Reduced Glutathione Protects against Cisplatin-induced Neurotoxicity in Rats

Frank P. T. Hamers, Jan H. Brakkee, Ennio Cavalletti, Michele Tedeschi, Laura Marmonti, Gabriella Pezzoni, Jan P. Neijt, and Willem H. Gispen

ABSTRACT

Reduced glutathione (GSH) is reported to diminish cisplatin-induced neurotoxicity, and it was for this reason that we studied GSH in an animal model of cisplatin neuropathy. The neuropathy was evaluated by measuring the sensory nerve conduction velocity (SNCV) in young adult Wistar rats. GSH injections (i.v.) were given twice weekly, within five minutes before cisplatin was injected (i.p.). In a first experiment GSH (500 mg/kg) in combination with a low-dose cisplatin treatment (1 mg/kg, 10 weeks) was investigated. Animals treated with cisplatin and placebo developed a neuropathy (SNCV at week 10: age controls, 61.9 m/s; cisplatin alone, 44.2 m/s), whereas rats treated with cisplatin and GSH did not (SNCV, 61.9 m/s). The same dose of GSH was used in combination with a high-dose cisplatin schedule (2 mg/kg, 5 weeks' treatment plus 5 weeks' recovery). Again, GSH protected animals against the development of neuropathy (SNCV at week 10: age controls, 61.9 m/s; cisplatin alone, 50.6 m/s; cisplatin plus GSH, 61.1 m/s). In another experiment four lower doses of GSH (25, 50, 100, and 200 mg/kg) were tested in combination with the low-dose cisplatin protocol (1 mg/kg, 11 weeks). The cisplatin group developed a neuropathy (SNCV at week 11: cisplatin alone, 50.2 m/s; age controls, 60.6 m/s). Only the dose of 200 mg GSH/kg was found to protect against the development of a neuropathy (SNCV, 61.0 m/s). In an antitumor study GSH administered at 300 mg/kg in combination with cisplatin at 1.5 mg/kg did not diminish the curative effect of cisplatin. We conclude that GSH prevents cisplatin-induced neuropathy and that it should be investigated further in the clinic.

INTRODUCTION

Since the emergence of prophylactic treatment for the nephrotoxicity of cisplatin, other side effects of this drug have become important. At present neurotoxicity is considered to be one of the major dose-limiting side effects (1). The neuropathy induced by cisplatin is of a purely sensory nature, with a preference for the thickest myelinated fibers (1A fibers). Disease of these fibers causes an invalidating sensory ataxia, eventually leading to wheelchair dependence. Other complaints include numbness, tingling sensations, and painful paresthesia. Even if treatment with cisplatin is discontinued when the first neuropathic symptoms become evident, the neuropathy may continue to develop for several months. Some of the symptoms will eventually subside, but total recovery may never be reached.

In the last decade some therapeutic options for cisplatin neuropathy have emerged. A rat model for cisplatin neuropathy has been developed and has been used to show that the neuroprotective ORG2766 and the calcium entry blocker nimodipine are competent protective agents against this neuropathy (2, 3). ORG 2766 has also been shown to be effective in patients, underscoring the predictive value of this animal model (4).

Apart from the drugs mentioned above, sulfhydryl group-containing drugs (originally developed for nephroprotection) have also been proposed as neuroprotective agents (1). Two interesting compounds in this respect are ethifos (WR-2721) (5, 6) and the tripeptide γ-Glu-

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2 To whom requests for reprints should be addressed.

Cys-Gly, GSH,3 the most abundant intracellular thiol (7, 8). In a clinical trial conducted to assess the nephroprotective efficacy of GSH in patients treated with cisplatin, GSH-treated patients were reported to exhibit less severe neuropathic symptoms than might have been expected, without any negative interference on oncolytic activity (7). Although these observations of Di Re are of a preliminary nature (no control group was involved and the neuropathy was not quantified), we deemed the observation to be of sufficient interest to investigate GSH in our animal model of cisplatin-induced neuropathy. In this paper we report that GSH provides protection against the cisplatin-induced slowing of sensory nerve conduction velocity induced by treatment with both a low and a high dose of cisplatin. Furthermore, we investigated the antitumor efficacy of the combination of GSH and cisplatin in the Walker 256/A rat mammary carcinoma model.

MATERIALS AND METHODS

Neuropathy Studies

Animals and Animal Care. These experiments were performed at the Rudolf Magnus Institute in Utrecht, on male Wistar rats of an inbred strain (originally obtained from TNO, Zeist, the Netherlands), weighing 250–290 g at the start of the experiments. The rats were housed on sawdust in Macrolon cages (3–4 rats/cage) and maintained on a 12 h:12 h light:dark cycle (lights on at 7:30 a.m.) with food and water provided ad libitum.

