Separate Mechanisms for Procarbazine Spermatotoxicity and Anticancer Activity

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ABSTRACT

Procarbazine causes dose-dependent decreases in sperm count after a single i.p. injection in (C57BL/6 × DBA/2)F1, male mice. Two antioxidants, N-acetylcysteine and sodium ascorbate, administered with equimolar doses of procarbazine decreased the spermatotoxicity of procarbazine. At the highest doses of procarbazine (400 mg/kg) that caused a 56% decrease in sperm count, equimolar doses of N-acetylcysteine coadministered with procarbazine caused only a 17% decrease in sperm count, and equimolar doses of ascorbate coadministered with procarbazine caused only a 13% decrease in sperm count. Thus, protection against the spermatotoxic effects of procarbazine was demonstrated with either antioxidant. The effect of the antioxidants on the chemotherapeutic efficacy of procarbazine against murine L1210 leukemia was also assessed. Procarbazine at the highest dose (600 mg/kg) increased mean survival time of mice inoculated i.p. with 1 x 106 L1210 leukemia cells by 31%. Simultaneous administration of equimolar doses of either N-acetylcysteine or ascorbate given with procarbazine caused no change in the increased mean survival time of tumor-baring mice. These results indicate a decrease in the toxicity of procarbazine when coadministered with antioxidants, via decreased spermatotoxicity without changing anticancer efficacy. The results also indicate that different mechanisms are involved in the spermatotoxicity and anticancer activity of procarbazine.

INTRODUCTION

Procarbazine, N-isopropyl-α-(2-methylhydrazino)-p-toluamide hydrochloride, has been widely utilized as an effective anticancer agent in the treatment of Hodgkin's disease (1) and several other types of cancer (2). An unfortunate side effect of procarbazine in combination chemotherapy is irreversible azoosperma in humans (3). The mechanism of damage to sperm cells of this important therapeutic agent has received considerable attention (4-6), but the precise mechanism of toxicity remains unknown. Bioactivation of procarbazine to an alkylating intermediate is a requisite process in the spermatoxicity as well as the anticancer activity of the drug (7).

We have recently demonstrated that the bioactivation of procarbazine to a spermatotoxic species involves benzylic oxidation of the compound rather than methyl oxidation (8). This work utilized deuterium-labeled analogues of procarbazine and, although the precise intermediate responsible for the spermatotoxic effect remains unknown, a general outline of the bioactivation of procarbazine can be postulated to proceed through azoprocarnazine and the azoxyprocarbazine isomers (Fig. 1) (7). In addition, the azoxyprocarbazine isomers have been shown to be more effective against murine L1210 than either procarbazine or azoprocarnazine (9).

The formation of methyl radical (10) or other radicals (11) during the metabolism of procarbazine and the possible involvement of these radicals in the anticancer activity of procarbazine have been described. Production of the methyl radical was indicated (10) during the microsomal metabolism of azoprocarnazine or the hydrazine but not from the metabolism of the azoxyprocarbazine isomers (Fig. 1). Thus, the azoxyprocarbazine isomers may be important intermediates in the spermatotoxic and anticancer processes via formation of alkylating intermediates, but not via the participation of the methyl radical.

In addition to our studies on the mechanisms of procarbazine bioactivation, the present study was designed to evaluate the hypothesis that antioxidants such as N-acetylcysteine and ascorbate could protect developing sperm cells from the toxic manifestations of procarbazine metabolites. Thiols have been used in cancer chemotherapy to protect against a number of toxicities (12) including bladder cystitis from cyclophosphamide, cardiac toxicity from doxorubicin, and renal toxicity from cisplatin (12, 13). In addition, we wished to assess the potential interference of the antioxidants on the anticancer efficacy of procarbazine, since treatments that decrease both spermatotoxicity and anticancer efficacy would not improve the therapeutic usefulness of procarbazine. Because our previous work suggested that different mechanisms are involved in the anticancer activity and spermatotoxicity of procarbazine, it was likely that antioxidants could protect against procarbazine toxicity without decreasing anticancer efficacy. This paper supports the hypothesis that separate toxic and antitumor mechanisms exist for procarbazine, and that the difference in mechanisms can be exploited to improve the therapeutic usefulness of this drug.

