PROTECTION BY SODIUM THIOSULFATE AND THIOUREA AGAINST LETHAL TOXICITY OF C/S- DIAMMINEDICHLOROPLATINUM (II) IN BACTERIA AND MICE

Minoru ISHIZAWA, Shun’ichiro TANIGUCHI and Tsuneo BABA
Cancer Research Institute, Faculty of Medicine, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812, Japan
Accepted June 10, 1981

Abstract—The protective effect of sodium thiosulfate and thiourea on the lethal toxicity of the antitumor drug, cis-diamminedichloroplatinum (II) (cis-DDP), was investigated in bacteria and mice. Initially, the agents capable of antagonizing bactericidal activity of cis-DDP were screened using WP2 uvrA, a strain of E. coli sensitive to this drug. Of the ten sulfur-containing compounds tested, thiourea and sodium thiosulfate exhibited potent protecting effects against cis-DDP cytotoxicity in bacteria. Propylthiouracil and methimazole showed intermediate levels of such protection, but the other 6 compounds had little or no protective effects. Thiourea and sodium thiosulfate were then subjected to the acute lethal toxicity test in mice to assess their protective activity in vivo. We found that cis-DDP i.v. lethality against mice can be blocked almost completely by excess amounts of thiourea or sodium thiosulfate. Thiourea protected against cis-DDP toxicity with a narrow range among the effective doses, while sodium thiosulfate was protective with a remarkably wide range of effective doses. The effectiveness of sodium thiosulfate was also indicated in experiments in which the LD50 dose of cis-DDP (16 mg/kg) i.p. increased over the level of >200 mg/kg with concomitant administration of sodium thiosulfate i.p.

With respect to improvements in the efficacy of cancer chemotherapy, it is essential to induce differential concentrations of antitumor drugs between tumor and non-tumor tissues, in vivo. We recently reported a special combination experiment using rats and an antitumor agent nitrogen mustard N-oxide and its antidote cysteine (1), each of which was administered through different vessels, on the basis of known special vascularization systems for certain tumors of the liver and the lung (2–5). However, only a few practically useful antidotes for antitumor drugs are available.

We have focused our attention on specific agents which not only antagonize activity of the potent antitumor drugs but also have the least toxicity to the host.

We selected for our experiments cis-diamminedichloroplatinum (II) (cis-DDP) as the antitumor drug because it has potent activity against a variety of experimental and human neoplasms (6, 7) and has the property of inorganic coordination complex that can easily bind with other compounds (8–10).

Thiourea is reportedly an antidote for cis-DDP and protects tumor-bearing mice from the host toxicity caused by cis-DDP (11). A
radioprotective agent, WR-2721 ((S-2-(3-aminopropylamino) -ethyl phosphorothioic acid)) (12) has also been reported to antagonize the activity of cis-DDP.

We report herein that sodium thiosulfate and thiourea are potent antagonizing agents against cis-DDP-induced lethal toxicity, in bacteria and mice.

MATERIALS AND METHODS

Chemicals: Chemicals used were obtained from following sources: cis-DDP, Nippon Kayaku Co., Ltd., Tokyo; propylthiouracil and methimazole (1-methyl-2-mercaptooimidazole), Chugai Seiyaku Co., Tokyo; DL-penicillamine, Aldrich Chemical Co., Inc., Milwaukee, Wis.; cysteamine hydrochloride and L-cysteine, Sigma Chemical Co., St. Louis, Mo.; and all other chemicals (analytical grade), Wako Pure Chemical Industries, Ltd., Osaka.

Cytotoxicity assay in bacteria: All chemicals were dissolved in 0.1 M NaCl (saline), and the solutions were passed through a Millipore filter (pore size, 0.45 \( \mu \)m), and diluted with sterile saline to appropriate concentrations immediately prior to use. *Escherichia coli* B/r strains, WP2 (uvrA+ trp-) and WP2 uvrA (uvrA- trp-) (13), were used. The bacterial cells grown at 37°C in a nutrient broth (Oxoid nutrient broth, No. 2) to a log phase were harvested by centrifugation, washed twice with saline and suspended in saline to give approx. 3\( \times 10^9 \) cells/ml. Test tubes containing 1 ml of cis-DDP and/or sulfur compound, at various concentrations, were incubated in saline for 5 min at 37°C, then 0.1 ml bacterial suspension was added to each tube, and incubations were continued for a further 30 min with shaking. At a total of 35 min, the mixtures were diluted to 100-fold into PBS (0.067 M phosphate buffer, pH 7.0, plus 0.1 M NaCl), and aliquots (0.1–0.4 ml) of serial dilutions were plated with 2.5 ml soft agar overlay (5 \( \mu \)g/ml L-tryptophan, 0.1 M NaCl, and 0.7% agar) onto Petri plates containing Davis-Mingioli minimal medium (14) supplemented with 0.4% glucose and 1.5% agar. The survival colonies were scored after incubation of the plates at 37°C for 2 days. Control tubes containing no chemicals were treated and assayed in a similar manner.

