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Selective alkylation of β_{II}-tubulin and thioredoxin-1 by structurally related subsets of aryl chloroethylureas leading to either anti-microtubules or redox modulating agents

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ABSTRACT

Aryl chloroethylureas (CEUs) are potent anti-neoplastic agents alkylating specific intracellular proteins such as β_{II}-tubulin. Recently we have identified a new subset of CEU derived from compound **36** that alkylates thioredoxin isoform 1 (Trx-1), inhibits the nuclear translocation of Trx-1, and favors the accumulation of cells in G_0/G_1 phase. We have evaluated the effects of various substituents and their position on the aromatic ring of a series of derivatives of **36** on (i) the anti-proliferative activity, (ii) the cell cycle progression, (iii) the nuclear translocation of Trx-1, and (iv) their covalent binding to β-tubulin. The same experiments were performed on representative CEU derivatives where the 2-chloroethyl amino moiety is replaced by either an ethyl, a 2-aminooxazolinyl or a 2-chloroacetyl group. On one hand, our results suggest that CEUs substituted on the phenyl ring at position 3 or 4 by cycloalkyl and substituted cycloalkyl or cycloalkoxy groups inhibit the nuclear translocation of Trx-1 and arrest the cell cycle progression in G_0/G_1. On the other hand, CEUs substituted by a fused aromatic ring, an aliphatic chain, or a fused aliphatic ring are alkylating β_{II}-tubulin but not Trx-1. Beside the expected inactivity of the ethylurea derivatives, none of the modification to the electrophilic moiety led to cross-selectivity of the drugs toward β-tubulin but increased the anti-proliferative activity and resulted in mitigated effects on Trx-1 translocation.

1. Introduction

N-Aryl-3-(2-chloroethyl)ureas (CEUs) are potential anti-cancer drugs that have been developed in our laboratory over the past two decades.[1–12] At first, structure–activity relationship studies have identified a subset of CEU binding covalently to the colchicine-binding site on β_{II}-tubulin through the acylation of the glutamic acid-198 residue.[3,4] The covalent binding of CEU to β-tubulin results in a complex sequence of events initiated by microtubule depolymerization and leading to cytoskeleton disruption, arrest of the cell cycle progression in G_2/M phase.[3–5,9,11] These events are paralleled by disorganization of the focal adhesion points and the actin stress fibers, cell rounding, detachment of the cells from the extracellular matrix and death by anoikis.[9]

CEUs of the first generation are active anti-microtubule agents on tumor cells having developed chemoresistance through mechanisms such as increased P-glycoprotein expression, increased DNA repair, increased intracellular glutathione-*S*-transferase activity, alteration of topoisomerase II activity and even cell adhesion-mediated drug resistance.[2,5] Two prototypical CEUs, namely tBCEU and ICEU, were shown to inhibit angiogenesis in vitro in Boyden' chambers and in vivo in the CAM assay and in mice using the Matrigel assay.[5] Furthermore [^{14}C]tBCEU and [^{125}I]ICEU were also shown to be preferentially biodistributed in organs of the gastro-intestinal tract, notably, duodenum and colon. ICEU was demonstrated as active as 5-fluorouracil on mice bearing CT-26 mouse colon carcinoma.[11,12]

Recently, the widening of our structure–activity relationship studies uncovered a compound designated as 1-(2-chloroethyl)-3-(4-cyclohexylphenyl)urea, (compound **36**) that exhibited a dramatic effect on the cell cycle progression when compared to tBCEU or ICEU.[13–16] The latter molecules arrested the cell cycle in G_2/M

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phase as the majority of anti-microtubules agents do while **36** was arresting the cell cycle in G_0/G_1 phase instead.[13–16] Interestingly, using [^{14}C]**36**, we confirmed that the drug does not covalently bind to the colchicine-binding site.[13–15] However, the drug was shown to covalently bind to thioredoxin isoform-1 (Trx-1) and to the aspartic acid residue in position 40 of prohibitin.[14] Compound **36** is binding also, to a lesser extent, to galectin-1 and -3 and to the mitochondrial voltage-dependent anion channel.

Thioredoxin-1 (Trx-1) represents an important target for the development of new anti-cancer drugs. Trx-1 is a 12-kDa protein involved in the control of the redox homeostasis of the cell.[17–30] Trx-1 is located normally in the cytoplasm and the nucleus.[18] However, Trx-1 is known to translocate to the nucleus when the cell is under a number of physiological and stress conditions such as UV radiations, H_2O_2, and cisplatinum leading to the stimulation of various transcription factors such as Nf-κB and AP-1.[17–19] In addition, Trx binds to PTEN and ASK-1 and contributes significantly to the cell survival signaling.[23–25] Trx-1 is overexpressed in a large number of cancer cell lines and correlates with poor patient prognostics.[19–22,25] Moreover, Trx-1 is involved in the chemoresistance to anti-neoplastic agents and radioactive radiations.[19–22] In that context, several studies showed that abrogating the cellular activity of Trx-1 reversed the chemoresistance to anti-neoplastics, such as cisplatinum, mitomycine C, doxorubicin, etoposide, and docetaxel, and raised the sensibility of tumor cells to radiotherapy.[18–22]

Such information prompted us to expand our structure-relationship studies to develop a new subset of CEU based on the molecular structure of the prototypical compound **36** that arrests the cell cycle in G_0/G_1 and covalently binds to Trx-1. Therefore, in this study, we have evaluated the nature and the position of different substituents on the aromatic ring of a series of derivatives of compound **36** on (i) the anti-proliferative activity, (ii) the cell cycle progression, (iii) the nuclear translocation of Trx-1, and (iv) their covalent binding to β-tubulin. We have also assessed the effects of modulating the electrophilic character of the 2-chloroethyl amino moiety of selected derivatives on the same parameters.

2. Results and discussion

2.1. Chemistry

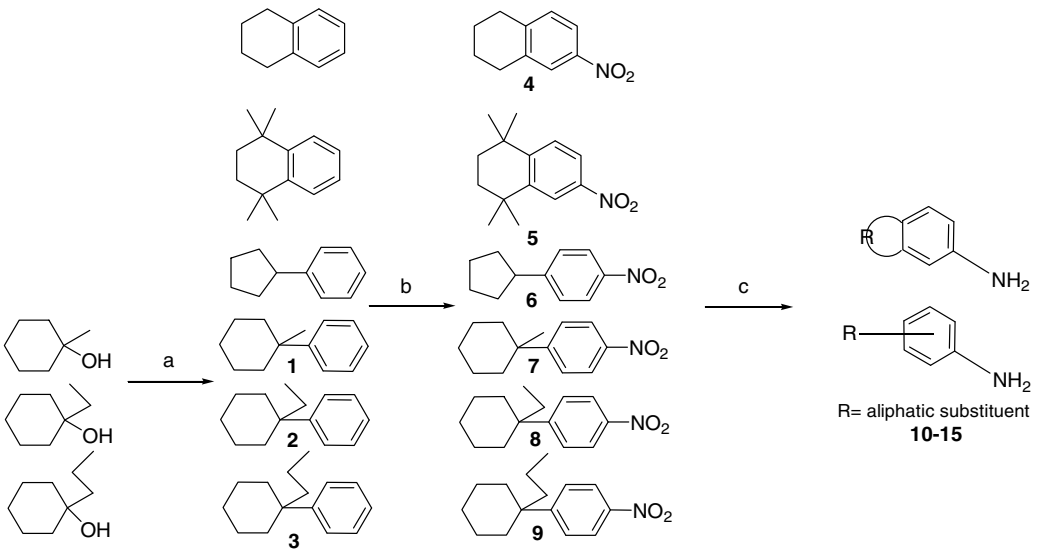
Schemes 1–3 illustrate the synthesis of CEU, *N*-aryl amino-2-oxazolines (4,5-dihydro-*N*-phenyloxazol-2-amines, OXAs) and 2-chloroacetylureas (CAUs) and 2-ethylureas (EUs), respectively. These compounds were prepared by the nucleophilic addition of either 2-chloroethylisocyanate, ethylisocyanate, or 2-chloroacetylisocyanate, respectively, on the corresponding anilines (Scheme 3).[3,6–8,10,15,16] Anilines substituted by cycloalkyl moieties were prepared using the Friedel–Craft alkylation followed by the nitration of the aromatic moiety (Scheme 1). Anilines substituted by cycloalkyloxy moieties were prepared using the Williamson' reaction followed by the reduction of the aromatic nitro group using $SnCl_2$ (Scheme 2).[15,16] OXA derivatives were prepared from the catalytic cyclization of CEU in presence of KF adsorbed on SiO_2 (ratio 4:6) in refluxing acetonitrile overnight (Scheme 3).

2.2. Anti-proliferative activity

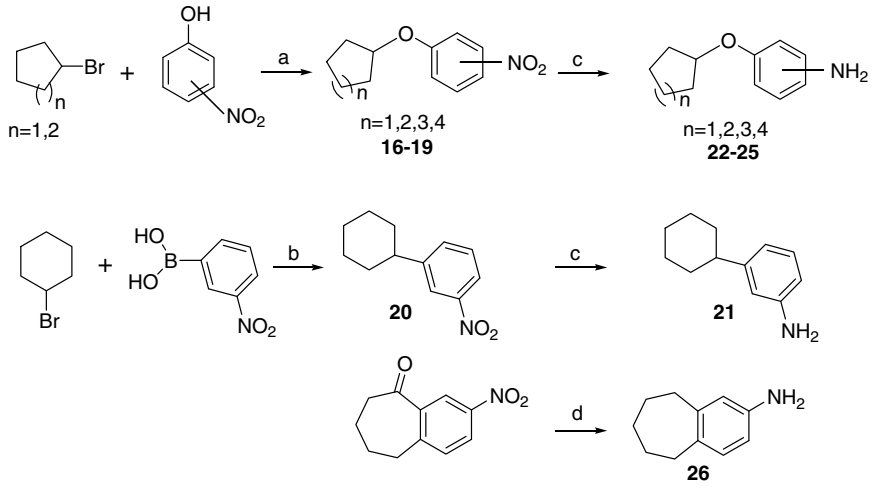
The anti-proliferative activity of CEU, OXA, CAU, and EU was evaluated on human cell lines: MCF-7 breast carcinoma, M21 skin melanoma, and HT-29 colon carcinoma cells. Cell growth inhibition was assessed according to the sulforhodamine method used at the NCI/NIH Developmental Therapeutics Program.[31] As depicted in Tables 1 and 2 compounds **28**–**37** and **39**–**56** have exhibited significant cell growth inhibition. CEU **27**, **38**, and EU **57** displayed a GI_{50} higher than 50 μM and thus were considered as inactive.

