Relevance of the Fanconi anemia pathway in the response of human cells to trabectedin

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Abstract

Trabectedin (Yondelis; ET-743) is a potent anticancer drug that binds to DNA by forming a covalent bond with a guanine in one strand and one or more hydrogen bonds with the opposite strand. Using a fluorescence-based melting assay, we show that one single trabectedin-DNA adduct increases the thermal stability of the double helix by >20°C. As deduced from the analysis of phosphorylated H2AX and Rad51 foci, we observed that clinically relevant doses of trabectedin induce the formation of DNA double-strand breaks in human cells and activate homologous recombination repair in a manner similar to that evoked by the DNA interstrand cross-linking agent mitomycin C (MMC). Because one important characteristic of this drug is its marked cytotoxicity on cells lacking a functional Fanconi anemia (FA) pathway, we compared the response of different subtypes of FA cells to MMC and trabectedin. Our data clearly show that human cells with mutations in *FANCA, FANCC, FANCF, FANCG*, or *FANCD1* genes are highly sensitive to both MMC and trabectedin. However, in marked contrast to MMC, trabectedin does not induce any significant accumulation of FA cells in G_2-M. The critical relevance of FA proteins in the response of human cells to trabectedin reported herein, together with observations showing the role of the FA pathway in cancer suppression, strongly suggest that screening for mutations in FA genes may facilitate the identification of tumors displaying enhanced sensitivity to this novel anticancer drug. [Mol Cancer Ther 2008;7(5):1309–18]

Introduction

Trabectedin (ET-743; Yondelis; Fig. 1A) is a potent antitumor drug currently undergoing phase II/III clinical trials that has recently received regulatory approval from the European Medicines Agency for the treatment of metastatic or advanced soft-tissue sarcoma after failure to anthracyclines and ifosfamide (1). Trabectedin binds in the DNA minor groove by establishing a covalent bond with the exocyclic 2-amino group of a guanine in one strand and one or two hydrogen bonds with other DNA bases in the opposite strand (Fig. 1B) depending on sequence context (2). This type of interaction differs from that observed for other DNA-binding antitumor drugs and could account for some of the distinctive pharmacologic features of trabectedin, such as the potent and rapid inhibition of activated but not constitutive transcription (3, 4) and the atypical response detected in cells with deficiencies in nucleotide excision repair (NER). In this latter respect, whereas all known DNA-interacting drugs are either more effective or equally effective in NER-deficient cells relative to their NER-proficient counterparts (5–8), trabectedin is paradoxically less cytotoxic in NER-deficient cells (9–12), particularly in those harboring a deleterious mutation in the XPG endonuclease (11, 13). On the other hand, experimental evidence from different laboratories has shown that both yeast mutants with gene deletions in the *RAD52* epistasis group [e.g., *Saccharomyces cerevisiae rad52Δ* (9) and *Schizosaccharomyces pombe rad51Δ* and *rad54Δ* cells (13, 14)] and mammalian cells deficient in *XRCC3, BRCA2, RAD51C*, and *XRCC2* (15) are much more sensitive to trabectedin than their normal counterparts. Because proteins encoded by these genes are components of the homologous recombination (HR) machinery, which is required for the repair of double-strand breaks (DSBs) associated with replication forks (16), all these data indicate that HR participates in the processing of the DNA lesions induced by trabectedin, including the generation and repair of DSBs (15, 17, 18). Given the unique mode of binding of trabectedin to DNA and the feasibility of having two or more trabectedin molecules simultaneously targeting adjacent or closely spaced sites on opposing strands (19, 20), we reasoned that this type of covalent adduct might functionally mimic an interstrand cross-link (ICL) such as that produced by the potent antitumor and bifunctional cross-linking agent mitomycin

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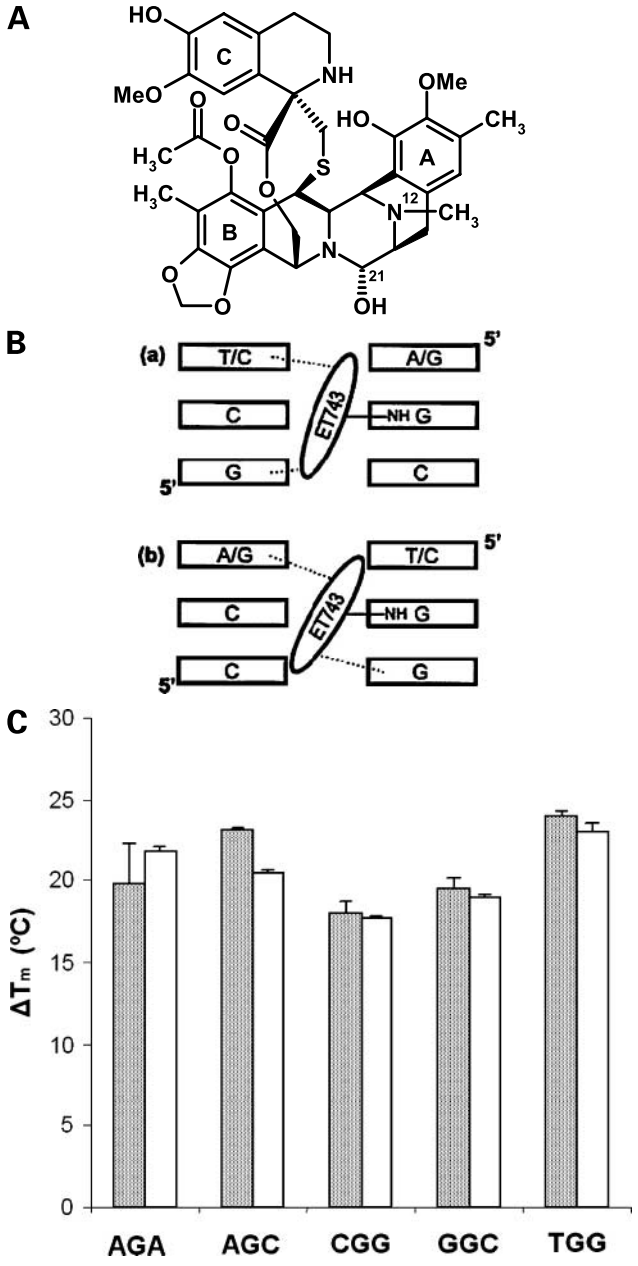

