British Journal of Pharmacology (2007) 152, 449–455
© 2007 Nature Publishing Group All rights reserved 0007–1188/07 $30.00
www.brjpharmacol.org

RESEARCH PAPER

Alkylation of prohibitin by cyclohexylphenyl-chloroethyl urea on an aspartyl residue is associated with cell cycle G_1 arrest in B16 cells

B Bouchon[1], J Papon[2], Y Communal[3], J-C Madelmont[2] and F Degoul[2]

[1]*Univ Clermont 1, UFR Pharmacie, Clermont-Ferrand, France;* [2]*Inserm, U484, Clermont-Ferrand, France and* [3]*Centre Jean Perrin, Clermont-Ferrand, France*

Background and purpose: Phenyl-chloroethyl ureas (CEUs) are a class of anticancer drugs that mainly react with proteins. Two molecules of this family, cyclohexylphenyl-chloroethyl urea (CCEU) and iodophenyl-chloroethyl urea (ICEU) induced G_1/S and G_2/M cell cycle blocks, respectively. We hypothesised that these observations were linked to a differential protein alkylation pattern.
Experimental approach: Proteins from B16 cells incubated with [^{14}C-urea]-CCEU and [^{125}I]-ICEU were compared by 2D-analyses followed by MALDI-TOF identification of modified proteins and characterisation of the CCEU binding. Protein expression was investigated by Western blot analyses and cell cycle data were obtained by flow cytometry.
Key results: Several proteins (PDIA1, PDIA3, PDIA6, TRX, VDAC2) were alkylated by both ICEU and CCEU but β-tubulin and prohibitin (PHB) were specifically alkylated by either ICEU or CCEU respectively. Specific alkylation of these two proteins might explain the observed difference in B16 cell cycle arrest in G_2 and G_1 phases respectively. Mass spectrometry studies on the alkylated prohibitin localised the modified peptide and identified Asp-40 as the target for CCEU. This alkylation induced an increased cellular content of PHB that should contribute to the accumulation of cells in G_1 phase.
Conclusions and implications: This study reinforces our findings that CEUs alkylate proteins through an ester linkage with an acidic amino acid and shows that PHB alkylation contributes to G_1/S arrest in CCEU treated B16 cells. Modification of PHB status and/or activity is an open route for new cancer therapeutics.
British Journal of Pharmacology (2007) **152,** 449–455; doi:10.1038/sj.bjp.0707415; published online 20 August 2007

Keywords: protein targets; anticancer drugs; cell cycle; protein alkylation; prohibitin; mass spectrometry

Abbreviations: CCEU, cyclohexylphenyl-chloroethyl urea; CEU, phenyl-chloroethyl urea; ICEU, iodophenyl-chloroethyl urea; PDI, protein disulphide isomerase; PHB, prohibitin; TRX, thioredoxin; VDAC, voltage-dependent anion channel

Introduction

Phenyl-chloroethyl ureas (CEUs), resulting from the fusion of the chlorambucil aromatic moiety and the carmustine non-nitrosated pharmacophore, are developed as new anti-tumor drugs for chemotherapy. Several CEUs display cytotoxic activity in various cancer cell lines including those characterised by resistance to conventional chemotherapy (Gaudreault *et al*., 1994; Mounetou *et al*., 2001, 2003) and also in experimental tumour models (Lacroix *et al*., 1988; Miot-Noirault *et al*., 2004; Petitclerc *et al*., 2004). Interestingly, CEUs mediate their cytotoxicity by covalent binding to cell proteins, not by modifying the DNA, and the main alkylated protein for antimitotic CEUs is β-tubulin (Legault *et al*., 2000). The electrophilic 2-chloroethylamino moiety of the molecule was initially proposed to interfere with the cysteinyl-239 residue of human β-tubulin isoform 2 (Legault *et al*., 2000), but the migrational modification of β-tubulin following treatment with antimitotic CEUs was shown to be related to the esterification of glutamic acid-198 of mouse β-tubulin isoform 5 (Bouchon *et al*., 2005), located near the colchicine-binding site (Ravelli *et al*., 2004). This modification blocks the entry of tubulin in the mitotic spindle or destabilises it, leading to a G_2/M cell-cycle arrest. Among the other CEU derivatives, cyclohexylphenyl-chloroethyl urea (CCEU) has been shown to have an IC_{50} around 20 μM on a broad range of tumour cells (Mounetou *et al*., 2001).

We first found that CCEU toxicity on B16 cells was associated with an increase of cells in G_1 phase, without the alkylation of β-tubulin. We then identified the proteins alkylated by CCEU and by the microtubule disrupter ICEU and among them, we focused on prohibitin (PHB). PHB has

Correspondence: Dr F Degoul, INSERM UMR484, BP184, Rue Montalembert, Clermont-Ferrand-Cedex F-63005, France.
E-mail: degoul@inserm484.u-clermont1.fr
Received 3 May 2007; revised 29 June 2007; accepted 12 July 2007; published online 20 August 2007

been first described to play an important role in the G_1/S transition (Mishra *et al*., 2006). Its alkylation by CCEU might explain the G_1 cell-cycle arrest. We showed that PHB alkylation occurs on Asp 40 following the same chemical mechanisms as alkylation of β-tubulin and leads to an increased amount of PHB protein.

