**Induction of Apoptosis in MCF-7:WS8 Breast Cancer Cells by β-Lapachone**

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**ABSTRACT**

β-Lapachone (β-lap) affects a number of enzymes in vitro, including type I topoisomerases (Topo I); however, its exact intracellular target(s) and mechanism of cell killing remain unknown. We compared the cytotoxic responses of MCF-7:WS8 (MCF-7) human breast cancer cells after 4-h pulses of β-lap or camptothecin (CPT), a known Topo I poison. A direct correlation between loss of survival and apoptosis was seen after β-lap treatment (LD₅₀ = 2.5 μM). A concentration-dependent, transient sub-2 N preapoptotic cell population was observed at 4–8 h. Estragon deprivation-induced synchronization and bromodeoxyuridine-labeling studies revealed an apoptotic exit point near the G₁-S border. Apoptosis activated by β-lap was closely correlated with cleavage of lamin B but not with increases in p53/p21 or decreases in bcl-2. Loss of hyperphosphorylated forms of the retinoblastoma protein was observed within 5 h, but cyclins A, B1, and E were unaltered for up to 72 h after 5 μM β-lap. Topo I and Topo IIα levels decreased at >24 h. Logarithmic-phase MCF-7 cells were not affected by ≤1 μM β-lap.

In contrast, dramatic and irreversible G₁-M arrest with no apoptosis was observed in MCF-7 cells treated with 1 μM CPT, monitored for 6–10 days posttreatment. MCF-7 cells treated with supralethal doses of CPT (5 μM) resulted in only ~20% apoptosis. No correlation between apoptosis and loss of survival was observed. MCF-7 cells exposed to >5 μM CPT arrested at key cell cycle checkpoints (i.e., G₁-S, G₂-M), with little or no movement for 6 days. Ten-fold increases in p53/p21 and 2–5-fold decreases in bcl-2, Topo I, Topo IIα, and cyclins A and B1, with no change in cyclin E, were observed. Temporal decreases in bcl-2 and cleavage of lamin B corresponded to the minimal apoptotic response observed.

β-Lapachone activated apoptosis without inducing p53/p21 or cell cycle arrest responses and killed MCF-7 cells solely by apoptosis. In contrast, concentration-dependent increases in nuclear p53/p21 and various cell cycle checkpoint arrests were seen in MCF-7 cells after CPT. Despite dramatic p53/p21 protein induction responses, CPT-treated MCF-7 cells showed low levels of apoptosis, possibly due to protective cell cycle checkpoints or the lack of specific CPT-activated apoptotic pathways in MCF-7 cells.

**INTRODUCTION**

β-Lap (3,4-dihydro-2,2-dimethyl-2H-naphthol[1,2-b]pyran-5,6-dione) is a naturally occurring compound that is present in the bark of the Lapacho tree (Tabebuia avellanedae), which is native to South America. The compound has a number of concentration-dependent effects in vivo, including antitumor, antiviral, and antipryanoosomal activities (1–3). β-Lap has significant antineoplastic activity against a variety of human cancer cells, including prostate cancer and promyelocytic leukemia cells (4, 5), and has reported activity against the S-180 mouse tumor line (6).

β-Lap was the first reported catalytic inhibitor in vitro of human DNA Topo I (4, 7, 8), although its exact intracellular target(s) in vivo and mechanism of cytotoxicity remain unknown. β-Lap was identified by our laboratory as a radiosensitizer and chemosensitizer of confluence-arrested as well as log-phase human or rodent cancer cells (7, 9, 10). We demonstrated synergistic cytotoxicity in X-irradiated rodent cells, with concomitantly suppressed neoplastic transformation, presumably involving DNA repair inhibition (9). Using enzyme assays in vitro, β-lap inhibited Topo I by a distinctly different mechanism from other Topo I poisons, such as CPT and its derivatives (4, 8, 11). Additionally, enzyme assays demonstrated in vitro that β-lap could poison the DNA-unwinding enzyme, Topo IIα, resulting in DNA breakage (i.e., DNA DSBs) through DNA-Topo IIα complex formation, similar to other poisons (12). Furthermore, β-lap suppressed the growth of yeast, which lacked Topo I expression, raising doubts about the exact mechanism of action of this compound and suggesting that, in addition to Topo I inhibition, it may have other intracellular targets. However, yeast do not express apoptotic caspases (13) and may not undergo apoptosis, limiting their use as a cytotoxicity model system. Prior data in vivo indicated that exposure of human or hamster cells to β-lap did not cause covalent Topo I-DNA or other protein-DNA complexes, as measured by SDS-K⁺ precipitation assays (8, 11). In addition, exposure of various human or hamster cells to β-lap did not result in single-strand breaks or DSBs, as measured by alkaline or neutral filter elutions, respectively (9). Because the compound acts on growth-arrested as well as log-phase cells (10), its intracellular target is probably not cell cycle regulated, arguing against Topo IIα as a target in vivo (12). These contradictory in vitro and in vivo data underscore the need to determine the exact intracellular target and mechanism of cell killing for β-lap. To date, few studies have directly examined the cytotoxic responses of human cancer cells after β-lap without prior treatment with ionizing radiation. A better understanding of the mechanism of action of β-lap is warranted due to its anticarcinogenic activity and its ability to radiosensitize cells. Because it is possible that the cytotoxic (antitumor, antiviral, and antipryanoosomal) and anticarcinogenic properties of β-lap are a direct result of apoptosis (4, 5), defining the apoptotic mechanisms induced by this drug may allow us to manipulate cell death pathways for improved chemotherapy or radiotherapy against breast cancer, while protecting normal cells.

