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14. ABSTRACT The objective of this investigation is to examine the protective effects of prolactin (PRL) against anti-cancer drugs. Three specific aims were formulated: 1) To characterize the protective effects of PRL against several anticancer drugs, 2) To determine anti-apoptotic mechanism(s), and 3) To examine the protective effects of PRL in vivo. Good progress has been made in all objectives as follows. First, we have shown the protective effects of PRL against taxol, cisplatin and vinblastine in more than one breast cancer cell line. Two, we identified increased activity of a detoxification enzyme as the mechanism by which PRL antagonizes the cytotoxic effects of cisplatin. Ongoing studies are examining to what extent this mechanism of chemoresistance applies to other cytotoxic drugs. Future research will also examine the roles of circulating and breast-derived PRL in the resistance to chemotherapy in live animals.					
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Introduction

Chemotherapy is the mainstay treatment for advanced and metastatic disease. DNA damaging agents have a proven record as anti-cancer drugs. Cisplatin, a platinum-based drug, is one of the most potent antitumor agents that is highly effective against lung, ovarian and prostate cancer but not breast cancer. Cisplatin interacts with DNA, forms adducts and intrastrand crosslinks and induces cell cycle arrest. Damaged DNA can either be repaired via several repair mechanisms or the cell is destined to die (1). While the choice of chemotherapeutic agents has increased in recent years, tumor resistance remains a major obstacle. Some tumors are intrinsically resistant to drugs while others acquire resistance following treatment. Resistance results from many causes, including drug efflux by transporters, inactivation by detoxifying enzymes, altered expression of pro/anti-apoptotic proteins or tumor suppressors, and increased DNA-repair mechanisms (2). Among detoxification enzymes, glutathione-S-transferase (GST) conjugates electrophilic drugs to glutathione, thus targeting them for extrusion by an ATP-dependent pump. GST inactivates platinum drugs, thereby increasing resistance to cisplatin in breast cancer (3).

Although breast tumors are among the few cancers that are hormone-sensitive, the role of hormones in chemoresistance has received little attention. Prolactin (PRL) is a 23kDa protein hormone which binds to a single-span membrane receptor and exerts its action via several interacting pathways, including Jak2-Stat5a/b, MAP kinase and PI3 kinase/Akt.. Both PRL and its receptor (PRL-R) are expressed in breast tissue and in many breast cancer cell lines (4). Autocrine PRL is mitogenic, as shown by the suppression of T47D cell proliferation following inhibition of PRL (5). PRL also functions as a survival factor in both malignant and non-malignant cells. This raises the prospect that PRL antagonizes apoptosis by anti-cancer drugs, thereby contributing to chemotherapeutic resistance. Indeed, a PRL-R antagonist enhanced cisplatin-induced apoptosis in T47D cells (6), and increased the suppressive effects of doxorubicin and taxol on colony formation in MCF-7 cells (7). Furthermore, breast cancer cells that produce PRL were more resistant to ceramide-induced apoptosis than those with low or no PRL (8). However, previous studies have not addressed the mechanism by which PRL confers chemoresistance, which was the major objective of the present studies.

Body

For these studies, we have selected MDA-MB-468 (468) breast cancer cells, which are both aggressive and metastatic and produce little or no detectable PRL. For the chemotherapeutic drug, we have selected cisplatin, a platinum-based drug that is highly effective against a wide variety of cancers including lung and ovarian cancers, lymphomas and germ cells tumors. We were intrigued by the fact that cisplatin is ineffective in breast cancer and hypothesized that this could be due to antagonism of its actions by PRL.

Specific Aims:

- 1) Compare the effects of PRL against cisplatin in T47D and 468 cells
- 2) Determine the signaling pathways that mediate the action of PRL
- 3) Examine the role of the detoxification enzyme GST
- 4) Determine whether PRL induces GST activity and/or expression

Results and discussion

PRL differentially affects cisplatin-induced cytotoxicity in T47D and 468 cells.

