0026-895X/00/020296-12$3.00/0

MOLECULAR PHARMACOLOGY, **57**:296–307 (2000).

Selection of Human Leukemic CEM Cells for Resistance to the DNA Topoisomerase II Catalytic Inhibitor ICRF-187 Results in Increased Levels of Topoisomerase IIα and Altered G_2/M Checkpoint and Apoptotic Responses

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Received August 11, 1999; accepted October 20, 1999

This paper is available online at http://www.molpharm.org

ABSTRACT

ICRF-187 is a bisdioxopiperazine anticancer drug that inhibits the catalytic activity of DNA topoisomerase (topo) II without stabilizing DNA-topoII cleavable complexes. To better understand the mechanisms of action of and resistance to topoII catalytic inhibitors, human leukemic CEM cells were selected for resistance to ICRF-187. The clones CEM/ICRF-8 and CEM/ICRF-18 are approximately 40- and 69-fold resistant to ICRF-187, and 12- and 67-fold cross-resistant to ICRF-193, respectively, but are sensitive to other topoII catalytic inhibitors (merbarone and aclarubicin), as well as collaterally sensitive to the DNA-topoII complex-stabilizing drug etoposide (VP-16). Both the number of VP-16- induced DNA-topoII complexes formed and the amount of in vitro topoII catalytic activity are enhanced in the drug-resistant cells. The ICRF-187-resistant clones contain ~5-fold increase in topoIIα protein levels and ~2.2-fold increase in topoIIα mRNA levels. Furthermore, CEM/ICRF-8 expresses ~3.5-fold increase in topoIIα promoter activity, suggesting that up-regulation of topoIIα in this clone occurs at the transcriptional level. Treatment of the drug-resistant or -sensitive cells with equitoxic doses of merbarone or teniposide results in a G_2/M arrest. In marked contrast, when treated with equitoxic ICRF-187 doses, the drug-resistant clones exhibit either a transient arrest or completely lack the G_2/M checkpoint compared with the drug-sensitive cells. This aberrant cell cycle profile is associated with a 48-h delay in drug-induced apoptotic cell death, as revealed by fluorescent-end labeling of DNA and poly (ADP-ribose) polymerase cleavage. In summary, resistance to ICRF-187 in CEM cells is associated with increased levels of catalytically active topoIIα and altered G_2/M checkpoint and apoptotic responses.

DNA topoisomerase II (topoII) is a nuclear enzyme that resolves DNA supercoiling and catenation by the breakage, strand-passage, and rejoining of double-stranded DNA (Champoux, 1990), thereby relieving topological constraints that occur during essential cellular processes such as DNA replication, transcription, cell division, and repair (Nelson et al., 1986; Brill et al., 1987). TopoII can also serve as a structural component of mitotic chromosome scaffolding (Uemura et al., 1987), playing an essential role in chromatin condensation during prometaphase and in sister chromatid segregation during anaphase (Adachi et al., 1991).

DNA topoII is a target for a number of clinically useful antitumor agents, in part because it is essential for cell survival. To date, there are two general classes of topoII inhibitors that interfere with enzyme catalysis at distinct points of the enzyme reaction. DNA topoII inhibitors, such as teniposide (VM-26), etoposide (VP-16), and the anthracyclines (daunorubicin and doxorubicin), stabilize cleaved DNA-topoII complexes (Chen et al., 1984; Robinson and Osheroff, 1991). In contrast to the complex-stabilizing topoII inhibitors, merbarone, aclarubicin, and the bisdioxopiperazines (e.g., ICRF-187 and -193) block the catalytic activity of the enzyme. Specifically, the bisdioxopiperazines have been reported to stabilize topoII in a closed-clamp configuration around the DNA (for reviews, see Andoh, 1998; Andoh and Ishida, 1998), whereas agents such as merbarone have been implicated recently in blocking the topoII-mediated DNA cleavage reaction (Fortune and Osheroff, 1998). Because

This work was supported in part by research Grants CA40570 and CA30103 from the National Cancer Institute (to W.T.B.), a research fellowship from the American Medical Association and Research Foundation (to S.E.M.), Cancer Center of the University of Illinois at Chicago, and research Grant CA31566 from the American Lebanese Syrian Associated Charities (ALSAC; to S.C.R.). S.E.M. was the (1998) Florence A. Carter Fellow of the American Medical Association.

ABBREVIATIONS: topo, topoisomerase; VM-26, teniposide; VP-16, etoposide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; FBS, fetal bovine serum; PARP, poly (ADP-ribose) polymerase; PCR, polymerase chain reaction; RT, reverse transcriptase; BrdU, 5-bromo-2-deoxyuridine; FITC, fluorescein isothiocyanate; CPT, camptothecin.

these drugs do not stabilize DNA-topoII complexes (i.e., they do not induce DNA strand breaks), they are termed "catalytic inhibitors" of topoII (Andoh and Ishida, 1998).

Inactivation of topoII by inhibitors, particularly the bisdioxopiperazines, is associated with abnormalities in chromosome condensation and sister chromatid segregation during the mitotic phase of the cell cycle, resulting in enhanced chromosomal breakage and cell death (Uemura et al., 1987; Adachi et al., 1991). These results are consistent with the fact that topoII plays an active role in kinetochore assembly, is a major structural component of the chromosome axis during mitosis, and is localized over interphase chromosomes (Wartburton and Earnshaw, 1997). Inhibition of topoII by bisdioxopiperazines such as ICRF-193 also results in a G_2/M checkpoint that is sensitive to the decatenation state of DNA; this checkpoint is believed to be distinct from a DNA damage-induced G_2 checkpoint (Downes et al., 1994). Bisdioxopiperazine-induced cell death is believed to be mediated through a programmed cell death pathway because treatment of murine thymocytes with ICRF-193 or ICRF-154 or CEM cells with ICRF-187 results in enhanced DNA laddering and reduced cell viability (Kizaki and Onishi, 1997; Khelifa and Beck, 1999). These types of topoII catalytic inhibitors may thus serve as useful tools to elucidate the biochemical mechanisms involved in the G_2/M checkpoint and apoptotic cell death pathways in response to abnormal chromosomal processing.

ICRF-187, originally developed as an antitumor agent (Creighton et al., 1969), is now used for the protection of cells against doxorubicin-induced cardiotoxicity (Speyer et al., 1992) and is a powerful nontoxic protector against VP-16-induced toxicity in treatment of brain tumors and metastases (Holm et al., 1996). To better understand the mechanisms of cellular response to bisdioxopiperazines, we selected human leukemic CEM cells for resistance to ICRF-187 and biochemically and pharmacologically characterized some of the ICRF-187-resistant clones. In the present study, we characterized two of our novel ICRF-187-resistant cell lines in terms of their drug responsiveness, karyotypes, DNA-topoII complex-forming and -decatenation activities, topoIIα expression levels, and cell cycle and cell death responses. We report herein that the resistance of these cells differs from that of previously reported ICRF-187-resistant Chinese hamster cell lines (Hasinoff et al., 1997; Sehested et al., 1998) and ICRF-187-resistant human small-cell lung cancer cells (Wessel et al., 1999) and is associated with increased topoIIα protein levels and altered G_2/M checkpoint and apoptotic responses.

Materials and Methods

Cell Lines and Drugs. Human leukemic CEM cells, a merbarone-resistant subline CEM/B1 (Kusumoto et al., 1996), and the novel ICRF-187-resistant sublines CEM/ICRF-8 and CEM/ICRF-18 were cultured in minimal essential medium for suspension cells (BioWhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS; Sigma Chemical Co., St. Louis, MO), and 2 mM L-glutamine (Life Technologies, Gaithersburg, MD). All cell lines were incubated at 37°C in a humidified chamber containing 5% CO_2/95% air. ICRF-187-resistant cells were selected from the original parent CCRF-CEM cell line by continuous incubation with increasing concentrations of ICRF-187, generously provided by Pharmacia and Upjohn (Kalamazoo, MI) and Dr. Ellen Friche (Riggs Hospital, Copenhagen, Denmark). Forty-four CEM/ICRF resistant clones were isolated from the polyclonal populations at different concentrations of ICRF-187 by limiting dilution using 96-well plates. Two of these 44 CEM/ICRF clones that retain resistance in the absence of ICRF-187 were used for further characterization. Other drugs, including VM-26, VP-16, 7-ethyl-10-hydroxycamptothecin (SN-38), CPT, merbarone, and aclarubicin, were obtained from sources described previously (Kusumoto et al., 1996). ICRF-193 was provided by Dr. John Nitiss (St. Jude Children's Research Hospital, Memphis, TN) and vinblastine was from either Sigma or Eli Lilly and Co. (Indianapolis, IN).

Growth Inhibition and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) Assays. For growth inhibition assays, exponentially growing cells were incubated with several different concentrations of drug for 48 h. The ratio of drug-treated to control cells was determined, and the concentration of drug required to inhibit cell growth by 50% (IC_{50}) was calculated. Fold-resistance was calculated by dividing the IC_{50} value of the resistant cells by the IC_{50} value of the drug-sensitive parental cell line. Growth inhibition of CEM cells and their drug-resistant sublines was shown to correlate with clonogenic survival (Kusumoto et al., 1996).

