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Lack of cross-resistance to a new cytotoxic arylchloroethyl urea in various drug-resistant tumor cells

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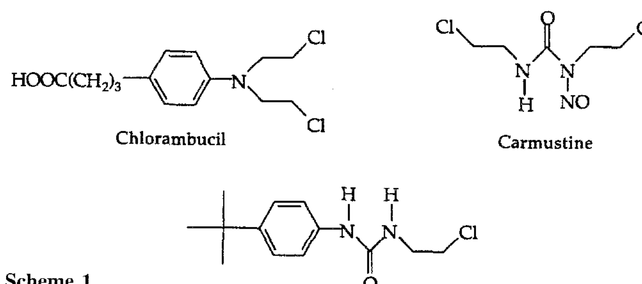
Abstract. 1-Aryl 3-(2-chloroethyl) ureas (CEUs), a new class of potent antineoplastic agents, were recently developed in our laboratory. These compounds were designed from the aromatic moiety of chlorambucil and the unnitrosated pharmacophore of carmustine. In the present study we investigated the effect of the potent CEU derivative 4-tert-butyl-[3-(2-chloroethyl)ureido] benzene (tBCEU) on tumor cell lines selected for resistance to a wide range of anticancer drugs. The resistance mechanisms found in these cells included increased expression of P-glycoprotein, increased intracellular concentration of glutathione and/or glutathione-S-transferase activity, alteration of topoisomerase II, and increased DNA repair. Whereas the resistant cell lines were found to be highly resistant to a panel of clinically known anticancer drugs, tBCEU was found to be equally cytotoxic to both resistant and parental cells. The nitrobenzylpyridine assay indicated that tBCEU is a weaker alkylating agent than chlorambucil. This lack of cross-resistance in various resistant tumor cells suggests that tBCEU could be potentially useful in the treatment of cancers resistant to conventional anticancer drugs.

Introduction

The development of tumor drug resistance has been well documented with the major classes of clinically available chemotherapeutic drugs, including anthracyclinediones, vinca alkaloids, alkylating agents, antimetabolites, and epipodophyllotoxins, as well as X-irradiation [15]. This phenomenon contributes significantly to treatment failure and relapses following initial responses to cancer chemotherapy. Strategies based on the combination of multiple drugs with different mechanisms of action have been limited since the development of a broad cross-resistance to structurally and functionally unrelated drugs is common both in experimental models and in the clinical setting. Sensitization of resistant cells using modulators directed toward the inhibition of a specific mechanism of resistance may be of limited success since accumulating evidence suggests that clinical resistance is a multifactorial process and rarely involves a single mechanism. The development of drugs with lower in vivo toxicity, a higher therapeutic index, and a lower capacity to induce a resistant phenotype would greatly improve anticancer therapy.

We have recently reported the synthesis and the biological activity of new antineoplastic agents derived from the aromatic moiety of chlorambucil (CLB) and the unnitrosated pharmacophore of carmustine (BCNU) [11]. Derivatives such as 4-tert-butyl-[3-(2-chloroethyl)ureido] benzene (tBCEU) (Scheme 1) have shown potent antitumor activity in vivo without producing significant side effects, particularly as compared with nitrosoureas or nitrogen mustards [7, 11]. In contrast to most alkylating agents, tBCEU is not mutagenic in the Ames test. Because drug resistance is a major limitation in cancer therapy, we report herein the cytotoxic effect of one of the most promising compounds we have synthesized, tBCEU, on a variety of drug-resistant human tumor cell lines deriving from lung, breast, ovary, and other animal tumor cell lines such as



Scheme 1

Abbreviations: ADR, Adriamycin; BCNU, carmustine; CDDP, cisplatin; CEUs, arylchloroethyl ureas; CLB, chlorambucil; MLN, melphalan; NBP, 4-(4-nitrobenzyl)pyridine; ND, not determined; tBCEU, 4-tert-butyl-[3-(2-chloroethyl) ureido] benzene; VBL, vinblastine; WT, wild type

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Table 1. Major characteristics of resistant cell lines used in the experiments

