Time- and Concentration-dependent Inhibition of the Clonogenic Growth of N-[4-(5-Nitro-2-furyl)-2-thiazolyl]formamide-induced Murine Bladder Tumor Cell Lines by cis-Diamminedichloroplatinum(II)\(^1\)

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ABSTRACT

The influence of the concentration and time of exposure to cis-diamminedichloroplatinum on the inhibition of the clonogenic growth of three N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide mouse bladder tumor cell lines was evaluated in a colony assay. Drug testing was performed in the murine model, and tumor cells were removed from the animals for in vitro testing. Murine drug testing revealed marked cis-diamminedichloroplatinum sensitivity of all three mouse bladder tumor lines. One-hr incubation in cis-diamminedichloroplatinum was an inadequate time of drug exposure to produce in vitro colony survival curves predictive of in vivo sensitivity to the drug. Furthermore, it was found that 6- to greater than 24-hr exposure to the drug was required to produce colony survival curves in the tumor colony assay predictive of tumor sensitivity. High drug concentrations using 1-hr drug incubation or continuous incubation in drug both produced colony survival curves predictive of tumor sensitivity. Both methods, however, would require higher products of the drug concentration multiplied by time curves than could theoretically be clinically achievable in the murine model. Until pharmacokinetic data on cis-diamminedichloroplatinum are available in this murine model, higher drug sensitivity boundaries than are presently being used for other chemotherapeutic agents will have to be utilized when testing these mouse bladder tumor cell lines for their sensitivity to cis-diamminedichloroplatinum in a tumor colony assay.

INTRODUCTION

In vitro culture systems are being evaluated for their ability to predict in advance whether a neoplasm will respond clinically to a particular anticancer drug. Recently, Hamburger and Salmon (4) devised a 2-layer agar culture system capable of supporting the clonogenic growth of a wide variety of human tumors. Utilizing this TCA,\(^3\) anticancer drug sensitivity testing has correlated closely with observed clinical responsiveness to the drug. Our early in vitro attempts at testing DDP against the MBT lines indicated resistance to DDP. The purposes of this study were to evaluate the effect of DDP on clonal growth by varying the time and the concentration of the drug and to determine the optimal method of testing DDP against the FANFT-induced MBT cell lines in a TCA.

MATERIALS AND METHODS

MBT Cell Lines. Three MBT cell lines (MBT-2, MBT-8, and MBT-683) were used for in vivo and in vitro drug testing. Each of these tumors originated as an invasive bladder cancer in a female C3H/He mouse after ingestion of FANFT for an average of 11 months. Tumor lines have been maintained by serial transplantation of syngeneic mice.

Murine Drug Testing. The methods for murine drug testing have been reviewed recently (12, 15). Briefly, 1 x 10\(^6\) viable MBT tumor cells were injected into the hind limbs of female C3H/He mice. Mice were randomized into control (10 to 15 mice) and treatment (10 to 12 mice) groups. DDP (6 mg/kg) was given by i.p. injection on Days 7, 14, and 21. Tumors were usually palpable by 12 days after tumor cell inoculation. Tumor sizes were monitored by biweekly measurements of the tumor diameters. The 2 measuring days after the last injection of drug was given were used for comparing tumor sizes in control and treated groups. The differences between control and treatment groups were determined by Student's t test.

TCA Culture Methods. Cells were cultured using a modified method of Hamburger and Salmon (4). One ml of underlayer without BALB/c mouse conditioned spleen media in 0.5% agar was plated on 35-mm plastic Petri dishes. Tumor cells were suspended in 0.3% agar with enriched CMRL (Grand Island Biological Co., Grand Island, N. Y.) and 15% horse serum (Flow Laboratories, Newbury Park, Calif.). The number of cells plated was 20,000 to 50,000/plate when removed from short-term culture and 500,000/plate when tumor cells were removed directly from the mouse. Cultures were incubated at 37\(^\circ\) in 5% CO\(_2\) and 100% humidified atmosphere. Cultures were examined with an Olympus CK inverted microscope at x40 and x100. Final colony counts were made to 14 days after plating. Aggregates of 30 or more cells were scored as colonies.

Preparation of Cell Suspensions. Tumor tissue was aseptically excised from the mouse, minced with scissors, and resuspended in enriched CMRL with 15% horse serum (4). Cell clumps were further separated with scalpels and passed through 21- to 25-gauge needles.

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\(^3\) The abbreviations used are: TCA, tumor-cloning assay; FANFT, N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide; MBT, mouse bladder tumor; DDP, cis-diamminedichloroplatinum(II); CMRL, Connaught Medical Research Laboratories Medium 1066; C x t, area under the drug concentration (\(\mu g/ml\)) versus time (hr) curve (\(\mu g/hr/ml\)).

