SV40 DNA Replication Inhibition by the Monofunctional DNA Alkylator Et743[†]

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ABSTRACT: Ecteinascidin 743 (Et743) is a highly cytotoxic anticancer agent isolated from the squirt *Ecteinascidia turbinate*, which alkylates DNA in the minor groove at GC-rich sequences resulting in an unusual bending toward the major groove. The ability of Et743 to block DNA replication was studied using the well-established simian virus (SV40) model for mammalian DNA replication in cells and cell-free extracts. Intracellular SV40 DNA isolated from Et743-treated BSC-1 cells was analyzed by native, two-dimensional agarose gel electrophoresis. A low frequency of Et743 adducts detected at 30−100 nM drug concentrations inhibited SV40 origin activity and induced formation of unusual DNA replication intermediates. Under cell-free conditions, only a high Et743 adduct frequency reduced SV40 DNA synthesis. Comparative studies involving related DNA alkylators, tomamycin and saframycin A, revealed inhibition of SV40 DNA replication in cells at concentrations approximately 10 times higher than Et743. Under cell-free conditions tomamycin- or saframycin-A-adducted DNA templates inhibited DNA synthesis similarly to Et743. Et743 appears to be unusual among other alkylators, because its adducts strongly inhibit intracellular SV40 DNA replication but are relatively weak as cis inhibitors as measured under cell-free conditions.

Ecteinascidin 743 (Et743)[1] (Figure 1), a natural antibiotic isolated from the Caribbean sea squirt *Ecteinascidia turbinate*, has shown remarkable anticancer activity *in vitro* and *in vivo* (*2*−*4*) and is currently in phase II clinical trials in the U.S. and Europe (*5*). Et743 binds to the DNA minor groove in GC-rich regions and alkylates one DNA strand at N2 of guanine through a carbinolamine moiety in a mechanism described previously for the pyrrolo[1,4]benzodiazepine group of DNA alkylators (i.e., anthramycin, tomamycin, and saframycin A) (*6*, *7*). Et743 is unique among other DNA alkylators because it bends the DNA minor groove toward the major groove. Upon binding to DNA, the wedge-shaped C subunit of Et743 (Figure 1) is positioned outside the minor groove, being accessible for possible DNA−drug−protein interactions (*8*). Whether the unique DNA-binding properties of Et743 contribute to its effectiveness as an antitumor agent remains unknown. Typical of DNA alkylators, Et743 inhibits processes associated with DNA metabolism, especially RNA transcription and DNA repair. Et743 interferes with protein binding to specific promoters and disrupts DNA−transcription factor complexes possibly by inducing structural distortions to DNA (*9*, *10*). Moreover, Et743−DNA adducts are not properly repaired by nucleotide excision repair (NER), particularly during transcription, causing accumulation of single-strand breaks that might contribute to cell death (*11*, *12*).

While Et743 also effectively inhibits [^{3}H]thymidine incorporation into DNA and slows S-phase progression, the underlying mechanism of replication inhibition remains unknown (*13*−*15*). DNA-damage-induced DNA replication inhibition might occur in either a cis-acting manner by direct action on the template through the formation of drug−DNA adducts (*16*−*18*) and/or through trans-acting factors (i.e., aggravated checkpoint responses by blocked replication forks that further decrease DNA synthesis activity). Intra-S-phase checkpoints involve activation of the major protein kinases related to PI3K (phosphatidylinositol-3-kinase), ATM, ATR, or DNA-dependent protein kinase (DNA-PK), which slow DNA synthesis, block new replicons from firing, and activate DNA repair (*19*, *20*). Several DNA-damaging agents, such as the topoisomerase I inhibitor camptothecin, induce intra-S-phase checkpoints as a consequence of accumulated DNA double-strand breaks generated by replication fork collisions with drug−DNA−enzyme complexes (*21*, *22*). Likewise, Et743 is able to induce single-strand breaks with an accumulation of protein−DNA cross links, but whether Et743 generates DNA damage as a result of destabilized replication forks or as a biproduct of incomplete DNA repair is not known (*13*, *23*).

The simian virus (SV40) model system has been used to determine whether DNA-damaging agents interfere with

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[1] Abbreviations: ATM, ataxia telangiectasia mutated; DNA-PK, DNA-dependent protein kinase; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Et743, ecteinascidin 743; ExoIII, exonuclease III; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; IC_{50}, 50% inhibitory concentration; MEM, minimal essential medium; NER, nucleotide excision repair; PI3K, phosphoinositol (3) kinase; RI, replication intermediate; SDS, sodium dodecyl sulfate; SV40, simian virus; S1, S1 nuclease; TAE, Tris−acetic acid−EDTA electrophoresis buffer; T-ag, SV40 large T antigen; TBE, Tris-borate electrophoresis buffer; TI, plasmid topoisomers; [^{3}H]TdR, [methyl-^{3}H]thymidine.

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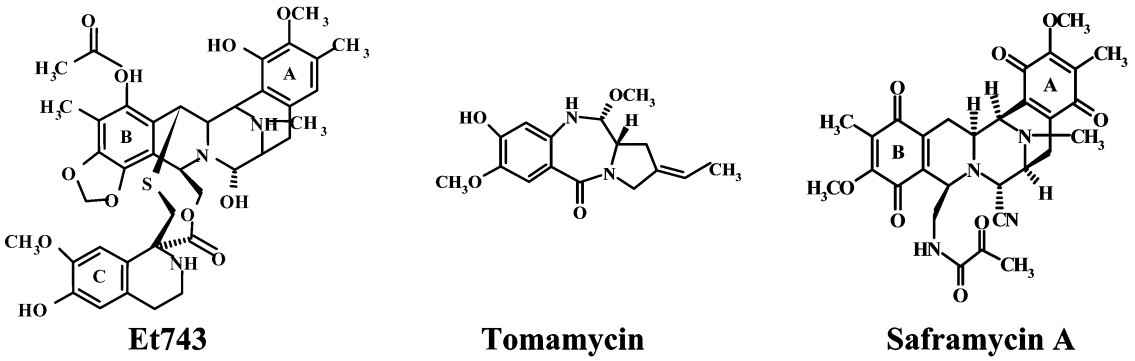

FIGURE 1: Chemical structures of Et743, tomamycin, and saframycin A.

DNA replication at the level of initiation and/or elongation or by cis- or trans-acting mechanism(s) (*24*, *25*). SV40 is an attractive model for studying the DNA-damaging agents effect on mammalian DNA replication because its DNA replication employs all of the mammalian replication factors except viral T antigen (T-ag) and can be monitored in intracellular as well as in cell-free environments (*26*). Analysis by neutral/neutral two-dimensional (2D) gel electrophoresis of intracellular SV40 replication intermediates (RIs) isolated from infected cells not only permits visualization of replication origin activity, but also can reveal DNA structural changes that occur during replication of damaged DNA.

