Sequence-dependent Enhancement of Cytotoxicity Produced by Ecteinascidin 743 (ET-743) with Doxorubicin or Paclitaxel in Soft Tissue Sarcoma Cells

Naoto Takahashi, Wei Wei Li, Debabrata Banerjee, Kathleen W. Scotto, and Joseph R. Bertino
Program of Molecular Pharmacology and Therapeutics [N. T., W. W. L., D. B., K. W. S., J. R. B.] and Department of Surgery [N. T., Memorial Sloan-Kettering Cancer Center, New York, New York 10021, and Department of Surgery, The Jikei University School of Medicine, Tokyo 105-8461, Japan [N. T.]

ABSTRACT

Ecteinascidin 743 (ET-743) is a potent antitumor agent from the Caribbean tunicate Ecteinascidin turbinata and is presently in clinical trials for human cancers. To better understand how ET-743 might be used clinically, the present study used SRB assays to examine the cytotoxicity resulting from combining ET-743 with three other antineoplastic agents: doxorubicin (DXR), trimetrexate, and paclitaxel in different administration schedules in two soft tissue sarcoma cell lines, HT-1080 and HS-18, in vitro. Concurrent exposure of ET-743 with DXR resulted in synergistic interactions in both cell lines. Addition of ET-743 for 24 h before DXR was the most effective cytotoxic regimen against both cell lines. Morphological study by fluorescence microscopy revealed that combination treatment of both cells with ET-743 and DXR induced apoptosis. Exposure to paclitaxel before ET-743 was also an effective regimen. These results encourage studies of the combination of ET-743 and DXR in the treatment of soft tissue sarcoma, because each of these agents have activity in this disease.

INTRODUCTION

STSs remain one of the most difficult human neoplasms to treat by chemotherapy. For example, many chemotherapeutic agents have only marginal activity against this disease, although they are effective in the treatment of other cancers. Developing new therapeutic strategies based on understanding of the alteration of biochemical pathways and genetic abnormalities in STSs may provide information for improving chemotherapy in this disease as well as searching for novel drugs that potentially kill STS cells.

The antitumor activity of extracts from the Caribbean tunicate E. turbinata was first discovered in the late 1960s. However, the purification of the active compounds was not completed until 1990 (1). One of them, ET-743, displays remarkable antitumor activities in in vitro and in vivo models (2–4), and clinical activity in STS patients has been reported (5).

We reported recently that ET-743 was highly active against STS cell lines, especially against malignant fibrohistiocytoma cell lines (6). DXR is one of the most effective drugs against STS (7, 8), and TMTX is a powerful anticancer agent, which has potential advantages over the more commonly used antifolate, methotrexate (9), and has activity in osteosarcomas (10). Paclitaxel has also been reported to have modest antitumor activity against STSs (11). Therefore, we examined whether ET-743 enhances the cytotoxicity of these three antineoplastic drugs and determined whether the combination of ET-743 with these drugs is schedule-dependent. These combination studies may be helpful in designing additional clinical studies in patients with STSs. The work presented in this paper investigates the in vitro activity of ET-743 alone as well as in combination with DXR, TMTX, or paclitaxel against two human STS cell lines, HT-1080 and HS-18. The combinations were analyzed by the CI method of Chou et al. (12–17).

MATERIALS AND METHODS

Chemicals. ET-743 was provided by Pharma-Mar s. a. (Tres Cantos, Madrid, Spain), and was prepared as a 2 mM stock solution in DMSO. Paclitaxel and DXR were obtained from Sigma Chemical Co. (St. Louis, MO). TMTX was supplied by U.S. Bioscience (Conshohocken, PA).

Cell Culture. HT-1080, a fibrosarcoma cell line, was purchased from American Type Culture Collection, and HS-18, a liposarcoma cell line, was established in this laboratory (18). These two cell lines were maintained as monolayer cultures in RPMI 1640 containing 10% fetal bovine serum.

SRB Cytotoxicity Assay. Cytotoxicity was determined by the SRB cytotoxicity assay using 96-well microtiter plates as described (19). Cells were plated in duplicate wells (5000 cells/well) and exposed to drugs at different concentrations. After 72 h of incubation, cells were fixed with 50% 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, and dye were dissolved by 10 mM Tris buffer (pH 7.5) and read at 570 nm on a BioWhittaker microplate reader 2001 (BioWhittaker Inc., Walkersville, MD). The wells with cells containing...
no drugs and wells with medium plus drugs but without cells were used as positive and negative controls, respectively.