Methods

Cell cultures

Mouse melanoma B16 cells were cultured in MEM medium supplemented with 10% fetal bovine serum, 1 mM non-essential amino acids, 1 mM sodium pyruvate and 4 μg ml^{-1} gentamycin. CCEU and ICEU (Figure 1) were synthesised as described previously (Mounetou *et al*., 2001). They were dissolved in dimethylsulphoxide (DMSO) and diluted in the medium to a final concentration not exceeding 0.5% of DMSO. IC_{50}, the value at which 50% of the cells are alive, was determined by at least three experiments performed in triplicate as described previously (Debiton *et al*., 2003) using Hoechst 333342 for DNA quantification. For kinetic studies, independent dishes were treated with 100 μM CCEU in 0.5% DMSO or 0.5% DMSO as a control for the indicated periods.

Flow cytometry

Cell cycle was analysed by flow cytometry with propidium iodide staining. Cells were scraped in their medium to recover both floating and adherent cells, and centrifuged at 800 *g*, the pellets were frozen in liquid nitrogen and stored at −80°C until analysis. Cells were resuspended in phosphate-buffered saline containing RNAse (0.5 mg ml^{-1}) for 15 min. After adding propidium iodide at a final concentration of 0.05 mg ml^{-1}, analysis was performed on three different cell cultures on a Coulter EPICS XL (λ_{exc}, 488 nm; λ_{em}, 620 nm).

One- or two-dimensional electrophoresis and immunoblotting

Protein extracts and one- (1D) or two-dimensional (2D) electrophoresis were prepared as described previously (Bouchon *et al*., 2005). The Western blot analyses were performed with anti-PHB antibodies (II-14-10, Abcam, Paris, France) and with anti-β-tubulin antibodies (Tub2-1, Sigma, France). 2D gel analyses and protein quantification were performed with PD Quest software (Bio-Rad, Marnes-la-Coquette, St Quentin, Fallavier, France).

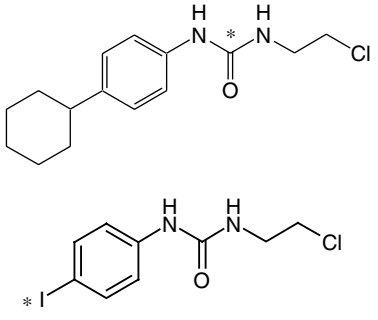

Figure 1 Chemical structure of CCEU and ICEU. The calculated monoisotopic masses for CCEU and ICEU are 280.13 and 323.95 Da, respectively, for the complete molecules, and 245.16 and 288.98 Da without the chlorine atom. *Shows the positions of the radiolabel. CCEU, cyclohexylphenyl-chloroethyl urea; ICEU, iodophenyl-chloroethyl urea.

Matrix-assisted laser desorption/ionisation–time of flight–mass spectrometry analysis of protein digests

Matrix-assisted laser desorption/ionisation–time of flight–mass spectrometry (MALDI-TOF-MS) analysis was performed on a Voyager DE-PRO mass spectrometer (Applied Biosystems, Framingham, MA, USA) as described previously (Bouchon *et al*., 2005). Trypsin digestion was performed with 100 ng trypsin (Promega, Madison, WI, USA) per gel piece, for 3 h at 36°C. The resulting peptides were analysed using cyano-4-hydroxycinnamic acid as a matrix in a positive reflector or linear 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). Post –source decay (PSD) analysis was performed at an accelerating voltage of 20 kV, using a timed ion gating for the selection of the precursor ion.

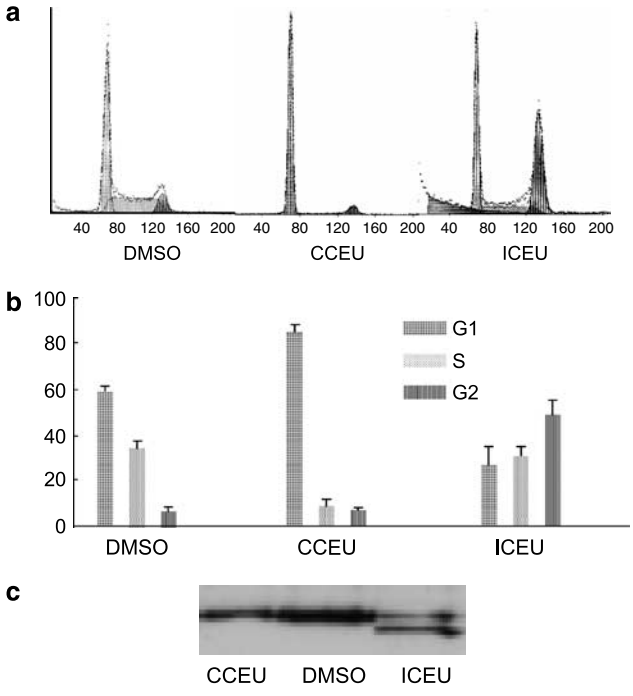

Figure 2 CCEU and ICEU block B16 cell cycle at G_1/S and at G_2/M transition, respectively. (**a**) Typical flow cytometry analysis with propidium iodide showing a decrease of cells in S phase, whereas the number of cells in G_1 phase increased after CCEU treatment. In contrast, ICEU treatment leads to a major increase of cells in G_2 phase due to a block in the G_2/M transition, when compared to DMSO-treated cells (control). (**b**) Distribution of cells in the different cell-cycle phases expressed as percentages. Statistical differences are found between controls and treated cells by the χ^2 test ($P<0.05$). (**c**) Western blot analysis: ICEU, but not CCEU, modified the migration properties of β-tubulin in extracts of B16 cells. CCEU, cyclohexyl-phenyl-chloroethyl urea; ICEU, iodophenyl-chloroethyl urea.

