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TITLE: The Role of Src Tyrosine Kinase Signaling Networks in the
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       Model

PRINCIPAL INVESTIGATOR: Christopher C. Taylor, Ph.D.
                        Ting Chen

CONTRACTING ORGANIZATION: Georgetown University School of Medicine
                          Washington, DC 20057

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Christopher C. Taylor, Ph.D.
Ting Chen

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Georgetown University School of Medicine
Washington, DC 20057

E-Mail: cct5@georgetown.edu

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Ovarian cancer is among the most common and deadly malignancies in women, accounting for nearly 15,000 deaths per year in the United States. We have developed a mouse model of ovarian cancer, which will allow for new studies into the biochemical changes that occur in the tumorigenic process. We have determined that Src tyrosine kinase is over expressed and constitutively activated in these mouse ovarian cancer cells. This leads to constitutive activation of downstream kinases such as phosphatidylinositol-3-kinase (PI3-kinase) and focal adhesion kinase (FAK). Pharmacologic inhibition of Src suppresses cell migration, alters localization of FAK, decreases protein tyrosine kinase phosphorylation, inhibits the PI3-kinase/Akt survival pathway and enhances the cell killing effects of two different classes of chemotherapeutics, taxol, and cisplatin, commonly used chemotherapeutic agents in women with ovarian cancer. Combination treatment of mouse ovarian cancer cells with Src inhibition and taxol activates a cell-killing pathway involving caspase 3 that is not activated by either treatment alone. We have also generated taxol resistant mouse ovarian cancer cells. Pharmacologic inhibition of Src restores taxol sensitivity to taxol resistant cells. Thus it appears that Src provides a potential new target for small molecule, targeted, combinatorial chemotherapy.

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INTRODUCTION

Ovarian cancer is among the most common and deadly malignancies in women, accounting for nearly 15,000 deaths per year in the United States. The prognosis for ovarian cancer is generally very poor due to late presentation of patients and the aggressive nature of the disease. The biochemical changes that occur in the tumorigenic process in these cancers are not well defined but most undoubtedly include aberrant growth factor signaling cascades. A better understanding of signal transduction cascades inappropriately activated in ovarian cancer cells will provide insights into the pathophysiology of ovarian cancer and may ultimately provide targets for new therapeutic interventions. We have developed a mouse model of ovarian cancer, which will allow for new studies into the biochemical changes that occur in the tumorigenic process. Several cell lines derived from mouse ovarian surface epithelial cells that have spontaneously transformed *in vitro* have been developed (1). One such cell line (ID8) has been partially characterized. Intraperitoneal injection of ID8 cells in syngeneic, immune competent mice results in the formation of multiple abdominal mesotheliomas and accumulation of ascites fluid very reminiscent of what is seen in ovarian cancer in women. Src tyrosine kinase, an intracellular signaling molecule that is aberrantly activated in a large proportion of ovarian cancers in women, is constitutively activated in ID8 cells (manuscript submitted, see Appendix). In addition, we have found that several other signal transduction molecules important in regulating cell growth and survival (phosphatidylinositol-3-kinase, Akt/PKB) and morphology (focal adhesion kinase) and which directly interact with Src, are constitutively activated. Blockade of tyrosine kinase activity in ID8 tumor cells with the Src selective inhibitor, herbimycin A, is cytostatic. Furthermore, herbimycin A enhances the cytotoxic effects of Taxol. It is hypothesized that disruption of specific c-Src protein-protein interactions will change the growth, aggressiveness and phenotype of ID8 tumor cells. The research proposed within this project is aimed at determining the role of Src and its specific interactions in ID8 tumor cell malignancy. This will be accomplished by both general and specific disruption of Src protein-protein interactions and determining the effect on tumor cell growth, morphology and tumor characteristics. In Specific Aim 1, Src activity in ID8 cells will be disrupted through the expression of a dominant negative (kinase inactive) Src kinase. Cell growth rate, anchorage independence, and chemotherapeutic sensitivity will be determined. In addition, the ability to form tumors in syngeneic mice will be assessed. Growth rate, tumor morphology and metastatic potential will be determined. The second aim of the research is to determine if Src interacts with and phosphorylates tubulin, the target of the antineoplastic agent Taxol, and how blockade of this interaction enhances Taxol's cytotoxicity. This will be accomplished by *in situ* immunolocalization and expression of kinase inactive Src. The goal of Aim 3 is to disrupt specific Src protein-protein interactions in ID8 cells. Src - PI3-kinase and Src - FAK interactions will be disrupted through expression of Src binding domains. Cell growth and anchorage independence and metastatic potential will be assessed. The information gained may provide important and novel new insights into the roles of Src, phosphatidylinositol-3-kinase and focal adhesion kinase in the neoplastic transformation of ovarian surface epithelial cells. This may ultimately provide information regarding the utility of these kinases as targets for new therapeutic strategies.

