Effectiveness of Ecteinascidin-743 against Drug-sensitive and -resistant Bone Tumor Cells

Katia Scotlandi, Stefania Perdichizzi, Maria Cristina Manara, Massimo Serra, Stefania Benini, Vanessa Cerisano, Rosaria Strammiello, Mario Mercuri, Gemma Reverter-Branchat, Glynn Faircloth, Maurizio D’Incalci, and Piero Picci


ABSTRACT

Purpose: The identification of new drugs is strongly needed for bone tumors. Ecteinascidin-743 (ET-743), a highly promising antitumor agent isolated from the marine tunicate Ecteinascidia turbinata, is currently under Phase II clinical investigation in Europe and the United States for treatment of soft tissue sarcoma. In this study, we analyzed the preclinical effectiveness of this drug in osteosarcoma and Ewing’s sarcoma.

Experimental Design: The effects of ET-743 were evaluated against a panel of human osteosarcoma and Ewing’s sarcoma cell lines characterized by different drug resistances and compared with the effects of standard antitumor agents. In addition, combination treatments with ET-743 and the other standard chemotherapy agents for sarcoma were analyzed to highlight the best drug-to-drug interaction.

Results: A potent activity of ET-743 was clearly observed against both drug-sensitive and drug-resistant (multidrug-resistant, methotrexate- and cisplatin-resistant) bone tumor cells at concentrations that are easily achievable in patients (pm to ns range). Ewing’s sarcoma cells appeared to be particularly sensitive to the effects of this drug. The analysis of the effects of ET-743 on cell cycle, apoptosis, and differentiation indicated that both osteosarcoma and Ewing’s sarcoma cells had a slower progression through the different phases of the cell cycle after treatment with ET-743. However, the drug was able to induce a massive apoptosis in Ewing’s sarcoma but not in osteosarcoma cells. In the latter neoplasm, ET-743 showed a differential effect, as indicated by the significant increase in the expression and activity of alkaline phosphatase, a marker of osteoblastic differentiation. Concurrent exposure of cells to ET-743 and other chemotherapeutic agents resulted in greater than additive interactions when doxorubicin and cisplatin were used, whereas subadditive effects were observed with methotrexate, vincristine, and doxorubicin.

Conclusions: Overall, these results encourage the inclusion of this drug in the treatment of patients with bone tumors, although a careful design of new regimens is required to identify the best therapeutic conditions.

INTRODUCTION

Osteosarcoma and Ewing’s sarcoma are the two most frequent bone tumors (1). Although the two neoplasms show several differences with respect to their origin and biological and molecular features, they share a similar clinical history. Both are very aggressive tumors with a marked tendency to recur and metastasize to the lungs and/or the skeleton (1). The introduction of chemotherapy has significantly improved the chance of survival of nonmetastatic patients with bone tumors, shifting the 5-year survival rate to around 50–60% (2–5). However, despite advances in therapy, one-third of patients with nonmetastatic disease and the great majority of patients with metastases at diagnosis do not survive, regardless of therapy (6, 7). Furthermore, recent clinical studies have indicated that the survival rate of osteosarcoma or Ewing’s sarcoma patients has reached a plateau phase and has possibly reached the highest levels achievable by conventional regimens (8–13). Successful treatment of therapy-resistant disease therefore requires new strategies as well as novel drugs from natural and other sources that are potently effective against bone tumor cells.

ET-743, a marine-derived agent isolated from Ecteinascidia turbinata, is a novel, potent cytotoxic agent currently under Phase II clinical trails in Europe and in the United States. The mechanisms by which ET-743 exerts its cytotoxic effects have not yet been elucidated, but studies in several laboratories suggest a novel spectrum of activities, including binding to the minor groove of DNA and alkylation of the N2 position of guanine, lower effectiveness in cells that are defective in nucleoside transporter, and molecular features, they share a similar clinical history. Both are very aggressive tumors with a marked tendency to recur and metastasize to the lungs and/or the skeleton (1). The introduction of chemotherapy has significantly improved the chance of survival of nonmetastatic patients with bone tumors, shifting the 5-year survival rate to around 50–60% (2–5). However, despite advances in therapy, one-third of patients with nonmetastatic disease and the great majority of patients with metastases at diagnosis do not survive, regardless of therapy (6, 7). Furthermore, recent clinical studies have indicated that the survival rate of osteosarcoma or Ewing’s sarcoma patients has reached a plateau phase and has possibly reached the highest levels achievable by conventional regimens (8–13). Successful treatment of therapy-resistant disease therefore requires new strategies as well as novel drugs from natural and other sources that are potently effective against bone tumor cells.

