Bryostatin 1 Down-Regulates mdr1 and Potentiates Vincristine Cytotoxicity in Diffuse Large Cell Lymphoma Xenografts

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ABSTRACT

The down-regulation of multidrug resistance (mdr1) gene expression as detected by competitive reverse transcription-PCR and the antitumor activity of bryostatin 1 (Bryol) in a newly established cell line from a patient with relapsed diffuse large cell lymphoma (DLCL) is investigated in a newly established cell line from a patient with relapsed diffuse large cell lymphoma (DLCL). The cell line (WSU-DLCL2) grows in liquid culture and forms s.c. tumors in mice with severe combined immune deficiency. WSU-DLCL2 is a mature B-cell line (IgGX) that is negative for EBV nuclear antigen, expresses the multidrug resistance phenotype, and has t(14;18)(q32;q21) plus other chromosomal aberrations. Exposure of the WSU-DLCL2 cells to Bryol in culture reversed the multidrug resistance phenotype within 24 h. A functional assay revealed a 4-fold increase in [3H]vincristine accumulation in Bryol-treated cells compared with control. Vincristine (VCR), doxorubicin, Bryol, and 1-β-D-arabinofuranosylcytosine showed no clinically significant activity when given alone to WSU-DLCL2-bearing severe combined immune deficiency mice. However, when given 24 h before each cytotoxic agent, Bryol substantially increased the antitumor activity of VCR but not 1-β-D-arabinofuranosylcytosine. There was a statistically significant (P < 0.001) decrease in the expression of P-glycoprotein in WSU-DLCL2 tumors taken from Bryol-treated animals compared with untreated controls. In vivo, a competitive reverse transcription-PCR assay revealed decreased mdr1 RNA expression 24 h after Bryol treatment. These findings taken together indicate that Bryol-induced down-regulation of mdr1 might be one mechanism by which Bryol potentiates VCR activity. The sequential use of both agents resulted in clinically significant antitumor activity in this lymphoma model.

INTRODUCTION

Expression of the mdr1 (mdr1) gene (mdr1) has been shown in a wide variety of human tumors and normal tissues (1). This gene is differentially expressed in normal tissues, particularly along the secretory epithelium of the bowel, bile canaliculi, tubular epithelium of the kidney, and pancreatic small ductule epithelium (2). Moreover, it is highly expressed in adrenal gland, placenta, capillary endothelium of the brain and testis, as well as hematopoietic precursors and lymphocytes (3–5). The gene encodes a M, 170,000 transmembrane glycoprotein (Pgp: Ref. 6). The expression pattern of mdr1 has suggested that Pgp might be involved in the secretion of metabolites and toxic substances into the excretory pathways of bile, urine, and gastrointestinal tract (3, 7). Within human tumors, mdr1 expression is high in solid tumors arising from tissues that are known to have a high level of expression, such as carcinoma of the colon, kidney, adrenal gland, and pancreas (1, 8). In lymphoma, mdr1 is generally expressed in 20% or fewer cases at presentation and in 50–65% of relapsed or refractory cases (9, 10). The expression of this mdr phenotype is shown to be associated with poor prognosis and resistance to chemotherapy (11). Such resistance is due to decreased intracellular accumulation of cytotoxic agents, resulting from the Pgp-mediated efflux mechanism (12).

Because of the clinical relevance of the mdr1 function, attempts at interfering with the Pgp mechanism are being made. Several compounds are capable of reversing the drug efflux mechanism by binding with Pgp (13). The doses of such compounds required to achieve clinical effect have been prohibitive due to toxicity (14). No agents have yet been reported to interfere with the mdr1 mechanism through down-regulation of gene expression. Bryol, a macrocyclic lactone and a natural marine product, is isolated from the marine byzozan Bugula neritina. It has antitumor, immune modulating, and differentiating effects on a number of B-cell lymphomas (15–17). Previously, we have shown that Bryol augments the inhibitory effect of VCR in WSU-DLCL2 in vitro (18). The use of RT-PCR allows for the amplification of individual RNA molecules. The