Figure 1. Analysis of the trabectedin adducts formed after incubation with selected duplex oligodeoxynucleotides. **A,** chemical structure of trabectedin. **B,** predicted mode of binding of trabectedin (ET-743) to dsDNA. Drug-DNA covalent bonds and hydrogen-bonding interactions are drawn, respectively, as *solid* and *dotted lines* in the two main types of complexes (**a** and **b**). **C,** increases in the melting temperatures of selected duplex oligodeoxynucleotides containing a single trabectedin covalent adduct in their central region following incubation with the drug for either 15 min (*shaded columns*) or 24 h (*open columns*). Mean ± SE of three independent experiments.

C (MMC; Supplementary Fig. S1;[6] ref. 21). ICLs represent a major threat to cell viability, insofar as they are highly effective in blocking replication and transcription forks (5). ICL resolution is known to occur through the coordinated action of multiple DNA repair pathways, including NER and HR, in a process that gives rise to DSBs because both DNA strands are covalently modified and need to be incised (16, 22).

Because of the reported structural characteristics of trabectedin-DNA adducts (2) we decided to investigate the extent to which a single drug molecule is able to stabilize dsDNA using a highly sensitive fluorescence-based melting assay (23). Increases in the thermal stability of the double helix suggested that trabectedin might mimic the cellular effects induced by ICL drugs. In this respect, previous studies have shown that cells deficient in the Fanconi anemia (FA) pathway display high sensitivity and show a characteristic accumulation in the G_2-M phase of the cell cycle after treatment with ICL drugs (24). Thus far, 13 different FA genes have been cloned, which code for proteins forming part of the upstream FA core complex (FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, and FANCM), for proteins of the FA-ID complex (FANCI and FANCD2), or for proteins functioning downstream the FA-ID complex (FANCD1/BRCA2, FANCJ/BRIP1, and FANCN/PALB2; see review in ref. 25). Significantly, proteins participating in all of these different steps of the FA pathway have been related to DNA repair and cancer suppression (26–30).

Our data show for the first time that cells with mutations in any of the FA genes tested are extraordinarily sensitive to trabectedin. This finding provides new clues for understanding the mechanism of action of trabectedin in human cells and strongly suggests that tumors incapable of activating the FA pathway will be particularly sensitive to this anticancer drug.

Materials and Methods

Fluorescence Melting Experiments

Synthetic oligodeoxynucleotides with one strand 5′-end labeled with the fluorophore 6-carboxyfluorescein (F) and the complementary strand 3′-end labeled with the fluorescence quencher tetramethylrhodamine (Q) were synthesized and purified by high-performance liquid chromatography as a single peak by Bonsai Technologies Group. Annealing of each F-oligonucleotide with its complementary Q-oligonucleotide at a final duplex concentration of 0.1 μmol/L in TAE buffer [0.08 mol/L Tris-acetate, 2 mmol/L EDTA (pH 7.5)] was accomplished in a PTC-100 thermocycler (MJ Research) by first heating to 95°C for 5 min and then gradually cooling down to 20°C at a rate of 1°C/min. Correct annealing was confirmed by monitoring the quenching in fluorescence that takes place when F and Q are placed in proximity on formation of a double-stranded structure. The melting reactions were carried out in 96-well plates loaded with the preannealed double-stranded oligonucleotides in a total volume of 20 μL TAE buffer at a final concentration of 0.1 μmol/L either in the absence or in the presence of the drug. Trabectedin was dissolved in DMSO and serially diluted in the same solvent to yield final concentrations from 0.01 to 0.1 μmol/L. The

[6] Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

mixtures were incubated for either 15 min or 24 h at 37°C before the melting assay was started in a 7500 Fast Real-Time PCR System (ABI Prism, Applied Biosystems) by increasing the temperature in small steps of 1°C/min up to 95°C. By recording the changes in fluorescence at 517 nm in the FAM channel obtained as a function of the temperature and calculating the midpoint of the transition (T_m), we were able to estimate the increases in melting temperatures (ΔT_m) brought about by drug binding. A series of custom-designed oligonucleotides were designed to provide a single occurrence of all possible binding sites for trabectedin in a single central triplet (Supplementary Fig. S2),[6] and all measurements were done in triplicate. The raw data recovered from the instrument for each plate consisted of an array of FAM fluorescence values for each of the 96 wells at each temperature. A complete analysis was then carried out using an in-house Visual Basic Application running on Microsoft Excel (23).

Cell Lines

Human fibroblastic cells FA-A (PD220), FA-C (PD331), and FA-G (PD352) were obtained from the Oregon Health Sciences University Fanconi Anemia Cell Repository. FA-D1 fibroblasts (HSC62) were kindly provided by Helmut Hanenberg. The normal diploid human lung fibroblast-like cell line, MRC-5, was kindly provided by J. Surrallés. Fibroblastic human cell lines derived from healthy donors and FA patients were cultured in DMEM supplemented with 10% fetal bovine serum (Lonza), 2 mmol/L L-glutamine (Life Technologies), 100 units/mL penicillin, and 100 μg/mL streptomycin (Pen-Strep, Life Technologies) and kept at 37°C in a humidified atmosphere with 5% CO_2. EBV-transformed lymphoblast cell lines (LCLs) were generated from healthy donors and from FA-A (FA88; ref. 31), FA-C (HSC526), and FA-F (EUFA698) patients. FA-C and FA-F LCLs were kindly provided by Helmut Hanenberg. LCLs were maintained in RPMI (Life Technologies) supplemented with 20% fetal bovine serum (Life Technologies) and antibiotics.

