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ORIGINAL ARTICLE

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Citrate enhances the protective effect of orally administered bismuth subnitrate against the nephrotoxicity of *cis*-diamminedichloroplatinum

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Abstract Attenuation of the renal toxicity of *cis*-diamminedichloroplatinum (CDDP) is important in the use of this effective but cytotoxic anticancer agent. We have previously shown that the renal toxicity of CDDP can be efficiently reduced by the induction of metallothionein (MT) by preadministration of bismuth compounds in mice. Bismuth subnitrate (BSN) is used as an antigastric ulcer agent and as an antidiarrheic agent, and is suitable for inducing MT in the kidney in cancer patients. However, due to the low absorption rate of Bi from the gastrointestinal tract, the efficacy of BSN in inducing renal MT is low. In the present study, we examined the effects of citrate as a vehicle for oral administration of BSN on the tissue distribution of Bi and induction of MT in the kidneys and tumors in mice inoculated with Meth-A fibrosarcoma. Renal levels of MT and Bi were markedly increased in the mice given BSN dissolved in citrate solution compared with those given BSN suspended in saline. On the other hand, the use of citrate increased Bi accumulation in the tumor only slightly and did not increase tumor MT levels. Administration of BSN with citrate efficiently depressed the renal toxicity of CDDP, but did not affect its antitumor activity. Since both BSN and citrate are used clinically as pharmaceuticals, the combination regimen of BSN and citrate may be readily applicable as a countermeasure against the adverse side effects of CDDP without affecting its antitumor activity.

Keywords Cisplatin · Metallothionein · Bismuth · Nephrotoxicity

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Introduction

cis-Diamminedichloroplatinum (CDDP) is an effective anticancer agent used for various types of tumor. However, adverse side effects of CDDP including renal toxicity, emesis, ototoxicity, and bone marrow toxicity have hindered its effective utilization in cancer patients [3, 25, 27, 29]. Since nephrotoxicity is a dose-limiting toxicity of CDDP, many trials investigating, for example, the use of hydration have been performed to improve renal function in CDDP-treated patients. Nevertheless, it is still difficult to administer high doses of CDDP to achieve its full therapeutic advantage owing to its nephrotoxicity.

In animal experiments, we have demonstrated that the induction of metallothionein (MT) in the kidneys by preadministration of bismuth (Bi) compounds markedly decreases the renal toxicity of CDDP without affecting its antitumor activity [10]. MT is a cysteine-rich protein of low molecular weight, and is known to play an important role in protection against the toxicity of heavy metals, alkylating agents and free radicals [5, 7, 9, 13, 22]. The most prominent feature of MT is its inducibility by various agents including heavy metals, cytokines, hormones and some organic chemicals [5, 7]. We have also shown that the induction of MT in the kidneys and bone marrow can suppress the renal and bone marrow toxicity of CDDP, doxorubicin and γ-irradiation [10, 16, 18, 24]. Thus, MT induction in target organs by an appropriate inducer could be used as a countermeasure against the adverse side effects of anticancer agents in cancer chemotherapy.

Among MT-inducing heavy metals, Bi is unique since the Bi-containing compound bismuth subnitrate (BSN) is used to protect the gastric mucosa and is used as an antidiarrheal agent in the clinic and by the general population [1, 21, 30]. Therefore, Bi compounds could be readily introduced into cancer chemotherapy as an adjunct for inducing MT in the target organs of anticancer agents. In addition, Bi has been shown to accumulate preferentially

in the kidney, suggesting that Bi compounds are good inducers of MT which may prevent the renal toxicity of CDDP. However, although parenteral injection of bismuth nitrate (BN) efficiently induces MT in the kidney, BN is not used as a pharmaceutical agent. On the other hand, BSN can be used in patients to protect the gastric mucosa, but the efficiency of BSN treatment for inducing renal MT is low because of its low absorption rate from the gastrointestinal tract, this property being beneficial for the protection of the gastric mucosa. Indeed, in some clinical trials, the absorption rate of Bi from orally administered BSN has been found not to be high enough to induce MT in the kidneys of cancer patients, indicating the need to develop a regimen that would show increased absorption of orally administered BSN [4, 17].

