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

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ABSTRACT

Certain \( N \)-alkylated analogues of the natural polyamine spermine, such as \( N^1, N^{11}\)-diethyl norspermine (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 \( N^1\)-acetyltransferase. On the basis of previously reported antitumor activity in human tumor xenograft model systems, DENSPM is currently undergoing Phase I 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 \( N^1\)-acetyltransferase activity occurred similarly in both parent and variant lines, polyamine depletion was greater in the variant lines. Collateral sensitivity of the MDR sublines to DENSPM is partially attributable to the finding that analogues (and spermine) 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, 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 ng/ml vincristine (VCR-4.5/4), 3 ng/ml vinblastine (VBL-4), or 8 ng/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.
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 acetylating spermidine. However, in the case of DENSPM-treated MALME-3M human melanoma cells, we have found that the preponderance (>97%) of the total acetylating 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...
retention by the sublines increased to levels similar to that retained by the parent line. Of the three sublines, VBL-4 showed the greatest heterogeneity in P-glycoprotein expression, as indicated by the width of the peak of antibody labeled cells (Fig. 1, right column). By contrast, the VBL-4 line showed the least heterogeneity in P-glycoprotein function, as indicated by the narrowness of the peak in rhodamine 123-labeled cells.

**Growth Sensitivity to DENSPM.** Importantly for comparison studies, all three sublines retained the approximate same growth rate under control conditions as the parent line (Fig. 2). Their growth sensitivity to DENSPM, however, was considerably greater than RPMI-7932 cells. When treated with the 10 μM analogue, the growth of the parent line was only marginally affected, while that of the sublines was cytotoxically affected after 48 h, as indicated by a sharp and significant decline in cell number (Fig. 2). The differential in growth sensitivity is also apparent in dose-dependence studies (Fig. 3). The parent line showed a flat curve, which decreased slowly from 0.01 μM analogue and then remained constant at about 40% control growth. This is a very typical response curve for DENSPM among most other cell lines examined. In contrast to the parent line, 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/10^6 cells, while those of the parent line were more typical of other cell types (480 pmol/10^6 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

### Table 1 Comparison of polyamine metabolism and DENSPM effects in RPMI-7932 parent and MDR human melanoma cells

<table>
<thead>
<tr>
<th>Cell line and treatment (48 h)</th>
<th>% control growth</th>
<th>Enzyme activities</th>
<th>Polyamine pools</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ODC (nmol CO₂/h/mg)</td>
<td>SAMDC</td>
</tr>
<tr>
<td>RPMI 7932</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
<td>4.85</td>
<td>2.58</td>
</tr>
<tr>
<td>10 μM DENSPM</td>
<td>78</td>
<td>0.31</td>
<td>0.06</td>
</tr>
<tr>
<td>VCR 4.5/4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
<td>10.81</td>
<td>1.11</td>
</tr>
<tr>
<td>10 μM DENSPM</td>
<td>43</td>
<td>0.35</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>VBL-4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
<td>13.69</td>
<td>1.54</td>
</tr>
<tr>
<td>10 μM DENSPM</td>
<td>31</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>BMCO-1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
<td>5.74</td>
<td>0.82</td>
</tr>
<tr>
<td>10 μM DENSPM</td>
<td>30</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

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

a Parent line; MDR lines derived by Lemont *et al.* (20).
Sensitivity of Melanoma MDR Variants to Polyamines Analouges

Table 2. DENSPM accumulation and spermidine uptake in RPMI-7932 and MDR variants

<table>
<thead>
<tr>
<th>Cells (2 h)</th>
<th>DENSPM (pmol/10E6 cells)</th>
<th>SPM (pmol/μg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI-7932</td>
<td>597 ± 65</td>
<td>9.1 ± 1.0</td>
</tr>
<tr>
<td>VCR 4.5/4</td>
<td>1257 ± 84</td>
<td>23.8 ± 2.0</td>
</tr>
<tr>
<td>VBL-4</td>
<td>1307 ± 121</td>
<td>29.7 ± 7.8</td>
</tr>
<tr>
<td>BMDOL-1</td>
<td>1171 ± 101</td>
<td>16.3 ± 2.0</td>
</tr>
</tbody>
</table>

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,N2-diethylspermine (also known as N1,N2-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. Lemoine 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 DENSMP 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 profiles, 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 polyamine transport seems to be decreased in MDR variants. The difference might be related to the different extents of resistance since both the K562 human erythroleukemia variants and the DX5 variants were >50-fold resistant to MDR susceptible agents (41) and since the polyamine transporter is known to show biphasic responses (19). A number of biochemical and cellular changes have been noted in MDR cells (43, 44) which, although not directly related to the mdr gene, may contribute to the MDR phenotype. Interestingly, at least one of these changes involves the increase of a MDR-unrelated plasma membrane protein (45) which could have membrane transport activity. Obviously, both the changes in polyamine biosynthesis and uptake and the increased sensitivity need to be further confirmed in other MDR cells, giving attention to the possibility that it may be unique to melanoma cells since they have already been found to have unusual polyamine properties.

Although MDR has been recognized as a laboratory phenomenon for two decades and although there is a growing indication of its contribution to clinical resistance, there are still no effective approaches to overcome resistance which can be used in standard oncology practice (16, 17, 44). Considerable effort is being dedicated to the use of MDR modulators which, in general, act as competitive inhibitors of P-glycoprotein (16, 44). Although clinical successes have been reported against acquired resistance in hematolymphoid malignancies, MDR modulation has yet to find a clearly defined role in cancer chemotherapy. Problems include the toxicity of such modulators towards normal tissue bearing P-glycoprotein, toxicities unrelated to P-glycoprotein, enhancement of anticancer drug toxicities, and pharmacokinetic complications when combined with anticancer agents.

If found to be widespread among MDR cells, the unaffected or collagen-free resistance of MDR tumor cells to CGF-48664 and DENSPM, respectively, could have potential clinical significance. At a minimum, these antagonists represent new agents which retain effectiveness against the MDR phenotype as a single agent to treat MDR malignancies or in combination with other cytotoxic agents. In the case of DENSPM, the acquisition of the MDR phenotype and the apparently related collateral growth sensitivity to the analogue may actually enhance the chemotherapeutic window and hence the effectiveness of the analogue. Unlike the use of MDR modulators, where additional toxicities and complications may be introduced, the use of DENSPM to exploit a possibly coincidental feature of the MDR phenotype (if only in melanoma) may provide a straightforward chemotherapeutic approach to the resistance problem.

ACKNOWLEDGMENTS

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REFERENCES

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