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# Differential Phosphoprotein Profiling of Tamoxifen Response

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## 14. ABSTRACT

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## 15. SUBJECT TERMS

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abstract

Tamoxifen revolutionized breast cancer treatment when it came into use some three decades ago. In estrogen receptor(ER)-$\alpha$ positive breast cancer cells, tamoxifen blocks cancer growth by competing for binding to ER and cuts recurrence risk in half. However, some advanced breast cancers that initially respond well to tamoxifen eventually become resistant. Several mechanisms of resistance have been hypothesized, including crosstalk between ER and growth factor receptor tyrosine kinase pathways. Several studies suggest that overexpression of HER2, and high levels of phosphorylated Akt or ERK, contribute to tamoxifen resistance. By cataloging global phosphorylation events in response to tamoxifen treatment in tamoxifen sensitive and resistant cells we will provide better understanding of the mechanism of acquired resistance and/or identify novel biomarkers for tamoxifen response. We have developed a method for comparison of global phosphoprotein profiles. Our methodology involves stable isotope labeling, a phosphoprotein affinity step, 1-D SDS-PAGE and LC-MS/MS. I have shown differential phosphoprotein patterns in MCF7/Chicago (tamoxifen sensitive) and MCF7/HER2 (tamoxifen resistant) cells as a result of tamoxifen treatment. I present preliminary data towards phosphoprotein profiling of tamoxifen response in the cytoplasmic fraction of MCF7/Chicago cells. Over 1250 proteins were identified using an Orbitrap (Thermo) including over 40 kinases and 20 phosphatases. A subset of the proteins is isotopically labeled and quantitative revealed that a majority of the proteins did not change in abundance, as expected. However, about 60 proteins were identified with Xpress ratio less than 0.6 and about 28 proteins with ratio larger than 1.66. Manual analysis is underway to confirm the protein abundance ratios. Manual validation will be performed on each of these proteins to confirm the ratio, compare the protein hits to tamoxifen responsive proteins identified in the literature and finally confirmed by biological validation.
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Introduction

Breast cancer remains the most common malignancy affecting women in the United States. About 80% of breast cancers are estrogen-receptor-alpha-positive (ER\textsubscript{α+}), some of which respond to estrogen hormone therapy. ERs is a ligand-activated transcription factor that plays a critical role in the etiology of breast cancer [1-3]. Selective estrogen receptor modulators (SERMs) have variable agonistic and/or antagonistic activities, depending on the type of ER (\textalpha versus \textbeta), tissue context, and interactions with different proteins such as transcriptional co-activator or co-repressors [4]. The first SERM, tamoxifen, revolutionized breast cancer treatment when it came into use some three decades ago. In ER\textalpha breast cancer cells, tamoxifen blocks cancer growth by competing for binding to ER and cuts recurrence risk in half [5, 6]. More recently, tamoxifen has been shown to prevent breast cancer in high-risk women [7-9]. Even in patients with ER\textalpha-positive breast cancer, only 40–50% of patients benefit from tamoxifen treatment, suggesting that a substantial fraction of ER-positive cancers are resistant to this drug. Additionally, advanced breast cancers that initially respond well to tamoxifen eventually become refractory to this compound. In some cases, tamoxifen can even act as a growth stimulatory signal. Several mechanisms of resistance have been hypothesized, including crosstalk between ER and other proliferative signals, such as growth factor receptor tyrosine kinase pathways [10-13]. The cumulative data from clinical studies show that overexpression of HER-2 and/or EGFR, and high levels of phosphorylated Akt or ERK, contribute to tamoxifen resistance in some patients [14-17]. HER-2, EGFR, Akt and ERK are all kinases and components of signaling pathways critical to cell growth and survival, highlighting the need for global phosphoproteome analysis.

