IN VITRO ANTITUMOR ACTIVITY OF A NEW PLATINUM ANALOGUE, NK 121 AGAINST FRESH HUMAN TUMOR CELLS AND ESTABLISHED TUMOR CELL LINES BY SUCCINATE DEHYDROGENASE INHIBITION TEST

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We analyzed the antitumor effect of a new platinum analogue, NK 121, against fresh human tumor cells and established tumor cell lines, and compared it with CDDP using the succinate dehydrogenase inhibition (SDI) chemosensitivity test. As the target cells, highly purified fresh tumor cells were obtained from three patients with malignant ascites due to gastric adenocarcinoma, two patients with pancreatic cancer, and two patients with ovarian cancer. The established KATO-III and C-1 cell lines were used for comparison. Tumor cells were cultured with 0.01-80 μg/ml of NK 121 or CDDP for 4 days, and following incubation the antitumor effect of NK 121 and CDDP was evaluated by the SDI test.

For fresh human tumor cells from patients treated with CDDP, the antitumor effect of NK 121 was superior to that of CDDP at concentrations below 5-10 μg/ml. In addition, for tumor cells from patients not treated with CDDP, the inhibition rate of NK 121 was similar to that of CDDP at concentrations less than 1-5 μg/ml. For KATO-III cells, the inhibition rate of CDDP was higher than that of NK 121 at concentrations above 20 μg/ml, but there was no difference in antitumor activity between the two agents under 10 μg/ml. For C-1 cells, there was also no difference between the two agents at concentrations less than 1 μg/ml.

Thus, NK 121 had a similar antitumor potency to CDDP at concentrations corresponding to the clinically achievable plasma levels which had been found in phase I trials.

Key words: Platinum analogue, NK 121, Succinate dehydrogenase inhibition test, Cancer chemotherapy, Fresh human tumor cells

INTRODUCTION

CDDP (cis-diamminedichloroplatinum II) is one of a group of platinum coordination complexes that was first described by Rosenberg et al.1, and it has been widely used for cancer therapy because of its potential value as a chemotherapeutic agent2-4. The side effects of CDDP include renal toxicity5, emetic reaction6, ototoxicity7 and myelosuppression8, and the dose-limiting side effect is renal toxicity9.

NK 121 is a novel platinum analogue (structural formula: cis-1,1-cyclobutane dicarboxylate- (R) -2-methyl-1,4-bu- tane diammine platinum II) developed for the purpose of reducing the side effects of CDDP (Fig. 1). In the past, it has generally been reported that the antitumor effects of CDDP analogues are inferior to that of CDDP itself10.

Recently, the succinate dehydrogenase inhibition (SDI) test has been used to select effective antican- cer agents for patients on an individual basis11. SDI
Antitumor activity of NK 121 by SDI test

Fig. 1. Chemical structures of NK 121 and CDDP

activity has been assayed in whole cell preparations without separation of tumor cells\(^{11}\), and although the purity of tumor cells in resected specimens obviously varies widely. It is considered that the SDI test should be performed using highly purified tumor cells to improve its accuracy.

In this study, we compared the antitumor effects of NK 121 and CDDP by the SDI test, and NK 121 in clinical doses was shown to have in vitro antitumor activity against both highly purified human tumor cells and established tumor cell lines.

**MATERIALS AND METHODS**

1. **Chemicals**

   CDDP and NK 121 were supplied by Nippon Kayaku Co., Ltd (Tokyo, Japan). NK 121 was provided as a lyophilized powder with a molecular weight of 439.37, which was dissolved in phosphate buffered saline. Both drugs were diluted in complete medium at a concentration of 0.01–80 μg/ml. The complete medium consisted of RPMI-1640 (Nissui Co., Tokyo, Japan) supplemented with 10 % heat-inactivated fetal calf serum (GIBCO, New York, USA), 2 mM L-glutamine, 100 units of penicillin/ml, and 100 μg of streptomycin/ml. The medium was adjusted to pH 7.2–7.4 using NaHCO\(_3\).

2. **Patient profiles**

   Case 1. A 57-year-old male was admitted with advanced gastric cancer (T\(_2\),N\(_0\),M\(_1\)) in May 1988. Four months later, he developed malignant ascites, and was treated by intraperitoneal administration of CDDP on five occasions (total dose 500 mg). The clinical response to CDDP was evaluated as a partial response. He was re-admitted because of an increase in his ascites in February 1989.