Ethyl urea derivatives, exemplified in Table 1 by compound **57**, are the non-electrophilic counterparts of CEU and have no noticeable impacts on cell growth and on the intracellular localization of Trx-1.[10,15,16] This confirmed that the 2-chloroethyl amino moiety of CEU is prerequisite to the anti-proliferative activity of these molecules. The replacement of the 2-chloroethyl amino moiety by the bioisosteric 2-aminooxazolinyl moiety (e.g., compounds **58**–**62**) was conducted similarly to anti-proliferative molecules (Table 2). The bioisosteric OXA derivatives **58**–**62** exhibited GI_{50} that were generally similar to their CEU counterparts **54**, **36**–**38**. However, CAU derivatives **63**–**67** bearing the potent electrophilic 2-chloroacetylamino moiety were 10 times more potent than their corresponding CEU and OXA derivatives. The increase of the electrophilic character of CEU through the replacement of the 2-chloroethyl amino group by a 2-chloroacetylamino moiety (e.g., compounds **27**, **36**–**38**) led to molecules having a higher anti-proliferative activity than CEU and OXA.

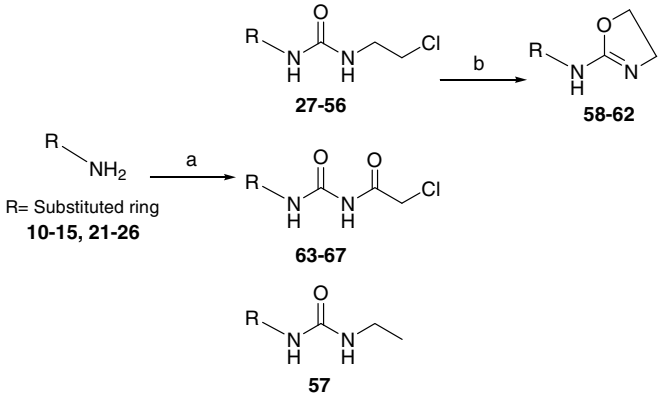
The aromatic ring of CEU must be substituted to initiate any anti-proliferative activity. To that end, the aryl 3-(2-chloroeth-



Scheme 1. Synthesis of selected anilines, Part 1. Reagents: (a) benzene, H_2SO_4; (b) acetic anhydride, HNO_3; (c) $SnCl_2{\cdot}2H_2O$, EtOH, reflux.



Scheme 2. Synthesis of selected anilines, Part 2. Reagents: (a) NaOH, DMF, reflux; (b) NiI_2, *trans*-2-aminocyclohexanol, NaHMDS, N_2, 2-PrOH; (c) $SnCl_2{\cdot}2H_2O$, EtOH, reflux; (d) AcOH, H_2SO_4, Pd/C, ethanol, H_2.



Scheme 3. Synthesis of aryl-substituted ethylureas. Reagents: (a) 2-chloroethylisocyanate, 2-chloroacetylisocyanate, or ethylisocyanate, CH_2Cl_2; (b) $SiO_2{\cdot}KF$, CH_3CN.

yl)urea **27** does not exhibit any anti-proliferative activity (Table 1). In addition, previous studies have clearly established that substitution on either position 2 and 6 abrogates also the biological activity.[1,6–8,10] From this study, it is not possible to predict any potential advantages to substitute the aromatic ring in position 3 versus position 4. However, the substitution of the aromatic ring on these positions by cycloalkyl or oxocycloalkyl groups having more than four carbon atoms leads to a significant anti-proliferative activity on all tumor cell lines tested. However, monosubstituted CEU appeared less active than 'bisubstituted' CEUs on positions 3 and 4 through non-heterocyclic fused rings such as the 2,3-dihydro-1H-indenyl **28**, the 1,2,3,4-tetrahydronaphthalenyls **29** and **30**, the 6,7,8,9-tetrahydro-5H-benzo[7]annulenyl **31**, the 9H-fluorenyl **33**, and the phenyl ring **32**. These CEUs exhibited similar anti-proliferative activities to several CAU tested. The presence of atoms such as oxygen and nitrogen in the alicyclic ring (e.g., **38**) decreases dramatically the anti-proliferative activity.

2.3. Effects of aromatic chloroethylureas and derivatives on the cell cycle

Table 1 shows percentage of MCF-7 cells in G_0/G_1, S, and G_2/M phases, respectively, after treatment with CEUs **27–57** and EU **58** for 24 h at two times their respective GI_{50}. Control cells (DMSO) were 59%, 22%, and 19% in G_0/G_1, S, and G_2/M phases, respectively. CEU **34–36**, **40**, **41**, **42**, **43**, **46**, **47**, **50–52** arrested cell in G_0/G_1. The treatment of cells with the aforementioned CEU resulted in the increase of cells population in G_0/G_1 phase by 4–16%. Figure 1 illustrates the accumulation of cells in G_0/G_1 induced by **36** and compound **64**. Conversely, the anti-microtubule CEUs **29**, **31**, **32**, **33**, **49**, **50**, and **55–57** increased the percentage of cells in G_2/M by 11–55%.

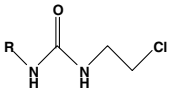
Treatment of M21 cells with compounds **54** and **55** for 24 h resulted in their accumulation in G_2/M, destabilization of the cytoskeleton, interruption of the β-tubulin entry in the mitotic spindle, and apoptosis.[3–5,14] On the other hand, **36** arrested the cell cycle in G_0/G_1 phase. Compound **36** has been shown using mass spectrometry to covalently bind to Trx-1. CEUs **28**, **29**, **30**, **32**, **33**, **49**, **56** target β-tubulin, leading to the arrest of the cell cycle progression in G_2/M. CEUs **36**, **39**, **40**, **41**, **42**, **46**, **47**, **50–53**, and OXA **59** lead to the abrogation of the nuclear translocation of Trx-1 and in the accumulation of cells in G_0/G_1 phase. These phenomena might be related to the fact that nuclear Trx-1 induces cell transcription and proliferation through the reduction of Ref-1 and the induction in parallel of AP-1 activity, which is a powerful transcription factor promoting cell growth and survival.[33–35] AP-1 is a key element in G_0 to G_1 transition via the AP-1-responsive cyclin D1 gene.[33–35] We hypothesized that CEUs, which confine Trx-1 to the cytoplasmic compartment, inhibit important pathways of nuclear transcriptional activity required for cell cycle regulation and cell proliferation.

2.4. β-Tubulin alkylation on M21-treated human melanoma cancer cells

Previous studies had shown that β-tubulin is a major target for tBCEU (**54**) and ICEU (**55**).[3–5,14] The covalent binding of CEU to β-tubulin increases the electrophoretic mobility of the β_{II}-tubulin by-product protein on SDS–PAGE electrophoresis, resulting in the appearance of a second band of β_{II}-tubulin band exhibiting an apparent lower molecular weight.[3]

To assess the intracellular alkylation of the colchicine-binding site on β_{II}-tubulin by selected CEU (**28–38**, **32–33**, **34–37**, **39–41**, **42–57**, **59**, **60**, **62–67**), M21 human melanoma cancer cells were treated with two times the GI_{50} of CEU for 16, 24 and 48 h using molecules having GI_{50} ranging from 1 to 50 μM. Proteins were extracted and separated by SDS–PAGE. Tables 1 and 2 show Western blot analysis using a monoclonal anti-β_{II}-tubulin antibody. Compound **30** induced the production of the alkylated

Table 1
Evaluation of the anti-proliferative activity on HT-29, M21, and MCF-7 cells and the effect of cell cycle progression on MCF-7 cells treated with 2-times the GI_{50} of selected CEU derivatives, PX-12, cisplatinum, colchicine, vinblastine and paclitaxel



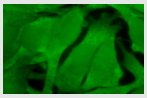
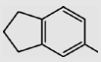
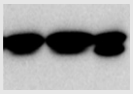
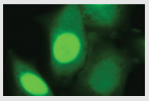
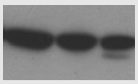
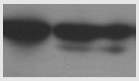
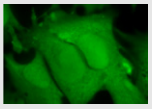
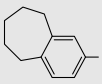
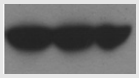
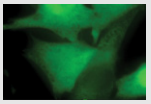
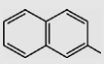
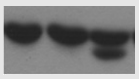
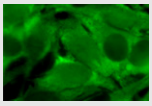
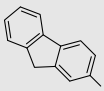
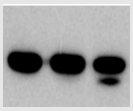
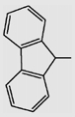
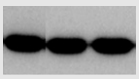
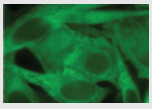
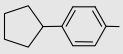
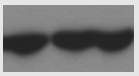
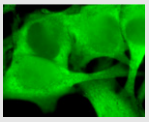
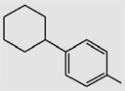
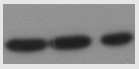
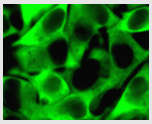
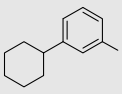
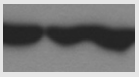
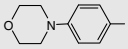
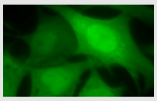
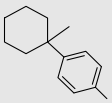
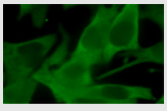
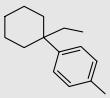
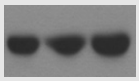
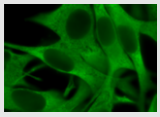
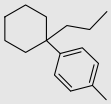
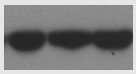
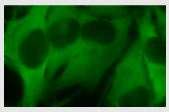
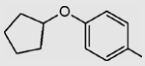
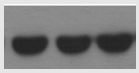
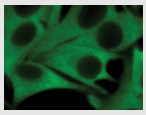
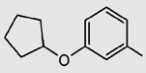
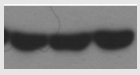
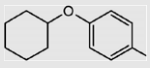
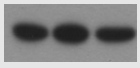
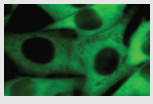
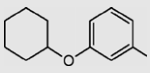
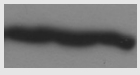
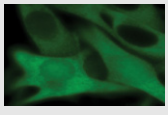
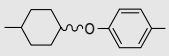
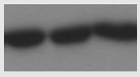
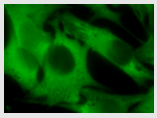
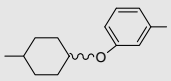
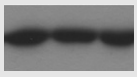
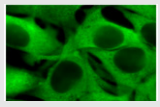
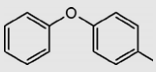
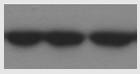
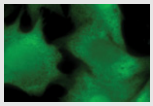
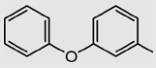
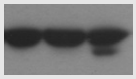
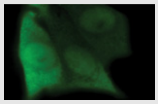
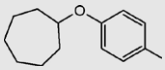
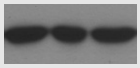
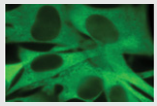
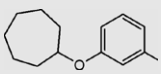
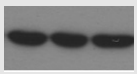
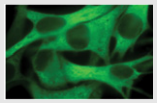
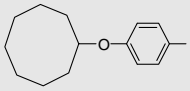
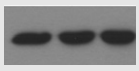
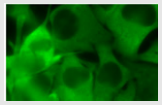
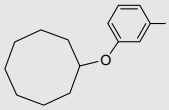
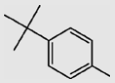
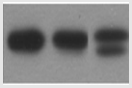
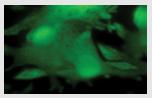
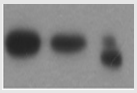
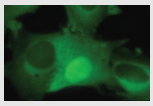
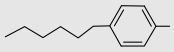
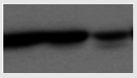
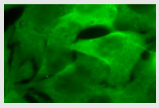
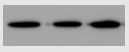
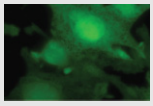
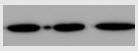
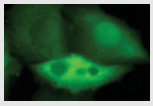
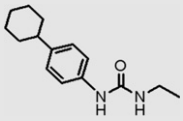
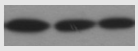
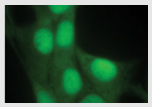
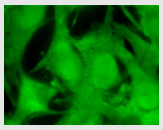
Compound	Substituent	GI_{50} (μM)			Cell cycle progression (%) on MCF-7[a]			β-Tubulin alkylationM21[a]	Trx-1 localization in M21[a]
		HT-29	M21	MCF-7	G_0/G_1	S	G_2/M	16, 24, and 48 h	
27[7,41]		n.a.	n.a.	n.a.	59	22	19	n.e.	
28[6,7,10]		3.6	8.0	14.1	31	29	40		
29[42]		2.0	3.7	7.3	10	49	41		
30		1.3	2.6	4.2	6	20	74		
31		3.2	7.2	12	33	24	43		
32[6,7]		1.9	4.1	5.6	17	38	45		
33[10]		2.3	5.1	14.2	11	57	32		
34[10]		19	27	22	63	29	8		
35		18	27	26	63	24	13		
36[7,15,16]		12.6	21	21	71	16	13		
37[43]		11.7	18	20	52	32	16		
38[44]		n.a.	n.a.	n.a.	n.e	n.e	n.e	n.e.	
39		7.8	16	18	60	9	31		