Although many biomarkers for breast cancer prognosis and therapy initially appeared attractive, over the years most of them have failed to become clinically useful, with the exception of hormone receptors (ER and PR) and the HER-2 tyrosine kinase receptor [18, 19]. Although ER status provides prognostic information, the major clinical value is to assess the likelihood that a patient will respond to endocrine therapy [2, 20]. HER2 is overexpressed in 25 to 30 percent of breast cancers, increasing the aggressiveness of the tumor [21]. The drug Trastuzumab (Herceptin) is a monoclonal antibody directed against the HER-2 and has a survival benefit when combined with chemotherapy in patients with metastatic breast cancer that overexpress HER-2 [22]. However, tumors that overexpress HER2 tend to be ER\textalpha negative and thus represent a separate treatment group. Current prognostic classifications are thus not enough to represent the broad clinical heterogeneity of breast cancer, making it difficult to target therapeutic strategies to each patient. A major component of prognosis for patients undergoing endocrine therapy is the acquired resistance to tamoxifen. Finding biomarkers for tamoxifen resistance and/or drugs that could help overcome the resistance is a very important topic.

Combining proteomics, systems biology and cancer biology, I am interrogating the status of signaling pathways in breast cancer cells with the goal of better understanding responses to tamoxifen and to identify biomarkers that predict therapy outcome.
Optimizing tamoxifen treatment conditions (Aim 1)

Previously, I have developed and published a method for enrichment of phosphoproteins [23]. The methodology involves a phosphoprotein affinity step, 1-dimensional SDS-PAGE and ESI LC-MS/MS and is termed PA-GeLC-MS/MS. The overall goal of this proposal is combine the phosphoprotein enrichment method with stable isotope labeling to obtain relative quantitation between two samples and thus be able to compare global phosphoprotein profiles of tamoxifen response. Before performing this phosphoprotein profiling, it is important to identify optimal conditions for tamoxifen treatment. The MCF7 breast cancer cell line is estrogen receptor positive, sensitive to tamoxifen and estrogen response and, to a lesser degree, tamoxifen response have been studied extensively in this cell line. The “normal” MCF7 cell line (MCF7/Chicago) is sensitive to tamoxifen treatment. A MCF7 cell line was transfected with full-length HER2 cDNA HER2 (MCF7/HER2) has acquired tamoxifen resistance but retains at least a partial response to estrogen [21]. Tamoxifen resistance was measured by treating the cells with high concentrations of tamoxifen (1 µM) and measuring cell death over several days. In addition, MCF7/HER2 and MCF7 control cells were implanted into nude mice. Both cells only produced tumors when stimulated with estrogen, but MCF7/HER2 grew much more rapidly. Tamoxifen inhibited growth in the MCF7-derived tumors but not in the MCF7/HER2 derived tumor [21].

Phosphoprotein profiling requires conditions where the tamoxifen resistant and sensitive cell lines differ in their phosphorylation profile but no cell death has occurred. In addition, an optimal sample for phosphoprotein profiling would have minimal changes in gene expression but substantial changes in phosphorylation patterns. Gene expression changes are usually “late” events (>24 hours). Changes in phosphorylation can occur in tens of minutes depending on the protein and growth conditions. To meet these requirements tamoxifen sensitive (MCF7/Chicago) and tamoxifen resistant (MCF7/HER2) cell lines were treated with low concentrations of tamoxifen (10 nM) and/or estrogen (10 nM) for 30 min to 24 hours allowing for identification of “early events”. After treatment, cells were scraped from the plates, lysed and proteins separated on SDS-PAGE. The proteins were transferred to nitrocellulose membranes and the membranes probed with antibodies to several signaling proteins proposed to be involved in tamoxifen response/resistance and known to be phosphorylated including Akt, HER-2 and ERK. Tubulin was used as a loading control. Results are shown in Figure 1A and Figure 1B. Significant phosphorylation of proteins was detected at the earliest timepoint (30 minutes) in Figure 1A and at the 2 h timepoint in Figure 1B indicating that phosphoprotein profiling would be feasible with these cells under these conditions. The two experiments differ in the time of incubation with serum free media (serum starvation) prior to treatment (Figure 1A for 2 hours, Figure 1B overnight). This might explain the change in peak time of phosphorylation with the cells that were serum starved overnight (Figure 1B) taking longer to grow and respond to stimulus (peak at 2h or later, Figure 1B) than the ones serum starved for only 2 hours (peak at 30 minutes, 1A).

