Potential Interactions between Antitubulin Agents and Temperature: Implications for Modulation of Multidrug Resistance

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ABSTRACT
We analyzed the effect of high temperature (a 1-h incubation at 43°C) on the accumulation and cytotoxicity of vinblastine and docetaxel in two model cell lines, K562 and MESSA, and their multidrug resistance (MDR) counterparts, K562/R7 and MESSA/Dx5. High temperature increased the amount of intracellular vinblastine and docetaxel significantly in MESSA cells and, to a much lesser extent, in K562 cells. MDR-positive cells retained a profound drug accumulation defect at 43°C. Hyperthermia enhances the cytotoxic effect of vinblastine (but not docetaxel) in both K562 and MESSA cells, but not in the MDR-positive variants. PSC833, a potent modulator of P-glycoprotein, induced high levels of drug accumulation in the two MDR-positive cell lines at both 37°C and at 43°C. PSC833 also significantly reduced the resistance levels of the two MDR-positive lines at both 37°C and at 43°C. The effect of hyperthermia on drug accumulation thus seems to depend on the cell line, whereas the effect on cytotoxicity depends on the type of compound. The MDR phenotype remains a therapeutic obstacle at 43°C but is accessible to modulation.

INTRODUCTION
Hyperthermia is a therapeutic option in patients with neoplasia. Of itself, it may exert antitumor activity and has been shown to potentiate the effect of radiotherapy and of a number of antimitotic drugs both in vitro and in vivo (1). Currently, attention has focused on melphalan and platin derivatives, two drugs that have been used in hyperthermia protocols in humans. Clinical trials have suggested the potential benefit of whole-body or local hyperthermia in combination with these drugs (2, 3).

Antitubulin agents are particularly interesting agents to examine in combination with therapeutic hyperthermia, because their intracellular target, the soluble tubulin/microtubule complex, is highly temperature dependent, at least in vitro (4). Although the polymerization of soluble tubulin dimers in vitro has been extensively studied between 4°C and 37°C, less data are available concerning the effect of supraphysiological temperatures on the tubulin/microtubule complex. It has been shown that hyperthermia results in an increase of the microtubule polymer mass in the toad (5) and in spontaneous tubulin polymerization in sea urchin eggs (6). Dermietzel and Streffer (7) have reported that a 60-min exposure of melanoma cells to 44°C induces severe microtubular alterations. Knox et al. (8) have attributed the reduced cytolytic activity of heated (42°C for 30 min) murine cytotoxic lymphocytes to the disruption of the microtubule organizing center, an effect that is prevented by preincubation with Taxol (9).

Recent reports have suggested that the critical issue determining the cytotoxic effects of antitubulin agents in vivo is not the soluble tubulin:polymerized tubulin ratio in cells but rather the dynamics of this complex and the ability of microtubule to grow and shorten rapidly to suit the needs of the cells (10). In this model, clinically achievable concentrations of Vinca alkaloids and taxanes reduce the dynamics of this complex without modifying the gross microtubule mass of the cell. It is therefore possible that an alteration of microtubule dynamics may influence the cytotoxic effect of antitubulin agents.

Hyperthermia has been shown to increase the intracellular accumulation of doxorubicin (11). Hyperthermia may therefore influence the effect of antitubulin agents at various levels, including drug accumulation and target interaction. To address this issue, we analyzed the effect of a 60-min exposure to 43°C, conditions that may be obtained in a given body compartment in vivo (12), on vinblastine and docetaxel accumulation and cytotoxicity in vitro. We studied two human model cell lines, adherent sarcoma MESSA and leukemic K562 that grows in suspension. Vinca alkaloids and taxanes are good substrates for the Pgp3 efflux pump responsible for the classical MDR phenotype (13, 14). Therefore, we also used the MDR-positive variants of these cell lines, K562/R7 and MESSA/Dx5, and examined the effect of the Pgp modulator PSC833 at 37°C and 43°C.