Table 1 (*continued*)

Compound	Substituent	GI_{50} (μM)			Cell cycle progression (%) on MCF-7[a]			β-Tubulin alkylationM21[a]	Trx-1 localization in M21[a]
		HT-29	M21	MCF-7	G_0/G_1	S	G_2/M	16, 24, and 48 h	
40		9.1	19	17	66	17	17		
41		18	36	23	73	14	13		
42[15,16]		31	38	49	72	19	9		
43[15,16]		9.9	22	35	64	24	12		
44[15,16]		23	36	45	57	29	14		
45[15,16]		7.9	20	25	59	33	8		
46		16	23	21	63	13	25		
47		19	32	33	65	20	15		
48[15,16]		2.4	5.2	9.1	15	20	65		
49[15,16]		5.6	16	24	44	26	30		
50[15,16]		13	18	19	75	19	6		
51[15,16]		10.7	20	21	72	22	6		
52		14	26	18	70	14	16		
53		20	30	23	61	29	10		

(*continued on next page*)

Table 1 (*continued*)

Compound	Substituent	GI_{50} (μM)			Cell cycle progression (%) on MCF-7[a]			β-Tubulin alkylationM21[a]	Trx-1 localization in M21[a]
		HT-29	M21	MCF-7	G_0/G_1	S	G_2/M	16, 24, and 48 h	
54[6,7,10] **tBCEU**		2.3	4.3	6.2	17	24	58		
55[6,7,10] **ICEU**		1.9	3.9	6.0	25	33	42		
56[6,7]		2.9	3.8	6.5	16	41	43		
PX-12[30]		25	8.3	8.3	29	13	58		
cDDP		10.1	12.9	6.4	53	27	20		
COL		0.004	0.015	0.009	19	19	62		
Pac		0.015	0.037	0.054	9	11	80		
57[15,16]		n.a.	n.a.	n.a.	55	23	22		
DMSO		n.a.	n.a.	n.a.	59	22	19		

In addition, the drug was assessed for β-tubulin alkylation using SDS–PAGE and the thioredoxin-1 intracellular localization was performed using a monoclonal anti-Trx-1 antibody.

n.a., non-active; n.e., not evaluated; cDDP, cisplatinum; Col, colchicine; Pac, paclitaxel.

[a] Drugs were tested at two times their respective GI_{50}.

band of β_{II}-tubulin after 24 h while CEUs **28**, **29**, **32**, **33**, **49**, **54–56** needed 48 h of incubation with M21 cells to exhibit a similar level of alkylation. Interestingly, the other molecules tested did not show the production of any alkylated β_{II}-tubulin by-products on SDS–PAGE.

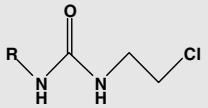
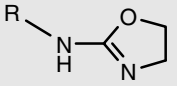
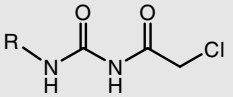
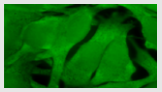
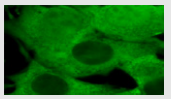
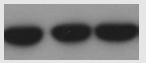
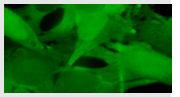
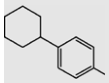
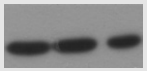
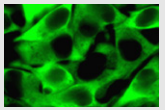
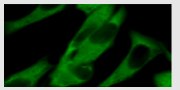
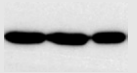
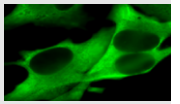
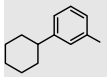
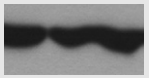
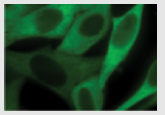
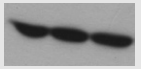
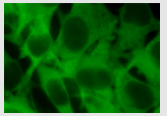
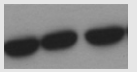
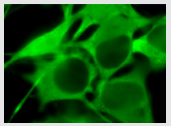
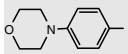
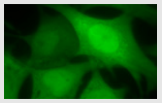
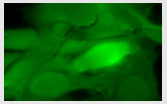
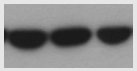
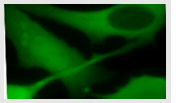
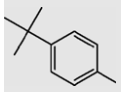
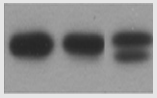
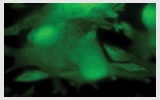
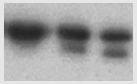
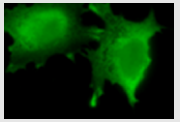
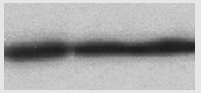
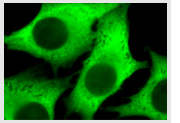
2.5. Changes in the intracellular location of thioredoxin-1

We previously showed that compound **36** inhibited the translocation of Trx-1 from the cytosol to the nucleus.[15,16] The influence of the new subset of CEU derivatives on the translocation of Trx-1 was assessed by immunocytofluorescence on M21 melanoma cells for treated for 16 h with concentrations two times the GI_{50} of the selected CEUs. Tables 1 and 2 show that compounds **29**, **36**, **37**, **39–42**, **44**, **46**, **47**, **50–53**, OXA **59**, **60**, CAU **64**, **65**, and **67** inhibited significantly the translocation of Trx-1 from the cytosol into the nucleus. Compounds **35**, **32**, **34**, **35**, **43**, **45**, **55**, and **66** induced only a partial inhibition of the nuclear translocation of Trx-1. Compounds **27**, **30–34**, **48**, **49**, **54**, **56–58**, **61–63**, colchicine, vinblastine, paclitaxel, and DMSO did not affect the intracellular location of Trx-1. Cisplatinum, as expected, favor significantly the nuclear translocation of Trx-1[32] but unexpectedly compounds **28**, **38**, and PX-12 also stimulated the nuclear translocation of Trx-1 from the cytosol. The mechanisms by which PX-12, **28** and **29** stimulate the nuclear translocation of Trx-1 are still unknown.

2.6. Selectivity of CEU toward Trx-1 versus β-tubulin

Our results obtained from previous and current experiments demonstrate that slight modifications of the structure of the substituents on the aromatic ring of CEU may lead to dramatic modification of the pattern of the proteins alkylated by the drugs.[1,3,6–10,13–16] For example (i) aliphatic and branched aliphatic CEUs having between 1 and 6 carbon atoms were shown

Table 2
Evaluation of the anti-proliferative activity, the ability to alkylate β-tubulin, and the intracellular localization of thioredoxin-1 of selected CEU, OXA, and CAU derivatives on M21 cells

R												
	Mol #	GI_{50} (μM)	β-tubulin alkylation	Trx-1 localization	Mol #	GI_{50} (μM)	β-tubulin alkylation	Trx-1 localization	Mol #	GI_{50} (μM)	β-tubulin alkylation	Trx-1 localization
			16, 24, and 48 h				16, 24, and 48 h				16, 24, and 48 h	
	**27**[7,41]	n.a.	n.a.		**58**	81	n.a.		**63**	6.9		
	**36**[15,16]	21			**59**[16]	18			**64**[16]	0.9		
	**37**[43]	18			**60**	45			**65**	2.4		
	**38**[44]	n.a.	n.a.		**61**	n.a.	n.a.		**66**	8.3		
	**54**[10]	4.3			**62**[10]	2.4			**67**[10]	1.1		

n.a., non-active.

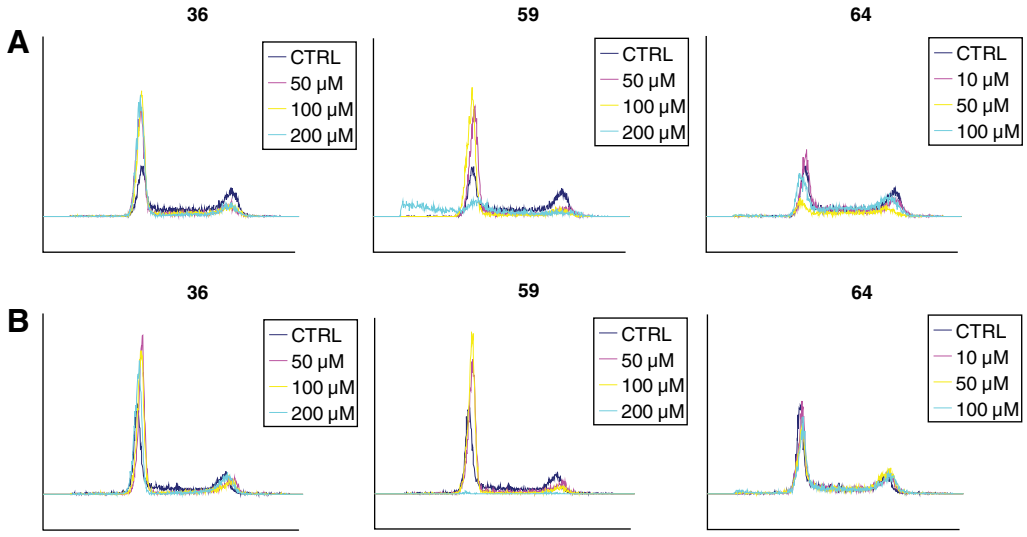


Figure 1. Effect of CEU **36**, OXA **59** and CAU **64** on cell cycle progression. Exponentially growing (A) MCF-7 or (B) HT-29 cells were incubated in absence or presence of **36**, **59** at 50, 100 and 200 μM (approximately two, four, and eight times their respective GI_{50}) or **64** at 10 or 50 μM for 24 h at 37 °C. The cell cycle was evaluated by flow cytometry using propidium iodide staining.

previously to covalently bind to β_{II}-tubulin,[1,3,6–10] (ii) cyclization of the *n*-hexyl CEU derivative into its cyclohexyl isomer led predominantly to the covalent binding of the molecule to Trx-1 without binding to β_{II}-tubulin (Table 3),[14–16] and (iii) homologation of the indanyl CEU **28**, that favors the nuclear translocation of Trx-1, into its tetrahydronaphthalenyl homolog **29** decreased the nuclear translocation of Trx-1 while both molecules are weakly alkylating β_{II}-tubulin. Tables 1 and 2 illustrate that CEUs derived from compound **36** are exhibiting a strong inhibition of the translocation of Trx-1 from the cytosol into the nucleus without any apparent covalent binding to β_{II}-tubulin.