One of the things to consider when examining tamoxifen response is whether cells should be treated with estrogen prior to tamoxifen treatment or not. To examine whether the tamoxifen response changes between an estrogen and estrogen free background I treated the cells with tamoxifen alone, estrogen alone or a mix of
tamoxifen and estrogen (Figure 1B). After 30 minute treatment very little differences were seen. After 120 minute treatment in the MCF7/HER2 cell line combined tamoxifen and estrogen treatment resulted in a robust pAkt, pHER2 and pERK signal whereas tamoxifen alone had significantly reduced pAkt, pHER2 and pERK signal. The difference between combined tamoxifen and estrogen treatment and tamoxifen alone was less pronounced in MCF7/Chicago cells but seemed to follow the same trend.

Protein abundance of ERK, Akt and HER2 proteins was measured with pan antibodies (Figure 1B). The proteins did not change significantly in abundance, in fact, if anything there appears to be more protein present in the 120 minute samples treated with tamoxifen than in the combined tamoxifen and estrogen treatment sample. Thus, the changes in phosphorylation of Erk, Akt and HER2 are caused by changes in the phosphorylation status of the protein, not changes in protein abundance. These conditions thus provide good samples for phosphoprotein profiling.

Future directions include more detailed analysis of the phosphoprotein response by examining other signaling proteins including EGFR, estrogen receptor and p38. Control samples (no treatment and estrogen only treatment) that were missing in the later time points will be included. Finally, other time points will be examined to better
pinpoint the peak of phosphorylation. The results show that phosphoprotein profiling is a feasible option as phosphorylation patterns change in as little as 30-120 minutes and differ significantly between tamoxifen resistant and sensitive cell lines in both timing and phosphoprotein pattern. However, care must be taken to make sure the cell lines are treated the same way and several replicates must be done to ensure that these results are reproducible.

Recently, I have obtained other MCF7-based cell lines. MCF7/C412 does not express estrogen receptor and will identify non-estrogen specific signaling events and serve as a control cell line. A cell line overexpressing estrogen receptor, MCF7/K1, which results in the cell line being non-responsive to estrogen, will further allow for identification of estrogen specific signaling events. Finally, these cell lines will never fully replicate conditions in breast cancer patients but these experiments could result in identification of several potential biomarkers that will then be tested in both other cell lines and ultimately, in patient samples.

Sample fractionation and phosphoprotein enrichment (Aim 2)
A mass spectrometer has a limited dynamic range. Thus, sample fractionation is critical to obtain information from low abundance proteins. MCF7/Chicago cells were separated into three fractions: cytoplasmic, nuclear loose and nuclear matrix using a protocol published in [24] (Figure 2). Coomassie staining of the gel reveals differences in protein patterns and Western Blotting confirms correct and specific localization of the cytoplasmic protein p44/p42 MAPK and the nuclear protein Histone H1.