   Case 2. A 56-year-old male underwent total gastrectomy with lymph node dissection for gastric cancer (T\(_3\),N\(_2\),M\(_0\)) in March 1987. In October 1988, he was diagnosed as having multiple liver metastases, and was treated by the intravenous administration of CDDP on four occasions (total dose 300 mg). No clinical response to CDDP was recognized, and malignant ascites developed from February 1989.

   Case 3. A 34-year-old female underwent 3 courses of intensive chemotherapy consisting of CDDP, adriamycin and cyclophosphamide (CAP) to treat peritoneal dissemination of ovarian cancer (T\(_3\),N\(_0\),M\(_1\)). In May 1989, she developed intestinal obstruction with ascites due to intra-abdominal recurrence, which was resistant to chemotherapy using CDDP.

   Case 4. A 59-year-old female suffered from ovarian cancer and received 3 courses of CAP therapy. In June 1989, a metastasis to the hepatic hilum was detected, and this tumor was resected with partial hepatectomy (T\(_2\),N\(_0\),M\(_1\)).

   Case 5. A 52-year-old female underwent partial gastrectomy with lymph node dissection (T\(_3\),N\(_3\),M\(_1\)) in February 1989. At operation, malignant ascites was noted to be present. As there had been no previous treatment with chemotherapeutic agents, ascitic fluid was collected during the operation.

   Case 6. A 70-year-old male underwent pancreatoduodenectomy for pancreatic cancer (T\(_2\),N\(_0\),M\(_0\)) in February 1989. He had not previously been treated with chemotherapeutic agents. The resected tumor tissue was prepared for the following experiment.

   Case 7. A 58-year-old male underwent distal pancreatectomy for pancreatic cancer (T\(_2\),N\(_0\),M\(_0\)) in September 1989. He also had not previously been treated by chemotherapeutic agents.

   Thus, treatment with CDDP was performed in 4 of the 7 patients (cases 1–4), and no treatment had been performed in cases 5–7.

   The ascites collected from cases 1, 2, 3 and 5, and the resected solid tumors from cases 4, 6 and 7 were used as the source of tumor cells.
3. Purification of fresh human tumor cells

Malignant ascites was collected sterilely from four patients, immediately centrifuged at 400 g for 5 min, and then suspended in complete medium.

Freshly excised tumor tissues obtained from the other three patients were processed using enzymatic digestion, as described by Itoh et al. Briefly, tumor tissues were dissected into pieces smaller than 5 mm³ which were immersed in complete medium containing collagenase (2 mg/ml, type V-S; Sigma), hyaluronidase (10 units/ml, type VI-S; Sigma), and DNase-I (0.4 mg/ml; Sigma). After a 40 min incubation at 37°C, the cells were harvested, washed three times, and suspended in complete medium.

The technique for purification of autologous tumor cells conformed to that of Uchida et al. Tumor cells obtained from ascites and solid tumor specimens were centrifuged on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) gradients at 400 g for 30 min. Mononuclear cells and tumor cells at the interface were collected, washed, and suspended at a concentration of 1 x 10⁶/ml in complete medium. The cells were then layered on discontinuous gradients containing 4 ml each of 25%, 15%, and 10% Percoll (Pharmacia, Uppsala, Sweden) in complete medium. Centrifugation was performed at 25 g for 7 min, and cells were washed and suspended in complete medium at a concentration of 1 x 10⁶/ml. The cells thus prepared were mainly tumor cells, with less than 10−15% contamination by nonmalignant cells as judged by morphological examination. They were more than 90−95% viable by the trypan blue dye exclusion test.

4. Established tumor cell lines

The KATO-III (human signet ring cell gastric carcinoma) and C-1 (human differentiated colon carcinoma) were used as established tumor cell lines. These cell lines were maintained in complete medium. On the day before the experiment, tumor cells were adjusted to a concentration of 5 x 10⁵/ml in fresh complete medium and incubation was then continued at 37°C.