BODY

Task 1.   To determine the effect of expression of a Src dominant negative tyrosine kinase on ID8 tumor cell growth, anchorage independence, metastatic potential and chemotherapeutic resistance (months 1-18).

a. develop clonal cell lines derived from ID8 cells transfected with an expression vector encoding an epitope tagged Src dominant negative and a G418 resistance gene (this vector is already in hand; months 1-3).

b. determine FAK and PI3-kinase activity of ID8 cells expressing the Src dominant negative compared to control transfected cells (months 4-9).

c. determine *in vitro* growth characteristics of ID8 cells expressing the Src dominant negative compared to control transfected cells utilizing cell counts, ^{3}H-thymidine incorporation and colony formation in soft agar (months 4-12).

d. determine chemotherapeutic sensitivity of ID8 cells expressing the Src dominant negative compared to control transfected cells by way of *in vitro* cytotoxicity assays (months 13-18).

e. determine *in vivo* tumor formation of ID8 cells expressing the Src dominant negative compared to control transfected cells by intraperitoneal injection of cell lines into syngeneic mice (7 mice per cell line, 4 different cell lines) and monitoring tumor formation (months 4-18).

Development of an inducible Src dominant negative

A Src dominant negative (Srcdn) construct was obtained from Upstate Biotechnology (Waltham, MA). The Srcdn insert was excised from the plasmid and subcloned into a pCDNA3.1 *Myc-His* expression plasmid (Invitrogen, Carlsbad, CA) in order to attach an epitope tag to the Srcdn (Srcdn$^{Myc-His}$), providing a means to distinguish the Srcdn protein from endogenous Src protein. This construct was then used to transfect ID8 mouse ovarian surface epithelial cancer cells (ID8 MOSEC cells). Transient expression of the Srcdn$^{Myc-His}$ was achieved as demonstrated by immunofluorescence and immunoblot analysis (data not shown, see below for explanation). Stable transfectants were selected with G418. Subsequent analysis failed to demonstrate expression of Srcdn$^{Myc-His}$. Thus we suspect that Srcdn$^{Myc-His}$ has been silenced. We are currently confirming that the Srcdn$^{Myc-His}$ construct was integrated into the genome. Additionally we are determining whether demethylation or histone acetylation will restore Srcdn$^{Myc-His}$ expression. While this has cost us a fair amount of time we do not see it in a negative light, it has forced us to pursue a strategy using an inducible system (see below) that in the end should prove to be more flexible and informative. In addition, if indeed the Srcdn$^{Myc-His}$ was silenced, this may suggest that cancer cells may be able to silence transgenes that are detrimental to their survival. This may have important implications with regard to the use of gene therapy in the treatment of cancers. We intend to pursue this further.

As a result of the inability to produce stable cells lines expressing the Srcdn$^{Myc-His}$ we are pursuing a strategy using an inducible system. The Srcdn$^{Myc-His}$ was excised from the pDNA 3.1 *Myc-His* vector and subcloned into an ecdysone inducible pInd vector (Invitrogen). This construct when cotransfected into cells with a vector encoding the ecdysone receptor and RXR (pVgRXR, Invitrogen) will be induced in the presence of the steroid hormone ponasterone. In the absence of ponasterone the Srcdn$^{Myc-His}$ is not expressed so there should be no selective pressure to silence the transgene. Initial transient transfections demonstrate the inducible system is working (Figure 1). We are currently in the process of selecting stable cell lines. Stable inducible lines will provide us with a very good *in vitro* and *in vivo* model system with a great deal of flexibility. We will be able to grow cells *in vitro* under certain conditions and then induce expression of Srcdn$^{Myc-His}$ and determine the effect on tumor characteristics. Similarly, we will be able to use the cells *in vivo*, allow tumors to form and develop to different stages, induce expression of the Srcdn$^{Myc-His}$ by providing animals with ponasterone in their drinking water and monitor further tumor growth development and metastasis. Similarly we will be able to turn off Srcdn$^{Myc-His}$

expression by withdrawal of ponasterone. Thus we believe this system will prove to be far superior to our original attempt to produce lines which constitutively express the Srcdn[Myc-His]. As soon as clonal cell lines have been established in vivo studies will be initiated.

Figure 1. Expression of an inducible Srcdn[Myc-His]. Srcdn was first subcloned into pCDNA Myc-His in order to provide an epitope tag. The resulting Srcdn[Myc-His] was then excised and subcloned into an ecdysone inducible construct. ID8 cells were cotransfected with a pVgRXR and the inducible Src. 48 hours post transfection cells were treated with ponasterone for eighteen hours in order to induce Srcdn[Myc-His] expression. Cells were fixed and immunofluorescence performed using an antibody directed against the His epitope tag. The image is a merged image of fluorescence and DIC optics.

In addition, small interfering RNA's (siRNAs) are being designed and synthesized as an alternative method to specifically inhibit Src activity (2). Cells will be transfected with the double stranded siRNA complementary to Src mRNA in order to block or knockdown Src protein expression. This provides a very specific and reversible way of blocking or knocking down translation of message. We plan to perform growth and anchorage independence assays in ID8 cells that have been transfected with the Src siRNAs.