Analysis of the Cellular Sensitivity to Trabectedin, MMC, and Ethylnitrosourea

For assessing the sensitivity of fibroblast cell lines to the drugs, aliquots of 10,000 cells per well were seeded in 96-well plates (BD Falcon) in subconfluent conditions. On the next day, fresh medium containing increasing concentrations of the drugs was added. Twenty-four hours later, the culture medium was removed and replaced with fresh medium without the drugs. Three days after, the cell viability was tested using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide assay, which is based on the protocol described by Mossmann (32). Briefly, cells were incubated for 3 h with 0.45 mg/mL 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (Sigma), dissolved previously in serum-free medium. Cultures were washed with PBS followed by the addition of DMSO (Sigma), and gentle agitation for the complete dissolution of the insoluble purple formazan product into a colored solution. The absorbance of this colored solution was quantified by measuring in a microplate reader (GeniosPro, Tecan). Cell viability was expressed as the percentage of the absorbance value determined in wells in which there was no drug (considered as 100% of viability). Analyses of the viability of LCLs were determined essentially as described previously (31). Five days after treatment with the drugs, cells were resuspended in PBS-bovine serum albumin (BSA; 0.05%) containing 0.5 μg/mL propidium iodide (Sigma) and incubated for 10 min at 4°C. Cell viability was determined by flow cytometry (EPICS, Coulter Electronics) based on the propidium iodide exclusion test using a dot blot of forward scatter versus propidium iodide fluorescence. MMC (Life Technologies) and trabectedin (PharmaMar) were kept at -80°C as 1 mmol/L stock solutions and ethylnitrosourea (Sigma) as a 0.1 mol/L stock solution.

Cytometric Analysis of γH2AX as a Surrogate Marker of DNA DSBs

Histone H2AX phosphorylated on Ser^{139} (γH2AX) was quantitated by flow cytometry as reported previously (33). Aliquots of 10^5 cells were washed in PBS and fixed in suspension in 4.5 mL ice-cold 1% methanol-free formaldehyde in PBS for 15 min on ice. After centrifugation, cells were resuspended in 5 mL of 70% ethanol (0-4°C) and stored at -20°C at least for 2 h. Cells were washed twice in PBS, centrifuged, and blocked with 1 mL of 1% BSA and 0.2% Triton X-100 in PBS (BSA-T-PBS) for 30 min at room temperature. After centrifugation cells were incubated in BSA-T-PBS containing 1 μg γH2AX antibody (Upstate Biotechnology) overnight at 4°C. Cells were washed twice in BSA-T-PBS and incubated in BSA-T-PBS with Alexa Fluor 488–conjugated $F(ab)_2$ fragment of goat anti-mouse secondary antibody (1:100 dilution; Molecular Probes) for 1 h at room temperature with gentle shaking. After washing, cells were resuspended in 1 mL propidium iodide solution 5 μg/mL (Molecular Probes) and 100 μg/mL DNase-free RNase A (Sigma) and incubated for 30 min at 37°C. Finally, cells were analyzed by flow cytometry (Coulter XL) with bivariate dot-plot analysis of γH2AX fluorescence and linear fluorescence of propidium iodide (DNA content) from 10,000 events with doublet discrimination. Analyses of cell cycle phases were done using the MODFIT LT software (Verity Software House). The background percentage of γH2AX in untreated cells was set between 3% and 7%. Increases over background values were determined in each experimental group.

Immunofluorescence Studies of γH2AX and RAD51 Foci Formation

Cells were seeded in cover slides treated previously with 2 μg/cm^2 retronectin (Takara Shuzo). After treatment with MMC or trabectedin, cells were washed and fixed with 3.7% paraformaldehyde in PBS. Fixation was followed by permeabilization with 0.5% Triton X-100 in PBS for 5 min. After 30 min in blocking buffer (10% fetal bovine serum, 0.1% NP-40 in PBS), cells were incubated overnight with either anti-RAD51 rabbit antibody (PC130; 1:200 dilution; Calbiochem) and/or anti-γH2AX mouse antibody (1:2,000 dilution). Cells were then washed three times in TBS and subsequently incubated with secondary antibodies: Texas

