

Replication and homologous recombination repair regulate DNA double-strand break formation by the antitumor alkylator ecteinascidin 743

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Adducts induced by the antitumor alkylator ecteinascidin 743 (ET-743, Yondelis, trabectedin) represent a unique challenge to the DNA repair machinery because no pathway examined to date is able to remove the ET adducts, whereas cells deficient in nucleotide excision repair show increased resistance. We here describe the processing of the initial ET adducts into cytotoxic lesions and characterize the influence of cellular repair pathways on this process. Our findings show that exposure of proliferating mammalian cells to pharmacologically relevant concentrations of ET-743 is accompanied by rapid formation of DNA double-strand breaks (DSBs), as shown by the neutral comet assay and induction of focalized phosphorylated H2AX. The ET adducts are stable and can be converted into DSBs hours after the drug has been removed. Loss of homologous recombination repair has no influence on the initial levels of DSBs but is associated with the persistence of unrepaired DSBs after ET-743 is removed, resulting in extensive chromosomal abnormalities and pronounced sensitivity to the drug. In comparison, loss of nonhomologous end-joining had only modest effect on the sensitivity. The identification of DSB formation as a key step in the processing of ET-743 lesions represents a novel mechanism of action for the drug that is in agreement with its unusual potency. Because loss of repair proteins is common in human tumors, expression levels of selected repair factors may be useful in identifying patients particularly likely to benefit, or not, from treatment with ET-743.

cancer therapy | natural products | lesion processing | DNA repair | response prediction

Compounds forming covalent DNA adducts (hereafter named DNA alkylators) are currently included in virtually all clinical drug regimens for the treatment of both leukemias and solid tumors. Lately, this group of compounds has attracted renewed interest because of the introduction of a novel class of antitumor agents with potent/unusual clinical activities, comprised of monofunctional DNA alkylators derived from natural sources. This group includes ecteinascidin-743 (ET-743, Yondelis, trabectedin), irifolven, hedamycin, and the acronycine derivative S23906. Mechanistic studies have revealed several differences in the way these compounds react with DNA-metabolizing enzymes as well as with the repair machinery (1–11), which could, at least in part, explain their different activity spectra. A better understanding of the factors controlling the induction and removal of DNA damage induced by these compounds should not only facilitate their clinical application, but may also contribute to our general understanding of the structural and biological features governing adduct processing in mammalian cells.

ET-743 has been subject to particular scrutiny linked to its source, clinical activity, and molecular mechanisms. ET-743 is a tetrahydroisoquinoline alkaloid isolated from the Caribbean sea squirt *Ecteinascidia turbinata* (12), pointing to marine products as a

potential new source of molecules with original chemical structures and activities. ET-743 shows a broad activity spectrum toward tumor cell lines at pM and low nM concentrations (6, 13) and has clinical activity toward ovarian cancer as well as soft tissue and bone sarcomas in heavily pretreated patients (14–16). In particular, ET-743 treatment is associated with a striking, long-term response in a subset of patients with otherwise chemoresistant soft tissue sarcomas (reviewed in ref. 17).

ET-743 binds to the minor groove of DNA with preference for GC-rich triplets and subsequently forms covalent adducts with the N2-position of guanine through its carbinolamine moiety (18). As a result, the minor groove is opened up and bended toward the major groove (19). The direction of bending is a novel feature among DNA minor groove-interactive agents, making ET-743 unique.

A particularly interesting aspect of the ET adducts is their interaction with cellular DNA repair pathways. ET lesions are not recognized by the global genome protein XPC but are recognized by transcription-coupled nucleotide excision repair (NER) as well as by proteins involved in the common NER pathway (1–3). Mammalian cells deficient in NER proteins such as the XPD and XPB helicases, and the XPG and ERCC1/XPF endonucleases showed 2- to 8-fold increased resistance to ET-743 in comparison with repair-proficient WT cells (1–3). Increased resistance to ET-743 was also reported for yeast mutants lacking the APN1 endonuclease, which particularly is involved in base excision repair (20). The unusual effect of these repair proteins on the cytotoxicity of ET-743 has been explained by the stabilization of repair complexes by the ET adducts (1–4, 21). Mismatch repair status had no detectable influence on the sensitivity to ET-743 (2, 6) whereas loss of the DNA-dependent kinase (DNA-PK) was reported to sensitize cells to ET-743 (2). Conflicting data have been reported for proteins involved in homologous recombination (HR) repair, because loss of repair function was associated with increased resistance to ET-743 in *Saccharomyces cerevisiae* (20) but increased sensitivity in *Schizosaccharomyces pombe* (21).

