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Heat enhances the cytotoxicity of cis-diamminedichloroplatinum(II)and its analogues cis-1, 1-cyclobutane-dicarboxylato(2R)-2-methyl-1, 4-butanediammineplatinum(II) and cis-diammine(glycolato)platinum in vitro

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Heat enhances the cytotoxicity of *cis*-diamminedichloroplatinum(II) and its analogues *cis*-1,1-cyclobutanedicarboxylato(2R)-2-methyl-1,4-butanediammineplatinum(II) and *cis*-diammine(glycolato)platinum in vitro

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Abstract. *cis*-1,1-Cyclobutanedicarboxylato(2R)-2-methyl-1,4-butanediammineplatinum(II) (NK121) and *cis*-diammine(glycolato)platinum (254-S), analogues of *cis*-diamminedichloroplatinum (II) (CDDP) with reduced nephrotoxicity, are under clinical phase trial in Japan. Since CDDP has been shown to be more cytotoxic under conditions of an elevated temperature, we tested the cytotoxicity and cellular uptake of these analogues at 37° and 43°C using EMT6/KU cells in vitro. The cytotoxicity of CDDP was enhanced at 43°C, and that of NK121 and 254-S was also enhanced, in a dose- and time-dependent manner. The 90% cytotoxic concentration (IC_{90}) of each drug was reduced 2.9-fold for CDDP, 2.5-fold for NK121, and 2.2-fold for 254-S. Cytotoxicity was maximal when the two modalities were used simultaneously for all three drugs. The intracellular platinum concentration was assayed using flameless atomic absorption spectrophotometry. When exposed to IC_{90} drug concentration at 43°C for 2 h simultaneously, the intracellular platinum concentration increased to 0.095 ± 0.007 μg/10^7 cells (a 1.9-fold increase) for CDDP, to 0.198 ± 0.012 μg/10^7 cells (a 1.3-fold increase) for NK121, and to 0.090 ± 0.014 μg/10^7 cells (a 1.3-fold increase) for 254-S; respectively, as compared with the level measured after drug exposure at 37°C ($P < 0.05$ for all drugs). The elevation in platinum concentration may be one of mechanism related to a synergistic effect of the two treatment modalities. The concomitant use of CDDP analogues and heat shows potential for possible clinical application.

Introduction

Hyperthermia has been reported to enhance the cytotoxicity of various anticancer drugs, including bleomycin [9], mitomycin C [29], adriamycin [24], and cisplatin (CDDP) [1, 2, 19, 29, 30]. Moreover, hyperthermia has been reported to overcome drug resistance, one cause of the ineffectiveness of chemotherapy [10, 30]. For these reasons, many investigators have used hyperthermia to treat patients with various forms of malignancies, locally [12] or systemically [6, 8, 16]. In our institute, we apply local hyperthermia in combination with chemotherapy (CDDP or bleomycin) and radiation [20, 26] for the treatment of patients with esophageal cancer. Application of hyperthermia has improved the survival of patients with advanced esophageal cancer, the 2-year survival of patients with incompletely resected esophageal cancer being increased from 15.6% to 34.3%, as compared with the results obtained in patients not given hyperthermia [20].

CDDP has proved to be effective for patients with a solid tumor, but nephrotoxicity is a major complication [5]. New analogues of CDDP, *cis*-1,1-cyclobutanedicarboxylato(2R)-2-methyl-1,4-butanediammineplatinum(II) (NK121) [7, 27] and *cis*-diammine(glycolato)platinum (254-S; see Fig. 1) [25, 28], have been synthesized in an attempt to reduce nephrotoxicity, and preclinical studies have shown that these drugs are potently active against rodent solid tumors [17, 25]. These drugs are under clinical phase II trials in Japan, and clinical effectiveness equal to or better than that of CDDP has been reported against some forms of malignancies [13, 14]. We report the interaction of these drugs with heat in vitro, and putative mechanisms are discussed.

Materials and methods

Drugs. CDDP powder, supplied by Nippon Kayaku Company, Ltd. (Tokyo, Japan), was dissolved in normal saline at a 5 mM concentration and stored at room temperature. NK121 was also supplied by Nippon Kayaku Company, Ltd. (Tokyo, Japan). 254-S was obtained from Shionogi Pharmaceutical Company Ltd. (Osaka, Japan; Fig. 1). NK121 and 254-S were dissolved in normal saline just before use and then diluted to appropriate concentrations.

