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Extreme Sensitivity to Yondelis® (Trabectedin, ET-743) in Low Passaged Sarcoma Cell Lines Correlates With Mutated p53

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Abstract Yondelis® (Trabectedin, ET-743) is a marine anticancer agent currently in Phase II/III development in patients with advanced pretreated soft tissue sarcoma. In the present study, we generated a panel of low passaged tumor cell lines from samples explanted from chemonaive sarcoma patients with different tumor types. We assessed in vitro sensitivity/resistance to Trabectedin and doxorubicin in a panel of sarcoma cell lines and examined the correlation between molecular alterations in DNA repair genes and sensitivity to Trabectedin. We treated cell lines with Trabectedin and doxorubicin in both 96-h and clonogenic assays. In both assays, well-defined groups of resistant and sensitive cell lines were observed. Resistance to Trabectedin did not correlate with resistance to doxorubicin, indicating that the two drugs may have different mechanisms of resistance. p53 mutations and deletions correlated with extreme sensitivity (IC50 < 1 nM) to Trabectedin ($P < 0.01$). In a pair of isogenic cell lines differing only in the presence or absence of wild-type p53, the absence of p53 rendered cells threefold more sensitive to Trabectedin. J. Cell. Biochem. 100: 339–348, 2007.

Key words: Trabectedin; ET-743; p53; sarcoma

Sarcomas are relatively rare malignant tumors derived from the mesenchymal, none-pithelial tissue developed from the embryonic mesoderm. Sarcomas comprise less than 10% of all cancers [Helman and Meltzer, 2003] and account for a higher percentage of overall cancer morbidity and mortality in children and young adults than in adults. The overwhelming majority of sarcomas are sporadic with unknown etiology, but there are several well-described genetic-predisposition syndromes and well-documented environmental exposures associated with specific sarcomas [Mackall et al., 2002].

Surgery is the accepted treatment for most sarcomas. However, for those patients with unresectable disease or residual tumors following surgery, radiation therapy is also used. Patients with metastatic disease are treated with systemic chemotherapy, usually consisting of doxorubicin and ifosfamide [Singer et al., 2000]. For certain chemosensitive sarcomas—particularly pediatric sarcomas—treatment includes chemotherapy followed by surgery and/or radiotherapy. This approach has proven to be effective in patients who have localized tumors, with increasing long-term survival rates [Blay et al., 2003]. However, for many

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tumors that have marginal levels of chemoresponsiveness and for patients with metastatic disease, new approaches are needed.

Yondelis® (trabectidin, ET-743) is a marine-derived product that has shown cytotoxic activity against various human tumor cell lines both in vitro and in vivo, including cell lines resistant to other chemotherapeutic agents [Jimeno et al., 1996; Izbicka et al., 1998; Valoti et al., 1998], although the basis for response to this drug remains unclear. The mechanism of action of Trabectedin may involve binding to the minor groove of DNA with some degree of sequence specificity and DNA cross-linking [Pommier et al., 1996; Bonfanti et al., 1999; Takebayashi et al., 2001]. The extensive inhibition of transcription indicates that this drug may also have a novel mechanism of action [Jin et al., 2000a; Minuzzo et al., 2000]. An in vitro correlation between gene expression and sensitivity to Trabectedin has been assessed by DNA microarray [Martınez et al., 2005]. Yondelis® has also probed to be active in clinical trials for the treatment of soft tissue sarcomas [Lecesne et al., 2005], ovarian [Sessa et al., 2005], breast [Zelek et al., 2000], and prostate [Michaelson et al., 2005] cancer that are resistant to previous chemotherapies and is under active Phase II/III clinical development in these tumor types, both as single agent and in combination therapy.

In order to gain knowledge on the molecular basis of sensitivity/resistance to Trabectedin, we have generated new cell lines from untreated sarcoma tumor samples, keeping the cell lines low passaged to avoid extensive genetic alterations as a consequence of genomic instability of the tumor. We assessed sensitivity to Trabectedin and doxorubicin, a cytotoxic drug used as first-line therapy in sarcomas [O'Byme and Steward, 1999]. We then examined the relation between molecular alterations in DNA repair genes and sensitivity to Trabectedin. We found well-defined differences in sensitivity to the two drugs tested. The differential sensitivity to Trabectedin and doxorubicin indicates that they may have different mechanisms of resistance. We observed a significant relationship between the direct alteration of p53, either by mutation or deletion, and extreme sensitivity to Trabectedin ($P < 0.01$) and between genomic instability and sensitivity to Trabectedin, suggesting that Trabectedin induces a DNA damage-like response involving p53.

