N-acetylcysteine as a chemoprotectant against ifosfamide nephrotoxicity; from mechanism to prevention

Lauren Hanly, The University of Western Ontario

Supervisor: Dr. Gideon Koren, The University of Western Ontario
A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Pharmacology and Toxicology
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N-ACETYLCYSTEINE AS A CHEMOPROTECTANT AGAINST IFOSFAMIDE NEPHROTOXICITY; FROM MECHANISM TO PREVENTION

(Thesis Format: Integrated-Article)

by

Lauren N. Hanly

Graduate Program in Pharmacology and Toxicology

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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Abstract and Keywords

The chemotherapy drug ifosfamide is used in the treatment of several childhood cancers. While effective, its use in children results in a 30% incidence of nephrotoxicity, and 5% incidence of Fanconi syndrome. This late effect is caused by oxidative damage, generated by chloroacetaldehyde, a toxic metabolite of ifosfamide cytochrome P450-mediated bioactivation in the kidney tubules. N-acetylcysteine has been identified as a promising strategy to mitigate nephrotoxicity through its antioxidant and glutathione stimulating properties. Furthermore, with current use in children for acetaminophen poisoning, its clinical utility is evident. Both cell and animal models have demonstrated n-acetylcysteine’s effectiveness in mitigating ifosfamide kidney toxicity. However, there is no data available to suggest the safe use of n-acetylcysteine with respect to maintenance of ifosfamide’s chemotherapeutic integrity. There is also a lack of information suggesting that the current dose for acetaminophen overdose will be sufficient for the alternative indication of renal protection. To address this gap in knowledge, which is critical in moving forward with the use of n-acetylcysteine in a clinical setting, herein I describe three studies, which focus on the translational pharmacokinetics of n-acetylcysteine, as well as its effect on the efficacy of ifosfamide. Using a comparison of our therapeutically effective rodent model to children receiving the 21-hour intravenous dose of n-acetylcysteine for acetaminophen overdose, we were able to demonstrate similar systemic exposures are achieved in both groups. This corroborates that the dose currently used in children is an excellent choice for renal protection. To demonstrate the impact of n-acetylcysteine on the antitumor efficacy of ifosfamide, we evaluated the combination of n-acetylcysteine and ifosfamide
mustard, the active antineoplastic agent *in vitro* and n-acetylcysteine and ifosfamide in a mouse xenograft model. Our investigations provide evidence to suggest n-acetylcysteine does not interfere with ifosfamide activity, further supporting its safe use. Based on existing evidence, including that presented in this thesis, we have developed clinical protocol recommendations, for the safe use of n-acetylcysteine for children who present with renal toxicity due to ifosfamide. I also detail a randomized prospective double-blinded study designed to assess the effectiveness of n-acetylcysteine as a prophylactic strategy, in a control fashion.

**Keywords:** ifosfamide, nephrotoxicity, n-acetylcysteine, pharmacokinetics, antitumour efficacy, translational research
Co-authorship

Chapter 1: Introduction and Rationale
Drs. Koren, Rieder and Chen gave support during manuscript preparation, provided feedback, and revised the manuscript before submission

Chapter 2: N-acetylcysteine as a treatment against nephrotoxicity caused by ifosfamide: Translational pharmacokinetics
Drs. Bajcetic, Palassery, Regueira, Turner, and Vasyleva were treating physicians of patients in the case reports. Dr. Baw and Becky Malkin identified cases of pediatric/adolescent acetaminophen overdose. Drs. Freeman and Cutler provided expertise and/or assistance as well as the facility to aid in analyzing serum n-acetylcysteine concentrations with HPLC. Dr. Aleksa aided in analyzing ifosfamide serum concentrations. Dr. Chen began recruitment in this study. Drs Chen and Aleska contributed to the methodological sections of this manuscript. Drs. Korens, Rieder and Chen gave support during manuscript preparation, provided feedback, and revised the manuscript before submission

Chapter 3: The effect of n-acetylcysteine on ifosfamide antitumour efficacy: in vitro
Dr. Chen provided assistance and expertise in order to carry experimental design and is co-first author on this paper. Drs. Koren, Reider, Chen and Yeger aided in experimental design, gave support during manuscript preparation, provided feedback, and revised the manuscript before submission

Chapter 4: The effect of n-acetylcysteine on ifosfamide antitumour activity: in vivo
Rene Figueredo aided and provided expertise in working with mice. Dr. Koropatnick provided facility and expertise to aid in experimental design. Drs. Koren, Rieder and Koropatnick gave support during manuscript preparation, provided feedback, and revised the manuscript before submission
Chapter 5: Future directions and overall perspectives

Drs. Vasylyeva, Shah and Regueira were treating physicians of the patient treated in the case report. Drs. Koren, Rieder, Huang and Vasylyeva gave support during manuscript preparation, provided feedback, and revised the manuscript before submission.

* All manuscripts in this thesis were written principally by Lauren Hanly.
Dedication

This thesis is dedicated to my parents.

You have provided me every opportunity to achieve success, and lead me to believe that anything and everything is possible.

Your love, support and encouragement have motivated me every step of the way;

I am forever grateful.
Acknowledgements

This thesis has been made possible only through the help, support and guidance of many. First thank you to the funding agencies which contributed to my research: The Paediatric Oncology Group of Ontario, The Canadian Institutes of Health Research and The Children’s Health Research Institute. Thank you to Dr. Nancy Chen, whose hard work prior to mine has given way to my project. You were a wonderful teacher, both showing me the ropes and passing your passion for this research on to me. I couldn’t have asked for a better mentor.

I would like to thank my committee Dr. James Koropatnick, Dr. James Hammond and Dr. Qingping Feng, and the Bend-Freeman-Rieder-Urqhuart Thursday morning lab meeting group. Your advice, suggestions and time you gave have been invaluable.

Best wishes and gratitude to Maud Rouleau, all of the members of the Rieder and Koren labs, both in London and Toronto. I blessed to have been of part of such a wonderful team.

To my sisters, thank you for your continued words of encouragement, love, and support. I can only hope to that you are as proud of my accomplishments, as I am of yours. To Tim, having you by my side from beginning to end of this journey has made everything a little easier. Your belief in me has kept me going, your humor kept me sane, and your love kept me fulfilled. Thank you.

Thank you to Dr. Michael Rieder for your kind greetings in the hallway and willingness to make time to chat. Your mentorship has been invaluable to my growth here at Western. Your thirst for knowledge inspires me.
And last, but most certainly not least, I owe a huge debt of gratitude to Dr. Gideon Koren. Your intelligence, your passion for your research, and your ability to teach never ceases to amaze me. You have given me the opportunity to reach my full potential, and shown me that I am capable of even more. I only hope I can begin to make the same kind of differences you make every day; you are an inspiration.
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<tr>
<td>$^{51}$Cr EDTA</td>
<td>$^{51}$Cr EDTA</td>
</tr>
<tr>
<td>ADR</td>
<td>Adverse drug reaction</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>b.i.d.</td>
<td>Twice a day</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>Calcium</td>
</tr>
<tr>
<td>CAA</td>
<td>Chloroacetaldehyde</td>
</tr>
<tr>
<td>CF</td>
<td>Cyclophosphamide</td>
</tr>
<tr>
<td>Cl</td>
<td>Chloride</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>D$_6$-2-DCEI</td>
<td>Deuterated 2-dechloroethylifosfamide</td>
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<td>D$_3$-3-DCEI</td>
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<tr>
<td>DCEI</td>
<td>Dechloroethylifosfamide</td>
</tr>
<tr>
<td>DIMESNA</td>
<td>Dithio-bis-mercaptopohtanosulphonate</td>
</tr>
<tr>
<td>EW-7</td>
<td>Ewing’s sarcoma</td>
</tr>
<tr>
<td>GFR</td>
<td>Glomerular filtration rate</td>
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<tr>
<td>GLY</td>
<td>Glycine</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>HIF 1$\alpha$</td>
<td>Hypoxia inducible factor</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>IFO</td>
<td>Ifosfamide</td>
</tr>
<tr>
<td>IFM</td>
<td>Ifosfamide mustard</td>
</tr>
<tr>
<td>IL-1$\beta$</td>
<td>Interleukin 1</td>
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<tr>
<td>IL6</td>
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<td>IP</td>
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</tr>
<tr>
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<td>Intravenous</td>
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<tr>
<td>K$^+$</td>
<td>Potassium</td>
</tr>
<tr>
<td>$K_{el}$</td>
<td>Elimination rate constant</td>
</tr>
<tr>
<td>LH</td>
<td>L-histidinol</td>
</tr>
<tr>
<td>LLCPK-1</td>
<td>Porcine renal proximal tubular cell</td>
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<tr>
<td>LPO</td>
<td>Lipid peroxidation</td>
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<tr>
<td>MBB</td>
<td>Monobromobimane</td>
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<td>Sodium 2-mercaptoethanosulphonate</td>
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<tr>
<td>MEL</td>
<td>Melatonin</td>
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<tr>
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<td>3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
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<td>Na$^+$</td>
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<td>Resveratrol</td>
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<tr>
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<tr>
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<td>Reactive oxygen species</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<td>---------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>TAU</td>
<td>Taurine</td>
</tr>
<tr>
<td>TQ</td>
<td>Thymoquinone</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
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Chapter 1: Introduction and Rationale

Part of the chapter has been published:

1.1 Overview

Ifosfamide (IFO) is a chemotherapeutic agent used over the past 2 decades for therapy of various adult and pediatric solid tumors [1-3]. In recent years, its use in pediatric solid tumors such as Ewing’s sarcoma, rhabdomyosarcoma, lymphoma, neuroblastoma, osteosarcoma, soft tissue sarcomas and Wilms tumor has rapidly increased [3-6] owing to greater antineoplastic activity than its analogue cyclophosphamide (CF) [7], greater cure rate and a higher therapeutic index, with relatively rare cross-resistance [8]. Patients who may not respond to CF treatment may still benefit from IFO [9,10]. However, IFO versus CF treatment still needs further investigation clinically. Its mode of cytotoxic effect is by acting as a bifunctional DNA alkylating agent, causing both inter and intra DNA strand cross-links. It can also cause cross-links between protein and DNA with these events ultimately preventing cellular replication, leading to cell death [11].

IFO is a pro-drug that needs to be oxidized intracellularly to the cytoactive IFO mustard. In addition, when metabolized by the cytochrome P450 (CYP) monooxygenase system in both liver [1] and kidney [12,13], it gives rise to the toxic metabolites acrolein and chloroacetaldehyde (CAA), the reactive metabolites responsible for IFO associated urotoxicity and nephrotoxicity, respectively [14-16]. The urotoxic effects of acrolein have been well mitigated by sodium 2-mercaptoethanesulfonate (MESNA) given concurrently with IFO. Presently, nephrotoxicity is the biggest concern during IFO treatment [6], especially in children. Up to 30% of children treated with IFO may suffer from some degree of renal damage. Although studies have yielded conflicting estimates of the risk involved with young age and IFO treatment, current research suggests that age [17,18]
along with cumulative dose [19-23], prior/concurrent cisplatin treatment and unilateral nephrectomy [23-25] are all risk factors for IFO-induced renal injury. Although it has shown potent effects as an antineoplastic agent, severe renal damage may seriously impact the quality of life and well being of children who survive cancer.

1.2 Ifosfamide metabolism

IFO is almost identical in structure to CF (Figure 1.1), varying only in the position of one chloroethyl group [26,27]. Metabolism of both occurs by the CYP isozymes 3A4, 3A5 and 2B6 [9,10,28], resulting in similar metabolites. There are only few quantitative differences due to variation in the rates of biotransformation [14]. Ring hydroxylation of ifosfamide produces 4-hydroxy-ifosfamide, in equilibrium with aldo-ifosfamide (Figure 1.2). Aldo-ifosfamide then spontaneously converts to the active metabolite IFO mustard and equimolar amounts of acrolein, or becomes dehydrogenated to inactive carboxy-ifosfamide. Acrolein is recognized as the toxic metabolite responsible for IFO-induced urotoxicity [15].

A certain percentage of IFO and CF also undergoes metabolism through an alternative pathway: oxidation of the chloroethyl side chain leads to 2-and 3-dechloroethyl-ifosfamide or 2- and 3-dechloroethyl-cyclofosfamide and equal amounts of CAA [16]. There is a large body of evidence that CAA is responsible for IFO-induced nephrotoxicity [29-31].
Figure 1.1 CF and IFO chemical structures, differing in the position of one chloroethyl group.
Cyclophosphamide

Ifosfamide
**Figure 1.2** IFO metabolism. Ring hydroxylation and side-chain oxidation of IFO results in ifosfamide mustard, and 2- and 3-dechloroethyl IFO and CAA, respectively.
1.2.1 Qualitative difference in ifosfamide versus cyclophosphamide metabolism

Although both CF and IFO undergo the ring hydroxylation pathway, it occurs much slower in IFO owing to steric hindrance caused by the differentially positioned chloroethyl group [32]. This allows for a greater proportion of IFO to be metabolized by an alternative side chain pathway. Hence up to 50% of IFO metabolism occurs through the side chain pathway producing CAA, although it only accounts for 10% of metabolism in CF [16]. Consequently, IFO also requires a greater therapeutic dose (up to threefold or more) to produce the same amount of alkylating agent when compared to CF. This leads to CAA quantities that are as much as 100-fold higher with IFO than the amounts being produced from CF [32].

1.2.1 Chloroacetaldehyde

First postulated to be the proximate nephrotoxic agent, studies of CAA area under the curve (AUC) argued that systemic exposure to CAA alone cannot explain this serious toxicity. Boddy et al. [33] showed in patients that those with a lower AUC of CAA exhibited a greater degree of nephrotoxicity. These results led to the conclusion that there was no correlation between CAA plasma concentrations and kidney damage. However, a key caveat is that the presence of a metabolite in urine provides no indication of where this metabolite has been produced. Woodland et al. [14] then hypothesized that local renal metabolism of IFO to CAA – not systemic exposure – produces renal tubular damage.

Although other toxic species such as acrolein are produced from IFO, metabolism studies by Springate and Taub [34] have demonstrated CAA to be the main nephrotoxic IFO metabolite. Although metabolism is often thought of as hepatic, the kidney possess
an impressive repertoire of xenobiotic-metabolizing enzymes [12,35], and thus the potential for production of high concentrations of toxic species by the kidney itself has come to light [12,13]. The ability of the kidney to ‘produce its own poison’ is, therefore, a new concept that deserves serious focus in understanding IFO-induced nephrotoxicity and possibly other drug-induced renal damage. At clinically relevant concentrations, IFO metabolism occurs in both porcine and human kidney microsomes. This metabolism occurs consistently with CYP-mediated drug biotransformation [14]. Recent studies have also shown that both pig and human renal tubules specifically possess the CYP enzymes, 3A4 and 2B6, necessary for CAA production [14,36,37].

Although previous findings demonstrate the capability of renal IFO metabolism, evidence of clinically relevant amounts of CAA being produced have also been shown. *In vitro*, CAA concentrations ranging from 10 to 50 μmol can cause renal tubular toxicity [30]. Aleksa et al. [38] used pharmacokinetic modeling to evaluate renal tubule concentrations of CAA. Their findings demonstrate renal tubule cell production of CAA at levels known to cause tubular damage (50 μmol). This supports the hypothesis that local renal production of CAA can be responsible for IFO-induced nephrotoxicity. These findings may also explain how age differences, shown later in this review, may affect renal metabolism and play a role in inter-individual variability in nephrotoxicity [14].

### 1.3 Ifosfamide-induced nephrotoxicity

Following the introduction of IFO in the 1970s, interest in its use was reduced owing to severe cases of hemorrhagic cystitis caused by acrolein [39]. This consequence of IFO treatment has since been addressed with the concomitant treatment of MESNA.
The synthetic thiol MESNA chelates acrolein, allowing for protection of the bladder’s epithelium. Prevention of urotoxicity has allowed for both higher and more frequent dose schedules of IFO [6]. The elimination of the dose-limiting effect of urotoxicity has subsequently revealed nephrotoxicity as a serious complication associated with IFO treatment.

Adults were initially the first group of patients observed to present with IFO-induced nephrotoxicity [18]. In the past decade, however, an awareness of the severity of nephrotoxicity in children treated with IFO has unfolded [40], causing a shift in this area of research, with a predominant focus on a younger population. Approximately 30% [17,18] of children treated with IFO suffer from chronic renal dysfunction with reports as low as 15% [41] and as high as 60% [40]. Around 1.4 – 5% of these children suffer from its most severe form, Fanconi syndrome [17,18,41].

1.3.1 Clinical presentation of ifosfamide-induced nephrotoxicity

Nephrotoxicity can present in any one segment or a combination of segments in the nephron: glomerulus, proximal or distal tubule, or the collecting duct [40]. In the case of IFO, it most commonly manifests as proximal tubule damage. Glomerular toxicity is normally secondary to that of tubular [42], although serious distal toxicity is rare [43]. The severity of chronic renal damage can vary from subclinical to severe tubular and/or glomerular toxicity (Table 1.1). Subclinical features may include glycosuria, aminoaciduria and increased excretion of low molecular mass proteins [44-48]. More severe proximal tubule toxicity can result in Fanconi syndrome with clinically relevant hypophosphatemic rickets, proximal renal tubule acidosis and hypokalemia [19,49-51]. Chronic glomerular dysfunction, evident by reduced glomerular filtration rate (GFR) and
elevated serum creatinine, may coexist [19,42,49]. Although relatively uncommon [43],
severe distal toxicity, when present, can lead to diabetes insipidus and distal renal tubular
acidosis [51,52].
Table 1.1 Clinical features and relevant sequelae of IFO-induced nephrotoxicity.
**Proximal Tubular Toxicity**

Fanconi syndrome including:
- Urinary loss of glucose, amino acids, and Low Molecular Weight proteins
- Phosphaturia
- Hypophosphatemic Rickets
- Kaluria
- Hypokalemia
- Bicarbonaturia
- Proximal Renal Tubular Acidosis

**Glomerular Toxicity**

- Reduced GFR
- Increased serum urea and creatinine concentrations
- Chronic Renal Failure

**Distal Tubular Toxicity**

- Nephrogenic Diabetes Insipidus
- Distal Renal Tubular Acidosis
1.3.2 Fanconi syndrome

Fanconi syndrome is described as a proximal tubule dysfunction. It consists of urinary loss of glucose, amino acids and low molecular mass protein as well as phosphate, bicarbonate and potassium [51,53,54]. Hypophosphatemia resulting from urinary phosphate loss can develop into hypophosphatemic rickets, a serious metabolic bone disease [50,54-57]. Proximal renal tubular acidosis, resulting from bicarbonaturia [20,58], along with hypophosphatemic rickets can lead to serious impairment of growth, which is especially detrimental to young children [59,60].

1.3.3 Inter-individual variability

Renal toxicity following IFO treatment is subject to great inter-individual variability. Onset, nature and severity can differ from one patient to the next, with many suffering no kidney damage although a few suffer from its most severe forms [23,40,50]. Evidence of renal damage may become apparent immediately after starting treatment [55,56] or not until after the treatment is complete [50,59,61,62]. A follow-up study in 75 patients who had completed IFO treatment found that subclinical damages developed in the first 2 years after completion of treatment [63]. A diagnosis of Fanconi syndrome was documented as far as 3 years following cessation of treatment [63]. This has been supported by previous studies, which found that most patients received a diagnosis of Fanconi syndrome within 2 years after treatment [49,64].

1.3.4 Long-term outcome and treatment

There is great uncertainty as to the degree of reversibility of renal damage as a result of IFO treatment [18]. Although some improvement has been seen in tubular damage [65], most cases are not resolved [17]. In fact, deterioration of tubular [53,56,62]
and glomerular function [19,42] is commonly seen following cessation of IFO therapy. There has been one documented case of a child completely recovering from severe Fanconi syndrome over a 2-year period [66]; no similar reports are yet to be seen in cases of serious glomerular toxicity. Most cases, however, demonstrate no change, slight improvement or slight deterioration. A study monitoring glomerular and tubular function over 10 years in 12 children, showed overall no significant change in either tubular or glomerular toxicity in the group. However, several individuals showed improvement in either glomerular filtration rate (GFR) or the severity of phosphaturia, although one demonstrated deterioration in both. On the whole, nephrotoxicity persisted and there was no evidence to allow for prediction of how the nephrotoxicity of individuals would progress [18].

At the present time, treatment of severe proximal tubule toxicity includes supportive therapy with oral supplementation of phosphate and bicarbonate. In the rare circumstance of chronic renal failure owing to glomerular toxicity, dialysis or renal replacement therapy may be required [17]. Treatment of young children is especially important, as 80% of pediatric cancer patients become long-term cancer survivors [67]. The severe consequences of IFO-induced nephrotoxicity can have a great impact on their quality of life and well-being.

1.3.5 *Ifosfamide-induced nephrotoxicity at a cellular level*

Understanding the mechanisms in which CAA exerts its toxic effect on renal tubule cells is critical to developing effective prevention. CAA has been implicated in the depletion of ATP levels [68-70], the collapse of the protein gradient through opening of the mitochondrial permeability transition pores [70,71] and the generation of reactive
oxygen species (ROS) [72]. It also causes decrease of Na\(^+\)/K\(^+\) ATPase activity [73,74] and elevation of intracellular Ca\(^{2+}\) [75]. A release of pro-inflammatory cytokines is also associated with IFO-induced nephrotoxicity [76].

