Metabolism of 1,3-Bis(2-chloroethyl)-1-nitrosourea by Rat Hepatic Microsomes

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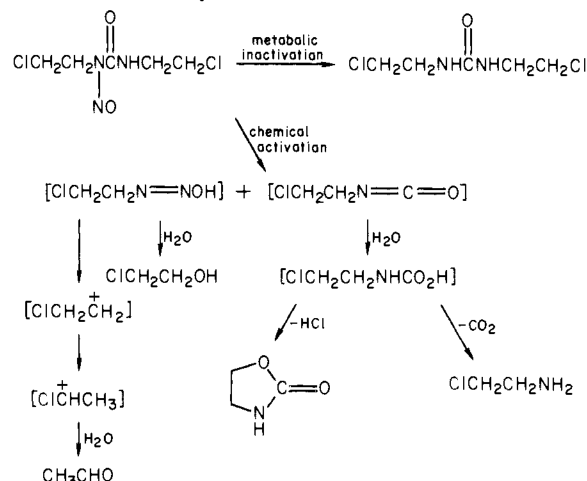
The in vitro metabolism of the anticancer agent 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) has been studied in male Fisher 344 rat liver microsomal preparations. The previously identified product, 1,3-bis(2-chloroethyl)urea (BCU), has been shown to be the major metabolite. Stable isotope labeling and mass spectral analysis of isolated metabolites indicate that BCU is formed exclusively from the metabolic denitrosation of BCNU. The rate of BCNU chemical decomposition in rat liver microsomal preparations deficient in NADPH and the metabolic disappearance rate in preparations containing added NADPH were measured and compared with the measured rate of metabolic formation of BCU under the same conditions. The rates of NADPH-dependent BCNU metabolism and BCU formation are equal within experimental error. BCNU was found to inhibit the rat liver 9000*g* supernatant metabolism of 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU).

The lipophilic chloroethylnitrosourea BCNU is used in the treatment of brain tumors and other malignant diseases.[3-5] The antitumor activity of BCNU and other chloroethylnitrosoureas is thought to be due to chemical decomposition products. BCNU reacts to give chloroethylating, $ClCH_2CH_2N{=}NOH$, and carbamoylating, $O{=}C{=}NCH_2CH_2Cl$, intermediates.[6] The suggested 2-chloroethyl azohydroxide intermediate is thought to produce both chemotherapeutic and toxic effects,[6,7] while there is little evidence that the carbamoylating species produces detectable cytotoxicity at therapeutic doses.[6-10]

Plasma clearance of BCNU is rapid.[11,12] Tissue distribution, chemical degradation, as well as serum protein catalyzed decomposition[13] and serum lipid partitioning[14] are possible biodisposition factors. Hepatic metabolism also affects BCNU plasma clearance and antitumor activity.[15] The liver has been identified as the primary site of BCNU metabolism.[16] Lung tissue homogenate has 30% of liver tissue metabolic activity, while no activity is detectable in kidney, spleen, brain, muscle, intestine, or serum. Hill has identified 1,3-bis(2-chloroethyl)urea as an NADPH-dependent product of mouse liver homogenate metabolism.[16] Some evidence has been presented for the formation of a glutathione conjugate of BCNU or BCU.[17] The chemical and metabolic reactions of BCNU are shown in Scheme I.

Scheme I. Chemical Activation and Metabolic Deactivation Pathways for BCNU



This report confirms the identification of BCU as a BCNU metabolite and describes investigations of the pathway of BCU formation that utilized stable isotope labeling and mass spectrometry. Rates of BCNU metabolism and BCU formation were determined and indicate that BCU is a major metabolite. BCNU is also shown to inhibit the liver microsomal metabolism of CCNU. These observations and the previously reported effects of phenobarbital pretreatment on the antitumor activity of BCNU and CCNU are explained in terms of an unusual structure–activity relationship in which BCNU is metabolically deactivated by hepatic cytochrome P-450 while CCNU is metabolized by the same enzyme to products that retain antitumor activity.

