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Biochemical Pharmacology, Vol. 29, pp. 652–654.

0006-2952/80/0215–0652 $02.00/0

Inhibition of tubulin polymerization by nitrosourea-derived isocyanates

(*Received* 30 *June* 1979; *accepted* 12 *September* 1979)

Antitumor active chloroethylnitrosoureas are chemically unstable and upon degradation yield reactive intermediates capable of alkylation by the 2-chloroethyl moiety and of carbamylation by the isocyanate moiety [1]. Alkylation of certain proteins by bis-1,3-(2-chloroethyl)-1-nitrosourea (BCNU) or 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) has been shown to occur non-selectively, compared to highly specific carbamylation by an active site-directed mechanism [2,3].

Preliminary studies in this laboratory have shown that treatment of L5178Y cells with certain 2-chloroethyl-nitrosoureas during growth in culture resulted in a decreased mitotic index. We have explored the question of whether drug-microtubule interactions may participate to some extent in antitumor activity. Several 2-chloroethyl-nitrosoureas that yield varying amounts of isocyanate upon degradation were studied for their effects upon the polymerization of isolated tubulin. Inhibition of polymerization was observed, and the stoichiometry of the inhibition was related to the nitrosourea-derived isocyanates.

Tubulin was isolated from freshly slaughtered cow or pig brain, using 0.1 M Pipes buffer (pH 6.9) and 1 mM guanosine triphosphate, according to the procedure of Borisy *et al.* [4]. Two polymerization cycles were performed, with the final pellet stored at −80° and used within 1 week. All protein concentrations were determined using the assay of Lowry *et al.* [5]. CCNU, BCNU, methyl CCNU (MeCCNU), chlorozotocin (2-[3-(2-chloroethyl)-3-nitrosoureido]-D-glucopyranose) (all supplied by the National Cancer Institute, Bethesda, MD), cyclohexyl isocyanate (CHI, supplied by the Eastern Kodak Co., Rochester, NY), *cis*-2-hydroxy CCNU (*cis*-2-OH CCNU) [6], and *trans*-4-hydroxy CCNU (*trans*-4-OH CCNU) [6] were freshly dissolved in acetone before adding to the tubulin. Acetone only was added to tubulin solutions for control experiments.

Thawed tubulin was depolymerized in 0.1 M Pipes + 1 mM EGTA [ethylene glycol-bis-(β-aminoethyl ether) *N,N'* tetra acetic acid] + 5×10^{-4} M $MgCl_2$ (pH 6.9). After centrifuging at 100,000 *g*, 4°, for 30 min, the pellet was discarded and GTP (10^{-3} M) was added to the supernatant fraction containing 2 or 3 mg/ml tubulin. The tubulin was then incubated at 15° for 2 hr with or without nitrosourea. Following this incubation, the method of Gaskin *et al.* [7] was used to monitor the extent of tubulin polymerization. By this procedure, the increase in turbidity at 350 nm was measured at 37°, using a Gilford model 220 spectrophotometer equipped with a thermostated sample changer and a chart recorder. The effect of CHI on tubulin polymerization was measured by the above method after incubation with the drug for 5 min at room temperature.

For electron microscopy, samples were removed from the polymerizing tubulin preparations. A drop was placed on a formvar-coated grid for 1 min and then stained for 1 min with six drops of 1% uranyl acetate before blotting dry. The grids were observed in a Phillips EM300.

1 - (2 - Chloroethyl) - 3 - ([1 - ^{14}C]cyclohexyl) - 1 - nitrosourea (134 nmoles) reacted with purified microtubule protein (27 nmoles) in 0.1 M Tris–HCl (pH 7.5) for 4 hr at room temperature. After overnight dialysis [5 M urea in 0.1 M Tris–HCl (pH 7.5) followed by water], the sample was incubated at 37° for 2 hr in 8 M urea, 0.1% sodium dodecylsulfate (SDS), and 0.5% mercaptoethanol. The tubulin was separated by gel electrophoresis, as described by Eipper [8]. The gels were cut, digested overnight with protosol, and counted in toluene 2,5-diphenyloxazole (PPO) fluor on a Beckman liquid scintillation counter LS-230. A similar gel was stained with Coomassie and scanned on a Gilford scanner on a Beckman DU spectrophotometer.

Within 8–10 min after placing the tubulin dimers at 37° to initiate polymerization, maximum turbidity was reached. This level was maintained for at least 30 min at 37°, similar to that seen by Gaskin *et al.* [7]. Normal tubules were observed in the electron microscope from samples removed at 6 and 30 min. Thus, turbidity correlated with polymerization of tubulin.

