Comparative Metabolism of 2-[Bis(2-chloroethyl)amino]tetrahydro-2H-1,3,2-oxazaphosphorine-2-oxide (Cyclophosphamide) and Its Enantiomers in Humans


ABSTRACT

The comparative metabolism of the enantiomers of cyclophosphamide and of the racemate has been studied in humans. Four patients were each given, sequentially, the racemate, the (+)-enantiomer, and its (-)-antipode. The plasma levels of parent drug and the urinary output (24 hr) of unchanged drug and of two enzymatically produced metabolites, 4-ketocyclophosphamide and carboxyphosphamide, were determined using mass spectrometry-stable isotope dilution. There was no significant difference between the three forms of cyclophosphamide with respect to plasma half-life (\( \beta \) phase) or in the urinary outputs of the drug or of carboxyphosphamide. The output of 4-ketocyclophosphamide after administration of (+)-cyclophosphamide was significantly greater than that produced from the racemate. Cyclophosphamide recovered from the urine of patients given the racemate was either racemic or only slightly enriched in the (-)-enantiomer. The two enantiomers were almost equally bound to plasma protein.

Based on these metabolic studies alone, there is little reason to predict that the enantiomers will differ from each other or from the racemate in their therapeutic effects in humans, but there are other factors, e.g., stereoselective uptake of the intermediary 4-hydroxylated metabolites by neoplastic cells, which could elicit such differences.

INTRODUCTION

Since its introduction into clinical practice 20 years ago, cyclophosphamide has become one of the most commonly used antineoplastic drugs with a broad spectrum of activity against solid tumors and hematological cancers. Despite the wealth of clinical and experimental knowledge generated during this time, one feature of this compound that has not previously been explored in humans relates to the structure of the molecule itself. Cyclophosphamide is a dissymmetrical molecule by virtue of the chiral phosphorus atom, and optical isomers are therefore possible. Hitherto, the drug has been administered clinically as the racemate. However, there are numerous reports of therapeutic differences between enantiomers of drugs, and this fact has prompted the synthesis and biological evaluation of the enantiomers of cyclophosphamide.

As examples of this phenomenon may be cited the greater central nervous system stimulant activity of (+)-amphetamine compared with that of its (-)-isomer (23), the superior anticoagulant activity of S-(-)-phenprocoumon compared with the R-(+)-isomer (10), the inactivity of (+)-methadone in the maintenance of opiate-dependent patients (21), and the superior analgesic activity of (+)-propoxyphene compared with the (-)-antipode (4). In the area of cancer chemotherapy, melphalan (3-[4-bis(2-chloroethyl)aminophenyl]-L-alanine) was selected for clinical use in preference to the \( \delta \) isomer (medphalan) on the basis of the superior potency of the L isomer against the Walker 256 carcinoma in rats (2). The L-mannitol analog of mannotyl myleran (1,6-dimethanesulfonyl-1,6-dideoxy-D-mannitol) has been reported to have very low antitumor activity compared with the \( \delta \) antipode (3). These examples concern molecules which are dissymmetrical by virtue of a chiral carbon atom. As an example of a biological difference based on chirality at phosphorus may be cited the greater enzyme inhibition by the S-(-)-isomers of some anticholinesterases, the alkyl S-alkylmethylphosphonothioates compared with their R-(+)-antipodes (9).

Recent syntheses of the enantiomers of cyclophosphamide (14) and of isotopically labeled (deuterated) variants thereof (5) have enabled their antitumor activity and metabolism to be separately evaluated. Therapeutic differences have been reported in tests against 2 murine tumors. Against the ADJ/PC6A plasma cell tumor, (-)-cyclophosphamide had approximately twice the therapeutic index (50% lethal dose/90% inhibitory dose) of the (+)-antipode (6). Smaller differences were observed in tests against the L1210 leukemia; male mice survived longest when treated with (-)-cyclophosphamide, whereas female mice showed the best response with the (+)-isomer (24). The metabolism of the enantiomers has been compared in several species (mouse, rabbit, and rat), both in vitro, using liver microsomes, and in vivo (5, 7). Particular attention has been paid to the influence of chirality upon the rate of the initial 4-hydroxylation step (Chart 1) and upon the yields of the enzymatically produced detoxification products 4-ketocyclophosphamide and carboxyphosphamide. It has been suggested that the relatively selective toxicity of cyclophosphamide toward tumor tissue is related to the more efficient detoxification thereto of 4-hydroxycyclophosphamide and aldophosphamide by normal host tissues (8). If this hypothesis is valid, then differences between the enantiomers regarding the relative yields of these products could result in different therapeutic efficacies, as could differences between the rates of metabolism of the enantiomers in the initial 4-hydroxylation step.

