Sensitivity of Soft Tissue Sarcoma Cell Lines to Chemotherapeutic Agents: Identification of Ecteinascidin-743 as a Potent Cytotoxic Agent

Wei Wei Li, Naoto Takahashi, Suresh Jhanwar, Carlos Cordon-Cardo, Yaroslav Elsabeeff, Jose Jimeno, Glynn Faircloth, and Joseph R. Bertino

Laboratories of Molecular Pharmacology [W. W. L., N. T., Y. E., J. R. B.], Human Genetics [S. J.], and Molecular Pathology [C. C-C.], Memorial Sloan-Kettering Cancer Center, New York, New York 10021, and Pharma Mar, s.a., Madrid, Spain [J. J., G. F.]

ABSTRACT

The cytotoxic effects of ecteinascidin-743 (ET-743), a novel marine natural product, were evaluated and compared with that of clinically used anticancer agents methotrexate, doxorubicin, etoposide, and paclitaxel in eight human soft tissue sarcoma (STS) cell lines. HT-1080, a fibrosarcoma cell line, and HS-42, a malignant mesodermal cell line, were the most sensitive of the cell lines to methotrexate, doxorubicin, etoposide, and paclitaxel. Other cell lines (IC₅₀s) varied considerably and were more resistant to these agents. ET-743 was more potent than any of these agents, with IC₅₀s in the pM range in all of the cell lines. Cytotoxicity of ET-743 was dose- and time-related (4–72 h exposure). Cytotoxic concentrations of ET-743 produced a S/G2 block in all of the cell lines tested. Three colon adenocarcinoma cell lines, HCT-8, HT-29, and HCT-116, and one breast cancer cell line, MCF-7, were 1–2 logs less sensitive to ET-743 than the STS cell lines. Cell lines were also characterized as to expression of oncogenes and tumor suppressor genes to attempt to correlate sensitivity of these cell lines to ET-743 and other chemotherapeutic agents. All of the cell lines except M8805, a malignant fibrous histiocytoma cell line, had mutations in p53 and/or overexpressed the MDM2 protein. Only HS-18, a liposarcoma cell line, lacked expression of the retinoblastoma protein. None of the cell lines had detectable expression of P-glycoprotein as measured by immunohistochemistry.

ET-743 is an extremely potent cytotoxic agent against human STS cell lines and is being evaluated as an antitumor agent in this disease.

INTRODUCTION

STSs remain one of the most refractory human cancers to chemotherapy (1). This laboratory is attempting to develop new therapeutic strategies based on an understanding of the genetic abnormalities in STSs (2), as well as searching for novel drugs from natural and other sources.

ET-743, a marine tetrahydroisoquinoline alkaloid isolated from Ecteinascidia turbinata, a potent cytotoxic agent against various human tumor cell lines, is currently undergoing clinical trials (3–5). The mechanism of action of ET-743 may involve binding to the minor groove of DNA with some degree of sequence specificity and DNA-protein cross-linking (6–10). Inhibition of transcriptional induction of proteins indicates that this drug may also have a novel mechanism of action (11, 12).

In this study we examined the effect of ET-743 and the anticancer agents, MTX, DOX, VP-16, and paclitaxel on eight STS cell lines. Compared with these clinically useful anticancer agents, ET-743 was more potent against STS cell lines, especially against MFH cell lines. The effect of ET-743 on these STS cell lines is dose- and time-related.

MATERIALS AND METHODS

Chemicals and Antibodies. ET-743 was obtained from Pharma Mar (Madrid, Spain) and prepared as a 2-mM stock solution dissolved in DMSO and stored at −80°C. For in vitro testing, the drug was diluted in RPMI 1640 to the desired concentration. MTX was obtained from Lederle Laboratories (Pearl River, NY). DOX and VP-16 were purchased from Sigma Chemical Co. (St. Louis, MO). Paclitaxel was obtained from Bristol-Myers Squibb Co. (Princeton, NJ). Polyclonal antibodies to bcl-2, bcl-XL, and bax and monoclonal antibodies to p53, pRb, cyclin D1, and E2F-1 were from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibody to Pgp was obtained from Neomarkers (Union City, CA). Monoclonal antibody to MRP-1 was from Signet (Dedham, MA).

