Induction of Heat-Labile Sites in DNA of Mammalian Cells by the Antitumor Alkylating Drug CC-1065†

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ABSTRACT: CC-1065 is a very potent antitumor antibiotic capable of covalent and noncovalent binding to the minor groove of naked DNA. Upon thermal treatment, covalent adducts formed between CC-1065 and DNA generate strand breaks [Reynolds, R. L., Molineux, I. J., Kaplan, D. J., Swenson, D. H., & Hurley, L. H. (1985) *Biochemistry 24*, 6228–6237]. We have shown that this molecular damage can be detected following CC-1065 treatment of mammalian whole cells. Using alkaline sucrose gradient analysis, we observe thermally induced breakage of [^{14}C]thymidine-prelabeled DNA from drug-treated African green monkey kidney BSC-1 cells. Very little damage to cellular DNA by CC-1065 can be detected without first heating the drug-treated samples. CC-1065 can also generate heat-labile sites within DNA during cell lysis and heating, subsequent to the exposure of cells to drug, suggesting that a pool of free and noncovalently bound drug is available for posttreatment adduct formation. This effect was controlled for by mixing [^{3}H]thymidine-labeled untreated cells with the [^{14}C]thymidine-labeled drug-treated samples. The lowest drug dose at which heat-labile sites were detected was 3 nM CC-1065 (3 single-stranded breaks/10^6 base pairs). This concentration reduced survival of BSC-1 cells to 0.1% in cytotoxicity assays. The generation of CC-1065-induced lesions in cellular DNA is time dependent (the frequency of lesions caused by a 60 nM treatment reaching a plateau at 2 h) and is not readily reversible. The induction of heat-labile sites in cellular DNA was confirmed by gel electrophoretic analyses of the damage to intracellular simian virus 40 (SV40) DNA in SV40-infected BSC-1 cells. Approximately 10-fold higher drug concentrations were needed to induce lesions in SV40 DNA compared to that in genomic DNA. The results of this study demonstrate that CC-1065 does generate heat-labile sites with the cellular DNA of intact cells and suggest that a mechanism of cytotoxic action of CC-1065 involves formation of covalent adducts to DNA.

The drug CC-1065, a fermentation product of *Streptomyces zelensis* that was isolated by the Upjohn Co. (Hanka et al., 1978; Martin et al., 1981), is one of the most cytotoxic antitumor antibiotics known. For example, CC-1065 was found to be 400 times more potent than adriamycin in inhibiting the growth of L1210 cells (Li et al., 1982) and was up to 100 times more potent in tumor cell cloning assays against a broad panel of human tumor lines (Bhuyan et al., 1982). It has also been found to be very active in vivo against a panel of experimental murine tumors (Martin et al., 1978; Neil et al., 1981). CC-1065 was withheld from clinical trials, however, when it was found to cause delayed death at subtherapeutic doses (McGovren et al., 1984). Recently, analogues of this antibiotic, possessing its extreme potency yet lacking delayed toxicities, have been synthesized (Warpehoski et al., 1985; Petzold et al., 1985; Warpehoski, 1986; Wierenga et al., 1986). Some of these analogues are entering phase I clinical trials (J. P. McGovren, personal communication). Since most of the information known regarding the mode of action of these agents is based on studies with the parent drug, CC-1065 remains a useful model drug for studying the mechanisms of this growing class of compounds.

CC-1065 (Figure 1) is comprised of three benzodipyrrole subunits (one subunit possessing a reactive cyclopropane ring) linked by amide bonds (Chidester et al., 1981). The out-of-plane projection of the amide bonds subscribes a right-handed twist along the length of the CC-1065 molecule that facilitates a nonintercalative interaction with DNA in which the drug fits snugly within the minor groove (Swenson et al., 1982; Li et al., 1982). This interaction consists of both hydrophobic interactions and Van der Waal's forces (Krueger et al., 1985). Unlike most other minor groove binders, CC-1065 does not form hydrogen bonds with the bases or sugars within the DNA groove (Zakrzewska et al., 1987). Upon association, the reactive cyclopropane group may bind covalently with DNA components. The alkylation is very specific for certain adenine- and thymine-rich sequences (Chidester et al., 1981; Swenson et al., 1982; Reynolds et al., 1985) in which adduct formation always occurs through N-3 of adenine (Hurley et al., 1984). These covalent adducts give rise to heat-labile sites that, when subjected to thermal treatment, lead to the release of a CC-1065-modified adenine and cleavage of the DNA backbone at the resulting apurinic site (Hurley et al., 1984; Reynolds et al., 1985). Thus, generation of heat-labile sites can be used as a measure of covalent modification of DNA by CC-1065.

