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ORIGINAL ARTICLE

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Cytocidal effect and DNA damage of nedaplatin in vitro by simulating pharmacokinetic parameters

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Abstract *Purpose*: The pharmacodynamic effects of cis-diammine(glycolato)platinum (nedaplatin, 254-S) in vitro have been reported, but the dosage and exposure time in vitro have not always been based on clinical observations of the drug's actions in vivo. Regardless of the actual exposure conditions used, the effect of cell-cycle nonspecific anticancer agents such as nedaplatin is believed to depend on the area under the drug concentration-time curve (AUC). In this study, we evaluated the pharmacodynamics of nedaplatin in vitro, especially in relation to its AUC dependency, in terms of cell survival and DNA crosslinking. *Methods*: BG-1 human ovarian cancer cells were treated with various concentrations of nedaplatin to simulate the pharmacokinetics of administration in a clinical setting. The BG-1 cells were exposed to nedaplatin dissolved in medium containing serum using constant concentration conditions, either high (maximum 7.69 mg/l) or low (average 1.33 mg/l). These concentrations were based on doses used in clinical studies. We then adjusted the exposure conditions in vitro to simulate the elimination of the drug from serum in vivo as follows: $T_{1/2\alpha}$ 1.20 h and $T_{1/2\beta}$ 2.70 h. The AUC values were set at 4, 8, 16, 25 and 40 mg · h/l for all exposure conditions. A colony-formation assay for the surviving fraction and an alkaline-elution assay for DNA crosslink measurement were done for the pharmacodynamic evaluation with comparison on the basis of the AUC value. *Results*: Exposure to a low concentration for a long time was the most effective of the exposure conditions at the same AUC value. The greater the AUC value, the higher the crosslink index under all exposure conditions. This index tended to increase particularly after exposure to the low concentration. The natural logarithm of the surviving fraction (Y') was a linear function of the crosslink index regardless of the drug-exposure condition: $ln(Y') = -87.2x + \ln(5.79)$, $R^2 = 0.89$. The threshold cytocidal effect was associated with a crosslink index of 0.02. *Conclusion*: There was a strong correlation between the cytocidal effect of nedaplatin and DNA crosslink formation. The cytocidal effect and DNA crosslinking in vitro depended on the exposure conditions used to define the AUC. Therefore, a new pharmacokinetic-pharmacodynamic model for nedaplatin must be constructed to investigate the most effective administration procedure in vivo.

Key words Simulation · 254-S · Nedaplatin · DNA · Crosslink · AUC

This study is the first in a series of pharmacodynamic studies of nedaplatin.

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Introduction

Many types of anticancer agent are in wide clinical use and there are many reports of the in vitro pharmacodynamic cytotoxicity of these anticancer agents. However, the dosages of drugs in some of these in vitro studies differ from those used in actual clinical practice; usually, the in vitro dosages are high. In general, the higher the drug concentration, the clearer the experimental results. In clinical practice, however, such high dosages cannot be given to patients because of severe toxicity. However, we believe that the experimental parameters of in vitro studies, such as drug concentration and exposure time, should approximate the in vivo pharmacokinetic parameters as closely as possible. In other words, in vivo conditions

should be simulated in vitro so that the results of the in vitro experiments can be applied to patients as efficiently as possible to improve existing clinical drug regimens.

In gynecologic oncology, platinum analogs are important chemotherapeutic drugs. The pharmacodynamics of platinum analogs are said to depend on the value of the area under the drug concentration-time curve (AUC). This belief is based on both experimental data in vitro and mathematical modeling [1, 2]. On the other hand, clinical observations suggest that the antitumor effect of platinum analogs, such as cis-diamminedichloroplatinum(II) (CDDP), is not influenced by the dosage [3], although the pharmacodynamic effect should theoretically be proportional to the AUC if the drug shows pharmacologic linearity. This suggests that there is some discrepancy between the experimental data supported by the model and actual clinical data. Therefore, it is important to investigate whether the AUC-dependency of platinum analogs in vitro occurs under constant concentration exposure conditions by simulating the in vivo concentration. To simulate the in vivo pharmacodynamics, the drug concentration in vitro should mimic the active form of the drug in a time-dependent manner, that is as a two-compartment model of the form $Ae^{-\alpha t} + Be^{-\beta t}$.

