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Discovery of Tamoxifen and N-Desmethyl Tamoxifen Protein Targets in MCF-7 Cells Using Large-Scale Protein Folding and Stability Measurements

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Abstract

The proteins in an MCF-7 cell line were probed for tamoxifen (TAM) and n-desmethyl tamoxifen (NDT) induced stability changes using the Stability of Proteins from Rates of Oxidation (SPROX) technique in combination with two different quantitative proteomics strategies, including one based on SILAC and one based on isobaric mass tags. Over 1000 proteins were assayed for TAM- and NDT- induced protein stability changes, and a total of 163 and 200 protein hits were identified in the TAM and NDT studies, respectively. A subset of 27 high confidence protein hits were reproducibly identified with both proteomics strategies and/or with multiple peptide probes. One-third of the high confidence hits have previously established experimental links to the estrogen receptor, and nearly all of the high confidence hits have established links to breast cancer. One high confidence protein hit that has known estrogen receptor binding properties, Y-box binding protein 1 (YBX1), was further validated as a direct binding target of TAM using both the SPROX and pulse proteolysis techniques. Proteins with TAM- and/or NDT-induced expression level changes were also identified in the SILAC-SPROX experiments. These proteins with expression level changes included only a small fraction of those with TAM- and/or NDT-induced stability changes.

TOC image

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SUPPORTING INFORMATION. The following files are available free of charge.

Keywords

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INTRODUCTION

It is becoming increasingly clear that pharmaceutical agents can act as more promiscuous ligands than once was assumed, and that such agents can interact with multiple protein targets potentially impacting many protein pathways.²⁻³ In some cases such ‘off-target’ interactions can contribute to the desired therapeutic outcome. However, in other cases such off-target interactions can be responsible for side effects, or have implications for new therapeutic applications. This realization of drug promiscuity has created the need to comprehensively characterize protein-drug interactions. This is especially true for the most abundant metabolites of therapeutics that are taken over long periods of time, such as the case with the breast cancer pro-drug tamoxifen.

The known therapeutic target of tamoxifen and its active metabolites is the estrogen receptor. However, the affinity of tamoxifen for the estrogen receptor is much lower than its active metabolites⁴ and biological activities of tamoxifen have been observed that appear to be independent of its binding interaction with the estrogen receptor.⁵ These additional biological activities include stimulation of cell programmed death and cell proliferation inhibition among others.⁵ The proteome-wide effects of tamoxifen treatment on MCF-7 cells, other breast cancer cell lines, and breast cancer tumor tissues have been previously studied with gene⁶ and protein expression level analyses.⁷⁻¹⁰ A drawback to the use of such expression level analyses to understand drug action is that the connection between a protein’s altered expression level and its altered function is dubious. Recently, several energetics-based methods for thermodynamic stability profiling (such as DARTS,¹¹ pulse proteolysis,¹² limited proteolysis,¹³ thermal proteome profiling¹⁴ and the Stability of Proteins from Rates of Oxidation (SPROX) technique¹⁵) have been developed for the large-scale and unbiased search for protein targets of ligands, such as small molecule therapeutics. Because of the close link between protein folding stability and protein function, such thermodynamic stability profiling methods have the potential to identify more functionally relevant protein targets of drugs than gene and protein expression level profiling methods.

Described here is the application of SPROX to identify novel protein targets of tamoxifen (TAM) and its most abundant^{16,17} but least therapeutically active⁴ metabolite, n-desmethyl tamoxifen (NDT). The SPROX approach was used in combination with two different quantitative proteomics strategies, including one involving isobaric tags for relative and absolute quantitation (iTRAQ) and one involving stable isotope labelling with amino acids in cell culture (SILAC). The so-called iTRAQ-SPROX and SILAC-SPROX techniques have been previously established for the detection and quantitation of protein-ligand interactions, both direct and indirect, in complex biological mixtures.¹⁶⁻¹⁹ The iTRAQ-SPROX experiments performed here involved making protein folding stability measurements on the proteins in an MCF-7 cell lysate both in the presence and in the absence of TAM and NDT.

The SILAC-SPROX experiments described here also involved making protein folding stability measurements on proteins in MCF-7 cell lysates. However, the cell lysates in the SILAC-SPROX experiments were derived from MCF-7 cells that were cultured both in the presence and in the absence of TAM and NDT. The latter cell culture study was modeled after a previous protein expression level study.⁹

The goal of this work was to identify proteins from MCF-7 cells with TAM- and/or NDT-induced stability changes. These proteins with ligand-induced stability changes are expected to have direct or indirect interactions with TAM and/or NDT. A total of 936 and 783 proteins were assayed for drug-induced stability changes using the iTRAQ- and SILAC-SPROX techniques (respectively), and 163 and 200 protein hits were identified in the TAM and NDT experiments (respectively). A subset of 27 high confidence protein hits were reproducibly identified with both proteomics strategies and/or with multiple peptide probes. The biological significance of these hits is discussed. Also reported is a novel data analysis strategy to identify protein targets of ligands using the iTRAQ-SPROX technique.

MATERIALS AND METHODS

Cell Culture

MCF-7 Lysate for iTRAQ-SPROX—MCF-7 cells were obtained from ATCC through the Duke Cell Culture Facility. The MCF-7 cells were cultured in a humidified 37°C incubator with 5% CO₂ according to ATCC guidelines. Briefly, the cells were grown in DMEM (Gibco) medium that was supplemented to contain 1mM sodium pyruvate (Gibco), 1× Anti-Anti (Gibco), 1x MEM non-essential amino acid solution (Gibco), 10 µg/ml insulin and 10% FBS (Hyclone). The cells were washed twice with PBS and harvested with HyQtase (Hyclone) in a 15 mL tube. Cells were pelleted at 1000 rpm for 5 min. The pelleted cells were resuspended in PBS, and pelleted at 1000 rpm for 5 min. The supernatant was discarded and pellets were stored at −20°C.