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If clumps were still present, the cell suspension was passed through sterile gauze. Viable nucleated cells were counted in a hemocytometer, and the viability as determined by trypan blue exclusion was between 70 and 90%.

MBT cells were also maintained in long-term culture. Cells were maintained in 100-mm plastic Petri dishes in Roswell Park Memorial Institute Tissue Culture Medium 1640 (Grand Island Biological Co.): 0.2% sodium bicarbonate:20% newborn calf serum (Grand Island Biological Co.): Fungizone (0.2 μg/ml) (Grand Island Biological Co.): Garamycin (50 μg/ml) (Schering, Kenilworth, N. J.). Confluent cells were subcultured at 1:10 dilutions after 10-min incubation with 0.175% trypsin in calcium- and magnesium-free phosphate-buffered saline (Grand Island Biological Co.). Cells grown in culture were harvested for testing by incubation in 0.175% trypsin in Hanks’ balanced salt solution for 10 min. The cells were centrifuged at 1100 rpm for min, the supernatant was removed, and the cells were resuspended in media. The viable nucleated cell counts as determined in a hemocytometer with trypan blue were greater than 90%.

Drug Preparation. DDP (Bristol Laboratories, Syracuse, N. Y.) was utilized in all drug-testing experiments. DDP was reconstituted in sterile water, diluted to the desired concentration, placed in 1.5-ml volumes in 6-ml plastic Falcon centrifuge tubes, and stored at —80°C. Prior to use, drugs were thawed in a 37°C water bath. Drug testing was done at 3 concentrations (0.9, 0.15, and 0.38 μg/ml). Other experiments utilized 1-log (3.8 μg/ml) and 2-log (38 μg/ml) doses above the standard high DDP (0.38 μg/ml) concentration. Continuous incubation experiments utilized doses of 0.0001, 0.0011, 0.011, and 0.38 μg/ml. Single-cell suspensions of MBT cells were prepared as described above and adjusted to a final desired number of cells/ml in CMRL and 15% horse serum.

Drug-testing Experiments. Drug studies were performed to compare colony survival curves after 1-hr drug incubation versus continuous drug incubation. At the end of in vivo drug studies using MBT-2, MBT-8, and MBT-683, primary tumors from 3 mice in each control group were removed, pooled, and either drug tested immediately or put in short-term culture for testing after 1 to 5 passages. One hr incubation was performed by incubating tumor cells in media for 1 hr in a 37°C water bath and then washing the drug from the cells 3 times with CMRL. After washing, cells were plated in triplicate and incubated at 37°C in 5% CO2. Simultaneous DDP drug studies were done in which cells were put into culture in the presence of drug without washing the drug off (continuous incubation). The in vivo versus in vitro tumor cell sensitivities to DDP were compared. In all studies, DDP was deemed effective if greater than 80% colony inhibition occurred at the high DDP drug concentration.

Results Table 1 lists the results of the murine drug studies using the 3 MBT cell lines (MBT-2, MBT-8, and MBT-683). DDP (6 mg/kg) significantly inhibited the growth of all 3 MBT cell lines when compared with the controls. In vitro drug studies were done using cells derived from the control mice sacrificed at the end of the murine drug study. Drug assays were performed using 1-hr and continuous incubation with DDP. Table 2 represents colony survival data from these in vitro studies. Using the standard 1-hr drug incubation, colony survival curves predictive of tumor resistance were produced in all 3 MBT cell lines. Continuous incubation with DDP resulted in marked inhibition of clonogenic growth even at the lowest drug concentrations in the 3 MBT cell lines resulting in colony survival data predictive of tumor sensitivity.

Using MBT cells derived from long-term culture, drug studies were performed to determine the optimal DDP exposure time required to produce colony survival curves predictive of tumor sensitivity. Tumor cells from each MBT cell line were exposed to DDP for 1, 6, 12, and 24 hr and continuously. Chart 1 represents colony survival curves produced from increasing

**Table 1**

<table>
<thead>
<tr>
<th>MBT cell line</th>
<th>Treated group</th>
<th>Total no. of mice</th>
<th>No. of tumors</th>
<th>Tumor diameter</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBT-2 Controls</td>
<td>11</td>
<td>11 (100)</td>
<td>13 (93)</td>
<td>10.7 ± 2.60</td>
<td>0.001</td>
</tr>
<tr>
<td>MBT-2 DDP treated</td>
<td>11</td>
<td>11 (100)</td>
<td>13 (93)</td>
<td>10.7 ± 2.60</td>
<td>0.001</td>
</tr>
<tr>
<td>MBT-8 Controls</td>
<td>14</td>
<td>14 (96)</td>
<td>14 (96)</td>
<td>12.0 ± 2.20</td>
<td>0.0001</td>
</tr>
<tr>
<td>MBT-8 DDP treated</td>
<td>11</td>
<td>11 (100)</td>
<td>14 (100)</td>
<td>12.0 ± 2.20</td>
<td>0.0001</td>
</tr>
<tr>
<td>MBT-683 Controls</td>
<td>14</td>
<td>14 (96)</td>
<td>14 (96)</td>
<td>12.0 ± 2.20</td>
<td>0.0001</td>
</tr>
<tr>
<td>MBT-683 DDP treated</td>
<td>11</td>
<td>11 (100)</td>
<td>14 (100)</td>
<td>12.0 ± 2.20</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