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The abbreviations used are: mdr, multidrug resistance; Pgp, P-glycoprotein; Bryol, bryostatin 1; RT-PCR, reverse transcription-PCR; DLCL, diffuse large cell lymphoma; FCM, flow cytometric analysis; CIg, cytoplasmic Ig; SCID, severe combined immune deficiency; VCR, vincristine; Ara-C, 1-β-D-arabinofuranosylcytosine.
method has been shown to be 1,000–10,000-fold more sensitive compared with the RNA blot techniques. The ultrasensitivity of RT-PCR makes it unique in detecting mRNA in small number of cells or in small amounts of tissue and mRNA level in mixed cells. In competitive PCR, a DNA fragment containing the same primer template sequences as the target competes for primer binding and amplification. The PCR products are distinguished by size, hybridization, or change in a restriction site. Competitive RT-PCR can be used to obtain quantitative information of mRNA levels comparable with traditional RNA blot techniques, with the added advantages of PCR.

In this study, we present evidence that Bryol decreases the Pgp and mdr1 RNA expression of a human lymphoma, and that this down-regulation is associated with increased tumor response to the Vinca alkaloid VCR.

PATIENT, MATERIALS, AND METHODS

Case Report. A white male, S. B., was diagnosed in 1989, at age 40, with non-Hodgkin’s lymphoma. He presented with abdominal discomfort and was found to have left lower quadrant mass on physical examination and computerized tomography. Exploratory laparotomy revealed lymphadenopathy throughout the small bowel mesentery, retroperitonium, and left lower quadrant mass on physical examination and computerized tomography. Exploratory laparotomy revealed lymphadenopathy throughout the small bowel mesentery, retroperitonium, and left lower quadrant mass on physical examination and computerized tomography. Histopathological examination showed malignant lymphoma, the diffuse large cell non-cleaved type of intermediate grade. This lymphoma was felt to represent transformation of follicular small cleaved cell (low grade), which was still identifiable in the intestinal wall. Immunoperoxidase staining on frozen tissue revealed the cell (low grade), which was still identifiable in the intestinal wall. Immunoperoxidase staining on frozen tissue revealed the majority of the cells to be monoclonal B-cells (κ−λ+). The patient received six cycles of cyclophosphamide (750 mg/m² iv), doxorubicin (50 mg/m² iv), VCR (1.4 mg/m² iv), and prednisone (40 mg/m² po, days 1–5; Ref. 19) and achieved complete remission. However, 3 months later, the abdominal mass recurred. At that time, the patient received radiation therapy in preparation for bone marrow transplantation. While on radiation, new evidence of disease was seen in the abdomen and lung with a pleural effusion. S. B. received further chemotherapy with cyclophosphamide (650 mg/m² iv), doxorubicin (25 mg/m² iv), and etoposide (120 mg/m² iv) on day 1; prednisone (60 mg/m² po) on days 1–14; and 1β-d-arabinofuranosylcytosine (300 mg/m² iv), bleomycin (5 U/m² iv), VCR (1.4 mg/m² iv), and methotrexate (120 mg/m² iv, followed by leucovorin rescue) on day 8 (Promace-CytoBOM; Ref. 20). After one cycle, the patient received high-dose cyclophosphamide and total body irradiation, followed by autologous stem cell rescue in April of 1990. Unfortunately, the disease progressed 5 weeks later with GI involvement and pleural effusions, from which the cell line WSU-DLCL2 was established. The patient expired shortly afterward of progressive disease ~14 months from the time of diagnosis.

Establishment of the Cell Line. Mononuclear cells were isolated by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) density centrifugation of the malignant pleural effusion. Cells were washed twice with HBSS and resuspended at a concentration of approximately 5 × 10⁶ cells/ml in RPMI 1640 containing 20% heat-inactivated fetal bovine serum, 1% t-glutamine, 100 units/ml penicillin G, and 100 μg/ml streptomycin. Cells were then cultured in a 25-cm² flask and incubated in a humidified 5% CO₂ atmosphere at 37°C. The medium was partially replaced as needed until steady cell growth was noted. After establishment, cells were adapted to grow in RPMI 1640 with 10% fetal bovine serum and designated as WSU-DLCL2. No growth factors, mitogens, or EBV were added to the cell culture medium.