The main objective of this study in humans has therefore...
Human Metabolism of Cyclophosphamide Enantiomers

been to determine whether stereoselectivity in these enzymatic pathways of metabolism is sufficiently pronounced to justify a more extended clinical evaluation of either enantiomer for therapeutic advantage over the racemate. To this end, the pharmacokinetic parameters of each enantiomer and the racemate have been compared, as have the urinary outputs of unchanged drug and the detoxification products 4-ketocyclophosphamide and carboxyphosphamide. Furthermore, the enantiomeric composition of unchanged cyclophosphamide recovered from the urine following administration of racemic cyclophosphamide has been determined to provide additional evidence regarding stereoselective metabolism in the initial 4-hydroxylation step.

Differences in affinity for plasma proteins is another factor which could result in differential metabolism of the cyclophosphamide enantiomers. Thus glifumide, a β-cytotropic antidiabetic drug, differs in its clinical properties from its enantiomer glisopexide, and this difference has been ascribed to much higher affinity of glifumide for human plasma protein (11). Since cyclophosphamide is appreciably bound (24%) to human plasma proteins (11), it seemed desirable to determine the relative binding efficiencies of its enantiomers.

MATERIALS AND METHODS

Patients. The 4 patients in this study had squamous cell carcinoma of the lung. Their mean age was 61 (range 54 to 68). Patient E. D. was female. All patients had normal renal and hepatic function as measured by standard biochemical tests. None had received cyclophosphamide previously.

Doses (1 g i.v.) of racemic cyclophosphamide, the (+)- and the (—)-enantiomer, were administered as a bolus, sequentially, with a 3-week interval between doses. Blood samples were taken at 5, 10, and 30 min and at 1, 2, 4, 6, and 12 hr. Urine was collected between 0 and 24 hr after administration of the drug. Patients were catheterized, and urine was passed into collection bags which were emptied at intervals of approximately 2 hr into a refrigerated (4°C) container. Plasma and urine samples were stored at −30°C prior to assay.

Quantification of Cyclophosphamide in Plasma. Drug levels were measured by mass spectrometry-stable isotope dilution using tetraduterated cyclophosphamide as an internal standard. The method was similar to that described previously for the determination of cyclophosphamide in blood (12). Cyclophosphamide-4,6-d4 (10 μl; 1.00 mg/ml in methanol) was added to duplicate aliquots (1 ml) of plasma which was then extracted with ethyl acetate (2 ml). The organic phase (separated by centrifugation where necessary) was concentrated to a small volume before final concentration onto the direct insertion probe of the mass spectrometer. The ratios between cyclophosphamide and its d4 analog were determined by comparison of the peak heights at m/e 211 and 215 (from at least 10 scans) in the mass spectrum ([M—CH2Cl]+ ions for the d0 and d4 forms, respectively). Plasma levels were fitted (by Dr. L. I. Hart, Institute of Cancer Research, Sutton, England) to a 2-compartment open model by digital computer using a nonlinear least-squares regression program (18), and pharmacokinetic parameters were calculated therefrom.