Inhibition of Src inhibits ID8 cell migration

Our previous studies had demonstrated that Src activation appears to be associated with activation of PI3-kinase. PI3-kinase plays an important role in cell motility and migration. Thus we undertook experiments to determine the role of Src and PI3-kinase in ID8 MOSEC cell migration. ID8 cells were plated on 5 μm tissue culture treated transwell inserts (5, 000 cells/insert) in serum free media. Cells were allowed to attach for 1.5 hours and then pretreated with the following kinase inhibitors; PP2 (Src specific inhibitor, 5 μM), LY294002 (PI3-kinase inhibitor, 50 μM) and PD98059 (MEK inhibitor, 50 μM). Following a 30-minute incubation with the inhibitors, serum-containing media was added to the bottom of the transwells. Cells were then allowed to culture overnight. At the end of the culture period cells were fixed with methanol and stained with Giemsa stain. Cell migration was determined by first counting the total number of cells attached to the membranes and then scraping the cells off the top of the membrane (ie. the side on which the cells were initially plated). The remaining cells (those that migrated through the membrane) were then counted. As represented in Figure 2 both the Src inhibitor PP2 and the PI3-kinase inhibitor LY294002 suppressed ID8 cell migration. The MEK inhibitor PD98059 had little effect on ID8 cell migration. This finding indicates that both PI3-kinase and Src can stimulate cell migration. As inhibition of Src inhibits PI3 kinase activity as measured by Akt phosphorylation we believe that Src is acting to stimulate cell motility via activation of PI3-kinase.
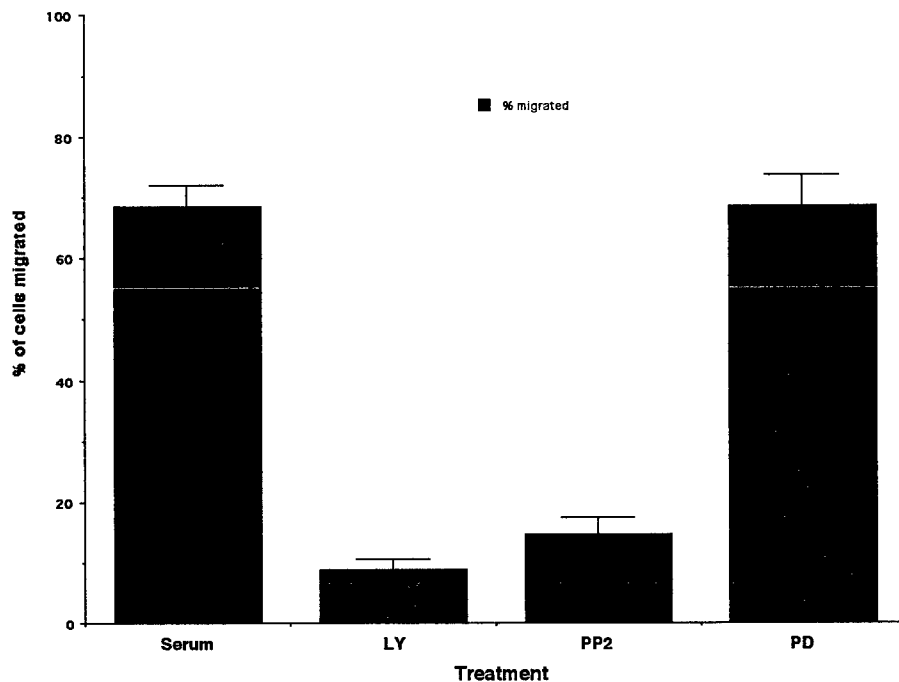
Figure 2. ID8 cell migration is blocked by Src or PI3-kinase inhibition. ID8 cells were plated on 5 µm tissue culture treated transwell inserts (5,000 cells/ insert) in serum free media. Cells were allowed to attach for 1.5 hours and then pretreated with the following kinase inhibitors; PP2 (5 µM), LY294002 (50 µM) and PD98059 (50 µM). Following a 30-minute incubation with the inhibitors, serum-containing media was added to the bottom of the transwells. Cells were then allowed to culture overnight. At the end of the culture period cells were fixed with methanol and stained with Giemsa stain. Cell migration was determined by first counting the total number of cells attached to the membranes and then scraping the cells off the top of the membrane (ie. the side on which the cells were initially plated). The remaining cells (those that migrated through the membrane) were then counted. The data provided is the mean ± the SEM of 4 replicates from a representative experiment. The experiment was repeated three times with consistent results.

Inhibition of Src disrupts peripheral focal adhesions and protein tyrosine phosphorylation

We have found that Src will co-immunoprecipitate focal adhesion kinase (FAK), an important regulator of cell growth and metastatic potential. Over expression of activated FAK has been associated with loss of anchorage dependence and resistance to anoikis (apoptosis resulting from loss of contact with extracellular matrix), a hallmark of cell transformation. Phosphorylation of tyrosine 397 of FAK, which serves as the binding site for the SH2 domain of Src, is necessary for this effect (3). Therefore the constitutive activation of FAK may be a very important event in ID8 cell transformation. In order to determine whether inhibition of Src will alter focal adhesions, cells were treated with PP2, a Src specific inhibitor, and then cells were fixed and analyzed for FAK expression and localization by immunofluorescence microscopy. We found that treatment of ID8 cells with PP2 led to a loss of periphral membrane FAK localization (Figure 3). In addition there was an increase in the number of small rounded, presumably proapoptotic cells. Treatment with PP2 also led to an overall decrease in

