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Aromatic 2-chloroethyl urea derivatives and bioisosteres. Part 2: Cytocidal activity and effects on the nuclear translocation of thioredoxin-1, and the cell cycle progression

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ABSTRACT

Recently, a subset of *N*-phenyl-*N*′-(2-chloroethyl)ureas (CEU) was found abrogating the nuclear translocation of thioredoxin-1 and arresting the cell cycle in G_0/G_1 phase. Several derivatives were prepared to assess their effect on cell cycle progression and on the intracellular location of Trx-1. Compounds **1**–**20**, **21**–**40**, and **41**–**60** exhibited GI_{50} between 1 and 80 μM. Immunocytochemistry analysis showed compounds **4**, **6**, **8**, **10**, **11**, **23**, **24**, **26**–**31**, **34**, **37**, **41**, **44**, **46**–**51**, **53**, **56**, and **57** inhibiting the nuclear translocation of Trx-1. Our results suggest that increasing the electrophilic character of these molecules might enhance the antiproliferative activity at the expense of the selectivity toward thioredoxin-1 and the G_0/G_1 phase arrest.

1. Introduction

N-Phenyl-*N*′-(2-chloroethyl)ureas (CEU) are soft alkylating agents developed in our laboratory as potential anticancer drugs.[1–12] These molecules were designed initially from the aromatic moiety of nitrogen mustards such as chlorambucil, and the non-nitrosated pharmacophore of aliphatic nitrosoureas such as carmustine. CEUs cytotoxicity has been shown to circumvent a large number of mechanisms of chemoresistance, including increased P-glycoprotein expression, increased DNA repair, increased intracellular gluthatione-*S*-transferase activity, and alteration of topoisomerase II activity.[2] Prototypical CEUs such as 1-(2-chloroethyl)-3-(4-*tert*-butylphenyl)urea (tBCEU) and 1-(2-chloroethyl)-3-(4-iodophenyl)ureas (ICEU) covalently bind to the colchicine-binding site on β-tubulin isoform 2 via a unique acylation of the glutamic acid residue in position 198 that leads to the arrest of the cell cycle progression in G_2/M transition and to apoptosis.[3,4,13]

Our structure–activity relationships and molecular pharmacology studies unexpectedly showed that a CEU derivative named 1-(2-chloroethyl)-3-(4-cyclohexylphenyl)ureas (cHCEU) was blocking the cell cycle progression in G_0/G_1 phase instead of G_2/M as all other antimicrotubules do.[13] In addition, studies using [^{14}C]cHCEU confirmed that the cHCEU does not bind to the colchicine-binding site but instead to the thioredoxin isoform-1 (Trx-1), the mitochondrial voltage-dependent anion channel isoform-1, and to a lesser extent to a few other unidentified cytosolic proteins. Interestingly, Trx-1 is an important protein in the G_1 to G_2/M phase transition and its alkylation may lead, at least partly, to the inhibition of its presence in the cell's nucleus and to the arrest of the cell cycle progression into the G_0/G_1 phase.[13] Interestingly, the arrest of the cell cycle progression in G_0/G_1 phase is the hallmark of drugs such as vitamin E,[14] acetylsalicylic acid,[15] sulindac,[16] celecoxib,[17] and troglitazone[18] or in dietary agents such as epigallocatechin-3-gallate,[19] resveratrol,[20,21] capsaicin,[22] and benzyl isothiocyanate[23] that are exhibiting chemopreventive activities against various cancers.

That observation prompted us to developed new molecules exhibiting similar properties.[24,25] To that end, a new subset of cytostatic CEU derivatives has been prepared to study the effect of the nature and the position of the substituents on the aromatic ring in regard to the inhibition of the intracellular translocation of Trx-1.[25] In addition, we have prepared also a series of analogues and bioisosteres of CEU, namely, *N*-phenyl-*N*′-(2-ethyl)ureas (EU), *N*-phenyl-*N*′-(2-chloroacetyl)ureas (CAU), and *N*-aryl amino-2-oxazoline (4,5-dihydro-*N*-phenyloxazol-2-amines: OXA) derivatives to study the effect of different alkylating moieties on the translocation of Trx-1 and their antiproliferative activity on three human tumor cell lines, namely, HT-29, M21, and MCF-7 tumor cells.

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2. Results and discussion

2.1. Chemistry

Scheme 1 illustrates the synthesis of CEU derivatives, EU derivatives, and CAU derivatives, respectively. These compounds were prepared by the nucleophilic addition of either 2-chloroethylisocyanate, ethylisocyanate or 2-chloroacetylisocyanate, respectively, on the corresponding anilines.[3,2] Anilines substituted by cycloalkyloxy moieties were prepared using the Williamson reaction followed by the reduction of the aromatic nitro group using $SnCl_2$.[2,26] OXA derivatives were prepared from the catalytic cyclization of CEU in presence of KF adsorbed on SiO_2 (ratio 4:6) in acetonitrile at room temperature (Scheme 1).

2.2. Antiproliferative activity

Tumor cell growth inhibition activity by CEU and classical antineoplastics was assessed on three human cancer cell lines: breast carcinoma MCF-7, skin melanoma M21, and colon carcinoma HT-29 cells. Cell growth inhibition was assessed according to the NCI/NIH Developmental Therapeutics Program. The results are summarized in Table 1 and expressed as the concentration of drug inhibiting cell growth by 50% (GI_{50}).

The results in Table 1 show that the antiproliferative activity of the drugs prerequisite the presence of an electrophilic group on the urea moiety of the molecules. Accordingly, EU derivatives, which are devoid of any electrophilic character, did not inhibit the growth of the tumor cells tested in this study at concentrations up to 100 μM (data not shown). However, all CAU derivatives (**41**–**60**) bearing the potent electrophilic 2-chloroacetylamino moiety displayed antiproliferative activity ranging from 1 to 10 μM on all tumor cells tested. The GI_{50} of CAU are in general significantly higher than their CEU counterparts. Interestingly, the bioisosteric OXA (**21**–**40**) were less active than CAU (**41**–**60**), but their antiproliferative activities were nevertheless similar to their corresponding CEU (**1**–**20**) counterparts. It is of interest to point out that the substitution of the aromatic ring on position 3 of CEU (**7**, **9**) and OXA derivatives (e.g., **29**, **31**, and **35**) exhibited a significant improvement of the antiproliferative activity of the drugs when compared to molecules substituted on position 4 (e.g., **6**, **8**, **28**, and **30**).

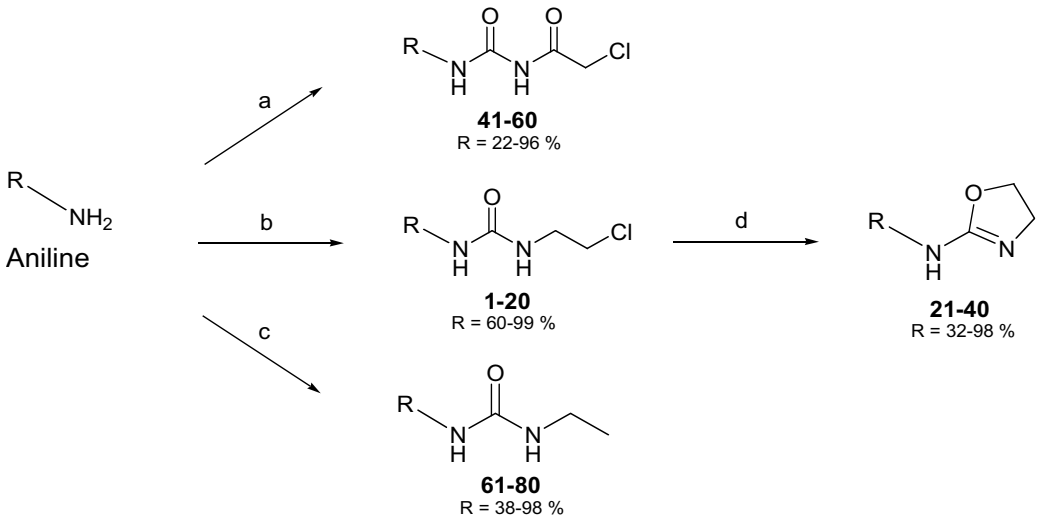
2.3. Effects of aromatic chloroethylureas and derivatives on the cell cycle

The results obtained are suggesting that CEU **4**, **6**, **10**, **11** and their OXA counterparts **24**, **26**, **30** and **31** are acting similarly on the cell cycle, arresting its progression in G_0/G_1 phase. However, the increase of the electrophilicity of their CAU counterparts **44**, **46**, **50** and **51** leads to significantly more potent antiproliferative molecules that still abrogating the presence of nuclear thioredoxin-1 but having lost the ability to arrest the cell cycle progression in G_0/G_1 (Table 2).

2.4. Effects of cHCEU derivatives on Trx-1 translocation

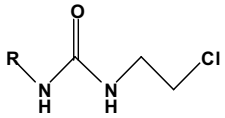
Previously, we have demonstrated the inhibition of the translocation of thioredoxin-1 induced by cHCEU and some of its derivatives.[25] To complete our structure–activity relationship studies, we analyzed the intracellular distribution of Trx-1 in M21 cancer cell line using an anti-Trx-1 monoclonal antibody with the new derivatives bearing various (EU, OXA, CAU) alkylating moieties. Table 1 shows that OXA **23**–**31**, **34**, **37** and CAU **41**, **44**, **46**–**51**, **56**, **57** derivatives were potent antiproliferative agents and modified dramatically the intracellular location of Trx-1 in a similar fashion as previously reported with CEU **4** (cHCEU), **6**, **8**, **10**, and **11**.[15] The CEU derivatives **2**, **3**, **5**, **7**, **9**, **17** (ICEU), **18**, the OXA derivatives **22**, **25**, **39**, **40**, and the CAU derivative **45**, **47**, **53**, **58** were still exhibiting a significant antiproliferative activity on all three tumor cell lines tested. However, their effect on the intracellular distribution of thioredoxin-1 was mitigated. Finally, the CEU derivatives **12**, **13**–**15**, **16** (tBCEU), **19**, **20**, the OXA derivatives **32**, **33**, **35**, **36**, **38**, and the CAU derivatives **42**, **43**, **52**, **54**, **55**, **59** did not modify the intracellular distribution of Trx-1 while exhibiting also antiproliferative activities suggesting that their antiproliferative activity is not solely related to thioredoxin-1 alkylation.