MATERIALS AND METHODS

Generation of Cell Lines and Culture Conditions

Sterile fragments from the resected tumor were minced in culture medium and then disaggregated by 1–2 h incubation with collagenase (100 μ/ml) at 37°C. After 24 h, the medium was changed to F-10 Ham (Gibco) supplemented with 1% Ultroser G (Biosepra). Cell lines generated were cultured in F-10 Ham supplemented with 1% Ultroser G. A673 cells were cultured in RPMI (Sigma) and SW872 cells in Leibovitz L-15 (Sigma). All media were supplemented with 10% FBS, fungizone, and penicillin/streptomycin.

Cytogenetic Analysis

Conventional cytogenetic analysis was performed by standard methods. Briefly, cultured cells were exposed to colcemid (Sigma) for 4–6 h at a final concentration of 0.01 ng/ml. Cells were then removed from flasks and underwent hypotonic treatment (KCl, 0.07 M) and fixation in methanolacetic solution (3:1 v/v). Giemsa-trypsin banding was used to elaborate the karyotype.

RT-PCR

Total RNA was collected using the TRI-REAGENT (Molecular Research Center, Inc.). RT-PCR was performed with 1 μg of RNasy (Promega) following the manufacturer's instructions. The following primers were used to amplify the regions: p73, forward 5′-TTTAACAGGATTGGGGTGTCC-3′, reverse 5″-CGTGAACTCCTCCTTGATGG-3′; MSH2, forward 5′-CATTTTCTTCAACCAGGAGGT-3′, reverse 5″-TGCCAACAATAATTTCTGGT GT-3′; MLH1, forward 5′-GCATCTAGACGTTTCCTTG G-3′, reverse 5″-GAGGAATTGGAGCCCAGGA-3′; β-actin, forward 5′-AGGCCAACCGCGAGAAGATGAC-3′, reverse 5″-GAAGTCCAGGGCGACGTAGCA-3′. cDNA was subjected to PCR, and products were analyzed by electrophoresis on a 1% agarose gel. It should be noted that the oligos for p73 identify only α, γ, and ζ alternate splicings.

p53 Sequence Determination

The complete CDS of human p53 was amplified from the cell line cDNAs using the specific primers 5′-ATGGAGGAGCCGCAGTCAGATC C-3′ and 5′-TCAGTCTGAGTCAGGCCCTTCT G-3′. PCR amplification was carried out with a combination of Taq and Pfu polymerases to ensure copy fidelity. The 1,281 bp fragment was

cloned into a pGEM-T-easy vector (Promega), and more than five positive clones for each sample were sequenced with the M13 forward and reverse primers.

Protein Determination

Cells were washed twice and sonicated in lysis-buffer (50 mM Tris-HCl pH 7.5, 1% NP-40, 10% glycerol, 150 mM NaCl, 2 mM, Complete protease inhibitor cocktail Roche). The protein content of the lysates was determined by the modified method of Bradford. Samples were separated on 7.5% SDS–PAGE gels, transferred onto Immobilon-P membrane (Millipore), and immunostained. To identify total p53 we used: PAb FL-393 anti-p53 of Santa Cruz, anti-Bax (N-20), anti-ERK2 (c-14), and pERK (Tyr 204) of Santa Cruz Biotechnology; anti-p21 (OP64F) of Oncogene Sci., as primary antibodies. Proteins were visualized using the adequate secondary antibody coupled to ECL detection system (Amersham).