Results and Discussion

BCNU decomposes chemically in rat liver microsomal preparations in the absence of added NADPH. At 37 °C and pH 7.4, the half-time for BCNU disappearance was 90 min. A small amount of BCU was formed in these incubation mixtures as a result of either chemical reaction or residual metabolic activity. Addition of NADPH significantly increased both the rate of BCNU disappearance and formation of BCU. The results reported as NADPH-dependent metabolism in this paper were obtained from the difference observed between NADPH free and added incubations. BCNU metabolic products were analyzed by

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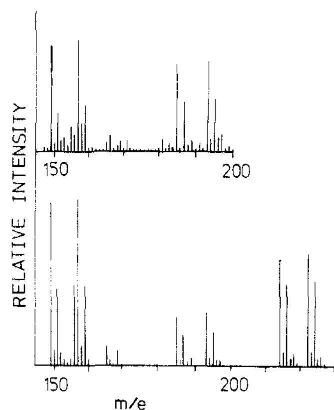

Figure 1. Chemical-ionization mass spectra of BCU isolated from the in vitro rat liver metabolism of a 1:1 mixture of BCNU and BCNU-d_8 (upper) and unreacted BCNU and BCNU-d_8 recovered from the same mixture (lower).

HPLC following ether extraction of the microsomal incubation mixture.

Metabolism of BCNU in rat liver 100000g microsomal preparation produced a single product with the same retention time as synthetic BCU. This structure was confirmed by comparison of the chemical ionization mass spectra of the isolated metabolite with that of the known compound. This is the same metabolite reported by Hill following mouse liver microsomal metabolism.[16,17] Analogous metabolic reaction products have been reported for 1-butyl-1-nitrosourea,[18] 1-methyl-1-nitrosourea,[16] and MeCCNU.[26]

The pathway for formation of BCU was investigated by metabolism of a mixture of 0.73 mM BCNU-d_0 and 0.73 mM BCNU-d_8 labeled on the methylene positions. BCU may be formed by two routes. Chemical or enzyme-catalyzed decomposition of BCNU to 2-chloroethyl isocyanate and combination of this intermediate with its hydrolysis–decarboxylation product, 2-chloroethylamine, will yield BCU.[20,21] BCU formed through this pathway would consist of a mixture of BCU-d_0, -d_4, and -d_8 in a ratio of 1:2:1.[21] Only BCU-d_0 and BCU-d_8 would be formed through the alternate pathway of metabolic denitrosation of BCNU-d_0 and BCNU-d_8. Chemical-ionization mass spectra of unreacted BCNU-d_0, BCNU-d_8, and metabolite BCU recovered from the HPLC eluate of the same incubation mixture are shown in Figure 1. The lower trace shows the chemical-ionization mass spectrum of unreacted BCNU-d_0 and BCNU-d_8 isolated from the incubation mixture after 30 min or 30% reaction. Protonated molecular ions are observed from m/z 214 to 219 for the natural abundance chlorine and carbon isotopes of BCNU-d_0 and an identical pattern appears at m/z 222 to 227 for the BCNU-d_8 variant. The ratio of BCNU-d_0 and BCNU-d_8 peak heights is the same as the ratio of amounts added to the incubation mixture, indicating that there is no deuterium isotope effect in BCNU metabolism. The upper trace shows the spectrum of metabolically formed BCU isolated from the incubation mixture. BCU-d_0 and BCU-d_8 are observed at m/z 185 to 190 and 193 to 198, respectively. The BCU-d_0/BCU-d_8 peak height ratio is the same as the initial BCNU-d_0/BCNU-d_8 ratio, so that no deuterium isotope effect was present in BCU formation. No BCU-d_4 isotope peaks were observed at m/z 189 to 194, indicating that BCU was formed with the urea structure intact, probably through cytochrome P-450 mediated denitrosation of BCNU. These data indicate that no BCU is formed by the chemical reaction pathway involving combination of 2-chloroethyl isocyanate and 2-chloroethylamine.

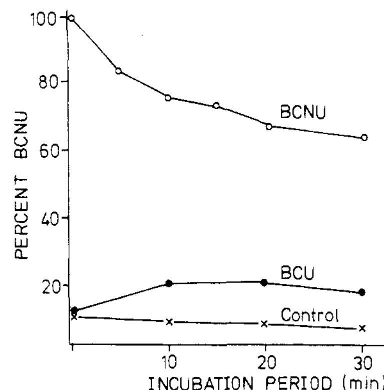

Figure 2. Disappearance of BCNU and formation of BCU during rat liver microsomal metabolism. The control monitors the amount of BCU present in the absence of added NADPH.

