Molecular Pharmacology Fast Forward. Published on September 1, 2005 as doi:10.1124/mol.105.014753

MOL 14753

Activation of the UPR is necessary and sufficient for reducing topoisomerase II α protein levels and decreasing sensitivity to topoisomerase targeted drugs

Miranda D. Gray, Melissa Mann, John L. Nitiss, and Linda M. Hendershot

Departments of Molecular Pharmacology (MDG, JLN) and Genetics & Tumor Cell Biology (MM, LMH), St. Jude Children's Research Hospital, 332 N. Lauderdale, Memphis, TN 38105

Molecular Pharmacology Fast Forward. Published on September 1, 2005 as DOI: 10.1124/mol.105.014753
This article has not been copyedited and formatted. The final version may differ from this version.

a) Running Title: UPR induction leads to etoposide resistance

b) Address correspondence to:

John L. Nitiss
St. Jude Children's Research Hospital
Molecular Pharmacology Dept.
332 N. Lauderdale
Memphis TN 38105
Phone 901-495-2794
E-mail john.nitiss@stjude.org

c) Number of pages: 29
Number of tables: 1
Number of figures: 6
Number of references: 63
Number of words in *Abstract*: 246
Number of words in *Introduction*: 870
Number of words in *Discussion*: 1508

d) ABBREVIATIONS: UPR, unfolded protein response, ER, endoplasmic reticulum, 2-DG, 2-deoxyglucose, GFP, green fluorescent protein, CHO, Chinese hamster ovary, CHO-BiP^{OE}, CHO cells over-expressing BiP

Molecular Pharmacology Fast Forward. Published on September 1, 2005 as DOI: 10.1124/mol.105.014753
This article has not been copyedited and formatted. The final version may differ from this version.

ABSTRACT

A wide range of chemotherapeutic agents have been identified that are active against solid tumors. However, resistance remains an important obstacle to the development of curative regimens. While much attention has been paid to acquired drug resistance, a variety of physiological pathways have also been described that reduce the sensitivity of previously untreated tumors to cytotoxic anti-tumor agents. Treatment of cells with pharmacological agents that alter the environment of the ER and activate the unfolded protein response (UPR) can render cells resistant to topoisomerase II poisons. We describe experiments showing that activation of the mammalian ER stress response is both necessary and sufficient to decrease topoisomerase II α protein levels and to render cells resistant to etoposide, a topoisomerase II-targeting drug. This is not due to the elevated levels of BiP that are a hallmark of this response, since a cell line that has been engineered to over express BiP does not show increased resistance to etoposide. The UPR was shown to be required for altered drug sensitivity, as the BiP over-expressing cell line, which is unable to activate the UPR, did not show decreased topoisomerase II levels or increased resistance to etoposide in response to stress conditions. The transient over-expression of an unfolded protein activated the UPR and lead to the concomitant loss of topoisomerase II α protein from the cells, demonstrating that UPR activation is sufficient for the changes in topoisomerase II levels that had previously been observed with pharmacological induction of the UPR.

Molecular Pharmacology Fast Forward. Published on September 1, 2005 as DOI: 10.1124/mol.105.014753
This article has not been copyedited and formatted. The final version may differ from this version.

INTRODUCTION

Due to their rapid growth and inadequate vascularization, the microenvironment of tumor cells can become limiting (Ma and Hendershot, 2004). The decreased levels of oxygen and nutrients can alter the homeostatis of the endoplasmic reticulum (ER), causing unfolded proteins to accumulate in the ER, which activate the unfolded protein response (UPR) pathway. The initial phases of this response, which can occur in all cells, are designed to protect the cell until the stress subsides. Mammalian cells respond to ER stress by increasing the transcription of resident ER chaperones to prevent protein aggregation (Lee, 1987), decreasing protein translation to limit the accumulation of unfolded proteins (Brostrom *et al.*, 1996), arresting cells in the G1 phase of cell cycle (Melero and Fincham, 1978) to prevent the propagation of cells experiencing physiological stress, and increasing the degradative capacity of the cell (Hampton, 2000) to decrease the load of unfolded proteins. If the ER stress is prolonged, apoptotic pathways are activated (Nakagawa *et al.*, 2000;Hitomi *et al.*, 2004) to destroy chronically affected cells. It is assumed that cancer cells take advantage of the cytoprotective elements of the response and disable the cytotoxic ones, although this has not been carefully studied.

Changes in the ER environment are sensed by three ER-localized transmembrane proteins Ire1, PERK, and ATF6. The ER chaperone BiP binds to the luminal portions of these three proteins in the absence of stress and maintains them in an inactive state (Bertolotti *et al.*, 2000;Shen *et al.*, 2002). When unfolded proteins begin to accumulate, BiP is released from the transducers, leading to their dimerization and activation in the case of Ire1 and PERK or transport to the Golgi for processing in the case of ATF6. ATF6 is liberated from the ER membrane by the S1P and S2P proteases (Ye *et al.*, 2000) and translocated to the nucleus where it activates the transcription of its targets (*e.g.*, ER chaperones like BiP and GRP94, and transcription factors liked XBP-1 and CHOP). Ire1 possesses an endoribonuclease activity that

Molecular Pharmacology Fast Forward. Published on September 1, 2005 as DOI: 10.1124/mol.105.014753
This article has not been copyedited and formatted. The final version may differ from this version.

is activated during ER stress to remove 26 nucleotides from the XBP-1 transcript. This alters the reading frame of XBP-1, resulting in a remodeled transcription factor with both a DNA binding and transactivation domain (Yoshida *et al.*, 2001;Calfon *et al.*, 2002). The first target of spliced XBP-1 to be identified is EDEM, which is a component of the ER degradation machinery (Yoshida *et al.*, 2003). PERK is an ER localized member of the eIF-2α kinase family (Harding *et al.*, 1999;Shi *et al.*, 1998). Phosphorylation of eIF-2α prevents the formation of translation initiation complexes, thereby blocking protein synthesis. In addition to preventing the accumulation of proteins, this block in protein synthesis leads to the rapid loss of D1 cyclin from cells causing them to arrest in G1 (Brewer and Diehl, 2000). Activation of eIF-2α kinases also leads to increased translation of ATF4 (Harding *et al.*, 2000), which transactivates another group of stress-inducible genes *(e.g.*, GADD34 and CHOP) (Ma and Hendershot, 2003).

DNA topoisomerases play essential roles in replication, transcription and chromosome segregation (Wang, 1996;Nitiss, 1998). The two major families of topoisomerases; type I enzymes that introduce transient single strand cuts in DNA, and type II enzymes that make double stranded breaks are both targets of clinically important anti-cancer agents (Wang, 1996;Osheroff, 1998). Mammalian cells have two topoisomerase II isozymes, topoisomerase II α and II β, and both enzymes are targeted by most topoisomerase II targeting agents (Walker and Nitiss, 2002). Drugs targeting either class of topoisomerase act by blocking the religation of the normally transient intermediate cleaved intermediate of the topoisomerase reaction (Chen and Liu, 1994), thus activating DNA damage checkpoints, which can lead to apoptosis using the same pathways as other DNA damaging agents (Kaufmann, 1998). Consequently, reduced levels of topoisomerases can lead to drug resistance, because they reduce the amount of topoisomerase:DNA complexes, and therefore the amount of drug-induced DNA damage (Nitiss

Molecular Pharmacology Fast Forward. Published on September 1, 2005 as DOI: 10.1124/mol.105.014753
This article has not been copyedited and formatted. The final version may differ from this version.

et al., 1993). Frequently, the reduction in topoisomerase II enzyme levels is accompanied by a reduction in their mRNA levels, suggesting alterations in the transcriptional regulation of the proteins (Nitiss and Beck, 1996). In addition, post-translational modifications such as phosphorylation, ubiquitination, and sumoylation can lead to changes in protein stability, or protein localization (Li and Liu, 2001;Mao *et al.*, 2000;Chikamori *et al.*, 2003) resulting in reduced levels of topoisomerase mediated DNA damage.

The cellular levels of topoisomerase II α can also be regulated by some stress conditions. Treatment of cells with UPR-inducing agents induces resistance to doxorubicin and other topoisomerase II targeting agents (Hughes *et al.*, 1989;Shen *et al.*, 1987), due to a dramatic reduction in topoisomerase II levels (Shen *et al.*, 1989;Yun *et al.*, 1995). In these studies, pharmacological agents were used to induce ER stress making it difficult to determine if the resistance to these poisons is a specific response of the UPR pathway or an indirect effect of the drugs used to activate the response. We demonstrate here that activation of the mammalian UPR is both necessary and sufficient to reduce the sensitivity of cells to topoisomerase II poisons. This will allow determination of the arm(s) of the pathway responsible for changes in drug sensitivity and may allow a targeted intervention to increase the chemotherapeutic efficacy of drugs that target topoisomerase II α.

MATERIALS AND METHODS

Cell lines

The derivation of CHO cells that over-express BiP has been previously described (Dorner *et al.*, 1992). The NIH3T3 murine fibroblast line, 293 human embryonic kidney cell line, COS-1 African Green monkey fibroblast line, CHO Chinese hamster ovary cell line, and CHO-BiP^{OE},

Molecular Pharmacology Fast Forward. Published on September 1, 2005 as DOI: 10.1124/mol.105.014753
This article has not been copyedited and formatted. The final version may differ from this version.

CHO cells that have been engineered to over-express hamster BiP were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 1% Fungizone (Biowhittaker, Walkersville, MD).

Western blotting

Equal numbers of cells were lysed directly in SDS-sample buffer (62.5 mM Tris HCl, pH 6.8, 10% (v/v) glycerol, 2% SDS, 5% β-mercaptoethanol, 0.0025% bromophenol blue), and proteins were separated on SDS-polyacrylamide gels under reducing conditions and transferred to membranes for Western analysis. The membranes were blocked with 0.1% gelatin, 0.1% Triton X-100 or 5% powdered instant milk, 0.1% Tween in Tris saline and then probed with indicated antiserum. For rodent BiP, a rabbit polyclonal anti-BiP antiserum (Hendershot *et al.*, 1995) was used at a 2 µl/ml. For detection of primate BiP, a monoclonal rat anti-BiP antibody (Bole *et al.*, 1986) was used at 60 µl/ml, Ig heavy chains were detected with a polyclonal rabbit anti-human γ heavy chain antiserum (Southern Biotechnology Associates) at 2 µg/ml, and CHOP was observed with a polyclonal rabbit anti-CHOP antisera (Brewer *et al.*, 1999) at 4 µg/ml. Antisera directed against actin and Hsc70 were obtained from Abcam, Inc., Cambridge, MA and Santa Cruz Biotechnology, Santa Cruz, CA respectively. In all cases the appropriate species-specific secondary antiserum (Southern Biotechnology Associates, Birmingham, AL) was used, followed by incubation with protein A-conjugated HRP (EY Laboratories, San Mateo, CA), and visualization with the ECL reagent (Amersham Biosciences, Piscataway, NJ).

