Increased *MDR1* Expression in Normal and Malignant Peripheral Blood Mononuclear Cells Obtained from Patients Receiving Depsipeptide (FR901228, FK228, NSC630176)

Robert W. Robey, Zhirong Zhan, Richard L. Piekarz, Ganesh L. Kayastha, Tito Fojo, and Susan E. Bates

Abstract

The increased expression of markers associated with a differentiated phenotype, such as P-glycoprotein (Pgp), follows treatment with histone deacetylase inhibitors. Because depsipeptide (FR901228, FK228, NSC630176) is a substrate for Pgp, up-regulation of the gene that encodes it, *MDR1*, would mean that depsipeptide induces its own mechanism of resistance. To examine the effect of depsipeptide on expression of ATP-binding cassette transporters associated with multidrug resistance, the kidney cancer cell lines 108, 121, 127, and 143 were treated with depsipeptide and evaluated by quantitative reverse transcription-PCR. Increased levels of *MDR1* (1.3- to 6.3-fold) and *ABCG2* (3.2- to 11.1-fold) but not *MRP1* (0.9- to 1.3-fold) were observed. The induced Pgp transported the fluorescent substrates rhodamine 123, bisantrene, calcein-AM, BODIPY-vinblastine, and BODIPY-paclitaxel. In normal peripheral blood mononuclear cells (PBMC) and circulating tumor cells obtained from patients receiving depsipeptide, increased levels of histone H3 acetylation were found. We next examined *MDR1* levels in normal and malignant PBMCs obtained from 15 patients enrolled in clinical trials with depsipeptide and detected up to a 6-fold increase in normal PBMCs and up to an 8-fold increase in circulating tumor cells after depsipeptide administration. In one patient with Sézary syndrome, increased *MDR1* gene expression was accompanied by increased cell surface Pgp expression in circulating Sézary cells as determined by measurement of MRK-16 staining by flow cytometry. These studies suggest that depsipeptide induces its own mechanism of resistance and thus provide a basis for clinical trials evaluating depsipeptide in combination with a Pgp inhibitor.

Histone deacetylase inhibitors (HDI) are a novel class of chemotherapeutic agents that have shown promise in the treatment of cancer (1, 2). The HDIs block histone deacetylase activity, thus increasing chromatin acetylation and, in turn, altering gene expression (3). HDIs may have a positive or negative effect on gene expression, although array studies have suggested that only 2% to 5% of genes are affected (4–6). Among the cell cycle effects, HDIs may also block cell proliferation by up-regulating the cyclin inhibitor p21WAF1/CIP1. Several HDIs are currently in phase I and II clinical trials, including suberoylanilide hydroxamic acid (5, 7), MS-275 (8), phenylbutyrate (6, 9), and depsipeptide (FR901228, FK228, NSC630176; refs. 10–12).

A cyclic peptide isolated from the fermentation broth of *Chromobacterium violaceum* (13), depsipeptide, like other HDIs, has been shown to induce a p21-dependent G1 arrest and a p21-independent G2-M arrest, with the G2-M arrest appearing more toxic (14). *In vitro* data suggest that depsipeptide may be effective in the treatment of breast cancer (15), B-cell chronic lymphocytic leukemia (16), and malignant lymphoid cells (17). In phase I and II clinical trials at the NIH, depsipeptide has displayed activity in patients with peripheral and cutaneous T-cell lymphomas (12). However, not all patients respond to therapy and some patients experience disease progression despite an initial response.

We have shown previously that the HDI sodium butyrate is capable of inducing a differentiated phenotype in cancer cells and increasing expression of the *MDR1* gene and its product, P-glycoprotein (Pgp; refs. 18, 19). Pgp is an ATP-binding cassette (ABC) transporter that mediates drug resistance by reducing intracellular drug concentrations via energy-dependent efflux (20). Subsequent studies have shown that the transcription factor NF-Y plays a key role in the up-regulation of *MDR1* gene expression by HDIs (21). Using COMPARE analysis and the National Cancer Institute Drug Screen database, depsipeptide was shown to be a Pgp substrate (14, 22). Further, it was shown to be highly susceptible to Pgp-mediated drug resistance. This raised the possibility that depsipeptide could up-regulate its own mechanism of resistance.
To determine the effect of depsipeptide on ABC transporters associated with drug resistance, we treated kidney cancer cell lines with depsipeptide and observed increased levels of MDR1 and ABCG2 but not MRP1 as measured by reverse transcription-PCR (RT-PCR). SW620 cells, known to readily up-regulate Pgp after HDI treatment, showed increased expression of a functional Pgp after treatment with 10 ng/mL depsipeptide for 4 or 24 hours. Additionally, SW620 and S1 colon cancer cells selected with depsipeptide were shown to overexpress Pgp as their mechanism of resistance. In normal and malignant peripheral blood mononuclear cells (PBMC) obtained from patients enrolled on phase I and II studies with the drug, increased levels of acetylated histone H3 were noted by immunoblot analysis. We also show for the first time increased levels of MDR1 gene expression as determined by PCR analysis in normal and malignant PBMCs obtained from patients receiving depsipeptide. The results presented here suggest that depsipeptide may induce its own mechanism of resistance and provide impetus for the addition of a Pgp inhibitor in chemotherapeutic treatment regimens with depsipeptide.