Several studies have demonstrated a significant decrease of ATP in renal tubular cells following treatment with CAA [68-70]. Not only does ATP have a critical role in sustaining normal kidney function [77], but also low levels of it initiate necrotic cell death [78,79], the predominant form of cell death in CAA toxicity [70,75,80]. This depletion of ATP may be the result of the collapse of the proton gradient in mitochondria [70]. Following increased intracellular Ca\(^{2+}\) and ROS due to CAA, there is subsequent induction of the mitochondrial permeability transition pores and, therefore, loss of mitochondrial membrane potential [71,81]. The ROS responsible are produced by the reactive metabolite (CAA). These reactive species damage macromolecules, proteins and DNA, as well as propagate further production of lipid radicals [72]. More specifically in the context of CAA toxicity, ROS can oxidize proteins on ATPase pumps, rendering them dysfunctional [82]. Studies have shown that inhibition of Na\(^+\)/K\(^+\) ATPase occurs in the renal cells of rats treated with IFO [73,74]. Maintenance of low intracellular levels of Na\(^+\) is critical for normal reabsorption of solutes. Loss of normal Na\(^+\) concentration may, therefore, contribute to urinary loss of solutes in Fanconi syndrome [82]. The increase in Ca\(^{2+}\) concentration is suggested to occur through the impairment of the Ca\(^{2+}\)/Na\(^+\) exchanger, therefore, inhibiting its export [75]. Ultimately leading to cell death, elevated levels of Ca\(^{2+}\) first cause damage to the cell cytoskeleton and membranes, as well as cause cellular degradation [83].

With respect to the inflammatory response present in IFO-induced nephrotoxicity,
Sehirli et al. [76] demonstrated an increase in serum concentration of cytokines TNF-α, IL-β2 and IL6. TNF-α has been suggested to play a role in increasing oxidative stress through depletion of glutathione (GSH) as seen in pulmonary vascular endothelial cells [84]. GSH as well as glutathione S-transferase activity has been reported to be depleted in a number of studies in IFO treated patients [30,85]. TNF-α also increases oxidative stress through ROS production in human endothelial cells [86], which may, therefore, also occur in kidney cells subject to CAA toxicity. In necrotic death during the inflammatory response, release of these cytokines is an expected feature [87]. By clearly defining these features of IFO-induced nephrotoxicity, caused primarily by oxidative stress, a more clear direction for future studies toward attenuating nephrotoxicity becomes apparent.

1.4 Risk Factors for ifosfamide-induced nephrotoxicity

There are several risk factors that predispose patients to nephrotoxicity. Age, cumulative IFO dose [19-22], previous and/or concurrent treatment with cisplatin, and previous unilateral nephrectomy [23-25] have been suggested as factors that will increase a patient’s risk of suffering from nephrotoxicity while on IFO treatment regimens. As age is an important risk factor, many of the published studies have been centered around children, as they are a more vulnerable population for both nephrotoxicity and its long-term consequences [18].

1.4.1 Age

Several studies have tried to explain the effect of age on nephrotoxicity. Although Loebstein et al. [88] and Skinner et al. [89] found conflicting results when correlation nephrotoxicity with age, recent studies on the ontogeny of CYP enzymes 3A and 2B6
have clarified this issue.

1.4.1.1 Loebstein vs. Skinner

In 2000, Skinner et al. [89] found that in a group of 76 children and adolescents, for whom all study end points were fully evaluable, there was no correlation between age and nephrotoxicity associated with IFO. They concluded that in the treatment of IFO, cumulative dose was the only risk factor in the development of renal toxicity. In contrast, in a study of 174 evaluable children and young adults, Loebstein et al. [88] found a negative correlation between age and nephrotoxicity. They found that younger children were at greater risk for IFO-induced kidney damage, with 41.4% of the 174 patients demonstrating nephrotoxicity. Although the latter study included children who had received or were concurrently receiving cisplatin treatment, 51 patients in their study did not, allowing for statistical analysis of this subgroup. After exclusion of previous or current cisplatin treatment, 33.5% of children were still observed to suffer from nephrotoxicity. In this study, renal function was graded as non-nephrotoxic, mildly, moderately or severely nephrotoxic based on preset criteria. Of the 9.2% of patients who were graded as having severe nephrotoxicity, all but one was under the age of five. This study concluded that age is a predictor of both development and severity of nephrotoxicity and that children younger than 3 years of age are at a greater risk of developing nephrotoxicity while being treated with IFO. Moreover, there was an apparent interaction between age and dose rendering children younger than 3 years of age more sensitive to nephrotoxicity of a given cumulative dose than older children.
1.4.1.2 Critical review of two opposing studies

A critical review of these two opposing viewpoints has been conducted by Aleksa et al. (Table 1.2) [90]. Both studies included patients of similar age and health status [88,89]; however, that of Skinner et al. [89] may not have been adequately powered with only 67 evaluable patients with pre and post IFO GFR measurements. The time of renal function assessment differed between the two studies. Patient’s renal function was assessed before IFO treatment to serve as a baseline, during and 3 months after treatment in the study by Loebstein et al. [88]. This study also included a 5 year follow-up after the patients’ first diagnosis. Skinner et al. [89] used a baseline of 105 healthy children and renal assessment was done only once at an average of 6 months. Aleksa et al. [90] pointed out that with no baseline and only one assessment, nephrotoxicity as either age or dose-dependent may be difficult to determine. No baseline data prevents one from concluding whether biomarkers of nephrotoxicity have increased or decreased in a specific individual. Follow-up in the study by Skinner et al. [89] may also have been too short to detect IFO-induced nephrotoxicity [90]. In terms of renal assessment, Skinner et al. [89] used a more advantageous method by directly measuring GFR by looking at clearance of $^{51}$Cr-labeled ethylenediaminetetraacetic acid ($^{51}$Cr EDTA) and $^{99m}$Tc-labeled diethylenetriaminepentaacetic acid. However, although Loebstein et al. [88] used the less reliable Schwartz formula, it is commonly used in outpatients and is a much easier procedure to carry out [90]. Aleksa et al. [90] concluded that young age as a risk factor in the development of IFO-induced nephrotoxicity should be considered for patients receiving concomitant cisplatin and that further research in this area needs to be carried out.
Table 1.2 Comparison of opposing studies of Loebstein et al. [88] and Skinner et al. [89].
<table>
<thead>
<tr>
<th><strong>Criterion</strong></th>
<th><strong>Loesten et al.</strong></th>
<th><strong>Skinner et al.</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients (Fully evaluable)</td>
<td>174 children and adolescents</td>
<td>76 children and adolescents</td>
</tr>
<tr>
<td>Concurrent cisplatin treatment</td>
<td>123</td>
<td>3</td>
</tr>
<tr>
<td>Baseline</td>
<td>Patients before treatment</td>
<td>106 healthy children and adults</td>
</tr>
<tr>
<td>Time of renal function assessment</td>
<td>Before, during and after (3 months) treatment, 5 year follow-up (from initial diagnosis)</td>
<td>After treatment (6 months)</td>
</tr>
<tr>
<td>Glomerular Filtration Rate Assessment</td>
<td>Schwartz Formula</td>
<td>Plasma clearance of Cr-EDTA</td>
</tr>
<tr>
<td>Nephrotoxicity grading parameters</td>
<td>GFR, hypophosphatemia, hypcarbia with acidosis, glucosuria, and proteinuria</td>
<td>GFR, tubular threshold for phosphorus serum bicarbonate morning urine osmolality</td>
</tr>
<tr>
<td>Conclusion on age</td>
<td>Age-dependant effect as a risk factor found with 41.4% patients demonstrating renal damage</td>
<td>No age-dependant effect</td>
</tr>
</tbody>
</table>
1.4.2 Renal ontogeny of CYP enzymes

The effect of age on IFO-induced renal toxicity has since been clarified. Young children have greater renal metabolism of IFO and, therefore, their kidneys are exposed to higher concentrations of CAA. Young murine animals corresponding to human toddlers show higher expression of CYP enzymes responsible for IFO metabolism in the kidney as well as show increased CAA production [91]. The ontogeny of CYP3A and 2B22, which have been cloned and characterized in pig renal tissue [35], were studied by Aleksa et al. [91] as they are analogues of the two major CYP enzymes 3A4 and 2B6 involved in IFO metabolism. There is 60% homology between the human CYP 3A4 and porcine 3A [92,93].

Aleksa et al. [91] confirmed the presence of 3A protein in porcine kidneys. Levels started low between days 0 and 15, significantly increased between days 15 and 60 and subsequently decreased to adult levels. This corroborates an apparent age dependence of CYP3A enzyme expression. CYP 2B22 did not show any significant ontogeny from newborn to adult. Aleksa et al. [91] also measured IFO metabolites to assess the ontogeny of its metabolism. This was done because ontogeny in protein expression measured does not necessarily reflect activity, in this case metabolism. CAA levels demonstrated a trend matching that of CYP3A protein levels. Through days 15 – 60, metabolism of IFO was significantly greater than newborn (days 0 – 10) and adult (days > 75) metabolism.

Ultimately, a significant increase in both CYP 3A expression and IFO metabolism in pigs of age 15 – 60 days was demonstrated, which supports an age-dependant effect on IFO metabolism. They concluded the effect is due to CYP 3A4 rather than CYP 2B6, as
2B6 does not show any significant differences with age [91].

The hypothesis that the ontogeny of CYP enzymes may be responsible, at least in part, for the age-dependent risk associated with IFO treatment has been strengthened by a study that concluded it is not due to differences in pediatric versus adult tubule susceptibility to CAA toxicity [68]. Dubourg et al. [68] compared the effects of various concentrations of CAA on isolated pediatric and adult proximal tubule cells. ATP levels reflecting cellular metabolism and lactate dehydrogenase released into media as a lysis marker were measured. GSH and acetyl-CoA plus CoA concentrations, and lactate metabolism were also evaluated. All measures were compared against each other in both cell groups following CAA incubation. Both pediatric and adult proximal tubule cells respond to various CAA concentrations (0.1 – 0.5 mM) in the same way. These results suggest that there is not a greater sensitivity of pediatric tubules to CAA as once hypothesized; therefore, it does not explain why younger children are more at risk of suffering from nephrotoxicity. This further reinforces that it is the ontogeny of CYP enzymes that is in fact responsible.

1.4.3 Cumulative dose, prior/concurrent nephrotoxin treatment and unilateral nephrectomy

The dose-dependent effect of IFO treatment is important and well established. Numerous reports support the suggestion that a higher cumulative IFO dose increases risk for nephrotoxicity [19,44,88,89]. Although Skinner et al. [89] and Lobstein et al. [88] had opposing arguments on the role of age in IFO nephrotoxicity; both concluded that total cumulative dose is important in the development of nephrotoxicity. It has been suggested that cumulative doses > 60 – 100 g/m² [19,48] should be avoided in children;
however, significant renal damage occurred in a cumulative dose as low as 45 g/m². This dose caused significant renal damage in patients who were also receiving cisplatin treatment, another known risk factor for IFO-induced nephrotoxicity. Cisplatin potentiates the effects of renal damage during IFO treatment, which might explain the discrepancy in significant cumulative doses seen. This, however, highlights the importance of considering adverse effects when more than one risk variable comes into play. Cumulative dose, age [88] and other potentially nephrotoxic treatments when considered together rather than separate are the best way of assessing the potential of suffering from nephrotoxicity. As an illustration, Loebstein et al. [88] documented a significant interaction between dose and age younger than 3 years, rendering younger children to disproportionately more nephrotoxicity than older children and adults with similar doses per surface area.

Serious kidney damage due to IFO treatment with previous [24,57] and/or concomitant [62,94] cisplatin has been repeatedly documented. Responsible for reduced GFR [95,96], cisplatin treatment may reduce IFO clearance by the kidneys. Other suggestions include that tubular secretion may be ineffective owing to cisplatin-induced tubular damage and that there is an inability to convert MESNA to free-thiol form. Decreased clearance and/or secretion allows for prolonged exposure of the tubules to toxic metabolites. One study has demonstrated the occurrence of IFO-induced nephrotoxicity increasing to 41.4 from 33.5% when cisplatin-treated patients were included in the statistical analysis [88]; however, this increase was not analyzed for significance.

Severe nephrotoxicity in IFO treatment has also been reported in patients after
unilateral nephrectomy [23]. Rossi et al. [23] assessed renal function in 120 patients following IFO treatment to identify risk factors in the development of nephrotoxicity. Ten of these patients had previously undergone a unilateral nephrectomy. This study concluded that of all potential risk factors associated with IFO-induced nephrotoxicity, unilateral nephrectomy was the most important. This observation is well explained by the single kidney being exposed to the whole body load of circulating IFO rather than to only 50% of it. Although each one of these factors is important to take into account individually, consideration must also be given to the cumulative and potentially synergistic effects of the combination of several risk factors. Similarly, consideration of which risk factor is present is critical, as each one will vary in how it may be dealt with clinically.

1.5 Prevention of ifosfamide-induced nephrotoxicity

There have been a number of experimental studies conducted in the recent years in an effort to prevent or attenuate nephrotoxicity associated with IFO treatment. Although MESNA has been considered as a potential therapy for attenuating nephrotoxicity as it demonstrates uroprotective properties, it has failed to provide the same protection \textit{in vivo} for kidneys [97]. Rapidly oxidizing to DIMESNA on administration, reduction of its oxidized form in the kidney further depletes GSH [98]. At present, most of the research in this area has focused on the use of antioxidants as a protective measure. GSH, a natural antioxidant, is an important defense mechanism of the cell, providing protection against toxic metabolites such as CAA [99]. Depletion of GSH has been demonstrated in kidney cells treated with IFO [30], which may already possess
a greater sensitivity to toxic damage owing to inherently low levels of GSH as compared to the liver [12]. A recent study by Chen et al. [98] has documented that the depletion of GSH by L-buthionine sulfoximine greatly increases renal tubular damage of IFO. Use of antioxidants as a way of compensating for, or inhibiting the depletion of GSH shows great promise. The antioxidants thymoquinone (TQ) [100], taurine (TAU) [101], resveratrol (RES) [76], melatonin (MEL) [73], l-histidinol (LH) [102] and n-acetylcysteine (NAC) [85] have all been studied in experimental animal models to assess their ability in preventing IFO-induced nephrotoxicity. Glycine (GLY) has also been assessed for its protective effects against IFO-induced nephrotoxicity in an animal model [103].

5.1 Glycine

Demonstrating substantial cellular protection, GLY, when depleted in proximal tubule cells may predispose them to necrotic damage [104]. GLY depletion has been proposed to occur as a result of reduced amino acid reabsorption in Fanconi syndrome [105]. A study by Nissim and Weinberg [103] has demonstrated the nephrotoxicity attenuating effects of GLY in IFO-treated rats, with plasma concentrations two to threefold greater than normal. GLY protected renal cells from the phospholipid losses as seen during IFO treatment, as well as decreased creatinine excretion. Aminoaciduria and Na⁺ wasting seemed to improve in GLY treated rats, as was the decrease in plasma HCO₃⁻ and PO₄³⁻ seen during IFO treatment. GLY did not, however, protect against all parameters of IFO-induced nephrotoxicity, more specifically, oxidative stress. ATP and GSH depletion, and an increase of oxidized GSH during IFO treatment as compared to controls, showed no change with GLY supplementation. However, importantly, GLY did
attenuate the severity of tubular function abnormalities. These results suggest that rather than acting as an antioxidant, GLY acts through alternative mechanisms to provide cytoprotection; cell integrity and metabolic function preservation occurred through diminished structural and functional abnormalities. Baines et al. [106] also demonstrated that GLY improved proximal tubule function in perfused kidneys in areas in which damage is not usually present. These studies suggest further research on clarifying the protective mechanisms of GLY as well as its influence on IFO’s antitumor activity is required.

5.2 Antioxidants

Other compounds that have protected against nephrotoxicity in a rat model seem to do so through antioxidant properties (Table 1.3). All of the antioxidants studied attenuated the depletion of GSH and prevented increases in lipid peroxidation in IFO-treated animals [73,76,85,100-102]. TAU, TQ and NAC also prevented the decrease in glutathione S-transferase activity [85,100,101]. Prevention of free-radical generation was demonstrated with RES as seen through depression of high luminol, lucigenen Cl and collagen levels, as seen to occur in renal damage in a study by Sehirli et al. [76]. MEL also prevented increased levels of collagen, as well as preserved Na⁺ / K⁺ ATPase activity [73].

Although all of the antioxidants prevented urinary loss of at least some of the solutes seen to occur during nephrotoxicity, only some of the antioxidants prevented low serum levels of solutes [73,76,85,100-102]. However, when contrasting the protective effects of each antioxidant on low serum concentrations and urinary loss of solutes, comparison becomes difficult as each study had slightly different features of
nephrotoxicity. Some found serum concentrations were not affected by IFO treatment, whereas others did. As well urine profiles of lost solutes were not the same in each case.

Reduction of both elevated serum creatinine and urea as compared to IFO treated animals was seen with all antioxidants with the exception of NAC, which did not affect urea levels [73,76,85,100-102]. However, NAC along with RES and MEL demonstrated an ability to attenuate the inflammatory response associated with renal damage. Prevention of interstitial inflammation, increased TNF-α, IL-β2 and IL-6, and neutrophil infiltrate in IFO treated animals was seen in NAC, RES and MEL respectively. Finally, both NAC and MEL maintained tubular and glomerular integrity, although RES allowed for tubular regeneration [73,76,85]. Importantly, the influence of these compounds on IFO antitumor activity has only been tested in TAU and TQ. TAU does not affect IFO activity, whereas TQ enhances it [100,101]. The effect of these compounds on the IFO activity is critical and should be studied in those showing promise for treatment in IFO-induced nephrotoxicity.
Table 1.3 Comparison of compounds shown to attenuate nephrotoxicity in rats.
<table>
<thead>
<tr>
<th>Chemical</th>
<th>Renal protection</th>
<th>Influence on IFO antitumor effect</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Glycine</strong> <em>(Endogenous amino acid)</em></td>
<td>Protection from phospholipid loss</td>
<td>Effect on IFO activity has not been shown</td>
</tr>
<tr>
<td></td>
<td>Reduction of urinary solute loss</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reduction of elevated serum creatinine and urea</td>
<td></td>
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<tr>
<td></td>
<td>Maintenance of cell integrity</td>
<td></td>
</tr>
<tr>
<td><strong>Taurine</strong> <em>(Endogenous amino acid)</em></td>
<td>Cellular protection from oxidative stress</td>
<td>No effect of IFO antitumor activity</td>
</tr>
<tr>
<td></td>
<td>- Prevention of GSH depletion</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Prevention of glutathione S-transferase activity decrease</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Prevention in rise of lipid peroxidation (LPO)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reduction of urinary solute loss</td>
<td></td>
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<tr>
<td></td>
<td>Prevention of low serum phosphate</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reduction of elevated serum creatinine and urea</td>
<td></td>
</tr>
<tr>
<td><strong>Thymoquinone</strong> <em>(active constituent in the volatile oil of the black seed)</em></td>
<td>Cellular protection from oxidative stress</td>
<td>Enhancement of the antitumor activity of IFO, improving IFO therapeutic index.</td>
</tr>
<tr>
<td></td>
<td>- Prevention of GSH depletion</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Prevention of glutathione S-transferase activity decrease</td>
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<td>- Prevention in rise of LPO</td>
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<td></td>
<td>Reduction of urinary solute loss</td>
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<td></td>
<td>Prevention in low serum phosphate and albumin</td>
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<td></td>
<td>Reduction of elevated serum creatinine and urea</td>
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</tr>
<tr>
<td><strong>L-histidinol</strong> <em>(Structural analogy of essential amino acid L-histidine)</em></td>
<td>Cellular protection from oxidative stress</td>
<td>Effect on IFO activity has not been shown</td>
</tr>
<tr>
<td></td>
<td>- Prevention of GSH depletion</td>
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<td></td>
<td>- Prevention in rise of LPO</td>
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<td></td>
<td>Reduction of urinary solute loss</td>
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<td></td>
<td>Prevention in low serum phosphate and albumin</td>
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<td></td>
<td>Reduction of elevated serum creatinine and urea</td>
<td></td>
</tr>
<tr>
<td><strong>Melatonin</strong> <em>(Indolamine produced in the pineal gland)</em></td>
<td>Cellular protection from oxidative stress</td>
<td>Melatonin has been shown to not interfere with antitumor effects of cisplatin and methotrexate. Its effects on IFO activity have not yet been shown.</td>
</tr>
<tr>
<td></td>
<td>- Prevention of GSH depletion</td>
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<td>- Prevention in rise of LPO</td>
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<td>Reduction of urinary solute loss</td>
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<td>Prevention in low serum phosphate and albumin</td>
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<td>Reduction of elevated serum creatinine and urea</td>
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<td></td>
<td>Prevention of neutrophil infiltration</td>
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<td></td>
<td>Prevention of collagen increase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Maintenance of Na’/K’-ATPase activity</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Improved tubular and glomerular maintenance</td>
<td></td>
</tr>
<tr>
<td><strong>Resveratrol</strong> <em>(Natural phytoalexin found in grapes, peanuts, mulberries and red wine)</em></td>
<td>Cellular protection from oxidative stress</td>
<td>Effect on IFO activity has not been shown</td>
</tr>
<tr>
<td></td>
<td>- Prevention of GSH depletion</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Prevention in rise of LPO</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Prevention of free radical generation</td>
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<tr>
<td></td>
<td>Reduction of urinary solute loss</td>
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<tr>
<td></td>
<td>Prevention of low serum phosphate, glucose and albumin</td>
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<td></td>
<td>Reduction of elevated serum creatinine, urea, lactate dehydrogenase activity</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reduction of elevated serum pro-inflammatory cytokines</td>
<td></td>
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<tr>
<td></td>
<td>Tubular regeneration</td>
<td></td>
</tr>
<tr>
<td><strong>NAC</strong> <em>(Synthetic precursor of GSH)</em></td>
<td>Cellular protection from oxidative stress</td>
<td>Effect on IFO activity has not been shown</td>
</tr>
<tr>
<td></td>
<td>- Prevention of GSH depletion</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Prevention of glutathione S-transferase activity decrease</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Prevention in rise of LPO</td>
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<td>Reduction of urinary solute</td>
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<td>Prevention of low serum potassium and magnesium</td>
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<td></td>
<td>Prevention of elevated serum creatinine</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Prevent interstitial inflammation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Prevented tubular and glomerular damage</td>
<td></td>
</tr>
</tbody>
</table>
1.5.3 **N-acetylcysteine**

Although all of these compounds have shown promise in the attenuation of IFO-induced nephrotoxicity, NAC has a major advantage over other therapies, as it is the only one presently approved for clinical use in children for other indications. Chen *et al.* [85,98] have studied the experimental effectiveness of NAC in the attenuation of IFO-induced nephrotoxicity *in vitro* and *in vivo* in a rat model. The choice of NAC in the treatment of nephrotoxicity of IFO was not only owing to its antioxidant properties; although NAC shares similar antioxidant properties to many of the others tested in attenuation of IFO-induced nephrotoxicity (stimulation of GSH, nucleophilic ROS scavenging properties and enhancement of glutathione S-transferase activity), it is also a synthetic precursor for GSH and most importantly is now used clinically in children [107-109] (Figure 1.3). Presently, NAC is used as an antidote for acetaminophen poisoning; it is commonly used both orally and intravenously with an apparently wide safety window. There has not been clinical use of the other antioxidants and, therefore, their safety in children remains in question.