Cell Lines and Cell Culture. HT-1080, a human fibrosarcoma cell line, HCT-8, HT-29, and HCT-116, human colon cancer cell lines, and MCF-7, a human breast cell line, were obtained from American Type Culture Collection. HS-16, a mesenchymal chondrosarcoma cell line, HS-18, a liposarcoma cell line, HS-30, a malignant hemangiopericytoma cell line,

3 The abbreviations used are: STS, soft tissue sarcoma; ET-743, ecteinascidin-743; MFH, malignant fibrous histiocytoma; MTX, methotrexate; DOX, doxorubicin; VP-16, etoposide; Pgp, P-glycoprotein.
HS-42, a mixed mesodermal sarcoma cell line, M8805, M9110, and HS-90, MFH cell lines, were established in this laboratory (13). All of the cell lines were maintained as monolayer cultures in RPMI 1640 containing 10% fetal bovine serum.

**Cytoxicity Assays.** Cytotoxicity to ET-743 and the other drugs was determined by the SRB cytotoxicity assay carried out in 96-well microtiter plates as described (14). Cells were plated in duplicate wells (5000 cells/well) and exposed to ET-743 at different concentrations at different times (4–72 h) and to other drugs for 5 days. Cells were fixed with 40% TCA solution for 1 h and 0.4% SRB (Sigma Chemical Co.) was added to each well. After a 30-min incubation, the plates were washed with 1% acetic acid and read at 610 nm on a BioWhittaker microplate reader 2001. The wells with cells containing no drug and with medium plus drug but no cells were used as positive and negative controls, respectively.

**Cell Cycle Analysis.** Exponentially growing cells were treated with or without drug for 24 h. Cells were then collected and fixed with ice-cold 70% methanol. DNA was stained with propidium iodide (Calbiochem, San Diego, CA) as described previously (15). Ten thousand stained cells were analyzed on a Becton Dickinson fluorescence-activated cell sorter.

**Immunological Analysis.** Cells were incubated with the appropriate antibodies, and immunohistochemical analysis was performed after cytopsin centrifugation using a standard avidin-biotin-peroxidase technique (16). Expression of various proteins in tumor cells was classified in one of three categories by estimating cultured cell nuclei staining: negative (−), positive (+), and strong positive (2+). For immunoblotting, cell extracts (100 μg) were subjected to 10% SDS-PAGE and transferred to nitrocellulose membranes. The blots were probed with various primary antibodies, and protein was detected using an enhanced chemiluminescence detection kit.

**RESULTS**

**Potent Activity of ET-743 against STS Cell Lines.** With the use of the SRB assay we examined the cytotoxic effect of ET-743 on eight STS cell lines. As shown in Fig. 1, after 3 days of continuous drug exposure, dose-dependent cell killing was observed in all cell lines. HT-1080 and three MFH cell lines were exquisitely sensitive to ET-743. The IC50s for these cell lines were 0.2–1 pM. HS-16, HS-18, HS-30, and HS-42 cell lines were less sensitive, yet the IC50s for these cell lines were still in the pM range (4–300 pM). To determine whether ET-743 is particularly potent against STS cells, we also examined cell killing induced by ET-743 in nonsarcoma cell lines that included colon cancer cell lines HT-29, HCT-8, and HCT-116 and a breast cancer cell line, MCF-7. These nonsarcoma cell lines were relatively resistant to ET-743 compared with STS cell lines. The IC50s ranged from 3 to 20 nM, 1–4-log higher than that obtained in STS cell lines. To determine whether cell killing induced by ET-743 is time-dependent, we measured cell survival as a function of exposure time (Fig. 2). Increased cytotoxicity was observed with increased time of exposure.