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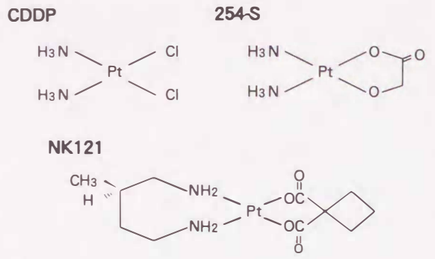

Fig. 1. Structures of CDDP, NK121, and 254-S

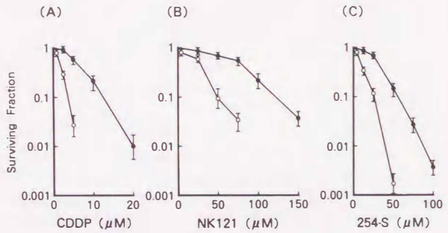

Fig. 2 A – C. Survival of EMT6/KU cells treated with various concentrations of **A** CDDP, **B** NK121, and **C** 254-S at 37° (●) or 43°C (○) for 1 h. *Points*, Means of three independent experiments; *bars*, SE

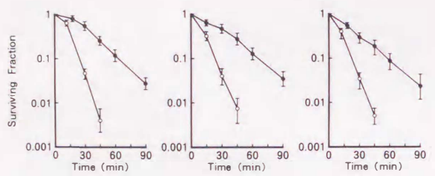

Fig. 3 A – C. Survival of EMT6/KU cells treated with IC_{90} concentrations determined from Fig. 2. Cells were exposed to **A** 12.5 μ*M* CDDP, **B** 115 μ*M* NK121, and **C** 55 μ*M* 254-S at 37° (●) or 43°C (○). *Points*, Means of three independent experiments; *bars*, SE

Cells. The EMT6/KU cells (mouse mammary tumor cells) were a gift from Prof. M. Abe (Department of Radiology, Kyoto University, Kyoto, Japan). These cells were cultured in minimum essential medium (Nissui Corporation, Tokyo, Japan) supplemented with 15% heat-inactivated fetal calf serum, 2.92 g/l L-glutamine, and antibiotics.

Drug and heat treatment. In vitro cell viability was assayed using colony formation [11]. Briefly, 2×10^5 cells were put in 100-mm plates (Corning, N. Y., USA), and at 2 days after implantation, exponentially growing cells were simultaneously exposed for 1 h to various concentrations of CDDP, NK121, or 254-S at 37° or 43°C. After this drug exposure, the dishes were washed three times with phosphate-buffered saline (PBS) trypsinized, counted, plated, and incubated at 37°C in a humidified atmosphere of 5% CO_2. After 7 days, the colonies were stained with Giemsa and counted. The plating efficiency of the untreated control was 80%–90%. The surviving fraction was expressed as the ratio of the numbers of surviving colonies counted on treated plates to those counted on control plates.

For evaluation of the cytotoxicity of each drug used for various periods of exposure, the cells were treated for 1 h at 37° or 43°C with the 90% cytotoxic concentration (IC_{90}) of the drugs, i.e., the concentration that yielded a surviving fraction of 0.1 (CDDP, 12.5 μ*M*; NK121, 115 μ*M*; 254-S, 55 μ*M*). Sequential treatment with drugs and heat was also evaluated using a 1-h exposure to each drug and/or heat treatment under the following conditions: 1 h drug exposure at a concentration that gave a surviving fraction of about 0.7 following 37° and 43°C heat treatment for 1 h. Heat treatment consisted of preheating the drug-containing medium, immediately exchanging the medium, and placing the dishes in a 43°C incubator (Napco Scientific Co., model 6200, N. Y., USA).

Intracellular platinum concentration. The intracellular platinum concentration was assayed using flameless atomic absorption spectrophotometry [18]. EMT6/KU cells were plated at 2×10^5/100-mm dish (Corning), and 4 days later the cells were exposed to medium containing the indicated concentrations of drugs at 37° or 43°C for the indicated periods, washed 3 times with PBS, and centrifuged. Samples were stored at –80°C until assay. The intracellular platinum concentration was assayed using a spectrophotometer (Hitachi model Z-7000, Japan). The platinum concentration was expressed in micrograms per 10^7 cells. All experiments were done in triplicate.

Statistical analysis. The differences observed in the intracellular platinum concentration were analyzed for statistical significance using Student's *t*-test. A *P* value of <0.05 was considered to represent statistical significance.

