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ALKYLATION OF β-TUBULIN ON GLU 198 BY A MICROTUBULE DISRUPTER

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Running title : ICEU alkylation of β-tubulin

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Abbreviations: CEUs, N-aryl-N'-(2-chloroethyl)ureas ; ICEU, N-4-iodophenyl-N'-(2-chloroethyl)urea) ; MALDI-TOF-MS, matrix assisted laser desorption ionisation time of flight mass spectrometry ; ESI-MS, electro spray ionisation mass spectrometry.

ABSTRACT

We have shown that β-tubulin was alkylated by a microtubule disrupter, ICEU, on a glutamic acid residue at position 198 and not on the previously proposed reactive cysteine 239. ICEU belongs to the 4-substituted-phenyl-N'-(2-chloroethyl) urea class (CEU) that alkylates mainly cellular proteins. Previous studies have shown that the *tert*-butyl (tBCEU) and iodo (ICEU) derivatives induce microtubule disruption due to β-tubulin alkykation. tBCEU was supposed to bind covalently to cysteine 239 of β-tubulin, but this binding site was not clearly confirmed (Legault, J., *et al*., 2000, Cancer Res., 60, 985-992). We have isolated and analysed β-tubulin after two-dimensional gel electrophoresis of proteins from B16 cells incubated with ICEU. Alkylated β-tubulin had a lower apparent molecular weight and a more basic isoelectric point than the unmodified protein. Labelled N-4-^{125}ICEU was effectively bound to the modified β-tubulin but using MALDI-TOF MS, we demonstrated that none of the cysteine residues of β-tubulin was linked to the alkylating agent. In contrast, peptide masses at m/z 4883 and 1792 in trypsin or Asp-N digestions of β-tubulin confirmed binding of iodophenylethylureido moiety to peptides [175-213] or [197-208] respectively. Fragmentation analyses by electrospray mass spectrometry using triply charged ions of peptide [175-213] identified a glutamic acid at position 198 as target for alkylation via an ester bond with ICEU. This amino acid located in the intermediate domain of the β-tubulin should play an essential role in the conformational structure necessary for the interaction between dimers in the protofilament .

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INTRODUCTION

Due to their major role in cell proliferation, microtubules constitute a non specific but efficient target for anti-tumor drugs. Among several compounds, two major products, colchicine and taxol, respectively impede microtubule polymerisation and depolymerisation properties by binding mainly to β-tubulin (Wilson et al., 1999). Among the class of N-aryl-N'-(2-chloroethyl)ureas (CEUs) (Mounetou et al., 2001), several molecules mimic the colchicine action and lead to cell death of treated cells by blocking the cell progression cycle at G2/M transition (Legault et al., 2000; Petitclerc et al., 2004). In vitro studies confirmed that this cytotoxic effect is based on a cytoskeleton modification with depolymerised microtubules due to β-tubulin modification (Petitclerc et al., 2004). Two β-tubulin alkylating CEUs [N-4-iodophenyl-N'-(2-chloroethyl)urea (ICEU) and N-4-*tert*-butylphenyl-N'-(2-chloroethyl)urea (tBCEU)] induce a specific SDS-PAGE pattern with a faster running additional band, immunoreactive for β-tubulin. This fast migrating protein is linked covalently to the tBCEU as shown with the labelled drug (Legault et al., 2000). Binding of CEUs has been attributed to the reactivity of cysteine residues within the target protein, which could form a covalent link by the mean of a nucleophilic substitution with the chloroethyl moiety of the CEU. Indeed, one of the isoforms of β-tubulin (β 3), which displays a serine instead of the cysteine at position 239, was not alkylated by tBCEU (Legault et al., 2000). Cysteines 239 and 354 are the best candidates for colchicine-like drugs target (Bai et al., 2000) and crystallography data of β-tubulin-colchicine-stathmin binding protein complex showed that they are buried within the colchicine binding site (Ravelli et al., 2004).

In the present study, we performed comparative protein and peptide analyses by matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS) and electrospray ionization tandem mass spectrometry **(**ESI-MS/MS) on ICEU alkylated β–tubulin isolated on a 2D-electrophoresis gel. We first showed that among ^{125}ICEU labelled proteins, β-tubulin was recovered in a major spot. MS analyses demonstrated that this ICEU-β-tubulin displays a higher molecular weight

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although it migrated faster than the native form. In addition, this modified tubulin had a more basic isoelectric point. Analyses of β-tubulin peptides indicated that the CEU molecule was not bound to C239 or any other cysteine residue of the protein. ESI-MS/MS demonstrated that ICEU was linked to β-tubulin-5 within peptide [175-213] and was covalently bound to a glutamic acid at position 198.

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MATERIALS AND METHODS

Materials

N-aryl-N'-(2-chloroethyl)ureas (CEUs, Fig. 1) were synthesised according to reference (Mounetou et al., 2001). ^{125}I -CEU was labelled starting from [^{125}I] iodide (Amersham, Orsay, France) with a specific activity of 0.2 mCi/µmol (Mounetou et *al.*, submitted). Cells culture media and additives were from Invitrogen (Cergy-Pontoise, France); foetal bovine serum from Sigma-Aldrich (St-Quentin-Fallavier, France). 2D electrophoresis were carried with Biorad products (Marnes la Coquette, France).

Cell culture treatment and protein extracts

Mouse melanoma B16 cells were cultured in MEM medium supplemented with 10% foetal bovine serum, 1 mM non essential amino-acids, 1mM sodium pyruvate and 4µg/ml gentamicin. Cells were subcultured every two days and plated at 3 to 5.10^5 cells/ml 24 hours before treatment. The CEUs solubilized in DMSO were added to the medium at 100µM for 24 hours; corresponding controls were performed with 0.5% DMSO.