Studies of CC-1065 adduct formation have been restricted primarily to naked DNA and synthetic oligonucleotides. Recently, analysis of circular dichroism spectra has implied that CC-1065 forms fewer covalent adducts with mouse chromatin than with isolated DNA (Moy et al., 1989). Thus, nucleosomal structure appears to influence formation of CC-1065–DNA adducts. To date, no investigation has addressed the question of whether covalent adducts are formed in drug-treated intact cells. We have developed systems to detect and characterize CC-1065-induced lesions in cellular DNA and report here the results of such studies.

Materials and Methods

Chemicals. CC-1065, generously supplied by the Upjohn Co. (Kalamazoo, MI), was dissolved in dimethylacetamide

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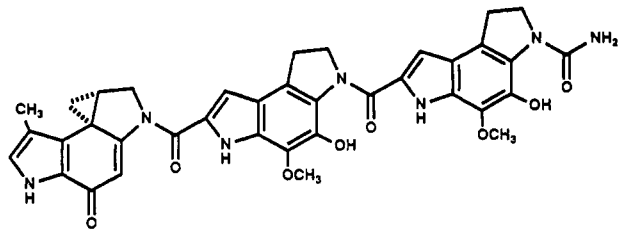

CC-1065

FIGURE 1: Structure of CC-1065.

(Aldrich Chemical, Milwaukee, WI) and stored at −20 °C. [*methyl*-^{3}H]Thymidine (48 Ci/mmol) was from CEA (France). [2-^{14}C]Thymidine (56 mCi/mmol) was purchased from Moravek Biochemicals, Inc. (Brea, CA).

Cell Culture. BSC-1 African green monkey kidney cells were maintained in Minimal Essential Medium supplemented with 8% calf serum, 2% fetal bovine serum, 0.2% sodium bicarbonate, and 2 mM glutamine (all from GIBCO, Grand Island, NY). For measurement of damage to intracellular SV40,[1] BSC-1 cells were infected with 5–15 pfu of SV40 virus in MEM containing 2% fetal bovine serum as described by Grimwade and Beerman (1986). The SV40-infected cells were used for drug treatment 40 h after infection.

CC-1065 Treatment of Whole Cells. BSC-1 cultures were initiated at 2×10^5 cells/25-cm^2 flask in 5 mL of media and labeled with [^{14}C]thymidine (dT) at a final concentration of 0.1 μCi/mL or with [^{3}H]dT at 0.4 μCi/mL. After 48 h, the labeled medium was replaced by fresh medium and the monolayers were incubated for 1 h and then exposed to CC-1065 for 2 h. The drug treatment was followed by two washes with PBS and trypsinization. Unless otherwise indicated, the suspensions were diluted 1:10 in ethanol (to extract both noncovalently bound and free CC-1065) and held on ice for 15 min. All following steps, prior to lysis, were carried out at 4 °C. The samples were centrifuged at 1000 rpm, and the pellets were resuspended in PBS. These were centrifuged again, and pellets were resuspended in PBS at approximately 5×10^5 cells/mL and mixed with an equal number of the nontreated [^{3}H]dT-labeled cells to detect the contribution of posttreatment damage. The mixture was then lysed with 1% sarkosyl and 2.5% sucrose in gradient buffer containing 0.7 M NaCl, 0.3 M NaOH, and 0.01 M EDTA, pH 8.6. The lysates were heated at 90 °C for 15 min. (It was determined that a 15-min heating period was sufficient to generate maximal damage.) Aliquots were loaded onto alkaline sucrose gradients as described below. Other aliquots were used to determine total radioactivity for each sample.

Sucrose Gradient Centrifugation. Each alkaline gradient consisted of 10 mL of 5–30% sucrose in gradient buffer (0.7 M NaCl, 0.3 M NaOH, 0.01 M EDTA, pH 13) underlaid with 0.5 mL of 60% sucrose in gradient buffer. An alkaline lysis layer (0.3 mL) composed of 1% sarkosyl and 2.5% sucrose in the gradient buffer overlaid the top of each gradient. Aliquots of drug-treated cell lysates were applied to the tops of these gradients. Samples were centrifuged at 20 °C in an SW41 Ti rotor for 18 h at 16000 rpm and then were fractionated at 10-s intervals from the top. The fractions were neutralized with 1 mL of 0.15 N HCl, and then their radioactivity was determined. The counts were converted to disintegrations per minute (dpm) and sedimentation profiles calculated as percent recovered radioactivity.