MATERIALS AND METHODS
Drugs and Reagents. Unlabeled vinblastine was obtained from Lilly (Saint Cloud, France), and [3H]vinblastine was purchased from Amersham (Little Chalfont, United Kingdom). Unlabeled and 3H-labeled docetaxel were generous gifts from...
Fig. 1 Accumulation of tritiated vinblastine and docetaxel in MESSA, MESSA/Dx5, K562, and K562/R7 cells at 37°C and 43°C. Cells were exposed to drug for 1 h and washed briefly in ice-cold PBS, and the intracellular drug was quantified as the ratio of dpm/mg protein. The results shown are the ratios of drug accumulated relative to that in the MDR-negative cell line at 37°C (MESSA or K562).

RESULTS

Drug Accumulation

Effect of Temperature on Drug Accumulation. In MESSA cells, the accumulation of intracellular vinblastine and docetaxel at 43°C was higher by 57 and 76%, respectively, in comparison to that at 37°C (Fig. 1). In K562 cells, intracellular vinblastine and docetaxel increased by 16 and 7% at 43°C, respectively (Fig. 1). As expected, the Pgp-expressing cell lines accumulated less labeled compounds than their Pgp-negative counterparts at 37°C. This defect was more profound for docetaxel (with only 7–11% of the amount of drug accumulated in the parental cell line) than for vinblastine (29–37% of the parental cell line). At 43°C, drug accumulation in K562/R7 and MESSA/Dx5 cells increased slightly in most cases but remained

Cell Culture. The human erythroleukemic cell line K562 was purchased from the American Type Culture Collection. The K562/R7 MDR cell line was derived by selecting K562 cells for resistance to doxorubicin (15). The human sarcoma cell line MESSA and its Pgp-expressing variant Dx5 were generously provided by B. I. Sikic (Stanford University, Stanford, CA; Ref. 16). All cell lines were cultured in RPMI 1640 supplemented with 10% newborn calf serum, 2 mM glutamine, 200 units/ml penicillin, and 100 µg/ml streptomycin. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

Growth Inhibition Assay. Approximately 10,000 cells/well were seeded in 96-well plates and incubated with and without drugs for 72 h at 37°C in an atmosphere of 5% CO₂. Growth inhibition was evaluated by the MTT colorimetric method on triplicate assays as described previously (17). The 540/690 absorbance was quantitated with a Titertek Multiscan microplate reader. The IC₅₀ was determined directly from semi-logarithmic dose-response curves.

Drug Accumulation Assays. Intracellular docetaxel and vinblastine accumulation were quantitated using radiolabeled drugs as described previously (18). Cells were incubated at 37°C or 43°C in serum-free RPMI 1640 in the presence of 50 nM [³H]docetaxel (19 Ci/mmol; DuPont New England Nuclear) or 50 nM [³H]vinblastine. After 1 h, cells were rapidly washed three times with ice-cold PBS, and the pellet was lysed with PBS buffer supplemented with 2% SDS. The radioactivity was quantified using a scintillation mixture, and the protein content was measured using the Lowry assay (19). Results were expressed as dpm/mg protein and compared to the accumulation in the parental cell line at 37°C.
Table 1 Effect of temperature and PSC833 on the reversal of the drug accumulation defect in the MDR positive lines K562/R7 and MESSA/Dx5

Results are expressed as the ratios of drug accumulated in these lines (dpm/mg protein): the amount accumulated in the parental line (K562 and MESSA, respectively) at the same temperature. Results are the mean of three experiments ± SD.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>VBL* 37°C</th>
<th>VBL 43°C</th>
<th>DOC* 37°C</th>
<th>DOC 43°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>K562/R7</td>
<td>37 ± 12</td>
<td>26 ± 7</td>
<td>11 ± 3</td>
<td>21 ± 5</td>
</tr>
<tr>
<td>K562/R7 + PSC833</td>
<td>133 ± 25</td>
<td>85 ± 10</td>
<td>109 ± 22</td>
<td>76 ± 10</td>
</tr>
<tr>
<td>MESSA/Dx5</td>
<td>29 ± 6</td>
<td>32 ± 5</td>
<td>7 ± 2</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>MESSA/Dx5 + PSC833</td>
<td>98 ± 19</td>
<td>96 ± 15</td>
<td>108 ± 11</td>
<td>73 ± 14</td>
</tr>
</tbody>
</table>

*VBL, vinblastine.  
**DOC, docetaxel.

