Cell Cycle Progression and Chromosome Segregation in Mammalian Cells Cultured in the Presence of the Topoisomerase II Inhibitors ICRF-187 and ICRF-159 (Razoxane) 1

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ABSTRACT

Certain bis(2,6-dioxopiperazine) derivatives, which include ICRF-187 [(+)-1,2-bis(3,5-dioxopiperazinyl-1-yl)propane; ADR-529] and its racemic compound ICRF 159 (Razoxygen), have been investigated as antineoplastic agents. In addition, ICRF-187 is currently under intense study as an agent to ameliorate the cardiac toxicity of antitumoral therapy. These agents have recently been identified as inhibitors of topoisomerase II. We studied the effects of ICRF-187 and ICRF-159 on the progression of cultured epithelial cells through M phase. Beginning approximately 1.5 h after drug addition, chromosome condensation was significantly inhibited. Cells entered and progressed through M phase at near normal rates, but the lack of complete chromosome separation during anaphase resulted in catastrophic effects on normal chromosome distribution. Immunolabeling with centromeric sera, which recognizes centromere proteins, and with MP-2 monoclonal antibody, which recognizes mitotic phosphoproteins, indicated that the centromeres of the chromosomes assembled a normal metaphase array in the presence of ICRF-187 and ICRF-159. Centromere separation in anaphase was initiated normally but was not completed because the chromatid arms failed to disengage from each other. Massive chromosome bridges were formed, and the chromatid mass became trapped in the cleavage furrow leading to its unequal distribution to the daughter cells. In many cases, all the chromatid was pushed into one of the two dividing cells. It is likely that previous studies, based on flow cytometry, indicating that bis(2,6-dioxopiperazine) derivatives cause an accumulation of cells with a 4N DNA content, reflect the incomplete segregation of chromosomes in mitosis rather than a block in G2 of the cell cycle as had been proposed.

INTRODUCTION

DNA topoisomerase II catalyzes the cleavage of a double-stranded DNA molecule, its passage through another such molecule, and its religation. This activity is thought to be an essential aspect in several cellular events such as DNA replication, RNA transcription, chromosome condensation, and chromosome segregation (reviewed in Ref. 1). Topoisomerase II may play a structural role as part of the nuclear matrix anchoring and organizing the chromatin in interphase nuclei (2, 3). In addition, it has been proposed to act as a “scaffold” for the assembly of mitotic chromosomes (4).

Topoisomerase II is a major target for many antineoplastic agents including the acridines (m-amesacrine), anthracycines (Adriamycin), ellipticine derivatives (9-hydroxylellipticine), and epidiphyllotoxins (VP-16 and VM-26) (5, 6). These drugs hyperstabilize the covalent enzyme-DNA intermediate. It is believed that subsequent cellular events, such as the passage of replication forks through these cleavable complexes, leads to irreparable DNA damage and cell death (7).

In the mitotic phase of the cell cycle, topoisomerase II is required for normal chromosome condensation and chromatid separation during anaphase (8–12). Topoisomerase II inhibitors cause the greatest number of DNA strand breaks to cells traversing the G2 to M phase boundary, although cytotoxicity induced by these agents appears to be highest during S (13). Studies involving immunolocalization and electron microscopy indicate that in mitosis, topoisomerase II is concentrated into a structural, proteinaceous backbone or “scaffold” around which the mitotic chromatin is arranged (14). In this way, topoisomerase II may play a structural as well as enzymatic role in chromosome condensation. However, recently this view of topoisomerase II as a significant structural component of mitotic chromosomes has been challenged (15, 16).

In mammalian cells, two forms of topoisomerase II are known, one with a molecular weight of 170,000 daltons (topoisomerase IIα) and one with a weight of 180,000 daltons (topoisomerase IIβ) (Refs. 17 and 18). These molecules are coded by separate genes on different chromosomes in humans (19). While the proteins encoded by the two genes show extensive homology at the amino acid level, several studies indicate that major differences exist in their expression and regulation. Topoisomerase IIβ is expressed at a relatively low, unchanged level at all stages of the cell cycle and is found in both proliferating and differentiated cells (20). In contrast, topoisomerase IIα is found in high concentration in proliferating cells and appears to be regulated during the cell cycle (20–22). Certain antineoplastic drugs show greater inhibition of the enzymatic activity of topoisomerase IIα than of topoisomerase IIβ (17).