ET-743, a marine-derived agent isolated from Ecteinascidia turbinata, is a novel, potent cytotoxic agent currently under Phase II clinical trails in Europe and in the United States. The mechanisms by which ET-743 exerts its cytotoxic effects have not yet been elucidated, but studies in several laboratories suggest a novel spectrum of activities, including binding to the minor groove of DNA and alkylation of the N2 position of guanine, lower effectiveness in cells that are defective in nucleoside transporter, and molecular features, they share a similar clinical history. Both are very aggressive tumors with a marked tendency to recur and metastasize to the lungs and/or the skeleton (1). The introduction of chemotherapy has significantly improved the chance of survival of nonmetastatic patients with bone tumors, shifting the 5-year survival rate to around 50–60% (2–5). However, despite advances in therapy, one-third of patients with nonmetastatic disease and the great majority of patients with metastases at diagnosis do not survive, regardless of therapy (6, 7). Furthermore, recent clinical studies have indicated that the survival rate of osteosarcoma or Ewing’s sarcoma patients has reached a plateau phase and has possibly reached the highest levels achievable by conventional regimens (8–13). Successful treatment of therapy-resistant disease therefore requires new strategies as well as novel drugs from natural and other sources that are potently effective against bone tumor cells.

3 The abbreviations used are: ET-743, ecteinascidin-743; MDR, multidrug-resistant; DXR, doxorubicin; MTX, methotrexate; CDDP, cisplatin; VCR, vincristine; ACT-D, actinomycin-D; MAB, monoclonal antibody; IMDM, Iscove’s modified Dulbecco’s medium; FBS, fetal bovine serum; ALP, alkaline phosphatase; CI, coefficient of interaction.
Table 1  Sensitivity of parental and resistant osteosarcoma and Ewing’s sarcoma cells to ET-743*

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>ET-743</th>
<th>DXR</th>
<th>MTX</th>
<th>CDDP</th>
</tr>
</thead>
<tbody>
<tr>
<td>U-2 OS</td>
<td>0.42 ± 0.02</td>
<td>10.31 ± 2.93</td>
<td>13.80 ± 1.56</td>
<td>581.67 ± 54.33</td>
</tr>
<tr>
<td>U-2/MDR 117.2</td>
<td>1.20 ± 0.20</td>
<td>79.14 ± 17.93</td>
<td>21.23 ± 8.49</td>
<td>570.00 ± 110.00</td>
</tr>
<tr>
<td>U-2/MDR 117.1</td>
<td>2.50 ± 1.10</td>
<td>259.14 ± 2.93</td>
<td>25.48 ± 4.32</td>
<td>610.00 ± 76.67</td>
</tr>
<tr>
<td>U-2/DOXO35</td>
<td>3.27 ± 1.47</td>
<td>1796.35 ± 503.62</td>
<td>29.72 ± 2.33</td>
<td>483.33 ± 135.67</td>
</tr>
<tr>
<td>U-2/MTX300</td>
<td>0.34 ± 0.07</td>
<td>6.72 ± 2.59</td>
<td>1000.00 ± 21.23</td>
<td>534.00 ± 74.33</td>
</tr>
<tr>
<td>U-2/CDPP300</td>
<td>0.08 ± 0.01</td>
<td>ND &amp;</td>
<td>ND</td>
<td>3733.33 ± 208.67</td>
</tr>
<tr>
<td>Saos-2</td>
<td>0.15 ± 0.01</td>
<td>12.93 ± 0.17</td>
<td>28.24 ± 6.37</td>
<td>506.67 ± 186.66</td>
</tr>
<tr>
<td>Sa/DOXO26</td>
<td>0.92 ± 0.10</td>
<td>1008.28 ± 25.52</td>
<td>33.97 ± 4.13</td>
<td>743.33 ± 290.00</td>
</tr>
<tr>
<td>Sa/MTX300</td>
<td>0.17 ± 0.02</td>
<td>11.90 ± 1.56</td>
<td>3087.47 ± 88.11</td>
<td>592.67 ± 168.00</td>
</tr>
<tr>
<td>Sa/CDDP300</td>
<td>0.04 ± 0.01</td>
<td>ND</td>
<td>ND</td>
<td>6333.33 ± 447.00</td>
</tr>
<tr>
<td>TC-71</td>
<td>0.25 ± 0.05</td>
<td>22.76 ± 2.07</td>
<td>9.77 ± 1.23</td>
<td>733.33 ± 66.66</td>
</tr>
<tr>
<td>TC/DOXO8</td>
<td>0.70 ± 0.28</td>
<td>1372.41 ± 175.86</td>
<td>11.25 ± 3.45</td>
<td>940.00 ± 246.66</td>
</tr>
</tbody>
</table>

* Cells were treated with different drugs for 72 h for Ewing’s sarcoma cell line (TC-71 and TC/DOXO8) or for 96 h for osteosarcoma cells (U-2 OS and Saos-2 parental cell lines and resistant variants; see “Materials and Methods” for details).