SILAC Labeled MCF-7 Growth for SILAC-SPROX—MCF-7 cells were cultured using heavy-labeled or light-labeled lysine and arginine according to established protocol.^{20–21} The heavy-labeled lysine was enriched with six ¹³C and two ¹⁵N atoms. The heavy-labeled arginine was enriched with six ¹³C atoms (Cambridge Isotope Laboratories). MCF-7 heavy-labeled cells were treated with drug and MCF-7 light-labeled cells were treated with vehicle as previously described.⁹ Briefly, NDT (Sigma) and TAM (Sigma) stock solutions were prepared in DMSO at a concentration of 1 mM. The heavy-labeled MCF-7 cell line was incubated with a final concentration of either 1 µM NDT/(1:1000 v/v)DMSO or 1 µM TAM/(1:1000 v/v)DMSO. Light-labeled MCF-7 was incubated with 1:1000 v/v DMSO. MCF-7 cells were incubated with NDT/DMSO, TAM/DMSO or DMSO for a total of 96 hours. The media and drug solutions were replaced after 48 hours. The cells were washed twice with PBS and harvested with HyQtase (Hyclone) in a 15 mL tube. Cells were pelleted at 1000 rpm for 5 min. The pelleted cells were re-suspended in PBS, and pelleted at 1000 rpm for 5 min. The supernatant was discarded and pellets were stored at −20°C.

Cell Lysate Preparation

Briefly, a frozen cell pellet was thawed on ice before 500 μ L of 20 mM phosphate buffer (pH 7.4) and 25 μ L of a 20x protease inhibitor cocktail were added to the pellet. The 20x protease inhibitor cocktail contained: 1 mM AEBSF, 10 μ M Pepstatin A, 500 μ M Bestatin, 20 μ M Leupeptin and 15 μ M E-64 (ThermoFisher Scientific, Waltham, MA). The pellet was lysed with 1.0 mm diameter zirconia/silica beads (BioSpec) using a Disruptor Genie (Scientific Industries) for 20 seconds with a 1 minute break on ice between disruptions. This was repeated 15–20 times. The sample was centrifuged for 10 minutes at 4°C and 15000 rcf, the supernatant was used as the lysate, and a Bradford assay was used to measure the protein concentration in each lysate preparation. The total protein concentration in each lysate was between 3–8 mg/mL, depending on the biological replicate.

iTRAQ-SPROX Protocol

The iTRAQ-SPROX analysis was performed similarly to previously described.²² Briefly, aliquots containing 200 μ L of the MCF-7 lysate were incubated with 300 μ M TAM/10% DMSO (v/v), 300 μ M NDT/10% DMSO (v/v) or 10% DMSO to generate the +TAM, +NDT, and control samples, respectively. Each sample was distributed into a series of buffers containing 20 mM phosphate (pH 7.4) and increasing concentrations of GdmCl. The SPROX reaction was performed by treatment with 3% H_2O_2 (v/v) and was quenched with excess methionine. The final GdmCl concentrations in the buffers were 0.5 M, 1.0 M, 1.3 M, 1.5 M, 1.7 M, 2.0 M, 2.7 M and 3.0 M. The protein in each denaturant-containing buffer was precipitated with TCA and the sample were submitted to a bottom-up proteomics analysis using iTRAQ quantitation. The protein samples from the 0.5 M, 1.0 M, 1.3 M, 1.5 M, 1.7 M, 2.0 M, 2.7 M and 3.0 M GdmCl were labeled with 113, 114, 115, 116, 117, 118, 119 and 121 iTRAQ reagents, respectively. The labeling reaction was performed according to the manufacturer's protocol, with the exception that 0.5 units of each iTRAQ reagent was used in the labeling reactions. The iTRAQ labeled samples were combined for the +TAM, +NDT and control samples. Methionine-containing peptides were enriched using a Pi^3TM Methionine Reagent kit (Nest Group) according to the manufacturer's protocol. In total, three iTRAQ-SPROX analyses were performed on the control, +TAM, and +NDT samples.

SILAC-SPROX Protocol

The SILAC-SPROX analyses were performed on proteins from the NDT treated heavy-labeled MCF-7 sample, on proteins from the TAM treated heavy-labeled MCF-7 sample, and on proteins from a light-labeled MCF-7 control sample according to previously established protocols.^{16, 21} Briefly, aliquots of the heavy- and light-labeled MCF-7 cell lysates were distributed into a series of 10 buffers containing 20 mM phosphate (pH 7.4) and increasing concentrations of GdmCl. The SPROX reaction was performed by treatment of the lysate samples in the denaturant-containing buffers with 3% H_2O_2 (v/v), and the oxidation reaction was quenched with excess methionine. The final denaturant concentrations were 0 M, 0.5 M, 0.75 M, 1.0 M, 1.25 M, 1.5 M, 1.75 M, 2.0 M, 2.5 M and 3.0 M. After the SPROX reaction was performed, the heavy- and light-labeled samples from the same denaturant concentration in the (+) and (–) drug samples were combined. The proteins in the combined samples were precipitated with TCA, and the samples were

subjected to a quantitative, bottom-up proteomics analysis using SILAC quantitation. In total, three SILAC-SPROX analyses were performed on the +/-TAM and +/-NDT samples.

Pulse Proteolysis Protocol

A pulse proteolysis experiment was performed on a purified recombinant human YBX1 construct (Novus Biologicals, #NBP2-30101) both in the presence and absence of TAM. Recombinant YBX1 was buffer exchanged on a 10 kDa MWCO filter (Amicon Ultra-0.5, EMD Millipore) with 100 mM Tris pH 8.0 to a protein concentration of ~0.5 µg/µL. Protease inhibitor was added to sample (100x protease inhibitor cocktail included pepstatin A (0.2 mM), leupeptin (0.4 mM), E-64 (0.3 nM), bestatin (1 mM), and AEBSF (20 mM) protease). Aliquots containing 50 µg of the YBX1 protein were equilibrated with either 300 µM TAM/10% DMSO (v/v) or 10% DMSO (v/v) for 1 hour at room temperature. Ten µL volumes of the (+) and (-) drug samples (containing 5 µg each) were distributed into 30 µL of buffers containing 100 mM Tris and increasing concentrations of urea. The protein samples in the urea-containing buffers were incubated at room temperature for 1 hour before the pulse proteolysis reaction was performed by treating the samples with 0.5 µg of thermolysin (Sigma), which was prepared in 100 mM Tris buffer, pH 8.0, containing 50 mM NaCl and 10 mM CaCl₂. After 1 minute the pulse proteolysis reactions were quenched upon addition of 5 µL of a 0.5 M EDTA solution. A volume of 15 µL of BME/loading dye (1:1 v/v) was added to each sample. Samples were heated to 95°C for 5 minutes before a 40 µL aliquot of each sample was loaded onto a 4%–20% midsize polyacrylamide gel (BioRad Criterion, Hercules, CA, USA) and subject to an electrophoretic separation and visualization with coomassie staining.