The first phosphoprotein enrichment was performed on the cytoplasmic fraction of MCF7/Chicago cells either treated with estradiol only or tamoxifen and estradiol together for a period of 2 hours. Whole cell lysates were prepared from 3 x 10^7 treated and untreated cells in 1.5 ml of lysis buffer (ProQ lysis buffer with endonuclease, phosphatase and protease inhibitors). The supernatant was collected, and protein yields were determined by Bradford analysis using Bio-Rad protein assay reagent. The lysate was diluted and loaded onto pre-equilibrated Pro-Q Diamond resin, the column washed and phosphoproteins eluted. The lysate, flow-through and eluate were concentrated in 10 kDa MWCO Vivaspin concentrators at 4 °C and washed with 50 mM Tris, pH 7.5. The proteins were boiled for 10 min before loading on NuPAGE 2-12% gradient gels. The gel was stained for phosphoproteins using Pro-Q Diamond stain and subsequently for proteins with Imperial Coomassie stain. Coomassie stained protein was visible in all three fractions including the flow through (Figure 3). The scarcity of phosphoproteins in the flowthrough fraction shows that the Pro-Q Diamond resin selectively binds phosphoproteins.
For analysis of the ProQ elution gel lane, the molecular weight region above 10 kDa was divided into 18 sections, each ~1 mm³. Sections were washed in water and completely destained using 100 mM ammonium bicarbonate in 50% acetonitrile. A reduction step was performed by addition of 100 µl of 50 mM ammonium bicarbonate pH 8.9 and 10 µl of 10 µM TCEP and allowed to reduce in 37 °C for 30 min. The proteins were alkylated by adding 100 µl of 50 mM iodoacetamide and allowed to react in the dark for 40 min. Gel sections were washed in water, initially dried with acetonitrile followed by a SpeedVac step of 30 min. Digestion was carried out using sequencing grade modified trypsin (40 ng/ml, Promega) in 50 mM ammonium bicarbonate. Sufficient trypsin solution was added to swell the gel pieces, which were kept in 4° C for 45 min and then incubated at 37° C overnight. Sections containing proteins larger than 150 kDa were pre-digested with Lys-C (0.25 mg/ml, Princeton Separations) in 6-8 M Urea overnight at 25° C, diluted to final concentration of less than 2 M Urea then digested with trypsin as described above. Peptides were extracted from the gel pieces with 5% formic acid.

The samples were then labeled with stable isotope by ¹⁸O labeling via trypsin exchange as has been described extensively in literature [25-27]. In short, the eluted peptides were dried in a vacuum centrifuge and dissolved in 10 µl acetonitrile. Using the Sigma ¹⁸O Proteome Profiler Kit, 40 µl of H₂¹⁸O or H₂¹⁶O was added to solubilize an aliquot of stabilized trypsin which was mixed with the peptides and incubated at 37° C for 12-24 h. The oxygen exchange reaction was stopped with 5 µl of 1.0% TFA in H₂O.

**Mass spectrometry**

All mass spectrometry was performed in the Mayo Proteomics Research Center, on Thermo LTQ-Orbitrap Hybrid FT Mass Spectrometers. The peptide samples were loaded to a 0.25 µl C8 trapping cartridge OptiPak custom-packed with Michrom BioResources Magic C8, 5 µm, 200A, washed, then switched in-line with a 20 cm by 75 um C18 ‘packed spray tip’ nano column packed with Magic C18AQ, 5 µm, 200A, for a 2-step gradient, where mobile phase A is water/acetonitrile/formic acid 98/2/0.2 and mobile phase B is acetonitrile/isopropanol/water/formic acid 80/10/10/0.2. Using a flow rate of 350 nl/min, a 90 min. 2-step LC gradient was run from 5% B to 50% B in 60 min, followed by 50%-95% B over the next 10 min, hold 10 min at 95% B, back to starting conditions and re-equilibrated. The samples were analyzed via electrospray tandem mass spectrometry (LC-MS/MS) on the LTQ-Orbitrap using a 60,000 RP Orbi survey scan, m/z 375-1950, with lock masses, followed by 5 LTQ CAD scans with isolation.
Figure 4. Identification of several (phospho)proteins that increased or decreased as a results of tamoxifen treatment.

MCF7/Chicago cells treated with estrogen and tamoxifen or just estrogen. The cells were lysed, the cytoplasmic fraction isolated, phosphoprotein enrichment performed and the samples run on 1-D SDS PAGE. The elutions lanes from the estrogen only and tamoxifen and estrogen samples were cut into 18 sections. Each section was digested with trypsin and the peptides extracted from the gel. Peptides from the tamoxifen and estrogen sample were O18 labeled (heavy) while the peptides from the estrogen only sample kept the O16 (light). The samples were then mixed and the peptides separated and identified by LC-MS/MS analysis on an Orbitrap. Protein identification was performed with X!Tandem and quantitation using CPAS. 1275 proteins were identified with a minimum of 2 peptides and could be quantitated. The graph is a frequency distribution of log2 Fold-Change. Proteins that did not change have a value of 1, proteins that go up are >1, proteins that go down are <1.