Protein extracts for electrophoresis were prepared essentially as described (Rabilloud, 1999). The cells were harvested by scraping and were subsequently pelleted and washed two times with PBS. The resulting pellet was resuspended in one volume of 10mM Tris-HCl pH 7.5 buffer containing 0.25M sucrose and 10mM EDTA, and 4 volumes of solubilization buffer (8.4M Urea, 2.4M Thiourea, 50mM DTT, 5% CHAPS) were added with a cocktail of protease inhibitors (Roche diagnostics, Meylan, France). Extraction was carried out for 30 minutes at room temperature by vigorous shaking and was followed by ultra centrifugation at 100 000 g for 30 minutes. Supernatants were recovered and protein concentration was measured by coomassie blue using bovine serum albumin (BSA) as standard.

One or two dimensional electrophoresis, immunoblotting.

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SDS-PAGE electrophoresis was performed on 10% polyacrylamide gel by loading samples of protein extracts without heat denaturation.

Proteins were transferred to nitrocellulose membrane (Immobilon NC, Millipore, St-Quentin-en-Yvelines, France) and were subjected to an anti β-tubulin antibody (Anti TUB2.1, Sigma, St-Quentin-Fallavier, France). Further incubation with an HRP labelled anti mouse antibody (Dako, Trappes, France) allowed to localize the detected proteins by chemiluminescence (Amersham, Orsay, France).

Analytical 2D gels (100 µg proteins on 7cm IPG strip, focused at 8000 Vhours) were prepared to determine modification of β-tubulin migration by western blot analysis.

Preparative 2D gels were performed with 350-500 µg of total cellular proteins loaded on 17 cm IPG-strip nl 3-10 or 4-7 in solubilization buffer containing 0.8 % ampholytes pH 3-10 and focused at 40 000 Vhours. IPG strips were further treated in a buffer at pH 8.8 containing 6M Urea, 2% SDS, 30 % glycerol, 50mM Tris-HCl, and including 1% DTT for the first 15 minutes and 4% Iodoacetamide for the next 15 minutes. The IPG-strips were then loaded onto 12.5% SDS polyacrylamide gel. Following migration, gels were stained with colloidal blue. For autoradiography, gels were dried and exposed to films. Protein spots encompassing β-tubulin area were excised and stored at –20°C for mass spectrometry analysis.

MALDI-TOF-MS analysis of intact protein

Passive elution from polyacrylamide gels was performed according to Claverol et al (Claverol et al., 2003). Briefly, protein spots were incubated overnight at 37°C in 30 µL of 0.1M sodium acetate, 0.1% SDS, pH 8.2. The samples were then sonicated for 15 min to facilitate elution. The eluted proteins were further cleaned by removing contaminants with a $ZipTip_{HPL}$ (Millipore). For this, the protein was loaded onto the $ZipTip_{HPL}$ in 90% acetonitrile (ACN) containing 0.1% aqueous acetic acid, and eluted in 1 µL of 50% ACN containing 0.1% aqueous acetic acid. The eluted protein was deposited directly onto the target, and the matrix solution (sinapinic acid) was further added onto it.

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MALDI-TOF-MS analysis was performed on a Voyager DE-PRO mass spectrometer (Applied Biosystems, Fragmingham, MA, USA) equipped with a delayed extraction MALDI source and a pulsed nitrogen laser (337 nm). Analysis of the intact molecules was performed in a positive linear mode. Calibration was performed in a close external mode using BSA (singly charged M^+ and doubly charged M^{2+}) ions.

MALDI-TOF-MS analysis of protein digests

Proteins were digested in-gel with proteases. For this, the gel pieces were extensively washed with 50 mM ammonium bicarbonate in 50 % aqueous ACN, and the enzyme was added to the dried gel pieces.

Trypsin digestion was performed with 120 ng trypsin (Promega, Madison, WI, USA) per gel piece. After 18 h at 36°C, the resulting peptides were extracted with 70% ACN in 0.5% aqueous trifluoroacetic acid (TFA) and the peptide mixture was analyzed by Maldi-Tof-MS using cyano-4-hydroxycinnamic acid (CHCA) as a matrix in a positive reflector mode. Internal calibration of samples was done using tryptic autolytic peptides (m/z at 842.510 and 2211.104). Identification of the protein using these mass fingerprinting data was carried out using the MS-FIT software (http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm). Analyses in a positive linear mode were performed for higher sensitivity in larger peptide analysis using trypsin autolytic peptides at m/z 3339.85 and 5561.32 (average masses) for calibration.

For Asp-N endoprotease treatment (Roche diagnostics, Meylan, France), 15 ng of enzyme was added to each protein spot, and incubated in ammonium bicarbonate buffer, containing 10 % ACN. The reaction was stopped after 3 hr, the resulting peptides extracted with 70% ACN in 0.5% aqueous TFA. The peptide mixture was analyzed by MALDI-TOF-MS using CHCA as a matrix in positive and negative reflector modes.

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Nano-ESI MS/MS analyses of protein digests

Nano-ESI-MS/MS analyses of trypsin digests were carried out on a LCQ ion trap mass spectrometer equipped with a nanoelectrospray source (Thermo Finnigan, San Jose, CA, USA). The nanoelectrospray capillaries (Protana, Odense, Denmark) were loaded with 3µL of peptide mixture in 50 % ACN in water containing 0.1% TFA. The peptides were directly analyzed by infusion and the ionization was performed with liquid junction with a non coated capillary probe (New Objective, Woburn, MA, USA). Data acquisition was performed in a manual mode and the Collision Induced Dissociation of selected precursor ions was performed using 30% relative collision energy. The MS/MS data from the non alkylated peptide were searched against the mammalian database subset from the NCBI database (with the SEQUEST search engine, LCQDeca software package). MS^2 and MS^3 data from alkylated peptide were interpreted manually, assuming the modified masses (see Fig. 7-A). Fragments are assigned according to Roepstorff (Roepstorff and Fohlman, 1984).

