Application Note: 445

Quantitative Profiling of DNA Damage Response Proteins Using iTRAQ Labeling and the LTQ Orbitrap XL

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Key Words
- LTQ Orbitrap XL
- HCD
- iTRAQ
- Peptides
- Phosphorylation
- Proteomics
- Quantitation

Introduction

The DNA damage response pathway is critical to maintaining genome stability, and proteins within this pathway are commonly misregulated in cancer cells. Camptothecin, an anti-cancer drug, 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, specifically phosphorylation.[1]

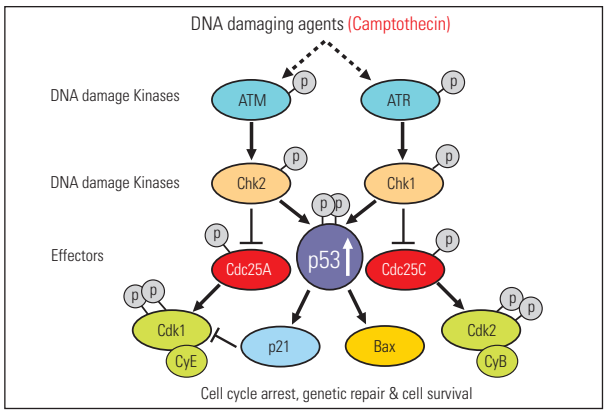

Figure 1. Schematic of the DNA damage response pathway. Activation of the DNA damage response pathway by camptothecin results in phosphorylation of multiple DNA damage kinases and downstream effectors. Phosphorylation of p53 by ATM, ATR, Chk1, and Chk2 results in its stabilization and an increase in the expression of biological stress response genes such as p21 and BAX, which impact cell cycle progression and apoptosis, respectively. Additional regulation of p53 and the cyclin-dependent kinases (Cdk1 and Cdk2) though Chk1 and Chk2 are also critical to DNA damage checkpoints in G1/S and G2/M transitions.

Depending on the type of DNA damaging agent, phosphorylation can be rapid—reaching a maximum 2 hours after treatment—or more robust and prolonged with a maximum between 8 and 24 hours after treatment[2] (Figure 2). Quantitation of the responses of different proteins or phosphorylation sites to camptothecin or other agents may help to elucidate the signaling pathways critical to the development of new anti-cancer drugs.

Previous studies indicated that the Thermo Scientific LTQ Orbitrap XL mass spectrometer with an HCD cell can be successfully used to accurately and precisely quantify iTRAQ™-labeled peptides.[3] Here, we extended

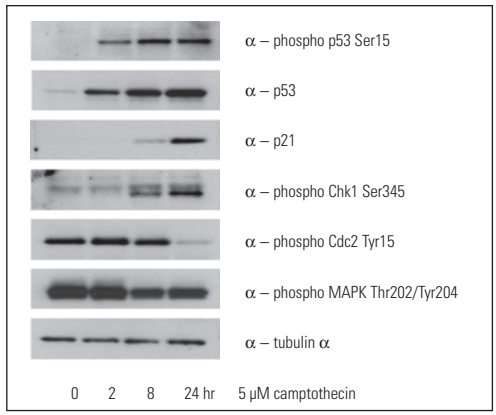

Figure 2. Comparison of A549 protein levels by Western blot after camptothecin treatment. A549 cells were treated with 5 µM camptothecin and harvested at 0, 2, 8, and 24 hours after treatment. Whole cell lysate (10 µg) of each sample was analyzed by 4%-20% SDS-PAGE and Western blotted using specific antibodies.

our efforts to use iTRAQ labeling and the LTQ Orbitrap XL™ equipped with an HCD cell to identify and characterize the dynamics of DNA damage response proteins in A549 cells over a period of 24 hours after treatment with camptothecin.

Goal

To identify and determine relative amounts of DNA damage response proteins in A549 cells over 24 hours after treatment with camptothecin using iTRAQ labeling and the LTQ Orbitrap XL.