Cytotoxicity

96-h assay. Trabectedin and doxorubicin were tested on 96-well trays. Cells were seeded in the 96-well trays at a density between 1,000 and 4,000 cells/well, depending on the cell size. The cells were left to plate down and grow for 24 h before adding each drug. The compound was diluted at 200× the final concentrations. The appropriate volume of the compound solution (usually 1 μl) was added automatically (Beckman FX 96 tip) to the medium to make it up to the final concentration for each drug. Each concentration was assayed in triplicate. Two sets of control wells were left on each plate, containing medium without drug or medium with the same concentration of DMSO. A third control set was obtained with the cells untreated just before adding the drugs (seeding control, number of cells starting the culture). Cells were exposed to the drugs for 96 h and then washed twice with phosphate buffered saline before being fixed with 1% glutaraldehyde. Cells were washed twice and fixed with crystal violet 0.5% for 30 min, then washed extensively, solubilized with 15% acetic acid, and absorbance measured at 595 nM.

Clonogenic assay. Trabectedin and doxorubicin were tested on six-well trays. Cells were seeded at a density of 1,000 cells/well. The cells were left to plate down and grow for 24 h before adding the drug. The medium was removed from the cells and replaced with 5 ml of medium dosed with drug. Each concentration was assayed in triplicate. The medium was changed every 3 days adding fresh drug. When colonies were observed in control untreated plates, cells were harvested, fixed with 0.5% glutaraldehyde and stained with crystal violet, and the colonies were counted.

Statistical analysis. Statistical analysis were performed using the SPSS (SPSS, Chicago IL) software package. Correlation was established by Student's T analysis. In all cases we show the statistical significance (P) of the correlation indicated.

RESULTS

Generation of Low Passaged Tumor Cell Lines

A total of 16 cell lines were directly established from surgical tumor tissue specimens from chemonaive sarcoma patients (Table I). Tumor origin of samples was confirmed by standard pathology analysis. Cell lines CNIO AW and CNIO AX were derived from the same primary tumor sample and isolated according to their different phenotypic features. We included four commercial cell lines to complement our panel, SW872 and 1455 (liposarcomas), A673 (Ewing's sarcoma), and Saos 2 (osteosarcoma).

To avoid undesired alterations in the newly generated cell lines due to the inherent genetic instability of the tumor, the lines were amplified up to passage 14–16 and cultures were frozen. Cell lines beyond passage 18 were not included in the analyses reported here. However, a control population of each cell line was kept in culture to ensure unrestricted proliferation capability. Cell lines SW872, 1455, A673, and Saos 2 are established cell lines [Richardson et al., 1996; Martinez-Ramirez et al., 2003] and therefore cannot be considered low passaged. Table I shows the tumor origin of each cell line, its morphological characteristics and its doubling time.

Response to Cytotoxic Drugs

We measured the response of the different cell lines to Trabectedin. Cells were cultured as indicated in Materials and Methods, and treated with different doses of Trabectedin over 4 days. Exposure time was based on the clinical pharmacokinetic profile; the recommended dose of 1.5 mg/m^2 given as 24-h infusion has an

TABLE I. Characteristics of the Cell Lines Generated in this Study and Their Response to ET-743 and Doxorubicin in 96-h and Clonogenic Assays

Cell line	Tumourorigin	Phenotype	Doubling time (h)	ET-743 (nM)		Doxorrubicin (nM)	
				96 h	Clonogenic	96 h	Clonogenic
CNIO AW	Liposarcoma	Fibroblastic	48 h	0.7	ND	45	ND
CNIO AX	Liposarcoma	Epithelial	48 h	0.7	0.09	44	28
SW872	Liposarcoma	Fibroblastic	24 h	0.5	0.05	>300	140
CNIO BK	Liposarcoma	Fibroblastic	96 h	9	0.3	>300	>300
1455	Liposarcoma	Fibroblastic	48 h	0.1	0.05	>300	27
CNIO AA	Leyomyosarcoma	Epithelial	36 h	0.4	0.05	21.5	12
CNIO AY	Leyomyosarcoma	Fibroblastic	48 h	9	0.1	44	55
CNIO AZ	Fibrous Tumor	Fibroblastic	96 h	5	0.06	14	37
CNIO BC	MPNST	Epithelial	96 h	>100	0.3	>300	109
CNIO BB	MPNST	Fibroblastic	72 h	>100	0.1	232	16
A673	Ewing sarcoma	Fibroblastic	24 h	1	0.1	50	52
CNIO BJ	Osteosarcoma	Epithelial	96 h	2	0.5	>300	30
CNIO BF	Osteosarcoma	Epithelial	24 h	0.3	0.07	15	14
CNIO BP	Osteosarcoma	Epithelial	48 h	0.3	0.2	>300	250
SAOS-2	Osteosarcoma	Epithelial	48 h	0.11	0.06	>300	24
CNIO BG	Mixoid fibrosarcoma	Fibroblastic	24 h	0.3	0.01	22	4
CNIO BM	Hibernoma	Fibroblastic	96 h	10	0.8	>300	>300
CNIO BN	Fibrohistiocitoma	Fibroblastic	72 h	>100	0.36	>300	>300
CNIO CE	Rabdomiosarcoma	Fibroblastic	48 h	>100	0.6	>300	>300
CNIO BI	GIST	Epithelial	24 h	0.1	0.09	50	25