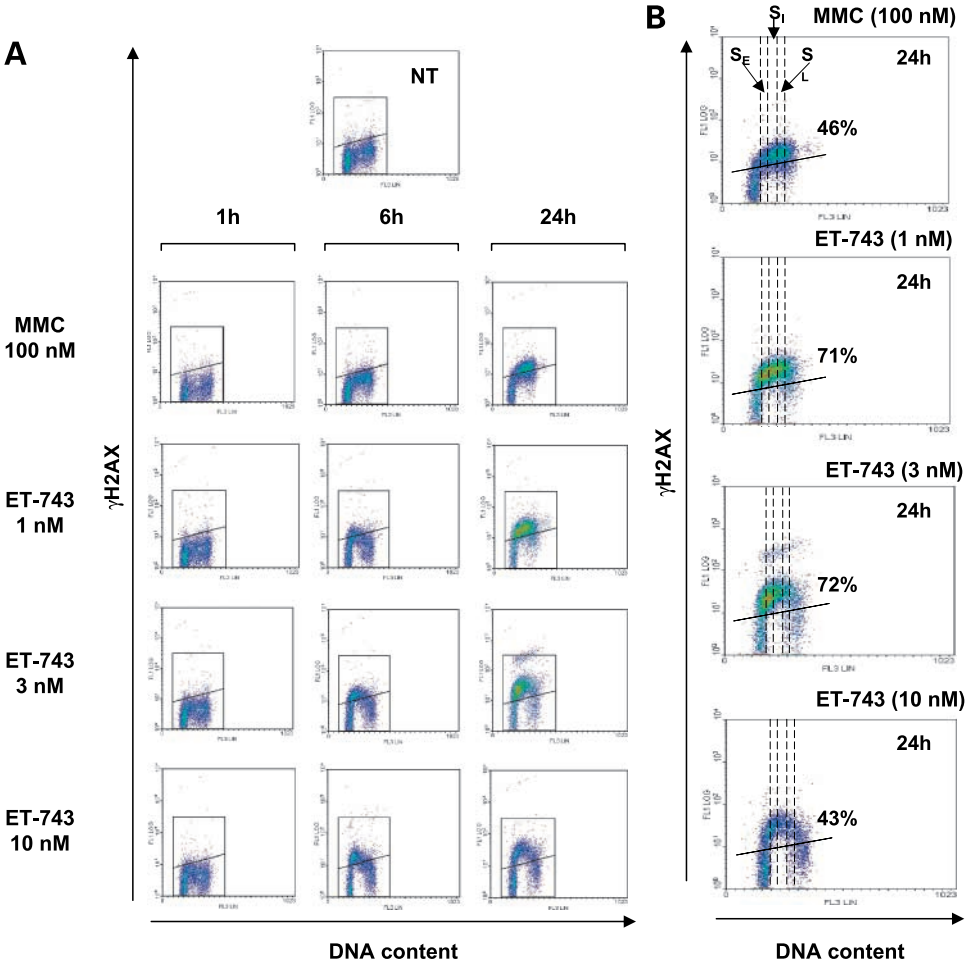

Figure 2. Generation of DNA DSBs in human MRC-5 fibroblasts treated with either MMC or trabectedin. **A,** representative flow cytometry analysis of γH2AX in MRC-5 cells treated with MMC and trabectedin (ET-743). **B,** detailed analysis of the generation of γH2AX in cells treated for 24 h with MMC or trabectedin (ET-743). SE, SI, SL; early, intermediate, and late S phase, respectively.

Red–conjugated anti-rabbit polyclonal antibody (Jackson Immunoresearch Laboratories) to detect RAD51 foci and Alexa Fluor 488 nm anti-mouse monoclonal antibody (Molecular Probes) for γH2AX detection. After 45 min, cells were washed for three times with TBS and slides were mounted in Moviol with 4,6-diamidino-2-phenylindole. Two hundred cells were scored using a fluorescence microscope (Zeiss Axioplan2), and cells with >10 foci of each marker protein were scored as positive (31).

Statistical Methods

Results from 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide assays were calculated as survival percentages respect to control cultures exposed to DMSO 0.1% (final concentration of drug vehicle in cultures). In Figs. 4 and 5, statistical differences between means were evaluated by using Student's t test. Differences were considered significant when $P \leq 0.05$. Results are reported as mean ± SE of at least three independent experiments.

Results

A Single Trabectedin Adduct Greatly Stabilizes dsDNA

The thermal stability of different duplex oligodeoxynucleotides, each containing a unique binding site for trabectedin in the central region, was assessed by means of a previously reported method based on molecular beacons (23). These duplexes encompass the two-hydrogen-bond-compliant purine-G-C and pyrimidine-G-G combinations shown in Fig. 1B as well as the more recently characterized AGA triplet for which only one hydrogen bond is possible (34, 35). The consistently high increment in melting temperature of dsDNA (average $\Delta T_m \geq 20°C$) was at least 2-fold greater than that brought about by a tight-binding bisintercalating agent such as echinomycin or thiocoraline (23) and was essentially identical after 15 min or 24 h of incubation in the whole range of drug/DNA ratios studied (1:10 to 1:1). The extra DNA stabilization was very similar for all the duplexes that embedded one of the preferred binding sequences in the central triplet irrespective of the number of hydrogen bonds (Fig. 1C), whereas the disfavored sequence studied, CGA (34, 35), was not stabilized at all (data not shown). These results are consistent with previous findings using plasmid DNA molecules (35, 36) and argues strongly in favor of the view that the hampering of DNA strand separation brought about by the covalent adduct may result in a strong blockade of transcription and replication forks in living cells. This behavior is reminiscent of that elicited by

other antineoplastic drugs that give rise to true ICLs such as MMC (37).

Therapeutic Doses of Trabectedin Induce DNA DSBs and Activate HR Repair in Human Cells

Because a single trabectedin adduct was very effective in hampering DNA strand separation, we asked whether low concentrations of trabectedin, similar to those observed in the plasma of patients treated with this drug (38), could result in the generation of DSBs in the DNA of cultured cells.

As deduced from the analysis of the γH2AX, treatment of MRC5 fibroblasts with either trabectedin or MMC induced DSBs (Fig. 2). Of note, doses of trabectedin as low as 3 nmol/L and even 1 nmol/L were sufficient to generate DSBs. Nonetheless, whereas MMC induced a predominant γH2AX marking in the intermediate/late S phase of the cell cycle, in trabectedin-treated cells, a high proportion of γH2AX staining could be observed in the early stages of the S phase (see representative analyses in Fig. 2B). Interestingly, the percentages of γH2AX-positive cells after 24-h exposures were lower in cells treated with 10 nmol/L trabectedin than in cells exposed to the lower concentrations of 1 or 3 nmol/L trabectedin (43% versus 71% and 72%, respectively).

The generation of DSBs in human cells treated with low doses of trabectedin was confirmed in human LCLs (see representative analyses in Fig. 3A). Moreover, kinetic analyses of γH2AX marking in these cells confirmed the higher efficacy of low (3 nmol/L) versus higher (10 nmol/L) doses of trabectedin to generate DSBs in these cells (Fig. 3B). Because differences in the generation of DSBs by 1 to 10 nmol/L trabectedin could be related to differences in the cell cycle status of the cells, we also determined the kinetics of γH2AX marking within the S phase of trabectedin-treated cells (Fig. 3B). Once again, these analyses confirmed the very high efficacy of trabectedin for generating DSBs within the S phase (90% of LCLs in S phase were positive for γH2AX after a 72-h exposure to 1 nmol/L of the drug) and showed that the relatively high dose of 10 nmol/L gives rise to a plateau effect when cells are exposed to the drug for 6 to 72 h.

