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Characterization of a new trabectedin-resistant myxoid liposarcoma cell line that shows collateral sensitivity to methylating agents

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Myxoid Liposarcomas (MLS), characterized by the expression of FUS-CHOP fusion gene are clinically very sensitive to the DNA binding antitumor agent, trabectedin. However, resistance eventually occurs, preventing disease eradication. To investigate the mechanisms of resistance, a trabectedin resistant cell line, 402-91/ET, was developed. The resistance to trabectedin was not related to the expression of MDR related proteins, uptake/efflux of trabectedin or GSH levels that were similar in parental and resistant cells. The 402-91/ET cells were hypersensitive to UV light because of a nucleotide excision repair defect: XPG complementation decreased sensitivity to UV rays, but only partially to trabectedin. 402-91/ET cells showed collateral sensitivity to temozolomide due to the lack of O^6-methylguanine-DNA-methyltransferase (MGMT) activity, related to the hypermethylation of MGMT promoter. In 402-91 cells chromatin immunoprecipitation (ChIP) assays showed that FUS-CHOP was bound to the PTX3 and FN1 gene promoters, as previously described, and trabectedin caused FUS-CHOP detachment from DNA. Here we report that, in contrast, in 402-91/ET cells, FUS-CHOP was not bound to these promoters. Differences in the modulation of transcription of genes involved in different pathways including signal transduction, apoptosis and stress response between the two cell lines were found. Trabectedin activates the transcription of genes involved in the adipogenic-program such as c/EBPα and β, in 402-91 but not in 402-91/ET cell lines. The collateral sensitivity of 402-91/ET to temozolomide provides the rationale to investigate the potential use of methylating agents in MLS patients resistant to trabectedin.

Trabectedin is a marine alkaloid isolated from the tunicate *Ecteinascidia turbinata* that has shown striking antitumor activity in a variety of preclinical models, including some that are insensitive to conventional chemotherapeutics.[1–5] Clinical investigations have shown that trabectedin is effective in soft tissue sarcomas,[6–12] and in several other human malignancies, and it was approved by EMEA in 2007 for second line therapy of soft tissue sarcoma and in 2009 for second line therapy of ovarian cancer patients.[13–17]

Key words: myxoid liposarcoma, trabectedin, resistance, FUS-CHOP
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Trabectedin binds to the N_2 of guanine in the minor groove of DNA, causing bending of the minor groove towards the major groove.[18–21]

Trabectedin affects transcription regulation in a promoter and gene-specific manner,[22,23] but the mechanisms underlining these effects are not elucidated. A peculiar aspect of trabectedin's mode of action is related to the fact that cells that are defective in transcription-coupled nucleotide excision repair (TC-NER), and are hypersensitive to UV light, are partially resistant to the drug.[24–28] It has therefore been hypothesized that in the presence of trabectedin, NER does not lead restoration of normal DNA function, but instead

enhances drug cytotoxicity. Defects of homologous recombination were instead associated with high sensitivity to trabectedin.[29,30]

Recently, an extraordinary sensitivity of myxoid liposarcoma (MLS) to trabectedin has been reported, with a rate of objective responses greater than 50% in patients who were resistant to primary chemotherapy with Doxorubicin and alkylating agents.[9,10]

The mechanism of sensitivity appears to be related to the ability of trabectedin to block the transactivating function of the fusion gene product (FUS-CHOP),[31] that represent the pathogenic lesion of this particular type of liposarcoma. However, after a response to trabectedin that can last for several months, patients become resistant to the drug and no information is available on the mechanisms leading to resistance of this exquisitely sensitive tumor.

In this study we derived, for the first time, a trabectedin-resistant cell line from the myxoid liposarcoma 402-91 cell line previously described for its sensitivity to trabectedin.[31,32] To better characterize the resistant cell line, both cellular and molecular approaches were used; we also investigated the pattern of sensitivity to other drugs and tried to explained the possible mechanisms involved in the resistance to trabectedin.

Material and Methods

Cells

To generate a trabectedin-resistant cell line, we exposed the MLS 402-91 cells (kindly supplied by Dr P. Aman)[33] to a stepwise increase in drug concentration using a short (1 hr) exposure, applied at 10–20 day intervals over 14 months, during which time the concentration of trabectedin was increased by a factor of 1.5–2. The resistant line was termed 402-91/ET: resistance was verified by drug sensitivity (clonogenic) assay every 6 months and was stable over 24 months in the absence of the compound.

Drugs

Trabectedin was kindly provided by PharmaMar, S.A. Tres Cantos, Spain. Melphalan, cis-diammineplatinum (II) dichloride (Cis-DDP), etoposide (VP16), campothecin (CPT), doxorubicin, temozolomide and *N*-acetyl-L-cysteine (NAC) were purchased Sigma Aldrich, (St. Louis). All drugs were diluted in RPMI 1640 medium just before use.

Clonogenicity assay

The 402-91 and 402-91/ET cells were seeded in 6 well-plates (IWAKI, Bibby Sterilin, Staffordshire, UK) at concentration of 700 cells ml^{-1}. The clonogenic assay was performed as previously described.[30]

Flow cytometric cell cycle analysis

DNA content and cell cycle were evaluated by standard flow cytometric methods.[25]

Fluorescence in situ hybridization (FISH) analyses

Exponentially growing cells were used for metaphase preparations, after exposure to colchicine (Sigma Aldrich) for 4–5 hr. For FISH bacterial artificial chromosome (BAC) clones RP11-196G11 (FUS gene) and RP11-181L23 (CHOP gene), were used to prepare probes for FISH analysis according to standard procedures.[34]

Microarray experiment and data analysis

Gene expression was analyzed using Agilent slides on which 44,000 oligonucleotides were spotted (Agilent Technologies, Santa Clara, USA). Experiments were performed in five replicates, with dye-swappings, using protocols and methods previously standardized and published.[35] The arrays were washed and scanned with a laser confocal scanner (G2565BA, Agilent Technologies) according to manufacturer's instructions. miRNA microarrays underwent standard post hybridization processing and the intensities of fluorescence were calculated by Feature Extraction software v.11 (Agilent Technologies). The microarray raw data is deposited in GEO (http://www.ncbi.nlm.nih.gov/geo, experiment number: GSE20350) according to MIAME (minimum information about a microarray experiment) guidelines. Data were analyzed using R-Cran 2.9 with Bioconductor and in particular using the Limma statistical package for a customized script microarray analysis, as previously described.[35] Pathways analysis was performed using "Metacore" software, a commercially available online tool (https://portal.genego.com).

RNA purification and semi-quantitative and quantitative RT-PCR

Total RNA was purified using the SV-total RNA kit according to the manufacturer's instructions (Promega, Milan, Italy). Reverse-transcription to cDNA was performed using a High Capacity cDNA Archive Kit starting from 1 μg of total RNA following the manufacturer's instructions (Applied Biosystem, Foster City, USA). Primer pairs are listed in Table 1. Differences in gene expression were determined by real time RT-PCR (ABI-7900, Applied Biosystems) using Sybr Green (Qiagen, Milan, Italy) or TaqMan probes (Applied Biosystems). Analysis was performed using the 2^{-DDCT} method.[36]

Western blot analysis

Whole protein extracts were obtained by using a lysis buffer containing 10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Nonidet NP-40, 5 mM EDTA, 50 mM NaF in the presence of protease inhibitors (Roche, Basel CH). For cytoplasmic and nuclear fractions, whole cells were prepared in buffer A (10 mM Hepes pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF) containing protease inhibitors (Roche) and nuclear extractions were obtained using Buffer C (20 mM Hepes pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF).

