Ecteinascidin 743 Induces Protein-linked DNA Breaks in Human Colon Carcinoma HCT116 Cells and Is Cytotoxic Independently of Topoisomerase I Expression

Yuji Takebayashi, François Goldwasser, Yoshimasa Urasaki, Glenda Kohlhagen, and Yves Pommier

Laboratory of Molecular Pharmacology, Division of Basic Sciences, National Cancer Institute, NIH, Bethesda, Maryland 20892-4255

ABSTRACT

Ecteinascidin 743 (Et743; NSC 648766) is a potent antitumor agent presently in clinical trials. Et743 selectively alkylates guanine N2 from the minor groove of duplex DNA and bends the DNA toward the major groove. This differentiates Et743 from other DNA-alkylating agents presently in the clinic. To date, the cellular effects of Et743 have not been elucidated. Recently, Et743 DNA adducts have been found to suppress gene expression selectively and to induce topoisomerase I (top1) cleavage complexes in vitro and top1-DNA complexes in cell culture. In the present study, we characterized the DNA damage and the cell cycle response induced by Et743 in human colon carcinoma HCT116 cells. Alkaline elution experiments demonstrated that micromolar concentrations of Et743 produced comparable frequencies of DNA-protein cross-links and DNA single-strand breaks. The single-strand breaks were protein-linked SSBs and were not associated with detectable DNA double-strand breaks. By contrast with camptothecin, these lesions persisted for several hours after drug removal and were not formed at 4°C. Et743 treatment induced transient p53 elevation, dose-dependent cell cycle accumulation in G2-M and in G1-S, and S-phase, and inhibition of DNA synthesis. The sensitivity of camptothecin-resistant mouse leukemia P388/CPT45 cells, which fail to express detectable top1, was similar to the sensitivity of wild-type P388 cells, suggesting that top1 is not a critical target for the antiproliferative activity of Et743.

INTRODUCTION

The potent cytotoxicity of extracts from the Caribbean tunicate Ecteinascidia turbinata was first discovered in the late 1960s. However, the purification of active compounds was not completed until 1990 (1). Et7432 is one of several related marine alkaloids isolated from the mangrove Caribbean tunicate E. turbinata (Fig. 1). Et743 displays remarkable activities. Cytotoxicity is observed in vitro at low doses in various tumor cell lines, including melanoma, non-small cell lung, ovarian, renal, prostate, and breast cancers (1–3). Et743 is also a potent drug against human tumor xenografts, being equally active as, or more efficacious than, cisplatin (4, 5). Et743 is in Phase I/II clinical trials and recent results indicate that it represents a promising anticancer drug (6, 7).

Et743 (Fig. 1) binds to the DNA minor groove by alkylating the N2 amino group of guanine at specific sequences (8–11). The mechanism of covalent adduct formation involves the formation of an iminium intermediate caused by the intramolecular acid-catalyzed dehydration of the carboline amine functional group (at position 2 of Et743; Fig. 1). Thus, elimination of the adjacent hydroxyl group (at position 21) results in a Schiff base vulnerable to nucleophilic attack, leading to alkylation of the exocyclic 2-amino group of guanine in the DNA minor groove (Fig. 1). This alkylation reaction is DNA sequence-specific with preference for some guanine dinucleotide sequences. It sets Et743 apart from other known DNA alkylating agents (10, 11).

Recent studies have shown that Et743 can enhance the formation of top1-DNA complexes in cells (12, 13), and that Et743 enhances the formation of cleavage complexes with purified top1 (12). Et743 can also inhibit the binding of transcription factors such as NF-Y to DNA (14) and selectively inhibit gene transcription (15, 16).

The aim of the present study was to elucidate the cellular effects and DNA damage produced by Et743 in human carcinoma cells. Alkaline elution was used to detect various DNA lesions, including SSBs and DSBs, as well as DPCs (17). The only detectable DNA lesions induced by micromolar concentrations of Et743 were protein-linked SSBs. The significance of these results is discussed in light of cytotoxicity, top1 inhibition, and cell cycle data.

