



Intramolecular Cyclization of N-phenyl N'(2-chloroethyl)ureas leads to Active N-phenyl-4,5-dihydrooxazol-2-amines Alkylating β -Tubulin Glu198 and Prohibitin Asp40

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Chambon, Yves Communal, Jean-Michel Chezal, Elisabeth
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Intramolecular Cyclization of *N*-phenyl *N'*(2-chloroethyl)ureas leads to Active *N*-phenyl-4,5-dihydrooxazol-2-amines Alkylating β -Tubulin Glu198 and Prohibitin Asp40.

Anna Trzeciakiewicz^{1,2}, Sébastien Fortin^{1,2,3}, Emmanuel Moreau^{1,2}, René C.-Gaudreault³, Jacques Lacroix³, Christophe Chambon⁴, Yves Communal⁵, Jean-Michel Chezal^{1,2}, Elisabeth Miot-Noirault^{1,2}, Bernadette Bouchon^{1,2} and Françoise Degoul^{1,2*}

¹ U990 INSERM, Rue Montalembert, BP 184, 63000 Clermont-Ferrand, France

² Clermont Université, Université d'Auvergne, Imagerie moléculaire et thérapie vectorisée, BP 10448, 63000 Clermont-Ferrand, France

³ CR-CHUQ, Hôpital Saint-François d'Assise, Université Laval, Unité des Biotechnologies et de Bioingénierie, Québec, Canada, G1L 3L5

⁴ INRA Clermont-Ferrand/Theix, Plate-forme Protéomique, 63122 Saint-Genès-Champanelle, France

⁵ Centre Jean Perrin, Rue Montalembert, 63011 Clermont-Ferrand, France

* **Corresponding author:** francoise.degoul@inserm.fr, tel: +33(0)473150817;

fax : +33(0)473150801

Author e-mails addresses:

AT: trzeciakiewicz@gmail.com; SF: sebastien.fortin.1@ulaval.ca;

EM: Emmanuel.Moreau@u-clermont1.fr; R C-G: rene.c-gaudreault@crsfa.ulaval.ca

JL: Jacques.Lacroix@crsfa.ulaval.ca CC: Christophe.chambon@clermont.inra.fr

YC: Yves.COMMUNAL@cjp.fr; JM C: j-michel.chezal@u-clermont1.fr;

EMN: elisabeth.noirault@inserm.fr; BB: bernadette.bouchon@inserm.fr;

Abstract

1
2 The cyclization of anticancer drugs into active intermediates has been reported mainly for
3
4 DNA alkylating molecules including nitrosoureas. We previously defined the original
5
6 cytotoxic mechanism of anticancerous *N*-phenyl *N'*(2-chloroethyl)ureas (CEUs) that involves
7
8 their reactivity towards cellular proteins and not against DNA; two CEUs subsets have been
9
10 shown to alkylate β -tubulin and prohibitin leading to inhibition of cell proliferation by G₂/M
11
12 or G₁/S cell cycle arrest. In this study, we demonstrated that cyclic derivatives of CEUs, *N*-
13
14 phenyl-4,5-dihydrooxazol-2-amines (Oxas) are two to threefold more active than CEUs and
15
16 share the same cytotoxic properties in B16F0 melanoma cells. Moreover, the CEU original
17
18 covalent binding by an ester linkage on β -tubulin Glu198 and prohibitin Asp40 was
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20 maintained with Oxas. Surprisingly, we observed that Oxas were spontaneously formed from
21
22 CEUs in the cell culture medium and were also detected within the cells. Our results suggest
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24 that the intramolecular cyclization of CEUs leads to active Oxas that should then be
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26 considered as the key intermediates for protein alkylation. These results could be useful for
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28 the design of new prodrugs for cancer chemotherapy.
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39 **Keywords:** *N*-phenyl *N'*(2-chloroethyl)ureas, *N*-phenyl-4,5-dihydrooxazol-2-amines, β -
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41 tubulin, prohibitin, alkylation, cyclization
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1. Introduction

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2 A major challenge for improving cancer chemotherapy is to design and synthesize new
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4 drugs that can selectively target cancer cells and bypass cancer chemoresistance systems. In
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6 this context, we have developed protein alkylating agents referred to as *N*-phenyl-*N'*-(2-
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8 chloroethyl)ureas (CEUs) [1, 2]. Two CEU subsets have been shown to selectively alkylate
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10 proteins through a unique ester linkage involving Glu198 on β -tubulin and Asp40 on
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12 prohibitin [3, 4]. Both proteins are strongly involved in the regulation of cell growth and cell
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14 cycle progression [5, 6]. Tubulins are classical targets for antimitotic drugs such as colchicine,
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16 vinblastine and paclitaxel [7] blocking the G₂/M transition of the cell cycle. Prohibitin, a
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18 chaperon protein involved in gene expression, regulates cell cycle and apoptosis, and has been
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20 considered as a tumor suppressor [5, 8]. However, no consensus for prohibitin function has
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22 been established since prohibitin is also required for primary and cancer cell proliferation [9,
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24 10]. The decreased G₁/S transition in CEU treated cells, associated with prohibitin alkylation
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26 is not fully understood and could be linked to either a mitochondrial dysfunction [11] or a
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28 nuclear repression of E2F1-mediated transcription [12]. β -tubulin alkylating CEUs induce a
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30 G₂/M arrest and have obvious antimitotic effects on different types of tumors both *in vitro* and
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32 *in vivo* [13-17]. Hence; the molecular events underlying CEU antiproliferative activity rely on
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34 the functionality of the targeted protein [3].
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44 The requirement of the chlorine atom in the CEU β -tubulin alkylation mechanism was
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46 clearly established using conventional structure-activity relationship (SAR) studies showing
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48 that *N*-phenyl-*N'*-ethyl ureas were devoid of cytotoxicity and unable to alkylate β -tubulin [1].
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50 Recently, we unexpectedly found that *N*-phenyl-4,5-dihydrooxazol-2-amines (Oxas) were
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52 more active than their CEU counterparts and still alkylating β -tubulin as demonstrated by
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54 western blot analysis [18]. It has been reported previously that the presence of oxazolinic
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heterocycles improves the efficiency of molecules developed for pancreatic cancer treatment [19] as well as of drugs inhibiting angiogenesis [20].

Here, we have determined the cytotoxicity mechanisms of three different Oxas and characterize their covalent binding to target proteins in B16F0 melanoma cells at the molecular level. Our results clearly showed that Oxas and their parent CEUs have the same β -tubulin and prohibitin amino acid targets. We further observed, for the first time, that the cyclization of CEUs into Oxas occurs spontaneously in the culture medium and that these Oxa forms were present within the cells. These data suggest that Oxas could be the reactive intermediates of CEUs.

2. Material and methods

2.1 Chemicals

N-(4-iodophenyl)-*N'*-(2-chloroethyl)urea (ICEU), *N*-(4-iodophenyl)-4,5-dihydrooxazol-2-amine (IOxa), *N*-(4-tertbutylphenyl)-*N'*-(2-chloroethyl)urea (*t*BCEU), *N*-(4-tertbutylphenyl)-4,5-dihydrooxazol-2-amine (*t*BOxa), *N*-(4-cyclohexylphenyl)-*N'*-(2-chloroethyl)urea (cHCEU), *N*-(4-cyclohexylphenyl)-4,5-dihydrooxazol-2-amine (cHOxa) were synthesized as previously described [21, 22]. All compounds were dissolved in DMSO at a concentration of 40 mM and stored at -20°C. The experiments were all performed with a final concentration of 0.5% DMSO in culture medium.

