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Increased sensitivity to platinum drugs of cancer cells with acquired resistance to trabectedin

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Background: In order to investigate the mechanisms of acquired resistance to trabectedin, trabectedin-resistant human myxoid liposarcoma (402-91/T) and ovarian carcinoma (A2780/T) cell lines were derived and characterised *in vitro* and *in vivo*.

Methods: Resistant cell lines were obtained by repeated exposures to trabectedin. Characterisation was performed by evaluating drug sensitivity, cell cycle perturbations, DNA damage and DNA repair protein expression. *In vivo* experiments were performed on A2780 and A2780/T xenografts.

Results: 402-91/T and A2780/T cells were six-fold resistant to trabectedin compared with parental cells. Resistant cells were found to be hypersensitive to UV light and did not express specific proteins involved in the nucleotide excision repair (NER) pathway: XPF and ERCC1 in 402-91/T and XPG in A2780/T. NER deficiency in trabectedin-resistant cells was associated with the absence of a G_2/M arrest induced by trabectedin and with enhanced sensitivity (two-fold) to platinum drugs. In A2780/T, this collateral sensitivity, confirmed *in vivo*, was associated with an increased formation of DNA interstrand crosslinks.

Conclusions: Our finding that resistance to trabectedin is associated with the loss of NER function, with a consequent increased sensitivity to platinum drugs, provides the rational for sequential use of these drugs in patients who have acquired resistance to trabectedin.

Trabectedin is a marine-derived tetrahydroisoquinoline alkaloid with antitumour activity. Its peculiar pentacyclic structure allows the interaction (through covalent binding) with the N2-position of guanine in the minor groove of the DNA (Pommier *et al*, 1996; Zewail-Foote and Hurley, 2001). Once bound, trabectedin is believed to interact with DNA-binding molecules, including transcription factors and DNA repair proteins (Forni *et al*, 2009). This interaction affects the transcription of activated genes in a promoter- and gene-specific manner and also results in a bending of the helix towards the major groove. This distortion causes the activation of pathways involved in DNA repair. Homologous recombination (HR) is particularly important for

trabectedin efficacy: indeed HR-deficient cells are approximately 100 times more sensitive to the drug. In contrast, non-homologous end joining and mismatch repair deficiency do not appear to affect the cytotoxic activity of this drug (D'Incalci and Galmarini, 2010).

A peculiar aspect of the mechanism of action of trabectedin is its pattern of activity in nucleotide excision repair (NER)-deficient cells: the drug showed decreased activity (from 2- to 10-fold) in these cells compared with NER-proficient cells. It is thought that DNA-bound trabectedin prevents the correction of DNA lesions by transcription-coupled NER (TC-NER) by creating cytotoxic ternary complexes with DNA-binding proteins of the NER system, such as XPG (Damia *et al*, 2001; Erba *et al*, 2001; Tavecchio *et al*,

2007). The formation of such complexes would then induce both transcription- and replication-coupled DNA double-strand breaks (DSBs) that require HR to be repaired.

Despite trabectedin having a unique and complex mechanism of action, trabectedin-treated patients frequently develop resistance to the drug. The mechanism of trabectedin resistance *in vitro* is not completely understood as few acquired resistant cell lines have been described. Several *in vitro* studies (Kanzaki *et al*, 2002; Shao *et al*, 2003) showed that prolonged exposure to trabectedin induced the down-regulation of Pgp1 (multidrug resistance-associated protein) expression.

In contrast with these data, Erba *et al* (2000) described Pgp1 overexpression in an ovarian cancer cell line resistant to trabectedin, Igrov-1/25ET.

The study of mechanisms underlying trabectedin resistance must take into consideration that trabectedin impairs transcription regulation (Jin *et al*, 2000; Minuzzo *et al*, 2000). This ability appears to be correlated with the induction of resistance in cancer cell lines, as showed by Marchini *et al* (2005). They observed changes in the gene expression profile of several genes (coding for transcription factors, cytoskeleton reorganisation enzymes, signal transduction proteins and enzymes involved in cellular metabolism) between parental and trabectedin-resistant cells.

In this study, we developed a human myxoid liposarcoma and an ovarian cancer cell line (402-91/T and A2780/T, respectively) resistant to trabectedin. Based on the assumption that trabectedin efficacy is correlated to the activity of DNA repair systems and transcription regulation, we characterised the resistant cell lines with both cellular and molecular approaches. The finding that trabectedin resistance is associated with collateral sensitivity to other chemotherapeutic agents, both *in vitro* and *in vivo* systems, has potential clinical applications.