protein tyrosine phosphorylation. These results will be confirmed in cells expressing the inducible Src dominant negative once clonal lines have been isolated.
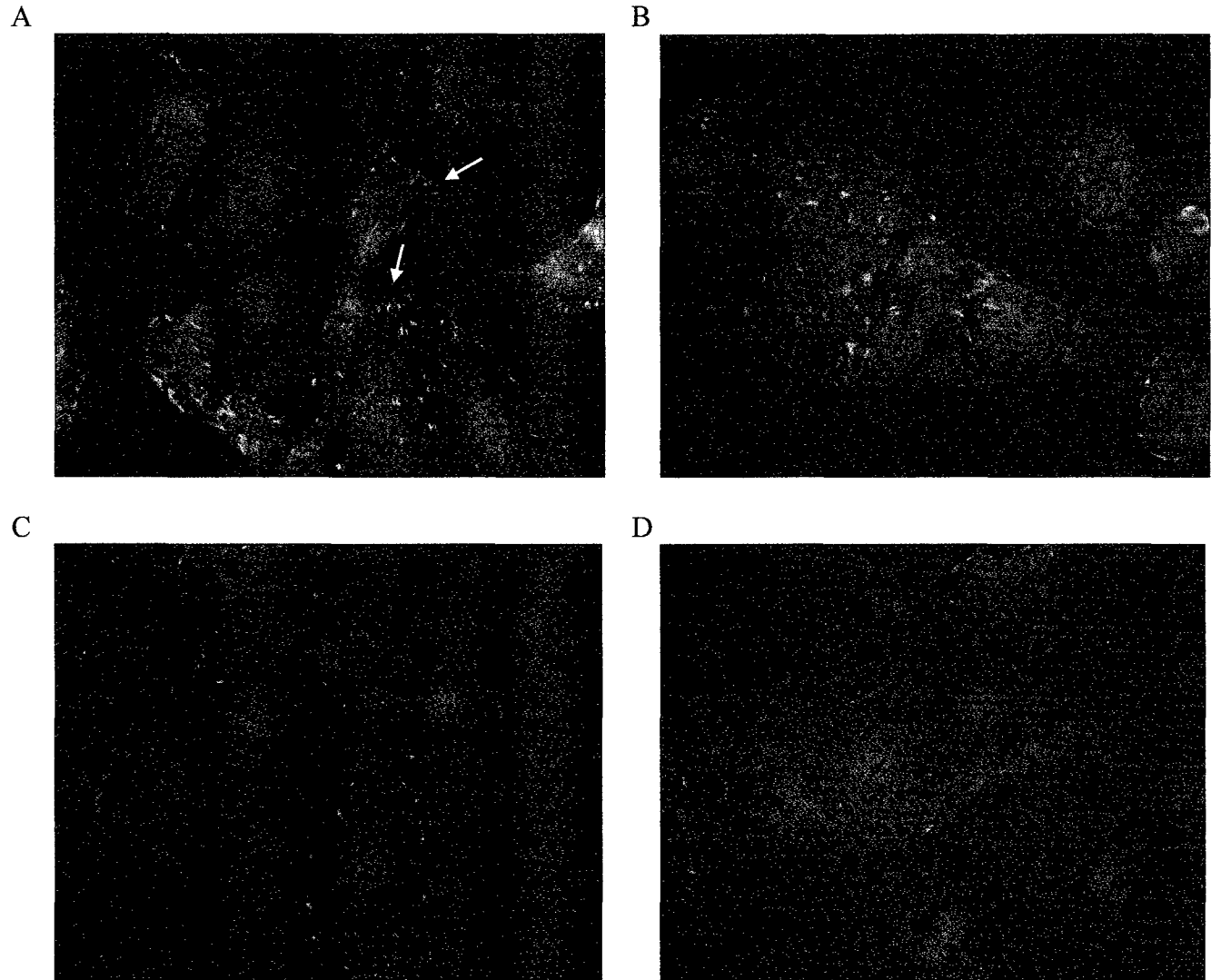

Figure 3. Loss of peripheral focal adhesion kinase and protein tyrosine phosphorylation following treatment with the Src specific inhibitor PP2. Cells were treated overnight with either vehicle or PP2 (10 μM), then fixed and analyzed for focal adhesion kinase (A and B) or tyrosine phosphoproteins (C and D) by immunofluorescence. Arrows indicate peripheral membrane focal adhesions.

Task 2.   To determine how disruption of Src activity enhances Taxol cytotoxicity of ID8 cells (months 1-24).

   a. determine tubulin and Src co-precipitation and tubulin tyrosine phosphorylation state in ID8 cells and ID8 cells treated with the Src selective inhibitors herbimycin A and PP2 (months 1-6).
   b. determine tubulin and Src co-precipitation and tubulin tyrosine phosphorylation state in ID8 cells expressing the Src dominant negative compared to control transfected cells (months 6-12).
   c. determine Src and tubulin co-localization and how disruption of Src activity alters Src and tubulin localization utilizing confocal immunofluorescence (months 12-24).