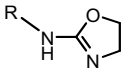
PX-12, colchicine, paclitaxel, and vinblastine used as controls were very potent antiproliferative agents blocking the cell cycle progression in G_2/M phase and did not change the location of thioredoxin when compared to DMSO (0.125%), used as excipient. Unexpectedly, CEU **1** and OXA **21** exhibited a significant increase of the presence of thioredoxin-1 in the nuclear compartment similar to the one observed with cisplatin. Such a nuclear increase of thioredoxin-1 with cisplatin has been already documented and is

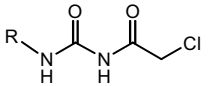


Scheme 1. Synthesis of aryl chloroethylureas; Reagents: (a) 2-chloroacetylisocyanate, CH_2Cl_2; (b) 2-chloroethylisocyanate, CH_2Cl_2; (c) ethylisocyanate, CH_2Cl_2; (d) SiO_2·KF, CH_3CN.

Table 1
Growth inhibition activity of compounds **1**–**60**, **61**, **64**, **68**, **71**, PX-12, cisplatin, colchicine, vinblastine, and paclitaxel on MCF-7, M21, and HT-29 cells, and immunocytochemistry performed with anti-thioredoxin on M21 cells







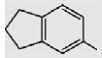
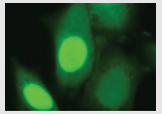
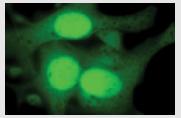
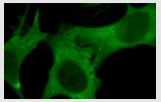
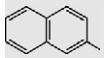
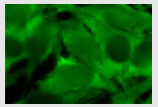
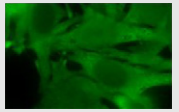
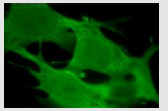
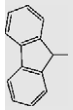
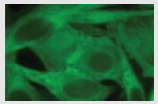
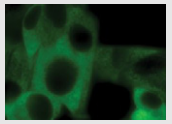
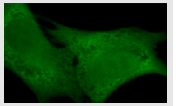
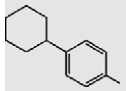
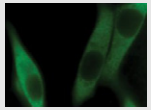
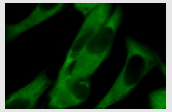
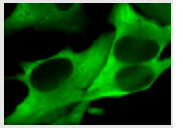
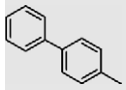
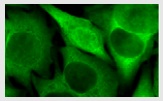
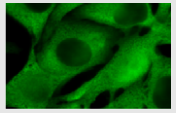
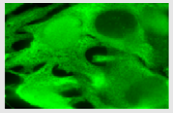
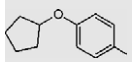
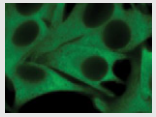
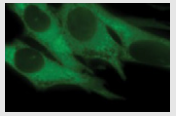
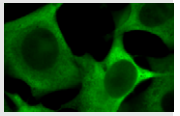
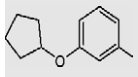
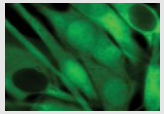
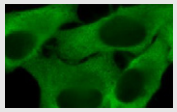
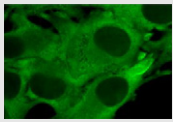
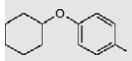
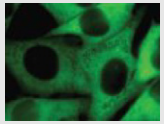
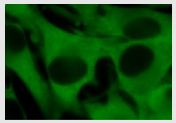
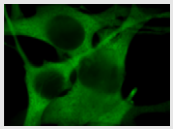
R	Mol#	GI_{50} (μM)			Trx-1 location	Mol#	GI_{50} (μM)			Trx-1 location	Mol#	GI_{50} (μM)			Trx-1 location
		HT-29	M21	MCF-7			HT-29	M21	MCF-7			HT-29	M21	MCF-7	
	**1**	3.6	8.0	14.1		**21**	4.9	8.1	9.1		**41**	7.0	1.7	4.0	
	**2**	1.9	4.1	5.6		**22**	2.0	3.5	4.5		**42**	4.9	3.7	7.6	
	**3**	19	27	22		**23**	15	17	11		**43**	8.6	3.4	4.8	
	**4**	12.6	21	21		**24**	12.8	17.8	17.5		**44**	2.3	0.9	1.5	
	**5**	8.3	14.5	42		**25**	5.8	8.8	10.6		**45**	2.3	1.4	3.9	
	**6**	31	38	49		**26**	16.1	21.1	23.1		**46**	3.1	2.9	3.9	
	**7**	9.9	22	35		**27**	52.2	76.9	62.1		**47**	3.1	3.5	6.2	
	**8**	23	36	45		**28**	12.6	20.8	19.1		**48**	3.1	5.0	4.7	

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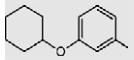
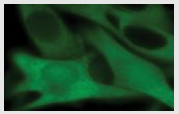
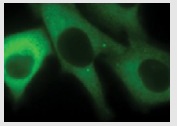
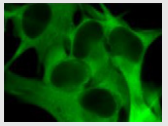
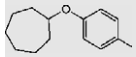
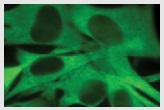
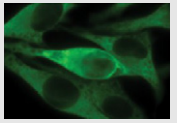
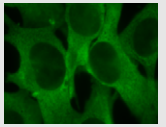
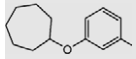
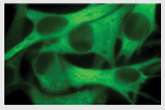
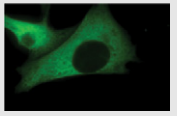
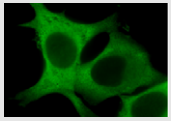
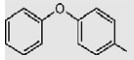
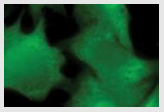
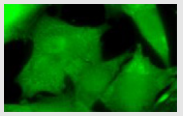
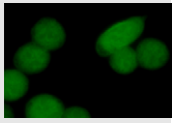
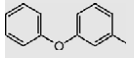
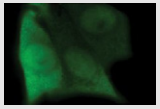
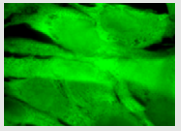
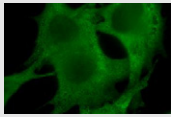
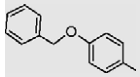
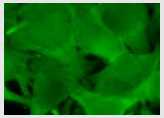
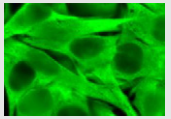
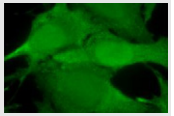
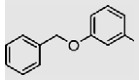
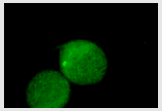
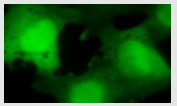
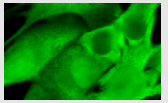
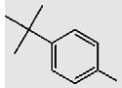
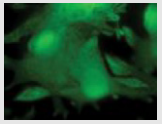
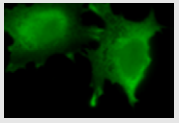
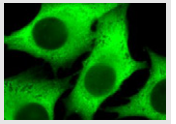
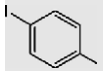
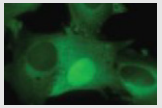
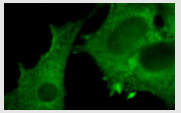
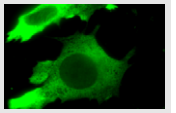
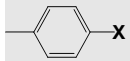
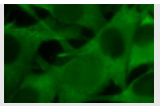
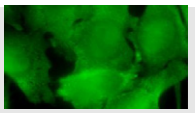
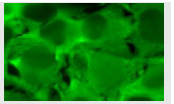
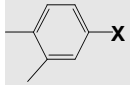
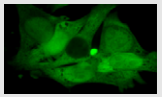
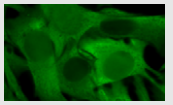
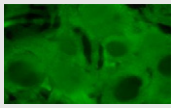
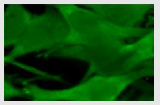
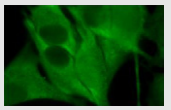
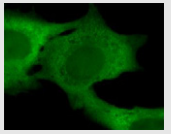
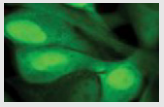
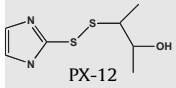
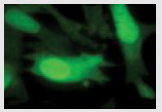
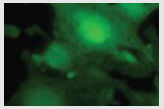
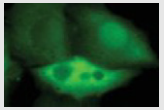
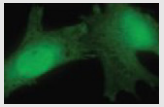
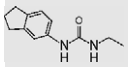
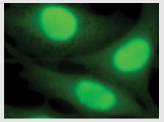
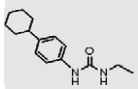
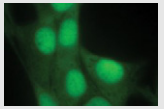
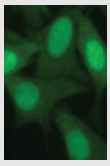
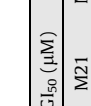
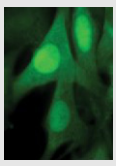
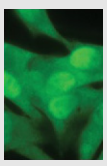
Table 1 (*continued*)

