Antimitotic Antitumor Agents: Synthesis, Structure−Activity Relationships, and Biological Characterization of *N*-Aryl-*N'*-(2-chloroethyl)ureas as New Selective Alkylating Agents

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A series of *N*-aryl-*N'*-(2-chloroethyl)ureas (CEUs) and derivatives were synthesized and evaluated for antiproliferative activity against a wide panel of tumor cell lines. Systematic structure−activity relationship (SAR) studies indicated that: (i) a branched alkyl chain or a halogen at the 4-position of the phenyl ring or a fluorenyl/indanyl group, (ii) an exocyclic urea function, and (iii) a *N'*-2-chloroethyl moiety were required to ensure significant cytotoxicity. Biological experiments, such as immunofluorescence microscopy, confirmed that these promising compounds alter the cytoskeleton by inducing microtubule depolymerization via selective alkylation of β-tubulin. Subsequent evaluations demonstrated that potent CEUs were weak alkylators, were non-DNA-damaging agents, and did not interact with the thiol function of either glutathione or glutathione reductase. Therefore, CEUs are part of a new class of antimitotic agents. Finally, among the series of CEUs evaluated, compounds **12**, **15**, **16**, and **27** were selected for further in vivo trials.

Introduction

Clinical cancer chemotherapy effectiveness is mostly limited by two major problems that still remain to be overcome: the lack of selectivity of anticancer agents and the occurrence of intrinsic or acquired resistances leading to significant side effects, and sometimes failures of treatments.[1] In the challenge to improve modern cancer chemotherapy, the search for new drugs with both higher therapeutic index and lower capacity to induce resistance is, therefore, an active field of investigation in medicinal chemistry.

Nitrogen mustards and chloroethylnitrosoureas are among the most powerful alkylating agents widely used in the chemotherapy of leukemias and carcinomas. On the one hand, nitrosoureas are very potent antineoplastic drugs but also highly toxic. On the other hand, nitrogen mustards are less active but well-tolerated by patients.[2,3] In our ongoing research program aimed at the discovery of potent and selective cytotoxic antitumor agents, we have designed new molecules based on the conjugation of the aromatic ring of chlorambucil (nitrogen mustard) as the prosthetic group and the cytotoxic 2-chloroethylnitrosourea moiety of carmustine as the pharmacophore. Only unnitrosated derivatives were found to be active.[4] Among them, *N*-(4-*tert*-butylphenyl)-*N'*-(2-chloroethyl)urea (**16**) exhibited better cytotoxicity than the parent drugs (i.e. chlorambucil, carmustine) and furthermore significant antineoplastic activity in vivo.[5] This compound was also nonmutagenic, nontoxic, and active even in cell lines that have developed resistance through P-glycoprotein overexpression, alteration in glutathione and/or glutathione-*S*-transferase activity, modification of topoisomerase II, or increase in DNA repair.[6] In a preliminary study, we suggested that the molecular mechanism of **16** involved an alteration of the cytoskeleton.[7] Generally, antimitotic drugs affect the cytoskeleton either by preventing the polymerization of tubulin to microtubules, necessary for the formation of the mitotic spindle in dividing cells (colchicine, vinblastine), or by stabilizing the polymeric structures (taxol).[2] Recently, we showed that **16** was an antimitotic agent since this drug induced arrest of cell growth in mitosis and interfered with β-tubulin polymerization.[8]

In this paper, we report the synthesis and cytotoxicity evaluation of a series of *N*-aryl-*N'*-(2-chloroethyl)urea (CEU) derivatives with various structural modifications in order to identify the essential structural elements required for the antiproliferative activity and to optimize the therapeutic index of these compounds. In addition, active CEUs were submitted to biological tests to demonstrate the target selectivity of these drugs. Finally, from the information generated, lead compounds were identified for further preclinical development.

Chemistry

CEUs **1**−**36**, *N*-phenyl-*N'*-alkylureas **39**−**48**, and derivatives **37** and **38**, listed in Tables 1−4, were synthesized from the corresponding anilines using 2-chloroethyl/alkyl isocyanate according to the standard procedure for the preparation of *N,N'*-unsymmetrical ureas, followed by acidic treatment for **37** and **38**.[9] The reaction conducted in either diethyl ether or ethanol provided rapidly the required ureas with high purity and yield.

Results and Discussion

Cytotoxicity Evaluation. Compounds **1**−**48** were evaluated for in vitro cytotoxic activity against seven

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Table 1. SAR Studies on *N*-Phenyl-*N'*-2-chloroethylureas: The Phenyl Ring

compd	R	IC_{50}[a] (μM)							mp (°C)	yield (%)
		LoVo	MDA-MB-231	P388D1	CHO	K562	L1210	HT 29		
1		NA[b]	140	NA	ND[c]	>100	57	>100	124–126	41
2	4-methyl	20	25	30	26	16	16	23	180–182	81
3	2-methyl	NA	NA	NA	ND	ND	ND	ND	150–152	57
4	3-methyl	60	60	75	ND	ND	ND	ND	130–132	38
5	2,6-dimethyl	NA	NA	NA	ND	ND	ND	ND	167–169	67
6	2,3-dimethyl	NA	NA	NA	ND	ND	ND	ND	152–154	53
7	3,4-dimethyl	3.5	7.2	4.2	8.7	3.5	3.8	5.4	151–153	78
8	3,5-dimethyl	8.8	20	8.6	24.7	10	8.8	20	153–155	55
9	2,4,6-trimethyl	NA	NA	NA	ND	ND	ND	ND	192–194	72
10	4-ethyl	3.2	5.0	5.2	6.4	2.5	2.7	6.4	161–163	53
11	4-*n*-propyl	10	18	11	19	8.2	9.0	23	154–156	69
12	4-isopropyl	1.0	2.5	2.1	3.2	1.3	1.2	5.0	140–142	65
13	4-*n*-butyl	10	19	10	18	10	12	22	139–141	56
14	4-isobutyl	ND	24	ND	23	11	11	21	105–107	60
15	4-*sec*-butyl	2.2	3.1	2.4	4.0	1.3	1.3	3.6	89–91	95
16	4-*tert*-butyl	4.1	6.2	5.2	6.3	3.5	3.0	9.1	134–136	60
17	4-*n*-pentyl	5.2	12	7.3	13	6.1	5.4	12	135–137	69
18	4-*neo*-pentyl	ND	34	ND	35	21	19	36	122–124	70
19	4-isopentyl	ND	32	ND	19	15	14	33	147–149	75
20	4-*n*-hexyl	6.1	15	8.2	15	7.1	5.0	12	130–132	66
21	4-cyclohexyl	22	44	22	23	18	16	38	164–166	63
22	4-*n*-heptyl	15	30	18	22	16	13	25	129–131	76
23	4-*n*-octyl	29	33	32	ND	ND	ND	ND	127–129	81
24	4-*n*-decyl	NA	NA	NA	ND	ND	ND	ND	124–126	53
25	4-*n*-dodecyl	NA	NA	NA	ND	ND	ND	ND	123–125	74
26	3-iodo	36	27	34	35	17	27	24	143–145	95
27	4-iodo	5.4	5.7	5.4	4.8	2.1	3.8	3.9	194–196	81
28	4-bromo	11	10	10	10	4.6	8.1	9.0	184–186	77
29	4-chloro	23	31	11	19	9.1	9.6	14	153–155	72
chlorambucil		9.0	80	7.0	30	11	3.0	>100		
carmustine		ND	7.4	5.9	8.2	9.0	4.5	17		

[a] Dose required to inhibit cell growth by 50%. Values are the means of at least three independent determinations. [b] NA: not active, $IC_{50} > 400$ μM. [c] ND: not determined.

cell lines, including human non-hormone-dependent breast carcinoma (MDA-MB-231), human colon adenocarcinoma (LoVo, HT 29), murine lymphocytotic leukemia (P388D1, L1210), chronic myelogenous leukemia (K562), and Chinese hamster ovary (CHO). The results, expressed as IC_{50} values, are reported in Tables 1–4. CEUs were considered significantly active when their IC_{50} values were inferior to 15 μM; i.e. they showed an enhanced cytotoxicity compared with the parent drugs (chlorambucil, carmustine).

Initial structure–activity relationship (SAR) studies focused on the effects of substitution on the aromatic ring of *N*-phenyl-*N'*-2-chloroethylureas (Table 1). Unsubstituted phenyl derivative **1** was found to be totally inactive. Moreover, the aromatic ring substitution with electron-donating groups significantly contributed to high cytotoxicity, whereas electron-withdrawing groups, regardless of the position, generally abolished activity.[4] Consequently, the *N*-(alkylphenyl)-*N'*-(2-chloroethyl)urea series (**2**–**25**) was first explored.