MATERIALS AND METHODS

Cell Culture and Chemicals. Human colon carcinoma HCT116 cell lines were cultured in RPMI 1640 (Life Technologies, Inc., Gaithersburg) containing 5% heat-inactivated FCS and 2 mM glutamine. No antibiotic was added to the medium. The cells were trypsinized and passaged once a week. P388 and P388/CPT45 mouse leukemia cells were a kind gift from Drs.

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2 Et743, Ecteinascidin 743; CPT, camptothecin; DPC, DNA-protein cross-links; SSB, DNA single-strand breaks; DSB, DNA double-strand breaks; top1, topoisomerase I; PCNA, proliferating cell nuclear antigen; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

To whom requests for reprints should be addressed, at Building 37, Room 4E28, NIH, Bethesda, MD 20892-4255. Phone: (301) 496-5944; Fax: (310) 402-0752; E-mail: pommier@nih.gov.

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Michael R. Mattern and Randal K. Johnson (SmithKline Beecham; Refs. 18 and 19). Et743 was provided by Dr. Glynn T. Faircloth, Pharma Mar USA, Inc., Cambridge, MA. CPT and etopside were provided by the National Cancer Institute Drug Synthesis and Chemistry Branch (Rockville, MD). Drug stock solutions were prepared in DMSO at a concentration of 10 mM. Additional dilutions were made in distilled water immediately before use. [methyl-3H]Thymidine and [14C]thymidine were purchased from New England Nuclear Research Products (Boston, MA).

**Measurement of Cellular DNA Damage.** DNA damage was measured using alkaline elution assays as described previously (17, 20). HCT116 cells were labeled with [14C]thymidine (0.04 mCi/ml) for 24 h. HCT116 internal standard cells were labeled with [methyl-3H]thymidine (0.2 mCi/ml) for 16 h. Cells were chased for an additional 24 h with radioisotope-free medium before drug treatments and were harvested after drug treatment by scraping into ice-cold HBSS.

**SSBs.** SSBs were analyzed using DNA-denaturing (pH 12.1) alkaline elution carried out under deproteinizing conditions. Cell suspensions were layered onto polycarbonate filters (2 μm, Poretics, Livermore, CA) and lysed with a solution containing 2% SDS, 0.1 M glycine, 0.025 M disodium EDTA, and 0.5 mg/ml proteinase K (pH 10.0). DNA was eluted with tetrapropylammonium hydroxide-EDTA containing 0.1% SDS (pH 10.0) at a flow rate of 0.02–0.03 ml/min. Fractions were collected at 3-h intervals for 15 h. Fractions and filters were processed and counted.

DSBs. DSBs were analyzed using non-DNA-denaturing (pH 9.6) elution carried out under deproteinizing conditions. After drug treatments, cells in ice-cold HBSS were layered onto polycarbonate filters (2 μm; Poretics, Livermore, CA) and lysed with a solution containing 2% SDS, 0.1 M glycine, 0.025 M disodium EDTA, and 0.5 mg/ml proteinase K (pH 10.0). DNA was eluted with tetrapropylammonium hydroxide-EDTA containing 0.1% SDS (pH 9.6) at a flow rate of 0.02–0.03 ml/min. Fractions were collected at 3-h intervals for 15 h. Fractions and filters were processed and counted.

DPCs. After drug treatment and scraping in ice-cold HBSS, cells were irradiated with 30 Gy. DPCs were analyzed under non-deproteinizing and DNA-denaturing conditions using protein-absorbing filters (polyvinylchloride-acryl copolymer filters, 0.8 μm pore size; Gelman Science, Ann Harbor, MI), and LS 10 lysis solution [2 M NaCl, 0.2% Sarkosyl, and 0.04 M disodium EDTA (pH 10.0)]. The DNA was eluted from filters with tetrapropylammonium hydroxide-EDTA (pH 12.1), without SDS at a flow rate of 0.02–0.04 ml/min. DPCs were calculated according to the bound-to-one-terminus model formula (17, 20, 21):

\[
DPC = [(1 - r)^{-1} - (1 - r_0)^{-1}] \times 3000
\]

where \( r \) is the retention for drug-treated cells and \( r_0 \) is the retention for the untreated cells.