MATERIALS AND METHODS

Chemicals. Procarbazine hydrochloride was a gift from Hoffman-LaRoche, Inc. (Nutley, NJ). Other chemicals were purchased as follows: sodium chloride and formaldehyde, 37% solution, from J. T. Baker Chemical Company (Phillipsburg, NJ); sodium lauryl sulfate from Fisher Scientific Company (Fair Lawn, NJ); Ficoll (type 400), sodium ascorbate and N-acetylcysteine (N-acetyl-L-cysteine) from Sigma Chemical Company (St. Louis, MO). Azoprocarnazine, the hydrazine, and a mixture of the two azoxyprocarbazine isomers were synthesized according to published procedures (14).

Animals. Male DBA/2 (C57BL/6 × DBA/2)F1, mice (hereafter called B6DF1) were obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained as described previously (8) in facilities accredited by the American Association for Accreditation of Laboratory Animal Care. Mice used for either the spermatotoxicity assays or L1210 activities were 8–12 weeks old and weighed between 24 and 32 g.

Sperm Count Determinations. B6DF1, mice (four mice per treatment group) were treated i.p. with the following compounds: (a) procarbazine hydrazine (255.2 mg/kg); (b) azoprocarnazine (255.2 mg/kg); (c) azoxyprocarbazine isomers (237.8 mg/kg); or (d) procarbazine (100, 200, 300, and 400 mg/kg) and/or N-acetylcysteine (equimolar doses to procarbazine) or ascorbate (equimolar doses to procarbazine). When the antioxidants were coadministered with procarbazine, separate syringes were used for i.p. injections and the antioxidants were given within 5 min after procarbazine administration. Epididymal sperm counts were obtained 18 days later, as previously described (8). Briefly, this method involved the excision of the epididymides, suspension of spermatozoa in a 10% Ficoll and 0.5% sodium lauryl sulfate aqueous solution, and the determination of sperm counts with a hemacytometer.

L1210 Murine Leukemia Studies. L1210 lymphocytic leukemia was originally obtained from NIH. For each experiment, ascites tumors were initiated from frozen stock by i.p. inoculation into B6DF1 mice. The ascites fluid obtained 7 days later was centrifuged at 200 x g for 10 min and resuspended in Hanks' balanced 0.9% NaCl solution after lysis of contaminating erythrocytes by a 4-s exposure to sterile distilled...
MECHANISMS OF PROCARBAZINE ACTIVITIES

Fig. 1. Proposed scheme for metabolism and spermatotoxicity of procarbazine.

![Chemical structures](image)

**Fig. 2.** Dose-effect relationship between i.p. administration of procarbazine alone (○) or in combination with equimolar N-acetylcysteine (▲) or sodium ascorbate (●) on spermatogenesis in B6D2F1 mice. Values are the means ± SD, expressed as a percentage of control mice given 0.9% NaCl solution.

**Fig. 3.** Effect of i.p. administration of procarbazine alone (○) or in combination with 189.9 mg/kg N-acetylcysteine (▲) or 307.4 mg/kg sodium ascorbate (●) on survival time of male B6D2F1 mice inoculated with L1210 leukemia. Values are the means ± SD, expressed as a percentage of control mice given 0.9% NaCl solution.

**RESULTS**

As previously reported (8), procarbazine administration depressed spermatogenesis in a manner which was both dose-dependent and log linear. Similarly, Fig. 2 shows the dose-effect relationship on spermatogenesis depression in B6D2F1 mice between procarbazine given i.p. alone and in combination with equimolar N-acetylcysteine or ascorbate. The addition of equimolar N-acetylcysteine or ascorbate to the treatment afforded significant protection from spermatotoxicity at all doses, and the level of protection was similar for both agents. Neither antioxidant had any effect on sperm count when administered alone in control experiments (data not shown).