The IC_{50} values obtained for methyl derivatives **2**–**9** clearly show that the position of the substituent on the phenyl ring greatly affected the cytotoxic activity. Each compound methylated on the 2- and/or 6-position (e.g. **3**, **5**, **6**, **9**) was inactive. In contrast, substitution at the 3-position (e.g. **4**, **8**) was tolerated and substitution at the 4-position (e.g. **2**, **7**) was optimal. Moreover, the 3,4-dimethyl derivative **7** displayed an enhanced activity compared with the corresponding monomethylated analogues **2** and **4**. On the basis of these results, the *N*-(4-alkylphenyl)-*N'*-(2-chloroethyl)urea series (**10**–**25**) was further investigated. As shown in Table 1, the cytotoxic potency depended on both the length and the degree of ramification of the alkyl chain. The CEU cytotoxicity generally dropped as the length of the alkyl chain increased especially from *n*-heptyl and beyond (**22**–**25**), probably due to a significant decrease of the hydrosolubility. However, *n*-pentyl (**17**) and *n*-hexyl (**20**) derivatives were equipotent with *n*-propyl (**11**) and *n*-butyl (**13**) analogues. Moreover, branched alkyl chains greatly enhanced the cytotoxicity of CEUs, as for *sec*- and *tert*-butyl derivatives **15** and **16** ($1.3 \le IC_{50} \le 9.1$ μM) 2–5 times more potent than the *n*-butyl counterpart **13** ($10 \le IC_{50} \le 22$ μM). A similar effect was markedly observed for the isopropyl derivative **12** compared with the *n*-propyl homologue **11**. Nevertheless, this trend was reversed in the pentyl series as neo and iso congeners **18** and **19** were less effective than the *n*-pentyl analogue **17**. The replacement of 4-*n*-hexyl group (**20**) with 4-cyclohexyl (**21**) also markedly reduced the cytotoxicity. In addition, the introduction of an iodine atom, approximately isosteric to the *tert*-butyl group, afforded one of the most potent derivatives (**27**, $IC_{50} \le 6$ μM). However, movement of the iodine from the 4- to 3-position, as for **26**, or replacement with either bromine (**28**) or chlorine (**29**) decreased the cytotoxic activity.

In addition, variation of the aromatic ring moiety was next examined. From the various CEUs represented in

Table 2. SAR Studies on *N*-Aryl-*N'*-2-chloroethylureas

compd	R	IC_{50}[a] (μM) LoVo	MDA-MB-231	P388D1	CHO	K562	L1210	HT 29	mp (°C)	yield (%)
30		6.2	6.1	5.3	7.2	3.0	3.0	5.5	185−187	40
31		5.1	9.0	4.2	9.3	6.0	4.3	13	206−209	43
32		5.1	10	7.3	10	5.5	5.0	10	155−157	40
33		51	76	70	69	26	51	48		30
34		NA[b]	50	NA	NA	113	144	ND[c]	122−124	70
35		NA	NA	NA	56	76	55	NA	165−167	60
36		150	150	130	106	107	118	ND	153−155	55

[a] Dose required to inhibit cell growth by 50%. Values are the means of at least three independent determinations. [b] NA: not active, IC_{50} > 400 μM. [c] ND: not determined.

Table 3. SAR Sudies on the Urea Function: Characterization and Cytotoxic Activity of Benzoxazone **37** and Quinazolinedione **38**

compd	IC_{50}[a] (μM) LoVo	MDA-MB-231	P388D1	mp (°C)	yield (%)
37	NA[b]	NA	NA	210−212	60
38	NA	NA	NA	193−195	90

[a] Dose required to inhibit cell growth by 50%. Values are the means of at least three independent determinations. [b] NA: not active, IC_{50} > 400 μM.

Table 2, naphthyl (**30**), fluorenyl (**31**), and indanyl (**32**) derivatives were the most cytotoxic with IC_{50} values ranging from 3 to 13 μM. Introduction of a heterocycle, as for compounds **33** and **34**, resulted in a total loss of cytotoxicity. Moreover, as previously noticed, CEUs **35** and **36**, where the 2-position was substituted, were inactive. Finally, these data show that the replacement of the phenyl ring by a bulky polycyclic aromatic system was compatible with good potency.

The modifications of the urea function were then briefly explored, as reported in Table 3. The inclusion of this function in a heterocyclic ring such as benzoxazone (**37**) or quinazolinedione (**38**) abolished activity. An exocyclic urea function appeared therefore to be the most favorable to generate any significant cytotoxicity.

On the basis of these results, *tert*-butyl-, *sec*-butyl-, and isopropylphenyl derivatives were selected for further SAR investigation on the *N'*-2-chloroethyl moiety. As presented in Table 4, replacement with *N'*-2-chloropropyl (**39**) or *N'*-alkyl (**40**−**48**) groups afforded inactive compounds. The important role played by the chlorine atom was not so obvious in the propyl subseries (compound **39** vs **46**), and a minor loss of cytotoxicity was observed as the *N'*-2-chloropropyl derivative (**39**) was already poorly active. Finally, the *N'*-2-chloroethyl moiety seemed to be essential for preserving cytotoxicity.

Biological Reactivity. Representative CEUs (**12**, **15**−**17**, **20**, **27**, **30**−**32**) were selected to evaluate their reactivity and determine their interaction with β-tubulin and also various biomolecules known as targets for anticancer drugs.

First, the evaluation of the alkylating activity of CEUs was performed by a colorimetric assay using *p*-nitrobenzylpyridine (NBP) as a nucleophilic scavenger.[10,11] The results, reported in Table 5, clearly show that CEUs exhibited poor alkylating ability, at least 10-fold weaker than chlorambucil while their cytotoxic potencies were higher (CEUs, 1.0 ≤ IC_{50} ≤ 13 μM; chlorambucil, carmustine, 3.0 ≤ IC_{50} ≤ 100 μM). However, as previously noticed in the SAR study, the alkylating moiety (i.e. the *N'*-2-chloroethyl group) is a prerequisite for the cytotoxic activity.

Indeed, we recently demonstrated that **16** was an antimitotic agent acting through alkylation of β-tubulin.[8] The effects of this CEU on the cytoskeleton can be visualized by indirect immunofluorescence microscopy. In Figure 1, the microtubule network of untreated MDA-MB-231 cells appeared to radiate outward from the perinuclear region to the plasma membrane. In contrast, both **16** and vinblastine altered the cellular network of

Table 4. SAR Studies on *N*-Phenyl-*N*′-alkylureas: The *N*′-2-Chloroethyl Moiety

compd	R_1	R_2	IC_{50}[a] (μM)					mp (°C)	yield (%)
			MDA-MB-231	K562	L1210	CHO	HT 29		
39	*tert*-butyl	2-chloropropyl	52	23	6	52	21	141–143	44
40	*tert*-butyl	methyl	NA[b]	NA	80	NA	196	182–184	48
41	*sec*-butyl	methyl	NA	142	62	NA	NA	109–111	55
42	isopropyl	methyl	NA	198	135	NA	NA	129–131	53
43	*tert*-butyl	ethyl	NA	84	16	NA	83	144–146	53
44	*sec*-butyl	ethyl	NA	101	43	NA	132	124–126	51
45	isopropyl	ethyl	NA	142	35	NA	NA	129–131	81
46	*tert*-butyl	propyl	131	30	5.4	132	35	153–155	64
47	*sec*-butyl	propyl	NA	106	30	NA	133	107–109	72
48	isopropyl	propyl	NA	52	14	133	76	133–135	78

[a] Dose required to inhibit cell growth by 50%. Values are the means of at least three independent determinations. [b] NA: not active, IC_{50} > 400 μM.