R	Mol#	GI_{50} (μM)			Trx-1 location	Mol#	GI_{50} (μM)			Trx-1 location	Mol#	GI_{50} (μM)			Trx-1 location
		HT-29	M21	MCF-7			HT-29	M21	MCF-7			HT-29	M21	MCF-7	
	**9**	7.9	20	25		**29**	4.4	12.0	15.9		**49**	3.8	2.7	6.3	
	**10**	12.5	18	19		**30**	16.6	27.1	26.8		**50**	2.3	5.0	3.6	
	**11**	10.7	20	21		**31**	6.9	14.4	14.8		**51**	6.9	4.2	7.2	
	**12**	2.4	5.2	9.1		**32**	2.0	3.1	3.5		**52**	3.6	4.2	4.2	
	**13**	5.6	16	24		**33**	5.5	9.6	12.2		**53**	5.2	5.3	8.1	
	**14**	37.5	na	79.7		**34**	10.3	15.9	11.1		**54**	2.6	5.1	4.6	
	**15**	2.9	6.5	10.0		**35**	0.8	1.5	2.6		**55**	6.5	4.5	6.2	
	**16**	2.3	4.3	6.2		**36**	1.5	2.4	3.0		**56**	3.3	1.1	4.8	
	**17**	1.9	3.9	6.0		**37**	1.3	2.0	2.9		**57**	6.9	4.2	6.8	

	**18**	16	33	44		**38**	13	22	27		**58**	10	4.9	7.5	
	**19**	4.2	8.4	12		**39**	2.7	5.1	5.7		**59**	6.9	4.3	5.5	
	**20**	50.6	na	11.3		**40**	53.4	60.7	24.1		**60**	5.1	3.2	6.2	
Cisplatin		23.5	22	21											
 PX-12		18.2	9.1	10.2											
Colchicine		0.004	0.015	0.009											
Paclitaxel		0.015	0.037	0.054											
Vinblastine		0.002	0.010	0.002											
	**61**	na	na	na											
	**64**	na	na	na											

(*continued on next page*)

Table 1 (*continued*)

R	Mol#	GI_{50} (μM)			Trx-1 location	Mol#	GI_{50} (μM)			Trx-1 location	Mol#	GI_{50} (μM)			Trx-1 location
		HT-29	M21	MCF-7			HT-29	M21	MCF-7			HT-29	M21	MCF-7	
	68	na	na	na											
	**71**	na	na	na											
DMSO 0.125%		na	na	na											

na, not active.
CEU derivatives were tested for thioredoxin-1 localization by immunocytochemistry on M21 cells at: (a) 50 μM for compounds **1**–**11**, **13**–**18**, **20**, **21**–**34**, **38**, **40**, **61**, **64**, **68**, and **71**, (b) 25 μM for compounds **1**, **5**, **12**, **19**, **21**, **25**, **29**, **31**, **33**, and **34**, (c) 10 μM for compounds **2**, **12**, **15**–**17**, **19**, and **39**, (d) 5 μM for compounds **22**, **32**, **35**–**37**, **41**–**60**.
Cisplatin, vinblastine, colchicine, paclitaxel, and PX-12 were used at 30, 0.1, 0.1, 0.1, and 40 μM, respectively.

related to chemoresistance mechanisms of cancer cells toward that drug.[27] Whether or not that molecules alkylating thioredoxin-1 could decrease cellular chemoresistance to cisplatin remains to be demonstrated.

3. Conclusion

This study was designed to assess the importance of the nature and the position of the substituents on the aromatic ring together with the role of the electrophilic moiety on tumor cell proliferation, cell cycle progression, and the intracellular distribution of Trx-1. Most new CEU derivatives bearing electrophilic moieties such as (2-chloroethyl)urea, 2-aminooxazoline and (2-chloroacetyl)urea exhibited potent antiproliferative activity and several of them initiated the disappearance of Trx-1 into the nucleus and arrested the cell cycle progression in G_0/G_1 phase. The absence of an electrophilic moiety on the molecule clearly resulted in pharmacologically inactive molecules. Anecdotally, CEU **1** and OXA **21** were favoring the nuclear distribution of thioredoxin. The effects of such an increase of the nuclear concentration of Trx-1 are still unknown.

4. Experimental

4.1. Chemistry and chemical methods

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 determined for the most potent compounds at the Proteomic and Mass Spectrometry Centre, University of Toronto (Canada). All reactions were conducted under a dried nitrogen atmosphere. All chemicals were supplied by Aldrich Chemicals (Milwaukee, WI). Non-commercially available aniline substituted by a cycloalkyloxy, a phenoxy or a benzyloxy moiety was prepared using a Williamson reaction followed by the reduction of the aromatic nitro group using $SnCl_2$ as published previously.[6,7] Liquid flash chromatography was performed on silica gel 60 A (American Chemicals Ltd, Montreal, Canada). 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}). Chromatograms were viewed under UV light at 254 nm.

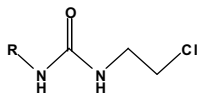
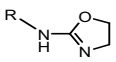
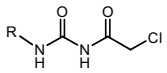
4.2. General procedure for the preparation of the substituted *N*-phenyl-*N′*-(2-chloroethyl)ureas (CEU) 1–20

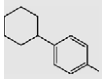
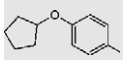
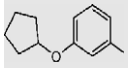
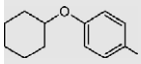
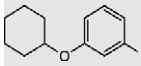
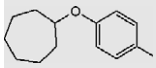
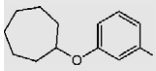
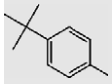
CEU **1**,[6,7,10] **2**–**3**,[10] **4**,[7] **6**–**13**[25], and **16**–**19**[6,7,10] were prepared as published previously. Briefly, 2-chloroethylisocyanate (3.6 mmol) was added dropwise to a stirred solution of the appropriate aniline (3 mmol) in dichloromethane (15 mL). The reaction mixture was stirred overnight at room temperature. The resulting crude precipitate was filtered, washed with cold ether, and recrystallized from ethanol and water.

4.2.1. 1-(2-Chloroethyl)-3-(4-biphenyl)urea (5)[30,31]

Compound **5** was synthesized from the nucleophilic addition of 4-phenylaniline to 2-chloroethylisocyanate. Yield: 91%; mp: 181–186 °C; IR (KBr) ν: 3319 (NH), 1641 (C=O) cm^{-1}; ^1H NMR (DMSO-d_6) δ: 8.79 (s, 1H, NH), 7.47 (m, 8H, Ar), 7.31 (t, 1H, J = 9.8 Hz, Ar), 6.47 (s, 1H, NH), 3.69 (t, 2H, J = 6.0 Hz, CH_2), 3.47 (t, 2H, J = 5.6 Hz, CH_2); ^{13}C NMR (DMSO-d_6) δ: 155.0, 139.97, 139.88, 133.0, 128.9, 126.9, 126.7, 126.1, 118.1, 44.5, 41.2. MS (ESI) m/z: 275.1 (M^++1).

Table 2
Effect of selected CEU derivatives, colchicine, and DMSO on the cell cycle progression

Substituent	% of cells				% of cells				% of cells			
	M	MG_0/G_1	S	G_2/M	M	G_0/G_1	S	G_2/M	M	G_0/G_1	S	G_2/M
	**4**	71	13	16	**24**	73	16	11	**44**	25	51	24
	**6**	72	19	9	**26**	70	23	7	**46**	58	33	9
	**7**	64	24	12	**27**	77	17	6	**47**	53	34	13
	**8**	57	29	14	**28**	82	13	5	**48**	70	20	10
	**9**	59	33	8	**29**	43	19	38	**49**	73	20	7
	**10**	75	19	6	**30**	82	14	4	**50**	59	30	11
	**11**	72	22	6	**31**	48	28	24	**51**	58	31	11
	**16**	17	24	58	**36**	15	11	74	**56**	61	20	19
	**17**	25	33	42	**37**	14	10	76	**57**	68	22	10
Colchicine		19	19	62								
DMSO		59	22	19								

Exponentially growing MCF-7 cells were incubated in the absence or presence of the drug for 24 h at 37 °C.
The cell cycle was evaluated using propidium iodide staining and flow cytometry analysis.
This table shows the % of MCF-7 cells in the different phases of the cell cycle progression ($G_0/G1$, S, and G_2/M, respectively) upon treatment with two-times their GI_{50} either with compound **4**, **6**, **7**, **8**, **9**, **10**, **11**, **16**, **17** or colchicine.

4.2.2. 1-(4-(Benzyloxy)phenyl)-3-(2-chloroethyl)urea (14)

Compound **14** was synthesized from the nucleophilic addition of 4-(benzyloxy)aniline to 2-chloroethylisocyanate. Yield: 93%; mp: 180–186 °C; IR (KBr) *v*: 3297 (NH), 1646 (C═O) cm^{-1}; ^1H NMR (DMSO-d_6) δ: 8.47 (s, 1H, NH), 7.35 (m, 7H, Ar), 6.91 (d, 2H, J = 8.8 Hz, Ar), 6.32 (s, 1H, NH), 5.05 (s, 2H, CH_2), 3.66 (t, 2H, J = 6.1 Hz, CH_2), 3.43 (t, 2H, J = 5.6 Hz, CH_2); ^{13}C NMR (DMSO-d_6) δ: 155.3, 153.1, 137.4, 133.7, 128.4, 127.8, 127.7, 119.5, 115.0, 69.5, 44.5, 41.2. MS (ESI) *m/z*: 305.1 (M^++1).