In this study, we used cis-diammine(glycolato)platinum (nedaplatin), a new platinum analog that does not bond with protein in serum [4–6]. We investigated the cell survival and DNA crosslinking following exposure to nedaplatin in vitro using a cell line to simulate in vivo pharmacokinetics, in which the nedaplatin concentration was varied to provide constant exposure.

Materials and methods

Cell growth and maintenance

The cultured cells were derived from human ovarian cancer and designated cell line BG-1. They were grown in modified McCoy's 5A medium (GIBCO, Grand Island, N.Y.) supplemented with 0.05% L-glutamine (GIBCO), 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate (GIBCO) [7] and 10% v/v heat-inactivated fetal calf serum (GIBCO) in a humidified atmosphere of 5% CO_2 in air at 37 °C. The culture was generated as a single layer. The cells were taken off by the addition of 0.17% trypsin (GIBCO) in 20 mg% EDTA for 15 min. Exponentially proliferating BG-1 cells growing for 3–4 days were used.

Drug exposure

Nedaplatin powder, kindly provided by Shionogi Research Laboratories (Osaka, Japan), was dissolved in 5% xylite, and was then diluted with modified McCoy's 5A medium containing serum at 37 °C, as described elsewhere [8–11]. The solution was warmed to 37 °C so that all exposures were performed at the same temperature. Four T75 flasks (Becton-Dickinson, Franklin Lakes, N.J.) each containing approximately 3×10^6 cells in 15 ml medium were used in each experiment.

Simulated exposure

The simulated exposure was based on clinical pharmacokinetic parameters. The AUC value of the normal nedaplatin dose (100 mg/m^2) is 16 mg · h/l administered as a 30-min infusion [5]. The AUC values obtained from clinical studies range from 16 to 25 mg · h/l [5, 12]. In the simulated exposure, the nedaplatin exposure time was 12 h in all experiments. The AUC values of nedaplatin were 4, 8, 16, 25 and 40 mg · h/l. The changes in nedaplatin concentration were the same as those found in vivo: $T_{1/2\alpha}$ 1.20 h from 0 to 2 h and $T_{1/2\beta}$ 2.70 h from 2 to 12 h (Fig. 1). Other parameters, such as maximum concentration, were calculated based on the AUC value and half-lives using an original program developed by Mathematica (Wolfram Research, Champaign, Ill.) on a Macintosh computer. With an AUC value of 16 mg · h/l, the nedaplatin concentrations were 7.690, 3.963, 2.511, 1.873, 1.315, 0.891, and 0.544 mg/l at 0–0.5, 0.5–1, 1–1.5, 1.5–2.5, 2.5–4, 4–6, and 6–12 h, respectively. The maximum nedaplatin concentration of 7.69 mg/l was the same as that in the 30-min infusion in vivo [5]. To obtain other concentration/time combinations for the AUC values of 4, 8, 25 and 40 mg · h/l, the concentration/time combinations for the AUC value of 16 mg · h/l were divided by 4, 2, 0.64, and 0.4, respectively.

Constant concentration exposure

The maximum nedaplatin concentration obtained by 30-min infusion in vivo is 7.69 mg/l [5], which was used for the high-dose exposure condition in this study. Since the AUC values investigated were 4, 8, 16, 25 and 40 mg · h/l, exposure times with 7.69 mg/l as the high dose were set at 0.5, 1.0, 2.0, 3.3 and 5.2 h, respectively. Since nedaplatin is eliminated and its level is too low to detect after 12 h in vivo, the low dose was set at 1.33 mg/l, which resulted in an AUC of 16 mg · h/l over 12 h. Therefore, the exposure times at 1.33 mg/l were set at 3.0, 6.0, 12.0, 18.8 and 30.1 h, resulting in AUC values of 4, 8, 16, 25 and 40 mg · h/l, respectively.

