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# Physicochemical factors for cytotoxic activity in platinum dinuclear complexes with pyrimidine and imide ligands

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**Abstract**

The relationship between cytotoxic activity of platinum dinuclear complexes toward cancer cells and their intrinsic properties (electrophilicity and hydrophobicity) have been examined. It is shown that the reactivity with chloride anion is a good index of the electrophilicity of the platinum complexes. By assuming a cell kill pharmacodynamic model, the relation between $IC_{50}$ value and the electrophilicity is obtained. It is concluded that inside cells the reactivity of the platinum complexes having imide ligands is higher than that of CDDP. The other important factor which affects the cytotoxic activity is hydrophobicity. The log $k'$ values ($k'$: capacity factor) are found useful to estimate the hydrophobicity of platinum complexes. The accumulation of platinum into cells is dominated by the hydrophobicity and the charge of platinum complexes. Highly hydrophobic complexes are thought to be adsorbed in cell membranes, resulting in low cytotoxic activity since they cannot reach DNA. A schematic model of the interaction between platinum complexes and serum proteins reveals that more hydrophobic complexes tend to bind to serum proteins more stably. At least three possible paths of the cellular platinum accumulation are suggested: direct accumulation of the platinum complexes, incorporation in the form of CDDP produced from the complexes, and incorporation through protein–platinum complexes, although the contribution of the third one may be small.


*Keywords:* Electrophilicity; Hydrophobicity; Effect of serum protein

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## 1. Introduction

The well-known anticancer platinum complex, CDDP [cisplatin, *cis*-diamminedichloroplatinum(II)] [1], is still an incomparably important substance for clinical use. Despite a great deal of exploration to find better activity and less toxicity [2], none with validity superior to CDDP have been discovered. Recently we showed that a head-to-head (HH) platinum dinuclear complex with 1-methylthymine ligand was active against Sarcoma 180 cell lines, while a head-to-tail (HT) derivative with 1-ethylthymine ligand was inactive [3]. We also found that HH complexes with imide ligands gave stronger cytotoxic activity [4,5]. It was, therefore, challenging to focus on controlling factors affecting the cytotoxic activity in the design and development of more advantageous complexes. Two physicochemical factors are considered here, electrophilicity (chemical reactivity) and hydrophobicity, which are generally thought to be the fundamental parameters of drugs [6]. These two factors are explained below, (Section 1.1 and Section 1.2), followed by closely related phenomena (Section 1.3 and Section 1.4).

### *1.1. Electrophilicity*

There are many kinds of nucleophiles for platinum complexes in vivo. Chloride anion is one of the most important of these, and CDDP alters the number of chloride ligands inside and outside of cells [7]. Since the concentration of chloride anion is much lower inside the cell (4 mM inside vs. 103 mM outside), CDDP releases its chloride ligands in the cell. Proteins and water are also significant candidates for alternative nucleophiles in vivo. We therefore focused on ligand exchange reactions (viz. electrophilicity) of the platinum complexes. Imide analogues appeared suitable for use in investigating the electronic effect of a free carbonyl group not engaged in the coordination, since a platinum complex with $\alpha$-pyrrolidone ligands is known to be inactive toward cancer cells [8]. The coordinating ability of nitrogen and oxygen atoms is probably changed by electron-withdrawing effect and/or resonance effect of the free carbonyl group.

### *1.2. Hydrophobicity*

This property of biologically active compounds is certainly one of the important features as is the chemical reactivity [6]. Since CDDP is believed to react with DNA [9], it must pass through cell membranes, which is thought to be closely related to the hydrophobicity of platinum complexes.

### *1.3. Accumulation of platinum in the cells*

The observed amount of platinum is composed of the species located inside cells and those adsorbed in cell membranes. It is reasonable to consider that this quantity is primarily dominated by the hydrophobicity and charge of platinum complexes.

### *1.4. Binding with serum proteins*

Platinum complexes are assumed to interact with proteins in serum, so their effect is also examined in vitro using bovine serum albumin (BSA) and fetal bovine serum (FCS) containing various proteins.

In the present study, the series of platinum complexes with 1-alkyluracil, 1-alkylthymine, and cyclic imide derivatives were chosen as ligands, as these were found to have cytotoxic activity toward cancer cells in previous studies [3–5].

## 2. Materials and methods

### *2.1. Reagents*

All reagents, unless otherwise noted, were purchased from the commercial suppliers specified in parentheses and used without further purification. *cis*-Diamminedichloroplatinum(II) (CDDP; Sigma), fetal bovine serum (FCS; Sankou Junyaku), bovine serum albumin (BSA, Fraction V, protease free; MILES), MEM medium (Nissui, Japan), non-essential amino acid (Gibco). BCA protein assay reagent was a product of Pierce, and all other reagents were of the highest grade commercially available.

### 2.2. Platinum complexes

The following platinum complexes were synthesized previously [3–5]. The structures of the dinuclear complexes and the ligands are shown in Scheme 1.

#### 2.2.1. Dinuclear Pt(II) complexes with 1-alkyluracil ligands (1–5)

$[Pt_2(MeUra)_2(NH_3)_4](NO_3)_2$ (HH) (**1**), $[Pt_2(EtUra)_2(NH_3)_4](NO_3)_2$ (HH) (**2**), $[Pt_2(n\text{-}BuUra)_2(NH_3)_4](NO_3)_2$ (HH) (**3**), $[Pt_2(BzlUra)_2(NH_3)_4](NO_3)_2$ (HH) (**4**), $[Pt_2(NaphCH_2Ura)_2(NH_3)_4](NO_3)_2$ (HH) (**5**).

#### 2.2.2. Dinuclear Pt(II) complexes with 1-alkylthymine ligands (6–10)

$[Pt_2(MeThy)_2(NH_3)_4](NO_3)_2$ (HH) (**6**), $[Pt_2(MeThy)_2(NH_3)_4](NO_3)_2 \cdot H_2O$ (HT) (**7**), $[Pt_2(EtThy)_2(NH_3)_4](NO_3)_2 \cdot 2H_2O$ (HT) (**8**), $[Pt_2(n\text{-}PrThy)_2(NH_3)_4](NO_3)_2$ (HH) (**9**), $[Pt_2(n\text{-}PrThy)_2(NH_3)_4](NO_3)_2 \cdot 2H_2O$ (HT) (**10**).

