretion of “total mesna” by coadministration of probenecid.

Upon entering the circulation mesna undergoes spontaneous oxidation to its dimer, dimesna (Brock \textit{et al.}, 1981; Ormstad \textit{et al.}, 1983). Similarly, mesna can also form a disulfide bond with Cys\textsuperscript{34}-albumin or low-molecular weight endogenous thiols: cysteine, homocysteine, or glutathione (GSH) (Urquhart \textit{et al.}, 2006). By convention, due to the technical difficulties of stabilizing and distinguishing low-molecular weight disulfides of mesna, no standardized methodology for quantification of its metabolites exist, which can make interpretation of results difficult. The mixed disulfides mesna-mesna (i.e. dimesna), mesna-cysteine, mesna-homocysteine, and mesna-GSH have been collectively termed “dimesna” in some studies (Ormstad & Uehara, 1982; Shaw & Weeks, 1987), while others refer to the mesna-cysteine, mesna-homocysteine, and mesna-GSH as “mesna”, quantifying dimesna (i.e. mesna-mesna) separately (Goren \textit{et al.}, 1998a; Verschraagen \textit{et al.}, 2003b). Due to the redox cycling between both forms of mesna and dimesna, either can lead to chemoprotection, thus the pharmacokinetic parameters of the sum of all mesna moieties termed “total mesna” were estimated to gain a more accurate estimation of drug disposition.
Non-compartmental pharmacokinetic analysis of the modulation of total mesna kinetics by probenecid revealed a significant decrease in CL$_{u,\text{renal}}$ (55.1%), without alteration of elimination half-life. To reconcile this, a corresponding decrease in V$_{ss}$ of 45.2% was also observed. This decrease in apparent volume may represent restriction of total mesna distribution to kidney parenchyma due to inhibition of renal uptake transporters, and suggests plasma dimesna concentrations do not rapidly equilibrate with the kidney, despite being highly perfused. Unlike the liver, renal transporter-mediated interactions rarely alter a drug’s volume of distribution (Grover & Benet, 2009).

Previously, clinical observations of decreases in volumes of distribution had been limited to only famotidine and procainamide following inhibition by probenecid and cimetidine, respectively (Inotsume et al., 1990; Somogyi et al., 1983). Oat3$^{-/-}$ mice have also demonstrated a decrease in steady-state volume of distribution of penicillin G and corresponding decrease in renal clearance (Vanwert et al., 2007). Thus, the distribution of total mesna represents a relatively unique case where inhibition of renal transporters leads to a significant lowering of the volume of distribution, presumably by restricting entry to the kidneys.

Estimation of the magnitude of mesna-probenecid interaction by calculation of CL$_{u,\text{renal,i}}$ from in vitro $K_i$ data and maximum unbound probenecid plasma concentrations assuming parallel pathways of elimination by OAT1 and OAT3 approximated the observed decrease in CL$_{u,\text{renal}}$ in presence of inhibitor (CL$_{u,\text{renal}} = 4.7 \pm 1.1$ versus CL$_{u,\text{renal,i}} = 5.33 \text{ L/h/1.73 m}^2$). However the concentration-time profile of drug at its site of action cannot be captured by this ratio, nor can the time-course of urine exposure be
generated by non-compartmental methods for the purpose of estimating the pharmacodynamic effect. Future modeling of changes in transporter-mediated disposition, whether due to drug interactions or genetic variation, should not only account for alterations in transporter-mediated clearance, but also volume of distribution in order to accurately predict resultant drug concentration-time profiles.

The observed changes in plasma exposure and average urinary excretion rate of 91% and 17%, respectively, imply the interaction between probenecid and total mesna to be a modest DDI. However, in the context of transporter mediated-DDI, the observed 55.1% decrease in time-averaged renal clearance of total mesna is in line with the observed decreases in the clearance of other OAT substrates acyclovir (32%), cidofovir (32%), and furosemide (66%) by probenecid interaction (Li et al., 2006). Despite current dosing regimes, the incidence of moderate (hematuria) to severe (renal failure) ifosfamide-induced urotoxicity remains 3.5 – 6.7% per chemotherapy cycle (Hensley et al., 2008). Considering the disposition of mesna is essential to chemotherapeutic efficacy, such a decline in secretion may raise the risk of hematuria, but could be easily compensated by an increased dose because of mesna’s wide margin of safety. Most importantly, coadministration of probenecid significantly decreased the fraction of total mesna cleared by renal transporter-mediated secretion (67.0%). Given that OAT1 and OAT3 function as uptake transporters enabling access to the kidney parenchyma which represents a significant proportion of total mesna’s volume of distribution, a decrease in tissue concentration of this magnitude would likely lower the clinical efficacy of dimesna to mitigate cisplatin induced nephrotoxicity. This observation also brings to light findings
that drugs reliant on secretion for their clinical efficacy, such as diuretics, may be particularly susceptible to transporter-mediated DDIs because of the inability of glomerular filtration to adequately compensate for the loss of urinary clearance (Eraly et al., 2006; Vallon et al., 2008).

The results presented here suggest the apical uptake transporter OAT4 in concert with apical efflux transporters MATE1, MRP2, and Pgp represent the previously hypothesized mechanism of renal reabsorption and re-secretion required for intracellular reduction of dimesna and excretion of mesna by the proximal tubule cells. In addition, basolateral uptake transporters OAT1 and OAT3 in concert with apical efflux transporters facilitate the transcellular flux of total mesna into the urine. The coordinated uptake of dimesna and efflux of mesna by renal drug transporters may play an important role in the activation of dimesna and subsequent inactivation of toxic metabolites of ifosfamide and cisplatin. Alteration of drug transporter activity due to SNPs or drug interactions may contribute to the incidence of dose delay due to toxicity in patients receiving such chemotherapies.
2.5 References


CHAPTER 3: ENZYMATIC AND NON-ENZYMATIC MECHANISMS OF
DIMESNA METABOLISM
3.1 Introduction

The metabolism of the chemoprotectant dimesna to its constituent mesna moieties is an essential step in the mitigation of ifosfamide-induced hemorrhagic cystitis and cisplatin-induced nephrotoxicity (Boven et al., 2002; Hensley et al., 2008; Kurowski & Wagner, 1997). Interestingly, mesna does not attenuate the anti-cancer efficacy of concurrently administered ifosfamide. This has been attributed to the rapid metal-catalyzed oxidation of mesna to its disulfide dimer, dimesna (Brock et al., 1981b; Brock et al., 1981a; Brock et al., 1982). Similarly, co-administration of dimesna has no effect on the pharmacokinetics of cisplatin (Boven et al., 2002).

In addition to homodimerization, mesna may form mixed disulfides with the low-molecular weight thiols cysteine, reduced glutathione (GSH), homocysteine, \( \gamma \)-glutamyl-cysteine, or cysteinyl-glycine, or become covalently protein bound to Cys\(^{34} \)-albumin. Recent work by our laboratory and others has demonstrated that the formation of mesna-Cys\(^{34} \)-albumin following mesna and dimesna dosing can be exploited to increase the fraction of homocysteine available for clearance (Lauterburg et al., 1994; Pendyala et al., 2000; Pendyala et al., 2003; Stofer-Vogel et al., 1993; Urquhart et al., 2006; Urquhart et al., 2007b; Urquhart et al., 2007a).

Homocysteine is a non protein-forming amino acid intermediate of the methionine cycle and precursor of GSH biosynthesis (Finkelstein, 1998; Selhub, 1999). Elevated plasma total homocysteine (defined as the sum of all sulphydryl, low molecular-weight disulfide, and protein bound homocysteine (Mudd et al., 2000)) is a graded, independent risk factor for the development of cardiovascular disease and vascular access thrombosis.
amongst patients requiring chronic hemodialysis (Bostom et al., 1997; Mallamaci et al., 2002; Moustapha et al., 1998; Robinson et al., 1996; Shemin et al., 1999).

The ability of mesna to increase the dialyzable fraction of homocysteine by thiol-disulfide exchange at Cys$^{34}$-albumin in uremic plasma has motivated further expansion of the therapeutic applications of mesna to the treatment of hyperhomocysteinemia in end-stage renal disease (ESRD) (Urquhart et al., 2006; Urquhart et al., 2007b).

The redox equilibrium and metabolism of sulfhydryl mesna, and its disulfide dimesna, are key determinants of their therapeutic applications. The identification of renal uptake and efflux transporters of dimesna (chapter two) has raised the possibility of active secretion of mesna into the urine following intracellular reduction. Cellular redox homeostasis is maintained by members of the oxidoreductase family of enzymes, notably the thioredoxin and glutaredoxin systems, and the abundant redox buffers GSH and cyst(e)ine (Cys; CySSyC) (Holmgren, 1989; Meister & Anderson, 1983). These systems have previously been implicated in the metabolism of dimesna, yet the kinetics and contributions of enzymatic and non-enzymatic mechanisms remain to be elucidated (Shanmugarajah et al., 2009; Verschraagen et al., 2004).

The objective of this study was to investigate potential enzymatic and non-enzymatic mechanisms of dimesna metabolism. To determine the mechanisms underlying dimesna redox equilibrium in the presence of the two most abundant biological thiols, Cys and GSH, we sought to measure the kinetics of their respective reactions using a model of two sequential bimolecular nucleophilic substitution ($S_N$2) reactions. An $S_N$2 reaction proceeds by a nucleophilic thiol (e.g. mesna or GSH) displacing disulfide bonded thiol moieties (e.g. mesna-mesna, mesna-GSH). Enzymatic activities of
recombinant enzymes of the thioredoxin and glutaredoxin systems, mouse kidney and liver homogenates, and HeLa cell lysate were also measured in the presence of dimesna and the known substrates oxidized glutathione (GSSG) and 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB). Finally, the contribution of both enzymatic and non-enzymatic activities present in HeLa cell lysates was characterized by a significant loss of mesna production that was evaluated following lysate protein denaturation. This approach permitted an accurate prediction of mesna production by denatured cell lysate due to non-enzymatic thiol-disulfide exchange alone.
3.2 Materials and Methods

3.2.1 Materials

5,5'-dithio-bis-2-nitrobenzoic acid (DTNB), bovine insulin, oxidized glutathione (GSSG), reduced glutathione (GSH), mesna, nicotinamide adenine dinucleotide phosphate (NADPH), recombinant human glutaredoxin (GLRX1; thioltransferase-1, GRX1, UniProtKB ID # P35754, EC 1.8.4.2), glutathione reductase (GSR; GR, UniProtKB ID # P00390, EC 1.8.1.7), thioredoxin (TXN1; TRX1, UniProtKB ID # P10599, EC 1.8.4.10), and purified rat thioredoxin reductase 1 (Txnrd1; Trxr1, UniProtKB ID # O89049, EC 1.8.1.9) were purchased from Sigma-Aldrich Canada Ltd. (Oakville, Ontario). Monobromobimane (Thiolyte, MBB) was obtained from EMD Biosciences (Gibbstown, NJ). Mesna was a kind gift from Dr. Gideon Koren (University of Toronto, Canada).

3.2.2 Enzymatic Activity of Purified Glutaredoxin and Thioredoxin Systems

Activity of the glutaredoxin system comprised of 0.01 U glutaredoxin (approximately 14.15 nM), 0.01 U glutathione reductase (approximately 80.59 nM), and 1 mM NADPH was measured spectrophotometrically at 340 nm by the initial rate of oxidation of NADPH as previously described (Mavis & Stellwagen, 1968). Endogenous substrate GSSG (1 mM) was included in each assay as a positive control.

Activity of the thioredoxin system comprised of 0.35 U thioredoxin (approximately 0.5 µM), 0.05 U thioredoxin reductase (approximately 11.75 nM), and 0.2 mM NADPH was measured spectrophotometrically at 340 nm by the initial rate of oxidation of NADPH as previously described (Arner et al., 1999). Insulin (0.016 mM) was included in each assay as a positive control.
Inhibition of the reduction of 1 mM DTNB by thioredoxin reductase in the presence of 1 mM dimesna and 1 mM NADPH was examined by monitoring the production of TNB at 412 nm as previously described (Arner et al., 1999).

3.2.3 Enzymatic Activity of Tissue Homogenates and Cell Lysate

Five Female DBA/lacJ mice were anesthetised by isofluorane inhalation. Liver and kidneys were removed and homogenized on ice with a motorized Tissue Tearor in PBS with 1 mM EDTA. Human cervical adenocarcinoma (HeLa) cells (ATCC # CCL-2) were cultured in DMEM (Lonza, Walkersville, Maryland) containing 10% FBS (Invitrogen, Carlsbad, CA), penicillin (50 U/mL) (Invitrogen), and streptomycin (50 µg/mL) (Invitrogen) at 37°C in a humidified 5% CO2 atmosphere. Three passages of cells were grown to 80% confluence, harvested by scraping, and lysed by repetitive freeze-thaw in PBS containing 1 mM EDTA. Total protein was measured by Pierce BCA protein kit (Pierce, Rockford, IL) and samples diluted in PBS containing 1 mM EDTA, aliquotted, and stored at -70°C.

Enzymatic activities of 1 mg/mL tissue homogenates and cell lysate supplemented with 1 mM NADPH were measured spectrophotometrically at 340 nm by the initial rate of oxidation of NADPH with and without 1 mM dimesna and 1 mM GSSG, alone and in combination.

3.2.4 Spectrophotometric Assay of Enzymatic Activity

Assays of enzymatic activity were carried out using a Thermo Multiskan Spectrum spectrophotometer (Thermo Electron, Waltham, MA). Enzymatic activities of experiments utilizing NADPH as a cofactor were monitored by a decrease in absorbance at 340 nm at one minute intervals for 60 min. Enzymatic activities of experiments
utilizing DTNB as a substrate were monitored by an increase in absorbance at 412 nm due to the production of TNB at 10 s intervals for 5 min. All reaction mixtures were dissolved in PBS (pH = 7.0) containing 1 mM ethylenediaminetetraacetic acid (EDTA) and pipetted in triplicate into a clear 96-well plate warmed to 37°C on a Zipvac 96 evaporator heating block (Glas-Col, Terre Haute, IN) to a final reaction volume of 200 µL. NADPH was added immediately prior to spectrophotometric measurements at 37°C. Negative controls containing all reagents except dimesna or endogenous substrates were included in each assay to control for background absorbance.

3.2.5 Reduction of Dimesna by Non-Enzymatic Thiol-Disulfide Exchange and Cell Lysates

To detect the presence of an enzymatic reduction of dimesna in cell lysates, untreated and denatured lysates were incubated with 1 mM dimesna and mesna production measured following fluorescence derivatization. Redox enzymes were inactivated and removed by heating lysate at 95°C for 5 min followed by centrifugation at 14,000 g for 10 min. Dimesna was added to a final concentration of 1 mM immediately prior to incubation of lysates in 1.7 mL microcentrifuge tubes at 37°C. Non-enzymatic thiol-dimesna exchange was examined using final concentrations of dimesna, cysteine, and GSH ranging from 10 to 3000 µM diluted in PBS containing 1 mM EDTA and incubated in 1.7 mL microcentrifuge tubes at 37°C. Aliquots of 50 µL were collected at 0, 5, 10, 15, 30, 60, and 90 min and transferred to a second microcentrifuge tube containing 25 µL of 12.5 mM MBB (25% acetonitrile, 3 mM EDTA). Derivatization was initiated by addition of 25 µL of 50 mM Tris buffer (pH = 9.0) and incubation at 37°C for 15 min. Cell lysates were additionally treated with 25 µL of 15% perchloric acid and
centrifuged at 9,000 g for 5 min to precipitate proteins. Supernatant pH was adjusted to approximately 4.0 by addition of 20 µL of 0.5 M citrate: 2.5 M sodium hydroxide solution. Samples were diluted with 4% acetonitrile: 96% 25 mM ammonium formate buffer (pH = 3.75) as necessary prior to analysis.