After exposure to nedaplatin under both exposure conditions, the cells were rinsed three times with nedaplatin-free modified McCoy's 5A medium with 10% v/v fetal calf serum at 37 °C. The cells were then removed with trypsin and resuspended in the medium at 37 °C.

Estimation of anticancer effect

The estimation of the anticancer effect was based on cell survival in a colony-formation assay, and DNA analysis was done with an alkaline-elution assay.

Colony formation

A colony-formation assay was used in this study to determine cell survival. In brief, 100–3000 cells suspended in modified McCoy's medium with 10% v/v fetal calf serum were seeded into culture

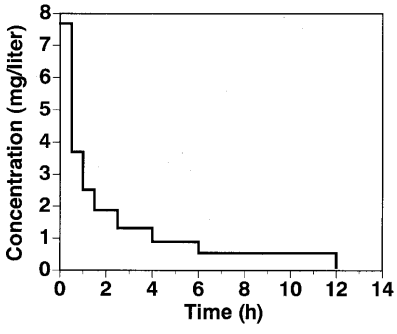

Fig. 1 The method used for simulation of in vivo exposure to nedaplatin. The nedaplatin concentration was simulated, based on the results of a clinical study [5] as a two-compartment model. Exposure at an AUC value of 16 mg · h/l is shown. For other AUC values, the exposure time and half-life of both α and β phases were kept constant

dishes of 60 mm diameter (Becton-Dickinson). The cells were incubated in a humidified atmosphere of 5% CO_2 in air at 37 °C for 14 days. The colonies were then stained with 0.1% crystal violet. Colonies of more than 16 cells were counted. The percentage cell growth for each exposure condition was calculated by dividing the number of cells in the drug-treated culture by the number of cells in the untreated culture.

DNA analysis

DNA crosslinking was measured using the alkaline-elution assay [13–16] described by Miyagi et al. with modifications [7]. After drug treatment, cell suspensions at 37 °C were irradiated with X-rays at 7.5 Gy with an MBR-1520 irradiator (Hitachi-Medico, Tokyo, Japan). The cells were then cooled immediately in ice. The cells (3×10^6) for each channel were loaded onto polycarbonate filters of 47 mm diameter with a pore size of 2 μm (Costar Scientific Corporation, Cambridge, Mass.) in Swinnex filter holders (Millipore Corporation, Bedford, Mass.). The cells were then lysed in 2 *M* NaCl (Wako, Osaka, Japan), 0.2% *N*-lauroylsarcosine (Sigma Chemical Company, St. Louis, Mo.), and 0.04 M EDTA (Ishizu Seiyaku, Osaka, Japan), pH 10.0, to remove most of the cell proteins, membranes, and RNA. The filters were then rinsed with 0.76% Na_4-EDTA (Ishizu Seiyaku) and 33 ml of a solution of 0.02 *M* EDTA (Sigma) and 20% tetrapropylammonium hydroxide, pH 12.3 (Tokyokasei, Tokyo, Japan). The cells were then overlaid in the dark and pumped through a peristaltic pump (Gilson Medical Electronics, Villiers-le-Bel, France) to a fraction collector (Advantec Toyo, Osaka, Japan) at a rate of 0.0389 ml/min. Ten fractions were collected at 90-min intervals for 15 h. At least three channels were used for each cell suspension specimen. Each fraction was mixed with 0.08 *M* KH_2PO_4 (Katayamakagaku, Osaka, Japan) and Hoechst 33258 dye (Sigma). DNA was then detected with a fluorometric assay [17–19]. Each DNA analysis was repeated three times. We did not use proteinase K in the lysis solution because dispersal by proteinase K produced no differences in the elution profiles in preliminary experiments (data not shown).