**DNA Synthesis Inhibition.** Cellular DNA of exponentially growing HCT116 cells was labeled with 0.05 μCi [14C]thymidine for 48 h at 37°C. The rate of DNA synthesis was measured by 10-min pulses with 1 μCi/ml [methyl-3H]thymidine. [methyl-3H]Thymidine incorporation was stopped by washing cell cultures twice in ice-cold HBSS. After scraping the cells in 4 ml of ice-cold HBSS, 1-ml aliquots were transferred to 1.5-ml microcentrifuge tubes and precipitated with 100 μl of

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**Fig. 1** Chemical structure of Et743 (NSC 648766) and proposed reaction of its reactive iminium intermediate with the exocyclic 2-amino group of guanine in the DNA minor groove.
100% trichloroacetic acid. Samples were dissolved overnight at 37°C in 0.5 ml of 0.4 M NaOH and counted by dual-label liquid scintillation. ³H values were normalized using ¹⁴C counts. Inhibition of DNA synthesis was calculated as the ratio ³H:¹⁴C in treated cells over ³H:¹⁴C in untreated control cells.

**Flow Cytometry.** Samples were prepared for flow cytometry as described previously (22). Briefly, cells were harvested, washed in ice-cold PBS (pH 7.4), fixed in ice-cold 70% ethanol, and treated with RNase (500 units/ml; Sigma Chemical Co., St. Louis, MO) at 37°C for 30 min. Cellular DNA was stained with 50 µg/ml of propidium iodide (Sigma Chemical Co.), and cells were stored at 4°C before analysis. Cell cycle analyses were performed using a Becton Dickinson fluorescence-activated cell analyzer. Cells (1 × 10⁶) were analyzed for each point, and quantitation of cell cycle distribution was performed using the Cell-Quest software (Becton Dickinson, San Jose, CA).

**p53 and PCNA Immunoblotting.** Proteins from SDS-PAGE gel were electrophoretically transferred to immunobilon membrane (Bio-Rad, Hercules, CA) for 30 min at 15 V. The membrane was blocked for 1 h in PBS-Tween 20 containing 5% nonfat dried milk. p53 monoclonal antibody (DO-1) and PCNA monoclonal antibody (PC10) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Reactions were performed overnight and followed by incubation with horseradish peroxidase-labeled antimouse IgG (1:1000). After washing in PBS-Tween 20, membranes were developed using the enhanced chemiluminescence detection system (NEN Life Science Products).

**MTT Assays.** Cytotoxicity of Et743 in P388 and its resistant clone P388/CPT45 was measured using the MTT (Sigma Chemical Co.) colorimetric assay performed in 96-well plates. Cells were seeded (10⁴ and 1.5 × 10⁵ for P388 and P388/CPT45, respectively) into each well with 90 µl of RPMI 1640 containing 20% FCS. Ten µl of Et743 or CPT at each concentration were added to the wells, and incubations were continued for 3 days, after which 10 µl of MTT (5 mg/ml PBS) were added to each well. After an additional 4-h incubation, the resulting formazan was dissolved in 100 µl of 2-propanol containing 0.04 N HCl. Optical densities were read immediately at 570 nm using a Micro Plate Reader. The calculated IC₅₀ correspond to drug concentration required to inhibit cell growth to 50% of the untreated controls.

**RESULTS**

**Et743 Induces Temperature-sensitive and Persistent SSBs and DPCs.** The ability of Et743 to induce DNA damage in HCT116 cells was assayed by alkaline elution. SSBs and DPCs were measured after 1 h of exposure to Et743. Both lesions increased linearly with drug concentration (Fig. 2, A and C). These lesions were below detection level at Et743 concentrations <1 µM. Interestingly, both SSBs and DPCs failed to form at 4°C (Fig. 2, A and C), which is in contrast with the CPT-induced SSBs and DPCs, which are detectable at 4°C (23).
Kinetics experiments showed that both SSBs and DPCs increased progressively in drug-treated cells (Fig. 2, B and D). After washing the cells in drug-free culture medium, both SSBs and DPCs persisted and even increased (Fig. 2, B and D). These results indicate that Et743 induces temperature-sensitive and persistent SSBs and DPCs.