Protein was quantified using the Bradford assay reagent (Bio-Rad Laboratories, Melville, USA), separated on a SDS

Table 1. List of primer pair sequences, Genebank accession number and annealing temperature (T_a) of genes analysed by real time RT-PCR

Gene name	GenBank acc. number	Sense	Antisense	T_a (°C)
TNFrsf25	NM_148965	CGTCGGAGGGCTATGGAG	TTCAGGTAGTGCCCCGC	60
IAP2	NM_001166	AACAATTGACATGTGTGCTTCC	TGGTTAAAGGAAATGCTGCG	60
IAP3	NM_182962.1	ATCCTGGGCAGCATACTGAG	CATTTTAGGGACATGGTGTTTTT	60
HSP27	NM_001540	AAGTTTCCTCCTCCCTGTCC	GAGACTGCCGCCAAGTAAAG	60
FOSL	NM_005438	GCCCACTGTTTCTCTTGAGC	AATCCTGGCAGCCCAGAG	60
HSP70	NM_005345	GGGCCTTTCCAAGATTGCTG	TTCAACATTGCAAACACAGGAAA	60
PTX3	NM_002852	ATGATTCCCTGTGGGACAAG	CCACTACCCCTTCCAGGACT	60
YAP65	NM_006106	CCGTTTCCCAGACTACCTTG	ATGGAGTCTGTTTTGGCTGC	60
IGFBP3	NM_001013398	CCAGCTCCAGGTGAGCC	CGGCAGGGACCATATTCTGT	60
TRIB3	NM_021158.3	ATGATTCCCTGTGGGACAAG	AGTCCTGGAAGGGGTAGTGG	60
HMGA2	NM_003483	GCAAGTTAGGTATGTTTGCAGGA	TTTGAATCGCTTGCTTGTTG	60
CSF3	NM_172220	CTCAGGTGGTAGGGAACAGC	TTGTAGGTGGCACACAGCTT	60
14-3-3e	XR_041291	CGCTGAAATGTTGCTGAAAA	TGTTTCTTCTCCTTCCCCCT	60
FUS-CHOP	NM_004960(FUS); NM_004083(CHOP)	TGGCTATGAACCCAGAGGTC	TCCCGAAGGAGAAAGGCAAT	52
XPG unmeth.	NM_000123	GTGTTGTTTTTTATTGGAAAATTGT	AACACTTCAAACTAACCTACCACC-	52
XPG met	NM_000123	TAGTGTCGTTTTTTATTGGAAAATC	GCTTCGAAACTAACCTACCG	52
MGMT unmeth.	NM_002412	TTTGTGTTTTGATGTTTGTAGGTTTTTGT	AACTCCACACTCTTCCAAAAACAAAACA	59
MGMT met.	NM_002412	TTTCGACGTTCGTAGGTTTTCGC	GCACTCTTCCGAAAACGAAACG	59
Calponin	NM_001299	GGAAGGTAGTTGAGGTTGTG	CCCAAACTCAAAACTCTAACCTAAC	63
28S	NR_003287	TTGAAAATCCGGGGGAGAG	ACATTGTTCCAACATGCCAG	60
GAPDH	NM_002046	GGACTCATGACCACAGTCCAT	GCCATCACGCCACAGTTT	60
Cyclophillin A	NM_021130	GCGTCTCCTTTGAGCTGTTT	GTCTTGGCAGTGCAGATGAA	60
Actin B	XR_019170	CACCCACACTGTGCCCATCTA	CAGCGGAACCGCTCATTGCCAATGG	60
Chip analysis				
PTX3	NM_002852	CCCACCAAATTCAGGGGAACT	GCATTGCTGGAGAGACGCAAA	63
FN1	NC_000002.11	CTTCGCTTCACACAAGTCCA	GCAGCGAACAAAAGAGATGC	63
SATCEN1		GGCGACCAATAGCCAAAAAGTGAG	CAATTATCCCTTCGGGGAATCGG	63

PAGE gel, transferred to a PVDF membrane (Immobilon, Millipore, Billerica, USA) and probed with the primary antibodies to XPG (Ab-1 (8H7) NeoMarkers, Westinghouse DriveFremont, USA), NF-YB (Diagenode, Liège, Belgium), MLH1 (C20, Santa Cruz Biotechnology, Santa Cruz, USA), XPA (FL273, Santa Cruz Biotechnology), ERCC1 (FL297, Santa Cruz Biotechnology), β-tubulin (H235, Santa Cruz Biotechnology) and GADD 153 (F-168, Santa Cruz Biotechnology).

Binding was detected using peroxidase labelled secondary antibodies and visualised using a chemiluminescence kit (Amersham, Milan, Italy).

Flow cytometric analyses of multidrug-related proteins

The expression of the multidrug-related protein, P-glycoprotein MDR-1, multidrug resistance-associated protein, MRP-1 and MRP-2 and lung resistance-related protein LRP-1, was evaluated by flow cytometric assay.[37]

HPLC-MS/MS method for the determination of trabectedin in cells

Trabectedin was quantified in cell extracts by a liquid chromatography tandem mass spectrometry (HPLC-MS/MS) method (Series 200, Perkin Elmer, Waltham; Applied Biosystems, API 4000, Carlsbad, CA), based on that of Rosing *et al.*[38]

HPLC determination of free cysteine and various forms of GSH

For free cysteine and free glutathione (GSH) determination, the cell lysates were deproteinized by treatment with 12% sulfosalicylic acid (2:1 vol/vol) and the analyte content determined in the acid-soluble fraction. Protein bound glutathione (GS-Pro) was determined in the protein pellet dissolved in 0.1 M NaOH. For GSSG determination, the cells were sonicated in the presence of 5 mM NEM and the analyte content was determined in cell lysates as described above. Protein

concentration was quantified by BCA-protein assay (BCA-protein assay, Pierce, Rockford, IL). Derivatization and chromatography procedures were performed essentially as previously reported.[39]

Mutational status of FUS-CHOP and XPG genes

FUS-CHOP mutational status was analyzed using primers pairs and conditions shown in Table 1. Amplification was performed with 33 cycles of 95°C for 1 min, 60°C for 30 sec and 72°C for 1 min with a proofreading Taq DNA polymerase (LA-Takara, Cambrex, Milan, Italy). XPG exon amplification used genomic DNA purified by the Maxwell 16 cell DNA purification kit (Promega) and using primer pairs spanning all the coding exons of *XPG* (Table 1). PCR involved incubation at 95°C for 2 min, followed by 35 cycles of 95°C for 30 sec, 54°C for 30 sec and 72°C for 1 min. Finally there was incubation at 72°C for 5 min. PCR products were separated on 1.5% agarose gels and visualized after ethidium bromide (SIGMA Aldrich) staining. Sequencing was performed at a local facility (http://www.primm.it) and sequence analysis was performed using ClustalW software (http://www.ebi.ac.uk/clustalw/).

MGMT activity determination

Oligonucleotide (2.5 pmoles, DNA Technology, Denmark) containing O^6meG within a PstI recognition site was radiolabeled with gamma ^{32}P-ATP (40 μCi, 6,000 Ci mmol^{-1}, GE Healthcare) by incubation with T4 polynucleotide kinase (Roche) for 30 min at 37°C. Complementary oligonucleotide (4 pmoles, SIGMA Aldrichs) containing a 5' biotin was annealed to the radiolabeled strand then diluted in PBS/0.5% BSA (SIGMA Aldrichs), aliquots [100 μl (~6 fmoles)] were added to each well of a preblocked (with PBS/0.5% BSA) streptavidin coated 96 well plate (biobind assembly 96 well plate, Thermo, Waltham, USA), incubated overnight at 4°C then washed three times with 400 μl PBS.

Cell-free sonicates were prepared according to Watson and Margison[40] and DNA concentration measured using a Quanti-it Picogreen dsDNA quantification kit (Molecular Probes, Invitrogen). Sonicate and standard samples [with known O^6 methylguanine-DNA-methyltransferase (MGMT) content] were diluted in 50 mM Tris-HCl, 1 mM EDTA, 3 mM dithiothreitol containing 1 mg ml^{-1} BSA to give a range of DNA concentrations, transferred into the oligo-bound plates and incubated for 4 hr at 37°C. Plates were then washed as above and 100 μl of PstI digestion mix (10 U/100 μL, New England BioLabs, Ipswich, UK) added to each well followed by incubation for 1 hr at 37°C. The supernatant was transferred onto a LumaPlate (Packard, Australia), dried overnight at 37°C and radioactivity quantified in a TOP-COUNT machine (Perkin Elmer, Waltham, USA). MGMT specific activity was expressed as Fmoles MGMT/μg DNA.

Methylation analysis

Genomic DNA was modified with sodium bisulfite using the Epitect Bisulfite kit (Qiagen). Methylation-specific PCR (MSP) amplification was carried out in a total volume of 25 μl using AmpliTaq Gold (Applied Biosystems). MSP products were separated on 2% agarose gels and visualized after ethidium bromide staining. To verify successful bisulfite modification of the DNA, a region of the calponin gene promoter was amplified alongside each modified DNA sample. XPG, MGMT and calponin primer pair sequences with their annealing temperatures are presented in Table 1.