**Concurrent Exposure to ET-743 and DXR, TMTX, or Paclitaxel.** Cells were seeded into 96-well plates as described previously. Cells were treated with seven different concentrations of the single drugs or a combination mixture at 1:100 (ET-743: DXR, TMTX, or paclitaxel) molar ratio. The molar ratio used reflects the potency of ET-743 versus the three drugs also studied. After a 72-h exposure, growth inhibition was measured using the SRB assay. (Fig. 1)

**Sequential Exposure to ET-743 and DXR, TMTX, or Paclitaxel.** Fig. 1 shows the sequence experiments diagrammatically. Using the same experimental setup described above, we tested three different concentrations of drugs, i.e., IC25, IC50, and IC75 of ET-743, DXR, TMTX, and paclitaxel, respectively. After a 24-h exposure to the first drug, the second drug was added to the relative wells for an additional 48 h. At the end of this time, growth inhibition was determined using the SRB assay.

**Determination of Synergism and Antagonism and Construction of Isobolograms.** The CI was calculated by the Chou-Talalay equation, which takes into account both potency (Dm or IC50) and the shape of the dose effect curve (the m value; Refs. 12–14). The general equation for the classic isobologram (CI = 1) is given by:

\[
\frac{(D_1)}{(D_2)} + \frac{(D_2)}{(D_1)} = \frac{(D_1)}{(D_2)}
\]

where \((D_1)\) and \((D_2)\) in the denominators are the doses (or concentrations) for \(D_1\) (ET-743) and \(D_2\) (another drug) alone that gives x% inhibition, whereas \((D_1)\) and \((D_2)\) in the numerators are the doses of ET-743 and another drug in combination that also inhibited x% (i.e., isoeffective). CI < 1, CI = 1, CI > 1 indicate synergism, additive effect, and antagonism, respectively.

The \((D_1)\) or \((D_2)\) can be readily calculated from the median-effect equation of Chou (14) and Chou (15) et al.:

\[
Dx = Dm \left[ \frac{fa}{(1 - fa)} \right]^{1/m}
\]

where \(Dm\) is the median-effect dose that is obtained from the antilog of the X-intercept of the median-effect plot, X-log \((D)\) versus \(Y = \log [fa/(1 - fa)]\) or \(Dm = 10^{- \text{X-intercept} / m}\), and \(m\) is the slope of the median-effect plot. Computer software of Chou and Chou (16, 17) allows automated calculation of \(m, Dm, Dx,\) and CI values. From \((Dm)\), \((Dx)\), and \(D1 + D2\), the
Fig. 3  The effect of sequence of administration on the cytotoxic interaction between ET-743 and three other agents in two sarcoma cell lines. A, DXR plus ET-743; B, TMTX plus ET-743; C, paclitaxel plus ET-743. Results are representative of three experiments in each sequence; bars, ± SD.
isobolograms can be automatically constructed based on Eq. A (12, 16).

For conservative mutually nonexclusive isobolograms of two agents, a third term,

\[
\frac{(D1)(D2)}{(D3)_{1}(D3)_{2}}
\]

is added to Eq. A (12, 14).

For simplicity, the third term is usually omitted, and, thus, the mutually exclusive assumption or classic isobologram is indicated (12, 14). In Figs. 2 and 3, the CI values obtained from the classic (mutually exclusive) calculation are given.

**Apoptosis Assay.** The cells isolated by exposure to trypsin were washed twice with PBS and fixed with 4% formaldehyde for 1 h. The fixed cells then washed again with PBS and stained with 200 μM of Hoechst 33342 for 15 min. The cells were examined under a fluorescence microscope. Cells (>100) were counted to quantify apoptotic cells (condensed or fragmented nuclei) in three different experiments.

**Flow Cytometric Studies of DXR Uptake.** After exponentially growing HT-1080 cells were preincubated with or without 1 nM of ET-743 or 10 μM of verapamil for 24 h, 10 μM of DXR was added to medium. At different time intervals during DXR incubation, the total fluorescence was measured by Becton Dickinson fluorescence-activated cell sorter. DXR fluorescence was detected with excitation at 488 nm and emission above 530 nm. The analyses were performed on ≥50,000 cells, and the data are the mean of three independent replicates. Measurements were corrected for the contribution of fluorescence of untreated cells (20).

**Statistics.** Statistical analysis was performed with StatView software (SAS Institute, Cary, NC). Results are expressed as mean ± SE, and Student’s t test was used for statistical analysis with \( P < 0.01 \) as the level of significance.