RESULTS

Modification of β-tubulin migration properties by ICEU

The modification of β-tubulin migration in SDS-PAGE by tBCEU or ICEU (Fig 1) was previously demonstrated in several cell lines (Legault et al., 2000; Mounetou et al., 2003; Petitclerc et al., 2004). To assess that this phenomenon was also present in B16 melanoma cells, we performed western blot analysis using a monoclonal antibody against rat purified β-tubulin (Fig. 2A). We observed an additional lower band in proteins extracted from cells incubated for 24 hours with 100µM ICEU (lane 2), absent in control cells (lane 1). After 2D gel electrophoresis on 7 cm IPG strip 4-7, controls and ICEU B16 proteins were coomassie stained (Fig. 2B upper images) or immunodetected using the same antibody (Fig 2B, lower images). An additional spot characterized by a lower apparent molecular weight and a more basic isoelectric point in ICEU treated cells was observed (right panel) compared to the control

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extracts (left panel). 2D electrophoresis analysis allowed thus to discriminate two β-tubulin spots after ICEU treatment .

To ascertain that this additional β-tubulin was linked to ICEU, B16 cells were incubated with ^{125}ICEU in the same conditions as with the non-radioactive molecule. On 1D electrophoresis gel, we observed a major radioactive band at 50KDa (Fig.3 A, upper panel). This labelled band could be superposed to the lower band of β-tubulin as revealed by western blotting (Fig. 3A, lower panel).

The labelled proteins were analyzed in 2D-electrophoresis on 17 cm IPG strip 4-7 and detected by autoradiography (Fig. 3B, upper panel); the spot in the β-tubulin area (Fig. 3B, lower panel) was excised and further identified as β-tubulin by MALDI-TOF-MS after at least 2 periods of radioactivity decay. This protein was identified as mouse tubulin β-5 chain (Swiss-Prot P99024). These data clearly demonstrated that β-tubulin was alkylated by ICEU and that ICEU was still fixed to the protein after 2D-electrophoresis.

β-tubulin alkylation demonstrated by MALDI-TOF-MS analysis of intact proteins

To verify that modified β-tubulin was not truncated as a result of alkylation by ICEU, an analysis by MALDI-TOF-MS of the intact proteins was performed after passive elution of the two corresponding spots from the 2D-gel. A mass of 49990 was obtained for the upper spot, while a mass of 50320 was measured for the lower one (Fig. 4-A) indicating that the protein was complete in both cases, and that the alkylating agent was effectively bound to the protein. The difference between the two masses was in agreement with ICEU structure (Fig. 1) in spite of a poor resolution due to the low recovery of a 50 kDa protein from the gel. In a tryptic digest, N-terminal and C-terminal peptides could be clearly identified thus confirming that β-tubulin is not truncated (data not shown). It should be noticed that the fast migrating spot (lower spot) actually had a higher mass than the slow migrating one (upper spot).

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β-tubulin alkylation does not result from Cys modification.

Upper and lower β-tubulin 2D-spots were further analyzed by MALDI-TOF-MS to characterize CEU binding to the protein. For this, a comparative study was performed on the tryptic digest of the two spots (Fig. 4-B). In the m/z range from 1000 to 3000, 60% of β-5 tubulin sequence was covered, including 4 cysteine residues out of the eight present along the molecule. This was the case especially for C239 which has been proposed as the main target for tBCEU (Legault et al., 2000). The corresponding peptide [217-241] was measured at m/z 2708.30, which corresponded to the mass of the peptide, carbamidomethylated by iodoacetamide during the 2D process, but not alkylated by ICEU. The same result was obtained for the peptides containing the three other cysteine residues (C354 on peptide [351-359] at m/z 1028.5, C303 on peptide [298-306] at m/z 1065.4 and C12 on peptide [3-19] at m/z 1822.9). As demonstrated ahead, ICEU was not removed by the different treatments of the gel, it was thus clear that neither C239 nor any of these 3 other cysteine residues were the binding site of ICEU.

Since in this analysis only four cysteine residues of β-5 tubulin could be detected, peptide analysis was extended to m/z 6000 in a linear mode to determine if a different cysteine could be the target for ICEU. The four other Cys residues were detected in two peptides in upper and lower β-tubulin spots, each of these Cys being carbamidomethylated (m/z 3313.7 as compared to 3199.6 for unmodified cysteine residues in peptide [123-154] (data not shown), and m/z 4596.0 as compared to 4481.9 for unmodified cysteine residues in peptide [175-213] (Fig. 5A). It was thus evident that ICEU did not bind to a cysteine residue of β-tubulin. One extra unidentified peptide at m/z 4883.8 was detected in the lower spot (Fig. 5-A). Although the peptide at m/z = 4596 was still present in this sample, the mass difference between these two peptides ($\Delta m = 288$) corresponded to the expected mass difference due to alkylation by ICEU (Fig. 1) and was thus a good candidate for further analysis.

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β-tubulin alkylation generates an additional tryptic peptide

Using ICEU, an extra tryptic peptide was observed exhibiting a Δm = 288 with the peptide [175-213] at m/z 4596 (Fig. 5-A). A comparable experiment was performed with tBCEU which had been shown to alkylate β tubulin in a similar way (Legault et al., 2000). An extra tryptic peptide at m/z 4814.6 was also observed, and the mass difference to peptide [175-213] was 218 (Fig. 5-B), which corresponded to the expected mass difference for this tBCEU structure (Fig. 1). These data indicated that ICEU as well as tBCEU were bound to peptide [175-213].