### Materials and Methods

**Materials.** Depsipeptide was obtained from the National Cancer Institute Anticancer Drug Screen (Bethesda, MD).

**Cell lines.** The 108, 121, 127, and 143 renal cell carcinoma cell lines were kindly provided by Dr. Marston Linehan (National Institute of Health, Bethesda, MI) (23). SW620 colon carcinoma cells were obtained from the National Cancer Institute Anticancer Drug Screen. S1 colon carcinoma cells were provided by Dr. Lee Greenberger (Johnson and Johnson Pharmaceutical Research, Raritan, NJ) (24). All cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. Pgp-overexpressing SW620 Ad300 cells were additionally maintained in 300 ng/mL doxorubicin (25). The S1 DP50 and SW620 DP50 sublines were generated by stepwise selection in depsipeptide and both were maintained in 50 ng/mL depsipeptide.

**Patient samples.** PBMCs were obtained by density-gradient separation of whole blood obtained from patients enrolled on a phase I or II trial with depsipeptide and were viably frozen until ready for use. All patients gave informed consent. Patients were treated with

### Table 1. Increase in ABC transporter gene expression in depsipeptide-treated renal carcinoma cell lines

<table>
<thead>
<tr>
<th></th>
<th>MDR1 (fold increase)</th>
<th>MRP1 (fold increase)</th>
<th>ABCG2 (fold increase)</th>
</tr>
</thead>
<tbody>
<tr>
<td>108</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>108 5 ng/mL depsipeptide (3 d)</td>
<td>6.3 ± 3</td>
<td>1.3 ± 0.5</td>
<td>3.2 ± 1.0</td>
</tr>
<tr>
<td>121</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>121 10 ng/mL depsipeptide (3 d)</td>
<td>3.4 ± 0.6</td>
<td>1.1 ± 0.06</td>
<td>5.0 ± 1.6</td>
</tr>
<tr>
<td>121 10 ng/mL depsipeptide (7 d)</td>
<td>2.6 ± 0.1</td>
<td>1.0 ± 0.0</td>
<td>4.7 ± 1.6</td>
</tr>
<tr>
<td>127</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>127 5 ng/mL depsipeptide (7 d)</td>
<td>1.3 ± 0.2</td>
<td>1.1 ± 0.2</td>
<td>3.9 ± 0.8</td>
</tr>
<tr>
<td>143</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>143 10 ng/mL depsipeptide (3 d)</td>
<td>1.4 ± 0.2</td>
<td>0.9 ± 0.2</td>
<td>11.1 ± 4.4</td>
</tr>
</tbody>
</table>

NOTE: ABC transporter gene expression was normalized to rRNA levels as determined by RT-PCR. Gene expression values for treated cells were then divided by expression levels for untreated cells to obtain fold increase. At least two independent rounds of depsipeptide treatment and RT-PCR analysis were done.

**Fig. 1.** ABC transporter expression and function in renal carcinoma cells treated with depsipeptide. A, renal carcinoma cells were incubated with depsipeptide (DP) for varying times after which RNA was extracted and RT-PCR analysis for MDR1, MRP1, ABCG2 and rRNA was done. Representative results from one round of treatment. B, untreated or depsipeptide-treated 108 and 121 cells were trypsinized and incubated in complete medium with 0.5 μg/mL rhodamine 123 with or without valsaparod for 30 minutes, washed, and allowed to efflux for 1 hour in complete medium continuing with (dashed line) or without (solid line) valsaparod.
17.8 mg/m² depsipeptide on days 1 and 5 of a 21-day schedule (10).

Of the patients examined, 3 were diagnosed with Sézary syndrome, whereas the other 12 patients had no apparent blood involvement and had normal circulating T cells. Three of the patients had not received any prior therapy, 2 received topical or other directed therapy, and the remaining patients received systemic chemotherapy with Pgp substrates, such as CHOP, EPOCH, or single-agent doxorubicin.

