Methylazoxyprocarbazine, the Active Metabolite Responsible for the Anticancer Activity of Procarbazine against L1210 Leukemia


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ABSTRACT

Procarbazine is a 1,2-disubstituted hydrazine derivative that is used to treat human leukemias. The anticancer activity of procarbazine results from bioactivation to reactive intermediates. It is first oxidized to azoprocarbazine and further N-oxidized to a mixture of methylazoxyprocarbazine and benzyazoxyprocarbazine isomers. In this study the azoxyprocarbazine isomers were synthesized and purified. The cytotoxic effect of the metabolites on the L1210 murine leukemia cell line were then evaluated in vitro by use of a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide. The results of this study showed that the methylazoxyprocarbazine isomer was the most cytotoxic metabolite (IC50 0.2 mM). The benzyazoxy isomer had an insignificant cytotoxic effect, and a mixture of the two isomers was intermediate in effectiveness. This assay, however, could not be used to determine the cytotoxicity of procarbazine since the drug itself (not the live cells) reduced the dye. A soft-agar clonogenic assay demonstrated that procarbazine was cytotoxic only at higher concentrations (IC50 1.5 mM) than methylazoxyprocarbazine (IC50 0.15 mM). The effect of procarbazine and its metabolites on the survival of L1210 tumor-bearing mice was determined, and methylazoxyprocarbazine was again the most effective compound. These studies demonstrate that the methylazoxyprocarbazine metabolite is probably the major cytotoxic intermediate involved in the mechanism of anticancer action of procarbazine.

INTRODUCTION

Procarbazine, N-isopropyl-o-(2-methylhydrazino)-p-toluamide hydrochloride, was originally developed as a monoamine oxidase inhibitor. It was later found to have anticancer activity and was used successfully in clinical trials by Martz (1) and coworkers in Switzerland in 1963. It is currently used in combination therapy and as a single agent in the treatment of Hodgkin's disease, leukemias, malignant lymphomas, small-cell carcinoma of the lung, and brain tumors (2–7).

Procarbazine itself is probably not active, but has been found by Weinkam and Shiba (8) to be bioactivated to cytotoxic species. Fig. 1 shows a proposed scheme for the metabolism of procarbazine to active cytotoxic compounds. It is first oxidized to azoprocarbazine via a process which is mediated by cytochrome P-450 and monoamine oxidase (9–11). The azoprocarbazine metabolite is further oxidized to a mixture of azoxyprocarbazine isomers (methylazoxyprocarbazine and benzyazoxyprocarbazine) through a cytochrome P-450-mediated process (12–14). Azoprocarbazine can also undergo tautomerization to a hydrazone which could lead to the formation of methyl free radicals, although tautomerization is probably not fast enough to produce the observed amounts of methane (15–16). Prough et al. (17) reported that thiols such as glutathione could increase the formation of methane, which led them to propose that thiols may be responsible for the conversion of methyl free radicals to methane which was measured in the expired air of procarbazine-treated rats (18). Moloney and Prough (19) have demonstrated that when the azoxy isomers were incubated with microsomes, methane was not produced, so methyl free radicals are probably not formed via metabolism of the azoxyprocarbazine.

In the past both hydrogen peroxide and formaldehyde have been proposed as potential cytotoxic byproducts of procarbazine metabolism (20–21), but subsequent studies have demonstrated that these compounds are probably not active (21). In vivo studies by Shiba and Weinkam (22) have demonstrated that a mixture of the azoxyprocarbazine metabolites was significantly more cytotoxic than procarbazine or azoprocarbazine against L1210 in tumor-bearing mice. Recent work from our laboratory (23) has demonstrated that the azoxyprocarbazine mixture was the most spermatotoxic of procarbazine or several metabolites in mice. Erikson et al. (24) have recently demonstrated that the methylazoxy isomer is probably the intermediate responsible for the in vivo cytotoxic activity of procarbazine against murine L1210 cells. These investigators have suggested that further metabolism of the azoxy metabolites may occur via cytosolic enzymes.

In the studies reported here we have compared the in vitro cytotoxicity of procarbazine with that of the two azoxyprocarbazine metabolites against murine L1210 cells in an attempt to provide definitive evidence for the participation of these metabolites in the action of procarbazine. In addition, procarbazine and the metabolites were administered to L1210-bearing mice in order to provide a correlation between the in vivo and in vitro antineoplastic activities of these agents.