Results

Cell-cycle arrest results from alkylation of different proteins

The IC_{50} of ICEU and CCEU in B16 cells was 9.9 ± 2.1 and $17.8 \pm 2.3\,\mu$M, respectively, as determined by Hoechst DNA quantification. Flow cytometry analysis of B16 cells treated for 24 h with $100\,\mu$M ICEU or $100\,\mu$M CCEU displayed a different cell-cycle pattern as compared to cells treated with DMSO as control (Figure 2a); ICEU increased the percentage of cells in G_2 phase whereas CCEU-treated cells were predominantly found in G_1 phase. This G_1 increase resulted in a drastic reduction of S phase cells (Figure 2b). Twenty percent of ICEU-treated B16 cells were apoptotic after 24 h of treatment as determined by sub-G_1 analyses with flow cytometry, compared to 10% in control cells, whereas in the same conditions no apoptotic cells were found in CCEU-treated B16 cells (data not shown). The incubation of B16 with CCEU did not modify the β-tubulin migration in contrast to ICEU that alkylated this protein as revealed by Western blot analysis (Figure 2c).

To understand the molecular mechanisms underlying such a difference, B16 cells were incubated with [^{14}C-urea]-CCEU or [^{125}I]-ICEU, and resulting protein extracts were analysed by 2D electrophoresis, followed by autoradiography. The large nonlinear 3–10 pH range strip revealed a similar number of radiolabelled proteins in the two cases. $\gamma-$Radiation from ^{125}I-labelled ICEU produced an autoradiogram with intense spots (Figure 3b, right), whereas β-radiation obtained by ^{14}C labelling of CCEU was responsible for relatively weak signals (Figure 3a, right). In CCEU extracts (Figure 3a), at least six labelled spots could be observed and nine in ICEU-treated cells (Figure 3b). These spots were recovered from non-radioactive gels (Figures 3a and b, left) and were identified by MS following trypsin digestion. Among the six identified spots on each gel (Table 1), five proteins were common in both treatments. They were identified as oxidoreductase protein disulphide isomerases (PDIA1, PDIA3 and PDIA6), thioredoxin (TRX), and the mitochondrial voltage-dependent anion channel-2 (VDAC2). The two specifically alkylated proteins were PHB for CCEU and β-tubulin for ICEU. Furthermore, determination of the specific alkylation activity (Table 1) showed that β-tubulin and PHB were the main targets for ICEU and CCEU, respectively, whereas VDAC2 was the second one for each molecule. PDIs and TRX were alkylated at a lower degree (except PDIA6 for ICEU).