Murine topoisomerase II α was detected with either a commercial reagent specific for this isoform (TopoGEN, Columbus, OH) or with an affinity purified rabbit anti-human topoisomerase II α antiserum produced in our lab. The topoisomerase II α antiserum was raised

against three peptides (MEVSPLQPVNEN, VEAKEKQDEQVGLPG, and KRAAPKGTKRDPAL), which are conserved in human and mouse topoisomerase II α but which are not found in topoisomerase II β from either species. The serum was affinity purified against the three peptides and was found to specifically recognize recombinant topoisomerase II α but not topoisomerase II β by Western blot analysis. Human topoisomerase II α (TopoGEN, Columbus, OH) and β (Becton-Dickenson (San Diego, CA) were detected with commercial reagents specific for each isoform.

Northern analysis

Total cellular RNA was isolated from approximately 10^7 cells using the RNAeasy kit (Qiagen, Valencia, CA). Isolated RNA was separated by agarose gel electrophoresis and transferred for hybridization (Brewer *et al.*, 1997). A 1.5 kb *PstI-EcoRI* fragment was isolated from a hamster BiP cDNA clone and used to detect BiP, a 1.7 kB Top2 PCR product amplified from mouse cDNA was used to detect Topoisomerase II α, a 600 bp *EcoRI-XbaI* fragment isolated from murine CHOP cDNA was used to detect CHOP, and a 1.1 kb fragment purchased (Clonetech, Palo Alto, CA) to detect GAPDH, which served as a loading control.

Clonogenic survival assays

Cells were plated at 4 $X10^5$ cells per 100mm plate in 10ml media. After 16 hours, the indicated ER stress-inducing drug was added to the plates at the specified concentration and times. Cells were then incubated with the indicated concentrations of etoposide for two hours, washed with 10ml PBS, and trypsinized. Varying numbers of cells were plated in triplicate on 100mm plates to ensure that between 30 and 300 colonies would be present on the plate for each treatment regimen. Plates were incubated 7-10 days to allow colony formation. The plates were then

Molecular Pharmacology Fast Forward. Published on September 1, 2005 as DOI: 10.1124/mol.105.014753
This article has not been copyedited and formatted. The final version may differ from this version.

washed with PBS and stained with crystal violet. Colonies were counted, and survival was determined by multiplying the number of colonies by the dilution factor and expressing this number as percent survival relative to surviving colonies that were not treated with etoposide. For determining sensitivity to ionizing radiation, cells were plated, and irradiated in 100mM dishes in culture media. Following irradiation, appropriate dilutions were plated, and cells were incubated for 7-10 days as above prior to determining surviving percentages. The total time required for irradiation (the time cells were not in an incubator) was approximately 10 minutes.

Expression of Ig heavy chain to induce ER stress response

Stratagene's pSG5 vector was altered to create an expanded multi-cloning site into which a humanized gamma heavy chain (Liu *et al.*, 1987) was inserted. COS-1 cells were co-transfected with the pGreen Lantern vector, which contains a cDNA encoding the green fluorescent protein, GFP (Invitrogen, Carlsbad, CA), along with either an empty vector (pSG5) or the pSG5-γ vector using the FuGENE 6 reagent (Roche Diagnostics, Indianapolis, IN). Forty eight hours post transfection, cells were trypsinized and GFP^+ cells were isolated using a Becton Dickinson FACS Vantage/SE Cell Sorter (San Jose, CA). Consistently, we observed approximately 15% GFP^+ cells in the cells transfected with pGreen Lantern alone and approximately 11% GFP^+ cells in the doubly transfected cells (data not shown). An aliquot of doubly transfected GFP^+ cells were co-stained with TRITC-conjugated anti-human γ and found to be ~95% positive for γ heavy chain (data not shown). The sorted cells were counted and $5x10^5$ of each were rinsed with PBS and immediately lysed in SDS-sample buffer. The lysates were electrophoresed on reducing SDS-polyacrylamide gels, transferred to nitrocellulose paper and analyzed by Western blotting as indicated.

Molecular Pharmacology Fast Forward. Published on September 1, 2005 as DOI: 10.1124/mol.105.014753
This article has not been copyedited and formatted. The final version may differ from this version.

RESULTS

Induction of the UPR results in etoposide resistance

Previous studies have shown that pharmacological induction of the UPR reduces cellular levels of topoisomerase II α protein and decreases their sensitivity to topoisomerase II targeting drugs. Since one aspect of the mammalian UPR is an arrest of cells in the G1 phase of cell cycle, which is known to lead to reduced levels of topoisomerase II α transcripts, we first determined the kinetics of topoisomerase II α loss in response to UPR activation. Thapsigargin inhibits the ER calcium ATPase, which leads to a depletion of ER calcium, thus altering the folding of proteins in this organelle and leading to a rapid induction of the UPR (Lee, 1992). NIH3T3 cells were treated with thapsigargin for the indicated times, and topoisomerase II α protein and mRNA levels were determined. As early as four hours after thapsigargin treatment, topoisomerase II α protein levels were dramatically decreased (Figure 1A), which corresponded to UPR induction as judged by the increased transcription of the *BiP* gene (Figure 1C). However, this was well before topoisomerase II α transcript levels were affected (Figure 1C), and too early for a significant number of the cells to arrest in G1 (data not shown and (Brewer *et al.*, 1999;Shen *et al.*, 1989). NIH3T3 cells were also treated with 2-deoxyglucose and tunicamycin (data not shown), both of which lead to inhibition of N-linked glycosylation and interfere with protein folding. Unlike thapsigargin, which can affect existing ER proteins, 2-deoxyglucose (DG) and tunicamycin only affect newly synthesized proteins, and therefore take somewhat longer to accumulate sufficient levels of unfolded proteins to activate the UPR. Accordingly, the loss of topoisomerase II α protein from 2-DG and tunicamycin treated cells was slower than that observed with thapsigargin (data not shown).

Molecular Pharmacology Fast Forward. Published on September 1, 2005 as DOI: 10.1124/mol.105.014753
This article has not been copyedited and formatted. The final version may differ from this version.

To determine the effects of thapsigargin on the sensitivity of NIH3T3 cells to etoposide, cells were cultured in the presence or absence of thapsigargin for 6 hrs and then treated with the indicated amount of etoposide for 2 hrs before reculturing them in medium lacking thapsigargin and etoposide. Cell number was determined 72 hr after etoposide treatment and expressed as a percent of cells remaining compared to cells that were not exposed to etoposide treatment (Figure 1B). For NIH3T3 cells not pretreated with thapsigargin (control), 10 μM etoposide reduced cell number to 16% of that found in the culture without etoposide, and 50 μM etoposide reduced the relative cell number to 8%. Pretreatment of the NIH3T3 cells with thapsigargin increased the relative cell number to 63% with 10 μM and 40% with 50 μM etoposide. At 100 μM etoposide, a concentration that reduced the number of non-thapsigargin-treated cells to <5%, nearly 50% of the thapsigargin-treated cells persisted after 72 hrs. Thus, thapsigargin treatment led to the loss of topoisomerase II α protein, but not transcripts, from NIH3T3 cells and afforded protection from etoposide-mediated killing. The loss of topoisomerase II protein is consistent with previous data obtained from various other cell lines with a variety of different agents known to induce ER stress (Shen *et al.*, 1989;Yun *et al.*, 1995), and our data suggest that this is mediated through post-transcriptional mechanisms, in agreement with an earlier study (Shen *et al.*, 1989).

UPR-induced loss of topoisomerase II from cells is restricted to the α form

Mammalian cells contain two different topoisomerase II isoforms, termed α and β (Drake *et al.*, 1987). Both isoforms are sensitive to etoposide *in vitro*, and confer etoposide sensitivity *in vivo* (Kaufmann *et al.*, 1998). Thus, we wished to determine if the effects of the UPR were specific to topoisomerase II α or targeted both topoisomerase II isozymes. The sequence similarity between the two isoforms and the lack of antibodies uniquely specific for the murine β

Molecular Pharmacology Fast Forward. Published on September 1, 2005 as DOI: 10.1124/mol.105.014753
This article has not been copyedited and formatted. The final version may differ from this version.

form made it necessary to perform this experiment in a human cell line. The human 293 embryonic kidney cell line was treated with thapsigargin for the indicated times, and cell lysates were prepared for western blotting (Figure 1D). Within 4 hr, topoisomerase II α levels had dropped dramatically, whereas topoisomerase II β levels remained unchanged even after 8 hr of thapsigargin treatment. These data demonstrate that the loss of topoisomerase II α during UPR activation is specific for the α isoform and is consistent with the possibility that the increased resistance of NIH3T3 cells to etoposide could to be due to changes in topoisomerase II α.

Increased levels of BiP are not sufficient to protect cells from etoposide or other DNA damaging agents

It has been suggested that the increased levels of BiP, a resident ER chaperone and the hallmark of the UPR, are responsible for protecting cells from etoposide during ER stress (Rao *et al.*, 2002;Reddy *et al.*, 2003). To examine this directly, we used a Chinese hamster ovary (CHO) cell line that had been engineered to over-express hamster BiP transcripts at levels comparable to those obtained during ER stress (Dorner *et al.*, 1992). As these cells are somewhat unstable due to either the over-expression of BiP or their inability to activate the UPR, we characterized our clone to ensure that it was deficient in UPR induction following treatment with agents that induce ER stress. As anticipated, when the signals were normalized for loading, we found that the CHO-BiP^{OE} cells were expressing the BiP transgene mRNA at levels similar to those observed in the parental line after 6 hr of thapsigargin treatment, and this level did not increase significantly after ER stress (Figure 2A). When protein levels were similarly analyzed, we found the BiP over-expressing cells actually had slightly higher levels of BiP than those achieved in the parental cell line after UPR induction, and as expected they were defective in their ability to induce CHOP protein (Figure 2B).

Molecular Pharmacology Fast Forward. Published on September 1, 2005 as DOI: 10.1124/mol.105.014753
This article has not been copyedited and formatted. The final version may differ from this version.