Topo poisons have not been adequately explored as antitumor agents for the treatment of breast cancer for multiple reasons, including (but not limited to) a lack of understanding of the molecular biology and cell cycle regulation underlying CPT-induced stress responses. Topo I carries out a single-strand DNA breakage, passage, and rejoining reaction that unwinds DNA. The enzyme is involved in transcription, replication, and recombination (14, 15) and is constitutively expressed during the cell cycle (16). The enzyme is elevated in many human cancers, such as prostate and colon (17), and is an important chemotherapeutic target for the specific elimination of tumor cells, irrespective of cell cycle status. Topo I poisons, such as...
CPT, cause damage to cells by creating Topo I-mediated DNA lesions. Once damaged, CPT-treated, wild type-expressing human cancer cells exhibit alterations in cell cycle distribution and induction of both p53 and p21 protein levels and can activate apoptosis (18, 19). Although there are few assessments of Topo I levels in human breast cancer compared to normal tissue, there are reports of efficacious anticancer activity of Topo I poisons against human breast cancers (20–22).

β-Lap-activated apoptosis in human myeloid leukemia (HL-60) and prostate cancer cells was independent of p53 status (4, 5). Because agents that induce apoptosis irrespective of p53 status may have potentially great therapeutic value, we investigated the apoptotic responses of MCF-7 human breast cancer cells after transient (4 h) β-lap or CPT treatments. β-Lap killed quiescent or log-phase MCF-7 cells primarily by apoptosis, causing the rapid loss of hyperphosphorylated forms of pRb (within 3–5 h), with no apparent p53/p21 induction responses. In contrast, CPT-treated MCF-7 cells showed dramatic p53/p21 responses and concentration-dependent cell-cycle checkpoint arrests but induced low levels of apoptosis, with little change in the phosphorylation status of pRb within 24 h posttreatment, even at supralethal doses.

MATERIALS AND METHODS

Chemicals and Antibodies. β-Lap (M, 242) and various naphthoquinone derivatives were either made by us or supplied by Drs. Donald T. Witiak (University of Wisconsin-Madison) or A. V. Pinto (Universidade Federal de Rio de Janeiro, Rio De Janeiro, Brazil). CPT (M, 348.4) was obtained from Sigma Chemical Co. (St. Louis, MO). All chemicals were dissolved as 10 mM stock solutions in DMSO, and aliquots were stored at −20°C. Stock solution concentrations were confirmed by spectrophotometric analyses using an extinction coefficient (ε) of 25,790 for β-lap. β-Lap and CPT treatments were always performed in a final DMSO concentration of 0.1%, the amount of DMSO equal to the highest percentage of solvent used in experimental conditions. DMSO at 0.1% did not affect log-phase MCF-7 cells in terms of doubling time, cell growth kinetics, or apoptosis (as monitored by flow cytometry and Hoechst dye staining).

Goat serum and goat FITC-conjugated antibodies were purchased from Sigma Chemical Co. (St. Louis, MO). Antimouse monoclonal anti-BrdUrd antibodies were obtained from Becton Dickinson (San Jose, CA) and used according to the manufacturer's instructions. Antimouse and antirabbit p53, bcl-2, and cyclins B1, E, and A antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antimouse p21 and α-tubulin antibodies were obtained from Oncogene Research Products (Cambridge, MA). Antibodies from scleroderma patient serum, which detect human Topo I protein, were purchased from TopoGEN, Inc. (Columbus, OH). Antihuman Topo IIα antibodies were obtained from Genosys, Inc. (The Woodlands, TX).

Cell Lines and Culture Conditions. The human breast cancer cell line MCF-7 was obtained from Dr. V. Craig Jordan (Northwestern University, Chicago, IL). All cells were grown in DMEM with 10% fetal bovine serum in a humidified 10% CO2-90% air atmosphere at 37°C. MCF-7 cells were seeded at low density (1.0 × 106 cells/mL) and then resuspended in 2.0 N HCl. Samples were then washed with PBS-TB, and RNA was digested with 10 μg/ml RNase A at 37°C for 1 h. Cells were collected by centrifugation at 500 × g and stored at −70°C until use. DNA-PI fluorescence was measured using a FACScan flow cytometer at a laser setting of 36 mW and an excitation wavelength of 488 nm. Resulting DNA distributions were analyzed for the proportion of cells in apoptosis and in G1/G0, S, and G2/M phases of the cell cycle. Data were analyzed by Modfit (Verity Software House, Inc., Topsham, ME), and all experiments were repeated at least three times, each in duplicate. Graphed data represent mean ± SE.

Estrogen Deprivation-induced Synchronization and BrdUrd-labeling Studies. MCF-7 cells were deprived of estrogen for 1 week to arrest cells in early G1 (23). Cells were then treated with 5 μM CPT or β-lap in estrogen-free medium for 4 h and subsequently released into estrogen-containing complete DMEM to initiate synchronous growth with or without prior drug treatment. At various posttreatment times, control and drug-treated MCF-7 cells were incubated with medium containing 10 μM BrdUrd (Sigma) for 30 min to label S-phase cells. Cells were then trypsinized, collected (500 × g), washed once with ice-cold PBS, and fixed (60% ethanol and 0.3% Tween 20). Samples were obtained at the indicated times for up to 96 h and stored in the dark at 4°C. Cell suspensions were incubated with 0.04% pepsin in 0.01 N HCl for 30 min at room temperature. Cells were collected as before and resuspended in 2.0 N HCl. Samples were then washed with PBS-TB, and DNA was digested with 10 μg/ml RNase A at 37°C for 1 h. Cells were washed with PBS-TB and incubated overnight with mouse monoclonal anti-BrdUrd antibodies (1:2.5 dilution). Samples were then washed with PBS-TB and incubated with goat antimouse IgG FITC-conjugated antibody (1:30 dilution) in PBS, 0.5% (v/v) Tween 20, and 0.1% goat serum for 20 min at room temperature. Cells were collected (500 × g), washed twice in PBS, labeled in 50 μg/ml PI, and analyzed using flow cytometry.

