In Vitro Cytotoxicity of a Novel Antitumor Antibiotic, Spicamycin Derivative, in Human Lung Cancer Cell Lines

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ABSTRACT

Spicamycin (SPM), produced by Streptomyces alanosinicus, induces potent differentiation in a human leukemia cell line, HL60. One of the derivatives of SPM (SPM-D), KRNS500, has a wide range of antitumor activity against human cancer cell lines. We examined the cytotoxicity of SPM-D in small and non-small cell lung cancer cell lines using 3-(4,5-dimethylthiadiazon-2-yl)-2,5-diphenyltetrazolium bromide and colony assays. SPM-D was active against a wide range of lung cancer cell lines. All three cisplatin (CDDP)-resistant cell lines established in our laboratory (PC-9/CDDP, PC-14/CDDP, and H69/CDDP) showed collateral sensitivity to SPM-D with relative resistance values of 0.43, 0.34, and 0.32, respectively. Intracellular SPM-D in PC-14/CDDP was 35% higher than that for PC-14 suggesting that intracellular accumulation can explain the collateral sensitivity to SPM-D at least in PC-14/CDDP. On the other hand, in PC-9/CDDP cells, no increase of intracellular SPM-D accumulation was observed, but the conversion ratio of a metabolite (the amino nucleoside moiety of spicamycin binding with glycine, SAN-G) from SPM-D evaluated by TLC was higher as compared with that of parental PC-9 cells (45.5% versus 37%; PC-9/CDDP versus PC-9). The increased intracellular metabolism of SPM-D could explain the mechanism of collateral sensitivity in PC-9/CDDP spicamycin-resistant cell lines. To elucidate the determinant of the SPM-D-induced cytotoxicity, we established SPM-D-resistant cell lines, PC-9/SPM-D, PC-14/SPM-D, and H69/SPM-D, by exposing cells to stepwise increases in SPM-D concentration. The relative resistances of these sublines were more than 5000, 46.6, and 37.8 times those of the parental cell lines, respectively. The intracellular concentration of the active metabolite, SAN-G, was found to be decreased in the SPM-D-resistant sublines. This result indicates that the intracellular metabolism of SPM-D to SAN-G is one of the determinants of cellular sensitivity to SPM-D in these SPM-D-resistant cell lines. In conclusion, both drug accumulation and metabolism may contribute to the sensitivity/resistance to SPM-D and both may merit investigation.

INTRODUCTION

Cisplatin is one of the most effective antitumor agents presently available. However, acquired resistance to cisplatin occurs frequently and some tumors are naturally resistant to the drug. Both types of cellular resistance to cisplatin are major obstacles to the successful treatment of solid tumors; thus there is still an urgent need for more effective and safer drugs. The antibiotic spicamycin, isolated from a culture broth of a streptomycete, Streptomyces alanosinicus, is a potent inducer of differentiation of human promyelocytic leukemia cells (HL60) and M1 myeloid leukemia cells (1, 2). The compound is composed of a long-chain fatty acid, glycine, aminohexose, and adenine. Thus it appears to be one of a family of unusual nucleotides with a variety of fatty acids. The chemical structure of this compound is quite similar to that of septacidin, an antitumor and antifungal antibiotic (3); spicamycin is probably the 2′ epimer of septacidin. Semisynthetic spicamycin derivatives which differ in the chain length of the fatty acid moiety show strong antitumor activity in a human tumor xenograft model (4). In vivo, spicamycin derivatives show antitumor activity against various human stomach and breast cancer cell lines (5). The aims of this study were to examine the antitumor activity of SPM-D against human lung cancer cell lines including cisplatin-resistant sublines and to elucidate the determinant of cellular sensitivity to this promising agent. Interestingly, all three cisplatin-resistant cell lines, PC-9/CDDP, PC-14/CDDP, and H69/CDDP, showed collateral sensitivity to SPM-D.

