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Inhibition of cisplatin protein binding: a possible therapeutic advantage

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ABSTRACT

Inhibiting the protein binding of cisplatin may increase the concentration of free cisplatin in the blood and improve its transition to target tissues. However, the types of protein binding to which cisplatin is subject and the kinds of proteins to which it binds in the blood are unknown. This study investigated the time course of binding between cisplatin and blood proteins. Cisplatin was found to bind mainly to human serum albumin (HSA). In addition, the binding ability of cisplatin increased in a time-dependent manner, which suggests the involvement of covalent binding. Sites I and II which are noncovalent-binding sites on HSA were involved to a limited extent. Subsequently, this study investigated bucillamine, D-penicillamine, and L-cysteine, all of which have an SH group, as protein binding inhibitors. An inhibitory effect was noted with bucillamine and D-penicillamine after 5 hours of preincubation. Differences were noted for L-cysteine at all time points. Thus, L-cysteine reduced the covalent binding of cisplatin. It is suggested that a drug administration plan that utilizes the inhibition of cisplatin protein binding may contribute to a reduction in dosage which hopefully will result in a decrease in the severity of cisplatin-induced adverse side effects.

Keywords: cisplatin; protein binding; covalent binding; D-penicillamine; L-cysteine

INTRODUCTION

The effects of drug therapy generally depend on the concentration of drug that is not bound to human serum proteins and thus remains free to exert its therapeutic effect. Takamura *et al.* (2005) reported that the diuretic resistance of furosemide, which is strongly bound to human serum albumin (HSA), recovers after increasing the blood and urine levels of free furosemide by inhibiting its protein binding with Bucolome, a non-steroidal anti-inflammatory drug (NSAID). We also observed that by inhibiting protein binding of the NSAID flurbiprofen with endogenous free fatty acids, blood levels of free flurbiprofen increased transiently resulting in potentiation of its analgesic effects (Ogata *et al.*, 2008). In planning a cancer chemotherapy regimen, the use of such protein binding inhibition could enhance the therapeutic effects of even low-dose therapy. However, determination of generalised tissue distribution requires careful investigation of therapeutic agent transition to organ tissues. Thus, the usefulness of protein binding inhibition in cancer chemotherapy requires a comprehensive understanding of serum proteins and the sites to which the chemotherapeutic agent binds. In addition, the substance that effectively suppresses protein binding must be identified, thereafter the drug concentrations in tumours under investigation, other tissues, and blood must be investigated.

In attempting to develop a drug administration plan based on protein binding inhibition by a third agent, we decided to work with the anticancer agent, cisplatin (*cis*-diamminedichloroplatinum [II]). This chemotherapeutic agent was selected because approximately 90% of cisplatin is protein bound in the blood (Bannister *et al.*, 1977; Vermorken *et al.*, 1984), and because it is administered locally to regions surrounding a tumour, such as in hepatic infusional therapy. However, no consensus has been reached on the characteristics of the binding between cisplatin and blood proteins. For example, no one has investigated cisplatin binding to the human serum proteins, α_1-acid glycoprotein (AGP) and γ-globulin (immunoglobulin). For this reason, we investigated the inhibition of cisplatin binding to serum proteins and devised a drug administration plan designed to enhance the pharmacological effects of cisplatin. First, changes in the amount of protein binding of cisplatin to human serum, HSA, AGP, and γ-globulin were evaluated over time to identify the main protein to which cisplatin binds. In addition, noncovalent binding of cisplatin was investigated using a fluorescent probe method. After administering a drug which we predicted would inhibit cisplatin protein binding, the effects on protein binding rates were re-investigated using human serum. We used bucillamine, D-penicillamine, and L-cysteine, all of which have a sulfhydryl (SH) group, as potential inhibitors of cisplatin protein binding.

MATERIALS AND METHODS

Reagents

Cisplatin powder was supplied by Nippon Kayaku Co., Ltd. HSA, AGP, γ-globulin, and dansylsarcosine piperidinium salt (DNSS) were purchased from SIGMA. Warfarin (WF) and bucillamine were supplied by Eisai Co., Ltd. and Santen Pharmaceutical Co., Ltd., respectively. D-penicillamine and L-cysteine were purchased from Wako Pure Chemical Industries, Ltd. All other reagents used were special grade. Human serum was obtained from a healthy adult man who consented to participate in this study. Blood was collected according to the ethical principles of the Helsinki Declaration.

