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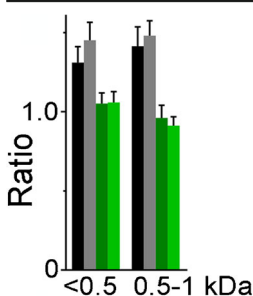
Quantitative Peptidomics with Five-plex Reductive Methylation labels

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Abstract. Quantitative peptidomics and proteomics often use chemical tags to covalently modify peptides with reagents that differ in the number of stable isotopes, allowing for quantitation of the relative peptide levels in the original sample based on the peak height of each isotopic form. Different chemical reagents have been used as tags for quantitative peptidomics and proteomics, and all have strengths and weaknesses. One of the simplest approaches uses formaldehyde and sodium cyanoborohydride to methylate amines, converting primary and secondary amines into tertiary amines. Up to five different isotopic forms can be generated, depending on the isotopic forms of formaldehyde and cyanoborohydride reagents, allowing for five-plex quantitation. However, the mass difference between each of these forms is only 1 Da per methyl group incorporated into the peptide, and for many peptides there is substantial overlap from the natural abundance of ^{13}C and other isotopes. In this study, we calculated the contribution from the natural isotopes for 26 native peptides and derived equations to correct the peak intensities. These equations were applied to data from a study using human embryonic kidney HEK293T cells in which five replicates were treated with 100 nM vinblastine for 3 h and compared with five replicates of cells treated with control medium. The correction equations brought the replicates to the expected 1:1 ratios and revealed significant decreases in levels of 21 peptides upon vinblastine treatment. These equations enable accurate quantitation of small changes in peptide levels using the reductive methylation labeling approach.

Keywords: Isotopic labels, Formaldehyde, Cyanoborohydride

Introduction

A large number of quantitative peptidomics and proteomics approaches have been developed to provide information about the absolute or relative levels of peptides and proteins in biological samples [1–10]. Some of these approaches incorporate stable isotopes into the peptides and proteins, either by labeling of cells or organisms or by post-extraction covalent modification of the peptides using chemical reagents. The peptides from different samples are then combined and analyzed, usually by liquid chromatography/mass spectrometry (LC/MS), with tandem mass spectrometry (MS/MS) to identify the peptides. The post-extraction labeling approach is widely used and a number of reagents have been described [1, 3–10]. The ideal isotopic tags react with all peptides, are commercially available for a moderate price, and are able to generate four or more isotopic forms to allow for multi-plex labeling. For optimal quantitation, it is useful if peptides labeled with the different isotopic forms of the reagent co-elute from reverse phase LC columns and have a sufficient difference in mass to avoid overlap from the natural abundance of ^{13}C and other isotopes. While a number of distinct reagents have been employed for quantitative proteomics and peptidomics, none is truly ideal. All of the commonly used reagents target free amines, and while most peptides have these groups, a small number are not labeled because of N-terminal acetylation or other modifications. In addition to this, each of the reagents has other problems that limit its widespread utility.

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A commonly used reagent for quantitative peptidomics is trimethylaminobutyrate N-hydroxysuccinimide (TMAB-NHS), which can be synthesized in five different mass forms that differ by 3 Da per form [3, 5, 11, 12]. However, the TMAB-NHS labels are not commercially available, thus limiting their widespread use. Commercially available isobaric tags, such as the widely used iTRAQ (isobaric tags for relative and absolute quantitation) and TMT (tandem mass tag) reagents are expensive and only provide quantitation of peptides selected for MS/MS analysis [4, 13]. The heavy isotopes within isobaric tags are distributed in different positions along the tags and quantitation is performed at the MS2 level when unique reporter ions are produced by fragmentation. The TMT six-plex produces reporter ions with 1 Da differences in the low mass range of 126–131 Da [8]. TMT ten-plex relies on other differential combinations of ^{13}C and ^{15}N isotopes in the mass tags, resulting in low mass differences (6.3 mDa) for some of the tags and requiring high resolving power for quantification [14].

Reductive methylation of amines using formaldehyde and sodium cyanoborohydride allows for the isotopic labeling of amines using relatively inexpensive reagents [7, 15–17]. The chemical reaction is efficient and can be conducted in a standard biochemical laboratory, without the need for special equipment. Primary amines are converted through a two-step process into tertiary amines with two methyl (Me) groups (Figure 1). Secondary amines, such as peptides with N-terminal proline residues, are converted into tertiary amines with a single Me group. Because proline residues are extremely rare on the N-terminus of tryptic peptides, nearly all peptides with a free N-terminal amine are labeled with pairs of methyl groups, leading to the term "dimethyl labeling" to refer to this approach, although the term "reductive methylation" is technically more accurate because it includes the addition of one Me group to secondary amines.

Depending on the isotopic forms of formaldehyde and sodium cyanoborohydride/deuteride, five different isotopic forms of the peptide can be generated, each differing by 1 Da per Me group incorporated (Figure 1). However, all peptides isolated from biological sources have naturally occurring stable isotopes such as ^{13}C, ^{2}H, ^{18}O, and ^{15}N, and also ^{34}S for those peptides containing Cys and/or Met residues. Thus, many peptides labeled with only one or two Me groups will show overlap between the different isotopic forms of the Me tags and the naturally occurring stable isotopes. Unless the contribution from the naturally occurring stable isotopes is taken into account, the relative quantitation will not be accurate.

To reduce the problem with peak overlap, some studies that used reductive methylation of amines included only two- or three-plex sets of tags [15, 18]; with three-plex tags it is possible to achieve mass shifts of 4 Da for each pair of methyl groups incorporated. For the mass range of tryptic peptides, which averages 8.4 residues [19], a 4 Da mass shift is sufficient to resolve the methylated peaks from the natural isotopes of most peptides. For proteomic applications, cleavage of proteins with Lys-C instead of trypsin produces peptides with two labeling sites, the N-terminal α-amine and the C-terminal ε-amine. These peptides incorporate four Me groups, allowing for accurate quantitation of most peptides when used with four-plex [20] or five-plex [21] reductive methylation labels. However, peptidomic studies do not usually digest the peptides with enzymes and instead are focused on the native molecules present in a biological sample. Compared with tryptic peptides, the mass range of endogenous peptides is much wider and there are no constraints for the N- and C-terminal residues. Therefore, the five-plex protocol for peptidomics results in mass differences of 1 Da for peptides with N-terminal proline and no internal lysine residues, and 2 Da for peptides with a single primary amine. Because peptides with a single primary amine represent approximately half of all peptides detected in peptidomics studies on human cell lines [22], deconvolution of the peaks to account for natural isotopic abundance is necessary for accurate five-plex quantitative peptidomics using reductive methylation.

Some mathematical and computational approaches have been used to resolve the overlapping of labeled peptides for quantitative proteomics [23–25]. Yoon and colleagues [25] developed quadratic equations to account for isotopic overlaps in dimethyl labeling in the N-terminal amines of peptides.
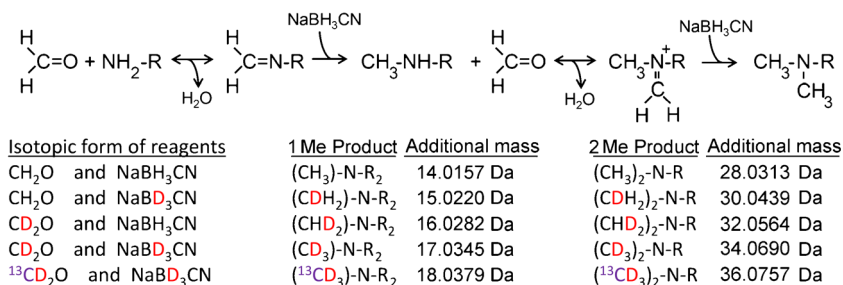

Isotopic form of reagents	1 Me Product	Additional mass	2 Me Product	Additional mass
CH_2O and $NaBH_3CN$	(CH_3)-N-R_2	14.0157 Da	$(CH_3)_2$-N-R	28.0313 Da
CH_2O and $NaBD_3CN$	(CDH_2)-N-R_2	15.0220 Da	$(CDH_2)_2$-N-R	30.0439 Da
CD_2O and $NaBH_3CN$	(CHD_2)-N-R_2	16.0282 Da	$(CHD_2)_2$-N-R	32.0564 Da
CD_2O and $NaBD_3CN$	(CD_3)-N-R_2	17.0345 Da	$(CD_3)_2$-N-R	34.0690 Da
$^{13}CD_2O$ and $NaBD_3CN$	$(^{13}CD_3)$-N-R_2	18.0379 Da	$(^{13}CD_3)_2$-N-R	36.0757 Da

Figure 1. Reductive methylation reaction scheme. Formaldehyde reversibly forms a Schiff's base with primary (left) and secondary amines (middle). When sodium cyanoborohydride is present, the imine group is reduced to the amine. Of the three hydrogens in each methyl (Me) group added by this reaction, two are provided by the formaldehyde and one by the cyanoborohydride. Therefore, the indicated combination of light and/or heavy forms of each reagent will result in products that differ by 1 Da per Me group. Because most peptides have free primary amines, they incorporate two Me groups per amine. Only peptides with secondary amines, such as N-terminal prolines, will incorporate an odd number of Me groups, with one Me group if no internal lysines and two more Me groups for each internal lysine