#### 2.2.3. Dinuclear Pt(II) complexes with imide ligands (11–14)

$[Pt_2(SI)_2(NH_3)_4](NO_3)_2 \cdot H_2O$ (HH) (**11**), $[Pt_2(DMGI)_2(NH_3)_4](NO_3)_2 \cdot H_2O$ (HH) (**12**) [22], $[Pt_2(DMGI)_2(NH_3)_4](NO_3)_2$ (HT) (**13**) [10], $[Pt_2(EMGI)_2(NH_3)_4](NO_3)_2$ (HH) (**14**).

#### 2.2.4. Mononuclear Pt(II) complexes of SI (15 and 16)

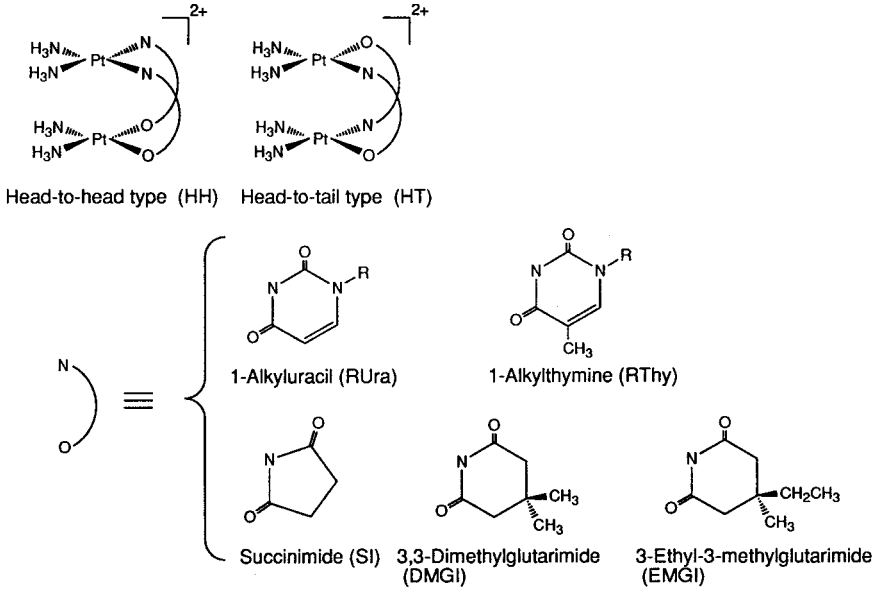
*cis*-$[Pt(SI)_2(NH_3)_2] \cdot 2H_2O$ (**15**), *cis*-$[PtCl(SI)(NH_3)_2]$ (**16**).

#### 2.2.5. Mononuclear Pt(II) complex of 1-alkyluracil ligand (17)

*cis*-$[Pt(MeUra)_2(NH_3)_2]$ (**17**).

### 2.3. Instruments

UV-VIS spectra were recorded on a Hitachi U-3200 or a Shimadzu MPS-2000 spectrophotometer. Capacity factor ($k'$) of Pt complexes was calculated from the retention time measured by HPLC (LC-6A, Shimadzu). The CAChe log $P$ values of ligands were calculated on a desk top computer (Macintosh Quadra 800, Apple Computer) using the physical properties calculated by AM1 (MOPAC ver. 6 in CAChe system, Sony Tektronix). Quantitative analysis of platinum was carried out with a Zeeman atomic absorption




Scheme 1.

spectrometer (SpectrAA-400, Varian). Cytotoxic activity was evaluated by counting the cell number directly by a Coulter Counter equipped with a Channelyzer (Coulter Electronics) or by MTT method [11].

### 2.4. Assessment of electrophilicity

The CDDP formation in the reaction between platinum complexes and chloride anions was used as an index of electrophilicity of platinum complexes. Platinum complexes (1 $\mu$mol) were completely dissolved in saline (0.15 M, 1 ml), and kept at constant temperature. Twenty $\mu$l of each solution were then chromatographed on HPLC (ODS column: Unisil Pack 250B, $6.0 \times 250$ mm, GL Sciences Inc.; eluant: $H_2O$, flow rate: 0.8 ml/min, detector: $\lambda = 230$ nm). The yield of CDDP was determined from the peak area (retention time: 6.5 min) as a function of time.

### 2.5. Assessment of hydrophobicity

Capacity factor defined as $k' = (t_R - t_0)/t_0$ was used to estimate hydrophobicity of the platinum complexes, where $t_R$ and $t_0$ were, respectively, the retention times of a platinum complex and a standard substance (KI) in HPLC (ODS column: Unisil NQ C18, $5.0 \times 250$ mm, GL Sciences Inc.; eluant: $CH_3CN$:$HNO_3$ (1 mM) = 45:55; flow rate: 0.8 ml/min; detector: $\lambda = 230$ nm). Each sample was dissolved in water (ca. 0.1 mg/ml) and 20 $\mu$l of the solution were injected. The CAChe log $P$ value of ligands was calculated as follows. Ligand structures were first optimized by MM2 method (CAChe ver. 3.6), then further optimized by AM1 method, and the heat of formation in vacuo ($HOF_{vac}$) and in water ($HOF_{water}$) and the solvent accessible surface area (SAS) were calculated. The solvent effect of water was estimated by a conductor-like screening model (COSMO) with default values of a dielectric constant (78.4) and an effective radius (1.00 Å) in water. The CAChe log $P$ was then calculated from the above physical quantities using the following relation:

$$\begin{aligned} \text{CAChelog}P = & -0.33263 + 0.048959\ \text{SAS} \\ & -0.027225\ \text{HOF}_{vac} \\ & +0.03116\ \text{HOF}_{water} \\ & -1.4003\sqrt{\text{N}} - 1.0241\sqrt{\text{O}} \end{aligned} \quad (1)$$

where N and O were the numbers of nitrogen and oxygen atoms.

### 2.6. Platinum accumulation into cancer cells

Experiments on cellular platinum accumulation were carried out with mouse Sarcoma 180 cells employing the method of Mistry et al. [12]. Cells were cultured in MEM medium containing 5% FCS, 20 mM HEPES, 1% non-essential amino acid. The cells ($3-4 \times 10^6$/well) in an exponential growth phase were exposed to a single concentration (100 $\mu$M) of a platinum complex with various incubation times (1–3 h) at 37°C in 5% $CO_2$. Immediately after exposure, the medium was aspirated, and the cells were washed with PBS (3.5 ml + 1.5 ml), harvested in PBS (0.5 ml) and sonicated for 30 min at 4°C. To determine protein content, the sonicated cells (50 $\mu$l) were incubated with 1 N sodium hydroxide (200 $\mu$l) overnight at room temperature before analysis. Protein concentration was determined using BCA protein assay reagent [13]. Cellular platinum concentrations were measured directly from the sonicated cells by a Zeeman atomic absorption spectrometer using the internal additions method and platinum standard solutions in 0.2% $HNO_3$. The platinum concentrations were expressed as nmol/mg protein.

