1208–1215 *Nucleic Acids Research, 2003, Vol. 31, No. 4*
DOI: 10.1093/nar/gkg215

DNA crosslinking and biological activity of a hairpin polyamide–chlorambucil conjugate

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Received as resubmission October 28, 2002; Accepted December 10, 2002

ABSTRACT

A prototype of a novel class of DNA alkylating agents, which combines the DNA crosslinking moiety chlorambucil (Chl) with a sequence-selective hairpin pyrrole–imidazole polyamide ImPy-β-ImPy-γ-ImPy-β-Dp (polyamide 1), was evaluated for its ability to damage DNA and induce biological responses. Polyamide 1-Chl conjugate (1-Chl) alkylates and interstrand crosslinks DNA in cell-free systems. The alkylation occurs predominantly at 5′-AGCTGCA-3′ sequence, which represents the polyamide binding site. Conjugate-induced lesions were first detected on DNA treated for 1 h with 0.1 μM 1-Chl, indicating that the conjugate is at least 100-fold more potent than Chl. Prolonged incubation allowed for DNA damage detection even at 0.01 μM concentration. Treatment with 1-Chl decreased DNA template activity in simian virus 40 (SV40) *in vitro* replication assays. 1-Chl inhibited mammalian cell growth, genomic DNA replication and cell cycle progression, and arrested cells in the G_2/M phase. Moreover, cellular effects were observed at 1-Chl concentrations similar to those needed for DNA damage in cell-free systems. Neither of the parent compounds, unconjugated Chl or polyamide 1, demonstrated any cellular activity in the same concentration range. The conjugate molecule 1-Chl possesses the sequence-selectivity of a polyamide and the enhanced DNA reactivity of Chl.

INTRODUCTION

DNA alkylating agents, and in particular the nitrogen mustards, remain an important class of antitumor drugs. For example, the aromatic nitrogen mustard chlorambucil (Chl) is routinely used in the chemotherapy of leukemias and lymphomas, and in other tumors (1). The biological activity of Chl and other nitrogen mustards is mediated through alkylation of guanine N7 position in the DNA major groove, and subsequent DNA interstrand crosslinking. The reaction is determined largely by the molecular electrostatic potential of the DNA, with alkylation occurring preferentially in the middle of runs of guanines (2). These covalent modifications of DNA, and especially interstrand crosslinks, lead to cell death if not repaired. Because of their relatively simple structure and high reactivity, nitrogen mustards have a very limited DNA sequence selectivity and rapidly lose their activity by non-specific interaction with a number of nucleophilic groups on cellular biomolecules other than DNA. Nitrogen mustards have been shown to be potentially carcinogenic, mutagenic and teratogenic, and to promote secondary malignancies (3,4), probably due to the non-specific interactions.

Attempts to improve the selectivity of nitrogen mustards, and to better target them to cancer cells in particular, have led to development of several new strategies. One is to create hybrid molecules that combine DNA targeting and DNA damaging moieties. One of the most successful applications of this scheme was the design of tallimustine (FCE 24517), a hybrid molecule consisting of the benzoyl nitrogen mustard and the AT-specific DNA minor groove binder distamycin. Tallimustine possesses enhanced sequence-selectivity, alkylates adenine in the DNA minor groove, and is cytotoxic to cancer cells (5). In order to further increase the sequence selectivity of DNA damage, we have used synthetic polyamides as DNA-targeting carriers [for recent review on polyamides development see (6)]. The hairpin polyamides are able to distinguish all four base pair combinations (GC, CG, AT and TA) in the minor groove of DNA (7). Polyamides can be designed to read DNA sequences up to 16 base pairs in length (8,9).

To combine DNA targeting and damaging activities, polyamide 1 which binds to the sequence 5′-(A/T)GC(A/T)GC(A/T)-3′ with high affinity (K_a~10^{10} M^{-1}), was conjugated with the DNA alkylating/crosslinking agent Chl (10). The resulting polyamide 1-Chl conjugate was shown to alkylate single sites within a 241-bp DNA fragment (under the cell-free conditions) (10). We test here whether such an activity would be maintained on complex DNA molecules and, importantly, whether 1-Chl possesses intracellular activity, in particular in comparison to that of the parent Chl, which is an anticancer clinical drug.

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We compared the mode of DNA interaction and biological activities of 1-Chl with the parent compounds, polyamide 1 (DNA recognizing/targeting moiety) and Chl (DNA alkylating moiety). 1-Chl and its parent compounds were tested for their ability to induce DNA damage, interfere with DNA replication under cell-free and cellular conditions, and inhibit cell growth and cell cycle progression. We find that 1-Chl alkylates and crosslinks DNA *in vitro* with high efficiency, and that alkylation occurs predominantly at the sequence targeted by the polyamide. Treatment of 293 cells with 1-Chl resulted in G_2/M phase arrest and DNA synthesis and growth inhibition. Conjugate 1-Chl exhibited higher activity (at least 100-fold) than its separate components, polyamide 1 and Chl, in every cell-free or cellular system tested, and demonstrated cytotoxic effects in several mammalian cell lines.

MATERIALS AND METHODS

Chemicals

1-Chl (ImPy-β-ImPy-(*R*) $^{\mathrm{CHL}}$-γ-ImPy-β-Dp), polyamide 1 (10) and Chl (Sigma, Saint Louis, MO) stock solutions were prepared in distilled water, and diluted in reaction buffer or cell culture medium before use. Simian virus 40 (SV40) DNA was purchased from Gibco/Life Technologies (Grand Island, NY), as were all cell culture materials. [α-^{32}P]dCTP (3000 Ci/mmol), [α-^{32}P]dATP (3000 Ci/mmol) and [γ-^{32}P]ATP (5000 Ci/mmol) were from Amersham (Piscataway, NJ).