Western Immunoblot Analyses. Whole-cell or nuclear extracts from control and drug-treated MCF-7 cells were isolated at various times for altered expression of key regulatory proteins. The immediate extraction of MCF-7 cells after 4 h drug incubation was made by "centrifugation at 4°C" on all Western immunoblots. Briefly, cells were washed twice with ice-cold PBS, scraped from 10-cm2 tissue culture dishes (Corning, Cambridge, MA), and collected by centrifugation at 500 × g. For nuclear extracts, cell pellets were washed once with ice-cold PBS, resuspended in ice-cold hypotonic solution [10 mM Tris-HCl (pH 7.5), 25 mM KCl, 2 mM Mg acetate, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride] and allowed to incubate on ice for 15 min. Samples were then passed through a 25-gauge needle to break cell membranes. Nuclei were then pelleted at 500 × g at 4°C for 5 min, resuspended in hypertonic solution [10 mM Tris-HCl (pH 7.5), 400 mM KCl, 2 mM Mg acetate, 20% glycerol, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride] and
incubated on ice for 10 min. Insoluble nuclear debris was removed by centrifugation at 800 × g for 10 min, and supernatants were aliquotted and stored at −80°C. For whole-cell extracts, cell pellets were washed twice with ice-cold PBS, lysed in loading buffer [62.5 mM Tris (pH 6.8), 6 M urea, 10% glycerol, 2% SDS, 0.003% bromphenol blue, and 5% β-mercaptoethanol], and sonicated on ice for 20 s. Protein concentrations were determined using a Bradford assay (Bio-Rad, Hercules, CA), and 10-μg protein samples were separated by 9 or 6% SDS-PAGE. Separated proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA) for 3 h at 150 V in a Bio-Rad Mini Trans-Blot Electrophoretic Transfer cell (Richmond, CA). Blots were blocked with PBS containing 10% calf serum and 0.2% Tween 20, probed with various primary antibodies as indicated (using 1:500 to 1:5000 dilutions) for at least 2 h, and washed in PBS-0.2% Tween 20 for 1 h. Blots were incubated with various secondary antibodies (using 1:500 to 1:1000 dilutions), and specific antibody-labeled proteins were detected using the ECL chemiluminescence detection system (Amersham, Buckinghamshire, England). Western immunoblot images were obtained by autoradiography using Fuji Medical X-ray film (Tokyo, Japan) as described (24). Induction levels were quantified using either densitometry or gel image analyses on a Bio-Rad Gel Doc 1000 System.

RESULTS

β-Lap Elicited Greater Apoptotic Responses but CPT Was More Cytotoxic. Survival after a 4-h pulse of various concentrations of β-lap or CPT was assessed as described in “Materials and Methods.” LD_{50}s were 2.5 μM and 55 nm for β-lap and CPT, respectively (Fig. 1). MCF-7 cells treated with 5 μM β-lap showed extensive apoptotic-like nuclear condensation at 24 h posttreatment, whereas CPT-treated cells (up to 10 μM) failed to show significant apoptosis (Fig. 2) for up to 10 days posttreatment (Figs. 3 and 4). A direct correlation between apoptosis (quantified at 48 h by flow cytometry) and loss of survival (measured at 6 days posttreatment; Fig. 1) after β-lap was noted. We observed extensive nuclear condensation in MCF-7 cells treated with β-lap; however, classical nuclear blebbing and DNA laddering, as seen with human prostate cancer cells after β-lap (4, 5), were not observed (Fig. 2).

No correlation between loss of survival and apoptosis (Fig. 1B) was noted in MCF-7 cells treated with CPT. MCF-7 cells treated with 5 μM CPT resulted in ~20–25% apoptosis (Fig. 2), as quantified by flow cytometry (see Figs. 2 and 3). Apoptosis was not observed in MCF-7 cells treated with 1 μM CPT at 48 h posttreatment using flow cytometry or Hoescht dye staining, although <1% survival was observed (Fig. 1B). Thus, although CPT was dramatically more cytotoxic to MCF-7 cells than β-lap, the lethality caused by CPT did not correlate with apoptosis (compare Fig. 1, A and B); the amount of apoptosis seen in Fig. 1 correlated with that noted in 6–10-day flow cytometric assays (Figs. 3 and 4).

Kinetics of β-Lap-Induced Apoptosis in MCF-7 Cells. Untreated log-phase MCF-7 cells replicated with an average population of 28% S, 18% G₂-M, and <1% apoptosis over 96 h following low cell density seeding, as described in “Materials and Methods” (Fig. 4A). Gradual increases in G₀/G₁ from 53% to ~75% were observed within 96 h following initial cell plating (Fig. 3A). Log-phase MCF-7 cells treated with 1 μM β-lap progressed through the cell cycle with no apoptosis or cell cycle delays (Fig. 3A). MCF-7 cells treated with β-lap for 4 h (at levels ≥ LD_{50}) resulted in the concentration-dependent appearance of preapoptotic, sub-G₀/G₁ (<2 N) cells, which peaked at 8–10 h and disappeared by 24 h after drug removal (Fig. 3B). Preapoptotic (<2 N) cells were subsequently replaced by apoptotic cells after 24 h (Fig. 3B). At 72 h, >60% of the population was apoptotic. Apoptosis in β-lap-treated MCF-7 cells was confirmed by morphological changes, including classical nuclear condensation (Fig. 2B). Typical G₀ and/or G₂-M cell cycle checkpoint accumulation, observed after the addition of DNA-damaging agents (e.g., 1 μM CPT,
were not observed following doses up to 1 μM CPT for up to 6 days (Figs. 1B and 3C).