**Sensitivity of STS Cell Lines to Other Chemotherapeutic Agents as Compared with ET-743.** To compare the activity of ET-743 to clinically used chemotherapeutic agents, we examined the effect of MTX, DOX, VP-16, and paclitaxel on STS cell lines. The IC50s for these drugs were calculated based on dose-response curve at each time point. HT-1080 and three MFH cell lines were exquisitely sensitive to ET-743. The IC50s for these cell lines were 0.2–1 pM. These nonsarcoma cell lines were relatively resistant to ET-743 compared with STS cell lines. The IC50s ranged from 3 to 20 nM, 1–4-log higher than that obtained in STS cell lines. To determine whether cell killing induced by ET-743 is time-dependent, we measured cell survival as a function of exposure time (Fig. 2). Increased cytotoxicity was observed with increased time of exposure.

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4 J. R. Bertino, unpublished data.
ET-743 Activity in Human STS Cell Lines

Table 1  Sensitivity of STS cells to MTX, DOX, VP-16, paclitaxel, and ET-743

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>IC50 (nm) MTX</th>
<th>DOX</th>
<th>VP-16</th>
<th>paclitaxel</th>
<th>ET-743</th>
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<tbody>
<tr>
<td>HT-1080</td>
<td>15</td>
<td>20</td>
<td>18</td>
<td>0.2</td>
<td>0.0002</td>
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<tr>
<td>M8805</td>
<td>230</td>
<td>110</td>
<td>90</td>
<td>0.8</td>
<td>0.0006</td>
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<tr>
<td>HS-90</td>
<td>45</td>
<td>30</td>
<td>22</td>
<td>0.3</td>
<td>0.0004</td>
</tr>
<tr>
<td>M9110</td>
<td>180</td>
<td>120</td>
<td>55</td>
<td>1.2</td>
<td>0.02</td>
</tr>
<tr>
<td>HS-16</td>
<td>36</td>
<td>160</td>
<td>48</td>
<td>0.8</td>
<td>0.06</td>
</tr>
<tr>
<td>HS-18</td>
<td>120</td>
<td>60</td>
<td>39</td>
<td>0.8</td>
<td>0.06</td>
</tr>
<tr>
<td>HS-30</td>
<td>95</td>
<td>50</td>
<td>18</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>HS-42</td>
<td>25</td>
<td>10</td>
<td>16</td>
<td>0.2</td>
<td>0.004</td>
</tr>
</tbody>
</table>

* Cells were treated with different drugs for 5 days (See “Materials and Methods” for details). Results are mean of three different experiments.


day exposure.

Table 2  Effect of ET-743 on cell cycle distribution in STS cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Control</th>
<th>G1</th>
<th>G2/M</th>
<th>G1</th>
<th>G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT-1080</td>
<td>37.2</td>
<td>39.1</td>
<td>23.7</td>
<td>33.9</td>
<td>36.6</td>
</tr>
<tr>
<td>M8805</td>
<td>40.5</td>
<td>21.1</td>
<td>30.4</td>
<td>13.2</td>
<td>69.5</td>
</tr>
<tr>
<td>HS-90</td>
<td>51.9</td>
<td>28.9</td>
<td>19.3</td>
<td>10.4</td>
<td>70.5</td>
</tr>
<tr>
<td>HS-16</td>
<td>52.7</td>
<td>28.1</td>
<td>19.2</td>
<td>10.4</td>
<td>70.5</td>
</tr>
<tr>
<td>HS-18</td>
<td>49.5</td>
<td>23.8</td>
<td>26.7</td>
<td>53.4</td>
<td>25.4</td>
</tr>
<tr>
<td>HS-30</td>
<td>68.9</td>
<td>19.5</td>
<td>11.6</td>
<td>17.2</td>
<td>69.6</td>
</tr>
<tr>
<td>HS-42</td>
<td>35.6</td>
<td>24.2</td>
<td>18.4</td>
<td>41.0</td>
<td>49.1</td>
</tr>
</tbody>
</table>