### 2.7. Interaction between platinum complex and serum proteins

Interactions of the platinum complexes with FCS including several proteins (BSA (17 g/l), globulin (16 g/l), etc.) were examined. Pure BSA and proteins in FCS solution were used as serum proteins. The complex (1 $\mu$mol) was dissolved in 2 ml of FCS/saline solution (the concentration was varied in the range of 0.0125–0.2 (v/v)) and reacted at 37°C. CDDP formation was then fol-

lowed by HPLC as described above. In the case of BSA, after mixing 0.4 ml of a platinum complex solution (0.20 mM in water) and 0.4 ml of a BSA solution (0.025–0.20 mM in 0.30 M aq. NaCl), the mixture was immediately incubated at 37°C. The CDDP formation was then measured in the same manner.

### 2.8. Assessment of cytotoxic activity

The cells ($1 \times 10^5$/well) were exposed to various concentrations of the complexes for 2 days (37°C, 5% $CO_2$) in a 24-well plate (Costar). Culture medium was used as a negative control and mitomycin C (MMC, 200 $\mu$g/ml) as a positive control. Following the exposure the medium was removed and trypsin-EDTA (200 $\mu$l) was added to each well. After confirming detachment of cells from the bottom of the well, culture medium (700 $\mu$l) was added. The cell suspension was then transferred to a test tube containing saline (4 ml) for counting by a Coulter Channelyzer. A MTT assay method was also employed to evaluate the activity.

## 3. Results

### 3.1. Electrophilicity of complexes

A time course of CDDP formation from various platinum complexes of HH dinuclear complexes with 1-alkyluracil ligands (**1**, **3**, **5**) in saline at 25°C is illustrated in Fig. 1. The observed rate constants ($k_{obs} = k[Cl^-]^2$, $[Cl^-] = 0.15$ M) of **1**, **3** and **5** are almost the same (ca. 0.1 $h^{-1}$ at 25°C), which indicates that the modification of 1-alkyluracil ligands does not influence the electrophilicity of the complexes to a great extent. The time course from HH dinuclear complexes with imide ligands (**11**, **12**, **14**) in saline at 25°C and 37°C is shown in Fig. 2. The $k_{obs}$ values for **11**, **12** and **14** were almost the same (0.9–1.3 $h^{-1}$ at 25°C and 4.3–4.7 $h^{-1}$ at 37°C) but were much larger than those of **1**, **3** and **5**. Interestingly, there was a difference in the final CDDP yield among HH dinuclear complexes having the imide ligands. That is, although the final yields from **12** and **14** were almost quantitative, that of **11** remained 80%. This suggests that **11** gives not only CDDP but other by-products. In fact, one candidate was separated by HPLC and identified as *cis*-[PtCl(SI)$(NH_3)_2$] (**16**) by elemental analysis. A time course of the formation of **15**, **16** and CDDP is shown in Fig. 3; the final yield of **16** was 40% and those of CDDP and **15** were 80%.

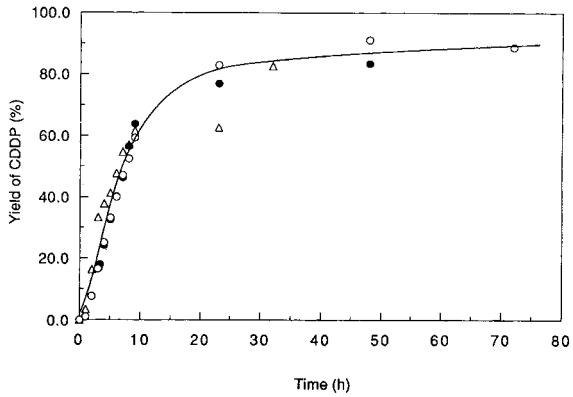



Fig. 1. Time course of CDDP formation from platinum dinuclear complexes with 1-alkyluracil ligands in an aqueous NaCl solution ([NaCl] = 0.15 M) at 25°C; [**1**] = [**3**] = $1.0 \times 10^{-3}$ M, [**5**] = $5.0 \times 10^{-4}$ M; (○) **1**, (●) **3**, (△) **5** (the data of **1** were cited from [3]).

### 3.2. Hydrophobicity of complexes

Hydrophilic drugs are easy to administer orally in clinical use, but little of them passes through

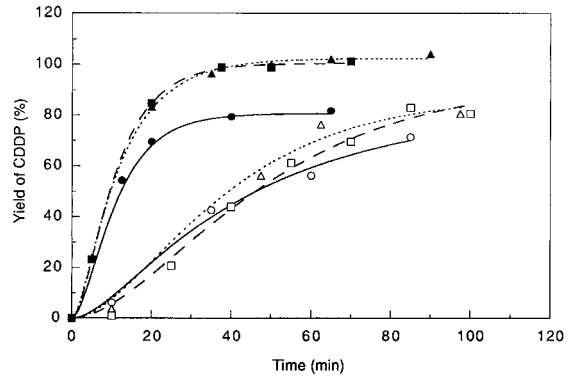



Fig. 2. Time course of CDDP formation from platinum dinuclear complexes with imide ligands in an aqueous NaCl solution ([NaCl] = 0.15 M); [**11**] = [**12**] = [**14**] = $1.0 \times 10^{-3}$ M; (○) **11**, 25°C, (●) **11**, 37°C, (△) **12**, 25°C, (▲) **12**, 37°C, (□) **14**, 25°C, (■) **14**, 37°C.

