Characterization of the Covalent Binding of N-Phenyl-N’-(2-chloroethyl)ureas to β-Tubulin: Importance of Glu198 in Microtubule Stability

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ABSTRACT

N-Phenyl-N’-(2-chloroethyl)ureas (CEUs) are antimicrotubule agents interacting covalently with β-tubulin near the colchicine-binding site (C-BS). Glutaryl 198 residue in β-tubulin (Glu198), which is adjacent to the C-BS behind the two potent nucleophilic residues, Cys239 and Cys354, has been shown to covalently react with 1-(2-chloroethyl)-3-(4-iodophenyl)urea (ICEU). By use of mass spectrometry, we have now identified residues in β-tubulin that have become modified irreversibly by 1-(2-chloroethyl)-3-[3-(5-hydroxy-pentyl)phenyl]urea (HPCEU), 1-[4-(3-hydroxy-4-methoxy-styryl)phenyl]-3-(2-chloroethyl)urea (4ZCombCEU), and N,N’-ethylenbis(iodoacetamide) (EBI). The binding of HPCEU and 4ZCombCEU to β-tubulin resulted in the acylation of Glu198, a protein modification of uncommon occurrence in living cells. Prototypical CEUs then were used as molecular probes to assess, in mouse B16F0 and human MDA-MB-231 cells, the role of Glu198 in microtubule stability. For that purpose, we studied the effect of Glu198 modification by ICEU, HPCEU, and 4ZCombCEU on the acetylation of Lys40 on α-tubulin, a key indicator of microtubule stability. We show that modification of Glu198 by prototypical CEUs correlates with a decrease in Lys40 acetylation, as observed also with other microtubule depolymerizing agents. Therefore, CEU affects the stability and the dynamics of microtubule, likewise a E198G mutation, which is unusual for xenobiotics. We demonstrate for the first time that EBI forms an intramolecular cross-link between Cys239 and Cys354 of β-tubulin in living cells. This work establishes a novel basis for the design of future chemotherapeutic agents and provides a framework for the design of molecules useful for studying the role of Asp and Glu residues in the structure/function and the biological activity of several cellular proteins under physiological conditions.

Introduction

The importance of tubulins and microtubules as targets for cancer management is clearly illustrated by the value of antimicrotubules, such as paclitaxel, vinca alkaloids, epothilone, colchicine, and combretastatin A-4 in the anticancer armamentarium (Rowinsky and Donehower, 1991). However, the effectiveness of these chemotherapeutic agents is impeded by the development of chemoresistance and by the selectivity of these drugs to a limited subset of cancers due to their inappropriate biopharmaceutical properties (Giaccone and Pinedo, 2009; Rowinsky and Donehower, 1991). The development of new chemotherapeutic agents is therefore necessary to overcome these limitations.
1996). To design new and selective antimitotics that are able to circumvent chemoresistance and have minimal pharmacodynamic and pharmacokinetic limitations, laboratories have been extensively studying the mechanisms underlying the biological behavior of tubulins and microtubules in cells. At the molecular level, mutations of residues such as αSer379 (e.g., αS379R) have been shown to inhibit interactions with microtubule-associated proteins and to destabilize microtubule structure (Martello et al., 2003). In addition, mutations, such as βF270V and βT74I, in the paclitaxel (Taxol)-binding site inhibit the binding of paclitaxel (Giannakakou et al., 2000), thus leading to chemoresistance to the drug. Moreover, modification of βCys239 and βCys354 by alkylating drugs, such as 2-chloroacetyl-2-demethylthiochlodochine (Bai et al., 2000), otellione A (RPR112378) (Combeau et al., 2000), and 2-fluoro-1-methoxyacetyl-2-demethylthiocolchicine (Buey et al., 2007). The demonstration that the alkylation of Cys239 by other groups (Ludueña and Roach, 1981b). However, by using MALDI-TOF mass spectrometry and 1-(2-chloroethyl)-3-[3-(5-hydroxypentyl)phenyl]-3-(2-chloroethyl)urea (4ZCombCEU) (Fortin et al., 2007a), we have selected 1-(2-chloroethyl)-3-[3-(5-hydroxypentyl)phenyl]-3-(2-chloroethyl)urea (βBCEU) was irreversibly binding to Cys239 in β-tubulin, which triggers microtubule disruption and anoikis (Legault et al., 2000; Deschesnes et al., 2007). The demonstration that the alkylation of Cys239 by βBCEU was indirect and based on the previous work by other groups (Ludueña and Roach, 1981b). However, by using MALDI-TOF mass spectrometry and 1-(2-chloroethyl)-3-[3-(5-hydroxypentyl)phenyl]-3-(2-chloroethyl)urea (ICEU), a bioisostere of βBCEU (Bouchon et al., 2005), we were unable to confirm this proposed mechanism of action. The mass spectrometric analysis completely excluded thioalkylation of Cys239 by ICEU as initially proposed but instead uncovered the unique and rather unexpected covalent binding of the drug onto Glu198 through the formation of an ester bond. The intracellular formation of an ester linkage by xenobiotics such as CEUs is to our knowledge unique. The only prior report of a similar nucleophilic addition of a prototypical CEU to a protein under living conditions was the addition of lower tert-butylthioester to a protein by Luduena et al. (1985) to characterize β-tubulins isolated from bovine brain. In vitro, EBI generates two intramolecular cross-links involving Cys239 and Cys354 as well as Cys12 and Cys201/Cys211, respectively. The intramolecular cross-link involving Cys12 and Cys211 appears in the absence of guanine nucleotides in the in vitro setting (Ludueña and Roach, 1991). The product of such cross-linking generates an immunoreacting band that migrates faster than the native β-tubulin on SDS-PAGE and corresponds to the EBI-β-tubulin adduct (Ludueña and Roach, 1981b).