3. Conclusion

This study aimed to understand the importance of the electrophilicity and the presence of substituting groups on the aromatic moiety of CEU on the 'specificity' of CEU toward β-tubulin and thioredoxin-1, respectively, and also their effect on cell cycle progression. At first, our results confirmed our previous observation[10] that non-electrophilic EU derivatives were pharmacologically inactive showing no effect on cell proliferation, cell cycle progression, and thioredoxin translocation and β-tubulin alkylation. Previous experiments had clearly established[10] that CEUs are substituted in position 3 or 4 or disubstituted in positions 3 and 4 by alkyl groups bearing between 1 and 6 carbon atoms and their bioisosteres are potent alkylators of the colchicine-binding site on tubulin. Unexpectedly, the cyclization of the *n*-hexyl group of **56** into **36** changed a molecule that is arresting the cell cycle in G_2/M phase, alkylating β-tubulin without modifying significantly the intracellular localization of thioredoxin-1 into a compound that is arresting the cell cycle progression at the G_0/G_1 phase, abrogating the nuclear translocation of thioredoxin-1, and that does not alkylate β-tubulin. These results suggest that three simple structural modifications of the aryl 2-chloroethyl pharmacophore may lead to the alkylation and the modulation of the activity of two different proteins or groups of proteins and their respective signaling pathways and then exhibiting different pharmacological properties. Our results suggest also that the inhibition of thioredoxin translocation by CEU requires the presence of an alicyclic ring alone or linked to the aromatic ring by an ether group either on position 3 or 4 of the aromatic ring. These CEU subsets are also active when substituted by lower alkyl groups having 1–3 carbon atoms on position 1 of the alicyclic ring. Modifications of the electrophilic character of CEU by replacing the 2-chloroethyl amino portion of the pharmacophore by a 2-aminooxazolinyl or a 2-chloroacetylamino moiety led also to compounds exhibiting slightly higher anti-proliferative activity without significantly modifying the end-points assessed in this study. We believe that besides sodium dichromate[23] these CEUs are among the first molecules reported to alkylate Trx-1 and to exhibit dramatic effects on its intracellular distribution. Thioredoxin-1 being comprised in powerful mechanisms of chemoresistance involving its translocation into the nucleus, this new subset of CEU could be useful as adjuvant chemo- or radio-sensitizing agents for cancer chemotherapy.

4. Experimental

4.1. Chemistry and chemical methods

All drugs were dissolved in DMSO and used at final concentrations lower than 0.12% (v/v) to avoid any potential toxicity. tBCEU, ICEU, and cHCEU were prepared as published previously.[10,15,16] Vinblastine sulfate, cisplatinum, colchicine, and paclitaxel were obtained from Sigma Chemicals (St. Louis, MO). 1-methylpropyl 2-imidazolyl disulfide (PX-12) was prepared as described by Kirkpatrick.[30] Proton NMR spectra were recorded on a Bruker AM-300 spectrometer (Bruker, Germany). Chemical shifts (δ) are reported in parts per million, relative to the internal tetramethylsilane standard. IR spectra were recorded on a Unicam spectrometer. Uncorrected melting points were determined on an electrothermal melting point apparatus. Mass spectra were performed on the most potent CEU derivatives, at the University of Montréal, Montréal, Canada. Chemicals and reagents were supplied by Aldrich Chemicals (Milwaukee, WI). 7-nitro-1-tetralone, 1,2,3,4-tetrahydronaphthalene and 1,2,3,4-tetrahydro-1,1,4,4-tetramethylnaphthalene, (±)-*trans*-2-aminocyclohexanol hydrochloride were purchased from Alfa Aesar (Ward Hill, MA, USA). Liquid flash chromatography was performed on silica gel 60 A (American Chemicals Ltd, Montreal, Canada), using the indicated solvent mixture expressed as volume/volume ratios. Solvents and reagents were used without purification unless specified otherwise. The progress of all reactions was monitored using TLC on precoated silica gel plates (Merck Silica Gel 60 F_{254}). The chromatograms were viewed under UV light at 254 nm.

4.2. General preparation of cycloalkylbenzenes (1–3)

A solution of 1-*n*-methylcyclohexanol or 1-*n*-ethylcyclohexanol or 1-*n*-propylcyclohexanol (20 mmol) in benzene (15 mL) was added dropwise at 0 °C to 10 mL of H_2SO_4 (12 M).[36] The mixture was stirred at room temperature for 3 h. The organic layer was separated, washed with brine and water, dried over anhydrous $MgSO_4$, and evaporated. The crude product was purified by liquid flash chromatography using petroleum ether.

4.2.1. 1-(1-Methylcyclohexyl)benzene (1)[36]

Compound **1** was synthesized from the alkylation of benzene with 1-*n*-methylcyclohexanol. Yield: 68%; ^{1}H NMR ($CDCl_3$) δ: 7.55 (d, 2H, J = 8.2 Hz, Ar), 7.50 (m, 2H, Ar), 7.36 (t, 1H, J = 7.6 Hz, Ar), 2.18 (m, 2H, CH_2), 1.76 (m, 8H, 4× CH_2), 1.39 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$) δ: 150.0, 147.0, 128.4, 125.7, 125.4, 38.2, 37.5, 30.8, 26.9, 22.9.

4.2.2. 1-(1-Ethylcyclohexyl)benzene (2)[36]

Compound **2** was synthesized from the alkylation of benzene with 1-*n*-ethylcyclohexanol. Yield: 41%; ^{1}H NMR ($CDCl_3$) δ: 7.39 (m, 5H, Ar), 2.17 (m, 3H, CH, CH_2), 1.79–0.99 (m, 9H, CH, 4× CH_2), 0.64 (t, 3H, J = 7.3 Hz, CH_3); ^{13}C NMR ($CDCl_{33}$) δ: 146.9, 127.1, 126.9, 126.5, 41.5, 38.0, 35.9, 26.9, 22.6, 8.1.

4.2.3. 1-(1-Propylcyclohexyl)benzene (3)[36]

Compound **3** was synthesized from the alkylation of benzene with 1-*n*-propylcyclohexanol. Yield: 56%; ^{1}H NMR ($CDCl_3$) δ: 7.43 (m, 5H, Ar), 2.20 (m, 3H, CH, CH_2), 2.1–0.93 (m, 11H, CH, CH_2), 0.88 (t, 3H, J = 7.1 Hz, CH_3); ^{13}C NMR ($CDCl_3$) δ: 147.3, 128.4, 127.1, 124.9, 46.6, 40.1, 36.5, 26.9, 22.6, 16.8, 14.6.

4.3. General preparation of cycloalkylnitrobenzenes (4–9)

To a solution of either **1**, **2**, **3**, 1,2,3,4-tetrahydronaphthalene, 1,2,3,4-tetrahydro-1,1,4,4-tetramethylnaphthalene, or 1-cyclopentylbenzene (10 mmol) in 10 mL of acetic anhydride was added dropwise a solution of HNO_3 (0.05 M) in 1 mL of acetic anhydride at 0 °C.[36] The mixture was stirred at room temperature for 2 h, poured on ice, and extracted with ether. The combined ether extracts were washed with a saturated $NaHCO_3$ solution, water, and dried over anhydrous $MgSO_4$. The solvent was evaporated under reduced pressure, and the oily crude product was purified by flash liquid chromatography with petroleum ether: CH_2Cl_2 (3:1).

4.3.1. 1,2,3,4-Tetrahydro-6-nitronaphthalene (4)

Compound **4** was synthesized from the nitration of 1,2,3,4-tetrahydronaphthalene. Yield: 55%; ^{1}H NMR ($CDCl_3$) δ: 7.96 (s, 1H, Ar), 7.67 (d, 1H, J = 8.1 Hz, Ar), 7.33 (d, 1H, J = 7.5 Hz, Ar), 2.88 (m, 4H, 2× CH_2), 1.85 (m, 4H, 2× CH_2); ^{13}C NMR ($CDCl_3$) δ: 145.2, 137.2, 133.8, 129.9, 125.8, 121.8, 29.7, 26.1, 22.6, 22.4.

4.3.2. 1,2,3,4-Tetrahydro-1,1,4,4-tetramethyl-6-nitronaphthalene (5)[37]

Compound **5** was synthesized from the nitration of 1,2,3,4-tetrahydro-1,1,4,4-tetramethylnaphthalene. Yield: 77%; ^{1}H NMR ($CDCl_3$) δ: 7.50 (s, 1H, Ar), 7.32 (d, 1H, J = 8.7 Hz, Ar), 7.17 (d, 1H, J = 8.7 Hz, Ar), 1.58 (m, 4H, 2× CH_2), 1.40 (s, 12H, 4× CH_3); ^{13}C NMR ($CDCl_3$) δ: 157.8, 152.8, 149.8, 126.5, 125.1, 123.1, 31.5, 29.0, 22.5.

4.3.3. 1-Cyclopentyl-4-nitrobenzene (6)

Compound **6** was synthesized from the nitration of 1-cyclopentylbenzene. Yield: 34%; ^{1}H NMR ($CDCl_3$) δ: 8.20 (d, 2H, J = 8.7 Hz, Ar), 7.79 (d, 2H, J = 8.6 Hz, Ar), 2.75 (m, 1H, CH), 1.93 (m, 2H, CH_2), 1.77–1.46 (m, 6H, 3× CH_2); ^{13}C NMR ($CDCl_3$) δ: 145.0, 138.7, 126.4, 115.2, 40.6, 29.7, 21.8.

4.3.4. 1-(1-Methylcyclohexyl)-4-nitrobenzene (7)[36]

Compound **7** was synthesized from the nitration of compound **1**. Yield: 88%; ^{1}H NMR ($CDCl_3$) δ: 8.14 (d, 2H, J = 8.9 Hz, Ar), 7.51 (d, 2H, J = 8.8 Hz, Ar), 1.98 (m, 2H, CH_2), 1.51 (m, 8H, 4× CH_2), 1.38 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$) δ: 149.5, 145.8, 128.2, 120.5, 37.9, 30.7, 26.5, 22.9.

4.3.5. 1-(1-Ethylcyclohexyl)-4-nitrobenzene (8)[36]

Compound **8** was synthesized from the nitration of compound **2**. Yield: 84%; ^{1}H NMR ($CDCl_3$) δ: 8.15 (d, 2H, J = 7.3 Hz, Ar), 7.48 (d, 2H, J = 8.8 Hz, Ar), 2.19 (m, 2H, CH, CH_2), 1.82–0.80 (m, 9H, CH, CH_2), 0.54 (t, 3H, J = 7.5 Hz, CH_3); ^{13}C NMR ($CDCl_3$) δ: 155.4, 145.7, 128.0, 123.2, 42.4, 37.7, 35.8, 26.7, 22.5, 7.8.