The kinetics of BCNU disappearance and BCU appearance during rat liver microsomal metabolism are shown in Figure 2. BCNU disappearance is due to both chemical and metabolic reactions. Under the conditions of these experiments, 60% of BCNU disappearance is due to NADPH-dependent metabolism. BCNU is metabolized at a rate of 56 ± 11 nmol (mg of protein)$^{-1}$ (10 min)$^{-1}$, so that 15 ± 3% of initial BCNU is consumed within 10 min. BCU appearance following addition of NADPH occurs at approximately the same rate, 54 ± 23 nmol (mg of protein)$^{-1}$ (10 min)$^{-1}$, so that 14.5 ± 6.2% of the initial amount of BCNU appears as BCU within 10 min. This indicates that BCU is the major metabolic product, but the formation of minor products may not be excluded. The concentration of BCU in incubation mixtures is observed to decrease after longer periods. This rate of decrease was approximately 32 nmol (mg of protein)$^{-1}$ (10 min)$^{-1}$. The rather large standard deviation in these results is due to experimental variation in the rate of microsomal BCNU metabolism. In each experiment there was a corresponding variation in the BCU appearance rate so that the ratio of BCNU metabolized to BCU formed remained near unity.

Incubation of BCNU with microsomes prepared from phenobarbital-pretreated rats gave greater amounts of BCU than incubation with microsomes from normal rats. An increase from 26 to 41 nmol (mg of protein)$^{-1}$ (15 min)$^{-1}$ was observed on incubation with induced rather than normal microsomes under an air atmosphere. Under nitrogen, where BCU disappearance is slow, an increase from 33 to 63 nmol (mg of protein)$^{-1}$ (15 min)$^{-1}$ was observed. The mechanism of metabolic denitrosation is not known. These studies indicate that the reaction may occur under oxic and anoxic conditions. Further studies on the mechanism were not pursued with this agent, since the rate of BCNU denitrosation is slow and the comparable rates of BCNU chemical degradation and BCU disappearance complicate data acquisition.

All of the above data are consistent with the previously reported effect of phenobarbital on the antitumor activity of BCNU in Fisher 344 rats.[15] These data support the suggestion that phenobarbital-induced loss of activity and

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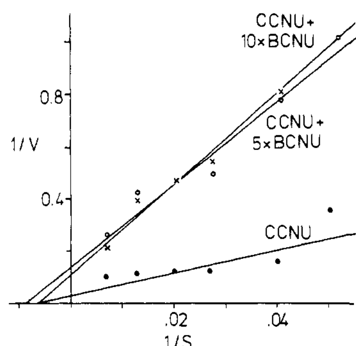

Figure 3. A double-reciprocal plot showing the inhibiton of in vitro rat liver 9000*g* supernatant metabolism of 190 μM CCNU [V_{max} = 30 μmol min^{-1} (mg of $protein)^{-1}$] by 5- and 10-fold molar excesses of BCNU. Incubations were for 10 min, with the protein content of the incubation mixture ranging from 0.65 to 10.2 mg/mL.

increased plasma clearance were related to a metabolic reaction that lead to an inactive product. This is potentially a clinically significant observation, since many brain tumor patients who are treated with BCNU are also treated with phenobarbital to control tumor side effects. However, we have been unable to detect BCU in the urine of patients that had received BCNU. These analyses used mass spectrometric and HPLC methods that would have detected <2% of the administered dose excreted as BCU in urine collected during 24 h following BCNU administration.

