The hepatotoxicity of 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) in rats

Ultrastructural evidence of a delayed microtubular toxicity

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Summary. A few cases of liver involvement have been reported in patients receiving treatment with the antineoplastic nitrosourea CCNU. A single oral dose of 20 or 50 mg/kg CCNU in female Wistar rats induced an important increase in transaminases between day 2 and day 6, followed by a second, moderate increase between day 21 and day 28. Alkaline phosphatases and conjugated hyperbilirubinemia (threelfold-increase) were noted for the two doses and were greater for the highest dose. Histological and ultrastructural studies disclosed hepatic lesions of two types: during the first phase of transaminase increase, inflammatory of the portal tracts; during the second phase marked dilation of bile canaliculi and numerous filamentous bundles distributed at random throughout the liver cell cytoplasm like normal microtubules. Thus, CCNU induced pericholangitis and intrahepatic cholestasis with microtubular abnormalities. The long-term evolution of hepatic alterations revealed that in the 3rd month after a single oral dose of 20 mg/kg CCNU, lesions were persistent but stable; no reversibility was observed in the 3rd month after 50 mg/kg CCNU, and evolution towards cholangiolyis and biliary cirrhosis was noted. We suggest that CCNU causes a bimodal hepatotoxicity in rats: an early and prolonged ductal injury and a delayed anti-liver cell microtubule toxicity.

Introduction

Many nitrosourea derivatives have been synthesized and screened for potential antineoplastic activity; only three have exhibited exceptional carcinostatic potential and are in current clinical use: 1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU), 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) and 1-(2-chloroethyl)-3-(4-methyl cyclohexyl)-1-nitrosourea (MeCCNU). These drugs are highly lipid-soluble, cross the blood-brain barrier, are distributed widely to tissues, and have an extremely short plasma half-life. They decompose nonenzymatically at relatively rapid rates, and their biotransformation products are bound to macromolecules through alkylation of nucleic acids and proteins as well as through carbamylation of proteins in intact cells.

CCNU is at least as or more active than BCNU against L1210 leukemia cells implanted intraperitoneally or intracerebrally in mice. It is more lipid-soluble, which might enhance passage across the blood-brain barrier. In contrast to BCNU, CCNU has only a single chloroethyl group and a cyclohexyl group.

Although the clinically used nitrosoureas are not potent hepatotoxins at the therapeutic doses, the liver toxicity of these drugs is well known, particularly when BCNU is used in high doses followed by autologous bone marrow transplantation [18, 37]. Liver damage was observed in experimental animals with inoculation of a single oral dose of BCNU [38] and was confirmed in humans during phase I and II trials. In an early phase I trial of BCNU, De Vita et al. [11] reported changes in hepatic functional values occurring in up to 26% of patients. In later phase II trials [24, 27], a few patients developed hepatic abnormalities with usual doses of BCNU; these were transient in some cases, whereas in the others the damage may have contributed to death.

CCNU was first evaluated in clinical trials in the late 1960s; it has since been used on a broad spectrum of tumors, especially those of the brain (for review see [39]). Clinical tolerance is relatively good; however, toxicity is manifested as acute nausea and vomiting and as delayed, dose-limiting bone marrow suppression. In humans, CCNU is less hepatotoxic than BCNU at clinically used doses; however, cases of liver toxicity have also been reported [10, 20, 21, 28]. The hepatic toxicity of CCNU in dogs and monkeys has been noted by Carter and Newman [7], and recent studies in rats [1, 14, 22] have revealed by light and electron microscopy that CCNU causes interlobular bile duct and common bile duct injuries associated with cholestasis at early times after treatment with a single oral dose of this drug.

The mechanism of nitrosourea-induced hepatic toxicity is still not clear. The objective of the present study was to evaluate the experimental hepatotoxicity of CCNU at later stages following the inoculation of a single oral dose of CCNU.

Materials and methods

Experimental procedures. Female Wistar rats weighing approximately 200 g with free access to food and water throughout the study were given a single dose of CCNU by gastric intubation. CCNU was dissolved in 1% carboxy-
Methyl cellulose solution so that the final volume given was 1 ml. Control rats (n = 20) received only carboxymethyl cellulose solution in an equivalent volume. A total of 216 animals were treated: 108 received 20 mg/kg CCNU (equivalent to 120 mg/m²), and 108 received 50 mg/kg (300 mg/m²). The animals were sacrificed at regular intervals: 2, 4, 6, 8, 15, 21, 28, and 90 days posttreatment.