**Immunophenotyping of WSU-DLCL2.** The fresh pleural fluid cells, the established cell line, and the s.c. xenograft cells underwent immunophenotyping by FCM on a FACScan (Becton Dickinson Immunodiagnostics, San Jose, CA) as described previously (17). Monoclonal antibodies used in this study were as follows: anti-CD10, CD19, CD20, CD22, HLA-DR, and Leu10 for B lineage; and CD2, CD5, and CD8 for T lineage. All monoclonal antibodies were obtained from Becton Dickinson. For Clgs, cells were treated with saponin (0.25%) and paraformaldehyde (1%), prior to staining as described previously (21). Assay for the EBV nuclear antigen was kindly performed by the EBV laboratory at the Joseph Stroke Research Institute (Philadelphia, PA).

**Xenografts.** Four-week-old female Fox Chase C.B.17 SCID mice were obtained from Taconic Laboratory (Germantown, NY). The animals became adapted, and WSU-DLCL2 xenografts were developed as described previously (22). Each mouse received 10⁷ WSU-DLCL2 cells (in serum-free RPMI 1640) s.c. in each flank area. When s.c. tumors developed, mice were sacrificed, and tumors were dissected and mechanically dissociated into single-cell suspension. Mononuclear cells were separated by Ficoll-Hypaque density centrifugation and washed twice with RPMI 1640. These cells were subjected to phenotypic and karyotypic analyses for comparison with the established tumor line to insure the human origin and its stability. For the subsequent drug-efficacy trials, small fragments of the WSU-DLCL2 xenograft (~30 mg) were transplanted s.c. into similarly conditioned animals, using a 12-gauge trocar. Mice were checked three times each week for tumor development. Once palpable tumors developed, groups of five animals were removed randomly for different treatments and a control. Each experimental group received i.v. injection of cytotoxic agents via a tail vein every fourth day for a total of three doses. For Bryol, the injections were given i.p. The combination groups were treated sequentially with Bryol i.p. followed 24 h later by the cytotoxic agent as described previously (22) and as shown in Table 2. Animals were observed for measurement of s.c. tumors, changes in weight, and side effects of the drugs and were euthanized when their total tumor burden reached 2400 mg to avoid discomfort. All studies involving mice were performed under Institutional Review Board-approved protocol. Tumor weights in SCID mice were plotted against time on a semilog sheet. The growth pattern was close to an S-shape. Tumor doubling (Td) is the time (in days) required for tumor to double its weight during the exponential growth phase.

**Assessment of Tumor Response.** The end points for assessing antitumor activity were according to standard procedures used in our laboratories (22) and as follows: tumor weight (mg) = (A × B²)/2, where A and B are the tumor length and width (in mm), respectively; Tumor growth inhibition (TIC) is the median tumor weight in the treated group (T) when the median tumor weight in the control group (C) reached ~1500
the Pgp transmembrane molecule, was used. Cells were first RNA frozen at -70°C. Cells were fixed in cold ethanol for 5 mm and stained with indirect immunofluorescence procedure as described previously (25).

CD21c ND 8.1 ± 0.8 13.7 ± 2.9
HLA-DR 92.1 99.3 ± 0.3 95.4 ± 0.6
CD2 1.0 5.8 ± 0.1 8.9 ± 1.4
CD5 1.4 5.3 ± 0.5 ND
CD8 1.8 5.4 ± 0.8 ND
LEU10 ND 98.3 ± 0.8 95.4 ± 1.4
SlaG 96.8 90.8 ± 5.9 88.8 ± 9.9
Slaλ 92 97.1 ± 1.5 91.3 ± 5.6
Sldx 2.3 11.7 ± 0.4 11.6 ± 1.5
SldD ND 5.5 ± 0.7 9.2 ± 1.3
SldM ND 4.9 ± 0.3 ND

a Numbers represent percentage of positive cells for the given marker. ±SD.
ND, not done.

mg. Tumor growth delay \((T - C)\) is the difference between the median time (in days) required for the treatment group tumor \((T)\) to reach 1500 mg and the median time (in days) for the control group tumors \((C)\) to reach same weight: tumor cell kill net \((\log_{10} K) = (T - C) / \text{duration of treatment in days} \times 3.32\). In this study, an agent is considered highly active \((+++++)\) when \(\log_{10} K\) (net) is >2.0 and gross is >2.8. An activity rating score of \((++++)\) is needed to effect complete tumor regression. A score of +++ is assigned for \(\log_{10} K\) (net) of 0.8–2.0 and (gross) of 2.0–2.8. This score is equivalent to partial remission in clinical terms. A score of either + or ++ is not considered active by usual clinical criteria (23).