It has recently been reported that the absorption rate of Bi from perfused rat intestine is relatively high when Bi compounds are dissolved in a citrate buffer solution [6, 28]. However, the increase in Bi absorption may enhance MT induction not only in the kidneys but also in tumor tissues, leading to the acquisition of drug resistance against various anticancer drugs. It remains unclear whether the use of citrate as a vehicle can enhance in vivo absorption of Bi and induction of MT in the kidneys and tumor tissues.

In the present study, we investigated the effects of citrate solution as a vehicle for the oral administration of BSN on tissue accumulation of Bi, induction of MT in the kidneys and tumor tissues, and the anticancer effects and nephrotoxicity of CDDP using tumor-bearing mice. Administration of BSN dissolved in citrate solution resulted in enhanced accumulation of Bi in the kidneys but only slight accumulation in tumor, leading to a preferential induction of MT in the kidney and, consequently, a reduction in nephrotoxicity without affecting the antitumor effect of CDDP.

Materials and methods

Chemicals

CDDP was supplied by Nippon Kayaku Company, Tokyo, Japan. BSN and citrate were purchased from Iwaki Company, Tokyo, Japan. Other chemicals were purchased from Wako Pure Chemical Industries, Tokyo, Japan. BSN was suspended in saline or dissolved in citrate buffer solution (pH 6.3). CDDP was dissolved in saline. All solutions and suspensions were prepared just prior to each treatment.

Tumor

Meth-A fibrosarcoma cells were supplied by Prof. Y. Kumazawa, Kitasato University, Tokyo, Japan, and maintained by intraperitoneal transplantation in male CDF1 mice. The viability of the tumor cells was tested by trypan blue exclusion.

Treatment of animals

Male CDF1 mice aged 6 weeks were purchased from Japan SLC. Five mice (23–25 g) per cage were housed under specific pathogen-free conditions and were given free access to water and food. All animal experiments were done in accordance with the Kitasato University Guideline for Animal Care and Experimentation. Meth-A fibrosarcoma cells (2×10^6 cells) were injected subcutaneously into the back of the mice, and 7 days later the mice were treated orally with a saline suspension of BSN (0 and 200 mg/kg) or citrate buffer solution of BSN (0, 50 and 100 mg/kg) using a stomach tube once a day for 5 days.

To examine the effects of vehicle on tissue distribution of Bi and induction of MT by BSN, mice were killed 24 h after the last administration of BSN, and samples of blood, urine, kidney and tumor tissues were collected. For collecting urine samples, mice were housed in metabolic cages for 8 h. Blood plasma samples were separated from heparinized blood by centrifugation. To examine the antitumor activity and renal toxicity of CDDP, mice were injected intraperitoneally with CDDP (45 μmol/kg) 24 h after the last administration of BSN. The mice were killed 5 days after CDDP administration, and blood samples and tumor tissues were collected. Antitumor activity was evaluated by measuring tumor weight, and the blood urea nitrogen (BUN) value was determined as an indicator of renal toxicity.

Analyses

For the measurement of Bi, samples of plasma, urine and tumor were digested in nitric acid. Concentrations of Bi were determined by a hydride generation method using a flame atomic absorption spectrophotometer (Shimazu model AA-640-12) with a Varian VGA-76 vapor generation accessory [10]. Concentrations of MT in the kidneys and tumor tissues were determined by a ^{203}Hg-binding assay [10]. BUN values were measured spectrophotometrically using a BUN assay kit (Urea-N-test; Wako).

Statistics

Student's *t*-test was used for statistical analysis of the data for the different groups of animals.

