Transcriptional signature of Ecteinascidin 743 (Yondelis, Trabectedin) in human sarcoma cells explanted from chemo-naïve patients

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Abstract
Ecteinascidin 743 (ET-743; Yondelis, Trabectedin) is a marine anticancer agent that induces long-lasting objective remissions and tumor control in a subset of patients with pretreated/resistant soft-tissue sarcoma. Drug-induced tumor control is achievable in 22% of such patients, but there is no clear indication of the molecular features correlated with clinical sensitivity/resistance to ET-743. Nine low-passage, soft-tissue sarcoma cell lines, explanted from chemo-naïve patients with different patterns of sensitivity, have been profiled with a cDNA microarray containing 6,700 cancer-related genes. The molecular signature of these cell lines was analyzed at baseline and at four different times after ET-743 exposure. The association of levels of TP53 mutation and TP73 expression with ET-743 sensitivity and cell cycle kinetics after treatment was also analyzed. Gene expression profile analysis revealed up-regulation of 86 genes and down-regulation of 244 genes in response to ET-743. The ET-743 gene expression signature identified a group of genes related with cell cycle control, stress, and DNA-damage response (JUNB, ATF3, CS-1, SAT, GADD45B, and ID2) that were up-regulated in all the cell lines studied. The transcriptional signature 72 hours after ET-743 administration, associated with ET-743 sensitivity, showed a more efficient induction of genes involved in DNA-damage response and apoptosis, such as RAD17, BRCA1, PAR4, CDKN1A, and P53DINP1, in the sensitive cell line group. The transcriptional signature described here may lead to the identification of ET-743 downstream mediators and transcription regulators and the proposal of strategies by which ET-743–sensitive tumors may be identified. [Mol Cancer Ther 2005;4(5):814–23]

Introduction
Ecteinascidin 743 (ET-743; Yondelis, Trabectedin) is a tetrahydroisoquinoline alkaloid with antiproliferative effects, isolated from the tunicate Ecteinascidia turbinata (1). This compound binds to the minor groove of DNA and binds the N2 position of guanine (2–4), producing a bend towards the major groove of the DNA, in a way that differs from that of other known alkylating agents. This alteration in the DNA structure could be the cause of the broad inhibition of inducible transcription, previously described at pharmacologically achievable concentrations, probably interfering with transcription factors (5–7). In addition, an efficient DNA repair function is known to be required for ET-743 cytotoxicity. In fact, the mode of action of ET-743 is different from that of other DNA-interacting antitumors because nucleotide-excision-repair deficiencies that increase the susceptibility of cell lines to cisplatin, UV, and alkylating reagents are more resistant to ET-743.

At the cellular level, ET-743 produces cell cycle perturbations with a decreased rate of S-phase progression and a G2-M blockade (3, 8–11). Experiments with synchronized cells showed that cells in G1 phase are more sensitive to the cytotoxic effects of ET-743 than are cells in G2 or S phase.

Phase I clinical trials started back in 1996 and phase II and comparative trials are currently in progress (12). The data available thus far confirm a therapeutic potential in patients with advanced breast cancer pretreated with anthracyclines and taxanes and in women with ovarian cancer that is resistant, or has experienced relapse, to platin-taxane–based therapy (13–15). Additionally, a large phase II program, conducted in patients with advanced soft-tissue sarcoma that is resistant, or has undergone relapse, to conventional agents, has shown long-lasting objective responses and tumor control in a clinically significant proportion of cases (16–18); such a rate of ET-743–induced tumor control seems to have an effect on median survival and particularly on the proportion of patients (29%) who were alive after 2 or more years.

This clinical data set suggests major constitutive/molecular differences between a group of patients in whom the compound is able to modulate progression of the disease for very long periods and a cohort that is fully resistant to ET-743.
Given the shown clinical benefit of ET-743 treatment for soft-tissue sarcoma, we decided to investigate the mechanism of action of this drug in a panel of chemonaive, low-passage soft-tissue sarcoma cell lines established from biopsies of newly diagnosed patients with different sarcoma histology types. We characterized the expression profiles of those cell lines and estimated their correlation with \textit{in vitro} sensitivity to ET-743 both in the absence of the drug and in time-course experiments. This should make it possible to develop a predictive ceteinascidin sensitivity model for subsequent validation in tumor samples from patients treated with ET-743.