Cyto genetic Studies. Chromosomal analysis of the WSU-DLCL2 cells in culture and in SCID mice was done according to standard methods as described previously (24). The cells were exposed to Colcemid (0.05 μg/ml) for 1 h. Cells were then harvested and treated with 0.075 M citrate, pH 6.8, for 15 min, before they were fixed with 3:1 methanol:acetic acid. Slides were prepared by dropping cell suspension onto ethanol-cleaned glass microscope slides. G-banding methods were applied for chromosome identification. From each culture, 20 metaphase cells were analyzed microscopically, and karyotypes were prepared.

In Vitro and in Vivo Detection of the Pgp by Flow Cytometry. Single-cell suspensions of WSU-DLCL2 in culture or dissociated from xenografts were washed and stained with anti-Pgp monoclonal antibody. The antibody C219 (Cetus, Inc., Malvern, PA), which detects an internal epitope of the Pgp transmembrane molecule, was used. Cells were first fixed in cold ethanol for 5 min and stained with indirect immunofluorescence procedure as described previously (25).

Competitive RT-PCR. Competitive RT-PCR was used to obtain quantitative information of mRNA levels. Tumors were excised from SCID mice, and portions were immediately frozen at -70°C. RNA was isolated by the one-step acid guanidinium phenol method (RNA STAT-60; Tel-Test, Friendswood, TX; Ref. 26). Two animals, each with bilateral s.c. tumors were used per time point and received one, two, or three injections of Bryol, followed 24 h later by removal of tumors for mdr1 assays. This design provided the opportunity to analyze multiple tumor samples and determine the consistency of the findings. Reverse transcription was carried out per the manufacturer’s recommendations using oligo(dT)12-18 primers and avian leukosis virus reverse transcriptase (RNA PCR kit; PE-Cetus, Norwalk, CT). Competitive internal standards (MIMICs) were constructed by two rounds of PCR amplified from the v-erbB gene using a PCR Mimic construction kit (PCR MIMIC; Clontech Labs, Palo Alto, CA). By using first a primer set with an internal 17 nucleotides to amplify a 267-bp fragment of v-erbB and an external 17 nucleotides that correspond to the mdr primers, amplification of this mimic template in presence of mdr primers results in a 307-bp product of v-erbB gene. Our mdr primers amplify 500 bp of mdr-extracted cDNA. An analogous series of primers and constructs was made for β-actin (499-bp fragment) and a β-actin competitor (363-bp product).

The sequences of the primers are as follows: mdr-Mimic-sense, 5’-GGCTTGGCTGTTAACCCCGAGCTGAGGGGAGTT-3’; mdr-Mimic-antisense, 5’-TTGGCATAGTCAGGAGCGG- TCAATGCATTGTAG-3’; Actin-Mimic-sense, 5’-GGGCGCCGCCAGCATACAGGGAGATGAAAGG3’; Actin- Mimic-antisense, 5’-ATGTCAGCACGATTCTTGATTCTGACCCTGG-3’; mdr-sense, 5’-CAGGCTTGGTGTTAATTA CCCC-3’; mdr-antisense, 5’-GCTTTGCTGATTCGAGCA CGC-3’; Actin-sense, 5’-GTGGGGGCGCCACGAGGACCGCAACA-3’; and Actin-antisense, 5’-CTCCTTTATATGCACCGCATTTTCC-3’.

The concentration of a spin column-purified mdr and β-actin competitive MIMIC (templates) were determined by fluorometry with H33258 dye. Each 20-μl PCR reaction contained 2 μl of the synthesized cDNA, at the same concentrations before and after Bryol treatment, two μl of serial dilutions of a known amount of the competitive MIMIC, and 1.5 mM MgCl2. PCR was carried out for 40 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 90 s. Eight μl of the PCR reaction were separated by electrophoresis on 2% agarose gels in 40 mM Tris-acetate-1 mM EDTA buffer (1× TAE) in the presence of ethidium bromide. Initially, 10-fold serial dilutions of the competitor were used to determine approximate levels of expression, followed by a 2-fold serial dilutions in that approximate range. Negative photographs of the gels under UV transillumination were scanned on a Zenith densitometer (Advanced American Biotech, Fullerton, CA). The density ratio of the target cDNA PCR product to the competitive MIMIC, adjusted for the difference in product length, plotted against the concentration of the competitor and used to determine mass quality in attomoles. For each sample, the ratio of mdr1 cDNA to actin cDNA is determined to normalize for differing amounts and integrity of extracted RNA.

