Identification of Aldophosphamide as a Metabolite of Cyclophosphamide in Vitro and in Vivo in Humans

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SUMMARY

Aldophosphamide (NSC 254), a putative key metabolite of cyclophosphamide, has now been isolated as a cyanohydrin derivative from an incubation mixture of cyclophosphamide with mouse liver microsomes in vitro and from the plasma of a cyclophosphamide-treated patient. The cyanohydrin has been shown to be identical with an authentic synthetic sample by mass spectrometry and combined gas chromatography-mass spectrometry.

INTRODUCTION

The antitumor agent cyclophosphamide is not itself cytotoxic. Instead it is converted to an active metabolite(s) by oxidation by the hepatic microsomal enzyme system. The hepatic metabolites of cyclophosphamide have proven difficult to characterize; significantly, many of them are chemically unstable as well as highly polar.

In recent years, however, progress has been made, helped most notably by the application of mass spectrometry (1, 2, 13). Chart 1 shows the major points of the metabolic scheme that is now generally accepted. Enzymatic hydroxylation is postulated to lead to the carboline, 4-hydroxycyclophosphamide (II a), and this is believed to isomerize to aldophosphamide (II b). Major urinary metabolites are carboxyphosphamide (VII) and 4-ketocyclophosphamide (VI) which, however, show no significant cytotoxic activity. Carboxyphosphamide and 4-ketocyclophosphamide are thought to arise from the oxidation of aldophosphamide and 4-hydroxycyclophosphamide, respectively. Spontaneous decomposition of aldophosphamide is thought to lead to the cytotoxic alkylating metabolite phosphoramidemustard (III), which has been identified as a human metabolite and has been assayed in patient blood.5

Nornitrogen Mustard (V) is formed by decomposition of most of the other metabolites and, in particular, from carboxyphosphamide (VII).5

The keystones in this scheme are the tautomers II a and II b, 4-hydroxycyclophosphamide and aldophosphamide. Although 4-hydroxycyclophosphamide has been synthesized and found to be cytotoxic (14), aldophosphamide has proven notoriously resistant to synthesis (12), and neither compound has been unequivocally characterized as a human metabolite.

This paper describes the isolation and characterization of the cyanohydrin derivative of aldophosphamide from incubations of cyclophosphamide with mouse liver microsomes in the presence of appropriate trapping reagents. Aldophosphamide has also been identified in the plasma of a patient receiving cyclophosphamide, after treatment of the plasma with the trapping reagents.

MATERIALS AND METHODS

Standard Aldophosphamide Cyanohydrin. Aldophosphamide prepared by CrO3 oxidation of the corresponding alcohol was characterized on the basis of a positive Purpald test for aldehydes and an NBP test for alkylation, IR spectum, and the preparation of well-characterized semicarbazone and cyanohydrin derivatives. The structural assignment was confirmed by mass spectrometry and by gas chromatography mass spectrometry. The details of synthesis and structural assignment are reported elsewhere (5, 8).

Gas Chromatography Mass Spectrometry. The cyanohydrin derivatives of synthetic and biologically produced aldophosphamide were characterized mass spectrometrically, with a DuPont Model 491 instrument using chemical ionization with the source temperature at 190°. Samples were inserted on the direct probe, or they were prepared for combined gas chromatography mass spectrometry by treatment with bis(trimethylsilyl)trifluoroacetamide and injected onto a 5% OV-17 column (Supelcoport 80/100 mesh), 2 ft x 1/4-inch inside diameter, connected to the mass spectrometer through a single-stage glass jet separator. Column temperature was programmed from 190° to 300° at 6°/min. The injection port was at 280°, and the interface line was at 220°. Selected ion monitoring was carried out with the DuPont Model 491 system.

Microsomal Incubation. The preparation of the microsomes and the incubation were carried out as reported earlier (2), except that the NADPH generating system was replaced with 13.0 mM NADPH added in 2 aliquots. Sodium

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bisulfite (5 mM) was present in the incubation mixture as a trapping agent for carbonyl compounds. At the end of the 60-min incubation the reaction mixture was placed on ice and centrifuged at 100,000 x g for 30 min to remove the microsomes. The supernatant was then extracted twice with equal volumes of chloroform. Sodium cyanide (2 mM) was added to the aqueous portion, and the reaction mixture was extracted twice with diethyl ether. The combined ether solution was evaporated, and its residue was analyzed. Control experiments were also run in which the sodium bisulfite, sodium cyanide, or both were omitted in incubation.

Clinical Sample. Blood (24 ml) was drawn from a patient 1 hr after the i.v. infusion of 4500 mg of cyclophosphamide (50 mg/kg). After addition of heparin, the sample was centrifuged, and the plasma was decanted. Sodium bisulfite (15 mg) was added and mixed. Ten min later, sodium cyanide (10 mg) was added, and the reaction mixture was extracted with an equal volume of diethyl ether. The ether solution was evaporated, and its residue was examined by gas chromatography-mass spectrometry.