Although it is widely believed that the cytotoxic activity of ET-743 is based on its interaction with the NER machinery

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Abbreviations: DSB, double-strand break; HR, homologous recombination; NER, nucleotide excision repair; DNA-PK, DNA-dependent kinase.

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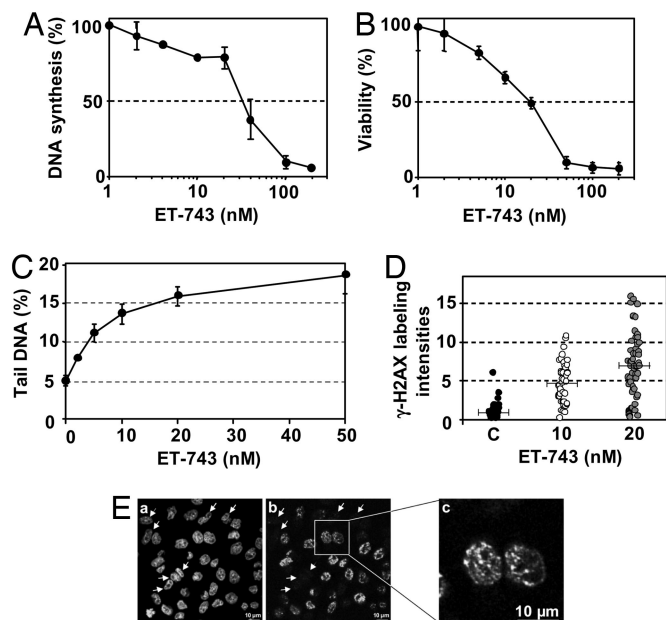


Fig. 1. Influence of ET-743 on DNA synthesis, cellular survival, and DSB formation. (A) HeLa cells were incubated with the indicated concentrations of ET-743 for 1 h, and the influence on DNA synthesis was measured by incorporation of radiolabeled thymidine. (B) HeLa cells were exposed to ET-743 for 1 h followed by postincubation in drug-free media for four doubling times and the MTT viability assay. (C) HeLa cells were incubated with the indicated concentrations of ET-743 for 1 h, and the formation of DSBs was determined by the neutral comet assay. The points indicate the average amounts of DNA in the comet tail. Error bars represent standard errors and are indicated when they exceed the symbol size. (D) Cells were incubated with the indicated concentrations of ET-743 for 1 h, and the formation of γ -H2AX foci was revealed by immunostaining. Total fluorescent intensities were determined by MetaMorph image analysis. Each point represents the γ -H2AX staining in an individual cell. Bars represent the average fluorescence intensities. (E) Confocal fluorescence microscopy of nuclear DNA (a) and γ -H2AX (b and c). Cells were incubated with 10 nM ET-743 for 1 h, fixed, and labeled with a γ -H2AX-directed antibody. DNA was counterstained with Topro-3. Arrows indicate cells with no detectable γ -H2AX signal.

(reviewed in ref. 17), the high residual activity of the drug toward NER-deficient cells as well as the rather modest resistance levels (2- to 8-fold) suggest that this cannot be the only mechanism of action of ET-743. We speculated that the ET adducts would be unusually stable because of the absence of DNA repair and thus able to interfere with the replication machinery whenever the treated cells entered S phase, possibly triggering the formation of secondary DNA lesions with increased toxicity.

We here report that exposure to pharmacologically relevant concentrations of ET-743 is associated with the induction of replication-dependent double-strand breaks (DSBs). Cells deficient for HR repair showed pronounced sensitivity to the drug due to the continued presence of unrepaired DSBs that gave rise to chromosomal abnormalities. Our findings characterize the processing of the monofunctional ET adducts into cytotoxic lesions and identify replication and HR repair as key modulators of the cellular activity of this unique compound.

Results

ET-743 Exposure Is Associated with Potent Inhibition of DNA Synthesis.

To establish whether unrepaired ET adducts affect DNA synthesis, the influence of ET-743 on the incorporation of radiolabeled thymidine was determined. The results indicate that DNA synthesis is reduced by 50% after 1-h exposure to 30 nM ET-743 (Fig. 1A). Next, cells were exposed to ET-743 for 1 h followed by postincubation in drug-free media for four cell doubling times, and the

cellular viability was determined by the MTT assay (Fig. 1B). The results show that the cellular viability is reduced by 50% after exposure to 20 nM ET-743. Therefore, inhibition of DNA synthesis and loss of cellular viability take place within the same dose range.

ET-743 Exposure Is Associated with a Dose-Dependent Formation of DNA DSBs.

