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The effect of a novel taurine nitrosourea, 1-(2-chloroethyl)-3-[2-(dimethylaminosulfonyl)ethyl] -1-nitrosourea (TCNU) on cytotoxicity, DNA crosslinking and glutathione reductase in lung carcinoma cell lines*

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Summary. A novel nitrosourea, 1-(2-chloroethyl)-3-[2-(dimethylaminosulfonyl)ethyl]-1-nitrosourea (TCNU) has been investigated with respect to cytotoxic mechanisms in rat and human cell lines which either possess (Mer+) or lack (Mer−) 0^6-alkylguanine transferase activity. TCNU produced significantly greater cytotoxicity in the Mer− cells (Walker 256 rat breast carcinoma resistant to nitrogen mustards; human lung carcinoma A427) than in the Mer+ cells (Walker 256 wild-type; human lung carcinoma A594). This correlated with results generated by alkaline elution studies which showed that TCNU caused DNA interstrand crosslinks in A427 but not in A549 cells. Inhibition of glutathione reductase activity by TCNU demonstrated that in carbamoylating activity the drug was intermediate between chlorozotocin and 1,(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) in both A427 and A549 cells. These data suggest that the presence of taurine in the drug structure does little to alter the cytotoxicity or the alkylating or carbamoylating properties of TCNU, and that any clinical advantages with TCNU will be the consequence of other factors.

Introduction

Taurine (2-aminoethanesulfonic acid) is a ubiquitous sulfur-containing amino acid in mammalian tissues. Its physiological functions appear to be relatively diverse and contingent upon the organ location [16]. In addition, there are indications that taurine uptake may be through either active or passive mechanisms [13] and may be selective with respect to a range of different tumor cells [2, 3, 13, 18]. Because of the possibility of aberrant uptake and metabolism of taurine by tumor cells, a nitrogen mustard derivative of the amino acid, 2-[bis-(2-chloroethyl)-amino] ethanesulfonic acid or taumustine, has recently received attention [10]. Taumustine was synthesized over three decades ago and although little antitumor effect was reported at the time [7], recent studies have demonstrated marked activity in a number of murine tumors [10].

The utility of taurine as a carrier molecule has been extended by attaching a nitrosourea moiety using the constituent nitrogen of the amino group of taurine. The resultant compound, 1-(2-chloroethyl)-3[2-(dimethylaminosulfonyl)-ethyl]-1-nitrosourea, or TCNU, has advantages over many of the existing nitrosoureas in its increased water solubility. Preliminary clinical applications of this agent in Europe have suggested encouraging activity in a number of different disease states [12, 17]. These studies, which have included breast cancer, melanoma and ovarian carcinoma, have revealed both partial and complete remissions in pretreated and untreated lung cancer patients.

In this study we have tested the concept that TCNU acts on human lung carcinoma and rat mammary carcinoma cells as a chloroethylating agent analogous to other chloroethylnitrosoureas. We find that like chloroethylnitrosoureas, TCNU produces DNA interstrand crosslinks in a guanine 0^6-alkyltransferase-deficient (Mer−) human cell line but not in a proficient (Mer+) cell line, and selectively kills the Mer− cells. In addition, we have studied the effects of TCNU on glutathione reductase, an enzyme that has previously been suggested to be a critical target for carbamoylating chloroethylnitrosoureas [1, 15].

Materials and methods

Cell culture. Walker 256 rat breast carcinoma cell lines were maintained as previously described [14]. The parent cell line (WS) although sensitive to nitrogen mustards compared with the resistant line (WR) demonstrates collateral sensitivity to nitrosoureas [4, 14].

A427 and A549 human lung carcinoma cells have been maintained in this laboratory for several years. Stock cell cultures were grown at 37 °C as monolayers in 75-cm^2 tissue culture flasks in MEM. The medium was supplemented with the following components: 10% fetal bovine serum, gentamicin (0.05 mg/ml), glutamine (0.3 mg/ml), 0.1 m*M* nonessential amino acids, 1 m*M* sodium pyruvate and 0.02 *M* 4(2-hydroxyethyl)-piperazine ethane sulfonic acid.