Analysis of alkaline elution data

The amount of DNA from the cells on the filter was calculated as a percentage of the amount retained after elution. The frequency of nedaplatin-induced DNA crosslinking in this study was calculated using the formula [20]:

$$\mathrm{CLI} = [(1 - R_0)/(1 - R_n)]^{1/2} - 1 \ ,$$

where CLI is the crosslink index of the DNA crosslinking in nedaplatin-treated cells, R_0 is the relative retention of irradiated cells in samples not exposed to nedaplatin, and R_n is the relative retention of nedaplatin-treated cells after a 7.5-Gy irradiation.

Statistical analysis

All values were analyzed using the paired *t*-test, ANOVA, and least-squares regression analysis.

Results

The colony-forming efficiency of untreated cells was 50.0 ± 3.9% (mean ± SE). Cell survival was likely to be a function of the AUC value under each exposure condition. In spite of the same AUC value, however, cell survival varied with various exposure conditions (Fig. 2). Exposure at the low concentration was the most effective of the procedures. In general, the simulated exposure was more effective than the high concentration exposure and less effective than the low concentration exposure. Statistically significant differences among these exposure conditions were not observed at AUC values of 4–25 mg · h/l, which are below the normal clinical level.

The maximum value of DNA crosslink formation occurred just after removal of the nedaplatin (data not shown). Therefore, the alkaline-elution assay was carried out just after removal of the nedaplatin. Figure 3 shows the kinetics of DNA crosslink formation as a function of the AUC value. The greater the AUC value, the higher the total crosslink index under all exposure conditions. This index tended to increase particularly with the low concentration exposure. The high concentration exposure led to the fewest DNA crosslinks at the same AUC value in all procedures. The simulated exposure conditions resulted in intermediate levels of crosslink formation.

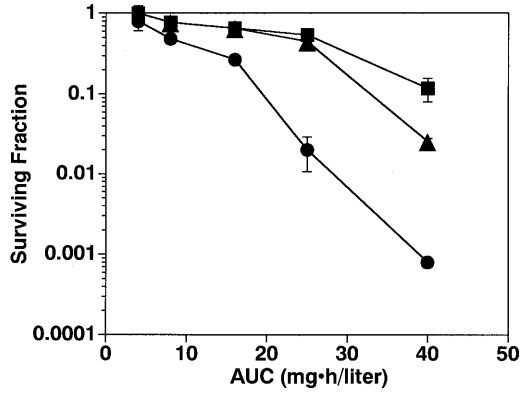

Fig. 2 Correlation between the AUC value and the surviving fraction. Cell survival varied with various exposure conditions in spite of the same AUC value. The low concentration exposure was the most effective at the same AUC value, especially at an AUC of 40 mg · h/l. The values are mean ± SE. *Error bars* (SE) are shown where they are larger than the symbols (■ 7.69 mg/l, ● 1.33 mg/l, ▲ simulated)

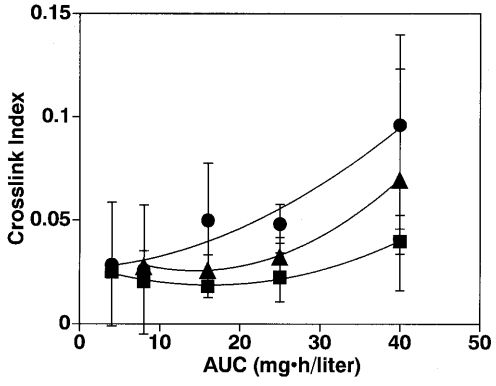

Fig. 3 Correlation between DNA crosslinks and the AUC value. The greater the AUC value, the higher the total crosslink index under all exposure conditions. This index tended to increase particularly under the low concentration exposure condition. The values are means ± SE. *Error bars* (SE) are shown where they are larger than the symbols. (■ 7.69 mg/l, ● 1.33 mg/l, ▲ simulated)