Previously, the SV40 model system has revealed that both the mono- and bifunctional cyclopropylpyrroloindole DNA-alkylating agents adozelesin and bizelesin respectively inhibit SV40 origin activity in a mechanism(s) associated with the activation of an intra-S-phase checkpoint (*24*, *25*, *27*, *28*).

The effect of Et743 on DNA replication and whether it inhibits replication in a trans- or cis-acting manner is unknown. In this study, we determined that Et743 inhibits intracellular SV40 origin activity and induces unusual RIs. SV40 DNA templates isolated from drug-treated cells were assessed for the presence of Et743 lesions. In addition, the ability of Et743 adducts on a defined DNA template to hinder DNA replication directly was measured. Finally, other Et743 family members were used to determine if analogous mechanisms of DNA replication inhibition are induced by alkylators that bind to DNA in the same manner as Et743 but lack its unique ability to bend the double helix (*8*, *29*).

MATERIALS AND METHODS

Drugs, Reagents, and Radiochemicals. Et743, tomamycin, and saframycin A were provided by NCI. Tomamycin (46 mM), Et743, and saframycin A stock solutions (10 mM) were dissolved in dimethyl sulfoxide (DMSO), stored at −20°C, and protected from light. [Methyl-^{3}H]thymidine ([^{3}H]-TdR, 48 Ci/mmol) and [^{14}C]thymidine (55 mCi/mmol) were obtained from Moravek Biochemichals Inc. (Brea, CA) and [γ-^{32}P]ATP (10 μCi/μL, 6000 Ci/mmol), [α-^{32}P]dATP (10 μCi/μL, 3000 Ci/mmol), and [α-^{32}P]dCTP and Sephadex G-50 columns were purchased from Amersham Bioscience Corp. (Piscataway, NJ). High-strength analytical-grade agarose used for 2D gels was obtained from BioRad Life Science (Hercules, CA). SYBR-Green was from Molecular Probes, (Eugene, OR). Genescreen membranes were from Perkin−Elmer Life Sciences Inc. (Boston, MA). DecaprimeII DNA-labeling kit was from Ambion (Austin, TX). S1 nuclease, exonuclease III (ExoIII), and restriction endonucleases *Bam*HI and *Bsp*147II (*Hae*II) were purchased from Fermentas Inc. (Hanover, MD), while *Nru*I and *Sph*I restriction endonucleases were from New England Biolabs (Beverly, MA). λ *Hin*dIII DNA size marker was from Invitrogen Corporation (Carlsbad, CA). All other chemicals were of reagent grade or better.

SV40 large T-ag was purified from baculovirus-infected High Five cells (Invitrogen Corporation) according to a published procedure (*30*). pSV011 plasmid containing the SV40 origin of replication has been described elsewhere (*31*). pPB12 kindly provided by Dr. Piero Bianco, University at Buffalo, was isolated from *Escherichia coli* using a Qiagen Maxi prep kit (QIAGEN Inc., Santa Clarita, CA).

Cell Growth Inhibition. BSC-1, green monkey kidney cells, were grown in minimal essential medium (MEM) with 8% fetal calf serum and 2% fetal bovine serum (MEM-10). HCT116, human colorectal carcinoma cells, were kindly provided by Dr. B. Vogelstein and were grown in McCoy 5A medium supplemented with 10% fetal bovine serum. All cell lines were maintained at 37 °C in a 5:95% CO_2/air incubator.

For growth inhibition, uninfected BSC-1 or HCT116 cells were seeded 22 h before drug treatment in 24-well plates (5000 cells/well). Logarithmically growing cells were then treated in quadruplicate for 4 h with either diluted solvent (0.1% DMSO) or drug (Et743 or tomamycin in 0.1% DMSO). After incubation for 4 h, the drug was removed and cells were washed once with warm phosphate-buffered saline (PBS) and incubated for an additional 4 days (96 h) in drug-free media. After 4 days, cells were trypsinized and counted using a Coulter Particle Counter. The average mean cell growth was graphed as a function of the increased drug concentration.

Drug Treatment of SV40-Infected Cells and Isolation of Viral DNA. BSC-1 cells were seeded at 2.5×10^5 cells per 60 mm dish and grown for 48 h until 70−80% confluent. Cells were infected with SV40 virus (multiplicity of infection > 1) in MEM containing 2% calf serum (MEM-2) for 2 h at 37 °C. After removal of the virus-containing medium, cells were incubated for an additional 24 h in fresh MEM-2. After infection for 26 h, Et743 or DMSO (as a solvent control, 0.1%) was added to the medium. After 3.5 h, [^{3}H]TdR was added (10μCi/mL) and the incubation was continued for 30 min. After drug incubation, SV40 DNA was isolated using a Hirt extraction procedure (*32*). Briefly, cells were washed

3 times with PBS and incubated for 1 h at 37 °C in 1 mL of 100 mM EDTA and 1% sodium dodecyl sulfate (SDS) containing proteinase K (0.2 mg/mL). Cellular proteins were precipitated by addition of 0.35 mL of 5 M NaCl and incubated at 4 °C overnight. Supernatants recovered by centrifugation for 30 min at 13000*g* were extracted twice with 10 mM Tris-HCl, 1 mM EDTA (TE at pH 7.6) buffered phenol and twice with chloroform/isoamyl alcohol (24:1). DNA was precipitated with a 0.7 volume of 2-propanol, recovered by centrifugation, followed by a wash with 70% of ethanol, recentrifuged, and dissolved in 100 μL of TE.

Inhibition of Intracellular SV40 DNA Synthesis. To establish the inhibitory concentrations of Et743 on intracellular SV40 DNA synthesis, SV40 DNA isolated from nondrug- and drug-treated BSC-1 cells, labeled for the last 30 min of the 4 h drug treatment with [^{3}H]TdR, was resolved in 1% agarose gel electrophoresis in 1× Tris−acetic acid−EDTA electrophoresis buffer (TAE) (30 V, 17 h). After electrophoresis gels were stained with SYBR-Green and photographed, fluorographic analysis was performed as described below. DNA synthesis was determined based on the signals of [^{3}H]TdR-labeled DNA quantitated as a sum of full-length SV40 forms (form I, II, and III) scanned from the film. The level of SV40 DNA synthesis inhibition was based on the [^{3}H]TdR signal of SV40 DNA in the gel normalized to the total SV40 DNA loaded, determined by the intensity of the SV40 genome bands stained with SYBR-Green, compared to the nondrug-treated control.

