Cell-free and Cellular Activities of a DNA Sequence Selective Hairpin Polyamide-CBI Conjugate*

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Alkylation agents are generally highly reactive with DNA but demonstrate limited DNA sequence selectivity. In contrast, synthetic pyrrole-imidazole polyamides recognize specific DNA sequences with high affinity but are unable to permanently damage DNA. An eight-ring hairpin polyamide conjugated to the alkylation moiety cyclopropylpyrroloindole, related to the natural product CC-1065, affords a conjugate 1-CBI (polyamide 1-CBI (1-(chloromethyl)-5-hydroxy-1,2-dihydro-3H-benz[e]indole) conjugate) which binds to specific sequences in the minor groove of DNA and alkylates a single adenine flanking the polyamide binding site. In this study, we show that 1-CBI alkylates DNA in both plasmid and intracellular minichromosomal form and inhibits DNA replication under both cell-free and cellular conditions. In addition, it inhibits cell growth and arrests cells in the G2/M phase of the cell cycle.

Polymides are synthetic DNA binding molecules designed to recognize and bind to specific sequences in the minor groove of the double helix with affinities comparable with those of DNA-binding proteins (1, 2). Polymides comprised of pyrrole (Py) and imidazole (Im) amino acids are connected through a γ-amino butyric acid (γ) linker to create a hairpin structure. An Im/Py pair recognizes a G-C base pair, and Py/Im recognizes a C-G base pair (3), whereas Py/Py is partially degenerate and recognizes both A-T and T-A base pairs (4). Hairpin polyamides have the potential to affect gene transcription by specifically targeting promoters or other regulatory sequences. It has been demonstrated that polyamides can bind TFIIA binding sites and interfere with 5 S RNA expression (5), inhibit replication of human immunodeficiency virus type 1 virus (6), or block the Ets binding site of the HER2/neu promoter (7). To date, there have been few reports describing polyamide-induced down-regulation of endogenous gene expression in intact cells, which may be related to the observation that nuclear localization is cell type-dependent (8). Likewise, inhibition of cell growth or anti-tumor activity has not been reported. The fact that binding of polyamides to DNA is reversible, and their inability to permanently damage DNA may account for the absence of cellular activities.

In contrast to polyamides, the cyclopropylpyrroloindole (CPI) DNA alkylation agents represent a class of antitumor drugs whose members include some of the most biologically active agents (9). CPI drugs interact with the DNA minor groove in A/T-rich sequences and alkylate the N3 position in adenine at the 3' end of the binding site. They inhibit DNA replication through cis- or trans-acting mechanisms, mainly at the stage of initiation (10–16).

In an effort to design hybrid DNA-binding compounds that covalently interact with DNA in a sequence-specific fashion, polyamide 1 was conjugated with a DNA alkylation group secO-CBI, an analog to the reactive moiety of CPI drugs (17). The conjugate 1-CBI (ImImPyPyPy-(R))Polyamide 1-CBI (ImImPyPyPy-(R))Polyamide 1-CBI was synthesized as described previously (17). A base pairs (4). Hairpin polyamides have the potential to affect gene transcription by specifically targeting promoters or other regulatory sequences. It has been demonstrated that polyamides can bind TFIIA binding sites and interfere with 5 S RNA expression (5), inhibit replication of human immunodeficiency virus type 1 virus (6), or block the Ets binding site of the HER2/neu promoter (7). To date, there have been few reports describing polyamide-induced down-regulation of endogenous gene expression in intact cells, which may be related to the observation that nuclear localization is cell type-dependent (8). Likewise, inhibition of cell growth or anti-tumor activity has not been reported. The fact that binding of polyamides to DNA is reversible, and their inability to permanently damage DNA may account for the absence of cellular activities.