A strong correlation was observed between the colony-forming efficiency and the crosslink index (Fig. 4). The natural logarithm of the surviving fraction (Y′) was a linear function of the crosslink index regardless of the drug exposure conditions: $ln(Y') = -87.2x + ln(5.79)$, $R^2 = 0.89$. The calculated threshold of the crosslink index for cell killing was 0.02.

Discussion

In this study, the most important factor influencing the cell-killing effect of nedaplatin was the number of DNA crosslinks. The greater the AUC value, the higher the total crosslink index under all the exposure conditions. Furthermore, the natural logarithm of the surviving fraction was a linear function of the crosslink index regardless of the exposure conditions. The challenge became how to form the greatest number of crosslinks.

Although cell survival was likely to be a function of the AUC value, the cell-killing effect could not be evaluated using only the AUC value. The cell survival varied among the various exposure conditions in spite of the same AUC value. The low concentration exposure produced the best results of all exposure conditions in vitro, followed by the simulated exposure, and the high concentration exposure produced the poorest results. These outcomes cannot be explained by the current pharmacokinetic-pharmacodynamic theory for cell-cycle nonspecific drugs such as platinum analogs [1, 2], although this theory can explain the cell-killing effect of CDDP. The theory for cell-cycle nonspecific drugs, which must penetrate the cell membrane and react with targets quickly, indicates that the cell-killing effect is a function of the AUC value regardless of the drug exposure conditions used. Additionally, the term "quickly" is not defined as an absolute time but as a relative time, which is usually the doubling time. The platinum analogs are regarded as type I drugs, which are concentration-dependent. The effect of these drugs is not affected by exposure time but is influenced by the AUC value [21–23]. Therefore, Ozawa's mathematical model of the pharmacokinetic-pharmacodynamics of cell-cycle nonspecific anticancer agents should not be applied to nedaplatin. As a result, the cell-killing effect of platinum analogs is not always dependent on the AUC value.

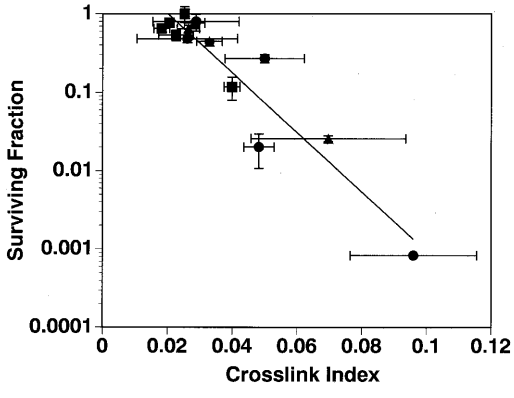

Fig. 4 Correlation between DNA crosslinks and cell survival. The logarithm of colony-formation efficiency is a function of the DNA crosslink index (CLI), regardless of the exposure conditions: $ln(Y') = -87.2x + ln(5.79)$, $R^2 = 0.89$ (where Y′ is the surviving fraction). The cell killing effect did not depend as much on the AUC value as on the level of DNA crosslinking in this cell line. The values are means ± SE. *Error bars* (SE) are shown where they are larger than the symbols. (■ 7.69 mg/l, ● 1.33 mg/l, ▲ simulated)

