A Role for Altered Microtubule Polymer Levels in Vincristine Resistance of Childhood Acute Lymphoblastic Leukemia Xenografts


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Received July 19, 2007; accepted November 5, 2007

ABSTRACT

The microtubule-depolymerizing drug, vincristine, is effective in the treatment of acute lymphoblastic leukemia (ALL). Although vincristine resistance mechanisms have been extensively characterized in cell lines, their clinical relevance is poorly understood. The aim of the current study was to define clinically relevant mechanisms of vincristine resistance in a panel of childhood ALL xenografts established in immune-deficient (nonobese diabetic/severe combined immunodeficient) mice. We also studied two independent xenograft sublines that were selected by in vivo vincristine exposure. In vitro vincristine sensitivity determined by a stromal coculture, murine bone marrow stromal cell line (MS-5), assay, but not methyl-thiazolyl-tetrazolium metabolic activity assay, significantly correlated (P = 0.05) with the length of the patients’ first remission. Investigations into mechanisms of resistance revealed no association with steady-state vincristine accumulation or increased activity and/or expression of ATP-binding cassette transporters, although increased intracellular levels of polymerized tubulin significantly correlated with resistance (r = 0.85; P = 0.0019). Two xenograft sublines selected by in vivo vincristine exposure exhibited a 2-fold increase in polymerized tubulin levels compared with the parental subline (P < 0.05), reflecting their in vivo vincristine resistance. In this study, a vincristine-resistant xenograft with high levels of polymerized tubulin was relatively sensitive to the microtubule-polymerizing drug paclitaxel. These results indicate that the balance between polymerized and nonpolymerized tubulin may be an important determinant of response to Vinca alkaloid-based chemotherapy regimens in childhood ALL.

Acute lymphoblastic leukemia (ALL) is the most common pediatric malignancy, accounting for more than 25% of all childhood cancers (Pui et al., 2002, 2004). Over the past 40 years, refinements in multiagent chemotherapy regimens and supportive care have resulted in a dramatic improvement in survival rates of children with ALL (Pui et al., 2002, 2004). At this time, remission is induced in over 95% of patients, with the likelihood of cure being greater than 70% (Pui et al., 2002, 2004). Despite such success, relapse still occurs in 25 to 30% of children with ALL, and those who suffer a bone-marrow relapse within 2 years of diagnosis experience less than a 20% likelihood of long-term survival (Roy et al., 2005). The major cause of treatment failure is intrinsic or acquired drug resistance, although the mechanisms by which this occurs remain poorly defined.

The Vinca alkaloid vincristine is one of the most active single agents used in the treatment of childhood ALL, and the in vitro responses of primary childhood ALL cells to vincristine are predictive of treatment outcome (Kaspers et al., 1997). Vincristine binds to the β subunit of the αβ-tubulin

ABBREVIATIONS: ALL, acute lymphoblastic leukemia; ABC, ATP-binding cassette; CEM, CCRF-CEM; QBSF, Quality Biological Serum Free; RPMI, Roswell Park Memorial Institute; PBS, phosphate-buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; PI, propidium iodide; calcein-AM, calcein-acetoxymethyl ester; MS-5, murine bone marrow stromal cell line; VCR, vincristine; WT, wild type; MK571, (R)-3-{[2-(7-chloro-2-quinolinyl)ethenyl][phenyl]-[3-dimethylamino]-3-oxopropyl[thio]methyl}[thio]-propanoic acid, sodium salt.
heterodimer, inducing microtubule destabilization, and at substoichiometric doses (less than one molecule of drug per tubulin heterodimer), it suppresses microtubule dynamics (Jordan and Wilson, 2004). Microtubules are key components of the cytoskeleton and play an important role in mitosis, and interference in microtubule dynamics by vincristine causes mitotic arrest and cell death (Jordan and Wilson, 2004). Microtubules are in a continual state of assembly and disassembly, which is controlled by complex interactions with microtubule-associated proteins, such as MAP4 and stathmin (OP18), and post-translational modifications of tubulin subunits (Amos and Schlieper, 2005). Increased levels of polymerized tubulin have been associated with increased microtubule stability, decreased sensitivity to the cytotoxic effects of microtubule-depolymerizing drugs such as the Vinca alkaloids, and increased sensitivity to the microtubule-polymerizing drug paclitaxel (Hari et al., 2003). This evidence is supported by elevated polymerized tubulin levels in vincristine-resistant human leukemia cell lines (Kavallaris et al., 2001). Studies using leukemia cell lines in vitro have shown that another common mechanism of vincristine resistance involves reduced intracellular drug accumulation due to overexpression of ATP-binding cassette (ABC) transporters (Broxterman et al., 1995; Dumontet and Sikic, 1999). However, investigations using patient biopsy specimens that implicate altered expression of drug efflux pumps, including P-glycoprotein (MDR1, ABCB1) and multidrug resistance-associated protein 1 (ABCC1), in the treatment outcome of childhood ALL remain controversial (Dhooge et al., 1999; Kanerva et al., 2001; Olson et al., 2005; Swerts et al., 2006).