Evaluation of Neuropathy. The neuropathy was quantified by electrophysiological measurements of the H reflex-related SNCV. These measurements were carried out under general anesthesia (Hypnorm; Janssen Pharmaceuticals BV, Tilburg, the Netherlands; containing 10 mg/ml fluanison and 0.315 mg/ml fentanyllactate; dose, 0.4 ml/kg body weight, administered s.c.). The experimenter who evaluated the neuropathy was unaware of the treatment the animals had received. The technique is described in detail elsewhere (2) and is summarized in Fig. 1.

Drugs. Glutathione (Boehringer Mannheim Italia, Monza, Italy) was delivered in sterile vials containing 2.5 g of lyophilized GSH and dissolved fresh every day in 10 ml sterile water (final concentration, 250 mg/ml). In the first two experiments this solution was administered undiluted, but in the third experiment this stock was diluted further with sterile water to final concentrations of 100, 50, 25, and 12.5 mg/ml. The GSH solution (0.2 ml/100 g body weight) was injected into the lateral tail vein. Equivalent volumes of sterile water were injected into those animals not receiving GSH.

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Experimental Design. Body weight was measured twice weekly in all animals, before the cisplatin and/or GSH injections. Cisplatin and GSH were injected individually. GSH was administered in the lateral tail vein, whereas cisplatin was injected i.p. within 5 min of the GSH injection. The SNCV was measured just before the start of and at several times during the experiment, as indicated in the next section. For all experiments, each experimental group initially consisted of 15 animals.

In the first experiment the effects of GSH treatment on the SNCV and body weight were evaluated in animals receiving a low-dose cisplatin treatment and in age-matched controls. The cisplatin dose used was 1 mg/kg body weight, 18 U.S.C. Section 1734 solely to indicate this fact.

2 The abbreviations used are: GSH, reduced glutathione; SNCV, sensory nerve conduction velocity; ANOVAR, analysis of variance.

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two times a week. The GSH dose was 500 mg/kg body weight, two times a week. Treatment was continued for 10 weeks. A total of 60 rats divided into 4 experimental groups were used. One group was treated with cisplatin and GSH, one group with cisplatin and water (neuropathy control), one group with saline and GSH (GSH control), and one group with saline and water (age-matched control). Total dosages administered in the cisplatin-treated groups were 20 mg cisplatin/kg, and in the GSH-treated groups, 10 g GSH/kg. Electrophysiological measurements were performed at 0, 4, 6, 8, and 10 weeks (cumulative cisplatin doses: 0, 8, 12, 16, and 20 mg/kg body weight).

In the second experiment the same dose of GSH (500 mg/kg body weight/injection) was tested in rats subjected to a high-dose cisplatin treatment (2 mg cisplatin/kg body weight/injection). Treatment was continued for 5 weeks, after which the animals were allowed to recover for another 5 weeks. No additional treatment was given during the second part of the experiment. Two groups of 15 rats received cisplatin as indicated and were cotreated with either GSH or sterile water (neuropathy control). As in the first experiment the total cisplatin dose administered was 20 mg cisplatin/kg, whereas the total GSH dose administered was 5 g GSH/kg. Electrophysiological measurements were performed at 0, 2, 3, 4, and 5 weeks (cumulative cisplatin doses: 0, 8, 12, 16, and 20 mg/kg body weight). An extra measurement was included at 10 weeks, to investigate possible delayed effects of cisplatin or GSH. This experiment was performed parallel to experiment 1, and thus it was possible to compare the SNCV of these animals with that of age-matched controls at weeks 0, 4, and 10.

In the third experiment we investigated the neuroprotective efficacy of four doses of GSH (25, 50, 100, and 200 mg/kg body weight) in rats subjected to the low-dose cisplatin treatment (1 mg/kg body weight/injection). Extra groups were included to control for neuropathy (cisplatin plus sterile water) and age (saline plus sterile water). Thus, 90 animals were divided into 6 treatment groups. The treatment was continued for 11 weeks, and SNCV was measured at 0, 4, 6, 8, 10, and 11 weeks (cumulative cisplatin doses: 0, 8, 12, 16, 20, and 22 mg/kg body weight, respectively).

**Antitumor Activity Study**

**Animals and Animal Care.** This experiment was performed at the Boehringer Mannheim Italia Research Centre in Monza on male Crl CD(SD)BR rats 6 weeks old (150 ± 2 g body weight) supplied by Charles River Co. (Monza, Italy). The rats were housed on sawdust in Macrolon cages (3–4 rats/cage) and maintained on a 12 h:12 h light:dark cycle (lights on at 7:00 a.m.) with food and water provided ad libitum.