2.2 Cell culture

B16F0 murine melanoma cell line was purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in DMEM (Invitrogen, Cergy-Pontoise, France) supplemented with 10% fetal bovine serum (Biowest, Paris, France), 1 mM non-essential amino acids (Invitrogen), 1 mM sodium pyruvate (Invitrogen), and 4 μ g/mL gentamicin

1 (Invitrogen). The cells were maintained at 37 °C in a moisture-saturated atmosphere
2 containing 5% CO₂.
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4 **2.3 Antiproliferative activity**

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7 Cell cytotoxicity was assessed using the resazurin assay as previously described [23]. To
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9 determine growth inhibitory concentrations, which is the concentration of the drug required to
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11 inhibit 50% of the tumor cell growth (GI₅₀), 3.5 x 10³ B16F0 cells were seeded into 96-well
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13 microtiter plates (Nunclon™, Nunc, Roskilde, Denmark) and maintained at 37°C in a
14
15 moisture-saturated atmosphere containing 5% CO₂ for 24 hours and then incubated with
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17 escalating drug concentrations (1-50 μM) for 48 hours. Fluorescence (excitation/emission:
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19 530/590 nm) was measured with a microtiter plate fluorescence reader (Fluoroskan Ascent
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21 FL, Thermo Labsystems, Helsinki, Finland).
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25 **2.4 Cell cycle progression analysis - flow cytometry**

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27 Cell cycle progression was analysed by flow cytometry with propidium iodide staining as
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29 previously described [3].
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33 **2.5 Determination of β-tubulin and prohibitin alkylation by western blot analysis**

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35 β-tubulin alkylation was determined by immunoblotting. Briefly, B16F0 cells were harvested
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37 from 60 mm Petri dishes (6X 10⁵ cells/dish), proteins were extracted in RIPA buffer with
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39 EDTA-free protease inhibitor cocktail (Roche Diagnostics, Meylan, France). Protein
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41 concentration was determined by Bradford assay using a CooAssay Protein Determination Kit
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43 (Interchim, Montluçon, France). For β-tubulin alkylation, fifty μg of total protein were
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45 separated on 10% SDS-polyacrylamide gels. For prohibitin alkylation, one hundred μg of urea
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47 extracted proteins (see 2DE paragraph) were submitted to IEF on 7-cm IPG strips pH 3 to 10
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49 (BioRad, Hercules, CA, USA) at 8000V/h, followed by a 12% SDS-polyacrylamide gel
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51 electrophoresis. Gels were transferred onto nitrocellulose membranes (Immobilon NC;
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53 Millipore, St-Quentin-en-Yvelines, France). The membranes were blocked in 5% non-fat dry
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1 milk in TBS-T (0.1% Tween 20) buffer and the blots were then incubated with a monoclonal
2 anti- β -tubulin antibody (Anti TUB2.1; Sigma, St-Quentin Fallavier, France) or a monoclonal
3 anti-prohibitin antibody (Abcam, Paris, France), then probed with anti-mouse horseradish
4 peroxidase (HRP) conjugated secondary antibody (Dako, Glostrup, Denmark). The blot
5 signals were detected by chemiluminescence (ECL, Amersham GE Healthcare,
6 Buckinghamshire, UK).
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13 **2.5 Microtubule structure – immunofluorescence**

14 B16F0 cells were seeded in 8-well culture glass slides (BD Biosciences, Erembodegem,
15 Belgium) at a density of 1×10^5 cells/ml for 24 hours. Cells were then treated with 0.5- to 1-
16 fold GI_{50} corresponding to 10 μ M ICEU, 3 μ M IOxa, 8 μ M tBCEU, 8 μ M tBOxa, 10 μ M
17 cHCEU or 5 μ M cHOxa in vehicle (0.5% DMSO) for 24 hours. They were washed twice with
18 PBS and fixed in methanol for 10 min at -20°C . Non-specific sites are blocked using 5% goat
19 serum for 30 min. Cells were incubated in 1/1000 diluted mouse monoclonal anti- α -tubulin
20 IgG (Sigma-Aldrich, St. Louis, MO, USA) for 2 hours at room temperature before incubating
21 with anti-mouse IgG labelled with Alexa Fluor 488 (Invitrogen) for 1 hour. Slides were
22 washed in PBS and then incubated in 10 μ g/ml Hoechst 33342 for 5 min to visualize the
23 nuclei. Microtubules and nuclei were observed with a fluorescent microscope (Olympus,
24 Center Valley, PA, USA) using FITC and DAPI filter sets, respectively.
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44 **2.6 Two-dimensional electrophoresis (2DE)**

45 For analysis of β -tubulin or prohibitin alkylation, two-dimensional electrophoresis was
46 conducted as described by Bouchon *et al.* [4].
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51 **2.7 Mass spectrometry analyses of protein digests**

52 MALDI-TOF MS analyses were performed according to the published procedure on trypsin
53 digests [4] on a Voyager-DE Pro mass spectrometer (ABSciex, Les Ulis, France).
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59 MALDI/PSD analysis was performed to characterize CEU binding on prohibitin [3].
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1 Nano ESI-MS/MS was used to localize and characterize β -tubulin alkylation. Analyses
2 were performed essentially as described previously [4] by direct infusion of the peptide
3 digest. The MS/MS data from the non-alkylated and alkylated peptides were interpreted
4 manually, assuming the modified masses. Fragments are assigned according to the
5 nomenclature of Roepstorff and Fohlman [24].
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11 **2.8 Stability and cellular uptake of drugs - HPLC**

12 Stability and cellular uptake in B16F0 cells was determined by HPLC. Cells were seeded in
13 100 mm Petri dishes ($2 \cdot 10^6$ cells/dish) and cultured for 24 hours. They were then exposed to
14 5- to 10-fold GI_{50} concentration of drugs *i.e.* 100 μ M (1 μ mol) ICEU, 30 μ M (0.3 μ mol) IOxa,
15 80 μ M (0.8 μ mol) *t*BCEU, 80 μ M (0.8 μ mol) *t*BOxa, 100 μ M (1 μ mol) cHCEU, 25 μ M (0.25
16 μ mol) cHOxa in 0.5% DMSO in Opti-MEM medium supplemented with $CaCl_2$ for 24 hours
17 (T24). Culture medium aliquots were collected in the presence or absence of cells, extracted
18 with 1 volume 0.2% trifluoroacetic acid (TFA) in acetonitrile (ACN) and centrifuged at
19 13000xg for 5 min. For cell lysates, cells were rinsed three times using PBS, scraped,
20 centrifuged at 800xg for 10 min and the pellets were stored at $-80^\circ C$. Free-intracellular drugs
21 were then extracted in 0.1% TFA in ACN / H_2O (V/V) and this solution was further frozen,
22 thawed and sonicated for 15 min. This cycle was repeated twice. After centrifugation at
23 13000xg for 5 min, cellular extracts and previously prepared culture media were analysed on
24 a HP 1100 series Agilent HPLC (Agilent, Les Ulis, France) equipped with diode array
25 detector using a Kromasil 5 μ particles C18 column (Interchim, Montluçon, France) at a flow
26 rate of 0.4 mL/min. The gradient used was: 0-3 min: 0.1% TFA/40% ACN in H_2O ; 3-13 min;
27 linear gradient to 0.1% TFA/100% ACN; 13-23 min 0.1% TFA/100% ACN. The column was
28 reequilibrated after each run for 10 min. Drug concentration was assessed at 255 nm and
29 calculated by the peak area compared to standard calibration curve. Each UV peak obtained in
30 HPLC was monitored at least once by ESI-MS/MS to confirm the structure of the product.
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3. Results

3.1 Antiproliferative and alkylating properties of CEUs and Oxas in B16F0 melanoma cells

Both CEUs and Oxas exhibited antiproliferative activity in the micro molar range (3-17 μ M) with GI_{50} being lower for Oxas when compared to the respective CEUs (**Table 1**). Similarly to their CEU counterparts, IOxa and *t*BOxa induced a cell cycle arrest at the G_2/M transition while cHOxa lead to G_1/S arrest with 90% of cells accumulated in G_0/G_1 phase (**Supplementary Figure 1**). On western blots, both ICEU/IOxa and *t*BCEU/*t*BOxa induced modification of β -tubulin migration, which was split into two bands suggesting protein alkylation (**Table 1**). Incubation of cells with cHCEU/cHOxa changed the pI of prohibitin, indicating its alkylation (**Table 1**). β -tubulin alkylating agents such as ICEU, IOxa, *t*BCEU and *t*BOxa altered microtubule structure as compared to control conditions. Immunofluorescent staining showed disorganization of the cytoskeleton architecture and the presence of punctuated tubulin aggregates that were not observed in controls (**Table 1**).