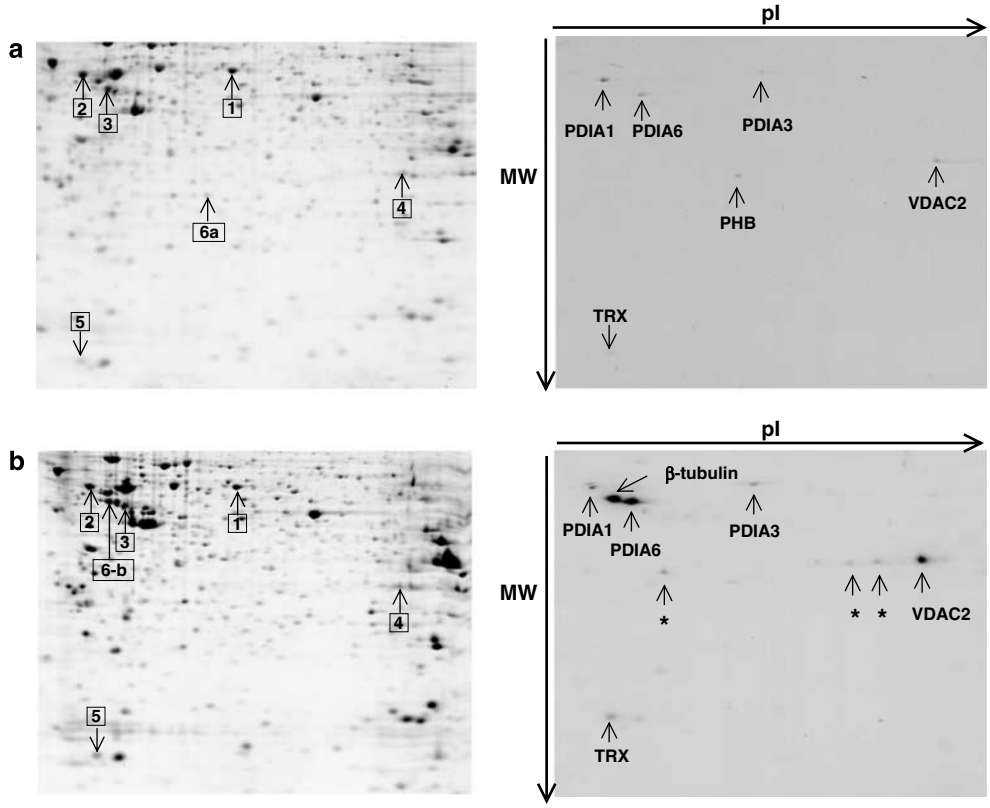

Figure 3 CCEU and ICEU alkylate common and specific B16 cell proteins. 2D electrophoresis on 3–10 nonlinear pH range strips: proteins were extracted from B16 cells treated for 24 h with $100\,\mu$M CCEU (**a**) or $100\,\mu$M ICEU (**b**), unlabelled (left) or radiolabelled ([^{14}C]-CCEU, 19 GBq/mmol and [^{125}I]-ICEU, 1.5 GBq/mmol) (right). They were stained with colloidal blue (left) or dried before autoradiography of ^{14}C or ^{125}I (right, **a** and **b,** respectively). Spots equivalent to the radioactive spots (numbers) were recovered on Coomassie-stained gels and identified by MALDI-TOF-MS. From the six major labelled spots, spots 1–5 were alkylated by both CEUs whereas spots 6-a and 6-b were specifically identified after CCEU or ICEU treatment, respectively. * Corresponds to radiolabelled materials that could not be identified, as they did not correspond to defined spots on a coloured gel. CCEU, cyclohexylphenyl-chloroethyl urea; ICEU, iodophenyl-chloroethyl urea; MALDI-TOF-MS, matrix-assisted laser desorption/ionisation–time of flight–mass spectrometry.

CCEU binds to PHB on Asp 40

After incubation of B16 cells with 100 μM CCEU, the protein pattern on 2D gels was modified in the PHB area as compared to the same area from DMSO-treated cells (Figure 4a). Western blot analysis showed that two spots were recognised by anti-PHB antibody and that only the basic one was radioactive (Figure 4b); no detectable additional spot could be found in B16 cells treated with DMSO and the identified PHB spot (P2) corresponding to the ^{14}C PHB (Figure 3a right and Figure 4b bottom) was absent in the control, whereas the P1 spot with the same apparent MW but with a more acidic pI was present in both samples.

MALDI-TOF-MS analyses of these two spots from the CEU-treated cells were performed, P1 as well as P2 were identified as PHB. The comparison of these spectra indicated that the single Cys residue was observed unmodified in the peptide at *m/z* 3125 in both cases (Figure 4c), but showed the presence of an unexplained ion at *m/z* 964 in the basic protein that was absent in the acidic one. In contrast, the acidic PHB contained a *m/z* 720, identified as [Ala 36-Arg 41] which was completely absent from the basic protein (Figure 4d). The mass difference between the two ions ($\Delta m = 244$) corresponded to the expected mass shift for binding of a cyclohexylphenyl-ethyl urea moiety. This indicated that

Table 1 Identification of the proteins alkylated by CCEU and ICEU

Spot number in CCEU B16 extracts	*Spot number in ICEU B16 extracts*	*Protein identification*	*Swiss Prot accession number*
1 *(3)*	1 *(5)*	PDIA3	P 27773
2 *(5)*	2 *(6)*	PDIA1	P 09103
3 *(4)*	3 *(3)*	PDIA6	Q 922R8
4 *(2)*	4 *(2)*	VDAC2	Q 60930
5 *(nd)*	5 *(4)*	TRX	P 10369
6-a *(1)*		Prohibitin	P 67778
	6-b *(1)*	β-Tubulin	P 99024

CCEU, cyclohexylphenyl-chloroethyl urea; ICEU, iodophenyl-chloroethyl urea; nd: not determined; PDI, protein disulphide isomerase; TRX, thioredoxin; VDAC2, voltage-dependent anion channel.
The spot numbers correspond to Figure 3. The numbers in the brackets represent the degree of protein alkylation, estimated for each protein spot by the ratio 'c.p.m./estimated protein amount in pmol' (1: highest, 6: lowest alkylation rate).