Results

Accumulation of Bi

Figure 1 shows the concentrations of Bi in kidney, tumor, plasma and urine determined 24 h after the last administration of BSN in mice inoculated with Meth-A fibrosarcoma. Mice receiving BSN (50 and 100 mg/kg per day) dissolved in citrate (BSN-citrate) showed 15 to 20 times higher concentrations of Bi in the kidney than those receiving BSN (200 mg/kg per day) suspended in saline (BSN-saline). Although Bi concentrations in blood plasma and urine in the BSN-saline group were slightly higher than those in the saline-alone or citrate-alone groups, Bi concentrations in the BSN-citrate groups were three to five times and four to five times higher in plasma and urine, respectively, than in the BSN-saline group. In tumor, Bi concentrations increased significantly in the BSN-citrate groups compared with the BSN-saline group, but the average Bi concentrations in tumor in the BSN-citrate groups were 20% to 30% of those in the kidney. These results suggest that gastrointestinal absorption of Bi was markedly enhanced by the use of citrate as a vehicle for BSN.

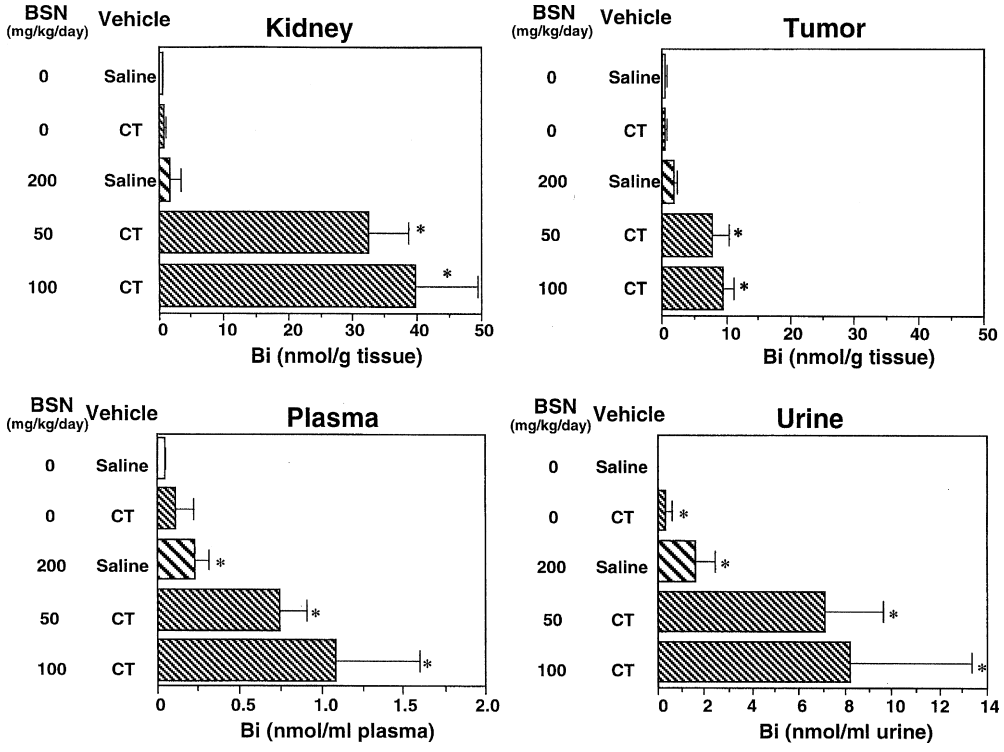


Fig. 1 Concentrations of Bi in plasma, urine, kidney and tumor tissues of mice treated with saline suspension or citrate buffer solution of BSN. Mice were inoculated subcutaneously with Meth-A fibrosarcoma on day 0. Animals received BSN suspension in saline or a solution in citrate buffer (0.5 *M*, pH 6.3) orally once a day for 5 days from day 7. Levels of Bi in the plasma, urine, kidney and tumor tissues were determined 24 h after the last administration of BSN. Each value for Bi content represents the mean ± SD for five mice. $*P < 0.01$ vs "saline alone" values

Meanwhile, the effect of citrate on Bi accumulation in tumor was less than its effect in kidney.

