Collateral Sensitivity of Human Melanoma Multidrug-resistant Variants to the Polyamine Analogue, \( N^1,N^{11}\)-Diethylnorspermine\(^1\)

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ABSTRACT

Certain \( N \)-alkylated analogues of the natural polyamine spermine, such as \( N^1,N^{11}\)-diethylnorspermine (DENSPM), rapidly deplete intracellular polyamine pools by down-regulating the biosynthetic enzymes, ornithine decarboxylase and \( S \)-adenosylmethionine decarboxylase, and by potently up-regulating the polyamine catabolizing enzyme, spermidine/spermine \( N^3\)-acyetyltransferase. On the basis of previously reported antiproliferative activity in human tumor xenograft model systems, DENSPM is currently undergoing Phase 1 clinical trials against human melanoma and other solid tumors. The antiproliferative activity of this analogue against the multidrug resistance (MDR) phenotype was examined in three MDR sublines of human melanoma RPMI-7932 cells, which were shown to be 2- to 10-fold resistant to classical MDR agents. These MDR lines had been separately derived using different selecting agents (Lemontt et al., Cancer Res., 48: 6344–6353, 1988). Subline functional resistance due to P-glycoprotein was confirmed by decreased retention of rhodamine 123 relative to parent cells as detected by flow cytometry. Although the three sublines were 2- to 10-fold less sensitive than the parent line to classical MDR-type agents, they were found in dose-response studies to be significantly more sensitive to DENSPM than the parent line. In addition, they showed a distinct cytotoxic response after a 48-h treatment with 10 \( \mu \)M DENSPM, which was not apparent in the parent line. Growth sensitivity of the sublines to the ornithine decarboxylase inhibitor, \( \alpha \)-difluoromethylornithine, or the \( S \)-adenosylmethionine decarboxylase inhibitor, CGP-48664, was found to be similar to parent cells. The ratio of the key biosynthetic enzyme activities for ornithine decarboxylase and \( S \)-adenosylmethionine decarboxylase was found to be 3.5- to 5-fold higher in all three sublines, due mainly to increases in the former enzyme. This imbalance produced unusually high putrescine pools. Although DENSPM down-regulation of decarboxylase activities and potent up-regulation of spermidine/spermine \( N^3\)-acyetyltransferase activity occurred similarly in both parent and variant lines, polyamine depletions were greater in the variant lines. Collateral sensitivity of the MDR sublines to DENSPM is partially attributable to the finding that analogue (and spermidine) uptake in the sublines was about 2-fold higher (after 2 h) than in the parent cells. The presence of disturbances in polyamine homeostasis and decreased sensitivity to DENSPM in three independently selected cell line variants suggests that they may be generally associated with the MDR phenotype in human melanoma and possibly other tumor cells. The collateral sensitivity of human melanoma MDR variants to DENSPM represents a possible therapeutic indication which should be considered during the ongoing clinical evaluation of this drug.

INTRODUCTION

Sustained increases in polyamine biosynthesis are a well-recognized component of neoplastic and neoplastic tissues and, therefore, constitute an attractive target for anticancer therapeutic intervention (1, 2). Drug discovery efforts by various academic and industrial programs have lead to the recent or imminent entry of at least five polyamine analogues into clinical trials: three polyamine analogues having potent antitumor activity based on apparently different modes of action (3–6), a new inhibitor of a polyamine biosynthetic enzyme (7, 8), and a reevaluation of the ornithine decarboxylase inhibitor (9), DFMO,\(^3\) as a chemopreventive agent (10, 11).

Our own interests have focused on the polyamine analogue DENSPM (12, 13) and CGP-48664 an inhibitor of \( S \)-adenosylmethionine decarboxylase (7, 8). DENSPM depletes polyamine pools and inhibits tumor cell growth by down-regulating polyamine biosynthesis and inducing polyamine excretion and catabolism (12, 14). On the basis of antitumor activity against human tumor xenografts (3, 4, 15), DENSPM is currently undergoing clinical trial at three institutions. In contrast to these analogues, CGP-48664 depletes intracellular polyamine pools by inhibiting the biosynthetic enzyme, SAMDC (8). Its selectivity for the enzyme is strongly indicated by the finding that Chinese hamster ovary cells, made >600-fold resistant to the drug, overexpressed SAMDC at the level of gene amplification (8). The inhibitor is currently undergoing preclinical development for trials against solid tumors. In human tumor model systems, both DENSPM and CGP-48664 seem to be most effective against solid tumors, especially melanoma (3, 4, 8).