MATERIALS AND METHODS

Cells. In order to generate trabectedin-resistant cell lines, myxoid liposarcoma 402-91 and ovarian carcinoma A2780 cells were exposed to a stepwise increase in drug concentration using a short (1 h) exposure for 10 treatments. Cells were grown in RPMI-1640, 10% FBS and 1% L-glutamine and maintained at $37\,^\circ C$ in a humidified atmosphere at 5% CO_2. The resistant cell lines were termed 402-91/T and A2780/T, respectively, and the resistance index was evaluated by clonogenic assay (Tavecchio *et al*, 2008).

Drugs. Trabectedin was kindly provided by PharmaMar, S.A. (Colmenar Viejo, Spain), stocked in DMSO at a concentration of 1 mM and stored at $-20\,^\circ C$. Cis-diammineplatinum (II) dichloride (cis-DDP) and carboplatin were purchased from Sigma Aldrich, St Louis, MO, USA. All drugs were diluted in RPMI-1640 medium just before use.

Proliferation assay. The evaluation of cisplatin and carboplatin cross-resistance on 402-91/T and A2780/T cell lines was assessed by WST-1 cell proliferation assay (Roche, Basel, Switzerland) (Romano *et al*, 2013).

Flow cytometric cell cycle analysis. Cell cycle perturbations induced by trabectedin and cis-DDP were evaluated by standard flow cytometric methods. Control and treated cells were counted by using Coulter Counter (ZM, Beckman Coulter, Brea, CA, USA) every 24 h after drug washout and fixed in 70% ethanol before DNA staining (Erba *et al*, 2001).

Flow cytometric γ-H2AX detection. At the end of treatment and at different times after drug washout, the cells were fixed and stained for γ-H2AX detection as previously described (Tavecchio *et al*, 2008).

RNA purification and semi-quantitative RT–PCR. Total RNA was purified using the RNeasy Mini Kit (Qiagen, Milan, Italy)

according to the manufacturer's instructions. Reverse transcription to cDNA was performed using a High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA) starting from 500 ng of total RNA following the manufacturer's instructions. Differences in ERCC1, XPF and XPG expression were determined by real-time RT-PCR (ABI-7900, Applied Biosystems, Carlsbad, CA, USA) using Sybr Green (Qiagen) and the following primers (ERCC1 FW: 5′-CCAACAGCATCATTGTGAGC-3′; ERCC1 RV: 5′-CGGGAATTACGTCGCCAAAT-3′; XPF FW: 5′-TTGTGAGGAAACTGTATCTGTGG-3′; XPF RV: 5′-AGCAA GCATGGTAGGTGTCA-3′; XPG FW: 5′-TCTGGAAGCTGCTGG AGTG-3′; XPG RV: 5′-TGCTAATATCAACAGCCAGGAT-3′). The analysis was performed using the 2^{-DDCT} method (Livak and Schmittgen, 2001).

Western blotting analysis. Whole-protein extracts were obtained using a lysis buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA and 1% Triton in the presence of protease inhibitors (Roche). Proteins were quantified using the Bradford assay reagent (Bio-Rad Laboratories, Melville, NY, USA), separated on a SDS–PAGE gel, transferred to a nitrocellulose membrane (Bio-Rad) and probed with the primary antibodies to XPG (Bethyl, Montgomery, TX, USA), ERCC1 (D6G6 XP, Cell Signaling Technology, Danvers, MA, USA), XPF (D3G8C, Cell Signaling Technology) and β-tubulin (H235, Santa Cruz Biotechnology, Dallas, TX, USA). Binding was detected using peroxidase-labeled secondary antibodies and visualised using a Chemiluminescence Kit (Thermo Scientific, Waltham, MA, USA).

Western blotting analysis was performed periodically (at least every 5 months) and for every new batch of cells that were defrosted.

