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ORIGINAL ARTICLE

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Cytocidal effect and DNA damage of nedaplatin: a mathematical model and analysis of experimental data

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Abstract *Purpose*: Cell cycle non-specific anticancer agents such as cis-diamminedichloroplatinum(II) are believed to depend linearly on the value of the area under the drug concentration time curve, which is supported by a mathematical model. However, the quantitative non-linear phenomena of both the cytocidal effect and DNA crosslink formation by cis-diammine(glycolato)platinum (nedaplatin) have been shown in vitro. Therefore, we developed a new mathematical model to explain these phenomena. *Methods*: We assumed that nedaplatin enters intracellular fluid from medium through simple diffusion to form DNA crosslinks that kill cells. We developed a mathematical model to represent this assumption using differential equations that we then solved using an original computer program. The calculated results were compared with the experimental data. *Results*: The drug's simple diffusion rate constant, the DNA crosslink formation rate constant, and the crosslink-dependent cell death rate constant in the model were 1.8×10^{-14} (l h^{-1}), 1.6×10^{8} (l $mol^{-1/2}$ h^{-1}), 5.45×10^{1} (mol^{-1}), respectively. The model fits the experimental results statistically. The model also demonstrated theoretical proof that continuous exposure at a low dose was superior to the short exposure at a high dose seen in published experimental data. *Conclusions*: We developed a mathematical model to describe the non-linear pharmacodynamic effect of nedaplatin in vitro. This model may provide a novel drug infusion procedure for cancer patients.

Key words Simulation · 254-S · Nedaplatin · Model · Computer-aided

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

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Introduction

Cell cycle non-specific anticancer agents such as cis-diamminedichloroplatinum(II) (CDDP) are believed to depend on the value of the area under the drug concentration time curve (AUC). This concept has been supported by a mathematical model developed by Ozawa et al. [1, 2] and experimental data [3, 4]. As a consequence, a certain AUC value of these drugs must demonstrate the same, or at least a similar, pharmacodynamic effect irrespective of the drug administration procedure used. Kawanishi et al. [5], however, reported that cis-diammine(glycolato)platinum (254-S or nedaplatin), an analog of platinum compounds, administered at a low concentration over a long time showed a better pharmacodymanic effect in vitro than the same administered at a high concentration for a short time, with regard to the cytocidal effect based on the AUC value. The number of DNA crosslinks created by nedaplatin also had a strong correlation with the cytocidal effect. These findings suggest that a new mathematical model is necessary to describe the pharmacodynamic effect of platinum analogs, especially nedaplatin.

We developed a mathematical model to explain the pharmacodynamic effect of nedaplatin in vitro, and compared it with the experimental results of previously published studies [5].

Materials and methods

Chemicals

Nedaplatin was kindly provided by Shionogi Research Laboratories, Osaka, Japan.

Cell growth and maintenance

The procedures were described by Kawanishi et al [5]. In brief, 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., USA) supplemented with 0.05% L-glutamine (GIBCO), 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate (GIBCO) [6], 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 stratum. The cells were removed with 0.17% trypsin (GIBCO) and added to 20 mg/dl EDTA for 15 min. Exponentially proliferating BG-1 cells that were growing for 3–4 days were used.

Drug exposure

The drug exposure procedures used were those described by Kawanishi et al [5]. In brief, powdered nedaplatin was dissolved in 5% xylite [7, 8, 9, 10] and was diluted with modified McCoy's 5A media with sera at 37°C.

The solution was warmed to 37°C so that all exposures were performed at a constant temperature. Four 75-cm^2 flasks (Becton-Dickinson, Franklin Lakes, N.J., USA) containing approximately 3×10^6 cells in 15 ml of medium per flask were used for each experiment.

The maximum nedaplatin concentration obtained with 30-min infusion in vivo was 2.53×10^{-5} M [11], which was defined as the high-dose exposure in this study. Since the AUC values investigated should be 1.32×10^{-5}, 2.64×10^{-5}, 5.27×10^{-5}, 8.25×10^{-5}, 1.32×10^{-4}, and 3.30×10^{-4} mol·h/l, the exposure times for 2.53×10^{-5} M 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 is too low to detect after 12 h in vivo, the low-dose concentration was set at 4.39×10^{-6} M, which achieved 5.27×10^{-5} mol·h/l of the AUC in 12 h. Therefore, the exposure times for 5.27×10^{-5} M were set at 3.0, 6.0, 12.0, 18.8, and 30.1 h, resulting in 1.32×10^{-5}, 2.64×10^{-5}, 5.27×10^{-5}, 8.25×10^{-5}, and 1.32×10^{-4} mol·h/l of the AUC value, respectively.

