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An ion-current-based, comprehensive and reproducible proteomic strategy for comparative characterization of the cellular responses to novel anti-cancer agents in a prostate cell model

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Abstract

Proteome-level investigation of the molecular targets in anticancer action of promising pharmaceutical candidates is highly desirable but remains challenging due to the insufficient proteome coverage, limited capacity for biological replicates, and largely unregulated false positive biomarker discovery of current methods. This study described a practical platform strategy to address these challenges, using comparison of drug response proteomic signatures by two promising anti-cancer agents (KX01/KX02) as the model system for method development/optimization. Drug-treated samples were efficiently extracted followed by precipitation/on-pellet-digestion procedure that provides high, reproducible peptide recovery. High-resolution separations were performed on a 75-cm-long, heated nano-LC column with a 7-hr gradient, with a highly reproducible nano-LC/nanospray configuration. An LTQ Orbitrap hybrid mass spectrometer with a charge overfilling approach to enhance sensitivity was used for detection. Analytical procedures were optimized and well-controlled to achieve high run-to-run reproducibility that permits numerous replicates in one set, and an ion-current-based approach was utilized for quantification. The false positives of biomarker discovery arising from technical variability was controlled based on FBDR measurement by comparing biomarker numbers in each drug-treated group vs. “sham samples”, which were analyzed in an order randomly interleaved with the analysis drug-treated samples. More than 1500 unique protein groups were quantified under stringent criteria, and of

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which about 30% displayed differential expression with FBDR of 0.3-2.1% across groups. Comparison of drug-response proteomic signatures and the subsequent immunoassay revealed that the action mechanisms of KX01/KX02 are similar but significantly different from vinblastine, which correlates well with clinical and pre-clinical observations. Furthermore, the results strongly supported the hypothesis that KX01/KX02 are dual-action agents (through inhibition of tubulin and Src). Moreover, informative insights into the drug-actions on cell cycle, growth/proliferation, and apoptosis were obtained. This platform technology provides extensive evaluation of drug candidates and facilitates in-depth mechanism studies.

Introduction

Understanding the detailed molecular mechanisms underlying drug actions is critical to predict/evaluate efficacy, specificity and safety, and to direct developmental therapeutic efforts[1]. Moreover, such knowledge is also highly valuable to the pharmaceutical industry, e.g. to examine whether the mechanisms of action of a new candidate largely overlaps those of existing drugs, which will greatly expedite pipeline decisions on further development, and thus help to lower both target attrition rate and overall development costs[1, 2]. Unfortunately, owing to the high complexity of biological systems, a strategy that is capable of comprehensive investigation of drug mechanisms of action remains elusive.

Traditionally, the study of drug mechanisms of action are carried out by examining drug effects on hypothesized targets with traditional biochemical approaches such as immune-based assays, enzymatic assays, binding protein analyses and siRNA-based genetic methods[3, 4]. Though considerable successes have been achieved, these approaches are often highly laborious and time-consuming, and can be biased in that the unexpected off-target effects of a drug and the collective effects by multiple targets may not be revealed[5].

Recently, strategies based on genomics expression profiling constitute a powerful tool for discovery-based investigations of drug mechanisms of action on molecular level. The genomics signatures of drug effects and the subsequent pathway analyses have been widely utilized for drug sensitivity prediction, molecular target identification and pathway modulator analysis[6-8]. By profiling numerous genes for their drug-induced expression level changes, these studies are conventionally believed to be capable of generating testable hypotheses related to the mechanisms of action and side effects of a drug, as well as discovering drug response biomarkers. However, gene expression level changes may not accurately reflect the drug effects on protein levels, because the majority of pathway modulators and drug targets are known to be proteins rather than genes[9]. Previous studies have shown that the changes in transcriptional patterns do not correlate well with the changes of protein patterns in biological systems[10-12]. By comparison, proteomic profiling approaches are capable of comparing the protein expression levels under different physiological and pharmaceutical conditions, and thus afford directly relevant information on the drug-induced biological cascades[13, 14]. Nevertheless, owing to many technical limitations, accurate and comprehensive elucidation of mechanisms of drug action using proteomic approaches constitutes a daunting challenge.

The typical approaches for cell-based proteomic expression profiling include two-dimensional gel electrophoresis (2-DE)[15] and various LC/MS-based methods such as isotope labeling by metabolic incorporation (e.g. SILAC)[16] or chemical/enzymatic derivatization (e.g. ICAT, iTRAQ and ^{18}O -incorporation)[17, 18], and more recently, label-free protein expression profiling approaches[19-23]. Nonetheless, none of the above techniques provides a facile means for an extensive investigation of drug actions on proteome levels. First, in order to comprehensively reveal the mechanisms of drug actions, the ability to obtain a high proteomic coverage of the target proteomes (e.g. tissue or cellular samples), especially for these low-abundance drug response proteins, is critical but difficult to achieve. This is due to the high complexity of tissue or cellular proteomes and high dynamic ranges in protein concentrations[24]. Moreover, many potential drug response proteins are membrane-bound/associated[25], which are difficult to efficiently extract and to accurately analyze using conventional proteomic approaches[26].

Second, to elucidate drug actions, especially when the comparison of the mechanisms of action of multiple agents is required, it is desirable to compare a relatively large number of biological replicates (e.g. 5 biological replicates per group), in order to reduce the incidence of false-positives of biomarker discovery due to biological variability, and to increase the quantitative reliability and statistical power of the results [19, 21, 22]. The label-free methods appeared to be a logical choice in these cases due to its potential capacity of analyzing a relatively large number of biological replicates[27, 28]. Nevertheless, because the label-free approach does not employ any internal standard, to minimize analytical bias and variation, highly quantitative and reproducible sample preparation and LC/MS analysis are required but often difficult to achieve especially for a large-scale quantification[21, 29].

Finally, current profiling strategies are plagued by false-positive discoveries of significantly-altered protein expression (i.e. biomarkers), due to either technical or biological variations [30-32]. The false-positive discovery of drug response proteins would result in misleading biological information and mask the valuable clues to drug mechanisms of action. Unfortunately, the false-positive biomarker discoveries in LC/MS-based proteomic profiling are often overlooked[30, 31] and so far there is no practical method to *experimentally* evaluate the false-positive discovery rate.