Results

Cytotoxicity of the drugs

Figure 2 shows the survival curves plotted for EMT6/KU cells following a 1-h incubation with each drug. The application of 1 h heat treatment alone produced a surviving fraction of 0.8–0.9. The cytotoxicity of these drugs was enhanced by 43°C heat treatment. The IC_{90} value determined for each drug from Fig. 2 at 37°C was reduced at 43°C: CDDP, from 12.5 to 4.2 μ*M* (a 2.9-fold decrease); NK121, from 115 to 48 μ*M* (a 2.5-fold decrease); and 254-S, from 55 to 25 μ*M* (a 2.2-fold decrease). All drugs showed enhanced cytotoxicity in a time-dependent manner (Fig. 3).

Sequential treatment with drugs and heat

For each compound, simultaneous heat and drug exposure caused maximal cytotoxicity. There was little heat-drug interaction when cells were heated before or after drug exposure (Fig. 4).

Intracellular platinum concentration

Figure 5 shows the intracellular platinum concentration measured at 37° and 43°C. After incubation for 1 h at various concentrations of the drugs, the intracellular platinum concentration determined at 43°C was elevated over that measured at 37°C, and the difference was statistically significant for exposure to CDDP at 100 μ*M*; to NK121 at

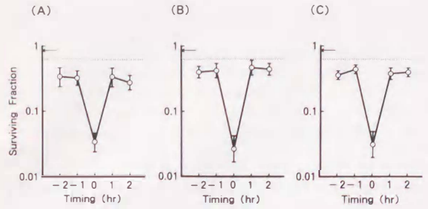

Fig. 4 A – C. Effect of treatment sequence on cell survival following exposure to drugs and heat. EMT6/KU cells were heated for 1 h at 43°C at varying times before (negative time value), during (time 0), and after (positive time value) 1 h drug treatment. **A** CDDP (5 μ*M*). **B** NK121 (75 μ*M*). **C** 254-S (30 μ*M*). *Arrows* indicate the surviving fraction in case of a single exposure to heat; *broken lines* indicate the surviving fraction in case of a single drug treatment. *Points*, Means of three independent experiments; *bars*, SE

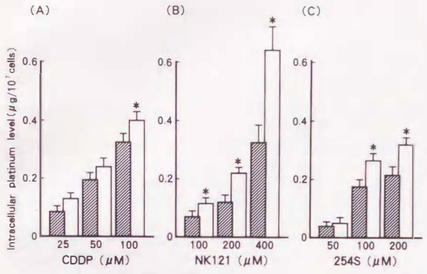

Fig. 5 A – C. Intracellular platinum concentration after exposure of cells to various concentrations of each drug for 1 h at 37° or 43°C. **A** CDDP, **B** NK121, **C** 254-S. ▨, 37°C for 1 h; ▭, 43°C for 1 h; *bars*, SE. * $P < 0.05$ as compared with 37°C

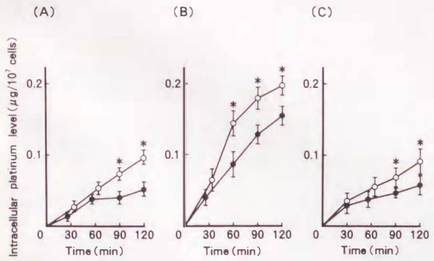

Fig. 6 A – C. Intracellular platinum concentration after exposure of cells to IC_{90} drug concentrations determined from Fig. 1 for various intervals at 37° (●) or 43°C (○). **A** CDDP (12.5 μ*M*). **B** NK121 (115 μ*M*). **C** 254-S (55 μM). *Points*, Means of three independent experiments; *bars*, SE. * $P < 0.05$ as compared with 37°C

100, 200, and 400 μ*M*; and to 254-S at 100 and 200 μ*M* ($P < 0.05$).

The platinum concentration was followed as a function of the duration of IC_{90} drug exposure (Fig. 6). Simultaneous heat treatment enhanced the intracellular accumulation of drugs in a time-dependent manner. Heat of 43°C increased the platinum concentration 1.4-fold, 1.7-fold, and 1.5-fold after exposure for 1 h, to the IC_{90} of CDDP, NK121, and 254-S, respectively, as compared with that measured at 37°C ($P < 0.05$ for NK121). The platinum concentration increased to 0.095 ± 0.007 μg/10^7 cells (a 1.9-fold increase) in CDDP-treated cells, to 0.198 ± 0.012 μg/10^7 cells (a 1.3-fold increase) in NK121-treated cells, and to 0.090 ± 0.014 μg/10^7 cells (a 1.3-fold increase) in 254-S-treated cells after exposure to the respective IC_{90} for 2 h at 43°C ($P < 0.05$ for all drugs).