ICRF-187-induced cytotoxicity was also measured by the MTT assay. Exponentially growing cells (CEM, CEM/ICRF-8, and CEM/ICRF-18) were plated at 500 cells/well in 96-well microtiter plates (100 μl/well). ICRF-187 was added to the cells at various concentrations in a final volume of 200 μl/well, and the cells were incubated at 37°C for 96 h. After drug exposure, 25 μl of MTT compound (Sigma; 4 mg/ml in SMEM without FBS) were added to each well and the cells were incubated at 37°C for 4 h. The plates were centrifuged in a swinging bucket rotor (1000*g*, 5 min), and the cells were incubated with 200 μl of dimethyl sulfoxide for 15 min at 25°C. The metabolic activity of the cells was measured by quantifying the conversion of the yellow MTT to a purple metabolite, MTT-formazan. Absorbance was read at 540 nm using a microplate reader. Four replicates were measured for each drug concentration and the experiments were done in triplicate. The IC_{50} value was calculated as the concentration of drug that killed 50% of the cells.

Chromosome Analysis. Karyotype analysis was performed as described previously (Kusumoto et al., 1996). Approximately five metaphases were examined for each cell line and the karyotype was written according to the International System for Human Cytogenetic Nomenclature (ISCN 1995).

DNA-Protein Complex Formation Assay in Intact Cells. TopoII-DNA covalent complex formation in intact cells was measured as described previously (Kusumoto et al., 1996). Briefly, cellular DNA and protein were labeled by incubating exponentially growing cells at 37°C with [^{14}C]leucine (0.2 μCi/ml) and [^{3}H]thymidine (0.6 μCi/ml) for approximately 16 to 24 h. The labeled cells were then incubated with various concentrations of VP-16 for 30 min, after which time the cells were disrupted, the DNA sheared, and DNA-topoII complexes measured as described previously (Kusumoto et al., 1996). To examine the effect of ICRF-187 on VP-16-mediated complex formation, cells were pretreated with ICRF-187 at the indicated concentrations for 2 h before the addition of VP-16. Results are expressed as the ratio of ^{3}HDNA to ^{14}C-protein, using the counts of protein precipitated as the internal control for all samples.

Decatenation Activity Assay. TopoII catalytic activity was analyzed in nuclear cell extracts by decatenation of kinetoplast (k) DNA using a topoII activity assay kit according to the manufacturer's instructons (TopoGEN, Columbus, OH). Briefly, appropriate dilutions of nuclear cell extracts were incubated with 200 ng of kDNA in a 1× reaction buffer (50 mM Tris-HCl, pH 8.0, 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM ATP, and 30 μg of BSA/ml) for 30 min at 37°C. The reactions were terminated with 0.1 volume stop buffer (0.025% bromphenol blue, 50% glycerol) and the decatenation products were separated in a 1% ethidium bromide-stained agarose gel at 100 V.

Western and Northern Blot Analysis. Whole-cell lysates or nuclear cell extracts were prepared from logarithmically growing cells (5×10^5 cells/ml) as described previously (Mo and Beck, 1997).

Proteins (50 μg/well) were separated in 7.5% SDS-polyacrylamide gels, electrophoretically transferred onto nitrocellulose, and incubated with either antiserum Ab-284, specific to the amino terminus of human topoIIα (Boege et al., 1995), or a polyclonal anti-poly(ADP-ribose) polymerase (PARP) antibody (Upstate Biotechnology, Lake Placid, NY). Bound antibody was detected using the enhanced chemiluminescence detection method (Amersham, Arlington Heights, IL) according to the manufacturer's instructions. Autoradiographic signals were quantified by densitometric scanning using a GS-700 Imaging Densitometer and Molecular Analyst Software (Bio-Rad, Hercules, CA).

Total RNA derived from the parental and drug-resistant cells was isolated using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. Total RNA (30 μg/lane) was separated in a 1.2% formaldehyde-denatured agarose gel and transferred to a Hybond membrane (Amersham). The membrane was hybridized using a previously constructed topoIIα probe (Tsai-Pflugfelder et al., 1988) that was labeled with [α-^{32}P]dCTP using the random prime II kit (Stratagene, La Jolla, CA). Total RNA content was quantified using a GS-700 Imaging Densitometer (Bio-Rad).

Luciferase Reporter Assay. Transient transfections were performed using plasmid p557 (Wang et al., 1997) that contains the full-length topoIIα promoter (nucleotides −577 to +90) (Hochhauser et al., 1992) subcloned upstream to the luciferase reporter gene in a pGL2-Basic vector (Promega, Madison, WI). The p557 luciferase plasmid was cotransfected with pSV-β-galactosidase control vector (Promega) to normalize for the transfection efficiency. DNA was introduced into the cells by electroporation using the Gene Pulser II apparatus with an extender (Bio-Rad), according to the manufacturer's instructions. After electroporation, the cells were incubated for approximately 17 h at 37°C and cell extracts were prepared using a 1× reporter lysis buffer (Promega). Luciferase activity was measured by a luminometer with an auto-injector (Model TD-20/20; Turner Designs, Sunnyvale, CA). Luciferase activities were normalized to β-galactosidase activities.

Nucleotide Sequencing. Polymerase chain reaction (PCR) amplification of the topoIIα promoter region (nucleotides −577 to +90) was performed as described previously (Mo et al., 1997) using a commercial kit (Ampli-*Taq*; Perkin-Elmer Corp., Foster City, CA). For amplification of defined regions within the topoIIα cDNA, single-stranded topoIIα cDNAs were synthesized from total RNA (5 μg) derived from CEM, CEM/ICRF-8, and CEM/ICRF-18 cells using Superscript II Reverse Transcriptase (RT; Life Technologies) in the presence of 3′ gene-specific primers (described below). After the RT reaction, cDNAs were PCR-amplified according to standard conditions (Danks et al., 1993). Primer sequences used for the PCR-amplification of motif B/dinucleotide binding site (nucleotides 1318–1603) as well as the tyrosine 805 active site (nucleotides 2264–2518), have been described previously (Danks et al., 1993). Primer sequences used for the PCR-amplification of the proximal amino-terminal region of topoIIα (nucleotides 1–165) were the following: topoIIα-5.1 (5′-ACCATGGAAGTGTCACCATTGCA) and topoIIα-3.1 (5′-GGTGGATCCAGCAATATCAT). PCR clones were sequenced by the dideoxy chain termination method with the Sequenase kit version 2 (Amersham) using universal, reverse, or specific primers when necessary. The sequences obtained from the PCR clones were compared with the published sequence of the human topoIIα cDNA (Tsai-Pflugfelder et al., 1988).

Cell Cycle Analyses. Cellular DNA content and cell cycle distribution were assessed by propidium iodide labeling. Approximately 1 × 10^6 cells (untreated or treated with drug) were harvested, washed with 1× PBS pH 7.4, fixed in 1 ml of 70% methanol, and incubated on ice for 30 min. The fixed cells were centrifuged (1200*g*, 5 min at 4°C), resuspended in 800 μl of 1× PBS, 200 μl of propidium iodide (0.1 mg/ml), and 5 μl of RNase (10 mg/ml) (Sigma), incubated in the dark (25°C, 30 min), and analyzed by flow cytometry. All cells were analyzed on a Becton Dickinson FACScan flow cytometer and the percentage of cell cycle distribution was determined by either the CellQuest or MODFIT programs (Verity Software House, Topsham, ME).

DNA synthesis was assessed by measuring the incorporation of BrdU and flow cytometric analysis. Approximately 0.5 to 1 × 10^6 cells were pulse-labeled with 30 μM BrdU (Sigma) for 1 h at the selected times after drug treatment and then fixed in 70% methanol. The methanol-fixed cells were resuspended in 1 ml of 2 N HCl/0.2 mg pepsin/ml for 30 min at 25°C, after which the suspension was neutralized with the addition of 3 ml of 0.1 M sodium tetraborate, pH 8.5. Cells were washed once with 1× washing buffer (0.01 M HEPES, 0.15 M NaCl, 4% FBS, 10% sodium azide, and 0.5% Tween 20). Replicative DNA synthesis was detected by staining the 5-bromo-2-deoxyuridine (BrdU)-containing cells with 0.1 ml of fluorescein isothiocyanate (FITC)-conjugated anti-BrdU antibody (Becton Dickinson, San Jose, CA) (1:5 in washing buffer, 30 min, 4°C). For simultaneous analysis of replicative DNA synthesis and DNA content, the cells were also treated with 50 μg of RNase/ml and 25 μg of propidium iodide/ml as described above.

Detection of Apoptosis. Drug-induced PARP cleavage in the drug-sensitive and -resistant cells was detected by Western blot analysis as described above. Detection of drug-induced programmed cell death was based on a single-step fluorescent labeling and flow cytometry method using the APO-DIRECT Kit (PharMingen, San Diego, CA), according to the manufacturer's instructions. Briefly, at selected times after drug treatment, approximately 3 to 4 × 10^6 cells were washed with 1× PBS (1200*g*, 5 min at 4°C), and incubated in 2.5 ml of 1% paraformaldehyde for 15 min on ice. After re-centrifugation, the cells were washed twice in 5 ml of 1× PBS and the cell pellets were fixed with 2 ml of ice-cold 70% methanol. The methanol-fixed cells were treated with FITC-labeled deoxyribonucleotide triphosphates (FITC-dUTP) (PharMingen) in the presence of the terminal deoxynucleotidyltransferase enzyme (PharMingen), which catalyzes the incorporation of FITC-dUTP onto the 3′-hydroxyl ends of DNA fragments. Cells were stained with propidium iodide and analyzed for total DNA content on a Becton Dickinson FACScan.

