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ORIGINAL ARTICLE

Effect of *N*-acetylcysteine route of administration on chemoprotection against cisplatin-induced toxicity in rat models

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Abstract Dosing and route of administration of *N*-acetylcysteine (NAC) for protection against cisplatin (CDDP) nephrotoxicity was investigated in rats. Two models of toxicity were tested: a single high dose of CDDP (10 mg/kg intraperitoneally (IP)), and multiple low dose treatments (1 mg/kg IP twice a day for 4 days, 10 days rest, then repeated). NAC (50–1,200 mg/kg) was given to the rats by IP, oral (PO), intravenous (IV) and intra-arterial (IA) routes. Renal toxicity was determined by blood urea nitrogen (BUN) and creatinine (CR) levels 3 days after treatment. Blood collected 15 min after NAC was analyzed for total NAC. Both models of CDDP administration produced renal toxicity. In the single dose CDDP model, NAC 400 mg/kg given IP and PO produced no renal protection as measured by BUN (131.8 ± 8.2 and 123.3 ± 8.2, respectively) or CR (2.3 ± 0.38 and 1.77 ± 0.21, respectively). IV NAC reduced nephrotoxicity, (BUN 26.3 ± 6.8, CR 0.47 ± 0.15). NAC 50 mg/kg IA gave better protection than IV. In the repeated-dose CDDP model, nephrotoxicity was blocked by 800 mg/kg NAC given IV but not IP. Blood concentrations of total NAC showed a dose response after IV NAC, but high dose NAC (1,200 mg/kg) by the PO route gave very low levels of NAC. Thus the protective properties of NAC are affected by the dose and route of administration.

Keywords *N*-acetylcysteine · Cisplatin · Chemoprotection · Route · Rats

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Introduction

N-acetylcysteine (NAC) is a cysteine analog with free radical scavenging activity that is gaining use as a chemoprotective agent [13]. In vitro, NAC has been shown to block cisplatin (CDDP)-induced apoptosis through the caspase-signaling pathway [23]. In animals and in human trials, NAC is protective against CDDP-induced toxicities, such as ototoxicity [2, 6, 8, 9, 17] and nephrotoxicity [1, 7, 20]. High doses of NAC, given intravenously or intra-arterially are protective even when administered significantly before or after chemotherapy [7]. In animals with brain tumors, thiols are protective against chemotherapy side effects

without reducing anti-tumor efficacy [16, 18, 19]. The thiols were given pre- or post-chemotherapy according to the two-compartment model, in which the reduction of tumoricidal effects are avoided by separating chemoprotectant and chemotherapy treatments in time or space, and the various systems are protected while the tumor is not [17, 19].

In addition to chemotherapy toxicity, NAC is also being evaluated for protection against imaging contrast-induced nephropathy [10, 14, 21, 24]. Multiple clinical trials for this indication have been reported, as well as two meta-analyses, but the efficacy remains unclear. These trials have generally used very low doses of NAC (~10 mg/kg) given by oral administration with inconsistent results. Shalansky et al. reported that certain clinical trials indicate the importance of dose and route of administration of NAC for prevention of contrast-induced nephrotoxicity [21]. We hypothesize that the dose and routes of administration of thiols given for protection could affect the level of protection achieved in chemotherapy toxicity.

The current studies utilize a rat model of CDDP-induced nephrotoxicity that has been shown to result consistently in renal tubule damage. Nephrotoxicity is reflected in high values of blood urea nitrogen (BUN) and creatinine (CR) when CDDP 10 mg/kg is given IP [7]. We believe that this CDDP toxicity provides a model for other nephrotoxicity, including contrast-induced nephrotoxicity. NAC given at 400 mg/kg IV was shown to be protective against nephrotoxicity in this model, but the effects of lower doses and different routes of administration have not previously been investigated. We have also developed a new model of nephrotoxicity induced by repeated small doses of CDDP over an 18-day period. In the present study we evaluated chemoprotection and pharmacology after NAC was given by multiple routes of administration. Serum levels of total NAC were also assessed in rats.

Methods

Animals

Animal studies were performed in accordance with guidelines established by the Oregon Health & Science University Institutional Animal Care Committee (IACUC) and with their approval of the protocols. Animals were housed at the Oregon Health & Science University Animal Facility and under the care of staff veterinarians.

Pharmacological agents

Cisplatin (Platinol) was obtained from Bristol-Myers Squibb (New York, NY, USA) and *N*-acetylcysteine was obtained from Abbott Laboratories (North Chicago, IL, USA) via the OHSU Hospital pharmacy.

