Bizelesin, a Bifunctional Cyclopropylpyrroloindole Alkylating Agent, Inhibits Simian Virus 40 Replication in Trans by Induction of an Inhibitor†

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ABSTRACT: Bizelesin, a bifunctional DNA minor groove alkylating agent, inhibits both cellular and viral (SV40) DNA replication in whole cells. Bizelesin inhibition of SV40 DNA replication was analyzed in SV40-infected cells, using two-dimensional (2D) neutral agarose gel electrophoresis, and in a cell-free SV40 DNA replication assay. Within 1 h of bizelesin addition to infected cells, a similar rapid decrease in both the level of SV40 replication intermediates and replication activity was observed, indicating inhibition of initiation of SV40 DNA replication. However, prolonged bizelesin treatment (≥2 h) was associated with a reduced extent of elongation of SV40 replicons, as well as the appearance on 2D gels of intense spots, suggestive of replication pause sites. Inhibition of elongation and induction of replication pause sites may result from the formation of bizelesin covalent bonds on replicating SV40 molecules. The level of in vitro replication of SV40 DNA also was reduced when extracts from bizelesin-treated HeLa cells were used. This effect was not dependent upon the formation of bizelesin covalent bonds with the template DNA. Mixing experiments, using extracts from control and bizelesin-treated cells, indicated that reduced DNA replication competence was due to the presence of a trans-acting DNA replication inhibitor, rather than to decreased levels or inactivation of essential replication factor(s).

Bizelesin (see Figure 1) is a highly cytotoxic cyclopropylpyrroloindole (CPI) agent that has been examined in preclinical trials (*1*, *2*) and has exhibited activity against colorectal cancer (*3*). Bizelesin interacts with isolated DNA by forming both noncovalent and covalent (alkylated) bonds in the minor groove at AT-rich regions (*4*−*6*). While monofunctional CPIs, such as adozelesin and CC-1065, alkylate a single DNA strand at N3 of adenine (*7*−*9*), bizelesin is a bifunctional CPI, with two reactive chloromethyl moieties that can convert to cyclopropyl alkylating species, allowing the formation of DNA cross-links with adenines on opposite DNA strands (*10*−*14*). The extent of bizelesin covalent bond formation can be determined by heating the alkylated DNA at ≥70 °C to induce DNA single- or double-strand breaks which are readily quantitated (*15*, *16*). In contrast to studies with isolated DNA, bizelesin's intracellular mechanism of action is unclear. Studies in cell culture showed that bizelesin was more highly cytotoxic than either CC-1065 or its derivative adozelesin (*14*, *17*). In whole animal studies, by contrast, bizelesin induced fewer lesions (*17*) and was less hepatotoxic than the monoalkylator parent CC-1065 (*18*).

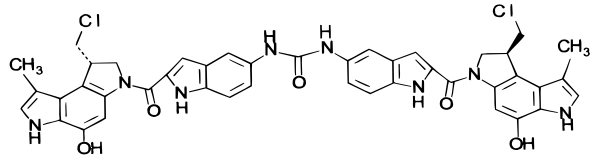

FIGURE 1: Chemical structure of bizelesin.

Earlier, bizelesin was reported to induce site-specific lesions in SV40 DNA in lytically infected BSC-1 cells (*16*, *19*). As with isolated DNA, bizelesin induced DNA alkylation of either two strands (resulting in a double-strand cross-link) or a single strand in the DNA of infected cells. The bonding sites were mapped to functionally important AT-rich regions of the genome.

Bizelesin also reduced the level of synthesis of viral DNA molecules in simian virus 40 (SV40) lytically infected BSC-1 cells (*20*). However, the SV40 DNA lesion frequency was limited at bizelesin concentrations which dramatically reduced the extent of replication of SV40 molecules, suggesting a trans mechanism of replication inhibition (i.e., one not directly related to levels of template adduction). A trans mechanism has been implicated in the inhibition of DNA synthesis by other DNA-damaging agents. Classic inducers of trans replication inhibition include X irradiation (*21*) and UV irradiation (*22*, *23*). More recently, studies with the monofunctional CPI adozelesin, as well as with the unrelated DNA strand scission agent C-1027, showed a decreased level

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of initiation of SV40 replication at doses which caused damage to only a minor fraction of viral DNA (*24*, *25*). While each of these agents acts in trans to inhibit replication, direct cis acting inhibition (e.g., due to the presence of a lesion on the replicating template) also may be observed at higher doses.

While bizelesin is a potent inhibitor of DNA replication, effects on specific molecular events in the replication process [i.e., initiation or elongation (or both) of nascent DNA chains] have not been reported. DNA electrophoresis patterns on two-dimensional (2D) agarose gels (*24*, *26*−*28*) can indicate whether agents which block DNA replication exert their inhibitory effect at the level of initiation or elongation of nascent DNA chains. SV40 provides a facile system for studying such effects, as parameters for its synthesis are well-defined (*29*−*31*).

