Isolation of cis- and trans-4-Methylcyclophosphamide and Antitumor Evaluation in Vivo

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SUMMARY

4-Methylcyclophosphamide was synthesized and separated into cis and trans isomers by column chromatography. Isolation of these isomers permitted individual evaluation against murine leukemia L1210 in vivo and assessment of possible differences in antileukemic activity. Results indicate no appreciable difference in activity of the isomers, suggesting essentially equal facility for activation by mouse liver microsomes in vivo.

INTRODUCTION

A synthesis program in our laboratory has resulted in the preparation and antitumor evaluation of several "preactivated" derivatives of cyclophosphamide and isophosphamide (12, 13). Upon our initiation of synthesis of related analogs of 4-methylcyclophosphamide, Feil and Lamoureux (5) reported spectroscopic evidence for the detection of cis and trans isomers of this cyclophosphamide derivative. Consequently, we undertook the separation of the isomers in order to permit evaluation of each isomer separately against the experimental tumor L1210 leukemia. Such evaluation could reveal an enzymatic preference for 1 of the isomers, thereby suggesting structural features that might enhance or inhibit activation of this type of structure in vivo by the mixed-function oxidase of mouse liver cells. This is the 1st report of biological evaluation of 4-substituted isomers of cyclophosphamide in vivo.

MATERIALS AND METHODS

Thin-Layer Chromatography. Thin-layer chromatography was performed on Analtech (Newark, Del.) precoated silica gel G plates (250 μm thick) in acetone:chloroform (3:1). The plates were activated at 120°C for 1 hr and stored in a desiccated chamber.

Alkylating Activity. Thin-layer chromatograms were sprayed with a 1% solution of 4-(p-nitrobenzyl)pyridine (Aldrich Chemical Co., Milwaukee, Wis.) in acetone, heated in an oven for 15 min at 140°C, and sprayed with a 3% solution of potassium hydroxide in methanol. Alkylating components yielded blue spots.

Column Chromatography. Column chromatography was performed on Silica Gel 40 (70 to 230 mesh, EM Laboratories, Elmsford, N. Y.) in acetone:chloroform (3:1).

Instrumentation. Mass spectral analysis was performed with a Hitachi Model RMU-6D mass spectrometer (Perkin-Elmer Corp., Norwalk, Conn.) and NMR (both 1H and 13C) measurements were performed with a Varian XL-100-15 spectrometer (Varian, Inc., Palo Alto, Calif.).

Evaluation against L1210 Leukemia. 4-Methylcyclophosphamide isomers were administered i.p. in 0.9% NaCl solution on the 1st day of inoculation of 106 leukemia cells in C57BL X DBA/2 (hereafter called BD2F1) mice; 6 mice were used for each dose.

RESULTS

4-Methylcyclophosphamide was prepared as described by Feil and Lamoureux (5). The crude product was separated into its 2 isomers by column chromatography. Fractions containing a single isomer, as indicated by thin-layer chromatography, were combined and evaporated. Both isomers crystallized on standing at room temperature. Trituration of the faster-migrating isomer with cold ethanol and of the slower-migrating isomer with cold ether, followed by filtration, gave white crystalline solids with melting points of 72-74°C and 102°C, respectively. Thin-layer chromatography indicated that each isomer was uncontaminated with the other, Rv 0.57 and Rv 0.48, respectively. Elemental analysis (carbon, hydrogen, nitrogen) of each isomer was as follows: 35.14, 6.19, 10.24 (fast) and 35.03, 6.08, 10.08 (slow); 34.94, 6.23, 10.19 (theory).

The assignment of configuration to the 2 isomers (Chart I) was based on a consideration of carbon-phosphorus coupling constants obtained from their 13C NMR spectra. We assumed that the P → O bond is axial, and the bulky bis(2-chloroethyl)amino group is equatorial. This has, indeed, been shown to be the case in the solid state for cyclophosphamide (III) (7) and for 4-ketocyclophosphamide (2) and 4-peroxycyclophosphamide (8) by X-ray crystallography. Since an equatorial methyl group at C-4 would be expected to exert a smaller change on the ring

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Antitumor Evaluation of Isomers of 4-Methylcyclophosphamide

bond angles than an axial 4-methyl, we expected the carbon-phosphorus couplings of C-4 and C-5 of the faster-migrating trans isomer, I, to be more like those of cyclophosphamide than the corresponding couplings of the slower-migrating cis isomer, II, would be. This appears to be substantiated by the data, and the assignments were made.