Analysis of Intracellular SV40 DNA RIs. The effects of Et743 on intracellular SV40 DNA replication were analyzed by neutral/neutral 2D gel electrophoresis as described previously (*33*). SV40 DNA (20 μL) isolated from drug-treated virus-infected BSC-1 cells, was linearized with *Bam*HI and electrophoresed in 0.6% agarose gels in 1× TAE and 0.1 μg/mL ethidium bromide for 24 h at 0.7 V/cm. The lanes were excised and placed into enlarged preparative wells perpendicular to the direction of the first dimension gel along with excess agarose [1% agarose and 0.5 μg/mL ethidinum bromide in 0.5× Tris-borate electrophoresis buffer (TBE)]. Second-dimension gels were run at 4 °C in 1× TBE and 0.5 μg/mL ethidinum bromide for 19 h at 4 V/cm. Gels were subjected to Southern blotting, and blots were hybridized to an [α-^{32}P]-labeled full-length linear SV40 DNA probe (~1 × 10^6 cpm/mL) prepared by random priming of *Eco*RI-linearized SV40 DNA (Ambion, Austin, TX). Blots were hybridized in 4× SSC, 2× Denhardt's reagents, 0.1% SDS, 0.1% sodium pyrophosphate, 0.01 M disodium EDTA, 0.1 g (w/v) dextran sulfate, and 0.12 mg/mL salmon sperm DNA, probed for 16 h at 65 °C, washed, and exposed to a phosphor screen (Molecular Dynamics, Sunnyvale, CA). The mass of SV40 RIs was determined from the two-dimensional phosphor images using ImageQuant software (Molecular Dynamics).

For fluorographic analysis to determine SV40 DNA replication activity, gels were dehydrated by agitation twice for 1 h in 95% ethanol. Gels were then impregnated with 5% 2,5-diphenyloxasole in 100% ethanol for 1 h with gentle agitation. Fluor was precipitated during incubation for 45 min in distilled water, and gels were dried on Whatman no. 3 mm paper using a gel drier for >4 h at 60 °C. Dried gels were exposed to Kodak XAR-5 film at −80 °C with exposure time adjusted within a linear response range of the film. Replication activity was determined from the [^{3}H]TdR-labeled DNA signals quantitated from fluorographic patterns scanned by a computing laser densitometer (Molecular Dynamics). The fraction of the total population of SV40 DNA molecules actively replicating in nondrug-treated cells was measured by comparing the mass of SV40 RIs with mass of nonreplicating SV40 DNA.

Cell Extracts and Cell-free SV40 DNA Replication Assay. Cell extracts were prepared for studies of drug inhibition of cell-free SV40 replication using HCT116 cells. Cell extracts were prepared as described previously (*26*, *30*) with minor modifications. Briefly, after washing cells 3 times with ice-cold PBS and once in hypotonic buffer [20 mM HEPES/KOH (pH 7.5), 1.5 mM $MgCl_2$, 5 mM KCl, and 1 mM dithiothreitol (DTT)], cells were suspended in hypotonic buffer for 10 min before lysing by three freeze−thaw cycles. Cell lysates were centrifuged at 9000*g* for 10 min at 4 °C. The soluble fraction of cell extracts was frozen on dry ice and stored at −80 °C. Protein concentrations were determined using the Bio-Rad protein assay reagent (Bio-Rad Laboratories Inc.). Conditions used for SV40 cell-free DNA replication were as described previously by others (*31*). Briefly, 30 ng of drug-pretreated SV40 origin-containing plasmid pSV011, 600 ng of T-ag, and 30 μg of protein control extract (no drug treatment) were combined with replication assay buffer (4 mM ATP, 0.2 mM CTP, 0.2 mM GTP, 0.2 mM UTP, 0.1 mM dCTP, 0.1 mM dGTP, 0.1 mM dTTP, 0.025 mM dATP), 7 mM $MgCl_2$, 0.024 unit of creatinine phosphokinase, 40 mM phosphocreatinine, and 2 μCi of [α-^{32}P]dATP in a final volume of 20 μL. Mixtures were incubated at 37 °C for 1 h, and reactions were stopped by addition of 10 mM Na_2EDTA, 1% SDS, and 50 mg/mL proteinase K. After proteinase K digestion for 1 h at 37 °C and phenol/chloroform extraction, DNA products were separated from unincorporated [α-^{32}P]dATP using Sephadex G-50 columns (Amersham Bioscience). DNA products were separated on 1% (w/v) agarose gels in 1× TAE by electrophoresis at 0.7 V/cm for 16 h. Gels were vacuum-dried on filter paper, and radioisotope incorporation into replication products was quantified using ImageQuant software.

Detection of Drug Lesions Using ExoIII/S1 Stop Assay. Optimal conditions for Et743 adduct detection were determined using purified pPB12 plasmid DNA. Because ExoIII does not act on 3′ overhangs and on single-stranded DNA, substrate DNA was prepared with only one end available for degradation to monitor ExoIII digestion. Briefly, pPB12 (~60 μg) was cut with two restriction endonucleases to produce asymmetric ends, a 3′ overhang (SphI) and a blunt end (NruI), and two DNA fragments 3697 and 412 bp. One-half of the restricted DNA (~30 μg) was dephosphorylated, radiolabeled with [γ-^{32}P]ATP using polynucleotide kinase (New England Biolabs) and separated from unincorporated nucleotides using Sephadex G-50 columns. Radioactively labeled and unlabeled DNA were mixed, and 10 μg split into fresh tubes for *in vitro* drug incubation (0.1−3 μM) at 37 °C for 1 h, in buffer containing 10 mM Tris (pH 7.5), 25 mM NaCl. For saframycin A, to ensure optimal drug binding to DNA, 9.5 mM DTT was added (*34*). DNA was separated from free drug using Sephadex G-50 columns. Samples were equilibrated at 30 °C for 2 min, followed by addition of ExoIII (500 units, Fermentas) at 30 °C for 4 min, and transferred to fresh tubes on ice containing 3× S1 nuclease

buffer, which inactivates ExoIII in a low-pH and high-salt environment. All samples were digested with S1 nuclease (3 units) at room temperature for 20 min. The S1 reaction was stopped by addition of 3 μL of STOP solution (300 mM Tris base and 50 mM EDTA), and 100 ng of each DNA sample was electrophoresed on 1% agarose gel in 1× TAE at 1 V for 18 h. Gels were vacuum-dried for 1 h at 60 °C and exposed to Phosphorimager screens. Drug presence on naked DNA was quantified based on the intensity of the product resolved by gel electrophoresis. The ExoIII reaction rate was based on the size of DNA products formed after 4 min using nondrug-treated DNA and was ~160 bp/min.

Intracellular DNA isolated from drug-treated cells was evaluated for the presence of Et743 adducts by the ExoIII/S1 stop assay. Briefly, intracellular SV40 DNA (~100 μg) was treated with enzymes *Bsp*143II (producing a 3′ overhang, Fermentas), *Bam*HI (producing a 5′ overhang, Fermentas), and RNase A (50 μg/mL, Roche Diagnostics, GmbH) to create two SV40 DNA fragments, 3546 and 1697 bp. After the reaction, DNA was phenol/chloroform-extracted and ethanol-precipitated. The DNA concentration was determined based on absorption at 260 nm (conversion factor $A_{260} = 1 = 50$ μg/mL). Subsequently, 10 μg of DNA was incubated with ExoIII (500 units) in a total volume of 20 μL at 30 °C for 4 or 8 min. Reaction aliquots (~10 μL) were transferred to fresh tubes containing S1 mix. Samples were then treated with S1 nuclease as described previously. Products were resolved by 1% agarose gel electrophoresis in 1× TAE, at 5 V/cm for 4 h. After electrophoresis, gels were stained with SYBR-Green, photographed, and transferred to Genescreen membranes. The rate of ExoIII digestion was determined based upon size of the ExoIII nondrug-treated product in gel, compared to a λ *Hin*dIII DNA size marker, and was ~260 bp/min. Southern blots were hybridized using the full-length SV40 [α-^{32}P]dCTP-labeled probe at 65 °C for 16 h and exposed to a Phosphorimager screen.