Quantification of Cyclophosphamide, 4-Ketocyclophosphamide, and Carboxyphosphamide in Urine. The following deuterated internal standards (1.00 mg/ml in methanol) were added to duplicate aliquots (1 ml) of the urine: cyclophosphamide-d4 (50 μl); 4-ketocyclophosphamide-d4 (10 μl); and carboxyphosphamide-d4 (50 μl). The procedure used for the isolation of cyclophosphamide and its metabolites was essentially that applied to their isolation and quantification in mouse urine (5). After extraction with ethyl acetate (2 ml), the organic phase was concentrated before subjection to TLC on glass plates (20 x 20 or 5 x 20 cm) coated with silicic acid (Merck Keiselgel G, 0.25 mm thick) developing with CHCl3:CH3OH (9:1). Cyclophosphamide was run as a standard (cyclophosphamide and 4-ketocyclophosphamide have the same Rf in this solvent) and the band corresponding to the cyclophosphamide marker was detected by exposure to iodine vapor and eluted with methanol (approximately 0.5 ml). The methanol eluate was treated for 10 min with ethereal diazomethane (0.5 ml) to convert 4-ketocyclophosphamide into its N-methyl derivative. After concentration, the ratio between 4-ketocyclophosphamide and 4-ketocyclophosphamide-d4 analogs were measured by mass spectrometry as above ([M—CH2Cl]+ ions at m/e 239 and 243 for the d0 and d4 forms, respectively, of the 4-ketocyclophosphamide derivatives).

The aqueous phase remaining after the above extraction with ethyl acetate was acidified to approximately pH 2 by the addition of 0.1 M HCl (1 ml) and extracted with ethyl acetate (4 ml). The organic phase was concentrated to dryness, and a solution of the residue in methanol was treated with ethereal diazomethane as above to yield the methyl esters of carboxyphosphamide and its d4 analog. The concentrate was subjected to TLC as above, alongside authentic carboxyphosphamide which had been similarly methylated. Bands were located by iodine staining. Where several bands of closely similar Rf's were present at the appropriate region, the major portion of each was removed and the location of the desired component was confirmed using the Epstein reagent (20). The ratio of carboxyphosphamide to its d4 analog was determined by mass spectrometry ([M—CH2Cl]+ ions at m/e 257 and 261 for the methyl esters and the d0 and d4 forms, respectively). A linear

* The abbreviation used is: TLC, thin-layer chromatography.
relationship was demonstrated for the concentration range encountered between the composition of mixtures of \(d_1\) and \(d_3\) forms and peak intensities for cyclophosphamide, 4-ketocyclophosphamide, and carboxyphosphamide after recovery from urine by the procedures described above.

**Mass Spectrometry.** Electron impact mass spectra were determined by the direct insertion technique with an AEI MS12 spectrometer, using an ionizing voltage of 70 eV, a trap current of 100 \(\mu\)A, and an ion source temperature of 80–100°C.

**Determination of the Enantiomeric Composition of Cyclophosphamide Recovered from Patients’ Urine.** Unmetabolized cyclophosphamide was recovered from the urine of all 4 patients treated with the racemate and from one patient (F. R.) after treatment with each enantiomer. Urine (0 to 24 hr, after removal of an aliquot for the above quantitative studies) was extracted with chloroform (2 \(\times\) 500 ml). Where an emulsion formed, this was dispersed by filtration under vacuum through a bed of Hyflo on a sintered funnel. The dried (Na\(_2\)SO\(_4\)) organic phase was concentrated, and a solution of the residue in chloroform was applied to the entire width of a plate coated with Silica Gel G (2000 \(\mu\)m thick; Anachem, Luton, Bedfordshire, England) which was developed in CHCl\(_3\):CH\(_3\)OH (9:1). The band containing cyclophosphamide (\(R_f\) 0.5) was detected by exposure to iodine vapor and its location confirmed by edge spraying with the Dragendorff reagent (Bismuth trinitride, 10% w/v in S\(_5\)HCl) which gave a positive reaction (orange color against a yellow background) with cyclophosphamide. The band was removed, eluted with methanol, and the concentrated eluate was treated with charcoal before concentration to dryness. The residues from urines of patients receiving the racemate were crystallized from water (0.3 ml) and those recovered from urine following administration of the enantiomers were dissolved in CCl\(_4\) (0.1 ml) and the solutions were treated with n-hexane (0.2 ml). In each case, large crystals were slowly deposited (usually during 2 days) and these were transferred onto filter paper and washed with the appropriate solvent (water or CCl\(_4\):hexane). The products were chromatographically homogeneous on TLC in CHCl\(_3\):CH\(_3\)OH (9:1), but TLC in ethyl acetate, which resolves cyclophosphamide (\(R_f\) 0.1) from 4-ketocyclophosphamide (\(R_f\) 0.3), revealed a trace of the latter (blue color with the Epstein reagent) as contaminant. The enantiomeric composition of the cyclophosphamide in each sample was determined by the method of Zon et al. (27) utilizing nuclear magnetic resonance spectroscopy in the presence of a chiral shift reagent (see below).