In vitro ligand–DNA reaction

All conjugate–DNA reactions were carried out at 37°C with 0.01 µg/µl SV40 DNA (linear, supercoiled or minichromosomal) in 25 mM triethanolamine, 1 mM EDTA, pH 7.2 (total vol 30 µl), for the time indicated, and terminated by addition of stop solution (final concentrations 1% SDS, 0.3 M sodium acetate, 5 mM EDTA). Samples were subsequently extracted with phenol/chloroform/isoamyl alcohol (PCI) mixture (25:24:1), precipitated with 3 vol 95% ethanol, washed with 70% ethanol, and dissolved in 10 mM Tris–HCl, 1 mM EDTA, pH 7.5 (TE) buffer.

SV40 DNA cross-linking *in vitro*

A cross-link assay was carried out according to Hartley *et al.* (11) with some modifications. SV40 DNA, linearized (*Bam*HI, Boeheringer Mannheim, Indianapolis, IN) and dephosphorylated (calf intestinal phosphatase, Sigma), was ^{32}P-5′-end-labeled using T4 polynucleotide kinase (Boehringer Mannheim). Following purification on G50 Quick Spin columns (Boehringer Mannheim) and precipitation, the DNA was resuspended in TE buffer at 1 mg/ml, and specific activity was quantitated by liquid scintillation. Labeled DNA (~10 000 c.p.m.) was mixed with 0.3 µg of 'cold' SV40 DNA, incubated with the studied compounds and purified as described above. Dried samples were dissolved in strand separation buffer (65% formamide, 1 mM EDTA, 0.04% of each bromophenol blue/xylene cyanol), heated at 55°C for 10 min and chilled immediately on ice. DNA was resolved on 1% agarose gels in 1× Tris–acetate electrophoresis (TAE) buffer at 1 V/cm for 16 h. The DNA bands were visualized and quantitated with a PhosphorImager Scanner and ImageQuant v.5 software (Molecular Dynamics, Sunnyvale, CA).

SV40 DNA forms conversion

Ligand-induced DNA damage was measured using a forms conversion assay (12). Supercoiled SV40 DNA (0.3 µg) was treated with the studied compounds and then purified as described above. In some experiments, SV40 DNA minichromosomes were used according to a previously described method (13). Ligand-treated and precipitated DNA was resuspended in TE, heated at 70°C for 2 h to convert alkylation sites into strand breaks, and resolved on 1% agarose gels in 1× TAE at 1 V/cm for 16 h. Separated SV40 DNA forms were visualized and quantitated with the ChemiImager 4400 System (AlphaInnotech, San Leandro, CA).

Repetitive primer extension (RPE)

SV40 DNA treated with the studied agents or untreated controls were used in RPE experiments (14). The primer (5′-CCCAAGGCTCATTTCAGGC-3′, starting from position 2818/reverse on the SV40 genome, synthesized by the Biopolymer Facility at Roswell Park Cancer Institute), was ^{32}P-5′-end-labeled with T4 polynucleotide kinase (Boehringer Mannheim), purified and used directly in thermal cycling. A linear amplification reaction (25 µl) consisted of 100 ng of SV40 DNA (control or drug-treated), labeled primer (0.5 µM), 1 U *Taq* DNA polymerase, 1.5 mM $MgCl_2$ and 0.2 mM of each dNTP, in 1× *Taq* polymerase buffer (Life Technologies). The polymerase reaction was started with 5 min denaturation at 95°C and carried out for 35 cycles of 30 s at 95°C, 30 s at 55°C and 55 s at 72°C. The standard sequencing reactions were performed according to the manufacturer's instructions using SequiTherm Excel II kit (Epicentre Technologies, Madison, WI) and the same primer. DNA fragments were separated on 8% polyacrylamide/8.3 M urea sequencing gels in 1× Tris–borate electrophoresis (TBE) buffer. Gels were dried and DNA replication products were visualized by phosphorimaging.

Cell-free SV40 DNA replication

The method and conditions used for SV40 cell-free DNA replication were as described previously (15). The total cellular protein extracts were prepared from non-treated, exponentially growing 293 cells by hypotonic buffer extraction, and protein concentrations were determined using the Bradford method (Bio-Rad Laboratories, Hercules, CA). Ligand-modified template DNA was prepared by incubating SV40 DNA (150 ng, form I) with 1-Chl at 37°C for 1 h, followed by phenol-chloroform extraction and precipitation as described above. 1-Chl-treated template DNA (50 ng) was mixed with 293 cell extracts (50 µg proteins), 600 ng of T antigen, and 2 µCi of [α-^{32}P]dATP in the replication buffer. The reactions (final vol 20 µl) were incubated at 37°C for 1 h, and then stopped by addition of 1% SDS, 10 mM EDTA and 0.1 mg/ml proteinase K. Following 30 min incubation at 37°C and PCI extraction, DNA was purified on G50 Quick Spin column and separated on 0.8% agarose gels in 1× TAE for 16 h at 1 V/cm. The DNA replication products were visualized and quantitated by phosphorimaging.

Cell culture and cell growth inhibition

HeLa S3, HCT-116, 293 and BSC-1 cells were grown as a monolayer, and Jurkat cells as suspension culture, in appropriate media at 37°C in a 5% CO_2 incubator. Exponentially growing cells were plated in 96-well plates at a density of 1×10^3 cells/well. After overnight incubation, the cells were treated with the studied compounds for 4 days, followed by washing and fixing with 50% cold trichloroacetic acid. Cell growth was quantitated based upon Sulphorhodamine B staining of cellular proteins and absorbance measurements at 570 nm.

Inhibition of DNA, RNA and protein synthesis

Inhibition of cellular macromolecule synthesis was measured as [^{3}H]-precursors incorporation into cells as described previously (15). Briefly, 293 cells were pre-labeled with [^{14}C]thymidine (0.01 µCi/ml) for 48 h, followed by a 24 h chase in fresh medium. Cells plated in 6-well plates (1×10^5 cells/well) were treated with 1-Chl for 18 h, and during the last 1 h [^{3}H]thymidine, uridine or leucine were added to a final concentration 0.5 µCi/ml. Following washes in phosphate-buffered saline, pH 7.4, and macromolecules precipitation with cold perchloric acid, the acid-insoluble radioactivity in samples was quantitated using liquid scintillation. Inhibition of precursors incorporation into cellular macromolecules was calculated as the ratio of [^{3}H] to [^{14}C] in drug-treated sample compared with non-treated control.