Northern Blot Analysis. WSU-DLCL2 and CCRF- CEM/R cells were exposed to Bryol at 200 nm for up to 72 h. The CCRF-CEM/R line is T-cell acute lymphoblastic leukemia with induced mdr1 amplification (150-fold) and Adria mycin resistance. The cells are used in this study as positive control. Total cellular RNA was isolated at 24-h intervals from treated and untreated (control) cells using RNA STAT-60 reagent (Tel-Test “B” Inc., Friendswood, TX) according to the manufacturer’s instructions. Twenty μg of each RNA sample were denatured and separated on a formaldehyde-agarose (1%) gel using a Northern-Max kit (Ambion, Inc., Austin, TX) and was trans-
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Fig. 1  G-banded karyotype of WSU-DLCL2 showing t(4;14) and t(14;18)(arrows). The composite karyotype is: 48,XY,t(1;2)(p36.1;q37),der(3)t(3;7)(q13:p15),t(4;14)(q27;132),t(7;21)(p11.2;q22),+i(7p),der(15)(q26),del(16)(q22),del(17)(q25).

Table 2  Activity of selected cytotoxic agents and Bryol in WSU-DLCL2 SCID xenografts

<table>
<thead>
<tr>
<th>Agent</th>
<th>Dose*</th>
<th>Route</th>
<th>No. of animals</th>
<th>T/C (%)</th>
<th>T - C(d)</th>
<th>Log10 kill (net)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluent (control)</td>
<td>0.0</td>
<td>i.v.</td>
<td>5</td>
<td>100</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>VCR</td>
<td>0.5 mg/kg</td>
<td>i.v.</td>
<td>5</td>
<td>29.6</td>
<td>16</td>
<td>0.7</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>3.3 mg/kg</td>
<td>i.v.</td>
<td>5</td>
<td>26.3</td>
<td>19</td>
<td>1.0</td>
</tr>
<tr>
<td>Ara-C</td>
<td>450 mg/kg</td>
<td>i.v.</td>
<td>5</td>
<td>38.2</td>
<td>16</td>
<td>0.7</td>
</tr>
<tr>
<td>Bryol</td>
<td>50 μg/kg</td>
<td>i.p./i.v.</td>
<td>5</td>
<td>100</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Bryol/</td>
<td>75 μg/kg</td>
<td>i.p./i.v.</td>
<td>5</td>
<td>39</td>
<td>7</td>
<td>0.5</td>
</tr>
<tr>
<td>VCR</td>
<td>0.5 mg/kg</td>
<td>i.v.</td>
<td>5</td>
<td>11.3</td>
<td>35</td>
<td>2.0</td>
</tr>
<tr>
<td>Bryol/</td>
<td>75 μg/kg</td>
<td>i.p./i.v.</td>
<td>5</td>
<td>6</td>
<td>38</td>
<td>2.5</td>
</tr>
<tr>
<td>VCR</td>
<td>0.5 mg/kg</td>
<td>i.v.</td>
<td>5</td>
<td>45.6</td>
<td>16</td>
<td>0.6</td>
</tr>
<tr>
<td>Bryol/</td>
<td>50 μg/kg</td>
<td>i.p./i.v.</td>
<td>5</td>
<td>Died of toxicity*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ara-C</td>
<td>450 mg/kg</td>
<td>i.v.</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bryol/</td>
<td>50 μg/kg</td>
<td>i.p./i.v.</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>doxorubicin</td>
<td>3.3 mg/kg</td>
<td>i.v.</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Doses are determined based on previous experiments with these drugs.

* This experiment was repeated using a lower dose of doxorubicin (2.3 mg/kg), where four of five mice also died of toxicity.

ferred to BrightStar-Plus nylon membrane (Ambion, Inc.). RNA transcripts were prepared as probes using PRTI-MDR1-human and PRTI GAPDH-human antisense probe templates and MAXIscriptr T7Kit (Ambion, Inc.). These RNA transcripts were labeled using BrightStar psoralen-biotin nonisotopic labeling kit (Ambion, Inc.). Northern blot analysis was carried out by hybridization at 69°C for mdr1 probe and at 65°C for glyceraldehyde-3-phosphate dehydrogenase probe. The BrightStar Biodeflect kit (Ambion, Inc.) was used in the detection process.