3.2 Oxas alkylate β -tubulin and prohibitin on the same amino acids as CEUs

The presence of two β -tubulin bands after SDS-PAGE of proteins extracted from B16F0 cells treated with IOxa and *t*BOxa (**Table 1**) suggested that the drugs bound covalently to the protein. To characterize the site of interaction, MS analyses were performed on β -tubulin treated respectively with ICEU, IOxa, *t*BCEU, *t*BOxa (**Figure 1A**). Alkylated tubulin spots isolated from 2DE gels were digested by trypsin and analyzed using MALDI-TOF-MS as previously described [4]. The native β -tubulin peptide [Val175-Arg213] on which alkylation had been shown to occur had an apparent molecular weight of 4595.7 (**Figure 1A**), while the peptide alkylated by ICEU presented a Δm of 288, as already observed [4]. The same m/z shift was observed on tubulin extracted from cells treated with IOxa (**Figure 1A**). To localize

1 the alkylation site, a nano ESI-MS/MS analysis was performed on this modified peptide
2 (**Figure 1B**). The fragmentation pattern allowed localizing the urea derivative on Glu24 of
3
4 the analyzed peptide corresponding to β -tubulin Glu198 (**Figure 1C**), as previously shown for
5
6 ICEU itself [4]. An intense fragmentation (m/z 1546.4, **Figure 1B**) occurred at the peptide-
7
8 like bond of the urea chain bound to Glu198 thus demonstrating the linearity of the added
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10 structure (**Figure 1D**).
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14 The same approach was used for *t*BCEU and *t*BOxa. For both drugs a m/z shift corresponding
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16 to the addition of the *t*Burea derivative was observed ($\Delta m = 218$) indicating alkylation on the
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18 same [175-213] β -tubulin peptide (**Figure 1A**).
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22 Prohibitin alkylation was determined using prohibitin spots isolated from 2DE gels (**Figure**
23
24 **2A**). When cHCEU is bound to its target, prohibitin exhibits a modified pI, as compared to the
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26 native protein [3]. The $m/z = 720$ corresponding to prohibitin peptide [Ala36-Arg41]
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28 observed in the native protein of control cells was shifted in cHCEU- and cHOxa- treated
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30 cells to $m/z = 964$ (**Figure 2B**). The two modified peptides were further characterized by
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32 MALDI-PSD analysis (**Figure 2C**). Their fragmentation patterns were identical in cHCEU
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34 and cHOxa treated cells, and revealed a modification on Asp5 of this peptide, corresponding
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36 to Asp40 of the protein.
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41 **3.3 CEU and Oxa stability in culture medium and uptake in B16F0 cells**

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43 ICEU/IOxa, *t*BCEU/*t*BOxa and cHCEU/cHOxa were separated by HPLC analysis and
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45 exhibited retention times of 12.9/4.3 min, 14.2/6.3 min and 16.2/9.8 min, respectively (**Figure**
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47 **3A**). CEUs were spontaneously transformed into Oxas, which remained stable for 24 hours in
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49 culture media in presence (T24+) or in absence (T24-) of cells (**Figure 3B, Table 2**). All the
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51 labelled peaks were recovered and analyzed by MS to confirm the identity. The HPLC
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53 protocol allowed the recovery of 70 to 98 % of the parent molecules incubated for 24 hours in
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55 the cell culture medium without cells (**Table 2**). Using the same HPLC conditions in the
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presence of cells, the percentage of detected molecules in the medium was lower suggesting an uptake of 7.4 to 36.5 % of CEUs and/or Oxas by B16F0 cells (**Table 2**). However, the levels of the intracellular free drug were low (<10%) suggesting that the major part of CEUs and Oxas was bound to proteins or biomolecules [25]. Drugs embedded in the cellular membranes and/or covalently bound to proteins were not detected by this procedure optimized to recover the free compounds. Another possibility was the metabolism of these drugs as our analyses were focused only on CEU and Oxa detection. We can also observe that the percentage of spontaneous CEU cyclization into Oxa was dependent on the molecular structure of the drug (6%, 26 % and 49% for cHOxa, IOxa and tBOxa formation, respectively (T24-) (**Table 2**).

4. Discussion

The study of the alkylating properties of CEU represents an interesting field of research since xenobiotics able to bind covalently to cellular proteins are relatively uncommon, especially when generating ester linkages with acidic amino acids such as glutamic and aspartic acids [3]. Here, we have demonstrated that Oxa derivatives are covalently linked to the same proteins and the same amino acids as their parent CEUs notably Glu198 for β -tubulin and Asp40 for prohibitin (**Figure 1 and 2**). CEUs and Oxas exhibited the same antiproliferative properties (cell cycle arrest, microtubule disruption specifically for antimitotics) (**Table 1, Sup Figure 1**), however, Oxas were, more active than their CEU counterparts. Altogether, these data suggest that both CEUs and Oxas exert their cytotoxic activity through similar mechanisms and that the pharmacological effects of CEUs could be linked to their intramolecular cyclization into Oxas.

Due to the absence of differences in the molecular masses of β -tubulin or prohibitin adducts obtained either with CEUs or their corresponding Oxas, we could not determine which molecule was involved in the alkylation reaction. However, fragmentation of the

1 modified peptide allowed to show that bound Oxa had a linear structure (**Figure 1 and 2**).
2 Moreover, in culture media CEUs were spontaneously transformed into Oxas. The level of
3 these spontaneously formed Oxas in the medium in presence or in absence of cells was
4 similar, unlike the concentration of CEUs that was decreased in the presence of cells
5 suggesting that CEUs can go through the membranes more easily than Oxas, even if they
6 display quite similar logP value (data from ALOGPS (<http://www.vcclab.org/lab/alogps/>, data
7 not shown). However, the Oxa level in the culture medium for IOxa-, tBOxa- and cHOxa-
8 treated cells was reduced compared to that without cells (26%, 10% and 33%, respectively)
9 showing that Oxas can penetrate into the cells. The low presence of free Oxas in cells and
10 their higher antiproliferative index (GI_{50}) strongly suggest that Oxas are significantly more
11 reactive with biomolecules than CEUs.
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26 The chlorine atom being necessary to maintain CEU β -tubulin capacity to alkylate proteins
27 [1], we can speculate that cyclization of CEUs into Oxas required this atom to confer electro-
28 attractor property. This cyclization step could involve the formation of a carbocation and/or
29 an aziridinic intermediate (**Figure 4**) as recent studies demonstrated that *N*-acyl-2,2-
30 dimethylaziridines could evolve into oxazolinic forms [26]. These two potential intermediates
31 characterized by very short half-lives, are difficult to observe in a biological context but it is
32 established that the aziridinium structures were clearly involved in DNA alkylation by *N*-
33 nitrosoureas and nitrogen mustards [27]. Interactions between β -tubulin and CEUs were
34 studied using CoMFA and CoMSIA models [28]. From these studies, it was deduced that the
35 ethylene chloride group of CEU was essential for the alkylation of Glu198 and that the
36 stabilization of a complex involving the colchicine-binding site and the CEU preceded the
37 completion of the Glu198 alkylation [29]. This intermediate complex should involve the
38 stabilizing contribution of Cys239 among other amino acids [29]. We are unable to conclude
39 if antimitotic CEUs are transformed into oxazolines in the colchicine-binding site of β -
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1 tubulin, when the urea group interacting with Cys239 [29] stabilizes the molecules, or react
2 spontaneously with acidic amino acids in a nucleophilic substitution reaction. Recent SAR
3 studies modifying the urea moiety of CEUs indicates that only 2-chloroacetamides and 2-
4 chloroacetylureas retained the cytotoxic properties without β -tubulin alkylation [30]. In these
5 two series of molecules, the CEU cyclization into Oxa could not occur reinforcing the
6 hypothesis that cyclization might be necessary for β -tubulin alkylation.
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14 New CEUs will be synthesized to elucidate this interesting point concerning the alkylation
15 process of Glu and Asp residues and to enhance our knowledge about the specific interactions
16 occurring in the colchicine-binding site. From a biological point of view, previous *in vivo* data
17 did not allow detecting oxazolinic forms or dechlorinated CEUs in urines of mice treated with
18 *t*BCEU, [31] while some unidentified metabolites were found in blood, tumors and colon of
19 mice treated with ICEU [32]. *In vivo* investigations will be pursued to identify these
20 metabolites and to determine precisely the active forms of CEUs or Oxas.
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31 In conclusion, one difficulty of this study was the absence of a mass difference between the
32 CEU or Oxa protein adducts revealed by mass spectrometry analyses, another one was based
33 on the fact that *N*-phenyl *N'*(2-chloroethyl)ureas cyclised spontaneously in cell culture
34 medium. However, we demonstrated that Oxas had similar mechanisms of cell cytotoxicity
35 and were present in the cells treated with CEU derivatives. We therefore suggest that Oxas
36 constitute key structures for the alkylation of cellular proteins. This study confers new
37 insights for drug conception through the cyclization of chlorinated forms into more active
38 oxazolinic ones.
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References