The rats were placed in diuresis cages for two consecutive 24-h periods before sacrifice. The presence of bilirubin in the urine was evaluated using Multistix strips. Blood specimens were taken from the aorta at the time of sacrifice: 2–3 ml for hematologic study was collected on 0.06 ml 8.5% EDTA K3 and agitated immediately; 2–3 ml was collected in a dry tube.

Blood counts were carried out using a Coulter Electronics Counter (Inc, Hialeah, Florida, USA). The erythrocyte count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration were determined at regular intervals between the 1st and 90th days in both groups. Assays of total and direct-reacting plasma bilirubin were carried out using a colorimetric assay with a Boehringer-Mannheim Diagnostica kit; glutamic-pyruvic transaminase activity was assayed colorimetrically with a BioMerieux test kit. Alkaline phosphatase activity was assayed colorimetrically with a p-nitrophenylphosphatase substrate [5]. Statistical analysis was carried out using Student’s t-test for the comparison of means.

Morphological studies. Histological and ultrastructural studies were carried out with groups of 12 animals, 4 of which were chosen at random for sacrifice at the times indicated above. Two of these animals were used for light microscopic study and the other two for electron microscopic study.

For light microscopy, the animals were perfused with 100 mM phosphate buffer (PBS) (pH 7.4) and liver specimens were fixed in MFA solution (methanol, 85 mL; formal, 10 mL; acetic acid, 5 mL/100 mL) for 3–4 h. After dehydration in graded methanol solutions, specimens were embedded in paraffin and 3-μm sections were stained with hematoxylin-eosin, periodic acid-Schiff, and Masson trichromic stain.

For electron microscopy, the animals were perfused with PBS solution, followed by 2.5% glutaraldehyde solution in 0.1 M cacodylate buffer (saccharose 5%, pH 7.3). Fixation was continued for 1 h after the dissection of 1-mm³ blocks. Postfixation was carried out with 2% osmium tetroxide for 1 h. After dehydration in a graded ethanol series and embedding in epoxy resin (glycidether 100, Merck, Darmstadt, FRG), ultrathin sections were cut on a Reichert OM U-2 ultramicrotome, stained with uranyl acetate and lead citrate, and examined with a Philips CM 10 transmission electron microscope.

Results

No death was seen with any of the treatments.

Changes in the blood count

There was no significant change in the erythrocyte count, hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin, or mean hemoglobin concentration in groups receiving 20 or 50 mg/kg CCNU. There was a significant decrease in hematocrit in animals receiving 20 mg/kg in comparison with controls (39.3 ± 2.7% vs 42.9 ± 1.7% in controls P < 0.05).

Specific liver function tests

(a) Hepatic transaminases (Fig. 1). At a dose of 20 mg/kg, treated animals showed a biphasic rise in transaminase activity (SGPT). There was a clear increase between the 2nd and 6th days, reaching a value of 403 ± 68 IU/l on the 4th day (48 ± 24 IU/l in the controls), with a return to normal between the 8th and 15th days. There was a second rise between the 21st and 28th days (215 ± 32 IU/l).

At a dose of 50 mg/kg, a major increase in SGPT activity (809 ± 80 IU/l) was seen beginning on the 2nd day of treatment. This sudden rise was followed by a rapid decrease, however, renormalization was not attained (167 ± 74 IU/l). A second rise was noted on the 15th day (400 ± 20 IU/l), followed by a new decrease, at no time returning to normal values.

(b) Serum alkaline phosphatase (Fig. 2). At a dose of 20 mg/kg CCNU, there was a two-fold rise in serum alkaline phosphatase between the 4th and 8th days. The greatest increase was seen on the 6th day (111.2 ± 20.7 IU/l in treated animals vs 61.1 ± 11.3 IU/l in controls). Between the 8th and 28th days alkaline phosphatase decreased, returning to normal in this group.

At a dose of 50 mg/kg CCNU, serum alkaline phosphatase showed an increase beginning on the 2nd day of treatment and remained significantly elevated beyond the 28th day.

(c) Serum bilirubin. A slight elevation in serum bilirubin levels developed between the 6th and 8th days of treatment with 20 mg/kg CCNU; there was no bilirubinuria.

At a dose of 50 mg/kg CCNU (Fig. 3), conjugated hyperbilirubinemia was noted beginning on the 2nd day and reaching a maximum on the 8th day (43 ± 18 mg/l vs 2.6 ± 1.0 mg/l in controls). Although there was a subsequent decrease in values, they remained very high between the 15th and 28th days. A parallel but lower rise in free bi-

![Fig. 1](https://example.com/fig1.png) Changes in serum transaminase activity (SGPT) with doses of 20 or 50 mg/kg p.o. CCNU as a function of time (mean for 6 experimental points)