RESULTS

Treatment of aldophosphamide with sodium bisulfite and sodium cyanide produced a cyanohydrin derivative identified primarily on the basis of the chemical ionization mass spectrum shown in Chart 2. The molecular weights (M + H at m/e 304, 306, and 308) are consistent with the proposed dichlorinated structure. In this spectrum ions comprising the (protonated) nor-nitrogen mustard part of the molecule are seen (m/e 142, 144, and 146). The elimination of HCN is detected as a fragmentation process, common to the spectra of N-ethylaldophosphamide cyanohydrin and N,N-dimethylaldophosphamide cyanohydrin as well.

When the cyanohydrin derivative was trimethylsilylated and introduced into the mass spectrometer via the gas chromatograph, a single major compound was found to be present (apart from silylating reagent) with a retention time of 9.0 min under the conditions used. The mass spectrum (Chart 3) of the material eluted from the gas chromatograph confirms that the cyanohydrin derivative carries 2 trimethylsilyl groups and that HCl is readily eliminated after ionization of molecules heated by passage through the gas chromatograph and interface. The symmetry of the peak in the gas chromatogram suggests that pyrolysis does not occur before ionization in the mass spectrometer.

Once authentic aldophosphamide had been characterized by gas chromatography and mass spectrometry, it was possible to look for that compound as a metabolite of cyclophosphamide. Initially, an in vitro system was examined from which larger amounts of the metabolite could be obtained with less contamination than from in vivo sources. Thus cyclophosphamide was incubated with mouse liver microsomes, with sodium bisulfite present as a trapping agent for aldehydes and for ketones (4). After chloroform extraction to remove unmetabolized cyclophosphamide and other lipophilic contaminants, any bisulfite derivatives present were converted to cyanohydrins (7, 9), and the mixture was extracted with ether. The spectrum of the residue of this ether extract obtained via the direct probe is shown in Chart 4. The high-mass end of this spectrum is similar to that obtained from authentic aldophosphamide.
Chart 2. Chemical ionization mass spectrum of aldophosphamide cyanohydrin introduced on the direct probe.

Chart 3. Chemical ionization mass spectrum of bis(trimethylsilyl)aldophosphamide cyanohydrin introduced through the gas chromatograph.

Chart 4. Chemical ionization mass spectrum of aldophosphamide cyanohydrin extracted from microsomal incubation mixture and introduced on the direct probe.
cyanohydrin. In particular, 3 molecular ion species (M + H) with masses 304, 306, and 308 are present in relative intensities of about 9:6:1. These represent the combinations of $^{35}\text{Cl}$ and $^{37}\text{Cl}$ at natural abundance in aldophosphamide cyanohydrin. The 3-peak dichloro pattern is seen again in the M + H – HCN fragment ions of masses 277, 279, and 281. The relative abundances of M + H (m/e 304) and M + H – HCN (m/e 277) are different in the 2 spectra and vary in repetitive scans of the same microsomal extract sample. This kind of variation of relative intensities of peaks with time is usually considered characteristic of a mixture, and the possibility cannot be eliminated that some 4-hydroxycyclophosphamide is present in this sample. Unmetabolized cyclophosphamide contributes the M + H peaks at m/e 261, 263, and 265 in the spectrum in Chart 4.

The cyanohydrin in the ether extract was also characterized by combined gas chromatograph mass spectrometry after trimethylsilylation. The gas chromatogram is shown in Chart 5. The partial mass spectrum presented in Chart 6 was obtained from the major component in the mixture (Chart 5, Peak A), which eluted from the gas chromatograph at the identical retention time (9.0 min) as the bis(trimethylsilyl) derivative of authentic aldophosphamide cyanohydrin. The similarity of the mass spectra (Charts 3 and 6) speaks for itself, particularly the chlorine isotope patterns in the protonated molecular ion species and the major fragment ion. Thus the identification of aldophosphamide as a metabolite formed in vitro is based on its chemical behavior with the trapping agent, on the gas chromatographic retention time of its trimethylsilylated cyanohydrin derivative, on the mass spectra of 2 derivatives measured under different conditions, and on the identity or similarity of these with the same measurements made on authentic material.

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The compound characterized as aldophosphamide cyanohydrin was also found when cyclophosphamide was incubated with microsomes in the presence of sodium cyanide instead of sodium bisulfite, although in a smaller amount. Similarly, it was obtained from incubations done in the absence of both sodium bisulfite and sodium cyanide, to which sodium cyanide was added at the end. This compound could not be detected in extracts from incubations that were not treated at some point with sodium cyanide.