RESULTS

1. Et743 Inhibits SV40 DNA Synthesis in BSC-1 Cells. The drug inhibitory effect on intracellular SV40 DNA synthesis was first assessed based on the incorporation of radioactively labeled DNA macromolecule precursor ([^{3}H]-TdR) into full-length SV40 DNA during the last 30 min of a 4 h Et743 treatment as described in the Materials and Methods. Concentration-dependent inhibition of SV40 DNA synthesis was observed as a decrease in the signal of fully replicated SV40 forms. The data from experiments are presented in Figure 2A. A 50% decrease in SV40 DNA synthesis was induced by 20 nM of Et743. To evaluate how rapidly Et743 inhibits DNA synthesis, infected BSC-1 cells were drug-treated with 100 nM Et743 for different times. After 60 min, intracellular SV40 DNA synthesis was reduced by 50% (Figure 2B). Thus, Et743 inhibits intracellular SV40 DNA synthesis rapidly and at nanomolar drug concentrations.

2. Et743 Inhibits Replication Origin Activity of SV40 DNA and Induces Unusual RIs. To monitor the effects of Et743 on intracellular SV40 replication, neutral/neutral 2D gel electrophoresis analysis of SV40 DNA RIs was utilized (*33*). This method separates branched RIs from linear nonreplicating molecules on the basis of size in the first dimension and shape in the second dimension. The nonlinear shape of

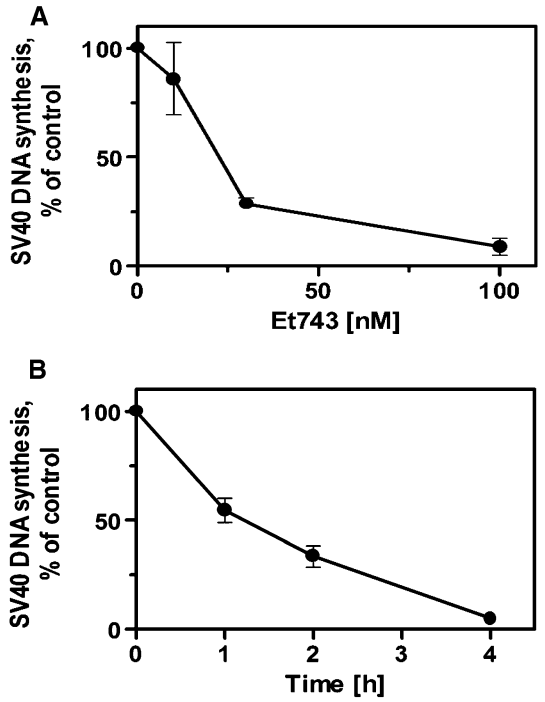

FIGURE 2: Et743 inhibits intracellular SV40 synthesis in a concentration- and time-dependent manner. SV40-infected BSC-1 cells were treated with (A) varying Et743 concentrations (0, 10, 30, and 100 nM) for 4 h or (B) at 100 nM of Et743 for varying times. The [^{3}H]TdR label was included for the last 30 min of a 4 h incubation period. Intracellular SV40 DNA forms I, II, and III were resolved by agarose gel electrophoresis, and the amount of SV40 DNA synthesis was determined from the sum of the signal intensities of the SV40 DNA forms as described in the Materials and Methods. Each data point was normalized for the total SV40 DNA signal in the gel and depicts the percent of nondrug-treated controls ± standard error (SE).

replicating molecules retards their migration in the second dimension relative to nonreplicating or fully replicated linear molecules. Replicating SV40 DNA molecules cleaved in the proximity of the termination region with *Bam*HI restriction endonuclease contain two replication forks that move in the opposite directions from the origin, where DNA replication initiates. A mixed population of these molecules replicated to various extents will produce an arc ("bubble arc") that rises upward and to one side of the "1n spot", which is where linearized full-length nonreplicating molecules of SV40 DNA migrate (Figure 3A). The bubble arc signal indicates origin activity and reflects both initiation and elongation of SV40 DNA replication. The signal sometimes detected underneath the bubble arc is a broken bubble arc (Figure 3A).

SV40 RIs isolated from virally infected BSC-1 cells treated for 4 h with various Et743 concentrations were analyzed by 2D gel electrophoresis. The drug concentrations were the same as those used for treatment of infected BSC-1 cells to examine [^{3}H]TdR incorporation into full-length SV40 DNA (see above). Two-dimensional gels resolve a bubble arc of total replicating DNA (from Southern blots probed with ^{32}P-labeled SV40 DNA), as well as signals on the bubble arc formed by newly replicated DNA (from fluorography of [^{3}H]-TdR-labeled DNA). While Southern analysis detects all SV40 RIs, fluorography depicts only the newly replicated DNA chains that incorporated [^{3}H]TdR during the last 30 min of a 4 h Et743 treatment. The bubble arc signal from DNA isolated from drug-treated cells was quantified and compared

