Cells resistant to HTI-286 do not overexpress P-glycoprotein but have reduced drug accumulation and a point mutation in \( \alpha \)-tubulin

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Abstract

HTI-286, a synthetic analogue of hemiasterlin, depolymerizes microtubules and is proposed to bind at the Vinca peptide site in tubulin. It has excellent in vivo antitumor activity in human xenograft models, including tumors that express P-glycoprotein, and is in phase II clinical evaluation. To identify potential mechanisms of resistance induced by HTI-286, KB-3-1 epidermoid carcinoma cells were exposed to increasing drug concentrations. When maintained in 4.0 nmol/L HTI-286, cells had 12-fold resistance to HTI-286. Cross-resistance was observed to other Vinca peptide-binding agents, including hemiasterlin A, dolastatin-10, and vinblastine (7- to 28-fold), and DNA-damaging drugs, including Adriamycin and mitoxantrone (16- to 57-fold), but minimal resistance was seen to taxanes, epothilones, or colchicine (1- to 4-fold). Resistance to HTI-286 was retained when KB-HTI-resistant cells were grown in athymic mice. Accumulation of \([^{3}H]\)HTI-286 was lower in cells selected in intermediate (2.5 nmol/L) and high (4.0 nmol/L) concentrations of HTI-286 compared with parental cells, whereas accumulation of \([^{14}C]\)paclitaxel was unchanged. Sodium azide treatment partially reversed low HTI-286 accumulation, suggesting involvement of an ATP-dependent drug pump. KB-HTI-resistant cells did not overexpress P-glycoprotein, breast cancer resistance protein (BCRP/ABCG2/MXR), MRP1, or MRP3. No mutations were found in the major \( \beta \)-tubulin isoform. However, 4.0 nmol/L HTI-286-selected cells had a point mutation in \( \alpha \)-tubulin that substitutes Ser for Ala\(^{12} \) near the nonexchangeable GTP-binding site of \( \alpha \)-tubulin. KB-HTI-resistant cells removed from drug became less resistant to HTI-286, no longer had low HTI-286 accumulation, and retained the Ala\(^{12} \) mutation. These data suggest that HTI-286 resistance may be partially mediated by mutation of \( \alpha \)-tubulin and by an ATP-binding cassette drug pump distinct from P-glycoprotein, ABCG2, MRP1, or MRP3. [Mol Cancer Ther 2004;3(10):1319 – 27]

Introduction

HTI-286 is a synthetic analogue of a family of naturally occurring tripeptides known as hemiasterliners (1). Like Vinca alkaloids and other peptides such as dolastatins and cryptophycins (2), HTI-286 and other hemiasterlins bind to tubulin with high affinity, cause depolymerization of microtubules, induce mitotic arrest, and block cell growth (3–5). However, the mechanism of action of peptidyl inhibitors is distinct from Vinca alkaloids based on many lines of evidence. First, peptides such as hemiasterlin and dolastatin competitively inhibit the binding of each other to tubulin, whereas they noncompetitively inhibit the binding of Vinca alkaloids to tubulin (2). Second, peptidyl antimicrotubule agents and Vinca alkaloids have different binding affinities for tubulin and produce morphologically distinct tubule aggregates (6). Finally, HTI-286 inhibits the growth of human tumor cells that have resistance associated with (a) overexpression of the drug transporters P-glycoprotein (encoded by MDR1), MRP1, or ABCG2 or (b) tubulin mutations in the taxane- or epothilone-binding sites (4). Because P-glycoprotein mediates resistance to a variety of chemotherapeutic drugs in experimental systems, and the protein may play a role in resistance in some tumor types in the clinic (7), HTI-286 may have clinical utility. As a result of its excellent preclinical efficacy profile and its novel interaction with tubulin, clinical evaluation of HTI-286 is currently under way (8).

Resistance exists or develops to nearly all chemotherapeutic drugs used in the clinic, including antimicrotubule agents. In experimental models, resistance to antimicrotubule drugs has been attributed to overexpression of drug efflux pumps, mutations in tubulin, expression of alternate tubulin isoforms, and alteration of apoptotic regulation (9, 10). The contribution of these mechanisms to chemotherapy failure in patients is not well understood but is under investigation (11–13).