**Materials and Methods**

**Cell Lines**

Eleven cell lines were studied, eight of which were directly established from surgical tumor-tissue specimens from sarcoma patients (Table 1). Sensitive and isogenic resistant chondrosarcoma cell lines (CS-1S and CS-R) were kindly provided by Dr. Lawrence Weissbach (Massachusetts General Hospital, Boston, MA). The sarcoma cell line SW872 was obtained from the American Type Culture Collection (Rockville, MD). The \textit{in vitro} sensitivity of each cell line to ET-743 was previously established.\(^4\) Cells were maintained in RPMI medium supplemented with 10% fetal bovine serum, fungizone, and penicillin/streptomycin. CS-1R and CS-1S were grown without antibiotics or fungizone.

For time-course analysis, cells were treated with 10 nmol/L ET-743 for 6, 24, 48, and 72 hours. \(TP53\) gene mutations were analyzed in accordance with described protocols (19).

**Real-time Reverse Transcription-PCR**

Quantitative reverse transcription-PCR (RT-PCR) was done for \(ATF3\), \(BRCA1\), \(GADD45B\), \(GAPDH\), \(JUNB\), \(RAD17\), \(SAT\), \(TAp73\), and \(\Delta Np73\) using the Applied Biosystems 7900HT (Applied Biosystems, Foster City, CA). \(GADPH\) expression was used as a reference for calculating expression levels of the other genes.

**Flow Cytometry Analysis**

Cells were cultured for 24 to 72 hours in the absence of the drug. ET-743, 10 nmol/L, was then added to the medium and incubation was continued for 6, 24, 48, and 72 hours. For flow cytometry analysis, cells corresponding to each treatment time were fixed in 70% ethanol, and RNase (0.6 \(\mu\)g/\(\mu\)L, final concentration) and propidium iodide (50 \(\mu\)g/mL) were added to the fixed cells. The DNA content was quantified with a flow cytometer (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ).

**RNA Extraction and Amplification**

Total RNA from cells was extracted in two steps after 6, 24, 48, and 72 hours of treatment using Trizol (Life Technologies, Inc., Grand Island, NY) and then purified with RNaseasy (Qiagen, Inc., Valencia, CA). Double-strand cDNA was synthesized from 4 \(\mu\)g of total RNA using the Superscript System for cDNA synthesis (Life Technologies). \textit{In vitro} transcription was carried out using the T7 Megascript \textit{in vitro} transcription kit (Ambion, Austin, TX). The quality of the amplified RNA produced was checked by electrophoresis and its concentration was measured.

**cDNA Microarray Hybridizations and Analysis**

Amplified RNA was hybridized on the CNIO-Oncochip as previously described (20). Briefly, 5 \(\mu\)g of amplified RNA were directly labeled with cyanine 3–conjugated dUTP (Cy3), whereas 5 \(\mu\)g of aRNA from the Universal Human Reference RNA (Stratagene) were labeled with cyanine 5–conjugated dUTP (Cy5) as reference. Hybridizations were done as described (21). Hybridized slides were scanned and analyzed using the Scanarray 5000 XL (GSI Lumonics, Kanata, Ontario, Canada) and GenePix Pro 4.0 software (Axon Instruments, Inc., Union City, CA), respectively.

**Data Analysis**

Raw microarray data were processed as previously described. Data obtained from each hybridization were stored in a database for analysis. Cy3/Cy5 ratios were normalized with respect to the median ratio value of all of the spots in the array. After normalization, spots with intensities for both channels (sum of medians) less than that of the local background were discarded. The ratios of the remaining spots were log transformed (base 2), and duplicated spots on the OncoChip were averaged to the median (20). After this first processing, results at all treatment times were compared with those in untreated cells, and genes were deemed to be up-regulated or down-regulated if the ratio of the difference with untreated cells was more than twice that at any of the four treatment times studied in at least 72% (8 of 11) of the cell lines analyzed. Biological functions of selected genes were assigned using the GeneCards\(^5\) and Gene Ontology\(^6\).

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\(^4\) V. Moneo and A. Carnero et al., unpublished data.