Feedback mechanisms referred to as checkpoints assure the proper timing of cell cycle events such as DNA replication, mitotic spindle assembly, chromosome segregation, and cell cleavage. One checkpoint system monitors the integrity of DNA during the G2 phase of the cell cycle. DNA damage induced by chemicals or radiation causes normal cells to arrest in G2 until the damage has been repaired (23). Cells treated with drugs that cause DNA strand breaks, such as the epipodophyllotoxins VP-16 (etoposide) and VM-26 (teniposide), do not progress from G2 to M (24), and the cyclin-dependent kinase, p34cdc2, is not activated (25). By contrast, cells already in M at the time of drug application proceed through mitosis but undergo abnormal chromosome segregation (10).

ICRF-159 was first described as an antiproliferative drug that blocked cells in the G2 phase of the cell cycle (26, 27). Recently, ICRF-187 has become of particular interest as an agent to counteract the cardiopathic effects of doxorubicin (Adriamycin). Several reports suggest that ICRF-187 may potentiate the chemotherapeutic effects of doxorubicin (reviewed in Ref. 28). Sehested et al. (29) reported that ICRF-187 interferes with the cytotoxic effects of daunorubicin but not that of doxorubicin. Recently, the bis(2,6-dioxopiperazine) derivatives, the class of compounds that includes ICRF-187 and ICRF-159, were found to inhibit topoisomerase II activity without the formation of cleavable DNA-enzyme complexes (30). One derivative, ICRF-193, has been demonstrated to inhibit normal chromosome segregation (31, 32).
We have performed a careful cytological analysis of the effects of the bis(2,6-dioxopiperazine) derivatives, ICRF-187 and ICRF-159, on the progress of cultured mammalian cells through the G1–M transition and through mitosis. We find that treatment of cells with ICRF-187 or ICRF-159 allows normal entry into M phase and the normal function of centromeres and the mitotic spindles. However, the drugs produce severe defects in chromosome condensation and separation during anaphase.

MATERIALS AND METHODS

Topoisomerase II Inhibition Assay. Purified human topoisomerase II, catenated kinetoplast DNA, and decatenated marker DNA were purchased from Topogen, Inc. (Columbus, OH). ICRF-187 (ADR-529) and ICRF-159 were gifts from Adria Laboratories (Dublin, OH). For topoisomerase II inhibition assays, stock solutions of 1 mg/ml in H2O were prepared just before use. VM-26 (teniposide) was a gift from Bristol-Myers Squibb Pharmaceutical Research Institute (Wallington, CT). It was stored as a stock of 10 mg/ml in DMSO at −20°C. Assays were performed in the presence and absence of drugs by mixing 1 unit of topoisomerase II with 0.2 μg of kinetoplast DNA in 20 μl of a buffer consisting of 50 mM Tris-Cl (pH 8.0), 120 mM KCl, 10 mM MgCl2, 0.5 mM ATP, 0.5 mM dithiothreitol, and 30 μg/ml bovine serum albumin. After 15 min at 37°C, reactions were terminated by the addition of 5 μl of a stop solution consisting of 5% sarkosyl, 0.0025% bromophenol blue, and 25% glycerol. The DNA products and decatenated marker DNA were electrophoresed on 1% agarose gels in 0.45 mM Tris-borate (pH 8.0)-1 mM EDTA containing 0.5 μg/ml ethidium bromide. The gel was photographed on an ultraviolet light box through an amber filter with Polaroid type 667 film.

Cell Culture and Fixation. Ptk1 cells, originally derived from rat kangaroo kidney, were obtained from the American Type Culture Collection (Rockville, MD). Ptk1 cells were cultured in Ham's F-12 medium (Gibco-BRL, Gaithersburg, MD) containing 0.5 μg/ml ethidium bromide. The gel was photographed on an ultraviolet light box through an amber filter with Polaroid type 667 film.