Nephroprotection studies

For the single high dose CDDP nephrotoxicity model, female Long Evans rats received CDDP 10 mg/kg IP, a dose that has been shown to produce renal tubule damage [7]. In the first study, rats were given either no NAC ($n = 7$) or NAC at 400 mg/kg 30 min prior to CDDP by the following routes of administration: oral (through an 18 French feeding tube, $n = 7$), IP ($n = 6$), or IV (through a 27GA butterfly needle in the femoral vein, $n = 6$). In the second study, rats were given NAC 50 mg/kg IV ($n = 6$) or IA (through a catheter down the descending aorta, $n = 7$) 30 min before CDDP, to serve as a model of renal arterial perfusion. Four rats were given CDDP 10 mg/kg IV for comparison to the IP group.

Three days after treatment, the rats were weighed, anesthetized with isoflurane, and blood samples were taken by intracardiac puncture. Blood chemistry analysis for BUN and creatinine concentrations in undiluted blood samples was performed with the I-Stat portable Clinical Analyzer (Heska Corp., Waukesha, WI, USA). The Heska analyzer BUN measurement peaks at 140 mg/dl, a level that has been shown histologically to be consistent with renal tubule damage [7]. The animals were then sacrificed while still anesthetized using an intracardiac injection of sodium pentobarbital, a method approved by OHSU Animal Guidelines.

A third study evaluated repeated CDDP doses. These rats were given a regimen of CDDP 1 mg/kg IP twice a day for 4 days. After 10 days with no treatment, the CDDP dosing was repeated. Rats received either no NAC ($n = 5$) or were treated with NAC 800 mg/kg IP ($n = 5$) or IV ($n = 5$) 15 min before each CDDP injection during the second phase of CDDP treatment. Blood samples were taken 3 days after the final CDDP dosage and analyzed for BUN as described above.

Pharmacology study

To determine serum levels of NAC and its metabolites following various doses, Long Evans female rats were anesthetized with an isoflurane/O_2 mixture and infused with NAC 100, 400 and 1,200 mg/kg IV, with three rats per group. Blood samples were taken by cardiac puncture 15 min post-inoculation and the animals sacrificed. Another group of rats was given NAC 1,200 mg/kg PO, with blood samples taken after 15 min in two rats and after 60 min in one rat post-NAC. The samples were allowed to clot, centrifuged at 3,000 rpm for 10 min (Beckman/Coulter Allegra 6R centrifuge) and the serum drawn off and frozen at −80°C prior to analysis by HPLC.

For chromatography, serum samples were deproteinated by treating them with trichloroacetic acid and passing the supernatants through an Amicon Biospheres Ultrafree-MC Centrifugal Filter Unit (Millipore Corporation, Billerica, MA, USA), Five thousand molecular weight limit (NMWL). NAC was measured by electrochemical detection, using a Waters radial compression module with a 10 μm C18 column (Waters, Inc., Milford, MA, USA), an ESA 5010 analytical cell, and an ESA 5100A coulochem detector (ESA, Inc., Chelmsfor, MA, USA). The samples were diluted in phosphate buffer and injected at 1.0 ml/min in 100 mM KH2PO4, pH 3. Area under the curve was compared to known concentrations in control sera.

To confirm the pharmacology findings, additional rats were treated with NAC 1,200 mg/kg administered PO ($n = 3$). Blood was taken 15 min post-treatment and allowed to clot. These sera were analyzed for total NAC using the Bioxytech GSH-400 colorimetric assay for glutathione (OxisResearch, Foster, CA, USA), which we have previously demonstrated gives equivalent values for total thiols as HPLC [7]. These data were analyzed in conjunction with the HPLC results.

Statistics

The individual studies compared independent groups (two to four groups per study). For creatinine and weight loss, an analysis of variance was performed. Residuals were evaluated using frequency histograms and the Shapiro–Wilks test. The assumption of homogeneity of variances across groups was evaluated using Levene's test (significance level 0.05) and, if the variances were not homogeneous, Welch's ANOVA was used in place of the usual ANOVA model. If there were significant differences among the groups based on the overall test, pair-wise comparisons were made using Bonferroni adjustment for multiple comparisons. If Welch's ANOVA was used for a particular parameter, pair-wise Welch's ANOVA's were used for pair-wise comparisons with Bonferroni adjustment. For BUN, several measurements were above detectable limits (140 mg/dl) and were censored at this value. A generalized Wilcoxon test was used to compare groups and medians are used rather than means. Confidence intervals for the medians are only defined if more than half of the observed values are uncensored (less than 140 mg/dl). If there was a difference among the groups, groups were compared pair-wise using the generalized Wilcoxon test with Bonferroni adjustment. The comparisons of each NAC-route combination individually with no NAC were deemed a priori to be the pair-wise comparisons of interest.