4.2.3. 1-(3-(Benzyloxy)phenyl)-3-(2-chloroethyl)urea (15)

3-(Benzyloxy)nitrobenzene was prepared by the nucleophilic substitution of benzyl chloride (14 mmol) with 3-nitrophenol (7 mmol) using sodium hydroxide (17 mM) in acetonitrile for 24 h. Yield: 98%; IR (KBr) *v*: 1529, 1351 (N═O) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 7.83 (m, 2H, Ar), 7.40 (m, 7H, Ar), 5.12 (s, 2H, CH_2); ^{13}C NMR ($CDCl_3$) δ: 159.3, 149.3, 135.9, 130.1, 128.6, 128.5, 127.5, 121.9, 116.0, 109.4, 70.6. 3-(Benzyloxy)aniline was prepared by the reduction of the nitro group of 3-(benzyloxy)nitrobenzene (6 mmol) with $SnCl_2$ (39 mmol) in refluxing ethanol for 12 h. Yield: 91%; IR (KBr) *v*: 3353 (NH); ^1H NMR (DMSO-d_6) δ: 7.50 (m, 5H, Ar), 7.18 (t, 1H, J = 8.0 Hz, Ar), 6.52 (d, 1H, J = 8.1 Hz, Ar), 6.4 (m, 1H, Ar), 5.10 (s, 2H, CH_2); ^{13}C NMR (DMSO-d_6) δ: 160.2, 148.2, 137.5, 130.3, 128.7, 128.0, 127.9, 108.4, 104.9, 102.1, 70.0. Compound **15** was synthesized from the nucleophilic addition of 3-(benzyloxy)aniline to 2-chloroethylisocyanate. Yield: 64%; mp: 117–120 °C; IR (KBr) *v*: 3353, 3326 (NH), 1644 (C═O) cm^{-1}; ^1H NMR (DMSO-d_6) δ: 8.66 (s, 1H, NH), 7.38 (m, 5H, Ar), 7.27 (s, 1H, Ar), 7.11 (t, 1H, J = 8.1 Hz, Ar), 6.89 (d, 1H, J = 8.0 Hz, Ar), 6.59 (d, 1H, J = 8.1 Hz, Ar), 6.41 (s, 1H, NH), 5.06 (s, 2H, CH_2), 3.67 (t, 2H, J = 6.1 Hz, CH_2), 3.44 (t, 2H, J = 5.6 Hz, CH_2); ^{13}C NMR (DMSO-d_6) δ: 158.8, 154.9, 141.5, 137.2, 129.5, 128.4, 127.8, 127.6, 110.3, 107.5, 104.5, 69.1, 44.4, 40.9. MS (ESI) *m/z*: 305.1 (M^++1).

4.2.4. 1-Adamantan-2-yl-3-(2-chloro-ethyl)urea (20)

Compound **20** was synthesized from the nucleophilic addition of 2-adamantanamine to 2-chloroethylisocyanate. Yield: 86%;

mp: 202–204 °C; IR (KBr) *v*: 3334 (NH), 1620 (C=O) cm^{-1}; ^1H NMR (DMSO-d_6) δ: 6.33 (s, 1H, NH), 6.20 (s, 1H, NH), 3.67 (d, 1H, J = 7.7 Hz, CH), 3.58 (t, 2H, J = 6.0 Hz, CH_2), 3.33 (t, 2H, J = 6.1 Hz, CH_2), 1.76 (m, 12H, CH), 1.51 (d, 2H, J = 12.1 Hz, CH); ^{13}C NMR (DMSO-d_6) δ: 157.2, 52.9, 44.9, 41.3, 37.26, 36.9, 32.3, 31.4, 26.8. MS (ESI) *m/z*: 257.1 (M^++1).

4.3. General procedure for the preparation of the substituted *N*-aryl amino-2-oxazoline (4,5-dihydro-*N*-phenyloxazol-2-amine: OXA derivatives) 21–40

OXA **21**, **23**, and **36–39** were prepared as published previously.[10] To a stirred solution of the appropriate *N*-phenyl-*N*′-(2-chloroethyl)urea derivative (0.35 mmol) in acetonitrile (10 mL) was added a mixture of SiO_2·KF (60:40%) (3.5 mmol). 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).

4.3.1. 4,5-Dihydro-*N*-(naphthalen-7-yl)oxazol-2-amine (22)

Compound **22** was synthesized from **2** and SiO_2·KF. Yield: 98%; mp: 168–178 °C; IR (KBr) *v*: 3226 (NH) cm^{-1}; ^1H NMR (DMSO-d_6) δ: 7.76 (m, 4H, Ar), 7.40 (m, 3H, Ar), 4.42 (t, 2H, J = 8.3 Hz, CH_2), 3.89 (t, 2H, J = 7.9 Hz, CH_2); ^{13}C NMR (DMSO-d_6) δ: 157.4, 134.2, 128.7, 127.6, 127.4, 127.2, 126.3, 124.2, 120.9, 115.3, 67.3, 50.0. MS (ESI) *m/z*: 213.1 (M^++1).

4.3.2. *N*-(4-Cyclohexylphenyl)-4,5-dihydrooxazol-2-amine (24)[24]

Compound **24** was synthesized from **4** and SiO_2·KF. Yield: 32%; mp: 118–120 °C; IR (KBr) *v*: 3397 (NH) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 7.20 (d, 2H, J = 8.1 Hz, Ar), 7.11 (d, 2H, J = 8.4 Hz, Ar), 4.35 (t, 2H, J = 8.3 Hz, CH_2), 3.83 (t, 2H, J = 8.2 Hz, CH_2), 2.43 (s, 1H, CH), 1.82 (m, 5H), 1.40 (m, 5H); ^{13}C NMR ($CDCl_3$) δ: 157.7, 142.4, 127.3, 119.5, 119.4, 67.2, 46.2, 34.6, 26.9, 26.2. MS (ESI) *m/z*: 245.2 (M^++1).

4.3.3. *N*-(4-Biphenyl)-4,5-dihydrooxazol-2-amine (25)[32]

Compound **25** was synthesized from **5** and SiO_2·KF. Yield: 98%; mp: 142–148 °C; IR (KBr) *v*: 3361 (NH) cm^{-1}; ^1H NMR (DMSO-d_6) δ: 7.54 (m, 4H, Ar), 7.31 (m, 5H, Ar), 6.30 (s, 1H, NH), 4.41 (t, 2H, J = 8.4 Hz, CH_2), 3.85 (t, 2H, J = 8.3 Hz, CH_2); ^{13}C NMR (DMSO-d_6) δ: 157.7, 140.9, 135.2, 128.7, 127.6, 126.8, 120.3, 67.4, 49.3. MS (ESI) *m/z*: 239.1 (M^++1).

4.3.4. *N*-(4-(Cyclopentyloxy)phenyl)-4,5-dihydrooxazol-2-amine (26)

Compound **26** was synthesized from **6** and SiO_2·KF. Yield: 64%; mp: 118–121 °C; IR (KBr) *v*: 2956 (NH) cm^{-1}; ^1H NMR (CDCl3) δ: 7.53 (s, 1H, NH), 7.12 (d, 2H, J = 8.8 Hz, Ar), 6.76 (d, 2H, J = 8.8 Hz, Ar), 4.66 (t, 1H, CH, J = 3.2 Hz), 4.32 (t, 2H, J = 8.3 Hz, CH_2), 3.75 (t, 2H, J = 8.2 Hz, CH_2), 1.78 (m, 6H), 1.58 (m, 2H); ^{13}C NMR ($CDCl_3$) δ: 158.4, 153.6, 135.0, 121.5, 116.0, 79.5, 67.4, 49.1, 32.8, 24.0. MS (ESI) *m/z*: 247.1 (M^++1).

4.3.5. *N*-(3-(Cyclopentyloxy)phenyl)-4,5-dihydrooxazol-2-amine (27)

Compound **27** was synthesized from **7** and SiO_2·KF. Yield: 52%; mp: 48–54 °C; IR (KBr) *v*: 3353 (NH) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 10.0 (s, 1H, NH), 7.11 (t, 1H, J = 5.0 Hz, Ar), 6.77 (s, 1H, Ar), 6.75 (d, 2H, J = 7.0 Hz, Ar), 6.52 (d, 1H, J = 7.4 Hz, Ar), 4.69 (s, 1H, CH), 4.44 (t, 2H, J = 8.2 Hz, CH_2), 3.79 (t, 2H, J = 8.2, CH_2), 1.87 (m, 6H, 3× CH_2), 1.76 (m, 2H, CH_2); ^{13}C NMR ($CDCl_3$) δ: 179.4, 158.8, 141.5, 129.4, 112.1, 110.2, 107.8, 79.2, 68.2, 47.7, 32.9, 24.1. MS (ESI) *m/z*: 247.1 (M^++1).

4.3.6. *N*-(4-(Cyclohexyloxy)phenyl)-4,5-dihydrooxazol-2-amine (28)

Compound **28** was synthesized from **8** and SiO_2·KF. Yield: 56%; mp: 115–121 °C; IR (KBr) *v*: 2934 (NH) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 7.15 (d, 2H, J = 7.3 Hz, Ar), 6.82 (d, 2H, J = 8.8 Hz, Ar), 4.35 (t, 2H, J = 8.3 Hz, CH_2), 4.14 (m, 1H, CH), 3.80 (t, 2H, J = 7.4 Hz, CH_2), 1.96 (m, 2H, CH_2), 1.79 (m, 2H, CH_2), 1.45 (m, 6H, 3× CH_2); ^{13}C NMR ($CDCl_3$) δ: 157.8, 153.3, 136.9, 121.1, 116.9, 76.13, 67.3, 50.2, 31.9, 25.7, 24.0. MS (ESI) *m/z*: 261.2 (M^++1).

4.3.7. *N*-(3-(Cyclohexyloxy)phenyl)-4,5-dihydrooxazol-2-amine (29)

Compound **29** was synthesized from **9** and SiO_2·KF. Yield: 64%; mp: 110–114 °C; IR (KBr) *v*: 2933 (NH) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 7.12 (t, 1H, J = 8.1 Hz, Ar), 6.84 (s, 1H, Ar), 6.79 (d, 1H, J = 7.9 Hz, Ar), 6.51 (d, 1H, J = 8.0 Hz, Ar), 4.36 (t, 2H, J = 8.3 Hz, CH_2), 4.20 (m, 1H, CH), 3.76 (t, 2H, J = 7.2 Hz, CH_2), 1.94 (m, 2H, CH_2), 1.77 (m, 2H, CH_2), 1.43 (m, 6H, 3× CH_2); ^{13}C NMR ($CDCl_3$) δ: 158.4, 158.1, 143.3, 129.5, 112.2, 109.9, 107.9, 75.3, 67.5, 48.7, 31.9, 25.7, 23.8. MS (ESI) *m/z*: 261.2 (M^++1).