**RESULTS**

**Determination of IC\(_{50}\) Values.** IC\(_{50}\) values for both HT-1080 and HS-18 cell lines after treatment with ET-743 and the other three antineoplastic drugs were first determined. For HT-1080 cells, the IC\(_{50}\)s of ET-743, DXR, TMTX, and paclitaxel were 0.01 ± 0.02 nM, 25 ± 3.2 nM, 6 ± 1.1 nM, and 1.3 ± 0.5 nM, respectively. For HS-18 cell lines, the values were 0.27 ± 0.06 nM, 225 ± 85 nM, 70 ± 15 μM, and 10 ± 1.9 nM, respectively.

**Concurrent Exposure to ET-743 and DXR, TMTX, or Paclitaxel.** The mutually exclusive CI values of HT-1080 and HS-18 cells, respectively, when simultaneously exposed to ET-743 and one of the other antineoplastic drugs such as DXR, TMTX, or paclitaxel at a 1:100 molar ratio combination mixture are presented in Fig. 2.

When either HT-1080 or HS-18 cells were treated with ET-743 and DXR, all of the CI values were below 1, indicating a synergistic cytotoxic effect. The CI values (mean ± SD) with this schedule were 0.86 ± 0.10, 0.83 ± 0.01, 0.84 ± 0.07, and 0.85 ± 0.12 at 50, 75, 90, and 95% cell kill, respectively, in HT-1080 cells, and 0.89 ± 0.16, 0.74 ± 0.05, 0.64 ± 0.03, and 0.60 ± 0.08 at 50, 75, 90, and 95% cell kill, respectively, in HS-18 cells. In contrast, when cells were treated with ET-743 and TMTX concomitantly, antagonism was observed (CI > 1). When cells were exposed to ET-743 and paclitaxel simultaneously, the CI value at IC\(_{50}\) was slightly >1 indicating a weak antagonism, whereas the CI value at the IC\(_{50}\) was <1 suggesting synergism.

**Sequenced Exposure to ET-743 and DXR, TMTX, or Paclitaxel.** Fig. 3A illustrates the CI plot obtained from both cell lines exposed initially to ET-743 for 24 h followed by DXR for 48 h. In both cell lines, ET-743 followed by DXR treatment showed a synergistic cytotoxic effect; the CI value of HT-1080 cells at 95% fraction killed was 0.49 ± 0.09 and that of HS-18 cells at 95% fraction killed was 0.41 ± 0.5. In contrast, DXR followed by ET-743 treatment had a CI value of 1.28 ± 0.92 at 95% fraction killed in HT-1080 cells, indicating additive effects or antagonism. The CI at 95% fraction killed was greater than that at the middle fraction killed in both cell lines, a situation more relevant for the clinical use of the combinations. Both results suggested that ET-743 followed by DXR treatment was better than the reverse sequence treatment.

When cells were exposed to ET-743 followed by TMTX (Fig. 3B), the CI values of HT-1080 cells were nearly 1 or >1 indicating that the effect of the two agents is antagonistic or additive. In contrast, all of the CI values for this combination in HS-18 cells were <0.6 indicating that in this cell line these two drugs had a synergistic effect. When cells were treated with TMTX followed by ET-743, an antagonistic effect was observed in HT-1080 cells; however, a synergistic effect was observed in HS-18 cell lines.

When cells were exposed to paclitaxel followed by ET-743 (Fig. 3C), the CI value of HT-1080 cells at 95% fraction killed was 0.73 ± 0.14 and that of HS-18 cells at 95% fraction killed showed modest synergy (CI = 0.83 ± 0.33).

**Effects of Combination of ET-743 and DXR on Apoptosis.** To determine the mechanisms of synergy between ET-743 and DXR, we first analyzed the effects of this combination therapy on induction of apoptosis using Hoechst fluorescence. Table 1 is a summary of percentage of apoptosis, which was induced by combination treatment with ET-743 and DXR, and Fig. 4 is a photograph, which shows morphological apoptotic cells in combination therapy. HT-1080 cells were treated with 100 μM of ET-743 and 10 μM of DXR individually or concomitantly for 48 h. As determined by quantitative fluorescence microscopy, the combination of ET-743 and DXR concurrently for 48 h increased the percentage of cells undergoing apoptosis from 2.0 ± 1.7% with DXR alone to 11.0 ± 3.6%
Fig. 4 Effect of ET-743 and DXR treatment on induction of apoptosis in HT-1080 and HS-18 cells. HT-1080 cells (A–D) were treated with 100 pm of ET-743 and 10 nm of DXR individually or concomitantly for 48 h, and HS-18 cells (E–H) were exposed to 400 pm of ET-743 and 40 nm of DXR individually or concomitantly for 48 h. Cells were harvested and stained with Hoechst 33342 as described in “Materials and Methods.” Cells were examined under a fluorescence microscope. Arrows, cells showing condensed or fragmented nuclei.
ET-743 and DXR in STS Cells

with the two drugs together. Approximately the same results were obtained from HS-18 cells that were treated with 400 pm of ET-743 and 40 nm of DXR individually or concomitantly for 48 h.