The CCNU and BCNU inhibition of maximum polymerization, as shown in Table 1, was dose dependent. MeCCNU and *trans*-4-OH CCNU at 5×10^{-7} moles behaved similarly, inhibiting 55 and 62 percent of control,

Table 1. Inhibition of polymerization

	Absolute amount (nmoles)	Percent inhibition	Standard deviation	Number of experiments
CCNU	250	26	13	4
	500	40	17	5
	750	70	11	3
BCNU	250	34	17	3
	500	51	12	4
	750	77	2	3

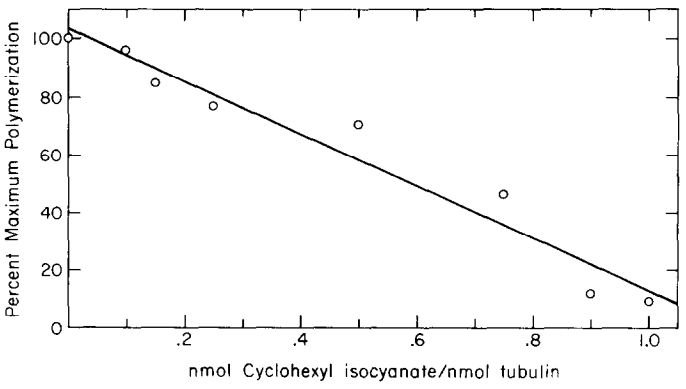

Fig. 1. Stoichiometry of inhibition of polymerization by CHI (a typical experiment). CHI (5–50 nmoles) was added to purified brain tubulin (50 nmoles) 5 min before initiating polymerization. The percent of polymerization is compared to that of the control after 30 min.

respectively. Chlorozotocin and *cis*-2-OH CCNU, however, were far less inhibitory at 5×10^{-7} moles inhibiting 7 and 2 percent, respectively.

As incubation with CCNU, BCNU, MeCCNU and *trans*-4-OH CCNU continued, the turbidity occasionally decreased rather than maintaining the maximum level. Generally this decrease began at about the time the control reached its maximum plateau. The decline was slow and steady until the experiment was terminated at 30 min. The two drugs which were only slightly inhibitory (*cis*-2-OH CCNU and chlorozotocin) did not cause this decrease in turbidity. Gaskin *et al.* [9] demonstrated that disassembly could be induced by the addition of calcium or colchicine. Our results could reflect a similar, delayed effect.

Samples were taken from all of the drug-treated tubulin preparations from two experiments after 6 min and observed by electron microscopy. In all cases, normal microtubules were observed, with no evidence of aggregation. After the turbidity decrease (30 min), electron microscope examination also revealed normal tubules. The concentration of tubules appeared lower, but no systematic study was performed.

Cyclohexylisocyanate (CHI) at concentrations between 5 and 50 nmoles (0.5 to 5×10^{-5} M) inhibited polymerization. The maximum inhibition of polymerization resulting from the drug treatment was linearly related to the dose (Fig. 1). In four similar experiments, half-maximal inhibition required 0.54 ± 0.05 nmole CHI/nmole tubulin monomer. This would suggest that the interaction between nucleophilic sites on the tubulin and the isocyanates has a considerable degree of specificity and occurs rapidly, as the half-life of CHI is less than 2 min [2]. Once the maximum plateau was reached (8–10 min), no decrease in the turbidity occurred in the remaining 30-min incubation.

The top of Fig. 2 is a scan of the absorbance of the Coomassie stain from one gel containing the tubulin labeled with [^{14}C]CCNU and below is the d.p.m. from a similar gel which was cut and counted. The α and β monomers are two very close bands which are not well resolved by the scanner. The ^{14}C is primarily located with the mono-

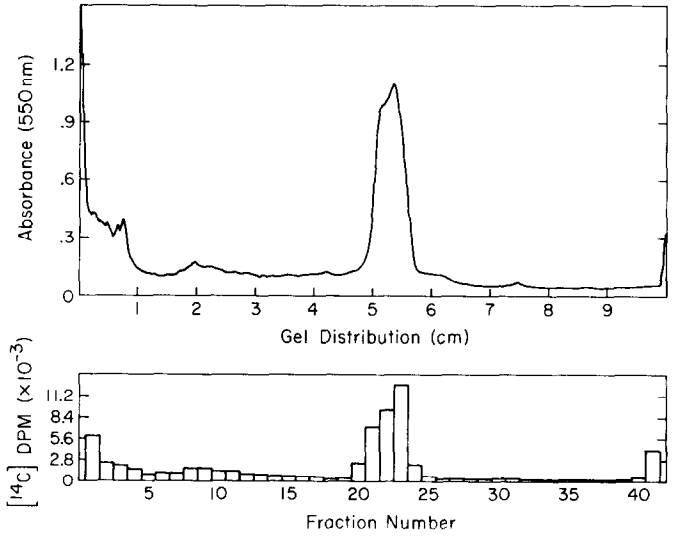

Fig. 2. [^{14}C]CCNU binding to purified brain tubulin. After incubation of purified brain tubulin with [^{14}C]CCNU, the protein was processed and run on gel electrophoresis. The top scan is the absorbance of the closely migrating α and β monomers on a Coomassie stained gel; the bottom shows the d.p.m. from a similar gel, cut and assayed for radioactivity.

mers; a small amount is present at the solvent front and with the high molecular weight protein at the beginning of the gel.