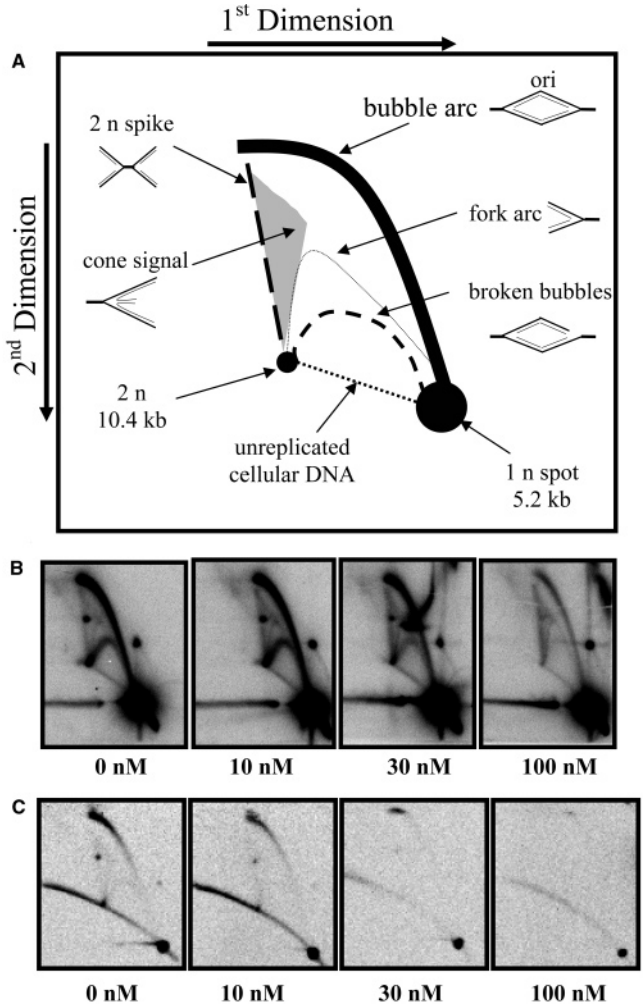

FIGURE 3: Et743 inhibits replication origin activity of SV40 DNA and induces unusual RIs. (A) Schematic representation of SV40 RI pattern resolved by 2D gel electrophoresis adapted from Cobuzzi et al. and Lopes et al. (*24*, *35*). SV40-infected BSC-1 cells were treated for 4 h with Et743 at the indicated concentrations (0, 10, 30, or 100 nM) and radiolabeled with [^{3}H]TdR for 30 min followed by SV40 DNA isolation. Linearized intracellular SV40 DNA was separated by neutral/neutral 2D gel electrophoresis and detected by (B) Southern blot hybridization with ^{32}P-labeled SV40 DNA or by (C) fluorography of [^{3}H]TdR-labeled DNA as described in the Materials and Methods. The gels are representative of the data from at least two independent experiments. Nonspecific signals seen in B (30 and 100 nM) to the right or overlapping the bubble arcs were not reproducible and considered insignificant.

to the control as described previously (*27*). Treatment of infected cells with Et743 resulted in a concentration-dependent decrease in the SV40 RI bubble arc signal detected in Southern blots (Figure 3B). The decrease in the ^{32}P signal in bubble arcs is consistent with a reduced level of replicating molecules resulting from decreased origin activity. A broken bubble arc signal (Figure 3A) seen in the nontreated cells diminished with increasing drug concentrations (Figure 3B). Interestingly, unusual DNA RIs were induced by Et743. A cone signal (Figure 3A), which was barely detectable in the control increased with increasing drug concentrations (Figure 3B). Such a cone signal has been reported by others in chromosomal DNA, during hydroxyurea-induced inhibition of replication fork progression in checkpoint-deficient mutants, and has been attributed to the formation of regressed replication forks in chicken-foot-like structures (*35*, *36*). The cone signal in Figure 3B is most prominent at the highest drug concentration tested (100 nM). Detection of the cone signal suggests that Et743 may inhibit fork elongation particularly at the higher drug concentrations (30−100 nM, Figure 3B).

To evaluate drug effects on newly replicated DNA, incorporation of [^{3}H]TdR into RIs was measured near the end of the drug treatment as described in the Materials and Methods. The reduction in newly replicated SV40 DNA was most readily detectable in the upper portion of the bubble arc, indicating disappearance of long DNA molecules (Figure 3C). The decreased incorporation of [^{3}H]TdR into the bubble arc was accompanied by simultaneous reduction of the full-length [^{3}H]-labeled SV40 DNA (1n spot, Figure 3A), indicating diminished origin activity (Figure 3C). The unusual cone signal noted in the Southern blot was observed in the fluorographic analysis of nondrug-treated SV40 RIs. The cone signal increased with drug concentrations in the Southern blot analysis (30−100 nM, Figure 3B); however, it was not detectable in the fluorographic analysis of newly replicated DNA (30−100 nM, Figure 3C). The lack of DNA structures that comprise the cone signal in the fluorogram may suggest that either such events occur early during drug treatment (i.e., prior to [^{3}H]TdR labeling) or require more than the 30 min labeling time for formation. Alternatively, DNA structures in the cone signal might be inactive in DNA replication and therefore fail to incorporate [^{3}H]TdR. When the results are taken together, they indicate that Et743 inhibits SV40 replication origin activity and induces accumulation of unusual DNA structures that comprise the cone signal.

3. Detection of Et743 Adducts in Intracellular SV40 DNA by a Unidirectional Deletion Assay Using Nucleolytic Enzymes. Intracellular SV40 DNA isolated from Et743-treated BSC-1 cells was evaluated for the presence of Et743−DNA adducts to test whether the drug was present on the SV40 template when replication was inhibited. Standard techniques used to detect and quantitate drug adducts on DNA often rely on heat treatment to convert adducts to strand breaks (*37*). Et743−DNA lesions are more difficult to measure because the covalently bound drug dissociates from DNA upon heat treatment without forming strand breaks (*6*). To measure drug adducts on intracellular SV40 DNA, ExoIII activity was monitored on the DNA template isolated from drug-treated cells under mild treatment conditions optimized for retention of the DNA-bound drug.

A schematic representation of the Et743 adduct detection procedure is presented in Figure 4A. SV40 DNA replicated in cells was isolated and digested with two restriction endonucleases to produce the ExoIII substrate (3.5 and 1.7 kb, lane 1 of Figure 4B) as described in the Materials and Methods. After ExoIII degradation, the resulting single-stranded DNA was removed by a subsequent reaction with S1 nuclease. The DNA was then size-fractionated by neutral gel electrophoresis, Southern-blotted, and detected using the ^{32}P-labeled full-length SV40 specific probe as described in the Materials and Methods. Intracellular SV40 DNA isolated from nondrug-treated and Et743-treated cells (10, 30, and 100 nM) was evaluated for Et743 adducts according to the procedure described in the Materials and Methods.

Restricted intracellular SV40 DNA templates, consisting of a mixture of two restriction fragments of intracellular SV40 DNA (3546 and 1697 bp, lane 1 of Figure 4B), were

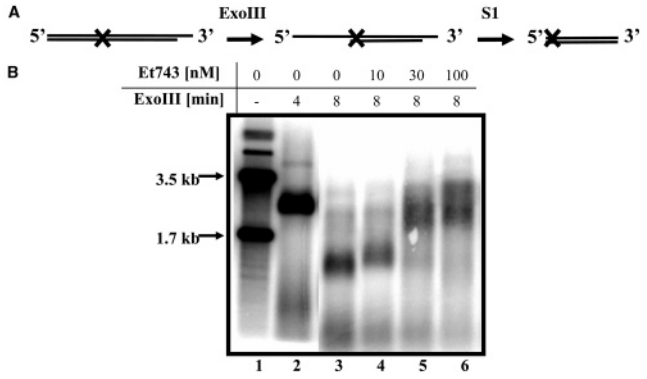

FIGURE 4: *In vitro* Et743 adducts detection in intracellular SV40 DNA by the ExoIII/S1 stop assay. Et743 adducts were detected using the ExoIII/S1 stop assay shown in A. First, ExoIII was used to degrade the restricted DNA fragment from one end. The presence of adducts (X) hinder ExoIII activity, resulting in incomplete DNA digestion. In a second step, ExoIII products are trimmed with S1 single-stranded DNA nuclease. (B) Intracellular SV40 DNA isolated from nondrug-treated and Et743-treated BSC-1 cells was digested as in A, using the procedure described in the Materials and Methods. The products were resolved by neutral agarose gel electrophoresis, after which DNA was transferred to a Genescreen membrane and hybridized to the full-length radiolabeled SV40 DNA. The radioactive signal was detected using a Phosphorimager screen. Lane 1, nondrug-treated restricted intracellular SV40 DNA; lanes 2 and 3, restricted intracellular SV40 DNA isolated from nondrug-treated cells digested with ExoIII nuclease for 4 and 8 min, respectively; and lanes 4−6, restricted intracellular SV40 DNA isolated from drug-treated cells digested with ExoIII nuclease for 8 min.