For the NAC pharmacology study, there are several values of serum NAC that are below detectable limits (BDL). For values BDL (which occurred only in the 1,200 mg/kg PO group), the limit of detection of 0.05 mg/mg was used in place of zero. This is a conservative approach as it minimizes the differences between these BDL values and values greater than the detection limits. There are also outliers in three of the four groups for NAC concentration indicating substantial skewness (confirmed by evaluation of residuals). Due to these outliers, a non-parametric approach ANOVA model (the Kruskal Wallis test) was used. For pair-wise comparisons, Wilcoxon two-sample tests were used with Bonferroni-adjustment for all six possible comparisons (an adjusted significance level of 0.0083 is used for each comparison). A Spearman non-parametric correlation coefficient was estimated to check for a monotonic association between dose and serum NAC. All analyses were run using SAS® Version 9.1 running in Windows XP.

Results

All but one of the rats receiving a single high dose of CDDP alone (10 mg/kg IP) had an abnormally high BUN (Fig. 1a). We have previously demonstrated that this high level of BUN correlates with renal tubule damage seen histologically [7]. NAC was chemoprotective against the CDDP nephrotoxicity, depending on the route of administration. For BUN data from Study 1 (Fig. 1a), there was a significant difference among the groups ($P < 0.0001$). The median BUN values were 140 mg/dl or greater for NAC IP (five of six values 140 mg/dl or greater), 17.5 mg/dl for NAC IV (95% CI: 16–47 mg/dl), 140 or greater for NAC PO (four of seven values 140 or greater), and 140 mg/dl or greater for no NAC (six of seven values 140 mg/dl or greater). The difference between NAC IV and no NAC was statistically significant ($P = 0.0133$, Bonferroni-adjusted significance level 0.0167) while the other two NAC groups did not differ from no NAC ($P = 0.380$ for PO and $P = 1.0$ for IP). For creatinine, the variances were heterogeneous ($P = 0.0383$). There was an overall difference among the four groups ($P = 0.0004$) with a significant difference between NAC IV and no NAC ($P = 0.0048$) but no differences between no NAC and either NAC PO ($P = 0.426$) or NAC IP ($P = 0.106$). Mean (SD) creatinine values were 2.89 (1.51) for no NAC, 2.32 (0.94) for NAC IP, 1.77 (0.56) for NAC PO, and 0.47 (0.37)) for NAC IV. The analysis of weight loss data in Fig. 1c indicated that the variances were heterogeneous ($P = 0.0060$) and there was a significant difference among the groups ($P = 0.0044$). There was a significant difference between NAC IV and no NAC ($P = 0.0021$) but not between NAC IP ($P = 0.791$) or NAC PO ($P = 0.774$) and no NAC. Mean percent weight loss was 18.3% (4.5%) for no NAC, 18.8% (2.5%) for NAC IP, 18.8% (1.2%) for NAC PO, and 5.8% (5.9%) for NAC IV.