**31P Nuclear Magnetic Resonance Spectroscopy.** The cyclophosphamide samples (3.45 to 10.5 mg) and [tris(3-trifluoromethylhydroxymethylene)-d-camphorato]europium(III) were dissolved in CDCl\(_3\) (2 ml). Spectra were recorded on a Jeol FX-60 spectrometer at 24.3 MHz with proton noise decoupling. Cyclic shifts were relative to external 85% H\(_3\)PO\(_4\) using high-frequency positive convention.

**Determination of Plasma Protein Binding of Cyclophosphamide and Its Enantiomers.** Membrane ultrafiltration (Amicon Centrillo CF25; M.W., 25,000 cut off) was performed by centrifugation (2500 rpm; 20 min). Levels of cyclophosphamide in plasma and ultrafiltrate were determined using “Quantification of Cyclophosphamide in Plasma.” (+)-Cyclophosphamide (20 \(\mu\)l; 0.89 mg/ml in water) was added to normal plasma (2 ml; Dr. M. Jarman) which was then incubated at 37°C for 20 min. After ultrafiltration, cyclophosphamide-d\(_4\) (10 \(\mu\)l; 1.00 mg/ml in methanol) was added to the ultrafiltrate (1 ml), and the level of cyclophosphamide was determined. The experiment was repeated using (–)-cyclophosphamide (20 \(\mu\)l; 0.87 mg/ml in water). In a further experiment, (–)-cyclophosphamide (30 \(\mu\)l) and (+)-cyclophosphamide-d\(_4\) (30 \(\mu\)l; 1.19 mg/ml) were added to normal plasma (3 ml). After incubation and ultrafiltration as above, the ratio of cyclophosphamide-d\(_4\) to cyclophosphamide-d\(_4\) was determined in both plasma and ultrafiltrate.

**RESULTS**

The pharmacokinetic parameters (Table 1) and the urinary outputs of cyclophosphamide, 4-ketocyclophosphamide, and carboxyphosphamide (Table 2), following sequential administration of racemic cyclophosphamide and its enantiomers to each of the 4 patients, are presented. With the exception of 2 sets of plasma level determinations, the data points were fitted to a biexponential function, \(C = Ae^{-\alpha t} + Be^{-\beta t}\), by computer (18). For the remaining sets, data points after 30 min were fitted to a monoexponential function, \(C = Be^{-\beta t}\), by least-squares regression (\(r = 0.92, 0.98\)).

There was a remarkable similarity in the mean \(t_{1/2}\) values (Table I) for the racemate, the (+)-enantiomer, and the (–)-enantiomer. There was no significant overall difference between the 3 forms in the outputs of cyclophosphamide (range, 39 to 104 mg) or of the principal detoxification product carboxyphosphamide (range, 22 to 114 mg). The output of 4-ketocyclophosphamide (range, 6 to 29 mg) was significantly greater (\(p < 0.05\)) from (+)-cyclophosphamide than from the racemate.

### Table 1

**Pharmacokinetic parameters for racemic (±)-cyclophosphamide and the (+)- and the (–)-enantiomer**

<table>
<thead>
<tr>
<th>Composition of administered cyclophosphamide</th>
<th>(A) ((\mu)g/ml)</th>
<th>(\alpha) (hr(^{-1}))</th>
<th>(B) ((\mu)g/ml)</th>
<th>(\beta) (hr(^{-1}))</th>
<th>(t,\beta) (hr)</th>
<th>(V_d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(±)</td>
<td>19.2 ± 2.6(^a)</td>
<td>4.1 ± 0.3</td>
<td>23.6 ± 6.1</td>
<td>0.096 ± 0.018</td>
<td>7.45 ± 1.4</td>
<td>45.9 ± 0.0</td>
</tr>
<tr>
<td>(+)</td>
<td>21.0 ± 2.6</td>
<td>4.0 ± 1.8</td>
<td>20.0 ± 4.5</td>
<td>0.100 ± 0.024</td>
<td>7.25 ± 1.75</td>
<td>50.6 ± 11.4</td>
</tr>
<tr>
<td>(–)</td>
<td>22.6 ± 9.9</td>
<td>4.7 ± 3.6</td>
<td>20.0 ± 3.3</td>
<td>0.092 ± 0.024</td>
<td>7.9 ± 1.7</td>
<td>49.6 ± 8.5</td>
</tr>
</tbody>
</table>

