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Biochemistry

Poisoning of human DNA topoisomerase I by ecteinascidin 743, an anticancer drug that selectively alkylates DNA in the minor groove

(minor groove ligand/DNA–protein crosslink/DNA damage/DNA alkylation)

YUJI TAKEBAYASHI, PHILIPPE POURQUIER, AKIRA YOSHIDA, GLENDA KOHLHAGEN, AND YVES POMMIER*

Laboratory of Molecular Pharmacology, Division of Basic Sciences, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892-4255

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ABSTRACT **Ecteinascidin 743 (Et743, National Service Center 648766) is a potent antitumor agent from the Caribbean tunicate *Ecteinascidia turbinata*. Although Et743 is presently in clinical trials for human cancers, the mechanisms of antitumor activity of Et743 have not been elucidated. Et743 can alkylate selectively guanine N2 from the DNA minor groove, and this alkylation is reversed by DNA denaturation. Thus, Et743 differs from other DNA alkylating agents presently in the clinic (by both its biochemical activities and its profile of antitumor activity in preclinical models). In this study, we investigated cellular proteins that can bind to DNA alkylated by Et743. By using an oligonucleotide containing high-affinity Et743 binding sites and nuclear extracts from human leukemia CEM cells, we purified a 100-kDa protein as a cellular target of Et743 and identified it as topoisomerase I (top1). Purified top1 was then tested and found to produce cleavage complexes in the presence of Et743, whereas topoisomerase II had no effect. DNA alkylation was essential for the formation of top1-mediated cleavage complexes by Et743, and the distribution of the drug-induced top1 sites was different for Et743 and camptothecin. top1–DNA complexes were also detected in Et743-treated CEM cells by using cesium chloride gradient centrifugation followed by top1 immunoblotting. These data indicate that DNA minor groove alkylation by Et743 induces top1-mediated protein-linked DNA breaks and that top1 is a target for Et743 *in vitro* and *in vivo*.**

The cytotoxicity and antitumor activity of extracts from the Caribbean tunicate *Ecteinascidia turbinata* were first discovered in the late of 1960s. However, the purification of the active compounds was not completed until 1990 (1). One of them, ecteinascidin 743 (Et743) (Fig. 1), has recently been selected as a novel anticancer agent because it displays remarkable antitumor activities in *in vitro* and *in vivo* models (1–5). Data from the National Cancer Institute Drug Discovery Screen by using the 60 human tumor cell line panel indicated the high potency of Et743 and its unique activity profile when compared with standard agents presently used in cancer chemotherapy (Y.T. and Y.P., unpublished work). Et743 is presently in Phase I/II clinical trials (6).

Interactions of ecteinascidins with DNA have been proposed on the basis of structural similarities with other tetrahydroisoquinoline-containing antibiotics, biochemistry, x-ray crystallography, NMR spectroscopy, and molecular modeling data (2, 5, 7–9). The pattern of potential hydrogen bond acceptors and donors indicates that the drug is likely to bind to the DNA minor groove (5, 8, 9). Et743 contains a carbinolamine center at the N2 position, and elimination of the adjacent hydroxyl group (at position 21) results in a Schiff base vulnerable to nucleophilic attack, leading to DNA alkylation (Fig. 1). The alkylation site has been assigned to the exocyclic 2-amino group of guanine in the DNA minor groove (Fig. 1). Remarkably, the alkylation reaction is DNA sequence specific (10). It requires noncovalent binding of Et743 in the DNA minor groove (5, 8, 9), and it is reversed on DNA denaturation (7). These characteristics set Et743 apart from the DNA alkylating agents presently used in cancer chemotherapy.

In spite of the exceedingly potent activity of Et743 *in vivo* against a variety of tumor models in mice (5) and responses in Phase I clinical trials (François Goldwasser, personal communication), the mechanisms of antitumor activity of Et743 have not yet been identified. Using a DNA–protein crosslink (DPC) assay, we found a 100-kDa protein that forms DNA crosslinks in the presence of Et743 DNA adducts and identified it to DNA topoisomerase I (top1). To our knowledge, this work is the first report for top1 as a cellular target of Et743 and for the trapping of top1 by a DNA-alkylating drug.

