Enhanced Therapeutic Efficacy of Cisplatin by Combination with Diethyldithiocarbamate and Hyperthermia in a Mouse Model

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ABSTRACT

A spontaneously metastasizing solid tumor model derived by transplanting the TA3Ha murine mammary carcinoma into the s.c. tail tissue of mice was used to develop a treatment strategy for enhancing the therapeutic efficacy of cisplatin (CDDP). This strategy was based on the findings that diethyldithiocarbamate (DDTC) reduces the toxicity of CDDP, and that localized hyperthermia (HT) augments the antitumor efficacy of CDDP. DDTC (500 mg/kg) reduced the CDDP-induced nephrotoxicity and gastrointestinal toxicity as well as increased the CDDP LD10 from 8 to 20 mg/kg in strain A mice. When CDDP and DDTC were used in multiple treatment schedules at 5-day intervals, DDTC protected the hosts but not the tumors against the toxicity of CDDP. HT administered locally to the tumor 1 h after the injection of CDDP (8 mg/kg) in 1 ml Hanks’ balanced salt solution increased the antitumor effect but not the host toxicity. While administration of 8 mg/kg CDDP alone or with HT three times at 5-day intervals caused 100% host mortality, this dose of CDDP could be used with no mortality by combining it with HT. A combination of 8 mg/kg CDDP with DDTC (750 mg/kg) and HT (43°C for 60 min), administered three times at 5-day intervals, retarded the local tumor growth significantly compared to the untreated, CDDP plus DDTC and DDTC plus HT control groups of mice. The frequency of lung metastasis in these groups on day 30 of tumor inoculation were 0, 90, 90, and 80%, respectively. The mean survival days of the mice treated with CDDP plus DDTC plus HT was 61 ± 6 compared to 34 ± 5 in the controls. The results presented here demonstrate that by combining CDDP with DDTC, high doses of CDDP can be safely administered. When localized HT is combined with high dose CDDP and DDTC, the tumor growth retardation and the host survival prolongation are significantly better than those obtained with the highest tolerable dose of CDDP alone or CDDP plus HT.

INTRODUCTION

cis-Dichlorodiammineplatinum(II) is an important cytotoxic drug in the treatment of a variety of human neoplasms (1). Due to its dose limiting toxic side effects (1,2) there is a clinically important need to develop procedures that enhance the therapeutic efficacy of CDDP.3 In response to this need, we examined the usefulness of a treatment regimen that combines large doses of CDDP with DDTC and localized HT in a murine tumor model. This study was based on the independent observations that DDTC reduces the host toxicity of CDDP (3–10), and that HT enhances the cytotoxic effects of CDDP (11–15). We have used a resectable, spontaneously metastasizing murine tumor model (16–18) to examine the effects of CDDP in combination with DDTC and HT on the local tumors, metastases, and on the host survival periods.

MATERIALS AND METHODS

Reagents. Nutrient medium F-10 and HBSS were purchased from the Grand Island Biological Company, Grand Island, NY. Fetal bovine serum was obtained from the HyClone Laboratories, Sterile Systems, Inc., Logan, UT. Ficoll-Paque was purchased from Pharmacia Chemicals, Piscataway, NJ. CDDP (Platinol) was purchased from the Bristol Laboratories, Syracuse, NY. DDTC was obtained from Sigma Chemical Co., St Louis, MO.

Mice. Female strain A mice were obtained from The Jackson Laboratory, Bar Harbor, ME. They were 6–8 weeks of age and weighed on an average 20 g at the beginning of each experiment.

Tumor Cells. Transplantable mammary adenocarcinoma line TA3Ha syngeneic to strain A mice (19) was used. Routinely, these cells are maintained in vivo as i.p. implants in strain A mice. Freshly harvested ascites cells were prepared and injected s.c. into the tails of mice as has been described previously (16–18).

Treatment Procedure. CDDP was freshly dissolved in HBSS and injected i.p. into strain A mice in 1-ml volume, unless otherwise stated, to give a final dose of 4 to 32 mg/kg. When DDTC was used, a freshly prepared solution in HBSS was injected (0.1 ml) i.p. 1 h after the CDDP injection. Localized HT, 1 h after CDDP (i.e., immediately after DDTC) was administered by suspending the tails of anesthetized mice in a water bath at 43°C for 60 min as has been described (17).

