Metabolism of Iphosphamide [2-(2-Chloroethylamino)-3-(2-chloroethyl)tetrahydro-2H-1,3,2-oxazaphosphorine 2-Oxide] and Production of a Toxic Iphosphamide Metabolite

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SUMMARY

Iphosphamide [2-(2-chloroethylamino)-3-(2-chloroethyl)tetrahydro-2H-1,3,2-oxazaphosphorine 2-oxide] is metabolized by mouse liver microsomes in the presence of reduced triphosphopyridine nucleotide and oxygen. When purified aldehyde oxidase is added, the initial metabolites do not appear in quantity; but a single, anionic product is present. After chemical methylation, the mass spectrum of the product of the aldehyde oxidase reaction is identical to that of 2-carbomethoxyethyl N,N'-bis(2-chloroethyl)phosphorodiamidate.

The initial oxidation reaction for Iphosphamide has a Km of 1.0 mM, and cyclophosphamide is a competitive inhibitor with a Ki of 1.1 mM. Iphosphamide is a competitive inhibitor of cyclophosphamide oxidation, with a Ki of 1.0 mM compared to a Km of 0.5 mM.

The initial metabolites of Iphosphamide are toxic to L1210 cells. Mice given injections of these cells, which have been exposed to the initial metabolites, have a longer lifespan than those inoculated with untreated cells. The product of the aldehyde oxidase reaction is not toxic in such tests.

Dogs rapidly metabolize Iphosphamide, and only a small amount of unchanged compound appears in the urine. Metabolites that have been isolated from dog urine are 4-keto-Iphosphamide and (after chemical methylation) 2-carbomethoxyethyl N,N'-bis(2-chloroethyl)phosphorodiamidate.

INTRODUCTION

Iphosphamide [2-(2-chloroethylamino)-3-(2-chloroethyl)tetrahydro-2H-1,3,2-oxazaphosphorine 2-oxide] differs from cyclophosphamide and other nitrogen mustard compounds in that the 2 functional chloroethyl groups are not attached to the same nitrogen atom. As is true for cyclophosphamide, Iphosphamide is not toxic as such but must be enzymatically activated to exert its effect (5). In studies with experimental animal tumors, Iphosphamide is generally more active than cyclophosphamide (5, 23, 24), but it is not quite as effective as cyclophosphamide in suppressing the immune response to injected antigens (8). A study of Iphosphamide as an antitumor agent in the treatment of human cancer shows that some patients with either bronchial carcinoma, ovarian carcinoma, lymphosarcoma, or Ewing's sarcoma respond to a single, large dose of the agent (22). The therapeutic effect is limited by nephrotoxicity.

The structural and biological similarities between Iphosphamide and cyclophosphamide led us to study the metabolism of Iphosphamide. Our results indicate that it is metabolized by mouse liver microsomes in vitro and by the intact dog to compounds analogous to those previously found for cyclophosphamide (9).

MATERIALS AND METHODS

Iphosphamide-14C, labeled in the carbons of the 2-chloroethylamine group attached to the phosphorus atom, was obtained from Dr. Robert Engle, Drug Development Branch, Cancer Chemotherapy National Service Center, National Cancer Institute, Bethesda, Md. There were no detectable impurities on paper chromatography of the sample. Ring-labeled cyclophosphamide-6-14C was a gift from the Mead-Johnson Research Center, Evansville, Ind., and was supplied to us by Dr. G. P. Wheeler. It was further purified by column chromatography on DEAE-Sephadex A-25 and was found to contain no detectable impurities on paper chromatography or by mass spectrometry.

Mass spectra were obtained with a Hitachi high-resolution, double-focusing RMU-6-D-3 mass spectrometer. Procedures for toxicity tests with L1210 cells have been described (18).