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DNA or for 4 h (minichromosomes). No DNA strand breakage was observed in controls for up to 16 h incubation at 37 °C. Following treatment, minichromosomes were incubated with 1% SDS for 30 min at 37 °C to dissociate nucleosomal-like structures. All samples were extracted with phenol/chloroform/isooamyl alcohol (PCI, 25:24:1), precipitated with ethanol, and resuspended in TE buffer containing 150 mM NaCl. Samples were then incubated at 70 °C for 2 h to convert alkylated bases to DNA strand breaks. DNA was electrophoresed on 0.8% agarose gels in 1× Tris-acetate electrophoresis (TAE) buffer for 16 h at 1 V/cm, stained with 1 μg/ml ethidium bromide, and quantitated using densitometry and ImageQuant program (Amersham Biosciences).

**Repetitive Primer Extension (RPE) Assay**—The reaction was carried out according to the published procedure (22, 23). The oligonucleotide primer (5′-GCGACTCTGAACTTCCCT-3′) starting from position 1991 (reverse) on the SV40 genome was synthesized by the Biopolymer Facility at Roswell Park Cancer Institute. The primer was end-labeled (reverse) on the SV40 genome was synthetized by the Biopolymer Facility at Roswell Park Cancer Institute. The primer was end-labeled with [γ-32P]ATP using T4 polynucleotide kinase, and excess radioactive label was removed using a G25 MicroSpin column (Roche Molecular Biochemicals). The amplification mixture consisted of 50 ng of treated SV40 DNA, 0.5 μCi of [γ-32P]ATP, and 1 unit of TaqDNA polymerase in 1× polymerase buffer (Bio-Rad). Amplified template DNA was prepared by incubating SV40 DNA (0.3 μg, form I) with 1-CBI at 37 °C for 16 h followed by PCI extraction and ethanol precipitation as described. The replication reaction mixture contained 50 μg of 293 cell extracts, 30 ng of drug-treated SV40 DNA, 0.6% SDS, 50 μl of 5× PCR buffer (Promega), 0.2 μCi of [γ-32P]ATP in replication buffer (4 mM ATP, 0.2 mM CTP, 0.2 mM GTP, 0.2 mM UTP, 0.1 mM dGTP, 0.1 mM dCTP, 0.025 mM dATP, 7 μM MgCl2, 0.024 units of Taq DNA polymerase, and 1 unit of T4 polymerase in 1× polymerase buffer (Invitrogen). Reaction was carried out using 35 cycles of 30 s at 95 °C, 30 s at 55 °C, and 55 s at 72 °C. The dideoxycycle sequencing reaction was performed according to manufacturer’s instructions (SequiTherm Excell II sequencing kit, Epicentre Technologies, Madison, WI) using the same primer and non-treated SV40 DNA template. DNA fragments were separated on an 8% polyacrylamide/8.3M urea sequencing gel and then visualized and quantitated using phosphorimaging techniques.

**Cell-free SV40 DNA Replication Assay**—Cell extracts were prepared from exponentially growing 293 cells as described earlier (18). Briefly, cells were harvested, washed twice with phosphate-buffered saline, resuspended in hypotonic buffer (20 mM Hepes, pH 7.5, 1.5 mM MgCl2, 5 mM KCl, 1 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride), disrupted by three rapid freeze/thaw cycles, and incubated on ice for an additional 30 min. After removing the cell debris by centrifugation at 12,000 × g for 15 min, protein concentration of the resulting extract was determined using the Bradford method (Bio-Rad). Agent-modified template DNA was prepared by incubating SV40 DNA (0.3 μg, form I) with 1-CBI at 37 °C for 16 h followed by PCI extraction and ethanol precipitation as described. The replication reaction mixture contained 50 μg of 293 cell extracts, 30 ng of drug-treated SV40 DNA, 0.6% SDS, 50 μl of 5× PCR buffer (Promega), 0.2 μCi of [γ-32P]ATP in replication buffer (4 mM ATP, 0.2 mM CTP, 0.2 mM GTP, 0.2 mM UTP, 0.1 mM dGTP, 0.1 mM dCTP, 0.025 mM dATP, 7 μM MgCl2, 0.024 units of creatine phosphokinase, and 40 μM phosphocreatine). After incubation for 1 h at 37 °C, the replication products were isolated on a G50 MicroSpin column (Roche Molecular Biochemicals) and separated by electrophoresis on a 0.8% agarose gel at 1 V/cm in 1× TAE for 16 h. Gel slices were excised and replication products were quantitated following exposure to a phosphorimaging screen.