Cell line	Selective agents	Histological type	Major mechanism(s) associated with resistance	References
MCF-7-ADRr	ADR	Human breast carcinoma	P-glycoprotein-mediated decrease in drug accumulation	[6]
HT-69-ADRr	ADR	Human small-cell lung carcinoma	Unknown, but topoisomerase II is partially involved	[8, 13]
LR-73-mdr3	mdr-3[a] transfected	Chinese hamster ovary carcinoma	P-glycoprotein-mediated decrease in drug accumulation	[10]
MCF-7-MLN	MLN	Human breast carcinoma	Increased removal of druginduced DNA cross-links	[7]
MatB-MLN	MLN	Rat mammary adenocarcinoma	Increased GSH/GST	[9, 16]
A-2780-CDDPr	CDDP	Human ovarian carcinoma	Increased repair capacity	[5, 18]

[a] This cell line was transfected with an expression-vector DNA for the multidrug-resistance gene 3 (mdr3)

MatB rat mammary adenocarcinoma and ovary LR-73 hamster cells. A number of distinct mechanisms were found to be associated with resistance in these cells, including P-glycoprotein expression, alteration in glutathione metabolism and/or glutathione-S-transferase activity, alteration of topoisomerases, and increased DNA repair capacity.

Materials and methods

Drugs. tBCEU was prepared as described previously [7]. Adriamycin, vinblastine (VBL), melphalan (MLN), CLB, and BCNU were obtained from Sigma Chemical Company (St. Louis, Mo., USA). Cisplatin (CDDP) was obtained from David Bull Laboratories (Horner, Canada). tBCEU was dissolved in dimethylsulfoxide (DMSO). Melphalan was dissolved in 99% ethanol containing 4% 1 *M* HCl. The final concentration of organic solvent was less than or equal to 0.5% of medium (v/v). Adriamycin, colchicine, CDDP, VBL, and BCNU were dissolved in distilled water. All these drugs were prepared freshly prior to each experiment.

Cell lines and culture. A-2780 cells were established from an untreated patient with ovarian carcinoma. CDDP-resistant sublines (obtained from Dr. T. C. Hamilton, National Cancer Institute, Bethesda, Md.) were selected in vitro by a dose-escalation procedure [12, 16]. MCF-7 cells were established from an untreated patient with breast cancer. Adriamycin (ADR)- and MLN-resistant cell lines were established by continuous exposure to ADR and MLN, respectively, as described elsewhere [3, 4]. Both A-2780 and MCF-7 cells were cultured in RPMI-1640 supplemented with 2 m*M* glutamine, 10% fetal bovine serum (Gibco, Burlington, Ontario, Canada) and 100 U gentamycin/ml.

Human small-cell lung cancer HT-69 and its ADR-resistant subline (HT-69-ADRr) [9] (kindly provided by Dr. Susan P. C. Cole, Cancer Research Laboratories, Queen's University, Kingston, Ontario, Canada) were grown in RPMI-1640 medium supplemented with 5% heat-inactivated bovine serum (HyClone, Logan, Utah), 4 m*M* glutamine, and 100 U gentamycin/ml.

The parental and MLN-resistant rat mammary carcinoma (MatB) cell lines were selected as described previously [18]. Cells were maintained in monolayer culture in alpha-minimal essential medium (α-MEM, Gibco) supplemented with 1.3% sodium pyruvate, 1.3% nonessential amino acids, 10% fetal bovine serum, and 100 U gentamycin/ml.

Chinese hamster ovary LR-73 (WT) and LR-73-mdr3 cell lines were generously provided by Dr. P. Gros (Department of Biochemistry, McGill University, Montreal, Québec, Canada). LR-73-mdr3 overexpresses the mdr3 gene, which confers resistance to ADR, VBL, and colchicine [10]. These cells were grown in RPMI-1640 medium supplemented with 10% fetal calf serum, 2 m*M* glutamine, and 64 U gentamycin/ml. All cells were maintained in a humidified atmosphere containing 5% CO_2.