The therapeutic concentration in children receiving NAC for acetaminophen overdose is 0.4 mM of NAC [107]. This clinically relevant concentration of NAC (0.4 mM) was sufficient in the prevention of IFO-induced nephrotoxicity *in vitro* [98]. In the only study of the kinetics of NAC in children, steady-state plasma concentration values of 0.51 mM have also been reported [110]. Further studies on NAC have been done to demonstrate that doses of NAC now given to children are well within serum concentrations necessary to prevent nephrotoxicity. Carrying out a systematic review of published NAC pharmacokinetic studies, Chen *et al.* [111] found steady-state plasma
concentration to range from 0.04 to 0.9 mM and calculated NAC urinary concentration to be 2 mM in healthy adults. They concluded that even at the lowest plasma steady-state (0.04 mM), this concentration, along with the 2 mM in the tubular lumen, is sufficient to expose renal tubular cells to the concentration of NAC previously demonstrated to confer protection. NAC has also been shown to prevent IFO-induced nephrotoxicity in rats at clinically relevant doses of IFO (50 mg/kg for 5 days) [85]. These studies demonstrate the great potential for NAC to be used clinically in the prevention of IFO-induced nephrotoxicity as evidence suggests the clinically relevant dose used in acetaminophen overdose in children is sufficient to provide renal protection.

A major issue in using NAC for prevention of IFO-induced nephrotoxicity is in ensuring that this antioxidant does have an effect on the cytotoxic activity of IFO; an issue which must be addressed before the clinical use of NAC.
Figure 1.3 Mechanisms of NAC.
1.6 Conclusion

Severe nephrotoxicity is a serious consideration when using IFO, especially in the vulnerable population of children with cancer. This renal damage is owing to the toxic metabolite produced locally in the kidney, CAA. Exerting its toxic effects through generating oxidative stress and depletion of the cellular protection mechanism GSH, the effects of CAA towards nephrotoxicity can be attenuated with antioxidant treatment. Although GLY and other antioxidants have all shown to attenuate IFO-induced nephrotoxicity in rats, NAC proves to be a clinically relevant choice, as it has a current therapeutic use in children.

1.7 Preamble

IFO that is commonly used to treat childhood pediatric solid tumors is widely used in combination therapies or alone. IFO has proven to be at better choice than CF in many cases as it has a greater cure rate, greater therapeutic index and greater antineoplastic activity. It also shows little cross-resistance and can, therefore, be used to treat patients who do not respond to CF treatment.

In an attempt to prevent IFO-induced nephrotoxicity, research has been conducted in recent years to clarify the mechanisms responsible for this serious adverse effect. A major breakthrough has been realizing that local production of a toxic species, rather than from systemic exposure, may explain the nephrotoxicity. Of great importance in the development in this field are the investigations that support local renal production of toxic levels of CAA and its mechanisms of toxicity to renal tubular cells, age as relevant risk factor and the use of antioxidants in the attenuation of IFO-induced
nephrotoxicity. Recognizing CAA as the metabolite responsible, as well as its mode of toxicity, is beneficial in developing strategies for protection against its detrimental effects. In knowing which cellular mechanisms have been disrupted, it becomes easier to define strategies for mechanistic-based treatments to protect these cellular processes. Progression of research on the use of antioxidants is supported by the knowledge that those cellular functions that have been altered are either protected or corrected. On the research being done with antioxidants, our opinion is that NAC shows the most promise, as we have demonstrated it to protect renal tubular cells from CAA toxicity. It has a current clinical use in children and is known to be safe. For a new molecule to enter pediatric use, many years of preclinical safety studies and multimillion dollars are needed. Such resources are not likely to become available in the hostile economic environment today for a drug that treats relatively few children.

According to the American Physiological Society the definition of translational research is “the transfer of knowledge gained from basic research to new and improved methods of preventing, diagnosing, or treating disease, as well as the transfer of clinical insights into hypotheses that can be tested and validated in the basic research laboratory” [112]. The recent research on the use of NAC in the attenuation of IFO-induced nephrotoxicity fulfills these different perspectives of translational research. The long known clinical presentation of IFO nephrotoxicity has been scrutinized in preclinical studies in different experimental models including renal proximal tubular cell lines, perfused animal kidneys, and ex vivo human and pig kidneys in the hope that the etiology can be clarified and that this knowledge will facilitate the discovery of possible pharmacological interventions. Considerable evidence has pointed to GSH
depletion as a major mechanism involved in impairing endogenous protective mechanisms and resulting in an enhanced renal insult.

The logical remedy would be then to replenish GSH levels; hence, NAC was selected for our studies as one of its main mechanisms of action is to act as a precursor for GSH synthesis. Because we proposed a new therapeutic usage for NAC, preclinical studies are required to determine both efficacy and safety in early development before any clinical trials can be undertaken. Throughout the preclinical studies, we ensured that the studies mimicked the human condition, as much as possible, such as by using clinically relevant drug concentrations and correlating the animal nephrotoxicity model with the human clinical experience. These considerations are likely to enhance the appropriate transition from preclinical discoveries to clinical development of NAC used as a complementary therapy with IFO.

We follow the concepts of translational research, as outlined in this thesis, by bridging the pharmacokinetic studies of NAC in the successful rat experiment with NAC concentrations in clinical patient samples. This will help in dose selection for future clinical trials. Overall, we have implemented different early phases of clinical trial designs to validate a new biological therapeutic practice of NAC in children treated with IFO. The effect of NAC on the antitumor activity of IFO both \textit{in vitro} and \textit{in vivo} is also described, as the integrity of IFO therapy must be maintained during NAC therapy, in order for it to be used a chemoprotectant. This research bridges the scientific and operational gaps between preclinical research and early-stage clinical studies, which provide essential benefits for developing NAC as a complementary therapeutic agent in the near future.
1.8 Hypothesis and objectives

**Overall hypothesis:** The current dose of NAC used for acetaminophen overdose will be clinically effective for renal protection against ifosfamide-induced toxicity in children, without interfering with IFO efficacy.

**Objectives:**

1- To investigate the translational pharmacokinetics of the standard NAC protocol for acetaminophen overdose in children.

2- To investigate the possible negative effects of NAC on the antitumour efficacy of ifosfamide in a cell model.

3- To investigate the possible negative effects of NAC on the antitumour efficacy of ifosfamide in a rodent model.

4- To develop clinical practice recommendations for the safe and effective use of NAC for IFO-induced nephrotoxicity.
1.9 References


40. Skinner R, Sharkey IM, Pearson ADJ, Craft AW. Ifosfamide, mesna, and


54. Sangster G, Kaye SB, Calman KC, Dalton JF. Failure of 2-mercaptoethane sulphonate sodium (mesna) to protect against ifosfamide nephrotoxicity. Eur J


Chapter 2: N-acetylcysteine as a treatment against nephrotoxicity caused by ifosfamide: Translational pharmacokinetics

Part of the chapter has been published:

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2.1 Introduction

The chemotherapeutic agent ifosfamide (IFO) is widely used for the treatment of solid tumors in children [1,2]. Although highly effective, it is associated with high rates of both urotoxicity and nephrotoxicity [3,4]. While severe hemorrhagic cystitis can be effectively mitigated with the concurrent administration of 2-mercaptoenthansulphonate (MESNA)[5,6], there is currently no preventive measure available for the serious nephrotoxicity. The importance of such prophylactic strategy becomes evident when considering the high rate of adverse renal effects occurring as a consequence of IFO therapy. It has been demonstrated that as many as 30% of children treated with IFO will have some degree of renal impairment, and 5% of children will develop full-fledged Fanconi syndrome [7]. Although surviving their cancer, these children will continue to suffer from renal impairment throughout their lives [8].

Ifosfamide is a pro-drug metabolized by cytochrome P450 enzymes 3A4, 3A5, and 2B6, resulting in the production of several metabolites including the active antineoplastic agent IFO mustard, the urotoxic metabolite acrolein, and the nephrotoxic metabolite chloroacetaldehyde (CAA)[9-11]. The nephrotoxic CAA has been identified by us to be produced in toxic quantities by the kidney [12-15]. The mechanism of CAA toxicity is believed to be primarily through oxidative stress with a critical depletion of glutathione (GSH). As a result of exposure to CAA, ATP depletion, increased lipid peroxidation, and decreased glutathione-S-transferase activity also occur in kidney cells [16,17]. Increases in intracellular Ca\(^{2+}\) and pro-inflammatory cytokines TNF-\(\alpha\), IL-\(\beta\)2, and IL-6 [18,19], as well as decreased Na\(^+\)/K\(^+\) exchanger activity [20], have also been
described. As a result of this mode of toxicity, the use of an antioxidant in the prevention of IFO-induced nephrotoxicity is biologically plausible.

N-acetylcysteine (NAC) is an antioxidant varying from the amino acid L-cysteine by one acetyl group (Figure 2.1). NAC provides protection as a GSH precursor, increasing its synthesis and replenishing its stores. It also increases glutathione-S-transferase activity, as well as neutralizes free radicals through its nucleophilic activity [21-23]. It has current clinical use as a topical antimucolytic agent but primarily as an antidote for acetaminophen overdose [24,25]. Importantly, it has also been shown to have a protective effect on the kidney in ischemia/reperfusion injury [26] and cisplatin and cyclosporine-induced nephrotoxicity in experimental systems [27,28]. All of these characteristics suggest NAC as a potentially effective candidate for renal protection against IFO.

Using both in vitro and in vivo models, our group has successfully shown NAC to exhibit a protective effect against IFO-induced renal cell damage [17] and nephrotoxicity [16]. Attenuating both cell death and GSH depletion, NAC demonstrated the ability to protect LLCPK-1 cells against IFO-induced damage at a clinically relevant concentration (0.4 mM) [17]. Similarly in a rodent model, NAC conferred protection against depleted GSH levels, diminished glutathione S-transferase activity, elevated lipid peroxide levels, and morphological damage to the proximal tubules and glomeruli, all seen to occur as a result of IFO therapy. NAC also diminished the severity of tubule dysfunction with significant attenuation of elevated serum creatinine, as well as elevated beta 2-microglobulin and magnesium urinary excretion [16].
However, despite being the drug of choice for acetaminophen overdose, there are very limited data on the pharmacokinetics of NAC in children, knowledge that is of critical importance in determining an effective therapeutic dose for preventing IFO-induced nephrotoxicity. The routine clinical use of intravenous (IV) NAC as a standard therapy in children poisoned by acetaminophen overdose provides not only information about its safety and efficacy in children but also an opportunity to explore its use in children affected by acetaminophen overdose, in the context of preventing IFO-induced nephrotoxicity. Blood samples are routinely drawn during treatment to assess acetaminophen levels and liver function, allowing us the opportunity to characterize the systemic exposure of NAC in children. Our objective was to compare the systemic exposure to NAC in children treated for acetaminophen overdose (in terms of area under the curve [AUC] over time) to the systemic exposure to NAC associated with prevention of nephrotoxicity in our experimental rat model. This pharmacokinetic comparison is critical in translating the systemic exposure associated with a therapeutic effect in the rat to the needed systemic exposure in children.
2.1 The chemical structure of NAC, varying from L-cysteine by one acetyl group.
2.1 Materials and Methods

2.1.1 Chemicals

NAC, sodium borohydride, and butylated hydroxytoluene were purchased from Sigma-Aldrich Canada Ltd (Oakville, Ontario, Canada). Monobromobimane was purchased from EMD Chemicals Inc. (Gibbstown, New Jersey). IFO, 2-dechloroethylifosfamide (2-DCEI), and 3-DCEI were purchased from Niomech (Bielefeld, Germany). Deuterated 2-dechloroethylifosfamide (d_6-2-DCEI) and deuterated 3-DCEI (d_4-3-DCEI) were kindly provided by Dr Susan Ludeman of Duke University (Durham, North Carolina).

2.1.2 Pharmacokinetic study of n-acetylcysteine in rats

All experimental protocols were approved by the University of Western Ontario Animal Care and Use Council. Male Wistar albino rats, 225 to 250 g, were purchased from Charles River Canada (Montreal, Quebec, Canada). They were housed at a constant temperature (22° ± 1°C) with a regular 12-hour light and dark cycle and fed a standard rat chow and water ad libitum.

A single bolus dose of 1.2 g/kg of NAC was given intraperitoneally. Blood samples were collected by intracardiac puncture at 5, 7.5, 10, 15, 30, and 45 minutes, as well as 1 and 1.5 hours. Six animals were studied for each time point. Blood samples were immediately centrifuged for 15 minutes at room temperature at 3000 rpm. Serum samples were stored at –80°C until analysis.

2.1.3 Pharmacokinetic study of n-acetylcysteine in children

The study was approved by the Research Ethics Board (REB) at the University of Western Ontario (Health Sciences REB No. 13625E).
Blood samples were obtained from children and adolescents who were admitted for acetaminophen overdose while being treated with IV NAC at the Children’s Hospital of Western Ontario (London, Ontario) and from hospitals surrounding the Greater Toronto Area counseled by the Ontario Poison Control Center in Toronto. All children received the recommended dosing schedule for acetaminophen overdose at 150 mg/kg for 60 minutes, 50 mg/kg for the next 4 hours, and 100 mg/kg for another 16 hours. Blood samples were collected during routine measurements of acetaminophen levels and liver function tests according to the management schedule at the hospital. We received discarded blood samples from these patients after these routine measurements were completed. The number of blood samples varied among patients as per the discretion of their treating physicians. It is a standard practice to keep the blood samples that have already been analyzed for 1 week in the core laboratory. We were notified of any cases. In a parallel line of investigation, we have documented the stability of NAC in frozen samples for more than a month.

Two pediatric case reports were also obtained describing two children receiving IFO in combination therapy who developed acute renal failure and were subsequently treated with NAC. Blood samples were obtained from one case for analysis of NAC and IFO concentrations, whereas serum creatinine and glomerular filtration rate were reported for the second case.

2.1.4 Analysis of plasma N-acetylcysteine concentrations

Total NAC concentrations were analyzed by high performance liquid chromatography, modified from the method of Jacobsen et al. [29]. To reduce surface tension, N-isoamyl alcohol was added to each sample, followed by sodium borohydride,
which was used as a reducing agent for dithiol bonds. After a brief incubation,
hydrochloric acid was added to neutralize excess sodium borohydride. Thiols in the
samples were derivatized with monobromobimane, a fluorescent labeling agent. To
prevent any large proteins from eluting through the chromatography column, perchloric
acid was used to precipitate the proteins, after which the samples were centrifuged at
9000 g for 5 minutes. To neutralize the acid, the sample pH was raised to 3.5 by adding
a solution of 2 M citrate in 10 M sodium hydroxide. The samples were centrifuged at
9000 g for an additional 3 minutes. One hundred microliters of the supernatant of each
sample were transferred into a glass insert, and 5 uL was injected into a 15 cm x 3.9
mm, 5 u Novapak C18 column maintained at 40° C in a Hewlett Packard 1090 LC. The
mobile phase (Pump A: 4% acetonitrile/25 mM ammonium formate buffer, pH = 3.8
with formic acid; pump B: 70% acetonitrile/10 mM KH2PO4 buffer, pH = 3.0 with
phosphoric acid) was run at 0.5 mL/min, and the eluent was detected by a Waters 474
scanning fluorescence detector with the excitation set to 390 nm and the emission set at
480 nm. The accuracy and precision of the assay were 1.3% and 2.0%, respectively.

2.1.5 Pharmacokinetic calculations

The AUC for plasma concentration-time curve was calculated according to the
trapezoidal rule during the period of sample collection, where:

$$\text{AUC}_{c_0-c_1} = C_0 + C_1/2 \times (T_1 - T_0).$$

A sum of AUC from each time interval gave the total AUC. Extrapolation of
AUC to infinity was made by \( \text{AUC}_{t-\infty} = \frac{C_t}{K_e} \), where \( C_t \) is the last time point the
sample was measured and $K_e$ is the elimination rate constant calculated from the descending slope of the plasma concentration curve. The final AUC in children is the sum of AUC from the definite area plus the residual area. The final AUC in rats is the sum of AUC from the definite area plus the residual area, multiplied by 6, as the therapeutically effective model consisted of 6 daily doses of 1.2 g/kg.

2.1.6 Analysis of ifosfamide plasma concentrations

Plasma samples were analyzed using our previously published method by Aleksa et al. [30]. All analyses were carried out under ambient temperatures. Briefly, 100 µL of plasma was used for analysis, and 100 µL blank plasma was spiked with IFO (5-100 ng/mL) for the standard curve. All samples were spiked with 10 µL of internal standard mix (10 µM trofosfamide). Methylene chloride was added for liquid-liquid extraction. First, we vortexed the samples for 45 seconds, following which they were centrifuged for 10 minutes at 4000 rpm at 4°C. The top aqueous layer was removed and using a stream of nitrogen gas, the organic layer was dried. Reconstitution of the samples was done using 30 µL of 10 mM ammonium acetate (pH, 7.0), and 5 µL was injected into the LC/MS/MS for analysis.

The samples were analyzed on an Agilent 1100 Series high-performance liquid chromatography (Agilent Technologies, Mississauga, Ontario) equipped with an Agilent 1100 Series binary pump. The separation of analytes was done with a CHIRAL-AGP column (150 x 4.0 mm, 5 µM, Chrom Tech, Apple Valley, Minnesota) using 10 mM ammonium acetate in water (pH, 7.00) (A) and 30 mM ammonium acetate in water (pH 4.00) (B) with the following gradient: time 0.0 minutes, A 5 100%, flow 400 mL/min; time 4.5 minutes, A 5 50%, flow 400 mL/min; time 7.10 minutes, A 5 100%,
flow 600 mL/minute. Introduction of the samples to the chromatographic system was completed with an Agilent 1100 Series Autosampler (Agilent Technologies). An LC-MS/MS API4000 Triple-Quadrupole was used for mass spectrometric detection (Applied Biosystems, MDS SCIEX, Foster City, California). LC-MS/MS API4000 Triple-Quadrupole was set with an API turbo ion spray ionization (PIS) source and functioned in positive ion mode. Analytes were quantified using multiple reactions monitored, with transitions monitored for IFO being m/z 261.1 to 154. Voltage of the ion spray was set at 5.5 kV, with source temperature was kept constant at 500°C. Data acquisition was performed using MDS-SCIEX Analyst software (version 1.4).