Materials and Methods

Cell Lines. The osteosarcoma cell lines Saos-2, U-2 OS, and MG-63 and the Ewing’s sarcoma cell lines SK-ES-1 and SK-N-MC were obtained from the American Type Culture Collection (Manassas, VA). The TC-71 and 6647 Ewing’s sarcoma cell lines were a kind gift from T. J. Triche (Children’s Hospital, Los Angeles, CA). The other osteosarcoma and Ewing’s sarcoma cell lines were obtained by cotransfection of the U-2 OS cell line with the MDR1 gene and the neomycin resistance neo gene as described previously (30). The U-2/MTX300, Saos-2/MTX300, and the U-2/CDPP300 and Saos-2/CDPP300 cell variants were obtained in our laboratory by stepwise increasing concentrations of MTX and CDDP up to 300 ng/ml MTX and CDDP, respectively, starting from the parental U-2OS and Saos-2 osteosarcoma cell lines. For all of the following experiments reported, the cell lines were maintained in IMDM supplemented with penicillin (100 units/ml), streptomycin (100 µg/ml; Life Technologies, Inc., Paisley, Scotland, United Kingdom), and 10% heat-inactivated FBS (Biowhittaker Europe, Verviers, Belgium) at 37°C in a humidified 5% CO2 atmosphere.

Drugs. ET-743 was kindly provided by Pharma Mar, S.A. (Tres Cantos, Spain). The stock solution of this drug was prepared in DMSO and stored at –20°C. DXR, CDDP, MTX, VCR, and ACT-D were purchased from Sigma (St. Louis, MO). Working dilutions of all drugs were prepared immediately before use.

In Vitro Cytotoxicity. The IC50 and IC70 (drug concentration resulting in 50% and 70% inhibition of growth, respectively) values were determined by seeding 20,000 cells/cm2 in IMDM + 10% FBS. After 24 h, medium was changed in IMDM with 10% FBS, without (control) or with increasing doses of the drugs. After 72 h for Ewing’s sarcoma cell lines and 96 h for osteosarcoma cell lines, cells were harvested by trypsin-0.02% EDTA (Sigma) and counted by trypan blue vital cell count to estimate the percentage of growth inhibition compared with the appropriate controls.

Combined in Vitro Treatments with ET-743 and DXR, MTX, CDDP, VCR, and ACT-D. Cells (20,000 cells/cm2) of the U-2 OS and Saos-2 osteosarcoma cell lines were seeded in IMDM + 10% FBS. After 24 h, cells were treated with varying concentrations of DXR (range, 0.3–10 ng/ml), MTX (range, 0.1–10 ng/ml), CDDP (range, 0.1–300 ng/ml), VCR (range, 0.1–3 ng/ml) and ACT-D (range, 10 µg/ml to 3 ng/ml) without (control) or with ET-743 [300 pm for U-2 OS cell line and 100 pm for Saos-2 cell line (doses corresponding to the dose that gives around 30% growth inhibition in each cell line)]. After 96 h of treatment, cell growth was evaluated on harvested cultures by trypan blue vital cell count.

Effectiveness of ET-743 in Osteosarcoma and Ewing’s Sarcoma Tumors. In this study, we examined the strong activity of ET-743 in soft tissue sarcoma cell lines (25, 26) and patients (27), raising new hopes for the treatment of these tumors. In this study, we examined the in vitro effects of ET-743 (cytotoxicity and effects on cell cycle and apoptosis) by using a panel of 13 osteosarcoma cell lines and 8 Ewing’s sarcoma cell lines. Cytotoxicity effects were analyzed in comparison with the other conventional anticancer agents currently used in the treatment of patients with bone tumors. Moreover, ET-743 activity was tested against MDR cells and osteosarcoma cells resistant to MTX or CDDP. Finally, combined treatments with ET-743 and the other standard chemotherapy agents for sarcoma (DXR, MTX, CDDP, VCR, and ACT-D) were analyzed to highlight the best drug-to-drug interactions and help design new multidrug chemotherapeutic regimens for patients with bone tumors.
Cell Cycle Analysis. U2-OS, U2/DOXO35, Saos-2, TC-71, and TC/DOXO8 cells (20,000 cells/cm²) were seeded in IMDM/H11001 10% FBS. The next day, medium was changed in IMDM/H11001 10% FBS without (control) or with the IC₅₀ dose of each cell line. After 24, 48, and 72 h, cell cultures were incubated with 10μM bromodeoxyuridine (Sigma) for 1 h in CO₂ atmosphere at 37°C. Harvested cells were fixed in 70% ethanol for 30 min. After DNA denaturation with 2N HCl, 1 x 10⁶ cells were processed for indirect immunofluorescence staining using α-bromodeoxyuridine MAb diluted 1:4 as a primary antibody (Becton Dickinson, San Jose, CA) and analyzed by flow cytometry (FACScan; Becton Dickinson). For the analysis of DNA content, cells were fixed with cold 70% ethanol, treated with 0.5 mg/ml RNase, and stained with 20 μg/ml propidium iodide.

Morphological Assessment of Apoptotic Nuclei. U2-OS, U2/DOXO35, Saos-2, TC-71, and TC/DOXO8 cells were seeded in IMDM + 10% FBS in 60-mm Petri dishes and treated the next day with the ET-743 IC₅₀ doses for each cell line. At 24, 48, and 72 h after treatment, cells were fixed in methanol/acetic acid (3:1) for 15 min and stained with 50 ng/ml Hoechst 33258 (Sigma). Cells with three or more chromatin fragments were considered apoptotic. The percentage of apoptotic nuclei was evaluated out of 1000 nuclei.