Table 2 Effect of temperature and PSC833 on the IC_{50} of vinblastine (VBL) and docetaxel (DOC) in cell lines K562, K562/R7, MESSA, and MESSA/Dx5

IC_{50} values were determined using a 72-h proliferation assay. Values shown are the median of three to six experiments ± SD.

<table>
<thead>
<tr>
<th>IC_{50}</th>
<th>37°C</th>
<th>37°C + PSC833</th>
<th>43°C</th>
<th>43°C + PSC833</th>
</tr>
</thead>
<tbody>
<tr>
<td>K562 + VBL</td>
<td>24 ± 6.4</td>
<td>10 ± 1.8</td>
<td>5.5 ± 1.2</td>
<td>4.7 ± 2.0</td>
</tr>
<tr>
<td>K562/R7 + VBL</td>
<td>420 ± 27</td>
<td>18 ± 4.8</td>
<td>240 ± 35</td>
<td>22 ± 5.1</td>
</tr>
<tr>
<td>K562 + DOC</td>
<td>6.5 ± 1.3</td>
<td>3.0 ± 1.1</td>
<td>5.8 ± 2.1</td>
<td>5.0 ± 3.2</td>
</tr>
<tr>
<td>K562/R7 + DOC</td>
<td>1025 ± 153</td>
<td>12 ± 2.9</td>
<td>977 ± 140</td>
<td>18 ± 4.2</td>
</tr>
<tr>
<td>MESSA + VBL</td>
<td>3.2 ± 0.7</td>
<td>2.8 ± 1.0</td>
<td>0.9 ± 0.2</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>MESSA/Dx5 + VBL</td>
<td>275 ± 20</td>
<td>12 ± 3.0</td>
<td>240 ± 34</td>
<td>20 ± 5.9</td>
</tr>
<tr>
<td>MESSA + DOC</td>
<td>2.1 ± 0.8</td>
<td>2.3 ± 1.0</td>
<td>2.8 ± 0.9</td>
<td>2.4 ± 1.4</td>
</tr>
<tr>
<td>MESSA/Dx5 + DOC</td>
<td>230 ± 35</td>
<td>8.5 ± 3.2</td>
<td>279 ± 15</td>
<td>10 ± 2.7</td>
</tr>
</tbody>
</table>

much smaller than the amount observed in K562 and MESSA cells, respectively.

Effect of PSC833 on Drug Accumulation. At 37°C, PSC833 efficiently reverted the accumulation defect of vinblastine and docetaxel in the Pgp-expressing cells K562/R7 and MESSA/Dx5 (Table 1). At 43°C, the reversal of the drug accumulation defect in K562/R7 and MESSA/Dx5 was more efficient for vinblastine than it was for docetaxel (Table 1).

Cellular Proliferation Assays

Duration of Exposure. MTT proliferation assays were performed after 72 h on cells that had been exposed to 37°C or 43°C for 60 min (Table 2). This duration of exposure was chosen because it is similar to the period that is achievable in the clinic. The effect of various durations of exposure to hyperthermia at 43°C was analyzed by comparing cell survival by the MIT proliferation assay with cells continuously grown at 37°C. Exposure to 43°C for 60 min decreased cell proliferation at 72 h by less than 5%, whereas 3-h exposures resulted in decreases of 10–15% (data not shown). The cell doubling time at 37°C was 24 h for K562 and K562/R7 cells and 22 h for MESSA and MESSA/Dx5 cells.

Effect of Hyperthermia on the Antimitotic Effect of Vinblastine and Docetaxel. A 60-min exposure to 43°C reduced the IC_{50} values of vinblastine on K562 cells by almost 5-fold (from 24 ± 5.5 nm) and on MESSA cells by 4-fold (from 3.2 to 0.9 nm) (Table 2). There was no significant alteration of the IC_{50} of vinblastine at 43°C in the MDR-positive cell line MESSA/Dx5, and there was a modest decrease from 420 nm at 37°C to 240 nm at 43°C in K562/R7 cells. The IC_{50} values for docetaxel were not significantly different with a 43°C exposure in any of the four cell lines examined.