Sedimentation constants, $S_{20,w}$, for each fraction were determined according to Young and Krumlauf (1981). DNA molecular weights corresponding to these $S_{20,w}$ values were determined by Studier's equations (Studier, 1965). Frequencies of single-stranded breaks were computed by the equation

$$f = \frac{(MW_n)_A/(MW_n)_B - 1}{(MW_n)_A/640} \quad (1)$$

where f is the number of breaks per 10^6 base pairs and $(MW_n)_A$ and $(MW_n)_B$ are the number average molecular weights of the internal control (^{3}H-labeled) and drug-treated (^{14}C-labeled) DNA peaks, respectively. The value 640 represents the average molecular weight of one DNA base pair.

Number average molecular weight was determined by the equation

$$(MW_n) = \frac{\sum \% \text{ Rec}_i}{\sum \% \text{ Rec}_i/MW_i} \quad (2)$$

where % Rec$_i$ is the percent of recovered radioactivity for each fraction of a given peak and MW$_i$ is the molecular weight of the corresponding fraction as similarly described by Ehman and Lett (1973). Peak limits were set at the fractions that contained typically greater than 1% recovered radioactivity. Figures shown are representative profiles of at least two separate experiments.

Appropriate controls showed that the highest level of dimethylacetamide used (0.01%) had no effect on adduct formation.

CC-1065-Induced Lesions in Intracellular SV40 DNA. Monolayers of the SV40-infected BSC-1 cells were treated with CC-1065 for a period of 2 h at 37 °C. The cells were then washed twice with cold PBS and detached with a rubber policeman, and suspensions were alcohol extracted as described for the drug treatment of uninfected cells. Pellets were resuspended in 0.25 mL of a buffer of 10 mM Tris and 10 mM EDTA (pH 7.6) and mixed 1:1 with 2% SDS in the same buffer. Samples were heated at 90 °C for 15 min. To assess drug-induced heat-labile sites, the samples were electrophoresed through 1% agarose gel at 30 V for 20 h in a running buffer of 50 mM Tris-HCl, (pH 8.3), 20 mM sodium acetate, and 2 mM EDTA. The gels were stained in 0.5 μg/mL ethidium bromide, and relative amounts of supercoiled (form I), nicked (form II), and linear (form III) SV40 DNA were quantitated.

Cell Survival Determination. Cell survival was determined by the relative plating efficiency of BSC-1 cells after treatment with CC-1065. Cultures were seeded with 4×10^5 cells/100-mm dish and incubated for 48 h, yielding a cell population density similar to that for the whole cell DNA damage assay. Each dish was then refed with fresh medium and treated with CC-1065 for 2 h. Following drug treatment, monolayers were washed twice with PBS and trypsinized. Each sample was plated in triplicate at 2×10^2, 10^3, and 10^4 cells/60-mm dish and 10^5 cells/100-mm dish. Following incubation of the dishes at 37 °C and 5% CO_2 for 10 days, the colonies were stained with methylene blue and counted and relative plating efficiencies were calculated.

Appropriate controls indicated that the highest level (<0.001%) of dimethylacetamide used had no effect on plating efficiencies.

RESULTS

Cytotoxicity of CC-1065 to BSC-1 Cells. The ability of BSC-1 cells to form colonies after treatment with CC-1065

[1] Abbreviations: D_{10}, drug concentration that gives 10% cell survival in clonogenic assays; SV40, simian virus 40; PBS, phosphate-buffered saline (0.14 M NaCl, 2 mM KCl, 6 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 7.2); dT, thymidine; pfu, plaque-forming units.

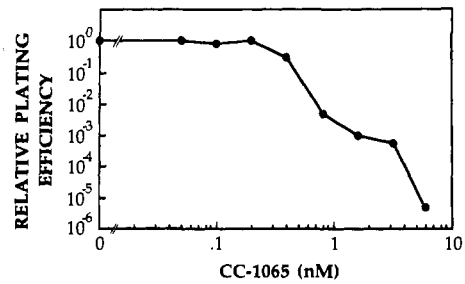

FIGURE 2: Survival of BSC-1 cells exposed to CC-1065 for 2 h. Relative plating efficiency was measured by colony formation 8 days after drug treatment.

was examined. In this assay, cells were treated with the drug for 2 h, followed by PBS washes, trypsinization, and replating for colony formation. Under these conditions, CC-1065 treatment of BSC-1 cells yielded a steep dose–response profile. Relative plating efficiency was decreased by 3 orders of magnitude within the range of 0.2–3.0 nM CC-1065. The concentration at which 10% of the cells survived drug treatment (D_{10}) was 0.5 nM CC-1065 (Figure 2). CC-1065 at 3 nM reduced cell survival to 0.1%.