To confirm that this extra peptide was effectively related to peptide at m/z 4596 and to locate the alkylation site along the peptide [175-213] more precisely, a digestion was performed using an enzyme with a different specificity. For this, unmodified or ICEU modified β-tubulin from two 2D gel spots were digested with endoprotease Asp-N. Due to the poor ionization of peptides having no basic residue, MALDI-TOF-MS analyses had to be performed in a negative reflector mode. A peptide at m/z 1791.53 was present in the lower spot, while m/z 1503.58 corresponding to peptide [197-208] in the upper spot was absent from the lower one (Fig. 5-C). The mass difference between these two peptides was consistent with the presence of ICEU, and thus, these data delimitated CEU alkylation site between Asp-197 and Tyr-208.

β-tubulin alkylation localization

To further localize the CEU molecule along the peptidic chain and to identify the modified amino acid, the two tryptic peptides at m/z 4596 and 4884 (measured at 4593 and 4881 in a monoisotopic resolution) obtained in the lower band were fragmented using a nano-ESI source. The triply charged precursor ion at m/z 1532 ($[M+3H]^{3+}$) from peptide 4593 ($[M+H]^{+}$) was isolated, fragmented and b (amino-terminal) as well as y (carboxy-terminal) ions were obtained (Fig. 6-A). y_3 to y_{14}, b_5 and b_7 singly-charged fragments as well as y_{16}, y_{26} to y_{33}, b_{23} and b_{25} doubly-charged fragments obtained from peptide 4593 allowed the identification of peptide [175-213] from β-tubulin .

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A comparable analysis was performed from the triply charged precursor ion at m/z 1628 ($[M+3H]^{3+}$) from peptide 4881 ($[M+H]^{+}$), (Fig. 6-B). The fragments obtained were compared to fragments from peptide 4593, and fragments y_4 to y_{14} indicated that peptide 4881 derived from peptide 4593. On the contrary, fragments with higher masses (doubly and triply charged fragments, boxed in Fig.6-B) could be assigned assuming an alkylation. Specific m/z observed in the two series of fragments allowed the localization of this alkylation : on the one hand the mass difference between m/z 1852.4 (y_{14}) and m/z 1186.0, attributed to y_{16}^{2+}, corresponded to the mass of E plus T plus 288; on the other hand, m/z 1256.6, 1465.4 and 1515.6 were assigned to the fragmentations framing an alkylated E (+288) and a T respectively, along the peptidic chain, and were thus identified as b_{23}^{2+}, b_{24}^{2+} and b_{25}^{2+}. Alkylation occurred thus on E24 of this peptide

As important fragments were present at m/z 1546.2, an MS^3 experiment was performed on this triply charged precursor ion (Fig. 6 C). It corresponded to a fragmentation within the ICEU molecule, at its "peptidic bond" (Fig. 7 A) and contained modified fragments (Δm 42) starting from y_{16} on the one hand and from b_{23} on the other hand (Fig. 7 B). It was thus confirmed from this MS^3 experiment that the iodophenylethylureido moiety of ICEU was localized on E24 residue of this peptide, which corresponds to E198 of β-tubulin.

DISCUSSION

We have demonstrated in B16 melanoma cells that ICEU alkylates β-tubulin 5 isoform by binding covalently to a glutamic acid residue at position 198. β-tubulin 5 represents the major isoform in B16 cells (present study), while we identified β3 and β4 isoforms (which display E at position 198) in too low amounts to detect alkylated counterparts . Mouse β5-tubulin isoform is homologous to human β2 tubulin, the two of them are expressed ubiquitously .

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The alkylation of E198 is in agreement with the more basic isoelectric point observed for tubulin after ICEU treatment (Fig. 1 and 2) due to the loss of an acidic charge; in addition, this amino acid is characterized by a pKa value of 4 which is compatible with an esterification reaction at physiological pH. In alkali conditions, trypsin digestion at pH 8.2 for 16 hr leads to partial loss of the ester linkage, giving rise to the non modified peptide [175-213] which was detected by MS in addition to the modified peptide (Fig. 5A). Identical alkylation of E198 probably occurs with tBCEU as the same data were observed in tryptic digests from modified tBCEU β-tubulin (Fig. 5B). This artifactual loss of alkylation which permitted the analysis of both peptides in the same sample was not observed in the case of a 2-3 hr incubation with endoprotease Asp-N (Fig. 5C) and resulted from the lability of this link.The involvement of an acidic amino acid in an alkylation reaction via an ester bond has been established for chlorambucil (a CEU parent drug) and angiotensin I-converting enzyme by an enzymatic approach (Harris and Wilson, 1982). In our study, in spite of the large molecular mass of the interesting peptides (m/z 4596 and 4884, average masses), we performed comparative nano-ESI MS/MS experiments with the triply charged ions of the native and modified peptides to determine which of the 8 acidic amino acids within peptide [175-213] was alkylated. This allowed to localize the alkylated amino acid between fragments b_{23} and b_{24} of the peptide (Fig. 7A) and thus identified E198 as a candidate for alkylation in MS^2 experiments (precursor ion at 1628). ICEU contains an amide bond which is fragmented as easily as any peptidic bond giving rise to a truncated ion at 1546. MS^3 fragmentation of this ion clearly confirmed that E198 is the target for the fixation of ICEU molecule. Mutation of yeast β-tubulin at position 198 conferred a resistance phenotype towards a microtubule disrupter, showing that this residue is important in the interaction between this protein and anti microtubules drugs (Richards et al., 2000). E198 is located in the T6-loop fragment of β-tubulin, essential to the interface between α and β subunits (Lowe et al., 2001; Nogales et al., 1998), near the buried colchicin site. This location is consistent with the competition experiment performed earlier (Legault et al., 2000). In this latter study, tBCEU could not alkylate tubulin in cells pre-treated with colchicine, suggesting that the targets for both drugs were identical or located close to each other within