In vitro protein binding experiment

To examine the rate of protein binding of cisplatin to various human serum proteins, cisplatin was added to serum proteins at clinical concentrations, followed by incubation for 1, 3, or 5 hours at 37°C (in the case of human serum, for 3 hours only). Ultrafilters (ULTRACENT-10, TOSOH) were filled with 0.5 mL of these test solutions, followed by centrifugation at 3200 rpm for 15 minutes. The concentration of free cisplatin in the obtained filtrate was measured with an atomic absorption photometer (Hitachi, Z-5010). The protein binding rate of cisplatin was calculated according to the following formula:

Protein binding rate (%) = (total cisplatin concentration – free cisplatin concentration) x 100 / total cisplatin concentration

Identification of binding sites on HSA by the fluorescent probe method

3μL of probe drug (WF or DNSS) was added to 3 mL of HSA solution. Cisplatin was then added and mixed, and measured immediately with a fluorophotometer (Jasco, FP-6300), as non-covalent binding of WF or DNSS to site I or site II, respectively, may occur very quickly. On fluorophotometry, the excitation wavelength of WF was 320 nm and the fluorescence wavelength was 400 nm; the excitation wavelength of DNSS was 350 nm and the fluorescence wavelength was 480 nm (Yamasaki *et al.*, 2005).

Measurement of the protein binding rate of cisplatin after adding the SH compound

To determine the effects of the SH compound on the covalent binding rate of cisplatin, the SH compound was prepared at a final concentration of 600μM and, after addition of human serum, preincubation was conducted at 37°C for 1 or 5 hours. Cisplatin was then added, followed by incubation at 37°C for 1, 3, or 5 hours. Alternatively, SH compound and cisplatin were added at the same time and incubated for 1 hour. Subsequently, 0.5mL of acetonitrile was added to 0.5mL of test solution for deproteinization, followed by centrifugation at 3000rpm for 10 minutes (the deproteinization method). The concentration of noncovalently-bound cisplatin plus free cisplatin in the resulting filtrate was measured with an atomic absorption photometer. The covalent binding rate of cisplatin was calculated according to the following formula:

Covalent binding rate (%) = (total cisplatin concentration – (concentration of noncovalently-bound cisplatin + free cisplatin)) x 100 / total cisplatin concentration

Statistical analysis

The unpaired Student *t*-test, Bonferroni test, and SNK test were employed for two-group, control, and multiple- group comparisons, respectively.

RESULTS

Binding between cisplatin and human serum or various human serum proteins

The protein binding of cisplatin to human serum, HSA, AGP, and γ-globulin was examined. Cisplatin binding to proteins was found to be time-dependent: the extent of protein binding was found to be approximately 18% at 0 hours, 41% at 1 hour, 76% at 3 hours, and 90% at 5 hours (**Figure 1**). Cisplatin protein binding to the various human serum proteins increased in a similar time-dependent manner; however, the binding to HSA was higher than those of other proteins (**Figure 2**).

Involvement of cisplatin at sites I and II on HSA

The binding properties of cisplatin to each site in the presence of a given concentration of warfarin and DNSS are shown in **Figure 3.** The fluorescence intensities of warfarin and DNSS showed a slightly decreasing trend; however, no significant difference was noted.

Effects of preincubation with SH compound on the protein binding of cisplatin

The SH compound was preincubated in advance with human serum for 1 or 5 hours before the administration of cisplatin. The reaction solution was incubated for 1 hour after adding the cisplatin solution. The inhibitory effect on protein binding was then examined using the deproteinization method. An incubation period of 1 hour for cisplatin was selected because that is the time at which the protein binding of cisplatin shows linearity, as may be seen in **Figure 1**. For each drug concentration, the amount of cisplatin was converted to 10μM from a normal blood level, and the concentration of SH was set at 600μM, the same concentration of HSA that is observed in healthy individuals. No change was noted in the inhibitory effects of bucillamine and D-penicillamine following 1 hour of preincubation; however, inhibitory effects due to bucillamine and D-penicillamine were noted following 5 hours preincubation. Differences were noted for L-cysteine at all time points (**Figure 4**).

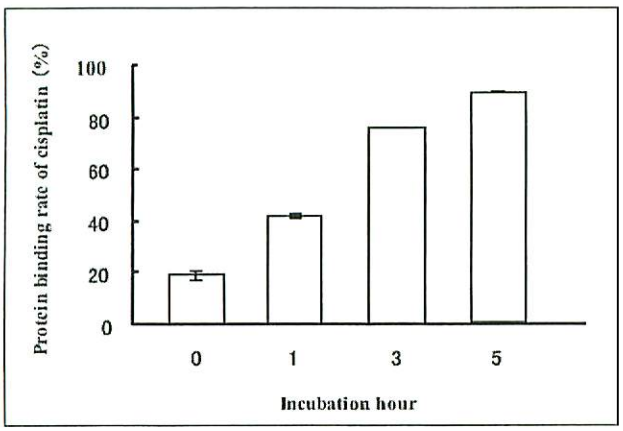

Figure 1. Protein binding of cisplatin to human serum. (each value represents the mean (SD) of three experiments)