In this study, we used 2D neutral agarose gel electrophoresis to determine the ability of bizelesin to inhibit initiation or elongation of SV40 DNA replication. The percentage of SV40 molecules containing a bizelesin adduct was quantitated, and the question of whether lesion induction directly or indirectly inhibited SV40 replication (i.e., in cis, by blocking progress of the replication fork, or in trans, by modulation of crucial replication factors) was examined. SV40 DNA replication was also assayed in vitro using extracts from bizelesin-treated HeLa cells. Mixing experiments were performed to evaluate whether the inhibition of SV40 DNA replication in vitro was due to decreased levels of an essential DNA replication factor(s) or to the induction of trans-acting inhibitory activity.

EXPERIMENTAL PROCEDURES

Chemicals. Bizelesin was provided by the Pharmacia & Upjohn Co. (Kalamazoo, MI). Stock solutions in dimethylacetamide (Aldrich Chemical Co., Milwaukee, WI) were stored at −20 °C and diluted in dimethyl sulfoxide (DMSO) prior to addition to cells. [α-^{32}P]dCTP and GeneScreen membranes were obtained from Dupont NEN (Boston, MA). [^{3}H]TdR ([*methyl*-^{3}H]thymidine, 48 Ci/mmol) was from CEA. [α-^{32}P]dATP was obtained from Amersham Pharmacia Biotech (Piscataway, NJ). The DECAprime II DNA labeling kit was from Ambion (Austin, TX). Proteinase K was from Boehringer Mannheim (Indianapolis, IN). All other chemicals were reagent grade.

Plasmids and Proteins. SV40 large T antigen (Tag) was purified from recombinant baculovirus-infected Sf9 cells using immunoaffinity chromatography (*32*). SV40 origin-containing plasmid pSV011 has been described elsewhere (*33*).

Bizelesin Treatment of SV40-Infected BSC-1 Cells and Preparation of DNA. Maintenance of BSC-1 (African green monkey kidney) cells in minimal essential medium and 10% calf serum (MEM) and conditions for infection with the SV40 virus were described previously (*24*, *34*). For SV40 replication studies, cells were plated at a density of 5×10^5/100 mm plate, and grown for 48 h until they were 80−90% confluent. Medium was removed, and cells were infected with SV40 virus in MEM containing 2% bovine calf serum (MEM-2). Two hours after infection, the virus-containing medium was replaced with 10 mL of virus-free MEM-2. Twenty-four hours after infection, 5 mL of medium was removed from each plate, and 25 μL of bizelesin at the appropriate concentration was added to the remaining 5 mL. For measurement of replication activity (i.e., replication of newly synthesized DNA), [^{3}H]TdR (10 μCi/mL of medium) was added during the last 30 min of the bizelesin incubation. Plates were rinsed twice with phosphate-buffered saline (PBS), and cells were removed from the plates by trypsinization. DNA was isolated using the QIAamp blood kit (QIAGEN Inc., Santa Clarita, CA) according to the manufacturer's protocol for blood and body fluids, except that samples were digested with QIAgen proteinase K for 1.5 h at 37 °C, instead of at 70 °C. DNA was stored in 0.1 mL QIAGEN AE buffer at −20 °C.

2D Gel Electrophoresis. Isolated DNA was digested with the restriction enzyme *Bam*HI which cuts the SV40 molecule once. Samples were assayed by neutral/neutral 2D gel electrophoresis as described previously (*24*). Briefly, samples were electrophoresed on 0.6% agarose gels in 1× TAE and 0.1 μg/mL ethidium bromide for 24 h at 0.7 V/cm. Ethidium bromide-stained DNA was visualized by placing the gel over an ultraviolet light source, and lanes were cut out and placed in a slot cut into the top of the second-dimension gel (1% agarose) perpendicular to the direction of the current. The second-dimension gels were electrophoresed at 4 °C in 1× TBE and 0.5 μg/mL ethidium bromide for 19 h at 4 V/cm.

Southern Blots and Fluorography. Duplicate 2D gels were electrophoresed, and were either Southern blotted for measurement of the mass of replication intermediates or processed for fluorography for measurement of replication activity as described previously (*24*). Briefly, one gel was Southern blotted to GeneScreen and hybridized to ^{32}P-radiolabeled full-length SV40 DNA. The DECAprime II DNA labeling kit was used for radiolabeling the SV40 DNA probe. The other gel was fluorographed by dehydration in ethanol (two washes in 95% ethanol for 1 h at room temperature), followed by soaking in absolute ethanol containing 5% 2,5-diphenyloxazole (PPO). After 1 h, the PPO/ethanol solution was removed and the gel was washed once for 1 h in H_2O to precipitate the PPO, and then dried.

Detection of SV40 DNA on Southern Blots. ^{32}P-radiolabeled SV40 DNA replication intermediates were detected by phosphorimaging as described previously (*24*, *25*). Briefly, to assay the mass of replication intermediates, phosphorimages of ^{32}P-radiolabeled Southern blots of 2D gels were scanned using a Molecular Dynamics phosphorimager (Sunnyvale, CA). To measure replication activity, X-ray film was exposed to fluorograms of ^{3}H-radiolabeled gels. Films were scanned using a Molecular Dynamics densitometer. Images in which either mass or replication activity was detected were analyzed using Molecular Dynamics ImageQuant software. Changes in replication intermediates were determined in ImageQuant by inserting identical ellipses over the tips of the bubble arc (i.e., to include the most intense signal in the upper left-hand portion of the bubble arc), the 1N spot (1N), and over a portion of the blot in which no DNA was located [to provide a background value (BG)]. The volumes of the ellipses were quantitated, and the amounts of the replication intermediates associated with the bubble arc were calculated