Fig. 3 shows the effects of procarbazine given i.p. alone and in combination with either N-acetylcysteine (189.9 mg/kg, equimolar with 300 mg/kg procarbazine) or ascorbate (307.4 mg/kg, equimolar with 400 mg/kg procarbazine) on survival of male B6D2F1 mice inoculated with 1 × 10⁵ L1210 murine leukemia cells. Doses of N-acetylcysteine higher than approximately 200 mg/kg were acutely lethal when coadministered to tumor-bearing mice with equimolar procarbazine doses. Therefore, a maximum-tolerated dose of 189.9 mg/kg of N-acetylcysteine was used for coadministration with all doses of procarbazine in L1210 leukemia studies. Lower doses of N-acetylcysteine did not change the antitumor effectiveness of procarbazine (data not shown). Equimolar doses of ascorbate with procarbazine did not change the effectiveness of the drug (data not shown), so a single high dose (equivalent to 400 mg/kg procarbazine) was used for all L1210 experiments.

Procarbazine treatment significantly prolonged mean survival at all doses. Moreover, increases in procarbazine doses produced increases in survival in a dose-dependent manner. Neither N-acetylcysteine nor ascorbate alone affected survival of mice bearing L1210 leukemia, nor did they alter the chemotherapeutic effectiveness of procarbazine. Further, the L1210 tumor did not affect sperm counts over the 9-day lifespan of control animals, nor did the tumor alter the sperm count

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*Figures and data are not reprinted here due to the limitations of text-based representation. For detailed visual content, please refer to the original source.*

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**Statistics.** All values in Figs. 2 and 3 are the mean ± SD of at least four animals for the spermatotoxicity assays and six animals for the L1210 assays. Statistical significance is based on the Student's t test with confidence limits, P < 0.05.
depression produced by moderate (300 mg/kg) procarbazine doses (data not shown).

Table 1 shows the effects of i.p. administration of 300 mg/kg of procarbazine or equimolar amounts of several of the stable metabolites of procarbazine on spermatogenesis in mice. Azoprocarbazine, the major circulating metabolite of procarbazine, produced significantly greater depression of sperm count than procarbazine. The mixture of the two azoxyprocarbazine metabolites was significantly more toxic than procarbazine or azoprocarbazine. Administration of the hydrazone metabolite resulted in only minor spermatotoxicity.

**DISCUSSION**

The susceptibility of developing sperm cells to repeated cytotoxic insult by procarbazine results in irreversible azoospermia in animals (15) and humans (3). One major goal of our studies was to block azoospermia without concomitant blockage of the anticancer efficacy of procarbazine. Our studies have shown that either N-acetylcysteine or ascorbate can be used to effectively protect early spermatids from the cytotoxicity of procarbazine. Since we utilized an 18-day waiting period after procarbazine treatment to count mature sperm, our protocol assessed the cytotoxicity of procarbazine on the early spermatid stage of developing sperm cells (16). Other studies have demonstrated that early spermatocytes are also susceptible to procarbazine cytotoxicity (17).

Although a single treatment with the protective agents significantly decreased spermatotoxicity, the protection was not absolute. It is possible that considerably greater protection may be achieved with chronic administration of ascorbate or N-acetylcysteine during drug treatment. Studies are in progress which will evaluate this hypothesis.

The protection of sperm cells by N-acetylcysteine or ascorbate was achieved without loss in anticancer activity of procarbazine. We have previously shown that ascorbate lacks antitumor activity in the L1210 murine model (18). Maintenance of the murine L1210 anticancer activity of procarbazine, coupled with significant decreases in spermatotoxicity, may provide protocols which will lead to safer use of this drug.

Our work has also provided information on the differences in mechanisms of toxicity and anticancer activity of procarbazine, although we are not able at this time to unequivocally explain the reasons for these differences. Moloney and Prough (10) have established that methane (presumably produced from the one-electron reduction of methyl radical) is formed via the microsomal oxidation of azoprocarbazine or, less likely, the hydrazone. Since the azoxyprocarbazine isomers did not produce methane in the in vitro system (10), the authors concluded that the azoxyprocarbazine isomers were not involved in the anticancer activity of procarbazine, if indeed the methyl radical is the crucial alkylating intermediate. Shiba and Weinkam (9) have established, however, that the azoxyprocarbazine isomers are more effective anticancer agents than procarbazine in the L1210 model. Thus, some doubt remains as to the role of the azoxyprocarbazine isomers in the anticancer activity of procarbazine, and the involvement of the methyl radical in the anticancer activity of procarbazine is yet to be established.