Detection of CC-1065-Induced DNA Damage in Whole Cells by Sedimentation in Alkaline Sucrose Gradients. The ability of CC-1065 to induce DNA damage in whole cells was determined with the use of alkaline sucrose gradient analysis of [^{14}C]dT-labeled BSC-1 cells. The assays for DNA damage employed essentially the same drug treatment conditions as used to determine plating efficiency. Since CC-1065 covalent adducts create heat-labile sites in isolated DNA, we analyzed DNA from drug-treated cells with and without heating of the samples.

In the absence of heating, even at doses as high as 900 nM CC-1065, the DNA of drug-treated cells remained intact (Figure 3a). Thus, CC-1065 itself does not induce direct breaks or alkali-labile sites. This is consistent with findings by Swenson et al. (1982), who first showed this result with naked DNA. Upon heating to 90 °C (15 min), however, degradation of genomic DNA was seen in CC-1065-exposed cells as shown by the shift in the peak of radioactivity to fractions of smaller MW (Figure 3b). In comparison, just heating by itself does very little damage to the DNA, as shown by the relatively small shift in the sedimentation profile for the samples not exposed to the drug (Figure 3b).

When CC-1065 was added to whole cells, it was not clear whether all the observed heat-labile sites were generated solely by drug that was covalently bound to the DNA inside the cell or if covalent adducts continued to form subsequent to cell lysis as a consequence of a remaining pool of noncovalently bound or free drug. To see if free drug can form covalent adducts under the latter conditions, we measured the generation of heat-labile sites by CC-1065 added directly to cell lysates. Our results demonstrated that exogenously added CC-1065 readily formed covalent adducts in the lysates (Figure 3c). The fact that there was even more damage to DNA in cell lysate than to whole cell DNA at the same drug to DNA ratio could be due to several factors, including (1) drug uptake properties of whole cells may limit CC-1065 available for adduct formation, (2) CC-1065 may be deactivated in a cellular environment, and (3) DNA dissociated from protein may be more accessible to the drug. Thus, if a reservoir of noncovalently bound drug remained after drug treatment of whole cells, posttreatment damage could contribute significantly to the total number of heat-labile sites observed.

Detection of Posttreatment Damage to Genomic DNA. We wished to determine the damage that is induced by CC-1065

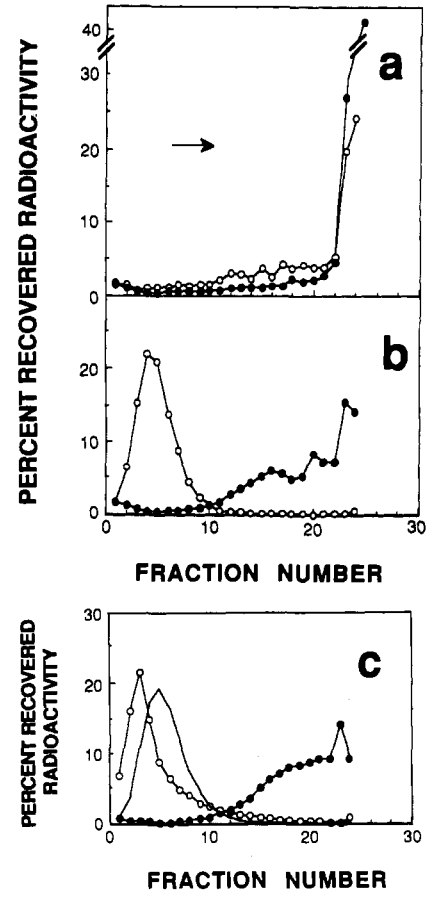

FIGURE 3: Sedimentation profiles of DNA damage from whole BSC-1 cells (panels a and b) or cellular lysates (panel c). Cells were labeled with [^{14}C]dT. Drug-exposed cells were incubated in lysing solution at 4 °C (panel a) or 90 °C (panel b) before being loaded onto alkaline sucrose gradients for centrifugation. Samples either were treated with 900 nM CC-1065 (○) or were control samples without the drug (●). Panel c shows results from lysates treated with 710 nM CC-1065 (○) and nontreated lysates (●) heated at 90 °C before being loaded onto alkaline sucrose gradients for centrifugation. The solid line represents results for whole cells treated with 710 nM CC-1065 (from the same experiment).