Table 5. Biological Data for *N*-Aryl-*N*′-2-chloroethylureas

compd	R	alkylating activity[a,b] (A_{570}/min)	β-tubulin alkylation[d] (%)	DNA damage[a,e] (exchanged dCps/ng DNA × 10^7)	GSH activity[g] (%)	GR activity[g] (%)
12	4-isopropylphenyl	3.0 ± 0.1	65	ND[c]	100	100
15	4-*sec*-butylphenyl	3.1 ± 0.3	76	ND	100	100
16	4-*tert*-butylphenyl	3.1 ± 0.3	44	2.22 ± 0.34	100	100
17	4-*n*-pentylphenyl	2.9 ± 0.1	40	1.93 ± 0.03	100	99
20	4-*n*-hexylphenyl	3.1 ± 0.2	34	2.32 ± 0.10	100	99
27	4-iodophenyl	3.1 ± 0.3	44	2.66 ± 0.41	100	98
30	naphthyl	ND	47	1.89 ± 0.31	ND	ND
31	fluorenyl	2.6 ± 0.1	23	1.98 ± 0.22	ND	ND
32	indanyl	3.5 ± 0.2	43	2.37 ± 0.25	ND	ND
1		ND	0	ND	ND	ND
43		0.07 ± 0.02	0	ND	ND	ND
chlorambucil		39 ± 5		12.7 ± 0.9[f]	ND	ND
carmustine		ND		19.9 ± 1.1[f]	50	20

[a] Values are means ± SD of at least three independent determinations. [b] Alkylation rate determined by the NBP assay at 570 nm (see Experimental Section). [c] ND: not determined. [d] Alkylation of β-tubulin in MDA-MB-231 cells incubated with the drug (30 μM) for 24 h, at 37 °C. Values are representative of three independent determinations. [e] DNA damages caused in MDA-MB-231 cells after a 2-h drug exposure (400 μM), at 37 °C, expressed as exchanged dCps/ng DNA (see Experimental Section). [f] Significantly different from control (2.30 ± 0.30 10^7 dCps/ng DNA); $P \leq 0.05$, Student's *t*-test. [g] Glutathione (GSH) and glutathione reductase (GR) activities measured after incubation with the drug (500 μM) expressed as percent of control (no drug added). Each experiment consisted of triplicate measurements.

the microtubules and led to a tubulin condensation around the nucleus, while cells treated with taxol displayed cytoplasmic microtubules and mitotic spindle. As **16** greatly affects microtubule assembly, it was essential to evaluate the alkylation of β-tubulin induced by the novel CEUs. To this aim, the total protein fraction of MDA-MB-231 cells treated with CEUs was isolated, separated by SDS–PAGE, and the β-tubulin revealed by Western blotting with an anti-β-tubulin antibody. The alkylated β-tubulin, migrating faster than the native protein, was then separated and quantified.[8] Results are reported in Table 5. The alkylation rate of active CEUs (**12**, **15**–**17**, **20**, **27**, **30**, **32**) ranged from 34–76% of the total β-tubulin except for **31** which exhibited a slightly lower percentage. Inactive CEUs (**1**, **43**) did not alkylate this protein. These data confirm that the antiproliferative activity of CEUs can be related to β-tubulin alkylation.

However, structural similarities with 2-chloroethylnitrosoureas and nitrogen mustards suggested that CEUs could also interact with other target biomolecules such as DNA, glutathione (GSH), or glutathione reductase (GR). Actually, the cytotoxic effects of 2-chloroethylnitrosoureas derive primarily from their ability to alter DNA.[1,2] To detect any DNA damages resulting from alkylation by CEUs, a DNA 3′-end labeling assay was carried out.[12] The data, presented in Table 5, demonstrate no significant difference between DNA isolated from cells either treated with CEUs (**16**, **17**, **20, 27**, **30**–**32**) or not treated (control). In contrast, chlorambucil and carmustine both produced significant DNA damage. Moreover, as previously reported, CEUs neither display nor induce mutagenicity or resistance in various tumor cells with increased DNA repair capacity.[6] These results indicate that DNA is not a cellular target of CEUs.

Moreover, carmustine is also known to inactivate GR and carbamoylate GSH, two key molecules involved in the cell's defense mechanism and essential for cellular function.[13–15] Thiol alteration may contribute to the antitumor effects of 2-chloroethylnitrosoureas but also induce major resistances to treatments.[16,17] To determine the CEUs reactivity, GSH and GR activities were measured after incubation with **12**, **15**–**17**, **20**, and **27**.[15,18] As shown in Table 5, the initial GSH and GR activities were maintained after treatment with CEUs, while they dropped to 50% and 20%, respectively, for carmustine. Therefore, CEUs did not react with the thiol function of these biomolecules. These data are consistent with the lack of resistance of CEUs previously observed in various tumor cells with elevated GSH or GR levels.[6]

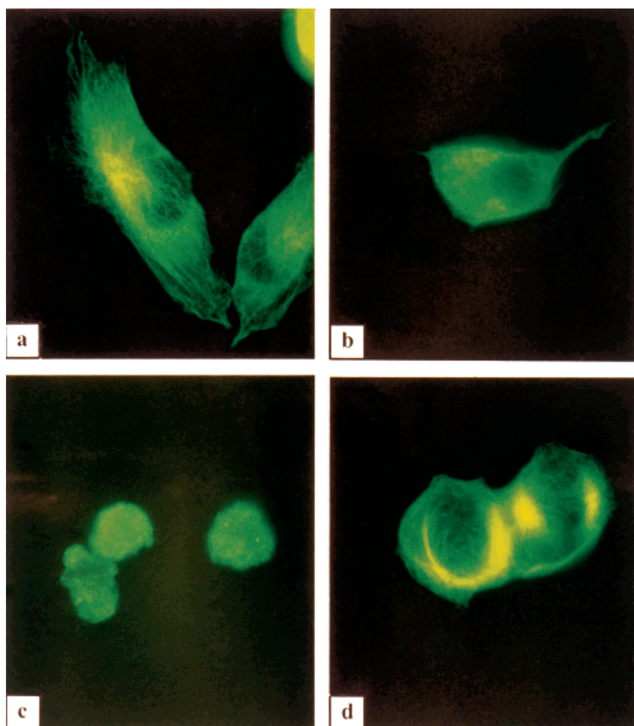

Figure 1. Effect of *N*′-(4-*tert*-butylphenyl)-*N*-(2-chloroethyl)urea (**16**) on microtubule network revealed by indirect immunofluorescence microscopy. MDA-MB-231 cells were (a) untreated, (b) incubated with **16** (30 μM), (c) incubated with vinblastine (5 μM), and (d) incubated with taxol (5 μM) for 3 h at 37 °C. After fixation, microtubules were visualized by sequential treatment with murine β-tubulin monoclonal antibody and IgG FITC conjugated antibody. Data are representative of three different experiments.

Finally, the weak alkylating activity of CEUs clearly prevented the reaction with DNA, GSH, and GR but was essential for displaying any cytotoxicity. Moreover, we showed that **16** alkylated β-tubulin on the cysteine 239 residue, indicating that this weak alkylating activity was sufficient to allow reaction with some specific sulfhydryl groups after a relevant period of incubation. These data indicate that the decreased alkylating potency of CEUs, compared to the parent drugs, was balanced by an enhanced selectivity toward cellular targets.

Conclusion

The SARs for the series of CEU derivatives, defined through variations of the three regions of the core structure, showed that the exocyclic urea function and the 2-chloroethyl group were essential. The most favorable aryl groupments were (i) a phenyl ring substituted at the 4-position with a branched alkyl chain or a halogen atom and (ii) an indanyl or fluorenyl group. These potent CEUs were weak but selective alkylating agents since they did not interact with either DNA or GSH or GR and appeared as antimitotic drugs acting by alkylation of β-tubulin. Among the CEUs active on a wide spectrum of tumor cell lines, **12**, **15**, **16**, and **27** exhibited a good profile for preclinical antitumoral investigations. Moreover, compound **27**, labeled with [^{125}I]iodine, would provide a useful tool for further biological studies.

Experimental Section

Chemistry. Chemical Methods. Proton nuclear magnetic resonance (NMR) spectra were performed on a Bruker AM-400 spectrometer. Chemical shifts (δ) are reported in parts per million relative to the internal tetramethylsilane standard. Infrared (IR) spectra were recorded on an Unicam spectrometer. Melting points (mp), uncorrected, were determined on an Electrothermal melting point apparatus. Elemental analysis were performed by the Chemistry Department, University of Montréal (Canada), and were within ±0.4% of theory for the formulas given unless otherwise indicated.

All reactions were conducted under a rigorously dried argon atmosphere, using oven-dried glassware. Diethyl ether was dried over sodium and stored over 4 Å molecular sieves. Chemicals were supplied by Aldrich Chemical Co. (Milwaukee, WI).

The synthesis of the 2-chloroethylurea **2** has been reported previously.[4] This compound has been synthesized herein according to the general procedure described below and then fully characterized.