In contrast, MCF-7 cells treated with 5 μM CPT demonstrated rapid and near permanent G1 and S-phase cell cycle arrests (Fig. 3D). Very few MCF-7 cells treated with 5 μM CPT entered G2, in contrast to the dramatic G2 block observed with 1 μM CPT. Low-level apoptosis (~10–20%, Fig. 3D) was observed for up to 144 h (6 days) in MCF-7 cells treated with 5 μM CPT. A significant amount of apparently nondividing MCF-7 cells were visible on the tissue culture plates after 5 μM CPT treatment. The changes in cell cycle distribution and apoptosis for CPT-treated MCF-7 cells discussed above were confirmed using temporal, 20-min BrdUrd pulse labeling (data not shown).

CPT, but not β-Lap, Repressed Cyclin A and B1 Protein Levels in MCF-7 Cells. We then examined alterations in the steady-state levels of a number of proteins that regulate or are regulated by the cell cycle at various times following β-lap or CPT exposures. Cyclin A is expressed during S and G2 phases of the cell cycle, and decreased expression of cyclin A may correlate with apoptosis (29). Repression of cyclin A levels in human cells after CPT exposure were noted (30). Steady-state nuclear protein levels of cyclins A, B1, and E were not altered in MCF-7 cells following 5 μM β-lap (Fig. 4). <2 μM β-lap (data not shown), or up to 1.0 μM CPT (data not shown). In contrast, cyclin A and B1 levels dramatically decreased in MCF-7 cells treated with 5 μM CPT, at times (i.e., 24–48 h, Fig. 4) when ~20% apoptosis, significant G1 delay, and decreases in S-phase were observed (Fig. 3D). Decreased steady-state levels of cyclin A [and, presumably, loss of cyclin-dependent kinase activity (30)] after 5 μM CPT correlated well with the delay of treated cells in G1, S and S phases. In contrast, levels of cyclin E, which is expressed in G2/G1 and S phase (31, 32), were unchanged for up to 72 h after 5 μM CPT, consistent with the constant level of G1 cells (Fig. 3D).

Unlike CPT, β-Lap Exposure Did Not Induce p53 or p21 Protein Levels in MCF-7 Cells. Exposure of wild-type p53-expressing rodent or human cells to CPT caused a dramatic increase in nuclear p53 protein levels (25, 33). CPT-mediated p53 induction is thought to be caused by the creation of DSBs and/or single-strand DNA breaks because cells attempt to replicate past Topo I-DNA "cleavable complexes" (25, 33). p53 levels can also be induced by the creation of free radicals (34). Because we previously reported that β-lap catalytically inhibited Topo I in vitro (4) and did not induce DNA breaks (9) or stabilize Topo I-DNA complexes (9, 11), we examined changes in p53 and p21 protein levels in MCF-7 cells after β-lap or CPT exposures. MCF-7 cells exposed to 0.1–10 μM β-lap did not induce nuclear or whole-cell p53 or p21 protein levels (Fig. 4 shows p53/p21 expression after 5 μM β-lap). In fact, nuclear levels of p53 actually dropped below basal levels following 10 μM β-lap (data not shown).

In contrast, treatment of MCF-7 cells with CPT (0.1–10 μM, 4 h)
Fig. 3. Cell cycle distribution of log-phase MCF-7 cells after β-lap or CPT exposures. Log-phase MCF-7 cells were treated with DMEM alone (A) or with DMEM containing 5 μM β-lap (B), 1 μM CPT (C), or 5 μM CPT (D) for 4 h, after which media were replaced with fresh DMEM. All treatments contained 0.25% DMSO, including DMEM alone. Cells were then monitored for cell cycle changes at various times for 72–144 h (up to 6 days). Preapoptotic cells (B) appeared at 4 h only after β-lap treatment and peaked between 8–12 h. Percentages, quantifications of DNA amounts. Apoplotic cells (which contained the least amount of DNA) were confirmed by morphological examination (Fig. 2). O, G1/G0 cells; □, S-phase cells; □, G2/M cells; ■, preapoptotic cells (only found in β-lap treatment); ▼, apoptotic cells. ———, actual flow cytometric data. Data are representative of cell cycle distribution trends observed in at least six separate experiments.
Fig. 4. Changes in protein expression after exposure of MCF-7 cells to β-lap or CPT. Log-phase MCF-7 cells were treated with 5 μM β-lap or CPT (4 h, 37°C) and monitored over time (in h) for the steady-state expression of various proteins. Changes in whole-cell (i.e., p53, p21, Topo I, Topo IIα, bcl-2, and α-tubulin) and nuclear (i.e., cyclins A, B1, and E) protein levels were quantified as described in “Materials and Methods.” Fluctuations in protein loading between samples were monitored by α-tubulin levels. Multiple low molecular weight protein bands derived from Topo I or Topo IIα degradation and previously observed after Western immunoblot analyses during apoptosis (39) were observed only in β-lap-treated extracts after extremely long exposures of X-ray film (data not shown). Similar degradation products were not observed after CRT-treated log-phase MCF-7 cells. Lower C, untreated control samples. For the steady-state levels of p53, both nuclear (data not shown) and whole-cell (shown) levels demonstrated p53 protein induction by CPT but not by β-lap.

caused 5–10-fold induction of p53 within 4–24 h (Fig. 4). As expected, delayed (with respect to p53) and dramatic increases (peaking at 48 h, Fig. 4) in the steady-state levels of p21 protein were observed in CPT-treated MCF-7 cells, as reported (35, 36).