Immunoblot analysis. PBMCs were resuspended in lysis buffer [0.02 mol/L Tris (pH 7.4), 0.2 mmol/L Triton X-100, 0.02% 2-mercaptoethanol] with 2 ng/mL aprotinin and solubilized by sonication to generate whole-cell lysates that were then subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were probed with anti-acetylated histone H3 antibody (Upstate Biotechnology, Lake Placid, NY), stripped in 0.2 mol/L NaOH, and probed with an anti-glyceraldehyde-3-phosphate dehydrogenase antibody (American Research Products, Belmont, MA). To detect Pgp in resistant cell lines, microsomal membrane protein (30 µg) was obtained by nitrogen cavitation and was electrophoretically separated and transferred to a polyvinylidene difluoride membrane that was then probed with the anti-Pgp antibody C219 (Signet Laboratories, Dedham, MA). Membranes were subsequently subjected to enhanced chemiluminescence with SuperSignal West Pico (Pierce Chemical, Rockford, IL).

RNA isolation and PCR analyses. RNA was extracted from PBMCs and circulating Sézary cells using the RNeasy kit (Qiagen, Valencia, CA) and from cell lines using RNASTAT-60 (Tel Test, Inc., Friendswood, TX) according to the manufacturer’s instructions. Quantitative PCR for MDR1 (ref. 26; forward GCTTGCGAGTTGAAGCAAAATT and reverse CAGACAGCTGACGAGTTCAAGAACTGACGAGGCTAATC and reverse ACCTTCCTCAATGGGCTAATG), and ABCG2 (ref. 28; forward TGCCCAAGTCAATGGCAACGATG and reverse GACTGAAAGGCCTAAACC) was done as described previously. MDR1, MRP1, and ABCG2 levels were normalized to rRNA (forward AACTCTGGTGGAGGTCCGT and reverse CTTACCAAAAGTGGCCCACTA) or β2-microglobulin (forward TTCACTCAATCCAAATGCGG CATCTTC and reverse GTGGAGCATTCAGACTTGTCTTTCAGCA) as described previously (26).

Flow cytometry analyses. Detection of Pgp expression and function by flow cytometry was done as described previously (29). Single-cell suspensions were obtained by trypanosinization and cells were incubated with either the anti-Pgp monoclonal antibody MRK-16 (Kamiya Biomedical, Seattle, WA) or an IgG2a negative control antibody (Becton Dickinson, San Jose, CA) for 30 minutes in Dulbecco’s PBS with 2% bovine serum albumin, centrifuged, and washed twice with Dulbecco’s PBS/bovine serum albumin. Cells were subsequently incubated with phycocerythrin-labeled horse anti-mouse secondary antibody (Vector Laboratories, Burlingame, CA). Pgp function was detected as described previously by resuspending cells in complete medium (phenol red-free Iscove’s modified MEM with 10% FCS) containing the fluorescent compounds 0.5 µg/mL rhodamine 123, 200 nmol/L calcein-AM, 50 nmol/L BODIPY-paclitaxel, 250 nmol/L BODIPY-vinblastine, or 25 µmol/L bisantrene with or without...
3 µg/mL of the Pgp inhibitor valspodar (PSC 833) and incubating them at 37°C in 5% CO₂ for 30 minutes. Cells were subsequently resuspended in drug-free medium continuing with or without 3 µg/mL valspodar for 1 hour at 37°C in 5% CO₂ to generate the PSC/efflux or efflux histograms, respectively.

A Becton Dickinson FACSort flow cytometer equipped with a 488 nm argon laser and a 530 nm bandpass filter was used to detect rhodamine 123, calcein-AM, BODIPY, and bisantrene fluorescence, whereas a 585 nm bandpass filter was used to detect phycoerythrin fluorescence. Dead cells were excluded based on propidium iodide staining.

**Cytotoxicity assays.** Four-day cytotoxicity assays were done as described previously (30). Briefly, cells were seeded in 96-well plates and allowed to attach overnight. Chemotherapeutic agents were added to cells at the desired concentration and incubated for 4 days at 37°C. Drugs were tested in quadruplicate, whereas untreated controls were tested in replicates of eight. Cellular proteins were fixed in 50% trichloroacetic acid and stained in 0.4% sulforhodamine B dissolved in 1% acetic acid. The plates were washed in 1% acetic acid and bound dye was solubilized with 10 mmol/L unbuffered Tris base (pH 10.5). Cell density was determined by measuring absorbance at 570 nmol/L. IC₅₀ values were determined by comparing average absorbance of drug-treated wells to that of untreated controls.