To examine the effects of BiP over-expression on sensitivity to topoisomerase II targeting drugs in the absence of ER stress, cells were exposed to etoposide briefly, then cellular sensitivity was assessed by determining cell number or by clonogenic survival. In the first experiments, CHO cells and CHO-BiP^{OE} cells were treated with etoposide for two hours. After etoposide exposure, cells were washed and recultured for 48 hrs. The number of cells present was determined by Coulter counting. For both CHO cells and CHO-BiP^{OE} cells, etoposide exposure caused a dose-dependent reduction in cell number, with no significant difference between the two cell lines (data not shown).

We also examined the sensitivity of CHO cells and CHO-BiP^{OE} cells to etoposide by clonogenic survival. Cells were treated with the indicated amounts of etoposide for 2 hrs, washed, and recultured in complete medium. After 7-10 days, the number of colonies were determined and plotted as a percent survival relative to untreated cells (Figure 3A). At concentrations less than10 μM etoposide, BiP over-expressing cells appeared slightly more sensitive to etoposide than the parental cells, whereas at the highest concentration of etoposide they were somewhat more resistant to etoposide sensitivity between the CHO and CHO-BiP^{OE} cells. These differences were not statistically significant (data not shown). Thus, increased levels of BiP that occur during UPR activation, and which are significantly less than that observed in the CHO-BiP^{OE} cell line, do not significantly protect against etoposide-mediated cell killing. Our results are the opposite of those reported previously where significant protection against etoposide mediated cell killing was observed in BiP over-expressing CHO cells (Reddy *et al.*, 2003).

Molecular Pharmacology Fast Forward. Published on September 1, 2005 as DOI: 10.1124/mol.105.014753
This article has not been copyedited and formatted. The final version may differ from this version.

Since BiP over-expression was hypothesized to provide protection against a broad range of apoptotic stimuli, we decided to examine the effect of BiP over-expression on sensitivity to other types of DNA damage that result in apoptotic cell death. We chose ionizing radiation as a DNA damaging agent because previous studies had suggested that UPR stress induction does not alter sensitivity to ionizing radiation (Hughes *et al.*, 1989). CHO and CHO-BiP^{OE} cells were exposed to ionizing radiation, as described in the materials and methods section, and sensitivity was assessed by clonogenic survival assays (Figure 3B). The sensitivity of the two cell lines to ionizing radiation was similar, with the CHO-BiP^{OE} cells actually showing slightly greater sensitivity to ionizing radiation. This is most apparent at higher doses, although after exposure to 10 Gy, there was less than a threefold difference in cell survival between the two cell lines. In results to be presented elsewhere, we also observed no difference in sensitivity between CHO and CHO-BiP^{OE} cells treated with the alkylating agents melphalan or methyl methane sulfonate. These results indicate that CHO-BiP^{OE} cells are not generally resistant to DNA damaging agents.

UPR activation is essential for both loss of topoisomerase II α and resistance to etoposide

The CHO cells that over-express BiP do not activate the UPR cascade in response to ER stress, since there are sufficient amounts of BiP to keep the UPR signal transducers in an inactive state (Bertolotti *et al.*, 2000;Shen *et al.*, 2002). This provided us with an opportunity to determine if UPR activation was required to reduce topoisomerase II α levels during ER stress conditions and provide protection from etoposide. CHO and CHO-BiP^{OE} cells were treated with thapsigargin for 6 or 16 hours, and, and cell lysates were prepared for western blotting. Similar to other cell lines examined, the parental CHO cells demonstrated decreased expression of topoisomerase II α in response to thapsigargin, (Figure 4A). When the CHO-BiP^{OE} cells that are unable to induce the ER stress response (Dorner *et al.*, 1992) were similarly treated,

Molecular Pharmacology Fast Forward. Published on September 1, 2005 as DOI: 10.1124/mol.105.014753
This article has not been copyedited and formatted. The final version may differ from this version.

topoisomerase II α levels remained unchanged, or slightly increased in response to thapsigargin. We also examined the effect of two other ER stress inducing agents on topoisomerase II α levels. Cells were treated with differing concentrations of either 2-deoxyglucose (Figure 4B) or dithiothreitol (Figure 4C) for six hours, and topoisomerase II α levels were examined. As was observed with thapsigargin, topoisomerase II α levels decreased in CHO cells following exposure to ER stress, while topoisomerase II α levels remained unchanged in CHO-BiP^{OE} cells. Since ER stress was still occurring in both cell lines, and unfolded proteins accumulated in both cell lines (Dorner *et al.*, 1992), these experiments demonstrate that the loss of topoisomerase II α from cells requires the activation of the UPR and is not occurring via a secondary effect of the drugs used to activate the UPR or to changes in the ER environment.

UPR activation is required for altered sensitivity of cells to etoposide in response to agents that cause ER stress

In order to determine the requirements for UPR activation in protecting cells from etoposide-mediated killing, we induced ER stress in CHO and CHO-BiP^{OE} cells before treating with etoposide and performed clonogenic survival assays. Both cell lines were pretreated with 1.0 μM thapsigargin for 6 hours before treating them with varying concentrations of etoposide for 2 h. After 7-10 days, surviving clones in each treatment group were counted and expressed as a percent of cells surviving after the appropriate UPR induction alone. CHO cells showed enhanced resistance to etoposide after induction of the UPR with both thapsigargin (Figure 5A). In both cases, induction of the ER stress prior to etoposide treatment increased clonal survival from ~0.2% to nearly 10% at the maximum dose of etoposide used. Although pre-treatment of CHO-BiP^{OE} cells with both UPR inducing agents did increase resistance of these cells to etoposide (Figure 5B), the level of protection was not as dramatic. The alterations in

Molecular Pharmacology Fast Forward. Published on September 1, 2005 as DOI: 10.1124/mol.105.014753
This article has not been copyedited and formatted. The final version may differ from this version.

chemosensitivity were also examined by comparing IC_{50} and IC_{90} for both cell lines (Table 1). Thapsigargin had little effect on the IC_{50} of etoposide treated CHO cells, but led to a 2 fold increase in the IC_{90} for etoposide. In contrast, there was no significant change seen in the IC_{50} or IC_{90} for etoposide with the CHO-BiP^{OE} cells. For comparison, Table 1 also shows that the IC_{50} or IC_{90} for ionizing radiation was unaffected by BiP over-expression. These experiments demonstrate that both loss of topoisomerase II and the decreased sensitivity of cells experiencing ER stress are dependent on activation of the UPR and are not an indirect effect of the drugs used to activate the UPR.

Our experiments demonstrated that UPR activation is essential for alterations in chemosensitivity of cells to topoisomerase II targeting agents, but they do not indicate if UPR activation is sufficient. Instead of using drugs to activate the UPR, which can have additional effects in the cells, we chose to induce the ER stress response by over-expressing an incompletely folded and unassembled Ig heavy chain in cells. The heavy chain binds to BiP (Haas and Wabl, 1983) and like many other BiP binding proteins is able to induce the UPR (Lenny and Green, 1991), perhaps by triggering the release of BiP from the ER stress transducers (Bertolotti *et al.*, 2000;Shen *et al.*, 2002). COS-1 cells were mock-transfected (control), transfected with GFP alone, or co-transfected with GFP and a human Ig γ heavy chain. Forty hours post-transfection the latter two were harvested and sorted for GFP$^+$ cells. Equal cell numbers for all three experimental sets were lysed in SDS-sample buffer and separated on SDS-polyacrylamide gels before transfer and western blotting with the indicated antibodies (Figure 6). The mock transfected cells served as a control for BiP and topoisomerase II levels, and as expected they did not express either γ heavy chains or CHOP, since they were not experiencing ER stress. The cells that were only expressing GFP were also negative for γ heavy chains, but

Molecular Pharmacology Fast Forward. Published on September 1, 2005 as DOI: 10.1124/mol.105.014753
This article has not been copyedited and formatted. The final version may differ from this version.

revealed a very minor induction of CHOP, perhaps due to the stress of transfection or cell sorting. The levels of BiP and topoisomerase II did not appear to be significantly affected. However, when cells expressing both GFP and γ heavy chains were examined, we found readily detectable levels of γ heavy chain, a concomitant induction of both BiP and CHOP demonstrating that the heavy chains had activated the UPR in these cells, and finally a dramatic loss of topoisomerase II α from the transfected cells. Thus, activation of the UPR without drugs is sufficient to induce loss of topoisomerase II α from cells.

Molecular Pharmacology Fast Forward. Published on September 1, 2005 as DOI: 10.1124/mol.105.014753
This article has not been copyedited and formatted. The final version may differ from this version.

DISCUSSION

A number of studies have shown that treatment of cells with agents that induce ER stress renders cells more resistant to treatment with drugs targeting topoisomerase II (Hughes *et al.*, 1989;Shen *et al.*, 1987). These studies demonstrated that ER stress did not lead to resistance to all cytotoxic agents, but were specific for drugs targeting topoisomerase II. In this work, we found that resistance to topoisomerase II targeting agents is a direct effect of UPR activation because it fails to occur in cells in which UPR activation is genetically blocked. Cells over-expressing BiP are unable to activate the UPR in response to ER stress, and we observed that these cells remained sensitive to etoposide even though they had been pre-incubated with ER stress-inducing agents. Although UPR inducing conditions do not alter drug accumulation (Shen *et al.*, 1987), it remained possible that ER stress caused the loss of critical proteins that abrogated the effects of topoisomerase II targeting drugs. By expressing an unfolded Ig heavy chain in the ER of cells, we obtained activation of the UPR without affecting the maturation of other secretory pathway proteins. These data demonstrated that expression of an unfolded protein was sufficient to reduce topoisomerase II protein levels, linking loss of topoisomerase II directly to UPR activation.

A critical aspect of drug resistance induced by UPR inducing agents is that the resistance is relatively specific for topoisomerase II targeting drugs. Resistance to DNA damage induced by ionizing radiation does not occur with UPR induction (Shen *et al.*, 1987). Some resistance to camptothecins has been reported (Tomida *et al.*, 1996), however, camptothecin resistance likely arises because of the G1 arrest induced by UPR inducing agents (Brewer *et al.*, 1999). Camptothecin cytotoxicity requires ongoing DNA replication (D'Arpa *et al.*, 1990;Holm *et al.*, 1989;Nitiss and Wang, 1996), therefore G1 arrest leads to camptothecin resistance. Importantly, for drugs such as cisplatin and some other alkylating agents, UPR induction leads to enhanced

Molecular Pharmacology Fast Forward. Published on September 1, 2005 as DOI: 10.1124/mol.105.014753
This article has not been copyedited and formatted. The final version may differ from this version.

drug sensitivity. These results suggest that UPR induction does not alter the ability of cells to commit to cell death, rather, induction of UPR provokes cellular responses that alter cell killing by specific classes of agents. Consistent with these results, we find that blocking UPR induction by BiP over-expression prevents the induction of resistance to topoisomerase II targeting agents, but does not affect sensitivity to ionizing radiation.