CPT, but not β-Lap, Repressed bcl-2 Protein Levels in MCF-7 Cells. The mitochondrial protein, bcl-2, plays an important role in protecting cells from apoptosis in various human cells (37). Treatment of MCF-7 cells with 5 μM (or 10 μM) β-lap did not affect the levels of bcl-2 at any time (Fig. 4), although 40–60% apoptosis was noted at 48 h (Fig. 3B). In contrast, MCF-7 cells treated with 5 μM CPT demonstrated a decrease in bcl-2 protein levels by 48 h. The Western blot in Fig. 5 used the same protein samples but was loaded differently. Levels of α-tubulin did not change after β-lap or CPT treatments, as shown in Fig. 4. Our results with bcl-2 (i.e., loss of bcl-2 following CPT and no alterations in this protein after β-lap) were consistent with previous data using human leukemia cells (5, 37).

Altered Topo I Levels in MCF-7 Cells after β-Lap or CPT. Decreases in Topo I protein levels were reported in human cells following Topo I poisons (38, 39). Because both CPT and β-lap inhibited Topo I in vitro (4, 8, 11) but killed MCF-7 cells by very different mechanisms (Figs. 1–4), we examined changes in cellular Topo I protein levels in MCF-7 cells after 5 μM β-lap or CPT (Fig. 4). Neither drug treatment, given as 4-h pulses, affected total cellular Topo I protein levels for up to 8 h. However, Topo I protein levels decreased ~70% by 24 h (Fig. 4) following 5 μM β-lap or CPT. By 48 h after 5 μM β-lap or CPT, Topo I protein was undetectable. Decreases in cellular Topo I levels in MCF-7 cells treated with β-lap (Fig. 4) coincided with the appearance of apoptosis (compare Fig. 4 to Fig. 3B), and apoptotic cleavage fragments, similar to those described previously (39), were observed after longer exposures of Western blots. In contrast, decreased Topo I levels in MCF-7 cells after CPT (Fig. 4) did not coincide with apoptosis because only 10–20% apoptotic cells were noted at 72–144 h (Fig. 3D). Topo I-related apoptotic cleavage fragments were not observed after CPT, presumably because low levels of apoptosis were induced.

Repression of Topo IIα Levels in MCF-7 Cells after β-Lap or CPT Treatments. We also examined alterations in total cellular levels of Topo IIα at various times after 5 μM β-lap or CPT (Fig. 4). Topo IIα protein and transcript are expressed in a cell cycle-dependent manner in late S and through G2-M phases of the cell cycle, with little expression in G0/G1 or early S phase (40). Similar decreases in Topo IIα protein levels were observed after β-lap or
CPT. The total cellular levels of Topo IIα decreased (by ~30%) at 8 h and remained at ~10% control levels from 24–48 h following β-lap (Fig. 4). At 72 h, there was only ~1% control levels remaining after CPT, and Topo IIα was not detected at 72 h after β-lap. Loss of Topo IIα coincided with the loss of Topo I in MCF-7 cells treated with 5 μM CPT or β-lap. The loss of Topo I and Topo IIα was very specific because cyclin E and α-tubulin levels remained unchanged after CPT or β-lap and p53/p21 levels increased after CPT (Fig. 4).

Loss of Hyperphosphorylated Forms of pRb and Cleavage of Lamin B in MCF-7 Cells after β-Lap Treatment. We previously found that β-lap-mediated apoptosis was p53 independent, occurring equally in null or mutant p53-expressing human cancer cells (4). Phosphorylation of pRb is needed for progression through the cell cycle (41), and changes in the phosphorylation status of pRb (easily observed by Western immunoblot analyses) were noted after apoptosis (42). We, therefore, examined the phosphorylation status of pRb in MCF-7 cells following treatment with 1 or 5 μM β-lap for up to 5 h (Fig. 5A). No changes in the phosphorylation state of pRb were noted following 1 μM β-lap, consistent with the lack of changes in cell cycle distribution (Fig. 3A). However, MCF-7 cells treated with 5 μM β-lap showed a dramatic loss of hyperphosphorylated forms of pRb and a drop in overall levels of pRb from 4 to 5 h (Fig. 5A). Loss of the hyperphosphorylated forms of pRb coincided with the induction of apoptosis (Fig. 3). These data suggest that, in fact, a delay at the G2/M phase of the cell cycle may have occurred following β-lap exposure, but accumulations were not observed due to the rapid loss of cells by apoptosis (Fig. 4). These data are consistent with a G2/M apoptotic exit point induced by β-lap in log-phase MCF-7 cells.

Lamin B, a nuclear envelope protein, is a known apoptotic substrate for caspases (43). Cleavage of lamin B was seen in MCF-7 cells treated with a 4-h pulse of 5 μM CPT (Fig. 5B), in proportion (~20%) to apoptosis (Fig. 3D). Similarly, MCF-7 cells treated with a 4 h pulse of 5 μM β-lap showed complete cleavage of lamin B by 72 h (Fig. 5B). Initial cleavage of lamin B was apparent in 4 h following β-lap. Interestingly, cleavage of lamin B occurred at the same time that peak levels of preapoptotic cells appeared in Fig. 3B.