3.2.6 Thiol Analysis

Thiol analysis was conducted by high performance liquid chromatography with fluorescence detection (HPLC-FD) with slight modification to methods previously described. (Urquhart et al., 2006) Thiols derivatized with monobromobimane were injected onto a Zorbax SB-C18 column (150 x 3.2 mm, 5 µm particle) maintained at 40°C in a Hewlett Packard 1090 LC (Agilent Technologies, Santa Clara, CA). Analytes were eluted with a gradient of 4% acetonitrile: 96% ammonium formate containing 0.75 mM dibutylamine (pH = 3.75) to 17% acetonitrile: 83% ammonium formate containing 0.75 mM dibutylamine (pH = 3.75) over 20 min at a flow rate of 0.5 mL/min. Retention times of derivatized cysteine, GSH, and mesna were approximately 5.3, 8.3, and 12.5 min, respectively. Peaks were detected by a Waters 474 scanning fluorescence detector ($\lambda_{\text{excitation}} = 390$ nm, $\lambda_{\text{emission}} = 480$ nm; Waters, Milford, MA). The accuracy and precision of the assay were 1.3% and 2.0%, respectively.

3.2.7 Determination of Non-Enzymatic Thiol-Disulfide Exchange Micro-Rate Constants

Reduction of dimesna by cysteine and glutathione can be summarized by the reaction scheme outlined in Figure 3.1. Given the mass balance equation:

$$\text{MM}_0 = \text{M}/2 + \text{MM} + \text{RM}/2$$  \hspace{1cm} (Eq. 3.1)
Where $MM_0$ is the starting dimesna concentration, and $M$, $MM$, and $RM$ represent mesna, dimesna, and mixed mesna disulfide species, respectively. The reaction scheme can be quantified by a system of ordinary differential equations:

\[ R' = k_2*RM*M - k_1*R*MM - k_3*R*RM + k_4*RR*M \]  
\[ MM' = k_2*RM*M - k_1*R*MM \]  
\[ RR' = k_3*R*RM - k_4*RR*M \]  
\[ RM' = k_1*R*MM - k_1*RM*M - k_3*R*RM + k_4*RR*M \]  
\[ M' = 2*(k_1*R*MM - k_2*RM*M + k_3*R*RM - k_4*RR*M) \]

where $RR$ represents the oxidized species of the starting thiol. Second-order micro-rate constants $k_1$, $k_2$, $k_3$, and $k_4$ were estimated by simultaneous regression to both mesna and either Cys or GSH data presented in Figures 3.2 and 3.3. Least-squares fitting was performed using Scientist software (Micromath Research, Salt Lake City, UT).

3.2.8 Modeling of Non-Enzymatic Reduction of Dimesna in HeLa cell Incubations

Dimesna, mesna, mesna-Cys, Cys, CySSyC, mesna-GSH, GSH, and GSSG concentration-time courses of untreated and denatured cell lysates due to non-enzymatic reactions were predicted using basal Cys and GSH concentrations and supplemented dimesna concentration as initial values of differential Equations 3.2 – 3.6. Dimesna consumption and mesna production due to Cys and GSH exchange were summed. Mesna, Cys, and GSH concentration-time courses were regressed to observed values for calculation of coefficients of determination using Scientist software (Micromath Research).
3.2.9 Calculation of Dimesna/Mesna Equilibrium Constant

At equilibrium, the magnitude of flux between species, as determined by the product of the second-order rate constants and concentrations of species, is equal, thus the equilibrium constant for the dependent reactions outlined in Figure 3.5 can be derived as:

\[
\begin{align*}
    k_1 R^* M M &= k_2 R M^* M \\
    k_3 R^* R M &= k_4 R R^* M \\
    R M &= (k_4 R R^* M)/(k_3 R)
\end{align*}
\]

(Eq. 3.7)

Substituting Equation 3.8 into Equation 3.7 yields:

\[
\begin{align*}
    k_1 R^* M M &= (k_2 k_4 R R^* M^2)/(k_3 R) \\
    k_1 k_3 R^2 M M &= k_2 k_4 R R^* M^2 \\
    (k_1 k_3)/(k_2 k_4) &= (R R^* M^2)/(R^2 M M) \\
    K_{eq} &= (R R^* M^2)/(R^2 M M) \\
         &= (k_1 k_3)/(k_2 k_4)
\end{align*}
\]

(Eq. 9)

where \( K_{eq} \) represents the equilibrium constant.

3.2.10 Calculation of Dimesna/Mesna Half-Cell Potential

Calculation of \( K_{eq} \) for dimesna in the presence of the standard biological reducing agent GSH facilitates indirect calculation of the half-cell potential of the dimesna/mesna redox system \( (E^{\circ, MSSM/MSH}) \) by the Nernst equation:

\[
E^{\circ, MSSM/MSH} = E^{\circ, GSSG/GSH} + (R/T/nF)*\ln(K_{eq})
\]

(Eq. 3.10)

Where \( E^{\circ, GSSG/GSH} \) is the half-cell potential of glutathione at pH 7.0 (-0.262 V (Millis et al., 1993)), \( R \) is the gas constant (8.314 J K\(^{-1}\) mol\(^{-1}\)), \( T \) is the absolute temperature of the
reaction (310 K), n is the number of electrons transferred (2), and F is the Faraday constant (96485 J V⁻¹ mol⁻¹).

3.2.11 Data Analysis and Statistics

Observed pseudo-first-order rate constants \( k_{\text{obs}} = k [\text{dimesna}] \) were determined by fitting the first-order exponential association and decay functions to mesna and Cys/GSH concentration-time data, respectively. Significant differences between enzymatic activity of kidney and liver homogenates and cell lysates were determined using one-way ANOVA followed by Tukey’s multiple comparison tests. Pairwise analysis of enzymatic data was performed using Student’s \( t \) test. Pairwise analysis of fitted micro-rate constants was performed non-parametrically by Mann-Whitney \( U \) test, due to non-normal distribution. A \( P < 0.05 \) was considered a statistically significant difference. Curve fitting and statistics were conducted using Prism (GraphPad Software, Inc., San Diego, CA).
Figure 3.1  Production of mesna by bimolecular nucleophilic substitution (S\textsubscript{N}2) reactions whereby a nucleophilic thiol (e.g. Cys or GSH) first displaces a mesna moiety of dimesna yielding mesna and a mixed disulfide, followed by substitution of the mesna moiety of the mixed disulfide, producing a second molecule of mesna and a homogeneous disulfide. Rate constants $k_1$, $k_2$, $k_3$, $k_4$ and species $R$, $MM$, $RM$, $RR$, and $M$ denote parameters and variables (respectively) utilized to describe equilibrium kinetics in Equations 3.1 – 3.6.
3.3. Results

3.3.1 Non-Enzymatic Reduction of Dimesna

Fluorescence derivatization followed by HPLC-FD allowed for the simultaneous measurement of mesna, Cys, and GSH thiols following incubation in PBS containing 1 mM EDTA at 37°C, pH = 7.0. The addition of EDTA facilitated the stabilization of thiols prior to the start of the experiment, but had no effect on the rate of thiol exchange in the presence of disulfide (data not shown), consistent with previously published observations (Verschraagen et al., 2004).

3.3.1.1 Estimation of Reaction Order and Macro-Rate Constants

Concentration-time courses of mesna production and corresponding thiol consumption following incubation of 1000 µM dimesna with a range of Cys and GSH concentrations (10 – 3000 µM) (Figure 3.2) and 1000 µM Cys or GSH with a range of dimesna concentrations (10 – 3000 µM; Figure 3.3) obeyed pseudo-first-order kinetics. The observed molar ratio of mesna production was approximately 2:1 for each mole of Cys or GSH consumed across all concentrations (mesna/cysteine = 2.12 ± 0.27, mesna/GSH = 2.06 ± 0.14).

Plots of estimated velocities (0 – 10 min or 0 – 15 min) were linear with slopes approximating unity at or below equimolar concentrations of reactants, indicating the rate of mesna production is first-order for each of the reactants (Figure 3.4). The slope of the secondary plot of observed pseudo-first-order rate constants of mesna production yielded apparent second-order rate constants of 1.394 x 10^{-4} µM^{-1} min^{-1} and 0.275 x 10^{-4} µM^{-1} min^{-1} for reduction of dimesna by Cys and GSH, respectively (Figure 3.5).
3.3.1.2 Estimation of Micro-Rate Constants

Measurement of both reactant (i.e. Cys and GSH) and product (i.e. mesna) sulfhydryls allowed for estimation of the micro-rate constants of Equations 3.2-3.6 describing the proposed thiol-disulfide reaction scheme (Figure 3.1). Micro-rate constants simultaneously fitted to mesna and Cys or GSH concentration-time courses are summarized in Table 3.1. Coefficients of determination were consistently high for predicted time courses of the reduction of dimesna by Cys (mesna $r^2 = 0.995 \pm 0.002$, Cys $r^2 = 0.983 \pm 0.011$) and GSH (mesna $r^2 = 0.969 \pm 0.022$, Cys $r^2 = 0.965 \pm 0.023$). Rate constants $k_1$, $k_2$, and $k_3$ were significantly different between reactants Cys and GSH ($P < 0.05$, Table 3.1).

3.3.1.3 Calculation of Dimesna Redox Equilibrium Constants and Half-cell Potential of Disulfide Bond

Using rate constants listed in Table 3.1, the equilibrium constants of dimesna in the presence of Cys and GSH were calculated by Equation 3.9 to be 0.200 and 1.697, respectively. Calculation of the half-cell potential of the dimesna disulfide bond in the presence of GSH (Equation 3.10) resulted in a dimesna/mesna redox potential of -0.255 V.

3.3.2 Enzymatic Reduction of Dimesna

Enzymatic activities of purified enzymes and homogenates incubated at 37°C in PBS containing 1 mM EDTA resulting in the oxidation of NADPH were monitored spectrophotometrically in the presence and absence of dimesna and known disulfide-containing substrates.
3.3.2.1 Direct Reduction of Dimesna by Thioredoxin System

An equimolar concentration of dimesna was unable to inhibit reduction of DTNB to 2-nitro-5-thiobenzoic acid (TNB) by thioredoxin reductase (1.64 ± 0.10 versus 1.67 ± 0.07 mAU TNB s\(^{-1}\), \(P = 0.733\)). However, the thioredoxin system demonstrated a significant dimesna concentration-dependent increase in enzyme velocity, in addition to prototypical substrate bovine insulin (Figure 3.6). A proposed mechanism of dimesna reduction by the thioredoxin system is summarized in Figure 3.7.

3.3.2.2 Indirect Reduction of Dimesna by Glutaredoxin System

Upon addition of dimesna to the glutaredoxin system, no significant change in NADPH concentration was detected. However, co-incubation of dimesna with GSH facilitated a significant increase in enzymatic activity by the glutaredoxin system (3.48 ± 0.75 nmol NADPH min\(^{-1}\)) compared to either dimesna or GSH alone (-0.183 ± 0.570 and 0.297 ± 0.187 nmol NADPH min\(^{-1}\), respectively, \(P < 0.001\), Figure 3.8).

Incubation of glutathione reductase with its endogenous substrate, GSSG, resulted in rapid oxidation of NADPH that was not inhibitable by co-incubation with equimolar dimesna (17.97 ± 1.79 versus 18.49 ± 4.79 nmol NADPH min\(^{-1}\), \(P = 0.873\)) suggesting dimesna does not bind to the catalytic site of glutathione reductase. A proposed mechanism of dimesna reduction by the glutaredoxin system is summarized in Figure 3.9.

3.3.2.3 Enzymatic Reduction of Dimesna by Tissue and Cell Homogenates

Enzymatic activity of mouse kidney and liver homogenates and HeLa cell lysate for dimesna reduction are presented in Figure 3.10. No activity was observed with dimesna alone in any homogenate. The rate of GSSG reduction with and without dimesna was greater in kidney than liver and HeLa cell homogenates when normalized to protein
concentration ($P < 0.001$, Figure 3.10). Enzymatic reduction of GSSG in all homogenates was unaltered by the presence of dimesna (Figure 3.10). Co-incubation of dimesna with increasing concentrations of GSH showed a saturable, concentration-dependent increase in enzymatic activity that was equivalent between kidney and liver homogenates (kidney $k_{\text{obs}} = 0.782 \pm 0.04$ versus liver $k_{\text{obs}} = 8.527 \pm 0.465 \text{ mM}^{-1}$, $P = 0.231$; kidney plateau = $8.527 \pm 0.465$ versus liver plateau $6.896 \pm 0.647 \text{ nmol NADPH min}^{-1} \text{ mg protein}^{-1}$, $P = 0.074$, Figure 3.11).

### 3.3.3 Contribution of Enzymatic and Non-Enzymatic Mechanisms of Reduction of Dimesna in HeLa Cell Lysates

Fluorescence derivatization of mesna and reactant sulphydryls of Cys and GSH enabled direct quantitation of rates of production and depletion (respectively) by HPLC-FD.

#### 3.3.3.1 Reduction of Dimesna by HeLa Cell Lysate Before and After Denaturation

Incubation of HeLa cell lysate with 1 mM dimesna yielded saturable mesna production ($k_{\text{obs}} = 0.057 \pm 0.002 \text{ min}^{-1}$, plateau = $87.7 \pm 1.3 \text{ µM}$) and concurrent decrease of endogenous Cys and GSH at observed rates of $0.141 \pm 0.006 \text{ min}^{-1}$ and $0.033 \pm 0.001 \text{ min}^{-1}$, respectively (Figure 3.12). Following denaturation, the observed rate of mesna production significantly increased ($0.095 \pm 0.001 \text{ min}^{-1}$, $P < 0.001$, Figure 3.12) while the concentration of mesna produced significantly decreased ($29.7 \pm 1.2 \text{ µM}$, $P < 0.001$, Figure 3.12). Denaturation significantly decreased the basal GSH concentration ($1.70 \pm 0.04 \text{ µM}$ versus $13.0 \pm 0.08 \text{ µM}$, $P < 0.001$, Figure 3.12). No change in GSH concentration of denatured lysate was detected in the presence of dimesna ($P = 0.079$, Figure 3.12).
Figure 3.12). Low molecular-weight thiols cysteinylglycine, cysteinylglutamate, and homocysteine were not detected in HeLa cell lysates.

3.3.3.2 Modeling of Sulfhydryl and Disulfide Species in HeLa Cell Lysate

Application of Equations 3.1-3.6 describing a two-step thiol-disulfide exchange reaction (Figure 3.1) enabled predictive modeling of the absolute contribution of non-enzymatic reduction of dimesna by cell lysate. Using fitted micro-rate constants listed in Table 3.1, basal concentrations of endogenous thiols (i.e. Cys and GSH) concentration-time courses of mesna, Cys, and GSH following incubation of HeLa lysates were predicted (Figure 3.13). Cys and GSH concentrations during incubation of HeLa lysate were well predicted by r-squared values of 0.822 and 0.937, respectively. Resultant mesna production, due to non-enzymatic thiol-disulfide exchange alone, accounted for only 58% of total sulfhydryl mesna produced (50.98 versus 87.89 µM, r² = 0.383, Figure 3.13). Removal of cytosolic enzyme activity resulted in significantly lower total sulfhydryl mesna production (30.83 ± 1.74 versus 87.89 ± 2.07 µM, P < 0.001, Figure 3.13) that was largely predicted by Cys-mediated non-enzymatic reduction of dimesna with coefficients of determination of mesna and Cys concentration-time courses of 0.993 and 0.802, respectively.
Table 3.1  Estimated second-order micro-rate constants of $S_N2$ reaction scheme in Figure 3.5, $n = 12$, $P$-value calculated non-parametrically by Mann-Whitney $U$ test.