Cappadona and colleagues [23] used the model of isotopic distribution of Senko and colleagues [26] to calculate and correct the isotopic overlaps for dimethyl labeling. However, these previous works were limited to labels on a single primary amine and to three-plex dimethyl labeling. Dasari and colleagues also used a model of isotopic distributions to account for isotopic overlaps, but it is only applicable to determine the extent of peptide deamidations [24]. In the present study, we developed a simple model to adjust isotopic overlapping based on cubic polynomial equations. Differently from the previous works, which were restricted to three-plex dimethylation, the equations we developed can model the isotopic overlaps of all five channels of reductive methylation. In addition, the set of equations are general and can be applied to any number of reductive methylations from 1 to 9. We tested the equations with data from an experiment in which HEK293T cells were treated with vinblastine or control medium lacking the drug. Vinblastine is an anticancer drug that destabilizes microtubules [27]. It was not predicted to have a major effect on the level of intracellular peptides, which are primarily generated by proteasome activity. However, mice that are defective in the enzyme named Nna1/CCP1, which modifies tubulin post-translational modifications, have greatly elevated levels of many intracellular peptides [28, 29]. Furthermore, two proteasome inhibitors that are anticancer drugs (bortezomib and carfilzomib) also elevate many intracellular peptides, whereas several other proteasome inhibitors which are not used as drugs fail to produce this effect [30–32]. Therefore, it was conceivable that vinblastine would alter the cellular peptidome, and this was tested in the present study.

The equations developed in our study are able to correct the quantitative results for the peptide mass range used in peptidomics. Because these equations were designed using the basis of mass shifts and peptide mass isotopic distribution, in principle this approach can be applied to any isotopic labeling protocol for quantitative proteomics and peptidomics.

Experimental

Cell Culture

HEK293T cells, obtained from American Type Culture Collection, were cultured in 150 mM dishes as previously described [30]. Altogether 10 plates of cells were used for the experiments, all plated at the same time from the same stock of cells and grown to the same confluency (~70%–80% confluent). Medium was removed and the cells were briefly washed in serum-free Dulbecco's Modified Eagle's medium (DMEM). Five of the plates were incubated for 3 h in serum-free DMEM containing 100 nM vinblastine diluted 1:10,000 from a 1 mM stock solution in DMSO. The other five plates were incubated for the same length of time in serum-free DMEM containing a comparable amount of DMSO (i.e., 0.01%). After 3 h incubation, the media were removed, cells were scraped from the plate with phosphate buffered saline, pelleted by low-speed centrifugation ($800 \times g$ for 5 min), and the cell pellets combined with

1 mL of 80 °C water and incubated for 20 min in an 80 °C water bath. After heating, the homogenates were centrifuged ($13,000 \times g$, 30 min, 4 °C) and frozen at –80 °C. To extract peptides, samples were thawed and centrifuged again, as above. The supernatant was cooled in an ice bath, acidified with HCl (final concentration 10 mM), incubated 15 min on ice, and centrifuged at $13,000 \times g$ for 30 min at 4 °C. The supernatant was removed and stored at –80°C until labeling.

Isotopic Labeling of Peptides by Dimethylation

The following procedure for reductive methylation was adapted from several published procedures [7, 33] and the modified procedure is described in more detail (Sayani Dasgupta, Leandro M. Castro, Alexandre K. Tashima, Lloyd D. Fricker: Quantitative peptidomics using reductive methylation of amines. Methods in Molecular Biology, Springer, in press 2018). In brief, samples were thawed at room temperature, 100 µL of 1 M sodium acetate buffer pH 6.0 was added, and the pH was checked with indicator paper. All of the following steps were performed in a ventilated fume hood, and all reagents were purchased from Sigma Aldrich. An 8 µL aliquot of freshly prepared 4% formaldehyde solution (light, CH_2O; intermediate, CD_2O; or heavy, $^{13}CD_2O$) was added, according to Figure 1. Immediately after the addition of formaldehyde, 8 µL of freshly prepared $NaBH_3CN$ 0.6 M or $NaBD_3CN$ 0.6 M was added, according to Figure 1. The samples were mixed, the pH was checked with indicator paper, and adjusted with 0.1 M HCl or NaOH to 6.0. After 2 h incubation at room temperature, the addition of formaldehyde and sodium cyanoborohydride was repeated, samples were mixed, the pH was adjusted to 6.0 (if necessary), and incubated overnight at room temperature. The reaction was quenched by the addition of 200 µL of freshly prepared 1% ammonium bicarbonate, mixed, and incubated for 2 h at room temperature. Then, 100 µL of 5% formic acid was added to each sample, mixed, and incubated for 10 min. Five samples labeled with different combinations of reagents (Figure 1) were combined, as described in Figure 2. In the first labeling set, the first three channels were from vinblastine treated samples, whereas the last two were from controls. In the second set, the first three channels were from controls, whereas the last two were from vinblastine treated samples. In total, five biological replicates of each condition were analyzed in two LC-MS/MS runs.

Peptides were separated from proteins using centrifugal filter units with a 10 kDa molecular weight cut-off cellulose membrane (Amicon Ultra-4 Centrifugal Filter unit with Ultracel-10 membrane; Millapore, Sigma). After centrifugation at $2300 \times g$ for 30 min, the flow-through was recovered and desalted on a C18 reverse phase spin column (Pierce, ThermoFisher). The C18 eluate was dried in a vacuum centrifuge and stored frozen until analysis on LC/MS, at which time it was reconstituted in 10 µL water.
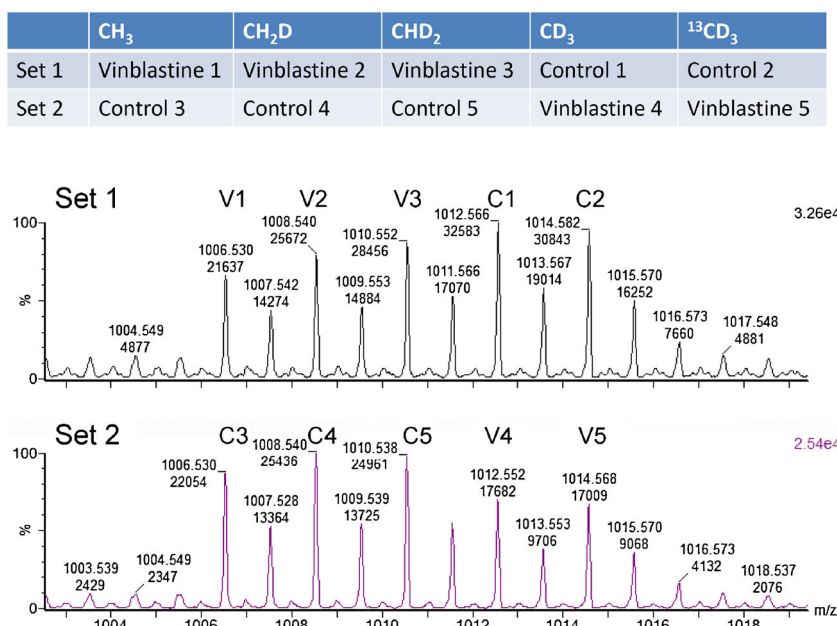
	CH$_3$	CH$_2$D	CHD$_2$	CD$_3$	^{13}CD$_3$
Set 1	Vinblastine 1	Vinblastine 2	Vinblastine 3	Control 1	Control 2
Set 2	Control 3	Control 4	Control 5	Vinblastine 4	Vinblastine 5

Figure 2. Labeling scheme used in the present study and representative data showing overlap between the natural isotopes and the heavy forms of Me groups. Ten plates of HEK293T cells were randomly divided into two groups. Half were treated with serum-free medium containing 100 nM vinblastine (V) in 0.01% DMSO, and the others were treated with medium containing 0.01% DMSO (control; C). After 3 h of incubation at 37 °C, medium was removed, cells were washed, and peptides extracted. To generate peptides with the indicated composition of light and/or heavy isotopes in each Me group, the samples were labeled using the reagent combinations described in Figure 1. Following labeling and quenching of the reagent, as described in Methods, five replicates were pooled to create two sets of samples as indicated. Note that the two sets have each label reversed for control and treated groups; this is done to control for potential problems in the labeling efficiency of one set of reagents. Each of the two sets was analyzed on LC/MS. Representative data shows a peptide with monoisotopic mass 977.47 Da (after subtraction of the mass of the added Me groups) that was labeled with two Me groups, determined from the mass difference of the peaks. This peptide was not identified by MS/MS analysis

Mass Spectrometry

The LC-MS/MS experiments were performed on a Synapt G2 mass spectrometer coupled to a nanoAcquity capillary liquid nano-chromatography (LC) system (Waters, Milford, MA, USA). The peptide mixture (5 μL) was desalted online for 5 min at a flow rate of 8 μL/min of phase A (0.1% formic acid) using a Symmetry C18 trapping column (5-μm particles, 180-μm inner diameters, 20-mm length; Waters). The mixture of trapped peptides was subsequently separated by elution with a gradient of 7%–65% of phase B (0.1% formic acid in acetonitrile) through a BEH 130 C18 column (1.7 μm particles, 75 μm inner diameter, 200 mm length; Waters) in 60 min. The data were acquired in the data-dependent mode and the MS spectra of multiple-charged protonated peptides generated by electrospray ionization were acquired for 0.3 s from m/z 300–1600. The three most intense ions exceeding base peak intensity threshold of 2500 counts were automatically selected and dissociated in MS/MS by 15- to 60-eV collisions with argon for 0.3 s. The typical LC and electrospray ionization conditions consisted of a flow rate of 250 nL/min, a capillary voltage of 3.0 kV, a block temperature of 70 °C, and a cone voltage of 50 V. The dynamic peak exclusion window was set to 90 s.

