

Quantitation of DNA Damage Response Signaling Proteins Using iTRAQ Labeling and LTQ Orbitrap XL

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Overview

Purpose: Identification and relative quantitation using iTRAQ™ of camptothecin-induced DNA damage response proteins in A549 cells over time.
Methods: A549 cells were treated with 5 μM camptothecin for 0, 2, 8 and 24 hours. Phosphoproteins were enriched using PMAC (Phosphoprotein Metal Affinity Chromatography), digested, labeled with iTRAQ and analyzed by nanoLC-ESI-MS/MS with the LTQ Orbitrap XL.
Results: Over 400 proteins were identified and quantified with at least two unique peptides (FPR<2%) in whole cell lysate. PMAC enrichment for phosphoproteins, followed by IMAC enrichment for phosphopeptides enabled identification and quantitation of additional known and novel proteins involved in DNA damage signaling and DNA repair.

Introduction

The DNA damage response pathway is critical in maintaining genome stability, and proteins within this pathway are commonly mis-regulated in cancer cells¹. Camptothecin is an anti-cancer drug that inhibits topoisomerase I DNA unwinding and leads to DNA damage in cells undergoing DNA replication (Figure 1). It has been shown that protein concentrations change in response to DNA damage and other cellular stresses through post-translational modifications – phosphorylation, in particular. Depending on the type of DNA damaging agents, phosphorylation can be rapid – reaching a maximum 2 h after treatment, or more robust and prolonged – reaching a maximum between 8 and 24 h after treatment (Figure 2)². Quantitation of the responses of different proteins or phosphorylation sites to camptothecin or other agents will help in the elucidation of the signaling pathways and development of new anti-cancer drugs. Here, we employed a mass spectrometry-based proteomics approach to identify and characterize camptothecin-induced DNA damage response proteins in A549 cells over 24 hr after treatment.

FIGURE 1. Model System–DNA Damage Response Pathway

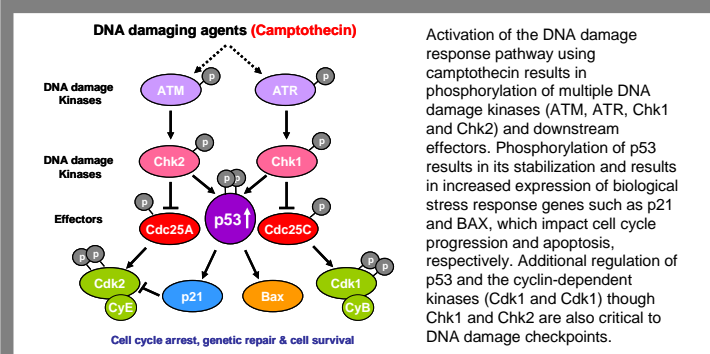
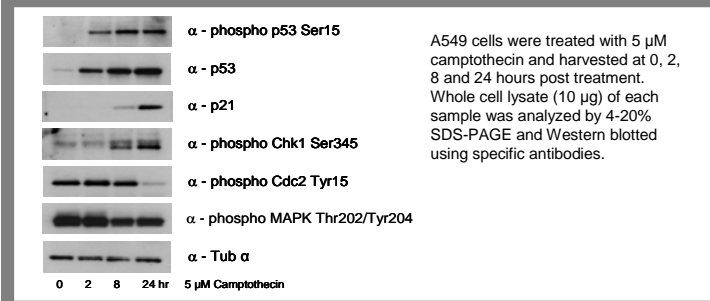


FIGURE 2. Comparison of A549 Protein Levels by Western Blot after Camptothecin Treatment



Methods

Cell treatment, phosphoprotein enrichment and iTRAQ labeling:

A549 cells (1 x 10⁷) treated with 5 μM camptothecin (SIGMA™) for 0, 2, 8 and 24 hours were harvested and were lysed with 2 mL of Thermo Scientific PMAC lysis buffer (whole cell lysate) containing Halt Protease and Halt Phosphatase inhibitors (Thermo Scientific). Samples were normalized for protein concentration using BCA Protein Assay (Thermo Scientific). 2 mg of each sample was incubated with 1 mL of PMAC resin (Thermo Scientific) for 30 minutes at 4 °C followed by five washes with 5 mL of PMAC lysis/binding/wash buffer. Phosphorylated proteins were eluted from the PMAC resin using the PMAC elution buffer (5 x 1 mL) and concentrated using an iCON concentrator (Thermo Scientific).