to intracellular DNA as opposed to damage occurring post-treatment. With naked DNA, noncovalently bound CC-1065 can be removed with alcohol extraction (Reynolds et al., 1985), implying that no significant further damage would be generated. The situation might be different for whole cell systems. With naked DNA, following extraction of CC-1065, at least 45% of the input drug has interacted with DNA (Swenson et al., 1982). From our whole cell experiments, by contrast, we estimate that less than 1% of input drug is responsible for damage. Hence, we might expect residual unextracted CC-1065 to contribute more significantly to total damage for cellular DNA than for naked DNA.

CC-1065-treated cells were extracted with ethanol following the treatment period to determine if we could diminish damage that might occur after the drug incubation. Moreover, to quantitate posttreatment damage caused by any remaining drug, a differently labeled [^{3}H]dT control cell population was added to each drug-treated ^{14}C-labeled sample just prior to lysis and heating. We reasoned that any adduct formation occurring after drug incubation and ethanol extraction might affect the DNA of this marker population and the drug-exposed cells to similar extents. Thus, break frequencies determined for ^{14}C-labeled DNA and corrected for damage registered by the ^{3}H-labeled DNA as an internal control should yield an estimate of the adducts formed inside the cell.

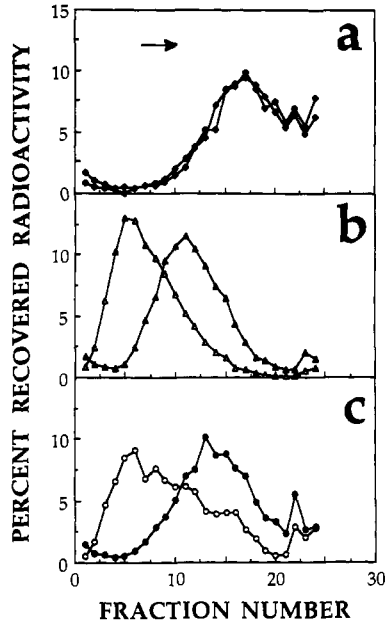

FIGURE 4: Effect of ethyl alcohol extraction on DNA damage of CC-1065-treated whole cells determined by sedimentation analysis. ^{14}C-labeled drug-treated cells (open symbols) were mixed with ^{3}H-labeled nontreated cells (closed symbols) prior to incubation at 90 °C. Fragmentation of ^{3}H-labeled DNA reflects posttreatment damage. Key: panel a (◇), no drug (control); panel b (△), whole cells treated with 710 nM CC-1065; panel c (○), whole cells treated with 710 nM CC-1065 followed by a 15-min extraction with ethanol prior to mixing with internal control.

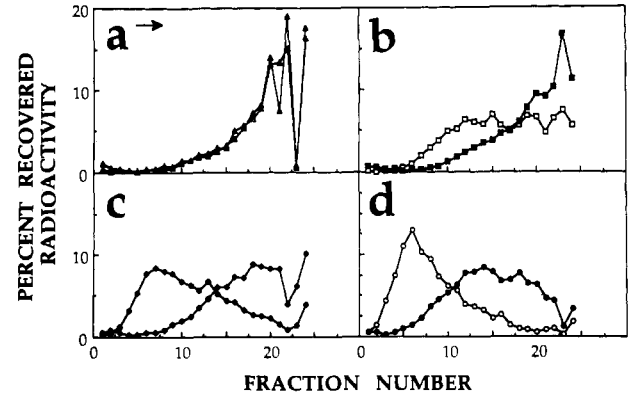

FIGURE 5: Alkaline sucrose gradient profiles of concentration-dependent damage by CC-1065 to cellular DNA. Open symbols represent CC-1065-treated, [^{14}C]dT-labeled BSC-1 cells. Closed symbols represent nontreated, [^{3}H]dT-labeled cells, which were used as internal controls. All drug-treated cells were extracted with ethanol prior to mixing with ^{3}H-labeled cells. ^{14}C-labeled cells were treated with the following concentrations: panel a (△), 0 nM CC-1065; panel b (□), 10 nM CC-1065; panel c (◇), 60 nM CC-1065; panel d (○), 300 nM CC-1065. In panels b–d, the frequencies of CC-1065-induced damage to BSC-1 genomic DNA were 21, 123, and 293 breaks/10^6 base pairs, respectively (frequency of heat-labile sites calculated as described in Materials and Methods).