A simple hypothesis for resistance to topoisomerase II targeting agents by UPR induction is the observed reduction in topoisomerase II (Hughes *et al.*, 1989;Shen *et al.*, 1989;Yun *et al.*, 1995). Cell killing by topoisomerase targeting drugs occurs through DNA damage arising from the trapping of the enzyme in a drug:enzyme:DNA ternary complex. When enzyme levels are reduced, DNA damage is diminished, resulting in reduced cell killing (Nitiss *et al.*, 1992;Potmesil *et al.*, 1988). We observed that BiP over-expressing cells failed to reduce topoisomerase II α protein, and the cells remained sensitive to etoposide. Conversely, induction of UPR by expression of an unfolded protein results in the loss of topoisomerase II (Figure 6), demonstrating that UPR induction by an unfolded protein, as opposed to a drug, also alters topoisomerase II levels. Furthermore, we showed that UPR induction leads to the loss of topoisomerase II α, but not topoisomerase II β. This suggests that some sensitivity to topoisomerase II targeting agents should persist, even when the UPR is induced. It may be possible to specifically target the β isoform of topoisomerase II (Gao *et al.*, 1999), which could allow targeting of cells undergoing ER stress.

Several effects of UPR activation could lead to decreases in topoisomerase II protein. Topoisomerase II α is transcriptionally regulated during the cell cycle; with highest mRNA levels occurring during G2/M phase (Swedlow and Hirano, 2003). UPR activation initiates G1

Molecular Pharmacology Fast Forward. Published on September 1, 2005 as DOI: 10.1124/mol.105.014753
This article has not been copyedited and formatted. The final version may differ from this version.

arrest in mammalian cells due to a PERK-dependent loss of cyclin D1 protein translation (Brewer and Diehl, 2000). Thus, cell cycle arrest could cause topoisomerase II α loss by blocking its transcription. However, our data showed that topoisomerase II α loss occurs before cells accumulate in G1 and before topoisomerase II transcript levels are unaffected, in keeping with earlier studies (Shen *et al.*, 1989). These results suggest that loss of topoisomerase IIα during ER stress involves post-transcriptional mechanisms. One possibility is that the translational arrest occurring downstream of PERK could block topoisomerase II synthesis. Like cyclin D1, topoisomerase II α translation could be affected longer than most proteins in cells experiencing ER stress. Another mechanism for depleting topoisomerase II α could be via enhanced protein turnover. UPR activation leads to nuclear accumulation of proteasomes (Ogiso *et al.*, 2002) and proteasomal inhibitors attenuate both stress-induced resistance to etoposide and loss of topoisomerase II α (Carlsson *et al.*, 1978). Recently, a glucose-regulated destruction domain (GRDD) was identified on topoisomerase II α that provides a binding site for Jab1/CNS5, which may mediate the degradation of topoisomerase II during ER stress (Yun *et al.*, 2004). Jab1 binds to the cytosolic domain of inactive Ire1 in non-stressed cells, but it is released when Ire1 is activated (Oono *et al.*, 2004). It is unclear why loss of topoisomerase II α should be a consequence of UPR activation. BiP over-expressing cells do not show a survival disadvantage when exposed to agents that lead to ER stress (Morris *et al.*, 1997), indicating that topoisomerase II α probably does not negatively affect cell survival under these stress conditions. Unfortunately it has not been possible to ectopically express topoisomerase II α in mammalian cells (Salmena *et al.*, 2001) in order to determine whether enforced expression of this enzyme diminishes survival under UPR inducing conditions.

Molecular Pharmacology Fast Forward. Published on September 1, 2005 as DOI: 10.1124/mol.105.014753
This article has not been copyedited and formatted. The final version may differ from this version.

It has also been suggested that elevated expression of the ER chaperone BiP plays a direct role in resistance to topoisomerase II targeting agents (Rao *et al.*, 2002;Gosky and Chatterjee, 2003;Reddy *et al.*, 2003). Although BiP is normally localized in the ER, some studies have suggested that when high levels of BiP are synthesized, either due to transgene expression (Reddy *et al.*, 2003) or during later phases of the ER stress response (Rao *et al.*, 2002), BiP may also be found in the cytosol, where it inhibits caspase cleavage and apoptosis in response to etoposide. When BiP over-expressing cells were compared to the parental cell line, we saw no resistance to etoposide, even though they express somewhat more BiP protein than cells in which the UPR has been induced. This result is in direct contradiction to a recent report showing that BiP over-expression BiP was sufficient to confer resistance to topoisomerase II targeting drugs and camptothecin (Reddy *et al.*, 2003). Their experiments were carried out in CHO cells that were engineered to over-express BiP and were derived from the same source as our lines. While we cannot explain why our results differ from those reported, it is possible that the two BiP over-expressing cell lines do not express the same amount of BiP. We demonstrated that our BiP over-expressing cells remained impaired in UPR activation as expected (Morris *et al.*, 1997). In the other study, it was suggested that BiP over-expression protected cells from etoposide treatment by inhibiting caspase cleavage. This model is difficult to reconcile with the patterns of drug sensitivity previously reported upon UPR induction. First, if BiP over-expression blocks caspase activation, it might be expected that BiP over-expression would also affect sensitivity to many other type of DNA damage. However, we found that BiP over-expression did not affect sensitivity to ionizing radiation, which is consistent with a previous study where UPR induction did not affect the sensitivity of cells to this type of DNA damage (Hughes *et al.*, 1989). Second, UPR induction actually increases cell killing by certain DNA damaging agents like cisplatin (Chatterjee *et al.*, 1997), which is also inconsistent with increased levels of BiP directly inhibiting apoptosis. Finally, it is clear from the results of Lock and

Molecular Pharmacology Fast Forward. Published on September 1, 2005 as DOI: 10.1124/mol.105.014753
This article has not been copyedited and formatted. The final version may differ from this version.

colleagues that topoisomerase II mediated DNA damage is correlated with topo II protein levels, which are reduced upon exposure to UPR inducing conditions (Shen *et al.*, 1989;Webb *et al.*, 1991). Our results show that topoisomerase II levels are similar between the two cell lines. Topoisomerase II protein is reduced in wild type CHO cells upon UPR induction but not in BiP over-expressing cells, which similarly show a continued sensitivity to etoposide during UPR activation. Together, our results are most consistent with some aspect of UPR induction other than BiP over-expression *per se* causing resistance to topoisomerase II targeting agents.

A resolution of the differences between our results and the results reported by Lee and colleagues will require a finer dissection of the UPR pathway, and identification of the branch(es) that are directly responsible for UPR induced loss of topoisomerase II. It should be possible to block steps in the UPR pathway that lead to loss of topoisomerase II, but leave other parts of the pathway intact, and determine whether loss of topoisomerase II is necessary and sufficient for UPR mediated resistance to topoisomerase II agents. These studies should also be useful in identifying potential mechanisms of increasing the sensitivity of tumor cells to chemotherapeutic agents, or minimizing their effects on normal cells.

Molecular Pharmacology Fast Forward. Published on September 1, 2005 as DOI: 10.1124/mol.105.014753
This article has not been copyedited and formatted. The final version may differ from this version.

REFERENCES

Bertolotti A, Zhang Y, Hendershot L M, Harding H P and Ron D (2000) Dynamic Interaction of BiP and ER Stress Transducers in the Unfolded-Protein Response. *Nat Cell Biol* **2**:326-332.

Bole DG, Hendershot L M and Kearney J F (1986) Posttranslational Association of Immunoglobulin Heavy Chain Binding Protein With Nascent Heavy Chains in Nonsecreting and Secreting Hybridomas. *J Cell Biol* **102**:1558-1566.

Brewer JW, Cleveland J L and Hendershot L M (1997) A Pathway Distinct From the Mammalian Unfolded Protein Response Regulates Expression of Endoplasmic Reticulum Chaperones in Non-Stressed Cells. *EMBO J* **16**:7207-7216.

Brewer JW and Diehl J A (2000) PERK Mediates Cell-Cycle Exit During the Mammalian Unfolded Protein Response. *Proc Natl Acad Sci U S A* **97**:12625-12630.

Brewer JW, Hendershot L M, Sherr C J and Diehl J A (1999) Mammalian Unfolded Protein Response Inhibits Cyclin D1 Translation and Cell-Cycle Progression. *Proc Natl Acad Sci U S A* **96**:8505-8510.

Brostrom CO, Prostko C R, Kaufman R J and Brostrom M A (1996) Inhibition of Translational Initiation by Activators of the Glucose-Regulated Stress Protein and Heat Shock Protein Stress Response Systems. Role of the Interferon-Inducible Double-Stranded RNA-Activated Eukaryotic Initiation Factor 2alpha Kinase. *J Biol Chem* **271**:24995-25002.

Calfon M, Zeng H, Urano F, Till J H, Hubbard S R, Harding H P, Clask S G and Ron D (2002) IRE1 Couples Endoplasmic Reticulum Load to Secretory Capacity by Processing the *XBP-1* MRNA. *Nature* **415**:92-96.

Carlsson J, Drevin H and Axen R (1978) Protein Thiolation and Reversible Protein-Protein Conjugation. N-Succinimidyl 3-(2-Pyridyldithio)Propionate, a New Heterobifunctional Reagent. *Biochem J* **173**:723-737.

Chatterjee S, Hirota H, Belfi C A, Berger S J and Berger N A (1997) Hypersensitivity to DNA Cross-Linking Agents Associated With Up-Regulation of Glucose-Regulated Stress Protein GRP78. *Cancer Res* **57**:5112-5116.

Chen AY and Liu L F (1994) DNA Topoisomerases: Essential Enzymes and Lethal Targets. *Annu Rev Pharmacol Toxicol* **34**:191-218.