average half-life in plasma of 89 h [Van Kesteren et al., 2000]. During exposure, the medium was changed twice, and fresh drug was added each time (Table I). Cells showed two well-differentiated responses to the treatment. One subset of cell lines was extremely sensitive to Trabectedin, showing an abrupt decrease in survival with $IC50 < 1$ nM, a concentration reachable in patients' plasma well below the recommended dose [Van Kesteren et al., 2000]. The second subset showed resistance to Trabectedin treatment with $IC50 > 1$ nM, including four cell lines where IC50 was not reached at 100 nM (Table I). The cell line A673 exhibited intermediate resistance to Trabectedin, with an IC50 of 1 nM. The differential sensitivity to Trabectedin was not related to the type of sarcoma.

In general, sensitivity to Trabectedin in the 96-h assay appears to correlate with shorter doubling time (Table I). Therefore, to avoid possible misinterpretations, we calculated the IC50 in clonogenic assays, where 1,000 cells were plated in a 2.5 cm well and cultured until individual clones appeared. Cells were treated with the same concentration of drugs as in the 96-h assay, and the medium was changed every 2 days. Cells that were resistant in the 96-h assay had an $IC50 > 0.1$ nM in the clonogenic assay (Table I). Similarly, cells that were sensitive in the 96-h assay had an $IC50 < 0.1$ nM in the clonogenic assay. A673 again showed intermediate sensitivity with an $IC50 = 0.1$ nM (Table I). Only two of 20 cell lines did not show correlation between the clonogenic and 96-h assays. The CNIO BP cell line showed higher resistance in the clonogenic assay, while the AZ cell line showed sensitivity in the clonogenic but not in the 96-h assay. The results in the BP cell line may have been due to the large number of long-time resistant clones, which could account for the high IC50 in the clonogenic assay. The inconsistent results in the AZ cell line may be attributable to the fact that Trabectedin toxicity takes more than 96 h to be observed. The IC50 values of the cell line A673 (1 nM in the 96-h assay; 0.1 nM in the clonogenic assay) were taken as the cut-off between extreme sensitive and resistant cell lines, both because these were the median values of IC50 of the cell line panel and because these values are clinically meaningful.

We also measured the response of the different cell lines to doxorubicin. Cells were cultured as for the Trabectedin assay and treated with different doses of doxorubicin in either 96-h or clonogenic assays (Table I). Cells showed three well-differentiated responses to the treatment. Approximately 50% of the cell lines were sensitive to doxorubicin, with similar IC50 values in both assays. A second group of cell lines were resistant to the drug, with

IC50 > 100 nM in both assays. Finally, a third group of cell lines showed sensitivity only in the clonogenic assay (1455, BB, BJ, and SAOS2) (Table I). In general, resistance to doxorubicin did not correlate with resistance to Trabectedin, suggesting different mechanisms of action for the two drugs.