\(^5\) http://bioinfo.cnio.es/gene_cards/index.html

\(^6\) http://fatigo.bioinfo.cnio.es/

**Table 1. Details of the cell lines treated with ET-743 and analyzed by cDNA microarrays**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tumor origin</th>
<th>IC(_{50}) (nmol/L) 96 h</th>
<th>Doubling time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR2103/1A</td>
<td>Liposarcoma</td>
<td>0.7</td>
<td>48</td>
</tr>
<tr>
<td>SR2103/1B</td>
<td>Liposarcoma</td>
<td>0.7</td>
<td>48</td>
</tr>
<tr>
<td>SW872</td>
<td>Liposarcoma</td>
<td>0.5</td>
<td>24</td>
</tr>
<tr>
<td>CA1010</td>
<td>Leiomyosarcoma</td>
<td>0.4</td>
<td>36</td>
</tr>
<tr>
<td>SR2205</td>
<td>Solitary Fibrous Tumor</td>
<td>1</td>
<td>96</td>
</tr>
<tr>
<td>SR2410</td>
<td>MPNST</td>
<td>&gt;100</td>
<td>96</td>
</tr>
<tr>
<td>SR2910</td>
<td>Ewing Sarcoma</td>
<td>1</td>
<td>24</td>
</tr>
<tr>
<td>SR0312</td>
<td>Osteosarcoma</td>
<td>0.4</td>
<td>24</td>
</tr>
<tr>
<td>SR0306</td>
<td>Nephroblastoma(^*)</td>
<td>&gt;100</td>
<td>96</td>
</tr>
<tr>
<td>CS-1R</td>
<td>Chondrosarcoma</td>
<td>34</td>
<td>ND</td>
</tr>
<tr>
<td>CS-1S</td>
<td>Chondrosarcoma</td>
<td>0.9</td>
<td>ND</td>
</tr>
</tbody>
</table>

Abbreviations: MPNST, malignant peripheral nerve sheath tumor; ND, not determined.

\(^*\)Rhabdomyoblastic differentiation.

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Mol Cancer Ther 2005;4(5). May 2005

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databases. Sensitivity/resistance signatures were obtained by dividing the median expression level for each gene in all sensitive cell lines by that of all resistant cell lines under basal conditions (i.e., in the absence of the drug) and throughout the time course of treatment with ET-743. Selected genes were defined as those with at least 2-fold up-regulation or down-regulation in the sensitive group of cell lines relative to the resistant group.

**Clustering Analysis**

To classify the temporal profiles of gene expression, cluster analysis was done using the Self-Organizing Tree Algorithm (22), assuming Euclidian distances between genes.

**Results**

**Gene Signature of ET-743**

Among the 11 human sarcoma cell lines studied, the SR2103/1A, SR2103/1B, CA1010, SR2205, SR2410, SR2910, SR0312, and SR0306 lines had been established at the Centro Nacional de Investigaciones Oncológicas from tissue samples of sarcoma patients and are considered low-passage cell lines. Cellular phenotype and ET-743 sensitivity data of all the cell lines are detailed in Table 1. The 11 human sarcoma cell lines were exposed to 10 nmol/L ET-743; this concentration may be achieved and maintained for more than 72 hours in patients plasma below the recommended clinical dose incorporated in the phase II trials in sarcoma and is therefore considered to be therapeutically appropriate for this experimental study (23).

ET-743 in vitro treatment produced deregulation of 330 genes in the majority of these cell lines (8 of 11; 72% of samples). Most of the deregulated genes were down-regulated (244; 74%) and only 86 genes (26%) were up-regulated (Fig. 1; raw data and selected gene data can be found in Table 1 at the website http://redlinfomas.cnio.es/publications/ET743). The number of genes of which expression level changed increased with the time elapsed after treatment. Thus, early on (6 hours of treatment), only four genes were noted as being consistently changed in most of the cell lines in response to treatment with ET-743: two genes (SAT and EST) were up-regulated and two genes (BCAR3 and DDX20) were down-regulated. The upward or downward tendency of these genes was constant throughout the treatment.

The genes up-regulated after treatment with ET-743 are mainly involved in signal transduction, molecule and ion transport, and energy pathways, whereas the down-regulated genes are predominantly associated with nucleic acid binding, gene transcription, signal transduction, and cell cycle control. Notably, the most significantly overexpressed downstream mediators after treatment with ET-743 were CS-1, ATF3, SAT, JUNB, GADD45, and ID2, all of which are involved in cell cycle arrest and apoptosis (Fig. 2A).

**Figure 1.** Expression profile of deregulated genes after treatment with ET-743. Eighty-six genes were up-regulated and 244 genes were down-regulated as a consequence of the treatment.
SSAT for apoptosis induction after treatment with polyamines analogues (25). The induction of SSAT after treatment with polyamine analogues has been proposed as a prognostic indicator of drug response (26) because it is known to be induced by 5-fluorouracil, TDX, cisplatin, and oxaliplatin (27, 28). It has also been shown that over-expression of SSAT, which is dependent on p53 status (27), produces cell cycle arrest in G2-M (29).

ATF3. ATF3 was up-regulated after 24 hours of treatment with ET-743 in 8 of 11 cell lines. ATF3 is a stress-inducible transcriptional repressor of which levels are dramatically induced in response to a variety of stress conditions in many different tissues, such as toxic chemicals, anticancer drugs, proteasome inhibitors, and genotoxic agents. This p53 target is involved in drug-induced apoptosis, specifically after treatment with DNA topoisomerase inhibitors, such as camptothecin or etoposide, inducing activation of caspase proteases (30, 31).