DNA content determination by flow cytometry

Exponentially growing 293 cells were treated with 1-Chl or Chl for 18 h followed by an additional 24 h incubation in drug-free medium. Harvested cells were fixed in 75% ethanol at –20°C for at least 18 h. Cellular DNA was stained for 30 min in the dark at room temperature in 0.1% sodium citrate containing 50 µg/ml propidium iodide (PI) (Sigma) and 100 µg/ml RNase A (Boehringer Mannheim). The DNA content in 10^4 cells/sample was measured by flow cytometry on a FACSscan apparatus (Becton-Dickinson, Franklin Lakes, NJ), and analyzed with WinList 5.0 program (Verity Software House, Topsham, ME).

RESULTS

Conjugate-induced effects on SV40 DNA in cell-free systems

Induction of DNA interstrand crosslinks. The Chl moiety attached to a polyamide in 1-Chl should cause DNA alkylation and crosslinking (Fig. 1). Alkylation of complementary DNA strands by one Chl molecule could result in interstrand crosslinking that prevents the separation of DNA strands. The formation of crosslinks in DNA can therefore be measured by denaturation of conjugate-treated linear DNA, followed by separation on neutral agarose gels (11). However, methods often employed in the detection of DNA interstrand crosslinks involve the use of harsh procedures (high temperature or pH) to denature the DNA duplex. Since the stability of DNA alkylation/crosslinks could be compromised under such conditions, we employed a mild method that involved heating the linear DNA in 65% formamide at 55°C for 10 min. Under these conditions, control DNA can be fully denatured (Fig. 2A and B, lanes 2) while at the same time alkali- or heat-labile crosslinks are preserved.

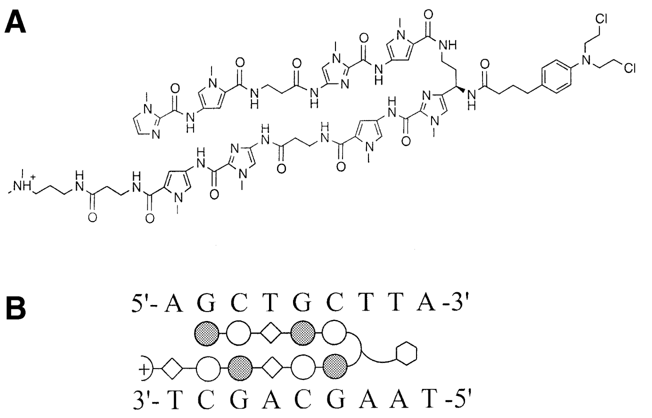

Figure 1. Structure of 1-Chl and model of its binding site. (**A**) Chemical structure of polyamide–Chl conjugate 1-Chl. (**B**) Model of polyamide conjugate bound to the match site 5′-AGCTGCT-3′. Shaded and non-shaded circles represent imidazole and pyrrole rings, respectively, diamonds represent β-alanine and hexagon represents Chl.

Treatment with increasing concentration of 1-Chl increased the fraction of double-stranded DNA that was resistant to denaturation (Fig. 2A, lanes 5–7). Crosslinks could be detected at agent concentrations as low as 0.1 µM, and at 1 µM 1-Chl ~50% of the plasmid was crosslinked and migrated as double-stranded DNA (Fig. 2A, chart). Under similar conditions, none of the parent agents (Fig. 2A, Chl, lane 3 or polyamide 1, lane 4) induced DNA interstrand crosslinks. The time dependence of 1-Chl-induced DNA crosslinking was investigated by incubating SV40 DNA with 10 µM conjugate for up to 24 h at 37°C. As shown in Figure 2B, the level of DNA crosslinks increased rapidly and reached the value of 75% of total DNA after 60 min treatment (Fig. 2B, chart). Longer exposures to 1-Chl resulted in DNA degradation, which manifested as a low molecular 'smear' on the gels (data not shown).

Sequence selectivity of DNA damage in cell-free systems. 1-Chl and its parent polyamide 1 were designed to target the specific DNA sequences, 5′-(A/T)GC(A/T)GC(A/T)-3′. Previous study confirmed the sequence preference of 1-Chl using a small DNA fragment. In this work, we further tested the agent's sequence selectivity on a relatively large (5243 bp) circular SV40 DNA using the RPE assay (also known as the 'polymerase stop assay') (14). Here, the conjugate-induced lesions (covalent adducts, crosslinks, strand breaks, etc.) on template DNA prematurely stop *Taq* polymerase-catalyzed primer extension, resulting in partially extended products whose length indicate the positions of the DNA damage with a 1–2 bp resolution.

First, the heat-induced conversion of agent–DNA adducts to DNA strand breaks was used to establish the proper conditions for 1-Chl–DNA interactions. In this assay, a single strand break results in the conversion of DNA form I (supercoiled) to form II (open circular), while the formation of DNA form III (linear) requires a frank double strand break or two closely spaced single strand breaks on opposite strands (12). To enhance the sequence selectivity of DNA alkylation by 1-Chl, relatively low agent concentrations and long incubation times (up to 24 h) were tested (10).