the protein scaffold. In the same experiments, β-tubulin 3 (which has a serine at position 239 instead of a cysteine residue) was not alkylated, and it was thus hypothesized that cysteine 239 was the binding site. This is consistent with the stronger reactivity of cysteine 239 and cysteine 354 than the other β-tubulin cysteine residues to colchicin and its derivatives (Bai et al., 2000) and to reducing compounds (Britto et al., 2002). Actually, changing cysteine 239 to serine should modify the structural folding of the protein so strongly that an interaction with exogenous products or with protein ligands can no longer occur. Our MALDI-TOF-MS analyses have shown that all cysteine residues were free in the control β-tubulin, as it had been shown previously in the rat brain α/β tubulin dimer (Britto et al., 2002) but also in ICEU β-tubulin. This clearly eliminates cysteine 239 or any other cysteine residue as a target in the β-tubulin 5 alkylation process. We presume that pre-binding of colchicines or the conformational changes induced by replacing cysteine 239 with serine prohibit alkylation of glutamate 198 within the T6 loop.

The intermediate fragment containing T6-loop should be implicated in the rotation movement from straight to curved structures in protofilaments (Ravelli et al., 2004). The observed effects of microtubule depolymerisation in cells treated with ICEU or tBCEU (Petitclerc et al., 2004) are in agreement with a potential role of E198 in lateral contacts in the microtubules. Alkylation of β-tubulin with ICEU and tBCEU lead to a faster migrating protein (Fig. 2 and 3) although the protein was complete and the alkylkation agent still present (Fig. 4). We suggest that in treated B16 cells, β-tubulin adopts a curved structure, inducing depolymerisation of microtubules, but also keeps this conformation after protein extraction and in SDS PAGE under denaturing conditions thus allowing faster migration. The basis of this conformational change has to be further elucidated.

β-tubulin is one of the main target proteins for ICEU alkylation. Identification of the E198 as an alkylation site refutes previous hypothesis that reacting amino acids in tubulins would be mainly cysteine residues. Current work will be helpful to evaluate the interactions of other β-tubulin alkylating drugs and for modelling studies based on crystallography data. Identification of ICEU binding sites in other alkylated proteins should help to elucidate if the nature of the reacting amino-acid participates more to the

CEU alkylation mechanism than the folding of the protein. This is of particular interest since ICEU is a potential drug for the treatment of colon cancer (Miot-Noirault et al., 2004).

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FIGURE LEGENDS

FIG. 1. Chemical structure of N-4-iodophenyl-N'-(2-chloroethyl)urea (ICEU) and N-4-*tert*-butylphenyl-N'-(2-chloroethyl)urea (tBCEU) . The calculated monoisotopic masses for ICEU and tBCEU are 323.95 and 254.12 respectively for the complete molecules, and 288.98 and 219.15 without the chlorine atom.

FIG. 2. ICEU treatment modifies 1D and 2D migration pattern of B16 β-tubulin. After incubation for 24 hours with 100µM ICEU, B16 proteins were extracted and loaded on a 10% SDS PAGE or submitted to 2D electrophoresis using 4-7 IPG strip and 12.5% SDS PAGE for the first and second dimension respectively. After protein transfer on membrane and hybridisation with an anti-rat tubulin antibody , 1D analysis (**2A**) revealed two bands in ICEU treated B16 cells (lane 2) while one band was present in control cells (lane 1). These extracts were studied by 2D electrophoresis in a 4-7 pI range and gels stained with colloidal coomasie blue **(2B**, upper images) were subsequently studied by western blot (**2B**, lower images). Two spots appeared in the treated samples (right panel), corresponding to the upper and lower band observed in the 1D approach. The lower spot displayed a more basic pI than the upper spot. Arrows indicate ICEU β-tubulin spots.

FIG. 3. ^{125}ICEU binds to β-tubulin. B16 cells were cultured for 24 hours with 100µM ^{125}ICEU or 100µM unlabelled ICEU. Protein extracts (**3A**) were analysed by autoradiography of the gel (upper panel) and immunoblotting (lower panel). Lane 1 corresponds to control cells, lane 2 to 100 µM ICEU treated cells , lane 3 to 100 µM ^{125}ICEU treated cells. The lower additional band observed on the western blot of treated ICEU cells (lane 3) overlaps the radioactive band in the 50KDa area in the ^{125}ICEU extracts (lane 3). 2D electrophoresis performed on ^{125}ICEU extracts (**3 B**) allowed detecting 1 major spot in the 50 KDa

range, which was identified as mouse β-5 tubulin (Swiss-Prot P99024) by Maldi-Tof MS analysis (autoradiogram and coomassie stained gel, upper and lower panels respectively). Arrows indicate ICEU β-tubulin spots.

FIG. 4. ICEU modifies β-tubulin mass but is not linked to Cys 239. Proteins from the upper and lower β-tubulin spots were eluted from the 2D-gel, and analyzed by Maldi-Tof MS (4A). The upper spot (top panel) had a mass of 49990 and the lower one (bottom panel) a mass of 50320. These masses are in agreement with the fixation of ICEU to β-tubulin. Tryptic peptides extracted from upper and lower β-tubulin spots (4**B,** upper and lower panels respectively) were analyzed by Maldi-Tof MS. Four cysteine containing peptides were identified and are localized on the spectra. They corresponded to C354 from peptide [351-359], C303 from peptide [298-306], C12 from peptide [3-19] and C239 from peptide [217-241]. All of them were detected as carbamidomethylated, and thus could not be the binding sites for ICEU.