Measurement of Intratumoral Temperature. The intratumoral temperatures of a set of tumors (using 12 mice) in response to localized HT were determined. Mice bearing s.c. tail implants of TA3Ha tumors were lightly anesthetized using sodium pentobarbital (Nembutal, Abbott Laboratories, North Chicago, IL). A small incision was made in the skin at the base of the tail and a 0.8-mm fluoroptic thermocouple probe was inserted under the skin to reach the tumor. Temperatures were measured using the Luxtron Fluoroptic Thermometer Model 2000B at 1-min intervals before, during, and after HT. The water bath temperature was constantly monitored using a second probe connected to the thermometer.

Toxicity Studies. In all the toxicity studies, the number of dead mice by day 15 after the last treatment was used as the end point. The nephrotoxicity and the gastrointestinal toxicity of CDDP was evaluated by examining the histological damage to the kidney and the small intestine. In these studies, 2 to 3 mice were sacrificed 5 days after the treatment, kidney and a piece of the jejunum were collected, fixed overnight in 10% neutral buffered formalin, and processed by routine histological methods.

Morphological damage to the kidney was quantitated by the procedure described by Gale et al. (20). At least 600 tubules around the subcortical glomeruli were evaluated for tubular necrosis, karyorrhexis, and pyknosis of cells. Tubules with one or more of the above features were considered abnormal.

The intestinal damage was quantitated by the procedure described by Dunnill and Whitehead (21). This procedure uses a template of short lines of equal lengths (l) in different directions superimposed randomly on the image of the sections. The number of lines (l), the end points of which fall within the villi, and the number of intersections (c) made by the lines with the villi are recorded. The surface to volume ratio is given by the equation,

\[ s/v = 4c/l^3 . \]
where \( s \) and \( v \) are the surface and volume, respectively. The index of the surface to volume ratio has been shown to provide an accurate and reproducible measure of the degree of villions atrophy (21).

Antitumor Effects. The TA3Ha tail tumors were measured in cm starting from the day of treatment (day 0) at three orthogonal diameters. The geometric mean diameters and the relative tumor sizes were reproducible measure of the degree of vilions atrophy (21).

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The question whether DDTC also protects the tumor against the CDDP cytotoxicity was addressed. The rate of change of RTS in the mice treated with 4 mg/kg CDDP alone was 0.117 ± 0.03 and that in the mice treated with 4 mg/kg CDDP plus 500 mg/kg DDTC was 0.139 ± 0.02. These values were different at \( P = 0.03 \). A similar tumor protective effect by high doses of DDTC has been reported in a rat model (9) which was suggested to be due to an early recovery of the tumors from the cytostatic effects of CDDP. When these treatments were repeated 3 times at 5-day intervals, the rate of change of RTS in the untreated controls (slope = 0.8; \( r = 0.996 \)). When DDTC was incorporated, the \( s/v \) was no different from that in the untreated controls. These results demonstrate that DDTC protects the hosts against CDDP-induced nephrotoxicity and gastrointestinal toxicity, as well as lethality. Thus by reducing the toxicity of CDDP, larger doses of it could be administered.

Antitumor effects of CDDP in Combination with DDTC. Tumor bearing mice were treated with 0, 4, 8, and 16 mg/kg CDDP plus 500 mg/kg DDTC and the rate of change of RTS was determined by the Student's \( t \) test. The results (Table 3) show a linear relationship between the rate of change of RTS and the dose of CDDP (slope = -0.005; \( r = 0.97 \)). These results demonstrate that DDTC permits the administration of large doses of CDDP and that increasing doses of CDDP produce increasing antitumor effects.

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When the mice were treated 3 times with 750 mg/kg DDTC in strain A female mice, the rate of change of RTS was determined by the Student's \( t \) test. The results (Table 3) show a linear relationship between the rate of change of RTS and the dose of CDDP (slope = -0.005; \( r = 0.97 \)). These results demonstrate that DDTC protects the hosts against CDDP-induced nephrotoxicity and gastrointestinal toxicity, as well as lethality. Thus by reducing the toxicity of CDDP, larger doses of it could be administered.

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11.0 ± 0.5 compared to 3.08 ± 0.5 in the untreated controls. The survival periods in these groups of mice were 61 ± 6 and 34 ± 5 days, respectively \( (P < 0.0001) \). However, a 10% toxicity related death was noted. Three treatments with 4 mg/kg CDDP plus HT also resulted in 10% host mortality. With this treatment regimen, the day 15 RTS was 1.41 ± 0.2 and the host survival period was 34 ± 10 days.