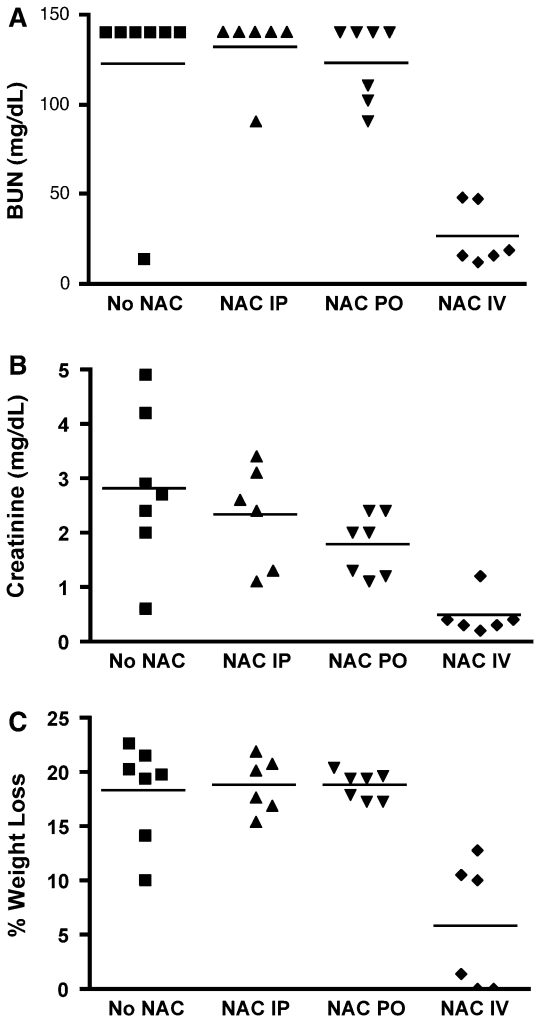

Fig. 1 Effect of route of administration on nephroprotection with NAC. Female Long-Evans rats were treated with CDDP 10 mg/kg IP 30 min after NAC 400 mg/kg given by IP, PO or IV routes, compared with CDDP alone (no NAC). Data, taken 3 days post-treatment, were **a** BUN, **b** creatinine, and **c** % weight loss. *Symbols* indicate individual animals, with the mean indicated by the *line*

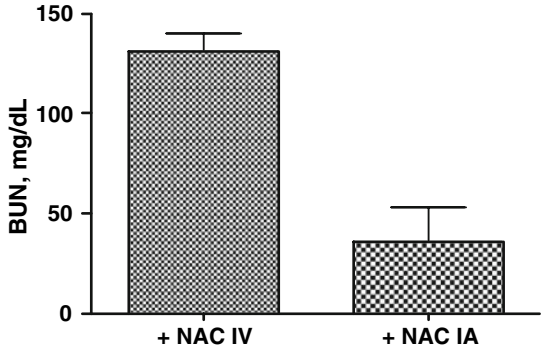

Fig. 2 Nephroprotection with low-dose NAC given IV or IA. Female Long-Evans rats were treated with CDDP 10 mg/kg IP 30 min after NAC 50 mg/kg administered IV or IA. The data shown are mean ± SEM

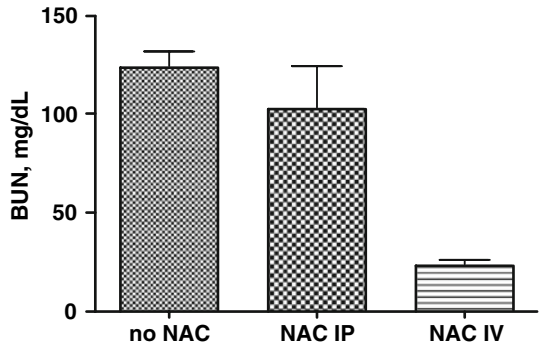

Fig. 3 Effect of repeated low-dose CDDP on renal function and NAC chemoprotection. Female Long-Evans rats were treated with CDDP 1 mg/kg twice daily for 4 days, rested 10 days, and then the CDDP dosing was repeated. Rats received either no chemoprotection (no NAC), or were pre-treated with NAC 800 mg/kg IP or IV 15 min prior to CDDP during the second CDDP treatment phase. Data taken 3 days after the final treatment indicate mean ± SEM

A second study was done to evaluate chemoprotection with a low dose of NAC (Fig. 2). IV administration of 50 mg/kg NAC was compared with administration of NAC through renal artery catheterization, as a model of protection against contrast-induced nephropathy. The analysis showed a significant difference between NAC IV and NAC IA ($P = 0.0101$). Median BUN was 20 mg/dl for NAC IA and 140 mg/dl or greater for NAC IV (four of seven values were 140 mg/dl or greater), indicating a significantly reduced rate of nephrotoxicity for the IA delivery (Fig. 3)

The third study evaluated repeated dosing with low dose CDDP, to more closely mimic clinical CDDP treatment regimens. The rats in the repeated CDDP dose group had no nephrotoxicity after the first round of treatment, but developed toxicity after the second round. The analysis of the third study showed an overall significant difference among the three groups ($P < 0.0001$). The difference between NAC IV and no NAC was statistically significant ($P = 0.0005$, Bonferroni-adjusted significance level 0.0250) while the difference between NAC IP and no NAC was not significant ($P = 0.386$). The median BUN values was at least 140 mg/dl for no NAC (BUN was 140 mg/dl or greater for four of seven rats), at least 105.5 for NAC IP (BUN was 140 mg/dl for two of four rats and 71 mg/dl was the higher value for the other two rats), and 19.5 mg/dl for NAC IV (95% CI: 18–28 mg/dl).