Chromatin immunoprecipitation (ChIP)

ChIP assays were performed as previously described[41] with the following antibodies: NF-YB (Pab001,Gene Spin srl, Milan, ItalyDiagenode, B), CHOP (F-168, Santa Cruz), FUS (A300-302, Bethyl Laboratories, Edison, NJ) and Flag control antibody, clone F7425 (Sigma Aldrich). The immunoprecipitated DNAs were analyzed by quantitative Real Time PCR (primer pair sequences shown in Table 1—ChIP analysis). Values are reported as fold enrichment over the control antibody.

Data analysis

The results are expressed as mean ± SD. Differences were analyzed by Student's *t* test for unpaired observations and two-way ANOVA complemented with Bonferroni-post test.

Results

Characterization and differential effects of trabectedin in 402-91 and 402-91/ET cell lines

Exposure of the parental cell line 402-91 to stepwise increasing concentrations of trabectedin generated a resistant cell line, 402-91/ET, for which the trabectedin IC_{50} was 50 nM, *i.e.*, a ten-fold increased resistance compared to the parental cells; the resistance was irreversible over at least 2 years of continuous culture. Parental and 402-91/ET cell lines did not differ with respect to doubling times (~24 hr for both).

Because the extraordinary sensitivity of MLS to trabectedin appears to be related to the expression of the FUS-CHOP fusion gene,[9,10] we examined if the *in vitro* selection of the 402-91/ET cell line altered FUS-CHOP expression, compared to the parental cell line. RT-PCR with primers able to discriminate between the different FUS-CHOP isoforms, showed that both cell lines expressed the same Type I splicing variant. The presence of FUS-CHOP chimera in both cell lines was also detected by FISH analysis (Fig. 1*a*) which also demonstrated that 402-91/ET cells have a higher chromosome number than the parental cells. The sequence of the FUS-CHOP cDNA was wild type in both 402-91 and 402-91/ET cells, suggesting that *in vitro* selection of trabectedin resistant cells did not mutate the FUS-CHOP coding sequence (data not shown).

The possibility that 1-hr trabectedin treatment might modulate chimera expression differently in 402-91 and 402-91/ET cells was assessed by RT-PCR and western blot analysis performed at different times after drug-washout. Figure 1*b* shows that, with the exception of 10 nM trabectedin at 6 hr

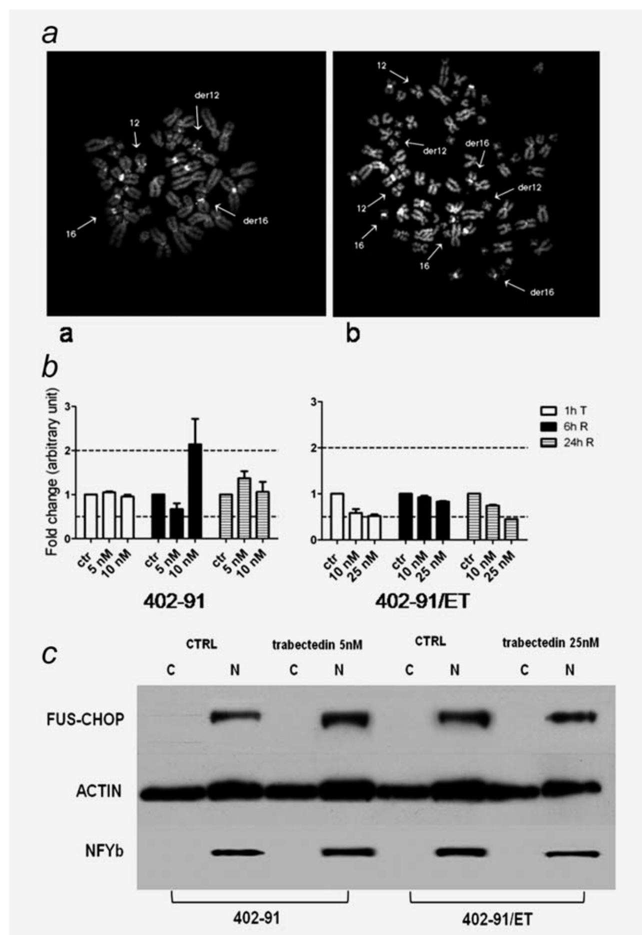


Figure 1. (*a*) FISH analysis of 402-91 (*a*) and 402-91/ET (*b*) metaphase cells using the BAC probe RP11-196G11 for the FUS gene and RP11-181L23 for the CHOP gene. The FUS-CHOP chimera was revealed by the presence of the two probes in derivative Chromosome 12. This analysis also showed that the 402-91/ET cell line had a significant increase in chromosome number compared with the parental cell line. (*b*) FUS-CHOP Real time RT-PCR analysis performed on 402-91 and 402-91/ET cell mRNA at different times of recovery in drug free medium after 1 hr of treatment with different doses of trabectedin. Data were analysed by the DDCT method and expressed as fold changes (arbitrary unit) compared to their untreated control, set as 1. Data are the mean of three independent experiments performed in triplicate; bars represent S.D. (*c*) Western blot analysis of 402-91 and 402-91/ET cell lines carrying the translocation *t*(12; 16) (q13; p11), using anti- CHOP antibodies (GAD153). The cells were harvested after 1 hr of treatment with the indicated concentrations of trabectedin. NF-YB was used as nuclear loading marker for data normalization. C = cytosol; N = nuclear.

after drug-washout in 402-91 cells, the FUS-CHOP chimera expression levels were closely similar in the two cell lines and to the control cells.

In vitro selection of 402-91/ET cells did not alter the FUS-CHOP subcellular localization: western blots (panel C) showed that the FUS-CHOP protein was in the nuclear fraction in both 402-91 and 402-91/ET treated and untreated cells at 72 hr after drug-washout. These blots also indicated that FUS-CHOP protein levels were comparable between untreated 402-91 and 402-91/ET cells, excluding the possibility that resistance could be explained by differences in chimeric protein expression.

To better characterize the resistant cell line we investigated the common mechanisms of drug resistance. The resistance to trabectedin was not due to the different expression of multi-drug resistance related proteins, such as MDR-1, LRP-1, MRP-1 and MRP-2, or to the trabectedin uptake and retention in 402-91/ET cell line (Supporting Information Figs. 1*a* and 1*b*).

We evaluated the total GSH content showing a weak, non significant, difference between the two cell lines: 62.5 $\pm$ 7.7 nmol mg^{-1} protein in 402-91 and 75.1 $\pm$ 1.3 nmol mg^{-1} protein in 402-91/ET cells. Instead the cysteine content was significantly different (*t* test, $p < 0.05$) in the two cell lines, being 2.6 and 9.36 nmol mg^{-1} protein in parental and resistant cell lines, respectively. However, inducing an increase in cysteine level by 24-hr pretreatment with non toxic concentration of 10 mM NAC (*N*-acetyl-L-cysteine), no decrease of the sensitivity of 402-91 cell line to trabectedin was found (data not shown). Taking together these data suggest that the resistance to trabectedin of 402-91/ET cells was not related to both GSH and cysteine content.

Cross resistance pattern

We compared the sensitivity of the 402-91 and 402-91/ET cell lines to a panel of DNA-damaging anticancer drugs to determine cross-resistance. As shown in Figure 2, 402-91/ET cells were found to be more sensitive to UV rays (very substantially) and to temozolomide (at high doses), and slightly less sensitive to Melphalan, but with other agents there were no or only slight differences.

DNA repair in 402-91 and 402-91/ET cells

The collateral sensitivity to UV light prompted us to investigate if there were differences in the expression of DNA repair genes. We used RT-PCR to assess the expression of genes involved in base excision repair (BER), nucleotide excision repair (NER), Fanconi anemia (FA) and translesion repair (polEta). All repair genes were similarly expressed in both the cell lines or over-expressed in the 402-91/ET cells with the exception of XPG, which was substantially downregulated in the resistant cell line (Fig. 3*a*). Western blots confirmed no detectable XPG expression in the 402-91/ET cells, but maintained expression of all of the other tested proteins (Supporting Information Fig. 2*a*).