Discussion

In our institute, we prescribe local hyperthermia in combination with chemotherapy and radiation to treat patients with esophageal cancer [20, 26]. We use a specially designed endoluminal electrode, which elevates the tumor temperature to 42°–45°C. We have observed no severe side effect, but the results have been less than satisfactory. Ideally, hyperthermia should enhance the killing of neoplastic cells while having a much weaker effect on normal tissue. However, nephrotoxicity, a dose-limiting factor of CDDP, is also enhanced by whole-body hyperthermia [2, 8, 23].

Figure 1 shows the structures of the three drugs we investigated. These drugs have a common feature: a central platinum atom linked to a carrier ligand and a leaving group [7, 25, 28, 31]. NK121 [7, 27] has (2R)-2-methylbutanediammine as the carrier ligand and cyclobutanedicarboxylate as the leaving group, whereas 254-S [25, 28] has amine as the former and glycolate as the latter. In principle, these drugs are thought to detach the leaving group inside the cell by hydrolysis, and a positively charged, aquated platinum species reacts with nucleophilic sites in the DNA. The carrier ligands are strongly bound to the platinum but have some relationship with the cytotoxicity of these drugs. The differences between the leaving group and carrier ligands of these two drugs may account for the differences in their cytotoxic effects or their side actions [4]. For carboplatin, which has the same leaving group as NK121, the difference in the cytotoxic mechanism as compared with CDDP has been elucidated. Micetich et al. [22] noted a weaker cytotoxic effect and a delay in DNA cross-linking for carboplatin as compared with CDDP in L1210 mouse leukemia cells in vitro. These authors concluded that the difference in cytotoxicity seen following exposure to equimolar concentrations of the drugs was due to their different patterns of DNA interaction and to considerable differences in their rates of hydrolysis. Knox et al. [15] also reported a difference in the pattern of detachment of leaving groups between CDDP and carboplatin.

The IC_{90} values obtained at 37°C were 12.5 μ*M* for CDDP, 115 μ*M* for NK121, and 55 μ*M* for 254-S (Fig. 2). The intracellular concentration of NK121 was higher than that of the other drugs after exposure for 1 h to the IC_{90}. After exposure of the cells to 100 μ*M* of each drug, NK121 showed the lowest platinum concentration among the three

drugs tested (Fig. 5). Thus, NK121 accumulates the least, possibly because of the heavier ligands or a different mode of forming active mono- or bifunctional adducts.

The mechanism of potentiation of various anticancer drugs by heat remains unclear. One possibility is that heat increases intracellular drug accumulation, as has been suggested by in vitro studies [30] and by in vivo [1, 19] hyperthermia studies. Wallner et al. [30] reported that resistance against CDDP was overcome in Chinese hamster ovary cells and that heat increased the intracellular accumulation of this drug 1.5- to 2.2-fold in vitro. Alberts et al. [1] demonstrated therapeutic synergism between hyperthermia and CDDP in a murine tumor model and reported that hyperthermia enhanced the uptake of CDDP by tumor cells. Our data on exposure of EMT6/KU cells to CDDP are consistent with the results of these prior studies in terms of both drug uptake and thermal enhancement of CDDP toxicity. Similar results were also observed for NK121 and 254-S in terms of intracellular drug accumulation. Heat treatment enhanced the ratio of cytotoxicity 2.9-fold for CDDP, 2.5-fold for NK121, and 2.2-fold for 254-S, and the platinum concentration measured after exposure of the cells to the IC_{90} of each drug for 1 h was elevated 1.4-fold, 1.6-fold, and 1.5-fold, respectively. Thus, the mechanism of hyperthermic enhancement cannot be explained only by an elevated concentration of platinum.

Another mechanism that may be responsible for the hyperthermic synergy is increased DNA damage. Meyn et al. [21] reported that CDDP treatment combined with heat yielded greater DNA cross-linking than that produced by CDDP alone. Although there may be a difference in the pattern of detachment of the leaving groups of NK121 and 254-S, these drugs are thought to show cytotoxicity by inducing the formation of DNA cross-links in the same fashion as CDDP. Thus, DNA damage may be increased when NK121 or 254-S is used at 43°C.

CDDP was more cytotoxic when given simultaneously with hyperthermia, as has been observed in prior studies in vitro [29] and in vivo [2]. Cohen and Robins [3] reported that hyperthermic enhancement of cytotoxicity was maximal when heat treatment was given during or just before carboplatin exposure in vitro. In our study, NK121 and 254-S produced results similar to those obtained with CDDP in terms of the sequence of drug application and heat treatment. Thus, these drugs should be used simultaneously in in vivo experiments.

We obtained evidence that the cytotoxicity of NK121 and 254-S was enhanced by heat. Investigation of their antineoplastic efficacy and normal-tissue toxicity is required, especially as related to their nephrotoxicity.

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