Glutathione Depletion by L-Buthionine Sulfoximine Antagonizes Taxol Cytotoxicity

James E. Liebmann, Stephen M. Hahn, John A. Cook, Claudia Lipschultz, James B. Mitchell, and Dwight C. Kaufman

ABSTRACT

Taxol is a naturally occurring chemotherapeutic agent that is active against a variety of tumors. Taxol is believed to act by binding tightly to microtubules and preventing their disaggregation. Others have shown that depletion of cellular glutathione results in the disaggregation of microtubules, presumably by allowing the oxidation of some or all of the cysteine residues in tubulins. We studied the effect of glutathione (GSH) depletion by L-buthionine sulfoximine (L-BSO) on taxol cytotoxicity in two human tumor lines. After a 24-h incubation in 5 mM L-BSO, the breast adenocarcinoma line MCF-7 and the lung adenocarcinoma line A549 were exposed to varying concentrations of taxol for 24 h. GSH levels were undetectable in cells treated with L-BSO. At the highest concentrations of taxol (50 mM), control MCF-7 cells had 10% cell survival and control A549 cells had only 1% cell survival as assessed by clonogenic assay. Pretreatment with 5 mM L-BSO resulted in a 3-fold increase in survival of MCF-7 cells and a 10-fold increase in survival of A549 cells. Pretreatment with L-BSO had no effect on taxol uptake into A549 or MCF-7 cells, as assessed by measurement of binding of [3H]taxol to cells. Following exposure to 37 mM taxol for 24 h, both cell lines had over 80% of their population in G2/M and bromodeoxyuridine labeling showed that taxol markedly reduced the percentage of cells in S phase. L-BSO pretreatment had no effect on the cell cycle in either cell line in the absence of taxol. However, in cells treated with taxol, L-BSO increased the percentage of cells in S phase by 3-fold in both cell lines. We conclude that depletion of cellular GSH by L-BSO results in resistance to taxol in MCF-7 and A549 cells. Resistance to taxol mediated by GSH depletion is not due to alterations in cellular uptake of taxol by L-BSO. L-BSO increased the S-phase fraction of taxol-treated cells in both cell lines. These data suggest that GSH depletion interferes with cell cycle changes induced by taxol. The alteration in taxol-induced cell cycle changes may account for the resistance to taxol produced by L-BSO.

INTRODUCTION

Natural products have been actively pursued as potential anticancer agents. Taxol, a diterpene derived from the yew tree Taxus brevifolia, has demonstrated activity against breast and lung tumors in preclinical animal models (1). Clinical trials have shown activity against ovarian and breast cancer (2, 3). While the exact mechanism of taxol cytotoxicity is unknown, there is evidence that its antitumor effects result from cell cycle effects may account for the resistance to taxol produced by L-BSO.

[1H]Taxol Binding. Cells were plated into 24-well plates at a concentration of 50,000 cells/well. Cells were plated in either control medium or medium containing 5 mM L-BSO. Twenty-four h later, 0.1 μCi of [3H]taxol was added to each well (8.7 μCi final concentration). Unlabeled taxol, in final concentration polymerization of tubulin and the formation of microtubules (9). Subsequent reduction of sulfhydryl moieties with mercaptoethanol or dithiothreitol restores the ability of tubulin monomers to polymerize and form microtubules. Further evidence of a crucial role for sulfhydryls in microtubule assembly comes from studies using L-BSO, a potent inhibitor of glutathione synthesis, and CDNB, a compound which causes the rapid consumption of GSH. Exposure of the human lymphoid cell line 3T3 to both L-BSO and CDNB resulted in a complete loss of microtubules within the cells (10). Cells actively depleted of GSH by CDNB without L-BSO present gradually regained normal GSH levels accompanied by microtubule reassembly (10). Thus, GSH depletion can markedly alter microtubule structure, presumably by permitting oxidation of sulfhydryl groups in tubulin.

Interestingly, preincubation of 3T3 cells in taxol prevents the disaggregation of microtubules when GSH is subsequently lowered by CDNB (11).