DDTC at a dose level of 750 mg/kg abolished the lethality of CDDP (8 mg/kg) in a 3 treatment schedule. Fig. 1 and Table 5 illustrate the effect of 8 mg/kg CDDP plus 750 mg/kg DDTC and HT (43°C for 60 min) administered 3 times at 5-day intervals. The rate of change of RTS in the mice treated with CDDP plus DDTC plus HT (0.017 ± 0.01) was 10 times less than in the untreated controls (0.177 ± 0.03), 7 times less than in the mice treated with CDDP plus DDTC (0.120 ± 0.03), and 5 times less than in the mice treated with DDTC plus HT (0.084 ± 0.001). The rate of change of RTS in CDDP plus DDTC plus HT group was 1.6-fold less than the calculated additive (slope = 0.028) effects of CDDP and HT. The mice in the above experiment were sacrificed on day 18 of treatment (30 days after tumor implantation) and the frequency of metabolic

<table>
<thead>
<tr>
<th>Table 4 Effect of single and multiple treatments with 8 mg/kg CDDP combined with 500 mg/kg DDTC on the change in the RTS of the TA3HA tail tumors in strain A mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Difference in the Δ RTS values of various experimental groups were: group 2 versus group 3, ( P = 0.0026 ); group 2 versus group 4, ( P &lt; 0.0001 ); groups 3 and 4, ( P &lt; 0.0001 ). The ( P ) values were determined by the Student's ( t ) test.</td>
</tr>
<tr>
<td>Group</td>
</tr>
<tr>
<td>-------</td>
</tr>
<tr>
<td>1. Control</td>
</tr>
<tr>
<td>2. 1 treatment</td>
</tr>
<tr>
<td>3. 2 treatments (on days 0 and 10)</td>
</tr>
<tr>
<td>4. 2 treatments (on days 0 and 5)</td>
</tr>
</tbody>
</table>

* Mean ± SD.

<table>
<thead>
<tr>
<th>Table 5 Effect of cisplatin (8 mg/kg) in combination with DDTC (750 mg/kg) and heat (43°C for 60 min) on the rate of growth of TA3HA tail tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Differences in the Δ RTS values compared to the controls were: group 2, ( P &gt; 0.001 ); groups 3 and 4, ( P &lt; 0.0001 ); the CDDP alone and CDDP + HT under similar treatment conditions caused 100% host mortality; RTS differences between groups 2 and 4, and between 3 and 4 were significant at ( P &lt; 0.0001 ). CDDP alone and CDDP + HT under similar treatment conditions resulted in 100% host mortality. The ( P ) values were determined by the Student's ( t ) test. The expected additive effect change in the RTS in the combination of CDDP, DDTC, and HT group is 0.028, which is 1.6-fold greater than the observed value.</td>
</tr>
<tr>
<td>Group</td>
</tr>
<tr>
<td>-------</td>
</tr>
<tr>
<td>1. Control</td>
</tr>
<tr>
<td>2. CDDP + DDTC</td>
</tr>
<tr>
<td>3. HT + DDTC</td>
</tr>
<tr>
<td>4. CDDP + DDTC + HT</td>
</tr>
</tbody>
</table>

* Mean ± SD.
tasis in the major organs was examined. The results summarized in Table 6 show that the mice treated with CDDP, DDTC, and HT had no lung metastasis, whereas the untreated controls, CDDP plus DDTC, and DDTC plus HT-treated mice had 90, 90, and 80% lung metastasis, respectively. Metastasis at other major sites followed a similar pattern (Table 6). Thus, a combination of CDDP, DDTC, and HT controlled the growth of not only the local tumors but also prevented and/or retarded the formation of metastases.

The effects of a combination of CDDP with DDTC and HT on the host survival prolongation were examined. The results summarized in Table 7 are from an experiment in which the mice were treated with 4 mg/kg CDDP, 500 mg/kg DDTC, and HT (43°C for 60 min) on days 0, 5, and 10. On day 11, the tail tumors were surgically excised and the mice were examined for 60 days to assess the tumor-induced host deaths. The group treated with CDDP, DDTC, and HT had 2 (20%) long-term survivors and 2 (20%) toxic deaths. The groups treated with only CDDP or only HT had 20 and 10% long-term survivors, respectively. The frequency of lung metastasis (major cause of tumor-induced host death) in the mice treated with CDDP plus DDTC plus HT, CDDP plus HT, CDDP plus DDTC, HT, and the untreated controls was 70, 75 100, 100, and 90%, respectively. Thus, 30% of the mice treated with CDDP plus DDTC plus HT were potentially cured.

The survival benefit seen in the groups treated with CDDP, DDTC, and HT does not appear to be attributable to the small size of the local tumors at the time of excision, since excision of comparable or even smaller size tumors in untreated control mice resulted in significantly fewer long-term survivors (Table 7).