For DNA alkaline elution studies 2.5×10^5 cells were seeded into 25-cm^2 flasks in 10 ml MEM and labeled for 24 h with 0.02 μCi/ml [^{14}C]thymidine (New England Nuclear; sp. act. 50 mCi/m*M*). The labeling period was followed by an 18-h incubation in fresh medium to allow for the incorporation of labeled thymidine into high-molecular-weight DNA.

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L1210 mouse leukemia cells were grown in suspension culture in RPMI 1640 medium supplemented with 15% heat-inactivated (60 °C, 45 min) fetal bovine serum. The DNA of L1210 cells was labeled by growing 3×10^5 cells/ml for 20 h in RPMI 1630 medium supplemented with 0.05 μCi/ml [^{3}H]thymidine (New England Nuclear; sp. act. 20 Ci/m*M*) and 10^{-6} *M* unlabeled thymidine.

Colony-forming assays. For A427 and A549 cell survival assays, cells were seeded at 1 and 5×10^3 cells per 25-cm^2 plastic flask. The flasks were incubated for 12–20 h to allow the cells to attach to the bottom of the flask. The cells were then exposed to the drug for 1 h. After 10–14 days of incubation in fresh medium the flasks were rinsed with Hanks, balanced salt solution, fixed with methanol and then stained with a solution containing 1 ml methylene blue, 1 ml 0.15 *M* Na_2HPO_4, and 1 ml 0.15 *M* KH_2PO_4 diluted to 50 ml with distilled water. Colonies were counted and the observed plating efficiencies were 45% for A549 cells and 14% for A427 cells.

Colony-forming assays using soft agar have been described previously for the Walker 256 cell lines [14]. Drug exposures were for 1 h, followed by replacement of the drug-containing media with fresh media.

Assay of DNA damage by alkaline elution. The basic principles involved in the detection of DNA damage by the alkaline elution assay have already been published, and the methodology has recently been reviewed in detail [8]. For analysis of strand breaks and DNA interstrand crosslinks, cells were lysed on 0.8 μ*M* pore size polycarbonate (Nucleopore) filters with 2% sodium dodecyl sulfate, 0.02 *M* EDTA, pH 10.0, and proteinase K (0.5 mg/ml), and were eluted at 2 ml/h with tetrapropylammonium hydroxide/EDTA (pH 12.1) containing 0.1% sodium dodecylsulfate. Cells for the interstrand crosslinking assay were irradiated with 300 rad ^{137}Cs gamma rays in the cold prior to lysis. Internal standards were [^{3}H]thymidine-labeled L1210 cells irradiated with 300 rad.

For assay of DNA-protein crosslinks, cells were irradiated with 300 rad ^{137}Cs gamma rays in the cold. Cells were lysed on 2-μm pore size polyvinylchloride filters with 2% sodium dodecylsulfate, 0.025 *M* EDTA, pH 10.0. The detergent was then washed away with 5 ml 0.02 *M* disodium EDTA, pH 10.0. Elution was with tetrapropyl ammonium hydroxide/EDTA pH 12.1. Internal standards were [^{3}H]thymidine-labeled L1210 cells irradiated with 300 rad.

Glutathione reductase assays. Glutathione reductase activity was measured by the method of Mize and Langdau [9]. Enzyme was prepared from either drug-treated or untreated log phase cells suspended in 0.1 *M* potassium phosphate (pH 7.6): 0.2 *M* KCl: 1m*M* KCl. This suspension was sonicated at a power setting of 60 on a sonic dismembrator (Artek, NY), using three 30-s exposures and centrifuged at 34000 rpm for 1 h. The crude enzyme was adjusted to 1.5 mg protein per ml prior to use. All data were expressed as percentages of untreated enzyme activity. Protein values were determined by the Bio-Rad protein assay (Bio-Rad, Richmond, Calif), with bovine γ-globulin as a standard.