FIG. 5. β-tubulin is alkylated within peptide [197-208]. Tryptic peptides were obtained after digestion of ICEU treated (**5A**) or tBCEU treated (**5B**) B16 cell tubulin. Peptides extracted from slow migrating (upper panels) and fast migrating (lower panels) tubulin spots were analyzed by Maldi-Tof in a positive linear mode. The part of the spectra showing differences between the two proteins are presented. Peptide at m/z 4595.8 - 4596.4 was identified as peptide [175-213]. An additional peptide was observed in both cases with a mass difference of 288 and 218 respectively to the peptide [175-213]. These mass differences corresponded to expected mass differences for ICEU and tBCEU respectively, and indicated the binding of CEU molecules on peptide [175-213]. Peptides extracted from upper and lower β–tubulin spots were digested using endoprotease AspN (5**C**, upper and lower panels respectively) and analyzed by Maldi-Tof MS in a negative reflector mode. A peptide at m/z 1503.58, present in the upper spot was absent in the lower one, while a peptide at m/z 1791.53 appeared in the lower spot. This mass shift corresponded to the

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binding of ICEU on peptide [197-208]. A minor peptide at m/z 1790.92 (▼) corresponding to the oxidized form of peptide [161-176] at m/z 1774.87 (▽) was present in the untreated protein and thus partially overlapped the modified peptide at m/z 1791.53.

FIG. 6. ICEU binds to E198 β-tubulin. β-tubulin was digested from a fast migrating spot (lower spot). Peptides at m/z 4593 and m/z 4881 were analyzed by nanoESI MS/MS. The MS^2 spectrum of the precursor ions at m/z 1532^{3+} (**6A**) allowed to identify peptide [175-213] of mouse β–tubulin-5. In the MS^2 spectrum of the precursor ion at m/z 1628^{3+} (**6B**) singly charged fragments allowed the identification of peptide [175-213] of β-tubulin, while doubly charged fragments with higher masses than b_{23} and y_{15} corresponded to modified fragments as compared to the original spectrum in 6A. These modified fragments were assigned assuming an alkylation at E24 (Δm 288). They are presented in boxes. The MS^3 spectrum of a precursor ion at m/z 1546.2^{3+} (**6C**) from the MS^2 spectrum (precursor ion at m/z 1628^{3+}) confirmed peptide [175-213] identity as well as the alkylation position. The alkylating agent had been fragmented at its amide bond (see Fig 7) and several b and y fragments, mostly doubly charged (in boxes) exhibited a Δm value of 42, as compared to the unalkylated peptide fragments in 6A. "y" refers to carboxy-terminal ions while "b" refers to amino-terminal ions. "M" corresponds to the non-fragmented peptide, * indicates the loss of one water molecule, ° indicates the loss of one NH_3 molecule and $CEU^{\#}$ indicates the fragmented alkylated molecule (see Fig. 7).

FIG. 7. Peptide [175-213] is alkylated by ICEU through an ester linkage. The sequence of peptide [175-213] possessing 2 cysteine residues and 8 putative alkylation sites at glutamic and aspartic acid residues is presented, including most fragmentations observed in MS/MS analyses (**7A**). The acidic moiety of E198 reacted with ICEU to create an ester bond (**7B**). ICEU could be fragmented at its peptidic linkage (1546.2^{3+}) giving rise to a fragmented CEU. Due to the lability of the ester bond in alkaline

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conditions, this linkage could be lost during tryptic incubation, and the unmodified peptide could thus be analyzed (1532^{3+}) in the same tryptic digest.

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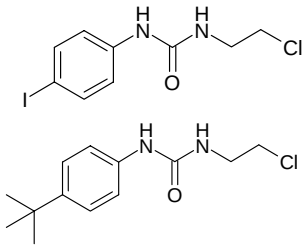

Figure 1

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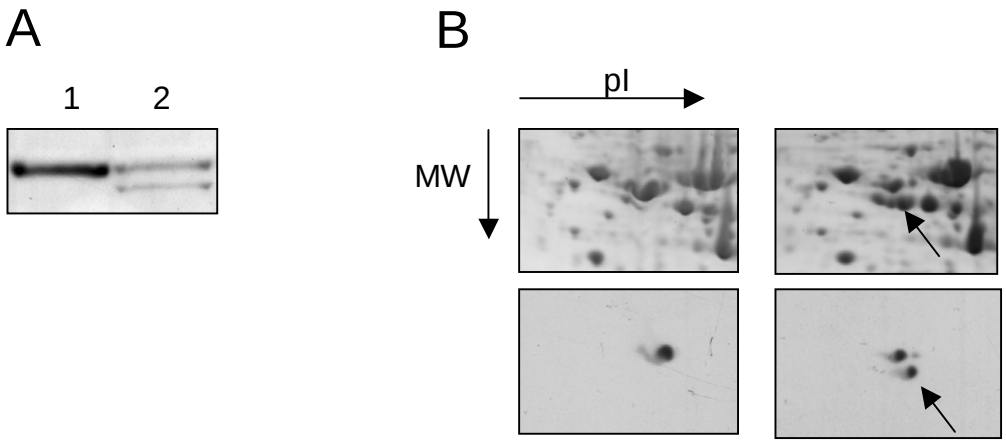

Figure 2

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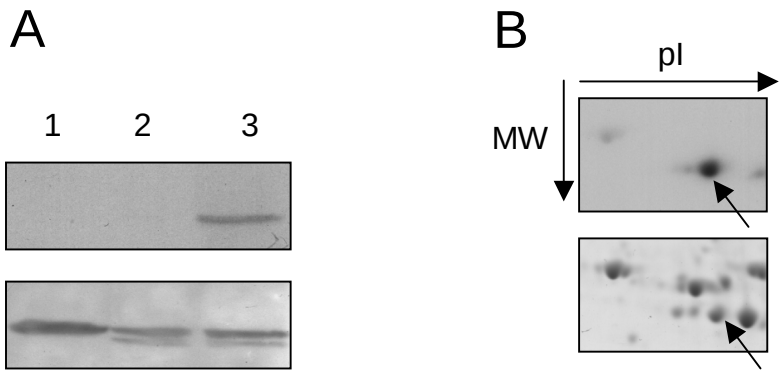