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- [1] Legault J, Gaulin JF, Mounetou E, Bolduc S, Lacroix J, Poyet P, et al. 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* 2000;60:985-92.
- [2] Mounetou E, Legault J, Lacroix J, C- Gaudreault R. 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* 2003;46:5055-63.
- [3] Bouchon B, Papon J, Communal Y, Madelmont JC, Degoul F. Alkylation of prohibitin by cyclohexylphenyl-chloroethyl urea on an aspartyl residue is associated with cell cycle G(1) arrest in B16 cells. *Br J Pharmacol* 2007;152:449-55.
- [4] Bouchon B, Chambon C, Mounetou E, Papon J, Miot-Noirault E, C-Gaudreault R, et al. Alkylation of beta-tubulin on Glu 198 by a microtubule disrupter. *Mol Pharmacol* 2005;68:1415-22.
- [5] Mishra S, Murphy LC, Murphy LJ. The Prohibitins: emerging roles in diverse functions. *J Cell Mol Med* 2006;10:353-63.
- [6] Hammond JW, Cai D, Verhey KJ. Tubulin modifications and their cellular functions. *Curr Opin Cell Biol* 2008;20:71-6.
- [7] Zhou J, Giannakakou P. Targeting microtubules for cancer chemotherapy. *Curr Med Chem Anticancer Agents* 2005;5:65-71.
- [8] Mishra S, Murphy LC, Nyomba BL, Murphy LJ. Prohibitin: a potential target for new therapeutics. *Trends Mol Med* 2005;11:192-7.
- [9] Sievers C, Billig G, Gottschalk K, Rudel T. Prohibitins are required for cancer cell proliferation and adhesion. *PloS One* 2010;5:e12735.

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- [10] Schleicher M, Shepherd BR, Suarez Y, Fernandez-Hernando C, Yu J, Pan Y, et al. Prohibitin-1 maintains the angiogenic capacity of endothelial cells by regulating mitochondrial function and senescence. *J Cell Biol* 2008;180:101-12.
- [11] Gregory-Bass RC, Olatinwo M, Xu W, Matthews R, Stiles JK, Thomas K, et al. Prohibitin silencing reverses stabilization of mitochondrial integrity and chemoresistance in ovarian cancer cells by increasing their sensitivity to apoptosis. *Int J Cancer* 2008;122:1923-30.
- [12] Joshi B, Ko D, Ordonez-Ercan D, Chellappan SP. A putative coiled-coil domain of prohibitin is sufficient to repress E2F1-mediated transcription and induce apoptosis. *Biochem Biophys Res Comm* 2003;312:459-66.
- [13] Petitclerc E, Deschesnes RG, Cote MF, Marquis C, Janvier R, Lacroix J, et al. 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* 2004;64:4654-63.
- [14] Moreau E, Fortin S, Desjardins M, Rousseau JL, Petitclerc E, C-Gaudreault R. Optimized N-phenyl-N'-(2-chloroethyl)ureas as potential antineoplastic agents: synthesis and growth inhibition activity. *Bioorg Med Chem* 2005;13:6703-12.
- [15] Borel M, Degoul F, Communal Y, Mounetou E, Bouchon B, C-Gaudreault R, et al. N-(4-iodophenyl)-N'-(2-chloroethyl)urea as a microtubule disrupter: in vitro and in vivo profiling of antitumoral activity on CT-26 murine colon carcinoma cell line cultured and grafted to mice. *Br J Cancer* 2007;96:1684-91.
- [16] Fortin S, Moreau E, Patenaude A, Desjardins M, Lacroix J, Rousseau JL, et al. 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* 2007;15:1430-8.

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- [17] Moreau E, Fortin S, Lacroix J, Patenaude A, Rousseau JL, C-Gaudreault R. N-Phenyl-N'-(2-chloroethyl)ureas (CEUs) as potential antineoplastic agents. Part 3: role of carbonyl groups in the covalent binding to the colchicine-binding site. *Bioorg Med Chem* 2008;16:1206-17.
- [18] Fortin JS, Cote MF, Lacroix J, Desjardins M, Petitclerc E, C-Gaudreault R. Selective alkylation of beta(II)-tubulin and thioredoxin-1 by structurally related subsets of aryl chloroethylureas leading to either anti-microtubules or redox modulating agents. *Bioorg Med Chem* 2008;16:7277-90.
- [19] Shaw AY, Henderson MC, Flynn G, Samulitis B, Han H, Stratton SP, et al. Characterization of novel diaryl oxazole-based compounds as potential agents to treat pancreatic cancer. *J Pharmacol Exp Ther* 2009;331:636-47.
- [20] Harris PA, Cheung M, Hunter RN, 3rd, Brown ML, Veal JM, Nolte RT, et al. Discovery and evaluation of 2-anilino-5-aryloxazoles as a novel class of VEGFR2 kinase inhibitors. *J Med Chem* 2005;48:1610-9.
- [21] Fortin JS, Lacroix J, Desjardins M, Patenaude A, Petitclerc E, C-Gaudreault R. Alkylation potency and protein specificity of aromatic urea derivatives and bioisosteres as potential irreversible antagonists of the colchicine-binding site. *Bioorg Med Chem* 2007;15:4456-69.
- [22] Mounetou E, Legault J, Lacroix J, C.-Gaudreault R. 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* 2001;44:694-702.
- [23] Debiton E, Madelmont JC, Legault J, Barthomeuf C. Sanguinarine-induced apoptosis is associated with an early and severe cellular glutathione depletion. *Cancer Chemother Pharmacol* 2003;51:474-82.

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- [24] Roepstorff P, Fohlman J. Proposal for a common nomenclature for sequence ions in mass spectra of peptides. *Biomed Mass Spectrom* 1984;11:601.
- [25] Saint-Laurent A, Boudreau N, Lariviere D, Legault J, Gaudreault RC, Auger M. Membrane interactions of a new class of anticancer agents derived from arylchloroethylurea: a FTIR spectroscopic study. *Chem Phys Lipids* 2001;111:163-75.
- [26] Besbes N, Jellali H, Pale P, Srasra E, Lofti Effrit M. Transformations de N-acylaziridines catalyees par des supports a base de silice et d'alumine : une elucidation mecanistique. *Comptes Rendus Chimie* 2010;13:358-64.
- [27] Gnewuch CT, Sosnovsky G. A Critical Appraisal of the Evolution of N-Nitrosoureas as Anticancer Drugs. *Chem Rev* 1997;97:829-1014.
- [28] Fortin S, Labrie P, Moreau E, Wei L, Kotra LP, C-Gaudreault R. A comparative molecular field and comparative molecular similarity indices analyses (CoMFA and CoMSIA) of N-phenyl-N'-(2-chloroethyl)ureas targeting the colchicine-binding site as anticancer agents. *Bioorg Med Chem* 2008;16:1914-26.
- [29] Fortin S, Wei L, Moreau E, Labrie P, Petitclerc E, Kotra LP, et al. Mechanism of action of N-phenyl-N'-(2-chloroethyl)ureas in the colchicine-binding site at the interface between alpha- and beta-tubulin. *Bioorg Med Chem* 2009;17:3690-7.
- [30] Fortin S, Moreau E, Lacroix J, Cote MF, Petitclerc E, C-Gaudreault R. Synthesis, antiproliferative activity evaluation and structure-activity relationships of novel aromatic urea and amide analogues of N-phenyl-N'-(2-chloroethyl)ureas. *Eur J Med Chem* 2010;45:2928-37.
- [31] Maurizis JC, Rapp M, Azim EM, Gaudreault RC, Veyre A, Madelmont JC. Disposition and metabolism of a novel antineoplastic agent, 4-tert-butyl-[3-(2-chloroethyl)ureido]benzene, in mice. *Drug Metab Dispos* 1998;26:146-51.

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- [32] Mounetou E, Miot-Noirault E, Gaudreault RC, Madelmont JC. N-4-iodophenyl-N'-2-chloroethylurea, a novel potential anticancer agent with colon-specific accumulation: radioiodination and comparative in vivo biodistribution profiles. *Invest New Drugs* 2009;28:124-31.

Figure Legends

Figure 1

β -tubulin alkylation by CEUs and their corresponding Oxas. (A) Comparative analyses by MALDI-TOF-MS of tubulin high mass peptides obtained in tubulin tryptic digest after DMSO treatment of cells or after ICEU, IOxa, *t*BCEU or *t*BOxa treatment. The low peak intensity observed in the case of ICEU was a partial dealkylation before analysis due to alkaline conditions during trypsin digestion. (B) ESI-MS/MS spectrum of the alkylated peptide obtained by IOxa. (C) Fragmentation pattern along the peptidic chain with localization of the alkylated amino acid on E23 of this peptide. (D) Fragmentation pattern in the bound structure confirming the opening of the oxazolinic heterocycle and its resulting linear structure. Z stands for the bound oxazoline derivative, whose structure is developed in scheme D with a grey background. Z* corresponds to a fragmentation along the bound structure, as indicated on the scheme. °, loss of one water molecule.