Data analysis (Aim 3)

The mass spectrometry data were converted to .mgf files via .mzXML intermediates and searched using Mascot using the O18 (MD) quantitation parameter. A fragment ion mass tolerance of 1.0 Da and a parent ion tolerance of 0.6 Da were specified. Oxidation of methionine, phosphorylation (S, T, Y) and Carbamidomethyl (C) were specified as variable modifications. Mascot results were loaded into Scaffold (Proteome Software), which uses Peptide and Protein prophet to calculate probabilities. Scaffold also conducted an X!Tandem search using the parameters used for Mascot. Through Scaffold, we identified 1275 proteins (protein probability >99, peptide probability >95, requiring a minimum of 2 unique peptides per protein identification). These were distributed across many functional groups, including a number of signaling proteins. We found 20 phosphatases and 42 kinases including Akt kinase.

Quantitation analysis is currently underway to identify changes in phosphoprotein abundance. Comparative Proteomics Analysis System (CPAS) is a open-source analytic system for evaluating and publishing proteomic data based on the modules developed in the Trans Proteomic Pipeline from Institute of Systems Biology (Seattle). CPAS was used to perform quantitation on the data from mzXML files. The analysis pipeline involved performing X!Tandem searches (using the parameters described above), converting the results to .pepXML format, processing by Peptide Prophet for statistical evaluation of peptide identifications and Xpress software for relative peptide quantification. For Xpress, the accurate masses were input: Double O18 = 4.008491, Single O18 = 2.004245. The peptide results from all 18 sections were exported and combined into one excel file. Proteins were compiled and protein averages calculated using a Perl script provided by the Hanash lab at Fred Hutch (Seattle). Through CPAS, we identified 1759 proteins and out of these 1224 proteins width of 1.6 Da on doubly and triply charged-only precursors between 375 Da and 1500 Da. Ions selected for MS/MS were placed on an exclusion list for 60 s using low mass exclusion of 1.0 Da, high mass exclusion of 1.6 Da.
were identified with more than one peptide. In preliminary analysis, the vast majority of proteins did not change substantially in abundance. However, about 60 proteins were identified with Xpress ratio less than 0.6 and about 28 proteins with ratio larger than 1.66 (Figure 4). Manual validation will be performed on each of these proteins to confirm the ratio, compare the protein hits to tamoxifen responsive proteins identified in the literature and finally confirmed by biological validation (Western Blots, siRNA and overexpression studies).

SILAC labeling and further phosphoprotein profiling

Although $^{18}$O labeling via trypsin exchange did result in labeling of peptides and allowed for quantitation it did not result in 100% labeling and some of the labeling was only partial (i.e. only one $^{18}$O was added) confounding quantitation. I have thus turned to stable isotope labeling in cell culture (SILAC). Another advantage of SILAC is that the cells are labeled with stable isotope in cell culture, allowing samples to be mixed before lysis and to be processed together through the phosphoprotein enrichment. This decreases differences in samples due to sample handling. Two populations of MCF7/Chicago and MCF7/HER2 cells were grown in DMEM “light” or “heavy” (13C6 L-lysine and 13C6,15N4 L-Arginine (Invitrogen)) media with 10% heat-inactivated fetal bovine serum, 1% penicillin/streptomycin and 0.3 mg/ml L-glutamine. Cells were incubated at 37 °C and 5% CO2 for at least 6 doublings to incorporate the amino acids to over 95%. In this experiment, I only serum starved for 2 hours and thus only treated cells for 30 minutes with tamoxifen or estrogen (same as in Figure 1A). I did three conditions: cells treated with a) ethanol (control) b) estrogen or c) tamoxifen for 30 minutes. I did the control versus estrogen and control versus tamoxifen both in the light/heavy and heavy/light combinations. The frozen cell pellets will be subjected to phosphoprotein profiling as described above and mass spectrometry and data analysis.
Key Research Accomplishments