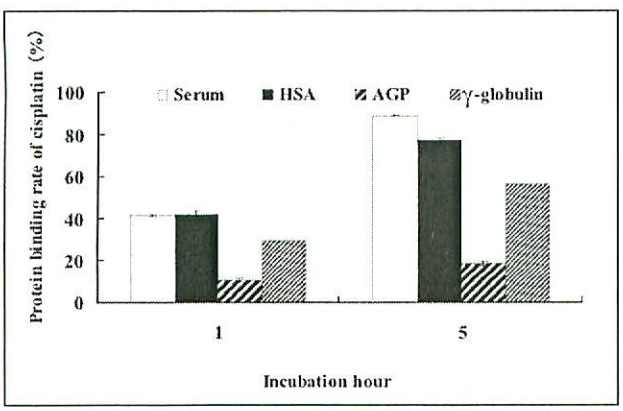

Figure 2. Protein binding of cisplatin to various human serum proteins. (each value represents the mean(SD) of three experiments)

Effects of the simultaneous addition of the SH compound and cisplatin on protein binding

The SH compound and cisplatin were added simultaneously, and then incubated for 1 hour, to investigate their inhibitory effects on protein binding. Protein binding was not inhibited (**Figure 5**).

Effects of changes in cisplatin incubation time on protein binding after preincubation with L-cysteine

After a fixed preincubation period, the incubation time was varied to investigate the inhibitory effects of cisplatin on the degree of protein binding. L-cysteine was preincubated in human serum 1 hour before the administration of cisplatin, after which

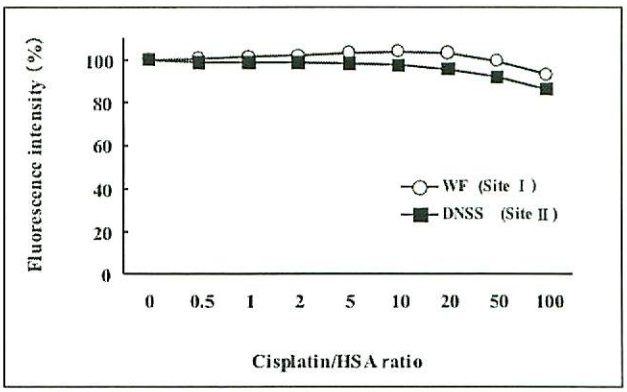

Figure 3. Involvement of cisplatin binding at sites I and II on HSA

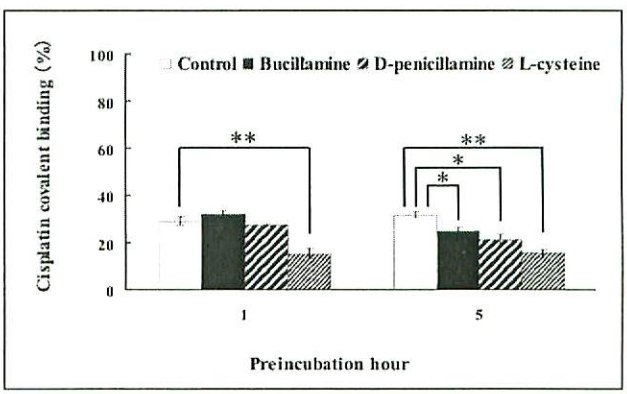

Figure 4. Effects of preincubation with SH compound on protein binding of cisplatin
(each value represents the mean (SD) of four experiments $*p<0.05$: $**p<0.01$).

the reaction solution was incubated for 1, 3, or 5 hours. The inhibitory effects on protein binding were examined by the deproteinization method. A significant difference was noted in covalent-binding inhibition at all time points (**Figure 6**).

DISCUSSION

In these studies, we demonstrated that cisplatin is bound mainly to HSA, and that the extent of protein binding increased in a time dependent manner. For the *in vitro* experiment, we determined that covalent binding is involved in the binding between cisplatin and HSA, and that L-cysteine of the SH compound functions as an inhibitor. However, because the extent of cisplatin protein binding was approximately 18% after 0 hours of incubation, the presence of a binding type other than covalent binding appears likely in the binding of cisplatin to HSA.

In general, the representative sites on HSA to which

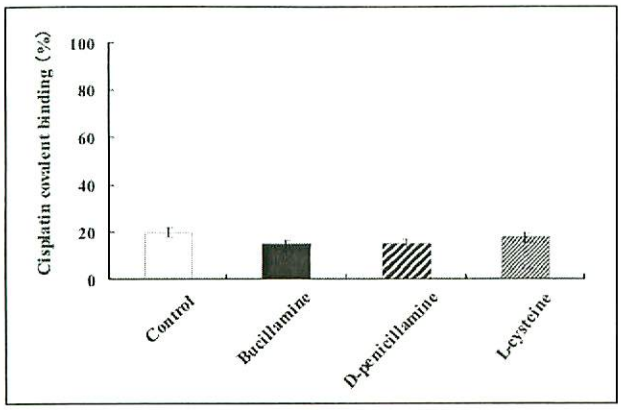

Figure 5. Effects of the simultaneous addition of SH compound and cisplatin on protein binding (each value represents the mean (SD) of four experiments)