After both methods of nedaplatin exposure, the cells were rinsed three times with nedaplatin-free modified McCoy's 5A medium with sera at 37°C. The cells were then removed with trypsin and resuspended in the medium at 37°C.

Intracellular accumulation studies

BG-1 cells were exposed to nedaplatin as described above. Following the procedure, the cells were harvested immediately and counted using a hemocytometer. The cell pellets were stored at –20°C until analysis. The pellets were then dissolved by incubation with both nitric acid and hydrogen peroxide at 100°C for 1 h. The resulting cellular digests were analyzed for platinum with a Z-5000 Zeeman atomic absorption spectrophotometer (Hitachi, Tokyo, Japan). The platinum content of the samples was determined by comparison with an external standard prepared by dilution with both nitric acid and hydrogen peroxide.

Estimation of anticancer effect

The anticancer effect was estimated by assessment of cell survival with a colony formation assay; DNA analysis was performed with an alkaline elution assay.

Colony formation

A colony formation assay was used for the survival assay in this study. In brief, 100–3,000 of the cells suspended in modified McCoy's media with 10% v/v fetal calf serum were seeded into culture dishes 60 mm in diameter (Becton-Dickinson). The cells were incubated in a humidified atmosphere of 5% CO_2 in air at 37°C for 14 days, and the colonies were stained with 0.1% crystal violet. Colonies of >16 cells were counted. The percentage of cell growth for each exposure was calculated by dividing the number of cells in the drug-treated culture by the number of cells in the untreated culture. The colony-forming efficiency of untreated cells was $50.0 \pm 3.9\%$ (mean ± SE).

DNA analysis

The DNA crosslinks were measured with the alkaline elution assay [12, 13, 14, 15] described by Miyagi et al. [6] with modifications. In brief, after drug treatment, cell suspensions at 37°C were irradiated with X-rays of 7.5 Gy with an MBR-1520 irradiator (Hitachi-Medico, Tokyo, Japan) and then cooled immediately in ice. After 3×10^6 of the cells for each channel were loaded on polycarbonate filters 47 mm in diameter with a pore size of 2 μm (Costar Scientific, Cambridge, Mass., USA) in Swinnex filter holders (Millipore, Bedford, Mass., USA), the cells were then lyzed in a solution consisting of 2 M NaCl (Wako, Osaka, Japan), 0.2% *N*-lauroylsarcosine (Sigma, St. Louis, Mo., USA), and 0.04 M EDTA (pH 10.0, Ishizu Seiyaku, Osaka, Japan) to remove most of the cell protein, membranes, and RNA. The filter was then rinsed with 0.76% sodium EDTA (Ishizu Seiyaku) and 33 ml of a solution of 0.02 M EDTA acid form (Sigma), and 20% tetrapropylammonium hydroxide (Tokyokasei, Tokyo, Japan), pH 12.3. The cells were 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. Each fraction was mixed with 0.08 M KH_2PO_4 (Katayamakagaku, Osaka, Japan) and Hoechst 33258 dye (Sigma). DNA was detected with a fluorometric assay. Each DNA analysis was repeated three times. We did not use proteinase K in the lysis solution because proteinase K use demonstrated no differences in the elution profiles in our preliminary experiments (data not shown).

Mathematical model and analysis

The mathematical model of the reaction to nedaplatin were created by using differential equations. This model was solved by using the Runge-Kutta method with an original program using Mathematica software (Wolfram Research, Champaign, Ill., USA) on a Macintosh computer. The simple diffusion rate constant and the DNA crosslink formation rate constant were determined using the least square regression method with the program. The calculated data and the observed experimental data were analyzed using the paired *t*-test. The crosslink-dependent cell death rate constant was determined using the colony formation experimental data with linear regression analysis.