**Intracellular SV40 DNA Replication Assay**—BSC-1 cells were seeded at 3 × 105 cells per 60-mm dish and grown for 24 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 virus-containing medium was removed, cells were incubated an additional 22 h in fresh MEM-2. At 24 h after infection, 1-CBI was added to the medium for 16 h treatment, and the SV40 DNA was extracted from cells according to the modified Hirt extraction procedure (24). In short, phosphate-buffered saline-washed cells were incubated for 10 min at 37 °C in 0.5 ml of 25 mM Tris-HCl, 5 mM EDTA, and 1% SDS containing 0.1 mg/ml RNAse followed by an additional 30-min treatment with proteinase K (final concentration, 1 mg/ml). Cellular proteins were precipitated by the addition of 0.35 ml of 3 μg/ml trichloroacetic acid, 1 μg/ml sodium acetate, and 0.67 μM acetic acid and incubation for 15 min on ice. Supernatants recovered by centrifugation for 15 min at 13,000 × g were loaded onto Qiagen spin columns (Valencia, CA). Columns were washed with 0.75 ml of 80 μM potassium acetate, 10 mM Tris-HCl, 40 μM EDTA, and 60% ethanol, and SV40 DNA was recovered with 50 μl of TE.

The effects of 1-CBI on SV40 DNA intracellular replication were analyzed using neutral/neutral two-dimensional isotope exchange using a previously described method (12). Briefly, SV40 DNA isolated from virus-infected and 1-CBI-treated BSC-1 cells was digested with the restriction enzyme BamHI that cuts the SV40 molecule only once. The replication reaction mixture contained 50 ng of SV40 T-antigen, and 2 μCi of [3H]-labeled primer, 1.5 mM MgCl2, 0.2 mM of each dNTP, and 1 unit of T4 polymerase in 1× polymerase buffer (Invitrogen). The reaction was carried out using 35 cycles of 30 s at 95 °C, 30 s at 55 °C, and 55 s at 72 °C. The dideoxycycle sequencing reaction was performed according to manufacturer’s instructions (SequiTherm Excell II sequencing kit, Epicentre Technologies, Madison, WI) using the same primer and non-treated SV40 DNA template. DNA fragments were separated on an 8% polyacrylamide/8.3M urea sequencing gel and then visualized and quantitated using phosphorimaging techniques.

**Cell Growth Inhibition Assay**—Conjugate-induced cell growth inhibition was determined by assaying the cell number after 4 days of continuous exposure. BSC-1 cells were plated on a 96-well plate at 105 cells/well and incubated for 24 h. Exponentially growing cells were treated with the tested agents for 4 days. Cells were then fixed in 1.6 trichloroacetic acid at 4 °C for 1 h, stained with 0.4% saponin, and exposed to 0°C for 15 min on ice. Supernatants recovered by centrifugation for 15 min at 13,000 × g were loaded onto Qiagen spin columns (Valencia, CA). Columns were washed with 0.75 ml of 80 μM potassium acetate, 10 mM Tris-HCl, 40 μM EDTA, and 60% ethanol, and SV40 DNA was recovered with 50 μl of TE.

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**Cell Cycle Progression Assay**—BSC-1 cells were treated with 1-CBI for 16 h followed by an additional 24-h incubation in drug-free medium. Cells fixed in 5 ml of ice-cold 75% ethanol at ~20 °C overnight were stained in sodium citrate buffer containing propidium iodide (50 μg/ml, Sigma) and RNaseA (100 μg/ml, Roche Molecular Biochemicals) for 30 min at room temperature. The DNA content of the stained cells (10 cells/sample) was analyzed by flow cytometry using a FACScan (BD Biosciences) with an excitation wavelength of 488 nm and a long pass (>600 nm) emission filter and WinList 5.0 program (Verity Software House, Topsham, ME).