<table>
<thead>
<tr>
<th></th>
<th>Cys + dimesna $10^{-4}$ (µM min$^{-1}$)</th>
<th>GSH + dimesna $10^{-4}$ (µM min$^{-1}$)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SE</td>
<td>Mean</td>
</tr>
<tr>
<td>k1</td>
<td>0.681</td>
<td>0.033</td>
<td>0.408</td>
</tr>
<tr>
<td>k2</td>
<td>1.648</td>
<td>0.275</td>
<td>3.218</td>
</tr>
<tr>
<td>k3</td>
<td>1.160</td>
<td>0.130</td>
<td>16.509</td>
</tr>
<tr>
<td>k4</td>
<td>2.399</td>
<td>0.605</td>
<td>1.233</td>
</tr>
</tbody>
</table>
Figure 3.2  Non-enzymatic reduction of 1 mM dimesna by cysteine (a, b) and glutathione (c, d) mediated thiol exchange in PBS, 1 mM EDTA at 37°C, pH = 7.0. Free thiol was measured by MBB derivatization followed by liquid chromatography with fluorescence detection. Fitted curves (solid lines) correspond to one-phase exponential association and one-phase exponential degradation of mesna production and thiol (Cys or GSH) loss. Starting thiol concentrations are in micromolar. Data represented as mean ± SE, n = 3.
Figure 3.2

(a) 

(b) 

(c) 

(d) 

Legend:
- ○ 3000
- ◆ 1000
- ▼ 300
- ▲ 100
- □ 30
- ● 10
Figure 3.3  Non-enzymatic reduction of dimesna by 1 mM cysteine (a, b) and 1mM glutathione (c, d) mediated thiol exchange in PBS, 1 mM EDTA at 37°C, pH = 7.0. Free thiol was measured by MBB derivatization followed by liquid chromatography with fluorescence detection. Fitted curves (solid lines) correspond to one-phase exponential association and one-phase exponential degradation of mesna production and thiol (Cys or GSH) loss. Starting dimesna concentrations are in micromolar. Data represented as mean ± SE, n = 3.
Figure 3.3

(a)

(b)

(c)

(d)
Figure 3.4  Initial velocities of mesna production by dimesna with excess Cys (a), Cys with excess dimesna (b), dimesna with excess GSH (c), and GSH with excess dimesna (d). Slope of the lines indicate thiol exchange of dimesna is first order for each of the reactants. Data represented as mean ± SE, n = 3.
Figure 3.4

(a) $y = 0.915x - 2.701$

(b) $y = 0.958x - 2.260$

(c) $y = 0.813x - 1.332$

(d) $y = 0.836x - 2.148$
Figure 3.5 Observed first-order rate constants ($k_{obs}$) of mesna production in the presence of excess Cys (a) and GSH (b) versus starting dimesna concentrations. Slope of the line indicates second-order rate constant for the reduction of dimesna by cysteine (a) and GSH (b). Data represented as mean ± SE, n = 3.
Figure 3.6  Reduction of dimesna by purified recombinant thioredoxin (500 nM) and thioredoxin reductase (11.75 nM) in PBS, 1 mM EDTA at 37°C, pH = 7.0. Enzyme velocities were measured by analysis of NAD formed spectrophotometrically at 340 nm. Data represented as mean ± SE, n = 3. Differences between incubations determined by ANOVA *, **, *** $P < 0.01$. 


Figure 3.7  Scheme of dimesna (MSSM) reduction by the thioredoxin system. The N-terminal active site Cys residue of thioredoxin(SH)$_2$ reduces protein disulfides by forming a transient mixed disulfide followed by fast thiol-disulfide exchange oxidizing a pair of reactive Cys at the active site producing thioredoxin(S)$_2$. Alternatively, reduced thioredoxin(SH)$_2$ may directly reduce dimesna to form two mesna moieties and thioredoxin(S)$_2$. Reduction of thioredoxin(S)$_2$ is facilitated by the flavoprotein thioredoxin reductase via electron transfer from NADPH.
Figure 3.7

Thioredoxin system

MSSM → 2 MSH

\[ \text{thioredoxin} \quad \text{SH} \quad \text{SH} \quad \text{thioredoxin} \quad S \quad S \]

FAD → FADH\textsubscript{2}

NADPH + H\textsuperscript{+} → NADP\textsuperscript{+}
Figure 3.8  Reduction of dimesna by purified recombinant glutaredoxin (14.15 nM) and glutathione reductase (80.59 nM) in PBS, 1 mM EDTA at 37°C, pH = 7.0. Enzyme velocities were measured spectrophotometrically at 340 nm. Data represented as mean ± SE, n = 3. Differences between incubations determined by ANOVA, * P < 0.01.
Figure 3.9  Scheme of dimesna (MSSM) reduction by the glutaredoxin system. Similar to thioredoxin, the N-terminal active site Cys residue of glutaredoxin(SH)$_2$ reduces protein disulfides by forming a transient mixed disulfide followed by fast thiol-disulfide exchange oxidizing a pair of reactive cysteines at the active site producing oxidized glutaredoxin(S)$_2$. Additionally, glutaredoxin(SH)$_2$ specifically reduces S-glutathionylated proteins and mixed disulfides, yielding a glutaredoxin-GSH conjugate. Dimesna (MSSM), although not a substrate of glutaredoxin, can first undergo non-enzymatic thiol exchange with GSH to produce the mixed mesna-glutathione disulfide (MSSG). MSSG can subsequently be reduced by glutaredoxin producing mesna and a glutaredoxin-GSH conjugate. The glutaredoxin-GSH conjugate can be reduced to glutaredoxin(SH)$_2$ by an additional molecule of GSH, forming oxidized glutathione (GSSG). Glutathione reductase recycles GSSG to two molecules of GSH via electron transfer from NADPH.
Figure 3.9

Glutaredoxin system

GSH + MSSM \rightarrow MSSG + MSH

\[ \text{glutaredoxin} \quad \text{SH} \quad \text{SH} \]

\[ \text{glutaredoxin} \quad \text{SSG} \quad \text{SH} \]

\[ \text{glutaredoxin} \quad \text{S} \quad \text{S} \quad + \text{GSH} \]

GSH \quad \text{GSSG}

2GSH

\text{glutathione reductase}

\[ \text{NADPH} + \text{H}^+ \quad \text{NADP}^+ \]
Figure 3.10 Enzymatic activity of mouse kidney and liver homogenates, and HeLa cell lysates in the presence of 1 mM NADPH, 1 mM oxidized glutathione (GSSG) and/or 1 mM dimesna (MSSM). Data represented as mean ± SE, kidney and liver; n = 5, HeLa; n = 3. *, ** P < 0.01.
Figure 3.11  Enzymatic activity of mouse kidney (○) and liver (□) homogenates incubated with 1 mM NADPH, 1 mM dimesna, and increasing concentrations of reduced glutathione (GSH). Data represented as mean ± SE, n = 5.
Figure 3.12  Mesna (a), Cys (b), and GSH (c) concentrations following incubation of HeLa cell lysate (1 mg/mL in PBS, 1 mM EDTA) with 1 mM dimesna at 37°C before (○) or after (□) protein denaturation. Data represented as mesna ± SE, n = 3.
Figure 3.12

(a) Mesna (µM) vs. Time (min)

(b) Cysteine (µM) vs. Time (min)

(c) GSH (µM) vs. Time (min)
Figure 3.13  Predicted concentrations of thiol species due to non-enzymatic thiol exchange as described by Equations 3.1-3.6 using second-order rate constants listed in Table 3.1 and basal endogenous thiol concentrations of HeLa lysate before (a) or after (b) denaturation. Observed mesna (Δ), Cys (○), and GSH (□) concentrations presented in Figure 3.12 are plotted as reference.
Figure 3.13

(a) and (b) illustrate the change in sulfhydryl concentration (µM) over time (min) for different compounds. The graphs show the concentration of Mesna, Cysteine, GSH, Cys-Mesna, GS-Mesna, GSSG, and Cys-Cys over time.
3.4 Discussion

The reduction of the disulfide dimesna to its two mesna thiol moieties is essential for conjugation of reactive metabolites of cisplatin and ifosfamide during chemotherapy (Boven et al., 2002; Hensley et al., 2008; Kurowski & Wagner, 1997). Furthermore, in vitro experiments have shown that sulfhydryl mesna is capable of increasing the dialyzable fraction of homocysteine by thiol-disulfide exchange at albumin-Cys\textsuperscript{34} in uremic plasma (Urquhart et al., 2006). Thus, the redox equilibrium and metabolism of sulfhydryl mesna and its disulfide dimesna play an important role in their pharmacology.

In this chapter, we quantify the non-enzymatic reduction of dimesna by the two most abundant endogenous thiols, Cys and GSH, and determine their contribution to the overall capacity of the common HeLa cell line to metabolize dimesna. Enzymatic reduction of dimesna is also demonstrated by two purified members of the oxidoreductase family of redox enzymes. Measurements of enzymatic activity of mouse kidney and liver homogenates and modeling of non-enzymatic thiol-disulfide exchange reactions of cell lysates demonstrate that enzymatic and non-enzymatic mechanisms work in concert to reduce dimesna to mesna.

Circulating mesna exists primarily as low molecular-weight and albumin-Cys\textsuperscript{34}-bound disulfides due to rapid metal-catalyzed oxidation facilitated by the rich availability of oxygen as an electron acceptor within the blood (Brock et al., 1981a; Brock et al., 1981b; Ormstad et al., 1983). Intracellularly, the oxidoreductase enzymes thioredoxin and glutaredoxin and thiol-based redox buffers cyst(e)ine and glutathione are responsible for maintaining redox homeostasis, and have been implicated in potential pathways of dimesna metabolism (Shanmugarajah et al., 2009; Verschraagen et al., 2004).
Similar to endogenous intracellular low molecular-weight disulfides cystine, homocystine, and GSSG, we hypothesized that dimesna undergoes thiol-disulfide exchange with thiolate anion-forming sulfhydryl groups via an $S_N2$ reaction. This reaction is commonly presented as a two-step reaction. Each step describes the transfer of a single electron and subsequently the formation and consumption of the mixed disulfide intermediate (i.e. RSSM, Figure 3.1) (Jocelyn, 1972).

To examine the validity of this mechanism of thiol-dimesna exchange we measured the concentration-time courses of mesna production and consumption of Cys and GSH using 12 different combinations. Semi-logarithmic plots of combinations of dimesna and thiols indicated more than one linear phase and thus an overall reaction order greater than one. Logarithmic plots of first-order exponential functions describing initial reaction velocities and reactant concentrations yielded linear slopes with respect to each reactant indicated the velocity is directly proportional to the concentration of each reactant (i.e. first-order). As previously reported for other thiols (Gilbert, 1995), the rate of thiol-disulfide exchange is dependent on the concentrations of both reactants (i.e. the ‘thiol’ and ‘disulfide’) hence the overall rate of dimesna consumption was expected to proceed via a second-order reaction. This is confirmed by the positive linear slopes of the plots of the observed pseudo-first-order rate constants with respect to dimesna in the presence of excess thiol. As anticipated from the steeper concentration-time courses of Cys compared to those of GSH, the second-order rate constant of Cys was approximately 5-fold greater than that of GSH. This difference in reactivity is likely due to the lower acid dissociation constant of cysteine ($pK_a = 8.2-8.5$ (Benesch & Benesch R., 1955)) than
that of GSH (pK\textsubscript{a} = 8.7 (Srinivasan \textit{et al.}, 1997)) ensuring greater ionization of the Cys thiol to a thiolate anion under physiological conditions.

Rates of thiol-disulfide exchange between Cys and GSH with dimesna in phosphate buffer were recently evaluated (Shanmugarajah \textit{et al.}, 2009; Verschraagen \textit{et al.}, 2004). Verschraagen \textit{et al.} reported first-order rate constants for the net loss of 1000 µM dimesna when co-incubated with single concentrations of Cys or GSH (500 µM each). Consistent with our results, the authors reported the rate of loss of dimesna to be significantly higher when mixed with Cys than GSH (Verschraagen \textit{et al.}, 2004). Shanmugarajah \textit{et al.} later expanded on the kinetics of non-enzymatic dimesna reduction by determining the forward and reverse second-order rate constants describing the transfer of the first electron and formation of the mixed disulfide. The values of forward and reverse rate constants, although consistent with the single concentration of dimesna and thiol used (100 µM), are likely only apparent rate constants because of the omission of the transfer of the second electron and formation of the final oxidized thiol (i.e. CySSyC and GSSG) (Shanmugarajah \textit{et al.}, 2009). Whether or not only half of the reaction can accurately predict the quantity of mixed disulfide produced at equilibrium remains to be determined by fully characterizing the combined reactions of thiol-dimesna exchange.

In the absence of data to support a more complicated reaction mechanism accounting for transient hydrogen bond and dipole formation of elemental reactions, the stoichiometry of the overall production of mesna was determined empirically to be approximately double for each mole of Cys or GSH consumed. Intuitively, this ratio arises because dimesna is a homodimer of two mesna moieties. This allowed for
formulation of differential Equations 3.1 – 3.6 to quantify the species following the reaction scheme outlined in Figure 3.1.

To fully characterize the non-enzymatic mechanism of dimesna reduction by Cys and GSH, we sought to determine the four second-order micro-rate constants of the differential equations describing the two reversible redox reactions. It should be noted that these rate constants describe the flux of sulfhydryl species and not their reactive thiolate anion form. However, the fraction of thiol in the thiolate anion form is directly proportional to its pKₐ and thus conditional on the pH of the system, which remained constant at pH = 7.0.

A number of observations regarding the tendency of thiol species to exchange with one another can be made from fitted rate-constants. First, the rate of reaction between Cys and dimesna is likely the rate limiting step and may explain why Shanmugarajah et al. were able to obtain an acceptable coefficient of determination using only this first reaction (Shanmugarajah et al., 2009). The simple equilibrium constant of this first reaction (k1/k2) is approximately 3-fold greater for Cys than GSH, and within the 2-5-fold range of apparent first- and second-order rate constants reported here and previously (Shanmugarajah et al., 2009; Verschraagen et al., 2004). Also, the rate constants of the second reaction suggest different affinities of cysteine and GSH species for mesna. Whereas formation of the mesna-Cys mixed disulfide is favoured, and thus more stable than CySSyC, the reverse is true of mesna-GSH, with GSSG existing as a more stable product. This pattern may be related to the relative differences in half-cell potentials. The redox potential of GSH ($E^{\circ'}_{RSH/RSR} = -0.262$) is slightly lower than that
of cysteine \((E^\circ_{RSS/RSH} = -0.245)\) at \(pH = 7.0\), thus GSSG possesses a more stable disulfide bond than cystine (Jocelyn, 1972; Millis et al., 1993).