MATERIALS AND METHODS

Drugs and Chemicals. SPM-D, 14C-labeled SPM-D, and its active metabolite SAN-G were supplied by Kirin Brewery Co., Tokyo, Japan (Fig. 1). SPM-D was dissolved in 100% DMSO and stored at −80°C. It was diluted with RPMI 1640 (Nikken Biomedical Laboratories, Kyoto, Japan) just before each experiment. 14C-Labeled SPM-D was dissolved in methanol:DMSO (95:5). The specific activity of labeled SPM-D was 1.28 Bq/mmol (34.6 mCi)/mmol. Digitonin was purchased from Sigma Chemical Co., St. Louis, MO. Cisplatin was provided from Bristol-Meyers Squibb Japan (Tokyo, Japan). TLC plates, Silica Gel 60F254, were purchased from Merck, Darmstadt, Germany.

Cell Lines and Tissue Culture. Among the human lung cancer cell lines, H69 was a small cell lung cancer cell line and PC-9 and PC-14 were adenocarcinoan lung cancer cell lines. Cell lines were obtained from the Japanese Cancer Research Resource Bank (Tokyo, Japan) with the exception of PC-9, PC-14 (from Professor Y. Hayata, Tokyo Medical College), and H69 (from the National Cancer Institute, Bethesda, MD). Cisplatin-resistant PC-9/CDDP (5), PC-14/CDDP (6), and H69/CDDP (7) were established by exposing PC-9/0.5, PC-14/1.5 and H69/0.4 cells, respectively, to cisplatin for 1 year. These cell lines were established in our laboratory by exposing PC-9, PC-14, and H69 cells continuously to gradually increasing concentrations of cisplatin and use of the limiting dilution technique (8). The cisplatin resistance of each cell line was confirmed to be maintained at a constant level in cisplatin-free medium for at least 1 year (data not shown). The cells were incubated with RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin at 37°C in the presence of 5% CO2.

Growth Inhibition Assay. To determine the cytotoxicity of the drugs, we performed the tetrazolium dye assay of Mosmann (9) modified as described previously (10). Briefly, 1 to 4 × 105 cells were seeded and incubated overnight in each well of a 96-well plate and 20 µl of SPM-D at each concentration were added. After 72 h of incubation at 37°C in the presence of 5% CO2, 20 µl of MTT dye (Sigma) were added to each well and the plates were incubated for 4 h under the same conditions as above. After centrifugation of the plates at 800 × g for 5 min, the medium was discarded completely. Formazan crystals were solubilized by addition of 200 µl of DMSO to each well. Absorbances were recorded at 562 and 603 nm on an EL-340 microplate reader (Bio-Tek Instruments, Inc., Winooski, VT). Wells containing only medium and MTT were used as a negative control. Each experiment was performed using 6 replicate wells for each drug concentration and more than

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2 Recipient of a resident research fellowship from the Foundation for Promotion of Cancer Research.
3 To whom requests for reprints should be addressed.

The abbreviations used are: SPM-D, spicamycin derivative; SAN-G, the amino nucleoside moiety of spicamycin binding with glycine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; IC50, drug concentration inhibiting cell growth by 50%; CDDP, cisplatin.
The IC₅₀ was defined as the drug concentration required for 50% reduction of the absorbance in each test and determined graphically from dose-response curves. The relative resistance was defined as IC₅₀ of the resistant subline/IC₅₀ of the parental cell line.

**Colony Assay.** To confirm the data obtained from the MTT assay, we performed colony assays with PC-9, PC-9/CDDP, PC-14, and PC-14/CDDP. The colony assay performed in this study was a modification of Hamburger-Salmon’s double agar method (11). In brief, cells cultured in RPMI 1640 supplemented with 10% fetal bovine serum were harvested, and cell viability was checked by trypan blue exclusion. One ml of 1 × 10⁶ cells (PC-14 and PC-14/CDDP) or 5 × 10⁵ cells (PC-9 and PC-9/CDDP) in 0.3% agar in RPMI 1640 with 10% fetal bovine serum was seeded onto 6-well plates on the underlayer of 0.5% agar containing enriched McCoy’s 5A medium (Gibco, Tokyo, Japan) in the presence of each concentration of SPM-D. The plates were incubated at 37°C in the presence of 5% CO₂. From the 7th day of incubation the numbers of colonies were counted every day. Colonies larger than 1000 cells were counted as one colony. Twenty colonies were randomly selected for each concentration of SPM-D or SAN-G. The plates were dried and the image was developed with BAS 2000 (Fuji Photo Film Co., Tokyo, Japan). The radioactivity of each band was calculated and normalized by the content of total protein for each cell line. Protein content was determined using the BCA protein assay reagent.