Single-cell gel electrophoresis (comet) assay. The modified single-cell gel electrophoresis (comet) assay (Hartley *et al*, 2011) was used to measure DNA interstrand crosslinking (ICL) induced by platinum drug treatment in parental and resistant cells. Exponentially growing A2780 cells were treated for 1 h with different concentrations of cis-DDP and collected after 9 h. All procedures were carried out on ice. Cells were diluted to a final concentration of 2.5×10^4 cells ml^{-1} and irradiated with X-ray (15 Gy) in order to deliver a fixed number of random DNA strand breaks (except for the untreated unirradiated control). Cells were embedded in 1% agarose on a precoated microscope slide, and each sample was prepared in duplicate. In subdued light, cells were lysed for 1 h in lysis buffer (100 mM disodium EDTA, 2.5 M NaCl, 10 mM Tris–HCl pH 10.5 and 1% Triton X-100 added at use) and then washed four times in distilled water every 15 min. Slides were incubated in alkali buffer (50 mM NaOH, 1 mM disodium EDTA, pH12.5) for 45 min followed by electrophoresis in the same buffer for 25 min at 18 V (0.6 V cm^{-1}), 250 mA. Finally, the slides were rinsed in neutralising buffer (0.5 M Tris–HCl, pH 7.5) then in saline and allowed to dry overnight at room temperature. Re-hydrated slides were stained with propidium iodide (2.5 μg ml^{-1}) for 30 min, rinsed in distilled water and oven-dried. Images were visualised using a NIKON inverted microscope (Chiyoda, Tokyo, Japan) with high-pressure mercury light source, 510–560 nm excitation filter and 590 nm barrier filter at $\times 20$ magnification. Images were captured using an on-line CCD camera and analysed using the Komet Analysis software 4.02 (Andor Technology, Belfast, UK). For each duplicate slide, 25 cells were analysed. The tail moment for each image was calculated as the product of the percentage of DNA in the comet tail and the distance between the means of the head and tail distributions (Olive *et al*, 1990). DNA ICL after drug treatment was expressed as the percentage decrease in tail moment compared with irradiated controls calculated by the formula:

$$\% \text{ decrease in tail moment} = \left[1 - \left(\frac{TMdi - TMcu}{TMci - TMcu}\right)\right] \times 100,$$

where $TMdi$ = tail moment of drug-treated irradiated sample; $TMcu$ = tail moment of untreated, unirradiated control; and $TMci$ = tail moment of untreated, irradiated control.

***In vivo* study.** Procedures involving animals and their care were conducted in conformity with the following laws, regulations and policies governing the care and use of laboratory animals: Italian Governing Law (D.lgs 26/2014; Authorisation n.19/2008-A issued 6 March 2008 by Ministry of Health); Mario Negri Institutional Regulations and Policies providing internal authorisation for persons conducting animal experiments (Quality Management System Certificate—UNI EN ISO 9001:2008—Reg. No. 6121); the NIH Guide for the Care and Use of Laboratory Animals (2011 edition); EU directives and guidelines (EEC Council Directive 2010/63/UE); and in line with Guidelines for the welfare and use of animals in cancer research (Workman *et al*, 2010).

Animal experiments has been reviewed and approved by the IRFMN Animal Care and Use Committee (IACUC) that includes members '*ad hoc*' for ethical issues. Animals were housed in the Institute's Animal Care Facilities, which meet international standards; they were regularly checked by a certified veterinarian who is responsible for health monitoring, animal welfare supervision, experimental protocols and procedure revision.

For *in vivo* studies, 4–6-week-old female athymic nude mice (Harlan Laboratories, Milan, Italy) maintained under specific pathogen-free conditions were used. Human ovarian A2780 cancer cells (10×10^6 cell per mouse) were implanted s.c. into the flank of recipient mice. When tumour was palpable (mean tumour weight value for the groups was about 100 mg), animals were divided randomly into treatment groups consisting at least of eight mice each. Tumour growth was measured three times a week using a caliper, and the tumour weights were calculated by the formula: length $\times$ (width)^2/2. Antitumour activity was expressed as $T/C\%$, where T and C were the mean tumour weights of treated and control groups, respectively. Treatment was considered active when $T/C < 42\%$.

Trabectedin, cisplatin and carboplatin were administered intravenously once a week for three consecutive weeks (q7dx3) at the doses of 0.15, 5 and 50 mg kg^{-1}, respectively. We used these doses and this schedule as they are the well-tolerated commonly used doses in mice and they are very close to the MTD (maximum tolerated dose) (Romano *et al*, 2013; Ricci *et al*, 2014).