Molecular Assessment

Cell lines were analyzed at the molecular level for mRNA expression of p53, p73, MSH2, and MLH1, DNA repair genes commonly altered in human tumors. The mutational status of p53 was analyzed by sequencing and then verified by Western blot. We analyzed p53 status by sequencing the mRNA in each cell line. We detected p53 mutations in 9 of 20 cell lines (Table II), while 2 of 20 cell lines showed deletions in one or two alleles of the gene. The 273H mutation was the most prevalent; however, we also detected one 251N mutation and one R175H mutation, and two instances of the R72P polymorphism (Table II). In most cases, we found both mutated and wild-type alleles, indicating that the mutation was produced in only one of the alleles. The CNIO BP cell line harbored the 175H mutation and the polymorphism R72P in one allele and wild-type p53 in the other allele. The CNIO BG cell line showed complete loss of one allele and deletion of two exons with loss of ORF in the other. Only wild-type transcripts were found in the remaining cell lines except in Saos2 where deletions were found. p53 protein was detected only in cell lines with mutated p53 (Fig. 1A) probably due to the stabilizing properties of the p53 inactivating mutations. The appearance of complex karyotypes appears to correlate with the presence of mutations or deletions of the p53 gene (Fig. 1A and Table II). We detected p73 mRNA loss in 11 of 20 cell lines (Fig. 1B). The loss of p73 did not correlate with either the presence of wild-type p53 or complex karyotypes. No loss of MLH1 or MSH2 was observed although decreased levels of MSH2 were found in CNIO AX and CNIO AZ cell lines (Fig. 1B).

We found a strong correlation between sensitivity to Trabectedin and p53 mutations ($P < 0.01$) (Table II). To confirm these findings, we analyzed isogenic cell lines differing only in the presence of wild-type p53. We treated HCT116 harboring wild-type p53 and HCT116 with deleted p53 by double homologous recombination with different doses of Trabectedin. Absence of p53 rendered cells more sensitive to Trabectedin (Fig. 2A). Analysis of the IC50 values in three independent experiments indicated that the absence of wild-type p53 led to a threefold increase in sensitivity to Trabectedin (Table III). Similar results were obtained with MCF7 a human breast tumor cell line carrying wild-type p53 and a derivative carrying a shRNA of p53 in three different experiments (Fig. 2B and Table IV). MCF7 with wild-type

TABLE II. Relationship Between Response to ET-743 and Molecular Alterations in p53, MSH2, MLH1, p73, and Karyotype

Cell line	Tumor origin	Et-743 response	P53 Mut	MSH2	MLH1	P73	Complex karyotype
CNIO AW	Liposarcoma	S	273H	+	+	+	+
CNIO AX	Liposarcoma	S	273H	+/–	+	+	+
SW872	Liposarcoma	S	251N	+	+	–	+
1455	Liposarcoma	S	R72P	+	+	–	ND
CNIO AA	Leiomyosarcoma	S	273H	+	+	+	+
CNIO BF	Osteosarcoma	S	273H	+	+	+	+
CNIO BP	Osteosarcoma	S	R72P, 175H	+	+	–	+
SAOS-2	Osteosarcoma	S	Del	+	+	+	+
CNIO BG	Mixoid Fibrosarcoma	S	Del	+	+	+	+
CNIO BI	GIST	S	273H	+	+	+	+
A673	Ewing's Sarcoma	C-O	WT	+	+	+	+
CNIO AZ	Fibrous tumor	R/S	WT	+/–	+	–	+
CNIO BK	Liposarcoma	R	WT	+	+	–	+
CNIO AY	Leiomyosarcoma	R	WT	+	+	–	–
CNIO BC	MPNST	R	WT	+	+	+	–
CNIO BB	MPNST	R	WT	+	+	+	–
CNIO BJ	Osteosarcoma	R	WT	+	+	–	ND
CNIO BM	Hibernoma	R	WT	+	+	–	ND
CNIO BN	Fibrohistocitioma	R	WT	+	+	–	ND
CNIO CE	Rabdomyosarcoma	R	WT	+	+	–	–

S, Sensitive; R, resistant; C-O, cut-off values; ND, Not determined; WT, wild type.