Microsomes were prepared from the livers of female DBA/2 mice as previously reported (9). The mice, weighing 20 to 25 g, were killed by cervical dislocation; the livers were excised, cooled, and homogenized in 3 volumes of 0.25 M sucrose. The homogenate was centrifuged at 9,000 x g for 15 min, and microsomes were obtained from the resulting supernatant by centrifugation at 100,000 x g for 45 min and were washed with 0.25 M sucrose.
Aldehyde oxidase\textsuperscript{2} was purified to near homogeneity from rabbit liver by the method of Rajagopalan and Handler (16). The specific activity was 2.5 units/mg, with 1 unit being defined as the amount of enzyme producing a change at 300 nm of 1 absorbance unit/min at 25° with 10\textsuperscript{–}mole sodium-potassium phosphate buffer (pH 7.3), 6.9 \textmu molar; Iphosphamide-\textsuperscript{14}C, 200 \textmu molar (0.1 \textmu Ci); and water to a final volume of 175 \textmu l. The amounts of microsomes and TPNH were optimal. The reaction was initiated by addition of the micromolar preparation and was stopped by streaking a 50-\textmu l portion on paper strips. Incubation of the preparation was for 20 min in an open tube at 37°. The reaction was linear for this period of time. Substrate and metabolites were separated by paper chromatography with isopropyl alcohol: NH\textsubscript{4}H\textsubscript{2}O (80:5:15, by volume) and measured by scanning with a Packard 7201 radiochromatogram scanner. Each assay was performed in duplicate. For oxidation of cyclophosphamide-\textsuperscript{14}C, the reaction was similar, except that the incubation period was 30 min (9). The optimum pH was 7.3.

Protein was determined by the method of Lowry et al. (12).

A beagle dog (9.2 kg) was given an i.v. injection of 188 mg (46 \textmu Ci) of Iphosphamide-\textsuperscript{14}C, and blood samples and urine were collected for 24 hr. The blood samples and portions of the urine were extracted with ethylene dichloride to separate the unchanged drug from its metabolites (13).

Thin-layer chromatography was performed on Analtech preparative silica gel plates. Column chromatography of urinary Iphosphamide metabolites was accomplished with DEAE-Sephadex A-50 and a gradient of NH\textsubscript{4}HCO\textsubscript{3}, as described previously (20).

RESULTS

Chemical Synthesis of 2-Carbomethoxyethyl N, N'-Bis(2-chloroethyl)phosphorodiamidate (Carbomethoxy-Iphosphamide). A solution of methyl \beta-hydroxypropionate (1.8 g, 17 \textmu molar) and triethylamine (2.5 ml) in ether (30 ml) was added dropwise with stirring at room temperature to 2-chloroethylphosphorodiamicid chloride (4.8 g, 24 \textmu molar) in ether (100 ml). The mixture was stirred for 16 hr at room temperature and filtered. The filtrate was treated with a solution of 2-chloroethylamine free base (prepared as described below) and triethylamine (2.5 ml) in ether (100 ml). The mixture was stirred for 3 days at room temperature and filtered. Evaporation of the filtrate gave a syrup, which was purified by preparative thin-layer chromatography in acetone:chloroform (3:1, \textnu v/\textnu v). The alkylation band of \textnu f \textnu 0.7, as judged by reaction with NBP\textsuperscript{•} (6), was scraped from the thin-layer plate, washed with acetone, and filtered. Evaporation of the filtrate gave a syrup the mass spectrum of which was consistent with that expected for carbomethoxy-Iphosphamide. The spectrum also contained a strong peak (m/e 199), presumably arising from M\textsuperscript{+} 248 – CH\textsubscript{2}Cl. A reasonable structure for a contaminant with a mass number of 248 is C\textsubscript{14}H\textsubscript{14}O\textsubscript{7}P(\textnu)\textnu\textnu\textnu\textnu(NH\textsubscript{2})\textnu\textnu\textnu\textnu\textnu\textnu\textnu\textnu\textnu\textnu\textnu\textnu•(CH\textsubscript{2}CH\textsubscript{2}Cl)\textsubscript{2}. The sample was purified further by repeated, preparative thin-layer chromatography in chloroform:methanol (95:5), and the strongest alkylating band (R\textsubscript{f} 0.23) was scraped from the thin-layer plate and washed with acetone. Filtration and subsequent evaporation of the filtrate gave purified carbomethoxy-Iphosphamide (Chart 4). The infrared spectrum showed strong bands at 3210, 2955, 1735, 1435, 1200, 1110, 1035, 950, and 660 cm\textsuperscript{–}1.