General Procedure for the Preparation of *N*-Aryl-*N*′-2-chloroethylureas and *N*-Phenyl-*N*′-alkylureas 1−36, 39−48. To a stirred solution of the relevant aniline (3 mmol) in diethyl ether or absolute ethanol (10 mL) was added dropwise 2-chloroethyl/alkyl isocyanate (3.6 mmol), as required. The reaction mixture was stirred overnight at ambient temperature. The resulting crude precipitate was filtered, washed with cold solvent and further purified, if necessary, by recrystallization from ethanol to afford the urea as a white solid.

***N*-Phenyl-*N*′-2-chloroethylurea (1):** yield 41%; mp 124−126 °C; ^{1}H NMR ($CDCl_3$) δ 3.45−3.55 (m, 4H, CH_2CH_2Cl), 6.15 (br s, 1H, N*H*CH_2, exchanges with D_2O), 6.89−7.30 (m, 5H, Ar−H), 8.00 (br s, 1H, ArN*H*, exchanges with D_2O); IR (KBr) ν 3320 (NH), 1640 (C=O) cm^{-1}. Anal. ($C_9H_{11}ClN_2O$) C, H, N.

***N*-(4-Methylphenyl)-*N*′-2-chloroethylurea (2):** yield 81%; mp 180−182 °C; ^{1}H NMR ($CDCl_3$) δ 2.13 (s, 3H, CH_3), 3.40−3.51 (m, 4H, CH_2CH_2Cl), 6.08 (br s, 1H, N*H*CH_2, exchanges with D_2O), 6.90−7.12 (dd, 4H, Ar−H), 7.90 (br s, 1H, ArN*H*, exchanges with D_2O); IR (KBr) ν 3340 (NH), 1640 (C=O) cm^{-1}. Anal. ($C_{10}H_{13}ClN_2O$) C, H, N.

***N*-(2-Methylphenyl)-*N*′-2-chloroethylurea (3):** yield 57%; mp 150−152 °C; ^{1}H NMR ($CDCl_3$) δ 2.17 (s, 3H, CH_3), 3.47−3.54 (m, 4H, CH_2CH_2Cl), 6.22 (br s, 1H, N*H*CH_2, exchanges with D_2O), 6.91−7.17 (m, 4H, Ar−H), 7.63 (br s, 1H, ArN*H*, exchanges with D_2O); IR (KBr) ν 3330 (NH), 1630 (C=O) cm^{-1}. Anal. ($C_{10}H_{13}ClN_2O$) C, H, N.

***N*-(3-Methylphenyl)-*N*′-2-chloroethylurea (4):** yield 38%; mp 130−132 °C; ^{1}H NMR ($CDCl_3$) δ 2.18 (s, 3H, CH_3), 3.46−3.52 (m, 4H, CH_2CH_2Cl), 6.15 (br s, 1H, N*H*CH_2, exchanges with D_2O), 6.66−7.15 (m, 4H, Ar−H), 7.95 (br s, 1H, ArN*H*, exchanges with D_2O); IR (KBr) ν 3340 (NH), 1630 (C=O) cm^{-1}. Anal. ($C_{10}H_{13}ClN_2O$) C, H, N.

***N*-(2,6-Dimethylphenyl)-*N*′-2-chloroethylurea (5):** yield 67%; mp 167−169 °C; ^{1}H NMR ($CDCl_3$) δ 2.04 (s, 6H, CH_3), 3.30−3.34 (m, 4H, CH_2CH_2Cl), 5.62 (br s, 1H, N*H*CH_2, exchanges with D_2O), 6.80−6.86 (m, 3H, Ar−H), 6.87 (br s, 1H, ArN*H*, exchanges with D_2O); IR (KBr) ν 3300 (NH), 1630 (C=O) cm^{-1}. Anal. ($C_{11}H_{15}ClN_2O$) C, H, N.

***N*-(2,3-Dimethylphenyl)-*N*′-2-chloroethylurea (6):** yield 53%; mp 152−154 °C; ^{1}H NMR ($CDCl_3$) δ 2.07, 2.19 (2s, 6H, CH_3), 3.47−3.52 (m, 4H, CH_2CH_2Cl), 5.93 (br s, 1H, N*H*CH_2, exchanges with D_2O), 6.86−7.07 (m, 3H, Ar−H), 7.25 (br s, 1H, ArN*H*, exchanges with D_2O); IR (KBr) ν 3320 (NH), 1640 (C=O) cm^{-1}. Anal. ($C_{11}H_{15}ClN_2O$) C, H, N.

***N*-(3,4-Dimethylphenyl)-*N*′-2-chloroethylurea (7):** yield 78%; mp 151−153 °C; ^{1}H NMR ($CDCl_3$) δ 1.95, 1.97 (2s, 6H, CH_3), 3.32−3.36 (m, 4H, CH_2CH_2Cl), 6.02 (br s, 1H, N*H*CH_2, exchanges with D_2O), 6.75−6.98 (m, 3H, Ar−H), 7.80 (br s, 1H, ArN*H*, exchanges with D_2O); IR (KBr) ν 3320 (NH), 1640 (C=O) cm^{-1}. Anal. ($C_{11}H_{15}ClN_2O$) C, H, N.

***N*-(3,5-Dimethylphenyl)-*N*′-2-chloroethylurea (8):** yield 55%; mp 153−155 °C; ^{1}H NMR ($CDCl_3$) δ 2.14 (s, 6H, CH_3), 3.45−3.49 (m, 4H, CH_2CH_2Cl), 6.10 (br s, 1H, N*H*CH_2,

exchanges with D_2O), 6.51−6.90 (m, 3H, Ar−H), 7.81 (br s, 1H, ArN*H*, exchanges with D_2O); IR (KBr) ν 3320 (NH), 1640 (C=O) cm^{-1}. Anal. ($C_{11}H_{15}ClN_2O$) C, H, N.

***N*-(2,4,6-Trimethylphenyl)-*N'*-2-chloroethylurea (9):** yield 72%; mp 192−194 °C; ^1H NMR ($CDCl_3$) δ 2.04, 2.08 (2s, 9H, CH_3), 3.36−3.39 (m, 4H, CH_2CH_2Cl), 6.60 (br s, 1H, N*H*CH_2, exchanges with D_2O), 6.68−6.72 (s, 2H, Ar−H), 6.74 (br s, 1H, ArN*H*, exchanges with D_2O); IR (KBr) ν 3300 (NH), 1640 (C=O) cm^{-1}. Anal. ($C_{12}H_{17}ClN_2O$) C, H, N.

***N*-(4-Ethylphenyl)-*N'*-2-chloroethylurea (10):** yield 53%; mp 161−163 °C; ^1H NMR ($CDCl_3$) δ 1.08 (t, *J* = 7.2 Hz, 3H, CH_3), 2.46 (q, *J* = 7.1 Hz, 2H, C*H*$_2$$CH_3$), 3.45−3.49 (m, 4H, CH_2CH_2Cl), 6.10 (br s, 1H, N*H*CH_2, exchanges with D_2O), 6.96−7.16 (dd, 4H, Ar−H), 7.90 (br s, 1H, ArN*H*, exchanges with D_2O); IR (KBr) ν 3320 (NH), 1640 (C=O) cm^{-1}. Anal. ($C_{11}H_{15}ClN_2O$) C, H, N.

***N*-(4-Propylphenyl)-*N'*-2-chloroethylurea (11):** yield 69%; mp 154−156 °C; ^1H NMR ($CDCl_3$) δ 0.75 (t, *J* = 7.2 Hz, 3H, CH_3), 1.39−1.41 (m, 2H, C*H*$_2$$CH_3$), 2.46 (t, *J* = 7.3 Hz, 2H, C*H*$_2$ CH_2CH_3), 3.39−3.43 (m, 4H, CH_2CH_2Cl), 6.08 (br s, 1H, N*H*CH_2, exchanges with D_2O), 6.87−7.10 (dd, 4H, Ar−H), 7.92 (br s, 1H, ArN*H*, exchanges with D_2O); IR (KBr) ν 3320 (NH), 1640 (C=O) cm^{-1}. Anal. ($C_{12}H_{17}ClN_2O$) C, H, N.