Protein-linkage of the SSBs Induced by Et743. The results of the alkaline elution assays described in Fig. 2 indicated that Et743 induced both SSBs and DPCs with comparable frequencies, temperature-dependence, and kinetics. To analyze further the relationship between the SSBs and DPCs induced by Et743, the frequencies of SSBs were plotted against those of DPCs in samples obtained from single cell cultures. Fig. 3A shows the results obtained for a total of 11 independent determinations. The points are within an area encompassed by ratios of 1:2 and 2:1 in the relative frequencies of SSB and DPC. This finding indicates that Et743 produces near equivalence of SSB and DPC. To examine further the relationship between SSB and DPC, alkaline elution assays were performed under deproteinizing or non-deproteinizing conditions (23). Five μM Et743 concentration was used in these experiments to investigate the protein-concealment of the SSBs under conditions where the SSB signal would be unambiguous. Et743-induced SSBs were detectable under deproteinizing conditions (compare triangles in Fig. 3, B and C). Together, these results indicate that Et743-induced SSBs appeared to be protein-linked.

Et743 Does Not Produce Detectable DSBs. The ability of Et743 to induce DSBs was assayed by alkaline elution at pH 9.6 (17). DSBs induced by 50 Gy of ionizing radiation or 50 μM etopside were used as positive controls. Et743 at the concentrations of 1, 5, or 10 μM, which produced high frequencies of SSBs, did not produce detectable DSBs (data not shown).

Cell Cycle Alterations and Inhibition of DNA Synthesis by Et743. Cell cycle response of human colon carcinoma HCT116 cells was examined. Fig. 4A shows that low concentrations of Et743 (10 nM) induced a marked accumulation of cells in the S and G2-M phases at 14 h and in the G2-M phase at 24 h after drug removal. Such a profile is consistent with agents that produce DNA damage. At 0.1 μM, Et743 induced a G1 arrest that was observed most clearly at the earliest time (6 h) after drug removal. At later times, G1 arrest was associated with an accumulation of cells in S-phase. At 1 μM, the G1 arrest was more pronounced and more persistent.

The cell cycle alterations produced by Et743 prompted us to examine the effect of Et743 on DNA synthesis during and after drug exposure (Fig. 4B). Pulse-labeling experiments with [methyl-3H]-thymidine showed that DNA synthesis was rapidly inhibited during Et743 treatments at 1 or 10 μM, reaching 20–30% of control levels. Inhibition of [3H]-thymidine incorporation by 1 μM and 10 μM Et743 persisted for at least 3 h after drug removal. Lower concentration of Et743 (0.1 μM) for 1 h produced no significant inhibition of thymidine incorporation during drug exposure but led to a delayed inhibition after drug removal.

The Et743 G1 arrest led us to investigate the effects of the drug on p53 expression. Fig. 5 shows that p53 protein levels were transiently elevated after Et743 treatment.

Activity of Et743 in the Absence of Cellular Top1. To determine the role of top1 for the cytotoxicity of Et743, we used a subline of mouse leukemia P388 cells, P388/CPT45, which do...
not express detectable top1 and are highly resistant (≥1000-fold) to CPT (18, 19, 24). Fig. 6 shows that Et743 had the same activity in both cell lines, which indicates that top1 is not required for the cytotoxicity of Et743.

COMPARE Analysis of Et743 (NSC 648766). The COMPARE algorithm was developed to compare the cytotoxicity profile of a given compound over the 60 cell lines of the National Cancer Institute Anticancer Drug Screen to those of established chemotherapeutic agents with known pharmacological mechanisms (25, 26). If the cytotoxicity profile of the agent is correlated with the patterns of one or more agents of known mechanism, then the hypothesis is that this agent may have the similar mechanism of action as those agents of known mechanism. Table 1 demonstrates Et743 exhibited the highest correlation with DNA binders. No significant correlation was found with CPT and its derivatives.