To expand upon these observations we have examined the effects of L-BSO on the cytotoxicity of taxol using clonogenic assays. We report that pretreatment of cells with L-BSO to reduce GSH levels to below detectable values results in marked antagonism of taxol-induced cytotoxicity.

MATERIALS AND METHODS

Chemicals. Taxol was supplied by the Cancer Therapy Evaluation Program, National Cancer Institute. L-BSO was purchased from Schieferhalle, Inc. (South Plainfield, NJ). [3H]Taxol was obtained from Research Triangle Institute (Research Triangle Park, NC). The [3H]taxol was found to be 96% pure by thin layer chromatography and 95% pure by high pressure liquid chromatography. BrdUrd, PI, anti-murine immunoglobulin labeled with FITC, and pepsin were purchased from Sigma Chemical Co. (St. Louis, MO). Monoclonal anti-BrdUrd was purchased from Becton-Dickinson (Mountain View, CA). Monoclonal murine anti-β-tubulin was purchased from Boehringer-Mannheim (Indianapolis, IN).

Cell Culture. Cell lines used in this study included the human breast adenocarcinoma line MCF-7 and the human lung adenocarcinoma line A549, both obtained from the American Type Culture Collection (Rockville, MD). Both cell lines were maintained as stock cultures in RPMI 1640 supplemented with 10% fetal calf serum and antibiotics. A number of 100-mm Petri dishes were plated with 5 × 10^6 cells in medium or medium containing L-BSO and incubated for 24 h. Taxol was then added directly to the plates over a final concentration range of 10–50 nM. Following a 24-h exposure to taxol, the cells were rinsed, trypsinized, counted, plated, and incubated for macroscopic colony formation. Following a 2-week incubation, colonies were fixed with methanol:acetic acid (3:1) and stained with crystal violet, and colonies with >50 cells were counted. All survival points were done in triplicate, and experiments were conducted a minimum of two times. Error bars shown in the figures represent SD and are shown when larger than the symbol. Plating efficiencies for MCF-7 and A549 cells ranged between 40–50% and 35–45%, respectively. Cells treated with L-BSO had lower plating efficiencies. L-BSO treatment (5 mM) resulted in plating efficiencies of 30–35% and 12–20% in MCF-7 and A549 cells, respectively.

[3H]Taxol Binding. Cells were plated into 24-well plates at a concentration of 50,000 cells/well. Cells were plated in either control medium or medium containing 5 mM L-BSO. Twenty-four h later, 0.1 μCi of [3H]taxol was added to each well (8.7 μCi final concentration). Unlabeled taxol, in final concentration...
tions of 10, 50, 100, 250, or 1000 nM was also added to wells containing [3H]taxol. Twelve hours later, medium was aspirated from the plates, and the cells were then lysed with 0.5 ml of a solution containing 1% Triton X-100 and 1% sodium dodecyl sulfate. The lysates were collected and counted in a liquid scintillation counter. Cells that had been exposed to unlabeled taxol alone were trypsinized and counted. Results are expressed as cpm/10^5 cells.

DNA Flow Cytometry and BrdUrd Analysis. MCF-7 and A549 cells were plated in medium or medium containing 5 mM L-BSO. After 24 h, cells were exposed to taxol for an additional 24 h; 10 μg BrdUrd was added to the plates in the last hour of taxol exposure. For DNA flow analysis, cells were trypsinized, washed in PBS, fixed in 70% ice-cold ethanol, and stored for analysis. For BrdUrd detection, cells were centrifuged out of ethanol and resuspended in a 1:1 Triton X-100 and 1% sodium dodecyl sulfate solution for 15 min at room temperature. The HCl treatment was halted by the addition of 1 ml 10 mM borate buffer (pH 8.6). Cells were centrifuged, resuspended in 0.5 ml PBS along with 10 μl of an anti-BrdUrd monoclonal antibody, and incubated for 30 min at room temperature. Cells were centrifuged out of the primary antibody, resuspended in 0.5 ml PBS, and incubated with 5 μg of an anti-mouse IgG-FITC labeled antibody for 30 min at room temperature. Finally, cells were centrifuged out of the secondary antibody and resuspended in 1 ml PBS + 10 μg/ml PI.