We have previously established a panel of continuous childhood ALL xenografts in nonobese diabetic/severe combined immunodeficient mice that were derived from biopsy specimens of patients who experienced diverse treatment outcomes (Liem et al., 2004). The in vivo responses of this panel of xenografts to vincristine significantly correlated with patient outcome (Liem et al., 2004). The present study reports a detailed assessment of the in vivo vincristine sensitivity of this panel of xenografts using two methods to assess the chemosensitivity of primary childhood ALL cells, and it investigates mechanisms associated with differential vincristine response. We show that the proportion of polymerized tubulin in the xenograft cells, but not steady-state vincristine accumulation or expression of ABCB1 or ABCC1, significantly correlates with in vivo vincristine sensitivity. Furthermore, we demonstrate that polymerized tubulin levels are significantly increased in two xenografts that were independently selected for vincristine resistance by in vivo treatment of nonobese diabetic/severe combined immunodeficient mice, whereas a vincristine-resistant xenograft with high levels of polymerized tubulin exhibits relative sensitivity to paclitaxel. Together, these findings highlight an important role for levels of tubulin polymer in vincristine response and indicate that alterations in microtubule stability are likely to influence clinical response to vincristine-based chemotherapy regimens in childhood ALL.

Materials and Methods

Childhood ALL Xenografts. The development, characterization, and in vivo vincristine responses of a series of childhood ALL xenografts derived from patient biopsies have been described previously (Lock et al., 2002; Liem et al., 2004). All children were treated at the Centre for Children’s Cancer and Blood Disorders and Sydney Children’s Hospital, and a detailed account of the patients’ clinical characteristics, risk stratification, and clinical trials on which they were enrolled was provided previously (Lock et al., 2002). Approval was obtained from the University of New South Wales Institutional Review Board for these studies. Informed consent was obtained according to the Declaration of Helsinki. Mononuclear cells used in this study were previously purified from the spleens of engrafted mice by Ficoll-density gradient centrifugation and cryopreserved in 90% (v/v) fetal bovine serum and 10% (v/v) dimethyl sulfoxide.

The in vivo selection of vincristine-resistant xenografts derived from xenograft ALL-17 (ALL-17-VCR<sup>1</sup> and ALL-17-VCR<sup>2</sup>) was described in detail elsewhere (Verrillas et al., 2006). After initial selection through the in vivo vincristine treatments, leukemia cells were harvested from the spleens of engrafted mice and expanded by secondary passage through recipient mice, also under vincristine selection, using established procedures (Lock et al., 2002; Liem et al., 2004). To compare the in vivo responses of these xenografts, the median event-free survival for control mice was subtracted from the median event-free survival of vincristine-treated (0.5 mg/kg i.p. every 7 days for 4 weeks) mice to generate a growth delay factor, exactly as described previously (Liem et al., 2004). All experimental studies were approved by the Animal Care and Ethics Committee of the University of New South Wales.

Cell Culture. For all experiments described in this study, xenograft cells were retrieved from cryostorage and resuspended in Quality Biological Serum Free (QBSF)-60 medium (Quality Biological, Inc., Gaithersburg, MD) supplemented with fms-like tyrosine kinase-3 ligand (20 ng/ml; kindly provided by Amgen, Thousand Oaks, CA), penicillin (100 U/ml), streptomycin (100 μg/ml), and l-glutamine (2 mM) (QBSF-60/F). Viability was determined by the exclusion of 0.2% trypan blue.

The human T-lineage ALL cell line, CEM-WT, and its vincristine-resistant subline, CEM/VCR R (Haber et al., 1989), were maintained as static suspension cultures at 37°C in a 5% CO<sub>2</sub> atmosphere in RPMI 1640 medium (Invitrogen Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Invitrogen Life Technologies), penicillin (100 U/ml), streptomycin (100 μg/ml), and l-glutamine (2 mM) (Complete RPMI 1640 medium). The murine bone marrowstromal cell line (MS-5) (kindly provided by Prof. K. John Mori, Niigata University, Niigata, Japan) was maintained in alpha minimum essential medium (Invitrogen Life Technologies) supplemented with 10% fetal bovine serum in a humidified atmosphere at 37°C and 5% CO<sub>2</sub> (Suzuki et al., 1992).