Inhibition of Src does not alter microtubule dynamics in ID8 mouse ovarian cancer cells

We have shown that inhibition of Src activity synergizes with both Taxol and colchisine induced ID8 MOSEC cell death. Although there is literature to suggest that Src and tubulin directly interact, to date we have been unable to detect any effect of Src inhibition on microtubule dynamics. In the last reporting period we reported that inhibition of Src had no gross effect on microtubule depolymerization or repolymerization in response to nocodozole and nocodozole washout. In addition, we have been unable to convincingly demonstrate that Src will co-precipitate with tubulin. Thus to date4 we have no convincing evidence for a Src – tubulin interaction. However, we have consistently observed that taxol or colchisine and PP2 have a synergistic cell killing effect, therefore we have continued to pursue the mechanism by which inhibition of Src synergises with microtubule disruption to induce cell death.

Identification of Cell Death Pathway

In an effort to better understand the synergy between taxol and Src inhibition, the cell death pathways activated by the combinatorial treatment were determined. The combination of paclitaxel and PP2 treatment was associated with cleavage and activation of Caspase-3 (Fig. 4a), a so-called executioner caspase. Neither paclitaxel (2 μM) nor PP2 (10 μM) alone led to the activation of Caspase-3, suggesting the combination treatment activates a cell death pathway not activated by either treatment alone. This effect was seen at very low paclitaxel concentrations (0.02 μM). Caspase-9, an upstream activator of Caspase-3 that is activated in response to mitochondrial dysfunction and release of mitochondrial cytochrome C does not appear to be activated in response to paclitaxel, PP2 or the combination treatment (Fig. 4c). In a preliminary experiment, Caspase-9 protein expression was knocked down using small interfering RNA (siRNA) specific for Caspase-9 (Fig. 4d). This approach was undertaken due to the lack of specificity of Caspase inhibitors and the cytotoxicity we noted with their use. Caspase-9 knockdown did not rescue paclitaxel plus PP2 treated cells (30 ± 3% for Caspase-9 intact and 38 ± 7% for Caspase-9 knockdown compared control cell number, 100%). Finally, mitochondrial function appears not to be disrupted as demonstrated by sequestration of mitochondrial dyes and an absence of cytochrome C release (Figure 5). Thus, the activation of Caspase-3 appears to be Caspase-9 independent.

Survivin is a member of the inhibitor of apoptosis (IAP) family of proteins. It associates with microtubules and has been proposed to be a possible inhibitor of Caspase-3, and is commonly overexpressed in cancers. We have found that Survivin is highly expressed in ID8 mouse ovarian cancer cells and is down regulated in response to Src inhibition by PP2 or PI3-kinase inhibition by LY294002 (Figure 4), therefore the down regulation of Survivin in response to Src inhibition may lead to the activation of Caspase-3, however, this has yet to be confirmed. We have obtained constructs encoding wild-type and dominant negative Survivin-EGFP (obtained from Dr. Dario Altieri, Yale University) that will allow us to pursue this possibility. Preliminary results indicate that Src inhibition may be associated with decreased Survivin stability (data not shown), possibly resulting in a lifting of Survivin inhibition of Caspase -3. Studies are currently underway to test this hypothesis.
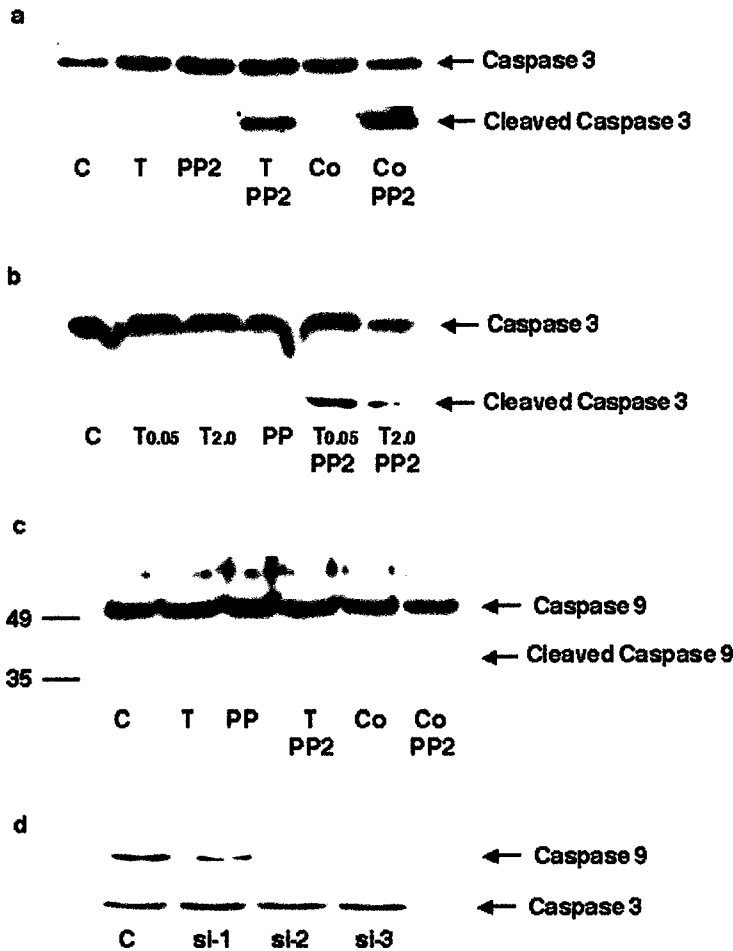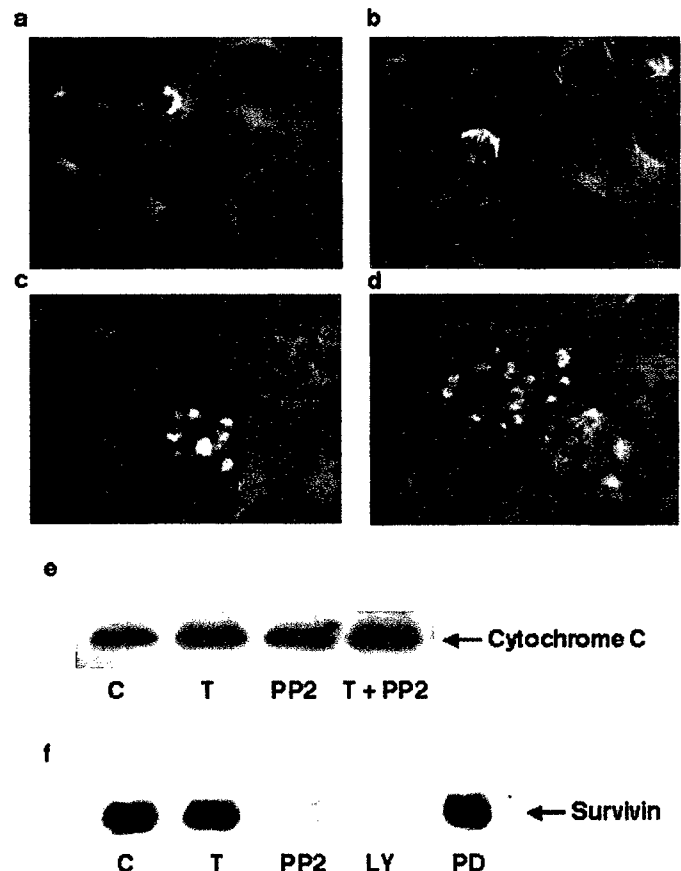
a