All samples were analyzed using the EPICS cell sorter (Coulter Electronics, Hialeah, FL). For BrdUrd analysis, the FITC labeled cells were detected by excitation at 488 nm and collection of the emitted light using a 525 nm band pass filter. The PI labeled cells were detected by excitation at 488 nm and collection of emitted light using a 630 nm long pass filter.

GSH Assay. For each survival and growth experiment extra plates were seeded for GSH determination. Following trypsinization cells were rinsed with PBS, and 1-5 × 10^6 cells were pelleted by centrifugation. The cell pellets were resuspended in 0.6% sulfosalicylic acid. GSH was assayed by the method of Tietze (12). This assay retains linearity at concentrations of GSH as low as 100 ng/ml.

Microtubule Immunofluorescence. Cells were cultured in chamber slides and treated with taxol and/or L-BSO for 24 h. They were then rinsed with PBS and fixed in methanol at −20°C for 30 min. Cells were probed with murine anti-β-tubulin antibody according to the procedure supplied by the manufacturer. Anti-murine immunoglobulin conjugated with FITC was subsequently applied to the cells. Epifluorescence photomicrographs were made with a Zeiss axioplan microscope.

RESULTS

Effect of Taxol on Cell Survival. Figs. 1 and 2 show the survival of cells after 24 h of exposure to taxol as assessed by clonogenic assay. Both cell lines were sensitive to taxol at 10 nM, the lowest concentration tested. However, MCF-7 cells had only about one log of cell death at 10 nM, while A549 cells had between one and two logs of cell kill. Both cell lines exhibited a plateau in cell kill with little additional cytotoxicity at taxol concentrations up to 50 nM.

Control A549 and MCF-7 cells had mean GSH concentrations of 39 μg/mg protein and 42 μg/mg protein, respectively. Treatment of both cell lines with 5 mM L-BSO for 24 h caused GSH levels to fall to below the detectable limits of our assay. L-BSO treatment also reduced the plating efficiency of both cell lines, as noted in "Materials and Methods." Despite reducing plating efficiency, L-BSO treatment resulted in a striking antagonism of the cytotoxic effects of taxol. At a taxol concentration of 50 nM, L-BSO-treated MCF-7 cells had a 3-fold increase in survival compared with control cells (Fig. 1). A549 cells treated with L-BSO demonstrated a 10-fold increase in survival in 50 nM taxol compared with control A549 cells (Fig. 2).

In an attempt to exclude the possibility that L-BSO antagonized taxol cytotoxicity independently of GSH depletion, A549 cells were exposed to 50 nM taxol after pretreatment for 24 h with 0.05, 0.5, or 5 mM L-BSO. Five mM and 0.5 mM L-BSO both reduced GSH to below detectable levels. GSH fell by 99% in cells treated with 0.05 mM L-BSO. In all cases, L-BSO pretreatment increased the survival of A549 cells 5-fold over control A549 cells. Additionally, A549 cells were incubated simultaneously in 50 nM taxol and 5 mM L-BSO without any pretreatment in L-BSO. In the absence of L-BSO pretreatment, no difference in taxol cytotoxicity between control and L-BSO-treated cells was observed.

Effect of Taxol on the Cell Cycle. L-BSO had no effect on the DNA histograms of either cell line. As reported by others (13), taxol produced a block in the cell cycle at G2/M in both cell lines (>80% of cells in G2/M; data not shown). Cell cycle analysis using BrdUrd labeling further defined the effects on the cell cycle induced by taxol and GSH depletion by L-BSO. Fig. 3 shows the two-dimensional BrdUrd/DNA contour plots of A549 and MCF-7 cells incubated with either taxol (for 24 h) or taxol and L-BSO (for 48 h). Control cells had
an S-phase fraction of 28% and 45% in the A549 and MCF-7 lines, respectively. L-BSO alone did not affect the percentage of cells in S phase. As shown in Fig. 3, taxol lowered the percentage of cells in S phase to 7.2% and 2.7% in the A549 and MCF-7 lines, respectively. L-BSO increased the S-phase fraction in taxol-treated cells to 25.2% and 6.6% in the A549 and MCF-7 lines.