ID2. ID2 is a member of a family of helix-loop-helix transcription factors that regulate cell differentiation and proliferation (32–34). Id2 binds to Rb family members, abolishing their growth-suppressing function. It has been

![Figure 2. ET-743 downstream genes. A, average of gene expression log-ratios (base 2) of the most significantly up-regulated genes in response to treatment with ET-743 in 11 sarcoma cell lines. The ratio for each time point is calculated by dividing the average expression at the corresponding time by the average of expression before treatment. B, expression pattern of four genes identified as ET-743 downstream genes were validated in two cell lines by RT-PCR.](image-url)
shown that trichostatin A, a histone deacetylase inhibitor, induces the expression of ID2 (35–37). Overexpression of ID2 augments apoptosis in myeloid and osteogenic sarcoma-derived cell lines (38).

**CS-1.** CS-1 is a calcineurin-binding protein. Calcineurin is a calcium- and calmodulin-dependent serine/threonine phosphatase that plays an important role in transducing calcium-dependent signals in a variety of cell types. This enzyme is required for G1 progression and it was recently identified as an important link between Ca\(^{2+}\) signaling and NF-κB activation in response to mitochondrial stress (39). Overexpression of Cs-1 inhibits calcineurin activity, producing cell cycle arrest (40).

**JUNB.** Activator protein 1 is composed of homodimers or heterodimers formed by related Jun (c-Jun, JunB, and JunD), Fos (c-Fos, FosB, Fra-1, and Fra-2), and ATF family proteins (41). Jun is known to be up-regulated in response to the exposure to genotoxic stress, such as alkylating agents or short-wavelength UV radiation (42, 43).

GADD45B. Gadd45 proteins cooperate in the activation of S and G2-M checkpoints following exposure of cells to UV irradiation and other genotoxic stresses, producing growth arrest and apoptosis (8, 44).

The expression changes for four selected genes were verified by real-time RT-PCR (Fig. 2B) in two cell lines. The pattern of gene expression modulation was comparable for real-time RT-PCR and microarray results, although the former technique was more sensitive in detecting gene expression changes (Fig. 2B).

**Differences between ET-743–Sensitive and ET-743–Resistant Sarcoma Primary Cell Lines**

As shown in Table 1, six cell lines were considered as ET-743 sensitive (those with LC\(_{50}\) < 1 nmol/L), and three were resistant to ET-743.

**Effect of ET-743 on the Cell Cycle of Resistant and Sensitive Cell Lines**

All cell lines were analyzed by flow cytometry at different treatment times with ET-743. The cell cycle of resistant cell lines was undisturbed throughout treatment (data not shown). A cytotoxic effect of ET-743 was revealed in sensitive cell lines as a G2-M blockade, or apoptosis. These effects varied among the cell lines studied, thereby confirming previous observations (8).

**Transcriptional Signature Associated with ET-743 Sensitivity**

The gene expression profiles of the sensitive and resistant cell lines were obtained under basal conditions (i.e., in the absence of the drug). The median expression in sensitive cell lines was compared with the corresponding median expression of the resistant cell lines by way of the S/R expression ratio. Differences between resistant and sensitive cell lines were also reflected in changes of the expression level of several genes (Table 2). The results at each time for each cell line and the median ratios and SDs are presented in Table 3 at the website (http://redinformas.cnio.es/publications/ET743). These data represent a mixture of signals concerning cell differentiation and molecular alterations that lead to ecteinascidin sensitivity model resistance. Thus, ET-743–sensitive sarcoma cells constitutively express the following markers: IL-13, MMP3, SAMSNI, EDG1, IGFBP3, and PRKAG2, whereas ecteinascidin-resistant cells show increased expression of PTPRM, PCDH7, NID2, CDH6, CCND1, NBL1, AHR, TIMP3, and PDGFR\(A\) (Table 2).

Interestingly, matrix metalloproteinase 3 and its inhibitor, tissue inhibitor of metalloproteinase 3, are differentially expressed in the sensitive and resistant cell lines, respectively. Metalloproteinase activity influences cellular sensitivity to extrinsic death in different cell types through proteolytic shedding of cell-surface signaling molecules. Metalloproteinase defective expression has already been described in the resistant chondrosarcoma cell line although its role in ET-743 resistance has not been elucidated (45). Matrix metalloproteinase 3, a regulator of matrix remodeling, plays a role in tumor metastasis through the degradation of fibronectin, laminin, gelatins, collagens, and cartilage proteoglycans. Matrix metalloproteinase 3 has been shown to selectively modulate Fas-mediated neuronal apoptosis induced by doxorubicin (46) and induces apoptosis when overexpressed in mammary epithelial cells (47). On the other hand, tissue inhibitor of metalloproteinase 3, which acts as an inhibitor of matrix metalloproteinase 3, was expressed at higher levels in the resistant cell lines. This inhibitor of metalloproteinase can either enhance or suppress apoptosis, depending on the cell type or context analyzed (48–50).