Inherent and acquired resistance of tumors to classical chemotherapeutic agents presents a major problem in cancer chemotherapy (16, 17). Of the many mechanisms known to be potentially responsible, MDR mediated by P-glycoprotein expression is one of the best understood mechanisms and is the most widely implicated due to its ability to confer cross-resistance to a number of clinically effective drugs (18). P-glycoprotein is encoded by the \( mdr-1 \) gene and acts as a pump to transport cytotoxic drugs out of the cell. On the basis of structure and uptake characteristics (8, 19), neither DENSPM nor CGP-48664 would be expected to be affected by P-glycoprotein function. In an attempt to determine whether they may have therapeutic usefulness in treating MDR tumors, we examined the growth sensitivity of MDR human melanoma cell line variants to these agents.

Three independently isolated MDR clones of the drug-sensitive RPMI-7932 human melanoma were obtained from Lemontt et al. (20) and studied. The lines had been derived by single-step selection in culture medium containing 4.5 \( \mu \)g/ml vincristine (VCR-4.5/4), 3 \( \mu \)g/ml vinblastine (VBL-4), or 8 \( \mu \)g/ml colchicine (BMCOL-1). Relative to the parent line, the MDR sublines were found to be unaffected in their sensitivity to CGP-48664 and collaterally sensitive to the analogue DENSPM, due apparently to distinctive shifts in polyamine homeostasis. Thus, while either drug may prove useful in therapeutic strategies targeting MDR, it would appear that the chemotherapeutic effectiveness of DENSPM may actually be improved in tumors displaying the MDR phenotype.

\(^{1}\) The abbreviations used are: DFMO, \( \alpha \)-difluoromethylornithine; DENSPM, \( N^1,N^{11}\)-diethylnorspermine; CGP-48664, 4-aminooindan-1-one 2'-amidinohydrazone; SAMDC, \( S \)-adenosylmethionine decarboxylase; MDR, multidrug resistance; VCR, vincristine; VBL, vinblastine; DMDP, \( N(3,4\text{-dimethoxyphenethyl})N\text{-methyl-2'-}\text{naphthyl} \)m-dithiane-2-propylamine; ODC, ornithine decarboxylase; SSAT, spermidine/spermine \( N^3\)-acyetyltransferase.

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\(^{3}\) The abbreviations used are: DENSPM, diethylnorspermine; CGP-48664, 4-aminooindan-1-one 2' amidinohydrazone; SAMDC, S-adenosylmethionine decarboxylase; MDR, multidrug resistance; VCR, vincristine; VBL, vinblastine; DMDP, N(3,4-dimethoxyphenethyl)-N'-methyl-2'-naphthylm-dithiane-2-propylamine; ODC, ornithine decarboxylase; SSAT, spermidine/spermine N³-acyetyltransferase.

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MATERIALS AND METHODS

Materials. The RPMI-7932 human melanoma cell lines and the three resistant lines were previously derived and characterized by Lemontt et al. (20) and generously provided by Genzyme Corp. (Framingham, MA). The specific inhibitor of ornithine decarboxylase (9), DFMO, was kindly provided by Marion Merrill Dow (Cincinnati, OH); the specific inhibitor of S-adenosylmethionine decarboxylase (8), CGP-48664, by Ciba Geigy (Basel, Switzerland); and the inhibitor of P-glycoprotein function (21, 22), DMDP, by Hoffman-Roche (Basel, Switzerland). The spermine analogue DENSPM was synthesized as described earlier (23, 24). [3H]Spermidine used in uptake studies was purchased from DuPont NEN (Boston, MA). Rhodamine 123 was obtained from Molecular Probe (Eugene, OR).

Cell Culture. RPMI-7932 cells and the MDR sublines, VCR-4.5/4, VBL-4, and BMCOL-1 were grown in α-minimal essential medium containing 10% fetal calf serum at 37°C in the presence of 5% CO2. Cultures were maintained under exponential growth conditions during the course of all experiments. Before being used for experimental purposes, the resistance of each cell line to the different selecting agents was confirmed to be as reported in their initial characterization (20).