***N*-(4-Isopropylphenyl)-*N'*-2-chloroethylurea (12):** yield 65%; mp 140−142 °C; ^1H NMR ($CDCl_3$) δ 1.10 (d, *J* = 6.8 Hz, 6H, CH_3), 2.70−2.72 (m, 1H, C*H*CH_3), 3.43−3.46 (m, 4H, CH_2CH_2Cl), 6.06 (br s, 1H, N*H*CH_2, exchanges with D_2O), 6.98−7.16 (dd, 4H, Ar−H), 7.87 (br s, 1H, ArN*H*, exchanges with D_2O); IR (KBr) ν 3320 (NH), 1640 (C=O) cm^{-1}. Anal. ($C_{12}H_{17}ClN_2O$) C, H, N.

***N*-(4-Butylphenyl)-*N'*-2-chloroethylurea (13):** yield 56%; mp 139−141 °C; ^1H NMR ($CDCl_3$) δ 0.84 (t, *J* = 7.2 Hz, 3H, CH_3), 1.24−1.27 (m, 2H, C*H*$_2$$CH_3$), 1.49−1.52 (m, 2H, C*H*$_2$$CH_2CH_3$), 2.47−2.49 (t, *J* = 7.3 Hz, 2H, C*H*$_2$ $CH_2CH_2CH_3$), 3.49−3.52 (m, 4H, CH_2CH_2Cl), 6.08 (br s, 1H, N*H*CH_2, exchanges with D_2O), 6.98−7.20 (dd, 4H, Ar−H), 7.81 (br s, 1H, ArN*H*, exchanges with D_2O); IR (KBr) ν 3330 (NH), 1640 (C=O) cm^{-1}. Anal. ($C_{13}H_{19}ClN_2O$) C, H, N.

***N*-(4-Isobutylphenyl)-*N'*-2-chloroethylurea (14):** yield 60%; mp 105−107 °C; ^1H NMR ($CDCl_3$) δ 1.07 (d, *J* = 7.1 Hz, 6H, CH_3), 1.50−1.53 (m, 1H, C*H*CH_3), 2.40−2.43 (m, 2H, C*H*$_2$CH), 3.51−3.54 (m, 4H, CH_2CH_2Cl), 6.08 (br s, 1H, N*H*CH_2, exchanges with D_2O), 7.00−7.20 (dd, 4H, Ar−H), 7.81 (br s, 1H, ArN*H*, exchanges with D_2O); IR (KBr) ν 3320 (NH), 1640 (C=O) cm^{-1}. Anal. ($C_{13}H_{19}ClN_2O$) C, H, N.

***N*-(4-*sec*-Butylphenyl)-*N'*-2-chloroethylurea (15):** yield 95%; mp 89−91 °C; ^1H NMR ($CDCl_3$) δ 0.74 (t, *J* = 7.4 Hz, 3H, CH_2C*H*$_3$), 1.14 (d, *J* = 7.0 Hz, 3H, CHC*H*$_3$), 1.48−1.52 (m, 2H, C*H*$_2$$CH_3$), 2.44−2.47 (m, 1H, C*H*CH_3), 3.51−3.54 (m, 4H, CH_2CH_2Cl), 6.06 (br s, 1H, N*H*CH_2, exchanges with D_2O), 7.00−7.21 (dd, 4H, Ar−H), 7.80 (br s, 1H, ArN*H*, exchanges with D_2O); IR (KBr) ν 3320 (NH), 1640 (C=O) cm^{-1}. Anal. ($C_{13}H_{19}ClN_2O$) C, H, N.

***N*-(4-*tert*-Butylphenyl)-*N'*-2-chloroethylurea (16):** yield 60%; mp 134−136 °C; ^1H NMR ($CDCl_3$) δ 1.20 (s, 9H, CH_3), 3.49−3.53 (m, 4H, CH_2CH_2Cl), 6.09 (br s, 1H, N*H*CH_2, exchanges with D_2O), 7.15−7.21 (dd, 4H, Ar−H), 7.85 (br s, 1H, ArN*H*, exchanges with D_2O); IR (KBr) ν 3300 (NH), 1640 (C=O) cm^{-1}. Anal. ($C_{13}H_{19}ClN_2O$) C, H, N.

***N*-(4-Pentylphenyl)-*N'*-2-chloroethylurea (17):** yield 69%; mp 135−137 °C; ^1H NMR ($CDCl_3$) δ 0.88 (t, *J* = 7.2 Hz, 3H, CH_3), 1.21−1.26, 1.58-1.62 (m, 6H, CH_2), 2.53-2.58 (m, 2H, ArC*H*$_2$), 3.58-3.70 (m, 4H, CH_2CH_2Cl), 6.33 (br s, 1H, N*H*CH_2, exchanges with D_2O), 7.10−7.17 (dd, 4H, Ar−H), 7.25 (br s, 1H, ArN*H*, exchanges with D_2O); IR (KBr) ν 3320 (NH), 1640 (C=O) cm^{-1}. Anal. ($C_{14}H_{21}ClN_2O$) C, H, N.

***N*-(4-Neopentylphenyl)-*N'*-2-chloroethylurea (18):** yield 70%; mp 122−124 °C; ^1H NMR ($CDCl_3$) δ 0.88 (s, 9H, CH_3), 2.45 (s, 2H, CH_2), 3.59−3.73 (m, 4H, CH_2CH_2Cl), 5.21 (br s, 1H, N*H*CH_2, exchanges with D_2O), 7.07−7.19 (dd, 4H, Ar−H), 7.25 (br s, 1H, ArN*H*, exchanges with D_2O); IR (KBr) ν 3330 (NH), 1640 (C=O) cm^{-1}. Anal. ($C_{14}H_{21}ClN_2O$) C, H, N.

***N*-(4-Isopentylphenyl)-*N'*-2-chloroethylurea (19):** yield 75%; mp 147−149 °C; ^1H NMR ($CDCl_3$) δ 0.92 (s, 6H, CH_3), 1.43-1.55 (m, 3H, CH, CH_2), 2.55−2.58 (m, 2H, ArC*H*$_2$), 3.57-3.65 (m, 4H, CH_2CH_2Cl), 5.21 (br s, 1H, N*H*CH_2, exchanges with D_2O), 7.07−7.19 (dd, 4H, Ar−H), 7.25 (br s, 1H, ArN*H*, exchanges with D_2O); IR (KBr) ν 3330 (NH), 1640 (C=O) cm^{-1}. Anal. ($C_{14}H_{21}ClN_2O$) C, H, N.

***N*-(4-Hexylphenyl)-*N'*-2-chloroethylurea (20):** yield 66%; mp 130−132 °C; ^1H NMR ($CDCl_3$) δ 0.79 (t, *J* = 7.1 Hz, 3H, CH_3), 1.18−1.21, 1.47−1.49 (2m, 8H, CH_2), 2.44−2.47 (m, 2H, ArC*H*$_2$), 3.49−3.54 (m, 4H, CH_2CH_2Cl), 6.08 (br s, 1H, N*H*CH_2, exchanges with D_2O), 6.97−7.18 (dd, 4H, Ar−H), 7.86 (br s, 1H, ArN*H*, exchanges with D_2O); IR (KBr) ν 3320 (NH), 1640 (C=O) cm^{-1}. Anal. ($C_{15}H_{23}ClN_2O$) C, H, N.

***N*-(4-Cyclohexylphenyl)-*N'*-2-chloroethylurea (21):** yield 63%; mp 164−166 °C; ^1H NMR ($CDCl_3$) δ 1.23−1.25, 1.68−1.73, 2.28−2.32 (3m, 11H, cyclohexyl), 3.46−3.49 (m, 4H, CH_2CH_2Cl), 6.07 (br s, 1H, N*H*CH_2, exchanges with D_2O), 6.97−7.16 (dd, 4H, Ar−H), 7.87 (br s, 1H, ArN*H*, exchanges with D_2O); IR (KBr) ν 3340 (NH), 1640 (C=O) cm^{-1}. Anal. ($C_{15}H_{21}ClN_2O$) C, H, N.

***N*-(4-Heptylphenyl)-*N'*-2-chloroethylurea (22):** yield 76%; mp 129−131 °C; ^1H NMR ($CDCl_3$) δ 0.77 (t, *J* = 7.2 Hz, 3H, CH_3), 1.16−1.19, 1.45−1.47 (2m, 10H, CH_2), 2.40−2.44 (m, 2H, ArC*H*$_2$), 3.45−3.49 (m, 4H, CH_2CH_2Cl), 6.09 (br s, 1H, N*H*CH_2, exchanges with D_2O), 6.94−7.18 (dd, 4H, Ar−H), 7.86 (br s, 1H, ArN*H*, exchanges with D_2O); IR (KBr) ν 3320 (NH), 1640 (C=O) cm^{-1}. Anal. ($C_{16}H_{25}ClN_2O$) C, H, N.