Use of Estrogen Deprivation-induced Synchronization to Elucidate Apoptotic and Cell Cycle Checkpoint Responses in MCF-7 Cells after β-Lap or CPT. Semisynchronized MCF-7 cells grown in estrogen-depleted medium were then used to further elucidate cell cycle redistribution and apoptosis following 5 μM β-lap or CPT. Estrogen-deprived, MCF-7 cells consisted of 74% G0/G1, 12% S, and 14% G2-M before release into estrogen-containing complete medium (Fig. 6A), compared to 55% G0/G1, 30% S, and 15% G2-M observed in log-phase MCF-7 cells grown in complete medium (Fig. 3). Estrogen depletion alone did not increase apoptosis (Fig. 6A). After release, MCF-7 cells, as expected, entered S phase between 8 and 24 h (Fig. 6A). These data are in agreement with previous studies that demonstrated that estrogen-depleted MCF-7 cells entered S phase ~12 h after release in estrogen-containing medium (23). These data were expected for MCF-7 cells with a doubling time of ~24 h. By >48 h, MCF-7 cells resumed log-phase asynchronous growth (Fig. 6A), with cell populations approximating those of the control (Fig. 3A). By Western immunoblot analyses, Topo I but not Topo Ila was observed in the first 18 h following release from estrogen blockage, consistent with the flow cytometry data in Fig. 6.

Semisynchronized MCF-7 cells were treated with or without 5 μM β-lap or CPT in estrogen-deprived medium and released in complete estrogen-containing medium beginning at t = 0. Consistent with results in Fig. 3B, estrogen-deprived MCF-7 cells treated with 5 μM β-lap for 4 h demonstrated a significant, near synchronous loss of cells due to apoptosis at or about the G2-S border (Fig. 6B). Dramatic and steady decreases in G2 cells were observed between 24 and 48 h posttreatment with concomitant increases in preapoptotic, followed closely by apoptotic, cells (Fig. 6B). BrdUrd-labeling analyses indicated that MCF-7 cells in G2 at the time of β-lap treatment never synthesized DNA and never entered S phase. Note the lowered percentage of S phase cells following β-lap treatment compared to control cells at t = 0, 4 h after drug treatment. MCF-7 cells in S phase at the time of 5 μM β-lap treatment incorporated BrdUrd and disappeared from the population within the first 4 h posttreatment [compare the 18% S-phase cells present in the untreated population (Fig. 6A) to the <2% S-phase cells present after a 4-h β-lap treatment (Fig. 6B)].
Estrogen-deprived MCF-7 cells were then treated for 4 h with or without 5 μM ß-lap or CPT. All treatments, including controls, were performed in estrogen-deprived medium containing 0.25% DMSO. At t = 0, cells were then washed free of drug, and complete media containing or CPT treatments. MCF-7 cells were grown in estrogen-deprived medium for 4 days. Apoptotic and apoptotic MCF-7 cells following ß-lap treatment (Fig. 6A). Some cases, symbols were larger than the corresponding error bars.

DISCUSSION

ß-Lap Caused Cell Death in MCF-7 Cells Exclusively by Apoptosis. ß-Lap killed growing or quiescent (estrogen-deprived) MCF-7 cells through an as yet undefined apoptotic mechanism(s). An inverse correlation between survival and induction of apoptosis (measured at 48 h) was noted (Fig. 1). Although it was difficult to accurately quantify apoptosis after >2 μM ß-lap treatments due to variable responses, the curves for apoptosis and loss of survival appeared to intersect at the LD₅₀ of the drug (i.e., 2.5 μM). Flow cytometric analyses at 24-h intervals over a 6-day period (i.e., at a time equal to survival assays, Fig. 3) confirmed the correlation seen in Fig. 1. Therefore, we conclude that MCF-7 cells die exclusively by apoptosis following ß-lap. Similar correlations between ß-lap-mediated apoptosis and loss of survival have been observed in a variety of human cancer cell lines, including cancers of the colon (HT-29 and HCT-116), prostate (LNCaP, PC-3, and DU-145), and other human breast cancer cell lines (i.e., MDA-MB-468 and T47D:A18; data not shown). MCF-7 cells appear to be the most sensitive breast cancer cell line to ß-lap-mediated apoptosis (44). Apoptosis in MCF-7 cells after ß-lap was confirmed by nuclear condensation and lamin B cleavage and quantified by flow cytometry (4). Preliminary data also revealed cleavage of other known cell death substrates, such as poly(ADP-ribose) polymerase, in ß-lap-treated (or CPT-treated) MCF-7 cells at 6–8 h posttreatment (44), in direct proportion to levels of apoptosis quantified by flow cytometry (Fig. 1), as observed with lamin B (Fig. 5A).