### Table 2. Genes constitutively overexpressed or down-regulated in sensitive compared with resistant cell lines

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene description</th>
<th>S/R</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL13RA2</td>
<td>Interleukin-13 receptor α2</td>
<td>5.25</td>
</tr>
<tr>
<td>MMP3</td>
<td>Matrix metalloproteinase 3</td>
<td>3.43</td>
</tr>
<tr>
<td>SAMSNI</td>
<td>SAM-domain protein SAMS-N1</td>
<td>2.94</td>
</tr>
<tr>
<td>EDG1</td>
<td>Endothelial differentiation, sphingolipid G-protein–coupled receptor 1</td>
<td>1.95</td>
</tr>
<tr>
<td>IGFBP3</td>
<td>Insulin-like growth factor-binding protein 3</td>
<td>1.74</td>
</tr>
<tr>
<td>PRKAG2</td>
<td>Protein kinase, AMP-activated, γ2 noncatalytic subunit</td>
<td>1.71</td>
</tr>
<tr>
<td>PDGFR(A)</td>
<td>Platelet-derived growth factor receptor α polypeptide</td>
<td>–3.13</td>
</tr>
<tr>
<td>TIMP3</td>
<td>Tissue inhibitor of metalloproteinase 3</td>
<td>–2.83</td>
</tr>
<tr>
<td>AHR</td>
<td>Aryl hydrocarbon receptor</td>
<td>–2.65</td>
</tr>
<tr>
<td>NBL1</td>
<td>Neuroblastoma, suppression of tumorigenicity 1</td>
<td>–1.93</td>
</tr>
<tr>
<td>CCND1</td>
<td>Cyclin D1</td>
<td>–1.90</td>
</tr>
<tr>
<td>CDH6</td>
<td>Cadherin 6, type 2, K-cadherin</td>
<td>–1.84</td>
</tr>
<tr>
<td>NID2</td>
<td>Nidogen 2 (osteoidogen)</td>
<td>–1.81</td>
</tr>
<tr>
<td>PCDH7</td>
<td>BH-protocadherin</td>
<td>–1.77</td>
</tr>
<tr>
<td>PTPRM</td>
<td>Protein tyrosine phosphatase receptor-type M</td>
<td>–1.69</td>
</tr>
</tbody>
</table>

**NOTE:** S/R is the log-ratio (base 2) of median expression in the sensitive versus resistant cell lines.
expression level increased in the sensitive cell lines after treatment with ET-743. Thus, Table 3 shows a group of genes of which expression of a series of genes in response to treatment between ET-743–sensitive and ET-743–resistant cells, the survival, and chemotaxis (54–56).

Platelet-derived growth factor receptor A, which is more strongly expressed in the resistant cell lines, enhances the p53-dependent apoptotic response of colorectal cells to DNA damage (51). The aryl hydrocarbon receptor is a ubiquitously expressed transcriptional regulatory protein, which induces the transcription of CYP1A1, CYP1A2, and CYPBI, which are genes that encode different enzyme isoforms of cytochrome P-450. These enzymes catalyze the oxidation of certain classes of xenobiotics, resulting in the generation of highly reactive electrophilic metabolites that bind specific residues of DNA, causing DNA-adduct formation that may lead to mutation and subsequent cellular transformation (52). The findings described here are consistent with those previously published showing ET-743 to be metabolized by P450, most predominantly by the cytochrome P-450 3A subfamily (53). Additionally, the higher AHR expression values observed here in the ET-743–resistant cell lines are consistent with the previous observation of an increased aryl hydrocarbon receptor expression associated with an adverse outcome in soft-tissue sarcoma patients (16).

Platelet-derived growth factor receptor A, which is more strongly expressed in the resistant cell lines, is a receptor tyrosine kinase overexpressed in a subset of solid tumors, and is therefore the target of drugs inhibiting this function, such as imatinib mesylate (Gleevec). Platelet-derived growth factor A selectively binds to platelet-derived growth factor receptor A, regulating cell proliferation, survival, and chemotaxis (54–56).