**RESULTS**

1-CBI Induces Thermolabile Lesions in SV40 DNA—1-CBI is a polyamide-based ligand (Fig. 1) that contains a CBI moiety related to the natural alkylating agent CC-1065 that binds and alkylates DNA. Heating of alkylated DNA at 70 °C resulted in strand breaks at the drug binding site (25–27). Whether similar thermolabile DNA lesion resulted from 1-CBI treatment was determined using an SV40 forms conversion assay. In this assay, induction of DNA strand breaks can be detected by changes in DNA forms distribution using agarose gel electrophoresis. Conversion of fast migrating SV40 DNA form I (supercoiled circular) to slow migrating form II (nicked circular) requires one strand break per DNA molecule, whereas the
formation of form III (linear) requires a frank double-strand break or at least two single-strand breaks, closely spaced on opposite strands.

The ability of 1-CBI to damage SV40 DNA is shown in Fig. 2. After incubating DNA for 16 h with 1 μM 1-CBI at 37 °C, DNA lesions can be converted to strand breaks by heating at 70 °C. The 2 h of heating resulted in the quantitative conversion of the alkylated form I SV40 DNA to form II and form III, while leaving non-treated control DNA undamaged (Fig. 2A, lane 2). First, the time dependence of DNA alkylation by 1-CBI was determined at 37 °C. As shown in Fig. 2A, DNA damage can be detected after a 1–2-h incubation at 37 °C, as indicated by a loss of form I molecules and increases in form II and form III (lanes 5 and 6). A complete loss of form I and significant increase in form III were observed only after a 16-h incubation. The results demonstrate that DNA alkylation is time-dependent and that a 16-h 1-CBI incubation produces maximum DNA lesions. Therefore, unless otherwise indicated, a 16-h treatment with 1-CBI was used in the subsequent studies.

Next, the dose dependence of 1-CBI-induced DNA damage was evaluated (Fig. 2B). SV40 DNA was treated for 16 h at 37 °C with increasing concentrations of 1-CBI. The quantitation of the gels showed that at 0.1 μM, the amount of supercoiled DNA (form I) was reduced by ~50%, whereas 0.2 μM 1-CBI converted more than 90% of the supercoiled DNA to forms II and III. Treatment with higher concentrations of the agent resulted in a significant increase in the form III band (2 μM, lane 7), suggesting alkylation of the closely spaced sites, which can be converted to double-strand break. Under the same experimental conditions, no DNA strand damage was observed with parent polyamide 1, which binds reversibly to DNA but cannot alkylate it (data not shown).

1-CBI Alkylates SV40 Minichromosomal DNA—1-CBI alkylation of naked SV40 DNA and SV40 minichromosomes was compared to determine whether DNA association with nucleoproteins affected alkylation. SV40 minichromosomes are formed in cells during lytic infection, when the viral genome associates with histones and other nuclear proteins to form circular molecules of nucleosomal chromatin. Minichromosomes provide a good model of mammalian chromatin in vitro because the virus uses the chromatin components of the host, and the minichromosomes with their full complement of DNA-associated proteins can be separated from the cellular genome. However, to examine DNA alkylation activity on chromatin structure, it is necessary to define a condition that can prevent 1-CBI from binding to DNA during the post-lysis period. Since it is experimentally difficult to remove free agent from minichromosomes after the reaction, there is a possibility that 1-CBI could bind naked DNA after dissociating nucleoprotein from the minichromosomal DNA. A previous study showed that some detergents, including SDS, are able to prevent CPI drugs from binding to DNA (27). Likewise, 1% SDS effectively prevented 1-CBI from binding to SV40 DNA without causing removal of 1-CBI already covalently bind to DNA (data not shown).