RESULTS

Trabectedin-resistant cell line derivation and characterisation. In order to investigate the mechanism of trabectedin resistance, we derived two trabectedin-resistant cell lines: 402-91/T and A2780/T. These sublines were generated from a myxoid liposarcoma and an ovarian cancer cell line, respectively, that were exposed to stepwise increasing concentrations of trabectedin for 10 1-h treatments. The sensitivity to trabectedin of the parental and resistant cell lines was tested by clonogenic assay as shown in Figure 1. We first considered the parental cell lines and observed that 402-91 cells are more sensitive to trabectedin than A2780 cells, with IC$_{50}$ values 1.5 and 8.2 nM, respectively. Second, we compared the IC$_{50}$ values between parental and resistant cells and we revealed that 402-91/T cells (Figure 1A) and A2780/T cells (Figure 1B) were six-fold less sensitive to trabectedin than the respective parental cell lines (1.5 *vs* 8.3 nM and 8.2 *vs* 48 nM, respectively; $P \leqslant 0.0001$, Student's *t*-test). For both cell lines, the resistance was irreversible over 1 year of continuous culture in drug-free medium, and no changes in morphology and growth features, compared with the parental cell lines, were observed.

Sensitivity to UV and involvement of NER. As demonstrated in previous studies (Takebayashi *et al*, 2001; Uboldi *et al*, 2012), trabectedin resistance is frequently associated with defects in the NER pathway and with increased sensitivity to UV light. We analysed the effects induced by UV light exposure in the resistant and parental cell lines. Figures 2A and B showed that both the 402-91/T and A2780/T cells were more sensitive (four- and six-fold, respectively) to UV light than the respective parental cells.

The high sensitivity of the resistant cell lines to UV light prompted us to investigate the expression of key proteins of the NER pathway, including XPG, XPF and ERCC1 (Figures 2C and D). In the A2780/T cell line, western blotting analysis revealed no detectable XPG expression, while RT–PCR analysis showed a slight downregulation of mRNA expression (fold change 0.43) when compared with parental cells. In contrast, 402-91/T did not express XPF and ERCC1, but no differences were observed in mRNA level for both genes (fold change 0.70 and 0.84, respectively).

Cell cycle effects and platinum drug sensitivity. In order to better characterise the resistant cell lines, we analysed the cell cycle perturbations induced by trabectedin using standard flow cytometric methods. DNA cell cycle analysis was performed on controls and cells treated for 1 h with different concentrations of trabectedin and collected 24, 48 or 72 h after drug washout. As shown in Supplementary Figures S1A and C, trabectedin caused accumulation of cells in G$_2$/M phase in 402-91 at 2.5 nM and in A2780 at 5 nM, already detectable at 24 h. The analysis performed at 48 and 72 h demonstrated that the cells overcame the block and progressed through the cell cycle. In contrast, the cells treated with a higher drug concentration remained blocked in the G$_2$/M phase of the cell cycle up to 72 h. In resistant cell lines, trabectedin did not induce the G$_2$/M block, even at higher drug concentrations (Supplementary Figures S1B and D). These data were in agreement with previous studies showing that NER status affected trabectedin efficacy: NER-proficient cells were more sensitive to the drug than NER-deficient ones (Damia *et al*, 2001) and displayed different perturbations of the
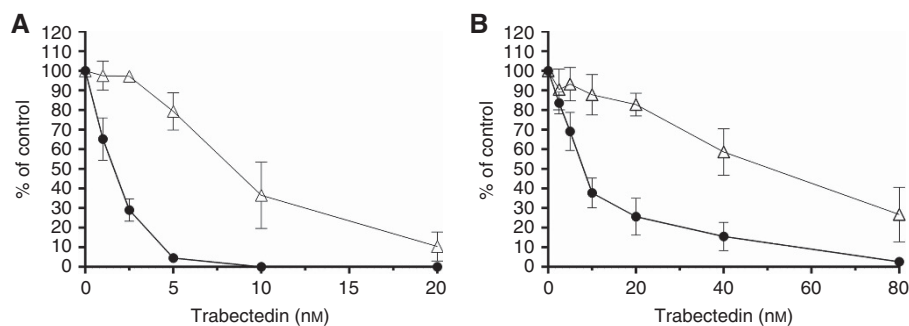

Figure 1. Sensitivity to trabectedin of the parental and resistant cell line tested by clonogenic assay. (**A**) 402-91 (●) *vs* 402-91/T (△); (**B**) A2780 (●) *vs* A2780/T (△). Each point is the mean of five replicates of three independent experiments; bars represent s.d.

cell cycle consisting of a greater accumulation of cells in G_2/M phases after trabectedin treatment (Tavecchio et al, 2007).