Figure 2

Prohibitin alkylation by cHCEU and its corresponding cHOxa. (A) Prohibitin spots localized on 2D gels (B) MALDI-TOF-MS analysis of these spots (C) Fragmentation spectra obtained from the two peptides having a *m/z* 964 (D) Fragmentation of peptide [AVIFDR], D being alkylated by a cyclo-hexyl-phenyl urea. The bound developed cyclohexyl structure is shown with a grey background to localize M* and M** fragments along the urea chain. “b” and “y” fragments were obtained from N and C-terminal fragmentation, respectively.

Figure 3

Quantitative HPLC analysis of ICEU, *t*BCEU or cHCEU in B16F0 cells. (A) Analysis of standard compounds (B) Analysis of cell culture medium after 24 hours of ICEU, *t*BCEU or

1 cHCEU exposure in absence (T24-) or presence (T24+) of B16F0 cells (C) Analysis of cell
2 lysates obtained from B16F0 cells cultured for 24 hours in serum-free Opti-MEM medium
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4 (T24+c) in the presence of 100 μ M ICEU, 80 μ M *t*BCEU or 100 μ M cHCEU. White arrows
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6 indicate CEUs, black arrows Oxas.
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9 **Figure 4**

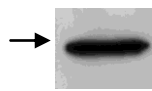
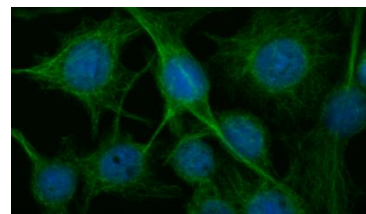
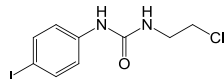
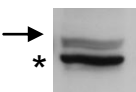
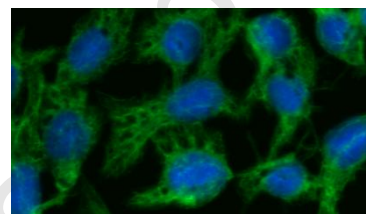
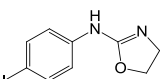
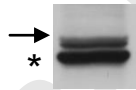
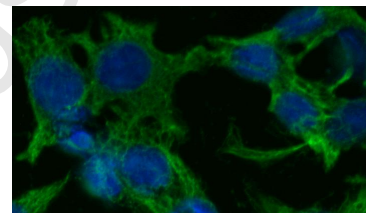
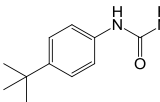
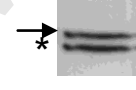
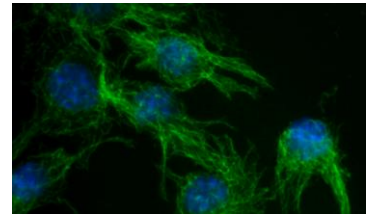
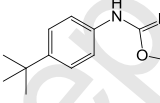
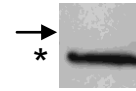
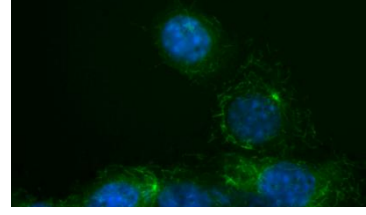
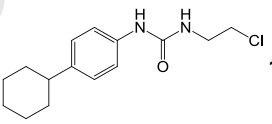
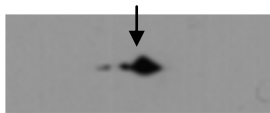
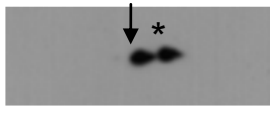
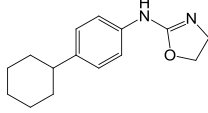

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11 **Scheme of the putative mechanisms leading to CEU cyclization into Oxas and reactivity**
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13 **of the protagonists with proteins.** CEUs, Oxas and their intermediates could react with
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15 acidic part of Glu/Asp residues of target proteins.
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22 **Supplementary Figures**

23 **Figure 1**

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25 **ICEU/IOxa and *t*BCEU/*t*BOxa block B16F0 cell cycle at G₂/M and cHCEU/cHOxa at**
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27 **G₁/S transitions.** Distribution of cells in different cell-cycle phases is expressed as the
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29 percentage of total cell number. Dotted lines indicate the percentage of each phase in control
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Table 1. Anti-proliferative and alkylating properties of CEUs and Oxas on B16F0 melanoma cells.

Compound	Structure	GI ₅₀ (μM)	β-tubulin alkylation	Microtubule structure
DMSO				
ICEU <i>N</i> -(4-iodophenyl)- <i>N'</i> -(2-chloroethyl)urea		10.6		
IOxa <i>N</i> -(4-iodophenyl)- 4,5-dihydrooxazol-2- amine		2.9		
tBCEU <i>N</i> -(4-tertbutylphenyl)- <i>N'</i> -(2-chloroethyl)urea		13.1		
tBOxa <i>N</i> -(4-tertbutylphenyl)- 4,5- dihydrooxazol-2-amine		7.1		
Compound	Structure	GI ₅₀	Prohibitin alkylation	
cHCEU <i>N</i> -(4-cyclohexylphenyl)- <i>N'</i> -(2-chloroethyl)urea		17.0	DMSO	
			cHCEU	
cHOxa <i>N</i> -(4-cyclohexylphenyl)- 4,5-dihydrooxazol-2- amine		7.2	cHOxa	

GI₅₀: drug concentration inhibiting 50% of cell growth; **β-tubulin alkylation**: determined by western blot in B16F0 cells treated or not (DMSO) for 24 hours at 5-10 x GI₅₀ drug concentration; **Microtubule structure**: visualised by immunofluorescence in B16F0 cells

treated or not (DMSO) for 24 hours at one GI_{50} drug concentration; **prohibitin alkylation:** determined by 2D western blot in B16F0 cells treated or not for 24 hours at a 5-10 x GI_{50} drug concentration. Arrows indicate the native form of the protein, asterisks indicate the alkylated form of the protein.

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Table 2. Drug recovery in culture medium and in B16F0 cells.

Drug treatment	Drug recovery in culture medium (%)				Drug recovery in cell lysates (%)		Calculated Drug content in cell pellets (%)
	T24-		T24+		T24+		
	CEU	Oxa	CEU	Oxa	CEU	Oxa	CEU and Oxa
ICEU	71.7	25.9	40.9	24.1	3.7	0.35	28.55
IOxa	-	95.1	-	68.7	-	1.0	25.4
fBCEU	34.0	48.6	24.5	50.4	nd	0.29	7.41
fBOxa	-	90.0	-	80.0	-	0.45	9.55
cHCEU	82.8	6.0	36.1	5.9	7.3	3.0	36.5
cHOxa	-	72.3	-	39.4	-	5.3	27.6