4.3.6. 1-Nitro-4-(1-propylcyclohexyl)benzene (9)[36]

Compound **9** was synthesized from the nitration of compound **3**. Yield: 75%; ^{1}H NMR ($CDCl_3$) δ: 8.12 (d, 2H, J = 8.7 Hz, Ar), 7.47 (d, 2H, J = 8.9 Hz, Ar), 2.06 (m, 3H, CH, CH_2), 1.85–0.86 (m, 11H, CH, CH_2), 0.71 (t, 3H, J = 6.9 Hz, CH_3); ^{13}C NMR ($CDCl_3$) δ: 155.8, 145.7, 127.8, 123.2, 46.2, 42.3, 36.1, 25.5, 22.3, 16.6, 14.6.

4.4. General preparation of 1-cycloalkyloxy-4-nitrophenols (16, 18) and 1-cycloalkyloxy-3-nitrophenols (17, 19)

The appropriate cycloalkylbromide (2 equiv, 14.4 mmol) was added to a solution of 3-nitrophenol or 4-nitrophenol (1 equiv, 7.19 mmol) and NaOH (2.5 equiv, 17.9 mmol) in 10 mL of DMF.[38] The mixture was stirred under reflux for 48 h. After completion of the reaction (TLC), water (200 mL) was added. The mixture was extracted with dichloromethane. The organic phase was washed with 1 N NaOH, water, and 1 N HCl, water, respectively. Afterward, the solution was dried over anhydrous Na_2SO_4, filtered, and evaporated under reduced pressure.

4.4.1. 1-(4-Methylcyclohexyloxy)-4-nitrobenzene (16)

Compound **16** was synthesized from the nucleophilic substitution of 4-nitrophenol to 1-bromo-4-methylcyclohexane (*cis* + *trans*). Yield: 14%; ^{1}H NMR ($CDCl_3$) δ: 8.16 (m, 2H, J = 9.0 Hz, Ar), 6.92 (m, 2H, J = 8.9 Hz, Ar), 4.28 (m, 1H, CH), 2.10–1.24 (m, 7H, CH, CH_2), 1.17–0.94 (m, 5H, CH, CH_3); ^{13}C NMR ($CDCl_3$) δ: 163.2, 141.1, 125.9, 115.4, 73.1, 34.8, 32.2, 30.8, 22.2.

4.4.2. 1-(4-Methylcyclohexyloxy)-3-nitrobenzene (17)

Compound **17** was synthesized from the nucleophilic substitution of 3-nitrophenol to 1-bromo-4methylcyclohexane (*cis* + *trans*). Yield: 12%; ^{1}H NMR ($CDCl_3$) δ: 7.73 (d, 1H, J = 7.8 Hz, Ar), 7.71 (s, 1H, Ar), 7.40 (t, 1H, J = 8.1 Hz, Ar), 7.18 (d, 1H, J = 7.2 Hz, Ar), 4.19 (m, 1H, CH), 2.10–1.34 (m, 7H, CH, CH_2), 1.12–0.89 (m, 5H, CH, CH_3); ^{13}C NMR ($CDCl_3$) δ: 158.5, 149.3, 129.9, 122.8, 115.4, 110.2, 72.9, 34.2, 32.9, 31.6, 22.3.

4.4.3. (4-Nitrophenoxy)cyclooctane (18)

Compound **18** was synthesized from the nucleophilic substitution of 4-nitrophenol to bromocyclooctane. Yield: 49%; ^{1}H NMR ($CDCl_3$) δ: 8.13 (d, 2H, J = 9.0 Hz, Ar), 6.84 (d, 2H, J = 9.2 Hz, Ar), 4.50 (m, 1H, CH), 1.86 (m, 4H, 2× CH_2), 1.59 (m, 10 H, 5× CH_2); ^{13}C NMR ($CDCl_3$) δ: 163.2, 140.9, 125.9, 115.4, 78.9, 31.5, 27.0, 25.6, 23.0.

4.4.4. (3-Nitrophenoxy)cyclooctane (19)

Compound **19** was synthesized from the nucleophilic substitution of 3-nitrophenol to bromocyclooctane. Yield: 33%; ^{1}H NMR

($CDCl_3$) δ: 7.75 (d, 1H, J = 9.0 Hz, Ar), 7.67 (s, 1H, Ar), 7.37 (t, 1H, J = 8.2 Hz, Ar), 7.16 (d, 1H, J = 8.1 Hz, Ar), 4.49 (m, 1H, CH), 1.89 (m, 4H, 2× CH_2), 1.63 (m, 10H, 5× CH_2); ^{13}C NMR ($CDCl_3$) δ: 158.4, 149.3, 129.9, 122.8, 115.3, 110.2, 78.9, 31.4, 27.0, 25.6, 23.0.

4.5. Preparation of 1-cyclohexyl-3-nitrobenzene (20)

Nickel iodide (0.06 mmol), *trans*-2-aminocyclohexanol hydrochloride (0.06 mmol), 3-nitrophenylboronic acid (1.2 mmol), and NaHMDS (2 mmol) were placed in a 10-mL round-bottomed flask.[39] The flask was purged with dry nitrogen for 5 min. 2-PrOH (5 mL) was added using a syringe, and the resulting mixture was stirred for 5 min at room temperature.[39] Cyclohexylbromide (1 mmol) was added using a syringe, and the flask was heated at 60 °C in an oil bath for 6 h. The reaction mixture was cooled to room temperature, and then filtered through a short pad of silica gel. Silica gel was washed with 200 mL of a mixture of hexanes/ether (1:1). The filtrate was concentrated under reduced pressure, and the residue was purified by flash liquid chromatography using a mixture of petroleum ether/CH_2Cl_2 (3:1).

Yield: 5%; ^{1}H NMR ($CDCl_3$) δ: 7.96 (m, 2H, Ar), 7.70 (t, 1H, J = 7.6 Hz, Ar), 7.54 (t, 1H, J = 7.8 Hz, Ar), 2.44 (m, 1H, CH), 1.76 (m, 5H, CH, CH_2), 1.36 (s, 5H, CH, CH_2); ^{13}C NMR ($CDCl_3$) δ: 153.6, 140.4, 133.1, 130.3, 123.3, 122.1, 45.3, 33.4, 25.1, 24.6.

4.6. General preparation of the anilines 10–15 and 21–25

Nitro derivatives **4–9**, **16–19**, and **21** (1 equiv, 4.97 mmol) were added to a solution of $SnCl_2{\cdot}2H_2O$ (6 equiv, 29.8 mmol) in 10 mL of ethanol. The mixture was stirred under reflux for 12 h. Then, 1 N NaOH (20 mL) was added. The mixture was extracted with ether. The organic phases were pooled, and washed with brine and afterward with water. The ether solution was dried over anhydrous $MgSO_4$, filtered, and evaporated under reduced pressure.

4.6.1. 5,6,7,8-Tetrahydronaphthalen-2-amine (10)

Compound **10** was synthesized by the reduction of **4**. Yield: 75%; IR (KBr) ν: 3353 (NH) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ: 6.96 (d, 1H, J = 6.2 Hz, Ar), 6.67 (s, 1H, Ar), 6.62 (d, 1H, J = 6.2 Hz, Ar), 3.58 (s, 1H, NH), 2.80 (m, 4H, 2× CH_2), 1.97 (m, 4H, 2× CH_2); ^{13}C NMR ($CDCl_3$) δ: 144.0, 137.2, 129.9, 125.5, 119.7, 115.6, 29.5, 23.5.

4.6.2. 5,6,7,8-Tetrahydro-5,5,8,8-tetramethylnaphthalen-2-amine (11)[37]

Compound **11** was synthesized by the reduction of **5**. Yield: 98%; IR (KBr) ν: 3392 (NH) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ: 7.31 (d, 1H, Ar), 7.15 (s, 1H, Ar), 6.65 (d, 1H, J = 8.2 Hz, Ar), 3.50 (s, 1H, NH), 1.51 (m, 4H, 2× CH_2), 1.39 (s, 12H, 4× CH_3); ^{13}C NMR ($CDCl_3$) δ: 149.8, 143.6, 137.7, 127.4, 114.1, 112.9, 31.8, 28.7, 22.8.

4.6.3. 4-Cyclopentylbenzenamine (12)

Compound **12** was synthesized by the reduction of **6**. Yield: 53%; IR (KBr) ν: 3362 (NH) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ: 7.37 (d, 1H, J = 8.5 Hz, Ar), 7.15 (d, 1H, J = 7.8 Hz, Ar), 3.55 (s, 1H, NH), 2.80 (m, 1H, CH), 1.98 (m, 2H, CH_2), 1.72–1.45 (m, 6H, 3× CH_2); ^{13}C NMR ($CDCl_3$) δ: 140.2, 139.8, 129.3, 116.4, 41.1, 32.8, 24.0.

4.6.4. 4-(1-Methylcyclohexyl)benzenamine (13)[36]

Compound **13** was synthesized by the reduction of **7**. Yield: 98%; IR (KBr) ν: 3362 (NH) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ: 7.16 (d, 1H, J = 8.5 Hz, Ar), 6.66 (d, 2H, J = 8.4 Hz, Ar), 3.56 (s, 1H, NH), 1.96 (m, 2H, CH_2), 1.48 (m, 8H, 4× CH_2), 1.35 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$) δ: 143.6, 140.3, 126.7, 116.1, 38.0, 37.0, 30.6, 26.5, 22.7.

4.6.5. 4-(1-Ethylcyclohexyl)benzenamine (14)[36]

Compound **14** was synthesized by the reduction of **8**. Yield: 98%; IR (KBr) ν: 3323 (NH) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ: 7.11 (d, 2H, J = 8.6 Hz, Ar), 6.68 (d, 2H, J = 8.6 Hz, Ar), 3.48 (s, 1H, NH), 2.01 (m, 3H, CH, CH_2), 1.71–091 (m, 9H, CH, CH_2), 0.54 (t, 3H, J = 7.9 Hz, CH_3); ^{13}C NMR ($CDCl_3$) δ: 143.4, 136.9, 126.4, 115.0, 40.6, 38.1, 35.9, 26.7, 22.5, 7.9.

4.6.6. 4-(1-Propylcyclohexyl)benzenamine (15)[36]

Compound **15** was synthesized by the reduction of **9**. Yield: 82%; IR (KBr) ν: 3372 (NH) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ: 7.08 (d, 2H, J = 8.6 Hz, Ar), 6.65 (d, 2H, J = 8.3 Hz, Ar), 3.50 (s, 1H, NH), 2.01 (m, 3H, CH, CH_2), 1.78–0.88 (m, 11H, CH, CH_2), 0.76 (s, 3H, J = 7.1 Hz, CH_3); ^{13}C NMR ($CDCl_3$) δ: 143.4, 137.4, 127.7, 115.0, 40.5, 36.4, 26.7, 22.5, 16.7, 14.8.