SPROX Protocol with Phenacyl Bromide Labelling (PAB-SPROX)

A PAB-SPROX experiment was performed on a purified recombinant human YBX1 construct (Novus Biologicals, #NBP2-30101) both in the presence and absence of TAM according to a previously established protocol²³. Briefly, 70 µL aliquots of a 1 mg/mL solution of recombinant YBX1 in 20mM phosphate buffer (pH 7.4) were combined with either 7.7 µL of a 3 mM TAM solution prepared in DMSO or 7.7 µL of DMSO to generate the (+) and (-) ligand samples, respectively. The (+) and (-) ligand samples were equilibrated for 1 hr at room temperature before 10 µL aliquots of the (+) and (-) ligand samples were each combined with 12.5 µL of a series of solutions containing 20 mM phosphate buffer (pH 7.4) and concentrations of GdmCl ranging from 0 to 2 M. The final GdmCl concentrations in the 7 GdmCl buffers used in the experiment were 0, 0.3, 0.5, 0.7, 1.0, 1.5 and 2.0 M. The YBX1 protein samples in the GdmCl-containing buffers were equilibrated for 1 hr at room temperature before the methionine oxidation reaction in SPROX was initiated with the addition of 2.5 µL of 9.8 M H₂O₂. The oxidation reactions were quenched with 500 µL of solution of methionine (300 mM). After 3 min, the proteins in each GdmCl containing buffer were precipitated upon addition of 125 µL of TCA and overnight incubation on ice. Approximately, ~90 µg of cell lysate proteins was added into each sample to enhance the yield of YBX1 protein in the TCA precipitation step. The samples were centrifuged at 14000 rcf for 30 min at 4 °C, and each supernatant was decanted. The protein pellets were each washed three times with 300 µL of ice-cold ethanol. The protein pellets were each dissolved in 30 µL of 0.5 M TEAB with 0.1 % final

concentration of SDS. The samples were vortexed, heated at 60 °C, and sonicated for 10 min at a time, for 2–3 cycles. The disulfide bonds were reduced with a final concentration of 5 mM TCEP for 1 hr at 60 °C. Cysteine residues were reacted in the presence of 10 mM MMTS for 10 min at RT.

The protein samples were digested overnight with 1.0 µL of 1 mg/mL trypsin at 37 °C. After addition of 20 µL HOAc and 30 µL ACN, the tryptic peptides generated in the (+) and (–) ligand samples were derivatized with light ($^{12}\text{C}_6\text{-PAB}$) and heavy ($^{13}\text{C}_6\text{-PAB}$) (respectively) phenacyl bromide, both of which were chemically synthesized as previously described.²⁴ For the derivatization reactions, 10 µL of freshly prepared $^{12}\text{C}_6\text{-PAB}$ or $^{13}\text{C}_6\text{-PAB}$ solutions (1 M) in acetonitrile were added to the (+) and (–) ligand samples (respectively). After 24 hr, the (+) and (–) ligand samples at the same denaturant concentration were pooled, each of the pooled samples was desalted using C18 resin and submitted to an LC-MS/MS analysis (see below).

LC-MS/MS Data Acquisition and Analysis

The iTRAQ- and SILAC-SPROX samples were analyzed on an Orbitrap Elite ETD mass spectrometer equipped with an EASY-nLC system at the Proteomics Resource at the Fred Hutchinson Cancer Research Center, an Orbitrap Fusion mass spectrometer (Thermo Scientific) equipped with Easy-nLC 1000 system (Thermo Scientific) also at the Proteomics Resource at the Fred Hutchinson Cancer Research Center, or on a Q-Exactive Plus high-resolution mass spectrometer equipped with a nanoAcuity UPLC system (Waters Corp) and a nanoelectrospray ionization source at the Duke Proteomics Facility. Summarized in Table S-1 are the various iTRAQ-SPROX and SILAC-SPROX samples generated in this work and the LC-MS/MS instrumentation on which they were analyzed. Detailed descriptions of the chromatographic conditions and instrumental parameters used for the LC-MS/MS analyses on the above instruments are also provided in the Supporting Information.

The LC-MS/MS data generated in the iTRAQ-SPROX experiments was searched against the SwissProt Human database version 2016-04-13 with Proteome Discoverer (version 2.1.1.21). All data was searched with the fixed modifications cysteine MMTS modification and iTRAQ 8-plex lysine and N-termini modification. The oxidation of methionine was searched as a variable modification. Two missed cleavages were allowed. The parameters for the Orbitrap Elite included a 10 ppm mass tolerance window for precursor masses and 0.8 Da for fragment mass tolerance. The parameters for the Q-Exactive included a 10 ppm mass tolerance window for precursor masses and 0.02 Da for fragment mass tolerance. The parameters for the Orbitrap Fusion included a 10 ppm mass tolerance window for precursor masses and 0.6 Da for fragment mass tolerance. The LC-MS/MS data generated in the SILAC-SPROX experiments were searched on MaxQuant 1.5.2.8²⁵ against the 201-04 release of the UniProt Knowledgebase (downloaded on 5/16/2014 at ftp://ftp.uniprot.org/pub/databases/uniprot/current_releases/release2014_04/knowledgebase/). Modifications searched included: two SILAC labels of the heavy sample (Arg6 and Lys8), variable acetylation of protein N-terminus, variable oxidation of methionine, variable deamidation of asparagine and glutamine, and fixed MMTS modification of cysteines. Trypsin was set as a specific enzyme with up to two missed cleavages with matching from

and to. The default settings of mass tolerances for the orbitrap were employed. Match between runs and re-quantification of searched peptides was checked. The mass spectrometry proteomics data from the SILAC- and iTRAQ-SPROX experiments has been deposited in ProteomeXchange (<http://proteomexchange.org>) via the PRIDE²⁶ partner repository with the data set identifiers PXD006732 and PXD006747.

The PAB-SPROX samples were analyzed on a Q-Exactive Plus HF MS instrument using parallel reaction monitoring (PRM) method. The method was based on a 30 minute 5–40% ACN gradient and a CE of 30 V was used. Selected precursors for the light and heavy PAB labeled YBX1 peptide, RPQYSNPPVQGEVMEGADNQGAGEQGRPVR, and a control peptide, VIPDFMLQGGDFTAGNGTGGK from a yeast protein (Cyclophilin A) were targeted (see Supporting Information for more detailed information about PAB-SPROX data acquisition and analysis). Approximately, 200 ng of total material was loaded on column for each LC-MS/MS run in the PAB-SPROX analysis.

iTRAQ-SPROX Data Analysis

The iTRAQ-SPROX data from the TAM and NDT studies was subjected to two normalizations. The iTRAQ reporter ion intensities in each product ion mass spectra were initially normalized to the average reporter ion intensity in each product ion mass spectra. These so-called N1-normalized values were subjected to a second normalization. For the second normalization, the N1 values generated for a given reporter ion were averaged for the non-methionine containing PSMs. The N1 normalized reporter ion intensities of the methionine containing PSMs were divided by these averaged values, or so-called N2 normalization factors. The N2 normalization factors and the corresponding standard deviations for each experiment are provided in Table S-2.