1-CBI alkylating activity on minichromosomal SV40 DNA was determined by the forms conversion assay (Fig. 2C), using SDS to prevent free agent from binding to DNA once nucleoproteins had been dissociated from minichromosomes. SV40 minichromosomes were treated with increasing concentrations of 1-CBI at 37 °C for 4 h (longer incubation at 37 °C resulted in disruption of minichromosomal structure) followed by the addition of 1% SDS for an additional 30 min. After free agent and proteins were removed by PCI extraction and ethanol precipi-
tation, strand breaks were induced by heating the alkylated DNA at 70°C for 2 h. 1-CBI damages minichromosomal DNA in a dose-dependent manner, as demonstrated in Fig. 2C. Fifty percent conversion of DNA form I to II and III was calculated from quantitated gels at 0.18 μM 1-CBI, the range similar to that observed for naked SV40 DNA. Thus, 1-CBI appears to be equally effective at alkylating both naked and minichromosomal DNA. Confirmation that 1-CBI could be prevented from binding DNA by 1% SDS was obtained when the detergent was added to the minichromosomes prior to the agent, resulting in no detectable increase in DNA damage (Fig. 2C, lane 8).

Sequence Specificity of 1-CBI-induced DNA Damage—Naturally occurring CPI compounds alkylate N-3 of adenine within AT-rich regions, thereby exhibiting sequence preference but not sequence specificity (28). The polyamide portion of 1-CBI is designed to bind a specific DNA sequence, 5'-(A/T)GG(A/T)C(A/T)-3'. This binding positions the agent to alkylate a single adenine located 2 bp away from the polyamide-targeting site. The sequence-specific alkylation by 1-CBI has been confirmed previously on a 277-bp DNA fragment (17). To further test this characteristic on a more complex DNA molecule (5243 bp), the RPE assay was performed using agent-treated SV40 DNA as a template. In this assay, DNA nascent chain elongation driven by polymerase stops at sites of the damage on template DNA, and the partial DNA synthesis products indicate the positions of DNA damage (22).

Initially, naked SV40 DNA was treated with 1-CBI for 16 h at 37°C and then extracted and precipitated to remove free 1-CBI. Agent-modified and control non-treated DNA was used as template in linear amplification reactions. The experiments demonstrated that 1-CBI induced DNA damage in vitro at the specific site, the polyamide-targeted site (Fig. 3). One μM 1-CBI effectively damaged DNA at an adenine site 2 bp adjacent to the polyamide binding site, 5'-AGGACTTA-3' (Fig. 3, lane 7, the arrow indicates presumed alkylated adenine). The parent polyamide 1 (at 3 μM) did not induce DNA damage under the same experimental conditions (data not shown). It was also noted that 1-CBI introduced DNA damage at a site composed of six contiguous adenines but not at other AT-rich regions in the analyzed DNA fragment.

To compare 1-CBI sequence specificity in cellular and cell-free environments, the RPE assays were performed using SV40 DNA isolated from virus-infected and then agent-treated BSC-1 cells (Fig. 3, lane 6). At 24 h after infection with SV40, BSC-1 cells were treated with 1-CBI for 16 h, and SV40 DNA was isolated as described under “Experimental Procedures.”
The experiments showed that 1-CBI alkylates the same DNA sequence on intracellular SV40 as on naked DNA (lanes 6 and 7). Since cell lysis was performed in the presence of 1% SDS, which prevents rebinding to DNA as described earlier in the cell-free study, we conclude that post-lysis 1-CBI-DNA interactions did not contribute to the SV40 DNA damage observed.