Here we employed a suite of technical advances to address the above challenges and thereby enabling comprehensive characterization of drug response proteomes via reproducible and large-scale expression profiling. Samples were extracted uniformly and efficiently with a strong buffer, followed by a precipitation/on-pellet-digestion procedure that provides high and reproducible peptide recovery, including that of membrane proteins[22, 33]. A nano-LC/nanospray flow path developed in house[22, 33, 34], which features a low void-volume, large loading capacity, high separation efficiency and high chromatographic reproducibility, was utilized for LC/MS analysis. The complex proteomic samples were resolved efficiently on a long, heated nano-LC column (75-cm in length) with a 7-hr gradient before LTQ/Orbitrap detection, which greatly increased the number of protein quantifiable. An Orbitrap MS enhanced with a charge-overfilling strategy[22, 33], which markedly improved the analytical reproducibility, sensitivity and dynamic range for peptide quantification without compromising the mass accuracy and resolution, was employed to acquire peptide precursor

signals. The preparation and analytical procedures were carefully optimized and controlled to ensure high run-to-run reproducibility and protein quantification was carried out using an ion-current-based, label-free method. The false-positive rates of biomarker discovery were estimated experimentally by analyzing a “sham” sample set in parallel with the analysis of drug-treated samples, and the experimental design was optimized for minimal false-positive biomarker discovery rate. To show a proof of concept, this strategy was applied in the molecular-level elucidation of the anti-cancer mechanisms of two promising drug candidates (KX01 and KX02, structure shown in SI Fig. 1) vs. these of an existing agent (Vinblastine).

KX01 and KX02 were developed to target the peptide-binding domain of Src Family Kinase (SFK) and have been shown to suppress oncogenic proliferation *in vitro* and *in vivo*[35-37]. Both of the compounds are currently progressing in clinical trials. While the structures of the two compounds are quite similar, KX02 is engineered to have physical properties more conducive to crossing the blood brain barrier in order to be applied to brain cancer. Though the two compounds were initially designed as peptide substrate competitive inhibitors of the tyrosine kinase Src, preclinical studies indicated that they also have significant inhibition of the polymerization of tubulin by binding to a novel site, and a novel conformation, on the tubulin heterodimer, which implied dual-mechanisms of action[37, 38]. Consequently, investigation of the drug actions on a proteome level will afford novel insights into the underlying anti-cancer effects exerted by these new candidates and thus helping to direct the further efforts on therapy and commercial development. In this study, using inhibition of the growth of prostate cancer PC3-LN4 cells as the model system, we acquired the drug response proteomic signatures respectively for the two compounds, which were then contrasted against these induced by vinblastine, a well-established microtubule-polymerization inhibition agent.

Materials and methods

Cell culture and cell growth assay

The PC3-LN4 human prostate cancer cells were cultured in RPMI-1640 medium, supplemented with 10% fetal bovine serum, at 37 °C in a humidified 5% CO₂ incubator. We did not use any antibiotics in the culture medium. All the test compounds were dissolved in DMSO to 10mM as a stock solution. Cells were seeded at 3×10^3 cells per well in a 96-well culture plate and incubated overnight. KX01, KX02 and Vinblastine were added to the medium at various concentrations. After incubation for 24, 48 and 72 hours cell number was assessed by incubating with MTT and measuring the absorbance at 570 nm on a microplate reader (Molecular Devices). The cell growth curve and GI₅₀ value (the concentration required to achieve 50% growth inhibition) for compounds were determined by using GraphPad Prism 5 (La Jolla, CA) software. Then PC3-LN4 cells were treated with KX01, KX02 and Vinblastine with respective double GI₅₀ concentrations; DMSO-treated cells served as controls. Cells were harvested at 24 hr and 72 hr of treatment (n=5 different dishes per group), respectively. Multiple washes with cold PBS buffers were performed in order to remove proteins from culture media.

Highly efficient and reproducible extraction and precipitation/on-pellet digestion

Cell pellets were dissolved in 1000 μ L of ice-cold lysis buffer (50 mM Tris-FA, 150 mM NaCl, 0.5% sodium deoxycholate, 1% SDS, 2% NP-40, pH8.0). And the resulted buffer was homogenized for 5-10 s bursts at 15, 000 rpm with a Polytron homogenizer (Kinematica AG, Switzerland), then followed by a 20 s cooling till the foam was settled and repeat it for 5-10 times. Adequate sonication was applied till the solution was clear, then centrifuged at 140,000g for 1 hour at 4 °C. The supernatant was carefully transferred and store at – 80 °C till further analysis.

In order to remove undesirable components in the samples while maintaining a high peptide recovery, we employed a precipitation/on-pellet-digestion protocol as we previously described[34]. After reduction and alkylation, the protein mixture was precipitated by stepwise addition of 9 volumes of cold acetone with continuous vortexing and then incubated overnight at –20 °C. After centrifugation at 12,000 g for 20 min at 4 °C, the supernatant was removed and the pellet was allowed to air-dry. Two consecutive digestion steps were employed with sequencing-grade trypsin (Sigma-Aldrich, St. Louis, MO): the step-1 digestion took 5 h to completely dissolve the protein pellet while the step-2 digestion enabled complete cleavage by incubation overnight (14 h). The optimal enzyme/substrate ratios for step-1 and step-2 digestions were determined as 1:40 and 1:25 (w/w), respectively.

Long gradient nano-RPLC/Mass Spectrometry

The Nano-RPLC system consisted of a Spark Endurance autosampler (Emmen, Holland) and a set of ultra-high pressure Eksigent (Dublin, CA) Nano-2D Ultra capillary/nano-LC systems. To achieve a comprehensive separation of the complex peptide mixture, a nano-LC/nanospray setup, which features low void volume, high loading capacity and high chromatographic reproducibility[22], was employed. Mobile phase A was 2% acetonitrile in 0.1% formic acid and mobile phase B was 98% acetonitrile with 0.1% formic acid. Samples containing 6 μ g of digested peptides were loaded onto a large-ID trap (300 μ m ID $\times$ 0.5 cm, packed with Zorbax 3- μ m C18 material) with 1% B at a flow rate of 10 μ L/min, and the trap was washed for 3 min. A series of nanoflow gradients (flow rate was 250 nL/min) was used to back-flush the trapped samples onto the nano-LC column (75 μ m ID $\times$ 75 cm, packed with Pepmap 3- μ m C18 material) for separation. The nano-LC column was heated at 52 °C to greatly improve both chromatographic resolution and reproducibility. A 7-hr shallow gradient was used to achieve sufficient peptide separation. The optimized gradient profile was as following: 3 to 8% B over 15 min; 8 to 24% B over 215 min; 24 to 38% B over 115 min; 38 to 63% B over 55 min; 63 to 97% B in 5 min, and finally isocratic at 97% B for 15 min. Efficient equilibration of the long column at 3% B for 35 min was performed before the next injection.

An LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA) was used for protein identification. The instrument was operating under data-dependent product ion mode. One scan cycle included an MS1 scan (m/z 310-2000) at a resolution of 60,000 followed by seven MS2 scans by CID activation mode, to fragment the seven most abundant precursors found in the MS1 spectrum. The overfilled target value for MS1 by Orbitrap was 8×10^6 , under which the Orbitrap was calibrated carefully for mass accuracy and FT

transmission, as described in previous works (34). The use of high target value on the Orbitrap enabled a highly sensitive detection without compromising the mass accuracy and resolution. The activation time was 30 ms, the isolation width was 1.5 Da for ITMS, the normalized activation energy was 35%, and the activation q was 0.25. Five biological replicates of each group (KX01, KX02, Vinblastine, and DMSO) per time point (24-hr or 72-hr) were analyzed randomly. In order to experimentally control and evaluate false-positive biomarker rate in the current experiment set, 10 sham samples (10 identical aliquots of pool sample obtained by pooling all biological samples) were analyzed in a sequence randomly interleaved with the runs of biological samples.

Protein identification and quantification

The MS/MS spectra were searched against the Swiss-Prot human protein database (released June 2, 2010) with a total of 20,295 protein entries using the SEQUEST Sorcerer 2 (Sage-N Research, Inc.). The search parameters used were as follows: 15 ppm tolerance for precursor ion mass and 1.0 Da for fragment ion mass with fully tryptic peptides restraint and a maximum of two missed cleavages. Static carbamidomethylation of cysteine and dynamic oxidation of methionine were used. The false discovery rate was detected by using a target-decoy search strategy[39]. The sequence database was doubled to contain each sequence in both forward and reversed orientations, enabling false discovery rate (FDR) estimation. Scaffold 2.0[40] (Proteome Software, Portland, OR) was used to validate MS/MS-based peptide and protein identification based on cross-correlation (Xcorr) and delta correlation (ΔC_n) values. The identification of at least two distinct peptides for required for any proteins or protein group. The method for determining the optimal parameter for identification is specified in a previous publication [34]. A false discovery rate of ~0.5% at peptide level was achieved in this study.

Ion current based label-free quantification was performed to gain the quantitative results of these confident protein identifications using the SIEVE v1.2 (Thermo Scientific, San Jose, CA). SIEVE is a label-free differential expression package that included chromatographic alignment, global intensity-based MS1 feature extraction and aggregate protein identification assignment[41]. The LC-MS/MS runs were aligned with ChromAlign algorithm[42] in SIEVE package. After completion of the alignment step, quantitative frames were created based on m/z and retention time. Peptide ion current areas were calculated for each frame by SIEVE to assess relative expression ratios. The statistical analysis to determine the p values of the expression level differences in expression levels between the drug-treated vs. DMSO control groups were carried out using Fisher's Combined Probability Test. Subsequent to frame creation, MS2 fragment scans associated with each frame were processed with SEQUEST with the same search parameters as above. The following parameters were set to align the retention time and m/z to generate the frames. We used Correlation Bin Width = 1, Tile Increment = 150, Tile Maximum = 300, Tile Size = 300, Tile Threshold = 0.6 in alignment parameter settings; m/z Min = 310, m/z Max = 2000, Frame Time Width = 3.5 minutes, Frame m/z width = 0.02 Da, Peak Intensity Threshold = 4,000 in frame parameter settings. The relative ratios were further normalized against the mean ratios of beta-actin and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

False-positive biomarker discovery rate (FBDR) Control

To experimentally investigate the false-positive biomarker discovery rate (FBDR), a sham sample set, which is consisted of 10 identical aliquots of pool sample obtained by pooling all biological samples, were created. Among the 10 aliquots, 5 were randomly assigned as the “experimental group” and the other 5 were assigned as the “control group”. The FBDR was calculated under a given set of cutoff thresholds, by firstly applying the thresholds uniformly to both the biological sample sets and the sham sample sets, and then compute the ratio of the number of differentially expressed proteins in sham set (i.e. false-positives arising from technical variability) vs. that in the biological set. Volcano plots (fold changes vs. *p*-values) were created for each dataset to visualize the effects of different cutoff criteria the distribution of biomarkers. Finally, the cutoff criteria of >1.4-fold changes to either direction and *p*<0.05 between control and treated samples (by Fisher’s Combined Probability Test) were determined as optimal for the drug-treated groups. With these criteria, a maximum of ~2% FBDR in both differentially expressed protein lists (24 hr-treatment and 72 hr-treatment) were achieved.

Western Blot analysis

For Western blot analyses, protein samples (30 µg/each) in lysis buffer (50 mM Tris-FA, 150 mM NaCl, 0.5% sodium deoxycholate, 1% SDS, 2% NP-40, pH8.0) and protein molecular weight standards (Santa Cruz, CA) were separated in small-sized 4-12% polyacrylamide gels by SDS-PAGE (Invitrogen). Proteins were transferred to nitrocellulose (NC) membranes, which was then blocked for 1 h with blocking solution (Invitrogen) and sequentially incubated with a primary antibody followed by an appropriate secondary antibody conjugated with horseradish peroxidase (HRP) (Santa Cruz, CA). The positive immunoreactions were detected with x-ray film by chemiluminescence using the ECL Western blotting kit (Pierce, Rockford, IL) and developed by a Kodak X-OMAT 2000A Processor. While reprobing a western blot, the stripping buffer from Invitrogen was used. Several proteins involved in M phase of mitotic cell cycle and cell survival/proliferation were selected to perform Western-blot analysis. The primary antibodies used in this study were as follows: rabbit polyclonal anti-fatty acid desaturase 2 (FADS2) (1:1000; Santa Cruz Biotechnology), mouse monoclonal anti-N-acetyltransferase 10 (NAT10) (1:1000; Santa Cruz Biotechnology), mouse monoclonal anti-cyclin dependent kinase 1/2 (CDK1/CDK2) (1:500; Cell Signaling Technology), rabbit polyclonal anti-phospho-cyclin dependent kinase 1(p-CDC2 or p-CDK1) (1:1000; Cell Signaling Technology), rabbit monoclonal anti-phospho-proto-oncogene tyrosine-protein family (p-Src) (1:500; Cell Signaling Technology), mouse monoclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:5,000; Santa Cruz Biotechnology), and mouse monoclonal anti-beta-actin (1:2,000; Santa Cruz Biotechnology).

Bioinformatics analysis

Gene Ontology (GO) annotation was performed using the online tool the Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resources v6.7 (<http://david.abcc.ncifcrf.gov/>)[43]. And the GO enrichment analysis was further analyzed to deep understanding the drug-altered proteins and all the proteins quantified in

this study were used as a reference. Hierarchical cluster analysis was performed using Cluster 3.0[44] and displayed by TreeView supporting tree-based and image-based browsing of hierarchical trees (<http://www.eisenlab.org>). In the heat map generated by TreeView, the up-regulated proteins are indicated in red, and the down-regulated proteins are indicated in green.

Results and Discussion

1. The development, optimization and evaluation of the proteomics strategy

The detailed procedure as well as the scheme for the comparison of the drug-action proteomic signatures of the three drugs, is illustrated in Fig. 1. With the purpose of achieving high sensitivity, selectivity, reproducibility and quantitative reliability to the extent possible, each step was thoroughly optimized using the treated PC3-LN4 prostate cancer cells as the model system.