Chemical shifts and the appropriate carbon-phosphorus coupling constants are given in Table 1 for the 2 4-methylcyclophosphamides and for cyclophosphamide itself. Assignments of the individual peaks were confirmed in all cases by the 1H-coupled spectra.

Except in the methyl region, the 1H NMR spectra were less informative because of very complex, overlapping multiplets. Our spectra of the separated isomers appear to be in fair agreement with the methyl data of Feil and Lamoureux (5) obtained on a mixture. Chemical shifts and methyl coupling constants are listed in Table 2.

Mass spectral analysis of the isomers indicated significant differences between the 2 (Table 3), thus permitting isomer differentiation by this method. The trans isomer exhibits a higher ratio of relative intensities of the M-CH₃:M fragments than does the cis isomer. Application of this observation to the 4-ethoxy derivatives reported by Connors et al. (3) and Cox et al. (4) suggests the possibility that the fast product in each case may be of trans configuration.

Table 1

13C NMR spectra of cis- and trans-4-methylcyclophosphamide

Spectra were determined in the pulsed Fourier transform mode on a Varian XL-100-15 NMR spectrometer operating at 25.16 MHz and equipped with a Digital NMR-3 data system. Solutions were of 100 mg compound per 0.4 ml CDCl₃. Chemical shifts are in ppm downfield from internal tetramethylsilane on solutions in CDCl₃ and are correct to ±0.02 ppm.

<table>
<thead>
<tr>
<th>Compound</th>
<th>I</th>
<th>II</th>
<th>III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon</td>
<td>δ</td>
<td>J(çP)</td>
<td>δ</td>
</tr>
<tr>
<td>4</td>
<td>48.35</td>
<td>2.7</td>
<td>48.44</td>
</tr>
<tr>
<td>5</td>
<td>33.53</td>
<td>7.3</td>
<td>33.70</td>
</tr>
<tr>
<td>6</td>
<td>66.47</td>
<td>7.3</td>
<td>66.42</td>
</tr>
<tr>
<td>7</td>
<td>49.05</td>
<td>4.9</td>
<td>49.19</td>
</tr>
<tr>
<td>8</td>
<td>42.21</td>
<td>2.4</td>
<td>42.40</td>
</tr>
<tr>
<td>CH₃</td>
<td>23.19</td>
<td>7.3</td>
<td>24.11</td>
</tr>
</tbody>
</table>

* Coupling constants are correct to ±0.3 for I and II and to ±0.6 for III.
* NR, not resolved.

Table 2

1H NMR spectra of cis- and trans-4-methylcyclophosphamide

Spectra were determined on a Varian XL-100-15 NMR spectrometer operating at 100 MHz. Solutions were of 20 mg compound per 0.4 ml CDCl₃. Chemical shifts are in ppm downfield from internal tetramethylsilane and are estimated to be accurate to ±0.01 ppm.

<table>
<thead>
<tr>
<th>Compound</th>
<th>I</th>
<th>II</th>
</tr>
</thead>
<tbody>
<tr>
<td>δ</td>
<td>J(çH)</td>
<td>δ</td>
</tr>
<tr>
<td>CH₃ on C₁</td>
<td>1.29</td>
<td>J(çNCH₃) = 1.95</td>
</tr>
<tr>
<td>H₁</td>
<td>1.5</td>
<td>2.0</td>
</tr>
<tr>
<td>H₂, H₃, H₄</td>
<td>3.1</td>
<td>3.9</td>
</tr>
<tr>
<td>H₄</td>
<td>4.0</td>
<td>4.6</td>
</tr>
</tbody>
</table>

* Coupling constants are correct to ±0.1.
Tentative identifications of certain fragments observed in the spectra are listed in Table 3.