MATERIALS AND METHODS

Synthesis of Metabolites. Procarbazine hydrochloride was a gift from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. Azoprocarbazine and the two isomeric azoxy derivatives were synthesized according to the following procedure. Procarbazine (2.00 g, 7.75 mmol) was oxidized with an equimolar amount of yellow mercuric oxide (Aldrich). After addition of 60 ml of ether and 15 ml of ethanol, the mixture was stirred for 40 min at room temperature. It was then vacuum filtered, evaporated, redissolved in a small amount of chloroform, and refiltered. Following evacuation to dryness, azoprocarbazine (1.3 g, 5.92 mmol) was produced in 76% yield. Purity was determined by NMR spectrometry (IBM NR 200) using deuterated chloroform.

The product of the reaction was then dissolved in 50 ml chloroform, and 95% m-chloroperoxybenzoic acid (Aldrich) was added slowly in three equal portions. This mixture was then stirred for 15 h at room temperature following which the reaction was terminated by the addition of 40 ml of 1% sodium hydroxide. The chloroform layer was then washed three times with 25 ml of water. The aqueous layers were subsequently combined, washed with 25 ml chloroform, and the chloroform washes added to the previous organic phase. The chloroform...
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\begin{align*}
\text{procarbazine} & \quad \text{azoprocarbazine} \\
\text{methyazoxyprocarbazine} & \quad \text{benzylazoxyprocarbazine} \\
\text{benzyldiazoion} & \quad \text{formaldehyde} \\
\text{methyliazoion} & \quad \text{N-isopropyl-p-formylbenzamide}
\end{align*}
\]

Fig. 1. A proposed metabolic pathway for procarbazine. Metabolism through the azoxy-procarbazine isomers has been well documented. Metabolism of the azxy isomers to the carbinols and diazonium intermediates is speculative.

was dried over anhydrous magnesium sulfate, vacuum filtered, and evaporated to dryness. A mixture of the azoxy isomers (approximately 2:1 ratio of methylazoxyprocarbazine to benzylazoxyprocarbazine) was produced in 97% yield from azoprocarbazine. This mixture of the azoxy isomers was utilized for evaluations of in vitro and in vivo LI210 activities.

The isomers were separated by dissolution in a small amount of 1:1 (v/v) ethyl acetate-hexane and layered onto a 120 g silica gel chromatography column which was eluted with 1:1 (v/v) ethyl acetate-hexane. Fractions of 25 ml were collected and analyzed for the presence of azoxy isomers by thin layer chromatography with a mobile phase of 3:5 (v/v) ethyl acetate-hexane. Fractions corresponding to each isomer (Rf of the benzylazoxy isomer = 0.19, and that of the methylazoxy isomer = 0.16) were combined and evaporated to dryness. Purity was then determined by NMR spectrometry using deuterated chloroform. The final yield from procarbazine was 60%, of which 55% was methylazoxyprocarbazine and 45% was benzylazoxyprocarbazine.

Cell Culture. Murine leukemia LI210 cells were obtained from Alexander Tseng, MD, University of California, Cancer Research Center, San Francisco, CA. The medium for cell growth consisted of RPMI 1640 media (GIBCO Laboratories, Plainview, NY) supplemented with penicillin/streptomycin solution (10,000 units/ml and 10,000 µg/ml, respectively, GIBCO), 10% newborn calf serum (Hyclone Laboratories, Logan, UT), and 1% L-glutamine (GIBCO). The cells were maintained at 37°C in a humidified 5% CO2 atmosphere and subcultured every 2 to 3 days.