In Vitro Cytotoxicity Assays. The colorimetric MTT assay, which measures a combination of inhibition of proliferation and cell death, was used to assess the in vitro vincristine sensitivity of xenograft cells and cell lines. After retrieval from cryostorage, xenograft cells were resuspended at densities previously optimized for each xenograft (2–5 × 10<sup>4</sup> cells/ml) and equilibrated overnight at 37°C in 5% CO<sub>2</sub> air. For each vincristine concentration, 100 μl of cell suspension was seeded in triplicate in 96-well U-bottomed microtiter plates (Greiner Bio-one, Frickenhausen, Germany) and equilibrated overnight at 37°C in 5% CO<sub>2</sub> air. Vincristine sulfate (Sigma-Aldrich, St. Louis, MO) was added in QBSF-60/F at the indicated concentrations. The plates were incubated for 48 h at 37°C in a 5% CO<sub>2</sub> atmosphere, and 12 μl of filter-sterilized MTT-labelling reagent (Sigma-Aldrich) was added to each well. The formazan crystals that formed after 6 h were dissolved in 100 μl of 10% (w/v) sodium dodecyl sulfate in 0.01 M HCl. The optical density of each well was measured at 570 nm, with reference to 655 nm. Cell viability was calculated as a percentage of solvent-treated controls. The CEM-WT and VCR R cell lines were similarly studied using the MTT assay, with exception that Complete RPMI 1640 medium (Invitrogen Life Technologies) was used throughout and the initial seeding density was 5 × 10<sup>4</sup> cells/ml.

Xenograft cell sensitivity to vincristine and paclitaxel was also
assessed using a stroma/fibroblast coculture system originally developed by Campana and co-workers (Campana et al., 1993; Kumagai et al., 1994) and adapted for use with MS-5 cells (Liem et al., 2004), hereafter referred to as the MS-5 assay. This assay provides a direct measurement of viable cell number. MS-5 cells were seeded into 96-well U-bottomed microtiter plates at a density of 12,000 cells/well and grown to confluence (72 h). Xenograft cells were resuspended at a density of 2 × 10^6 cells/ml in qBSP-60/F medium, and 200 µl of the cell suspension was seeded in duplicate wells onto the MS-5 cells, which had been prewashed with sterile phosphate-buffered saline (PBS). After overnight equilibration at 37°C in 5% CO₂, air, vincristine sulfate or paclitaxel was added to a range of final concentrations. Forty-eight hours later, cells were harvested and stained with allophtocyanin-conjugated antithuman CD45 (BD PharMingen, San Jose, CA) and propidium iodide (PI) (10 µg/ml; BD PharMingen). Viable human leukocytes (CD45+/PI−) were enumerated using a FACScalibur flow cytometer (BD Immunocytometry Systems, San Jose, CA) with reference to 20,000 10-µm latex beads (Beckman Coulter, Fullerton, CA) that had been added to each well before harvest. Cell viability was calculated as a percentage of solvent-treated controls.

High-Resolution Cell Division Tracking. The methodology for monitoring the proliferation of xenograft cells in short-term culture using the fluorescent dye carboxyfluorescein diacetate succinimidyl ester (Molecular Probes, Eugene, OR) has been described in detail elsewhere (Nordon et al., 1997; Liem et al., 2004). In brief, freshly thawed xenograft cells in qBSP-60/F medium were incubated with 0.75 µM carboxyfluorescein diacetate succinimidyl ester for 10 min at 37°C in the dark. Cells were resuspended in carboxyfluorescein diacetate succinimidyl ester-free medium, incubated overnight, and sorted using a FACSVantage SE with DiVa option (BD Immunocytometry Systems). Sorting gates were set around the central histogram peak at a width of 34 to 40 channels out of a total of 1024 on a linear scale to obtain the most homogeneously staining cell populations. Sorted cells were then centrifuged, resuspended in qBSP-60/F medium at a density of 2 × 10^6 cells/ml, and plated into 96-well plates with or without MS-5 coculture, as described above. At various times thereafter, cells were harvested by vigorous pipetting and enumerated using the FACScalibur flow cytometer as described above. Cells that had divided were identified by halving of fluorescein intensity (relative to colcemid-treated undivided cells) at each generation. Independent experiments verified that 100% of divided cells were human CD45+ and not contaminated with murine cells (data not shown). The Proliferation Index at each time point was calculated using the ModFit LT version 2.0 Proliferation Wizard software (Verity Software House, Topsham, ME), as described in detail elsewhere (Nordon et al., 1997; Liem et al., 2004).

Vincristine Accumulation Assay. Accumulation of vincristine in leukemia cells is known to reach a steady state within 2 h of incubation (Haber et al., 1989). Xenograft cells (pre-equilibrated in culture overnight) and CEM cell lines were incubated at a density of 12,000 cells/well in 96-well U-bottomed microtiter plates at a density of 12,000 cells/well and grown to confluence (72 h). Xenograft cells were resuspended in qBSP-60/F medium, and 200 µl of the cell suspension was added before the addition of the following inhibitors: verapamil (Sigma-Aldrich) or MK571 (Molecular Probes), each at a final concentration of 50 µM. Verapamil blocks the activity of both ABCB1 and ABCB1, whereas MK571 inhibits ABCB1 alone. After incubation at 37°C in the dark for 10 min, calcine-AM was added to a final concentration of 0.25 µM. Cells were incubated at 37°C in the dark for 10 min, and a total of 10,000 events per sample were acquired using a FACSCalibur flow cytometer with an excitation wavelength of 488 nm and detection at 530 nm. PI-positive cells were excluded from analysis. Cell populations that express increased ABCB1 and/or ABCB1 activity exhibit increased calcine-AM fluorescence (right shift) when preincubated with verapamil or MK571, compared with nontreated cells. Data presented are representative of at least three separate experiments.

