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METABOLISM OF CYCLOPHOSPHAMIDE BY PURIFIED CYTOCHROME P-450 FROM MICROSOMES OF PHENOBARBITAL-TREATED RATS[1]

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Incubation of [^{3}H]-sidechain-labeled and [^{14}C]-C(4)-ring-labeled cyclophosphamide (CPA) with purified cytochrome P-450 from liver microsomes of rats treated with phenobarbital resulted in the production of a major metabolite that contained both labels, was unaffected by diazomethane, possessed high polarity, was identical in TLC and HPLC behavior to a synthetic standard, didechlorodihydroxy-CPA, and was converted to CPA and bis(2-chloroethyl)amine by thionyl choloride. These results indicate that phenobarbital-inducible cytochrome P-450 is able to dechlorinate CPA and may account, in part, for the inability of phenobarbital to enhance the therapeutic activity and toxicity of this important anticancer and immunosuppressive agent.

Cyclophosphamide (CPA), an effective cancer chemotherapeutic drug and immunosuppressive agent, is activated by liver microsomal cytochrome P-450. The initial product, 4-hydroxy-CPA, undergoes ring-opening to form aldophosphamide, which spontaneously decomposes to yield acrolein and phosphoramide mustard (PM), the single biologically-active alkylating metabolite of CPA (1).

Previous studies (2,3) indicated that CPA produces a depression of microsomal cytochrome P-450 oxidase and the reductase activities in phenobarbital (PB)-treated rats, and this effect was shown to be due to the CPA metabolite acrolein. In this report we present data which indicate that the major cytochrome P-450 band, designated as cytochrome P-450 peak B_2 by Guengerich and Martin (12) and isolated from hepatic microsomes of PB-treated rats, metabolizes CPA by dechlorination, a pathway different from the commonly recognized pathway of 4-hydroxylation (1). Since dechlorination leads to products incapable of functioning as biological alkylating agents, elevated levels of the

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cytochrome P450 responsible for this deactivation pathway may account, in part, for the reported ineffectiveness of PB in improving the therapeutic effects of CPA (4-9). Our results therefore demonstrate that PB-inducible cytochrome P450 metabolizes CPA via a previously unreported pathway. These results also suggest the desirability of assessing the relative contribution of this new metabolic pathway in patients being treated with CPA, as a means of predicting the relative extent of activation and of accounting, in part, for the variability in response.

MATERIALS AND METHODS:

Chemicals:

[^{3}H] Cyclophosphamide (specific activity 440 mCi/mmole) labeled in both 2-chloroethyl groups was purchased from Amersham, Arlington Heights, IL, and [^{14}C]-cyclophosphamide (specific activity 11.25 mCi/mmole) labeled at carbon-4 in the oxazaphosphorine ring was purchased from New England Nuclear, Boston, MA. The sources of other chemicals have either been reported previously (2), or the chemicals and solvents were obtained in the highest available purity from commercial suppliers.

Preparation of Phenobarbital-Inducible Cytochrome P-450 Fraction (Peak B_2):

Animal treatment with phenobarbital and isolation of hepatic microsomes followed previously reported methods (10,11). In these and other studies, PB pretreatment of rats and mice was found to enhance CPA metabolism greater than two-fold and cytochrome P450 content from 1.8 to 3-fold. Furthermore, while hepatic microsomes from PB-pretreated rats produced the dechlorinated CPA metabolite, hepatic microsomes from control rats either failed to produce this metabolite or produced barely detectable amounts of this metabolite. These initial results confirmed induction by PB of hepatic microsomal cytochrome P-450 and the metabolism of CPA. Purified cytochrome P-450 (Peak B_2) and NADPH cytochrome P-450 reductase were isolated according to the procedure of Guengerich and Martin (12). The specific activity of the purified cytochrome P-450 obtained in this way, based on the measurement of the reduced CO-complexed difference spectrum, according to the method of Omura and Sato (13), ranged between 15-17 nmoles cytochrome P-450/mg protein. This PB-inducible cytochrome P-450 was found to migrate as a single spot on SDS-gel electrophoresis; however, HPLC of this protein band revealed at least three cytochrome P-450-containing proteins (14). Specific activity of the purified NADPH-cytochrome P-450 reductase was 57 units/mg protein (1 unit = 1 mol of cytochrome C reduced/min).