Initial studies showed that PRL antagonized the cytotoxic effects of the microtubule altering drugs taxol and vinblastine, and the DNA damaging drugs cisplatin and doxorubicin. To verify that such protection is not confined to one cell line, the effects of PRL on cisplatin-induced cytotoxicity were compared in 468 and T47D cells. Unlike the strong suppressive effect of cisplatin on 468 cells (**Fig 1, left panel**), it was only moderately effective in T47D cells (**Fig 1, right panel**). We postulate that the poorer responsiveness of T47D cells to cisplatin is due to their higher level of endogenous PRL. Moreover, the complete antagonism of all doses of cisplatin in T47D cells by PRL, as opposed to its partial efficacy against the high drug doses in 468 cells, could be attributed to the very high density of the PRL-R in T47D cells. To study the mechanism by which PRL confers chemoresistance, we selected the prototypical DNA-damaging agent cisplatin, and took advantage of low endogenous PRL production in 468 cells.

The protective effect of PRL in 468 cells is mediated by the Jak/Stat and MAPK pathways.

The action of PRL as a survival factor can be mediated through several signaling pathways. As shown in **Fig 2**, treatment of 468 cells with PRL caused activation of MAPK, Jak/Stat5 and PI3K pathways, albeit with a somewhat different dynamics. PRL induced robust activation of ERK1/2 that began at 5 min and progressively increased thereafter. Stat 5 was also immediately phosphorylated by PRL, and was slightly decreased by 240 min. On the other hand, Akt was only marginally activated by PRL. These data confirm that the three major PRL signaling pathways are intact in 468 cells. To determine which of these pathways mediate protection by PRL against cisplatin, we used selective inhibitors. As shown in **Fig 3**, cisplatin decreased cell viability by 50%, an effect that was abrogated by pretreatment with PRL. However, PRL did not protect the cells when either the Jak or MAPK pathways were blocked by AG490 and U0126, respectively. In contrast, inhibition of the PI3K pathway with wortmannin did not prevent PRL from antagonizing cisplatin. These results suggest that protection by PRL in 468 cells involves Jak-Stat and MAPK signaling rather than the PI3K-Akt survival pathway.

Charting the actions of PRL to a lower binding of cisplatin to DNA.

We postulated that PRL may inhibit cisplatin-induced apoptosis by reducing its availability for interacting with DNA. To this end, we used inductively coupled plasma mass spectroscopy (ICP-MS) which can measure platinum, to quantify binding of cisplatin to DNA. **Fig 4** shows that PRL reduced the amount of platinum bound to DNA by 50%, as compared to cisplatin alone. These results suggest that PRL most likely acts by reducing cisplatin binding to DNA rather than by antagonizing its actions through alterations in pro-/anti-apoptotic proteins which are downstream of DNA damage.

PRL antagonizes the actions of cisplatin by activating GST.

The next objective was to identify the mechanism by which PRL prevents cisplatin from entering the nucleus and binding to DNA. Potential candidates are: 1) membrane transporters that extrude cisplatin from the cells, and 2) detoxification mechanisms which inactivate cisplatin. Multidrug resistance-associated proteins (MRPs) play a role in resistance to cisplatin. There is also evidence implicating thiol-containing compounds, such as glutathione, in the resistance to cisplatin. GST catalyzes the conjugation of glutathione to cisplatin, making it more water soluble

and thus readily excretable. To examine which of these mechanisms mediates the protective effects of PRL, we employed inhibitors of MRP (probenecid) or GST (ethacrynic acid). Whereas PRL effectively antagonized cisplatin cytotoxicity in the presence of the MRP inhibitor, it failed to protect the cells when GST was blocked (**Fig 5**), implicating GST, rather than MRP transporters, in its protective actions.