Single-cell electrophoresis under neutral conditions (the neutral comet assay), which almost uniquely detects DSBs (22, 23), was used to determine whether ET-743 exposure is accompanied by formation of DNA strand breaks. The results (Fig. 1C) show that 1-h exposure of HeLa cells to ET-743 was associated with a dose-dependent increase in DSBs, which could be detected at doses as low as 2 nM. The rapid formation of DSBs indicates that they are a direct result of drug action and not due to apoptotic DNA fragmentation. In agreement, exposure to 10 nM ET-743 for up to 24 h was not accompanied by any detectable increase in the fraction of apoptotic cells as determined by annexin V labeling (data not shown).

ET-743 Exposure Is Accompanied by γ -H2AX Formation.

The formation of phosphorylated histone H2AX (γ -H2AX) is considered as a sensitive surrogate marker for the formation of DSBs. Cells were exposed to ET-743 for 1 h followed by immunostaining with a γ -H2AX-directed antibody. The results show that ET-743 exposure is accompanied by a dose-dependent increase in γ -H2AX formation [Fig. 1D and supporting information (SI) Fig. 5]. Interestingly, focalized γ -H2AX staining was observed only in a subset of the cell population, indicating a possible role of the cell cycle (Fig. 1E, compare a and b; arrows indicate cells without γ -H2AX staining). ET-associated induction of γ -H2AX was observed in all cell lines examined, including HeLa cells, Chinese hamster cells, and CEM human leukemia cells (data not shown).

Influence of DNA and RNA Synthesis. To determine whether the ET-induced DSBs were linked to macromolecular synthesis, HeLa cells were first incubated with different doses of ET-743 in the absence or presence of aphidicolin, a DNA synthesis inhibitor. The results show that aphidicolin substantially reduced the induction of DSBs even at doses as high as 50 nM ET-743 (Fig. 2A). In contrast, 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole, a selective inhibitor of RNA polymerase II, had no detectable influence on the formation of DSBs (data not shown).

To further characterize the role of DNA synthesis, HeLa cells were incubated with ET-743 in the presence of aphidicolin for 1 h followed by postincubation in drug-free media for 4 h (Fig. 2B). The results show that reinitiation of DNA synthesis during the postincubation period, as determined by incorporation of radiolabeled thymidine (data not shown), was accompanied by a gradual formation of DSBs. By 4 h after incubation, the DNA synthesis had recovered totally and the levels of DSBs had reached approximately the same levels as observed after 1 h in the presence of ET-743 alone. These results clearly indicate that the formation of ET-induced DSBs strongly, if not entirely, depends on active DNA synthesis. The results also suggest that even brief exposure to ET-743 results in the formation of long-lasting ET adducts, which are difficult to repair and can be converted into DSBs later.

ET-743-Mediated DNA Damage in Synchronized Cells.

To further confirm the role of DNA synthesis in the formation of DSBs, nonproliferating lymphocytes (that are in the G_0 phase of the cell cycle) were isolated from the peripheral venous blood of healthy donors and exposed to ET-743. The results showed no formation of DNA strand breaks, even at doses as high as 100 nM ET-743 as determined by the highly sensitive alkaline comet assay, in marked contrast to what was observed for cycling CEM lymphoblastic leukemia cells (SI Fig. 6).

Next, the influence of ET-743 toward unsynchronized, G_1 -synchronized, or S-synchronized HeLa cells was compared. The

the initial drug exposure. Unrepaired adducts led to formation of DSBs as determined by single-cell electrophoresis under neutral conditions and by γ -H2AX induction. Interestingly, γ -H2AX staining was completely absent in some cells, suggesting a role for the cell cycle in the formation of the ET-induced DSBs. In agreement, no DNA strand breaks were observed in resting lymphocytes or in cells synchronized in G₁, whereas cells synchronized in S showed increased levels of DSBs. These findings suggest a strict requirement for DNA synthesis in the formation of the ET-induced DSBs.