nd: not detected; - :not applicable

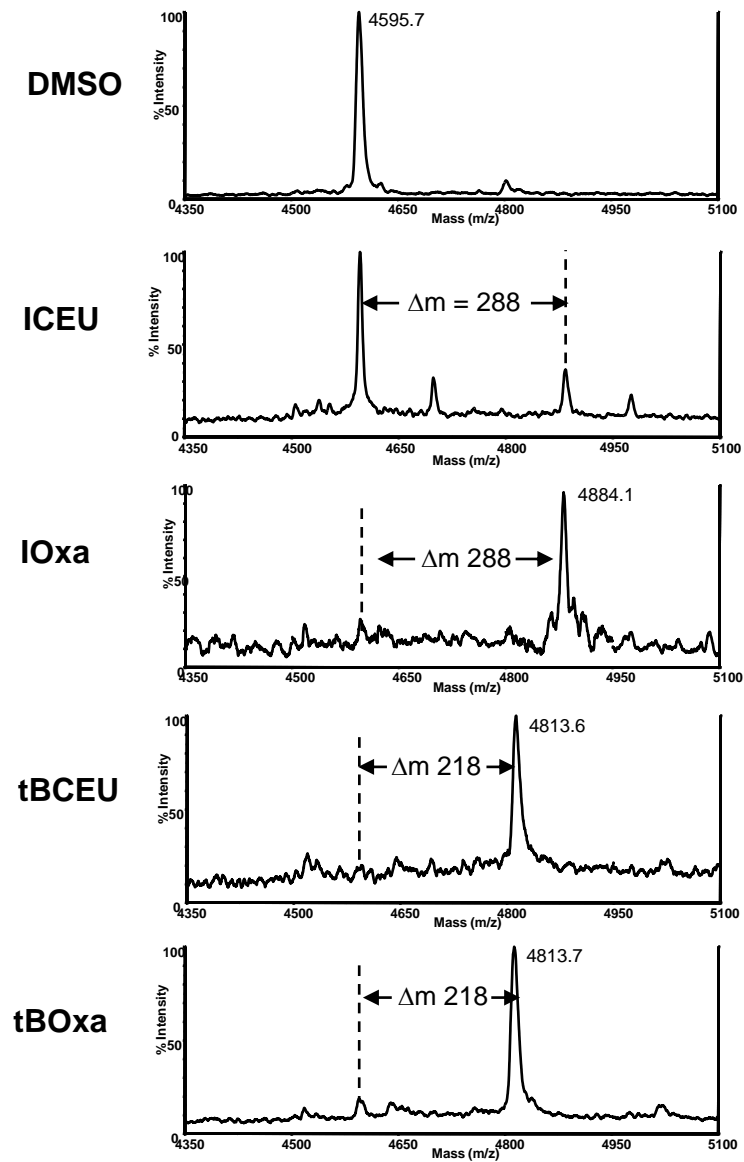
Concentrations of each compound were determined by HPLC and expressed as the percentages of the initial drug input. The culture medium was collected after 24 hours of incubation in the absence (T24-) or presence of cells (T24+). Drug recovery in cell pellets was calculated from drug recovery in other compartments and reported to the maximal amount of drugs recovered without cells (T24-). The values represent the mean of at least 2 independent experiments.

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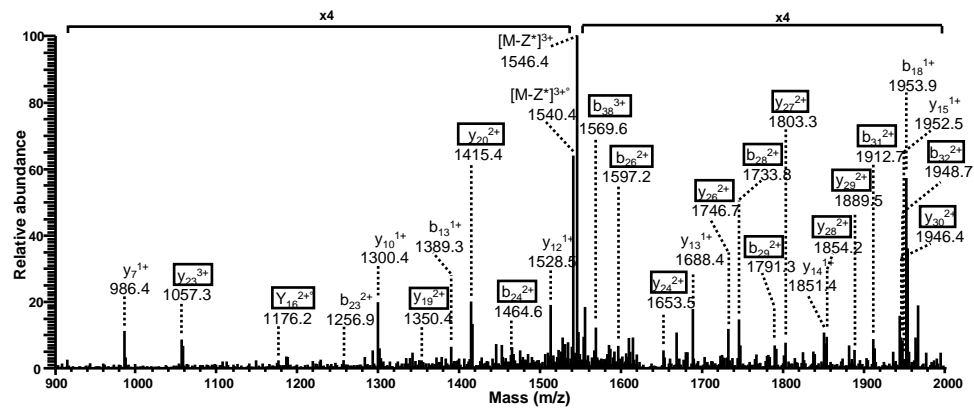
Figure

Figure 1

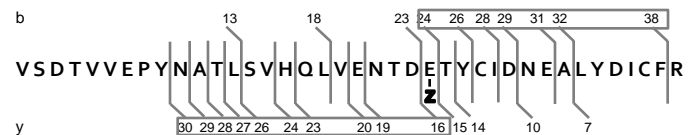
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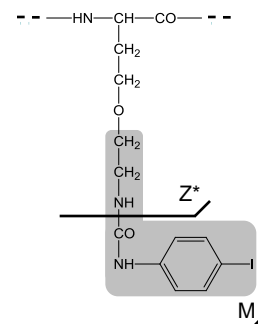
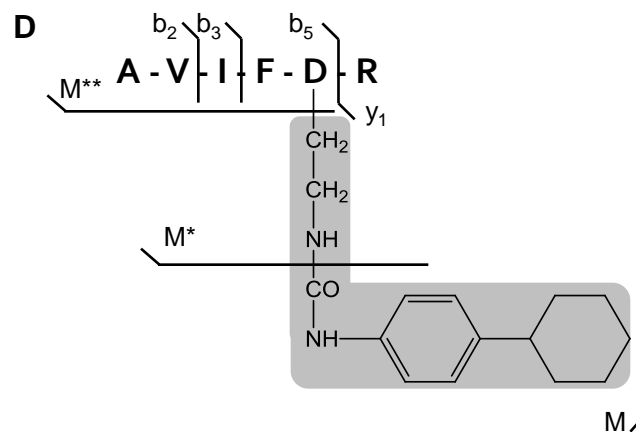
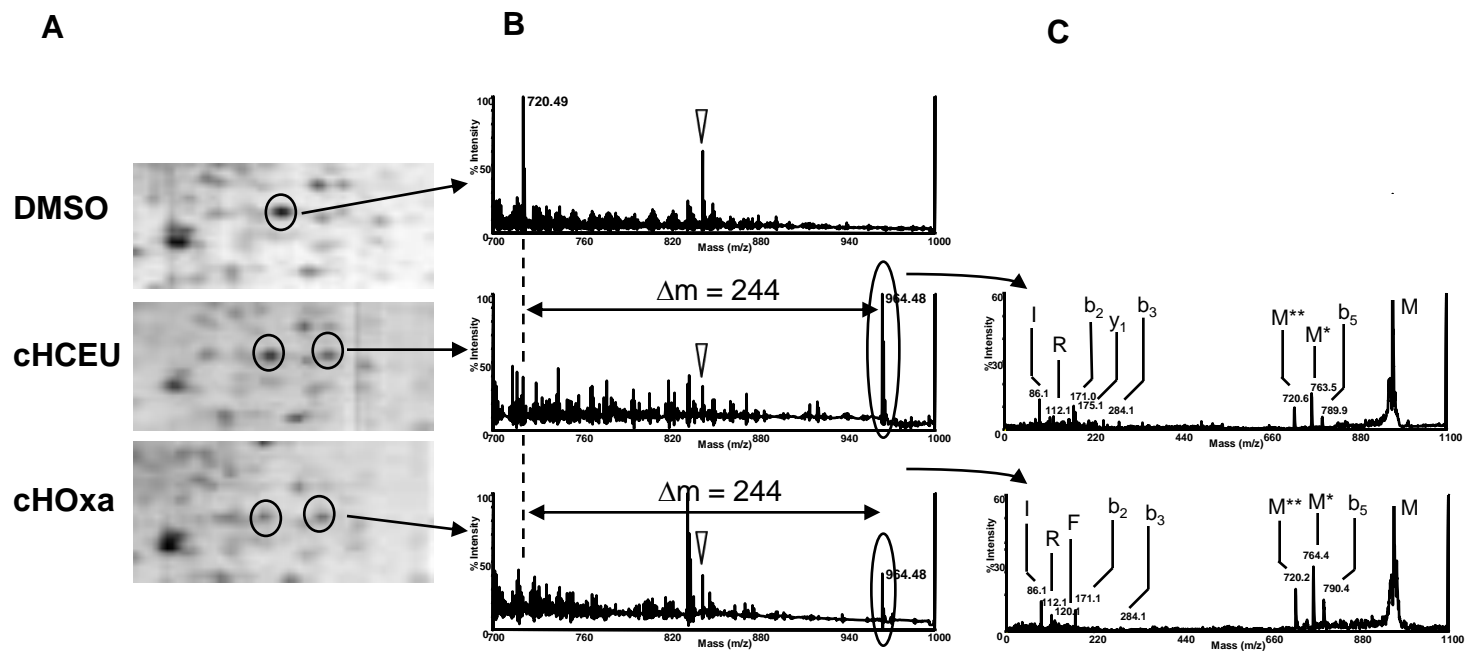