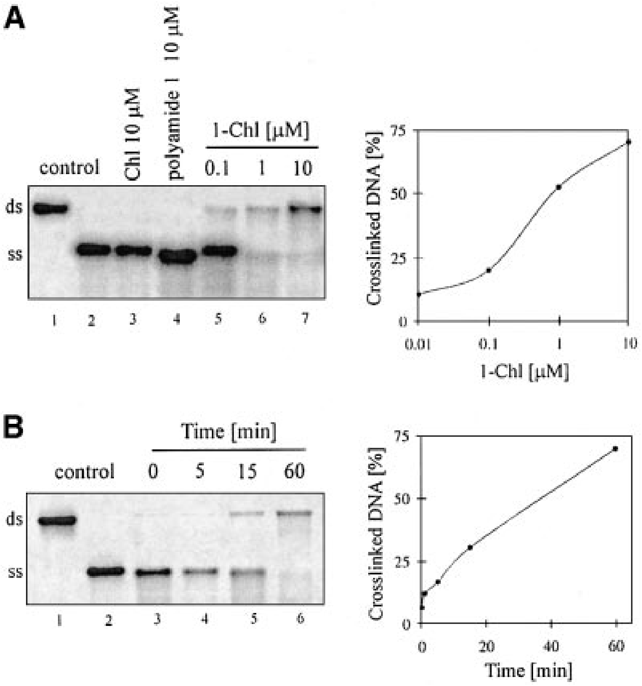

Figure 2. Formation of DNA interstrand cross-links on SV40 DNA by 1-Chl, polyamide 1 and Chl. (**A**) Dose dependence. SV40 DNA was incubated at 37°C for 1 h with 10 µM of Chl (lane 3) polyamide 1 (lane 4) or 0.1–10 µM 1-Chl (lanes 5–7). (**B**) Time dependence. SV40 DNA was incubated at 37°C with 10 µM 1-Chl for 0, 5, 15 and 60 min (lanes 3–6). Lanes 1 and 2 represent native, double-stranded (ds) and denatured, single-stranded (ss) control SV40 DNA, respectively. Linearized and [^{32}P] end-labeled SV40 DNA was treated with agents, purified, denatured and resolved on neutral agarose gels as described in Materials and Methods. The percentage of double-stranded and resistant to denaturation cross-linked DNA was determined by phosphoimaging analysis of dried gels. The data points in the charts represent the average of at least two independent experiments.

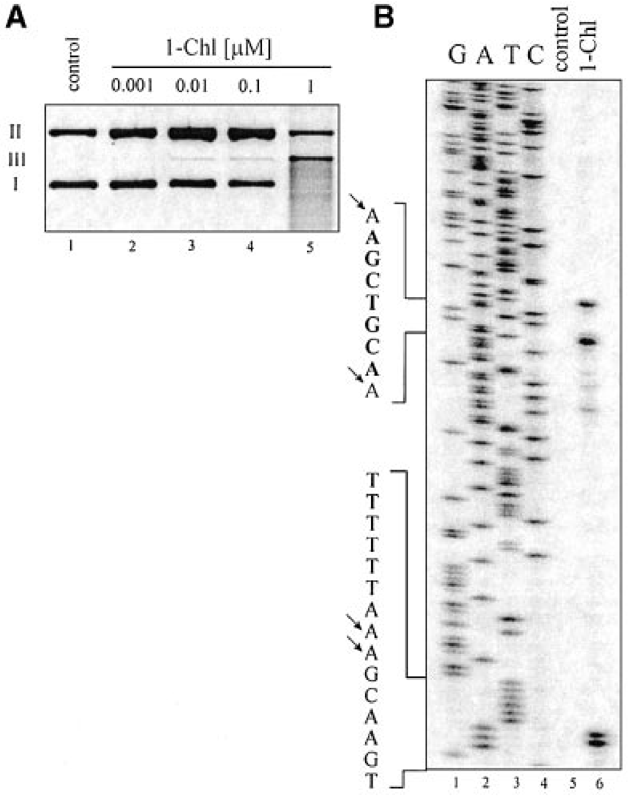

Figure 3. Sequence selectivity of 1-Chl-induced DNA alkylation. (**A**) SV40 DNA alkylation induced by 1-Chl (0.001–1 µM) following prolonged incubation (18 h at 37°C). DNA alkylation lesions were converted to strand breaks as described in Materials and Methods. (**B**) Sequence selectivity of 1-Chl-induced SV40 DNA alkylation. Template DNA (0.3 µg) was either reacted with 1 µM 1-Chl for 18 h at 37°C (lane 6) or untreated (control, lane 5). Following extraction and ethanol precipitation, DNA samples (0.1 µg) were subjected to the RPE reaction using ^{32}P-end-labeled primers (starting from position 2818/reverse on the SV40 genome) and resolved on sequencing polyacrylamide gels as described in Materials and Methods. DNA fragments were visualized by phosphoimaging analysis. Arrows indicate 1-Chl binding sites, and GATC (lanes 1–4) represent results of sequencing reactions using the same DNA primers.

Forms conversion assay was carried out using naked SV40 DNA incubated with 1-Chl (0.001–1 µM) for 18 h at 37°C (Fig. 3A). After heating at 70°C, increase in linear (form III) and nicked-circular (form II) DNA could be detected at 0.01 µM 1-Chl (Fig. 3A, lane 3), and a significantly higher level of DNA damage was observed at 1 µM conjugate (Fig. 3A, lane 5). In contrast, the parent compounds, polyamide 1 and Chl, did not induce measurable DNA damage at concentrations up to 10 µM (data not shown). DNA template prepared by reaction of 1 µM 1-Chl with SV40 DNA for 18 h was used in subsequent RPE experiments.

Amplification of non-treated SV40 DNA (lane 5) in RPE experiment resulted in long extension products that migrated near the top of the gel (beyond the region shown in Fig. 3B). The parent compounds including Chl and polyamide 1 that were inactive in previous assays showed virtually no inhibition of primer extension at concentrations as high as 10 µM (data not shown). In contrast, SV40 DNA treated with 1 µM 1-Chl for 18 h at 37°C and subjected to the RPE reaction produced several distinct bands corresponding to shorter DNA fragments (Fig. 3B, lane 6). The intense bands at A$^{\leftarrow}$AGCTGCAA$^{\leftarrow}$ sequence (the arrows indicate presumed alkylated bases) demonstrate that under these conditions, 1-Chl targets its designed sequence. However, besides the polyamide match site, 1-Chl also damaged one of the downstream mismatch sites (Fig. 3B, lane 6, lower part, A$^{\leftarrow}$A$^{\leftarrow}$GCA$\underline{\text{AG}}$T; the arrows indicate presumed alkylated bases and the mismatched bases are underlined).

Inhibition of cell-free SV40 DNA replication. A cell-free SV40 DNA replication assay was employed to examine the functional effects of 1-Chl-induced DNA damage. In this assay, the template SV40 DNA was combined with replication factors isolated from exponentially growing 293 cells and supplemented by SV40 large T antigen. Under these conditions, it is possible to fully replicate the SV40 genome in a cell-free environment (16).