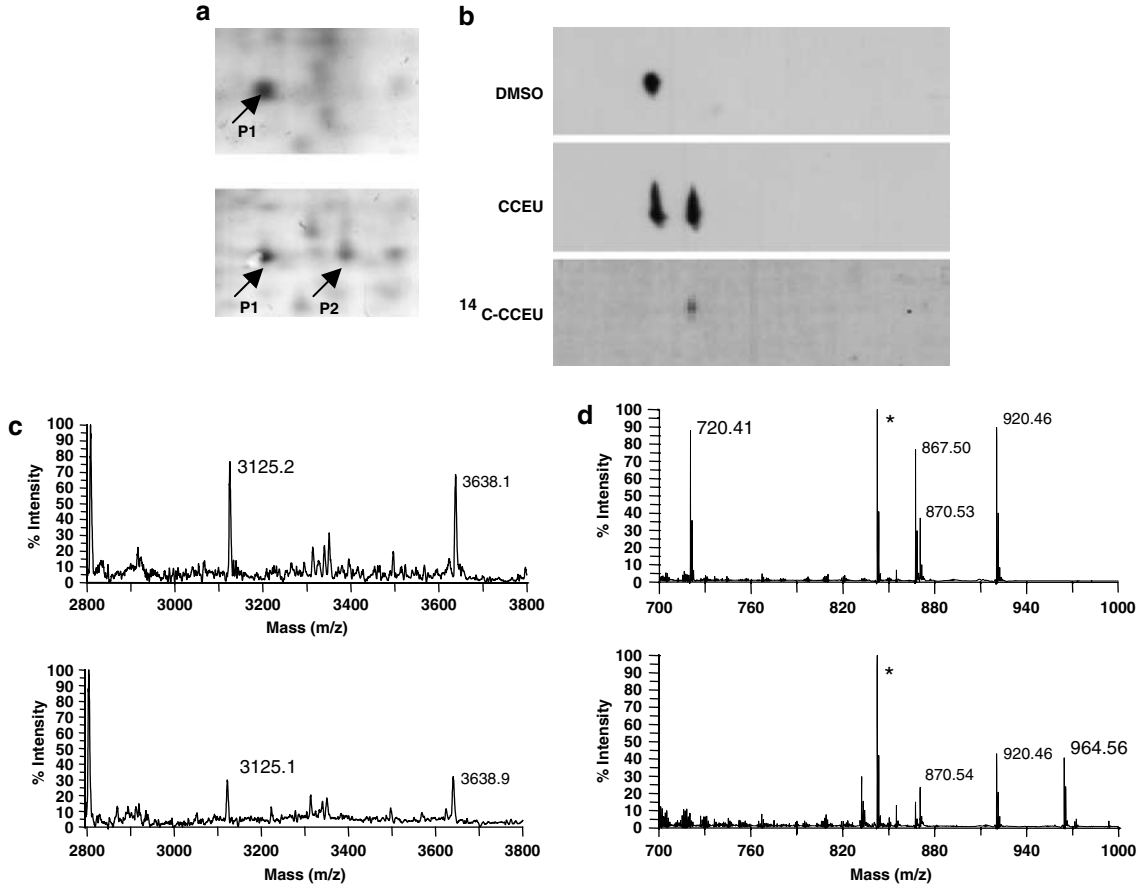

Figure 4 CCEU covalently alkylates PHB on an Asp residue. (**a**) 2D gel analysis of proteins extracted from cells treated for 24 h with 100 μM CCEU (bottom) revealed one additional spot (P2) when compared to DMSO-treated cells (top). (**b**) Western blot analysis showed that two PHB spots were only present in CCEU-treated B16 cells, the basic one being labelled by [^{14}C]-CCEU as demonstrated by autoradiography of the membrane. (**c**) MALDI-TOF-MS comparison of the two proteins identified as PHB. The ion at *m/z* 3125 corresponds to the peptide [Gly 44-Arg 70] containing the only Cys residue of PHB for the acidic PHB (top) or basic PHB (bottom). (**d**) MALDI-TOF-MS comparison of the PHB spots. An ion at *m/z* 720.41, corresponding to peptide [Ala 36-Arg 41] present in the original acidic PHB (top) was absent in the basic PHB, which in contrast contained an ion at *m/z* 964.56 (bottom). The mass difference ($\Delta m = 244.15$) was in accordance with the presence of a CCEU residue on peptide [Ala 36-Arg 41]. * Corresponds to trypsin autolysis peptide (*m/z* = 842.5100). CCEU, cyclohexylphenyl-chloroethyl urea; DMSO, dimethylsulphoxide; MALDI-TOF-MS, matrix-assisted laser desorption/ionisation–time of flight–mass spectrometry; PHB, prohibitin.

the basic PHB was alkylated, whereas the acidic one corresponded to an unalkylated protein. To characterise this ion unambiguously, a PSD analysis was performed directly on the same sample (Figure 5a). The fragmentation pattern confirmed the identity of [Ala 36-Arg 41] peptide (b and y fragments), and indicated clearly the presence of the urea derivative on the Asp 40 residue. The mass difference between b3 and b5 fragments corresponds to Phe and Asp residues plus the CEU derivative. This CEU derivative could be fragmented at the ester bond, giving rise to the unmodified peptide ([M-CEU]), or fragmented at its peptide-like bond, giving rise to a characteristic ion ([M-CEU#], $m/z = 763.7$, see Figures 5a and b).

Alkylation of PHB is associated with cell-cycle arrest

The number of B16 cells in G_1 phase increased from 50 to 80% after treatment for 8–16 h with 100 μM of CCEU (Figure 6a), whereas control cells treated with DMSO showed no change. This increase was concomitant with the appearance of the basic spot identified as the modified PHB (Figure 6b). As PHB was present in two forms, the total amount of PHB in the cellular extracts was estimated by Western blotting and compared to β-tubulin content (Figure 6c). It was clear that the total PHB content increased in CCEU-treated cells in a time-dependent manner.

