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Effect of nitrosoureas on calmodulin activity in vitro and in mouse intestine in vivo

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Summary. *The effects of BCNU, CCNU, methyl-CCNU, streptozotocin, and chlorozotocin on calmodulin activity were studied in vitro and in vivo. Preincubation of BCNU, CCNU, and methyl-CCNU with calmodulin produced a concentration-dependent inhibition of in vitro calmodulin activity expressed as stimulation of cyclic nucleotide phosphodiesterase. Cyclohexylisocyanate produced a similar inhibition. Streptozotocin and chlorozotocin had no effect. Calmodulin inhibition by methyl-CCNU was dependent on the concentration of calcium in the preincubation mixture. Administration of methyl-CCNU or chlorozotocin IP to CF_1 mice produced a dose-dependent inhibition of calmodulin activity in the small intestine. Methyl-CCNU produced a significant decrease in intestinal calmodulin activity as early as 1 h after treatment, an effect that persisted up to 52 h. Morphologic changes in the intestinal crypt eptihelial cells were evident between 27 h and 5 days after treatment, but not earlier than 27 h. Renal and testicular calmodulin activity and morphology were unaffected. Although it was not possible to correlate the extent of calmodulin inhibition with severity of the intestinal lesions, the data suggested a relationship between reduced activity of calmodulin in a tissue and the ultimate appearance of lesions. This apparent interaction between an antitumor drug and calmodulin in vivo could have multiple implications for cancer chemotherapy.*

Introduction

The clinical use of antitumor drugs is limited by a wide spectrum of toxicities, including gastrointestinal epithelial cell lesions and bone marrow destruction. Effective design of new antitumor drugs must be based on known or potential mechanisms of toxicity and of efficacy. The nitrosoureas, which are reactive antitumor drugs, alkylate DNA as their putative mechanism of action [15]. Several drugs in this class also possess the ability to carbamoylate cellular proteins [24]. Because carbamoylation is thought to be unrelated to antitumor efficacy, a reasonable concern exists over unnecessary side-effects produced clinically by this action [8].

Several antitumor drugs [12, 20, 22] have been reported to interact in vitro with calmodulin, a ubiquitous calcium receptor and regulatory protein [9]. Preliminary studies [10] suggested that nitrosoureas might also interact with calmodulin. In the studies reported here, BCNU, CCNU, and methyl-CCNU, which have both carbamoylating and alkylating activities, were compared with streptozotocin and chlorozotocin, which have only alkylating activity, for their degree of inhibition of calmodulin-stimulated phosphodiesterase activity in vitro. For in vivo evaluation, mouse duodenum was selected as a known target of nitrosourea toxicity. Active calmodulin levels were determined following IP administration of methyl-CCNU and chlorozotocin to mice. The results suggest that calmodulin may be a biochemical target of nitrosourea toxicity.

Materials and methods

For in vitro studies, calmodulin and phosphodiesterase were isolated from fresh bovine brain [21]. To measure the effect of nitrosoureas on calcium-dependent calmodulin-stimulated phosphodiesterase activity, the procedure described by Wallace et al. [19] was altered to include a 45-min preincubation at 30° C to allow the reactive nitrosoureas to degrade in the presence of calmodulin. The preincubation mixture contained 880 ng calmodulin, 80 m*M* Tris (pH 8.0), 3 m*M* $MgSO_4$, and 50 μM $CaCl_2$. The remaining assay reagents were added following the preincubation, as described by Wallace et al. [19]. BCNU, CCNU, and methyl-CCNU were dissolved in ethanol and added to the preincubation mixture in a minimum volume. Chlorozotocin and streptozotocin were dissolved in 5 m*M* citrate buffer (pH 5.0). All experiments were carried out in duplicate. Antitumor drugs were provided by the Drug Synthesis and Chemistry Branch, National Cancer Institute, Bethesda, Md. One sample of methyl-CCNU was generously provided by Dr John A. Montgomery, Southern Research Institute, Birmingham, Ala. Cyclohexylisocyanate was obtained from the Aldrich Chemical Co., Milwaukee, Wisc.

The in vivo experiments were performed in young adult male CF_1 mice purchased from Harlan Industries Inc., Indianapolis, Ind. Mice were placed in polycarbonate cages with hardwood bedding and were provided with Purina rodent chow and tap water ad libitum. All drug treatments were on the basis of individual body weights. Methyl-CCNU was diluted in a minimum volume of ethanol and homogenized in 1% cremophor (Sigma Chemical Co.) in 0.85% sodium chloride solution (Celline, Fisher Chemical Co.). Chlorozotocin was diluted for injection in 5 m*M* citrate buffer (pH 5.0). At selected times following an IP injection of a nitrosourea, mice were killed by cervical dislocation, and approximately

Abbreviations: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; methyl-CCNU, 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea

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2 in. of small intestine proximal to the stomach was removed, washed in ice-cold 20 m*M* Tris buffer (pH 8.0), and frozen at −18° C. Kidneys and testes were removed and treated similarly.