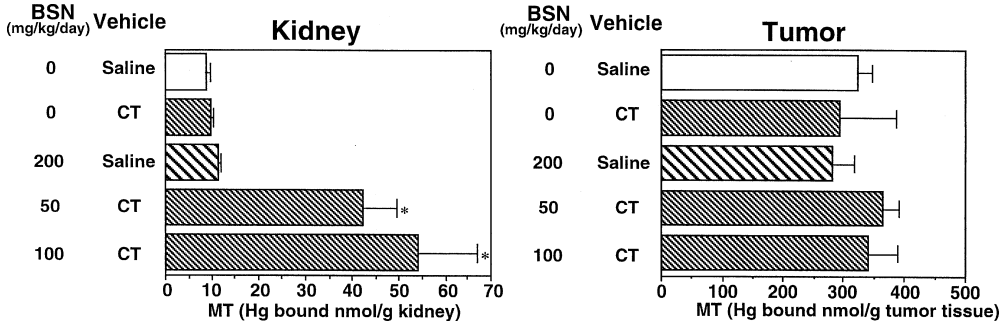
Induction of metallothionein

As shown in Fig. 2, the effect of citrate on MT induction by BSN was different between the kidney and tumor. In the kidney, the MT concentrations in the BSN-saline group were similar to those in the saline-alone and citrate-alone groups, while the BSN-citrate group showed markedly increased concentrations of MT in the kidney, reflecting the increase in renal Bi accumulation (Fig. 1). In tumor, basal MT concentrations in the saline-alone and citrate-alone groups were about 30 times higher than those in the kidney of the saline-alone and citrate-alone groups. Administration of BSN-saline or BSN-citrate did not cause significant increases in tumor MT concentrations compared with basal levels. These results suggest that the slight increase in the tumor Bi concentration in the BSN-citrate groups was not sufficient to induce levels of MT significantly higher than the basal levels in the tumor.

Fig. 2 Concentrations of MT in kidney and tumor tissues of mice treated with saline suspension or citrate buffer solution of BSN. Mice were inoculated subcutaneously with Meth-A fibrosarcoma on day 0. Animals received BSN suspension in saline or BSN solution in citrate buffer (0.5 *M*, pH 6.3) orally once a day for 5 days from day 7. Levels of MT in the kidney and tumor tissues were determined 24 h after the last administration of BSN. Each value for MT content represents the mean ± SD for five mice. $*P < 0.01$ vs "saline alone" values

Changes in renal toxicity of CDDP

Administration of CDDP increased BUN levels in mice receiving saline or citrate alone (Fig. 3). Pretreatment of mice with BSN-citrate reduced BUN levels to control levels, whereas pretreatment with BSN-saline showed no protective effect. These results reflect the difference in Bi accumulation in the kidney between the BSN-citrate and



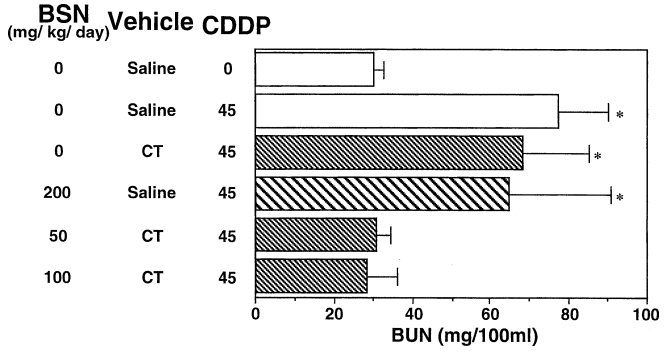


Fig. 3 Effect of pretreatment with saline suspension or citrate buffer (0.5 *M*, pH 6.3) solution of BSN on renal toxicity of CDDP in mice inoculated with Meth-A fibrosarcoma. Mice were pretreated orally with saline suspension or citrate buffer solution of BSN at 24-h intervals for 5 days. Mice were injected intraperitoneally with CDDP (45 μmol/kg) 24 h after the last administration of BSN. BUN levels were determined 5 days after the CDDP injection. Each value for BUN represents the mean ± SD for five mice. $*P < 0.01$ vs "saline alone" values