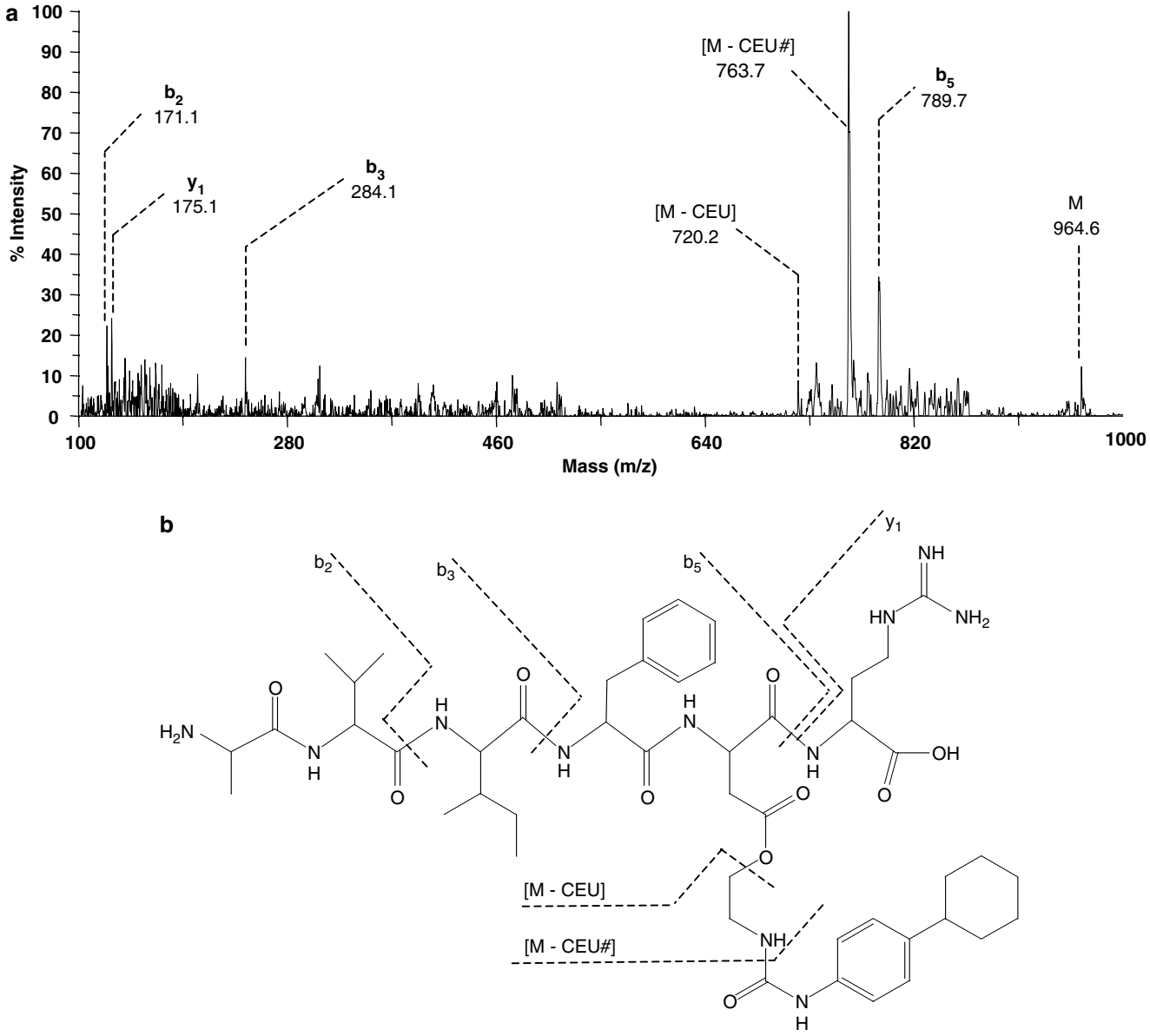

Figure 5 CCEU covalently alkylates PHB by an ester bond. (**a**) Fragmentation spectrum of the modified peptide [Ala 36-Arg 41]. Specific fragmentation ions are indicated in the figure. (**b**) Structure of the alkylated peptide, CEU# indicates a fragmented CEU. CEU, phenyl-chloroethyl urea; CCEU, cyclohexylphenyl-chloroethyl urea; PHB, prohibitin.

Discussion

CCEU and ICEU covalently alkylated six proteins in B16 cells that were identified. ICEU and CCEU particularly bound to the same PDI family proteins (PDIA, PDIA3 and PDIA6), to TRX and to VDAC2. According to the alkylation rates (Table 1), VDAC2 is the second target for the two drugs and PDIs are alkylated in a different order depending on the compound that was used. CCEU-TRX spot was too small and too diffuse to be counted in the ^{14}CCEU experiments and thus was not ranked. These common targets VDAC2 and PDI (and probably TRX) could present a quite similar nucleophilic structure. Moreover, PDIs and TRX share some amino-acid sequence homologies (see below). Besides these common targets, β-tubulin was found to be specifically alkylated by ICEU, whereas PHB was a major target for CCEU (Figure 3, Table 1). The lack of β-tubulin alkylation by CCEU may result from the chemical or physical properties of the lateral group which should impede this covalent link, as it has been demonstrated for related compounds (Fortin *et al*., 2007). On the other hand, the absence of PHB alkylation by ICEU should rather be a consequence of an increased affinity for β-tubulin leading to a G_2/M arrest and potentially to a decrease of all regulatory proteins from the G_1 and/or S phase. PHB alkylation leads to a shift in isoelectric point (Figure 4a) consistent with the loss of an acidic charge, indicating that alkylation should occur on a charged residue. Indeed, the peptide implicated in the binding of CCEU on PHB was identified close to its N-terminal region, and MS analyses demonstrated that CCEU bound to PHB at Asp 40. This link was made by an ester bond, in the same manner as the binding of ICEU to β-tubulin occurred at position 198 on a Glu residue (Bouchon *et al*., 2005). Alkylation of β-tubulin and PHB followed the same mechanism, no mass modification of peptides carrying Cys residue could be found, thus showing an original way of protein binding. Determination of other alkylation sites for VDAC2, PDI and TRX are currently ongoing to confirm a general mechanism for CEU alkylation.

PHB and β-tubulin alkylation could account for the observed modification of the cell cycle following CCEU and ICEU treatments, respectively. Indeed as reported previously, β-tubulin alkylation lead to a G_2/M blockage by inducing a depolymerisation of microtubules. PHB may participate in regulating the G_1/S phase transition. As observed in 2D and 1D gels, PHB content increases in CCEU-treated cells. The amount of PHB could modulate proliferation of cell lines (Peng *et al*., 2006) even if its first described action (control of G_1/S transition) seemed to be performed by the PHB mRNA rather than by the protein (Manjeshwar *et al*., 2003). This is still a matter of debate which is complicated by the pleiotropic location of PHB in nucleus and cytoplasm (Mishra *et al*., 2005). Mitochondrial PHB is dedicated to chaperone function (Nijtmans *et al*., 2000, 2002), the nuclear PHB is implicated in the transcription regulation of various genes (Fusaro *et al*., 2003) and cytosolic PHB could be linked to the transduction signal from epidermal growth factor receptors and activation of the