4.3.8. *N*-(4-(Cycloheptyloxy)phenyl)-4,5-dihydrooxazol-2-amine (30)

Compound **30** was synthesized from **10** and SiO_2·KF. Yield: 70%; mp: 62–68 °C; IR (KBr) *v*: 3338 (NH) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 7.13 (d, 2H, J = 8.8 Hz, Ar), 6.75 (d, 2H, J = 7.3 Hz, Ar), 4.31 (m, 3H, CH, CH_2), 3.75 (t, 2H, J = 8.3 Hz, CH_2), 1.66 (m, 5H, CH), 1.57 (m, 5H, CH), 1.54 (m, 2H, CH); ^{13}C NMR ($CDCl_3$) δ: 158.0, 153.4, 135.0, 121.5, 116.4, 78.6, 67.3, 49.2, 33.8, 28.3, 23.0, 22.6. MS (ESI) *m/z*: 275.2 (M^++1).

4.3.9. *N*-(3-(Cycloheptyloxy)phenyl)-4,5-dihydrooxazol-2-amine (31)

Compound **31** was synthesized from **11** and SiO_2·KF. Yield: 78%; mp: 83–86 °C; IR (KBr) *v*: 3411 (NH) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 7.13 (t, 1H, J = 8.1 Hz, Ar), 6.84 (s, 1H, Ar, NH), 6.78 (d, 1H, J = 8.0 Hz, Ar), 6.48 (d, 1H, J = 8.1 Hz, Ar), 4.34 (m, 3H, CH, CH_2), 3.81 (t, 2H, J = 8.3 Hz, CH_2), 1.94 (m, 2H, CH_2), 1.70 (m, 4H, 2× CH_2), 1.58 (m, 4H, 2× CH_2), 1.48 (m, 2H, CH_2); ^{13}C NMR ($CDCl_3$) δ: 158.6, 157.8, 143.1, 129.5, 111.9, 109.9, 107.3, 77.8, 66.8, 49.7, 33.7, 28.6, 23.3. MS (ESI) *m/z*: 275.2 (M^++1).

4.3.10. 4,5-Dihydro-*N*-(4-phenoxyphenyl)oxazol-2-amine (32)[33]

Compound **32** was synthesized from **12** and SiO_2·KF. Yield: 97%; mp: 149–155 °C; IR (KBr) *v*: 3120 (NH) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 7.27 (m, 4H, Ar), 7.05 (t, 1H, J = 7.3 Hz, Ar), 6.95 (t, 4H, J = 6.4 Hz, Ar), 4.40 (t, 2H, J = 8.3 Hz, CH_2), 3.82 (t, 2H, J = 8.2 Hz, CH_2); ^{13}C NMR ($CDCl_3$) δ: 158.1, 157.7, 151.8, 138.1, 130.0, 122.6, 121.5, 120.1, 118.0, 67.3, 49.2. MS (ESI) *m/z*: 277.1 (M^++Na).

4.3.11. 4,5-Dihydro-*N*-(3-phenoxyphenyl)oxazol-2-amine (33)

Compound **33** was synthesized from **13** and SiO_2·KF. Yield: 98%; mp: 92–95 °C; IR (KBr) *v*: 3060 (NH) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 7.29 (t, 2H, J = 7.3 Hz, Ar), 7.20 (t, 1H, J = 8.1 Hz, Ar), 7.03 (m, 4H, Ar), 6.92 (s, 1H, Ar), 6.63 (d, 1H, J = 8.1 Hz, Ar), 4.36 (t, 2H, J = 8.2 Hz, CH_2), 3.73 (t, 2H, J = 8.3 Hz, CH_2); ^{13}C NMR ($CDCl_3$) δ: 157.8, 157.2, 144.0, 130.0, 129.7, 123.2, 119.0, 115.1, 112.6, 110.6, 67.5, 48.4. MS (ESI) *m/z*: 255.1 (M^++1).

4.3.12. *N*-(4-(Benzyloxy)phenyl)-4,5-dihydrooxazol-2-amine (34)

Compound **34** was synthesized from 14 and SiO_2·KF. Yield: 98%; mp: 139–143 °C; IR (KBr) *v*: 3041 (NH) cm^{-1}; ^1H NMR ($CDCl_3$) δ:

7.41 (m, 5H, Ar), 7.20 (d, 2H, J = 8.4 Hz, Ar), 6.90 (d, 2H, J = 8.9 Hz, Ar), 5.03 (s, 2H, CH), 4.35 (d, 2H, J = 8.2 Hz, CH_2), 3.79 (d, 2H, J = 8.1 Hz, CH_2); ^{13}C NMR ($CDCl_3$) δ: 157.9, 154.5, 137.3, 135.9, 128.6, 127.7, 127.5, 121.1, 115.4, 70.4, 67.3, 49.9. MS (ESI) m/z: 269.1 (M^++1).

4.3.13. *N*-(3-(Benzyloxy)phenyl)-4,5-dihydrooxazol-2-amine (35)

Compound **35** was synthesized from 15 and SiO_2·KF. Yield: 98%; mp: 117–128 °C; IR (KBr) v: 3051 (NH) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ: 7.38 (m, 5H, Ar), 7.26 (t, 1H, J = 7.4 Hz, Ar), 6.70 (s, 1H, Ar), 6.85 (d, 1H, J = 6.7 Hz, Ar), 6.63 (d, 1H, J = 8.0 Hz, Ar), 5.08 (s, 2H, CH_2), 4.36 (t, 2H, J = 8.2 Hz, CH_2), 3.79 (t, 2H, J = 8.1 Hz, CH_2); ^{13}C NMR ($CDCl_3$) δ: 159.5, 157.7, 137.2, 129.8, 128.7, 127.6, 127.5, 112.5, 108.9, 106.7, 69.9, 67.3, 49.4. MS (ESI) m/z: 269.1 (M^++1).

4.3.14. *N*-(1-Adamantan-2-yl)-4,5-dihydrooxazol-2-amine (40)

Compound **40** was synthesized from **20** and SiO_2·KF. Yield: 98%; mp: 168–169 °C; IR (KBr) v: 3170 (NH) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ: 4.46 (s, 1H, NH), 4.23 (t, 2H, J = 8.6 Hz, CH_2), 3.76 (t, 2H, J = 8.6 Hz, CH_2), 3.70 (s, 1H, CH), 1.96 (s, 2H, CH), 1.80 (s, 8H, CH), 1.70 (s, 2H, CH), 1.56 (m, 2H, CH); ^{13}C NMR ($CDCl_3$) δ: 160.7, 67.6, 56.9, 52.6, 37.6, 37.1, 32.7, 31.7, 27.2. MS (ESI) m/z: 221.2 (M^++1).

4.4. General procedure for the preparation of the substituted *N*-phenyl-*N′*-(2-chloroacetyl)urea derivatives (CAU) 41–60

CAU **41**, **43**, and **56–59** were prepared as published previously.[10] 2-Chloroacetylisocyanate (3.6 mmol) was added dropwise to a stirred solution of the relevant aniline (3 mmol) in dichloromethane (15 mL). The reaction mixture was stirred overnight at ambient temperature. The resulting crude precipitate was filtered, washed with cold ether, and purified by recrystallization from ethanol and water.

4.4.1. 1-(2-Chloroacethyl)-3-(naphthalen-2-yl)urea (42)[34]

Compound **42** was synthesized from the nucleophilic addition of naphthalen-2-amine to 2-chloroacethylisocyanate. Yield: 79%; mp: 204–208 °C; IR (KBr) v: 3235 (NH), 1698 (C=O) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ: 10.99 (s, 1H, NH), 10.36 (s, 1H, NH), 8.19 (s, 1H, Ar), 7.84 (m, 3H, Ar), 7.46 (m, 3H, Ar), 4.44 (s, 2H, CH_2); ^{13}C NMR (DMSO-d_6) δ: 168.8, 150.4, 135.1, 133.4, 130.0, 128.7, 127.5, 127.3, 126.5, 124.8, 120.3, 115.9, 43.3. MS (ESI) m/z: 263.1 (M^++1).

4.4.2. 1-(2-Chloroacethyl)-3-(4-cyclohexylphenyl)urea (44)

Compound **44** was synthesized from the nucleophilic addition of 4-cyclohexylbenzenamine to 2-chloroacethylisocyanate. Yield: 93%; mp: 183–186 °C; IR (KBr) v: 3235 (NH), 1711 (C=O) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ: 10.15 (s, 1H, NH), 9.19 (s, 1H, NH), 7.41 (d, 2H, J = 8.5 Hz, Ar), 7.19 (d, 2H, J = 8.34 Hz, Ar), 4.18 (s, 2H, CH_2), 2.48 (s, 1H, CH), 1.85 (m, 5H), 1.43 (m, 5H); ^{13}C NMR (DMSO-d_6) δ: 167.8, 150.0, 144.9, 134.2, 127.4, 120.6, 44.0, 42.5, 34.5, 26.9, 26.1. MS (ESI) m/z: 295.2 (M^++1).