Chikamori K, Grabowski D R, Kinter M, Willard B B, Yadav S, Aebersold R H, Bukowski R M, Hickson I D, Andersen A H, Ganapathi R and Ganapathi M K (2003) Phosphorylation of Serine 1106 in the Catalytic Domain of Topoisomerase II Alpha Regulates Enzymatic Activity and Drug Sensitivity. *J Biol Chem* **278**:12696-12702.

D'Arpa P, Beardmore C and Liu L F (1990) Involvement of Nucleic Acid Synthesis in Cell Killing Mechanisms of Topoisomerase Poisons. *Cancer Res* **50**:6919-6924.

Dorner AJ, Wasley L C and Kaufman R J (1992) Overexpression of GRP78 Mitigates Stress Induction of Glucose Regulated Proteins and Blocks Secretion of Selective Proteins in Chinese Hamster Ovary Cells. *EMBO J* **11**:1563-1571.

Drake FH, Zimmerman J P, McCabe F L, Bartus H F, Per S R, Sullivan D M, Ross W E, Mattern M R, Johnson R K, Crooke S T and . (1987) Purification of Topoisomerase II From Amsacrine-Resistant P388 Leukemia Cells. Evidence for Two Forms of the Enzyme. *J Biol Chem* **262**:16739-16747.

Gao H, Huang K C, Yamasaki E F, Chan K K, Chohan L and Snapka R M (1999) XK469, a Selective Topoisomerase IIbeta Poison. *Proc Natl Acad Sci U S A* **96**:12168-12173.

Molecular Pharmacology Fast Forward. Published on September 1, 2005 as DOI: 10.1124/mol.105.014753
This article has not been copyedited and formatted. The final version may differ from this version.

Gosky D and Chatterjee S (2003) Down-Regulation of Topoisomerase II Alpha Is Caused by Up-Regulation of GRP78. *Biochem Biophys Res Commun* **300**:327-332.

Haas IG and Wabl M (1983) Immunoglobulin Heavy Chain Binding Protein. *Nature* **306**:387-389.

Hampton RY (2000) ER Stress Response: Getting the UPR Hand on Misfolded Proteins. *Curr Biol* **10**:R518-R521.

Harding HP, Novoa I, Zhang Y, Zeng H, Wek R, Schapira M and Ron D (2000) Regulated Translation Initiation Controls Stress-Induced Gene Expression in Mammalian Cells. *Mol Cell* **6**:1099-1108.

Harding HP, Zhang Y and Ron D (1999) Protein Translation and Folding Are Coupled by an Endoplasmic-Reticulum-Resident Kinase. *Nature* **397**:271-274.

Hendershot LM, Wei J-Y, Gaut J R, Lawson B, Freiden P J and Murti K G (1995) In Vivo Expression of Mammalian BiP ATPase Mutants Causes Disruption of the Endoplasmic Reticulum. *Mol Biol Cell* **6**:283-296.

Hitomi J, Katayama T, Eguchi Y, Kudo T, Taniguchi M, Koyama Y, Manabe T, Yamagishi S, Bando Y, Imaizumi K, Tsujimoto Y and Tohyama M (2004) Involvement of Caspase-4 in Endoplasmic Reticulum Stress-Induced Apoptosis and A{Beta}-Induced Cell Death. *J Cell Biol* **165**:347-356.

Holm C, Covey J M, Kerrigan D and Pommier Y (1989) Differential Requirement of DNA Replication for the Cytotoxicity of DNA Topoisomerase I and II Inhibitors in Chinese Hamster DC3F Cells. *Cancer Res* **49**:6365-6368.

Hughes CS, Shen J W and Subjeck J R (1989) Resistance to Etoposide Induced by Three Glucose-Regulated Stresses in Chinese Hamster Ovary Cells. *Cancer Res* **49**: pp 4452-4454.

Kaufmann SH (1998) Cell Death Induced by Topoisomerase-Targeted Drugs: More Questions Than Answers. *Biochim Biophys Acta* **1400**:195-211.

Kaufmann SH, Gore S D, Miller C B, Jones R J, Zwelling L A, Schneider E, Burke P J and Karp J E (1998) Topoisomerase II and the Response to Antileukemic Therapy. *Leuk Lymphoma* **29**:217-237.

Lee AS (1987) Coordinated Regulation of a Set of Genes by Glucose and Calcium Ionophores in Mammalian Cells. *Trends Biochem Sci* **12**:20-23.

Lee AS (1992) Mammalian Stress Response:Induction of the Glucose-Regulated Protein Family. *Curr Opin Cell Biol* **4**:267-273.

Lenny N and Green M (1991) Regulation of Endoplasmic Reticulum Stress Proteins in COS Cells Transfected With Immunoglobulin Mu Heavy Chain CDNA. *J Biol Chem* **266**:20532-20537.

Li TK and Liu L F (2001) Tumor Cell Death Induced by Topoisomerase-Targeting Drugs. *Annu Rev Pharmacol Toxicol* **41**:53-77.

Liu AY, Mack P W, Champion C I and Robinson R R (1987) Expression of Mouse::Human Immunoglobulin Heavy-Chain CDNA in Lymphoid Cells. *Gene* **54**:33-40.

Ma Y and Hendershot L M (2003) Delineation of the Negative Feedback Regulatory Loop That Controls Protein Translation During ER Stress. *J Biol Chem* **278**:34864-34873.

Ma Y and Hendershot L M (2004) The Role of the Unfolded Protein Response in Tumour Development: Friend or Foe? *Nat Rev Cancer* **4**:966-977.

Mao Y, Desai S D and Liu L F (2000) SUMO-1 Conjugation to Human DNA Topoisomerase II Isozymes. *J Biol Chem* **275**:26066-26073.

Melero JA and Fincham V (1978) Enhancement of the Synthesis of Specific Cellular Polypeptides in a Temperature-Sensitive Chinese Hamster Cell Line (K12) Defective for Entry into S Phase. *J Cell Physiol* **95**:295-306.

Molecular Pharmacology Fast Forward. Published on September 1, 2005 as DOI: 10.1124/mol.105.014753
This article has not been copyedited and formatted. The final version may differ from this version.

Morris JA, Dorner A J, Edwards C A, Hendershot L M and Kaufman R J (1997) Immunoglobulin Binding Protein (BiP) Function Is Required to Protect Cells From Endoplasmic Reticulum Stress but Is Not Required for the Secretion of Selective Proteins. *J Biol Chem* **272**:4327-4334.

Nakagawa T, Zhu H, Morishima N, Li E, Xu J, Yankner B A and Yuan J (2000) Caspase-12 Mediates Endoplasmic-Reticulum-Specific Apoptosis and Cytotoxicity by Amyloid-Beta. *Nature* **403**:98-103.

Nitiss JL (1998) Investigating the Biological Functions of DNA Topoisomerases in Eukaryotic Cells. *Biochim Biophys Acta* **1400**:63-81.

Nitiss JL and Beck W T (1996) Antitopoisomerase Drug Action and Resistance. *Eur J Cancer* **32A**:958-966.

Nitiss JL, Liu Y X, Harbury P, Jannatipour M, Wasserman R and Wang J C (1992) Amsacrine and Etoposide Hypersensitivity of Yeast Cells Overexpressing DNA Topoisomerase II. *Cancer Res* **52**:4467-4472.

Nitiss JL, Liu Y X and Hsiung Y (1993) A Temperature Sensitive Topoisomerase II Allele Confers Temperature Dependent Drug Resistance on Amsacrine and Etoposide: a Genetic System for Determining the Targets of Topoisomerase II Inhibitors. *Cancer Res* **53**:89-93.

Nitiss JL and Wang J C (1996) Mechanisms of Cell Killing by Drugs That Trap Covalent Complexes Between DNA Topoisomerases and DNA. *Mol Pharmacol* **50**:1095-1102.

Ogiso Y, Tomida A and Tsuruo T (2002) Nuclear Localization of Proteasomes Participates in Stress-Inducible Resistance of Solid Tumor Cells to Topoisomerase II-Directed Drugs. *Cancer Res* **62**:5008-5012.

Oono K, Yoneda T, Manabe T, Yamagishi S, Matsuda S, Hitomi J, Miyata S, Mizuno T, Imaizumi K, Katayama T and Tohyama M (2004) JAB1 Participates in Unfolded Protein Responses by Association and Dissociation With IRE1. *Neurochem Int* **45**:765-772.

Osheroff N (1998) DNA Topoisomerases. *Biochim Biophys Acta* **1400**:1-2.

Potmesil M, Hsiang Y H, Liu L F, Bank B, Grossberg H, Kirschenbaum S, Forlenza T J, Penziner A, Kanganis D and . (1988) Resistance of Human Leukemic and Normal Lymphocytes to Drug-Induced DNA Cleavage and Low Levels of DNA Topoisomerase II. *Cancer Res* **48**:3537-3543.

Rao RV, Peel A, Logvinova A, Del Rio G, Hermel E, Yokota T, Goldsmith P C, Ellerby L M, Ellerby H M and Bredesen D E (2002) Coupling Endoplasmic Reticulum Stress to the Cell Death Program: Role of the ER Chaperone GRP78. *FEBS Lett* **514**:122-128.

Reddy RK, Mao C, Baumeister P, Austin R C, Kaufman R J and Lee A S (2003) Endoplasmic Reticulum Chaperone Protein GRP78 Protects Cells From Apoptosis Induced by Topoisomerase Inhibitors: Role of ATP Binding Site in Suppression of Caspase-7 Activation. *J Biol Chem* **278**:20915-20924.

Salmena L, Lam V, McPherson J P and Goldenberg G J (2001) Role of Proteasomal Degradation in the Cell Cycle-Dependent Regulation of DNA Topoisomerase IIalpha Expression. *Biochem Pharmacol* **61**:795-802.

Shen J, Chen X, Hendershot L and Prywes R (2002) ER Stress Regulation of ATF6 Localization by Dissociation of BiP/GRP78 Binding and Unmasking of Golgi Localization Signals. *Dev Cell* **3**:99-111.

Shen J, Hughes C, Chao C, Cai J, Bartels C, Gessner T and Subjeck J (1987) Coinduction of Glucose-Regulated Proteins and Doxorubicin Resistance in Chinese Hamster Cells. *Proc Natl Acad Sci U S A* **84**:3278-3282.

Shen JW, Subjeck J R, Lock R B and Ross W E (1989) Depletion of Topoisomerase II in Isolated Nuclei During a Glucose-Regulated Stress Response. *Mol Cell Biol* **9**:3284-3291.