**Results**

**Cells treated with depsipeptide show increased expression of MDR1 and ABCG2 at the RNA level.** To determine the effect of depsipeptide treatment on the expression of ABC transporters associated with multidrug resistance, the renal cell carcinoma lines 108, 121, 127, and 143 were treated with depsipeptide for varying periods of time. After treatment, RNA was extracted and the expression of MDR1, MRP1, and ABCG2 was determined by quantitative PCR analysis. Average values from at least two independent experiments are shown in Table 1 and representative PCR results are shown in Fig. 1A. We found increased expression of MDR1 and ABCG2 in cells treated with depsipeptide. No induction of MRP1 was observed. We have shown previously that overexpression of ABCG2, in contrast to Pgp, does not confer resistance to depsipeptide (31).

To determine if the increase in MDR1 expression was accompanied by induction of a functional Pgp, the ability of the cells to transport rhodamine 123 was examined. As shown in Fig. 1B, untreated 108 and 121 cells or cells treated with depsipeptide for 3 days were incubated with rhodamine 123 with (dashed line) or without (solid line) valspodar. The increased difference between dashed and solid histograms after treatment is indicative of a functional Pgp.

**Time course and characterization of Pgp induced by depsipeptide.** We showed previously induction of Pgp in SW620 colon cancer cells by sodium butyrate, unrecognized as a HDI at the time (18). These cells were therefore used to perform dose-response and time-course studies to follow the induction of Pgp. SW620 cells were treated overnight in 0.1, 1, 10, or 100 ng/mL depsipeptide, and Pgp expression and function was examined by flow cytometry. Whereas low levels of MRP-16 staining, denoted by the small difference between the MRK-16 histogram (dashed line) and the negative control antibody histogram (solid line), were observed in untreated and SW620 cells treated with 0.1 ng/mL depsipeptide, cells treated with 1, 10, or 100 ng/mL depsipeptide showed increased MRK-16 staining (Fig. 2A). Interestingly, increasing the concentration of depsipeptide did not result in higher levels of Pgp. Increased rhodamine 123 efflux, shown by the greater distance between solid and dashed histograms, was observed in the same treated cells where increased MRK-16 staining was observed. Subsequently, a time course was done and cells were treated with 10 ng/mL depsipeptide for 2, 4, 6, 12, and 24 hours. MRK-16 staining was determined by flow cytometry (Fig. 2B). Increased levels of Pgp were evident after at least a 12-hour incubation period in depsipeptide.

**Fig. 3.** Pgp induced by depsipeptide treatment transports diverse Pgp substrates. A, untreated SW620 cells and SW620 cells treated with 10 ng/mL depsipeptide for 24 hours were incubated with MRK-16 (dashed line) or negative control (IgG2b) antibody (solid line) for 30 minutes, washed, and incubated with phycoerythrin-labeled secondary antibody for 30 minutes (first row). Untreated and treated SW620 cells were also incubated in 25 µmol/L bisantrene (second row), 50 µmol/L BODIPY-paclitaxel (third row), 200 nmol/L calcein-AM (fourth row), or 250 nmol/L BODIPY-verapamil (fifth row) with or without 3 µg/mL valspodar for 30 minutes, washed, and allowed to efflux for 1 hour continuing with (dashed line) or without (solid line) valspodar. Representative results from two independent experiments. B, MRK-16 staining (top row) and transport of rhodamine 123 (middle row) and BODIPY-paclitaxel (bottom row) was determined as outlined in (A) on untreated SW620 cells or cells treated with 10 ng/mL depsipeptide for 4 hours, washed, and incubated in drug-free medium for 20 additional hours before assay.

**Table 1.** Time course of expression of ABC transporters in SW620 cells treated with depsipeptide.

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>MDR1</th>
<th>ABCG2</th>
<th>MRP1</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.1</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>1</td>
<td>0.5</td>
<td>0.4</td>
<td>0.6</td>
</tr>
<tr>
<td>2</td>
<td>0.8</td>
<td>0.7</td>
<td>0.9</td>
</tr>
<tr>
<td>4</td>
<td>1.2</td>
<td>1.1</td>
<td>1.3</td>
</tr>
<tr>
<td>6</td>
<td>1.5</td>
<td>1.4</td>
<td>1.6</td>
</tr>
<tr>
<td>12</td>
<td>2.0</td>
<td>1.9</td>
<td>2.1</td>
</tr>
<tr>
<td>24</td>
<td>2.5</td>
<td>2.4</td>
<td>2.6</td>
</tr>
</tbody>
</table>
To determine if the induced Pgp could transport other known Pgp substrates, untreated SW620 cells and SW620 cells treated with 10 ng/mL depsipeptide for 24 hours were incubated in the fluorescent compounds bisantrene, BODIPY-verapamil, BODIPY-paclitaxel, or calcein-AM. These compounds have been shown previously to be substrates for Pgp (32). Representative results from two independent experiments are shown in Fig. 3A.