MS spectra were analyzed using the MassLynx 4.1 software (Waters). Peak groups representing peptides labeled with different isotopic labels were identified (as in Figure 2) and spectra corresponding to the elution profile were combined for quantitation (as in Figure 3). The relative intensity of each monoisotopic peak was entered into the spreadsheet. The adjusting equations used to account and correct isotopic overlapping of labeled peaks are described in the next section. To quantify relative peptide levels between the vinblastine-treated and control replicates, the adjusted peak intensity of each treated group was compared with the average of the control replicates in each experiment.

To identify peptides, MS/MS data were analyzed using both the Mascot search engine (Matrix Science Ltd, UK) and Peaks Studio 7.5 (Bioinformatics Solutions Inc., Canada); these two approaches gave largely the same results, but each found a small number of unique peptides. Databases searched included IPI_human (91,464 sequences; 36,355,611 residues) and human SwissProt (92,904 sequences). No cleavage site was specified. Modifications included the reductive Me labels and also N-terminal protein acetylation, methionine oxidation, and cyanylation of Cys (which was not detected in the present study, but was found in previous studies that used TMAB tags). The same parameters were used for Peaks Studio for de novo analysis of MS/MS data and database search. All search results were manually interpreted to eliminate false positives, as described [11, 34]. In brief, the criteria included (1) observed
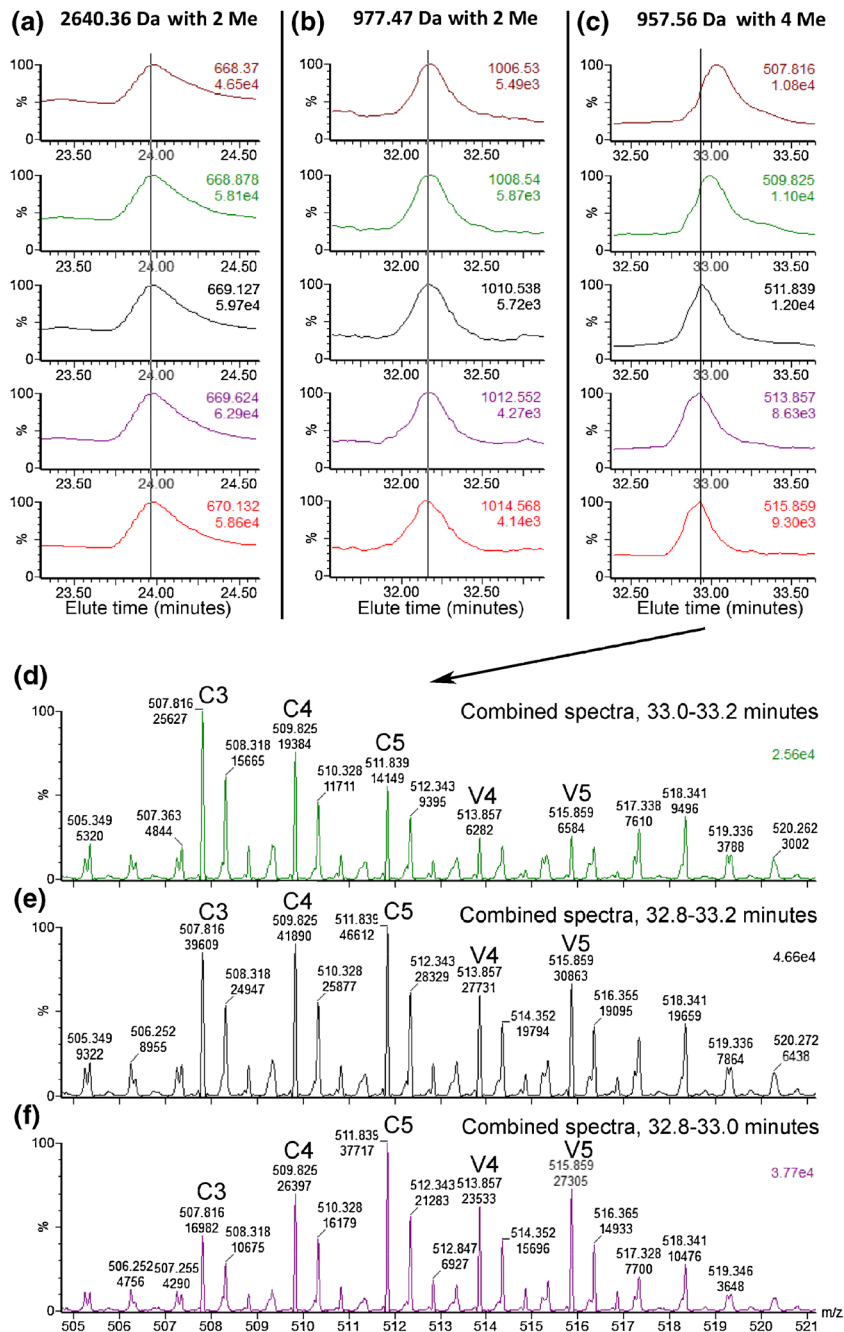
Figure 3. Representative data showing that some, but not all peptides co-elute from reverse phase LC columns. **a–c**: Elution profiles of the five labeled forms of different peptides, with top panels showing peptides labeled with the lightest form of the labels and bottom panels showing peptide labeled with the heaviest form. (**a**): Ions with *m/z* of 668.37 to 670.13, representing the 4+ ions of a 2640.36 Da peptide labeled with two Me groups. This peptide was identified by MS/MS analysis as an internal fragment of vimentin (sequence SLGSALRPSTSRSLYASSPGGVYATR). (**b**): Ions with *m/z* 1006.53 to 1014.57, representing the 1+ ions of a 977.47 Da peptide labeled with two Me groups. This peptide was not identified from MS/MS analysis, and is the same peptide shown in Figure 2. (**c**): Ions with *m/z* 507.82 to 515.86, representing the 2+ ions of a 957.56 Da peptide labeled with four Me groups. This peptide was identified by MS/MS analysis as a C-terminal fragment of FK506 Binding Protein (sequence DVELLKLE). Note that the light and heavy forms of the peptides in panels a and b co-elute, whereas the heavier forms of the peptide in panel c elute slightly ahead of the lighter forms. **d–f**: MS spectra of different subsets of the peak shown in panel c, representing either the second half (33.0 to 33.2 min; panel d), the first half (32.8 to 33.0 min; panel f), or the entire range of all forms (32.8 to 33.2 min; Panel e). Note that the relative peak heights of each form of Me group varies considerably among the spectra, and the full range (i.e., panel e) shows the smallest variation among the triplicates of the control-treated cells, using the numbering scheme shown in Figure 2. The y-axes in all panels show the relative intensity of the signal

mass within 40 ppm (preferably 20 ppm) of the theoretical mass; (2) the number of Me tags observed matched the number of free amines available (i.e., Lys residue and N-terminus if not acetylated); (3) the observed charge state(s) of the peptide was consistent with the expected number of positive charges; (4) ≥80% of the major fragments observed in MS/MS matched b or y ions, with a minimum of five matches. The mass window for MS spectra for both Mascot and Peaks searches was typically set as 1.1 Da, and only search results that were within 0.1 Da of either 0 or ±1.0 Da were further considered by manual inspection. This was done because our preliminary searches found that ~30% of the MS/MS spectra did not correspond to the monoisotopic peak of the peptide with one of the five isotopic forms of the Me tags, and instead corresponded to a peptide that had one or two heavier natural isotopes (not considering the isotopes in the Me tags). Thus, to maximize the results, it was important to use a wide window of the Mascot or Peaks searches and then manually check those peptides "off" by 1 Da to see if the computer had incorrectly assigned their monoisotopic masses, as described [35].