Cytotoxicity assay. Cytotoxicity was examined by [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium salt] (MTT) assay (HT-69, MatB, and A-2780 cells) or by clonogenic assay [4] on monolayers (MCF-7 cells). The MTT assay was carried out essentially as described elsewhere [6]. Briefly, $1-5\times10^3$ cells/100 µl were seeded in 96-well plates and preincubated for 18–24 h. Then, 100 µl fresh medium containing various concentrations of drug was added to the cultures. The cells were incubated with the drug for 72 h. Cell survival was evaluated by MTT by replacing the culture media with 20 µl of a solution containing 2.5 mg MTT/ml in PBS (pH 7.4):RPMI-1640 (1:1, v/v). After 4 h incubation at 37° C, 100 µl DMSO was added to dissolve the precipitate of reduced MTT. Plates were then shaken for 15 min and the absorbance was determined at 570 nm with a Behring Elisa Processor II (Behring, Marburg, Germany).

For clonogenic assay, 400 cells were plated on 6-well plates. After 20 h the cells were treated in complete RPMI-1640 medium with the selected drug for 1 week. Colonies were stained with 0.1% Coomassie blue, 50% methanol, and 10% acetic acid. The cloning efficiency was determined as the number of colonies formed divided by the initial number of cells seeded ×100 to obtain the percentage of colonies formed.

Kinetics of alkylation of 4-(4-nitrobenzyl)pyridine by CLB and tBCEU. The kinetics of alkylation of tBCEU was investigated by a colorimetric assay developed by Bardos et al. [2]. Briefly, 1 ml ethanol containing 400 nmol CLB or tBCEU, 1 ml of a 10% (v/v) solution of NBP in ethanol, 1 ml 50 m*M* acetate buffer (pH 4.3), and 1 ml ethanol were mixed together and kept on ice prior to the initiation of the reaction. The reaction was initiated by heating the solutions at 80° C in a shaking water bath. After various periods of incubation the reaction was stopped by cooling down the mixtures on ice for 5 min. Then, 1.5 ml 0.1 *M* KOH:ethanol (1:2, v/v), was added to the reaction mixture. Tubes were vortexed for 12 s and 2.5 min later the optical density was read at 570 nm. The values were compared with those obtained using a blank containing all the reagents except the alkylating agent. The constant of alkylation was evaluated by linear regression of the respective curves generated for each drug.

Results and discussion

To investigate the effectiveness of tBCEU in resistant cells, we screened a panel of resistant cell lines expressing the most common mechanisms associated with tumor resistance. These include (a) decreased cellular drug accumulation through the expression of membrane P-glycoproteins coded by mdr genes (i. e., MCF-7-ADRr, LR-73-mdr3); (b) other non-P-glycoprotein-mediated mechanisms, including alteration of topoisomerases II (HT-69-ADRr); (c) elevation of intracellular glutathione (GSH) and/or glutathione-S-transferase (GST) activity (MatB-MLNr); and (d) increased DNA repair capacity (A-2780-CDDPr, MCF-7-MLNr; Table 1).

Multidrug-resistant (MDR) cell lines (43- to 200-fold resistance to ADR and, usually, cross-resistance to ADR analogs, epipodophyllotoxins, vinca alkaloids, taxol, and mitoxantrone, among other agents) exhibit either increased

Table 2. Cytotoxicity of tBCEU in multidrug-resistant cells

Cell lines/drug	IC50[μM][a]	
	ADR	tBCEU
MCF-7-WT	0.02 ± 0.01	7.3 ± 0.9
MCF-7-ADRr	4.6 ± 0.6	5.3 ± 1.6
	(209)	(0.7)
HT 69-WT	0.08 ± 0.01	28.3 ± 5
HT 69-ADRr	5.7 ± 1.1	33 ± 3.2
	(68)	(1.2)
LR-73-WT	35 ± 4	1.5 ± 1.5
LR-73-MDR3	1500 ± 50	0.9 ± 1
	(43)	(0.6)

[a] Concentration inhibiting 50% of cell growth as determined graphically from the survival curves. Data represent mean values ± SD for at least 3 independent experiments. Given in parentheses are the factors of resistance that correspond to the ratio of the IC50 in resistant cells to that in parental sensitive cells