Because epigenetic modification of gene promoter regions has been shown to influence transcription, we examined if hypermethylation in the XPG promoter region could explain the lack of its expression. Both methylated and unmethylated bands were seen in the parental cell line, but we were not

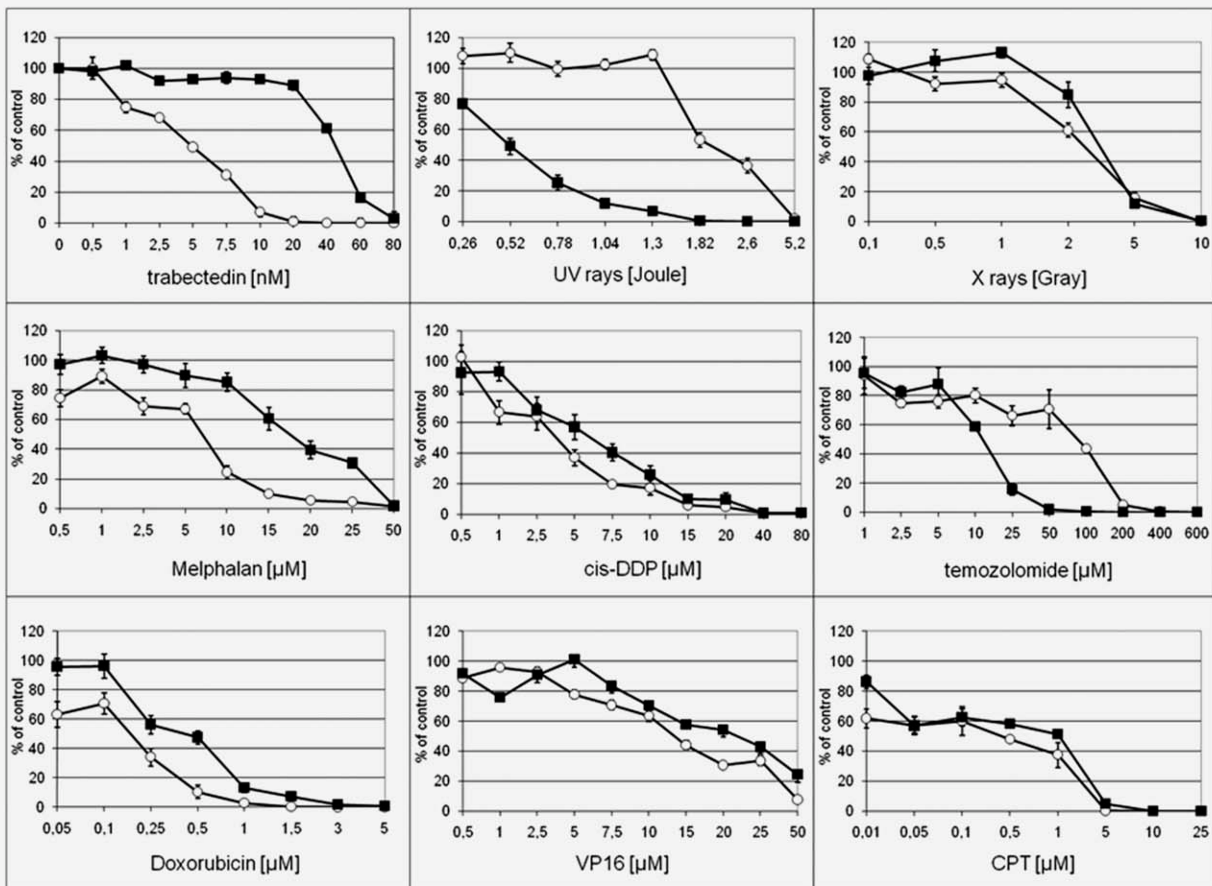
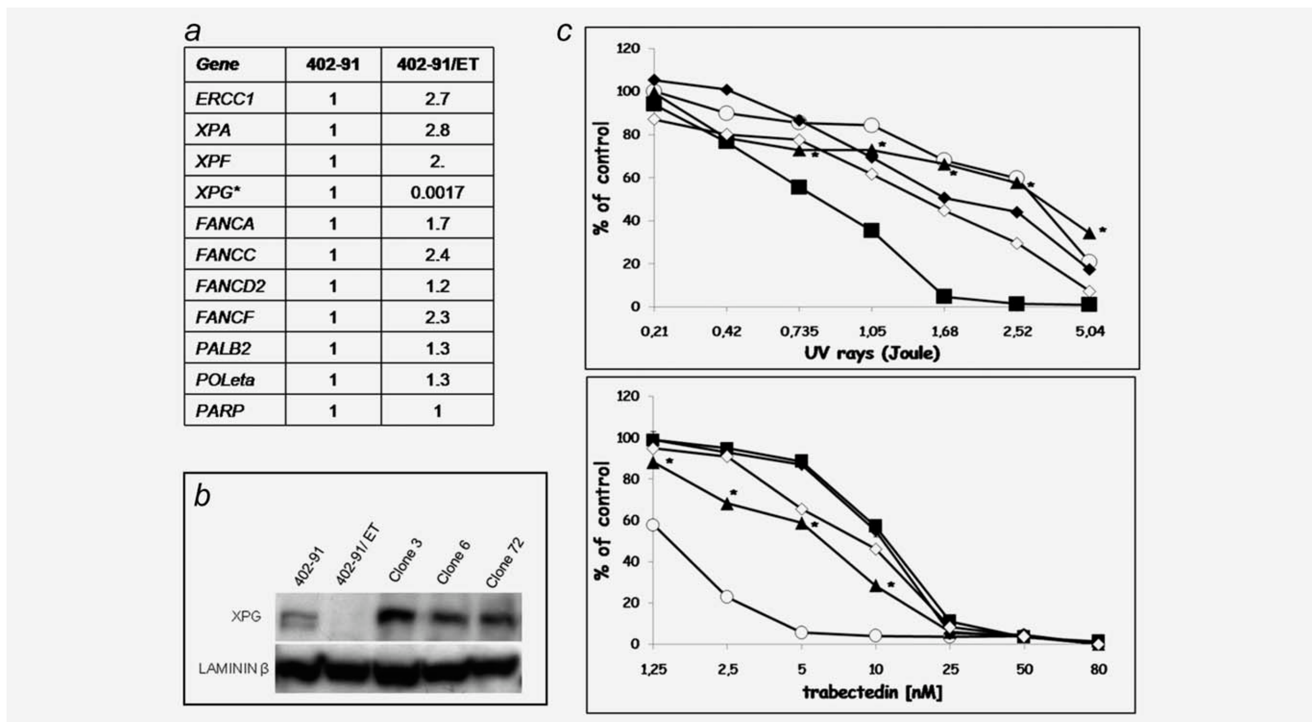


Figure 2. Effect of trabectedin, UV rays, X rays and various anticancer drugs on the clonogenicity of 402-91 (○) and 402-91/ET (■) cell lines. Each point is the mean of six replicates of three independent experiments; bars represent S.D.

able to amplify these regions in the resistant subline, although the amplification of the *calponin* promoter region was clearly demonstrated (Supporting Information Fig. 2*b*). Sequencing of the XPG gene revealed that in 402-91/ET cell line, Exon 1, containing the ATG, was absent along with the 5′ upstream region, where the MSP primers were designed to anneal, thus explaining the lack of amplified bands. There were no other mutations (Supporting Information Fig. 2*c*).

To further explore the possibility that the absence of the XPG gene product was crucial for resistance to trabectedin, we transfected 402-91/ET cells with an expression vector encoding the XPG cDNA and produced three clones with different levels of expression of the XPG protein (Fig. 3*b*). The expression of transfected XPG complemented the UV sensitivity to levels that were closer to that of the 402-91 cells (Fig. 3*c*). However, only in the case of Clone 3 cells were sensitivity to trabectedin consistently restored ($p < 0.001$, two-way ANOVA test), and even in this case complementation was incomplete. This suggests that the mechanism of resistance to trabectedin is not completely accounted for by attenuation of XPG expression.