Results

The cytotoxicity of TCNU was compared in both human and rat carcinoma cell lines which possessed either a Mer+ or Mer− phenotype. Figure 1 shows the response of WR (Mer−) and WS (Mer+) following a 1-h exposure to TCNU. The survival was significantly greater in the wild-type (WS) cells than in the WR. This result was consistent with the survival of the human Mer+ (A549) and Mer− (A427) cell lines (Fig. 2), where TCNU was preferentially cytotoxic towards the A427 cell line compared with the A549 cell line.

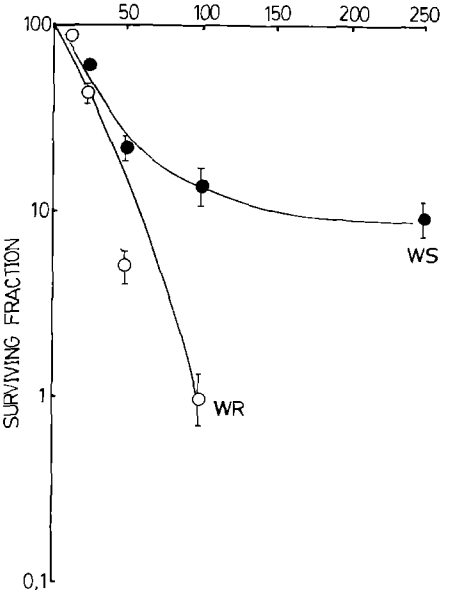

Fig. 1. Comparative survival of WS and WR cells following exposure to various concentrations of TCNU. Cells were exposed to the drug for 1 h prior to replacement of media. Data are means ±SD of three experiments. *WS*, Walker 256 wild-type; *WR*, Walker 256 nitrogen mustard-resistant

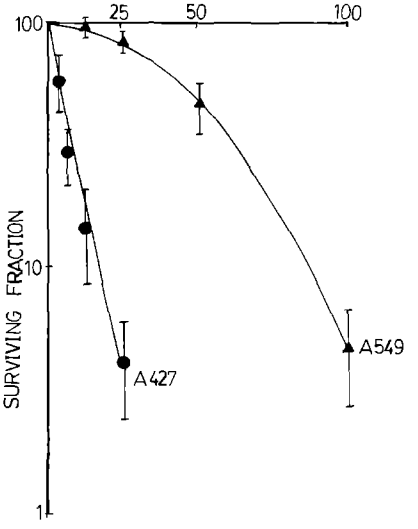

Fig. 2. Inhibition of colony-forming ability of A427 (●) and A549 (▲) cells by a 1-h treatment with various concentrations of TCNU

The comparative inhibition of glutathione reductase by TCNU is shown in Fig. 3. Inhibition of glutathione reductase by nitrosoureas has been shown to reflect their capacity to generate carbamoylating isocyanates [1, 15]. CCNU decomposes to form cyclohexyl isocyanate, which at con-