Figure 3

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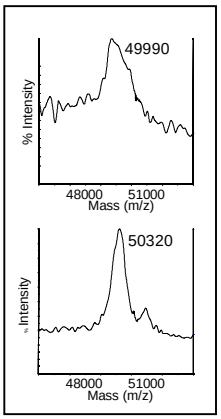
A



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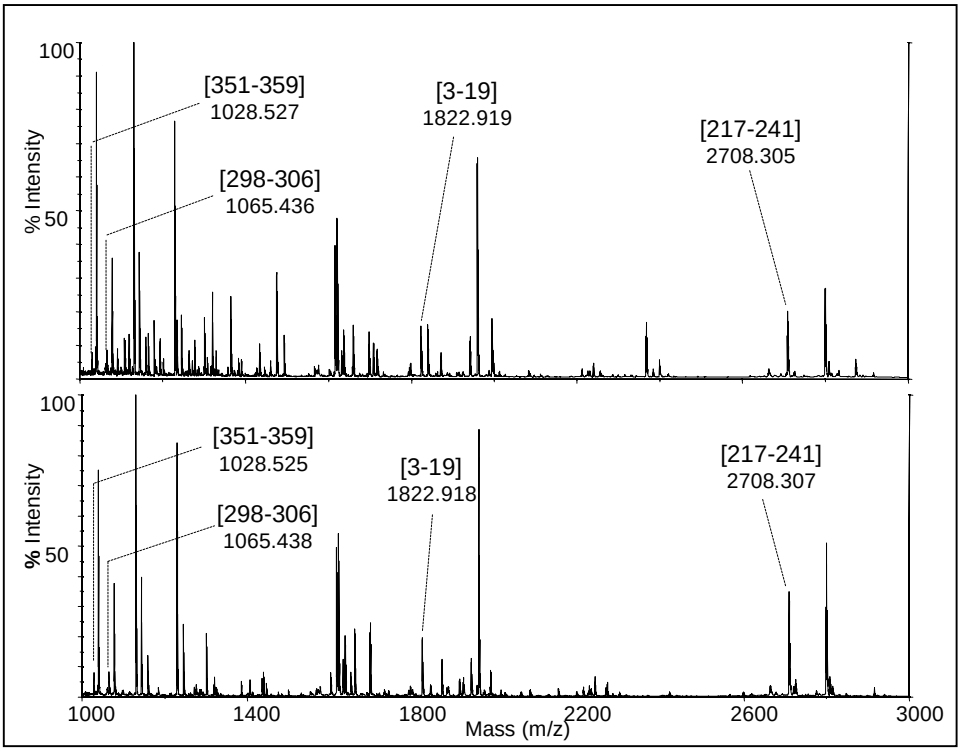

Figure 4

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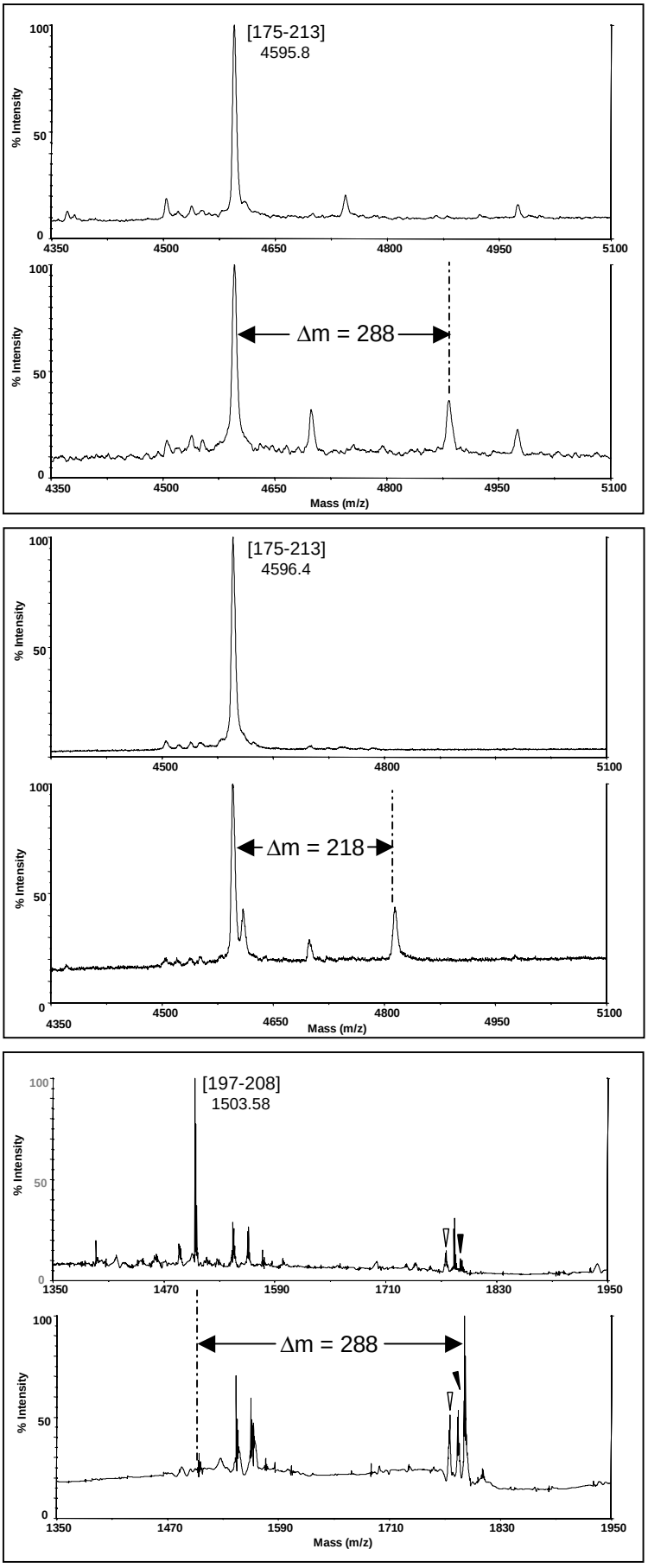