Previous studies have demonstrated that cell lines with NER deficiencies were more sensitive to cisplatin treatment (Stevens et al, 2008; Wood, 2010). As shown in Figure 3, we evaluated the sensitivity of the trabectedin-resistant and parental cell lines to platinum drugs. We treated the cells with cisplatin (1-h treatment) or carboplatin (24-h treatment) and observed, for both cancer cell lines, that trabectedin-resistant sublines were two-fold more sensitive to platinum drugs than the parental lines.

γ-**H2AX DNA damage response.** The formation of phophory-lated histone H2AX (γ-H2AX) is considered a sensitive marker for the detection of DSBs (Dickey et al, 2009). As shown in previous studies (Soares et al, 2005; Guirouilh-Barbat et al, 2008; Tavecchio et al, 2008) trabectedin induces replication and transcription-coupled DSBs whose formation can be influenced by NER activity. H2AX phophorylation can also act as a sensitive and general marker of DNA damage induced by cisplatin in several cell models (Clingen et al, 2008; Spanswick et al, 2012). To measure possible differences in γ-H2AX formation between the A2780 and A2780/T
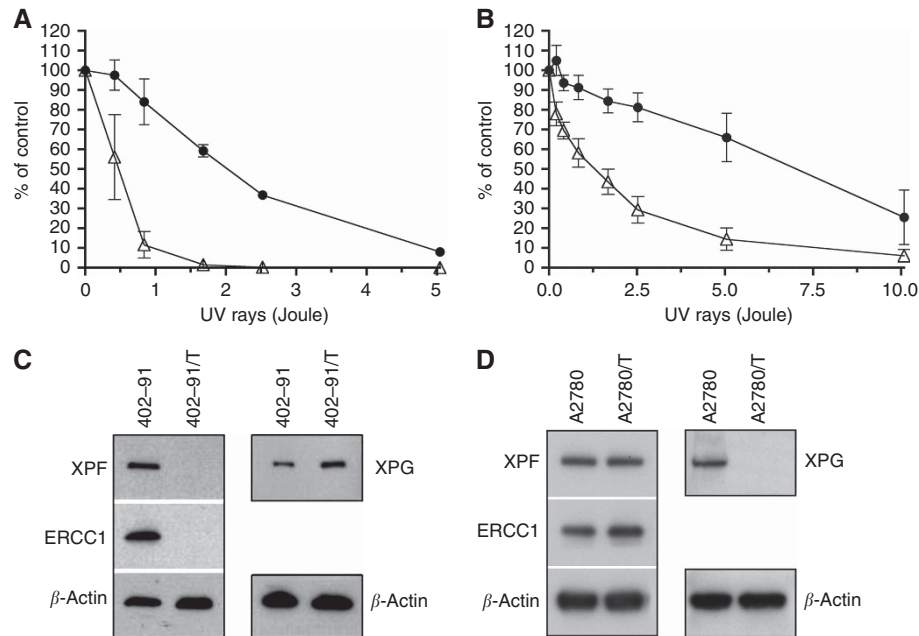

Figure 2. Effect of UV rays on the clonogenicity of (**A**) 402-91 (●) and 402-91/T (Δ) cell lines; (**B**) A2780 (●) and A2780/T (Δ) cell lines. Each point is the mean of five replicates of three independent experiments; bars represent s.d. Western blotting analysis of ERCC1, XPF and XPG protein expression in (**C**) 402-91 and 402-91/T; (**D**) A2780 and A2780/T.
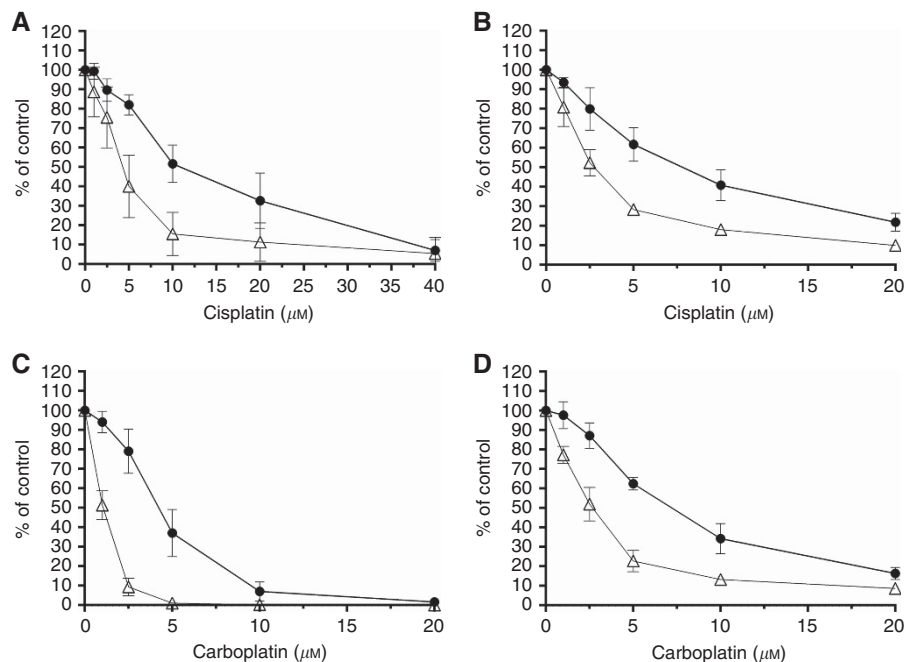