**Inhibition of Cell-free SV40 DNA Replication—**1-CBI induces DNA lesions that could stop DNA polymerase, as shown in RPE experiments. Therefore, whether 1-CBI-alkylated SV40 DNA could serve as a template in the more complex DNA synthesis system (such as cell-free SV40 DNA replication) was examined (Fig. 4A). Naked SV40 DNA was treated with 1-CBI for 16 h at 37°C followed by removal of free 1-CBI. The replication reaction containing extracts of 293 cells, control, or agent-treated SV40 DNA template and SV40 T-antigen was carried out as described under "Experimental Procedures." Reduced DNA replication competence was noted when the SV40 DNA template was treated with 0.1 μM 1-CBI (lane 2), and the reduction was maximal with 0.5–1 μM 1-CBI (lanes 4 and 5). The inhibition curve (Fig. 4B) shows that 0.25 μM 1-CBI decreased SV40 DNA replication by ~50%. Since the cell extract was not agent-treated and free agent was removed after template treatment, the inhibition of replication activity is likely due to 1-CBI-induced covalent damage to the DNA template. No inhibition of SV40 DNA replication was detected when the SV40 DNA template was pretreated with up to 10 μM polyamide 1 (data not shown).

**Inhibition of SV40 DNA Replication in BSC-1 Cells—**Since it was found that 1-CBI inhibited cell-free SV40 replication, we examined whether similar activity can be observed in the intact cells. It has been shown by two-dimensional gel electrophoresis that CPI drugs can block intracellular SV40 replication at the level of initiation (12, 14). At 24 h after infection with SV40, BSC-1 cells were treated with 1-CBI for 16 h, and the isolation of SV40 DNA was carried out as described. Naked DNA was separated on two-dimensional gel electrophoresis and analyzed as described under "Experimental Procedures." 1-CBI treatment caused a concentration-dependent decrease in intracellular SV40 replication intermediates detected in the form of bubble arcs, as indicated by the arrows in Fig. 5; the bubble arc intensity was reduced at 0.1 μM and diminished completely at 3–10 μM agent concentration.

**Cell Cycle Arrest Induced by 1-CBI—**One of the cellular responses following DNA damage induced by CPI agents is a delay in S phase and block at the G2/M transition in the cell cycle (29). Flow cytometry was used to evaluate the cell cycle progression of BSC-1 cells treated with 1-CBI. Asynchronously growing cells were treated with 1-CBI for 16 h and postincubated in agent-free medium for 24 h, and then cellular DNA was stained with propidium iodide, and DNA content was analyzed by flow cytometry (Fig. 6). In control non-treated samples, the majority of cells were in G1 (~62%) with the remaining distributed through S and G2/M. With the increase of 1-CBI concentration, the delayed progression through S and the G2/M arrest were observed. Twenty-four h after treatment with 1 μM 1-CBI, the majority of cells were in late S (~55%) and G2/M (30%). Under similar conditions, no effects on cell cycle were observed in BSC-1 cells treated with polyamide 1 (data not shown).

**Cell Growth Inhibition by 1-CBI—**Since 1-CBI exhibited the ability to damage and interfere with DNA functions in whole cells, one can expect that it is capable of inhibiting cell growth. Growth of BSC-1 cells following 1-CBI treatment was measured using standard sulforhodamine-B staining of cellular proteins. The cell growth curve (Fig. 7) shows that ~0.3 μM 1-CBI inhibits cell growth by 50%, whereas polyamide 1 has no effect up to 10 μM. To address the possible cell line specificity of this activity, the cell growth inhibition induced by 1-CBI was also analyzed using human 293 and NIH3T3 (mouse embryonic fibroblasts) cells. Similar levels of growth inhibition were observed in all tested cell lines at 0.1–10 μM 1-CBI concentrations (data not shown).

In addition, the ability of 1-CBI to inhibit cell growth was evaluated using a pair of human breast cancer cell lines, MDA435/LCC6 wild type and MDA435/LCC6 multidrug-resistant (MDR) (30). As compared with the wild type (sensitive) cell line, ~3.5-fold higher concentration of 1-CBI is required to inhibit the growth of MDR cells by 50% (IC50) (Table 1). By comparison, a 12-fold higher concentration of the DNA cross-linking CPI agent bizelesin was required to inhibit growth of the MDR cell line as compared with the wild type cell line, whereas both cell lines were equally sensitive to CC-1065 and adzelesin (monoalkylating CPI agents).

