Depletion of glutathione by L-butathione sulfoximine antagonizes taxol cytotoxicity in MCF-7 human breast adenocarcinoma cell line
Several natural agents have been found to have potential as anticancer agent. Taxol is a naturally occurring chemotherapeutic agent and has a role against a variety of tumors. Taxol is known to act by binding tightly to microtubules and preventing their disaggregation (330). Multidrug resistant human tumor cells possess a membrane bound high molecular weight glycoprotein analogous to the p170 glycoprotein product of the multidrug resistant gene. These cells have reduced steady state of accumulation of taxol. Therefore, one of the mechanism by which cells become resistant to taxol could be due to altered uptake and efflux (335). Other means by which cells become resistant to taxol could be alteration of tubulin structure as is observed in Chinese hamster ovary cell mutants that have abnormal tubulin structure and are known to require taxol for proper tubulin polymerization and normal growth (336).

However, there may be other possible mechanisms of resistance to taxol. Looking at the structure of microtubule shows that they are composed of two peptides, α-tubulin and β-tubulin, which have 12 and 8 cysteine residues, respectively (337,338). Polymerization of tubulin and formation of microtubules is prevented by oxidation of tubulin sulfahdyryl groups (339). If these sulfahdyryl moieties are reduced with mercaptoethanol or dithiothreitol, the polymerization of microtubules is achieved by tubulin monomers. This is further supported by use of L-butathione sulfoximine (L-BSO), which plays a crucial role in depletion of glutathione. L-BSO is a potent inhibitor of glutathione (GSH) synthesis, and CDNB, a compound which causes the rapid consumption of GSH. Human lymphoid cell line 3T3 when exposed
to both L-BSO and CDNB resulted in complete loss of microtubules within the cell (340). GSH depletion, therefore, can markedly alter microtubule structure, presumably by permitting oxidation of sulfahydryl group in tubulin. Preincubation of 3T3 cells in taxol prevents the disaggregation of microtubules when GSH is subsequently lowered by CDNB (341).

This work deals with the glutathione depletion by BSO on taxol cytotoxicity in MCF-7 human adenocarcinoma cell line.

Material and Methods

Chemicals: Taxol (pacilitaxel), L-butathione sulfoximine (L-BSO) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other tissue culture chemicals were also purchased from Sigma Chemical Co.. Fetal calf serum was purchased from Biological Industries (Kibbutz Beit Haemek, Israel). All other chemicals were of analytical grade.

Cell Culture: MCF-7, a human breast adenocarcinoma cell line, was obtained from the National Facility for Animal Tissue and Cell Culture (NFATCC) Pune, India. The cell line was maintained in RPMI-1640 supplemented with 0.2% sodium bicarbonate and 10% FCS and antibiotics as described earlier in chapter 2.

Drug Study: A number of 90mm petridishes were plated with $5 \times 10^5$ cells in medium or medium containing L-BSO and incubated for 24h. Taxol was added directly to the plates over a final concentration range of 0.05 - 5.0 μM. Following exposure to drugs, the cells were harvested and cell growth was monitored by
counting the viable cells after trypan blue dye exclusion test using a hemocytometer. Each experiment was repeated at least 3-4 times and each treatment was in triplicate. The percentage of trypan blue excluded (live cells) were more than 95% in untreated group.

**Glutathione (GSH) Assay:** For measuring of GSH content, following trypsinization cells were rinsed with PBS and $1 \times 10^6$ cells were pelleted by centrifugation. GSH was assayed by the method of Moron et al. (1979) as described in chapter 2.

**Cell cycle analysis:** MCF-7 cells were plated in medium or medium containing 5 mM L-BSO. After 24h cells were exposed to taxol for an additional 24h. Cells were harvested, washed with cold PBS and pelleted by centrifugation at 2000xg for 10 min at 4°C. The pelleted cells were used for flow cytometric analysis as mentioned earlier in chapter 2.

**Results**

Effect of different concentrations of L-BSO on the growth of MCF-7 cells is shown in Fig.1. A dose dependent effect of different concentrations of L-BSO on growth of MCF-7 cells was observed as assessed by trypan blue exclusion test. MCF-7 cell line was sensitive to 1 mM BSO, the lowest concentration tested. A dose dependent inhibition of cell growth was observed with L-BSO. Effect of taxol on cell survival is shown in Fig. 2. Exposure to different concentrations of taxol to MCF-7 cells showed that lowest concentration of taxol that resulted in inhibition of growth was 0.05 µM. A dose dependent inhibition on growth was observed after 48h of exposure as assessed by trypan blue exclusion test. Pretreatment of MCF-7 cells with
5 mM L-BSO for 24h followed by treatment with different concentrations of taxol for 48h resulted in striking antagonism of the cytotoxic effect of taxol. At taxol concentration of 5.0 μM, L-BSO treated MCF-7 cells had 4 fold increase in survival compared with control cells.

Control MCF-7 cells had mean GSH concentration of 10.7 μM ± 0.7. Treatment with 5mM L-BSO for 24h caused depletion of GSH content to 3.2 μM ± 0.2 (Fig.3).