The formation of secondary DNA lesions in term of DSBs is not restricted to ET-743 but has previously been described for a number of other DNA-interacting agents including the DNA cross-linking agent mitomycin C (24) and the monofunctional DNA alkylator S23906 (11) and in NER-deficient cells after UV radiation (25) and is believed to occur when the advancing DNA replication fork runs into the DNA adducts. ET-induced DSBs were accompanied by the formation of focalized γ -H2AX, which is believed to promote the assembly of HR repair complexes around the DSB (26, 27). Mammalian cells deficient in HR showed pronounced sensitivity to ET-743 whereas nonhomologous end joining repair had minor influence on the sensitivity to ET-743. Although the relation between DNA synthesis and HR is well established (for recent review, see ref. 28), their respective links to DSB formation is more ambiguous, and several possibilities have been suggested. First, we might have a sequential model where the DSBs are created by the replication fork (or its collapse) thereby triggering HR. In this case we would expect little, if any, differences in the initial levels of DSBs between HR-proficient or -deficient cells. Second, HR might be needed to prevent replication fork collapse and to promote replication restart. In this case we would expect more initial levels of DSBs in HR-deficient cells. Third, the DSBs may be reaction intermediates formed during the recombination process that had become activated by the stalled replication forks. In this case we would expect more initial levels of DSBs in HR-proficient cells. Our experimental results provide clear evidence in favor of the first model. Strikingly, although HR status had little, if any, influence on the initial levels of DSBs after short exposure to ET-743, marked differences were revealed during the postincubation period, where no detectable repair was observed in the HR-deficient cells. The prolonged presence of unrepaired DSBs was followed by the appearance of mitotic chromosomes with chromatid aberrations in terms of gaps and breaks, in agreement with the S-phase-dependent induction of DSBs.

Previous studies showed that the ET adducts were recognized by transcription-coupled NER, a process that is triggered by arrest of the elongating RNA polymerase II by the DNA adducts. Surprisingly, 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole, a selective RNA polymerase II inhibitor, showed no detectable influence on the formation of ET-induced DSBs, suggesting that RNA synthesis does not contribute to DSB formation. Intriguingly, similar findings have previously been reported for mitomycin C, another DNA minor groove binder (29) associated with the formation of replication-dependent DSBs (24).

HR appears to be at least as important as NER in terms of modulating the response to ET, positively or negatively. Findings with mammalian repair mutants indicate that loss of NER function was associated with 2- to 8-fold increased resistance (1–3), whereas loss of proteins needed for HR repair was accompanied by 8- to 23-fold increased sensitivity (this study). The substantial residual activity of ET-743 toward NER-deficient cells suggests that the effects of NER and HR repair are, at least in part, independent events. In agreement, we observed a dose-dependent formation of DSBs in XPG-CS-deficient cells, which are deficient in both transcription-coupled NER and the common NER pathway (SI Fig. 7). In comparison, the levels of ET-induced DSBs were only slightly higher in repair-proficient WT and XPC cells, indicating that the putative DNA/ET/NER complexes are unlikely to be a major contributor to the formation of DSBs.

The conversion of the ET adducts into DSBs may, at least in part, be explained by the influence of the adducts on the properties of the local duplex DNA. Biochemical evidence as well as molecular modeling studies suggest that the ET adducts strongly stabilize the helical structure of duplex DNA, which is likely to slow down DNA separation during macromolecular synthesis (30, 31). In agreement, ET adducts were shown to strongly hamper DNA unwinding by the RecBCD helicase (9). These findings suggest that ET-743 may have functional similarities to cross-linking agents like mitomycin C, an established inducer of replication-dependent DSBs (24).

The important influence of different DNA repair pathways on the cytotoxic activity of ET-743 indicates that DNA repair functionality may be useful to assist in selection of the patients most likely to benefit from treatment with ET-743. This notion is supported by a preliminary clinical study with 92 sarcoma patients.¹ This study reports that ET-treated patients could be divided into three subgroups: one group responding with long-lasting progression-free survival, one group with an intermediate response, and one group of patients who did not benefit from the treatment. Importantly, long-term progression-free survival was associated with high expression of mRNA coding for the NER protein ERCC1 and low expression levels of the recombination proteins BRCA1 and BRCA2. In marked contrast, high expression of BRCA1/BRCA2 and low expression of ERCC1 were associated with lack of clinical response to ET-743 in every one of the 14 patients in this group.

Taken together, we have identified the formation of replication-dependent DSBs as a crucial component of the cytotoxic activity of ET-743 and provided a model explaining the involvement of replication and HR repair in the processing of ET-induced DNA lesions. Our data provide a rationale to use expression levels of selected repair factors for clinical response prediction and underline the interplay between macromolecular synthesis and repair pathways in DNA lesion processing.

Experimental Procedures

Chemicals and Cells. ET-743 was generously provided by the National Cancer Institute (Frederick, MD), and thymidine, aphidicolin, and nocodazole were purchased from Sigma (Saint-Quentin Fallavier, France). 5,6-Dichloro-1- β -D-ribofuranosylbenzimidazole was obtained from Calbiochem (La Jolla, CA).