**Drugs.** Glutathione (Boehringer Mannheim Italia) was dissolved in saline (final concentration, 60 mg/ml). The GSH solution (0.50 ml/100 g body weight) was injected into the lateral tail vein. Equivalent volumes of saline were injected into those animals not receiving GSH.

Cisplatin (Rhône Poulenc, Pharma, Italy) was dissolved in sterile water to a final concentration of 0.30 mg/ml. Sterile water served as a placebo. Cisplatin or placebo solution was injected i.v. (0.5 ml/100 g body weight) 15 min after the GSH injection.

**Tumor.** Walker 256/A was maintained by periodic s.c. transplantation as described elsewhere (9). In this study about 100 mg of proliferating tumor tissue were implanted into the right subaxillary region. Tumor diameters were measured twice a week with Vernier calipers, starting at day 7, when tumor mass became palpable.

**Evaluation of Antitumor Activity.** Primary tumor growth was assessed by caliper measurement, and tumor weight was estimated according to the method of Geran et al. (10). The antitumor effect was determined by the mean tumor weight, calculated twice a week. The number of long-term survivors was recorded at the end of the experiment at day 60.

**Treatment Schedule.** Four groups of 10 animals each were used in this experiment. Tumor was implanted on day 0. Treatment with GSH and/or cisplatin started on day 1 after tumor implantation and was repeated twice a week over 3 weeks (at days 1, 4, 7, 11, 14, and 17). One group served as age-matched controls, one group was treated with cisplatin (1.5 mg/kg), one group was treated with GSH (300 mg/kg), and one group was treated with both cisplatin (1.5 mg/kg) and GSH (300 mg/kg).

**Data Analysis**

Data were analyzed by an ANOVAR for multiple measurements (all time points), followed by supplemental t tests. Data from animals that died during the experiment were omitted from the statistical analysis.

**RESULTS**

**Neurotoxicity Experiments**

**General Toxicity**

In the first experiment (20 mg cisplatin/kg administered over 10 weeks) three animals died during anesthesia. Otherwise, all rats survived until the end of the experiment.

In the second experiment (20 mg cisplatin/kg administered over 5 weeks) two animals in the GSH cotreated group died following anesthesia. The dose of cisplatin used was very debilitating for the rats cotreated with water. The maximum cisplatin dose that could be tolerated relatively well by this group was 20 mg/kg (10 of 15 rats survived), but in the next 5 weeks, prior to the final electrophysiological measurement at week 10, another 4 animals (of 10) died.

In the third experiment three animals from the age-matched control group died after anesthesia. In the cisplatin control group two animals died after anesthesia and one animal died in the tenth week. Of the rats cotreated with cisplatin and 25 mg GSH/kg, three died in the last week of the experiment, one of these during anesthesia. In the group cotreated with cisplatin and 50 mg GSH/kg one animal died during anesthesia at the second measurement and two more died in the last treatment week. Of the animals cotreated with cisplatin and 100 mg GSH/kg, two died in the eighth week, followed by two more in the eleventh and last week. In the group cotreated with cisplatin and 200 GSH/kg one animal died during anesthesia. These data are summarized in Table 1.
Body Weight

In all experiments cisplatin influenced body weight in a dose-dependent manner. Animals injected with saline instead of cisplatin continued to grow, whereas cisplatin-treated animals gained less weight or even lost weight. GSH treatment did not influence weight gain in age-matched controls (Fig. 2).

In the first experiment the rats subjected to cisplatin treatment (low dose) continued to grow, albeit at a slower rate than the controls for the first few weeks; thereafter their body weight decreased from 6 weeks onward. This weight loss, however, was partly inhibited by cotreatment with GSH (Fig. 2).

In the second experiment (high-dose cisplatin) weight gain was inhibited by cisplatin from the start of the experiment, followed by a decrease after 4 weeks in the animals not treated with GSH. The weight loss was reversible upon discontinuation of cisplatin treatment from 5 weeks onward in the group cotreated with GSH (Fig. 3).

In the third experiment (low-dose cisplatin with various doses of GSH) age-matched controls continued to grow, and cisplatin-treated rats gained some weight until week 4 but lost weight after week 8. GSH did not significantly influence body weight in any treatment group (Fig. 4).