Interaction of CPA with Phenobarbital-Induced Cytochrome P-450 Fraction:

[^{3}H]-Sidechain-labeled-CPA ([^{3}H] CPA) or [^{14}C]-C(4)-ring-labeled-CPA ([^{14}C]-CPA) was metabolized with the reconstituted cytochrome P-450 system. The incubation mixture contained per ml, 0.1 mmol potassium phosphate buffer (pH 7.4), 0.1 nmol cytochrome P-450, 0.4 unit NADPH-cytochrome P-450 reductase, 10 μg dilauroyl phosphatidylcholine, 7.5 μmoles $MgCl_2$ and 0.3 μmol [^{3}H] or [^{14}C] CPA. The reaction mixture was incubated for 5 min at 37°C and the reaction was started by the addition of 0.2 μmol NADPH. The incubation at 37°C was continued and at 20 and 40 min of incubation the mixture was fortified with 0.1 nmol cytochrome P-450 and 0.2 μmol NADPH. The reaction was terminated at 60 min by freezing the incubation mixture in dry ice. Usually 1 ml incubations were performed and the number of tubes, 10-50 in a batch, were processed according to need. No aqueous soluble metabolites of [^{3}H] or [^{14}C] CPA were formed in the absence of cytochrome P-450 or NADPH.

Isolation of CPA Metabolites:

Incubates of [^{3}H] CPA or [^{14}C] CPA were allowed to thaw and immediately extracted with chloroform (3 x 10 ml). The aqueous solution was lyophilized, and the residue was extracted with chloroform (3 x 10 ml). The chloroform insoluble fraction was extracted with methanol (3 x 10 ml) with filtration after extraction with each 10 ml aliquot of methanol. A small methanol-insoluble residue remained.

Thin-Layer (TLC) and High-Pressure-Liquid Chromatography (HPLC)

TLC was performed on Analtech (Newark, DE) silica gel G (100 microns) plates in various one or two-component solvents. HPLC was performed with a Waters (Waltham, MA) ALC-242 chromatograph on a Partisil PAC column in an acetonitrile:methanol gradient.

Radioactivity Determination

Radioactive samples were counted in Aquasol (New England Nuclear, Boston, MA) in a Packard Tri-Carb model 3315 liquid scintillation spectrometer with external standard. Radiochromatogram scanning was performed with a Packard model 7220/21 scanner.

RESULTS AND DISCUSSION

Chloroform extracts of incubates of [^{3}H] CPA or [^{14}C] CPA with the isolated cytochrome P-450 consistently contained less than half (20-35%) of the total radioactivity in the incubates while methanol extracts contained most (60-70%) of the total. Methanol-insoluble residues accounted for 5-10% of the total.

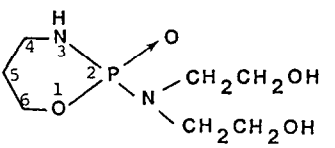
Since we originally anticipated that the isolated isozyme was the specific 4-hydroxylating isozyme, sodium bisulfite and sodium cyanide were added at the end of incubation to trap 4-hydroxy-CPA as a stable, chloroform-extractable derivative, aldophosphamide cyanohydrin (15). Thin-layer chromatography on silica gel (TLC) of chloroform extracts, along with synthetic standards of norHN2, 4-keto-CPA, aldophosphamide cyanohydrin, dechloroethyl-CPA, and alcophosphamide (16), in acetone:chloroform (3:1), consistently failed to demonstrate the presence of significant amounts of these or any other non-polar metabolites. Approxmately 90% of the radioactivity in chloroform extracts co-chromatographed with CPA, while small amounts co-chromatographed with alcophosphamide (ca. 5%), aldophosphamide cyanohydrin (ca. 4%), and dechloroethyl-CPA (ca. 2%) (1). 4-Hydroxy-CPA has been shown to be stable under identical conditions of pH and temperature (Dr. R. F. Borch, unpublished results), and trapping of 4-hydroxy-CPA with cyanide under closely similar conditions has been demonstrated (17).