Liver/Bone/Kidney ALP Activity. The percentage of osteosarcoma cells displaying ALP activity at their cell surface was evaluated by using the Sigma ALP kit, according to the manufacturer’s instructions, on cytospins obtained from cells treated for 96 h with 500 pM ET-743. The percentage was calculated out of 300 cells. In addition, ALP activity in the conditioned medium of osteosarcoma cells was also measured by using p-nitrophenylphosphate as a substrate, in accordance with the instructions of the manufacturer (Roche Diagnostic GmbH, Mannheim, Germany).

Expression and Activity of P-Glycoprotein in Cells after Treatment with ET-743. The cell surface expression of P-glycoprotein in cells treated or not treated (control) with ET-743 (IC₅₀ values) for 96 h was analyzed by indirect immunofluorescence and flow cytometry (FACSCalibur; Becton Dickinson) using the MRK-16 MAb (dilution, 1:100; Kamiya, Thousand Oaks, CA). To evaluate the extrusion activity of P-glycoprotein after ET-743 treatment, cells were exposed to the drug for 96 h, incubated in medium containing 10 μg/ml DXR, and washed twice with PBS solution before observation and measurement of DXR intracellular fluorescence by cyttofluorometry (FACScalibur; Becton Dickinson). The analysis was performed on vital cells identified through fluorescein diacetate staining (1 mg/ml, 10-min incubation).
Expression of P-Glycoprotein, Rb, p53, and MDM2 in Osteosarcoma Cell Lines. P-glycoprotein and p53 expression was evaluated by flow cytometry (FACS Calibur; Becton Dickinson) using the MRK-16 (dilution, 1:100; Kamiya) and pAb1801 (dilution, 1:1500; Calbiochem-Novabiochem Co., San Diego, CA) MAb, respectively. Rb and MDM2 expression was analyzed on methanol-acetone-fixed cells by indirect immunofluorescence by using the anti-Rb G3–245 (dilution, 1:40; BD PharMingen, San Diego, CA) and anti-MDM2 IF2 (dilution, 1:20; Calbiochem-Novabiochem Co.) MAb.

Statistical Analysis. Differences among means were analyzed using a two-sided Student’s t test. The IC50 for each particular drug was defined as the concentration of drug that reduces growth of 50% of untreated control cells and was calculated from linear transformation of the dose-response curves. A relative resistance index was expressed as the ratio of the IC50 of the drug-resistant cells to the IC50 of the drug-sensitive parental cell line. The analysis of drug combination effects was performed by using the fractional product method and the Chou-Talalay equation for the determination of synergism, additive effect, and antagonism, respectively. The analyses of resistant variants indicated that ET-743 was able to completely abrogate resistance to MTX and CDDP; MTX-resistant cells showed a level of sensitivity comparable to that of parental cell lines, and CDDP-resistant cells were even more sensitive to ET-743 than parental cell lines.

RESULTS

Activity of ET-743 against Drug-sensitive and -resistant Osteosarcoma and Ewing’s Sarcoma Cells. The in vitro cytotoxic effects of ET-743 were examined, after long continuous drug exposure (72 h for Ewing’s sarcoma cells and 96 h for osteosarcoma cell lines; differences are due to the different mean doubling times of the different tumors), on two osteosarcoma cell lines, one Ewing’s sarcoma cell line, and different drug-resistant variants obtained from the parental cells according to the procedure described in “Materials and Methods.” Table 1 shows the IC50 values obtained for all these cell lines. The sensitivity of the U-2 OS and Saos-2 osteosarcoma cells and the TC-71 Ewing’s sarcoma cell line to ET-743 was in the nM range, confirming the potent activity of ET-743 against sarcoma cells (25).

Fig. 3 Flow cytometric analysis of DXR (10 μg/ml) intracellular incorporation in drug-sensitive and MDR sarcoma cells after 72 h of treatment with ET-743. Broken line represents negative control; Thin black line represents DXR incorporation in cells not exposed to ET-743; Thick black line represents DXR incorporation in cells exposed to ET-743 (400 nm for U-2 OS, 4 nm for U2/DOXO35, 150 nm for Saos-1, 1 nm for Sa/DOXO26 and TC/DOXO8, and 250 pm for TC-71). Data are from one experiment representative of two similar experiments.

For simplicity, however, the third term of the Chou-Talalay equation is usually omitted (25). Only the CI values obtained from the classic (mutually exclusive) calculation are therefore given in the “Results.” However, similar results were also obtained when the complete equation was used for combination studies with ET-743 and DXR or CDDP (data not shown).