digested with ExoIII for 4 or 8 min. While 4 min digestion of intracellular SV40 DNA resulted in ~1000 bp degradation, ExoIII after 8 min traversed ~2100 bp at a rate 260 bp/min and produced a final fragment size of ~1450 bp (Figure 4B). The probability of meeting the drug lesion on the DNA (sensitivity) increased with increased time of ExoIII treatment. Adduct frequency was based on the assumption that one adduct is sufficient to hinder ExoIII activity measured after 8 min of DNA digestion. The ExoIII/S1 assay detected approximately one drug−DNA adduct per 2100 bp. Because ~2100 bp covers almost half of the full-length SV40 DNA (5243 bp), approximately 2 drug molecules per SV40 genome could be detected by this technique.

SV40 DNA isolated from cells treated with 30 nM of Et743 and digested with ExoIII resulted in considerably slower mobility than the control, indicating the presence of Et743−DNA adducts (lane 5 of Figure 4B). Almost complete ExoIII inhibition was observed at 100 nM (lane 6 of Figure 4B). Random drug distribution within intracellular SV40 DNA likely contributed to the observed DNA smearing.[2] Et743 adducts were first detected on the intracellular SV40 DNA template at 30 nM (~2 per SV40 genome, lane 5 of Figure 4B), a concentration that caused reduction of intracellular SV40 RI formation (30 nM, parts B and C of Figure 3).

4. Et743-Treated DNA Templates Are Impaired in Their Ability to Support DNA Synthesis under Cell-free Conditions. Because Et743 binding uniquely distorts DNA and could affect enzymes involved in DNA processing (*8*) including those involved in DNA repair, we next tested the effectiveness of Et743−DNA adducts to inhibit cell-free replication. Purified DNA template was drug-treated and used for cell-free SV40 DNA synthesis assays. Plasmid DNA containing the SV40 origin of replication, pSV011, was incubated *in vitro* with a range of drug concentrations for 1 h at 37 °C. The DNA was then precipitated to remove free drug prior to use in cell-free SV40 DNA synthesis assays. Cell extracts isolated from nondrug-treated HCT116 cells were used to support SV40 DNA replication under conditions described in the Materials and Methods. Replication products were electrophoresed, and the radioactivity incorporated into DNA (RI, replication intermediates and TI, topoisomers, Figure 5A) was quantified. Et743-treated DNA template reduced cell-free SV40 replication in a concentration-dependent manner with an IC_{50} of 2 μM, while 10 μM treatments resulted in an almost total loss of DNA synthesis activity (parts A and B of Figure 5).

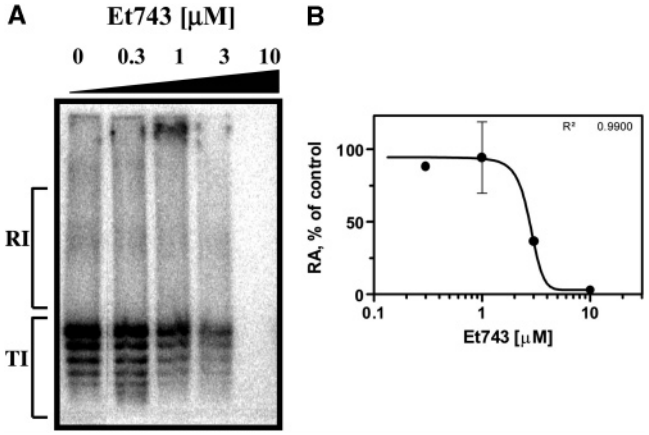

FIGURE 5: Et743-treated DNA template decreases SV40 cell-free DNA replication activity. After drug treatment of pSV011 with increased Et743 concentrations (0, 0.3, 1, 3, and 10 μM), the DNA template (30 ng) was used in a cell-free SV40 DNA replication assay. Cell-free replication was carried out for 1 h at 37 °C. Replication products were purified, (A) resolved on neutral agarose gels, and exposed to a Phosphorimager screen. Positions of plasmid topoisomers (TI) and replication intermediates (RI) are indicated. (B) Total radioactive signal from each lane was quantified and compared to a signal from a nondrug-treated control and plotted as replication activity (RA), percent of control ± SE.

5. Adducts on Et743-Treated DNA Template Detected by an ExoIII/S1 Assay. Next, whether the ability of Et743 to inhibit cell-free SV40 DNA replication correlates with the number of drug adducts present on the DNA template was tested. The ExoIII/S1 assay was used to detect Et743 adducts on purified radioactively end-labeled pPB12 plasmid. After treatment with the drug, free Et743 was removed and the plasmid was digested with ExoIII and S1 nucleases as described in the Materials and Methods.

The gel migration of the ExoIII digestion products of Et743-treated DNA was visualized after electrophoresis by exposure to a Phosphorimager screen and compared to the signal from the nondrug-treated control. Significant inhibition of ExoIII activity was observed with 0.3 μM Et743, while 1 μM caused almost complete enzyme inhibition (Figure 6A). In Figure 6B, Et743−DNA adducts are expressed as a percent of the ExoIII product formation compared to a nondrug-treated control.

[2] Because S1 nuclease could introduce breaks at sites of bulged DNA (*1*), drug-treated DNA was incubated with S1 alone and resolved on agarose gel electrophoresis together with nondrug-treated DNA to check for possible degradation (data not shown). Even at the highest drug concentration tested (100 nM), no degradation was observed.