2-Chloroethylphosphoramic dichloride was prepared by the following procedure. 2-Chloroethylamine hydrochloride (30 g) in freshly distilled phosphorus oxychloride (150 ml) was refluxed for 16 hr with stirring. Excess phosphorus oxychloride was removed by evaporation in a vacuum, giving a clear, colorless syrup the mass spectrum of which was as expected for 2-chloroethylphosphoramic dichloride. A portion of the product was used without further purification in the procedure described above.

2-Chloroethylamine free base was prepared from the hydrochloride (3.5 g) by treating a stirred, concentrated, aqueous solution of the hydrochloride with concentrated potassium hydroxide solution to pH 8 to 9 in the presence of ether (50 ml) while cooling the materials in an ice bath. The ether layer was separated, and the aqueous layer was extracted with ether (50 ml). The ether layers were combined, dried over anhydrous sodium sulfate, and filtered. The ether solution of the free base was used in the procedure described above.

Chemical Synthesis of 4-Keto-Iphosphamide. Iphosphamide (100 mg) in water (5 ml) was added to a solution of ferric sulfate heptahydrate (400 mg) in water (4 ml). The solution was cooled in an ice bath and treated dropwise with stirring in 10 min in a nitrogen atmosphere with a solution of 30% hydrogen peroxide (200 mg) in water (1 ml). The mixture was stirred for 1 hr at 0° and filtered. The filtrate was extracted 4 times with chloroform, 25 ml each time. The extract was dried over anhydrous sodium sulfate, filtered, and evaporated in a vacuum. The residue was purified by thin-layer chromatography in acetone:chloroform (1:1), giving NBP-positive bands at R\textsubscript{f} 0.55 and 0.35. Elution with acetone and mass spectral analysis of the residues indicated that the faster-moving band was 4-keto-Iphosphamide and the other was Iphosphamide. The specimen of 4-keto-Iphosphamide crystallized upon standing. Trituration with a small amount of ethanol gave crystalline 4-keto-Iphosphamide: m.p., 110°; mass spectrum, M\textsuperscript{+} 274, m/e 239 (M – Cl, 1 Cl), m/e 225 (M – CH\textsubscript{2}Cl, 1 Cl, base peak); infrared spectrum (cm\textsuperscript{–}1): 3230 (NH); 2950, 2920, 2850 (CH); 1690 (strong, C=O); 1445, 1370, 1320, 1290, 1250, 1230, 1210, 1100, 1035, 965, 945, 810, 790, 690, 580, 570, 505.

Enzymatic Production of Metabolites. After incubation...
of Iphosphamide-14C with microsomes and TPNH, 4 radioactive peaks with $R_F$ values of 0.03, 0.55, 0.63, and 0.82 consistently appeared in addition to the substrate on paper chromatography of the preparation (Chart 1). No metabolic peaks appeared in the absence of either microsomes or TPNH. However, if purified aldehyde oxidase (0.4 unit) was present initially in the reaction mixture, each of these peaks was either greatly reduced or absent, and a new peak with an $R_F$ of 0.68 appeared. This product was formed quantitatively at the expense of the initial metabolites and was moved toward the positive pole on electrophoresis at pH 7.0.

A survey of various mouse tissues, performed as described previously (9), showed that liver and lung could but kidney, spleen, muscle, brain, intestine, L1210 lymphoma, and Sarcoma 180 could not metabolize Iphosphamide. The rate of reaction for 900 x g supernatants of liver homogenates was 9.0 nmoles of products per mg protein per 30 min; that for lung tissue was 2.0 nmoles per mg per 30 min. For all other tissues, the rate was less than 0.5 n mole per mg per 30 min.