← Caspase 3

← Cleaved Caspase 3

C   T   PP2   T   Co   Co
              PP2   PP2

b

← Caspase 3

← Cleaved Caspase 3

C   To.o5   T2.0   PP   To.o5   T2.0
                        PP2   PP2

c

49 —

← Caspase 9

← Cleaved Caspase 9

35 —

C   T   PP   T   Co   Co
             PP2   PP2

d

← Caspase 9

← Caspase 3

C   si-1   si-2   si-3

Figure 4. Src inhibition and paclitaxel combinatorial treatment activates caspase 3. ID8 cells were grown in serum containing media and treated for 20 hours with paclitaxel (T, 1 μM), PP2 (10 μM), colchicine (Co, 10 μM) or combinations of paclitaxel and PP2 or colchicine and PP2. Controls received vehicle only (DMSO, 0.1%). Cleavage of Caspase-3 and Caspase-9 were determined by immunoblot analysis with specific antibodies (a-c). siRNA was used to knockdown Caspase-9 protein expression (d). Three different Caspase-9 specific sequences were used (si-1, si-2, si-3).

Figure 5. Src inhibition and paclitaxel combinatorial treatment does not induce mitochondrial dysfunction. ID8 cells were grown in serum containing media and treated for 20 hours with paclitaxel (T, 1 μM), PP2 (10 μM) or paclitaxel and PP2. Controls received vehicle only (DMSO, 0.1%). Mitochondrial function was determined by uptake and mitochondrial sequestration of Mitotracker dye (red) in live cells. Cells were then fixed and immunofluoresence with an antibody specific for tubulin (green) performed. Nuclei were stained with Dapi (blue). Release of cytochrome C (e) and Survivin expression (f) as determined by immunoblot analysis of the cytosolic fraction (e). Cells were treated with paclitaxel (T, 1 μM), the Src inhibitor PP2 (10 μM), the PI3-kinase inhibitor LY294002 (50 μM), or the MEK inhibitor PD98059 (50 μM).

a

b

c

d

e

← Cytochrome C

C   T   PP2   T + PP2

f

← Survivin

C   T   PP2   LY   PD

10

Task 3.   To determine how specific disruption of Src - FAK and Src - PI3-kinase interactions alters ID8 cell growth, anchorage independence, metastatic potential and chemotherapeutic resistance (months 13-36).

    a. Subclone sequences corresponding to the region of Src that interacts with FAK and the region that binds with PI3-kinase into expression vectors containing a G418 resistance gene (months 13-15).

    b. develop clonal cell lines derived from ID8 cells transfected with the above expression vectors (months 16-18).

    c. determine *in vitro* growth characteristics of ID8 cells expressing the FAK and PI3-kinase binding sequences compared to control transfected cells utilizing cell counts, ^{3}H-thymidine incorporation and colony formation in soft agar (months 19-27).

    d. determine chemotherapeutic sensitivity of ID8 cells expressing the FAK and PI3-kinase binding sequences compared to control transfected cells by way of *in vitro* cytotoxicity assays (months 28-36).

    e. determine *in vivo* tumor formation of ID8 cells expressing the FAK and PI3-kinase binding sequences compared to control transfected cells by intraperitoneal injection of cell lines into syngeneic mice (7 mice per cell line, 4 different cell lines) and monitoring tumor formation (months 15-36).