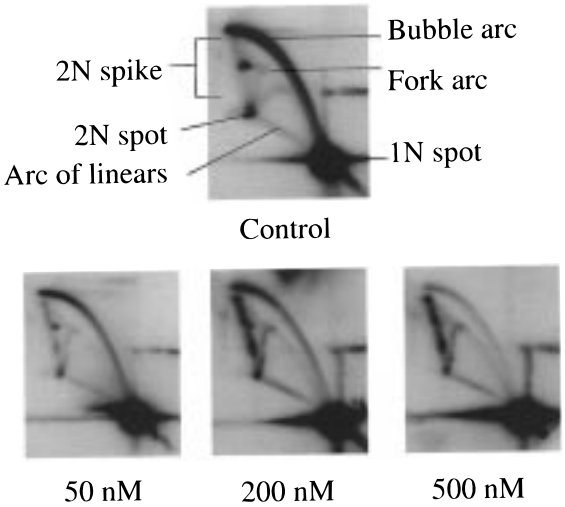

FIGURE 2: Bizelesin effects on the electrophoretic migration pattern of SV40 replication intermediates on 2D neutral agarose gels. SV40-infected BSC-1 cells were treated for 4 h with differing concentrations of bizelesin. DNA was isolated, restricted with *Bam*HI, electrophoresed, Southern blotted, and hybridized with ^{32}P-radiolabeled full-length SV40 DNA as described in Experimental Procedures. Panel 1 (top) is a phosphorimage of a Southern blot showing the pattern of replication intermediates observed in the absence of drug treatment. The indicated replication intermediates are described in the Results. In panels 2−4 (bottom), cells were incubated with the bizelesin concentrations that are shown.

according to the formula

$$\text{bubble arc} = \frac{(\text{bubble arc signal} - \text{BG})}{(1\text{N} - \text{BG})/(1\text{N}_{\text{control}} - \text{BG})}$$

where 1N and $1N_{control}$ are the amounts of unreplicated *Bam*HI-linearized SV40 DNA in the sample and control, respectively (see Figure 2).

Detection of Bizelesin-Induced DNA Lesions. A 0.01 mL aliquot of DNA isolated as described above was incubated at 70 °C for 2 h to convert bizelesin-alkylated lesions into DNA strand breaks. After being loaded into the wells of a 1.0% agarose gel, samples were electrophoresed for 20 h at 1.5 V/cm to permit separation of SV40 intact supercoiled form I (FI) from nicked circular form II (FII) and linear form III (FIII) DNA. The gel was Southern blotted and hybridized to the ^{32}P-radiolabeled full-length SV40 probe described above, and the ^{32}P radiolabel was detected by phosphorimaging. Increases in the amounts of SV40 form II and III DNA were indicative of DNA single-strand and double-strand break damage, respectively, and were quantitated as described previously (*35*).

Cell-Free Replication. (1) Cell Extracts. Cervical carcinoma HeLa S3 cells were grown in suspension culture in Dulbecco's minimal essential medium (DMEM) and 10% fetal bovine serum. Final bizelesin concentrations of 100−800 nM were added to the culture medium 4 h before the cells were harvested. Equivalent amounts of drug vehicle (DMA and DMSO) were added to control cultures. Cell extracts were prepared as described by others (*30*, *32*) with minor modifications. Briefly, after being washed once with cold PBS and once with hypotonic buffer [20 mM HEPES/KOH (pH 7.5), 1.5 mM $MgCl_2$, 5 mM KCl, and 1 mM dithiothreitol], cells were suspended in hypotonic buffer for 10 min before lysing by seven passages through a 25 gauge needle. Cell lysates were left on ice for 30 min before separating the soluble fraction from cell debris by centrifugation at 9000*g* for 10 min at 4 °C. The cell extracts were aliquoted, frozen in liquid nitrogen, and stored at −80 °C. The protein concentrations of the extracts were determined using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Inc., Hercules, CA).

(2) Cell-Free SV40 DNA Replication Assays. The conditions used for SV40 cell-free DNA replication were as described elsewhere by others (*32*). Briefly, 30 ng of SV40 origin-containing plasmid pSV011, 500 ng of T-antigen, and 40 μg of protein from extracts of control (no drug treatment) or bizelesin-treated cells (or as indicated in the figure legends) 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, 7.0 mM $MgCl_2$, 0.024 unit of creatine phosphokinase, 40 mM phosphocreatine, and 2 μCi of [α-^{32}P]dATP) in a final volume 10 μL, and the mixture was incubated at 37 °C for 60 min. Reactions were stopped by the addition of 10 mM Na_2EDTA, 1% SDS, and 50 μg/mL proteinase K. Following a 20 min (37 °C) proteinase K digestion and phenol/chloroform extraction, DNA products were ethanol precipitated, dissolved in TE, and separated on a 0.8% (w/v) agarose gel in 1× TAE by electrophoresis at 5 V/cm for 3 h. Gels were vacuum-dried onto filter paper, and radioisotope incorporation into replicative forms was analyzed using a Bio-Rad Molecular Analyst Phosphorimager. The total amount of DNA synthesis represents the radioactivity present in each lane, including the replication intermediates (RIs) and the replicated monomeric plasmids from form I through II.