Sensitivity of the U-2 OS and Saos-2 osteosarcoma cells and the TC-71 Ewing’s sarcoma cell line to ET-743 was in the nM range, confirming the potent activity of ET-743 against sarcoma cells (25). The analyses of resistant variants indicated that ET-743 was able to completely abrogate resistance to MTX and CDDP; MTX-resistant cells showed a level of sensitivity comparable to that of parental cell lines, and CDDP-resistant cells were even more sensitive to ET-743 than parental cell lines. With respect to MDR cells, ET-743 was dramatically more active in comparison with DXR, although a low level of resistance was maintained in MDR cells with respect to their parental cell lines (Fig. 1). The activity of ET-743 appeared to be slightly reduced in relation to the level of MDR and expression of P-glycoprotein (30), confirming that ET-743 may be partially affected by the P-glycoprotein phenotype (33). Nevertheless, the IC50 values of MDR osteosarcoma and Ewing’s sarcoma cells were still in nM range (1.2–3.3 nM), lower than that obtained in drug-sensitive nonsarcoma cells, such as colon and breast cancer cell lines (25). Exposure of MDR cells to ET-743 for 72 h did not
modulate the expression of P-glycoprotein on the surface of these cells (Fig. 2), whereas a slight reduction of the DXR extrusion activity of P-glycoprotein was observed (Fig. 3). Increased intracellular DXR accumulation was observed in all three MDR cell lines and in U-2 OS parental cells, which showed a low level of P-glycoprotein expression, but was not observed in the two P-glycoprotein-negative parental cells after ET-743 treatment.

Activity of ET-743 in Osteosarcoma and Ewing’s Sarcoma Cells in Comparison with Other Chemotherapeutic Drugs. Thirteen osteosarcoma cell lines and eight Ewing’s sarcoma cell lines were analyzed to evaluate their sensitivity to ET-743 and to the other drugs that are currently included in the chemotherapeutic regimens of osteosarcoma and Ewing’s sarcoma patients (DXR, MTX, and CDDP for osteosarcoma; DXR, VCR, and ACT-D for Ewing’s sarcoma). Cells were exposed to a concentration of chemotherapeutic agent that was able to give a 70% growth inhibition in U-2 OS and TC-71 cells, used as reference for osteosarcoma and Ewing’s sarcoma cell lines, respectively. Almost none of the cell lines express P-glycoprotein, with the exceptions of U-2 OS and IOR/OS-17, which did express a low level of the protein (data not shown). With regard to osteosarcoma, Fig. 4 reveals that the sensitivity of the 13 cell lines to DXR, MTX, and CDDP varied considerably, illustrating the heterogeneity of these tumor cells in their response to the agents that are usually given in osteosarcoma chemotherapy. In particular, 5 cell lines were resistant to DXR, 10 were resistant to MTX, and only 2 were resistant to CDDP. With respect to ET-743, eight cell lines were clearly sensitive, two were marginally resistant, and three markedly resistant. Exposure of these three cell lines to a higher concentration of ET-743 (1 nM) confirmed their relative level of resistance because the percentage of growth inhibition at this dose was still well below 50% (data not shown). No correlation was suggested between ET-743 sensitivity and sensitivity to DXR, MTX, and CDDP. Nor did we observe any relationship between drug sensitivity of osteosarcoma cell lines and the expression of genes such as p53, Rb, and MDM2 (data not shown), in agreement with other reports (18, 24). With regard to Ewing’s sarcoma, heterogeneity was observed for ACT-D and VCR, whereas all eight cell lines appeared to be sensitive to DXR and ET-743 (Fig. 5). For ET-743, in particular, all cell lines considered here were highly sensitive (growth inhibition higher than 70%) at a very low concentration of 400 pm.

Effects of ET-743 on Cell Cycle, Apoptosis, and Differentiation. The analyses of cell cycle phase perturbations induced by ET-743 were examined in two osteosarcoma cell lines (U-2 OS and Saos-2) and one Ewing’s sarcoma cell line (TC-71). In addition, we also examined the U2/DOXO35 and TC/DOXO8 MDR subline variants. Fig. 6 shows the percentage of cells in the different cell cycle phases after different time exposures to ET-743. Treatment of the cells with IC_{50} doses of ET-743 induced a marked accumulation of cells in the S phase after 24 h and in G_{2}-M after 48 h. However, after 72 h of exposure, although a blockade in G_{2}-M was still evident, particularly for MDR cells, we also observed that the percentage of cells in G_{1} phase shifted back toward the levels of the controls. These data suggested that ET-743 induces a delay of cell progression from G_{1} to G_{2}-M rather than a permanent blockade of the cells in the different cell cycle phases. The effects were similar in both osteosarcoma and Ewing’s sarcoma cells. However, when we analyzed the effects of ET-743 on apoptosis, a marked difference was observed between osteosarcoma cells and Ewing’s sarcoma cells. In fact, the morphological evaluation of cells exposed to IC_{50} doses of ET-743 for 24–72 h showed a significant induction of apoptosis in Ewing’s sarcoma cells, both in TC-71 and TC/DOXO8, whereas no differences were observed in osteosarcoma (Fig. 7, A and B), suggesting a cytostatic rather than a cytotoxic effect of ET-743 in these cells. As a consequence, we decided to evaluate the possibility that this agent may act as a differentiating drug in osteosarcoma. Indeed, the expression and activity of bone/liver/kidney ALP, a
marker of osteoblastic differentiation (34), were significantly enhanced by treatment with ET-743 in several osteosarcoma cell lines (Table 2). This effect appeared to be more evident in the cells most sensitive to the drug.