The 402-91/ET cell line showed collateral sensitivity to temozolomide, a methylating agent that generates a number of adducts. The majority of the toxic effects of temozolomide are probably due to O^6-methylguanine (O^6-meG) that constitutes only the 6% of the DNA lesions caused by the drug. The DNA repair protein MGMT, by removing the methyl group from O^6-meG in DNA, can protect cells against the toxic effects of temozolomide-induced damage.[42,43] The collateral sensitivity to temozolomide in 402-91/ET cells prompted us to study the MGMT expression. In contrast to 402-91 cells, the trabectedin resistant cell line showed undetectable MGMT activity (Fig. 4*a*). This lack of MGMT activity was related to the hypermethylation of the MGMT promoter again in contrast to the largely unmethylated status of the promoter in 402-91 cells (Fig. 4*b*). To confirm that the sensitivity of 402-91/ET cells to temozolomide was correlated with the absence of MGMT, we incubated the MGMT-expressing 402-91 cell line for 2 hr with a non toxic concentration (5 μM) of Lomeguatrib, an MGMT inactivating agent that completely ablated the activity of the protein (data not shown), and then analyzed, by clonogenic assay, its sensitivity to temozolomide. Figure 4*c* shows that the decreased level of MGMT made 402-91 cells more sensitive to temozolomide ($p < 0.001$ two-way ANOVA test). However, inactivation of MGMT did not affect the sensitivity of 402-91 cells to



Gene	402-91	402-91/ET
ERCC1	1	2.7
XPA	1	2.8
XPF	1	2.
XPG*	1	0.0017
FANCA	1	1.7
FANCC	1	2.4
FANCD2	1	1.2
FANCF	1	2.3
PALB2	1	1.3
POLeta	1	1.3
PARP	1	1

Figure 3. (*a*) mRNA expression of various repair genes in the two cell lines. Data are expressed as fold expression relative to the expression in the sensitive cell line. (*b*) Western blot analysis of XPG protein expression in 402-91, 402-91/ET, and clones 3, 6 and 72 cells. (*c*) Sensitivity to UV or trabectedin of 402-91 (○), 402-91/ET (■), Clone 3 (▲), Clone 6 (◆) and Clone 72 (◇) cell lines evaluated by colony forming assay. Each point is the mean of six replicates of three independent experiments; bars represent S.D. *$p < 0.001$ two-way-ANOVA test.

trabectedin (Fig. 4*d*), indicating that MGMT was not involved in the mechanism of resistance to trabectedin.

ChIP analysis and gene expression of 402-91/ET cells

By using the 402-91 cell line as an *in vitro* model of the MLS tumor, we previously demonstrated that trabectedin's mechanism of action is related to the detachment of the FUS-CHOP chimera from the promoter of some of its target genes.[31] To test the hypothesis that FUS-CHOP transcriptional activity was impaired in the 402-91/ET resistant model, we used ChIP analysis to monitor the binding of the chimera to two well known genes target, PTX3 and FN1, previously studied in the parental cell line. The analysis was developed using anti FUS and anti-CHOP antibodies.[31] Antibodies to both FUS and CHOP produced positive signals in ChIP assays of the PTX3 and FN1 promoters in untreated 402-91 cells. This association was decreased by 5 nM trabectedin treatment, already after 1h of treatment and for at least 6 h after drug-washout (Fig. 5*a*). By contrast, ChIP analysis performed in the 402-91/ET cell line, revealed no binding of the chimera to the PTX3 and FN1 promoters, in both untreated and trabectedin treated cells. In keeping with these findings, real time RT-PCR analysis showed that PTX3 and FN1 expression levels in untreated 402-91/ET cells were downregulated relative to untreated 402-91 cells (Fig. 5*b*).

Because trabectedin-mediated transcription modulation in 402-91 cells activates the adipogenic program by increasing the expression levels of c/EBPα and c/EBPβ,[31] we reasoned that in 402-91/ET cells, impaired FUS-CHOP chimera binding to its promoters might attenuate this pathway. RT-PCR analysis (Fig. 5*b*) confirmed that in 402-91 cells trabectedin activates the transcription of both c/EBPα and β in a time and dose dependent manner. In contrast, in 402-91/ET cells this effect was not evident: c/EBPα and c/EBPβ expression levels were comparable in treated and untreated cells. In addition, c/EBPβ expression levels were similar in the two cell lines while c/EBP α expression in resistant cell line was markedly reduced relative to 402-91 cells.

Whole genome analysis of 402-91/ET cells

Microarray analysis identified a subset of 1,563 probes ID that were differentially expressed between 402-91/ET and 402-91 cells. We focused our analysis on those IDs with lgFC > 1.5 (233 ID) and <-1.5 (279 ID), with a *p*-value threshold < 0.05. These 512 ID (corresponding to 464 known genes) represent a mixture of different molecular pathways and networks. To identify biological processes that may underlie the mechanism of resistance to trabectedin, we used commercially available software ("METACORE" https://portal.genego.com/cgi/data_manager.cgi) to group the selected 512 probes. Selected genes were statistically compared to the entire set of genes spotted onto the array to look for under- and over-represented functional categories. Supporting Information Table 1 shows the 10 biological processes selected with a *p*-value < 0.05. To validate the microarray data, a

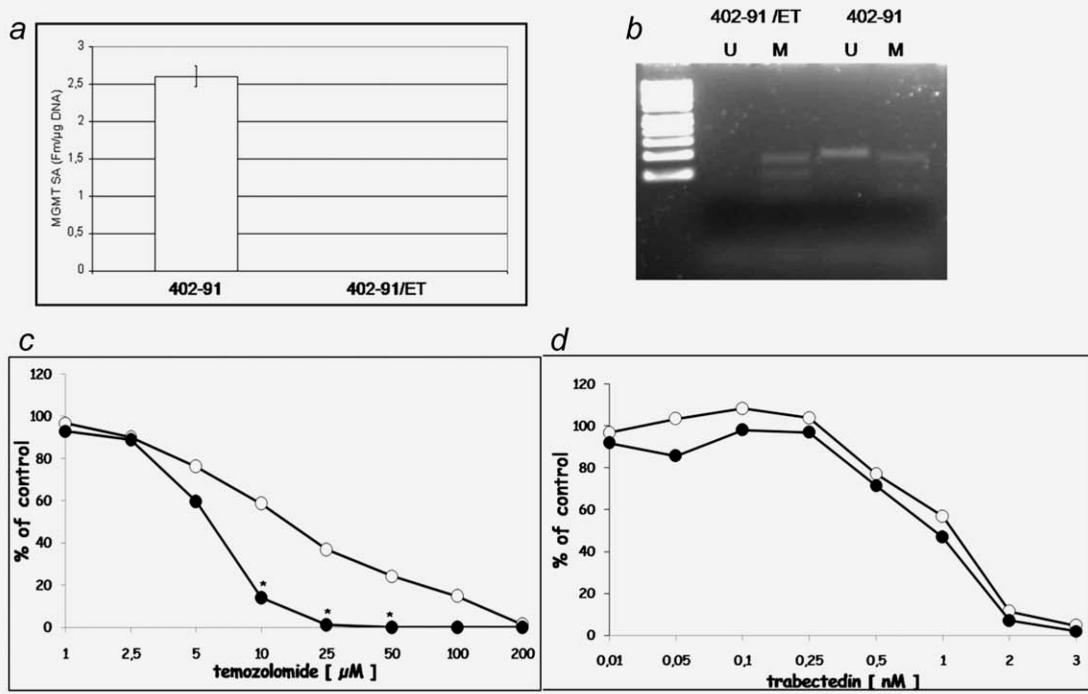


Figure 4. (*a*) Activity of MGMT. (*b*) Methylation pattern of the MGMT promoter. (*c*) Sensitivity to temozolomide alone (○), or after preincubation with Lomeguatrib (●) of the 402-91 cell line determined by colony forming assay. Each point is the mean of six replicates of three independent experiments; bars represent S.D. *$p < 0.001$ two-way ANOVA test. (*d*) Sensitivity to trabectedin alone (○) or after preincubation with Lomeguatrib (●) of the 402-91 cell line determined by colony forming assay. Each point is the mean of six replicates of three independent experiments; bars represent S.D.

selection of 16 genes belonging to four different biological processes including "Apoptosis and survival" (BOX 1), "stress response" (BOX 2), "signal transduction" (BOX 3) and "transcription-CREB pathway" (BOX 4) were analyzed by RT-PCR (Supporting Information Fig. 3). To correct for batch effects, analyses were performed on independent pools of RNA purified from the same exponentially growing cells. Results obtained by qRT-PCR (empty columns) reflected those obtained by microarray (full columns) in both qualitative and quantitative terms. This suggests that most array probe sets are likely to accurately measure single transcript levels within a complex mixture of transcripts.