When glutathione was tested, the original solution was adjusted to pH 6 with dilute NaOH solution, just before starting experiments. The pH values of the original solutions of other tested chemicals were within a range of 5.8–6.2 which was nearly that of saline.

Lethal toxicity tests in mice: Female ddY mice weighing 24–28 g, at 6 to 8 weeks of age, were divided into groups of 10. cis-DDP, sodium thiosulfate, and thiourea were freshly prepared by dissolution in 0.9% NaCl solution and passed through a Millipore filter before use.

For i.v. administration, a solution of each sulfur compound was first administered to mice to give 0.5 ml per 25 g body weight via the tail vein at various doses. Two min later, a solution of cis-DDP was given via the same i.v. route at a fixed dose to give 0.5 ml per 25 g body weight. For i.p. experiments, a solution of sulfur compound was given i.p. at a fixed dose to give 1 ml per 25 g body weight. One min later, a solution of cis-DDP was given i.p. at various doses with varying volumes of 1.25–5.0 ml per 25 g body weight. In both experiments, mice given cis-DDP or a sulfur compound alone were assessed in a similar way. The survivors were counted 30 days after these treatments.

RESULTS

Protective effect of sulfur-containing compounds on cis-DDP cytotoxicity in *E. coli* cells: Figure 1 shows survival rates of WP2 and WP2 uvrA cells treated with cis-DDP for 30 min at 37°C. The strain WP2 uvrA,
lacking the capacity to repair UV-induced damage in the DNA, was much more sensitive to cis-DDP-induced cytotoxicity compared to the wild type strain WP2, possessing the repair capacity.

Thus, we attempted to screen agents capable of protecting against the cytotoxicity caused by cis-DDP, using the sensitive strain WP2 uvrA. Figures 2, 3 and 4 show the results of assay for ten sulfur compounds, using WP2 uvrA cells in which a fixed concentration of 75 μM cis-DDP (22.5 μg/ml, which kills approximately 99% cells) was mixed with each sulfur compound at 5- to 100-fold excess molar ratios to cis-DDP. Viabilities determined as colony-forming units were plotted against increasing molar ratios of each sulfur compound to cis-DDP. It can be seen that thiourea and sodium thiosulfate most significantly protected the cells against cis-DDP cytotoxicity, depending on the increase in their molar ratios to cis-DDP (Fig. 2). Propylthiouracil and methimazole showed intermediate levels of such protection (Fig. 3).
L-methionine, penicillamine (Fig. 3), glutathione (reduced form), cysteamine, potassium thiocyanate, and L-cysteine (Fig. 4) had little or no effect. None of the sulfur compounds exhibited toxicity in the absence of cis-DDP, except for L-cysteine. Thiourea and sodium thiosulfate, when added to the cells which had been exposed to cis-DDP and washed free of cis-DDP, exhibited no rescue (data not shown).

Protective effect of thiourea and sodium thiosulfate on cis-DDP-induced lethal toxicity in mice: Since the bacterial assay described above suggested the possibility that thiourea and sodium thiosulfate may also protect against cis-DDP-induced lethal toxicity in animals, tests for their protecting ability were performed using mice.

Figure 5 shows survival rates determined 30 days after i.v. treatment with cis-DDP at a fixed dose of 90 μmol/kg (27 mg/kg) (equivalent dose of 9.5 × LD50) in combination with thiourea or sodium thiosulfate at varying excess molar ratios to cis-DDP. In this combination test, both sulfur compounds were given i.v. 2 min prior to cis-DDP administration. In a preliminary experiment, this timing gave the best protection by thiourea or sodium thiosulfate among the groups given 2 and 15 min before or after cis-DDP administration. The observed values of i.v. LD50 for thiourea and sodium thiosulfate were 5,000 and >5,000 mg/kg, respectively. All the mice given cis-DDP alone at the lethal dose died within 7 days after the treatment and the principal side effect was nephrotoxicity, as has also been observed by other workers (15). On the other hand, 90 and 80% of the mice given cis-DDP with thiourea survived, at the combination ratio of 7.5 and 15, respectively (Fig. 5a). However, if mice were given
thiourea at molar ratios higher than 15, the toxicity was greatly increased (Fig. 5a). All such mice died under conditions of acute uremia.