The aims of this study were to identify the specific site of the nucleophilic addition of prototypical CEUs on β-tubulin and to evaluate the importance of Glu198 in microtubule stability via the acetylation of Lys40 of α-tubulin, a post-translational modification that correlates with the state of stability of microtubules (Matsuyama et al., 2002).

### Materials and Methods

**Materials.** Biochemicals and the anti-β-tubulin antibody (clone TUB 2.1) were purchased from Sigma-Aldrich (St. Louis, MO) unless specified otherwise. Anti-α-tubulin antibody (H-300) and anti-acetylated tubulin antibody (6-11B-1) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). ICEU, HPCEU, and 4ZCombCEU were synthesized as described previously by Mountou et al. (2001), Moreau et al. (2005), and Fortin et al. (2007a), respectively (Fig. 1). EBI was purchased from TRC Biomedical Research Chemicals (North York, ON, Canada) (Fig. 1). Colchicine, vinblastine, and paclitaxel were purchased from Sigma-Aldrich. Combretastatin A-4 was synthesized according to Pettit et al. (1995). M4Beu, a human melanoma cell line, was kindly provided by Dr. J.-F. Doré (INSERM, Unit 218, Lyon, France). B16F0 murine melanoma cells and MDA-MB-231 human breast carcinoma cells were obtained from the American Type Culture Collection (Manassas, VA). Two-dimensional electrophoresis was performed using Bio-Rad reagents (Marnes-la-Coquette, France). MALDI-TOF mass spectrometry was performed using Voyager DE PRO (Applied Biosystems Inc., Foster City, CA) with postsource decay. Nano-ESI-MS/MS analyses were performed using an LCQ ion trap mass spectrometer equipped with a nanoelectrospray ion source (Thermo Fisher Scientific, Waltham, MA).

**Cell Culture, Tumor Cell Growth Inhibition and Protein Extracts.** M4Beu, B16F0, and MDA-MB-231 cells were cultured in Dulbecco’s modified minimal essential medium (Invitrogen, Cergy-Pontoise, France). All medium supplements were from Invitrogen, with the exception of fetal bovine serum, which was purchased from Biowest S.A.S. (Nuaillé, France). Culture media were supplemented with 10% fetal bovine serum, 1 mM nonessential amino acids, 1 mM sodium pyruvate, and 4 μg/ml gentamicin. Cells were maintained at 37°C in a moisture-saturated atmosphere containing 5% CO₂. GC₅₀ were determined as follows: 3.5 × 10⁻³ B16F0 and 5.0 × 10⁻³ M4Beu cells were seeded into 96-well microtiter plates (NunclonTM, Nunc, 