However, simultaneous treatment with 5mM L-BSO and taxol, no difference in taxol cytotoxicity between control cells and cells co-incubated with L-BSO was observed (data not shown).

Effect of taxol on cell cycle after pretreatment with L-BSO is shown in Fig.4 and Table 1. Effect of L-BSO on the DNA histogram showed that pretreatment with L-BSO for 24 h had no effect on G2/M, however, inhibition of percentage of cells in S-phase was observed. As earlier reported, taxol produced a block in cell cycle at G2/M phase in MCF-7 cell line. Cell cycle analysis after taxol and GSH depletion is shown in Fig.4 and Table 1. BSO decreased the G2/M fraction in taxol treated cells to 21% in MCF-7 cell thereby showing G2/M block of cell cycle produced by taxol is partially reversed by L-BSO.

Discussion

The results in this chapter show that depletion of glutathione by pretreatment of MCF-7 human adenocarcinoma cell line with L-BSO results in marked resistance
to the cytotoxic effect of taxol. GSH depletion resulted in marked improvement in cell survival at all taxol concentrations. The data presented here emphasizes the protective effect of GSH depletion over a range of taxol concentration.

The cell cycle analysis data suggest that there is partial reversal by L-BSO of G2/M block produced by taxol. This suggests that the mechanism of protection against taxol provided by GSH depletion may be partly due to change in the cell cycle. A mutant Chinese hamster ovary cell line has been found which has abnormal tubulin structure and requires taxol for growth (336). It is possible that cellular glutathione depletion by L-BSO could result in abnormal tubulin and then taxol would help in normal cell growth.

L-BSO has been used together with chemotherapy in clinical trials for treatment of ovarian cancer (342). It has a possible use as a chemotherapy sensitizer. Taxol is very promising in use for breast cancer and ovarian cancer. The present data indicates that the use of L-BSO as a chemotherapy sensitizer with taxol in clinical trials would result in a decrease in tumor response to taxol.

The present data shows that treatment of MCF-7 cell line with L-BSO to deplete cellular GSH levels protects the cells from cytotoxicity caused by taxol. Mechanism of protection against taxol provided by GSH depletion could be partly due to change in the cell cycle. The exact mechanism and significance of L-BSO on cell cycle changes is not very clear. Additional studies are required to throw light on the mechanism by which GSH depletion reverses taxol cytotoxicity. It is possible that GSH depletion may affect the binding of taxol to the cells which can be explained by using \([^3]H\) taxol.
Fig. 1  Effect of different concentrations of L-BSO on the rate of proliferation of MCF-7 cells. Cells were plated in triplicate in 24-well culture plates containing $5 \times 10^4$ cells/well. Two days after plating the medium was replaced with the same medium (control) or medium containing indicated concentrations of L-BSO. Triplicate wells were counted for cell number after 48h and means $\pm$ SD of triplicates were plotted. SD < 5% of means are not shown.
Fig. 2  Pretreatment of MCF-7 cells with L-BSO induces protection against taxol-induced cell kill. Cultures were pretreated with 5 mM L-BSO for 24h after seeding 5 x 10^4 cells in 24-well culture plate. After additional 24 h at 37°C taxol was added at indicated concentrations. The cells were harvested 48h later and counted by trypan blue exclusion using hemocytometer. Taxol only (○), Taxol and 5.0 mM L-BSO (●).
Fig. 3  Glutathione content in L-BSO (5 mM) treated and untreated MCF-7 cells. 5 x 10⁴ cells were plated in 60 mm culture plates. Two days after plating, the medium was replaced with the same medium or medium containing 5 mM of L-BSO, incubated for 24h. About 10⁶ cells were harvested and processed for glutathione assay as described earlier. Results are mean ± SD values of triplicate samples.
Fig. 4 Flow cytometry analysis of MCF-7 cells. A: Control (untreated cells), B: 0.5 μM taxol treated cells, C: 5 mM L-BSO treated cells, D: Cells pretreated with 5 mM L-BSO for 24h then treated with 0.5 μM taxol. Cells were harvested 48h later for cell cycle analysis.
Table 1  Flow cytometric analysis of MCF-7 cells

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<thead>
<tr>
<th>Treatment</th>
<th>Distribution (%) of cells in</th>
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<tbody>
<tr>
<td></td>
<td>G₁</td>
</tr>
<tr>
<td>Control</td>
<td>64</td>
</tr>
<tr>
<td>L-BSO (5 mM)</td>
<td>78</td>
</tr>
<tr>
<td>Taxol (0.5 μM)</td>
<td>50</td>
</tr>
<tr>
<td>BSO (5 mM) + Taxol (0.5 μM)</td>
<td>69</td>
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Cell cycle phase distribution of human breast adenocarcinoma cell line MCF-7 after 48h of treatment with either 5 mM L-BSO, 0.5 μM taxol or cells pretreated with L-BSO for 24h and then incubated with taxol for 48h. Phase distribution was estimated after computer analysis of DNA histogram obtained by flow cytometry of cells fixed with 70% ethanol and stained with propidium iodide.