To measure active calmodulin in these tissues, the samples were thawed, weighed, and placed in two volumes of 50 m*M* Tris (pH 8.0) containing 3 m*M* $MgSO_4$ and 1 m*M* EGTA. Tissues were homogenized by 12 passes in a teflon-glass grinder using a Sorvall Omni-mixer at a setting of 4. The remainder of the calmodulin isolation followed the procedure outlined in a report by Wallace et al. [21]. The heat-stable supernatant derived from this procedure was used as a calmodulin source in the phosphodiesterase stimulation assay cited previously. Protein concentrations were determined by the method of Schacterle and Pollack [13].

Tissue samples to be evaluated histopathologically were placed in 10% formalin buffered in 0.1 *M* phosphate (pH 7.0). Duodenum was identified as the proximal 1-cm segment of intestine attached to the stomach. Samples were embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin.

Statistical analyses were used to demonstrate differences between control and treated levels of active calmodulin in vivo and for analysis of calcium dependency data. Student's *t*-test and linear regression analysis were performed as indicated [14].

Results

The ability of nitrosoureas to inhibit calcium-dependent calmodulin-stimulated phosphodiesterase activity in vitro is summarized in Fig. 1. BCNU, methyl-CCNU and streptozotocin, and CCNU and chlorozotocin were preincubated with calmodulin at concentrations ranging from 10^{-8} *M* to 10^{-4} *M*. Control values were determined in the absence of nitrosourea. All in vitro data points are the average of duplicate experiments. The drugs with greater carbamoylating activity, CCNU and methyl-CCNU, were more effective calmodulin inhibitors than BCNU, which produces a less-reactive isocyanate [8]. Chlorozotocin and streptozotocin, which lack carbamoylating activity [2], were unable to inhibit calmodulin-stimulated phosphodiesterase activity. Cyclohexylisocyanate, a carbamoylating breakdown product of CCNU, was also an effective calmodulin inhibitor (Fig. 1D).

Additional in vitro experiments suggested that the inhibition of calmodulin activity by methyl-CCNU was partially calcium-dependent. Using calcium-free calmodulin preincubated with 0.2 m*M* methyl-CCNU for 45 min at 30° C, calmodulin activity (nanomoles of cyclic AMP hydrolyzed per minute and per milligram of phosphodiesterase) was 839 ± 19, compared with 416 ± 51 ($P < 0.005$) when calcium was added to the preincubation mixture. This is approximately a 2-fold greater inhibition of calmodulin in the presence of calcium. At 0.02 m*M* methyl-CCNU in the preincubation mixture, the calmodulin activity was 1,174 ± 21 for calcium-free calmodulin, compared with 944 ± 49 ($P < 0.025$) for calcium-replete calmodulin.

Figure 2 shows the effect on intestinal calmodulin activity of a 100 mg/kg dose of methyl-CCNU injected IP into male CF_1 mice. The LD_{10} of methyl-CCNU is about 33 mg/kg. At all times studied, ranging from 1 to 52 h after treatment, a significant ($P < 0.01$) decrease in active levels of intestinal calmodulin was observed. Histopathologic evaluation of intestinal tissue from mice treated similarly demonstrated

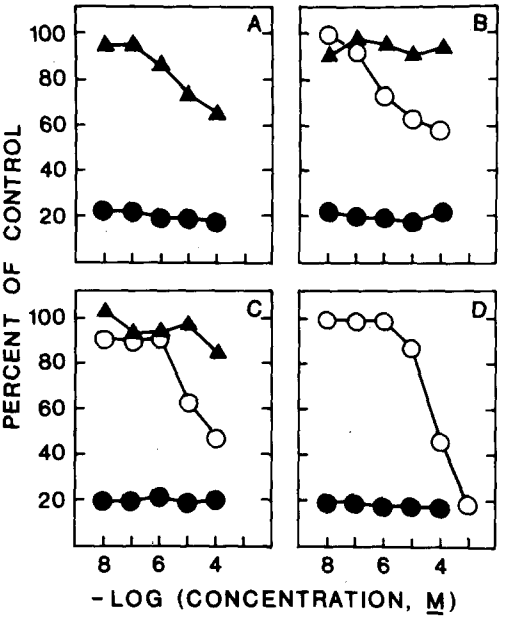

Fig. 1A–D. Effects of selected nitrosoureas and cyclohexylisocyanate on calcium-dependent calmodulin-stimulated phosphodiesterase activity in vitro. **A** BCNU; **B** streptozotocin (▲) and methyl-CCNU (○); **C** chlorozotocin (▲) and CCNU (○); **D** cyclohexylisocyanate. *Closed circles* (●) at the bottom of each graph represent the effect of the nitrosoureas on basal levels of phosphodiesterase activity (no calmodulin added). Control values were determined with no nitrosourea added