In contrast to the work on methane production, our studies with the azoxyprocarbazine isomers on the mechanism of spermatotoxicity have shown that these metabolites are at least twice as potent as procarbazine as spermatotoxic agents (Table 1). In addition, the hydrazone, a possible precursor for methane production (10), caused very little spermatotoxicity. Thus, we conclude that the spermatotoxicity of procarbazine is caused by an alkylating agent that is produced through the sequential formation of azoprocarbazine and the azoxyprocarbazine isomers, without the intervention of the methyl radical (see Fig. 1).

Since both N-acetylcysteine and ascorbate protect spermatids against procarbazine toxicity, a mechanism of protection likely involves either reduction of oxidized intermediates, or interception of electrophilic alkylating intermediates by protective nucleophilic biomolecules such as glutathione. N-Acetylcysteine is well known as a precursor of glutathione (12, 13, 19), and ascorbate may increase glutathione levels via the reduction of oxidized glutathione (20). Glutathione or N-acetylcysteine may intercept an electrophilic intermediate such as methyl diazine (21) in the spermatotoxic process, but it is unlikely that glutathione or N-acetylcysteine may也可能 inactivate the cytotoxic intermediate which is responsible for L1210 activity, since survival times of tumor-bearing mice were unaffected by N-acetylcysteine or ascorbate coadministration.

Since glutathione has been shown to increase methane production and to decrease DNA binding of the methyl moiety in vitro (22), we might have expected ascorbate and/or N-acetylcysteine would decrease the in vivo efficacy of procarbazine, especially if the methyl radical were involved. Since the anticancer activity was not affected, we believe that the methyl radical probably is not involved in the anticancer activity of procarbazine.

Reduction of an electrophilic intermediate such as methyl diazine by N-acetylcysteine, ascorbate, glutathione, or other antioxidants, is the most likely process in the protection of spermatids. The exact nature of the mechanism of protection will be difficult to determine, since the intermediate is probably short-lived and several of the reducing agents might interact with it in different ways. Since the methyl radical is not produced from the azoxyprocarbazines, and since the azoxyprocarbazines are the most potent spermatotoxic agents, it seems clear that the spermatotoxic intermediate is not the methyl radical and that the intermediate is most likely formed via the chemical or enzymatic metabolism of the azoxyprocarbazine intermediates. The precise nature of the intermediate has yet to be determined, but it is clear from our previous work (8) that the oxidation of the benzylic C—H bond is necessary for formation of the intermediate. Thus, oxidation of the methylazoxy procarbazine isomer at the benzylic position to produce an electrophilic intermediate is the most likely mechanism of procarbazine spermatotoxicity. Our results imply that the spermatotoxic intermediate is different from the antitumor intermediate, but we are not able to fully explain at this time what that difference is.

**Table 1** Depression of spermatogenesis in B6D2F1 mice by equimolar doses of procarbazine or its metabolites

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose (mg/kg)</th>
<th>Sperm count* (% control)</th>
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<tbody>
<tr>
<td>Procarbazine</td>
<td>300.0</td>
<td>56.4 ± 6.3</td>
</tr>
<tr>
<td>Hydrazone</td>
<td>255.2</td>
<td>84.5 ± 2.8</td>
</tr>
<tr>
<td>Azoprocarbazine</td>
<td>255.2</td>
<td>34.0 ± 4.3*</td>
</tr>
<tr>
<td>Azoxyprocarbazine</td>
<td>273.8</td>
<td>18.8 ± 2.2</td>
</tr>
</tbody>
</table>

*Mean ± SD of three determinations from each of four mice.
*Significantly lower than the sperm count for mice given procarbazine.
REFERENCES

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