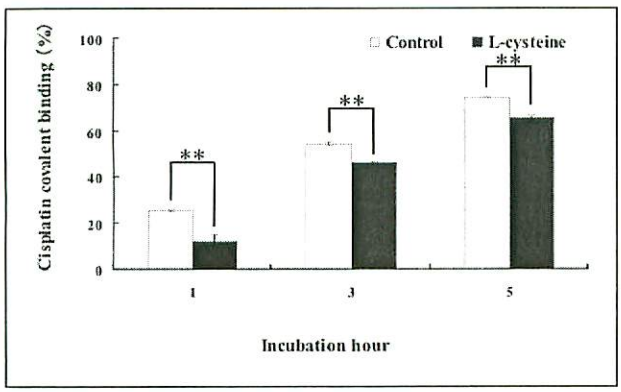

Figure 6. Effects of variation in cisplatin incubation time on protein binding after preincubation with L-cysteine (each value represents the mean(SD) of four experiments $^{**}p<0.01$)

a drug binds instantly and noncovalently to protein, are sites I, II (Kuragh-Hansen *et al.*, 2002; Otagiri *et al.*, 2005)). We used a fluorescent probe to elucidate the binding properties of these binding sites and the drug. As a result, it was shown that the involvement of sites I and II were very small. The protein binding of cisplatin and HSA was mostly covalent binding; however, the involvement of noncovalent binding was also suggested to account for the immediate binding effect.

In a previous report, Andrei *et al.* (1998) indicated that the covalent binding site with HSA is a free SH group in Cys34. We hypothesized that it is important to bind free SH groups with a safe substance, in order to increase the concentration of free cisplatin circulating at its target sites by inhibiting the binding of cisplatin. Therefore, we used bucillamine, D-penicillamine, and L-cysteine as potential inhibitors of protein binding, because these carry an -SH group and appear to allow covalent binding at this site. It has been reported that the antirheumatic drugs bucillamine and D-penicillamine undergo disulfide bonding with plasma proteins (Anraku *et al.*, 2004), and that the protein binding increases with time reaching nearly 100% at 24 hours after administration (Nozu *et al.*, 1977). We selected an amino acid, L-cysteine as a safe potential inhibitory agent because it also carries an -SH group. No inhibitory effect of bucillamine or D-penicillamine was seen following 1 hour of preincubation; however, an inhibitory effect of bucillamine and D-penicillamine was noted following 5 hours of preincubation. Differences were noted for L-cysteine at all time points, which demonstates the inhibitory effect of L-cysteine on cisplatin protein binding. Because the adverse effects of bucillamine and D-penicillamine include renal failure, clearly the concomitant administration of either of these drugs with cisplatin would be extremely harmful.

A subsequent experiment investigating the inhibitory effects on protein binding due to the simultaneous addition of an SH compound and cisplatin, followed by 1 hour of incubation, demonstrated that neither of these substances exhibited a protein inhibitory effect. Because the disulfide binding reaction tends to progress slowly between the SH compound and the Cys34 of HSA, we consider that the simultaneous addition of cisplatin was not effective and that pre-incubation may be necessary. Furthermore, our study on the inhibitory effects of L-cysteine on cisplatin protein binding over time with the period of pre-incubation kept constant and the period of incubation varied, demonstrated significant differences in covalent-binding inhibition at all time points. Thus, L-cysteine was shown to reduce covalent binding of cisplatin during both preincubation and incubation. Cisplatin is a drug that plays an important role in a number of tumours including its use as hepatic intra-arterial chemotherapy for liver cell cancer. Particularly in transcatheter hepatic arterial chemo-embolization (TACE), the concomitant use of an embolic substance increases retention of cisplatin around the tumour and arrests nutrient supply to the

tumour. However, we think that cisplatin will not exert its full effects, even when administered by TACE, because its transport across tumour tissue is limited by its binding to blood proteins, resulting in low concentrations of free cisplatin. Therefore, we assumed that discovering safe substances that inhibit cisplatin protein binding might increase the concentration of free cisplatin circulating in the blood and thus improve its transition to tumour tissue. Development of drug regimens that employ inhibition of protein binding may lead to a decrease in the frequency and severity of adverse effects and may be useful for hepatic infusion therapy.

We think it would be useful to investigate further the effect of cisplatin administered with L-cysteine and to examine possible applications for hepatic infusion therapy and the possibility of an alternative pathway of cisplatin protein binding.

Declaration of interests

The authors declare that no financial assistance or grant funding was received for this research.

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CONTENTS

Volume 7 No 4, 2010