5. Method of SDI test

Chemosensitivity was assessed by the SDI test, using the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2, 5-diphenyl tetrazolium bromide, Sigma No. M 2128) to measure the viability of tumor cells. MTT was dissolved in phosphate buffered saline at 5 mg/ml and filtered for sterilization. Then, 100 µl of tumor cell suspension (5 x 10⁵ cells/ml) was added to 25 µl of serial 2-fold dilutions of NK 121 or CDDP (0.01−80 µg/ml at final concentrations) in 96-well flat-bottomed microtiter plates (Corning No. 25860), and incubated at 37°C in a humidified 5% CO₂ atmosphere for 24−96 h. Each drug dilution was
assessed in triplicate. Three microtiter wells containing tumor cells suspended in 125 μl of complete medium (total tumor cell number was equivalent to that in test wells) were used as a control for cell viability, and three wells containing only complete medium were used as controls for nonspecific dye reduction. After incubation, 15 μl/well of MTT solution was added to all the wells, and plates were incubated for a further 4 h. Then acid-isopropanol (100 μl of 0.04 N HCl in isopropanol) was added to all the wells and the mixtures were pipetted thoroughly to dissolve the dark blue crystals.

The plates were then read on a microplate reader (Corona Electric, MTP-32) using a test wavelength of 570 nm and a reference wavelength of 630 nm. The control wells without tumor cells had an OD of less than 0.005. For the established tumor cell lines, the assay was performed three times, and the mean and standard deviation of the values obtained were determined. The inhibition rate was calculated as follows:

\[
\text{inhibition rate} = \left(1 - \frac{\text{OD drug treated}}{\text{OD control}}\right) \times 100
\]

**RESULTS**

1. Purity of fresh human tumor cells

The purity of tumor cells immediately after enzymatic digestion alone or centrifugation alone was 22.2–51.9 % (mean ± SD, 37.2 ± 11.0 %), while after Table 2. Purity of fresh human tumor cells

<table>
<thead>
<tr>
<th>Patient</th>
<th>Tumor cell source</th>
<th>Purity of tumor cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ascites</td>
<td>22.2</td>
</tr>
<tr>
<td>2</td>
<td>ascites</td>
<td>50.6</td>
</tr>
<tr>
<td>3</td>
<td>ascites</td>
<td>37.5</td>
</tr>
<tr>
<td>4</td>
<td>primary tumor</td>
<td>23.5</td>
</tr>
<tr>
<td>5</td>
<td>ascites</td>
<td>51.9</td>
</tr>
<tr>
<td>6</td>
<td>primary tumor</td>
<td>33.3</td>
</tr>
<tr>
<td>7</td>
<td>primary tumor</td>
<td>41.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Before purification</th>
<th>After purification</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>37.2 ± 11.0</td>
<td>91.0 ± 1.5**</td>
</tr>
</tbody>
</table>

The tumor cells obtained from solid tumors or ascites were enriched to a purity of 91.0 ± 1.5% by purification.

The purity of tumor cells before purification (enzymatic digestion alone in solid tumors and centrifugation alone in ascites) was 37.2 ± 11.0% (**p<0.01**). Table 3. Optimal incubation time for the SDI test

<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>OD without drugs</th>
<th>Inhibition rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CDDP (80 μg/ml)</td>
<td>NK121 (80 μg/ml)</td>
</tr>
<tr>
<td>24</td>
<td>0.39</td>
<td>40.4</td>
</tr>
<tr>
<td></td>
<td>26.1</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>0.39</td>
<td>84.0</td>
</tr>
<tr>
<td></td>
<td>65.4</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>0.36</td>
<td>82.3</td>
</tr>
<tr>
<td></td>
<td>50.1</td>
<td></td>
</tr>
<tr>
<td>96</td>
<td>0.41</td>
<td>90.4</td>
</tr>
<tr>
<td></td>
<td>62.2</td>
<td></td>
</tr>
</tbody>
</table>

For fresh human tumor cells without drugs the OD at 700 nm was equivalent at each incubation time. The inhibition rate increased with incubation time.
processing on the Ficoll-Hypaque discontinuous gradients the purity was $70.5 \pm 6.8\%$. Tumor cells were enriched to $91.0 \pm 1.5\%$ by use of the Percoll discontinuous gradients (Table 2).