* Cells were exposed to ET-743 at IC50 for each cell line for 24 h. Cells were then collected and fixed with 70% methanol and stained with propidium iodide. Stained cells (10,000) were then analyzed for each sample by flow cytometric analysis.

chemotherapeutic agents, and no correlation was observed between ET-743 sensitivity and sensitivity to MTX, DOX, VP-16, and paclitaxel.

**ET-743-induced Cell Cycle Arrest.** To understand why ET-743 is highly active against STS cell lines, we examined the effect of ET-743 on the cell cycle. As shown in Table 2, treatment of the cells with ET-743 at IC50 for each cell line for 24 h resulted in decreased G1 and increased S phase or G2/M accumulation in all cell lines tested in this study except HT-1080 and HS-18 cells.

**Relationship between ET-743-induced Cell Killing and p53 and pRb Status.** Because alterations of oncogene and tumor suppression gene expression may affect drug sensitivity, we determined the expression of certain oncogenes and tumor suppression genes. For example, p53 and pRb, tumor suppression proteins, play an essential role in regulating cellular response to DNA damage in human tumor cells. We examined whether cell killing induced by this drug in these cell lines is dependent on p53 (± MDM2 overexpression) and/or pRb status. As shown in Table 3, all cell lines except the M8805 cell line had mutations of p53 and/or overexpressed MDM2 protein. Only the HS-18 cell line lacked expression of pRb. Neither p53 status nor pRb status of these cell lines correlated with sensitivity to the drugs tested, although as noted, only one cell line had wild-type p53, and only one cell line lacked pRb.

**DISCUSSION**

In this study we demonstrated that ET-743 is highly active against human STS cell lines. A comparison of antitumor activity of ET-743 between STS cell lines and nonsarcoma cell lines showed that cell killing induced by ET-743 was much lower than in STS cell lines. Further studies are needed to determine the mechanism of action of ET-743 in human tumor cells.
greater in STS cell lines, and the cytotoxic effect of ET-743 was more potent than that of several clinically used anticancer drugs, namely, MTX, DOX, VP-16, and paclitaxel. The potency of ET-743 in STS cell lines may not be so easily explained by the currently described mechanisms of action for this drug such as inhibition of DNA synthesis or topoisomerase I, because inhibitory concentrations of ET-743 required for these effects was nm to \( \mu M \) levels (8, 10). It may involve other novel mechanisms, especially that of a cell type/tissue-specific inhibition of transcription induction of proteins by ET-743 (11, 12).

Alterations of pathways involving drug sensitivity in most STS, especially in MFH, include p53, pRb, E2F, MDM-2, and cyclin D1/cdk4 (17, 18). Increased levels of the antiapoptotic protein bcl-2 and Pgp were also reported in some STSs (18, 19). However, we did not observe a correlation among p53, pRb, E2F, MDM2, and cyclin D1 expression (Table 2) with the degree of cell killing induced by ET-743 as well with sensitivity to the other chemotherapeutic agents reported here. A major mechanism affecting sensitivity of tumor cells to some DNA-damaging agents is expression of Pgp. Pgp and Pgp-related protein (MRP1) expression in these STS cell lines was not detected. In addition, there was no cross-resistance between ET-743 and DOX, VP-16, or paclitaxel.

In summary, ET-743 has been identified as a potent cytotoxic agent against STS cell lines although the detailed mechanism of antitumor activity of this drug, in general, and the basis for the sensitivity of STS cells to this drug remain to be elucidated. In accord with these in vitro findings, encouraging preliminary results have been reported in STS patients treated with ET-743 (20, 21).

REFERENCES

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