In Vitro Cell Growth Assays. The in vitro cytotoxicity of the azoxy metabolites of procarbazine with LI210 cells was evaluated with the MTT3 colorimetric assay described by Mossman (25). In brief, the azoxy metabolites were dissolved in 5% DMSO, and 11-µl aliquots (resulting in a final DMSO concentration of 0.5%) were pipetted into quadruplicate wells of 96-well microtiter plates (Nunc Corp., Denmark). Wells in the first column were left blank while wells in the second column were drug-free, receiving only diluent as a control. LI210 cells in exponential phase of growth were harvested by centrifugation and counted by hemocytometer using Trypan blue exclusion to check viability. After adjusting the cell concentration to 1 x 10⁶ cells/ml, 100 µl of the cell suspension (1 x 10⁶ cells) was added to each of the wells of the plate using a multichannel pipette (Titertek, Flow Laboratories, Inc., McLean, VA). The plate was then incubated for 3 days at 37°C in a humidified 5% CO2 atmosphere. MTT (Sigma Laboratories, St. Louis, MO) was prepared as a stock solution (5 mg/ml) and filtered, sonicated. Eleven µl of the MTT solution were then added to all wells of the plate except the first column (blanks). After incubating the plate for 4 h in 5% CO2 at 37°C, 100 µl of 0.04 N HCl in isopropanol was added to all wells of the plate and thoroughly mixed in order to solubilize the formazan crystals. Well absorbances at 540 nm were then measured on a multiwell scanning spectrophotometer (Dynatech MR600, Alexandria, VA). The mean absorbance of quadruplicate drug-treated wells was compared to that of control wells, and the results were expressed as a percentage of control absorbance ± SD.

The effect of procarbazine and its azoxy metabolites on LI210 leukemia cell soft agar colony formation was determined with a system

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3 The abbreviations used are: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; DMSO, dimethyl sulfoxide; NMR, nuclear magnetic resonance; IC₅₀, concentration resulting in 50% inhibition; HPLC, high-performance liquid chromatography.
described by Salmon et al. (26) and modified by Harker et al. (27). Comparisons were made of the cytotoxicity induced by each drug or metabolite for L1210 cells which had been exposed to the agent for either a 1-h period or on a continuous basis. For studies utilizing the continuous exposure technique, stock solutions containing 25 times the final desired procarbazine concentration were prepared in sterile 0.9% NaCl solution. Similar solutions of the azoxy metabolites were diluted in 12.5% DMSO (resulting in final concentrations of DMSO of 0.5%). L1210 cells in exponential phase of growth were harvested by centrifugation, counted by hemocytometer, and adjusted to a concentration of 1 x 10^4 cells/ml. Two ml of cells were then added to tubes containing 80-1 aliquots of the 25x stock drug solutions. Melted 3% (w/v) Noble agar (Difco Laboratories, Detroit, MI) at 42°C was then added to the drug-containing cell suspensions to bring the final agar concentration to 0.3% (w/v), and 0.5 ml of the mixture was pipetted onto a previously poured feeder layer (containing 0.5% w/v) in triplicate 16.6-mm diameter wells of a 24-well Linbro (Flow Laboratories, Inc.) culture plate. After agar solidification, the plates were incubated for 10 days at 37°C in a humidified atmosphere containing 5% CO₂. Colonies, defined as cell aggregates of at least 30 cells measuring at least 50 μm in diameter, were then counted on an inverted-phase microscope (Olympus Optical Co., Tokyo, Japan) at 40x. The mean total colony count from triplicate wells divided by the number of cells plated per well represented the plating efficiency. Colony growth in drug-treated wells was expressed as a percentage of control growth ± SD.

For the cytotoxicity studies utilizing 1-h drug exposures, cells were added to tubes containing 40-μl aliquots of the concentrated drug solutions and incubated for 1 h at 37°C in a shaking water bath. The cells were then washed with 3 ml Hank’s balanced salt solution (GIBCO) containing 10% newborn calf serum then centrifuged for 10 min at 200 x g. The wash step was then repeated and the cells resuspended in 2 ml of fresh growth medium (RPMI 1640 + P/S + 10% newborn calf serum + 1% L-glutamine). Warmed agar was then added as described above (to a final concentration of 0.3% w/v) and 0.5 ml of the agar-cell mixture was pipetted onto a feeder layer containing 0.5% (w/v) agar. The plates were incubated at 37°C in a humidified atmosphere containing 5% CO₂ for 10 days and colonies identified and counted as described above. Animals. Eight- to twelve-week-old male DBA/2 and (C57BL/6 × DBA/2)F1, (hereafter called B6D2F1) mice weighing 22–30 g were obtained from Simonsen Laboratories (Gilroy, CA) and were maintained as previously described (28).