**Ecteinascidin-Inducible Genes in the Sensitive Cell Lines**

In addition to the constitutive differences that exist between ET-743–sensitive and ET-743–resistant cells, the soft-tissue sarcoma cell lines studied showed variable expression of a series of genes in response to treatment with ET-743. Thus, Table 3 shows a group of genes of which expression level increased in the sensitive cell lines after 72 hours of treatment with ET-743. By contrast, Table 4 illustrates a group of genes of which expression increased in the resistant cell lines after 72 hours of treatment with ET-743, and decreased in the sensitive cell lines.

**RAD17.** RAD17 is a component of the DNA damage G2-M checkpoint, interacting with RAD1 (protein-protein interaction in the cell cycle checkpoint machinery), and is required for S-phase and G2-M arrest in response to DNA damage or incomplete DNA replication (57, 58).

**BRCA1.** BRCA1 has been purified as part of a large protein complex known as BRCA1-associated genome surveillance complex, which contains a wide range of DNA repair and replication proteins (44, 59). An interaction with p53-BRCA1 (60) has been noted in the repair after DNA damage.

**PAR4.** PAR4 (PAWR) is a cell death modulator that inhibits the atypical protein kinase C isoforms, leading to reduced cellular NF-κB activation and increased apoptosis (61).

**CASP8AP2.** CASP8AP2 is involved in Fas-mediated activation of caspase 8 during apoptosis.

The transcription of TP53DINP1 is increased during cellular stress by p53-mediated activation of transcription. TP53DINP1, in association with homeodomain-interacting protein kinase 2, regulates p53 transcriptional activity on P21, MDM2, PIG3, and BAX promoters, inducing G1 arrest and increasing p53-mediated apoptosis (62). Homeodomain-interacting protein kinase has been shown to play a critical role in triggering p53-dependent apoptosis in response to the antineoplastic drug cisplatin (63).

### Table 3. Genes with a higher level of expression in sensitive than resistant cell lines after 72 hours of treatment

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene description</th>
<th>S/R</th>
<th>Trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAD17</td>
<td>RAD17 (S. pombe) homologue</td>
<td>1.81</td>
<td><img src="image1.png" alt="image" /></td>
</tr>
<tr>
<td>PTPN3</td>
<td>Protein tyrosine phosphatase, nonreceptor type 3</td>
<td>1.78</td>
<td><img src="image2.png" alt="image" /></td>
</tr>
<tr>
<td>RANBP2L1</td>
<td>RAN-binding protein 2-like 1</td>
<td>1.76</td>
<td><img src="image3.png" alt="image" /></td>
</tr>
<tr>
<td>BRCA1</td>
<td>Breast cancer 1, early onset</td>
<td>1.65</td>
<td><img src="image4.png" alt="image" /></td>
</tr>
<tr>
<td>PAWR</td>
<td>PRKC, apoptosis, WT1, regulator</td>
<td>1.28</td>
<td><img src="image5.png" alt="image" /></td>
</tr>
<tr>
<td>p53DINP1</td>
<td>p53-inducible p53DINP1</td>
<td>2.06</td>
<td><img src="image6.png" alt="image" /></td>
</tr>
<tr>
<td>CASP8AP2</td>
<td>Caspase 8–associated protein 2</td>
<td>1.77</td>
<td><img src="image7.png" alt="image" /></td>
</tr>
<tr>
<td>HSA250839</td>
<td>Gene for serine/threonine protein kinase</td>
<td>1.65</td>
<td><img src="image8.png" alt="image" /></td>
</tr>
<tr>
<td>CDKN1A</td>
<td>Cyclin-dependent kinase inhibitor 1A (p21, Cip1)</td>
<td>1.61</td>
<td><img src="image9.png" alt="image" /></td>
</tr>
</tbody>
</table>

**NOTE:** S/R column shows the log-ratio (base 2) of the median expression level of sensitive and resistant cell lines at 72 hours; trend column shows the change in the level of expression of these genes during the treatment.

IGFBP-3, which is also overexpressed in sensitive cell lines, enhances the p53-dependent apoptotic response of colorectal cells to DNA damage (51).

**Table 4. Genes with a higher level of expression in resistant than in sensitive cell lines after 72 hours of treatment**