Development of Src Homology Domain Constructs

The individual Src homology domains (SH domains) have been cloned into EGFP fusion plasmids. This allows for the visualization of these domains within live cells as well as providing an epitope for immunoprecipitation and immunoblot studies.   To date constructs for Src dominant negative-EGFP, SH3-SH2-EGFP, and SH3-EGFP have been made. This strategy will allow us to inhibit subsets of Src protein protein interactions in order to determine which interactions are important for individual aspects of the cancer cell's phenotype. We are currently making stable transfectants expressing the various constructs.

Preliminary results using transient expression demonstrates that the SH domain – EGFP fusions are regionally localized within the cell and co-localize with tyrosine phosphorylated proteins and focal adhesion kinase (Figure 6). This demonstrates that the EGFP does not interfere with proper localization. We are currently determining the effects of the expression of these domains on cell characteristics. Preliminary results indicate that expression of the SH3-SH2 domain alters focal adhesion kinase localization in a manner similar to that seen with Src inhibition by PP2 (data not hown). The ultimate goal is to disrupt individual Src protein-protein interactions as a chempotherapeutic approach in an effort to decrease cytotxicity to non-target cells.
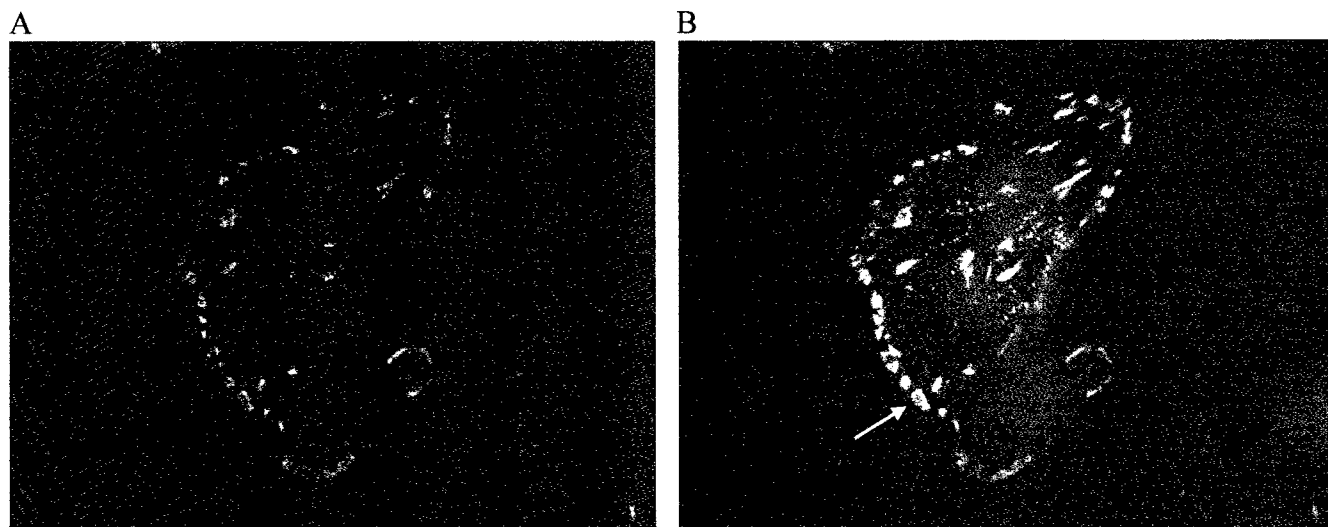
A

B

Figure 6.   Co-localization of SH3-SH2-EGFP with tyrosine phosphorylated proteins.   Mouse ID8 ovarian cancer cells were transfected with an expression vector encoding the Src SH3-SH2 domains fused to EGFP (green).   Cells were then fixed and tyrosine phosphorylated proteins were visualized by immunofluorecence (A, red).   Note extensive overlap of SH3-SH2EGFP with tyrosine phosphorylated proteins (arrow, yellow).