Iphosphamide was a competitive inhibitor of the oxidation of cyclophosphamide by the microsomal system (Chart 2). In a single experiment, the inhibition constant ($K_i$) was 1.0 mM, compared to a Michaelis constant ($K_m$) for cyclophosphamide of 0.5 mM. The kinetic values for cyclophosphamide oxidation by this system have been reported (8). For the reverse experiment (Chart 3), cyclophosphamide had a $K_i$ of $1.1 \pm 0.3$ mM (standard deviation for 3 separate determinations) and Iphosphamide had a $K_m$ of $1.0 \pm 0.1$ mM (standard deviation for 4 separate determinations).

Identification of the Product Formed by Aldehyde Oxidase. The fact that addition of aldehyde oxidase to the

Chart 1. Paper chromatography of Iphosphamide and metabolites. A, standard assay with mouse liver microsomes; B, standard assay with microsomes and added aldehyde oxidase (0.4 unit).

Chart 2. Inhibition of cyclophosphamide metabolism by Iphosphamide. The standard assay for cyclophosphamide oxidation was used, except that the concentration of cyclophosphamide was varied as indicated. Ordinate, reciprocals of the $\mu$moles of products formed.

Chart 3. Inhibition of Iphosphamide by cyclophosphamide. The standard assay for Iphosphamide oxidation was used, except that the concentration of cyclophosphamide was varied as indicated. Ordinate, reciprocals of the $\mu$moles of products formed. The $K_m$ and $K_i$ values displayed are for this particular experiment. X, 1.43 mM cyclophosphamide; A, 2.29 mM cyclophosphamide; O, no inhibitor.
Metabolism of Iphosphamide

**Biological Evaluation of the Initial Metabolites.** The evaluation of toxicity of the initial oxidation products of Iphosphamide involved using the microsomal system to generate the products and adding 4 ml of L1210 cells to 1 ml of reaction mixture for a final concentration of $4 \times 10^7$ cells/ml. The exposure period was 1 hr at 35°. For the complete system, 21 μg of initial metabolites were generated in 1 ml, so that the final concentration in the cell suspension was 4.2 μg/ml. The initial metabolites were converted to a single product when aldehyde oxidase was present. The cells were washed by centrifugation with 0.9% NaCl solution and then injected into C57 x DBAF, mice at $2 \times 10^8$ cells/mouse with 10 animals in each test group. Their life-span was observed. The results of varying the reaction components are in Table 1. The initial metabolites of Iphosphamide increased the life-span from 8 to 12 days, an indication that 99.9% of the treated cells were killed (18). Removal of these products by aldehyde oxidase returned the value to that of the controls. Neither Iphosphamide, nor microsomes, nor aldehyde oxidase alone had any effect on the life-span. Microsomes plus aldehyde oxidase and Iphosphamide plus aldehyde oxidase were also ineffective. We determined that Iphosphamide was not altered by aldehyde oxidase. The entire experiment was monitored by taking samples for paper chromatography to determine that the appropriate changes had occurred.

**Serum Levels and Urinary Excretion of Iphosphamide.** Iphosphamide-$^{14}$C was metabolized rapidly by a beagle dog. The half-life of the agent in the serum was less than 30 min. Within 1 hr after injection, less than 10% of the radioactivity in the serum was unchanged Iphosphamide. At 6 hr, none could be detected. One hr after injection of the drug, 95% of the material in the urine was in the form of metabolites. Within 24 hr, 84% of the dose was recovered in the urine; of this, only about 2% was unchanged Iphosphamide.

**Characterization of the Urinary Metabolites.** Urine (0 to 12 hr) from a beagle dog given side-chain-labeled Iphosphamide-$^{14}$C was collected by a catheter and frozen immediately upon collection. The urine was filtered through Celite and extracted 4 times with chloroform, 100 ml each time, giving an extract (see below) and an aqueous layer, which was divided into 2 portions (one-fourth and three-fourths) and lyophilized. The solid residue from the smaller fraction was extracted successively with acetone and methanol. The remaining solid was dissolved in water and acidified to pH 3, and the solution was lyophilized. The residue was subjected to mass spectral analysis and was found to give a spectrum corresponding to the fragmentation of carboxymethoxy-Iphosphamide Chart 4).