Cell survival in this study was not dependent on the AUC value only. Because the low concentration and a long exposure resulted in the largest number of DNA crosslinks, the reaction time is likely to be an important factor in nedaplatin's ability to form DNA crosslinks. That is to say, nedaplatin requires more time than CDDP to penetrate the cell membrane and to form DNA crosslinks. Nakajima et al. have reported that nedaplatin passes through the cell membrane by simple diffusion, which is dependent on the drug concentration, and then forms irreversible DNA crosslinks [24]. Another study, in which the reactivity of nucleosides was examined using high-speed chromatography, has revealed that nedaplatin reacts with guanine mainly in nucleosides, as does CDDP [25], but it requires a reaction time about twenty times that required by CDDP (Totani et al., unpublished data). In terms of the chemical structure of platinum analogs, the opening speed of the leaving group strongly correlates with the rate of protein binding [26–28]. Although it has glycolato residues as leaving groups, nedaplatin exists in serum and passes through the cell membrane in unbound form. Because nedaplatin is a relatively stable drug that shows slow opening of the leaving group, its transport from the cell membrane to the drug target and its binding kinetics with guanines in the nucleus likely require a longer time than that required by CDDP. These findings suggest that nedaplatin requires more time to penetrate the cell membrane than CDDP.

Because DNA crosslinks created by platinum analogs are believed to have a cytocidal effect, we investigated the formation of DNA crosslinks as one of the most important pharmacodynamic factors in the activity of nedaplatin. DNA crosslinks created by platinum analogs are classified as DNA-DNA crosslinks and DNA-protein crosslinks. DNA-DNA crosslinks are classified as either interstrand crosslinks or intrastrand crosslinks. Cytotoxicity is believed to result from the formation of DNA-interstrand crosslinks [29–35]. Plooy et al. have demonstrated that interstrand crosslinks and DNA-protein crosslinks require a long time to repair, unlike intrastrand crosslinks [30]. On the other hand, Pérez et al. have reported that interstrand crosslinks created by CDDP can be rearranged into intrastrand crosslinks on oligodeoxyribonucleotides [36]. Therefore, we measured interstrand DNA crosslinks.

Cell survival showed a strong correlation with the DNA crosslink index. The logarithm of cell survival was a linear function of the crosslink index ($R^2 = 0.89$). Therefore, the DNA crosslink index is a good parameter

to use for predicting cell survival statistically. In other words, the number of DNA crosslinks can be used as an objective measure in the study of the pharmacodynamic effects of nedaplatin as well as CDDP [37]. Both the AUC value or the AUC intensity, which is defined as the clinical AUC value per week, and the number of DNA crosslinks should be considered when a chemotherapy regimen that includes platinum analogs is planned. Higher AUC value for CDDP can be obtained by using consecutive low-dose administrations [38–42]. When the AUC value for CDDP is the same, low-dose consecutive administrations might be superior to high-dose bolus administration because low-dose administration produces fewer side effects in patients. These factors must also be studied for nedaplatin. The threshold for DNA crosslinking and the AUC value should also be considered when planning chemotherapy regimens.

In this study, we measured and evaluated cell survival and DNA crosslink formation in the BG-1 cell line in response to different concentrations of nedaplatin administered with various exposure times to simulate the pharmacokinetic parameters defined in clinical studies. We therefore simulated the in vivo pharmacodynamics of nedaplatin in an in vitro setting. Since the simplest model of pharmacodynamics in vitro is considered to be a function of both the drug concentration and the drug exposure time, in vitro simulation of in vivo exposure is an adequate experimental procedure. Yamamoto et al. have demonstrated that the pharmacodynamic effect of CDDP, with regard to DNA crosslinking and cell survival in vitro in a simulated in vivo exposure, is identical to that of constant exposure on the basis of AUC [37]. Ma et al. have also reported an in vitro simulation of in vivo exposure to CDDP [43]. In our simulated in vivo exposure, both the cell-killing effect and the amount of DNA crosslink formation fell between those of the low-dose and high-dose exposure conditions. Thus, the antitumor effects in vitro should be measured not by exposure to a constant concentration but by simulating the pharmacokinetics because nedaplatin seems to be a nonlinear agent. In vivo exposure should now be simulated in vitro to evaluate the antitumor effect of nedaplatin.

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