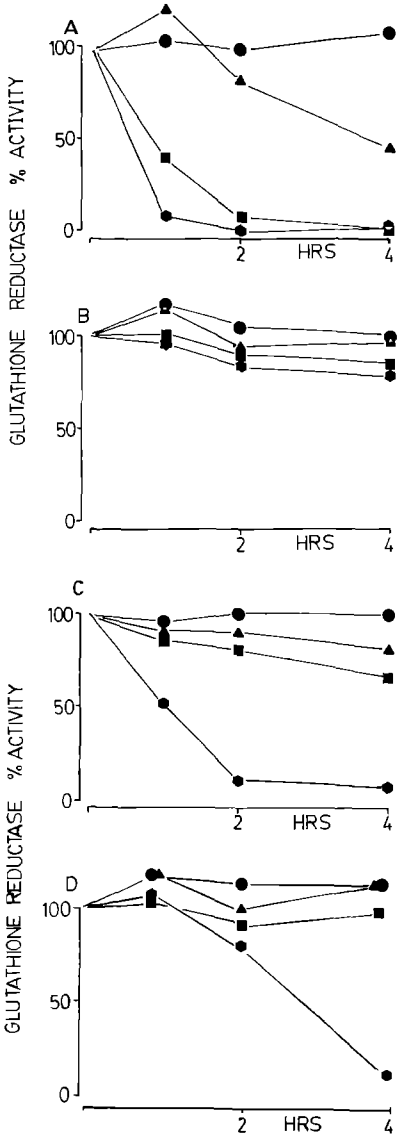

Fig. 3A–D. Inhibition of glutathione reductase by CCNU, CLZ, and TCNU. Treatment of A549 cells was for the times shown using four different drug concentrations: ● 100 μ*M*, ▲ 250 μ*M*, ■ 500 μ*M*, ◆ 1000 μ*M*.
Data are expressed as percentages of control glutathione reductase activity. For A549 this was 20.7, for A427 15.3 nmol NADPH oxidized/mg protein/min. **A** CCNU in A549 cells; **B** chlorozotocin in A549 cells; **C** TCNU in A549 cells; **D** TCNU in A427 cells

centrations of 250 μ*M* and above caused significant short-term inhibition of enzyme activity (Fig. 3A). Chlorozotocin (Fig. 3B), because of intramolecular considerations, is not an effective carbamoylating agent [6] and demonstrated little or no inhibition of glutathione reductase activity. Figure 3C shows that TCNU has activity which is intermediary to that of CCNU and chlorozotocin. Additionally, there is little difference between the effects of TCNU on A549 and on A427 cells (Fig. 3D) in terms of degree of inhibition. However, approximately 90% inhibition occurred by 2 h in A549, as against 4 h for A427.

DNA interstrand crosslinking by TCNU in A549 and A427 cells was examined by the alkaline elution technique. TCNU produced DNA interstrand crosslinks in A427 cells but not in A549 cells, and the peak of crosslink formation was delayed 12 h following drug treatment (Fig. 4). There was evidence of removal of some of the interstrand crosslinks between 12 and 24 h after treatment with TCNU in A427 cells. The formation of interstrand crosslinks by TCNU in A427 cells, measured at the time of maximum crosslinking, was proportional to drug concentration. In contrast, there were no detectable interstrand crosslinks at any time or any concentration in A549 cells. The increased rates of elution of drug-treated A549 cells relative to 300 rad-treated A549 cells was attributable to the formation of DNA strand breaks in this cell line.

The ability of TCNU to induce single-strand breaks in both cell lines can be seen in Table 1. The fraction of DNA retained on the filter is inversely related to the frequency of single-strand breaks. Initially a large quantity of breaks was apparent in the resistant A549 cell line, but after 6–24 h incubation in drug-free medium the majority of these breaks had been repaired. That is, immediately after a 200-μ*M* drug treatment 54.5% of the DNA of A549 cells remained on the filter, whereas 18 h after the same drug treatment 84.5% of the DNA remained on the filter (Table 1). There is little evidence of any significant strand breakage in the A427 cells at any time after drug treat-

Table 1. Fraction of DNA retained on filter after a 1-h exposure of TCNU in A427 and A549 human lung carcinoma cells

Time after drug treatment (h)	Concentration μM	A427[a]	A549[a]
0	50	0.955	0.905
	100	0.930	0.740
	200	0.815	0.545
6	50	0.980	0.930
	100	0.905	0.885
	200	0.795	0.835
12	50	0.895	0.950
	100	0.850	0.905
	200	0.785	0.840
18	50	0.975	0.915
	100	0.980	0.890
	200	0.940	0.845
24	50	0.970	0.945
	100	0.965	0.895
	200	0.940	0.795

[a] Fraction of DNA retained on filter is inversely related to frequency of strand breaks

Table 2. DNA-protein crosslinking index in A427 and A549 human lung carcinoma cells after a 1-h exposure (×300 for rad equivalents)

Time after drug treatment (h)	Concentration μM	A427		A549	
0	50	0.064	0.024	0.098	0.101
	100	0.084	0.059	0.162	0.138
	200	0.149	0.171	0.302	0.287
6	50	0.140	0.097	0.156	0.171
	100	0.286	0.145	0.354	0.367
	200	0.519	0.402	0.597	0.549