Effect of GSH Depletion on Taxol Binding. Figs. 4 and 5 show the binding of [3H]taxol to MCF-7 and A549 cells. Depletion of cellular GSH by L-BSO had no effect on the binding of taxol to either of the two cell lines.

Effect of Taxol and L-BSO on Microtubule Morphology. Fig. 6 shows the results of immunofluorescence studies performed on A549 cells exposed to 37 nM taxol and/or 5 mM L-BSO for 24 h. L-BSO alone had no effect on the appearance of the microtubules compared with control cells. As others have also noted (4), taxol caused marked condensation and thickening of microtubules. Cells treated with L-BSO and taxol exhibited microtubule structure that appeared very similar to that of control cells. MCF-7 cells exhibited microtubule changes after treatment with taxol and/or L-BSO that were akin to those seen in A549 cells.

DISCUSSION

We have shown that the depletion of glutathione with L-BSO pretreatment of two different human tumor cell lines results in marked resistance to the cytotoxic effects of taxol. The L-BSO-mediated resistance was demonstrated in clonogenic assays, which measure cell survival. Although the degree of protection afforded by L-BSO against taxol differed between the two cell lines, GSH depletion resulted in significant improvement in cell survival at all taxol concentrations.

The concentrations of taxol that were used in these studies are lower than those used by other investigators. However, nearly all previous in vitro studies of taxol have utilized growth inhibition assays (14, 15). We have found, using clonogenic assays in a number of human tumor cell lines, that taxol has an unusual dose-response curve. This is manifested by a sharp decline in cell survival (to about 1% of control cells) over a narrow range of taxol concentration (10-30 nM exposure for 24 h) with a subsequent plateau in survival that extends across taxol concentrations up to 2 μM. 3 The killing of both MCF-7 and A549 cells by exposure to taxol for 24 h is maximal at a taxol concentration of about 20 nM. The data presented here emphasize the protective effect of GSH depletion over the range of taxol concentrations where cytotoxicity is first evident and in the early part of the “plateau” of cell survival. Higher concentrations of taxol do not further enhance killing of these cells. Similarly, the protection against taxol afforded by L-BSO-induced GSH depletion is not altered at higher concentrations of taxol.

The biphasic nature of the dose-response curves for taxol is puzzling. Biphasic dose-response curves could arise if two subpopulations of cells are present, one of which is exquisitely sensitive and the other essentially resistant to a particular agent. This is somewhat

3 J. Liebmann, J. A. Cook, and J. B. Mitchell, personal observation.
unlikely in the present study given that two completely different human tumor cell lines were used, yet both exhibited the same overall response. The dose-response curves for these two cell lines to other chemotherapy drugs (e.g., doxorubicin, cisplatin, melphalan) tested in our laboratory have yielded exponential dose-response curves through 3 logs of survival. We cannot rule out, however, the possibility that in both cell lines there are two subpopulations which differ with respect to taxol sensitivity. Regardless of the nature of the dose-response curve, GSH depletion results in both cell lines being much less responsive to taxol at all taxol concentrations.

It is unlikely that L-BSO antagonized taxol independently of GSH depletion. Cells pretreated with concentrations of L-BSO that ranged from 0.05 to 5 mM had extremely low or undetectable GSH levels. Similar degrees of taxol antagonism were seen at all concentrations of L-BSO used. Furthermore, when pretreatment with L-BSO was eliminated, no antagonism of taxol in A549 cells was seen by coincidental incubation of cells in 5 mM L-BSO. Because GSH levels fall with a half-life of 4 h in A549 cells (16), it takes almost 24 h for 5 mM L-BSO to deplete GSH by about 99%. The lack of antagonism of taxol by L-BSO when cells had not been previously depleted of GSH supports the notion that it is GSH depletion and not simply the presence of L-BSO that is responsible for taxol antagonism.