Discussion

Trabectedin has recently been shown to possess a significant antitumor activity in MLS with an objective response rate of ∼50%, median progression-free survival of almost 1.5 years and 2-years progression free survival exceeding 30%. However, even after prolonged responses, drug resistance occurs, patients thus requiring alternative treatments.[9,10]

To investigate the mechanism of trabectedin resistance in MLS, we exposed the sensitive 402-91 cell line to stepwise increases in trabectedin concentrations to generate the resistant cell line, 402-91/ET which is ∼10 times less sensitive to the drug. Resistance was irreversible over more than 2 years. The 402-91/ET cell line maintains the typical translocation *t*(12;16)(q13;p11) that results in the expression of the FUS-CHOP fusion gene, which represents the hallmark and the principal pathogenic lesion of MLS.[44–46]

To our knowledge this is the first trabectedin-resistant MLS cell line that has been obtained and characterized.

From our present studies, we can exclude that the mechanism of trabectedin resistance was due to a low intracellular drug retention. Not only the expression levels of the multidrug resistant transporters, but also the intracellular drug concentrations were closely similar in the two cell lines. Furthermore, the reduced sensitivity to trabectedin was not related to the higher GSH or cysteine level found in the 402-91/ET cell line.

Previous data from this[24,25,47] and other laboratories[26–28] have indicated that NER deficiency confers resistance to trabectedin. Takebayashi *et al.*[26] characterized a cell line, HCT-116/ER5, which was selected for resistance to trabectedin. This cell line did not express functional XPG because of a point mutation in the XPG gene which resulted in a premature termination codon. The cell line was also hypersensitive to UV light. Transient transfection of HCT-116/ER5 with an XPG cDNA-containing expression vector restored its sensitivity to trabectedin. These reports support our finding that the trabectedin resistant 402-91/ET cell line was hypersensitive to UV-radiation and we now show that 402-91/ET cells do not express XPG because of the loss of Exon 1. However, when 402-91/ET cells were transfected with an expression vector

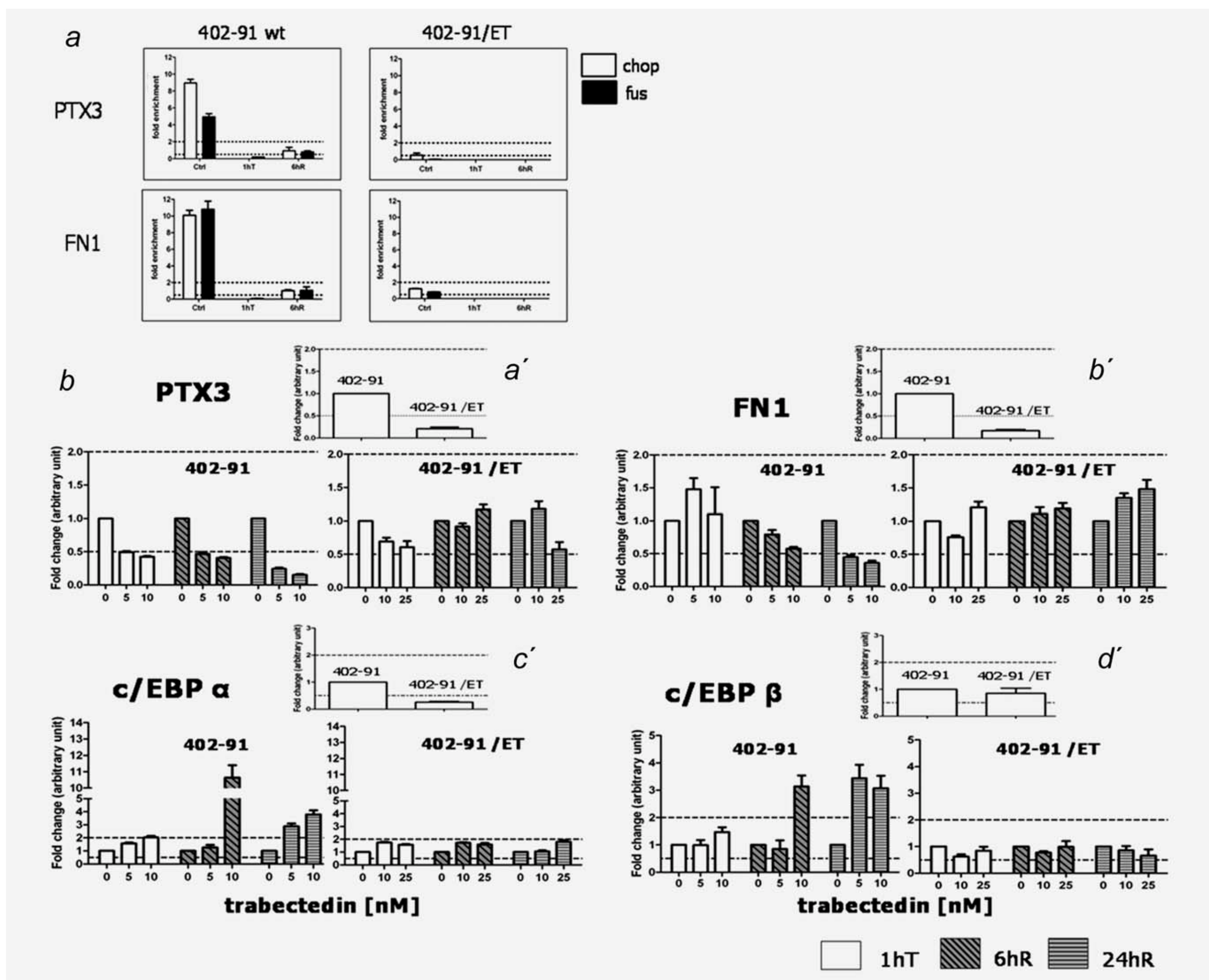


Figure 5. (*a*) Chip analysis performed on 402-91 and 402-91/ET cells of two target genes of FUS-CHOP (PTX3 and FN1). (*b*) Real time RT-PCR analysis of PTX3, FN1, c/EBPα and c/EBPβ genes, performed in 402-91 and 402-91/ET cells. The assay was performed after treatment (1 hT) and at different time intervals after drug-wash-out (6 hr and 24 hr), with different equitoxic trabectedin concentrations; 5 and 10 nM for 402-91 and 10–25 nM for 402-91/ET cells. [b(*a*′–*d*′)] m-RNA basal expression of PTX3 (*a*′), FN1 (*b*′), c/EBPα (*c*′) and c/EBPβ (*d*′), in 402-91 and 402-91/ET cell lines.

encoding the XPG cDNA, although the hypersensitivity to UV light was markedly reduced, the trabectedin sensitivity was only partially restored in the isolated clones. Thus only a portion of the trabectedin resistance could be attributable to the XPG mutation and other mechanisms unrelated to NER function operates in 402-91/ET cells.

One hint as to the resistance mechanism comes from analysis of FUS-CHOP binding *in vitro*. Forni *et al.*[31] showed that in 402-91cell line FUS-CHOP was bound to the promoters of PTX3 and FN1 and that trabectedin caused a detachment of the chimera. For this reason we investigated if FUS-CHOP chimera was able to bind to the promoters of PTX3 and FN1 in 402-91/ET cells. Here we report that in this resistant cell line FUS-CHOP was not bound to the promoters of target genes.

This might explain the important differences that exist between 402-91 and 402-91/ET cell lines in the expression of many genes that are regulated by FUS-CHOP. Among them there are genes associated to adipocytic differentiation. C/EBPα and β, "master" genes in adipogenesis, are upregulated both in 402-91 and in clinical MLS specimens after trabectedin treatment[31] and their induction probably triggers adipocytic terminal differentiation. Trabectedin induction of C/EBPα and β in 402-91, but not in the 402-91/ETcell line, is therefore in line with a fundamental change in FUS-CHOP promoter targeting in the resistant cell line. This is further reinforced by gene profiling analysis, indicating that there are several potentially important differences between the 402-91 and 402-91/ET cell lines.