Numbers in parentheses, percentage. Mean ± S.D.
time of exposure to DDP in MBT-2, MBT-8, and MBT-683. From 6- to greater than 24-hr incubation in DDP was required to produce colony survival curves predictive of sensitivity to DDP. Increasing the length of exposure to DDP without changing the drug concentrations increasingly inhibited colony survival.

Table 3 represents colony survival curves produced after 1-hr incubation of MBT cells in standard and ultrahigh concentrations of DDP. One-log (3.80 µg/ml) drug concentrations in the MBT-683 and MBT-8 studies and 2-log (38.00 µg/ml) drug concentrations in MBT-2 were required to produce colony survival curves predictive of in vivo sensitivity to DDP. These drug concentrations multiplied by time would represent a C x t of 3.6 µg·hr/ml for MBT-683 and MBT-8 and 38.0 µg·hr/ml for MBT-2.

In order to determine the lowest DDP drug concentration with continuous incubation that would produce colony survival curves predictive of sensitivity to DDP, we performed drug assays using continuous exposure to drug concentrations from 0.0001 to 0.38 µg/ml. Table 4 represents the colony survival data from that study. MBT-2 and MBT-683 required a DDP concentration of between 0.11 and 0.38 µg/ml in order to produce significant inhibition of colony formation with continuous exposure to the drug. These drug C x t values would represent a C x t of between 36.0 and 127.6 µg·hr/ml. MBT-8 required a DDP concentration between 0.011 and 0.11 µg/ml or a C x t between 3.6 and 36.0 µg·hr/ml. In order to evaluate the effect of horse serum on the activity of DDP in inhibiting the clonogenic growth of these tumors, we performed 1-hr drug incubations in media containing increasing concentrations of horse serum from 0 to 15%. The concentration of horse serum had no effect on the colony survival curves produced after 1-hr exposure to DDP in any of the MBT cell lines. In all experiments, colony survival curves remained in the resistant range regardless of the concentration of horse serum.

**DISCUSSION**

One-hr drug incubation has been used as a standard time of exposure to anticancer agents in the TCA when drug testing human tumors (11). The clonogenic growth of the MBT cell lines included in this study was not significantly inhibited by DDP after 1-hr drug incubation. Simultaneous murine drug

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**Table 2**

<table>
<thead>
<tr>
<th>MBT cell line</th>
<th>Source of tumor cells</th>
<th>Time of DDP exposure</th>
<th>% of colony survival at DDP concentration (µg/ml)</th>
<th>Interpretation of colony survival data</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBT-2</td>
<td>From primary tumor</td>
<td>1 hr</td>
<td>Control 0.09 100 185 109 77 R</td>
<td></td>
</tr>
<tr>
<td>MBT-8</td>
<td>Short-term culture</td>
<td>1 hr</td>
<td>Continuous 100 6 3 0 S</td>
<td></td>
</tr>
<tr>
<td>MBT-683</td>
<td>Short-term culture</td>
<td>Continuous</td>
<td>100 85 46 44 R</td>
<td></td>
</tr>
</tbody>
</table>

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\( ^a \) R, resistant; S, sensitive.

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Chart 1. Colony survival curves produced using MBT cell lines. A, MBT-2; B, MBT-8; and C, MBT-683. Increasing the time of exposure to DDP in the face of constant drug concentrations (0.09, 0.15, and 0.38 µg/ml) resulted in increasing inhibition of clonogenic growth of these murine tumor cell lines. ○, 1 hr; ●, 6 hr; ▲, 12 hr; ▲, 24 hr; □, continuous.
Effect of 1-hr incubation on colony survival using ultrahigh drug concentrations of DDP

Table 3

<table>
<thead>
<tr>
<th>MBT cell line</th>
<th>Time of DDP exposure (hr)</th>
<th>% of colony survival at following DDP concentration</th>
<th>Interpretation of colony survival data</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBT-2</td>
<td>Control</td>
<td>0.09 µg/ml 0.15 µg/ml 0.38 µg/ml 3.80 µg/ml 38.00 µg/ml</td>
<td>S (at 38.00 µg/ml)</td>
</tr>
<tr>
<td>MBT-8</td>
<td>1</td>
<td>100 91 94 78 37 19</td>
<td>S (at 3.80 µg/ml)</td>
</tr>
<tr>
<td>MBT-683</td>
<td>1</td>
<td>100 92 107 40 2 2</td>
<td>S (at 3.80 µg/ml)</td>
</tr>
</tbody>
</table>

* S, sensitive.