### Table 6 Effect of 8 mg/kg CDDP, 750 mg/kg DDTC, and HT (43°C for 60 min) on distribution of spontaneous metastasis by the TASH tumor implanted s.c. into the tails of strain A mice

<table>
<thead>
<tr>
<th>Group</th>
<th># Lungs</th>
<th>LLN*</th>
<th>RLN</th>
<th>MLN</th>
<th>Spleen</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>90</td>
<td>95</td>
<td>65</td>
<td>20/30</td>
<td>30/0</td>
</tr>
<tr>
<td>CDDP + DDTC</td>
<td>10</td>
<td>90</td>
<td>78</td>
<td>91</td>
<td>22/11</td>
<td>0/10</td>
</tr>
<tr>
<td>HT + DDTC</td>
<td>10</td>
<td>80</td>
<td>80</td>
<td>55</td>
<td>40/0</td>
<td>0/0</td>
</tr>
<tr>
<td>CDDP + DDTC + HT</td>
<td>10</td>
<td>10</td>
<td>15</td>
<td>0</td>
<td>0/0</td>
<td>0/0</td>
</tr>
</tbody>
</table>

* LLN, lumbar lymph node; RLN, renal lymph node; MLN, mediastinal lymph node.

### Table 7 Effect of CDDP, DDTC, HT, and primary tail tumor excision on survival of TASH tumor-bearing mice

Long-term survivors are mice surviving for >60 days. Groups 4, 5, 6, and 7, received treatments on days 0, 5, and 10. HT, localized hyperthermia at 43°C for 60 min in a water bath; CDDP, 4 mg/kg; DDTC, 500 mg/kg.

<table>
<thead>
<tr>
<th>Tail tumor geometric mean diameter at time of excision</th>
<th>Long-term survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Groups</td>
<td></td>
</tr>
<tr>
<td>1. Control</td>
<td>0.39 ± 0.03*</td>
</tr>
<tr>
<td>2. Control</td>
<td>0.48 ± 0.06</td>
</tr>
<tr>
<td>3. Control</td>
<td>0.75 ± 0.19</td>
</tr>
<tr>
<td>4. HT alone</td>
<td>0.59 ± 0.02</td>
</tr>
<tr>
<td>5. CDDP alone</td>
<td>0.57 ± 0.13</td>
</tr>
<tr>
<td>6. CDDP + HT</td>
<td>0.42 ± 0.07</td>
</tr>
<tr>
<td>7. CDDP + DDTC</td>
<td>0.44 ± 0.10</td>
</tr>
<tr>
<td>DDTC + HT</td>
<td>0.44 ± 0.10</td>
</tr>
</tbody>
</table>

* Mean ± SD.

The above study was extended to investigate if the host survival prolongation benefit can be achieved in mice without the surgical intervention. The results are presented in Table 8. When the tumor-bearing mice were treated 3 times at 10-day intervals with 8 mg/kg CDDP, 500 mg/kg DDTC, and HT, 20% of the mice survived for 60 or more days (mean survival days, 51 ± 8); in the group similarly treated at 5-day intervals, 56% of the mice survived for 60 or more days with mean survival period of 61 ± 6 days. In the group treated with CDDP and DDTC, and in the untreated control groups, no long-term survivors were found.

### DISCUSSION

This study constitutes an attempt to improve the therapeutic efficacy of CDDP in a well characterized, spontaneously metastasizing experimental solid tumor model. For enhancing the therapeutic efficacy of CDDP we integrated the two important and independent findings reported in the literature, that DDTC selectively reduces the CDDP-induced host toxicity (3-10), and that HT produces a synergistic cytotoxicity when combined with CDDP (11-15).

DDTC is a sulfur-containing metal-chelating agent of proven clinical efficacy as an antidote for acute nickel-carbonyl poisoning (23). The results from the studies by Borch et al. (3-6) as well as others (7, 8, 10), demonstrate that DDTC is also effective in reducing the platinum toxicity. Based on these findings, DDTC has been entered into clinical trials for escalating the dose of CDDP that could be administered (9). The usefulness of increased doses of CDDP is well demonstrated by Ozols et al. (24). These authors report that CDDP at 120 mg/m² produced a 55% response rate in the ovarian carcinoma patients and a 50% response rate in the testicular cancer patients who had failed a standard dose of 50-75 mg/m² CDDP.

Our present studies demonstrate a linear relationship between the dose of CDDP administered and the tumor response in an experimental model.