[^3]H]VCR Accumulation. WSU-DLCL2 cells at 5 X 10^5/ml were cultured with or without Bryol (200 nm) for 24 h and washed with RPMI 1640. Cells (2.5 X 10^5) in 1 ml of glucose-free RPMI 1640 were then exposed to 10 nm of[^3]H]VCR (specific activity, 4.5 Ci/mmol; Amersham Life Science, Arlington Heights, IL) for 10 min and incubated at 37°C. At various time points, cells were washed three times with chilled PBS, and the pellets were suspended in 1 ml of scintillation liquid cocktail and analyzed on a Beckman scintillation counter (model LS-3801). Results were then converted to pmol/10^6 cells and plotted against time.

Anthracycline Efflux Analysis by Flow Cytometry. WSU-DLCL2 cells (5 X 10^5) from either control culture or following exposure to Bryol (200 nm) for 24 h were aliquoted in 12 X 75-mm tubes. After washing, cells were incubated with daunorubicin (at a final concentration of 2 μg/ml), Adriamycin (20 μg/ml), or no treatment for 30 min at 37°C. Cells were then washed, placed on ice, and immediately analyzed on Coulter-XL flow cytometer, where 10^4 cells were acquired per test and results were expressed on semilog scale.
RESULTS

Characterization of WSU-DLCL2. After 4 weeks of culture, some of the fresh pleural fluid mononuclear cells began to proliferate. Those cells achieved a steady state of growth by 10 weeks. The cell line, designated WSU-DLCL2, has a doubling time of 1.8 h. Examination of the smears under a light microscope revealed large cells with primitive lymphoblastoid features, including a large irregular nucleus composed of fine chromatin with one or more nucleoli and varying degrees of indentation. WSU-DLCL2 tested negative for EBV nuclear antigen.

Comparison of the phenotypes of the primary pleural fluid cells, established WSU-DLCL2 line in vitro, and in the SCID mice as a xenograft is presented in Table 1. The cell line and the xenograft showed the same characteristics as the original lymphoma. Cells expressed B-cell markers with a monoclonal IgG\alpha. There was no expression of T-lymphoid markers. Other monoclonal antibodies tested on the cell line, not shown in Table 1, were as follows: BL1, 52.0%; CD45, 99.7%; CD21, 6.3%; CDw14, 7.2%; BL7, 12.1%; CDw13, 6.9%; CD33, 13.9%; CD37, 98.4%; ClG\gamma, 54.3%; ClG\alpha, 94.0%; ClG\epsilon, 4.9%; ClG\mu, 6.9%; and ClG\delta, 4.5%.

In Vitro and in Vivo Cytogenetic Analysis. Cytogenetic analysis of the cell line revealed an abnormal male karyotype with several clonal aberrations (Fig. 1). The composite karyotype was: 48.XY,t(1;2)(p36.1;q37),der(3)t(3;7)(q13;p15),t(4;14)(q27;p32),+i(7)(p10),der(7)t(3;7)(q21;p11.2),+8,t(14;18)(q32;q21),del(15)(q26.1),del(16)(q22),del(17)(q25). The t(14;18) is usually associated with follicular B-cell lymphoma. The same chromosomal abnormalities were observed in the s.c. tumor taken from SCID mice.

In Vivo Drug Efficacy. When 25 SCID mice were injected s.c. with equal numbers of WSU-DLCL2 cells (10^5) in each flank, 20 developed tumors (80% take rate). The tumors were palpable by the end of the third week. Tumor volume doubling time (Td) in SCID mice was 3.8 days. When WSU-DLCL2 was passaged in SCID mice, the take rate was 100%. Drug efficacy trials were conducted on animals with palpable tumors.

Table 2 shows the activity of Bryol and selected cytotoxic...
agents given alone or sequentially to WSU-DLCL2-bearing SCID mice. When tumor responses are determined by the T/C value, VCR, doxorubicin, Bryol (in the higher dose), and Ara-C are considered active against this type of human tumor (T/C values, <42%). However, if log10 kill values are added as a criterion, none of the agents had a clinically significant activity except doxorubicin. It should be noted that activity rating of [++] or [+++] is needed to effect partial or complete tumor regressions, respectively. Thus, a score of [ +, log10 kill (net) 0.4-0.79] or [ +, log10 kill (net) >2.0] is not considered active by usual clinical criteria (23).