2.3 Results

2.3.1 N-acetylcysteine pharmacokinetics in a rodent model

The mean systemic exposure of a 1.2 g/kg intraperitoneal dose of NAC in rats was 3.12 mM·h (Figure 2.2). In our rat model, in which NAC therapy conferred protection against IFO-induced nephrotoxicity, we gave NAC at 1.2 g/kg intraperitoneally daily for 6 days [16]. Therefore, the total mean systemic exposure of the rat model demonstrating therapeutic efficacy was 18.72 mM·h. The lower and upper limits of systemic exposure in the rat model were 9.92 and 30.02 mM·h, respectively (Table 2.1).
**Figure 2.2** The systemic exposure of NAC in a rat model receiving a 1.2 g/kg intraperitoneal dose of NAC (n = 3 for each time point). AUC is 3.12 mM·h, where the dotted line represents the residual area calculated. Total systemic exposure is 18.72 mM·h, as 6 doses were given.
Table 2.1 A comparison of mean, higher and lowest systemic exposures of NAC between children/adolescents and the rat model.
<table>
<thead>
<tr>
<th></th>
<th>Mean Systemic Exposure, mM·h</th>
<th>Highest Systemic Exposure, mM·h</th>
<th>Lowest Systemic Exposure, mM·h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Children/adolescents</td>
<td>14.48</td>
<td>32.96</td>
<td>6.22</td>
</tr>
<tr>
<td>Rat Model</td>
<td>18.72</td>
<td>30.02</td>
<td>9.92</td>
</tr>
</tbody>
</table>
2.3.2 \textit{N-acetylcysteine pharmacokinetics in children and adolescents}

Discarded blood samples were collected from 16 children and adolescents treated for acetaminophen overdose. Six children were excluded from AUC calculations either because there was no information on the time blood samples were drawn or because they were not given the standard 21-hour NAC IV therapy. Ten of the 16 children were given the standard NAC protocol for acetaminophen overdose and had a mean systemic exposure of 14.48 mM·h (Table 2.2). The lowest systemic exposure seen in children was 6.22 mM·h, and the highest was 32.96 mM·h (Figure 2.3 and Table 2.1).
Table 2.2 AUC calculations for patients receiving the standard NAC protocol for acetaminophen overdose.
<table>
<thead>
<tr>
<th>Subject</th>
<th>Systemic Exposure of Children/Adolescents Treated With the Standard NAC Protocol for Acetaminophen Overdose (mM·Hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>32.96</td>
</tr>
<tr>
<td>2</td>
<td>20.57</td>
</tr>
<tr>
<td>3</td>
<td>17.58</td>
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<tr>
<td>4</td>
<td>8.01</td>
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<tr>
<td>7</td>
<td>10.93</td>
</tr>
<tr>
<td>8</td>
<td>6.22</td>
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<td>9</td>
<td>7.46</td>
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<td>11</td>
<td>13.27</td>
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<td>13</td>
<td>16.95</td>
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<tr>
<td>14</td>
<td>10.38</td>
</tr>
<tr>
<td>Mean</td>
<td>14.48 ± 7.62</td>
</tr>
</tbody>
</table>
**Figure 2.3** The systemic exposure of NAC in a child (subject 1) receiving the standard NAC IV protocol, 150 mg/kg for 60 minutes, 50 mg/kg for 4 hours, and 100 mg/kg for 16 hours. Area under the curve is 32.96 mM·h, where the dotted line represents the residual area calculated.
2.4 Case reports

2.4.1 Case report 1

A 10-year-old girl was hospitalized at the University Children’s Hospital Belgrade, Serbia, with primitive neuroectodermal tumor and acute renal failure. Her tumor was aggressive with liver metastases. She underwent surgery; however, some tumor masses could not be removed. The patient received the following protocol:

*Day 1.* Ifosfamide 3 g/m², vincristine 1.5 mg/m², dactinomycin 1.5 mg/m², and MESNA and NAC (IV dosage for acetaminophen overdose: 150 mg/kg loading dose over 60 minutes of continuous infusion, 50 mg/kg over 4 hours, 100 mg/kg over 16 hours). She underwent dialysis after 24 hours. There were no adverse reactions apart from vomiting.

*Day 2.* Ifosfamide 3 g/m² and MESNA and NAC (IV dosage for acetaminophen overdose). She underwent dialysis after 19 hours. There were no adverse reactions.

*Day 3.* IFO 3 g/m² and MESNA without NAC. After 12 hours, face fasciculations appeared, and electroencephalogram confirmed encephalopathy, so she underwent dialysis after 15 hours. After continuous dialysis, the patient recovered. She also received ondansetron, meropenem, furosemide, and morphine as needed. After the first dose, she exhibited unexpected diuresis of 200 mL/kg, whereas after the third dose the diuresis was completely recovered.

She showed all signs of tumor lysis. She did not show either clinical or
laboratory evidence of renal failure. The child’s systemic exposure to NAC was 21.68 mM·h after the first NAC dose and 14.92 mM·h after the second, resulting in a total systemic exposure of 36.60 mM·h (Figure 2.4). Ifosfamide exposure was also calculated (Figure 2.5).
Figure 2.4 The systemic exposure of NAC in patient from case report 1, where the patient received 2 doses of the standard NAC IV protocol, 150 mg/kg for 60 minutes, 50 mg/kg for 4 hours, and 100 mg/kg for 16 hours, during her IFO therapy. AUC is 36.60 mM·h.
Figure 2.5 Total systemic exposure of IFO in patient from case 1, where the patient received 3 doses of 3 g/m².
2.4.2 Case report 2

A 15-year-old Hispanic male was admitted to the hospital for chemotherapy for abdominal recurrence of mixed germ cell testicular tumor. He was diagnosed with the primary tumor 8 months earlier and underwent right orchiectomy and chemotherapy per protocol ACTZ0132, which included cisplatin, etoposide, and bleomycin. His serum creatinine was elevated after the first chemotherapeutic cycle and remained in the higher ranges of 1.2 to 1.6 mg/dL throughout. His serum alpha fetoprotein and beta HCG levels showed a rise 7 months after initial induction chemotherapy; he was reassessed and was found to have an abdominal relapse on computed tomography. On the day of admission, his serum creatinine was 1.23 mg/dL, sodium 137 mmol/L, potassium 4.1 mmol/L, magnesium 1.8 mg/dL, and alpha fetoprotein 1204.7 ng/mL. The next day, after hydration with fluids, the patient received the following protocol:

*Day 1.* Paclitaxel 257 mg, carboplatin 653 mg, IFO 3400 mg, and MESNA 684 mg.

*Days 2-5* IFO 3420 mg and MESNA 684 mg, along with appropriate prehydration and posthydration. Creatinine started rising by day 2 and steadily increased up to 2.06 mg/dL by day 7, along with decrease in urinary output (Figure 2.6).

*Day 7-10.* Oral NAC 600 mg BID. NAC was discontinued on day 11.

After reaching a peak of 2.25 mg/dL on day 8, serum creatinine showed a steady decrease to 1.12 mg/dL on the day of discharge, which was day 15. The urine output also showed similar improvement on initiation of NAC. His serum magnesium throughout this period stayed between 1.7 and 1.9 mg/dL.
Figure 2.6 Serum creatinine levels of patient from case report 2 in response to NAC therapy.
2.5 Discussion

Nephrotoxicity is a serious adverse effect of IFO therapy, affecting 30% of children treated [7]. As this renal toxicity is likely mediated primarily by oxidative stress, the use of NAC, an antioxidant, as a prophylactic treatment for its prevention has been proposed. This study is one of the first to examine the pharmacokinetics of NAC in the pediatric age group. The goal of this study was to determine whether NAC plasma concentrations (in terms of AUC) in patients are producing adequate systemic exposure compared to our experimental rodent model. Our results show that therapeutically effective levels of NAC in preventing IFO-induced nephrotoxicity in a rat model are comparable to systemic exposure in children being treated with the 21-hour IV NAC protocol for acetaminophen overdose. Mean systemic exposure in the rat model was 18.72 mM·h, compared to 14.48 mM·h in children. The distribution of systemic exposures in the rat model and in children was also similar, with the lowest exposures being 9.92 mM·h and 6.22 mM·h in the rat model and in children, respectively. Similarly, the highest range systemic exposure seen in the rat model and in children was 30.02 mM·h and 32.96 mM·h, respectively. These results suggest that the current therapy of NAC for pediatric acetaminophen overdose shows promise in providing renal protection for children who are on IFO therapy. We have corroborated these results with two cases of IFO-induced nephrotoxicity, where NAC appeared to mitigate nephrotoxicity. Measurement of NAC in case 1 further supports the level of systemic exposure to NAC needed to protect against nephrotoxicity. Out of these two cases described by us, one child was dialyzed, and hence in said case clinical improvement may not be directly related to NAC. A randomized control trial is needed
to quantify the efficacy of NAC for IFO-induced nephrotoxicity.

Of importance, in comparing the systemic exposure of the rats and children, the systemic exposure in the pediatric acetaminophen overdose cases might have been an underestimate. Blood obtained for calculating the AUC of NAC in children was based on discarded samples; therefore, we were limited in our ability to obtain a specific preset number of samples or samples at predetermined time points. On AUC calculation, we had to assume that the highest NAC concentration measured was the peak concentration, although blood levels could potentially still be increasing; hence, our calculated AUC may be an underestimation of the actual systemic exposure. In addition, the difference in length of treatment between the rat model and the children has to be considered. Our therapeutically effective rat model was treated with NAC for 6 days, whereas children treated for acetaminophen overdose were treated only once for a 21-hour period. Our goal was to conclude if the 21-hour IV protocol provides an overall systemic exposure equivalent to the rat model, in terms of total body exposure to NAC not length of exposure. However, we may also consider that children receiving a similar number of NAC treatments as the rat model would achieve a systemic exposure that well exceeds the AUCs found to be therapeutically effective in our rodent model. Moreover, there are descriptions of NAC protocols for paediatric acetaminophen toxicity that extend several days.

Although several antioxidants, including resveratrol [19], melatonin [20], taurine [31], glycine [32], L-histidinol [33], and thymoquinone [34], have also been shown to prevent IFO-induced nephrotoxicity in rat models, in the clinical setting, NAC is currently the only one approved for and commonly used in children. Although the
antioxidants melatonin and resveratrol are being assessed for use in children for sleep disorders [35,36] and obesity prevention [37], little to no information on their safety in children is available, making their use for an alternative indication, such as renal protection during IFO therapy, years from being realized. NAC is widely used clinically in both children and adults as treatment for acetaminophen overdose. Acetaminophen is the most regularly used pain-relieving agent worldwide [38,39] and is the most common cause of both intentional and unintentional drug overdoses. In the United States, more than 30,000 hospitalizations occur annually involving acetaminophen overdose [40]. Because NAC is used as an antidote under these circumstances, this provides a wealth of information on the safety and efficacy of NAC in children. This information is critical when assessing the use of NAC for an alternative indication in children, in this case renal protective strategies when using nephrotoxic agent, and from a time perspective makes NAC the most relevant choice.

The most commonly used NAC protocol for acetaminophen overdose is the 21-hour IV protocol, which has been used for decades by Canada, Europe, and Australia and since 2004 by the United States. The 21-hour IV NAC protocol includes a 150 mg/kg loading dose infused for 60 minutes and 2 maintenance IV doses: 50 mg/kg for 4 hours and 100 mg/kg for 16 hours [41,42]. As this is the standard therapy associated with acetaminophen overdose in Canada, the systemic exposures of NAC measured in our patients adequately represent this course of therapy. Overall, it provides a total of 300 mg/kg of NAC over 21 hours (Table 2.3). Before 2004 in the United States, a 72-hour oral protocol for NAC was the standard therapy in cases of acetaminophen overdose [42]. This protocol includes a 140 mg/kg loading dose, followed by seventeen
70 mg/kg maintenance doses every 4 hours [43]. It allows a total of 1330 mg/kg over 72 hours or 53.2 to 133 mg/kg systemically over 72 hours in consideration of the oral bioavailability of NAC, found to range from 4% to 10% [44]. Alternatively, a 36-hour oral protocol and 48-hour IV protocol have also been described [45,46]. Although the 36-hour protocol is a truncated version of the 72-hour oral protocol, the 48-hour IV protocol involves a 140 mg/kg loading dose and twelve 70 mg/kg maintenance doses every 4 hours, providing a total of 980 mg/kg over 48 hours. When compared in terms of safety and efficacy in treating acetaminophen overdose, all 3 protocols, the 21-hour IV, 48-hour IV, and 72-hour oral protocols, were found to be equivalent, with the 48-hour and 72-hour protocols appearing superior in later treated patients. In the context of this translational research, a 2- to 3-fold higher systemic exposure to NAC than the one used by us has also been shown to be safe in children. This may be of importance if the pharmacodynamics of NAC in IFO-induced nephrotoxicity necessitates more NAC than was effective in the rat model.

In terms of safety, both oral NAC and IV NAC have been shown to be safe with an uncommon incidence of adverse events [47]. Nausea and vomiting are common side effects associated with administration of both oral and IV NAC [48,49]. Anaphylactoid reactions are more commonly associated with IV NAC, as opposed to oral NAC, occurring at a rate of 0% to 48% [47]. This is thought to be attributed to the infusion rate; however, most cases are minor and easily manageable. Under most circumstances, NAC can be resumed and the treatment protocol completed. Life-threatening or serious events have an incidence of less than 5% [47,50-53]. One report of an anaphylactoid reaction occurring during oral NAC treatment has been documented, although after
treatment of symptoms the patient resumed and completed the NAC protocol [54].

To the best of our knowledge, this is the first attempt to determine NAC pharmacokinetics in a pediatric age group. The conventional dosage schedule used in the 21-hour IV NAC treatment for acetaminophen overdose in children produced systemic exposure comparable to that of our rat model associated with therapeutic effect in nephrotoxicity prevention. Similarly, with NAC protocols that provide an overall higher dose existing, the ability to achieve systemic exposures closer to or greater than our successfully treated rodent model becomes possible, even further supporting the use of NAC for this alternative indication. We describe here the first two case reports of NAC given during IFO therapy, associated with successful prevention of acute renal toxicity. Case report 1 presents the successful use of the 21-hour IV protocol, and case 2 shows the successful use of oral NAC (600 mg orally, twice per day), despite its low oral bioavailability (4%-10%). These cases suggest not only that the current NAC protocol used for acetaminophen overdose, for which pharmacokinetics in children was assessed in this study, will be successful as a prophylactic treatment for IFO-induced nephrotoxicity but that other formulations and doses may also provide adequate protection. These cases also corroborate the safe and effective use of NAC in children, as reported by previous studies. Together with our recent data on the lack of effect of NAC on IFO efficacy, described later in this thesis, the present study, as well as the case reports, further strengthens the choice of NAC as a therapeutic option to prevent IFO nephrotoxicity in children. Our results support a randomized control trial to quantify the effects of NAC on IFO nephrotoxicity. Based on the preliminary clinical results available to us, it is reasonable to consider NAC treatment when the first signs of IFO-
induced nephrotoxicity emerge. Furthermore, our study looks at the systemic exposure in children treated only with the standard 21-hour IV protocol; the existence of protocols varying in route of administration, overall dose, and length affords future opportunity to assess the utility of such protocols.
Table 2.3 A comparison of total systemic dose received (mg/kg) by each NAC protocol described in the literature.
<table>
<thead>
<tr>
<th>Protocol Dose</th>
<th>Loading Dose mg/kg</th>
<th>Maintenance Dose</th>
<th>Total Systemic mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>72-hour oral</td>
<td>140</td>
<td>70 every 4 hours x 17</td>
<td>53.2-133</td>
</tr>
<tr>
<td>48-hour intravenous</td>
<td>140</td>
<td>70 every 4 hours x 12</td>
<td>980</td>
</tr>
<tr>
<td>36-hour oral</td>
<td>140</td>
<td>70 every 4 hours x 9</td>
<td>30.8-77</td>
</tr>
<tr>
<td>21-hour oral</td>
<td>150</td>
<td>50 for 4 hours followed by 100 for 16 hours</td>
<td>300</td>
</tr>
</tbody>
</table>

Oral bioavailability (4-10%) is accounted for in total systemic dose for oral protocols.
2.6 References


27. Mishima K, Baba A, Matsuo M, et al. Protective effect of cyclic AMP against...


Chapter 3: The effect of n-acetylcysteine on ifosfamide antitumour efficacy: *in vitro*

Part of the chapter has been published:


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* Chen N and Hanly L are co-first authors.
3.1 Introduction

With its use today in many pediatric and adult cancers including rhabdomyosarcoma, osteosarcoma, neuroblastoma, Ewing’s sarcoma, leukemia and non-hodgkins lymphoma, ifosfamide (IFO) is an important chemotherapeutic agent in a large repertoire of available antineoplastic drugs [1-5]. IFO was developed in the 1970s as an analogue of the nitrogen mustard cyclophosphamide [6]. It is a DNA alkylating agent, causing cytotoxicity through its ability to form both inter- and intra-DNA strand cross links, preventing cell replication and in time leading to cell death [7]. Despite its high therapeutic index, rather rare cross-resistance, and treatment success for certain cancers that do not respond to cyclophosphamide [8, 9], IFO use does not come without serious health risks. Adverse effects common to many cancer drugs including gastrointestinal toxicity, myelosuppression, and neurotoxicity occur with IFO therapy, along with two dose limiting late effects: urotoxicity and nephrotoxicity [10-13].

The use of IFO was first limited due to extremely frequent and severe bladder toxicity [14]. This is now successfully treated with sodium 2-mercaptoethanosulphonate (MESNA), routinely given in conjunction with IFO, in the amount of 40% (if given orally) or 20% (if given intravenously), of the ifosfamide dose. Following successful use of this chemoprotectant, treatment protocols were able to include both higher and more frequent IFO dose schedules, leading to the awareness of a new dose limiting toxicity, that of the kidney [2, 15, 16].

Kidney toxicity associated with IFO is an age-dependent issue, with 30% of children who are treated with IFO developing some form of nephrotoxicity [17-19]. The nephrotoxicity can vary from minor tubular dysfunctions to full blown proximal tubular
dysfunction in the form for Fanconi syndrome, which affects 5% of children treated [20-22]. Glomerular toxicity may also be present in as many as 30% of children [11]. These dysfunctions may result in disorders such as hypophosphatemic rickets, renal tubular acidosis and chronic renal failure. Severely impacting the quality of life of those affected, their toxicity may lead to the need for lifelong supplementations, renal dialysis, renal transplant, or in some case death [11, 20-24].

It is not IFO itself that is responsible for urotoxicity and nephrotoxicity, rather products of its metabolism, acrolein and chloroacetaldehyde (CAA) respectively [25-27]. Metabolized by cytochrome P450's 3A4, 3A5 and 2B6, IFO is a pro-drug requiring biotransformation to produce the active antineoplastic agent, ifosfamide mustard (IFM). This occurs through ring hydroxylation, resulting in both IFM and acrolein. 50% of IFO is metabolized through this pathway, while the remainder undergoes side chain oxidation, producing the metabolites 2- or 3- dechloethylifosfamide with equal amounts of CAA [28-30]. CAA is currently understood to be responsible for nephrotoxicity associated with IFO. Early studies demonstrated that systemic exposure of CAA, as measured by area under the curve, does not have a positive correlation with degree of nephrotoxicity. Those having a higher AUC showed a lesser degree of nephrotoxicity [31]. However, more recent findings suggest that renal, not hepatic metabolism of IFO is responsible for the production of renal toxic quantities of IFO. These studies demonstrated that the kidney not only possess the CYP P450s required for metabolism, but that they kidney is capable of producing quantities shown to be nephrotoxic [27, 32-34].
CAA functions as a nephrotoxin primarily through the generation of oxidative stress. Exposure to CAA results in several insults including increases in ROS, depletion of ATP, increases in intracellular calcium, increases in intracellular sodium, release of pro-inflammatory cytokines, and importantly, depletion of GSH [35-43]. These mechanisms of toxicity, resulting in increased oxidative stress, aid in defining a clear direction for protective strategies: antioxidants.

As MESNA, an antioxidant, is currently used alongside IFO, its renal protective effects have been assessed. While *in vitro* work has successfully shown MESNA will also protect against IFO-induced nephrotoxicity, rodent studies bring to light that this is not the case *in vivo* [44]. MESNA rapidly auto-oxidizes to diMESNA in the blood and is then reduced back to its free thiol form in kidney; in its reduced state, it is able to protect the bladder epithelium. Reduction back to MESNA occurs at the expense of renal glutathione (GSH), which becomes oxidized and requires its own reduction before it can again protect cells against oxidative damage [45]. Exhaustion of GSH stores within the kidney, an organ that already has lower GSH levels than the liver, may predispose the kidney to damage from oxidative insult, which as discussed above, is the primary mechanism of IFO toxicity.

N-acetylcysteine, however, in an antioxidant that has shown great promise protecting against IFO kidney toxicity. NAC is an effective renal protectant in both proximal tubule cell and rodent models, using clinically relevant concentrations [46-48]. And while several other antioxidants have also shown protective properties against IFO-induced nephrotoxicity in animal models [40, 41, 49-52], NAC is the most promising choice. First, like the other antioxidants, NAC is a nucleophile with ROS-scavenging
properties. However unlike the others, NAC is also a precursor to GSH synthesis [53-55]. This becomes important when evaluating the significant reduction of GSH in the kidney, caused by IFO. Second, NAC is currently the only antioxidant to have shown protection, which is used clinically. As an antidote for acetaminophen overdose, NAC is currently both effectively and safely used in children for hepatic protection against oxidative damage [56]. Furthermore, the 21-hour IV dose used in acetaminophen overdose, as discussed earlier in this thesis, should also be sufficient to provide renal protection [57].

However, despite the growing support in favor of the effective use of NAC to prevent IFO-induced nephrotoxicity, it must first be demonstrated that NAC does not interfere with the anticancer effects of IFO. The purpose of this study is to evaluate the effects NAC on IFM in two relevant cancer cell lines. The addition of MESNA was also included to assess if the NAC + MESNA combination has any effect on IFM efficacy.

3.2 Materials and Methods

3.2.1 Reagents

MESNA, NAC and the tetrazolium salt MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were bought from Sigma–Aldrich Canada Ltd. (Oakville, ON., Canada); IFO from Baxter Oncology GmbH and Baxter Corporation, Hallen-Westfalen, Germany) and IFM from Eno Research and Development (Research Triangle Park, Durham, N.C., USA).