Microarray Analysis of Gene Expression. Total RNA was prepared from xenograft cells using the RNeasy Kit (QiAGEN, Valencia, CA). Gene-expression analysis was performed in the Hartwell Center core laboratory (St. Jude Children’s Research Hospital, Memphis, TN) using the Affymetrix HG-U133Plus2 GeneChip (54,613 probe sets) (Affymetrix, Inc., Santa Clara, CA). RNA quality was confirmed by UV spectrophotometry and by analysis on an Agilent 2100 Bioanalyzer (Agilent Technologies, Foster City, CA). Processing of RNA samples was performed according to the Affymetrix gene expression protocol (http://www.affymetrix.com/support/technical/manual/expressionmanual.affx). Expression signals were calculated using the MASS statistical algorithm within the Affymetrix GCSOS software (version 1.4). Signal values were scaled using the global normalization method with the 2% trimmed mean set to 500. Detection calls for each transcript (absent, marginal, or present) were determined using the default parameters within the GCSOS software. Expression data were visualized using GeneSifter software (VizX Labs, Seattle, WA). Gray shading in Fig. 2 indicates an absent call from Affymetrix quality control.

Tubulin Polymerization Assay. Levels of soluble (cytosolic) and polymerized (cytoskeletal) tubulins were determined in the panel of xenografts and cell lines CEM-WT and CEM/VCR R, using an assay previously established in our laboratory for leukemia cells (Kavalari et al., 2001). Cells were washed twice in prewarmed PBS, and 2 × 10^6 cells were centrifuged (3000g for 5 min). The PBS was aspirated, and the cell pellet was resuspended in 200 µl of hypotonic buffer [1 mM MgCl₂, 2 mM EGTA, 0.5% (v/v) Nonidet P-40, and 20 mM Tris-HCl, pH 6.8] containing 1% (v/v) protease inhibitors (Sigma-Aldrich). This hypotonic buffer has been previously shown to maintain the integrity of microtubules (Minotti et al., 1991). The resuspended cells were incubated at 37°C for 10 min in the dark, after which an additional 200 µl of hypotonic buffer was added. The cell lysates were vortexed, and the fractions were separated by centrifugation (18,000g for 10 min at room temperature). The supernatant containing the unpolymerized (soluble) tubulin was transferred to a fresh tube. The pellet fraction containing the polymerized (insoluble) tubulins was resuspended in 400 µl of hypotonic buffer. Proteins were solubilized in 135 µl of sample buffer [45% (v/v) glycercol, 0.3 M Tris-HCl, pH 6.8, 9.2% (v/v) SDS, 0.04% (v/v) saturated bromophenol blue, and 20% (v/v) β-mercaptoethanol], and the polymerized fraction was sonicated thoroughly on ice before heating all samples at 100°C for 5 min. Proteins (30 µl) were separated using Criterion 4 to 15% ready gels (Bio-Rad, Hercules, CA), transferred to nitrocellulose membranes, and probed for α-tubulin using standard immunoblotting techniques. The tubulin signal was quantified, and polymerized tubulin was expressed as a percentage of total polymerized and soluble tubulin, as described previously (Kavallari et al., 2001).

Statistical Analysis. All numerical data presented are the mean ± S.E.M. of at least three independent experiments, unless stated otherwise. For the cytotoxicity experiments, IC₅₀ values were
calculated from cumulative survival curves. Correlations between quantitative variables were determined by regression analysis using GraphPad Prism 4.00 (GraphPad Software, Inc., San Diego, CA). For each analysis, a Pearson correlation coefficient (R) was calculated, and a Fisher R-to-Z transition was carried out to calculate a probability level (P value) for the null hypothesis that the correlation was equal to zero. For all statistical tests, the level of significance was set to 0.05.

Results

Vincristine Sensitivity of Childhood ALL Xenografts. We previously established a panel of 10 continuous xenografts derived from childhood ALL biopsies (Table 1), documented their relevance with respect to the clinical characteristics of the disease, and demonstrated that their in vivo responses to vincristine significantly correlated with the clinical outcome of the patients from whom they were derived (Lock et al., 2002; Liem et al., 2004). The two principal methods reported to assess the in vitro sensitivity of primary childhood ALL cells to chemotherapeutic drugs involve the following: 1) estimation of cell viability by mitochondrial function (usually MTT assay) (Kaspers et al., 1997); and 2) coculture of ALL cells with stromal or fibroblast cell support and direct enumeration of viable cells (Kumagai et al., 1994). In this study, both methods were used to determine the in vitro vincristine sensitivity of the panel of 10 xenografts, with IC_{50} values reported in Table 1, and representative survival curves are shown in Fig. 1.