RESULTS

Inhibition of Topoisomerase II in Vitro. Incubation of purified topoisomerase IIα with ICRF-159 and ICRF-187 inhibited, in a dose-dependent manner, the ability of the enzyme to decatenate kinetoplast DNA (Fig. 1). As expected, a similar inhibition of enzyme activity was seen with VM-26.

Cell Cycle Progression. Cells that entered mitosis within 1 h after the addition of ICRF-187 or ICRF-159 showed no changes in chromosome condensation or segregation. Beginning at 1.5 h after the

Fig. 1. ICRF-187 and ICRF-159 inhibit topoisomerase II activity in vitro. Arrowheads, position of migration of the untreated, catenated kinetoplast DNA. Arrows, migration positions of the decatenated products formed as a result of topoisomerase II activity. Lane 1, decatenated standard; Lane 2, reaction mixture without topoisomerase II; Lane 3, control complete reaction mixture in the absence of drug; Lanes 4 and 7, ICRF-159 at 50 and 20 μg/ml, respectively; Lanes 5 and 8, ICRF-187 at 50 and 20 μg/ml, respectively; Lanes 6 and 9, VM-26 at 50 and 20 μg/ml, respectively.

[Note: The citations are not included in the natural text representation.]
addition of drugs, cytological effects became apparent. We examined the effects of the drugs on the progression of cells from G₂ to mitosis. Because topoisomerase II is necessary for normal chromosome condensation, we used an immunofluorescent assay for counting M phase cells that was not dependent upon detecting condensed chromosomes. Coverslips containing Ptk1 cells were scored for the presence of mitotic cells as revealed by labeling with the MPM-2 antibody and by the presence of a mitotic microtubule pattern at 1, 2, and 4 h after the addition of VM-26 or ICRF-187. As shown in Table 1, cultures treated with VM-26 showed a noticeable diminution in the number of mitotic cells at 1 h and, by 4 h, contained no cells in M phase. In contrast, cultures treated with ICRF-187 contained a slightly elevated proportion of mitotic cells at 4 h in comparison to the normal level of mitotic cells in untreated cultures.

### Chromosome Morphology and Segregation in Cells Treated with ICRF-159 and ICRF 187.

The Ptk1 cell line is comprised of epithelial cells that contain a small number of large chromosomes (2n = 12) and remain relatively flat during mitosis, making them useful for light microscopic analysis of the assembly of chromosomes and the progression of mitosis. LLC-PK cells (2n = 38) remain exceedingly flat in mitosis and have a regular and well-developed microtubule array, rendering them ideal for discerning subtle alterations induced by drugs in the structure of the mitotic spindle.

In cultures treated with ICRF-159 and ICRF-187 for periods longer than 1 h, the chromatin in mitotic cells exhibited only partial condensation, resulting in abnormally long and tangled chromosomes (Fig. 2). ICRF-159 and ICRF-187 showed no apparent differences in their effects on metaphase chromosomes. As a result, metaphase cells treated with these drugs were scored for the number of stained chromosomes and for the presence of normal metaphase figures, as revealed by labeling with the MPM-2 antibody and by the presence of a mitotic microtubule pattern. Control cultures of Ptk1 cells had a mitotic index of 1.2%.

### Table 1  Cell cycle progression of Ptk1 cells from G₂ to M in the presence of inhibitors of topoisomerase II.

<table>
<thead>
<tr>
<th>Length of treatment (h)</th>
<th>VM-26* (%)</th>
<th>ICRF-187 (%)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>20/3420 (0.6)</td>
<td>43/5780 (1.1)</td>
</tr>
<tr>
<td>2</td>
<td>9/3480 (0.3)</td>
<td>31/5080 (1.0)</td>
</tr>
<tr>
<td>4</td>
<td>0/3830 (0)</td>
<td>75/2530 (2.9)</td>
</tr>
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* Treatment with VM-26 inhibits progression of Ptk1 cells from G₂ to M while treatment with ICRF-187 does not. Ptk1 cells were treated with VM-26 (20 μg/ml) or ICRF-187 (20 μg/ml) for the indicated length of time. The cells were detergent extracted, fixed, and then labeled for immunofluorescence with the MPM-2 monoclonal antibody to mitotic phosphoepitopes and with anti-tubulin. The cultures were scored for the number of cells in mitosis as defined by labeling with MPM-2 and by the presence of a mitotic spindle. Control cultures of Ptk1 cells had a mitotic index of 1.2%.