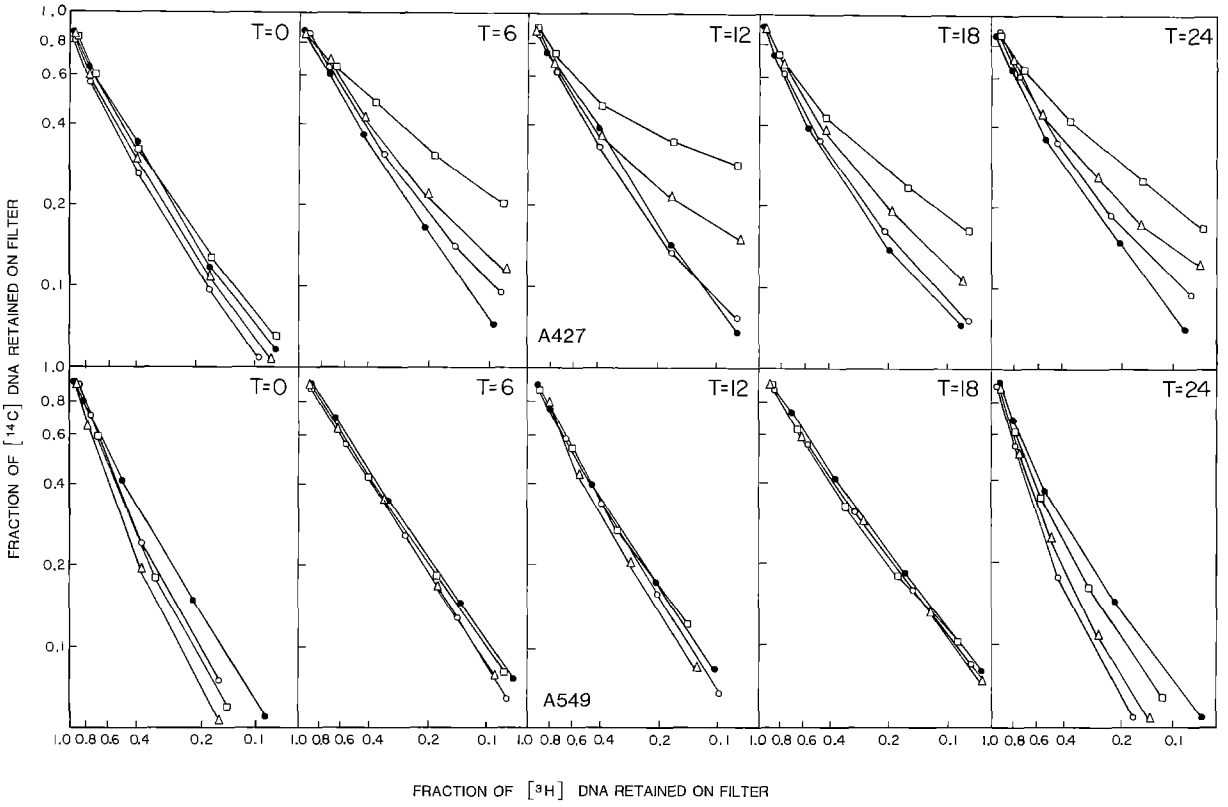

Fig. 4. DNA interstrand crosslinking in A427 *(upper panels)* and A549 *(lower panels)* cells treated with TCNU; ●, 300 rad control; ○, 50 μ*M*; △, 100 μ*M*; □ 200 μ*M*. After a 1-h treatment cells were incubated in drug-free medium for the times indicated

ment. At most times after drug treatment 80%–95% of the DNA of A427 cells remained on the filter.