The N2-normalized values generated for each PSM containing methionine were fit to Equation 1 using the Nelder and Mead Simplex method for regression.²⁷

$$y = A + \frac{(B - A)}{1 + e^{-\frac{C_{1/2} - x}{b}}} \quad \text{Equation (1)}$$

In Equation 1: A is the pre-transition baseline, B is the post-transition baseline, b is a measure of the steepness of the transition and $C_{1/2}$ is the transition midpoint of the denaturation data. The data were fit to Equation 1 using a JAVA-based program, which was developed in house. The program fit the set of N2-normalized values from each PSM a total of nine times including one time using all of the data as well as one time after the removal of each N2-normalized value. The highest quality fit, according to the adjusted R^2 value, was selected as the program output. PSMs with poor data (adjusted $R^2 < 0.8$) were removed from the analysis. PSMs with isolation interference > 30% or isolation purity < 70% were also removed from the analysis. For peptides with multiple PSMs (i.e., multiple chemical denaturation data sets that were well fit to Equation 1 the normalized reporter ion intensities for the same denaturant concentration were averaged to produce one denaturation data set

per peptide identified. The JAVA-based program was used to fit this averaged data set to generate a single $C_{1/2}$ value for each peptide in the +TAM, +NDT, and control samples.

Peptide (and the corresponding protein) hits in the iTRAQ-SPROX experiments were selected based on the magnitude of the $C_{1/2}$ value determined between the (–) and (+) ligand samples and the magnitude of the N2 normalization value differences at or between the transition regions of the chemical denaturation curves generated for the (–) and (+) ligand samples. Statistically significant $C_{1/2}$ values were taken to be those greater than or equal to two times the pooled standard deviation (or ‘s-pooled’ value) of all the $C_{1/2}$ values generated from all the PSMs. The s-pooled value was determined to be 0.25 (see Table S-3). Statistically significant N2 reporter ion differences were taken as those with $-\log_{10}(\text{Diff}_{\text{prob}}) > 1$, where the $\text{Diff}_{\text{prob}}$ corresponds to the probability that the measured N2 reporter ion difference would randomly occur (see Supporting Information). The specific hit criteria used in each biological replicate of the iTRAQ-SPROX experiment are summarized in Table S-4. The averaged and fitted chemical denaturation data sets obtained for all the assayed peptides in the NDT and TAM analysis are summarized in Table S-5 and S-6. The iTRAQ-SPROX data analysis procedure described here was validated using previously published iTRAQ-SPROX data on selected model systems (see Supporting Information).

SILAC-SPROX Data Analysis

The output files from Maxquant 1.5.2.8 were analyzed using a Mathematica 11.0 script. Briefly, only peptides identified in the output files with $H/L > 0$ were included in the analysis. To account for protein expression level differences, the H/L from non-methionine-containing peptides were normalized by the median H/L value from non-methionine-containing peptides. The cutoff values for determining a significant H/L shift corresponded to those at the 5th and 95th percentile of the normalized H/L ratios recorded for all the non-methionine-containing peptides. Methionine-containing peptides were also normalized by the protein medians. The H/L ratios of methionine-containing peptides were averaged within the same sequence and charge state within a denaturant concentration; and the resulting H/L ratios were $\log_2$ transformed. Methionine-containing peptides were required to be identified in 7 or more denaturant concentrations to be assayed. Assayed peptides with 2 or more consecutive H/L ratio values outside the cutoffs in the same direction (either high or low) were categorized as potential hits. The H/L versus [GdmCl] data obtained for oxidized and wild-type methionine-containing peptides that were potential hits were fit to Equations 2 and 3, respectively. Equations 2 and 3 represent the ratio of the two SPROX curves expected for the light and heavy labeled peptide. In each case, A is the extent of the oxidation (i.e., the amplitude of the SPROX curve), k_{ox} is the pseudo-first order rate constant associated with the oxidation reaction of an unprotected methionine residue, t is the reaction time, G is the protein folding free energy, ΔG is the change in the folding free energy, m_1 and m_2 are

$\frac{\delta \Delta G}{\delta [GdmCl]}$ representing the steepness of the transition regions of the two SPROX curves, R is the ideal gas constant, and T is temperature. In order to avoid overfitting and ensure physical significance of the parameters, constraints were applied (see Table S-7) to the fitting.

$$\text{Log}_2(H/L) = \text{Log}_2 \left(\frac{1 - A * e^{\frac{-k_{ox}t}{1 + e^{-\frac{\Delta G + \Delta\Delta G + m_1[D]}{RT}}}}}{1 - A * e^{\frac{-k_{ox}t}{1 + e^{-\frac{\Delta G + m_2[D]}{RT}}}}} \right) \quad \text{Equation (2)}$$

$$\text{Log}_2(H/L) = \frac{1.44k_{ox}t}{1 + e^{-\frac{\Delta G + m_2[D]}{RT}}} - \frac{1.44k_{ox}t}{1 + e^{-\frac{\Delta G + \Delta\Delta G + m_1[D]}{RT}}} \quad \text{Equation (3)}$$

The goodness of the fitting was evaluated by the R^2 extracted from the fitting and a customized relative standard deviation using Equation 4. In Equation 4 N is the number of denaturant concentrations identified, $A_{Exp,i}$ is the H/L for the i^{th} data point from experiment and $A_{Fit,i}$ is the H/L for the i^{th} data point calculated from the fitted model. A smaller RSD indicates a better fitting. Typically, $R^2 > 0.8$ and $RSD < 0.2$ were categorized as a good fitting.

$$RSD = \frac{\sqrt{\frac{\sum_{i=1}^n (A_{Fit,i} - A_{Exp,i})^2}{n-1}}}{\text{Max}(A_{Exp,i}) - \text{Min}(A_{Exp,i})} \quad \text{Equation (4)}$$

In order to achieve optimal fitting results, the data were fitted an additional n times with the removal of one point, where n is the number of denaturant concentrations identified. The best fitted curve was selected by highest R^2 value.