L1210 In Vivo Survival Studies. These investigations were performed as previously described (23). Briefly, the generation of L1210 ascites tumors was initiated by i.p. inoculation of L1210 cells into DBA/2 mice. After a period of 7 days, ascites fluid was removed, centrifuged, resuspended in Hank’s balanced 0.9% NaCl solution after lysis of contaminating erythrocytes, and 0.1 ml containing 1 x 10^6 viable cells was inoculated i.p. into six B6D2F1 mice per treatment group. For these studies, procarbazine was dissolved in 0.9% NaCl solution, while each metabolite was dissolved with sonication in 5% DMSO in corn oil. Initial experiments with azoxy isomers were performed at a dose of 273.8 mg/kg, which is equimolar with 300 mg/kg procarbazine (for the azoprocabarzone metabolite and its hydrazonic tautomer, a dose of 255.2 mg/kg is an equal molar dose to 300 mg/kg procarbazine). Later studies to obtain dose-effect data with the methylazoxy compound used doses of 91.3, 182.6, and 273.8 mg/kg (equimolar with 100, 200, and 300 mg/kg procarbazine, respectively). One day after inoculation of the tumor cells, the compounds were injected i.p. into mice in a volume equal to 1% of the body weight. Equivalent injections of 5% DMSO in corn oil were given to control tumor-bearing mice. Animals were checked at 8-h intervals and survival times recorded accordingly.

Statistical Methods. The Mann-Whitney U test was used to assess statistical significance for the L1210 survival time data. Confidence limits were set at p < 0.05.

RESULTS

Metabolite Synthesis. NMR spectrometric analysis of the separated azoxy isomers showed them to be essentially pure.

Fig. 2 shows the 'H-NMR spectrum of the benzylazoxyprocarbazine (Fig. 2A) and methylazoxyprocarbazine (Fig. 2B) metabolites. The structural assignments were based on work by Weinkam and Shiba (8) who assigned the structures based on differences in the chemical shifts of the methyl and methylene protons after N-oxidation. The presence of oxygen on either nitrogen changes the chemical shift of the adjacent protons to a further downfield position. The chemical shifts for benzylazoxyprocarbazine were 3.23 ppm for the methyl protons, while that of the methylene protons were 5.38. Corresponding shifts for the methylazoxy isomer were 4.20 and 4.70 ppm, respectively.

MTT Assay. Because of the poor solubility of the procarbazine metabolites in aqueous solution, DMSO at concentrations of 1–4% was required to keep the drugs in solution. Initial studies were performed to determine the effects of these concentrations of DMSO on the growth of L1210 cells. Cell exposures (72 h) for media containing 1, 2, and 4% DMSO resulted in the loss of 30, 75, and 90% of the plated cells, respectively. Seventy-two-hour cell exposures to the lowest concentration of DMSO which solubilized the metabolites in solution (0.5%) resulted in a 10% reduction in cell number. Control wells for subsequent assays utilizing DMSO to solubilize the metabolites contained 0.5% DMSO.

As noted in Fig. 3, the methylazoxyprocarbazine metabolite was far more cytotoxic to the L1210 cells than the benzylazoxyprocarbazine or azoxyprocabarzone mixture. It can be seen that 0.5 mM methylazoxyprocarbazine killed approximately 90% of the cells while the same concentration of benzylazoxyprocarbazine killed only 10% of the cells. The IC₅₀ of the methylazoxy isomer was approximately 0.2 mM, whereas the
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The L1210 cells were less sensitive to procarbazine after shorter periods of exposure. Fig. 5 displays the results of experiments in which L1210 cells were exposed to procarbazine or the azoxy metabolites for a period of 1 h then plated for subsequent colony growth. The procarbazine IC₅₀ for L1210 cells following 1-h exposures was 3.5 mM.

Clonogenic assays utilizing the continuous exposure technique demonstrated that in L1210 cells methylazoxyprocarbazine was the most cytotoxic metabolite with an IC₅₀ of 0.15 mM. As was demonstrated for procarbazine, the cytotoxicity of the metabolite is dependent on the duration of cell exposure. The methylazoxyprocarbazine IC₅₀ for L1210 cells was 0.5 mM after a 1-h exposure versus 0.15 mM in continuous exposure studies. The same concentration that killed 50% of the cells during continuous exposure killed less than 10% of cells after a 1-h exposure.

Benzylazoxyprocarbazine was much less cytotoxic than methylazoxyprocarbazine to L1210 cells. The IC₅₀ of benzylazoxyprocarbazine following continuous cell exposure was 0.8 mM while concentrations of the metabolite up to 1.0 mM were inactive after 1-h exposures.

The plating efficiency of the L1210 cells throughout all the clonogenic assay studies was found to vary between 20 and 68%, averaging approximately 44%. Thus, results of the clonogenic assay should be very representative of the true reproductive capacity of the L1210 murine leukemia cell line.