Shi Y, Vattem K M, Sood R, An J, Liang J, Stramm L and Wek R C (1998) Identification and Characterization of Pancreatic Eukaryotic Initiation Factor 2 Alpha-Subunit Kinase, PEK, Involved in Translational Control. *Mol Cell Biol* **18**:7499-7509.

Swedlow JR and Hirano T (2003) The Making of the Mitotic Chromosome: Modern Insights into Classical Questions. *Mol Cell* **11**:557-569.

Tomida A, Yun J and Tsuruo T (1996) Glucose-Regulated Stresses Induce Resistance to Camptothecin in Human Cancer Cells. *International Journal of Cancer* **68**:391-396.

Walker JV and Nitiss J L (2002) DNA Topoisomerase II As a Target for Cancer Chemotherapy. *Cancer Invest* **20**:570-589.

Wang JC (1996) DNA Topoisomerases. *Annu Rev Biochem* **65**:635-692.

Webb CD, Latham M D, Lock R B and Sullivan D M (1991) Attenuated Topoisomerase II Content Directly Correlates With a Low Level of Drug Resistance in a Chinese Hamster Ovary Cell Line. *Cancer Res* **51**:6543-6549.

Ye J, Rawson R B, Komuro R, Chen X, Dave U P, Prywes R, Brown M S and Goldstein J L (2000) ER Stress Induces Cleavage of Membrane-Bound ATF6 by the Same Proteases That Process SREBPs. *Mol Cell* **6**:1355-1364.

Yoshida H, Matsui T, Yamamoto A, Okada T and Mori K (2001) XBP1 mRNA is Induced by ATF6 and Spliced by IRE1 in Response to ER Stress to Produce a Highly Active Transcription Factor. *Cell* **107**:881-891.

Yoshida H, Matsui T, Hosokawa N, Kaufman R J, Nagata K and Mori K (2003) A Time-Dependent Phase Shift in the Mammalian Unfolded Protein Response. *Dev Cell* **4**:265-271.

Yun J, Tomida A, Andoh T and Tsuruo T (2004) Interaction Between Glucose-Regulated Destruction Domain of DNA Topoisomerase IIalpha and MPN Domain of Jab1/CSN5. *J Biol Chem* **279**:31296-31303.

Yun J, Tomida A, Nagata K and Tsuruo T (1995) Glucose-Regulated Stresses Confer Resistance to VP-16 in Human Cancer Cells Through a Decreased Expression of DNA Topoisomerase II. *Oncol Res* **7**:583-590.

Molecular Pharmacology Fast Forward. Published on September 1, 2005 as DOI: 10.1124/mol.105.014753
This article has not been copyedited and formatted. The final version may differ from this version.

Footnotes:

This work was supported by NCI Grant CA23099, the Cancer Center CORE Grant CA21765 and the American Lebanese Syrian Associated Charities of St. Jude Children's Research Hospital.

Molecular Pharmacology Fast Forward. Published on September 1, 2005 as DOI: 10.1124/mol.105.014753
This article has not been copyedited and formatted. The final version may differ from this version.

FIGURE LEGENDS

Figure 1. Treatment of cells with thapsigargin induces resistance to etoposide and rapid depletion of topoisomerase II α. A. NIH3T3 cells were treated with 1.0 μM thapsigargin for indicated times. The cell extracts were prepared and topoisomerase II levels were assessed by western blotting using an anti-topoisomerase II α specific antibody. β-actin was probed as a loading control. **B.** NIH3T3 cells were pre-treated with 2.0 μM thapsigargin for 6 hrs and then incubated with the indicated etoposide concentrations for 2 hours. Cells were washed and recultured for three days at which time the cell number was assessed and expressed as a percent of cells surviving with no etoposide treatment. **C.** NIH3T3 cells were treated with either 2.5 μg/ml tunicamycin (Tunic) or 2.0 μM thapsigargin (Thaps) for the indicated number of hours. RNA was isolated, separated by gel electrophoresis and probed for BiP and topoisomerase II α transcripts. A probe directed against G3DPH mRNA was used as a loading control. **D.** 293 cells were treated with 2.0 μM thapsigargin for indicated times. Cell extracts were prepared and protein levels were assessed by western blotting using an anti-human topoisomerase II α antibody and an anti-human topoisomerase II β antibody. β-actin was probed as a loading control.

Figure 2. Over-expression of BiP prevents UPR induction. A. CHO and CHO-BiP^{OE} cells were treated with 5.0 μM thapsigargin for 6 hours. RNA was isolated and a northern blot was hybridized with a 1.5 kB *PstI-EcoRI* fragment from a hamster BiP cDNA clone. The positions of the endogenous BiP mRNA and the larger exogenous form are indicated to the right. GAPDH levels were hybridized as a loading control. **B.** CHO and CHO-BiP^{OE} cells were treated with 1.0 μM thapsigargin for 6 hours. Cell extracts were prepared and protein levels were determined by western blotting using a rabbit polyclonal anti-BiP antiserum. β-actin was probed as a loading control.

Figure 3. Over-expression of BiP does not alter sensitivity to etoposide or ionizing radiation as determined by clonogenic survival. A. CHO and CHO-BiP^{OE} cells were treated with the indicated etoposide concentrations for 2 hours, after which cells were washed, trypsinized, and recultured. Varying numbers of cells were plated in triplicate for each drug dosage and re-incubated for 10 days. Survival is expressed as percent of colonies formed with no etoposide treatment. **B.** CHO and CHO-BiP^{OE} cells were exposed to increasing doses of ionizing radiation. Appropriate concentrations of cells depending on radiation dosage were plated in triplicate. Colonies were stained and counted after 10 days of incubation.

Figure 4. Induction of the UPR pathway is necessary to induce depletion of topoisomerase II α. A. CHO and CHO-BiP^{OE} cells were treated with 1.0 μM thapsigargin for varying amounts of time. Cell extracts were prepared, separated by electrophoresis, and transferred for western blotting. Topoisomerase II levels were assessed using a polyclonal anti-topoisomerase II α antibody. Hsc70 protein levels were detected with a polyclonal anti-Hsc70 antibody, which served as a control for sample loading. These two cell lines were also treated with increasing concentrations of either 2-deoxyglucose (**B.**) or dithiothreitol (**C.**) for 6 hours. Cell extracts were prepared and topoisomerase II levels were assessed using an anti-topoisomerase II α antibody. β-actin levels were determined and served as a loading control.

Molecular Pharmacology Fast Forward. Published on September 1, 2005 as DOI: 10.1124/mol.105.014753
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 5. Induction of the UPR is necessary for increased resistance to etoposide as determined by clonogenic survival. CHO (**A.**) and CHO-BiP^{OE} (**B.**) cells were pre-treated with 1.0 μM thapsigargin for 6 hours followed by the indicated etoposide concentrations for 2 hours. Cells were washed, trypsinized, and varying numbers of cells were re-plated in triplicate. After re-incubating for 10 days,, colonies were stained, counted and expressed as a percent of cells surviving after treatment with thapsigargin alone.

Figure 6. UPR induction is sufficient to induce reduction in topoisomerase II α levels. COS-1 cells were co-transfected with an empty pSG5 vector and pGreen Lantern or with a pSG5 vector encoding the Ig γ heavy chain plus pGreen Lantern. Forty-eight hrs post-transfection, GFP-positive cells were isolated and cell lysates were prepared and analyzed by western blotting with the indicated antisera.

Molecular Pharmacology Fast Forward. Published on September 1, 2005 as DOI: 10.1124/mol.105.014753
This article has not been copyedited and formatted. The final version may differ from this version.

Table 1. IC50's and IC90's for CHO and CHO-BiP^{OE} clonogenic assays

Cell Line	Treatment	IC_{50}	IC_{90}
CHO	Etoposide 2 h	3.4 μM	9.5 μM
	10 μM Tg 6 h + etoposide 2 h	4.1 μM	18.5 μM
	Irradiation	1.6 Gy	5.0 Gy
CHO-BiP^{OE}	Etoposide 2 h	1.5 μM	7.5 μM
	10 μM Tg 6 h + etoposide 2 h	0.9 μM	7.5 μM
	Irradiation	1.6 Gy	4.2 Gy