In contrast to the thiourea combination, the viability of mice given cis-DDP in combination with sodium thiosulfate increased depending on increase in the combination ratio, reaching 100% viability at the ratio of 42 and retaining it up to the ratio of 105 (Fig. 5b).

Other tests using the i.p. system were done to compare cis-DDP itself and cis-DDP plus sodium thiosulfate for LD50. As shown in Fig. 6, the LD50 of cis-DDP i.p. was approx. 16 mg/kg, whereas the i.p. value of the combination could not be obtained in this experiment. However, the value is expected to exceed 200 mg/kg since there was no incidence of lethality even when 200 mg/kg cis-DDP and 2,500 mg/kg sodium thiosulfate were given concomitantly. Tests using doses over 200 mg/kg of cis-DDP could not be done because of the poor solubility of this compound (1 mg/ml).

Our findings strongly indicate that unlike thiourea which protects only within a narrow range of its effectiveness, sodium thiosulfate can safely be applied in combination studies for cis-DDP because of a wide range of protectability against cis-DDP toxicity and also because of its low toxicity.

**DISCUSSION**

The results of our study clearly demonstrate that sodium thiosulfate and thiourea can significantly prevent the lethal effect of cis-DDP in *E. coli* cells and in mice. The bacterial screening system we used seems to be useful as a simple and rapid testing tool for the detection of protective agents for a large number of toxic antitumor drugs. The possibility remains that sulfur compounds (other than thiourea and sodium thiosulfate) which showed moderate to low protective ability against cis-DDP cytotoxicity in bacterial cells (Figs. 3 and 4) may exhibit efficient protection in animals. However, since only a weak level of protection by cysteine against lethality of cis-DDP in mice was obtained in our preliminary tests (unpublished results), it seems likely that there is a positive correlation between the two kinds of assays we used in this study.

It should, however, be emphasized from the results of our animal toxicity tests that there are notable differences between sodium thiosulfate and thiourea. In the case of sodium thiosulfate, toxicity never occurred even when an excess high molarity of sodium thiosulfate was given to mice with a lethal dose of cis-DDP (Figs. 5b and 6). Rather the higher the dose of the antidote, the more potent was the protective action against cis-DDP in mice. In the case of thiourea, toxicity due to a combination of an excess dose with a lethal dose of cis-DDP did appear (Fig. 5a) and almost all of the treated mice died earlier than did the mice given a corresponding dose of cis-DDP alone (data not shown). It still remains a question of why such a severe toxicity can be produced if thiourea is administered concomitantly beyond a certain dose though the same levels
of the antidote alone revealed no toxicity. Production of free ammonia or other toxic compounds (presumably some of the platinum-containing coordination complex other than cis-DDP) as the result of in vivo interaction between cis-DDP and the excess thiourea may be considered as the most probable cause. Recently, Burchenal et al. (11) demonstrated the action of thiourea in the protection against cis-DDP-induced toxicity in mouse leukemia cells in vitro and in vivo. They observed that the presence of at least 100 times of thiourea to cis-DDP (at the molar basis) was necessary to achieve a complete protection against cytotoxicity caused by cis-DDP in the mouse leukemia cells in vitro. The levels of thiourea requirement were similar to those in our bacterial system (Fig. 2).

Thiourea is known to reverse cis-DDP-induced DNA cross-links in mouse leukemia cells in culture (16) or in bacteriophage DNA in vitro (17) at the concentrations of 0.1–1 M, the levels being about 100- to 1,000-fold larger molarity than those effective for the bacterial cells. Thiourea given chronically reportedly induces hepatic tumors in rats (18). All these findings taken together suggest that application of thiourea to cis-DDP chemotherapy, though effective, would not be practical.