In the sequential treatment experiments, Bryol/Ara-C was not superior to Ara-C alone. Bryol/doxorubicin was too toxic because all of the animals lost weight, became listless, and died. Reducing the dose of doxorubicin to 2.3 mg/kg was also lethal to the animals. The Bryol/VCR, however, was well tolerated at both dose levels of Bryol and was associated with significant antitumor activity, as measured by tumor growth inhibition, tumor growth delay, and tumor log10 kill (Table 2).

Bryol Effect on Pgp Expression. Incubation of WSU-DLCL2 cells in the presence of Bryol (200 nM) in vitro resulted in decreased expression of Pgp as detected by flow cytometry. In control cultures, 60–70% of WSU-DLCL2 cells expressed detectable Pgp, whereas no significant expression was detected after 24 h of exposure to Bryol (Fig. 2 A and B). This effect was
Pgp expression was then assayed in WSU-DLCL2 xenografts in SCID mice before and after Bryol treatment. WSU-DLCL2 tumors in control animals showed a comparable level of Pgp expression to that seen in vitro (Fig. 2, D and F). Twenty-four h after first injection of Bryol (50 μg/kg), Pgp expression decreased from 62 to 9% (P < 0.001; Fig. 2E). This expression decreased further (to 2.5%) when the animal received two Bryol injections 72 h apart (Fig. 2F).

Down-Regulation of mdr1 RNA by Bryol. Photographs of agarose gel electrophoresis of PCR reactions containing 2-fold serial dilutions of mdr1 competitor with a constant amount of cDNA from WSU-DLCL2 xenografts excised from SCID mice, either untreated or after Bryol injections, are shown in Fig. 3A. These data demonstrate a decrease in mdr1 cDNA after Bryol treatment. The mdr1-β-actin-normalized expression for the various samples is shown in Fig. 3B. The data show that mdr1 RNA was down-regulated in WSU-DLCL2 tumors after treatment of SCID mice with Bryol. Thus, the down-regulation of mdr protein assessed by flow cytometry occurs, at least in part, at the RNA level. The mdr1 mRNA, however, was not detectable in WSU-DLCL2 cells using conventional Northern blot technique. In these experiments, Bryol showed a 33%...
Bryostatin 1 and Diffuse Large Cell Lymphoma Xenografts

reduction in mdr1 mRNA in the CCRF-CEM/R cell line used as a positive control (Fig. 4).

**Functional Drug Efflux Assays.** Functional assays were used to demonstrate the impact of Bryol-induced mdr1 down-regulation on drug retention. Two classes of mdr1-dependent compounds were evaluated: Vinca alkaloids and anthracyclines. Prior exposure of WSU-DLCL2 cells to Bryol for 24 h led to increased intracellular accumulation of [3H]VCR (Fig. 5). There was a 4-fold increase in [3H]VCR in bryol-treated cells compared with untreated controls. Such a difference was apparent after 30 min of exposure to [3H]VCR. Bryol-treated cells also showed increased intracellular accumulation of [3H]VCR (Fig. 5).

**DISCUSSION**

In this report, we show that administering VCR 24 h after Bryol to SCID mice bearing DLCL tumors improves antitumor activity. Bryol by itself showed some activity when given at the maximum tolerated dose by the SCID mice (75 μg/kg). In previous work, we have demonstrated that a Bryol dose of 100 μg/kg was toxic to SCID mice (22). However, even at a lower dose of 50 μg/kg, which had no significant antitumor activity, Bryol decreased Pgp and down-regulated mdr1 RNA expression, as measured by flow cytometry and quantitative PCR assay. This effect on mdr1 expression may be a mechanism by which Bryol potentiates VCR action. This conclusion is supported by the increased uptake of [3H]VCR and daunorubicin fluorescence by WSU-DLCL2 cells after prior exposure to Bryol compared with control cells.