- Acquired 4 cell lines
  - MCF7/Chicago (tamoxifen sensitive)
  - MCF7/HER2 (overexpress HER2, tamoxifen resistant)
  - MCF7/K1 (overexpress ER, non-responsive to estrogen)
  - MCF7/C412 (no ER expression)
- Identification of differential phosphorylation patterns in response to estrogen, tamoxifen and combined estrogen and tamoxifen treatment in tamoxifen sensitive (MCF7/Chicago) and tamoxifen resistant (MCF7/HER2) cell lines.
- Phosphoprotein enrichment from tamoxifen treated and control untreated cytoplasmic proteins from tamoxifen sensitive (MCF7/Chicago) cell line. Over 1200 proteins were identified from the combined extract. Stable isotope labeling allowed for quantitation of a subset of the proteins. The vast majority of proteins did not change in abundance, however, about 60 proteins were identified with Xpress ratio less than 0.6 and about 28 proteins with ratio larger than 1.66.
- SILAC labeling of MCF7/Chicago and MCF7/HER2 cell lines. Each cell line was grown as heavy or light and treated with tamoxifen or left untreated. The cells were harvested and are awaiting phosphoprotein enrichment and mass spectrometry analysis.
Reportable Outcomes


Conclusion

We have developed a method for comparison of global phosphoprotein profiles. Our methodology involves stable isotope labeling, a phosphoprotein affinity step, 1-D SDS-PAGE and LC-MS/MS. I have shown differential phosphoprotein patterns in MCF7/Chicago (tamoxifen sensitive) and MCF7/HER2 (tamoxifen resistant) cells as a result of tamoxifen treatment. HER2, Akt and ERK are all phosphorylated as a result of tamoxifen treatment within 30 to 120 minutes depending on the length of serum starvation pretreatment. Protein abundance changes are minimal and thus the change in phosphorylation is due to protein modification not degradation or synthesis. The results show that phosphoprotein profiling is a feasible option as phosphorylation patterns change in as little as 30-120 minutes and differ significantly between tamoxifen resistant and sensitive cell lines in both timing and phosphoprotein pattern. Interestingly, combined tamoxifen and estrogen treatment resulted in a robust pAkt, pHER2 and pERK signal in MCF7/HER2 cells whereas tamoxifen alone had significantly reduced pAkt, pHER2 and pERK signal. This difference was less pronounced in MCF7/Chicago cells but seemed to follow the same trend. To my knowledge this is the first comparison of tamoxifen versus combined tamoxifen and estrogen treatment. Further analysis is underway. I have also obtained cell lines that either lack estrogen receptor or overexpress it and do not respond to estrogen stimuli. These cell lines will be used to examine estrogen response and identify estrogen induced signaling events.

I present preliminary data towards phosphoprotein profiling of tamoxifen response in the cytoplasmic fraction of MCF7/Chicago cells. Over 1200 proteins were identified using an Orbitrap (Thermo) including over 40 kinases and 20 phosphatases. A subset of the proteins is isotopically labeled and quantitative revealed that a majority of the proteins did not change in abundance, as expected. However, about 60 proteins were identified with Xpress ratio less than 0.6 and about 28 proteins with ratio larger than 1.66. Manual analysis is underway to confirm the protein abundance ratios. Manual validation will be performed on each of these proteins to confirm the ratio, compare the protein hits to tamoxifen responsive proteins identified in the literature and finally confirmed by biological validation (Western Blots, siRNA and overexpression studies). Results will reveal the proteins that change in phosphorylation and/or abundance in response to tamoxifen in a tamoxifen sensitive cell line (MCF7/Chicago).

Next, I have prepared stable isotope labeled cells using SILAC labeling for both MCF7/Chicago and MCF7/HER2 cells untreated or treated with tamoxifen. These samples will be subjected to phosphoprotein profiling, mass spectrometry and data analysis as described above. This will allow for comparison of the tamoxifen response in tamoxifen sensitive (MCF7/Chicago) and tamoxifen resistant (MCF7/HER2) cell lines. Differences in response will elucidate the mechanism and/or markers of tamoxifen resistance. Findings will be confirmed in other cell line pairs (sensitive and resistant) to establish a common mechanism of tamoxifen resistance.
References


Appendices

None