To provide insight into the thermodynamic stability of dimesna compared to CySSyC and GSSG, the \(K_{eq}\) of the proposed reaction mechanism was derived. Calculation of \(K_{eq}\) of dimesna when mixed with Cys or GSH revealed \(K_{eq}\)s of 0.200 and 1.697, respectively. A \(K_{eq}\) < 1 implies the reverse reaction is more favoured, suggesting dimesna is a more stable species than CySSyC. A \(K_{eq}\) > 1 of dimesna when mixed with GSH implies a tendency of the forward reaction to be favoured, because it yields the more stable disulfide product GSSG (Millis et al., 1993). These observations are consistent with the existence of mesna moieties predominately as dimesna in patient plasma, despite circulating total Cys concentrations of approximately 300 µM. In contrast, only approximately 3 µM of GSH is present in plasma to facilitate reduction of dimesna by a more favourable reaction (Masuda et al., 2010; Pendyala et al., 2000; Verschraagen et al., 2003).

Direct measurement of oxidation potentials of thiols by potentiometric methods is complicated due to formation of metal-thiol complexes at electrode surfaces, hence the half-cell potential of thiols are inferred using their \(K_{eq}\)s when mixed with a common redox buffer of known half-cell potential, such as GSH; representing the oxidation of mesna by GSSG (Jocelyn, 1972; Szajewski & Whitesides, 1980). For comparison to previously established half-cell potentials of other endogenous and common therapeutic thiols, the half-cell potential of the disulfide bond of dimesna was determined indirectly using the equilibrium constant of dimesna in the presence of GSH and its established half-cell potential. The half-cell potential of mesna (-0.255 V) is similar to the redox
potentials of therapeutic and endogenous thiols captopril (-0.287 V), cysteamine (-0.260 V), Cys (-0.245 V), GSH (-0.262 V), homocysteine (-0.256 V), and penicillamine (-0.243 V) and much like the redox potentials of these thiols, favours oxidation \( (E^{o'}_{\text{RSSR/RSH}} < 0) \) (Jocelyn, 1972; Millis et al., 1993). Thus, our findings provide a mechanistic explanation for the greater reduction of CySSyC and homocystine by mesna than for GSSG \textit{in vitro} (Pendyala et al., 2000), and clinical observations of dramatic Cys and homocysteine depletion with only a modest decline in GSH during mesna therapy (Pendyala et al., 2000; Lauterburg et al., 1994; Stofer-Vogel et al., 1993; Smith et al., 2003).

Remarkably, the ability of mesna to deplete circulating homocysteine levels by thiol-disulfide exchange has recently lead to its use in patients with end-stage renal disease (ESRD). Elevated plasma total homocysteine is a graded, independent risk factor for the development of cardiovascular disease and vascular access thrombosis amongst patients requiring chronic hemodialysis (Bostom et al., 1997; Mallamaci et al., 2002; Moustapha et al., 1998; Robinson et al., 1996; Shemin et al., 1999). Earlier work by our laboratory demonstrated that mesna can act as an effective thiol exchange agent to increase the free fraction of plasma total homocysteine (Urquhart et al., 2006), and increased its dialytic clearance (Urquhart et al., 2007b). The calculated redox potential of dimesna/mesna further supports the development of mesna as a therapeutic thiol exchange agent for lowering total plasma homocysteine without depletion of the endogenous antioxidant GSH.

Thiol-disulfide exchange of dimesna may be catalyzed by the ubiquitous thioredoxin and glutaredoxin systems of the oxidoreductase family of redox proteins (Holmgren, 1989). Both thioredoxins and glutaredoxins catalyze the reversible reduction
of disulfide bonds by means of a dithiol/disulfide motif, Cys-X-X-Cys, known as the '
'thioredoxin fold' (Holmgren et al., 1975; Martin, 1995). The reactive N-terminal Cys
residue readily forms a thiolate anion which acts as a strong nucleophile attacking the
substrate disulfide to form a transient mixed disulfide. This mixed redoxin-substrate
disulfide is then reduced by the, now deprotonated, C-terminal Cys residue of the
thioredoxin fold producing oxidized redoxin and reduced substrate (Berndt et al., 2007;
Holmgren, 1995). Thioredoxins are then specifically reduced by the selenocysteine of the
active site of the flavoprotein thioredoxin reductase (Gladyshev et al., 1996; Zhong et al.,
2000). Oxidized glutaredoxins, on the other hand, are chemically reduced by GSH,
forming GSSG which is then reduced by the flavoprotein glutathione reductase (Meister
& Anderson, 1983). Both systems actively maintain thiol redox homeostasis through use
of NADPH as a final electron donor (Holmgren, 1989).

Human thioredoxin systems cytosolic/nuclear thioredoxin 1/thioredoxin reductase
1 and mitochondrial thioredoxin 2/thioredoxin reductase 2 are ubiquitously expressed and
essential for ribonucleotide synthesis (Engstrom et al., 1974), ROS signalling,(Rhee et
al., 2005) nuclear receptor signalling (Grippo et al., 1985; Matthews et al., 1992),
apoptosis (Ueno et al., 1999), and embryonic development (Nonn et al., 2003).

Enzymatic reduction of dimesna has been proposed to proceed via the thioredoxin
system consisting of the 12 kDa E. coli protein thioredoxin (trxA; Trx1, UniProtKB ID #
P0AA25) and 55 kDa bovine thioredoxin reductase (TXNRD1; TR1, UniProtKB ID #
O62768, EC 1.8.1.9.) however the significance of the findings relative to controls was
not reported (Verschraagen et al., 2004). In the present study, we demonstrate a
concentration-dependent increase in enzymatic activity of a thioredoxin system
consisting of recombinant human thioredoxin and purified rat thioredoxin reductase in
the presence of clinically relevant dimesna concentrations. Given that dimesna was
unable to lower the rate of reduction of DTNB by thioredoxin reductase, dimesna is most
likely reduced directly by thioredoxin (Figure 3.7).

The glutaredoxin system, in concert with GSH, has also been implicated as a
Isoforms of the human glutaredoxins include cytosolic glutaredoxin 1, and mitochondrial
 glutaredoxins 2 and 5 (Berndt *et al.*, 2007). The catalytic activities of these redoxins are
dependent on the availability of GSH and so are inexorably linked to the activity of
 glutathione reductase to maintain a high cellular GSH/GSSG ratio (Meister & Anderson,
1983). Together with cofactors GSH and NADPH, the glutaredoxin system facilitates
ribonucleotide synthesis (Holmgren, 1979), dehydroascorbate reduction (Wells *et al*.,
1990), redox signalling (Shelton *et al.*, 2005), nuclear receptor signalling
(Bandyopadhyay *et al.*, 1998; Hirota *et al.*, 2000), apoptosis (Chrestensen *et al.*, 2000;
Daily *et al.*, 2001), and cellular differentiation (Takashima *et al.*, 1999).

Incubation of dimesna with 12 kDa glutaredoxin from rat liver (Glrx; Grx1,
UniProtKB ID # Q9ESH6, EC 1.8.4.2; formerly thiol transferase) and 53 kDa yeast
 glutathione reductase (*GLR1*; GR, UniProtKB ID # P41921, EC 1.8.1.7, formerly EC
1.6.4.2) increased the sulfhydryl concentration of the mixture at a rate similar to dialyzed
cytosolic fractions of kidney homogenate (Ormstad *et al.*, 1983). Reduction of dimesna
by purified enzymes of the glutaredoxin system consisting of the 10 kDa *E. coli* protein
glutaredoxin (grxA; Grx1, UniProtKB ID # P68688, EC 1.8.4.2) and yeast glutathione
 reductase when supplemented with GSH has also been reported using spectrophotometric
measurement of NADPH oxidation (Verschraagen M, 2004). Consistent with the results of Verschraagen et al. using non-mammalian enzymes (Verschraagen et al., 2004), enzymatic activity of the human cytosolic glutaredoxin system was only observed following co-incubation of GSH with dimesna. This was corroborated by the inability of 1 mM dimesna to inhibit reduction of equimolar GSSG by glutathione reductase. Taken together, dimesna is not a clinically relevant substrate of either of the enzymes of the glutaredoxin system. The catalytic site of glutathione reductase is believed to be highly specific to the γ-glutamylcysteine residues of GSH (Meister & Anderson, 1983), thus, the formation of glutathionylated mesna moieties (i.e. mixed disulfides) by chemical thiol-disulfide exchange of GSH with dimesna facilitates enzymatic activity.

Similar to findings from the purified glutaredoxin system, supplementing kidney and liver homogenates with GSH dramatically increased the enzymatic activity. The equivalency between kidney and liver homogenates, despite the greater reductive capacity of the kidney, suggests that formation of the glutathione-mesna mixed disulfide represents the rate limiting step in GSH-mediated enzymatic reduction of dimesna. Our results suggest that GSH-dependent reduction of dimesna by kidney and liver reported here and elsewhere (Goren et al., 1998; Ormstad & Uehara, 1982; Ormstad et al., 1983) derives from a combination of non-enzymatic GSH-dimesna exchange and glutaredoxin-catalyzed reduction of mesna-GSH disulfides.

Perfused organ experiments have implicated the kidneys and liver as sites of dimesna metabolism (Goren et al., 1998; Ormstad & Uehara, 1982; Ormstad et al., 1983). In addition, work presented in chapter two demonstrated secretion of mesna by a HeLa cell heterologous expression system following dimesna incubation. To measure the
relative capacities of these tissue types to enzymatically reduce dimesna, the oxidation of NADPH by HeLa cell and mouse kidney and liver homogenates was evaluated. Kidney homogenate possessed the greatest capacity to enzymatically reduce GSSG, presumably due to the high expression of glutathione reductase by renal tissue. Similar to purified enzyme systems, the addition of dimesna to GSSG-supplemented homogenates had no effect on enzymatic activity. Interestingly, the addition of dimesna alone to homogenates had no significant effect on basal enzymatic activity. Using the more sensitive method of liquid chromatography-electrochemical detection to directly measure dimesna reduction, Verschraagen et al. were able to demonstrate an increase in dimesna reduction with the addition of NADPH to kidney homogenate, suggesting the existence of enzyme-facilitated reduction (Verschraagen et al., 2004). The apparent lack of enzymatic activity in our system may be due to the low sensitivity of spectrophotometric methodology. Although one mole dimesna or GSSG is reduced for every mole of NADPH consumed, the limit of quantitation of the spectrophotometric measurement of NADPH oxidation is 25 to 50 fold higher than detection of thiols by liquid chromatography-coupled electrochemical detection or fluorescence derivatization (Shanmugarajah et al., 2009; Urquhart et al., 2006).

As an alternative approach to test for the presence of enzyme-dependent reduction of dimesna in HeLa cells, the rates of mesna production and Cys or GSH consumption were simultaneously measured in HeLa cell lysates before and after inactivation of enzymes by denaturation and centrifugation. Incubation of dimesna with untreated cell lysate resulted in production of mesna and concurrent decline in basal Cys and GSH concentrations. Following denaturation of enzymes, mesna production was significantly
decreased. Although the concentration-time course of Cys remained unaffected, basal GSH was significantly lower, presumably due to the loss of glutathione reductase. Although dimesna reduction was clearly lowered by denaturation, whether the observed decrease in mesna production was due to a loss of redox enzymes or a consequence of a loss of non-enzymatic GSH-mediated thiol exchange alone, due to lowered basal GSH concentrations, was not immediately apparent.

To determine the contributions of enzymatic and non-enzymatic mechanisms of dimesna reduction in HeLa cells, the concentration-time courses of thiols species were predicted in silico by our model of thiol-dimesna exchange using parameters fitted in previous in vitro experiments. Application of the model using basal Cys and GSH concentrations resulted in good fits ($r^2 > 0.82$) to observed data for both untreated and denatured lysates. Interestingly, predicted mesna production due to non-enzymatic thiol exchange alone could only account for just over half of the mesna found in untreated lysate, suggesting the existence of an enzymatic mechanism of dimesna reduction. Upon removal of cellular enzymes by denaturation, the residual mesna production was almost entirely accounted for by non-enzymatic cysteine-dimesna exchange, further supporting the hypothesis that enzymatic reduction significantly contributes to the intracellular metabolism of dimesna. Deviation of predicted concentrations from observed values may be due to the contribution of additional intracellular low molecular weight thiols such as homocysteine, γ-glutamylcysteine, and cysteinylglycine.

In addition to prediction of product and reactant thiols, the use of micro-rate constants allows for calculation of concentrations of mixed disulfide reaction intermediates. The importance of the production of mesna mixed disulfides containing a
terminal γ-glutamate moiety in the mitigation of cisplatin-induced nephrotoxicity has recently been proposed (Hausheer et al., 2010a; Hausheer et al., 2010b). Toxication of cisplatin proceeds by S-glutathionylation and sequential cleavage of glutamate, glycine, and cysteine residues by γ-glutamyltranspeptidase (GGT), aminopeptidase N (APN), and finally cysteine-S-conjugate-β-lyase (CCBL) to form reactive thiolate-platinum species (Hanigan et al., 1994; Hanigan et al., 1996; Hanigan et al., 2001; Townsend et al., 2003). Mesna was previously believed to be the sole active metabolite of dimesna; able to directly conjugate to cisplatin, thereby preventing its metabolic activation. Hausheer et al. has since demonstrated that mesna mixed disulfides containing a terminal γ-glutamate moiety, such as mesna-glutathione and mesna-cysteinyl-glutamate, inhibit GGT and APN activity (Hausheer et al., 2010a; Hausheer et al., 2010b). Our finding that mesna-GSH mixed disulfide is formed by non-enzymatic GSH-dimesna exchange at concentrations approximately 30% of intracellular mesna, suggests that inhibition of the toxication pathway of cisplatin by dimesna represents an additional clinically relevant mechanism of chemoprotection.
3.5 References


Chrestensen,C.A., Starke,D.W. & Mieyal,J.J. (2000) Acute cadmium exposure inactivates thioltransferase (Glutaredoxin), inhibits intracellular reduction of...


CHAPTER 4: MESNA FOR THE TREATMENT OF
HYPERHOMOCYSTEINEMIA IN HEMODIALYSIS PATIENTS
4.1 Introduction

Homocysteine (Hcy) is a thiol containing amino acid ubiquitously formed by the demethylation of methionine. Homocysteine represents a key branch point in the methionine cycle and its concentration is tightly regulated intracellularly. Once formed, Hcy can be recycled back to methionine via the vitamin B_{12} and folic acid dependent or betaine dependent remethylation pathways, catabolized to cystathionine via the vitamin B_{6} dependent transsulfuration pathway, or exported from cells into the vascular space (Figure 4.1) (Finkelstein, 1998). Once in the bloodstream, Hcy rapidly undergoes thiol exchange with circulating low-molecular weight thiols, covalent protein binding with -Cys^{34}-albumin, and auto-oxidation. Thus, Hcy within the plasma is comprised of reduced (sulphydryl) Hcy (~1%) low-molecular weight disulfides (20 - 30%) and protein bound Hcy (70 - 80%) collectively termed total homocysteine (tHcy) (Hortin et al., 2006; Mansoor et al., 1992).