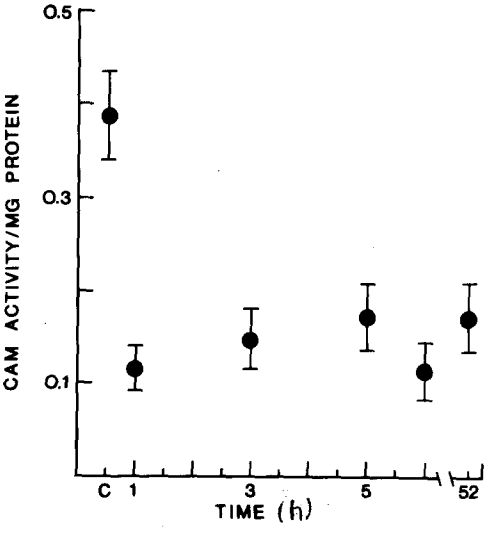

Fig. 2. Effects of a single IP dose (100 mg/kg) of methyl-CCNU on intestinal calmodulin activity at selected times after treatment in CF_1 mice. CAM activity refers to nanomoles of cyclic AMP hydrolyzed per minute and per milligram of phosphodiesterase. Protein refers to heat-stable protein extracted from samples of intestine. Data points are means ± SE. All posttreatment values were significantly different from control (*C*) values ($P < 0.05$)

severe degeneration of the intestinal crypt epithelial cells on day 5 after treatment. No lesions were evident in any samples earlier than 27 h after treatment. Calmodulin activity was not measured at times later than 52 h because of the concern that cell loss would confuse interpretation of whole-tissue calmodulin data. The dose-response relationship between methyl-CCNU injected IP and the level of active intestinal

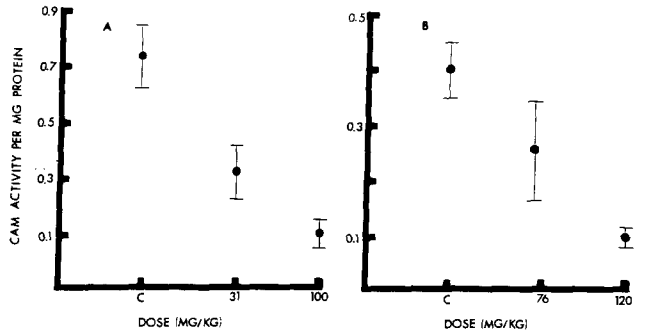

Fig. 3A, B. Dose-response of methyl-CCNU (**A**) and chlorozotocin (**B**) inhibition of intestinal calmodulin activity in CF_1 mice. All determinations were made at 3 h after treatment. CAM activity refers to nmoles of cyclic-AMP hydrolyzed per minute and per milligram of phosphodiesterase. Protein refers to heat-stable protein extracted from samples of intestine. Data points are means ± SE. Control *(C)* mice were treated with diluent. For methyl-CCNU (**A**), linear regression analysis demonstrated a negative correlation between dose and degree of calmodulin inhibition ($r = -0.67$, $P < 0.01$). Linear regression analysis of the chlorozotocin data (**B**) demonstrated a negative correlation between dose and degree of calmodulin inhibition ($r = -0.74$, $P < 0.01$)

calmodulin measured at 3 h after treatment is shown in Fig. 3A. These data are compared with results obtained in concurrent diluent-treated controls, and they represent several pooled experiments. At a dose of 31 mg/kg methyl-CCNU, there were no morphologic changes in the intestinal epithelium.

Chlorozotocin, a noncarbamoylating nitrosourea, was also studied in vivo for comparison of its effect on calmodulin activity at approximately equitoxic (LD_{10} = 25 mg/kg) and equimolar doses with respect to 100 mg/kg methyl-CCNU. As shown in Fig. 3B, 120 mg/kg chlorozotocin, the equimolar dose, produced an inhibition of intestinal calmodulin activity resembling that produced by 100 mg/kg methyl-CCNU. The approximately equitoxic dose of 76 mg/kg chlorozotocin yielded a moderate and variable inhibition of intestinal calmodulin activity. Mice treated with 120 mg/kg chlorozotocin did not survive long enough for intestinal lesions to become evident microscopically. The 76-mg/kg dose, however, did produce intestinal crypt cell lesions of variable severity by day 5.

Other tissues evaluated for alterations in the levels of active calmodulin included the testes and the kidney of CF_1 mice treated with 100 mg/kg methyl-CCNU IP. In the testes, a nontarget tissue, there was no decrease in the levels of active calmodulin, and no lesions were evident on histopathologic evaluation. The kidney, a target organ of methyl-CCNU toxicity in the BDF_1 mouse [5], failed to yield consistent histopathologic evidence of necrosis following 100 mg/kg methyl-CCNU in CF_1 mice. Similar strain-related differences in toxicity are known, and therefore this observation was not surprising. There was no depression in renal calmodulin activity at any time point studied following treatment of CF_1 mice with 100 mg/kg methyl-CCNU.