The study of resistance mechanisms to HTI-286 has broad utility. First, it provides insight into the mechanism of action of the drug; no such study for any peptidyl
Materials and Methods

Compounds

HTI-286 was synthesized at Wyeth Research (Pearl River, NY) using modifications (14) of methods reported previously (1). Hemiasterlin A, rhizoxin, and maytansine were generously provided by Dr. R.J. Andersen (University of British Columbia, Vancouver, British Columbia, Canada). Paclitaxel, vincristine, vinblastine, colchicine, Adriamycin, and sodium azide were obtained from Sigma Chemical Co. (St. Louis, MO). Docetaxel and vinorelbine were obtained from MedWorld Pharmacy (Valley Cottage, NY). Mitoxantrone and bisantrene were manufactured by Wyeth Research. Epothilone B and phosphomycin A were obtained from Calbiochem (San Diego, CA). Dolastatin-10, topotecan, and camptothecin were generously provided by the National Cancer Institute (Bethesda, MD). [3H]HTI-286 (25 Ci/mmol) was prepared by the Wyeth Research Radiosynthesis Group and Chemical and Screening Sciences. [14C]Paclitaxel (74 mCi/mmol) was obtained from Moravek Biochemicals, Inc. (Brea, CA). Nonradioactive compounds were solubilized as 1 or 10 nmol/L stocks in DMSO for in vitro studies.

Selection of HTI-286-Resistant Cell Lines

The KB-3-1 epidermoid carcinoma cell line was generously provided by Dr. M. Gottesman (National Cancer Institute) and was maintained in DMEM (Invitrogen, Carlsbad, CA) supplemented with 16.5% FCS, 2 mmol/L L-glutamine, 10 μmol/L sodium pyruvate, 10 units/mL penicillin, and 10 μg/mL streptomycin. KB-3-1 cells were selected for resistance to HTI-286 by continuous exposure to the drug. The first selection concentration of HTI-286 was 0.7 nmol/L, which was approximately the IC50 for KB-3-1 cells after 1 month of exposure (1). Cells adapted to HTI-286 at successive concentrations of 1.0, 1.2, 1.5, 2.0, 2.5, 3.0, 4.0, and 6.0 nmol/L (≈ 1.2- to 1.5-fold steps) Cells adapted to 2.5 nmol/L HTI-286 (sixth step; 5 months after the initial selection) were designated KB-2.5-HTI cells. Cells were further exposed to 4.0 nmol/L HTI-286 (eighth dose step; 5 months after initial selection) and were named KB-4.0-HTI. Thereafter, cells were adapted to 6.0 nmol/L HTI-286, but poor viability was observed after 5 months at this concentration, and these cells were excluded from further analysis. Four months after initially selecting the KB-4.0-HTI cells, a subset of these cells were removed from HTI-286 and maintained in normal growth medium. After 3 to 7 months growth in the absence of HTI-286, this cell line was analyzed and designated KB-4.0-HTI(−). A parallel series of KB-3-1 cells were also incubated with stepwise increasing concentrations of paclitaxel beginning at 2.0 nmol/L and, after 3 months, were stable when maintained at 15.0 nmol/L paclitaxel (designated KB-15.0-PTX).

Proliferation Assays

Cell survival was assessed by the sulforhodamine B assay following 3-day treatments with candidate agents as described previously (4).

Drug Accumulation Studies

Cells were plated overnight in triplicate wells of a 24-well dish (2 × 104 cells/well) in the absence of any drugs. After a wash with prewarmed serum-free medium, 1 nmol/L [3H]HTI (≈ 0.008 μCi) or 500 nmol/L [14C]paclitaxel (≈ 0.01 μCi) in serum-free medium was added. Cells were incubated at 37°C for 2 hours. Drug-containing medium was then aspirated and cells were washed three times with cold PBS. Cells were lysed in 2 N NaOH for 1 hour at 37°C and an aliquot was removed, neutralized with an equal volume of 2 N HCl, mixed with scintillation cocktail, and counted. The protein content in each sample was quantitated using the Bio-Rad (Hercules, CA) detergent-compatible protein assay. Inhibition of ATP-dependent transporters was accomplished by incubating cells in 2.5 mmol/L sodium azide in glucose-free medium (15).