Results

Drug Responsiveness of ICRF-187-Resistant Cells. Growth inhibition and/or cell cytotoxicity induced by topoI or topoII inhibitors or by vinblastine was determined for CEM, CEM/ICRF-8, and CEM/ICRF-18 cell lines. By a growth inhibition assay, the IC_{50} values of ICRF-187 for CEM/ICRF-8 and CEM/ICRF-18 could only be estimated to be greater than 200 μM and 400 μM, respectively, compared with an IC_{50} value of 10 μM for the parental CEM cells (S.E.M., R.S.C., and W.T.B., unpublished observations). For reasons not yet clear, the growth inhibition curves for the ICRF-187-resistant cell lines seemed to reach plateau at about 60 to 70% of control even at high concentrations of drug (>1.0 mM ICRF-187). The survival curves drop to near zero, however, when assessing these same treated cells for cytotoxicity using the trypan blue exclusion method (data not shown). Therefore, to more accurately define the level of resistance of these cell lines to ICRF-187, an MTT assay was performed. According to this cytotoxicity assay, and as shown in Table 1, the IC_{50} values of ICRF-187 for CEM/ICRF-8 and CEM/ICRF-18 were 250 and 466 μM, respectively, compared with an IC_{50} value of 6.8 μM for CEM. The values for CEM were in close agreement to those estimated from the growth inhibition assays (S.E.M., R.S.C., and W.T.B., unpublished observations). We conclude that CEM/ICRF-8 is 40-fold resistant and CEM/ICRF-18 is 69-fold resistant to ICRF-187. In addition to ICRF-187, clones 8 and 18 were resistant to its analog, ICRF-193, by 12- and 67-fold, respectively. These CEM/ICRF cell

lines were significantly resistant to these bisdioxopiperazines, as determined by Student's *t* test.

We determined by the use of a growth inhibition assay, however, that these clones were not cross-resistant to other catalytic inhibitors of topoII such as merbarone and aclarubicin. For the complex-stabilizing topoII inhibitors, CEM/ICRF-8 showed statistically significant collateral sensitivity to VP-16 and CEM/ICRF-18 demonstrated statistically significant collateral sensitivity to the topoI inhibitor camptothecin (CPT) and to the microtubule inhibitor vinblastine. These data indicate that cellular changes that occurred during the selection process are distinct from those responsible for resistance to other catalytic inhibitors of topoII (merbarone and aclarubicin) and from complex-stabilizing agents such as VP-16 and VM-26. Furthermore, these drug-resistant clones seem to have different phenotypes.

Chromosome Analysis. The doubling times of CEM and each CEM/ICRF-187-resistant subline were all approximately 16 h. In our observations, however, the drug-resistant cells seemed to reach plateau at a higher cell density compared with the parental CEM cells (data not shown). According to karyotype analysis (Fig. 1A), all cell lines were near tetraploid (containing approximately 94 chromosomes), and exhibited common, multiple chromosomal abnormalities, most of which have been documented in earlier studies for drug-resistant CEM sublines (Beck et al., 1987; Kusumoto et al., 1996). In comparing the present cell lines, no major variables were present except for CEM/ICRF-8, which, in addition to containing two to three distinct markers, also had a loss of an extra X chromosome. The karyotypes, determined from a total of five metaphases from each cell line, were, for CEM- and CEM/ICRF-18: 94, XXX, −X, dup(1)(p32p36), −8, −8, add(9)(p22)x2, +14, +14, +20, +20, +mar1; and for CEM/ICRF-8: 94, XX, −X, **−X**, dup(1)(p32p36), −8, −8, add(9)(p22)x2, +14, +14, +20, +20, +mar1, +**mar2**.

VP-16-Induced DNA-Protein Complex Formation is Increased in ICRF-187-Resistant Cells. To assess the activities of topoII in these novel ICRF-resistant cell lines, we measured the complex-stabilizing activity of topoII in intact cells after treatment with VP-16. Without drug treatment, the basal levels of complex formation in the resistant cell lines were similar to those seen in the CEM cells, whereas treatment with 100 μM VP-16 revealed an increase in the number of DNA-protein complexes formed in these cells (Table 2). CEM/ICRF-8, in particular, formed 3.3-fold more DNA-protein-cleavable complexes compared with the CEM cell line after treatment with 100 μM VP-16. This increase was statistically significant as determined by the Student's *t* test. This result is consistent with the fact that this clone is collaterally sensitive to VP-16 (Table 1). By contrast, the VP-16-induced DNA-protein complexes in clone 18 were approximately the same as those seen in the parental CEM (~2-fold increase), suggesting clonal variability in the resistant cell lines. As a control, the number of DNA-protein complexes stabilized by VP-16 in the merbarone resistant cell line, CEM/B1, was lower compared with the number formed in the CEM cells, as reported previously (Kusumoto et al., 1996). This is primarily explained by the fact that CEM/B1 is cross-resistant to VP-16 and contains reduced topoIIα levels (Kusumoto et al., 1996).

Inhibition of VP-16-Stabilized DNA-topoII Complexes by ICRF-187 in the ICRF-Resistant Cells. The effect of ICRF-187 on VP-16-stabilized DNA-topoII complex formation was also studied to determine whether the resistance of our cells may be caused in part by altered drug transport. It has been well documented in several studies that topoII catalytic inhibitors, through selective inhibition of topoII activity, interfere with topoII binding to its DNA substrate, thus preventing epipodophyllotoxins (VP-16, VM-26) from stabilizing complexes between topoII and DNA (Jensen et al., 1990; Chen and Beck, 1993) and can result in the reduction of drug-induced single-strand DNA breaks (Beere et al., 1996). As revealed in Table 3, treatment of CEM and CEM/ICRF-18 cells with VP-16 resulted in a ~2-fold increase in formation of DNA-protein cleavable complexes compared with untreated cells or cells treated with ICRF-187 alone. However, pretreatment of these cells with ICRF-187 resulted in a significant reduction in VP-16-stabilized complex formation (Table 3). Because the extent of inhibition of VP-16-induced complexes by ICRF-187 is the same in both the drug-sensitive and -resistant cell lines, then ICRF-187 probably enters the cell and targets topoII directly in both cell lines alike.

TopoII Decatenation Activity is Increased in ICRF-187-Resistant Cells. Changes in the amount of VP-16-induced DNA-cleavable complex formation in the drug-resistant cells suggest that topoII catalytic activity may also be altered in these cells. The ability of topoII to decatenate

TABLE 1
Sensitivity of CEM and ICRF-187-resistant CEM cells to different drugs

Drug	IC_{50}			Fold Resistance	
	CEM	CEM/ICRF-8	CEM/ICRF-18	Clone-8	Clone-18
		μM[a]			
ICRF-187	6.8 ± 0.7[b]	250 ± 29[b]	466 ± 17[b]	40[c]	69[c]
ICRF-193	0.4 ± 0.1	4.9 ± 1.0	27 ± 4.0	12[c]	67[c]
VM-26	0.04 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	0.8	1.0
VP-16	0.46 ± 0.09	0.24 ± 0.01	0.34 ± 0.06	0.5[c]	0.7
Merbarone	13.5 ± 1.3	11.2 ± 2.9	12.2 ± 2.7	0.8	0.9
Aclarubicin	0.16 ± 0.05	0.12 ± 0.03	0.09 ± 0.03	0.8	0.6
SN-38	1.7 ± 0.3[d]	1.8 ± 0.6[d]	1.0 ± 0.4[d]	1.1	0.6
CPT	2.8 ± 0.04[d]	2.4 ± 0.54[d]	1.5 ± 0.12[d]	0.9	0.5[c]
Vinblastine	2.8 ± 0.3[d]	2.7 ± 0.4[d]	1.5 ± 0.2[d]	1.0	0.5[c]

SN-38, 7-ethyl-10-hydroxycamptothecin.
Data are presented as mean ± S.E. of at least three independent experiments.
[a] As determined from 48-h growth inhibition assays.
[b] As determined from the MTT assay.
[c] $P \leq .05$ by Student's *t* test.
[d] Expressed in nanomolar range.

kDNA in the ICRF-resistant cell lines was determined after incubation of nuclear extracts derived from CEM, CEM/ICRF-8, or CEM/ICRF-18 cells with kDNA. TopoII, but not topoI, has the ability to decatenate kDNA, generating monomeric DNA, which can be either in a nicked or closed circular form but not in a linear DNA form (Fig. 2). As shown in Fig. 2, nuclear extracts (500 ng) derived from ICRF-187-resistant cells contain more topoII activity (~75–95% decatenated kDNA) compared with an equal amount of extract derived from the parental CEM cells (only ~5–10% decatenated kDNA), suggesting that ICRF-resistance is also associated with enhanced catalytic activity.

Increased Levels of TopoIIα Protein and mRNA in ICRF-187-Resistant Cells. We examined the levels of topoIIα protein in the ICRF-187 resistant cells using an antibody that is specific to the amino terminus of topoIIα (Ab 284) (Boege et al., 1995). Figure 3A shows a representative immunoblot of topoIIα in drug-sensitive and -resistant CEM cells. As determined in at least five independent experiments, the topoIIα protein levels (170-kDa band) were increased ~5- and 3-fold in CEM/ICRF-8 and CEM/ICRF-18, respectively, compared with the CEM parental cell line (Fig. 3A). Moreover, in addition to the full-length 170-kDa topoIIα protein, several other isoforms of topoIIα have been detected in total cellular extracts of CEM cells and have been shown to be alternatively spliced transcripts (Mo and Beck, 1997) (Fig. 3A). All isoforms are expressed at higher levels in the ICRF-resistant cells compared with CEM. As reported previously and serving as a control, expression of topoIIα in the merbarone-resistant cell line CEM/B1 was decreased to ~40% of that in the CEM cells (Kusumoto et al., 1996). Both topoI and topoIIβ protein levels in the ICRF-resistant cells remained unchanged compared with the CEM cells (data not shown).