MATERIALS AND METHODS

Cell Culture, Chemicals, and Enzymes. Human colon carcinoma HCT 116, HT-29, and leukemia CEM cells were cultured in RPMI medium 1640 (Life Technologies, Gaithersburg, MD) containing 10% heat-inactivated fetal calf serum and 2 mM glutamine in a 5% CO_2 incubator at 37°C. No antibiotic was added to the medium. The cells were trypsinized and passaged once a week. Et743, camptothecin (CPT), and etoposide (VP-16) were provided by the National Cancer Institute Drug Synthesis and Chemistry Branch (Rockville, MD). Stock solutions of drugs were prepared in dimethyl sulfoxide at a concentration of 10 mM. Further dilutions were made in distilled water immediately before use. [γ-^{32}P]ATP and [α-^{32}P]dGTP were purchased from New England Nuclear. Human top1 was purified from Sf9 cells by using a baculovirus construct for the full length N terminus truncated human top1 cDNA (11). In some experiments, human top1 was purchased from TopoGEN (Columbus, OH). Human topoisomerase II (top2) was obtained from John L. Nitiss (St. Jude Children's Research Hospital, Memphis, TN).

Preparation of Nuclear Extracts. The method used is a modification of that previously described (12). Briefly, log-phase cultures containing 5×10^9 cells were washed twice at 4°C by using nucleus buffer (150 mM NaCl/1 mM KH_2PO_4/5 mM $MgCl_2$/1 mM EGTA) and recovered by centrifugation at $200 \times g$ for 10 min. Cell pellets were resuspended in nucleus buffer containing 0.03% Triton X-100. After incubation at 4°C

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Abbreviations: CPT, camptothecin; DPC, DNA–protein crosslink; Et743, ecteinascidin 743; top1, topoisomerase I; top2, topoisomerase II; VP-16, etoposide; SV40, simian virus 40; pSK, pBluescript; ICE, *in vivo* complex of enzyme.

*To whom reprint requests should be addressed at: Building 37, Room 5D02, National Institutes of Health, Bethesda, MD 20892-4255. e-mail: pommier@nih.gov.

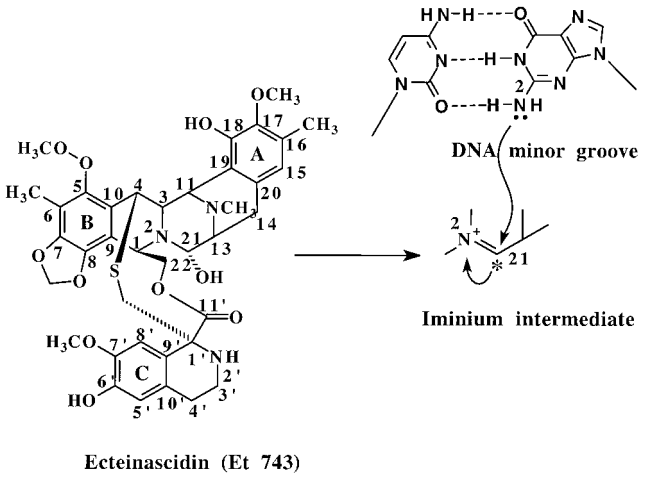

FIG. 1. Chemical structure of Et743 (National Service Center 648766) and proposed reaction of its reactive iminium intermediate with the guanine 2-amino group (7).

for 10 min, nucleus pellets were recovered by centrifugation at 350 × *g* for 10 min. After washing with ice-cold nucleus buffer, pellets were recovered by centrifugation at 350 × *g* for 10 min. Salt extraction of the nuclear pellet was achieved by adjusting the final NaCl concentration to 0.35 M and gentle mixing at 4°C for 30 min. After centrifugation at 12,000 × *g* for 30 min, supernatants containing salt-soluble material were collected as nuclear extracts that were immediately used for protein purification.

DPC Assay. Oligonucleotide (Fig. 2*A*) with high-affinity binding sites for Et743 (7) (Midland Certified Reagent, Midland, TX) was 5′-end labeled with T4 polynucleotide kinase (GIBCO/BRL) and [γ-^{32}P]ATP. Kinase reaction and annealing with unlabeled lower DNA strand were performed as described (7). Labeled duplex oligonucleotide and nuclear extracts were incubated with or without Et743 in reaction buffer (10 mM Tris·HCl/5 mM $MgCl_2$/50 mM NaCl) for 1 hr at 25°C. After adding Laemmli loading buffer, samples were electrophoresed at 100 V on 8% SDS/PAGE (63 mM Tris·HCl/10% glycerol/2% SDS/0.0025% bromophenol blue, pH 6.8) gels (Fig. 2*A*). Gels were dried on 3-MM paper sheets and analyzed with a PhosphorImager by using IMAGEQUANT software (Molecular Dynamics).