HeLa cervical carcinoma cells and CEM lymphoblastic leukemia cells have been described previously (6). Recombination-deficient cell lines and their corresponding repair-proficient parental cells were generously provided by Malgorzata Zdzienicka (Leiden University Medical Center, Leiden, The Netherlands). The cells include *irs1*, defective in XRCC2 (32); *irs1SF*, defective in XRCC3 (33, 34); CL-V4B, defective in RAD51C (35); V-C8, defective in BRCA2/XRCC1/FANCD1 (36); and XR-V 15B, defective in Ku80/XRCC5 (37, 38).

DNA-PK-deficient Fus9 human M059J glioblastoma cells and DNA-PK-proficient Fus1 cells (39) were kindly provided by Bernard Salles (Centre National de la Recherche Scientifique/University Paul Sabatier, Toulouse, France). All cell lines were tested regularly for *Mycoplasma* contamination by PCR analysis.

Cell Synchronization. To synchronize HeLa cells in G₁, cells were treated with nocodazole (75 ng/ml) for 18 h followed by mitotic shake-off (70–90% mitotic cells) and postincubation in drug-free media for 3 h. A double thymidine block was used to synchronize cells at the G₁/S border. Cells were incubated with 2 mM thymidine for 16 h followed by 8 h of recovery and a second 16-h thymidine

¹Schöffski, P., Casali, P. G., Taron, M., Van Oosterom, A. T., Judson, I. R., Grosso, F., Blay, J.-Y., Maki, R. G., Tercero, J. C., Jimeno, J. M., Rosell, R. (2006) 42nd Annual Meeting of the American Society of Clinical Oncology, June 2–6, 2006, Atlanta, GA, abstr. 9522.

exposure followed by 3 h postincubation in drug-free media to permit the cells to enter S (>80% S-phase cells).

For “biological” synchronization, nonproliferating lymphocytes (that are in the G₀ phase of the cell cycle) were isolated from the peripheral venous blood of healthy donors by Ficoll centrifugation according to standard procedures (40).

Cell Cycle Analysis and Inhibition of DNA Synthesis. Cell cycle analysis was determined by flow cytometry analysis, and inhibition of DNA synthesis was determined by incorporation of radioactive thymidine as described previously (7, 41). Values are given as the mean of at least two independent experiments, each done in duplicate.

Viability Assays. The cytotoxic activity of ET-743 was measured by using the MTT colorimetric assay as previously described (6). All values are averages of at least three independent experiments, each done in duplicate.

For annexin V labeling, ET-treated cells were rinsed and stained with annexin V-FITC (Molecular Probes Invitrogen, Cergy-Pontoise, France) for 15 min at room temperature in the dark followed immediately by flow cytometry on an Epics XL/MCL flow cytometer (Beckman Coulter, Roissy, France).

Single-Cell Electrophoresis. Cells for comet analysis were exposed to the indicated drug concentrations at 37°C in the dark and analyzed immediately according to previously published procedures (21, 23, 42). Image analysis was performed by using Komet 5.5 software (Kinetic Imaging, Nottingham, U.K.). At least 100 cells were analyzed per sample. Values represent the average of at least two independent experiments.

Immunofluorescence and Microscopy. HeLa, AA8, or Irs1SF cells were plated onto coverslips and grown overnight before treatment with ET-743. CEM cells were treated with ET-743 and fixed onto

coverslips by cytospin (4 min at 265 × g, Cytofuge 2; Statspin, Norwood, MA). After treatment, cells were fixed in freshly prepared 4% paraformaldehyde for 20 min and permeabilized with PBS/Triton X-100 (0.5%) for 20 min. Coverslips were saturated in presence of PBS⁺ (PBS containing 1% BSA and 0.2% gelatin; Sigma), and antigens were revealed by immunolabeling using primary mouse antibodies directed against γ-H2AX (1:100 dilution; catalog no. 05-636; Upstate Biotechnology). The secondary antibody used in this study was Cy3-conjugated donkey anti-mouse IgG antibody (1:100 dilution; catalog no. 715-165-151; Jackson ImmunoResearch, Bar Harbor, ME). All antibodies were diluted in PBS⁺. Topro-3 was added to label DNA (1:5,000 dilution, T3605; Molecular Probes Invitrogen), and images were collected by using either a TCS SP confocal microscope (Leica) or a Retiga 1300 camera (QImaging, Burnaby, Canada) attached to a Nikon TS 100 microscope. Fluorescence intensities were measured by using MetaMorph software (Universal Imaging, Downingtown, PA). Background over noncellular regions was subtracted.

Karyotype Analysis. V79 parental cells and V-C8 mutant cells (BRCA2/XRCC1/FANCD1⁻) were exposed for 1 h to the indicated doses of ET-743. Cells were washed with PBS and postincubated in drug-free medium for 24 h, and chromosome spreads were prepared as described (22). One hundred metaphases per treatment condition were evaluated.

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