4.4.3. 1-(2-Chloroacethyl)-3-(4-biphenyl)urea (45)[35]

Compound **45** was synthesized from the nucleophilic addition of 4-phenylaniline to 2-chloroacethylisocyanate. Yield: 71%; mp: 214–217 °C; IR (KBr) v: 3232 (NH), 1697 (C=O) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ: 10.96 (s, 1H, NH), 10.25 (s, 1H, NH), 7.65 (m, 6H, Ar), 7.46 (t, 2H, J = 7.4 Hz, Ar), 7.35 (t, 1H, J = 7.2 Hz, Ar), 4.43 (s, 2H, CH_2); ^{13}C NMR (DMSO-d_6) δ: 168.7, 150.2, 139.6, 136.9, 135.6, 129.0, 127.2, 126.4, 120.2, 43.3. MS (ESI) m/z: 289.1 (M^++1).

4.4.4. 1-(2-Chloroacethyl)-3-(4-(cyclopentyloxy)phenyl)urea (46)

Compound **46** was synthesized from the nucleophilic addition of 4-(cyclopentyloxy)benzenamine to 2-chloroacethylisocyanate. Yield: 80%; mp: 170–174 °C; IR (KBr) v: 3119 (NH), 1715 (C=O) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ: 10.11 (s, 1H, NH), 9.62 (s, 1H, NH), 7.37 (d, 2H, J = 8.8 Hz, Ar), 6.85 (d, 2H, J = 8.8 Hz, Ar), 4.73 (s, 1H, CH), 4.17 (s, 2H, CH_2), 1.85 (m, 6H, CH_2), 1.62 (m, 2H, CH_2); ^{13}C NMR ($CDCl_3$) δ: 168.0, 155.5, 150.6, 129.1, 122.3, 116.0, 79.5, 42.5, 32.8, 24.0. MS (ESI) m/z: 297.1 (M^++1).

4.4.5. 1-(2-Chloroacethyl)-3-(3-(cyclopentyloxy)phenyl)urea (47)

Compound **47** was synthesized from the nucleophilic addition of 3-(cyclopentyloxy)benzenamine to 2-chloroacethylisocyanate. Yield: 96%; mp: 129–131 °C; IR (KBr) v: 3249 (NH), 1719 (C=O) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ: 10.19 (s, 1H, NH), 9.06 (s, 1H, NH), 7.19 (m, 3H, Ar), 6.99 (d, 1H, J = 7.9 Hz, Ar), 6.67 (d, 1H, J = 8.2 Hz, Ar), 4.76 (s, 1H, CH), 4.19 (s, 2H, CH_2), 1.87 (m, 6H, CH_2), 1.65 (m, 2H, CH_2); ^{13}C NMR ($CDCl_3$) δ: 167.8, 158.8, 149.7, 137.7, 129.8, 112.2, 107.9, 102.6, 79.4, 42.5, 32.8, 24.0. MS (ESI) m/z: 297.1 (M^++1).

4.4.6. 1-(2-Chloroacethyl)-3-(4-(cyclohexyloxy)phenyl)urea (48)

Compound **48** was synthesized from the nucleophilic addition of 4-(cyclohexyloxy)benzenamine to 2-chloroacethylisocyanate. Yield: 93%; mp: 153–159 °C; IR (KBr) v: 3236 (NH), 1710 (C=O) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ: 10.04 (s, 1H, NH), 9.0 (s, 1H, NH), 7.38 (d, 2H, J = 8.8 Hz, Ar), 6.88 (d, 2H, J = 8.9 Hz, Ar), 4.21 (m, 3H, CH, CH_2), 1.98 (m, 2H, CH_2), 1.79 (m, 2H, CH_2), 1.52 (m, 6H, 3× CH_2); ^{13}C NMR ($CDCl_3$) δ: 167.8, 155.1, 150.0, 129.4, 122.3, 116.7, 75.9, 42.5, 31.8, 25.6, 23.7. MS (ESI) m/z: 311.1 (M^++1).

4.4.7. 1-(2-Chloroacethyl)-3-(3-(cyclohexyloxy)phenyl)urea (49)

Compound **49** was synthesized from the nucleophilic addition of 3-(cyclohexyloxy)benzenamine to 2-chloroacethylisocyanate. Yield: 98%; mp: 122–130 °C; IR (KBr) v: 3147 (NH), 1705 (C=O) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ: 10.26 (s, 1H, NH), 9.68 (s, 1H, NH), 7.19 (m, 2H, Ar), 6.99 (d, 1H, J = 8.0 Hz, Ar), 6.71 (d, 1H, J = 8.1 Hz, Ar), 4.20 (m, 3H, Ar), 1.94 (m, 2H, CH_2), 1.78 (m, 2H, CH_2), 1.55 (m, 2H, CH_2), 1.47 (m, 4H, 2× CH_2); ^{13}C NMR ($CDCl_3$) δ: 168.2, 158.5, 150.5, 137.7, 129.8, 112.6, 108.4, 75.5, 42.5, 31.7, 25.6, 23.7. MS (ESI) m/z: 311.1 (M^++1).

4.4.8. 1-(2-Chloroacethyl)-3-(4-(cycloheptyloxy)phenyl)urea (50)

Compound **50** was synthesized from the nucleophilic addition of 4-(cycloheptyloxy)benzenamine to 2-chloroacethylisocyanate. Yield: 87%; mp: 145–149 °C; IR (KBr) v: 3125 (NH), 1705 (C=O) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ: 10.06 (s, 1H, NH), 9.16 (s, 1H, NH), 7.38 (d, 2H, J = 8.9 Hz, Ar), 6.84 (d, 2H, J = 8.8 Hz, Ar), 4.37 (m, 1H, CH), 4.18 (s, 2H, CH_2), 2.01 (m, 2H, CH_2), 1.79 (m, 4H, 2× CH_2), 1.72 (m, 4H, 2× CH_2), 1.47 (m, 2H, CH_2); ^{13}C NMR ($CDCl_3$) δ: 167.8, 155.2, 150.1, 129.2, 122.3, 116.5, 78.4, 42.5, 33.7, 28.4, 23.0. MS (ESI) m/z: 325.1 (M^++1).

4.4.9. 1-(2-Chloroacethyl)-3-(3-(cycloheptyloxy)phenyl)urea (51)

Compound **51** was synthesized from the nucleophilic addition of 3-(cycloheptyloxy)benzenamine to 2-chloroacethylisocyanate. Yield: 98%; mp: 135–138 °C; IR (KBr) v: 3129 (NH), 1701 (C=O) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ: 10.2 (s, 1H, NH), 9.1 (s, 1H, NH), 7.22 (m, 2H, Ar), 6.99 (d, 1H, J = 7.7 Hz, Ar), 6.66 (d, 1H, J = 7.2 Hz, Ar), 4.42 (m, 1H, CH), 4.19 (s, 2H, CH_2), 2.01 (m, 2H, CH_2), 1.79 (m, 4H, 2× CH_2), 1.61 (m, 4H, 2× CH_2), 1.51 (m, 2H, CH_2); ^{13}C NMR ($CDCl_3$) δ: 167.9, 158.5, 149.8, 137.7, 129.8, 112.4, 108.3, 102.7, 78.0, 42.5, 33.7, 28.4, 23.0. MS (ESI) m/z: 325.1 (M^++1).

4.4.10. 1-(2-Chloroacethyl)-3-(4-phenoxyphenyl)urea (52)[36]

Compound **52** was synthesized from the nucleophilic addition of 4-phenoxybenzenamine to 2-chloroacethylisocyanate. Yield:

78%; mp: 157–163 °C; IR (KBr) *v*: 3133 (NH), 1704 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 10.21 (s, 1H, NH), 9.30 (s, 1H, NH), 7.47 (d, 2H, *J* = 8.9 Hz, Ar), 7.34 (t, 2H, *J* = 7.9 Hz, Ar), 7.11 (t, 1H, *J* = 7.4 Hz, Ar), 7.00 (d, 4H, *J* = 6.9 Hz, Ar), 4.19 (s, 2H, CH_2); ^{13}C NMR ($CDCl_3$) δ: 168.0, 157.4, 154.1, 150.1, 131.9, 129.8, 123.3, 122.2, 119.6, 118.7, 42.5. MS (ESI) *m/z*: 305.1 (M^++1).

4.4.11. 1-(2-Chloroacethyl)-3-(3-phenoxyphenyl)urea (53)

Compound **53** was prepared from the nucleophilic addition of 3-phenoxybenzenamine to 2-chloroacethylisocyanate. Yield: 77%; mp: 140–144 °C; IR (KBr) *v*: 3269 (NH), 1708 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 10.27 (s, 1H, NH), 9.39 (s, 1H, NH), 7.34 (t, 2H, *J* = 7.7 Hz, Ar), 7.27 (d, 2H, *J* = 8.1 Hz, Ar), 7.16 (m, 2H, Ar), 7.10 (d, 2H, *J* = 8.1 Hz, Ar), 6.81 (d, 1H, *J* = 8.2 Hz, Ar), 6.00 (s, 2H, CH_2); ^{13}C NMR ($CDCl_3$) δ: 168.1, 158.1, 156.7, 150.2, 138.0, 130.2, 129.8, 123.7, 119.3, 115.0, 110.9, 42.4. MS (ESI) *m/z*: 305.1 (M^++1).

4.4.12. 1-(4-(Benzyloxy)phenyl)-3-(2-chloroacetyl)urea (54)

Compound **54** was synthesized from the nucleophilic addition of 4-(benzyloxy)aniline to 2-chloroacethylisocyanate. Yield: 97%; mp: 198–202 °C; IR (KBr) *v*: 3226 (NH), 1709 (C=O) cm^{-1}; ^1H NMR (DMSO-d_6) δ: 10.86 (s, 1H, NH), 10.02 (s, 1H, NH), 7.40 (m, 7H, Ar), 7.00 (d, 2H, *J* = 8.8 Hz, Ar), 5.09 (s, 2H, CH_2), 4.39 (s, 2H, CH_2); ^{13}C NMR (DMSO-d_6) δ: 168.6, 154.9, 150.3, 137.2, 130.6, 128.4, 127.8, 127.7, 121.6, 115.1, 69.4, 43.2. MS (ESI) *m/z*: 319.1 (M^++1).