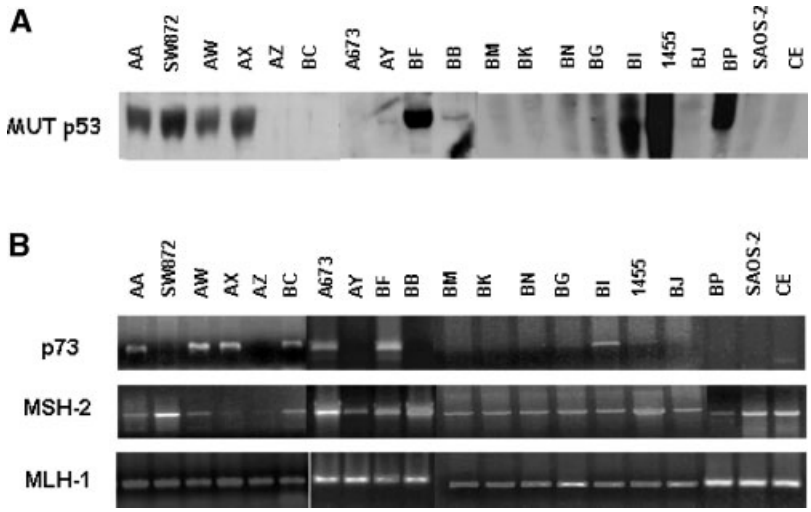

Fig. 1. **A**: Analysis of the levels of p53 in the cell lines. **B**: mRNA expression analysis by RT-PCR of p73, MSH2, MLH1 in the cell lines used in this study.

p53 were resistant to Trabectedin treatment whereas MCF7 with constitutive expression of p53 shRNA were highly sensitive to Trabectedin.

To explore whether DNA-damage induced by Trabectedin was responsible for some p53-derived resistance, we treated HCT116 cells with Trabectedin at IC50 concentrations during 18 h. Then we measured the response to Trabectedin by analyzing the activation of genes that might contribute to resistance to apoptosis (Fig. 3). We observed that the compound induces a DNA damage like response by accumulating p53 in the p53 wild-type HCT116 cells. However, no induction of p21 or bax was observed. Moreover, Trabectedin alto triggered an unexpected activation of the MEK pathway by increasing the phosphorylation of MAPK which might act as survival signal. Even more surprising is the specific activation of MEK by Trabectedin only in the cells carrying wild-type p53, indicating that this is a p53-dependent response.

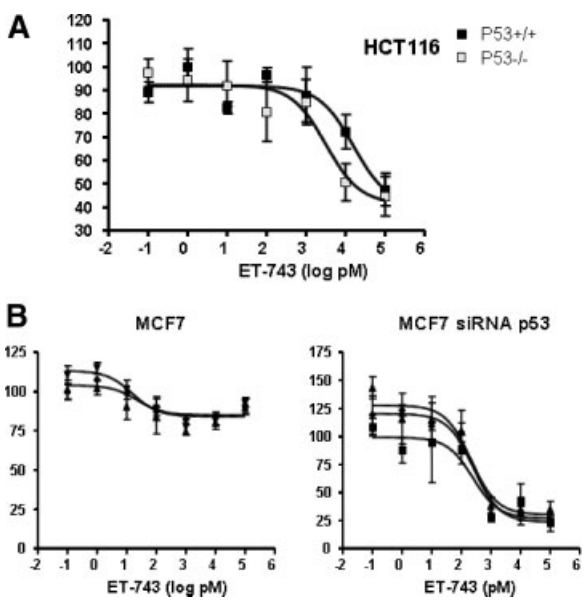

Fig. 2. Effect of ET-743 in (**A**) HCT116 (■) and HCT116 (p53 null) (□) cell lines, or (**B**) MCF7 (left) and MCF7-p53siRNA (p53 null) (right) cell lines. Cells were treated at different concentrations of ET-743 for 96 h. Graphs represent the growth of each cell line relative to cells treated only with carrier (100%). Each curve is the average of two independent experiments performed in triplicate samples. Three independent curves are represented in each graph.

DISCUSSION

We generated and analyzed cell lines derived from biopsies of chemonaive soft tissue sarcoma patients and assessed response to Trabectedin

TABLE III. IC50 of ET-743 in HCT116 and HCT116 (p53 null) Cell Lines in Three Independent Experiments

	IC50 (nM)		
HCT116	Expt 1	Expt 2	Expt3
p53 wt	16.63	11.30	15.89
P53 null	4.92	3.22	5.05

Each experiment is the average of two independent curves performed in triplicate samples. Cells were treated at different concentrations of ET-743 for 96 h. IC50 values were calculated using the PRISM program.