PRL increases GST activity and induces the expression of the GST μ isoform

To verify involvement of GST, we used an enzymatic assay to determine if PRL increases GST activity in 468 cells. **Fig 6, left panel**, reveals that PRL induced a 4-fold increase in GST activity, which was abrogated by Jak or MAPK inhibitors. Furthermore, treatment of the cells with ethacrynic acid abolished the ability of PRL to decrease cisplatin binding to DNA (**Fig 6, right panel**). Since ethacrynic acid alone did not increase nuclear cisplatin levels, the rate of transport of cisplatin into the nucleus may not be affected by basal, non-stimulated GST activity. GSTs are divided into several distinct classes, based on sequence similarities and substrate specificity, with much attention paid to the roles of GST μ , GST π and GST θ polymorphism in cancer incidence and response to therapy. Using real-time PCR, we found that PRL increased the expression of GST μ by 3-fold without affecting GST π (**Fig 7**).

Summary of the mechanism by which PRL antagonizes cisplatin-induced apoptosis.

Fig 8 conceptualized the mechanism by which PRL confers resistance against cisplatin in breast cancer cells. Cisplatin enters the cell via passive diffusion and goes into the nucleus, where it binds to DNA and causes apoptosis. Binding of PRL to its receptor results in its dimerization, an association of Jak2 and Shc and subsequent signaling via Stat5a/b and ERK1/2 pathways. It is through either or both of these signaling pathways that PRL increases transcription of the detoxification enzyme GST. Increased GST activity promotes the conjugation of glutathione to cisplatin, followed by extrusion of the conjugate from the cell via transporters. Consequently, less cisplatin is available for entry into the nucleus, resulting in decreased apoptosis.

Key Research Accomplishments

- ❖ This is the first demonstration that PRL, a natural hormone, confers a broad range chemoresistance in breast cancer cells.
- ❖ Protection by PRL is not limited to one breast cancer cell line and extends to several anti-cancer drugs.
- ❖ One mechanism by which PRL confers chemoresistance is by increasing expression and activity of a detoxification enzyme resulting in reduced drug entry into the nucleus.

Reportable Outcomes

Publications

1. LaPensee C, Hugo E, Ben-Jonathan N. Insulin stimulates interleukin-6 expression and release in LS14 human adipocytes through multiple signaling pathways. *Endocrinology* 149:5415-5422, 2008
2. Hugo ER, Borcharding DC, Gersin KS, Loftus J, Ben-Jonathan N. Prolactin release by

adipose explants, primary adipocytes and LS14 adipocytes. Journal of Clinical Endocrinology and Metabolism 93:4006-4012, 2008.

3. Hugo E, Brandebourg T, Woo J, Loftus J, Alexander J, Ben-Jonathan N. Bisphenol A at environmentally relevant doses inhibits adiponectin release from human adipose tissue explants and adipocytes. Environmental Health Perspectives 116:1642-1647, 2008.
4. Ben-Jonathan, N, Chen S, Dunckley J, LaPensee C, Kansra S. Estrogen receptor α mediates the epidermal growth factor-induced prolactin expression and release in lactotrophs. Endocrinology 150:705-802, 2009.
5. LaPensee E, Tuttle T, Fox S, Ben-Jonathan N. Bisphenol A at low nanomolar doses confers chemoresistance on estrogen receptor alpha positive and negative breast cancer cells. Environmental Health Perspectives 117:175-180, 2009.
6. Ben-Jonathan N, Hugo E, Brandebourg T. Effects of bisphenol A on adipokine release from human adipose tissue: Implications for the metabolic syndrome. Molecular and Cellular Endocrinology (in press).
7. LaPensee E, Schwemberger S, Afton S, LaPensee C, Bahassi M, Ben-Jonathan N. Prolactin confers chemoresistance against cisplatin in breast cancer cells by activating glutathione-S-transferase (submitted).

Presentations in meetings

1. Borcharding D, Brandebourg T, Ben-Jonathan N. Regulation of prolactin expression and release in human adipocytes by insulin and catecholamines. Endo 2008, San Francisco, CA (abs # p2-626).
2. Brandebourg T, Ben-Jonathan N. Expression of dopamine receptors in human adipose tissue: Augmentation of isoproterenol-stimulated lipolysis by dopamine. Workshop on the establishment, maintenance & turnover of fat depots, Bethesda, MD, May 2008.
3. LaPensee E, Ben-Jonathan N. Prolactin antagonizes chemotherapeutic-induced cytotoxicity in breast cancer cells. Era of Hope Breast Cancer Meeting, Baltimore MD, June 2008.