To determine whether the increased levels of topoIIα protein are caused by enhanced levels of topoIIα mRNA, total RNA was extracted from CEM and the ICRF-187 resistant clones, and analyzed by Northern blotting. Representative expression levels of topoIIα mRNA in the drug-resistant and -sensitive cell lines are shown in Fig. 3B. Unlike the topoIIα immunoblot, alternatively spliced transcripts are not seen in Fig. 3B because the cDNA probe we used recognizes the 3′ region of topoIIα that is deleted in these splice variants. Compared with the CEM control, ICRF-resistant cell lines contained ~1.5- to 2.2-fold increased topoIIα mRNA, with CEM/ICRF-8 containing the highest levels (2.2-fold increase). As previously reported, the merbarone-resistant cell line (CEM/B1) expressed approximately 50% less topoIIα mRNA than CEM (Fig. 3B) (Mo et al., 1997).

To determine whether the increased levels of topoIIα mRNA may be caused by transcriptional up-regulation, basal

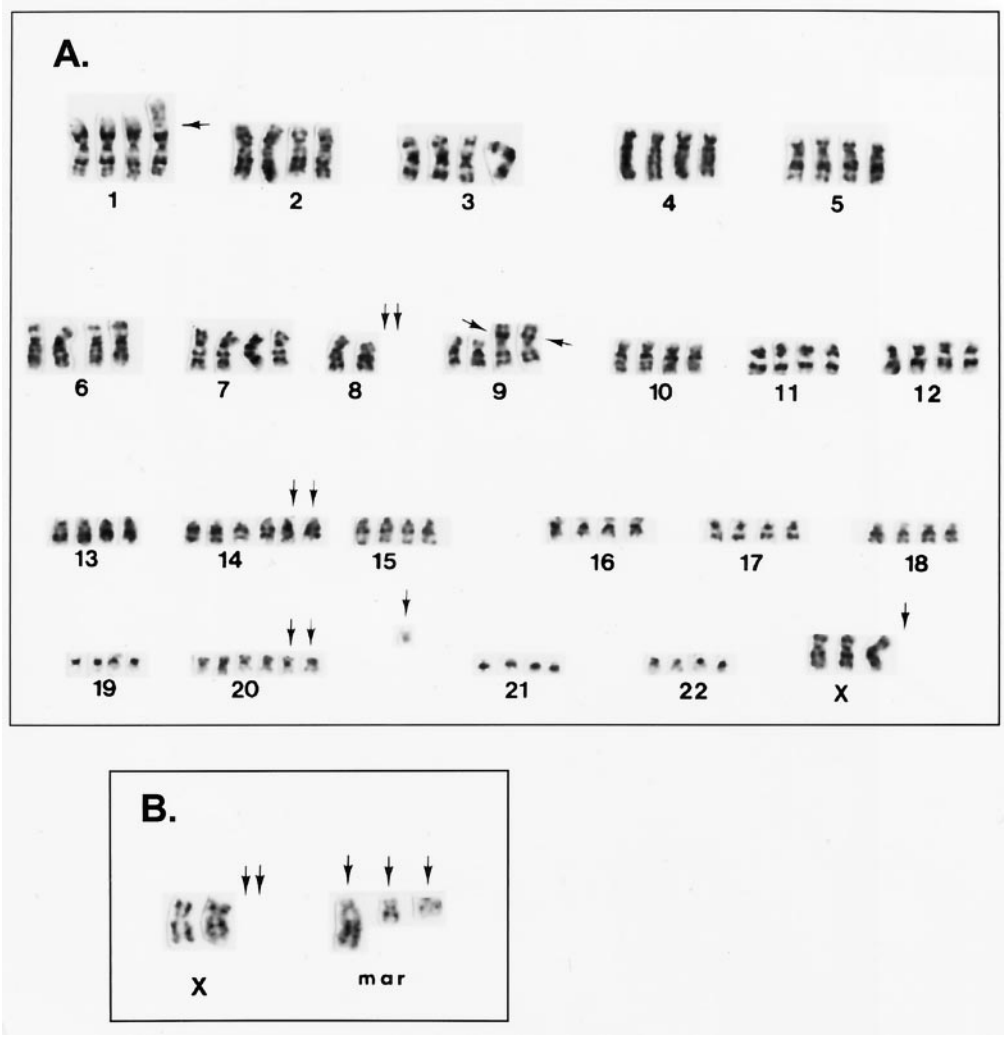

Fig. 1. Karyotypes of CEM and CEM/ICRF-resistant cells. A, representative karyotype of the drug-sensitive CEM and drug-resistant CEM cell lines: 94, XXX, −X, dup(1) (p32p36), −8, −8, add(9)(p22)x2, +14, +14, +20, +20, +mar1. Shown is the karyotype for CEM, which is identical with that of CEM/ICRF-8 and CEM/ICRF-18. Arrows indicate consistent structural or numerical chromosomal abnormalities. B, CEM/ICRF-8 cell line containing, in addition, two distinct chromosomal changes not seen in the other cell lines: loss of an X-chromosome and distinct markers.

topoIIα promoter activity was also analyzed by transfecting a full-length topoIIα promoter-luciferase reporter construct into drug-sensitive and -resistant cells and testing for luciferase activity. Transient transfection experiments revealed that CEM/ICRF-8 and CEM/ICRF-18 cells expressed approximately 3.5-fold and 1.3-fold more promoter activity, respectively, than the parental CEM cells (Fig. 3C). CEM/ICRF-8, in particular, expressed statistically significantly higher luciferase activity than any of the other cell lines examined, suggesting that the up-regulation of topoIIα in this clone occurs at the transcriptional level. Interestingly, although topoIIα protein is increased in clone −18, this may be caused by factors other than or in addition to transcriptional activation (e.g., increased stability or half-life of mRNA).

Sequence Analysis of the TopoIIα Promoter and cDNA. Sequence analysis of the full-length topoIIα promoter from CEM/ICRF-8 and CEM/ICRF-18, compared with the promoter sequence of the CEM parental cells, revealed no mutations (data not shown). Similarly, sequence analysis of conserved regions of the topoIIα cDNA derived from each drug-resistant cell line, which included the motif B/dinucleotide binding site (amino acids 436–522 sequenced) and the catalytic Tyr805 region (amino acids 755–840 sequenced) (Danks et al., 1993) also revealed no mutations compared with the published sequence (Tsai-Pflugfelder et al., 1988). Recent studies have also described a point mutation found in the proximal amino-terminal region of topoIIα in a Chinese hamster ovary cell line selected for resistance to ICRF-159 (Sehested et al., 1998). In our ICRF-187-resistant cell lines, however, this amino-terminal region (amino acids 1–55 sequenced) was wild-type compared with the CEM parental cells. In summary, our data suggest that the resistance of these cells to ICRF-187 is unique and does not depend on changes in the highly conserved areas of the topoIIα cDNA sequence.

TABLE 2

VP-16-stabilized DNA-protein complex formation in drug-sensitive and -resistant cell lines. The number of complexes is expressed as the ratio of ^{3}H-labeled DNA to ^{14}C-labeled protein (see text for details)

Cell Line	Number of DNA-Protein Complexes		
	No drug	100 μM VP-16	Fold increase
CEM	1.6 ± 0.1	3.4 ± 0.4	2.0
CEM/B1	1.3 ± 0.2	2.0 ± 0.2	1.5
CEM/ICRF-8	1.4 ± 0.1	4.6 ± 0.4	3.3[a]
CEM/ICRF-18	1.9 ± 0.1	3.6 ± 0.1	2.0

Data are presented as mean ± S.E. of at least three independent experiments each.
[a] $P \leq .05$ by Student's t-test.

TABLE 3

VP-16-stabilized DNA-protein complex formation in drug-sensitive and -resistant cell lines. The number of complexes is expressed as the ratio of ^{3}H-labeled DNA to ^{14}C-labeled protein (see text for details)

Cell Line	Number of DNA-Protein Complexes			
	No drug	ICRF-187	VP-16	ICRF-187 + VP-16
CEM	1.1 ± 0.2	1.1 ± 0.2	1.9 ± 0.1	1.4 ± 0.1[a]
CEM/ICRF-18	1.0 ± 0.2	1.2 ± 0.2	1.8 ± 0.2	1.4 ± 0.1[a]

Data are presented as mean ± S.E. of at least three independent experiments each.
[a] $P \leq .05$ by Student's t-test for cells treated with ICRF-187 + VP-16 compared with cells treated only with VP-16.