Enzyme Assays. ODC and SAMDC activities were assayed according to the methods of Seely and Pegg (25) and Pegg and Poso (26), as described previously (12, 13). For the SSAT enzyme assay, the cell extract was thawed and centrifuged for 1 h at 35,000 rpm using a Spinco 40 rotor. SSAT activity was determined on the resultant supernatant according to the method of Libby et al. (27). In the case of DENSPM-treated cell samples, the enzyme extract was diluted so that SSAT activity fell within the linear range of the assay. It should be noted that this assay also measures enzyme activities other than SSAT that are capable of acetyling spermidine. However, in the case of DENSPM-treated MALME-3M human melanoma cells, we have found that the preponderance (>97%) of the total acetyling activity is SSAT (13).

Polyamine Pools. Cell samples were extracted with 0.6 M perchloric acid and centrifuged, and the supernatant extract was assayed for polyamines and DENSPM by high pressure liquid chromatography using a precolumn dansylation system (28) as described previously by this laboratory (13, 14). Polyamine levels were expressed on the basis of nmol per 10⁶ cells.

Flow Cytometry. To compare P-glycoprotein expression at the cell surface of MDR and parent cells, cells were incubated with MRK-16 antibody (Ref. 30; obtained from Dr. Tsuruo, Tokyo, Japan) and subsequently labeled with R-phycoerytherin-conjugated goat anti-mouse antibody. The assay for P-glycoprotein function (31–34) makes use of the fact that the dye rhodamine 123 behaves like MDR drugs such as doxorubicin and is effluxed out of the cell via the P-glycoprotein system. Thus, dye retention is an indication of P-glycoprotein function. Parent and subline melanoma cells were incubated for 20 min in 5 μg/ml rhodamine 123, washed, and incubated for 60 min in media to allow efflux of the dye; after which, cellular fluorescence was analyzed by flow cytometry. In variations of this scheme, cells were sometimes pretreated with 10 μM DENSPM or 10 μM CGP-48664 for 24 h to deplete polyamine pools, or they were treated with 2 μM DMDP during the dye uptake incubation, wash,
and efflux period to inhibit P-glycoprotein function and prevent dye efflux (21, 22). Rhodamine 123-containing cells were then analyzed on a FACScan (Becton Dickinson, San Jose, CA; dye emission was collected through a 530/30 band pass filter).

RESULTS

P-Glycoprotein Expression and Function. P-Glycoprotein expression of the various sublines was confirmed by whole cell labeling with MRK-16 antibody (30, 34) and quantitated by flow cytometry (Fig. 1). By peak fluorescence values, the sublines stained about 4- to 10-fold more intensely than the parent line (10.5 versus 45 to 110). The functional resistance of the sublines was then confirmed by a rhodamine 123 retention assay in which dye content was quantitated by flow cytometry (Fig. 1). During the 1-h postlabeling period, the sublines effluxed at least 10 times more rhodamine 123 than the parent line (both contained similar amounts of the dye following the 20 min labeling period). To demonstrate that this loss was mediated by P-glycoprotein, cells were treated with the calcium channel blocker DMDP during the dye uptake incubation, wash, and efflux period. While the parent lines were unaffected by this treatment, rhodamine

Fig. 2. Effects of 10 μM DENSPM on the growth of RPMI-7932 and the three MDR variants VCR 4.5, VBL 4.5/4, and BMCOL-1 over a 72 h time course. Cells were plated at −24 h and treated with analogue at 0 h. Note that, under control conditions, the growth rate of the parental cells was similar to that of the sublines, with the exception of BMCOL-1, which lagged 2 days instead of 1 after seeding before growth resumed. While the parent cells were only marginally growth inhibited by DENSPM, the three sublines were affected by cytotoxicity after 48 h. Points, the mean of three experiments; bars, SD.
Table 1: Comparison of polyamine metabolism and DENSPM effects in RPMI-7932 parent and MDR human melanoma cell lines and treatment (48 h)