Compared to non-drug-treated cells (Figure 4a), posttreatment damage is evident by the shift of the ^{3}H-labeled curve (internal control) to fractions of smaller DNA sizes (Figure 4b). Alcohol extraction of free and reversibly bound drug reduces posttreatment damage as seen by the shift toward larger DNA sizes of both the ^{3}H-labeled cell curve and the drug-treated cell curve (Figure 4c). By calculating the frequency of breaks per 10^6 base pairs for each set of conditions, we can estimate that in the absence of extraction of unreacted CC-1065 with alcohol the posttreatment damage in drug-treated DNA is typically about 20% of the total measured heat-labile sites. With extraction, posttreatment damage is typically reduced to approximately 9% of the total. Alcohol extraction and the mixing of differently labeled internal control cell populations were employed in all further gradient sedimentation studies unless otherwise noted.

Concentration Dependency of CC-1065-Induced Damage to Genomic DNA. The refined assay described in the preceding section was used to determine the concentration dependence of CC-1065-induced DNA damage to whole cells. In a comparison with results from the external control (^{14}C-labeled nontreated cells in Figure 5a), we detected some heat-labile sites on the DNA of cells that were treated with 10 nM CC-1065 (Figure 5b). Very little damage was evident in the ^{3}H-labeled internal control cells of the same sample. At higher CC-1065 concentrations (60 and 300 nM), we observed a gradual increase in the production of heat-labile sites in ^{14}C-labeled material. There is also an increase in the damage to the ^{3}H-labeled internal controls (Figure 5c,d). The frequency of breaks (per 10^6 base pairs) based on the molecular weights of the drug-treated cellular and the internal control DNA for each concentration (calculations described in Materials and Methods) is believed to be a measure of covalent adducts of CC-1065 formed intracellularly. DNA damage is first detected at 3.0 nM CC-1065 (3 single-stranded breaks/10^6 base pairs) and approaches 300 breaks/10^6 base pairs (at 300 nM CC-1065). There was no further increase in the number of strand breaks detected for CC-1065 above 300 nM (data not shown), but these concentrations exceeded the optimal range for detection of additional damage under the centrifugation conditions employed.

We also assayed for the ability of CC-1065 to generate thermally induced double-stranded breaks by employing nondenaturing gradient conditions. (Double-stranded breaks would be produced if two or more CC-1065 molecules were covalently bound in close proximity to each other but on opposing strands of the DNA helix.) Our results indicated that thermally induced double-stranded breaks are generated, but at very low frequencies (data not shown). Thus, covalent adducts of CC-1065 do not show a tendency to juxtapose on opposing strands of the DNA helix.

We additionally examined CC-1065 effects using isolated nuclei as a target. The induction of heat-labile sites in nuclei could be detected after a very short treatment, and damage was dependent on drug concentration (data not shown). Moreover, at similar drug concentrations, the level of damage in nuclei exceeds (by approximately 2-fold) damage seen with whole cells. It is possible that cellular membrane and/or cytoplasmic components are affecting the ability of CC-1065 to induce lesions in the DNA of whole cells.

Kinetics of CC-1065-Induced Damage to Genomic DNA and Its Reversal. In order to determine the time course of formation of CC-1065-DNA adducts in whole cells, monolayers were treated for intervals ranging from $^1/_4$ to 4 h with 60 nM CC-1065 and processed for gradient analysis. As seen in Figure 6, damage increased linearly with time of exposure until 2 h, at which time damage reached a plateau at about 240 breaks/10^6 base pairs.

We also addressed the question of the reversibility of CC-1065-induced lesions. BSC-1 monolayers exposed for 2 h to 60 nM CC-1065 were washed twice with PBS and incubated further (0–4 h) in fresh media. Virtually the same number of strand breaks were obtained upon thermal treatment of cell samples after up to 4 h postincubation in drug-free medium (Figure 6, inset). Thus, CC-1065-induced heat-labile sites represent lesions that are apparently irreversible within this time frame.