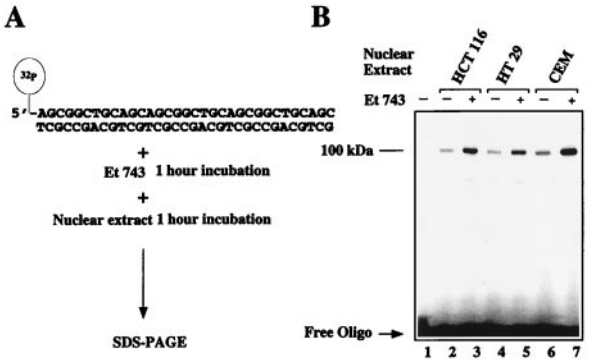

FIG. 2. Detection of covalent DPCs with a 100-kDa protein from nuclear extracts from human colon carcinoma HCT 116, HT 29, and human leukemia CEM cells. (*A*) A DNA oligonucleotide (33 mer) containing Et743 preferential alkylation sequences was 5′-end-labeled on the upper strand. The oligonucleotide was incubated as indicated with nuclear extracts in the absence or presence of 0.1 μM Et743 for 1 hr at 25°C. (*B*) Samples were electrophoresed (8% SDS/PAGE) and analyzed by PhosphorImager. Lane 1, oligonucleotide alone; lanes 2–7, oligonucleotide + nuclear extracts from the indicated cells; lanes 3, 5, and 7, reactions with 0.1 μM Et743.

Purification of Protein Involved in the DPC Induced by Et743. All procedures were performed at 4°C by using FPLC (Amersham Pharmacia). Active fractions were examined by DPC assay. Purification steps are summarized in Fig. 3*A*. Nuclear extracts were passed over a PD-10 column for desalting and exchanging of buffer A (10 mM Tris·HCl/1 mM DTT/10 mM NaCl). Samples were next applied to a Q-Sepharose column (Hitrap-Q, 5 ml) equilibrated with buffer A. The column was washed with buffer A and eluted with a linear gradient of 0.01–0.5 M NaCl in buffer A. Active fractions were collected and loaded onto an Heparine–Sepharose column (Hitrap–Heparine, 5 ml) equilibrated with buffer A containing 0.5 M NaCl and eluted with a linear gradient of 0.5–1.0 M NaCl in buffer A. Finally, active fractions were applied to a Mono S (Amersham Pharmacia) HR5/5 column equilibrated with buffer A and eluted with a linear gradient of 0.01–1.0 M NaCl in buffer A. Purified proteins were analyzed by Silver staining by using Silver Stain Plus kit (Bio-Rad).

top1 Immunoblotting. Proteins from SDS/PAGE gel were electrophoretically transferred to Immobilon-P membrane (Millipore) for 30 min at 15 V. The membrane was blocked for 1 hr in PBS–Tween 20 (PBS-T) containing 5% nonfat dried milk. top1 monoclonal antibody was obtained from Yungchi Cheng (Yale University, New Haven, CT). Reactions were performed overnight and followed by incubation with horseradish peroxidase-labeled anti-mouse IgG (1:1,000 dilution). After washing in PBS-T, membranes were developed by using the enhanced chemiluminescence detection system (New England Nuclear/Life Science Products).

SV40 DNA Nicking Assay. Each reaction (10 μl final volume) in 10 mM Tris·HCl, pH 7.5/50 mM KCl/5 mM $MgCl_2$/0.1 mM EDTA/1 mM ATP/15 μg/ml BSA contained 0.3 μg supercoiled simian virus 40 (SV40) DNA (GIBCO/BRL). Relaxed DNA was prepared by first incubating native SV40 DNA with excess top1 for 1 hr at 25°C. Reactions were performed at 25°C (top1) or 37°C (top2) for 30 min and terminated by adding 0.5% SDS and 0.5 mg proteinase K (final