3.2.2 Cell lines and cell cultures

Two different pediatric cancer cell lines that are sensitive to IFO treatment were
used: neuroblastoma SK-N-BE(2) (ATCC No. CRL-2271, Cedarlane Laboratories Inc., Burlington, ON, Canada) and rhabdomyosarcoma RD114-B (ATCC No. CRL-7763, Cedarlane Laboratories Inc.). Both neuroblastoma cells and rhabdomyosarcoma cells were cultured as subconfluent monolayers at 37 °C in a humidified atmosphere with 5% CO₂. Neuroblastoma cells were cultured in a 1:1 mixture of Eagle’s minimum essential medium and F12 medium with 10% fetal bovine serum (Invitrogen, Burlington, ON, Canada) and 1% penicillin–streptomycin (Invitrogen). Rhabdomyosarcoma cells were cultured in Dulbecco’s modified eagle’s medium with 10% fetal bovine serum, 1% penicillin–streptomycin, and 2 mmol/L L-glutamine (Invitrogen). Both cell lines were supplied with fresh media and subcultured 2–3 times each week by trypsinization with 0.25% trypsin with EDTA.

3.2.3 Experimental Design

First, a dose response curve for both IFO and IFM was established by incubating both rhabdomyosarcoma and neuroblastoma for 24 hours at various concentrations, followed by MTT assay at 24 hours.

Next, the different drug treatment groups were added to each well of the microplate on day one. Both pretreatment and concurrent regimens were employed to each treatment group. Pretreatment groups were treated with a 4-hour incubation of NAC alone on day 0. The treatment groups were as follows:

1) 100 uM IFM alone. Concentration as informed by previous experiment
2) 0.4 mM NAC + 100 uM IFM
3) 0.3 mM MESNA + 100 uM IFM
4) 0.4 mM NAC + 0.3 mM MESNA + 100 uM IFM
Cells were incubated for 4 hours, following which drug containing media was removed and fresh medium was added. Cells were cultivated for 4 days, with MTT being added on day 5.

Concentrations of both NAC and MESNA are clinically relevant, with 0.4 mM of NAC being the estimated steady state concentration in children [46] and 0.3 mM being the minimum urinary concentration of MESNA in children and adolescents [13].

### 3.2.4 MTT assay

Cell viability was measured using the MTT assay as described by Mosmann [58]. Cells were incubated in 96-well plates containing either $1.5 \times 10^4$ cells per 100 μL adherent rhabdomyosarcoma cells or $4 \times 10^4$ cells per 100 μL mixture of suspension and adherent neuroblastoma cells. Depending on the treatment group, a drug or combination of drug solution was added to each well of the microplate, and incubated for the appropriate amount of time. On the specified day, as informed by the treatment regimen, 10 μL of sterile MTT solution (5mg/mL MTT) in phosphate buffered saline was added to each well, followed by incubation at 37°C for 2.5 hours. The reaction was stopped with 100 μL of acidic isopropanol solution. Difference in absorbance values at 570 nm and 690 nm were determined using Tecan Safire™ microplate reader (Durham, NC).

### 3.3.5 Statistical analysis

Studies were replicated at least 3 times or more. Comparison of treatment groups was done using a one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls post hoc test. Values are presented as means±standard error.
3.3 Results

3.3.1 Concentration dependant effects of ifosfamide and ifosfamide mustard

We first determined the effect of the parent compound IFO and the pharmacologically active metabolite IFM on cell growth of RD114-B and SK-N-BE(2) cell lines, two relevant pediatric cancers (Figure 3.1 & 3.2). In either cell lines, the parent drug ifosfamide did not result in decreased cell growth until non-clinically relevant concentration of 5 mM. On the other hand, IFM produced a dose-dependent decrease in cell viability at low micromolar concentrations in both cell lines. At 100 μmol/L IFM more than 50% cell death was observed (n=3).
**Figure 3.1** The effect of IFO and IFM on RH cell growth, expressed as a percentage of control. (*, p < 0.05  **, p < 0.01).
Figure 3.2 The effect of IFO and IFM on NR cell growth, expressed as a percentage of control. (**, p < 0.01).
3.3.2 The effect of pre-treatment with n-acetylcysteine on tumor cells treated with ifosfamide mustard

A single concentration of each metabolite was used for the combined treatments that included NAC, MESNA, and NAC plus MESNA. In both cancer cell lines, as anticipated, 100 μmol/L of the antineoplastic metabolite IFM significantly decreased cellular viability (p < 0.001) (Figure 3.3 & 3.4). When NAC alone was given together with IFM, there were no significant changes in viability as compared with the effect of IFM treatment alone. MESNA has been routinely used as part of concurrent treatment with ifosfamide. In accordance with clinical experience, MESNA did not significantly alter cell viability when it was given concurrently with IFM. When both thiols MESNA and NAC were given together with IFM, there was no further increase in the viability of these cancer cells (n=5).
Figure 3.3 The effect of pre-treatment with NAC + IFM on RH cell growth, expressed as a percentage of control. (***, p < 0.001). NAC and N, n-acetylcysteine; MES and M, sodium 2 mercaptoethanesulfonate.
Figure 3.4 The effect of pre-treatment with NAC + IFM on NR cell growth, expressed as a percentage of control. (***, p < 0.001). NAC and N, n-acetylcysteine; MES and M, sodium 2-mercaptoethanesulfonate.
3.3.3 Effect of concurrent treatment of n-acetylcysteine on tumor cells treated with ifosfamide mustard

We compared the 2 different treatment regimens to determine whether pretreatment of the cancer cells with NAC would produce a result different from that achieved by concurrently treating them with NAC and the metabolites. As most patients with malignancies appear to have markedly lower plasma GSH levels than patients in good health [59], addition of a modulating agent like NAC, which has the ability to increase intracellular and plasma GSH [53], may increase the endogenous protective capacity to deal with reactive toxic metabolites. As shown in both cancer cell lines, the different types of combined treatments with IFM produced no significant changes in cellular viability as compared with the effect of IFM treatment alone (n=4) (Figure 3.5 & 3.6).
Figure 3.5 The effect of concurrent NAC + IFM on RH cell growth, expressed as a percentage of control (***, p < 0.001). NAC and N, n-acetylcysteine; MES and M, sodium 2-mercaptoethanesulfonate.
Relative RH Cell Survival (% of Control)

Control
IFM 100 μmol/L
IFM 100 μmol/L + NAC
IFM 100 μmol/L + MES
IFM 100 μmol/L + N + M

***
**Figure 3.6** The effect of concurrent NAC + IFM on RH cell growth, expressed as a percentage of controls. (***, p < 0.001.) NAC and N, n-acetylcysteine; MES and M, sodium 2-mercaptoethanesulfonate.
3.4 Discussion

With the 5 year survival rate for childhood cancer climbing above 80%, and over 270,000 childhood cancer survivors in the United States, more focused efforts to prevent late effects of chemotherapy are required [60, 61]. Two thirds of pediatric cancer survivors will develop health conditions as a result of their cancer therapy with one third dealing with conditions that are either severe or life-threatening [62]. These debilitating conditions hinder the quality of life of these survivors, and while they may be able to live a life that is cancer free, their health continues to suffer.

Our group has extensively studied the use of NAC as a chemoprotectant against IFO-induced nephrotoxicity. We have previously demonstrated that NAC will protect against IFO cytotoxicity and depleted GSH in LLCPK-1, a porcine renal proximal tubule cell line, at concentrations shown to be therapeutically relevant in children based on pharmacokinetic modeling [46, 47]. NAC also protects against a rodent model of IFO-induced nephrotoxicity, in which depleted glutathione, impaired glutathione S-transferase and lipid peroxidation were all observed and protected against with NAC. Also observed was increased serum creatinine, and urinary loss of β2 microglobulin and magnesium, as well as loss of nephron morphological integrity, again prevented with treatment of NAC [48]. However, despite the evidence in support of NAC to prevent the nephrotoxicity caused by IFO, its use has not been adopted clinically. This is most likely owing to the lack of information on the potential interaction between NAC and the chemotherapeutic integrity of IFO. While some studies have suggested thiol chemoprotectants such as NAC do not block the antitumor efficacy of antineoplastic
drugs during chemotherapy [63-65]), others suggest NAC may protect against the cytotoxicity resulting from some chemotherapy treatments.

NAC has been used in a number of in vitro experiments as a way of assessing potential chemotherapeutic agents’ mechanisms of antitumor activity, including studies with MSFTZ, a flavanone derivative [66]; isoobtusilactone A from Cinnamomum kotoense [67]; aclarubicin [68]; sanguinarine [69]); asteriscunolide [70]; and [71]. These compounds possess antitumor activity in a variety of cell lines, through a variety of mechanisms. In these studies, NAC demonstrated the ability to protect against mechanisms such as mitochondrial dysfunction [66, 68], Bcl-2/Bax modulation [66], apoptosis [66, 68, 69], increased levels of reactive oxygen [65, 68, 70, 71], caspase activation [69], reduced ERK phosphorylation [71], and ultimately cell cytotoxicity. The ability of NAC to protect through one or more of these mechanisms leads to the common conclusion that reactive oxygen species played a pivotal role in the antitumor activity of the compound being tested [66-71]. Despite this, only one study assessing NAC’s antitumor activity in consideration of its role as a chemoprotectant found negative results. Wu et al. [65] showed that NAC blocks the cytotoxic effects of cisplatin when given concurrently with or up to 2 h after treatment. However, the same study suggested that the timing of NAC administration might potentially be adjusted in order for it to act as a chemoprotectant without affecting antitumor activity. Similar conclusions were reported by Muldoon et al. [63] and Neuwelt et al. [64], who demonstrated that separation of spatial and temporal administration of chemoprotectants and chemotherapeutic drugs can protect the integrity of both treatments. Also of significance is a study by Heaney et al. [73], who report that NAC does not affect the
cytotoxicity of the antineoplastic drugs vincristine, doxorubicin, methotrexate, and imatinib, although it did attenuate cytotoxicity caused by cisplatin. Of importance, in our study we did not measure the production of reactive oxygen species, as it is well established that the majority of cell death caused by IFM is due to apoptosis, with some cells dying of necrosis (Becker et al. 2002; Zhang et al. 2005). Previous studies by our group and others have established that NAC does in fact decrease oxidative stress [48]. However, the efficacy of vincristine, doxorubicin, methotrexate, and imatinib, which also elicit their antitumor effects through apoptosis, was not negatively affected by NAC [74-77]. For this reason we focused our paper only on the cell death caused by IFM and the possible effects of NAC on its cytotoxicity.

Of similar interest are reports that antioxidants may act synergistically when added to chemotherapy treatments, resulting in greater antitumor activity than with treatment with the antineoplastic drug alone. Thymoquinone potentiates IFO and cisplatin antitumor activity [50], while melatonin acts synergistically with IFO and vincristine tumor kill [78], both of which have been shown in animal models. While similar studies have not been carried out with NAC and IFO, Gao et al. [79] demonstrated that NAC does have antitumorigenic effects. NAC demonstrated antitumor effects in 3 tumorigenic models in vivo through increased degradation of a protein involved in tumorigenic pathways, HIF-1, which regulates genes involved in angiogenesis, mitochondrial function, and glycolysis.

While there are conflicting data on the role of NAC and its antitumor effects, most of the data looking at NAC as a chemoprotectant suggest that it will not interfere with IFO activity. Therefore, the aim of this study was to determine if NAC has a
negative effect on the ability of IFM, to kill cancer cells. We looked at two relevant cancer cell lines, in which IFO is often included for their treatment. Our results demonstrated that NAC does not affect the tumor kill of IFM in either neuroblastoma or rhabdomyosarcoma cells lines. This was true for both concurrent and pretreatments, either with NAC alone or NAC in combination with MESNA.

It is important to note that IFM rather than the parent compound IFO was used in these experiments. IFM is in fact the active antineoplastic form of IFO. It is cytotoxic through actions as cell cycle non-specific DNA alkylating agent. IFM has two highly reactive alkyl groups, which are both able to form covalent bonds with 7-nitrogen of purine bases in DNA. With two moieties capable of forming these covalent bonds, both intra- and inter-DNA strand cross links are possible, resulting in an impaired ability of the cell to undergo DNA relocation, signaling cell death [80, 81]. Rapidly dividing cells, such as cancer cells, are most affected by this type of agent.

The formation of IFM occurs through ring hydroxylation of IFO. Enzymatic conversion of IFO occurs to form 4-hydroxyIFO, which then spontaneously converts to aldo-IFO, which may also exist in its tautomeric form carboxyl-IFO. Aldo-IFO then undergoes β-elimination to form IFM. The enzymes required for the first bioactivation are CYP 3A4, 3A5 and 2B6 [28-30]. Tumor cells in general, have inherently lower levels of cytochrome P450 enzymes [82-85]. Specifically, studies demonstrate that neuroblastoma cells are unable to activate IFO, resulting in no cytotoxicity[84], while there was considerable cytotoxicity when either rat liver microsomes or pre-activated 4-hydroxyIFO were used. Consequently, in vitro, IFO should result in little to no toxicity in both rhabdomyosarcoma and neuroblastoma cell lines. As expected our results
corroborated these findings, with IFO showing no death in either cell line until extremely high concentrations of 5 mM. While cell death was observed at high concentrations, normal plasma concentrations of IFO are between 50 and 800 uM [86], suggesting us that such high concentrations are not clinically relevant.

*In vitro* then, the use of liver microsomes to bioactivate IFO or a pre-activated form such as IFM itself is required; we chose the latter. As expected, our results showed a dose dependant decline in tumor cell viability with increasing concentrations of IFM. It is important to note that in humans, the majority of IFO metabolism occurs hepatically, following which IFM is transported in erythrocytes to the tumor tissues, where passive diffusion or transporter mediated process allow entry into tumor cells [87]. Therefore the lack of bioactivating enzymes in cancer cells will not prevent their exposure to IFM *in vivo*.

The dose of IFM used when assessing the potential effects of NAC was one that produced greater than 50% cell death. However cell kill was not 100%. This is important for being able to assess both the potential positive and negative effects NAC might have. In terms of clinical relevance, this dose is also within the reported range of plasma concentrations of IFM [87-89]. As discussed previously, NAC, both in the form a concurrent and pretreatments, had no impact on the cancer cell cytotoxicity of IFM. Futhermore NAC + MESNA treatments also had no impact of IFM activity. This is important, as MESNA is a standard adjuvant to IFO therapy for the protection of bladder toxicity [15, 45]. Therefore these results suggest that potential combination of both chemoprotectants NAC and MESNA will not affect IFM efficacy. The fact that neither NAC nor MESNA interact with the efficacy of IFM can be explained by a lack
of complete understanding as to the mechanism of cell death caused by IFM. It has widely been assumed that since apoptosis is the main form of cell death caused by IFO, ROS must play a pivotal role, and therefore suggesting that addition of any antioxidant might interrupt the apoptotic pathway. However, to our knowledge, no study has looked at the presence of ROS during IFO-mediated cell death and in fact, little is known regarding how cell death occurs following DNA alkylation. Further to this point, Sentruker et al. [90] demonstrate that upon evaluation of two chemotherapy drugs commonly associated with oxidative damage as their mechanism of cancer cell death, neither in fact produced any increases in ROS. Therefore, both etoposide and cisplatin antitumor effects may not involve oxidative stress as once accepted. Similarly, the lack of inhibitory effects of NAC, as well as other antioxidants such as MESNA, thymoquinone and melatonin on IFO cancer cell kill, might suggest the same is true for IFO.

This study is the first to demonstrate that NAC does not interfere with the antitumour efficacy of IFM and is an important first step in demonstrating the clinical utility of NAC. The next reasonable step includes assessing the effects of NAC in tumor xenograft animal model, which will further our understanding of any effects NAC might have on IFO efficacy. This is a critical step, which will provide important information regarding the NAC’s ability to improve the risk/benefit ratio of IFO use and may further support its use in preventing nephrotoxicity in children, in a clinical setting.
3.5 References


Chapter 4: The effect of n-acetylcysteine on ifosfamide antitumour efficacy: *in vivo*

A version of this chapter has been published:

4.1 Introduction

Currently, the 5-year survival rate for children diagnosed with cancer is 82%, compared to 50% thirty years ago [1, 2]. It is now estimated that 1 in 1000 adults living in the United States are survivors of childhood cancer [2]. The increasing numbers of childhood cancer survivors has highlighted the previously underappreciated problem of potential long-term adverse effects of chemotherapy. As survival rates in this population increase, so does the overall number of people suffering from late effects and/or suboptimal health conditions, secondary to initial presentation of cancer and therapy [3]. Two thirds of the adults who have survived their childhood cancer suffer from an adverse condition as a result of their cancer treatment. Late effects occurring in one third of these adults may be severe or life threatening [4, 5].

Nephrotoxicity is an adverse effect that can be caused by the antineoplastic drug ifosfamide (IFO). This is an age dependent problem, with up to 30% of children who receive IFO therapy suffering varying degrees of nephrotoxicity. In the most severe cases, IFO treatment may result in Fanconi syndrome, a proximal tubule dysfunction that can produce hypophosphatemic rickets, necessitating lifelong supplementation with phosphate and bicarbonate [6, 7]. It may also result in severe glomerular toxicity leading to acute or chronic renal failure, requiring renal dialysis and/or renal transplantation [8, 9]. IFO is used in children primarily for the treatment of Ewing’s sarcoma, rhabdomyosarcoma, osteosarcoma, neuroblastoma, as well as acute lymphoblastic leukemia and non-Hodgkin’s lymphoma [10-14]. While severe urotoxicity was previously the dose-limiting toxicity associated with IFO, concomitant administration of sodium 2- mercapto-ethanosulphonate now provides uro-protection.
Consequently, nephrotoxicity has emerged as the most severe toxicity associated with IFO use [14, 15].

IFO is a pro-drug that undergoes metabolic activation to produce the active cytotoxic agent ifosfamide-mustard: the relatively low cytotoxicity of the parent drug reduces undesirable toxicity in most non-tumour tissues. However, the toxic metabolite chloroacetaldehyde (CAA), produced during IFO metabolism, appears to be responsible for toxicity against the kidney [16-19]. Produced locally in the kidney [20], CAA is generated via side-chain oxidation of IFO. The alternative pathway to ring hydroxylation, that produces the active antineoplastic agent IFO mustard, and acrolein (Figure 1.2). Acrolein is the metabolite responsible for bladder toxicity [16, 21, 22].

Both side chain oxidation and ring hydroxylation metabolism of IFO (via the cytochrome P450 isoymes 3A4, 3A5 and 2B6) occur in equimolar amounts in both the liver and in the renal tubule cells. Thus, the kidney is capable of producing toxic metabolites intra-renally [20, 23-25].

CAA toxicity occurs through oxidative stress, suggesting the potential for the use of an antioxidant to mitigate IFO-induced renal toxicity. While several antioxidants have been investigated as potential therapeutics for renal toxicity associated with IFO, n-acetylcysteine (NAC) is the most suitable agent. Although other antioxidants such as thymoquinone [26], resveratrol [27], taurine [28], l-histidinol [29], and melatonin [30] act much like NAC and provide protection through their activity as nucleophiles, by scavenging toxic reactive oxygen species, NAC is clinically distinct from the others. NAC acts as a precursor to glutathione, depletion of which is a key characteristic of IFO-induced nephrotoxicity. Importantly, NAC is also commonly used clinically [31,
as the drug of choice in children and adults for treatment of acetaminophen overdose using a 21-hour IV protocol (5, 33). This provides valuable information supporting future use of NAC in combination with IFO, since the previous use of NAC in children provides a reassuring record of safety in this vulnerable population in which safety data is often difficult to obtain. Furthermore, our group has generated evidence supporting the therapeutic efficacy of NAC as a promising mitigator of renal toxicity secondary to IFO. Both in vitro [34] and in vivo [35] studies in rats have demonstrated that clinically relevant concentrations of NAC are able to mitigate IFO-induced renal toxicity in models of Fanconi syndrome. We have further demonstrated that NAC doses described above for acetaminophen overdose are likely to be sufficient for renal protection [36].

When considering the contribution of IFO to late effects it is important to recognize the extent of its use. Based on Canadian pediatric cancer statistics between 2000 and 2004, we have estimated that IFO may have been part of chemotherapy protocols used to treat as high as 25% of children with cancer [1]. This highlights the need for approaches that protect the long-term health of childhood cancer survivors, and emphasizes the extent to which IFO may contribute to such late effects. However, any concurrent therapy must be demonstrated not to interfere with eradication of neoplastic cells and cancer cure rates. While NAC shows promise in the protection against late effects caused by IFO, it should first be shown that it does not interfere with the ability of IFO to be an effective chemotherapeutic agent before it can be used clinically. In this study we assessed the potential of NAC to affect the efficacy of IFO.
4.2 Materials and Methods

All experimental protocols described here were approved by the University of Western Ontario Animal Care and Use Council.

4.2.1 Mice

Female NIH-III (nude homozygous) mice (28-42 days) were purchased from Charles River Canada (Montreal, QC, Canada). Mice were kept at a constant temperature and regular light cycles of 12-h light and dark, under pathogen-free conditions. They were fed Harlan-Teklad diet 2919 and water ad libitum.