The data in Table 1 indicate that there was a broad range of vincristine sensitivity using both the MTT and MS-5 assays. Using the MTT assay, there was a >3000-fold difference between the most sensitive (ALL-8, IC_{50} = 0.18 nM) and the most resistant (ALL-2, IC_{50} = 578 nM) xenografts, whereas this range was >500-fold for the MS-5 assay (ALL-10 IC_{50} = 0.15 nM, ALL-19 IC_{50} = 81 nM). Moreover, whereas MTT IC_{50} values for the xenografts did not correlate with clinical outcome, there was a significant correlation between IC_{50} by MS-5 assay and the length of the patients’ CR1 (r = 0.79; P = 0.05). For data obtained using the MS-5 assay, the five most vincristine-resistant xenografts were all derived from patients who died from their disease, whereas four of five patients with vincristine-sensitive xenografts remained alive for up to 11 years after diagnosis. Therefore, the MS-5 assay seems to better reflect clinical outcome compared with the MTT assay, consistent with the in vivo vincristine sensitivity of the xenografts (Lock et al., 2002; Liem et al., 2004).

MS-5 and/or stromal cells have been shown to enhance the in vitro survival of normal and malignant hematopoietic cells and confer resistance to cytotoxic drugs (Itoh et al., 1989; Manabe et al., 1992; Mudry et al., 2000; Konopleva et al., 2002; Liem et al., 2004). However, the ability of MS-5 cells to protect against vincristine cytotoxicity varied in this panel of xenografts. Three distinct effects were observed when xenograft cells were cocultured with MS-5 cells compared with the MTT assay: 1) decreased vincristine sensitivity (IC_{50} ratio >2-fold) in xenografts ALL-8, ALL-17, and ALL-19 (Table 1; Fig. 1A); 2) increased vincristine sensitivity (IC_{50} ratio <0.5-fold) in xenografts ALL-2, ALL-10, and ALL-11 (Table 1; Fig. 1B); and 3) little difference in vincristine sensitivity (IC_{50} ratio between 0.5- and 2-fold) in xenografts ALL-3, ALL-4, ALL-7, and ALL-16 (Table 1; Fig. 1C).

Vincristine is a drug that is known to preferentially target proliferating cells. However, the variable effects of MS-5 cells on xenograft cell sensitivity to vincristine could not simply be attributed to the effects on cell proliferation, as determined by high-resolution cell division tracking (see Supplementary Fig. S1). Figure S1 depicts the Proliferation Index of four xenografts, cultured with or without MS-5 cells, over a comparable time period used for the vincristine cytotoxicity assays. Whereas MS-5 cells exerted minimal effects on the proliferation of ALL-7 (Fig. S1A) and ALL-19 (Fig. S1B) cells, their influence on the vincristine sensitivity of these two xenografts was disparate, with ALL-7 exhibiting no difference and ALL-19 becoming 15-fold resistant (Table 1; Fig. 1A). Furthermore, whereas MS-5 cells stimulated the proliferation of ALL-11 (Fig. S1C) and ALL-17 (Fig. S1D) cells, again their influence on vincristine sensitivity was disparate, with ALL-11 cells becoming 95-fold more sensitive and ALL-17 exhibiting 2.4-fold resistance. Thus, the effects of MS-5 cell coculture on ALL cell sensitivity to vincristine seem complex and not merely reflective of changes in cell proliferation.

### Table 1

Clinical and biological characteristics of childhood ALL xenografts and cell lines

<table>
<thead>
<tr>
<th>Xenograft or Cell Line</th>
<th>Length of CR1</th>
<th>Survival after First Relapse</th>
<th>Current Clinical Status</th>
<th>VCR IC_{50} by MTT Assay</th>
<th>VCR IC_{50} by MS-5 Assay</th>
<th>Steady-State VCR Uptake</th>
<th>Polymerized Tubulin</th>
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<tbody>
<tr>
<td></td>
<td>nmo.</td>
<td></td>
<td></td>
<td>nM</td>
<td>nM</td>
<td>pmol/10^6 cells</td>
<td>%</td>
</tr>
<tr>
<td>ALL-2</td>
<td>30</td>
<td>46</td>
<td>DOD</td>
<td>578</td>
<td>5.1</td>
<td>0.34 ± 0.13</td>
<td>8.5 ± 2.2</td>
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<tr>
<td>ALL-3</td>
<td>38</td>
<td>115*</td>
<td>CR2</td>
<td>1.6</td>
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<td>0.91 ± 0.34</td>
<td>5.6 ± 1.9</td>
</tr>
<tr>
<td>ALL-4</td>
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<td>1</td>
<td>DOD</td>
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<td>DOD</td>
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<td>5.0 ± 2.3</td>
</tr>
<tr>
<td>ALL-8</td>
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<td>16</td>
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<td>ALL-10</td>
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<td>CR1</td>
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<tr>
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<td>CR1</td>
<td>398</td>
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<tr>
<td>ALL-16</td>
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<td>CR1</td>
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<td>1.0 ± 0.4</td>
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<tr>
<td>ALL-17</td>
<td>25</td>
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<tr>
<td>ALL-19</td>
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<td>N.A.</td>
<td>N.A.</td>
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<td>N.A.</td>
<td>N.A.</td>
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<td>2.8 ± 0.2</td>
<td>34.1 ± 0.7</td>
</tr>
</tbody>
</table>