![Fig. 1. Molecular structures of ICEU, βBCEU, HPCEU, 4ZCombCEU, and EBI.](image-url)
Roskilde, Denmark) and incubated with escalating concentrations of the drug for 48 h. Resazurin assay was used as described by Debenton et al. (2003) to assess the number of cells. GI50 is the concentration of the drug required to inhibit 50% of the tumor cell growth. For MALDI-TOF mass spectrometry studies, B16F0 and M4Beu cell lines were plated at 3.0 × 10^5 cells/ml 24 h before incubation with the drugs. The drugs were added to the cells at concentrations equivalent to 10 × their respective GI50 values and incubated for 24 h before β-tubulin isolation and analysis. For nano-ESI MS/MS, cells were treated with a 100 μM concentration of the drugs for 16 h. For EBI analysis, cells were incubated with a 100 μM concentration of the reagent for 2 h (Legault et al., 2000). Protein extracts for two-dimensional electrophoresis were prepared essentially as described by Bouchon et al. (2005). In brief, after incubation with the drugs at the indicated concentration and time, cells were harvested by scraping, pelleted by centrifugation, and washed twice with phosphate-buffered saline. The resulting pellet was resuspended in 1 volume of 10 mM Tris-HCl buffer, pH 7.5, containing 0.25 M sucrose and 10 mM EDTA, and 4 volumes of solubilization buffer (8.4 M urea, 2.4 M thiourea, 50 mM dithiothreitol, and 5% CHAPS were added with a cocktail of protease inhibitors (Roche Diagnostics, Meylan, France). Extraction was carried out for 30 min at room temperature by vigorous shaking and was followed by ultracentrifugation at 100,000 × g for 30 min. Supernatants were recovered, and protein concentration was measured by Coomassie Brilliant Blue staining using bovine serum albumin as standard (Bradford, 1976). For immunoblotting, MDA-MB-231 and B16F0 cells were plated at 3.0 × 10^5 cells/ml for 24 h before incubation with the drugs. Drugs were added to the MDA-MB-231 cells at 30, 2.5, and 10 μM for ICEU, HPCEU, and 4ZCombCEU, respectively, and they were added to the B16F0 cells at 30, 5, and 20 μM for ICEU, HPCEU, and 4ZCombCEU, respectively. Colchicine, combretastatin A-4, vinblastine, and paclitaxel were added to the MDA-MB-231 cells at 25, 10, 5, and 5 nM, respectively. Cells were then incubated for 0, 8, 16, 24, and 36 h for ICEU, HPCEU, and 4ZCombCEU and for 0, 8, 24, and 48 h for colchicine, combretastatin A-4, vinblastine, and paclitaxel. Thereafter, floating and adherent cells were harvested using a rubber policeman and centrifuged for 3 min at 8000 rpm. Cell pellets were washed with 500 μl of ice-cold phosphate-buffered saline and stored at −80°C until use. Protein extracts for electrophoresis were prepared essentially as described by Fortin et al. (2007b). In brief, the cell pellets were lysed by the addition of 100 μl of 5X Laemmli sample buffer (60 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% mercaptoethanol, and 0.01% bromphenol blue). Cell extracts were boiled for 5 min. The protein concentration was determined using the Bradford method (Bradford, 1976).

Two-Dimensional Electrophoresis. Preparative two-dimensional gels were performed using 350 to 500 μg of total cellular protein extracts focialized on a 17-cm IPG linear strip gradient (pH 4 to 7) in solubilization buffer containing 0.8% ampholytes for 60 000 V/h. IPG strips were further treated in a buffer containing 6 M urea, 2% SDS, 30% glycerol, 50 mM Tris-HCl (pH 8.8), and 1% dithiothreitol for the first 15 min, which was replaced with 4% iodoacetamide for the next 15 min. The IPG strips were then loaded onto 12.5% SDS polyacrylamide gels. After migration, gels were stained with Colloidal Blue. Protein spots encompassing the β-tubulin area were excised and stored at −20°C until mass spectrometry analysis.

One-Dimensional Electrophoresis and Immunoblotting. Twenty micrograms of protein extracts were subjected to electrophoresis using 0.1% SDS/10% polyacrylamide gels. After migration, proteins were transferred onto nitrocellulose membranes that were incubated with TBS/TMT (Tris-buffered saline, pH 7.4, 5% fat-free dry milk and 0.1% Tween 20) for 1 h at room temperature and then with the anti-β-tubulin (clone TUB 2.1), anti-α-tubulin (H-300), or anti-acetylated α-tubulin primary antibody (H-11B-1) (1:500, TBST with 5% bovine serum albumin) for 16 h at 4°C. Membranes were then washed with TBST and incubated with 1:2500 peroxidase-conjugated anti-mouse (anti-α- and anti-β-tubulin antibodies) or anti-rabbit (anti-acetylated α-tubulin antibody) immunoglobulin (1:2500 in TBST) for 2.5 h at room temperature. After washing the membranes with TBST, detection of the immunoblotted proteins was carried out with an enhanced chemiluminescence detection reagent kit.