Experimental Conditions

Cell treatment, phosphoprotein enrichment and iTRAQ labeling

A549 cells (10^7) were treated with 5 µM camptothecin (Sigma, St. Louis, MO) and harvested at 0, 2, 8, and 24 hours post treatment. The cells were lysed with 2 mL of lysis/binding/wash buffer (whole cell lysate) containing the Thermo Scientific Protease and Halt Phosphatase inhibitors. Samples were normalized for protein concentration using a Thermo Scientific BCA Protein Assay. At each time point, from the total cell extract, 2 mg of sample was diluted to 0.5 mg/ml in lysis/binding/wash buffer. The diluted samples were added to pre-equilibrated Thermo Scientific Phosphoprotein Enrichment columns and incubated for 30 minutes at 4 °C. The incubation was

followed by five washes with 5 mL of lysis/binding/wash buffer. Phosphorylated proteins were eluted from the columns using the elution buffer (5 x 1 mL) and concentrated using a Thermo Scientific iCON concentrator.

Whole or phosphoprotein-enriched (PE) cell lysates (50 µg from each time point) were reduced with 5 mM DTT and alkylated with 25 mM iodoacetamide. The lysates were then precipitated with acetone, digested, and individually labeled with iTRAQ reagents (Applied Biosystems, Foster City, CA) as follows: 0 hr (114), 2 hr (115), 8 hr (116), and 24 hr (117).

The labeled samples were combined in a 1:1:1:1 ratio and applied to Thermo Scientific PepClean C-18 spin columns. Phosphopeptides were further purified from 15 µg of iTRAQ labeled phosphoprotein-enriched fractions by using a Thermo Scientific Phosphopeptide Isolation Kit according to the manufacturer's instructions.

LC

Pump: NanoLC-2D (Eksigent Technologies, Dublin, CA)
Column: PepMap™ C18 column, 75 µm ID × 15 cm (Dionex, Sunnyvale, CA)
Mobile phase A: 0.1% Formic Acid in Water
Mobile phase B: 0.1% Formic Acid in Acetonitrile
Flow rate: 200 nL/min
Gradient: 5% to 30% B in 180 min

Mass Spectrometry

LTQ Orbitrap XL with nanospray ion source was operated as follows:*

MS Resolution: 30,000 FWHM at *m/z* 400
FT MS AGC target: 5e5
FT MS/MS AGC target: 2e5, 2 amu isolation width
Top 3 HCD MS2: at 35% normalized collision energy
MS/MS Resolution: 7,500 FWHM at *m/z* 400, 2 microscans
Monoisotopic precursor selection
Exclusion mass tolerance: 10 ppm
MS/MS threshold: 8000 (~S/N 2:1)
Max ion time FT MS: 100 ms
Max ion time FT MS/MS: 300 ms
Full MS range: 400-1500 *m/z*
MS/MS mass range: 80-2000 *m/z*

The experiment was run as top three data dependent HCD MS/MS scans in parallel with three CID MS/MS scans in the LTQ (35% collision energy, 5000 AGC target).

DYNAMIC EXCLUSION:

Repeat count: 2
Duration: 30 s
Exclusion duration: 120 s

**Additional studies indicated that 1e5 FT MS/MS target, 1 microscan and precursor isolation width of 2 amu are sufficient to produce high-quality HCD spectra. For more details on optimal experimental conditions, see application note #421[3].*

Data Processing

Thermo Scientific BioWorks 3.3.1 software with the SEQUEST® search algorithm was used for protein identification based on a protein probability filter of $1x10^{-3}$ and a peptide mass accuracy of 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.

Thermo Scientific Proteome Discoverer 1.0 with SEQUEST or MASCOT™ search engines was used for spectra-preprocessing, protein ID and quantitation based on iTRAQ reporter ions.

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 Mascot and Sequest searches.

Results and Discussion

Two types of samples were analyzed: whole cell lysates and phosphoprotein enriched fractions. A total of 539 proteins were identified in the whole cell lysate digests by using a Human IPI 3.26 database and the search filters specified above. In the PE fraction, a total of 303 proteins were identified. Only 158 proteins were similar between two samples, thus demonstrating an increase in sample diversity after enrichment (Figure 3). Based on Swiss Prot annotations, 90% of proteins identified after enrichment were phosphoproteins with an increase in kinase content from 0.5% to 4%.