Because HR constitutes an error-free repair mechanism that can participate in the processing of DNA ICLs and DSBs, we investigated whether therapeutic doses of trabectedin could activate the HR machinery of the cell. To this aim, the generation of nuclear RAD51 foci was determined immediately after and 40 h after a 6-h treatment with trabectedin (Fig. 4). As was the case in MMC-treated cells, a significant increase in the proportion of RAD51-positive cells was observed immediately after the 6-h exposure with trabectedin, an effect that persisted at least 40 h later. As shown in the representative pictures

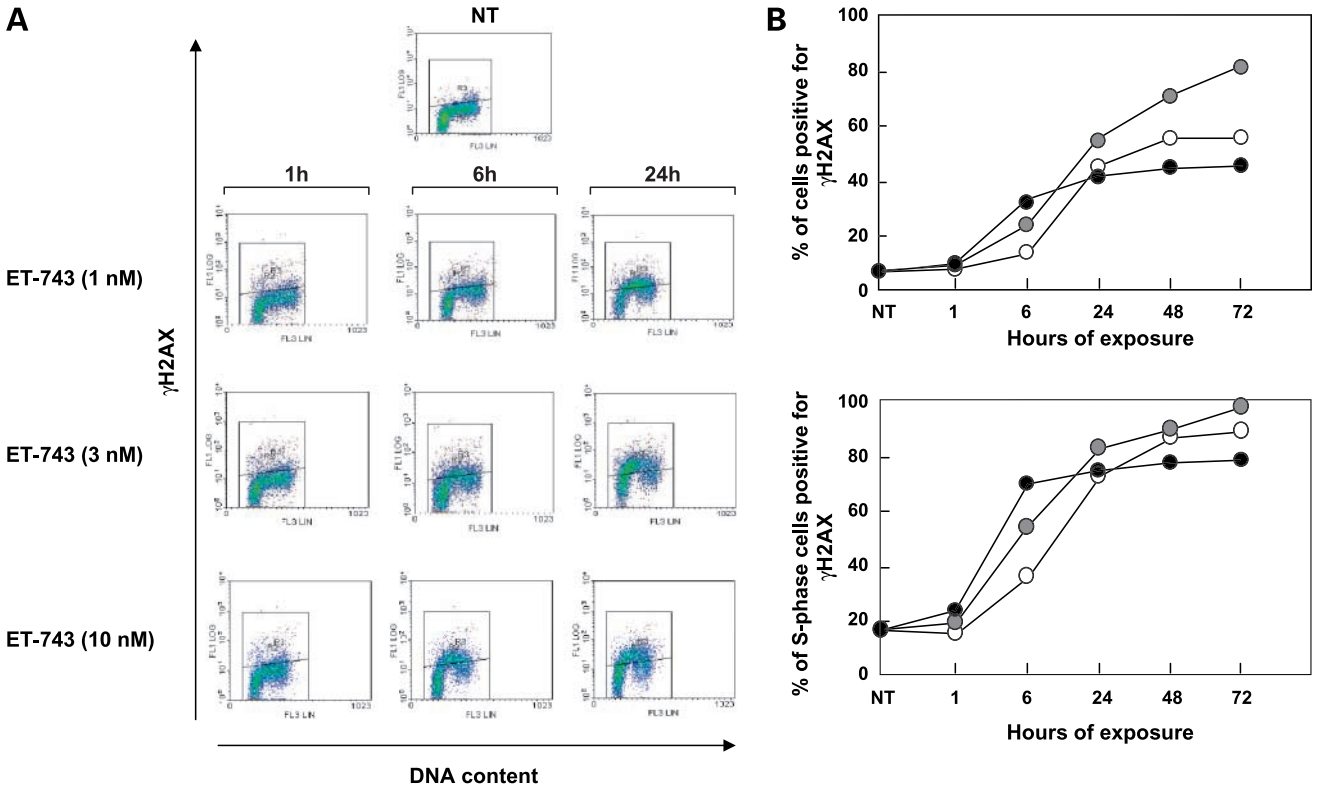

Figure 3. Generation of DNA DSBs in human LCLs treated with trabectedin. **A,** representative analyses of γH2AX in human LCLs treated with trabectedin (ET-743) for 1, 6, and 24 h. **B,** time course analysis of γH2AX in human LCLs treated with 1 nmol/L (○), 3 nmol/L (◍), or 10 nmol/L (●) trabectedin for 1 to 72 h. *Bottom,* proportion of cells in S phase that were positive for γH2AX staining. Representative experiment.

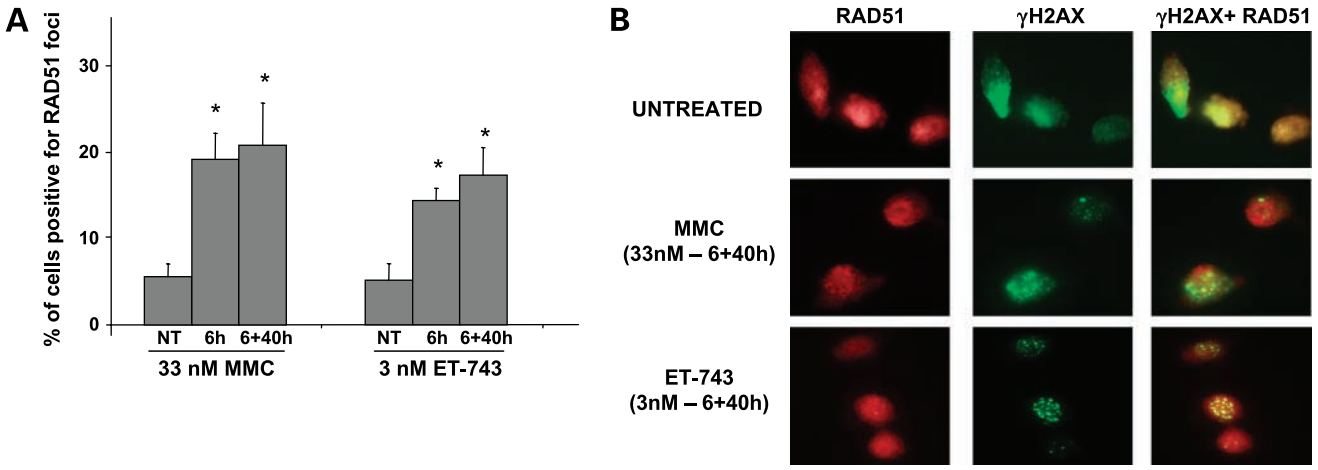

Figure 4. Induction of nuclear Rad51 foci in human LCLs treated with MMC and trabectedin (ET-743). **A,** proportion of RAD51-positive cells in untreated cells (*NT*) and in cells exposed for 6 h to MMC (33 nmol/L) or trabectedin (ET-743; 3 nmol/L) and analyzed immediately after treatment (6 h) or 40 h later (6 + 40 h). **B,** representative pictures showing RAD51-foci and γH2AX-foci in LCLs treated with MMC and trabectedin. Original magnification, ×1,000. Mean ± SE of three independent experiments. *, $P < 0.05$, significant differences between the proportion of cells positive for RAD51 foci in samples not exposed to the drugs and samples exposed to MMC or ET-743.

of Fig. 4, RAD51 always colocalized with γH2AX, in good consistency with the notion that RAD51 is recruited at sites of DSBs to facilitate their repair via HR.