Increased surface expression of Pgp was confirmed in the depsipeptide-treated cells as shown in the top row of histograms in Fig. 3. Accordingly, the treated cells showed increased levels of valspodar-inhibitable efflux of bisantrene, BODIPY-paclitaxel, BODIPY-verapamil, and calcein-AM compared with untreated cells (Fig. 4).

Pgp overexpression prevents depsipeptide-mediated histone acetylation and confers resistance to Pgp substrate drugs in depsipeptide-resistant S1 and SW620 cells. A. total cell lysate from untreated SW620 cells, SW620 cells treated with 10 ng/mL depsipeptide for 24 hours, untreated Pgp-overexpressing SW620 Ad300 cells, SW620 Ad300 cells treated with 10 ng/mL depsipeptide for 24 hours, or SW620 Ad300 cells treated concomitantly with 10 ng/mL depsipeptide and 3 μg/mL valspodar was electrophoretically separated and transferred to a polyvinylidene difluoride membrane. The membrane was subsequently sequentially probed with anti-acetylated histone H3 (AcH3) antibody and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody. B. microsomal membrane protein (30 μg) from parental (S1 and SW620) and depsipeptide-resistant (S1 DP50 and SW620 DP50) cells was subjected to electrophoresis and transferred to a polyvinylidene difluoride membrane, which was probed with the anti-Pgp antibody C219. Membrane protein from Pgp-overexpressing SW620 Ad300 cells was included as a positive control. C. parental and depsipeptide-resistant cells were incubated in MRK-16 antibody (dashed line) or negative control antibody (solid line) for 30 minutes, washed, and incubated with phycoerythrin-labeled secondary antibody (left column). Alternatively, cells were incubated in complete medium with 0.5 μg/mL rhodamine 123 in the presence or absence of valspodar, washed, and allowed to incubate in substrate-free medium continuing with (dashed line) or without (solid line) valspodar (right column). Representative results for S1 DP50 (top row) and SW620 DP50 cells. Pgp-overexpressing SW620 Ad300 cells were included as a positive control. D. 4-day cytotoxicity assays were done on S1 (○, left column), S1 DP50 (●, left column), SW620 (○, right column), and SW620 DP50 (●, right column) cells with depsipeptide and vinblastine. Representative results.

To determine if the induced Pgp could transport other known Pgp substrates, untreated SW620 cells and SW620 cells treated with 10 ng/mL depsipeptide for 24 hours were incubated in the fluorescent compounds bisantrene, BODIPY-verapamil, BODIPY-paclitaxel, or calcein-AM. These compounds have been shown previously to be substrates for Pgp (32). Representative results from two independent experiments are shown in Fig. 3A. Increased surface expression of Pgp was confirmed in the depsipeptide-treated cells as shown in the top row of histograms in Fig. 3. Accordingly, the treated cells showed increased levels of valspodar-inhibitable efflux of bisantrene, BODIPY-paclitaxel, BODIPY-verapamil, and calcein-AM compared with untreated cells (Fig. 4).

Table 2. Cross-resistance profile of depsipeptide-selected cell lines

<table>
<thead>
<tr>
<th>Drug</th>
<th>S1</th>
<th>S1 DP50</th>
<th>Relative resistance</th>
<th>SW620</th>
<th>SW620 DP50</th>
<th>Relative resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dumpsipeptide</td>
<td>0.5 ± 0.03</td>
<td>450 ± 71</td>
<td>900</td>
<td>0.5 ± 0.2</td>
<td>150 ± 71</td>
<td>300</td>
</tr>
<tr>
<td>Adriamycin</td>
<td>0.01 ± 0.008</td>
<td>0.2 ± 0.7</td>
<td>20</td>
<td>0.03 ± 0.004</td>
<td>0.4 ± 0.1</td>
<td>13</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>0.01 ± 0.005</td>
<td>0.6 ± 0.5</td>
<td>60</td>
<td>0.004 ± 0.0007</td>
<td>0.1 ± 0.007</td>
<td>25</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>0.001 ± 0.0007</td>
<td>0.04 ± 0.04</td>
<td>40</td>
<td>0.002 ± 0.001</td>
<td>0.04 ± 0.02</td>
<td>20</td>
</tr>
</tbody>
</table>