Degrees obtained

Dr. Elizabeth LaPensee, my graduate student, completed her dissertation entitled: Prolactin and Estrogens Confer Chemoresistance in Breast Cancer Cells: Mechanism of Action. She graduated in October 10, 2008 and has a position of a postdoctoral fellow at the University of Michigan.

Conclusion

Drug resistance is a challenging issue for breast cancer patients, being the main reason for treatment failure in advanced and metastatic disease. Our data suggest that a reduction in

circulating PRL levels or blockade of the PRL-R should improve the efficacy of chemotherapy in breast cancer patients. Understanding the mechanism by which PRL exerts its anti-cytotoxic actions is especially relevant to cisplatin, a widely used and highly effective chemotherapeutic agent which nonetheless has shown little success in the treatment of breast cancer. Long acting, orally administered dopamine agonists such as bromocriptine and cabergoline that reduce PRL have an excellent record of safety and efficacy in the treatment of pituitary prolactinomas and in restoring fertility in PRL-dependent reproductive dysfunctions. Since these drugs are FDA-approved, clinical trials for their effectiveness in breast cancer can commence without a delay. The projected benefits of lowering the PRL input to breast tumors include a wider choice of effective chemotherapeutic agents, increased effectiveness of commonly-used anti-cancer drugs and decreased toxicity and side effects normally associated with high dose chemotherapy.

Future studies should examine whether certain drug combinations or increased dosages can overcome the tumor promoting effects of PRL. The results of this research should inspire innovative clinical interventions since protection from drug-induced tumor shrinkage by PRL can explain chemoresistance in certain patients. Such patients may have slightly elevated serum PRL levels, increased breast PRL production or increased PRL-R expression. Testing PRL serum levels or breast PRL-R expression in biopsy specimens may predict chemosensitivity. Moreover, agents that block PRL production or action should improve the efficacy of chemotherapy. Importantly, the involvement of PRL in chemoresistance may extend to other types of cancers.

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7. Howell SJ, Anderson E, Hunter T, Farnie G, Clarke RB 2008 Prolactin receptor antagonism reduces the clonogenic capacity of breast cancer cells and potentiates doxorubicin and paclitaxel cytotoxicity. *Breast Cancer Res* 10:R68
8. Perks CM, Keith AJ, Goodhew KL, Savage PB, Winters ZE, Holly JM 2004 Prolactin acts as a potent survival factor for human breast cancer cell lines. *Br J Cancer* 91:305-311