1.1. Reproducible and efficient extraction, cleanup and digestion—In order to accurately identify and characterize drug response proteins from the cellular matrices, it is critical to achieve a *quantitative and reproducible* sample preparation procedure that selectively remove detergents and matrix components while providing high and reproducible peptide recovery. To address these requirements, we employed a strong extraction buffer to extract the cellular proteins with high efficiency, and then a highly efficient precipitation/onpellet-digestion procedure [22] to cleanup and digest the extracts (Material and methods).

The use of the strong extraction buffer (Material and methods) permits the efficient extraction of total proteins including the hydrophobic proteins from organelles and plasma membrane[22, 33, 45]. As expressed by the high and reproducible protein yield per million of cells (SI Fig. 2A), a highly reproducible and efficient extraction was achieved.

The procedure for precipitation and two-phase on-pellet-digestion were optimized for high recovery and reproducibility using PC3-LN4 extracts. Under the optimized conditions (Material and methods), complete tryptic digestion was achieved, as indicated by the fact that more than 98% of the identified peptides were completely cleaved. We assessed the reproducibility of peptide recoveries from PC3-LN4 extracts by measuring the peptide yields of 10 aliquots of a pooled extract. A mean peptide recovery at 85.7% and a precision at 4.6% (RSD%) were achieved and day-to-day reproducibility were excellent (SI Fig. 2B). Such a high level of recovery and reproducibility would greatly contribute to a reliable ion-current-based quantification.

1.2 Extensive, sensitive and reproducible nano-LC/MS method for in-depth proteomic analysis—A comprehensive, in-depth proteomic comparison of drug-treated vs. control cellular proteomes is essential for this study but is challenging due to the high complexity of the proteomes. A highly efficient chromatographic separation would help to achieve comprehensive quantification, especially for lower abundance peptides that may represent the regulatory, drug response proteins. Furthermore, because the ion-current-based strategy does not use internal standard, high run-to-run reproducibility of retention times and MS signal intensities is critical in order for an reliable matching and quantification[19]. Here

we employed a unique nano-LC/nanospray configuration that features a low void volume, high separation efficiency and reproducibility, and a long, heated nano-LC column with a shallow, 7-hr gradient, for efficient separation of the digest mixtures. As to the MS detection, an Orbitrap analyzer operated under an “ion-overfilling” condition was employed to achieve high quantitative sensitivity and wide dynamic range.

The chromatographic conditions were thoroughly optimized for the efficient and reproducible separation of the tryptic peptides derived from PC3-LN4 extract. The optimized chromatographic strategy achieved high-resolution and highly reproducible separation of the cell samples by employing i) a low-void-volume nano-LC/nanospray configuration [22, 33], ii) a long reversed-phase nano-column (75-cm in length and packed with 3- μ m materials) with a 7-hr gradient, and iii) the use of large-ID trap (300 μ m I.D.) with a bi-direction flow path that provides improved gradient mixing and dampened pump noise. An elevated temperature at 50 °C was used for separation to further improve chromatographic resolution and separation reproducibility .

An Orbitrap MS was employed to acquire the ion currents of peptide precursors using an overfilling strategy to improve sensitivity[22, 34]. Under the optimized conditions, an approximately 8-fold gain in sensitivity was achieved by the overfilling strategy (i.e. 8×10^6 charges for AGC, Automatic Gain Control) over that by default AGC (5×10^5) without sacrificing mass resolution and accuracy (<3 ppm without internal calibration). The increased sensitivity considerably enhanced the ability for biomarker discovery of low-abundance, regulatory proteins.

A representative base peak chromatogram for the analysis of PC3-LN4 samples under the optimized LC/MS conditions is shown in Fig. 2. An extensive chromatographic resolution of the PC3-LN4-driven peptides was achieved, as expressed by the extended peptide elution window of >320 min and a peak capacity >700.

We assessed the run-to-run reproducibility for both chromatographic separation and quantification at the proposed large replicates (i.e. n=20 per treatment time point) by performing 20 consecutive analyses of a pooled PC3-LN4 sample over a 6-day period. Fourteen representative peptides that were randomly selected to represent each 20-min segments of the elution window, were used for the evaluation (SI Table S1). The reproducibility for the twenty 7-h runs was excellent, as expressed by the low variations of 0.19-1.57% and 6.1-13.8% respectively for the retention times and area-under-the-curves (AUC) across the 14 peptides. This high level of reproducibility can be attributed to the highly reproducible LC/MS strategy as well as the high efficiency of the precipitation/on-pellet-digestion procedure.

1.3 The ion-current-based quantification strategy—Previous study demonstrated that the ion-current-based methods may be superior to the spectral-counting in that it may provide higher sensitivity for detecting altered proteins and affords better quantitative accuracy when conducted properly [21, 28, 46]. Consequently, in order to achieve an accurate characterization of proteomic signatures of drug actions, the ion-current-based method was utilized in the current study.

For ion-current-based profiling, a stable ionization efficiency, a sensitive, selective and high-resolution MS1 detection, as well as a highly reproducible and efficient chromatographic separation are required to ensure quantitative accuracy and precision[19, 47-49]. Here we developed an analytical strategy to address the above challenges, which allows an accurate, reproducible and extensive ion-current-based expression profiling of the PC3-LN4 proteomes.

Several ion-current-based bioinformatics tools were evaluated with the raw data of pooled samples, and finally Sieve is chosen as it was the only package that is capable to smoothly and robustly analyze the very large datasets of precursor ion currents generated by the long gradient nano-LC separation. The optimal parameter settings for alignment, frame generation, normalization and relative quantification were identified by analyzing a test sample set containing 20 repetitive runs of a pooled PC3-LN4 extract (shown in *Experimental* section).

The actual cutoffs (i.e. p value and fold of change) for biomarker discovery were determined individually for each control/experimental set based on the experimentally determined false-positive biomarker discovery rate (FBDR, discussed below).

1.4 Approach for experimental assessment of false-positive biomarker discovery rate (FBDR)—Currently, the high false-positive rates of biomarker discovery[50-52], which root from the biological variability and technical variability, represent a prevalent problem for quantitative proteomics [30, 32]. In order to alleviate the biological variability, the current study conducted well-controlled cell experiments (cf. the *Experimental*) to minimize the individual variability, and employed a relatively large number of biological replicates ($n=5$ for each group at each time point). To address the false-positive biomarker discovery arising from technical variability, the highly reproducible sample preparation and LC/MS strategy discussed above were employed to minimize the quantitative variations. Furthermore, we employed an experimental means to measure the false-positive biomarker discovery rate (FBDR) and thus to assess and control the false-positive discovery of the drug response proteins in each experimental sets.