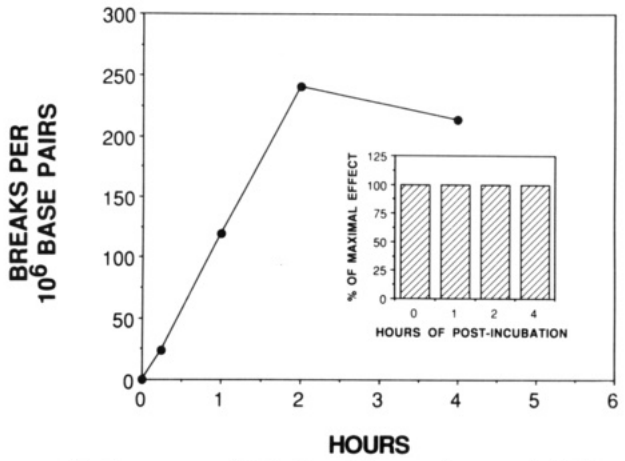

FIGURE 6: Time course of induction of damage to genomic DNA of BSC-1 whole cells by 60 nM CC-1065. The inset shows the lack of reversal of this damage. Breaks frequencies were quantitated by alkaline sucrose gradient analysis of ^{14}C-labeled drug-treated DNA and ^{3}H-labeled internal control DNA (as described in the legend to Figure 5). Monolayers used for the reversal assay were washed twice with PBS following drug treatment and then incubated in fresh media for the indicated time periods before analysis. Maximal effect at 60 nM CC-1065 for 2 h is based on 241 breaks/10^6 base pairs.

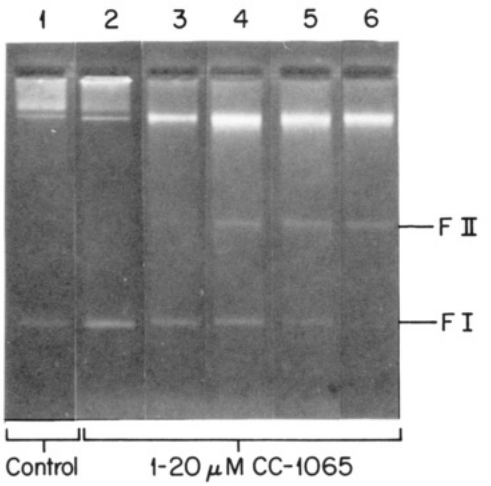

FIGURE 7: CC-1065 damage to intracellular SV40 DNA. SV40-infected BSC-1 cells were treated for 2 h with CC-1065. Cells were extracted with ethanol prior to a 15-min 90 °C treatment. Gels were stained with ethidium bromide. Individual lanes represent the results from CC-1065 concentrations of (1) 0 nM, (2) 1 μM, (3) 2 μM, (4) 5 μM, (5) 10 μM, and (6) 20 μM.

CC-1065-Induced Damage to Intracellular SV40 DNA. SV40 DNA, which exists in cells as minichromosomes, is a useful model target for drug-induced damage, as it is a defined molecule that can be electrophoretically separated from genomic DNA. We used the SV40 system to confirm the DNA-damaging ability of CC-1065 on an intracellular target. Thermally induced DNA strand breaks in intracellular SV40 minichromosomes (in SV40-infected BSC-1 cells) were monitored for conversion of supercoiled form I DNA to relaxed (nicked) form II DNA. Separation of CC-1065-treated SV40 DNA on agarose gels demonstrated a concentration-dependent decrease in the amount of form I SV40 DNA with a concurrent increase in the amount of form II. This effect is first seen at 1 μM CC-1065. A 50% conversion from form I to form II occurs at 5–10 μM CC-1065 (Figure 7). Generation of linear form III DNA was not evident.

Discussion

Our study has shown for the first time that CC-1065 can react with cellular DNA to form covalent adducts that produce strand breaks upon heat treatment. Alkaline sucrose gradient analysis of CC-1065-treated BSC-1 cells revealed a concentration- and time-dependent increase in thermally induced breaks in genomic DNA. The observation that maximal damage requires 2 h of drug exposure is consistent with reports by Swenson et al. (1982), who noted the same length of exposure for maximal covalent drug binding to naked calf thymus DNA. Sedimentation analysis proved to be a sensitive assay for the quantitation of lesions induced by CC-1065. We were able to measure damage at concentrations as low as 3 nM. Extrapolation from our data indicates that 1 break/10^6 base pairs of genomic DNA would be generated by 1.4 nM CC-1065 during a 2-h treatment.