The cis isomer (NSC 241531) and the trans isomer (NSC 241532) were evaluated against an inoculum of 10^6 L1210 leukemia cells at doses of 200 and 100 mg/kg. The higher dose of both isomers was toxic (>LD_{50}), and the lower dose gave an increase in life-span of 45% and 46%, respectively. The average day of death of the control animals was 10.1 days. Cyclophosphamide administered at a dose of 300 mg/kg (LD_{50} = 312 mg/kg) routinely produces approximately 70% 30-day survivors and an increase in life-span of approximately 250% of the non-survivors under identical conditions (11).

**DISCUSSION**

Arnold et al. (1) synthesized numerous ring-alkylated analogs of cyclophosphamide many years ago, but only recently Feil and Lamoreux (5) demonstrated the existence of cis and trans isomers of this type of derivative. Because of the interest in the metabolism of cyclophosphamide and its analogs, biological evaluation of both cis and trans isomers of a representative of this type of structure would provide information on a stereochemical preference for activation by the mixed function oxidase of liver microsomes. Although evaluation of any of the alkyl isomers of both endo- and exocyclic-substituted analogs would be an aid to our understanding of the metabolic process of oxidation and consequent or subsequent generation, spontaneously or enzymatically, of the important alkylating product, analogs substituted on C_4 of the oxazaphosphorine ring, the important site of enzymatic oxidation, would be expected to influence the critical enzymatic reaction more directly. We, therefore, separated 4-methylcyclophosphamide into its cis and trans isomers. The existence of cis- and trans-4-ethoxy-4-methylcyclophosphamide (3) and cis and trans-4-ethoxy-6-methylcyclophosphamide (4) has been reported. The cis, trans-designation in the latter case probably refers, by analogy with cyclophosphamide, to the 4-ethoxy substituents and not to the 6-methyl substituents; the added asymmetric center would allow for 4-cis-6-cis, 4-cis-6-trans, 4-trans-6-cis, and 4-trans-6-trans isomers, provided the P→O bond remains in the axial position.

Evaluation of cis- and trans-4-methylcyclophosphamide against L1210 leukemia in vivo failed to reveal any difference between the 2, thus indicating that both axial and equatorial configurations of the 4-methyl substituent have a similar effect on liver microsomal oxidation of C_4 in the cyclophosphamide ring. Both isomers are less active than cyclophosphamide against this experimental tumor system and not to the 6-methyl substituents; the added O bond remains in the axial position.

Consequently, degradation in vivo to phosphoramidate mustard (4, 6, 15) and methyl vinyl ketone (4, 15) would be anticipated. An interesting difference is noted between BALB/c mice used by Cox et al. (4) and BD2F_1 mice used in our experiments; whereas the toxic dose of the isomeric mixture of 4-methylcyclophosphamide is greater than that of cyclophosphamide in BALB/c mice, the reverse is true for either isomer of 4-methylcyclophosphamide in BD2F_1 mice. The same order of toxicity was observed in sheep (5) and in rats (1) as we observed in BD2F_1 mice. Administration of isolated 4-hydroxy-4-methylcyclophosphamide resulted in a smaller LD_{50}, suggesting less efficient oxidation of 4-methylcyclophosphamide in vivo (4).

The observation of similar activity against L1210 leukemia by both cis- and trans-4-methylcyclophosphamide is not only consistent with a similar ease of enzymatic oxidation but also of similar rates of degradation of the intermediate 4-hydroxy metabolites. Indeed, Cox et al. (4) observed a single product, the acyclic tautomer of 4-hydroxy-4-methylcyclophosphamide, from both chemical and enzymatic oxidation of 4-methylcyclophosphamide, indicating the extreme instability of the cyclic structure. Such a property appears to be in direct contrast to cyclophosphamide itself, where 4-hydroxy-4-methylcyclophosphamide can be isolated (14), but aldophosphamide, the acyclic isomer, apparently cannot, being characterized physically only transiently (10, 14) or as a stabilized derivative (9). A report to the contrary has appeared (16), but no definitive physical data (infrared, NMR, mass spectral) for the existence of aldophosphamide itself were included; consequently, it seems probable that the product described as aldophosphamide may be 1 of the cis or trans isomers of 4-hydroxy-4-methylcyclophosphamide, being analogous, therefore, to the recently reported ethoxy derivatives (3, 4).

**REFERENCES**

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