Effect of Hyperthermia on Modulation of the MDR Phenotype by PSC833. PSC833 potently enhanced the antimitotic effect of vinblastine and docetaxel on MDR-positive cell lines K562/R7 and MESSA/Dx5 at 37°C, with 25-fold reductions of the IC_{50} values for vinblastine, and 30–70-fold reductions of the IC_{50} values for docetaxel (Table 2). A slight reduction of the IC_{50} values of K562 was observed both for vinblastine and docetaxel at 37°C in the presence of PSC833, which is compatible with a low level of expression of Pgp in this cell line. MESSA IC_{50} values were not influenced by PSC833. At 43°C, PSC833 efficiently reduced the IC_{50} of the MDR-positive cell lines to the same extent as that observed at 37°C.

DISCUSSION

Our results obtained in two MDR-negative cell lines show that transient exposure to 43°C increases the intracellular accumulation of vinblastine and docetaxel and enhances the cytotoxic effect of vinblastine but not docetaxel. Therefore, there was no strict correlation between the accumulation of drugs within cells and the cytotoxic effect of the compounds. In the MDR-positive cell lines, hyperthermia did not potentiate the cytotoxicity of vinblastine, even when drug accumulation was increased by PSC833. These data suggest that hyperthermia may potentiate the cytotoxicity of Vinca alkaloids by a mechanism that is unrelated to intracellular drug concentration and that is not efficient in Pgp-expressing cells.

The MDR-positive cell lines K562/R7 and MESSA/Dx5 displayed the same drug accumulation deficit at 43°C and 37°C, suggesting that the Pgp efflux pump was fully efficient under hyperthermic conditions. Pgp is an ATP-dependent active efflux pump that is inhibited at low temperatures; however, to our knowledge, its performance at higher temperatures has not been
described in the literature. The Pgp modulator PSC833 efficiently reduced the vinblastine and docetaxel accumulation defect and enhanced the cytotoxicity of these compounds in both Pgp-expressing cell lines at both 37°C and 43°C. These data suggest that the MDR phenotype persists at 43°C but may be inhibited by potent modulators.

Vinca alkaloids have classically been considered to be microtubule depolymerizing agents, and taxanes have classically been considered to be stabilizing agents, on the basis of in vitro results obtained with very high concentrations of drugs. At clinically relevant drug concentrations, the ratio of soluble tubulin:polymerized tubulin is not significantly modified by either Vinca alkaloids or taxanes (20). Conversely, microtubule dynamics are reduced by both types of drugs. A possible explanation for the enhancement of vinblastine cytotoxicity but not docetaxel cytotoxicity by hyperthermia may be that enhanced microtubule dynamics increases the free tubulin dimer fraction that is, over a given period of time, effectively accessible to vinblastine, whereas docetaxel only binds to polymerized tubulin, the mass of which is not modified under these conditions (21).

Cells undergo a number of alterations at supraphysiological temperatures. Dyson et al. (22) have observed apoptotic changes up to temperatures of 43°C. The synthesis of heat shock proteins such as hsp74 may be inhibited by Taxol (23). Hyperthermia with melphalan has been shown to inhibit p34cdc2 kinase (24), an enzyme involved in the regulation of mitotic microtubule networks (25). It is therefore conceivable that structural modifications of the mitotic spindle occur under hyperthermic conditions, altering the sensitivity of spindle microtubules to antitubulin agents. Both Vinca alkaloids and taxanes have been shown to induce apoptosis of tumor cells. Because the proliferation assay used in our studies evaluates the inhibition of cell growth rather than cell kill, additional studies evaluating the induction of apoptosis under hyperthermic conditions would be of interest.

Our results suggest that the combination of hyperthermia with vinblastine, but not with docetaxel, may result in enhanced antitumor activity in MDR-negative cells. The MDR phenotype remains active at 43°C but may be efficiently inhibited by potent modulators such as PSC833.

REFERENCES

Potential interactions between antitubulin agents and temperature: implications for modulation of multidrug resistance.

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