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Fig. 2. Chromosome condensation is inhibited in Ptk1 cells treated with ICRF-187 and ICRF-159. Ptk1 cells incubated for 3 h in the presence of drugs were detergent lysed in chromosome stabilization buffer, fixed, and processed for immunofluorescent labeling with MPM-2 antibody. A late prometaphase cell in a culture incubated in ICRF-159 (A and B) shows a normal distribution of centromeres (arrows) and mitotic poles (arrowheads) labeled with the MPM-2 antibody (A). The same cell imaged by phase contrast microscopy (B) reveals long, extended chromosomes that have undergone only partial condensation. A control, late prometaphase cell incubated without drug shows the normal pattern of labeling of centromeres (arrows) and mitotic poles (arrowheads) by the MPM-2 antibody (C) and the normal condensed state of the chromosomes visible by phase contrast microscopy (D). Bar, 5 μm.

Fig. 3. Microtubules of the mitotic spindle are assembled normally in the presence of ICRF-187 and ICRF-159. LLC-PK cells were incubated 1.5 h in ICRF-187, lysed in detergent, fixed, and immunolabeled with antibodies to tubulin. A metaphase cell imaged by fluorescence microscopy (A) reveals a normal spindle structure consisting of bundles of microtubules (arrow) converging on the mitotic poles (arrowheads). A phase contrast image of the same cell (B) shows the abnormal, undercondensed chromosomes extending well into the peripheral cytoplasm (curved arrows). Bar, 5 μm.
CELL CYCLE PROGRESSION IN ICRF-187 AND ICRF-159

Effects upon cells. Cells were found at all stages of mitosis, and no structural alterations in the microtubules of the mitotic spindle were detected (Fig. 3). Interestingly, the centromeres of drug-treated cells were routinely found to form precise metaphase alignments even when the bulk of the chromosome arms were tangled far off the metaphase plate (Fig. 4). Labeling of cells with antibodies to topoisomerase II indicated that the protein was incorporated into the undercondensed chromosomes but did not concentrate at the centromeres as it normally does in the chromosomes of untreated cells (Fig. 5).

Cells at various stages of anaphase were commonly seen in cultures treated with ICRF-187 and ICRF-159 for more than 1 h, but these cells were never seen to separate their chromatids normally. In most instances, the chromatin remained tangled in a large mass near the middle of the dividing cell (Fig. 6). In late anaphase, the cleavage furrow was often found to cut through the chromosome mass. In telophase cells near the completion of cytokinesis, the pair of daughter cells often contained unequal amounts of chromatin. Often the cleavage furrow would segregate all or nearly all of the chromatin into one of the two daughter cells (Fig. 7). Abnormal cleavages were rarely seen in control cultures.

Cells that had moved into M phase in the presence of ICRF-159 and ICRF-187 were unable to recover if placed into fresh medium. However, cells that were treated with drugs for 3 h in G2 and then placed for 2 h in fresh medium lacking drugs were able to enter mitosis and undergo normal chromosome condensation and segregation (Fig. 8). This evidence suggests that, in order to manifest the chromosomal abnormalities, a cell must be exposed to the drug at the time of chromosome condensation during the entry into M phase.

DISCUSSION

We have performed a detailed cytological analysis of the effects of the compounds ICRF-187 and ICRF-159 on progression through mitosis in epithelial cell lines in which the morphological events of mitotic chromosome assembly and segregation can be clearly imaged. The PtK1 and LLC-Pk1 cell lines responded identically to all of the drug treatments. To avoid the complicating effects of additional drugs used for cell synchronization procedures, we used random populations of cells treated for relatively brief periods of from 15 min to 4 h. In this manner we specifically examined the effects of the drugs on progression from G2 to M phase. As expected from previous work, cells in the presence of the epidophyllotoxin VM-26 ceased in their progression from G2 into mitosis as judged by the absence of any chromosome condensation, the lack of expression of the mitotic phosphoproteins recognized by the MPM-2 antibody, and the failure to