RESULTS

Electrophoresis on 2D agarose gels separates DNA replication intermediates on the basis of size in the first dimension and both size and shape in the second dimension. Replicating SV40 DNA was linearized with the restriction enzyme *Bam*HI and electrophoresed on 2D gels. For detection of SV40 DNA, gels were Southern blotted, and blots were hybridized to full-length ^{32}P-radiolabeled SV40 DNA as described in Experimental Procedures. Panel 1 of Figure 2 is a phosphorimage of a Southern blot of a representative gel showing 2D migration of SV40 DNA replication intermediates. SV40 replicates bidirectionally from the origin of replication. Because *Bam*HI cuts the circular SV40 molecule once at a point in the termination region opposite the origin of replication, the majority of replicating molecules consist of bubbles of various sizes resulting from fork elongation toward the termination region and the *Bam*HI cut site. These bubbles are visualized as an intense band (the bubble arc) curving upward from the 1N spot (the unreplicated molecule) and represent 7−12% of all SV40 molecules (*24*, *25*). The 2N spike consists of nearly fully replicated molecules differing in the position of meeting forks or, in the case of recombination, of their crossed strands (*36*). Molecules migrating at the 2N spot may be nearly completely replicated SV40 molecules which have slowed near the termination point and consist predominantly of linear replicated DNA (*36*, *37*). Alternatively, signal in this region may be contributed by partial restriction enzyme digestion of multimeric (e.g., dimeric) SV40 DNA molecules. A very

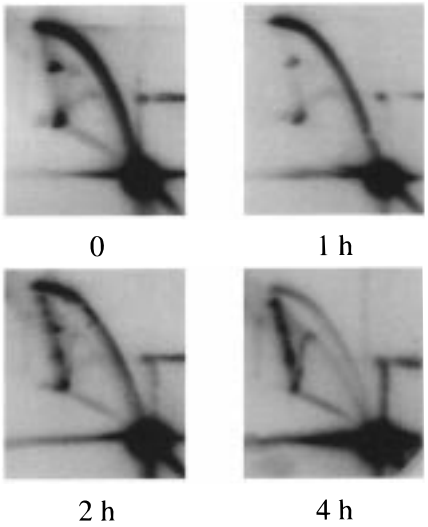

FIGURE 3: Time course of bizelesin-induced changes in SV40 replication intermediates. SV40-infected BSC-1 cells were incubated with 500 nM bizelesin. DNA was isolated, electrophoresed, blotted, and hybridized as described in the legend of Figure 2. The figure shows phosphorimages of DNA from cells treated with bizelesin for 0−4 h.

faint fork arc was observed, indicating the presence of very limited numbers of molecules replicating through non-cairns intermediates. A description of the electrophoretic migration of replicating DNA intermediates on neutral 2D gels is detailed elsewhere by others (*36*, *38*).

Changes in SV40 replication intermediates after incubation for 4 h with increasing concentrations of bizelesin are shown in Figure 2. The intensity of SV40 replication intermediates decreased progressively in bizelesin-treated compared to that in control (untreated) cells (panel 1). The bubble arc signal was reduced by at least 60 and 90% by 50 and 500 nM bizelesin, respectively (see panels 2 and 4, respectively). Such a loss in the bubble arc would be expected if bizelesin treatment inhibited initiation of new replicons and the already initiated molecules continued to elongate. While an increase in the fork arc also was observed, it was minimal compared to the loss of the bubble arc. By contrast, an increase in signal at discrete positions along the 2N spike was detected with as little as 50 nM bizelesin and intensified as the bizelesin concentration was increased to 500 nM. At least three distinct spots were evident with 500 nM bizelesin (panel 4). These spots accounted for 50% of the signal from SV40 replication intermediates remaining after the drug treatment. Since a characteristic of replicating DNA structures migrating on 2D gels is that the DNA signal is especially strong at positions where the rate of replication is reduced (i.e., at replication pause sites) (*39*), the appearance of these spots on the 2N spike is suggestive of replication fork pausing.

Figure 3 is a phosphorimage of a representative 2D gel showing SV40 replication intermediates from cells exposed to 500 nM bizelesin for increasing times of up to 4 h. A decrease in bubble arc intensity compared to the control (panel 1) was observed after treatment for 1 h (panel 2), and the bubble arc continued to decrease 2 and 4 h after addition of bizelesin (panels 3 and 4). Spots along the 2N spike (see Figure 2) not evident at 1 h were present by 2 h and remained pronounced 4 h after bizelesin addition.

Bizelesin induced a concentration- and time-dependent decrease in the amount of SV40 DNA replication intermediates (see Figures 2 and 3) which could result from a decreased initiation of replicating DNA. If initiation and not elongation were inhibited, the number of replicating intermediates detected by [^{32}P]SV40 hybridization of Southern blots (i.e., the bubble arc mass) would be reduced due to elongation in the absence of additional initiation events. Because of the decreased numbers of replicating intermediates, the extent of incorporation of [^{3}H]thymidine into these intermediates (i.e., the replication activity) also would decrease. By contrast, if elongation were inhibited, movement of the replication fork would be blocked, and the bubble arc mass would not decrease. However, the extent of [^{3}H]-thymidine incorporation into the bubble arc would decrease dramatically (*25*).