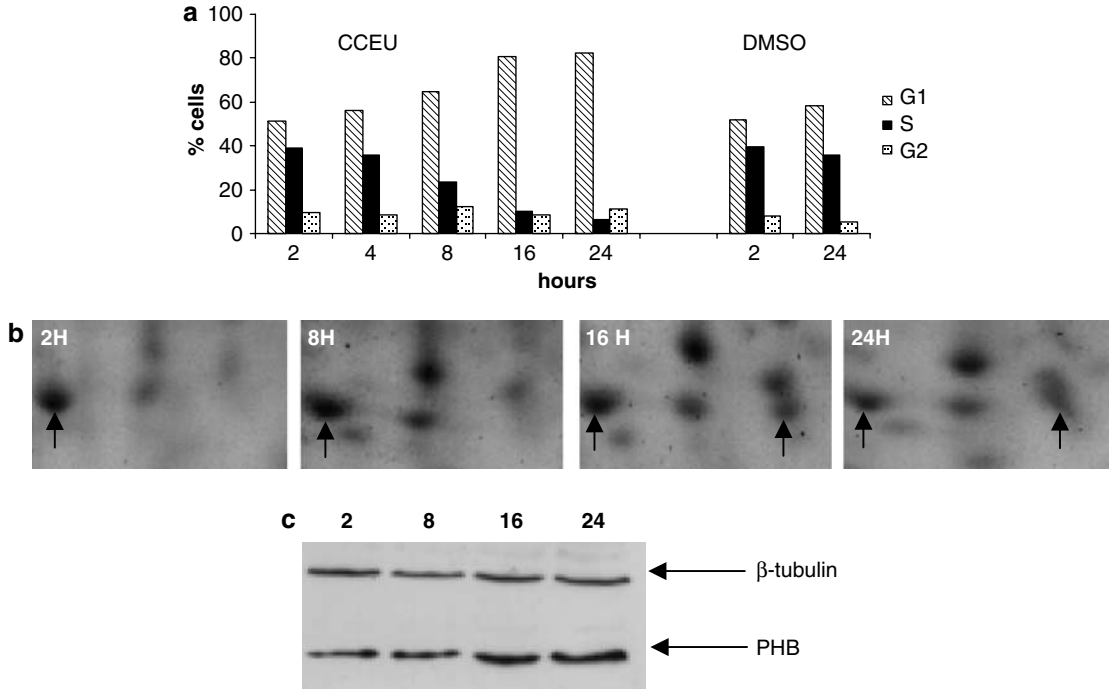

Figure 6 Time course of CCEU-induced cell-cycle arrest paralleled PHB accumulation in cellular extracts. (**a**) A G_1 increase, determined in kinetic studies of B16 cells treated with 100 μM CCEU occurred after 8 h and remained stable (75–80%) at 24 h, compared to DMSO-treated cells. (**b**) 2D electrophoreses of CCEU-treated B16 cell protein extracts showed that the additional spot appeared between 8 and 16 h incubation. (**c**) Analysis of PHB content in whole-cell extracts by Western blotting: an increased PHB amount was detected after 8–16 h of incubation, using anti-PHB and anti-β-tubulin antibodies for normalisation. Data presented above are representative of at least two independent experiments. CCEU, cyclohexylphenyl-chloroethyl urea; DMSO, dimethylsulphoxide; PHB, prohibitin.

MAP kinase (Wang *et al*., 2004). From the present study we cannot tell if all or only a fraction, of cellular PHB is modified. Nevertheless, we still can hypothesise that the alkylation in the N-terminal portion of PHB modifies its trafficking, thereby impairing mitochondrial import, whereas the C-terminal portion is implicated in the translocation to nuclear membranes (Rastogi *et al*., 2006). Alkylation by CCEU could increase the nuclear fraction of PHB which interacts with transcription factors such as E2F1 and P53 and so modify the cell cycle (Joshi *et al*., 2003).

VDAC2, the second target for both CEUs, is also located in mitochondria, suggesting a subcellular concentration of CEUs in mitochondria. CEUs are small molecules that do not need a transporter and they should move freely through the endoplasmic reticulum (ER) to alkylate PDIs. Interestingly, these ER proteins exhibit a TRX pattern (Freedman *et al*., 2002), suggesting that they present a common target where the CEUs could bind covalently. PDIs display two TRX motifs and alignment studies show a strong homology for a stretch of 12 amino acids encompassing a cysteinyl doublet and interestingly an acidic residue (Asp or Glu), which could be the potential target for CEUs. Other studies are currently ongoing to identify amino acids involved in the binding of CEUs to other major proteins, and to clarify the detailed mechanism of this binding.

In conclusion, our data has shown some interesting properties of CEUs in alkylating proteins crucially involved in the cell cycle, through the formation of ester bonds on acidic amino acids. These findings encourage the assessment of the functional consequences of these alkylations with the aim of developing non-mutagenic chemotherapy, with specific protein targets.

Acknowledgements

We thank Dr E Debiton for protocols of microplate assays, Dr E Mounetou and Dr M Azim for molecule labelling and Dr M Theisen for critical reading of this paper.

Conflict of interest

The authors state no conflict of interest.

References

Bouchon B, Chambon C, Mounetou E, Papon J, Miot-Noirault E, Gaudreault RC *et al*. (2005). Alkylation of beta-tubulin on Glu 198 by a microtubule disrupter. *Mol Pharmacol* **68**: 1415–1422.