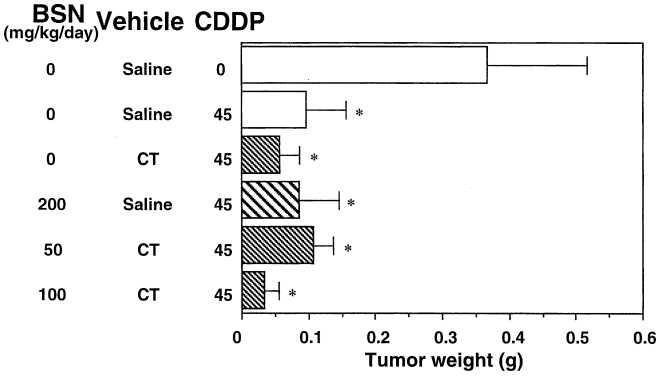


Fig. 4 Effect of pretreatment with saline suspension or citrate buffer solution of BSN on antitumor activity of CDDP in mice inoculated with Meth-A fibrosarcoma. Mice were pretreated orally with saline suspension or citrate buffer solution of BSN at 24-h intervals for 5 days. Mice were injected intraperitoneally with CDDP (45 μmol/kg) 24 h after the last administration of BSN. Tumor weights were measured 5 days after the CDDP injection. Each value for tumor weight represents the mean ± SD for five mice. $*P < 0.01$ vs "saline alone" values

BSN-saline groups. Although administration of high doses of Bi compounds has been reported to cause nephrotoxicity, the increased renal accumulation of Bi at these levels did not elicit renal toxicity as judged by BUN values.

Changes in antitumor activity of CDDP

The antitumor activity of CDDP was evaluated in terms of tumor weight determined 5 days after CDDP treatment in mice inoculated with Meth-A fibrosarcoma. As shown in Fig. 4, pretreatment with BSN-citrate or with BSN-saline did not affect antitumor activity of CDDP. These results may reflect the fact that tumor MT levels were not altered by BSN treatment either in the BSN-citrate or in the BSN-saline group (Fig. 3). Thus, pretreatment of tumor-bearing mice with BSN-citrate did not affect the antitumor activity of CDDP due to the low accumulation of Bi in tumor tissues, while preferential accumulation of Bi in the kidney led to an efficient reduction of CDDP-induced renal toxicity.

Discussion

In the present study, we investigated the effect of the vehicle for oral administration of BSN in inducing renal MT. The results obtained clearly demonstrated that the administration of BSN dissolved in citrate increased gastrointestinal absorption of Bi in mice, leading to increases in Bi accumulation (Fig. 1) and MT induction (Fig. 2) in the kidney compared to mice treated with BSN suspended in saline. Enhanced induction of renal MT resulted in an efficient reduction in the nephrotoxicity of CDDP (Fig. 3). Meanwhile, the anticancer effect of CDDP was not affected by pretreatment with BSN-citrate (Fig. 4), probably due to the fact that tumor MT levels were not significantly increased by pretreatment with BSN-citrate (Fig. 2). Thus, utilization of citrate as a vehicle for oral administration of BSN may be an ideal way to reduce the adverse side effects of CDDP without affecting its anticancer effect.