Continuous exposure to DDP using low drug level

Table 4

<table>
<thead>
<tr>
<th>MBT cell line</th>
<th>% of colony survival at following DDP concentration</th>
<th>Interpretation of colony survival data</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBT-2</td>
<td>Control 0.0011 0.0111 0.011 0.038</td>
<td>S (at 0.38 µg/ml)</td>
</tr>
<tr>
<td>MBT-8</td>
<td>100 µg/ml 99 104 88 36 3</td>
<td>S (at 0.11 µg/ml)</td>
</tr>
<tr>
<td>MBT-683</td>
<td>100 105 84 110 62 3</td>
<td>S (at 0.38 µg/ml)</td>
</tr>
</tbody>
</table>

* S, colony inhibition in the sensitive range (<20% colony survival).

C × t 1 to 2 logs higher than a theoretically achievable C × t (0.38 µg/hr/ml). Drug concentrations required for the production of sensitive colony survival curves using continuous drug exposure also required C × t 1 to 2 logs higher than would theoretically be clinically achievable. These C × t values, produced by either 1-hr or continuous drug incubation, could not be achieved in vivo and therefore would not reflect accurately clinical sensitivity of MBT cells to DDP. Caution must be used, however, in using pharmacokinetic data derived from human drug data when dealing with a murine model. Similar DDP pharmacokinetic data are not presently available in this murine model.

It has been demonstrated that DDP acts through the inhibition of DNA synthesis as a result of interstrand and intrastrand cross-linking of the DNA molecule (9, 17). Bergerat et al. (1) have demonstrated previously that, in monolayer cultures of human colon carcinoma (LoVo) cells, the DDP exposure time was directly related to cell kill with maximal cell kill occurring after 24-hr exposure to drug. Using these human colon cancer cells, they later demonstrated (2) that, at a concentration of 0.5 µg/ml, significant DNA distributional changes occurred in vitro only after 24- and 48-hr exposure to DDP. With increasing time of exposure to the drug, a slow decline in early S-phase accumulation was accompanied by an increased accumulation of cells in the G2 phase of the cell cycle. S- and G2-phase blocks were progressively converted to complete blocks by increasing the exposure times or increasing the drug concentrations to 5 to 10 µg/ml. Our findings of time-dependent DDP inhibition of the clonogenic growth of the MBT cell lines could reflect such time-dependent DNA distributional changes in these cell lines.

Using a human leukemia cell line, Takahashi et al. (16) demonstrated that the activity of DDP was inhibited by human serum albumin. We examined the effect of horse serum on the activity of DDP by performing 1-hr incubations in DDP with increasing concentrations of horse serum from 0 to 15% prior to doing drug assays. We were unable to demonstrate an inhibition of the biological activity of DDP by these concentrations of horse serum. This is not surprising since the low concentrations of serum albumin in our media would not approach 2.5 g of serum albumin per dl utilized in the above-mentioned experiments.

Using FANFT-induced MBT cell lines, we have demonstrated that the sensitivity to DDP in the TCA is both time and concentration dependent. Either increasing the 1-hr in vitro DDP concentration to high levels or utilizing continuous DDP incubation will produce colony survival curves which correlate with the tumor sensitivity found in vivo. At the present time, however, neither method can be recommended, since both methods entail utilizing C × t values that may be too high to be clinically achievable. Utilizing either method may represent a cytotoxic effect of high concentrations of DDP rather than a clinical tumor sensitivity to the chemotherapeutic effect of the drug.

Finally, our criterion for drug sensitivity, for DDP in the TCA of 20% colony survival may be too rigid for this drug. Moon (8) has developed drug sensitivity index boundaries for many chemotherapeutic agents used in human drug testing using a TCA. The sensitivity index is described as the area under the colony survival curve between 0 and the upper drug concentration. A boundary of sensitivity indexes has then been determined from clinical data below which the patient would be predicted to respond to a given drug. The drug sensitivity index...
boundary for DDP in their hands is one of the highest of any of the drugs they studied. Therefore, we recommend using higher drug sensitivity boundaries for DDP or using a drug sensitivity cutoff less than 80% (60 to 65%) when drug testing FANFT-induced MBT cell lines in this clonogenic assay. Further investigation is necessary to determine why the drug sensitivity boundary is higher for DDP than for other chemotherapeutic agents when drug testing FANFT-induced MBT cell lines in a TCA.

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