4.6.7. 3-Cyclohexylbenzenamine (21)

Compound **21** was synthesized by the reduction of **20**. Yield: 75%; IR (KBr) ν: 3356 (NH) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ: 7.11 (t, 1H, J = 7.5 Hz, Ar), 6.65 (d, 1H, J = 7.5 Hz, Ar), 6.53 (m, 2H, Ar), 3.56 (s, 1H, NH), 2.44 (m, 1H, CH), 1.80 (m, 5H, CH, CH_2), 1.39 (m, 5H, CH, CH_2); ^{13}C NMR (DMSO-d_6) δ: 149.5, 146.4, 129.2, 117.4, 113.8, 112.8, 44.7, 34.4, 27.0, 26.3.

4.6.8. 4-(4-Methylcyclohexyloxy)benzenamine (22)

Compound **22** was synthesized by the reduction of **16**. Yield: 42%; IR (KBr) ν: 3421 (NH) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ: 6.77 (d, 2H, J = 8.3 Hz, Ar), 6.62 (d, 2H, J = 8.5 Hz, Ar), 3.97 (m, 1H, CH), 3.42 (s, 1H, NH), 2.07–1.23 (m, 7H, CH, CH_2), 1.02–0.83 (m, 5H, CH, CH_3); ^{13}C NMR ($CDCl_3$) δ: 150.8, 140.3, 118.2, 116.4, 73.4, 34.4, 33.3, 32.2, 22.0.

4.6.9. 3-(4-Methylcyclohexyloxy)benzenamine (23)

Compound **23** was synthesized by the reduction of **17**. Yield: 84%; IR (KBr) ν: 3416 (NH) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ: 7.04 (t, 1H, J = 7.5 Hz, Ar), 6.28 (m, 3H, Ar), 4.11 (m, 1H, CH), 3.62 (s, 1H, NH), 2.13–1.27 (m, 7H, CH, CH_2), 1.05–0.87 (m, 5H, CH, CH_3); ^{13}C NMR ($CDCl_3$) δ: 147.8, 130.0, 107.7, 106.2, 103.3, 71.6, 34.2, 32.8, 30.3, 22.3.

4.6.10. 4-(Cyclooctyloxy)benzenamine (24)

Compound **24** was synthesized by the reduction of **18**. Yield: 98%; IR (KBr) ν: 3411 (NH) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ: 6.72 (d, 2H, J = 8.8 Hz, Ar), 6.62 (d, 2H, J = 8.7 Hz, Ar), 4.24 (m, 1H, CH), 3.41 (s, 1H, NH), 1.90 (m, 4H, 2× CH_2), 1.58 (m, 10H, 5× CH_2); ^{13}C NMR ($CDCl_3$) δ: 150.8, 140.0, 117.9, 116.4, 79.1, 31.7, 27.2, 25.7, 23.1.

4.6.11. 3-(Cyclooctyloxy)benzenamine (25)

Compound **25** was synthesized by the reduction of **19**. Yield: 61%; IR (KBr) ν: 3431 (NH) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ: 7.05 (t, 1H, J = 8.0 Hz, Ar), 6.29 (m, 3H, Ar), 4.38 (m, 1H, CH), 3.64 (s, 1H, NH), 1.91 (s, 4H, 2× CH_2), 1.60 (m, 10H, 5× CH_2); ^{13}C NMR ($CDCl_3$) δ: 159.0, 147.9, 130.1, 107.7, 106.1, 103.3, 77.6, 31.9, 27.2, 25.8, 23.2.

4.7. Preparation of 6,7,8,9-tetrahydro-5H-benzo[7]annulen-2-amine (26)[40]

Palladium on 10% charcoal (55 mg) was added to a solution of 7-nitro-1-tetralone, 1,2,3,4-tetrahydronaphthalene (1 mmol) in 10 mL of acetic acid containing four drops of H_2SO_4 (12 M).[40] The suspension was shaken under 40 psi of hydrogen in a Parr apparatus for 12 h. The mixture was filtered on Celite, and the filtrate was evaporated under reduced pressure. The crude product was mixed with HCl (1 N) and washed with ethyl acetate. The aqueous phase was neutralized at pH 8 with a solution of NaOH (2 N). The product

was extracted with ethyl acetate. The organic layer was washed with brine, dried over anhydrous Na_2SO_4, and evaporated under vacuum.

Yield: 76%; IR (KBr) ν: 3445 (NH) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 6.95 (d, 1H, J = 7.8 Hz, Ar), 6.53 (s, 1H, Ar), 6.47 (d, 1H, J = 7.7 Hz, Ar), 3.50 (s, 1H, NH), 2.76 (t, 4H, J = 4.6 Hz, 2× CH_2), 1.88 (m, 2H, CH_2), 1.71 (m, 4H, 2× CH_2); ^{13}C NMR ($CDCl_3$) δ: 144.5, 144.4, 133.8, 129.9, 116.5, 112.4, 37.0, 35.9, 32.9, 29.0, 28.6.

4.8. General procedure for the preparation of substituted *N*-phenyl-*N*′-(2-chloroethyl)ureas (CEUs, 27–56), *N*-aryl amino-2-oxazolines (4,5-dihydro-*N*-phenyloxazol-2-amine: OXA derivatives, 58–62), *N*-phenyl-*N*′-(2-chloroacetyl)urea derivatives (CAU, 63–67)

EU **57**,[15,16] CEUs **28**,[6,7,10] **32**,[6,7] **33**,[10] **34**,[10] **36** (cHCEU),[7,15,16] **42**–45,[15,16] **48**–51,[15,16] **54** (tBCEU),[6,7,10] **55** (ICEU),[6,7,10] OXA **59**,[16] **62**,[10] and CAU **64**,[16] **67**[10] were prepared as published previously. The corresponding isocyanate (3.6 mmol), either ethylisocyanate, 2-chloroethylisocyanate, or 2-chloroacetylisocyanate, was added dropwise to a stirred solution of the relevant aniline (3 mmol) in dichloromethane (15 mL). The reaction mixture was stirred under a dried nitrogen atmosphere overnight at ambient temperature. The resulting precipitate was filtered, washed with cold ether, and purified by recrystallization from ethanol and water. For the preparation of the 2-amino oxazoline derivatives, a mixture of SiO_2.KF (60:40%) (3.5 mmol) was added to a stirred solution of the appropriate *N*-phenyl-*N*′-(2-chloroethyl)urea derivatives (0.35 mmol) in acetonitrile (10 mL). The suspension was refluxed overnight. After cooling, the mixture was filtered and the solvent was evaporated under reduced pressure. The residue was purified by flash chromatography on silica gel (dichloromethane/methanol 95:5).

4.8.1. 1-(2-Chloroethyl)-3-phenylurea (27)[7,41]

Compound **27** was synthesized from the nucleophilic addition of aniline to 2-chloroethylisocyanate. Yield: 36%; mp: 126–129 °C; IR (KBr) ν: 3320 (NH), 1640 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 7.30 (m, 4H, Ar), 7.09 (m, 1H, Ar), 6.96 (s, 1H, NH), 5.57 (s, 1H, NH), 3.65 (m, 4H, 2× CH_2); ^{13}C NMR ($CDCl_3$) δ: 155.9, 138.3, 129.4, 124.1, 121.2, 44.7, 42.0. MS (ESI) m/z: 199.1 $(M+1)^+$.

4.8.2. 1-(2-Chloroethyl)-3-(1,2,3,4-tetrahydronaphthalen-6-yl)urea (29)[42]

Compound **29** was synthesized from the nucleophilic addition of **10** to 2-chloroethylisocyanate. Yield: 15%; mp: 175–183 °C; IR (KBr) ν: 3334 (NH), 1644 (C=O) cm^{-1}; ^1H NMR (DMSO-d_6) δ: 8.44 (s, 1H, NH), 7.10 (d, 1H, J = 7.1 Hz, Ar), 7.06 (s, 1H, Ar), 6.90 (d, 1H, J = 8.2 Hz, Ar), 6.33 (s, 1H, NH), 3.66 (t, 2H, J = 6.1 Hz, CH_2), 3.40 (m, 2H, CH_2), 2.64 (m, 4H, CH_2), 1.71 (m, 4H, CH_2); ^{13}C NMR (DMSO-d_6) δ: 155.2, 137.6, 136.6, 129.5, 129.0, 118.1, 115.7, 44.5, 41.2, 29.1, 28.2, 23.0, 22.8. MS (ESI) m/z: 253.1 $(M+1)^+$.

4.8.3. 1-(2-Chloroethyl)-3-(1,2,3,4-tetrahydro-1,1,4,4-tetramethylnaphthalen-6-yl)urea (30)

Compound **30** was synthesized from the nucleophilic addition of **11** to 2-chloroethylisocyanate. Yield: 23%; IR (KBr) ν: 3337 (NH), 1660 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 7.60 (d, 1H, J = 8.3 Hz, Ar), 7.41 (s, 1H, Ar), 7.12 (s, 1H, NH), 7.00 (d, 1H, J = 8.0 Hz, Ar), 6.02 (s, 1H, NH), 3.51 (m, 4H, 2× CH_2), 1.52 (m, 4H, 2× CH_2), 1.39 (s, 12H, 4× CH_3); ^{13}C NMR ($CDCl_3$) δ: 156.4, 145.1, 140.7, 135.7, 126.6, 120.5, 119.1, 44.5, 42.1, 31.9, 29.0, 22.6. MS (ESI) m/z: 309.2 $(M+1)^+$.

4.8.4. 1-(2-Chloroethyl)-3-(6,7,8,9-tetrahydro-5H-benzo[7]annulen-3-yl)urea (31)

Compound **31** was synthesized from the nucleophilic addition of **26** to 2-chloroethylisocyanate. Yield: 98%; mp: 159–165 °C; IR (KBr) ν: 3320 (NH), 1638 (C=O) cm^{-1}; ^1H NMR (DMSO-d_6) δ: 8.49 (s, 1H, NH), 7.13 (s, 1H, Ar), 7.09 (s, 1H, Ar), 6.95 (d, 1H, J = 7.9 Hz, Ar), 6.38 (s, 1H, NH), 3.66 (t, 2H, J = 6.2 Hz, CH_2), 3.41 (m, 2H, CH_2), 2.70 (m, 4H, 2× CH_2), 1.77 (m, 2H, CH_2), 1.56 (m, 4H, 2× CH_2); ^{13}C NMR (DMSO-d_6) δ: 155.3, 143.3, 137.8, 136.2, 129.1, 118.9, 115.4, 44.5, 41.2, 36.1, 35.2, 32.1, 28.4, 28.1. MS (ESI) m/z: 267.1 $(M+1)^+$.