1-Chl-treated SV40 DNA (0.3 and 3 µM 1-Chl for 1 h at 37°C) was used as the template in the replication reaction carried out as described in Materials and Methods. As shown in Figure 4, treatment of SV40 DNA with as low as 0.3 µM 1-Chl decreased the template competency for replication reaction by ~25% (lane 2), whereas DNA treated with 3 µM 1-Chl was virtually inactive in the replication reaction (lane 3).

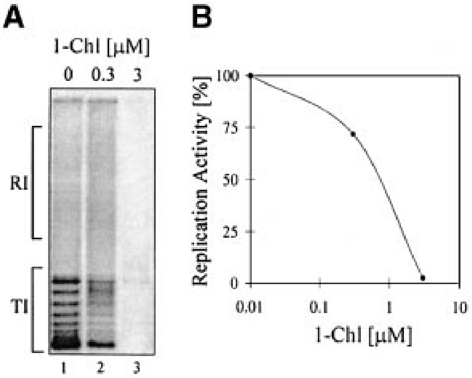

Figure 4. 1-Chl-treated DNA is a poor template for replication in cell-free system. Naked SV40 DNA was treated with 1-Chl at the indicated concentrations for 1 h at 37°C. Replication reactions were assembled using 30 μg of 293 cells extract, 50 ng of drug-treated SV40 DNA and 0.6 μg of SV40 T-antigen, and incubated at 37°C for 1 h. Replication products were purified, resolved on 0.8% neutral agarose gels (**A**), and quantitated (**B**) as described in Materials and Methods. Positions of plasmid topoisomers (TI) and replication intermediates (RI) are indicated. The data points in the chart represent the average of two independent experiments.

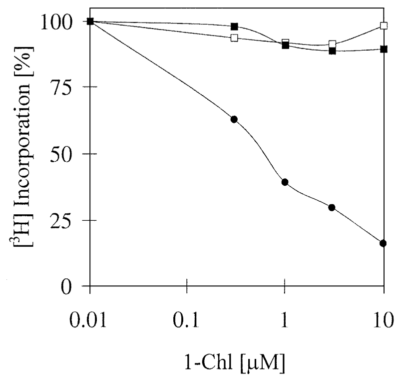

Figure 5. Inhibition of DNA, RNA and protein synthesis in 293 cells. Exponentially growing 293 cells were treated for 18 h with 1-Chl (0.01–10 μM), followed by 1 h incubation with [^{3}H]thymidine (DNA, shaded circles), [^{3}H]uridine (RNA, shaded squares) and [^{3}H]leucine (proteins, open squares). The percentages of inhibition of the precursors incorporation into DNA, RNA and proteins were determined as described in Materials and Methods. The data points represent the average of two independent experiments.

The IC$_{50}$ (50% inhibitory concentration) calculated from the graph was 0.9 μM. Since the free agent was presumably completely removed from DNA by extraction and precipitation, the loss of replication activity can be attributed to 1-Chl-induced irreversible damage to DNA template.

Cellular effects

DNA, RNA and protein synthesis inhibition. The results presented so far demonstrated that 1-Chl alkylates and crosslinks DNA with the desired sequence selectivity, and inhibits DNA replication in cell-free systems *in vitro*. However, the question remains how this *in vitro* activity, detected on naked SV40 DNA, translates into cellular effects. Since 1-Chl inhibited DNA replication in cell-free system, we have started evaluation of the agent cellular activities by testing the macromolecules synthesis in human 293 cells after 18 h treatment with 1-Chl.

The conjugate-treated cells were examined for bulk DNA, RNA and protein synthesis by measurement of [^{3}H]-thymidine, [^{3}H]uridine and [^{3}H]leucine incorporation, respectively. As shown in Figure 5, agent dose-dependent decrease in thymidine incorporation into genomic DNA was observed after 18 h incubation with 0–10 μM of 1-Chl. The highest studied concentration (10 μM) was sufficient to inhibit DNA synthesis by ~85% (shaded circles), however, there was virtually no inhibition of RNA (shaded squares) or protein synthesis (open squares). Under similar conditions, neither polyamide 1 nor Chl was able to inhibit DNA, RNA or protein synthesis (data not shown).

Cell growth inhibition. Exponentially growing 293 cells were treated for 4 days at 37°C with 1-Chl concentrations ranging from 0.001 to 10 μM. The IC$_{50}$ was observed at ~0.013 μM 1-Chl (Fig. 6A, shaded circles). No significant growth inhibition was detected in 293 cells treated with similar concentrations of Chl (open squares) or polyamide 1 (shaded squares). This observation corroborates with and extends our previous results showing the highly enhanced activity of 1-Chl compared with its parent compounds.

Additional studies conducted with several cell lines produced interesting observations that 1-Chl growth inhibition activities are cell line-dependent (Table 1). The concentration which inhibited cell growth by 50% varied from 2 nM (Jurkat cell line) to >2 μM (BSC-1 and HCT-116 cell lines). In addition to evaluating growth inhibition of 1-Chl in tumor lines, normal human diploid fibroblast cells (IMR 90) were also found to be insensitive up to concentrations as high as 2 μM (personal communication from Athena Lin and Tonya Jackson). In addition, IMR 90 cells neoplastically transformed with ras, which greatly enhances their susceptibility to DNA-damaging drugs, were found to be only slightly sensitive to 1-Chl (~50% growth inhibition required 2 μM treatment). Unlike 1-Chl, Chl showed similarly low activity against 1-Chl sensitive (293) or insensitive (BSC-1) cells. The extraordinary differences in sensitivity of cells to 1-Chl cannot be explained by differences in media composition or culture conditions (data not shown).

Cell cycle perturbations. DNA interacting/damaging agents are known to induce cell cycle perturbations and arrest the cell cycle progression predominantly at the G$_2$/M boundary (17). The monoparametric flow cytometry method was used to evaluate 1-Chl effects on cell cycle progression. Exponentially and asynchronously growing 293 cells were treated with 0.01–1 μM 1-Chl or Chl for 18 h, followed by 24 h incubation in agent-free medium. After the cellular DNA was stained with PI, DNA content was analyzed by flow cytometry as described in Materials and Methods.