L1210 in Vivo Survival Studies. The two azoxy compounds had quite different effects on the survival of mice inoculated i.p. with L1210 leukemia (Table 1). The benzylazoxy isomer was essentially without effect, whereas the methylazoxy isomer was more effective against L1210 leukemia than either procarbazine (18.2% increase in lifespan) or azoprocarbazine (21.8% increase in lifespan) at equimolar doses. The hydrazone compound, which is a tautomer of azoprocarbazine, was almost inert, producing only a 3.6% increase in lifespan over that of control. In addition, the highest dose of methylazoxyprocarbazine was lethal to approximately half of the mice within the first 48 h after drug treatment.

All three doses of methylazoxyprocarbazine proved quite effective against the L1210 murine leukemia system, with a marked and significant increase in efficacy as the dose was...
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Table 1 Prolongation of L1210 survival time produced by procarbazine and its metabolites

Male B6D2F1 mice were inoculated with L1210 ascites leukemia obtained from the ascitic fluid of DBA/2 mice. One day later mice were injected with a compound in 0.9% NaCl solution or 5% DMSO in corn oil (survival data were identical with either 0.9% NaCl or corn oil controls). Animals were examined at 8-h intervals.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Survival time</th>
<th>Dose (mg/kg)</th>
<th>Days</th>
<th>%ILS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>9.1 (8.5-10.2)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Procarbazine</td>
<td>6</td>
<td>300</td>
<td>10.8 (10.2-11.8)</td>
<td>18.2e</td>
</tr>
<tr>
<td>Hydrazozone</td>
<td>6</td>
<td>255.2</td>
<td>9.5 (8.5-10.2)</td>
<td>3.6</td>
</tr>
<tr>
<td>Azoprocbarbazine</td>
<td>6</td>
<td>255.2</td>
<td>11.2 (9.5-12.5)</td>
<td>21.8</td>
</tr>
<tr>
<td>Azody mixture</td>
<td>12 (7)</td>
<td>237.8</td>
<td>13.5 (12.8-14.5)</td>
<td>44.6e</td>
</tr>
<tr>
<td>Benzyaloxy</td>
<td>12</td>
<td>237.8</td>
<td>9.5 (8.8-10.2)</td>
<td>3.6</td>
</tr>
<tr>
<td>Methylazoxy</td>
<td>12 (6)</td>
<td>237.8</td>
<td>14.4 (10.1-18.4)</td>
<td>58.7</td>
</tr>
<tr>
<td>Methylazoxy</td>
<td>12 (11)</td>
<td>182.6</td>
<td>12.8 (12.1-14.8)</td>
<td>40.4d</td>
</tr>
<tr>
<td>Methylazoxy</td>
<td>12</td>
<td>91.3</td>
<td>10.8 (10.1-13.4)</td>
<td>18.4e</td>
</tr>
</tbody>
</table>

a Number of mice treated per group (number surviving more than 48 h and included in data).

b In days, median (range).
c Percentage increased lifespan.
d Significantly greater than control (P < 0.05).
e Equimolar with 300 mg/kg dose of procarbazine.
f Equimolar with 200 mg/kg dose of procarbazine.
g Equimolar with 100 mg/kg dose of procarbazine.

Increased. Indeed, the highest dose (273.8 mg/kg) was much more effective than the highest dose of procarbazine (600 mg/kg which produced a 31% lifespan increase) that was tested in our previous studies (23).

DISCUSSION

In this report we have demonstrated that the purity of the azoxy isomers can easily be confirmed by 1H-NMR spectrometry after separation by column chromatography and analysis by thin-layer chromatography. Previously reported methods of synthesis (8–9) used HPLC to separate and purify the azoxy isomers. We have found that our synthetic method produced greater yields of pure metabolites than these previously reported methods.

Results of both the in vitro cytotoxicity assays and in vivo survival studies indicate that methylazoxyprocarbazine is the active species involved in the cytotoxic activity of procarbazine. Previous investigators have postulated that methyl radicals, indicated by the production of methane, were involved in the cytotoxic action of procarbazine (18, 29), but Moloney and Prough (19) have demonstrated that methane is not generated from the azoxy isomers. Since we have shown that the methylazoxy isomer is probably the cytotoxic species, we believe that methyl radicals are not linked to procarbazine’s cytotoxicity to cancer cells. Erikson et al. (24), using Ducore and Barth’s doubling time assay (30) (which determines how long it takes for control and drug-treated cells to resume growth at the normal doubling time), have also recently demonstrated that methylazoxyprocarbazine is the active cytotoxic species to L1210 cells. They also found that exposures of L1210 cells to methylazoxyprocarbazine resulted in the production of DNA single-strand breaks (as measured by alkaline filter elution) which could potentially lead to cell death. They postulated that these single-strand breaks were possibly due to direct strand scission and spontaneous depurination of methylated DNA (24).