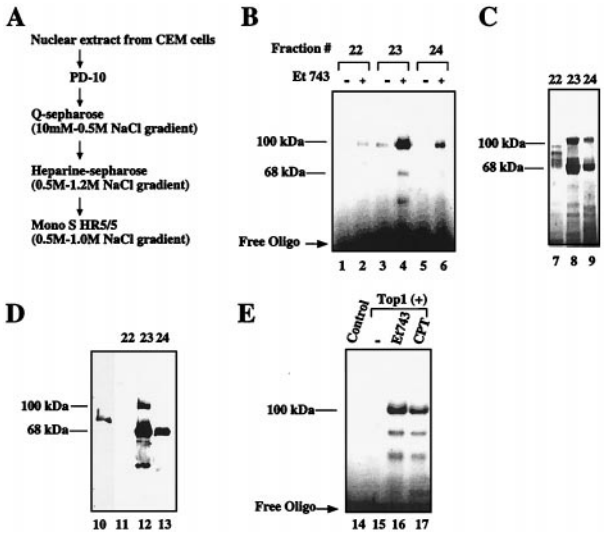

FIG. 3. Identification of top1 as Et743-inducible DPC protein. (*A*) Purification strategy (see details in *Materials and Methods*). (*B*) SDS/PAGE DPC assay of fraction 22–24 after Mono S column chromatography. Lanes 1, 3, and 5 are without Et743; lanes 2, 4, and 6 are with 0.1 μM Et743. (*C*) Typical 8% SDS-polyacrylamide gel and silver staining of aliquots from fractions 22–24 (lanes 7–9, respectively) after Mono S chromatography. (*D*) top1 immunoblotting of the post-Mono S fractions 22–24. Lane 10 corresponds to a truncated form (86 kDa) of purified top1. (*E*) Covalent complexes formed by purified recombinant top1 in the presence of Et743. Autoradiography of a typical SDS/PAGE: lane 14, DNA alone; lane 15, + top1; lanes 16, + top1 + 0.1 μM Et743; lane 17, + top1 + 10 μM CPT.

concentrations) for 1 hr at 50°C. After adding 1 μl of 10× loading buffer (20% Ficol 400/0.1 M Tris·HCL, pH 7.8/100 mM NaCl/1 mM Na_2EDTA, pH 8.0/1% SDS/0.25% bromophenol blue), samples were loaded onto 1% agarose gels in 1× TBE (90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3) containing 0.05 μg/ml ethidium bromide. DNA was visualized by transillumination with ultraviolet light (300 nm).

Sequencing of top1- and top2-Mediated DNA Cleavage Sites. top1-mediated cleavage sites were mapped in two different single end-labeled DNA substrates: the 161-bp *Pvu*II-*Hin*dIII fragment of pBluescript (pSK) plasmid (Stratagene) and the 5,191-bp *Ban*I-*Hpa*II fragment of SV40 DNA (GIBCO/BRL). Both fragments were single-end labeled by a fill-in reaction (13, 14). Briefly, linearized pSK (200 ng) or SV40 (50 ng) were incubated with [γ-^{32}P]dGTP in 1× labeling buffer (0.5 mM dATP/dCTP/dTTP in 50 mM Tris·HCl, pH 8.0/100 mM MgCl/50 mM NaCl) in the presence of 0.5 units of the Klenow fragment of DNA polymerase I. Labeled DNA was purified by phenol/chloroform extraction followed by ethanol precipitation and resuspension in water. For cleavage assays, labeled DNA (approximately 50 fmol per reaction) were incubated with purified top1 for 30 min at 25°C with or without drug in 1× reaction buffer (10 mM Tris·HCl, pH 7.5/50 mM KCl/5 mM $MgCl_2$/0.1 mM EDTA/15 μg/ml BSA). Reactions were stopped by adding 0.5% SDS (final concentration), ethanol precipitated, and resuspended in loading buffer (80% formamide/45 mM sodium hydroxide/1 mM sodium EDTA/0.1% xylene cyanol/0.1% bromophenol blue, pH 8.0). Reaction products were separated in a 7% denaturing polyacrylamide gel (7 M urea) in 1× TBE for 2 h at 40 V/cm at 50°C. Imaging and quantitations were performed by using a PhosphorImager.

Analysis of top1- and top2-DNA Covalent Complexes in Drug-Treated CEM Cells. The *in vivo* complex of enzyme (ICE) bioassay was used (15, 16). Briefly, after drug treatment, ≈1 × 10^6 CEM cells were collected by centrifugation (1,500 rpm, 10 min) and lysed in 1 ml of sarkosyl 1% followed by 30 strokes of Dounce homogenizer. Lysates were loaded on top of CsCl gradient containing four densities (1.37, 1.50, 1.72, and 1.82 g/ml) (16). After ultracentrifugation (30,700 rpm × 24 hr at 20°C), fractions (0.5 ml) were collected from the bottom of the tubes. DNA was detected in each fraction by agarose gel. For topoisomerase detection, aliquots from each fraction (100 μl) were diluted with an equal volume of 25 mM sodium phosphate buffer, pH 6.5, and applied to Immobilon-P membrane (Millipore) by using a slot-blot vacuum manifold. Immunoblotting for top1 or top2 was performed as described above. top2 monoclonal antibody was purchased from TopoGEN.