MT is known to reduce the toxic effects of heavy metals, alkylating agents, inducers of reactive oxygen species (ROS), and γ-irradiation by virtue of its high content of sulfhydryl groups [5, 7, 22]. Since CDDP is a Pt-containing drug and has the characteristics of an alkylating agent and also an ROS inducer, the toxic effects of CDDP could be greatly affected by MT through several mechanisms [8, 9, 20]. We have already shown that renal, intestinal and bone marrow toxicity of CDDP can be prevented by MT induction in the target organs by preadministration of Bi compounds in mice, although the tissue Pt concentrations are not changed [10, 18, 24]. As to non-MT factors involved in CDDP detoxification, it has been shown that Bi pretreatment does not increase the concentrations of GSH, the activity of antioxidant enzymes in bone marrow cells [16], or the activity of SOD in the kidney [2]. Furthermore, in agreement with the findings of previous studies [2, 16], the administration of BSN hardly induced MT in Meth-A tumor tissue. Although the basal level of MT in Meth-A tumors was high (Fig. 2), this may not have been the reason for the inability of BSN to induce tumor MT, since other MT inducers such as zinc and X-rays have been shown to induce MT in Meth-A cells inoculated in mice [26, 27].

Among Bi compounds, BN is an efficient inducer of renal MT when administered parenterally [10], but is not used as a medicine. On the other hand, BSN has already been used as a medicine, but its major disadvantage for inducing renal MT is the low rate of absorption of Bi from the gastrointestinal tract. The present study has overcome this drawback by using citrate as the vehicle for BSN. Although the precise mechanism underlying

the increased intestinal absorption of Bi when BSN and citrate are administered simultaneously is unclear, one possibility is the formation of Bi-citrate [28]. Furthermore, the merit of using citrate with BSN is that citrate is also used as a medicine for adjusting urinary pH. Thus, the combination regimen of BSN and citrate for enhancing MT induction in the kidney may be readily applicable via the oral route in patients receiving CDDP chemotherapy.

We should be careful in increasing the gastrointestinal absorption of Bi since overdosing of Bi compounds is known to cause nephrotoxicity [14, 15]. However, as shown in Fig. 4, the combination regimen of BSN and citrate did not increase BUN values, but rather reduced the elevated levels of BUN caused by CDDP treatment to control levels. Therefore, the enhanced absorption of Bi when BSN was administered with citrate in this experiment did not elicit Bi toxicity at least in terms of renal function. In addition, we used two doses of BSN, 50 and 100 mg/kg, but 50 mg/kg BSN was enough to induce renal MT and suppress the nephrotoxicity of CDDP, while 200 mg/kg BSN suspended in saline could not induce renal MT. Therefore, the dose of BSN could be decreased by using citrate as the vehicle, which may reduce the possibility of Bi intoxication.

Another disadvantage of increasing Bi absorption may be undesirable induction of MT in tumor tissues, which may lead to acquisition of resistance against various anticancer agents. We have already reported that the elevated levels of tumor MT induced by Zn treatment confers resistance against CDDP, doxorubicin and melphalan in mice inoculated with human bladder tumor [19, 23]. On the other hand, the cells derived from MT knockout mice were shown to be more sensitive to anticancer drugs compared with control cells, suggesting that even a low concentration of MT plays an important role in protection against the tumoricidal effects of anticancer drugs, probably through the inhibition of apoptosis [11, 12]. In this study, the administration of BSN-citrate increased concentrations of Bi in the kidney, but only slightly in tumor (Fig. 1), and consequently, MT induction in tumor was negligible (Fig. 2). We also confirmed no diminution of the antitumor effects of CDDP by pretreatment with BSN-citrate (Fig. 4). In accordance with our previously reported results [10], Bi accumulated preferentially in the kidney but only slightly in tumor even when Bi absorption was enhanced by citrate.

The results of this study in mice raise the possibility that the use of BSN and citrate, both of which are used generally in the clinical situation, may improve the efficacy of CDDP chemotherapy by reducing toxic side effects without affecting the antitumor actions of CDDP. Another intriguing possibility is that, with this regimen, higher doses of CDDP could be administered to cancer patients. Further study with multiple doses of CDDP in combination with Bi and citrate is warranted. Furthermore, as MT is known to reduce the adverse side effects of other anticancer drugs such as doxorubicin and melphalan, this regimen may be applicable widely in cancer chemotherapy.

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