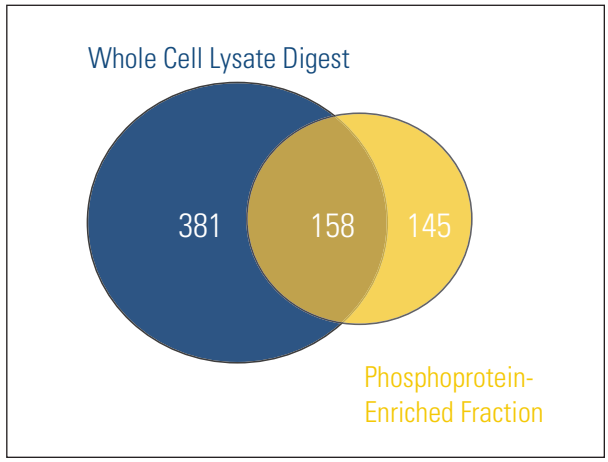

Figure 3. Venn diagram of proteins identified in whole cell lysate digest versus PE fraction

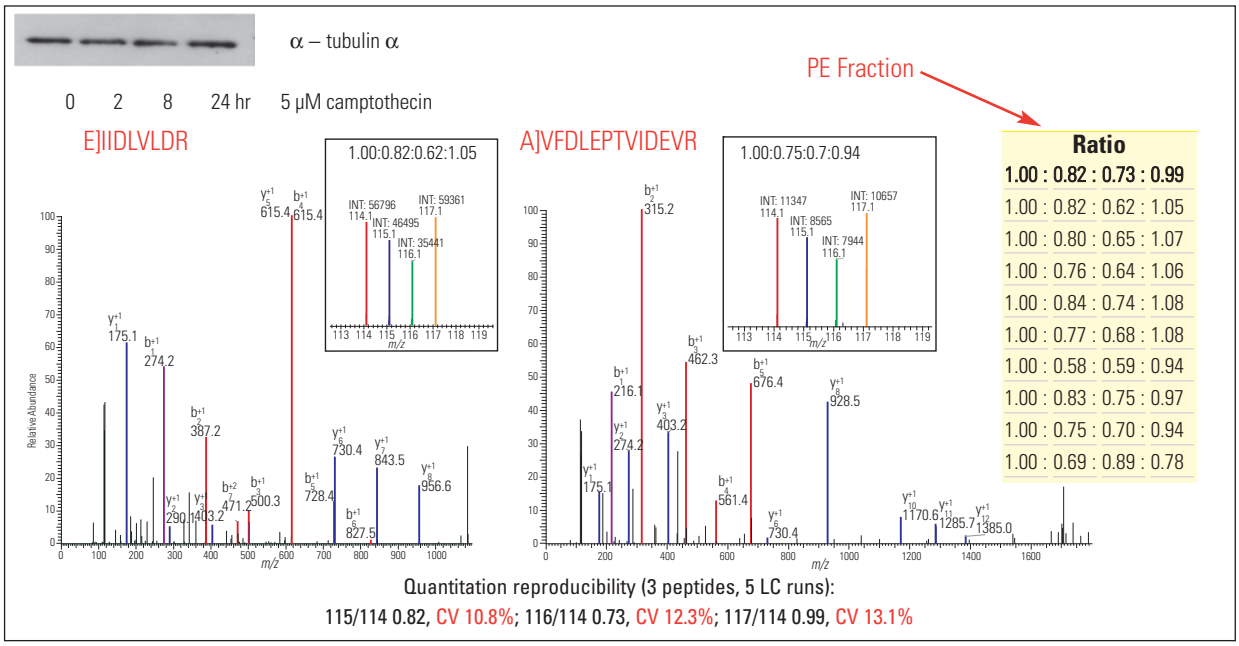

Figure 4. Normalization for iTRAQ labels using alpha-tubulin

Alpha-tubulin is an abundant constitutive protein; therefore, its relative abundance reflected in the iTRAQ reporter ions was chosen as an internal standard for the normalization of the iTRAQ labeled peptides (Figure 4). The ratio of the iTRAQ reporter ions from the tubulin peptides should be 1:1:1:1; the deviation from unity is due to variations in the protein concentration and in the sample preparation at each time point. Normalization coefficients were based on iTRAQ reporter ion ratios from three different peptides in tubulin from five technical replicates. The following normalization coefficients were used: for the whole cell lysate, 1:2.3:1.9:2.5; and for the PE fraction, 1:0.8:0.7:1.