In early experiments, methanol extracts were treated with diazomethane to stabilize PM as its methyl ester (16). PM was expected to be present as a result of some

spontaneous degradation of 4-hydroxy-CPA and aldophosphamide during incubation. TLC of methanol extracts along with synthetic standards of PM methyl ester and carboxyphosphamide methyl ester in chloroform:methanol (9:1) revealed only trace amounts of PM while the major radioactivity remained at the origin (16), thus indicating the high polarity of the major metabolite. The polarity of the major metabolite was illustrated further by evaporation of a methanol extract and extraction of the residue with acetone; only 6% of the total radioactivity in the extract residue was solubilized by acetone.

Side-by-side TLC of methanol extracts of incubates from [^{3}H] CPA and [^{14}C] CPA in a variety of TLC solvent systems (chloroform:methanol [1:1 and 1:2], methanol, and methanol:water [9:1]) demonstrated by radioscanning that the R_f's of the major radioactive component in extracts of both labels were identical. Diazomethane treatment of both the [^{3}H]- and [^{14}C]-labeled major metabolite, either in total methanol extracts or after TLC isolation, had no effect on TLC mobility; this property demonstrates the absence of an acidic function in the metabolite and indicates that the metabolite has retained the oxazaphosphorine ring intact.

Consideration of the foregoing data led to the tentative assignment of the following structure to the major metabolite:



Such a structure would be expected to be polar and to be unaffected by diazomethane and would retain both [^{3}H] and [^{14}C] labels.

A synthetic standard of didechlorodihydroxy-CPA was prepared by selective chlorine displacement from [^{3}H] CPA and [^{14}C] CPA (separately) by hydroxide ion in dilute potassium hydroxide, and the dihydroxy analog of CPA was isolated by TLC in methanol. Structural confirmation was obtained by mass spectral analysis using the fast atom bombardment technique: normal (positive) mode, m/z (mass to charge ratio) 225 (strongest peak in spectrum), [M(molecular ion) + 1]$^{+}$; negative mode, m/z 223 (strongest peak in spectrum), [M-1]$^{-}$. Several attempts to obtain a definitive mass spectrum of the

metabolite by analysis of TLC- and HPLC- purified fractions were unsuccessful, presumably because of interfering impurities.

Synthetic didechlorodihydroxy-CPA was compared by TLC with the major metabolite generated in incubates of the isolated cytochrome P-450 fraction with [^{3}H] CPA and [^{14}C] CPA. Identical R_f's were obtained in the following systems upon side-by-side TLC on siliga gel (solvent, R_f): acetone, 0.0; methanol, 0.35; ethanol, 0.08; methanol:water (9:1), 0.50; methanol:water (1:1), 0.60.

High pressure liquid chromatography on a Partisil PAC column in an acetonitrile:methanol gradient (HPLC) of synthetic [^{3}H] didechlorodihydroxy-CPA and the TLC-isolated major metabolite from incubates of the purified cytochrome P-450 with [^{3}H] CPA and [^{14}C] CPA, under precisely identical conditions on the same day with collection of 40 1-ml fractions followed by radioassay, gave the major radioactivity in the same fraction (#19) in every case. In order to eliminate the possibility of slight variation in HPLC retention times of the major radioactive metabolite in [^{3}H]- and [^{14}C]-incubates, [^{3}H]- and [^{14}C]-TLC – isolated fractions were mixed and separated by HPLC with fraction collection and radioassay for [^{3}H] and [^{14}C]. The major radioactivity observed in both [^{3}H] and [^{14}C] channels appeared in the same fraction (#18).