Figure 3. Collateral sensitivity to cisplatin or carboplatin assessed by WST-1 cell proliferation assay: (**A** and **C**) 402-91 (●) vs 402-91/T (Δ); (**B** and **D**) A2780 (●) vs A2780/T. Each point is the mean of five replicates of three independent experiments; bars represent s.d.

cell lines, we performed flow cytometric γ-H2AX analysis after trabectedin or cisplatin treatment. As shown in Figure 4B, treatment with equitoxic doses of trabectedin (20 nM for A2780 and 80 nM for A2780/T) generated a higher percentage of γ-H2AX-positive cells in the A2780 cell line. The induction of this DNA damage marker was time dependent in both cell lines but the distribution in the cell cycle phases of γ-H2AX-positive cells was different (Figure 4A). Starting from 8 h after drug washout, A2780-damaged cells were principally in early S phase while in A2780/T positive cells were detected in all the cell cycle phases with an accumulation in late S–G$_2$/M phase. Figure 4D shows that induction of H2AX phophorylation after treatment with equitoxic doses of cis-DDP (30 μM in A2780 and 15 μM in A2780/T) was time dependent and comparable for both cell lines. The analysis of the cell cycle phase distribution of cis-DDP-treated cells (Figure 4C) revealed that 24 h after drug washout the γ-H2AX-positive cells were principally accumulated in S–G$_2$/M phases both in A2780 and A2780/T cell lines.

DNA interstrand crosslinking. Spanswick *et al* (2012) demonstrated that A2780 cells were not able to 'unhook' cisplatin-induced DNA ICLs from DNA over a 48-h period. For this reason, we performed a modified comet assay to compare the levels of ICL formation after cisplatin treatment in the parental and trabectedin-resistant cell lines. Cells were treated for 1 h followed by 9-h incubation in drug-free medium to allow the peak of ICL formation, as previously described (Spanswick *et al*, 2012). As shown in Figure 5, treatment with equimolar doses of drug produced a higher level of ICL formation in trabectedin-resistant cells than in the parental ones.

***In vivo* collateral sensitivity to platinum drugs.** We performed *in vivo* experiments only for the ovarian cancer cell lines as the s.c. implantation of 402-91 cells did not generate an appreciable tumour mass. The collateral sensitivity to platinum drugs observed *in vitro* was confirmed *in vivo* (Figure 6). In spite of the fact that *in vivo* A2780 xenografts were unexpectedly found to be only marginally sensitive to trabectedin – possibly owing to pharmacokinetic reasons – A2780/T xenografts displayed a marked increase in sensitivity to cisplatin (*T/C* 23%) and carboplatin treatment (*T/C* 40%) compared with those obtained from parental xenografts (*T/C*: 61% and 60%, respectively).