Figure 1

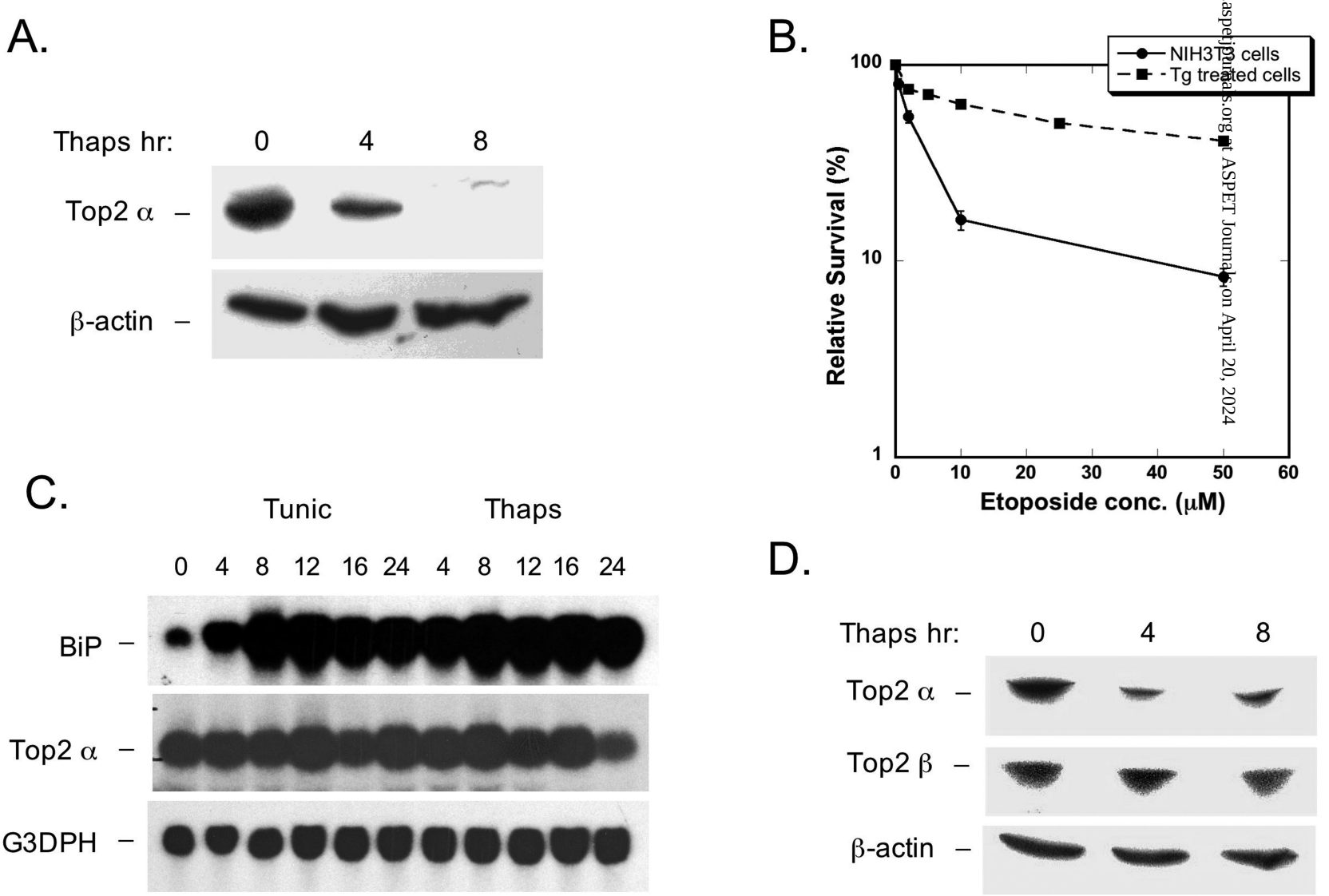


Figure 2

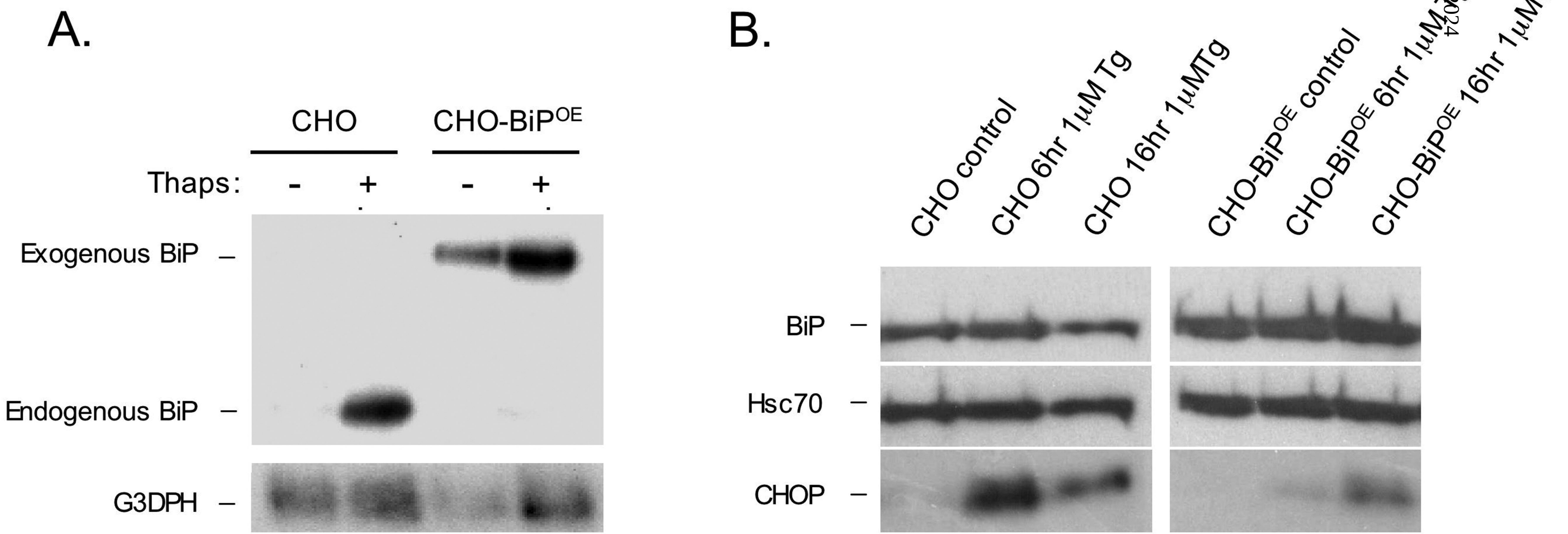


Figure 3

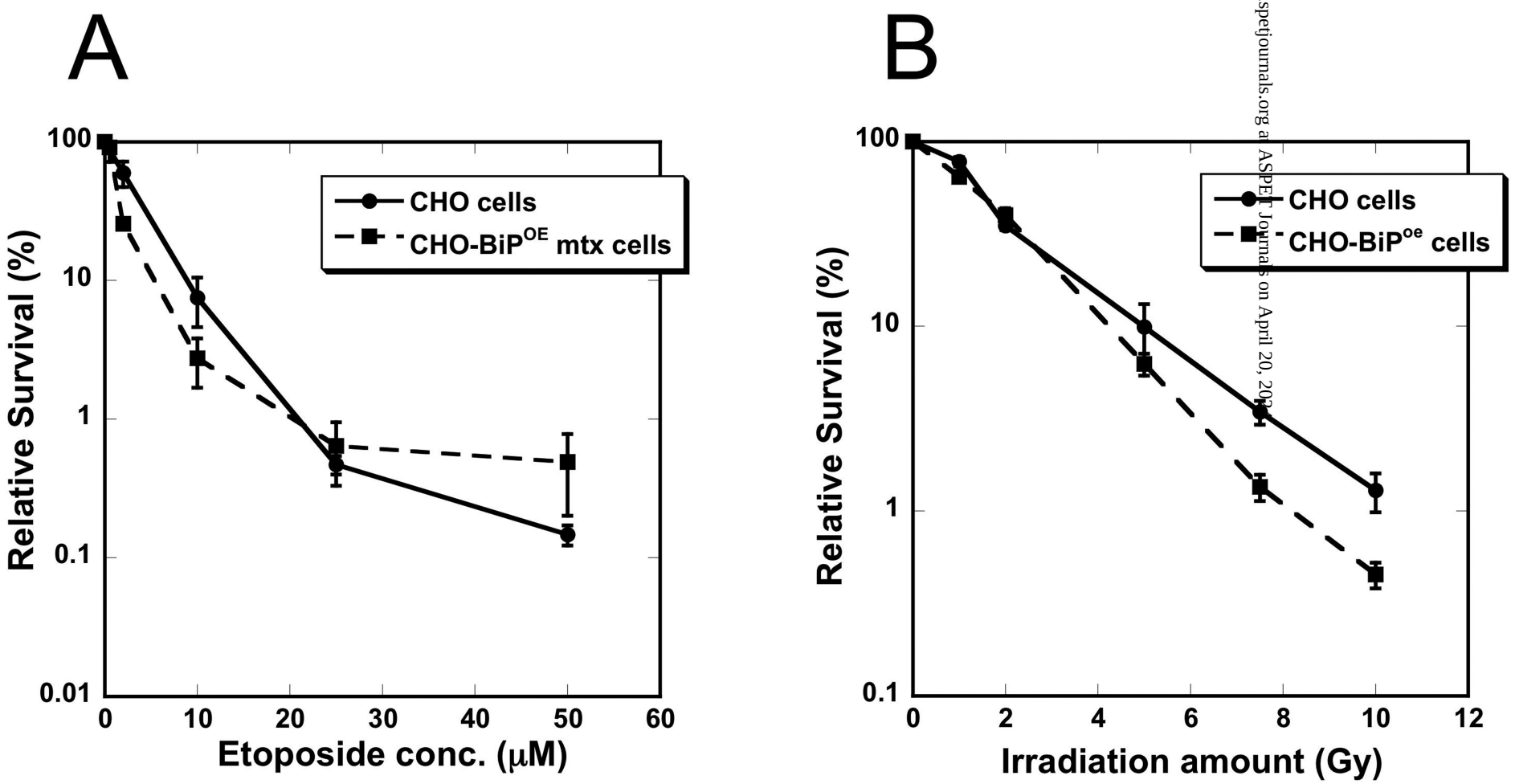


Figure 4

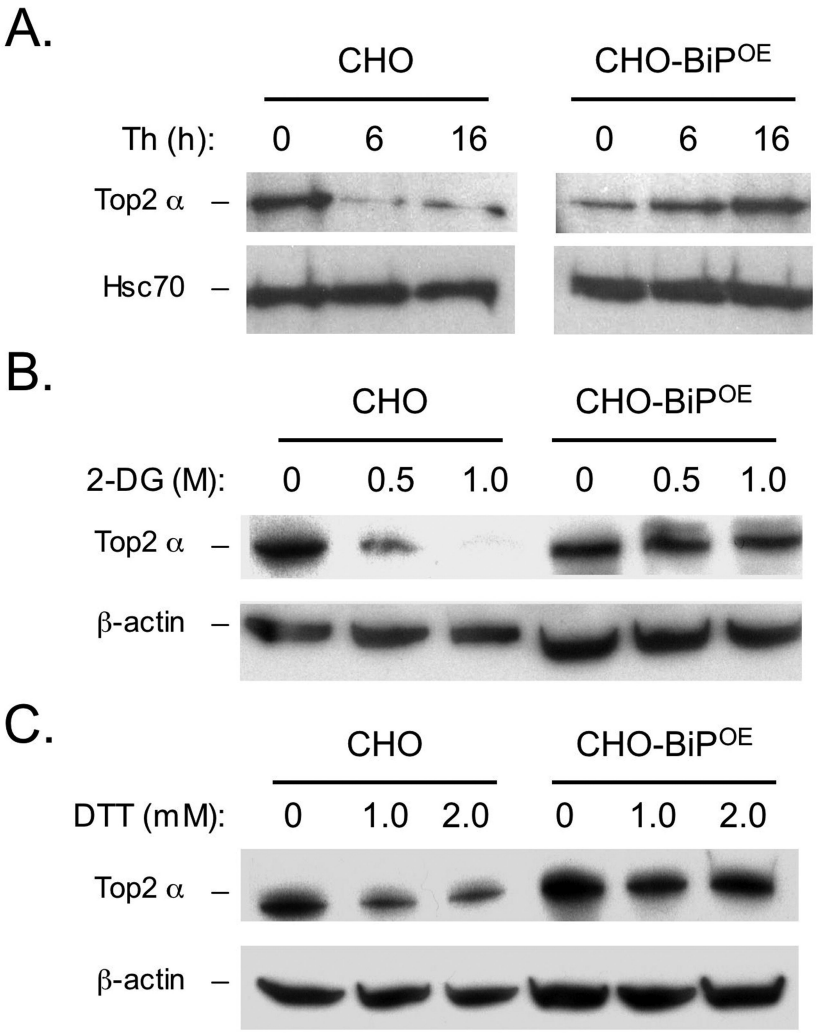
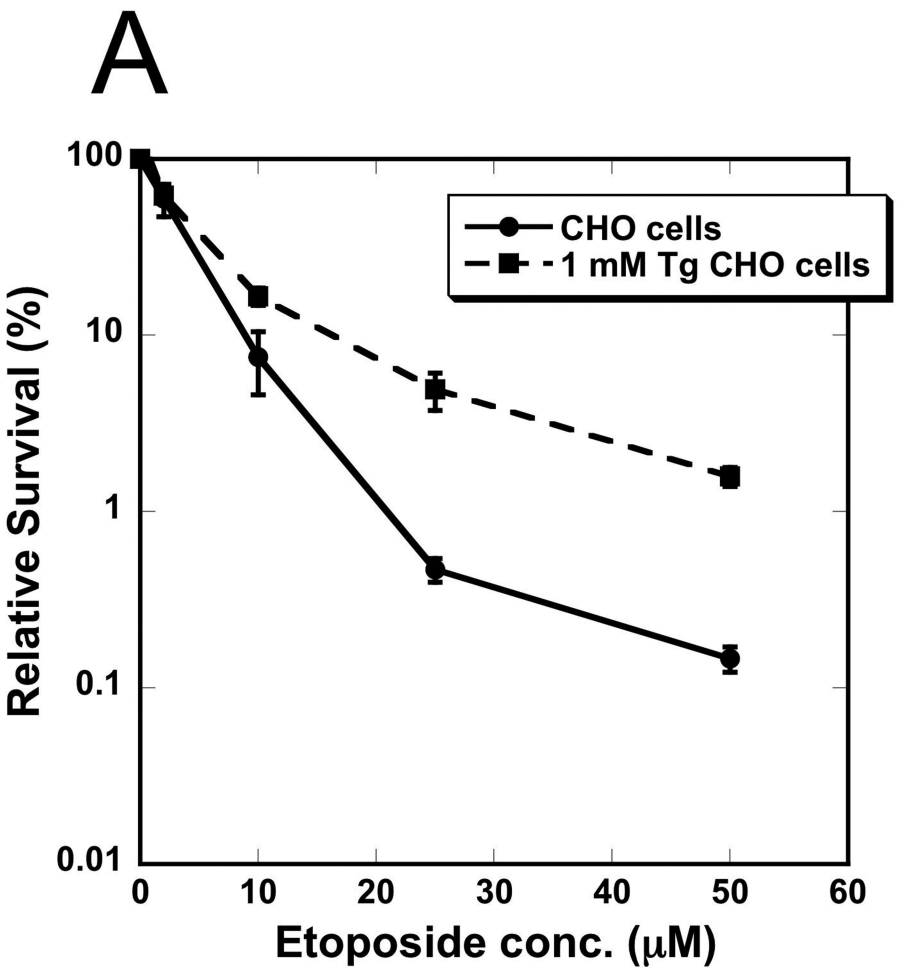

A.
CHO
CHO-BiP^OE
Th (h):
0
6
16
0
6
16
Top2 α
Hsc70
B.