After fitting each curve in each biological replicate, the data sets from all biological replicates with good fits were averaged for each unique peptide sequence, charge and sample type to generate a single data set for each peptide. The averaged data set was fitted again by the described procedure. The $C_{1/2}$ values were calculated from the fitted parameters using Equation 5 and Equation 6 for heavy and light labeled peptides respectively, and the $C_{1/2}$ value was calculated by the difference between heavy and light labeled peptides. If the $C_{1/2}$ value was calculated as outside of the denaturant concentration range, the $C_{1/2}$ value was set as the margin value of the range.

$$C_{1/2, Heavy} = \frac{-0.28 - \Delta G - \Delta\Delta G}{m_1} \quad \text{Equation (5)}$$

$$C_{1/2, Light} = \frac{-0.28 - \Delta G}{m_2} \quad \text{Equation (6)}$$

All of the assayed peptides and proteins for the TAM and NDT experiments are summarized in Table S-8. The fitting results of all the hits with calculated G , and $C_{1/2}$ values are summarized in Table S-9.

Protein Expression Level Analysis

Protein expression level ratios were generated from the SILAC-SPROX data sets. Non-methionine containing peptide H/L ratios reported on the relative protein expression between vehicle-treated and NDT- or TAM-treated MCF-7 proteins. Median H/L ratios for non-methionine containing peptides were calculated for each unique protein in each biological replicate of SILAC-SPROX. Proteins with significant changes in expression were required to be quantified in all three biological replicates and were determined with a two-tailed Student's t-test. Proteins with a p-value < 0.05 and average H/L values outside the 5 and 95% frequency distribution (0.66 and 1.42 for TAM and 0.67 and 1.48 for NDT) were deemed significant.

RESULTS

Experimental Proteomic Coverage

The experimental workflows employed in the iTRAQ- and SILAC-SPROX experiments described here are summarized in Figures 1 and 2. In total three biological replicates of the iTRAQ-SPROX and SILAC-SPROX experiments using the MCF-7 lysate and MCF-7 cell culture (respectively) were performed with both TAM and NDT. The number of peptides and proteins that were probed for stability changes with TAM and NDT treatment with the iTRAQ-SPROX and SILAC-SPROX experiments are summarized in Table 1. These experiments enabled over 1000 proteins to be probed for TAM and NDT induced stability changes. Ultimately, 163 and 200 unique protein hits were identified in the TAM and NDT binding experiments using the SPROX methodologies applied here, respectively. In some cases, protein hits were identified using multiple methionine-containing peptide probes.

Drug-Induced Protein Stability Changes

In total, 186 peptides from 163 proteins (Table S-10) were identified as hits with TAM treatment and 254 peptides from 200 proteins (Table S-10) were identified as hits with NDT treatment. Shown in Figure 3 are the data obtained for a methionine-containing peptide hit identified in both the iTRAQ- and SILAC-SPROX experiments. Shown in Figure 4 is a volcano plot of the iTRAQ-SPROX data. From the total number of iTRAQ- and SILAC-SPROX hits identified with drug-induced stability changes in the presence of TAM and NDT, a subset of high confidence hits was determined. High confidence peptide hits were identified as those that were a hit in both the iTRAQ- and SILAC-SPROX experiments. Protein hits were also considered high confidence if more than one peptide mapping to the same protein was identified as a hit with similar behavior with either drug (stabilization or destabilization). The high confidence hits are summarized in Table 2 and 3. In total, 44 peptides from 20 proteins and 24 peptides from 11 proteins were identified as high confidence hits in the NDT and TAM experiments, respectively. The reproducibility of the hits in both the iTRAQ and SILAC readouts suggests that these protein hits are likely true positives. The overlapping hits between the iTRAQ- and SILAC-SPROX experiments are

also expected to be the most biologically relevant hits as the drug treatment in the SILAC-SPROX experiment took place in the cell culture.

The TAM and NDT-induced stability changes observed in the hit proteins may be due to direct interactions of these folding domains with TAM/NDT or due to allosteric effects and/or indirect interactions. Further studies are needed in order to characterize whether a protein hit results from a direct or indirect interaction. One way to differentiate direct interactions from indirect interactions is to perform ligand binding studies on purified protein constructs. Direct drug binding interactions with a protein target should be observable with the purified protein target, whereas indirect interactions should not. Indirect interactions can result, for example, when the direct binding of a drug to a target protein either induces or precludes a protein-protein interaction between the target protein and another protein.

One high confidence protein hit, Y-box protein binding 1 (YBX1), was further probed for direct interactions with TAM using a purified recombinant YBX1 construct and both the PAB-SPROX²³ and pulse proteolysis techniques.¹² This protein was selected for further study because of its known link to the estrogen receptor (see STRING analysis below) and it is commercially available. In the pulse proteolysis technique the protease susceptibility is evaluated as a function of denaturant concentration both in the presence and in the absence of ligand. The results of the pulse proteolysis assay (Figure 5) revealed that TAM altered the protease susceptibility of purified recombinant YBX1 (see 0.75 M urea lane in Figure 5A). However, it is noteworthy that the purified YBX1 protein exhibited unusual behavior in the pulse proteolysis assay. YBX1 was readily susceptible to proteolysis even in the absence of denaturant, and increasing amount of denaturant appeared to protect the protein from proteolysis (Figure 5B). This is in contrast to more typical proteins that are protected from proteolysis at low denaturant concentrations, and readily proteolyzed at high denaturant concentrations. The C-terminal domain of YBX1 is known to be disordered,²⁸ therefore it is not surprising that the protein was proteolyzed in the absence of denaturant. The apparent protection of YBX1 from proteolysis at high denaturant concentrations is likely due to urea-induced aggregation of YBX1. Despite this unusual behavior of YBX1 in the pulse proteolysis experiment, TAM-induced changes were observed (see 0.75 M lanes in Figure 5A), suggesting that YBX1 does directly interact with TAM.

The PAB-SPROX experiment, which is fundamentally identical to SILAC-SPROX, was also used to validate the YBX1 protein as a direct target of TAM. The denaturant dependence of the H/L ratios observed for the PAB-labelled YBX1 peptide RPQYSNPPVQGEVMEGADNQGAGEQGRPVR were consistent with a TAM-induced stabilization. Such a TAM-induced stabilization was also observed with the same methionine-containing peptide probe (albeit in its oxidized form) in the SILAC-SPROX analysis of unpurified YBX1 in the MCF-7 cell lysate (see Figure 6). The SILAC (and/or PAB)-SPROX data sets generated on the wild-type and oxidized forms of a given hit peptide should be mirror images of each other. This is not the case for the data shown in Figure 6 because (i) the +TAM samples were *light*-labelled in the PAB-SPROX experiment whereas they were *heavy*-labelled in the SILAC-SPROX experiment and (ii) the baseline Log₂(H/L)

values in the PAB-SPROX experiment appear to be shifted up ~1.5 units. In theory, the baseline $\text{Log}_2(\text{H/L})$ values in PAB- and SILAC-SPROX should be close to 0.