RESULTS

Et743 Induces the Covalent Binding of a 100-kDa Nuclear Protein to an Oligonucleotide Containing Et743 Alkylating Sites. To detect proteins involved in DNA crosslinking in the presence of Et743, we used an SDS/PAGE DPC assay. Based on our previous knowledge of sequence-specific DNA alkylation by Et743 (7), we designed an oligonucleotide (Fig. 2*A*) containing high-affinity binding sites for Et743 (5′-CGG, -AGG, GGG, TGG, or AGC) (7). Covalent DPCs were detected by SDS/PAGE and autoradiography (Fig. 2*B*). Et743 enhanced a single crosslinking band running with a molecular mass of approximately 100 kDa. Covalent DPC was enhanced by Et743 with nuclear extracts from various human cell lines (colon carcinoma HCT 116, HT 29, and leukemia CEM cells) (Fig. 2*B*). SDS/PAGE DPC assay was used to purify the 100-kDa protein that formed DPCs in the presence of Et743.

Purification of top1 as the 100-kDa Target Molecule of Et743. We purified the protein(s) involved in the DPCs induced by Et743 in three steps (Q-Sepharose, Heparine-Sepharose, and Mono S chromatography) (Fig. 3*A*). The activity peaks were 0.25 M for Q-Sepharose and 0.85 M for Heparine–Sepharose. The results of the last step of purification by using Mono S chromatography are shown in Fig. 3*B*. The Et743-inducible DPC activity was eluted at molecular mass of 100 kDa and 68 kDa after 0.5 M NaCl (fraction 23) (Fig. 3*B*) and corresponded to two major bands with silver staining of SDS/PAGE (8%) (Fig. 3*C*). It is noticeable that the elution conditions for chromatography of the Q-Sepharose and Heparine–Sepharose were similar to those reported for top1 purification (17, 18), and that the molecular masses of 100 kDa and 68 kDa also coincided to those of mammalian top1. The 68-kDa band is known to correspond to a proteolytic product of the native 100-kDa top1. Thus, immunoblotting with top1 antibody was performed with Mono S fractions. top1 was detected only in fractions 23 and 24 by immunoblotting (Fig. 3*D*), and the top1 immunoreactive bands corresponded to the silver staining pattern (Fig. 3*C*). We then performed SDS/PAGE DPC assay with recombinant top1 to determine whether purified top1 could form Et743-inducible DPCs in our assay. As shown in Fig. 3*E*, recombinant top1 produced the 100-kDa DPCs in the presence of Et743. CPT, a specific top1 inhibitor, was used as a positive control and also produced the 100-kDa DPC. Taken together, these data demonstrate that top1 is a DPC target of Et743 and that Et743 induces the formation of top1-mediated DPCs *in vitro*.

Unique Features of the Et743-Induced top1 Cleavage Complexes. We determined next whether purified top1 (or top2) could form cleavage complexes in the presence of Et743. Cleavage complexes consist of enzyme-linked and mediated DNA strand breaks. In the case of top1, the enzyme is reversibly linked to the 3′-DNA terminus of each DNA single-strand break that it generates (19, 20). Using SV40 as a substrate, we found that Et743 induced top1-mediated DNA nicking (Fig. 4*A*). We next compared the activity of Et743 in supercoiled native SV40 DNA and in relaxed circular SV40 DNA. Fig. 4*B* shows that induction of top1-mediated DNA cleavage was more efficient in supercoiled than in relaxed circular DNA (93% cleavage vs. 41%). This could be attributed at least in part to the preferential binding and activity of top1 on supercoiled DNA. We also tested whether Et743 had comparable effects on top2 cleavage complexes and found it not to be the case (Fig. 4*C*). Up to this point, the top1 data indicated that Et743 can induce top1 cleavage complexes.

To further analyze the Et743–top1 interactions, we performed DNA sequencing analysis. Fig. 5 shows top1-mediated DNA cleavage sites induced by Et743 and CPT. top1-mediated DNA cleavage sites were induced by Et743 at different locations than those induced by CPT in the two DNA fragments examined: a DNA fragment adjacent to the SV40 origin and a fragment from pSK known to contain many sites for top1 cleavage complexes (13, 21). CPT enhances top1 cleavage complexes by inhibiting top1-mediated DNA ligation (19). This inhibition is reversible, and increasing salt concentration reverses the CPT-induced cleavage complexes (22, 23). Fig. 5*B* shows that top1-mediated DNA cleavage was rapidly reversed in the presence 0.35 M NaCl for Et743 as well as CPT.