Development of Taxol Resistant Mouse Ovarian Cancer Cells

Although not part of the original application we have developed ID8 mouse ovarian cancer cells that are resistant to paclitaxel (ID8^{TaxR} cells).   This was accomplished by chronic exposure of cells to sub-lethal and increasing concentrations of paclitaxel.   ID8^{TaxR} cells have upregulated the multidrug resistance gene (Mdr).   Interestingly, inhibition of Src tyrosine kinase with PP2 resensitizes cells to paclitaxel. This is in the absence of any change in Mdr expression (Figure 7).   Preliminary experiments with a fluorescent- tagged paclitaxel also suggest that PP2 inhibition of Src does not interfere with paclitaxel uptake or sequestration (data not shown) suggesting that Mdr function is not altered by Src inhibition. Further experiments are required to determine the mechanism by which Src inhibition restores paclitaxel sensitivity.   The finding that Src inhibition restores sensitivity of resistant cells to paclitaxel underscores the potential importance of Src as a target for small molecule chemotherapy.   Chemotherapeutic resistance in general and paclitaxel resistance in particular are major problems in advanced and recurrent ovarian cancer.
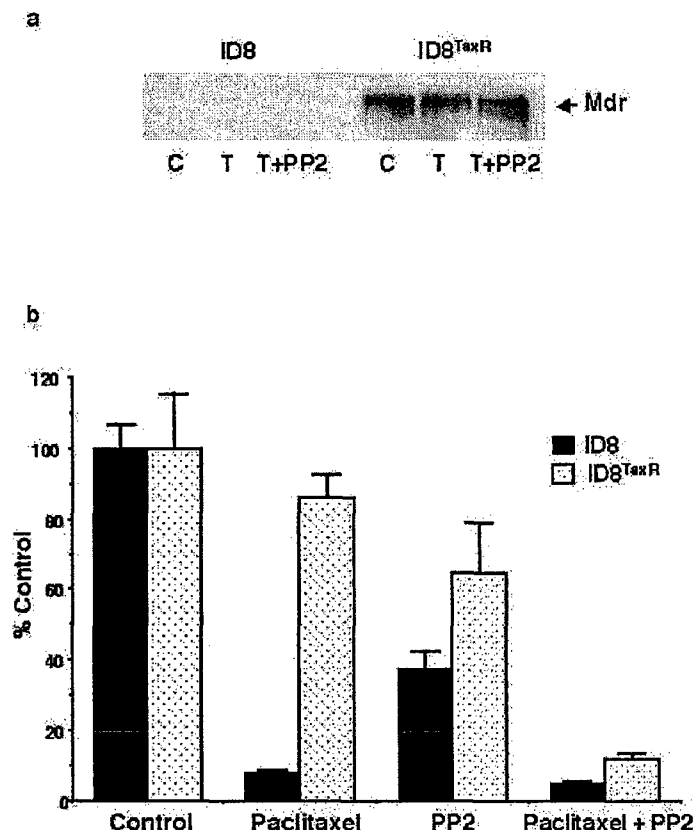
Fig. 8. ID8 paclitaxel resistant cells are resensitized to paclitaxel independent of Mdr expression. ID8 cells were made resistant to paclitaxel by continuous culture in increasing concentrations sub cyto-toxic concentrations of paclitaxel. Mdr1 expression in ID8 and ID8 paclitaxel resistant cells (ID8^{TaxR}) was determined by immunoblot analysis. Cell survival was determined 48 hours after the addition of paclitaxel (1 μM), PP2 (10 μM) or both paclitaxel and PP2. Viable cell number was determined by direct cell counts and trypan blue exclusion. Data represents the mean ± SEM of 4 replicates in a representative experiment.

KEY RESEARCH ACCOMPLISHMENTS:

- Production of mouse model of ovarian cancer (in collaboration with Paul Terranova, University of Kansas Medical Center).

- Construction of an inducible Src dominant negative.

- Identification of aberrant signaling networks contributing to tumor characteristics in mouse ovarian cancer cells.

- Identification of signaling pathways involved in cell migration in mouse ovarian cancer cells.

- Identification of a possible chemotherapeutic target for ovarian cancer (Src tyrosine kinase).

- Development of Taxol resistant mouse ovarian cancer cells.

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REPORTABLE OUTCOMES:

1. Development of spontaneously transformed mouse ovarian surface epithelial cell line (Ovarian cancer cells).

2. Development of an inducible Src dominant negative system.

3. Presented findings at AACR special meeting "Apoptosis and Cancer: Basic Mechanisms and Therapeutic Opportunities in the Post-Genomic Era.

4. Submitted Grant Application to DOD – OCRP (June 18, 2002): "Combinatorial Chemotherapy in a Mouse Model of Ovarian Cancer" (#OC020085).

5. Submitted grant application to NIH (May 31, 2002): "Src tyrosine kinase in ovarian cancer" (1RO1 CA100580-01).

6. Two manuscripts in preparation.

OVERALL CONCLUSIONS

The results obtained from the performed research extend our previous findings with regard to Src – FAK and Src – PI3-kinase. Inhibition of PI3 kinase inhibits ID8 cell migration. Inhibition of Src also inhibits ID8 cell migration and Akt phosphorylation. Akt is a PI3-kinase target, thus it appears that Src may activate PI3-kinase in ID8 cells, potentially stimulating cell migration. In addition, inhibition of Src activity appears to alter focal adhesion kinase localization. Focal adhesions play an important role in cell migration and attachment to the extracellular matrix. Therefore, Src activation may play a role in cell migration (metastasis?) by multiple mechanisms, ie. by activating PI3-kinase, and by stimulating assembly of new focal adhesions. With the development of the inducible Src dominant negative we will be able to confirm and extend these results, using both *in vitro* and *in vivo* models.

We have also identified the cell death pathway that is activated by the combination treatment of paclitaxel and Src inhibition that is not activated by either treatment alone. This pathway involves the activation of Caspase-3, an executioner caspase, possibly by a Survivin mediated mechanism. As mentioned above we have also developed paclitaxel resistant mouse ovarian cancer cells. Inhibition of Src restores paclitaxel sensitivity thus providing a rationale for further study of Src as a potential chemotherapeutic target.

Finally, much of the work that is performed in the mouse ovarian cells is being replicated in human ovarian cancer cell lines. To date, the results from the human cells are consistent with what we have found in the mouse. The great advantage with the mouse cell line is that we can use these in immune intact animals.

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APPENDIX

No appendix attached