Debiton E, Madelmont JC, Legault J, Barthomeuf C (2003). Sanguinarine-induced apoptosis is associated with an early and severe cellular glutathione depletion. *Cancer Chemother Pharmacol* **51**: 474–482.

Fortin S, Moreau E, Patenaude A, Desjardins M, Lacroix J, Rousseau JL *et al.* (2007). *N*-Phenyl-*N*′-(2-chloroethyl)ureas (CEU) as potential antineoplastic agents. Part 2: role of omega-hydroxyl group in the covalent binding to beta-tubulin. *Bioorg Med Chem* **15**: 1430–1438.

Freedman RB, Klappa P, Ruddock LW (2002). Protein disulfide isomerases exploit synergy between catalytic and specific binding domains. *EMBO Rep* **3**: 136–140.

Fusaro G, Dasgupta P, Rastogi S, Joshi B, Chellappan S (2003). Prohibitin induces the transcriptional activity of p53 and is exported from the nucleus upon apoptotic signaling. *J Biol Chem* **278**: 47853–47861.

Gaudreault RC, Alaui-Jamali MA, Batist G, Bechard P, Lacroix J, Poyet P (1994). Lack of cross-resistance to a new cytotoxic arylchloroethyl urea in various drug-resistant tumor cells. *Cancer Chemother Pharmacol* **33**: 489–492.

Joshi B, Ko D, Ordonez-Ercan D, Chellappan SP (2003). A putative coiled-coil domain of prohibitin is sufficient to repress E2F1-mediated transcription and induce apoptosis. *Biochem Biophys Res Commun* **312**: 459–466.

Lacroix J, Gaudreault RC, Page M, Joly LP (1988). *In vitro* and *in vivo* activity of 1-aryl-3-(2-chloroethyl) urea derivatives as new antineoplastic agents. *Anticancer Res* **8**: 595–598.

Legault J, Gaulin JF, Mounetou E, Bolduc S, Lacroix J, Poyet P *et al.* (2000). Microtubule disruption induced *in vivo* by alkylation of beta-tubulin by 1-aryl-3-(2-chloroethyl)ureas, a novel class of soft alkylating agents. *Cancer Res* **60**: 985–992.

Manjeshwar S, Branam DE, Lerner MR, Brackett DJ, Jupe ER (2003). Tumor suppression by the prohibitin gene 3′ untranslated region RNA in human breast cancer. *Cancer Res* **63**: 5251–5256.

Miot-Noirault E, Legault J, Cachin F, Mounetou E, Degoul F, Gaudreault RC *et al.* (2004). Antineoplastic potency of arylchloroethylurea derivatives in murine colon carcinoma. *Invest New Drugs* **22**: 369–378.

Mishra S, Murphy LC, Murphy LJ (2006). The Prohibitins: emerging roles in diverse functions. *J Cell Mol Med* **10**: 353–363.

Mishra S, Murphy LC, Nyomba BL, Murphy LJ (2005). Prohibitin: a potential target for new therapeutics. *Trends Mol Med* **11**: 192–197.

Mounetou E, Legault J, Lacroix J, Gaudreault RC (2001). Antimitotic antitumor agents: synthesis, structure-activity relationships, and biological characterization of *N*-aryl-*N*′-(2-chloroethyl)ureas as new selective alkylating agents. *J Med Chem* **44**: 694–702.

Mounetou E, Legault J, Lacroix J, Gaudreault RC (2003). A new generation of *N*-aryl-*N*′-(1-alkyl-2-chloroethyl)ureas as microtubule disrupters: synthesis, antiproliferative activity, and beta-tubulin alkylation kinetics. *J Med Chem* **46**: 5055–5063.

Nijtmans LG, Artal SM, Grivell LA, Coates PJ (2002). The mitochondrial PHB complex: roles in mitochondrial respiratory complex assembly, ageing and degenerative disease. *Cell Mol Life Sci* **59**: 143–155.

Nijtmans LG, de Jong L, Artal Sanz M, Coates PJ, Berden JA, Back JW *et al.* (2000). Prohibitins act as a membrane-bound chaperone for the stabilization of mitochondrial proteins. *EMBO J* **19**: 2444–2451.

Peng X, Mehta R, Wang S, Chellappan S, Mehta RG (2006). Prohibitin is a novel target gene of vitamin D involved in its antiproliferative action in breast cancer cells. *Cancer Res* **66**: 7361–7369.

Petitclerc E, Deschesnes RG, Cote MF, Marquis C, Janvier R, Lacroix J *et al.* (2004). Antiangiogenic and antitumoral activity of phenyl-3-(2-chloroethyl)ureas: a class of soft alkylating agents disrupting microtubules that are unaffected by cell adhesion-mediated drug resistance. *Cancer Res* **64**: 4654–4663.

Rastogi S, Joshi B, Fusaro G, Chellappan S (2006). Camptothecin induces nuclear export of prohibitin preferentially in transformed cells through a CRM-1-dependent mechanism. *J Biol Chem* **281**: 2951–2959.

Ravelli RB, Gigant B, Curmi PA, Jourdain I, Lachkar S, Sobel A *et al.* (2004). Insight into tubulin regulation from a complex with colchicine and a stathmin-like domain. *Nature* **428**: 198–202.

Wang S, Zhang B, Faller DV (2004). BRG1/BRM and prohibitin are required for growth suppression by estrogen antagonists. *EMBO J* **23**: 2293–2303.