To determine the role of DNA alkylation by Et743 in the formation of top1 cleavage complexes, experiments were performed with DNA alkylated with Et743 after removal of free Et743 by ethanol precipitation. top1-mediated DNA cleavage was observed under these conditions (Fig. 5*C*, lane 5), indicating that top1 can be trapped by Et743-DNA adducts. By contrast, pretreatment of the DNA with CPT (lane 9) failed to trap top1, which is consistent with CPT binding to top1–DNA complexes and formation of reversible ternary complexes. Finally, we also found that overnight incubation of DNA with Et743 at 0°C failed to induce top1 cleavage complexes, which suggests that DNA alkylation by Et743 is temperature sensitive. These data suggest that the alkylation of DNA by Et743

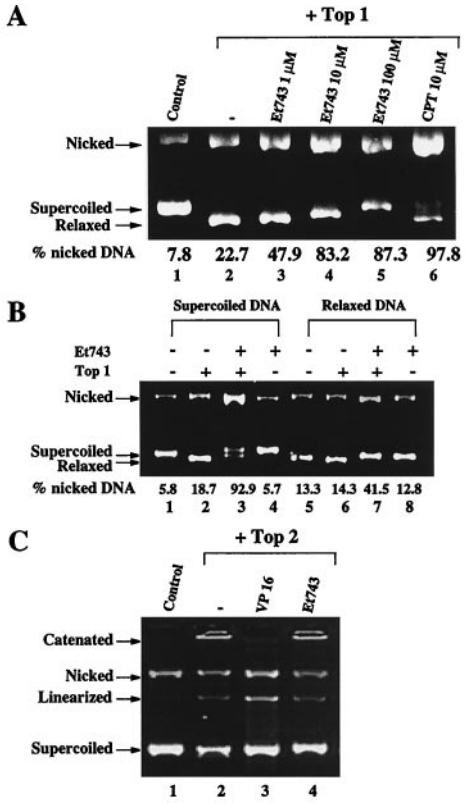

FIG. 4. Et743 induces top1-mediated DNA single-strand breaks. SV40 DNA was reacted with top1 in the absence or presence of drugs, as indicated. Reactions were stopped with 0.5% SDS followed by proteinase K incubation. Samples were run in 1% agarose gels containing ethidium bromide. Percent nicked DNA was computed after FluorImager analysis (Molecular Dynamics) by using the IMAGEQUANT software and is indicated below each lane. (*A*) top1-mediated nicking assay by using supercoiled negative SV40 DNA. Lane 1, DNA alone; lane 2, + top1; and lanes 3–6, + top1 and the indicated concentrations of Et743 or CPT. (*B*) Et743 induces more top1-mediated DNA nicking in supercoiled than in linear SV40 DNA. Lanes 1 and 5, DNA alone; lanes 2 and 6, DNA + top1; lanes 3 and 7, DNA + top1 + Et743 (0.1 μM); lanes 4 and 8, Et743 without top1. (*C*) Et743 does not induce detectable top2 cleavage complexes. Lane 1, DNA alone; lane 2, + top2; and lanes 3–4, + top2 and the indicated drug concentrations.

is sufficient for induction of top1-mediated DNA cleavage by Et743.

Et743 Induces top1–DNA Complexes in Cells. The ICE bioassay can detect topoisomerase–DNA covalent complexes in tissue culture cells or *in vivo* samples (15, 16). We used this assay to evaluate whether top1–DNA complexes were detectable in Et743-treated cells. Exponentially growing CEM cells were treated with either Et743, CPT, or VP-16 for 1 hr and processed in the ICE bioassay. Fractionation of the CsCl gradient showed DNA bands in fractions 7–10 (Fig. 6*B*), and immunoblotting revealed the presence of top1 signals in these DNA fractions for the Et743- and CPT-treated cells, but not in the untreated or VP-16-treated cells (Fig. 6*A*). Immunoblotting against top2 was positive in the DNA fractions of the VP-16-treated cells, but not in the untreated or CPT- or Et743-treated cells (Fig. 6*C*). These data indicate that Et743 produces top1- but not top2-DNA covalent complexes in drug-treated cells and demonstrate that top1 is a cellular target for Et743.