Appendices

Figure Legends

- Fig. 1. PRL differentially protects T47D and MDA-MB-468 cells from cisplatin-induced cytotoxicity. MDA-MB-468 (*left panel*) or T47D (*right panel*) cells were treated with cisplatin (0-800 ng/ml) or pretreated with 25 ng/ml PRL followed by cisplatin. After 4 days, cell viability was determined by the MTT assay. Each value is a mean $\pm$ SEM of six replicates. * significant differences ($p < 0.05$) between PRL treatment and drug alone.
- Fig. 2. Activation of several signaling pathways by PRL. MDA-MB-468 cells were treated with PRL (100 ng/ml) for 0-240 min. Cell lysates were analyzed by Western blotting, using antibodies against phospho ERK1/2 (p-ERK1/2), ERK1/2, phospho-Stat 5 (p-Stat 5), Stat 5 or phospho-Akt (p-Akt) and Akt. Shown are representative blots, repeated three times.
- Fig. 3. Both Jak-Stat and MAPK signaling mediate the protective effects of PRL. MDA-MB-468 cells were treated with AG490 (10 μ M), UO126 (10 μ M) or wortmannin (Wort; 250 nM) 1 hr before addition of 100 ng/ml PRL for 24 hrs. The next day, cisplatin (800 ng/ml) was added for 24 hrs. Cell viability was determined by the MTT assay. Each value is a mean $\pm$ SEM of six replicates. * significant difference ($p < 0.05$) vs control; ** significant difference vs cisplatin.
- Fig. 4. PRL reduces cisplatin binding to DNA. MDA-MB-468 cells were treated with PRL (100 ng/ml) for 24 hrs followed by 800 ng/ml cisplatin for 8 or 24 hrs. DNA was isolated and platinum (Pt) content was analyzed by inductively coupled plasma mass spectroscopy. * significant difference ($p < 0.05$) vs the corresponding cisplatin treatment.
- Fig. 5. PRL antagonizes cisplatin cytotoxicity via GST rather than MRP transporters. MDA-MB-468 cells were treated with probenecid (10 μ M) or ethacrynic acid (10 μ M) for 1 hr before PRL (100 ng/ml) for 24 hrs. This was followed by treatment with 800 ng/ml cisplatin for 24 hrs. Cell viability was determined by the MTT assay. Each value is a mean $\pm$ SEM of six replicates. * significant difference ($p < 0.05$) vs control; ** significant difference vs cisplatin treatment.
- Fig. 6. PRL increases GST activity and blockade of GST reduces cisplatin entry into the nucleus. *Left panel*: cells were treated with AG490 (10 μ M) or UO126 (10 μ M) for 1 hr before PRL (100 ng/ml) for an additional 24 hrs. GST activity was determined spectrophotometrically by measuring the rate of 1-chloro-2,4-dinitrobenzene (CDNB) conjugation to glutathione. * significant difference vs control. *Right panel*: cells were treated with ethacrynic acid (EA; 10 μ M) for 1 hr before addition of PRL (100 ng/ml) for 24 hrs. The following day, cisplatin (800 ng/ml) was added for 24 hrs. DNA was isolated and platinum (Pt) content was analyzed by inductively coupled plasma mass spectroscopy. * significant vs control; ** significant vs cisplatin+PRL treatment.
- Fig. 7. PRL induces the expression of GST μ but not GST π . Cells were treated with 100 ng/ml

PRL for 0, 24 or 48 hrs. Expression of GST μ and GST π were determined by real-time PCR. Values were calculated as fold change over time 0 and represent an average of three separate experiments.

- Fig. 8. A model depicting the proposed mechanism by which PRL antagonizes cisplatin-induced apoptosis. After entering the cell by diffusion, cisplatin (Pt) enters the nucleus and binds to DNA. This results in intrastrand cross links, adduct formation and apoptosis. PRL binds to its receptor and activate the Jak2/Stat5a/b and ERK1/2 pathways. These lead to increased transcription of the detoxification enzyme glutathione-s-transferase (GST). Higher GST activity promotes conjugation of glutathione (GSH) to cisplatin. The conjugate is then extruded from the cell via transporters. Since less cisplatin is available for entry into the nucleus, PRL treatment results in decreased apoptosis and resistance to cell killing by cisplatin.