**Establishment of SPM-D-resistant Sublines.** We established the SPM-D-resistant sublines PC-9/SPM-D, PC-9/SPM-D, and H69/SPM-D by exposing PC-9, PC-14, and H69 cells to stepwise increases in SPM-D concentration. In brief, each parental cell line was exposed continuously to SPM-D starting from 10 nM and then increasing to 40 μM by stepwise increases in drug concentration. The resistant sublines were isolated by the limiting dilution method as described previously (8). The resistant sublines were stable for at least 1 month in drug-free medium.

**Digitonin Permeabilization of Cells.** To clarify the cytotoxic effect of SPM-D, PC-9, PC-9/CDDP, and H69/SPM-D were made permeable to SAN-G by digitonin. The MTT assay was then performed with the permeabilized cells, by a modification of Smolen’s method (12). In brief, 10 μl of 2 mg/ml digitonin (Sigma) were added to 1 ml of cell suspension containing 1 × 10⁶ cells with a final digitonin concentration of 20 μg/ml. The cells were washed with 10 ml of warm medium twice 1 min later and dispersed in warm medium again, and the permeabilized cells were seeded at 1 × 10⁶/well in 96-well plate. Each concentration of SPM-D or SAN-G was then added. Growth inhibition was assayed after 72 h of incubation using the MTT assay.

### RESULTS

**Collateral Sensitivity of Cisplatin-resistant Sublines to SPM-D by Colony Assay.** There was a wide range of IC₅₀ values among the human lung cancer cell lines, ranging from 0.01 to 2 μM by MTT assay (data not shown). The IC₅₀ values were relatively lower than the IC₅₀ values for the so-called “key agent” cisplatin.

We evaluated the sensitivity of several drug-resistant cell lines to SPM-D. Interestingly, cisplatin-resistant sublines showed collateral sensitivity to SPM-D (Table 1). The relative resistance ratios of PC-9/CDDP, PC-9/CDDP, and H69/CDDP for cisplatin were 8.59, 7.63, and 2.48, respectively, whereas those for SPM-D were 0.33, 0.34, and 0.32, respectively. The collateral sensitivity to SPM-D was confirmed by colony assay in these cisplatin-resistant cell lines (Fig. 2). Although the IC₅₀ of SPM-D in the colony assay was higher than that in the 3-day MTT assay, the cisplatin-resistant sublines also showed collateral sensitivity to SPM-D.

### Table 1 Growth-inhibitory effect of CDDP and SPM-D in cisplatin-resistant cell lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>IC₅₀ of CDDP (μM)</th>
<th>RR</th>
<th>IC₅₀ of SPM-D (μM)</th>
<th>RR</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC-9</td>
<td>0.53 ± 0.24</td>
<td>1.00</td>
<td>0.06 ± 0.01</td>
<td>1.00</td>
</tr>
<tr>
<td>PC-9/CDDP</td>
<td>4.92 ± 0.93</td>
<td>8.59</td>
<td>0.02 ± 0.01</td>
<td>0.33</td>
</tr>
<tr>
<td>PC-14</td>
<td>1.6 ± 0.25</td>
<td>1.00</td>
<td>0.44 ± 0.11</td>
<td>1.00</td>
</tr>
<tr>
<td>PC-14/CDDP</td>
<td>7.63 ± 1.19</td>
<td>7.63</td>
<td>0.15 ± 0.05</td>
<td>0.34</td>
</tr>
<tr>
<td>H69</td>
<td>3.77 ± 0.25</td>
<td>1.00</td>
<td>2.0 ± 0.1</td>
<td>0.60</td>
</tr>
<tr>
<td>H69/CDDP</td>
<td>9.33 ± 1.15</td>
<td>2.49</td>
<td>0.63 ± 0.15</td>
<td>0.32</td>
</tr>
</tbody>
</table>

* Cells were exposed to cisplatin at the indicated concentration, followed by incubation for 3 days. Results are presented as means ± SD of 3 independent experiments.