Effect of Different Classes of TopoII Inhibitors on Cell Cycle Distribution in ICRF-187-Resistant Cells. The increased levels of catalytically active topoIIα in the ICRF-187-resistant cells suggest that they could affect the cell cycle and the response of the cells to drugs. Grossly, the doubling time of the resistant cells was the same as that of the sensitive cells (~16 h). Moreover, the distribution of logarithmically growing cells was the same for the drug-resistant and -sensitive cells alike: G_1, 39.8 ± 2.2%; S, 44.7 ± 1.6%; G_2/M, 9.4 ± 1.4%. However, differences were seen in the cell cycle distribution after drug treatment. We treated CEM, CEM/ICRF-8, and CEM/ICRF-18 cells with either catalytic inhibitors (ICRF-187 or merbarone) or the DNA-topoII-stabilizing drug VM-26 and analyzed the cell cycle distribution at several time points post-treatment by flow cytometric analysis of DNA content. Thus, treatment of drug-sensitive and -resistant cells with 0.15 μM VM-26 (Fig. 4), or 30 μM merbarone (Fig. 5), resulted in a drug-induced G_2/M cell cycle checkpoint arrest by 24 h. Mitotic index measurements of drug-treated CEM cells revealed that the drug-induced block was either at G_2 or before the start of prometaphase (Chen and Beck, 1993). Treatment with VM-26 blocked more than 85% of the cells in the G_2/M phase of the cell cycle within 18 to 24 h and was associated with a sub-2N DNA (i.e., containing less than 2N DNA content) peak, indicative of apoptotic cells (Fig. 4). This observation is consistent with the fact that VM-26, a DNA damaging drug, arrests cells in G_2, thereby targeting them for cell death (Solary et al., 1993). Treatment with merbarone blocked ~90% of the cells in the G_2/M phase by 24 h (Fig. 5). A small percentage (10%) of these G_2/M-blocked cells began a second round of DNA replication without dividing; this is represented in Fig. 5 by the 8N (polyploid) DNA content apparent in all cell lines by 24 h post-treatment. This is a common effect that has been reported previously (Chen and Beck, 1993; Gorbsky, 1994) and seems to be unique to catalytic inhibitors of topoII.

In contrast to VM-26 and merbarone, the effect of ICRF-187 on cell cycle distribution of the drug-resistant cells was

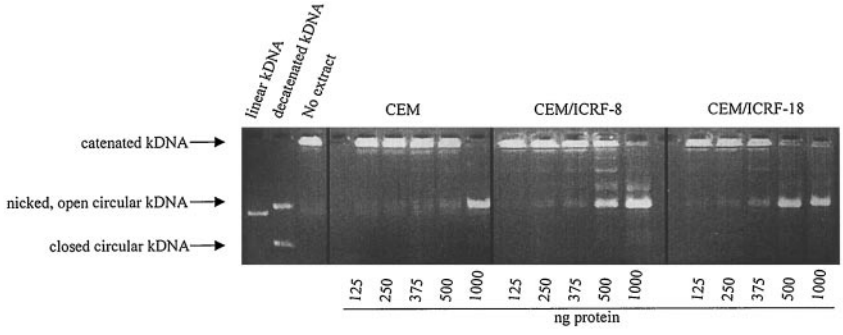

Fig. 2. TopoII decatenation activity is increased in ICRF-187-resistant cell lines. TopoII catalytic activity in CEM cells and ICRF-187-resistant cells (clones −8 and −18) was determined by decatenation of kinetoplast DNA. Varying amounts of nuclear cell extracts (125–1000 ng) were incubated with kDNA and the reactions were electrophoresed in a 1% ethidium bromide-stained agarose gel as described in *Materials and Methods*. Decatenated DNA is shown by the generation of nicked or closed circular kDNA. This gel is representative of at least three independent experiments.

markedly different. Thus, although approximately 93% of CEM cells exhibited a G_2/M arrest by 14 h in response to an IC_{80} dose of ICRF-187 (55 μM) (Fig. 6), by 48 h, ~50% of the CEM cells escaped the G_2/M block to form an 8N DNA population. Microscopic examination revealed that the ICRF-187-treated CEM cells were enlarged and the cultures had moderate cellular debris by 48 h (data not shown), which is consistent with the observed drug-induced polyploidy (8N) and sub-2N DNA populations (Fig. 6). However, in contrast to the drug-sensitive CEM cells, and also in contrast to the effects of VM-26 and merbarone, treatment of CEM/ICRF-8 and CEM/ICRF-18 with equitoxic IC_{80} doses of ICRF-187 (550 and 800 μM, respectively) failed to result in a complete drug-induced G_2/M cell cycle arrest (Fig. 6). Specifically, CEM/ICRF-8 transiently arrested in the G_2/M phase by 14 h (78% of cells in G_2/M) in response to drug but then re-entered the cell cycle so that by 24 h, approximately 40% of the cells were in the G_1 phase. Interestingly, CEM/ICRF-18 cells completely lacked an ICRF-187-induced G_2/M cell cycle checkpoint and did not have any 8N DNA (Fig. 6), although by 72 to 96 h after ICRF-187 treatment, the drug-resistant cell cultures exhibited extensive cellular debris and cell blebbing, indicative of apoptotic cell death (S.E.M. and W.T.B., unpublished observations). This defect in an ICRF-187-inducible G_2/M checkpoint in the drug-resistant cells is not simply caused by failure of the drug to reach the topoII target, because our data in Table 2 demonstrated that ICRF-187 probably targets topoII directly in both the drug-sensitive and -resistant cells alike.

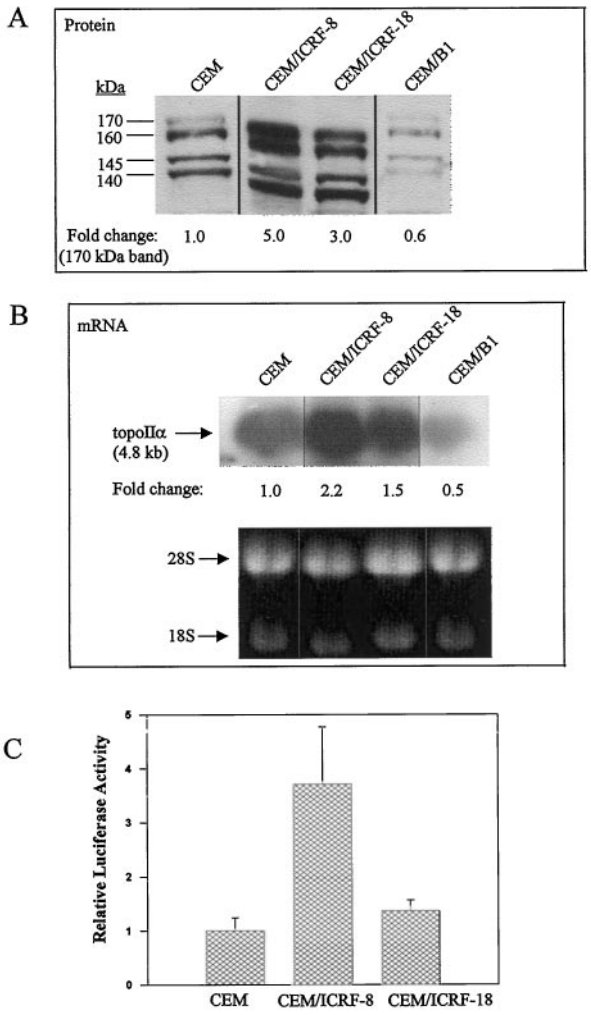

Fig. 3. Increased levels of topoIIα protein and mRNA and enhanced promoter activity in CEM cells selected for resistance to ICRF-187. A, immunoblot analysis of topoIIα was done on drug-sensitive and -resistant cell lines as shown. Nuclear cell extracts were electrophoresed in 7.5% SDS-polyacrylamide gel electrophoresis and topoIIα was detected using Ab-284 as described in *Materials and Methods*. Shown are four topoIIα isoforms (Mo and Beck, 1997) with their respective molecular masses. Equal loading of total cellular protein was determined by blotting for β-tubulin. This Western blot is representative of at least five separate experiments. B, total RNA was extracted from drug-sensitive and -resistant cell lines for Northern blot analysis of topoIIα as described in *Materials and Methods*. The 28-S ribosomal RNA was used for normalization of gel loading. This Northern blot is representative of three independent experiments. C, the full-length topoIIα promoter-luciferase construct along with the pSV-β-galactosidase construct were cotransfected into CEM, CEM/ICRF-8, and CEM/ICRF-18 cells. topoIIα promoter activity was expressed as relative (to β-gal) luciferase activity measured 17 h after electroporation as described in *Materials and Methods*. Data are averages of four independent experiments, each done in duplicate (error bars, S.E.). $P < .05$ for CEM/ICRF-8 compared with CEM.

Effect of ICRF-187 on S-Phase Progression. To better understand the effects of ICRF-187 on cell cycle progression, DNA synthesis was assessed by measuring the incorporation of the thymidine analog, BrdU, into DNA derived from logarithmically growing CEM, CEM/ICRF-8, or CEM/ICRF-18 cells treated with or without equivalent cytotoxic doses of drug. As seen in Figs. 7, A and B, the parental CEM cells, harvested at various times after ICRF-187 treatment, exhibited a marked decrease in the number of cells entering the S-phase by 9 to 12 h, with a corresponding increase in the percentage of cells entering the G_2/M phase (only 0, 12, and 24 h time points shown for Fig. 7A). By 24 h after ICRF-187 treatment, a large percentage of CEM cells, which arrested at G_2/M, escaped the block to re-replicate their DNA and form an 8N DNA population (Fig. 7A). These results are consistent with the flow cytometry data shown in Fig. 6.