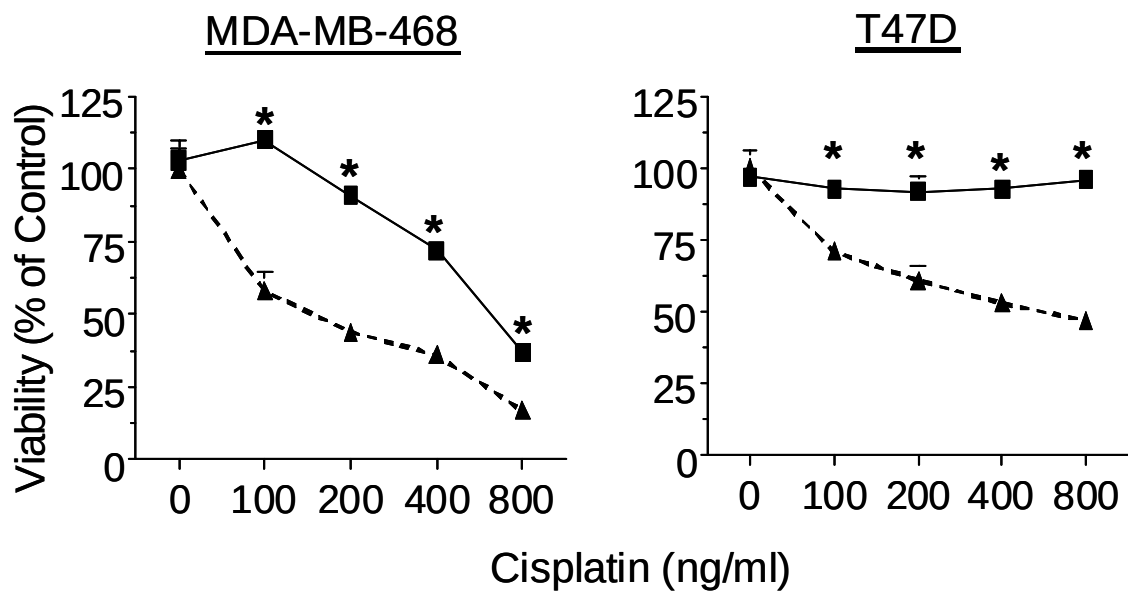


Figure 1

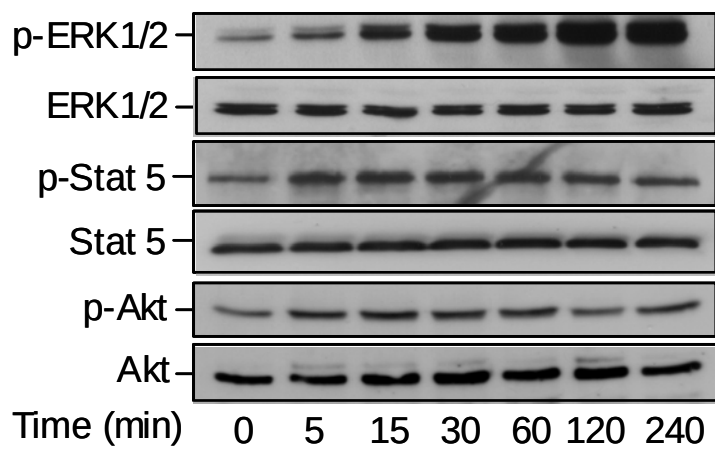


Figure 2

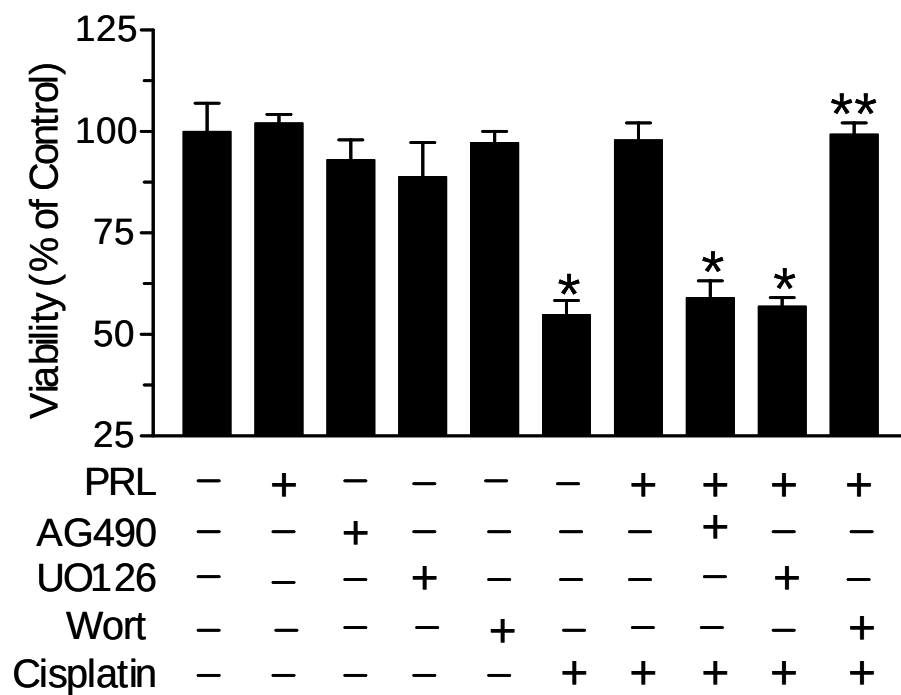