**Cytotoxicity of Combined in Vitro Treatments.** Experiments were carried out to determine the effects on the growth of bone tumor cells of conventional chemotherapeutic drugs (DXR, MTX, CDDP, ACT-D, and VCR) with ET-743. U-2 OS and Saos-2 cells were simultaneously exposed to increasing concentrations of conventional agents and to a concentration of ET-743 that gave a 30% growth inhibition after 96 h (300 pM for U-2 OS, 4 nM for U2/Doxo35, and 150 pM for Saos-2). Results are the means of two independent experiments and are expressed as the percentage of cells in the different cell cycle phases as determined by flow cytometry.

**DISCUSSION**

The identification of novel therapeutic strategies and new potent drugs effective against sarcomas is a high-priority goal. In fact, since the identification of ifosfamide, no new agents for sarcoma therapy have proved to be effective. Moreover, recent...
clinical studies have indicated that the survival rate of sarcoma patients has reached a plateau phase, and no significant improvements have been obtained in the last few years (8–13). In this study, we investigated the in vitro effectiveness of ET-743, a very promising agent discovered in 1990 (35) and recently entered in several clinical studies against solid tumors (24, 27, 36–39), on drug-sensitive and drug-resistant bone tumor cells. In addition, we analyzed whether ET-743 enhances cytotoxicity of the other antineoplastic agents that are currently used in the treatment of osteosarcoma and/or Ewing’s sarcoma patients to identify the best drug-drug combinations.

ET-743 showed a remarkable activity not only on drug-sensitive osteosarcoma and Ewing’s sarcoma cells but also on drug-resistant cell variants. Although the effectiveness of this compound against tumors resistant to chemotherapy has been observed previously in xenografts (21), this is the first time, to the best of our knowledge, that the activity of ET-743 was analyzed in a wide spectrum of well-characterized cell lines.

Fig. 7. ET-743 significantly induces apoptosis in Ewing’s sarcoma cells but not in osteosarcoma cells, as indicated by the morphological analyses of apoptotic nuclei after different time exposure to ET-743. Cells were plated on plastic dishes and exposed 24 h later to the corresponding IC_{50} dose of the drug (250 pm for TC-71, 1 nm for TC/DOXO8, 400 pm for U-2 OS, 4 nm for U2/DOXO35, and 150 pm for Saos-2). A, Morphological appearance of osteosarcoma and Ewing’s sarcoma nuclei stained with Hoechst 33258 after ET-743 treatment. B, percentage of apoptotic nuclei at different time exposure to the drug. Results are expressed as means of triplicate plates ± SE. *P < 0.05, Student’s t test.
with different sensitivities to conventional chemotherapeutic agents. ET-743 was extremely active on CDDP-resistant cell lines, showing even greater effectiveness in these cells than in parental cell lines. MTX-resistant variants were found to be equally sensitive to ET-743 with respect to parental cells. These data are consistent with the findings that ET-743 acts by a different mechanism of action from that of CDDP (21) and MTX. Because CDDP-resistant cells appeared to be more sensitive to ET-743 than parental cell lines, it would be interesting to verify whether this phenomenon was due to the alterations in the DNA mismatch repair proteins that have been associated with an increased resistance of many cancer cell lines to CDDP. With regard to MDR, P-glycoprotein-expressing cell lines, we found that ET-743 was slightly less effective against these cells with respect to their parental cell lines. The level of resistance appears to be enhanced according to the increase in MDR.

Table 2  In vitro ALP activity of osteosarcoma cells after 96 h of treatment with ET-743

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Expression of membrane-bound ALP (% positive cells)</th>
<th>ALP activity in conditioned medium (mU/ml/10^6 cells)</th>
<th>IC_{so} value ET-743 (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saos-2</td>
<td>Control: 100, ET-743: 100</td>
<td>Control: 73 ± 5, ET-743: 374 ± 8^a</td>
<td>0.15</td>
</tr>
<tr>
<td>SARG</td>
<td>Control: 100, ET-743: 100</td>
<td>Control: 357 ± 64, ET-743: 1072 ± 45^a</td>
<td>0.10</td>
</tr>
<tr>
<td>IOR/OS-7</td>
<td>Control: 100, ET-743: 100</td>
<td>Control: 123 ± 11, ET-743: 789 ± 130^a</td>
<td>0.30</td>
</tr>
<tr>
<td>IOR/OS-14</td>
<td>Control: 35 ± 8, ET-743: 83 ± 5</td>
<td>Control: 28 ± 6, ET-743: 86 ± 10^a</td>
<td>0.56</td>
</tr>
<tr>
<td>IOR/OS-17</td>
<td>Control: 40 ± 2, ET-743: 70 ± 8</td>
<td>Control: 38 ± 11, ET-743: 76 ± 33</td>
<td>1.28</td>
</tr>
</tbody>
</table>

^a Cells were treated with 500 pm ET-743 for 96 h. Cytospins were prepared to evaluate the expression of ALP on the cell surface. Conditioned medium was collected, and ALP activity was measured by using p-nitrophenylphosphate as a substrate.