A similar correlation between loss of survival and apoptosis was not observed in MCF-7 cells following CPT exposures (Fig. 1). Irreversible G₂-M arrest responses and a lack of apoptosis for up to 10 days in MCF-7 cells treated with ≤1 μM CPT were observed. At ≥5 μM CPT, only 15–20% apoptosis was observed, and cells were permanently arrested in G₂ and S phase. Estrogen-deprived MCF-7 cells treated with 5 μM CPT did not progress into G₂-M and demonstrated significantly less apoptosis. Thus, lethality of MCF-7 cells treated with CPT correlated well with loss of reproductive potential and irreversible G₂-M cell cycle arrest, rather than with apoptosis. Apoptosis following CPT but not ß-lap was also influenced by growth status or the presence of estrogen, because estrogen deprivation significantly reduced apoptosis in MCF-7 cells following a 4-h exposure to 5 μM CPT (compare apoptosis in log-phase MCF-7 cells at 6–8 h posttreatment (44), to levels observed in estrogen-deprived cells, Figs. 3D and 6C, respectively). Similar estrogen-related protection of MCF-7 cells from apoptosis has been noted (45).

ß-Lap-mediated Apoptosis Occurred at a Cell Cycle Exit Point between G₀/G₁ and G₂-S. ß-lap-treated MCF-7 cells progressed through the cell cycle with no apparent accumulations in G₀/G₁, S, or G₂-M. By 24–48 h, ß-lap-exposed log-phase MCF-7 cells were removed by apoptosis from the cell cycle somewhere between G₀/G₁ and the G₂-S phases of the cell cycle (Fig. 6C). Very little apoptosis was induced (1–2%, Fig. 6C) compared to CPT-treated log-phase MCF-7 cells (up to 22%, Fig. 1B). The proportion of estrogen-deprived MCF-7 cells in S and G₂-M after CPT treatment remained constant but was lower than S and G₂-M levels in untreated cells. DNA synthesis labeling analyses revealed very little BrdUrd incorporation, suggesting that CPT-treated MCF-7 cells were stationary in the cell cycle or progressed very little. MCF-7 cells in S phase at the time of treatment appeared to stall in S phase without incorporating BrdUrd. These data indicate that treatment of estrogen-deprived MCF-7 cells with >5 μM CPT resulted in G₀ and S-phase delays, and cells were apparently more resistant to apoptosis.

The loss of cells from G₀/G₁ correlated well with increases in pre-apoptotic and apoptotic MCF-7 cells following ß-lap treatment (Fig. 6B). These data are consistent with an apoptotic loss of cells from the cell cycle prior to initiation of S phase. By 48 h, over 70% of MCF-7 cells exited the cell cycle and entered apoptosis. Estrogen-depleted cells showed a greater level of apoptosis in 48 h after 5 μM ß-lap than did log-phase MCF-7 cells (Fig. 6B).

Estrogen-deprived MCF-7 cells treated with 5 μM CPT arrested in G₀ and S phases of the cell cycle (Fig. 6C). Very little apoptosis was induced (1–2%, Fig. 6C) compared to CPT-treated log-phase MCF-7 cells (up to 22%, Fig. 1B). The proportion of estrogen-deprived MCF-7 cells in S and G₂-M after CPT treatment remained constant but was lower than S and G₂-M levels in untreated cells. DNA synthesis labeling analyses revealed very little BrdUrd incorporation, suggesting that CPT-treated MCF-7 cells were stationary in the cell cycle or progressed very little. MCF-7 cells in S phase at the time of treatment appeared to stall in S phase without incorporating BrdUrd. These data indicate that treatment of estrogen-deprived MCF-7 cells with >5 μM CPT resulted in G₀ and S-phase delays, and cells were apparently more resistant to apoptosis.

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Fig. 6. Cell cycle and apoptosis in estrogen-deprived/synchronized MCF-7 cells after ß-lap or CPT treatments. MCF-7 cells were grown in estrogen-deprived medium for 4 days. Estrogen-deprived MCF-7 cells were then treated for 4 h with or without 5 μM ß-lap or CPT. All treatments, including controls, were performed in estrogen-deprived medium containing 0.25% DMSO. At t = 0, cells were then washed free of drug, and complete media containing 10% FCS were added. Samples were monitored for cell cycle changes and apoptosis for various time points (as described in Materials and Methods). Four-h treatments were untreated control MCF-7 cells (A), 5 μM ß-lap (B), and 5 μM CPT (C). Data points, mean percentages of G₀/G₁ cells (A), preapoptotic cells (B), G₂-M cells (C), and apoptotic cells (D) from three separate experiments; bars, SE. In some cases, symbols were larger than the corresponding error bars.
pRb is a tumor suppressor gene that controls transit of cells through the G1-S border. The pRb protein undergoes a series of phosphorylation events that alter its function to allow cell cycle progression (41). Loss of hyperphosphorylated forms of pRb occurs during apoptosis (42). Treatment of MCF-7 cells with 5 μM β-lap caused the apparent rapid (within 3–5 h posttreatment) dephosphorylation of pRb (Fig. 5). In temporal terms, loss of hyperphosphorylated pRb occurred prior to the apparent activation of a caspase that cleaved lamin B, presumably caspase 6 (39). The loss of hyperphosphorylated pRb and the synchronous loss of G1/G0 cells, implicate the G1/G0 border as the apoptotic exit point for β-lap-induced apoptosis. Further analyses of the loss of hyperphosphorylated forms of pRb and degradation of this cell cycle control protein is under investigation (44).