There were no significant differences in cross resistance of 402-91 and 402-91/ET cell lines to other drugs. There was,

however, a very clear collateral sensitivity to temozolomide and to other methylating agents such as MNNG (data not shown). This finding was intriguing as the toxicity of methylating agents is generally related to their reaction at the O^6-position of guanine and resistance is mediated by the action of MGMT. In contrast, cell killing by the dihaloalkanes requires the functional activity of MGMT and MGMT deficient cells are more resistant to these agents.[48] To investigate if the mechanism of trabectedin killing resembled that of dihaloalkanes, we quantified the expression of MGMT in the 402-91 and 402-91/ET cell lines. MGMT activity was easily measurable in extracts of the 402-91 but not detected in the 402-91/ET cell line. Furthermore, inactivation of MGMT by Lomeguatrib, sensitized 402-91 but not the resistant cell line to temozolomide. The MGMT gene was found to be silenced by methylation of its promoter in the 402-91/ET cells thus explaining the absence of MGMT activity and the sensitivity to temozolomide in 402-91/ET cells. If downregulation of MGMT was responsible for the resistance of 402-91/ET cells to trabectedin, it would be expected that inactivation of MGMT in 402-91 cells would increase their resistance. This was not the case: Lomeguatrib had no effect on the sensitivity to trabectedin in either 402-91 or 402-91/ET cell lines, so it can be concluded that the mechanism of action of trabectedin does not resemble that of the dihaloalkanes. These results also indicate that trabectedin might instigate not only genetic (for example, deletion of part of the XPG gene) but also epigenetic modifications that will be interesting to explore in future studies. The finding that prolonged treatment with trabectedin might determine changes in MGMT promoter methylation, invite us to speculate that patients resistant to trabectedin might be very sensitive to methylating agents; this clinically interestingly hypothesis requires more experimental supports. Tumor biopsies from relapsed patients need to be assayed for MGMT activity or promoter methylation before a clinical trial could be proposed. It would also be important to establish that MGMT expression was not downregulated in normal tissues.

References

1. Valoti G, Nicoletti MI, Pellegrino A, Jimeno J, Hendriks H, D'Incalci M, Faircloth G, Giavazzi R. Ecteinascidin-743, a new marine natural product with potent antitumor activity on human ovarian carcinoma xenografts. *Clin Cancer Res* 1998;4:1977–83.
2. Izbicka E, Lawrence R, Raymond E, Eckhardt G, Faircloth G, Jimeno J, Clark G, Von Hoff DD. In vitro antitumor activity of the novel marine agent, ecteinascidin-743 (ET-743, NSC-648766) against human tumors explanted from patients. *Ann Oncol* 1998;9: 981–7.
3. Hendriks HR, Fiebig HH, Giavazzi R, Langdon SP, Jimeno JM, Faircloth GT. High antitumour activity of ET743 against human tumour xenografts from melanoma, non-small-cell lung and ovarian cancer. *Ann Oncol* 1999;10:1233–40.
4. Meco D, Colombo T, Ubezio P, Zucchetti M, Zaffaroni M, Riccardi A, Faircloth G, Jose J, D'Incalci M, Riccardi R. Effective combination of ET-743 and doxorubicin in sarcoma: preclinical studies. *Cancer Chemother Pharmacol* 2003;52:131–8.
5. D'Incalci M, Colombo T, Ubezio P, Nicoletti I, Giavazzi R, Erba E, Ferrarese L, Meco D, Riccardi R, Sessa C, Cavallini E, Jimeno J, et al. The combination of yondelis and cisplatin is synergistic against human tumor xenografts. *Eur J Cancer* 2003;39:1920–6.
6. Yovine A, Riofrio M, Blay JY, Brain E, Alexandre J, Kahatt C, Taamma A, Jimeno J, Martin C, Salhi Y, Cvitkovic E, Misset JL. Phase II study of ecteinascidin-743 in advanced pretreated soft tissue sarcoma patients. *J Clin Oncol* 2004;22: 890–9.
7. Le Cesne A, Blay JY, Judson I, Van Oosterom A, Verweij J, Radford J, Lorigan P, Rodenhuis S, Ray-Coquard I, Bonvalot S, Collin F, Jimeno J, et al. Phase II study of ET-743 in advanced soft tissue sarcomas: a European organisation for the research and treatment of cancer (EORTC) soft tissue and bone sarcoma group trial. *J Clin Oncol* 2005;23:576–84.
8. Garcia-Carbonero R, Supko JG, Maki RG, Manola J, Ryan DP, Harmon D, Puchalski TA, Goss G, Seiden MV, Waxman A, Quigley MT, Lopez T, et al. Ecteinascidin-743 (ET-743) for chemotherapy-naive patients with advanced soft tissue sarcomas: multicenter phase II and pharmacokinetic study. *J Clin Oncol* 2005; 23:5484–92.
9. Grosso F, Jones RL, Demetri GD, Judson IR, Blay JY, Le Cesne A, Sanfilippo R, Casieri P, Collini P, Dileo P, Spreafico C, Stacchiotti S, et al. Efficacy of trabectedin (ecteinascidin-743) in advanced pretreated myxoid liposarcomas: a retrospective study. *Lancet Oncol* 2007;8:595–602.
10. Grosso F, Sanfilippo R, Virdis E, Piovesan C, Collini P, Dileo P, Morosi C, Tercero JC, Jimeno J, D'Incalci M, Gronchi A, Pilotti S, et al. Trabectedin in myxoid liposarcomas (MLS): a long-term analysis of a single-institution series. *Ann Oncol* 2009;20:1439–44.
11. Demetri GD, Chawla SP, von Mehren M, Ritch P, Baker LH, Blay JY, Hande KR, Keohan ML, Samuels BL, Schuetze S, Lebedinsky C, Elsayed YA, et al. Efficacy and safety of trabectedin in patients with advanced or metastatic liposarcoma or leiomyosarcoma after failure of prior anthracyclines and ifosfamide: results of a randomized phase II study of two different schedules. *J Clin Oncol* 2009;27:4188–96.
12. Casali PG, Sanfilippo R, D'Incalci M. Trabectedin therapy for sarcomas. *Curr Opin Oncol* 2010;22:342–6.
13. Sessa C, De Braud F, Perotti A, Bauer J, Curigliano G, Noberasco C, Zanaboni F, Gianni L, Marsoni S, Jimeno J, D'Incalci M, Dall'o E, et al. Trabectedin for women with ovarian carcinoma after treatment with platinum and taxanes fails. *J Clin Oncol* 2005;23:1867–74.
14. Krasner CN, McMeekin DS, Chan S, Braly PS, Renshaw FG, Kaye S, Provencher DM, Campos S, Gore ME. A Phase II study of trabectedin single agent in patients with recurrent ovarian cancer previously treated with platinum-based regimens. *Br J Cancer* 2007;97:1618–24.
15. Del Campo JM, Roszak A, Bidzinski M, Ciuleanu TE, Hogberg T, Wojtukiewicz MZ, Poveda A, Boman K, Westermann AM, Lebedinsky C. Phase II randomized study of trabectedin given as two different every 3 weeks dose schedules (1.5 mg/m^2 24 h or 1.3 mg/m^2 3 h) to patients with relapsed, platinum-sensitive, advanced ovarian cancer. *Ann Oncol* 2009;20: 1794–802.
16. Carter NJ, Keam SJ. Trabectedin: a review of its use in soft tissue sarcoma and ovarian cancer. *Drugs* 2010;70:355–76.
17. Monk BJ, Herzog TJ, Kaye SB, Krasner CN, Vermorken JB, Muggia FM, Pujade-Lauraine E, Lisyanskaya AS, Makhson AN, Rolski J, Gorbounova VA, Ghatage P, et al.