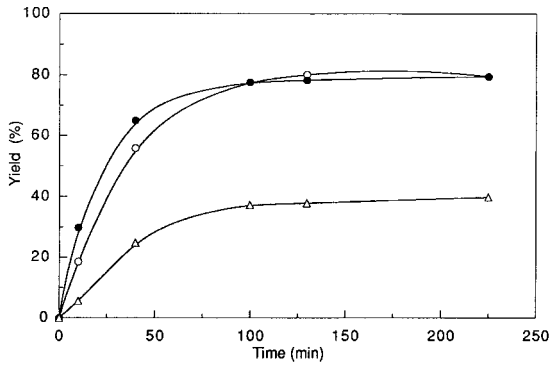



Fig. 3. Time course of the formation of CDDP (○), **15** (●) and **16** (△) from **11** in an aqueous NaCl solution ([NaCl] = 0.15 M) at 25°C; [**11**] = $1.0 \times 10^{-3}$ M.

the hydrophobic cell membrane. On the contrary, if the hydrophobicity of a drug rises above a certain level, it is not only difficult to use clinically but is also inactivated by interaction with hydrophobic species and cell membranes in a biological condition. Thus, in principle, drugs should have an optimum hydrophobicity for their general activity [6]. The log $P$ value has been most commonly used as an index of hydrophobicity. In the traditional measurement of log $P$, the conventional shaking-flask method has been employed, but this is very laborious and time-consuming. A convenient alternative method is to measure capacity factor ($k'$) by HPLC and evaluate the hydrophobic property [14]. Table 1 summarizes log $k'$ of the platinum complexes and CAChe log $P$ of the ligands together with the observed retention time ($t_R$) and the other physical quantities used for the calculation ($HOF_{vac}$, $HOF_{water}$, SAS). Notably, a linear relationship exists between calculated CAChe log $P$ and experimentally obtained log $k'$ (Fig. 4). For example, Eq. (2) holds for the HH platinum complexes with 1-alkyluracil ligand series.

$$\log k' = 0.19045 + 0.43159\ \text{CAChe log}\ P. \qquad (2)$$

It is apparent that the modification of the alkyl groups of the ligands contributed regularly to the overall hydrophobicity of the corresponding complexes.

### 3.3. Accumulation of platinum to cancer cells

Drug accumulation in cancer cells is an important property for an antitumor agent, since lower

Table 1
Observed log $k'$ of platinum complexes, calculated CAChe log $P$ of ligands, and other physical quantities necessary for the calculation

| Complex | Retention time ($t_R$)[a] (min) | log $k'$[b] | $HOF_{vacuo}$[c] (kcal $mol^{-1}$) | $HOF_{water}$[c] (kcal $mol^{-1}$) | SAS[d] ($Å^2$) | CAChe log $P$ |
|---|---|---|---|---|---|---|
| **1** | 7.10 | −0.29 | −48.26 | −68.91 | 70.77 | −1.127 |
| **2** | 8.17 | −0.13 | −54.50 | −74.79 | 77.84 | −0.794 |
| **3** | 11.30 | 0.15 | −68.19 | −88.30 | 93.09 | −0.095 |
| **4** | 14.63 | 0.33 | −21.49 | −42.78 | 102.18 | 0.495 |
| **5** | 35.0[e] | 0.81 | −0.962 | −23.27 | 118.23 | 1.329 |
| **6** | 7.18 | −0.29 | −55.51 | −74.83 | 77.11 | −0.803 |
| **7** | 7.86 | −0.18 | −55.51 | −74.83 | 77.11 | −0.803 |
| **8** | 9.43 | −0.01 | −61.70 | −80.67 | 83.99 | −0.479 |
| **9** | 9.27 | −0.02 | −68.59 | −87.36 | 91.26 | −0.144 |
| **10** | 11.58 | 0.16 | −68.59 | −87.36 | 91.26 | −0.144 |
| **11** | 7.06 | −0.31 | −77.37 | −96.12 | 61.20 | −1.070 |
| **12** | 8.77 | −0.07 | −97.27 | −115.32 | 75.92 | −0.405 |
| **14** | 11.30 | 0.14 | −102.17 | −120.20 | 81.73 | −0.138 |

[a] Retention time of a standard substance KI ($t_0$) was measued in each experiment ($t_0$ was located in the range 4.74–4.76 min).
[b] $k' = (t_R - t_0)/t_0$; $k'$ values for **1**–**5**, **11**, **12** and **14** were cited from [5].
[c] Heats of formation of ligands in vacuo and in water.
[d] Solvent accessible surface area.
[e] Peak was broad.

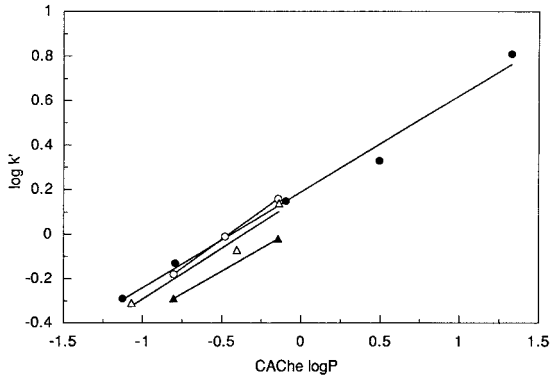



Fig. 4. Relation between log $k'$ and CAChe log $P$; (●) 1-alkyluracil ligands, (▲) 1-alkylthymine ligands (HH), (○) 1-alkylthymine ligands (HT), (△) imide ligands.

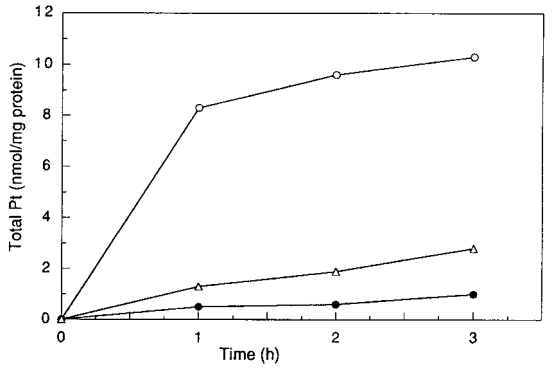



Fig. 5. Accumulation of platinum to Sarcoma 180 cells at 37°C; (△) CDDP, (●) **1**, (○) **5**.

drug accumulation caused by reduced uptake has been reported to be responsible for acquired resistance to CDDP in several cell lines [15]. The precise mechanism by which CDDP enters cells is unclear, but both passive diffusion and carrier mediated mechanisms have been implicated [16]. Thus a full analysis of factors affecting cytotoxic activity should take into account the relative uptake of various platinum complexes to cells [12]. A time course of cellular accumulation of typical 1-alkyluracil platinum complexes (**1** and **5**) and CDDP is given in Fig. 5, where the accumulation amount reflects their hydrophobicity and charge; the accumulation of hydrophobic complex **5** was three times higher than CDDP and 10 times higher than **1**. It was also found that mononuclear complex **17** was accumulated to much less a degree than **1** (not shown in Fig. 5).