<table>
<thead>
<tr>
<th>Cell line and treatment (48 h)</th>
<th>% control growth</th>
<th>ODC (nmol CO₂/h/mg)</th>
<th>SAMDC (pmol/min/mg)</th>
<th>Ratio</th>
<th>SSAT (pmol/min/mg)</th>
<th>PUT (pmol/10⁶ Cells)</th>
<th>SPD (pmol/10⁶ Cells)</th>
<th>SPM (pmol/10⁶ Cells)</th>
<th>DENSPM</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI 7932⑥</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Control</td>
<td>100</td>
<td>4.85</td>
<td>2.58</td>
<td>1.9</td>
<td>20</td>
<td>480</td>
<td>1700</td>
<td>2185</td>
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<tr>
<td>10 μM DENSPM</td>
<td>78</td>
<td>0.31</td>
<td>0.06</td>
<td>&lt;0.05</td>
<td>32605</td>
<td>5580</td>
<td>2940</td>
<td>3080</td>
<td>6745</td>
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<tr>
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<tr>
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<td>10.81</td>
<td>1.11</td>
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<td>5580</td>
<td>2940</td>
<td>3080</td>
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<tr>
<td>10 μM DENSPM</td>
<td>43</td>
<td>0.35</td>
<td>&lt;0.05</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>150</td>
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<td>VBL-4</td>
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<td></td>
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<tr>
<td>Control</td>
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<td>13.69</td>
<td>1.54</td>
<td>8.9</td>
<td>35</td>
<td>3110</td>
<td>2650</td>
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<tr>
<td>10 μM DENSPM</td>
<td>31</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>110</td>
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<tr>
<td>BMCOL-1</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Control</td>
<td>100</td>
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<td>0.82</td>
<td>7.1</td>
<td>45</td>
<td>2655</td>
<td>1865</td>
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<tr>
<td>10 μM DENSPM</td>
<td>30</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;5</td>
<td>25</td>
<td>95</td>
<td>5340</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

⑥ Data are means from two experiments performed in duplicate; PUT, putrescine; SPD, spermidine; SPM, spermine.

Parent line; MDR lines derived by Lemont et al. (20).

Sensitivity of melanoma MDR variants to polyamines analogues

The sublines exhibited very steep dose-response curves, all of which decreased sharply in the range of 0.001 to 0.1 μM DENSPM and extended down to 0% control growth. By 10 μM, the sublines were completely growth inhibited, while the parent line was growing at 40% of control (Fig. 3).

Polyamine Perturbations. Various aspects of polyamine metabolism were compared in the RPMI-7932 cells and their sublines. Of significance was the finding that the ratio between the two key biosynthetic enzyme activities, ODC and SAMDC, were very different (Table 1). In the parent line, the ODC:SAMDC ratio was ~1.9, while in the sublines, it ranged from 7.1 to 9.8, due mainly to increases in the activity of ODC. Consistent with these enzyme profiles, putrescine pools in the sublines were markedly elevated and ranged from 2655 to 5580 pmol/10⁶ cells, while those of the parent line were more typical of other cell types (480 pmol/10⁶ cells). Spermidine and spermine pools were similar for parent and sublines. The possibility that the high putrescine pools might be due to cadaverine produced by an undetected Mycoplasma infection (35) was discounted by spiking the samples with cadaverine and observing that two separate peaks appeared on the
chromatogram. Thus, the high putrescine peak did not contain cadaverine.

Treatment with DENSPM had similar enzyme effects in parent and MDR sublines at 48 h (Table 1). Typically, ODC and SAMDC were down-regulated, and SSAT was markedly induced. Two of the cell lines accumulated more analogue than the parent line and induced higher levels of SSAT. Polyamine pools were similarly depleted in all cell lines following analogue treatment. On the suspicion that differences in DENSPM accumulation between parent cells and the sublines might be more apparent during shorter exposures (i.e., assuming that analogue levels would equilibrate in culture after 48 h), we treated cell lines for 2 h and found that uptake into all three sublines was about twice that of the parent line (Table 2). This altered analogue uptake can be extrapolated to polyamine uptake in general since previous studies strongly suggest that spermine analogues such as DENSPM enters cells via a transporter protein (36) which is specific for polyamines (37) and since the analogue cannot penetrate polyamine transport-deficient CHO cells (Ref. 38; Fig. 4). To confirm this probability, the uptake of [3H]spermidine over the same 2-h period was compared in the various cell lines. All three sublines accumulated about 1.8 to 3 times as much [3H]spermidine as the parent line (Table 2), indicating that differences in DENSPM accumulation parallel differences in polyamine accumulation and, hence, are probably related.