*CR1, alive in first complete remission; CR2, alive in second complete remission; DOD, dead of disease; N.A., not applicable; N.D., not done.

* No event (censored).
Analysis of Mechanisms Associated with Vincristine Resistance. Reduced intracellular steady-state vincristine levels, due to increased activity of drug efflux pumps, were frequently associated with vincristine resistance (Broxterman et al., 1995; Dumontet and Sikic, 1999). Vincristine accumulation assays were carried out on xenograft cells to determine whether differences in steady-state levels could account for their varied sensitivity to vincristine. Two control ALL cell lines were also used, the vincristine-sensitive CEM-WT cell line and its vincristine-resistant CEM/VCR R subline, the latter exhibiting a well-characterized defect in vincristine uptake (Kavalalaris et al., 2001). Table 1 highlights the difference (>17-fold) in vincristine uptake between these two cell lines.

Table 1 illustrates the range of steady-state vincristine accumulation levels in the panel of xenografts, and all values falling between those for CEM-WT and VCR R cells are shown. Vincristine accumulation in the xenograft cells varied from <0.5 to 1.9 pmol/10^6 cells. Moreover, ALL-2 accumulated the least vincristine of all xenografts, which seemed to reflect its relative resistance to the drug in both MTT and MS-5 assays (Table 1). However, vincristine uptake was highest in ALL-17 and -19, which were also relatively vincristine-resistant in both assays. Subsequent analysis revealed no significant correlations between vincristine uptake and vincristine sensitivity assessed by either the MTT or MS-5 assays, even when sensitivity was expressed in terms of IC50 or relative metabolic activity/cell survival at 10 or 100 nM vincristine (data not shown). Therefore, it is unlikely that differences in drug uptake account for the varied sensitivity of the panel of xenografts to vincristine.

Experiments were also carried out using the calcein-AM assay to determine the activity of drug efflux pumps in xenograft cells. Figure 2A demonstrates that VCR R cells exhibited high activity of both ABCB1 and ABCC1, indicated by the dramatic right shift (increased intracellular calcein-AM) of cells preincubated with MK571 (green line) or verapamil (red line) compared with nontreated cells (blue line). In contrast, the xenograft that exhibited the lowest steady-state vincristine accumulation (ALL-2; Table 1) showed no evidence of drug efflux pump activity using this assay. Analysis of a total of seven xenografts using the calcein-AM assay showed no indication of drug efflux pump activity (data not shown).
The role of drug-efflux activity in vincristine resistance of xenograft cells was further examined by microarray analysis of gene expression. Whereas expression of ABCC1 was detected in all xenografts (Fig. 2B), ABCB1 expression was low as indicated by a high proportion of absent calls in the analysis. Moreover, the relative expression of ABCC1 and ABCB1 (absent calls excluded) showed no significant correlation with steady-state vincristine accumulation in the xenografts. ABCC1 expression seemed highest in the two T-lineage ALL xenografts, ALL-8 and ALL-16.

Increased levels of polymerized tubulin could also account for resistance to vincristine by counteracting the depolymerizing effects of the drug (Kavallaris et al., 2001). Immunoblots of soluble and polymerized tubulin fractions from xenografts are represented in Fig. 3A, which indicate the difference in the level of polymerized tubulin between the xenografts. Table 1 and Fig. 3B depict the levels of polymerized tubulin in all xenografts in three separate experiments. Consistent with a previous study (Kavallaris et al., 2001), vincristine-resistant CEM/VCR R cells had significantly more polymerized tubulin (45 ± 1.5%) compared to CEM-WT cells (34.1 ± 0.7%). Unlike the high fraction of tubulin polymer observed in leukemia cell lines (Kavallaris et al., 2001; Dumontet et al., 2004), levels of polymer were relatively lower in ALL xenografts using the same assay as that used for cell lines. It is of interest to note that the highest levels of polymerized tubulin in xenograft cells were encountered in ALL-2, -4, -17, and -19, xenografts that were relatively vincristine-resistant in both the MTT and MS-5 assays (Table 1). A subsequent analysis revealed a statistically significant correlation between the proportion of polymerized tubulin and vincristine sensitivity assessed by the MS-5 assay, either as IC_{50} (r = 0.85; P = 0.0019; Fig. 3C) or as cell survival at 10 nM vincristine (r = 0.81; P = 0.0049; data not shown) or 100 nM vincristine (r = 0.64; P = 0.047; data not shown). Moreover, the correlation between the proportion of polymerized tubulin and the patients’ CR1 approached significance (P = 0.07). Statistically significant correlations were not observed between the proportion of polymerized tubulin and vincristine sensitivity assessed by the MTT assay (data not shown).