***N*-(4-Octylphenyl)-*N'*-2-chloroethylurea (23):** yield 81%; mp 127−129 °C; ^1H NMR ($CDCl_3$) δ 0.78 (t, *J* = 7.2 Hz, 3H, CH_3), 1.15−1.18, 1.46−1.49 (2m, 12H, CH_2), 2.41−2.44 (m, 2H, ArC*H*$_2$), 3.48−3.52 (m, 4H, CH_2CH_2Cl), 6.07 (br s, 1H, N*H*CH_2, exchanges with D_2O), 6.96−7.18 (dd, 4H, Ar−H), 7.85 (br s, 1H, ArN*H*, exchanges with D_2O); IR (KBr) ν 3330 (NH), 1640 (C=O) cm^{-1}. Anal. ($C_{17}H_{27}ClN_2O$) C, H, N.

***N*-(4-Decylphenyl)-*N'*-2-chloroethylurea (24):** yield 53%; mp 124−126 °C; ^1H NMR ($CDCl_3$) δ 0.78 (t, *J* = 7.1 Hz, 3H, CH_3), 1.14−1.17, 1.45−1.48 (2m, 16H, CH_2), 2.40−2.43 (m, 2H, ArC*H*$_2$), 3.47−3.51 (m, 4H, CH_2CH_2Cl), 6.05 (br s, 1H, N*H*CH_2, exchanges with D_2O), 6.95−7.17 (dd, 4H, Ar−H), 7.83 (br s, 1H, ArN*H*, exchanges with D_2O); IR (KBr) ν 3320 (NH), 1640 (C=O) cm^{-1}. Anal. ($C_{19}H_{31}ClN_2O$) C, H, N.

***N*-(4-Dodecylphenyl)-*N'*-2-chloroethylurea (25):** yield 74%; mp 123−125 °C; ^1H NMR ($CDCl_3$) δ 0.79 (t, *J* = 7.1 Hz, 3H, CH_3), 1.15−1.18, 1.44−1.47 (2m, 20H, CH_2), 2.41−2.44 (m, 2H, ArC*H*$_2$), 3.48−3.52 (m, 4H, CH_2CH_2Cl), 6.05 (br s, 1H, N*H*CH_2, exchanges with D_2O), 6.95−7.18 (dd, 4H, Ar−H), 7.82 (br s, 1H, ArN*H*, exchanges with D_2O); IR (KBr) ν 3330 (NH), 1640 (C=O) cm^{-1}. Anal. ($C_{21}H_{35}ClN_2O$) C, H, N.

***N*-(3-Iodophenyl)-*N'*-2-chloroethylurea (26):** yield 95%; mp 143−145 °C; ^1H NMR (DMSO-d_6) δ 3.40−3.42, 3.64−3.66 (2m, 4H, CH_2CH_2Cl), 6.98 (br s, 1H, N*H*CH_2, exchanges with D_2O), 7.03−7.95 (m, 4H, Ar−H), 8.88 (br s, 1H, ArN*H*, exchanges with D_2O); IR (KBr) ν 3320 (NH), 1640 (C=O) cm^{-1}. Anal. ($C_9H_{10}ClIN_2O$) C, H, N.

***N*-(4-Iodophenyl)-*N'*-2-chloroethylurea (27):** yield 81%; mp 194−196 °C; ^1H NMR (DMSO-d_6) δ 3.40−3.42, 3.62−3.64 (2m, 4H, CH_2CH_2Cl), 6.43 (br s, 1H, N*H*CH_2, exchanges with D_2O), 7.22−7.54 (dd, 4H, Ar−H), 8.77 (br s, 1H, ArN*H*, exchanges with D_2O); IR (KBr) ν 3320 (NH), 1635 (C=O) cm^{-1}. Anal. ($C_9H_{10}ClIN_2O$) C, H, N.

***N*-(4-Bromophenyl)-*N'*-2-chloroethylurea (28):** yield 77%; mp 184−186 °C; ^1H NMR (DMSO-d_6) δ 3.39−3.41, 3.61−3.66 (2m, 4H, CH_2CH_2Cl), 5.15 (br s, 1H, N*H*CH_2, exchanges with D_2O), 6.27 (br s, 1H, ArN*H*, exchanges with D_2O), 7.19−7.43 (dd, 4H, Ar−H); IR (KBr) ν 3320 (NH), 1635 (C=O) cm^{-1}. Anal. ($C_9H_{10}BrClN_2O$) C, H, N.

***N*-(4-Chlorophenyl)-*N'*-2-chloroethylurea (29):** yield 72%; mp 153−155 °C; ^1H NMR (DMSO-d_6) δ 3.39−3.41−3.62−3.66 (m, 4H, CH_2CH_2Cl), 6.44 (br s, 1H, N*H*CH_2, exchanges with D_2O), 7.24−7.43 (dd, 4H, Ar−H), 8.80 (br s, 1H, ArN*H*, exchanges with D_2O); IR (KBr) ν 3330 (NH), 1635 (C=O) cm^{-1}. Anal. ($C_9H_{10}Cl_2N_2O$) C, H, N.

***N*-(2-Naphthyl)-*N'*-2-chloroethylurea (30):** yield 40%; mp 185−187 °C; ^1H NMR (DMSO-d_6) δ 3.45−3.53 (2m, 4H, CH_2CH_2Cl), 6.25 (br s, 1H, N*H*CH_2, exchanges with D_2O),

7.17–7.86 (m, 7H, Ar–H), 8.23 (br s, 1H, ArN*H*, exchanges with D_2O); IR (KBr) ν 3310 (NH), 1630 (C=O) cm^{-1}. Anal. ($C_{13}H_{13}ClN_2O$) C, H, N.

***N*-(2-Fluorenyl)-*N'*-2-chloroethylurea (31):** yield 43%; mp 206–208 °C; ^1H NMR (DMSO-d_6) δ 3.25–3.33 (2m, 4H, CH_2CH_2Cl), 3.50 (s, 2H, CH_2 fluorenyl), 6.02 (br s, 1H, N*H*CH_2, exchanges with D_2O), 6.93–7.44 (m, 7H, Ar–H), 8.08 (br s, 1H, ArN*H*, exchanges with D_2O); IR (KBr) ν 3320 (NH), 1630 (C=O) cm^{-1}. Anal. ($C_{16}H_{15}ClN_2O$) C, H, N.

***N*-(5-Indanyl)-*N'*-2-chloroethylurea (32):** yield 40%; mp 155–157 °C; ^1H NMR (DMSO-d_6) δ 1.90–1.95, 2.69–2.73 (2 m, 6H, CH_2 indanyl), 3.45–3.49 (2m, 4H, CH_2CH_2Cl), 6.93–7.25 (m, 3H, Ar–H) 6.02 (br s, 1H, N*H*CH_2, exchanges with D_2O), 8.08 (br s, 1H, ArN*H*, exchanges with D_2O); IR (KBr) ν 3300 (NH), 1640 (C=O) cm^{-1}. Anal. ($C_{12}H_{15}ClN_2O$) C, H, N.

***N*-(6-Indazolyl)-*N'*-2-chloroethylurea (33):** yield 30%; ^1H NMR (DMSO-d_6) δ 3.15–3.19 (m, 4H, CH_2CH_2Cl), 5.98 (br s, 1H, N*H*CH_2, exchanges with D_2O), 6.40–7.56 (m, 4H, Ar–H, C*H* indazole), 8.12 (br s, 1H, ArN*H*, exchanges with D_2O), 12.02 (s, 1H, NH indazolyl, exchanges with D_2O); IR (KBr) ν 3200 (NH), 1630 (C=O) cm^{-1}. Anal. ($C_{10}H_{11}ClN_4O$) C, H, N.

***N*-[2-(4,6-Dimethylpyridyl)]-*N'*-2-chloroethylurea (34):** yield 70%; mp 122–124 °C; ^1H NMR (DMSO-d_6) δ 2.11, 2.27 (2 s, 6H, CH_3), 3.57–3.63 (m, 4H, CH_2CH_2Cl), 6.40–6.45 (m, 2H, Ar–H), 8.92, 9.95 (2 br s, 2H, ArN*H*, N*H*CH_2, exchanges with D_2O); IR (KBr) ν 3140 (NH), 1670 (C=O) cm^{-1}. Anal. ($C_{10}H_{14}ClN_3O$) C, H, N.