TABLE IV. IC50 of ET-743 in MCF/ and MCF7p53siRNA (p53 Null by p53siRNA Expression) Cell Lines in three Independent Experiments

	IC50 (nM)		
MCF7	Expt 1	Expt 2	Expt 3
P53 wt	>100	>100	>100
P53 siRNA	0.24	0.21	0.25

Each experiment is the average of two independent curves performed in triplicate samples. Cells were treated at different concentrations of ET-743 for 96 h. IC50 values were calculated using the PRISM program.

and to doxorubicin. We also examined the correlation between different molecular alterations and Trabectedin sensitivity. In general, patterns of sensitivity and resistance were similar in the clonogenic and 96-h assays (Table I). Response to Trabectedin did not correlate with response to doxorubicin, indicating that the two drugs have different mechanisms of resistance. These findings are along the lines of those of Li et al. [2000b] and Izbicka et al. [1998] showing an absence of cross-resistance between Trabectedin and doxorubicin. Moreover, long-lasting responses to Trabectedin have been reported in advanced sarcoma patients who are resistant to doxorubicin [Lecesne et al., 2003].

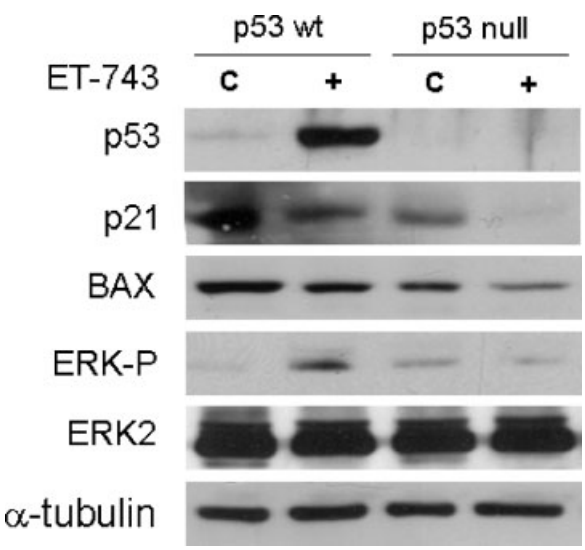

Fig. 3. Effect of ET-743 in HCT116 (p53 wt) and HCT116 (p53 null) (p53 null) cell lines. Cells were treated at IC50 concentrations of ET-743 for 18 h, then harvested and total protein extracted and PAGE run. Protein was determined by Western blot as indicated in the Materials and Methods.

Half of the cell lines harbored p53 mutations. p53 mutations and deletions were associated with large karyotypic abnormalities, which can be explained by the established relationship between absence of p53 function and genomic instability [Overholtzer et al., 2003]. A strong correlation was also seen between extreme sensitivity to Trabectedin (IC50 < 1 nM) and p53 mutations, but no correlation was found with any other molecular marker. Inactivation of p53 by mutation or truncation is one of the most common molecular alterations in soft tissue sarcoma, occurring in 30%–50% of tumors [Toguchida et al., 1992]. Overexpression of mutated p53 by immunohistochemistry is more frequent in high grade and metastatic tumors than in low grade and primary sarcomas [Latres et al., 1994]. In addition, patients with p53-mutated soft tissue sarcoma have worse clinical outcome and significantly lower survival than patients having wild-type p53 tumors [Drobnjak et al., 1994]. We found a significant association between the direct alteration of p53 and extreme in vitro sensitivity to Trabectedin ($P < 0.01$).

Bcl-2 overexpression and p53 mutations have recently been reported to be involved in drug resistance in soft tissue sarcoma [Fayette and Blay, 2005]. However, other studies [Pakos et al., 2004; Wunder et al., 2005] failed to find a correlation between p53 mutations and outcome in osteosarcoma patients. In soft tissue sarcoma [Zhan et al., 2001], wild-type p53 sensitizes cells to doxorubicin by downregulating the expression of multidrug-resistance-1 (MDR-1) which is involved in the efflux of chemotherapeutic agents.