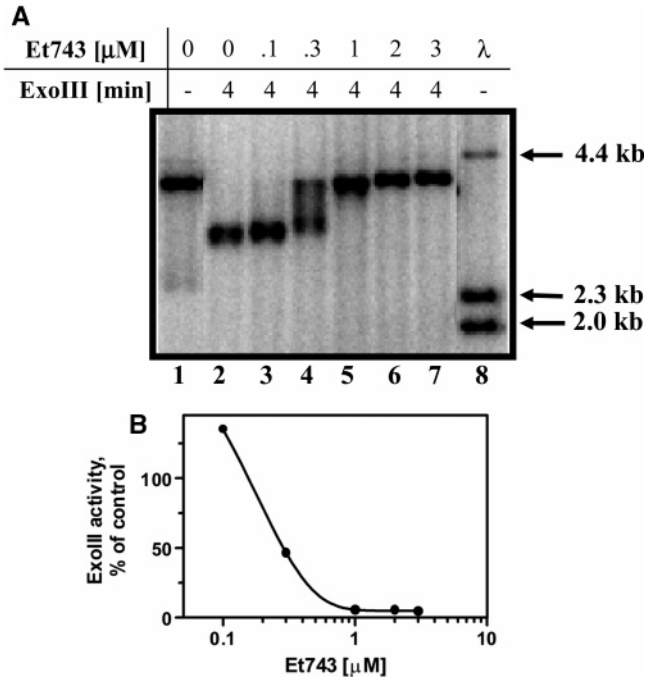

FIGURE 6: Et743 adducts on cell-free plasmid DNA detected by the ExoIII/S1 stop assay. Restricted and radiolabeled pPB12 was incubated with the drug, and adducts were detected as described in Figure 4. (A) ExoIII/S1 reaction products were electrophoresed together with a DNA marker (λ symbol, a size marker from *Hin*dIII digest of λ DNA, lane 8) on neutral agarose gels and detected using a Phosphorimager screen. Arrows indicate the expected size of DNA fragments of marker DNA. (B) ExoIII products were quantified from gels and compared to a nondrug-treated control. Lane 1, substrate DNA for ExoIII/S1 digestion; lanes 2−7, nondrug- and drug-treated DNA digested with ExoIII/S1 nucleases; and lane 8, λ *Hin*dIII DNA marker.

To compare drug concentrations that induced adducts on the DNA template with those needed to block cell-free DNA replication, the drug concentrations used in each assay were expressed as *r* values,[3] to normalize for differences in DNA content. While ExoIII activity was decreased by 50% at $r = 0.002$ (1 free drug molecule every 520 bp, 0.3 μM), no inhibition of cell-free mammalian replication was detected. Reduction in replication activity by 50% was observed at $r = 0.08$ (1 free drug molecule per every 12 bp, 2 μM), which is 40 times greater than the drug concentration needed to induce 50% reduction in ExoIII digestion of drug-treated templates.

6. Comparison of DNA Replication Inhibition by Other Pyrrolo[1,4]benzodiazepines. Tomamycin was used to assess whether the activity of Et743 as a DNA replication inhibitor extends to other DNA alkylators. Tomamycin has the same DNA sequence selectivity as Et743 but bends DNA toward the minor groove distorting the double helix to a lesser extent than Et743 (*29*). The data in Table 1 compares drug inhibitory concentrations that block cell growth and SV40 DNA synthesis by 50%. Tomamycin at 10 times higher drug concentrations than Et743 decreased cell growth and intracellular SV40 DNA synthesis. The ratios of the concentrations needed to block cell growth and SV40 DNA synthesis by 50% were similar with tomamycin and Et743. The results presented in Table 1 suggest that, even though Et743 and tomamycin differ in potency at the cellular level to block replication, both agents inhibit intracellular SV40 replication at equicytotoxic concentrations.

[3] *r*-value drug molecules/bp DNA (4109 bp for pPB12 and 2880 bp for pSV011).

Table 1: Summary of Drug Concentrations Inhibiting Cell Growth and Intracellular SV40 DNA Replication by 50% (IC_{50} Values)

	IC_{50} values		
drug	cell growth[a] (μM)	intracellular SV40 DNA synthesis[b] (μM)	formation of intracellular SV40 RIs[c] (μM)
Et743	0.02	0.02	0.04
tomamycin	0.2	0.15	0.2

[a] Cell growth of uninfected BSC-1 cells was measured 4 days after a 4 h drug treatment as described in the Materials and Methods. The IC_{50} is derived from two independent experiments. [b] Intracellular SV40 DNA synthesis was evaluated based on [^{3}H]TdR incorporated into fully replicated SV40 genomes resolved by agarose gel electrophoresis and fluorographed as described in the Materials and Methods. [c] Formation of intracellular SV40 RIs isolated from nondrug-and drug-treated cells was determined based on 2D gel agarose electrophoretic analysis. Quantification of SV40 RIs from Southern blotting assays as described in the Materials and Methods.

Table 2: Correlation of Drug−DNA Adducts Required to Inhibit DNA Replication and ExoIII Activity (IC_{50} Values)

	IC_{50} values	
drug	ExoIII activity[a] (μM)	cell-free SV40 DNA replication[b] (μM)
Et743	0.3	2
tomamycin	0.4	2
saframycin A	0.3	2

[a] ExoIII activity was assessed using drug-treated linearized plasmid DNA as described in Figures 4A and 6. [b] Cell-free SV40 replication activity was assessed using drug-treated DNA template as described in the Materials and Methods.

7. Inhibition of Cell-free DNA Replication Correlates with Adduct Formation on the DNA Template. Because Et743 is unique in its ability to bend the DNA minor groove toward the major groove (*8*), we investigated whether inhibition of SV40 DNA synthesis by Et743 could be influenced by distortion of the DNA template. Tomamycin and saframycin A, a compound structurally similar to Et743 but lacking the C subunit that determines the Et743 ability to bend DNA (see Figure 1), were used in *in vitro* studies to determine cis inhibition of cell-free SV40 DNA synthesis. The conditions for *in vitro* drug treatment were as described in the Materials and Methods.

The ExoIII/S1 assay described previously (Figure 6) was utilized to determine whether the drugs induce similar levels of DNA adducts. Saframycin A and tomamycin, which bind DNA as efficiently as Et743, interfere with ExoIII activity at concentrations similar to Et743, with an IC_{50} near 0.3 μM (Table 2). For all drugs, almost complete ExoIII inhibition was observed at 1 μM drug (1 free drug molecule per every 173 bp, $r = 0.006$, data not shown).

Next, pSV011 DNA templates containing the SV40 origin of replication were treated with varying concentrations of each drug and assayed for their ability to support cell-free SV40 DNA replication. Et743, saframycin A, and tomamycin at 2 μM (at 1 free drug molecule per every 12 bp, $r = 0.08$) all inhibited cell-free DNA replication by 50%, while complete inhibition (>90%) was achieved at 10 μM for each

drug (data not shown), demonstrating that these three agents decreased replication activity to a similar extent (Table 2).

DISCUSSION

In this study, the SV40 model system was used to examine the effects of Et743 on DNA synthesis and replication origin activity. At drug concentrations that inhibit cell growth, Et743 decreased intracellular SV40 DNA synthesis (Table 1), which agrees with previously published reports on cellular DNA synthesis inhibition by Et743 (*13*, *14*). In addition, Et743 reduced genomic DNA synthesis in BSC-1 and HCT116 as measured by [^{3}H]TdR incorporation, at concentrations similar to those used for intracellular SV40 replication inhibition (data not shown).