BCNU, CCNU and MeCCNU caused cell-cycle-related sensitivity differences in the human lymphoma cell line, T_1 [10]. The increased sensitivity to BCNU and MeCCNU in G2 and to CCNU and MeCCNU in the early S phase of the cell cycle could reflect, in part, disturbance by the drugs of the synthesis or polymerization and degradation of tubulin which occurs at these two times, respectively [11].

In summary, several nitrosoureas (CCNU, BCNU, MeCCNU, CHI and *trans*-4-OH CCNU) which degrade to form isocyanates have been shown to inhibit the polymerization of purified brain tubulin in a dose-dependent manner. Failure to yield appreciable isocyanate due to intramolecular carbamylation (chlorozotocin and *cis*-2-OH CCNU) resulted in a lack of inhibition of polymerization. The CHI inhibition of tubulin polymerization is stoichiometric. The [^{14}C]-cyclohexyl moiety derived from CCNU appears to bind covalently to the α and β tubulin monomers.

Acknowledgements—This work was aided by Grant CH-109 from the American Cancer Society. We thank Joanne Potter for technical assistance.

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Biochemical Pharmacology, Vol. 29, pp. 654–657.

0006-2952/80/0215-0654 $02.00/0

Effects of lithium chloride on the cholinergic system in different brain regions in mice

(*Received* 5 *May* 1979; *accepted* 4 *September* 1979)

Lithium is a potent therapeutic and prophylactic agent in the treatment of affective disorders, although its mode of action is still unknown [1]. In comparison with a number of reports on the effects of lithium on the brain monoaminergic system, relatively little work has been carried out on the *in vivo* actions of lithium on the central cholinergic system [2,3]. In relation to the role of acetylcholine (ACh) in the control of mood and behavior, it has been hypothesized by Janowsky *et al.* [4] that an imbalance between the central cholinergic and adrenergic systems is the etiology of affective disorders. They considered depression to be a disease of cholinergic dominance and mania to be the converse. Also, an antimuscarinic action of antidepressants in sympathetic ganglia [5] and a correlation between behavioral excitation and decreased brain ACh [6] or between depression and increased ACh level in brain [7] have been reported. Therefore, in the present experiments we examined the effects of a single dose and of chronic administration of lithium chloride (LiCl) on (1) steady state levels of ACh and choline (Ch) and (2) ACh turnover by an indirect method using hemicholinium-3 (HC-3) [8], in different brain regions in mice.

Male ddY mice weighing 20–30 g, were used in all experiments. In the single dose schedule, 4.72 m-equiv./kg of LiCl were injected i.p., and the animals were killed 2 hr later. In the chronic administration schedule, mice were treated twice daily (8:00 a.m. and 8:00 p.m.) for 5 days with 2.36 m-equiv./kg, i.p., of LiCl. On day 6 (7:00 a.m.), mice were given the last dose and killed 2 hr later. LiCl was administered in an isotonic solution. The controls consisted of animals that received an equivalent amount of isotonic sodium chloride.

For the experiments using an intraventricular injection of HC-3, mice were anesthetized with ether the day before they were killed. The skin above the skull was dissected away and the skull around the bregma was exposed; then a point 1 mm lateral from the bregma was marked. On the next day, immediately before the intraventricular injection, the mouse was grasped firmly and a transparent rubber disc (*ca.* 3 mm in diameter, 0.6 mm thick) was stuck on the marked point on the skull to prevent leakage of the drug when the intraventricular injection was made. After this a hypodermic needle (28 gauge, 1.7 cm, cut off from the hub), which was attached to the holder and connected to the microsyringe (50 μl) with polyethylene tubing, was inserted perpedicularly, through the rubber disc, 3 mm into the skull, with the help of a stop attached to the needle. Intraventricular injection of saline caused no apparent behavioral change except for 10–20 sec of immobility immediately after the injection. Saline solution (5 μl) containing 7 μg of HC-3 was injected intraventricularly 30 min before the animals were killed. The NaCl and LiCl groups were always treated concurrently and killed between 9:00 and 11:00 a.m.

For the assays of ACh and Ch, mice were killed by immersion into liquid nitrogen for 2.5–3.0 sec according to their weights. The brains were dissected in a cold box (−2°) into five regions (cortex, hippocampus, striatum, diencephalon and midbrain), basically according to the method of Schubert and Sedvall [9], wrapped individually