In our studies with L1210 leukemia cells, cellular metabolic inactivation by the compounds, as measured by the MTT assay, correlated well with drug inhibition of soft agar colony formation. Harker et al. (31) and other investigators (32) have demonstrated that the clonogenic assay is usually a more sensitive measure of cytotoxicity than the MTT assay. The MTT assay measures the activity of the enzyme, succinic dehydrogenase. It has been shown that MTT itself is nonlethal to cells (33). Mossman (25) has shown that within 30 min of lysis, dead cells are not able to reduce MTT. However, some damaged cells may not divide, but they are still able to reduce the tetrazolium dye. In the clonogenic assay these cells can’t divide, so no colonies would be seen. Clonogenicity measures the capacity for self-renewal of the stem cells, therefore any injury to the cell should be reflected in its clonogenicity.

Erikson et al. (24) have determined that procarbazine at concentrations up to 20 μM was not cytotoxic to L1210 leukemia cells in vitro. Our own studies utilizing the clonogenic assay, which is considered to be a more sensitive assay than the doubling time assay (34) that these investigators used, have provided evidence that procarbazine is cytotoxic to L1210 cells during 1-h exposures with an IC50 of 3.5 μM. Differences in the sensitivities of the two assays used for these studies, (clonogenic and doubling time) could possibly account for the differences noted.

We have noted slightly greater cytotoxicity of both procarbazine and its azoxy metabolites when cells are exposed on a continuous basis (10 days) compared to 1-h exposures. Theoretically, the difference could be related to ease of drug entry into cells. This seems unlikely however, since procarbazine is highly lipophilic and enters cells readily by passive diffusion, even entering the blood-brain barrier. Its metabolites have also been detected in the brain (2, 8). Another explanation for increased cytotoxicity with longer exposures might be that procarbazine or its metabolites are degraded or metabolized further during the longer time periods.

Shiba and Weinkam (35) have demonstrated the rapid metabolic transformation of procarbazine within 40 min of incubations with a 9000-g rat liver supernatant preparation. HPLC analysis of the products of those incubations demonstrated the disappearance of procarbazine and appearance of azoprocarbazine and the azoxy metabolites. Results of our experiments, in which cytotoxicity was demonstrated in L1210 cells following 1-h procarbazine exposures, raises the possibility that procarbazine may be converted to azoprocarbazine and then subsequently to the azoxy metabolites. In order to explain these findings, one must postulate that metabolic activation of procarbazine may not be necessary for in vitro cytotoxicity or, more likely, that the L1210 leukemia cells may possess the necessary metabolic machinery for cellular activation of the drug. Erickson et al. (24) have shown, however, that L1210 cells contain little or no cytochrome P-450 or monoamine oxidase activity, so the process of bioactivation of procarbazine in these isolated cells is difficult to explain.

Both azoxy metabolites of procarbazine were found to be slightly more cytotoxic to L1210 cells during continuous cell exposures than during 1-h exposures, which suggests that further metabolic activation of the azoxy metabolites may occur in vitro to produce the carbinol intermediates (Fig. 1) leading to ultimate methylating agents. This observation has been supported by in vivo studies of 1,2-dimethylhydrazine by Fiala (36) who has proposed that methylazoxymethane may be further transformed to methylazoxymethanol, and converted to methylidiazonium ions which may be the ultimate methylating agent. Erikson et al. (24) demonstrated that methylazoxyprocarbazine cytotoxicity to L1210 leukemia cells is dependent on time of exposure, with cells exposed for 1-h demonstrating 50% survival versus only 5% survival after 3-h exposures.

Weinkam and Shiba (8) have also proposed that the azoxy
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isolomers may undergo further metabolism via a carbon-hydroxylation reaction leading to the formation of an ultimate metabolite of the azoxy-procarbazine. Drug Metab. Dispos., 10: 474-482, 1982.


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