* RR, relative resistance: RR = IC₅₀ of resistant cell/IC₅₀ of parental cell.
showed collateral sensitivity to SPM-D in the colony assay. The relative resistance ratios of PC-9/CDDP and PC-14/CDDP were 0.55 and 0.29, respectively. These results suggest that at least cell lines with acquired cisplatin resistance are hypersensitive to SPM-D in vitro. To clarify whether there was a negative relationship between cisplatin sensitivity and SPM-D sensitivity, the sensitivities of 15 human lung cell lines to both drugs were examined. However, there was no relationship between sensitivity to cisplatin and SPM-D (data not shown).

Intracellular Accumulation of SPM-D in Cisplatin-resistant Cell Lines. To elucidate the mechanism of the collateral sensitivity to SPM-D, the intracellular accumulation of SPM-D was compared between two pairs of cisplatin-resistant and parental cells (Fig. 3). The relative intracellular concentration of SPM-D, which was defined as total uptake of $^{14}$C-labeled SPM-D/cell volume (1 x $10^3$ cells), in PC-9/CDDP was 62% of that for PC-9. Intracellular SPM-D in PC-14/CDDP was 35% higher than that for PC-14. These results suggest that intracellular accumulation can explain the collateral sensitivity of to SPM-D at least in PC-14/CDDP, because the colony-forming assay with PC-9 and PC-14 demonstrates a very steep dose-response curve for SPM-D. A 10% increase in concentration is observed between approximately IC$_{90}$ and IC$_{10}$. In light of this, it would seem entirely plausible that a 30% increase in intracellular accumulation alone could account for the observed collateral sensitivity in at least PC-14/CDDP. However, the mechanism for the collateral sensitivity in PC-9/CDDP remained unclear.

Intracellular Metabolism of SPM-D in Cisplatin-resistant Cells. To elucidate the mechanism of the collateral sensitivity, the intracellular metabolism of SPM-D was analyzed by TLC in PC-9 and PC-9/CDDP. After incubation of the cells with $[14C]$SPM-D for 2 h, the cell extract was loaded onto a TLC plate. Three radioactive bands were identified on the plate. The top band ($R_f = 0.9$) was identified as SPM-D, and the bottom one ($R_f = 0.1$) as SAN-G, which is an active metabolite composed of adenine, an unusual amino sugar, and glycine. Because the central carbon of glycine is labeled with $^{14}$C in labeled SPM-D, this metabolite was detected easily. Using nonlabeled SAN-G as a marker, we confirmed that the bottom band coincided with the SAN-G band in a TLC experiment (data not shown). The chemical structure corresponding to the middle band ($R_f = 0.45$), which was the faintest one, has not yet been determined. Most of the SPM-D was extracted by the chloroform/methanol mixture, and all of the metabolite was extracted by water:methanol. In PC-9/CDDP, the intracellular conversion ratio from SPM-D to SAN-G were increased from 37% to 46% as compared with those of PC-9. This result suggests that the increased sensitivity of PC-9/CDDP might be due to increased activation of SPM-D to SAN-G.

SPM-D-resistant Sublines. We established the SPM-D-resistant subline, PC-9/SPM-D, from PC-9 to elucidate the mechanism of SPM-D cytotoxicity. PC-9/SPM-D showed more than 5000-fold resistance to SPM-D as compared with PC-9. It was impossible to calculate the IC$_{50}$ for PC-9/SPM-D within the concentration of SPM-D that we used. The resistance of PC-9/SPM-D was stable for at

Intracellular accumulation of SPM-D in cisplatin-resistant and parental cells. The cells were incubated for 30 min with 1 $\mu$l of $[14C]$SPM-D (20 $\mu$Ci/ml). The relative concentration of SPM-D was determined by

\[
\text{Relative conc. of SPM-D (DPM)} = \frac{\text{Total uptake of SPM-D}}{\text{Volume of each cell}} \times \frac{\text{Volume of PC-9}}{10^4}
\]
Table 2 Cytotoxicity of various drugs in SPM-D-resistant cell lines