**DISCUSSION**

In the current study, we evaluated the DNA binding agent 1-CBI, which combines the sequence specificity of a polyamide

![Fig. 5. Effects of 1-CBI on intracellular SV40 DNA replication. SV40-infectected BSC-1 cells were treated for 16 h with the indicated concentrations of 1-CBI (0–10 μM). Purified SV40 replication intermediates were separated by two-dimensional agarose gel electrophoresis, detected by Southern blotting and hybridization to [32P]-SV40 probe, and analyzed as described under "Experimental Procedures." The positions of replication intermediates (bubble arcs) are indicated by arrows.](image)
Table I

<table>
<thead>
<tr>
<th>Agent</th>
<th>MDA435/LCC6 IC50</th>
<th>MDA435/LCC6 IC50</th>
<th>Ratio (MDR/sensitive)</th>
</tr>
</thead>
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<tr>
<td>1-CBI</td>
<td>0.36 µM</td>
<td>1.23 µM</td>
<td>3.5</td>
</tr>
<tr>
<td>CC-1065</td>
<td>6.3 nM</td>
<td>6.1 nM</td>
<td>0.9</td>
</tr>
<tr>
<td>Adozelesin</td>
<td>6.1 nM</td>
<td>6.9 nM</td>
<td>1.1</td>
</tr>
<tr>
<td>Bizelesin</td>
<td>11 µM</td>
<td>132 µM</td>
<td>12</td>
</tr>
</tbody>
</table>

a Cells were agent-treated for 4 days, and then the growth was quantitated based upon sulforhodamine-B staining of cellular proteins and absorbance measurements at 570 nm as described under “Experimental Procedures.”

DNA translates into its biological activity are described in this study.

Analysis of DNA damage using an SV40 forms conversion assay showed that 1-CBI alkylates DNA after incubation at 37 °C and that lesions can be converted to strand breaks by heating alkylated DNA at 70 °C for 2 h. The DNA damaging activity of 1-CBI is only 2–3-fold less than that of its parent alkylating agent CC-1065 based on the conversion of 50% of form I SV40 (−0.1 versus −0.05 µM) (Fig. 2, A and B, and Ref. 27). However, a 16-h incubation is required to produce the maximum amount of DNA damage, whereas only 15 min is needed for maximal CC-1065-induced damage (27). Examination of the form conversion data obtained using naked (Fig. 2, A and B) and minichromosomal (Fig. 2C) DNA indicated that the presence of chromatin structure components had little effect on the level of damage introduced by 1-CBI. In addition, supercoiled DNA (form I) was converted predominantly to nicked circular DNA (form II), and a significant increase in linear form III was observed only at higher 1-CBI concentrations (>1 µM). Since conversion from form I to II requires one single-strand break, these results indirectly confirm the monoalkylating activity of 1-CBI.

Classical CPI agents target the N-3 of adenine on DNA (28), and their DNA sequence selectivity is limited to the AT-rich region (27). The polyamide moiety of 1-CBI, however, is designed to target the agent to specifically alkylate a single adenine flanking the polyamide binding site 5′-(A/T)GG(A/T)CA(T)-3′. Our cell-free RPE experiments using 1-CBI-treated DNA showed that a single adenine was alkylated in a dose-dependent fashion in the analyzed SV40 DNA region. The alkylated adenine is 2 bp away from the 3′-terminal of the polyamide-binding sequence 5′-AGGACT-3′ (Fig. 3, lane 7). Furthermore, similar sequence specificity was observed on an intracellular SV40 DNA target that was isolated from 1-CBI-treated, SV40 virus-infected cells (Fig. 3, lane 6). Therefore, the ability of 1-CBI to alkylate a polyamide-specific DNA sequence in both cell-free and intracellular environments has been demonstrated. These results are consistent with earlier findings which showed that CPI agents produce similar DNA damage profiles regardless of whether the target is naked or nucleosomal DNA (11, 27). We note in the RPE analysis (Fig. 3, lower part of the gel) that 1-CBI introduces lesions at two adenines in the 5′-AAAAA-3′ tract proximal to a polyamide match sequence 5′-GGACA-3′.