4.2.2 Tumor cells

Experiments were carried out using a Ewing’s sarcoma xenograft model, as described by Sanceau et al. [37]. EW-7 cells (wild-type p53 primary tumours localized to the scapula) [37] were a kind gift from Dr. O. Delattre (Institute Curie, Paris, France). PCR-based testing for pathogen, confirmed that cells were free of mycoplasma or common rodent viruses (IMPACT II test, IDEXX RADIL, Columbia, Missouri, USA). Cells were cultured in standard RPMI tissue culture medium supplemented with 10% fetal bovine serum and L-glutamine (2 mM) and grown in collagen coated tissue culture flasks. They were initially established as transplantable tumours (maximum volume of 1000 mm³) by subcutaneous injection of 20 x 10⁶ cells into the flanks of female NIH-III mice. Thereafter, xenografts were maintained in vivo by sequential passaging of subcutaneous implants of tumour fragments, as described below in Experimental Design, with an engraftment success rate exceeding 90%.

4.2.3 Reagents

NAC was purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada).
Ifosfamide was purchased from Baxter Oncology GmbH and Baxter Corporation (Mississauga, ON, Canada). Collagen I, bovine was manufactured by Gibco and purchased from Invitrogen Corporation (Burlington, ON, Canada). RPMI1640 was purchased from Wisent Bioproducts (St. Bruno, QC, Canada).

4.2.4 Experimental Design

EW-7 tumour xenograft fragments (approximately 10-20 mm³) were surgically implanted into the flank of mice according to a protocol described by Morton and Houghton (ref. 38; procedures 1, 2b, 3, 4, 5a and 6 described therein) under Avertin (tribomethanol)-induced anaesthesia. When tumours reached a volume of 50-100 mm³, the mice were randomly assigned into one of the following groups (n=5 per group).

1. Saline-treated control group: 0.9% saline (in 0.3 ml, intraperitoneally (IP)) daily for 6 days.
2. NAC group: NAC (1.2 g/kg in 0.3 ml 0.9% saline, pH 7.2 IP) daily for 6 days. This dose was identical to that capable of preventing IFO-induced nephrotoxicity in rats [35]. NAC doses as high as 1.2 g/kg have been reported not to induce any deleterious effects [39].
3. IFO group: IFO (60 mg/kg in 0.3 ml 0.9% saline, pH 7.2 IP) daily for 3 days.
4. Concurrent NAC + IFO group: IFO plus NAC (60 mg/kg IFO and 1.2 g/kg NAC, both in 0.3 ml 0.9% saline, pH 7.2 IP) daily for 3 days, followed by 3 additional days of NAC treatment alone.
5. Pretreatment NAC + IFO group: NAC (1.2 g/kg in 0.3 ml 0.9% saline, pH 7.2 IP) daily for 6 days, followed by IFO (60 mg/kg in 0.3 ml 0.9% saline, pH 7.2 IP) daily for 3 days.
Tumours were allowed to grow until the fastest-growing tumor of each mouse reached a volume of 1000 mm$^3$, at which point the mouse was euthanized.

4.2.5 Outcome measures

Tumour volumes were estimated every 2 days by caliper measurements (volume=0.52 (length x width$^2$)) until euthanasia.

4.2.6 Statistical analysis

Control and treatment groups were compared using the Mann-Whitney U-test, as indicated. Values are presented as medians±interquartile range.

4.3 Results

4.3.1 Effect of ifosfamide on EW-7 Ewing xenograft growth

We investigated the effects of NAC on IFO efficacy in a tumor xenograft mouse model. When EW-7 tumours in host mice reached the target size (approximately 100 mm$^3$) they were subsequently treated with IFO. Tumour growth was significantly inhibited in the IFO treatment group (n=8) compared to growth of tumours in control mice treated with saline alone (n=6) (Figure 4.1).
**Figure 4.1** The effect of ifosfamide on EW-7 xenograft growth. Starting at day 1, mice received IFO (60 mg/kg IP) daily for 3 days or 0.9% saline alone, daily for 6 days (control). Values plotted are median ± interquartile range; medians were significantly different when compared by the Mann Whitney U-test (+p<0.05; ++p<0.01).
4.3.2 Effect of concurrent n-acetylcysteine + ifosfamide on EW-7 xenograft growth

Mice with established EW-7 tumours were treated concurrently with both NAC and IFO, and with follow-on treatment with NAC alone, in order to determine whether the growth-inhibiting capacity of IFO was affected by NAC. When treated with both IFO and NAC daily for 3 days, followed by 3 days of NAC alone (n=7), EW-7 xenograft tumour volumes were significantly smaller than in control, saline-treated mice (n=6) (Figure 4.2). When tumours in mice treated with IFO alone (n=8) were compared with those in mice treated concurrently with both NAC and IFO (n=7), it was clear that NAC had no effect on the capacity of IFO to inhibit tumour growth: IFO inhibited tumour growth with equal efficiency in both NAC-treated mice and in mice without NAC treatment (Figure 4.2). In fact, tumours in mice treated concurrently with both NAC and IFO had median volumes that were numerically lower (although not significantly different) than in mice treated with IFO alone.
**Figure 4.2** The effect of concurrent NAC + IFO on EW-7 xenograft growth and IFO efficacy. Starting at day 1, mice received IFO (60 mg/kg IP) + NAC (1.2 g/kg IP) daily for 3 days, followed by 3 days of daily treatment with NAC alone, IFO alone (60 mg/kg IP) daily for 3 days or 0.9% saline alone daily for 6 days (control). Plotted values are median ± interquartile range; medians were significantly different when compared by the Mann Whitney U-test) (+p<0.05 and ++p<0.01 when IFO is compared to control; *p<0.05 when NAC + IFO is compared to control).
4.3.3 Effect of pre-treatment n-acetylcysteine + ifosfamide on EW-7 xenograft growth

Mice were also pre-treated with NAC, followed by IFO, in order to determine whether pre-existing NAC in whole animals and/or xenografted tumour environment affected the capacity of IFO to inhibit EW-7 xenograft growth. Pretreatment with NAC daily for 6 days followed by IFO daily for 3 days (n=6), resulted in inhibition of EW-7 xenograft tumour growth and, furthermore, comparison of tumors in mice treated with IFO alone with those in mice pre-treated with NAC before IFO treatment revealed that NAC pretreatment had no effect on the capacity of IFO to inhibit EW-7 tumor growth (Figure 4.3). As with concurrent NAC and IFO therapy, tumors in mice pre-treated with NAC before IFO administration had median volumes that were numerically lower (although not significantly different) than in mice treated with IFO alone.
Figure 4.3 The effect of pre-treatment with NAC followed by IFO treatment on EW-7 xenograft growth and IFO efficacy. Starting at day 1, mice were treated with NAC alone (1.2 g/kg IP) daily for 6 days, followed by IFO (60 mg/kg IP) daily for 3 days, IFO alone (60 mg/kg IP) daily for 3 days or 0.9% saline alone daily for 6 days (control). Plotted values are median ± interquartile range; medians were significantly different when compared by the Mann Whitney U-test (+p<0.05 and ++p<0.01 when IFO is compared to control; *p<0.05 and **<p<0.01 when pre-treatment with NAC + IFO is compared to control).
4.3.4 Effect of n-acetylcysteine alone on EW-7 xenograft growth

The effect of NAC alone on EW-7 xenograft growth was assessed in comparison with saline-treated controls. While NAC alone resulted in lower median tumor volumes on days 65 and 68 following treatment, tumor volumes at all other time points were not significantly different between NAC- and saline- treated mice, suggesting that the effects of NAC alone on tumor growth in this test system are either negligible or non-existent (Figure 4.4).
Figure 4.4 The effect of NAC on tumor volumes. Starting at day 1, mice were treated with NAC (1.2 g/kg IP) daily for 6 days, or daily with 0.9% saline for 6 days (control). Values plotted are median ± interquartile range; medians were significantly different when compared by the Mann Whitney U-test (*p<0.05).
4.4 Discussion

NAC possesses characteristics that make it a promising chemoprotectant against IFO-induced renal injury. As it is currently approved against acetaminophen overdose in children [5, 33], safety data are available to allow its safe use for this potential new indication. Children with cancer are a vulnerable population and therefore sufficient evidence for the safety of NAC for this population is imperative before its clinical use as a renal protectant. Critically, there is a large body of evidence supporting the effectiveness of NAC in renal protection during IFO therapy. Our group has demonstrated the therapeutic efficacy of NAC in protecting against IFO-induced cytotoxicity and glutathione depletion in a proximal tubule cell line, as well as IFO-induced nephrotoxicity in a rodent model of Fanconi syndrome [34, 35]. In vivo, NAC prevented renal tubular morphological damage, reduced glutathione (GSH) levels, glutathione S-transferase activity and lipid peroxidation, as well as prevented elevated levels of serum creatinine, magnesium and β2-microglobulin in rats treated with IFO [35]. Furthermore, regarding the clinical utility of NAC, we demonstrated that the current dose of NAC used in acetaminophen poisoning (the 21-hour IV dose protocol) should provide a sufficient dose for renal protection based on our translational pharmacokinetic work [36]. Our recent pharmacokinetic study compared the systemic exposure of NAC between our therapeutically effective rodent model and children receiving NAC for acetaminophen overdose, showing similar systemic exposures between both groups. We also analyzed 2 cases of children with cancer experiencing IFO-induced renal injury, following which, NAC was added concurrently to their treatment plan. In both cases, following NAC treatment, the renal impairment was
reversed, further supporting the experimental findings [36]. However, while all the
studies to date support the effectiveness of NAC, it must first be confirmed that it does
not interfere with the antitumor activity of IFO before it can be evaluated in the context
of a clinical trial. *In vitro* studies using rhabdomyosarcoma and neuroblastoma cell lines
have demonstrated that NAC does not interfere with the cytotoxicity of the active
metabolite IFO mustard [40]. To further support this, the results of this *in vivo* study
demonstrated that both pre-treatment and concurrent therapy with NAC show no
evidence of interference with IFO efficacy. As discussed in greater detail below, both
treatments not only significantly reduced human EW-7 tumor xenograft growth
compared to saline treated mice, but more importantly, there was no significant effect of
NAC (at doses expected to reduce renal toxicity) on the potential of IFO to treat EW-7
tumors.

While our study did not assess the effects of either IFO and/or NAC on the renal
function of the mice, both doses of NAC and IFO have been investigated in this context
in animal models. Of greatest importance, the dose of NAC in this study (1.2 g/kg daily
for 6 days) is the dose that our group previously demonstrated to provide protection to
the kidneys of rats treated with IFO [35]. As discussed above, this dose of NAC
prevented urinary loss of solutes such as magnesium and β2-microglobulin, and serum
increases in creatinine, and protected against GSH depletion, lipid peroxidation,
decreased glutathione S-transferase activity, and morphological damage to renal tubules
[35]. Furthermore, doses of IFO as low as 40 mg/kg daily for 3 days have been shown
to produce Fanconi syndrome resulting in phosphaturia, glucosuria, aminoaciduria,
changes in membrane phospholipid composition, and glutathione depletion [41]. Our
group used an IFO dose of 60 mg/kg daily for 3 days. These data suggest that both the
dose of IFO and NAC are sufficient to induce Fanconi syndrome and protect against it,
respectively.

We chose the EW-7 Ewing xenograft model based on the successful use of
those cells by Sanceau et al. [37], and the high relevance of Ewing’s sarcoma as the
second most common bone cancer among children [42, 43]. In order to provide a useful
experimental model, the concentration of IFO selected for treatment of xenografts must
be effective in moderately reducing tumor volumes, but should not result in complete
ablation of tumors. The capacity of NAC to either inhibit or enhance IFO efficacy,
would therefore be ascertainable. The concentration of IFO selected for this study (60
mg/kg daily for 3 days) fulfilled this requirement (Figure 4.1). IFO treatment alone for 3
days reduced median EW-7 tumor volume compared to saline treatment but did not
induce complete tumor disappearance. Thus, our model was suitable to assess either the
positive or negative effects of NAC on IFO activity.

Concurrent NAC and IFO treatment of mice bearing EW-7 tumors led to
significantly lower median tumor volumes compared to control mice (Figure 4.2). This
demonstrated that NAC plus IFO therapy is effective in reducing EW-7 tumor volumes.
However, while important, these data did not evaluate the potential effect of concurrent
NAC treatment on IFO efficacy. The comparison between NAC plus IFO, and IFO
alone, on EW-7 tumor growth allowed this evaluation. We showed that IFO reduced
tumor growth, and that its potential was unaffected by concurrent NAC treatment
(Figure 4.2). NAC had therefore no negative effect on the ability of IFO to reduce tumor
volumes.
The effect of pre-treatment with NAC was also assessed. Evaluation of both concurrent and pre-treatment strategies allows for flexibility when determining treatment regimens in a clinical setting. Similar results were seen in mice pre-treated with NAC, followed by IFO therapy. A comparison of pretreatment with NAC plus IFO to control saline-treated mice shows that this therapy is capable of reducing EW-7 tumor volumes (Figure 4.3). Furthermore, a comparison between pretreatment NAC plus IFO with IFO alone showed no significant difference between the two with respect to median tumor volumes (Figure 4.3). Therefore, NAC administered concurrently with IFO, or prior to IFO treatment, had no effect on IFO efficacy; there was a similar IFO-induced reduction in EW-7 tumor volumes regardless of addition of NAC to the treatment. This further reinforces the fact that it is unlikely that NAC will interfere with the clinical efficacy of IFO. These results are in accordance with other studies reporting a lack of effect of NAC on the capacity of other chemotherapeutic agents to inhibit tumor growth. Carboplatin, etoposide, and doxorubicin, along with several other agents, have all been shown to maintain their chemotherapeutic efficacy in the presence of NAC [44, 45]. This further strengthens the implication of this study that NAC is unlikely to negatively affect the efficacy of these agents.

As outlined above, there is a large body of evidence supporting the use of NAC as a prophylactic treatment for IFO-induced nephrotoxicity. While the data presented in this article are important in realizing the use of NAC clinically, the fact that IFO is not generally used as a single-agent raises the concern that NAC may interfere with the efficacy of other chemotherapy agents used in combination with IFO. It therefore becomes important to review protocols in which IFO combinations exist. IFO is
identified for use in combination with etoposide, doxorubicin, cisplatin, cytarabine, cyclophosphamide, and carboplatin [46-50, 51,52]. Data assessing the potential effects of NAC on the efficacy of these drugs are already available. Etoposide, doxorubicin or carboplatin, when administered concurrently with NAC, induce no changes in cytotoxic activity [45, 53]. While cyclophosphamide has yet to be evaluated with NAC, it is an analogue of IFO, differing only in one chloroethyl group and sharing the same qualitative metabolic profile. It is therefore reasonable to predict that it will act in the same manner as IFO and, when administered in combination with NAC, it will not have reduced efficacy. Finally, while there is evidence to suggest that NAC interferes with cisplatin by conjugating and rendering it incapable of entering the cell, it has also been demonstrated that altering the time and site of administration of NAC and cisplatin allows for effectiveness of both treatments [44, 54].

4.5 Conclusion

We present data suggesting that concurrent or pre-treatment with NAC at concentrations capable of preventing IFO-induced renal toxicity does not affect IFO antitumor activity in vivo. While IFO is commonly used in combination therapies, numerous available studies suggest that these combinations will not be negatively impacted by NAC therapy, similar to the lack of inhibitory effects of NAC on IFO efficacy, demonstrated in the present study. We conclude that there is a strong basis on which we can proceed in testing the use of NAC to prevent IFO-induced nephrotoxicity in children, even among combination therapies. This study further strengthens the need for a randomized clinical trial of NAC as a renal protectant for children with cancer.
4.6 References


28. Badary OA. Taurine attenuates fanconi syndrome induced by ifosfamide without


54. Wu YJ, Muldoon LL, Neuwelt EA. The chemoprotective agent N-acetylcysteine
Chapter 5: Overall discussion and future directions

Part of this chapter has been published:

5.1 Preamble: Future directions and overall perspective

In this alternate thesis structure, each chapter has been already published in the peer review literature with its own discussion section. Considering the evidence described in this thesis, along with the immediate need to address the issue of how clinicians and hospitals should prevent and treat nephrotoxicity caused by IFO, I have selected to focus this overall discussion section on the synthesis of clinical protocol recommendations for NAC rescue therapy to prevent IFO nephrotoxicity in children. While we do believe there is sufficient evidence to treat those patients who present with kidney toxicity, this protocol does not obviate the need for a randomized control trial (RCT). We have designed a prospective RCT to determine the effectiveness of NAC as a prophylactic strategy against IFO-induced nephrotoxicity. This chapter concludes with a discussion and overall perspective.

5.2. N-acetylcysteine rescue protocol for nephrotoxicity in children caused by ifosfamide

5.2.1 Introduction

In the United States alone, there are over 270,000 adult survivors of childhood cancer [1], among whom a majority are suffering from late effects secondary to their cancer treatment. These survivors have health problems that may seriously affect their quality of life, and/or may be life threatening [2]. Nephrotoxicity, primarily manifesting as proximal tubule dysfunction, in particular can be a devastating late effect which may necessitate lifelong supplementations, renal dialysis and renal transplant, and in severe cases may result in death [3]. The chemotherapy agent ifosfamide (IFO) is among the
most common causes of chemotherapy-related renal damage [4] and despite a large body of preclinical evidence supporting the effectiveness of n-acetylcysteine (NAC) as a concurrent therapy for IFO-related permanent kidney damage, its use has yet to be adopted regularly in a clinical setting. We summarize below two published case reports wherein NAC was successfully used as renal protectant during IFO treatment, and report for the first time, a third successful case. The objective of this document is to summarize the evidence supporting the use of NAC as a treatment for children suffering from IFO nephrotoxicity and to present a NAC rescue protocol. It is hoped that additional clinicians will join those who have followed this protocol in order to develop a prospective randomized trial.

5.2.1.1 Ifosfamide

IFO is an antineoplastic agent used in the treatment of pediatric solid tumours, including Ewing’s tumours, osteosarcoma, rhabdomyosarcoma, and non-hodgkins lymphoma, as well as acute lymphoblastic leukemia [5, 6]. While a proven treatment in the battle against childhood cancer, with regimens including IFO having a higher cure rate for most cancers than its analogue cyclophosphamide [7], IFO use does not come without consequences. Nephrotoxicity affects up to 30% of children who are treated with this agent [3, 8]. While these children may benefit from its antineoplastic potential and be cured of their cancer, their health and quality of life may be seriously impacted by this late effect.

IFO, a nitrogen mustard, is a prodrug, which is metabolized to the active antineoplastic agent IFO-mustard [9]. Metabolic activation to IFO mustard occurs via ring hydroxylation by CYP P450 3A4, 3A5, and 2B6 yielding both IFO mustard and
acrolein [9] (Figure 1.2). IFO may also be metabolized via the alternate metabolic pathway, side chain oxidation, producing 2- and 3-dechloroethyl IFO and chloroacetaldehyde (CAA)[9, 10]. Both acrolein and CAA are toxic reactive metabolites that are known to be responsible for some of the toxicities associated with IFO use [10, 11].

Neurotoxicity, myelosuppression, GI toxicity, nephrotoxicity and urotoxicity have all been identified as adverse effects of IFO chemotherapy, with the latter two being the most severe [4]. In the past, IFO use was limited by the commonly occurring and severe urotoxicity. However this late effects has been overcome with the co-administration of 2-mercaptoethanosulphonate (MESNA), a synthetic thiol which protects the bladder epithelium against the reactive metabolite acrolein, allowing for an almost complete reduction in the incidence of urotoxicity. This allowed for the ability to increase both dose and frequency with which IFO was given, following which nephrotoxicity emerged as the most severe, and dose limiting toxicity [4].

5.2.1.2 Nephrotoxicity

Cumulative dose, unilateral nephrectomy, prior platinum therapy and age, are all risk factors predisposing patients to kidney toxicity [3]. With respect to those affected, nephrotoxicity presents in each child to varying degrees. 30% of children who are treated with IFO will suffer mild to moderate renal dysfunction with 5% suffering from its most severe form Fanconi syndrome [3, 8]. Presenting as a proximal tubule dysfunction, Fanconi syndrome results in urinary loss of important solutes such as glucose, amino acids, B2 micoglobulin, phosphate and bicarbonate[8]. It may lead to renal tubular acidosis and/or hypophosphatemic rickets, and the need for lifelong
supplementation with phosphate, bicarbonate and potassium [12, 13]. Up to 30% of children may also suffer from declining glomerular function, resulting in the need for dialysis, renal transplant, and/or causing death [8]. Treatment options are limited to supportive care with most children seeing little to no improvement in their renal function overtime and many seeing persistent declines in renal function [8] (Table 1.1).

The metabolite CAA, formed by side chain oxidation of IFO, is responsible for this late effect associated with IFO [14]. However, studies show that systemic concentrations of CAA do not correlate with severity of nephrotoxicity, suggesting hepatic metabolism of IFO does not play a role in kidney toxicity [15]. Of importance, while the majority of xenobiotic metabolism is thought to be hepatic, there is evidence that the kidney is also capable of such biotransformation [11]. Moreover, there are studies available that demonstrate that in the case of IFO, the kidney possesses the enzymes responsible for its metabolism and is capable to producing levels of CAA which in turn have been shown to be toxic to kidney tubules [11, 14, 16]. Therefore the kidney “creates its own poison”, and when unable to detoxify at the same rate with which it is being produced, this results in toxicity.