A study of NAC pharmacology and metabolism was performed to assess the effect of dose and route of administration on serum thiol concentrations (Fig. 4). The data in Fig. 4 include outliers that clearly violate the assumption of normality. For this reason, non-parametric analyses were performed. There is a significant difference among the four groups ($P = 0.0023$). Comparisons of the IV NAC doses to the PO dose indicate no significant difference between

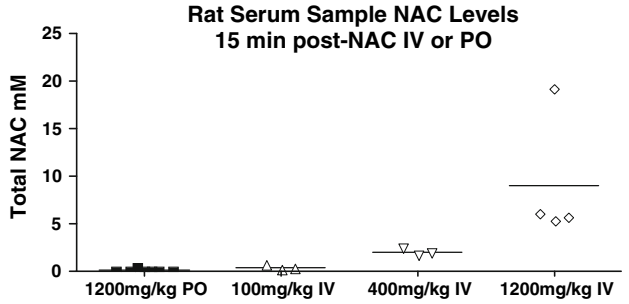

Fig. 4 Effect of route of administration on NAC pharmacology. Female Long Evans female rats received NAC infusion at 100, 400 and 1,200 mg/kg IV ($n = 3$ rats per group). Blood samples were taken 15 min post-inoculation. Another group of 3 rats was given NAC 1,200 mg/kg PO, with blood samples taken after 15 min in 2 rats and after 60 min in 1 rat. Total NAC concentrations were analyzed by HPLC. The NAC 1,200 mg/kg PO administration was repeated in three rats, with one rat receiving NAC 1,200 mg/kg IV. These sera were analyzed using the Bioxytech GSH-400 colorimetric assay for glutathione, and the results included with the HPLC data. *Symbols* indicate individual animals, with the mean indicated by the *line*

100 mg/kg IV and PO ($P = 0.0284$), a significant difference between 400 mg/kg IV and PO ($P = 0.0070$), and a significant difference between 1,200 mg/kg IV and PO ($P = 0.0039$). The difference between 100 and 400 mg/kg was not significant ($P = 0.0463$), between 100 and 1,200 was not significant ($P = 0.0323$), and between 400 and 1,200 was not significant ($P = 0.0339$). The lack of differences may reflect the small group sizes. For many of these pair-wise comparisons, the values in one group are all less than the values in the other group (e.g. 100–400 and 400–1,200), however, the sample size is too small to achieve significance. The Spearman correlation coefficient comparing IV dose to serum NAC is 0.947 ($P < 0.0001$) indicating a strong monotonic (that is, dose–response) association. The Pearson correlation coefficient was 0.722 ($P = 0.0184$) indicating that a significant dose–response relationship that may not be linear as the Spearman correlation is closer to one.

Discussion

We have previously demonstrated that CDDP 10 mg/kg IP resulted in a consistently high BUN and CR in 3 days and that these blood values correlated well with renal tubule damage seen histologically [7]. The nephrotoxic rat model is used in this study to evaluate the effect of delivering NAC by different routes and is not meant to evaluate antitumor effects of CDDP, which is the subject of previous studies in our lab [16, 18, 19]. Pretreatment with NAC given at 400 mg/kg IV provided protection from the renal toxicity. The present study demonstrates that the route of administration can have a profound effect on the efficacy of protection. Those rats given NAC at 400 mg/kg IV had significantly less occurrence of abnormally high BUN, creatinine and weight loss than those groups given the same dose of NAC by the IP and PO routes. Further, the groups given IP and PO NAC showed the same toxicity as the group with no thiol protection.

Repeated doses of CDDP over an 18 day period resulted in rats with nephrotoxicity, as indicated by a high BUN, showing that a low dose (1 mg/kg) of CDDP can induce nephrotoxicity when given to rats as a long-term regimen. This toxicity was blocked by pre-treatment with NAC 800 mg/kg IV, but not when the NAC was administered IP. Thus, the difference in protection of IP versus IV NAC was consistent with the single high dose CDDP models.