DISCUSSION

The present study reports the identification of top1 as a cellular target for Et743, a potent anticancer agent presently in clinical

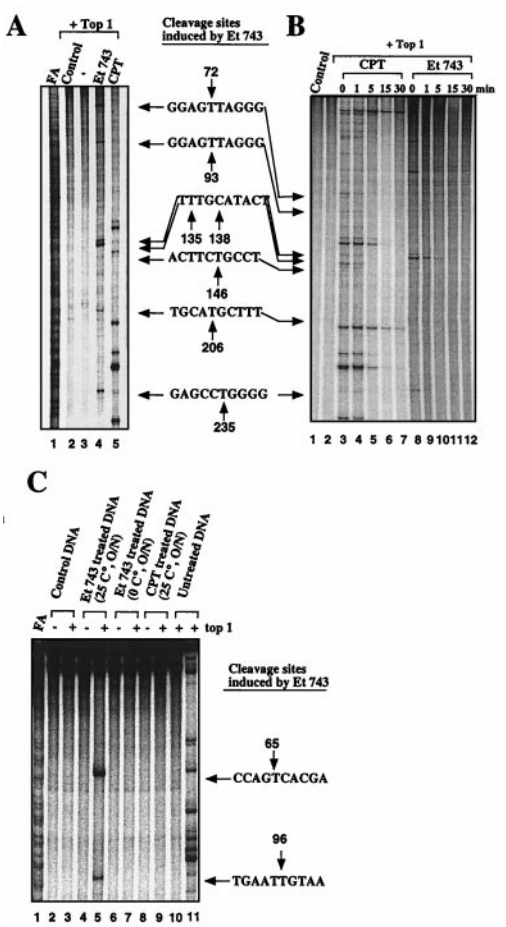

FIG. 5. DNA alkylation by Et743 induces salt-reversible top1-mediated DNA breaks at different sites from CPT. (*A*) Differences in sequence selectivity of top1-mediated DNA breaks for Et743 and CPT. The long *Ban*I-*Hpa*II fragments of SV40 DNA 3′-end-labeled at the *Ban*I Site was used. Lane 1, formic acid (FA); lane 2, DNA alone; lane 3, + top1 without drug; lane 4, top1 + Et743 (10 μM); lane 5, top1 + CPT (10 μM). (*B*) Salt reversibility of the top1-mediated DNA breaks induced by Et743. Drug-induced DNA cleavage was reversed by adding 0.35 M NaCl (final concentration) (at time 0 corresponding to a 30-min incubation of top1 with Et743 or CPT; lanes 3 and 8, respectively) and by further incubation at 25°C for the indicated times. (*C*) Et743-DNA adducts induce the top1 cleavage complexes. 3′-end-labeled *Pvu*II/*Hin*dIII fragment of pSK(−) phagemid DNA was used for these reactions. In lanes 2–9, reactions were performed in two consecutive steps. First, the DNA was treated overnight (O/N ≈16 hr) with or without drug, as indicated above pairs of lanes, then DNA was ethanol precipitated to remove free drug. Secondly, the DNA was reacted ± top1 in the absence of added drug (lanes 2–10) or in the presence of 10 μM CPT (lane 11). Lane 1, FA-sequencing lane.

trials. The identification of eukaryotic top1 is based on the following observations: (*i*) purification of nuclear proteins that form DNA–protein complexes in the presence of Et743 yields 70- and 100-kDa proteins identified as top1 (Fig. 2); (*ii*) recombinant top1 forms DNA–protein complexes and cleavage complexes with DNA alkylated with Et743 (Figs. 3–5); and (*iii*) top1–DNA complexes can be detected by using the ICE bioassay in cells treated with Et743 (Fig. 6).

DNA topoisomerases have been recognized as key targets for some of the most active anticancer drugs presently used in the clinic. In general, the drugs convert the enzymes into a cellular poison by stabilizing the topoisomerase cleavage complexes, which are the catalytic intermediate for topoisomerization reactions. This is why topoisomerase inhibitors that induce topoisomerase cleavage complexes are commonly referred to as "topoisomerase poisons" (20, 24, 25). Two of the

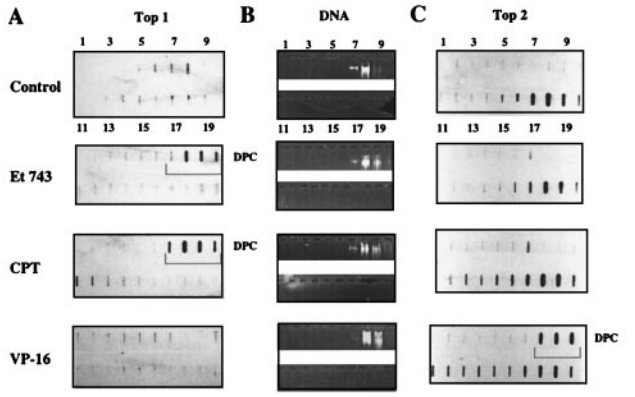

FIG. 6. Induction of top1 cleavage complexes by Et743 in human leukemia CEM cells by using the ICE bioassay. Cells ($\approx 1 \times 10^6$) were treated with 10 μM Et743, 10 μM CPT, or 100 μM VP-16 for 1 hr at 37°C, after which they were lysed with 1% sarkosyl and subjected to the ICE bioassay. Cesium chloride fractions are indicated above and under the control for each set. (*A*) top1 immunoblotting; (*B*) DNA staining after electrophoresis; (*C*) top2 immunoblotting of each fraction.