Table 3. Cytotoxicity of tBCEU in alkylating-drug-resistant cells

Cell lines/drug	IC50[μM][a]			
	CLB	MLN	CDDP	tBCEU
MCF-7-WT	ND	3.5 ± 0.6	1.3 ± 0.3	7.3 ± 0.9
MCF-7-MLNr	ND	12.8 ± 2.3	2.1 ± 0.3	6.5 ± 1.5
		(3.6)	(1.6)	(0.9)
MatB-WT	0.6 ± 0.1	0.3 ± 0.04	0.2 ± 0.02	7.6 ± 1.6
MatB-MLNr	38.5 ± 2.6	30.3 ± 34.1	17.5 ± 1.9	8.2 ± 1.6
	(61)	(98)	(80)	(1.1)
A 2780-WT	ND	ND	3.8 ± 0.9	6.3 ± 1.7
A 2780-CDDP	ND	ND	27.7 ± 5.7	7.3 ± 1.7
			(7.3)	(1.2)

[a] Concentration inhibiting 50% of cell growth as determined graphically from the survival curves. Data represent mean values ± SD for at least 3 independent experiments. Given in parentheses are the factors of resistance that correspond to the ratio of the IC50 in resistant cells to that in parental sensitive cells

expression of the mdr1 (MCF-7-ADRr) or mdr3 (LR-73-ADRr) genes encoding membrane-associated drug-efflux pump P-glycoprotein [3, 10] or other non-P-glycoprotein-mediated mechanisms (HT-69-ADRr) [9]. In addition to the mechanisms described above, MCF-7-ADRr cells have been found to have increased anionic GST and selenium-dependent GSH peroxidase [3]. Furthermore, a new transporter gene has been characterized in HT-69-ADRr cells that has been suggested to be involved in the intracellular distribution of ADR [8], although its role in resistance remains unclear. Alteration of topoisomerase II is also associated with MDR in HT-69ADRr cells [9]. Unlike drugs involved in the MDR phenotype, tBCEU was found to be equally active in both the resistant cells and their respective drug-unselected cells (Table 2). This indicates that the activity of tBCEU does not involve either a P-glycoprotein mechanism or MDR mechanisms not associated with P-glycoprotein such as those described in HT-69-ADRr cells.

Increased GSH levels and GST activity are important mechanisms of resistance to many alkylating drugs, including nitrogen mustards, nitrosoureas, and CDDP

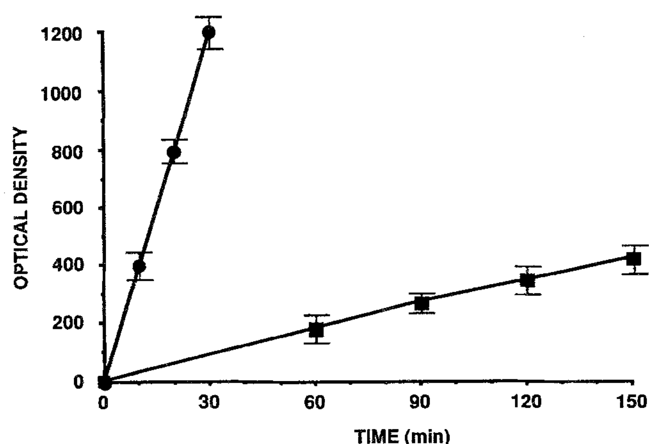

Fig. 1. relative alkylation of NBP by CBL and tBCEU. In all, 4 nmol drug was reacted with NBP at 80° C for various periods followed by alkalinization with a 1:2 (v/v) mixture of 0.1 *M* KOH:ethanol. The tubes were vortexed for 12 s and the optical density was read 2.5 min later at 570 nm. ■, tBCEU; ●, CLB. Each point represents the mean value for 3 separate measurements. The coefficients of correlation for these linear correlation curves were 0.997 and 0.990 for tBCEU and CLB, respectively