Results

We assumed that nedaplatin enters intracellular fluid, especially the nucleus, from medium through simple diffusion and forms DNA crosslinks that kill cells (Fig. 1). Because each nedaplatin molecule has two bonds that can bind with DNA, mainly guanine residues, the average number of DNA sites bound with

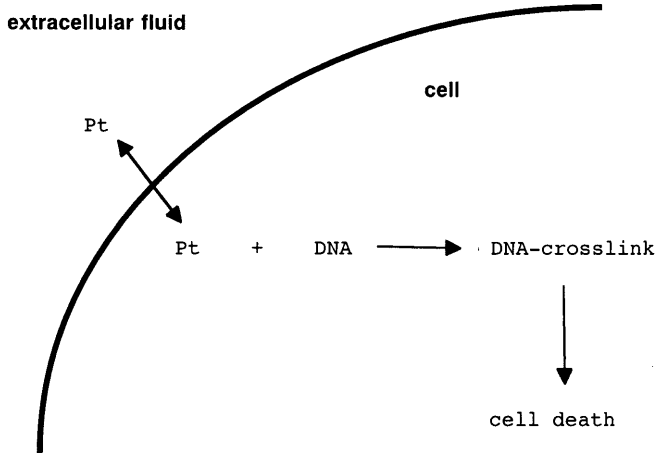

Fig. 1 Scheme of the mathematical model of the reaction of nedaplatin (*Pt*) with DNA. Nedaplatin in extracellular fluid enters cells through simple diffusion and then binds with DNA, creating DNA crosslinks that induce cell death

nedaplatin is believed to be between 1 and 2 per platinum atom. The DNA crosslink formation is considered irreversible. Hence the nedaplatin dose (mol) in a cell can be expressed as follows:

$$x - p^{-1}y \tag{1}$$

in which p = the average number of DNA bound sites per platinum atom; x = the drug dose (mol) per cell; y = the number of DNA crosslinks (mol) per cell.

The presumed maximum number of DNA (guanine) crosslinks per cell observed after the alkaline elution assay can be expressed as follows:

$$D \cdot g \cdot R^{-1} \tag{2}$$

in which D = the number of DNA bases per cell (mol); g = the guanine mol proportion in DNA; R = the ratio of the DNA crosslinks per platinum atom observed after the alkaline elution assay.

Therefore, the reactions can be expressed as follows:

$$\frac{dx_1}{dt} = -k_0 \frac{x_1}{V_1} + k_0 \frac{x_2 - p^{-1}y}{V_2}, \tag{3}$$

$$\frac{dx_2}{dt} = k_0 \frac{x_1}{V_1} - k_0 \frac{x_2 - p^{-1}y}{V_2}, \tag{4}$$

$$\frac{dy}{dt} = k_1 \left(x_2 - p^{-1}y\right)^{p-1} \left(D \cdot g \cdot R^{-1} - y\right), \tag{5}$$

in which D = the number of DNA bases per cell (mol); g = the guanine mol proportion in DNA; k_0 = the drug's simple diffusion rate constant; k_1 = the DNA crosslink formation rate constant; k_2 = the crosslink-dependent cell death rate constant; R = the ratio of the DNA crosslinks per platinum atom observed after the alkaline elution assay; t = exposure time (h); V_1 = the volume (l) in the medium; V_2 = the volume (l) of a cell; p = the number of bound sites per platinum atom; x_1 = the drug dose (mol) in the medium; x_2 = the drug dose (mol) in a cell; and y = the number of DNA crosslinks per cell (mol). We used the following constants for a supposed human cell line: $D = 9.489 \times 10^{-15}$ (mol) [16], g = 0.20 [17], p = 2, which means all of the platinum bonds of each nedaplatin molecule bind with guanines, $V_1 = 1.5 \times 10^{-2}$ (l), $V_2 = 1.12 \times 10^{-12}$(l) [18].

We also assumed that the cytocidal effect of nedaplatin depends not on time but on the percentage of DNA crosslinks. This phenomenon can be expressed as follows:

$$\frac{dz}{dy} = -k_2 z, \tag{6}$$

in which z = the surviving fraction.