The data presented in this communication and those reported by Borch et al. (3) clearly demonstrate that the CDDP-induced renal and gastrointestinal toxicity are significantly reduced by DDTC. Further studies to examine the influence of DDTC on the CDDP-induced neurotoxicity in an appropriate experimental model are warranted.

Borch and Pleasant (5) followed by others (7, 8) have demonstrated that DDTC does not reduce the cytotoxic effects of CDDP against the rat and mouse ascites tumors. However, this aspect has not been well investigated in solid tumor systems. Recent studies in a solid tumor system have shown that at high dose of DDTC (750 mg/kg) reduces the antitumor efficacy of CDDP (3). Our present results are in agreement with these findings. For instance, the antitumor effect produced by three treatments of 8 mg/kg CDDP plus 750 mg/kg DDTC, was less than that produced by three treatments of 4 mg/kg CDDP plus 500 mg/kg DDTC. With 4 mg/kg CDDP, 500 mg/kg DDTC given in a single treatment schedule, the antitumor efficacy of CDDP was slightly but significantly reduced. However, when a multiple treatment schedule (3 times at 5-day intervals) is used, the antitumor effects of 4 mg/kg CDDP were not altered by 500 mg/kg DDTC. Thus, at moderate doses, in a multiple treatment schedule, DDTC appears to exhibit a selective host protective effect.

The administration of CDDP with DDTC appears to be more effective when repeated at short intervals. Two treatments with 8 mg/kg CDDP plus 500 mg/kg DDTC given at 10-day...
This is evidenced by the finding that when the untreated tumors was decreased by 90% compared to the untreated controls, and the survival benefit seen in the group treated with CDDP, CDDP plus HT, 20% host mortality was seen which negated of similar or smaller sizes were resected, fewer than 10% of the geometric mean diameter) of the tumors at the time of resection. mice. This compared very favorably with the untreated (0%), was possible to obtain long-term survival benefits in 70% of the demonstrated an increased tumor response and suggested the possibility of DDTC when combined with CDDP and given in multiple treatments at relatively short intervals protect the normal host tissue but not the tumors against the toxicity of CDDP. Although DDTC permitted the administration of high doses of CDDP, which resulted in pronounced local tumor control, the survival prolongation of TA3Ha tumor-bearing mice was less than satisfactory.

In view of the demonstrated efficacy of CDDP and HT combination in controlling the tumor growth and metastasis (13), we combined localized HT with CDDP and DDTC. This combination treatment protocol which included DDTC, permitted the use of high doses of CDDP which had not been hitherto possible. When 8 mg/kg CDDP were combined with 750 mg/kg DDTC and HT, the rate of growth of local tumors was decreased by 90% compared to the untreated controls, and by >75% compared to the CDDP plus DDTC or HT plus DDTC treatments. A similarly impressive effect was observed against macroscopic metastases. These results thus demonstrated an increased tumor response and suggested the possibility that host survival benefit may ensue.

By using a 3-treatment schedule at 5-day intervals consisting of 4 mg/kg CDDP, 500 mg/kg DDTC, and 60 min of HT (43°C), followed by surgical excision of the local tail tumor, it was possible to obtain long-term survival benefits in 70% of the mice. This compared very favorably with the untreated (0%), DDTC plus HT (10%), and CDDP (20%) controls. With CDDP plus HT, 20% host mortality was seen which negated any benefit that was noted in the remaining mice in this group. The survival benefit seen in the group treated with CDDP, DDTC, and HT followed by surgical excision of the local tumor was not merely due to the relatively small size (0.44 ± 0.1 cm geometric mean diameter) of the tumors at the time of resection. This is evidenced by the finding that when the untreated tumors of similar or smaller sizes were resected, fewer than 10% of the mice lived for over 60 days.

The survival benefits obtained by the triple combination were not restricted only to the mice that underwent surgical treatment. Three treatments with 8 mg/kg CDDP with DDTC and HT repeated at 5-day intervals prolonged the host survival from 34 ± 5 days in the controls to 61 ± 6 days. One of the mice treated 4 times with 8 mg/kg CDDP and HT survived for over 80 days, which was never seen before. Thus, high dose CDDP in combination with DDTC and HT prolonged the host survival far beyond what could be achieved by the highest tolerable dose of CDDP with or without HT.

In conclusion, DDTC protects the hosts against CDDP toxicity and under appropriate treatment conditions, it does not alter the antitumor effects of CDDP. Combination of CDDP with localized HT selectively increases the tumor response. By integrating the procedures that reduce the CDDP-induced host toxicity and those that selectively increase the tumor response, a significant improvement in the therapeutic efficacy of CDDP may be obtained.

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