^b Concentration of ET-743 resulting in 50% inhibition of cell growth. Results of individual experiments, representative of two similar experiments, are shown.

^c P < 0.001, Student’s t test.

^d P < 0.05, Student’s t test.

Fig. 8 Inhibitory effects of DXR, CDDP, MTX, VCR, or ACT-D in combination with ET-743 (300 pm for U-2 OS cells, 100 pm for Saos-2 cells) after simultaneous and continuous treatments. Cells were treated with the drugs at the indicated concentrations alone or in association with ET-743 on the first day after cell seeding for a total of 96 h. Results represent the mean ± SE of duplicate or triplicate experiments.
P-glycoprotein expression level, substantially confirming the idea that the activity of ET-743 may be partly affected by the presence of P-glycoprotein on the cell surface (33). However, the levels of resistance to ET-743 of MDR cells were remarkably lower than those observed for DXR (7–8-fold in comparison with more than 100-fold), and the IC50 values of these highly resistant cells were still as low as 1–4 nM, a concentration that is normally achieved and maintained in the plasma of patients treated with ET-743 (24). Exposure of MDR cells to equally toxic ET-743 doses did not modulate the expression of P-glycoprotein on their cell surface. This is in agreement with previous studies reporting only a minimal, if any, effect of ET-743 on constitutive MDR1 expression (40, 41). On the contrary, these studies indicated that nanomolar concentrations of ET-743 (25–50 nM) inhibited transcriptional activation of the MDR1 promoter by multiple inducers (40, 41). Although the mechanisms responsible for the block of the MDR1 promoter are still under investigation, with the steroid and xenobiotic receptor as well as the minor groove-interacting transcription factor NF-Y proposed as candidate target genes of ET-743, these data and our findings have important clinical implications. In fact, ET-743 may be the first pharmacologically relevant agent that prevents activation of MDR1 transcription by multiple stress inducers, including toxic agents such as chemotherapy and radiation, which were reported to rapidly enhance tumor MDR1 RNA levels during the course of cytotoxic therapy (42). ET-743 is therefore the first agent with the potential for blocking this activation. Its effectiveness against cells that are resistant to the principal drugs currently used in chemotherapy further increases the clinical attractiveness of this drug. Furthermore, we showed, in agreement with a previous observation (26), that treatment with ET-743 slightly reduced the extrusion activity of

Table 3  The effect of simultaneous exposure to ET-743 and chemotherapeutic agents in two osteosarcoma cell lines evaluated by Chou-Talalay equation (31, 32)

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Other drugs</th>
<th>D1</th>
<th>D2</th>
<th>(D1)1</th>
<th>(D1)2</th>
<th>CI values</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>U-2 OS DXR</td>
<td>0.23</td>
<td>0.3</td>
<td>0.23</td>
<td>4.90</td>
<td>1.06</td>
<td>Additive</td>
<td></td>
</tr>
<tr>
<td>CDDP</td>
<td>0.23</td>
<td>10</td>
<td>0.28</td>
<td>245.50</td>
<td>0.86</td>
<td>Synergy</td>
<td></td>
</tr>
<tr>
<td>VCR</td>
<td>0.23</td>
<td>0.3</td>
<td>0.20</td>
<td>0.36</td>
<td>1.98</td>
<td>Antagonism</td>
<td></td>
</tr>
<tr>
<td>ACT-D</td>
<td>0.23</td>
<td>0.3</td>
<td>0.11</td>
<td>0.81</td>
<td>2.46</td>
<td>Antagonism</td>
<td></td>
</tr>
</tbody>
</table>

Saos-2

D1, doses of ET-743, expressed in ng/ml, used for combined treatments; D2, doses of other drugs, expressed in ng/ml, used for combined treatment; (D1)1, doses of ET-743 that alone gives the same percentages of inhibition recorded in combined treatments; (D1)2, doses of other drugs that alone give the same percentages of inhibition recorded in combined treatments.