The histograms of the DNA content distribution in cells treated with 1-Chl or Chl are shown in Figure 6B. In control, non-treated samples, the majority of cells are in the G$_1$ phase. However, treatment with increased doses of 1-Chl resulted in a gradual increase in the proportion of the G$_2$/M peak (up to 53% at 1 μM 1-Chl), thus indicating that 1-Chl is able to arrest cell cycle progression in G$_2$/M phase. Under similar

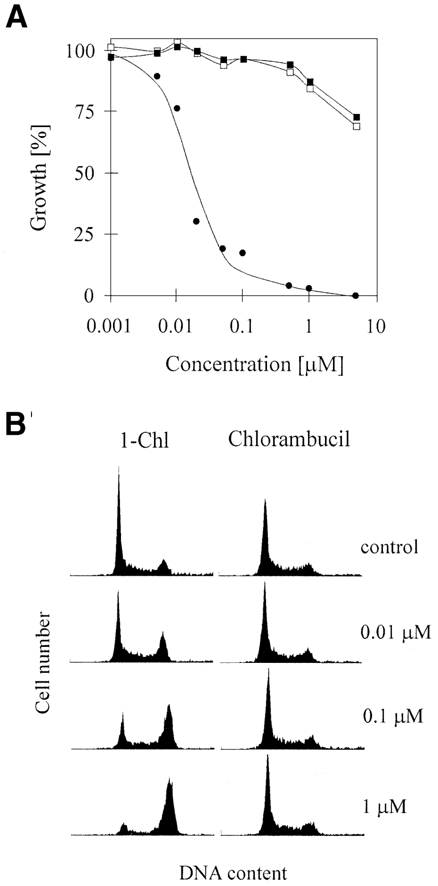

Figure 6. Inhibition of cell growth and cell cycle progression induced by 1-Chl and its parent compounds in 293 cells. (**A**) Growth inhibition of 293 cells treated for 96 h with 0.001–5 µM of 1-Chl (shaded circles), Chl (open squares) or polyamide 1 (shaded squares). The percentage inhibition of cell growth was determined using standard sulphorhodamine method as described in Materials and Methods. The data points represent the average of at least two independent experiments done in triplicate. (**B**) Drug-induced cell cycle perturbation in 293 cells. Cellular DNA content in cells treated for 18 h with 0.01, 0.1 and 1 µM of 1-Chl (left panel) or Chl (right panel), and then post-incubated in fresh medium for 24 h was evaluated by flow cytometry as described in Materials and Methods.

Table 1. 1-Chl-induced cell growth inhibition

Cell line	Cell line description	IC_{50} [nM][a]
Jurkat	Human T cell leukemia	2.2
293	Human transformed embryonic kidney	13
HeLa S3	Human epithelial carcinoma	200
BSC-1	African green monkey kidney	>2000
HCT-116	Human colon carcinoma	>2000

[a]Cells were drug treated for 4 days, then the growth was quantitated based upon sulphorhodamine B staining of cellular proteins and absorbance measurements at 570 nm as described in Materials and Methods.

conditions, Chl did not alter cell cycle distribution (Fig. 6B, right panel). In a similar study using BSC-1 cells, 1-Chl concentrations as high as 20 µM and incubation times up to 48 h, resulted in no effect on cell cycle progression essentially mirroring the resistance seen for growth inhibition (data not shown).

DISCUSSION

It was shown previously, that 1-Chl alkylates N3 adenines in a polyamide-targeted, sequence-selective manner (10). However, it was not known if the drug-induced DNA alkylation/damage lesions were single-stranded (DNA monoadducts) or double-stranded (DNA interstrand crosslinks). Therefore, we tested whether the ability of Chl to crosslink DNA was altered because of coupling with a polyamide. The lack of strand separation of full length linear SV40 treated with 1-Chl under denaturing conditions proved the formation and covalent nature of 1-Chl-induced crosslinks. The parent polyamide 1, which lacks Chl moiety, was unable to crosslink double-stranded DNA under these conditions, thus excluding the possibility that the strong, but non-covalent binding of polyamide to DNA contributes to crosslinking appearance. Furthermore, 1-Chl was a much more efficient DNA crosslinking agent than its parent nitrogen mustard Chl, which under the same conditions did not induce crosslinks at concentrations up to 10 µM, although some crosslinks were observed at higher concentrations (data not shown). It has been noted previously that Chl–distamycin conjugates crosslink naked and cellular DNA more efficiently than Chl alone (18), probably due to the more efficient and faster delivery of Chl alkylating groups to the DNA reactive sites.

The DNA crosslinking assay demonstrated the improved DNA damaging activity of 1-Chl, compared to Chl. Since this assay is based on ligand interactions with simple linear DNA molecules, 1-Chl alkylation potency was also studied using more complex, supercoiled SV40 DNA. Neither Chl nor polyamide 1 produced DNA damage at concentrations up to 10 µM, while DNA damage (demonstrated as conversion of supercoiled form I DNA to forms II and III) was observed at 1-Chl concentrations ranging from 0.1 to 10 µM (1 h treatment at 37°C). Longer treatments with 1-Chl further increased the level of drug-induced DNA damage, and the first DNA strand breaks could be detected at concentrations as low as 0.01 µM following 18 h exposure (Fig. 3A). However, prolonged incubation and higher 1-Chl concentrations led to extensive DNA damage, resulting in smaller fragments.

The DNA forms conversion assay was also employed to study drug interactions with SV40 minichromosomal DNA, e.g. DNA protected by nucleosomal histones and other chromatin proteins. Studies using SV40 minichromosomal DNA demonstrated that the presence of chromatin structure has no effect on the amount of DNA damage produced by 1-Chl (data not shown). Overall, 1-Chl DNA damaging activity as tested in crosslinking and forms conversion assays were similarly independent of DNA structure (linear, circular or minichromosomal) and occurred at concentrations significantly lower than concentrations needed for Chl to damage DNA.