### *3.4. Effect of serum proteins*

The interaction of the platinum complexes with serum proteins seems important because of their high concentrations in serum [17]. In fact, serum proteins are known to interact with CDDP [18], while it is unclear whether certain serum protein–platinum complexes are therapeutically effective and contribute to the cytotoxic activity. The CDDP–BSA complexes do not significantly dissociate during dialysis experiments [17,19]. We expected that this interaction might be strengthened by the higher hydrophobic property of dinuclear complexes. Thus we sought to learn whether proteins in the culture medium interact with HH dinuclear complexes of 1-alkyluracil derivatives (**2** and **5**) in an FCS-saline solution whose concentration was varied in a similar range as that of the culturing medium. The CDDP formation from **2** and **3** in saline with and without FCS was observed, and the time-yield curve fitted the first order rate equation. The amount of CDDP produced after 8 h in an FCS-saline solution was reduced to 85% of that in saline alone, which indicates that the interaction between dinuclear complexes (**2** and **3**) and the proteins in FCS leads to the suppression of CDDP formation. Fig. 6a and b show the relationship between the concentration of serum proteins and the yield of CDDP after 8 h reaction of **2** and **5**. In both cases the CDDP yield decreases with increase in protein concentration.

## 4. Discussion

If aquated complexes of platinum compounds are truly active species against cancer cells, the ligands of a complex must leave the metal center. Dissociation of ligands in saline is accompanied by the stepwise exchange of water and/or $Cl^-$ [20]. If both ligands are substituted by water, the dinuclear complex should produce an aquated complex. This complex would then be exchanged

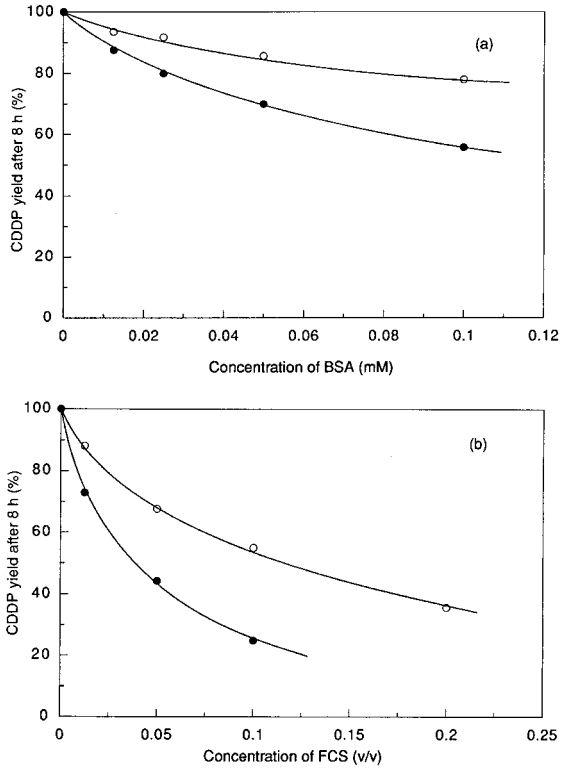



Fig. 6. Effect of serum proteins on the yield of CDDP after 8 h reaction of **2** (○) and **5** (●) in an aqueous NaCl solution ([NaCl] = 0.15 M) at 37°C; (a) BSA, (b) FCS.

stepwise by $Cl^-$, resulting in CDDP. The equilibrium between aquated complex and CDDP can be shown as follows:

$$\text{cis-}[Pt(NH_3)_2(OH_2)_2]^{2+} + Cl^- \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \text{cis-}[PtCl(NH_3)_2(OH_2)]^+$$

$$\text{cis-}[PtCl(NH_3)_2(OH_2)] + Cl^- \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} \text{cis-}[PtCl_2(NH_3)_2]$$

The following rate constants ($k_{+1}$, $k_{-1}$, $k_{+2}$, $k_{-2}$) and equilibrium constants ($K_a$, $K_b$) were reported by Miller and House [21]: $k_{+1} = 9.09 \times 10^{-2}$ $M^{-1}$ $s^{-1}$, $k_{-1} = 2.5 \times 10^{-5}$ $s^{-1}$, $K_a = k_{+1}/k_{-1} = 3.6 \times 10^3$ $M^{-1}$; $k_{+2} = 6.26 \times 10^{-3}$ $M^{-1}$ $s^{-1}$, $k_{-2} = 6.32 \times 10^{-5}$ $s^{-1}$, $K_b = k_{+2}/k_{-2} = 9.91 \times 10$ $M^{-1}$ at 25°C ($\mu = 0.1$ M, $NaClO_4$) in an acidic condition. In our examination, chloride anion (0.15 M, $[Cl^-] \gg$ [platinum complex]) was selected as one of the nucleophiles for HH dinuclear complexes in water. Since total equilibrium constant, $K = K_a \times K_b = 3.6 \times 10^5$ $M^{-2}$, suggests that the equilibrium is almost inclined to CDDP under the present conditions, the rate of reverse reaction to the original dinuclear complex seems much slower than that of the CDDP formation [22]. The rate of CDDP generation can therefore be used as an index of the electrophilicity of the complexes.

Contrary to **12** and **14**, **11** did not give CDDP quantitatively at 37°C (Fig. 2), due to the formation of by-product **16**. To produce **16**, a scission must occur at the Pt–N coordination site of **15**. The isomerization of HH to HT forms was reported by $^{195}Pt$ NMR measurements in dinuclear complex with glutarimide ligands (GI) [22]. The HT dinuclear complex then gave $[Pt(GI)(NH_3)_2(OH_2)]^+$ in nucleophilic attack by water (path D). It was reported that $[Pt(GI)(NH_3)_2(OH_2)]^+$ was also formed from the diaqua complex and a mononuclear complex, $[Pt(GI)_2(NH_3)_2]$, through another path (path E). Therefore, it is very likely that **11** may produce **16** through a similar path. For **12**, however, the $^{195}Pt$ NMR experiment gave no indication of HT form (**13**) in water, probably because of the bulkiness of two methyl groups of the ligand [22]. The same logic may be applicable to **14**.