DISCUSSION

In this paper, we report the development and characterisation of two trabectedin-resistant cell lines, myxoid liposarcoma 402-91/T and ovarian A2780/T cells that were six-fold less sensitive to the
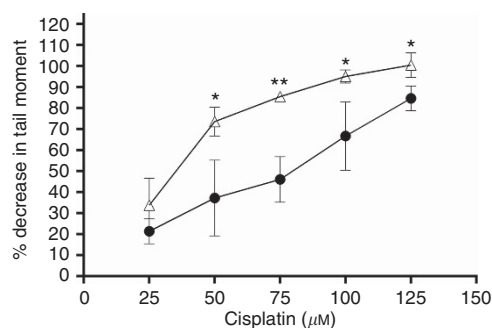
Figure 5. DNA ICL formation in A2780 (●) and A2780/T (Δ) cells following treatment with cisplatin. Samples were taken at 9 h after drug washout and ICLs were measured using the modified comet assay. Data are the mean ± s.d. from at least three independent experiments. *$P \leqslant 0.5$; **$P \leqslant 0.01$, Student's *t*-test.
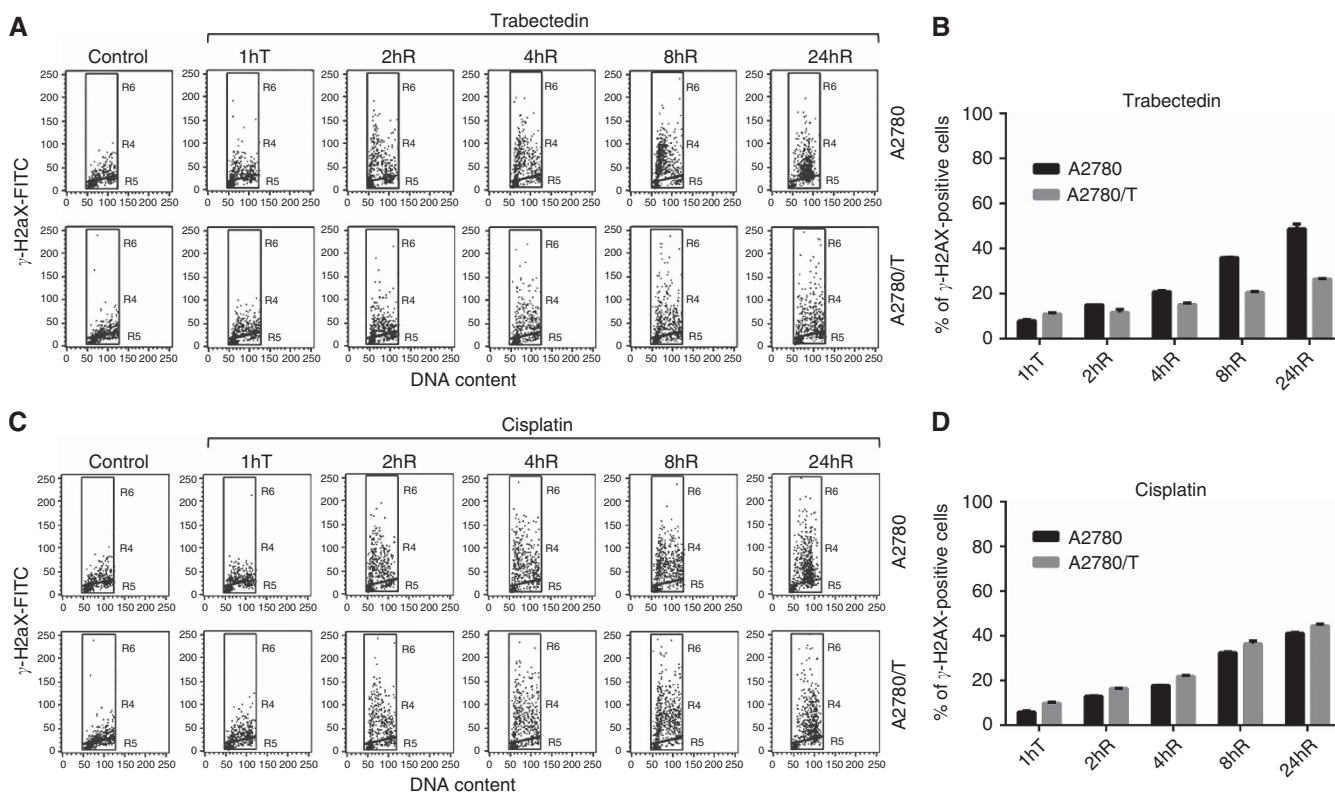
Figure 4. Analysis of γ-H2AX levels in A2780 and A2780/T cell lines after 1 h (**A** and **B**) trabectedin and (**C** and **D**) cisplatin treatment at different time points: 1hT (treatment), 2, 4, 8, and 24hR (drug washout). Each point is the mean of three independent experiments; bars represent s.d.
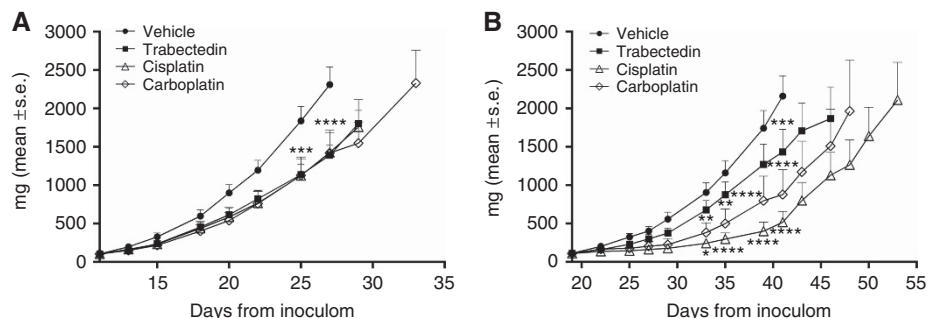
Figure 6. *In vivo* antitumour activity of trabectedin, cisplatin and carboplatin administered intravenously q7dx3 at the doses of 0.15, 5 and 50 mg kg^{-1}, respectively, in (**A**) A2780 and (**B**) A2780/T. *$P \leqslant 0.5$; **$P \leqslant 0.01$; ***$P \leqslant 0.001$; ****$P \leqslant 0.0001$, ANOVA test.