NOTE: IC50s are in μmol/L, except for depsipeptide values, which are in ng/mL. Relative resistance values were calculated by dividing the IC50 of the selected cell lines by the IC50 of the respective parental cell lines.
Samples were collected from A and SW620 cells treated with 10 ng/mL depsipeptide for 2 hours, rhodamine 123 and BODIPY-paclitaxel were also observed in increased surface expression of Pgp and increased transport to that seen in SW620 cells exposed continuously for 24 hours. The magnitude was similar (Fig. 3) cycle 1, cycle 2, and cycle 3. Increased levels of acetylated histone H3 are observed in normal PBMCs and circulating tumor cells obtained from patients post-depsipeptide treatment. Whole-cell lysates of PBMCs (5 μg) were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were probed with anti-glyceraldehyde-3-phosphate dehydrogenase antibody (GAPDH), stripped with 0.2 mol/L NaOH, and probed with anti-acetylated histone H3 antibody (AcH3). Samples were collected from (A) cycles 1 and 23, (B) and (C) cycle 1, (D) cycle 12, and (E) cycle 13.

Untreated SW620 cells as evidenced by the increased distance between solid and dashed histograms.

Because patients are treated with a 4-hour infusion of depsipeptide, SW620 cells were treated with 10 ng/mL depsipeptide for 4 hours, washed, and incubated in drug-free medium for an additional 20 hours. Subsequently, cells were harvested and Pgp expression was measured. As shown in Fig. 3B, cells treated with depsipeptide for 4 hours showed increased MRK-16 staining compared with untreated SW620 cells (top row). Increased levels of valspodar-inhibitable rhodamine 123 (Fig. 3B, center row) and BODIPY-paclitaxel (Fig. 3B, bottom row) transport were also observed following short-term depsipeptide treatment. The magnitude was similar to that seen in SW620 cells exposed continuously for 24 hours. Increased surface expression of Pgp and increased transport of rhodamine 123 and BODIPY-paclitaxel were also observed in SW620 cells treated with 10 ng/mL depsipeptide for 2 hours, washed, and incubated for 22 hours in drug-free medium (data not shown). These results suggest that even short-term treatment with depsipeptide can induce a functional Pgp.

Pgp expression prevents depsipeptide-mediated histone H3 acetylation. The potential of Pgp overexpression to limit the activity of depsipeptide has been noted previously (22). Figure 4A shows the effect of Pgp expression on the acetylation of histone H3 in parental SW620 cells or Pgp-overexpressing SW620 Ad300 cells following overnight treatment with 10 ng/mL depsipeptide. Low levels of acetylated histone H3 were observed in untreated SW620 cells (lane 1), whereas high levels were observed in SW620 cells incubated with depsipeptide (lane 2). Low levels of acetylated histone H3 were also observed in SW620 AD300 cells (lane 3) and remained low when treated with depsipeptide (lane 4) unless incubated with depsipeptide in the presence of 3 μg/mL of the Pgp inhibitor valsaparod, when an increase in acetylated histone H3 was readily observed (lane 5). These results confirm that Pgp expression abrogates depsipeptide activity.

Chronic exposure to a Pgp substrate is also known to induce Pgp as a resistance mechanism (26). SW620 and S1 colon carcinoma cells were incubated in increasing amounts of depsipeptide to generate the resistant sublines SW620 DP50 and S1 DP50, which are maintained in 50 ng/mL depsipeptide. When examined for Pgp expression, both resistant sublines exhibited high expression of Pgp as shown by immublot analysis of microsomal membrane protein (Fig. 4B). Additionally, increased staining of the anti-Pgp antibody MRK-16 as well as increased rhodamine 123 efflux was noted (Fig. 4C). In 4-day cytotoxicity assays, both resistant sublines were found to be cross-resistant to depsipeptide and to the Pgp substrates doxorubicin, paclitaxel, and vinblastine. The cross-resistance data for the depsipeptide-selected sublines are summarized in Table 2, whereas representative results with some of the drugs are shown in Fig. 4D. When the cytotoxicity assays with depsipeptide were repeated with the Pgp antagonist valsaparod, resistance to the drug was abrogated, again indicative of Pgp being the mechanism of resistance to depsipeptide in these cells (data not shown). These results suggest that repeated exposure to depsipeptide may increase Pgp levels and confer resistance to other chemotherapeutic agents that are Pgp substrates.