4.4.13. 1-(3-(Benzyloxy)phenyl)-3-(2-chloroacetyl)urea (55)

Compound **55** was synthesized from the nucleophilic addition of 3-(benzyloxy)aniline to 2-chloroacethylisocyanate. Yield: 22%; mp: 156–161 °C; IR (KBr) *v*: 3132 (NH), 1704 (C=O) cm^{-1}; ^1H NMR (DMSO-d_6) δ: 10.91 (s, 1H, NH), 10.16 (s, 1H, NH), 7.34 (m, 7H, Ar), 7.10 (d, 1H, *J* = 8.0 Hz, Ar), 6.79 (d, 1H, *J* = 8.1 Hz, Ar), 5.11 (m, 2H, CH_2), 4.41 (m, 2H, CH_2); ^{13}C NMR (DMSO-d_6) δ: 168.7, 158.8, 150.1, 138.6, 137.0, 129.9, 128.5, 127.9, 127.7, 112.2, 110.2, 106.4, 69.3, 43.3. MS (ESI) *m/z*: 319.1 (M^++1).

4.4.14. 1-Adamantan-2-yl-3-(2-chloroacetyl)-urea (60)

Compound **60** was synthesized from the nucleophilic addition of 2-adamantanamine to 2-chloroacethylisocyanate. Yield: 75%; mp: 187–192 °C; IR (KBr) *v*: 3241 (NH), 1694 (C=O) cm^{-1}; ^1H NMR (DMSO-d_6) δ: 10.66 (s, 1H, NH), 8.59 (s, 1H, NH), 4.30 (s, 2H, CH_2), 3.86 (d, 1H, *J* = 7.2 Hz, CH), 1.80 (m, 12H, CH), 1.64 (m, 2H, CH); ^{13}C NMR (DMSO-d_6) δ: 168.7, 151.7, 53.1, 43.1, 37.0, 36.5, 31.6, 31.4, 26.5. MS (ESI) *m/z*: 271.1 (M^++1).

4.5. General procedure for the preparation of the substituted *N*-phenyl-*N'*-(2-ethyl)ureas 61–80

Compounds **61** (1-ethyl-3-(2,3-dihydro-1H-inden-6-yl)urea),[10] **63** (1-ethyl-3-(9H-fluoren-9-yl)urea),[10] **64** (1-(4-cyclohexylphenyl)-3-ethylurea),[25] **76** (1-(4-*tert*-butylphenyl)-3-ethylurea),[10] **77** (1-ethyl-3-(4-iodophenyl)urea),[10] **78** (1-ethyl-3-*p*-tolylurea)[10], and **79** (1-ethyl-3-(3,4-dimethylphenyl)urea)[10] were prepared as published previously. Ethylisocyanate (3.6 mmol) was added dropwise to a stirred solution of the relevant aniline (3 mmol) in dichloromethane (15 mL). The reaction mixture was stirred overnight at ambient temperature. The resulting crude precipitate was filtered, washed with cold ether, and purified by recrystallization from ethanol and water.

4.5.1. 1-Ethyl-3-(naphthalen-7-yl)urea (62)

Compound **62** was synthesized from the nucleophilic addition of naphthalen-2-amine to ethylisocyanate. Yield: 50%; mp: 183–187 °C; IR (KBr) *v*: 3326 (NH), 1714 (C=O) cm^{-1}; ^1H NMR (DMSO-d_6) δ: 8.65 (s, 1H, NH), 8.05 (s, 1H, Ar), 7.75 (t, 3H, *J* = 8.4 Hz, Ar), 7.31 (m, 1H, Ar), 6.21 (t, 1H, *J* = 7.2 Hz, Ar), 6.21 (s, 1H, NH), 3.16 (m, 2H, CH_2), 1.09 (t, 3H, CH_3); ^{13}C NMR (DMSO-d_6) δ: 155.3, 138.3, 133.9, 128.7, 128.2, 127.4, 126.8, 126.2, 123.5, 119.6, 112.5, 34.0, 15.5.

4.5.2. 1-(2-Chloroacethyl)-3-(4-biphenyl)urea (65)

Compound **65** was synthesized from the nucleophilic addition of 4-phenylaniline to ethylisocyanate. Yield: 86%; mp: 205–208 °C; IR (KBr) *v*: 3364 (NH), 1712 (C=O) cm^{-1}; ^1H NMR (DMSO-d_6) δ: 8.53 (s, 1H, NH), 7.61 (d, 2H, *J* = 7.7 Hz, Ar), 7.50 (m, 4H, Ar), 7.42 (t, 2H, J = 7.4 Hz, Ar), 7.32 (t, 1H, *J* = 7.2 Hz, Ar), 6.14 (s, 1H, NH), 3.13 (m, 2H, CH_2), 1.07 (t, 3H, CH_3); ^{13}C NMR (DMSO-d_6) δ: 155.1, 140.2, 140.0, 133.7, 128.9, 126.9, 126.6, 126.0, 118.0, 34.0, 15.5.

4.5.3. 1-(4-(Cyclopentyloxy)phenyl)-3-ethylurea (66)

Compound **66** was synthesized from the nucleophilic addition of 4-(cyclopentyloxy)benzenamine to ethylisocyanate. Yield: 91%; mp: 166–168 °C; IR (KBr) *v*: 3334 (NH), 1638 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 7.14 (d, 2H, *J* = 8.8 Hz, Ar), 6.77 (m, 3H, Ar, NH), 5.08 (s, 1H, NH), 4.68 (s, 1H, CH), 3.22 (m, 2H, CH_2), 1.82 (m, 6H, CH_2), 1.63 (m, 2H, CH_2), 1.09 (t, 3H, *J* = 7.2 Hz, CH_3); ^{13}C NMR ($CDCl_3$) δ: 156.9, 155.3, 130.9, 124.4, 116.2, 79.6, 35.1, 32.8, 24.0, 15.5.

4.5.4. 1-(3-(Cyclopentyloxy)phenyl)-3-ethylurea (67)

Compound **67** was synthesized from the nucleophilic addition of 3-(cyclopentyloxy)benzenamine to ethylisocyanate. Yield: 77%; mp: 134–139 °C; IR (KBr) *v*: 3311 (NH), 1644 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 7.12 (m, 2H, Ar, NH), 6.98 (s, 1H, Ar), 6.74 (d, 1H, *J* = 7.9 Hz, Ar), 6.55 (d, 1H, *J* = 8.1 Hz, Ar), 5.39 (s, 1H, NH), 4.69 (s, 1H, CH), 3.24 (m, 2H, CH_2), 1.77 (m, 6H, CH_2), 1.72 (m, 2H, CH_2), 1.10 (t, 3H, *J* = 7.3 Hz, CH_3); ^{13}C NMR ($CDCl_3$) δ: 158.9, 156.2, 140.1, 129.8, 112.4, 110.8, 108.0, 79.3, 35.1, 32.8, 24.0, 15.4.

4.5.5. 1-(4-(Cyclohexyloxy)phenyl)-3-ethylurea (68)

Compound **68** was synthesized from the nucleophilic addition of 4-(cyclohexyloxy)benzenamine to ethylisocyanate. Yield: 44%; mp: 139–145 °C; IR (KBr) *v*: 3306 (NH), 1636 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 7.14 (d, 2H, *J* = 8.8 Hz, Ar), 6.79 (m, 3H, Ar, NH), 5.08 (s, 1H, NH), 4.15 (m, 1H, CH), 3.22 (m, 2H, CH_2), 1.94 (m, 2H, CH_2), 1.79 (m, 2H, CH_2), 1.40 (m, 6H, 3× CH_2), 1.08 (t, 3H, *J* = 7.2 Hz, CH_3); ^{13}C NMR ($CDCl_3$) δ: 156.9, 154.9, 131.1, 124.3, 116.9, 76.0, 35.1, 31.8, 25.6, 23.8, 15.4.

4.5.6. 1-(3-(Cyclohexyloxy)phenyl)-3-ethylurea (69)

Compound **69** was synthesized from the nucleophilic addition of 3-(cyclohexyloxy)benzenamine to ethylisocyanate. Yield: 38%; mp: 128–131 °C; IR (KBr) *v*: 3337 (NH), 1645 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 7.10 (m, 2H, Ar, NH), 7.00 (s, 1H, Ar), 6.75 (d, 1H, *J* = 7.7 Hz, Ar), 6.57 (d, 1H, *J* = 8.2 Hz, Ar), 5.40 (s, 1H, NH), 4.20 (s, 1H, CH), 3.20 (m, 2H, CH_2), 1.91 (m, 2H, CH_2), 1.74 (m, 2H), 1.49 (m, 3H), 1.49 (m, 3H), 1.11 (t, 3H, *J* = 7.2 Hz, CH_3); ^{13}C NMR ($CDCl_3$) δ: 158.6, 156.2, 140.1, 129.8, 112.7, 111.3, 108.5, 75.4, 35.1, 31.8, 25.6, 23.7, 15.4.

4.5.7. 1-(4-(Cycloheptyloxy)phenyl)-3-ethylurea (70)

Compound **70** was synthesized from the nucleophilic addition of 4-(cycloheptyloxy)benzenamine to ethylisocyanate. Yield: 80%; mp: 143–146 °C; IR (KBr) *v*: 3338 (NH), 1640 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 7.15 (d, 2H, *J* = 8.8 Hz, Ar), 6.81 (d, 2H, *J* = 8.8 Hz, Ar), 6.45 (s, 1H, NH), 4.84 (s, 1H, NH), 4.34 (m, 1H, CH), 3.25 (m, 2H, 2 CH_2), 1.95 (m, 2H, CH_2), 1.69 (m, 4H, 2× CH_2), 1.61 (m, 4H, 2× CH_2), 1.50 (m, 2H, CH_2), 1.11 (t, 3H, *J* = 7.3 Hz, CH_3); ^{13}C NMR ($CDCl_3$) δ: 156.8, 155.3, 130.7, 125.0, 116.8, 78.4, 35.2, 33.7, 28.4, 23.0, 15.5.