With intracellular SV40 DNA, we demonstrate 50% conversion (1 break/SV40 molecule = 100% conversion from form I supercoiled to form II relaxed) at concentrations between 5 and 10 μM CC-1065. Solely on the basis of size difference between SV40 and genomic DNA, 0.2 μM drug should be needed to generate 1 break/molecule of intracellular SV40 DNA ($\approx 5 \times 10^3$ base pairs). Thus, this system is less sensitive than alkaline sucrose gradient analysis of lesions in genomic DNA or viral DNA appears less susceptible to drug action. Recent data from our laboratory suggest that intracellular synthesis of SV40 DNA is severalfold less sensitive to CC-1065 inhibition than genomic DNA replication (Woynarowski et al., unpublished data).

The detection of thermally labile sites when drug was added to cellular lysates indicated that CC-1065 could continue to induce formation of adducts even after cell lysis. Posttreatment damage may originate from two sources: (i) nonbound intracellular CC-1065 could bind covalently to DNA or (ii) reversibly interacting CC-1065 could convert to the irreversibly bound covalent product. It should be noted that the lysing procedure uses high salt concentration and a strong detergent—that is, conditions generally unfavorable for the formation and stability of noncovalent interactions. In such a milieu, noncovalently bound CC-1065 might dissociate from the DNA. At the same time, deproteinization could render DNA more accessible for the alkylation by CC-1065. Therefore, it is possible that covalent adducts formed during the posttreatment period may result from direct reactions of CC-1065 molecules with DNA rather than from the conversion of noncovalently bound drug.

The refined assay that we applied allowed us to distinguish posttreatment lesions from the products of true intracellular or intranuclear reactions. This was achieved by extracting treated cells with ethanol to remove noncovalently bound drug and free drug and then mixing these cells with untreated ^{3}H-labeled cells, which served as internal controls. On the basis of damage to the internal controls, we estimate that posttreatment damage to treated whole cell DNA (following extraction) was typically about 10% of the total damage.

Specific CC-1065 covalent binding to cellular DNA may be responsible for drug-induced growth inhibition, as we observed CC-1065-induced heat-labile sites at drug concentrations comparable to those inhibiting cell growth. There are less than 10^4 CC-1065 adducts/cell at its D_{10}, as estimated by extrapolation of the frequencies calculated from Figure 5 (see legend). Formation of covalent adducts in whole cells at such low frequencies coupled with the high sequence specificity of CC-1065 binding suggests that the drug might exert its biochemical mechanism at critical sites on the genome. Preliminary results from our laboratory indicate that CC-1065 reacts with intracellular SV40 DNA at unique sites (Beerman

et al., unpublished data). With regard to the mechanism of cytotoxicity, it is interesting to note that CC-1065 inhibited DNA synthesis in BSC-1 cells at concentrations similar to those producing heat-labile sites, with 50% inhibition occurring at 3 nM (Woynarowski et al., unpublished data). Thus, it is likely that formation of CC-1065 covalent adducts in the cell brings about inhibition of DNA replication and, consequently, blocks cell proliferation.

Adduct formation by CC-1065 does not appear to be readily repaired (Figure 6, inset). This is consistent with observations by Tang et al. (1988), who have shown that CC-1065-induced lesions are not recognized by isolated repair nucleases. It is possible that the adducts may be difficult for repair enzymes to recognize because CC-1065 does not markedly distort local DNA structure and does not produce a bulky lesion. This apparent irreversibility may be a factor in the extreme cytotoxic potential of CC-1065.

The fact that CC-1065 can form heat-labile lesions when added to whole cells suggests that formation of covalent adducts, the mechanism ascribed to CC-1065 through previous cell-free analyses, may indeed play a major role in drug cytotoxicity. Moreover, it has been observed that analogues of CC-1065 lacking the ability to form covalent adducts with isolated DNA show much diminished cytotoxicity, implying that the antiproliferative properties of this drug are related to its potential to form covalent lesions (Hurley et al., 1988; Warpehoski & Hurley, 1988). Successful detection of lesions caused by CC-1065 in genomic DNA by alkaline sucrose gradient analysis shows that the same approach may be useful for investigating the mechanisms of CC-1065 analogues. Using this method, we have detected and quantitated DNA lesions in whole cells with U-73,975 (adozelesin), a CC-1065 analogue that has been accepted for phase I clinical trials (unpublished data). These studies may help to understand which aspects of the molecular actions of CC-1065 and its analogues are important for drug cytotoxicity.

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