MALDI-TOF MS Analysis of Protein Digests. Proteins were digested in-gel with trypsin. In brief, the pieces of gel were washed thrice with 50 mM ammonium bicarbonate in 50% aqueous acetonitrile for 15 min. Digestion was performed using 120 ng of trypsin (Promega, Madison, WI) per piece of gel. After 3 h at 36°C, the resulting peptides were extracted with aqueous acetonitrile (70%), and the peptide mixture was analyzed by MALDI-TOF MS using cyano-4-hydroxy-cinnamic acid as a matrix in a positive reflector (m/z 800-3000). Internal calibration of samples was done using trypsin autolytic peptides (m/z at 842.510, 1940.935, 2211.104, monoisotopic masses). Identification of the protein using these mass fingerprinting data was carried out using ProteinProspector MS-FIT software (http://prospector.ucsf.edu/prospector/mshome.htm). Analyses were performed in a positive linear mode for higher sensitivity for the analysis of larger peptides (m/z >3000), and sinapinic acid (3,5-dimethoxy-4-hydroxy-cinnamic acid) was used as a matrix. Internal calibration was done using trypsin autolytic peptides at m/z 3339.9 and 5561.3 (average masses) for calibration. When the trypsin autolytic peptides were not present in the spectra, internal calibration was done using β-tubulin peptides already identified in the control sample.

Nano-ESI-MS/MS Analyses of Protein Digests. Analyses were performed essentially as described previously (Bouchon et al., 2005) by direct infusion of the peptide digest. The MS/MS data from the monoisotopic nonalkylated and alkylated peptides were interpreted manually, assuming the modified masses. Fragments are assigned according to the nomenclature of Roepstorff and Fohlman (1984).

Results

EBI, HPCEU, and 4ZCombCEU Inhibit Melanoma Cell Proliferation and Modify β-Tubulin Migration on SDS-PAGE. Cell growth inhibition (GI50) of EBI, HPCEU, and 4ZCombCEU was determined in mouse B16F0 and human M4Beu melanoma cell lines, respectively. As indicated in Table 1, the GI50 in both cell lines was approximately 250 nM for EBI and in the 0.5 to 5 μM range for CEU. Modified β-tubulin appeared as an extra immunoreactive band migrating faster than the native protein on SDS-PAGE. That additional β-tubulin band was observed on Western blots in both M4Beu and B16F0 melanoma cells after 2 h of incubation with EBI and after 24 h with CEU.

Mass Spectrometric Characterization of the β-Tubulin Adduct Obtained after Incubation of Cells with EBI. Figure 2, A, B, and C (left insets), shows that, after incubation of B16F0 cells with DMSO and EBI, β-tubulins were analyzed using two-dimensional electrophoresis. Native β-tubulin (Fig. 2A, right insets) and β-tubulin thioalkylated by EBI (Fig. 2B, right insets) were analyzed by MALDI-TOF mass spectrometry after digestion by trypsin. First, the MALDI-TOF reflector spectra (m/z 800-3000) of the tryptic digests covered 53.8 and 47.3% of the amino acid sequence of the murine tubulin β5-chain after DMSO and EBI treatment, respectively. The MALDI-TOF spectra of the corresponding spot obtained from M4Beu cells also identified the human tubulin β5-chain (Supplemental Fig. 1). The MALDI-TOF reflector spectra (m/z 800-3000) of the tryptic digest covered 60.4 and 50.7% of the amino acid sequence murine tubulin β5-chain after DMSO and EBI treatment, respectively. The spectra of the two murine
TABLE 1
Effects of CEUs and EBI on the proliferation of melanoma cell lines and on tubulin

<table>
<thead>
<tr>
<th>Compound</th>
<th>GI50 (µM)</th>
<th>β-Tubulin</th>
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<tr>
<td></td>
<td>B16F0</td>
<td>M4Beu</td>
</tr>
<tr>
<td></td>
<td>B16F0</td>
<td>M4Beu</td>
</tr>
<tr>
<td>HPCEU</td>
<td>1.08</td>
<td>0.68</td>
</tr>
<tr>
<td>4ZCombCEU</td>
<td>3.28</td>
<td>2.80</td>
</tr>
<tr>
<td>EBI</td>
<td>0.28</td>
<td>0.25</td>
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![Fig. 2.](image)