Analysis of P-Glycoprotein, ABCG2, MRP3, and MRP1 Expression

Drug transporter protein levels were determined by isolating cell membranes followed by standard SDS-PAGE, immunoblot, and chemiluminescent analyses as described previously (4). Proteins were detected by incubating blots with the following antibodies: P-glycoprotein with PC03 antibody at 1:500 dilution (Oncogene Science, Uniondale, NY), ABCG2 with BXP-21 antibody at 1:50 dilution (a generous gift of Dr. G.L. Scheffer, Free University Hospital, Amsterdam, Netherlands; ref. 16), MRP3 with M3II-9 antibody at 1:50 dilution (generously provided by Dr. G.L. Scheffer and represent the parental S1, which expresses low levels of wild-type ABCG2, were used as positive and negative control cell lines for ABCG2 (18). The secondary antibodies used were goat anti-rabbit (P-glycoprotein and ABCG2) or goat anti-mouse (MRP3 and MRP1) IgG horseradish peroxidase–conjugated antibodies (1:1,000-1:2,000 dilution, Bio-Rad). Signal was detected with enhanced chemiluminescence reagents (Amersham, Piscataway, NJ).

Several cell lines were used as positive controls for drug transporter overexpression. S1-M1-3,2, which overexpress a mutant ABCG2 (Arg285Gly),5 and parental S1, which express low levels of wild-type ABCG2, were used as positive and negative control cell lines for ABCG2 (18). The 2008 and 2008/3MPT3-8 cell line pair was generously provided by Dr. G.L. Scheffer and represent the parental and MRP3-overexpressing controls, respectively (19). The HL60 and HL60/ADR cell pair was generously provided

5 S. Bates, personal communication.
by Dr. M. Center and represent the parental and MRP1-overexpressing controls, respectively (20). Levels of mRNA for drug transporter proteins were determined in parental and resistant cell lines by quantitative reverse transcription-PCR (21).

cDNA Sequencing

Total RNA was isolated with the RNAsena Total Isolation System (Promega, Madison, WI) and cDNA was obtained with the SuperScript First-Strand Synthesis System (Invitrogen). Tubulin transcripts were amplified using Pfx DNA polymerase (Invitrogen) plus 5' and 3' primers specific for human class I (hM40) β-tubulin (Genbank accession no. J00314, forward primers ATGGAGGAAATCTGACATCT-CAGGCTGGT or CTTGCCCATACATACCTTGA; reverse primer TTAGCCTCTCTTTGCTTCTACCCGAA) and human α-tubulin (Genbank accession no. BC017004, forward primers ATGCGTGAATGCAATCCATCCTCACCCGTAACAG and reverse primer TTAGATATCTCTCTCTATCTTACCCGTC; reverse primer TTAGCCTCTCTTTGCTTCTACCCGAA) and cDNA was obtained with the SuperScript First-Strand Synthesis System (Promega, Madison, WI) and cDNA was obtained with the SuperScript First-Strand Synthesis System (Promega, Madison, WI) and cDNA was obtained.

Results

Resistance Profiles of HTI-Resistant Cell Lines

KB-2.5-HTI and KB-4.0-HTI cells were 9- to 14-fold resistant to HTI-286 and to the highly related analogue hemiasterlin A (Table 1). Moderate to high cross-resistance was observed for drugs that have been reported to bind to the Vinca peptide-binding domain in tubulin (6, 22) including dolastatin-10 (18- to 28-fold) and phomopsin A (5-fold). Cross-resistance (5- to 14-fold) was also found to the Vinca alkaloids, vinblastine and vinorelbine, and to drugs that competitively inhibit Vinca alkaloid binding to tubulin (maytansine and rhizoxin; ref. 22). Minimal resistance was observed to colchicine (2- to 3-fold), which binds to a site distinct from these other depolymerizing agents (23). Minimal or no resistance was observed for agents that promote microtubule stabilization, including paclitaxel, docetaxel, eleutherobin, and epothilone B (1- to 4-fold). Interestingly, high levels of cross-resistance (14- to 57-fold) were observed to cytotoxic agents such as camptothecin, topotecan, and mitoxantrone, which are known to interact with DNA, with the exception of bisantrene where resistance increased only ~2.5-fold. The doubling time was determined to be ~23, 26, and 30 hours for KB-3-1, KB-2.5-HTI, and KB-4.0-HTI cells, respectively. These minimal differences are unlikely to explain the observed resistance profile in a 72-hour cell growth assay.