<table>
<thead>
<tr>
<th>Components of reaction system</th>
<th>Metabolites (μg/ml) in cell suspension</th>
<th>Median day of death for 10 mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (saline control)</td>
<td>0.0</td>
<td>8.0</td>
</tr>
<tr>
<td>Complete system</td>
<td>4.2</td>
<td>12.0</td>
</tr>
<tr>
<td>Minus microsomes</td>
<td>0.0</td>
<td>7.5</td>
</tr>
<tr>
<td>Minus Iphosphamide</td>
<td>0.0</td>
<td>7.0</td>
</tr>
<tr>
<td>Minus Iphosphamide plus aldehyde oxidase</td>
<td>0.0</td>
<td>8.0</td>
</tr>
<tr>
<td>Plus aldehyde oxidase</td>
<td>0.0</td>
<td>8.0</td>
</tr>
<tr>
<td>Minus Iphosphamide minus microsomes plus aldehyde oxidase</td>
<td>0.0</td>
<td>8.0</td>
</tr>
<tr>
<td>Minus microsomes plus aldehyde oxidase</td>
<td>0.0</td>
<td>8.0</td>
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stances as judged by reaction with NBP. The major alkylation component of the neutral methanol extract (Rf 0.2) was separated by preparative thin-layer chromatography in chloroform: methanol (3:1) and eluted with methanol. The eluted material was treated with excess ethereal diazomethane and evaporated. The methylated metabolite was purified further by thin-layer chromatography in chloroform: methanol (95:5). The NBP-positive band at Rf 0.15 was eluted with methanol and filtered; the solvent was evaporated, giving the purified, methylated metabolite. Thin-layer chromatographic comparison with synthetic carbamethoxy-Iphosphamide in 3 solvent systems (acetone: chloroform, 3:1; chloroform: methanol, 95:5; and chloroform: methanol, 3:1) on silica gel indicated identical rates of migration for the two. Mass spectral analysis identified carbamethoxy-Iphosphamide (Chart 4) as well as some methyl N,N′-bis(2-chloroethyl)phosphorodiamidate [CH3OP(O)(NHCH2CH2Cl)] [M + 234 (2 Cl); m/e 199 (1 Cl) (M – Cl); m/e 185 (1 Cl, M – CH2Cl)], in the methylated metabolite fraction. The precise source of methyl N,N'-bis(2-chloroethyl)phosphorodiamidate is not known.

The remaining portion of the dog urine was separated by column chromatography on DEAE-Sephadex A-25 with an NH4HCO3 gradient as previously described (20), and 3 radioactive fractions (A, B, and C) were observed. Thin-layer chromatography on silica gel of the residue obtained upon lyophilization of Fraction A indicated an NBP-positive component with an Rf identical to that of Iphosphamide in acetone: chloroform (1:1). Fractions B and C were lyophilized. The residues were triturated with methanol and filtered, and the filtrate was treated with excess ethereal diazomethane. After evaporation, the methylated fractions were analyzed by mass spectrometry. Thin-layer and mass spectral analysis of methylated Fraction B identified carbamethoxy-Iphosphamide. The mass spectrum of the methylated Fraction C revealed no chlorine-containing fragments, and thin-layer chromatography indicated only weak alkylation activity in comparison with Fraction B. This fraction was not further characterized.

A chloroform extract of 0-12 hr urine from a beagle dog given Iphosphamide-14C was evaporated, giving a small residue (10 mg). Analysis of the material on a thin-layer plate in chloroform: methanol (95:5) gave a major NBP-positive band at Rf 0.25 (identical by thin-layer and mass spectral analysis to Iphosphamide) and a minor NBP-positive band at Rf 0.1, which was eluted with acetone, filtered, and evaporated. Thin-layer chromatography of the residue in chloroform: methanol (95:5) along with synthetic 4-keto-Iphosphamide showed that the two were identical. Mass spectral analysis gave a fragment of m/e 225 (1 Cl, M + 274 – CH2Cl) along with a very weak fragment of m/e 211, presumably arising from a small amount of Iphosphamide in the sample. Although the mass spectrum of Iphosphamide also contains a peak of m/e 225 (1 Cl), this peak (m/e 225) is much weaker (ca. 2%) than the peak of m/e 211. Mass spectral analysis of crystalline, synthetic 4-keto-Iphosphamide showed a base peak of m/e 225 (1 Cl), with the 2nd most abundant fragment corresponding to loss of chlorine (m/e 239). A weak molecular ion peak (M+ 274, 2 Cl) was also observed.