Kawanishi et al. [5] found that there was a threshold of DNA crosslinks created to induce the cytocidal effect. The threshold was determined to be 4.68% of DNA crosslinks by linear regression analysis. The surviving fraction was then solved as follows:

$$\begin{cases} z = z_0 \cdot e^{-k_2 \cdot \%\mathrm{DNAcrosslink}}, \{\%\mathrm{DNAcrosslink} \geq 0.468\} \\ z = 1, \{\%\mathrm{DNAcrosslink} < 0468\} \end{cases} \tag{7}$$

The calculated rate constants were obtained as follows:

$$k_0 = 1.8 \times 10^{-14}(\mathrm{lh}^{-1}),\ k_1 = 1.6 \times 10^{8}(\mathrm{lmol}^{-1/2}\mathrm{h}^{-1}),$$
$$k_2 = 5.45 \times 10^{1}(\mathrm{mol}^{-1}),\ z_0 = 1.285 \times 10^{1}.$$

The calculated results and observed experimental results are shown in Figs. 2, 3, 4, 5, 6, and 7. There were no significant differences between the calculated data and experimental data. This suggested that our mathematical model represents the experimental data. This model also indicates that the pharmacodynamic effect of nedaplatin is a non-linear function of both time and dose and that the cytocidal effect does not depend on the AUC value.

Discussion

We developed an in vitro mathematical model of nedaplatin. This model explains the results of experiments showing that nedaplatin acts as a non-linear agent in conditions that mimic an in vivo environment [5] based on clinical studies [11, 19]. The model also supports the clinical findings that consecutive low-dose administration of CDDP, which has been believed to have an AUC-dependent cytocidal effect, yields the best outcome [20, 21, 22, 23, 24].

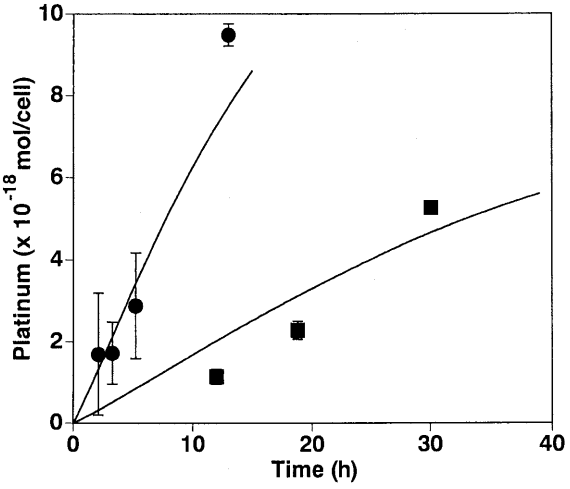

Fig. 2 The amount of platinum per cell as a function of time. Experimental results with high-dose (2.53×10^{-5} M, ●), and low-dose (4.39×10^{-6} M, ■) nedaplatin are statistically identical to the calculated results, which are shown by the *lines*. Values represent the mean ± SD. *Error bars* (SE) are shown where they are larger than the symbols

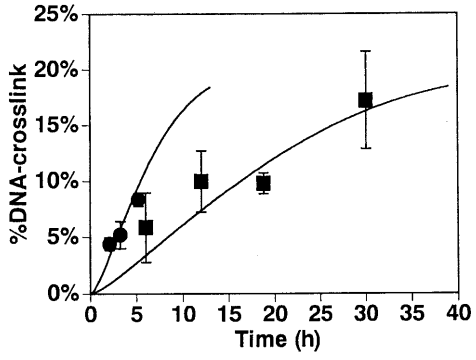

Fig. 3 The percentage of crosslinks per cell as a function of time. Experimental results with high-dose (2.53×10^{-5} M, ●), and low-dose (4.39×10^{-6} M, ■) nedaplatin are statistically identical to the calculated results, which are shown by the *lines*. Values represent the mean ± SE. *Error bars* (SE) are shown where they are larger than the symbols

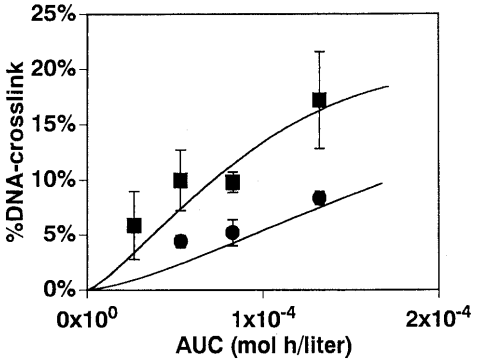

Fig. 4 The percentage of crosslinks per cell as a function of the value of the area under the time concentration curve (*AUC*). Experimental results with high-dose (2.53×10^{-5} M, ●) and low-dose (4.39×10^{-6} M, ■) nedaplatin are statistically identical to the calculated results, which are shown by the *lines*. Values represent the mean ± SE. *Error bars* (SE) are shown where they are larger than the symbols