Resistance to HTI-286 Decreases on Removal of Drug

After the KB-HTI cells showed stable growth for 4 months, cells were grown in the absence of drug for ~5 months. The resultant cell line, KB-4.0-HTI(-), had a doubling time of ~24 hours. They were 5- to 7-fold resistant to HTI-286, dolastatin-10, and vinblastine and 16-fold resistant to mitoxantrone (Table 1). Hence, the sensitivity of KB-4.0-HTI(-) cells to HTI-286, dolastatin-10, and mitoxantrone increased by 3- to 5-fold on removal from HTI-selection. In contrast, these cells remained resistant to vinblastine but sensitive to paclitaxel in vitro.

KB-2.5-HTI Cells Are Resistant to HTI-286 In vivo but Are Sensitive to Paclitaxel

To determine whether KB-HTI-resistant cells remain refractory to HTI-286 but sensitive to paclitaxel in vivo, KB-2.5-HTI cells were evaluated in athymic mice. After the tumors reached a predetermined size, animals were given i.v. doses of 60 mg/kg paclitaxel or 1.25 mg/kg HTI-286 given approximately once a week for 3 weeks. On this schedule, the doses of both drugs were ~80% of the maximum tolerated dose (4). Paclitaxel completely inhibited the growth of tumors derived from both KB-3-1 (parental) and KB-2.5-HTI cells (Fig. 1A and B). HTI-286 inhibited the growth of parental KB-3-1 tumors by 75% compared with saline-treated animals on day 26 (Fig. 1A). However, HTI-286 caused a minimal decrease in growth in tumors derived from KB-2.5-HTI cells (Fig. 1B), suggesting that these cells retain physiologically relevant resistance to HTI-286 in vivo.

KB-4.0-HTI Cells Have Low Accumulation of HTI-286 but not Paclitaxel

To determine if a drug transporter may contribute to resistance in KB-HTI cells, the cellular accumulation of radiolabeled HTI-286 and paclitaxel was measured. The amount of [3H]HTI-286 remaining in KB-2.5HTI and KB-4.0-HTI cells after the incubation period was 38% and 61%, respectively, lower than the amount in parental KB-3-1 cells (Fig. 2A). In contrast, [3H]HTI-286 accumulation was unchanged in KB-4.0-HTI(-) cells compared with parental KB-3-1. No change in accumulation of [14C]paclitaxel was observed in any of the three KB-HTI-resistant cell lines.

Low Accumulation of HTI-286 in KB-4.0-HTI Cells Is ATP Dependent

ATP-binding cassette (ABC) protein pumps require ATP to supply the energy for active drug transport (7). To determine if low accumulation of HTI-286 in KB-HTI-resistant cells was dependent on ATP, accumulation studies were conducted in the presence of sodium azide to inhibit the function of a putative ABC transporter. Low accumulation of HTI-286 in KB-4.0-HTI cells was nearly completely reversed in the presence of 2.5 mmol/L sodium azide.
Mechanisms of Resistance to HTI-286