Disruption of the FA Pathway Hypersensitizes Human Cells to Trabectedin

To study in more detail whether trabectedin mimicked the action of classic ICL drugs, we investigated whether one of the characteristic effects of these drugs, in particular their exacerbated cytotoxicity on cells deficient in the FA pathway, was also applicable to trabectedin. To this end, the effect of trabectedin on human fibroblasts competent in the FA pathway (MRC-5 cells) was compared with that on FA-A, FA-C, and FA-G fibroblasts (deficient in proteins of the FA core complex: FANCA, FANCC, and FANCG) and also on FA-D1 fibroblasts (deficient in FANCD1/BRCA2, a downstream protein in the FA pathway). MMC and ethylnitrosourea were used as positive and negative controls because ethylnitrosourea can only form monoadducts in a single DNA strand. As expected, all the FA fibroblasts tested were hypersensitive to MMC. Interestingly, fibroblasts deficient in proteins of the FA core (FA-A, FA-C, and FA-G cells) as well as FA-D1 fibroblasts were much more sensitive to trabectedin than wild-type (WT) cells (IC_{50} values were on average 8-fold lower; Fig. 5). In contrast to this observation, FA cells were not hypersensitive to ethylnitrosourea (Fig. 5). To confirm these findings, the sensitivity to trabectedin of FA-A, FA-C, and FA-F LCLs was also tested. Once again, FA cells (in these case, human FA LCLs) were highly sensitive to trabectedin compared with LCLs from healthy donors (IC_{50} values were 10-fold lower; Fig. 5). Taken together, these results show a similar dependence on the FA pathway to process the DNA damage induced by trabectedin and the classic ICL drug MMC but not by the agent that gives rise exclusively to monoadducts. The fact that mutations in genes encoding for proteins of the FA core complex (FANCA, FANCC, FANCF, and FANCG) as well as in the *FANCD1/BRCA2* gene confer cell hypersensitivity to trabectedin unequivocally establishes the critical relevance of the FA/BRCA pathway in the repair of the DNA damage induced by this drug.

Comparative Cell Cycle Analysis in Cells Treated with Trabectedin or MMC

To further investigate the cellular effects induced by trabectedin in comparison with those evoked by classic ICL drugs, we determined the cell cycle kinetics of WT and FA LCLs treated with trabectedin or MMC (Fig. 6). In WT cells, short exposures of 1 h with either drug induced an accumulation of cells in the S phase. On the other hand, prolonged exposures (48-72 h) to MMC induced cell accumulations in G_1, whereas the same exposures to 1, 3, and 10 nmol/L trabectedin induced cell accumulations in G_2-M, S, and G_1-S, respectively.

The cell cycle differences observed in cells treated with either MMC or trabectedin were even more striking when FA cells were used. Although the expected accumulation of FA-A cells in G_2-M was observed after MMC treatment (24-72 h exposures), none of the tested doses of trabectedin was capable of inducing any significant accumulation in G_2-M of FA-A cells, which were instead blocked in G_1 (see representative analyses in Fig. 6).

To investigate whether this striking difference between MMC and trabectedin was exclusively produced in FA-A cells, similar experiments were conducted in FA-C cells subjected to either acute (6 h) or chronic (up to 72 h) exposures to the drug. Data in Supplementary Fig. S3[6] confirmed the previously observed differences in the cell cycle response evoked by both drugs in FA cells. As happened in FA-A cells (see Fig. 6), chronic or acute exposures to trabectedin did not induce accumulation of FA-C cells in G_2-M (Supplementary Fig. S3[6]).

This final set of experiments clearly shows that the FA pathway is playing different roles in cell cycle control in the cells treated with either MMC or trabectedin. Thus, although our previous data in this study suggested that trabectedin mimics many of the DNA and cellular effects induced by classic ICL drugs, the characteristic blockade of FA cells in G_2-M elicited by the representative drug MMC is not reproduced by trabectedin.

Discussion

Although previous studies have investigated the mechanism(s) by which trabectedin inhibits cell growth (3, 9–13, 35, 36), the primary mechanism accounting for the potent antiproliferative effects of trabectedin in some cancer cell types is still poorly understood. A cancer cell line selected *in vitro* for its enhanced resistance to trabectedin (11) lacked a functional XPG (ERCC5), the structure-specific DNA endonuclease that makes the first 3′ incision on ssDNA during NER and transcription-coupled NER (39). Similarly, *S. pombe* cells deficient in *rad13* (the yeast orthologue of *XPG*) also displayed increased resistance to trabectedin and underwent much less DNA damage than the corresponding isogenic WT strain. However, additional experiments showed that it is not the missing endonuclease activity of this protein that confers resistance to trabectedin but the lack of a highly conserved basic residue in the DNA-binding domain that can presumably stabilize a rad13/DNA/trabectedin ternary complex through a direct interaction with the drug (13). This rather unique mechanism can account for the unusual behavior of these mutant cells because defects in NER proteins, which are involved in the repair of DNA damage caused by many DNA-binding anticancer drugs (e.g., the nitrogen mustard mechlorethamine, cisplatin, or MMC; refs. 5, 6), typically lead to increased drug sensitivity (5, 40, 41) and not the reverse, as is the case for trabectedin.