Fig. 2. Modification of β-tubulin by EBI. Control and EBI-treated B16F0 cell proteins were separated using two-dimensional electrophoresis, and the corresponding β-tubulin spots were excised from the gel. Digested proteins were then extracted and analyzed by MALDI-TOF-MS. Analysis was performed in two acquisition modes. A, the six peptides containing the eight Cys residues of native β-tubulin are identified and mapped on its peptide mass fingerprint. B, after EBI treatment, only four of these six peptides were present, whereas one new peptide was detected at m/z 3764.3. Missing peptides are positioned by dotted squares on the spectra. C, nano-ESI-MS/MS analysis of the newly occurring peptide allowed the localization of EBI binding on two Cys-containing peptides, namely [351–359] and [217–241]. Fragments corresponding to the cross-linked sections are in boxes. This experiment was repeated two times with similar results. 1y, C-terminal ions of peptide [351–359]; 2y, C-terminal ions of peptide [217–241]; 1b, N-terminal ions of peptide [351–359]; 2b, N-terminal ions of peptide [217–241]. D, EBI bisthioalkylation of these two peptides and their corresponding fragmentation pattern are presented.
Fig. 3. β-Tubulin acylation by 4ZCombCEU and HPCEU. A, MALDI-TOF-MS spectra of β-tubulin after treatment by 4ZCombCEU or HPCEU. Spectra obtained in B16F0 cell line may be compared with the spectrum from DMSO-treated cells (Fig. 3A). An identical spectrum was obtained from DMSO-treated M4Beu cells. Peptide masses at m/z 4906 and 4844, with mass differences of 310 and 248, respectively, with peptide [175–213] corresponding to the mass expected for that peptide when modified by the corresponding CEU. Missing peptides are positioned by dotted squares. B, peptides at m/z 4844.1 from mouse B16F0 (top) and human M4Beu (bottom) β-tubulin were analyzed by nano-ESI-MS/MS. The fragmentation pattern confirms peptide [175–213] identity and shows the acylation position. In both cases, Glu198, corresponding to E24 in the peptide was the acylation target. The portion of each spectrum identifying a maximum of relevant fragmentation is presented. In particular, fragments y10, y12, and y15 allowed us to confirm the position of the modified residue. Peptide fragments containing a CEU portion are in boxes. This experiment was repeated two times with similar results. These fragmentation positions along the peptide chain are indicated under each respective spectrum. y, C-terminal ions; b, N-terminal ions; *, loss of one water molecule.

β-tubulin spots were compared in a lower mass range (m/z 800-3000 in a reflector mode) of monoisotopic masses and in an upper mass range (m/z 3000–6000 in a linear mode) of average masses, allowing the observation of eight cysteinyl residues present in β-tubulin (Fig. 2A) (m/z 1028.5: peptide [351–359] containing Cys354; m/z 1065.4: peptide [298–306] containing Cys303, m/z 1822.9: peptide [3–19] containing Cys12; m/z 2708.4: peptide [217–241] containing Cys239; m/z 3313.7: peptide [123–154] containing Cys127 and Cys129; m/z 4596.0: peptide [175–213] containing Cys201 and Cys211). In β-tubulin treated with EBI (Fig. 2B), the peptides at m/z 1028.5 and 2708.4, respectively, were absent; and one unidentified peptide at m/z 3764.3 was found. The spectra of the two human β-tubulin spots were also compared in the two mass ranges (m/z 800-3000 and 3000−6000), and the eight cysteinyl residues present in human β-tubulin were also detected (Fig. 3A; Supplemental Fig. 1). Peptides at m/z 1028.5 and 2708.4, respectively, were absent, and one unidentified peptide at m/z 3764.9 was present (Supplemental Fig. 2). A complementary nano-ESI-MS/MS analysis was performed on β-tubulin treated with EBI to identify the newly formed peptide and to elucidate its structure. The spectrum shown in Fig. 2C identifies the two missing peptides. All of the main fragments were identified from peptides [351–359] and [217–241] obtained from β-tubulin. In peptide [351–359], fragments 1b4, 1b5, 1b6, 1y6, and 1y8 corresponded to fragments containing Cys354 linked to Cys239 via EBI. In peptide [217–241], fragments 2b22 and 2b23 allowed the localization of the modification on Cys239. Figure 2D presented the peptides and their corresponding fragmentation pattern. In conclusion, these fragmentation pattern data localized the EBI-induced modification of β-tubulin to a bisalkylation to EBI and involving peptides [351–359] and [217–241] on Cys354 and Cys239, respectively.