Table 1. Profile of KB-3-1 cells selected for resistance to HTI-286

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC_{50} (nmol/L)*</th>
<th>RR'</th>
<th>IC_{50} (nmol/L)</th>
<th>RR'</th>
<th>IC_{50} (nmol/L)</th>
<th>RR'</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>KB-3-1</td>
<td></td>
<td>KB-2.5-HTI</td>
<td></td>
<td>KB-4.0-HTI</td>
<td></td>
</tr>
<tr>
<td>Microtubule depolymerizing agents</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>HTI-286</td>
<td>0.53 ± 0.05</td>
<td>9.4</td>
<td>6.50 ± 1.60</td>
<td>12.3</td>
<td>2.52 ± 0.04</td>
<td>4.8</td>
</tr>
<tr>
<td>Hemisterlin A</td>
<td>0.33 ± 0.05</td>
<td>14.8</td>
<td>4.38 ± 3.37</td>
<td>13.3</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Dolastatin-10</td>
<td>0.03 ± 0.02</td>
<td>18.3</td>
<td>0.83 ± 0.33</td>
<td>27.7</td>
<td>0.15 ± 0.05</td>
<td>5.1</td>
</tr>
<tr>
<td>Phomopsin A</td>
<td>671 ± 55</td>
<td></td>
<td>3,529 ± 2,988</td>
<td>5.3</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Vinblastine</td>
<td>0.73 ± 0.03</td>
<td>5.1</td>
<td>6.19 ± 1.26</td>
<td>8.5</td>
<td>5.49 ± 1.84</td>
<td>7.5</td>
</tr>
<tr>
<td>Vinorelbine</td>
<td>1.22 ± 0.33</td>
<td>5.2</td>
<td>8.64 ± 2.94</td>
<td>7.1</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Maytansine</td>
<td>0.06 ± 0.001</td>
<td>7.5</td>
<td>0.84 ± 0.19</td>
<td>14.0</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Rhizoxin</td>
<td>9.13 ± 2.17</td>
<td>10.2</td>
<td>98.4 ± 37.6</td>
<td>10.8</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Colchicine</td>
<td>6.64 ± 1.02</td>
<td>2.3</td>
<td>22.4 ± 0.07</td>
<td>3.4</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Microtubule polymerizing agents</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Paclitaxel</td>
<td>2.66 ± 0.92</td>
<td>1.9</td>
<td>3.18 ± 0.62</td>
<td>1.2</td>
<td>4.75 ± 0.53</td>
<td>1.8</td>
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<tr>
<td>Docetaxel</td>
<td>0.44 ± 0.20</td>
<td>3.2</td>
<td>1.25 ± 0.76</td>
<td>2.8</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Epothilone B</td>
<td>0.63 ± 0.08</td>
<td>3.2</td>
<td>2.37 ± 2.20</td>
<td>3.8</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Eleutheroxin</td>
<td>17.4 ± 1.63</td>
<td>2.1</td>
<td>21.3 ± 4.40</td>
<td>1.2</td>
<td>ND</td>
<td></td>
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<tr>
<td>DNA active drugs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Topotecin</td>
<td>18.1 ± 2.98</td>
<td>44.3</td>
<td>1,029 ± 541</td>
<td>56.8</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>10.0 ± 6.50</td>
<td>44.5</td>
<td>430 ± 159</td>
<td>43.0</td>
<td>161 ± 47</td>
<td>16.1</td>
</tr>
<tr>
<td>Camptothecin</td>
<td>14.9 ± 6.11</td>
<td>18.0</td>
<td>335 ± 157</td>
<td>22.5</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Adriamycin</td>
<td>33.9 ± 21.2</td>
<td>13.7</td>
<td>552 ± 111</td>
<td>16.3</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Bisantrine</td>
<td>119 ± 47</td>
<td></td>
<td>299 ± 91</td>
<td>2.5</td>
<td>ND</td>
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</tr>
</tbody>
</table>

*Values are mean IC_{50} (nmol/L) ± SD from two to six independent determinations.

1RR (relative resistance) = ratio of IC_{50} of the resistant cell line to IC_{50} of the parental line.

ND, not determined.

azide (Fig. 2B). This was not due to a toxic effect because this concentration of sodium azide did not kill cells during the experiment. No effect of sodium azide was observed on KB-4.0-HTI(−) cells, which did not have low accumulation of HTI-286. As a positive control for the reversible effect of sodium azide on an ABC transporter, KB-V1 cells were used. Whereas HTI-286 is a poor substrate for P-glycoprotein, KB-V1 resistance to HTI-286 is likely due to the extremely high expression of P-glycoprotein in these cells (4). Consistent with this, low accumulation of HTI-286 was observed in KB-V1 cells and sodium azide partially reversed this effect (Fig. 2B). These data suggest that an ABC drug transporter may mediate low accumulation of HTI-286 in KB-4.0-HTI cells.

The Drug Transport Proteins P-Glycoprotein, ABCG2, MRP3, or MRP1 Are Not Overexpressed in KB-4.0-HTI Cells

Four ABC transporters known to induce drug resistance and low drug accumulation (7) were investigated in KB-HTI-selected cells. Levels of mRNA and protein were determined by real-time reverse transcription-PCR and/or immunoblot analyses, respectively. Levels of P-glycoprotein mRNA decreased nearly 10-fold in KB-4.0-HTI cells, whereas levels in KB-4.0-HTI(−) were unchanged compared with parental cells (data not shown). No P-glycoprotein was detected by immunoblot in the KB-3-1 or KB-HTI-resistant cells (Fig. 3A). In contrast, high levels of P-glycoprotein mRNA and protein were measured in paclitaxel-resistant KB-8-5 cells as reported previously (21). Similarly, P-glycoprotein is highly expressed in KB-15.0-PTX cells after <5-month stepwise exposure to paclitaxel from 2.0 to 15.0 nmol/L. These cells have 32-fold resistance to paclitaxel but retain sensitivity to HTI-286 (24).