As expected, the baseline $\text{Log}_2(\text{H/L})$ values for a control peptide in the PAB-SPROX experiment were close to 0 (see Supporting Information). The baseline shift observed for the YBX1 peptide RPQYSNPPVQGEVMEGADNQGAGEQGRPVR in the PAB-SPROX experiment on the purified protein is likely due to differential aggregation/precipitation of the YBX1 protein in the presence and absence of TAM. One explanation for the baseline shift in the PAB-SPROX experiment on the purified protein may be that YBX1 more readily aggregates/precipitates in the absence of TAM. This is consistent with the aggregation phenomenon observed in the pulse proteolysis experiment on the purified protein (see above), and it is likely a result of the unfolded/disordered structure that the C-terminal domain of the unliganded protein is known to adopt in solution.²⁸ The absence of such aggregation phenomena in the SILAC-SPROX experiment is likely due to the relatively low YBX1 protein concentration in the unpurified MCF-7 lysate.

Drug-Induced Protein Expression Level Changes

The SILAC-SPROX data generated here also enabled drug-induced protein expression level changes to be determined. Such drug-induced protein expression level data was generated for 2838 and 2870 proteins in the TAM and NDT studies described here. However, only 799 and 671 proteins (Table S-11) were successfully probed for TAM and NDT-induced expression level changes (respectively) in all three biological replicates of the SILAC-SPROX experiment. A t-test analysis of these 799 and 671 proteins ultimately identified 49 proteins with significant ($p < 0.05$) TAM-induced expression level changes and 33 proteins with significant ($p < 0.05$) NDT-induced expression level changes (Table S-11).

The SILAC-SPROX study performed here was modeled after an MCF-7 expression level study previously reported by Hengel and co-workers.⁹ The expression levels obtained here were compared to those previously reported by Hengel and co-workers.⁹ In this earlier study a total of 83 proteins were identified with TAM-induced protein expression level changes. A total of 34 of these 83 proteins were assayed for TAM-induced expression level changes here, and only 1 protein was found to have a significant expression level change. Interestingly, this protein was AGR2, the protein hit found to be the most upregulated by TAM treatment by Hengel and co-workers⁹ and the protein for which the protein expression level change was validated by immunoblotting.

DISCUSSION

Correlation between Drug-Induced Stability Changes and Drug-Induced Expression Level Changes

One goal of this work was to determine what, if any, correlation exists between drug-induced stability changes and drug-induced expression level changes. Out of the 163 proteins identified here with TAM-induced stability changes, 99 were successfully probed for TAM-induced expression level changes and 14 were determined to have significant TAM-induced expression level changes based on the SILAC-SPROX data. Out of the 200

proteins with NDT-induced stability changes, 96 were probed for NDT-induced expression changes and 4 were determined to have significant NDT-induced expression level changes based on the SILAC-SPROX data.

Out of the 11 proteins with high confidence TAM-induced stability changes, 7 were probed for TAM-induced expression changes and none were determined to have a significant TAM-induced expression level change. Out of the 20 proteins with high confidence NDT-induced stability changes, 16 were probed for NDT-induced expression level changes and only 1 was determined to have a significant NDT-induced expression level change (DYNC1H1; NDT/Control=0.62). Our results indicate that most MCF-7 proteins with TAM or NDT induced stability changes do not have a significant expression level change with TAM or NDT treatment versus treatment with vehicle. These results suggest that that proteome-wide stability profiling is complementary to proteome-wide expression level profiling.

Overlap between TAM and NDT Hits

TAM and NDT differ by a single methyl group. Therefore, some overlap in the protein targets of these two molecules is expected. Nearly all of the TAM and NDT-induced protein stability hits were assayed for drug-induced protein stability changes in both the TAM and NDT analyses. Out of the 163 TAM-induced and 200 NDT-induced protein stability changes, 58 proteins (~36%) were found to have both TAM and NDT-induced stability changes. Out of the 11 high confidence TAM-induced and 20 NDT-induced protein stability changes, 4 proteins displayed both TAM and NDT-induced stability changes. These results indicate that TAM and NDT do share some protein targets, but most of the protein targets detected here are unique to one drug or the other. It is also possible that some of the protein hits unique to one drug or the other, are false positives.

Interestingly, the protein overlap between the TAM- and NDT-induced protein expression level hits was somewhat higher than that observed for the protein stability hits. Out of the 49 proteins with TAM-induced expression changes and 33 proteins with NDT-induced expression changes, 23 proteins had similar upregulation or downregulation with TAM and NDT treatment. Included in the proteins with TAM and NDT-induced upregulation was Selenium-binding protein 1, which displayed one of the higher Drug/Control expression ratios of ~2.2 for both drugs. The expression of this protein has previously been shown to be downregulated in MCF-7 cells treated with estrogen.²⁹ The treatment of MCF-7 cells with TAM or NDT appears to have the opposite effect of estrogen treatment in MCF-7 cell line.

Proteins with High Confidence Drug-Induced Stability Changes and the Estrogen Receptor

The 27 high confidence protein hits with either TAM- and/or NDT-induced stability changes were investigated for experimental connections to MAPK1, AKT1 and ESR1 in a protein association network analysis using STRING.³⁰ ESR1 is the known binding target of tamoxifen and its active metabolites. The MAPK1 and AKT proteins are downstream of the estrogen receptor binding signaling and upstream of estrogen receptor element activation.³¹ The ESR1, MAPK1, and AKT genes were included in the STRING analysis in order to identify experimental links between the hit proteins and the estrogen receptor and ligand-induced estrogen receptor signaling. The output of this STRING analysis is summarized in

Figure 7. A relatively large fraction (~33%) of the 27 high confidence protein hits are experimentally linked to the estrogen receptor through either direct or indirect interactions.