\(^a\) Mean ± S.D.
DISCUSSION

Inasmuch as the enantiomers of cyclophosphamide differed little from each other and from the racemate in their plasma half-lives (β phase) and in the urinary outputs of unchanged drug and of the principal detoxification product, carboxyphosphamide, there is little evidence that metabolism of cyclophosphamide is stereoselective in humans.

The results may be compared with those for the 3 animal species thus far studied (mouse, rabbit, and rat), since these studies were undertaken, in part, to determine whether there exists a good animal model for metabolism of the enantiomers of cyclophosphamide in humans. Evidence for stereoselectivity in the initial 4-hydroxylation step in these animals was afforded by comparing the rates of metabolism of the enantiomers by liver microsomes (7). On this evidence, metabolism by the mouse is only marginally stereoselective, (+)-cyclophosphamide being slightly more rapidly metabolized than (−)-cyclophosphamide. Rat microsomes showed some stereoselectivity for the (−)-enantiomer, whereas those from the rabbit metabolized this isomer 3- to 5-fold more rapidly than the (+)-form. The mouse and the rat exhibited stereoselectivity in the formation of urinary metabolites (5), particularly 4-ketocyclophosphamide. Thus, the yield of 4-ketocyclophosphamide from (−)-cyclophosphamide is greater than from the (+)-isomer, whereas those from the rabbit racemate and 4-ketocyclophosphamide were observed following the administration of the 3 forms of cyclophosphamide used in this study.

Unchanged cyclophosphamide, recovered from the urine after administration of the racemate, was little different from the racemate in enantiomeric composition (Table 3). Cyclophosphamide recovered from the urines of one patient after administration of each enantiomer remained enantiomerically pure.

Plasma protein binding of cyclophosphamide was not stereoselective at the concentrations chosen. The percentage binding of the enantiomers when incubated individually was almost identical, 35% for the (+)-cyclophosphamide and 36% for the (−)-isomer. When unlabeled (−)-cyclophosphamide and (+)-cyclophosphamide-d4 were incubated in admixture (total concentration of the "racemate", 20.6 μg/ml), the intensity ratio of the ions m/e 211 and 215 was the same in the mass spectra of extracts from both plasma and ultrafiltrate.

No intrapatient differences in either clinical antitumor response or host toxicity were observed following the administration of the 3 forms of cyclophosphamide used in this study.

| Table 2 |

Human Metabolism of Cyclophosphamide Enantiomers

| Table 3 |

Enantiomeric composition of cyclophosphamide recovered from patients' urines (24-hr collections)

<table>
<thead>
<tr>
<th>Enantiomeric composition of administered cyclophosphamide</th>
<th>% of (−)-cyclophosphamide in recovered drug</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient</td>
<td>F. R.</td>
</tr>
<tr>
<td>(±)</td>
<td>50.7</td>
</tr>
<tr>
<td>(+)</td>
<td></td>
</tr>
<tr>
<td>(−)</td>
<td></td>
</tr>
</tbody>
</table>

| Table 4 |

Urinary outputs of cyclophosphamide, 4-ketocyclophosphamide, and carboxyphosphamide over 24 hr following sequential i.v. administration (1 g, 3 weeks between doses) of racemic (±)-cyclophosphamide and the (+) and the (−)-enantiomers to each of 4 patients