[19, 20]. GSTs are a family of detoxification enzymes that catalyze the conjugation of GSH to various reactive metabolites or to drug-DNA adducts. The resistant model (MatB-MLNr) used in this investigation involves both GSH- and GST-selected mechanisms [18]. MatB-MLNr cells are 98-fold resistant to MLN and are cross-resistant to other alkylating drugs as well as to X-irradiation [1, 18]. They exhibit 2-fold increases in intracellular GSH concentration, 4-fold increases in selenium-dependent GSH peroxidase activity, and about 5-fold increases in GST activity associated with the overexpression of Yc and Yp GST mRNAs. MatB-MLNr cells are highly resistant not only to MLN (98-fold), mechlorethamine (96-fold, not shown), CLB (61-fold), CDDP (80-fold), BCNU (50-fold; data not shown), and mitomycin C (9-fold; data not shown) but also to ADR and VBL (2-fold [18]). Interestingly, no cross-resistance was observed with tBCEU, which was found to be equally effective in both parental and drug-resistant cells (Table 3). This could imply that tBCEU is not deactivated by a GSH/GST-catalyzed conjugation reaction and is not a substrate for GST. A 25% decrease in MLN transport has also been found in MatB-MLNr cells [1] that contributes in part to the high resistance of these cells to MLN. MLN is carried by two separate amino-acid transporter systems: the sodium-dependent ASC-like (alanine-serine-cysteine) system and the sodium-independent L-system (leucine-preferring) [5]. The observed lack of resistance to tBCEU also suggests that this drug may pass through the cytoplasmic membrane by a mechanism different from the two amino-acid carrier systems, but this has to be confirmed.

Finally, no cross-resistance to tBCEU was detected in A-2780-CDDPr or MCF-7-MLNr cells (Table 3). In both of these cell lines, there is indirect evidence of enhanced DNA repair to prevent drug-induced DNA damage [4, 12, 14]. Although the mechanisms of repair of CDDP- and MLN-induced DNA damage remain unknown, the observed lack

of resistance to tBCEU suggests that this compound may act differently or may utilize a different repair pathway.

In a previous investigation [17], we have shown that tBCEU alters the synthesis of vimentin and tubulin in a human breast-cancer cell line (MDA-MB-231). These proteins are important in the organization of the cytoskeleton and in cell proliferation and differentiation [13]. The action of tBCEU might be mediated by an alteration of the cytoskeleton, for example, by modulating the polymerization or depolymerization of the microtubules in manners such as those described for a number of antimitotic agents such as vinca alcaloids, colchicine, podophyllotoxin derivatives, and taxol. However, unlike tBCEU, the activity of these drugs was found to be affected by the expression of P-glycoprotein.

The molecular structure of tBCEU is analogous to that of nitrogen mustards such as CLB [7]. It contains a 2-chloroethyl group, which is part of the pharmacophore moiety of nitrosoureas and nitrogen mustards such as BCNU and MLN, respectively [11]. From these similarities in structure, it would be tempting to suggest that tBCEU acts through similar alkylation and/or carbamoylation mechanisms that are responsible for the activity of many alkylating drugs. However, experiments using 4-(4-nitrobenzyl)pyridine (NBP) as a nucleophile demonstrated that tBCEU was a much weaker alkylating agent (by a factor of 13.9) than CLB (Fig. 1). In these experiments the rate constant of the alkylation of NBP by CLB and tBCEU was 38.9 and 2.8, respectively. This observation suggests that alkylation potency is not a prerequisite for the biological activity of tBCEU and may explain the absence of genotoxicity in bacteria and the high tolerance in vivo [7].

The cytotoxicity of tBCEU [7, 11] appears to be lower than that of ADR and VBL in tumor cells. However, the cytotoxic activity of tBCEU could be favorably compared with that of alkylating agents such as CLB. Furthermore, the drug has a low in vivo toxicity in both acute and chronic administration (unpublished results) that might be an indication of a high therapeutic index.

The present evidence that tBCEU is effective in a number of resistant tumor cells exhibiting the most common mechanisms of chemotherapy resistance adds to the attractiveness of this novel class of drugs for ultimate clinical development as well as for probing for new mechanisms of cellular and molecular pharmacology in cancer cells.