![Fig. 4. Centromeres assemble a regular array at metaphase in ICRF-187 and ICRF-159 in the absence of normally condensed chromosomes. PtK1 cells incubated for 3 h in the presence of ICRF-159 were detergent lysed, fixed, and processed for immunofluorescent labeling with MPM-2 antibody. A fluorescent image of a metaphase cell (A) shows complete alignment of the immunolabeled centromeres (arrows) to the metaphase plate. The mitotic poles (arrowheads) are also labeled. The phase contrast image of the same cell (B) shows the undercondensed chromosomes (curved arrows) extending into the peripheral cytoplasm. Bar, 5 μm.](image-url)

![Fig. 5. Topoisomerase II is incorporated into mitotic chromosomes but not concentrated at the centromeres in the presence of ICRF-187 and ICRF-159. PtK1 cells incubated for 3 h in the presence or absence of ICRF-159 were detergent lysed, fixed, and processed for immunofluorescent labeling with antibody to topoisomerase II. Immunofluorescent labeling of a drug-treated metaphase cell (A) reveals that the chromosome arms contain detergent-insoluble topoisomerase II (arrowheads) with little concentration at the centromeric regions. The same cell imaged by phase contrast microscopy (B) shows the typical, undercondensed chromosomes (arrowheads). Immunofluorescent labeling of a control cell (C) reveals anti-topoisomerase II labeling of chromosome arms (arrowheads) with an intense concentration of the antibody label at the centromeres (arrows). The phase contrast image of the same cell (D) shows the normal appearance of the condensed chromosomes (arrowheads). Bar, 5 μm.](image-url)
form a mitotic array of microtubules. In contrast, cells treated with ICRF-159 or ICRF-187 did not exhibit blockage in G2 and, although abnormal, were found at all stages of mitosis. The unifying characteristic of those inhibitors that generate the G2 cell cycle block appears to be the stabilization of a cleavable enzyme-DNA complex that results in DNA damage. The DNA damage then activates a G2 checkpoint and blocks the cell cycle. The topoisomerase II inhibitors ICRF-187 and ICRF-159 do not induce cleavable complexes and DNA damage. Concomitantly, they do not produce a G2 cell cycle arrest. Thus it seems clear that there is not a G2 checkpoint control mechanism that directly monitors normal topoisomerase II function.

For cells treated longer than 1 h with ICRF-187 and ICRF-159, chromosome condensation and segregation were profoundly perturbed. In general, the chromosomes appeared incompletely condensed and were present as long strands. They were often difficult to resolve as individuals and tended to form a large, tangled mass at the middle of the cell. Interestingly, we found that the centromeres of cells treated with ICRF-187 and ICRF-159 could align precisely at the metaphase plate, independent of the tangled chromosome arms, suggesting that the drugs did not significantly impair the function and regulation of the centromeres. This result suggests that alignment of chromosomes at the metaphase plate is due to forces acting on the centromeres and that forces acting on the chromosome arms do not play a significant role. Thus, these data refute models invoking chromosome arm-directed forces to explain the alignment of chromosomes at the metaphase plate (35).

The increase in the mitotic index of cultures treated for 4 h with ICRF-187 suggests that the drug-treated cells required a somewhat longer time period to transit through mitosis. One explanation is that there exists an M phase checkpoint that monitors chromosome condensation or topoisomerase function and blocks progression through M. If such a checkpoint exists, it is relatively ineffective since cells seem to progress through M readily in the presence of ICRF-187 and ICRF-159. An alternative explanation is that the centromeres of the tangled chromosomes require a longer time to congress to a functional metaphase plate. Studies in mammalian cells (36, 37) and yeast (38) suggest the existence of a checkpoint that can detect misaligned chromosomes and block progression through mitosis. We suggest that this checkpoint monitors not the alignment of chromosomes but the alignment of the centromeres at the metaphase plate. In the presence of ICRF-187 and ICRF-159, more time is necessary to achieve alignment of the centromeres; thus, mitosis is prolonged.