Modeling of Peptide Isotopic Overlapping

The simulations of isotopic peak profiles were performed in the MS-Isotope module of Protein Prospector (http://prospector.ucsf.edu) with the sequences of 26 peptides identified in the peptidome of HEK293T cells, covering the mass range of 0.6–5 kDa (Supplementary Table S1). We consider the overlapping intensities up to the 9th isotope, designating the monoisotopic peak of each peptide as the 0th isotope. The overlapping peaks in peptidomics using reductive methylation depends on the combination of light/heavy formaldehyde and cyanoborohydride used (up to five combinations of sample groups) and on the number of Me groups incorporated into each amine group. We derived formulas to adjust the relative peak intensity and subtract the natural isotopic composition of the peptides containing reductive methylations on primary or secondary amines, up to nine reactions. We considered two Me for the ε-amine of lysines and for all N-terminal primary amines, and one Me for proline that has a secondary amine.

For consecutive samples, the isotopic overlapping contributions have a cumulative effect. For instance, for two Me in a primary amine, the first sample ($i = 0$) has no overlap, the second sample peak ($i = 1$) has the overlap of the 2nd isotope of sample "0", the third has the overlap of the 2nd isotope of the sample "1" plus the 4th isotope of sample "0", and so on. Therefore, the general equation for overlapping intensity calculations is:

$$I_{i,j} = \sum_{l=0}^{n=i} I_{l,j,k=(i-l)j} \qquad (1)$$

where $I_{i,j}$ is the observed peak intensity and $I_{l,j,k}$ is the individual peak contribution. The indexes i and l are related to sample group (i,l = 0 to 4) and j and k are number of Me (j = 1 to 9) and isotopic peak, respectively. A detailed example of how to compute intensities with Equation 1 is given in the

Supplementary text. The equations to adjust peak intensities based on the observed peptide mass (M, in Da) are:

$$S_{i,j} = \frac{I_{i,j}}{k_{i,j}} - I_b \qquad (2)$$

where $S_{i,j}$ is the adjusted intensity of sample i with j reductive methylations, $k_{i,j}$ is an adjusting factor, and I_b is the background intensity. The adimensional adjusting factor $k_{i,j}$ is given by the polynomial equation:

$$k_{i,j} = a_{i,j} + b_{i,j}M + c_{i,j}M^2 + d_{i,j}M^3 \qquad (3)$$

and $a_{i,j} - d_{i,j}$ are adjusted constants based on the isotopic distribution calculations; $k_{i,j}$ values were fitted to simulated data of $I_{i,j}$ with polynomial fit in OriginPro 2016 (Originlab, Northampton, MA, USA). We resolved the real roots of Equation 3 for each set of Me with scripts in Scilab 6.0.0 (Scilab Enterprises, www.scilab.org) to find the peptide masses giving theoretical deviation of 2.5% from expected 1:1 ratios due to isotopic overlapping (script in the Supplementary text).

Results

HEK293T cell extracts treated either with vinblastine or control medium were labeled with one of five combinations of isotopic forms of formaldehyde and sodium cyanoborohydride, as shown in Figure 1. Altogether five replicates of vinblastine-treated cells and five replicates of control cells were labeled and the 10 samples analyzed on two LC/MS runs using the scheme shown in Figure 2. Signals of unlabeled peptides containing free amines were not detected in our LC-MS/MS experiments, suggesting that the efficiency of the labeling was very high. Representative data for a peptide of approximately 1 kDa that was labeled with two Me groups shows a noticeable difference in peak intensity between the CH_3- and CH_2D-labeled peaks (Figure 2). This difference in peak intensity is consistent with the expected overlap due to naturally occurring ^{13}C and other isotopes. With the established maximum theoretical ratio deviation of 2.5%, we solved the cubic $k_{i,j}$ equations and found the respective peptide masses up to $j = 9$ (number of Me). The results show that we need the adjusting model equations for the entire mass range for 1–3 Me, while they are not needed for peptide masses below 1315.8 Da for 4 Me, 2048.4 Da for 5 Me, 2694.2 Da for 6 Me, 3128.1 Da for 7 Me, 3673.4 for 8 Me, and 4220.0 Da for 9 Me.

The difference in peak intensity for the isotopic forms in the representative data shown in Figure 2 is not due to differential co-elution of the heavy and light forms of this peptide; these forms of this peptide show identical elution profiles from the reverse phase column (Figure 3). Most other peptides detected in the study were also found to show co-elution of the heavy and light forms. However, small peptides that were labeled with four or more Me groups tended to show slight differences in the elution time from the LC column (Figure 3c). For example, the 957.56 Da peptide labeled with four Me groups

elutes with a peak at 33.1 min for the lightest form and at 32.9 min for the heaviest form (Figure 3c). As a result, if the peak is integrated over 33.0 to 33.2 min, there is a greater contribution from the lighter forms, and from 32.8 to 33.0 min a greater contribution from the heavier forms, compared with integration over the entire range of 32.8 to 33.2 min (Figure 3d–f). Thus, it is important to integrate the peak over the entire elute range for both the lightest and heaviest forms of peptides in order to insure accurate quantitation of peptides labeled with a large number of Me groups. Peptides that incorporate fewer Me groups (due to the absence of lysine residues) are less of a problem with co-elution issues, but have a much greater problem with peak overlap (e.g., the representative data shown in Figure 2).

Isotopic Overlapping Calculation

To address the problem of peak overlap due to naturally occurring isotopes, we simulated the isotopic intensities of 26 peptides identified in the HEK293T cell extracts (Supplementary Table S1). For this, the program MS-Isotope was used (on-line through the ProteinProspector site). This program considers the natural abundance of ^{13}C, ^{2}H, ^{15}N, ^{18}O, ^{33}S, ^{34}S, and ^{36}S in the peptides, based on the amino acid composition. The results from these 26 representative peptides were then used as reference values for the overlapping model calculations. The most significant overlapping effects are for the higher isotopic labels, fewer number of Me (lower mass differences), and higher peptide masses. The lowest possible nominal mass difference among labeled peptides is 1 Da, resulting from a single methylation ($j = 1$) of peptides that do not contain Lys residues and have a Pro on the N-terminus. The heaviest isotopic label in the series is from ^{13}CD$_3$ substitutions ($i = 4$). For the heaviest peptide mass considered in the model (5 kDa), supposing there was only one single ^{13}CD$_3$ substitution and 1:1:1:1:1 ratios for the peptides in each sample, we observe a theoretical $I_{4,1}:I_{0,1}$ ratio 13.8 times higher than the expected 1:1 (Figure 4, Supplementary Table S1). Even for the lowest peptide mass in the range (0.6 kDa), there is a 51% relative intensity increase due to overlapping for $i = 4$ and $j = 1$. As the number of Me on primary and secondary amines in the peptides increases, the isotopic overlapping effects decrease because of the higher mass shifts and lower abundances of higher isotopes. Considering a 5 kDa peptide with 2, 3, and 4 ^{13}CD$_3$ substitutions ($i = 4$), we observe $I_{4,j}:I_{0,j}$ ratio deviations by 8.6, 5.7, and 4.0 times, respectively (Figure 4). At 5 kDa, there are significant deviations of the 1:1 ratio up to 9 Me, where the ratio increases 7% due to overlapping (Supplementary Table S1).

The adjustments of isotopic overlapping with polynomial fits up to the third-order resulted in coefficient of determination values (R^2) for the polynomials > 0.99 for all equations (Table 1). Application of the polynomial models to correct the ratios to the expected 1:1:1:1:1 ratios resulted in root mean square deviations (RMSD) ranging from 0.1% to 3.3% with average of 2.0% (Table 1).

To test these adjustment formulas with experimental data, we focused on the first three-plex channels in the study comparing vinblastine-treated and control cells. In both LC/MS runs, the first three-plex channels represented identical replicates, either vinblastine-treated cells in Run 1, or control medium-treated cells in Run 2. This way, both runs could be used, without complications from the effect of the drug treatment on peptide levels. In theory, the ratio of the 2nd and 3rd channels to the 1st channel ($i = 0$) should be 1:1 but this was only found for small peptides with two or more Me groups (Figure 5). Peptides that incorporated a single Me group (due to a N-terminal Pro residue, and not incomplete labeling of primary amines) showed ratios of 1.25–1.50, even for peptides below 1 kDa (Figure 5). After adjustment with the formula based on the theoretical isotopic distribution, the ratios were very close to 1.00 (Figure 5, green bars). Peptides that incorporated two Me groups showed ratios close to 1.00 for peptides below 1 kDa, and got progressively larger for peptides of higher mass, with those in the 4.0–4.5 kDa range showing ratios of >5 (Figure 5). The adjustment formulas brought all ratios very close to the theoretical 1.00 ratio (Figure 5, green bars). Peptides that incorporated three Me groups (e.g., N-terminal Pro and one internal Lys) also required correction, mainly for peptides above 1.5 kDa, and the correction formulas brought the ratios close to 1.0. There were relatively few peptides in this group, compared with those that incorporated an even number of tags, which is consistent with the low fraction of peptides with N-terminal Pro. Thus, the error bars for the peptides with three Me groups (and also one Me group) are larger than for those that incorporated two or four Me groups, and one mass range (4.5–5.0 kDa) had only a single peptide and therefore no error bars were possible (Figure 5). Peptides that incorporated four Me groups were generally very close to 1.00 ratios for peptides below 2.0 kDa, and the largest mass detected for this set (3.5–4.5 kDa) showed ratios close to 3 (Figure 5). As with the other sets of peptides, the adjustment formulas brought the ratios close to the theoretical 1.00 ratio (Figure 5, green bars).