It was reported earlier that the active complexes (low $IC_{50}$) produce CDDP in saline but the inactive complexes (large $IC_{50}$) do not [3]. This means that all HH type complexes were active and all the HT type ones were inactive. The 1-alkylthymine ligand complexes (**6** and **9**) were less active than the 1-alkyluracil or imide series (**1**–**5** or **12**, **14**) [3,5]. Imide complexes (**12**, **14**) showed the highest activity among the three groups, the order of cytotoxic activity as a whole being imide > uracil > thymine complexes. The dominating factor to be considered first is the electrophilicity. To understand the relation between electrophilicity and cytotoxic activity, we proposed a possible reaction scheme based on a basic pharmacodynamic model of chemotherapeutic effects [23]. The cell kill pharmacodynamic

model of platinum dinuclear complexes is given in Scheme 2. Here, the meanings of the parameters are as follows: $C_1$, $C_2$, concentrations of the complexes in the medium; $C_{e1}$, $C_{e2}$, intracellular concentrations of the complexes; $k_{obs}$, pseudo-first-order rate constant for the formation of CDDP; $C_m$, total concentration of the complexes in the medium; $k_{d1}$, $k_{d2}$, rate constants of drug-induced irreversible cell death; $K_1$, $K_2$, equilibrium constants between intra- and extra-cellular concentrations; $k_s$, rate constant of cell proliferation; $k_r$, rate constant of physiological degradation of cells. This model obeys the following assumptions: $C_m$, $C_{e1}$ and $C_{e2}$ rapidly reach equilibrium; $C_m$ is constant during the experiment; conversion of dinuclear complex to CDDP is the pseudo-first-order reaction.

Eq. (3) is derived from the above scheme,

$$\begin{aligned} dC_s/dt &= k_s C_s - k_r C_s - k_{d1} C_s C_{e1} - k_{d2} C_s C_{e2} \\ &= [k_s - k_r - k_{d1} K_1 C_m \exp(-k_{obs} t) \\ &\quad - k_{d2} K_2 C_m \{1 - \exp(-k_{obs} t)\}] C_s \end{aligned} \tag{3}$$

where $C_s$ is the cell density. Solving Eq. (3), we obtain

$$\begin{aligned} \ln(C_s/C_{s0}) &= (k_s - k_r - k_{d2} K_2 C_m) t \\ &\quad + C_m (k_{d2} K_2 - K_{d1} K_1) \\ &\quad \times \{1 - \exp(-k_{obs} t)\}/k_{obs} \end{aligned} \tag{4}$$

where $C_{s0}$ is the cell density at $t = 0$. In the control experiment where the platinum complexes are absent,

$$\ln(C_s'/C_{s0}) = (k_s - k_r) t \tag{5}$$

where $C_s'$ is the cell density in the control experiment. Eq. (4) and Eq. (5) lead to

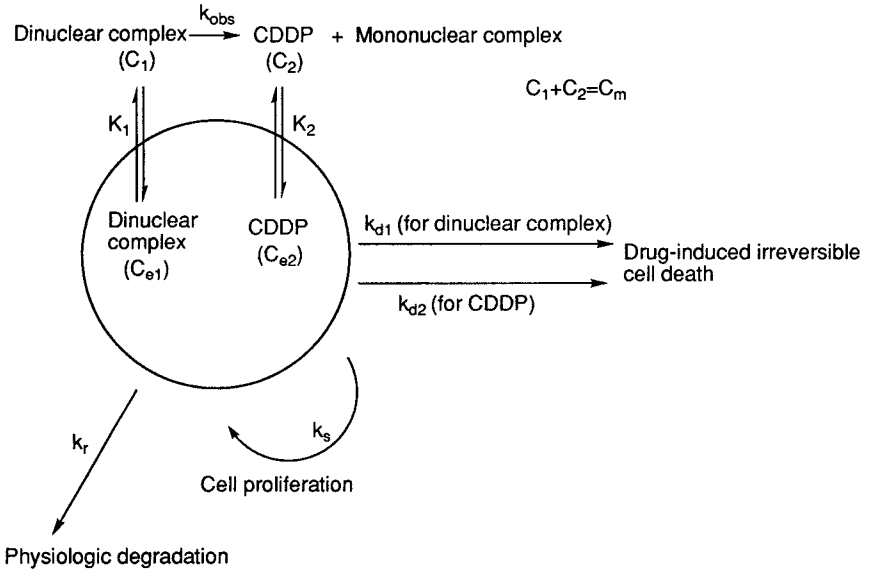
$$\begin{aligned} \ln(C_s/C_s') &= [-k_{d2} K_2 t + (k_{d2} K_2 - k_{d1} K_1) \\ &\quad \times \{1 - \exp(-k_{obs} t)\}/k_{obs}] C_m \end{aligned} \tag{6}$$

Surviving fraction ($f_{din}$) of cells exposed to dinuclear platinum complexes is given by

$$f_{din} = 100(C_s/C_s') = 100 \exp(-m_{din} C_m) \tag{7}$$

where

$$\begin{aligned} m_{din} &= k_{d2} K_2 t - (k_{d2} K_2 - k_{d1} K_1) \\ &\quad \times \{1 - \exp(-k_{obs} t)\}/k_{obs} \end{aligned} \tag{8}$$




Scheme 2.

If CDDP alone is present in the system (viz. $k_{obs}$ becomes infinite), the surviving fraction ($f_{CDDP}$) for CDDP is represented by

$$f_{CDDP} = 100(C_s/C_s') = 100\exp(-m_{CDDP}C_m) \quad (9)$$

where

$$m_{CDDP} = k_{d2}K_2t \quad (10)$$

Eqs. (7) and (9) give $IC_{50}$ as

$$IC_{50}(din) = -\ln 0.5/m_{din} \quad (11)$$

$$IC_{50}(CDDP) = -\ln 0.5/m_{CDDP} \quad (12)$$

The ratio of $IC_{50}$ values is thus given by Eq. (13),

$$IC_{50}(CDDP)/IC_{50}(din) = 1 - (1 - k_{d1}K_1/k_{d2}K_2) \times [\{1 - \exp(-k_{obs}t)\}/k_{obs}t] \quad (13)$$