Reaction of thionyl chloride at room temperature with a TLC-purified metabolite fraction from [^{3}H] CPA gave a product which was separated by TLC in chloroform:methanol (1:1) (R_f of metabolite = 0.4). Two radioactive bands (R_f 0.9 and 0.1) were obtained. The mobile band (R_f 0.9) was co-chromatographed by TLC with CPA and norHN2(bis[2-chloroethyl] amine) in acetone:chloroform (3:1), and the two standards were detected by color formation with 4-(p-nitrobenzyl)pyridine (NBP). CPA and norHN2 and the void areas on the TLC plate were collected for radioassay and gave the following results (band, identity, cpm): 1, front, 271; 2, norHN2, 945; 3, CPA, 2085; 4, void, 96; 5, void, 82; 6, origin, 129. An aqueous methanol eluate of the immobile band (R_f 0.1, presumably a mixture of the hydrochlorides of norHN2 and bis[2-hydroxyethyl] amine), after lyophilization, was treated with thionyl chloride in dimethylacetamide at 50° for 15 min. After removal of solvent and excess thionyl chloride in vacuo, the residue in water was extracted with chloroform to remove colored contaminants, adjusted to pH 9 with N KOH, and extracted with chloroform. The chloroform extract was co-chromatographed

with norHN2 by TLC in acetone:chloroform (3:1). A leading band, the single NBP-detectable band (norHN2), the remaining void area, and the origin were radioassayed, giving the following results (band, identity, cpm): 1,leading band, 304; 2, norHN2, 3023; 3, void area between norHN2 and origin, 364; 4, origin, 207. These data demonstrate the production of CPA and norHN2 from the metabolite by a chlorinating agent. Extensive cleavage of the phosphorus-mustard bond is reasonable in view of facile P-N bond cleavage by acid.

Collected data on the common, major [^{3}H]- and [^{14}C]-labeled metabolite (high polarity; unaffected by diazomethane; common to both labels; identical by side-by-side TLC in several solvent systems and by precisely identical HPLC profile to synthetic didechlorodihydroxy-CPA; identical by coelution HPLC; conversion to CPA and norHN2 by thionyl chloride) are consistent with the didechlorodihydroxy-CPA structure.

A precedent for replacement of chlorine by the hydroxyl group by cytochrome P-450 can be found in studies on mixed function oxidation of 1,2-dichloroethane by Guengerich et al. (18) in which 2-chloroethanol was produced. Additionally, in studies on the urinary metabolites of CPA in sheep, Bakke et al. (19) identified a minor metabolite whose structure is similar to that of the cytochrome P-450 metabolite; the sheep metabolite, 5- or 6-ketodidechlorodihydroxy-CPA, establishes a precedent for replacement of chlorine by the hydroxy group in CPA in vivo.

The significance of the observation for metabolism of CPA in vivo is not known. Investigations designed to determine the presence or absence of the metabolite in plasma and urine of control and phenobarbital-treated rats dosed with CPA is under investigation.

The existence of a phenobarbital-inducible cytochrome P-450 that is capable of dechlorinating CPA could account in part for the inability of phenobarbital to appreciably enhance the experimental antitumor effect of CPA (4-9). Although ring hydroxylation activity as well as cytosolic deactivation activity (10) may be enhanced simultaneously, dechlorination of either parent drug or preactivated intermediate (4-hydroxy-CPA) could lead to no net elevation of tissue levels of preactivated and activated (PM) metabolites in the host. Microsomal dechlorination in extra-hepatic host tissues and CPA-resistant tumor cells could also be a contributing factor in selective toxicity (20, 21) and drug resistance (22, 23), respectively. The relative contribution of dechlorination compared

with other proposed toxification (24, 25) or detoxification (20) mechanisms remains to be investigated. It is likely that the metabolite was not identified in numerous earlier studies on the metabolism of CPA in vivo because of the primary interest in alkylating metabolites. Previous studies demonstrated that alkylating metabolites do not account for all excreted radioactivity in rodents, dogs, or humans (1, 16, 26).

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