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene description</th>
<th>S/R</th>
<th>Trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>H4F3</td>
<td>H4 histone family, member G</td>
<td>−1.51</td>
<td><img src="image10.png" alt="image" /></td>
</tr>
<tr>
<td>PTPRK</td>
<td>Protein tyrosine phosphatase, receptor type, K</td>
<td>−1.53</td>
<td><img src="image11.png" alt="image" /></td>
</tr>
<tr>
<td>MAL</td>
<td>Mal, T-cell differentiation protein</td>
<td>−1.55</td>
<td><img src="image12.png" alt="image" /></td>
</tr>
<tr>
<td>TIA-2</td>
<td>Lung type-I cell membrane–associated glycoprotein</td>
<td>−1.88</td>
<td><img src="image13.png" alt="image" /></td>
</tr>
<tr>
<td>H4FI</td>
<td>H4 histone family, member I</td>
<td>−1.52</td>
<td><img src="image14.png" alt="image" /></td>
</tr>
<tr>
<td>APOC1</td>
<td>Apolipoprotein C-I</td>
<td>−1.53</td>
<td><img src="image15.png" alt="image" /></td>
</tr>
<tr>
<td>CAV1</td>
<td>Caveolin 1, caveolae protein, 22 kDa</td>
<td>−1.56</td>
<td><img src="image16.png" alt="image" /></td>
</tr>
<tr>
<td>ISG15</td>
<td>IFN-stimulated protein, 15 kDa</td>
<td>−1.68</td>
<td><img src="image17.png" alt="image" /></td>
</tr>
<tr>
<td>MMP10</td>
<td>Matrix metalloproteinase 10 (stromelysin 2)</td>
<td>−1.69</td>
<td><img src="image18.png" alt="image" /></td>
</tr>
</tbody>
</table>

**NOTE:** The S/R column shows the log-ratio (base 2) of sensitive to resistant cell lines at 72 hours; the trend column shows the change in the level of expression of these genes during the treatment.
CDKN1A. CDKN1A (p21) wild-type p53-activated fragment 1, p21, regulator of cell cycle, cyclin-dependent kinases 2 and 4 (CDK2/CDK4) inhibitor protein, inhibiting cell cycle progression by interacting with G1 cyclin/CDK complexes and proliferating cell nuclear antigen.

The expression levels of Rad17 and BRCA1 were verified by real-time RT-PCR (Fig. 3) in one sensitive and one resistant cell line, confirming the results obtained by cDNA microarray analysis.

Ecteinascidin-Inducible Genes in the Resistant Cell Lines

MAL and CAV1 are components of membrane rafts. These rafts are membrane microdomains that play a central role in signal transduction by acting as a scaffold in which molecules involved in signal transduction pathways can interact.

Analysis of TP53 Status

TP53 is a critical cellular gatekeeper that mediates DNA damage–induced cell cycle arrest and apoptosis. As time-course experiments reveal the expression in ET-743–sensitive cell lines of p53 targets, we tested TP53 status in this panel of cell lines by sequencing exons 5 to 8. As shown in Table 5, only three cell lines (SR2205, SR2910, and SR2410) had wild-type TP53, whereas all other cell lines featured mutated TP53. In this panel of cell lines, TP53 status did not correlate with ET-743 sensitivity, thereby confirming previous observations (64).

TP73 Expression

To determine whether TP73 expression was induced in response to treatment with ET-743, and to study a possible relation of TP73 expression with sensitivity to ET-743, quantitative PCR was done at all the time points studied (0, 6, 24, 48, and 72 hours).

Basal levels of TAp73 and ΔNp73 varied among cell lines, and did not correlate with sensitivity or resistance to ET-743 (see Fig. 4). ET-743 seems to induce a decrease in the level of expression of full-length TAp73, unlike other chemotherapeutic drugs inducing DNA damage (65). By contrast, three of the studied cell lines showed a striking increase in the expression of ΔNp73, which is derived from an alternative promoter in intron 3 and lacks the transactivation domain of full-length TAp73. ΔNp73 is known to counteract apoptosis and growth suppression mediated by wild-type p53 and TAp73, inducing drug resistance to wild-type p53–harboring tumor cells (66). No relation was observed between the induction of ΔNp73 and ET-743 sensitivity.

Discussion

ET-743 is a potent cytotoxic agent that acts as a transcription-interfering agent (5). Different clinical and experimental studies have begun to provide an explanation of the mechanism underlying the cytotoxic effect of this drug, although further effort is still necessary to fully understand its mechanism of action.

One of the most striking findings here is the massive down-regulation of gene transcription, confirming previous studies done with a smaller selection of genes (5). We have used a cDNA Oncochip, enriched in cancer-relevant genes, containing a total of 6,800 genes. Analysis of the changes induced by ET-743 revealed a common signature of 244 down-regulated and 86 up-regulated genes in the majority (8 of 11) of these cell lines.

High-throughput screening has allowed the identification of genes that are induced after treatment with ET-743. These genes (CS-1, ATF3, SAT, JUNB, GADD45, and ID2) have a common role as transcription regulators, leading to cell cycle arrest in G2-M and apoptosis (25, 29–31, 38, 40, 42–44), which can be correlated with the cell cycle arrest and apoptosis induced after treatment of different cell types with ET-743 in our experiments and those of others (3, 8–11).