***N*-[1-(5,6,7,8-Tetrahydronaphthyl)]-*N'*-2-chloroethylurea (35):** yield 60%; mp 165–167 °C; ^1H NMR (DMSO-d_6) δ 1.60–2.16, 2.75–2.84 (2m, 8H, CH_2), 3.47–3.52 (2m, 4H, CH_2CH_2Cl), 6.45 (br s, 1H, N*H*CH_2, exchanges with D_2O), 7.30–7.92 (m, 7H, Ar–H), 8.13 (br s, 1H, ArN*H*, exchanges with D_2O); IR (KBr) ν 3300 (NH), 1640 (C=O) cm^{-1}. Anal. ($C_{13}H_{17}ClN_2O$) C, H, N.

***N*-(1-Naphthyl)-*N'*-2-chloroethylurea (36):** yield 55%; mp 153–155 °C; ^1H NMR (DMSO-d_6) δ 3.47–3.52 (2m, 4H, CH_2CH_2Cl), 6.45 (br s, 1H, N*H*CH_2, exchanges with D_2O), 7.30–7.92 (m, 7H, Ar–H), 8.13 (br s, 1H, ArN*H*, exchanges with D_2O); IR (KBr) ν 3300 (NH), 1640 (C=O) cm^{-1}. Anal. ($C_{13}H_{13}ClN_2O$) C, H, N.

***N*-(4-*tert*-Butylphenyl)-*N'*-2-chloropropylurea (39):** yield 44%; mp 141–143 °C; ^1H NMR ($CDCl_3$) δ 0.99 (s, 9H, CH_3), 1.66–1.70 (m, 2H, C*H*$_2$$CH_2Cl$), 3.03–3.06, 3.30–3.34 (2m, 4H, NCH_2, CH_2Cl), 6.98–7.00 (m, 4H, Ar–H), 7.73 (br s, 1H, ArN*H*, exchanges with D_2O); IR (KBr) ν 3340 (NH), 1640 (C=O) cm^{-1}. Anal. ($C_{14}H_{21}ClN_2O$) C, H, N.

***N*-(4-*tert*-Butylphenyl)-*N'*-methylurea (40):** yield 48%; mp 182–184 °C; ^1H NMR ($CDCl_3$) δ 1.30 (s, 9H, CH_3), 2.82 (s, 3H, NHC*H*$_3$), 7.15–7.36 (m, 4H, Ar–H); IR (KBr) ν 3320 (NH), 1640 (C=O) cm^{-1}. Anal. ($C_{12}H_{18}N_2O$) H, N; C: calcd, 69.88; found, 67.97.

***N*-(4-*sec*-Butylphenyl)-*N'*-methylurea (41):** yield 55%; mp 109–111 °C; ^1H NMR ($CDCl_3$) δ 0.81 (t, J = 7.3 Hz, 3H, C*H*$_3$$CH_2$), 1.20 (s, 6H, CH_3), 1.51–1.61 (q, J = 7.3 Hz, 2H, CH_3C*H*$_2$), 2.53–2.60 (m, 1H, CH), 2.82 (s, 3H, NHC*H*$_3$, exchanges with D_2O), 6.20 (br s, 1H, ArN*H*, exchanges with D_2O), 7.11–7.25 (m, 4H, Ar–H); IR (KBr) ν 3320 (NH), 1640 (C=O) cm^{-1}. Anal. ($C_{12}H_{18}N_2O$) C, H, N.

***N*-(4-Isopropylphenyl)-*N'*-methylurea (42):** yield 53%; mp 129–131 °C; ^1H NMR ($CDCl_3$) δ 1.23 (d, J = 6.8 Hz, 6H, CH_3), 2.82 (s, 3H, NHC*H*$_3$, exchanges with D_2O), 2.85–2.92 (m, 1H, CH), 7.14–7.25 (m, 4H, Ar–H); IR (KBr) ν 3320 (NH), 1640 (C=O) cm^{-1}. Anal. ($C_{11}H_{16}N_2O$) H, N.

***N*-(4-*tert*-Butylphenyl)-*N'*-ethylurea (43):** yield 53%; mp 144–146 °C; ^1H NMR ($CDCl_3$) δ 1.14 (t, J = 7.2 Hz, 3H, CH_2C*H*$_3$), 1.30 (s, 9H, CH_3), 3.23–3.32 (q, J = 7.2 Hz, 2H, NCH_2), 7.15–7.35 (m, 4H, Ar–H); IR (KBr) ν 3320 (NH), 1640 (C=O) cm^{-1}. Anal. ($C_{13}H_{20}N_2O$) C, H, N.

***N*-(4-*sec*-Butylphenyl)-*N'*-ethylurea (44):** yield 51%; mp 124–126 °C; ^1H NMR ($CDCl_3$) δ 0.81 (t, J = 7.4 Hz, 3H, C*H*$_3$$CH_2$), 1.13 (t, J = 7.3 Hz, 3H, NCH_2C*H*$_3$), 1.20 (s, J = 7.1 Hz, 3H, CH_3), 1.51–1.61 (quint, J = 7.3 Hz, 2H, CH_3C*H*$_2$), 2.53–2.60 (m, 1H, CH), 3.25–3.32 (q, 3H, NCH_2), 7.11–7.20 (m, 4H, Ar–H); IR (KBr) ν 3320 (NH), 1640 (C=O) cm^{-1}. Anal. ($C_{13}H_{20}N_2O$) C, H, N.

***N*-(4-Isopropylphenyl)-*N'*-ethylurea (45):** yield 81%; mp 129–131 °C; ^1H NMR ($CDCl_3$) δ 1.13 (t, J = 7.2 Hz, 3H, C*H*$_3$$CH_2$), 1.22 (d, J = 7.1 Hz, 6H, CH_3), 2.82–2.92 (m, 1H, CH), 3.24–3.31 (q, J = 7.2 Hz, 2H, CH_3C*H*$_2$), 7.14–7.25 (m, 4H, Ar–H); IR (KBr) ν 3320 (NH), 1640 (C=O) cm^{-1}. Anal. ($C_{12}H_{18}N_2O$) C, H, N.

***N*-(4-*tert*-Butylphenyl)-*N'*-propylurea (46):** yield 64%; mp 153–155 °C; ^1H NMR ($CDCl_3$) δ 0.90 (t, J = 7.4 Hz, 3H, C*H*$_3$$CH_2$), 1.30 (s, 9H,$CH_3$), 1.49–1.55 (m, 2H, CH_3C*H*$_2$), 3.19 (t, J = 7.4 Hz, 2H, NCH_2), 7.15–7.34 (m, 4H, Ar–H), 6.20 (br s, 1H, AR–NH, exchanges with D_2O); IR (KBr) ν 3320 (NH), 1640 (C=O) cm^{-1}. Anal. ($C_{14}H_{22}N_2O$) C, H, N.

***N*-(4-*sec*-Butylphenyl)-*N'*-propylurea (47):** yield 72%; mp 107–109 °C; ^1H NMR ($CDCl_3$) δ 0.81 (t, J = 7.4 Hz, 3H, C*H*$_3$$CH_2$), 0.91 (t, J = 7.3 Hz, 3H, NCH_2CH_2C*H*$_3$), 1.20 (s, 3H, CH_3), 1.49–1.61 (m, J = 7.3 Hz, 4H, CH_3C*H*$_2$), 2.53–2.60 (m, 1H, CH), 3.18–3.22 (t, J = 7.4 Hz, 2H, NCH_2), 7.11–7.18 (m, 4H, Ar–H); IR (KBr) ν 3320 (NH), 1640 (C=O) cm^{-1}. Anal. ($C_{14}H_{22}N_2O$) C, H, N.

***N*-(4-Isopropylphenyl)-*N'*-propylurea (48):** yield 78%; mp 133–135 °C; ^1H NMR ($CDCl_3$) δ 0.89 (t, J = 7.4, 7.4 Hz, 3H, C*H*$_3$$CH_2$), 1.21 (d, J = 7.0 Hz, 6H, CH_3), 1.46–1.53 (m, 2H, C*H*$_2$$CH_3$), 2.83–2.88 (m, 1H, CH), 3.17 (t, J = 7.3 Hz, 2H, NCH_2), 6.51 (br s, 1H, ArN*H*, exchanges with D_2O), 7.16–7.25 (m, 4H, Ar–H); IR (KBr) ν 3320 (NH), 1640 (C=O) cm^{-1}. Anal. ($C_{13}H_{20}N_2O$) C, H, N.