Advanced-stage DLCL was found to be curable by multiagent chemotherapy more than two decades ago (19, 27). The original regimens resulted in cure of 30–40% of cases. Although numerous attempts were made to improve on these results, no further progress has thus far been made (20, 28). mdr1 expression in DLCL is usually low at presentation (29) but increases at relapse or in resistant cases (10). These observations suggest that mdr1 expression may be clinically important in this tumor. The WSU-DLCL2 line was established from a relapsed DLCL that was clinically resistant to therapy. The disease relapsed after high doses of chemotherapy and radiation, followed by bone marrow transplantation. The ability of these cells to grow as xenografts in SCID mice makes it a useful preclinical animal model for resistant lymphoma.

The bryostatins represent one of the most promising therapeutic agents isolated from marine animals (30). Bryol, the most extensively studied, has antineoplastic properties against a number of murine tumors including B16 melanoma (31), M5076 reticulum cell sarcoma, and L10A B-cell lymphoma (32). Recently, we have shown that Bryol can inhibit the phosphorylation of VCR in WSU-DLCL-2 cells *in vitro* (18). We hypothesized that such effect is through the mdr mechanism, because the cell line expresses mdr phenotype. To prove this, we first demonstrated a decrease in Pgp expression by WSU-DLCL-2 cells in the presence of Bryol *in vitro* (Fig. 2). Moreover, in *in vivo* studies, similar results were observed when WSU-DLCL-2 tumors were removed from SCID mice after Bryol treatment.

To determine whether the down-regulation of mdr1 expression is at the RNA level, we developed a competitive PCR method to quantitate the mdr1 RNA. These experiments showed a decrease in the mdr1 RNA of WSU-DLCL-2 tumors removed from SCID mice treated with Bryol compared with untreated controls (Fig. 3). The competitive PCR method has been shown to be quantitative in a number of systems (33, 34). Our modification of normalizing the input cDNA by using competitive PCR for β-actin allows for more accurate quantitation and is applicable to biopsy samples. After confirming that Bryol did indeed lead to a decrease in the amount of Pgp and mdr1 RNA, we tested whether there was a measurable decrease in Pgp function that would actually account for the Bryol enhancement of VCR efficacy. We demonstrated that Bryol treatment of WSU-DLCL2 cells leads to increased [3H]VCR and daunorubicin accumulation intracellularly (Figs. 4 and 6). Cyclosporin A, a known inhibitor of Pgp, led to increased rhodamine-123 fluorescence, mimicking Bryol effect (data not shown).

It is possible that Bryol has additional functions that can influence drug uptake besides down-regulation of mdr1 RNA. One such function is the phosphorylation of Pgp. Although we did not assay for Pgp phosphorylation by Bryol, PKC activators, in general, are known to phosphorylate Pgp (35). Some studies have shown that phosphorylation of Pgp leads to modulation of its function (36). However, more definitive studies using nonphosphorylatable Pgp mutants demonstrated that phosphorylation/dephosphorylation mechanisms do not play a significant role in the establishment of mdr phenotype mediated by human Pgp (37–39). Another function of Bryol that has relevance to drug sensitization is the modulatory effect on bcl2 and p53 gene expression. We have demonstrated previously that exposure of WSU-DLCL2 cells to Bryol *in vitro* led to decreased bcl2 expression and up-regulation of p53 (18). This change in phenotype is associated with drug sensitivity, as exemplified by quantitation of VCR cytotoxicity. It is likely, therefore, that reversal of resistance to chemotherapy by Bryol is quite complex and may involve more than one known resistance mechanism.

The clinical course of the lymphoma from which the WSU-DLCL2 line was established was extremely aggressive and resistant to the most intensive therapy available. The WSU-DLCL2 xenograft model was also resistant to single-agent therapy with the exception of modest activity of doxorubicin (Table 2). Only the sequential use of Bryol and VCR led to what is considered a significant activity in the mouse that is clinically meaningful. This finding is consistent with an earlier observation made in our laboratory that sequential use of Bryol and VCR was quite effective against a human Waldenstrom’s macroglobulinemia xenograft model (22).

Modulation of mdr1 is a previously unrecognized effect of Bryol, and the down-regulation of the mdr1 RNA is a novel mechanism for overcoming mdr function. Because Bryol has entered clinical trials in cancer patients, recognition of this function may be clinically useful.

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Bryostatin 1 down-regulates mdr1 and potentiates vincristine cytotoxicity in diffuse large cell lymphoma xenografts.


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