The fact that phenobarbital pretreatment of tumor-bearing rat produced a 100% decrease in BCNU antitumor activity contrasts with the absence of any decrease in CCNU activity.[15] CCNU has been shown to be a substrate for cytochrome P-450 in which the major metabolites are hydroxylated on the cyclohexyl ring.[16,22-24] These metabolites have nearly the same activity as the parent CCNU.[25] The above results suggest that BCNU is also a cytochrome P-450 substrate but that metabolism occurs through denitrosation. This is supported by the data of Figure 3. CCNU is metabolized more rapidly by rat liver 9000*g* supernate preparations than is BCNU. Addition of BCNU to the incubation mixture at a concentration that is 5 or 10 times greater than CCNU significantly slows the rate of CCNU metabolism. This indicates that both BCNU and CCNU may bind to the cytochrome P-450 active site but that the pathways of metabolism by this enzyme are different. It appears that there is an unusual structure–activity relationship within the chloroethylnitrosourea series of drugs in which the cyclohexyl group of CCNU serves as a favorable site of nondestructive cytochrome P-450 metabolism. The chloroethyl group of BCNU is not readily hydroxylated so that this agent is subject to the slower deactivating denitrosative metabolic pathway of the same enzyme. 1-(2-Chloroethyl)-2-(4-*trans*-methylcyclohexyl)-1-nitrosourea (MeCCNU) activity against i.c. 9L tumor in rat is reduced but not eliminated by phenobarbital pretreatment.[15] The favored position of cyclohexyl ring hydroxylation is blocked by the 4-methyl group in this analogue. The result is that the rate of metabolism via hydroxylation is slowed and denitrosation becomes a competing metabolic pathway.[16,26] It appears, therefore, that the N-2 substituent of chloroethylnitrosourea analogues may influence the extent of metabolic deactivation.

Experimental Section

BCNU and CCNU were obtained from the National Cancer Institute. BCNU-d_8 labeled in the methylene positions was obtained from the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute, through a contract with SRI International, Menlo Park, CA. Mass spectrometry was performed with a Finnigan 3200 instrument equipped with a dual electron impact/chemical ionization source and operated in the chemical ionization mode with isobutane as a reagent gas. High-performance liquid chromatography (HPLC) was performed on a Perkin-Elmer Model Series 2 liquid chromatograph with a C_{18} μBondapak column and a LC-55 variable wavelength detector. A solvent of 20% acetonitrile in water was used at a flow rate of 1 mL/min.

Hepatic microsomes and 9000*g* supernatant were prepared from the livers of 170 to 200 g male Fisher 344 rats and from rats pretreated with phenobarbital for 2 weeks by addition of 2.0 g of phenobarbital/L of drinking water. Liver homogenate fractions were prepared according to the method of Fouts.[27] Incubation mixtures consisted of substrate, 500–550 μg/g of rat liver (final concentration 0.7 mM); 1.6 mL of 9000*g* supernatant or 100000*g* microsomal pellet suspension; 0.1 mL of 0.1 M $MgSO_4$; 7.5 mg of NADPH; and 0.1 M phosphate buffer to give a final volume of 4.5 mL at a pH of 7.4. Incubations were conducted at 37 °C in a shaking water bath. Boiled 9000*g* supernatant or 100000*g* microsomal pellet suspensions were used as controls in these experiments. BCNU undergoes chemical degradation in the microsomal enzyme preparation. In order to distinguish between chemical and metabolic reactions, the decomposition and product formation rates were monitored in the absence and presence of added NADPH. The BCNU decomposition rate in the absence of added NADPH is subtracted from the more rapid rate that is observed following NADPH addition. The difference is reported as the NADPH-dependent rate of metabolism.

Analyses of metabolic reaction mixtures were performed from 250-μL aliquots of the incubation mixture taken between 0 and 30 min following NADPH addition. BCNU was quantitated using the chemical-ionization mass spectrometric method previously described.[28] In this assay a known amount of BCNU-d_8 is added to the reaction mixture aliquot, which is then extracted with ether to remove both BCNU and BCNU-d_8. The concentration of BCNU in the aliquot is determined from the BCNU/BCNU-d_8 peak height ratio obtained from the chemical-ionization mass spectrum of the ether extract. The metabolite BCU was analyzed from 1.0-mL aliquots of the incubation mixture. 1-(2-Chloroethyl)-3-cyclohexylurea (CCU) was added as a standard and the mixture was extracted with 3 × 3 mL of ether. The combined ether extracts were evaporated to a small volume and analyzed with HPLC on a C_{18} μBondapak column with 20% acetonitrile in water as a solvent. A wavelength of 205 nm was used to detect the urea chromophore. CCNU was analyzed using the previously reported HPLC method.[13]

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