The Effect of ET-743 on DXR Uptake. Because sequence treatment of ET-743 followed by DXR was the most effective regimen, we tested the possibility that sensitization to DXR by ET-743 could be attributable to increased intracellular drug accumulation. Such an increase could arise from alterations in drug influx, efflux, or intracellular binding. DXR uptake was studied initially as a function time in HT-1080 cells, which were in drug influx, efflux, or intracellular binding. DXR uptake was drug accumulation. Such an increase could arise from alterations DXR by ET-743 could be attributable to increased intracellular effective regimen, we tested the possibility that sensitization to sequence treatment of ET-743 followed by DXR was the most

In the present study, we have attempted to understand the mechanisms for synergistic interaction of ET-743 and DXR. Our results indicate that the mechanism of cell death by the combination treatment of ET-743 and DXR is apoptosis, greater than that seen with ET-743 or DXR alone treatment (Fig. 4). ET-743 also enhanced DXR accumulation (Fig. 5). While this work was being completed, Jin et al. (22) and Minuzzo et al. (23) reported that ET-743 blocked induction of mdr-1 and other proteins by inhibition of transcription. Other mechanisms of cell kill by ET-743 have been proposed including alklylation of DNA (24) and inhibition of topoisomerase I (25), but these effects are seen with higher concentrations of the drug as compared with inhibition of transcription. Although none of the cell lines tested here expressed P-glycoprotein, induction of P-glycoprotein by DXR (26) can be down-regulated by ET-743. To determine this possibility, mdr-1 mRNA in HT-1080 cells, before and after DXR treatment, was measured by quantitative reverse transcription-PCR (Taqman), and no message was detected after DXR exposure (data not shown).

Concomitant exposure of ET-743 and TMTX showed an antagonistic cytotoxic effect. When cells were exposed to ET-743 followed by TMTX (Fig. 3C), the CI values of HT-1080 cells showed an antagonistic effect. In contrast, those of HS-18 cells showed a synergistic effect. Expression of retinoblastoma protein may be one possible reason for the differences observed in the sequence study between HT-1080 and HS-18 cell lines. HS-18 cells, which lack retinoblastoma protein, are relatively resistant to TMTX (18). The synergism in cytotoxicity observed in HS-18 cell lines exposed to ET-743 followed by TMTX may be explained by the concentration of TMTX. In sequence studies, the molar ratio of ET-743 and TMTX at the IC_{50} was very different between two cell lines: 1:259,259 (ET-743:TMTX) in HS-18 cell lines compared with 1:600 in HT-1080 cell lines.

Paclitaxel is used to treat several solid tumors and has demonstrated activity in a series of human sarcoma cell lines. In this study, the importance of sequence of administration is strikingly illustrated by the combination of paclitaxel and ET-743. ET-743 and paclitaxel result in strong cytotoxic synergism when paclitaxel is administered before ET-743 (Fig. 3C). In contrast, the same agents cause less than additive cytotoxicity when ET-743 is administered concomitantly or before paclitaxel (Figs. 2 and 3C).

In summary, the present studies show a synergistic interaction between ET-743 and DXR occurs in two human STS cell lines. Synergy was evident for all of the drug combinations evaluated; however, sequencing with ET-743 followed by DXR treatment may be the most favorable regimen for future clinical trials because of the consistently high degree of synergy obtained for a range of doses evaluated. Exposure to paclitaxel...
followed by ET-743 is also an effective regimen against human STS cells, whereas concomitant exposure and reverse sequenc- ing are antagonistic.

ACKNOWLEDGMENTS

We thank Dr. Ting-Chao Chou for many helpful discussions.

REFERENCES


Sequence-dependent Enhancement of Cytotoxicity Produced by Ecteinascidin 743 (ET-743) with Doxorubicin or Paclitaxel in Soft Tissue Sarcoma Cells

Naoto Takahashi, Wei Wei Li, Debabrata Banerjee, et al.