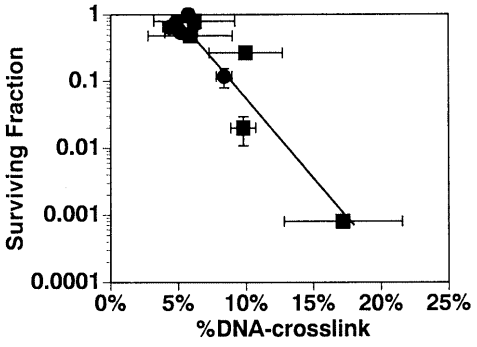

Fig. 5 The surviving fraction as a function of the value of the percentage crosslink. Experimental results with high-dose (2.53×10^{-5} M, ●) and low-dose (4.39×10^{-6} M, ■) nedaplatin are statistically identical to the calculated results, which are shown by the *lines*. The statistical results of the experimental data by least square regression analysis are $\log (Y') = 1.109\ (\pm 0.255) - 23.69\ (\pm 2.94) \times$, $r^2 = 0.89$, $P < 0.0001$. A threshold of the percentage crosslink to induce cell killing is observed, which allows investigation and improvement of the administration procedures used for patients. Values represent the mean ± SE. *Error bars* (SE) are shown where they are larger than the symbols

Ozawa et al. [1] developed a mathematical model to explain cell cycle non-specific agents, including platinum analogs. Their model tries to explain the cytocidal effect by using information about the extracellular action of the drug and the cell cycle, and the model requires the cell cycle to continue acting after the drug exposure. We believe that Ozawa's model applies only in an environment with an extremely low concentration exposure for a long time, which must be more than a few times the doubling time of the cells. In other words, the cell cycle has to continue during and for a long time after the drug exposure. However, cell cycle delay and phase blocks were often observed in vitro, and the model cannot explain apoptosis induced by p53 that results in a G_1 block. Our mathematical model, however, explains the experimental results based on clinical data.

One of the important differences between Ozawa's model and ours is the most-important independent variable for the cytocidal effect. In our model, the cytocidal effect of nedaplatin is considered to be a function not of the cell cycle but of the number of DNA crosslinks. We believe that cell cycle delay, phase block, and the cytocidal effect are induced by DNA crosslinks. Because cell death is the ultimate goal of anticancer agents, the concept of the cell cycle does not have to be incorporated into the mathematical model. The quantitative relationship between the cell cycle and the number of DNA crosslinks and the relationship between the cell cycle and the cytocidal effect are now under investigation.

Another difference between Ozawa's model and ours concerns the degradation of drugs. Our model explains

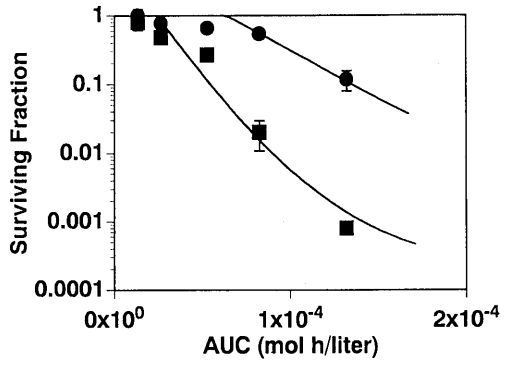

Fig. 6 The experimental data with high-dose (2.53×10^{-5} M, ●) and low-dose (4.39×10^{-6} M, ■) nedaplatin demonstrate that the surviving fraction is a non-linear function of the value of the AUC [5], although the surviving fraction of cell cycle non-specific anticancer agents such as nedaplatin have been thought to depend on the AUC value regardless of the exposure procedure used. The calculated results of the mathematical model, which are shown by *lines*, are identical to the experimental results statistically. Values represent the mean ± SE. *Error bars* (SE) are shown where they are larger than the symbols

the degradation phenomenon easily with the first equation by calculating the drug in medium as a function of time; for example, if the logarithm of the degradation is supposed to be a linear phenomenon, the drug dose in medium as a function of time can be expressed as follows: $x_1 = e^{-at+b}$, in which x_1, a, b, and t are the drug dose in medium, the degradation rate constant, the intercept, and time, respectively. The degradation of drugs such as CDDP seems to be a relatively slow phenomenon compared with cell doubling time [1, 2]. Furthermore, the degradation constant of nedaplatin was 1/20 times lower than that of CDDP (T. Totani, Y. Adachi, K. Aono, unpublished data). For those reasons, we did not think it mandatory to modify our model. Such a modification may be necessary for other anticancer agents.