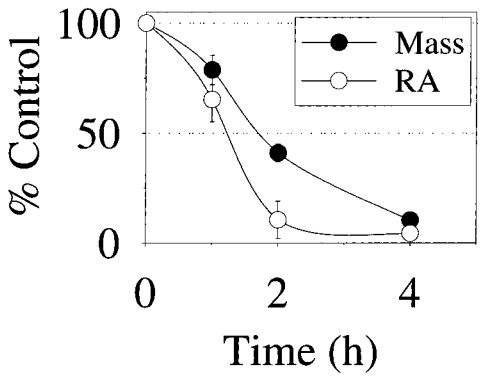

FIGURE 4: Summary of changes in the bubble arc mass and replication activity of SV40 DNA when infected BSC-1 cells were incubated with 500 nM bizelesin for differing periods of time. The procedure for calculating the mass from phosphorimages of ^{32}P-radiolabeled Southern blots and the replication activity from fluorograms of [^{3}H]thymidine 2D gels is described in Experimental Procedures.

Bizelesin-induced changes in the SV40 bubble arc mass and replication activity from duplicate samples were determined by Southern blotting and fluorography, respectively, as described in Experimental Procedures. Briefly, [^{3}H]-thymidine was added to cells 30 min prior to the termination of bizelesin treatment. After electrophoresis, one set of 2D gels was Southern blotted and hybridized with [^{32}P]SV40 for the detection of the bubble arc mass. The second set was dried, and ^{3}H-radiolabeled replication intermediates were fluorographed for the detection of replication activity. The bubble arc on Southern blots and fluorograms was detected by phosphorimaging and autoradiography, respectively, and quantitated as described in Experimental Procedures. Figure 4 summarizes the time course of 500 nM bizelesin effects on bubble arc mass and replication activity. Both mass and replication activity decreased with increasing bizelesin treatment time. After 1 h, both mass and replication activity had decreased by 25%. After 2 h, replication activity was inhibited to a much greater extent [i.e., by 90% (±8%)] than was the mass [i.e., by 59% [±4%, standard error of the mean (SEM)]]. After 4 h, the mass as well as the replication activity was reduced to 10% (±2%, SEM) of the control value. The coincident decrease in mass and replication activity 1 h after bizelesin addition is consistent with inhibition of initiation, while at later times (i.e., ≥2 h), the preferential decrease in replication activity may indicate a predominant effect on elongation.

Bizelesin forms DNA covalent bonds which are converted into strand breaks upon heating at ≥70 °C (*16*). To determine

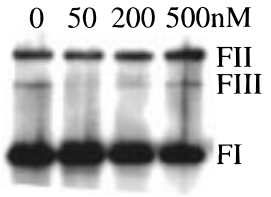

FIGURE 5: Intracellular SV40 DNA strand damage analysis. SV40-infected BSC-1 cells were incubated with bizelesin for 4 h. DNA was isolated and electrophoresed on an agarose gel as described in Experimental Procedures. A phosphorimage of a Southern blot of DNA from cells treated with 0−500 nM bizelesin is shown. The positions of intact supercoiled form I (FI), nicked circular form II (FII), and linear form III (FIII) SV40 DNA are denoted.

whether bizelesin bonding to DNA was directly responsible for inhibiting fork progression, the number of lesions in total (replicating and nonreplicating) SV40 DNA from bizelesin-treated cells was determined. Cells were treated with bizelesin, and DNA was isolated as described in Experimental Procedures, then heated at 75 °C for 2 h to induce DNA strand breaks, and electrophoresed on a one-dimensional agarose gel to separate undamaged supercoiled SV40 form I from nicked circular form II and linear form III DNA. Figure 5 is a phosphorimage of a Southern blot of a representative gel showing the distribution of SV40 topological forms with increasing bizelesin concentrations. In this experiment, no change in form distribution was apparent when cells were incubated for 4 h with 50−500 nM bizelesin. Additional experiments revealed that treatment for 4 h with 500 nM bizelesin affected only a 0.34% increase in the amount of form III, while an increase in the amount of form II and a decrease in the amount of form I of 9% were noted. However, no DNA strand damage was detected in the samples treated with 50 nM bizelesin in which a 50% decrease in the amount of SV40 RIs was observed. Thus, at concentrations which effected significant decreases in replication activity (see Figure 2), bizelesin-induced single- or double-strand damage to total cellular SV40 DNA was extremely limited.

While forms analysis indicates the frequency of strand damage in the entire population of SV40 DNA molecules, two-dimensional gel electrophoresis may provide a sensitive method for measuring the frequency of bizelesin adducts on replicating DNA. Examination of Figures 2 and 3 suggests that DNA lesions sufficient to induce pausing of replication forks arise after prolonged incubation times ($\geq$2 h) with $\geq$50 nM bizelesin. By comparison of the signal contributed by replication intermediates contained in the 2N spike after prolonged treatment with the total pool of available replication intermediates (i.e., the signal associated with the bubble arc from untreated cells), the fraction of bizelesin-adducted replicating SV40 DNA can be estimated. For this calculation, the signal contained in a region drawn around the newly arising pause sites on the 2N spike was quantitated in ImageQuant and compared to the signal associated with the bubble arc from control (untreated) samples according to the following formula:

$$\frac{[(\text{pause sites} - \text{BG})/(1\text{N} - \text{BG})]}{[(\text{bubble arc}_{\text{control}} - \text{BG})/(1\text{N}_{\text{control}} - \text{BG})]}$$

where BG is the background value for the same sized region on the image.