Summarized results are presented in Table 1. On average, 85% of the 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 response at 8 hours. In the phosphoprotein-enriched sample, more proteins were down-regulated than up-regulated, with maximum changes again occurring after 8 hours of camptothecin treatment. Differences in the responses can be explained by the cell functions of the identified proteins and the type and level of their post-translational modifications as shown in Figure 5A.

To quantify the phosphorylation sites specifically, we further purified phosphopeptides from the iTRAQ PE fraction by using IMAC (gallium-IDA) spin columns.

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

* Identification data are from FTMS/ITCID runs using the 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

Table 1. Kinetics of the cellular response to camptothecin treatment

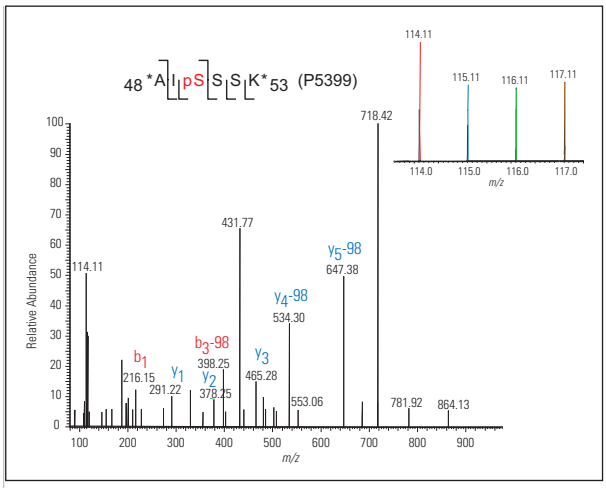

Figure 5A. Example of phosphopeptide response kinetics after treatment with camptothecin

After IMAC enrichment an additional 30 phosphorylation sites were identified and quantified from a very limited amount of sample (15 µg of PE fraction). Figure 5 shows an example of quantitative profiling for phosphopeptides (A) and examples of corresponding protein kinetics (B). The camptothecin treatment induces significant changes at the peptide level but not at the protein level in the presented example. A new phosphorylation site is found at S9 in the high mobility group (HMG) protein HMGA1 (P17096), which is well known to be phosphorylated upon DNA damage and hyperphosphorylated at early stages of apoptosis.[4] However, camptothecin treatment can also promote dephosphorylation. For example, as shown in fig 5A., at site S50 of activated RNA polymerase II transcriptional coactivator p15 protein (TCP4, P5399), whose activity is controlled by protein kinases, dephosphorylation promotes double-stranded DNA-binding and cofactor function.[5] These results illustrate that relative quantitation of specific sites can be more informative than median ratios of whole proteins for the study of cellular responses.

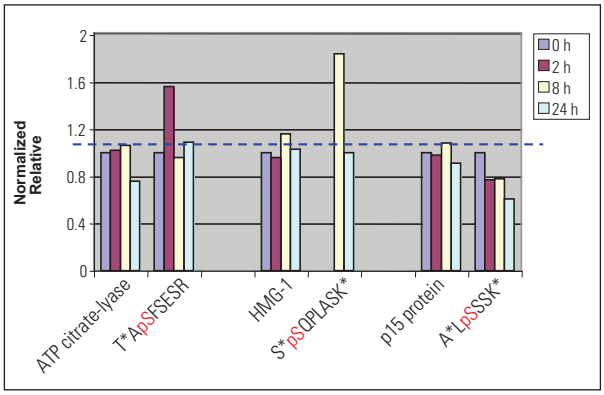

Figure 5B. Dynamics of total protein versus phosphopeptide response

Conclusion

Quantitation of iTRAQ-labeled peptides in complex mixtures can be successfully performed using HCD fragmentation. In this study, more than 500 proteins from A549 cell lysates were identified and quantified using iTRAQ labeling and an LTQ Orbitrap XL mass spectrometer equipped with an HCD collision cell. Phosphoprotein purification using the Thermo Scientific Phosphoprotein Enrichment Kit increased sample diversity by adding 145 additional proteins. After sequential IMAC purification of a 15 µg digested PE sample, an additional thirty phosphorylation sites were identified and quantified.

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