<table>
<thead>
<tr>
<th></th>
<th>PC-9</th>
<th>PC-9/SPM-D</th>
<th>Relative resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPM-D (μM)</td>
<td>0.06 ± 0.01</td>
<td>&gt;3000</td>
<td>&gt;5000</td>
</tr>
<tr>
<td>CDDP (μM)</td>
<td>0.53 ± 0.24</td>
<td>0.53 ± 0.16</td>
<td>0.99</td>
</tr>
<tr>
<td>Taxol (nm)</td>
<td>39.8 ± 26.4</td>
<td>68.67 ± 25.72</td>
<td>1.73</td>
</tr>
<tr>
<td>Vindesine (nm)</td>
<td>3.73 ± 1.05</td>
<td>4.57 ± 2.06</td>
<td>1.22</td>
</tr>
<tr>
<td>Doxorubicin (μM)</td>
<td>0.11 ± 0.07</td>
<td>0.19 ± 0.05</td>
<td>1.78</td>
</tr>
<tr>
<td>VP-16 (μg/ml)</td>
<td>1.9 ± 0.89</td>
<td>6.23 ± 3.91</td>
<td>3.28</td>
</tr>
<tr>
<td>CPT-11 (μM)</td>
<td>8.6 ± 1.64</td>
<td>10.15 ± 0.21</td>
<td>1.18</td>
</tr>
</tbody>
</table>

* See Table 1, Footnote b. Relative resistance = IC50 of resistant cell/IC50 of parental cell.

** Results are presented as means ± SD of 3 independent experiments.

Table 3 Intracellular accumulation of SPM-D in the resistant sublines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cellular content of SPM-D (dpm)</th>
<th>Relative concentration of SPM-D (dpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC-9</td>
<td>2405 ± 482</td>
<td>2405 ± 482</td>
</tr>
<tr>
<td>PC-9/SPM-D</td>
<td>1808 ± 441</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PC-14</td>
<td>1533 ± 189</td>
<td>1735 ± 214</td>
</tr>
<tr>
<td>PC-14/SPM-D</td>
<td>1574 ± 6</td>
<td>1781 ± 7</td>
</tr>
</tbody>
</table>

* NS, not significant.

least 3 months in the SPM-D-free medium. Table 2 shows the sensitivity of PC-9/SPM-D to several antitumor agents determined by MTT assay. PC-9/SPM-D showed no cross-resistance to any of the drugs except etoposide (a topoisomerase II inhibitor). Because the resistance ratio to etoposide was very small as compared with that for SPM-D (3.28 versus >5000), PC-9/SPM-D seemed to have specific resistance to SPM-D. Unexpectedly, collateral sensitivity for cisplatin was not observed. We also obtained another SPM-D-resistant stable clone, PC-14/SPM-D, from PC-14. PC-14/SPM-D showed 46.6-fold resistance to SPM-D. To elucidate the mechanism responsible for the decreased sensitivity to SPM-D, PC-9/SPM-D and PC-14/SPM-D were used in the following experiments as highly and moderately resistant sublines, along with their respective parental cell lines.

Intracellular Accumulation of SPM-D in SPM-D-resistant Sublines. To elucidate the mechanism of the resistance to SPM-D in PC-9/SPM-D and PC-14/SPM-D, we measured the intracellular accumulation of SPM-D. However, there was no significant difference in the intracellular accumulation of SPM-D between the two cell lines (Table 3). Although the intracellular concentration of SPM-D in PC-9/SPM-D was reduced by more than 30% compared with PC-9, it could not explain the extremely high resistance of PC-9/SPM-D (>5000). These results suggest that a change in SPM-D accumulation is not responsible for the mechanism of resistance.