Using this formula, 5% of the SV40 DNA-replicating molecules contained lesions after infected cells were treated with 200 nM bizelesin for 4 h. These lesions may reflect the presence of either single- or double-stranded covalent DNA adduct formation after prolonged incubation times and may contribute to a cis inhibition of the rate of replication fork elongation.

Spots on the 2N spike suggestive of replication pause sites were not detected after incubation with bizelesin for shorter times (i.e., $\leq$1 h). Thus, the early effects on initiation suggested by the rapid decrease in the bubble arc signal appear to be independent of bizelesin−DNA adduct formation. Since direct alkylation of the SV40 template probably was not responsible for the pronounced effect on DNA replication induced by short periods of exposure to bizelesin, early inhibition of replication initiation likely occurred in trans.

SV40 cell-free DNA replication assays were used to characterize this trans inhibition of DNA replication. Viral origin-containing plasmids were added to the in vitro assay as the DNA template. These exogenously added templates were not exposed to bizelesin during drug treatment of the cells. Therefore, any cis-acting effects due to lesions in replicating molecules are limited to damage which might occur during the reaction if bizelesin is present in the extract from drug-treated cells. HeLa S3 cell cultures were treated with 0, 100, 200, 400, or 800 nM bizelesin. After drug treatment for 4 h, all cultures had identical cell densities. Cells were harvested and subjected to extract preparation as described in Experimental Procedures, and the extracts were adjusted to 10 mg/mL protein. The gross protein patterns of these extracts, as analyzed by SDS−polyacrylamide gel electrophoresis and Coomassie blue staining, were indistinguishable (data not shown). The extracts were tested for their ability to support SV40 DNA replication in vitro. Figure 6A is a phosphorimage of an agarose gel showing the electrophoretic migration pattern of ^{32}P-radiolabeled SV40 DNA newly replicated by extracts from HeLa cells treated with 0−800 nM bizelesin. Twenty, thirty, or forty μg of total protein from each mock- or bizelesin-treated cell extract was used in SV40 cell-free DNA replication assays.

The extent of replication was greatly reduced in extracts from bizelesin-treated HeLa cells compared to extracts from control cells. A decrease in the total level of ^{32}P incorporation was observed with as little as 100 nM bizelesin at all three protein levels [compare lanes 1−3 (control) with lanes 4−6]. With 800 nM bizelesin, the radioactive signal throughout each of the lanes was reduced dramatically (lanes 13−15) compared to the control. Panel B is a graphic representation of the data shown in panel A. Treatment with 100 nM bizelesin reduced the level of replication 20−40%, while decreases of at least 50% in replication activity were observed with extracts from cells treated with 400 nM bizelesin. Thus, extracts from bizelesin-treated (100−800 nM) HeLa cells were deficient in their ability to support SV40 DNA replication.

It was possible that residual bizelesin retained during isolation of the cell extracts might have contributed to the replication inhibition observed in Figure 6. To test this

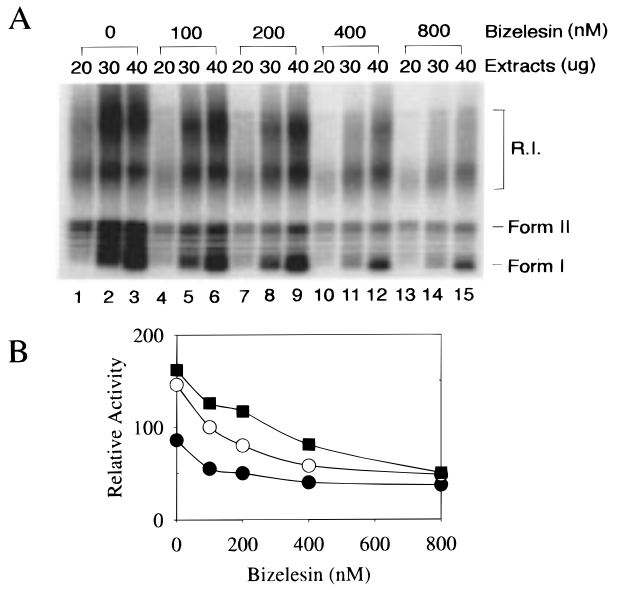

FIGURE 6: Cell-free SV40 DNA replication by extracts of bizelesin-treated HeLa cells. (A) Twenty, thirty, or forty micrograms of extract from 0 (lanes 1−3), 100 nM (lanes 4−6), 200 nM (lanes 7−9), 400 nM (lanes 10−12), and 800 nM (lanes 13−15) bizelesin-treated HeLa cells was assayed as described in Experimental Procedures. The migration positions of form I (FI), form II (FII), and θ form DNA replication intermediates (RI) are denoted. (B) Graphic representation of the data depicted in panel A. ^{32}P incorporation into newly replicated SV40 DNA by 20 (●), 30 (○), or 40 μg (■) of extract was quantitated by phosphorimage analysis.