The approach taken here, using low-passage sarcoma cell lines, makes this study of even greater interest because such cell lines have characteristics that are closest to those of tumors.

This study has also attempted to identify specific drug-induced genes of which expression is associated with ET-743 sensitivity. To this end, tumor cells were treated with ET-743, and basal and ET-743–induced genes were compared in sensitive and resistant cells. Although the cell lines display a constellation of ET-743–induced changes at several time intervals, at 72 hours, all the cells lines showed a relatively uniform signature induced by the drug, which also revealed a set of genes that were differentially expressed in sensitive and resistant cells. Thus, after 72 hours of treatment, ET-743–sensitive soft-tissue sarcoma cells expressed a set of DNA damage sensors, including RAD17 and BRCA1, which underlies the unique mechanism of ET-743 action. ET-743 is known to subvert normal nucleotide excision repair by generating...
lethal DNA breaks during transcription-coupled nucleotide excision repair (67). ET-743–induced adducts are recognized by the nucleotide excision repair system (67), which leads to apoptosis of the tumor cells, instead of repairing the damage caused by the adducts. The increased expression of both RAD17 and BRCA1 indicates the activation of a sensor for DNA damage including both proteins. Our results are consistent with an explanation whereby BRCA1 functions as a molecular determinant of response to a range of different chemotherapeutic agents (68), now including ET-743.

Genes found to be overexpressed in sensitive soft-tissue sarcoma cells after ET-743 treatment include apoptosis inducers, such as PAR4 and CASP8AP2, and some known p53 targets, such as TP53DINP1 and CDKN1A. This prompted us to investigate further the potential role of p53 and p73 in regulating apoptosis in response to ET-743 treatment. Cell lines harboring TP53 mutations showed no detectable changes in ET-743 sensitivity, confirming previous observations (3, 9, 11), although p21 induction was greater in the TP53 wild-type cell lines.

The confirmation in this series that ET-743 is not associated with TP53 status led us to investigate whether the p73 pathway is involved in the response to ET-743. We hypothesized that p73 might substitute the apoptosis-inducer role of p53, as has been shown in cells lacking functional p53 (65). Thus, we measured the expression levels of p73 throughout the treatment with ET-743 in sensitive and resistant cell lines. Treatment of the panel of human soft-tissue sarcoma cell lines with ET-743 resulted in the inhibition of TAp73 and the induction of the oncogenic form Np73, unlike what has been shown for cisplatinum and other DNA-damaging drugs. The expression levels of TAp73 and Np73 genes were not related to TP53 status or sensitivity to ET-743, which differs from the observations for other DNA-damaging drugs (65, 69–71). These results strongly suggest that the response of the sarcoma cells to ET-743 does not occur through p53/p73 pathways. The significance of p73 down-regulation after treatment with ET-743 is not yet clear, and functional experiments with p73 are needed to clarify the effect of different p73 transcripts on ET-743 response.

### Table 5. TP53 status in the cell lines studied

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>SW872</th>
<th>CS-R*</th>
<th>CS</th>
<th>SR0306*</th>
<th>SR2103A</th>
<th>SR2103B</th>
<th>SR0312</th>
<th>CA1010</th>
<th>SR2410*</th>
<th>SR2205</th>
<th>SR2910</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 5</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>Exon 6</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>Exon 7</td>
<td>25:1:T-A</td>
<td>245:G-A</td>
<td>245:G-A</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>Exon 9</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
</tbody>
</table>

**NOTE:** Mutations are indicated by the corresponding nucleotide change. Abbreviations: WT, wild-type TP53. *ET-743–resistant cell lines.

### Figure 4.
Quantitative RT-PCR showing the expression levels of TAp73 and ΔNp73 in different sensitive and resistant cell lines during ET-743 treatment. The level of expression is heterogeneous but there is a general inhibition of full-length p73, whereas ΔNp73 seems to be induced after treatment with ET-743.
The comparison of sensitive and resistant cells has also revealed a set of genes, including MMP3, AHR, and TIMP3, which are differentially expressed in the absence of the drug. Results obtained here for this panel of low-passage soft-tissue sarcoma cell lines need to be corroborated in tumor specimens before they are translated to clinical trials. This method may help to identify the subset of soft-tissue sarcoma tumors that potentially show an increased sensitivity to ET-743 (17).

References
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Molecular Cancer Therapeutics

Transcriptional signature of Ecteinascidin 743 (Yondelis, Trabectedin) in human sarcoma cells explanted from chemo-naïve patients

Nerea Martínez, Margarita Sánchez-Beato, Amancio Carnero, et al.


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