2. Optimal incubation time for the SDI test

When fresh human tumor cells were incubated without drugs (case 1), the OD$_{570}$ remained steady and there was no variation due to changes in incubation time. However, the inhibition rates for NK 121 and CDDP increased with longer incubation times (Table 3). Therefore, a 96-h incubation period was used for all subsequent studies.

3. Antitumor effects of NK 121 and CDDP against established tumor cell lines

For KA10 III cells, there was no significant difference in OD$_{570}$ between NK 121 and CDDP at under 10 $\mu$g/ml. However, 80 $\mu$g/ml of CDDP inhibited SD activity completely, although NK 121 inhibited it only 56% ($P<0.01$) (Fig. 2). For the C-1 line also, the inhibition of SD activity by NK 121 was

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Fig. 2. Antitumor effects of NK 121 and CDDP against KATO-III cells

Fig. 3. Antitumor effects of NK 121 and CDDP against C-1 cells
equivalent to that obtained by CDDP under 1 μg/ml, whereas the viability of tumor cells was completely abrogated by a high concentration of CDDP in contrast to the effect of NK 121 (P < 0.01 or 0.05) (Fig. 3).

4. Antitumor effects of NK 121 and CDDP against fresh human tumor cells

The purified tumor cells freshly isolated from the four patients treated with CDDP and the other three untreated patients were also evaluated for the inhibition of NK 121.

For tumor cells from patients treated with CDDP, the inhibition rate of NK 121 was higher than that of CDDP at concentrations less than 10 μg/ml (case 1) or 5 μg/ml (case 2 and 3). There was no difference between the inhibition rates of NK 121 and CDDP at concentrations less 5 μg/ml (case 4).

Fig. 4. Antitumor effects of NK 121 and CDDP against fresh human tumor cells
chemosensitivity. With tumor cells from case 1, the inhibition rate of CDDP was lower than that of NK-121 under 10 μg/ml, while it was higher at concentrations above 20 μg/ml. Similarly, in cases 2 and 3, the inhibition rate of NK-121 was better than that of CDDP under concentrations of 5 μg/ml. In addition, there was no difference between the inhibition rates of NK-121 and CDDP at concentrations less than 5 μg/ml in case 4 (Fig. 4).

In case 5, however, the inhibition rate of CDDP was nearly equivalent to that of NK-121 at concentrations under 5 μg/ml, while it was higher than that of NK-121 above 10 μg/ml. In case 6, the inhibition rate of CDDP was higher than that of NK.

For tumor cells from patients untreated with CDDP, the inhibition rate of NK-121 was nearly equivalent to that of CDDP at concentrations less than 5 μg/ml (case 5) and 1 μg/ml (case 7). However, the inhibition rate of CDDP was higher than that of NK-121 even at the concentrations less than 2 μg/ml in case 6.

Fig. 5. Antitumor effects of NK-121 and CDDP against fresh human tumor cells.
121 even at concentrations less than 2 μg/ml. In case 7, there was no difference between the antitumor effect obtained by NK 121 and CDDP at a concentration of less than 1 μg/ml (Fig. 5).

**DISCUSSION**

Recently, CDDP has become one of the major agents used in cancer chemotherapy. At present, renal toxicity remains the dose-limiting side effect, though other side effects have been reduced by the use of diuretics, anti-emetic agents, and fosfomycin16-18).

Because of its toxicity, several analogues of CDDP have been developed in an effort to ameliorate its side effects while retaining the antitumor activity of the parent compound.

The side effects of these new analogues of CDDP are less than those of the parent compound, but Russell et al. have demonstrated that at equivalent concentrations carboplatin and iproplatin were both about 10 times less toxic than CDDP for tumor cells19). Other investigators have also reported that aminooethylpyrrolidine-platinum complexes had a 2- to 3-fold weaker toxic effect than CDDP20).

A phase I study of NK 121 was performed in 38 cancer patients, and no significant nephrotoxicity was observed. Nausea and vomiting were noted in most of the patients who received ≥320 mg/m², but only three patients required anti-emetic agents21).

It was found that 80 % of NK 121 was not bound to protein until 8 h after a 3-h drip infusion. The Cmax at a dose of 80–120 mg/m² was 7–13 μg/ml, and at 200–280 mg/m² it was 20–26 μg/ml. Plasma levels of NK 121 were maintained at 1 μg/ml at 4 h or 8 h after the administration of 80–120 mg/m² or 200–280 mg/m² of NK 121, respectively (unpublished data).