The analysis described in Figure 5 focused only on the identically treated triplicate replicates in each LC/MS run (i.e., vinblastine-treated or control medium-treated). Because the adjustment formulas were able to bring the observed ratio of these identically treated triplicate replicates to within several percent of the theoretical ratio of 1.0, we could use this approach to examine if vinblastine treatment caused a change in the levels of peptides. For this, the relative level of a peptide in each of the five biological replicates was determined after applying the appropriate adjustment formula based on the number of Me groups incorporated (Supplementary Table S2). It was possible to examine relative levels of peptides that had not been identified by MS/MS, either because there were no data (i.e., the peptide was not selected for CID) or the data were not of sufficient quality for identification; the number of Me groups was determined from the MS spectra and did not depend on peptide identification. The relative levels of every peptide detected in MS spectra are shown in Supplementary
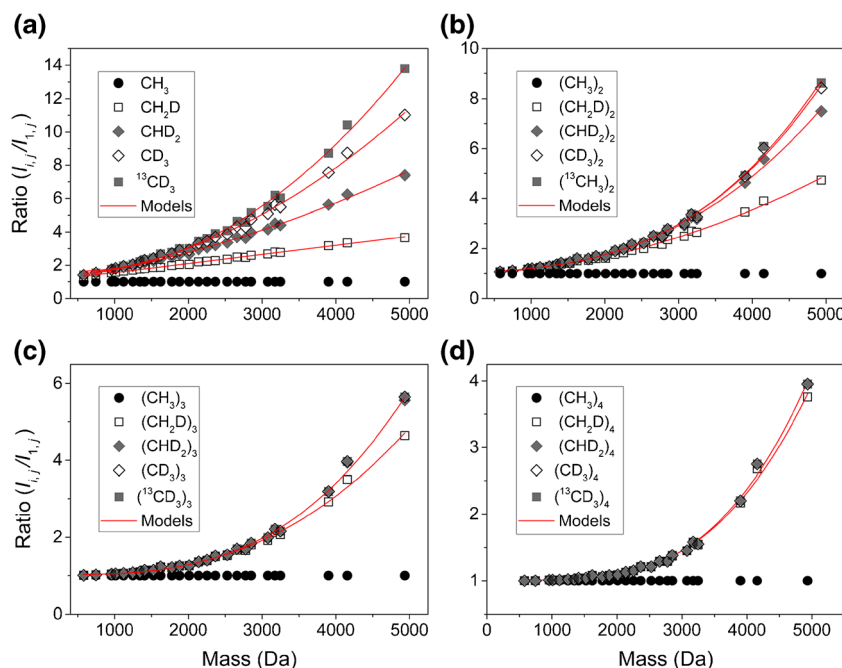
Figure 4. Relative levels of peptides in the 0.6–5 kDa mass range in HEK293T cells for the five-plex reductive methylation simulated in MS-Isotope (symbols) and adjusted by polynomials (—). The 0th isotopes of the first label (CH_3) are unaffected by peak overlap and are used as the references for quantitation, with theoretical ratios defined as 1. For peptides labeled with one Me group (panel **a**) or two Me groups (panel **b**), four equations are used for modeling, one for each isotopic label, as the overlapping contributions are cumulative and significant up to the last label. For peptides labeled with thee Me (panel **c**) or four Me groups (panel **d**), only two equations are needed. For example, for 3 Me the 4th peak of CH_3 overlaps with the 0th of CH_2D, needing one adjusting equation. The CHD_2 has the contribution of the 4th peak from CH_2D plus the 7th from CH_3, needing a second adjusting equation. The CD_3 has the contribution of the 4th peak from CHD_2 plus the 7th from CH_2D plus the 10th peak from CH_3, but this last contribution is already negligible. The heavier labels have the same overlapping behavior, thus the second equation in this case is valid for the remaining labels. Accordingly, for 5 Me and above, only one equation is needed

Table S2. The relative level was calculated by dividing the observed peak intensity (after correction using the formula) by the average of all control replicates in that run (i.e., two control replicates in Run 1, three control replicates in Run 2—see Figure 2 for labeling scheme, and Supplementary Table S2 for data). The overall trend was for a decrease in peptides upon vinblastine treatment, with the mean vinblastine:control ratio of 0.87 for all 642 ion sets that could be quantified (Supplementary Table S2). Some of the 642 ion sets listed in Supplementary Table S2 were only detected in one of the two LC/MS runs, and therefore had fewer than five replicates of each treatment group—these were not further considered in our

Table 1. Adjusting factor $k_{i,j}$ equation constants for each sample ($i = 1–4$, 0 is refers to the 0th sample, not affected by isotopic overlapping) and number of reductive methylations per peptide ($j = 1–9$)

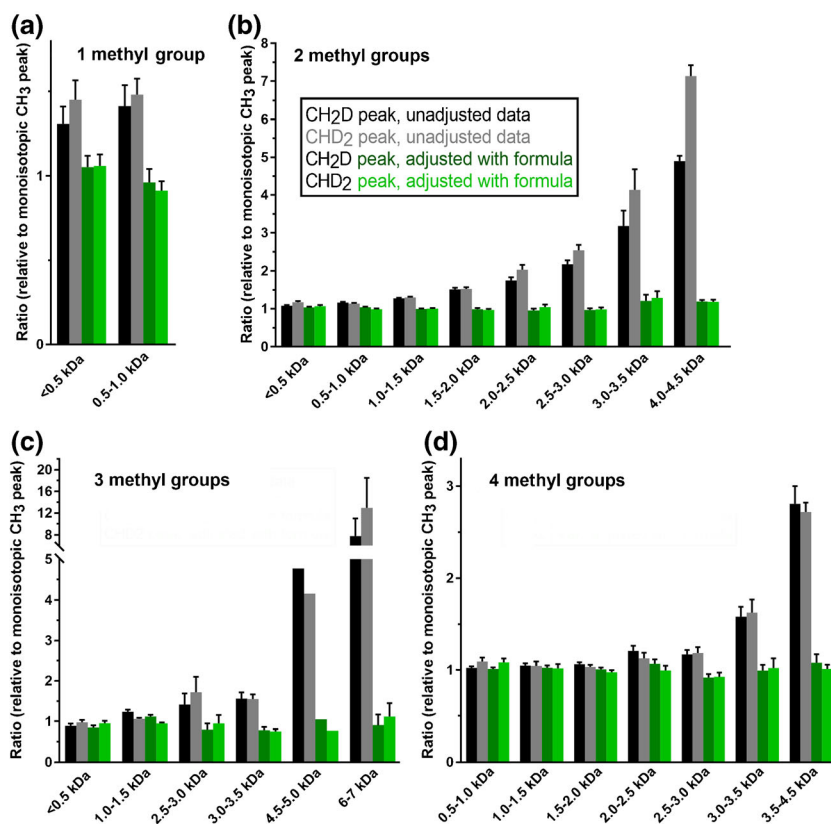| i	j	ΔM (Da)	$a_{i,j}$	$b_{i,j}$ ($\times 10^{-4}$)	$c_{i,j}$ ($\times 10^{-7}$)	$d_{i,j}$ ($\times 10^{-11}$)	R^2	RMSD (%)
1	1	1	1.0118	5.4639	0	0	0.9975	1.2
2	1	2	1.1934	2.9344	2.7551	−1.5622	0.9972	2.1
3	1	3	1.3020	1.2135	3.6862	0.2500	0.9967	2.9
4	1	4	1.2938	1.9533	2.7868	4.1206	0.9965	3.3
1	2	2	0.9907	0.4541	1.4830	0	0.9965	2.0
2	2	4	1.0890	−0.4493	1.2870	2.9970	0.9962	3.0
3	2	6	0.9930	1.6150	−0.0395	5.6970	0.9965	3.0
4	2	8	0.9580	2.3290	−0.4602	6.4410	0.9967	2.9
1	3	3	1.1039	−1.6446	0.8922	1.8709	0.9954	2.6
2–4	3	$3i$	1.0118	3.7006	−0.4143	4.5436	0.9961	2.6
1	4	4	0.9843	0.8273	−0.9258	3.8881	0.9952	2.1
2–4	4	$4i$	0.9470	1.5707	−1.3530	4.6344	0.9956	2.0
1–4	5	$5i$	0.9045	2.2550	−1.6112	3.8818	0.9960	1.5
1–4	6	$6i$	0.9027	2.1087	−1.3524	2.7400	0.9942	1.1
1–4	7	$7i$	0.9379	1.2976	−0.7959	1.5028	0.9933	0.7
1–4	8	$8i$	1.0218	0.6101	−0.4863	0.8763	0.9958	0.3
1–4	9	$9i$	0.9101	1.0811	−0.4279	0.5598	0.9959	0.1