Figure 5

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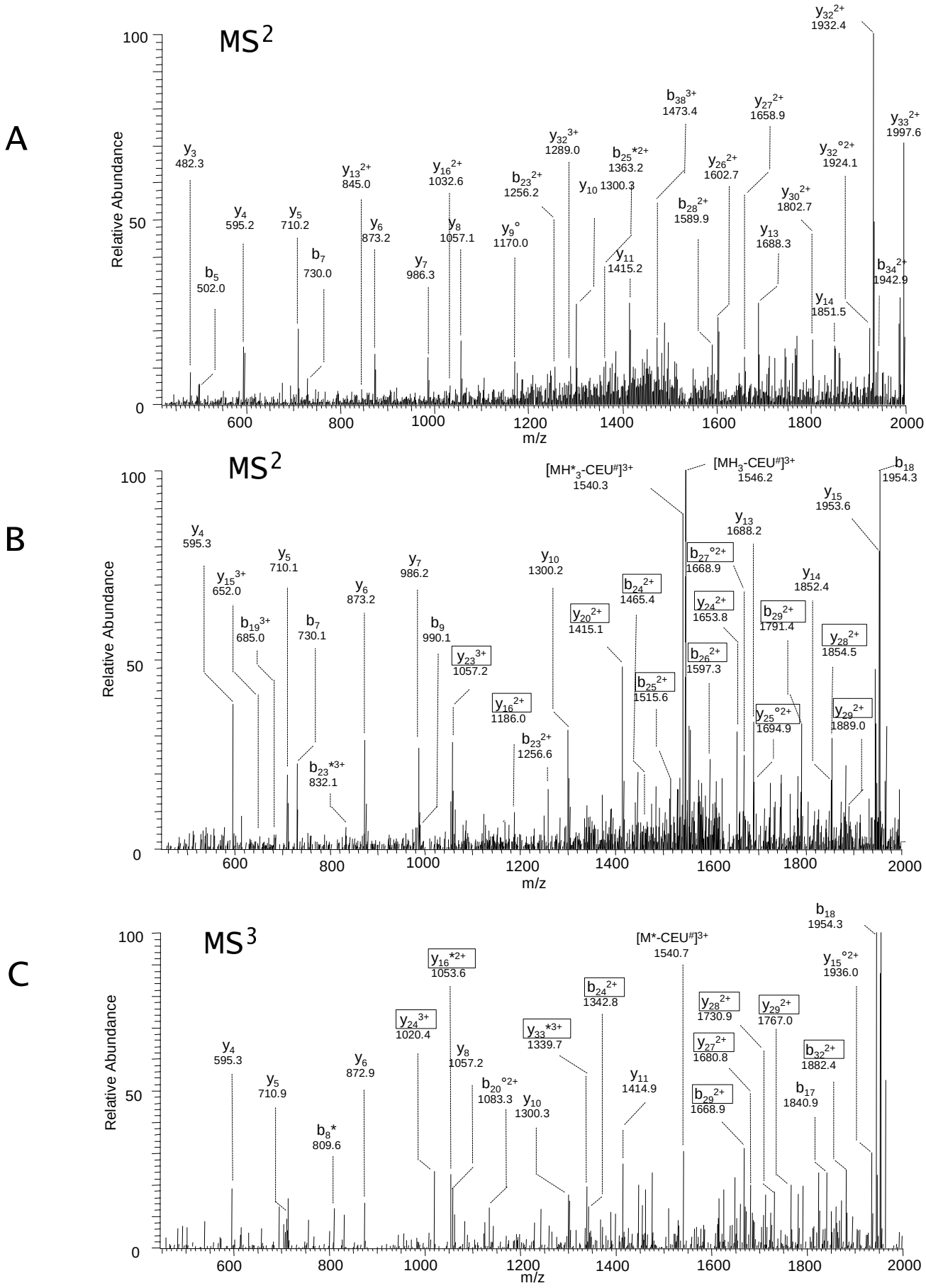

Figure 6

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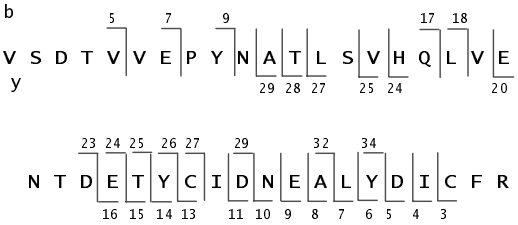

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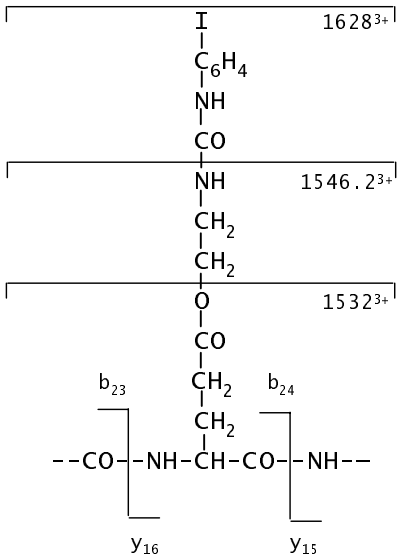

Figure 7