The drug-resistant cells, however, exhibited different S-phase synthesis profiles in response to drug treatment. As revealed in Figs. 7A and B, ICRF-187-treated CEM/ICRF-8 cells exhibited a transient decrease in S-phase synthesis with a corresponding increase in G_2/M cells (9–12 h) and, by 24 h, approximately 20% of the cells entered into the 8N DNA population (Fig. 7A). By contrast, CEM/ICRF-18 cells exhibited no significant decrease in the S-phase fraction in response to ICRF-187, nor was there an apparent 8N DNA population (Figs. 7A and B). As a positive control, VM-26 treatment induced a G_2/M arrest in both the drug-sensitive and -resistant cell lines (Fig. 7A). These results indicate that in response to equitoxic doses of ICRF-187, CEM/ICRF-8 cells transiently arrest at G_2/M; with some cells entering into 8N, whereas the CEM/ICRF-18 cells completely lack an ICRF-187-induced G_2/M checkpoint compared with the CEM parental cells.

ICRF-187-Induced DNA Fragmentation and Apoptosis Are Delayed in the ICRF-187-Resistant CEM Cell Lines. CEM, CEM/ICRF-8, and CEM/ICRF-18 cells were measured for apoptotic cell death in response to equivalent cytotoxic doses of ICRF-187 by detection of fluorescence-labeled 3′-hydroxyl ends of DNA fragments as described in *Materials and Methods*. By flow cytometric analysis, ICRF-187-induced DNA fragmentation was detected as increased fluorescence (FITC-dUTP) intensity in both drug-sensitive and -resistant cells (Fig. 8). The timing and amount of drug-

induced DNA fragmentation, however, varied widely between each cell line. In particular, drug-induced DNA fragmentation in the CEM cells was detected as early as 24 h, whereas for the CEM/ICRF-8 and CEM/ICRF-18 cells, ICRF-187-induced apoptosis was not detected until 48 to 72 h or 96 h, respectively (Fig. 8).

In addition to a delay in drug-induced DNA fragmentation, the drug-resistant cells also had a delay in ICRF-187-induced PARP cleavage, a commonly used indicator of apoptosis (Fig. 9). About 40% of cleaved PARP (85 kDa) was observed in the CEM cells at 24 h in response to ICRF-187 (Fig. 9), whereas in CEM/ICRF-18 cells, drug-induced PARP cleavage did not occur until 48 h, and this was only 30%. As a negative control, very little or no PARP cleavage was detected in the untreated cell lines at all time points (only 72 h control shown in Fig. 9). As a positive control, PARP cleavage was detected at 24 h for all cells in response to the DNA-damaging agent VM-26. Results from Figs. 8 and 9 therefore suggest that different mechanisms of ICRF-187-induced apoptotic cell death may exist between the drug-sensitive and -resistant cell lines. The drug-sensitive CEM cells in response to ICRF-187 seem to be targeted relatively quickly for apoptotic cell death, partly because these cells exit from the G_2/M checkpoint without dividing and become polyploid, whereas the ICRF-resistant cells, when treated with equitoxic doses of drug, exhibit a delayed apoptotic response that is independent of a G_2/M checkpoint. In summary, our results suggest that resistance to ICRF-187 in our human leukemic CEM cell lines is associated with increased levels of catalytically active topoIIα and altered G_2/M checkpoint and apoptotic responses.

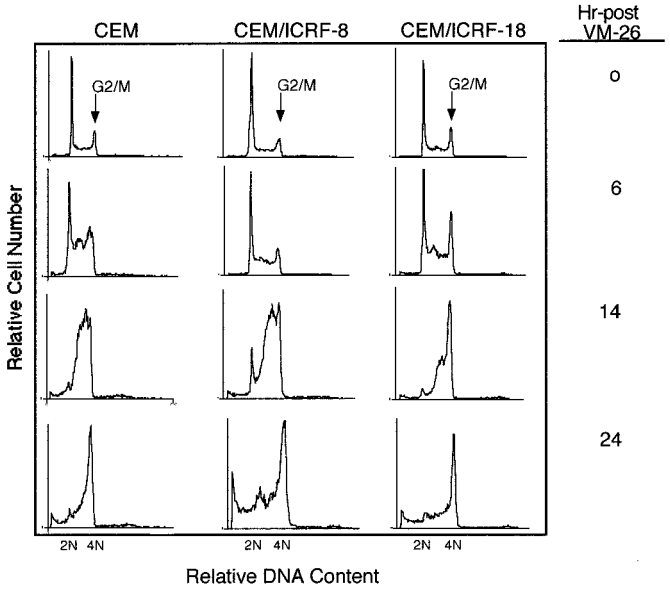

Fig. 4. Induction of G_2/M cell cycle checkpoint by VM-26 in ICRF-187-resistant CEM cells. CEM, CEM/ICRF-8, and CEM/ICRF-18 cells were treated with 0.15 μM VM-26 and then harvested at the indicated times for analysis of DNA content by flow cytometry as described in *Materials and Methods*. Cell cycle populations are characterized as 2N (G_1 phase) and 4N (G_2/M phase). All flow cytometry analyses were performed in at least three separate experiments. Shown are representative histograms from one experiment.

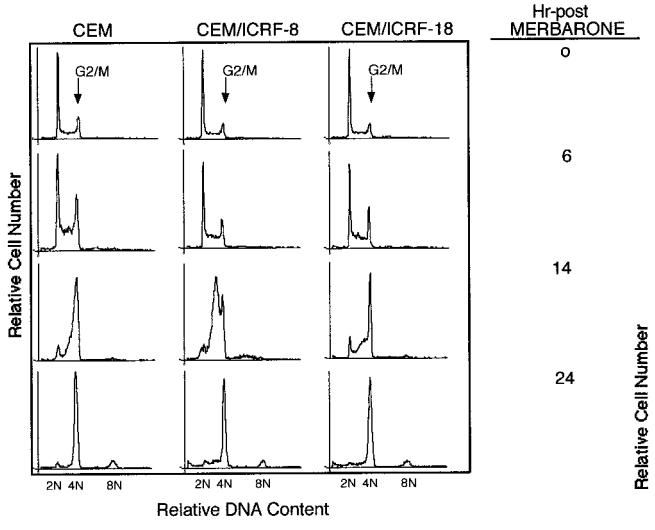

Fig. 5. Induction of G_2/M cell cycle checkpoint by merbarone in ICRF-187-resistant CEM cells. CEM, CEM/ICRF-8, and CEM/ICRF-18 cells were treated with 30 μM merbarone and then harvested at the indicated times for analysis of DNA content by flow cytometry as described in *Materials and Methods*. Cell cycle populations are characterized as 2N (G_1 phase), 4N (G_2/M phase), and 8N (polyploid stage). All flow cytometry analyses were performed in at least three separate experiments. Shown are representative histograms from one experiment.

Discussion

CEM leukemic cells selected for resistance to the topoII catalytic inhibitor ICRF-187 contain higher levels of topoIIα

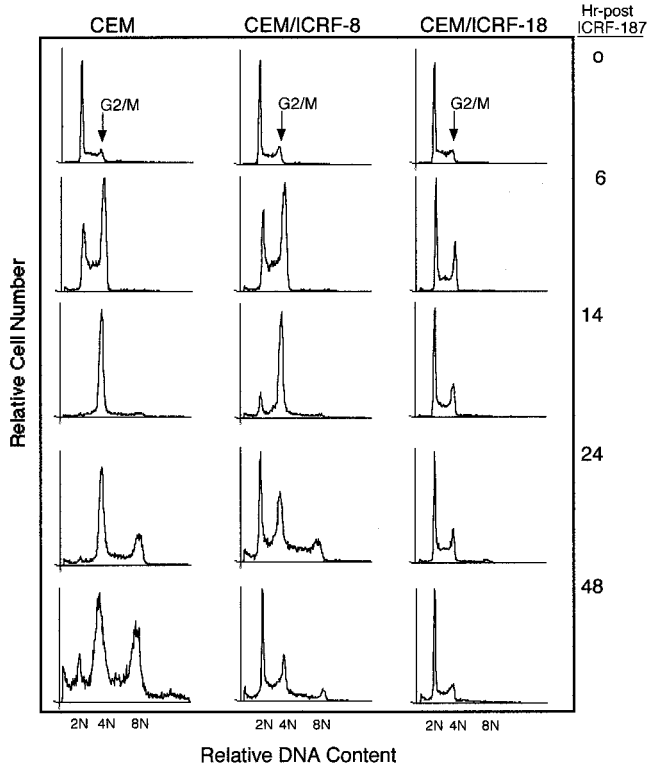

Fig. 6. Effects of the catalytic inhibitor, ICRF-187, on cell cycle distribution in drug-sensitive and -resistant CEM cells. CEM, CEM/ICRF-8, and CEM/ICRF-18 cells were treated with 55 μM, 550 μM, and 800 μM ICRF-187, respectively. After treatment, all samples were harvested at the indicated times for analysis of DNA content by flow cytometry as described in *Materials and Methods*. Cell cycle populations are characterized as 2N (G_1 phase), 4N (G_2/M phase), and 8N (polyploid stage). All flow cytometry analyses were performed in at least three separate experiments. Shown are representative histograms from one experiment.

protein and express increased in vitro topoII catalytic activity compared with the parental cells. To our knowledge, this is the first report of an ICRF-187-resistant cell line that expresses *increased* levels of catalytically active topoIIα protein. These findings are, in fact, in agreement with the mechanistic model of topoII catalytic inhibitors, such as ICRF-187, that bind to the closed clamp form of topoII, rendering topoII inactive and incapable of binding to DNA (for review, see Andoh, 1998). Indeed, topoII protein levels have been found to be inversely correlated with bisdioxopiperazine-induced cell cytotoxicity, supporting the idea that topoII is a significant cellular target for these catalytic inhibitors (Ishida et al., 1995; Andoh, 1998; Andoh and Ishida, 1998). In contrast to our novel findings, however, other studies have shown the opposite correlation: decreased levels of topoIIα protein corresponding with resistance to topoII catalytic inhibitors such