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References

1. Alaoui-Jamali MA, Panasci L, Centurioni MG, Shecter R, Lehnert S, Batist G (1992) Nitrogen mustard-DNA interaction in melphalan-resistant mammary carcinoma cells with elevated intracellular glutathione and glutathione-S-transferase activity. Cancer Chemother Pharmacol 30: 341
2. Bardos T, Datta-Gupta P, Hebborn P, Triggle DJ (1965) Study of comparison chemical and biological activities of alkylating agents. J Med Chem 8: 167
3. Batist G, Tulpule A, Sinha BK, Katki AG, Meyers CE, Cowan KH (1986) Overexpression of a novel anionic glutathione transferase in multidrug-resistant human breast cancer cells. J Biol Chem 261: 15544
4. Batist G, Torres-Garcia S, Deymuys JM, Greene D, Lehner S, Rochon M, Panasci L (1989) Enhanced DNA crosslink removal: the apparent mechanism of resistance in a clinically relevant melphalan-resistant human breast cancer cell line. Mol Pharmacol 36: 224
5. Begleiter A, Lam HY, Grover J, Froese E, Goldenberg GJ (1979) Evidence for active transport of melphalan by two amino acid carriers in L5178Y lymphoblasts in vitro. Cancer Res 39: 353
6. Carmichael J, De Graff WG, Gazdar AF, Minna ID, Mitchell JB (1987) Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. Cancer Res 47: 936
7. C-Gaudreault R, Lacroix J, Pagé M, Joly LP (1988) Synthesis and in vitro assay of 1-aryl 3-(2-chloroethyl) ureas as potential anticancer agents. J Pharm Sci 77: 185
8. Cole SPC, Bhardwaj G, Gerlach JH, Mackie JE, Grant CE, Almquist KC, Stewart AJ, Kurz EU, Duncan AMV, Deeley RG (1992) Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. Science 258: 1650
9. Cole SPC (1991) The 1991 Merck Froost award. Multidrug resistance in small cell lung cancer. Can J Physiol Pharmacol 70: 313
10. Gros P, Dhir R, Croop J, Talbot F (1991) A single amino acid substitution strongly modulates the activity and substrate specificity of the mouse mdr 1 and mdr 3 drug efflux pumps. Proc Natl Acad Sci USA 88: 7289
11. Lacroix J, C-Gaudreault R, Pagé M, Joly LP (1988) In vitro and in vivo activity of 1-aryl 3-(2-chloroethyl) urea derivatives as new antineoplastic agents. Anticancer Res 8: 595
12. Lai G, Ozols RF, Smyth JF, Young R, Hamilton TC (1988) Enhanced DNA repair and resistance to cisplatin in human ovarian cancer. Biochem Pharmacol 37: 4597
13. MacRae TH, Langdon CM (1989) Tubulin synthesis, structure, and function: what are the relationships? Biochem Cell Biol 67: 770
14. Matsuda H, Tanaka T, Kusaba I (1990) Increased removal of DNA-bound platinum in a human ovarian cell line resistant to *cis*-diammine-dichloroplatinum(II). Cancer Res 50: 1863
15. McClean S, Hill B (1992) An overview of membrane, cytosolic and nuclear proteins associated with the expression of resistance to multiple drugs in vitro. Biochim Biophys Acta 1114: 107
16. Hamilton TC, Young RC, Ozols RF (1984) Experimental model systems of ovarian cancer: applications to the design and evaluation of new treatment approaches. Semin Oncol 11: 285
17. Poyet P, Ritchot N, Béchard P, C-Gaudreault R (1993) Effect of an aryl chloroethyl urea on tubulin and vimentin syntheses in a human breast cancer cell line. Anticancer Res 13: 1447
18. Schecter RI, Woo A, Duong M, Batist G (1991) In vivo and in vitro mechanisms of drug resistance in a rat mammary carcinoma model. Cancer Res 51: 1434
19. Schecter R, Alaoui-Jamali M, Batist G (1992) Glutathione S-transferase in chemotherapy and carcinogenesis. Biochem Cell Biol 70: 349
20. Waxman D (1990) Glutathione S-transferases: role in alkylating agents resistance and possible target for modulation chemotherapy – a review. Cancer Res 50: 6449