Whole or PMAC-enriched cell lysates (50 μg from each time point) were reduced with 5 mM DTT, alkylated with 25 mM iodoacetamide, acetone precipitated, digested and individually labeled with iTRAQ™ reagents (Applied Biosystems) as follows: 0 hr - 114; 2 hr - 115; 8 hr - 116 and 24 hr - 117. Labeled samples were combined in 1:1:1:1 ratio and applied to PepClean™ C-18 spin columns (Thermo Scientific). Phosphopeptides were further purified from 50 μg of iTRAQ labeled PMAC-enriched fractions using a Pierce® phosphopeptide isolation kit (Thermo Scientific) according to manufacturer's instructions.

LC/MS:

An Eksigent® NanoLC-2D™ HPLC with a PepMap™ C₁₈ column 75μm ID x 15cm (Dionex) was used to separate peptides using a 5-30% gradient (A: water, 0.1% formic acid; B: acetonitrile, 0.1% formic acid) at 200 nL/min over 180 min. An LTQ Orbitrap XL™ mass spectrometer was used to detect peptides and generate MS/MS data under the following settings: MSn Target Orbitrap = 2e5; MSn Target Ion Trap = 1e4; MS/MS = 2 μscans, 300 ms max ion time; MS = 400-1500 m/z, 30 000 resolution; MS/MS = Top Three Data Dependent™ acquisition HCD/ Top Three Data Dependent acquisition CID IT; Dynamic Exclusion = Repeat count 2, Duration 30sec, Exclusion duration 120sec; HCD Parameters: Collision Energy = 35%.

Data Processing:

BioWorks™ 3.3.1 with SEQUEST® was used for protein ID based on a protein probability filter of 1e-002 and a peptide mass accuracy 10 ppm. Quantitation based on iTRAQ reporter ions was accomplished by PepQuan™ software within BioWorks 3.3.1. Only data points that fell within 2 standard deviations from the average ratio for each protein were considered.

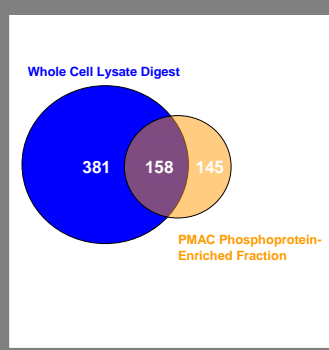
Mascot™ software 2.2 (Matrix Sciences) was also used for protein ID based on a probability of 99.9 (p<0.01) and a precursor mass accuracy of 10 ppm. For iTRAQ quantitation significant (95% t-test) normalized median ratios were used. The human IPI database was used for both search engines.

Results

To determine relative changes in overall protein abundance and phosphorylation state of proteins after camptothecin treatment, a 4-plex iTRAQ labeling method was employed before mass spec analysis. Two types of samples were analyzed: whole cell lysates and PMAC phosphoprotein enriched fractions. A total of 303 and 539 proteins were identified using a Human IPI 3.26 database and search filters as specified in "Methods" in PMAC-enriched and whole cell lysate tryptic digests, respectively (Figure 3). Only 158 proteins were similar between two samples demonstrating increase sample diversity after enrichment. Based on Swiss Prot annotations, 90% of proteins identified after PMAC enrichment are phosphoproteins.

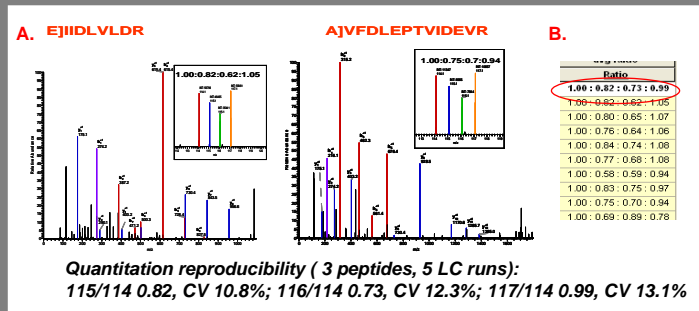
Quantitation of low mass reporter ions generated during MS/MS analysis of iTRAQ labeled peptides was performed using the HCD (High Energy Collision Dissociation) collision cell available on LTQ Orbitrap XL.

FIGURE 3. Venn Diagram of Proteins Identified before and after PMAC Enrichment



The HCD MS/MS spectra have high mass accuracy and resolution since they are acquired in the orbitrap and display similar fragmentation patterns to those from a quadrupole collision cell. This fragmentation technique produces rich "triple quad" like fragmentation patterns including fragments in the low m/z range. Figure 4 shows HCD MS/MS spectra for 2 out of 3 quantified peptides from tubulin alpha chain in the PMAC treated sample. This protein was used as a standard to normalize iTRAQ ratios based on Western Blot results (Figure 2). The iTRAQ reporter ion ratios were calculated using the 114 ion as a reference (0 hr time point). As shown in Figure 4, reproducible quantitation for five replicates was observed with a CV range of < 15%.