Homocysteine metabolism can be adversely affected by many genetic, disease, and dietary factors, resulting in a plasma tHcy concentration $\geq 15$ µmol /L (termed hyperhomocysteinemia; $\geq 2.03$ mg/L) (Brattstrom et al., 1998; Broxson, Jr. et al., 1989; Jacques et al., 1996; Mudd et al., 1969; Mudd et al., 1985). It is well established that decreased glomerular filtration rate, and therefore progression of chronic kidney disease (CKD), is inversely correlated with plasma tHcy (Anwar et al., 2001; Arnadottir et al., 1996). Culmination of CKD to end-stage renal disease (ESRD) results in hyperhomocysteinemia in the majority of patients undergoing maintenance hemodialysis (Bostom & Lathrop, 1997; House & Donnelly, 1999; House et al., 2000; Tamura et al., 1996).
Many studies have shown plasma tHcy to be a strong, independent, predictor of atherosclerosis and thrombosis in ESRD (Bostom & Culleton, 1999; Mallamaci et al., 2002; Moustapha et al., 1998; Robinson et al., 1996; Welch & Loscalzo, 1998). The relative risk of cardiovascular events, including mortality, was shown to increase by 1% per micromolar increase in plasma tHcy in a prospective study by Moustapha et al. (Moustapha et al., 1998). Another prospective trial by Mallamaci et al. reported patients in the third plasma tHcy tertile were 8.2 times more likely to suffer an atherothrombotic event than patients in the first tertile (Mallamaci et al., 2002).

In most patient populations plasma tHcy can be normalized by supplementation with combinations of folic acid, B12, and vitamin B6 (Ubbink et al., 1993; Ubbink et al., 1994). Unfortunately folic acid, betaine, serine, vitamin B6, and vitamin B12 supplements fail to normalize plasma tHcy in patients with ESRD (Bostom et al., 1995; Bostom et al., 1996a; Elian & Hoffer, 2002; House & Donnelly, 1999; Spence et al., 1999). Increasing Hcy removal by increasing dialysis membrane pore size have also proven ineffective (House et al., 2000; Vriese et al., 2003). The inability of high dose folic acid and B vitamins to normalize plasma tHcy or impact cardiovascular outcomes has been highlighted in the recently completed Homocysteinemia in Kidney and End-Stage Renal Disease (HOST) trial (Jamison et al., 2007). Taken together, these observations suggest investigation of alternate treatment modalities to lower tHcy in patient with ESRD is justified.

A promising strategy for lowering tHcy in ESRD is to increase Hcy excretion by thiol exchange. Thiol exchange utilizes a sulphydryl containing drug that is able to exchange with Cys\textsuperscript{34}-albumin bound Hcy thereby enhancing the dialyzable, sulphydryl
and low-molecular weight, fraction of plasma tHcy. Scholze et al. have used this strategy successfully with the mucolytic thiol agent N-acetylcysteine (NAC) (Scholze et al., 2004). Use of the chelating agent dimercaptosuccinic acid (DMSA) by our group proved ineffective (House et al., 2004). In vitro analysis of mesna, a drug indicated for the chelation of reactive oxazaphosphorine metabolites, would be efficacious at increasing dialytic clearance of Hcy (Urquhart et al., 2006). Lauterburg et al. previously demonstrated a remarkable decline of plasma cysteine and tHcy in patients receiving intravenous mesna as an adjunct to ifosfamide chemotherapy (Lauterburg et al., 1994).

Recently we demonstrated that a single, predialysis, 5 mg/kg intravenous bolus of mesna significantly increases the dialytic clearance of tHcy (Urquhart et al., 2007b). In addition, plasma tHcy remained significantly lower two days after mesna administration. Despite these initial positive findings, prolonged administration of 5 mg/kg mesna failed to normalize elevated plasma tHcy in a randomized, placebo-controlled trial (Urquhart et al., 2008). Therefore a larger dose of mesna would be required to correct plasma tHcy levels in ESRD. Since cancer patients undergoing ifosfamide chemotherapy routinely receive 10 – 12 mg/kg intravenous mesna thrice daily, we presumed 12 mg/kg intravenous mesna thrice weekly would be well tolerated in dialysis patients and have a greater impact in regard to lowering tHcy.

We hypothesized that 12 mg/kg intravenous mesna, at the beginning of each dialysis session, over the course of one week would lower plasma tHcy levels compared to placebo. Following positive results, a second study was conducted to evaluate the decline of tHcy during thrice weekly 12 mg/kg mesna, for four weeks. A cumulative tHcy
lowering effect may indicate mesna as an effective agent to normalize plasma tHcy in patients on dialysis.

The objective of this study was to determine if routine 12 mg/kg intravenous mesna will provide a cumulative decrease in plasma tHcy over a four week period in vitamin-replete hemodialysis patients.
Figure 4.1  Methionine pathway. Enzymes and vitamin cofactors indicated by open and shaded ellipses, respectively. Abbreviations used: 5,10-MTHF, 5,10-methylenetetrahydrofolate; 5-MTHF, 5-methyltetrahydrofolate; AdoHcy, S-adenosylhomocysteine; AdoMet, S-adenosylmethionine; BHMT, betaine-homocysteine methyltransferase; CBS, cystathionine β-synthase; Cys, cysteine; Cysta, cystathionine; DHF, dihydrofolate; DHFR, dihydrofolate reductase; DMG, dimethylglycine; GGCS, γ-glutamylcysteine synthase; γ-GluCys, γ-glutamylcysteine; GS, glutathione synthase; GSH, glutathione; Hcy, homocysteine; MAT, methionine adenosyl transferase; Met, methionine; MS, methionine synthase; MTHFR, methylenetetrahydrofolate reductase; SAHH, S-adenosylhomocysteine hydrolase; SHMT, serine hydroxymethyltransferase; THF, tetrahydrofolate; TS, thymidylate synthase.
Figure 4.1
4.2 Methods

4.2.1 Subjects

Eight patients on maintenance hemodialysis (7 males, 1 female) were recruited from the London Health Sciences Centre – University Campus and South Street Hemodialysis Units for the initial pilot study to evaluate mesna treatment for one week. Following analysis of the pilot study, a second trial of four week duration was conducted. Ten patients on maintenance hemodialysis (8 males, 2 females) were recruited from the London Health Sciences Centre – South Street Hemodialysis Unit. Inclusion criteria for both studies were: Replavite® daily multivitamin supplementation (containing 6 µg vitamin B_{12}, 10 mg vitamin B_{6}, and 1 mg folate; WN Pharmaceuticals, Mississauga, Canada), thrice weekly hemodialysis for at least 90 days with a high flux biocompatible membrane, and willingness to give written informed consent. Patients were excluded from the study if they had malnutrition (normalized protein catabolic rate < 1.0 g/kg/d), low serum albumin < 3.0 g/dL (< 30 g/L), were clinically unstable, or were women of child bearing potential who refused to practice adequate contraception. The Health Sciences Research Ethics Board at the University of Western Ontario approved the study protocol. The study protocol also received a No Objection Letter (NOL) from the Therapeutic Products Directorate of Health Canada, as required for clinical research with off-indication pharmaceuticals in Canada. Patients were dialyzed with Fresenius Optiflux hollow fiber dialyzers (Fresenius AG, Bad Homburg, Germany).

4.2.2 Design of Studies

Both studies were placebo-controlled, double-blind, randomized, cross-over trials. Subjects were randomized by a computerized randomization program to receive either
mesna or placebo (equal volume of saline) prior to crossing over to the alternate
treatment arm. Twelve-milligram per kilogram mesna was diluted four-fold in normal
saline and administered over five minutes at the beginning of dialysis by intravenous
injection into the venous return of the dialysis blood circuit. This was repeated three
times during each week of treatment, corresponding to the beginning of each patient’s
dialysis session.

During the initial pilot study mesna and placebo arms were one-week in length
and were interceded by a single week of washout during which time subjects received
only routine dialysis. Blood samples were drawn pre- and postdialysis at the beginning of
each treatment arm. Predialysis blood samples were drawn 44 hours after completing the
final cycle of each arm for comparison of plasma tHcy concentrations following one
week of mesna and placebo treatments.

Analysis of samples of the pilot study suggested a longer treatment would further
lower plasma tHcy. The second study was of similar design to the first, but extended the
treatment arms to four weeks. In addition, twelve-day washout periods followed each
treatment arm to measure the duration of detectable levels of plasma mesna within
subjects. Five millilitre blood samples were drawn predialysis once a week during the
twelve-week study period and postdialysis at the beginning and end of each treatment
arm. All blood samples were immediately placed on ice and centrifuged within 30
minutes to obtain plasma that was frozen until completion of the study.

4.2.3 Dialysate Collection

The sum of all spent dialysate was collected from three subjects during their first
dialytic sessions of placebo and mesna arms of the four-week study for calculation of the
total excretion of Hcy, Cys, and mesna. Dialysate was collected in 44 gallon (166.6 L) Rubbermaid® Brute® drums (Atlanta, GA) lined with disposable biohazard bags. Ten milliliter samples were drawn from the accumulated dialysate every 5 minutes until 30 minutes and at 1, 2, 3 hours and immediately after the end of dialysis (either 3.5 or 4 hours). Drums were continuously weighed using Longacre Racing Products, Inc. AccuSet™ Computerscales® (Monroe, WA). Net weight of dialysate (total weight minus drum weight) was recorded at each collection time to allow for calculation of volume and subsequent calculation of Hcy, Cys, and mesna excretion.

4.2.4 Sample Analysis & Statistical Methods

Plasma tHcy, tCys, and total mesna (sum of mesna sulfhydryl, low-molecular weight disulfides, and protein-bound mesna) were measured by modification of the chromatographic method described by Urquhart et al. (Urquhart et al., 2006). Citrate, sodium borohydride (NaBH₄), ethylenediaminetetraacetic acid (EDTA), hydrochloric acid (HCl), sodium hydroxide (NaOH), and perchloric acid were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO). Monobromobimane (MBB) was obtained from EMD Biosciences (Gibbstown, NJ). All solvents and reagents were of HPLC-grade or higher. Disulfide bonds were completely reduced by addition of 53 mM NaBH₄ in 0.05 M NaOH and incubation at 37°C for 5 minutes. Residual reducing agent was quenched by 1.2 M HCl and thiols were derivatized with 12.5 mM monobromobimane (25% acetonitrile, 3 mM EDTA) at 37°C for 15 minutes. Protein was precipitated by addition of 15% perchloric acid and centrifugation. Supernatant pH was raised to 4.0 with 0.5 M citrate: 2.5 M NaOH solution prior to injection onto a Zorbax SB-C18 column (150 x 3.2 mm, 5 µm particle) maintained at 40°C in a Hewlett Packard 1090 LC (Agilent
Technologies, Santa Clara, CA). Mesna was eluted at 12.5 minutes with a gradient of 4% acetonitrile: 96% ammonium formate: 0.1 % formic acid containing 0.75 mM dibutylamine (pH 3.75) to 17% acetonitrile: 83% ammonium formate: 0.1 % formic acid containing 0.75 mM dibutylamine (pH 3.75) over 20 minutes at a flow rate of 0.5 mL/min. tHcy and tCys were eluted at 7 and 15 minutes, respectively, with 4% acetonitrile: 96% ammonium formate: 0.1 % formic (pH 3.75) at a flow rate of 0.5 mL/min. Derivatized thiols were detected by a Waters 474 scanning fluorescence detector ($\lambda_{\text{excitation}} = 390$ nm, $\lambda_{\text{emission}} = 480$ nm; Waters, Milford, MA). The accuracy and precision of the assay were 1.3% and 2.0%, respectively. Results are mean ± SD. Inter-dialytic changes in plasma tHcy were tested for significance by repeated measures analysis of variance (ANOVA) followed by Tukey’s multiple comparison tests. Predialysis plasma tHcy concentrations at the end of each treatment arm of the one-week study were analyzed by paired $t$-test.

For determination of the sample size required for the four-week study, it was assumed that longer treatment would produce a difference in plasma tHcy equal to or larger than the previous pilot study. Sample size was calculated using the mean difference in plasma tHcy observed following one week of mesna (2.87 µmol/L), with a standard deviation of 1.97 µmol/L, and level of significance ($\alpha$) of 0.05. A sample size of 5 subjects would be sufficient to detect this difference with 90% power (Friedman LM et al., 1996). A total of 10 subjects were recruited to account for a dropout rate of 50%.

Amounts of tCys, tHcy, and total mesna excreted, predialysis plasma tHcy, and areas under the concentration-time curves (AUCs) of plasma tHcy throughout the four-week study during placebo and mesna arms were compared by paired $t$-test. Inter-dialytic
changes in plasma tHcy at the beginning and end of each treatment arm were tested for significance by repeated measures ANOVA followed by Tukey’s multiple comparison tests. Calculations of AUCs and statistical tests were completed using Prism (GraphPad Software, Inc., San Diego, CA). *P*-values less than 0.05 were considered statistically significant.
4.3 Results

4.3.1 One Week study

Seven of the eight subjects recruited for the one-week pilot study had hyperhomocysteinemia (plasma tHcy ≥ 15 µmol/L [≥ 2.0 mg/L]). Subjects’ baseline characteristics are listed in Table 4.1. Mesna significantly decreased the postdialysis plasma tHcy greater than placebo during the first dialysis cycle of each arm (% decline during dialysis, placebo = 45.2 ± 11.6%, mesna = 68.8 ± 13%, \( p < 0.001 \)). Following one week of 12 mg/kg IV mesna, predialysis plasma tHcy was significantly lowered by 12.8 ± 7.8% compared to placebo (placebo: 23.4 ± 8.0 µmol/L vs. mesna: 20.5 ± 7.6 µmol/L, \( P = 0.0044 \)).

4.3.2 Four Week study

The observation of a significant decline in plasma tHcy after only one week of mesna administration, motivated extension of the treatment period to four weeks. A second study was conducted with ten subjects with ESRD receiving thrice weekly 12 mg/kg mesna for four weeks. Over the course of the 12 week study period one subject received a kidney transplant, one subject was withdrawn due to vascular access complications, and one subject died from acute myocardial infarction due to co-morbidity. Each event was determined to be unrelated to mesna therapy. Five of the seven subjects to complete the second study had hyperhomocysteinemia. Subjects’ baseline characteristics are listed in Table 4.2.

Predialysis plasma total mesna concentrations were at steady-state between weeks one and four (Figure 4.2), and declined to 9.9 ± 4.9 µmol/L twelve days after the last dose. No significant decline in predialysis plasma tHcy was detected after four weeks.
(placebo: 18.3 ± 8.5 µmol/L versus mesna: 18.7 ± 6.3 µmol/L, \( P = 0.41 \)). Areas under the plasma tHcy concentration-time curves of four-week placebo and mesna treatments were also not significantly different (placebo: 547.6 ± 206.5 µmol/L · day vs. mesna: 515.2 ± 158.0 µmol/L · day, \( P = 0.2042 \), Figure 4.3).

The effect of dialysis on decreasing plasma tHcy was significantly greater after the first dose of mesna (day 0), compared to the inter-dialytic changes during placebo. However, the last dose of mesna (day 28) caused a drop in plasma tHcy that was not significantly different than placebo (% drop placebo, day 0 = 40.1 ± 10.1%; placebo, day 28 = 40.1 ± 10.1%; mesna, day 0 = 65.8 ± 8.3%; mesna, day 28 = 42.0 ± 7.7%, Figure 4.4).