HPCEU and 4ZCombCEU Acylate β-Tubulin on Glu198. The β-tubulin adducts obtained from the covalent binding of CEU were isolated by two-dimensional electrophoresis, and their corresponding spots were analyzed using MALDI-TOF mass spectrometry after trypsin digestion (Fig. 3A). The MALDI-TOF reflector spectra (m/z 800-3000) of the tryptic digest covered 38.7 and 57.7% of the amino acid
sequence of the murine tubulin β5-chain after HPCEU and 4ZCombCEU treatment, respectively (Supplemental Figs. 3 and 4). The tryptic digest covered 61.9 and 49.1% of the amino acid sequence of the human tubulin β5-chain after HPCEU and 4ZCombCEU treatment, respectively (Supplemental Figs. 3 and 4). In the m/z range of 3000 to 6000, all of the spectra showed the disappearance of the m/z 4596.0 corresponding to peptide [175–213] containing Glu198, Cys201, and Cys211. An additional peak at m/z 4906.7 appeared in the spectra when cells were treated with 4ZCombCEU (Fig. 3A; Supplemental Fig. 4). This additional peptide was also observed at m/z 4844.6 and 4844.1 when B16F0 and M4Beu cells were treated with in HPCEU (Fig. 3A). These additional peptides correspond to m/z of the peptide [175–213] plus the m/z of 4ZCombCEU or HPCEU minus the hydrogen and the chlorine atoms that are both involved in the nucleophilic reaction. We also looked for the amino acid residue involved in the covalent binding of HPCEU in both cell lines (Fig. 3B). The tryptic peptide at m/z 4841.3 of HPCEU (theoretical monoisotopic mass) obtained in the lower band was fragmented using a nano-ESI source. The triply charged precursor ion at m/z 1614.4 ([M + 3H]3+) from peptide 4841.3 ([M+H]+) was isolated and fragmented. All of the main fragments were retrieved in the two spectra confirming the β-tubulin peptide [175–213] identity. Moreover, we show that the modification occurs on Glu198 residue by retrieving fragments b23, b24, y15, and y16, which correspond to unmodified and modified fragments N and C termini relative to Glu198, respectively. Figure 3B presents the peptides obtained from both cell lines and their corresponding fragmentation pattern. By use of the same approach, we further showed that the acylation of β-tubulin by 4ZCombCEU also occurs on Glu198 (Supplemental Fig. 4). Taken together, the mass spectrometry experiments clearly confirm that ICEU, HPCEU, and 4ZCombCEU acylate peptide [175–213] on Glu198 in both tumor cell lines.

**Acylation of Glu198 by ICEU, HPCEU, and 4ZCombCEU Decreases the Level of Acetylation of Lys40 on α-Tubulin.** Immunoblots of β-tubulin, α-tubulin, and acetylated α-tubulin of protein extracts from untreated MDA-MB-231 and B16F0 cells revealed that the levels of β-tubulin and α-tubulin remained constant while the level of acetylated α-tubulin increased over time (Fig. 4, left insets). When MDA-MB-231 and B16F0 cells were treated with ICEU, HPCEU, and 4ZCombCEU, the formation of CEU-β-tubulin adduct increased in a time-dependent manner (Fig. 4, center and right insets). We observed that the decrease in the level of α-tubulin acetylation level was also time-dependent,

**Discussion**

We have used MALDI-TOF and ESI mass spectrometry to identify the amino acids targeted by the thioalkylating EBI and by CEUs as exemplified by the prototypical HPCEU and 4ZCombCEU, two classes of compounds that inhibit the polymerization of the α/β-tubulin heterodimers into microtubules. These experiments conducted on murine (B16F0) and human (M4Beu) cells demonstrate that the acylation of Glu198 is a general mechanism across mammalian species bearing β1- or β2-tubulins, which contain Glu198, Cys239, and Cys354 residues.