On the other hand, sodium thiosulfate has been used clinically in fairly large doses in renal function tests and as an antidote for human cyanide poisoning. Furthermore, we found that exceedingly high doses of sodium thiosulfate administered either i.v. or i.p. alone or in combination with the lethal dose of cis-DDP produced no toxicity in the host (Figs. 5b and 6). These observations suggest that sodium thiosulfate can safely be used in combination with cis-DDP, at least in experimental studies. Sodium thiosulfate may interact with cis-DDP outside the cells or tissues, because it did not rescue the bacterial cells already affected by cis-DDP. This is also likely from the fact that sodium thiosulfate is utilized in renal function tests because of its rapid clearance property. It would be of considerable interest to know the inactivated form of cis-DDP by sodium thiosulfate. Pt[(S2O3)2]2⁻ should be a candidate according to platinum coordination chemistry (8), but further studies of such a substance and the mechanism of formation in vivo remained to be determined.

We are currently applying sodium thiosulfate and cis-DDP to particular combination chemotherapy experiments in cases of liver tumor induced in rats.

Acknowledgements: This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan and by Fund for Research from IBM.

We thank Drs. K. Nishikawa and J. Uozumi, and Mr. K. Aoki for assistance with the animal experiments, Ms. M. Kimura for technical help and M. Ohara for critical reading of the manuscript.

REFERENCES
1) Baba, T. and Nishikawa, K.: Effective combination of anticancer drug with its antidote for chemotherapy of hepatic metastasis. Gann 71, 157–158 (1980)
2) Breedis, C. and Young, G.: The blood supply of neoplasms in the liver. Am. J. Pathol. 30, 969–985 (1954)
3) Healey, J.E.: Vascular patterns in human metastatic liver tumors. Surgery, Gynecol. Obstet. 120, 1187–1193 (1965)
4) Markowitz, J.: The hepatic artery. Surgery, Gynecol. Obstet. 95, 644–646 (1952)
5) Milne, E.N.C.: Circulation of primary and metastatic pulmonary neoplasms—A post-mortem microarteriographic study. Am. J. Roentgenol. 100, 603–619 (1967)
6) Rosenberg, B.: Anticancer activity of cis-dichlorodiammineplatinum(II) and some relevant chemistry. Cancer Treat. Rep. 63, 1433–1438 (1979)
7) Wolpert-Defilippes, M.K.: Antitumor activity of cis-dichlorodiammineplatinum(II). Cancer
8) Basole, F. and Pearson, R.G.: Mechanisms of Inorganic Reactions—A study of Metal Complexes in Solution—John Wiley & Sons, Inc., New York (1958)

9) Kelman, A.D. and Parese, H.J.: Mode of DNA binding of cis-platinum(II) antitumor drugs: A base sequence-dependent mechanism is proposed. Cancer Treat. Rep. 63, 1445–1452 (1979)

10) Zwelling, L.A. and Kohn, K.W.: Mechanism of action of cis-dichlorodiammineplatinum(II). Cancer Treat. Rep. 63, 1439–1444 (1979)

11) Burchenal, J.H., Kalaher, K., Dew, K., Lokys, L. and Gale, G.: Studies of cross-resistance, synergistic combinations and blocking of activity of platinum derivatives. Biochimie 60, 961–965 (1978)

12) Yuhas, J.M. and Culo, F.: Selective inhibition of the nephrotoxicity of cis-dichlorodiammineplatinum (II) by WR-2721 without altering its antitumor properties. Cancer Treat. Rep. 64, 57–64 (1980)

13) Green, M.H.L. and Muriel, W.J.: Mutagen testing using trp+ reversion in Escherichia coli. Mutation Res. 38, 3–32 (1976)

14) Davis, L. and Mingioli, E.S.: Mutants of Escherichia coli requiring methionine or vitamin B₁₂. J. Bacteriol. 60, 17–24 (1950)

15) Madias, N.E. and Harrington, J.T.: Platinum nephrotoxicity. Am. J. Med. 65, 307–314 (1978)

16) Zwelling, L.A., Filipski, J. and Kohn, K.W.: Effect of thiourea on survival and DNA cross-link formation in cells treated with platinum (II) complexes, L-phenylalanine mustard, and bis (2-chloroethyl)-methylamine. Cancer Res. 39, 4989–4995 (1979)

17) Filipski, J., Kohn, K.W., Prather, R. and Bonner, W.M.: Thiourea crosses cross-links and restores biological activity in DNA treated with dichlorodiammineplatinum(II). Science 204, 181–183 (1979)

18) Radomska, J.L., Deichmann, W.B., Macdonald, W.E. and Glass, E. M.: Synergism among oral carcinogens—1. Results of the simultaneous feeding of four tumorigens to rats. Toxicol. appl. Pharmacol. 7, 652–656 (1966)