Figure 3

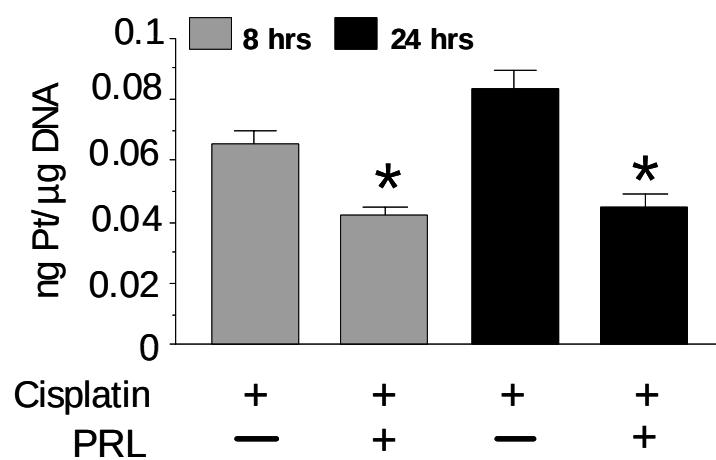


Figure 4

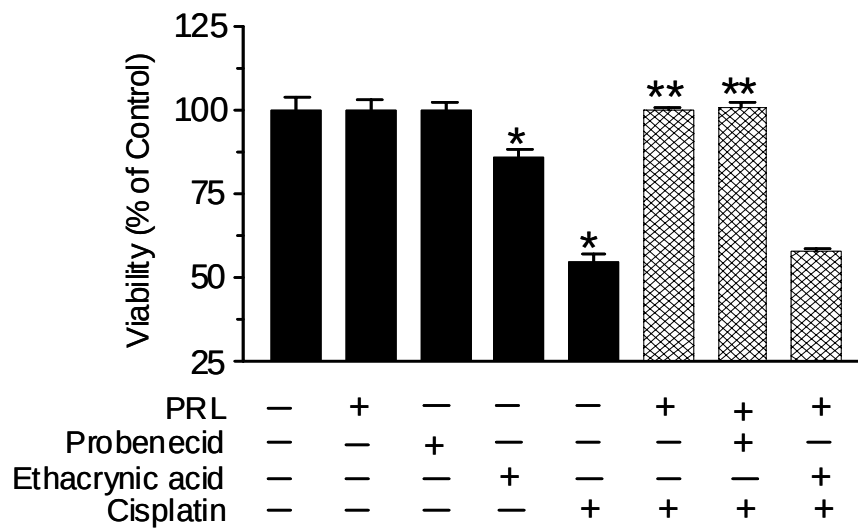


Figure 5