NAC was given to two more groups of rats at the lower dose of 50 mg/kg IV in the femoral vein and IA through a catheter placed so that the NAC was delivered down the descending aorta. This model was done to simulate the delivery of drugs in patients directly to the renal arteries through a pig-tail catheter. The data in Fig. 4 show that at this low level of NAC protection can be achieved by the appropriate route of delivery. The IV NAC at this dose was not protective. These data indicate that route of administration is even more important with low dose NAC.

The overall results are consistent with a significant difference between NAC IV and no NAC but no significant differences between no NAC and either NAC IP or NAC PO. Within each of the four studies, pair-wise comparisons are adjusted for the number of such comparisons using Bonferroni adjustment. No such adjustment are made for four experiments as these are independent experiments. The values for below detectable limits in Experiment 4 were not treated as censored observations as these all occurred within a single group. For the other three experiments, there were censored data in two or more groups so a censored data approach was used.

Several other clinical [3, 22] and animal [1, 6, 7, 11] studies have investigated the link between CDDP and renal tubule damage, as well as the protective effects of pretreatment with thiols. NAC is also commonly used for the prevention of contrast-induced nephropathy. The mechanism of prevention may be anti-oxidant and vasodilatory effects [10], and thus is similar to the protective actions of NAC against CDDP-induced renal toxicity. Although NAC dose and route of administration have been hypothesized to be key factors in the efficacy of chemoprotection against contrast-induced nephropathy [21], most clinical trials have used a low dose administered orally [14, 24]. In a recent clinical trial, patients benefited from pretreatment with a higher dose of NAC than is usually given for contrast-induced nephropathy [15], but even this dose was lower than in the present study. More specifically, the highest dose of NAC given in that report was, for a 70 kg patient, 17 mg/kg IV, followed by 17 mg/kg PO twice daily for

2 days. Our minimum renal protective dose of NAC was 50 mg/kg given IA. We believe that analogous to our CDDP toxicity results, NAC must be given at significantly higher doses by the IV or IA route to achieve chemoprotection against contrast-induced nephropathy.

Serum samples taken 15 min after IV administration in three groups of rats showed an escalation of concentration consistent with the doses of NAC at 100, 400 and 1,200 mg/kg. However, when given PO at the highest dose of 1,200 mg/kg NAC, there was little NAC found in the serum, even when the sample was taken 60 min post-NAC. Many patients are being treated by the PO route [14, 24], which does not appear to be the most effective.

Previously we have demonstrated that all NAC in the IV infusate was in the non-oxidized form. In contrast to the IV results, the group given NAC 1,200 mg/kg by the PO route had very low levels of total serum NAC even 60 min after infusion. Studies in our lab have found the half-life of NAC to be 11 min [18]. The NAC, while well absorbed intact from the small intestine, has been found to undergo extensive metabolism on first-pass through the liver [4]. There is also evidence of intestinal metabolism of NAC in the rat, which would further reduce NAC bioavailability after PO administration [5]. These findings are consistent with the data in the present study. The PO and IP routes lead directly to the hepatic portal system, and the NAC is metabolized by the liver before reaching the main circulation. This also explains the result of the low-dose NAC administration IV versus IA, as the protection is delivered directly to the target organ by the IA route. Directed treatment by thiols has previously been found to be effective against CDDP-induced ototoxicity [12]. This may be the best course of treatment for maximizing the protective effect of thiols.

In conclusion, the level of protection against CDDP-induced nephrotoxicity in rats is affected by the route of NAC administration. The rats given NAC 400 mg/kg IV showed a lower level of toxicity than those given the same dose of NAC by the IP and PO routes, which were not significantly different than the group given CDDP only. The rats given NAC 50 mg/kg IA before CDDP were protected, while those given the low dose NAC IV developed renal toxicity. Finally, the serum levels of NAC given IV rose consistently when the dose was increased from 100–400 to 1,200 mg/kg, but the NAC 1,200 mg/kg given PO had very low serum levels, even when sampled after 60 min. We conclude that the route of NAC delivery has an effect on the efficacy of NAC protection and could be due to the high level of NAC metabolized during the first pass through the liver.

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Conflict of interest statement Drs. Neuwelt and Muldoon, Oregon Health & Science University (OHSU), Portland Veterans Affairs Medical Center (PVAMC) and the Department of Veterans Affairs have a significant financial interest in Adherex, a company that may have a commercial interest in the results of this research and technology. This potential conflict of interest was reviewed and managed by the OHSU Integrity Program Oversight Council and the PVAMC Conflict of Interest in Research Committee. Dr. Neuwelt has divested his financial interests in Adherex.

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