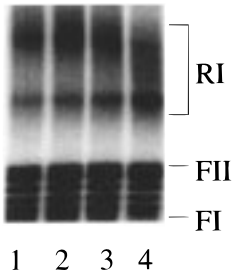

FIGURE 7: Cell-free DNA replication in the presence of bizelesin. Bizelesin was added to control cell extracts prior to assaying for cell-free replication competence. The assay was performed and samples were analyzed as described in the legend of Figure 6. Lanes show replication of SV40 DNA by (1) untreated cell extract (control), (2) untreated cell extracts to which vehicle (DMSO) alone was added, and (3 and 4) bizelesin-treated (200 and 800 nM) cell extracts.

possibility, bizelesin (200 or 800 nM)[1] was added to control extracts prior to performing cell-free SV40 DNA replication assays (Figure 7). The addition of exogenous bizelesin caused only minor changes in the electrophoretic pattern of the newly replicated DNA (see the slightly increased mobility of RIs in the 800 nM lane), and virtually no difference in the total amount of newly synthesized DNA, as compared to control reactions. Thus, the effects of exogenously added bizelesin on in vitro DNA replication are minor. These results indicate that the decrease in the extent of in vitro DNA replication with extracts from bizelesin-treated cells (57−70% in extracts from 800 nM bizelesin-treated cells; see Figure 6) cannot be due merely to the presence of free bizelesin in extracts from drug-treated cells.

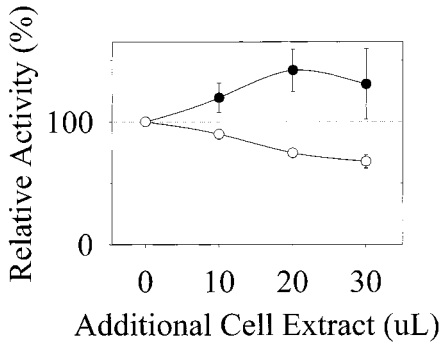

FIGURE 8: Changes in the in vitro replication competence of control cell extracts induced by extracts of bizelesin-treated cells. Ten to thirty micrograms of cell extract protein from mock-treated (●) or 800 nM bizelesin-treated cells (○) was added to 40 μg of control. Samples were analyzed as described in the legend of Figure 6. The drug treatment and mixing experiments were performed three times. Results from each experiment were normalized by setting the 40 μg control cell extract data point to 100% and are expressed ± the standard deviation.

In bizelesin-treated cells, inhibition of DNA replication may occur by inactivation or reduction of an essential DNA replication factor(s), or induction of a negative regulator or inhibitor of DNA replication. To distinguish between these possibilities, SV40 DNA replication reactions were performed in a constant volume (10 μL) using mixtures of extracts from control cells and cells treated with 800 nM bizelesin (Figure 8). Since the mixing experiment was performed in a constant reaction volume, no dilution of essential replication factors took place. DNA synthesis using 40 μg of mock-treated cell extract was vigorous but submaximal. Further addition of 10−30 μg of mock-treated cell extract further increased the level of SV40 DNA synthesis, showing an additive response.[2]

Data from Figure 6 show that extracts from cells treated with 800 nM bizelesin, while reduced in their replicative competence, still retained 30−40% of control activity. If a reduction in replication factors in the extracts of drug-treated cells alone was responsible for inhibition, addition of 10−30 μg of protein from extracts of bizelesin-treated cells would be expected to contribute a small (i.e., <10%) increase in the amount of newly replicated SV40 DNA synthesized by the control extracts. Instead, addition to the control cell extract of 10−30 μg of extracts from cells treated with 800 nM bizelesin decreased the SV40 DNA replication activity. This is characteristic of an antagonistic response. Since DNA replication by the control extract was suppressed by the drug-treated cell extract, bizelesin probably acts by inducing production of a DNA replication inhibitor. In addition to the apparent induction of an inhibitor of replication, whether bizelesin either inactivated or reduced the levels of any essential DNA replication factor(s) could not be established.

DISCUSSION

This study indicates that the bifunctional alkylator bizelesin inhibits both initiation and elongation of SV40 DNA replication. The SV40 molecule requires only 20−40 min to complete one round of replication. If initiation is inhibited while the replicating molecules continue to elongate, a rapid

[1] These concentrations were chosen since the effect on replication was seen with extracts from cells treated with 200−800 nM bizelesin.

[2] The increase in the level of synthesis was not linear at these levels as the reaction was approaching its plateau.

decrease (i.e., within 40 min of drug addition) in the level of SV40 replicating bubbles should be observed. Such a rapid decrease in the SV40 bubble arc was observed ≤1 h after bizelesin addition (see Figure 3). A selective effect on initiation also has been observed with the monofunctional CPI agents adozelesin (*24*) and CC-1065 (unpublished data). In addition, the DNA-reactive enediyne strand-scission agent C-1027 was reported to inhibit initiation both of SV40 DNA replication in lytically infected cells (*25*) and of EBV DNA replication in latently infected immortalized cultured human Raji cells (*40*). These observations suggest that inhibition of initiation of DNA replication in either lytically infected or immortalized cells may be a general response to treatment with diverse types of DNA damaging drugs.