Figure 2



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Figure 3

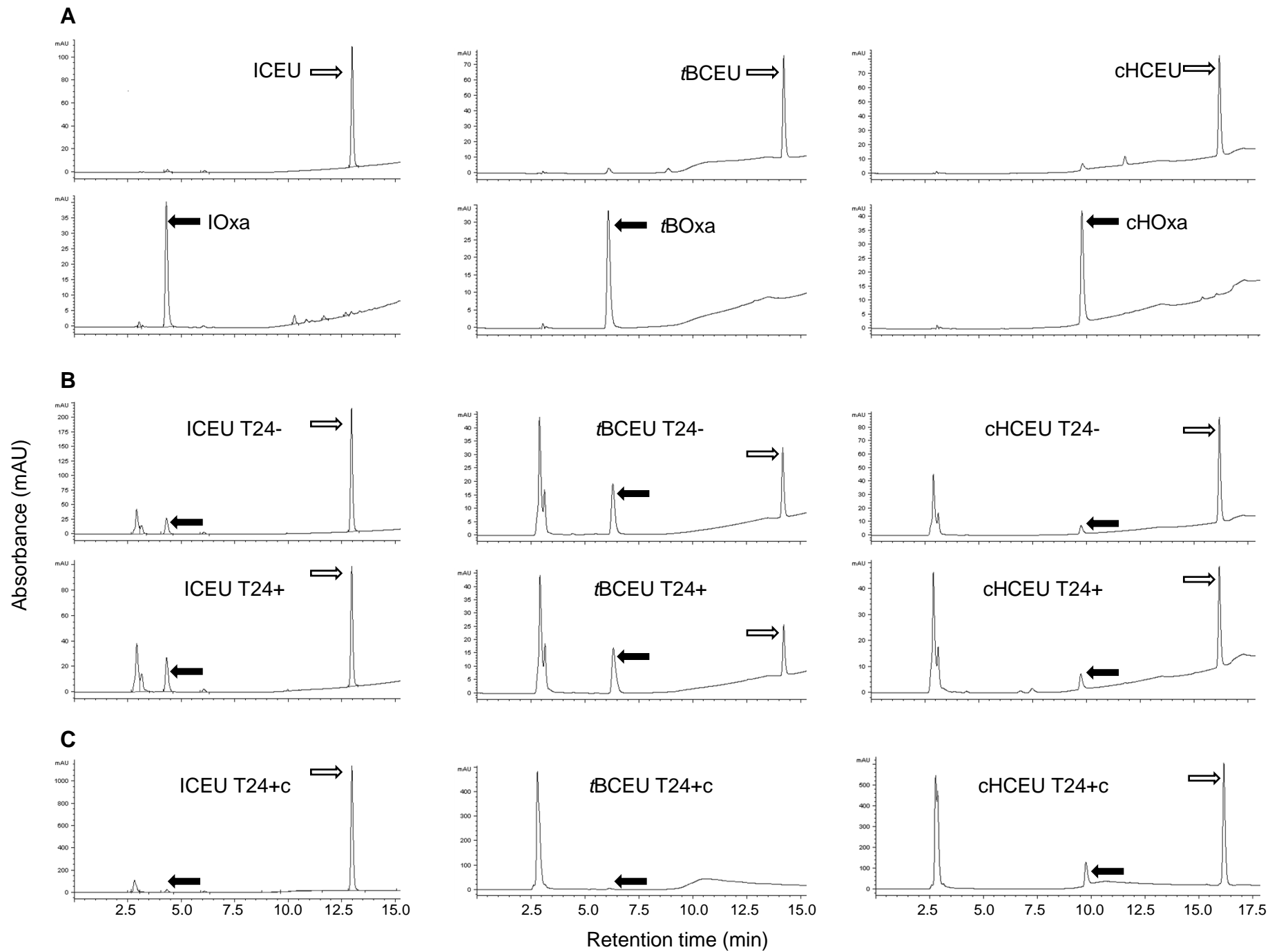
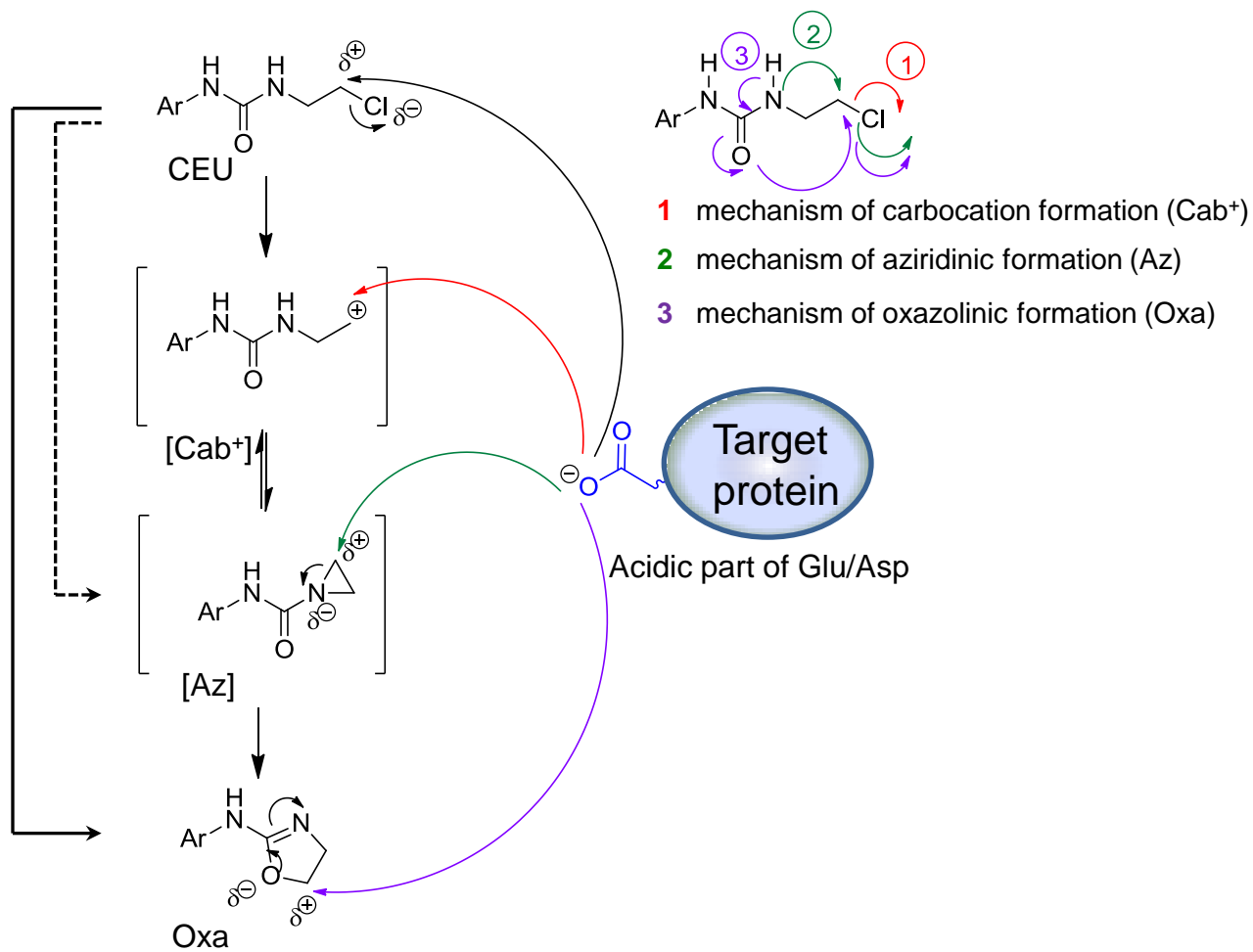