Topoisomerase II is incorporated into mitotic chromosome arms to play enzymatic and perhaps structural roles in chromosome segregation. We recently determined that in normal chromosomes, topoisomerase II is particularly concentrated at the centromeres (39). This concentration at the centromere was eliminated in the chromosomes of cells treated with ICRF-187 and ICRF-159. Such centromeres were still able to congress to the metaphase alignment and initiate separation at anaphase, suggesting that the high concentration of topoisomerase II at the centromeres is not essential for these functions. The high concentration of topoisomerase II may be required for proper organization of the centromere or to catalyze the rapid and complete separation of the centromeric DNA of the two chromatids at the onset of anaphase.

In the presence of ICRF-187 and ICRF-159, the separation and anaphase movements of the centromeres toward the opposite poles caused the tangled chromosome mass to stretch, but the chromosomes...
were unable to segregate. Often the cleavage furrow collapsed around the chromatin, and the division of the chromatin to the two daughter cells was unequal. Frequently, all the chromosomes segregated to one of the daughter cells. Flow cytometry studies of cells treated with bis(2,6-dioxopiperazine) derivatives showed that many 4N cells accumulated (26, 31). This led to the suggestion that the such drugs produce a temporary or permanent G2 block. For example, Ishida et al. (31) studied the effects of two related bis(2,6-dioxopiperazine) derivatives, ICRF-154 and ICRF-193, on cell cycle progression in a human T-cell line. Although they detected aberrant mitoses, the authors nonetheless concluded that the drugs induced a G2 block as well as inhibited chromosome segregation. We detected no evidence for a G2 block and feel that the appearance of a hyperdiploid population can be fully explained by the failure of the cytokinetic furrow to separate the chromosomes into the two daughter cells. It is likely that the failure of previous workers to detect an increased mitotic index for cells treated with bis(2,6-dioxopiperazine) derivatives is due to the method of counting cells with condensed chromosomes. Since the inhibitors diminish the degree of condensation, mitotic cells become more difficult to detect at early stages of mitosis, particularly in cells with relatively small chromosomes.

Our results stressing the importance of topoisomerase II in the normal segregation of chromosomes are in complete agreement with the genetic studies in the yeasts Saccharomyces cerevisiae and Schizosaccharomyces pombe. In S. cerevisiae, it has been shown that topoisomerase II mutants undergo elevated nondisjunction in mitosis (40) and improper segregation of recombined chromosomes during meiosis I (41). In S. pombe, topoisomerase II is required both for chromosome condensation in prophase and for chromosome separation at anaphase (11).

Our goal in these experiments was to examine the effects of the bis(2,6-dioxopiperazine) derivatives on G2 to M progression. It is possible that the drugs also affect other stages of the cell cycle. Ishida et al. (31) reported that the related compound, ICRF-154, caused a transient retardation of S phase in a leukemic human T-cell line. However, in preliminary studies of cells cultured for periods of 12–24 h in ICRF-187 and ICRF-159, we noted that mitotic figures were abundant and were qualitatively indistinguishable from those of cells treated for shorter time periods (data not shown). This suggests that disruption of normal chromosome condensation is a major consequence of treating cells with bis(2,6-dioxopiperazine) derivatives.

Topoisomerase II presumably performs multiple roles in the cell, and a number of currently used therapeutic drugs exert their antineoplastic effects by targeting this enzyme. In many instances, the precise mechanisms by which these drugs inhibit cell proliferation is uncertain. Greater understanding of the normal functions of topoisomerase II and the cell biological responses to its inhibition may contribute to the development of better schemes to suppress neoplastic cell proliferation.

ACKNOWLEDGMENTS

I thank Dr. Anthony Imondi of Adria Laboratories for providing ICRF-187 (ADR-529) and ICRF-159 and Dr. John D. Matiskella of the Bristol-Myers Squibb Pharmaceutical Research Institute for VM-26 (teniposide). I am grateful to Drs. Potu N. Rao, Fred Drake, and J. B. Rattner for supplying antibodies used in this study. I thank Bill Ricketts and Dave Lawrence for able technical assistance. I also thank Dr. Marie Hanigan for insight and for valuable criticism of the manuscript.

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