MCF-7 cells treated with CPT demonstrated very different cell cycle, protein expression, and apoptotic responses. CPT is a known Topo I poison (27) that results in DNA breaks (25), induction of p53/p21 (18), cell cycle arrest at G1, S, and G2-M cell cycle checkpoints (33), and apoptosis (50). Cellular responses to CPT occurred in specific dose-dependent patterns in MCF-7 cells after a 4-h pulse, as well as in a variety of human prostate cancer cells (4). In MCF-7 cells treated with ≤1 μM CPT, significant cell cycle arrest in G2-M but not in G1 or S phases was observed (Fig. 3C). Increases in p53/p21 nuclear protein levels were observed, presumably as a direct response to DSBs (25), but apoptosis was not noted. DSBs (51), as well as p53/p21 induction (26), may have accounted for the subsequent G2-M arrest following CPT. In contrast, MCF-7 cells treated with 5 μM CPT stalled primarily in G1 and S phases, where most cells were at the time of treatment, especially when deprived of estrogen (Fig. 6C). Formation of extensive Topo I-DNA complexes after such supralethal doses of CPT probably caused transcriptional interference (14, 15) and subsequent rapid and complete cell cycle arrest, possibly mediated by a rapid and extensive p53 response (Fig. 4). Apoptotic cells (i.e., ~20%) were then observed at 72 h posttreatment with decreases in bcl-2, cyclin B1, and cyclin A, but not cyclin E. Decreases in bcl-2 and cyclin A/cdk2 have been observed after CPT treatment (30, 37, 52). The protein expression data shown in Fig. 4, which demonstrated moderate decreases in bcl-2 in MCF-7 cells after CPT, correlated with the low level of apoptosis observed (37). Although CPT was more cytotoxic than β-lap on a molar ratio basis (Fig. 1), we speculate that the p53/p21 induction and subsequent cell cycle checkpoint arrest responses observed after CPT may have, in fact, promoted repair and prevented apoptosis in MCF-7 cells. Alternatively, but not mutually exclusively, CPT-mediated cell death signals may work through apoptotic pathways (e.g., caspase 3 activation) that are deficient in MCF-7 cells (44). Finally, estrogen-deprived MCF-7 cells treated with CPT appeared resistant to apoptosis, although no differences in survival in quiescent and growing cells were noted. The lack of apoptotic responses after CPT treatment may also participate in the potent carcinogenic properties of this agent (53).

β-Lap Is Not a DNA-damaging Agent. Our data are consistent with the hypothesis that β-lap exposure does not cause DNA breaks in exposed human cells, as reported (9). Exposure of MCF-7 cells to β-lap (up to 10 μM) did not elicit cell cycle checkpoint responses or p53/p21 induction, which can be a very sensitive measure of the appearance of DNA breaks in cells exposed to DNA-damaging agents (25, 33) or agents that generate free radicals (26, 51). In fact, at high doses of β-lap (≥10 μM), we noted decreases in the basal steady-state level of p53 protein. Further research is currently ongoing in our laboratory to examine this p53 degradation. Coordinate cell cycle and p53 responses indicative of DNA damage were observed in MCF-7 cells (which express wild-type p53 protein) after CPT treatment (Figs. 3 and 4). Collectively, our data suggest that β-lap induces a direct apoptotic response in MCF-7 cells, without the formation of DNA breaks, consistent with previous data (9) at a time when Topo I but not Topo IIa protein was expressed. Further research will be required to delineate whether β-lap works through Topo I in vivo, because the inhibition of Topo I by β-lap also did not produce DNA breaks or Topo I-DNA complexes (4, 5, 12). We speculate that β-lap may directly activate apoptotic proteases, possibly through a non-nuclear signaling mechanism.

It is interesting that similar and dramatic decreases in Topo I and Topo IIa protein levels occurred after β-lap or CPT (Fig. 4). Alterations in Topo I levels have been reported following poisons that target this enzyme (38, 54). We speculate that decreases in Topo I and/or Topo IIa after β-lap exposure may have resulted from a depletion of cells in G2-M due to a prior loss of cells at G1 (Figs. 3B and 4) and/or the activation of apoptotic proteases (e.g., caspaspe), which specifically target these enzymes (39). Consistent with this theory, we observed Topo-related, cleavage fragments that were consistent with previously described apoptotic products following longer exposures of Western blots (39). In contrast, Topo I and Topo IIa protein decreases after 5 μM CPT were not caused solely by apoptotic responses because only 20% apoptosis was observed. Decreases in Topo I may represent irreversible and near complete formation of covalent DNA-Topo I complexes (38, 54), and simultaneous loss of Topo IIa may have resulted from accumulation of exposed cells in G1 and S phases, with depleted G2-M cells over time (Figs. 3D and 4); Topo IIa is expressed during late S and throughout G2-M (40).

Manipulation of Apoptotic Pathways for Improved Human Breast Cancer Therapy. Topo I levels are elevated in human prostate and colon cancers (17), but very little data are available for human breast cancers compared to associated normal tissue. The overall levels of Topo I protein or enzyme do not, however, necessarily predict the cytotoxic outcome in many cancer cells (55). Therefore, we believe that it is crucial to better understand how cancer cells, particularly breast cancer cells, are killed following Topo I poisons or following other compounds that mediate apoptotic-dependent cytotoxicity (e.g., β-lap). Equally important are the mechanisms of protection against apoptosis, which may reduce efficacy. Understanding such mechanisms of cell death and cell death protection may allow us to alter apoptotic responses in human breast cancer cells for tumor regression. β-Lap and its derivatives hold great promise for chemotherapeutic approaches that exploit specific apoptotic pathways that are present in tumor cells but absent or weakly activated in normal tissue. We believe that this compound holds promise as a direct chemotherapeutic agent and as a radiosensitizer against human breast cancer, which may also suppress neoplastic transformation and genetic instability induced by damage in normal tissue by apoptotic processes (9). A better understanding of cell stress responses to this agent may lead to the identification of its exact intracellular target.

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REFERENCES


Induction of Apoptosis in MCF-7:WS8 Breast Cancer Cells by β-Lapachone


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