Electrophysiology

In all age-matched control groups (experiments 1 and 3), the SNCV increased from around 50 to about 60 m/s, as may be expected in maturing rats. As there was no significant difference with time in the SNCV of the GSH- and water-treated age-matched controls (ANOVAR: $F_{2,25} = 0.44; P < 0.51$), we concluded that GSH did not influence the age-related increase in SNCV by itself. We therefore combined these groups to form one age-matched control group in the further analyses of experiments 1 and 2. Neuropathy developed in both the low- and the high-dose cisplatin treatment groups, as evidenced by a decrease in SNCV in the later stage of intoxication (Figs. 5–7).

First Experiment. The group treated with cisplatin plus water developed a significant neuropathy (ANOVAR for age-matched controls versus cisplatin controls: $F_{1,27} = 52.30; P < 0.001$; $t$ test at week 10: $t = 8.63; df = 37; P < 0.001$), whereas in the group treated with cisplatin plus 500 mg GSH/kg the cisplatin-induced decrease in SNCV did not occur (ANOVAR for cisplatin plus water versus cisplatin plus 500 mg GSH/kg: $F_{1,22} = 20.60; P < 0.001$; $t$ test at week 10: $t = 8.43; df = 22; P < 0.001$). The SNCV of the last group (cisplatin plus 500 mg GSH/kg) did not differ significantly from that of the age-matched controls (ANOVAR: $F_{1,37} = 2.13; P < 0.153$; $t$ test at week 10: $t = 0.54; df = 37; P > 0.1$). See Fig. 5.

Second Experiment. A significant neuropathy developed in those animals not cotreated with GSH as compared to both controls (experiment 1) and animals cotreated with 500 mg GSH/kg (ANOVAR for cisplatin controls versus age controls: $F_{1,31} = 7.61; P < 0.010$; $t$ test
Cisplatin and GSH treatment effects on body weight were similar to those observed in neuropathy experiment 3 (data not shown).

**DISCUSSION**

In this study we demonstrated that GSH has protective effects against cisplatin-induced neuropathy in rats after treatment with low and high doses of cisplatin. In the first experiment we showed that GSH did not influence the SNCV in developing rats per se. Cotreatment with GSH (500 mg/kg) given before an injection of a low dose of cisplatin prevented the neuropathy completely. In the second experiment we demonstrated that GSH treatment also prevented the neuropathy induced by treatment with a high dose of cisplatin. The neuroprotective effect observed was reason to perform a study with GSH doses of 25, 50, 100, and 200 mg/kg (experiment 3). In this study only the dosage of 200 mg/kg was effective in countering the decrease in the SNCV induced by cisplatin. Moreover, GSH cotreatment (at a GSH:cisplatin ratio the same as that which was effective in preventing the neuropathy) was found not to affect the antitumor activity of cisplatin in the Walker/A mammary rat tumor model.

GSH was not developed as a neuroprotective agent but as a nephroprotectant. One of the first side effects of cisplatin encountered was nephrotoxicity, and in the last decade a few protective agents against this toxicity have been investigated. It is interesting to note that of these nephroprotective drugs, both GSH (7) and ethiofos (6) are also reported to protect against cisplatin neuropathy. These drugs have one factor in common: sulfhydryl groups. Cisplatin causes marked changes in the renal level of the most abundant intracellular thiol, glutathione (11), and since exogenously administered GSH accumulates in the kidney (12), it was thought that GSH could interfere with renal toxicity.

Although all tissues produce GSH, some synthesize more than others. The liver, for instance, is a net exporter of this peptide. The intracellular uptake of GSH from plasma almost always involves its degradation by membrane-bound γ-glutamyl transpeptidase, followed by transport into the cell and its resynthesis by γ-glutamylcysteine synthetase and glutathione synthetase. Glutamate is coupled by γ-glutamyl transferase to another amino acid (with a preference for cysteine), and this dipeptide is transported across the cell membrane. If the dipeptide is γ-glutamylcysteine it can be used directly for GSH synthesis by glutathione synthetase (13). Exogenously administered GSH is reported to increase intracellular GSH levels, but most often this increase can be effectively overcome by the administration of GSH was not developed as a neuroprotective agent but as a nephroprotectant. One of the first side effects of cisplatin encountered was nephrotoxicity, and in the last decade a few protective agents against this toxicity have been investigated. It is interesting to note that of these nephroprotective drugs, both GSH (7) and ethiofos (6) are also reported to protect against cisplatin neuropathy. These drugs have one factor in common: sulfhydryl groups. Cisplatin causes marked changes in the renal level of the most abundant intracellular thiol, glutathione (11), and since exogenously administered GSH accumulates in the kidney (12), it was thought that GSH could interfere with renal toxicity.