<table>
<thead>
<tr>
<th>Composition of administered cyclophosphamide</th>
<th>Patient</th>
<th>Urinary output (mg)</th>
<th>Urinary output (mg)</th>
<th>Urinary output (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cyclophosphamide</td>
<td>4-Ketocyclophosphamide</td>
<td>Carboxyphosphamide</td>
</tr>
<tr>
<td>(±)</td>
<td>F. R.</td>
<td>39</td>
<td>7.0</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>S. M.</td>
<td>76</td>
<td>16</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>J. R.</td>
<td>41</td>
<td>6.0</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>E. D.</td>
<td>50</td>
<td>18</td>
<td>56</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>51.5 ± 17.0a</td>
<td>11.75 ± 6.2b</td>
<td>62.04 ± 14.1</td>
</tr>
<tr>
<td>(+)</td>
<td>F. R.</td>
<td>63</td>
<td>12</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>S. M.</td>
<td>52</td>
<td>20</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>J. R.</td>
<td>63</td>
<td>11</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>E. D.</td>
<td>72</td>
<td>29</td>
<td>55</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>62.5 ± 8.2</td>
<td>18.04 ± 8.45b</td>
<td>61.75 ± 26.0</td>
</tr>
<tr>
<td>(−)</td>
<td>F. R.</td>
<td>94</td>
<td>6.9</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>S. M.</td>
<td>68</td>
<td>6.6</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>J. R.</td>
<td>105</td>
<td>12.5</td>
<td>115</td>
</tr>
<tr>
<td></td>
<td>E. D.</td>
<td>71</td>
<td>11</td>
<td>80</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>84.5 ± 17.9</td>
<td>9.25 ± 2.95</td>
<td>81.75 ± 42.7</td>
</tr>
</tbody>
</table>

a Mean ± S.D.

b These values differ significantly. One patient (J. R.) gave a slightly higher output of 4-ketocyclophosphamide from (−)-cyclophosphamide than from the (+)-enantiomer. Hence the levels from the enantiomers do not differ significantly although the difference in the mean outputs of 4-ketocyclophosphamide is greater between the enantiomers than between (+)-cyclophosphamide and the racemate.
in the initial metabolism of cyclophosphamide, the mouse could afford a model for human metabolism of the cyclophosphamide enantiomers. Because of large interpatient variations in metabolism, more patients would have to be examined to determine whether the trend toward greater output of 4-ketocyclophosphamide from (+)-cyclophosphamide is general in humans. However, even if confirmed, it seems unlikely that this relatively small degree of stereoselectivity in the formation of a minor detoxification product (the yield of the 4-keto derivative in no case represents >3% of the administered dose) could elicit clinically observable differences in response between the enantiomers.

The validity of the carboxyphosphamide levels requires comment, in view of evidence that this metabolite decomposes significantly to nitrogen mustard under mild conditions (11). Indeed, we found that carboxyphosphamide was 50% decomposed when a solution (concentration, 200 µg/ml) in urine (pH 5.5) was kept at room temperature for 24 hr. In the present study, catheterization prevented more than a brief exposure of the urine to in vivo temperatures, and samples were exposed for only 2 hr at room temperature before refrigeration. In the cited study (11), urine was collected on ice in 4-hr batches. Decomposition was thereby minimized, although probably not eliminated. In view, however, of the proven instability of carboxyphosphamide, it is highly probable that some of the carboxyphosphamide formed enzymatically from aldophosphamide (Chart 1) is subsequently lost by decomposition in vivo and that this detoxification pathway is more important in humans than measurements of urinary levels of carboxyphosphamide would indicate.

The finding (Table 3) that there was no marked preponderance of one enantiomer in the urine recovered after racemic cyclophosphamide was given requires further comment, in view of a previous report to the contrary from these laboratories (6). Thus, based on their specific optical rotations, samples of cyclophosphamide isolated from the urines of 2 of 3 patients in this earlier study were markedly enriched in the (−)-isomer, the percentage of this isomer being 83 and 91%. Since the specific optical rotation of the enantiomers of cyclophosphamide (α) D ± 2.3°) is small compared with that of many chiral substances of natural origin which may be present in urine, minor contamination by such substances could have been responsible for the observed optical rotation of these samples. Hence, the enantiomeric composition of 2 of the 3 samples from the earlier study, which had apparent (−)-cyclophosphamide contents based on optical rotation of 57 and 91%, was redetermined by the presently described method of 31P nuclear magnetic resonance spectroscopy in the presence of a chiral shift reagent, which is not subject to the possible source of error of the earlier method. The new values for the content of the (−)-enantiomer were, respectively, 50 and 55.2%. These findings provide further evidence that the initial metabolism (mainly 4-hydroxylation) of cyclophosphamide is either not stereoselective or only slightly so and show that the previously reported conclusion to the contrary was wrong.