drug. Only a few cell lines have been described that show specific acquired resistance to trabectedin (Erba *et al*, 2001; Kanzaki *et al*, 2002; Manara *et al*, 2005; Marchini *et al*, 2005; Duan *et al*, 2009; Uboldi *et al*, 2012).

402-91/T and A2780/T cells were hypersensitive to UV radiation, and we demonstrated that both cell lines had defects in the NER pathway because they lack the expression of key proteins of this repair mechanism: XPF and ERCC1 in 402-91/T and XPG in A2780/T. These data are in keeping with previous studies conducted on cell lines with defined defects of DNA repair mechanisms and which suggested that trabectedin efficacy can be affected by NER and HR activity (Damia *et al*, 2001; Erba *et al*, 2001). Defects in HR were associated with high sensitivity to the drug while cells that are defective in TC-NER are partially resistant to trabectedin and hypersensitive to UV light (Damia *et al*, 2001; Takebayashi *et al*, 2001). The role of XPG status in trabectedin efficacy is important as this protein is part of the ternary complex (trabectedin–DNA–XPG) that stalls the DNA replication fork during S-phase resulting in DNA breaks that need to be repaired by HR (Herrero *et al*, 2006). It is known that XPF and ERCC1 take part in a subsequent step in removing trabectedin from the DNA (de Laat *et al*, 1999) and the fact that 402-91/T cells were resistant to trabectedin confirms the importance of the NER pathway in the modulation of trabectedin efficacy. Even if the relationship between transcript and protein expression is unknown for NER molecules (Stevens *et al*, 2008), our RT–PCR data revealed no downregulation in mRNA expression of XPF, ERCC1 and XPG, suggesting that the lack of protein expression might be due to modification at the posttranscriptional level.

Differently from the mode of action of other alkylating agents, trabectedin causes strong perturbations of the cell cycle causing a delay of cells progressing from G_1 to G_2 and ultimately resulting in a G_2/M block in cells with functional NER (Erba *et al*, 2001; Minuzzo *et al*, 2005; Tavecchio *et al*, 2007). This paper supports this observation showing that trabectedin-resistant cells, which are defective for NER pathway, did not undergo the G_2/M block observed in parental cells (NER proficient).

As expected, cells deficient in NER were hypersensitive to UV light and particularly sensitive to platinum drugs. In the present paper, we show for the first time that trabectedin-resistant cells exposed to platinum drugs are more sensitive to these drugs because of an increased formation of DNA ICLs that represent the critical toxic lesion of the drugs. This finding was convincingly reproduced *in vivo* and thus could be potentially clinically relevant. It is in fact plausible that ovarian cancer patients receiving trabectedin may become more sensitive to subsequent platinum-based therapies. This hypothesis is supported by the retrospective analysis of the survival of patients who had participated in the OVA301 trial that was designed to compare the effectiveness of the combination of trabectedin and pegylated liposomal doxorubicin (PLD) with PLD alone (Monk *et al*, 2015). It was found that

patients who received platinum-based therapy at relapse following trabectedin survived much longer than those who received the same therapy after PLD alone (27.7 *vs* 18.7 months) (Poveda *et al*, 2014). Although this retrospective analysis requires prospective validation, the data shown in the present study provide a clear molecular rationale for the efficacy of the sequential treatment with trabectedin and platinum drugs.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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