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buthionine sulfoxime, a potent inhibitor of γ-glutamylcysteine synthetase. This indicates the importance of the transport mechanism outlined above. Although this mechanism is the most important mechanism in GSH transport, there is evidence that the tripeptide is taken up directly by carrier-mediated transport (14). The administration of GSH esters would be the most effective (and least specific) way to increase intracellular GSH levels, since it would be independent of the availability of γ-glutamyl transpeptidase or γ-glutamylcysteine synthetase (15, 16). Organs containing high levels of transpeptidase activity are the first to benefit from exogenously administered GSH. The kidney is the most important scavenger of plasma GSH, but peripheral nerves (e.g., the sciatic nerve of the rat) also contain quite high levels of γ-glutamyl transpeptidase (17), indicating that peripheral nerves could import exogenous GSH as well.

GSH might exert its nephro/neuroprotective effects in several ways. The formation of cisplatin-GSH complexes, although slow and incomplete (18), might be of importance. Litterst et al. (11) determined cisplatin and (reduced) GSH chromatographically in the cytosol of tissues from cisplatin-treated rats and found 30% of the recovered cisplatin to be associated with GSH. However, the formation of cisplatin-GSH complexes in plasma does not seem to occur in the dosage schemes used: Torti et al. (19) found that GSH administered 15 min before cisplatin did not induce the formation of cisplatin-GSH complexes in plasma in humans. In rats there is evidence that GSH decreases the platinum concentration in dorsal root ganglia (20). This observation would be in line with the observed reduction in neurotoxicity since cisplatin-induced neuropathy is thought to be secondary to dorsal root neuronal involvement (21, 22).

The glutathione redox cycle provides an important mechanism for protection against oxidative damage by free radicals. In the intracellular milieu, with its low chloride concentration (1/30 of extracellular), the chloride ions of cisplatin are replaced stepwise by water (23). The very reactive platinum complex that is formed in this manner can cause the formation of free radicals which could be responsible for some of the toxic effects of cisplatin (24). As such, free radical scavenger activity linked to GSH might provide protection against cisplatin-induced damage.

GSH plays a role in the regulation of K⁺-channel kinetics (25). This regulation is probably based upon the fast and reversible formation of disulfide bridges between different functional parts of the K⁺ channel. The depletion of intracellular GSH may have profound effects on the function of cells that are dependent on the normal kinetics of this ion channel. It is also possible that the kinetics of other ion channels are also regulated by intracellular GSH levels. This could be another mechanism by which GSH influences nerve cell function and health.

Finally, neuroprotection may be a consequence of the action of GSH on kidney function. Some data support this hypothesis. First, the excretion of cisplatin remains constant during treatment, as demonstrated by Bohm et al. (26). This indicates minimal kidney damage. Otherwise, GSH pretreatment does not influence the pharmacokinetic parameters of cisplatin and does not change the bound fraction of cisplatin in plasma (27).

As a result of its role in detoxification reactions, GSH might impair the oncolytic efficacy of cisplatin. Zunino (28), however, showed in experimental tumor models that exogenous GSH administration does not influence the oncolytic activity of cisplatin. In this study, we extend these data to the GSH:cisplatin ratio that was found to prevent the occurrence of a neuropathy. Under these experimental conditions no interference by GSH with cisplatin antitumor efficacy was observed. Modulation of GSH metabolism may enhance the therapeutic efficacy of cisplatin (29–31). GSH is reported to be involved in resistance to cisplatin (29–31). GSH is reported to be involved in resistance to cisplatin (29–31). GSH is reported to be involved in resistance to cisplatin (29–31). GSH is reported to be involved in resistance to cisplatin (29–31). GSH is reported to be involved in resistance to cisplatin (29–31). GSH is reported to be involved in resistance to cisplatin (29–31). GSH is reported to be involved in resistance to cisplatin (29–31). GSH is reported to be involved in resistance to cisplatin (29–31). GSH is reported to be involved in resistance to cisplatin (29–31). GSH is reported to be involved in resistance to cisplatin (29–31). GSH is reported to be involved in resistance to cisplatin (29–31). GSH is reported to be involved in resistance to cisplatin (29–31). GSH is reported to be involved in resistance to cisplatin (29–31). GSH is reported to be involved in resistance to cisplatin (29–31). GSH is reported to be involved in resistance to cisplatin (29–31). GSH is reported to be involved in resistance to cisplatin (29–31). GSH is reported to be involved in resistance to cisplatin (29–31).
clinical trial with GSH suggests a response rate even better than that obtained with traditional treatment regimes (43).

In conclusion, our study results indicate that GSH is another potential neuroprotective drug that probably exerts its action in a way different from that of ORG 2766 and nimodipine. This offers the possibility of combining these agents in future clinical trials.

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