Discussion

The studies reported here indicate that selected nitrosoureas are capable of inhibiting calmodulin activity in vitro and suggest that carbamoylation may be a prominent feature of the apparent nitrosourea-calmodulin interaction. CCNU and methyl-CCNU both possess significant carbamoylating activity [24], and both were effective calmodulin inhibitors. BCNU, which releases a less reactive chloroethylisocyanate, did not inhibit calmodulin activity to the extent that CCNU or methyl-CCNU did. Chlorozotocin and streptozotocin, both of which lack carbamoylating activity, demonstrated no ability to inhibit calmodulin activity. Cyclohexylisocyanate, a product of CCNU degradation [4], was also an active calmodulin inhibitor.

Calmodulin inhibition by methyl-CCNU in vitro seemed to be calcium-dependent. The inhibiton of calmodulin by vincristine [22] and phenothiazines [23] is also calcium-dependent. The reduced ability of methyl-CCNU to inhibit calmodulin under calcium-free conditions could be due to nonspecific carbamoylation and/or alkylation of less critical sites exposed when calmodulin is in its calcium-free conformation. When calmodulin binds calcium, a conformational change necessary for activity occurs in the protein molecule [17], increasing the α-helical content and exposing hydrophobic sites that may be more compatible for binding the hydrophobic nitrosoureas. The hydrophobic nature of these binding sites suggests an explanation for the lack of inhibitory activity of chlorozotocin and streptozotocin in vitro. These two nitrosoureas are relatively polar and hydrophilic. The observation that chlorozotocin inhibited intestinal calmodulin activity in vivo suggests that additional factors that influence the apparent chlorozotocin-calmodulin interaction were operative in vivo and that calmodulin inhibition may be more complex than carbamoylation. Experiments to identify the site of derivatization by methyl-CCNU indicate that one or more lysine residues are modified in vitro. These experiments should ultimately permit elucidation of the structure of the adduct.

The time-course of methyl-CCNU-induced intestinal calmodulin inhibition showed a significant and consistent depression in calmodulin activity at time points studied up to 52 h after treatment. At times earlier than 27 h, there were no epithelial cell lesions associated with the decreased levels of calmodulin activity. This observation suggested that impairment of calmodulin function preceded cell death and the development of intestinal lesions.

There was a dose-dependence between the degree of inhibition of calmodulin and the amount of chlorozotocin or methyl-CCNU that the mice received. It was not possible to correlate lesion severity with degree of inhibition of calmodulin for either drug. In the case of chlorozotocin, although 120 mg/kg yielded a consistent depression in calmodulin activity, no intestinal lesions developed because the mice died within 2 days, insufficient time for necrosis to become evident. For methyl-CCNU, 31 mg/kg produced a variable inhibition of calmodulin activity, and microscopic evidence of lesions was variable and difficult to quantitate, even on day 5 after treatment. The apparent dependence of this relationship on lethal doses leaves open the possibility that the nitrosourea-calmodulin interaction in vivo may be nonspecific. Studies in progress with radiolabeled nitrosoureas are designed to clarify the relationship between calmodulin inhibition and derivatization in vivo.

The gastrointestinal epithelium is a rapidly dividing tissue with a turnover time ranging from 2–4 days [11]. Because dividing cells require elevated concentrations of active calmodulin for successful cell division [1, 7], a greater proportion of the calmodulin present in intestinal epithelium would be in the

calcium-bound, active conformation. This could render it more susceptible to interaction with methyl-CCNU, as already discussed. Testes and kidney tissues showed no evidence of calmodulin inhibition, perhaps because they are not as rapidly dividing cell populations as the intestinal epithelium. Thus, a lower proportion of the intracellular calmodulin of testes and kidney would be in the active, calcium-bound conformation, reducing its vulnerability to attack by methyl-CCNU, and providing a rationalization for the observed tissue specificity of calmodulin inhibition.

Tumor cells may be expected to have higher levels of intracellular calmodulin than their normal counterparts [3, 18]. Moreover, recent work has suggested that tumor cell resistance may, in part for certain drugs, be due to the presence of calcium- or calmodulin-dependent drug efflux pumps [6, 16]. Taken together, the available evidence seems to suggest that interaction of an antitumor drug with cellular calmodulin may contribute mechanistically to drug-induced cytotoxicity. The pharmacologic, toxicologic, and therapeutic implications of the present observations, particularly with regard to new rationales for combination chemotherapy of drug-resistant tumor cell populations, warrant additional investigation.

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