Figure 4



Supplementary Figure 1

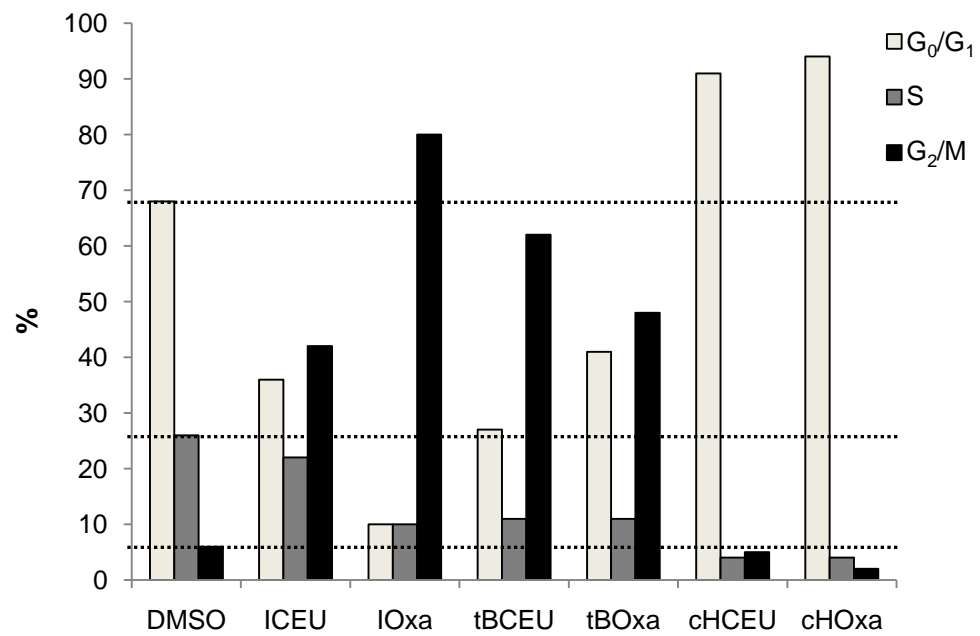
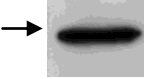
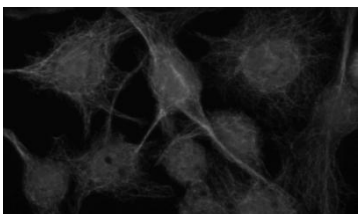
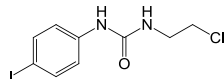
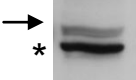
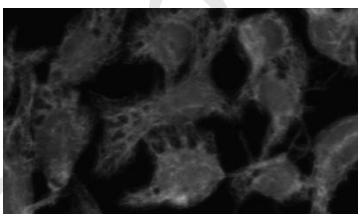
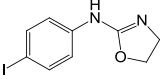
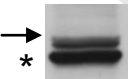
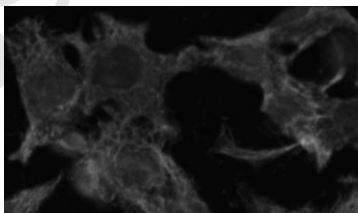
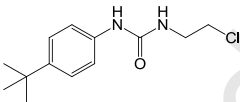
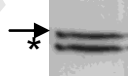
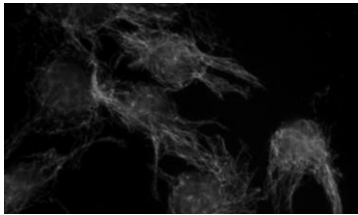
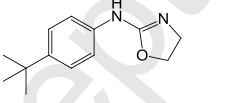
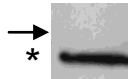
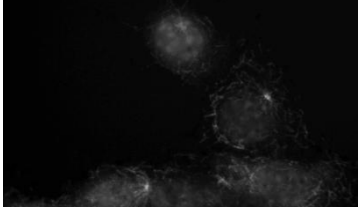
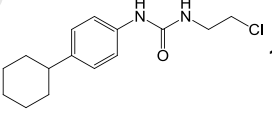
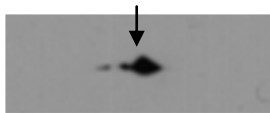
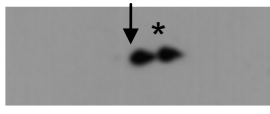
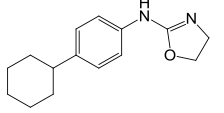



Table 1. Anti-proliferative and alkylating properties of CEUs and Oxas on B16F0 melanoma cells.

Compound	Structure	GI ₅₀ (μM)	β-tubulin alkylation	Microtubule structure
DMSO				
ICEU <i>N</i> -(4-iodophenyl)- <i>N'</i> -(2-chloroethyl)urea		10.6		
IOxa <i>N</i> -(4-iodophenyl)- 4,5-dihydrooxazol-2- amine		2.9		
tBCEU <i>N</i> -(4-tertbutylphenyl)- <i>N'</i> -(2-chloroethyl)urea		13.1		
tBOxa <i>N</i> -(4-tertbutylphenyl)- 4,5- dihydrooxazol-2-amine		7.1		
Compound	Structure	GI ₅₀	Prohibitin alkylation	
cHCEU <i>N</i> -(4-cyclohexylphenyl)- <i>N'</i> -(2-chloroethyl)urea		17.0	DMSO	
			cHCEU	
cHOxa <i>N</i> -(4-cyclohexylphenyl)- 4,5-dihydrooxazol-2- amine		7.2	cHOxa	

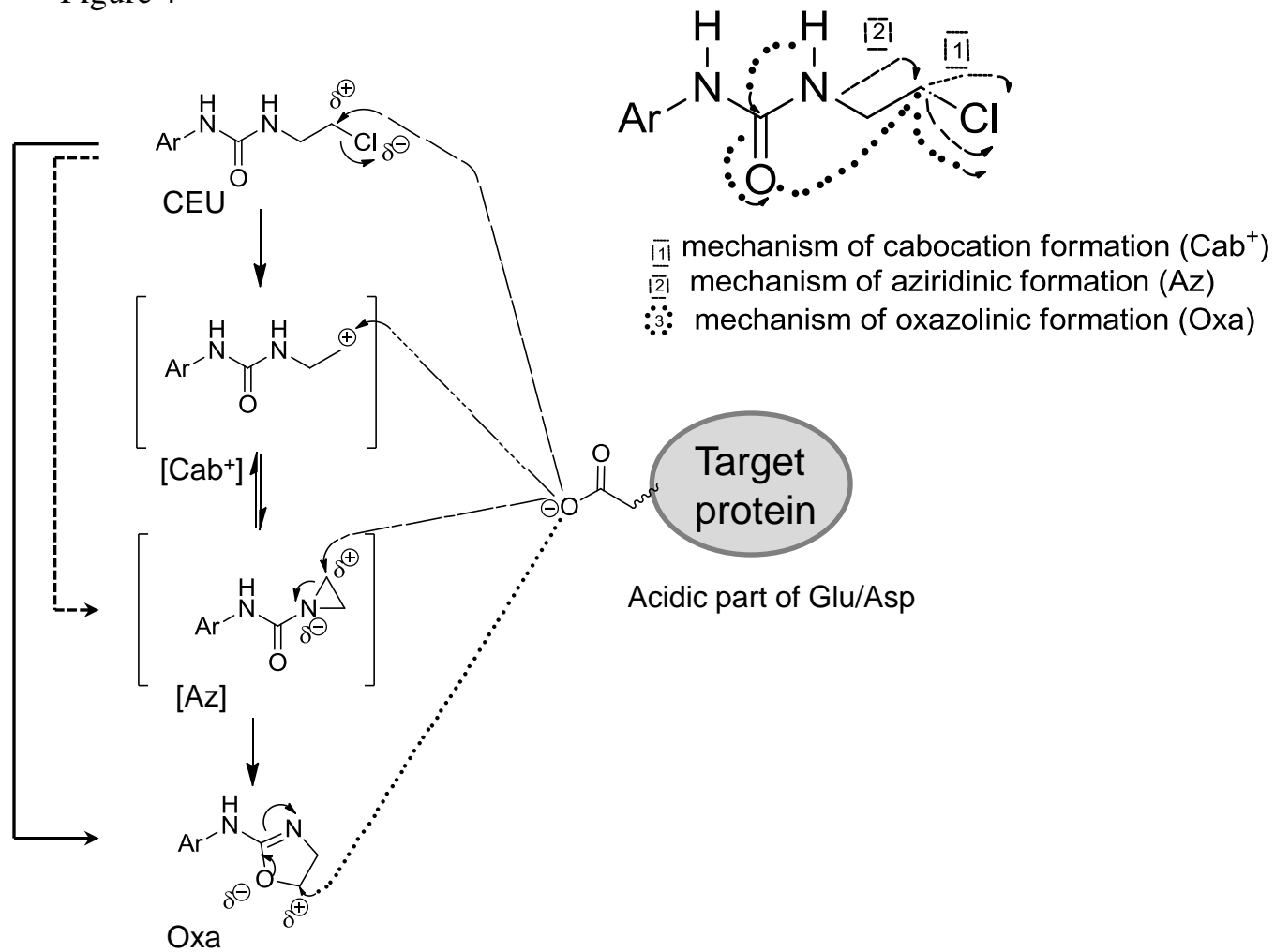
GI₅₀: drug concentration inhibiting 50% of cell growth; **β-tubulin alkylation**: determined by western blot in B16F0 cells treated or not (DMSO) for 24 hours at 5-10 x GI₅₀ drug concentration; **Microtubule structure**: visualised by immunofluorescence in B16F0 cells

treated or not (DMSO) for 24 hours at one GI_{50} drug concentration; **prohibitin alkylation:** determined by 2D western blot in B16F0 cells treated or not for 24 hours at a 5-10 x GI_{50} drug concentration. Arrows indicate the native form of the protein, asterisks indicate the alkylated form of the protein.

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Figure 4



Mechanisms of CEU/Oxa toxicity in melanoma cells