Included in the 9 high-confidence protein stability hits that have been experimentally linked to the estrogen receptor according to the STRING analysis is Y-box binding protein 1 (YBX1). YBX1 is also linked to both MAPK1 and AKT1 in the STRING analysis depicted in Figure 7. In a previous study, the expression of YBX1 in breast cancer tissues was monitored by immunostaining, and the upregulation of this protein was linked to poor outcomes and drug resistance.³² Overexpression of YBX1 in an MCF-7 cell line was also shown to decrease ER α expression and increase ERBB2 expression.³³ This earlier study also reported the overexpression of YBX1 in MCF-7 inhibited TAM-induced apoptosis based on data collected in a DNA fragmentation assay.³³ More recently, it was shown that YBX1 directly binds to the estrogen receptor and TAM treatment disrupts that binding.³⁴ It was also reported in this recent study that TAM treatment induces the binding of YBX1 to the ERBB2 response element.³⁴ Moreover, YBX1 and ER deletion mutants revealed that the cold shock domain of YBX1 binds to the ligand binding domain of the estrogen receptor and that that increased YBX1 levels are correlated to increased ubiquitination and proteasomal degradation of ER.³⁴ The direct interaction of TAM with YBX1 identified here (Figure 5 and 6) may contribute to the impact of YBX1 on ERBB2 and ER.

Protein Targets Linked to Breast Cancer

A large fraction (~85%) of the 27 high-confidence protein stability hits have been previously linked to breast cancer. For example, Elongation factor Tu and Tubulin beta 3 chain which were shown to be destabilized by NDT and stabilized by NDT, respectively. Elongation factor Tu has been linked as a regulator of the epithelial-to-mesenchymal transition (EMT), and therefore cancer metastasis. Decreased levels of Elongation factor Tu have been shown to increase invasiveness of MCF-7 cell line.³⁵ Tubulin beta 3 chain expression has been shown to increase in the presence of estradiol in MCF-7 cell line, which can be mitigated by TAM treatment.³⁶ It has also been shown that patients with lower levels of Tubulin beta 3 chain can have increased overall survival time when treated with taxanes.³⁷ The breast cancer literature links of the stability hits identified in this study (Table 2 and 3) help validate the biological significance of these protein hits.

CONCLUSION

Large-scale unbiased analyses of drug action have typically relied on gene and protein expression level profiling to identify the proteins and biological pathways targeted either directly or indirectly by drugs. Here we utilize thermodynamic stability measurements to identify novel protein targets of a currently used breast cancer therapeutic TAM, and its major metabolite, NDT. The results reported here suggest that the protein hits identified using such a thermodynamic stability profiling approach are largely different from those identified in protein expression level profiling studies.

As part of this work over 1000 proteins were assayed for TAM- and NDT- induced stability changes and a total of 163 and 200 unique protein hits of TAM and NDT were identified in this work, respectively. Not surprisingly a fraction (~36%) of the TAM and NDT hits were

overlapping. Moreover, a significant fraction (~33%) of the 27 high confidence protein hits identified here have previously known experimental links to the estrogen receptor, the known therapeutic target of TAM and NDT. Included in this subset of high confidence hits is YBX1. TAM was shown to directly interact with purified recombinant YBX1 construct using both SPROX and pulse proteolysis. Most of the high-confidence protein hits also have previously established links to breast cancer. These previously established links help validate the biological significance of the protein hits identified here. Similarly, the results of the work described here provide a biophysical basis for the role of these proteins in TAM and NDT drug action.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Schematic representation of the iTRAQ-SPROX experimental workflow used in this work. MCF-7 cell lysate was incubated with either DMSO, TAM/DMSO or NDT/DMSO before being incubated in an increasing series of denaturant containing buffer and subjected to a SPROX analysis with iTRAQ quantitation.

Figure 2.

Schematic representation of the SILAC-SPROX experimental workflow used in this work. MCF-7 cell line growth medium was supplemented with TAM/DMSO, NDT/DMSO or DMSO for a 3-day period. Cells were harvested and subjected to a SPROX analysis with SILAC quantitation.

Figure 3.

Shown in (A) and (B) are the iTRAQ- and SILAC-SPROX data (respectively) collected on the methionine-containing peptide LATPTYGDLNHLVSATMSGVTSLR from TUBB3 in the NDT binding study. In (A) the dark and light shaded data points represent the data collected in the presence and in the absence of NDT, respectively, and the solid lines represent the best fit of the data to Equation 1. The data points indicated with an “X” were not included in the fit. In (B) the data points were fit to Equation 3, and the solid and dotted curves represent the theoretical SPROX curves generated from the SILAC-SPROX data. In (A) and (B) the dotted vertical lines indicate the $C_{1/2}$ values of the SPROX curves.

Figure 4.

Shown in (A) and (B) are volcano plots showing the thermodynamic stability measurements and hits in the TAM and NDT binding study. The vertical lines represent the s-pooled hit criteria and the horizontal lines represent the $\text{Diff}_{\text{prob}}$ hit criteria. The light and dark shaded points represent the data for non-hit and hit peptides, respectively.

Figure 5.

Pulse proteolysis with purified recombinant YBX1 +/- TAM. A) Pulse proteolysis of YBX1 +/- TAM in increasing concentration of urea. Red box depicting TAM-induced difference at 0.75 M Urea. B) Intact YBX1 and pulse proteolysis of YBX1 in the absence of TAM at low and high denaturant.

Figure 6. SPROX data generated on YBX1 +/- TAM. The filled circles represent data generated on the YBX1 peptide RPQYSNPPVQGEVMEGADNQGAGEQGRPVR in the PAB-SPROX analysis of the purified recombinant YBX1 construct. The open circles represent data on the oxidized version of the same YBX1 peptide in the SILAC-SPROX analysis of the unpurified protein in the MCF-7 cell lysate. Both data sets are consistent with a TAM-induced stabilization of YBX1.

Figure 7.

STRING analysis of the 27 high-confidence TAM and NDT hits. The STRING analysis was conducted using medium confidence data from experiments. Also included in the STRING analysis were the MAPK1, AKT1 and ESR1 (see text). The red dashed line represents an experimentally observed direct interaction between ER and YBX1 that was recently reported in reference.³⁴

Table 1

Experimental coverage and number of hits identified in the SPROX experiments described here.

Drug	TAM		NDT	
Experiment	Proteomic Coverage Peptides(Proteins)	Hits Peptides(Proteins)	Proteomic Coverage Peptides(Proteins)	Hits Peptides(Proteins)
iTRAQ-SPROX	1806(811)	105(99)	1981(849)	149(132)
SILAC-SPROX	2046(629)	81(77)	2240(683)	105(88)
Total Unique	3244(1055)	186(163)	3556(1098)	254(200)

Table 2

High confidence TAM-induced protein stability hits.