Levels of Polymerized Tubulin in in Vivo-Selected Vincristine-Resistant Xenograft Sublines. To further explore the relationship between levels of polymerized tubulin and vincristine resistance, we studied two xenograft sublines of ALL-17 (VCRR1 and VCRR2) that were independently selected for vincristine resistance by weekly treatment of engrafted nonobese diabetic/severe combined immunodeficient mice (Verrills et al., 2006). Consistent with previous studies (Liem et al., 2004), mice engrafted with ALL-17 cells exhibited a growth delay factor of 42.8 days when exposed to vincristine. In contrast, mice engrafted with ALL-17-VCRR1 and ALL-17-VCRR2 exhibited growth delay factors of 22.9 and 21.3 days, respectively, an approximate 2-fold level of in vivo resistance. Representative immunoblots of subcellular fractions from xenograft sublines are shown in Fig. 4A, which indicate that both vincristine-resistant sublines contained...
increased levels of polymerized tubulin compared to the parental ALL-17 xenograft. Quantitative data indicated that ALL-17-VCR\textsuperscript{R1} and ALL-17-VCR\textsuperscript{R2} expressed a >2-fold increase in polymerized tubulin compared with ALL-17 cells, a statistically significant difference ($P < 0.005$ and $P < 0.05$ for ALL-17-VCR\textsuperscript{R1} and ALL-17-VCR\textsuperscript{R2} versus ALL-17, respectively) (Fig. 4B).

**Increased Paclitaxel Sensitivity in a Vincristine-Resistant Xenograft.** The relationship between levels of polymerized tubulin and in vitro vincristine sensitivity suggests that vincristine-resistant xenografts with high levels of polymerized tubulin would exhibit increased sensitivity to the microtubule-polymerizing drug paclitaxel (Hari et al., 2003). This possibility was explored using the xenografts with the highest (ALL-19) and lowest (ALL-10) levels of polymerized tubulin (Table 1). Figure 5 shows an apparent inverse relationship between the vincristine and paclitaxel sensitivity assessed by MS-5 assay at three paclitaxel concentrations in the two xenografts, consistent with their different levels of polymerized tubulin (Table 1).

**Discussion**

Vincristine was first incorporated into treatment regimens for childhood ALL in the 1960s, and it remains one of the most active single agents used to treat the disease. Despite this success, a significant proportion of patients relapse, and their leukemias develop resistance to the drug by mechanisms that remain poorly defined. In this study, we have used a heterogeneous panel of childhood ALL xenografts established from patient biopsies to show that the equilibrium between intracellular pools of polymerized and unpolymerized tubulin reflects in vitro vincristine sensitivity. These findings may have significant implications for the future management of vincristine-resistant ALL.

Another significant finding presented in this study was the lack of a consistent protective effect of stromal cell coculture on ALL cell sensitivity to vincristine. In fact, whereas three xenografts did exhibit reduced vincristine sensitivity when cocultured with MS-5 cells, four xenografts showed no difference and three demonstrated increased sensitivity. This finding was somewhat surprising, because we and other groups have shown that stromal cell support enhances the survival of ALL cells (Manabe et al., 1992; Mudry et al., 2000; Liem et al., 2004) and protects ALL cell lines against apoptosis induced by the chemotherapeutic drugs etoposide and cytarabine (Mudry et al., 2000). One possible explanation for the varied responses to vincristine when xenograft cells were cocultured with stromal cells is differential cell proliferation, because vincristine is preferentially cytotoxic to proliferating cells (Drewinko et al., 1981). Stromal cell coculture has been shown to both inhibit (Paraguassu-Braga et al., 2003) and stimulate (Mudry et al., 2000) ALL cell proliferation. However, a simplistic relationship between the effects of stromal cells on ALL cell proliferation and vincristine sensitivity was not supported in this study by cell division-tracking analysis of four xenografts. To our knowledge, this is the first report comparing the effects of stromal cell coculture on childhood ALL sensitivity with vincristine. Our results indicate that the factors controlling the responses of individual leukemias to stromal cell coculture are complex, and further generalization requires additional large-scale analysis of primary biopsy samples or xenografts.