It was important to show whether either enantiomer underwent inversion of configuration in vivo, and it was for this reason that the enantiomeric composition of cyclophosphamide recovered from the urine of one patient after administration of each enantiomer was determined. Several examples of metabolic inversions of configuration of compounds chiral at carbon have been reported (13, 16, 26), although none, as far as the present authors are aware, of inversion at phosphorus. This consideration is also important in connection with the use of pseudoracemates, in which one enantiomer is labeled with a stable isotope, which serves to distinguish its metabolic fate from that of its antipode. Such mixtures have been used in our studies on stereoselective metabolism of cyclophosphamide in animals (5, 7). The procedure is clearly valid only in the absence of metabolic inversion. The present study shows that, at least in humans, inversion at phosphorus in cyclophosphamide does not occur. This finding does not exclude the possibility that metabolites might undergo spontaneous inversion, particularly the acidic chiral metabolites carboxyphosphamide and aldophosphamide (Chart 1). Inversion of the latter would also affect the cyclic tautomer 4-hydroxycyclophosphamide and its oxidation product, 4-ketocyclophosphamide. However, the appropriate synthetic enantiomers required to test these possibilities were not available at the time of this study, although syntheses are reported to be in progress (24).

Binding of the enantiomers of cyclophosphamide to plasma proteins was compared because of a recent report (11) that 24% of racemic cyclophosphamide was bound in contrast with earlier reports that there was no detectable adsorption of cyclophosphamide by serum proteins (17) and that the drug was only 2.4% bound to human serum albumin (22). The presently determined values of between 35 and 44% binding are more consistent with the later report (11). It is noteworthy that these higher values for protein binding were found when drug concentrations were within the range of those encountered clinically, whereas concentrations used in the earlier studies were much higher, respectively, 0.7 to 1.4 × 10−2 M (1.9 to 3.9 mg/ml) (17) and 3 × 10−2 M (8.3 mg/ml) (22). At these high concentrations, protein binding might be saturated. The present results also show that there is no stereoselectivity in plasma protein binding of the enantiomers of cyclophosphamide, administered either separately or in a racemic mixture.

In summary, there is insufficient stereoselectivity in the enzymatic pathways of metabolism of cyclophosphamide in humans to support a prediction that either enantiomer would be superior to its antipode or to the racemate in cancer chemotherapy. However, other factors could elicit different therapeutic responses between the enantiomers; for example, preferential uptake of one of the stereoisomers of 4-hydroxycyclophosphamide into neoplastic cells could confer selective toxicity. In this connection, it is noteworthy that 4-hydroxylation introduces a second chiral center into the cyclophosphamide molecule and that one of the 4 stereoisomers of 4-methylcyclophosphamide, which is similarly additionally chiral at C-4, is metabolized differently from the other 3 by rat liver microsomes (1). The superior activity of melphalan against L1210 leukemia cells in culture compared with its D isomer [cf. the earlier report (2) cited herein] has been tentatively ascribed to a preferential uptake by the L1210 cells of the L antipode (25). Stereoselective uptake among the stereoisomers of 4-hydroxycyclophosphamide could result in different intracellular concentrations of the spontaneously formed cytotoxic decomposition product phosphoramidate mustard (see Chart 1) despite the absence of stereoselectivity in the enzymatic pathways of metabolism. Finally, the conclusions reached in the present study regarding
the relative lack of evidence for stereoselective metabolism of cyclophosphamide enantiomers in humans do not necessarily apply to analogs chiral at phosphorus, and the comparative metabolism of the enantiomers of one such analog, isophosphamide (15), is therefore currently under investigation.

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-1,3,2-oxazaphosphorine-2-oxide (Cyclophosphamide) and Its Enantiomers in Humans


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