SPM-D Metabolism in SPM-D-resistant Sublines. We compared the intracellular metabolites of SPM-D in these pairs. After 2 h of incubation, SAN-G was barely detected in PC-9 as band 3 (Fig. 4A). After 12 h of incubation, SAN-G in PC-9 increased remarkably but it could not be detected in PC-9/SPM-D. Band 2 was also detected, although there was no remarkable difference between PC-9 and PC-9/SPM-D. A significant decrease of SAN-G was noticed in PC-9/SPM-D. The decrease in the amount of SAN-G (Fig. 4B, Band 3) was also remarkable in PC-14/SPM-D, which was incubated with 14C-labeled SPM-D for 24 h, although SAN-G was very faint in the sample taken after 12 h of incubation (data not shown). These results suggest that intracellular SAN-G is the factor governing sensitivity to SPM-D.

Cytotoxicity of SAN-G in Digitonin-permeabilized Cell Lines. The above results suggested that SAN-G plays a major role in the cytotoxicity of SPM-D and that drug activation is primarily responsible for sensitivity to SPM-D. To confirm that intracellular metabolism of SPM-D to SAN-G is a determinant of cellular sensitivity to SPM-D, the cytotoxicity of SAN-G was examined. Because SAN-G hardly penetrates the cell membrane, the cells were permeabilized briefly with digitonin. Cell viability determined by the MTT assay and trypan blue staining was more than 95% of the control (data not shown). Under nonpermeabilized conditions, there was a clear difference in SPM-D IC50 among PC-9, PC-9/CDDP, and PC-9/SPM-D. On the other hand, there was no difference in IC50 values among these cell lines when they were permeabilized (Fig. 5). This finding was also demonstrated in PC-14 (data not shown).

DISCUSSION

SPM-D showed a wide range of cytotoxicity in various human lung cancer cell lines with IC50 values of 0.01 to 2 μM by 3-day MTT assay. This drug was effective against both small and non-small cell lung cancer cell lines. In this study, all three cisplatin-resistant cell lines, PC-9/CDDP, PC-14/CDDP, and H69/CDDP, showed collateral...
compounds in general having a fatty acid moiety. Compounds, intracellular metabolism, especially of the fatty acid known to possess a fatty acid moiety in their structure. For these within a few min (data not shown). High permeability is considered to be important for their antitumor activity. Therefore, the one of the advantageous features of SPM-D.

Several antitumor agents such as N4-acyl derivatives of 1-β-D-arabinofuranosylcytosine (13) and palmitoylrhizoxin (14, 15) are its long-chain fatty acid, the intracellular concentration reached a plateau of its fatty acid moiety varies. Among these heterogeneous compounds, one with a 14-carbon fatty acid named SPM-D has been found to be most active in PC-9/CDDP cells. This compound is heterogeneous because the length of its fatty acid moiety is important for their antitumor activity. Therefore, the one of the advantageous features of SPM-D.

There remain alternative explanations for sensitivity and resistance mechanisms. For example, the intracellular availability of the parent compound may be altered by biochemical and biophysical influences, including the cellular content of fatty acid-binding protein and other potential receptors.

Spicamycin was originally isolated from culture broth of S. alanosinicus. This compound is heterogeneous because the length of its fatty acid moiety varies. Among these heterogeneous compounds, one with a 14-carbon fatty acid named SPM-D has been found to be most active against various human solid tumor cell lines such as those of stomach and breast cancer (4). The fatty acid chain in the structure of spicamycin derivatives is responsible for their permeation across the membrane. In fact, we found rapid kinetics of intracellular uptake of SPM-D in the accumulation study. Because this compound is highly lipid soluble due to its long-chain fatty acid, the intracellular concentration reached a plateau within a few min (data not shown). High permeability is considered to be one of the advantageous features of SPM-D.

Several antitumor agents such as N4-acyl derivatives of 1-β-D-arabinofuranosylcytosine (13) and palmitoylrhizoxin (14, 15) are known to possess a fatty acid moiety in their structure. For these compounds, intracellular metabolism, especially of the fatty acid moiety, might be important for their antitumor activity. Therefore, the present results appear to be important not only for SPM-D but also for compounds in general having a fatty acid moiety.

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