Because KB-HTI-resistant cells had substantial cross-resistance to agents known to be substrates for the ABCG2 drug efflux pump (i.e., mitoxantrone and topotecan), the expression of this transporter was investigated in KB-HTI-resistant cells. When assessed with a highly sensitive reverse transcription-PCR method, the level of ABCG2 mRNA increased by only 2.2- and 2.3-fold in KB-4.0-HTI and KB-4.0-HTI(−) cells, respectively (data not shown). However, levels of ABCG2 protein remained unchanged in KB-4.0-HTI and KB-4.0-HTI(−) cells compared with parental KB-3-1, suggesting that ABCG2 protein overexpression is not involved in resistance to HTI-286 or DNA-damaging drugs. These data are consistent with a functional assay conducted to evaluate the potential reversibility of ABCG2-mediated drug efflux. Fumitremorgin C, which resensitizes ABCG2-expressing S1-M1-3.2 cells to mitoxantrone or topotecan (18), failed to significantly enhance the sensitivity of KB-2.5-HTI cells to HTI-286, mitoxantrone, or topotecan (1.2- to 1.8-fold reversal) but enhanced sensitivity of S1-M1-3.2 cells to mitoxantrone by 2.5-fold (data not shown).

Preliminary transcriptional profiling of the KB-HTI-resistant cell lines using DNA microarrays revealed that MRP3 mRNA was elevated ~5-fold in KB-4.0-HTI cells.
compared with parental KB-3-1 cells and returned approximately to parental levels in the revertant KB-4.0-HTI(−) cells. Consistent with this, quantitative reverse transcription-PCR showed a 3-fold increase in levels of MRP3 transcript in KB-4.0-HTI cells compared with parental KB-3-1 and revertant KB-4.0-HTI(−) cells (data not shown). However, no change in the level of MRP3 protein was detected between parental and KB-4.0-HTI cells (Fig. 3C). High levels of MRP3 protein were detected in the positive control cell lines 2008/MRP3-8 (transfected with MRP3) and A549, which have been shown to express high levels of the protein (19, 25). Therefore, whereas a small increase of MRP3 mRNA is observed in KB-4.0-HTI cells, there is no increase in protein levels detectable by immunoblot.

MRP1 is an ABC transporter associated with resistance to vincristine and DNA-damaging agents (7). MRP1 protein was undetectable by immunoblot analysis in KB-3-1 parental, KB-2.5-HTI, KB-4.0-HTI, KB-4.0-HTI(−), or parental HL60 leukemia cells but was observed in HL60/ADR cells shown previously to express high levels of MRP1 (ref. 20; Fig. 3D).

**α-Tubulin Is Mutated at Ala12 in KB-4.0-HTI Cells**

Mutation of tubulin has been reported in cell lines made resistant to several antimicrotubule agents (9, 10). No sequence differences were detected in the predominant hM40 β-tubulin isotype among KB-3-1, KB-2.5-HTI, KB-4.0-HTI, and KB-4.0-HTI(−) cells. However, a single nucleotide change (GCT to TCT) was observed in codon 12 of α-tubulin in both KB-4.0-HTI and KB-4.0-HTI(−) cells that converted Ala12 to Ser (Fig. 4). A mixture of both GCT and TCT was consistently observed on chromatograms using different sequencing primers, suggesting that both wild-type and mutant alleles are expressed in KB-4.0-HTI and KB-4.0-HTI(−) cell lines. Notably, this mutation was not detected in the KB-2.5-HTI cell line, suggesting that it appeared at higher doses of drug selection. Ala12 in α-tubulin is near the nonexchangeable GTP nucleotide-binding site of the tubulin dimer (Fig. 5).