EBI is a homobifunctional analog of iodoacetamide known to react specifically with nucleophilic sulfhydryl groups (Luduena and Roach, 1991). The high toxicity of EBI has restricted previously its use as an in vitro probe for identifying the binding site of antimitotics (e.g., colchicinoids) (Luduena and Roach, 1981a; Luduena and Roach, 1981b,c; Luduena et al., 1987), GTP, maytansine, and vinblastine (Luduena et al.,
increase of the level of the acetylation of Lys40 on the one used for the other drugs tested. Our results show involving Cys12 and Cys201/Cys211 residues in vitro. No cross-links lar cross-links involving Cys239 and Cys354, as well as 1985), or the structural and functional characterization of several alkylating agent-inactivating microtubules (Bai et al., 1989, 2000; Nguyen et al., 2005). On the other hand, Cys354 is described as an essential residue involved in the colchicine-binding activity of β-tubulin (Bai et al., 1996; Basusarkar et al., 1997). Roach and Luduena (1984) have reported that the two cross-links involving Cys239 and Cys354, as well as Cys12 and Cys201/Cys211, generate two β-tubulin adducts that migrate faster than native β-tubulin on SDS-PAGE gels. However, Legault et al. (2000) observed the formation of only one band migrating faster than the native β-tubulin when treating cells with EBI. To further document adduct formation between EBI and β-tubulin, we compared the β-tubulin adducts resulting from the covalent binding of EBI onto β-tubulin in living cells. The results shown in Fig. 2 confirm that both adducts obtained from B16F0 and M4Beu cells bear an identical cross-link involving Cys239 and Cys354. Under our experimental conditions, we were unable to detect the second cross-link reported by Roach and Luduena (1984) involving Cys12 and Cys201 or Cys211 obtained in absence of the guanine nucleotides in the in vitro settings. These results are consistent with those obtained by Roach and Luduena (1984) because guanine nucleotides are present in living cells. In addition, the results strongly suggest that Cys239 and Cys354 are the most nucleophilic and accessible cysteinyl residues on β-tubulin. We also assessed the effect of cross-linking Cys239 and Cys354 with EBI on the level of acetylation of Lys40 on α-tubulin. EBI is very toxic for the cells, which cannot be exposed more than 4 h to a 100 μM concentration of the compound. Higher time exposure results in a dramatic decrease of the tubulin signal in Western blot and the presence of numerous decaying cells. We consequently decided to perform the immunoblot experiments at 1.5 μM (5 × GI50), a GI50 in the same order of magnitude as the one used for the other drugs tested. Our results show increase of the level of the acetylation of Lys40 on α-tubulin in both B16F0 and MDA-MB-231 cells (data not shown). However, at that concentration, we did not observe the second immunoreactive β-tubulin band characteristic of the acylation of Cys239 and Cys354. Therefore, the increased level of acetylation of Lys40 does not correlate with the cross-link involving Cys239 and Cys354 by EBI.

CEUs are known to covalently bind to the colchicine-bind- ing site and to generate a β-tubulin adduct that also migrates faster than native β-tubulin in SDS-PAGE. Furthermore, mass spectrometric analyses confirmed that prototypical CEUs bind to the Glu198 residue, suggesting that all antimicrotubule CEUs might also acylate Glu198. Acylation of Glu198 was unexpected as a mechanism of protein inhibition because Glu198 is remotely located in an adjacent pocket behind the colchicine-binding site and also behind the nucleophilic Cys239 and Cys354 residues that are the targets of several alkylating agent-inactivating microtubules (Bai et al., 1989, 2000; Shan et al., 1999). We speculate that the acylation of Glu198 occurs instead of the alkylation of Cys239 due to the structure of CEUs, which is different from the other electrophilic agents studied, and because the 2-chloroethyl moiety of CEU is a much weaker electrophile than that of any other alkylation agent tested so far. According to computational simulations, the acylation of Glu198 by CEUs is favored over the alkylation of Cys239 because it requires less energy of stabilization between the electrophilic moiety of CEUs (i.e., C1 in the 2-chloroethyl moiety of CEUs) and the nucleophilic Glu198 (Fortin et al., 2009).