4.8.5. 1-(2-Chloroethyl)-3-(4-cyclopentylphenyl)urea (35)

Compound **35** was synthesized from the nucleophilic addition of **12** to 2-chloroethylisocyanate. Yield: 65%; mp: 154–157 °C; IR (KBr) ν: 3324 (NH), 1642 (C=O) cm^{-1}; ^1H NMR (DMSO-d_6) δ: 8.57 (s, 1H, NH), 7.30 (d, 2H, J = 8.3 Hz, Ar), 7.10 (d, 2H, J = 8.3 Hz, Ar), 6.38 (s, 1H, NH), 3.60 (t, 2H, J = 6.1 Hz, CH_2), 3.43 (m, 2H, CH_2), 2.89 (m, 1H, CH), 1.96 (m, 2H, CH_2), 1.73 (m, 2H, CH_2), 1.68 (m, 2H, CH_2), 1.46 (m, 2H, CH_2); ^{13}C NMR (DMSO-d_6) δ: 155.2, 138.7, 138.0, 127.1, 117.9, 44.7, 44.4, 41.3, 29.3, 25.0. MS (ESI) m/z: 267.1 $(M+1)^+$.

4.8.6. 1-(2-Chloroethyl)-3-(3-cyclohexylphenyl)urea (37)[43]

Compound **37** was synthesized from the nucleophilic addition of **21** to 2-chloroethylisocyanate. Yield: 70%; mp: 156–160 °C; IR (KBr) ν: 3323 (NH), 1660 (C=O) cm^{-1}; ^1H NMR (DMSO-d_6) δ: 8.61 (s, 1H, NH), 7.31 (s, 1H, Ar), 7.15 (m, 2H, Ar), 6.76 (d, 1H, J = 7.2 Hz, Ar), 6.41 (s, 1H, NH), 3.67 (t, 2H, J = 6.1 Hz, CH_2), 3.44 (m, 2H, CH_2), 2.43 (m, 1H, CH), 1.77 (m, 5H, CH, CH_2), 1.40 (m, 5H, CH, CH_2); ^{13}C NMR (DMSO-d_6) δ: 155.1, 148.1, 140.3, 128.5, 119.7, 116.2, 115.4, 44.4, 44.0, 41.3, 29.1, 26.4, 25.7. MS (ESI) m/z: 281.1 $(M+1)^+$.

4.8.7. 1-(2-Chloroethyl)-3-(4-morpholinophenyl)urea (38)[44]

Compound **38** was synthesized from the nucleophilic addition of 4-morpholinobenzenamine to 2-chloroethylisocyanate. Yield: 60%; mp: 184–189 °C; IR (KBr) ν: 3330 (NH), 1639 (C=O) cm^{-1}; ^1H NMR (DMSO-d_6) δ: 8.40 (s, 1H, NH), 7.26 (d, 2H, J = 8.5 Hz, Ar), 6.85 (d, 2H, J = 8.4 Hz, Ar), 6.30 (s, 1H, NH), 3.74 (m, 4H, 2× CH_2), 3.64 (m, 2H, CH_2), 3.43 (m, 2H, CH_2), 3.01 (m, 4H, 2× CH_2); ^{13}C NMR (DMSO-d_6) δ: 155.3, 146.1, 132.8, 119.2, 116.0, 66.2, 49.4, 44.5, 41.4. MS (ESI) m/z: 284.1 $(M+1)^+$.

4.8.8. 1-(2-Chloroethyl)-3-(4-(1-methylcyclohexyl)phenyl)urea (39)

Compound **39** was synthesized from the nucleophilic addition of **13** to 2-chloroethylisocyanate. Yield: 62%; IR (KBr) ν: 3315 (NH), 1699 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 7.95 (s, 1H, NH), 7.20 (s, 4H, Ar), 6.30 (s, 1H, NH), 3.55 (m, 4H, 2× CH_2), 1.50 (m, 10H, 5× CH_2), 1.29 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$) δ: 156.8, 144.3, 136.3, 126.2, 119.6, 43.9, 41.9, 38.2, 37.8, 30.4, 26.4, 22.7. MS (ESI) m/z: 295.2 $(M+1)^+$.

4.8.9. 1-(2-Chloroethyl)-3-(4-(1-ethylcyclohexyl)phenyl)urea (40)

Compound **40** was synthesized from the nucleophilic addition of **14** to 2-chloroethylisocyanate. Yield: 63%; IR (KBr) ν: 3333 (NH), 1711 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 7.72 (s, 1H, NH), 7.15 (m, 4H, Ar), 6.11 (s, 1H, NH), 3.52 (m, 4H, 2× CH_2), 1.23 (m, 12H, 6× CH_2), 0.50 (t, 3H, J = 7.4 Hz, CH_3); ^{13}C NMR ($CDCl_3$) δ: 156.4, 135.9, 127.6, 119.9, 44.7, 42.1, 36.2, 26.7, 22.4, 7.9. MS (ESI) m/z: 309.2 $(M+1)^+$.

4.8.10. 1-(2-Chloroethyl)-3-(4-(1-propylcyclohexyl)phenyl)urea (41)

Compound **41** was synthesized from the nucleophilic addition of **15** to 2-chloroethylisocyanate. Yield: 71%; IR (KBr) ν: 3372 (NH), 1714 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 7.81 (s, 1H, NH), 7.24 (m, 4H, Ar), 6.11 (s, 1H, NH), 3.54 (m, 4H, CH_2), 1.97–

0.88 (m, 14H, CH_2), 0.66 (t, 3H, J = 7.7 Hz, CH_3), 3.45, 2.21 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$) δ: 156.4, 142.4, 135.8, 127.4, 120.1, 44.7, 42.1, 40.9, 36.2, 26.6, 22.3, 16.7, 14.7. MS (ESI) m/z: 323.2 $(M+1)^+$.

4.8.11. 1-(4-(4-Methylcyclohexyloxy)phenyl)-3-(2-chloroethyl)urea (46)

Compound **46** was synthesized from the nucleophilic addition of **22** to 2-chloroethylisocyanate. Yield: 70%; mp: 112–118 °C; IR (KBr) v: 3304 (NH), 1635 (C=O) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ: 7.30 (s, 1H, NH), 7.13 (d, 2H, J = 8.7 Hz, Ar), 6.81 (d, 2H, J = 8.3 Hz, Ar), 5.80 (s, 1H, NH), 4.05 (s, 1H, CH), 3.51 (m, 4H, 2× CH_2), 2.06–1.22 (m, 7H,CH, CH_2), 1.08–0.86 (m, 5H, CH, CH_3); ^{13}C NMR ($CDCl_3$) δ: 156.8, 154.8, 131.0, 123.7, 116.9, 72.9, 44.6, 42.0, 33.2, 32.0, 31.4, 22.2. MS (ESI) m/z: 311.2 $(M+1)^+$.

4.8.12. 1-(3-(4-Methylcyclohexyloxy)phenyl)-3-(2-chloroethyl)urea (47)

Compound **47** was synthesized from the nucleophilic addition of **23** to 2-chloroethylisocyanate. Yield: 98%; IR (KBr) v: 3353 (NH), 1710 (C=O) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ: 7.62 (s, 1H, NH), 7.13 (t, 1H, J = 8.1 Hz, Ar), 7.00 (s, 1H, Ar), 6.78 (t, 1H, J = 6.6 Hz, Ar), 6.57 (d, 1H, J = 6.5 Hz, Ar), 6.00 (s, 1H, NH), 4.08 (s, 1H, CH), 3.46 (m, 4H, 2× CH_2), 2.06–1.21 (m, 7H, CH, CH_2), 0.97–0.82 (m, 5H, CH, CH_3); ^{13}C NMR ($CDCl_3$) δ: 158.2, 156.2, 139.9, 129.8, 112.5, 111.3, 108.5, 72.2, 44.4, 42.0, 32.0, 31.8, 31.4, 22.2. MS (ESI) m/z: 311.2 $(M+1)^+$.

4.8.13. 1-(2-Chloroethyl)-3-(4-(cyclooctyloxy)phenyl)urea (52)

Compound **52** was synthesized from the nucleophilic addition of **24** to 2-chloroethylisocyanate. Yield: 62%; mp: 106–110 °C; IR (KBr) v: 3293 (NH), 1710 (C=O) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ: 7.36 (s, 1H, NH), 7.12 (d, 2H, J = 8.8 Hz, Ar), 6.75 (d, 2H, J = 8.8 Hz, Ar), 5.85 (s, 1H, NH), 4.31 (m, 1H, CH), 3.48 (m, 4H, 2× CH_2), 1.71 (m, 6H, 3× CH_2), 1.60 (m, 8H, 4× CH_2); ^{13}C NMR ($CDCl_3$) δ: 156.9, 154.7, 130.9, 123.6, 116.8, 78.4, 44.4, 42.0, 31.7, 27.1, 25.7, 23.1. MS (ESI) m/z: 325.2 $(M+1)^+$.

4.8.14. 1-(2-Chloroethyl)-3-(3-(cyclooctyloxy)phenyl)urea (53)

Compound **53** was synthesized from the nucleophilic addition of **25** to 2-chloroethylisocyanate. Yield: 86%; mp: 98–103 °C; IR (KBr) v: 3392 (NH), 1710 (C=O) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ: 7.94 (s, 1H, NH), 7.10 (t, 1H, J = 8.1 Hz, Ar), 6.97 (s, 1H, Ar), 6.75 (d, 1H, J = 7.6 Hz, Ar), 6.51 (d, 1H, J = 8.1 Hz, Ar), 6.23 (s, 1H, NH), 4.34 (m, 1H, CH), 3.47 (m, 4H, 2× CH_2), 1.87 (m, 6H, 3× CH_2), 1.72 (m, 8H, 4× CH_2); ^{13}C NMR ($CDCl_3$) δ: 158.6, 156.4, 140.1, 129.7, 112.2, 110.9, 108.3, 78.0, 44.2, 41.9, 31.6, 27.1, 25.7, 23.1. MS (ESI) m/z: 325.2 $(M+1)^+$.

4.8.15. 1-(2-Chloroethyl)-3-(4-hexylphenyl)urea (56)[6,7]

Compound **56** was synthesized from the nucleophilic addition of 4-hexylbenzenamine to 2-chloroethylisocyanate. Yield: 14%; mp: 121–123 °C; IR (KBr) v: 3300 (NH), 1640 (C=O) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ: 8.53 (s, 1H, NH), 7.30 (d, 2H, J = 8.3 Hz, Ar), 7.03 (d, 2H, J = 8.3 Hz, Ar), 6.40 (s, 1H, NH), 3.66 (t, 2H, J = 6.0 Hz, CH_2), 3.42 (m, 2H, CH_2), 2.48 (t, 2H, J = 7.4 Hz, CH_2), 1.52 (m, 2H, CH_2), 1.26 (m, 6H, 3× CH_2), 0.86 (m, 3H, CH_3); ^{13}C NMR (DMSO-d_6) δ: 155.1, 138.0, 135.1, 128.4, 117.9, 44.4, 41.3, 34.5, 31.1, 28.3, 22.1, 13.9. MS (ESI) m/z: 283.2 $(M+1)^+$.

4.8.16. 4,5-Dihydro-*N*-phenyloxazol-2-amine (58)

Compound **58** was synthesized from **27** and SiO_2.KF. Yield: 98%; IR (KBr) v: 3309 (NH) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ: 7.24 (m, 4H, Ar), 6.99 (m, 1H, Ar), 6.58 (s, 1H, NH), 4.37 (t, 2H, J = 8.3 Hz, CH_2), 3.80 (t, 2H, J = 8.3 Hz, CH_2); ^{13}C NMR ($CDCl_3$) δ: 157.9, 129.0, 122.5, 120.1, 67.4, 49.2. MS (ESI) m/z: 163.1 $(M+1)^+$.