One biological activity often associated with DNA damaging agents is their ability to inhibit DNA replication via a cis-acting mechanism due to the presence of lesions on the replicating template. We studied drug-induced inhibition of DNA replication under cell-free conditions using SV40 templates treated with 1-CBI. Replication inhibition was observed at concentrations as low as 0.1 µM (Fig. 4). At the same concentration range, ~50% of SV40 DNA form I was converted to form II and form III in the forms conversion assay (Fig. 2), indicating that 50%
of SV40 DNA molecules acquired at least one DNA lesion, rendering them inactive in the replication assay. Our results showed that the 1-CBI concentrations required for DNA replication inhibition and DNA damage in cell-free system are similar, suggesting direct relationships between both events.

However, the accessibility of cellular DNA to the alkylation by 1-CBI might be limited by factors such as cell membrane permeability or protein interference. To explore 1-CBI-induced inhibition of DNA replication under cellular conditions, two-dimensional electrophoresis gel analysis was performed using the well defined SV40 DNA replication system in BSC-1 cells. This technique had been used in previous studies to demonstrate that CPI agents, adozelesin (12) and bizelesin (14), can inhibit cellular DNA replication at the level of initiation. Like these CPI agents, inhibition of intracellular SV40 DNA replication by 1-CBI was indicated by a loss of replication intermediates with increasing 1-CBI concentration (Fig. 5). Although this profile is consistent with a blockage of the initiation of DNA replication, further studies would be needed to verify the effects on elongation.

Another consequence of exposing cells to 1-CBI is interference with cell cycle progression. Previous studies showed that cells treated with DNA-alkylating CPI agents slow their progression through S phase and arrest in the G2/M phase of cell cycle (29). Our flow cytometry results clearly indicated that 1-CBI delayed S-phase progression and blocked cells in G2/M phase (Fig. 6).

The potency of a DNA damaging agent is related to its cytotoxic potential. Although 1-CBI shows the ability to inhibit cell growth (Fig. 7), it requires a 50-fold higher concentration of the hybrid compound to inhibit cell growth in comparison with the classical CPI agents (31). This can be partially explained by the relatively large molecular weight of 1-CBI, which may hinder its cellular uptake. Nevertheless, since the parent polyamide 1 was virtually inactive even at higher concentrations (>10 μM), this finding demonstrates that addition of the alkylating activity to the polyamide confers an ability to inhibit cell growth. The cell growth inhibition of 1-CBI was observed at levels similar to those required for the detection of cellular DNA damage, DNA replication inhibition, and cell cycle arrest in BSC-1 cells. This is distinct from previous reports with some CPI agents in which cell growth inhibition was observed at much lower levels than those required for the detection of either DNA damage or DNA synthesis inhibition (13, 32).

Previous studies showed that the MDR phenotype could affect the activity of CPI drugs (31). As compared with the wild type cell line, the 12-fold higher concentration of bizelesin was required to inhibit the growth of MDR cells in 50%. On the other hand, adozelesin or CC-1065 was equally active against sensitive and MDR-resistant cells. Interestingly, 1-CBI evoked intermediate differences between sensitive and resistant cells (3.5-fold). We hypothesize that the deviation of 1-CBI from patterns established for other mono-alkylating CPI drugs (adozelesin and CC-1065) could be partial related to its higher sequence selectivity.

The high binding affinity and sequence specificity of polyamides suggest that they may be useful in both basic research and drug development. Although the displacement of transcrip-