In contrast to observations made in NER-deficient cells, experiments on yeast and mammalian cells harboring specific deletions in HR genes (e.g. *RAD51C, XRCC2, XRCC3*, and *BRCA2*) have shown that HR mutants are highly sensitive to trabectedin (9, 13, 15). Because loss of HR repair was associated with the persistence of unrepaired DSBs, it has been proposed that DSBs play a key step in the processing of the DNA lesions produced by this drug (15). Furthermore, a strict requirement for DNA synthesis in the formation of these DSBs has been recently shown (15).

Based on these observations, and because of the particular structure of trabectedin, which endows it with the ability to form a covalent adduct with one DNA strand and simultaneously establish at least one hydrogen bond and extensive hydrophobic contacts with the complementary strand (Fig. 1B; refs. 2, 19, 20), we first investigated the extent to which trabectedin is able to stabilize dsDNA using a highly sensitive and accurate fluorescence-based method. Noteworthy, our analyses of the thermal stability of different duplex oligodeoxynucleotides each containing a single tailor-made trabectedin-DNA adduct showed a marked stabilization of the double helix following incubation with trabectedin (Fig. 1C). These results are compatible with the idea that DNA strand separation will be hampered intracellularly in the presence of the bonded drug, with the covalent adduct(s) behaving in practice similarly to an ICL, as reported for agents such as cisplatin (42) and the nitrogen mustard mechlorethamine (in a low percentage of lesions; ref. 43) or MMC (under reducing conditions; ref. 21). This hypothesis raised the interesting possibility that removal of the trabectedin-DNA adducts might require the same repair machinery that cells use for removing ICLs.

Although recent data have shown the generation of γH2AX foci after treatment with high doses of trabectedin (10-100 nmol/L; ref. 15), we were interested in understanding whether clinically relevant doses of trabectedin (~1-3 nmol/L; ref. 38) were also capable of inducing DSBs in human DNA compared with MMC.

Our γH2AX analyses showed that trabectedin recapitulates the DSBs induced by MMC in human cells even

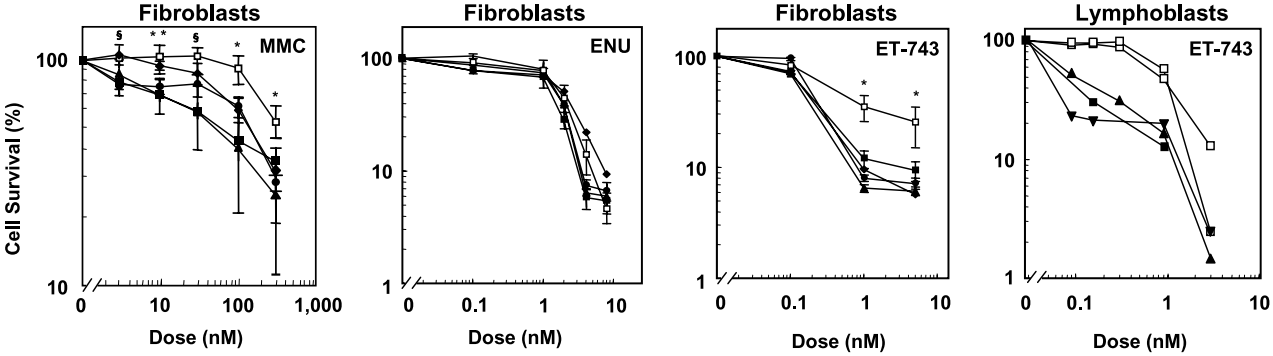

Figure 5. Increased sensitivity of human cells deficient in the FA/BRCA pathway to MMC and trabectedin (ET-743) but not to ethylnitrosourea (*ENU*). WT and FA fibroblastic and LCLs were treated with the drugs and analyzed for viability as indicated in Materials and Methods. Survival curves corresponding to WT (□), FA-A (▲), FA-C (■), FA-F (▼), FA-G (●), and FA-D1 (◆) are shown. Mean ± SE of three to five independent experiments. *, $P < 0.05$, significant differences between the control group and all groups of FA cells; §, significant differences between the control group and FA-A and FA-C cells; **, significant differences between the control group and FA-A, FA-C, and FA-G cells.