It is noteworthy that Wiesen et al. (2007) have shown that, in K20T cells deriving from a paclitaxel-resistant human breast cancer cell line, an E198G mutation of β-tubulin resulted in the formation of a β-tubulin band migrating faster than the native β-tubulin on SDS-PAGE gels. The latter β-tubulin mutation found in K20T cells confirms that Glu198 plays an important role in the resistance to Taxus spp. alkaloids. In addition, that mutation is presumably responsible for the decrease in α-tubulin acetylation and microtubule stability. A similar situation is observed when Glu198 is acylated by CEUs. First, the acylation of Glu198 by prototypical CEUs is shown by the formation of an adduct migrating faster than native β-tubulin in SDS-PAGE. Second, the acylation of Glu198 by antimicrotubule CEUs causes a decrease of α-tubulin acetylation without modifying the α-tubulin content. The latter phenomenon could be related either to a direct decrease in intrinsic α-tubulin acetylation, to a direct increase in its deacetylation by histone deacetylase 6 (Matsuyama et al., 2002), or to an indirect consequence of the microtubule depolymerization related to the acylation of Glu198 by CEUs.

Recently, Dorleans et al. (2009) have elegantly determined the structure of soluble tubulin unliganded at the colchicine-binding site and showed that the T7 loop occupies the colchicine site ligand, forcing the H8 helix and the intermediate domain β-sheet apart and preventing tubulin from adopting a straight conformation, as C-BS ligands do. Modifications of the molecular conformation of the C-BS by ligands, such as colchicine or podophyllotoxin, or the covalent binding of a drug to Cys239, Cys354 or Glu198 should lead to major conformational modifications preventing the α/β-tubulin heterodimer to adopt a straight conformation and therefore inhibiting microtubule assembly and probably favoring α-tubulin deacetylation or inhibiting α-tubulin acetylation by a still unidentified protein. As a consequence, we observed from Western blot analysis that the level of Lys40 acetylation decreases in cells treated by colchi- cine, combretastatin A-4, or vinblastine at 5 × their respective GI50. In the same conditions, paclitaxel increases the level of Lys40 acetylation (Fig. 5). These results confirm that α-tubulin acetylation is a key indicator of microtubule stability. In addi- tion, results obtained by Wiesen et al. (2007) using K20T cells support the hypothesis that the binding of drugs to the colchicine-binding site or in its vicinity (e.g., vinblastine, colchicine, or CEUs) results in the modification of the conformation of β-tubulin and incapacity of Glu198 to participate to the binding of a protein involved through an undetermined mechanism in the acetylation of Lys40.

In a broader perspective, other CEU subsets were shown to covalently bind to mitochondrial voltage-dependent anion channel (Patenaude et al., 2007), thioredoxin-1 (Fortin et al., 2008a,c) and prohibitin-1 (Bouchon et al., 2007) without binding to β-tubulin. The characterization of the binding of these CEUs to the aforementioned proteins has not been...
completed yet. However, Bouchon et al. (2007) have clearly demonstrated that a CEU derivative, named 4-cyclohexylphenoxy-n-chloroethylurea, acylates prohibitin-1 on Asp40, and we have shown that thioredoxin-1 and prohibitin-1 abrogates their subsequent translocation from the mitochondrial to the perinuclear compartments (Fortin et al., 2008a,b,c; J. Fortin, E. Petitclerc, and R. C.-Gaudreault, unpublished results). These results suggest that minor modifications to the aminothio moiety of CEUs may generate drugs that are able to selectively inhibit the biological activity of targeted proteins through acylation of key aspartic or glutamic acids. We believe that the present work opens a new area for the development of drugs acting through a totally new concept of acylation of acidic amino acids. Furthermore, the present work provides a framework for the design of molecules useful for studying the role of aspartyl and glutamyl residues in the biological activity of proteins under physiological conditions. The biological importance of phosphorylation of serine, threonine, or tyrosine residues, the role of cysteine and cystine residues in redox-based reactions, and the importance of the ε-amino group of lysyl residues in the activity of various proteins have been studied extensively. The present work provides support for the use of selectively modifying reagents such as CEUs for the specific study of the role of the β- and γ-carboxyl groups of Asp and Glu residues in structure/function of proteins.

**Authorship Contributions**

**Participated in research design:** Fortin, Bouchon, Moreau, Chezal, Degou, and C.-Gaudreault.

**Conducted experiments:** Fortin, Lacroix, and Degou.

**Contributed new reagents or analytic tools:** Chambron, Goutard, and T. Dollé.

**Performed data analysis:** Fortin, Bouchon, Lacroix, Moreau, Degou, and C.-Gaudreault.

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**References**


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