DISCUSSION

The present study reports the DNA-damaging and cellular effects of Et743. The only detectable DNA lesions induced by Et743 in human colon carcinoma HCT116 cells were protein-linked SSBs. Protein-linked DNA breaks are the hallmark of topoisomerase poisons, which trap the DNA cleavage intermediates of the enzymes (27, 28). These DNA cleavage intermediates are SSBs with the enzyme linked to the 3’-DNA terminus of the break for top1, and DNA single- and DSBs with the one enzyme monomer linked to each 5’-end of the breaks for topoisomerase II (28, 29). The present observation of protein-linked DNA breaks in Et743-treated cells is consistent with the recent detection of top1-DNA complexes in Et743-treated cells (12, 13). The protein-linked DNA breaks (top1 cleavage complexes) observed in Et743-treated cells exhibit some remarkable differences compared with those induced by CPT. First, the Et743-induced protein-linked DNA breaks persist after drug removal whereas the CPT-induced DNA breaks reverse extremely fast, even at 0°C (23). Possibly, this is because top1 is trapped by the Et743 adducts (12), whereas in the case of CPT, the drug binds reversibly to the top1-DNA complex (28, 30). A second difference between the protein-linked DNA breaks induced by Et743 and those induced by CPT is their temperature-dependence. Whereas the CPT-induced protein-linked DNA breaks can form at 0°C (23), those induced by Et743 were strongly suppressed at 0°C. This difference might be attributable to a temperature-sensitive drug uptake or to reduced DNA alkylation at low temperature (12). Alternatively, one cannot exclude the possibility that other cellular factors besides top1 generate the protein-linked DNA breaks induced by Et743.

On the basis of the cellular data obtained in the top1-deficient cell line (P388/CPT45 cells; Fig. 6), it appears that top1 inhibition is not required for Et743 activity. By contrast with CPT, Et743 was equally active in cells that lack detectable top1. Thus, the relevance of the top1 cleavage complexes for the cytotoxicity of Et743 is questionable. Because Et743 is cytotoxic at much lower concentrations than those required to detect top1 cleavage complexes, it is likely that only very few top1 cleavage complexes might accumulate in Et743-treated cells at pharmacological concentrations, and that Et743 probably targets other cellular molecules.

The COMPARE analysis indicates that Et743 is most closely related to other DNA-binding agents rather than to...
CPTs. Sequence-specific guanine N2 alkylation might affect the activity of various DNA-binding factors. However, the concentrations required in vitro to detect such effects on NF-Y, TBP and E2F are 10 μM (14), which, as in the case of top1, raises the question of the pharmacological relevance of such observations. Recently, two studies demonstrated that relatively low concentrations of Et743 (10 – 50 nM) effectively and selectively inhibited the transcriptional activation of cellular genes regulated by the NF-Y and possibly by Sp1 transcription factors (15, 16). Each of these factors binds to GC-rich sequences that are preferentially alkylated by Et743 (10). Thus, the steric hindrance produced by the C-ring of Et743 (Fig. 1) bulging in the DNA minor groove (31), and the associated bend of the DNA toward the major groove opposite to the adduct (32) might stabilize the binding (trapping) of transcription factors (15, 16) and/or top1 (12, 13) and interfere with the specific intermolecular interactions within transcription complexes, thereby resulting in their malfunction.

REFERENCES


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Table 1COMPARE analysis of Et743 (NSC 648766) using LC_{50} and TGI data from the National Cancer Institute in vitro anticancer drug screen

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Fig. 6 Cell survival of P388- and top1-deficient P388/CPT45 cells treated with Et743 or CPT. Cell survival was measured by MTT assay after continuous drug exposure for 3 days. ● P388 and △ P388/CPT45 cells, respectively.


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