The cytocidal and other pharmacodynamic effects of platinum agents, such as DNA crosslink formation, have been said to depend on the AUC value. Experimental results and our mathematical model, however, demonstrated that the cytocidal effect of nedaplatin is a function not of the AUC value but of the number of DNA crosslinks. The AUC dependency of cisplatin is supported by Ozawa's model theoretically and by many published experiments. Figure 8 shows that our model can also explain the AUC dependency if a simple diffusion rate constant (k_0) and a DNA crosslink formation rate (k_1) constant are proposed to be lower and higher, respectively. This calculated result indicates that the AUC-dependent cytocidal effect observed with cisplatin may also be explained by our model. The DNA crosslink formation of cisplatin has been reported to be 20 times faster than that of nedaplatin (T. Totani, Y. Adachi, K. Aono, unpublished data). This faster reaction of cisplatin with guanine residues seemed to result in pseudo-AUC dependency on DNA crosslink formation. Therefore, the cytocidal effect of cisplatin appeared to depend on the AUC value. We believe that the mathematical model in this study can be applied to other platinum agents, with some modifications.

The cytocidal effect as a function of time or dose for cell cycle phase non-specific agents does not increase if time or dose increase beyond a certain value (Fig. 9). This phenomenon has been demonstrated in many published papers [1, 2, 4, 16, 25, 26, 27, 28, 29, 30].

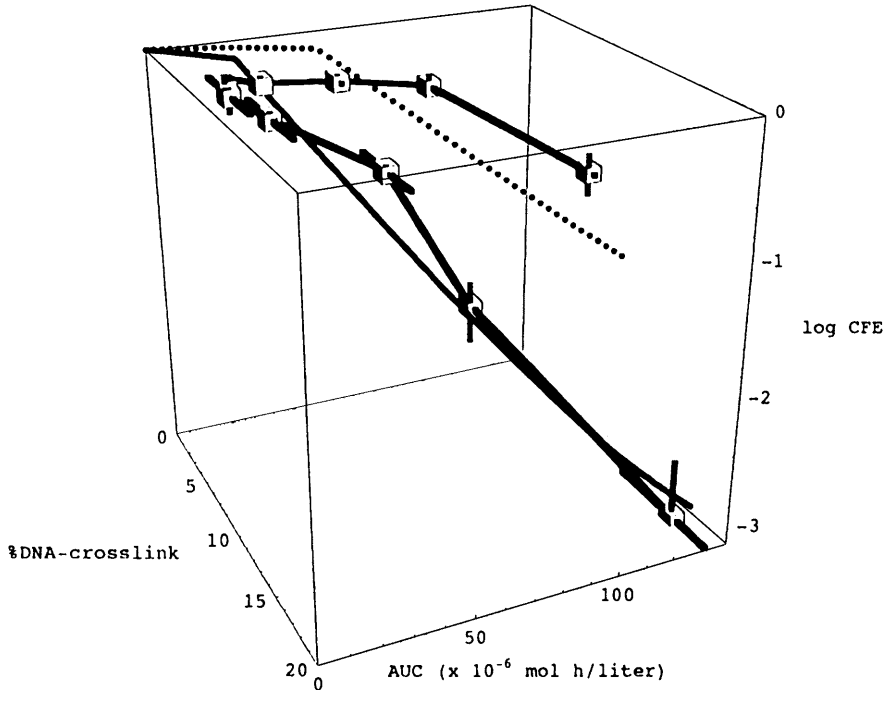

Fig. 7 Three-dimensional view of the reaction to nedaplatin. The experimental data of the high and low concentrations are shown by the cubes and are connected. Values represent the mean ± SE. *Error bars* (SE) in the three-dimensional space are shown where they are larger than the cubic symbols. The calculated results are shown by the *dotted line* (high concentration) and the *solid line* (low concentration). The mathematical model fits the experimental data. Whenever possible, the pharmacodynamic phenomena of anticancer drugs should be considered in a three-dimensional space, because pharmacodynamic effects, such as the cytocidal effect, are a function of, at least, the concentration and time