The relation between the $IC_{50}$ ratio and $k_{obs}$ is shown in Fig. 7 at various $k_{d1}K_1/k_{d2}K_2$ values ($t = 48$ h). Here we obtain the following noteworthy result. If $k_{d1}K_1/k_{d2}K_2$ is smaller than 1, the $IC_{50}$ ratio decreases as $k_{obs}$ decreases. However, if $k_{d1}K_1/k_{d2}K_2$ is larger than 1, the $IC_{50}$ ratio increases as $k_{obs}$ decreases. In the case that $k_{d1}K_1/k_{d2}K_2$ is equal to 1, the $IC_{50}$ ratio is always 1 (constant). The physical meaning of this result is that a platinum complex with high values of $K_1$ and/or $k_{d1}$ tends to offer higher cytotoxic activity especially when $k_{obs}$ is small. In Table 2 are summarized the observed $IC_{50}$ ratio and $k_{obs}$ for 1-alkyluracil, 1-alkylthymine, and imide complexes. The $IC_{50}$ ratio is smaller than 1 in the case of 1-alkyluracil and 1-alkylthymine complexes (**1** and **6**), but it somewhat exceeds 1 in the case of glutarimide derivative complexes (**12** and **14**). It is apparent from Fig. 7 and Table 2 that $k_{d1}K_1/k_{d2}K_2$ values of **12** and **14** are larger than 1. It is suggested from the data on accumulation of platinum complexes into cells that $K_2$ is larger than $K_1$ [3]. Therefore, $k_{d1}$ should be larger than $k_{d2}$ for **12** and **14**, which means that **12** and **14** have higher reactivity than CDDP inside cells.

Table 2
Observed $IC_{50}$ ratio and $k_{obs}$

| Complex | $IC_{50}(CDDP)/IC_{50}(din)$[a] | $k_{obs}$ ($h^{-1}$)[b] | |
|---|---|---|---|
| | | 25°C | 37°C |
| **1** | 0.66 | 0.097[c] | 0.058[c] |
| **6** | 0.61 | 0.23[c] | 0.79[c] |
| **12** | 1.01 | 1.3 | 4.7 |
| **14** | 1.08 | 1.3 | 4.7 |

[a] Ratio of $IC_{50}$ values for CDDP and dinuclear complexes.
[b] Pseudo-first-order rate constant for CDDP formation.
[c] Cited from [3].

This tendency seems reasonable, since the complexes with larger electrophilicity for chloride anion should have higher reactivity toward other nucleophiles such as water and DNA bases inside cells. The small $IC_{50}$ ratio for **1** and **6** is probably ascribable to small $k_{obs}$ and $k_{d1}$ values, although the ratio does not agree with the calculated one quantitatively.

We have succeeded in the estimation of hydrophobicity of platinum complexes using the capacity factor in HPLC. As far as we know, there has been no application of the capacity factor to metal complexes. The key point in this success was the use of nitric acid aqueous solution as an eluant. As nitrate anion is a hydrophobic ion, metal cationic complexes can be extracted into organic phases in the form of hydrophobic ion pairs. A similar mechanism can account for the separation of the platinum complexes according to their hydrophobicity by an ODS column. Since the principle of this method should be applicable to other metal complexes, it will be a tool of great promise in the field of inorganic medicinal chemistry.

It is not likely that much platinum accumulates inside cells in the case of **5** since its $IC_{50}$ is higher than **2**. It is very likely that **5** is adsorbed on the membranes, because it has high hydrophobicity and positive charge (+2). It is known that electrostatic attractive force is strengthened in the medium of low dielectric constant (in most cases, high hydrophobicity tends to bring about a low dielectric constant environment). Another important phenomenon relevant to the hydrophobicity of platinum complexes is the interaction with

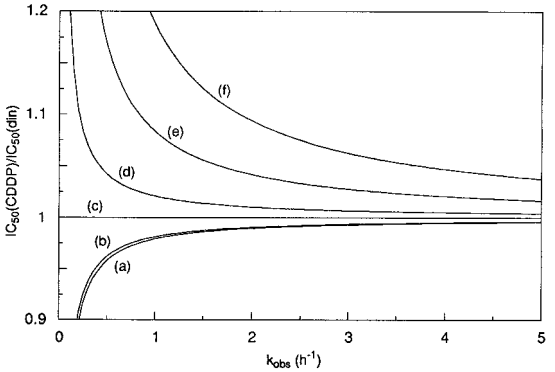



Fig. 7. Theoretical relation between $IC_{50}$ ratio and reaction rate constant ($k_{obs}$) of CDDP formation from platinum dinuclear complexes; (a) $k_{d1}K_1/k_{d2}K_2 = 0$, (b) 0.1, (c) 1, (d) 2, (e) 5, (f) 10.

serum proteins. In order to analyze this in detail, the following scheme was assumed,

$$\underset{(a_0-x-w)}{D} + \underset{(nb_0)}{S} \overset{K_m}{\rightleftharpoons} \underset{(w)}{D\cdot S} \overset{k_2}{\rightarrow} \underset{(y)}{D\text{–}S}$$

$$\underset{(a_0-x-w)}{D} + \underset{(c_0)}{2Cl^-} \overset{k_1}{\rightarrow} \underset{(z)}{CDDP}$$

$$\underset{(z)}{CDDP} + \underset{(nb_0)}{S} \overset{k_3}{\rightarrow} \underset{(r)}{CDDP\text{–}S}$$

where $D$ denotes a dinuclear complex, $S$ signifies a binding site on a protein, $n$ is the number of the binding site, $D\cdot S$ is a reversibly bound complex between a dinuclear complex and a binding site of a protein, *D-S* is an irreversibly associated complex, CDDP-*S* is an irreversibly associated complex between CDDP and a binding site, $a_0$, $b_0$ and $c_0$ are initial concentrations of a dinuclear complex, a protein and chloride anion, respectively, $x$ is the amount of a dinuclear complex converted to the other species, and $y$, $z$ and $w$ are respective concentrations of *D-S*, CDDP and $D\cdot S$. The above reaction scheme can be analyzed under the assumption that the total concentration of the binding site ($nb_0$) and the concentration of chloride anion ($c_0 = 0.15$ M) are constant during the reaction, since serum proteins such as BSA possess many hydrophobic adsorption sites and $c_0$ is also much larger than $a_0$ [17,19]. The following equations are obtained:

$$K_m = (a_0 - x - w)nb_0/w \tag{14}$$

$$dy/dt = k_2 w \tag{15}$$

$$dz/dt = k_1(a_0 - x - w)c_0^2 - k_3 znb_0 \tag{16}$$

$$dr/dt = k_3 znb_0 \tag{17}$$

Eqs. (14)–(17) lead to Eq. (18),

$$dz/dt + k_3 znb_0 = \{k_1 K_m c_0^2 a_0/(K_m + nb_0)\} \times \exp\{-(k_2 nb_0 + k_1 K_m c_0^2)t/(K_m + nb_0)\} \tag{18}$$