Also measured during the study, was the total mesna, tHcy, and tCys excreted by three patients throughout their dialysis sessions, following their first doses of saline and mesna. Interestingly, mesna significantly increased the area under the dialysate tHcy amount-time curve (placebo: 24.0 ± 12.4 mg · h vs. mesna: 30.4 ± 14.3 mg · h, \( P = 0.0206 \), Figure 4.5). More importantly the total tHcy excreted during dialysis was significantly improved following mesna administration (placebo: 12.2 ± 6.4 mg versus mesna: 14.7 ± 6.6 mg, \( P = 0.0068 \)) while creatinine excretion was unaffected (placebo: 1357 ± 189 mg vs. mesna: 1571 ± 528 mg, \( P = 0.28 \)). The amounts of tCys excreted during dialysis after mesna and placebo was not different (placebo: 354.4 ± 133.8 mg versus mesna: 303.4 ± 99.6 mg, \( P = 0.0823 \)).
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Table 4.2  Baseline Patient Characteristics; four-week study

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Figure 4.2  Predialysis plasma total mesna concentrations of seven subjects administered 12 mg/kg of mesna thrice weekly for four weeks and twelve days after the last dose.

Results are expressed as mean ± SD, n = 7.
Figure 4.3  Predialysis plasma tHcy concentrations of seven subjects administered 12 mg/kg of mesna (Δ) and an equal volume of saline (□) thrice weekly for four weeks and twelve days after the last dose. Results are expressed as mean ± SD, n = 7.
**Figure 4.4** Pre- and postdialysis plasma tHcy concentrations following the first (day 0) and last (day 28) doses of placebo and mesna. Results are expressed as mean ± SD, n = 7, *, ** P < 0.05.
Figure 4.5  tHcy excreted by three subjects administered 12 mg/kg of mesna (Δ) and an equal volume of saline (□) during the first dialytic session of each treatment arm. Results are expressed as mean ± SD, n = 1-3.
4.4 Discussion

The incidence of kidney failure is growing as the population ages. The only cure for ESRD is kidney transplantation; however adult patients in Canada must wait an average of ten years before transplantation (Zaltzman, 2006). Meanwhile, patients are confined to dialysis treatment. The incidence of cardiovascular mortality amongst ESRD patients 55 to 64 years of age is 15-fold higher than the general population (Collins et al., 2001). Effective treatment of cardiovascular disease in ESRD is crucial to aid in the survival of patients until transplantation.

Plasma tHcy concentrations observed in ESRD commonly exceed 20 µmol/L and may explain the discrepancy between the incidence of cardiovascular disease amongst dialysis patients and the general population (Becker et al., 1997). Elevated plasma tHcy is a predictor of death and atherothrombotic events within dialysis patients free of malnutrition (Bostom et al., 1997; Mallamaci et al., 2002; Moustapha et al., 1998). Recent meta-analysis followed by subgroup analysis of randomized controlled trials (Righetti et al., 2006; Wrone et al., 2004; Zoungas et al., 2006) using folic acid to lower plasma tHcy within the dialysis patient population reported null results (Bazzano et al., 2006). Furthermore, the addition of vitamin B6 and B12 to folic acid supplementation also failed to affect cardiovascular event rates (Mann et al., 2008). The inability of vitamin supplementation to normalize plasma tHcy in ESRD has previously been demonstrated, thus the Hcy lowering effect achievable by vitamins may not yield significant atherothrombotic benefit, especially in the advanced atherosclerosis seen in ESRD (Lindner et al., 1974).
An alternative strategy for lowing tHcy in ESRD is thiol exchange. Currently potential thiol exchange agents NAC, DMSA, and mesna have been tested in vivo with mixed results (Bostom et al., 1996b; Friedman et al., 2003; House et al., 2004; Scholze et al., 2004; Thaha et al., 2006; Urquhart et al., 2007b; Ventura et al., 1999; Ventura et al., 2003). N-acetylcysteine given orally (1.2 g twice daily for four weeks) produced a 19% decrease in tHcy levels, although this decline failed to reach significance (Friedman et al., 2003). When a single 5 g dose was given intravenously, a decline in predialysis plasma tHcy concentration was detected two days after dosing (Scholze et al., 2004). Using a sustained-release formulation of NAC given twice daily, Nolin et al. has recently shown both 0.6 g and 1.2 g doses significantly lower plasma tHcy in ESRD 21.5% and 24.8%, respectively, following two weeks of treatment (Nolin et al., 2010). Although the author’s rationale for using NAC was to reduce oxidative stress by providing a precursor for glutathione synthesis, no change in circulating tCys or glutathione was detected in healthy controls or ESRD (Nolin et al., 2010), suggesting that γ-glutamyl cysteine synthase, the rate limiting step in glutathione synthesis (Meister, 1988), is adequately saturated by baseline cysteine concentrations. Furthermore, no change in oxidative stress-markers asymmetric dimethylarginine and protein carbonyl concentrations were detected (Nolin et al., 2010). The observed decline in plasma tHcy was likely due to continuous NAC-mediated thiol exchange provided by the sustained-release formulation.

Our group has recently tested both DMSA and mesna as potential thiol exchange agents in vivo. In a well-powered study, oral DMSA (2.5 mg/kg/d, 4 weeks) failed to lower plasma tHcy levels (House et al., 2004). Conversely, we have shown that mesna can effectively increase the efficacy of Hcy removal by dialysis. Subjects who received a
single 5 mg/kg IV dose of mesna showed a significant decline in plasma tHcy of 2.3 ± 1.8 µmol/L (0.3 ± 0.2 mg/L) two days after administration. (Urquhart et al., 2007b) However, when dosing was extended to thrice weekly predialysis for eight weeks, no significant lowering effect was observed (Urquhart et al., 2008).

To further decrease plasma tHcy concentrations between dialysis sessions, both dose and duration of mesna were increased in the initial pilot study presented here. Eight subjects received 12 mg/kg at the beginning of each dialysis session for one week. The result of this study was a decrease in predialysis tHcy of 2.9 ± 2.0 µmol/L, significantly lower compared to a week of placebo, but a meager change compared to the single 5 mg/kg dose which yielded a decline of 2.3 ± 1.8 µmol/L. Unfortunately, due to sparse sampling it is unknown whether this further decline is the result of increased dose, duration, or both.

To test the ability of 12 mg/kg mesna to further lower plasma tHcy a second study of adequate statistical power and longer duration was conducted. Ten subjects were recruited and given mesna and saline thrice weekly for four weeks according to a cross-over trial design. Predialysis blood samples were drawn weekly for measurement of plasma total mesna, tHcy, and tCys throughout treatment. Plasma total mesna concentrations appeared to be at steady-state between days 7 and 28. Despite giving a dose of mesna over two-fold higher than our previous study, which utilized 5 mg/kg, the mean steady-state concentration of plasma total mesna was no higher during the four-week study presented here (12 mg/kg: 110.8 ± 43.6 µmol/L [18.16 ± 7.15 mg/L]; 5 mg/kg at 4 weeks: 104.8 ± 51.8 µmol/L [17.18 ± 8.49 mg/L], at 8 weeks: 106.2 ± 38.4 µmol/L [17.41 ± 6.30 mg/L]). The apparent loss of linear pharmacokinetics typically
observed in cancer patients, may be the result of first-order dialytic removal of mesna prior to distribution to plasma albumin.

It has previously been shown in ESRD that thiol exchange lowers tHcy by increasing clearance (Urquhart *et al.*, 2007b). This mechanism was reinforced by data gathered from urine tHcy measurements following 10 mg/kg oral mesna administration in healthy subjects (Urquhart *et al.*, 2007a). Based on previous studies of mesna in healthy controls and ESRD patients, it’s reasonable to conclude that the dialytic and/or urinary excretion of tHcy is enhanced by mesna. Collection of the total volume of dialysate spent during the dialytic session has confirmed this unequivocally. The total tHcy excreted during dialysis was significantly improved following mesna administration (placebo: 12.2 ± 6.4 mg versus mesna: 14.7 ± 6.6 mg, \( P = 0.0068 \)). Dialysate was collected during the first day of each treatment arm, thus during initial sampling each subject was naïve to treatment.

The failure to normalize plasma tHcy, in the present study following four weeks of mesna, and our previous study (Urquhart *et al.*, 2008), may be explained by the apparent loss of mesna efficacy. Postdialysis plasma tHcy on day 28 was significantly higher than day 0 of mesna therapy, and no difference was observed between placebo and mesna on day 28. Unfortunately dialysate collection was not repeated at the end of each arm, thus the decline in mesna’s efficacy was not confirmed by comparison of Hcy excretion on day 0 to day 28.

This apparent loss of efficacy may be explained by a change in the protein binding equilibrium of plasma thiols in the presence of mesna at steady-state. Increasing the mesna concentration within the plasma releases Hcy sulfhydryl by occupying protein
previously bound to Hcy. With successive doses of mesna, fewer Hcy-Cys\textsuperscript{34}-albumin sites are available for thiol exchange. Free, sulphhydril Hcy may then be sequestered to plasma protein non-specifically, preventing its dialytic clearance.

Additionally, patients may develop resistance to thiol exchange therapy over the course of treatment by a compensatory increase in Hcy production. Previous work by our laboratory has shown decreased Hcy production rates of erythrocytes and peripheral blood mononuclear cells (PBMCs) isolated from ESRD patients with hyperhomocysteinemia due to a significantly lower ratio of substrate S-adenosyl-methionine to inhibitor S-adenosyl-homocysteine (Cutler \textit{et al.}, 2006; Urquhart \textit{et al.}, 2002). Acutely increasing plasma tHcy excretion may serve to increase this ratio, and thus increase Hcy production, returning patients to a state of chronic hyperhomocysteinemia.

A number of groups have reported the ability of pharmacological parenteral vitamin B\textsubscript{12} doses to significantly lower plasma tHcy concentrations in most dialysis patients (Elian & Hoffer, 2002; Hoffer \textit{et al.}, 2000; Kaplan \textit{et al.}, 2001; Obeid \textit{et al.}, 2005b; Sombolos \textit{et al.}, 2002). Thiol exchange could be an important adjunctive therapy to parenteral B\textsubscript{12} to lower plasma tHcy of those patients who remain at risk.

Elevated levels of tHcy in patients with renal disease were first observed by Wilcken \textit{et al.} in 1979 (Wilcken & Gupta, 1979). They were also the first to note that supplementation with the cofactors of the methionine cycle can lower plasma tHcy (Wilcken \textit{et al.}, 1981). Using oral doses of folic acid (5 mg/d) and pyridoxine (100 mg/d) combined with intramuscular vitamin B\textsubscript{12} (1 mg/d) they were able to reduced plasma tHcy concentrations by about one-third. This led to the investigation of single doses of
individual vitamins. Wilcken et al. observed the greatest Hcy-lowering effect from folic acid, with a minor effect from B\(_{12}\), and no change after pyridoxine treatment (Wilcken et al., 1981). Following the conclusion that folic acid deficiency was the greatest determinant of elevated plasma tHcy, many studies have been carried out with a multitude of doses of folic acid (Arnadottir et al., 1993; Arnadottir et al., 2000; Bostom et al., 2000; Dierkes et al., 1999; Manns et al., 2001; Sunder-Plassmann et al., 2000; Tremblay et al., 2000). From these studies it can be concluded that 1 mg/d folic acid is equipotent in Hcy-lowering ability to 2.5, 5, and 15 mg/d; each producing a reduction in plasma tHcy of about one-third within vitamin naïve patients.

The clinical trial completed by Wrone et al. highlights the ineffectiveness of increasing folate doses above 1 mg/d on both plasma tHcy concentrations and incidence of cardiovascular morbidity and mortality in patients with ESRD. Patients received standard vitamin supplementation (12.5 mg pyridoxine, 6 µg B\(_{12}\)) plus 1, 5, or 15 mg folic acid daily. After a median follow-up of 24 months no significant differences in survival or cardiovascular events were observed between groups. In addition, the proportion of patients reaching normal tHcy levels (tHcy < 15 µmol/L) did not differ between folic acid dosages (Wrone et al., 2004). Prior to mandatory folic acid fortification of grains, Bostom et al. compared the effect low-dose versus high-dose oral vitamin supplements in 27 dialysis patients. Patients were randomized to receive either 1 mg/d folic acid, 10 mg/d pyridoxine, and 12 µg/d B\(_{12}\) or 15 mg/d folic acid, 100 mg/d pyridoxine, and 1 mg/d B\(_{12}\) for eight weeks. Following treatment, mean plasma tHcy was 30% lower in patients receiving high-dose vitamins, yet the authors concluded that folic acid alone accounted for the observed decline (Bostom et al., 1996a). It is likely that folic
acid was responsible for the decline in tHcy observe in both groups compared to baseline concentrations. However, in light of later studies outlining the equipotency of 1 mg and 15 mg doses, folic acid alone could not account for the observed decline in tHcy between groups.

Nowadays, oral multivitamin supplementation and intake of fortified grains is commonplace amongst patients with ESRD. For this reason most dialysis patients are not folate deficient, and despite this the prevalence of hyperhomocysteinemia remains high. Vitamin B_{12} may be the next limiting factor in Hcy metabolism catabolism.

Although standard oral vitamins, containing 6 – 12 µg of vitamin B_{12}, are sufficient to maintain normal serum cobalamin levels within dialysis patients (reference range: 200 – 672 pmol/L) recent evidence suggests that these levels do not necessarily lead to the required intracellular levels. Mononuclear cells of dialysis patients have a diminished vitamin B_{12} uptake capacity compared to healthy controls (Obeid et al., 2005a). This is likely due to down-regulation of holotranscobalamin (holoTC) receptors as a result of serum holoTC accumulation (Herrmann & Obeid, 2005). Combined with the low bioavailability of vitamin B_{12} (approximately 10-fold lower than folic acid) it should follow that parenteral dosing is necessary to achieve the supra-physiological serum concentrations required to restore the re-methylation pathway of Hcy in ESRD.

A handful of trials have evaluated the effect of intravenous vitamin B_{12} on plasma tHcy of dialysis patients (Elian & Hoffer, 2002; Hoffer & Elian, 2004; Hoffer et al., 2005b; Kaplan et al., 2001; Koyama et al., 2002). Forms of vitamin B_{12} used include those found in serum (hydroxocobalamin and its precursor cyanocobalamin) and intracellularly (methylcobalamin). In a prospective, randomized, controlled trial Hoffer et
observed a 32% decrease in patients receiving standard oral vitamin therapy (1 mg/d folic acid, 6 mg/d pyridoxine, and 10 µg/d vitamin B₁₂) plus 1 mg/wk subcutaneous hydroxocobalamin compared to those patients receiving standard vitamins following 16 weeks of treatment (Elian & Hoffer, 2002). When dosing was repeated at two week intervals in a separate trial their group only observed an 11.5% decline in plasma tHcy (Hoffer et al., 2005b). Methylcobalamin given as 500 mg intravenous doses after each dialysis session for three weeks by Koyama et al. produced a 50% decline in plasma tHcy; normalizing all patients within the treatment arm (Koyama et al., 2002). Although the results of Koyama et al. and Hoffer et al. are positive, costs of methylcobalamin and hydroxocobalamin are high due to their instability. Comparison of 1 mg/wk intravenous doses of hydroxocobalamin and cyanocobalamin (an inexpensive preparation commonly used to treat pernicious anemia) by Hoffer et al. demonstrated that both preparations are equally effective at lowering plasma tHcy in ESRD. Plasma tHcy was lowered approximately one-third by both treatments, despite cyanocobalamin producing cobalamin levels 3- to 5-fold lower than hydroxocobalamin treatment. It should be noted that even during cyanocobalamin treatment serum cobalamin levels were 10- to 20-fold higher than baseline levels (4,900 – 13,200 pmol/L compared to 531-625 pmol/L) (Hoffer et al., 2005a).