All cell samples used for the profiling are pooled and then ten aliquots of the pooled sample were procured, which constitute the sham samples. Among the ten aliquots, five were randomly designated as the “sham-control samples” while the rest were designated as the “sham-experimental samples”. The sham samples were prepared, analyzed by LC/MS and then the sham-control vs. sham-experimental groups were contrasted using exactly the same procedures and in the same batch as for the biological sample sets. The FBDR was calculated as the ratio of the number of “decoy biomarkers” in sham samples over the number of significantly altered proteins identified in a biological sample experiment set, under the same cutoff thresholds. The values of FBDR reflect the collective effects by the multiple-hypotheses-testing problem and the variations in preparation and LC/MS analyses, and thus providing a reliable, quantitative means to evaluate the credibility of the outcomes of a biomarker discovery experiment, and to determine the ideal cutoff thresholds for each biomarker dataset.

2. Characterization and Differentiation of the drug actions of KX01, KX02 and Vinblastine by comparing the drug response proteomic signatures

KX01 was designed as a novel inhibitor for Src substrate binding site, and has been found to inhibit the proliferation of human colon carcinoma, breast carcinoma and leukemia cells[53]. KX02 is a lipophilic analog of KX01 and a recent study conducted on a xenograft mice model of glioblastoma demonstrated it eliminated the tumors in 60% of treated animals[36]. Moreover, based on the results of pre-clinical and clinical studies, it could be speculated that KX01 and KX02 may exert their high efficacy in treating solid cancer tumors by a **dual-action** mechanism, i.e. by targeting the binding domain of Src Kinase and acting as a pre-tubulin inhibitor[37, 38, 54]. Therefore, a discovery-based, proteome-level investigation of the cellular responses to the two compounds would provide novel and extensive insights into the drug mechanisms of action. Here we applied the developed platform technology to the characterization of drug actions of KX01 and KX02. Furthermore, we attempted to differentiate the drug response proteomic signatures of the two compounds against that of Vinblastine (VBL), a well-established microtubule inhibitor that binds with high affinity to tubulin [55]. KX01 is currently undergoing Phase II trials for prostate cancer. The PC3-LN4 cell line was used as a cell model due to its highly metastatic potential, representing a tough cell type to achieve growth inhibition by anti-cancer drugs. The PC3-LN4 cell line was treated respectively with KX01, KX02, VBL and DMSO control, and then prepared and analyzed in parallel. To ensure the reliability of the result and to minimize biological variation, 5 biological replicates were employed for each group. In order to comprehensively evaluate the drug effects on a temporal dimension, two drug treatment durations at 24 and 72 hr, respectively mimicking the clinical conditions of the initial and prolonged exposures to the drugs, were investigated. At each time point, drug response proteomic signatures of individual drugs were acquired using the ion-current-based strategy, followed by functional annotation and preliminary validations of selected regulatory proteins with western blot method.

2.1 Dose normalization based on the inhibitive effects of KX01, KX02 and VBL on tumor cell proliferation—In order to warrant a fair comparison of the three agents, we normalized the dose amount of each based on their GI50 concentrations (the concentration required to reduce the growth of treated cells to half that of untreated cells) upon 72-hr drug treatments. The dose levels twice as high as the GI50 of each drug were used for the investigations. The GI50 values was determined by MTT assay with 72h drug treatment (Fig. 3). Based on the data, the dose levels used for proteomic profiling study were decided as 80, 100 and 2 nM accordingly for KX01, KX02 and VBL. The effects of all three agents on PC3-LN4 cell growth at varying multiples of their GI50s were also evaluated and the results are shown in SI Fig. 3. During the 72-hr treatment period, PC3-LN4 cell growth was not significantly affected at drug concentrations below half of GI50, whereas at higher dosing levels (2X GI50), pronounced inhibitions in cell proliferation vs. solvent control at both 24 and 72 hr were observed for all three agents. Moreover, treatments with KX01, KX02 and VBL at and above 2X GI50 levels resulted in enormous accumulation of cells in G2/M phase at 24 hr (SI Fig. 4), which agrees with previous reports that VBL and KX01 induced prominent G2/M-phase cell cycle arrest in cancer cells[35, 56]. The proteomics

study conducted here further suggested the three drugs may share some common molecular mechanisms of cell cycle arrest (*cf. discussion below*).

2.2 The ion-current-based quantification and the experimental assessment of FBDR in all drug-treated groups—

The reproducibility and efficiency of sample preparation steps, chromatographic separation and the AUC of peptide peaks for the analysis of drug-treated PC3-LN4 samples was carefully evaluated and controlled (*cf. the discussions in previous sections*). As a result, high analytical reproducibility were achieved, as expressed by the excellent sieve alignment scores (0.78-0.85) and the high precision of AUC of marker peptides (RSD were 7.4-13.3%) across the five biological replicates within each treatment or control group. For the 24- and 72-hr treatment sets, approximately 310 and 330 thousands quantification frames were generated and 1321 and 1598 protein groups were respectively quantified under a stringent set of identification and framing criteria, including high cutoffs to resulting in 0.5% peptide FDR and strict criteria for frame generation (Material and methods). Among these proteins, 510 were membrane associated, indicating the high recovery of membrane proteins by the overall proteomic procedure (SI Fig. 5)

In order to identify the proper cutoff thresholds for biomarker discovery, the FBDR for each drug-treated group were investigated under various sets of cutoff thresholds. Finally, the cutoff criteria of >1.4-fold changes to either directions and $p < 0.05$ between control and treated samples were determined optimal. The volcano plots (fold changes vs. p -values) for all groups under these thresholds are shown in Fig. 4. The colored data points represent proteins that were deemed as significantly-changed under the optimal cutoff criteria described above. While there is only one “decoy biomarker” in the sham dataset (SI Table S2) under the above-mentioned cutoff values, KX01, KX02 and VBL treatments for 24hr respectively resulted in 87, 47 and 52 significantly-altered proteins compared to the DMSO control (SI Table S3), and 363, 507 and 242 proteins were determined as significantly altered after 72-hr treatments by KX01, KX02 and VBL (SI Table S4) respectively. As a result, excellent FBDR ranging from 0.2-2.1% were achieved, which suggests the biomarkers discovered in all drug-treated groups are highly reliable. The low FBDR achieved in this study can be attributed to the well-controlled and reproducible sample preparation and LC/MS analysis, as well as the high sensitivity and reliability for biomarker discovery by the ion-current-based strategy optimized in this study.

2.3. Functional annotation of altered proteins—

Out of the total of 541 differentially expressed proteins, 495 DAVID Biological Process ID were matched. Top ten biological process groups encompass the majority of matched IDs (427): metabolic process (338 proteins), translation (92), microtubule-based process (20), cell cycle (53), cell proliferation (26), proteolysis (32), transport (81), regulation of apoptosis (33), cell differentiation (46) and RNA splicing (41). Due to the prominent G2/M phase arrest induced by these drugs, special attention was paid to investigate the proteins in the group of M phase of mitotic cell cycle, a sub-category of the cell cycle group; 17 proteins were found in this sub-category by GO annotation.