Inhibition of DNA replication by DNA damaging agents can be mediated by secondary factors, which act in trans. Trans inhibition may result either from the reduced availability or activity of crucial replication factors or from induction of a replication inhibitor. The former appears to be the case for inhibition of DNA replication by UV treatment of cells. When cultured cells were treated with UV light, extracts from these cells were unable to support SV40 DNA replication in vitro (*22*, *23*, *41*). Exogenous addition of a known cellular DNA replication factor, replication protein A (RPA), to extracts from UV-treated cells rendered them fully competent for SV40 DNA replication. These results were not consistent with the presence of an active inhibitor of replication in these extracts, but rather with a trans effect mediated by the inactivation of an essential DNA replication factor.

By contrast, the results reported herein indicate the presence of an active inhibitor of replication in extracts from bizelesin-treated cells (see Figure 8). In further support of differing mechanisms of trans inhibition induced by UV light and bizelesin treatment, addition of exogenous RPA to bizelesin-treated cell extracts did not restore their ability to support SV40 DNA synthesis to levels obtained with mock-treated cell extracts (data not shown). These differences suggest that bizelesin-mediated DNA replication arrest is unique from the replication arrest caused by UV treatment of cells. A trans replication inhibitor in extracts from cells treated with the topoisomerase I inhibitor camptothecin also was reported previously (*42*). While the camptothecin-induced inhibition also was not reversible with exogenous RPA addition, it is unknown whether the same factor is involved in camptothecin- and bizelesin-induced replication inhibition.

Extracts from cells treated with the monofunctional CPI agent adozelesin (100 nM) exhibit an even greater level of inhibition of SV40 DNA replication in vitro (∼85%) than extracts from cells treated with 800 nM bizelesin (57−70%; see Figure 6). However, unlike extracts from bizelesin-treated cells, extracts from adozelesin-treated cells did not inhibit DNA replication by control cell extracts upon mixing (S.-R. Kuo, J.-S. Liu, and T. Melendy, unpublished data).

Cells respond to the action of DNA-damaging agents by a decreased level of cell cycle progression and by checkpoint arrest (*43*−*46*). Slowed progression through S and checkpoint arrest in G2-M have been observed in mammalian cells treated with either of the monofunctional CPIs adozelesin or CC-1065 (*47*−*49*). It is unknown whether bizelesin alters cell cycle progression in normal cells. A bizelesin-induced DNA damage response also cannot be confirmed in the present study, since SV40 lytically infected cells normally cease to divide after undergoing only one doubling after viral infection (*29*). However, if the SV40 replication inhibitor identified in extracts from bizelesin-treated HeLa cells (see Figure 8) also functions as an inhibitor of genomic DNA replication, checkpoint arrest would be expected.

Cellular DNA damage and checkpoint arrest pathways may be mediated by protein phosphorylation. For example, UV, γ-irradiation, and double-strand DNA breaks all result in phosphorylation of p53 (for a review, see ref *50*). Studies for evaluating the effects of CPI DNA-damaging agents on the phosphorylation levels of various DNA replication factors are currently underway.

Inhibition of SV40 DNA elongation was observed when infected cells were exposed to bizelesin for longer treatment times (i.e., incubation for ≥2 h) (see Figure 4). Longer treatment times also reportedly promote formation of bizelesin−DNA covalent bonds (*16*, *51*), and the presence of these adducts may physically block SV40 replication in a cis-acting manner. CPI agents have been shown to inhibit the action of both DNA helicases and DNA polymerases (*52*, *53*). Inhibition of these enzymes would be consistent with decreased levels of SV40 DNA replication.

Cis inhibition of elongation also was indicated by the appearance of a series of intense spots (replication pause sites) on the 2N spike after prolonged treatment with bizelesin (see Figures 2 and 3). Spots were localized to discrete positions along the spike, suggesting an accumulation of lesions at specific sites. Earlier, the location of bizelesin covalent binding sites on the SV40 genome, including one within the termination region (at bp 2600), was reported (*16*). Bizelesin alkylation at discrete positions on replicating SV40 daughter molecules could impede elongation of the replication fork in cis at the site of the lesion and produce the pattern that is observed.

Cis inhibition of SV40 elongation was not observed with monofunctional CPIs. After 2D gel electrophoresis of SV40 replication intermediates from cells treated with adozelesin or CC-1065 at concentrations sufficient to reduce the amount of replication intermediates by ≥90%, no high-intensity spots were visualized on the 2N spike or elsewhere on the 2D gel pattern, and only very limited elongation inhibition were observed (ref *24* and unpublished data, respectively). By contrast, the signal associated with pause sites on the 2N spike after treatment with 200 nM bizelesin for 4 h equaled 5% of the signal associated with replication intermediates from untreated cells (see Figure 2). Bizelesin's ability to inhibit elongation in cis as indicated by the appearance of these pause sites may contribute to its increased cytotoxicity compared to those of the monofunctional CPIs adozelesin and CC-1065 (*14*, *17*).

In conclusion, bizelesin inhibited initiation of SV40 DNA replication in trans and induced an inhibitor of cell-free DNA replication. Future studies will attempt to define the nature of this inhibitor and answer the question of whether its production is a general response to the interaction of CPI agents with intracellular DNA. The elongation inhibition observed after longer treatment times may be a direct cis effect of bizelesin covalent bond formation on replicating SV40 DNA molecules.

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