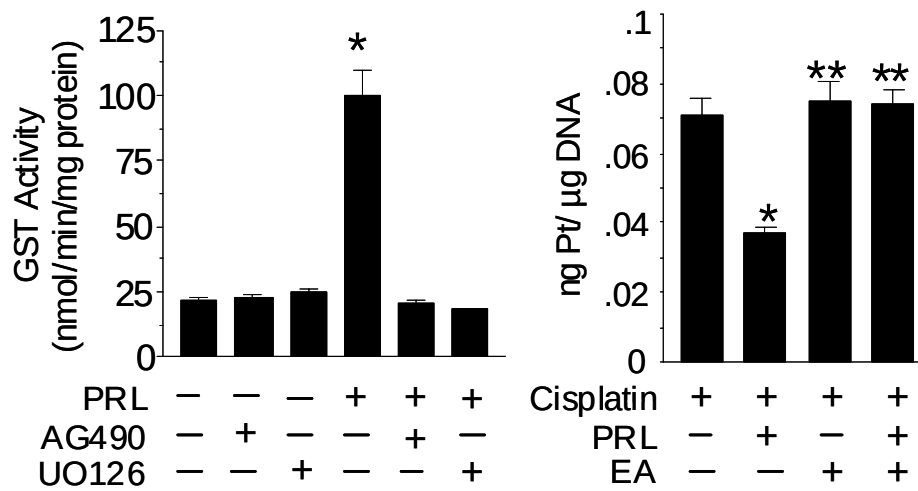


Figure 6

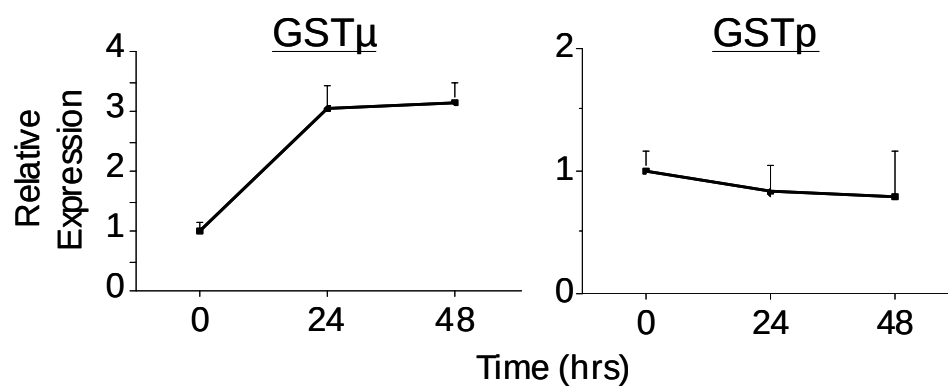


Figure 7

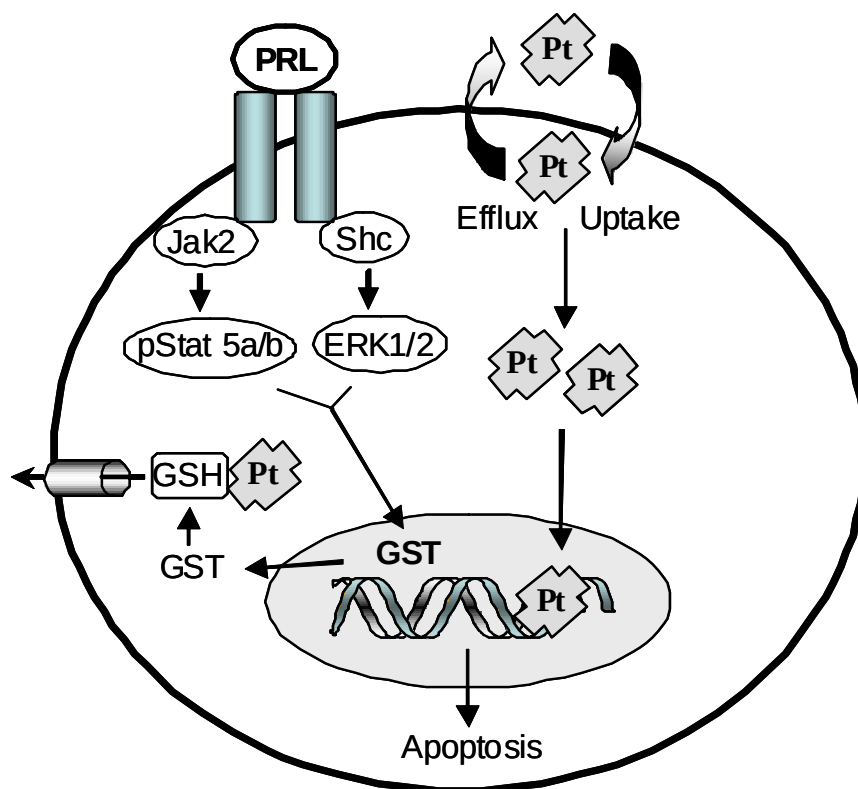


Figure 8