CHO
CHO-BiP^OE
2-DG (M):
0
0.5
1.0
0
0.5
1.0
Top2 α
β-actin
C.
CHO
CHO-BiP^OE
DTT (mM):
0
1.0
2.0
0
1.0
2.0
Top2 α
β-actin

Figure 5



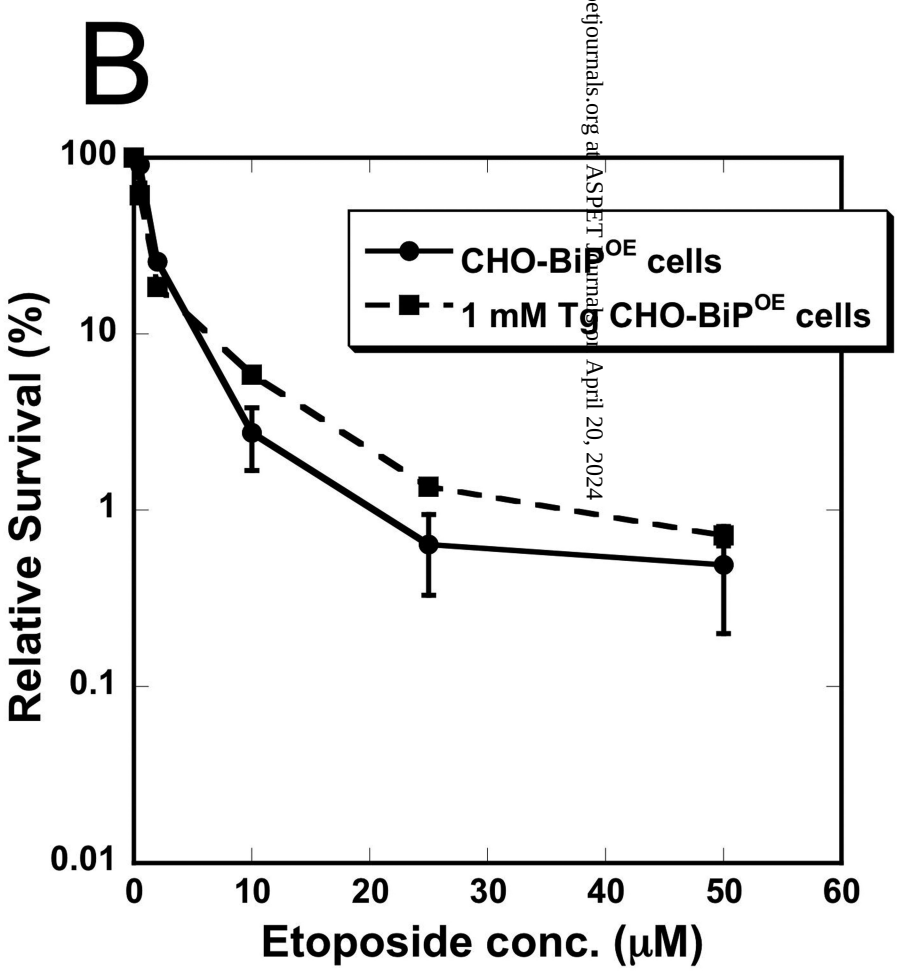
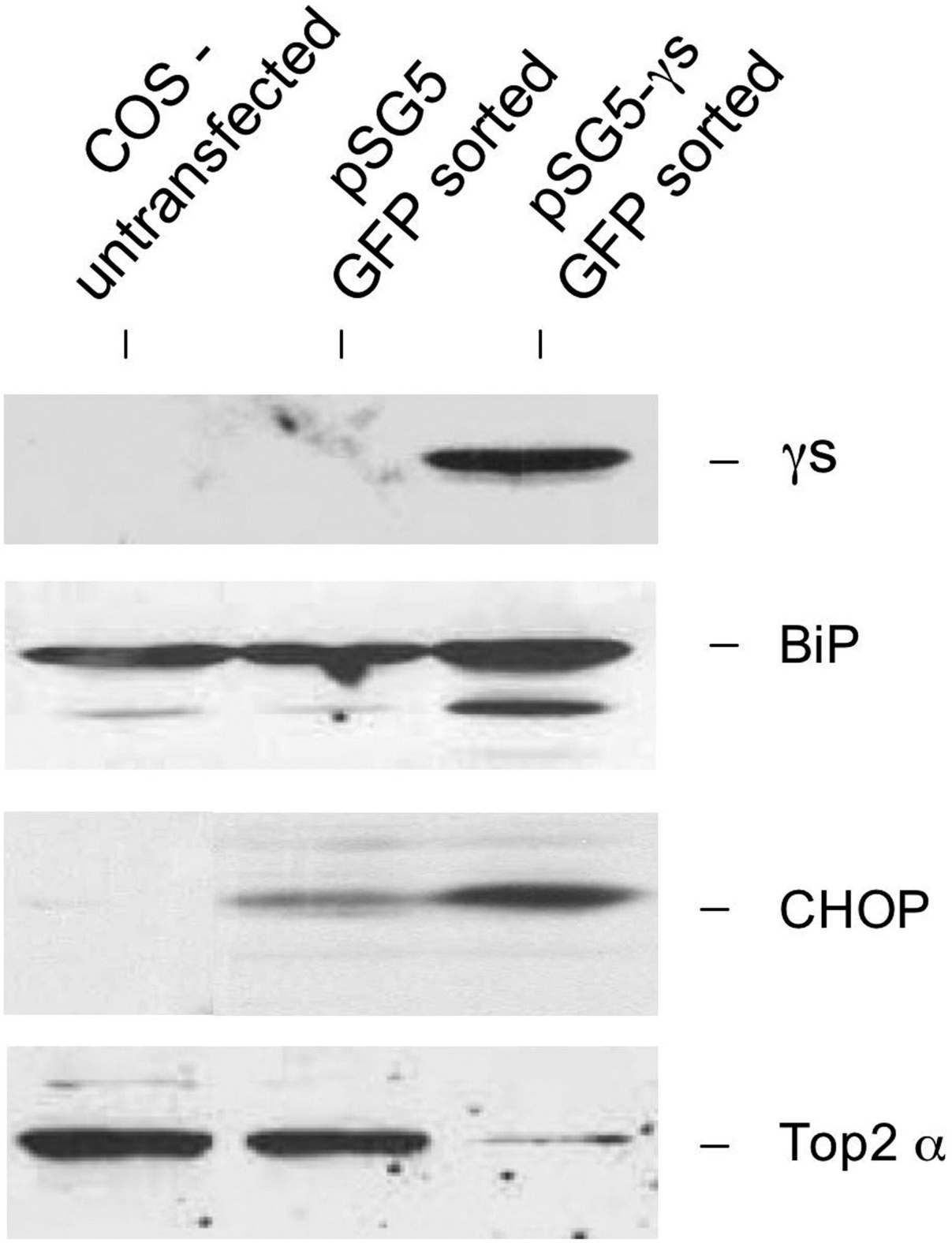


Figure 6


COS - untransfected
pSG5 GFP sorted
pSG5-γs GFP sorted
– γs
– BiP
– CHOP
– Top2 α