Depsipeptide increases levels of acetylated histone H3 in normal and malignant circulating PBMCs obtained from patients treated with depsipeptide. PBMCs from patients receiving depsipeptide were collected to determine if increased levels of histone acetylation could be detected, thus showing a biologically active dose of depsipeptide. Patients were enrolled in a phase I or II study and treated with 17.8 mg/m² depsipeptide on days 1 and 5 of a 21-day cycle (10). PBMCs were generally obtained pretreatment, 4 hours, and 24 hours after the start of the depsipeptide infusion on days 1 and 5 and at later time points when possible. In some instances, PBMCs were obtained in later cycles. The 4-hour time point was chosen because depsipeptide was infused over a 4-hour period and we wanted to determine whether histone acetylation would occur early in treatment. Although time-course data presented above suggested that a 12-hour time point would be optimal for detection of Pgp, the 24-hour time point was more easily obtained. Figure 5A to E presents results in five representative patients who received depsipeptide. An increase in histone H3.
acetylation was observed in PBMCs obtained 4 hours after the initiation of depsipeptide treatment and is still observed in some patients at 24 hours post-treatment.

**Depsipeptide induces MDR1 expression in PBMCs obtained from patients receiving depsipeptide.** Quantitative RT-PCR for MDR1 gene expression was subsequently done on RNA extracted from PBMCs obtained from patients receiving depsipeptide. Samples were obtained at the same time points noted above for detection of histone acetylation. As the treatment protocols did not require patients to have circulating tumor cells, we examined MDR1 expression in normal circulating mononuclear cells obtained from patients treated with depsipeptide. The small sample size did not allow us to confirm surface expression of Pgp in normal PBMCs. The results are summarized in Fig. 6. In the 12 patients with normal circulating mononuclear cells, up to a 6-fold increase in MDR1 gene expression was observed. Among the 3 patients diagnosed with Sézary syndrome (patients 2, 3, and 15), up to an 8-fold increase was noted. The induction seemed transient, as MDR1 levels returned to near pretreatment levels by 24 to 48 hours postinfusion. The transient nature of depsipeptide-induced MDR1 expression has recently been shown by Xiao et al. (33).

**Increased cell surface Pgp expression in circulating tumor cells obtained from a patient after treatment with depsipeptide.** We next looked for detectable Pgp up-regulation in circulating tumor samples obtained from patients receiving depsipeptide. Circulating Sézary cells were obtained from patient 15 (Fig. 6) before depsipeptide infusion and then at 4 hours, 24 hours, 48 hours, and 7 days postinfusion. Cells were incubated with the anti-Pgp antibody MRK-16 (dashed line) or negative control antibody (solid line) for 30 minutes, washed, and incubated with phycoerythrin-labeled secondary antibody to generate the histograms of Fig. 7. The Kolmogorov-Smirnov D value, a measure of the degree of difference between two histograms, is calculated for the negative control (solid line) and MRK-16 (dashed line) histograms and is shown for each of the samples. There is a slight increase in MRK-16 staining seen at 24 and 48 hours postinfusion of depsipeptide, which parallels the results for MDR1 expression determined by RT-PCR for patient 15 in Fig. 6. These results suggest that depsipeptide can up-regulate cell surface expression of Pgp in circulating tumor cells.

**Discussion**

Depsipeptide is a novel HDI that is effective in the treatment of cutaneous and peripheral T-cell lymphomas (12). It was first determined by our group to be a Pgp substrate using data generated from the National Cancer Institute Anticancer Drug Screen and was also shown to induce Pgp when incubated with cancer cells (22). When the T-cell lymphoma cell line, HuT78, was chronically exposed to depsipeptide, Pgp emerged as the mechanism of resistance (26). Others have also shown that Pgp is a major contributor to depsipeptide resistance in cancer cells (33–35). We have extended these observations by showing increased levels of MDR1 expression and, in one case, increased cell surface Pgp expression in normal PBMCs and circulating tumor cells obtained from patients receiving depsipeptide. Although it is not known what level of Pgp expression would confer clinically relevant levels of depsipeptide resistance, we postulate that Pgp expression is responsible for depsipeptide resistance in some patients.