As shown with RPE experiments, 1-Chl appears to target Chl to its polyamide DNA binding sequence. *Taq* polymerase was arrested at the sequences matching the polyamide 1 DNA-binding sequence 5′-(A/T)GC(A/T)GC(A/T)-3′ (Fig. 3B, upper part, AGCTGCA), as well as double-base pair mismatch site (Fig. 3B, lower part, AGCA$\underline{\text{AG}}$T). It has been demonstrated previously that the specificity for the alkylation at match sites over mismatch sites is ~20-fold (10). However, on the particular SV40 DNA fragment analyzed in this study both match and double-base pair mismatch sites appear to be alkylated. One could imagine lower occupancy at a mismatch site, but faster rate of alkylation affording similar yield of cleavage at two different sites.

Besides demonstrating the sequence selectivity of 1-Chl-induced DNA damage, the RPE experiments also revealed that the conjugate-induced lesions stop DNA polymerase in the PCR reaction, and could potentially inhibit DNA synthesis. Therefore, the cell-free *in vitro* SV40 DNA replication assay was used to study the effects of 1-Chl-induced DNA alkylation on DNA synthesis. The 1-Chl-treated DNA was unable to serve as a DNA synthesis template, with nearly complete replication inhibition observed at 3 µM 1-Chl. This inhibitory activity could be attributed to formation of DNA crosslinks between the complementary strands, which was observed in the range 1–10 µM (Fig. 2A), and the IC_{50} values calculated from both experiments are very close (0.9 and 1 µM). DNA interstrand crosslinks have been postulated to inhibit or prevent replication, as well as transcription of cellular DNA, because these processes require that the complementary DNA strands be free to separate and bind proteins, a process that is perturbed by the presence of covalent links.

A number of studies have shown a direct correlation between the degree of DNA interstrand crosslinking and biological responses to alkylating agents both *in vitro* and *in vivo* (19–21). Therefore, one can presume that 1-Chl, as an active and efficient DNA alkylating and crosslinking agent, would induce biological effects in mammalian cells. Short-term (4 days continuous incubation) assays showed that 1-Chl inhibits the growth of the 293 human cell line at doses similar to those active in cell-free DNA damaging experiments (IC_{50} = 0.013 µM). It is important to emphasize that Chl induces DNA interstrand crosslinks and inhibits cell growth at concentrations ranging from micromolar to millimolar depending on the cell line, therefore attachment of sequence-selective polyamide moiety can improve Chl activity by several orders of magnitude.

Factors that may enhance 1-Chl cytotoxicity as compared to Chl include the sequence selectivity of crosslinking and the degree of DNA structure distortion by the formation of larger adducts. Both factors may affect the cell's ability to recognize and repair DNA lesions. It has been shown for a series of nitrogen mustards–minor groove binders conjugates that, while the overall crosslinking efficiency did not dramatically change with the increase in the agents' length, the sequence selectivity of alkylation and the cytotoxicity were considerably enhanced (22,23).

Relatively low 1-Chl concentrations (1 µM for 18 h) were able to induce DNA synthesis inhibition (Fig. 5) and a G_2/M block in the cell cycle (Fig. 6B). Under the same conditions, the parent compounds, polyamide 1 and Chl, did not inhibit cell growth or cell cycle progression. DNA crosslinking agents, as well as other types of DNA damaging drugs, are known to inhibit cellular DNA replication and arrest cells in G_2/M (17,24). Thus, the preferential inhibition of DNA replication and G_2/M block suggest that 1-Chl damages intracellular, genomic DNA in a fashion similar to that observed for cell-free SV40 DNA.

This study demonstrates the cellular activity of a polyamide–Chl conjugate. Interestingly, our recent uptake and nuclear localization study of polyamide-dye conjugates using confocal laser scanning microscopy revealed that polyamides localize mainly in the cytoplasm, not the nucleus of most cell types (25). The fact that significant biological activity is seen in selected cell types with the conjugate polyamide, suggests that for 1-Chl, fewer DNA lesions may be needed to impair DNA template function compared to reversible binding polyamides. Similarly, a polyamide conjugated to a DNA alkylating group from the cyclopropylpyrroloindole family also showed significant enhancement of whole cell activity (26). The yet unexplained differences between the growth inhibitory activities of 1-Chl on various cell lines could be caused by the differences in cellular uptake or more likely intracellular transport of this agent as suggested by uptake studies in which polyamide-dye conjugates were restricted to certain T-cell lines (25).

In summary, studies presented here have demonstrated that the Chl-conjugated polyamide 1-Chl binds and alkylates DNA *in vitro*. The compound-induced DNA damage occurs predominantly at the designated polyamide-binding site and hinders DNA template functionality with regard to replication *in vitro*. Compared with its parent compounds, polyamide 1 and nitrogen mustard Chl, the conjugate molecule is far more effective in both *in vitro* cell-free and cellular tests. 1-Chl damages and crosslinks SV40 DNA at concentrations as low as 0.01 and 1 µM, respectively, whereas polyamide 1 and Chl did not demonstrate any activity up to 10 µM. In addition, 1-Chl arrests cells in the G_2/M phase and inhibits cell growth and DNA replication, while neither of the parent compounds showed activity at concentrations up to 10 µM.

ACKNOWLEDGEMENTS

We are very grateful to Loretta Gawron for technical assistance and to Drs Marry M. McHugh and Christine M. White from Roswell Park Cancer Institute for careful reading of this manuscript and insightful comments. This study was supported in part by NIH grants CA80939 and CA16056 to T.A.B., and GM57148 to P.B.D. N.R.W. was supported by predoctoral fellowships from Bristol-Myers Squibb and the Ralph M. Parsons Foundation.