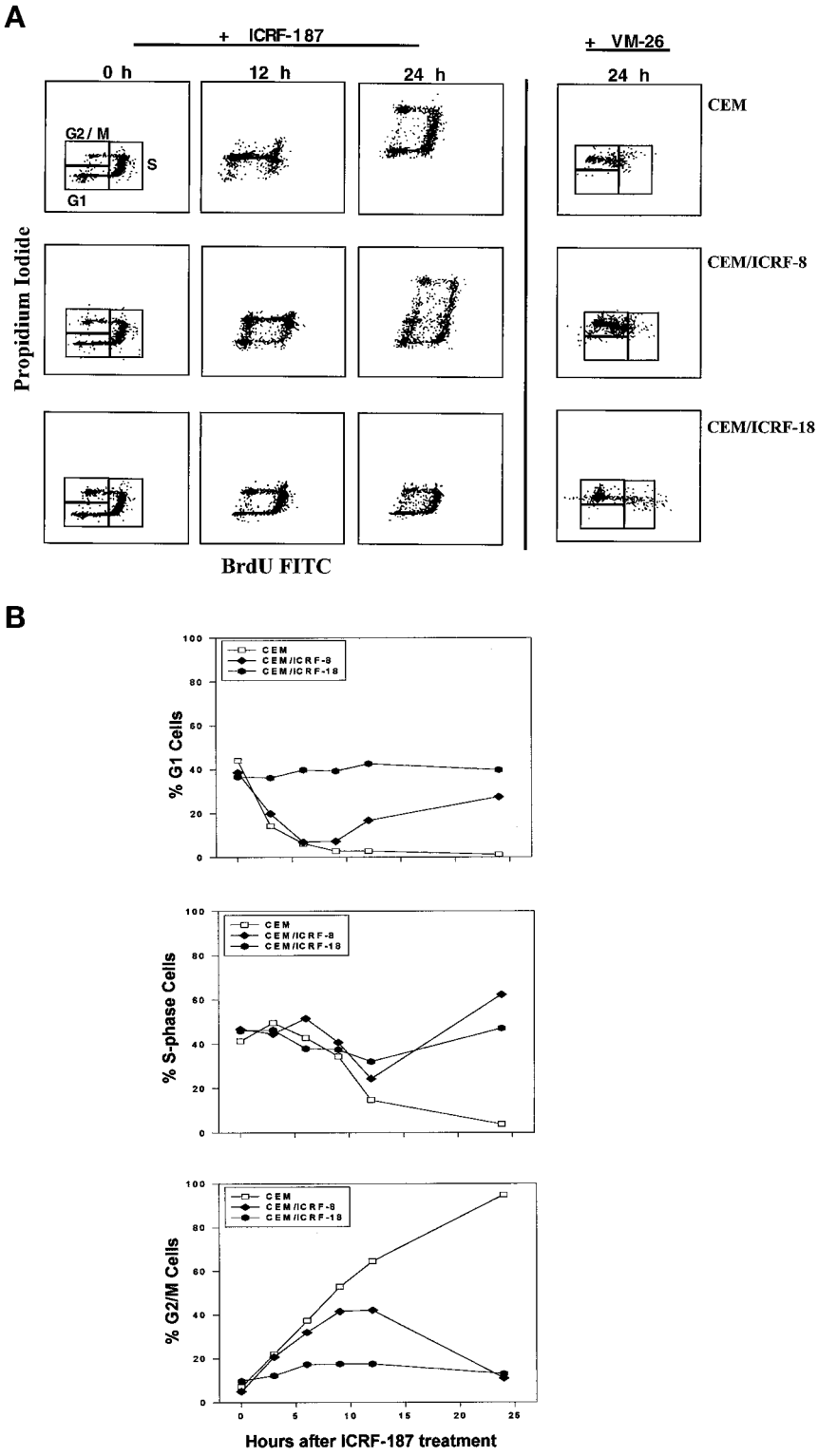

Fig. 7. Cell cycle distribution in drug-sensitive and -resistant CEM cells exposed to ICRF-187 or VM-26. A, CEM, CEM/ICRF-8, and CEM/ICRF-18 cells were treated with 55 μM, 550 μM, and 800 μM ICRF-187, respectively, or with 0.15 μM VM26 and cell cycle distribution was determined at 0, 12, and 24 h after drug treatment by flow cytometry analysis. The flow cytometric dot plots display simultaneous analysis of S-phase DNA synthesis (x-axis), as determined by a 1-h BrdU pulse-labeling of cells at the indicated times after drug treatment, and total DNA content (y-axis), as determined by propidium iodide staining. Cell cycle populations are characterized as G1 (2N DNA content), S-phase (BrdU-incorporation), and G_2/M (4N DNA content). See *Materials and Methods* for details. B, time course of cell cycle progression (% G1, S, or G_2/M cells) in CEM, CEM/ICRF-8, and CEM/ICRF-18 cells after treatment with ICRF-187 as described above. Shown is a representative of at least three separate experiments.

as merbarone (Kusumoto et al., 1996) or ICRF-187 (Hasinoff et al., 1997; Sehested et al., 1998). Such conflicting studies imply that these phenotypes may be cell-type specific and/or that these drugs may have other targets, suggesting the existence of multiple mechanisms of resistance to bisdioxopiperazines that do not necessarily correlate with the catalytic effect of these other types of topoII inhibitors. In fact, increased levels of topoIIα alone in our cell lines seem to be insufficient to account for the pattern of ICRF-187 resistance, because differing phenotypes exist between our drug-resistant clones. These include differences in resistance factors, cross-resistance profiles, topoIIα protein levels, alterations in the cell cycle, and in the delay of drug-induced cell death.

The observation that the bisdioxopiperazine-resistant CEM cells are not cross-resistant to other catalytic inhibitors of topoII (e.g., merbarone and aclarubicin) is not unexpected, given the fact that differential sensitivities to topoII catalytic inhibitors in cells expressing altered topoIIα levels have been reported previously by others (Hasinoff et al., 1997; Sehested et al., 1998). In those studies, a decrease in topoIIα protein correlated with resistance to bisdioxopiperazines but sensitivity to other catalytic inhibitors, such as aclarubicin and merbarone. These data strengthen the argument that in our resistant cells, cellular changes may have been incurred during the selection process that specifically interfere with bisdioxopiperazine-topoII interactions, suggesting that the mechanism of action of bisdioxopiperazines in our CEM cell lines differs from those of other classes of topoII catalytic inhibitors.

As indicated, although both CEM/ICRF-8 and CEM/ICRF-18 are resistant to bisdioxopiperazines, they express clonal variability, in that they have different phenotypes, as shown by their different resistance and cross-resistance profiles, variations in topoIIα overexpression, drug-induced cell cycle arrest, and apoptosis. CEM/ICRF-18 cells, which are the most resistant to ICRF-187 and ICRF-193 but express lower levels of topoIIα protein compared with CEM/ICRF-8, are also collaterally sensitive to the topoI drug CPT and to the microtubule inhibitor vinblastine. Collateral sensitivity to CPT cannot be explained by altered topoI expression, because Western blot analysis showed no changes in total topoI protein levels compared with the parental CEM cells. The basis of collateral sensitivity to the topoI inhibitor CPT is therefore unknown at present, although other studies have reported that cell lines resistant to topoII inhibitors are also hypersensitive to some topoI inhibitors (Tan et al., 1989).

In contrast to CEM/ICRF-18, CEM/ICRF-8 exhibits statistically significant collateral sensitivity to the complex-stabilizing topoII inhibitor, VP-16. This is consistent with the