Trabectedin plus pegylated liposomal doxorubicin in recurrent ovarian cancer. *J Clin Oncol* 2010;28:3107–14.
18. Pommier Y, Kohlhagen G, Bailly C, Waring M, Mazumder A, Kohn KW. DNA sequence- and structure-selective alkylation of guanine N2 in the DNA minor groove by ecteinascidin 743, a potent antitumor compound from the Caribbean tunicate *Ecteinascidia turbinata*. *Biochemistry* 1996; 35:13303–9.
19. Moore BM,II Seaman FC, Hurley LH. NMR-based model of an ecteinascidin 743-DNA adduct. *J Am Chem Soc* 1997;119: 5475–6.
20. Hurley LH, Zewail-Foote M. The antitumor agent ecteinascidin 743: characterization of its covalent DNA adducts and chemical stability. *Adv Exp Med Biol* 2001;500:289–99.
21. Gago F, Hurley LH. Devising a structural basis for the potent cytotoxic effects of ecteinascidin 743. In: Demeunynck M, Bailly C, Wilson WD, editors. Small molecule DNA and RNA binders: from synthesis to nuclear acid complexed. Weinheim, Germany: Wiley-VCH; 2002. 643–75.
22. Minuzzo M, Marchini S, Broggini M, Faircloth G, D'Incalci M, Mantovani R. Interference of transcriptional activation by the antineoplastic drug ecteinascidin-743. *Proc Natl Acad Sci USA* 2000;97:6780–4.
23. Jin S, Gorfajn B, Faircloth G, Scotto KW. Ecteinascidin 743, a transcription-targeted chemotherapeutic that inhibits MDR1 activation. *Proc Natl Acad Sci USA* 2000; 97:6775–9.
24. Damia G, Silvestri S, Carrassa L, Filiberti L, Faircloth GT, Liberi G, Foiani M, D'Incalci M. Unique pattern of ET-743 activity in different cellular systems with defined deficiencies in DNA-repair pathways. *Int J Cancer* 2001;92:583–8.
25. Erba E, Bergamaschi D, Bassano L, Damia G, Ronzoni S, Faircloth GT, D'Incalci M. Ecteinascidin-743 (ET-743), a natural marine compound, with a unique mechanism of action. *Eur J Cancer* 2001; 37:97–105.
26. Takebayashi Y, Pourquier P, Zimonjic DB, Nakayama K, Emmert S, Ueda T, Urasaki Y, Kanzaki A, Akiyama SI, Popescu N, Kraemer KH, Pommier Y. Antiproliferative activity of ecteinascidin 743 is dependent upon transcription-coupled nucleotide-excision repair. *Nat Med* 2001;7:961–6.
27. Zewail-Foote M, Li VS, Kohn H, Bearss D, Guzman M, Hurley LH. The inefficiency of incisions of ecteinascidin 743-DNA adducts by the UvrABC nuclease and the unique structural feature of the DNA adducts can be used to explain the repair-dependent toxicities of this antitumor agent. *Chem Biol* 2001;8:1033–49.
28. Stevens EV, Nishizuka S, Antony S, Reimers M, Varma S, Young L, Munson PJ, Weinstein JN, Kohn EC, Pommier Y. Predicting cisplatin and trabectedin drug sensitivity in ovarian and colon cancers. *Mol Cancer Ther* 2008;7:10–8.
29. Soares DG, Poletto NP, Bonatto D, Salvador M, Schwartsmann G, Henriques JA. Low cytotoxicity of ecteinascidin 743 in yeast lacking the major endonucleolytic enzymes of base and nucleotide excision repair pathways. *Biochem Pharmacol* 2005; 70:59–69.
30. Tavecchio M, Simone M, Erba E, Chiolo I, Liberi G, Foiani M, D'Incalci M, Damia G. Role of homologous recombination in trabectedin-induced DNA damage. *Eur J Cancer* 2008;44:609–18.
31. Forni C, Minuzzo M, Virdis E, Tamborini E, Simone M, Tavecchio M, Erba E, Grosso F, Gronchi A, Aman P, Casali P, D'Incalci M, et al. Trabectedin (ET-743) promotes differentiation in myxoid liposarcoma tumors. *Mol Cancer Ther* 2009;8:449–57.
32. Germano G, Frapolli R, Simone M, Tavecchio M, Erba E, Pesce S, Pasqualini F, Grosso F, Sanfilippo R, Casali PG, Gronchi A, Virdis E, et al. Antitumor and anti-inflammatory effects of trabectedin on human myxoid liposarcoma cells. *Cancer Res* 2010;70:2235–44.
33. Aman P, Ron D, Mandahl N, Fioretos T, Heim S, Arheden K, Willen H, Rydholm A, Mitelman F. Rearrangement of the transcription factor gene CHOP in myxoid liposarcomas with t(12;16)(q13;p11). *Genes Chromosomes Cancer* 1992;5:278–85.
34. Montefalcone G, Tempesta S, Rocchi M, Archidiacono N. Centromere repositioning. *Genome Res* 1999;9:1184–8.
35. Marchini S, Mariani P, Chiorino G, Marrazzo E, Bonomi R, Fruscio R, Clivio L, Garbi A, Torri V, Cinquini M, Dell'Anna T, Apolone G, et al. Analysis of gene expression in early-stage ovarian cancer. *Clin Cancer Res* 2008;14:7850–60.
36. Antonescu CR, Tschernyavsky SJ, Decuseara R, Leung DH, Woodruff JM, Brennan MF, Bridge JA, Neff JR, Goldblum JR, Ladanyi M. Prognostic impact of P53 status, TLS-CHOP fusion transcript structure, and histological grade in myxoid liposarcoma: a molecular and clinicopathologic study of 82 cases. *Clin Cancer Res* 2001;7:3977–87.
37. Erba E, Bergamaschi D, Bassano L, Ronzoni S, Di Liberti G, Muradore I, Vignati S, Faircloth G, Jimeno J, D'Incalci M. Isolation and characterization of an IGROV-1 human ovarian cancer cell line made resistant to Ecteinascidin-743 (ET-743). *Br J Cancer* 2000;82:1732–9.
38. Rosing H, Hillebrand MJ, Jimeno JM, Gomez A, Floriano P, Faircloth G, Henrar RE, Vermorken JB, Cvitkovic E, Bult A, Beijnen JH. Quantitative determination of Ecteinascidin 743 in human plasma by miniaturized high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry. *J Mass Spectrom* 1998;33: 1134–40.
39. Pastore A, Massoud R, Motti C, Lo Russo A, Fucci G, Cortese C, Federici G. Fully automated assay for total homocysteine, cysteine, cysteinylglycine, glutathione, cysteamine, and 2-mercaptopropionylglycine in plasma and urine. *Clin Chem* 1998;44: 825–32.
40. Watson AJ, Margison GP. O6-alkylguanine-DNA alkyltransferase assay. *Methods Mol Biol* 2000;152:49–61.
41. Caretti G, Salsi V, Vecchi C, Imbriano C, Mantovani R. Dynamic recruitment of NF-Y and histone acetyltransferases on cell-cycle promoters. *J Biol Chem* 2003;278: 30435–40.
42. Verbeek B, Southgate TD, Gilham DE, Margison GP. O6-methylguanine-DNA methyltransferase inactivation and chemotherapy. *Br Med Bull* 2008; 85:17–33.
43. van Nifterik KA, van den Berg J, van der Meide WF, Ameziane N, Wedekind LE, Steenbergen RD, Leenstra S, Lafleur MV, Slotman BJ, Stalpers LJ, Sminia P. Absence of the MGMT protein as well as methylation of the MGMT promoter predict the sensitivity for temozolomide. *Br J Cancer* 2010;103:29–35.
44. Panagopoulos I, Mertens F, Isaksson M, Mandahl N. A novel FUS/CHOP chimera in myxoid liposarcoma. *Biochem Biophys Res Commun* 2000;279:838–45.
45. Antonescu CR, Elahi A, Healey JH, Brennan MF, Lui MY, Lewis J, Jhanwar SC, Woodruff JM, Ladanyi M. Monoclonality of multifocal myxoid liposarcoma: confirmation by analysis of TLS-CHOP or EWS-CHOP rearrangements. *Clin Cancer Res* 2000;6: 2788–93.
46. Orvieto E, Furlanetto A, Laurino L, Dei Tos AP. Myxoid and round cell liposarcoma: a spectrum of myxoid adipocytic neoplasia. *Semin Diagn Pathol* 2001;18:267–73.
47. Tavecchio M, Natoli C, Ubezio P, Erba E, D'Incalci M. Dynamics of cell cycle phase perturbations by trabectedin (ET-743) in nucleotide excision repair (NER)-deficient and NER-proficient cells, unravelled by a novel mathematical simulation approach. *Cell Prolif* 2007;40:885–904.
48. Abril N, Margison GP. Mammalian cells expressing *Escherichia coli* O6-alkylguanine-DNA alkyltransferases are hypersensitive to dibromoalkanes. *Chem Res Toxicol* 1999;12:544–51.