The mechanism by which CAA exerts its toxicity within the kidney is due increased oxidative stress. Increases in reactive oxygen species and products of lipid peroxidation [17], as well as increased calcium and sodium concentrations have been demonstrated effects of CAA[18, 19]. While increased calcium can be detrimental to the cell, causing damage to the cytoskeleton, cell membrane, and potentially causing cell death, low levels of sodium are important for proper solute reabsorption, this being a hallmark of Fanconi syndrome [20, 21]. CAA may also result in loss of mitochondrial
membrane potential, depletion of ATP and increases in pro-inflammatory cytokines TNF-α, IL-1β and IL 6[22-24]. Given the primary mechanism of CAA toxicity is oxidative damage, most crucial, is the depletion of glutathione (GSH) as a result of CAA [14, 17, 25]. As our intracellular protective mechanism against oxidative stress, GSH’s ability to protect against CAA become critical to cell survival in environments with increased oxidative stress.

5.2.1.3 N-acetylcysteine

Much like in the way that MESNA, routinely given with IFO, protects the bladder, there is hope for such an antidote for kidney toxicity. With an awareness of the mechanism through which CAA exerts its toxicity it becomes easier to define this promising prophylactic and/or rescue strategies. With strong evidence supporting the main mechanism of kidney toxicity as oxidative stress, with a critical depletion in GSH [17, 25, 26], antioxidants are a promising strategy. While several antioxidants such as resveratrol, thymoquinone, melatonin, taurine, glycine and L-histidinol have been assessed in the prevention of IFO-induced nephrotoxicity[19, 24, 26-30], none of these options directly addresses the issue of glutathione depletion. More importantly, none of these compounds are currently used clinically in children for any indication. Our group has chosen to assess the protective potential of NAC against IFO nephrotoxicity. Not only does NAC function as a nucleophile, detoxifying ROS, it also acts as a precursor to glutathione synthesis by providing the cysteine essential for its formation [31, 32]. Even further to the advantage of NAC, is that it is currently used clinically in children for acetaminophen overdose[31, 32, 33 ]. This provides safety data that is not only
unavailable for alternative protective strategies, but can be very difficult to obtain in vulnerable populations such as children.

Currently used in the 21-hour IV schedule for acetaminophen overdose worldwide, this dose, along with several oral protocols (72 and 36 hours) have been demonstrated to exhibit similar efficacy when given within the 8 hour window following poisoning [34], with the 36 and 72 hour oral and 48 hour IV protocols being more effective for late treated patients [35, 36]. These protocols also demonstrate similar safety (Table 2.3). With respect to adverse events, they include anaphylactoid reactions when receiving IV formulations however more common side effects include nausea and vomiting when taken orally [37]. Anaphylactoid reactions are generally attributed to infusion rate. While they have been reported to occur in as little as 0% of patients to as many as 48%, they are generally minor and easily managed. [38]. In most cases NAC infusion can be continued and completed following treatment of the reaction and administration of diphenhydramine [39-41]. Also important, is the established safety of repeated or chronic NAC use. Oral NAC is often used in the treatment of chronic obstructive pulmonary disease (COPD) and is used safely for extended periods of time ranging from 22 weeks to 6 months. Chronic use of NAC in these patients resulted in minimal side effects, of which the most common were gastrointestinal; in all cases NAC therapy remained uninterrupted [42]. These characteristics make NAC not only a promising choice for kidney protection during IFO therapy but also the most readily available for immediate use.

5.2.2 Supporting Evidence
Prophylaxis is defined as prevention of disease, while treatment is defined as remedy of disease. We present below evidence for NAC use, both as prophylaxis and treatment, for IFO-induced nephrotoxicity. We reiterate here, that we choose to discuss NAC rather than other treatment options, which have also successfully protected against IFO-induced nephrotoxicity in animal models, because we believe that NAC is currently the most clinically relevant choice. This is not to suggest that the other agents may not be equally effective or even more potent than NAC, as a direct comparison of the chemicals has never been done. We do however argue that the lack of information of the safe use of these chemicals in children severely hinders their ability to be used in a clinical setting in the near future, until appropriate safety data is collected. The availability of this information for NAC, allows it to be used in a more immediate manner, allowing for prevention/treatment of IFO-nephrotoxicity sooner than with the other treatments. Should one of these other treatments be deemed superior to NAC and have adequate safety date, a re-evaluation of the best treatment for IFO-nephrotoxicity should be done.

5.2.2.1 Evidence supporting n-acetylcysteine for prophylaxis

There is large body of evidence to date to support the clinical use of NAC to prevent IFO renal toxicity. NAC has been efficacious in protecting against IFO-induced renal toxicity in both cell and rodent models [17, 25]. LLCPK-1, porcine renal proximal tubule cells, treated with IFO have depleted levels of glutathione and significant cell death. Treatment with NAC partially protects against glutathione depletion, and fully protects against decreased cell viability[25]. NAC also displays similar protective effects in a rodent model of nephrotoxicity. In these animals, IFO resulted in depletion
of glutathione, reduced glutathione-s-transferase activity and increases in products of lipid peroxidation. IFO also resulted in morphological damage to tubules and glomeruli, increased levels of serum creatinine and increased urinary excretion of β2-microglobulin and magnesium. With co-administration of NAC, rats were protected against all parameters of early stages of Fanconi syndrome. Lipid peroxidation as measured by malondialdehyde and 4-hydroxyalkenal increases, and decreases glutathione S-transferase activity were both restored to control levels. However in contrast to the in vitro model, full rather than partial protection against GSH depletion was seen [17]. Morphological damage including distorted tubules and degenerated glomeruli, as well as interstitial inflammation and oedema, seen with IFO treatment, was not present in NAC treated group. Our group has also done translational work demonstrating that the 21-hour IV dose of NAC used in children for acetaminophen poisoning, provides comparable systemic exposure when compared to our therapeutically effective rodent model, which as discussed above is protected at early stages of IFO-induced Fanconi syndrome. This suggests that the 21 hour IV dose of NAC should also be sufficient in providing renal protection [43] supporting the choice of 21 hour IV protocol over other oral protocols.

While studies of efficacy are critical to the use of NAC clinically, just as important is work assessing the effects of NAC on the antineoplastic ability of IFO. Both in vitro and in vivo work exists which shows no evidence that NAC interferes the chemotherapeutics effects of IFO. In two relevant cancer cell lines, rhabdomyosarcoma and neuroblastoma, IFM treated cancer cells retained their ability to kill with both the addition of NAC and NAC + MESNA combination [44]. The addition of MESNA,
routinely administered with IFO for uroprotection, was included to ensure that a combination of these chemoprotectant, would also not interfere with IFM efficacy. Furthermore, in a mouse xenograft model, where mice were implanted with Ewing’s sarcoma tumours, treatment with NAC and IFO, retained the same ability to inhibit tumour growth as IFO alone treated mice, when both were compared to control. While not significant, NAC + IFO treated mice has small tumor volumes, than mice treated with IFO alone. These studies suggest that ability of IFO to retain its alkylating properties against tumour cells, even in the presence of NAC [45]. Further supporting these findings is work which suggests that the addition of NAC to IFO treatment does not affect IFO pharmacokinetic parameters such as plasma half life, area under the curve, and plasma clearance [46].

5.2.2.2 Evidence supporting n-acetylcysteine for treatment

While the previous evidence suggests a prophylactic strategy, case reports, collected from circumstances in which treating physicians deemed NAC intervention a necessity following presentation of nephrotoxicity, also provide evidence that NAC can be used not only for prevention but treatment as well.

The most convincing support for the clinical use of NAC in children suffering from renal dysfunction are two case reports which have already been described in the literature, in addition to the third described below, of children who received NAC during, or just following, IFO containing chemotherapy regimens after demonstrating signs of renal dysfunction. The first case report describes a 10-year old girl who presented with primitive neuroectodermal tumour of the Ewing’s sarcoma family and acute renal failure. Following unsuccessful surgery, she was treated with IFO (3g/m²),
vincristine (1.5mg/m²), dactinomycin (1.5mg/m²), and MESNA on day 1, and IFO (3g/m²) and MESNA only on day 2 and 3, followed by dialysis on each day. She was also given the 21 hour IV dose of NAC on day 1 and 2. AUC’s of NAC calculated for these two days were similar to AUC’s observed in children treated with this dose of NAC for acetaminophen overdose. Following treatment this child had no clinical or laboratory signs of renal damage and she showed all signs of tumour lysis. The second case report describes a 15-year old male who had recurrence of mixed germ cell testicular cancer. He had elevated levels of serum creatinine (1.2-1.6mg/dl), which remained high following his chemotherapy regimen of cisplatin, etoposide and bleomycin. Following an abdominal relapse, he was treated with a protocol including Paclitaxel 257 mg, carboplatin 653 mg, IFO 3400 mg, and MESNA 684 mg on day 1, and IFO 3400mg and MESNA only on days 2-5. The patient’s serum creatinine steadily rose following treatment to a peak of 2.25mg/dl after the end of therapy. NAC was started 2 days following the end of the chemotherapy protocol at a dose of 600mg BID and was given for 5 days. Following treatment, serum creatinine declined to 1.12mg/dl, levels which were lower than observed creatinine levels at the start of treatment [45].

5.2.2.2.1 CASE REPORT

A 4-year-old girl was diagnosed with poorly differentiated epithelial Wilms tumor of favorable histology in April 2010. The tumor was stage 3 due to periaortic node involvement. She received radiation and AREN0532 protocol treatment. In May 2012 she had a relapse surrounding the pulmonary artery, which was confirmed by biopsy. She was started on ICE (Ifosfamide /Carboplatin /Etoposide) protocol and Topotecan/cyclophosphamide therapy for recurrent Wilms tumor. She received four
cycles of treatment with ICE, which included 5 days of 1.8g/m² of IFO admixed with MESNA 360 mg/m² over 2 hours daily for 5 days. During the 4th cycle, her serum creatinine remained in the range of 0.4-0.6 mg/dl during the ICE administration (day 1-5) but steadily began to increase following discharge on day 6 to a peak of 1.7mg/dl on day 10, when she was admitted to hospital for suspected sepsis (Figure 1). Following admission antibiotics (meropenum and vancomycin) and IV hydration were started. NAC was started on day 11 and was continued for 5 day at a dose of IV 1g per day. Following NAC administration, serum creatinine levels began to fall and within 1 week were within the normal ranges. Although suspected sepsis could have resulted in renal injury, and therefore its treatment in renal improvement, sepsis was ruled out as blood and urine cultures were negative. This would indicate the presence and treatment of sepsis was not responsible for changes in renal function. With respect to hydration status, following rehydration the patient did show slight improvement in serum creatinine, however her levels were still abnormally high suggesting kidney injury could not be ruled out. After starting NAC, there is steep decrease in creatinine values and clinical improvement, improvements which were not seen with rehydration. This would suggest improvements were due at least in part to NAC therapy, as renal dysfunction is multifactorial. Although her condition improved, the decision was made to withhold the 5th cycle of ICE. She continues her chemotherapy now with topotecan and cyclophosphamide only; all evidence to date suggests successful treatment of her cancer. She does not have any evidence of metastatic disease and her renal function remains within normal limits.
Currently there is no available approved drug to treat or prevent nephrotoxicity caused by IFO. There is however a good body of evidence, presented above, which suggests both the safe and effective use of NAC for treatment and prevention purposes. While a RCT is most certainly necessary to determine its effectiveness, we believe there is sufficient evidence to use NAC in cases where nephrotoxicity is already present; evidence which includes 3 case reports of children who were successfully treated with NAC for nephrotoxicity which presented during treatment, and whose chemotherapy was successful. Given that there are no alternative options, we believe it is irresponsible not to provide these children with the best possible chance they have to restore their renal function, especially in consideration of the minimal risk associated with NAC therapy and the potential enormous benefits. While we understand physicians may be reluctant to treat every child who requires IFO with NAC, we believe that until an RCT is carried out, the use of NAC is justified if and when nephrotoxicity is present. Following an RCT evaluating NAC given as standard adjuvant to IFO therapy, much in the same way MESNA is given for uroprotection, we would suggest NAC may be more appropriately used and accepted for prophylaxis.
Figure 5.1 Serum creatinine levels of 4-year old suffering from Wilms tumor.
5.2.3 Clinical protocol recommendations

The following guidelines have been developed with input from scientists, clinical pharmacologists and practicing physicians including nephrologists and pediatric oncologists. This section outlines the basic steps and considerations when treating kidney toxicity associated with IFO treatment.

5.2.3.1 Who should be treated? Grading nephrotoxicity

Adolescents and children who present to oncology with cancer requiring treatment with IFO should have baseline kidney function assessed. Both glomerular and tubular function should be assessed by measurements of serum creatinine, electrolytes, phosphate, bicarbonate, calcium magnesium and pH, and urinary glucose and protein. Patients should also have renal function assessed immediately and 1 week following each IFO containing cycle and immediately before each subsequent cycle begins. Follow up after completion of therapy should occur at 1 week, 3 months and 1 year to continue to monitor renal function. Patients declines of renal function can be measured one of two ways:

1- Using the following five criteria described by Lobstein et al. [47] with modifications, to defined abnormal renal function:

1) Hypophosphatemia (<2 SD for age)
2) Hypocarbia together with metabolic acidosis (bicarbonate<18mEq/L together with pH<7.32)
3) Glycosuria (any amount)
4) Proteinuria (1.0 g/L or greater)

5) Measured or estimated glomerular filtration rate (GFR) and/or creatinine clearance (CrCl) are < 80 ml/min/1.73m².*

* measured by isotopic nuclear GFR measurement, measured by creatinine clearance, estimated by creatinine-based equations (such as modified Schwartz formula), estimated by cystatin C-based equations or others

Each criterion is to be considered only if it appears as normal in the baseline renal function assessment and became abnormal following the start of IFO and only if it appeared as abnormal at least twice.

The patient can be classified in one of four categories based on their degree of nephrotoxicity (Table 5.1):

OR

2- As estimations of GFR by creatinine are often underestimated when patient creatinine levels are not in steady state, renal injury can be assessed according to pRIFLE criteria [48] (Table 5.2):

Any patient categorized as mildly, moderately or severely nephrotoxic, OR who meets any pRIFLE criteria of should be treated with NAC.
Table 5.1 Degree of nephrotoxicity based on Lobestein et al. [47] criteria.
<table>
<thead>
<tr>
<th>Degree of Nephrotoxicity</th>
<th># of Abnormal Criteria Met</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-nephrotoxic</td>
<td>None</td>
</tr>
<tr>
<td>Mildly nephrotoxic</td>
<td>1</td>
</tr>
<tr>
<td>Moderately nephrotoxic</td>
<td>2-3</td>
</tr>
<tr>
<td>Severely nephrotoxic</td>
<td>4-5</td>
</tr>
</tbody>
</table>
Table 5.2 pRIFLE criteria.
<table>
<thead>
<tr>
<th></th>
<th>Estimated CCL</th>
<th>Urine Output</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Risk</strong></td>
<td>eCCl decrease by 25%</td>
<td>&lt;0.5 ml/kg/h for 8 h</td>
</tr>
<tr>
<td><strong>Injury</strong></td>
<td>eCCl decrease by 50%</td>
<td>0.5 ml/kg/h for 16 h</td>
</tr>
<tr>
<td><strong>Failure</strong></td>
<td>eCCl decrease by 75% or eCCl &lt;35 ml/min/1.73 m</td>
<td>&lt;0.3 ml/kg/h for 24 h or anuric for 12 h</td>
</tr>
<tr>
<td><strong>Loss</strong></td>
<td>Persistent failure &gt;4 weeks</td>
<td></td>
</tr>
<tr>
<td><strong>Endstage</strong></td>
<td>End-stage renal disease (persistent failure &gt;3months)</td>
<td></td>
</tr>
</tbody>
</table>
5.2.3.2 How should n-acetylcysteine be given?

While both oral and IV NAC are approved protocols (for acetaminophen overdose) the use of IV NAC is recommended for the indication of renal protection for several reasons. Oral NAC has a low oral bioavailability of 4-10% [49], therefore a greater systemic dose can be achieved with IV NAC, even when total oral dose is much higher (Table 5.3). Furthermore oral NAC has a high rate of nausea and vomiting as a side effect making it difficult know what dose, if any at all, was administered [37]. However most importantly, the 21-hour IV dose that can be given safely provides the highest systemic dose, therefore assuring the patient has the highest level of renal protection.

As IFO cycles can range for 2-5 days [50], it is recommended that NAC be given in the 21-hour IV dose on all days following IFO administration. While no studies exist using repeated administration of the 21-hour IV protocol, as discussed above, oral NAC is routinely given daily for up to 6 month without any serious adverse events [42]. The 21-hour IV protocol is as follow should be administered as follows:

For patients over 40kg [51](Table 5.3):

For patients under 40kg [52](Table 5.4):

The 200mg/mL solution of NAC should be diluted to 40mg/mL with D5W to avoid excessive free water leading to hyponatremia and seizures.
Renal function should be measured each day following IV NAC and 1 week following completion of treatment. Ifosfamide treatment can be continued with special attention being paid to monitoring for changes in renal function. Concomitant NAC treatment should be considered in all future IFO therapy as a preventative measures.
Table 5.3 21-hour intravenous protocol for NAC.
<table>
<thead>
<tr>
<th></th>
<th>Dose</th>
<th>Volume of D5W</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>150 mg/kg</td>
<td>200mL</td>
<td>15-60 minutes</td>
</tr>
<tr>
<td>2</td>
<td>50 mg/kg</td>
<td>500mL</td>
<td>4 hours</td>
</tr>
<tr>
<td>3</td>
<td>100 mg/kg</td>
<td>1000mL</td>
<td>16 hours</td>
</tr>
</tbody>
</table>
Table 5.4 Dilution of 21-hour IV NAC protocol for patients under 40kg.
<table>
<thead>
<tr>
<th>Infusion</th>
<th>Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.75 ml/kg of NAC 200 mg/ml in 3 ml/kg of D5W with maximum of 200 ml of D5W administered over 1h</td>
</tr>
<tr>
<td>2</td>
<td>0.25 ml/kg of NAC 200 mg/ml in 10 ml/kg of D5W with maximum of 500 ml of D5W administered over 4h</td>
</tr>
<tr>
<td>3</td>
<td>0.5 ml/kg of NAC 200 mg/ml in 20 ml/kg of D5W with maximum of 1000 ml of D5W administered over 16h</td>
</tr>
</tbody>
</table>
5.2.3.3 Measurement of tumor response

Tumor response should be evaluated according to Jurgens et al. [4]. Tumors should be assessed for response with X-ray and/or computerized axial tomography at the end of treatment and will be classified as follows:

1) Complete remission: Lack of measurable evidence of disease
2) Partial remission: regression of more than 50% of tumor size
3) Non-responder: regression of less than 50% of tumor size and progressive disease

5.2.4 Considerations of n-acetylcysteine usage

Several considerations should be given when using NAC treatment for IFO-induced renal toxicity.

5.2.4.1 Dose and route of administration

IV NAC is the preferred treatment option as it is the standard protocol currently used by most and because it provides a higher systemic exposure, therefore ensuring the highest NAC dose possible. However, there may also be circumstances in which administration of IV NAC is either not practical or not possible. Under such circumstances we would recommend the use of oral NAC as opposed to no treatment at all. Our second case report suggests that the systemic doses achieved with oral NAC can be sufficient in providing renal protection. In such circumstances we recommend the use of 600mg BID in order to provide the patient with a minimum amount of renal protection. However, we again re-iterate, IV NAC is the preferred treatment option as it is the standard protocol used by most and because it provides a higher systemic exposure, therefore ensuring the highest NAC dose possible.
5.2.4.2 Adverse drug reactions

As previously discussed, there exists the potential for adverse drug reactions (ADRs) when using NAC, most commonly anaphylactoid reactions when administration is IV. Following presentation of such a reaction, we recommend following the guidelines published by Bailey et al. [41]. These guidelines are based on a 6-year retrospective case series and a literature review, and were assessed prospectively in a poison centre. Following an anaphylactoid reaction, they recommend reassessing the need for NAC. Should it be deemed necessary, they suggest IV administration of diphenhydramine 1mg/kg (maximum 50mg); NAC may be restarted 1 hour after [41].

5.2.4.3 Other

IFO is commonly given in combination therapy either as part of the ICE protocol including IFO, carboplatin and etoposide as well in combination with etoposide alone. It is important to be aware that work exploring the effects of NAC on both these chemotherapeutic agents has been assessed in animal models. These studies demonstrated that NAC had no effect on the antineoplastic effects of either carboplatin or etoposide [53]. Second, while the ideal patient treated with NAC would present only suffering from nephrotoxicity caused by IFO and would be treated with IV NAC during their IFO cycle, pediatric oncology is far from ideal. Patients may present with declining renal function as a result of previous treatment with nephrotoxic drugs, such as platinums or methotrexate. Under these circumstances, treatment with NAC is still recommended. As demonstrated in two of the case reports, a patient may present with declining renal function prior to any treatment with IFO. In both cases recovery of
kidney function was observed even when the primary renal insult may not have been IFO. Furthermore, renal dysfunction may develop following the completion of all IFO cycles, therefore preventing the administration of NAC during the IFO cycle as recommended above. As evidenced in 2 of the described cases, NAC also provided renal protection even after the IFO cycle was completed, suggesting children will still benefit from NAC therapy even when administration is delayed. We would therefore still recommend that patients with delayed onset of nephrotoxicity be administered at least one course of the 21 hour IV dose of NAC.

5.2.5. Future directions

The ultimate goal of NAC therapy would be to provide patients with renal protection, much in the way that MESNA is currently always given alongside IFO; or at a very minimum to susceptible individuals once the means of identifying them becomes available. The future direction of NAC therapy includes a RCT designed to assess the effectiveness of NAC as a prophylactic strategy for IFO-induced nephrotoxicity. In the meantime, NAC rescue therapy is the most promising strategy available to patients and is an important consideration in the maintenance of the health and well being of the patient.