Figure 5. Variation of peak intensity of experimental data before and after correction with the formula. For this analysis, identified and unidentified peptides that were labeled with one–four Me groups were considered. The peak intensity of peptides labeled with CH_2D and CHD_2 were compared with the monoisotopic peak labeled with CH_3. Because both sets of data included identically treated replicates for these three groups (either all vinblastine-treated in set 1 or control medium-treated in set 2), it was possible to combine data from both sets, thereby doubling the number of peptides in the analysis. Black bars show the ratio of the CH_2D peak to the CH_3 peak, grey bars show the ratio of the CHD_2 peak to the CH_3 peak. In theory, all ratios should be 1:1, but due to natural isotopes the ratios are 1.3 to 1.5 for all peptides labeled with one Me group detected in the study (top left panel a). After application of the formula to adjust the relative levels of each peptide (dark and light green bars), the ratios are within 10% of the expected 1.00 value. For peptides labeled with two Me groups (top right panel b), the lowest mass group (<0.5 kDa) is within 10%, but those in the 0.5 to 1.0 kDa range are 15% higher, and with each increasing mass range the experimentally observed ratios are dramatically elevated. Application of the adjustment formula brings the ratios within 10% of the theoretical 1.00 ratio for all peptides below 3.0 kDa. Similar analysis of peptides labeled with three Me groups (lower left panel c) or four Me groups (lower right panel d) show increasing observed ratios with mass of the peptide, and correction with the adjustment formula. Error bars show standard error of the mean. The number of peptides in each set are: **(a)** one Me group n = 4, 6; **(b)** two Me groups n = 23, 61, 99, 59, 40, 25, 7, 2; **(c)** three Me groups n = 4, 4, 2, 4, 1, 7; **(d)** four Me groups n = 34, 22, 46, 17, 24, 12, 6, with numbers referring to the groups indicated in the panel (left to right)

analysis. Others showed variability among the control-treated replicates that was greater than the range of values observed in the vinblastine-treated replicates—these were also not further considered in our analysis. Furthermore, some peptides could not be accurately quantified due to co-elution with another ion that had a similar *m/z* as one or more of the Me forms of the peptide—these were also not further considered in our analysis. Altogether, 22 peptides met these criteria and Student's *t*-test was used to determine if the vinblastine-treated replicates were statistically different from the control-treated replicates (Figure 6). All of these 22 peptides showed a statistically significant change, most of them decreased by vinblastine treatment, which is consistent with the overall trend observed with all peptides. Only seven of these peptides were identified by MS/

MS sequencing; for the others, the mass is indicated in Figure 6 so that it can be cross-referenced with the data in Supplementary Table S2. The identified peptides are from proteins that were previously found to be represented in the HEK293T cell peptidome, such as 40S ribosomal protein S21 and S28, peptidylprolyl isomerase A, RNA binding motif protein 3, protein QIL1, and splicing factor arginine/serine-rich 2. For some of these proteins, multiple peptides were detected in the peptidomic analysis, and not all peptides derived from the protein were significantly altered upon vinblastine treatment. For example, the two peptide fragments of peptidylprolyl isomerase A that are shown in Figure 6 (DIAVDGEPLGRVSF and ELFADKVPKTAENFRAL) have vinblastine:control ratios of 0.73 and 0.77, respectively, whereas the other 10
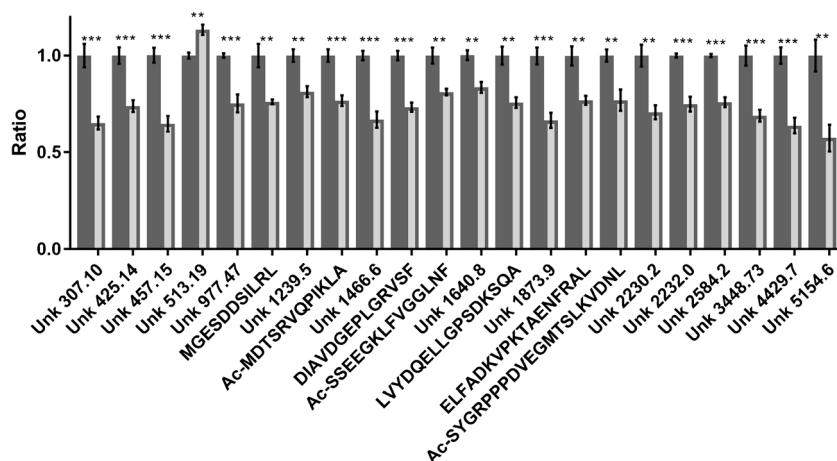
Figure 6. Effect of vinblastine treatment of relative levels of selected peptides in HEK293T cells. Peptides detected in both LC/MS runs that could be quantified in both runs (e.g., no peak overlap with co-eluting ions) were considered, so that five values of vinblastine-treated cells could be compared with an equal number of control replicates. Peak intensities were adjusted with the formula appropriate for the number of Me groups, up to eight Me groups; for peptides with a greater number of Me groups, no correction formula was applied. Within each LC/MS run, the control replicates were compared with the average control value, and the vinblastine-treated replicates were also compared with the average control value. Student's t-test (two tailed, unpaired) was used to compare the treated (light grey bar) and control group (dark grey bar) for each peptide. $**$ $p < 0.01$; $***$ $p < 0.001$. Error bars show standard error of the means with n = 5 for all peptides except for the peptide of mass 4429.74, which was detected in three charge states and therefore had n = 15

peptides detected in the present study that were derived from the same protein had an average vinblastine:control ratio of 0.84, which is close to the average of all 642 ion sets (0.87). Therefore, vinblastine treatment appears to affect some peptides more than others, possibly by altering the stability of the observed peptides.

Discussion

Many protocols for quantitative peptidomics and proteomics use chemical tags containing stable isotopes to introduce mass differences between various samples. Reductive methylation with formaldehyde and sodium cyanoborohydride is a simple procedure that uses inexpensive commercially available reagents, and with various combinations can perform five-plex quantitation. However, the major limitation of these tags is the small mass difference between five-plex isotopic forms, with only 1 Da difference per Me group added. Because approximately half of all peptides detected in typical peptidomics or proteomics experiment have a single primary amine, these peptides are labeled with only two Me groups and differ by 2 Da. Although it is possible to calculate the isotopic distribution of the naturally occurring stable isotopes for each peptide identified in a study and subtract the contribution due to the natural isotopes, this is time-consuming and cannot be used for unknown peptides that were detected in MS mode but not sequenced by MS/MS analysis. The major advance of the present study is the development of formulas to correct the contribution from the naturally occurring isotopes. Altogether, nine groups of formulas were created for peptides labeled with one–nine Me groups; beyond this, there is no need to consider

overlap between the naturally occurring isotopes and those from the added Me groups for peptides below 5 kDa.

With the advent of the formulas to subtract the contribution from naturally occurring isotopes, the reductive methylation approach is close to the ideal system for quantitative labeling. Although other approaches such as the isobaric iTRAQ and TMT tags allow for more than five-plex labeling, these reagents are expensive and provide a limited coverage of the peptidome, allowing quantitation only for samples that were selected for MS/MS. In contrast, reductive methylation is similar to other isotopic tags in that peptides are quantified from MS spectra and do not depend on MS/MS spectra. While it is important to eventually identify peptides by MS/MS sequencing, it is possible to perform additional runs targeting specific peptides for MS/MS and focus on those peptides found to show the largest changes. Thus, the ability to quantify both identified and unidentified peptides is an important advantage of the reductive methylation approach over the isobaric iTRAQ and TMT labels. Other isotopic tags such as TMAB also allow for five-plex quantitation of peptides from MS spectra [11]. However, these tags are not commercially available and difficult to synthesize in sufficient purity to avoid problems such as the iodination of His and Tyr residues [12]. Furthermore, TMAB is a quaternary amine that decomposes on MALDI and ion trap mass spectrometers, limiting its usefulness to Q-TOF mass spectrometers [12]. In contrast, peptides labeled by reductive methylation are tertiary amines and can be analyzed on a wide range of mass spectrometers [7, 15–18, 33, 36].