Both the MTT and stromal cell coculture assays have been used successfully to assess the chemosensitivity of primary childhood ALL cells. In a large study of 136 patients, vincristine sensitivity assessed by MTT assay was associated with significantly improved patient outcome compared with leukemia cells from patients who exhibited intermediate sensitivity or resistance (Kaspers et al., 1997). We did not observe a similar relationship in this study using xenografted cells, which may be attributed to a notably smaller sample size of 10 xenografts assessed by MTT assay. The long-term viability of primary childhood ALL cells when cocultured with stromal cells has been associated with patient clinical outcome (Kumagai et al., 1996), and stromal cell coculture has been used to test novel therapies in childhood ALL (Campana et al., 1993; Kumagai et al., 1994). However, to our knowledge, this is the first report that has used a stromal cell coculture assay to attempt to correlate the chemosensitivity of a series of childhood ALL samples with patient outcome. Whereas vincristine is only one of multiple drugs used in combination chemotherapy regimens to treat childhood ALL, it is used in the induction phase of therapy, and a patient’s initial response to treatment is considered one of the strongest predictors of outcome (Pui et al., 2001, 2002). Therefore, the MS-5 assay may provide cell culture conditions that more closely reflect the human bone marrow microenvironment, which may explain the closer relationship between vincristine sensitivity and clinical outcome for the MS-5 assay compared with the MTT assay shown in this study. However, the relatively small number of xenografts analyzed in this study precluded detailed statistical comparisons between in vitro vincristine sensitivity, mechanisms associated with vincristine resistance, and patient clinical outcome. Larger prospective studies using primary biopsy specimens now seem to be justified in accurately defining such relationships.

One of the most common mechanisms of vincristine resistance reported in cell line studies is reduced intracellular drug accumulation due to increased activity of ABC drug transporters, such as ABCB1 and ABCC1 (Broxterman et al., 1995; Dumontet and Sikic, 1999). However, the role of mul-
Vincristine Resistance Mechanisms in Childhood ALL

In the panel of xenografts tested, vincristine sensitivity closely correlated with the level of polymerized tubulin. Whereas high levels of polymerized tubulin have previously been identified in an ALL cell line selected for vincristine resistance in vitro (Kavallaris et al., 2001), such a correlation has not previously been observed in ALL cells exhibiting inherent sensitivity or resistance to vincristine. To date, studies demonstrating altered levels of polymerized tubulin have focused on cell lines, and our data have shown that the polymerized tubulin fraction is much lower in ALL xenograft samples compared to leukemia cell lines (Kavallaris et al., 2001; Dumontet et al., 2004). Further support that tubulin polymer levels may affect microtubule stability, and hence the action of vincristine, was demonstrated when our xenograft sublines that were selected for vincristine resistance in vivo also exhibited significantly increased levels of polymerized tubulin. These vincristine-selected xenografts have recently been shown to exhibit distinct changes in cytoskeletal proteins and factors that regulate these proteins (Verrills et al., 2006). Mechanisms that regulate microtubule stability are complex, involving post-translational modification of multiple tubulin isoforms, interaction with microtubule-associated proteins, and changes in microfilament- and cytoskeleton-regulating proteins (Verrills and Kavallaris, 2005). Future studies will focus on factors that control the differential tubulin polymerization between xenografts and how these influence drug/tubulin interactions.

In summary, this study has shown the following: 1) coulture of childhood ALL cells with stromal cells confers no consistent protective effects against vincristine cytotoxicity; 2) the proportion of tubulin present in its polymerized form in a panel of childhood ALL xenografts directly correlates with in vitro vincristine sensitivity when assessed using a stromal culture assay; and 3) two independent xenograft sublines selected for vincristine resistance in vivo also exhibit increased levels of polymerized tubulin. Increased microtubule stability has been associated with hypersensitivity to the microtubule-stabilizing drug paclitaxel in vincristine-resistant Chinese hamster ovary cells (Hari et al., 2003) and in an ALL xenograft (ALL-19) (this study). In contrast, decreased microtubule stability was associated with hypersensitivity to vincristine in CEM cells selected for resistance to the microtubule-stabilizing drug desoxyzopobialone B (Verrills et al., 2003). Therefore, selective manipulation of microtubule stability may enhance the therapeutic index of vincristine. Whereas our panel of xenografts is representative of the heterogeneous nature of childhood ALL, large-scale studies using primary biopsy material are now warranted to further define the relationship between microtubule stability and patient response to vincristine-based chemotherapy regimens.

Acknowledgments

The Children’s Cancer Institute Australia for Medical Research is affiliated with the University of New South Wales and Sydney Children’s Hospital. We thank the staff of the Centre for Children’s Cancer and Blood Disorders, Sydney Children’s Hospital (Sydney, NSW, Australia) for the provision of patient biopsies for xenograft studies and for information on patient outcome. We also thank Claudia Fleming for technical assistance with the calcine-AM assay.

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