Sequence	Gene	iTRAQ-SPROX ^a	SILAC-SPROX ^a	Breast Cancer Reference Number
IGNTGGM(ox)LDNILASK	ACLY	–	Yes	38
DEPSVAAMVYPFTGDHK	ACLY	Yes	–	38
MSINAEVVVVGDLVEVK	ATP1A1	–	Yes	39
DMTSEQLDDILK	ATP1A1	Yes	–	39
VTYTPM(OX)APGSYLISIK	FLNA	Yes	No	40–41
HTAM(ox)VSWGGSIPNSPFR	FLNA	–	Yes	40–41
GAGSYTIMVLFADQATPTSPIR	FLNA	Yes	–	40–41
TFEM(ox)SDFIVDTR	FLNB	–	Yes	10
GIEPTGNMVK	FLNB	Yes	–	10
ETMQSLNDR	KRT18	No	Yes	6, 42
EELLFMK	KRT18	Yes	–	6, 42
DVDEAYM(ox)NKVELESR	KRT8	No	Yes	
SNMDNM(OX)FESYINLRR	KRT8	Yes	–	
SNM(OX)DNM(OX)FESYINNLR	KRT8	Yes	No	
GSSAVGLTAYVMKDPETR	MCM4	Yes	–	43
NLNPEDIDQLITISGM(ox)VIR	MCM4	–	Yes	43
TQYSSAMLESLLPGIR	NPEPPS	Yes	–	
QMGFPLIYVEAEQVEDDR	NPEPPS	Yes	–	
VKEGM(ox)NIVEAMER	PPIA	No	Yes	44–45
HTGPGILSM(OX)ANAGPNTNGSQFFICTAK	PPIA	Yes	No	44–45
SLEDQVEM(OX)LR	PRKCSH	Yes	No	
ESLQQMAEVTR	PRKCSH	Yes	–	
RPQYSNPPVQGEVM(ox)EGADNQGAGEQGRPVR	YBX1	–	Yes	32–34
RPQYSNPPVQGEVM(ox)EGADNQGAGEQGR	YBX1	–	Yes	32–34

^aThe “–” indicates the peptide was not assayed; “No” indicates the peptide was assayed and not assigned as a hit; “Yes” indicates the peptide was assayed and assigned as a hit.

Table 3

High confidence NDT-induced protein stability hits.

Sequence	Gene	iTRAQ-SPROX ^a	SILAC-SPROX ^a	Breast Cancer Reference Number
ISMPDIDLNLKGPK	AHNAK	Yes	–	46–48
ISMPDIDLNLGTGPK	AHNAK	Yes	–	46–48
ASM(ox)GTlafDEYGRPFLLIKDQDR	CCT5	–	Yes	49
EKFEEM(ox)IQQIK	CCT5	–	Yes	49
MVVLSLPR	DYNC1H1	Yes	–	50
TM(ox)TLFSALR	DYNC1H1	–	Yes	50
SGDAAIVEM(ox)VPgKPMCVESFSQYPPLGR	EEF1A2	–	Yes	51–52
SGDAAIVEMVPgKPM(ox)CVESFSQYPPLGR	EEF1A2	–	Yes	51–52
LAM(OX)QEFMILPVGAANFR	ENO1	Yes	No	53
LAMQEFMILPVGAANFR	ENO1	No	Yes	53
LMIEMDGTEK	ENO1	Yes	–	53
GAGSYTIMVLFADQATPTSPIR	FLNA	Yes	No	40–41
LSPFMADIRDAPQDFHPDR	FLNA	Yes	–	40–41
YAPSEAGLHEMDIR	FLNA	No	Yes	40–41
MGLAM(OX)GGGGGASFDR	HNRNPM	Yes	–	54
VEVTYVELLMDAEGK	HNRNPM	Yes	–	54
IMQSSSEVGYDAM(OX)AGDFVNM(OX)VEK	HSPD1	Yes	–	55
IMQSSSEVGYDAM(OX)AGDFVNMVEK	HSPD1	Yes	–	55
ISGGSVVEMQGDEN(OX)TR	IDH1	Yes	–	56–57
LIDDMVAQAM(OX)K	IDH1	Yes	–	56–57
EVMPDLLAYLK	IPO4	Yes	No	
TLTMAPYLSTEDVPLAR	IPO4	Yes	–	
SSAYESLMEIVK	KPNB1	Yes	Yes	58
ETMQSLNDR	KRT18	Yes	–	6, 42
TVQSLEIDLDSMRNLK	KRT18	Yes	–	6, 42
DVDEAYM(ox)NKVELESR	KRT8	No	Yes	
ELQSQISDTSVVLSDNSR	KRT8	No	Yes	
LESGMQNM(ox)SIHTK	KRT8	No	Yes	
LNLEAINYM(ox)AADGDFK	LGALS1	–	Yes	8, 59
LNLEAINYMAADGDFK	LGALS1	Yes	–	8, 59
ALEQQVEEM(ox)K	MYH9	–	Yes	8, 60–61
SM(ox)AVAAAR	MYH9	–	Yes	8, 60–61
M(ox)GQM(ox)AM(ox)GGAM(ox)GINNR	NONO	–	Yes	62
PVTVEPM(ox)DQLDDEGLPEK	NONO	–	Yes	62

Sequence	Gene	iTRAQ-SPROX ^a	SILAC-SPROX ^a	Breast Cancer Reference Number
EGM(OX)NIVEAMER	PPIA	Yes	No	44–45
EGMNIVEAM(OX)ER	PPIA	Yes	No	44–45
M(OX)VNPTVFFDIAVDGEPLGR	PPIA	–	Yes	44–45
MVNPTVFFDIAVDGEPLGR	PPIA	No	Yes	44–45
QVLGQM(OX)VIDEELLGDGHSYSR	PSMB4	–	Yes	63–64
TQNPM(OX)VTGTSVLGVK	PSMB4	–	Yes	63–64
ALTVPELTQQM(OX)FDAK	TUBB3	Yes	No	37
LATPTYGDLNHLVSATMSGVTSLR	TUBB3	Yes	Yes	37
TIGTGLVTNTLAMTEEEK	TUFM	Yes	–	35–36
TVVTGIEMFHK	TUFM	No	Yes	35–36

^aThe “–” indicates the peptide was not assayed; “No” indicates the peptide was assayed and not assigned as a hit; “Yes” indicates the peptide was assayed and assigned as a hit.