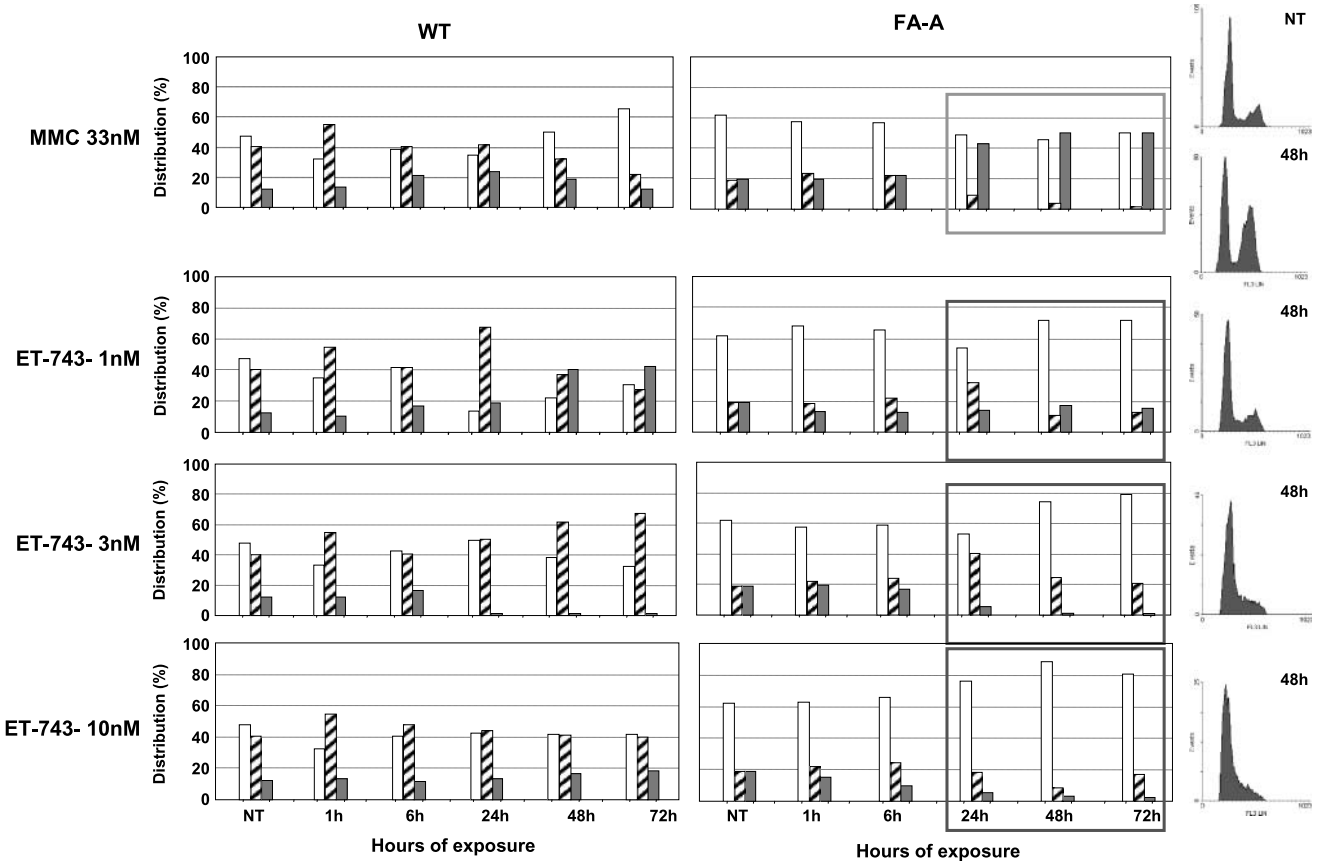

Figure 6. Cell cycle analysis of WT and FA-A human LCLs treated with either MMC or trabectedin. Cell cycle distribution of WT and FA-A LCLs exposed to 33 nmol/L MMC or to 1, 3, or 10 nmol/L trabectedin (ET-743) for 1 to 72 h. *Open columns,* G_1; *striped columns,* S; *gray columns,* G_2-M. Right panels show representative cell cycle histograms of FA-A cells either untreated (NT) or treated with MMC or ET-743 for 48 h (48 h).

when used at concentrations as low as 1 nmol/L (expected to be attained in the tumor cells of patients enrolled in the clinical studies; ref. 38). In good consistency with data reported for other cell types at a variety of concentrations (15), we observed the specific generation of DSBs during the S phase of the cell cycle (Fig. 3). Nonetheless, it is worth pointing out that our studies consistently show the relative inefficacy of 10 nmol/L trabectedin for inducing DSBs in human cells, compared with the lower, clinically relevant concentrations of 1 and 3 nmol/L, provided that sufficiently long incubation times (24-72 h) are allowed (Fig. 3B). Because the cytotoxic effects of trabectedin are clearly dose dependent (Fig. 5), the above-mentioned observation possibly points to some differences in the cytotoxic mechanism(s) at low and high concentrations of trabectedin. In fact, previous evidence has suggested two distinct and dose-dependent signaling routes that can be activated as a consequence of trabectedin treatment: a transcription-dependent pathway leading to cell cycle arrest and a transcription-independent pathway leading to rapid apoptosis (44).

As reported previously for MMC-treated cells (see Fig. 4A and data in ref. 45), trabectedin also promoted the translocation of RAD51 to the nucleus of cells in which DSBs had been generated, most likely as structural intermediates required for the correct processing of the trabectedin-DNA adducts (15). The observation that RAD51 was codetected with γH2AX in the same nuclear foci (Fig. 4B) is consistent with the idea that it is the DSB itself rather than the trabectedin-DNA adduct that induces the translocation of RAD51 to the site of the DNA damage to facilitate DNA repair via HR.

Based on the similarities in the generation of DSBs and activation of the HR machinery between trabectedin and MMC, we investigated whether the characteristic hypersensitivity of FA cells to ICL drugs was reproduced when FA cells were treated with trabectedin. In good consistency with recently published data (15), we observed that FA-D1 cells (lacking a functional BRCA2 protein that participates downstream the FANCI/FANCD2 complex; ref. 46) are hypersensitive to both trabectedin and MMC. Moreover, our data show for the first time that FA cells deficient in proteins of the FA core (upstream the FANCI/FAND2 complex; FANCA, FANCC, FANCF, and FANCG; ref. 47) are also hypersensitive to trabectedin (Fig. 5).

Data showing that cells deficient in FA/BRCA2 proteins are hypersensitive to both MMC and trabectedin not only provide a link between the FA pathway and the response of human cells to trabectedin but also disclose a hitherto unknown similarity in the mechanism of action of these two minor groove binding drugs.

Although trabectedin mimicked the action of MMC with respect to both generation of DSBs and participation of the FA pathway in the processing of the DNA damage, marked differences were noted in the effects of these two drugs on the cell cycle of WT cells, which were even more accentuated in FA cells. Thus, in contrast to the accumulation of FA cells in G_2-M after treatment with DNA ICL agents (48), our data in Fig. 6 and Supplementary Fig. S3[6] clearly show that trabectedin does not induce any significant blockade of FA-A and FA-C cells in G_2-M. The rationale for the mechanisms underlying these differential effects on cell cycle progression is currently unclear. Nevertheless, differences in the nature of the interaction of these drugs with the DNA, in the mechanism of checkpoint activation, or in the disruption of other cellular functions such as gene transcription may account for these discrepancies.

Finally, the mounting evidence showing that mutations in genes coding for proteins that participate in the FA pathway have a role in cancer (26–30) underscores the relevance of screening for mutations in FA genes in cancer cells from treatment-eligible patients because tumors with a disrupted FA pathway are likely to display enhanced sensitivity to trabectedin.

Disclosure of Potential Conflicts of Interest

L. Perez and J.C. Tercero: PharmaMar employees. J.A. Casado, P. Rio, E. Marco, B. Albella, F. Gago, and J.H. Bueren: PharmaMar grant support. The other authors reported no potential conflicts of interest.

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