DISCUSSION

We are not certain of the identity of the initial metabolites of Iphosphamide-14C, which appear as 4 radioactive peaks on paper chromatography. Their toxicity is similar to that for the initial metabolites of cyclophosphamide (9). Since none of these are found in quantity when aldehyde oxidase is added to the system, 1 of them is likely to be the open-chain aldehyde, for which we propose the trivial name "aldo-Iphosphamide" (Chart 5). This name is applied in accord with naming the product of cyclophosphamide oxidation "aldophosphamide" (9). The other peaks may represent the ring-closed, 4-hydroxy isomers of Iphosphamide and fragments arising from aldo-Iphosphamide, which could break down after formation in a system lacking aldehyde oxidase. For 2-carboxyethyl N,N'-bis(2-chloroethyl)phosphorodiamidate, the compound in the presence of aldehyde oxidase, we propose the trivial name "carboxy-Iphosphamide." This name is chosen to conform to that applied to a similar metabolite of cyclophosphamide (9, 19, 20).

The Km for Iphosphamide, 1.0 mM, is higher than that for cyclophosphamide, which is 0.5 mM. However, the Vmax, 4.5 umoles per g of liver per hr, is also higher than that for cyclophosphamide, which is 1.5 umoles per g per hr. Although the kinetic plots indicate that the inhibition between the 2 substrates is competitive, classical competitive inhibition is not apparent because the K for cyclophosphamide (1.1 ± 0.3 mM) is not equal to its Km (0.5 ± 0.1 mM).* Nevertheless, the enzyme oxidizing cyclophosphamide and nicotine (9) is likely to be the same as that oxidizing Iphosphamide, for the tissue distribution of the activities is the same; and similar substrates are involved. Using NBP in a colorimetric reaction, Allen and Creaven (3) have found a Km of 19.4 mM for the metabolism of Iphosphamide by rat liver microsomes. The difference between this value and the one reported here may be due to use of different animal species and to different assay procedures. Large differences in kinetic values have been reported for the oxidation of cyclophosphamide by rat liver microsomes (see Ref. 9).

Acrolein has recently been found as a metabolite of both Iphosphamide and cyclophosphamide, and the investigators propose that it contributes to the cytostatic activity of these compounds (1, 2). In our studies on the metabolism of ring-labeled cyclophosphamide, we did not detect acrolein, although its existence was not excluded (9). In the investigation reported here, acrolein could not have been detected, because we used side-chain-labeled Iphosphamide. Nevertheless, we do not think it likely that acrolein is the

* The Km for cyclophosphamide in this system is 0.5 ± 0.1 mM (S.D. for 17 separate experiments) (D. L. Hill, unpublished data).
metabolite that is principally responsible for the activity of either cyclophosphamide or Iphosphamide for the following reasons. (a) The analog of cyclophosphamide in which the side chain is replaced by a diethylamino group readily liberates acrolein in a microsomal system, as do Iphosphamide and cyclophosphamide (2). However, this analog is only one-third as toxic to rats as cyclophosphamide and has no detectable antitumor effect (4). (b) Cell lines that are resistant to cyclophosphamide are also resistant to other alkylating agents (7, 10, 11, 14, 17, 21), an indication that cyclophosphamide functions primarily as this type of compound. As judged by reaction with NBP, acrolein has no alkylating activity (R. F. Struck, unpublished material), and it also has no antitumor effect against L1210 leukemia in mice (W. R. Laster, Jr., unpublished material).

REFERENCES

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