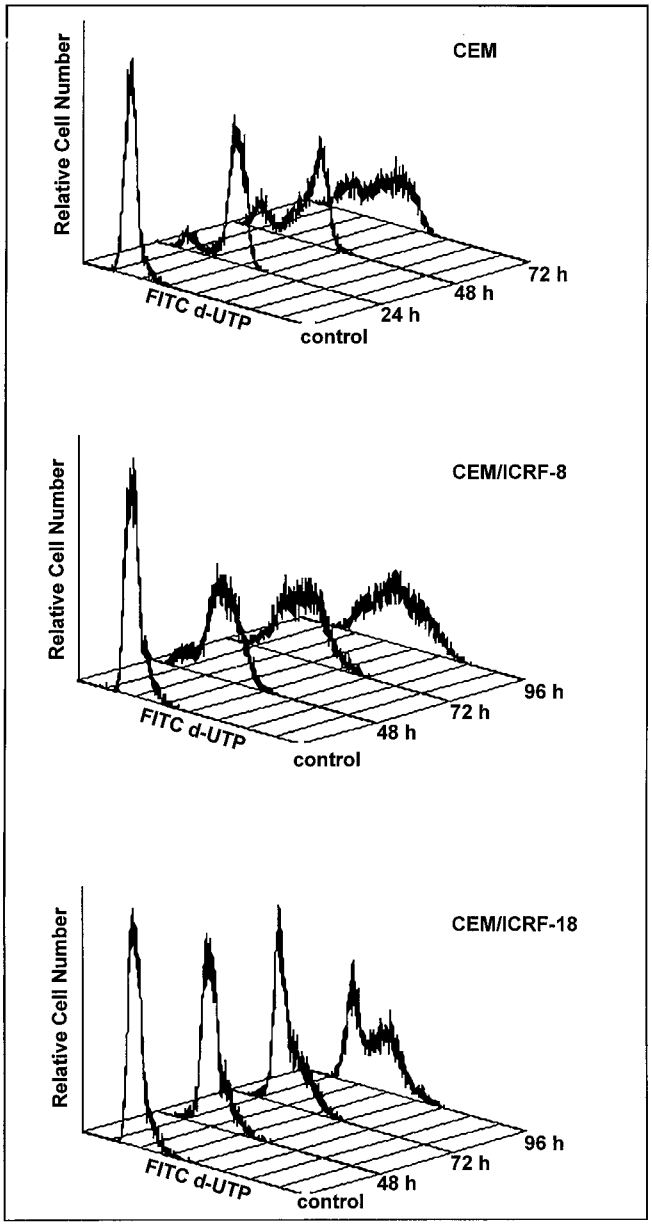

Fig. 8. ICRF-187-induced apoptosis is delayed in the ICRF-187-resistant CEM cell lines. CEM, CEM/ICRF-8, and CEM/ICRF-18 cells were treated with 55 μM, 550 μM, and 800 μM ICRF-187, respectively, after which they were harvested at the indicated times and analyzed for drug-induced apoptotic cell death by flow cytometry analysis as described in *Materials and Methods*. The histograms represent DNA fragmentation, as determined by increased incorporation of FITC-dUTP (x-axis) into DNA fragments of apoptotic cells. All flow cytometric analyses were done in at least three separate experiments. Shown are representative histograms from a single experiment.

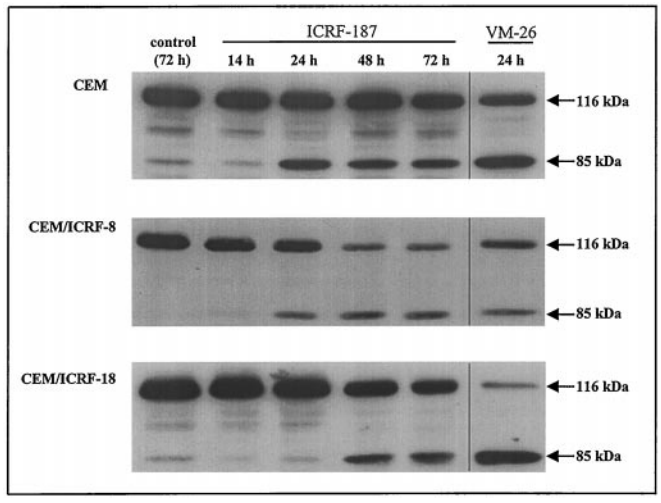

Fig. 9. Effect of ICRF-187 on PARP cleavage in drug-sensitive and ICRF-resistant cells. CEM, CEM/ICRF-8, and CEM/ICRF-18 cells were treated with 1% dimethyl sulfoxide or with 55 μM, 550 μM, and 800 μM ICRF-187, respectively, or with 0.15 μM VM-26 for 14 h, 24 h, 48 h, or 72 h before harvesting for protein as described in *Materials and Methods*. This Western blot is representative of three independent experiments.

observations that this particular clone contains the highest level of topoIIα expression (Fig. 3) and exhibits a significantly higher number of DNA-protein complexes (Table 2) compared with the other ICRF-resistant clones. It is unclear, however, why CEM/ICRF-8 cells are not collaterally sensitive to the complex-stabilizing topoII inhibitor, VM-26, although it is quite clear from our observations that there are other likely mechanisms of action of these topoII inihibitors that we do not yet fully understand. The increase in topoIIα protein levels in the CEM/ICRF-8 cells seems to be regulated at the transcriptional level, because these cells express both a 2.2-fold increase in topoIIα mRNA levels and a statistically significant 3.5-fold increase in topoIIα promoter activity compared with the parental CEM cells. Sequence analysis of the full-length topoIIα promoter in CEM/ICRF-8 revealed no mutations, suggesting that alterations in the expression of *trans*-acting factors may play a key role in regulating topoIIα gene expression. Studies are currently in progress to examine the expression levels of candidate transcription factors and their binding activities on the topoIIα promoter in our ICRF-187-resistant cell lines.

Before ICRF-187-induced apoptotic cell death, CEM/ICRF-8 cells transiently arrest in the G_2/M phase in response to ICRF-187, whereas the CEM/ICRF-18 cells lack a drug-induced G_2/M arrest compared with the drug-sensitive CEM cells. These novel observations suggest once again that multiple mechanisms of resistance to bisdioxopiperazines are likely to exist. Interestingly, these studies differ from another of our studies, which analyzed drug-induced cell cycle profiles in VM-26-resistant cells; VM-26 treatment resulted in a drug-induced G_2/M block in two VM-26-resistant sublines containing a mutated topoIIα (Chen and Beck, 1993). Lack of an ICRF-induced G_2/M arrest in the ICRF-resistant cells suggests that the CEM/ICRF-18 cells may be defective in the uptake and/or delivery of ICRF-187. However, we found no evidence of P-glycoprotein or multidrug resistance-associated protein overexpression in these resistant cells that might account for decreased drug transport (S.E.M. and W.T.B., unpublished observations). Moreover, pretreatment of CEM/ICRF-18 cells with ICRF-187 results in the significant reduction of VP-16-mediated complex formation (Table 3), suggesting that ICRF-187 enters into the drug-resistant CEM/ICRF-18 cells as well as in the parental CEM cells and directly interacts with topoII. If this is the case, then other proteins in these drug-resistant cells may compensate for topoII inactivation by compensatory facilitation of proper chromosome condensation/decondensation of sister chromatids during mitosis. If this were the case, ICRF-187-resistant cell lines may have adapted an alternative mechanism for the proper processing of catenated chromosomes during mitosis.

Changes in the *topoII* gene and/or changes in critical G_2/M checkpoint genes that are affected by ICRF-187 serve as possible explanations for the apparent alterations in the ICRF-187-induced G_2/M cell cycle checkpoint. In terms of bisdioxopiperazine-type drugs affecting the activation of G_2/M checkpoint genes, a study found that fostriecin, a topoII catalytic inhibitor, blocked cell growth at G_2/M through inhibition of a serine/threonine protein phosphatase activity that is necessary for the regulation of centrosome replication (Cheng et al., 1998). In terms of topoIIα, although we have not been able to detect any mutations in the highly conserved areas (motif B/dinucleotide binding site and catalytic Tyr-805 regions) nor in the amino-terminal region (amino acids 1–55), in which a specific topoII mutation was identified to confer specific resistance to bisdioxopiperazines (Sehested et al., 1998), further sequence analysis is needed. Importantly, our finding of no mutations in previously reported regions of topoIIα emphasize the unique nature of our ICRF-resistant cell lines compared with others that have been reported.

In addition to a defect in an ICRF-187-induced G_2/M checkpoint, our bisdioxopiperazine-resistant cells exhibit a delay in ICRF-187-induced apoptosis compared with the drug-sensitive cell line. Programmed cell death in response to the catalytic inhibitor ICRF-187 has been reported in earlier studies (Kizaki and Onishi, 1997), but ours is the first observation of a cell death mechanism induced by ICRF-187 that is independent of an initial G_2/M cell cycle checkpoint. This suggests that at least two different mechanisms of drug-induced cell death exist (i.e., one via G_2/M arrest and the other independent of the G_2/M checkpoint). For the sensitive CEM cells, drug-induced cell death may arise from the inability of the cell to proceed through the cell cycle because of prolonged chromosomal damage and polyploidy. Polyploidization is a result of cells passing from the transient drug-induced G_2/M block into mitosis but failing to divide because of damaged spindles and abnormalities in chromosome segregation (Ishida et al., 1994). Interestingly, the ability of cells to arrest in G_2/M and then bypass this checkpoint to become polyploid is believed to be functionally linked with apoptotic cell death (Kizaki and Onishi, 1997) and could account for differences in drug responsiveness. In contrast, for our ICRF-resistant cells, drug-induced cell death may be the result of compromised cell cycle checkpoints. Thus, the resistant cells would continue to progress through the cell cycle in the presence of drug and, consequently, may slowly accumulate DNA damage until a certain "DNA-damage threshold" is reached, at which time the cells are then targeted for cell death. Such an accumulation of genomic instability coupled with deregulated cell cycle checkpoints has been shown to result in programmed cell death (Bracey et al., 1997). Accordingly, further insight into the differences in signaling pathways that lead to cell death in our drug-sensitive and bisdioxopiperazine-resistant cells should enhance our knowledge of responsiveness to this interesting class of topoII inhibitors.

Acknowledgments

We thank Queen Rodgers (St. Jude Children's Research Hospital) for assistance in the initial selection of the ICRF-187-resistant cell lines, and Dr. F. Boege (University of Würzburg, Germany) for the generous gift of the anti-topoIIα antibody. We also thank Tina Cadre and Mohammed Thalji for their excellent technical assistance in this project. We are grateful to Drs. John Nitiss (St. Jude Children's Research Hospital, Memphis, TN) and Yin-Yuan Mo (Department of Molecular Genetics, University of Illinois at Chicago, Chicago, IL) for helpful advice and critical review of early drafts of the manuscript.

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