The present study showed that NK 121 had an equally potent antitumor effect against highly purified human tumor cells as did CDDP at concentrations less than 5 or 10 μg/ml in cases 1–5. These levels are similar to the plasma levels maintained for 4–8 h by a clinical dose of CDDP or NK 12121,22) Moreover, when the cut-off concentrations were set at 20 μg/ml for CDDP and 80 μg/ml for NK 121 on the basis of the drug pharmacokinetics, the inhibition rates for both drugs were over 50 %. Thus, it was suggested that the antitumor effects of CDDP and NK 121 were equivalent at clinically achievable concentrations.

In previous SDI tests used to determine chemosensitivity, tumor cells were not purified and whole cell preparations including lymphocytes and mesothelial cells were used10,11,23). We found that the purity of tumor cells was from 22 % to 52 % immediately after enzymatic digestion of tumor tissues, and was increased to 91 % by the purification technique described in this paper. To clarify the antitumor effect of a chemotherapeutic agent against fresh human tumor cells, it is important to exclude nonmalignant cells as far as possible when performing the SDI test.

In the established tumor cell lines which contained no nonmalignant cells (KATO-III and C-1), it was found that NK 121 inhibited the viability of tumor cells as potently as CDDP at concentrations less than 1–5 μg/ml.

Other investigators have demonstrated that the therapeutic efficacy of NK 121 was equivalent to that of CDDP in certain human tumor cell lines transplanted into nude mice24). In contrast, using a colony assay, Yonei et al. reported that the antitumor effect of NK 121 was weaker than that of CDDP against established tumor cell lines and fresh human tumor cells25).

Moreover, Lokich et al. demonstrated that the patterns and frequency of toxicity were greatly diminished, and the excellent antitumor effects were observed by continuous administration of CDDP26). Thus, it is suggested that NK 121 may offer the therapeutic efficacy by continuous venous infusion, preserving a relatively low plasma concentration.

It is of interest that the inhibition rate of NK 121 was higher than that of CDDP at concentrations less than 5–10 μg/ml in fresh tumor cells obtained from patients who had been treated with CDDP. Also, it was nearly equal to that of CDDP in tumor cells from case 5 and 7 not previously treated with CDDP (at concentrations less than 1–5 μg/ml). These results suggest that there is no cross-reactivity between NK 121 and CDDP, and we are currently investigating whether NK 121 has antitumor activity against CDDP-resistant tumor cell lines.
References


新しいCDDP誘導体NK121のSuccinate dehydrogenase inhibition法を用いたヒト新鮮分離腫瘍細胞および培養癌細胞に対するin vitro抗腫瘍効果の検討

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新しいCDDP誘導体であるNK121の新鮮ヒト腫瘍細胞および培養癌細胞に対する抗腫瘍効果をCDDPを対照としてSDI法による抗腫瘍薬感受性試験を用いて解析した。腫瘍による悪性腹水3例、卵巢癌2例および膀胱癌2例からFicoll-Hypaque 2段階・Percoll 3段階不連続密度勾配法により純度90％以上で単離した新鮮ヒト腫瘍細胞と、胃癌細胞癌KATO-III、大腸癌株化細胞C-1について、0.01～80 µg/mlのCDDPまたはNK121を加えて4日間のSDI法を施行した結果、CDDPの治療効力のない3例中2例では、1～5 µg/ml以下の濃度においてNK121とCDDPの抑制率に差を認めなかったが、CDDPによる治療効力のある4例中3例で、5～10 µg/ml以下の低濃度においてNK121の抑制率はCDDPのそれと同回った。これに対し、培養細胞では、KATO-IIIに対しては20 µg/ml以上の濃度でCDDPの抑制率はNK121よりも高値であったが、10 µg/ml以下で両薬剤に差を認めず、C-1に対しても1 µg/ml以下の濃度で両薬剤に差を認めなかった。

したがって、NK121の第1相試験で得られた薬剤の低い血中濃度の持続状態においては、NK121はCDDPと同等の抗腫瘍活性を発現し、臨床的有用性が特に発揮されると思われた。

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