Growth Sensitivity to Polyamine Enzyme Inhibitors. Since polyamine metabolism was similarly and distinctly altered in the MDR sublines, their relative sensitivity to two specific inhibitors of polyamine biosynthetic enzymes was examined. By dose-response analysis (Fig. 5), neither DFMO, a specific inhibitor of ODC (9), nor CGP-48664, a specific inhibitor of SAMDC (8), were found to differentially affect the growth of the parent line or its MDR sublines. As is typical of growth responses to polyamine antagonists, the dose curves for both inhibitors were relatively flat for all cell types. Thus, the MDR sublines were not cross-resistant to either inhibitor.

DISCUSSION

Preclinical in vivo antitumor studies have shown that the polyamine analogue DENSPM and the polyamine inhibitor CGP-48664 are particularly effective against melanoma and other solid tumors (3, 4). On the basis of these findings, melanoma is among the main solid tumors being targeted in the current Phase I clinical trials of DENSPM. Our interest in initiating the present study was to demonstrate that MDR human melanoma cells retain sensitivity to the polyamine antagonists, such as the inhibitor CGP-48664 and the analogue DENSPM. Indeed, MDR sublines were found to be similarly sensitive to DFMO and CGP-48664 as the parent line, indicating that polyamine inhibitors may be useful in treating MDR tumors. Since growth inhibition by DENSPM was shown to be dependent on a transporter (Fig. 4) that is specific for polyamines (36—38) and since there is no indication that polyamines or their analogues are exported by the P-glycoprotein (39), it was our expectation that the human MDR sublines would mimic the parent line in their sensitivity to DENSPM. They were found, however, to be collateral sensitive to the analogue. In particular, the sublines were cytotoxically affected, while the parent line was cytostatically inhibited. In addition, the cells were found to contain distinct perturbations in polyamine metabolism and transport, which may be at least partially responsible for the shift in growth sensitivity.

No similar observations have been reported previously in other MDR cell lines. Davidson et al. (40) found that doxorubicin resistance neither increased nor decreased the responsiveness of human breast cancer cells to a DENSPM-related analogue, N1,N12-diethyl spermine (also known as N1,N12-bis(ethyl)spermine), which is known to have similar uptake characteristics. Collateral sensitivity to such analogues may be novel to melanoma cell lines. We have shown previously that these cells are unique in certain aspects of polyamine metabolism,
such as high inducibility of SSAT by polyamine analogues (2, 13) and high growth sensitivity to polyamine analogues and inhibitors (2–4, 8, 13, 14). The generality of these observations among other human melanoma cells is further suggested by the similarity of findings in all three cell lines, a finding made more significant by the fact that, although the sublines originated from the same parent line, they were separately derived using three different selecting agents. Lemontt et al. (20) isolated five lines from the RPMI-7932 cells by single-step selection in culture medium containing either vincristine, vinblastine, or colchicine. All lines showed relatively low (4- to 24-fold) levels of drug resistance and were cross-resistant to standard MDR drugs.

In attempting to determine the basis for increased sensitivity to DENSPM in the sublines, we observed that all three sublines displayed an increased ratio in ODC activity to SAMDC activity, due
mainly to increases in the former enzyme. This was functionally confirmed by the presence of unusually high putrescine levels in the sublines, a disturbance which would be predicted by such enzyme profiles. Despite this difference, both enzymes were effectively downregulated by DENSPM. Of greater relevance to analogue sensitivity were >50-fold resistant to MDR susceptible agents and since the polyamine transport seems to be decreased in MDR variants. SSAT was induced to a greater extent in the MDR sublines. Polyamine analogues such as DENSPM are taken up by a specific polyamine transport system (Fig. 4) and there are no known functional linkages between polyamine transport and the MDR phenotype. Interestingly, two groups (41, 42) have recently reported that the membrane protein which could have membrane transport activity would be predicted by such enzyme variation in the structure of spermine on the association with DNA and the induction of DNA transformational changes. Biochem. J., 269: 329–334, 1990.

We gratefully acknowledge the skilled technical assistance of John Miller and Paula Diegelman. We are also indebted to Dr. Henry Hoppe of the Genzyme Corporation for providing the human melanoma parent and MDR cell lines used in this study.

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