Two-dimensional gel electrophoretic analysis of SV40 RIs isolated from Et743-treated SV40-infected BSC-1 cells showed a concentration-dependent decrease in the SV40 replication bubble arc, indicating the inhibition of replication origin activity (Figure 3B). Moreover, Et743 strongly inhibited the formation of newly replicated SV40 DNA measured as the [^{3}H]TdR signal incorporated into SV40 RIs during the last 30 min of drug treatment. Disappearance of the newly replicated DNA (Figure 3C) is consistent with the decreased rate of DNA synthesis (*24*, *27*, *38*).

Because the preferred binding sites of Et743, GC-rich sequences, occur uniquely inside the SV40 replication origin, within T-ag-specific DNA-binding sites (*39*), an initiation block might occur through the disruption of T-ag interaction with DNA. Alternatively, decreased replication might be due to the DNA-damage-aggravated intra-S-phase checkpoint(s) that prevent replication initiation. Reduction of SV40 RIs by the latter mechanism was previously observed for another highly cytotoxic monofunctional DNA-alkylating agent adozelesin, which preferentially blocks initiation of intracellular SV40 replication by activation of cellular checkpoints associated with the origin recognition complex (ORC) (*24*, *40*). Adozelesin-favored binding to AT-rich regions might further contribute to the replication block at the level of initiation because many DNA replication origins contain such target sites, including the SV40 origin (*39*, *41*).

Other mechanisms of replication inhibition, such as fork destabilization and decreased replication fork movement, might play a role in the reduction in the mass of SV40 RIs. Broken bubbles can arise as a consequence of collapsed forks, when replication is stopped by damage on the template or when checkpoints are unable to maintain fork stability (*36*). The broken bubble signal was detected in the control, but decreased with increasing Et743 concentrations (Figure 3B). Pause sites would indicate accumulated DNA molecules at spots difficult to pass by replication forks and have been associated previously with other cytotoxic DNA alkylators, such as adozelesin and bizelesin (*25*, *42*). However, in contrast to the situation in multiorigin chromosomes, it is difficult to detect pause sites in episomes that replicate from a single origin. The detection of a cone signal, particularly at the highest Et743 concentration (100 nM, Figure 3B), might indicate replication fork regression because of a block in elongation and fork collapse (*35*, *36*). Generation of Et743-induced fork reversal and possible replication recovery require further experimental evaluation. Studies are now underway to verify the role of fork regression in Et743-induced DNA replication inhibition.

Conversely, a decreased level of gene transcription caused by Et743 might play a role in the observed inhibitory effect on intracellular SV40 replication. Overall, only a slight decrease of the total cellular RNA synthesis was induced by Et743 in uninfected BSC-1 cells, as measured by [^{3}H]-uridine incorporation (data not shown), suggesting a modest Et743 effect on cellular RNA synthesis. However, specific inhibition of T-ag transcription, whose gene product is required for SV40 DNA synthesis, might lead to an indirect inhibition of both initiation and elongation of intracellular SV40 replication. Given that, after Et743 treatment of SV40-infected BSC-1 cells (100 nM), no significant change in T-ag expression was observed at the protein level, it is likely that the observed intracellular SV40 DNA replication block is not due to the inhibitory effects of the drug on T-ag synthesis (data not shown).

Impaired ability of extracts isolated from drug-treated cells to support cell-free replication provides additional confirmation for drug-depleted replication factors and/or induced trans-acting inhibitors. Interestingly, only high Et743 concentrations decreased cell extract competence to drive cell-free replication (>1 μM, data not shown), suggesting that, possibly at the conditions used to inhibit SV40 origin activity by 90%, replication factors are still fully competent.

Estimates of Et743 adducts on SV40 DNA isolated from virus-infected cells, treated with Et743 concentrations that cause significant inhibition of intracellular SV40 replication (30 nM, parts B and C of Figure 3), are approximately 2 drug molecules per every SV40 genome (5243 bp) (Figure 4B). Such a low adduct frequency might indicate that the decrease of replication is dependent on a single Et743 adduct encounter, suggesting a drug-induced cis effect on replication fork progression. Furthermore, such an effect could promote trans-acting replication inhibition. For instance, as reported previously, adozelesin, at concentrations that decreased intracellular SV40 replication, triggered S-phase checkpoint responses in cells (*24*, *28*).

Additional studies of Et743-induced DNA replication inhibition assessed the effectiveness of Et743−DNA adducts on the DNA template to block enzymes involved in DNA replication. A relatively high free Et743/DNA template ratio was required to directly block cell-free replication (IC_{50} at 1 free drug molecule per every 12 bp, at $r = 0.08$, Figure 5). At Et743 concentrations that induced detectable adducts (i.e., starting from 1 free drug molecule every 520 bp, $r \geq 0.002$, Figure 6), there was little decrease in replication activity. These findings indicate that Et743−DNA adducts are relatively poor inhibitors in regard to a direct block of cell-free DNA synthesis and may dissociate or be bypassed during ongoing DNA synthesis (*43*). Whether bypass synthesis could explain the high Et743 concentrations required to block cell-free DNA synthesis is currently under study. On the other hand, it is possible that SV40 replication under cell-free conditions is not dependent on all cellular replication factors essential in cells, so that Et743-induced inhibition of replication would be more readily observed in cells. For instance, Et743-induced inhibition of intracellular SV40 replication might depend on single-stranded DNA damage that occurs at sites of incompletely removed Et743−DNA adducts by the NER pathway (*11*, *12*). Because the conditions of cell-free SV40 DNA replication likely do not support NER activity, a high level of Et743 adducts could be required to

diminish DNA synthesis activity. Future studies using "NER repaired" Et743-treated DNA templates and cell extracts isolated from NER-proficient and -deficient cells might clarify the role of DNA repair in Et743-induced inhibition of cell-free replication.

Tomamycin, which also alkylates DNA but does not bend the minor groove toward the major groove (*29*) and is 10-fold less cytotoxic than Et743, reduced intracellular SV40 replication to the same degree as Et743 at equicytotoxic concentrations. Replication activity of DNA templates, pretreated with either tomamycin or saframycin A, the other Et743 family member, was decreased only at relatively high levels of DNA adducts (with an IC_{50} at 1 free drug molecule every 12 bp, Table 2). Despite differences in conformational changes induced in the DNA double helix by the tested agents, cell-free DNA synthesis was similarly reduced.

Overall, our study revealed that Et743 effectively inhibits intracellular SV40 DNA synthesis by decreasing replication origin activity and by inducing unusual RIs that may be blocked in fork progression. A direct effect of Et743 on replication was not recapitulated under cell-free conditions, because the lesions themselves were not unusually effective at halting cell-free SV40 DNA synthesis. The lack of certain essential cellular factors under cell-free conditions might explain the differences in Et743 concentrations required to inhibit intracellular versus cell-free replication. Studies are now underway to determine the mechanism(s) by which Et743 inhibits DNA replication in cells.

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