Parenteral vitamin B₁₂ supplementation (1 mg/wk) in addition to standard vitamin therapy (1 mg/d folic acid, 6 mg/d pyridoxine, and 10 µg/d vitamin B₁₂) can lower plasma tHcy concentrations of most dialysis patients below 15 µmol/L. However additional Hcy lowering therapies such as thiol exchange may be necessary to further treat patients who remain hyperhomocysteinemic. The target plasma tHcy concentration
is accepted as less than 15 µmol/L (Kang et al., 1992), however epidemiological evidence suggests that even levels above 9 µmol/L warrant treatment (Hackam et al., 2000). It has been recently shown that the odds ratios for venous thrombosis fail to reach significance only when plasma tHcy drops below 12 µmol/L. Subsequently, the authors proposed that the cut-off for hyperhomocysteinemia be lowered to 12 µmol/L (Castanon et al., 2007). To date, no treatment has been able to achieve this level in the majority of dialysis patients studied.

With the recently completed large vitamin trials (Norwegian Vitamin Trial (Bonaa et al., 2006), Heart Outcomes Prevention Evaluation 2 (Lonn et al., 2006), and Vitamin Intervention for Stroke Prevention (Toole et al., 2004)) showing no effect on cardiovascular disease, it has been suggested that alternate methods of Hcy lowering should developed (Loscalzo, 2006). In addition to parenteral vitamins, the appropriate dosing regimen of mesna may be the necessary tool to lower plasma tHcy and test the Hcy theory of cardiovascular disease in ESRD.
4.5 References


GENERAL DISCUSSION
5.1 Summary and Future Areas of Research

5.1.1 In vitro and in vivo Assessment of the Role of Organic Anion Transporters in the Disposition of Mesna and Dimesna

The successful therapeutic use of mesna for the mitigation of ifosfamide-induced hemorrhagic cystitis and, more recently, dimesna as a chemoprotectant against cisplatin-induced nephrotoxicity is in large part due to kidney-specific reduction of their circulating inert disulfides (Brock et al., 1981a; Verschraagen et al., 2003). Upon entering the circulation mesna is rapidly oxidized to its inactive dimer, dimesna (Brock et al., 1981a; Brock et al., 1981b). Circulating dimesna must pass into the kidney parenchyma, be reduced to mesna, and subsequently be excreted into the urine to exert its protective effects.

Dimesna was originally hypothesized to be confined to the vascular space due to its highly polar sulfonate groups (Brock et al., 1981a). Experiments using isolated perfused rat organs later demonstrated mesna accumulation in the kidneys, but not liver, following dimesna perfusion (Goren et al., 1998; Ormstad & Uehara, 1982). More recent experiments have corroborated these findings and further demonstrated a lack of accumulation of mesna into colorectal tumours and red blood cells (Verschraagen et al., 2004a).

Thus far, only Ormstad et al. have examined the saturable uptake of $[^{14}\text{C}]$dimesna by renal tissue. Temperature- and ATP-dependent uptake of dimesna was observed using isolated rat renal epithelial cells. Reduction of glomerular filtration of perfused rat kidneys resulted in a dramatic decline in $[^{14}\text{C}]$dimesna accumulation, leading the authors to conclude that the appearance of mesna in the urine occurs by filtration of plasma.
dimesna, re-absorption of dimesna at the apical (luminal) brush border membrane, and re-secretion into the urine by an as yet unidentified mechanism (Ormstad & Uehara, 1982). This conclusion, however, cannot account for renal clearance exceeding glomerular filtration rate (GFR) (James et al., 1987; Urquhart et al., 2007a) and thus suggests a mechanism involving renal transport proteins. The identity of these transporters remained to be determined.

In chapter two we elucidated the renal drug transporters responsible for reabsorption of dimesna into the proximal tubule cells and subsequent efflux of active mesna into the urine. In addition, we identified novel transcellular pathways of transporter-mediated active secretion of mesna by the proximal tubule cells. Interestingly, an initial screen of known drugs uptake transporters revealed only renally-expressed organic anion transports (OAT) 1, 3, and 4 were capable of dimesna uptake, supporting previous observations (Ormstad & Uehara, 1982; Ormstad et al., 1983; Verschraagen et al., 2004a). Kinetic analysis of these transporters suggested they are clinically relevant given the high doses of mesna and dimesna administered during chemotherapy. Efflux transporters breast cancer resistance protein (BCRP), multidrug and toxin extrusion 1 (MATE1), multidrug resistance proteins MRP1, MRP2, MRP4, MRP5, and P-glycoprotein (Pgp) significantly reduced dimesna accumulation. Apical renal transporters MATE1, MRP2, and Pgp were also capable of mesna efflux. Within the proximal tubule, therefore, dimesna may undergo reabsorption from the lumen by apically expressed OAT4 and, following intracellular reduction, re-secretion as mesna by MATE1, MRP2, and Pgp. This pathway accounts for the previously hypothesized activation of dimesna to mesna by renal epithelia (Ormstad & Uehara, 1982). Additionally, basolateral uptake
transporters OAT1 and OAT3 in concert with apically expressed efflux transporters MATE1, MRP2, MRP4, and Pgp represent novel pathways of dimesna and mesna transcellular secretion and potential mechanisms by which renal clearance of total mesna exceeds GFR.

The clinical relevance of our findings was highlighted by a controlled drug interaction study with probenecid in which inhibition of renal drug transporters significantly reduced both renal clearance and volume of distribution of mesna moieties, leading to a significantly decreased rate of urine excretion and subsequent increase in plasma exposure. The identification of mesna and dimesna transporters provides mechanisms by which genetic variability or drug-drug interactions may decrease the efficacy of these chemoprotectants, increasing the risk of ifosfamide- and cisplatin-induced toxicities and consequent reduction in cancer therapy.

As outlined in the discussion of chapter two, a number of functional polymorphisms exist for many of the renal transporters indentified. The significance of these polymorphisms, particularly in combinations of uptake and efflux transporters, can be tested by site-directed mutagenesis of wild-type clones previously screened using our heterologous expression system.

Fruitful in vitro results may lead to clinical study of total mesna pharmacokinetics of subjects stratified by genotype. It should be noted, however, that the frequencies of functional polymorphisms of renal drug transporters of interest are often very low, even within specific ethnic ancestries (Bleasby et al., 2005; Erdman et al., 2006; Fujita et al., 2005; Shima et al., 2010). MRP2 SNPs -24C>T and -1019A>G are an exception; with
frequencies of 0.15 to 0.19 and 0.54, respectively, in Caucasians (de Jong et al., 2007; Rau et al., 2006).

Diuretics are commonly administered with intravenous hydration to mitigate urinary tract irritation during ifosfamide and cisplatin chemotherapies (Hensley et al., 2008; Hogle, 2007). However, like dimesna, many loop diuretics (e.g. furosemide, torsemide) are substrates of OATs and their renal clearance and subsequent pharmacodynamics are therefore mediated predominantly by transporter-facilitated secretion (Vallon et al., 2008; Vormfelde et al., 2006). Whether co-administration of loop diuretics decreases mesna secretion could be assessed by urine collection during chemotherapy in a non-invasive observational study. Ultimately, a balance between adequate cytotoxic metabolite conjugation by mesna and dilution by diuretics must be determined to optimize patient outcome.

Clinically, our results demonstrate OATs contribute significantly to the pharmacokinetics of total mesna. Given the highly conserved nature these genes, the contribution of OATs to the pharmacodynamics of mesna and dimesna can be tested using knock-out animal models. The clearance and pharmacodynamics of diuretics have similarly been tested using Oat1\(^{-/-}\) and Oat3\(^{-/-}\) mice (Vallon et al., 2008). Comparison of biochemical markers of nephrotoxicity (e.g. blood urea nitrogen, kidney injury molecule-1 mRNA, micro-hematuria) and histology of wild-type and knock-out mice receiving mesna with ifosfamide or dimesna with cisplatin may demonstrate the importance of Oats in chemoprotection.

One of the most interesting pharmacokinetic findings following probenecid dosing was the decrease in the volume of distribution of total mesna. This may be due to
prevention of distribution within the kidney, necessitating the existence of basolateral efflux transporter(s) to facilitate distribution between kidney parenchyma and blood volume. However, our initial screen of efflux transporters included only those expressed at the apical membrane of the proximal tubule. Basolaterally expressed efflux transporters MRP3 and MRP6 are ideal candidates to facilitate the reabsorption of mesna, providing a mechanism of dimesna reduction during passage through the kidney (van de Water et al., 2005). Cloning these transporters will enable future screening of their dimesna transport capacity.

5.1.2 Enzymatic and Non-Enzymatic Mechanisms of Dimesna Metabolism

The flux of mesna moieties between sulphydryl (mesna) and disulfides (e.g. dimesna, mesna-GSH) allows for nephro- and uroprotection without attenuation of anti-cancer efficacy (Brock et al., 1981a; Verschraagen et al., 2003). Dimesna had previously been shown to undergo GSH- and Cys-mediated reduction (Shanmugarajah et al., 2009; Verschraagen et al., 2004b). In chapter three we provide evidence of non-enzymatic reduction of dimesna via bimolecular nucleophilic thiol-disulfide exchange with thiols GSH and Cys. Similar to other endogenous and therapeutic thiols, the redox potential of mesna/dimesna favours oxidation to the more stable disulfide species dimesna (Jocelyn, 1972; Millis et al., 1993). Detailed kinetic analysis of reactions of Cys with dimesna and GSH with dimesna revealed initial rapid reduction of dimesna by Cys, but an equilibrium constant favouring dimesna over CySSyC. In contrast, the initial formation of mesna-GSH disulfide was slower than that of cysteine; however, oxidized glutathione was a more favoured product, increasing the relative concentration of mesna at equilibrium. Thus, despite micromolar concentrations of thiols available to reduce dimesna in the
plasma, the presence of cysteine at concentrations 100-fold greater than glutathione facilitate the existence of dimesna as the predominant species in plasma.

Similar to endogenous disulfides, dimesna may also undergo enzyme-catalyzed reduction by the thioredoxin and glutaredoxin systems. Our work clearly demonstrates a dose-dependent increase in enzymatic activity of the thioredoxin system in the presence of dimesna, confirming a prior report of the systems possible involvement (Verschraagen et al., 2004b). No activity of the glutaredoxin system was observed in the presence of dimesna alone. However, addition of reduced GSH significantly increased enzymatic activity. This has been hypothesized to occur via the formation of glutathione-mesna mixed disulfide and subsequent reduction by the \( \gamma \)-glutamylcysteine-specific active site of glutaredoxin (Meister & Anderson, 1983; Verschraagen et al., 2004b). This hypothesis may be directly tested by addition of custom synthesized glutathione-mesna disulfide to the glutaredoxin system. Additionally, and perhaps more simply, the presence of mixed disulfide species in reaction mixtures may be quantified by liquid chromatography-mass spectrometry (LC-MS). Use of LC-MS will also serve to confirm concentrations of therapeutically important mixed disulfide mesna metabolites predicted by modeling of thiol-disulfide exchange reactions.

In addition to the formation of low molecular-weight disulfides, mesna also readily exchanges with endogenous thiols bound to Cys\(^{34}\)-albumin. Previous work by our laboratory has shown that the thiol-disulfide exchange of mesna with Cys\(^{34}\)-albumin can be used to increase the free fraction of plasma total homocysteine (tHcy) (Urquhart et al., 2006). Homocysteine is a graded, independent risk factor the atherosclerosis and subsequent cardiovascular disease (Chao et al., 1999; Graham et al., 1997; Nygard et al.,
The standard treatment for hyperhomocysteinemia (plasma tHcy > 15 µmol/L) is oral supplementation of the vitamin cofactors of homocysteine metabolism; pyridoxine, folic acid, and vitamin B12. This treatment can successfully lower tHcy concentrations in the vast majority of patients with normal renal function (Ubbink et al., 1993; Ubbink et al., 1994). Amongst those with diminished renal function however, oral vitamin supplementation has had limited success (Bostom et al., 1995; Bostom et al., 1996; Elian & Hoffer, 2002; House & Donnelly, 1999; Spence et al., 1999).

The use of thiol exchange agents to increase the free fraction of tHcy and thereby increase its dialytic clearance represents an alternative approach to the treatment of hyperhomocysteinemia in end-stage renal disease (ESRD). The results outlined in chapter three provide a mechanistic explanation of mesna’s greater reduction of CySSyC and homocystine than oxidized glutathione (GSSG) in vitro (Pendyala et al., 2000), and clinical observations of dramatic Cys and Hcy depletion with only a modest decline in GSH during mesna therapy (Pendyala et al., 2000; Lauterburg et al., 1994; Stofer-Vogel et al., 1993; Smith et al., 2003). The calculated redox potential of dimesna/mesna further supports the development of mesna as a therapeutic thiol exchange agent for lowering tHcy without depletion of the endogenous antioxidant GSH.

5.1.3 Mesna for the Treatment of Hyperhomocysteinemia in Hemodialysis Patients

Our laboratory has previously demonstrated that a single, predialysis, 5 mg/kg intravenous bolus of mesna can significantly increase the dialytic clearance of tHcy (Urquhart et al., 2007b). In addition, plasma tHcy remained significantly lower two days after mesna administration. Despite these initial positive findings, prolonged
administration of 5 mg/kg mesna failed to normalize elevated plasma tHcy in a randomized, placebo-controlled trial (Urquhart et al., 2008). Similar to the progress of these initial studies, in chapter four we demonstrate that a dose of 12 mg/kg had a significant tHcy-lowering effect at one week, but no effect following four weeks of treatment.

This apparent loss of efficacy may be explained by a change in equilibrium of plasma thiols in the presence of mesna at steady-state dosing. With successive doses of mesna, fewer Hcy-Cys\textsuperscript{34}-albumin sites are available for thiol exchange. Free, sulfhydryl Hcy may then be sequestered to plasma protein non-specifically, preventing its dialytic clearance. This alteration in protein binding at equilibrium can be evaluated in future studies using our established \textit{in vitro} thiol exchange assay by ‘preloading’ of uremic plasma with mesna (Urquhart et al., 2006).

Results of our \textit{in vivo} drug interaction study in subject with normal renal functions suggest mesna moieties are in flux between the total body water and kidney parenchyma. Coupled with its reductive capacity, this suggests the kidney may contribute to the maintenance of the sulfhydryl mesna fraction of total mesna within the plasma. Thus, the presence of functional renal transporters enables more efficient, continuous, thiol exchange. Studies evaluating the activity of organic anion transporters of uremic rats suggests renal transporter function may be impaired in chronic renal failure (Aoyama \textit{et al.}, 2003; Deguchi \textit{et al.}, 2004; Ji \textit{et al.}, 2002). Whether the function of these transporters is impaired in chronic renal failure or essential to the equilibrium of mesna and dimesna within the plasma remains to be determined.
The efficacy of thiol exchange to lower tHcy by increasing its clearance may also be attenuated by a concurrent increase in production. Plasma tHcy concentrations are tightly controlled by negative feedback via the reversible enzyme S-adenosyl-homocysteine hydrolase (SAHH) (Perna et al., 1995; Perna et al., 2001). Its substrate, S-adenosyl-homocysteine is a potent, competitive inhibitor of S-adenosylmethionine mediated methylation, resulting in a decrease in Hcy production and cellular methylation status. Prior work by our laboratory has shown decreased export of Hcy by erythrocytes and peripheral-blood mononuclear cells (PBMCs) isolated from patients with ESRD (Cutler et al., 2006; Urquhart et al., 2002). Alleviation of hyperhomocysteinemia by increased clearance may result in improved methylation status and compensation of Hcy production, until the rate of Hcy removal is at equilibrium with transmethylation. Thus, it is possible that the increased Hcy clearance provided by doses of mesna used thus far have yet to correct the methylation status of cells, and subsequently normalize plasma tHcy. Future, short-term dose-escalation studies of mesna may benefit from monitoring PBMC methylation status as an indicator of long-term efficacy.