The functional analysis of isogenic cell lines showed that the absence of wild-type p53 response increased sensitivity to Trabectedin. Therefore, restoration of wild-type p53 function should lead to an increased resistance to Trabectedin, comparable to the increased sensitivity to cisplatin and doxorubicin found in pediatric sarcoma cells treated with adenovirus-mediated wild-type p53 [Ganjavi et al., 2005]. However, Erba et al. [2001], using wild-type p53 nullizygous mouse embryonic fibroblasts in a pair of cell lines carrying wild-type p53 or the val143ala p53 mutant, found similar sensitivity to Trabectedin in the two cell lines. The cause for these different results is not clear, although Erba et al. [2001] reported that SW620 cells, which do not express wild-type p53, were

more sensitive to Trabectedin than LoVo cells, which carry wild-type p53. Furthermore, most rodent tissues are deficient in p53-regulated NER [Hanawalt, 2002].

At pharmacological concentrations, Trabectedin generates DNA adducts that distort the DNA structure and are repaired by the transcription-coupled nucleotide excision repair (TC-NER) pathway [Takebayashi et al., 2001]. During this process, single strand breaks (SSB) are generated that trigger the SSB-repair machinery, involving PPAR-1, and the base excision repair (BER) mechanism, at least in part facilitated by p53. In fact, p53 protein levels sharply increase in cells treated with Trabectedin [Erba et al., 2001; Martínez et al., 2001]. In cell lines with mutated p53 protein, SSB generated by TC-NER are not sensed and repaired, and p53-independent apoptotic mechanisms lead to cell death. In contrast, in cells with wild-type p53, SSB are sensed and properly repaired, thus reducing the apoptotic response to Trabectedin treatment. The extent of the DNA damage may determine whether the cell goes to cell-cycle arrest and repairs the DNA or to p53-independent apoptosis. In a recent study, the Trabectedin gene expression signature identified a group of genes related to cell-cycle control, stress, and DNA-damage response that were upregulated in all the cell lines studied [Martınez et al., 2005]. The molecular signature after Trabectedin administration was analyzed at baseline and at four different times after ET-743 exposure. Gene expression profile analysis revealed upregulation of 86 genes and downregulation 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 upregulated in all the cell lines studied. The transcriptional signature 72 h 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 [Martınez et al., 2005]. As previously suggested, Trabectedin transcriptional signature seems to be related to DNA-damage pathway activation. Our experiments in HCT116 with p53 wild-type confirms that Trabectedin induces the accumulation of p53, similar to a DNA-damage response. However, we did not observed bax nor p21 induction by this p53 accumulation. It is possible that the specific transcriptional repression documented for Trabectedin [Minuzzo et al., 2000, 2005] is the responsible for this apparently contradictory result.

Trabectedin was more effective in cells lacking DNA-PK; moreover, pre-treatment of HCT-116 colon carcinoma cells with wortmannin, a potent inhibitor of DNA-PK, sensitized cells to Trabectedin. An increase in Trabectedin sensitivity was also observed in ataxia talangiectasia-mutated cells [Damia et al., 2001]. Both proteins and ATM are upstream sensors of DNA damage in the p53 pathway [EI-Deiry, 2003]. Trabectedin may elicit a DNA damage-like response, and alteration in the proteins regulating that response (p53, DNA-PK, ATM, NER) might modulate response to Trabectedin. However, p53 appears not to be the only determinant of Trabectedin sensitivity or resistance, since in this pathway many other molecules are involved. We have also observed that Trabectedin triggers an unexpected activation of the MEK pathway by increasing the phosphorylation of MAPK and this seems to be a p53-dependent response. Although at this point we do not fully understand the relationship between p53 and MEK activation, it fully justify the increased sensitivity found in p53 mutant cells, since MEK pathway activation might enhances survival [Ballif and Blenis, 2001; Torii et al., 2004]. The mechanism of action of Trabectedin merits further investigation to clarify the role of p53 and AKT.

Our findings show a clear and significant relationship between p53 mutations and extreme sensitivity to Trabectedin in low passaged cell lines at a concentration easily reachable in patients well below the recommended dose, providing a useful tool to assess response/resistance to Trabectedin in patients. Cell lines are easy to handle and allow testing of predictive hypothesis. However, initial observations in vitro must be validated in vivo. Therefore, more research is needed to assess the validity of p53 as marker for Trabectedin extreme sensitivity in humans. Retrospective studies are currently ongoing to determine the p53 mutational and expression status in tumor samples and correlate results with clinical outcome in soft tissue sarcoma patients treated with Trabectedin.

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