FIGURE 4. HCD Spectra of PMAC Tubulin α Peptides (A) and iTRAQ Reporter Ion Quantitation Ratios from BioWorks 3.3.1 (B)



To estimate the relative abundance of A549 cell proteins, calculated median ratios were first normalized using iTRAQ reporter ratios for alpha tubulin (PMAC fraction: 1:0.82:0.72:0.99; whole cell lysate: 1:2.3:1.97:2.53) and then evaluated by t-test. A summary of results is presented in Table 1. On average, 85% of identified proteins were quantified in both samples. Interestingly, in the whole cell lysate sample more proteins were up-regulated than down-regulated after drug treatment with a maximum of response at 8 hr. In the phosphoprotein enriched sample, more proteins were down-regulated than up-regulated but most changes again occurred after 8 hr of camptothecin treatment. The difference in the response can be explained by cell function of identified proteins and type and level of its post-translational modifications as shown in Table 1.

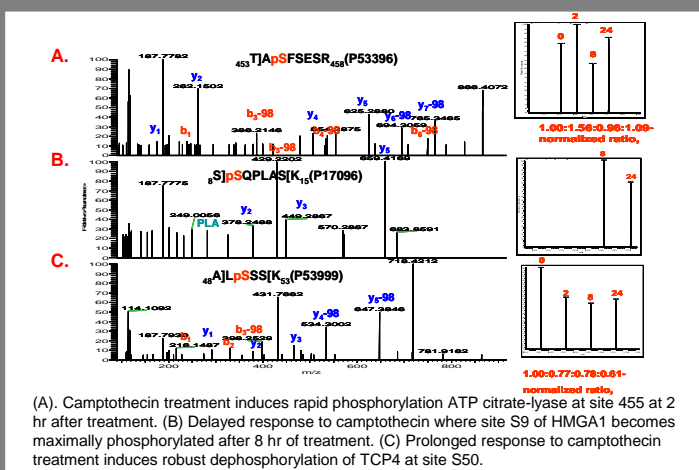
TABLE 1. Summary of iTRAQ Quantitation of A549 Proteins after Camptothecin Treatment

Time Point, hr	2		8		24		# Protein Identified* # Proteins Quantified**
Type of Sample/ Relative Abundance Ratios to 0 hr	↑	↓	↑	↓	↑	↓	
Whole Cell Lysate	70	49	90	17	68	19	536/447
PMAC-enriched Fraction	7	30	12	32	15	22	303/267

* Identification data are from FTMS/ITCID runs using Mascot search engine (p<0.01)
 **Quantitation data are significant normalized median ratios (95% t-test, more than 1.3 fold change, 5 LC runs) for proteins identified at FPR<2% with 2 peptides minimum

To specifically quantify the phospho sites, we further purified phosphopeptides from the iTRAQ PMAC fraction by IMAC (gallium-IDA) spin columns. After IMAC enrichment, we were able to identify and quantify an additional 30 phosphorylation sites from a very limited amount of sample (15 μg of PMAC fraction). Figure 5 shows an example of quantitation results for 3 phosphopeptides from three different proteins. We found that the camptothecin treatment creates a new phosphorylation site at S9 in the high mobility group protein HMGA1 (P17096) which is well known to be phosphorylated upon DNA damage and hyperphosphorylated at early stages of apoptosis³ (Figure 5B). However, camptothecin treatment can also promote dephosphorylation. For example, at site S50 of activated RNA polymerase II transcriptional coactivator p15 protein (TCP4, P5399), which activity is controlled by protein kinases, dephosphorylation will promote double stranded DNA-binding and cofactor function⁴ (Figure 5C).

FIGURE 5. MS/MS Spectra of iTRAQ Labeled Phosphorylated Peptides



Conclusions

- Quantitation of low mass reporter ions generated during MS/MS analysis of iTRAQ labeled peptides can be successfully performed using HCD on the LTQ Orbitrap with high precision (CV < 15%).
- More than 500 proteins were quantified by iTRAQ using the HCD method from A549 whole cell and PMAC lysates.
- Phosphoprotein enrichment by PMAC resin increased sample diversity by 145 additional proteins identified with > 30 phosphorylation sites quantified and identified after sequential IMAC purification of the 15 μg PMAC sample.

References

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