One possible explanation for the differential in both cytotoxicity and DNA interstrand crosslink formation between A427 and A549 cells would be that drug uptake is different in the two cell lines. Table 2 shows that the formation of DNA-protein crosslinks (in rad equivalents) is of a slightly higher level in the resistant A549 cells than in the A427 cells. For instance, 6 h after a 100-μ*M* drug treatment there was an average DNA-protein crosslink index of 0.215 (64.6 rad equivalents) in A427 cells and 0.36 (108.2 rad equivalents) in A549 cells.

Discussion

TCNU appears to possess pharmacological properties which are similar to other chloroethylnitrosoureas. Spontaneous decomposition of nitrosoureas is known to produce alkylating electrophilic species and carbamoylating isocyanates. The production of DNA damage and inhibition of critical cellular enzymes such as glutathione reductase are critical factors in determining drug efficacy. Previous studies with the Walker 256 rat breast carcinoma have shown that the WS line has twice the glutathione reductase activity found in the selected nitrogen mustard-resistant line [15]. In addition, WS cells are Mer+, while the resistant cells are Mer− [4]. Survival data recorded with TCNU show that the WS express a degree of resistance compared with WR and that the differential between the two is more pronounced at higher drug concentrations. This may reflect the importance of the elevated intracellular glutathione reductase and guanine-O^6-alkyltransferase.

Since preliminary data suggest that TCNU has a marked activity in a number of lung tumors [17], the drug's effects in two human lung carcinoma cell lines was compared. Inhibition of glutathione reductase by TCNU was compared with either CCNU or chlorozotocin to estimate the relative carbamoylating activity. Chlorozotocin is known to have limited carbamoylating potential because of the intramolecular reactivity [6]. CCNU is an effective carbamoylator, as evidenced by the considerable reductase inhibition. TCNU is intermediate. In terms of the kinetics of inhibition, although both A549 and A427 cells sustained a 90% inhibition of reductase activity, the inhibition occurred more rapidly in the former. This may reflect differences in the maintenance of enzyme levels within the two cell lines or differences in competitive intracellular nucleophilic targets for isocyanate attack. Glutathione reductase is especially crucial in the lung, because the pulmonary toxicity of some nitrosoureas is believed to be a function of the inhibition of glutathione reductase [11] and subsequent tissue damage by oxidative stress, through a depleted ability to reduce oxidized glutathione. These data would suggest that on a mole for mole basis TCNU is less likely than CCNU to cause pulmonary metabolic disruption. It remains possible that lung tumors have modified glutathione reductase activities compared with normal lung tissue, but it is likely that any therapeutic advantage for TCNU will not be a function of glutathione reductase inhibition.

As part of this study we also wished to determine whether TCNU affected cellular DNA in the manner expected for chloroethylation of guanine O^6-position within DNA. The answer to this question was clearly affirmative,

as indicated by the following observations: (a) TCNU produced DNA interstrand crosslinks in a cell line (A427) deficient in guanine-0^6-alkyltransferase activity, but not in a cell line (A549) having a high level of this activity. The guanine-0^6-alkyltransferase deficiency would be expected to impair the removal of chloroethyl groups from guanine 0^6-positions in A427 cells, and this results in an increased cytotoxic action against these cells. (b) DNA-protein crosslinks were produced in both cell types, with a slightly higher level of formation in the resistant A549 cells. Whether this increased level of DNA-protein crosslinking in the resistant cell line is a form of detoxification is unclear at this time. (c) DNA strand breaks were produced immediately after drug treatment in A549 cells and appeared to undergo some form of repair upon incubation in drug-free medium. Little strand breakage was observed in A427 cells. These results are in close agreement with those observed for chloroethylnitrosoureas against Mer+ and Mer− cell lines [5].

In conclusion, the therapeutic efficacy of TCNU in human lung cancer in comparison to other chloroethylnitrosoureas [17] does not appear to be related to any alteration in its reactivity to DNA. In addition, the limited inhibition of glutathione reductase relative to CCNU may reduce the pulmonary toxicities usually seen with carbamoylating nitrosoureas. It may be that the long plasma half-life of TCNU in humans [12], as well as other altered pharmacokinetic parameters relative to the previously used chloroethylnitrosoureas, determines the therapeutic effectiveness of this class of compound.

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