cellular DNA topoisomerases are targeted by anticancer drugs. In the case of top2, the cleavage complexes consist of DNA double-strand breaks linked at each of the 5′-DNA termini to a top2 molecule, whereas in the case of top1, the cleavage complexes are DNA single-strand breaks (Fig. 4) with the enzyme linked to the 3′–DNA termini. The list of top2 inhibitors is extensive and includes DNA intercalators such as anthracyclines (doxorubicin, adriamycin, idarubicin, epirubicin, etc.), anthrapyrazoles (mitoxantrone), acridines (amsacrine), and nonintercalative drugs such as the epipodophyllotoxins [VP-16 and teniposide (VM-26)] and the azatoxins (25, 26). For top1, the only class of inhibitors approved for clinical use are the CPT derivatives (20). Thus, the present study identifies Et743 as a novel class of clinical agents that target top1.

The molecular interactions between topoisomerases and their inhibitors remain generally ill defined. Based on DNA sequence analyses, it was proposed that intercalating agents and epipodophyllotoxins bind at the interface of the top2–DNA cleavage site. This hypothesis was referred to as the "stacking model" (27) and was extended to CPTs and top1 (13). More recently, crosslinking studies have provided further support for the binding of inhibitors at the interface of the topoisomerase–DNA cleavage site (10, 28). The case of Et743 is remarkable because this drug represents the first DNA alkylating agent that can trap top1 cleavage complexes.

Previous studies indicate that Et743 alkylates DNA from the minor groove at guanine N2 (Fig. 1). Wang and coworkers first proposed this after solving the crystal structure of Et743 and modeling the alkylation at guanine N2 (2, 5). More recently, biochemical evidence indicated that Et743 alkylates preferentially certain GC-rich sequences and that this alkylation requires a duplex DNA structure because it is reversible on DNA denaturation (7). Thus, it was proposed that Et743 displays an original mode of alkylation with formation of a covalent adduct between the exocyclic 2 amino group of guanine located in the DNA minor groove and the C2 atom of Et743. This interpretation is consistent with optimum minor groove binding of Et743 for efficient nucleophilic attack of the drug iminium intermediate by guanine N2 (Fig. 1). Using high-field NMR experiments, Hurley and coworkers recently provided direct evidence for this type of molecular interactions (8, 9).

The importance of minor groove interference for top1 poisoning by drugs was previously suggested by studies with reversible binders. The clearest example for this type of effect is for benzimidazoles, including the commonly used Hoechst dyes that are pure minor groove ligands (29, 30). Minor groove interactions have also been proposed for top1 poisoning by anthracyclines that bear sugar substitutions with extension in the DNA minor groove (31–33). Such anthracyclines are generally poor top2 poisons and act as top2 suppressors, suggesting that minor groove occupancy by small ligands tends to trap top1 rather than top2. The peculiarity of Et743 compared with the other minor groove binders is that Et743 forms covalent minor groove adducts. Thus, Et743 would be expected to form persistent top1 cleavage complexes. The fast reversibility of the Et743-induced top1-mediated DNA breaks under nonphysiological high salt or heat conditions (Fig. 5) does not imply that the cellular effects of ecteinascidin would be more transient and less damaging than those of CPT. In fact, in Et743-treated cells, the protein-linked DNA breaks detected by alkaline elution persist much longer than those induced by CPT (Y.T., F. Goldwasser and Y.P., unpublished work), which is consistent with the role of DNA alkylation by Et743 for the induction of persistent top1 cleavage complexes.

The finding that Et743 acts as a top1 poison both *in vitro* and in human cancer and leukemia cells suggests that top1 is one of the possible targets of the drug. Et743 differs from the only clinically used top1 inhibitors, the CPTs, by the persistence and location of the top1 cleavage complexes. This suggests that Et743 and CPTs differ by the distribution of their genotoxic effects. In addition, it is possible that the Et743–DNA adducts can interfere with other DNA-binding proteins whose inhibition contributes to the extraordinary potency of the drug as an anticancer agent.

Note Added in Proof: While this work was in press, topoisomerase I poisoning by Et743 was reported (34).

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