**Discussion**

HTI-286 is a peptidyl antimicrotubule agent that is being evaluated in cancer patients because it overcomes resistance to taxanes and Vinca alkaloids in preclinical models (4). Because resistance develops to nearly all hM40 β-tubulin isotype among KB-3-1, KB-2.5-HTI, KB-4.0-HTI, and KB-4.0-HTI(−) cells. However, a single nucleotide change (GCT to TCT) was observed in codon 12 of α-tubulin in both KB-4.0-HTI and KB-4.0-HTI(−) cells that converted Ala12 to Ser (Fig. 4). A mixture of both GCT and TCT was consistently observed on chromatograms using different sequencing primers, suggesting that both wild-type and mutant alleles are expressed in KB-4.0-HTI and KB-4.0-HTI(−) cell lines. Notably, this mutation was not detected in the KB-2.5-HTI cell line, suggesting that it appeared at higher doses of drug selection. Ala12 in α-tubulin is near the nonexchangeable GTP nucleotide-binding site of the tubulin dimer (Fig. 5).
Two resistance mechanisms are the most likely explanations for the observed profiles of KB-HTI-resistant cells: overexpression of an ABC transporter, other than P-glycoprotein, ABCG2, MRP3, or MRP1, and alterations in tubulin, the known target of HTI-286. In favor of the transporter hypothesis, (a) KB-2.5-HTI and KB-4.0-HTI cells have reduced intracellular accumulation of radiolabeled HTI-286, (b) revertant KB-4.0-HTI(−) cells do not have low drug accumulation, and (c) low drug accumulation was reversed by sodium azide, which reduces ATP levels required for transporter function. However, several ABC transporters that have been implicated in multidrug resistance to a wide variety of anticancer agents, including P-glycoprotein, ABCG2, MRP3, and MRP1, are not overexpressed in HTI-resistant cells. Consistent with this, (a) HTI-286-selected cells have little or no resistance to taxanes or bisantrone, two classes of agents that are excellent substrates for P-glycoprotein; (b) low accumulation of paclitaxel, which is often mediated by P-glycoprotein, is not found in HTI-resistant cells; (c) fumitremorgin C, a reversal agent for ABCG2, did not reverse high-level resistance to the three known ABCG2 substrates, mitoxantrone, topotecan, and Adriamycin; and (d) cells that overexpress P-glycoprotein, ABCG2, or MRP1 are not cross-resistant to HTI-286 (4). These data suggest that an ABC transporter other than P-glycoprotein, ABCG2, or MRP1 may be involved in resistance to HTI-286, Vinca alkaloids, peptidyl agents, and/or DNA-damaging drugs in KB-HTI-resistant cells. Additional work is needed to further identify putative transporters that may mediate resistance in these cells.

A second hypothesis that may explain resistance to HTI-286 is an alteration of tubulin structure or function. If tubulin changes contribute to HTI-286 resistance, then the cross-resistance profile of HTI-resistant cells to other tubulin-binding drugs may also change. Consistent with this, resistance is high to another peptidyl antimicrotubule agent, dolastatin-10, moderate for Vinca alkaloids, and minimal for colchicine or microtubule polymerizing agents. It is known that dolastatin-10 competitively inhibits the binding of hemiasterlin to tubulin, whereas Vinca alkaloids noncompetitively inhibit the binding of dolastatin-10 to tubulin. The binding domains of dolastatin-10 and Vinca alkaloids are thought to overlap within ε-tubulin (2), whereas the binding domains for colchicine or microtubule polymerizing agents are distinct from both of these agents (23). Our resistance profile data support a HTI-286 binding site at or near the Vinca peptide-binding domain.

A structural change in tubulin that has occurred in KB-4.0-HTI cells is a single nucleotide mutation in α-tubulin that encodes for conversion of Ala12 to Ser. Preliminary DNA sequencing using intron-specific primers suggests that this mutation also exists in genomic α-tubulin and protein sequencing suggests that both wild-type and mutant protein are expressed in these cells.6 Because

6 M. Hari, in preparation.
KB-4.0-HTI cells but not parental or KB-2.5-HTI cells express mutant α-tubulin, yet 9-fold resistance to HTI-286 is found in KB-2.5-HTI cells, mutant tubulin may influence but does not solely account for resistance. Additional work is required to show that the mutant form of tubulin has functional consequences as has been suggested in other resistant lines (27). Point mutations in tubulin may directly influence the integrity of drug-tubulin interactions and/or indirectly compromise interactions between microtubule subunits, thus altering microtubule dynamics and stability (10). It is not expected that Ala12 of α-tubulin directly interacts with the Vinca peptide-binding site. However, it is not yet known whether the α-Ala12 mutation has long-range effects on tubulin structure that impact the cross-resistance profile observed for Vinca and peptidyl agents. Numerous mutations in β-tubulin have been associated with resistance, particularly to drugs that bind to β-tubulin and stabilize microtubules (10). Several cell lines selected for resistance to antimicrotubule drugs express α-tubulin with mutations (28–31); however, mutation of α-Ala12 has not been reported previously. Ala12 resides near the nonexchangeable GTP-binding site (N-site) at the intradimer surface of the α and β subunits. This amino acid side