An interesting question is whether high resolution mass spectrometers could solve the problem of isotopic overlaps for reductive methylation. There are indeed small mass differences between the dimethyl tagged peptides from the CH_3 to

the CD_3 compared with the natural abundance isotopes. These mass differences are mainly due to the contribution of deuterium (from the labels) and ^{13}C (naturally occurring, except for one of the labels) and equal 5.8 mDa between subsequent channels for each 2 Me substitution. However, the monoisotopic $^{13}CD_3$ and the 2nd isotope of the CD_3 of each labeled peptide have exactly the same elements, which means their mass difference is zero. Thus, mass spectrometry cannot resolve the overlaps of the $^{13}CD_3$ and the 2nd isotope of the CD_3 for any Me substitution. In regard to the other quantitation channels in which small mass differences are observed, very high resolution is needed to differentiate the labels. For example, consider the 2nd isotope of the 2168.2 Da peptide Ac-HTILLVQPTK(Me)$_2$RPEGRTY, one of the peptides used to derive the equations. Based on the resolving power (given by M/ΔM), the MS resolution for this peptide should be 373,828 FWHM (full width at half maximum) to appropriately separate 5.8 mDa. While the Orbitrap Fusion (500,000 FWHM) or a Fourier Transform Ion Cyclotron Resonance mass spectrometer would be able to resolve these mass differences, typical bottom-up proteomics or peptidomics experiments are not usually performed at such high resolution. Other instruments commonly used for proteomics and peptidomics, such as the Q-Exactive Orbitrap and the Q-Exactive HF (Thermo Fischer Scientific) have maximum resolving powers of 140,000 and 240,000 FWHM, respectively. Therefore, the mass spectrometers present in most laboratories are not able to resolve the deuterated methyl tags from the natural ^{13}C peaks, and our equations are required to address the overlap problem for reductive methylation labels.

A minor drawback to the reductive methylation approach is that for some labeled peptides, the light and heavy forms do not precisely co-elute from reverse phase LC columns. A previous study using reductive methylation to label amino acids and other small amine-containing molecules noted the isotope effect [16], but other studies reported nearly precise co-elution of peptides labeled with light and heavy methyl groups [15, 18, 20]. In the present study, the isotope effect was negligible for the vast majority of peptides, and was only seen for small peptides labeled with four or more Me groups. For those peptides where the heavy and light forms do not precisely co-elute, it is important to combine spectra over the entire elution profile of all forms rather than use a single spectrum or subset of spectra. An alternative would be to use a sharper gradient to elute peptides over a shorter time and thereby provide better co-elution of the isotopic forms of small peptides labeled with multiple Me groups, although this would limit the number of peptides available for MS/MS analysis.

The adjustment formulas were based on 26 representative peptides that are commonly detected in peptidomic analysis of HEK293T cells [22, 30]. Because amino acids differ in the number of H, C, N, O, and S atoms, the amino acid composition will affect the relative intensity of the heavy forms, relative to the intensity of the monoisotopic form. However, for most amino acids, the difference in composition is relatively minor because the major contributor to the heavy peaks is due to ^{13}C,

based on its natural abundance (1.107% of all C atoms) and the large number of C atoms in most amino acids. Although H is more abundant than C in all amino acids, the natural abundance of ^2H (deuterium) is only 0.015% and so this is a minor contributor to the mass. All amino acids also contain at least one N and O, but the natural abundance of ^{15}N and ^{18}O is 0.366% and 0.204%, respectively, so their contribution is less than that of ^{13}C. The one atom with heavy isotopes that have a higher natural abundance than ^{13}C is sulfur. The light form (^{32}S) is 95.02% of the total sulfur, and there are several heavier forms: ^{33}S (0.75%), ^{34}S (4.21%), and ^{36}S (0.02%). Sulfur is only present in two amino acids, Met and Cys, both of which are relatively rare in the peptidome of HEK293T cells (Supplementary Table S2) and also rare in most proteins in UniProtKB/Swiss-Prot database (http://web.expasy.org/docs/relnotes/relstat.html). But because ^{34}S has an abundance of 4.21% and adds 2 Da to the mass of a peptide, peptides high in Met and/or Cys would need to adjust the relative peak intensity based on the actual amino acid composition of the peptide. Of the 26 peptides used in the present study for the derivation of the adjustments, four contained a single Met, one contained two Met, and none contained Cys (Supplementary Table S1). While the general formula derived from these peptides are useful for the rapid deconvolution of data, peptides with many Met and/or Cys residues would benefit from custom analysis. Peptides with multiple disulfide bonds are commonly found among snake and spider toxins [37, 38].

Rather than test the reductive methylation with 10 replicates of untreated HEK293T cells, we decided to combine the evaluation of the technique with an experiment to test the effect of a drug treatment. The choice of vinblastine was based on our previous findings that other anticancer drugs (i.e., bortezomib and carfilzomib) greatly altered the levels of most peptides in HEK293T cells [30, 31]. Although these drugs are proteasome inhibitors and would be expected to lower the levels of peptides that are products of proteasome-mediated cleavages, we unexpectedly found that both of these drugs elevated levels of many peptides [30, 31]. Other proteasome inhibitors were tested and most of them primarily reduced levels of intracellular peptides, as expected [30, 32]. Thus, the two proteasome inhibitors that are successful anticancer drugs seemed to have a distinct ability to elevate levels of intracellular peptides, and therefore we thought it important to test another anticancer drug that works through a completely different mechanism than the proteasome inhibitors. Vinblastine was selected because it disrupts microtubules [27]. A previous study on *pcd* mice, which lack CCP1/Nna1 activity also found large increases in the levels of many intracellular peptides [28, 29]. CCP1/Nna1 is a microtubule-modifying enzyme that removes Glu from the C-terminus of alpha tubulin and/or side chains of alpha and beta tubulin [39–41]. It is not clear how the absence of CCP1/Nna1 in the *pcd* mouse affects levels of intracellular peptides. Protein degradation by macroautophagy is sensitive to microtubule inhibitors such as vinblastine [42]. Therefore, we tested if vinblastine treatment of HEK293T cells altered peptide levels. While some peptides were found to significantly decrease by

~20%–50% (Figure 6), these changes are relatively small compared with the dramatic >5-fold changes seen in the studies examining bortezomib, carfilzomib, or *pcd* mice [28–31]. The mechanism behind the vinblastine-induced decrease in levels of some peptides is not known. Because all peptides derived from the same protein were not similarly affected by vinblastine, it does not appear to be a general change in protein degradation. Instead, it is possible that vinblastine affects the stability of some of the peptides. It has been proposed that the intracellular peptides detected in peptidomics analyses are stabilized by binding to proteins [30].

Conclusions

We applied cubic equations to correct isotopic overlap, thereby enabling the reductive methylation protocol to five-plex quantitative peptidomics. Although it was previously possible to calculate the isotopic contribution for each peptide, based on its amino acid composition, the formulas derived in our study allow for the rapid deconvolution of the data, and can also be applied to unidentified peptides. The low RMSD values of the polynomial fits show that the cubic equations can accurately model the isotopic overlaps. As the equations are based on general peptide mass isotopic distributions and mass shifts, they can be applied to any isotopic labeling protocol. The model can also indicate when overlapping become significant and when the adjusting equations can be neglected.