After determining that a derivative of aldophosphamide could be isolated from the in vitro system, the analytically more difficult task was addressed, that of detecting this metabolite in the blood of patients treated with cyclophosphamide. On the basis of previous experience with the active metabolite phosphoramide mustard, it was expected that greater sensitivity would be required for analysis of patient blood and, more especially, that greater selectivity would be required to detect aldophosphamide in the milieu of the many endogenous contaminants in plasma extracts. As in the earlier work with phosphoramide mustard, the mass spectrometer was used in the selected ion monitoring mode as a highly specific and sensitive gas chromatograph detector.

Chart 7 shows the selected ion profiles of the 2 abundant ions of masses 412 and 414, formed from authentic bis(trimethylsilyl)aldophosphamide cyanohydrin and the profiles of these same 2 ions monitored during the chromatographic separation of the plasma extract. These peaks were absent in ion profiles of the extract of plasma from a volunteer not receiving cyclophosphamide. The derivative of aldophosphamide is judged to be present, based on coincident retention time with the standard, on the formation of ions of the same masses as those characteristic of the standard, and on the occurrence of these ions with relative abundances (3:1) appropriate to the presence of a chlorine atom and the same as those observed in the selected ion profiles of the reference material.

**DISCUSSION**

Although the position of the postulated equilibrium between 4-hydroxycyclophosphamide II a and aldophosphamide II b is not known, formation of a derivative of II b will continuously displace the equilibrium, and the bisulfite and cyanohydrin formed will include both original isomer populations. Indeed, aldophosphamide cyanohydrin can be recovered in excellent yield when 4-hydroxycyclophosphamide is equilibrated in solution and treated with bisulfite and cyanide. Since a carbonyl group is required for derivatization (4), conversion of II a to II b must be facile. Thus the derivatization techniques and gas chromatography-mass spectrometry-selected ion monitoring method developed here could be used to assay levels of 4-hydroxycyclophosphamide-plus-aldophosphamide in physiological fluids, preferably with a stable isotope-labeled analog added as internal standard.

The facile formation of the bisulfite and cyanohydridn adducts demonstrates the reactivity of the aldehyde group in aldophosphamide. This reactivity must be kept in mind in evaluating the intrinsic toxicity of the compound and also
potential deactivation reactions that might compete with its decomposition to phosphoramid mustard.

A number of lines of evidence have suggested that the metabolic scheme shown in Chart 1 is correct and that 4-hydroxycyclophosphamide and its isomer, aldophosphamide, are the compounds initially formed in the metabolic activation of cyclophosphamide. (a) Carboxycyclophosphamide and 4-ketocyclophosphamide have been shown to be oxidation products not of cyclophosphamide, but of some intermediate metabolite (6, 13). Aldophosphamide and 4-hydroxycyclophosphamide were suggested as reasonable precursors to these products. (b) The metabolic product phosphoramid mustard would be expected to result from $\beta$ elimination of acrolein from aldophosphamide (2). (c) Sladek (10) demonstrated that the activation of cyclophosphamide by hepatic microsomes generated material that gave a positive color test for aldehydes and that activation of cyclophosphamide in the presence of semicarbazide produced a new product, detectable on thin-layer chromatographic analysis. Material with the same thin-layer mobility was obtained from rat blood treated with semicarbazide. Sladek interpreted this product to be the semicarbazone of aldophosphamide. (d) 4-Hydroxycyclophosphamide was synthesized by Takamizawa et al. (14) and shown to be cytotoxic in vitro. (e) Struck (11) treated synthetic 4-hydroxycyclophosphamide with semicarbazide in aqueous solution and was able to isolate and identify by IR spectrophotometry and proton magnetic resonance spectrometry the semicarbazone of aldophosphamide. In several thin-layer chromatographic systems this compound had the same mobility.
as did Sladek’s putative semicarbazone. (f) Voelcker et al. (15, 16) have described qualitative and quantitative analyses by thin-layer chromatography of 4-hydroxycyclophosphamide and aldophosphamide from both synthetic and biological sources (12).

Although the information cited above has strongly supported the occurrence of 4-hydroxycyclophosphamide and/or aldophosphamide as metabolites, the definitive identification of these compounds from biological systems has been difficult. Connors et al. (3) have described the isolation and identification by mass spectrometry of 4-ethoxycyclophosphamide from microsomal incubation mixtures of cyclophosphamide treated with ethanol. Although the authors suggested that this derivative arose from 4-hydroxycyclophosphamide by displacement of the hydroxyl group by the ethoxy group, it might alternatively arise from addition of ethanol across the double bond of imino intermediate IX (7, 9) or by addition of ethanol to the aldehyde and recyclization of the hemiacetal.

The identification reported here of aldophosphamide cyanohydrin from microsomal incubation and from the plasma of a patient treated with cyclophosphamide confirms aldophosphamide as a metabolic product of cyclophosphamide formed both in vitro and, in humans, in vivo. However, the relative chemical and biological properties of 4-hydroxycyclophosphamide, aldophosphamide, phosphoramid mustard, and the putative imino intermediate IX remain to be defined.

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