In order to further explore the significance of these identified functional groups, we performed an enrichment analysis, which sought for the drug-altered proteins that are

enriched in certain functional groups compared against these from all the proteins quantified. Some categories including translation (1.6-fold enrichment), microtubule-based process (3.0-fold enrichment), cell proliferation (1.3-fold enrichment) and M phase of mitotic cell cycle (1.4-fold enrichment) were enriched as shown in Supplementary SI Fig. 6. This result implied the proteins related to these specific functions were potentially the important targets for further investigations.

2.4 Hierarchical cluster analysis of the proteomic expression patterns induced by KX01, KX02 and VBL—

In order to examine the dissimilarity among the differentially expressed proteins induced by KX01, KX02 and VBL, a hierarchical cluster analysis was performed. The cluster analysis and heat map generation were carried out using Cluster 3.0 and TreeView with centroid-linkage method. The heat maps and tree diagram are shown in Fig. 5. The clustering was implemented in two manners: i) analysis all significantly-altered proteins (541 in total, as shown in the upper panel of Fig. 5) and ii) targeted analysis of the altered proteins in three Biological Process categories that are closely relevant to anti-cancer effects: cell cycle (53 proteins), apoptosis (21) and cell differentiation (46), as shown in the lower panels of Fig. 5. As indicated in the tree diagrams, treatments with KX01 and KX02 are classified into the same clusters in all categories and at either time points. Conversely, VBL-treated groups showed expression patterns different from these of KX01 and KX02, and are classified into separate clusters. These results imply that the mechanisms of action of VBL are significantly different from these of KX01 and KX02, which correlates well with the previous clinical and pre-clinical observations. Furthermore, clustering analysis revealed potentially similar drug actions of KX01 and KX02. This is likely due to the highly similar functional moieties of KX01 and KX02 (*c.f.* SI Fig.1). Therefore, these results suggest the technology developed here is capable of differentiating drug mechanisms of action by different compounds.

Interestingly, the cluster analysis shown in Fig. 5 showed significantly different expression patterns between 24 hr and 72 hr treatments. This result is supported by the fact that while there were 87 altered proteins after 24-hr KX01 treatment, only 30 (34%) of these proteins remained changed to the same trend at 72 hr. We speculate these drastic differences in the temporal expression patterns may reflect the contrasts of cellular responses to short-term vs. prolonged drug treatments, and thus deserve further investigations.

2.5 The differentially expressed proteins induced by KX01, KX02 and VBL—

The Venn diagram of the differentially expressed proteins induced by the three drugs was shown in Fig. 6. Obviously KX01 and KX02 shared more commonly altered proteins than did either of them with VBL (e.g. 23 vs. 17 and 12 for 24-hr treatments and 322 vs. 150 and 180 for 72-hr treatments), which supports the result of cluster analysis that the mechanisms of action of KX01 and KX02 are similar but different from these of VBL.

For 24-hr treatment, only 10 altered proteins were common for all drugs (detailed list shown in the SI Table S3). Among them, N-myc downstream-regulated gene 1 (NDRG1) is a metastasis suppressor that is down-regulated in a variety of cancer types including prostate cancer compared to normal cells[57]. In this study, it was observed NDRG1 was up-regulated in all drug groups of 24-hr treatments, which may suggest the possible role of anti-

metastasis by these drugs. As for 72-hr treatment, 142 altered proteins were in common for all three drugs (Fig. 6, detailed list is shown in SI Table S4). Among them, 133 were down-regulated and only 9 were up-regulated. The down-regulated proteins are mostly responsible for protein/RNA metabolic process, translation, cell cycle, transport, response to stimulus and transcription (SI Table S4). It is interesting that out of the 9 up-regulated proteins, 4 are members of S100 family: S100-A6, S100-A10, S100-A11 and S100-P. The S100 proteins belong to the calcium-binding EF-hand motif superfamily and are associated with multiple biological processes including the cell cycle, cell differentiation, apoptosis and cell motility[58]. For an example, decrease of S100-A6 (calcyclin) expression in prostate cancer compared with normal cells was observed and re-expression of S100-A6 mRNA were induced by treatment with an anti-cancer drug (5-Azacytidine) [59].

Among other altered proteins, we further validated selected key proteins that are closely relevant to the anti-cancer mechanisms of the three drugs, which are discussed in the following two sections.

2.6 The effects on tubulins and Src signaling pathway by KX01, KX02 and VBL

—VBL is an anticancer agent for the treatment of a wide range of solid tumors, and it has been well-established that VBL exerts its effects by binding to the β -tubulin subunit of α/β -tubulin heterodimers and thus inhibiting cell division[60]. By comparison, clinical and pre-clinical studies suggested the possibility that KX01 and KX02 inhibit the growth of cancer cells by a dual-action mechanism, i.e. both as tubulin polymerization inhibitor and Src inhibitor[35, 37, 38]. Nonetheless, this hypothesis had not been extensively tested before the current study. In this study, the ability to acquire highly informative drug response proteomic signatures enabled the examination as to whether KX01 and KX02 are dual-action agents. We investigated changes in the expression levels and/or phosphorylation states of tubulins and c-Src induced by KX01 and KX02, by both interrogating the proteomics data and performing western blot assays. Changes induced by VBL were employed as the control for a single-action agent.

Seven tubulin isotypes were uniquely identified and quantified (i.e. excluding common peptides) by the ion-current-based approach. As shown in Fig. 7, all the tubulin isotypes were down-regulated by treatments with the three drugs for 24 hr, and to even greater extents for treatments of 72 hr. An autoregulatory mechanism was previously proposed to explain the down-regulation of soluble tubulin dimers by tubulin depolymerizing agents such as VBL[61]: the tubulin depolymerization by these agents result in the increase in tubulin monomer pool, which subsequently lead to reduction of the tubulin synthesis by rapidly decreasing the expression in tubulin mRNA [61, 62]. We speculate the inhibition of tubulins by KX01 and KX02 may also follow a similar mechanism, which could find supports in the observations that: i) The down-regulation of tubulin mRNA was detected for all these three drugs (SI Fig. 7); ii) no isotype-specific regulation of tubulins at protein level was observed for any of the three drugs; iii) there was no perceivable difference in the time-dependent characteristics of tubulin inhibition among the three drugs (Fig. 7) and iv) in an *in vitro* experiment, using a photo-affinity linker as the docking agent and a nano-LC/Orbitrap/ETD for identification, we were able to confirm both KX01 and KX02 bind to tubulin with high efficiency (data not shown). As the main focus of this work is the

development and application of the proteomics-based platform strategy for the differentiation of action mechanisms among drugs rather than elucidating the exact mechanisms of certain biological cascades, this assumption has yet to be further examined.

