The Effects of N-acetylcysteine on Ifosfamide Efficacy in a Mouse Xenograft Model

L. Hanly, R. Figueredo, M.J. Rieder, J. Koropatnick and G. Koren

Background/Aim: Nephrotoxicity is observed in 30% of children treated with ifosfamide. We have shown that N-acetylcysteine (NAC) successfully mitigates nephrotoxicity of ifosfamide in cell and rodent models. However, before this treatment is evaluated clinically, it must be established that NAC does not interfere with the efficacy of ifosfamide.

Materials and Methods: Mice implanted with Ewing’s sarcoma tumours received the following treatments: saline, ifosfamide, ifosfamide + NAC concurrently, pre-treatment with NAC + ifosfamide, or NAC alone. Results: Median volumes of EW-7 tumour xenografts in mice treated with ifosfamide (n=8), ifosfamide with concurrent NAC therapy (n=7), and NAC pre-treatment (n=6) (p<0.05) were significantly reduced compared to median tumour volumes of control mice (n=6). None of the NAC treatments affected ifosfamide-mediated reduction in tumour volumes. Conclusion: NAC does not interfere with the efficacy of ifosfamide in a EW-7 xenograft model. These results support the clinical evaluation of NAC as a strategy against ifosfamide-induced nephrotoxicity in children.

Correspondence to: Dr. Gideon Koren, Department of Physiology and Pharmacology, Schulich School of Medicine and Dentistry, University of Western Ontario, London, Ontario, Canada. E-mail: gkoren@uwo.ca

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associated with IFO, concomitant administration of 2-mercapto-ethan sulphonate 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 produces the active anti-neoplastic agent IFO mustard, and acrolein (Figure 1). 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 isozymes 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-renal (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, 32), as the drug of choice in children and adults for treatment of acetaminophen overdose using a 21-h i.v. 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 paediatric 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.

Materials and Methods

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

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.

Tumour 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 20x10⁶ 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%.

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).

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, i.p.) daily for 6 days. (2) NAC group: NAC (1.2 g/kg in 0.3 ml 0.9% saline, pH 7.2 i.p.) 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 i.p.) daily for 3 days.

(4) Concurrent NAC + IFO group: IFO plus NAC (60 mg kg \(^{-1}\) IFO and 1.2 g/kg NAC, both in 0.3 ml 0.9% saline, pH 7.2 i.p.) 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 i.p.) daily for 6 days, followed by IFO (60 mg/kg in 0.3 ml 0.9% saline, pH 7.2 i.p.) daily for 3 days.

Tumours were allowed to grow until the fastest-growing tumour of each mouse reached a volume of 1000 mm\(^3\), at which point the mouse was euthanized.

**Outcome measures.** Tumour volumes were estimated every 2 days by caliper measurements (volume=0.52 (length\(\times\)width\(^2\))) until euthanasia.
Statistical analysis. Control and treatment groups were compared using the Mann-Whitney U-test, as indicated. Values are presented as median±interquartile range.

Results

Effect of ifosfamide on EW-7 Ewing xenograft growth. We investigated the effects of NAC on IFO efficacy in a tumour xenograft mouse model. When EW-7 tumours in host mice reached the target size (approximately 100 mm³) 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 2).

Effect of concurrent NAC+IFO 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 3). 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 3). 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.

Effect of pre-treatment NAC+IFO 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. Pre-treatment 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 tumours in mice treated with IFO alone with those in mice pre-treated with NAC before IFO treatment revealed that NAC pre-treatment had no effect on the capacity of IFO to inhibit EW-7 tumour growth (Figure 4). As with concurrent NAC and IFO therapy, tumours 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.

Effect of NAC 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 tumour volumes on days 65 and 68 following treatment, tumour volumes at all other time points were not significantly different between NAC- and saline-
treated mice, suggesting that the effects of NAC alone on tumour growth in this test system are either negligible or non-existent (Figure 5).

**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 GSH levels, GST 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-h i.v. 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 ifosfamide-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 antitumour 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 tumour 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 tumours.
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 which 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 glutathione depletion, lipid peroxidation, decreased GST 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, and 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 tumour volumes, but should not result in complete ablation of tumours. 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 2). IFO treatment alone for 3 days reduced median EW-7 tumour volume compared to saline treatment but did not induce complete tumour 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 tumours led to significantly lower median tumour volumes compared to control mice (Figure 3). This demonstrated that NAC plus IFO therapy is effective in reducing EW-7 tumour 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 tumour growth allowed this evaluation. We showed that IFO reduced tumour growth, and that its potential was unaffected by concurrent NAC treatment (Figure 3). NAC, had therefore no negative effect on the ability of IFO to reduce tumour 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 pre-treatment with NAC plus IFO to control saline-treated mice shows that this therapy is capable of reducing EW-7 tumour volumes (Figure 4). Furthermore, a comparison between pre-treatment NAC plus IFO with IFO alone showed no significant difference between the two with respect to median tumour volumes (Figure 4). 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 tumour 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 tumour 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).

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 antitumour 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 protective agent for children with cancer.

Conflicts of Interest

The Authors declare no conflicts of interest.

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References