Solving Eq. (18), we obtain Eq. (19),

$$a_0/z = \{1 + (k_2 - k_3 K_m)nb_0/k_1 K_m c_0^2\} \times \left[\exp(-k_3 nb_0 t) - \exp\{-(k_2 nb_0 + k_1 K_m c_0^2)t/(K_m + nb_0)\}\right] \tag{19}$$

Eq. (19) can be approximated by Eq. (20) under the present experimental conditions,

$$a_0/z = 1 + (k_2 - k_3 K_m)nb_0/k_1 K_m c_0^2 \tag{20}$$

The relation between $a_0/z$ and $b_0$ is shown in Fig. 8a and b, respectively, for BSA and FCS, where good linearity was observed in both cases. As these results are consistent with the above model, we can use the quantity $K$ ($= nk_2/K_m$) as an index for the interaction. Since $k_{obs}$ ($= k_1 c_0^2$) of **3** is 0.58 $h^{-1}$ (Table 2) and $k_3$ is $2.6 \times 10^2$ $M^{-1}$ $h^{-1}$, the $K$ values for **2**-BSA and **5**-BSA complexes are calculated as $1.8 \times 10^3$ $M^{-1}$ $h^{-1}$ and $4.7 \times 10^3$ $M^{-1}$ $h^{-1}$, respectively, from the slopes of the straight lines in Fig. 8a. We can conclude, therefore, that more hydrophobic platinum complexes form a more stable complex with serum proteins.

From the above discussion, three possible paths by which the platinum dinuclear complexes affect cancer cells are considered: incorporation in the form of (1) platinum dinuclear complexes in their intact form, (2) CDDP produced from the dinuclear complexes, and (3) platinum–protein complexes. Apparently the third path does not con-

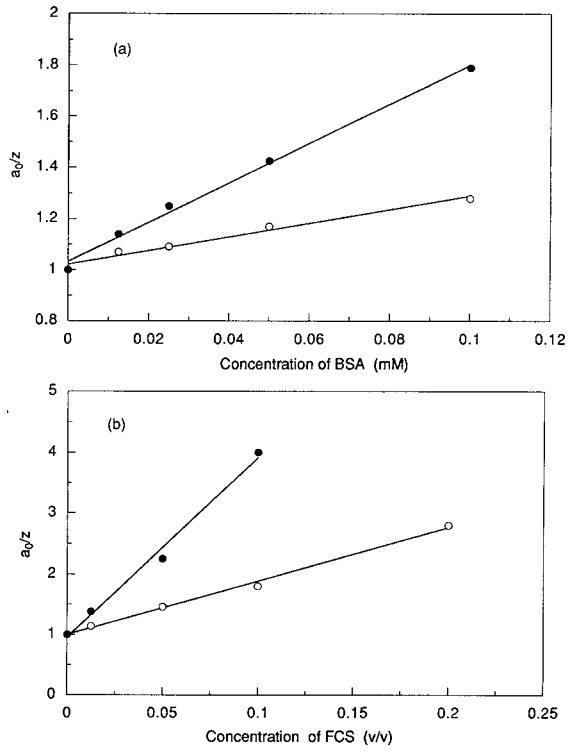



Fig. 8. Relation between $a_0/z$ and concentration of serum proteins; (a) BSA, (b) FCS; **2** (○) and **5** (●).

tribute so much in view of the large size of the complexes. Thus the net cytotoxic activity would be actually determined by the result of the competition of the above two species (1) and (2).

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## References

[1] B. Rosenberg, L. Van Camp, J.E. Trosco, V.H. Mansour, Nature 222 (1969) 385.
[2] J.D. Woollins, B. Rosenberg, J. Inorg. Biochem. 19 (1963) 41.
[3] P. Rekonen, Y. Dohta, M. Kodaka, T. Okada, K. Okamoto, H. Okuno, J. Med. Chem. 40 (1997) 515.
[4] Y. Dohta, M. Kodaka, T. Okada, K. Okamoto, H. Okuno, Inorg. Chim. Acta 252 (1996) 5.
[5] Y. Dohta, C.S. Browning, P. Rekonen, et al., Inorg. Chim. Acta 263 (1997) 69.
[6] C. Hansch, T. Fujita, J. Am. Chem. Soc. 86 (1964) 1616.
[7] L.R. Kelland, S.J. Clarke, M.J. McKeage, Platinum Metals Rev. 36 (1992) 178.
[8] T. Abe, H. Moriyama, K. Matsumoto, Chem. Lett. (1989) 1857.
[9] S.E. Sherman, S.J. Lippard, Chem. Rev. 87 (1987) 1153.
[10] H. Urata, H. Moriyama, K. Matsumoto, Inorg. Chem. 30 (1991) 3914.
[11] J. Carmichael, W.G. DeGraff, A.F. Gazdar, J.D. Minna, J.B. Mitchell, Cancer Res. 47 (1987) 936.
[12] P. Mistry, L.R. Kelland, S.Y. Loh, G. Abel, B.A. Murrer, K.R. Harrap, Cancer Res. 52 (1992) 6188.
[13] K.J. Wiechelman, R.D. Braun, J.D. Fitzpatrick, Anal. Biochem. 175 (1988) 231.
[14] C. Yamaguchi, H. Takami, K. Yamamoto, K. Miyoshi, N. Takao, Chem. Pharm. Bull. 32 (1984) 4994.
[15] T. Ohmori, T. Morikage, Y. Sugimoto, et al., Jpn. J. Cancer Res. 84 (1993) 83.
[16] P.A. Andrews, S.B. Howell, Cancer Cells 2 (1990) 35.
[17] S.L. Gonias, S.V. Pizzo, J. Biol. Chem. 258 (1983) 5764.
[18] P.T. Daley-Yates, D.C. McBrien, Biochem. Pharmacol. 33 (1984) 3063.
[19] T. Hoshino, M. Misaki, M. Yamamoto, H. Shimizu, Y. Ogawa, H. Toguchi, J. Pharm. Sci. 84 (1995) 216.
[20] L.E. Erickson, M. Godfrey, R.G. Larsen, Inorg. Chem. 26 (1987) 992.
[21] S.E. Miller, D.A. House, Inorg. Chim. Acta 166 (1989) 189.
[22] J. Matsunami, H. Urata, K. Matsumoto, Inorg. Chem. 34 (1995) 202.
[23] S. Ozawa, Y. Sugiyama, J. Mitsuhashi, T. Kobayashi, M. Inaba, Cancer Chemother. Pharmacol. 21 (1988) 185.