The 2nd proposed drug-action mechanism for KX01 and KX02 is the binding to Src and thus inhibiting Src pathway. Previously, KX01 has been reported to inhibit c-Src phosphorylation and the downstream signaling proteins in MCF-7 cells[35]. In the current study, the proteomics and western blot data suggested that none of the three drugs significantly affect total Src level or its functional phosphorylation state (Y416) at the early stage exposure (24 hr), as shown in Fig. 8. Conversely, after 72-hr treatments, KX01 and KX02 significantly decreased the total Src levels and its Y416-phosphorylation, but such decreases were not observed for VBL-treated cells (Fig. 8). Furthermore, we investigated the expression levels of two downstream signaling proteins, paxillin and cortactin. Paxillin is a multifunctional scaffold protein endowed with important roles in regulation of androgen- and epidermal growth factor-induced MAPK signaling and, and was found to regulate cell proliferation in prostate cancer cells[63]. Cortactin is overexpressed in malignant tumors and play important roles in cell migration and metastasis[64]. The proteomics data suggested both proteins were also significantly down-regulated by KX01 and KX02 at 72-hr, but not VBL (SI Table S4). Therefore, both KX01 and KX02 inhibit Src and its downstream proteins in PC3-LN4 cells, and therefore are likely to reduce cell proliferation and migration.

Collectively, the above results suggest that while KX01 and KX02 inhibit both tubulin polymerization and Src/Src-related pathways, VBL decreases tubulin but not significantly affecting Src. Therefore, the current study strongly support the hypothesis that KX01 and KX02 are dual-action agents.

2.7 Preliminary validations of additional key proteins involved in cell proliferation and fatty acid metabolism—In order to further explore the utility of the proteomic data generated from this study, we conducted preliminary validations on some key proteins involved in cell cycle and fatty acid metabolism.

The expression profiling results indicated that in total 17 proteins associated with M phase of mitotic cell cycle were down-regulated by drug treatments, such as N-acetyltransferase 10 (NAT10), cyclin-dependent kinase 1 (CDK1), pescadillo homolog 1 (PES1), mitotic checkpoint protein BuB3, nuclear mitotic apparatus protein 1 (NUMA1) and cyclin-dependent kinase 2 (CDK2) (SI Table S5). Down-regulation and/or changes of phosphorylation states of these proteins could potentially result in mitotic arrest of the PC3-LN4 cells, and therefore are important targets for investigating the drug actions of anti-cancer agents. Here we chose to validate two of the important regulators by western blot: the NAT10 and CDK1. NAT10 was primarily identified as an activator for up-regulation of telomerase activity[65], and recently it was found to regulate cell cytokinesis; the depletion of NAT10 induced G2/M cell cycle arrest or delay of mitotic exit[66]. Proteomic results showed that NAT10 was significantly down-regulated in PC3-LN4 cells treated with KX01 and KX02 at both 24 hr and 72 hr, and only at 72 hr for VBL-treated cells (*cf.* SI Table S5), which indicated all drugs decreased the expression of NAT10 but with different temporal

characteristics. These results were confirmed by western blot analysis (Fig. 9). The down-regulation of NAT10 may have played a role in the prominent G2/M cell cycle arrest observed in the cell experiments(*cf.* section 2.1).

Phosphorylation of CDK1 is an important regulator for the activation of mitosis and a previous study demonstrated decreased Y15 phosphorylation of CDK1 in MCF-7 cells treated with KX01[35]. In this study, significant down-regulations of CDK1 in PC3-LN4 by the treatments with KX01, KX02 and VBL were observed by the ion-current-based profiling (SI Table S5); furthermore, western blot analysis revealed significant decrease of the Y15-phosphorylation of CDK1 in all drug treatment groups at 72 hr (SI Fig. 8). Therefore, all three drugs altered both the expression and the Y15-phosphorylation of CDK1, which may contribute to the G2/M cycle arrest by restraining mitosis.

The fatty acid metabolism plays a critical role in cancer cell survival/proliferation and thus proteins in this category are often extensively investigated as either markers for mechanism study or therapeutic targets[67]. GO annotation revealed eight altered proteins as associated with fatty acid metabolism (SI Table S6). Among these, an interesting observation was the down regulation of fatty acid desaturase 2 (FADS2), a key player as the rate-limiting enzyme involved in the desaturation and elongation of linoleic and α -linolenic acid to long-chain polyunsaturated fatty acid. Ion-current-based profiling revealed decreases of FADS2 were observed in cells treated by KX01 and KX02 after both 24-hr and 72-hr treatments, as well as for VBL after 72-hr treatment. These changes were further confirmed by western blot (Fig. 9). The down-regulation of FADS2 could decrease the susceptibility of tumor cells to apoptosis by reducing the level of long-chain n3 PUFA [68]. As a result, the down-regulation of FADS2 in this study may reflect the cellular responses of anti-apoptosis in response to the drug treatments.

Conclusion

A discovery-based, non-biased proteomics strategy for comprehensive investigation of drug mechanisms of action is highly valuable but often difficult to achieve due to a number of technical difficulties. To address this challenge, we developed a comprehensive and reproducible ion-current-based profiling approach, which provides extensive proteomic coverage, high analytical reproducibility, low false-positive rates for biomarker discovery and the ability to employ a relatively large number of biological replicates. Well-controlled and highly reproducible sample preparation and LC/MS strategy were achieved by using strong-buffer extraction, precipitation/on-pellet-digestion and a reproducible nano-LC/nanospray setup, which helped to attain the quantitative accuracy and precision necessary to discover the drug-altered proteins among a relatively large number of biological replicates (e.g. 40 biological replicates in this study). A long, heated nano-LC column in conjunction with a 7-hr gradient efficiently resolved the peptide mixture and thereby permitting a comprehensive proteomics quantification especially for regulatory proteins of low-abundance, without using a multi-dimensional chromatographic strategy. The large loading capacity of the chromatographic system as well as the overfilling strategy on an Orbitrap enhanced analytical sensitivity.

A relatively large number of biological replicates per group were employed to alleviate the false-positive biomarker discoveries arising from biological variations. Moreover, we devised an innovative means to quantify the extent of false-positive biomarker discovery rooting from technical variations. The FBDR was measured by comparing the number of altered proteins discovered from a biological sample set vs. that discovered from a sham sample set, which was analyzed in a sequence randomly interleaved with the runs of biological samples. The measured FBDR values reflect the collective effects of both the multiple-hypotheses-testing problem and the variations in sample preparation and LC/MS analyses, and thus providing an accurate estimation. Furthermore, based on the FBDR values under various cutoff thresholds, the optimal cutoff criteria for biomarker discovery can be readily established.