P-glycoprotein expression level, substantially confirming the idea that the activity of ET-743 may be partly affected by the presence of P-glycoprotein on the cell surface (33). However, the levels of resistance to ET-743 of MDR cells were remarkably lower than those observed for DXR (7–8-fold in comparison with more than 100-fold), and the IC50 values of these highly resistant cells were still as low as 1–4 nM, a concentration that is normally achieved and maintained in the plasma of patients treated with ET-743 (24). Exposure of MDR cells to equally toxic ET-743 doses did not modulate the expression of P-glycoprotein on their cell surface. This is in agreement with previous studies reporting only a minimal, if any, effect of ET-743 on constitutive MDR1 expression (40, 41). On the contrary, these studies indicated that nanomolar concentrations of ET-743 (25–50 nM) inhibited transcriptional activation of the MDR1 promoter by multiple inducers (40, 41). Although the mechanisms responsible for the block of the MDR1 promoter are still under investigation, with the steroid and xenobiotic receptor as well as the minor groove-interacting transcription factor NF-Y proposed as candidate target genes of ET-743, these data and our findings have important clinical implications. In fact, ET-743 may be the first pharmacologically relevant agent that prevents activation of MDR1 transcription by multiple stress inducers, including toxic agents such as chemotherapy and radiation, which were reported to rapidly enhance tumor MDR1 RNA levels during the course of cytotoxic therapy (42). ET-743 is therefore the first agent with the potential for blocking this activation. Its effectiveness against cells that are resistant to the principal drugs currently used in chemotherapy further increases the clinical attractiveness of this drug. Furthermore, we showed, in agreement with a previous observation (26), that treatment with ET-743 slightly reduced the extrusion activity of
P-glycoprotein against DXR, indicating that ET-743 may increase the effectiveness of this drug also on MDR cells. All these aspects appear to be particularly important for osteosarcoma, in which around a third of patients overexpress P-glycoprotein at the onset and for whom expression of this protein has been significantly associated with a higher risk of recurrence and development of metachronous metastases (43–45).

The analyses of a panel of 13 osteosarcoma and 8 Ewing’s sarcoma cell lines have pointed out a higher sensitivity of Ewing’s sarcoma in comparison with osteosarcoma cells to ET-743. In particular, all Ewing’s sarcoma cell lines were found to be highly sensitive to 400 pm ET-743, whereas in osteosarcoma, a higher heterogeneity was generally observed, with at least three cell lines being markedly resistant to the concentration of 1 nM. As also found previously by others (18, 25), the differences in the level of sensitivity among osteosarcoma cell lines were not correlated with the expression of other proteins, such as wild-type p53, Rh, MRP, and MDM2. The analyses of the effects of ET-743 on cell cycle and apoptosis have indicated that both osteosarcoma and Ewing’s sarcoma cells progressed more slowly through the different phases of the cell cycle. However, when we analyzed the effects of ET-743 on apoptosis, we observed an important difference between Ewing’s sarcoma and osteosarcoma cells. In fact, prolonged exposure to the drug induced a significant apoptotic effect in Ewing’s sarcoma but not in osteosarcoma. A differential effect was observed in the latter cells, as shown by the significant increase in bone/liver/kidney ALP expression and activity, a marker of osteoblastic differentiation (34), after treatment with 500 pm ET-743. These findings may indicate that, besides the cytostatic effect, ET-743 is able to induce different intracellular signals in different cell histotypes (i.e., an apoptotic signal in Ewing’s sarcoma cells and a differentiative one in osteosarcoma), further supporting the idea that this agent is a promoter-specific, transcription-interfering drug (16). Microarray studies are in progress to highlight the molecular mechanisms that may be differentially induced by the drug in Ewing’s sarcoma and osteosarcoma cells. Very preliminary results confirmed a differential genetic induction of a series of genes involved in apoptosis in the two different neoplasms. A detailed and functional analysis of these genes will follow.

Finally, because from a clinical point of view, to be of significant therapeutic value, any new drug should be effectively combined with conventional anticancer agents in mediating their antitumor activity, we investigated whether ET-743 enhances the cytotoxicity of the other chemotherapeutic drugs that are currently used in the treatment of patients with bone tumors. Synergistic effects between ET-743 and DXR as well as CDDP were clearly observed in two cell lines, whereas a subadditive action was observed when cells were concomitantly exposed to ET-743 and VCR, ACT-D, or MTX. Our data partly confirmed the data reported on soft tissue sarcomas by Takahashi et al. (26) with respect to DXR and MTX.

Taken together, our findings further support the clinical attractiveness of ET-743 in the treatment of sarcomas. Its potent activity against drug-sensitive and -resistant cells renders this drug worthy of being included in the chemotherapeutic regimens against these neoplasms. Encouraging preliminary results have been reported in sarcoma patients treated with ET-743 (27, 36, 37). However, particular attention should be paid in the design of clinical protocols, due to the subadditive effect of ET-743 with several drugs that are currently and commonly used for bone tumor patients.

REFERENCES

guanine N2 in the DANN minor groove by ecteinascidin 743, a potent antitumor compound from the Caribbean tunicate Ecteinascidia turbinata. Biochemistry, 35: 13303–13309, 1996.


Effectiveness of Ecteinascidin-743 against Drug-sensitive and -resistant Bone Tumor Cells
Katia Scotlandi, Stefania Perdichizzi, Maria Cristina Manara, et al.

Updated version  Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/8/12/3893

Cited articles  This article cites 42 articles, 23 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/8/12/3893.full.html#ref-list-1

Citing articles  This article has been cited by 8 HighWire-hosted articles. Access the articles at:
/content/8/12/3893.full.html#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.