REFERENCES

1. Faguet,G.B. (1994) Chronic lymphocytic leukemia: an updated review. *J. Clin. Oncol.*, **12**, 1974–1990.
2. Mattes,W.B., Hartley,J.A. and Kohn,K.W. (1986) DNA sequence selectivity of guanine-N7 alkylation by nitrogen mustards. *Nucleic Acids Res.*, **14**, 2971–2987.
3. Saffhill,R., Margison,G.P. and O'Connor,P.J. (1985) Mechanisms of carcinogenesis induced by alkylating agents. *Biochim. Biophys. Acta*, **823**, 111–145.
4. Sanderson,B.J. and Shield,A.J. (1996) Mutagenic damage to mammalian cells by therapeutic alkylating agents. *Mutat. Res.*, **355**, 41–57.
5. Pezzoni,G., Grandi,M., Biasoli,G., Capolongo,L., Ballinari,D., Giuliani,F.C., Barbieri,B., Pastori,A., Pesenti,E. and Mongelli,N. (1991) Biological profile of FCE 24517, a novel benzoyl mustard analogue of distamycin A. *Br. J. Cancer*, **64**, 1047–1050.
6. Dervan,P.B. (2001) Molecular recognition of DNA by small molecules. *Bioorg. Med. Chem.*, **9**, 2215–2235.
7. Dervan,P.B. and Burli,R.W. (1999) Sequence-specific DNA recognition by polyamides. *Curr. Opin. Chem. Biol.*, **3**, 688–693.

8. Trauger,J.W., Baird,E.E. and Dervan,P.B. (1996) Recognition of DNA by designed ligands at subnanomolar concentrations. *Nature*, **382**, 559–561.
9. White,S., Szewczyk,J.W., Turner,J.M., Baird,E.E. and Dervan,P.B. (1998) Recognition of the four Watson–Crick base pairs in the DNA minor groove by synthetic ligands. *Nature*, **391**, 468–471.
10. Wurtz,N.R. and Dervan,P.B. (2000) Sequence specific alkylation of DNA by hairpin pyrrole-imidazole polyamide conjugates. *Chem. Biol.*, **7**, 153–161.
11. Hartley,J.A., Berardini,M.D. and Souhami,R.L. (1991) An agarose gel method for the determination of DNA interstrand crosslinking applicable to the measurement of the rate of total and ‘second-arm’ crosslink reactions. *Anal. Biochem.*, **193**, 131–134.
12. McHugh,M.M., Woynarowski,J.M., Mitchell,M.A., Gawron,L.S., Weiland,K.L. and Beerman,T.A. (1994) CC-1065 bonding to intracellular and purified SV40 DNA: site specificity and functional effects. *Biochemistry*, **33**, 9158–9168.
13. Grimwade,J.E. and Beerman,T.A. (1986) Measurement of bleomycin, neocarzinostatin, and auromomycin cleavage of cell-free and intracellular simian virus 40 DNA and chromatin. *Mol. Pharmacol.*, **30**, 358–363.
14. Woynarowski,J.M., Chapman,W.G., Napier,C. and Herzig,M.C. (1999) Induction of AT-specific DNA-interstrand crosslinks by bizelesin in genomic and simian virus 40 DNA. *Biochim. Biophys. Acta*, **1444**, 201–217.
15. Dziegielewski,J., Melendy,T. and Beerman,T.A. (2001) Bleomycin-induced alterations in DNA replication: relationship to DNA damage. *Biochemistry*, **40**, 704–711.
16. Stillman,B. (1986) Chromatin assembly during SV40 DNA replication *in vitro*. *Cell*, **45**, 555–565.
17. O’Connell,M.J., Walworth,N.C. and Carr,A.M. (2000) The G2-phase DNA-damage checkpoint. *Trends Cell Biol.*, **10**, 296–303.
18. Wyatt,M.D., Garbiras,B.J., Haskell,M.K., Lee,M., Souhami,R.L. and Hartley,J.A. (1994) Structure-activity relationship of a series of nitrogen mustard- and pyrrole-containing minor groove-binding agents related to distamycin. *Anticancer Drug Des.*, **9**, 511–525.
19. Thomas,C.B., Osieka,R. and Kohn,K.W. (1978) DNA cross-linking by *in vivo* treatment with 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea of sensitive and resistant human colon carcinoma xenograms in nude mice. *Cancer Res.*, **38**, 2448–2454.
20. Pawlak,K., Pawlak,J.W. and Konopa,J. (1984) Cytotoxic and antitumor activity of 1-nitroacridines as an aftereffect of their interstrand DNA cross-linking. *Cancer Res.*, **44**, 4289–4296.
21. Sunters,A., Springer,C.J., Bagshawe,K.D., Souhami,R.L. and Hartley,J.A. (1992) The cytotoxicity, DNA crosslinking ability and DNA sequence selectivity of the aniline mustards melphalan, chlorambucil and 4-[bis(2-chloroethyl)amino] benzoic acid. *Biochem. Pharmacol.*, **44**, 59–64.
22. Wyatt,M.D., Lee,M., Garbiras,B.J., Souhami,R.L. and Hartley,J.A. (1995) Sequence specificity of alkylation for a series of nitrogen mustard-containing analogues of distamycin of increasing binding site size: evidence for increased cytotoxicity with enhanced sequence specificity. *Biochemistry*, **34**, 13034–13041.
23. Brooks,N., McHugh,P.J., Lee,M. and Hartley,J.A. (1999) The role of base excision repair in the repair of DNA adducts formed by a series of nitrogen mustard-containing analogues of distamycin of increasing binding site size. *Anticancer Drug Des.*, **14**, 11–18.
24. Konopa,J. (1988) G2 block induced by DNA crosslinking agents and its possible consequences. *Biochem. Pharmacol.*, **37**, 2303–2309.
25. Belitsky,J.M., Leslie,S.J., Arora,P.S., Beerman,T.A. and Dervan,P.B. (2002) Cellular uptake of N-methylpyrrole/N-methylimidazole polyamide-dye conjugates. *Bioorg. Med. Chem.*, **10**, 3313–3318.
26. Wang,Y.D., Dziegielewski,J., Chang,A.Y., Dervan,P.B. and Beerman,T.A. (2002) Cell-free and cellular activities of a DNA sequence selective hairpin polyamide-CBI conjugate. *J. Biol. Chem.*, **277**, 42431–42437.