5.3. N-acetylcysteine as a prophylactic strategy against nephrotoxicity in children caused by ifosfamide; a randomized control trial protocol

5.3.1 Rationale

NAC is currently used clinically in children for acetaminophen overdose [33, 54, 55]. With this in mind it is clear that NAC is an excellent candidate in the
prophylactic treatment of renal toxicity caused by IFO, as safety and efficacy in children has been established. Children are a population for which this data is often limited and difficult to obtain. The availability of this data for NAC allows for a relatively short amount of time until it can be used, while compounds for which this data is not known would take many years to be realized clinically. NAC would provide immediate treatment of IFO-nephrotoxicity following evidence suggesting it will provide renal protection during IFO therapy. The current protocol used worldwide is the 21-hour IV protocol consisting of infusion of 150mg/kg over 60 minutes, 50 mg/kg for 4 hours and finally 100mg/kg for another 16 hours providing a total dose of 300mg/kg [54, 56]. In the past other protocols, providing both greater and lower total doses have been explored and used in the treatment of acetaminophen overdose and include a 72 and 36 hour oral dose and a 48 hour IV dose (Table 2.1).

To test the ability of NAC to protect against IFO-induced nephrotoxicity in a pediatric population, we propose to conduct a randomized controlled trial during which children receiving IFO therapy will be randomly assigned to one of two groups, one receiving IFO only and the other receiving IFO and concurrent NAC treatment. Preliminary data suggesting the effectiveness of NAC in the treatment of nephrotoxicity caused by ifosfamide includes 3 case reports, summarized above in supporting evidence section of the NAC rescue protocol. These cases suggest NAC is effective in providing treatment of renal toxicity during IFO therapy. However, before NAC can be approved for use in a clinical setting, its effectiveness must be shown in a controlled fashion that we propose to do with a RCT.

5.3.2 Hypothesis and aim
NAC, in the 21-hour IV formulation used for acetaminophen overdose, given as adjuvant to IFO chemotherapy, will decreased the incidence of nephrotoxicity in children being treated for their cancer.

Our aim is to examine the effectiveness of NAC in the prevention of IFO-induced nephrotoxic adverse events.

This study will provide evidence to support or counter the introduction of the 21-hour IV NAC dose, as standard of care, for children receiving IFO treatment.

5.3.3 Experimental approach

This phase III multicentre prospective double-blinded RCT will compare the effectiveness of the 21-hour IV protocol of n-acetylcysteine as a prophylactic treatment against renal dysfunction resulting from IFO.

5.3.3.1 Study design

In this randomized controlled trial, participants will be randomized according to block randomization. There will be four blocks represented by each cancer type in which patients will be assigned randomly to receive NAC concomitantly with IFO and MESNA infusion or to receive a placebo saline infusion. 100 patients will be randomized to each arm (total 200). Both patient and clinician will be blinded to treatment received.

5.3.3.2 Study population

Children and adolescents diagnosed with osteosarcoma, Ewing’s sarcoma, acute lymphoblastic leukemia and non-Hodgkin’s lymphoma, where IFO will be included in their chemotherapy treatment will be eligible for participation. Written, informed consent will be obtained from parents and legal guardians of all participants under the
age of 18. Assent will be obtained in all children over the age of 7. Patients will be recruited from the Division of Haematology/Oncology out of the Department of Pediatrics from both the Hospital for Sick Children in Toronto, Ontario and the London Health Sciences Centre in London, Ontario. Ethical approval will be obtained from institutional research ethics boards at both sites.

5.3.3.3 Inclusion criteria

1. Primary diagnosis with osteosarcoma, Ewing’s sarcoma, acute lymphoblastic leukemia and non-Hodgkin’s lymphoma treated with IFO alone or IFO in combination therapy
2. Between the ages of 0-21
3. Written informed consent from parent or guardian, or patients where applicable
4. Assent from children over the age of 7

5.3.3.4 Exclusion criteria

1. Prior or current renal dysfunction
2. Hepatic dysfunction
3. Prior chemotherapy
4. Failure to obtain a written consent/assent
5. Participation in other clinical trials

5.3.3.5 Primary Endpoints

**Renal function:** Renal function will be assessed as per protocol by Loebstein *et al.* [47].

**Glomerular filtration:** Shwartz formula, the ratio of height to plasma creatinine
Tubular function: serum concentration of electrolytes, phosphate, bicarbonate, calcium, magnesium and serum pH, and glycosuria and protienuria as assessed through spot urine sample.

The following five criteria were used to define abnormal renal function:

1) Hypophosphatemia (<2 SD for age)

2) Hypocarbia together with acidosis (bicarbonate<18mEq/L together with pH<7.32)

3) Glycosuria (any amount)

4) Proteinuria (1.0 g/L or greater)

5) Decreased creatinine clearance (80 mL/min/1.73 m²).

Each criterion was considered only if it appeared as normal in the baseline renal function assessment and became abnormal following the start of IFO and only if it appeared as abnormal at least twice. Each patient will be classified in one of four categories based on their degree of nephrotoxicity:

Non-nephrotoxic: no abnormal criteria recorded

Mildly nephrotoxic: one abnormal criterion recorded

Moderately nephrotoxic: 2-3 abnormal criteria

Severely nephrotoxic: 4-5 abnormal criteria

Tumor response: Tumor response will be evaluated according to the criteria of Jurgens et al. [4]. Tumors will be assessed for response with X-ray and/or computerized axial
tomography at the end of treatment and will be classified as follows:

*Complete remission:* Lack of measureable evidence of disease

*Partial remission:* regression of more than 50% of tumour size

*Non-responder:* regression of less than 50% of tumor size and progressive disease

5.3.3.6 Methodology

Following enrollment into the clinical trial, the patient and their parents will meet with the study coordinator at the Hospital for Sick Children or London Health Sciences Centre, 1 week prior to starting chemotherapy treatment. During this meeting, the study coordinator will become familiar with the patient and their family, providing them with information regarding the study, answering any questions they have in a private setting. Following consent to participate, a trial nurse will draw blood in order to assess baseline kidney function, as well as rule out any the presence of any exclusion criteria.

During chemotherapy treatment, the 21-hour IV NAC protocol or placebo will be given each day immediately following the chemotherapy session including IFO. Kidney function will be reassessed at the end of each chemotherapy cycle, as well as prior to the start of the next IFO containing cycle. Tumor response will be measured at the end of each cycle. Follow-up appointments, which will include measuring kidney function and tumor response, will occur every 3 months for 24 months, and every 6 months thereafter. Trial follow-up will end 3 years post-treatment.

Patients, who continue to suffer from grade 2 nephrotoxicity or higher for more than two successive renal function tests, will be switched to protocol containing less
nephrotoxic drugs and their nephrotoxicity treated according to the institutional standard of care.

Patients, who suffer from an anaphylactoid reaction resulting from IV NAC, will be given 1mg/kg of IV diphenhydramine (maximum 50 mg); one hour after NAC infusion can be resumed. This treatment is according to recommendations on how to manage anaphylatoid reactions caused by NAC [41]. Supportive care for all chemotherapy side effects will be treated as per the institutions standard of care.

5.3.3.7 Sample size and statistical calculations

A sample size of 50 per group was calculated a priori and designed to detect a clinically significant decrease in incidence of nephrotoxicity from 30% to 15% with 80% power and alpha of 0.05. This was defined by expert consensus as being clinically significant through consultation with clinicians and is based on the current rate of nephrotoxicity with current treatment protocols. Proportions will be compared by Chi square or Fisher exact test.

5.3.4 Ethical considerations

This study has been discussed and received input from practicing oncologists, nephrologists, and pharmacologists. While it may result in a small increase in baseline risk due to the chance of anaphylactoid reaction associated with IV NAC, this adverse event is non-life-threatening, is easily treated, and is less serious than nephrotoxicity; therefore its potential benefit in preventing nephrotoxicity far outweighs the risk associated with an anaphylactoid reaction. Due to the nature of oncology, and the timeliness with which treatment must commence, the study is discussed with patients one week prior to the start of therapy under non-stressful conditions. With the exception
of the baseline kidney function test, all blood sampling will be obtained as a part of routine care, which would occur regardless of enrollment in the trial.

5.4 Overall discussion and future directions

80% of children will survive their cancer; however, two-thirds of those who have survived their childhood cancer will suffer health problems secondary to their cancer treatment. This is a significant number of people who are unnecessarily suffering an impaired quality of life. These late effects can be protected against provided the opportunity to research prophylactic and treatment strategies. The use of chemoprotectants as means to prevent side effects caused by chemotherapy is not new. In oncology, these late effects are merely the cost of successful cancer therapy. In regards to IFO, treatment of these late effects has come a long way. MESNA is routinely given alongside IFO therapy in order to prevent urotoxicity and a wealth of information suggests NAC will prevent nephrotoxicity.

In order to identify NAC as a potential treatment strategy for nephrotoxicity associated with IFO, the first step is to understanding its role in kidney toxicity. Identifying CAA the nephrotoxic metabolite, allows for a clear definition of the mechanism through which the kidney was damaged; oxidative stress, suggesting a clear direction for potential treatment and prevention options. Antioxidants appear to be a logical solution to a problem resulting from oxidative damage. Further to that end, n-acetylcysteine, with its current use in children appears the most reasonable choice in a long list of antioxidant compounds. Equipped with a well-chosen candidate for renal protection, demonstrating the efficacy of NAC in both cell and animal models is the
next step in continuing this translational research. The studies described in this thesis provide the final step in defining a treatment that will allow for prevention of renal damage caused by ifosfamide treatment.

The work involving NAC truly has gone through the evolution of bench to bedside. Beginning with a lack of knowledge surrounding how nephrotoxicity might occur, to then a clear picture of the mechanisms involved, enabled potential treatment strategies to become clear. This will significantly impact the health of those at risk for developing nephrotoxicity. However it might also serve as an example and provide insight into the prevention of other late effects resulting from cancer therapy.

With respect to future work, there is still more that can be done to achieve a better understanding of nephrotoxicity. It is likely that genetics play some role in deciding which children will be affected by nephrotoxicity. Therefore we suggest future studies include assessing genetic variability that may contribute to the risk of developing nephrotoxicity. Aldehyde dehydrogenase, which is responsible for deactivation of CAA to chloroacetate in the tubule cells shows variability with both high and low expressing polymorphisms [57-59]. This information could define clearer strategies for use of NAC during IFO therapy such that only children at risk for or displaying signs of renal dysfunction will be given this treatment.
5.5 References


Appendices

Appendix 1: Copyright approval for published work

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Author: Lauren N. Hanly, Nancy Chen, Katarina Aleksa, Murray Cutler, Milica Bajcetic, Rasmi Palassery, Osvaldo Regueira, Curtis Turner, Bandar Baw, Becky Malkin, David Freeman, Michael J. Riede, Tetyana L. Vasyl'ova, Gideon Koren

Publication: Journal of Clinical Pharmacology
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PhD Candidate
Appendix 2: Ethics approval for clinical studies
Appendix 3: Ethics approval for animal studies

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Your Animal Use Protocol form entitled:

Renal Handling of Drugs during Development

has been approved by the Animal Use Subcommittee.
This approval is valid from 06.01.07 to 06.31.08.
The protocol number for this project remains as 2006-038-05.

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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.

c.c. Approved Protocol Approval Letter - G. Koren, M. Rieder, W. Lagerwerf, N. Chen
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PROTOCOL MODIFICATION FORM - #2008-069 Koropatnick

1. INVESTIGATOR CONTACT INFORMATION – See Section 1, AUP Reference
1. PI Full Name: James Koropatnick
2. Primary Institution/Department: LHFV/CHLIP
3. Office Location – Building/Room #: Victoria Research Building, A4-10/A
4. Weekday Work Hours Phone #: ext. 58854
5. PI After-Hours Emergency Contact: [Redacted]
6. Pager – Phone & Pager # (Optional): [Redacted]
7. UWO Email: [Redacted]
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<td>Yes [ ] No [ ]</td>
<td>Yes [ ] No [ ]</td>
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1. Has this project received scientific peer review resulting in funding identified in Section 5? Yes [ ] No [ ]
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3. If "NO" to Items 1 AND 2, and this is NOT a SCIENTIFIC TEACHING protocol, please provide a list of ONE TO THREE PUBLICATIONS (relevant to this project). This project is being undertaken in the my laboratory (Koropatnick) in collaboration with Dr. Gideon Koren. Consequently, the supporting publications are from his laboratory:

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<td>8. Please list control measures to be taken to minimize risk of exposure to animal facility staff (e.g. safety glasses, respiratory protection, protective clothing, barrier cabinet, etc.) and identify re-measure(s) or safety change(s).</td>
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<td>9. If animals are transported between buildings, please describe the procedures.</td>
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<td>10. Describe the decontamination process for cages, bedding and other exposed materials.</td>
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<td>11. Describe the carcass disposal method and outline the procedures taken.</td>
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<tr>
<th>Safety Officer Name</th>
<th>Institution</th>
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<tr>
<td>A.M. McCaskill</td>
<td>Western</td>
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I authorize the contents of this Section. Authorization Date (mm/dd/yy): 10/03/10
Appendix 4: Plasma AUC of NAC of patients treated for acetaminophen overdose
Appendix 4.1 The systemic exposure of NAC in subject 2, receiving the standard NAC intravenous protocol, 150 mg/kg for 60 minutes, 50 mg/kg for 4 hours, and 100 mg/kg for 16 hours. AUC is 20.57 mM·h, where the dotted line represents the residual area calculated.
Appendix 4.1
Appendix 4.2 The systemic exposure of NAC in subject 3, receiving the standard NAC intravenous protocol, 150 mg/kg for 60 minutes, 50 mg/kg for 4 hours, and 100 mg/kg for 16 hours. AUC is 17.58 mM·h, where the dotted line represents the residual area calculated.
Appendix 4.2

![Graph showing the relationship between NAC plasma concentration (umol/L) and time (hr). The concentration decreases over time.]
Appendix 4.3 The systemic exposure of NAC in subject 4, receiving the standard NAC intravenous protocol, 150 mg/kg for 60 minutes, 50 mg/kg for 4 hours, and 100 mg/kg for 16 hours. AUC is 8.01 mM·h, where the dotted line represents the residual area calculated.
Appendix 4.3

![Graph showing NAC Plasma Concentration (umol/L) over Time (hr)].

- The x-axis represents Time (hr) ranging from 0 to 40.
- The y-axis represents NAC Plasma Conc. (umol/L) ranging from 0 to 600.
- The graph illustrates the concentration profile of NAC in plasma over time, peaking at approximately 600 umol/L at 10 hours, then decreasing gradually.
Appendix 4.4 The systemic exposure of NAC in subject 7, receiving the standard NAC intravenous protocol, 150 mg/kg for 60 minutes, 50 mg/kg for 4 hours, and 100 mg/kg for 16 hours. AUC is 10.93 mM·h, where the dotted line represents the residual area calculated.
Appendix 4.4
Appendix 4.5 The systemic exposure of NAC in subject 8, receiving the standard NAC intravenous protocol, 150 mg/kg for 60 minutes, 50 mg/kg for 4 hours, and 100 mg/kg for 16 hours. AUC is 6.22 mM·h, where the dotted line represents the residual area calculated.
Appendix 4.5
Appendix 4.6 The systemic exposure of NAC in subject 9, receiving the standard NAC intravenous protocol, 150 mg/kg for 60 minutes, 50 mg/kg for 4 hours, and 100 mg/kg for 16 hours. AUC is 7.46 mM·h, where the dotted line represents the residual area calculated.
Appendix 4.6

![Graph showing the relationship between NAC plasma concentration (umol/L) and time (hr). The concentration increases linearly with time.]
Appendix 4.7 The systemic exposure of NAC in subject 11, receiving the standard NAC intravenous protocol, 150 mg/kg for 60 minutes, 50 mg/kg for 4 hours, and 100 mg/kg for 16 hours. AUC is 13.27 mM·h, where the dotted line represents the residual area calculated.
Appendix 4.7
Appendix 4.8 The systemic exposure of NAC in subject 13, receiving the standard NAC intravenous protocol, 150 mg/kg for 60 minutes, 50 mg/kg for 4 hours, and 100 mg/kg for 16 hours. AUC is 16.95 mM·hr, where the dotted line represents the residual area calculated.
Appendix 4.8

![Graph showing the relationship between NAC plasma concentration (umol/L) and time (hr). The concentration increases linearly with time.]
Appendix 4.9 The systemic exposure of NAC in subject 14, receiving the standard NAC intravenous protocol, 150 mg/kg for 60 minutes, 50 mg/kg for 4 hours, and 100 mg/kg for 16 hours. AUC is 10.38 mM·hr, where the dotted line represents the residual area calculated.
Appendix 4.9

![Graph showing N-Acetylcysteine (NAC) Plasma Conc. (umol/L) vs. Time (hr)](image-url)
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University of Western Ontario  
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EDUCATION

Doctor of Philosophy, Pharmacology and Toxicology.  
University of Western Ontario, London, Canada.  
2008-2013

Advisors: Gideon Koren (MD) and Michael Rieder (MD, PhD).  
Thesis: N-acetylcysteine as a chemoprotectant against ifosfamide  
Nephrotoxicity; from mechanism to prevention.

Bachelor of Medical Science (Honours), Faculty of Medicine  
University of Western Ontario, London, Canada.  
2003-2008

SCHOLARSHIPS AND AWARDS

Paediatric Research Day; 1st place Clinical Research Oral  
Presentation  
2013

Canadian Society of Pharmacology and Therapeutics; K.M.  
Piafsky Trainee Presentation Award.  
2012

Canadian Institute of Health Research; Doctoral Research Award  
Priority Announcement : Childhood Cancer (SHOPP).  
2011-2013

Canadian Society of Pharmacology and Therapeutics;  
Travel Bursary  
2009-2012

University of Western Ontario; Schulich Graduate Scholarship  
2008-2013

University of Western Ontario; Dean’s Honor Roll  

University of Western Ontario; Scholarship of Excellence  
2003

Royal Arch Masons; Masonic Bursary  
2003-2006

St. Michaels Catholic Secondary School; CFUW Stratford Club  
Scholarship  
2003

St. Michaels Catholic Secondary School; he Lions R.N. Bissonnette  
Scholarship  
2003

St. Michaels Catholic Secondary School; OAC Biology, Father  
2003
Joseph O’Rourke memorial award

AREAS OF RESEARCH INTEREST

Pharmacology, toxicology
Paediatric Oncology
Personalized Medicine
Drug Safety in Lactation
Pharmacogenomics
Nephrology
Drug Safety in Pregnancy
Clinical Trials

TEACHING EXPERIENCE

Department of Physiology and Pharmacology, University of Western Ontario:
• Pharmacology 4660a (Human Toxicology): Course Manager (2010-2012),
  Teaching Assistant (2009-2012).
• Medical Science 4930F/G (Selected topics in Medical Science (2008-2009).

Faculty of Medicine, University of Western Ontario;
• Meds IV Therapeutics and Diagnostics (2010-2013).

Canadian Institutes of Health Research- Let’s Talk Science Partnership Program; Teaching local elementary class and science fair judge (2008-2011).

PUBLICATIONS


Hanly L, Rieder MJ; Huang SS, Vasylyeva TL, Shah RK, Regueira O, and Koren G.

**INVITED SPEAKER**


Canadian Pediatric Society Annual Conference 2012: Drugs and the Kidney (2012).

**SELECTED PLATFORM PRESENTATIONS**

Canadian Society of Pharmacology and Therapeutics; The effect of n-acetylcysteine on the antitumour efficacy of ifosfamide in a mouse xenograft model

Paediatric Research Day: N-acetylcysteine protects against ifosfamide-induced nephrotoxicity; translational research

**CONFERENCES ATTENDED**

Canadian Society of Pharmacology and Therapeutics Annual Meeting
- Trainee Oral Presenter, Toronto, Ontario 2012
- Poster Presenter, Montreal, Quebec 2011
- Poster Presenter, Toronto, Ontario 2010
- Poster Presenter, Winnipeg, Manitoba 2009

Paediatric Oncology Group of Ontario Annual Conference
- Poster Presenter, Toronto, Ontario 2012
- Poster Presenter, Toronto, Ontario 2011

American Society
- Poster Presenter, Dallas, Texas 2011

Margaret Moffat Research Day
- Poster Presenter, London, Ontario 2012
- Poster Presenter, London, Ontario 2010
- Poster Presenter, London, Ontario 2009
Lawson Research Day
- Poster Presenter, London, Ontario 2010
- Poster Presenter, London, Ontario 2009

IUPHAR World Pharma 2010
- Poster Presenter, Copenhagen, Denmark 2010

RELATED PROFESSIONAL EXPERIENCE

The Motherisk Program; Hospital for Sick Children, Toronto, Canada. 2010- Present
Bilingual information counsellor.

The N-acetylcysteine Consortium: International Coordinator. 2012-2013

7th Annual IHDCYH Summer Institute in Maternal Fetal Pharmacology. 2011
Canadian Intitute of Health Research/Natioal Institute of Child and Health Development, Montreal Quebec.

Children’s Health Research Institute Representative: Counsel for Canadian Child Health Research Annual Symposium, Winnipeg, Manitoba. 2010

PROFESSIONAL AFFILIATIONS

Canadian Society of Pharmacology and Therapeutics; trainee 2009-present

April 2013