4.5.8. 1-(3-(Cycloheptyloxy)phenyl)-3-ethylurea (71)

Compound **71** was synthesized from the nucleophilic addition of 3-(cycloheptyloxy)benzenamine to ethylisocyanate. Yield: 64%; mp: 98–102 °C; IR (KBr) ν: 3416 (NH), 1715 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 7.15 (t, 1H, J = 8.1 Hz, Ar), 6.95 (s, 1H, NH), 6.76 (m, 2H, Ar), 6.57 (d, 1H, J = 8.2 Hz, Ar), 5.15 (s, 1H, NH), 4.39 (m, 1H, CH), 3.25 (m, 2H, CH_2), 1.94 (m, 2H, CH_2), 1.65 (m, 4H, 2× CH_2), 1.60 (m, 4H, 2× CH_2), 1.57 (m, 2H, CH_2), 1.12 (t, 3H, J = 7.3 Hz, CH_3); ^{13}C NMR ($CDCl_3$) δ: 158.7, 156.0, 140.0, 129.0, 112.9, 111.4, 108.9, 78.0, 35.1, 33.7, 28.4, 23.0, 15.4.

4.5.9. 1-Ethyl-3-(4-phenoxyphenyl)urea (72)

Compound **72** was synthesized from the nucleophilic addition of 4-phenoxybenzenamine to ethylisocyanate. Yield: 79%; mp: 150–153 °C; IR (KBr) ν: 3334 (NH), 1642 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 7.26 (m, 5H, NH, Ar), 7.06 (t, 1H, J = 7.3 Hz, Ar), 6.93 (t, 4H, J = 8.8 Hz, Ar), 5.47 (s, 1H, NH), 3.24 (m, 2H, CH_2), 1.11 (t, 3H, J = 7.1 Hz, CH_3); ^{13}C NMR ($CDCl_3$) δ: 157.6, 156.7, 153.1, 134.3, 129.7, 123.0, 122.7, 119.8, 118.4, 35.1, 15.4.

4.5.10. 1-Ethyl-3-(3-phenoxyphenyl)urea (73)

Compound **73** was synthesized from the nucleophilic addition of 3-phenoxybenzenamine to ethylisocyanate. Yield: 78%; mp: 110–112 °C; IR (KBr) ν: 3315 (NH), 1649 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 7.49 (s, 1H, NH), 7.28 (t, 2H, J = 7.6 Hz, Ar), 7.15 (t, 1H, J = 8.0 Hz, Ar), 7.03 (m, 5H, Ar), 6.62 (d, 1H, J = 8.0 Hz, Ar), 5.61 (s, 1H, NH), 3.18 (m, 2H, CH_2), 1.05 (t, 3H, J = 7.2 Hz, CH_3); ^{13}C NMR ($CDCl_3$) δ: 158.0, 156.9, 156.3, 140.7, 130.0, 129.7, 123.4, 119.1, 114.6, 113.0, 110.5, 35.0, 15.3.

4.5.11. 1-(4-(Benzyloxy)phenyl)-3-ethylurea (74)

Compound **74** was synthesized from the nucleophilic addition of 4-(benzyloxy)aniline to ethylisocyanate. Yield: 98%; mp: 168–171 °C; IR (KBr) ν: 3348 (NH), 1638 (C=O) cm^{-1}; ^1H NMR (DMSO-d_6) δ: 8.22 (s, 1H, NH), 7.37 (m, 7H, Ar), 6.90 (d, 2H, J = 8.8 Hz, Ar), 5.99 (s, 1H, NH), 5.04 (s, 2H, CH_2), 3.10 (m, 2H, CH_2), 1.05 (t, 3H, J = 7.2 Hz, CH_3); ^{13}C NMR (DMSO-d_6) δ: 155.4, 152.9, 137.4, 134.0, 128.4, 127.7, 119.4, 115.0, 69.5, 34.0, 15.6.

4.5.12. 1-(3-(Benzyloxy)phenyl)-3-ethylurea (75)

Compound **15** was synthesized from the nucleophilic addition of 3-(benzyloxy)aniline to ethylisocyanate. Yield: 30%; mp: 139–142 °C; IR (KBr) ν: 3330 (NH), 1652 (C=O) cm^{-1}; ^1H NMR (DMSO-d_6) δ: 8.42 (s, 1H, NH), 7.38 (m, 5H, Ar), 7.38 (s, 1H, Ar), 7.23 (t, 1H, J = 8.1 Hz, Ar), 7.10 (d, 1H, J = 8.0 Hz, Ar), 6.88 (d, 1H, J = 8.1 Hz, Ar), 6.56 (s, 1H, NH), 6.09 (s, 1H, NH), 5.06 (s, 2H, CH_2), 3.10 (m, 2H, CH_2), 1.06 (t, 3H, J = 7.1 Hz, CH_3); ^{13}C NMR (DMSO-d_6) δ: 158.8, 155.1, 141.9, 137.3, 129.4, 128.4, 127.8, 127.5, 110.3, 107.2, 104.3, 69.2, 33.9, 15.5.

4.5.13. 1-Adamantan-2-yl-3-ethyl-urea (80)

Compound **80** was synthesized from the nucleophilic addition of 2-adamantanamine to ethylisocyanate. Yield: 74%; mp: 190–195 °C; IR (KBr) ν: 3363 (NH), 1629 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 6.00 (s, 1H, NH), 5.76 (s, 1H, NH), 3.66 (d, 1H, J = 7.8 Hz, CH), 3.00 (m, 2H, CH_2), 1.72 (m, 13H, CH, CH_2), 1.50 (d, 2H, J = 12.2 Hz, 2× CH), 0.99 (t, 3H, J = 7.1 Hz, CH_3); ^{13}C NMR ($CDCl_3$) δ: 165.8, 52.7, 37.3, 37.0, 33.9, 32.34, 31.2, 26.7, 15.7.

4.6. Biological assays

4.6.1. Materials and reagents

Vinblastine sulfate, cisplatin, colchicine, paclitaxel, and the monoclonal antibody anti-β_{II}-tubulin (clone TUB 2.1) were obtained from Sigma Chemicals (St. Louis, MO). 1-Methylpropyl 2-imidazolyl disulfide (PX-12) was prepared as described by Kirkpatrick.[28] The monoclonal antibody anti-Trx-1 was purchased from BD Pharmigen (2G11, San Diego, CA). The peroxidase conjugated anti-mouse immunoglobulin and ECL Western blotting detection reagent kit were purchased from Amersham Canada (Oakville, Canada).

4.6.2. Cell culture

HT-29 human colon carcinoma, M21 human skin melanoma and MCF-7 human breast carcinoma cells were purchased from the American Type Culture Collection (Manassas, VA). The cells were cultured in calf serum iron supplemented (Hyclone, Mississauga, Ontario) medium containing $NaHCO_3$ (2.2 g/L), glucose (4.5 g/L), and glutamine (292 μg/mL). The cells were maintained at 37 °C in a moisture-saturated atmosphere containing 5% CO_2.

4.6.3. Growth inhibition assays

The growth inhibition potency of CEUs was assessed using the procedure described by the National Cancer Institute for its drug screening program.[29] 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 iron supplemented medium. Plates were incubated at 37 °C, in a moisture-saturated atmosphere containing 5% CO_2 for 24 h. Freshly solubilized drugs in DMSO were diluted in fresh medium and aliquots of 100 μL containing sequential dilution of drugs were added. Final drug concentrations ranged from 100 to 0.7 μM. DMSO concentration was maintained lower than 0.5% to avoid cell toxicity. Plates were incubated for 48 h. Assays were 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 μQuant 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 in reference to the control DMSO-treated cells for each drug concentrations. The experiments were performed at least in triplicate. 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.

4.6.4. Cell cycle analysis

After incubation of 3.5×10^5 MCF-7 cells with CEU derivatives and colchicine at 2- and 4-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).

4.6.5. Cellular distribution of thioredoxin-1 by immunocytochemistry

M21 cells were seeded at 1×10^5 cells per well in six-well plates that contained 22-mm glass coverslips coated with fibronectin (10 μg/mL) and incubated for 18 h at 37 °C. Tumor cells were incubated either with CEUs (**2**, **12**, **15**–**17**, **19** at 10 μM, **1**, **5**, **12**, **19** at 25 μM, **1**–**11**, **13**–**18**, **20**, **40** at 50 μM), OXAs (**22**, **32**, **35**–**37** at 5 μM, **16**–**17**, **19**, **39** at 10 μM, **21**, **25**, **29**, **31**, **33**, **34** at 25 μM, **21**–**34**, **38**, **40** at 50 μM), CAUs (**41**–**60** at 5 μM), EU (**61**, **64**, **68**,

71) 50 μM, cisplatin (30 μM), vinblastine sulfate (0.1 μM), colchicine (0.1 μM), paclitaxel (0.1 μM), PX-12 (40 μM), or DMSO (0.1%). 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 during 1 h at 37 °C. The cells were then additionally incubated for 1 h at 37 °C with the anti-thioredoxin-1 monoclonal antibody (2G11, San Diego, CA; 1/200) in a solution containing 0.1% saponin and 3% BSA in PBS. The cells were washed three times with PBS containing 0.05% Tween 20™ and incubated 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. The cellular distribution of the fluorescent thioredoxin isoform 1 was assessed using an Olympus BX51 microscope. Images were captured as a 8-bit tagged image file format files with a Q imaging RETIGA EXI digital camera driven by Imagi pro express software.

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