L-BSO depletion of GSH has been used by a number of investigators to define the role of intracellular thiols in mediating resistance to chemotherapeutic drugs. The cytotoxicity of a number of drugs, including melphalan, doxorubicin, and bleomycin, is enhanced when GSH is depleted by L-BSO (17, 18). In contrast, the polypeptide antibiotic neocarzinostatin appears to require reduced thiols for the activation of its chromophore in order to be cytoxic. Depletion of GSH by L-BSO protects cells from killing by neocarzinostatin (19). We are not aware, however, of any data that suggest that taxol requires intracellular reduction in order to bind tubulin and exert its cytotoxic effects.

It is not clear what the exact mechanism of L-BSO antagonism of taxol cytotoxicity might be. In all experiments, however, L-BSO pretreatment caused GSH levels to fall below the limits of detection in our assay. Profound depletion of GSH may directly affect other cellular thiols (20). In the absence of GSH, thiols may form thioesters or become oxidized to disulfides. The overall effect of oxidation of cellular thiols is unclear. Earlier work by others demonstrated a striking effect on microtubule disaggregation when cellular GSH levels were depleted by CDNB and L-BSO in 3T3 cells (10). It is possible that GSH depletion in MCF-7 and A549 cells may also affect microtubule structure in those cell lines. However, GSH depletion by L-BSO did not affect the morphology of microtubules as assessed by immunofluorescence microscopy of MCF-7 or A549 cells. Furthermore, binding of [3H]taxol to either MCF-7 or A549 cells was unaffected by GSH depletion. Any effect that GSH depletion might have on microtubule structure is unlikely, therefore, to involve the taxol binding site.

A characteristic effect of taxol on cells has been to cause a block in the cell cycle at G2/M (13). Over 80% of both A549 and MCF-7 cells were blocked in G2/M after exposure to 37 nM taxol for 24 h. BrdUrd labeling showed that L-BSO greatly increased the fraction of cells in

Fig. 6. Immunofluorescence study of microtubules in A549 cells. A, control A549 cells after staining of microtubules as described in “Materials and Methods.” B, A549 cells that have been incubated in 5 mM L-BSO for 48 h. C, A549 cells after a 24-h incubation in 37 nM taxol. D, A549 cells exposed to 37 nM taxol for 24 h after pretreatment with 5 mM L-BSO.
S phase after taxol treatment. This suggests that the mechanism of protection against taxol provided by GSH depletion may be due in part to changes in the cell cycle.

A Chinese hamster ovary cell line mutant has been described which has abnormal tubulin structure and requires taxol for growth (6). It is conceivable that cellular thiol depletion by L-BSO could result in abnormal tubulin and that taxol would then be required for normal cell growth. However, growth curves for A549 and MCF-7 cells in the presence of taxol and L-BSO fail to show any benefit from taxol supplementation of the L-BSO-treated cells (data not shown). In fact, both L-BSO and taxol either alone or in combination inhibit cell growth. This is in contrast to the Chinese hamster ovary cell mutants in which taxol supplementation restores normal growth. It is unlikely that the protective effect of L-BSO on taxol cytotoxicity is mediated by a change in tubulin structure which results in a need for taxol to restore normal tubulin function.

Clinical trials of L-BSO as a chemotherapy sensitizer are in progress (21). The use of L-BSO together with chemotherapy has been of particular interest in the treatment of ovarian cancer (22). Taxol shows considerable promise as an effective drug for ovarian cancer. Our data indicate that any attempt to modulate the activity of taxol with L-BSO in clinical trials may result in a decrease in tumor response to taxol.

In summary, we have shown that treatment of two human cancer cell lines with L-BSO to deplete cellular GSH markedly protects the cells from cytotoxicity caused by taxol. GSH depletion also prevented the condensation of microtubules that occurs after taxol exposure. GSH depletion did not affect the binding of [3H]taxol to these cells. However, GSH depletion increased the percentage of cells in S phase after exposure to taxol. The significance of the effect of L-BSO on the cell cycle changes induced by taxol is not clear. We are pursuing additional studies in an attempt to elucidate the mechanism of the interaction between GSH and taxol.

REFERENCES

Glutathione Depletion by L-Buthionine Sulfoximine Antagonizes Taxol Cytotoxicity

James E. Liebmann, Stephen M. Hahn, John A. Cook, et al.


Updated version Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/53/9/2066

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/53/9/2066. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.