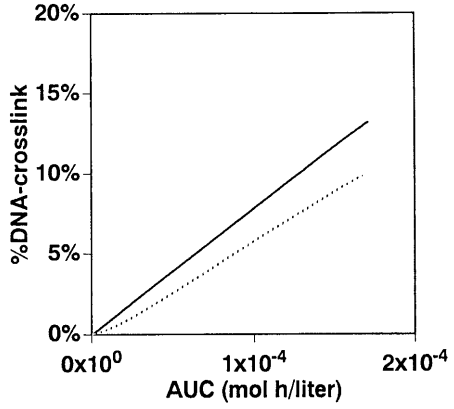

Fig. 8 The statistical AUC dependency of DNA crosslink formation using our mathematical model by modifying constant numbers. The DNA crosslink formation rate constant ($k_1 = 2.25 \times 10^8$) is higher, and the simple diffusion rate constant ($k_0 = 9.2 \times 10^{-15}$) is lower than those shown in Fig. 4. The calculated results of high-dose (2.53×10^{-5} M) and low-dose (4.39×10^{-6} M) conditions are shown by the *dotted* and *solid lines*, respectively. This mathematical model can be applied to other platinum analogs by modifying the constant numbers

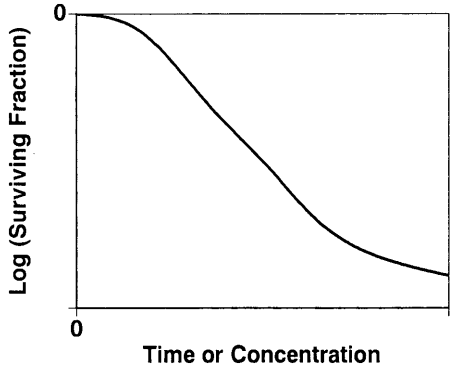

Fig. 9 The cytocidal effect of some kinds of drugs as a function of time and dose. The cytocidal effect is not increased as it was at the high values. This phenomenon has been reported often [1, 2, 4, 16, 25, 26, 27, 28, 29]. If there is a limited number of drug targets in the cells, this phenomenon is observed. This phenomenon cannot be explained by the current model [1, 2] but is explained by our model

Ozawa's model can only explain the time cytocidal effect through the degradation theory. Thus, their model cannot be applied to the dose cytocidal phenomenon. Our model, however, explains both phenomena at the same time with simulation because the number of DNA crosslinks determines the cytocidal effect. Our model suggests that, if most of the drug targets, such as guanines, for platinum analogs are occupied by drugs, the cytocidal effect does not increase.

Our model requires several constants, such as the DNA crosslink number per platinum molecule observed with the alkaline elution assay or the volume of a cell or the number of DNA bases per cell. These constants are determined in each laboratory and cell line. Therefore, the calculated data cannot be applied to clinical usage immediately. For example, the ratio of the DNA crosslink number per platinum molecule in this study was 2480.56, while that of another study was 631.39 [16]. Although these differences or experimental errors may still exist, the non-linearity of the pharmacodynamics of nedaplatin allow investigation of the optimal drug administration with regard to a threshold of cytocidal effect. Our model showed that the low-concentration, long exposure was superior to the high-concentration, short exposure. Moreover, our model suggests that there might be a better method of administering the drug, although the AUC values are the same. Increasing the number of DNA crosslinks at a defined AUC value is important to estimate the cytocidal effect and find a better means of administration. Therefore, we will incorporate the drug concentration change in vivo into our model despite the constants in order to simulate the pharmacokinetics/pharmacodynamics in vivo on a computer. Thus, a better administration method in vivo, including varying the drug infusion speed as well as using a conventional constant-speed infusion, should be investigated. Because platinum analogs have been believed to be AUC dependent, oncologists have paid attention only to the dose intensity. Because nedaplatin seems to be a non-linear agent, clinical studies must be performed to investigate a more-effective administration method. Our model may provide oncologists with useful information for more-appropriate protocols for clinical studies.

We developed a mathematical model to describe the non-linear pharmacodynamic effect of nedaplatin in vitro. This model may lead to a novel drug infusion procedure for cancer patients.

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