<table>
<thead>
<tr>
<th>Codon number in α-tubulin</th>
<th>Cell Line</th>
<th>Codon 12 Nucleotides</th>
<th>Codon12 Amino Acid</th>
</tr>
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<tbody>
<tr>
<td>11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td></td>
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<td>G</td>
<td>Ala</td>
</tr>
<tr>
<td>T</td>
<td>C</td>
<td>A</td>
<td>Ala</td>
</tr>
</tbody>
</table>

KB-4.0-HTI(-) and KB-4.0-HTI(+) mixtures of T/G are evident.

Figure 4. Sequencing chromatograms of α-tubulin cDNA around codon 12 derived from KB-3-1 parental and KB-HTI-resistant cell lines. The cDNA derived from α-tubulin mRNA of the indicated cell lines was sequenced using forward and reverse sequencing primers. Nucleotide signals from representative strands are displayed. Note that, for KB-4.0-HTI and KB-4.0-HTI(-), mixtures of T/G are evident.

Figure 5. Model of Ala12 in the α/β-tubulin dimer. Ala12 in the α-tubulin subunit (Ala side chain in green) is near the nonexchangeable GTP site at the intradimer interface. Gray, β-tubulin; teal, α-tubulin. GDP in the exchangeable site and paclitaxel are highlighted in the β-tubulin subunit. Colors of atoms: red, oxygen; orange, phosphorus; purple, nitrogen.
Mechanisms of Resistance to HTI-286

The current study supports other work suggesting that HTI-286 may have a novel interaction with α-tubulin. Mutations in α-tubulin distinct from Ala12 were also observed in 1A9 ovarian carcinoma cells selected for resistance to HTI-286 (34). In addition, we have recently shown that two radiolabeled photoaffinity analogues of HTI-286 exclusively label α-tubulin, one within amino acids 314 to 339 (35) and the other within amino acids 204 to 280 (36). It has been proposed previously that the peptidyl drugs hemiasterlin and dolastatin bind to the vinca peptide site that is predicted to reside near the E-site GTP and close to the α/β interdimer surface (6, 22). These combined data do not rule out the probability that HTI-286 also binds to the reported vinca peptide domain. However, we hypothesize that HTI-286 and possibly other peptidyl antimicrotubule agents interact with α-tubulin and that this effects longitudinal interactions between tubulin dimers.

Altered levels of total tubulin and expression of various tubulin isotypes have also been suggested as mechanisms of resistance to antimicrotubule agents (10). When the expression of α-tubulin was assessed as an overall indicator of the level of total tubulin expression in KB-3-1 and KB-2.5-HTI cells, tubulin levels were equivalent (data not shown). In addition, preliminary transcriptional profiling of the resistant lines has not shown significant changes in any tubulin isotypes.

The KB-HTI-resistant cells in the present study were selected by multistep selection with HTI-286 beginning with a concentration of drug that inhibits growth by 50% followed by increasing concentrations. It has been suggested that single-step exposure of cells to a high concentration of antimicrotubule agent may be more clinically relevant because patients are typically treated at a concentration of antimicrotubule agent may be more valuable and may parallel the multifactorial resistance observed during chronic exposure of human tumors to cytotoxic drugs in vivo (38).

In conclusion, we have shown that resistance to HTI-286 is likely to be caused by mechanisms that are distinct from those that mediate resistance to taxanes and the vinca alkaloids. Chronic exposure to HTI-286 may induce the expression of a drug transporter distinct from P-glycoprotein, ABCG2, MRPI, and MRP3. Although the mechanistic basis for resistance to HTI-286 requires further study, expression of a drug transporter and/or changes or mutations in α-tubulin seem to be involved. Given the difficulty in isolating HTI-resistant cells in these experiments and the observation that their resistance mechanisms may differ from those induced by taxanes, these data suggest that clinical resistance to HTI-286 may be distinct compared with currently approved drugs. Hence, HTI-286 may be a treatment alternative for those who have failed existing therapies.

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References

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Cells resistant to HTI-286 do not overexpress P-glycoprotein but have reduced drug accumulation and a point mutation in α-tubulin

Frank Loganzo, Malathi Hari, Tami Annable, et al.


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