4.8.17. *N*-(3-Cyclohexylphenyl)-4,5-dihydrooxazol-2-amine (60)

Compound **60** was synthesized from **37** and SiO_2. Yield: 50%; IR (KBr) v: 3323 (NH) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ: 7.35 (s, 1H, Ar), 7.25 (m, 2H, Ar), 7.08 (d, 1H, J = 7.1 Hz, Ar), 6.41 (s, 1H, NH), 4.43 (t, 2H, J = 8.0 Hz, CH_2), 3.70 (t, 2H, J = 8.0 Hz, CH_2), 2.45 (m, 1H, CH), 1.82 (m, 5H, CH, CH_2), 1.38 (m, 5H, CH, CH_2); ^{13}C NMR ($CDCl_3$) δ: 156.5, 149.0, 139.6, 128.9, 120.8, 119.2, 117.3, 66.5, 47.4, 44.2, 34.4, 26.7, 26.2. MS (ESI) m/z: 245.2 (M^++1).

4.8.18. 4,5-Dihydro-*N*-(4-morpholinophenyl)oxazol-2-amine (61)

Compound **61** was synthesized from **38** and SiO_2. Yield: 93%; mp: 149–157; IR (KBr) v: 3367 (NH) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ: 7.19 (d, 2H, J = 8.9 Hz, Ar), 6.84 (d, 2H, J = 8.9 Hz, Ar), 5.62 (s, 1H, NH), 4.36 (t, 2H, J = 8.3 Hz, CH_2), 3.83 (m, 6H, 3× CH_2), 3.07 (t, 4H, J = 4.7 Hz, 2× CH_2); ^{13}C NMR ($CDCl_3$) δ: 157.8, 147.1, 134.7 121.1, 116.8, 66.9, 66.8, 50.2, 50.1. MS (ESI) m/z: 248.1 (M^++1).

4.8.19. 1-(2-Chloroacetyl)-3-phenylurea (63)

Compound **63** was synthesized from the nucleophilic addition of aniline to 2-chloroacetylisocyanate. Yield: 73%; mp: 164–166 °C; IR (KBr) v: 3240 (NH), 1715 (C=O) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ: 10.25 (s, 1H, NH), 9.25 (s, 1H, NH), 7.51 (d, 2H, J = 7.9 Hz, Ar), 7.36 (t, 2H, J = 8.1 Hz, Ar), 7.16 (t, 1H, J = 7.4 Hz, Ar), 4.20 (s, 2H, CH_2); ^{13}C NMR ($CDCl_3$) δ: 167.9, 150.0, 136.6, 129.2, 124.9, 120.5, 42.5. MS (ESI) m/z: 163.1 $(M+1)^+$.

4.8.20. 1-(2-Chloroacetyl)-3-(3-cyclohexylphenyl)urea (65)

Compound **65** was synthesized from the nucleophilic addition of **21** to 2-chloroacetylisocyanate. Yield: 70%; mp: 119–124 °C; IR (KBr) v: 3226 (NH), 1717 (C=O) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ: 10.89 (s, 1H, NH), 10.15 (s, 1H, NH), 7.34 (d, 2H, J = 9.3 Hz, Ar), 7.25 (t, 1H, J = 7.1 Hz, Ar), 6.97 (d, 1H, J = 7.5 Hz, Ar), 4.42 (s, 2H, CH_2), 2.52 (m, 1H, CH), 1.74 (m, 5H, CH, CH_2), 1.36 (m, 5H, CH, CH_2); ^{13}C NMR (DMSO-d_6) δ: 168.7, 150.2, 148.6, 137.4, 128.9, 122.3, 118.1, 117.4, 43.8, 43.2, 33.9, 26.4, 25.6. MS (ESI) m/z: 295.1 $(M+1)^+$.

4.8.21. 1-(2-Chloroacetyl)-3-(4-morpholinophenyl)urea (66)

Compound **66** was synthesized from the nucleophilic addition of 4-morpholinobenzenamine to 2-chloroacetylisocyanate. Yield: 39%; mp: 175–179 °C; IR (KBr) v: 3362 (NH), 1702 (C=O) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ: 10.84 (s, 1H, NH), 9.99 (s, 1H, NH), 7.39 (d, 2H, J = 8.8 Hz, Ar), 6.94 (d, 2H, J = 8.8 Hz, Ar), 4.39 (s, 2H, CH_2), 3.75 (t, 4H, J = 4.7 Hz, 2× CH_2), 3.08 (t, 4H, J = 4.41 Hz, 2× CH_2); ^{13}C NMR (DMSO-d_6) δ: 168.5, 150.2, 147.7, 129.5, 121.1, 115.7, 66.1, 48.9, 43.2. MS (ESI) m/z: 298.1 $(M+1)^+$.

4.9. Biological assays

4.9.1. Materials and reagents

The monoclonal antibody anti-β_{II}-tubulin (clone TUB 2.1) and the mouse anti-thioredoxin (clone 2G11) were obtained from Sigma Chemicals (St. Louis, MO) and BD Transduction Laboratories (Mississauga, ON, Canada), respectively. The anti-mouse Alexa 488 was purchased from Invitrogen (Carlsbad, CA). The horseradish peroxidase-conjugated anti-mouse IgG was supplied by GE Healthcare (Little Chalfont, Buckinghamshire, UK).

4.9.2. Cell culture

Human cell lines HT-29 (colon carcinoma), MCF-7 (breast carcinoma) and M21 (skin melanoma) cells were purchased from the American Type Culture Collection (Manassas, VA). The cells were cultured in DMEM medium supplemented with 2.2 g/L sodium bicarbonate, 4.5 g/L glucose, 100 μg/mL streptomycin sulfate A,

100 U of penicillin G, 292 μg/mL glutamine, and 5% bovine calf serum (Hyclone laboratories, Logan, UT). Cells were maintained in a moisture saturated atmosphere at 37 °C in 5% CO_2.

4.9.3. Cell growth inhibition assay

The growth inhibition potency of CEUs, OXAs and CAUs was assessed using the procedure described by the National Cancer Institute for its drugs screening program.[31] 96-well microtiter plates were seeded with either 5×10^3 HT-29, 3.5×10^3 M21, or 3.5×10^3 MCF-7 cells suspended in 100 μL of calf serum. Freshly solubilized drugs in DMSO were diluted in culture medium and aliquots of 100 μL containing sequential dilution of drugs were added. Final drug concentrations ranged from 100 to 0.3 μM. DMSO concentration was maintained at 0.12% to avoid growth inhibition. Plates were incubated for 48 h. The incubation was stopped by addition of cold trichloroacetic acid to the wells (10% final concentration), followed by incubation for 1 h at 4 °C. Plates were washed five times with water. Sulforhodamine B solution (50 μL) at 0.1% (w/v) in 1% acetic acid was added to each well, and plates were incubated for 15 min at room temperature. After staining, unbounded dye was removed by washing five times with 1% acetic acid. Bonded dye was solubilized with 10 mM Tris base, and the absorbance was read using a μ Quant Universal Microplate Spectrophotometer (Biotek, Winooski, VT) at 585 nm. A background OD from a control reference plate fixed on the day of treatment was subtracted from the OD obtained with the 48-h growth period. The growth inhibition percentage was calculated with reference to DMSO-treated cells for each drug concentrations. The results were obtained from at least three separated experiments. The GI_{50} assay was considered valid when the variability among data for a given set of conditions, within the same experiment, was less than 10% with respect to the mean value.

4.9.4. Cell cycle analysis

After incubation of 3.5×10^5 MCF-7 cells with compounds **32–37, 39–57**, **59**, and **64** at two, four, and eight times their respective GI_{50} or DMSO for 24 h, the cells were trypsinized, washed with PBS, resuspended in 1 mL of PBS, and fixed by the addition of 2.4 mL of ice-cold anhydrous ethanol. Then, cells were centrifuged for 5 min at 1000*g*. Cell pellets were resuspended in PBS containing 50 μg/mL of propidium iodide and 200 μg/mL of RNase. Mixtures were incubated at room temperature for 30 min, and cell cycle distribution was analyzed using an Epics Elite ESP flow cytometer (Coulter Corporation, Miami, FL).

4.9.5. Confocal fluorescence microscopy

M21 cells were seeded at 1×10^5 cells per well in 6-well plates that contained 22-μm glass coverslips coated with fibronectin (10 μg/mL) and incubated for 24 h at 37 °C. Tumor cells were incubated either with CEUs (**27–56**), OXAs (**58–62**), CAUs (**63–67**), or EU (**57**) at two times the GI_{50} and cisplatin (25 μM), colchicine (50 nM), PX-12 (25 μM), vinblastine sulfate (50 nM), or DMSO (0.12%). Afterward, the cells were washed twice with PBS (pH 7.4), and then fixed with 3.7% formaldehyde in PBS for 20 min. After two washes with PBS, the cells were permeabilized with 0.1% saponin in PBS and blocked with 3% (w/v) BSA in PBS for 1 h at 37 °C. The cells were then incubated for 2 h at 37 °C with the anti-thioredoxin-1 monoclonal antibody (BD Transduction Laboratories) in a solution containing 0.1% saponin and 3% BSA in PBS (1:200). The cells were washed three times with PBS containing 0.05% Tween 20™ and incubated for 1 h at 37 °C in blocking buffer containing anti-mouse IgG Alexa-488 (1:1000), 4′,6-diamidino-2-phenylindole (2.5 μg/mL in PBS) to stain the cellular nuclei (1:2000). Cells were then processed as described above. The cellular distribution of the fluorescent thioredoxin-1 was assessed using an Olympus BX51 microscope. Images were captured as 8-bit tagged image format files with a Q imaging RETIGA EXI digital camera driven by Image Pro Express software.

4.9.6. Western blot analysis

Prior to drug exposure, approximately 5×10^5 M21 cells were seeded into 6-well plates and incubated for 12 h. Exponentially growing M21 cells were incubated in the presence of two times the GI_{50} of the selected CEU; vinblastine sulfate, colchicine, and paclitaxel were tested at 50 nM for 16, 24, and 48 h at 37 °C. The controls consisted of the final concentration of DMSO in the culture medium (maintained under 0.12% (v/v)). Afterward, floating and the adherent M21 cells were pooled, washed with ice-cold PBS, and then lysed by addition of 100 μL Laemmli sample buffer. The cell extracts were boiled for 5 min. The protein concentration was determined using the Lowry method. Twenty micrograms of proteins from the protein extracts were subjected to electrophoresis using 0.1% SDS and 10% polyacrylamide gels. The proteins were transferred onto nitrocellulose membranes that were incubated with TBSMT (TBS, pH 7.4, 5% fat-free dry milk or BSA or TBST and 0.1% Tween 20™) for 1 h at 37 °C, and then with the β-tubulin (1:500, 5% fat-free dry milk) primary antibody for 1 h at room temperature. Membranes were washed with TBST and incubated with 1:2500 peroxidase-conjugated anti-mouse immunoglobulin in TBSMT for 1 h at room temperature. After washing the membranes with TBST, detection of the immunoblot was carried out with an enhanced chemiluminescence (ECL) detection reagent kit.

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