The etiology of hyperhomocysteinemia in ESRD remains to be determined. Many studies point to a decline in metabolic clearance as the cause. Although plasma tHcy is inversely correlated to glomerular filtration rate, less than 1% of Hcy is excreted and no loss of plasma tHcy is seen between arterial and venous blood flow through the kidney (van Guldener et al., 1998). Homocysteine loading in dialysis patients produced an area under the concentration-time curve (AUC) that was four-fold greater than healthy controls (Guttormsen et al., 1997). This suggests extra-renal inhibition of Hcy metabolism as the cause of hyperhomocysteinemia in ESRD. An eloquent study by Stam
et al. using a stable isotope of methionine demonstrated that re-methylation and transsulfuration pathways of Hcy catabolism are 28% and 26% lower than healthy controls, respectively (Stam et al., 2004).

Mesna has been shown to effectively lower plasma tHcy in dialysis patients by a mechanism independent of B₁₂ status and may provide an important adjunct to vitamin therapy for the normalization of plasma tHcy (Urquhart et al., 2007b). A number of groups have reported the ability of pharmacological parenteral vitamin B₁₂ doses to significantly lower plasma tHcy concentrations in most dialysis patients (Elian & Hoffer, 2002; Hoffer et al., 2000; Hoffer & Elian, 2004; Kaplan et al., 2001; Obeid et al., 2005; Sombolos et al., 2002). Administration of parenteral doses of B₁₂ and oral vitamins to provide the necessary cofactors to restore Hcy re-methylation, combined with mesna during hemodialysis to increase Hcy excretion, may effectively normalize elevated tHcy in ESRD.

5.2 Conclusions

The unique pharmacological properties of mesna and dimesna have led to their continued development for new therapeutic uses over the past 45 years. The remarkable chemoprotection provided by mesna and its prodrug and primary metabolite, dimesna, without detriment to the efficacy of chemotherapy can be attributed to the redox equilibrium and differential transport of these drugs. Recent work by our laboratory has further expanded the application of mesna for the lowering of a known risk factor of atherosclerosis and thrombosis in ESRD with the hope of eventually improving patient outcome.
5.3 References


APPENDICES
Appendix 1

Office of Research Ethics
The University of Western Ontario
Room 4160 Support Services Building, London, ON, Canada N6A 5C1
Telephone: (519) 661-3035 Fax: (519) 661-2048 Email: ethics@uwo.ca
Website: www.uwo.ca/researchethics

Use of Human Subjects - Ethics Approval Notice

Principal Investigator: Dr. D.J. Freeman
Review Number: 15078
Review Date: March 4, 2009
Revision Number: 1
Review Level: Expedited
Protocol Title: Effect of probenecid on the pharmacokinetics of mesna in healthy subjects
Department and Institution: Physiology & Pharmacology, London Health Sciences Centre
Sponsor:
Ethics Approval Date: March 4, 2009
Documents Reviewed and Approved: Revised Study End Date
Documents Received for Information:
Expiry Date: March 31, 2010

This is to notify you that The University of Western Ontario Research Ethics Board (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans and the Health Canada/ICH Good Clinical Practice: Consolidated Guidelines; and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced revision(s) or amendment(s) on the approval date noted above. The membership of this REB also complies with the membership requirements for REB's as defined in Division 5 of the Food and Drug Regulations.

The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB’s periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the UWO Updated Approval Request Form.

During the course of the research, no deviations from, or changes to, the protocol or consent form may be initiated without prior written approval from the HSREB except when necessary to eliminate immediate hazards to the subject or when the change(s) involve only logistical or administrative aspects of the study (e.g. change of monitor, telephone number). Expedited review of minor change(s) in ongoing studies will be considered. Subjects must receive a copy of the signed information/consent documentation.

Investigators must promptly report to the HSREB:
(a) changes increasing the risk to the participant(s) and/or affecting significantly the conduct of the study;
(b) all adverse and unexpected experiences or events that are both serious and unexpected;
(c) any information that may adversely affect the safety of the participants or the conduct of the study.

If these changes/adverse events require a change to the information/consent documentation, and/or recruitment advertisement, the newly revised information/consent documentation, and/or advertisement, must be submitted to this office for approval.

Members of the HSREB who are named as investigators in research studies, or declare a conflict of interest, do not participate in discussion related to, nor vote on, such studies when they are presented to the HSREB.

Chair of HSREB: Dr. Joseph Gilbert

Ethics Officer to Contact for Further Information

☐ Janice Sutherland (jsutherl@uwo.ca)
☐ Elizabeth Wembolt (ewembolt@uwo.ca)
☐ Grace Kelly (gake10001@uwo.ca)
☐ Denise Graffon (dgaffon@uwo.ca)

This is an official document. Please retain the original in your files.
Appendix 2

January 12, 2009

*This is the Original Approval for this protocol*
*A Full Protocol submission will be required in 2013*

Dear Dr. Kim:

Your Animal Use Protocol form entitled:
Pharmacokinetic analysis of OATP1B substrates in Oatp1b2 knockout and wildtype mice
Funding Agency: CIHE - Grant #MOP-89753

has been approved by the University Council on Animal Care. This approval is valid from Jan. 12th, 2009 to January 31, 2010. The protocol number for this project is #2608-123.

1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears please contact this office when grant approval is received.

ANIMALS APPROVED FOR 4 Years

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REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

R. Kim, S. Lemay, W. Lagerwerf

The University of Western Ontario
Animal Use Subcommittee / University Council on Animal Care
Health Sciences Centre, London, Ontario • CANADA – N6A 5C1
PH: 519-661-2111 ext. 86770 • FL: 519-661-2828 • www.uwo.ca/animal
Office of Research Ethics
The University of Western Ontario
Room 00045 Dental Sciences Building, London, ON, Canada N6A 5C1
Telephone: (519) 661-3038 Fax: (519) 850-2466 Email: ethics@uwo.ca
Website: www.uwo.ca/research/ethics

Use of Human Subjects - Ethics Approval Notice

Principal Investigator: Dr. D.J. Freeman
Review Number: 11478
Revision Number: 3
Protocol Title: The Effect of Intravenous Aspirin on Plasma Total Homocysteine Concentration in Patients With End-Stage Renal Disease Requiring Hemodialysis
Department and Institution: Medicine, London Health Sciences Centre
Sponsor: 
Ethics Approval Date: June 5, 2006
Expiry Date: September 30, 2006
Documents Reviewed and Approved: Revised Study End Date, revised Letter of Information and Consent
Documents Received for Information:

This is to notify you that The University of Western Ontario Research Ethics Board for Health Sciences Research involving Human Subjects (HSREB) which is organized and operates according to the Tri-Council Policy Statement and the Health Canada/ICH Good Clinical Practice Practices: Consolidated Guidelines; and the applicable laws and regulations of Ontario has reviewed and granted full board approval to the above named research study on the approval date noted above. The membership of this REB also complies with the membership requirements for REB's as defined in Division 8 of the Food and Drug Regulations.

This approval shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB's periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the UWO Updated Approval Request Form.

During the course of the research, no deviations from, or changes to, the protocol or consent form may be initiated without prior written approval from the HSREB except when necessary to eliminate immediate hazards to the subject or when the change(s) involve only logistical or administrative aspects of the study (e.g. change of monitor, telephone number). Expedited review of minor change(s) in ongoing studies will be considered. Subjects must receive a copy of the signed information/consent documentation.

Investigators must promptly also report to the HSREB:
- changes increasing the risk to the participant(s) and/or affecting significantly the conduct of the study;
- all adverse and unexpected experiences or events that are both serious and unexpected;
- any new information that may adversely affect the safety of the subjects or the conduct of the study.

If these changes/adverse events require a change to the information/consent documentation, and/or recruitment advertisement, the newly revised information/consent documentation, and/or advertisement, must be submitted to this office for approval.

Members of the HSREB who are named as investigators in research studies, or declare a conflict of interest, do not participate in discussion related to, nor vote on, such studies when they are presented to the HSREB.

Chair of HSREB: Dr. John W. McDonald
Deputy Chair: Susan Hodkinson

Ethics Officer to Contact for Further Information
Janice Sutherland (suther@uwo.ca)
Jennifer McEwan (jmclean@uwo.ca)
Ethics Officer (ethics@uwo.ca)

This is an official document. Please retain the original in your files.
Appendix 4

Therapeutic Products Directorate

OUR MISSION: We contribute to the health of Canadians and to the effectiveness of the health care system by regulating pharmaceuticals and medical devices and by providing Canadians with access to information to make informed choices.

NOTRE MISSION: Nous contribuons à l’amélioration de la santé des Canadiens et à l’efficacité du système de soins de santé en réglementant les produits pharmaceutiques et les matériaux médicaux et en offrant aux Canadiens un accès à l’information pour qu’ils puissent faire des choix éclairés.

If you receive this fax in error, please advise the sender immediately.
Si vous recevez cette télécopie par erreur, veuillez en aviser immédiatement l’expéditeur.

TO/DA
Name/Nom: Dr. David J. Freeman
Organization/Organisme: Dr. David J. Freeman
Tel./Tel.: (519) 653-4234
Fax/Télécopieur: (519) 653-4232
No. of Pages, including this page/N° de pages, incluant cette page: 2

FROM/DE
Name/Nom: Yadinder Bhuller, M.Sc.
E-Mail/Courriel électronique: yadinder_bhuller@hc-sc.gc.ca
Tel./Tel.: 613-941-5010
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TITLE
Manager - Clinical Trials Group II / Gestionnaire - Programme des essais cliniques Groupe II
Office of Clinical Trials / Bureau des essais cliniques
Therapeutic Products Directorate / Direction des produits thérapeutiques

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1600 St.oma Street / 1600 Rue Stoma
2T0A, Ontario
K1A 0G9

Website/Site Web: http://www.hc-sc.gc.ca/dhp-psp/prodpharma/index_e.html
http://www.hc-sc.gc.ca/dhp-psp/prodpharma/index_f.html

MESSAGE

IMPORTANT: Please note that the following consultation packages have been posted online.
Veuillez prendre note que le document qui suit est disponible sur notre site internet.

Clinical Trials Manual/Maîtrise d’essais cliniques

11/22/2006 WED. 12:24 [TX/RX N 7022]
22 November 2006

Dr David J. Freeman
Scientist
London Health Sciences Centre, Canada
University Hospital
339 Windermere Road, Room C5-141
LONDON, Ontario
N6A 5A5

No Objection Letter RE: Protocol #122006

Dear Dr. Freeman,

I am pleased to inform you that the information and material to support your Clinical Trial Application for UROMITEXAN, control number 109385, received on October 23, 2006, have been reviewed and we have no objection to your proposed study.

I would remind you of the necessity of complying with the Food and Drug Regulations, Division 5, in the sale of this product for clinical testing. In addition, the regulations impose record keeping responsibilities on those conducting clinical trials.

You are also reminded that all clinical trials should be conducted in compliance with the Therapeutic Products Directorate's Guideline for Good Clinical Practice.

Should you have any questions concerning this letter, please contact the undersigned at (613) 941-0570.

Yours sincerely,

Yadvinder Bhuller, M.Sc.
Manager - Clinical Trials Group II
Office of Clinical Trials
Appendix 5

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Dear Dr. Cutler,

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Department of Physiology & Pharmacology
Graduate Program in Pharmacology and Toxicology
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Education:

2001 – 2005  BMSc. (Hons. with Distinction; Physiology & Pharmacology), The University of Western Ontario, London, Ontario, Canada

2005 – Present  Ph.D. Candidate (Pharmacology and Toxicology), The University of Western Ontario, London, Ontario, Canada

Thesis title: Pharmacokinetics and Therapeutic Uses of Mesna
Supervisors: Dr. David J. Freeman

Employment History:

May 2002 – August 2005  Research Assistant (Division of Clinical Pharmacology), Department of Medicine, London, Ontario, Canada.
Supervisor: Dr. David J. Freeman

May 2009 – August 2009  Intern (Drug Metabolism and Pharmacokinetics), Genentech Inc., South San Francisco, California,
Project title: Evaluation of Drug Disposition in Preclinical Models of Colorectal Cancer
Manager: Dr. Edna Choo

Scholarships and Awards:

2009 – Recipient of an Ontario Graduate Scholarship (OGS). This scholarship is a province-wide competition worth $15,000 per year. It is payable in monthly installments from September 2009 – August 2010.

2008/2009 – Nominated for a Graduate Teaching Award (GTA) for recognition of excellent efforts in teaching undergraduate students.
2008 – Awarded the Drs. Madge and Charles Macklin Fellowship from the Schulich School of Medicine & Dentistry worth $3,000 for excellence in both teaching and research. August 31, 2008.
2007 – Recipient of an Ontario Graduate Scholarship (OGS). This scholarship is a province-wide competition worth $15,000 per year. It is payable in monthly installments from September 2007 – August 2008.
2006 – Awarded the Hari and Gudrun Sharma Award presented annually to “an outstanding continuing student in the Graduate Program in Pharmacology and Toxicology” at the University of Western Ontario. A $1,000.00 cash award was presented at the Stevenson Banquet, November 15, 2006.
2006 – Recipient of an Ontario Graduate Scholarship (OGS). This scholarship is a province-wide competition worth $15,000 per year. It is payable in monthly installments from September 2006 – August 2007.
2005/2006 – Nominated for a Graduate Teaching Award (GTA) for recognition of excellent efforts in teaching undergraduate students.

**Publications:**

Published


Submitted

5) Cytochrome P450 2D6 phenotyping in an elderly demented population and response to galantamine in dementia. JA Clarke, MJ Cutler, I Gong, UI Schwarz, DJ Freeman, M Dasgupta.

In Preparation

6) Review: The role of drug uptake transporters in chemotherapy efficacy. MJ Cutler, EF Choo.


8) Enzymatic and non-enzymatic mechanisms of dimesna metabolism. MJ Cutler, TJ Velenosi, DJ Freeman.

Abstracts:


Presented at the 3rd Annual National Symposium of the Canadian Child Health Clinician Scientist Program (CCHCSP) in St. John’s, Newfoundland, October 14-16, 2005.


14) Mesna for the Treatment of Hyperhomocysteinemia in Hemodialysis Patients.


Invited Oral Presentations:


4) Homocysteine as a Risk Factor: Past, Present, and Future. **MJ Cutler**
   Presented at the Division of Clinical Pharmacology Grand Rounds, London Health Sciences Centre – University Hospital, London, Ontario. October 17, 2007


7) Drug Disposition in Preclinical Models of Oncology. **MJ Cutler**
   Presented at the Division of Clinical Pharmacology Grand Rounds, London Health Sciences Centre – University Hospital, London, Ontario. October 14, 2009


Teaching Activities:

September 2005 – April 2006 Teaching Assistant for Pharmacology 357 (3rd year laboratory course; Fundamentals of Pharmacology and Toxicology)
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