Acknowledgments

References

1. Gygi, S.P., Rist, B., Gerber, S.A., Turecek, F., Gelb, M.H., Aebersold, R.: Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. Nat. Biotechnol. **17**, 994–999 (1999)
2. Ong, S.E., Blagoev, B., Kratchmarova, I., Kristensen, D.B., Steen, H., Pandey, A., Mann, M.: Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. Mol. Cell Proteomics. **1**, 376–386 (2002)
3. Zhang, R., Sioma, C.S., Thompson, R.A., Xiong, L., Regnier, F.E.: Controlling deuterium isotope effects in comparative proteomics. Anal. Chem. **74**, 3662–3669 (2002)
4. Ross, P.L., Huang, Y.N., Marchese, J.N., Williamson, B., Parker, K., Hattan, S., Khainovski, N., Pillai, S., Dey, S., Daniels, S., Purkayastha, S., Juhasz, P., Martin, S., Bartlet-Jones, M., He, F., Jacobson, A., Pappin, D.J.: Multiplexed protein quantitation in Saccharomyces cerevisiae using amine-reactive isobaric tagging reagents. Mol. Cell. Proteomics. **3**, 1154–1169 (2004)
5. Che, F.Y., Fricker, L.D.: Quantitative peptidomics of mouse pituitary: Comparison of different stable isotopic tags. J. Mass Spectrom. **40**, 238–249 (2005)
6. Che, F.Y., Biswas, R., Fricker, L.D.: Relative quantitation of peptides in wild type and Cpe<fat/fat> mouse pituitary using stable isotopic tags and mass spectrometry. J. Mass Spectrom. **40**, 227–237 (2005)
7. Melanson, J.E., Avery, S.L., Pinto, D.M.: High-coverage quantitative proteomics using amine-specific isotopic labeling. Proteomics. **6**, 4466–4474 (2006)
8. Dayon, L., Hainard, A., Licker, V., Turck, N., Kuhn, K., Hochstrasser, D.F., Burkhard, P.R., Sanchez, J.C.: Relative quantification of proteins in human cerebrospinal fluids by MS/MS using six-plex isobaric tags. Anal. Chem. **80**, 2921–2931 (2008)
9. Xiang, F., Ye, H., Chen, R.B., Fu, Q., Li, L.J.: N,N-Dimethyl Leucines as Novel Isobaric Tandem Mass Tags for Quantitative Proteomics and Peptidomics. Anal. Chem. **82**, 2817–2825 (2010)
10. Frost, D.C., Greer, T., Xiang, F., Liang, Z., Li, L.: Development and characterization of novel eight-plex DiLeu isobaric labels for quantitative proteomics and peptidomics. Rapid Commun. Mass Spectrom. **29**, 1115–1124 (2015)
11. Morano, C., Zhang, X., Fricker, L.D.: Multiple isotopic labels for quantitative mass spectrometry. Anal. Chem. **80**, 9298–9309 (2008)
12. Fricker, L.D.: Limitations of Mass Spectrometry-Based Peptidomic Approaches. J. Am. Soc. Mass Spectrom. **26**, 1981–1991 (2015)
13. Thompson, A., Schafer, J., Kuhn, K., Kienle, S., Schwarz, J., Schmidt, G., Neumann, T., Johnstone, R., Mohammed, A.K., Hamon, C.: Tandem mass tags: a novel quantification strategy for comparative analysis of complex protein mixtures by MS/MS. Anal. Chem. **75**, 1895–1904 (2003)
14. McAlister, G.C., Huttlin, E.L., Haas, W., Ting, L., Jedrychowski, M.P., Rogers, J.C., Kuhn, K., Pike, I., Grothe, R.A., Blethrow, J.D., Gygi, S.P.: Increasing the multiplexing capacity of TMTs using reporter ion isotopologues with isobaric masses. Anal. Chem. **84**, 7469–7478 (2012)
15. Hsu, J.L., Huang, S.Y., Chow, N.H., Chen, S.H.: Stable-isotope dimethyl labeling for quantitative proteomics. Anal. Chem. **75**, 6843–6852 (2003)
16. Guo, K., Ji, C., Li, L.: Stable-isotope dimethylation labeling combined with LC-ESI MS for quantification of amine-containing metabolites in biological samples. Anal. Chem. **79**, 8631–8638 (2007)
17. Wang, J., Zhang, Y., Xiang, F., Zhang, Z., Li, L.: Combining capillary electrophoresis matrix-assisted laser desorption/ionization mass spectrometry and stable isotopic labeling techniques for comparative crustacean peptidomics. J. Chromatogr. A. **1217**, 4463–4470 (2010)
18. Boersema, P.J., Aye, T.T., van Veen, T.A., Heck, A.J., Mohammed, S.: Triplex protein quantification based on stable isotope labeling by peptide dimethylation applied to cell and tissue lysates. Proteomics. **8**, 4624–4632 (2008)
19. Swaney, D.L., Wenger, C.D., Coon, J.J.: Value of using multiple proteases for large-scale mass spectrometry-based proteomics. J. Proteome Res. **9**, 1323–1329 (2010)
20. Hsu, J.L., Huang, S.Y., Chen, S.H.: Dimethyl multiplexed labeling combined with microcolumn separation and MS analysis for time course study in proteomics. Electrophoresis. **27**, 3652–3660 (2006)
21. Wu, Y., Wang, F., Liu, Z., Qin, H., Song, C., Huang, J., Bian, Y., Wei, X., Dong, J., Zou, H.: Five-plex isotope dimethyl labeling for quantitative proteomics. Chem. Commun. (Camb.) **50**, 1708–1710 (2014)
22. Gelman, J.S., Sironi, J., Castro, L.M., Ferro, E.S., Fricker, L.D.: Peptidomic analysis of human cell lines. J. Proteome. Res. **10**, 1583–1592 (2011)
23. Cappadona, S., Munoz, J., Spee, W.P., Low, T.Y., Mohammed, S., van Breukelen, B., Heck, A.J.: Deconvolution of overlapping isotopic clusters improves quantification of stable isotope-labeled peptides. J. Proteome. **74**, 2204–2209 (2011)
24. Dasari, S., Wilmarth, P.A., Reddy, A.P., Robertson, L.J., Nagalla, S.R., David, L.L.: Quantification of isotopically overlapping deamidated and 18o-labeled peptides using isotopic envelope mixture modeling. J. Proteome Res. **8**, 1263–1270 (2009)
25. Yoon, J.Y., Lim, K.Y., Lee, S., Park, K., Paek, E., Kang, U.B., Yeom, J., Lee, C.: Improved quantitative analysis of mass spectrometry using quadratic equations. J. Proteome Res. **9**, 2775–2785 (2010)
26. Senko, M.W., Beu, S.C., McLaffertycor, F.W.: Determination of monoisotopic masses and ion populations for large biomolecules from resolved isotopic distributions. J. Am. Soc. Mass Spectrom. **6**, 229–233 (1995)
27. White, J.G.: Effects of colchicine and Vinca alkaloids on human platelets. I. Influence on platelet microtubules and contractile function. Am. J. Pathol. **53**, 281–291 (1968)
28. Berezniuk, I., Sironi, J.J., Wardman, J., Pasek, R.C., Berbari, N.F., Yoder, B.K., Fricker, L.D.: Quantitative peptidomics of Purkinje cell degeneration mice. PLoS One. **8**, e60981 (2013)
29. Berezniuk, I., Sironi, J., Callaway, M.B., Castro, L.M., Hirata, I.Y., Ferro, E.S., Fricker, L.D.: CCP1/Nna1 functions in protein turnover in mouse

brain: Implications for cell death in Purkinje cell degeneration mice. FASEB J. **24**, 1813–1823 (2010)

30. Dasgupta, S., Castro, L.M., Dulman, R., Yang, C., Schmidt, M., Ferro, E.S., Fricker, L.D.: Proteasome inhibitors alter levels of intracellular peptides in HEK293T and SH-SY5Y cells. PLoS One. **9**, e103604 (2014)

31. Gelman, J.S., Sironi, J., Berezniuk, I., Dasgupta, S., Castro, L.M., Gozzo, F.C., Ferro, E.S., Fricker, L.D.: Alterations of the intracellular peptidome in response to the proteasome inhibitor bortezomib. PLoS One. **8**, e53263 (2013)

32. Fricker, L.D., Gelman, J.S., Castro, L.M., Gozzo, F.C., Ferro, E.S.: Peptidomic analysis of HEK293T cells: effect of the proteasome inhibitor epoxomicin on intracellular peptides. J. Proteome Res. **11**, 1981–1990 (2012)

33. Boersema, P.J., Raijmakers, R., Lemeer, S., Mohammed, S., Heck, A.J.: Multiplex peptide stable isotope dimethyl labeling for quantitative proteomics. Nat. Protoc. **4**, 484–494 (2009)

34. Wardman, J., Fricker, L.D.: Quantitative peptidomics of mice lacking Peptide-processing enzymes. Methods Mol. Biol. **768**, 307–323 (2011)

35. Hsieh, E.J., Hoopmann, M.R., MacLean, B., MacCoss, M.J.: Comparison of database search strategies for high precursor mass accuracy MS/MS data. J. Proteome Res. **9**, 1138–1143 (2010)

36. Fu, Q., Li, L.: De novo sequencing of neuropeptides using reductive isotopic methylation and investigation of ESI QTOF MS/MS fragmentation pattern of neuropeptides with N-terminal dimethylation. Anal. Chem. **77**, 7783–7795 (2005)

37. Abreu, T.F., Sumitomo, B.N., Nishiyama Jr., M.Y., Oliveira, U.C., Souza, G.H., Kitano, E.S., Zelanis, A., Serrano, S.M., Junqueira-de-Azevedo, I., Silva Jr., P.I., Tashima, A.K.: Peptidomics of Acanthoscurria gomesiana spider venom reveals new toxins with potential antimicrobial activity. J. Proteome. **151**, 232–242 (2017)

38. Huang, S.Y., Chen, S.F., Chen, C.H., Huang, H.W., Wu, W.G., Sung, W.C.: Global disulfide bond profiling for crude snake venom using dimethyl labeling coupled with mass spectrometry and RADAR algorithm. Anal. Chem. **86**, 8742–8750 (2014)

39. Berezniuk, I., Vu, H.T., Lyons, P.J., Sironi, J.J., Xiao, H., Burd, B., Setou, M., Angeletti, R.H., Ikegami, K., Fricker, L.D.: Cytosolic Carboxypeptidase 1 Is Involved in Processing alpha- and beta-Tubulin. J. Biol. Chem. **287**, 6503–6517 (2012)

40. Rogowski, K., van Dijk, J., Magiera, M.M., Bosc, C., Deloulme, J.C., Bosson, A., Peris, L., Gold, N.D., Lacroix, B., Grau, M.B., Bec, N., Larroque, C., Desagher, S., Holzer, M., Andrieux, A., Moutin, M.J., Janke, C.: A family of protein-deglutamylating enzymes associated with neurodegeneration. Cell. **143**, 564–578 (2010)

41. Kalinina, E., Biswas, R., Berezniuk, I., Hermoso, A., Aviles, F.X., Fricker, L.D.: A novel subfamily of mouse cytosolic carboxypeptidases. FASEB J. **21**, 836–850 (2007)

42. Finn, P.F., Mesires, N.T., Vine, M., Dice, J.F.: Effects of small molecules on chaperone-mediated autophagy. Autophagy. **1**, 141–145 (2005)