2-(2-Chloroethyl)amino-7-chloro-1,3-benzoxaz-4-one (37). 2-(2-Chloroethylureido)-4-chlorobenzoic acid (0.5 g, 1.8 mmol) was obtained from 2-amino-4-chlorobenzoic acid according to the general procedure described above and vigorously stirred in concentrated sulfuric acid for 3 h at room temperature. The mixture was neutralized with a 10% aqueous solution of potassium carbonate. The resulting precipitate was filtered and dried to afford the benzoxazone **37** as a white solid (0.28 g, 60%): mp 210–212 °C; ^1H NMR ($CDCl_3$) δ 3.30–3.35 (m, 4H, CH_2CH_2Cl), 6.68–7.80 (m, 3H, Ar–H), 8.80 (br s, 1H, N*H*, exchanges with D_2O); IR (KBr) ν 1710 (C=O) cm^{-1}. Anal. ($C_{10}H_8N_2O_2Cl_2$) C, H, N.

3-(2-Chloroethyl)-2,4-(1*H*,3*H*)-quinazolinedione (38). 2-(2-Chloroethylureido)benzonitrile (0.25 g, 1.1 mmol) was obtained from 2-aminobenzonitrile according to the general procedure described above and vigorously stirred in a mixture of concentrated hydrochloric acid (2 mL) and absolute ethanol (5 mL) for 3 h at room temperature. The resulting precipitate was filtered and dried to afford the quinazolinedione **38** as a white solid (0.24 g, 90%): mp 193–195 °C; ^1H NMR ($CDCl_3$) δ 3.88–3.91, 4.60–4.65 (2 m, 4H, CH_2CH_2Cl), 7.40–7.74 (m, 4H, Ar–H), 11.5 (br s, 1H, N*H*, exchanges with D_2O); IR (KBr) ν 3340 (NH), 1780, 1705 (C=O) cm^{-1}. Anal. ($C_{10}H_9N_2O_2Cl$) C, H, N.

Biological Assays. Materials and Reagents. Biochemicals, chemicals, drugs and the monoclonal antibody anti-β-tubulin (clone TUB 2.1) were obtained from Sigma Chemical (St. Louis, MO), proteinase K from Gibco BRL. The peroxidase conjugated anti-mouse immunoglobulin and ECL Western blotting detection reagent kit were purchased from Amersham Canada (Oakville, Canada). Mixtures are expressed as volume: volume ratios.

Cytotoxicity Assay. Cytotoxicity was assessed using the Alamar Blue assay as described by Lancaster et al.[19] Briefly, $(1-5) \times 10^3$ cells in 100 μL were seeded in 96-well plates and preincubated for 24 h. After addition of 100 μL fresh medium containing increasing concentration of the test drug, cells were incubated at 37 °C for 72 h. The culture medium was replaced by 50 μL of PBS containing 20% Alamar Blue in RPMI-1640. Cell survival was calculated from fluorescence (excitation, 485 nm; emission, 590 nm) measured with a FL 600 Reader (Bio-Tek Instruments). Cytotoxicity was expressed as the dose of drug required to inhibit cell growth by 50% (IC_{50}). Values are the means of at least three independent determinations.

Determination of Alkylating Activity. The alkylation rate of test drug was evaluated by a the 4-(4-nitrobenzyl)-

pyridine (NBP) colorimetric assay described by Bardos et al.[10] Briefly, 1 mL of a NBP 10% solution in ethanol and 1 mL of 50 mM sodium acetate (pH 4.3) were added to an ethanol solution (1 mL) containing 400 nmol mL^{-1} of either chlorambucil or test derivative and heated to 80 °C in a shaking water bath for 60, 90, 120 or 150 min. After cooling on ice for 5 min, and addition of 1.5 mL of 0.1 M KOH/ethanol (1:2), samples were vortexed for 12 s, set aside for 2.5 min, and the absorbance measured at 570 nm with a UV1601 spectrophotometer (Shimadzu). Blank samples without drug gave the background values. The absorbance readings were plotted against time and the linear regression of the respective curves generated for each drug gave directly the alkylation rate. Data are given as mean values ± SD from at least three independent experiments.

Glutathione Activity Assay. Determination of glutathione (GSH) activity was performed using the 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) as described by Tietze et al. with minor modifications.[18] The reaction mixture (200 μL) containing 500 μM GSH, 1 mM EDTA and 500 μM test compound (2% solution in ethanol) in 0.1 M phosphate buffer (pH 7.4) was incubated for 60 min at room temperature. Then, 750 μL of 0.1 M phosphate buffer containing 0.133 mM DTNB and 1 mM EDTA were added. The formation of the chromophoric product was determined by measuring the absorbance at 412 nm with a UV1601 spectrophotometer (Shimadzu). Each experiment was conducted in triplicate. Results are expressed as percent of control (no drug added).

Glutathione Reductase Activity Assay. Determination of glutathione reductase (GR) was performed as described by Ellman et al. with minor modifications.[20] Briefly, 800 μL of 0.1 M phosphate buffer (pH 7.4) containing 0.016 U mL^{-1} GR, 100 μM NADPH, 1 mM EDTA, 1 mg mL^{-1} BSA and 500 μM test compound (2% solution in ethanol) were incubated for 60 min at 30 °C. Then, 200 μL of glutathione oxidized form (GSSG) were added to the mixture and incubated for 60 min, at 30 °C. The GR activity was determined by measuring the decrease in absorbance at 412 nm over a 3-min period with a UV1601 spectrophotometer (Shimadzu). Each experiment was conducted in triplicate. Results are expressed as percent of control (no drug added).

Detection of DNA Damage. MDA-MB-231 growing cells were incubated at 37 °C for 2 h in growth medium supplemented or not with 400 μM test derivative. Cells were then washed with cold phosphate-buffered saline (PBS) before lysis in buffer containing 50 mM Tris-HCl (pH 7.4), 1% SDS, 20 mM EDTA, 10 mM deferoxamine mesylate and 0.5 mg mL^{-1} proteinase K. The DNA fraction was isolated by protein salting out as described by Miller et al. and 3′-end labeled by a nucleotide exchange reaction catalyzed by T4 DNA polymerase as described by Legault et al.[12,21] The incorporated radioactivity measured by dot filtration and converted into number of dCps exchanged/ng of DNA accounted for quantification of DNA damage.[12]

Analysis of Alkylated β-Tubulin. Exponentially growing MDA-MB-231 cells (~5 × 10^6) were incubated in the presence of 30 μM test compound, except for the control, for 24 h at 37 °C. Cells were washed with PBS and lysed in 500 mL Laemmli sample buffer 1X.[22] Samples were analyzed by SDS−PAGE and immunoassay. Briefly, samples (1 × 10^5 cells) were separated by 10% SDS−PAGE using the Laemmli system.[22] Membranes were incubated with PBSMT (PBS, pH 7.4, 5% fat-free dry milk and 0.1% Tween-20) for 1 h at room temperature, and then with 1:500 monoclonal anti-β-tubulin (clone TUB 2.1) for 1 h. Membranes were washed with PBSMT and incubated with 1:2000 peroxidase-conjugated anti-mouse immunoglobulin in PBSMT for 30 min. Detection of the immunoblot was carried out with the ECL Western blotting detection reagent kit. Optic densities of the normal and alkylated β-tubulin band were integrated using a NIH imager (Scion Corporation, Frederick, MD) and the percentage of alkylated β-tubulin was calculated. Data are representative of three separate experiments.

Immunofluorescence Microscopy. MDA-MB-231 cells were grown in 2-well Lab-Teck coverglass chambers and allowed to attach for 24 h prior to the addition of drug in appropriate concentration. Cells were washed once in PBS and fixed in a solution of PBS containing 4% paraformaldehyde for 30 min at room temperature. The fixed cells were rehydrated in PBS, washed 6 times with PBS and then incubated for 15 min in PBS containing 0.25% Triton X-100. The slides were incubated in a blocking solution of PBS containing 1% goat serum for 15 min at 4 °C. Cells were then incubated for 3 h at 4 °C with an anti-mouse β-tubulin monoclonal antibody (1:1.500) in PBS solution containing 1% goat serum and washed three times 10 min in blocking solution followed by an incubation for 2 h at room temperature with the anti-mouse IgG FITC conjugated antibody (1:200). The coverslips were mounted using PBS (pH 8.5) containing 10 mg mL^{-1} *p*-phenylenediamine. Cell nuclei were stained in parallel with 10 mM propidium iodide. The coverslips were mounted on slides and examined with a fluorescence microscope Axiophot (Zeiss).

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