Previous studies with the HDI sodium butyrate in our laboratory showed increased levels of Pgp in SW620 cells incubated with the drug but were not accompanied by decreased intracellular accumulation of all known substrate drugs. Decreased intracellular accumulation of colchicine was noted, but intracellular levels of vinblastine and Adriamycin were not reduced, a finding we originally attributed to hypophosphorylation of Pgp (18). However, site-directed mutagenesis has shown that the phosphorylation state of Pgp does not affect transport per se (36). In the present study, cells treated overnight with depsipeptide exhibited increased transport...
of several known Pgp substrates, including rhodamine 123, bisantrene, calcine-AM, BODIPY-paclitaxel, and BODIPY-vinblastine. Depsipeptide-resistant cells that overexpress Pgp were also found to be cross-resistant to doxorubicin and vinblastine. The reason for the different results with sodium butyrate and depsipeptide is unclear and goes beyond the scope of the present report. Notably, cells treated with sodium butyrate are exposed to 100-fold higher drug levels (simulated versus 2 mmol/L sodium butyrate) and this may result in other cellular effects aside from histone acetylation. Other groups have shown that sodium butyrate potentiates doxorubicin cytotoxicity independent of the expression of ABC transporters, such as Pgp (37, 38). Interestingly, when SW620 cells were treated with sodium butyrate, we noted that the increased surface Pgp expression was accompanied by increased transport of rhodamine 123, a substrate not tested in the original study (data not shown).

The results presented here suggest that the efficacy of depsipeptide may be increased if combined with a Pgp inhibitor in future clinical trials. Several promising Pgp inhibitors are currently in clinical trials, including elacridar (GF120918; ref. 40), tariquidar (XR9576; ref. 41), and zosuquidar (LY335979; ref. 40). Tariquidar is a potent Pgp inhibitor that has been shown to inhibit Pgp at nanomolar concentrations (42, 43). Studies examining CD56+ cells obtained from patients receiving tariquidar have shown that a single i.v. dose of tariquidar effectively inhibits Pgp for >24 hours (41). Tariquidar has also been shown to prevent expression of Pgp in vitro (44). Minimal data are available regarding the expression of Pgp in cutaneous T-cell lymphomas. In a study by van Haselen et al., Pgp was detected by C219 staining in 4 of 10 cases of primary cutaneous large T-cell lymphoma (45). In 6 cases of mycosis fungoides, punch biopsy specimens were obtained before and after three or four courses of chemotherapy and no change in C219 staining was observed before and after treatment. Jillella et al. examined skin biopsies from 25 patients diagnosed with cutaneous T-cell lymphoma who at some point had circulating Sézary cells observed on a peripheral smear (46). Of these 25 biopsies, 18 were found to be positive for Pgp expression as determined by staining with MRK-16, U1C-2, or JSB-1. Ten biopsies stained positively with all three antibodies, whereas 8 stained positively with at least two of the three antibodies. All specimens were obtained after treatment with chemotherapy; notably, only 9 of the 18 biopsies were obtained from patients treated with chemotherapy regimens that included Pgp substrates. This would suggest that the patient tumors expressed Pgp de novo without prior treatment or that treatment with a chemotherapy regimen not including Pgp substrates may induce Pgp. If the latter were true, this could explain why some patient tumors showed intrinsic resistance to depsipeptide.

The ability of HDIs to influence gene expression makes them attractive for use in combination therapies, and several strategies have been proposed. Depsipeptide has been shown to increase expression of the Na+/I− symporter and to restore iodine accumulation in thyroid cell lines that have lost the ability to trap iodine. Thus, depsipeptide could be combined with radioiodine to treat thyroid carcinoma that no longer responds to radioiodine therapy (47). Sequential 5-aza-2-deoxycytidine/depsipeptide treatment has been shown to induce expression of NY-ESO-1, an attractive target for cancer immunotherapy (48). Combination therapies with interleukin-2 active drugs have also been suggested. We observed increased expression of the interleukin-2 receptor in circulating tumor cells in cutaneous T-cell lymphoma patients receiving depsipeptide (12). In the HuT78 cell line, we showed increased interleukin-2 receptor levels and increased sensitivity to the interleukin-2 receptor levels and increased sensitivity to the interleukin-2 targeted therapeutic Denileukin diftitox (Ontak) following pretreatment with depsipeptide (26). The HDI arginine butyrate has been shown to increase expression of the interleukin-2 receptor in leukemia and lymphoma, thus rendering Denileukin diftitox more effective (49). Depsipeptide could also potentially be combined with Denileukin diftitox to increase its effectiveness. Pretreatment of leukemic cells with the HDI sodium butyrate increases expression of topoisomerase I and confers hypersensitivity to topoisomerase II active drugs, such as etoposide (50). A similar effect would be expected with depsipeptide.

In summary, we report induction of the MDR1 gene and Pgp expression in normal and malignant cells obtained from patients after depsipeptide treatment. The increased expression of Pgp may limit the effectiveness of depsipeptide. Clinical trials in combination with a Pgp inhibitor are warranted.

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