To show a proof of concept, this strategy was applied in the differentiation of the mechanisms of action of KX01/KX02 vs. these of VBL. Drug response proteomic signatures in PC3-LN4 cells were investigated after treatments of 24 hr and 72 hr, and approximately 1500 unique protein groups were quantified reproducibly. The cutoff thresholds for biomarker discovery were determined as >1.4 fold up- or down- regulation and $p < 0.05$ between each drug treated group vs. DMSO control, under which low FBDR ranging from 0.3-2.1% were achieved for the six drug-treated groups. The low FBDR indicated the high reliability of the discovered drug response proteins, which is highly critical for the downstream data analysis and validation.

Cluster analysis of the altered proteins revealed KX01 and KX02 yielded similar proteomic signatures, which were easily distinguished from that of VBL. This observation is consistent with the hypothesis that the mechanisms of action of KX01 and KX02 are similar due to the alike functional groups, but distinct from these of VBL. Proteomics data showed all three drugs decreased the expression of various isoforms of tubulins in similar time-dependent manners, suggesting all three drugs inhibit tubulins by an alike mechanism. Conversely, proteomics and western blot data indicated that both KX01 and KX02 significantly inhibit Src after 72-hr treatments, but such inhibition was not observed in cells treated with VBL. Collectively, these results suggested that the anti-cancer effects of KX01 and KX02 are inflicted by a dual-action mechanism, i.e. inhibiting tubulin and Src kinase signaling, whereas VBL inhibits tubulin but not perceptibly affecting Src signaling pathway.

To further explore the proteomics data, we performed some preliminary studies on key proteins involved in cell cycles and lipid metabolism. The insights obtained from these studies may help to achieve a broader understanding of the molecular-level mechanism for these drugs. Additionally, interesting temporal characteristics of the expression of some drug response proteins were obtained, which may provide valuable insights to direct the studies of vehicle delivery and drug resistance.

In order to obtain more comprehensive and generalized information on cellular responses to anti-cancer drugs in prostate cancer cells, further investigation in more cell lines are desirable. Moreover, comparison of the drug response proteomes in sensitive vs. resistant cell lines, or sensitive vs. normal cell lines, may yield invaluable insights related to potential therapeutic targets and/or cancer-specific responses. Such works are on-going in our lab.

Collectively, this work demonstrated that the comprehensive, reproducible and ion-current-based strategy developed here is capable of differentiating the mechanisms of action of multiple drugs with high reliability. As the method reveals abundant and quantitative information on drug response proteins, it is capable of providing novel and in-depth insights into the biological cascades underlying drug effects. Therefore, this platform technique is highly valuable for evaluating new candidates (e.g. to examine whether further development is justifiable), directing therapeutic efforts and facilitating in-depth mechanism study. Moreover, the technology has high translational value as it is amendable for the identification of the markers for rational responses and pharmacodynamics in tumors, which are important for drug development and evaluation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- A practical platform technique to obtain drug-responsive proteomics signatures
- The method provides accurate and reliable evaluation of promising drug candidates
- The well-controlled and reproducible strategy allows multiplex capacity
- A novel method to control the false-positive rates of biomarker discovery
- Unique mechanisms of actions were revealed for two anticancer candidates

Fig. 1.

The scheme of the overall proteomics strategy for the high-resolution, comparative analysis of the drug response proteomic signatures induced by KX01 and KX02, two promising anti-cancer agents. Vinblastine (VBL) served as the control for a single-mechanism-of-action drug. A highly reproducible, sensitive and comprehensive ion-current-based quantification procedure was developed and optimized. The false-positive biomarker discovery rates (FBDR) arising from technical variability in each drug-treated group were measured by comparing numbers of biomarkers discovered in each group vs. “sham groups”, which were consisted of identical aliquots of the same pooled sample and analyzed in an order randomly interleaved with the analysis drug-treated samples. Based on FBDR, proper cut-off thresholds (p value and fold of change) were determined to ensure low false-positives in each biomarker list.

Fig. 2.

A typical base peak chromatogram illustrating the high-resolution separation of drug-treated PC3-LN4 samples. The separation was carried out on a long nano-LC column (75-cm in length and packed with 3- μ m C18 particles) heated at 52°C. An Orbitrap was employed as the detector. The MS acquisition was triggered 22 min after the start of the nano-LC gradient.

	KX01	KX02	vinblastine
GI50 (nM)	40	50	1

Fig. 3.

The determination of GI50 values of KX01, KX02 and vinblastine in PC3-LN4 cells. PC3-LN4 cells were incubated with increasing concentrations of the each compound for 72 hr and the growth inhibition was assessed by MTT assay. The results are presented as percentages of the vehicle control.

Fig. 4.

The volcano plots illustrating an experimental strategy to control false-positive biomarker discovery rates by analyzing a sham sample set along with the biological sets. PC3-LN4 prostate cancer cells treated with KXO1, KXO2 and Vinblastine at their corresponding 2xGI50 concentrations for 24 hr and 72 hr were profiled by the ion-current-based approach. The Y axis shows the average ratios ($n=5$ per group) of protein levels in a drug-treated group vs. the time-matched DMSO control group, while the X axis shows the p values for the comparison. Each dot represent a unique protein group and the dashed lines denote the selected cutoff thresholds ($p < 0.05$ and > 1.4 -fold change in either directions) that define significantly-altered proteins (i.e. biomarkers), which are shown as red dots. Only one false-positive “biomarker” was discovered in the sham set under the current cutoff criteria, and the measured FBDR for each drug-treated group under the same criteria is shown in the corresponding plots.

Fig. 5. Hierarchical clustering analysis of the differentially-expressed proteins induced by drug treatments in PC3-LN4 cells. Upper panel: the heat map for all differentially expressed proteins; lower panels: heat maps for proteins in the biological process categories of apoptosis, cell differentiation and cell cycle.

Fig. 6.
The Venn diagrams of differentially-regulated proteins following drug treatments of 24 hr (A) and 72 hr (B).

Fig. 7.

The relative expressions of tubulin isotypes in PC3-LN4 cells treated with VBL, KX02 and KX01 compared against DMSO controls. The expression ratios after (A) 24-hr treatments and (B) 72-hr treatments are shown. The ratios were obtained by the ion-current-based proteomics approach (n=5 biological replicates per group), and the expressions of two “house keeping” proteins, actin and GAPDH, are also shown.

Fig. 8.

The western blot validation of the relative expression of phosphorylated Src (p-Src) in PC3-LN4 cells following treatments with VBL, KX02 and KX01. Ratios are presented relative to DMSO-treated controls.

Fig. 9. Western blot validation of the down-regulations of FADS2 (fatty acid desaturase) and NAT10 (N-acetyltransferase 10) induced by drug treatments.